DETECTION OF *CERCOSPORA BETICOLA* BY PCR IN AMENDED AND NATURALLY INFESTED FIELD SOIL

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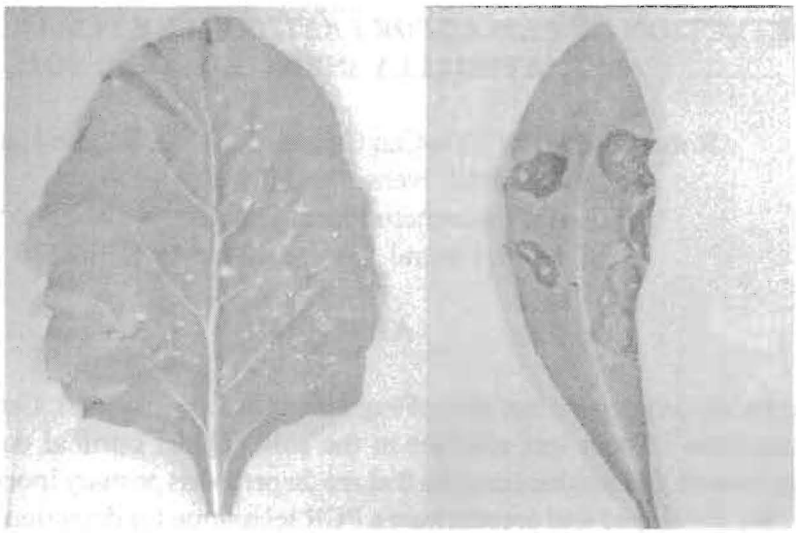
ABSTRACT

The causal agent of Cercospora leaf spot of sugarbeet (*Beta vulgaris* L), *Cercospora beticola*. Sacc. survives as stromata in beet leaf residues in the soil. Under optimal conditions, overwintering propagules germinate and produce conidia that are dispersed as primary inoculum to initiate infection in sugarbeet. We developed and present here a PCR technique for detection of *C. beticola* in the soil. The DNA was purified from soil amended with *C. beticola* and naturally infested soil using PowerSoil DNA Kit (MO BIO Lab. Carlsbad, CA) as per manufacture's instructions. The purified DNA was collected and subjected to PCR reaction in Extract-N-Amp PCR mix (Sigma Aldrich, St Louis MO) with CBACTIN based primers. 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, 72°C for 1 min extension and 5 min final extension at 72°C. The amplified products were resolved by electrophoresis in 1% agarose gels. The fragment sizes of *C. beticola* amended and the infected field soil products correlated with the expected size of the control DNA extracts from *C. beticola* cultures. Amplicons were sequenced and compared to pure culture *C. beticola* actin sequence. Alignment of sequences of the amplified products confirmed them to be those of *C. beticola*. The system will enable rapid post planting screening for inoculum potential of *C. beticola* in soil and determine the effect of soil applied biocontrol agents on *C. beticola* and inoculum potential.

Introduction

Sugarbeet (*Beta vulgaris* L.) and safflower (*Carthamus tinctorius* L.) are important crops in the Lower Yellowstone River Valley where sugarbeet is also rotated with irrigated safflower. Both crops are susceptible to *Cercospora beticola* Sacc., which causes Cercospora leaf spot (CLS) of sugarbeet (Fig. 1a) and leaf spot of Cercospora (LSC) on safflower (Fig. 1b). The pathogen overwinters on infected beet residue as stromata (Windels et al, 1998). Under optimal conditions, characterized by relatively high humidity or heavy dew, conidiophores and conidia are produced on the stromata (Ruppel, 1986). The conidiophores and conidia that serve as primary inoculum are dispersed by wind, irrigation and rain water and insects to sugarbeet to initiate primary infection. Thus the disease could be transmitted from post harvest inoculum reserve to the other crop in the following growing season. Currently, a method is available for direct detection and identification *C. beticola* in infected plant tissues (Lartey et al, 2003). Other plant hosts can therefore be detected and identified. As an additional step toward completely tracking headway of the pathogen in the field, we have developed and present here a protocol for detection of *C. beticola* in soil.



Figures 1. *Cercospora beticola* infected sugarbeet (A) and safflower (B)

Materials and Methods

Soil Samples

The test samples consisted of *C. beticola* amended field soil and soils from two sugarbeet fields under different sprinkler irrigation at Sidney, Montana. Additionally, a soil sample from Florida, which had not been exposed to sugarbeet, was also used as a control for development of the protocol.

DNA Templates

DNA was purified from soil samples with using The PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA) as per manufacturer's instructions. In manufacturer provided PowerBead Tubes, 0.25 gm of each test soil sample was added and gently vortex mixed. 60µl of the Solution C1 was then added to each soil sample, first vortex mixed briefly and then at maximum speed for additional 10 minutes. The PowerBead Tubes containing the samples then were centrifuged for 30 seconds at 10,000 x *g* at room temperature. The supernatant (400 to 500µl) was then transferred to manufacturer provided clean 2 ml collection tubes. 250µl of Solution C2 was added to each sample, vortex mixed for 5 seconds, incubated at 4°C for 5 minutes and centrifuged at room temperature for 1 minute at 10,000 x *g*. Up to 600µl of supernatant were then carefully transferred to a clean 2 ml collection tube. 200µl of Solution C3 was then added to each sample in the tubes, vortex mixed briefly and incubated at 4°C for 5 minutes. The tubes were then centrifuged at room temperature for 1 min at 10,000 x *g*. About 750µl of supernatant were then carefully transferred into clean 2 ml collection tubes and 1200µl of Solution C4 was added to the samples. The tubes were then vortex mixed for 5 seconds. Approximately 675µl of the sample were loaded onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. The filtrate was discarded and an additional 675µl of supernatant was transferred to the spin filter and centrifuged for 1 minute at room temperature at 10,000 x *g*. After discarding the filtrate, the remaining supernatant was transferred

onto the Spin Filter and centrifuge at 10,000 x *g* for another 1 minute at room temperature. The pellets were washed by transferring 500µl of Solution C5 into the tubes and centrifuging at room temperature for 30 seconds at 10,000 x *g*. The filtrate was discarded and centrifuged again at room temperature for an additional 1 minute at 10,000 x *g*. The Spin Filters were then carefully transferred to clean 2 ml Collection Tubes. The purified DNA was finally eluted with 100µl of Solution C6 that was added to the center of the white filter membrane and centrifuged at room temperature for 30 seconds at 10,000 x *g* and stored at -20° until ready to use.

PCR Amplification

The CBACTIN959 primer set from Cercospora actin gene (Lartey et al, 2003) was used for the PCR amplification. The primer set CBACTIN959L (5' AGCACAGTATCATGATTGGTATGG 3') and CBACTIN959R (5' CACTGATCCAGACGGAGTACTTG 3') was designed to amplify about 959 bp DNA fragment of *C. beticola* actin gene sequence. In addition to *C. beticola* amended soil, samples from the two field soils under MESA and LEPA irrigation and Florida which were subjected to soil kit extraction protocol were also subjected to PCR amplification. Additional controls were from purified DNA from pure *C. beticola* culture and blank reactions mixture. Amplifications were carried out using Extract-N-Amp PCR mix (SigmaAldrich, St Louis MO), as described by Lartey et al (2003). The 20 μl PCR reactions consisted of 10 μl Extract-N-Amp PCR mix, 4 μl PowerSoil™ DNA Isolation Kit based DNA extract, 1.5 μM each of the forward and reverse primers and deionized water. The 35 cycle amplification was carried out using Master gradient Thermal Cycler (Eppendorf Scientific Inc., Westbury, NY) at 94°C for 1 minute denaturation, 52°C for 30 seconds annealing and 72°C for 1 minute extension. The PCR products were electrophoresed through 1% agarose gels in Loening E buffer (Loening, 1969). The PCR product sizes were determined by comparing fragment mobilities to 1 KB ladder in adjacent lanes.

Sequencing and sequence comparison

The PCR fragments were excised from the gel, and the DNA fragments were purified with QIAquick Gel Extraction Kit (QIAGEN Inc. Valencia, CA) following the manufacturer's protocol. The DNA fragments were primed with CBACTIN959L and then subjected to dye terminator cycle sequencing using the CEQ DTCS Quick Start kit (Beckman Coulter, Fullerton, CA) as previously described by Lartey et al. (2005). The sequences were exported into the Vector NTI (Invitrogen Corp, Carlsbad, CA) and aligned for comparison.

Results and Discussion

Results of the PCR amplification of *C. beticola* from amended and natural soil are presented in Fig. 2. The *C. beticola* CBACTIN959 actin primers amplified the expected segments from all purified DNA samples from the tested soil (lanes 4-6). These samples, lane 4 from *C. beticola* amended soil and from sugarbeet fields lanes 5 and 6 correspond with the amplified DNA fragment from purified pure *C. beticola* culture in lane 3. On the contrary, amplification was not observed in the control blank lane 2 nor in the sample from the Friod research station.

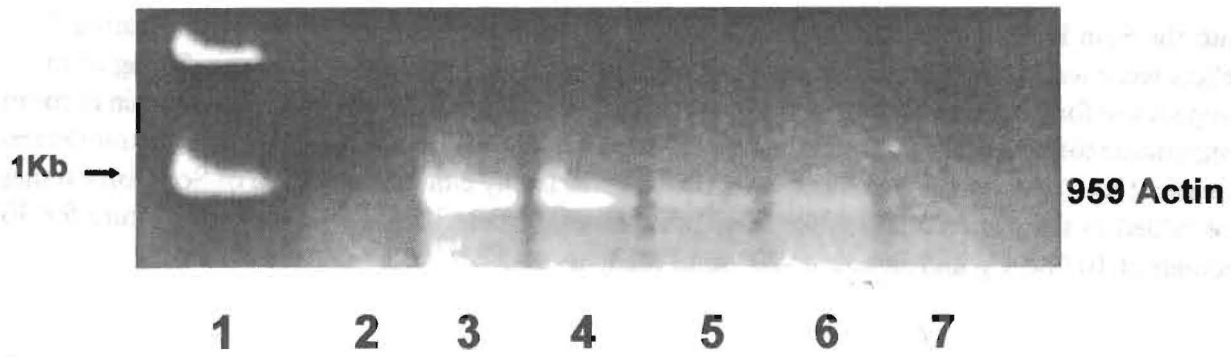
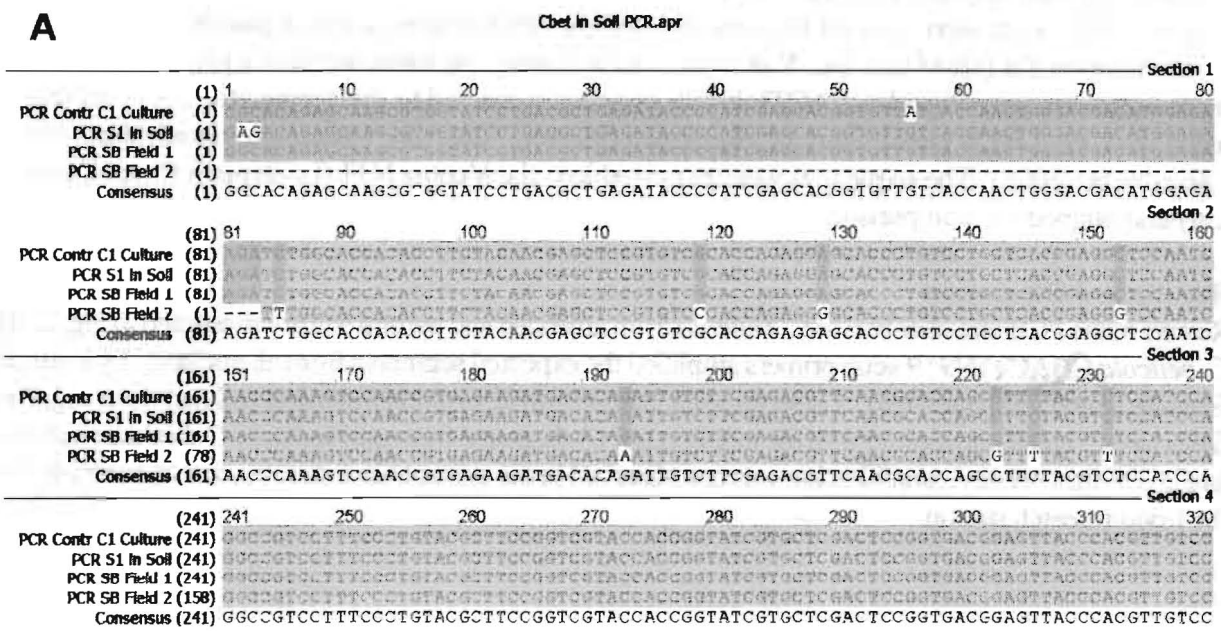
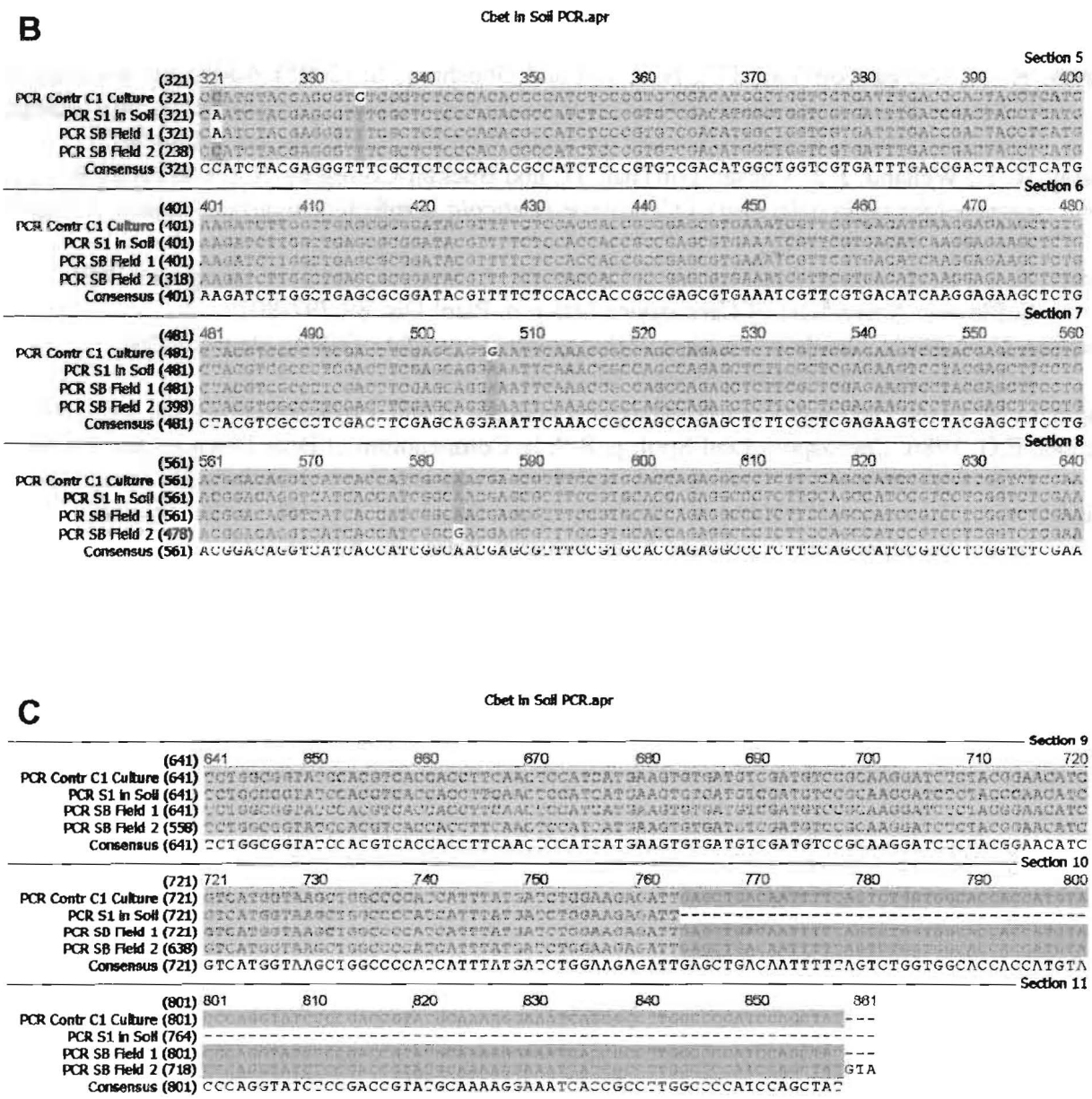


Figure 2. PCR detection of *Cercospora beticola* in amended natural field soils. Lane: 1=KB Ladder; 2=Blank control; 3=Control C2 culture; 4= Amended field soil; 5= Sugarbeet field soil 1; 6= Sugarbeet field soil 2; 7= Control field soil from Florida

Amplified DNA fragments were purified with QIAquick Gel Extraction Kit (QUIAGEN, Inc.) and subjected to fluorescent cycle sequencing using the Beckman Coulter CEQ 2000XL DNA Analysis System (Beckman Coulter). Comparison of the sequences using Vector NTI sequence analysis software is presented in Fig 3. The comparison of the sequences of amplicons from pure *C. beticola* culture, soil amended with *C. beticola* and two sugarbeet fields provided over 98.6 % sequence homology among the samples. Thus, providing evidence that the fragments were all from *C. beticola.*

Figure 3. Comparison of sequenced *C. beticola* actin gene amplicons from purified soil DNA samples. Samples were from *C. beticola* C1 Culture, S1 in Soil and two sugarbeet fields.





In this research, we have developed and presented a protocol for detection of *C. beticola* in field soils. Even where crop rotation is a standard practice as in most growing areas, severe incidence of Cercospora leaf spot has been observed under optimal environmental conditions. Additional control measures such as application of fungicides may therefore be required to prevent severe economic loss. These observations further suggest the presence of ample inoculum reservoir, most likely from soil where the pathogen has been reported to survive for up to 27 months (Nagel, 1938). Our protocol should enable rapid evaluation of field soil prior to planting for inoculum potential in the subsequent course of the growing season. The information together with eventual weather conditions should help in designing effective control strategy against *C. beticola*.

Literature Cited

Lartey, R. T., Caesar-TonThat, TC., N. I. Sol and Ghoshroy, S. (2005) Additional evidence of Safflower (*Carthamus tinctorius*) as an alternate host of *Cercospora beticola.* Proceedings, Biennial Meeting, American Society for Sugar Beet Technologists. pp. 144-150.

Lartey, R. T., Weiland, J. J., Caesar-TonThat, TC and Bucklin-Comiskey, S. (2003) A PCR protocol for rapid detection of *Cercospora beticola* in infected sugarbeet tissues. J. Sugar Beet Res. 40:1-10

Lartey, R. T., T. C. Caesar-TonThat, A. J. Caesar, W. L. Shelver, and J. W. Bergman. 2005. Safflower: A new host of *Cercospora beticola.* Plant Dis. 89:797-801.

Loening, U. E. 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide gel-electrophoresis. Biochem. J. 113:131-13

Nagel, C. A. 1938. The longevity of *Cercospora beticola* in soil. Phytopathology 28: 342-350.

Ruppel, E.G. 1986. Cercospora Leaf Spot. p. 8-9. In Compendium of Beet Diseases and Insects. E.D. Whitney and J.E. Duffus, eds. American Phytopathological Society: St. Paul, MN

Windels, C.E., Lamey, A.H., Hilde, H., Widner, J., and Knudsen, T. 1998. A Cercospora leaf spot model for sugar beet, in practice by an industry. Plant Dis. 82:716-725.