# PCR-BASED DETECTION OF BENZIMIDAZOLE RESISTANCE IN *CERCOSPORA BETICOLA*

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Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola* (Sacc.), is the most destructive foliar pathogen of sugarbeet worldwide. Control measures include the use of resistant varieties and crop rotation, but timely fungicide applications are essential for CLS management. *Cercospora beticola* represents a classic 'high-risk' pathogen for fungicide resistance development due to high genetic variability, abundant sporulation, polycyclic nature, and the high number of fungicide applications required for disease control. Resistance to sterol demethylation inhibitor (DMI) fungicides (FRAC group 3) has been increasing in *C. beticola* populations (Bolton et al. 2012), forcing growers to increase reliance on quinone outside inhibitor ($Q_O$I) fungicides (FRAC group 11). $Q_O$Is act by binding the quinol oxidation site of the cytochrome $bc_1$ enzyme complex, which leads to an energy deficit in sensitive fungi (Fisher and Meunier 2008). Point mutations in *cytb* that encode an amino acid exchange from phenylalanine to leucine at position 129 (F129L), glycine to arginine at position 137 (G137R), or glycine to alanine at position 143 (G143A) have been associated with resistance to $Q_O$I fungicides. We recently showed that the cytb G143A mutation in *C. beticola* was associated with QoI resistance in Italy (Birla et al. 2012), Michigan (Bolton et al. 2013), and the Red River Valley of Minnesota and North Dakota (Bolton et al. 2013).

Benzimidazole fungicides have been used extensively in CLS disease management. However, increased resistance to these fungicides has been documented in *C. beticola* (Weiland and Halloin 2001). Benzimidazoles act primarily by binding to fungal tubulin, which inhibits mitosis and interferes with the fungal cytoskeleton. As such, mutations in β-tubulin genes have been associated with resistance to benzimidazole fungicides. Davidson et al. (2006) cloned a fragment of the β-tubulin gene and showed that the E198A mutation in β-tubulin was associated with benzimidazole resistance in Michigan populations of *C. beticola*. The objective of this study was to (i) clone the full-length β-tubulin gene from *C. beticola*, (ii) sequence β-tubulin in isolates derived from the Red River Valley that are sensitive and resistant to benzimidazole, and (iii) develop a qPCR assay to identify resistant isolates.

## Materials and Methods

*Sample collection, fungal isolation, and fungicide sensitivity assays*
Sugarbeet leaves with CLS were received from Red River Valley fields. Conidia were harvested as described previously (Secor and Rivera 2012). Briefly, 50 µL of T-water (0.06% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO), 0.02% (w/v) filter-sterilized ampicillin (Sigma-Aldrich) added after the solution had been autoclaved) was added to the surface of a CLS lesion. The lesion was gently scraped with a pipette tip to liberate conidia into the T-water, which was transferred to a water agar plate amended with Topsin at 5 µg $ml^{-1}$ or to non-amended plates. This process was repeated for several lesions in the sample. After 16 h, spores that germinated on Topsin-amended plates were transferred to a clarified V8-medium plate and incubated at 22°C (±1°C) for two weeks. This was the source colony for subsequent experiments.

*Cercospora beticola β-tubulin sequence analysis*
DNA was isolated using the CTAB method from mycelia scraped from the V8-agar surface. A series of primers were designed to amplify β-tubulin using the GoTaq Flexi DNA Polymerase kit (Promega, Madison, WI) following the manufacturer's instructions. Consensus sequences were analyzed using Vector NTI (Invitrogen, Carlsbad, CA) software. The primers were used to amplify the β-tubulin gene from 25 sensitive and 25 resistant isolates.

*Detection of E198A mutation site by real-time PCR*
A real-time PCR procedure was developed to differentiate benzimidazole-resistant isolates harboring the E198A mutation from sensitive isolates. Pairwise comparisons of primer (200, 300, and 400 nM), probe (100, 200, and 300 nM), and $MgCl_2$ concentrations (3.5, 4.5, and 5.5 mM) were assessed. The optimized procedure used for screening isolates in this study utilized the GoTaq Flexi DNA Polymerase kit in a 25 µL volume containing 1X clear buffer, 5.5 mM $MgCl_2$, 0.2 mM of each dNTP, 300 nM of each primer and probe, 1.25 U of GoTaq DNA polymerase, and ~0.5 ng of genomic DNA. Real-time PCR was carried out in a PTC-200 thermal cycler (MJ Research, Waltham, MA) outfitted with a Chromo4 Real Time PCR Detector (Bio-Rad Laboratories, Hercules, CA) and analyzed with

MJ Opticon Monitor software version 3.1 (Bio-Rad Laboratories). For each dye, the threshold line was manually adjusted to the highest fluorescence value attained from the respective negative control. PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers were supplied by Invitrogen as HPLC-purified. Probes contained a locked nucleic acid on the mutation site and were supplied by Sigma-Aldrich.

**Results and Discussion**

*Identification of the E198A mutation*
Field isolates collected from the Red River Valley were assessed for *in vitro* sensitivity to the Topsin. Twenty-five isolates that were able to grow on Topsin-amended media were considered benzimidazole-resistant. We sequenced the β-tubulin gene from these 25 isolates and 25 isolates that were benzimidazole-sensitive and identified a single nucleotide polymorphism that encoded an E198A transition in the β-tubulin protein. No other mutations were identified that were associated with benzimidazole resistance.

*Differentiation of resistant and sensitive isolates using real-time PCR assay*
Several primer and probe sequences were tested for the ability to distinguish benzimidazole-resistant and -sensitive isolates (not shown). One primer and probe combination exhibited the highest efficiency, specificity, and fluorescence with the lowest Ct value per unit of DNA than all other tested combinations. The utility of the real-time PCR assay was validated by analyzing all isolates in this study. All real-time PCR results were concordant with *cytb* sequencing where a cycle threshold (Ct) value from the sensitive-specific probe was achieved only when using template from sensitive isolates, and conversely a Ct value from the E198A-specific probe only occurred when using template from resistant isolates. The use of real-time PCR to identify benzimidazole-resistant isolates is a valuable tool for fungicide resistance management. Compared to traditional spore germination assays, real-time PCR has the potential to increase throughput while decreasing the time needed to determine benzimidazole-sensitivity of a sample. Knowledge of benzimidazole resistance in a field may be especially important during the growing season when short fungicide application intervals combined with high CLS disease pressure demand a quick turnaround time to guide the choice of fungicide chemistry to apply.

Further research needs to be conducted to optimize PCR parameters so our G143A primer set that identifies QoI-resistant isolates can be multiplexed with the benzimidazole primer set. This would allow the investigator to identify resistance to two separate chemistries in a single PCR reaction, greatly increasing sample throughput.

**References**

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