A PCR Protocol for Rapid Detection of *Cercospora beticola* in Sugarbeet Tissues

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ABSTRACT

Cercospora leaf spot, caused by *Cercospora beticola*. Sacc. is the most important foliar disease of sugarbeet (*Beta vulgaris* L.), yet the progression of infection from soil to diseased leaves remains incompletely understood. A sensitive method for detection of *C. beticola* on disease-free plants could be used to determine how early in the growing season sugarbeet tissues are colonized by the fungus and to what extent asymptomatic weeds and non-beet crops may harbor the fungus. We present an Extract-N-Amp Plant PCR Kit (Sigma)-based protocol for rapid detection and identification of *C. beticola* in sugarbeet tissues. Leaf disks from field-sampled diseased tissues or disease-free greenhouse plants were homogenized and diluted with manufacturer-provided extraction and dilution solutions. Without further DNA purification, aliquots of the homogenate were added to PCR reactions and amplified using the *Cercospora* actin gene specific and ITS region based primers. Fragment sizes of the amplified products correlated with the size of that amplified from DNA extracted from *C. beticola* cultures. Sequence comparison of the amplified products confirmed them to be from *C. beticola*. The protocol will enable rapid detection and identification of *C. beticola* in asymptomatic and diseased sugarbeet, in alternate hosts and soil debris that harbor the fungus.

Additional Key Words: *Beta vulgaris*, extraction, fungus, DNA

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Cercospora leaf spot (Fig. 1A) is prevalent wherever sugarbeet (*Beta vulgaris* L.) is grown (Bleiholder and Weltzien, 1972). Heavy pressure from the disease, which is caused by *Cercospora beticola* Sacc., results in significant loss in root weight and reduction of recoverable sugar in sugarbeet (Smith and Ruppel, 1973). The pathogen survives on infected beet residue as stromata. Under optimal disease conditions, characterized by high relative humidity, heavy dew or wet conditions, conidiophores and conidia are produced on the stromata. Based on several decades of research, the current model of *C. beticola* infection indicates that conidiophores and conidia that serve as primary inoculum are dispersed by wind, irrigation, rain water and insects to sugarbeet to initiate primary infection (McKay and Pool, 1918; Ruppel, 1986; Windels *et al.*, 1998).

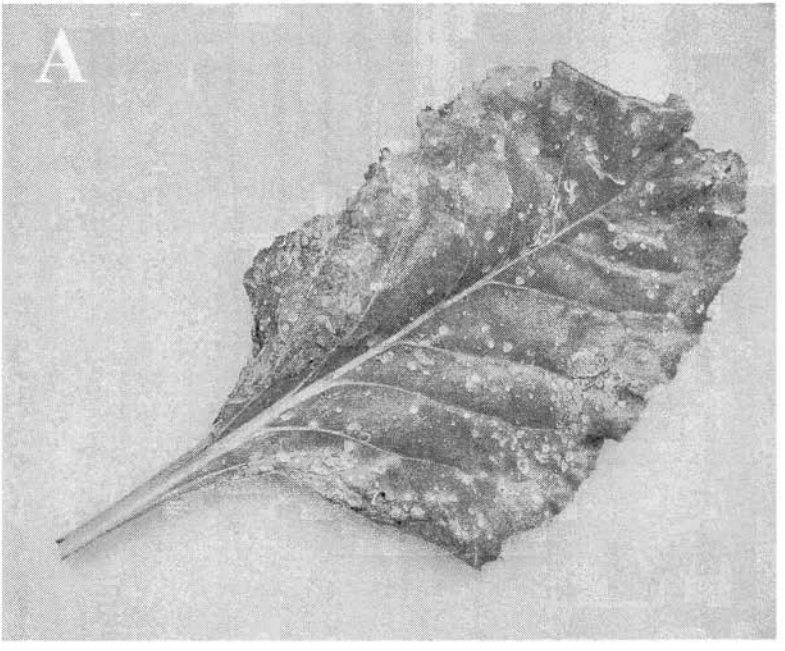

Fig. 1A: *Cercospora* infected sugarbeet leaf. Although lacking the characteristic red halo around the lesions, the disease shown was positively identified as Cercospora leaf spot.

Rapid detection of disease and accurate identification of the causal agent is necessary for the development of an effective control system. With the aid of a light microscope, the presence of conidiophores and conidia in lesions can be used to identify Cercospora leaf spot.

However in the absence of conidiophores and conidia in the lesion, Cercospora leaf spot could be confused with spots produced by *Phoma betae* Frank and *Ramularia beticola* Fautrey and Lambotte (Whitney and Duffus, 1986) and leaf blotches caused by abiotic factors or bacteria (Wolf and Vereet, 2002). Weiland and Sundsbak (2000) recently developed a PCR protocol for identification and differentiation of several fungal pathogens of sugarbeet including *C. beticola.* Although a protocol was outlined in that work for the detection of *Aphanomyces cochlioides* Drechsler in diseased sugarbeet seedlings, the plant extraction procedure reported was laborious and no reference to its use for the detection of *C. beticola* was presented. We present here a PCR protocol for the detection and identification of *C. beticola* that utilizes a simple and rapid extraction system from sugarbeet leaf tissue.

MATERIALS AND METHODS

Leaf and fungal samples

Leaves showing symptoms of Cercospora leaf spot were harvested from sugarbeet fields in the Sidney, MT area. Controls consisted of greenhouse-grown uninfected sugarbeet leaves, potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) cultures of *C. beticola* isolates C1 and C2 and total DNA extracts from *C. beticola* cultures. For PDA cultures, mycelial discs were transferred to fresh PDA plates and incubated at 22°C for 3 weeks.

DNA Primers

Two sets of primers were designed with Primer Premier (Premier Biosoft International, Palo Alto, CA) from the *Cercospora* actin gene sequence in GenBank (Lartey and Weiland, Accession # AF443281). The primer set CBACTIN915L (5' GTAAGTGCTGCCACAATCAGAC 3') and CBACTIN915R (5' TACCATGACGATGTTTCCGTAG 3') was designed to amplify an approximately 915 bp fragment. The other set CBACTIN959L (5' AGCACAGTATCATGATTGGTATGG 3') and CBACTIN959R (5' CACTGATCCAGACGGAGTACTTG 3') was designed to amplify an approximately 959 bp fragment of the *C. beticola* actin gene sequence. In addition, the primers ITS1 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' (Weiland and Sundsbak, 2000) were used.

Rapid generation of PCR Templates

Templates were prepared using a modification of the manufacturer's protocol for Extract-N-Amp Plant PCR Kits (Sigma

Chemical Co., St. Louis, MO). Leaf disks (0.6 cm in diameter) from lesions of *C. beticola* infected sugarbeet leaves (Fig. 1A) and from healthy control leaves (Fig. 1B) were homogenized in 100 µl extraction solution and incubated at 95°C for 10 min. Dilution solution (100 µl) was added to the reaction, vortex-mixed and stored at 4°C until ready to use. Samples from control fungal cultures were treated similarly but were rinsed in deionized water to remove excess agar and air-dried prior to homogenization.

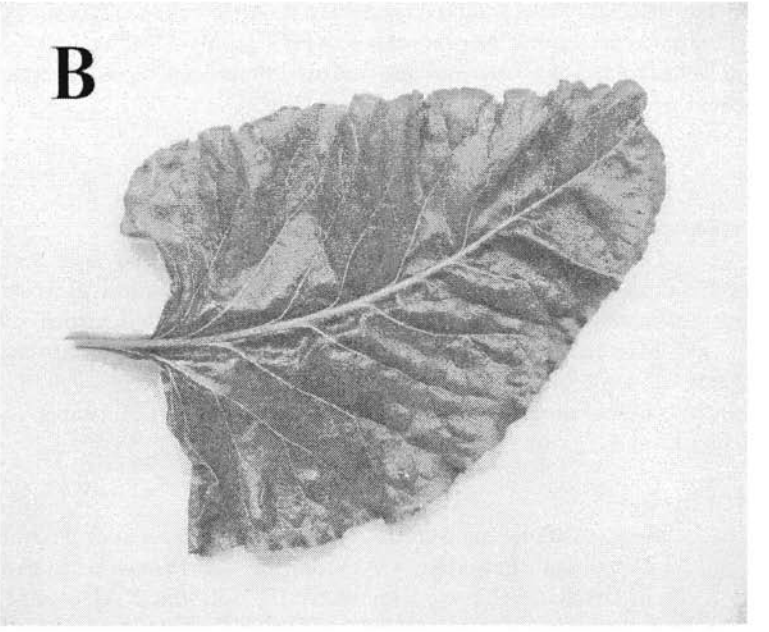

Fig. 1B: Healthy sugarbeet leaf.

To evaluate the potential of the protocol, two-year-old freeze dried-sugarbeet leaves (suspected to be infected with *C. beticola*) were tested. The old leaf samples were treated just as the fresh *C. beticola* infected leaf tissues.

Total genomic DNA was extracted from PDA cultures of *C. beticola* as described by Thon *et al.*(2000) for the DNA control template. Harvested mycelia were lyophilized and ground to powder. The mycelia powder was mixed with CTAB extraction solution and incubated at 65°C for 1 hr. The samples were extracted with chloroform. The DNA was precipitated from the upper phase with isopropanol, and washed with

70% ethanol. The DNA pellets were dried and resuspended in Extract-N-Amp Plant PCR extraction and dilution solutions.

PCR Amplification

The 20 µl PCR reaction consisted of 10 µl Extract-N-Amp PCR mix (a 2X PCR reaction mix containing buffer, salts, dNTPs, Taq polymerase and TaqStart antibody), 4 µl sample extraction solution and 1.5 µl each of the forward and reverse primers in deionized water. Other controls were a manufacturer provided control and a blank reaction, consisting of extraction solution without plant or fungal extract. Amplification was carried out over 35 cycles using a Mastercycler gradient thermocycler (Eppendorf Scientific Inc., Westbury, NY) at 94°C for 1 min denaturation, 52°C for 30 sec annealing and 72°C for 1 min extension. The PCR amplified products were resolved by electrophoresis in 1% agarose gels in Loening E buffer (Loening, 1969). The PCR product sizes were determined by comparing the relative mobility of the amplified fragments to the 1 KB ladder (New England Biolabs Inc., Beverly, MA) in adjacent lanes.

Cloning and Sequencing

PCR amplified fragments were cloned into pCR 2.1-TOPO plasmid vectors using the TOPO Cloning Kit (Invitrogen Corp., Carlsbad, CA) as described by the manufacturer. The cloning reaction consisted of 2 µl fresh PCR product, and 1 µl TOPO vector. The reaction volume was adjusted with deionized water to 6 µl, mixed gently and incubated for 5 min at room temperature (22 to 23°C). The ligated products were used to transform TOP10 One Shot Electrocomp *E. coli* using Electroporator 2510 (Eppendorf Scientific Inc., Westbury, NY) and cultured on LB amp plates (Difco, Becton, Dickinson & Co. Sparks, MD). Selected bacteria colonies were then cultured in LB broth (Difco) overnight at 37°C. The plasmid DNA was purified using the QIAprep mini kit and protocol as described by the manufacturer (QIAGEN Inc., Valencia, CA).

Dye terminator cycle sequencing was carried out on the plasmid DNA extracts using the CEQ DTCS Quick Start kit (Beckman Coulter, Fullerton, CA) for comparison of the amplified fragment with the *Cercospora* actin gene. The sequencing reaction consisted of 50 fmol of purified PCR products in deionized water, 1.6 µM CBACTIN959L or CBACTIN959R sequencing primer in water and 12 µL of DTCS premix. The reaction mixture was adjusted to 20 µl with deionized water. A control reaction consisted of a pUC18-based template with M13-47 sequencing primer, both provided by the manufacturer. The mixture was subjected

to thermal cycling at 90°C for 20 sec, 50°C for 20 sec and 60°C for 4 min for 30 cycles and was terminated with 4 µl of stop solution and 1 µl of 20 mg/ml glycogen. The reaction was subjected to ethanol precipitation and suspended in 40 µl of sample loading solution and sequenced with a Beckman Coulter CEQ 2000XL DNA Analysis System (Beckman Coulter, Inc., Fullerton, CA). Results were imported into the Vector NTI (InforMax, Bethesda, MD) and aligned to compare sequences with the *Cercospora* actin sequence from GenBank (Accession # AF443281).

RESULTS

Results of the amplification are presented in Fig 2. Of the two

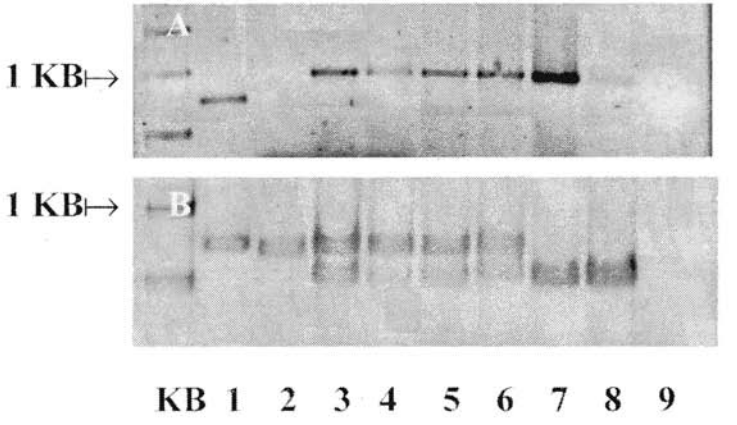

Fig. 2. PCR amplified fragments of *Cercospora beticola* genome. Fig.2A The fragments of the *C. beticola* genome were amplified with actin specific primers. Fig. 2B The fragments of *C. beticola* genome were amplified with ITS1 and ITS4 primers. KB = 1KB Ladder: 1=Manufacturer's control; 2=Uninfected sugarbeet leaf; 3 through 6= Infected sugarbeet leaves without *Cercospora* isolation, culture and genomic DNA extraction; 7 = Genomic DNA extract from fungal culture; 8=Fungal culture without standard genomic DNA extraction and 9= Control blank.

primers sets that were tested, the CBACTIN959L and CBACTIN959R set provided consistent amplification of the *C. beticola* actin gene segment (Fig. 2A). The expected fragment was about 1 kbp. Regardless of the source of the template; direct from infected sugarbeet tissues (lanes 3 through 6), DNA extract from fungal culture (lane 7) or direct from fungal cultures (lane 8); a fragment of the expected size was amplified. No

amplification was observed from the uninfected control sugarbeet leaves or the blank (lanes 2 and 9 respectively). An expected amplified fragment of 0.7 kbp also was observed from the manufacturer's control (lane 1).

Results of the amplification using the ITS-based primer pair are presented in Fig 2B. Two fragments were amplified from each tissue sample from the infected plant tissues (lanes 3 through 6). The upper fragments of about 0.7 kbp correspond to plant ITS fragments from uninfected the control (lane 2). The lower fragments of about 0.6 kbp characterize the expected fragments from the *C. beticola* ITS region (Weiland and Sundsbak, 2000). The lower fragment corresponds in size with amplified fragments from the DNA extract from fungal culture (lane 7) and direct amplified fragment from fungal colony (lane 8). No amplification was obtained from the blank control. An expected, amplified fragment of 0.7 kbp also was obtained from the manufacturer's control (lane 1).

The resolved DNA sequences from the cloned fragments were aligned with the *C. beticola* actin sequence from GenBank to validate that the PCR amplified fragments with CBACTIN959L and CBACTIN959R primers were indeed the encoded the actin gene. The sequences that were generated from the infected plant tissue and pure *C. beticola* culture showed 99.8% homology to that *C. beticola* actin sequence from GenBank (results not shown). The high level of homology between the sequences indicates that the amplified products from the infected plant lesions were indeed, those of *C. beticola.*

The results of the amplification from diseased twenty-two month old dried sugarbeet leaf tissues are presented in Fig. 3. Using

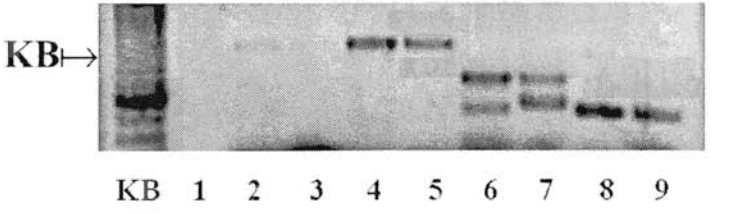

Fig. 3. PCR amplified fragments from twenty-two month old *Cercospora* infected sugarbeet leaves. Lanes 2 through 5 were amplified using actin primers and 6 through 9 using ITS1 and ITS4 primers. 100bp = 100 bp ladder; 1= Control blank; 2, 3, 6 and 7= Infected old leaves; 4 and 8 = Genomic DNA extract from fungal culture; 5 and 9=Fungal culture without genomic DNA purification.

CBACTIN959L and CBACTIN959R primers, the expected 1 kbp actin fragments were amplified (lanes 2 and 3). The fragments corresponded in size to fragments from genomic DNA extract from fungal culture and fungal culture without genomic DNA purification, respectively (lanes 4 and 8). The ITS1 and ITS4 primers also amplified the expected 0.7 kbp fragments (lanes 6 and 7), which also corresponded in size to fragments from genomic DNA extract from fungal culture and fungal culture without genomic DNA purification, respectively (lanes 5 and 9). No amplification was observed with the blank control (lane 1).

DISCUSSION

We present in this research a protocol for rapid detection and identification of *C. beticola*, the causal agent of Cercospora leaf spot of sugarbeet, without laborious manipulation of the sample. Indeed, without culture of fungal propagules and genomic DNA extraction, we were able to amplify unique fragments of the *C. beticola* genome from infected tissue. Alignment of the actin-fragment-derived sequence with the sequences of *C. beticola* actin from GenBank confirmed the fragment to be that of *C. beticola*. This technique also enabled us to detect *C. beticola* in twenty-two month old, dried, infected sugarbeet leaves. The successful amplification of the expected PCR fragments from these old tissues extends the potential use of the protocol beyond fresh plant tissue samples. Verification that any product from PCR amplification is due to the presence of *C. beticola* can be obtained by either restriction and fragment analysis such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) of the PCR product or DNA sequencing of the amplified product. Among these, DNA sequencing remains the most reliable, because it may sometimes be required to resolve verification of the other protocols. The fact that the present work was done using field samples suggests that the test is useful in the initial stages of disease diagnosis. Besides sugarbeet, *C. beticola* produces leaf spots on most *Beta vulgaris* such as red garden beets, Swiss Chard and mangel-wurzel (McKay and Pool, 1918). Several weeds have been described by Vestal (Vestal, 1933) as susceptible to *C. beticola*. This includes common lambsquarters (*Chenopodium album* L.), redroot pigweed (*Amaranthus retroflexus* L.), dwarf mallow (*Malva rotundifolia* L.), broadleaf plantain (*Plantago major* L.), great burdock (*Arctium lappa* L.) and lettuce (*Lactuca sativa* L.). Although additional species have been reported as host to *C. beticola* (Ruppel, 1986), the author cautioned that adequate cross-inoculation tests are needed for verification of those reports. Indeed

in recent years other common weeds such as field bindweed (*Convolvus arvensis* L.), (Windels *et al.*, 1998), winged pigweed (*Cycloloma atriplicifolium* (Spreng.) Coult.), wild buckwheat (*Polygonum convolvulus* L.) and devil's-claw (*Proboscidea louisianica* (Mill.) Thellung) (Jacobsen, 2000) have been named as hosts of *C. beticola.* Clearly these host plants could be a serious reservoir of inoculum able to maintain the organism through long periods in the absence of sugarbeet (Forsyth *et al.*, 1963). Even where crop rotation is a standard practice, severe incidence of Cercospora leaf spot has been observed under optimal environmental conditions and additional preventive measures such as application of fungicides may be required to prevent severe economic loss. The occurrence of severe disease incidence suggests the presence of other, yet-to-be-identified secondary hosts that may be important sources of inoculum. This may include other crops and weeds, some of which may not show symptoms in the field but still serve as a reservoir of *C. beticola.* Our protocol also will enable rapid screening, detection and identification of *C. beticola* on these putative hosts, ultimately leading to the development of more effective control strategies against Cercospora leaf spot and provide information about the epidemiology of the fungus.

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