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Studies on the inherent resistance risk to fenhexamid in *Botrytis cinerea*

B.N. Ziogas, A.N. Markoglou and A.A. Malandrakis
Laboratory of Pesticide Science, Agricultural University of Athens, Votanikos, 188 55 Athens, Greece (Phone: +210 5294507; Fax: +210 3422062; E-mail: ZIV@AUA.GR)

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Abstract

After chemical mutagenesis with *N*-methyl-*N*-nitrosoguanidine (MNNG) two phenotypes that were highly or moderately resistant to fenhexamid, were isolated from a wild-type strain of *Botrytis cinerea*, at a mutation frequency of 0.9×10^{-5}. Resistance factors, based on EC_{50} values, were 460–570 and 10–15, respectively. The mutation(s) for resistance to fenhexamid did not affect the sensitivity of mutant strains to the benzimidazole benomyl, the phenylpyridinamine fluazinam, the anilinopyrimidine cyprodinil, the guanidine iminoctadine or to the sterol-biosynthesis-inhibiting fungicides fenarimol, fenpropimorph and tridemorph. On the contrary, an increased sensitivity (EC_{50} ratios of 0.2–0.6) of fenhexamid-resistant strains to the phenylpyrrole fludioxonil and the dicarboximide iprodione was observed. Study of fitness parameters of fenhexamid-resistant isolates of both phenotypic classes showed that these mutation(s) had no effect on mycelial growth and sensitivity to high osmolarity, but they did affect one or more of some other characteristics, such as sporulation, conidial germination and sclerotia production. In tests on cucumber seedlings under greenhouse conditions, all highly fenhexamid-resistant isolates tested presented decreased infection ability compared with the wild-type. Preventive applications of a commercial formulation of fenhexamid, Teldor 50 WP, were effective against lesion development on cotyledons by the wild-type, but ineffective, even in high concentrations, against disease caused by the fenhexamid-resistant isolates. The risk of resistance problems arising during commercial use of fenhexamid is discussed.

Introduction

Fenhexamid, a hydroxyanilide derivative, is one of the recently introduced fungicides with a high preventive activity against grey mould disease caused by *Botrytis cinerea* in various crops (Suty et al., 1997). It is also active against other plant pathogens such as *Monilinia* spp. and *Sclerotinia sclerotiorum*. Moreover, this compound is easily degraded and presents a favourable toxicological profile and environmental behaviour (Rosslenbroich et al., 1998; Rosslenbroich and Stuebler, 2000).

Most investigations on the effect of the toxicant on fungal growth and morphology of *B. cinerea* showed that it suppresses the germination of fungal spores only at relatively high concentrations, but it is highly effective in inhibiting subsequent stages of disease development. Shortly after the initiation of spore germination, germ-tubes from treated spores stop growing, showing granular structures in the cytoplasm, resembling those induced by sterol biosynthesis inhibitors (Köller, 1992; Haenssler and Pontzen, 1999). Germ-tubes collapse and die before they are able to penetrate the plant surface. Treated hyphal tips frequently show abnormal excretion of what is thought to be cytoplasm or cell wall associated material. However, it is not clear whether these effects are directly associated with the mode of action of fenhexamid (Haenssler and Pontzen, 1999; Debieu et al., 2001). Early investigations on the mode of action have suggested that this fungicide has a mechanism of action different from that of all other botryticides known

(Rosslenbroich and Stuebler, 2000). Other reports have been suggested that it interferes with cell-wall biosynthesis (Haenssler and Pontzen, 1999), or inhibits the C-4 demethylation of sterol biosynthetic pathway (Debieu et al., 2001).

The development of resistance in fungi to fungicides with a specific mechanism of action has become a serious problem in the control of grey mould disease. *B. cinerea* is the classical 'high risk pathogen' from the view of resistance management (Brent and Hollomon, 1998), and the newly introduced botryticides face the possibility of resistance development. Such a resistance risk was demonstrated for the phenylpyrroles and anilinopyrimidines in recent reports (Faretra and Pollastro, 1993; Forster and Staub, 1996; Rueegg et al., 1997; Hilber and Hilber-Bodmer, 1998; Chapeland et al., 1999; Leroux et al., 1999; Ziogas and Kalamarakis, 2001). Early indications that fenhexamid is also at risk were provided by monitoring studies, which revealed the presence of field isolates of *B. cinerea* with reduced sensitivity to fenhexamid, before the commercial introduction of the fungicide (Suty et al., 1997; Leroux et al., 1999).

In order to increase our knowledge regarding the possibility of development of practical resistance to fenhexamid, *in vitro* and *in planta* tests were undertaken with laboratory mutants of *B. cinerea*. The specific objectives of the present study were: (a) to determine the mutation frequency and the level of resistance of mutant strains to fenhexamid; (b) to elucidate the cross-resistance relationships between fenhexamid and other botryticides; (c) to examine the impact of mutations for fenhexamid-resistance on the saprophytic and parasitic fitness of mutant strains; and (d) to examine the mutant response to fungicide treatments *in planta*.

Materials and methods

Fungal strains and culture conditions

The wild-type strain wt-B_1 of *B. cinerea* (teleomorph *Botryotinia fuckeliana*) isolated from a tomato crop in Greece was used to obtain fenhexamid-resistant mutant isolates (B/FNH). Mutant isolates B/$FLD_{osm/r}$-17 (osmotic resistant) and B/$FLD_{osm/s}$-42 (osmotic sensitive) that were resistant to the phenylpyrrole fludioxonil, obtained from the collection of the Plant Pathology Laboratory of Agricultural University of Athens, were used in fungitoxicity, cross-resistance and pathogenicity tests. All isolates were grown on potato dextrose agar (PDA) in a controlled climate cabinet at 22 °C with 14 h day^{-1} illumination provided by fluorescent tubes and 70% relative humidity. For long-term storage, the isolates were maintained in glass tubes on PDA at 10 °C in the dark and single tip transfers were made once a month.

Fungicides

The fungicides used *in vitro* were pure technical grade. Fenhexamid was kindly supplied by Bayer Crop Science AG (Leverkusen, Germany), cyprodinil and fludioxonil by Syngenta Crop Protection AG (Basle, Switzerland), fluazinam by ISK Biosciences Ltd (Kent, UK), iminoctadine by Dainippon Ink and Chemicals Inc. (Japan), iprodione by Rhone Poulenc Agro, Lyon, France (now Bayer Crop Science), benomyl by Du Pont de Nemours and Co. (Wilmington, DE, USA), fenarimol by Dow Agrosciences (Indianapolis, USA), fenpropimorph by Novartis, Basle, Switzerland (now Syngenta) and tridemorph by BASF AG (Limburgerhof, Germany). Stock solutions of fungicides were made in ethanol, with exceptions of benomyl and fenhexamid which were dissolved in acetone and in isopropyl-alcohol, respectively.

In pathogenicity tests, aqueous suspensions of the commercial formulations Teldor 50 WP (500 g kg^{-1} fenhexamid) and Saphire 50 WP (500 g kg^{-1} fludioxonil) were used. The fungicide concentrations were expressed as active substance (μg ml^{-1}).

Mutation induction

Conidial suspensions (1.8×10^7 conidia ml^{-1}) of the wild-type strain of *B. cinerea* in water were obtained from 8 to 10-day-old slant cultures. They were agitated on a rotary shaker at 22 °C and 100 rev min^{-1}, with 10 μg ml^{-1} *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (Ziogas and Girgis, 1993) for 4 h in darkness and washed twice with sterile distilled water. The mutagenic treatment resulted in 95% lethality. Conidia were resuspended in water and were plated on PDA medium containing 25 μg ml^{-1} fenhexamid and incubated at 22 °C for 15 days, to enable resistant colonies to appear. The selected resistant isolates were maintained on PDA slants containing 2.5 μg ml^{-1} fenhexamid, the minimal

inhibitory concentration (MIC) for the wild-type strain.

In vitro *fungitoxicity tests*

The fungicide sensitivity of the wild-type and mutant strains of *B. cinerea in vitro* was assessed by measuring the radial growth on PDA plates containing a range of concentrations of each fungicide. The experiments were conducted in 9-cm Petri dishes inoculated with 2-mm mycelial plugs, from water-agar (WA) medium on which conidia of *B. cinerea* had been allowed to germinate. The agar plugs were placed with the surface mycelium in direct contact with the medium. The effect of the fungicide on growth was determined by measuring the diameters of the mycelial colonies, after incubation for 4–5 days at 22 °C in the dark. The fungicides were added aseptically to sterilize growth medium from stock solutions, prior to inoculation. In all cases, the final amount of solvent never exceeded 1% (v : v) in treated and control samples. At least six concentrations with three replicas for each fungicide were used in order to obtain the respective fungitoxicity curves. The concentrations causing a 50% reduction in the growth rate (EC_{50}) were determined using the dose response curves after probit analysis. The ratio of EC_{50} or MIC for a resistant isolate to the EC_{50} or MIC for the parent sensitive strain gave an estimation of the resistance level (resistance factor, Rf).

Determination of fitness parameters

Mutants of *B. cinerea* were tested for mycelial growth rate, sporulation, spore germination, sclerotia production and osmotic pressure sensitivity on PDA medium. Three 2-mm mycelial WA-plugs for each strain were transferred to the centres of PDA-plates for radial growth measurements. After incubation at 22 °C in the dark, the colony diameter of each isolate was measured at 24 h intervals. To determine conidial production in the absence of fungicides, PDA-plates were inoculated with a conidial suspension (10^6 conidia ml^{-1}) and were incubated for 8–10 days at 22 °C with 14 h day^{-1} illumination. The total mycelial mass produced in each dish was transferred to a 250-ml Erlenmeyer flask with 20-ml de-ionized water. The flasks were agitated vigorously and the concentration of conidia in the resulting spore suspension, after filtration through cheesecloth, was evaluated measuring with a Neubauer haemocytometer and expressed as number of conidia per square centimetre of the plate culture. Spore germination and sclerotia production were determined after 6 h and 20 days of incubation, respectively, on PDA medium in the dark. The osmosensitivity of fenhexamid-resistant strains determined after 4-day inoculation on PDA medium amended with 2.5% KCl. Radial growth, sporulation, spore germination, sclerotia production and osmotic sensitivity measurements were subjected to analysis of variance using a Dunnett's multiple range test at $P = 0.05$.

Pathogenicity and resistance tests on cucumber seedlings

Pathogenicity bioassay and fungicide-resistance of various mutant strains of *B. cinerea* were determined by examining symptom severity caused by each strain on cucumber seedlings (*Cucumis sativus*, cv. Telegraph) according to the method described by Kato et al. (1984), with minor modifications (Ziogas and Girgis, 1993). Cucumber seedlings grown in plastic pots for 8–10 days (four seedlings per 17-cm pot, two pots per treatment) were used at the cotyledon stage. The formulated fungicides in aqueous suspensions were sprayed to run-off at the desired doses with a hand-sprayer 5 h before inoculation. Control plants were sprayed with de-ionized water. The centre of each cotyledon was punctured with a needle and a 2-mm mycelial plug from the margin of a young colony on PDA medium was placed on the wound. The inoculated plants were incubated in a moist chamber at 22 °C in the dark for 3–5 days and the infection was scored by evaluating the lesion of each cotyledon. Disease development was evaluated according to the following indices: 0, no infection; 0.5, rot only under inoculum; 1, less than 20%; 2, 21–50%; and 4, rot on more than 50% of cotyledon surface.

Statistical analysis

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

Results and discussion

Isolation and characterization of fenhexamid-resistant isolates

The mycelial growth of the wild-type isolate was inhibited 50% (EC_{50}) and 100% (MIC) at the concentrations of 0.19 and 2.5 μg fenhexamid ml^{-1}. A rather higher fungitoxicity of fenhexamid to *B. cinerea* was found by other researchers (Suty et al., 1997; Debieu et al., 2001).

Mutants resistant to fenhexamid were isolated at high frequency after chemical mutagenesis of conidia of *B. cinerea*. Approximately 5.6×10^6 conidia of the wild-type strain wt-B_1, which survived the mutagenic MNNG treatment, were plated on PDA containing 25 μg ml^{-1} fenhexamid. From this selection medium, 51 resistant colonies were obtained during the incubation period of 15 days, indicating a mutation frequency of 0.9×10^{-5}. Most of the resistant isolates appeared between the 3rd and 7th days of incubation. The presence of isolates with reduced sensitivity *in vitro* to fenhexamid has also observed in natural populations of the pathogen, which were never exposed to this fungicide (Suty et al., 1997; Leroux et al., 1999).

Tests on the response to fenhexamid of mutant strains resulted in the identification of two fenhexamid-resistant phenotypes. The major phenotypic class (95% of fenhexamid-resistant isolates) included mutants with high resistance to fenhexamid (Rf: 80–90 and 460–570 based on MIC or EC_{50} values, respectively). The minor phenotypic class, which included mutants with a moderate level of resistance (Rf: <20 and 10–15 based on MIC or EC_{50} values, respectively), appeared 10–14 days after inoculation, when the fungicide concentration may have decreased from the initial value. A dose-dependent decrease of growth was observed with the wild-type and the representative mutant strains tested from both phenotypic classes (Figure 1).

Fitness of mutant strains is an important parameter in assessing the risk for resistance development. Results from tests under laboratory conditions are very informative with regard to the subsequent response of pathogen population in the field (Dekker, 1995). In the present work, study of some fitness-determining characteristics in the wild-type and representative fenhexamid-resistant mutant isolates of both classes, showed that the mutation(s) leading to fenhexamid resistance, in most of the cases, are responsible for some fitness penalties, such as reduced conidial germination, sporulation and sclerotia production (Table 1). However, fenhexamid-resistance had no effect on mycelial growth or on the osmotic sensitivity of mutant isolates.

Cross-resistance

A study of the sensitivity of mutant isolates of *B. cinerea* to other fungicides in comparison with

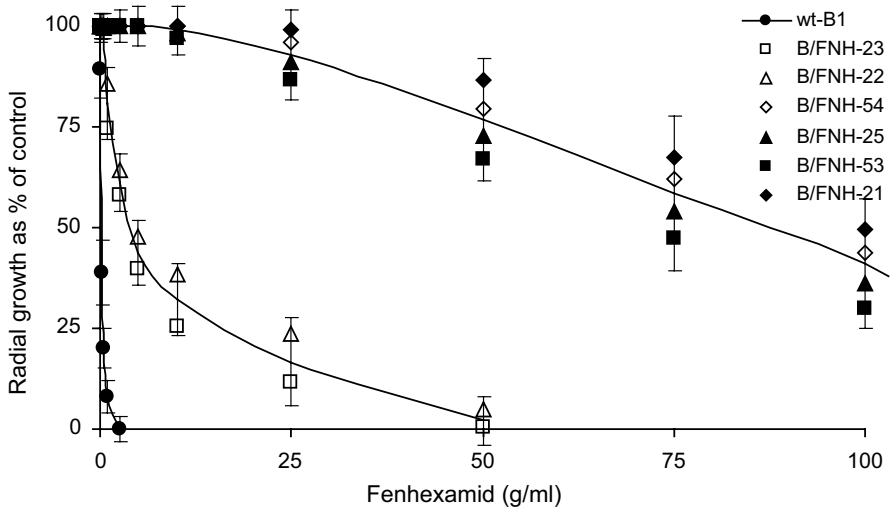

Figure 1. Sensitivity of the wild-type (wt-B_1) and six representative fenhexamid-resistant (B/FNH) isolates of *B. cinerea* to fenhexamid on PDA medium. Measurements were made after 4 days of incubation.

the wild-type strain did not reveal cross-resistance of fenhexamid with benzimidazole, anilinopyrimidine, phenylpyridinamine, guanidine and sterol biosynthesis inhibiting fungicides (Table 2). In contrast, the mutation(s) for resistance to fenhexamid were responsible for an increased sensitivity to fludioxonil and iprodione (Rf: 0.2–0.6 based on EC_{50} values), indicating a negative cross-resistance relationships between phenylpyrroles and dicarboximides with hydroxyanilides (Table 2). However, a cross-resistance relationship (negative or positive) between phenylpyrroles and hydroxyanilides was not revealed after testing the sensitivity of representative fludioxonil-resistant strains B/$FLD_{osm/r}$-17 and B/$FLD_{osm/s}$-42 of *B. cinerea* to fenhexamid. Various cross-resistance patterns between fenhexamid and other fungicides were observed with field isolates of *B. cinerea* (Leroux et al., 1999). It is difficult to find an explanation for the above pattern of cross-resistance between fenhexamid and phenylpyrroles. Genetic studies with an appropriate microorganism are required to unravel the situation.

Table 1. Comparison of mutants of *B. cinerea* resistant to fenhexamid with their parental wild-type strain in respect to some saprophytic fitness parameters on agar medium

Strains	Resistance factor[a] based on EC_{50}[b] (mean ± SE[c])	Radial growth[d]	Osmotic sensitivity[e]	Sporulation[f]	Spore germination[g]	Sclerotia production[h]
wt-B_1		49a[i]	46a[i]	8.4a[i]	87.2a[i]	97.4b[i]
B/FNH-23	11 ± 0.29	49a	46a	8.7a	75.7b	96.8b
B/FNH-22	13 ± 1.06	48a	45a	8.6a	81.3ab	68.4c
B/FNH-53	456 ± 8.67	50a	47a	6.2b	88.3a	99.7b
B/FNH-25	457 ± 11.13	51a	47a	5.8bc	52.8c	114.7a
B/FNH-54	526 ± 9.08	50a	48a	7.9ab	89.4a	95.3bc
B/FNH-21	547 ± 14.34	50a	48a	4.1c	90.7a	70.3c

[a]The ratio of EC_{50} for mutant: EC_{50} for wild-type.
[b]Effective concentration causing 50% reduction in growth rate.
[c]Pooled standard error; three replications.
[d]Mean colony diameter (mm) measurements after 4 days of incubation ($n = 3$).
[e]Mean colony diameter (mm) in presence of KCl (2.5%) after 4 days of inoculation ($n = 3$).
[f]Mean number ($\times 10^6$) of conidia per cm^2 of colony after 10 days of incubation ($n = 3$).
[g]Proportion (%) of germinated conidia after 6 h incubation ($n = 100$).
[h]Mean dry weight of sclerotia (mg) per colony after 10 days of incubation ($n = 3$).
[i]Within columns, values followed by the same letter do not different significantly according to Dunnett's multiple range test ($P = 0.05$).

Table 2. Reduced sensitivity to different fungicides of representative mutant isolates of *B. cinerea* resistant to fenhexamid or to fludioxonil

Fungicide	Wild-type EC_{50}[b] (mean ± SE[c])	Rf[a] based on EC_{50}[b] (mean ± SE[c])					
		B/FNH-21[d]	B/FNH-54[d]	B/FNH-25[d]	B/FNH-53[d]	B/$FLD_{osm/r}$-17[d]	B/$FLD_{osm/s}$-42[d]
Fenhexamid	0.19 ± 0.027	547 ± 19.3	526 ± 28.9	475 ± 13.4	456 ± 18.6	1.4 ± 0.13	1.2 ± 0.28
Fludioxonil	0.005 ± 0.0006	0.6 ± 0.08	0.2 ± 0.04	0.3 ± 0.01	0.4 ± 0.07	3000 ± 23.78	2980 ± 14.96
Iprodione	0.25 ± 0.01	0.3 ± 0.01	0.6 ± 0.01	0.4 ± 0.02	0.2 ± 0.03	100 ± 9.34	100 ± 12.47
Benomyl	0.04 ± 0.008	1.2 ± 0.15	1.4 ± 0.23	1.2 ± 0.27	1.3 ± 0.19	1.2 ± 0.11	1.4 ± 0.31
Fluazinam	0.025 ± 0.004	1.6 ± 0.34	1.4 ± 0.28	1.6 ± 0.12	1.3 ± 0.04	1.4 ± 0.18	1.3 ± 0.26
Cyprodinil	0.05 ± 0.012	1.2 ± 0.23	1.1 ± 0.33	1.3 ± 0.27	1.5 ± 0.02	1.3 ± 0.02	1.1 ± 0.43
Iminoctadine	0.23 ± 0.03	1.4 ± 0.18	1.2 ± 0.04	1.3 ± 0.03	1.1 ± 0.22	—[e]	—
Fenarimol	1.37 ± 0.17	1.6 ± 0.13	1.3 ± 0.01	1.1 ± 0.14	1.4 ± 0.11	—	—
Fenpropimorph	0.17 ± 0.033	2.3 ± 0.34	1.9 ± 0.23	1.7 ± 0.08	2.1 ± 0.27	—	—
Tridemorph	0.23 ± 0.017	1.6 ± 0.17	1.6 ± 0.07	1.3 ± 0.16	1.5 ± 0.17	—	—

[a]Resistance factor.
[b]Effective concentration causing 50% reduction in growth rate.
[c]Pooled standard error; three replications.
[d] B/FNH-mutant strains resistant to fenhexamid; B/FLD-mutant strains resistant to fludioxonil.
[e]Not tested.

Table 3. Effect of fungicides on lesion development following inoculation of cucumber seedlings with sensitive and mutant strains of *B. cinerea* resistant to fenhexamid or to fludioxonil

Fungicide concentration (μg/ml)	Infection of cotyledons (% of control)						
	wt-B_1	B/FNH-21	B/FNH-54	B/FNH-25	B/FNH-53	B/$FLD_{osm/r}$-17	B/$FLD_{osm/s}$-42
No Fungicide	100 (59a[b])[a]	100 (42b)[a]	100 (30bc)[a]	100 (22c)[a]	100 (18c)[a]	100 (56a)[a]	100 (48ab)[a]
Fenhexamid							
2.5	36c	97a	95a	90b	87b	38c	35c
25	27c	88a	90a	78b	75b	26c	24c
100	15c	76a	74a	68b	64b	17c	13c
750	1c	70a	66a	57b	53b	2c	1c
1000	0c	67a	50b	54b	49b	0c	0c
Fludioxonil							
1	28b	17c	21bc	14c	18c	100a	100a
5	6b	3b	5b	0c	4b	100a	96a
10	3b	1b	0b	2b	0b	97a	87a
100	0b	0b	0b	0b	0b	86a	74a

[a]The sum of indices in 16 cotyledons.
[b]Within rows, values followed by the same letter do not differ significantly according to Dunnett's multiple range test ($P = 0.05$).

Pathogenicity and fungicide response of fenhexamid-resistant strains on plants

Pathogenicity studies showed that none of the fenhexamid-resistant strains of *B. cinerea* tested lose their ability to cause infection on cotyledons of cucumber plants. However, all the highly resistant isolates presented a reduction of infection ability (30–70%) compared with the wild-type strain (Table 3).

The results of preventive applications of fenhexamid against the wild-type and the representative mutant strains of *B. cinerea* that were resistant to fenhexamid (B/FNH) or to fludioxonil (B/FLD) are shown in Table 3. Fenhexamid was effective against the wild-type and the mutant strains which were resistant to fludioxonil. However, the fenhexamid-resistant strains, were not controlled by preventive fenhexamid applications, even at the concentration of 1000 μg a.s. ml^{-1} (Table 3, Figure 2). Moreover, as in the case of *in vitro* tests, fludioxonil was slightly more effective against grey mould disease caused by fenhexamid-resistant mutants compared with the wild-type strain of *B. cinerea*.

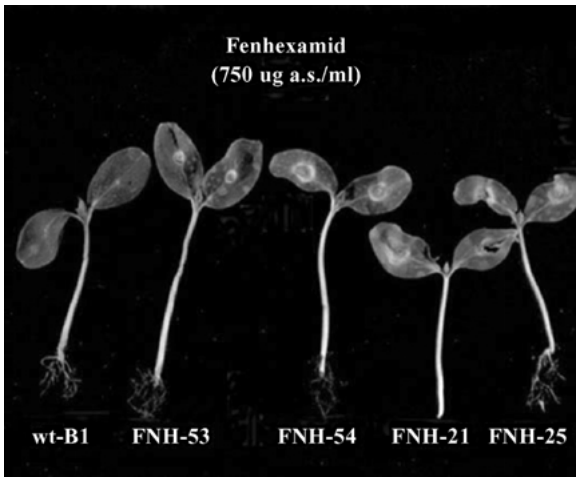

Figure 2. Effectiveness of Teldor 50 WP (fenhexamid) on lesion development by the wild-type (wt-B_1) and highly fenhexamid-resistant mutant isolates (B/FNH) of *B. cinerea* on cucumber seedlings.

Conclusions

Taking into consideration the high mutation frequency towards highly fenhexamid-resistant mutants, the expression of such resistance on plants, and moreover the high potential of *B. cinerea* for quick adaptation to changes in environmental conditions, a considerable inherent resistance risk to fenhexamid has to be considered for the control of grey mould disease in glasshouse crops. However, the reduction in pathogenicity and other fitness characteristics of mutant strains indicates that the risk of practical resistance problems may be lower under open-air conditions. A decrease of resistance population(s) must be expected during the intervals of the absence of

fenhexamid from the fungicide applications. Certainly, more data on the survival and competition of the fenhexamid-resistant mutants is needed to indicate the level of the risk for field failures of hydroxyanilides in the future. However, a loss in fitness could be overcome by continued selection in natural populations for both fenhexamid resistance and normal phytopathogenicity. A gradual development of resistance may occur as it was happen in the case of DMIs (De Waard, 1994; Brent and Hollomon, 1998). Therefore, it is necessary to use fenhexamid in carefully designed anti-resistance strategies to maintain its effectiveness.

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