Detection of *Cercospora beticola* and *Phoma betae* on Table Beet Seed using Quantitative PCR

Noel L. Knight, Lori B. Koenick, Sandeep Sharma, and Sarah J. Pethybridge†

Plant Pathology & Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell AgriTech at the New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456, U.S.A.
Accepted for publication 13 January 2020.

ABSTRACT

Cercospora beticola and *Phoma betae* are important pathogens of table beet, sugar beet, and Swiss chard (*Beta vulgaris* subsp. *vulgaris*), causing Cercospora leaf spot (CLS) and Phoma leaf spot, root rot, and damping-off, respectively. Both pathogens may be seedborne; however, limited evidence is available for seed infestation by *C. beticola*. Due to the limitations of culture-based seed assessment methods, detection of these pathogens was investigated using PCR. A *P. betae*-specific quantitative PCR assay was developed and used in conjunction with a *C. beticola*-specific assay to assess the presence of pathogen DNA in 12 table beet seed lots. DNA of *C. beticola* and *P. betae* was detected in four and eight seed lots, respectively. Plate tests and BIO-PCR confirmed the viability of each pathogen; however, competitive growth of other microbes and low incidence limited the frequency and sensitivity of detection in some seed

lots. The results for *P. betae* support previously described infestation of seed. Further investigation of *C. beticola*-infested seed lots indicated the ability of seedborne *C. beticola* to cause CLS on plants grown from infested seed. Detection of viable *C. beticola* on table beet seed demonstrates the potential for pathogen dispersal and disease initiation via infested seed, and provides valuable insight into the epidemiology of CLS. Surveys of commercial table beet seed are required to determine the frequency and source of *C. beticola* seed infestation and its role as primary inoculum for epidemics, and to evaluate the effectiveness of seed treatments.

Keywords: disease control and pest management, ecology and epidemiology, mycology, techniques

Movement of plant pathogens via seed can pose significant risks for spread of disease, changes in pathogen populations, and biosecurity (Munkvold 2009). The risks posed by seed infestation are increased due to asymptomatic seed, potential long-term pathogen survival, and low percentages of infestation that may result in severe disease (Mancini et al. 2016; Pellegrino et al. 2010). Although techniques for detection of seedborne pathogens are available, low incidences and nonuniform distribution of seed infestation present continuing challenges for robust detection and management (Mancini et al. 2016).

Phoma betae (syn. *Neocamarosporium betae*, *Pleospora betae*) (Ariyawansa et al. 2015) causes reduced seed germination and seedling emergence, damping-off, foliar lesions (Phoma leaf spot), root disease (Phoma root rot), and storage rot of table beet, sugar beet, and Swiss chard (*Beta vulgaris* subsp. *vulgaris* L.) (Byford and Gambogi 1985; Harveson 2009; Koenick et al. 2019b; Pethybridge et al. 2018). *Phoma betae* has been well documented as a seedborne pathogen of *B. vulgaris* (Byford and Gambogi 1985; Edson 1915; Leach 1946; Leach and MacDonald 1976; Pethybridge et al. 2018; Pool and McKay 1915), and was reported to survive in the soil on

colonized plant debris for up to 26 months (Bugbee 1974; Bugbee and Soine 1974). *P. betae* can be detected on seed by plating onto water agar (Mangan 1971), and may be internal or external within *B. vulgaris* seed (Leach and MacDonald 1976). Effective control of *P. betae* may be achieved through crop rotations, avoiding infested seed, selection of less susceptible cultivars (Koenick et al. 2019a), and the application of conventional fungicides on the seed and foliage (Maude et al. 1969; Pethybridge et al. 2018; Reinert et al. 2017). In conventional table beet fields in New York, Phoma leaf spot occurs at low incidence; however, in fields using organic production practices, it may be common (Koenick et al. 2019b; Pethybridge et al. 2018). The lack of effective seed treatment and disease management options for organic table beet producers has contributed to Phoma leaf spot reemerging as a significant issue (Koenick et al. 2019a; Pethybridge et al. 2018).

Cercospora beticola causes a foliar disease, Cercospora leaf spot (CLS), of table beet, sugar beet, and Swiss chard (Weiland and Koch 2004). In warm temperate climates, CLS can result in significant damage to leaves and defoliation (Koike et al. 2010; Weiland and Koch 2004). In table beet production in New York, defoliation leads to the inability to mechanically harvest roots using top-pulling machinery (Pethybridge et al. 2017, 2018). Foliar disease in the fresh market may also lead to consumer rejection (Pethybridge et al. 2017, 2018). Primary sources of *C. beticola* inoculum are considered to be colonized plant debris, with *C. beticola* detected up to 39 months after placement of infested plant debris on the soil surface (Khan et al. 2008; Solel 1970) and alternative hosts (Jacobsen and Franc 2009; Knight et al. 2019b; McKay and Pool 1918; Pool and McKay 1916). CLS management in table beet relies on crop rotations to reduce soilborne inoculum, control of alternative hosts, and application of fungicides to reduce the rate of CLS progress (Pethybridge et al. 2017).

Although *C. beticola* has been described as a potential seedborne pathogen of *B. vulgaris* (Jacobsen and Franc 2009; Navazio et al. 2010; Noble and Richardson 1968; Srivastava 2004), direct evidence is limited. McKay and Pool (1918) monitored sugar beet

†Corresponding author: S. J. Pethybridge; sjp277@cornell.edu

Current address of L. B. Koenick: Cornell Cooperative Extension Monroe County, Rochester, NY 14617, U.S.A.

Funding: This research was supported by the United States Department of Agriculture National Institute of Food and Agriculture Hatch project NYG-625424, and the Federal Capacity Funds Initiative Project 2018-19-113 managed by Cornell AgriTech at The New York State Agricultural Experiment Station, Cornell University, Geneva, NY, U.S.A.

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

The author(s) declare no conflict of interest.

seed for infestation with *C. beticola* by assessing rinsate from seed for viable conidia and plating macerated seed tissue. Viable *C. beticola* was detected on one domestic seed lot harvested from a diseased crop, and not detected in 28 imported seed lots. Seedlings grown from the infested seed lot exhibited CLS lesions on the cotyledons and greater disease than plants grown from the same seed treated with formaldehyde, demonstrating the ability of seedborne *C. beticola* to cause disease (McKay and Pool 1918). Sugar beet seed infestation with *C. beticola* was similarly described in European literature cited by Noble and Richardson (1968) and Vereijssen et al. (2004), and as a personal communication in Vereijssen et al. (2004), where conidia were found on processed seed and associated with the development of unexpected CLS epidemics. In sugar beet seed, *C. beticola* was suggested to be present inside the cork or close to the embryo (Vereijssen et al. 2004). However, there is limited direct evidence of *C. beticola*-infested seed since the original work of McKay and Pool (1918) and, although subsequent studies on seedborne fungi of beet have not detected *C. beticola* (Agarwal et al. 2006; Byford and Gambogi 1985), optimized methods for detection of *C. beticola* from seed have not been available.

Population biology studies of *C. beticola* have suggested the absence of regional genetic structure, supporting the potential role of a long-distance means of dispersal such as seedborne inoculum in CLS epidemics in table and sugar beet (Knight et al. 2018, 2019a; Moretti et al. 2006; Turgay et al. 2010; Vaghefi et al. 2017a,b). However, the incidence of infested seed would likely vary and may not play a role in every situation (Vaghefi et al. 2016a). Further evidence suggesting a role for seedborne inoculum was low genetic differentiation between *C. beticola* populations from monoculture table beet fields with shared seed sources, and greater genetic differentiation between *C. beticola* populations from mixed-cropping farms and monoculture fields using seed from different sources (Vaghefi et al. 2017b). A subsequent study on temporal genetic differentiation of *C. beticola* associated with CLS in table beet fields reported limited differentiation between populations from two locations which maintained the same seed source in each year, while a third location, which changed seed source between years, exhibited greater genetic differentiation of populations between years (Knight et al. 2018). Assessment of *C. beticola* populations from Europe and the United States have indicated potential gene flow between these regions (Knight et al. 2019a; Vaghefi et al. 2017a), which coincides with the movement of table beet seed between Europe and the United States (L. du Toit, personal communication). Table beet seed is also exported to the United States from seed production areas in Chile, New Zealand, and South Africa (L. du Toit, personal communication). Convoluted trade pathways and genetic studies suggesting the occurrence of seedborne *C. beticola* in modern agricultural systems (Knight et al. 2019a) support a need for more comprehensive investigation and surveillance of beet seed for such seedborne pathogens (L. du Toit, personal communication).

Sensitive, accurate, and rapid detection of pathogens is crucial for effective monitoring of seed health. Traditional methods for detection of seedborne fungi involve incubation and visual assessment of microbial growth. These methods are simple to apply and indicate pathogen viability but require expert mycological skills, are time consuming, and may not detect low levels of seed infestation (Mancini et al. 2016). The range of currently available pathogen detection techniques and their use for seed testing has been reviewed (Mancini et al. 2016; Munkvold 2009), with PCR identified as a valuable tool, especially when used in conjunction with methods that indicate pathogen viability. Quantitative PCR provides increased sensitivity of detection compared with conventional PCR, and allows quantification of target DNA within seed (Carneiro et al. 2017; Chen et al. 2013; Chilvers et al. 2007; Duressa et al. 2012; Guillemette et al. 2004). Conventional PCR assays have been designed to detect several beet

fungal pathogens (Lartey et al. 2003; Weiland and Sundsbak 2000) and, with increased availability of pathogen DNA sequences and phylogenetic investigations, improved assays have been developed (Knight and Pethybridge in press).

The objective of this study was to use PCR and culture-based techniques to evaluate the potential for table beet seed to be a source of *C. beticola* and *P. betae*. A complementary objective was to design and validate a *P. betae*-specific quantitative PCR assay.

MATERIALS AND METHODS

Design of *P. betae*-specific primers and probe. Partial sequences of the phylogenetically informative gene encoding the second largest subunit of RNA polymerase II (*rpb2*) (Větrovský et al. 2016) were aligned between nine *P. betae* isolates, closely related fungal species (de Gruyter et al. 2013; Vaghefi et al. 2019; Wanasinghe et al. 2017), and fungal species commonly associated with beet (Strausbaugh et al. 2015) to identify polymorphisms consistently discriminating between *P. betae* and other species (Supplementary Table S1). Sequences of reference isolates were obtained from the National Center for Biotechnology Information nucleotide database. Multiple sequence alignments were conducted using MAFFT v. 7.388 (Katoh and Standley 2013; Katoh et al. 2002) in Geneious Prime v. 2019.0.4 (<https://www.geneious.com>) (Kearse et al. 2012). Primer3 v. 4.1.0 (Koressaar and Remm 2007; Untergasser et al. 2012) was used to design potential primers and probes for suitable lengths and associated melting temperatures. Primers were positioned to align with at least one discriminatory polymorphism at the 3' terminus (Petruska et al. 1988). The probe was positioned to capture polymorphisms discriminating *P. betae* from other closely related fungal species. To allow for potential multiplexing, primer and probe melting temperatures were designed to be less than 2°C different from the *B. vulgaris* and *C. beticola* primers and probes reported by Knight and Pethybridge (in press). Specificity of the primer and probe sequences was assessed by a BLAST search of the GenBank nucleic acid database using the Others (nr and so on) database: Nucleotide Collection (nr/nt).

DNA extraction. DNA was extracted from fungal and table beet tissue using the Wizard Genomic DNA Extraction kit (Promega Corp., Madison, WI, U.S.A.) following the method described by Knight and Pethybridge (in press), with slight modifications for table beet leaf tissue. Leaf tissues were harvested from 4-week-old table beet plants (cultivar Ruby Queen), placed in a gravimetric drying oven at 60°C for 12 h, and subsequently ground in a 2-ml polypropylene tube (Eppendorf, Hamburg, Germany) using a polypropylene pestle and separated into 10-mg subsamples. Extracted DNA was dissolved into 100 µl of autoclaved high-purity water.

Conventional PCR conditions. The conventional PCR assay containing the Pb_R2 primers was performed in 20-µl volumes using a T100 thermal cycler (Bio-Rad, Hercules, CA, U.S.A.). Reactions contained autoclaved high-purity water, 0.4 U of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.), 1× standard *Taq* buffer (containing 1.5 mM MgCl₂), 250 µM dNTPs, 0.25 µM Pb_R2-F and Pb_R2-R primers (Integrated DNA Technologies, Coralville, IA, U.S.A.) (Table 1), and 5 µl of diluted DNA (10 ng/µl) template. Conventional PCR was performed as described by Knight and Pethybridge (in press).

Quantitative PCR conditions. The Pb_R2 primer/probe assay was assessed in duplex reactions with the SbEc1 (*B. vulgaris*) primer/probe assay designed by De Coninck et al. (2012) (Table 1). The CbCAL primer/probe assay was performed as described by Knight and Pethybridge (in press). Amplification of *B. vulgaris* DNA was included as an internal positive control for beet tissue samples. A mixed *P. betae* and *B. vulgaris* DNA sample (1:1 ratio of standard DNA) was assessed for intra- and interassay variability. The duplex assay was performed using white 96-well Multiplate PCR plates (Bio-Rad) and Optical 8-cap strips (Bio-Rad) in a C1000 Touch thermal cycler, CFX96 real-time system (Bio-Rad).

Reactions were performed in 20- μ l volumes containing 10 μ l of Immomix (Bioline Meridian Bioscience, Memphis, TN, U.S.A.) and 5 μ l of DNA template. The Pb_R2 assay contained 150 nM Pb_R2-P 5' CAL Fluor Red 610/3' BHQ-2-labeled hydrolysis probe; 150 nM SbEc1 5' FAM/3' BHQ-1-labeled hydrolysis probe (LGC Bioscience Technologies, Hoddessdon, UK); and 0.25 μ M Pb_R2-F, Pb_R2-R, SbEc1-F, and SbEc1-R primers (Integrated DNA Technologies). Thermal cycling was performed as described by Knight and Pethybridge (in press).

Each plate contained no-template controls and genomic DNA standards (positive and negative controls) for *C. beticola*, *P. betae*, and *B. vulgaris*. A duplicate 10-fold serial dilution of pure genomic *C. beticola* or *P. betae* DNA was included in each plate. Four 10-fold serial dilutions for *C. beticola* or *P. betae* DNA (approximately 10 ng to 1 pg) were used for quantification. DNA concentrations of *C. beticola*, *P. betae*, and *B. vulgaris* standards were determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) with the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific). Standard curves for *C. beticola* and *P. betae* DNA were generated using the Bio-Rad CFX Manager 3.1 software, as described by Knight and Pethybridge (in press).

Primer and probe specificity. The *P. betae* specificity of the Pb_R2 assay was determined in conventional and quantitative PCR. DNA of 17 fungal species present on farms in New York, including 40 *P. betae* isolates (Table 2), was assessed in duplicate. Conventional PCR products were visualized as described by Knight and Pethybridge (in press). Quantitative PCR results were assessed for increases in fluorescence above the background level prior to 35 cycles.

Selective detection of *C. beticola* and *P. betae* DNA from seed. Twelve seed lots were obtained from commercial suppliers and farmers that save seed within their own enterprise (Table 3). Metadata associated with these seed lots is proprietary. Seed lots 1 to 11 were produced using organic management, while seed lot 12 was produced using conventional practices. Seed lots 1 and 2 were included due to the appearance of CLS on crops grown from these seed in fields where the disease had not previously been observed (M. McGrath, personal communication). Seed lots 3 and 4 were obtained from a private organic farm which produces seed for its own use, previously described in population genetics studies of *C. beticola* by Knight et al. (2018) and Vaghefi et al. (2017b) as Farm 1. Seed lots 5 to 11 were included after commercial rejection due to *P. betae* infestation. Seed lot 12 was included to represent a conventionally produced and fungicide-treated seed lot using thiram (42-S Thiram; Bayer CropScience, Leverkusen, Germany), fludioxonil (as Maxim; Syngenta, Basel, Switzerland), and mefenoxam (as Apron XL; Syngenta). Thiram seed treatment was introduced primarily to control *P. betae* (Maude et al. 1969).

Seed samples of 20 g (approximately 1,200 seeds) were weighed out from available seed lots and ground to a powder in a coffee grinder (model 80335R; Hamilton Beach, Glen Allen, VA, U.S.A.),

and 20-mg subsamples ($n = 54$) of each ground seed sample were weighed into 1.5-ml microfuge tubes. After processing of each seed lot, the grinding apparatus was rinsed with water followed by 70% (vol/vol) ethanol. DNA was extracted from the subsamples using the method described by Knight and Pethybridge (in press), and the resultant DNA was diluted 10-fold. A 10-fold dilution of the original DNA extractions from ground seed reduced the potential effects of inhibitors (Knight and Pethybridge in press). The diluted DNA was assessed in duplicate using both the *C. beticola*-specific (Knight and Pethybridge in press) and *P. betae*-specific duplex quantitative PCR assays, and the quantification cycle values and DNA quantities for *C. beticola* and *P. betae* were determined as described above.

DNA extractions from single seeds of seed lots 1 and 12 were assessed for the incidence of *C. beticola* DNA. Single seeds ($n = 60$) of seed lots 1 and 12 were ground in 2-ml microfuge tubes with two 4.5-mm ball bearings (Daisy Outdoor Products, Rogers, AR, U.S.A.) using a TissueLyser (Qiagen, Hilden, Germany) for 20 s at 30 Hz. DNA was extracted using the Wizard method, and 10-fold-diluted DNA was assessed in duplicate using the *C. beticola* duplex quantitative PCR assay. A quantification cycle of less than 35 was considered a positive detection of *C. beticola* DNA (Knight and Pethybridge in press).

Pathogen presence and viability on seed. The presence of *P. betae* in the 12 table beet seed lots was assessed using plate tests (Mangan 1971). Two hundred seeds per seed lot were placed on 2% water agar (20 g/liter; Hardy Diagnostics, Santa Maria, CA, U.S.A.) + ampicillin at 0.2 g/liter (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 10-cm-diameter Petri dishes. Ten seeds were placed on each Petri dish and incubated in the dark at 20°C. *P. betae* was identified from morphological characteristics, including the presence of holdfasts, after 10 days of incubation (Bugbee 1974; Mangan 1971; Monte and García-Acha 1988). Seed germination was also quantified by counting the number of seed within each lot that had at least one seedling at least 2 cm long.

The presence of *C. beticola* on seed was assessed for seed lot 1 using a method similar to that described above. One hundred seeds were placed on 2% water agar + ampicillin and clarified V8 agar (10% [vol/vol] clarified V8 broth, agar at 15 g/liter, and ampicillin at 0.2 g/liter) (Miller 1955; Secor and Rivera 2012). Ground seed (15 mg) from lots with detectable *C. beticola* DNA was placed on each of 12 Petri dishes containing clarified V8 agar and spread with a polypropylene cell spreader. Fungal growth on agar was observed over 5 days, and hyphal tips of slow-growing fungi that resembled *Cercospora* spp. were subcultured onto clarified V8 agar. Cultures with morphological characteristics resembling *C. beticola* were subcultured into 50 ml of 10% (vol/vol) clarified V8 broth and incubated for 5 days on a shaker at 80 rpm, and the mycelial mass was removed and dried at 60°C for 48 h. Dry mycelial samples were ground in 2-ml microfuge tubes with two 4.5-mm ball bearings (Daisy Outdoor Products) using a TissueLyser (Qiagen) for 20 s at 30 Hz. DNA was extracted using the Wizard method and assessed in

TABLE 1. Primer and probe sequences for detection and quantification of *Phoma betae*, *Cercospora beticola*, and *Beta vulgaris* DNA

Primer, probe ^a	Sequence (5'→3')	Target	Fragment (bp) ^b
Pb_R2-F	CAAGGATACTAGCCGAAGGTTGTGA	<i>P. betae</i>	...
Pb_R2-R	GTGTTTTTCGGCAATCTCCTCG	<i>P. betae</i>	87
Pb_R2-P	CAGCAACTGGCTCAACAGCGCCAA	<i>P. betae</i>	...
CbCAL-F ^c	GGCAGAGCTAACGACAGCAA	<i>C. beticola</i>	...
CbCAL-R ^c	TTGTTGTCGGCGTCTGACT	<i>C. beticola</i>	199
CbCAL-P ^c	ACCCTCTGCGAATGTACTGAACCTCTG	<i>C. beticola</i>	...
SbEc1-F ^d	ACTTGCCTGGCTTTTGTCTTAGT	<i>B. vulgaris</i>	...
SbEc1-R ^d	GCCAGGTGCTGACTTGATTATT	<i>B. vulgaris</i>	90
SbEc1-P ^d	CCCARAGCATTTTCCAGTGCTCATATTGA	<i>B. vulgaris</i>	...

^a F = forward primer, R = reverse primer, and P = hydrolysis probe.

^b Base pairs of DNA.

^c Primers and hydrolysis probe for *C. beticola* reported by Knight and Pethybridge (in press).

^d Primers and hydrolysis probe for *B. vulgaris* reported by De Coninck et al. (2012).

duplicate using the *C. beticola* duplex quantitative PCR assay (Knight and Pethybridge in press).

BIO-PCR for detection of viable *C. beticola*. A modified BIO-PCR method (Mancini et al. 2016; Schaad et al. 1995) was used to determine viability of *C. beticola* in seed lots 1 to 4, which had detectable levels of *C. beticola* DNA with quantitative PCR, and seed lots 6 and 12, which did not have detectable *C. beticola* DNA. A single seed was placed in 50 ml of 10% (vol/vol) clarified V8 broth and incubated on a shaker at 80 rpm for 7 days. The seed was removed, and the remaining broth/biomass mixture was transferred to a 50-ml falcon tube. Tubes were centrifuged for 10 min at 4,500 rpm. Supernatant was discarded and the remaining pellet was placed in a 2-ml microfuge tube. The tissue was dried, ground, and used for DNA extraction as previously described for dry mycelia. Each sample was assessed in duplicate using the *C. beticola* duplex quantitative PCR assay, with a quantification cycle of less than 35 considered positive detection of *C. beticola* DNA (Knight and

Pethybridge in press). The percentage of detection was calculated by dividing the number of *C. beticola* DNA-positive samples by the total number of biomass samples assessed, multiplied by 100.

Seed to seedling transmission of *C. beticola*. Initially, 500 seeds of lot 1 and a control treatment of 100 treated seeds of lot 12 were planted into 50-cell (80 cm³) propagation trays at a depth of 1 cm in potting mix (SunshineMix number 8 Fafard-2; Sungro Horticulture, Agawam, MA, U.S.A.) and grown in a glasshouse for 4 weeks. Plants were fertilized with Peters Excel (ICL Fertilizers, Dublin, OH, U.S.A.) with nitrogen at 130 ppm using a Hozon siphon mixer (Phytotronics, Earth City, MO, U.S.A.) twice a week while in the glasshouse. In two subsequent replicated trials, 40 individual seeds of lots 1 and 12 were planted into round plastic pots (15 cm in diameter by 13 cm in depth) at 1 cm depth and grown in a glasshouse with a 14-h photoperiod and temperature of 21 and 15°C (light and dark, respectively) for 8 weeks. Subsequently, plants were placed in a growth chamber and a misting apparatus using municipal water

TABLE 2. Fungi used for specificity testing of the *Phoma betae*-specific Pb_R2 primers in conventional and quantitative PCR

Species	Location	Strain	Host
<i>Alternaria alternata</i>	United States	GAR17-214	<i>Allium sativum</i>
<i>Athelia rolfsii</i>	United States	17-006	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>
<i>A. rolfsii</i>	United States	BA-060	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>Botrytis cinerea</i>	United States	Number 899.1	<i>Phaseolus vulgaris</i>
<i>B. cinerea</i>	United States	Number 870	<i>P. vulgaris</i>
<i>Cercospora apii</i> ^a	Germany	CBS 116455; CPC 11556	<i>Apium graveolens</i>
<i>C. apii</i>	United States	Ch15-001	<i>Beta vulgaris</i> var. <i>cicla</i>
<i>C. beticola</i>	Chile	2-59	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	Chile	2-60	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	England	EU 11-2	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	Germany	EU 11-156	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i> ^a	Italy	CBS 116456; CPC 11557	<i>B. vulgaris</i>
<i>C. beticola</i>	Italy	EU 10-29	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	Sweden	EU 11-280	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	Turkey	TK-373	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb14-080	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb15-101	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb14-047	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb16-156	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb16-172	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb14-070	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb15-043	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb15-211	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb15-259	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	Tb15-215	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	HI-Tb-001	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	HI-Tb-064	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	C16-31	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. beticola</i>	United States	C16-32	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. carotae</i> ^a	Norway	CBS 101.65	<i>Daucus carota</i>
<i>C. carotae</i>	United States	Ca15-001	<i>D. carota</i> var. <i>sativa</i>
<i>C. carotae</i>	United States	Ca15-002	<i>D. carota</i> var. <i>sativa</i>
<i>Cercospora</i> cf. <i>flagellaris</i>	United States	Tb16-178	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. cf. flagellaris</i>	United States	Tb15-041	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. cf. flagellaris</i>	United States	Tb15-039	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. cf. flagellaris</i>	United States	Tb15-188	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. cf. flagellaris</i>	United States	Sp16-006	<i>Spinacia oleracea</i>
<i>Cercospora</i> cf. <i>chenopodii</i> ^a	France	CBS 132620; CPC 14237	<i>Chenopodium album</i>
<i>Cercospora</i> cf. <i>chenopodii</i>	United States	Lq15-012	<i>Chenopodium album</i>
<i>Cercospora</i> sp. G	United States	Pg16-04	<i>Amaranthus retroflexus</i>
<i>Cercospora</i> sp. G	United States	Tb15-005	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. zeae-maydis</i>	United States	Cz-m002NY12	<i>Zea mays</i>
<i>C. zeae-maydis</i>	United States	Cz-m003NY12	<i>Z. mays</i>
<i>C. zebrina</i>	United States	Tb16-177	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>C. zebrina</i>	United States	C.m.002NY-07	<i>Medicago sativa</i>
<i>C. asparagi</i>	United States	As-01	<i>Asparagus officinalis</i>
<i>C. asparagi</i>	United States	As-02	<i>A. officinalis</i>
<i>Colletotrichum incanum</i>	United States	Col3	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>Didymella americana</i>	United States	K004	<i>B. vulgaris</i> subsp. <i>vulgaris</i>

(Continued on next page)

^a The ex-type strains of *C. apii*, *C. beticola*, *C. carotae*, *C. cf. chenopodii*, and *P. betae* were obtained from the fungal collection of the Westerdijk Fungal Biodiversity Institute, the Netherlands.

was operated for 4 h on 5 days/week for 6 to 8 weeks. Plants were watered once every 4 days. Growth chamber conditions were 25°C with a 16-h photoperiod at 820 lux (110-W fluorescent mercury bulbs; Philips, Andover, MA, U.S.A.) and a mist water temperature of 22.7°C. These conditions maintained relative humidity at >90% for 4 h/day. Leaves were visually monitored for disease symptoms every second day for 4 to 8 weeks.

Leaves with CLS-like lesions were examined for signs of *C. beticola*. Lesions exhibiting pseudostromata and conidia with morphology and dimensions resembling *C. beticola* (Weiland and Koch 2004) were considered positive. The incidence of plants with CLS was recorded and lesions per leaf were counted. The presence of *C. beticola* DNA in leaf lesions was determined using the method described by Knight and Pethybridge (in press). Briefly, DNA was extracted from dried, ground lesion tissue using the Wizard method and assessed with the *C. beticola*-specific duplex quantitative PCR assay.

RESULTS

***P. betae* assay design and specificity.** Multiple sequence alignment of the *rpb2* gene region identified multiple polymorphisms as unique to *P. betae* compared with closely related

species (Fig. 1). Primers were designed to contain discriminatory nucleotide polymorphisms at the 3' terminus, while the probe sequence was aligned to a region containing multiple polymorphisms (Fig. 1; Table 1).

Conventional PCR using Pb_R2 primers and fungal DNA (1 to 5 ng/μl) of 95 isolates from 17 species (Table 2) amplified a single 87-bp fragment from DNA of each of the 40 *P. betae* isolates. The Pb_R2 duplex quantitative PCR assay reported quantification cycles for only *P. betae* DNA.

A triplex assay containing the CbCal, Pb_R2, and SbEc1 primers and probes produced nonspecific fluorescence (data not shown). Therefore, duplex assays of either CbCal or Pb_R2 combined with SbEc1 were used for assessment of DNA from table beet seed.

Detection thresholds for *P. betae* quantitative PCR assay.

Quantitative PCR assessment was limited to 35 cycles in order to avoid false positives due to nonspecific fluorescence, with 35 cycles sufficient for standard curve production and detection of small quantities of target DNA. *P. betae* DNA was quantifiable from 0.001 to 10 ng. The standard curve of the quantification cycle and log of standard DNA quantities was calculated for *P. betae* ($R^2 > 0.99$, $y = -3.87x + 25.7$, $E = 81.3$). The mean value ($n = 10$) of *P. betae* DNA in a mixed *P. betae* and *B. vulgaris* DNA sample assessed for intraassay variability was 3.3 ng of *P. betae* DNA (coefficient of

TABLE 2. (Continued from previous page)

Species	Location	Strain	Host
<i>D. americana</i>	United States	Pey20	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>D. americana</i>	United States	14098A	<i>P. lunatus</i>
<i>D. americana</i>	United States	14099DD	<i>P. lunatus</i>
<i>Phoma betae</i> ^a	...	CBS 109410	<i>B. vulgaris</i>
<i>P. betae</i>	Great Britain	IMI 156653	<i>Beta</i> sp.
<i>P. betae</i>	New Zealand	ICMP 10945	<i>B. vulgaris</i>
<i>P. betae</i>	United States	DN1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	DN5	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	ICMP 22602; Pb1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	JH-TG1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	JH-TG10	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	MF1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	MF2	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	MFG1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	MFG2	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	MFG3	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Pb2	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Pb3	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Pb4	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb044	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb045	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb103	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb104	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb121	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb145	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb146	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb205	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb206	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb228	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb229	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	Phb98	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	RN1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	RN18	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	RN21	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	RN35	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	RN6	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR1	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR10	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR16	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR21	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR32	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR35	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>P. betae</i>	United States	TR6	<i>B. vulgaris</i> subsp. <i>vulgaris</i>
<i>Rhizoctonia solani</i>	United States	GAR16-196	<i>Allium sativum</i>
<i>Sclerotinia sclerotiorum</i>	United States	178	<i>Phaseolus vulgaris</i>
<i>S. sclerotiorum</i>	United States	147	<i>P. vulgaris</i>

variation [CV] = 6%). The mean value ($n = 10$) of *P. betae* DNA in the mixed DNA sample assessed for interassay variability was 2.8 ng of *P. betae* DNA (CV = 10.9%).

Detection of *C. beticola* and *P. betae* DNA from seed. The *C. beticola* and *P. betae* quantitative PCR assays detected their respective DNA targets and were reproducible between replicate samples of each seed lot (Table 3). DNA of *C. beticola* was detected in seed lots 1, 2, 3, and 4, while *P. betae* DNA was detected in seed lots 3, 4, 7, 8, 9, 10, 11, and 12 (Table 3). Assessment of DNA from 60 individual seeds of seed lots 1 and 12 indicated that all seed samples from seed lot 1 had detectable *C. beticola* DNA, whereas no *C. beticola* DNA was detected in seed lot 12.

Pathogen presence and viability on seed. *P. betae* was detected by plate testing in seed lots 1, 2, 4, 5, 9, 10, and 11 (Table 3). The incidence of *P. betae* within these seed lots ranged from 0.5 to 10%. Germination rates were high for each commercial organic seed lot (72 to 94%), whereas seed from the private organic farm exhibited reduced germination for seed lots 3 (9.5%) and 4 (58%) (Table 3).

For observation of *C. beticola*, plating of intact seed from lots 1 to 4 resulted in the growth of multiple fungal species, many of which grew more rapidly than a reference culture of *C. beticola*. Slow-growing colonies were targeted for subculturing and, of 122 isolations from separate seeds, 4 *C. beticola* isolates were confirmed using PCR (3% isolation frequency).

Plating of ground seed from lots 1 to 4 resulted in the growth of numerous colonies of multiple fungal species, with variation observed between seed lots. Seed lots 1 and 2 exhibited multiple slow-growing colonies resembling *C. beticola*, which were subcultured before interference from faster-growing fungi. Seed lots 3 and 4 exhibited a mixture of slow- and fast-growing fungi, with slow-growing colonies overgrown prior to formation of features typical of *C. beticola*. PCR of subcultured isolates confirmed the presence of viable *C. beticola* in seed lots 1 and 2.

BIO-PCR for detection of viable *C. beticola*. Incubating seed in broth resulted in variable biomass consisting of fungal and bacterial components. DNA of *C. beticola* was detected in seed lots 1, 2, and 4 (ranging from 10 to 18.75%; Table 4). No *C. beticola* DNA was detected in biomass grown from seed lots 3, 6, and 12.

Seed-to-seedling transmission of *C. beticola*. CLS symptoms were observed on true leaves of plants from seed lot 1, grown

4 weeks in the glasshouse followed by 4 to 6 weeks in the high-humidity growth chamber. After 4 weeks in high humidity, these plants had begun to lodge and senesce. Control plants from seed lot 12 showed no signs of disease or lodging. Six lesions on individual plants from seed lot 1 were observed to develop pseudostromata and conidia resembling *C. beticola* (Weiland and Koch 2004). Quantitative PCR confirmed the presence of *C. beticola* DNA in each lesion.

In subsequent replicated trials using 8-week-old plants placed in the high-humidity chamber for 6 to 8 weeks, CLS-like symptoms were observed on true leaves of 15 and 12 plants in replicate one and 0 and 12 plants in replicate two from seed lots 1 and 12, respectively. The number of lesions on affected leaves was one or two. After 4 weeks, plants from seed lot 1 had begun to lodge and senesce. Control plants from seed lot 12 showed no signs of lodging. Pseudostromata and conidia were not observed in CLS-like lesions in replicate one. Pseudostromata and conidia were observed in CLS-like lesions on three of the leaves collected from seed lot 12 in replicate two. Quantitative PCR confirmed the presence of *C. beticola* DNA in one of the lesions exhibiting pseudostromata.

DISCUSSION

We believe that this study provides the first direct evidence of *C. beticola* infestation of table beet seed, and demonstrates the initiation of CLS in plants grown from infested seed. Quantitative PCR assays for *P. betae* and *C. beticola* were utilized for assessment of seed infestation, with detection of pathogen DNA supported by isolation of viable *P. betae* and *C. beticola*. The results demonstrate that infested table beet seed may be a means of dissemination for both *P. betae* and *C. beticola* in New York.

P. betae has been well documented as a seedborne pathogen of beet (Byford and Gambogi 1985; Edson 1915; Leach 1946; Pethybridge et al. 2018; Pool and McKay 1915); however, studies reporting isolation of *C. beticola* from seed are limited (McKay and Pool 1918). Traditional methods for isolation of fungi from seed involve incubation and visual identification (Mancini et al. 2016); however, the presence of diverse mycoflora which compete with the fungi of interest can limit detection. The slow growth of *C. beticola* in culture can impede detection (Khan et al. 2008; Vereijssen et al. 2004) and, although *P. betae* forms distinct structures (Mangan

TABLE 3. Detection and quantification of *Cercospora beticola* and *Phoma betae* DNA in 12 table beet seed lots

Seed lot	Germ (%) ^a	Year	<i>Cercospora beticola</i>		<i>Phoma betae</i>		
			C _q ^b	DNA (ng/mg) ^c	C _q ^b	DNA (ng/mg) ^c	Infested (%) ^d
1	80	2018	26.38 ± 0.05	0.67 ± 0.019	— ^e	—	0.5
2	87	2018	28.13 ± 0.03	0.23 ± 0.004	—	—	1
3 ^f	9.5	2016	30.07 ± 0.03	0.07 ± 0.002	28.92 ± 0.03	1.52 ± 0.028	0
4 ^f	58	2018	26.76 ± 0.04	0.53 ± 0.013	28.59 ± 0.04	1.85 ± 0.045	3.5
5	75	2015	—	—	—	—	1
6	94	2015	—	—	—	—	0
7	90	2003	—	—	34.31 ± 0.05 ^g	0.06 ± 0.002 ^g	0
8	92	2015	—	—	34.39 ± 0.06 ^g	0.06 ± 0.002 ^g	0
9	72	2016	—	—	32.54 ± 0.06	0.18 ± 0.006	10
10	85	2016	—	—	31.56 ± 0.11	0.34 ± 0.018	2.7
11	92	2016	—	—	31.07 ± 0.06	0.43 ± 0.016	3.7
12 ^h	88	2017	—	—	34.47 ± 0.05 ^g	0.06 ± 0.002 ^g	n/a

^a Germination was assessed across 200 seeds placed on water agar media after 10 days of incubation.

^b Mean ± standard error of the quantification cycle (C_q) ($n = 54$). Quantitative PCR was run for 35 cycles.

^c Mean ± standard error of pathogen DNA (ng/mg) detected in ground table beet seed tissue ($n = 54$).

^d Seed ($n = 200$) were visually assessed for *P. betae* after incubation for 10 days on water agar media; n/a = not applicable.

^e A dash (—) indicates the absence of a quantification cycle.

^f Seed lots 3 and 4 were produced on a private farm using organic practices.

^g DNA of *P. betae* was not detected in all 54 DNA samples. Mean ± standard error values were calculated only for samples with positive detection. For seed lot 7, $n = 46$; seed lot 8, $n = 7$; and seed lot 12, $n = 33$.

^h The crop producing seed lot 12 was managed using conventional strategies. Treatment of the seed with thiram (42-S Thiram; Bayer CropScience, Leverkusen, Germany), fludioxonil as Maxim (Syngenta, Basel Switzerland), and mefenoxam as Apron XL (Syngenta) precluded the ability to assess seed infestation on water agar.

1971), both were often overgrown by other fungi present on seed in the current study. Species-specific quantitative PCR was used as an alternative detection method for both *P. betae* and *C. beticola* DNA extracted from seed, with positive results providing guidance for further investigation of viability.

Quantitative PCR has been applied for sensitive and specific detection of pathogen DNA on seed in multiple pathosystems (Mancini et al. 2016; Munkvold 2009) but may have several potential limitations. Design of robust species-specific primers requires availability of phylogenetically diverse DNA sequences of both the target and closely related species, and subsequent PCR confirmation of specificity across DNA of a diverse set of species (Knight and Pethybridge in press; Schaad and Frederick 2002). Technical impediments may also occur due to poor-quality DNA, inhibitors from seed extracts, late nonspecific fluorescence, and detection of remnant DNA from nonviable pathogen propagules (Agarwal 2006; Munkvold 2009).

P. betae DNA was detected in extracts from seed and the results were supported by viable *P. betae* in plate tests. Low levels of infestation ($\leq 1\%$) resulted in no or limited detection of *P. betae* DNA, and *P. betae* DNA was also detected in seed where no viable *P. betae* was observed. The discrepancies between DNA detection and plate tests for *P. betae* may be explained by misidentification of Phoma-like fungi on beet seed (Byford and Gambogi 1985; Vaghefi et al. 2016b), the presence of remnant *P. betae* DNA, or differences in the sampling strategy for each method. The age and quality of seed can also affect pathogens, microflora, and their interactions (Neergaard 1973). Seed lots 3 and 4 contained the greatest quantities of *P. betae* DNA but, in plate tests, had nondetectable (0%) and 3.5% estimated *P. betae* incidences, respectively. Seed lots 3 and 4 also exhibited the lowest germination rates (9.5 and 58%, respectively). Decreased viability of the seed may have resulted in a shift in the populations of microflora, which affected the viability, or ability to observe, *P. betae*. These results demonstrate PCR-based detection of *P. betae* DNA in seed, and the potential for this method to complement current plate-based detection methods.

The detection of *C. beticola* DNA and viable *C. beticola* from multiple table beet seed lots supports the potential for *C. beticola* to be disseminated via infested seed. No peer-reviewed literature for the detection of *C. beticola* on beet seed has been reported since McKay and Pool (1918) observed the fungus on sugar beet seed over a century ago. However, extension factsheets frequently describe *C. beticola* as being seedborne (Jones and Windels 1991; Navazio et al. 2010; Reiners et al. 2017). A major limiting factor for the detection of *C. beticola* on seed has been the absence of an effective detection method. The slow growth of *C. beticola* compared with other fungi, along with the lack of a selective medium, has limited the ability of traditional methods to detect *C. beticola* (Khan et al. 2008; Vereijssen et al. 2004). The current study utilized several methods to detect *C. beticola* on seed, including quantitative PCR, plating methods, BIO-PCR, and disease initiation assessment.

Quantitative PCR successfully detected *C. beticola* DNA on four seed lots; however, pathogen viability cannot be confirmed with this technique. Plating of whole seeds resulted in detection of *C. beticola* at low frequency (3%). Plating of ground seed allowed multiple colonies of *C. beticola* to be observed in seed lots 1 and 2; however, no *C. beticola* colonies were observed in seed lots 3 and 4. The condition and age of seed at the time of testing may affect the frequency and viability of fungi on the seed (Neergaard 1973), suggesting that the reduced germination and, potentially, the on-farm storage conditions of these seed lots may have affected the viability of *C. beticola*. A gradual decline in the ability of *C. beticola* to sporulate on stored seed has been reported and associated with death of mycelia and conidia; however, conidia were still produced after 2 years of storage of heavily infested seed (Aebi and Rapin 1954; Wenzl 1959). BIO-PCR (Munkvold 2009; Phan et al. 2002; Schaad et al. 1995) supported the presence of viable *C. beticola* in three of six seed lots. However, late quantification cycles indicated that competition between *C. beticola* and other microbes may have reduced the ability to detect viable *C. beticola*. Because the location of *C. beticola* associated with the infested seed is currently unknown, surface sterilization was not attempted. Further optimization and integration of the methods, including development of a selective medium, surface sterilization, and grinding of seed, may improve the accuracy and precision of detection.

The presence of CLS symptoms on plants grown from infested seed provides further evidence of the potential role of infested table beet seed as a primary inoculum source, and supports the observation of CLS on plants grown from seed lots 1 and 2 in fields where the disease was not previously observed (M. McGrath, personal communication). Initiation of CLS from *C. beticola*-infested sugar beet seed was similarly demonstrated by McKay and Pool (1918). Further support for the association of CLS and table beet seed comes from seed lots 3 and 4, grown on a private, organic mixed-cropping farm in a closed production system. Seed from this farm, described by Knight et al. (2018) as Farm 1, was implicated in the low genetic differentiation of *C. beticola* populations between

TABLE 4. Detection of *Cercospora beticola* DNA in biomass grown in CV8 broth from individual seeds of six table beet seed lots

Seed lot	<i>C. beticola</i> DNA detection (%) ^a
1	18.75
2	10
3	0
4	10
6	0
12	0

^a A single seed was placed in 50 ml of CV8 broth ($n = 20$) for 7 days for seed lots 2, 3, 4, 6, and 12. Forty-eight seeds were individually assessed for seed lot 1. Seeds were removed prior to DNA extraction. Detection was positive when a quantification cycle occurred before 35 cycles.

Fig. 1. Alignment of Pb_R2 primer and probe sequences against partial RNA polymerase II subunit sequences (87 bp) of six fungal species. Polymorphisms between *Phoma betae* and the other species sequences are indicated by shading (white text on black indicates >3, black text on gray indicates 2 to 3, and white text on gray indicates 1 polymorphism). GenBank accession numbers for RNA polymerase II subunit sequences were *P. betae* (GU371774), *Neocamarosporium obiones* (KC584503), *Pleospora calvescens* (KC584500), *P. herbarum* (DQ247794), *P. typhicola* (KC584505), and *Cercospora beticola* (KT216555). *Phoma betae* (GU371774) was used for base pair reference positions.

years. This contrasted with another farm (Farm 2) which used two different seed sources between years, and exhibited significant genetic differentiation of *C. beticola* populations. Seed planted at Farm 2 was not available in the current study; however, the genetic similarity of the Farm 2 *C. beticola* population to European *C. beticola* populations suggested long-distance movement of seedborne inoculum (Knight et al. 2019a; Vaghefi et al. 2017a). Limited information regarding table beet seed sources and movement is available (Knight et al. 2019a).

The detection of *C. beticola* on seed may have major implications for disease management. Critical questions remain regarding the initiation of CLS on plants grown from infested seed and the minimal amount of *C. beticola* required to initiate a CLS epidemic. The polycyclic nature of CLS (Weiland and Koch 2004) suggests that relatively few instances of disease initiation from infested seed may result in a severe CLS epidemic in a susceptible crop under favorable environmental conditions. Seed and plant health are also factors to consider during disease observation. In the growth chamber, plants grown from seed lot 1 exhibited lodging and senescence, while plants from seed lot 12 did not exhibit these symptoms. The vigor of the plants may have been affected by chemical treatment of seed lot 12 (Khan et al. 1992), seed quality, and different genetic backgrounds. The environmental conditions required for CLS development from seedborne inoculum require further investigation.

The presence of *C. beticola* and *P. betae* on seed highlights the importance of effective seed treatment options, especially in organic table beet production systems (Koenick et al. 2019a; Pethybridge et al. 2018). Seed treatments such as thiram are applied for conventional management of *P. betae*; however, approved organic seed treatments are not currently available for *P. betae* on table beet seed (Pethybridge et al. 2018). In addition, although seed treatment such as soaking in formaldehyde (McKay and Pool 1918) may reduce *C. beticola* inoculum and the potential for CLS initiation, the efficacy of modern synthetic fungicide seed treatments is unknown.

ACKNOWLEDGMENTS

We thank C. Bowden, A. Klein, and S. Murphy for excellent technical support; and the seed companies and growers involved in this study.

LITERATURE CITED

- Aebi, H., and Rapin, J. 1954. Control of cercosporiosis of sugar beet. Rev. Appl. Mycol. 35:62-63 [English summary].
- Agarwal, P. C., Dev, U., Rani, I., and Khetarpal, R. K. 2006. Seed-borne fungi detected in sugar beet seeds imported into India during last three decades. Plant Health Prog. 7. doi:10.1094/PHP-2006-1211-01-RS
- Agarwal, V. K. 2006. Seed Health. International Book Distributing Company, Lucknow, India.
- Ariyawansa, H. A., Thambugala, K. M., Manamgoda, D. S., Jayawardena, R., Camporesi, E., Boonmee, S., Wanasinghe, D. N., Phookamsak, R., Hongsanan, S., Singtripop, C., Chukeatiro, E., Kang, J.-C., Jones, E. B. G., and Hyde, K. D. 2015. Towards a natural classification and backbone tree for *Pleiosporaceae*. Fungal Divers. 71:85-139.
- Bugbee, W. M. 1974. A selective medium for the isolation and enumeration of *Phoma betae* in soil and seed. Phytopathology 64:706-708.
- Bugbee, W. M., and Soine, O. C. 1974. Survival of *Phoma betae* in soil. Phytopathology 64:1258-1260.
- Byford, W., and Gambogi, P. 1985. *Phoma* and other fungi on beet seed. Trans. Br. Mycol. Soc. 84:21-28.
- Carneiro, G. A., Matic, S., Ortu, G., Garibaldi, A., Spadaro, D., and Gullino, M. L. 2017. Development and validation of a Taqman real-time PCR assay for the specific detection and quantification of *Fusarium fujikuroi* in rice plants and seeds. Phytopathology 107:885-892.
- Chen, Y. Y., Conner, R. L., Gillard, C. L., McLaren, D. L., Boland, G. J., Balasubramanian, P. M., Stasolla, C., Zhou, Q. X., Hwang, S. F., Chang, K. F., and Babcock, C. 2013. A quantitative real-time PCR assay for detection of *Colletotrichum lindemuthianum* in navy bean seeds. Plant Pathol. 62:900-907.
- Chilvers, M. I., du Toit, L. J., Akamatsu, H., and Peever, T. L. 2007. A real-time, quantitative PCR seed assay for *Botrytis* spp. that cause neck rot of onion. Plant Dis. 91:599-608.
- De Coninck, B. M. A., Amand, O., Delauré, S. L., Lucas, S., Hias, N., Weyens, G., Mathys, J., De Bruyne, E., and Cammue, B. P. A. 2012. The use of digital image analysis and real-time PCR fine-tunes bioassays for quantification of *Cercospora* leaf spot disease in sugar beet breeding. Plant Pathol. 61:76-84.
- de Gruyter, J., Woudenberg, J. H. C., Aveskamp, M. M., Verkley, G. J. M., Groenewald, J. Z., and Crous, P. W. 2013. Redisposition of phoma-like anamorphs in *Pleiosporales*. Stud. Mycol. 75:1-36.
- Duressa, D., Rauscher, G., Koike, S. T., Mou, B., Hayes, R. J., Maruthachalam, K., Subbarao, K. V., and Klosterman, S. J. 2012. A real-time PCR assay for detection and quantification of *Verticillium dahliae* in spinach seed. Phytopathology 102:443-451.
- Edson, H. A. 1915. Seedling diseases of sugar beets and their relation to root-rot and crown-rot. J. Agric. Res. 4:135-168.
- Guillemette, T., Iacomini-Vasilescu, B., and Simoneau, P. 2004. Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. Plant Dis. 88:490-496.
- Harveson, R. M. 2009. Phoma leaf spot. Pages 11-12 in: Compendium of Beet Diseases and Pests. R. M. Harveson, L. E. Hanson, and G. L. Hein, eds. American Phytopathological Society, St. Paul, MN, U.S.A.
- Jacobsen, B. J., and Franc, G. D. 2009. *Cercospora* leaf spot. Pages 7-10 in: Compendium of Beet Diseases and Pests. R. M. Harveson, L. E. Hanson, and G. L. Hein, eds. American Phytopathological Society, St. Paul, MN, U.S.A.
- Jones, R. K., and Windels, C. E. 1991. A Management Model for *Cercospora* Leaf Spot of Sugarbeets. AG-FO-5643-E. Minnesota Extension Service, University of Minnesota, St. Paul, MN, U.S.A.
- Katoh, K., Misawa, K., Kuma, K. i., and Miyata, T. 2002. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30:3059-3066.
- Katoh, K., and Standley, D. M. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Mol. Biol. Evol. 30:772-780.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., and Drummond, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647-1649.
- Khan, A. A., Abawi, G. S., and Maguire, J. D. 1992. Integrating matricconditioning and fungicidal treatment of table beet seed to improve stand establishment and yield. Crop Sci. 32:231-237.
- Khan, J., Rio, L. E., Nelson, R., Rivera-Varas, V., Secor, G. A., and Khan, M. F. R. 2008. Survival, dispersal, and primary infection site for *Cercospora beticola* in sugar beet. Plant Dis. 92:741-745.
- Knight, N. L., and Pethybridge, S. J. An improved PCR assay for species-specific detection and quantification of *Cercospora beticola*. Can. J. Plant Pathol. In press. doi.org/10.1080/07060661.2019.1621380
- Knight, N. L., Vaghefi, N., Hansen, Z. R., Kikkert, J. R., and Pethybridge, S. J. 2018. Temporal genetic differentiation of *Cercospora beticola* populations in New York table beet fields. Plant Dis. 102:2074-2082.
- Knight, N. L., Vaghefi, N., Kikkert, J. R., Bolton, M. D., Secor, G. A., Rivera, V. N., Hanson, L. E., Nelson, S., and Pethybridge, S. J. 2019a. Genetic diversity and structure in regional *Cercospora beticola* populations from *Beta vulgaris* ssp. *vulgaris* suggest two clusters of separate origin. Phytopathology 109:1280-1292.
- Knight, N. L., Vaghefi, N., Kikkert, J. R., and Pethybridge, S. J. 2019b. Alternative hosts of *Cercospora beticola* in field surveys and inoculation trials. Plant Dis. 103:1983-1990.
- Koenick, L. B., Kikkert, J. R., and Pethybridge, S. J. 2019a. Phoma leaf spot susceptibility and horticultural characteristics of table beet cultivars in New York. Plant Health Prog. 20:95-103.
- Koenick, L. B., Vaghefi, N., Knight, N. L., du Toit, L. J., and Pethybridge, S. J. 2019b. Genetic diversity and differentiation in *Phoma betae* populations on table beet in New York and Washington states. Plant Dis. 103:1487-1497.
- Koike, S. T., du Toit, L. J., and Abawi, G. S. 2010. *Cercospora* leaf spot of vegetable crops in the Chenopodiaceae. Pages 91-96 in: *Cercospora* Leaf Spot of Sugar Beet and Related Species. R. T. Larter, J. J. Weiland, L. Panella, P. W. Crous, and C. E. Windels, eds. American Phytopathological Society, St. Paul, MN, U.S.A.
- Koressa, T., and Remm, M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23:1289-1291.
- Larter, R. T., Weiland, J. J., Caesar-TonThat, T., and Bucklin-Comiskey, S. 2003. PCR protocol for rapid detection of *Cercospora beticola* in sugarbeet tissues. J. Sugar Beet Res. 40:1-10.
- Leach, L. 1946. Seed-borne *Phoma* and its relation to the origin of sugar beet seed lots. Proc. Am. Soc. Sugar Beet Technol. 4:381-388.
- Leach, L., and MacDonald, J. 1976. Seed-borne *Phoma betae* as influenced by area of sugar beet production, seed processing and fungicidal seed treatments. J. Am. Soc. Sugar Beet Technol. 19:4-15.

- Mancini, V., Murolo, S., and Romanazzi, G. 2016. Diagnostic methods for detecting fungal pathogens on vegetable seeds. *Plant Pathol.* 65:691-703.
- Mangan, A. 1971. A new method for the detection of *Pleospora bjoerlingii* infection of sugar beet seed. *Trans. Br. Mycol. Soc.* 57:169-172.
- Maude, R. B., Vizor, A. S., and Shuring, C. G. 1969. The control of fungal seed-borne diseases by means of a thiram seed soak. *Ann. Appl. Biol.* 64: 245-257.
- McKay, M. B., and Pool, V. W. 1918. Field studies of *Cercospora beticola*. *Phytopathology* 8:119-136.
- Miller, P. M. 1955. V-8 juice agar as a general purpose medium for fungi and bacteria. *Phytopathology* 45:461-462.
- Monte, E., and García-Acha, I. 1988. Vegetative and reproductive structures of *Phoma betae* in vitro. *Trans. Br. Mycol. Soc.* 90:233-245.
- Moretti, M., Karaoglanidis, G., Saracchi, M., Fontana, A., and Farina, G. 2006. Analysis of genotypic diversity in *Cercospora beticola* Sacc. field isolates. *Ann. Microbiol.* 56:215-221.
- Munkvold, G. P. 2009. Seed pathology progress in academia and industry. *Annu. Rev. Phytopathol.* 47:285-311.
- Navazio, J., Colley, M., and Zyskowski, J. 2010. Principles and Practices of Organic Beet Seed Production in the Pacific Northwest. Organic Seed Alliance, Port Townsend, WA, U.S.A.
- Neergaard, P. 1973. Detection of seed-borne pathogens by culture tests. *Seed Sci. Technol.* 1:217-254.
- Noble, M., and Richardson, M. J. 1968. An Annotated List of Seed-Borne Diseases. Commonwealth Mycological Institute, Surrey, England.
- Pellegrino, C., Gilardi, G., Gullino, M. L., and Garibaldi, A. 2010. Detection of *Phoma valerianellae* in lamb's lettuce seeds. *Phytoparasitica* 38:159-165.
- Pethybridge, S., Kikkert, J., Hanson, L., and Nelson, S. 2018. Challenges and prospects for building resilient disease management strategies and tactics for the New York table beet industry. *Agronomy (Basel)* 8:112.
- Pethybridge, S. J., Vaghefi, N., and Kikkert, J. R. 2017. Management of *Cercospora* leaf spot in conventional and organic table beet production. *Plant Dis.* 101:1642-1651.
- Petruska, J., Goodman, M. F., Boosalis, M. S., Sowers, L. C., Cheong, C., and Tinoco, I. 1988. Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc. Natl. Acad. Sci. U.S.A.* 85:6252-6256.
- Phan, H. T. T., Ford, R., Bretag, T., and Taylor, P. W. J. 2002. A rapid and sensitive polymerase chain reaction (PCR) assay for detection of *Ascochyta rabiei*, the cause of ascochyta blight of chickpea. *Australas. Plant Pathol.* 31:31-39.
- Pool, V. W., and McKay, M. 1915. *Phoma betae* on the leaves of the sugar beet. *J. Agric. Res.* 4:169-177.
- Pool, V. W., and McKay, M. 1916. Climatic conditions as related to *Cercospora beticola*. *J. Agric. Res.* 6:21-60.
- Reiners, S., Bellinder, R. R., Curtis, P. D., Helms, M., Landers, A. J., McGrath, M. T., Nault, B. A., and Seaman, A. 2017. Pages 114-121 in: Beets. Cornell Integrated Crop and Pest Management Guidelines for Commercial Vegetable Production Cornell Cooperative Extension, Ithaca, NY, U.S.A.
- Schaad, N. W., Cheong, S., Tamaki, S., Hatziloukas, E., and Panopoulos, N. J. 1995. A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. *Phytopathology* 85:243-246.
- Schaad, N. W., and Frederick, R. D. 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Can. J. Plant Pathol.* 24:250-258.
- Secor, G. A., and Rivera, V. V. 2012. Fungicide resistance assays for fungal plant pathogens. Pages 385-392 in: *Plant Fungal Pathogens. Methods in Molecular Biology (Methods and Protocols)*, Vol. 835. M. D. Bolton and B. P. H. J. Thomma, eds. Humana Press, Totowa, NJ, U.S.A.
- Solel, Z. 1970. Survival of *Cercospora beticola*, the causal agent of sugar beet leaf spot, in Israel. *Trans. Br. Mycol. Soc.* 54:504-506.
- Srivastava, S. N. 2004. Management of sugarbeet diseases. Pages 307-355 in: *Fruit and Vegetable Diseases*. K. G. Mukerji, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Strausbaugh, C. A., Neher, O., Rearick, E., and Eujayl, I. A. 2015. Influence of harvest timing, fungicides, and beet necrotic yellow vein virus on sugar beet storage. *Plant Dis.* 99:1296-1309.
- Turgay, E. B., Bakir, M., Ozeren, P., Katircioglu, Y. Z., and Maden, S. 2010. Detection of pathotypes and genetic diversity of *Cercospora beticola*. *Plant Pathol. J.* 26:306-312.
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., and Rozen, S. G. 2012. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* 40:e115.
- Vaghefi, N., Hay, F. S., Kikkert, J. R., and Pethybridge, S. J. 2016a. Genotypic diversity and resistance to azoxystrobin of *Cercospora beticola* on processing table beet in New York. *Plant Dis.* 100:1466-1473.
- Vaghefi, N., Kikkert, J. R., Bolton, M. D., Hanson, L. E., Secor, G. A., Nelson, S. C., and Pethybridge, S. J. 2017a. Global genotype flow in *Cercospora beticola* populations confirmed through genotyping-by-sequencing. *PLoS One* 12:e0186488.
- Vaghefi, N., Kikkert, J. R., and Pethybridge, S. J. 2016b. Incidence and pathogenicity of *Didymella americana* on table beet in New York. *Plant Health Prog.* 17:270-272.
- Vaghefi, N., Nelson, S. C., Kikkert, J. R., and Pethybridge, S. J. 2017b. Genetic structure of *Cercospora beticola* populations on *Beta vulgaris* in New York and Hawaii. *Sci. Rep.* 7:1726.
- Vaghefi, N., Silva, A., Koenick, L. B., and Pethybridge, S. J. 2019. Genome resource for *Neocamarosporium betae* (syn. *Pleospora betae*), the cause of Phoma leaf spot and root rot on *Beta vulgaris*. *Mol. Plant-Microbe Interact.* 32:787-789.
- Vereijssen, J., Schneider, H. J. H. M., and Termorshuizen, A. A. J. 2004. Possible root infection of *Cercospora beticola* in sugar beet. *Eur. J. Plant Pathol.* 110:103-106.
- Větrovský, T., Kolařík, M., Žifčáková, L., Zelenka, T., and Baldrian, P. 2016. The *rpb2* gene represents a viable alternative molecular marker for the analysis of environmental fungal communities. *Mol. Ecol. Resour.* 16:388-401.
- Wanasinghe, D. N., Hyde, K. D., Jeewon, R., Crous, P. W., Wijayawardene, N. N., Jones, E. B. G., Bhat, D. J., Phillips, A. J. L., Groenewald, J. Z., Dayaratne, M. C., Phukhamsakda, C., Thambugala, K. M., Bulgakov, T. S., Camporesi, E., Gafforov, Y. S., Mortimer, P. E., and Karunarathna, S. C. 2017. Phylogenetic revision of *Camarosporium* (*Pleosporineae*, *Dothideomycetes*) and allied genera. *Stud. Mycol.* 87:207-256.
- Weiland, J., and Koch, G. 2004. Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). *Mol. Plant Pathol.* 5:157-166.
- Weiland, J. J., and Sundsbak, J. L. 2000. Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rRNA gene. *Plant Dis.* 84:475-482.
- Wenzl, H. 1959. The importance of infection of beet seed by *C. beticola* and its control. *Rev. Appl. Mycol.* 39:360 [English summary].