VIII Cercospora leaf spot – a recent disease in sugar beet; fungicide resistance and variation in strains

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(part of text is taken from Lisa Schulz' master's thesis)

Cercospora leaf spot (CLS) in sugar beet is caused by the fungal pathogen *Cercospora beticola* and is the most destructive foliar disease of sugar beet worldwide (Skaracis et al., 2010). It can cause grave damage to the leaf canopy and thereby reduce the yield and quality of sugar beet.

In general, the severity of an infection with *C. beticola* depends greatly on environmental conditions as well as the resistance level of the cultivars used and agricultural practices (e.g. crop rotation and chemical treatments). Sugar yield losses have been reported to be up to 50% and more (Rossi et al., 2000b).

In Denmark, outbreaks of CLS have still been scarce and mostly local, primarily due to the current, less favourable climate conditions. However, the disease severity has increased in recent years, and it is anticipated that CLS will become a challenge in Northern climate regions in the years to come (Hansen, 2022).

Cercospora beticola primarily infects species of the genus *Beta* but can also cause symptoms on other species of the *Chenopodiaceae* family (like *Spinacea* and *Amaranthus*) (Weiland and Koch, 2004). Even though *C. beticola* is known to be a heterothallic fungus and occurs as one of two mating types (MAT1-1-1 or MAT1-2-1), there is no current knowledge of a sexual stage of *C. beticola* (Rangel et al., 2020). Nevertheless, *C. beticola* populations are generally characterised by high genetic diversity. It has therefore been suggested that hyphal anastomosis or different mating types within populations (MAT1-1-1 and MAT1-2-1) contribute to the sexual recombination within *C. beticola* populations. (Rangel et al., 2020).

Between the growing seasons *C. beticola* is known to overwinter in form of pseudostromata (persistent hyphal structures) on infected plant debris (Weiland and Koch, 2004). These structures have in the past been regarded as the main source of primary inoculum. More recent population studies have, however, reviewed the role of clonally reproduced primary inoculum as the source of infection (Groenewald et al., 2008) and stressed the potential role of imported inocula via plant material, agricultural equipment (Knight et al., 2018, 2019) as well as windborne conidia or stromata from other host plants (Khan et al., 2008; Knight et al., 2020). A study by Spanner et al. (2022) has recently confirmed the presence of viable *C. beticola* structures in sugar beet seed lots (in the pericarb of the fruit) and suggested the spreading of the pathogen, including strains carrying fungicide resistance via the trading of seeds. The life cycle of *C. beticola* is shown in Figure 1.

Life cycle and infection biology

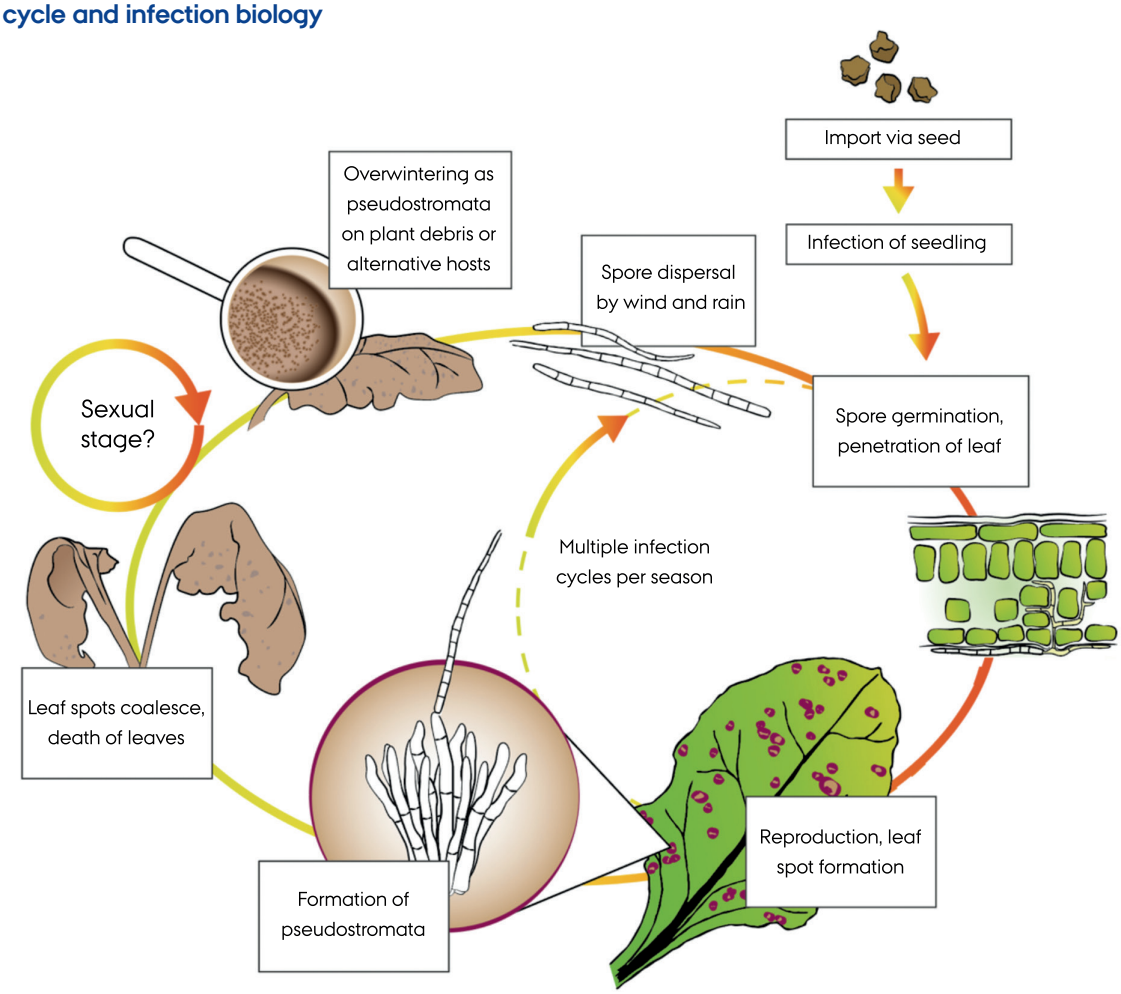

Figure 1. Life and disease cycle of *Cercospora beticola* on sugar beet (adapted from Rangel et al., 2020).

When conidia have formed, they are released and/or carried by wind or dispersed by water splashes to the sugar beet plants. Once landed on the host, they germinate and penetrate the leaves through its stomata and develop hyphae which grow intercellularly inside the parenchymatous leaf tissue (Rangel et al., 2020).

The appearance of the first symptoms depends on climatic conditions but can be typically expected 5 to 21 days after infection (Khan et al., 2009). Most *Cercospora* species are necrotrophs. The fungi produce phytotoxins and hydrolytic enzymes to kill cells in advance of mycelial growth (Weiland and Koch, 2004). This causes the formation of typically reddish-brown coloured leaf spots with a centre of grey-brown necrotic tissue (Figure 2). The lesions range between 0.5 mm and 6 mm in diameter. New pseudostromata develop and become visible as characteristic dark speckles within the grey centre of the leaf spots. They serve to identify *C. beticola* together with conidiophore structures and the long, thin septate conidia (from 2.5 µm to 4 µm wide and from 50 µm to 200 µm long) (Figure 3) (Weiland and Koch, 2004). The pseudostromata give rise to several following generations of asexually produced spores. The fungus is known to induce abundant sporulation about three days after the infected tissue dies (Rossi et al., 2000a).

Figure 2. Sugar beet leaf showing mild symptoms of *C. beticola*. (Photo taken on 10 March 2022). Photo: Lisa Schulz.

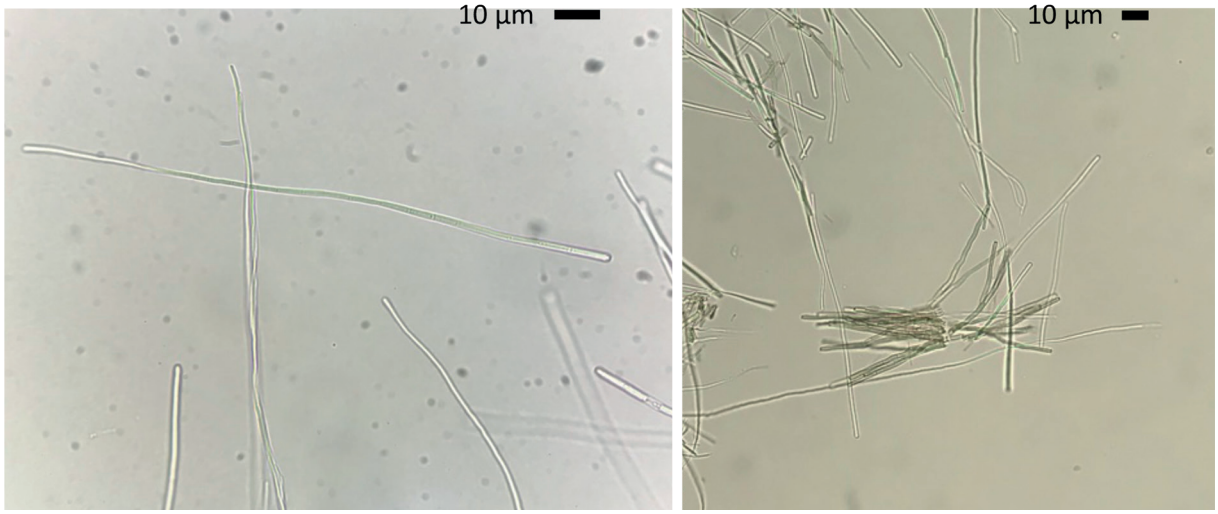

Figure 3. Micrographs of conidiospores (left) and conidiophores (right) of *C. beticola*. (Photos: Lisa Schulz).

One of the typically many sporulation cycles takes about 12 days, depending on how favourable weather conditions are. Optimal conditions are temperatures between 25°C and 35°C during the day and around 16°C at night and a very high relative humidity (RH) (between 90% and 95%) (Forsyth et al., 1963). Spore production is favoured by temperatures between 15°C and 23°C, but spores do not form at temperatures under 10°C or above 38°C (Pool and McKay, 1916). Conidia germination is highest at RH close to 100% and a temperature of 25°C (Khan et al., 2009).

In an advanced stage of infection, typically late in the season, the plant re-stimulates vegetative growth to compensate loss of foliage. This happens at the cost of sugars stored in the root. The consequence of this can be the loss of root weight, sucrose content as well as inferior juice quality, all of which will contribute to an overall lower sugar yield (Rossi et al., 2000b).

Control of *Cercospora beticola* using fungicides

In many sugar beet cultivations, fungicide applications are the primary tool to control CLS disease. A variety of fungicides are registered and can be used by growers in various parts of the world for the control of the fungus (Skaracis et al., 2010). The main active ingredients used against *C. beticola* belong to the strobilurins (QoI; FRAC group 3) and the demethylase inhibitors (DMI; FRAC group 11).

The high reliance on fungicides has given rise to fungicide-resistant *C. beticola* strains in several regions (Nikou et al., 2009; Kumar et al., 2021; Muellender et al., 2021), rendering the disease challenging to manage.

Fungicide resistance to QoI in *C. beticola* has been well described in the literature and associated with the G143A amino acid alteration in the *cytb* gene (Bolton et al., 2013). In two recent studies, Muellender and colleagues (2021) and Spanner and colleagues (2021) found evidence for the association of target-site resistance in the *cyp51* gene with reduced DMI sensitivity in European *C. beticola* populations. Traditionally, fungicide use in Denmark has been relatively restricted, also in sugar beet crops. Recent findings also confirmed that *C. beticola* is a seedborne disease, and fungicide resistance was found in seed lots destined for European farmers (Spanner et al., 2022). Therefore, the Danish *C. beticola* population might already be adapted to fungicides despite the rare occurrence of CLS and relatively lower fungicide exposure in Denmark (Heick et al., 2020).

The presented study set out to give a status of fungicide sensitivity and to screen for fungicide target-site resistance in Danish *C. beticola* isolates to determine the potential risk of fungicide resistance in the light of increasing disease severity in Denmark.

Testing for fungicide resistance

In-vitro sensitivity (EC_{50} values) of Danish *C. beticola* samples from 2021 (n = 33; three sites) was tested towards fungicides of the DMI (prothioconazole-desthio, difenoconazole), QoI (azoxystrobin) and SDHI (boscalid, fluopyram, fluxapyroxad) classes (FRAC group 7) using a microtitre assay. The isolates were produced as described by Secor et al. (2010). All isolates were resistant to azoxystrobin with EC_{50} values > 10 mg/l. The sensitivity levels towards DMIs were in line with the results of Muellender et al. (2021), indicating a similar DMI adaption in Danish *C. beticola* isolates as seen in other European countries (Table 1). SDHI fungicides were insensitive (EC_{50} > 10 mg/l) against *C. beticola*, which confirms previous findings in other *Cercospora* species (Sautua et al., 2020).

The samples from 2021 and an additional 41 samples collected in 2020 (from eleven sites) were analysed for the presence of amino acid alteration G143A, using qPCR (Bolton et al., 2013). G143A was found in 70% of the samples from 2020 and in all samples from 2021.

Further, the *cyp51* gene of samples from 2021 was amplified with a PCR and sequenced to find amino acid alterations associated with DMI insensitivity. Seven different CYP51 haplotypes were identified; the most frequent was harbouring L144F in combination with I309T and a synonymous mutation at amino acid position 170. An alteration at position 294, which led to an alteration from lysine to arginine (K294R), was found in three samples. K294R has not been previously described, and its impact on DMI sensitivity needs to be validated. Sequences of the *cyp51* gene obtained in this study were uploaded to the Nucleotide BLAST database for genome sequencing under the accession numbers: ON324109 - ON324115.

The results presented herein are the first report of QoI-resistant and DMI-adapted *C. beticola* isolated from Denmark. Furthermore, the ineffectiveness of SDHI fungicides against *C. beticola* was shown. Therefore, it is advocated that the management of *C. beticola* exploits the possibilities of fungicide resistance strategies such as applying lower doses, mixing active ingredients and alternating fungicides with different modes of action. Furthermore, a sustainable IPM approach should include agronomic practices such as crop rotation, the sowing of tolerant cultivars and the application of non-chemical biopesticides.

Genetic diversity of *Cercospora beticola* in Denmark

There is a broad base of scientific literature on the genetic structure and diversity as well as the population dynamics of *C. beticola* in other parts of the world. Tools used in these studies include microsatellite markers (also known as Simple Sequence Repeats, SSR), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and single nucleotide polymorphisms (SNPs) (Groenewald et al., 2007; Turgay et al., 2010; Vaghefi et al., 2017a).

C. beticola populations are described to have an overall high genetic and genotypic diversity (at allele, gene and genotype level). Other studies have aimed to quantify genetic homogeneity and differentiation between *C. beticola* populations to analyse whether and at which spatial scale gene flow is happening (Groenewald et al., 2008; Vaghefi et al., 2017a; Knight et al., 2019). Overall populations of the fungus are characterised by low intercontinental differentiation as well as high levels of gene flow (Groenewald et al., 2008; Knight et al., 2019; Rangel et al., 2020).

The genetic diversity of *C. beticola* in Denmark has not previously been investigated due to rare occurrences. It is relevant now to study the diversity of the Danish population of *C. beticola*, particularly in the light of increasing observations of CLS in Danish sugar beet fields and the recent *in vitro* detection of fungicide resistance. This study was initiated based on funding from Sukkerroeafgiftsfonden in 2021 and 2022 (projects: "Cercospora-bladplet – en risiko for dansk sukkerproduktion" and "Cercospora-blad-

plet – en risiko for dansk sukkerproduktion, del II"). The objective was to implement the method of SSR genotyping of *C. beticola* to be able to study genetic diversity and population structure in the Danish population of *C. beticola* in the future.

Table 1. EC_{50} (mg/l) values for prothioconazole-desthio (PTZ-desthio), difenoconazole (Dif), azoxystrobin (Azo), fluopyram (Flu), fluxapyroxad (Flux) and boscalid (Bos) and amino acid alterations found in the *cytb* and *cyp51* region of the *C. beticola* isolates used in this study.

Isolate	EC_{50} PTZ-desthio (mg/l)	EC_{50} Dif (mg/l)	EC_{50} Azo (mg/l)	EC_{50} Flu (mg/l)	EC_{50} Flux (mg/l)	EC_{50} Bos (mg/l)	Amino acid alteration found in *cytb*	Amino acid alteration found in *cyp51*
Wildtype strain	0.01	0.01	0.01	>10	>30	>30		
QoI-resistant strain	0.3196	0.18	>30	>10	>30	>30	G143A	
21-CB-DK-01-01	0.02	0.54	>30	>10	>30	>30	G143A	L144F
21-CB-DK-01-02	0.01	0.12	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-03	0.02	0.35	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-04	0.01	0.57	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-05	0.01	0.32	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-06	0,03	0.35	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-07	0.01	0.98	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-08	0.02	0.12	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-09	0.01	0.09	>30	>10	>30	>30	G143A	L144F + I309T, E170
21-CB-DK-01-10	0.02	0.49	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-11	0.00	0.07	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-01-12	0.04	0.18	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-02-01	0.32	4.22	>30	>10	>30	>30	G143A	L144F + I309T, E170
21-CB-DK-02-02	0.00	0.25	>30	>10	>30	>30	G143A	L144F, H306R
21-CB-DK-02-03	0.00	0.47	>30	>10			G143A	L144F, H306R
21-CB-DK-02-04	0.27	1.31	>30	>10	>30	>30	G143A	L144F + I309T, E170
21-CB-DK-02-05	0.01	0.29	>30	>10			G143A	L144F, H306R
21-CB-DK-02-06	0.01	0.19	>30	>10			G143A	Y464S
21-CB-DK-02-07	0.06	0.58	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-02-08	0.30	2.23	>30	>10	>30	>30	G143A	L144F + I309T, E170
21-CB-DK-02-09	0.01	0.12	>30	>10			G143A	L144F, K294R, H306R
21-CB-DK-02-10	0.02	0.24	>30	>10			G143A	L144F, K294R, H306R
21-CB-DK-02-11	0.02	0.11	>30	>10			G143A	L144F, H306R
21-CB-DK-03-01	0.04	0.72	>30	>10	>30	>30	G143A	L144F + I309T, E170
21-CB-DK-03-02	0.03	0.52	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-03-03	0.01	0.36	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-03-04	0.01	0.30	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-03-05	0.01	0.51	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-03-06	0.04	0.09	>30	>10	>30	>30	G143A	L144F + I309T, E170
21-CB-DK-03-07	0.01	0.02	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-03-08	0.11	0.18	>30	>10	>30	>30	G143A	L144F + I309T, E170, K294R
21-CB-DK-03-10	0.07	0.27	>30	>10			G143A	L144F + I309T, E170
21-CB-DK-03-11	0.12	0.03	>30	>10	>30	>30	G143A	L144F, E170
Mean	0.05	0.52	>30	>10	>30	>30		

Thirteen SSR markers previously developed for *C. beticola* by Groenewald et al. (2007) and Vaghefi et al. (2017b) were applied. In total, 114 Danish *C. beticola* isolates from diseased sugar beet leaves sampled at different sites in 2020-2022 were successfully SSR genotyped. Initial results showed the presence of a minimum of 37 Multi Locus Genotypes (MLG) in the Danish population of *C. beticola* across the three sampled years. In some field sites, only one MLG was detected, whereas other field sites contained multiple MLGs. The initial results indicate a high diversity to be further investigated. Future studies will include comparison of the genotypes identified in Denmark with genotypes identified in other countries to determine the level of differentiation among populations and possible gene flow to infer on the possible source of Cercospora leaf spot in Denmark.

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