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A high multi-drug resistance to chemically unrelated oomycete fungicides in *Phytophthora infestans*

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Abstract

Mutants of *Phytophthora infestans* with high resistance to the amidocarbamates iprovalicarb and benthiavalicarb and to the cyanoimidazole cyazofamid were isolated after UV-mutagenesis and selection on media containing one of the above fungicides. *In vitro* fungitoxicity tests showed that all resistant strains presented a highly reduced sensitivity to both cyazofamid and to the amidocarbamates. Cross-resistance studies with other oomycete fungicides from different chemical groups showed that the mutation(s) for resistance to iprovalicarb (IPV), benthiavalicarb (BVC) and cyazofamid (CZF) also greatly reduced the sensitivity of mutant strains to the phenylamide metalaxyl, acetamide cymoxanil, morpholine dimethomorph, benzamide zoxamide and to chlorothalonil. A lower reduction of sensitivity of mutant strains to the strobilurins azoxystrobin, kresoxim-methyl, pyraclostrobin and trifloxystrobin, azolones famoxadone and fenamidone and to antimycin A was observed. A resistance correlation was not apparent for the dithiocarbamate propineb and phenylpyridinamine fluazinam. Studies of fitness parameters in the wild-type and mutant strains of *P. infestans* showed that most resistant isolates had significantly reduced sporulation and sporangial germination, but not in the differentiation of sporangia into zoospores. Pathogenicity tests on tomato seedlings showed that most resistant isolates were significantly less pathogenic compared to the wild-type parent strain. However, experiments on the stability of the resistant phenotypes did not show a reduction in resistance when the mutants were grown for more than eleven generations on inhibitor-free medium. This is believed to be the first report of high level multi-drug resistance in fungal pathogens to chemically unrelated fungicides inhibiting different sites of cellular pathway.

Introduction

Phytophthora infestans is one of the most destructive and genetically dynamic pathogens with a worldwide distribution causing late blight disease on potato and tomato. Chemical control remains the main measure to reduce the incidence of the disease in most crops, especially under greenhouse conditions. On a worldwide basis, fungicidal control of potato late blight accounts for one-fourth of the total annual expenditure for fungicides on all crops (Erwin and Ribeiro, 1996). Phenylamides, the first systemic oomycete fungicides, have been widely used for many years to control *P. infestans* and other oomycetes. However, since the early 80s the efficacy of phenylamides has declined due to the emergence of resistant isolates in the field (Georgopoulos and

Grigoriou, 1981; Cohen and Reuveni, 1983; Cooke, 1991; Shattock, 2002).

During the last 10 years fungicide research has produced a diverse range of antifungal agents, which are expected to have a significant impact on the control of oomycetes. These new oomycete fungicides include the morpholine dimethomorph (Albert et al., 1988; Kuhn et al., 1990), strobilurin-related fungicides such as azoxystrobin, trifloxystrobin, famoxadone (Bartlett et al., 2002), the cyanoimidazole cyazofamid (Mitani et al., 2001a, c), the amidocarbamates iprovalicarb (IPV) and benthiavalicarb (BVC) (Suty and Stenzel, 1999; Reuveni, 2003) and the benzamides zoxamide and zarilamide (Egan et al., 1998). Biochemical studies on the mode of action showed that their fungitoxicity is based on different mechanisms of action from those of phenylamides and acetamides, which interfere with nucleic acid (RNA and DNA, respectively) biosynthesis (Davidse et al., 1983; Ziogas and Davidse, 1987). Cyazofamid (CZF) and strobilurin fungicides inhibit electron transfer in fungal mitochondria by binding at the ubiquinone reduction (Qi) and ubiquinol oxidation (Qo) centre of cytochrome *b*, respectively (Von Jagow and Link, 1986; Mitani et al., 2001b). Benzamides block nuclear division by binding to the *b*-subunit of tubulin in oomycetes (Young, 1991; Young and Slawecki, 2001). The site of action of dimethomorph and amidocarbamates has not yet been identified, although on the basis of morphological effects inhibition of cell wall and/or amino acid biosynthesis have been proposed (Kuhn et al., 1991; Suty and Stenzel, 1999; Reuveni, 2003). Due to the different mechanisms of action it is expected that the novel oomycetes fungicides will be widely used for disease control and resistance management in the field.

However, in the case of site-specific inhibitors, a serious concern is the duration of their effectiveness against the target pathogens. *Phytophthora infestans* is a classical 'high risk pathogen' with regard to resistance development (Brent and Hollomon, 1998), and almost all of the above mentioned newly introduced site-specific fungicides face the possibility of resistance development. Indeed, in the last few years field populations of certain downy mildews, such as *Plasmopara viticola* (Gisi et al., 2000), *Pseudoperonospora cubensis* (Ishii et al., 2001) and *Pythium aphanidermatum* (Bartlett et al., 2002) resistant to QoI have been detected in Europe and elsewhere.

Limited information is available concerning the risk for resistance development to cyazofamid, amidocarbamates, dimethomorph and zoxamide in oomycetes. In order to evaluate the potential risk of field resistance development to novel oomycete fungicides mutagenic, pathogenicity and cross-resistance studies were undertaken with laboratory mutants of *P. infestans*. The specific objectives of the present study were: (a) to explore the genetic potential of *P. infestans* to develop resistance to new oomycete fungicides; (b) to determine the level of resistance and the cross-resistance relationships between oomycete fungicides affecting different target sites, and (c) to assess the impact of resistant mutations on the ecological fitness characteristics of *P. infestans*.

Materials and methods

Fungal strains and culture conditions

A strain of *Phytophthora infestans* (A1 mating type), CBS 430.90, with wild-type sensitivity to metalaxyl, obtained form the Fungal Biodiversity Centre of Centraalbureau voor Schimmelcultures (The Netherlands), was used to obtain iprovalicarb (IPV), benthiavalicarb (BVC) and cyazofamid (CZF) resistant isolates. All isolates were grown and maintained on rye-agar medium (RAM) containing 2% sucrose, in a controlled climate cabinet at 20 °C with 14 h day^{-1} light and 70% relative humidity. For long-term storage the isolates were maintained in glass tubes on RAM 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. Iprovalicarb, trifloxystrobin, fenamidone and propineb were kindly supplied by Bayer CropScience AG (Leverkusen, Germany), benthiavalicarb by Kumiai Chemical (Japan), kresoxim-methyl and pyraclostrobin by BASF AG (Limburgerhof, Germany), zoxamide by Dow Agrosciences (Indianapolis, USA), cymoxanil and famoxadone by Du Pont de Nemours and Co. (Wilmington, DE, USA), metalaxyl and

azoxystrobin by Syngenta Crop Protection AG (Basle, Switzerland), dimethomorph by American Cyanamid Co. (New Jersey, USA), fluazinam and chlorothalonil by ISK Biosciences Ltd (Kent, UK). Cyazofamid and antimycin-A were purchased from Fluka and sigma-Aldrich, respectively. Stock solutions of fungicides were made in ethanol (IPV, BVC, metalaxyl, chlorothalonil, pyraclostrobin and fluazinam), methanol (CZF, trifloxystrobin, fenamidone and antimycin A), ethyl acetate (azoxystrobin, kresoxim-methyl and famoxadone) or acetone (cymoxanil, dimethomorph, zoxamide and propineb).

Mutation induction

Sporangial suspensions (approximately 10^8 sporangia ml^{-1}) of the wild-type strain of *P. infestans* in water were obtained from a 3 week-old culture. They were exposed with continuous agitation to ultraviolet irradiation (TUV Philips, 15 W, 254 nm) for 1 min, which resulted in 98% lethality. After irradiation they were kept for 30 min in the dark to minimize photorepair of radiation damage and then plated on RAM medium containing 5 $\mu g\ ml^{-1}$ IPV, 1 $\mu g\ ml^{-1}$ BVC, or 0.1 $\mu g\ ml^{-1}$ CZF and incubated at 20 °C for 20 days, to enable resistant colonies to appear. The selected resistant isolates IPV, BVC and CZF were maintained on RAM medium slants containing 0.5 $\mu g\ ml^{-1}$ IPV, 0.1 $\mu g\ ml^{-1}$ BVC or 0.01 $\mu g\ ml^{-1}$ CZF, the respective minimal inhibitory concentrations (MICs) for the wild-type parent strains.

In vitro *fungitoxicity tests*

At first, an initial qualitative assessment, using limited fungicide concentrations, was carried out with all isolates of *P. infestans*. Afterwards, fungitoxicity tests were made using several concentrations of each fungicide to determine the EC_{50}, the EC_{90} (the concentration causing 50 and 90% reduction of growth, respectively) and the MIC values. The fungicide sensitivity of the wild-type and mutant strains was assessed by inoculating RAM medium plates with mycelial inoculum consisting of 5 mm plugs cut from the growing edge of 1 week-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 6 days at 20 °C in the dark. The EC_{50} or EC_{90} for the wild-type isolate were determined from dose-response curves after probit analysis.

Determination of saprophytic and parasitic fitness parameters

Mutants of *P. infestans* were tested for mycelial growth rate, sporulation, sporangial differentiation into zoospores and germination and stability of resistant phenotype compared with the wild-type parent strain. Three 5 mm mycelial agar plugs for each strain were transferred to the centre of RAM medium plates for radial growth measurements. After incubation at 20 °C in the dark, the colony diameter of each isolate was measured at 24 h intervals up to 8 days. To determine sporangial production in the absence of fungicides, RAM plates were inoculated with 5 mm mycelial plugs and were incubated for 20 days at 20 °C in the dark. The total mycelial mass produced in each dish was transferred to a 250 ml Erlenmeyer flask with 10 ml deionized water. The flasks were agitated vigorously and the concentration of sporangia in the resulting spore suspension, after filtration through cheesecloth, was determined with a Neubauer haemocytometer and expressed as number of sporangia per cm^2 of the RAM culture. Germ tube emergence of sporangia was determined by seeding sporangia on clarified RAM medium after 15 h incubation at 20 °C. For the determination of the effect on the differentiation of sporangial cytoplasm into zoospores, sporangial suspensions were incubated in 5 ml distilled water in 50 ml Erlenmeyer flasks for 1 h at 4 °C and then for 10–12 h at 10 °C. Zoospore release started after 2 h incubation at 10 °C and maximum zoospore numbers occurred after 8–10 h. Sporangia of 2 week-old cultures on RAM were used in all germination experiments.

The stability of resistant phenotypes was examined by successive subcultures of each resistant strain on fungicide-free growth medium for at least 11 transfers. The sensitivity to IPV, BVC and CZF was estimated at each generation of mutant isolates by measuring the mycelial growth at the MIC for the wild-type and at 10 and 100 μg ml^{-1} IPV, BVC or CZF.

Pathogenicity of selected mutant isolates of *P. infestans* was determined by examining symptom severity caused by each strain on tomato (*Lycopersicum esculentum* cv. Packmore) seedlings. Tomato seedlings grown in plastic pots for 20 days (four seedlings per 17 cm pot, three pots per treatment) were inoculated at the 2–3 leaf stage. A wound was made on the stem near to the neck of the tomato plant. A mycelial plug (5 mm diam) was taken with a cork borer from the margin of a young colony on RAM medium and placed on the wound. The inoculated plants were incubated in a moist chamber at 20 °C for 10–15 days and the infection was recorded by evaluating the area of the infected tissue of each plant. Disease development was evaluated according to the following indices: 0, no apparent infection; 0.5, necrosis only under inoculum; 1, < 25% necrosis; 2, 25–50% necrosis and 4, necrosis on > 50% of the stem.

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} values 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, sporangial germination and differentiation and pathogenicity ratings of isolates.

Results

Selection of mutant strains of P. infestans *resistant to amidocarbamates and to cyazofamid*

Mutants of *P. infestans* resistant to IPV, BVC and CZF were isolated at a low mutation frequency after UV-mutagenesis and selection on fungicide-amended media, indicating the existence of a genetic and biochemical potential for development of field resistance towards these particular fungicides. Depending on the selection medium, the mutants were designated as IPV, BVC or CZF. Approximately 5×10^7 irradiated sporangia of the wild-type strain, which survived the mutagenic treatment (98% lethality), were plated on RAM medium containing IPV (5 μg ml^{-1}) or BVC (1 μg ml^{-1}) or CZF (0.1 μg ml^{-1}). From these selection media, 3, 6 and 4 resistant colonies were obtained, respectively, during the first 10–15 days of the incubation period, indicating a mutation frequency of approximately 1×10^{-7} for the above fungicides. Most of the resistant isolates appeared between 7 and 10 days incubation.

Preliminary tests on the response of mutant isolates to IPV, BVC and CZF at the MICs for the wild-type strain of *P. infestans* and at 10 and 100-fold higher concentrations, showed a high reduction in the sensitivity of all mutant strains. The growth of mutant strains was not inhibited even at the highest concentration used.

Level and stability of resistance

Fungitoxicity tests on the response of IPV, BVC and CZF isolates to the presence of IPV, BVC or CZF in the growth medium, showed that all mutants were highly resistant to these fungicides, independently of the fungicide used for their isolation. The mycelial growth of mutants was slightly inhibited even at 100 μg ml^{-1} (Figure 1). On the contrary, a dose-dependent decrease in growth was observed with the wild-type parent strain. The mycelial growth of the wild-type isolate was inhibited 50% (EC_{50}) and 90% (EC_{90}) at 0.05 and 0.25 μg ml^{-1} IPV, 0.025 and 0.075 μg ml^{-1} BVC and 0.0025 and 0.005 μg ml^{-1} CZF, respectively.

All mutant strains maintained their resistance after successive subculturing on fungicide-free medium. The resistance to IPV, BVC and CZF was not reduced even after 11 transfers in fungicide-free medium.

Cross-resistance with other oomycete fungicides

Fungitoxicity tests on the response of mutant strains to other oomycete fungicides showed that the mutated gene(s) also reduced the sensitivity of mutant isolates to the phenylamide metalaxyl, acetamide

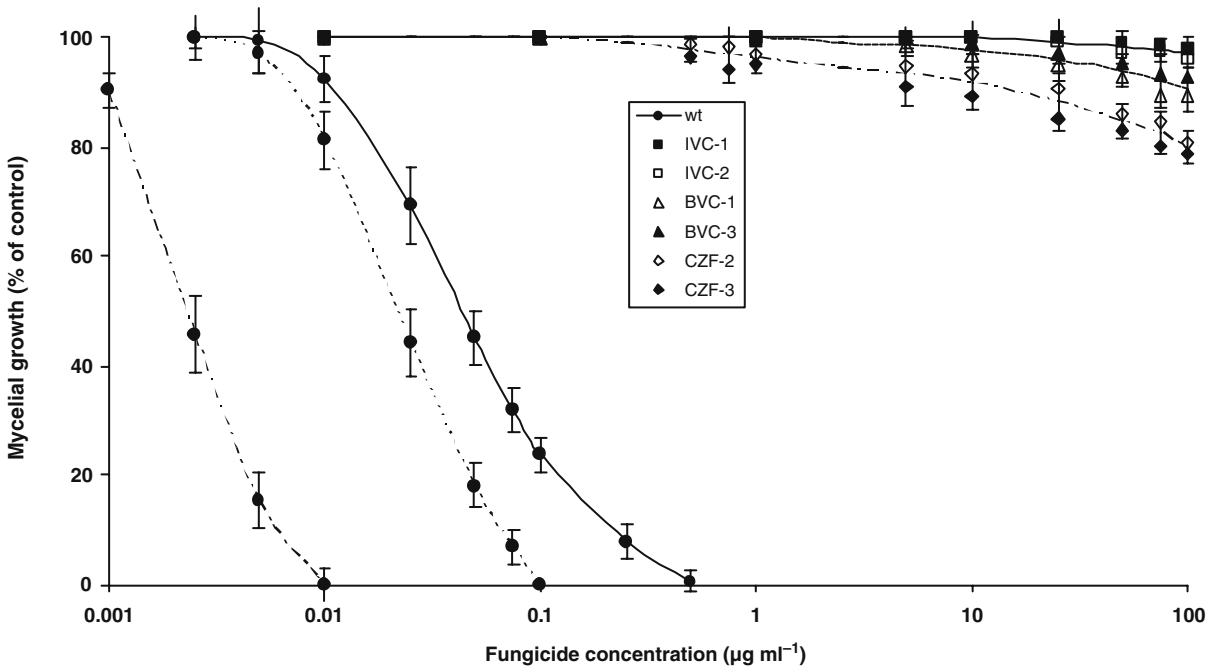

Figure 1. Sensitivity of the wild-type and representative resistant isolates of *Phytophthora infestans* to IPV (—/wt, IVC-1, IVC-2), BVC (·····/wt, BVC-1, BVC-3) and CZF (- · - · - /wt, CZF-2, CZF-3) on rye-agar medium. Measurements were made after 6 days incubation at 20 °C. Results are means of three replications with bars showing the standard errors.

cymoxanil, morpholine dimethomorph, benzamide zoxamide, QoIs azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin, famoxadone and fenamidone, chlorothalonil and to QiI antimycin A (Table 1). However, the resistance level to QoIs and antimycin A was lower than to other fungicides. A reduction in the sensitivity was not apparent in the case of dithiocarbamate propineb and phenylpyridinamine fluazinam (Table 1). Moreover, an interesting fungitoxicity profile was observed in the case of a mutant strain CZF-4, which was resistant to QoI fungicides azoxystrobin, pyraclostrobin, famoxadone and fenamidone, but was more sensitive than the wild-type to kresoxim-methyl and trifloxystrobin (Rf: 0.02 and 0.05, based on EC_{90} values, respectively). It is difficult at present to find a reasonable explanation for the response of this particular mutant strain.

Characterization of saprophytic and parasitic fitness parameters

Study of fitness parameters in the wild-type and mutant strains showed that the mutation(s) leading to amidocarbamates and CZF resistance affect the ecological fitness of most mutant isolates. The mycelial growth of the mutants varied; in some isolates (IPV-1, IPV-2, BVC-1, BVC-3, CZF-2 and CZF-4) the growth rate was similar to the wild-type parent strain, while some others grew slower (40–60% compared with the wild-type strain). Comparisons of other fitness parameters such as sporulation and sporangial germination between resistant mutants and the wild-type parent strain of *P. infestans* showed that with the exception of sporangial production by IPV-2, IPV-6 and BVC-3 and sporangial germination by IPV-2, BVC-3 and CZF-2, these fitness parameters were significantly affected in all other mutant isolates (Table 2). On the other hand, the differentiation of sporangial cytoplasm into zoospores was not affected or only slightly affected in all mutant isolates (Table 2).

The pathogenicity experiment showed that none of the resistant strains of *P. infestans* tested lost their ability to cause infection on tomato stems (Table 2). However, most mutant strains were less aggressive than the wild-type parent strain. With the exception of IPV-2, BVC-3 and CZF-2 all other mutant isolates had a significantly reduced

Table 1. Fungicide sensitivity of wild-type and representative mutant isolates of *Phytophthora infestans* selected by IPV, BVC or CZF-amended medium

Fungicide	Wild-type EC_{90}[a] (μg ml^{-1}) (mean ± SD[b])	Relative growth at 10 μg ml^{-1} (mean ± SD[b])										
		wt	IPV-1[c]	IPV-2[c]	BVC-1[c]	BVC-3[c]	BVC-5[c]	BVC-9[c]	BVC-11[c]	CZF-2[c]	CZF-3[c]	CZF-4[c]
IPV	0.25 ± 0.046	0	97 (±3)	100	93 (±6)	100	95 (±5)	100	97 (±3)	94 (±6)	95 (±4)	100
BVC	0.075 ± 0.004	0	96 (±3)	100	98 (±1)	94 (±5)	95 (±3)	97 (±4)	100	91 (±8)	100	87 (±12)
CZF	0.005 ± 0.001	0	81 (±8)	100	100	100	94 (±6)	97 (±1)	100	89 (±6)	100	91 (±8)
Zoxamide	0.15 ± 0.071	0	100	100	96 (±3)	100	100	100	86 (±2)	100	96 (±2)	98 (±3)
Dimethomorph	0.5 ± 0.14	0	100	100	98 (±2)	89 (±7)	100	100	100	100	94 (±5)	87 (±9)
Cymoxanil	2.5 ± 0.82	0	100	93 (±4)	90 (±8)	100	94 (±3)	98 (±2)	100	100	92 (±4)	90 (±11)
Metalaxyl	7.5 ± 1.32	0	97 (±2)	100	86 (±12)	100	98 (±1)	100	94 (±3)	100	100	100
Chlorothalonil	0.075 ± 0.06	0	93 (±6)	100	100	94 (±5)	80 (±17)	81 (±15)	92 (±8)	98 (±2)	100	83 (±17)
Azoxystrobin	0.75 ± 0.03	0	64 (±18)	49 (±5)	52 (±26)	60 (±6)	57 (±4)	52 (±16)	59 (±21)	52 (±12)	25 (±9)	34 (±18)
Kresoxim-methyl	5 ± 1.16	0	53 (±12)	64 (±17)	47 (±9)	60 (±21)	50 (±12)	58 (±14)	nt	65 (±23)	40 (±8)	0
Pyraclostrobin	1 ± 0.41	0	36 (±9)	54 (±13)	nt	62 (±7)	58 (±17)	nt	58 (±11)	nt	64 (±13)	43 (±6)
Trifloxystrobin	7.5 ± 0.65	0	66 (±19)	52 (±8)	nt	57 (±11)	64 (±21)	nt	nt	63 (±13)	42 (±7)	0
Famoxadone	37.5 ± 5.33	25 (±4)	65 (±18)	72 (±21)	60 (±8)	57 (±6)	50 (±12)	nt	nt	73 (±19)	55 (±3)	50 (±14)
Fenamidone	0.35 ± 0.08	0	nt[d]	57 (±4)	nt	60 (±12)	58 (±9)	nt	nt	64 (±15)	61 (±11)	44 (±9)
Fluazinam	7.5 ± 2.14	0	0	23 (±8)	0	29 (±11)	20 (±9)	0	18 (±7)	0	0	0
Antimycin-A	0.5 ± 0.13	0	45 (±6)	58 (±3)	nt	56 (±24)	60 (±17)	nt	nt	63 (±10)	48 (±5)	35 (±9)
Propineb	50 ± 7.82	40 (±7)	0	56 (±7)	48 (±6)	53 (±8)	50 (±11)	43 (±7)	50 ± 13	nt	52 (±13)	44 (±3)

[a]Effective concentration causing 90% reduction in growth rate of wild-type.
[b]Pooled standard deviation of the means ($n = 3$); where SD are not given, replicates were identical. Correlation coefficient ranged from 0.89 to 0.97.
[c]IPV mutant strains selected by IPV; BVC mutant strains selected by BVC; CZF mutant strains selected by CZF.
[d]nt: not tested.

Table 2. Comparison of *Phytophthora infestans* mutant isolates resistant to IPV, BVC or CZF with their parental wild-type strain with respect to some fitness parameters

Strains	Mycelial growth[a]	Sporangial production[b]	Sporangial germination[c]	Zoospore formation[d]	Pathogenicity[e]
Parent strain	49a[f]	5.2a[f]	96.4a[f]	97.2a[f]	100a[f]
Iprovalicarb-mutants					
IPV-1	50a	0d	–	–	36c
IPV-2	46ab	4.8a	100a	98.9a	89ab
IPV-6	28b	3.6ab	55.6b	89.4ab	52b
Benthiavalicarb-mutants					
BVC-1	48a	0d	–	–	57b
BVC-3	52a	4.3a	98.3a	96.7a	96a
BVC-5	25bc	2.7b	36.7c	88.4ab	17e
BVC-9	22c	0d	–	–	20de
BVC-10	18d	1.6bc	17.2d	90.2ab	43bc
BVC-11	20c	0d	–	–	30cd
Cyazofamid-mutants					
CZF-1	30b	0.4c	44.6bc	79.4b	40c
CZF-2	49a	2.9b	89.5ab	95.3a	100a
CZF-3	26b	0d	–	83.7b	55b
CZF-4	51a	0d	–	–	27d

[a]Mean colony diameter (mm) measurements after 8 days of incubation ($n = 3$).
[b]Mean number ($\times 10^4$) of sporangia per cm^2 of colony after 20 days of incubation ($n = 3$).
[c]Percentage of germinated sporangia after 15 h incubation ($n = 100$).
[d]Percentage of differentiated zoosporangia into zoospores after 8 h incubation ($n = 100$).
[e]Pathogenicity as % of wild-type. The sum of indices of 12 tomato plants for the wild-type was 42.
[f]Within columns, means followed by the same letter do not differ significantly according to Dunnett's multiple range test ($P = 0.05$).

infection ability (50–80%) compared with the wild-type parent strain.

Discussion

The appearance of oomycete mutant isolates resistant to phenylamide (Georgopoulos and Grigoriou, 1981; Cohen and Reuveni, 1983; Cooke, 1991; Shattock, 2002) and strobilurin fungicides (Gisi et al., 2000; Ishii et al., 2001; Bartlett et al., 2002) has been well documented. However, little information is available concerning isolation of mutants resistant to the other site-specific oomycete fungicides. Although the acetamide cymoxanil has been used intensively for more than 20 years, only a variation in the sensitivity distribution was observed in *Phytophthora infestans*, probably due to genetic diversity (Hamlen and Power, 1998). Attempts to generate mutants of *P. infestans* and *Phytophthora capsici* resistant to dimethomorph by mycelial adaptation on fungicide-amended media failed to produce resistant mutants (Young et al., 2001). However, moderately resistant mutants of *Phytophthora parasitica* (Chabane et al., 1993) and *P. capsici* (Young et al., 2001) were isolated using ultraviolet and chemical mutagenesis, respectively. The results of several efforts to identify strains of *P. infestans* and *Pythium sylvaticum* resistant to CZF, including field monitoring and mutation studies, supported the conclusion that the risk of resistance to CZF is probably low (Mitani et al., 2001c). Chemical or ultraviolet mutagenesis in *P. infestans*, *P. capsici* and *Phytophthora megasperma* was unsuccessful for producing mutants resistant to benzamides zoxamide or zarilamide (Young et al., 2001). The authors suggested that the failure to isolate mutants resistant to benzamides resulted from the diploid nature of oomycetes and the recessiveness of chromosomal resistant allelomorph(s) for target-site changes having little effect on the sensitivity of heterozygous diploid strains (Young et al., 2001). Furthermore, to our knowledge, there is no report for resistance development to amidocarbamates in any oomycete and to strobilurin fungicides in *P. infestans*.

Contrary to all above data, in the present study mutants of *P. infestans* highly resistant to amidocarbamates IPV and BVC and to cyanoimidazole

CZF were isolated from a wild-type strain after UV-mutagenesis and selection on fungicide-amended media. Our findings do not support the suggestion mentioned above by Young et al. (2001). The selection of resistant mutants clearly indicates a dominance of resistant allele(s) in *P. infestans*. Studies on the genetic control of resistance to phenylamides have recognized a dominance of resistant alleles in *P. infestans* (Lee et al., 1999; Shattock, 2002).

Studies on the sensitivity of mutant strains to fungicides from other chemical groups showed that the mutation(s) for resistance to IPV, BVC and CZF also greatly reduced the sensitivity of mutant isolates to the phenylamide metalaxyl, acetamide cymoxanil, morpholine dimethomorph, benzamide zoxamide and chlorothalonil. A lower reduction in the sensitivity of mutant strains to the strobilurin fungicides azoxystrobin, kresoxim-methyl, pyraclostrobin and trifloxystrobin, to azolones famoxadone and fenamidone and to antimycin A was found. Our data is the first report clearly indicating the existence of the genetic and biochemical potential for the development of a high level multi-drug resistance to chemically unrelated fungicides in oomycetes. Previous studies have shown cross-resistance only among phenylamide fungicides. Metalaxyl-resistant mutants of *P. infestans* (Diriwächter et al., 1987), *P. parasitica* (Chabane et al., 1993) and *Phytophthora citricola* (Joseph and Coffey, 1984) were resistant to other phenylamides but remained sensitive to oomycete fungicides inhibiting different target sites. Similar results have also been reported for metalaxyl-resistant mutants of *P. megasperma* and *Plasmopara viticola* (Albert et al., 1988; Kuhn et al., 1990). Phenylamide-resistant field isolates of *P. infestans* were sensitive to mancozeb, chlorothalonil and cymoxanil (Sedegui et al., 1999). Metalaxyl-resistant mutants of *Pseudoperonospora cubensis* and *P. infestans* did not exhibit cross-resistance to CZF (Mitani et al., 2001a). In addition, metalaxyl-resistant mutants of *P. infestans* were sensitive to IPV (Suty and Stenzel, 1999). A positive cross-resistance has also been detected among QoI in *P. viticola* and *P. cubensis* (Ishii et al., 2001). No correlation in cross-sensitivity between azoxystrobin and oxadixyl or between azoxystrobin and cymoxanil has been observed in *P. infestans* (Gisi et al., 1997). Furthermore, strobilurin-resistant mutants of *P. cubensis* were sensitive to the QiI fungicide CZF (Mitani et al., 2003). The amidocarbamate IPV was found to be effective against isolates of *P. viticola* resistant to cymoxanil or dimethomorph (Suty and Stenzel, 1999). The only other report presenting evidence of a correlation between chemically unrelated oomycete fungicides was by Cohen and Samaucha (1984) which found a lower effectiveness of cyprofuram + folpet, oxadixyl, propamocarb and phosetyl for the control of metalaxyl-resistant isolates of *P. infestans* and *P. cubensis* in greenhouse tests.

It is well accepted that the most important biochemical mechanism causing resistance of fungal pathogens to fungicides in practice results from a modification at the target-site of fungicidal action in the target pathogens (Brent and Hollomon, 1998). Biochemical studies have shown that resistance to phenylamides in *P. megasperma* f.sp. *medicaginis* is due to a change in RNA polymerase I (Davidse et al., 1983). Furthermore, in the case of inhibitors of cytochrome bc_1 complex, point mutations in the Qo-site of cytochrome *b* have been shown to substantially reduce the sensitivity of several fungal species to QoI fungicides. 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 QoI, has been found in *P. viticola* and *P. cubensis* (Gisi et al., 2000; Ishii et al., 2001; Sierotzki et al., 2004). 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) and *P. viticola* (Sierotzki et al., 2004). However, a target site change does not provide a reasonable explanation for the multi-drug resistance found in the present work. The reduction of sensitivity to inhibitors affecting different sites of cellular processes indicates that a mechanism other than target-site modification is the underlying biochemical mechanism of resistance. Mechanisms leading to reduced fungicide concentration at the target site, such as decreased accumulation of the inhibitor into the fungal mycelium by an efficient efflux pump or by an increased binding of the compound in the cell wall or degradation of the fungicide to non-fungitoxic compounds, may explain the multi-drug resistance phenomenon. However, the relatively lower level of resistance to energy production inhibitors

interfering with the mitochondrial electron transport at the cytochrome bc_1 complex (QoI and QiI) and also the absence of resistance to fluazinam, an inhibitor of ATP synthesis, indicates that the energy-dependent increased efflux pump is the most probable mechanism for the multi-drug resistance phenomenon in *P. infestans*. A low resistance to unrelated fungicides, due to the above mechanism, has already been observed in various non-oomycete fungal pathogens (De Waard, 1997; Del Sorbo et al., 2000). Future molecular and biochemical studies on these mutant strains may provide evidence for the molecular basis of the observed cross-resistance pattern and the underlying biochemical mechanism(s) of resistance.

Studies of fitness of mutant strains, showed that with the exception of stability of resistance *in vitro*, the mutation(s) appeared to be pleiotropic having a significant adverse effects on other fitness-determining characteristics in most mutant strains of *P. infestans*. However, in a few mutant strains the fitness parameters were practically unaffected. Conflicting reports, regarding the genetic link between fitness characteristics and resistance to phenylamides or to QoI fungicides in oomycetes were also found in the literature. Data supporting a significant reduction of fitness characteristics (Bruin and Edgington, 1982), or indicating stability of the resistant phenotype (Joseph and Coffey, 1984; Chabane et al., 1993) and/or pathogenicity of mutant isolates similar to the sensitive ones (Bruin and Edgington, 1982; Kadish et al., 1990; Chabane et al., 1993) have been reported. However, resistance to phenylamides was found to be under the control of a single gene in *P. infestans*, and correlation studies between resistance and fitness components did not reveal any association (Lee et al., 1999).

The data of the present study and the practical failures of phenylamides and more recently of strobilurin fungicides in the control of certain oomycetes emphasize the need to explore thoroughly the potential of different species of oomycetes for resistance development to the novel site-specific introduced fungicides. The commercial use of these fungicides requires careful implementation of appropriate anti-resistance strategies to preserve their effectiveness, including monitoring studies to detect any change in the sensitivity within pathogen populations.

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