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Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides

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Abstract

Mutants of *Botrytis cinerea* with moderate and high resistance to pyraclostrobin, a Qo inhibitor of mitochondrial electron transport at the cytochrome bc_1 complex, were isolated at a high mutation frequency, after nitrosoguanidine mutagenesis and selection on medium containing pyraclostrobin and salicylhydroxamate (SHAM), a specific inhibitor of cyanide-resistant (alternative) respiration. Oxygen uptake in whole cells was strongly inhibited in the wild-type strain by pyraclostrobin and SHAM, but not in the mutant isolates. Cross-resistance studies with other Qo and Qi inhibitors (QoIs and QiIs) of cytochrome bc_1 complex of mitochondrial respiration showed that the mutation(s) for resistance to pyraclostrobin also reduced the sensitivity of mutant strains to other QoIs as azoxystrobin, fluoxastrobin, trifloxystrobin and picoxystrobin, but not to famoxadone and to the QiIs cyazofamid and antimycin-A. An increased sensitivity of pyraclostrobin-resistant strains to the carboxamide boscalid, an inhibitor of complex II, and to the anilinopyrimidine cyprodinil, a methionine biosynthesis inhibitor, was observed. Moreover, no effect of pyraclostrobin resistance mutation(s) on fungitoxicity of the hydroxyanilide fenhexamid, the phenylpyrrole fludioxonil, the benzimidazole benomyl, and to the phenylpyridinamine fluazinam, which affect other cellular pathways, was observed. Study of fitness parameters in the wild-type and pyraclostrobin-resistant mutants of *B. cinerea* showed that most mutants had a significant reduction in the sporulation, conidial germination and sclerotia production. Experiments on the stability of the pyraclostrobin-resistant phenotype showed a reduction of resistance, mainly in moderate resistant strains, when the mutants were grown on inhibitor-free medium. However, a rapid recovery of the resistance level was observed after the mutants were returned to a selective medium. Studies on the competitive ability of mutant isolates against the wild-type parent strain, by applications of a mixed conidial population, showed that, *in vitro*, all mutants were less competitive than the wild-type strain. However, the competitive ability of high resistant mutants was higher than the moderate ones. Pathogenicity tests on cucumber seedlings showed that all mutant strains tested exhibited an infection ability similar with the wild-type parent strain. Preventive applications of the commercial product of F-500 25EC (pyraclostrobin) were effective against lesion development on cotyledons by the wild-type, but ineffective, even at high concentrations, against disease caused by the pyraclostrobin-resistant isolates. Boscalid (F-510 50WG) was found equally effective against the disease caused by the wild-type or pyraclostrobin-resistant mutants. This is the first report indicating the appearance of *B. cinerea* strains resistant to QoI fungicides by the biochemical mechanism of site modification and the risk for field resistance.

Introduction

Pyraclostrobin is one of the recently introduced strobilurin fungicides, with a broad spectrum of activity against fungal species among four major classes of Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes. It is highly effective against grey mould disease caused by *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*), and other serious fungal pathogens on a wide range of crops (Ammermann et al., 2000). Most investigation on the effect of the toxicant on fungal growth and morphology showed that it has a protective, curative and eradicative effect by inhibiting spore germination, mycelial growth and sporulation of the fungal pathogens. Its fungitoxicity is based on the inhibition of mitochondrial respiration by binding at the ubiquinol oxidation centre (Qo site) of cytochrome *b* (Bartlett et al., 2002).

The development of resistance to fungicides with specific mechanism of action is a serious problem in the control of grey mould disease. *Botrytis cinerea* is a classical 'high risk pathogen' from the view of resistance management (Brent and Hollomon, 1998), and almost all the newly introduced botryticides face the possibility of resistance development. Indeed, such resistance risk has already been demonstrated for phenylpyrroles, anilinopyrimidines and hydroxyanilides in recent reports (Faretra and Pollastro, 1993; Chapeland et al., 1999; Leroux et al., 1999; Ziogas and Kalamarakis, 2001; Ziogas et al., 2003, 2005). In the case of strobilurins and more generally of the inhibitors of cytochrome bc_1 complex, the inherent resistance risk was estimated to be medium (Brent and Hollomon, 1998). However, in the last few years there have been reports of a decreased sensitivity of certain plant pathogens, such as powdery and downy mildew, to QoIs in Europe and elsewhere (Heaney et al., 2000; Sierotzki et al., 2000a, b, 2004; Chin et al., 2001a; Ishii et al., 2001).

Defining the resistance risk is not easy, and important considerations are the genetic potential and possible mechanism(s) by which resistance is achieved in the pathogen population. Biochemical and molecular studies have shown that resistance to QoI fungicides could appear either by a target site modification through point mutations in the Qo site of cytochrome *b* (Di Rago and Colson, 1989; Zheng and Köller, 1997; Zheng et al., 2000) or by increased electron transfer through the alternative oxidase pathway (Ziogas et al., 1997; Olaya and Köller, 1999a; Tamura et al., 1999). However, alternative respiration appears to play no significant role in pathogenesis on QoI-treated plants in natural populations of pathogens controlled by these fungicides (Ziogas et al., 1997; Olaya and Köller, 1999b) possibly because host flavones released during infection interfere with induction of this pathway (Zheng et al., 2000).

Until now no information is available concerning the risk for resistance development to QoIs in *B. cinerea*. In order to improve the knowledge regarding the possibility of development of practical resistance to the above fungicides, pathogenicity, competition and cross-resistance studies were undertaken with laboratory mutants of *B. cinerea* resistant to pyraclostrobin. The specific objectives of the present study were: (a) to determine the mutation frequency and the level of resistance of mutant strains to pyraclostrobin; (b) to elucidate the cross-resistance relations between pyraclostrobin and other respiratory inhibitors; and (c) to assess the impact of mutations for resistance to pyraclostrobin on the ecological fitness characteristics, such as virulence, resistance expression *in planta*, stability of resistance and competitive ability of mutant strains of *B. cinerea*. The knowledge of the impact of mutations on the fitness of mutants will help to understand the resistance phenomenon and assist in predicting the risk related to the build-up of field resistance to QoIs in *B. cinerea*.

Materials and methods

Fungal strains and culture conditions

The wild-type strain wt-B_1 of *B. cinerea* (teleomorph *B. fuckeliana*) isolated from tomato in Greece was used to obtain pyraclostrobin-resistant isolates (B/PYR). All isolates were grown on potato dextrose agar (PDA) in a controlled climate cabinet at 22 °C with 14 h day^{-1} light and 70% relative humidity. For long-term storage the isolates were maintained in glass tubes on PDA at 10 °C in the dark and single tip transfers were made once a month.

Fungicides

The fungicides used in *in vitro* tests were pure technical grade. Pyraclostrobin, cyazofamid and boscalid were kindly supplied by BASF AG (Limburgerhof, Germany), azoxystrobin, picoxystrobin, fludioxonil and cyprodinil by Syngenta Crop Protection AG (Basle, Switzerland), fluazinam by ISK Biosciences Ltd (Kent, UK), famoxadone and benomyl by Du Pont de Nemours and Co. (Wilmington, DE, USA), trifloxystrobin, fluoxastrobin and fenhexamid by Bayer CropScience AG (Leverkusen, Germany). Antimycin-A and SHAM were purchased from Sigma and Aldrich, respectively. Stock solutions of fungicides were made in ethanol, with the exception of benomyl and fenhexamid which were dissolved in acetone and in isopropanol, respectively.

In the pathogenicity tests, under greenhouse conditions, aqueous suspensions of the commercial products F-500 25EC (250 g l^{-1} pyraclostrobin), F-510 50WG (500 g kg^{-1} boscalid), Chorus 75WP (750 g kg^{-1} cyprodinil) and Saphire 50WP (500 g kg^{-1} fludioxonil), were used. The fungicide concentrations were expressed as μg active ingredient ml^{-1}.

Mutation induction

Conidial suspensions (approximately 10^7 conidia ml^{-1}) of the wild-type strain of *B. cinerea* in water were obtained from 8- to 10-day-old slant cultures. They were agitated on a rotary shaker at 22 °C and 100 rev min^{-1}, with 10 μg ml^{-1} *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) for 4 h in the dark and washed twice with sterile distilled water. Conidia were re-suspended in water and were plated on PDA containing 15 μg ml^{-1} pyraclostrobin and 0.5 mM SHAM and incubated at 22 °C for 15 days, to enable resistant colonies to appear. The selected resistant isolates B/PYR were maintained on PDA agar slants containing 0.15 μg ml^{-1} pyraclostrobin, the minimal inhibitory concentration (MIC) for the wild-type parent strain of *B. cinerea* in addition to 0.5 mM SHAM. Additionally, pyraclostrobin-resistant spontaneous mutants of *B. cinerea* were obtained without mutagenesis on the same selection medium.

In vitro *fungitoxicity tests*

At first, an initial qualitative assessment (preliminary tests) was carried out with all monospore isolated mutants of *B. cinerea*. Afterwards, fungitoxicity tests were made with 10 representative mutant strains using several concentrations of each fungicide to determine the EC_{50} and the EC_{90} (the concentration causing 50% and 90% reduction of growth, respectively) values. All fungicide sensitivity tests were performed in the presence of salicylhydroxamic acid (SHAM) at the concentration of 0.5 mM, which was required to suppress resistance due to alternative respiration. The fungicide sensitivity of the wild-type and mutant strains was assessed by inoculating PDA plates with mycelial inoculum consisting of 2 mm plugs cut from water-agar (WA) medium on which conidia of *B. cinerea* had been allowed to germinate, after overnight incubation at 22 °C. The mycelial-agar plugs were placed with the surface mycelium in direct contact with the medium. The fungicides were added aseptically to sterilized growth medium from stock solutions, prior to inoculation. In all cases, the final amount of solvent never exceeded 1% (v:v) in treated and control samples. At least six concentrations with three replicas for each fungicide were used to obtain the respective fungitoxicity curves. Control plates without fungicide received an equivalent amount of solvent. The effect of the fungicide on growth was determined by measuring the diameter of mycelial colonies after incubation for 4 days at 22 °C in the dark. The EC_{50} or EC_{90} were determined from dose response curves after probit analysis. The ratio of EC_{50} or EC_{90} for a resistant isolate to the EC_{50} or EC_{90} for the parent sensitive strain gave an estimation of the resistance level (resistance factor, Rf).

Measurement of conidial substrate oxidation

For whole-cell respiration studies, germinated conidia in water were re-suspended in a respiration medium containing glucose (5 g l^{-1}), magnesium sulphate (2 mM) and yeast extract (5 g l^{-1}). The germinated conidia were adjusted to 5–6 mg dry weight ml^{-1} and the suspension shaken at 22 °C. After 1.5 h incubation in the presence of inhibitors, samples (2 ml) were withdrawn and the rate of oxygen uptake was determined polarographically

at 22 °C with a Clark-type (Rank Brothers) oxygen electrode inserted in a cuvette on a magnetic stirrer. SHAM (1 mM) was added to the reaction mixture as a specific inhibitor of cyanide-resistant respiration. The respiration rates were calculated on the basis of 265 μM O_2 in the air-saturated medium at 22 °C and were expressed in nmoles O_2 h^{-1} mg^{-1}.

Determination of saprophytic fitness parameters

Mutants of *B. cinerea* were tested for mycelial growth rate, sporulation, spore germination, sclerotia production, stability of resistant phenotypes and competitive ability compared with the wild-type parent strain. Three 2 mm mycelial WA-plugs for each strain were transferred to the centre of PDA plates for radial growth measurements. After incubation at 22 °C in the dark, the colony diameter of each isolate was measured at 24 h intervals. To determine conidial production in the absence of fungicides, PDA-plates were inoculated with a conidial suspension (10^5 conidia per plate) and were incubated for 10 days at 22 °C with 14 h day^{-1} light. The total mycelial mass produced in each dish was transferred to a 250 ml Erlenmeyer flask with 20 ml deionized water. The flasks were agitated vigorously and the concentration of conidia in the resulting spore suspension, after filtration through cheesecloth, was determined with a Neubauer haemocytometer and expressed as number of conidia per square centimetre of the PDA culture. Spore germination and sclerotia production were determined after 6 h and 20 days incubation, respectively, on PDA medium in the dark.

The stability of resistant phenotypes was assessed by successive transfers of selected mutants at first in fungicide-free growth medium, for at least seven generations, and afterwards on pyraclostrobin-containing medium. The sensitivity to pyraclostrobin was measured after every subculture of mutant isolates at the concentration of 0.15 μg ml^{-1} pyraclostrobin (the MIC for the wild-type) in the presence of 0.5 mM SHAM.

The *in vitro* competitive ability of B/PYR-mutant isolates was studied with mixed inocula of pyraclostrobin-resistant mutants and the wild-type parent strain of *B. cinerea*, in the absence of fungicide treatment. PDA plates were inoculated with a mixed conidial suspension (10^5 conidia per plate) of each mutant with the wild-type strain, at the proportions of 50:50 and 90:10, and were incubated for 8–10 days at 22 °C with 14 h day^{-1} light. At the end of the incubation period the total mycelial mass was harvested and the resulting spore suspension produced at each generation was used to re-inoculate new PDA plates. A random sample of at least 50 single conidial colonies, from the initial inoculation and those produced in each generation, was examined for pyraclostrobin sensitivity at the MIC (0.15 μg ml^{-1}) for the wild-type strain in the presence of 0.5 mM SHAM.

Study of pathogenicity and resistance expression in planta

Pathogenicity and *in planta* fungicide resistance of various mutant isolates of *B. cinerea*, were determined by examining symptom severity caused by each strain on cucumber seedlings (*Cucumis sativus*, cv. Telegraph) according to the method described previously by Ziogas and Girgis (1993). Cucumber seedlings grown in plastic pots for 8–10 days (four seedlings per 17 cm pot, two pots per treatment) were used at the cotyledon stage. The formulated fungicides in aqueous suspensions were sprayed to run-off at the desired doses with a hand-sprayer 5 h before inoculation. Control plants were sprayed with deionized water. The centre of each cotyledon was punctured with a needle and a 2 mm mycelial plug from the margin of a young colony on PDA was placed on the wound. The inoculated plants were incubated in a moist chamber at 22 °C for 3–5 days and the infection was scored by evaluating the lesion of each cotyledon. Disease development was evaluated according to the following indices: 0, no infection; 0.5, rot only under inoculum; 1, less than 20% rot; 2, 21–50% rot and 4, rot on more than 50% of cotyledon surface.

Statistical analysis

Data analyses were made with the Statistical Analysis System (JMP, SAS Institute, Inc., Cary, NC, USA). The growth rate and the EC_{50} or EC_{90} value for each isolate and fungicide were calculated from the data subjected to probit analysis. Dunnett's multiple range test was used to assess the differences between mycelial growth rates, sporulation, spore germination, sclerotia production, competitiveness and pathogenicity ratings of isolates.

Results

Selection of pyraclostrobin-resistant mutants

Mutant strains of *B. cinerea* resistant to pyraclostrobin were isolated at high frequency with or without chemical mutagenesis, indicating the existence of a genetical and biochemical potential for development of field resistance towards this particular fungicide. Approximately 7×10^5 mutated conidia of the wild-type strain, which survived the mutagenic treatment (97% lethality), were plated on PDA containing 15 μg ml^{-1} pyraclostrobin and 0.5 mM SHAM. From this selection medium, 428 resistant colonies were obtained during the first 12 days of incubation, indicating a mutation frequency of 6.2×10^{-4}. Most of the resistant isolates appeared between the 7th and 10th day of incubation. Pyraclostrobin-resistant mutants were also isolated without mutagenesis, at a slightly lower frequency of 4×10^{-4}.

Preliminary tests on the response of mutant isolates, obtained after mutagenesis, to pyraclostrobin at the MIC (0.15 μg ml^{-1} pyraclostrobin) for the wild-type strain of *B. cinerea*, resulted in the identification of two pyraclostrobin-resistant phenotypes (R_1 and R_2) regarding the level of resistance. The R_1 phenotypic class, which was observed at high frequency (80% of pyraclostrobin-resistant mutants), appeared after 7–9 days of incubation, and included mutants with high resistance to pyraclostrobin. The mutants of R_2 phenotypic class (approx. 20% of total isolates) were moderately resistant to pyraclostrobin and appeared after 10–12 days of incubation. From this initial screening, a representative sample of 10 mutant isolates, 6 from R_1 and 4 from R_2 resistant phenotype, were chosen for further studies. Fungitoxicity tests on the response of 50 spontaneous mutants to pyraclostrobin showed that all of these presented a low resistance level and were not included in further studies.

Level and stability of pyraclostrobin resistance

The mycelial growth of the wild-type isolate was inhibited 50% (EC_{50}) and 90% (EC90) at the concentrations of 0.015 and 0.075 μg ml^{-1} pyraclostrobin, in addition to 0.5 mM SHAM. Tests on the response of B/PYR isolates to the presence of pyraclostrobin and SHAM in growth medium, showed that the mutants of R_1 phenotype were

Table 1. Comparison of *Botrytis cinerea* isolates resistant to pyraclostrobin with their parental wild-type strain with respect to saprophytic fitness parameters on agar medium

Strains	Resistance factor[a] based on EC_{90}[b] (mean ± SE[c])	Radial growth[d]	Sporulation[e]	Spore germination[f]	Sclerotia production[g]
wt-B_1		48a[h]	8.6a[h]	87.4a[h]	97.2a[h]
R_1 phenoype					
B/PYR-34	178 ± 7.23	49a	9.2a	78.5b	100.3a
B/PYR-35	212 ± 6.07	48a	3.0d	55.1c	68.1bc
B/PYR-117	220 ± 9.68	49a	6.9b	48.7c	83.4b
B/PYR-121	135 ± 7.32	49a	6.7b	71.7b	64.2c
B/PYR-134	197 ± 8.26	48a	3.9cd	50.7c	13.6e
B/PYR-202	156 ± 9.98	48a	7.3b	22.6e	71.9bc
R_2 phenotype					
B/PYR-110	42 ± 4.14	48a	1.7e	40.2d	6.5f
B/PYR-113	34 ± 3.28	47ab	5.8c	27.6e	3.8f
B/PYR-122	50 ± 4.38	46ab	3.5d	43.1cd	33.9d
B/PYR-167	38 ± 3.17	45b	2.7d	38.5d	12.5e

[a]The ratio of EC_{90} for mutant:EC_{90} for wild-type.
[b]Effective concentration causing 90% reduction in growth rate.
[c]Pooled standard error; three replications.
[d]Mean colony diameter (mm) measurements after 4 days incubation ($n=3$).
[e]Mean number ($\times10^6$) of conidia per cm^2 of colony after 10 days incubation ($n=3$).
[f]Percentage of germinated conidia after 6 h incubation ($n=100$).
[g]Mean dry weight of sclerotia (mg) per plate after 20 days incubation ($n=3$).
[h]Within columns, values followed by the same letter do not differ significantly according to Dunnett's multiple range test ($P=0.05$).

highly resistant to pyraclostrobin, with a resistance factor (Rf) based on EC_{90} values ranging from 130 to 230. The R_2 resistant phenotype presented a moderate (Rf: 30–55) resistance level (Table 1). Moreover, a low to moderate resistance (Rf: 15–20, based on EC_{90} values) was observed, in the case of isolates that were obtained without mutagenic treatment (results not shown). A dose-dependent decrease in growth was observed with the wild-type (wt-B_1) and all mutant isolates.

Growth of mutant strains in fungicide-free medium resulted in a rapid decrease in pyraclostrobin resistance, almost from the first transfer, particularly in moderate resistant phenotype mutants. The resistance to pyraclostrobin was reduced by up to 90% after seven transfers of both phenotypes in pyraclostrobin-free medium. However, a rapid recovery of the initial level of resistance was observed when the mutants were subcultured (up to three transfers) on medium containing 0.15 μg ml^{-1} pyraclostrobin (Figure 1).

Inhibitor sensitivity of glucose oxidation by germinated conidia from the wild-type and the mutant isolates

The oxygen uptake by the wild-type and the mutants of R_1 phenotype were 620 and 610 nmoles O_2 h^{-1} mg^{-1}, respectively. However, in the case of mutant strains of R_2 phenotype the oxygen uptake was 2-fold lower (280 nmoles O_2 h^{-1} mg^{-1}). A dose-dependent inhibition of the respiration rate by pyraclostrobin in both wild-type and B/PYR-mutant isolates was observed (Figure 2). Nevertheless, there was a striking difference between the wild-type strain and pyraclostrobin-resistant mutants. The concentrations causing a 90% reduction in the respiration rate were 1.5 μg ml^{-1} for the wild-type and approximately 50 and 225 μg ml^{-1} pyraclostrobin for moderately and highly resistant phenotypes, respectively. A Rf of 33 and 150 based on EC_{90} values was calculated for R_1 and R_2 resistant phenotype, respectively (Figure 2).

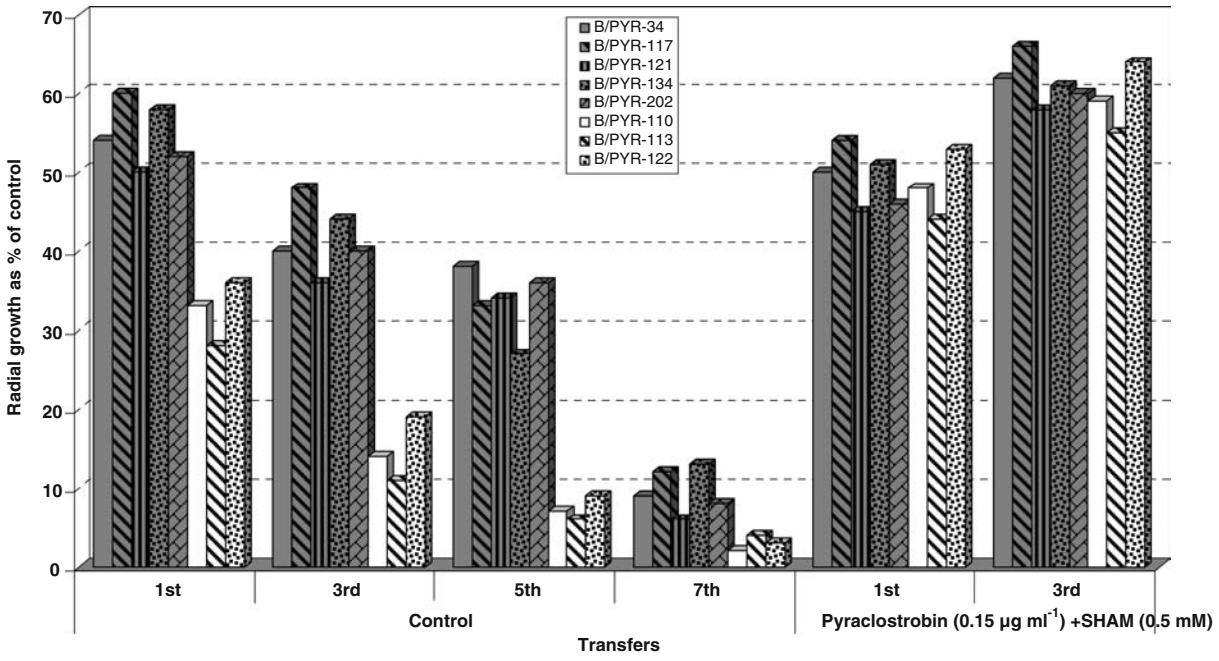

Figure 1. Growth of eight representative pyraclostrobin-resistant isolates of *Botrytis cinerea* at 0.15 μg ml^{-1} pyraclostrobin + 0.5 mM SHAM, after subculturing on PDA medium with or without pyraclostrobin + SHAM. Measurements were made after 4 days incubation at 22 °C.

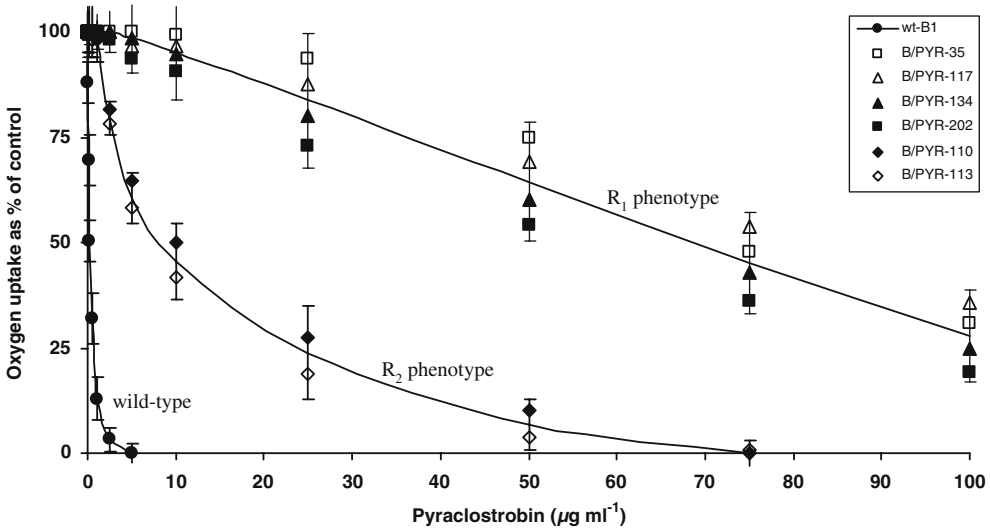

Figure 2. Effect of pyraclostrobin in addition to SHAM (1 mM) on oxygen uptake by the wild-type and four representative pyraclostrobin-resistant (B/PYR) mutant isolates of *Botrytis cinerea* utilizing glucose as substrate. Results are means of three replicates with bars showing the standard errors.

Saprophytic fitness and competitive ability in vitro *of pyraclostrobin-resistant strains*

Study of some fitness-determining characteristics in the wild-type and pyraclostrobin-resistant mutants from both phenotypic classes showed that the mutation(s) leading to pyraclostrobin resistance had no effect on mycelial growth of mutant isolates. Comparisons of other saprophytic fitness-determining parameters such as sporulation, spore germination and sclerotia production, between pyraclostrobin-resistant mutants and the parent wild-type strain of *B. cinerea* showed that, with the exception of B/PYR-34 mutant isolate, most of these fitness parameters were significantly reduced in all mutant isolates (Table 1).

The mutation(s) for resistance to pyraclostrobin had also significant adverse effects on the ability of mutants to compete with the wild-type strain (Figure 3). After three subcultures in fungicide-free medium, a rapid decline in the frequency of all moderate resistant isolates was observed, indicating that these isolates were weak competitors against the wild-type (pyraclostrobin-sensitive) strain, presumably, due to their reduced sporulation and/or spore germination (Table 1). The competitiveness of most R_1 resistant mutant isolates was higher than the R_2 ones. The proportion of R_1-resistant isolates remained stable or reduced slightly after three transfers in fungicide-free medium. However, after subculturing seven times, a high reduction in the proportion of all high resistant isolates was observed.

Cross-resistance

The cross-resistance patterns of pyraclostrobin with other inhibitors of bc_1 complex and with fungicides affecting other cellular pathways are shown in Table 2. The mutation(s) for resistance to pyraclostrobin reduced the sensitivity of mutant strains to other Qo inhibiting fungicides such as azoxystrobin (Rf: 25–165, based on EC_{90} values), trifloxystrobin (Rf: 30–130), picoxystrobin (Rf: 30–175) and fluoxastrobin (Rf: 25–110), but not to famoxadone or the Qi inhibitors cyazofamid and antimycin-A. An increased sensitivity of pyraclostrobin-resistant mutants of both phenotypic classes to the carboxamide boscalid (Rf: 0.2–0.4), an inhibitor of mitochondrial electron transport from succinate to ubiquinone (complex II), and to the anilopyrimidine cyprodinil (Rf: 0.2–0.3), a methionine biosynthesis inhibitor, was observed (Table 2). However, no change in the sensitivity to the phenylpyrrole fludioxonil, the hydroxyanilide fenhexamid, the benzimidazole benomyl or the phenylpyridinamine fluazinam was observed.

Pathogenicity and expression of resistance in planta

The effect of mutations for resistance to pyraclostrobin on the pathogenicity of *B. cinerea* was determined by comparing the symptom severity caused by wild-type and each mutant strain on cucumber seedlings. As shown in Table 3, none of the pyraclostrobin-resistant strains of *B. cinerea* tested lost the ability to cause infection on cotyledons of cucumber plants. Most mutant strains exhibited an infection ability similar with that of wild-type parent strain.

The results of preventive applications of pyraclostrobin against the wild-type and the representative pyraclostrobin-resistant mutants of *B. cinerea* are shown in Table 3. Pyraclostrobin was found to be highly effective against the wild-type, but ineffective, even at the high concentration of 1500 μg a.i. ml^{-1}, against lesion development caused by pyraclostrobin-resistant mutants of both phenotypes (Table 3). The negative cross-resistance relationship between pyraclostrobin and the fungicides boscalid and cyprodinil was not observed *in planta*. Preventive applications of the commercial products of F 510 50WG (boscalid), Chorus 75WP (cyprodinil) and Saphire 50WP (fludioxonil), at the fungicide concentrations of 750, 250 and 100 μg a.i. ml^{-1}, respectively, resulted in complete inhibition of lesion development on cucumber seedlings by both wild-type and pyraclostrobin-resistant mutants (Table 3).

Discussion

Mutants of *Botrytis cinerea* with high and moderate resistance to pyraclostrobin were readily isolated after chemical mutagenesis and selection on media containing pyraclostrobin. This is the first report indicating the existence of a genetical and biochemical potential for the development of field resistance to Qo inhibiting (QoI) fungicides in *B. cinerea*. Two different resistant phenotypes have also been observed in previous studies with perennial ryegrass-infecting strains of *Pyricularia grisea* (teleomorph *Magnaporthe grisea*) resistant to azoxystrobin and trifloxystrobin (Vincelli and Dixon, 2002) and with laboratory mutants of *Venturia inaequalis* resistant to kresoxim-methyl (Zheng et al., 2000).

Prior to the detection of resistant *Blumeria* (*Erysiphe*) *graminis* f.sp. *tritici* isolates in German field populations (Heaney et al., 2000), the inherent resistance risk for QoIs was estimated to be medium and any practical resistance was expected to arise in a step-wise manner, through a gradual increase in the proportion of resistant mitochondria (Brent and Hollomon, 1998). Cytochrome *b*, the target enzyme of the QoIs, is encoded by mitochondrial DNA (Di Rago and Colson, 1989; Zheng and Köller, 1997) and resistance to QoIs through a target-site modification is expected to be inherited in a non-Mendelian way. Detailed genetic analysis of QoI-resistance in *Ustilago maydis* provided a satisfaction of the three criteria (uniparental inheritance, vegetative segregation and intracellular selection) of cytoplasmic (non-Mendelian) heredity (Ziogas et al., 2002). Practical failures of QoIs were first recorded in the control of wheat powdery mildew (*B. graminis*) in northern areas of Germany during 1998 (Sierotzki et al., 2000a) and, more recently in the control of cucumber powdery mildew (*Podosphaera fusca*) and downy mildew (*Pseudoperonospora cubensis*) observed in Japan (Ishii et al., 2001) and *Plasmopara viticola* in Europe (Collina et al., 2004; Sierotzki et al., 2004). Since then, strobilurin resistance has been detected for several plant pathogens, and for some diseases practical resistance developed more rapidly than expected (Heaney et al., 2000; Sierotzki et al., 2000b; Steinfeld et al., 2001; Vincelli and Dixon, 2002; Ma et al., 2003).

Biochemical and molecular studies with laboratory and field isolates resistant to strobilurin-related fungicides showed that the mechanisms of site modification and of alternative respiration for the development of resistance to QoIs are mainly responsible. Point mutations in the Qo-site of cytochrome *b* have been shown to substantially reduce sensitivity of several fungi to fungicides that inhibit electron transport at the Qo site (Di Rago and Colson, 1989; Zheng and Köller, 1997; Zheng et al., 2000). A single point-mutation from glycine to alanine at position 143 (G143A) in the mitochondrial cytochrome *b* amino acid sequence, leading to a high level of resistance to all Qo inhibitors, has been found in phytopathogenic fungi such as *B. graminis*, *V. inaequalis*, *Mycosphaerella fijiensis*, *M. grisea*, *Didymella bryoniae*, *Sphaerotheca fuliginea*, *Plasmopara viticola*,

Table 2. Fungicide sensitivity of wild-type and pyraclostrobin-resistant isolates of *Botrytis cinerea*

Fungicide	Wild-type EC_{90}[b] ($\mu g\ ml^{-1}$) (mean ± SE[c])	Resistance factor[a] based on EC_{90}[b] (mean ± SE[c])							
		R_1 phenotype					R_2 phenotype		
		B/PYR-34[d]	B/PYR-117[d]	B/PYR-121[d]	B/PYR-134[d]	B/PYR-202[d]	B/PYR-110[d]	B/PYR-113[d]	B/PYR-122[d]
Pyraclostrobin	0.075 ± 0.046	178 ± 7.23	220 ± 9.68	135 ± 7.32	197 ± 8.26	156 ± 9.98	42 ± 4.14	34 ± 3.28	50 ± 4.38
Azoxystrobin	0.25 ± 0.061	165 ± 12.27	110 ± 8.28	147 ± 6.46	105 ± 7.89	120 ± 13.26	30 ± 2.36	25 ± 5.68	48 ± 7.28
Trifloxystrobin	0.1 ± 0.032	125 ± 6.62	120 ± 9.28	100 ± 10.92	128 ± 5.72	132 ± 7.86	27 ± 2.18	45 ± 8.34	35 ± 8.34
Picoxystrobin	0.075 ± 0.004	150 ± 3.37	175 ± 9.23	135 ± 6.17	120 ± 11.73	115 ± 5.23	42 ± 6.54	30 ± 7.53	50 ± 3.54
Fluoxastrobin	1.75 ± 0.033	100 ± 8.23	98 ± 5.13	105 ± 10.03	100 ± 8.37	110 ± 11.03	25 ± 9.77	50 ± 4.62	35 ± 6.34
Famoxadone	15 ± 2.683	1.2 ± 0.23	1.0 ± 0.08	1.4 ± 0.13	1.6 ± 0.17	1.0 ± 0.58	1.6 ± 0.06	1.4 ± 0.97	1.1 ± 0.97
Cyazofamid	8.75 ± 1.637	1.4 ± 0.31	1.1 ± 0.12	1.3 ± 0.27	1.3 ± 0.06	1.0 ± 0.02	1.4 ± 0.31	1.0 ± 0.16	1.2 ± 0.15
Antimycin-A	25 ± 2.721	1.2 ± 0.12	1.6 ± 0.13	–[e]	1.3 ± 0.43	1.6 ± 0.06	1.0 ± 0.04	1.0 ± 0.28	–
Boscalid	25 ± 2.372	0.4 ± 0.08	0.2 ± 0.04	0.3 ± 0.01	0.4 ± 0.07	–	0.4 ± 0.01	–	0.3 ± 0.06
Cyprodinil	10 ± 1.226	0.3 ± 0.01	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.03	–	0.3 ± 0.01	–	–
Fludioxonil	0.05 ± 0.008	1.1 ± 0.43	1.0 ± 0.07	–	–	1.2 ± 0.12	1.7 ± 0.29	–	1.2 ± 0.23
Fenhexamid	1.75 ± 0.477	1.3 ± 0.26	1.5 ± 0.72	1.1 ± 0.07	–	1.0 ± 0.23	1.3 ± 0.12	–	1.6 ± 0.34
Fluazinam	0.25 ± 0.043	1.2 ± 0.28	1.3 ± 0.18	1.2 ± 0.42	–	1.4 ± 0.21	1.3 ± 0.35	–	1.2 ± 0.04
Benomyl	0.075 ± 0.003	1.0 ± 0.02	1.2 ± 0.13	1.0 ± 0.05	–	2.1 ± 0.52	2.3 ± 0.78	–	–

[a]The ratio of EC_{90} for mutant:EC_{90} for wild-type.
[b]Effective concentration causing 90% reduction in growth rate.
[c]Pooled standard error; three replications.
[d]B/PYR mutant strains resistant to pyraclostrobin.
[e]Not tested.

Table 3. Effect of fungicides on lesion development following inoculation of cucumber seedlings with wild-type and pyraclostrobin-resistant strains of *Botrytis cinerea*

Fungicide concentration (μg a.i ml^{-1})	Infection of cotyledons (% of control)								
	R_1-phenotype						R_2-phenotype		
	wt-B_1	B/PYR-34[d]	B/PYR-117[d]	B/PYR-121[d]	B/PYR-134[d]	B/PYR-202[d]	B/PYR-110[d]	B/PYR-113[d]	B/PYR-122[d]
Control	100 (60a[b])[a]	100 (59a)[a]	100 (62a)[a]	100 (60a)[a]	100 (55b)[a]	100 (59a)[a]	100 (54b)[a]	100 (57ab)[a]	100 (61a)[a]
F-500 25 EC[c]									
25	56b	100a	100a	100a	100a	100a	100a	100a	100a
150	16c	100a	100a	98a	100a	100a	74b	82b	89ab
500	7c	92a	94a	90a	89a	97a	56b	42b	58b
1500	0c	82a	88a	80a	79a	86a	37b	23b	36b
F-510 50WG[c]									
25	66a	70a	74a	72a	68a	65a	71a	67a	–[e]
100	37a	34a	38a	40a	39a	36a	33a	37a	–
500	8a	7a	11a	8a	10a	6a	9a	6a	–
750	0a	1a	3a	0a	2a	0a	0a	0a	–
Chorus 75WP[c]									
100	20a	22a	19a	20a	23a	19a	20a	22a	16ab
250	2a	4a	0a	3a	0a	0a	0a	0a	0a
Saphire 50WP[c]									
1	30a	28a	27a	–	32a	25ab	30a	–	27a
5	9a	7a	7a	–	8a	10a	9a	–	6ab
10	3a	0a	2a	–	0	4a	3a	–	0a
100	0d	0a	0a	–		0a	0a	–	0a

[a]Value in parenthesis equals the sum of indices of 16 cotyledons for each control.
[b]Within rows, values followed by the same letter do not differ significantly according to Dunnett's multiple range test ($P=0.05$).
[c]Commercial formulations: F-500 (pyraclostrobin); F-510 (boscalid); Chorus (cyprodinil); Saphire (fludioxonil).
[d]B/PYR mutant strains resistant to pyraclostrobin.
[e]Not tested.

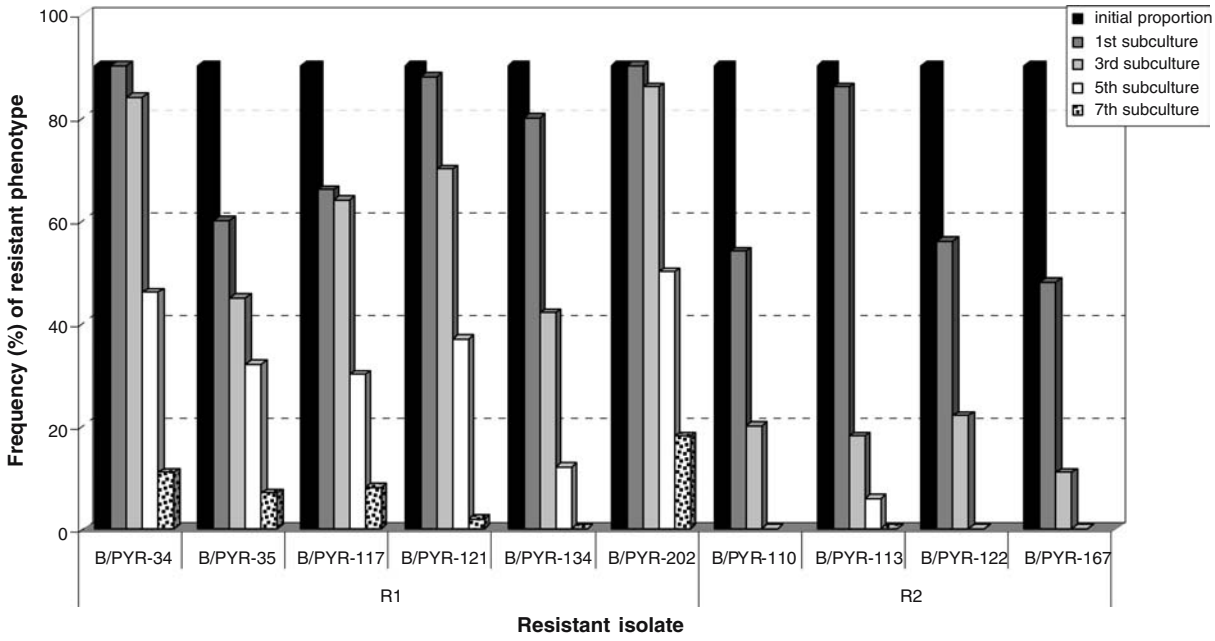

Figure 3. In vitro competition between pyraclostrobin-resistant isolates and the wild-type strain of *Botrytis cinerea*, co-inoculated on PDA medium at the initial ratio 90:10, respectively. Measurements were made after 10 days incubation at 22 °C.

P. cubensis, *Alternaria alternata*, *Alternaria tenuissima* and *Alternaria arborescens* (Heaney et al., 2000; Sierotzki et al., 2000a, b; Zheng et al., 2000; Ishii et al., 2001; Avila-Adame and Köller, 2003; Ma et al., 2003). A novel G143S amino-acid exchange has been identified for a spontaneous laboratory mutant of *M. grisea* (Avila-Adame and Köller, 2003). Recently, a second amino acid substitution of phenylalanine with leucine at position 129 (F129L) has been reported in QoI-resistant isolates of *Pythium aphanidermatum* (Bartlett et al., 2002), *P. grisea* (Farman, 2001; Kim et al., 2003), *P. viticola* (Sierotzki et al., 2004) and *Alternaria solani* (Pasche et al., 2005). Alternative respiration in response to the inhibition of cytochrome pathway has been described in *Septoria tritici* (*Mycosphaerella graminicola*) as a second potential mechanism of resistance to respiratory inhibitors acting at the Qo site of cytochrome bc_1 complex (Ziogas et al., 1997). This potential bypass of target site of strobilurin fungicides has also been described in other plant pathogens such as *M. grisea* (Yukioka et al., 1997) *Gaeumannomyces graminis* (Joseph-Horne et al., 1999), *B. cinerea* (Tamura et al., 1999) and *V. inaequalis* (Olaya and Köller, 1999a). However, the alternative respiration pathway is essential for fungal metabolism but probably not for field resistance to QoIs (Ziogas et al., 1997; Olaya and Köller, 1999b). *In vivo* experiments showed that the decreased ATP formation by alternative respiration was inadequate for efficient parasitic growth on the host (Ziogas et al., 1997).

In the present work, the oxygen uptake by germinated conidia was strongly inhibited in the wild-type strain by the mixture of pyraclostrobin and SHAM, a specific inhibitor of cyanide-resistant (alternative) respiration, but not that in the mutant isolates of *B. cinerea*, indicating that alternative respiration is apparently not the biochemical mechanism of resistance to pyraclostrobin in these mutant strains, and probably change(s) at the ubiquinol oxidation centre (Qo-site) of cytochrome *b* are responsible for the reduced sensitivity to pyraclostrobin of both mutant phenotypes. Similar results have also been reported with laboratory mutants of *U. maydis* resistant to azoxystrobin (Ziogas et al., 2002), and with field isolates of *M. fijiensis* (Sierotzki et al., 2000b) and *V. inaequalis* (Steinfeld et al., 2001). On the contrary, study of the mechanism of resistance to azoxystrobin in *S. tritici* indicated the presence of an efficient alternative pathway inhibited by SHAM in the mutant isolate (Ziogas et al., 1997). The

existence of two different phenotypes in *B. cinerea* regarding the level of resistance to pyraclostrobin and the rate of oxygen uptake, presumably indicates two different genotypes, each of which may have a distinct single nucleotide substitution resulting in different amino acid changes in the target site of pyraclostrobin which may or may not affect the bc_1 activity.

Cross-resistance studies between pyraclostrobin and other fungicides showed that the mutations for resistance to pyraclostrobin are responsible for reduced sensitivity of mutant strains to the QoIs azoxystrobin, trifloxystrobin, fluoxastrobin and picoxystrobin, but not to the QoI famoxadone and to the Qi inhibitors (QiI) cyazofamid and antimycin-A. An increased sensitivity (EC_{90s} ratio of 0.2–0.4) of pyraclostrobin-resistant strains to boscalid, an inhibitor of complex II of mitochondrial electron transport, and to cyprodinil, a methionine biosynthesis inhibitor was observed, indicating negative cross-resistance relationships between QoI fungicides with carboxamides and anilinopyrimidines. A similar negative correlation between azoxystrobin and boscalid was also found in mutant strains of *A. solani* (Pasche et al., 2005). However, at this time it is difficult to find an explanation for the negative cross-resistance relations between QoIs and the fungicides boscalid and cyprodinil. A positive cross-resistance, with high resistance factors, among QoIs has also been observed in many other phytopathogenic fungi (Heaney et al., 2000; Sierotzki et al., 2000a, b; Chin et al., 2001a, b; Ishii et al., 2001; Steinfeld et al., 2001; Vincelli and Dixon, 2002; Ziogas et al., 2002; Pasche et al., 2005). The absence of cross-resistance between Qo and Qi inhibitors in pyraclostrobin-resistant mutants of *B. cinerea*, would suggest changes only in the configuration at Qo-site of cytochrome *b* which do not transmit a structural change to the Qi-site. According to the current information it appears that the replacement of guanidine to cytosine in the GGT codon of glycine (GGT to GCT) at position 143 confers a high level of resistance among all QoI fungicides (Sierotzki et al., 2000a; Ishii et al., 2001; Avila-Adame and Köller, 2003), including the azolones famoxadone and fenamidone (Chin et al., 2001b). On the contrary, the F129L substitution conveys a lower level of resistance with different cross-resistance patterns among QoIs and a negative relation with boscalid (Kim et al., 2003; Pasche et al., 2005).

Fitness of mutant strains is an important parameter regarding the risk for practical resistance development. Results from studies to verify the overall cost of resistant mutations on fitness are informative in terms of determining the subsequent response of pathogen populations in the field. In the present work the study of fitness-determining characteristics in wild-type and pyraclostrobin-resistant mutants of *B. cinerea* showed that the mutation(s) leading to pyraclostrobin resistance carry some fitness penalties. Most isolates exhibited a significant reduction in the saprophytic fitness parameters, such as sporulation, conidial germination and sclerotia production. Reductions in fitness parameters indicate that these mutations can be induced in the laboratory, but should not cause a serious practical problem. However, the saprophytic fitness penalties were less in some mutant strains of R_1 phenotype. Most of the fitness characteristics were slightly affected or unaffected in these mutant strains. Pathogenicity tests showed that none of the pyraclostrobin-resistant strains of *B. cinerea* tested lost the ability to cause infection on cucumber plants. Most mutants exhibited infection ability similar with that of the wild-type parent strain. Similar results were obtained from laboratory mutants of *U. maydis* resistant to azoxystrobin (Ziogas et al., 2002). Fitness penalties have also been observed in resistant populations of *P. viticola* and *M. grisea*, carrying the G143A and G143S mutation for resistance to QoIs, respectively (Heaney et al., 2000; Avila-Adame and Köller, 2003). Contrary to the above, fitness penalties were not apparent in the case of G143A mutation in *B. graminis* and the resistant populations were widespread (Heaney et al., 2000).

Experiments on the stability of pyraclostrobin-resistant mutants of *B. cinerea* showed a significant reduction of resistance in both phenotypic classes (R_1 and R_2), when the mutants were grown on medium without pyraclostrobin, indicating intracellular selection of wild-type (sensitive) mitochondria. However, the high level of resistance to pyraclostrobin returned rapidly when the mutant strains were subcultured on pyraclostrobin-containing medium. Apparently, resistant mitochondria were less competitive than wild-type ones, but under fungicide selection pressure mutated mitochondria replicate faster than wild-type ones. Similar results were also observed with laboratory mutant strains of

U. maydis (Ziogas et al., 2002) and *V. inaequalis* (Zheng et al., 2000) resistant to azoxystrobin and kresoxim-methyl, respectively. Contrary to this, the resistance of target-site mutants of *M. grisea* carrying the G143A or G143S mutation remained stable during four consecutive disease cycles in the absence of azoxystrobin (Avila-Adame and Köller, 2003).

Competition experiments using mixed inocula of spores from sensitive and resistant strains of *B. cinerea* showed that *in vitro* all resistant strains were less competitive than the wild-type parent strain. However, the highly resistant strains were more competitive than the moderate ones. Obviously, the penalties in the ecological fitness characteristics are responsible for the reduced competitive ability of all mutant strains. On the contrary, competition experiments between resistant and sensitive field isolates of *B. graminis* f.sp. *tritici* showed that resistance to kresoxim-methyl and trifloxystrobin was stable in the absence of selection over three generations, and also that the resistant isolates appeared equal in fitness to the sensitive ones on untreated plants (Chin et al., 2001a).

The data of the present work indicate distinct target-site modifications at the Qo-site of cytochrome *b* in R_1 and R_2 phenotypes of *B. cinerea* which affect the bc_1 activity and the pathogen fitness differentially. Future molecular characterization of QoI-resistant mutants will confirm the presence of, and provide information on the change(s) of cytochrome *b* gene and their effect on ecological fitness of resistant strains. Although our results clearly show fitness penalties in most mutant strains, the field application of pyraclostrobin requires careful implementation of preventive anti-resistance strategies. Beyond the genetic variability and flexibility caused by sexual reproduction and heterokaryosis, the high reproductive rate, the wide host range, the ability of saprophytic growth and the intracellular selection of insensitive mitochondria create an apparent inherent resistance risk to pyraclostrobin and other QoI fungicides in *B. cinerea*.

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