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Biological and molecular characterization of laboratory mutants of *Cercospora beticola* resistant to Qo inhibitors

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Abstract

The resistance to strobilurin-related fungicides and its molecular basis in laboratory mutant isolates of *Cercospora beticola* was investigated. After ultraviolet mutagenesis, mutants with high, moderate or low resistance levels to pyraclostrobin were isolated from a wild-type strain of *C. beticola*. Fungitoxicity tests on the response of resistant isolates on medium containing pyraclostrobin and salicylhydroxamate (SHAM), a specific inhibitor of cyanide-resistant (alternative) respiration, indicated that the biochemical mechanism of alternative oxidase was not responsible for the reduced sensitivity to pyraclostrobin for half of the mutants. Cross-resistance studies with other inhibitors of the cytochrome bc_1 complex of the mitochondrial respiratory chain showed that the mutation(s) for resistance to pyraclostrobin also reduced the sensitivity of mutant strains to other Qo inhibitors such as azoxystrobin and fenamidone, but not to the Qi inhibitor cyazofamid. No effect of pyraclostrobin-resistant mutation(s) on fungitoxicity of the carboxamide boscalid, the triazoles epoxiconazole and flutriafol and to the benzimidazole benomyl, which affect other cellular pathways or other steps of the respiratory chain, was observed. Study of fitness parameters showed that most mutants had a significant reduction in sporulation and pathogenicity compared to the wild-type parental isolate. However, experiments on the stability of the resistant phenotype did not show a significant reduction of the resistance for half of the mutants when grown for at least four generations on pyraclostrobin-free medium. Molecular analysis of cytochrome *b* cDNA, isolated from the wild-type and the pyraclostrobin-resistant mutant isolates, revealed two novel amino acid replacements at positions involved in Qo resistance in other species. The glycine (GGT) to serine (AGT) replacement at position 143 (G143S) was found in the isolate with the highly resistant phenotype. The second amino acid change was the replacement of phenylalanine (TTC) by valine (GTC) at position 129 (F129V), which was found in a mutant strain with the moderately resistant phenotype. Four additional mutations located in conserved regions of the mitochondrial cytochrome *b* gene (I154L, N250D, E256G and V261D) were detected in some mutant isolates of *C. beticola* but their possible role in Qo-resistance needs further investigation. This is the first study reporting *C. beticola* strains resistant to Qo inhibitor fungicides due to the biochemical mechanism of target-site modification, resulting from amino acid changes in the mitochondrial cytochrome *b* gene.

Introduction

Cercospora leaf spot caused by *Cercospora beticola* is one of the most serious diseases of sugar beets with world-wide economic importance. Control of the fungus in Greece and other areas with a warm climate and irrigation is based mainly on frequent fungicide applications. However, serious problems have resulted from the extensive appearance of resistant isolates to the intensively used benzimidazoles, organotin fungicides and sterol biosynthesis inhibiting triazoles (Karaoglanidis et al., 2003). Isolates resistant to the benzimidazoles appeared in the early of 1970s resulting in complete failure of disease control (Georgopoulos and Dovas, 1973; Weiland and Halloin, 2001). The replacement of benzimidazoles with the triazole fungicides was only a temporary solution to the problem because in the last 10 years triazole-resistant isolates were also reported (Karaoglanidis et al., 2000). The organotin fungicide fentin-acetate, which was used in the anti-resistance strategies was withdrawn and the use of the dithiocarbamate maneb may eventually be restricted. Such restrictions intensified the need for new fungicides with novel site-specific mechanisms of action.

During the last decade fungicide research has produced a diverse range of anti-fungal agents with novel modes of action, which are expected to have a significant impact on the control of *C. beticola*. The Qo inhibitors (QoI) of the cytochrome bc_1 complex of the respiratory chain, is the most important new chemical group of fungicides with a broad spectrum of activity used against fungal species among the four major classes of Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes (Ammermann et al., 2000). However, QoI fungicides are also threatened by resistance development as indicated by recent reports. In the last few years field populations of certain pathogens, such as *Blumeria* (*Erysiphe*) *graminis*, *Plasmopara viticola*, *Venturia inaequalis*, *Pyricularia* (*Magnaporthe*) *grisea* and *Mycosphaerella fijiensis*, resistant to QoI have been detected in Europe and elsewhere (Heaney et al., 2000; Sierotzki et al., 2000a, b; Chin et al., 2001a; Ishii et al., 2001). Biochemical and molecular studies have shown that resistance to QoI fungicides would 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 alternative oxidase, a bypass of the complexes III and IV of the cytochrome pathway (Ziogas et al., 1997; Tamura et al., 1999).

Strobilurin fungicides such as trifloxystrobin and pyraclostrobin have recently been registered in Greece for the control of Cercospora leaf spot and a serious concern is the duration of their effectiveness. *Cercospora beticola* is a 'high risk' pathogen in terms of resistance and these fungicides face the possibility of control failure due to the appearance of field-resistant strains. To our knowledge no information is available concerning the risk for resistance development to QoI in *C. beticola*. Defining the resistance risk is not easy and important considerations for this are the genetic potential and the possible mechanism by which resistance is achieved in the pathogen population. Thus the specific objectives of the present study were: (a) to explore the genetic potential of *C. beticola* to develop resistance to QoI; (b) to identify the biochemical mechanism and the molecular basis for resistance to QoI and (c) to assess the impact of mutations for resistance to QoI on the ecological fitness characteristics of *C. beticola*.

Materials and methods

Fungal strains and culture conditions

The wild-type strain 222S of *C. beticola* isolated from sugar beet plants (*Beta vulgaris* var. *sacharifera* cv. Adrienne) in Greece was used to obtain pyraclostrobin-resistant mutants (C/PYR). All isolates were grown and maintained on Aspergillus Complete Medium (ACM) containing 1% glucose, 1% yeast extract and 2% agar, in a controlled climate cabinet at 27 °C with 14 h day^{-1} light and 70% relative humidity. For long-term storage the isolates were maintained in glass tubes on ACM medium 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, epoxiconazole and boscalid were kindly supplied by BASF AG

(Limburgerhof, Germany), azoxystrobin by Syngenta Crop Protection AG (Basle, Switzerland), fenamidone by Bayer CropScience AG (Leverkusen, Germany), flutriafol by Zeneca Agrochemicals (Farnham, UK) and benomyl by Du Pont de Nemours and Co. (Wilmington, DE, USA). Cyazofamid, antimycin A and salicylhydroxamate (SHAM) were purchased from Fluka, Sigma and Aldrich, respectively. Stock solutions of fungicides were made in acetone, with the exception of SHAM and antimycin A, which were dissolved in methanol and ethanol, respectively.

Mutation induction

Pyraclostrobin-resistant isolates were obtained via UV mutagenesis. Conidial suspensions (approximately 10^7 conidia ml^{-1}) of the wild-type strain of *C. beticola* in water were obtained from 8 to 10 day-old cultures and were exposed with continuous agitation to ultraviolet irradiation (TUV Philips, 15W, 254 nm) for 5 min. After irradiation the conidia were kept for 30 min in the dark to minimize photorepair of radiation damage, then plated on ACM medium containing 5 μg ml^{-1} pyraclostrobin and incubated at 27 °C for 15 days, to enable resistant colonies to appear. The selected resistant isolates C/PYR were maintained on ACM agar slants containing 2 μg ml^{-1} pyraclostrobin, the concentration causing 90% reduction (EC_{90}) of growth of the wild-type parent strain of *C. beticola*.

In vitro *fungitoxicity tests*

Fungitoxicity tests were made with six mutant strains using several concentrations of each fungicide to determine the EC_{50} and the EC_{90} values. The fungicide sensitivity of the wild-type and mutant strains was assessed by inoculating ACM medium plates with mycelial inoculum consisting of 2 mm plugs cut from the growing edge of 5 day-old cultures maintained on the same medium. 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 replicates 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 10 days at 27 °C in the dark. The EC_{50} or EC_{90} values were determined from dose-response curves after probit analysis.

Determination of ecological fitness parameters

Mutants of *C. beticola* were tested for mycelial growth rate, sporulation, stability and pathogenicity compared with the wild-type parent strain. Growth rates in fungicide-free ACM medium were calculated as previously described. To determine conidial production in the absence of fungicides, ACM plates were inoculated with a conidial suspension (10^5 conidia per plate) and were incubated for 10 days at 27 °C with 14 h day^{-1} light. The total mycelial mass produced was scraped from each dish and transferred to a 250 ml Erlenmeyer flask with 20 ml deionized water. The flasks were hand-agitated vigorously and conidia were filter-separated through cheesecloth. The concentration of the resulting spore suspension was determined with a Neubauer haemocytometer and expressed as number of conidia cm^{-2} of the ACM culture.

The stability of resistant phenotypes was assessed by successive transfers of selected mutants in fungicide-free growth medium, for at least four generations. The sensitivity to pyraclostrobin was measured after every subculture of mutant isolates at the EC_{90} for the wild-type strain.

Pathogenicity of the wild-type and mutant isolates of *C. beticola* was determined by examining symptom severity caused by each strain on sugar beet plants. Sugar beet seedlings grown in plastic pots for four weeks (three seedlings per 17 cm pot, two pots per treatment) were used at the 3–4 leaf stage. A suspension of 10^4 conidia ml^{-1} was sprayed to run-off. The inoculated plants were incubated in a moist chamber for 15 days at 27 °C with 14 h day^{-1} light, and the infection was recorded according to the 0–9 disease index rating scale of Kleinwanzlebener Saatzucht (Shane and Teng, 1992).

Isolation of genomic DNA and total RNA and synthesis of cDNA in C. beticola

Fungal cultures were grown on a 9 cm disc of sterile cellophane on ACM at 27 °C for 10 days. Mycelium was peeled off and ground in liquid nitrogen using a mortar and pestle. Genomic DNA and total RNA were isolated using TRI reagent (Sigma) according to manufacturer's instructions. The RNA was treated with DNase RQ1 (Promega) and first-strand cDNA was synthesized using SuperScript II RNase H^- reverse transcriptase (Invitrogen) and an oligo(dt) adaptor primer [5′-GACTCGAGTCGACATCGA $(-dT)_{17}$-3′].

Cloning and sequence analysis of a partial fragment of C. beticola *cytochrome b gene*

The degenerate oligonucleotide primers, cytb1F (designed from conserved regions of the cyt b protein) and cytbasco1R (adapted from Sierotzki et al., 2000a, b) (Table 1), were used to amplify a partial fragment (~705 bp) of the cyt *b* gene from gDNA and cDNA of the wild-type 222S *C. beticola* strain. The primers cytb1F and cytbasco1R (Table 1), at a concentration of 0.5 μM were used in PCR reaction containing 3 mM $MgCl_2$, 1 mM dNTPs, and 2.5 units of HotStar *Taq* DNA polymerase (Qiagen) in the manufacturer's PCR buffer. The PCR conditions were 95 °C for 15 min and then 35 cycles of 94 °C for 30 s, 48.5 °C for 1 min and 72 °C for 2 min with a final 10 min extension at 72 °C. Products of the expected size were ligated to pGEM-T easy vector (Promega), and several independent clones were sequenced in both directions. Sequence data were analyzed using Lasergene software (DNAstar, Madison, USA). The 3′ end of the cyt *b* cDNA was obtained by PCR with a specific forward primer, cytb3RACE (Table 1) and the adaptor primer [5′-GACTCGAGTCGACATCGA-3′]. The PCR reaction mixture was as above except it contained 2 mM $MgCl_2$, 1 mM dNTPs, 0.4 μM of each primer and 1.75 units of HotStar *Taq* polymerase. The PCR reaction was carried out at 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 40 s with a final 10 min extension at 72 °C. Products of the expected size (~480 bp) were sub-cloned, sequenced and analyzed as above.

Table 1. Primers used for the amplification of the cytochrome *b* gene fragment

Primer	Sequence (5′-3′)
cytb1F	ga(ag)ca(ct)at(agt)atg(ac)g(agt)ga(ct)gt
cytbasco1R	aaca(at)(agct)g(ct)(agt)at(agc)ac(at)ccta
cytb3RACE	cttcatgccgaacgttttagg
cytbSF	acagcttcagcatttttcttctt
cytbSR	tctatgaatggtgattcaacgtg

The primers cytbSF and cytbSR (Table 1) were used to amplify a 800 bp fragment encoding cyt *b* gene from the wild-type (222S) and the pyraclostrobin-resistant mutants C/PYR-C4, C/PYR-U2 and C/PYR-N7. The PCR reaction was as above except it contained 0.2 μM from each primer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, and 1.25 units of HotStar *Taq* DNA polymerase. The PCR reaction was carried out at 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 40 s with a final 7 min extension at 72 °C. The fragments were ligated to pGEM-T easy vector. At least 10 independent clones from each strain were sequenced in both directions and analyzed as previously described.

Statistical analysis

Data analysis was carried out 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. Duncan's multiple range test was used to assess the differences between mycelial growth rates, sporulation and pathogenicity ratings of isolates.

Results

Isolation and characterization of pyraclostrobin-resistant mutants of C. beticola

Mutant strains of *C. beticola* resistant to pyraclostrobin were isolated after UV mutagenesis and selection on medium containing pyraclostrobin, indicating the existence of a genetic and biochemical potential for development of resistance towards this particular fungicide. Approximately 4.5×10^5 mutated conidia of the wild-type strain, which survived the mutagenic treatment (98%

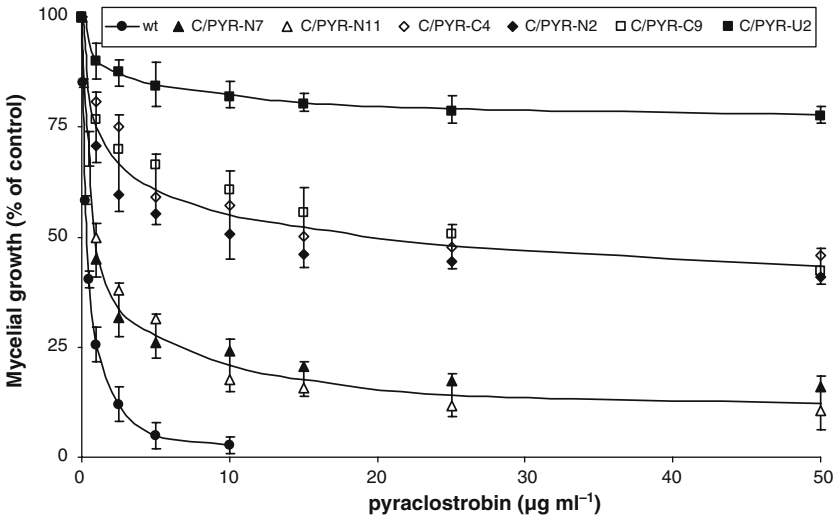

Figure 1. Sensitivity of the wild-type and mutant isolates (C/PYR) of *C. beticola* to pyraclostrobin on ACM medium. Measurements were made after 10 days incubation at 27 °C. Results are means of four replicates with bars showing the standard errors.

lethality), were plated on ACM containing 5 µg ml^{-1} pyraclostrobin. From this selection medium, six resistant colonies were obtained during the first 10 days of incubation, indicating a mutation frequency of 1.3×10^{-5}. A dose-dependent growth inhibition of the wild-type strain was observed and the EC_{50} and EC_{90} values were found at concentrations of 0.35 and 2 µg ml^{-1} pyraclostrobin (Figure 1). Fungitoxicity tests on the response of C/PYR isolates to the presence of pyraclostrobin in growth medium resulted in the identification of three pyraclostrobin-resistant phenotypes regarding the level of resistance observed. The resistance factors (Rf), based on EC_{50} values, were more than 200, 50–80 and 3–5 for the highly resistant (HR: C/PYR-U2), moderately resistant (MR: C/PYR-N2, C/PYR-C4 and C/PYR-C9) and low resistant (LR: C/PYR-N11 and C/PYR-N7) phenotype, respectively (Figure 1).

The sensitivity of the wild-type 222S and C/PYR-U2, C/PYR-C9 and C/PYR-N7 mutant strains to pyraclostrobin was not affected by the presence of SHAM in the growth medium (Figure 2). Fungitoxicity tests of pyraclostrobin in the presence of SHAM showed an increase of sensitivity in the case of C/PYR-N2 and C/PYR-N11 mutants indicating that alternative respiration is, at least partially, responsible for their resistance to pyraclostrobin. An interesting decreased fungitoxic effect of pyraclostrobin was observed in the case of C/PYR-C4 strain when SHAM was added in growth medium (Figure 2).

The cross-resistance patterns of pyraclostrobin with other inhibitors of bc_1 complex and fungicides affecting other cellular pathways or other sites of the cytochrome pathway 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 and fenamidone. No resistance relation between pyraclostrobin and the Qi inhibitor cyazofamid was observed. An interesting fungitoxicity profile was observed in the case of the mutant strains C/PYR-N2 and C/PYR-N11, which were more resistant than the wild-type to antimycin A in the absence of SHAM (Table 2). Resistance to antimycin A was also observed in the case of the C/PYR-C4 regardless of the presence of SHAM. A resistance correlation was not found for the benzimidazole benomyl, triazoles epoxiconazole and flutriafol and the carboximide boscalid, which affect other cellular processes or other steps of electron transport.

Stability of resistance and ecological fitness parameters

Growth of mutant isolates in fungicide-free medium showed, with the exception of C/PYR-U2 and C/PYR-N7, a reduction in pyraclostrobin

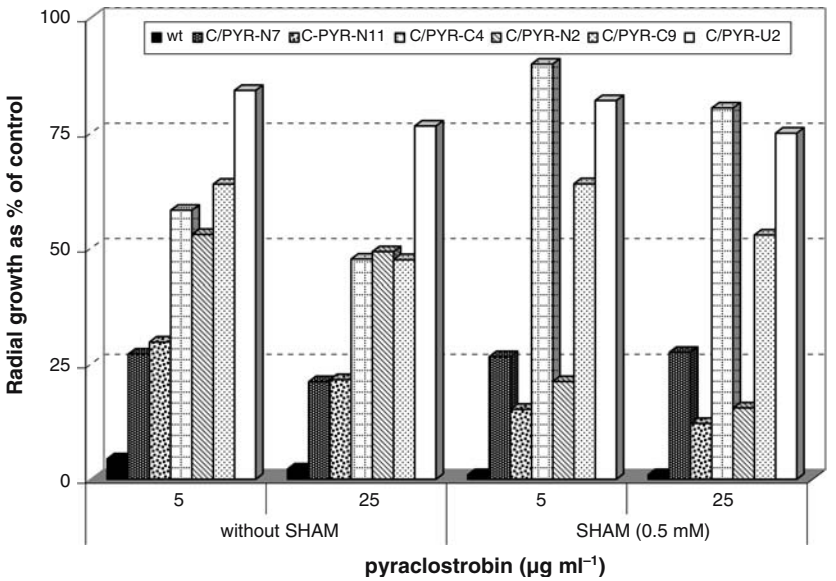

Figure 2. Sensitivity of the wild-type and mutant isolates (C/PYR, grouped by phenotype) of *C. beticola* to pyraclostrobin in absence or presence of SHAM (0.5 mM) on ACM medium. Measurements were made after 10 days incubation at 27 °C. Results are means four replicates.

resistance (Figure 3). However, a recovery of the level of resistance was observed when the mutants were sub-cultured (up to four transfers) on medium containing 2 µg ml^{-1} pyraclostrobin (Figure 3).

Study of fitness-determining characteristics in the wild-type and mutant strains showed that the mutation(s) leading to resistance to QoI fungicides affect the ecological fitness of most mutant isolates. The mycelial growth varied; the C/PYR-C9, C/PYR-N11, C/PYR-C4, C/PYR-N2 and C/PYR-N7 isolates grew approximately as the wild-type parent strain, while the growth rate of C/PYR-U2 was 30% lower compared to the wild-type strain. Comparisons of conidial production between resistant mutants and the wild-type parent strain of *C. beticola* showed a reduction in sporulation for half (C/PYR-N2, C/PYR-N11 and C/PYR-C4) of the mutant isolates tested (Table 3).

Study of the effect of resistance mutation(s) on the pathogenicity showed that none of the resistant

Table 2. Fungicide sensitivity of wild-type and pyraclostrobin-resistant isolates of *Cercospora beticola*

Fungicide	Mycelial relative growth[a] (mean ± SE[b])						
	222S (wt)	C/PYR-N7[c]	C/PYR-N11[c]	C/PYR-N2[d]	C/PYR-C4[d]	C/PYR-C9[d]	C/PYR-U2[e]
Pyraclostrobin (5 µg ml^{-1})	4.67 ± 0.35	27.2 ± 0.28	31.19 ± 1.75	52.95 ± 0.90	58.4 ± 1.25	65.38 ± 0.25	84.47 ± 0.31
Azoxystrobin (5 µg ml^{-1})	0	55.20 ± 1.90	46.92 ± 0.85	58.42 ± 1.30	66.36 ± 0.05	68.11 ± 1.25	74.68 ± 0.40
Fenamidone (10 µg ml^{-1})	12.5 ± 0.05	64.90 ± 0.18	60.22 ± 0.55	65.20 ± 0.35	71.91 ± 1.15	74.32 ± 0.20	75.25 ± 0.68
Cyazofamid (10 µg ml^{-1})	79.81 ± 0.20	78.68 ± 0.45	78.25 ± 1.55	80.10 ± 0.19	80.90 ± 1.20	78.90 ± 0.15	78.22 ± 0.90
Antimycin A (5 µg ml^{-1})	54.69 ± 1.05	52.63 ± 0.05	88.25 ± 0.85	83.77 ± 0.25	91.18 ± 0.95	52.70 ± 1.15	59.62 ± 0.82
Boscalid (5 µg ml^{-1})	74.50 ± 1.00	71.70 ± 1.86	73.25 ± 0.35	77.78 ± 0.49	69.66 ± 1.51	78.57 ± 1.00	72.28 ± 1.96
Epoxiconazole (10 µg ml^{-1})	12.40 ± 0.57	11.35 ± 0.07	12.76 ± 0.75	11.32 ± 0.97	9.96 ± 0.96	9.45 ± 1.08	nt
Flutriafol (5 µg ml^{-1})	21.03 ± 1.42	21.59 ± 1.43	20.51 ± 5.42	nt	22.78 ± 1.78	26.12 ± 1.75	19.40 ± 1.53
Benomyl (0.25 µg ml^{-1})	1.00 ± 0.05	nt[f]	nt	1.25 ± 0.10	1.25 ± 0.20	2.05 ± 0.50	1.00 ± 0.05

[a]Mycelial growth as percent of fungicide-free control at certain fungicide concentrations after 10 days of incubation at 27 °C (n = 4).
[b]Pooled standard error of the means (n = 4).
[c]Low resistant strains, [d]moderately resistant strains, [e]highly resistant strain.
[f]nt: not tested.

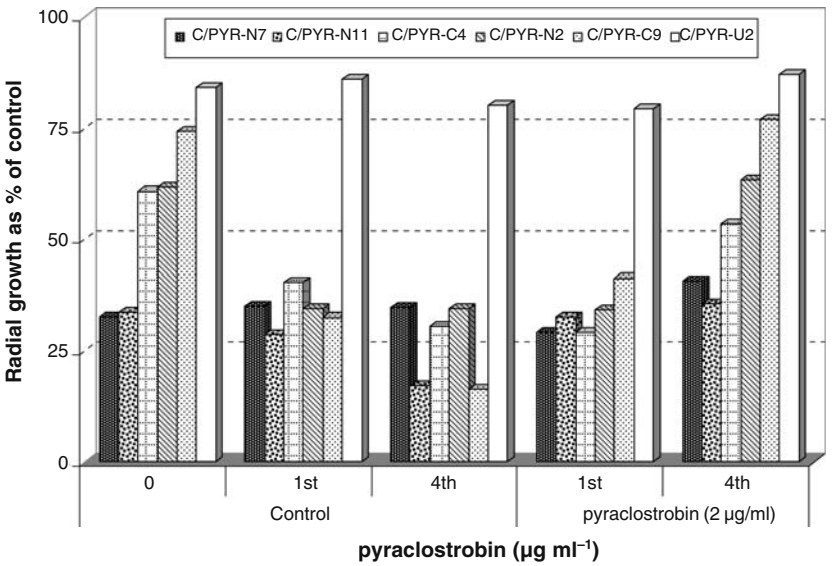

Figure 3. Growth of pyraclostrobin-resistant isolates of *C. beticola* at 2 µg ml^{-1} pyraclostrobin, after sub-culturing on ACM medium in presence or absence of pyraclostrobin. Measurements were made after 10 days incubation at 27 °C.

strains lost their ability to cause infection on sugar beet seedlings. However, all mutant strains were less aggressive than the wild-type parental strain (Table 3).

cDNA and deduced amino acid sequence of cyt b gene of C. beticola

The resistance in the presence of SHAM in most isolates indicated an altered target site resistance mechanism. In order to verify this hypothesis, a region of cyt *b* gene from the wild type strain (222S) was sequenced. A 963 bp fragment of *C. beticola* cyt *b* cDNA was obtained by using degenerate primers for fungal cyt *b*, as well as utilizing 3′RACE techniques. Comparison of the predicted cyt *b* amino acid sequence with amino acid sequences from other fungi is shown in Figure 4. The cyt *b* amino acid sequence showed highest similarity (93.1%) with the corresponding region of the cyt *b* gene from *M. graminicola*. All major amino acid components, such as the conserved PEWY and the NKL motives of the cyt *b* gene and the predicted transmembrane helices are present in the *C. beticola* cyt *b* gene (Figure 4). The PEWY loop, which is shown in bold at the

Table 3. Ecological fitness of *C. beticola isolates* resistant to pyraclostrobin compared to their parental wild-type strain

Strains	Resistance factor[a] (based on EC50[b])	Radial growth[c]	Sporulation[d]	Pathogenicity[e]
222S (wt)	1	34.50 ab[f]	5.06 a	100 a
C/PYR-U2	>200	25.13 d	4.37 ab	46.15 c
C/PYR-C9	80	35.37 a	5.18 a	69.65 bc
C/PYR-C4	70	33.06 ab	3.62 bc	64.10 bc
C/PYR-N2	50	31.87 bc	2.93 c	76.92 b
C/PYR-N7	5	31.34 bc	5.52 a	82.05 b
C/PYR-N11	3	33.50 ab	3.02 bc	79.43 b

[a]The ratio of EC_{50} of mutant : EC_{50} of the wild-type.
[b]Effective concentration causing 50% reduction in growth rate.
[c]Mean colony diam. (mm) measurements after 10 days incubation ($n = 4$).
[d]Mean number ($\times 10^4$) of conidia cm^{-2} of colony after 10 days incubation ($n = 4$).
[e]Pathogenicity as % of wild type. The sum of indices of six sugar beet plants for the wild-type isolate was 39.
[f]Within columns, values followed by the same letter do not differ significantly according to Duncan's multiple range test ($P = 0.05$).

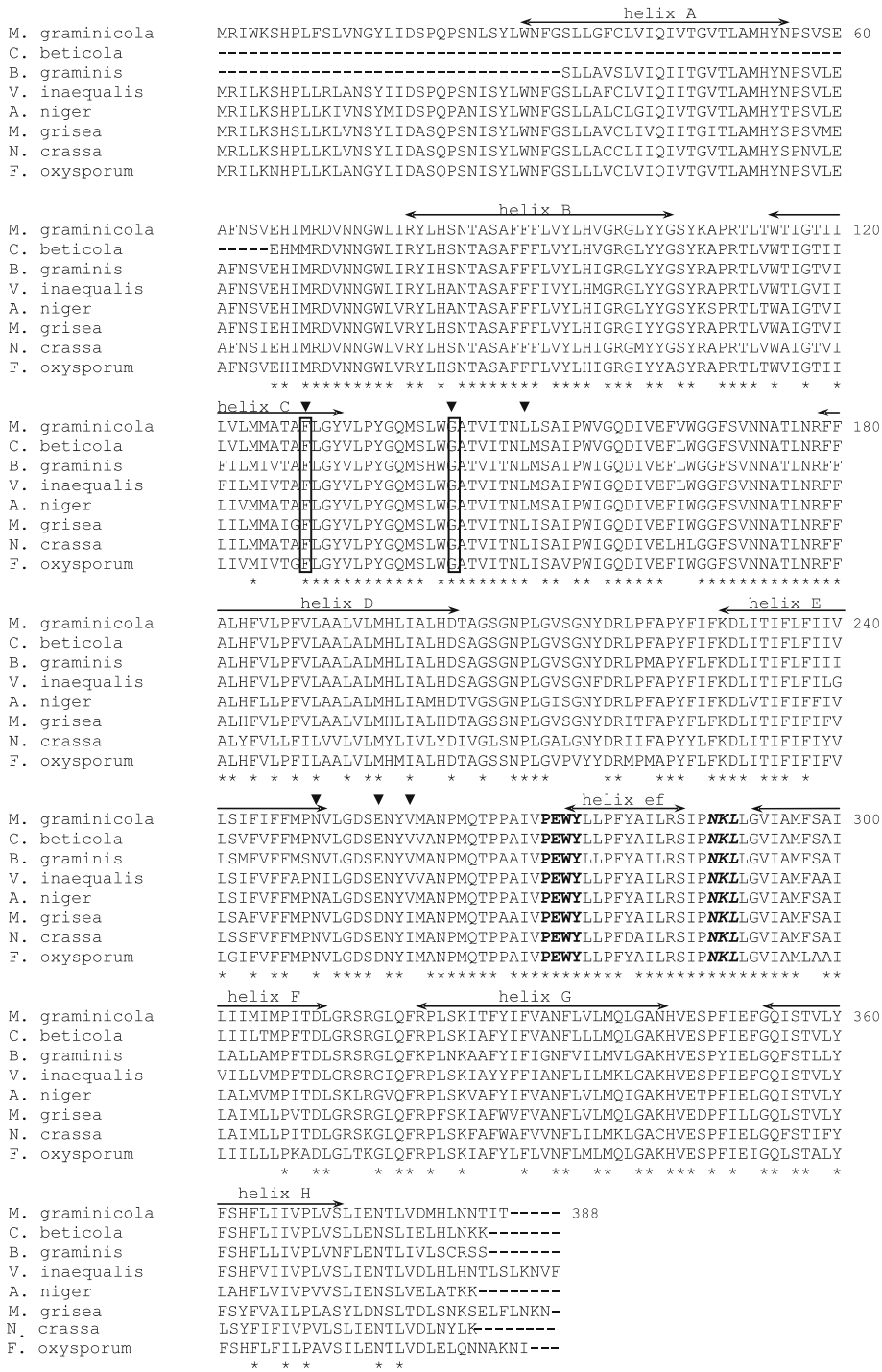

Figure 4. Amino acid alignment of putative fungal cyt *b* proteins. Alignment was performed using Clustal W. Gaps introduced to maximize sequence identity are shown by a horizontal dash. Identical amino acids are indicated by asterisks. Numbering of amino acid residues follows translation of *M. graminicola* cyt *b* gene (AY247413). The positions of previously reported amino acid substitution polymorphism (F129L, G143A) which affect fungicide sensitivity are boxed. A solid triangle indicates the positions where the novel mutations were found in this study (F129V, G143S, I154K, N250D, E256G, V259D). The predicted transmembrane helices A, B, C, D, E, ef, F, G and H are shown by arrows. The PEWY and NKL motives are shown in bold. Accession numbers: *B. graminis* AAK26621; *V. inaequalis* AAC03553; *A. niger* Q33798; *M. grisea* AA091628; *N. crassa* AAA31961; *F. oxysporum* AAW67488.

beginning of the ef helix is involved in inhibitor binding at the Qo site.

Identification of resistant mutations

Based on the sequence obtained from the wild-type strain, gene specific primers were designed and a ~800 bp fragment of cyt *b* DNA was obtained from the pyraclostrobin-resistant strains. The deduced cyt *b* amino acid sequences from mutant strains were then compared to that of the pyraclostrobin-sensitive strain.

Two amino acid replacements were identified at codons 129 and 143 (Table 4) in two resistant strains. In the first case, a TTC-to-GTC resistance mutation resulted in the replacement of a phenylalanine residue with a valine at codon 129 in the C/PYR-C4 (MR) strain. The second mutation, a glycine (GGT)-to-serine (AGT) substitution at codon 143 was identified in the C/PYR-U2 (HR) strain. Both mutations were located at positions previously implicated in QoI-resistance in other species.

Additional mutations were identified; an isoleucine (ATA)-to-lysine (AAA) substitution at codon 154 in C/PYR-C4 and C/PYR-N7 (LR) isolates, a glutamic acid (GAG)-to-glycine (GGG) substitution at codon 256 and a valine (GTT)-to-asparagine (GAT) substitution at codon 261 in the C/PYR-C4 isolate and an asparagine (AAC)-to-aspartic acid (GAC) substitution at codon 250 in the C/PYR-U2 QoI-resistant isolate.

Discussion

Mutants of *C. beticola* with high, moderate and low resistance to pyraclostrobin, an inhibitor of mitochondrial electron transport at the Qo-site of cytochrome bc_1 complex, were isolated after ultraviolet mutagenesis and selection on media containing pyraclostrobin. The existence of three different resistant phenotypes in *C. beticola* indicated three different genotypes, each of which may have different amino acid changes in the target site of QoI or coding different biochemical resistance mechanisms.

Study of the sensitivity of mutant strains to other fungicides showed that the mutation(s) for resistance to pyraclostrobin also reduced the sensitivity of mutant isolates to the QoI azoxystrobin and fenamidone, but not to the Qi inhibitor (QiI) cyazofamid, the carboxamide boscalid, the triazoles epoxiconazole and flutriafol and to the benzimidazole benomyl, which affect other cellular pathways or other target sites of the respiratory chain. The absence of cross-resistance between Qo and Qi inhibitors in pyraclostrobin-resistant mutants of *C. beticola* would suggest changes only in the configuration at the Qo-site of cytochrome *b*, which do not transmit a structural change to the Qi-site. A positive cross-resistance, with high resistance factors, among QoI 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; Markoglou et al., 2006).

In addition, the absence of any effect of SHAM on the fungitoxicity of pyraclostrobin in the mutants C/PYR-U2, C/PYR-N7 and C/PYR-C9 indicated that alternative respiration is not the biochemical mechanism of resistance to pyraclostrobin in the above mutant strains. However, the alternative oxidase may be, at least partially, the biochemical mechanism of resistance in the case of

Table 4. Mutations in the cyt *b* gene in pyraclostrobin-resistant strains of *C. beticola*

Isolate	Resistant phenotype[a]	Amino acid substitution					
		129[b]	143	154	250	256	261 (aa)
222S	wt	Phe (TTC)[c]	Gly (GGT)	Ile (ATA)	Asn (AAC)	Glu (GAG)	Val (GTT)
C/PYR-U2	HR	Phe (TTC)	Ser (**A**GT)	Ile (ATA)	Asp (**G**AC)	Glu (GAG)	Val (GTT)
C/PYR-C4	MR	Val (**G**TC)	Gly (GGT)	Lys (A**A**A)	Asn (AAC)	Gly (G**G**G)	Asp (G**A**T)
C/PYR-N7	LR	Phe (TTC)	Gly (GGT)	Lys (A**A**A)	Asn (AAC)	Glu (GAG)	Val (GTT)

[a]HR: Highly resistant strain, MR: Moderately resistant strain, LR: Low resistant strain.
[b]Numbering of amino acid residues follows the translation of *M. graminicola* cyt *b* gene (AY247413).
[c]The coding nucleotide sequence is given in parenthesis.

mutants C/PYR-N2 and C/PYR-N11 as indicated by the increase of fungitoxicity of pyraclostrobin in the presence of SHAM. Furthermore, an interesting fungitoxicity profile was observed in the case of the mutants C/PYR-C4, C/PYR-N2 and C/PYR-N11 which were as sensitive to cyazofamid as the wild-type isolate, but were resistant to antimycin A. The above strains, apparently, escape antimycin A inhibition by utilizing the alternate respiration pathway. The only explanation for the absence of resistance to cyazofamid of the above mutant isolates is that the binding of this fungicide at the ubiquinone reduction (Qi) centre of complex III also interferes with the branching point of the alternative and cytochrome pathways. However, such an hypothesis can be supported only by a differential binding of cyazofamid and antimycin A at the Qi centre of cyt *b*.

The function of the alternative oxidase as a resistance mechanism against QoI has been described in several phytopathogenic fungi (Ziogas et al., 1997; Yukioka et al., 1997; Joseph-Horne et al., 1999; Tamura et al., 1999; Olaya and Köller, 1999a) but does not appear to play a significant role in field resistance development due to either insufficient ATP formation needed for host infection (Ziogas et al., 1997) or plant flavones, which are released during infection and interfere with the induction of this pathway (Olaya and Köller, 1999b).

Certainly, it is well-accepted that the most important biochemical mechanism of resistance in fungal pathogens to fungicides in practice, results from a modification at the target-site of fungicidal action in the target pathogen (Brent and Hollomon, 1998). In the case of QoI fungicides, biochemical and molecular studies with laboratory and field isolates have shown that resistance to these fungicides could result from a target site modification through point mutations at the Qo site of cytochrome *b* (Di Rago and Colson, 1989; Zheng and Köller, 1997; Zheng et al., 2000).

To determine the molecular mechanism of resistance to QoI in the resistant isolates, a partial fragment of the mitochondrial cyt *b* gene was isolated from a wild-type strain of *C. beticola*. All major amino acid components, such as the conserved PEWY and NKL motives of the cyt *b* and the predicted transmembrane helices were found in the *C. beticola* cyt *b*. The nucleotide sequence was compared between pyraclostrobin-resistant and the wild-type parental isolate and two novel mutations at residues previously implicated in QoI resistance were identified. The G143S substitution was found in the highly resistant mutant C/PYR-U2. This mutation has previously been associated with high QoI resistance in *Magnaporthe grisea* (Avila-Adame and Köller, 2003), while an alternative substitution at this residue (G143A) is known to confer QoI resistance in numerous phytopathogenic fungi (Heaney et al., 2000; Sierotzki et al., 2000a, b; Zheng et al., 2000; Chin et al., 2001a, b; Ishii et al., 2001; Ma et al., 2003). Although an additional mutation the N250D (substitution between related small amino acids) was present in the C/PYR-U2, we speculate that the mutation G143S is the main cause of QoI resistance in this isolate, although functional expression will clarify the exact role of this mutation. The substitution F129V was found in the C/PYR-C4 resistant strain. This mutation has not been previously reported; however the substitution of the aromatic F129 by a closely related aliphatic amino acid (leucine) has been shown to confer QoI resistance in model systems (Fisher and Meunier, 2005) and phytopathogenic fungi (Farman, 2001; Bartlett et al., 2002; Kim et al., 2003; Sierotzki et al., 2004; Pashe et al., 2005). Our study indicates that the F129V may have a similar role in cyt b-mediated resistance in *C. beticola*.

In the present work, study of fitness-determining characteristics in the wild-type and the pyraclostrobin-resistant mutants of *C. beticola* showed that the mutation(s) leading to pyraclostrobin resistance appeared to be pleiotropic, having adverse effects on fitness-determining characteristics in most mutant strains. Prior to the detection of resistant *B. graminis* f.sp. *tritici* isolates in German field populations (Heaney et al., 2000), the inherent resistance risk for QoI was estimated to be medium and any practical resistance was expected to arise in a step-wise manner (Brent and Hollomon, 1998). Cytochrome *b*, the target enzyme of the QoI, is encoded by the mitochondrial DNA (Di Rago and Colson, 1989; Zheng and Köller, 1997) and the resistance to QoI through a target-site modification was found to be inherited in a non-Mendelian way (Ziogas et al., 2002).

The practical failures of strobilurin fungicides in the control of certain powdery and downy mildews due to a mutation at position 143 (G143A) in the target-encoding gene, indicate that the resistance

mutation at the same position found in this study (G143S), if present in field strains with comparable fitness characteristics to the wild-type, would cause a rapid development of high QoI-resistance in *C. beticola*.

Certainly, the commercial use of QoI for Cercospora leaf spot control requires careful implementation of appropriate anti-resistance strategies to preserve their effectiveness followed by monitoring studies to detect any changes in the sensitivity of the pathogen population.

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