Genetic analysis and molecular characterisation of laboratory and field mutants of *Botryotinia fuckeliana* (*Botrytis cinerea*) resistant to QoI fungicides

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Abstract

BACKGROUND: QoI fungicides, inhibitors of mitochondrial respiration, are considered to be at high risk of resistance development. In several phytopathogenic fungi, resistance is caused by mutations (most frequently G143A) in the mitochondrial cytochrome b (*cytb*) gene. The genetic and molecular basis of QoI resistance were investigated in laboratory and field mutants of *Botryotinia fuckeliana* (de Bary Whetz.) exhibiting *in vitro* reduced sensitivity to trifloxystrobin.

RESULTS: *B. fuckeliana* mutants highly resistant to trifloxystrobin were obtained in the laboratory by spontaneous mutations in wild-type strains, or from naturally infected plants on a medium amended with 1–3 mg L$^{-1}$ trifloxystrobin and 2 mM salicylhydroxamic acid, an inhibitor of alternative oxidase. No point mutations were detected, either in the complete nucleotide sequences of the *cytb* gene or in those of the *aox* and Rieske protein genes of laboratory mutants, whereas all field mutants carried the G143A mutation in the mitochondrial *cytb* gene. QoI resistance was always maternally inherited in ascospore progeny of sexual crosses of field mutants with sensitive reference strains.

CONCLUSIONS: The G143A mutation in *cytb* gene is confirmed to be responsible for field resistance to QoIs in *B. fuckeliana*. Maternal inheritance of resistance to QoIs in progeny of sexual crosses confirmed that it is caused by extranuclear genetic determinants. In laboratory mutants the heteroplasmic state of mutated mitochondria could likely hamper the G143A detection, otherwise other gene(s) underlying different mechanisms of resistance could be involved.

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Keywords: grey mould; fungicide resistance; quinol oxidation inhibitors; cytochrome b gene; G143A mutation; mitochondrial inheritance

1 INTRODUCTION

The development of field resistance to fungicides can seriously affect the effectiveness of chemical control of phytopathogenic fungi, as frequently experienced for several fungicide–pathogen combinations. It can be explained as the result of the adaptation of microbial populations to adverse conditions on account of genetic modifications that are hence transmissible to progenies.

The risk of resistance development to chemicals is greatly influenced by genetic variation and evolutionary plasticity of a fungal species. Pathogens considered at high risk by the Fungicide Resistance Action Committee (FRAC; www.frac.info) include *Botryotinia fuckeliana* (de Bary Whetz. (teleomorph of *Botrytis cinerea* Pers.), the fungus inducing grey mould on many economically important crops in all temperate areas of the world. Adequate disease control often requires repeated fungicide sprays, which cause a high selection pressure for resistance in the pathogen’s populations.

Resistance development to fungicides is indeed a very frequent phenomenon in *B. fuckeliana*, and dramatic reduction in the effectiveness of spray schedules has often been reported. Benzimidazoles lost their importance against grey mould a long time ago owing to very common resistance in field populations of the fungus. In the late 1970s they were replaced by dicarboximides, which initially displayed a very high effectiveness, but then the development of field resistance led to frequent decrease in fungicidal activity. Acquired resistance has already been reported for more recent fungicides belonging to different classes of chemicals, such as phenylpyrroles, anilinopyrimidines, hydroxyanilides and SDHI fungicides.

Clarification of the sexual behaviour and mating system of *B. fuckeliana* revealed information concerning the genetic bases

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of fungicide resistance. Rapid progress in molecular genetics has allowed more rapid clarification of the genetic mechanisms underlying acquired resistance in *B. fuckeliana* to the main fungicide groups used against grey mould.\textsuperscript{12,13}

Quinol oxidation inhibitors (QoIs) include antimicrobial compounds that specifically inhibit mitochondrial respiration by binding to the outer quinol oxidation site (Qo) of the cytochrome *bc*\textsubscript{1} complex (complex III) and hence interfering with the catalytic activity of the enzyme.\textsuperscript{14} This blocks the respiratory electron transfer chain, which leads to an energy deficiency by reducing notably ATP synthesis.\textsuperscript{15} In the past 10–20 years, several QoIs have been developed for both medical and agricultural applications.\textsuperscript{16}

The discovery of QoI fungicides was inspired by a group of natural substances, known as strobilurins, which are derivatives of β-methoxyacrylic acid and include strobilurin A, oudemansiin and myxothiazole, produced by basidiomycete wood-rooting fungi, such as *Strobilurus tenacellus* (Pers.) Singer and *Oudemansiella mucida* (Schrad.) Höhn., and by the myxobacterium *Myxococcus fulvus* Cohn.\textsuperscript{15,17} Nowadays, QoIs are registered in many countries for use on different crops, and they are frequently included in IPM strategies because of their broad spectrum of action against important fungal and oomycete diseases. However, the levels of biological activity of QoI fungicides are variable, and not all of them exhibit high levels of control. The usefulness of QoI fungicides in grey mould management is generally very limited and restricted to certain crops or a few compounds.\textsuperscript{12,18}

Unfortunately, the intrinsic risk of fungal resistance to QoIs is high. QoI-resistant isolates of *Blumeria graminis* (DC.) Speer f. sp. *tritici* Em. Marchal, the causal agent of wheat powdery mildew, were detected in Germany soon after their introduction into agricultural practice. Currently, QoI resistance has been detected worldwide in at least 40 different fungal pathogens (www.frac.info), and the consequent reduced effectiveness is a challenge in crop protection.

In the large majority of cases, a high level of resistance to QoIs is conferred by a single point mutation in the cytb gene, leading to the substitution of glycine by alanine at position 143 (G143A), as confirmed by functional genetic studies in *Mycosphaerella fijiensis* M. Morelet.\textsuperscript{19} A strong association between QoI resistance and the G143A substitution has been clearly shown for most of the fungal pathogens in which QoI resistance has been detected (www.frac.info).\textsuperscript{19–22} Furthermore, the G143A substitution is also found in strobilurin-producing basidiomyceetes such as *S. tenacellus* and *Mycena galopoda* (Pers.) P. Kumm.\textsuperscript{17}

A different amino acid substitution, from phenylalanine to leucine at position 129 (F129L), causing a lower level of resistance to QoIs than the G143A mutation, has been detected in cytochrome *b* of QoI-resistant isolates of several phytopathogenic fungi, such as *Magnaporthe grisea* (T.T. Hebert) M.E. Barr,\textsuperscript{22} *Pythium aphanidermatum* (Edson) Fitzp.,\textsuperscript{15} *Alternaria solani* Sorauer,\textsuperscript{23,24} *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni,\textsuperscript{25} *Podosphaera fusca* (Fr.) U. Braun & N. Shishkoff\textsuperscript{26} and *Pyrenophora teres* Drechsler.\textsuperscript{27} A third amino acid change from glycine to arginine at position 137 (G137R) has been more recently associated with a moderate level of QoI resistance in *Pyrenophora tritici-repentis* (Died.) Drechsler.\textsuperscript{27}

In some fungal species, QoI resistance is not supported by mutations in the cytb gene, while in others the structure of the gene is such that it is unlikely to undergo the G143A mutation.\textsuperscript{27–29} Other mechanisms, including alternative respiration and efflux transporters, may be exploited by pathogens to reduce their sensitivity to QoI fungicides, but their occurrence in nature and the impact on resistance seems so far to be limited. Alternative unknown mechanisms leading to resistance to QoIs have been hypothesised in *Venturia inaequalis* (Cooke) G. Winter\textsuperscript{30} and *P. fusca*.\textsuperscript{31}

The main objectives of the present study were: (i) to characterise *B. fuckeliana* isolates of different origin, and from several host plants, for their response to QoI fungicides; (ii) to compare the mechanism(s) leading to QoI resistance in laboratory and field mutants of the fungus; (iii) to verify the mode of inheritance of QoI resistance in sexual progeny; (iv) to validate rapid molecular methods to detect the G143A mutation in the *B. fuckeliana* cytochrome *b* gene.

## MATERIALS AND METHODS

### 2.1 Fungal isolates and culture conditions

Two monoascosporic isolates SAS56 and SAS405 with wild-type sensitivity to QoIs, and of opposite mating types (*MAT1-1* and *MAT1-2* respectively), were used in attempts to select QoI-resistant laboratory mutants and in sexual crosses.

A total of 55 monoconidial isolates of *B. fuckeliana* collected from table grape vineyards located in southern Italy during 2008 and 50 isolates of different geographic and host origins maintained in the authors’ collection (supporting information Table S1) were characterised for their response to trifloxystrobin and screened for the polymorphic structure of the cytb gene. Fifteen isolates collected from different hosts and locations in Japan and previously characterised for their response to QoIs as well as for the cytb gene\textsuperscript{32} were used in genetic and molecular analysis (supporting information Table S2).

All isolates were grown on malt extract agar (MEA: 20 g L\textsuperscript{−1} of malt extract, 20 g L\textsuperscript{−1} of agar) at 21 ± 1 °C in darkness. In order to promote sporulation, cultures were grown on potato dextrose agar (PDA: infusion from 200 g L\textsuperscript{−1} of peeled and sliced potatoes kept at 60 °C for 1 h, 20 g L\textsuperscript{−1} of dextrose, adjusted at pH 6.5, and 20 g L\textsuperscript{−1} of agar) at 21 ± 1 °C under 12 h per day exposure to a combination of two daylight (Osram, L36W/640) and two near-UV (Osram, L36/73) lamps. Conidial germination tests were carried out on dextrose agar (DA: 10 g L\textsuperscript{−1} of glucose, 20 g L\textsuperscript{−1} of agar) for 3–5 days at 20 °C.

QoI-resistant isolates were always maintained under selective pressure by growing them on fungicide-amended medium. Fugal stock cultures made up by suspensions of conidia and mycelial fragments in 10% glycerol (v/v) were established for long-term storage at −80 °C.

### 2.2 Chemicals

Stock solutions were prepared by dissolving trifloxystrobin or kresoxim-methyl (technical grade; gifts from BASF AG, Limburgerhof, Germany) in dimethylsulphoxide, while a commercial formulation of azoxystrobin (Ortiva, 23.2% AI; Syngenta Crop Protection, Basel, Switzerland) was dispersed in sterile distilled water. Each fungicide concentration to be tested was obtained from the stock solution or suspension and added to media cooled down to 45–50 °C after autoclaving. The final concentration of the solvent was the same in all media, including the fungicide-unamended controls, and never exceeded 1% (v/v).

Inhibitors of alternative oxidase, such as salicylhydroxamic acid (SHAM) or n-propyl gallate, enhance the biological activity of QoIs in several fungal species including *B. fuckeliana*.\textsuperscript{32–35} Therefore, media containing 2 mm SHAM, predissolved in methanol, were used to evaluate the fungal response to QoI fungicides.
2.3 Fungicide sensitivity tests

Sensitivity to trifloxystrobin, azoxystrobin and kresoxim-methyl of the wild-type strains of *B. fuckeliana*, SAS56 and SAS405, never exposed to QoI fungicides, was evaluated in both conidial germination and colony growth assays. The range of concentrations of each fungicide (0.01 – 300 mg Al L$^{-1}$) was adjusted to establish EC$_{50}$ (effective concentration causing 50% growth inhibition) and MIC (minimal inhibitory concentration) values.

Tests on conidial germination were carried out by spotting aliquots (10 µL) of suspension containing 5 × 10$^5$ conidia mL$^{-1}$ on discs (6 mm diameter) of DA, either without or amended with increasing concentrations of fungicide. The discs, placed on microscope glass slides, were incubated in a moist chamber at 21 ± 1°C in darkness. After 16 and 48 h, random samples of 100 conidia on each of three replicates per condition were observed at ×125 magnification to evaluate the inhibitory effect caused by each fungicide concentration on conidial germination and germ-tube elongation.

Shifts in QoI sensitivity of 14 laboratory and 11 field mutants collected from grapevines in Italy were then assessed through colony growth tests on trifloxystrobin-amended medium. Mycelial discs (4 mm diameter) from actively growing margins of 3–5-day-old cultures of 25 *B. fuckeliana* isolates were used to inoculate three replicated petri dishes (100 mm diameter) containing SHAM-supplemented MEA amended with increasing concentrations of the fungicide (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 mg Al L$^{-1}$). The effect of the fungicide on growth was determined by measuring the diameter of developing colonies after 3, 5 and 7 days of incubation at 21 ± 1°C in darkness. EC$_{50}$ values were determined using the dose–response curves through probit analysis. The resistance factor (RF) was calculated as the ratio between the EC$_{50}$ value of the resistant mutant and the mean value of EC$_{50}$ for wild-type isolates.

2.4 Selection of QoI-resistant mutants

Experiments aimed at obtaining laboratory mutants were carried out either by selecting spontaneous mutations or after physical or chemical treatments. For UV treatment, 10 mL of conidial suspensions (1.5–7.1 × 10$^7$ conidia mL$^{-1}$) from seven-day-old cultures of 25 *B. fuckeliana* isolates were used to inoculate three replicated petri dishes (100 mm diameter) containing MEA-supplemented MEA amended with increasing concentrations of the fungicide (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 mg Al L$^{-1}$). The effect of the fungicide on growth was determined by measuring the diameter of developing colonies after 3, 5 and 7 days of incubation at 21 ± 1°C in darkness. EC$_{50}$ values were determined using the dose–response curves through probit analysis. The resistance factor (RF) was calculated as the ratio between the EC$_{50}$ value of the resistant mutant and the mean value of EC$_{50}$ for wild-type isolates.

Field isolates and the reference strains SAS56 and SAS405 were grown on MEA in petri dishes (100 mm diameter) at 21 ± 1°C for 2–3 days, kept at 15 ± 1°C in the dark for 4 weeks to induce sclerotia differentiation and then conditioned at 0 ± 1°C for a further 4 weeks for breaking dormancy. In order to prevent problems related to the possible instability of fungicide resistance, all resistant isolates were also grown under selective conditions (MEA added with 1 mg L$^{-1}$ trifloxystrobin and 2 mM SHAM, or with 100 mg L$^{-1}$ azoxystrobin and 1 mM n-propyl gallate).

Eleven QoI-resistant field isolates (R) were crossed with SAS56 and SAS405 and with four sensitive Japanese field isolates (S), and with each other in all the combinations. Mutants were mated either as sclerotial or spermatising partner in each cross.

Ascospores from individual apothecia were spread at low density on MEA or on MEA-SHAM amended with trifloxystrobin (1 mg L$^{-1}$). After 18 h of incubation at 21 ± 1°C in darkness, the germination of five samples of 100 ascospores was assessed through observations under a dissecting microscope. The response to Qols was confirmed by collecting a sample of ten single ascospores from each apothecium and evaluating their ability to yield colony on MEA-SHAM containing trifloxystrobin (1 mg L$^{-1}$).

2.5 Genetic analysis of meiotic progenies

Fifteen field isolates collected in Japan (supporting information Table S2) and characterised for their response to Qols and for the cytb gene previously$^{32}$ were submitted to PCR analysis for assessing their mating type by using idiomorph-specific primer pairs, alfa.Y.23/alfa.Y.649 specific for MAT1-1 and hmg.sp.162/hmg.sp.1119 specific for MAT1-2 (paper in preparation).

Field isolates and the reference strains SAS56 and SAS405 were grown on MEA in petri dishes (100 mm diameter) at 21 ± 1°C for 2–3 days, kept at 15 ± 1°C in the dark for 4 weeks to induce sclerotia differentiation and then conditioned at 0 ± 1°C for a further 4 weeks for breaking dormancy. In order to prevent problems related to the possible instability of fungicide resistance, all resistant isolates were also grown under selective conditions (MEA added with 1 mg L$^{-1}$ trifloxystrobin and 2 mM SHAM, or with 100 mg L$^{-1}$ azoxystrobin and 1 mM n-propyl gallate).

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2.6 DNA extraction, PCR and sequencing

Total DNA was extracted and purified from mycelium of *B. fuckeliana* isolates as previously described.$^{11}$ Nucleotide and deduced amino acid sequences of the mitochondrial cytb gene of *B. fuckeliana* (accession number AB262969) were obtained from the EMBL GenBank (http://www.ebi.ac.uk), whereas the entire chromosomal regions, including the alternative oxi-dase (BC1G_05703.1) and the Rieske protein (BC1G_07481.6) genes, were obtained from the working draft of the *Botrytis cinerea* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu).$^{36}$

The PCR primer pairs used to amplify fragments of each target gene from total DNA were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and synthesised by Sigma-Life Science (Milan, Italy). The sequences of primers and the size of DNA amplified fragments are reported in Table 1.

Reaction mixtures (50 µL) contained 1.5 mM of MgCl$_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.1 µM of each primer, 0.1–0.3 µg of DNA template and 2 U of Takara LA Taq DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan). Amplifications were carried out in a Gene Amp PCR System 9700 thermal cycler (Perkin-Elmer, Norwalk, CT) programmed for 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C and a final extension for 7 min at 72°C. Negative controls in which water replaced the target DNA were always run to detect potential contamination. PCR products were purified with Quantum Prep PCR Kleen Spin columns (Bio-Rad Laboratories) and visualised by electrophoresis on 1.5% (w/v) agarose gel in 45 mM Tris-borate and 1 mM ethidium bromide staining.

Amplified products were then subjected to direct sequencing in both forward and reverse directions, using the same primers as for PCR, by external service (PRIMM, ‘Basic-Read’ DNA Sequencing Service, Milan Facility, Italy). BioEdit Sequence Alignment Editor (v.7.0.7.0; Tom Hall, North Carolina State University) and the Lasergene sequence analysis software package (v.8.0.2; DNASTAR, Inc., Madison, WI) were used to align the sequence data.

Validation of the idiomorph-specific and MAT1-1 specific primer pairs was performed by nest PCR with the internal primer pair alfa.Y.23/alfa.Y.649 specific for MAT1-1 and hmg.sp.162/hmg.sp.1119 specific for MAT1-2 (paper in preparation).
Table 1. PCR primer pairs designed on the sequences of the genes encoding the cytochrome b (Cytb), the alternative oxidase (AO) and the Rieske protein (RsP)

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Forward/reverse sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytb.139/</td>
<td>5′-ACGCGATTTCTCAGTCCAG-3′/</td>
<td>734</td>
</tr>
<tr>
<td>Cytb.872</td>
<td>5′-ATGCCCTCCTAATGCTGAG-3′/</td>
<td>1298</td>
</tr>
<tr>
<td>Cytb.757/</td>
<td>5′-CAGAGCTGATGACTCTGAGA-3′/</td>
<td>1205</td>
</tr>
<tr>
<td>Cytb.2054</td>
<td>5′-GGACAAAAAGGACAGCTTATAC-3′/</td>
<td>878</td>
</tr>
<tr>
<td>Cytb.1950/</td>
<td>5′-AACGACGATTTCTCAGTCCAG-3′/</td>
<td>1014</td>
</tr>
<tr>
<td>Cytb.3154</td>
<td>5′-TTCCCTCACTGTTCTGACCTC-3′/</td>
<td>1034</td>
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<td>Cytb.2408/</td>
<td>5′-TCATTTGCAGCGAAGTTTGA-3′/</td>
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<td>260a</td>
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<td>Cytb.4837/</td>
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<td>RsP.1179</td>
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* Primer pairs used in real-time AS-PCR.

Inc., Madison, WI) were used to edit, assemble and translate sequences.

2.7 Cleaved amplified polymorphic sequence (CAPS) assay

The single nucleotide polymorphism (SNP) from guanine to cytosine at codon 143 creates a recognition site for the restriction endonucleases in the cyt b sequence of B. fuckeliana, suitable for CAPS analysis.32,34,37 Primers Cytb.2408 and Cytb.3285 were used to amplify the region containing the SNP to yield an expected 878 bp fragment. The reaction mixture (25 µL) contained 100 ng of template DNA, 2 mM of MgCl2, 0.3 mM of dNTPs, 0.75 U of GoTaq® DNA polymerase (Promega, Madison, WI) and 0.5 µM of each primer. The cycling programme was essentially similar to that described above with 25 amplification cycles. The amplified fragments from total DNA of 55 QoI-resistant and 37 QoI-sensitive isolates were digested with Alul (Sigma-Aldrich, Milan, Italy)37 according to the manufacturer’s protocol, followed by electrophoretic separation on 2% agarose gel with TBE buffer and ethidium bromide staining.

2.8 Real-time PCR assay

In order to detect and quantify the G143A mutation in mitochondrial DNA of B. fuckeliana, the following two primer pairs were designed in the cyt b gene for a real-time allele-specific PCR assay (AS-PCR): (i) Cytb.4831/Cytb.5030, amplifying a region of the gene in both wild-type and resistant isolates; (ii) Cytb.3387/Cytb.3627, with the forward primer specific for the mutated codon 143 (Table 1). PCR was optimised in a reaction volume of 25 µL containing 1× IQ SYBR Green Supermix (Bio-Rad Laboratories), 0.5 µM of each primer and up to 100 ng of total DNA template. The assay was performed and results analysed on a CFX96 real-time PCR detection system (Bio-Rad Laboratories). Cycling parameters were 3 min at 95 °C, followed by 25 cycles of 10 s at 95 °C and 45 s at 65 °C. Each sample was assayed at least twice. The performance of the assay was evaluated using DNA from three sensitive (SAS56, SA5405 and WS259) and two field-resistant isolates (BAR476 and BAR483) carrying the G143A mutation, as previously verified by partial sequencing of the cyt b gene. For both primer pairs, standard curves were constructed using serial dilutions of DNA from the resistant field mutant BAR476 by plotting the threshold cycle (Ct) versus the amount of log quantity of template DNA. The presence of the G143A mutation in mtDNA was then verified in three resistant laboratory mutants derived from the reference strain SA5405.

3 RESULTS

3.1 Response of B. fuckeliana isolates to QoI fungicides

To determine discriminatory concentrations between B. fuckeliana isolates sensitive or resistant to Qols, the two reference strains SAS56 and SA5405 were initially tested for their response to triflouxistrobin, kresoxim-methyl and azoxystrobin in the presence of 2 mM SHAM by colony growth assays, and MIC and EC50 values were determined. Triflouxistrobin and kresoxim-methyl (EC50 < 0.01 mg L−1) proved to be more effective than azoxystrobin (EC50 = 0.1–0.3 mg L−1). Colony growth of wild-type isolates was totally inhibited by 1 mg L−1 (triflouxistrobin) or 3 mg L−1 (azoxystrobin and kresoxim-methyl).

Laboratory mutants of B. fuckeliana resistant to triflouxistrobin were obtained exclusively by selection of spontaneous mutations from both wild-type reference strains at a frequency of 5.0 × 10−7 (SAS56) or 1.3 × 10−8 (SA5405) conidia. Most of the resistant colonies appeared after 6–10 days of incubation. In the working conditions adopted, no resistant mutants were isolated from UV-irradiated or NTG-treated conidia, even after 15 days of incubation. Triflouxistrobin-resistant mutants able to grow on MEA amended with 1 mg L−1 triflouxistrobin (MIC for wild-type strains) were frequently selected among monoconidial isolates derived from naturally infected grape berries exposed to QOI treatments.

Fourteen laboratory mutants and eleven field isolates of B. fuckeliana resistant to triflouxistrobin were selected and characterised for their response to increasing concentrations of the fungicide in colony growth tests. All tested mutants exhibited a high level of resistance, with EC50 values of >100 mg L−1 (Fig. 1) and RF > 10 000. However, laboratory mutants showed a marked instability of resistance that was frequently lost, even after the first transfer, when grown under non-selective conditions on fungicide-unamended MEA (data not shown).

3.2 Genetic analysis

Japanese field isolates of B. fuckeliana, four sensitive and eleven resistant to QoI fungicides, were selected for experimental crosses with wild-type reference strains to study the inheritance of QoI resistance. All sensitive isolates (S-1-1, S-1-3, SA 4-2-4 and IBA-1-2) and five of the QoI-resistant isolates (R-1-2, R-1-4, IBA-1-1, SA-5-1-2 and SA-5-1-6) were unable to produce sclerotia and could only be used as the spermatising parent in sexual crosses. Three
of the QoI-resistant isolates (R-1-2, R-1-4 and IBA-1-1-1) showed anomalous morphological features and did not yield microconidia (Table 2).

Sclerotia of SA5-1-5, Nishimi 8-3 and Himi 8–4 isolates were much smaller than normal and were unable to produce apothecia. No apothecia were obtained when the Himi 8–4 and S-1-1 isolates were mated as spermatising partner. The remaining isolates were sexually fertile, with compatible reference strains of known mating type, as previously ascertained by PCR experiments using a primer specific for each mating-type gene (MAT1-1 and MAT1-2) (Table 2).

To determine the phenotype distribution in the progeny, ascospores from individual apothecia were spread on MEA amended with 2 mM SHAM, either alone or with added trifloxystrobin (1 mg L⁻¹), and observed under the microscope. Whenever possible, apothecia from reciprocal crosses, in which reference strains were used successfully, as either the sclerotial or the spermatising parent, were analysed. Samples of individual ascospores were subcultured to MEA, and the colonies obtained were tested for colony growth on fungicide-amended media.

A total of 43 apothecia from 15 different crosses involving ten field isolates were analysed. In all crosses in which resistant isolates were mated as sclerotial partner, the whole progeny inherited resistance, whereas in all the other crosses the whole progeny was sensitive (Table 3). No differences were observed when resistant isolates used in the cross had been grown under selective or non-selective conditions for obtaining sclerotia and microconidia.

3.3 Molecular analysis

The nucleotide sequence of the mitochondrial cytochrome b gene (cytb) of B. fuckeliana was obtained by direct sequencing of PCR amplification products of partially overlapping fragments of the gene from DNA of two wild-type strains and six putative laboratory mutants resistant to QoI fungicides. Seven

Table 2. Sclerotia and apothecia production from isolates of B. fuckeliana

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mating type</th>
<th>MEA</th>
<th>MEA + trifloxystrobin</th>
<th>MEA + azoxystrobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number</td>
<td>Size</td>
<td>Number</td>
</tr>
<tr>
<td>SAS-56</td>
<td>MAT-1-1</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>SAS-405</td>
<td>MAT-1-2</td>
<td>+</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>R-1-2</td>
<td>MAT-1-2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>R-1-4</td>
<td>MAT-1-2</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Arimi 4-3</td>
<td>MAT-1-2</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Arimi 4–5</td>
<td>MAT-1-2</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>SAS-1-2</td>
<td>MAT-1-1</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>SAS-1-5</td>
<td>MAT-1-1</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>SAS-1-6</td>
<td>MAT-1-1</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>IBA1-1-1</td>
<td>MAT-1-2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>S-1-1</td>
<td>MAT-1-2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>–</td>
</tr>
<tr>
<td>S-1-3</td>
<td>MAT-1-2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>–</td>
</tr>
<tr>
<td>SA4-2-4</td>
<td>MAT-1-1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>–</td>
</tr>
<tr>
<td>IBA1-2-1</td>
<td>MAT-1-1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>–</td>
</tr>
</tbody>
</table>

* n.s.: no sclerotia; +: small size and/or few sclerotia; . . . + + + + , big size and/or many sclerotia.
* n.a.: no apothecia.
Table 3. Resistance phenotype of ascospore progeny from different crosses

<table>
<thead>
<tr>
<th>Cross</th>
<th>Resistance phenotype of parental isolates</th>
<th>Number of apothecia tested</th>
<th>Phenotype of ascospore progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAS56 × Arimi 4–3</td>
<td>S R</td>
<td>6</td>
<td>S</td>
</tr>
<tr>
<td>Arimi 4–3 × SAS56</td>
<td>R S</td>
<td>3</td>
<td>R</td>
</tr>
<tr>
<td>SAS56 × Arimi 4–5</td>
<td>S R</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>Arimi 4–5 × SAS56</td>
<td>R S</td>
<td>6</td>
<td>R</td>
</tr>
<tr>
<td>SAS56 × Nishimi 8–3</td>
<td>S R</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>SAS56 × Nishimi 8–5</td>
<td>S R</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Nishimi 8–5 × SAS56</td>
<td>R S</td>
<td>2</td>
<td>R</td>
</tr>
<tr>
<td>SAS56 × S-1-3</td>
<td>S S</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>SAS405 × SA4-2-4</td>
<td>S S</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>SAS405 × SAS-1-5</td>
<td>S R</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>SAS405 × SAS-1-6</td>
<td>S R</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Arimi 4–5 × SAS-1-2</td>
<td>R R</td>
<td>2</td>
<td>R</td>
</tr>
<tr>
<td>Arimi 4–5 × SAS-1-5</td>
<td>R R</td>
<td>2</td>
<td>R</td>
</tr>
<tr>
<td>Arimi 4–5 × SAS-1-6</td>
<td>R R</td>
<td>2</td>
<td>R</td>
</tr>
<tr>
<td>Nishimi 8–5 × IBA1-2-1</td>
<td>R R</td>
<td>2</td>
<td>R</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

*Response on MEA-SHAM (2 mM) amended with trifloxystrobin (1 mg L⁻¹): S, sensitive; R, resistant.

Specific primer pairs (Cytb.139/Cytb.872, Cytb.757/Cytb.2054, Cytb.1950/Cytb.3154, Cytb.2408/Cytb.3285, Cytb.2974/Cytb.3887, Cytb.2857/Cytb.3870, and Cytb.3738/Cytb.4771) were designed from the reference cytb sequence (GenBank accession AB262969), and PCR conditions were optimised for obtaining the expected products (Table 1). PCR amplicons (734–1298 bp) were purified, sequenced and assembled to obtain the complete sequence of the cytb gene.

All primer pairs produced fragments of the expected sizes, the only exception being the pair Cytb.2408/Cytb.3285. When these primers were used to amplify the cytb gene from the parental strain SAS56 and mutants derived from it, a PCR product of about 2100 bp instead of 878 bp was generated. The amplicon was then sequenced and assembled to obtain the complete sequence of the cytb gene.

By comparing the nucleotide sequences obtained from the laboratory mutants resistant to trifloxystrobin and the parental wild-type strains did not reveal any of the mutations known to be responsible for Qol resistance (G143A, F129L and G137R). Except for the possible presence of the group-I intron, which was unrelated to the resistance to Qol fungicides, all the examined sequences were found to be identical in nucleotide composition.

A selection of B. fuckeliana isolates from different geographical locations and host plants, 64 sensitive and 55 resistant to Qol fungicides, were analysed. Only five isolates, all Qol sensitive, generated products corresponding in size to the presence of the G143-associated intron sequence, whereas amplicons derived from the remaining isolates were consistent in size with fragments lacking the intron. The sequencing of the 2083 bp amplicons confirmed the presence of the intron and its position in the cytb gene.

Specific primer pairs were designed on both the cytb and aox genes. DNA from SAS56 and SAS405 was used to test the primers and act as positive control in PCR assays.

A second approach used CAPS analysis to detect the G143A mutation in the cytb gene of 69 Qol-resistant mutants selected under laboratory or field conditions. The single nucleotide change at position 143 (GGT → GCT) creates an additional site for cleavage by the restriction enzyme AluI (S′-AG↓CT-3′) that allows the discrimination between wild-type and mutated genotypes according to specific restriction profiles on PCR-amplified DNA. Therefore, the primer pair Cytb.2408/Cytb.3285 was used to amplify a cytb fragment including the site of mutation that was
mutants produces five bands (363, 210, 136, 92 and 69 bp). (iii) The intron-lacking PCR amplicon of 878 bp size that, after digestion, produces four bands even in 0.1 ng of total DNA. Results of real-time AS-PCR were fully specific and was able to detect the G143A mutation in mtDNA allele-specific Cytb.3387C/Cytb.3627 primer pair showed high specificity and was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytb was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify was used to amplify total mtDNA, whereas the primer pair cytbCytb.3387C/Cytb.3627 was used specifically to detect and quantify.
conditions and found the G143A mutation only sporadically, but were able to demonstrate the heteroplasmic state of four isolates through cloning and sequencing of the cytb gene.

These findings support the hypothesis that resistant and sensitive mitochondria are frequently in the heteroplasmic state, their equilibrium depends on the strength of selective pressure and the isolate displays a resistant or sensitive phenotype, depending on their relative proportion.\(^5\) The heteroplasmic status has been demonstrated in several eukaryotic organisms.\(^1\)\(^9\)\(^5\) Heteroplasmy with respect to the G143A mutation of the mitochondrial cytb gene has been demonstrated in *V. inaequalis*,\(^4\)\(^2\) *P. leucotricha*,\(^4\)\(^3\) Cercospora cassicola S. Narayan, P.N. Singh & G.P. Rao, Mycolelosiella nattrassii Deighton, Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.\(^5\)\(^5\) and recently also in *B. fuckeliana*.\(^3\) In *P. leucotricha*, the relative proportion of mutated and wild-type mitochondria is associated with differences in QoI sensitivity levels of the isolates.\(^5\)\(^6\) In *B. fuckeliana*, fitness penalties were found in laboratory mutants resistant to pyraclostrobin that showed significant reduction in sporulation, conidial germination and sclerotia production, and as a result were less competitive than the parental strains, although pathogenicity and virulence on cucumber seedlings were unaffected.\(^4\)\(^6\) A similar phenomenon has been observed in QoI-resistant isolates of Cercospora beticola Sacc. and *U. maydis*, still with compromised pathogenicity.\(^4\)\(^3\)\(^4\)\(^5\)

Extrachromosomal inheritance of antibiotic resistance in bacteria is well documented,\(^5\)\(^7\) and extranuclear inheritance of fungicide resistance in fungi is known. In fungi, mutations in mitochondrial DNA (mtDNA) are commonly inherited in a fashion of maternal inheritance.\(^5\)\(^6\)\(^5\) Although the mode of inheritance of QoI resistance is always maternally transmitted, the segregation pattern of the trait in random ascospore progeny is in a ratio of 1:0 in the anisogamous *V. inaequalis* and in a ratio of 1:1 in the isogamously and hermaphroditic *B. graminis f. sp. tritici*.\(^1\)\(^5\)\(^6\)\(^9\) Genetic analysis of sexual progeny of *U. maydis* revealed a non-Mendelian inheritance of azoxytrobin resistance in laboratory mutants of the fungus.\(^4\)\(^3\)

*B. fuckeliana* isolates carrying the G143A mutation in the mitochondrial cytb gene were crossed with a sensitive isolate of opposite mating type having the wild-type allele of the gene. In all crosses, the whole progeny retained the phenotypic response to trioxystrobin of the sclerotal parent, while the phenotype of asexual fruiting body (fertile) parent was never detected. The uniparental (sclerotal) inheritance of resistance phenotype confirms the mitochondrial origin of QoI resistance.

Molecular methods for the detection and quantification of resistance alleles are useful in either monitoring fungal populations in the field or in characterising single isolates. A CAPS assay for detecting the G143A mutation in the *B. fuckeliana* cytb gene, set up previously,\(^3\)\(^7\) was validated on a total of 106 isolates. The results of CAPS analysis confirmed the sequencing data and proved to be a reliable, rapid and easy method to verify the occurrence of the SNP in the cytb gene associated with QoI resistance in a large number of isolates. Furthermore, a SYBR Green-based real-time AS-PCR was also set up and validated on QoI-resistant isolates. Such techniques will be useful in quantifying the resistant allele of the the cytb gene in fungal individuals or populations.

No point mutations associated with the resistant phenotype of the investigated laboratory mutants could be detected in the nucleotide sequences of either the alternative oxidase gene or the Rieske protein gene which has been supposed to be involved in QoI resistance of plant pathogens.\(^2\)\(^9\)\(^6\)\(^0\) Although the instability of the resistant phenotype strongly suggests a heteroplasmic status of mtDNA, other unknown resistance mechanisms could be involved.

**ACKNOWLEDGEMENTS**

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**SUPPORTING INFORMATION**

Supporting information may be found in the online version of this article.

**REFERENCES**


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