






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,

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weed host management, and cultivation to reduce infested residue levels, are widely used to manage disease.

Useful website: https://www.ncbi.nlm.nih.gov/genome/11237?genome_assembly_id=352037

KEYWORDS

cercosporin, effector, fungicide resistance, secondary metabolite

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 Marggraf showed that sucrose in both white and red beetroot was chemically identical to the prohibitively expensive tropical cane sugar (Marggraf, 1748). Marggraf'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 (Lartey *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).

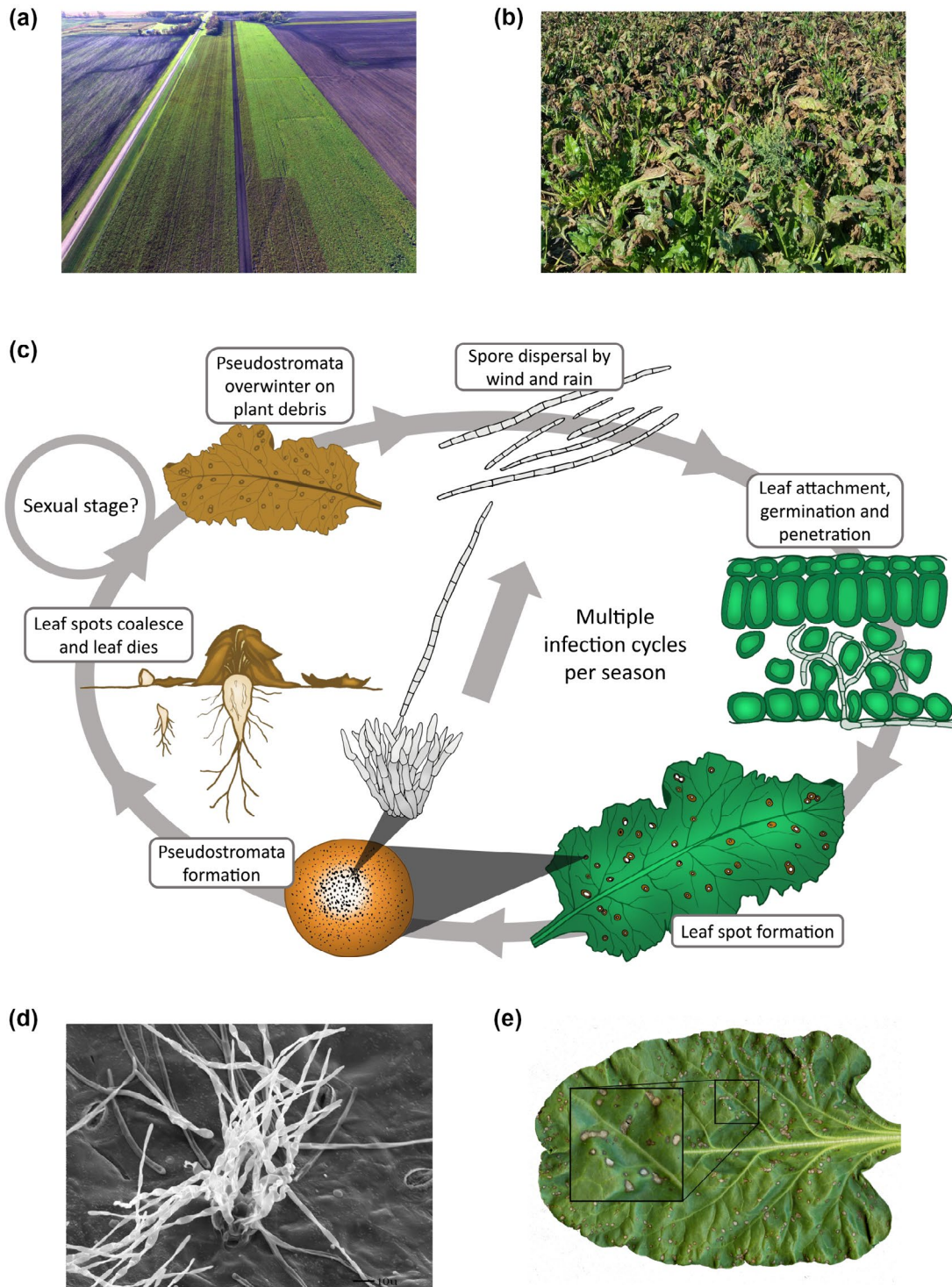


FIGURE 1 *Cercospora* leaf spot (CLS) of sugar beet. (a) Drone image highlighting the importance of fungicides for disease management. While the disease was well-managed on the right side of the field, an abrupt halt in fungicide application ultimately resulted in increased CLS disease as evidenced by 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 symptomatology 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 × 3–5 µm wide at the base (Figure 1d). Conidia are needle-shaped (2–3 × 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; Solel 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; Solel and Minz, 1971b). In the field, these characteristic CLS lesions are typically observed after sugar beet canopy closure (Khan *et al.*, 2008). 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.*, 2011) and one production region within the United States (Obuya *et al.*, 2011). In contrast, 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.*, 2017c). 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 Capnodiales 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 resequencing 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 π 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 ^a	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	1,616	1,468	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^b									
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^b									
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).

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

^bOnly 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 $\leq 1e-10$; percentage coverage of query $\geq 70\%$) 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 *C. beticola* 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; Ohm *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 (PKSs) and nonribosomal peptide synthases (NRPSs), 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 Ehrenshaft, 2000; de Jonge *et al.*, 2018) and beticolin (Goudet *et al.*, 1998). The genome of *C. beticola* possesses 15 type I PKSs, 23 NRPSs, 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 NRPSs (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 ($p < .0001$, 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.

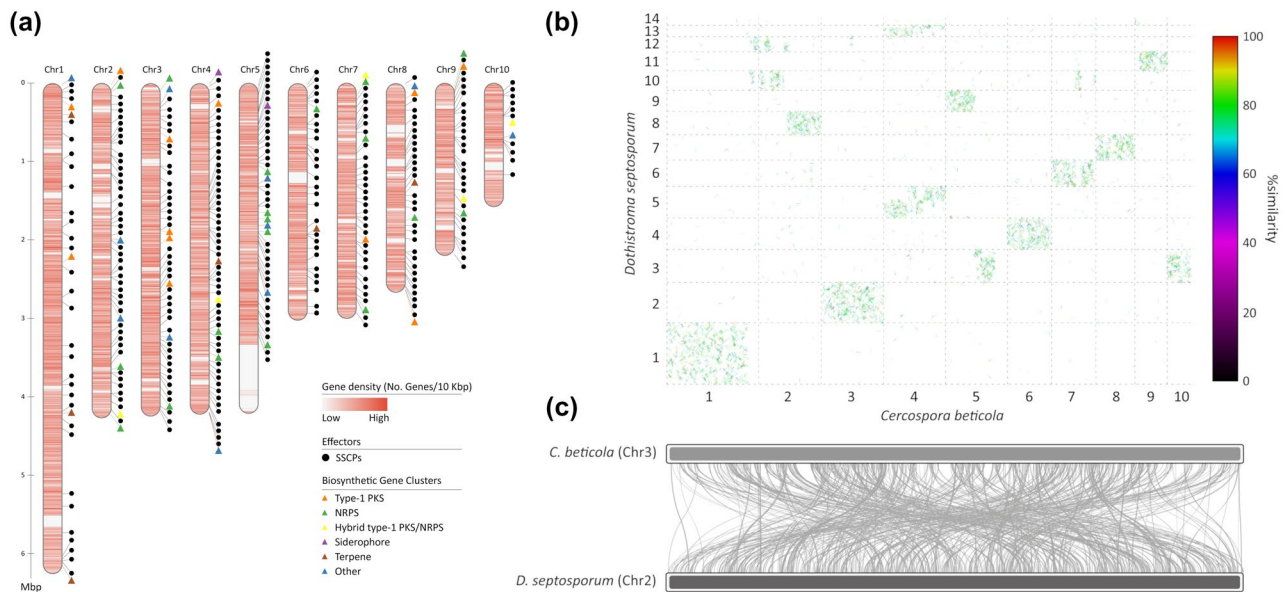


FIGURE 2 (a) Schematic representation of the 10 largest (pseudo)chromosomes and/or scaffolds of *Cercospora beticola*, highlighting the density of genes (genes/10 kb; ranging from 0 [white] to 10 [dark red]), the location of candidate effectors (the small, secreted cysteine-rich proteins or SSCPs; black circles) and the type and location of biosynthetic gene clusters (coloured triangles) for secondary metabolites. (b) Whole-genome alignment between *C. beticola* and *Dothistroma septosporum* highlighting extensive chromosome mesosynten. Whole-genome, protein sequence-based alignments between *C. beticola* and *D. septosporum* reveal many short syntenic regions that are spread over each scaffold or chromosome pair, exemplary for mesosynten. (c) Example of the shuffled homologous regions shared between chromosomes 3 and 2 from *C. beticola* and *D. septosporum*, respectively. (a) and (c) prepared using RIdeogram (Hao *et al.*, 2019); (b) prepared by *promer* in MUMMER3 (Kurtz *et al.*, 2004)

5.4 | *C. beticola* synteny with related fungi

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 macrosynten. Such macrosynten is only rarely observed among species in the fungal kingdom. In fungi, a type of synteny known as microsytent is common in which small segments of up to 10 conserved genes can be found. Moreover, a phenomenon termed mesosytent 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 mesosynten 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 mesosynten. 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, nonhost-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 fioriniae* 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 H_2O_2 and free radical forms such as $O_2^{\bullet-}$, HO_2^{\bullet} , and OH^{\bullet} . 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" (1O_2). 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, nonreducing 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 toralacetone and subsequently to cercoquinone C by CTB3, a predicted O-methyltransferase FAD-dependent monooxygenase (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).

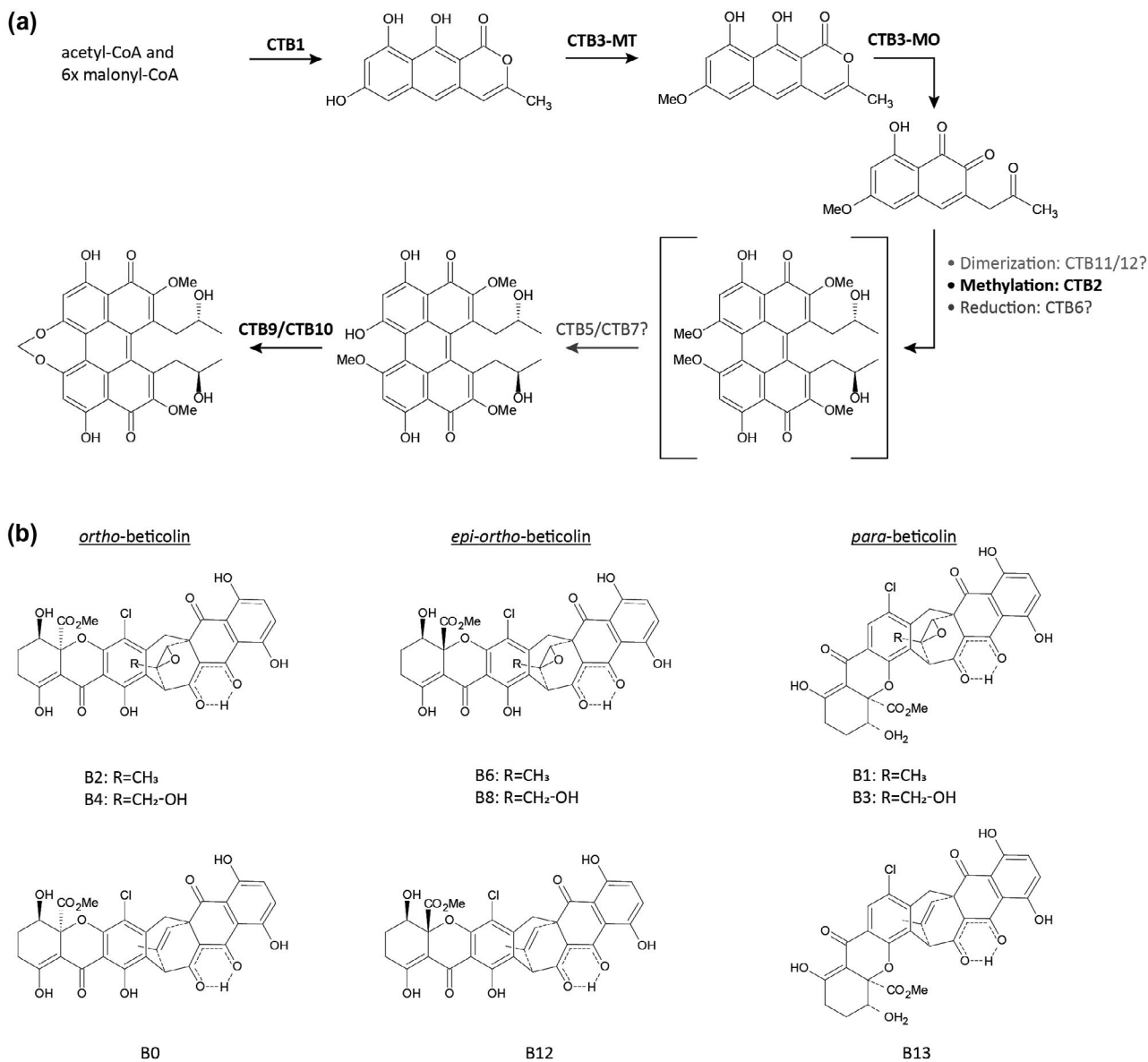


FIGURE 3 (a) Preliminary scheme of the cercosporin biosynthetic pathway consisting of 12 clustered genes. The polyketide synthase CTB1 forms *nor*-toralactone, which is processed to cercoquinone C by CTB3 methyltransferase (CTB3-MT) and monooxygenase (CTB3-MO). Further processing of this cercosporin intermediate might be mediated by CTB2, CTB6, CTB11, and CTB12 to yield the cercosporin intermediate displayed in the square brackets, which has not been directly observed but is rather logically inferred. CTB5 and CTB7 are hypothesized to prime the cercosporin molecule for methylenedioxy bridge formation by CTB9 and CTB10. (b) Beticolin structures and isoforms. Beticolins are structurally related but can differ by residues (R) and isoforms (*ortho*-, *epi-ortho*-, or *para*-beticolin). Beticolins that carry the same residues are able to transform into each other by switching isomery. For example, *ortho*-beticolin B2 can transform into the *epi-ortho*-beticolin B6 or *para*-beticolin B1 based on the position of the oxygen and chlorine atoms

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 phleiochrome 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 *Shiraia 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 CTB 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 ($^1\text{O}_2$) 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 CTB 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 (*Berteroa 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 Mg^{2+} , beticolins inhibit tumoral cell growth in mice (Ding *et al.*, 1996, 2001), interfere with 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 fungicide-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 CbAve1. CbAve1 is a homolog of VdAve1 (Avirulence on Ve1 tomato), an effector secreted by the vascular wilt pathogen *Verticillium dahliae*. In *V. dahliae* the VdAve1 effector is recognized by the tomato cell surface-localized immune receptor Ve1 and confers resistance to the pathogen. In the absence of Ve1, VdAve1 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, CbAve1 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 (Kusstascher *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.*, 2003). 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 State 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 Chrysai-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

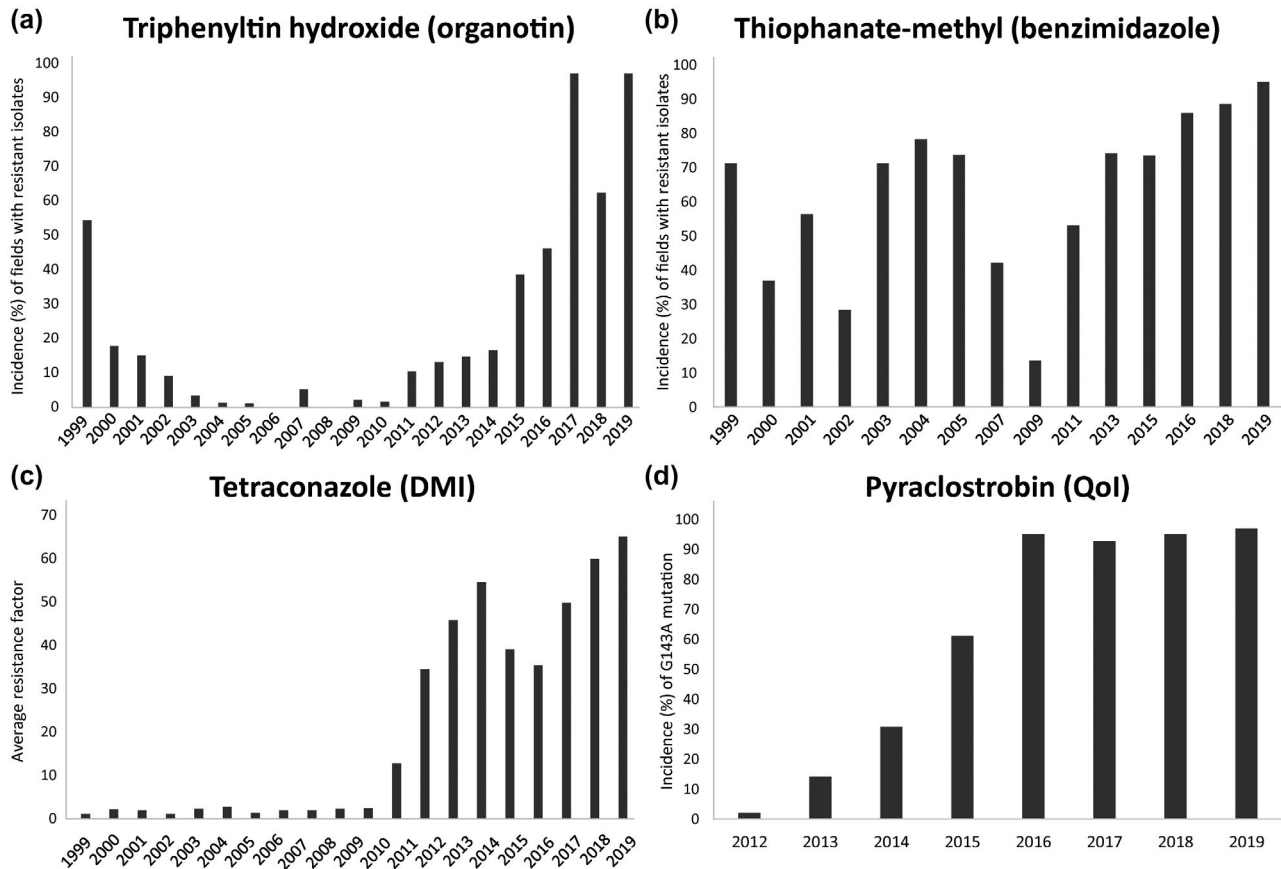


FIGURE 4 Fungicide resistance surveys for *Cercospora beticola* in the Red River Valley region. (a) Incidence (percentage) of fields sampled annually from 1999 to 2019 with isolates resistant to the organotin fungicide triphenyltin hydroxide at 1 $\mu\text{g}/\text{ml}$. (b) Incidence (percentage) of fields sampled from 1999 to 2019 with resistance to the benzimidazole fungicide thiophanate-methyl at 5 $\mu\text{g}/\text{ml}$. (c) Average annual resistance factor values to the demethylation inhibitor (DMI) fungicide tetraconazole for isolates sampled annually from 1999 to 2019, where the resistance factor values are the calculated EC_{50} values divided by the baseline sensitivity values. (d) Incidence (percentage) of sampled isolates annually from 2012 to 2019 harbouring the G143A mutation in cytochrome b, conferring resistance to the quinone outside inhibitor (QoI) fungicide pyraclostrobin

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 EC₅₀ values (Karaoglanidis and Ioannidis, 2010). *C. beticola* isolates with EC₅₀ values of greater than 1 ppm caused significantly more disease on sugar beet after application of a DMI fungicide than isolates with EC₅₀ 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 (EC₅₀ > 1 ppm) to tetraconazole 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 Thanassouloupoulos (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 Qols. DMIs target the lanosterol 14 α -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 (Leroux *et al.*, 2007; Ziogas and Malandrakis, 2015). In the RRV region of the United States, *CbCYP51* was overexpressed in several *C. beticola* isolates with EC₅₀ 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 (EC₅₀ values >50 ppm) from northern Greece (Nikou *et al.*, 2009) and the RRV region of the United States (EC₅₀ 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 EC₅₀ values >1 ppm.

12.4 | FRAC group 11

The Qol 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 Housni *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 Qols, 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 Qol pyraclostrobin and therefore its use is no longer recommended for CLS management in the region (Secor *et al.*, 2017).

Qols 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), Qol-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 Qol 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 Río *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.






ACKNOWLEDGEMENTS

The authors thank Dr Michael Metzger (Minn-Dak Farmers Cooperative) for images in Figure 1a and b and Dr John Weiland (USDA - ARS) for the image in Figure 1d. The contribution by S. Pethybridge was supported by the United States Department of Agriculture National Institute of Food and Agriculture Hatch project NYG-625424 and the Federal Capacity Funds Initiative Project 2018-19-113 managed by Cornell AgriTech at The New York State Agricultural Experiment Station, Cornell University, New York. The M. Bolton laboratory is supported by USDA project 3060-21000-044 and grants from the Sugarbeet Research & Education Board of Minnesota and North Dakota, and the Beet Sugar Development Foundation.

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.

How to cite this article: Rangel LI, Spanner RE, Ebert MK, et al. *Cercospora beticola*: The intoxicating lifestyle of the leaf spot pathogen of sugar beet. *Molecular Plant Pathology*. 2020;21:1020–1041. <https://doi.org/10.1111/mpp.12962>