

2007

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Abstract: Sugar beet and safflower are sometimes rotated or grown side by side in the Sidney, MT region of the Lower Yellowstone River Basin (LYRB). *Cercospora beticola* and *C. carthami* infect sugar beet (*Beta vulgaris*) and safflower (*Carthamus tinctorius*) respectively. *C. beticola* is ubiquitous in sugar beet, but *C. carthami* has not been reported in LYRB. Observations of unusual leaf spots on safflower in Sidney led to investigation and subsequent identification of safflower as a host of *C. beticola*. We describe a comparative structural study of progression of *C. beticola* infection and disease development in both sugar beet and safflower. The two crops were manually infected with two isolates of *C. beticola* (C2 and Sid1). Gradual development of the pathogen on the leaf surface and disease symptoms were investigated with scanning electron microscope operated at a variable pressure mode. Some specimens were sputter coated with gold to obtain higher resolution images. Lesions in sugar beet and safflower showed a substantial amount of hyphal mass. A number of stomatal apertures in lesion areas of both host plants and in splits in sugar beet lesions clearly showed protruding hyphae, indicating presence of internalized hyphae after establishment of infection. Substantial hyphal mass developed eventually and covered the lesions of both host plants. Assay of the symptoms by PCR provided evidence for *C. beticola* in the lesions, thus confirming it as the causal agent of the leaf spot of both sugar beet and safflower.

Key words: *Cercospora* leaf spots, *Cercospora carthami*, Electron microscopy, PCR, weed hosts

INTRODUCTION

Sugar beet (*Beta vulgaris*) is an important crop in the Lower Yellowstone River Basin (LYRB) of the United States. The annual oilseed crop, safflower (*C. tinctorius*) also is well adapted to this region and is sometimes rotated with sugar beet. Often, the two crops may be seen growing in adjacent fields. Both have been previously reported susceptible to *Cercospora* Leaf Spots (CLS) with safflower being susceptible to *Cercospora carthami* Sund and Ramak (Dajue and Mündel, 1996) and sugar beet being susceptible to *Cercospora beticola* Sacc. (Whitney and Duffus, 1986). CLS of safflower caused by *C. carthami* was first reported in 1924 in India and has since been reported in other countries in the old world including Iran and Israel (Ashri, 1971; Minz *et al.*, 1961; Patil, 1988; Zad, 1992). Worldwide, *Cercospora* Leaf Spot (CLS) is a major disease of sugar beet (Bleiholder and Weltzien, 1972) that reduces root yield and sugar content, while also increasing sugar impurities and storage losses

(Weiland and Sundsbak, 2000). Gross losses due to CLS can exceed 30%. The disease is well established and occurs frequently in the LYRB. While CLS of sugar beet is common in LYRB, CLS of safflower caused by *C. carthami* has not been previously observed in the LYRB. Therefore, the appearance of unusual spots on safflower leaves in the LYRB prompted investigation to determine the potential of safflower as a possible host of *C. beticola*. Subsequently, we identified and reported safflower as a new host of *C. beticola* (Lartey *et al.*, 2005a, b). This was the first report, to our knowledge, of infection of a member of the Asteraceae by *C. beticola*. Beside *Beta* species, previously reported hosts of *C. beticola* include species of *Atriplex*, *Cycloloma* and *Chenopodium* belonging to the family Chenopodiaceae and *Amaranthus* which belongs to the family Amaranthaceae. Unlike previously reported hosts of *C. beticola*, safflower is not just a common weed, but an important commercial oil and bird seed crop. This follow up report presents a Scanning Electron

Microscopic (SEM) comparison of disease progression of *C. beticola* infection in both safflower and sugar beet.

MATERIALS AND METHODS

Spore production and infection of safflower: *C. beticola* isolate C2 was provided by John J. Weil and ARS, Fargo, ND and Sid2 by Anthony J. Caesar, ARS, Sidney, MT. Inocula were produced on low sodium V-8 agar plates at 26°C under constant light. Spores were harvested between five and ten days and suspended at the concentration of 20,000 spores/mL of sterile water solution containing 0.1% Tween 20 for inoculation. The safflower (cv. Centennial) and sugar beet (cv. Thunder) plants were spray inoculated with an Atomizer (Sunrise Medical HHG Inc., Somerset, PA). The inoculated plants were first incubated under 90% minimum relative humidity (RH) and 8 h photoperiod at 32°C for 3-4 days. The plants were then transferred to and maintained in growth chamber at about 60% RH, 8 h photoperiod and at constant 26°C. Untreated controls were not inoculated. The plants were observed for development of symptoms.

Scanning electron microscopy: After inoculation, sample leaves from infected and uninfected safflower and sugar beet plants were harvested every three days and examined for disease and pathogen development. The freshly harvested leaves were observed under a Hitachi 3200N (Hitachi High Tech., Schaumburg, IL) variable pressure Scanning Electron Microscope (SEM) operated under variable pressure mode. Specimens were observed using a backscattered electron detector. Leaves of infected and uninfected safflower and sugar beet were also fixed in glutaraldehyde, post fixed in osmium tetroxide, dehydrated in ethanol series, critically dried using a Ladd critical point dryer and sputter coated with gold using a Desk II sputter coater (Denton Vacuum, Moorestown NJ) before viewing under SEM. Images were captured digitally using PCI Quartz image acquisition software (Hitachi High

Tech., Schaumburg, IL) and saved as JPEG formatted files in Photoshop and subsequently analyzed.

PCR assay for *C. beticola* infection in safflower and sugar beet: To confirm the presence of the pathogen in the observed lesions of both safflower and sugar beet, diseased leaves were assayed for infection by PCR (Lartey *et al.*, 2003) using *C. beticola* specific primers CBACTIN959L (5' AGCACAGTATCATGAT TGGTATGG 3') and CBACTIN959R (5' CACTGATCCA GACGGAGTACTTG 3') (Lartey *et al.*, 2003), which were designed to amplify a 959 bp fragment of *C. beticola* actin gene and ITS non-specific primers ITS1 5' TCCGTAGGT GAACCTGCGG 3' and ITS4 5' TCCTCCGCTT ATTGATATGC 3' (Weiland and Sundsbak, 2000). For DNA extractions leaf disks (0.6 cm diameter) cut from sample lesion tissues were homogenized in 100 µL N-Amp Plant PCR Kits (Sigma Chemical Co. St. Louis, MO) extraction solution incubated at 95°C for 10 min and diluted to 100 µL vol. The final PCR reaction mixture consisted of 10 µL Extract-N-Amp PCR mix, 4 µL extraction solution, 1.5 µL of each primer and 3 µL of deionized water for a total of 20 µL. Amplification was carried out over 35 cycles. Cycles included 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. Controls consisted of uninoculated plants, manufacturer provided control and a blank mixture. The amplified products were resolved by electrophoresis in 1% agarose gels in Loening E buffer (Loening, 1969). PCR product sizes were determined by comparing the relative mobility of the amplified fragments to 1 KB ladder (New England Biolabs Inc., Beverly, MA) in adjacent lanes.

RESULTS

Symptoms: Two weeks after inoculation, both C2 and Sid2 isolates of *C. beticola* induced spot lesions on safflower and sugar beet (Fig. 1A and B). The lesions were typical

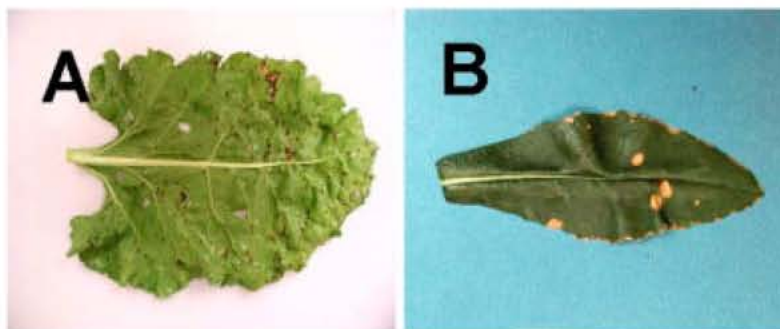


Fig. 1: Lesions of *Cercospora beticola* infection in sugar beet leaf (A) and safflower leaf (B)

of previously reported *C. beticola* spot symptoms in sugar beet (Weiland and Koch, 2004) and safflower (Lartey *et al.*, 2005). No lesions were observed on the uninoculated controls of either sugar beet or safflower.

Ultrastructural observations: Inoculum consisting of single spores was observed randomly distributed on the sprayed leaf surface, including guard cells (Fig. 2A). After growth on the leaf surface, hyphal strands were observed entering into stomatal openings of safflower (Fig. 2B). Entry of *C. beticola* through stomatal opening did not

appear to follow a particular pattern as some hyphal strands grew over some stomatal openings without apparent entry.

Two types of symptoms were observed. In both cases, the visual symptoms were characterized as typical spots. However, the SEM examination of the surface of the lesions in sugar beet exhibited deep splits (Fig. 3A). These splits were not observed in lesions of safflower tissues (Fig. 3B). No sign of the pathogen was observed in the early stages of lesion development which appeared first after about two weeks post inoculation. Further

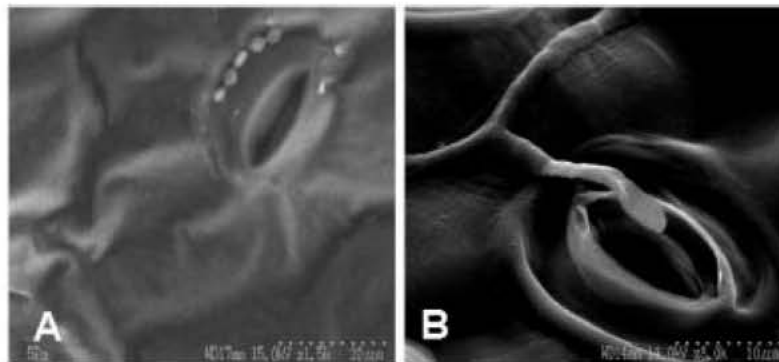


Fig. 2: Progression of *C. beticola* infection in sugar beet and safflower. A. Inoculum of *C. beticola* on guard cells of sugar beet. B. Entry of *C. beticola* through stomatal opening of a safflower leaf

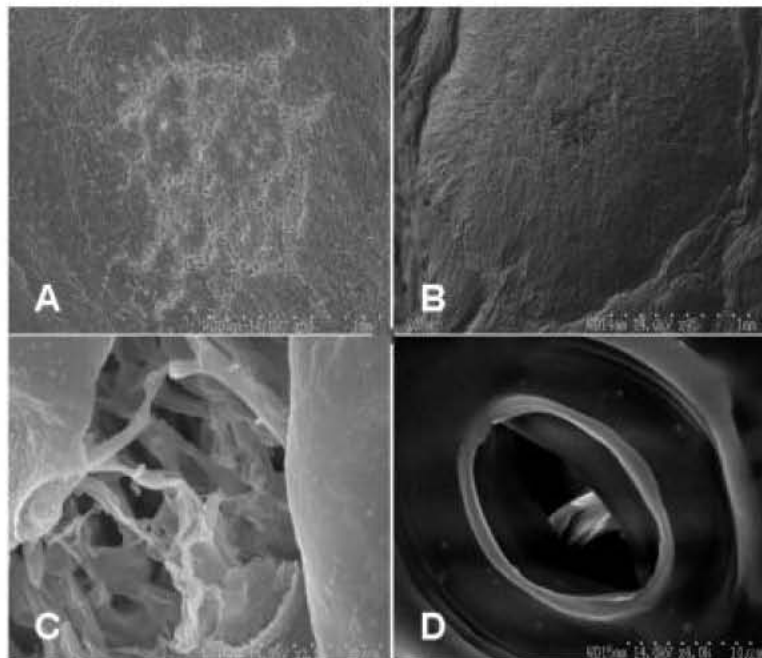


Fig. 3: Developed lesions of Cercospora Leaf Spot after infection of sugar beet and safflower with *C. beticola*. A. Leaf spot lesion induced by *C. beticola* in sugar beet A) and safflower B) (Notice splits in sugar beet lesion (arrows), but absent in safflower lesion. C) Mycelial mass of *C. beticola* in lesion tissues of sugar beet seen through splits in the lesions and D. Hypha of *C. beticola* in a stomatal opening of safflower lesion tissues

examination of the splits within the lesion tissues of sugar beet revealed dense hyphal mass (Fig. 3C). While splits were not observed in safflower lesions, hyphae of *C. beticola* were observed in stomatal openings of safflower lesion tissues (Fig. 3D).

Emerging hyphal structures of *C. beticola* were observed from lesions of sugar beet and safflower (Fig. 4). In addition to stomatal openings, emerging young hyphae were also observed piercing through tissues near and away from the stomatal openings within the sugar beet lesion tissues (Fig. 4A and 4C). Emergence of the pathogen from safflower was only through stomatal openings (Fig. 4B and D). Eventually, the emerged pathogen appeared as hyphal mass in both safflower and sugar beet (Fig. 5A and B, respectively).

PCR assay for *C. beticola* in sugar beet and safflower lesions:

The results of the PCR-based detection of *C. beticola* in sugar beet and safflower tissues are presented in Fig. 6. Control standards for the PCR detection include uninfected safflower (lanes 2 and 3), uninfected sugar beet (lanes 4 and 5) DNA extracts from C2 cultures (lanes 6 and 7) and DNA extracts from Sid2 cultures (lanes 8 and 9). No amplification was observed in lanes 2 and 4 of the safflower and sugar beet, respectively by the actin primers, CBACTIN959L and CBACTIN959R. However, ITS fragments of about 0.7 kbp were amplified from the two uninfected control crops and these are presented in lanes 3 for safflower and 5 for sugar beet. Fragments of about 1 kbp for actin and 0.6 kbp for ITS were observed, respectively in lanes 6 and 7 of the C2

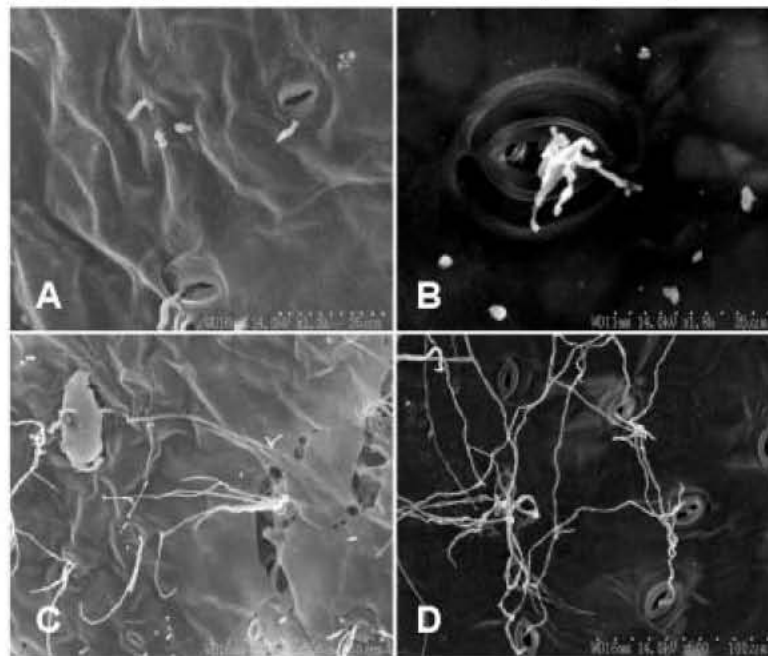


Fig. 4: Emerged hyphal structures of *C. beticola* from lesions of sugar beet and safflower, respectively. The pathogen emerged from both the necrotic splits and stomatal tissues of sugar beet A and C, but only from stomatal opening of safflower B and D

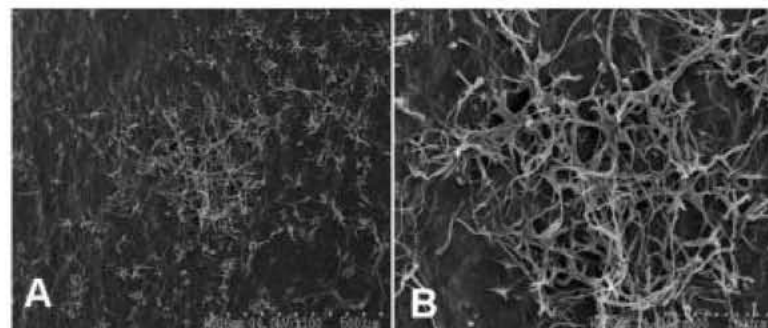


Fig. 5: The pathogen emerged from necrotic splits and stomatal tissues of sugar beet but only from stomatal openings of safflower and formed mycelial mass in a lesion of safflower A and sugar beet B

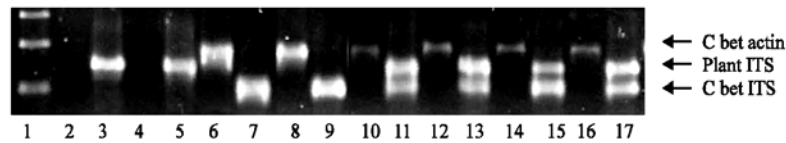


Fig. 6: Confirmation of *C. beticola* isolates C2 and Sid2 infection of safflower and sugar beet by PCR. 1 = Size marker; 2 and 3 = Uninfected safflower; 4 and 5 = Uninfected sugar beet; 6 and 7 = C2 Control cultures 8 and 9 = Sid2 Control cultures; 10-13 = Safflower lesions (C2 and Sid2); 14-17 = Sugar beet lesions (C2 and Sid2)

positive control. Similar fragments were observed, respectively in lanes 8 and 9 of the positive control Sid2. From safflower lesions, fragments of *C. beticola* isolates C2 and Sid2 were, respectively amplified with the *C. beticola* actin specific primers (lanes 10 and 12) and from sugar beet (lanes 14 and 16). The amplified 959 bp fragments correspond with the positive DNA control extracts from C2 and Sid2 pure culture in lanes 6 and 8, respectively. Using the ITS primers, fragments of about 0.6 kbp were also amplified from safflower leaf lesions, caused by the C2 and Sid2 isolates and these are shown in lanes 11 and 13, respectively and from sugar beet in lanes 15 and 17. The fragments correspond in size with the fragment from the C2 and Sid2 positive controls in lanes 7 and 9, respectively. Additional ITS fragments of about 0.7 kbp were also amplified from infected lesions and these corresponded to the amplified ITS fragments from the uninoculated control safflower plant (lane 3) and sugar beet (lane 5).

DISCUSSION

We recently provided evidence that safflower is a host of *C. beticola*, causing leaf spot disease symptoms (Lartey *et al.*, 2005a, b) which we hence forth refer to as leaf spot of cercosporer (LSC) as a constrict to CLS that is caused by *C. carthami*. In this study, we compared progression of infection of two *C. beticola* isolates, C2 and Sid2, in sugar beet and safflower. Spray inoculation of both isolates produced leaf spot symptoms in both crops. This observation is consistent with our previous observations in which four *Cercospora* isolates, C1, C2, Sid1 and Sid2 induced spot lesions in both safflower and sugar beet.

It has been previously reported that infection of sugar beet by *C. beticola* commences with stomatal penetration of the pathogen hyphae (Rathaiah, 1977). It is evident from our observations that penetration of the pathogen follows the same stomatal pathway during infection of safflower as hyphal strands of the pathogen are observed entering stomatal openings of safflower after the spray inoculation. In the early stages of development, splits were observed in the spot lesions of sugar beet leaf

tissues but not in the lesions of safflower. Subsequently, hyphal tissues of *C. beticola* were observed in the splits within the lesions of the diseased sugar beet leaves. Hyphal mass was observed in the stomatal openings of safflower, but no splits were observed. According to (Steinkamp *et al.*, 1979), penetration is followed by ramification and intercellular growth of fungal hyphae in the parenchymous tissue of the epidermis. Our observations strongly suggest similar spread in the safflower leaf tissues. However, the differences of lesion structure in safflower and sugar beet is noteworthy.

Following successful primary infection, it is by and large acknowledged that secondary infection cycles lead to subsequent spread of the disease. After the penetration of the pathogen and establishment of infection, conidia are produced on conidiophores, mostly on the abaxial surface of infected leaves. Subsequently, conidia are spread by water (rain and irrigation) splash, wind and insects to initiate secondary infections (Weiland and Koch, 2004). Certainly, the conidiophore development must commence with reemergence of *C. beticola* from lesion tissues. We provide evidence that the pathogen reemerged from both stomatal, necrotic tissues and splits openings in lesions of sugar beet. In this interesting observation, it appears that *C. beticola* ruptures the epidermis of sugar beet leaves and these points of emergence appear to be foci of later epidermal tearing. This is in contrast to safflower where the fungus only emerges from the stomata. This could be explained by absence of splits within the lesions of the safflower host. Following reemergence, extensive development of mycelial mat occurred in both sugar beet and safflower.

We concluded the SEM study by providing additional evidence that the observed fungal propagules are from *C. beticola*. To confirm that the observed hyphae were from *C. beticola*, we applied a recently developed modified PCR technique that allowed direct amplification of target DNA without the need for purification of the genomic DNA from the infected tissue. Our results confirmed the observed fungi from the necrotic lesions of both sugar beet and safflower were indeed from *C. beticola*.

It had been stated previously that, beside Beta species (McKay and Pool, 1918), common weeds such as *Chenopodium album* L., *Amaranthus retroflexus* L., *Malva rotundifolia* L., *Plantago major* L., *Arctium lappa* L. and *Lactuca sativa* L., (Vestal, 1931) could serve as hosts of *C. beticola*. More recently, common mallow (*Malva neglecta* Wallr.) and field bindweed (*Convolvulus arvensis* L.) (Ruppel 1986), winged pigweed (*Cycloloma atriplicifolium* (Spreng.) Coult) and wild buckwheat (*Polygonum convolvulus* L.) (Jacobsen *et al.*, 2000) have also been reported as weed hosts of *C. beticola*. These, however, serve only as minor source of inoculum (Windels *et al.*, 1998). The present study substantiates our previous findings that safflower, an important commercial crop is also a host of *C. beticola*. Thus, weeds are not the only potential source of inoculum, but other important crops such as safflower could also serve as a source of inoculum. In contrast to the weeds, safflower is cultivated on a broad scale and could therefore serve as a major source of inoculum. Because safflower is a major agronomic crop in the region, sugar beet could also serve as source of inoculum for infection of safflower. Our observation also raises an interesting question as to whether *C. beticola* cannot digest the cuticle of safflower in contrast to sugar beet. Understanding the genetics of this observation may offer a clue for breeding resistance sugar beet against *C. beticola*. CLS of sugar beet causes significant yield loss, reduced sugar content and beet rot during storage (Shane and Teng, 1992; Smith and Ruppel, 1971; Smith and Ruppel, 1973). The effect of LSC by *C. beticola* on yield of safflower oil seed, oil content and seed storage have not been determined. Beside the potential function as source of inoculum for infection of sugar beet, it will be crucial to determine the possible effects of LSC on commercial value of safflower crops in future research.

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