Additional evidence of Safflower (*Carthamus tinctorius*) as an alternate host to *Cercospora beticola*

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ABSTRACT

In the Northern Great Plains (NGP), safflower (*Carthamus tinctorius* L.) is increasingly being evaluated for rotation with sugarbeet (*Beta vulgaris* L). Safflower and sugarbeet are susceptible to two different species of Cercospora, *Cercospora carthami* and *Cercospora beticola* respectively. This investigation of safflower as an alternate host of *C. beticola* was prompted by observation of unusual spot lesions on safflower in the NGP at Sidney, Montana. Previous report from our laboratory indicated that safflower has potential to serve as an alternate host of *C. beticola*. We present in this report additional evidence that safflower indeed is an alternate host of *C. beticola*. Safflower plants were infected with four *C. beticola* isolates (C1, C2, Sid1 and Sid2). Sugarbeets were inoculated single spore cultures of the four isolates from infected safflower lesions. Lesions of the symptoms were assayed by PCR for presence of C. beticola. Amplified PCR products were sequenced, imported into the Vector NTI (InforMax, Bethesda, MD) and aligned to compare with the *C. beticola* sequence from GenBank (Accession # AF443281). The aligned sequences from all four isolates from safflower and sugarbeet showed significant homology with sequence from *C. beticola*. Our results confirm the presence of *C. beticola* in lesions of infected safflower and substantiate safflower as a host of *C. beticola*.

INTRODUCTION

Sugarbeet (*Beta vulgaris* L) is one of the most important irrigated crops in the Northern Great Plains (NGP) including Western North Dakota and Eastern Montana. Irrigated safflower (*Carthamus tinctorius* L.) an annual, broadleaf oilseed crop is increasingly being evaluated for rotation with sugarbeet at Sidney, Montana in the NGP (Fig.1) and the two crops are occasionally grown adjacent to each other (Figure 1B). Both crops are hosts of different species of *Cercospora*. Safflower is a host of *Cercospora carthami* Sundar and Ramakr while sugarbeet is susceptible to *Cercospora beticola* Sacc. Cercospora leaf spot, caused by *C. beticola* is one of the most important diseases of sugarbeet and occurs wherever the crop is grown (Bleiholder and Weltzien 1972). Without control, the disease results in significant root yield loss, reduced sugar content of sugarbeets, sugar extraction and root storage life (Smith and Ruppel 1971; Shane and Teng 1992). According to Shane and Teng (1992) gross losses to Cercospora leaf spot can be as high as 30%. Cercospora leaf spot of safflower caused by *C. carthami* was first reported in India in 1924 where epidemics occurred in some years (Ashri 1971). He cited several references that indicate that the disease has been observed only in the old world (Africa, Asia and Europe) with specific incidences in Pakistan, Iran, Israel and India (Mündel and Huang 2003). Symptoms are characterized by round to irregular slightly sunken brown black spots up to 1 cm diameter with occasional yellowing tinge at the border. Stromata of the pathogen appear as small black dots in

Figure 1A. A safflower field at Sidney, Montana. **B.** Sugarbeet growing adjacent to safflower at Sidney, Montana

concentric rings on diseased leaves. In severe cases, the infected leaves turn brown, became distorted and eventually disintegrate. The stem may blacken at the base of severely infected leaves and bending of the stem or die back may result from severe infection (Holdeman and McCartney, *personal communication*). Cercospora leaf spot of safflower by *C. carthami* has not been observed previously in the NGP or even in the United States (Mortensen and Bergman 1983; Mortensen et al. 1983). On the other hand Cercospora leaf spot of sugarbeet is well established in the NGP and there have been a significant increased disease incidence of in the region. The observation of unusual brown necrotic spots on safflower in the NGP followed by our previous report of safflower as a potential alternate host of *C. beticola* (Lartey et al. 2004), prompted this study to provide further evidence that safflower is an alternate host of *C. beticola.*

MATERIALS AND METHODS

Pathogenicity tests: Four isolates of *C. beticola* were evaluated for cross infection by pathogenicity tests in safflower and sugarbeet. The isolates race C1 and C2 (Whitney and Lewellen 1976) were provided by John J. Weiland, ARS, Fargo, North Dakota. Sid1 and Sid2 were isolated from infected sugarbeet at Sidney, Montana by Anthony J. Caesar, ARS/NPARL, Sidney, MT.

For safflower, inoculum spores of each of the 4 *C. beticola* isolates were produced on low sodium V-8 agar plates at 25°C, harvested after 5 to 10 days and suspended in sterile water containing 0.1 % Tween 20 as previously described in Lartey et al (2003). Each spore suspension was adjusted to 2 x 10^4 spore/ml of water and used to spray-inoculated 6 safflower plants cv Centennial (Bergman et al. 2001) with an Atomizer (Sunrise medical HHG Inc., Somerset, PA) at 6-8 leaf stage.

For sugarbeet, spores of all four test isolates of *C. beticola* were isolated from spot symptoms of safflower leaves by incubating infected leaves on wet paper towels in a closed plastic container at 25°C overnight. The spores then were harvested and transferred to PDA from which mycelial plugs were next transferred to V-8 agar to produce inoculum spores as previously described. Each spore suspension was then used to inoculate 6 sugarbeet plants as previously described for safflower. All the inoculated plants were incubated under 90% minimum relative

humidity (RH) and 8 h photoperiod at 32 °C for 2-3 days. Plants then were transferred to and maintained in growth chamber with *ca* 60 % RH, 8 h photoperiod and 26 °C. Untreated controls consisted of uninoculated plants which were maintained under the same conditions. All plants were observed over a period of six weeks for development of symptoms.

PCR assay for *C. beticola* infection in safflower and sugarbeet: A PCR protocol (Lartey et al. 2003) was used to examine lesions and cultures of spores from the lesions of safflower and sugarbeet plants for presence of *C. beticola*. Templates were prepared by mildly homogenizing sample leaf disks and air dried cultures of spores (0.6 cm diameter) from lesions of diseases leaves in 100 µl extraction solution of Extract N-Amp Plant PCR Kits (Sigma Chemical Co. St. Louis, MO). The homogenates were incubated at 95°C for 10 minutes followed by addition of 100 µl dilution solution. The reaction was then vortex-mixed and stored at 4°C.

The 20 µl PCR, reaction mixture consisted of the 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, 1.5 µM each of the forward and reverse primers in deionized water. The reactions were primed with CBACTIN959L (5' AGCACAGTATCATGATTGGTATGG 3') and CBACTIN959R (5' CACTGATCCAGACGGAGTACTTG 3'), which were designed to amplify about 959 bp fragment of *C. beticola* actin gene sequence. Controls were prepared DNA extracts of cultures of the original *C. beticola* isolates and uninoculated safflower and sugarbeet leaves. Amplifications were carried out over 40 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. All the above experiments were repeated at least twice.

The 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.

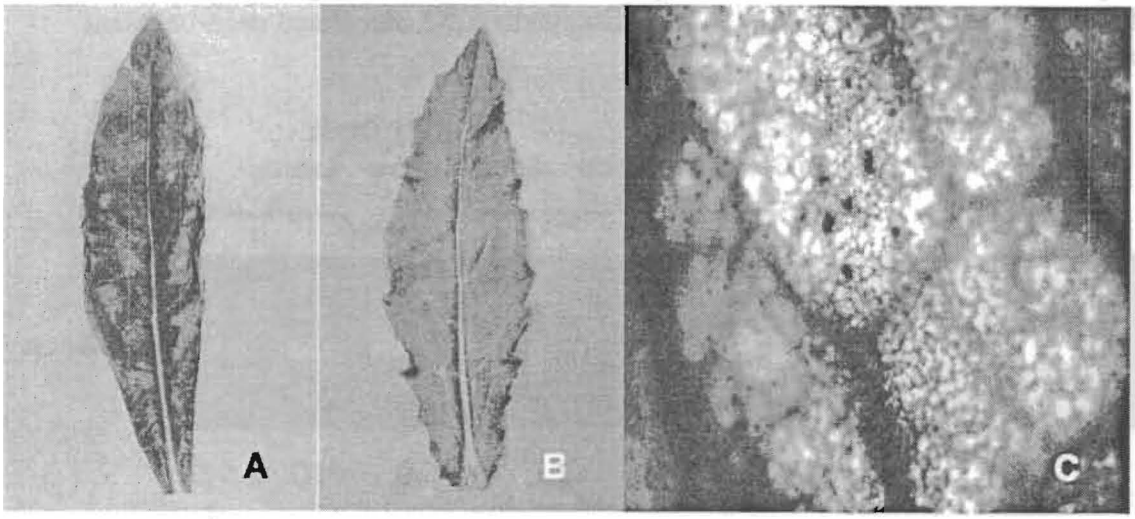
Sequencing and alignment: The PCR fragments were cleaned with QIAquick (QIAGEN Inc.) following the manufacturer's protocol. The fragments were then subjected to dye terminator cycle sequencing using the CEQ DTCS Quick Start kit (Beckman Coulter, Fullerton, CA) for comparison of the amplified fragment with the *Cercospora* actin gene. The sequencing reaction consisted of 50 fmol of the 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. The reaction 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 for stop solution and 1 µl of 20 mg/ml glycogen. The reaction was precipitated with ethanol and suspended in 40 µl of sample loading solution followed by sequencing with Beckman Coulter CEQ 2000XL DNA Analysis System (Beckman Coulter). Results were imported into the Vector NTI (InforMax, Bethesda, MD) and aligned to compare the sequences with *Cercospora* actin sequence from GenBank (Access # AF443281).

RESULTS AND DISCUSSIONS

Between 2 and 3 weeks after inoculation, the first leaf spot symptoms were observed on the safflower plants which were inoculated with each of the four *C. beticola* isolates (Figures 2A

and 2B). The symptoms appeared round to irregular light brown black spots, frequently with dark brown to black borders. Dark brown to black fructifications of the pathogen could be observed in some of the leaf spot lesions (Figure 2C), with the aid of a dissecting scope or at low magnification (10X) with light microscope

Figure 2. *Cercospora beticola* isolate Sid2 infected safflower. **A.** Safflower leaf showing lesions of *C. beticola* infection. **B.** Uninfected control safflower leaf. **C.** Fruiting bodies in spot lesion of an infected safflower leaf.



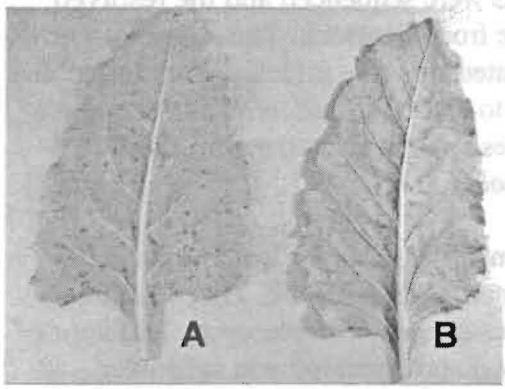


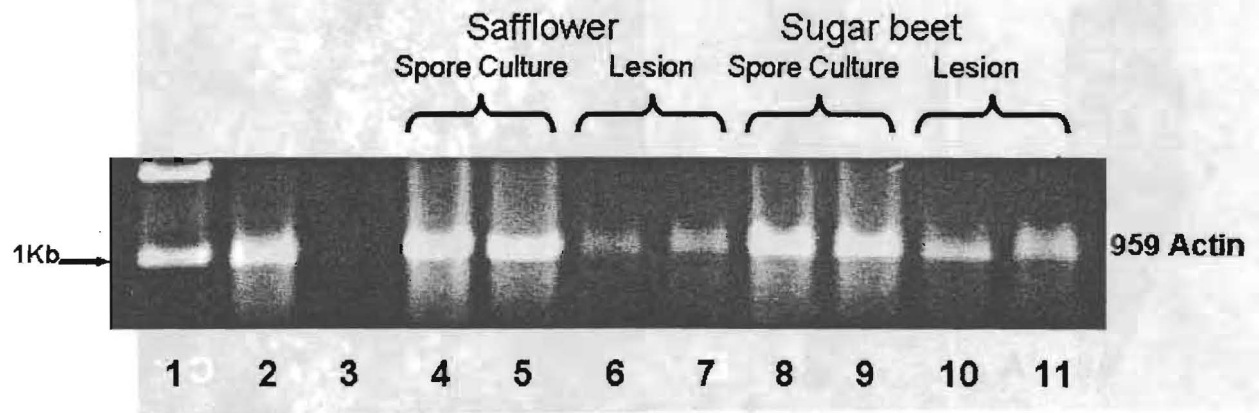
Figure 3. *Cercospora beticola* isolate C2 infected sugarbeet (**A**). Leaves were inoculated with isolated *C. beticola* from infected safflower. **B.** Uninfected control sugarbeet leaf.

For sugarbeet, first symptoms appeared at about 2 weeks post inoculation and were characteristic of Cercospora leaf spot of (Figure 3) Leaf spot symptoms were not respectively observed in the uninfected control safflower and sugarbeet plants (Figures 2B and 3B).

PCR assay for *C. beticola* in safflower and sugarbeet symptom tissues: The results of the PCR-based detection of *C. beticola* in infected safflower and sugarbeet tissues are presented in Figure 4. Expected fragments of all the *C. beticola* isolates (C1, Sid1, C2 and Sid 2) were amplified by the *C. beticola* actin specific primers, CBACTIN959L and CBACTIN959R. Lanes 4, 5, 6 and 7 are PCR fragments from safflower leaf lesion from infection with C2 and S1. The lanes 8, 9, 10 and 11 are fragments from infected sugarbeet. The 959 bp fragments correspond

with the positive control of DNA extract from original C1 pure culture (lane 2). No PCR fragment was observed in the uninfected control safflower sample using the 959 actin primers (lane 3).

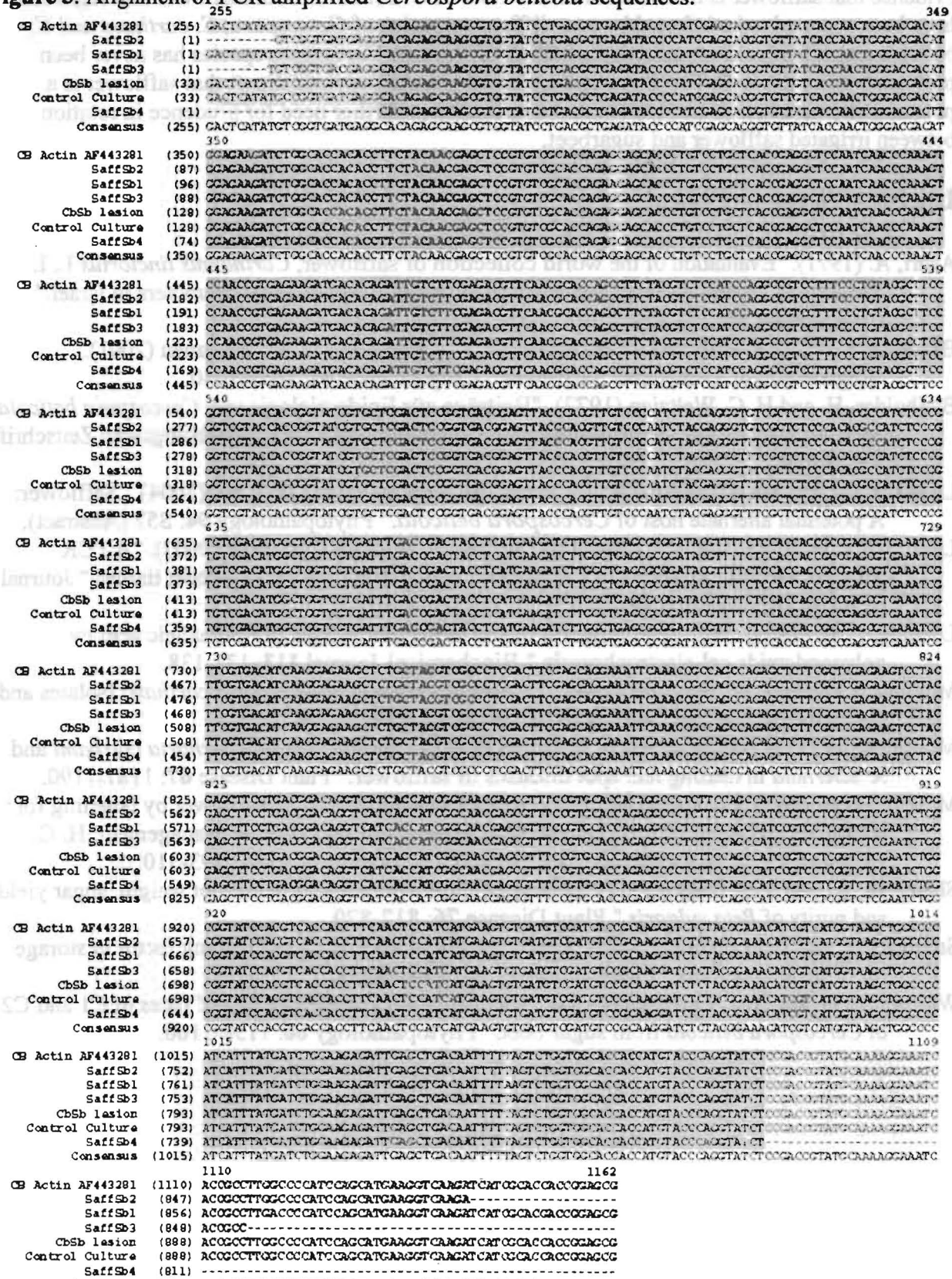
Figure 4. Detection of *Cercospora beticola* in safflower and sugarbeet leaf lesions by PCR. Lane: 1=KB Ladder; 2=Control C2 culture; 3=Control uninoculated safflower; 4= Spore culture from C2 inoculated safflower leaf lesion; 5= Spore culture from S2 inoculated safflower leaf lesion; 6= Amplification from C2 inoculated safflower leaf lesion tissue; 7= Amplification from S2 inoculated safflower leaf lesion tissue; 8= Spore culture from C1 inoculated sugarbeet leaf lesion; 9= Spore culture from S1 inoculated sugarbeet leaf lesion; 10= Amplification from C1 inoculated sugarbeet leaf lesion tissue; 11= Amplification from S1 inoculated sugarbeet leaf lesion tissue



To confirm that the PCR amplified fragments from inoculated safflower and sugarbeet leaf lesions were from *C. beticola*, the amplified PCR fragments were sequenced and the resolved DNA sequence was aligned with *C. beticola* actin sequence from GenBank. The alignment is presented in Figure 5 and showed that the sequences generated from the infected plant tissue and pure *C. beticola* culture were significantly similar (99.8%) to that of *C. beticola* actin sequence from GenBank. This level of similarity among the sequences indicates that the amplified products from the infected plant lesions were from *C. beticola*

We present evidence that the sequences of PCR fragments from *C. beticola* inoculated safflower plants are homologous to *C. beticola* actin sequence from GenBank. The, evidence confirms safflower is an alternate host of *C. beticola,* the causal agent of Cercospora leaf spot of sugarbeet. All four tested isolates of *C. beticola* produced leaf spot symptoms in safflower. Inocula of all four *C. beticola* isolates, which were produced from single spores cultures, reisolated from leaf lesions of diseased safflower caused Cercospora leaf spot in sugarbeet plants. We then amplified segments of *C. beticola* actin gene in the leaf spot lesions of safflower and culture of single spores from the symptoms by PCR. Assay of sugarbeet lesions which were infected with *C. beticola* from safflower leaf lesions by PCR of also indicated the presence of *C. beticola* in the lesions. By sequencing the PCR products and comparing the sequences, we confirmed finally the presence of *C. beticola* in both safflower and sugarbeet lesions. Thus, we were able to show that *C. beticola* can infect safflower under greenhouse conditions and that inoculum from infected safflower can also infect sugarbeet. This observation provides further

Figure 5. Alignment of PCR amplified *Cercospora beticola* sequences.



evidence that safflower is indeed an alternate host of *C. beticola*. Both safflower and sugarbeet have been reported to be infected by two different species of *Cercospora*, *C. carthami* and *C. beticola* respectively. Cross infection of either of the two Cercospora species has never been previously demonstrated. This investigation confirms our previous report that safflower is a potential alternate host of *C. beticola* and demonstrates further need for prudence in rotation between irrigated safflower and sugarbeet.

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