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e-Xtra*

Seedborne *Cercospora beticola* Can Initiate Cercospora Leaf Spot from Sugar Beet (*Beta vulgaris*) Fruit Tissue

Rebecca Spanner,[1,2] Jonathan Neubauer,[1] Thies M. Heick,[3] Michael A. Grusak,[1] Olivia Hamilton,[1,2] Viviana Rivera-Varas,[2] Ronnie de Jonge,[4] Sarah Pethybridge,[5] Kimberley M. Webb,[6] Gerhard Leubner-Metzger,[7] Gary A. Secor,[2] and Melvin D. Bolton[1,2,†]

[1] Edward T. Schafer Agricultural Research Center, United States Department of Agriculture–Agricultural Research Service, Fargo, ND, U.S.A.
[2] Department of Plant Pathology, North Dakota State University, Fargo, ND, U.S.A.
[3] Institute for Agroecology, Aarhus University, Slagelse, Denmark
[4] Plant-Microbe Interactions, Department of Biology, Science4Life, Utrecht University, Utrecht, The Netherlands
[5] Plant Pathology & Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell AgriTech, Cornell University, Geneva, NY, U.S.A.
[6] Soil Management and Sugar Beet Research Unit, United States Department of Agriculture–Agricultural Research Service, Fort Collins, CO, U.S.A.
[7] Department of Biological Sciences, Royal Holloway University of London, Egham, U.K.
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ABSTRACT

Cercospora leaf spot (CLS) is a globally important disease of sugar beet (*Beta vulgaris*) caused by the fungus *Cercospora beticola*. Long-distance movement of *C. beticola* has been indirectly evidenced in recent population genetic studies, suggesting potential dispersal via seed. Commercial sugar beet "seed" consists of the reproductive fruit (true seed surrounded by maternal pericarp tissue) coated in artificial pellet material. In this study, we confirmed the presence of viable *C. beticola* in sugar beet fruit for 10 of 37 tested seed lots. All isolates harbored the G143A mutation associated with quinone outside inhibitor resistance, and 32 of 38 isolates had reduced demethylation inhibitor sensitivity (EC_{50} > 1 µg/ml). Planting of commercial sugar beet seed demonstrated the ability of seedborne inoculum to initiate CLS in sugar beet. *C. beticola* DNA was detected in DNA isolated from xylem sap, suggesting the vascular system is used to systemically colonize the host. We established nuclear ribosomal internal transcribed spacer region amplicon sequencing using the MinION platform to detect fungi in sugar beet fruit. Fungal sequences from 19 different genera were identified from 11 different sugar beet seed lots, but *Fusarium*, *Alternaria*, and *Cercospora* were consistently the three most dominant taxa, comprising an average of 93% relative read abundance over 11 seed lots. We also present evidence that *C. beticola* resides in the pericarp of sugar beet fruit rather than the true seed. The presence of seedborne inoculum should be considered when implementing integrated disease management strategies for CLS of sugar beet in the future.

Keywords: etiology, fungal pathogens, microbiome

Cercospora leaf spot (CLS) is the most destructive foliar disease of sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) worldwide and is caused by the fungus *Cercospora beticola* (Rangel et al. 2020). In warm temperate growing regions, CLS can cause yield losses of 42% (Shane and Teng 1992) and up to 100% in the absence of fungicide treatment (Jacobsen and Franc 2009; Shane and Teng 1992). Furthermore, CLS-infected plants are more susceptible to postharvest disease in storage piles (Smith and Ruppel 1971). Disease management programs for CLS currently integrate cultural practices, host genetic resistance, and fungicide applications (Rangel et al. 2020). Cultural practices, such as rotation with non-host crops and tillage to bury infested plant debris, aim to reduce primary inoculum for the next season. The development of sugar beet varieties with CLS tolerance that are high yielding has historically been a challenge, but continual improvements are being made (Smith and Campbell 1996; Vogel et al. 2018). In growing regions where disease pressure is high, the current forms of host tolerance are insufficient to manage CLS alone (Rangel et al. 2020). Consequently, fungicide applications are required in a timely manner to inhibit the proliferation of *C. beticola* in sugar beet leaves. However, widespread and repeated use of the same fungicides over large growing areas has rapidly led to the development of resistance or reduced sensitivity to four chemical classes in *C. beticola* populations (Fungicide Resistance Action Committee [FRAC] groups 30 [organotins], 1 [benzimidazoles], 11 [quinone outside inhibitors], and 3 [sterol demethylation inhibitors]; Birla et al. 2012; Bolton et al. 2012a, 2013; Cerato and Grassi 1983; Karaoglanidis et al. 2001; Rangel et al. 2020; Secor et al. 2010). Management of fungicide resistance through mixing and rotation of different chemical classes is aiding in the retention of fungicide efficacy (Secor et al. 2010), but additional strategies are required to enhance the sustainability of this tactic.

†Corresponding author: M. D. Bolton; melvin.bolton@usda.gov

R. Spanner and J. Neubauer contributed equally to this work.

Current address of R. Spanner: Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; ANID–Millennium Science Initiative–Millennium Institute for Integrative Biology (iBIO), Santiago, Chile.

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The dominant primary inoculum for local CLS epidemics is considered to be specialized fungal overwintering structures on plant debris called pseudostromata (Khan et al. 2008; McKay and Pool 1918; Pool and McKay 1916). Because *C. beticola* can infect multiple plant species (Knight et al. 2019b), pseudostromata can persist either on sugar beet or alternative host plant tissue. Previous studies suggested that pseudostromata surviving for 22 months on the soil surface can initiate disease (Khan et al. 2008). With conducive environmental conditions, conidia can form on pseudostromata and move to new host plants to initiate the infection process, and multiple cycles of both asexual reproduction and infection can occur within a single growing season (McKay and Pool 1918; Nagel 1945; Vereijssen et al. 2007). A teleomorph has not been found for *C. beticola*, but genetic evidence supports panmictic populations (Bolton et al. 2012b; Groenewald et al. 2006, 2008; Vaghefi et al. 2017a). Several population genetics studies have suggested the initiation of CLS epidemics involves an alternative inoculum source(s) to clonally reproducing pseudostromata (Groenewald et al. 2008; Knight et al. 2018, 2019a; Vaghefi et al. 2017a, 2017c). Recurrent clonal lineages have also been found across continents (Knight et al. 2019a; Vaghefi et al. 2017a), suggesting the long-distance movement of clonal isolates of *C. beticola*. However, spore dispersal studies have indicated a limited range of *C. beticola* (Imbusch et al. 2019), suggesting that long-distance movement mediated by spores is not likely. Temporal and spatial shifts in multilocus genotypes in New York table beet *C. beticola* populations also suggests the existence of external primary inoculum sources to the fields in question, such as infested seed from different sources (Knight et al. 2018; Vaghefi et al. 2017c).

Humans have an extensive history of mediating the long-distance dispersal of pathogens. Some of the earliest reports of plant-pathogenic fungi associated with seed include *Claviceps purpurea* of rye as described by Hellwig in 1699 (Baker and Smith 1966) and *Colletotrichum lindemuthianum* of bean as described by Frank (1883). More recently, studies have begun to dissect the roles of seedborne pathogens in seed transmission. *Ramularia collo-cygni* is a seedborne pathogen that causes Ramularia leaf spot of barley (*Hordeum vulgare*), characterized by late season necrosis in host leaf tissue (Havis et al. 2014). *R. collo-cygni* is present in both embryo and nonembryo tissue (Matusinsky et al. 2011) and can colonize the developing plant without visible symptoms in an endophytic phase (Nyman et al. 2009). Sowing of infested seed in field trials indicated that the pathogen can move through the developing plant and into the subsequent generation of seed (Havis et al. 2014).

Fungal pathogens have previously been associated with sugar beet seed, including *Phoma betae* (syn. *Neocamarosporium betae*, *Pleospora betae*) causing Phoma leaf spot and root rot and *Uromyces betae* causing beet rust (Agarwal et al. 2006; Richardson 1990). *C. beticola* has also been reported to be seedborne in sugar beet (McKay and Pool 1918). McKay and Pool (1918) identified a sugar beet seed lot that was infested with viable *C. beticola* conidia. When sown, the seedlings produced from this seed source had CLS lesions on the cotyledons, and formaldehyde treatment of seed reduced disease incidence. Vereijssen et al. (2004) provided anecdotal evidence of infested seed (polished, processed, and pelleted) associated with CLS epidemics in Europe. Vereijssen et al. (2004, 2005) also demonstrated that sugar beet roots could act as a primary infection site for *C. beticola* conidia. Most recently, Knight et al. (2020) identified viable *C. beticola* in commercial table beet seed lots. Plants grown from these infested lots developed CLS, suggesting that seedborne *C. beticola* can cause disease in table beet. It remains unclear how *C. beticola* would colonize the host as seedborne inoculum, but it could spread to the leaves via endophytic, symptomless colonization of vascular tissue as shown in other pathosystems (Hammond et al. 1985; Sesma and Osbourn 2004; Sukno et al. 2008).

In sugar beet and other crops of the Amaranthaceae, harvested and processed "seed" would be defined botanically as a "fruit," consisting of the true seed surrounded by the fruit coat (pericarp) (Ignatz et al. 2019) (Fig. 1). The true seed is composed of a thin seed coat (testa), covering an embryo that surrounds a starch storage tissue (perisperm) (Hermann et al. 2007). The harvested sugar beet fruits are monogerm and are further processed by cleaning, polishing, priming, and pelleting as detailed by Kockelmann and Meyer (2006). In this study, processed sugar beet fruit that is surrounded by commercial pellet material is referred generically as "seed" to adhere to industry naming conventions (Fig. 1). Therefore, we use the term "seedborne" to refer to the concept that plant disease is initiated from infested sugar beet seed that was commercially prepared as polished fruit. If seedborne *C. beticola* is an important source of inoculum for CLS, it will be important to identify the precise location of the fungus within the sugar beet fruit for future remedial treatment strategies and detection. Moreover, knowledge of the location of the fungus in fruit tissue will provide insight into management of the disease in seed production areas.

The detection of fungal pathogens in seed has historically been through visual examination, media culture, or seedling grow-out assays (Etebu and Nwauzoma 2017). However, these methods are extremely time-consuming and often fail to identify pathogens accurately. Moreover, obligate biotrophic fungi cannot be cultured, and for the remaining culturable species, competition between fungi (and bacteria) and/or nonoptimal growth conditions can also limit identifiable taxa. The most rapid, sensitive, and accurate methods of pathogen detection and quantification are molecular assays (McCartney et al. 2003). Many species-specific PCR-based techniques now exist to facilitate the detection of seedborne pathogens (Mancini et al. 2016; Munkvold 2009), including for *C. beticola* (Knight et al. 2020; Shrestha et al. 2020). However, to investigate the entire seed microbiome, high-throughput amplicon sequencing can be performed, allowing the description of total taxa present and comparison of microbiome compositions between different environments (Eyre et al. 2019).

In order to shed light on the potential for CLS to be transmitted by commercially prepared seed sources, our first major objectives were to establish whether *C. beticola* is present and viable in processed sugar beet fruit through both culturing and DNA-based confirmation and, if so, determine if this inoculum can initiate CLS. To gain further insight into the seedborne pathology of CLS, we sought to determine the fruit tissue(s) where *C. beticola* resides and investigate whether the fungus uses xylem vessels to systemically colonize the plant. We also aimed to determine whether seedborne *C. beticola* isolates are resistant to widely used fungicide chemistries. Finally, we sought to characterize the fungal microbiome of sugar beet fruit using long-read MinION sequencing of fungal internal transcribed spacer (ITS) sequences to detect fungal genera in seed lots.

MATERIALS AND METHODS

Isolation and identification of fungi from sugar beet fruit. Commercial sugar beet seed producers typically incorporate antifungal and/or growth-inducing chemistries in the form of a "pellet" that surrounds the processed sugar beet fruit (Kockelmann et al. 2010). To initially assess whether sugar beet seed tissues may harbor *C. beticola*, we obtained 37 commercially available sugar beet seed lots derived from seed production areas in Europe and the United States. All seed lots were screened for fungal growth by placing pelleted seed on 39 g/liter potato dextrose agar (PDA; BD Biosciences; San Jose, CA, U.S.A.) Petri plates (size 15 mm × 60 mm) and incubating at 22°C for 14 days under continuous light. Initial growth assays used 10 pelleted seeds from each seed lot to see if fungal growth occurred. Because we identified several cases in which fungal growth was observed only after sugar beet seeds had germinated (see *Results*) or fast-growing nontarget fungi grew from the pellet material itself, we proceeded to remove pellet material in order to directly screen sugar beet fruit (Fig. 1).

To remove pellet material from fruit, seeds were placed in sterile water. After 5 min, a gentle vortex was used to remove the pellet

material from the fruit. The fruit was then surface-sterilized for 10 min by placing it in 10% bleach (vol/vol) followed by triple rinsing in sterile water. Fruit were air-dried in a laminar flow bench prior to plating. Fifty fruits were plated per seed lot (a single fruit on a PDA plate), and fungal isolates were only analyzed further from these depelleted fruit. Plates were monitored daily for 3 weeks for fungal growth. Fungal isolations were made by replating a single 5-mm plug excised from each distinct area of fungal growth onto a fresh PDA plate and incubating at 22°C for 14 days.

To identify all fruit-derived fungal isolates, DNA was extracted from a single 5-mm plug excised from the PDA plate described above via a sodium dodecyl sulfate (SDS) lysis prep as described by Dodhia et al. (2021). DNA was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, U.S.A.) with the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, U.S.A.). *C. beticola* isolates were confirmed by performing species-specific quantitative PCR (qPCR) of the calmodulin gene (*CbCAL*) using the primers and method described by Knight and Pethybridge (2020). DNA from isolate 09-40 (de Jonge et al. 2018) was included as a positive control. If fungal growth was not *C. beticola*, PCR amplification of the entire nuclear ribosomal ITS region (up to 1,500 bp), including ITS regions ITS1 and ITS2 and part of the large subunit (LSU), was performed with universal ITS primers ITS1f-Kyo2 and LR3-I and methodology described by Mafune et al. (2019).

PCR products were purified using SureClean Plus (Bioline, Memphis, TN, U.S.A.) according to manufacturer's instructions and sent to MCLAB (San Francisco, CA, U.S.A.) for Sanger sequencing using the same forward and reverse primers used in PCR. The resulting forward and reverse sequences obtained were aligned and assembled into a single contig using Geneious software version 9.1.8 (Biomatters, Ltd., San Diego, CA, U.S.A.) before performing a BLASTn search of the National Center for Biotechnology Information (NCBI) nucleotide collection (https://www.ncbi.nlm.nih.gov) to identify the most similar sequence using the lowest Expect (E) value.

Dissection of fruit and isolation of fungi. To gain insight into the location of *C. beticola* in sugar beet fruit tissue, a total of 173 seeds (91 from seed lot 6 and 82 from seed lot 10; two seed lots from which the highest frequency of *C. beticola* had grown in initial plate growth assays) were each separated into three components: a) pellet, b) pericarp, and c) true seed (Fig. 1). First, a small utility hammer was used to dislodge the fruit from the outer pellet material. A razor blade was then used to carefully separate the pericarp from the true seed. No surface sterilization of tissues or pellet was performed. All tools used for dissection were sterilized between samples. All three components were labeled according to the individual pelleted seed they were extracted from, allowing us to track the origin of each component. These components were plated on PDA Petri plates (as described above) supplemented with streptomycin (30 mg/ml), ampicillin (50 mg/ml), and neomycin (50 mg/ml) to suppress bacterial growth. Plates were incubated at 23°C under continuous light for 10 days. Fungal isolation, DNA extraction, and species identification were performed as described above.

Fungicide resistance profiling of *C. beticola*. Thirty-eight fungal isolates identified as *C. beticola* from the initial seed lot isolation were single spore purified (Secor and Rivera 2012) and assessed for fungicide resistance using both genotypic and phenotypic methods (Table 1). The presence of the G143A mutation in cytochrome b associated with QoI resistance was assessed using the real-time PCR method described by Bolton et al. (2013). To measure sensitivity to the DMI fungicide tetraconazole, EC_{50} values were obtained as described by Secor and Rivera (2012). Briefly, 4-mm plugs of the isolates were each placed on unamended clarified V8 (CV8) agar plates (10% vol/vol clarified V8 juice [Campbell's Soup Co.], 0.5% wt/vol $CaCO_3$, 1.5% wt/vol agar [Sigma-Aldrich; St. Louis, MO, U.S.A.]) and four CV8 plates amended with 10-fold dilutions of technical grade tetraconazole (active ingredient of Eminent 125SL [Sipcam Agro]), dissolved in methanol from 100 to 0.1 µg/ml. All plates were incubated in the dark at 20°C for 15 days after which two perpendicular measurements were made across the colonies to calculate an average diameter. For each tetraconazole concentration, the percentage reduction in growth compared with nonamended media was calculated. The EC_{50} value for each isolate was calculated by plotting the percentage reduction in growth against logarithmic tetraconazole concentration and using regression curve fitting to find the tetraconazole concentration that reduced growth by 50% (Secor and Rivera 2012).

To determine if any of the 38 *C. beticola* isolates obtained from the initial isolation of sugar beet seed lots were clonal, eight polymorphic markers SSRCb20, SSRCb21, SSRCb22, SSRCb23, SSRCb24, SSRCb25, SSRCb26, and SSRCb27 (Vaghefi et al. 2017b) were amplified in multiplex PCR and analyzed as described by Vaghefi et al. (2017b) using the *C. beticola* DNA detailed above. In brief, PCRs used 1× Multiplex PCR Master Mix (New England Biolabs, Ipswich, MA, U.S.A.) in a total volume of 17 µl with 0.2 µM of each primer, 1.25 U of GoTaq DNA polymerase, and approximately 10 ng of genomic DNA. DNA was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, U.S.A.) with the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, U.S.A.). PCR conditions were an initial denaturation at 95°C for 5 min, followed by 37 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 30 s, and with a final 5-min elongation step at 68°C. Separation of labeled DNA fragments was performed by MCLAB (South San Francisco, CA, U.S.A.) using a Genetic Analyzer 3730*xl* (Applied Biosystems, Foster City, CA, U.S.A.). Fragment size in base pairs was determined using Peak Scanner software (v.1.0; Applied Biosystems). Multilocus SSR genotypes were established manually based

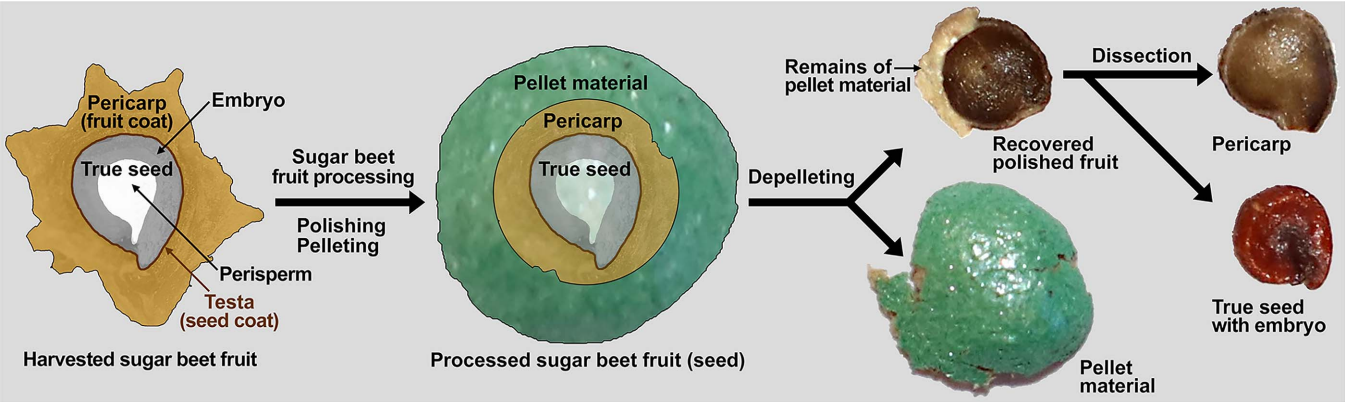

Fig. 1. The anatomy of commercially processed sugar beet seed before and after processing. Harvested sugar beet fruits contain the true seed encased by the pericarp, which is polished and further processed by pelleting (Hermann et al. 2007; Ignatz et al. 2019; Kockelmann et al. 2010). To analyze the location of *Cercospora beticola* in sugar beet fruits, depelleting and dissection of seed tissue was employed and each component assessed individually.

TABLE 1. Fungicide sensitivities of *Cercospora beticola* isolates grown from 37 sugar beet seed lots[a]

Seed lot number	*C. beticola* isolate number	Tetraconazole EC_{50}[b]	Q_oI genotype[c]
1	NA[d]	–	–
2	20-S066	68.897	R
	20-S075	40.080	R
3	20-S011	6.116	R
	20-S012	14.773	R
	20-S013	26.829	R
	20-S014	1.739	R
	20-S015	21.250	R
	20-S016	16.124	R
4	NA	–	–
5	20-S001	0.955	R
	20-S052	0.766	R
6	20-S004	5.288	R
	20-S005	63.095	R
	20-S006	11.765	R
	20-S007	26.108	R
	20-S008	5.173	R
	20-S009	21.964	R
	20-S010	0.541	R
7	NA	–	–
8	NA	–	–
9	20-S067	0.782	R
10	20-S019	7.794	R
	20-S020	5.814	R
	20-S021	68.036	R
	20-S022	10.000	R
	20-S023	62.063	R
	20-S024	51.636	R
	20-S025	67.363	R
	20-S026	23.991	R
	20-S027	0.667	R
	20-S028	77.734	R
	20-S029	3.714	R
	20-S030	26.254	R
	20-S036	86.309	R
	20-S065	26.720	R
	20-S035	45.848	R
11	NA	–	–
12	20-S038	46.595	R
13	NA	–	–
14	20-S040	53.095	R
	20-S046	7.604	R
15	NA	–	–
16	NA	–	–
17	NA	–	–
18	20-S074	25.530	R
19	NA	–	–
20	NA	–	–
21	NA	–	–
22	NA	–	–
23	NA	–	–
24	20-S031	0.422	R
25	NA	–	–
26	NA	–	–
27	NA	–	–
28	NA	–	–
29	NA	–	–
30	NA	–	–
31	NA	–	–
32	NA	–	–
33	NA	–	–
34	NA	–	–
35	NA	–	–
36	NA	–	–
37	NA	–	–

[a] A total of 38 *C. beticola* isolates grew from 10 of the 37 seed lots. Each isolate was assayed for demethylation inhibitor fungicide sensitivity (tetraconazole EC_{50} value measurements, µg/ml) and for the presence of the G143A quinone outside inhibitor (QoI) fungicide resistance mutation.

[b] Tetraconazole EC_{50} values calculated as described by Secor and Rivera (2012).

[c] QoI resistance genotype (G143A in cytB) was assessed as described by Bolton et al. (2013).

[d] Not applicable (NA) indicates that no *C. beticola* strain was identified from this seed lot.

on unique combinations of allele sizes obtained for the eight loci (Vaghefi et al. 2017b) (Supplementary Table S1).

Seed-to-seedling transmission efficiency assays. Twelve pelleted sugar beet seeds from seed lots 1, 3, and 10 were directly sown in 15 cm-diameter pots containing Pro-Mix BX potting soil (Quakertown, PA, U.S.A.). Pots were placed in a humidity chamber that maintained 90 to 95% relative humidity with a day temperature of 30°C, a night temperature of 26°C, and a 12-h photoperiod. Plants were watered as necessary. Twelve pots of each seed lot were randomly placed within the chamber at 20 cm spacing. This transmission experiment was conducted as two separate trials. Leaves were harvested at approximately 13 weeks after sowing. The chambers used in this study had not been used previously for sugar beet growth or associated *C. beticola* inoculations. Nonetheless, all chambers were thoroughly sanitized prior to seed-to-seedling experiments using 10% (vol/vol) bleach. Leaves that exhibited disease phenotypes characteristic of CLS circular lesions (gray in the center with black pseudostromata and a brown-red outer ring; Rangel et al. 2020) were considered as such. The number of CLS lesions on each leaf (all leaves considered) was counted and averaged on a per plant basis. Plants grown from all three seed lots developed at comparable rates. Transmission rate (%) was determined as the percentage of germinated seed in a seed lot with at least one CLS lesion after 13 weeks. A minimum of five lesions per seed lot underwent fungal reisolation as described by Secor and Rivera (2012). Isolates derived from CLS lesions were confirmed to be *C. beticola* using the qPCR methodology described by Knight and Pethybridge (2020).

Xylem sap collection and analysis. Twenty-two sugar beet seeds from each of seed lots 1, 3, and 10 were directly sown in 4-cm-diameter cones containing Pro-Mix BX potting soil (Quakertown). Cones were placed in humidity chambers as described above for seed-to-seedling transmission experiments. Plants were watered as needed. Plants were arranged in the same design as for the seed-to-seedling transmission experiments, but 22 plants were used in each trial. At 5 weeks after planting, which represents the stage at which at least six fully expanded leaves were present, plants were used for xylem sap collection. Xylem sap was collected following the methodology of Satoh et al. (1992). Briefly, stems were cut approximately 1 cm above the root, and the cut surface was surface sterilized with bleach (10% vol/vol). Xylem sap was collected as it pooled on the cut surface. Xylem sap was collected for 4 h with a pipette and placed into a sterile container on ice. Xylem sap from each plant in a seed lot was pooled together to form one sample of 250 µl per seed lot. After the collection period, xylem sap was frozen at –20°C until further analysis. To assess whether *C. beticola* could be identified in the collected xylem sap, DNA was isolated from the sap using the SDS lysis prep described by Dodhia et al. (2021) and used in *C. beticola*-specific qPCR analyses as described by Knight and Pethybridge (2020). qPCR products were subsequently purified using SureClean Plus (Bioline, Memphis, TN, U.S.A.), according to manufacturer's instructions, and sent to MCLAB (San Francisco, CA, U.S.A.) for Sanger sequencing using the same forward and reverse primers (CbCAL-F and CbCAL-R) used in qPCR (Knight and Pethybridge 2020). The resulting forward and reverse sequences obtained were aligned and assembled into a single contig using Geneious software version 9.8.1 (Biomatters, Ltd., San Diego, CA, U.S.A.) before performing a BLASTn search of the NCBI nucleotide collection (https://www.ncbi.nlm.nih.gov) to identify the most similar sequence using the lowest Expect (E) value.

MinION sequencing of sugar beet fruit DNA. Long-read sequencing of fungal ITS PCR products was performed using the MinION (Oxford Nanopore Technologies, Oxford, UK) sequencing platform for 11 sugar beet seed lots: 1, 3, 5, 6, 8, 10, 19, 24, 27, 29, and 30. For each seed lot, three replications were analyzed. Each replication was comprised of DNA isolated from 20 depelleted and surface-sterilized fruit. Sugar beet fruits (pericarp plus true seed) were ground in a mixer mill (Retsch USA, Newtown, PA, U.S.A.) using 4.5-mm ball bearings to grind and homogenize the samples.

DNA was isolated using the DNeasy Plant Mini Kit (Qiagen; Germantown, MD, U.S.A.) following the manufacturer's recommendations. DNA was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, U.S.A/) with the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, U.S.A.). The full-length internal transcribed spacer regions (ITS1 and ITS2) of ribosomal RNA genes were then amplified and sequenced using standard conditions with universal ITS primers ITS1f-Kyo2 and LR3-I, used by Mafune et al. (2019). The PCR included a peptide nucleic acid (PNA) blocking primer 5′-CTTTGGGTTGTGCCAGC-3′ that we designed to inhibit amplification of sugar beet sequence. We obtained the sugar beet DNA sequence between ITS primers ITS1f-Kyo2 and LR3-I and used a similar approach to Lundberg et al. (2013) to design an elongation arrest PNA primer. The sugar beet ITS sequence was split into short *k*-mers of 17 nucleotides in length using the str_split_fixed function in R version 3.6.3 (R Core Team 2017), and we queried for exact matches in the NCBI fungal ITS database. The chosen primer was a 17-nt sequence with no significant similarity to any NCBI fungal ITS sequences and an annealing temperature approximately 10°C higher than the ITS1f-Kyo2 primer, extension of which it would block. The PNA blocking primer was at 0.4 µM concentration in a 25-µl PCR. PCR products were purified using SureClean (Bioline, London, UK). To demonstrate that the sequencing method was robust with high reproducibility for a single biological sample, we additionally set up three ITS PCRs from the same DNA sample (seed lot 19, biological replicate number 1) and performed MinION sequencing. For all PCRs, agarose gel electrophoresis was used to confirm the presence of an amplicon at approximately 1,000 bp.

Barcodes were attached to each of 11 purified PCR products per run using the Rapid PCR Barcoding kit (Oxford Nanopore Technologies) and LongAmp Taq 2× master mix (New England BioLabs, MA, U.S.A.) according to the Oxford Nanopore Technologies protocol. Barcoded amplicon libraries were purified with AMPure XP magnetic beads (Beckman Coulter, CA, U.S.A.) and resuspended in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. Barcoded libraries were pooled in equimolar concentrations (approximately 9 ng/µl) to a total of approximately 100 ng, then loaded onto a MinION flow cell (R9.4.1, Oxford Nanopore Technologies) according to the manufacturer's instructions. MinKNOW software (version 2.0, Oxford Nanopore Technologies) was used to execute sequencing, and raw reads (fast5) were accumulated over 48 h with live base-calling (fast option) to output fastq files. Reads were demultiplexed in real-time using MinKNOW to output reads into a separate directory per barcode. Raw reads can be found at NCBI under BioProject PRJNA681640.

The downstream processing of data was performed based on the protocol by Mafune et al. (2019) with deviations outlined below and using the python scripts available at https://github.com/mycoophile/nanopore-ITS. Fastq files were filtered through NanoFilt version 2.6.0 (De Coster et al. 2018) for a Q-score of 10. Because the following pairwise sequence alignment step requires considerable memory and processing power, we randomly selected 5,000 sequences per barcode to analyze, using the sample function of seqtk (Li 2013). Sequences were aligned per barcode using the global pairwise alignment option, -gins1 in MAFFT v7.402 with reduced gap penalties using options –op 0.5 and –gop 0.5 (Katoh and Standley 2013). Seqret (part of EMBOSS suite of tools) (Rice et al. 2000) was used to convert the fasta alignment files to phylip format using the -osformat phylip option. A distance matrix was calculated for each alignment using the F84 model in PHYLIP version 3.697 (Felsenstein 2004) dnadist with default parameters. Operational taxonomic unit (OTU) clustering was performed using the OptiClust method in Mothur version 1.44.1 (Schloss et al. 2009) using 92% sequence similarity (cutoff = 0.08). OTUs were organized into clusters for subsequent MAFFT alignment (same options as before) using the fasta_otu_collater2.py script (Mafune et al. 2019). OTUs with <10 sequences were removed from further analysis, since BLASTn searches of consensus sequences from low abundance OTUs (under 10 sequences) tended to give uncertain results at the genus level (≤85% identity score). OTUs were aligned using MAFFT, and an ungapped consensus sequence was produced from the alignment using the OTU_UnGapCons_v4.py script. The consensus sequences were used to perform BLASTn of the NCBI database. Taxon identities were assigned based on the BLASTn hit(s) with highest query coverage, followed by lowest E value and highest percentage identity (>85%). The number of reads assigned to a particular taxon was converted to relative abundance (%) by calculating the percentage of total classified reads represented by that taxon. The mean relative abundance of each identified taxon over three biological replicates was calculated per seed lot. The resulting graphs were generated using the ggplot2 package version 3.3.3 (Wickham 2011) with R version 3.6.3 (R Core Team 2017). Pearson's product-moment correlation coefficient was calculated in R version 3.6.3 (R Core Team 2017).

For the seed lot DNAs mentioned above, the *C. beticola*-specific qPCR described by Knight and Pethybridge (2020) was performed using three biological replicates of each sample. The mass of *C. beticola* DNA in picograms (pg) within the sample was inferred using a linear standard curve of known DNA masses plotted against Ct values. DNA mass was converted to picograms of DNA per milligram of seed material (pg/mg) using the original mass of seed tissue used for DNA extraction. To determine correlations between the results obtained for detection methods (see Results section), Pearson's product moment correlation coefficients were calculated in R version 3.6.3 (R Core Team 2017).

RESULTS

Isolation and identification of fungi from sugar beet fruit tissues. After 7 to 10 days, fungal growth suggestive of *C. beticola* was identified in 27% of seed lots. Fungal growth was initiated from pelleted (Fig. 2A) or depelleted (Fig. 2B) fruit, or the hypocotyl of a germinated seed (not shown). Using species-specific qPCR of the calmodulin gene in fungal isolates obtained from depelleted fruit, 38 *C. beticola* isolates were confirmed from 10 seed lots (Table 1). Seed lots 3, 6, and 10 harbored the most *C. beticola* with isolation incidences of 12, 14, and 30%, respectively. The most common fungal species identified via media culture of depelleted fruit and ITS sequencing was *C. beticola* (*n* = 38), but we also recovered 30 isolates of *Alternaria* spp. from 12 seed lots (Supplementary Table S2). We also identified *Aspergillus* spp. (*n* = 8), *Fusarium* spp. (*n* = 8 isolates), *Cladosporium* spp. (*n* = 6), *Byssochlamys spectabilis* (*n* = 4), *Mucor* spp. (*n* = 3), *Phialemonium* spp. (*n* = 3), *Penicillium* spp. (*n* = 2), *Trichoderma* spp. (*n* = 2), *Actinomucor* spp. (*n* = 1), *Corticum* spp. (*n* = 1), and *Pseudozyma* spp. (*n* = 1).

Dissection of true sugar beet seed and isolation of fungi. To identify the compartment(s) that may harbor *C. beticola*, we dissected processed sugar beet fruit (seed) (Fig. 1) from lots 6 and 10 because they had the highest relative abundances of *C. beticola* in initial isolation studies. From seed lot 6, seven dissected fruits harbored *C. beticola* only in the pericarp (7.7% of those tested). We also recovered *C. beticola* from the pericarp and associated true seeds in two dissected fruits. From seed lot 10, eight dissected fruits had *C. beticola* exclusively in the pericarp (9.8% of those tested) and one dissected fruit grew *C. beticola* from both the pericarp and associated true seed. In no case were isolates recovered from only the true seed. No *C. beticola* isolates were recovered from the separated pellet material. *Alternaria* spp. isolates were additionally identified in the pericarp of one dissected fruit from seed lot 6 and three dissected fruits from seed lot 10.

Fungicide resistance profiling of *C. beticola*. We assayed 38 *C. beticola* isolates derived from sugar beet fruit for resistance to both QoI and DMI fungicide classes. SSR marker analyses revealed that all 38 *C. beticola* isolates were nonclonal, both within and between seed lots (Supplementary Table S1). All 38 isolates contained the G143A mutation associated with QoI resistance

(Bolton et al. 2013) and were therefore considered QoI-resistant (Table 1). For tetraconazole, six isolates had EC_{50} values below 1.0 µg/ml and were considered DMI-sensitive (Bolton et al. 2012a). The remaining 32 isolates had EC_{50} values over 1.0 µg/ml, demonstrating the majority (84%) had reduced sensitivity to DMI fungicides.

Seed-to-seedling transmission efficiency assays. To determine whether seedborne *C. beticola* could initiate disease in sugar beet, we planted pelleted sugar beet seed from seed lots 1, 3, and 10 and observed developing plants for CLS symptoms (Fig. 3). Seed lot 1 was chosen because no *C. beticola* isolates were recovered from this variety (Table 1) and, therefore, it acted as a negative control. In contrast, we were successful in isolating several *C. beticola* isolates from seed lots 3 and 10 (Table 1). Sugar beet plants were observed for 13 weeks, and the same experiment was conducted as two separate trials. In the first trial, 54 lesions were observed among the 12 plants in seed lot 3, with an average of 4.5 lesions per plant (Table 2). Likewise, 132 lesions were observed among the 12 plants in seed lot 10, with an average of 11 lesions per plant. No lesions were observed on plants sown from seed lot 1. In the second trial, 21 lesions were observed for 12 plants in seed lot 3, with an average of 1.75 lesions per plant (Table 2). For seed lot 10, 25% of the plants died (3 of 12), but the remaining 9 plants harbored 789 lesions and an average of 87.67 lesions per plant. The seed-to-seedling transmission rate was 0% for seed lot 1 in both trials, 75% for seed lot 3 in both trials, 75% for seed lot 10 in trial 1 and 100% for seed lot 10 in trial 2. Examples of sugar beet leaves with CLS lesions from seed lot 10 are shown in Figure 3. Isolations from 10 randomly selected CLS lesions per seed lot were all confirmed to be *C. beticola* using the species-specific qPCR described by Knight and Pethybridge (2020).

Xylem sap analysis from infected sugar beet plants. Using DNA extracted from xylem sap, we detected *C. beticola*-specific qPCR amplicons from seed lot 10, but not seed lots 1 or 3, in both trials. To ensure the obtained amplicons were from *C. beticola*, qPCR products were sequenced, and the resulting sequence (GenBank accession no. MW589637) that exhibited no sequence polymorphism from any sap-derived strain was used as a query at GenBank. All amplicons were 100% matches to *CbCAL* (partial calmodulin gene, GenBank accession number AY840425.1).

MinION sequencing of fruit DNA to identify seedborne fungi. To establish a rapid, nonculture-based technique for detecting *C. beticola* and other fungi present in seed lots, we performed long-read MinION sequencing of fungal ITS PCR products from 11 sugar beet seed lots (1, 3, 5, 6, 8, 10, 19, 24, 27, 29, and 30). Seed lot 1 was chosen because we had not been able to grow *C. beticola* from this seed lot, while seed lots 3, 6, and 10 were chosen because they harbored the most *C. beticola* isolates from seed. The remaining seven seed lots were chosen at random. For seed lot 1, biological replicate number 1, PCRs were performed both with and without a newly designed peptide nucleic acid (PNA) blocking primer, and we observed that its presence reduced the relative abundance of *B. vulgaris* reads substantially from 72.5 to 3.8% (Fig. 4). Therefore, we incorporated PNA-based blocking in the remainder of PCRs for the other seed lots and biological replicates. We accumulated enough reads (from approximately 20K to 150K per sample) for downstream analyses in under 24 h for 11 barcoded samples loaded simultaneously. We first attempted to utilize the "What's In My Pot" (WIMP) workflow (Juul et al. 2015) but found it was inappropriate for classifying reads of this length (approximately 1 kb) and often misclassified individual reads when compared with manual BLASTn analysis (not shown). Instead, we used a pipeline established by Mafune et al. (2019) for identifying fungal species from ITS amplicons sequenced with a MinION. The results obtained for each of three biological replicates are shown in Supplementary Table S3. The relative abundance of each species identified was highly consistent between technical replicates of seed lot 19 biological replicate 1 (Supplementary Fig. S1A), the differences in abundance ranging from 1.6 to 3.9% with the highest difference being in *B. vulgaris* reads. Three different subsamples of 5,000 reads were extracted from the same sequenced sample and downstream analysis produced near-identical results, with differences in relative species abundance ranging from just 0.9 to 1.6% (Supplementary Fig. S1B).

The mean proportion of reads classified as different taxonomic groups are shown for each of the 11 seed lots (Fig. 5). In total, we identified 19 different fungal genera present on sugar beet fruit. In all seed lots except 5 and 6, *Fusarium* was the most highly represented fungal genus. *Alternaria* was the second most represented genus and made up an average of 89% of classified reads in seed lot 5. *Cercospora* was the third most prevalent genus throughout seed lots. Reads matching to members of the genus *Cercospora* were found in every seed lot tested, although the relative abundance varied from 0.4 to 48.7%. There was a strong positive correlation between mean relative abundance of *Cercospora* reads and the number of *C. beticola* isolates grown from a seed lot (Pearson's product-moment correlation, $r = 0.82$, $P = 0.002$) (Fig. 6). There was also a positive correlation between mean relative abundance of *Alternaria* reads and the number of *Alternaria* spp. isolates grown from a seed lot (Pearson's product-moment correlation, $r = 0.71$, $P = 0.014$) (Fig. 6). However, there were no significant correlations between *C. beticola* DNA quantity measured using qPCR and relative read abundance (Pearson's product-moment correlation, $P = 0.2012$) (Fig. 6). There was a higher variability in the relative abundance of reads between biological replicates for seed lots harboring more *C. beticola* or *Alternaria* (Fig. 6). We also noticed consistent differences between biological replicates throughout seed lots. For example, the highest proportion of unclassified reads for each seed sample tended to be in biological replicate 3 (Supplementary Table S3).

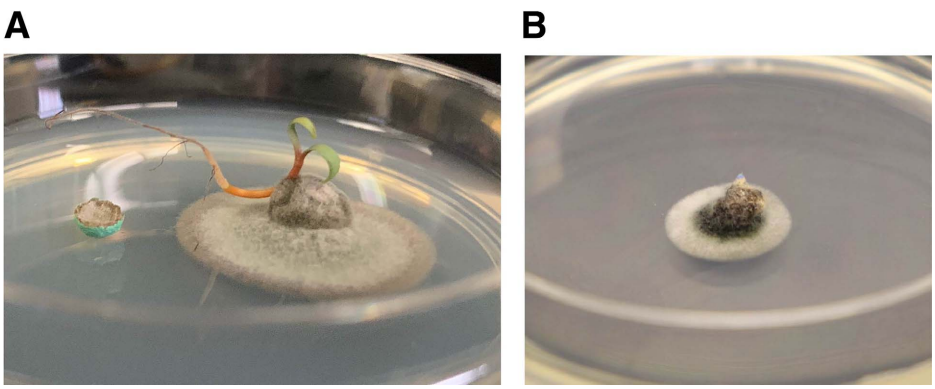

Fig. 2. Fungal growth from pelleted sugar beet fruit. Fungal growth was either **A,** monitored directly from pelleted seed or **B,** from surface-sterilized depelleted sugar beet fruit and was later confirmed to be *Cercospora beticola* using species-specific qPCR.

DISCUSSION

Seedborne pathogens present a considerable threat to agriculture in today's global trade scenario. By surviving in seed, pathogens can be widely distributed by humans across natural boundaries and consequently introduced into new areas (Kumar 2020). Therefore, rapid and reliable detection methods for seedborne pathogens and novel decontamination treatments of infested seed lots are important technological advances for managing seedborne diseases (Boelt et al. 2018; Mancini and Romanazzi 2014; Olesen et al. 2011). The observed recent spread of new pathogens of vegetable and salad crops, including spinach and Swiss chard (Amaranthaceae; relatives of sugar beet), via seedborne mechanisms was found to be favored by market globalization and/or global warming (Gilardi et al. 2018; Gullino et al. 2019). For sugar beet, the long-distance movement of *C. beticola* has been evidenced indirectly in several recent population genetic studies through the identification of recurrent clonal lineages across continents (Knight et al. 2019a; Vaghefi et al. 2017a). Spatial and temporal shifts in field genotypes of *C. beticola* have also been associated with the use of external seed sources (Knight et al. 2018; Vaghefi et al. 2017c). Furthermore, anecdotal evidence has long suggested the presence of seedborne *C. beticola* and its association with CLS epidemics in sugar beet (Jacobsen and Franc 2009; Richardson and Noble 1968; Schürnbrand 1952; Vereijssen et al. 2004). We suspected that seed transmission may play a role in CLS after we identified isolates of *C. beticola* originating from Sweden with quinone outside inhibitor (QoI) resistance in 2011 (G. A. Secor and M. D. Bolton, *unpublished data*), despite a low frequency of QoI fungicide applications for sugar beet disease management in Sweden before 2012 (Anne Lisbet Hansen, personal communication). Long-distance movement of these QoI-resistant isolates to Sweden via infested seed from seed production areas in a different country(s) where QoI fungicides were utilized was hypothesized as a logical explanation for these observations. Alternatively, QoI fungicide use in nonsugar beet crops may have been selected for QoI-resistant *C. beticola* strains growing saprophytically in Swedish soils or on alternative weed hosts.

Fig. 3. Cercospora leaf spot lesions on sugar beet that developed from seedborne *Cercospora beticola*. The photographs show the adaxial surfaces of two harvested leaves taken from 13-week-old plants that developed from seed lot 10.

In this study, we identified viable *C. beticola* in 10 distinct sugar beet seed lots and demonstrated the ability of seedborne *C. beticola* to act as primary inoculum and initiate disease. Similar findings were presented by McKay and Pool (1918) using unprocessed multigerm seed, but no other study has provided direct evidence for seedborne transmission in this pathosystem for more than 100 years. Seed transmission of *C. beticola* was demonstrated for table beet by Knight et al. (2020), who found that seedborne *C. beticola* could cause CLS in seedlings.

During industrial seed processing, sugar beet fruits are usually polished to remove parts of the outer pericarp (Fig. 1) to aid subsequent pelleting (Ignatz et al. 2019; Kockelmann et al. 2010). As an initial indication of the presence of *C. beticola* in commercially prepared sugar beet seed, we attempted to isolate the fungus from pelleted seed using plate growth assays. The pelleted seed yielded many fast-growing fungi that quickly outcompeted other slow-growing fungi, including *C. beticola*. Consequently, we opted to remove seed pellets for the remainder of the seed screening assays. Ten of the 37 sugar beet seed lots tested had fungal growth identified as *C. beticola*. In addition to *C. beticola*, several other fungal and bacterial species were also identified. In fact, the abundance of microflora in sugar beet seed often increased the amount of time necessary to purify *C. beticola* from other fungal species in isolation studies, and its slow-growing nature often led to it being overgrown by contaminating species. Consequently, the targeted and sensitive method of detecting *C. beticola* by qPCR, as described by Shrestha et al. (2020) and Knight and Pethybridge (2020), is useful for sequence-specific detection of this pathogen.

Historically, the culturing of fungi has been critical for detection and identification based on morphology. However, this can limit identification to culturable fungi present in relatively high abundance and/or are faster growing than the species of interest (Huffnagle and Noverr 2013). Because we were also interested in obtaining a comprehensive overview of the fungal species diversity present in sugar beet fruit, we sequenced the full-length ITS1 and ITS2 rRNA

TABLE 2. The results for seed-to-seedling transmission across two separate trials to test transmission of *Cercospora beticola* in sugar beet seed lots 1, 3, and 10[a]

Trial	Seed lot	Lesions[b]			Transmission frequency[d]
		Mean[c]	Standard deviation	Sum	
1	1	0.00	0.00	0.00	0.00
	3	4.50	4.50	54.00	0.75
	10	11.00	10.51	132.00	0.75
2	1	0.00	0.00	0.00	0.00
	3	1.75	1.42	21.00	0.75
	10	87.67	51.73	789.00	1.00

[a] The table reports the mean number of Cercospora leaf spot lesions per plant for 12 total plants for each seed lot, along with the standard deviation and total sum. The transmission frequency (%) for each seed lot is reported for each trial and is the frequency of 12 plants that went on to exhibit at least one lesion.
[b] Cercospora leaf spot lesions identified by characteristic signs and symptoms on sugar beet leaves.
[c] Mean number of cercospora leaf spot lesions identified per sugar beet plant.
[d] The frequency of sugar beet plants exhibiting at least one CLS lesion, therefore having undergone transmission of *C. beticola* from seed to seedling.

regions (approximately 1 kb) from seed-derived DNA using the MinION nanopore sequencing platform. Our results suggested that long-read amplicon sequencing largely replicates the results of culturing, in the case of the two dominant fungal genera *Cercospora* and *Alternaria*, and could be employed as an alternative detection method to simultaneously detect multiple fungal pathogens in fruit. We additionally identified *Cercospora* reads in seed lots that we could not isolate *C. beticola* from in plate growth assays, supporting the use of molecular-based assays to detect fungal contamination in seed lot batches that otherwise stay undetected. It is also possible these seed batches harbor unviable *C. beticola* and, consequently, do not represent potential risks for crop production. Such research will be the focus of future studies.

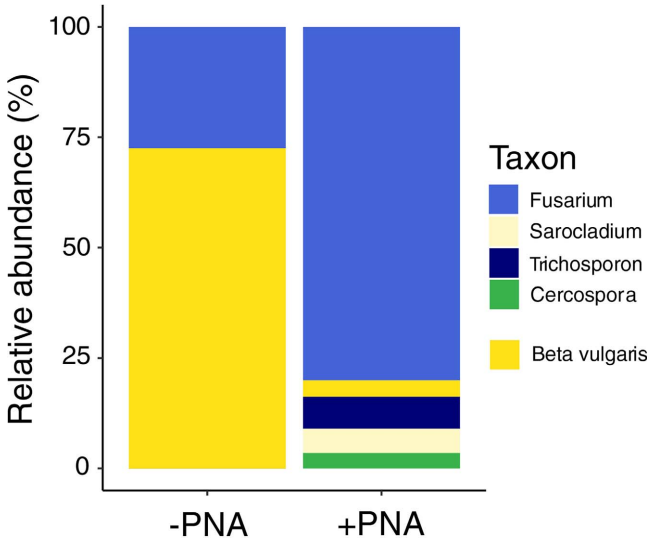

Fig. 4. Relative abundance of taxa identified through MinION sequencing of internal transcribed spacer (ITS) amplicons in the same biological sample (seed lot 1, biological replicate 1) both without (–) and with (+) a peptide nucleic acid (PNA) blocking primer included in the initial ITS PCR. The PNA blocking primer was designed to specifically inhibit amplification of sugar beet ITS sequence. The relative abundance is the proportion of total classified reads (%) assigned to a specific taxon.

To investigate seed-to-seedling transmission of *C. beticola*, we planted pelleted seed from two seed lots that were infested with *C. beticola* and seed lot 1 from which no *C. beticola* was isolated. The majority of plants that developed from the two infested seed lots developed CLS symptoms (75 to 100%), whereas the seed lot that was not apparently infested lacked any symptoms during the period of observation used in this study. Given the disease was specifically detected in seed lots 3 and 10, our results strongly suggest that seedborne *C. beticola* can initiate disease in sugar beet. Because we found 75 to 100% transmission rates for *C. beticola* in seed lots 3 and 10, it appears that seedborne inoculum could represent a substantial primary inoculum source in sugar beet fields. We note that this suggests that *C. beticola* was present on nearly all of the seed sown although *C. beticola* was isolated at a relatively low frequency from seed lots 3 and 10 in the plate growth assays (12 and 30%, respectively). If a very small amount of fungal material is present on seed and/or is not exposed sufficiently to the media or provided optimal environmental conditions for growth, it is unlikely to grow. There is also the issue of other organisms, such as bacteria and other fungi, outcompeting and overgrowing slow-growing *C. beticola* in vitro. Furthermore, *C. beticola* may be more competitive and transmissible within the tissues of its adapted host. Taken together, the transmission study results suggest that more sensitive detection methods, such as qPCR, are required to identify viable *C. beticola* in seed lots. Artificial seed inoculation and field experiments can be performed in the future to establish the threshold of seedborne *C. beticola* required, under conducive conditions, to initiate an economically relevant field epidemic. Although *Alternaria* was isolated from sugar beet fruit, no *Alternaria* lesions were identified in our seed to seedling assays. This may indicate that our humidity chamber conditions were suboptimal for this pathogen or that *Alternaria* inoculum in the seed is not able to cause disease. Future studies will be directed to assess whether *Alternaria* is also a seedborne pathogen of sugar beet.

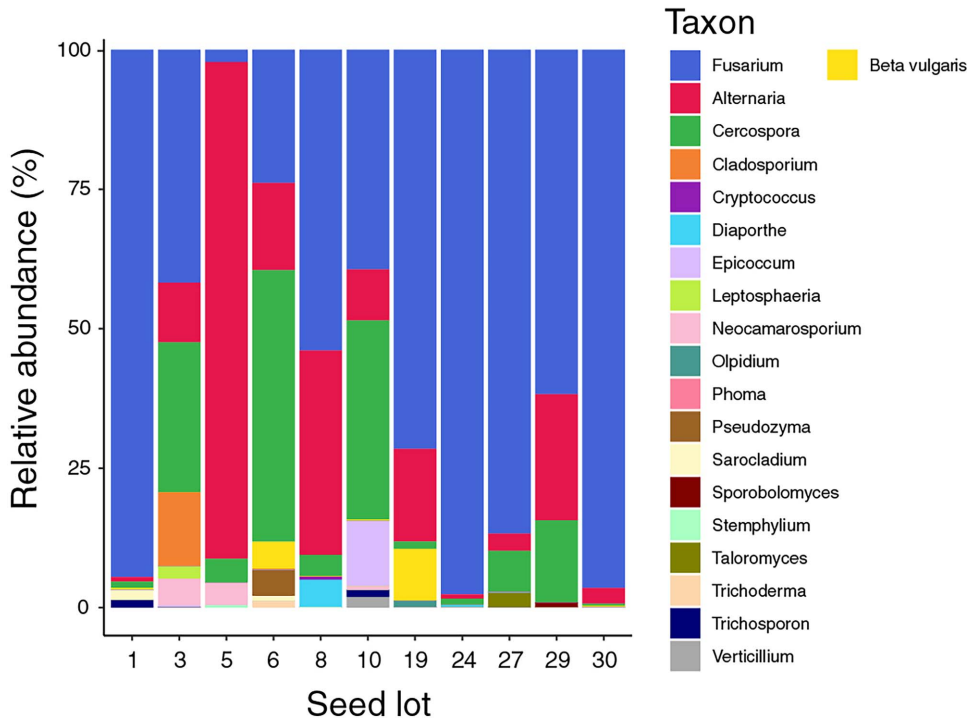

Fig. 5. Relative abundance of taxa identified in 11 sugar beet seed lots through MinION sequencing of internal transcribed spacer (ITS) amplicons. The relative abundance is the proportion of total classified reads (%) assigned to a specific taxon and is a mean value of three different biological samples from each seed lot.

We detected the presence of *C. beticola* in xylem sap via species-specific qPCR, suggesting the fungus may utilize the vascular system to spread upwards through the sugar beet plant to the foliage. It was previously demonstrated that root infection of sugar beet seedlings by *C. beticola* can give rise to leaf symptoms (Vereijssen et al. 2004, 2005), and it was deemed unlikely to have occurred through epiphytic growth due to the low frequency of stem lesions. Further microscopic and molecular studies are required to establish precisely how *C. beticola* spreads from the germinated seed to initiate foliar disease.

To remove *C. beticola* as a source of inoculum for CLS disease, sugar beet fruit may require additional treatment. The pellet from processed sugar beet fruit often contains fungicides for the management of various seedling diseases caused by pathogens such as *Pythium* spp., *P. betae*, *Aphanomyces cochlioides*, or *Rhizoctonia solani* (Harveson et al. 2009). Because seedborne *C. beticola* has not previously been documented from processed sugar beet seed, testing the efficacy of pellet fungicides or various mechanical seed processing procedures for CLS management has not been carried out to our knowledge. Seed treatment selection may depend on pathogen localization within the processed seed. In the present study, we identified *C. beticola* within fruit pericarp from two seed lots infested with the fungus. Three individual sugar beet fruits (of 17 harboring the fungus) had *C. beticola* growing from both the pericarp and the true seed, perhaps suggesting the fungus had transferred to the true seed's testa from the pericarp. It is also possible that the fungus moved to the testa via the xylem stream during seed development, as a vascular bundle has been shown to extend into the chalazal region of the seed coat (Esau 1967). To shed light on colonization events during seed production and germination, investigations could be performed to monitor a fluorescent-tagged *C. beticola* strain using confocal laser-scanning and electron microscopy (Maruthachalam et al. 2013).

Because our study suggests that *C. beticola* colonizes the pericarp of sugar beet fruit, physical treatment such as hot water or chemical treatment may be required to eliminate the fungus (Taylor and Harman 1990). Biological control methods such as *Pseudomonas* spp. and *Trichoderma* spp. reduced colonization of *Pythium ultimum* in sugar beet pericarps, suppressing seedling damping-off in a comparable manner to fungicides (Georgakopoulos et al. 2002; Osburn et al. 1989; Taylor and Harman 1990). Select beneficial microorganisms, with optimized pH and nutrient sources, may also function to suppress *C. beticola* colonization of the sugar beet pericarp as shown for other pathosystems (Taylor and Harman 1990). Soaking seed (fruit) in formaldehyde was demonstrated by McKay and Pool (1918) to reduce *C. beticola* inoculum, but efficacies of modern fungicidal seed treatments are, to our knowledge, yet to be explored.

The presence of the QoI-resistant genotype (100%) and reduced DMI sensitivity (84%) in fruit-derived *C. beticola* isolates suggest these isolates originated from growing areas where fungicides are routinely used, which drives selection for resistant strains in the population. It is also important to consider the seedborne movement of these strains could facilitate the spread of fungicide resistance across continents, reducing the efficacy of current fungicide chemistries. To our knowledge, no report has previously documented the movement of fungicide-resistant isolates of any species via seed. Ideally, sugar beet seed would be produced in areas where *C. beticola* does not thrive and disease is rare. However, if this cannot be the case, chemical treatments may need to be considered during seed processing to manage *C. beticola* in the future. Such seed treatments should be from effective FRAC groups with a low risk

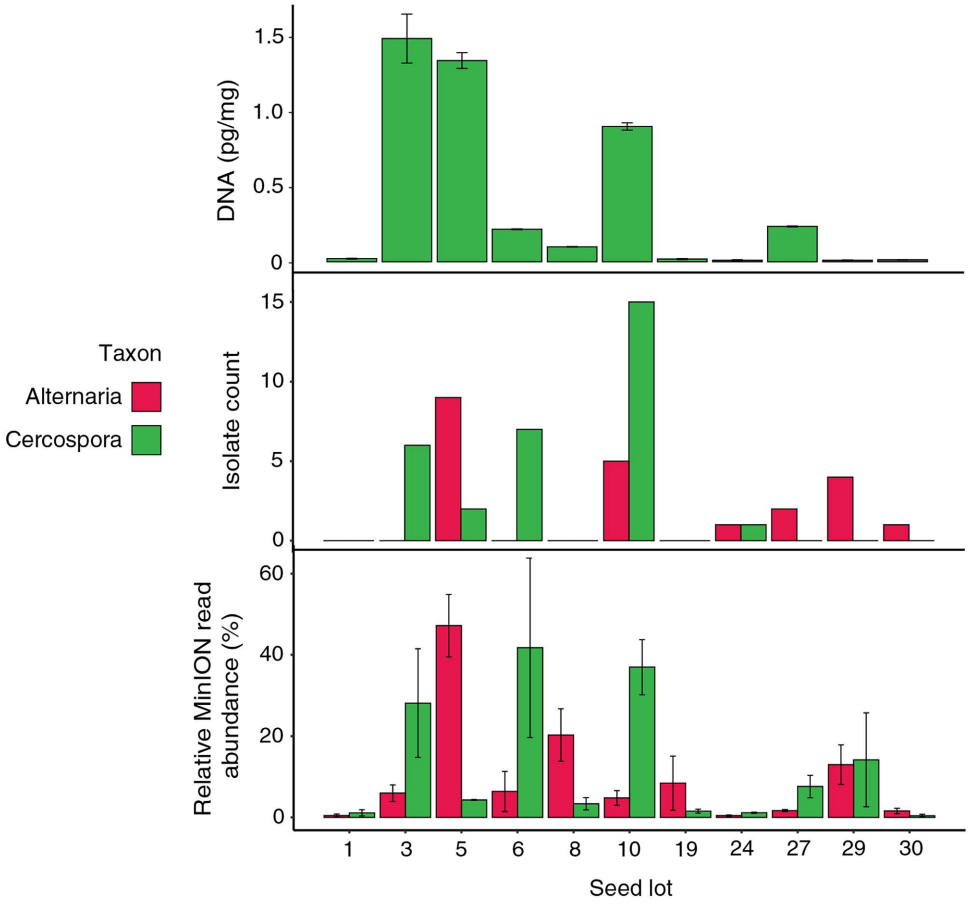

Fig. 6. The amount of *Cercospora beticola* DNA detected in 11 sugar beet seed lots (pg/mg of seed material) using qPCR (top panel), the number of *Cercospora* and *Alternaria* spp. isolates grown from each seed lot (middle panel), and the mean relative read abundance (± standard error, SE) of *Cercospora* and *Alternaria* spp. (%) identified through MinION sequencing of internal transcribed spacer (ITS) amplicons of three biological replicates (bottom panel). The relative read abundance is the proportion of total classified reads (%) assigned to the taxon.

of resistance development. In the current study, *C. beticola* grew directly from nine different commercial seed lots (European and United States) and seed-to-seedling transmission was demonstrated for two of these. Therefore, current seed production treatments may not be effective in managing seedborne *C. beticola*.

The dynamics of seedborne inoculum in field-based CLS epidemics should be investigated further. For example, it is currently not known what quantity of inoculum is required to initiate an epidemic. Further studies looking at the host and environmental factors affecting seed-to-seedling transmission rates will also need to be carried out. Because CLS is a polycyclic disease, it is likely that very few instances of seed-to-seedling transmission are required in a field to initiate a severe epidemic, granted that the host is susceptible and environmental conditions are conducive (Knight et al. 2020; McGee 1995). Rapid, sensitive, and accurate seed testing procedures, such as qPCR, should be implemented to be able to detect *C. beticola* at a sufficient threshold to initiate disease. Seed may need to be routinely tested and certified in the future to demonstrate that it is free of *C. beticola* inoculum.

The fungal microbiome of sugar beet fruit has previously been investigated using culturing techniques (Bugbee 1974; Kowalik and Lechowicz 1984; Singh et al. 1974), but to our knowledge, our work is the first study to generate a comprehensive profile of fruit mycoflora using deep sequencing. Intriguingly, we find the presence of other, potentially viable, fungal pathogens in addition to *C. beticola* in processed sugar beet seeds. In agreement with previous studies, we found long-read ITS amplicon sequencing using the MinION to be a rapid and cost-effective method for profiling microbial communities (Benítez-Páez et al. 2016; Calus et al. 2018; Kerkhof et al. 2017; Kilianski et al. 2015; Mafune et al. 2019). The three most abundant taxa found on sugar beet fruit were *Fusarium*, *Alternaria*, and *Cercospora* and were identified on every seed lot tested. In total, we identified 19 different fungal genera via long-read sequencing. In addition to *Cercospora*, some of these genera contain known pathogens of sugar beet, such as *Fusarium oxysporum* f. sp. *betae* causing *Fusarium* yellows (Webb et al. 2019), *Fusarium secorum* causing Fusarium yellowing decline (Secor et al. 2014), various *Alternaria* spp. causing Alternaria leaf spot (McFarlane et al. 1954), known seedborne pathogen *P. betae* causing Phoma leaf spot and root rot (Vaghefi et al. 2019), *Stemphylium* spp. causing Yellow leaf spot (Hanse et al. 2015) and *Verticillium dahliae* causing Verticillium wilt (Karadimos et al. 2000). In the present study, we identified viable *Alternaria* spp. within the pericarp of sugar beet fruit. However, further experiments will be needed to determine whether the remaining identified species are viable on processed seed and can cause disease in seed-to-seedling assays. As well as being present in the sugar beet fruit mycobiome, *Fusarium* spp. and *Alternaria* spp. were previously described in the sugar beet phyllosphere (Pusenkova et al. 2016; Thompson et al. 1993; Zachow et al. 2008) and rhizosphere (Huang et al. 2020; Pusenkova et al. 2016). Ascomycota is consistently the dominant phylum in each study, but the relative abundance of families varies, which may be dependent on the environment, tissue microenvironment, and/or identification method (culturing or direct tissue sequencing).

In our study, we were able to confidently assign OTUs to the genus level. Variation between different technical replicates from the same DNA sample was consistent but highest for *B. vulgaris*. This could be attributed to the varying efficacy of the sugar beet-specific ITS blocking primer between PCRs used to inhibit amplification of sugar beet ITS sequences. DNA from all seed lots was extracted, amplified, and sequenced in three separate batches, representing the three biological replicates. We observed consistent differences between biological replicates suggesting the procedure may be sensitive to variability between DNA extractions, individual PCR setups, and/or library preparations despite consistent parameters being used. For downstream analysis, we demonstrated that subsampling of 5,000 reads was sufficient to capture a reliable representation of the fungal profile. Several of the fungal genera that we detected in long-read sequencing are known for having identical ITS sequences shared among different species, such as *Cercospora* (Groenewald et al. 2005) and *Fusarium* (O'Donnell and Cigelnik 1997), and may require additional markers to define species. We also note that we identified *Fusarium* spp. to have the highest number of fungal reads in multiple seed lots, but this may not reflect the real relative abundance in terms of fungal biomass. ITS amplicon sequencing can result in overrepresentation or underrepresentation of certain groups when quantifying abundance (Loit et al. 2019; Mafune et al. 2019), caused by a variable number of rRNA genes between fungal species (Schoch et al. 2012) or PCR biases (Bellemain et al. 2010). To overcome this, a community standard can be included as described by Taylor et al. (2016), Benítez-Páez and Sanz (2017), Bakker (2018), and Mafune et al. (2019). Inclusion of a community standard (a mock community with known DNA quantities) for each sequencing run may also help to normalize variation in relative abundance between experiments. The method and pipeline used in this study also failed to confidently identify OTUs comprised of 10 or fewer reads. Because these low abundance OTUs could not be confidently classified, they could be taxa without available reference sequences in the database or with too few reads to obtain an accurate consensus sequence. Alternatively, these OTUs could be artifacts of a nonoptimal MinION read clustering process. Nanopore sequencing still has a low base-call accuracy of around 85% when compared with 99% in short read technologies (Jain et al. 2017; Rang et al. 2018), particularly in homopolymer regions, which can be challenging for clustering shorter amplicons (such as approximately 1 kb in this study) into OTUs. After executing the analysis pipeline established by Mafune et al. (2019), there were a large number of these low abundance OTUs (10 or fewer reads) and, therefore, a seemingly high percentage of reported unclassified reads (31 to 85%). Other nanopore mycobiome studies have also shown low proportions of classified reads: 7 and 24% of reads were reported as classified by Piñar et al. (2020) using the WIMP workflow, and multiple small unclassifiable OTUs were reported in other pipelines (Davidov et al. 2020; Hu et al. 2019; Mafune et al. 2019). Future improvements in nanopore chemistry to minimize base-calling errors could overcome some of these issues. Moreover, the development of new software specifically for aligning and clustering MinION reads of amplicons derived from complex communities would be helpful for more accurate classification and reduced computational load.

In summary, we propose that processed sugar beet seed should be considered as a primary inoculum source in the management of CLS in the future. New management strategies may include routine seed testing for the presence of *C. beticola* and treatment of raw or pelleted fruit to reduce the fungal density. Furthermore, through investigation of the fungal mycobiome of sugar beet fruit, we identified the presence of potentially viable seedborne fungi from multiple genera that harbor common sugar beet pathogens, including *Alternaria* and *Fusarium*, which could also play important roles in disease epidemics. Future investigations will clarify how *C. beticola* initiates disease from the processed seed and the epidemiological importance of seedborne inoculum in field epidemics of CLS in sugar beet.

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