Cercospora beticola: The intoxicating lifestyle of the leaf spot pathogen of sugar beet

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Abstract
Cercospora leaf spot, caused by the fungal pathogen Cercospora beticola, is the most destructive foliar disease of sugar beet worldwide. This review discusses C. beticola genetics, genomics, and biology and summarizes our current understanding of the molecular interactions that occur between C. beticola and its sugar beet host. We highlight the known virulence arsenal of C. beticola as well as its ability to overcome currently used disease management strategies. Finally, we discuss future prospects for the study and management of C. beticola infections in the context of newly employed molecular tools to uncover additional information regarding the biology of this pathogen.

Taxonomy: Cercospora beticola Sacc.; Kingdom Fungi, Phylum Ascomycota, Class Dothideomycetes, Order Capnodiales, Family Mycosphaerellaceae, Genus Cercospora.

Host range: Well-known pathogen of sugar beet (Beta vulgaris subsp. vulgaris) and most species of the Beta genus. Reported as pathogenic on other members of the Chenopodiaceae (e.g., lamb's quarters, spinach) as well as members of the Acanthaceae (e.g., bear's breeches), Apiaceae (e.g., Apium), Asteraceae (e.g., chrysanthemum, lettuce, safflower), Brassicaceae (e.g., wild mustard), Malvaceae (e.g., Malva), Plumbaginaceae (e.g., Limonium), and Polygonaceae (e.g., broad-leaved dock) families.

Disease symptoms: Leaves infected with C. beticola exhibit circular lesions that are coloured tan to grey in the centre and are often delimited by tan-brown to reddish-purple rings. As disease progresses, spots can coalesce to form larger necrotic areas, causing severely infected leaves to wither and die. At the centre of these spots are black spore-bearing structures (pseudostromata). Older leaves often show symptoms first and younger leaves become infected as the disease progresses.

Management: Application of a mixture of fungicides with different modes of action is currently performed although elevated resistance has been documented in most employed fungicide classes. Breeding for high-yielding cultivars with improved host resistance is an ongoing effort and prudent cultural practices, such as crop rotation,
1 | DOMESTICATION HISTORY OF BETA VULGARIS AND THE IMPACT OF CERCOSPORA BETICOLA ON MODERN FARMING

The world currently relies on sugar beet (Beta vulgaris subsp. vulgaris) for approximately one-fifth of its total sugar (ISO, 2018). While the other major source of sucrose, sugar cane, is grown in more tropical climates, sugar beet is largely grown in temperate regions with the northern tier states in the United States, France, Germany, and Russia being the top producers (FAOSTAT, 2019). All cultivated beets (fodder, chard, table beet, sugar beet) are regarded to be derived from wild Mediterranean sea beet (B. vulgaris subsp. maritima) (OECD, 2001). In the late 18th century, the German chemist Andreas Marggraff showed that sucrose in both white and red beetroot was chemically identical to the prohibitively expensive tropical cane sugar (Marggraff, 1748). Marggraff’s student Franz Karl Achard noticed that conical white fodder beets had high sugar content and began to grow them for sugar production (Fischer, 1989). Achard is credited with establishing the sugar beet industry in Europe by building the first processing factory in Silesia (now Poland) in 1801. Sugar beet was subsequently commercialized in the United States in 1879 (Magnuson, 1918). Continuous breeding over the last 200 years has led to an increase in sugar content from 8% to more than 18% in cultivars grown today (Dohm et al., 2014). Additionally, the discovery of male sterile cytoplasm permitted the development of hybrid varieties for yield increase (Biancardi et al., 2010). Although breeding and improved cultivation practices yielded significant increases in sugar beet production and sugar yield in recent decades, many abiotic and biotic stresses remain that continue to affect sugar beet growth and ultimately attenuate sugar production worldwide.

The most common and destructive foliar disease of sugar beet globally is cercospora leaf spot (CLS) (Holtschulte, 2000). The disease was first described on Beta cicla in Italy by Saccardo (1876) but now has been identified across the globe wherever sugar beet is grown. CLS is most pernicious in warm, humid growing regions (Larney et al., 2010), which make up almost one-third of the total sugar beet production area in the United States (USDA-NASS, 2010). The main adversity is the loss of recoverable sucrose, reaching almost 50% under uncontrolled moderate to high disease pressure (Lamey et al., 1987; Shane and Teng, 1992). Additional economic losses occur as a result of increased impurities that complicate sucrose recovery processes, leading to higher processing costs and reduced extractable sucrose (Shane and Teng, 1992). Diseased plants are also more susceptible to storage rot in winter storage piles (Smith and Ruppel, 1973). Large economic losses were seen in sugar beet crops in southern Germany in the late 1980s and early 1990s due to severe CLS epidemics (Wolf and Verreet, 2005). In the United States, Minnesota and North Dakota suffered losses from CLS in 1998 estimated at $113 million from yield reduction and fungicide application costs (Cattanach, 1999). In these areas, with conducive environmental conditions, it is crucial to appropriately apply fungicides otherwise substantial loss of foliar photosynthetic area can occur due to CLS (Figure 1a,b). The upper Midwest has seen epidemics in three out of the last four years in large part due to fungicide-resistant Cercospora beticola populations that have resulted in several hundred million dollars in lost revenue (Mike Metzger, Minn-Dak Farmers Cooperative, personal communication). A deeper understanding of the C. beticola–sugar beet interaction may allow for the development of innovative strategies to prevent the disease and ultimately cut much of these losses. Here, we discuss the biology of this pathogen and explore the recent advances in the molecular and genetic understanding of CLS.

2 | INFECTION BIOLOGY

The causal agent of CLS, C. beticola, has the ability to complete several asexual cycles within a single season under conducive conditions (McKay and Pool, 1918; Nagel, 1945; Vereijssen et al., 2007). Between sugar beet growing seasons, the fungus survives primarily as desiccation-resistant hyphal structures on infected plant residues within leaf substomatal cavities. These specialized overwintering structures are known as pseudostromata, or false stromata (conidia-producing hyphae), because they are composed of both fungal tissue and remnants of host tissue (Eriksson, 1981). Pseudostromata can persist on plant debris for 2 years and have long been regarded as the primary inoculum sources for infection (Pool and McKay, 1916; Khan et al., 2008). However, many population studies have questioned the role of clonally reproducing primary inoculum (Groenewald et al., 2006, 2008; Knight et al., 2018, 2019). Other potential sources of initial inocula include dispersal of C. beticola-infested plant material via tools or machinery (Knight et al., 2018, 2019), infested seed, windborne conidia, or stromata from other host plants (Khan et al., 2008; Franc, 2010; Skaracis et al., 2010; Tedford et al., 2018; Knight et al., 2020).
Cercospora leaf spot (CLS) of sugar beet. (a) Drone image highlighting the importance of fungicides for disease management. While the disease was well-managed on the right side of the field, an abrupt halt in fungicide application ultimately resulted in increased CLS disease as evidenced by the brown colour noted on the left side of the field. (b) Extensive CLS disease in a sugar beet field. (c) Disease cycle of Cercospora beticola on sugar beet. Infection is initiated by airborne or splash-dispersed conidia that penetrate the sugar beet leaf through stomata and give rise to intercellular hyphal growth. Leaf spots form on the leaves after the switch to necrotrophy, which typically occurs 7 days after infection. Pseudostromata develop in these lesions and asexually produce spores, leading to multiple infection cycles during the growing season. The pseudostromata are also the overwintering structures on plant debris at the end of the season. It is possible that C. beticola can sexually reproduce and produce ascospores, in a similar way to Zymoseptoria tritici, but this stage has not been observed. Extensive CLS disease in a sugar beet field. (d) Scanning electron micrograph exhibiting C. beticola conidiophores emerging from sugar beet. (e) CLS symptomology on sugar beet.
**C. beticola** is an ascomycete fungus only known to exist in the anamorphic (asexual) state (Crous et al., 2001; Groenewald et al., 2013). To reproduce, melanized conidiophores form from pseudostromata to produce conidia in the spring but this may be preceded by saprophytic, vegetative growth of fungal mycelia (Pool and McKay, 1916) (Figure 1c). Transmission electron microscopy has shown that bundles of 10–20 conidiophores (18–25 μm in diameter) often form on both upper and lower leaf surfaces and are produced from subepidermal or substomatal pseudostromata (Pons et al., 1985). Pseudostromata are three to six cells deep and up to eight to 10 cells wide. Conidiophores consist of one or two cells and are 10–25 μm long x 3–5 μm wide at the base (Figure 1d). Conidia are needle-shaped (2–3 x 36–107 μm) and colourless with several cross-walls (Weiland and Koch, 2004). The minimum requirements for conidial development are temperatures of at least 15°C and a relative humidity of 60% or higher (Pool and McKay, 1916; Sole and Minz, 1971a). Through wind, rain, irrigation, water splash, or insect transfer, spores are disseminated and make contact with the abaxial surface of sugar beet leaves or petioles to initiate infection (Lawrence and Meredith, 1970; Khan et al., 2007). Some studies have suggested that roots may also act as primary infection sites (Vereijssen et al., 2005), although the specific requirements for root infection remain elusive (Khan et al., 2008). Germination of conidia is optimal at high relative humidity (near 100%) and at temperatures of approximately 25°C (Ruppel, 1986; Khan et al., 2009). After germination, appressoria are produced, allowing hyphae to penetrate leaf tissue through stomata and spread intercellularly with no visible leaf symptoms (Rathaiah, 1977; Steinkamp et al., 1979). As the fungus switches to its necrotrophic phase, the production of phytotoxins and degradative enzyme activity leads to the necrotizing of infected cells (Steinkamp et al., 1979). Symptoms appear as circular spots 3–5 mm in size and tan to grey in colour, encircled by a tan-brown to red-purple border (Windels et al., 1998) (Figure 1e). Symptoms can develop on older leaves as quickly as 5 days after infection when there are favourable conditions of high humidity (>90%) and warm temperatures (day 27–32°C, night > 17°C) (Pool and McKay, 1916; Sole and Minz, 1971b). In the field, these characteristic CLS lesions are produced from subepidermal or substomatal pseudostromata (Pons et al., 1985). Pseudostromata formed within the lesions become the site for production of new conidia as early as 7 days after infection under favourable conditions (Jacobsen and Franc, 2009). Conidia are again disseminated by wind, rain splash, or insect transfer to initiate another infection cycle. Early studies show evidence for rain splash being the major factor in spore dispersal (Carlson, 1967) while studies by Khan et al. (2008) suggested that wind was the major dispersal factor for *C. beticola* inoculum because higher disease severity was observed on exposed plants when compared to plants in plastic cages or with ground cover. The distance that conidia can travel whilst retaining viability has not been reported, but genetic studies have provided evidence that long-range dispersal has occurred (Groenewald et al., 2008; Vaghefi et al., 2017a; Knight et al., 2018).

### 3 | POPULATION GENETICS

The population biology of *C. beticola* has been extensively explored to quantify genetic structure, gene flow, and genotypic and genetic diversity in space and time. Tools used to study *C. beticola*'s evolutionary ecology have included random amplified polymorphic DNA, amplified fragment length polymorphisms, microsatellites, and single nucleotide polymorphisms (Groenewald et al., 2007; Turgay et al., 2010; Vaghefi et al., 2017b). A consistent theme in these studies has been to provide indirect evidence for sexual recombination, migration, and mutation. These findings have supported the development of hypotheses surrounding pathogen biology and movement with important implications for disease epidemiology and management. For example, the lack of a known teleomorph for *C. beticola* means a substantial gap exists in the knowledge surrounding primary inoculum sources and between-field inoculum movement (Pethybridge et al., 2018).

*C. beticola* populations are generally characterized by high allelic, genetic, and genotypic diversity, typically coinciding with remarkably high phenotypic diversity (Ruppel, 1972; Moretti et al., 2004) that is often exhibited in in vitro colony morphology (Figure S1). Although *C. beticola* is heterothallic (self-sterile) where any one isolate has either mating type (MAT) gene (MAT1-1-1 or MAT1-2-1) at the MAT1 locus, additionally each isolate has MAT1-1-1 and MAT1-2-1 exon fragments located at ostensibly random loci across the genome (Bolton et al., 2014). Interestingly, these MAT fragments could largely reconstitute a hypothetical fused organization of MAT genes, suggesting a homothallic (self-fertile) ancestral state for *C. beticola* and other related species (Bolton et al., 2014). Ratios of isolates with different mating types (MAT1-1 and MAT1-2) within populations have been location-specific. Isolates of one mating type have dominated populations from sugar beet in Iran (Bakhshi et al., 2014) and table beet in New York (Vaghefi et al., 2017). Equal mating type ratios in equilibrium have been characterized in populations from sugar beet fields in Minnesota and North Dakota (Bolton et al., 2012c), and table beet in New York (Vaghefi et al., 2017). Equal mating type ratios together with high genotypic diversity have supported the potential for sexual recombination within populations (Groenewald et al., 2006, 2008; Bolton et al., 2012c). Additionally, microsatellite alleles in linkage disequilibrium when combined with equal mating type ratios and high genotypic diversity in certain *C. beticola* populations from table beet provide evidence to suggest that a mixed reproductive mode is more likely (Vaghefi et al., 2016, 2017c; Knight et al., 2018).

Several studies have also quantified genetic homogeneity and genetic differentiation between *C. beticola* populations on variable spatial scales ranging from different hosts in the same field (Vaghefi et al., 2017c), fields within and between regions (Vaghefi et al., 2016, 2017b), and between continents (Groenewald et al., 2008; Vaghefi et al., 2017a; Knight et al., 2019). High levels of gene flow and low differentiation between *C. beticola* populations on different continents have supported evidence for genetic similarity and panmictic populations and the potential role of infested plant material dissemination.
in pathogen movement and initiation of epidemics (Groenewald et al., 2008; Vaghefi et al., 2017b; Knight et al., 2019). Knight et al. (2019) reported genetic diversity, differentiation, and relationships among 948 C. beticola isolates from 28 populations in eight regions and identified two major clusters. One cluster was specific to New York, with evidence of recent expansion and divergence, and another was common across North and South America, Eurasia, Hawaii, and selected states within the United States (North Dakota and Michigan) with shared origin (Knight et al., 2019). Human-mediated dispersal of C. beticola-infested plant material may be responsible for primary inoculum within individual seasons, while findings from studies that evaluated the temporal stability of populations within fields have challenged the role of overwintering inoculum in epidemic initiation (Knight et al., 2018, 2019).

4 | POPULATION GENOMICS

Genome sequencing has provided valuable insight into the evolution and biology of diverse fungal plant pathogens (Soanes et al., 2007). Genome sequencing of C. beticola was initially aimed at identifying new polymorphic markers for genotyping of field isolates (Vaghefi et al., 2017b) and unravelling biosynthetic gene cluster evolution (de Jonge et al., 2018).

The genome size of C. beticola is comparable to other closely related Dothideomycetes, but the repeat content is considerably lower and includes less than 2% of the entire genome (de Jonge et al., 2018). In comparison, genomes of the two related species Mycosphaerella fijiensis and Cladosporium fulvum belonging to the same order Capnodiaceae have significantly higher repeat contents of 37% and 44%, respectively (Ohm et al., 2012). This indicates the presence of efficient genome defence mechanisms in C. beticola, such as repeat induced point mutations and DNA methylation, that may prevent the propagation of repetitive elements (Cambareri et al., 1989; Gladyshev, 2017).

Population genomic sequencing of some other fungal plant pathogens, including Leptosphaeria maculans and Zymoseptoria tritici, have demonstrated that repetitive elements can associate with the presence/absence variation of genes (Rouxel et al., 2011; Plissonneau et al., 2016). Despite the low repeat content in C. beticola (de Jonge et al., 2018), recent analyses of population genomic data have revealed a substantial number of genes showing presence/absence variation (Spanner and Bolton, unpublished data). It is possible that a dynamic repertoire of effector genes is driven by a coevolutionary arms race between plant and pathogen and facilitates the infection of C. beticola on different hosts.

We analysed North American field populations of C. beticola to assess the amount and distribution of genetic variation and infer about the recent demography of the pathogen. When analysing sequencing data from more than 100 field isolates, we identified approximately 500,000 single nucleotide polymorphisms from which we computed a mean nucleotide diversity of $\pi = 0.0035$ (Potgieter, Bolton and Stukenbrock, unpublished data). We found that $\pi$ varies considerably along the C. beticola genome, including regions with a 10-fold increase in genetic variation. Further analyses should scrutinize patterns of genetic variation along the genome to understand the functional relevance of variation “hotspots” and the underlying drivers of genetic variability. Research on the genome evolution of C. beticola could have great importance in the development of new management strategies, including the identification of new fungicide targets.

Population genomic data can also clarify the demographics within a species (Dutheil and Hobolth, 2012; Excoffier et al., 2013). Different demographic scenarios, such as population bottleneck and population expansion, affect the distribution of genetic variation in a population. A population bottleneck will significantly reduce genetic variation in the population while a population expansion will lead to an excess of newly derived mutations. Different measures, derived from the distribution of allele frequencies along the genome, reflect recent demographic events that have affected genetic variation in a population (Alcala et al., 2013). We used the population genomic data of C. beticola to compute the Tajima’s $D$ statistic (Tajima, 1989) along the genome to measure genetic variation at the DNA level via a standardized pairwise comparison of the number of nucleotide differences to the number of segregation sites. While the value varies significantly along chromosomes, we found an overall positive Tajima’s $D$ value of 1.1. Tajima’s $D > 0$ reflects an excess of alleles of intermediate frequencies, which is expected under a scenario of population contraction. While additional analyses are required to infer the recent demography of C. beticola in North America, this positive Tajima’s $D$ value could indicate a recent loss of genetic variation in the pathogen population due to demographic changes. Recent demographic changes in populations of C. beticola may result from selection on the fungus imposed by fungicide treatment and resistant beet cultivars. It may also reflect the recent emergence of the pathogen on sugar beet as a new host. Comparative population genomic analyses can provide an opportunity to date different demographic events that have shaped genetic variation in the pathogen.

Population genomic sequencing of C. beticola from other continents and from wild hosts will furthermore provide important information on the centre of origin and the global population genetic structure of the pathogen, as well as patterns of gene flow and signatures of selection in agricultural and wild ecosystems.

5 | DEVELOPMENT AND CHARACTERIZATION OF THE C. BETICOLA REFERENCE GENOME

We previously reported the C. beticola reference genome size to be 37 Mb (de Jonge et al., 2018). The final, high-quality annotation contained 12,281 genes and 12,495 transcripts, encompassing 28,389 unique exons. On average, protein-coding genes contained 2.3 exons and 1.3 introns that were approximately 780 and 73 bp long, respectively. A significant proportion of genes (4,384 genes or 35.7%) did not contain an intron and are referred to as single-exon genes. We assigned both 5’ untranslated regions (UTRs) and 3’ UTRs to 7,147
genes, 5′ or 3′ UTR to 1,561 genes, and 3,573 genes did not have an assigned UTR. The average length of the 5′ UTR was 188 bp and that of the 3′ UTR 300 bp. Most intronic sequences were found in the coding regions (15,195 or 96%). We identified 446 introns (2.8% of all introns) in 5′ UTRs and 181 (1.2% of all introns) in 3′ UTRs, representing a significant under-representation of 3′ UTR introns exemplified by the 25-fold lower intron density (7.6 × 10^{-5} introns/mRNA transcripts) as compared to 5′ UTR introns (3.0 × 10^{-4} introns/mRNA transcripts). In Arabidopsis and some mammals, a pronounced under-representation of 3′ UTR introns may be explained by a requirement for the nonsense-mediated decay (NMD) pathway (Lejeune and Maquat, 2005; Chung et al., 2006). NMD is a cellular process that is involved in the detection and decay of mRNA transcripts that contain premature stop codons. Our results suggest that filamentous fungi may use a similar NMD pathway. In this regard it is interesting to note that an examination of the C. beticola proteome resulted in the identification of three important protein components of the exon junction complex, which is essential for NMD (Lejeune and Maquat, 2005). Examination of UTR characteristics in other related fungi based on available gene structures supports this view, albeit limited by the number as well as the accuracy of these gene structures.

A detailed overview of all gene model statistics, previously reported in de Jonge et al. (2018), is shown in Table 1 as well as a comparison of these statistics with five related plant pathogenic Dothideomycete fungi with well-characterized genome sequences. These are Dothistroma septosporum (teleomorph Mycosphaerella pini), Z. tritici (previously known as Mycosphaerella graminicola), L. maculans, Parastagonospora nodorum (previously known as Stagonospora nodorum), and Pyrenophora tritici-repentis as well as a more distantly related Eurotiomycete Aspergillus nidulans.

### Table 1  Cercospora beticola genome statistics and comparison to other related fungi

| Species | Cbe | Cbr | Ccn | Dse | Ztr | Lma | Ptr | Pno | Ani |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Assembly statistics** | | | | | | | | | |
| Total assembly length (Mb) | 37.1 | 37.4 | 34.0 | 30.2 | 39.7 | 44.9 | 37.8 | 37.2 | 30.5 |
| Total length of gaps (Mb) | 3.2 | 0.3 | 0.0 | 0.1 | 0.0 | 1.1 | 0.6 | 0.2 | 0.7 |
| No. of scaffolds/contigs | 248 | 28,905 | 6,126 | 20 | 21 | 41 | 47 | 108 | 8 |
| NG50 scaffolds (no.) | 4 | 111 | 422 | 5 | 6 | 10 | 6 | 13 | 4 |
| LG50 scaffolds (Mbp) | 4.17 | 0.096 | 0.023 | 2.60 | 2.67 | 1.77 | 1.99 | 1.05 | 3.76 |
| NG95 scaffolds (no.) | 10 | 11,497 | 2,320 | 12 | 18 | 25 | 20 | 43 | 8 |
| GC content (%) | | | | | | | | | |
| Overall (excl. gaps) | 52.2 | 51.5 | 52.6 | 53.1 | 52.1 | 45.2 | 51.0 | 50.4 | 50.4 |
| Coding (CDS) | 53.8 | 53.9 | 54.3 | 54.6 | 55.6 | 54.1 | 53.6 | 54.6 | 53.4 |
| Repeat content (Mb) | 0.51 | ND | ND | 1.08 | 6.98 | 15.93 | 0.80 | 2.88 | 1.07 |
| **Protein-coding genes** | | | | | | | | | |
| Protein-coding genes (no.) | 12,281 | 11,972 | 11,556 | 12,580 | 10,951 | 12,469 | 12,169 | 12,380 | 10,680 |
| Mean gene length (bp) | 1,885 | 1,584 | 1,556 | 1,896 | 1,602 | 1,446 | 1,461 | 1,616 | 1,736 |
| Percentage coding | 68.4 | 51.1 | 52.9 | 79.3 | 44.2 | 41.2 | 52.9 | 49.1 | 62.2 |
| Mean gene density (no. genes/100 kb) | 36.3 | 32.3 | 34.0 | 41.8 | 27.6 | 28.5 | 32.7 | 33.4 | 35.8 |
| Mean CDS length (bp) | 1,469 | 1,473 | 1,450 | 1,223 | 1,310 | 1,258 | 1,349 | 1,271 | 1,456 |
| **Exons** | | | | | | | | | |
| No. of exons | 28,100 | 28,046 | 26,848 | 28,937 | 28,611 | 35,201 | 32,716 | 32,994 | 34,743 |
| Exons/gene | 2.3 | 2.3 | 2.3 | 2.3 | 2.6 | 2.8 | 2.7 | 2.7 | 3.3 |
| Mean exon length (bp) | 780 | 629 | 624 | 773 | 532 | 446 | 530 | 495 | 477 |
| **Introns** | | | | | | | | | |
| Introns (no. introns) | 15,819 | 16,074 | 15,292 | 16,356 | 17,660 | 22,732 | 20,547 | 20,614 | 24,062 |
| Introns/gene | 1.3 | 1.3 | 1.3 | 1.3 | 1.6 | 1.8 | 1.7 | 1.7 | 2.3 |
| Mean intron length (bp) | 73 | 83 | 80 | 91 | 133 | 103 | 114 | 90 | 82 |

ND, not determined (ND).

* Genomes are Cercospora beticola (Cbe), Cercospora berteroae (Cbr), Cercospora canescens (Ccn), Dothistroma septosporum (Dse), Zymoseptoria tritici (Ztr), Leptosphaeria maculans (Lma), Pyrenophora tritici-repentis (Ptr), Parastagonospora nodorum (Pno), and Aspergillus nidulans (Ani).

* Only considering the longest transcript (if alternatives exist).
5.1 | Proteome characterization of C. beticola and related fungi

We used BLAST and InterProScan to obtain functional annotation for the full C. beticola proteome encompassing in total 12,495 proteins. BLASTp analyses of all C. beticola proteins against the NCBI nonredundant database identified significant similarity (E-value ≤ 1e-10; percentage coverage of query ≥ 70%) for 9,580 proteins (77%) whereas similar analyses against a database containing all fungal protein sequences available at the Joint Genome Institute MycoCosm portal resulted in identification of 10,529 proteins (84%) with significant similarity. Similar results were obtained for the six other ascomycete species, ranging from 63% (L. maculans) to 86% (A. nidulans). InterProScan led to the identification of 12,965 Pfam domains, 5,039 SMART domains and 12,541 superfamily domains in the proteome. Integration of the BLASTp and InterProScan results by BLAST2GO yielded gene ontology (GO) terms for 7,380 C. beticola proteins (59%).

To compare the proteomes of these seven ascomycete fungi including C. beticola, all proteins were clustered into 12,167 orthologous protein families based on all-versus-all BLASTp similarity via orthoMCL analysis (Li et al., 2003). From the resulting 12,167 ortholog clusters, we selected 3,554 that contain one protein sequence from each genome. We further filtered these protein clusters by retaining only those that contain single-copy homologs in 39 ascomycete fungi using data from orthoDB (Waterhouse et al., 2012) for phylogenetic analyses, resulting in a reduced set of 850 protein families. Concatenated protein alignments were then used to build a well-supported phylogenetic tree that clusters C. beticola most closely with D. septosporum and secondly to Z. tritici (Figure S2a). This phylogeny is in congruence with the majority of other studies using single- and multilocus analyses that place the genus Zymoseptoria adjacent to the Cercospora, Pseudocercospora, Dothistroma, and Sphaerulina genera (Goodwin et al., 2001; de Wit et al., 2012; Ohn et al., 2012; Arango Isaza et al., 2016; Chang et al., 2016; de Jonge et al., 2018). Orthologous protein information was also used to further enhance the functional annotation of the C. beticola proteins. More specifically, GO terms from orthologous A. nidulans proteins, for which extensive GO-term annotation is available through AMIGO (Carbon et al., 2009), were replicated to C. beticola proteins. Using this approach, we were able to assign a GO term to a combined total of 8,608 C. beticola proteins.

5.2 | Secondary metabolite cluster expansion in C. beticola

Secondary metabolites (SMs) are small bioactive molecules produced by many organisms including bacteria, plants, and fungi (Stringlis et al., 2018). They are abundant in filamentous fungi in which they play crucial roles in the establishment of specific ecological niches. Unlike primary metabolites, SMs are not essential for fungal growth, development, or reproduction but contribute to adaptation (e.g., protection against environmental stresses) or pathogenicity. Many enzymes are involved in the synthesis of a single SM. Polyketide synthases (PKs) and nonribosomal peptide synthases (NRPs), which catalyse the elongation of polyketides and oligopeptides, respectively, are among the most prominent and well-studied SM biosynthetic genes. C. beticola is known to produce a number of SMs, including cercosporin (Daub and Ehreshaft, 2000; de Jonge et al., 2018) and beticolin (Goudet et al., 1998). The genome of C. beticola possesses 15 type I PKSs, 23 NRPs, three PKS-NRPS hybrids, six terpene cyclases, and one demethylallyl tryptophan synthase (de Jonge et al., 2018). The closely related dothideomycetes D. septosporum and Z. tritici possess a significantly lower number of both type I PKSs (3 and 10, respectively) and NRPs (5 and 8, respectively). The expansion of NRPSs in C. beticola is particularly notable when compared to L. maculans, P. nodorum, and P. tritici-repentis (this study) and C. fulvum (de Wit et al., 2012). The expansion in NRPSs is further exemplified by a high number of NRPS-related Pfam domains (PF00501, PF00550, PF00668, and PF13193). Interestingly, the predicted C. beticola SM clusters are preferentially found in subtelomeric regions (Figure 2a), a common feature of fungal SM clusters (Palmer and Keller, 2010). Probably, the observed co-regulation of expression among genes within SM clusters is achieved by epigenetic forces such as chromatin-level control (Palmer and Keller, 2010). Chromosomal location of gene clusters near centromeres and telomeres possibly correlates with regions that experience facultative heterochromatin, that is, large regions that can efficiently be silenced as well as activated by chromatin-mediating factors (Palmer and Keller, 2010).

5.3 | Definition and annotation of the C. beticola secretome

The arsenal of potentially secreted proteins (i.e., the secretome) among plant pathogens includes key pathogenicity molecules that are generally referred to as effectors (Kamoun, 2007). To identify candidate effectors, we scanned the proteome of C. beticola for proteins that are predicted to be secreted and lack transmembrane domains. We identified 1,087 such proteins of which 333 exhibited additional effector characteristics including small size (≤300 amino acids) and cysteine-rich (≥2 cysteine residues) (Figure 2a; SSCPs). Analysis of genomic localization revealed an under-representation of SSCPs on chromosome 1 (p < 0.001, chi-square test, Figure 2a) that might be an indication of genome compartmentalization, often referred to as the two-speed genome (Raffaele et al., 2010). Of the 333 candidate effectors, 62 (approximately 19%) had no BLASTp hits in the nonredundant database from NCBI nor in the collection of 347 fungal proteomes obtained from the MycoCosm fungal genomics portal and are therefore considered C. beticola-specific.
Conservation of sequence between chromosomes or scaffolds of different species or strains is typically referred to as synteny. Through gene loss, inversions, translocations, or other chromosomal rearrangements, synteny between homologous chromosomes (i.e., of common descent) can be broken. However, major chromosomal rearrangements are rare when comparing distantly related eukaryotes, and as a result large collinear regions with similar genes in similar order and orientation can be found across these species. This kind of “global” chromosome conservation is known as macrosynteny. Such macrosynteny is only rarely observed among species in the fungal kingdom. In fungi, a type of synteny known as microsynteny is common in which small segments of up to 10 conserved genes can be found. Moreover, a phenomenon termed mesosynteny in which homologous chromosomes display significant conservation of gene content but not gene order is typical in the ascomycete fungi and especially prominent in the Dothideomycete class to which *C. beticola* belongs (Hane et al., 2011). Conservation of gene content but not of gene order can be attributed to a large number of intrachromosomal rearrangements such as inversions and a very limited number of interchromosomal rearrangements. Hane et al. (2011) hypothesized that a high frequency of inversions might occur during meiosis, although this does not explain why Dothideomycetes in particular, and only ascomycete fungi, display such a striking conservation of chromosome gene content because many species in this class do not have a known sexual stage.

To investigate whether *C. beticola* chromosomes also display mesosynteny to related fungal species, we aligned *C. beticola* chromosomes with those of the closely related fungus, *D. septosporum* (Figure 2b,c). Notably, strict conservation of chromosome content but not order can be observed, the hallmark for mesosynteny. Considering a limited number of intrachromosomal rearrangements, it is expected that some collinear segments remain because insufficient rearrangements have occurred to randomize the complete gene order within a chromosome. On the other hand, some collinear segments might be the result of continuing selection pressure to keep particular genes involved in the same or similar biological processes tightly clustered on the genome, as we have previously observed is the case for biosynthetic gene clusters for cercosporin (de Jonge et al., 2018; Ebert et al., 2019) and melanin (Ebert et al., 2019).

6 | *C. BETICOLA* EFFECTOR REPERTOIRE

Effectors can be described as microbially secreted molecules such as proteins, SMs, and small RNAs that contribute to niche colonization (Rovenich et al., 2014; Snelders et al., 2018). This definition implies that effector functions are not necessarily restricted to interaction with a host plant but also include involvement in microbial competition and nutrition acquisition (Fatima and Senthil-Kumar, 2015; Snelders et al., 2018) that may have roles during a saprophytic growth period. Effectors that are secreted during host colonization may have the ability to modulate host biochemistry and physiology,
including defence responses, to facilitate host colonization (de Jonge et al., 2011). The type of effectors produced depends on the lifestyle of the pathogen and stage of infection. Biotrophic fungi obtain their nutrients from living plant tissues and their effectors typically act to inhibit plant defence responses and facilitate nutrient acquisition. However, necrotrophic fungi secrete toxins and proteins to evoke host cell death and induce nutrient release (Koeck et al., 2011). The hemibiotrophic lifestyle of C. beticola begins with a latent symptomless (biotrophic) phase where invasive hyphae grow within the host plant followed by a fine-tuned transition to necrotrophy. The two different phases of growth probably require different effector repertoires. Future time-course transcriptome studies of sugar beet infection may help to elucidate how phase transition occurs and identify candidate effectors contributing to biotrophy or necrotrophy, as has been shown in other pathosystems (Gan et al., 2013; Rudd et al., 2015; Zuluaga et al., 2016). To date, both SM and proteinaceous effectors have been identified in C. beticola and are discussed below.

7 | CERCOSPORIN

Cercosporin is a light-activated, non-host-specific toxin produced by most Cercospora species (Daub and Ehrenshaft, 2000). Mutant lines that are unable to produce this SM experience a virulence penalty, implicating cercosporin as a virulence factor for C. beticola, C. nicotianae, C. kikuchii, C. coffeicola, and Cercospora cf. flagellaris (Callahan et al., 1999; Choquer et al., 2005; Weiland et al., 2010; Rezende et al., 2020; Souza et al., 2019). However, there are Cercospora species that naturally lack the ability to produce this toxin and yet are virulent phytopathogens, demonstrating that cercosporin is not solely necessary for pathogen success in the genus (Weiland et al., 2010; Swart et al., 2017). The metabolic pathway genes involved in cercosporin formation are organized in a cercosporin toxin biosynthesis (CTB) cluster. While cercosporin was thought to be a unique feature of fungi that belong to the Cercospora genus, it has been shown that the CTB cluster experienced duplications and multiple horizontal gene transfers across a variety of taxa, including many Colletotrichum species, and Colletotrichum florianae has been confirmed to also produce this potent toxin (de Jonge et al., 2018).

7.1 | Toxicology

The photosensitizing nature of various perylenequinones, including cercosporin, has long been known (Brockmann et al., 1950; Weiss et al., 1957; Yamazaki et al., 1975). The essential structural feature responsible for photodynamic activity is the 3,10-dihydroxy-4,9-perylenequinone chromophore (Hudson et al., 1997), which allows absorption of visible and near-UV light that elevates the perylenequinone to an electronically excited triplet state (Foote, 1968; 1976). Once in this activated triplet state, two types of reactions are known to occur (DeRosa and Crutchley, 2002; Guedes and Eriksson, 2007): the excited perylenequinone can react with oxygen either indirectly (type I reaction) through a reducing substrate or directly (type II reaction) (Figure S3). Interaction with an electron donor leads to the formation of free radicals or radical ions that in turn react with oxygen to produce reactive oxygen species (ROS) such as H2O2 and free radical forms such as O2•−, HO•, and OH•. In a direct interaction between a triplet perylenequinone and oxygen, energy can be transferred from the excited triplet state perylenequinone to oxygen, resulting in an excited singlet state of oxygen, also known as “singlet oxygen” (1O2). Both type I and II reactions yield ROS that at high concentrations are harmful to cells as they can cause lipid peroxidation as well as protein and DNA damage (Blokhina et al., 2003; Birben et al., 2012).

7.2 | Biosynthesis

While extensive research on the CTB pathway has shed light on selected pathway steps, it has not yet been possible to determine a full biosynthesis scheme due to the extreme instability of most pathway intermediates and the potential occurrence of feedback inhibition (Newman and Townsend, 2016). To date, 12 genes have been found to be part of the CTB biosynthetic pathway, involved in either cercosporin production or export (Figure 3a). The iterative, non-reducing PKS CTB1 is essential for cercosporin production and acts as the keystone enzyme to initiate the biosynthetic process (Choquer et al., 2005; Crawford and Townsend, 2010; Newman et al., 2012). CTB1 harbours six functional domains that work together to form nor-toralactone, the first intermediate in the cercosporin assembly line using one acetyl-CoA and 6 × malonyl-CoA units as substrate (Choquer et al., 2005; Newman et al., 2012; Newman and Townsend, 2016). Next, the nor-toralactone intermediate is processed to toralacitone and subsequently to cercquinone C by CTB3, a predicted O-methyltransferase FAD-dependent monoxygenase (Dekkers et al., 2007; Newman and Townsend, 2016; de Jonge et al., 2018). Further processing of this intermediate may be mediated by CTB2, CTB6, and CTB11/CTB12, which are hypothesized to methylate, reduce, and dimerize the molecule, respectively (Staerkel et al., 2013; Newman and Townsend, 2016). However, the corresponding intermediates have not been directly observed but are rather logically inferred. The CTB5 and/or CTB7 gene products are hypothesized to prime the cercosporin intermediate for methylenedioxy bridge formation by CTB9 and CTB10 to yield the final cercosporin molecule (Chen et al., 2007; Newman and Townsend, 2016; Swart et al., 2017; de Jonge et al., 2018). The CTB8 gene product is a Zn(II)Cys6 zinc finger transcription factor that is not directly involved in the modification of the toxin itself but is responsible for mediation of the CTB gene cluster expression (Chen et al., 2007). Lastly, the gene cluster holds two major facilitator superfamily (MFS) transmembrane transporters, CTB4 and the cercosporin facilitator protein (CFP) (Chen et al., 2007; Choquer et al., 2007; de Jonge et al., 2018). C. nicotianae CTB4 mutant strains displayed a reduction in cercosporin production by at least 35% while cercosporin accumulated in fungal mycelium and was not secreted into the medium, suggesting that CTB4 mediates cercosporin export (Choquer et al., 2007).
When stimulated by high light conditions, CTB4 mutants secreted a dark brown compound of unknown nature that quickly diffused into the solid medium. Interestingly, a CTB4 homolog is also present in the putative phleichrome biosynthetic pathway, but is lacking in the predicted elsinochrome and hypocrellin clusters (Ebert et al., 2019). As elsinochrome and hypocrellin are secreted by Elsinoë fawcettii and Shiraiha bambusicola, respectively, despite the lack of a CTB4 homolog, the question arises whether CTB4 is indeed solely responsible for toxin export or whether other transporter proteins can functionally substitute toxin export in the absence of CTB4 (Choquer et al., 2007).

The CFP gene is incorporated in the CTB cluster of C. beticola (de Jonge et al., 2018) and is hypothesized to partially provide tolerance to cercosporin (auto-resistance via toxin export (Callahan et al., 1999).

### 7.3 Auto-resistance

It is essential for fungi that produce antifungal SMs to protect themselves from their own toxin. The phenomenon of avoiding self-intoxication is known as auto-resistance (AR). Genes with potential
AR roles have been identified in highly conserved toxin biosynthesis gene clusters, with no direct involvement in toxin production. Three self-resistance strategies have been highlighted in the literature: toxin export, detoxification, and duplication of the toxin target (Keller, 2015). However, to this date only toxin export via transporter proteins and detoxification of toxic compounds have been found in cercosporin-producing fungi.

Protection by toxin efflux relies on the transportation of toxic substances from the inside of a cell to the outside through membrane transport proteins. Transporters involved in self-resistance mainly belong to the MFS and ABC superfamilies (Cannon et al., 2009; Costa et al., 2014).

The MFS transporter protein, CFP, was first identified in C. kikuchii and has been shown to be involved in cercosporin AR as targeted gene disruption of CFP resulted in mutant strains with increased sensitivity to exogenous cercosporin (Callahan et al., 1999). With the recent discovery that the CBT cluster consists of additional genes (de Jonge et al., 2018), it was possible to demonstrate that the MFS transporter homolog in C. beticola, CbCFP, is incorporated in the cluster and flanked on both sides by genes necessary for cercosporin production (de Jonge et al., 2018). Mutants that lack CbCFP are more sensitive to exogenous cercosporin in vitro (Ebert and Bolton, unpublished data), suggesting that this AR mechanism is conserved in the Cercospora genus.

Besides toxin export, Cercospora species are known to have a second mechanism of AR through reductive detoxification of the cercosporin molecule (Daub et al., 1992, 2000; Leisman and Daub, 1992; Sollod et al., 1992). It has been shown that stably methylated and acetylated reduced cercosporin derivatives absorb less light and generate significantly less singlet oxygen (\(\text{O}_2^*\)) compared to wild-type cercosporin (Leisman and Daub, 1992). Further investigation has revealed that the cell surface of cercosporin-resistant strains is surrounded by a reducing environment (Leisman and Daub, 1992), showing that resistant fungi can reduce proximate cercosporin into its less reactive form and maintain this detoxification so long as the molecule is nearby. Although specific genes have been reported in other fungi that are linked to active cercosporin detoxification (Ververidis et al., 2001; Panagiotis et al., 2007), no CBT genes have yet been affiliated with AR via cercosporin reduction.

8 | BETICOLINS

Beticolins are a group of nonhost-specific phytotoxins of which 20 members (B0 to B19) have been identified to be produced by C. beticola (Milat and Blein, 1995; Goudet et al., 1998, 2000) and the hoary alyssum (Berteronia incana) pathogen Cercospora berteroeae (Assante et al., 1977). Alternative names for these toxins, such as Gelbe Fraktion (Schlösser, 1962; Langfelder et al., 2003), Cercospora beticola toxin (CBT) (Assante et al., 1977), and cebetins (Jalal et al., 1992; Robeson and Jalal, 1993), arose due to simultaneous research efforts by different groups and limited data concerning their structure during early research. Later, analyses of their chemical structures revealed that beticolins are structurally closely related (Figure 3b). All have a chlorine atom attached to the central aromatic ring, while their octocyclic basic structure is composed of two subunits: a partially hydrogenated anthraquinone and a partially hydrogenated xanthone that are connected through a seven-membered ring (Ducrot et al., 1994, 1996; Simon-Plas et al., 1996; Goudet et al., 1998, 2000). Structural differences between beticolins are due to different isomeric configurations (ortho-, para-, or epi-ortho-) and variable residues (Milat et al., 2010). Interestingly, beticolins can switch isomery. For example, ortho-beticolin B2 is able to transform into the para-beticolin B1 or epi-ortho-beticolin B6 and vice versa (Ducrot et al., 1994).

Early research on their biological function indicated that beticolins have antibacterial and phytotoxic properties (Schlösser, 1962). However, necrosis formation in plants upon beticolin application was only induced in the presence of light. Later it was found that due to their ability to form complexes with \(\text{Mg}^{2+}\), beticolins inhibit tumoral cell growth in mice (Ding et al., 1996, 2001), interfere with \(\text{H}^+\)-ATPase activity (Gomès et al., 1996a, 1996b, 1996c; Simon-Plas et al., 1996), and are able to incorporate themselves into lipid bilayers to form ion channels with poor ion selectivity (Goudet et al., 1998, 1999, 2000). The latter property led to the classification of beticolins as ion channel-forming toxins (Goudet et al., 2000). While chemical structures and biological activity have been evaluated in recent decades, the biosynthetic pathway of these toxins is unknown, therefore it is currently not possible to assess to what extent beticolin production and associated phytotoxic effects contribute to C. beticola virulence.

9 | MELANIN

Fungal phytopathogen melanin production has been implicated in appressorial penetration of host plants and pathogenesis as well as being an integral component of the cell wall that can be useful for tolerating environmental stresses (Langfelder et al., 2003; Liu and Nizet, 2009). Recent research mining the C. beticola genome for novel PKS genes has revealed the secondary metabolite production of melanin (Ebert et al., 2019). Phylogenetic analysis of the CbPKS1 gene belonging to the DHN-melanin clade grouped C. beticola with other well-characterized melanin biosynthetic clusters from various ascomycetes. Additionally, whole-cluster homology with other PKS genes that are involved in melanin production provided further evidence that these genes may be functional. Knockout mutants in melanin CbPKS1 resulted in albino phenotypes that did not affect the PKS biosynthetic cluster involved in cercosporin production (Ebert et al., 2019). Interestingly, a gene adjacent to CbPKS1 encoding for a predicted tetra-hydroxynaphthalene reductase (Cb4HNR) involved with melanin biosynthesis has been shown to be induced in fungus-resistant C. beticola strains (Bolton et al., 2016). This proposes a role for the SM production of melanin that is probably an adaptation involved in survival in a challenging environment.
10 | PROTEIN EFFECTORS

Effector proteins are known to be employed by a broad variety of plant pathogenic fungi to evade detection by the host during colonization. Upon pathogen recognition, the plant will initiate defence responses such as the production of chitinases that target fungal hyphae for degradation. To shield the fungal cell wall from plant-derived chitinases, the biotrophic fungus *C. fulvum*, which causes leaf mould on tomato, secretes the virulence factor *CfAvr4* (van Esse et al., 2007). This effector can bind to chitin in the fungal cell wall and protect fungal hyphae from hydrolysis by plant chitinases (van den Burg et al., 2003, 2004; van Esse et al., 2007; Chang and Stergiopoulos, 2015). A homologous gene was discovered in *C. beticola* (Stergiopoulos et al., 2010) and Cercospora *cf. flagellaris* (Rezende et al., 2020), and in vitro studies found that the *CbAvr4* and *Cfla-Avr4* gene products could functionally bind to chitin (Mesarich et al., 2016; Rezende et al., 2020). As chitin-binding appears to be a conserved biological trait between *CfAvr4* and *Avr4* homologs, it is hypothesized that *Avr4* homologs including *CbAvr4* also share the *CfAvr4* function of shielding fungal hyphae from lysis by plant chitinases.

Effector protein identification through comparative genomics has served as a useful tool to detect another *C. beticola* effector named *CbAv1*. *CbAv1* is a homolog of *VdAv1* (*Avr*ulence on *Ve1* tomato), an effector secreted by the vascular wilt pathogen *Verticillium dahliae*. In *V. dahliae* the *VdAv1* effector is recognized by the tomato cell surface-localized immune receptor *Ve1* and confers resistance to the pathogen. In the absence of *Ve1*, *VdAv1* has been shown to contribute to fungal virulence but the mechanism remains elusive (de Jonge et al., 2012). In the *C. beticola–B. vulgaris* system, *CbAv1* is expressed during infection and its product acts as a virulence factor (Boshoven et al., 2015).

Due to its hemibiotrophic lifestyle, it was hypothesized that *C. beticola* also secretes proteinaceous effectors that promote its necrotrophic phase. Using a phenotype-based forward genetics approach, a proteinaceous virulence factor named *CbNIP1* (*Cercospora beticola* necrosis-inducing protein 1) was identified due to its ability to necrotize sugar beet and *Nicotiana benthamiana* leaves (Ebert et al., 2018). Interestingly, *CbNIP1*’s ability to induce necrosis within 48 hr was highly regulated by light. While other necrosis-inducing proteins such as *ZtNIP1* and *ZtNIP2* of the wheat pathogen *Z. tritici* require light for full functionality (M’Barek et al., 2015), *CbNIP1* was most active in complete darkness as exposure of *CbNIP1*-infiltrated sugar beet leaves with a 12 hr light-dark cycle led initially to chlorosis formation that gradually turned necrotic over time (Ebert et al., 2018). Furthermore, *CbNIP1* appears to contribute to necrotic symptom development, as up-regulated *CbNIP1* expression in planta correlates with necrotic lesion appearance. At present, the mode of action of *CbNIP1* and its location during infection remains unknown.

11 | DISEASE MANAGEMENT

The integrated management of CLS comprises cultural practices, moderate host resistance, and the timely application of fungicides. Cultural practices aim to reduce the amount of initial inoculum for the following season through rotation with nonhost crops, tillage (burying infested debris), and avoiding planting next to fields previously sown with sugar beets. Epidemiological models regarding the disease progress of CLS have been established to predict disease severity and timing of fungicide application (Rossi and Battilani, 1991; Windels et al., 1998; Pitblado and Nichols, 2005; Racca and Jörg, 2007). Fungicides should be sprayed early in a protective manner to avoid the development of conidial populations, which can infect new unprotected foliage. Although there have been several studies regarding the potential of different bacteria and fungi as biocontrol agents for CLS, including *Trichoderma* and *Bacillus subtilis* (Collins and Jacobsen, 2003; Galletti et al., 2008), there have been no current reports of their success as a management tool in the field. Alternatively, the presence of several microbial groups has been correlated with disease incidence in sugar beet fields and these microbes may be useful as biological markers for predicting disease outbreaks (Kusstatscher et al., 2019).

11.1 | Host resistance

The improvement in CLS resistance in sugar beet varieties over the last few decades has been a concerted effort by geneticists and breeders. Wild sea beet, *B. vulgaris* subsp. *maritima*, has long been a source of CLS resistance genes (Rossi, 1995). Other wild *Beta* relatives, such as *Beta procumbens*, have displayed nonhost resistance but are sexually incompatible with *B. vulgaris* (Panella and Frese, 2000). Inheritance of CLS resistance in sugar beet lines after introgression from wild sea beet is complex and has low heritability (Smith and Ruppel, 1974), while at the same time incorporation into high-yielding commercial sugar beet hybrids remains a challenge (Smith and Campbell, 1996). Promisingly, recent field trials in Germany showed that several European sugar beet varieties with CLS resistance lacked a yield penalty in the absence of disease and had better economic performance than susceptible varieties (Vogel et al., 2018). CLS resistance is typically managed by at least four identifiable quantitative trait loci (QTLs) (Smith and Gaskill, 1970; Nilsson et al., 1999; Setiawan et al., 2000; Taguchi et al., 2011). Precise mapping of resistance QTLs helps marker-assisted selection (MAS) in breeding programmes to introgress CLS resistance (Taguchi et al., 2011). The more precise the mapping, the higher the chances of breaking the potential linkage between CLS resistance and unfavourable traits. The underlying gene products can also be identified and used as molecular markers to identify alleles associated with resistance (Hunger et al., 2013). Previously, monogenic resistance was identified to race C2 of *C. beticola* in a sugar beet cultivar (Lewellen and Whitney, 1976) but the resistance was shown to be unstable (Koch and Jung, 2000). No other examples of monogenic resistance to CLS have been described since in sugar beet. Current and future breeding efforts for CLS resistance can exploit the reference-quality 567 Mb genome sequence of sugar beet with 27,421 transcript-supported genes published by Dohm et al. (2014). An additional reference-quality genome was recently developed.
for a different sugar beet variety with focused annotation of nucleotide-binding (NB-ARC), leucine-rich repeat (NLR) disease resistance genes, including 231 tentative NB-ARC loci (Funk et al., 2018). When comparing these loci to validated resistance genes from monocots and eudicots, there appeared to be extensive B. vulgaris-specific subfamily expansions. Draft genomes of Beta patula and sea beet were also recently released (del Río et al., 2019) and represent valuable resources for sugar beet breeding research.

11.2 Molecular basis of host defence

Although few studies have focused on the molecular basis of resistance, there is some published work examining plant defences upon C. beticola infection. The interaction between sugar beet and C. beticola begins with an initial defence response by the plant up-regulating phenylalanine ammonia-lyase (PAL) involved in the various biosynthetic pathways for many plant-related SM compounds (i.e., lignins, flavonoids, and phytoalexins) used during biotic attacks (Liang et al., 1989). These plant defence mechanisms have been found to be suppressed by C. beticola via an interaction with a pathogen-induced molecule on the PAL core promoter (Schmidt et al., 2004). Later it was discovered that on initial infection with the pathogen and through mid- to late-stages of disease development, the plant hormone abscisic acid (ABA) was elevated (Schmidt et al., 2008). ABA was also found to reduce PAL gene expression in sugar beet through an unknown mechanism (Schmidt et al., 2008). ABA has been found to interfere with biotic stress signalling in other plants, including via suppression of PAL transcription and activity, which negatively impact disease resistance (Mauch-Mani and Mauch, 2005).

Additional research into the defence response of sugar beet cultivars to CLS showed that three cultivars with varying resistance genotypes (susceptible, polygenic partial resistance, or monogenic resistance) differed in timing and strength of defence reactions on infection (Weltmeier et al., 2011). In all three cultivars, genes were activated in hormone production (ethylene, jasmonic acid, and gibberellin), lignin and alkaloid synthesis, signalling, and pathogenesis-related (PR) genes by the time symptoms had appeared. The monogenic resistant genotype (resistant to C. beticola isolate C2) displayed strong defences (PR and WRKY gene expression) 1 day after inoculation and there was no significant increase in C. beticola biomass. The partial resistance genotype had a stronger defence response than the susceptible genotype and a 50% reduction in C. beticola biomass, but the pathogen was still able to infect and cause disease when there was late initiation of defence responses at 15 days after inoculation.

12 Fungicide usage and resistance development

The use of fungicides has been an integral part of CLS management primarily due to the lack of effective nonchemical alternatives. There are two main types of chemistries available for disease management: protectant fungicides with broad-spectrum activity and systemic fungicides that target a specific site in the fungus. Of the former, the most commonly used are the ethylene bisdithiocarbamate (EBDC, Fungicide Resistance Action Committee or FRAC Group M03) class of fungicides, copper-based fungicides (FRAC group M01), and, in the United States, the organotin class of compounds (FRAC Group 30) such as triphenyltin hydroxide and triphenyltin acetate. The three main classes of systemic fungicides that have been employed globally are the benzimidazoles (FRAC Group 1), triazoles (sterol demethylation inhibitors or DMIs, FRAC Group 3), and quinone outside inhibitors (Qols, FRAC Group 11).

The continued efficacy of these fungicide classes has been marred by the emergence of resistant strains in C. beticola populations over the last few decades (Figure 4). C. beticola resistance has been noted to occur after widespread and repeated use of the same fungicide classes (Giannopolitis, 1978; Secor et al., 2010; Rosenzweig et al., 2020). Other factors contributing to the development of fungicide resistance are the polycyclic nature of this pathogen, its high rate of sporulation and common spray programmes being used over large areas for disease management (Dekker, 1986). The rotated use of different fungicide classes has been implemented to suppress selection for fungicide-resistant C. beticola strains. Systemic fungicides (such as DMIs) are also commonly mixed with a protectant fungicide for higher efficacy, reduced costs, and as an additional step in resistance management (Ioannidis, 1994).

With the emergence of resistance to most available fungicide chemistries, many recent studies have focused on characterizing the molecular basis of resistance. For fungicides with single target sites, such as the benzimidazoles, Qols and DMIs, target genes can be sequenced and compared in both sensitive and resistant isolates to identify probable causal mutations.

12.1 FRAC group 30

Triphenyltin acetate was used in Europe in the 1970s, while triphenyltin hydroxide was used extensively in the United States throughout the 1980s (Windels et al., 1998). Tolerance to triphenyltin fungicides quickly emerged in Greece (Giannopolitis, 1978), Serbia (Marić et al., 1984), North Dakota, and Minnesota (Bugbee, 1995; Campbell et al., 1998). However, it was noted that triphenyltin-resistant strains of C. beticola were less competitive than sensitive strains in the absence of this fungicide (Giannopolitis and Chrysayi-Tokousbalides, 1980). Furthermore, annual surveys of triphenyltin hydroxide resistance in C. beticola isolates from central North America’s Red River Valley (RRV) region have also suggested that a fitness penalty is associated with triphenyltin hydroxide resistance (Secor et al., 2010). Recent surveys of this region have shown high incidence of triphenyltin resistance (97% of isolates surveyed in 2017) but the severity of resistance was low (average spore germination rate was still <30%), perhaps suggesting that organotin may still be effective fungicides (Secor et al., 2017). Organotins act by inhibiting ATP

Molecular basis of host defence

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synthase activity to stop oxidative phosphorylation in mitochondria but the genetic basis of resistance in fungi is unknown (Gadd, 2000). Although one of the most effective fungicide groups in use, organotins are no longer permitted for use within the European Union because of their associated consumer risks (Risk and Policy Analysts Limited, 2005).

12.2 | FRAC group 1

Benzimidazole fungicides were implemented in the early 1970s. The first report of economic losses due to benzimidazole resistance in *C. beticola* populations was in Greece in 1973 (Georgopoulos and Dovas, 1973), followed by appearances in other production areas worldwide such as the United States (Ruppel and Scott, 1974; Bugbee, 1982), China (Dafang and Shuzhi, 1982), and India (Pal and Mukhopadhyay, 1983). EBDC and DMI fungicides were subsequently introduced to manage these resistant populations alongside the organotins. Benzimidazoles inhibit microtubule assembly during mitosis by binding to β-tubulin subunits (Davidse, 1986). Sequencing of the target β-tubulin gene identified a glutamic acid to alanine amino acid change at codon 198 (designated E198A) associated with high benzimidazole resistance in multiple populations of *C. beticola* in the United States and Europe (Davidson et al., 2006; Trkulja et al., 2013). An additional β-tubulin mutation, phenylalanine to tyrosine at codon 167 (designated F167Y), has been found in low-to-moderate resistant isolates from Serbia (Trkulja et al., 2013), causing F167Y isolates to be more sensitive to low temperatures while E198A isolates had no detectable fitness penalty.

12.3 | FRAC group 3

Another important class of fungicides used to manage *C. beticola* are DMIs. They have both protective and curative activity against *Cercospora* spp. and low levels of phytotoxicity (Brown et al., 1986; Dahmen and Staub, 1992). Although DMIs were initially thought to have a moderate risk of resistance development (Brown
et al., 1986), C. beticola resistance has now been found in Europe (Karaoglanidis et al., 2001a), Morocco (El Housni et al., 2018), Canada (Trueman et al., 2017), and the United States (Secor et al., 2010; Bolton et al., 2012b; Rosenzweig et al., 2020). Resistance to DMIs is observed as a near-continuum, ranging from high to low EC$_{50}$ values (Karaoglanidis and Ioannidis, 2010). C. beticola isolates with EC$_{50}$ values of greater than 1 ppm caused significantly more disease on sugar beet after application of a DMI fungicide than isolates with EC$_{50}$ values below 1 ppm, implicating 1 ppm as a reasonable threshold value for DMI resistance (Bolton et al., 2012b).

In the 2017 RRV region survey, 25.9% of tested C. beticola isolates were resistant (EC$_{50}$ > 1 ppm) to tetoconazole while 47.1% of the same isolates were resistant to another DMI difenoconazole, which suggests that there is not strict cross-resistance within the DMIs (Secor et al., 2017) and supports the earlier findings of Karaoglanidis and Thanassoulopoulos (2003). One study suggested that there may be some fitness penalties associated with DMI resistance, namely reduced virulence and spore production (Karaoglanidis et al., 2001b), but this has yet to be evidenced in field surveys.

The mechanism of resistance to DMIs is typically more complex than to benzimidazoles and QoIs. DMIs target the lanosterol 14α-demethylase CYP51, which is a cytochrome P450 enzyme catalysing a key step in the fungal ergosterol biosynthesis pathway. Without the synthesis of the cell membrane sterol ergosterol, there is inhibition of fungal cell growth. Resistance can occur not only through target site modifications of CYP51, but also through overexpression of CYP51, increased active efflux of DMIs, and multiple copies of the target CYP51 gene (Le Roux et al., 2007; Ziogas and Malandrakis, 2015). In the RRV region of the United States, CbCYP51 was overexpressed in several C. beticola isolates with EC$_{50}$ values >1 ppm, in both quantitative PCR and RNA-Seq studies, but the genetic mechanism underpinning this expression is unknown (Bolton et al., 2012a, 2016). No evidence has been found for alternative splicing or differential methylation of CbCYP51 between sensitive and resistant isolates (Bolton et al., 2012a).

Interestingly, a silent mutation at codon 170 has been identified to be present only in highly resistant C. beticola isolates (EC$_{50}$ values >50 ppm) from northern Greece (Nikou et al., 2009) and the RRV region of the United States (EC$_{50}$ values >20 ppm) (Obuya et al., 2015). Recently, nonsynonymous polymorphisms in CbCYP51 have also been discovered that appear to be linked to DMI resistance (Trkulja et al., 2017; Shrestha et al., 2020). The amino acid substitutions L144F, I309T, I387M, and Y464S in isolates from the RRV region of the United States (Spanner and Bolton, unpublished data) are all associated with DMI EC$_{50}$ values >1 ppm.

12.4 | FRAC group 11

The QoI class of fungicides were introduced in 1996 and first used for CLS in 2002, proving to be highly effective fungicides against C. beticola (Karadimos et al., 2005; Secor et al., 2010). Pathogen surveys in Europe (Birla et al., 2012; Piszczek et al., 2018), Morocco (El Houssni et al., 2018), Japan (Kayamori et al., 2020), Canada (Trueman et al., 2013), and the United States (Secor et al., 2010; Kirk et al., 2012) have indicated the rapid development of resistance to QoIs, which appears to be stable. In 2017, 89.1% of C. beticola isolates surveyed in the RRV region of the United States were resistant to the QoI pyraclostrobin and therefore its use is no longer recommended for CLS management in the region (Secor et al., 2017).

QoIs act by binding the quinol oxidation site of the cytochrome bc1 complex in the mitochondria, which disrupts ATP production (Fernández-Ortuño et al., 2008). The membrane protein cytochrome b forms the core of the complex and is encoded by the cytochrome b (cytb) gene. Similar to other fungi (Fernández-Ortuño et al., 2008), QoI-resistant isolates of C. beticola found to date have the substitution of glycine by alanine at codon 143 (designated G143A) (Birla et al., 2012; Bolton et al., 2013; Trkulja et al., 2017; Piszczek et al., 2018).

The identification of mutations underlying resistance to fungicide classes enables the rapid detection of resistance via PCR methods. Real-time PCR methods are already being employed for QoI resistance in annual surveys of C. beticola isolates (Malandrakis et al., 2011; Bolton et al., 2013) and methods have been developed to detect benzimidazole and DMI-resistant isolates (Nikou et al., 2009; Trkulja et al., 2013; Rosenzweig et al., 2015; Shrestha et al., 2020). A method of amplifying DNA under isothermal conditions has been developed and termed loop-mediated isothermal amplification (LAMP) (Notomi et al., 2015). This tool may eventually allow for fungicide resistance profiling of a C. beticola field population prior to spraying to best determine the appropriate chemical regime.

13 | CONCLUSIONS AND FUTURE PERSPECTIVES

Future studies of CLS disease in sugar beet can exploit the wealth of genetic and genomic resources that have become available in recent years for both C. beticola (de Jonge et al., 2018) and its hosts (Dohm et al., 2014; del Rio et al., 2019). The development of gene-editing technologies such as CRISPR-Cas9 (Doudna and Charpentier, 2014), which also revolutionized genetic editing in a broad variety of filamentous fungi as highlighted in multiple reviews (Idnurm and Meyer, 2018; Schuster and Kahmann, 2019; Song et al., 2019; Vicente et al., 2019), could be employed to investigate gene function via allele replacement in addition to gene knockouts. This would facilitate the identification of important genes and mutations for virulence in C. beticola. Breeding for effective host resistance to CLS with minimal yield penalty will continue to be important and may be expedited by gene-editing techniques if effective gene targets are identified. Additionally, because cercosporin is a virulence factor for the fungus, transferring a cassette of fungal-derived cercosporin AR genes to sugar beet could be a method to establish durable resistance in the host. Molecular advances will continue to help us understand additional
aspects of CLS disease. The elucidation of the molecular basis of fungicide resistances is allowing us to better monitor and manage resistant populations in the field so that we can maintain the efficacy of current available fungicides. It will be imperative in the future to identify key primary sources of inoculum and establish biological mechanisms used by C. beticola to generate genetic diversity. Ultimately, improved knowledge of host–pathogen interactions will aid in successful integrated management of CLS.

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DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analysed in this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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