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ORIGINAL ARTICLE

Identification of *Colletotrichum spaethianum* causing sugar beet leaf spot in North Dakota, USA

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Abstract

Sugar beet leaves with dark brown to blackish, necrotic lesions were found in a grower's field in Casselton, North Dakota, USA in August 2021. Morphological features of the isolates obtained in growth media from the diseased samples were observed and documented. The pathogenicity of the randomly selected isolates developed identical disease symptoms on the inoculated leaves. Molecular characterization of the isolates was conducted by identifying homologies with sequences of the internal transcribed spacer, the largest subunit of RNA polymerase II (*rpb2*), β-tubulin (*β-tub*), calmodulin (*CaM*), and plasma membrane ATPase (*Pma1*) genes followed by multilocus phylogenic analyses. Based on morphological characteristics, pathogenicity, and molecular analyses, the causal organism was identified as *Colletotrichum spaethianum*. This is the first report of *C. spaethianum* causing leaf spot on sugar beet in North Dakota, USA. The report will help growers design an effective disease management for a novel pathogen in sugar beet in the Red River Valley of Minnesota and North Dakota, USA.

Keywords: *ATPase,* calmodulin, fungus, ITS, leaf disease, sugar beet, *β-tub*, *rpb2*

Introduction

In the Red River Valley of Minnesota and North Dakota, USA, sugar beet (*Beta vulgaris* L.) is one of the most significant sugar-yielding crops. According to ISO 2023, 34 million metric tons of beet sugar was produced worldwide in 2021. The USA is the third-largest producer of sugar beets in the world, accounting for 55 to 60% of the country's total sugar production (Lord and Vidalina 2021). *Cercospora beticola*, *Alternaria tenuissima*, *Stemplylium vesicarium*, *Phoma betae*, *Ramularia beticola*, *Colletotrichum incanum* (leaf spot/blight), *Erysiphe betae* (powdery mildew), and *Uromyces beticola* (rust) are the main pathogens that cause foliar diseases in sugar beet (Bublitz *et al.* 2019; Khan 2023). Sugar beet foliar infections cause a reduction in production and quality, which leads to management failure, increasing costs for growers, and the development of fungicide resistance mutations (Cortes *et al.* 2022; EL Housni *et al.* 2023;). Crop losses due to leaf spot diseases are evaluated to be 10 to 40% or more in serious disease conditions. *Plant growth, quality, fruit output, and marketability are all negatively impacted by Colletotrichum spp.* (Hanson *et al.* 2023).

In numerous crop groups, including ornamentals, medicinals, fruits, nursery, and vegetable crops, anthracnose caused by *Colletotrichum* spp. is one of the most prevalent diseases. The disease is characterized by leaf spots and blight, leading to wilting and frequent dieback of leaves as well as reddish brown to dark brown necrotic patches (Mukherji and Bhasin 1986). Early in the growth season, disease symptoms attack

the lower leaves first, then the sickness progresses to the upper leaves. Typically, disease signs are seen from summer till harvest. The fungus overwinters as mycelium in and on seeds, soil, and field detritus. This fungus also forms large hyphae known as stromata, which have conidiophores on the surface of the host. Conidia are produced at the tips of conidiophores and disseminated through wind, rain, insects, field workers, and agricultural tools. Humid, wet, rainy weather is necessary for infection to occur. Cool wet weather promotes fungal development and optimum temperature for spore growth is between 75 to 85°F (24 to 30°C). Moisture is required for germination and development of the fungus and infection of the plant. Conidia penetrate host tissue through specialized hyphae called appressoria. The incidence of disease in cultivated fields can vary depending on the level of the pathogen inoculum in the soil and other environmental factors.

The aim of this investigation was to describe the symptomology, and to morpho-molecularly characterize the *Colletotrichum* sp. which caused the leaf spot disease in a sugar beet field in Casselton, North Dakota.

Materials and Methods

Isolation and morphological characterization of the pathogen

In August 2021, sugar beet leaves showing disease symptoms were collected from a grower's field located in Casselton, North Dakota (46.54178 N, 97.12409 W). Samples had characteristic sunken, dark brown to blackish, necrotic lesions that were irregular in shape, including blighted leaves that resulted from the coalesced lesions (Fig. 1). The disease incidence was estimated at 10 to 15% and the disease severity was measured at 30 to 40% (n = 50 plants investigated). Fifteen symptomatic leaves from 15 plants were sampled. Symptomatic leaves (2 × 2 mm) were surface-disinfested in 1% NaOCl for 1 min, rinsed with sterile distilled water for 30 s three times, and allowed to dry in a laminar airflow cabinet followed by plating on potato dextrose agar (PDA) amended with 200 mg · l^{-1} streptomycin at 25°C in the dark. Five days post inoculation, whitish to pale grayish colored colonies grew. Single spores were transferred to a synthetic nutrient-poor agar medium (SNA) (Leslie and Summerell 2006). Twenty-five pure isolates were obtained. Morphological features of the isolates were observed under inverted fluorescence (VWR, USA) and a Leica microsystems stereomicroscope (Deerfield, IL, USA).

Fig. 1. Sugar beet leaf samples with dark brown to black irregular lesions were collected from a grower's field located in Casselton, North Dakota, USA

Molecular identification

For molecular identification, the genomic DNA of the five randomly selected isolates (ZCS, ZCS-1, ZCS-2, ZCS-3, and ZCS-4) was extracted using the Qiagen Plant DNeasy Plant Minikit (Hilden, Germany). The PCR amplification and sequence analyses were performed for the internal transcribed spacer region (ITS), the second largest subunit of RNA polymerase II (*rpb2*), β-tubulin (*β-tub*), calmodulin (*CaM*), and plasma membrane ATPase (*Pma1*) genes (Tab. 1). Primers were synthesized by Integrated DNA Technologies (IDT, Novato, CA, USA). For conventional PCR, 25 µl reactions were performed using 12.5 µl GoTag® Master Mix (Promega, WI, USA), 9.5 µl of nuclease-free water, 1.0 µl of each forward and reverse primer at a concentration of 10 µm, and 1 µl (10 ng · μl^{-1}) of DNA template. The PCR conditions were as follows: for ITS, the first cycle of initial denaturation was performed at 95°C for 10 min, followed by 30 cycles in a series at 95°C for 1 min, 57°C for 1 min, and 72°C for 90 s, with a final cycle at 72°C for 10 min; for *rpb2*, 1 cycle of 90 s at 94°C, followed by 40 cycles of 30 s at 94°C, 90 s at 55°C, and 2 min at 68°C, followed by final extension for 5 min at 68°C, and beta-tubulin: 94°C for 1 min; 58 or 68°C for 1 min; 72°C for 1 min; repeat protocol for 32 cycles, with a 5- to 10-s extension time per cycle, for calmodulin primers, initial denaturation for 3 min at 95°C, followed by 35 cycles at 95°C for 30 s, 55°C for 45 s and 72°C for 1 min, followed by a final chain elongation step at 72°C for 8 min, for ATPase primers, initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 58.5°C for 30 s, extension at 72°C

Table 1. Primers used for PCR identification of sugar beet anthracnose

Gene	Symbol	PCR Primers 5′ to 3′	References
Internal Transcribed Spaces	ITS	ITS1 [5′-TCCGTAGGTGAACCTGCGG-3′] ITS4 [5′-TCCTCCGCTTATTGATATGC-3′]	White *et al*. (1990)
Second largest subunit of RNA polymerase II	*rpb2*	5F2 [5′-GGGGWGAYCAGAAGAAGGC-3′] 7cR [5′-CCCATRGCTTGYTTRCCCAT-3′]	O'Donnell *et al*. (2010)
β-tubulin	*β-tub*	Bt_2a [5′-GGTAACCAAATCGGTGCTGCTTTC-3′] Bt_2b [5′-ACCCTCAGTGTAGTGACCCTT GGC-3′]	Glass and Donaldson (1995)
Calmodulin	*CaM*	cmd5 [5′-CCGAGTACAAGGAGGCCTTC-3′] cmd6 [5′-CCGATAGAGGTCATAACGTGG-3′]	Hong *et al*. (2006)
ATPase	*Pma1*	ATPD-F [5′-ATCGTCTCCATGACCGAGTTCG-3′] ATPD-R[5′-TCCGATGGAGTTCATGATAGCC-3′]	Lawrence *et al*. (2013)

for 1 min, and the final extension at 72°C for 5 min. The PCR products were run on 1.2% gels stained with ethidium bromide to visualize the amplicon size and treated with Exo-SAP II (ThermoFisher Scientific, MA, USA) following the manufacturer's protocol. The samples were sent to Molecular Cloning Laboratories (MCLAB, San Francisco, CA, USA) for Sanger sequencing.

Phylogenetic analyses

The sequences obtained with the forward and reverse primers were assembled using Sequencher 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA). The two sequences ITS and *β-tub* of the isolate ZCS-1 were selected for phylogenetic analyses based on the recommendations of Onestopshopfungi (https://onestopshopfungi.org/colletotrichum/), Jayawardena *et al.* (2021), and Talhinhas and Baroncelli (2021) (Tab. 2). The two sequences were subjected to Basic Local Alignment Search Tool available at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and were found to be identical to the sequences of *C. spaethianum*. BLAST search also identified the nearest relatives to this species that were obtained from published literature (Onestopshopfungi; Jayawardena *et al*. 2021; Talhinhas and Baroncelli 2021) and were downloaded from the GenBank. The downloaded sequences were combined with the respective sequences of ZAZ-1 isolate in two separate FASTA format files. The sequence files were aligned using multiple sequence alignment tool Clustal Omega available at https://www.ebi.ac.uk/Tools/msa/clustalo/. The alignment files for the two genes were concatenated and adjusted visually using the software Mesquite version 3.81 (Maddison 2007). The alignment file was used to construct phylogenetic trees using two methods: 1) The parsimony tree was obtained using PAUP version 4.0a (https://phylosolutions.com/paup-test/). The tree was produced using a heuristic search with a starting tree obtained by 1000 random stepwise addition of sequences, tree-bisection-reconnection (TBR) as the branch-swapping algorithm with MULTREES in effect. Gaps were treated as missing characters. Supports for branches were assessed with 1000 replates of bootstrap. 2) The Maximum Likelihood tree was obtained using MEGA X with the substitution model predetermined using MEGA X (Tamura *et al*. 2021). Support for the clades was assessed with 1000 bootstrap replicates.

Table 2. Accession numbers for building a *Colletotrichum spaethianum* phylogenetic tree

Species name	Isolate	ITS	*β-tub*
C. bletillum	CGMCC 3.15117	JX625178	JX625207
C. liriopes	CBS 119444	GU227804	GU228098
C. metake	CNUCC 311173	MT192601	MT192831
C. guizhouensis	CGMCC 3.15112	JX625158	JX625185
C. incanum	ATCC 64682	KC110789	KC110816
C. lilii	CBS 109214	GU227810	GU228104
C. liriopes	CBS 119444	GU227804	GU228098
C. riograndense	ICMP 20083	KM655299	KM655300
C. spaethianum	CBS 167.49	GU227807	GU228101
C. spaethianum	CBS 100063	GU227808	GU228102
C. spaethianum (Khan lab)	ZCS-1	OP601837	PP157931
C. tofieldiae	CBS 495.85	GU227801	GU228095
C. verruculosum	IMI 45525	GU227806	GU228100
C. rusci	CBS 119206	GU227818	GU228112
C. trichellum	CBS 217.64	GU227812	GU228106
C. lindemuthianum (Outlier)	CBS 144.31	JQ005779	JQ005863

Pathogenicity assay

The pathogenicity assays of the isolates were conducted under greenhouse conditions on sugar beet plants. Eight sugar beets (cv. Hilleshog 4302; Hilleshog Seed LLC, Longmont, CO, USA) plants (6 weeks old) were grown in 25 × 25 × 20 cm plastic pots (T.O. Plastics Inc., Clearwater, MN, USA.) containing Sunshine Mix #1 potting mix (Fison Horticulture, Vancouver, B.C., Canada) and 20 g of slow-release fertilizer Osmocote 15-9-12 (N-P-K) (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA). Seeds were commercially treated with Kabina ST (FRAC 7 fungicide), and Poncho® Beta (Bayer Crop Science, USA), which is a systemic insecticide and acts against damaging soil and foliar insect pressure. The pathogenicity assays of the *C. spaethianum* isolates were conducted by spraying spore suspension ($1 \times 10^4 \cdot ml^{-1}$) on 10 sugar beet plants (cv. Hilleshog 4302) at the 8-true leaf stage grown in plastic pots containing soilless mix. Spore suspension was sprayed on the upper surface of sugar beet leaves until runoff. Sugar beet plants inoculated with sterile autoclaved water were considered as non-inoculated controls. The experiment was conducted twice. Inoculated plants were kept in a humidity chamber for 5 days at 25°C, relative humidity >80%, and 14 h photoperiod and then transferred to the greenhouse with a 14-h photoperiod (600-W high-pressure sodium lamps; P.L. Light Systems, Inc., Beamsville, Ontario, Canada), maintained at a relative humidity of around 70% and at 23 ± 2°C (Argus Control Systems Ltd., British Columbia, Canada).

Results

Morphological features of the fungal isolates

Brown to blackish dot-like acervuli were observed under stereo- and compound microscopes. Cushion shaped brown to blackish dot-like asexual fruiting bodies (acervuli) showed numerous tiny and blackish, needle-like setae (Fig. 2). Pure colonies were grayish-white, cottony mycelia in the growth media. The conidia were hyaline, slightly curved, and aseptate (Fig. 3). No chlamydospores were observed. Based on symptoms, as well as morphological and microscopic observations, the fungus was identified as *Colletotrichum spaethianum* (Allesch.) Damm, P.F. Cannon & Crous (Damm *et al.* 2009; Cannon *et al.* 2012).

Pathogenicity assay of the *Colletotrichum spaethianum* isolates under greenhouse conditions

Two weeks later, all inoculated leaves developed gray to dark brown, necrotic lesions. Mock-inoculated

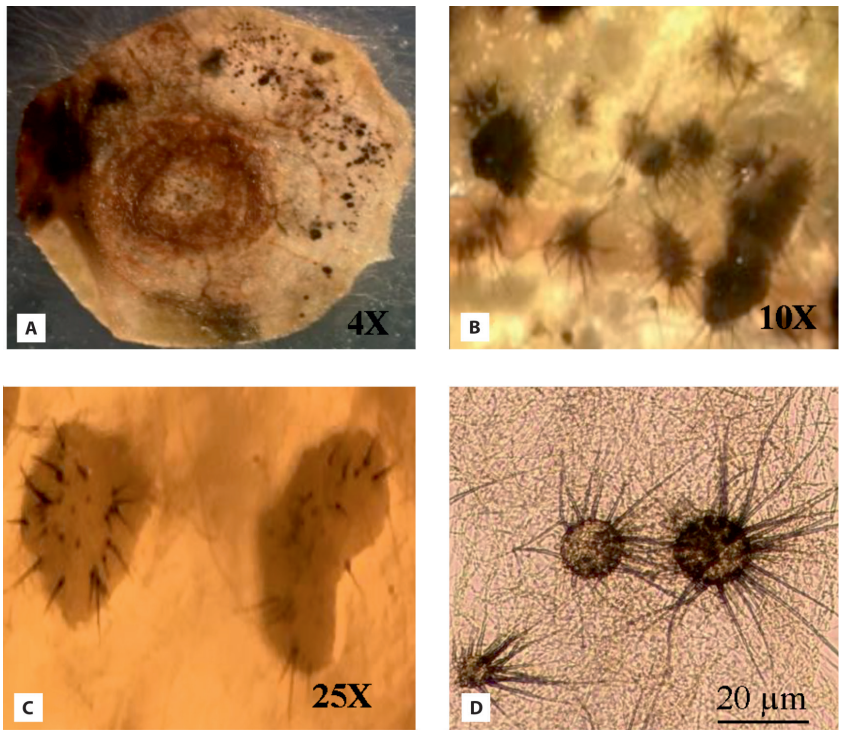

Fig. 2. Visualization of lesions on sugar beet leaves under a stereo microscope (4X, 10X, and 25X magnifications) and a compound microscope. A – brown to blackish dot-like acervuli; B–C – cushion shaped asexual fruiting bodies showed numerous tiny and D – blackish, needle-like setae (scale bar = 20 µm)

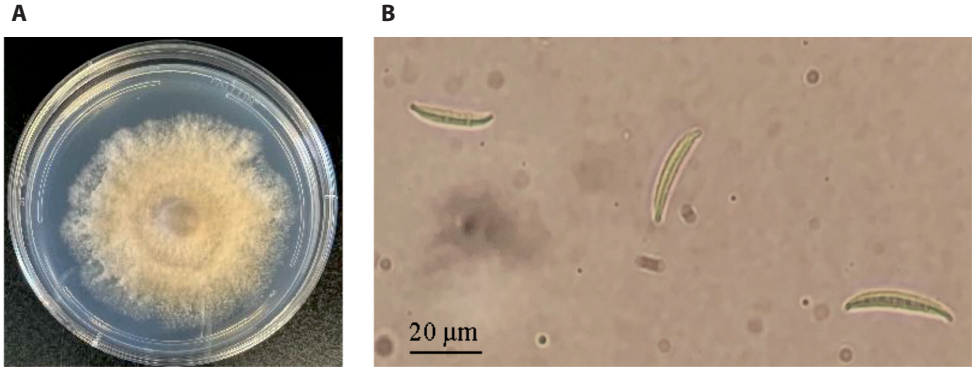

Fig. 3. A – Pure colony of *Colletotrichum spaethianum* growing in PDA media; B – conidia were slightly curved and aseptate

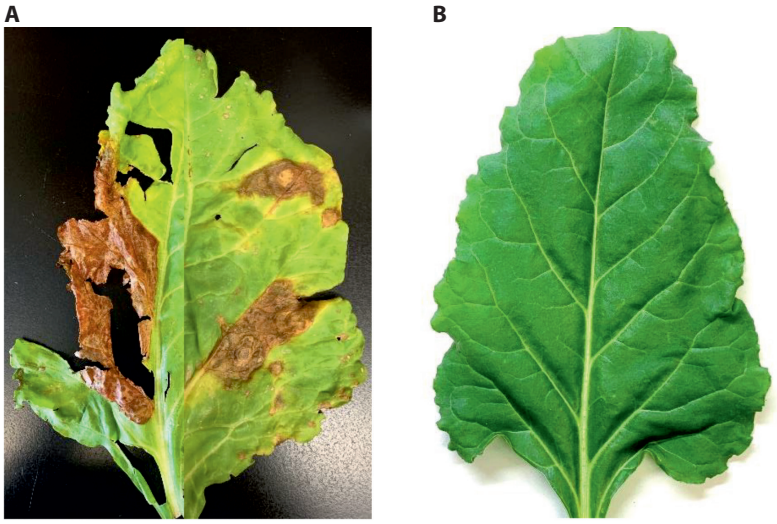

Fig. 4. A – sugar beet leaves were inoculated with *Colletotrichum spaethianum* developed dark brown lesions and blighted symptoms, while the B – mock-inoculated leaves were symptomless

plant leaves were symptomless. The isolates retrieved from the inoculated leaves were morphologically identical to the *C. spaethianum* field isolates (Fig. 4).

Sequence homologies of the *Colletotrichum spaethianum* isolates

The obtained sequences were submitted to GenBank OP601837 (ITS), OP622277 (*rpb2*), PP157931 (*β-tub*), PP157930(*CaM*),andPP157932(*Pma1*).BLASTNsearch showed 100% sequence homology with MN305792 (524/524 bp) (ITS), XM_049277277 (845/845 bp) (*rpb2*), MN307740 (391/391 bp) (*β-tub*), XM_049270604 (224/224 bp) (*CaM*), and XM_049277129 (516/516 bp) (*Pma1*) of *C. spaethianum*.

Phylogenic relationships of the *Colletotrichum spaethianum* isolates

The phylogenetic tree (Fig. 5) showed that ZCS-1 (OP601837) clustered with two reference strains of *C. spaethianum* [CBS 167.49 (GU227807) and CBS 100063 (GU227808)] in a highly supported clade. The latter two strains were obtained from hosts like *Hosta sieboldiana* (GU227807) and *Lilium* sp. (GU227808). The three isolates were from different geographic locations, Europe (GU227807), Asia (GU227808), and America (OP601837). The nearest relatives to this species are the species of *C. guizhounse* and *C. lilii* which formed a larger clade with the *C. spaethianum* with a high bootstrap value.

Discussion

C. spaethianum was originally described from dead stems of plantain lilies in Berlin, Germany in 1895. This fungus has a wide host range and is distributed throughout Europe, Asia, North America, and South America. The *C. spaethianum* has been reported to be pathogenic to many economically important crops

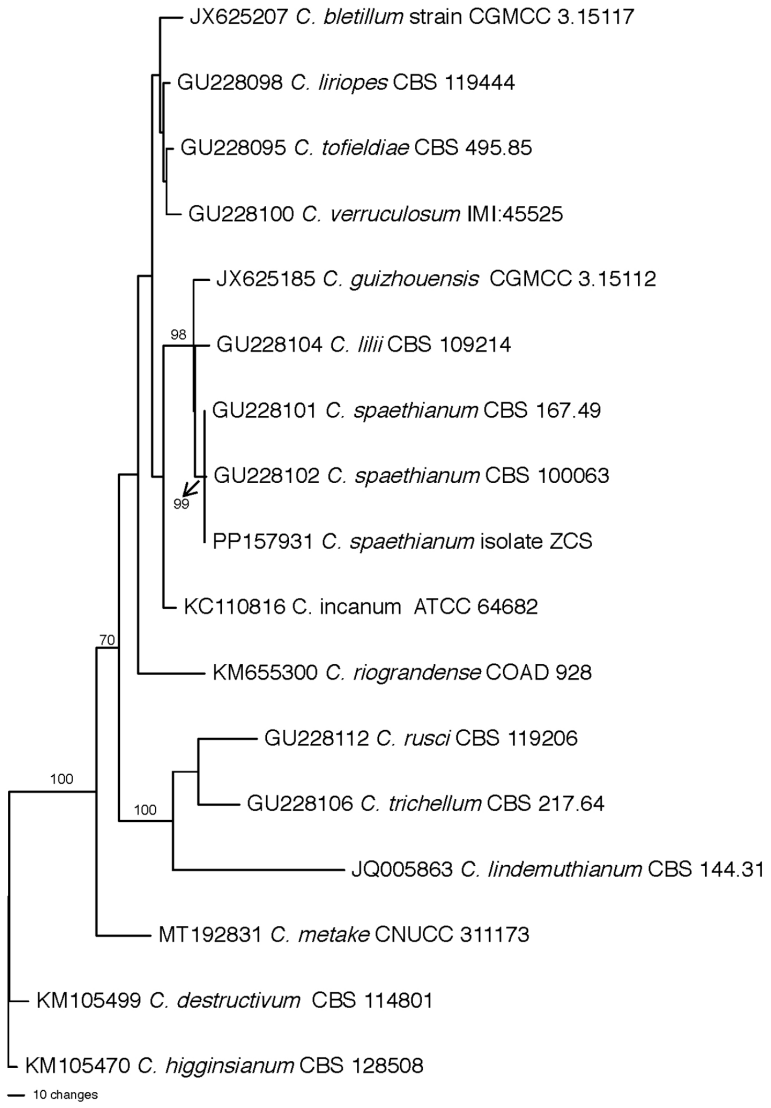

Fig. 5. Phylogenic tree based on DNA sequencing data of β-tubulin (*β-tub*) and internal transcribed spacer (ITS) resolving the position of *Collectortichjum spaethianum* isolate ZCS from this study within the phylogeny of related species of *Colletotrichum*. *C. higginsianum* strain CBS 1285008 was used as an outgroup taxon

including onion (Yu *et al*. 2024), lily (Cheon and Jeon 2016), soybean (Boufleur *et al*. 2021), loquat, common bean (Talhinhas and Baroncelli 2023), yellow daylily, asparagus (Moriwaki and Watanabe 2021) and medical herbs (Ma *et al*. 2020) worldwide. *C. incanum* has been reported in Michigan, USA to cause anthracnose in sugar beet (Hanson *et al.* 2023). Effective management of anthracnose disease relies on the integration of cultural, biological, and chemical control measures. Cultural practices may involve one or more of such practices as removal and destruction of crop residues from fields, avoiding overcrowding, overhead irrigation, and late afternoon watering, avoiding leaf injuries, avoiding handling plants when wet, and controlling pests and pathogens. Crop rotation with non-host crops for at least 3 years and cultivation of tolerant varieties if available, is also recommended. There are no registered fungicides for *Colletotrichum* spp. to manage sugar beet leaf spots. An enzyme named "Laccase" produced by the beneficial fungus *Laetisaria arvalis* is considered a possible natural cure for sugar beet leaf spot (Comis 2023). To the best of our knowledge, this is the first report of *C. spaethianum* causing leaf spot on sugar beet in North Dakota. This report will help growers to formulate effective disease management practices to control this fungus.

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