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Selection, characterization and genetic analysis of laboratory mutants of *Botryotinia fuckeliana* (*Botrytis cinerea*) resistant to the fungicide boscalid

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Abstract Resistance to the fungicide boscalid in laboratory mutants of *Botryotinia fuckeliana* (*Botrytis cinerea*) was investigated. The baseline sensitivity to boscalid was evaluated in terms of colony growth (EC_{50}=0.3–3 μg ml^{-1}; MIC=10–30 μg ml^{-1}) and conidial germination (EC_{50}=0.03–0.1 μg ml^{-1}; MIC=1–3 μg ml^{-1}) tests. Mutants were selected *in vitro* from wild-type strains of the fungus on a fungicide-amended medium containing acetate as a carbon source. Mutants showed two different levels of resistance to boscalid, distinguishable through the conidial germination tests: low (EC_{50}~0.3 μg ml^{-1}, ranging from 0.03 to 1 μg ml^{-1}; MIC>100 μg ml^{-1}) and high (EC_{50}> 100 μg ml^{-1}) resistance. Analysis of meiotic progeny from crosses between resistant mutants and sensitive reference strains showed that resistant phenotypes were due to mutations in single major gene(s) inherited in a Mendelian fashion, and linked with both the *Daf1* and *Mbc1* genes, responsible for resistance to dicarboximide and benzimidazole fungicides, respectively. Gene sequence analysis of the four sub-units of the boscalid-target protein, the succinate dehydrogenase enzyme, revealed that single or double point mutations in the highly conserved regions of the iron-sulphur protein (Ip) gene were associated with resistance. Mutations resulted in proline to leucine or phenylalanine replacements at position 225 (P225L or P225F) in high resistant mutants, and in a histidine to tyrosine replacement at position 272 (H272Y) in low resistant mutants. Sequences of the flavoprotein and the two transmembrane sub-units of succinate dehydrogenase were never affected.

Keywords *Botrytis cinerea* · Carboxamides · Fungicide resistance · Succinate-ubiquinone oxidoreductase

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Introduction

Botryotinia fuckeliana de Bary (Whetz.) (teleomorph of *Botrytis cinerea* Pers.) is an ubiquitous fungus inducing grey mould on more than 250 crops. Its broad genetic variability and its extreme adaptability often lead to the acquirement of resistance to fungicides and losses of effectiveness in the control of grey mould (Fungicide Resistance Action Committee, http://www.frac.info).

Fungicides still play an important role in Integrated Pest Management (IPM) against grey mould, especially on grapevine, horticultural and greenhouse crops. According to their mode of action they can be classified as affecting mitosis and cell division (i.e. benzimida-

zoles, N-phenylcarbamates), signal transduction (i.e. dicarboximides, phenylpyrroles), methionine biosynthesis (i.e. anilinopyrimidines), respiration [i.e. succinate dehydrogenase inhibitors (SDHI) or carboxamides, QoI fungicides)], sterol biosynthesis (i.e. hydroxyanilides), or having a multi-site activity (i.e. sulphamides) (http://www.frac.info). However, in many countries, the use of most of them has been revoked or is restricted in anti-resistance strategies.

The comprehension of the sexual behavior and mating system of the fungus (Faretra et al. 1988a) and the availability of classical and molecular methods for genetic analysis has allowed a clarification of genetic bases of resistance to several fungicides. One or few major genes are usually involved in fungicide resistance and different alleles often cause different resistance levels as well as different pleiotropic effects affecting fitness or causing cross-resistance to unrelated fungicides. For example, at least four alleles of the gene *Mbc1*, coding for β-tubulin, cause sensitivity/resistance to benzimidazoles variously accompanied by hypersensitivity to *N*-phenylcarbamates (Faretra and Pollastro 1991, 1993; Pollastro and Faretra 1992; Yarden and Katan 1993). Resistance to dicarboximides and phenylpyrroles is due to mutations in the polymorphic gene *Daf1*, coding for an histidine kinase, that is also involved in methanol tolerance and osmosensing (Leroux and Fritz 1984; Faretra and Pollastro 1991; Pollastro et al. 1996a; Cui et al. 2002; Oshima et al. 2002), and more rarely by the gene *Daf2* unlinked with the *Daf1* gene (Faretra and Pollastro 1993). High resistance to dicarboximides, associated or not with resistance to phenylpyrroles, negatively affects fitness and is generally undetectable in the field (Pollastro et al. 1996a). Two major genes (*Dic1* and *Dic2*) are responsible for high or low resistance to dichlofluanid, respectively (Pollastro et al. 1996b). Resistance to anilinopyrimidines is caused by at least three major genes, expressing the Ani^{R1}, Ani^{R2} and Ani^{R3} phenotypes (Chapeland et al. 1999). Ani^{R1} is involved into the building up of resistance to anilinopyrimidines, while Ani^{R2} and Ani^{R3}, often designed MDR1 and MDR2, are involved in multidrug resistance to anilinopyrimidines, dicarboximides, phenylpyrroles and several sterol biosynthesis inhibitors (Chapeland et al. 1999; Leroux et al. 2002). Different levels of resistance were detected among anilinopyrimidine-resistant field isolates; low resistance is lethal in homokaryon, while high resistance does not seem to affect the fitness of mutant strains (De Miccolis Angelini et al. 2002). Fenhexamid resistant mutants are not easily obtained under laboratory conditions, although low and high resistance are caused by single major gene(s) (De Guido et al. 2007). Field isolates with reduced sensitivity to fenhexamid were detected in France, Germany and Switzerland before its introduction in agricultural practice, but no or little effects on the effectiveness of the fungicide were observed (Leroux et al. 2002; Suty et al. 1999; Baroffio et al. 2003). It has been recently demonstrated that a few single mutations at the amino acid position 412 of the 3-ketoreductase enzyme (coded by *ERG27*) in highly resistant strains (namely HydR3 phenotype) of the fungus is responsible for fenhexamid resistance (Fillinger et al. 2008).

The pyridine carboxamide boscalid [2-chloro-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide] is a new broad-spectrum fungicide (FRAC group 7), recently introduced for the control of several fungi belonging to *Ascomycetes* and *Basidiomycetes*. In Italy, commercial formulations containing boscalid are allowed for use against *B. fuckeliana*, *Sclerotinia* spp., *Alternaria* spp., *Monilinia* spp., powdery mildews and other pathogens, on fruits, vegetables and grapevine.

Its mode of action is the inhibition of electron transport in mitochondrial respiration by binding to the complex II, also referred to as succinate dehydrogenase (SDH) complex or succinate:quinone reductase (SQR). Like the other enzymatic complexes of the respiratory chain (I, III and IV), the enzyme is a component of the inner mitochondrial membrane. However, it does not function as a proton pump and consists of four nucleus-encoded sub-units: the flavoprotein (SDHA) and the iron-sulphur protein (SDHB) sub-units are located in the peripheral part, and act as the dehydrogenase catalytic portion oxidating succinate to fumarate in the tricarboxylic acid cycle; whereas two membrane-anchored protein sub-units (QPs), known as cytochrome b (SDHC) and CybS protein (SDHD), anchoring SDHA and SDHB to the membrane, are responsible for the quinone reductase activity (Hägerhäll 1997).

Boscalid prevents energy production and makes unavailable the chemical building blocks for the synthesis of essential cell components and, hence, disrupts fungal growth with deleterious effect on spore germination, germ tube elongation, mycelial growth, and sporulation. In this respect, it resembles QoI

fungicides, but there is no cross-resistance between the two groups of fungicides due to their different sites of action.

Carboxamides or SDHI are rated by FRAC as fungicides at medium to high risk of resistance (http://www.frac.info). Field resistance to carboxin and other carboxamide fungicides, available since 1966, and primarily active against *Basidiomycetes*, have been reported in *Ustilago nuda* (C.N. Jensen) Rostr. (Leroux and Berthier 1988), *Puccinia horiana* Henn. (Abiko et al. 1977), and *Uromyces caryophyllinus* (Schrank) J. Schröt. (Gullino et al. 1986). In most cases, SDHI resistance has been associated with point mutations in the SDH genes that result in altered amino acid sequences at the fungicide binding site. Induced resistance to carboxamides in an isolate of *Ustilago maydis* (DC.) Corda was attributed to substitution of histidine with leucine in the iron-sulphur protein sub-unit (Broomfield and Hargreaves 1992). Skinner et al. (1998) showed that the expression of a mutant Ip (SDHB) allele, causing a substitution of histidine with either leucine or tyrosine, in *Mycosphaerella graminicola* (Fuckel) J. Schröt. is responsible of resistance to carboxin. A mutation in the cytochrome b sub-unit (SDHC), causing the replacement of an asparagine by a lysine, was found to be responsible of resistance to flutolanil and carboxin in *Coprinus cinereus* (Schaeff.) Gray (Ito et al. 2004). More recently, Shima et al. (2009) demonstrated that resistance to carboxin in laboratory mutants of *Aspergillus oryzae* (Ahlb.) E. Cohn can be conferred independently by single point mutations in each of the three genes coding for the B, C and D sub-units of SDH complex.

Reduced sensitivity to boscalid in phytopathogenic fungi was first reported in *Alternaria alternata* (Fr.) Keissl, on pistachio in California (Avenot and Michailides 2007) and its molecular basis has been clarified (Avenot et al. 2008). In *B. fuckeliana*, the baseline sensitivity to boscalid was determined in laboratory tests (Stammler and Speakman 2006). Boscalid-resistant mutants were obtained by UV-treatment (De Miccolis Angelini et al. 2006, 2007; Stammler et al. 2008; Zhang et al. 2007), whereas field resistance has been restricted to few European countries (De Miccolis Angelini et al. unpublished; Stammler 2008; Stammler et al. 2008). Point mutations in the iron-sulphur protein sub-unit (SDHB) were responsible of the resistance for the fungicide (Stammler et al. 2008).

The present paper deals with selection of boscalid-resistant laboratory-mutants of *B. fuckeliana* and their phenotypic and genetic characterization, with the aim to contributing to evaluate the risk of acquired field resistance to the fungicide and to define appropriate preventive measures. Preliminary results of the research work were previously reported by De Miccolis Angelini et al. (2006, 2007).

Materials and methods

Media

The following media were used (ingredients per litre of distilled water; media were added with 20 g l^{-1} agar Oxoid n. 3): water agar (WA; 25 g agar Oxoid n. 3), malt extract agar (MEA; 20 g Oxoid malt extract), potato dextrose agar (PDA; infusion from 200 g peeled and sliced potatoes kept at 60°C for 1 h, 20 g dextrose, adjusted at pH 6.5), acetate water agar (AcWA: 5 g sodium acetate, 0.01 M citrate, adjusted at pH 6.0 with KOH), acetate minimal medium [AcMM: 10 ml solution A (10 g KH_2PO_4, 100 ml^{-1} water), 10 ml solution B (20 g $NaNO_3$, 5 g KCl, 5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4$, 100 ml^{-1} water), 1 ml of micronutritive solution (Sanderson and Srb 1965), 5 g sodium acetate, 0.01 M citric acid, adjusted at pH 6.0 with KOH].

Fungicides

Commercial formulation of boscalid [Cantus WG, water-dispersible granules containing 50% active ingredient (a.i.), BASF AG, Limburgerhof, Germany) was suspended in sterile distilled water. Technical grade benomyl (Du Pont de Nemours and Co., Wilmington, DE, USA) and vinclozolin (BASF AG), representative of benzimidazoles and dicarboximides, respectively, were dissolved in dimethylsulfoxide. The fungicides were added to sterilized media cooled down to 45–50°C after autoclaving. The final concentration of the solvent was the same in all media, control included, and never exceeded 1% (v:v).

Fungal strains and culture conditions

Genetic nomenclature used here is according to genetic symbols and terminology described by

Faretra and Grindle (1992). Six reference isolates of *B. fuckeliana*, derived from single ascospores and never exposed to boscalid, were used in this investigation. The reference strains SAS56 (*MAT1-1 Mbc1S Daf1S*) and SAS405 (*MAT1-2 Mbc1HR Daf1LR*) were used as wild types to obtain boscalid-resistant mutants. Four near-isogenic strains with SAS56 [SAR10993 (*MAT1-2 Mbc1HR Daf1LR*), SAR10995 (*MAT1-1 Mbc1HR Daf1LR*)] or with SAS405 [SAR11004 (*MAT1-2 Mbc1S Daf1S*) and SAR11008 (*MAT1-1 Mbc1S Daf1S*)] (Pollastro et al. 1996a) were also used in determining baseline sensitivity and as parents in sexual crosses. The same wild-type sensitive reference strains, fourteen boscalid-resistant laboratory mutants, and twenty-five of their resistant monoascosporic progenies were used in molecular analysis.

All isolates were routinely grown on MEA at 21±1°C in the darkness. For long-term storage, suspensions of conidia and mycelium of each isolate were maintained at −80°C in 10% glycerol and revitalized on MEA just before use.

Baseline sensitivity

Sensitivity to the SDHI fungicide carboxin is known to be enhanced on media containing non-fermentable carbon sources for *U. maydis*, *Neurospora crassa* Shear & B.O. Dodge, *Saccharomyces pasteurianus* Reess *ex* E.C. Hansen, and *Aspergillus nidulans* (Eidam) G. Winter (Ragsdale and Sisler 1970). The same was observed in *B. fuckeliana* with regard to boscalid (Leroux 2004). Therefore, media containing acetate rather than glucose were used to evaluate the response to boscalid of *B. fuckeliana*.

Colony growth test

Three replicated Petri dishes (100 mm diam) containing AcMM, such as or amended with 9 increasing concentrations of the fungicide (0.01 to 100 μg ml^{-1}), were inoculated with mycelium plugs (4 mm) from the borders of actively growing colonies on MEA in order to determine EC_{50} (concentration causing 50% reduction of colony growth) and MIC (minimal inhibitory concentration) values. The orthogonal diameters of developing colonies were measured after 2, 4 and 6 days of incubation at 21±1°C in darkness.

Conidial germination test

Aliquots (10 μl) of conidial suspension ($5{\cdot}10^5$ units ml^{-1}) were spotted on disks (6 mm diam) of AcWA, such as or amended with 9 concentrations of boscalid (0.01 to 100 μg ml^{-1}), placed on sterile microscope slides in order to determine EC_{50} and MIC values. The disks were incubated in a moist chamber at 21± 1°C in darkness: After 16 h, conidia were fixed with lactophenol-cotton blue. Random samples of 100 conidia on each of three replicated spots per condition were observed at ×125 magnification, and germinated conidia were counted. Germ tubes length was measured in three replicated samples of 15 conidia per each condition by using a micrometer ocular.

Selection of boscalid resistant mutants and phenotypic characterization

Colonies of SAS405 and SAS56 were grown on PDA with 12 h per day exposure to a combination of 2 daylight (Osram, L36W/20) and 2 near-UV (Osram, L36/73) lamps to promote sporulation. Conidia were collected by scraping the surface of 7-day-old colonies with a sterile loop, suspended in sterile distilled water containing 0.05% Tween 20, and filtered through Miracloth (Calbiochem, La Jolla, Canada) to remove mycelial fragments.

For mutagenic treatment, aliquots (10 ml) of conidial suspensions were plated in Petri dishes (100 mm) and exposed to UV irradiation (150 mJ) in a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA, USA), conditions routinely killing about 99% of conidia. Irradiated or non-irradiated conidia were incorporated in AcMM and AcWA amended with 1 or 3 μg ml^{-1} of boscalid (a.i.), concentrations inhibiting the growth of wild-type strains. The final concentration of conidia was 1–5× 10^8 l^{-1} of medium. Petri dishes were incubated at 21 ±1°C in the dark, and colonies developing within 7 days were counted, individually transferred on fresh AcMM added with 3 μg ml^{-1} of boscalid (a.i.) for further analysis. Experiments were repeated twice.

Twenty three laboratory mutants were tested for colony growth on AcMM and conidia germination on AcWA such as or amended with boscalid, as described above. The orthogonal diameters of the fungal colony were measured every 2 days during 1 week of incubation at 21±1°C in darkness. EC_{50} was deter-

mined by using dose-response curves. The ratio of the EC_{50} of resistant strains/EC_{50} of sensitive strains yielded an estimation of the resistance factor (Rf).

Derivation of meiotic progenies and genetic analysis

The genetic basis of resistance to boscalid was investigated on the ground of the response to the fungicide of randomly collected monoascosporic progeny. Forty-four mutants were mated as either sclerotial or spermatizing partner with the sensitive strains SAS56, SAS405, and four near-isogenic strains to obtain apothecia, as described by Faretra et al. (1988b). Ascospores were spread at low density on WA, collected singly with the aid of dissecting microscope. Boscalid-resistant phenotypes were distinguished from sensitive ones on the basis of their colony growth on AcMM amended with 3 μg ml^{-1} (a.i.) of boscalid after 2–3 days of incubation at 21±1°C. In addition, response to dicarboximide and benzimidazole fungicides was determined on MEA amended with vinclozolin (5 μg ml^{-1}) or benomyl (10 μg ml^{-1}). Data obtained from single apothecia were statistically analyzed for segregation of phenotypic characters by means of the χ^2 test, as corrected by Yates.

Molecular analysis

All molecular techniques were essentially as described by Sambrook et al. (1989).

DNA extraction

Genomic DNA was extracted and purified from mycelium of *B. fuckeliana* strains grown on cellophane disks overlaid on MEA, basically according to Murray and Thompson (1980). In brief, following a two-day fungal growth, mycelium was collected by scraping the cellophane, powdered under liquid nitrogen and added with 600 μl of CTAB buffer [100 mM Tris-Cl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 2% cetyldimethylethylammonium bromide (w/v); 0.2% β-mercaptoethanol (v/v)]. Samples were frozen and thawed three times using liquid nitrogen and a water bath at 75°C, and then incubated at 75°C for 1 h. After chloroform extraction, the clear supernatant was transferred to a new tube and precipitated with isopropanol. After 30 min at −80°C the tube was centrifuged at 14,000 rpm for 15 min, then the pellet was washed with cold 70% ethanol, air-dried, dissolved in TE (10 mM Tris-Cl; 1 mM EDTA, pH 8), treated with 0.1 μg μl^{-1} DNAase-free pancreatic RNAase (Sigma, Milan, Italy) for 2 h at 37°C, and finally precipitated by the addition of 0.6 vol of 5 M ammonium acetate and 2 vol of cold absolute ethanol. The final DNA pellet, washed with 70% ethanol and air-dried, was dissolved in water and stored at −80°C until use.

Polymerase chain reaction

B. fuckeliana nucleotide and protein sequence predictions of the succinate dehydrogenase (SDH) genes were obtained from the *Botrytis cinerea* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu). PCR amplification of the SDH gene fragments of the four sub-units was carried out using pairs of 20-mer oligonucleotides and the genomic DNA of *B. fuckeliana* as a template. All primers were designed using Primer3 software (Rozen and Skaletsky 2000) and synthesized by Invitrogen (Carlsbad, CA, USA). The primer pairs selected and the size of DNA fragments amplified are reported in Table 1. Reaction mixtures (50 μl) contained 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.1 μM each primers, 0.1–0.3 μg of DNA template and 2 units 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, USA) set as follows: one initial stage at 95°C for 5 min followed by 25 cycles, each consisting of 1 min of denaturation at 95°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C, and then a final extension stage of 7 min at 72°C. Negative controls, in which water replaced the target DNA, were run each time to detect potential contamination.

DNA sequencing

Amplified DNA fragments were sequenced, assembled and aligned. In detail, PCR fragments, visualized on agarose gel, were standardized for concentration, quality and quantity and processed for direct sequencing by external services (PRIMM, "smart-read" DNA Sequencing Service, Naples Facility, Italy). Sequences were analysed using Bioedit software (version 7.0.5.3)

Table 1 PCR primer pairs

Succinate dehydrogenase sub-unit	Gene	Primer pairs	Sequences Forward / Reverse	PCR product (kbp)
Flavoprotein (Fp)	*SdhA*	A62/A1358	5′-AAACGCCCTTGGACTAACCT-3′ 5′-ACCAGCGCCATAAATACCAG-3′	1.297
		A771/A2132	5′-CGTCAAGGGTTCAGATTGGT-3′ 5′-ATCCGAGTTCCAGATCATGC-3′	1.362
		A1907/A3170	5′-CGATGCTGGTGCTGAATCTA-3′ 5′-TAGTTGCCCCTCCGTACATC-3′	1.264
Iron-sulphur protein (Ip)	*SdhB*	B103/B1403	5′-AGAGGTCACATACGCACACG-3′ 5′-ACCAGTAGGAGGGGCAAGAT-3′	1.301
		B1189/2346	5′-CCCACTACCCCACACCTATG-3′ 5′-ACAAGCATCGGTTTTGGAAC-3′	1.158
Cytochrome b	*SdhC*	C69/C1042	5′-ATACCTTCCGCATCAACGAC-3′ 5′-CCCAGGCCAGTAGGACATTA-3′	0.974
CybS protein	*SdhD*	D388/D1363	5′-ACCTTGCACGTGACTTGTTG-3′ 5′-GCTTGAATAAAGGCCACGAG-3′	0.976

(Tom Hall, North Carolina State University) for manual editing and translation, FASTA (The European Bioinformatics Institute, Cambridge, UK; http://www.ebi.ac.uk/fasta33/) and BLAST (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/BLAST/) softwares for similarity search and multiple sequence alignments. The sequences were aligned with those in gene and protein databases and compared with those obtained from the wild-type reference strains SAS56 and SAS405 using ClustalW (version 1.83) (www://www.ebi.ac.uk/clustalw) and the SeqMan Pro software of the Lasergene package (version 8.0.2, DNASTAR, Inc., Madison, WI, USA).

Results

Baseline sensitivity

Inhibition of conidial germination on AcWA amended with different concentrations of boscalid is shown in Fig. 1a. The EC_{50} of the tested strains ranged from 0.03 to 0.1 μg ml^{-1} of boscalid; 1 μg ml^{-1} of the fungicide inhibited conidial germination in most tested strains; the only exception was the reference strain SAS405 for which MIC was 3 μg ml^{-1}.

On the fungicide-unamended control medium, germ tube length ranged from 72 to 108 μm after 16 h. A notable reduction in the germ tube elongation was observed for the tested wild-type strains on boscalid-amended media, and EC_{50} ranged from 0.01 μg ml^{-1} (SAS56) to about 0.3 μg ml^{-1} (SAR10993) (Fig. 1b).

Colony growth was totally inhibited by 10 or 30 μg ml^{-1} of boscalid. Generally, EC_{50} was about 1–3 μg ml^{-1}; the reference strain SAS56 was more sensitive and showed an EC_{50} value between 0.3 and 1 μg ml^{-1} (Fig. 1c).

Phenotypic characterization of boscalid-resistant mutants

Boscalid-resistant mutants were obtained almost exclusively from UV-irradiated conidia of SAS56 reference strain, with a mutation rate of 1.2×10^{-5} of survivor conidia. Spontaneous mutations were never selected, with the only exception of one mutant obtained from 4.5×10^{7} conidia of SAS405 strain on AcMM added with 3 μg ml^{-1} boscalid.

Twenty-three putative resistant mutants were selected and characterized for their response to boscalid. Only one phenotype was discriminated by the colony growth test (EC_{50}>100 μg ml^{-1}) (Fig. 2a); whereas two different phenotypes with different level of resistance, high (named BosHR, EC_{50}>100 μg ml^{-1}) and low resistance (BosLR, mean EC_{50} value ~ 0.3 μg ml^{-1}, ranging from 0.03 to 1 μg ml^{-1}; MIC>100 μg ml^{-1}) were distinguished by conidial germination test (Fig. 2b).

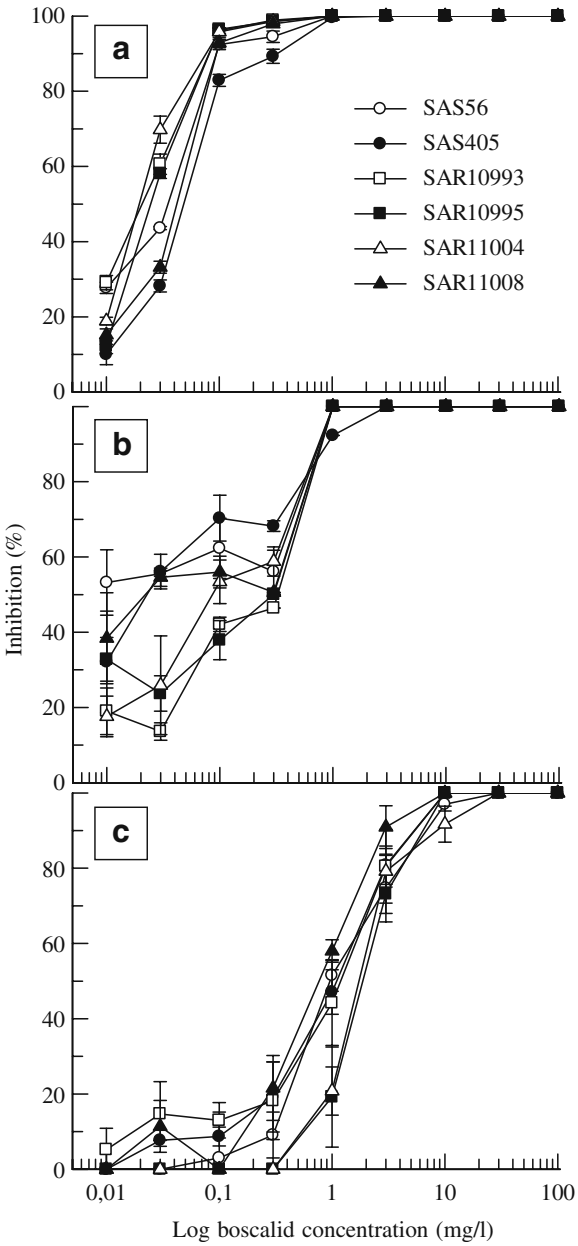


Fig. 1 Baseline sensitivity of 6 wild-type sensitive strains to boscalid: **a** percentage of inhibition of conidia germination on AcWA; **b** percentage of inhibition of germ tube elongation on AcWA; **c** percentage of inhibition of colony growth on AcMM. *Bars* represent standard error

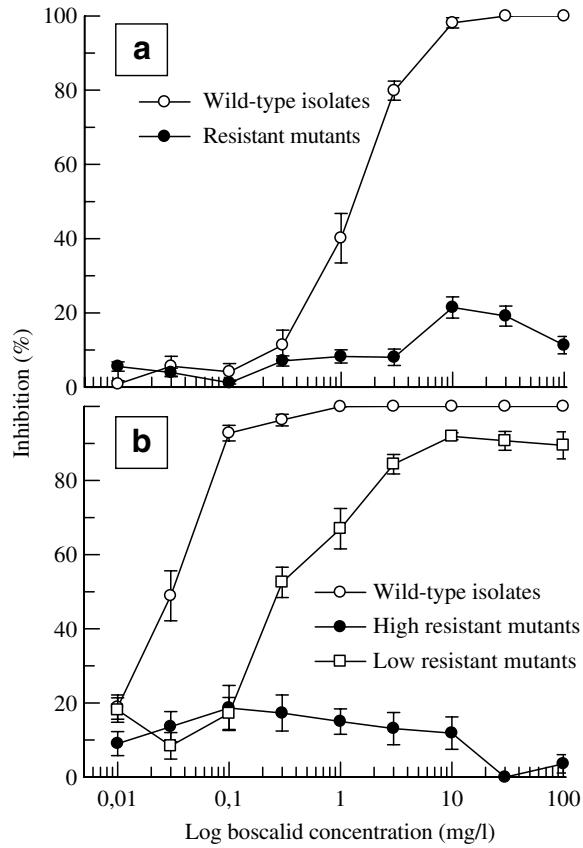


Fig. 2 Response of wild-type strains and resistant mutants of *B. fuckeliana*: **a** percentage of inhibition of colony growth on AcMM; figures are the average of 6 wild-type strains and 23 laboratory-resistant mutants; **b** percentage of inhibition of conidia germination on AcWA; figures are the average of 6 wild-type strains, 14 high resistant (BosHR) and 9 low resistant (BosLR) mutants. *Bars* represent standard error

Genetic analysis

Forty-four resistant mutants were mated with boscalid-sensitive reference strains. As expected, all were fertile in crosses with appropriate reference strains: 43 mutants derived from SAS56 behaved as MAT1-1, and only the mutant obtained from SAS405 behaved as MAT1-2 (data not shown). Ascospore progeny of 29 fertile crosses involving 27 mutants were analyzed (Table 2). Most apothecia yielded ascospore progenies in which sensitive (BosS) and resistant (BosR) phenotypes segregated in statistically significant 1:1 ratios. This suggests that resistance to boscalid was due to mutations in single major gene(s). Exceptions were the progenies obtained from the crosses between reference strains and the resistant mutants BAR338, BAR367, and BAR369, in which a lower proportion of resistant ascospores than sensitive ones were found. Fungicide-resistance traits of parental strains (BosR Mbc1S Daf1S × BosS Mbc1HR Daf1LR) were suitable for examining the re-assortment in ascospore progeny of response to boscalid, benzimidazoles and

Table 2 Genetic analysis of randomly collected ascospores

Cross	Apothecia number	Ascospore numbers			χ^2 corrected by Yates[a]
		Total	Resistant	Sensitive	
SAS405 × BAR334	3	127	66	61	0.13
BAR334 × SAS405	1	42	27	15	2.88
SAS405 × BAR336	1	43	17	26	1.49
SAS405 × BAR338	1	51	13	38	11.29
BAR340 × SAS405	1	55	22	33	1.82
SAS405 × BAR341	1	31	18	13	0.52
SAS405 × BAR342	1	44	22	22	0.02
SAS405 × BAR343	2	88	38	50	1.38
SAS405 × BAR344	2	103	55	48	0.35
SAS405 × BAR350	1	36	22	14	1.36
SAS405 × BAR352	2	92	42	50	0.53
SAS405 × BAR354	1	50	25	25	0.02
BAR355 × SAS405	1	50	28	22	0.50
SAS405 × BAR356	1	44	23	21	0.02
BAR358 × SAS405	1	45	29	16	3.20
SAS405 × BAR359	2	98	42	56	1.72
SAS405 × BAR360	2	72	40	32	0.68
SAS405 × BAR361	1	51	26	25	0.00
SAS405 × BAR364	1	49	25	24	0.00
BAR364 × SAS405	2	93	48	45	0.04
SAS405 × BAR365	1	27	8	19	3.70
SAS405 × BAR366	2	67	35	32	0.06
SAS405 × BAR367	3	145	86	59	4.66
SAS405 × BAR368	2	101	59	52	2.53
SAS405 × BAR369	1	45	13	32	7.20
SAS405 × BAR370	1	49	20	29	1.31
SAS405 × BAR372	2	106	47	59	1.14
SAS405 × BAR374	1	41	22	19	0.10
BAR390 × SAS56	1	25	10	15	0.64
Total	42	1,870	928	952	0.34

[a] $\chi 2$ values for 1 degree of freedom are 3.84 at P=0.05 and 6.63 at P=0.01 levels of probability

dicarboximides. In ascospore progenies, The Bos marker showed linkage to the *Daf1* gene, the ratio of parental to recombinant phenotypes (603:256) being significantly different from 1:1 (Table 3). A slighter but significant linkage was also observed between the Bos marker and the *Mbc1* gene (parental to recombinant ratio was 475:384) (Tables 4).

Molecular analysis

Nucleotide sequences of the genes coding for the four SDH sub-units were analyzed and specific primer pairs were designed on each of them: the Fp coding gene *SdhA*, the Ip gene *SdhB*, the cytochrome b gene *SdhC*, and the CybS gene *SdhD*. PCR conditions were optimized for obtaining the expected amplified products. DNA extracted by two wild-type strains and fourteen laboratory resistant mutants of *B. fuckeliana* was used as template in PCR amplifications. PCR amplicons were sequenced. Nucleotide sequences of each gene fragment from mutants were compared with those from boscalid-sensitive parental strains, SAS56 and SAS405, and sequences available in gene banks. A schematic representation of the four analysed SDH sub-unit genes is reported in Fig. 3.

Table 3 Recombination between traits of resistance to boscalid and vinclozolin in ascospore progenies of *B. fuckeliana*

Cross		N. of apothecia analysed	Ascospore progeny						χ^2 for independent segregation of markers[a]
Reference strain (BosS DafLR)	Resistant mutant (BosR DafS)		Total	Phenotypes (Bos-Daf)				Parental: recombinant	
				S-S	S-LR	R-S	R-LR		
SAS405	BAR334	2	72	11	18	24	19	42:30	1.68
SAS405	BAR338	1	51	16	22	12	1	34:17	11.29
SAS405	BAR340	1	55	19	14	10	12	24:31	0.65
SAS405	BAR343	2	88	6	44	25	13	69:19	27.28
SAS405	BAR350	1	36	4	10	16	6	26:10	6.25
SAS405	BAR354	1	50	4	21	14	11	35:15	7.22
SAS405	BAR355	1	50	9	13	18	10	31:19	2.42
SAS405	BAR356	1	44	10	11	18	5	29:15	3.84
SAS405	BAR358	1	45	5	11	22	7	33:12	8.89
SAS405	BAR360	1	32	5	10	14	3	24:8	7.03
SAS405	BAR361	1	51	4	21	17	9	38:13	11.29
SAS405	BAR364	1	49	7	17	20	5	37:12	11.76
SAS405	BAR365	1	27	4	15	6	2	21:6	7.26
SAS405	BAR366	2	67	3	30	25	9	55:12	26.33
SAS405	BAR368	2	101	11	31	43	16	74:27	20.95
SAS405	BAR374	1	41	1	18	13	9	31:10	9.76
Total		20	859	119	306	297	137	603:256	139.37

[a] χ^2 values for 1 degree of freedom are 3.84 at P=0.05 and 6.63 at P=0.01 levels of probability

In order to obtain the complete sequence of the flavoprotein sub-unit gene, three different primer pairs, yielding partially overlapping fragments of 1,297, 1,362, and 1,264 bp, were used (Table 1). The nucleotide sequence obtained by joining the three PCR fragments was 2,940-bp long and contained the 2,400-bp *SdhA* gene with six introns (Fig. 3a) coding for Fp (*Botrytis cinerea* Sequencing Project, accession number BC1G_07795.1). A 974-bp PCR fragment encompassing the whole cytochrome b sub-unit *SdhC* gene (BC1G_14785.1) was amplified using the primer pair C69/C1042 (Table 1). The 570-bp coding fragment contained two putative introns at nucleotide positions 41–128 and 217–270, respectively (Fig. 3b). DNA amplifications by using primer pair D388/D1363 (Table 1) yielded a PCR product of about 1.0 kb encompassing the 579-bp CybS trans-membrane sub-unit *SdhD* gene (BC1G_01333.1), as well as two introns both of 52 bp (Fig. 3c). Two partially overlapping DNA fragments containing the Fe-S protein sub-unit *SdhB* gene (accession BC1G_13286.1) were amplified using the primer pairs B103/B1403 and B1189/B2346 (Table 1). The 1,935 bp of the two overlapping PCR-amplified fragments included non-coding flanking regions of 503 bp (5′) and 473 bp (3′). The 959-bp coding sequence was interrupted by a unique 56-bp putative intron between codons 144 and 145 (nucleotide positions 431 and 486). Three Cys-rich clusters responsible for the ligation of the iron-sulphur centres (S1, S2 and S3), highly conserved throughout a broad range of organisms, were identified at amino-acid positions 121–142, 213–225 and 270–282, respectively (Fig. 3d).

Nucleotide sequences from boscalid-resistant mutants and wild-type strains were compared. The nucleotide sequences of the flavoprotein gene (*SdhA*) and the two membrane-anchoring sub-units genes (*SdhC* and *SdhD*) were identical in sensitive and resistant strains. Single or double point mutations in the *SdhB* gene coding the Fe-S protein sub-unit (Ip) of the succinate dehydrogenase were detected in resistant mutants. In detail, in all mutants with a high level of resistance (BosHR), whatever their origin (UV-induced or spontaneous), changes at codon 225 of the *SdhB* gene were observed.

Table 4 Recombination between traits of resistance to boscalid and benomyl in ascospore progenies of *B. fuckeliana*

Cross		N. of apothecia analysed	Ascospore progeny						χ^2 for independent segregation of markers[a]
Reference strain (BosS MbcHR)	Resistant mutant (BosR MbcS)		Total	Phenotypes (Bos-Mbc)				Parental: recombinant	
				S-S	S-HR	R-S	R-HR		
SAS405	BAR334	2	72	16	19	16	21	35:37	0.01
SAS405	BAR336	1	51	16	22	9	4	31:20	1.96
SAS405	BAR340	1	55	21	12	10	12	22:33	1.82
SAS405	BAR343	2	88	15	35	20	18	55:33	5.01
SAS405	BAR350	1	36	6	8	10	12	18:18	0.03
SAS405	BAR354	1	50	11	14	14	11	28:22	0.50
SAS405	BAR355	1	50	12	10	10	18	20:30	1.62
SAS405	BAR356	1	44	12	10	16	6	26:18	1.11
SAS405	BAR358	1	45	4	12	17	12	29:16	3.20
SAS405	BAR360	1	32	9	6	11	6	17:15	0.03
SAS405	BAR361	1	51	15	10	15	11	25:26	0.00
SAS405	BAR364	1	49	11	13	16	9	29:20	1.31
SAS405	BAR365	1	27	4	15	6	2	21:6	7.26
SAS405	BAR366	2	67	10	23	18	16	41:26	2.93
SAS405	BAR368	2	101	16	26	31	28	57:44	1.43
SAS405	BAR374	1	41	9	10	11	11	21:20	0.00
Total		20	859	187	245	230	197	475:384	9.43

[a] χ^2 values for 1 degree of freedom are 3.84 at $P=0.05$ and 6.63 at $P=0.01$ levels of probability

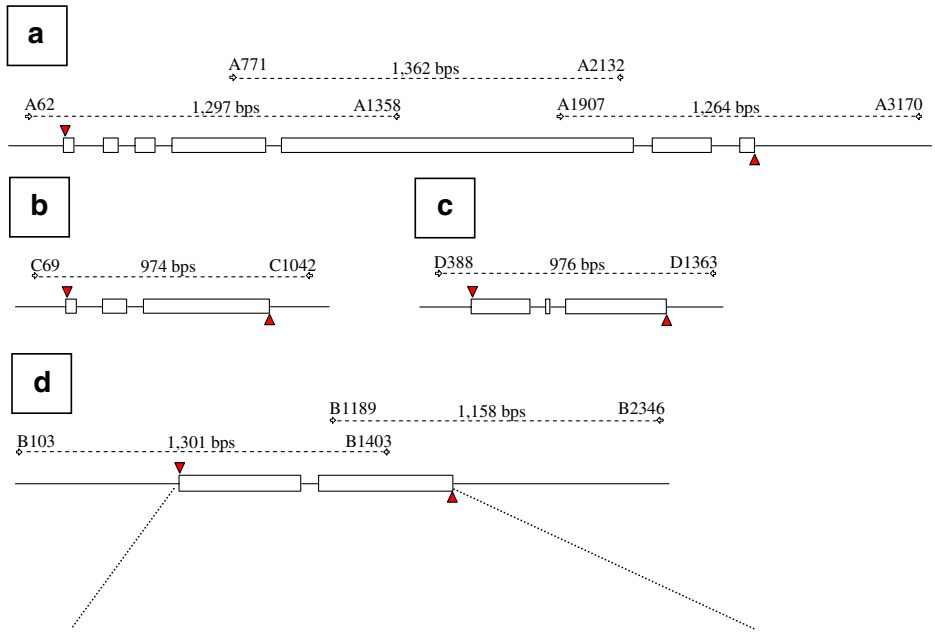
Most of them had a single nucleotide change, CTC instead of CCC, substituting a proline with a leucine in the second Cys-rich cluster. One BosHR mutant carried two consecutive mutations (CTT) leading to the same amino-acid change, and other two BosHR mutants had two consecutive C→T mutations, with a phenylalanine (TTC) replacing proline in the aminoacidic sequence (Table 5 and Fig. 3d). No differences in response to boscalid were found associated to the two different amino-acid substitutions. BosLR strains was modified into the codon 272 with TAC instead of CAC. These nucleotide changes led to the substitution of tyrosine with histidine within the third cysteine-rich cluster-Ip sub-unit (Table 5).

Discussion

Baseline sensitivity of *B. fuckeliana* to boscalid was assessed after setting up experimental conditions. Acetate rather than glucose was used as a source of carbon in media for evaluating boscalid-response in *B. fuckeliana*, and no significant effects on either conidial germination or colony growth were observed. Indeed, in presence of glucose, the pathogen can overcome the activity of the fungicide, via anaerobic fermentation, whereas acetate is directly used in the tricarboxylic acid cycle (Stammler and Speakman 2006).

Results showed that conidial germination and germ tube elongation were more sensitive to boscalid than was mycelial growth. Under the adopted experimental

Fig. 3 Schematic representation of the genes encoding for the four sub-units of the SDH complex: **a** the flavoprotein gene *SdhA*; **b** the cytochrome b gene *SdhC*; **c** the CybS protein gene *SdhD*; **d** the iron-sulphur protein gene *SdhB*. Positions of start and stop codons are given as *inverted triangles*, respectively, above and below the *solid bars* representing exons. *Dotted lines* indicate the sequence of PCR product obtained with reported primer pairs. Below the nucleotide and deduced amino-acid sequence of the *B. fuckeliana* iron-sulphur sub-unit gene *SdhB* (*Botrytis cinerea* Sequencing Project database, accession number: BC1G_13286.1) are represented. Intron is in lower case and Cys-rich clusters associated with the iron-sulphur centres (S1, S2, and S3) are in *bold*. The *squared codons* and *asterisks* identify the position of mutations and the changed amino-acid residues

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1     ATGGCTGCTCTCCGCACAGGTGCCCGCAGTGCACGCGCGATATTCGCCGCATCACGACCA    60
1      M  A  A  L  R  T  G  A  R  S  A  R  A  I  F  A  A  S  R  P     20

61    GCTTTCAGAACTCAGATGCGAACCATGGCATCAGTCGACAGCTCAGTACCTGAAAGTCCT   120
21     A  F  R  T  Q  M  R  T  M  A  S  V  D  S  S  V  P  E  S  P     40

121   ACCGTTTCTCCATCCCGTCCTGTCGAATCTGCTTCCAAGACCTCCACTGTCAAGGAACCT   180
41     T  V  S  P  S  S  R  P  V  E  S  A  S  K  T  S  T  V  K  E  P     60

181   GCTGCCGACTCGGAGTCTTTGATCAAGACATTCAACATTTACAGATGGAACCCAGATGAG   240
61     A  A  D  S  E  S  L  I  K  T  F  N  I  Y  R  W  N  P  D  E     80

241   CCAACCAGCAAGCCCCGCATGCAATCTTACACTTTGGATCTCAACAAGACTGGACCTATG   300
81     P  T  S  K  P  R  M  Q  S  Y  T  L  D  L  N  K  T  G  P  M    100

301   ATGTTGGATGCGCTTATTAGAATCAAGAATGAGGTCGACCCTACCCTTACATTCAGAAGA   360
101    M  L  D  A  L  I  R  I  K  N  E  V  D  P  T  L  T  F  R  R    120

361   TCTTGCAGAGAAGGTATCTGCGGCAGTTGTGCAATGAACATTGATGGAGTAAACACATTG   420
121    S  C  R  E  G  I  C  G  S  C  A  M  N  I  D  G  V  N  T  L    140

421   GCTTGCTTGTgtatgttaatcaactattcaatattaaaacacatttgctgaccatttatt   480
141    A  C  L                                                       143

481   ctacagGCCGCATTCCAAGAGACGCTAAGCACGAAACGAAGATCTACCCACTACCCCAC    539
144          C  R  I  P  R  D  A  K  H  E  T  K  I  Y  P  L  P  H     161

540   ACCTATGTCGTCAAGGATATTGTTCCAGATTTGACACAATTCTACAAGCAATACAAGTCC   599
162    T  Y  V  V  V  K  D  I  V  P  D  L  T  Q  F  Y  K  Q  Y  K  S    181

600   ATTAAGCCATATCTTCAACACACCGACCCAGCACCAGAAGGAAAAGAATACTTGCAATCT   659
182    I  K  P  Y  Y  L  Q  H  T  D  P  A  P  E  G  K  E  Y  L  Q  S    201

660   AAGGAGGATCGTAAGAAGCTTGATGGACTTTACGAATGTATTCTCTGCGCATGCTGCTCG   719
202    K  E  D  R  K  K  L  D  G  L  Y  E  C  I  L  C  A  C  C  S    221

720   ACATCTTGCCCCTCCTACTGGTGGAACAGTGAGGAGTACTTGGGACCAGCTATCTTGTTG   779
222    T  S  C  P*  S  Y  W  W  N  S  E  E  Y  L  G  P  A  I  L  L    241

780   CAGAGTTACAGATGGCTTGCAGATTCCCGTGATCAGAAGAAGGAAGAACGTAAGGCAGCT   839
242    Q  S  Y  R  W  L  A  D  S  R  D  Q  K  K  E  E  R  K  A  A    261

840   TTGGATAACAGCATGAGTTTGTACAGATGTCACACTATTCTCAACTGCTCGAGGACATGT   899
262    L  D  N  S  M  S  L  Y  R  C  H*  T  I  L  N  C  S  R  T  C    281

900   CCGAAGGGATTGAATCCTGGTTTGGCAATTGCGGAGATTAAGAAGGAAATGGCTTTCTAA   959
282    P  K  G  L  N  P  G  L  A  I  A  E  I  K  K  E  M  A  F  *     301
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Table 5 Sequence changes within the succinate dehydrogenase iron-sulphur protein (*SdhB*) gene and coded amino acid (*in bold*) in laboratory boscalid-resistant mutants as compared to wild-type sensitive strains of *B. fuckeliana*

		Resistance phenotype	Codon numbers	
			225	272
Wild-type strains	T4; B05.10; SAS56; SAS405	BosS	CCC Pro	CAC His
Laboratory mutants	BAR341; BAR344; BAR372	BosLR	CCC Pro	**TAC Tyr**
	BAR334; BAR343; BAR358; BAR360; BAR361; BAR367; BAR368; BAR390	BosHR	**CTC Leu**	CAC His
	BAR364	BosHR	**CTT Leu**	CAC His
	BAR330; BAR357	BosHR	**TTC Phe**	CAC His

conditions, conidial germination of the wild-type sensitive strains showed EC_{50}=0.03–0.1 μg ml^{-1} and MIC=1–3 μg ml^{-1}; colony-growth tests showed EC_{50}=0.3–3 μg ml^{-1} and MIC=10–30 μg ml^{-1}. These results confirmed those previously obtained by Stammler and Speakman (2006) and Zhang et al. (2007). Hence, 1 and 3 μg ml^{-1} of boscalid were the concentrations used for selecting laboratory mutants.

The experiments aimed at selecting boscalid-resistant mutants showed that resistance can be induced prevalently, if not exclusively, by UV exposure, since spontaneous mutations could not be selected with only one exception. These results fit well with those previously obtained by Stammler et al. (2008) and Zhang et al. (2007).

Two types of boscalid-resistant mutants were discriminated according to the response of conidial germination to the fungicide: low (BosLR, with mean EC_{50} value~ 0.3 μg ml^{-1}, ranging from 0.03 to 1 μg ml^{-1} and MIC> 100 μg ml^{-1}) and high resistance (BosHR, unaffected or only slightly inhibited by 300 μg ml^{-1} of boscalid). The BosHR phenotype was prevalent and, as opposite to the BosLR phenotype, it was distinguishable from the wild-type phenotype even in mycelial growth test.

For the first time, genetic analysis of ascospore progenies of crosses between boscalid-resistant mutants and wild-type sensitive strains was performed and showed that the mutation(s) responsible for resistance of laboratory mutants occurred in single major gene(s) inherited in a 1:1 ratio in ascospore progeny. The classical mendelian analyis proved that the resistance gene(s) is located in the nucleus, confirming data reported for the SDH genes in animals and fungi (Lang et al. 1999), and showed that in *B. fuckeliana* it is linked with the *Daf1* and *Mbc1* genes conferring resistance to dicarboximides and benzimidazoles, respectively. This is a finding with potential outcomes on the population biology of fungicide-resistant fungal populations.

Due to the different mode of action of boscalid from other fungicides active against the pathogen, such as benzimidazoles, dicarboximides, and anilinopyrimidines, cross-resistance was not expected and it has never been observed. A negative cross-resistance between boscalid and QoI fungicides, such as kresoxym-methyl and azoxystrobin, previously reported for *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout (Pasche et al. 2005), was recently observed in *B. fuckeliana* (Zhang et al. 2007). Pyraclostrobin-laboratory resistant mutants of *B. fuckeliana* showed an increased sensitivity to boscalid and to the anilinopyrimidine cyprodinil (Markoglou et al. 2006).

Succinate dehydrogenase is the established target for carboxamide fungicides. Resistance to carboxin and boscalid results from mutations within the iron-sulphur protein sub-unit (SDHB) responsible for a single amino-acid substitution within the third Cys-rich cluster (Broomfield and Hargreaves 1992; Skinner et al. 1998; Honda et al. 2000; Avenot et al. 2008; Shima et al. 2009). Nevertheless, another nuclear locus, named *oxr*-2, in *U. maydis* (White et al. 1978), and the two gene loci encoding the C and D sub-units of SDH in *A. oryzae* (Shima et al. 2009) can also be involved in carboxin resistance.

Sequencing of the SDH genes allowed us to study resistance to boscalid in laboratory mutants of *B. fuckeliana*. The results showed that fungicide resistance was linked to the SDH genotype, and that mutations leading to boscalid resistance occurred in the Ip protein, and not in the structural genes coding for the other complex II-sub-unit proteins. These findings are in agreement with those previously reported by Stammler et al. 2008. Molecular analysis demonstrated that each boscalid-resistant mutant differed from the wild-type strain by a point mutation leading to an amino-acid substitution in

two (S2 and S3) of the three cysteine-rich regions of the protein, associated with iron-sulphur centres that are involved in electron transfer to ubiquinone. This finding corroborates the hypothesis that carboxamide fungicides disrupt the function of the iron-redox centres of the Ip sub-unit preventing their reoxidation (Ackrell et al. 1977).

The mutants with low resistance (BosLR) carried the change of a histidine with tyrosine at position 272. Therefore, as found with other fungal pathogens, such as *U. maydis*, *M. graminicola* and *A. alternata* (Keon et al. 1994; Skinner et al. 1998; Avenot et al. 2008), the modification of a highly conserved histidine residue in the S3 Cys-rich cluster of the Ip sub-unit is associated with boscalid resistance in *B. fuckeliana*. Different mutations in the Ip sub-unit of the fungus may also play a role in fungicide resistance, since the replacement of proline at position 225 by leucine or phenylalanine in S2 was indeed found in BosHR mutants.

Resistance of *B. fuckeliana* mutants to boscalid may, therefore, be explained by conformational changes within the S2 and S3 iron-redox centres which allows electron transfer even in presence of the fungicide. These results provide an insight into the mechanism of resistance to boscalid in laboratory mutants. Nevertheless, it must be stressed out that different mechanisms may occur in the field under the selective pressure of fungicide sprays.

The well known ability of *B. fuckeliana* to acquire resistance to fungicides and the current knowledge on the mode of action of SDHI fungicides and on the mechanism of resistance recommends that appropriate anti-resistance strategies must be implemented in IPM schedules.

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