PCR-BASED DETECTION OF STROBILURIN RESISTANCE IN *CERCOSPORA BETICOLA**

Melvin D. Bolton[1], Keshav Birla[2], Viviana Rivera-Varas[2], Mohamed F. R. Khan[2], and Gary Secor[2]

[1]USDA – ARS, Northern Crop Science Laboratory, Fargo
[2]Department of Plant Pathology, North Dakota State University, Fargo

* The research presented here is modified from Bolton et al. 2013. Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. Pest Management Science 69: 35-39.

Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola* (Sacc.), is the most destructive foliar pathogen of sugarbeet worldwide.[1] Control measures include the use of resistant varieties and crop rotation, but timely fungicide applications are essential for CLS management.[2] Resistance to sterol demethylation inhibitor (DMI) fungicides (FRAC group 3) has been increasing in *C. beticola* populations,[3] forcing growers to increase reliance on quinone outside inhibitor (Q_OI) fungicides (FRAC group 11). Q_OIs act by binding the quinol oxidation site of the cytochrome bc_1 enzyme complex, which leads to an energy deficit in sensitive fungi.[4] 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_OI fungicides.[4]

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.[2, 3, 5] Although Q_OI fungicides have been widely used to control CLS since 2003, there have been no reports of Q_OI-control failures in the United States. However, growers in Michigan reported CLS control failures despite application of Q_OI fungicides during the 2011 growing season. The present work confirms the resistance phenotype of isolates collected in Michigan, describes the molecular mechanism associated with resistance, and provides a real-time PCR protocol to differentiate Q_OI-resistant isolates from sensitive isolates.

Materials and Methods

Sample collection and fungal isolation

In September 2011, sugarbeet leaves with CLS were received from fields exhibiting disease control problems in Bay, Gratiot, Huron, Saginaw, and Sanilac counties in Michigan, USA. Conidia were harvested as described previously.[6] 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[6] and incubated at 22°C (±1°C). After 24 h, one germinated conidium was transferred to a clarified V8-medium plate[6] and incubated at 22°C (±1°C) for two weeks. This was the source colony for subsequent experiments. This process was repeated for each sample.

Because all isolates collected in 2011 were Q_OI-resistant (see Results), we tested isolates in our culture collection harvested in 1998 and 1999 from Michigan sugarbeet fields for triazole sensitivity testing (G. Secor, *unpublished*) that had never been exposed to pyraclostrobin or trifloxystrobin. In 1998 and 1999, agar plugs were taken from the leading edge of source colony plates as described above and stored in 2.0 ml tubes at -20°C. To revive isolates, plugs were removed from storage, placed on V8-medium plates, and incubated at 22°C (±1°C) for two weeks. These were the source colonies for subsequent experiments.

Fungicide sensitivity assays

A conidia germination assay was used to calculate EC_{50} values (effective concentration of fungicide that reduces spore germination by 50%) to technical grade pyraclostrobin (BASF, Research Triangle Park, NC) and trifloxystrobin (Bayer Crop Science, Research Triangle Park, NC) as described by Secor and Rivera.[6] All fungicide-amended plates contained salicylhydroxamic acid (Sigma-Aldrich catalog #S607, St. Louis, MO) at 100 µg ml^{-1}. The percentage of germinated conidia on plates amended with serial ten-fold dilutions of fungicide from 0.001 to 100.0 µg ml^{-1} was used to calculate the EC_{50} value.[6]

Cercospora beticola cytb sequence analysis
DNA was isolated using the CTAB method[7] from mycelia scraped from the V8-agar surface. Primers 738 and 739 (Table 1), designed on GenBank Accession # EF176921,[8] amplified a fragment of *cytb* using the GoTaq Flexi DNA Polymerase kit (Promega, Madison, WI) following the manufacturer's instructions and a final $MgCl_2$ concentration of 2.5 mM. PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s, followed by 72°C for 5 min. PCR amplicons were sequenced directly (McLab, South San Francisco, CA). Consensus sequences were analyzed using Vector NTI (Invitrogen, Carlsbad, CA) software.

Table 1. Primers and probes used in this study

Primer	Role	Sequence (5' – 3')[a]	Amplicon size (bp)
738	*cytb* Forward	CAGCTTCAGCATTTTTCTTCTT	645
739	*cytb* Reverse	TGGCAGAAAACATAGCAATAACA	
735	*cytb* real-time Forward	GAGGTCTATACTATGGTTCTTA	181
736	*cytb* real-time Reverse	TGTCCTACTCATGGTATTG	
740-SEN	Sensitive-specific probe	6FAM-TGAG[G]TGCAACTGTTATTACTAA-BHQ-1	
741-RES	G143A-specific probe	HEX-TGAG[C]TGCAACTGTTATTACTAA-BHQ-1	

[a] 6-carboxyfluorescein (6FAM); hexachloro-6-carboxyfluorescein (HEX); Black Hole Quencher-1 (BHQ-1). Locked nucleic acid bases are shown in brackets ([]).

Simple sequence repeat (SSR) analysis
Genotype identification was assessed using the simple sequence repeat (SSR) primer set SSRCb3[9] as described by Bolton et al.[5] Briefly, PCR amplifications were conducted using the GoTaq Flexi DNA Polymerase kit in a total volume of 20 µL containing 1X Clear GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.2 µM of each primer, 1.0 U of GoTaq DNA polymerase, and ~25 ng of genomic DNA. PCR conditions were an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. A final seven min elongation step at 72 °C was included. Separation of labeled DNA fragments was performed by McLab using a Genetic Analyzer 3730*xl* (Applied Biosystems, Foster City, CA). Fragment size (base pairs) was determined using Peak Scanner Software (Version 1.0; Applied Biosystems).

Detection of G143A mutation site by real-time PCR
A real-time PCR procedure was developed to differentiate Q_OI-resistant isolates harboring the G143A 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 (735, 736; 740-SEN, and 741-RES; Table 1), 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 735 and 736 were supplied by Invitrogen as HPLC-purified. Probes 740-SEN and 741-RES contained a locked nucleic acid[10] on the mutation site and were supplied by Sigma-Aldrich. DNA from the sensitive isolate 98-10-M and resistant isolate 11-1046-2 (Table 2) was diluted in a 10X dilution series from 10.0 ng to 0.10 pg and used as template to assess sensitivity of the assay.

Results and Discussion

Survey of Michigan Cercospora beticola isolate sensitivity to pyraclostrobin and trifloxystrobin
Forty field isolates collected in 2011 from Michigan counties were assessed for *in vitro* sensitivity to the Q_OI fungicides pyraclostrobin and trifloxystrobin. All 40 isolates exhibited EC_{50} values > 0.92 µg ml^{-1} to both fungicides (Table 2). In contrast, isolates collected in Michigan in 1998 and 1999 with no previous exposure to trifloxystrobin or pyraclostrobin exhibited EC_{50} values ≤0.006 µg ml^{-1} (Table 2). Therefore, the 2011 Michigan isolates represent an approximate 1000-fold decrease in sensitivity compared to baseline EC_{50} values. Likewise, baseline EC_{50} values of *C. beticola* isolates collected in MN and ND were 0.003 and 0.005 µg ml^{-1} to pyraclostrobin and trifloxystrobin, respectively,[2] confirming that EC_{50} values associated with Q_OI-sensitivity are < 0.01 µg ml^{-1}. Our results suggest that cross resistance between pyraclostrobin and trifloxystrobin occurs in *C. beticola*, which is known to occur in other fungi (www.frac.info). To our knowledge, this is the first report of Q_OI control failures of

CLS in the United States. Since trifloxystrobin and pyraclostrobin were first used for CLS control in 2002 and 2003, respectively,[2] these resistant populations were detected within nine years of sustained and widespread use of Q_OIs. The number of Q_OI applications varies regionally and annually, but may include up to three Q_OI applications during the Michigan growing season (S. Poindexter, *personal communication*). Malandrakis et al. recently characterized pyraclostrobin resistance levels from isolates collected in Greece, but no resistant phenotypes were found.[11]

Pyraclostrobin is currently the most widely used Q_OI fungicide for CLS control in Michigan (J. Stewart, *personal communication*). This fungicide has been well adopted by growers because of protective and translaminar activity[12] as well as claimed plant health benefits. For example, pyraclostrobin is believed to provide yield benefit and increased frost tolerance when applied near the end of the growing season. The use of pyraclostrobin as the final fungicide reduces resistance management flexibility and may have contributed to the selection of Q_OI-resistant isolates in some locations. Isolates were collected after final Q_OI applications had occurred in fields with CLS control problems, which likely contributed to the dominance of Q_OI-resistant isolates found in this study. *Cercospora beticola* populations in Europe and the United States have characteristics typical of sexual reproduction even though no teleomorph has been described for this fungus.[5,9] If *C. beticola* has a sexual stage, it is likely to be involved with the evolution of fungicide resistance in this pathosystem.

Table 2. Sensitivity of *C. beticola* field isolates to Q_OI fungicides in correlation with mutations at codon 143 in *cytb* gene and real-time PCR Ct values

Isolate	Harvest location[a]	EC_{50} value[b] (μg ml^{-1}) pyraclostrobin	trifloxystrobin	SSR allele size (BP)[c]	Amino acid at codon 143	Ct value[d] 740-SEN	741-RES
98-9-M	MI	0.001	0.004	302	G	24.2	–
98-10-M	MI	0.001	0.001	257	G	23.9	–
98-12-M	MI	0.001	0.005	257	G	23.5	–
98-13-M	MI	0.006	0.006	257	G	23.3	–
98-19-M	MI	0.003	0.005	257	G	24.3	–
98-101-5-M	MI	0.001	0.006	309	G	23.7	–
98-150-M	MI	0.001	0.004	302	G	24.3	–
99-233-3-M	MI	0.001	0.004	309	G	23.4	–
99-521-29-M	MI	0.001	0.005	302	G	23.8	–
11-1003-2	Saginaw	0.915	1.000	307	A	–	21.8
11-1004-1	Saginaw	5.345	6.657	257	A	–	22.3
11-1005-1	Saginaw	46.015	9.544	265	A	–	20.2
11-1005-2	Saginaw	7.865	3.250	265	A	–	23.3
11-1006-2	Saginaw	9.538	8.456	265	A	–	21.7
11-1007-1	Bay	4.783	7.651	265	A	–	21.0
11-1007-2	Bay	16.113	6.845	257	A	–	22.8
11-1008-1	Bay	4.824	7.495	265	A	–	22.2
11-1009-1	Bay	47.419	39.382	257	A	–	21.9
11-1014-1	Huron	5.031	4.539	307	A	–	21.4
11-1015-1	Huron	72.226	7.303	307	A	–	21.2
11-1015-2	Huron	2.100	7.790	257	A	–	19.5
11-1016-1	Huron	51.723	5.893	265	A	–	19.4
11-1022-1	Tuscola	29.682	6.864	301	A	–	19.6
11-1022-2	Tuscola	6.371	4.978	301	A	–	20.2
11-1026-1	Huron	7.146	25.947	265	A	–	19.3
11-1026-2	Huron	7.557	8.547	257	A	–	19.2
11-1027-1	Huron	25.947	29.720	257	A	–	21.1
11-1027-2	Huron	4.121	6.064	257	A	–	19.6
11-1028-1	Bay	8.792	7.618	265	A	–	19.4
11-1028-2	Bay	4.528	4.965	257	A	–	19.6
11-1035-2	Sanilac	31.023	6.359	265	A	–	20.6
11-1036-1	Huron	29.682	3.625	265	A	–	21.3
11-1036-2	Huron	5.550	8.150	257	A	–	19.8
11-1037-1	Huron	40.863	6.785	257	A	–	20.4
11-1037-2	Huron	5.918	4.236	257	A	–	20.8
11-1038-1	Huron	39.880	19.857	265	A	–	19.2
11-1038-2	Huron	3.238	35.479	265	A	–	20.9
11-1039-1	Huron	20.422	6.788	265	A	–	21.8
11-1040-1	Gratiot	12.013	7.640	307	A	–	19.9
11-1040-2	Gratiot	22.328	13.658	307	A	–	21.8
11-1041-1	Gratiot	4.547	6.093	307	A	–	19.3

11-1042-1	Gratiot	31.393	8.833	257	A	–	19.3
11-1042-2	Gratiot	32.626	2.364	257	A	–	20.6
11-1043-2	Gratiot	16.925	3.269	257	A	–	20.1
11-1044-1	Gratiot	14.274	13.850	265	A	–	23.0
11-1044-2	Gratiot	5.231	8.562	265	A	–	20.3
11-1045-2	Gratiot	16.235	7.023	307	A	–	19.2
11-1046-1	Gratiot	41.713	5.484	265	A	–	21.3
11-1046-2	Gratiot	26.213	8.365	265	A	–	22.5

[a] Location refers to counties in Michigan from which *C. beticola* isolates were harvested with the exception of Q_OI-sensitive isolates collected in 1998 and 1999 where the county of harvest is not known. In the latter case, the location is labeled MI for Michigan.
[b] EC_{50} values calculated as described by Secor and Rivera.[6]
[c] Genotype identification was assessed using the SSR primer set SSRCb3[9] as described by Bolton et al.[5] Number indicates fragment size in base pairs (BP) .
[d] Dash line (–) refers to sample/probe combination where no cycle threshold (Ct) was achieved.

Identification of the G143A mutation
To investigate whether nonsynonymous mutations in *cytb* are associated with reduced Q_OI sensitivity, we sequenced the *cytb* region containing the three most-common mutation sites associated with Q_OI-resistance in fungi. Without exception, all isolates with EC_{50} values > 0.92 µg ml^{-1} harbored a point mutation at nucleotide position 428 that encoded a G143A mutation compared to *cytb* sequence from isolates with baseline sensitivity (Table 2). Analysis of sequence trace files did not indicate evidence of heteroplasmy. The two identified *cytb* haplotypes have been deposited in GenBank under accession numbers JQ619932 and JQ619933.

This is the first report of Q_OI resistance associated with the G143A mutation in isolates of *C. beticola* in the United States. Laboratory mutants encoding a G143S mutation exhibited reduced sensitivity to Q_OI fungicides,[8] but this mutation has not been found in nature.[11] The G143A mutation has been reported in most Q_OI-resistant pathogens to date.[4] When the G143A mutation dominates in a pathogen population, there is a consistent association with a loss of disease control.[13, 14] Moreover, many plant pathogens with the G143A mutation do not exhibit a significant disease fitness penalty,[15-17] suggesting the mutation will persist even in the absence of selection pressure imposed by Q_OI application. Future research directed towards the evaluation of disease fitness of Q_OI-resistant isolates will be important for fungicide resistance management in this pathosystem.

Genotype analysis
SSR markers are useful for assessing genetic diversity in *C. beticola*.[5, 9] In total, six SSR alleles were identified from 49 isolates in Michigan (Table 2), which is similar to the genetic diversity found in other *C. beticola* populations.[5] Our results demonstrate that four genotypes can be distinguished among the resistant isolates using a single SSR primer set (Table 2), suggesting that resistance developed independently in several genetic backgrounds in Michigan. Moreover, *C. beticola* is not known for inoculum movement across wide geographical areas, suggesting that Q_OI-resistance did not originate from a single location. Likewise, Q_OI resistance in other pathosystems has been shown to occur in several backgrounds at separate locations.[18, 19]

Differentiation of resistant and sensitive isolates using real-time PCR assay
Several primer and probe sequences were tested for the ability to distinguish Q_OI-resistant and -sensitive isolates (not shown). Primer pair 735/736 multiplexed with probes 740-SEN and 741-RES (Table 1) exhibited the highest efficiency, specificity, and fluorescence with the lowest Ct value per unit of DNA than all other tested combinations. Primer, probe, and $MgCl_2$ concentrations were optimal at 300 nM, 300 nM, and 5.5 mM, respectively (data not shown). PCR efficiencies[20] were 98.6% and 99.4% for the detection of sensitive and resistant isolates, respectively. 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 740-SEN was achieved only when using template from sensitive isolates, and conversely a Ct value from the G143A-specific probe 741-RES only occurred when using template from resistant isolates (Table 2). Q_OI sensitivity could be determined using DNA concentrations from 10 ng to 1.0 pg (not shown). The use of real-time PCR to identify Q_OI-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 Q_OI-sensitivity of a sample. Knowledge of Q_OI 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.

Several factors may have contributed to the development Q_OI resistance. Growers in Michigan typically rotate between DMI and Q_OI fungicides (J. Stewart, *personal communication*), even though other fungicide chemistries such as triphenyltin hydroxide or thiophanate-methyl are available. Moreover, the heavy use of Q_OI fungicides on sugarbeet as well as crops grown in rotation with sugarbeet may have selected for Q_OI-resistant strains of *C. beticola*. Finally, increased planting of varieties with high sugar content but increased susceptibility to CLS may have amplified disease pressure at a time when Q_OI fungicides were losing efficacy (S. Poindexter, *personal communication*), which may have increased levels of Q_OI-resistant *C. beticola* inoculum. Effective control of CLS requires an integrated approach involving crop rotation, planting varieties with tolerance to CLS, and timely application of fungicides from several fungicide classes to minimize fungicide resistance development. Fungicides from chemistries other than Q_OIs will need to be applied in fields with Q_OI-resistance problems to ensure that Q_OI-resistance does not become more damaging and spread further.

In conclusion, we identified Michigan isolates with high EC_{50} values compared to baseline sensitive isolates to two fungicides extensively applied for CLS management. All Q_OI-resistant isolates harbored the G143A mutation, which was exploited in a highly sensitive real-time PCR assay. Since Q_OI-resistance appears to have occurred at several locations in several genetic backgrounds, careful monitoring of G143A will be critical to slow the spread of Q_OI-resistance and ensure efficacy of Q_OI fungicides for CLS control.

Acknowledgements

The authors thank X. Wang for excellent technical assistance and Dr. J. Suttle (USDA – ARS) for critical review of the manuscript. The authors thank Drs. S. Poindexter (Michigan State University) and J. Stewart (Michigan Sugar Company) for helpful discussions. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. This research was supported by USDA-ARS CRIS project 5442-22000-042-00D and grants from the Beet Sugar Development Foundation and the Sugarbeet Research and Education Board of Minnesota and North Dakota.

References

1. Weiland J, Koch G, Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). *Mol Plant Pathol* **5**:157-166 (2004).
2. Secor GA, Rivera VV, Khan MFR, Gudmestad NC, Monitoring fungicide sensitivity of *Cercospora beticola* of sugar beet for disease management decisions. *Plant Dis* **94**:1272-1282 (2010).
3. Bolton MD, Birla K, Rivera-Varas V, Rudolph K, Secor GA, Characterization of *CbCyp51* from field isolates of *Cercospora beticola*. *Phytopathol* **102**:298-305 (2012).
4. Fisher N, Meunier B, Molecular basis of resistance to cytochrome *bc*1 inhibitors. *FEMS Yeast Res* **8**:183-192 (2008).
5. Bolton MD, Secor GA, Rivera V, Weiland JJ, Rudolph K, Birla K*, et al*, Evaluation of the potential for sexual reproduction in field populations of *Cercospora beticola* from USA. *Fungal Biol* **116**:511-521 (2012).
6. Secor GA, Rivera VV. Fungicide resistance assays for fungal plant pathogens. In: Bolton MD, Thomma BPHJ, editors. Plant Fungal Pathogens: Methods and Protocols. New York: Humana Press; 2012. p. 385-392.
7. Stewart C, Via LE, A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *BioTechniques* **14**:748-749 (1993).
8. Malandrakis A, Markoglou A, Nikou D, Vontas J, Ziogas B, Biological and molecular characterization of laboratory mutants of *Cercospora beticola* resistant to Qo inhibitors. *Eur J Plant Pathol* **116**:155-166 (2006).
9. Groenewald M, Groenewald JZ, Linde CC, Crous PW, Development of polymorphic microsatellite and single nucleotide polymorphism markers for *Cercospora beticola* (Mycosphaerellaceae). *Mol Ecol Notes* **7**:890-892 (2007).
10. Braasch DA, Corey DR, Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chemistry & Biology* **8**:1-7 (2001).
11. Malandrakis AA, Markoglou AN, Nikou DC, Vontas JG, Ziogas BN, Molecular diagnostic for detecting the cytochrome *b* G143S - QoI resistance mutation in *Cercospora beticola*. *Pestic Biochem Physiol* **100**:87-92 (2011).
12. Karadimos DA, Karaoglanidis GS, Tzavella–Klonari K, Biological activity and physical modes of action of the Qo inhibitor fungicides trifloxystrobin and pyraclostrobin against *Cercospora beticola*. *Crop Prot* **24**:23-29 (2005).

13. Gisi U, Sierotzki H, Cook A, McCaffery A, Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag Sci* **58**:859-867 (2002).
14. Sierotzki H, Frey R, Wullschleger J, Palermo S, Karlin S, Godwin J, *et al*, Cytochrome *b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Manag Sci* **63**:225-233 (2007).
15. Banno S, Yamashita K, Fukumori F, Okada K, Uekusa H, Takagaki M*, et al*, Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome *b* gene. *Plant Pathol* **58**:120-129 (2009).
16. Corio-Costet M-F, Dufour M-C, Cigna J, Abadie P, Chen W-J, Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. *Eur J Plant Pathol* **129**:315-329 (2011).
17. Karaoglanidis GS, Luo Y, Michailides TJ, Competitive Ability and Fitness of Alternaria alternata Isolates Resistant to QoI Fungicides. *Plant Dis* **95**:178-182 (2010).
18. Torriani SFF, Brunner PC, McDonald BA, Sierotzki H, QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. *Pest Manag Sci* **65**:155-162 (2009).
19. Kim YS, Dixon EW, Vincelli P, Farman ML, Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. *Phytopathol* **93**:891-900 (2003).
20. Pfaffl MW, A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**:2002-2007 (2001).