



First report of G143A strobilurin resistance in *Cercospora beticola* in sugar beet (*Beta vulgaris*) in Poland

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Abstract *Cercospora* leaf spot (CLS) caused by the fungus *Cercospora beticola* is the most common and destructive disease of sugar beet in Poland. Strobilurin (Quinone outside inhibitors—QoI) fungicides are widely used for CLS control. In Poland for several years, a gradual increase in QoI tolerance has been observed. In 2015, most of the tested isolates (56%) collected from several independent locations were able to grow in a PDA medium supplemented with 1 µg/ml of azoxystrobin. An additional test showed that the EC₅₀ value for the most resistant isolates was ≥100 µg/ml, while for the sensitive ones it was ≤0.01 µg/ml. The results of cytochrome b partial DNA sequencing revealed that all resistant isolates contained single guanine to cytosine substitution (G/C), predicting the conversion of glycine to alanine at the 143 codon position (G143A). To our knowledge, this is the first report in a peer-reviewed paper on G143A mutation determining the strobilurin resistance of *C. beticola* in Poland.

Keywords *Cercospora* leaf spot · *Cercospora beticola* · QoI resistance

Cercospora leaf spot (CLS) caused by the fungus *Cercospora beticola* Sacc. is one of the most common and destructive foliar diseases of sugar beet (*Beta vulgaris*) worldwide (Skaracis et al. 2010). The disease leads to premature death of leaves, and by reducing the assimilation area it causes a significant loss of root yield and diminished sucrose content (Skaracis et al. 2010). In Poland, the disease occurs annually, but its economic impact is particularly noticeable during years with warm and rainy summer and early autumn. In Poland, CLS is mainly controlled with single-site fungicides, such as sterol demethylation inhibitors (DMI) or quinone outside inhibitors (QoI) fungicides, used as single or mixed preparations. Unfortunately, frequent application of this class of fungicides leads to selective pressure and the development of resistance in plant pathogens (Ma and Michailides 2005).

QoI fungicides (strobilurins) are widely used in agricultural production because of their wide antifungal spectrum and low phytotoxicity. They act by binding the quinol oxidation site of cytochrome bc₁ protein. It disrupts electron transfer between cytochrome b and c₁ complexes and leads to ATP production deficiency in sensitive fungi (Bartlett et al. 2002; Fisher and Meunier 2008). The QoI resistance is usually conferred by single-point mutation in mitochondrial cytochrome b gene (*cytb*). Amino acid exchange from glycine to alanine at the 143 codon position (G143A) was detected as the main source of QoI resistance in many plant pathogenic fungi (Birla et al. 2012; Bolton et al. 2012; Jiang et al. 2009; Obuya and Franc 2016). Substitution phenylalanine to leucine (F129L) and glycine to arginine (G137R) are less common (Fisher and Meunier 2008).

Poland is one of the most important sugar beet producers in the European Union (<http://ec.europa.eu/eurostat>). Due to the significance of sugar beets in agricultural production,

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it is very important to know and monitor the occurrence of QoI-resistant isolates. The aim of the study was to detect the presence of mutations leading to QoI resistance in *C. beticola* in Poland.

Sugar beet leaves infected with *C. beticola* were randomly sampled from several fields located mainly in central (Dobrzelin, Kruszwica), eastern (Krasnystaw, Werbkowice) and western (Kluczewo) Poland in August and September 2015. The fields were owned by Krajowa Spółka Cukrowa S.A. and the crops were protected according to Polish plant protection recommendations. Individual, superficially sterilised leaf spot lesions were placed and grown on a PDA (Potato Dextrose Agar; Oxoid Ltd, Basingstoke, UK) medium. After 1 week of incubation at 21 °C, the emerging *C. beticola* cultures, identified by colony morphological features, were transferred into a new PDA medium. In total, 126 *C. beticola* isolates were obtained (Dobrzelin 12, Kruszwica 36, Krasnystaw 23, Werbkowice 25, Kluczewo 18, others locations 12 isolates). All of the cultures were incubated for 2–3 weeks at 21 °C. In order to assess strobilurin sensitivity mycelial plugs (0.5 cm²) taken from the edge of the colonies were placed on a PDA medium amended with 1 µg/ml azoxystrobin (Amistar 250 SC; Syngenta, Basel, Switzerland) and on PDA without amendment. The percentage of growth inhibition was measured according to the method described by Bugbee (1995). In order to estimate the EC₅₀ values (half maximal effective concentration), selected isolates were additionally tested on the PDA medium containing azoxystrobin (Sigma-Aldrich; Saint Louis, USA) at a concentration of: 0, 0.01, 0.1, 0.5, 1, 5, 10, 20, 50 and 100 µg/ml. To block the alternative oxidation pathway each medium was supplemented with 100 µg/ml salicylhydroxamic acid (SHAM; Sigma-Aldrich). The fungicide was dissolved in acetone at 100 mg/ml and diluted to 10 mg/ml in distilled water before being added to the medium. The samples were incubated for 7 days at 21 °C. The mean EC₅₀ values of the tested isolates were calculated. The assays were repeated three times.

Ten resistant (EC₅₀ value ≥10–100 µg/ml) and three sensitive isolates (EC₅₀ value ≤0.01 µg/ml) were chosen for cytochrome b DNA sequence analysis. Total genomic DNA was extracted from fresh mycelium using a Plant/Fungi DNA isolation Kit (Norgen Biotek Corp.; Thorold, Canada), following the instruction of the manufacturer. Fresh mycelium (100 mg) growing for 2–3 weeks on a PDA medium was gently scraped with a lancet and transferred into a 1.5 ml Eppendorf tube. The samples were precisely macerated with a micropestle, and sterile quartz sand was added. The quantity and quality of the extracted DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific; Waltham, USA). 20 ng/µl DNA solutions were prepared for each probe and preserved at

–20 °C. A DNA fragment of cytochrome b gene was PCR amplified with cytbSF and cytbSR primers (Malandrakis et al. 2011), followed by Sanger sequencing of the product obtained. The PCR mixture contained 40 ng of template DNA, 1 µM of each primer, 1 × Master MIX (Thermo Fisher Scientific). All reactions were carried out in total volumes of 20 µl. The PCR thermal cycling protocol was as follows: initial denaturation at 95 °C for 3 min, then 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and elongation for 60 s at 72 °C, followed by the final extension step for 5 min at 72 °C. All PCR reactions were conducted in a Mastercycler ep gradient S thermal cycler (Eppendorf; Hamburg, Germany). The amplification products were separated by gel electrophoresis in 1.5% agarose gel in 0.5 × TBE (Thermo Fisher Scientific) with Midori Green (Nippon Genetics; Dueren, Germany) staining and visualised under UV 360 nm light. The PCR products were commercially Sanger-sequenced at Genomed S.A. (www.genomed.pl).

Most of the tested isolates (56%) collected from several independent locations (Dobrzelin 6, Kruszwica 15, Krasnystaw 17, Werbkowice 17, Kluczewo 8, others locations 7 isolates) were able to grow (growth inhibition percentage >50%) on the medium supplemented with 1 µg/ml azoxystrobin. An additional test showed that the EC₅₀ for the selective, resistant isolates was ≥10–100 µg/ml, while for the sensitive ones it was ≤0.01 µg/ml. The sequencing analysis of the partial cytochrome b gene revealed that all of the ten tested resistant isolates contained single guanine to cytosine (G/C) substitution predicting the conversion of glycine to alanine at the 143 codon position (G143A). This mutation was not observed in any of the three sensitive isolates tested. F129L or other mutations were not found in any items. The partial DNA sequences of cytochrome b strobilurin-resistant and strobilurin-sensitive isolates were deposited in the NCBI database (GenBank Accession Nos. MF327259 and MF327260). The obtained *C. beticola* cytochrome b partial DNA sequences exhibited 100% homology to the *C. beticola* cytochrome b sequence (GenBank Accession Nos. JQ360626 and JQ360628).

In the USA, QoI fungicides have been used for management of sugar beet diseases since the late 1990s (Briere et al. 2003; Secor et al. 2010); in Europe and Poland—approximately since the late 2000s. There were no reports on QoI field resistance in the USA and Greece until 2011, and in France until 2012 (Birla et al. 2012; Malandrakis et al. 2011; Secor et al. 2010). The first QoI-resistant isolates of *C. beticola* containing the substitution of glycine to serine at the 143 codon position (G143S) and others were detected under artificial conditions only (Malandrakis et al. 2006). However, due to the widespread use of strobilurin in CLS management QoI resistance is developing rapidly in many sugar beet growing areas. Nowadays QoI-resistant

field isolates of *C. beticola* with the G143A mutation have been detected in many sugar beet growing areas in Europe (mostly the Mediterranean area) and the USA (Bolton et al. 2012; Birla et al. 2012; Obuya and Franc 2016). Recently the F129L mutation was identified as a moderate source of QoI resistance in *C. beticola* isolates obtained from table beets in the USA (New York) (Vaghefi et al. 2016). Until 2015 the G143A mutation was not reported in Poland, but increased strobilurin tolerance was observed (Pieczul and Perek 2013). The phenomenon was attributed to the additional supply of energy due to increased alternative oxidase (AOX) activity or due to other reasons (Pieczul and Perek 2015). Previous studies indicated that AOX can be induced by fungicides causing ubiquinone inhibition (QoI), what increased survival of fungi under QoI treatment (Wood and Hollomon 2003).

In comparison with warmer sugar beet growing areas, in Poland the increase in QoI-resistant *C. beticola* isolates was delayed. We found it as a result of less frequent application of fungicides for sugar beet protection in this climate zone. The data confirmed resistant G143A phenotypes of *C. beticola* isolates collected from fields at independent locations. To our knowledge, this is the first report in a peer-reviewed paper of the *C. beticola* strobilurin resistance induced by G143A mutation in Poland. To conclude, the research findings indicate that QoI fungicides can be less efficient for CLS control in Poland and new disease management strategies must be implemented. The choice of fungicides for CLS control in Poland is limited mainly to DMI and QoI, which makes effective crop protection more difficult.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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