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# Effect of phenylpyrrole-resistance mutations on ecological fitness of *Botrytis cinerea* and their genetical basis in *Ustilago maydis*

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## Abstract

Mutants of *Botrytis cinerea* and *Ustilago maydis* highly resistant to fludioxonil were isolated at a high frequency, after nitrosoguanidine or UV mutagenesis, respectively, and selection on media containing fludioxonil. Tests on the response of mutant strains to high osmotic pressure resulted in the identification of two fludioxonil-resistant phenotypes ($FLD_{osm/s}$ and $FLD_{osm/r}$), regarding the sensitivity to high osmolarity. Approximately 95% of fludioxonil-resistant mutants were found to be more sensitive to high osmotic pressure than the wild-type parent strains. Genetic analysis of phenylpyrrole-resistance in the phytopathogenic basidiomycete *U. maydis*, showed that fludioxonil-resistance was coded by three unlinked chromosomal loci (*U*/*fld*-1, *U*/*fld*-2 and *U*/*fld*-3), from which only the *U*/*fld*-1 mutation coded an osmotic sensitivity similar to that of the wild-types. Cross-resistance studies with fungicides from other chemical groups showed that the mutations for resistance to phenylpyrroles affect the sensitivity of mutant strains to the aromatic hydrocarbon and dicarboximide fungicides, but not to the benzimidazoles, anilinopyrimidines, phenylpyridinamines, hydroxyanilides or the sterol biosynthesis inhibiting fungicides. A study of fitness parameters in the wild-type and fludioxonil-resistant mutants of *B. cinerea*, showed that all osmotic sensitive ($B/FLD_{osm/s}$) isolates had significant reductions in the characteristics determining saprophytic fitness such as mycelial growth, sporulation, conidial germination and sclerotial production. Contrary to that, with the exception of mycelial growth, the fitness parameters were unaffected or only slightly affected in most of the osmotic resistant ($B/FLD_{osm/r}$) isolates. Tests on cucumber seedlings showed that the osmotic-sensitive strains were significantly less pathogenic compared with the wild-type and $B/FLD_{osm/r}$ strains of *B. cinerea*. Preventative applications of the commercial products Saphire 50 WP (fludioxonil) and Rovral 50 WP (iprodione) were effective against lesion development on cotyledons by the wild-type and the mutant strains of *B. cinerea* that were resistant to the anilinopyrimidine cyprodinil (B/CPL-27) and to the hydroxyanilide fenhexamid (B/FNH-21), but ineffective, even at high concentrations, against disease caused by the fludioxonil-resistant isolates (B/FLD) and a mutant strain resistant to the dicarboximide iprodione (B/IPR-1). Experiments on the stability of the fludioxonil-resistant phenotype showed a reduction of resistance, mainly in osmotic-sensitive isolates, when the mutants were grown on inhibitor-free medium. A rapid recovery of the high resistance was observed after mutants were returned to the selection medium. Studies on the competitive ability of mutant isolates against the wild-type parent strain of *B. cinerea*, by applications of a mixed conidial population, showed that, *in vitro*, all mutants were less competitive than the wild-type strain. However, the competitive ability of osmotic-resistant mutants was higher than the osmotic-sensitive ones. Furthermore, competition tests, *in planta*, showed a significant reduction of the frequency of both phenylpyrrole-resistant phenotypes, with a respective increase in the population of the wild-type strain of the pathogen.

## Introduction

Grey mould caused by *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) is one of the most serious diseases on a wide range of crops with world-wide economic importance (Jarvis, 1980). Chemical control remains the main measure to reduce the incidence of grey mould in most crops, especially under greenhouse conditions. However, in the last three decades, chemical control of the pathogen has suffered heavily from the development of resistance to the intensively used benzimidazoles and dicarboximides (Lorenz, 1988; Smith, 1988).

During the last few years, fungicide research has produced a diverse range of antifungal agents with novel modes of action, which are expected to have a significant impact on the control of grey mould disease (Leroux, 1996; Rosslenbroich and Stuebler, 2000). These new classes of fungicides include anilinopyrimidines, phenylpyridinamines, hydroxyanilides, phenylpyrroles and strobilurins. However, as *B. cinerea* is a classical 'high risk' pathogen from the view of resistance management (Brent and Hollomon, 1998), almost all the newly introduced botryticides face the possibility of resistance development. Indeed, such resistance risk has already been demonstrated for phenylpyrroles, anilinopyrimidines and hydroxyanilides with laboratory mutant strains of the fungus (Faretra and Pollastro, 1993; Hilber and Hilber-Bodmer, 1998; Chapeland et al., 1999; Leroux et al., 1999; Ziogas and Kalamarakis, 2001; Ziogas et al., 2003).

Fludioxonil and fenpiclonil are phenylpyrrole fungicides derived from the natural antibiotic pyrrolnitrin produced by *Pseudomonas pyrociniae* (Nishida et al., 1965). They are broad-spectrum fungicides used against fungal species among Ascomycetes, Basidiomycetes and Deuteromycetes (Koch and Leadbeater, 1992). Fenpiclonil is commercially available as a seed dressing, while fludioxonil, due to its photostability, is used as a foliar fungicide, with a high preventive activity against *B. cinerea* (Gehmann et al., 1990).

Laboratory mutants resistant to both phenylpyrroles and dicarboximides have been reported for *B. cinerea* and other fungal species (Leroux et al., 1992, Faretra and Pollastro, 1993; Hilber et al., 1995; Ziogas and Kalamarakis, 2001). Interestingly, *B. cinerea* field strains resistant to dicarboximides do not show resistance to phenylpyrroles and practical resistance problems have not yet been reported (Leroux et al., 1999). Due to the differences observed between laboratory and field isolates of *B. cinerea* the question of resistance risk to phenylpyrroles is still unclear. Reduced fitness has been suggested as a possible explanation for the absence of field isolates resistant to phenylpyrroles. However, little information is available on the effect of phenylpyrrole-resistance mutations on fitness of mutant strains, and many questions have to be answered. Thus, the first objective of the present study was to assess the impact of mutations for resistance to phenylpyrroles on the fitness characteristics, such as virulence, stability of resistance and competitive ability of mutant strains of *B. cinerea*. Furthermore, to expand our knowledge of the genetic basis of resistance to phenylpyrroles, the genetic control of resistance to fludioxonil in the phytopathogenic basidiomycete *U. maydis* was studied. The knowledge of the genetic control of resistance and the impact of mutations on the ecological fitness of mutants will help to understand the resistance phenomenon and assist in predicting the risk related to the build-up of field resistance to phenylpyrroles in *B. cinerea*.

## Materials and methods

### *Fungal strains and culture conditions*

The wild-type strain wt-$B_1$ of *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) isolated from tomato in Greece was used to obtain fludioxonil-resistant isolates (B/FLD). Mutant isolates B/$IPR_{osm/s}$-1, B/CPL-27 and B/FNH-21 that were resistant to the dicarboximide iprodione, the anilinopyrimidine cyprodinil and the hydroxyanilide fenhexamid respectively, were obtained from the collection of the Laboratory of Pesticide Science of Agricultural University of Athens. These isolates 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 $day^{-1}$ light and 70% relative humidity (RH). 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.

For the study of genetic control of phenylpyrrole-resistance, two compatible wild-type strains 201 and 501 of the heterothallic basidiomycete *Ustilago maydis* isolated from infected corn plants, were used to obtain fludioxonil-resistant isolates (U/FLD). Monosporidial isolates of *U. maydis* were grown on a simplified Ustilago Complete Agar medium (UCM) according to Holliday (1961) in a controlled climate cabinet at 29 °C and 70% RH in the dark. Sporidia were obtained by growing the strains in complete liquid medium at 29 °C on a rotary shaker at 150 rpm. For long-term storage the isolates were maintained on UCM agar slants at 10 °C in the dark and single-spore transfers were made once a month.

*Fungicides*

The fungicides used in *in vitro* tests were pure technical grade. Fludioxonil, fenpiclonil and cyprodinil were kindly supplied by Syngenta Crop Protection AG (Basle, Switzerland), fluazinam and chlorothalonil by ISK Biosciences Ltd. (Kent, UK), iprodione by Rhone Poulenc Agrochimie S.A. (Lyon, France), benomyl and chloroneb by Du Pont de Nemours and Co. (Wilmington, DE, USA), chlozolinate by Enichem Agricultura (Milano, Italy), tecnazene by Zeneca Agrochemicals (Farnham, UK), procymidone, tolclofos-methyl and the wettable powder formulation 'Sumico' (carbendazim + diethofencarb) by Sumitomo Chemical Co. (Osaka, Japan), quintozene (PCNB) by Uniroyal Chemical Ltd. (Slough, Berkshire, UK), fenhexamid and triadimenol by Bayer CropScience AG (Leverkusen, Germany) and fenpropimorph by Dr. R. Maag Ltd. (Dielsdorf, Switzerland). Stock solutions of fungicides were made in ethanol, with the exception of benomyl and fenhexamid which were dissolved in acetone and in isopropanol, respectively.

In the pathogenicity tests, aqueous suspensions of the commercial products Saphire 50 WP (500 g $kg^{-1}$ fludioxonil), Rovral 50 WP (500 g $kg^{-1}$ iprodione), Teldor 50 WP (500 g $kg^{-1}$ fenhexamid), Chorus 75 WP (750 g $kg^{-1}$ cyprodinil), Benlate 50 WP (500 g $kg^{-1}$ benomyl) and Sumico 50 WP (250 g $kg^{-1}$ carbendazim and 250 g $kg^{-1}$ diethofencarb) were used. The fungicide concentrations were expressed as $\mu$g active ingredient (a.i.) $ml^{-1}$.

*Mutation induction*

Conidial suspensions ($1.8 \times 10^7$ 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 rpm, with 10 $\mu$g $ml^{-1}$ *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (Ziogas and Girgis, 1993) for 4 h in the dark and washed twice with sterile distilled water. Conidia were resuspended in water and were plated on PDA containing 50 $\mu$g $ml^{-1}$ fludioxonil and incubated at 22 °C for 15 days.

In the case of *U. maydis* a suspension of $10^7$ $ml^{-1}$ log phase sporidia was exposed, with continuous agitation, to ultraviolet irradiation (TUV Philips, 15W, 253.7 nm) for 3.5 min. After irradiation they were kept for 30 min in the dark to minimize photorepair of radiation damage, and then plated on UCM agar medium amended with 75 $\mu$g $ml^{-1}$ fludioxonil and incubated at 29 °C for 20 days.

Selected resistant isolates B/FLD and U/FLD were maintained on PDA or UCM agar slants containing 0.1 $\mu$g $ml^{-1}$ and 0.25 $\mu$g $ml^{-1}$ fludioxonil, the minimal inhibitory concentration (MIC) for the wild-type parent strains of *B. cinerea* and *U. maydis*, respectively.

In vitro *fungitoxicity tests*

The *in vitro* fungicide sensitivity of the wild-type and mutant strains of *B. cinerea* was assessed by measuring the radial growth on PDA plates containing a range of concentrations of each fungicide to determine the $EC_{50}$ (the concentration causing a 50% reduction of growth) and MIC (minimal inhibitory concentration) values. PDA plates were inoculated with 2 mm mycelial plugs, from water–agar (WA) medium on which conidia of *B. cinerea* had been allowed to germinate, after overnight incubation at 22 °C. 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 diameter of mycelial colonies after incubation for 4–5 days at 22 °C in the dark.

For *U. maydis*, the toxicity of fungicides on the wild-type and mutant strains was measured on both agar and liquid media. In the first case the colony forming ability was determined by

spreading sporidia on the surface of UCM containing various concentrations of the fungicides to determine the $EC_{50}$ and MIC values. The number of sporidia capable of producing colonies in control and treated media was recorded after 4 days. For toxicity measurements in liquid culture, approximately $5 \times 10^4$ actively growing sporidia per ml were added to 50 ml of the synthetic glucose liquid medium of Coursen and Sisler (1960) containing the fungicide at the desirable concentration. The cultures were incubated at 29 °C on a rotary shaker (150 rpm) for 22–24 h. Growth rates were determined as changes in optical density (OD) (at 450 nm) of sporidial suspensions, or by dry weight measurements.

The fungicides were added aseptically to sterilized growth medium from stock solutions prior to inoculation. In all cases, the final amount of solvent never exceeded 1% (v:v) in treated and control samples. At least six concentrations with three replicas for each fungicide were used to obtain the respective fungitoxicity curves. The $EC_{50}$ 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 saprophytic fitness of phenylpyrrole-resistant mutants of Botrytis cinerea*

Mutants of *B. cinerea* were tested for mycelial growth rate, sporulation, spore germination, sclerotial production, sensitivity to osmotic pressure, stability of resistant phenotypes and competitive ability compared with the wild-type parent strain. Three 2 mm mycelial WA-plugs for each strain were transferred to the centre of PDA plates for radial growth measurements. After incubation at 22 °C in the dark, the colony diameter of each isolate was measured at 24 h intervals. To determine conidial production in the absence of fungicides, PDA-plates were inoculated with a conidial suspension ($10^5$ conidia per plate) and were incubated for 8–10 days at 22 °C with 14 h $day^{-1}$ light. The total mycelial mass produced in each dish was transferred to a 250 ml Erlenmeyer flask with 20 ml de-ionized water. The flasks were agitated vigorously and the concentration of conidia in the resulting spore suspension, after filtration through cheesecloth, was determined with a Neubauer haemocytometer and expressed as number of conidia $cm^{-2}$of the PDA culture. Spore germination and sclerotial production were determined after 6 h and 20 days incubation, respectively, on PDA medium in the dark. The sensitivity of fludioxonil-resistant isolates to high osmotic pressure as compared to that of the wild-type strain was determined after 4 days incubation on PDA amended with 25 mg $ml^{-1}$ KCl.

The stability of resistant phenotypes was assessed by successive transfers of selected mutants in fungicide-free growth medium. The sensitivity to fludioxonil was measured after every subculture of mutant isolates at the concentration of 10 $\mu$g $ml^{-1}$ fludioxonil, which resulted in approximately 40% growth inhibition. The *in vitro* competitive ability of B/FLD-mutant isolates was studied with mixed inocula of fludioxonil-resistant isolates and the wild-type parent strain of *B. cinerea*, in the absence of fungicide treatment. PDA plates were inoculated with a mixed conidial suspension ($10^5$ conidia per plate) of each mutant with the wild-type strain, at the proportions of 20:80, 50:50 and 80:20, and were incubated for 8–10 days at 22 °C with 14 h $day^{-1}$ light. At the end of the incubation period the total mycelial mass was harvested and the resulting spore suspension was used to inoculate new PDA plates. A random sample of at least 50 single conidial colonies was examined for fludioxonil sensitivity at the MIC (0.1 $\mu$g $ml^{-1}$) for the wild-type strain.

*Study of pathogenicity, resistance expression and competitive ability in planta of phenylpyrrole-resistant mutant isolates of* Botrytis cinerea

Pathogenicity and *in planta* fungicide resistance of various mutant isolates of *B. cinerea*, were determined by examining symptom severity caused by each strain on cucumber seedlings (*Cucumis sativus*, cv. Telegraph) according to the method described previously (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 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% rot; 2, 21–50% rot and 4, rot on more than 50% of cotyledon surface. To determine the competitive ability of B/FLD-mutant strains *in planta*, mixed sporidial suspensions of each mutant isolate with the wild-type parent strain, at three different ratios (20:80, 50:50 and 80:20), were sprayed on cucumber plants. The inoculated plants were placed in a humidity chamber (100% RH at 22 °C with 14 h light) for 10–12 days. The total conidial mass produced on each plant was transferred to a flask with 20 ml sterile water. The flasks were agitated vigorously and after filtration through cheesecloth, the resulting conidial suspension was used to inoculate new plants. At the same time a conidial sample was plated on PDA, a random sample of at least 50 single colonies was tested for their sensitivity to fludioxonil on PDA plates at the MIC for the wild-type strain.

*Genetic studies of phenylpyrrole-resistance in* Ustilago maydis

The techniques described by Holliday (1961), with minor modifications (Markoglou and Ziogas, 1999), were used for crossing each mutant with the compatible wild-type and other mutant strains on 10-day-old maize seedlings (*Zea mays*, cv. B-73) and for random genetic analysis of progeny. Each U/FLD mutant isolate was crossed first with the compatible wild-type strain and then all possible $R \times R$ crosses were made. The mixed sporidial suspension ($10^6$ sporidia $ml^{-1}$) of each cross was injected just above the first node of the seedlings. The inoculated seedlings (four seedlings per 17-cm pot, three pots per cross) were placed in a humidity chamber (100% RH at 29 °C) for 48 h, after which they were returned to the growth room (14 h $day^{-1}$ light at 29 °C and 60% RH) for 20 days. The recognition of genetic control of resistance to phenylpyrroles and sensitivity to osmotic pressure was based on the random analysis of at least 100 progeny for their sensitivity to fludioxonil and to 25 mg $ml^{-1}$ KCl, on UCM agar medium using a 26-pin replicator. In order to avoid any progeny preference in the isolation procedure, all of the colonies that originated from single spores that appeared in a plate or part of the plate were collected. The progeny segregation was statistically analysed by the means of the Chi-square ($x^2$) tests with 1 degree of freedom and at $p = 0.05$ level of significance.

Disease development was evaluated according to: (a) the number of infected or dead plants; (b) the time taken for galls to appear and mature; and (c) the production of the mature teliospores. Approximately 10 g dry weight of gall from each cross were ground in a mortar after addition of water, and were used for the measurement of teliospore production. The maturity of galls was determined by light microscopy of thin sections.

*Statistical analysis*

Data analyses were made with the Statistical Analysis System (JMP, SAS Institute, Inc., Cary, NC, USA). The growth rate and the $EC_{50}$ 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, sclerotial production, osmotic sensitivity, competitiveness and pathogenicity ratings of isolates.

## Results

*Isolation and characterization of fludioxonil-resistant isolates of* B. cinerea

Mutant strains of *B. cinerea* resistant to fludioxonil were readily isolated at high frequency after nitrosoguanidine mutagenesis, indicating the existence of a genetical potential for development of field resistance towards this particular fungicide. Approximately $1.2 \times 10^6$ mutated conidia of the wild-type strain, wt-$B_1$, which survived the mutagenic treatment (95% lethality), were plated onto PDA containing 50 $\mu g$ $ml^{-1}$ fludioxonil. From this selection medium, 1573 resistant colonies were obtained during the first 10 days of incubation, indicating a mutation frequency of $1.3 \times 10^{-3}$. Most of the resistant isolates appeared between the 5th and 7th day of incubation. The mycelial growth of the wild-type isolate was inhibited 50%

($EC_{50}$) and 100% (MIC) at the concentrations of 0.005 and 0.1 $\mu$g ml$^{-1}$ fludioxonil. Tests on the response of B/FLD isolates to the presence of fludioxonil in growth medium, showed that all isolates were highly resistant to fludioxonil, with a resistance factor (Rf) based on $EC_{50}$ values ranging from $2980 \pm 15$ to $5080 \pm 11$ (Table 1). A dose-dependent decrease in growth was observed with the wild-type (wt-$B_1$) and mutant isolates.

*Saprophytic fitness of fludioxonil-resistant strains of* B. cinerea

Tests for evaluation of osmosensitivity of isolates on PDA amended with 25 mg ml$^{-1}$ KCl, resulted in the identification of two fludioxonil-resistant phenotypes, osmotic-resistant (B/$FLD_{osm/r}$) and osmotic-sensitive (B/$FLD_{osm/s}$) (Table 1). The B/$FLD_{osm/s}$ phenotypic class was observed at a high frequency (approximately 95% of fludioxonil-resistant mutants). The mycelial growth of these mutants was highly inhibited (80–90% compared with the wild-type strain) on medium containing 25 mg ml$^{-1}$ KCl. The B/$FLD_{osm/r}$ phenotypic class appeared during the first 5–6 days of incubation and included mutants with tolerance to increased osmotic pressure equivalent to the wild-type. Comparison of other fitness determining parameters such as rate of mycelial growth, sporulation, spore germination and sclerotial production, between fludioxonil-resistant mutants and the parent strain of *B. cinerea*, showed that these characteristics were significantly reduced in all osmotic-sensitive (B/$FLD_{osm/s}$) isolates. Contrary to that, fitness parameters, except radial growth, were unaffected or only slightly affected in most osmotic-resistant isolates (B/$FLD_{osm/r}$) (Table 1).

*Cross-resistance*

To recognize cross-resistance relationships of phenylpyrroles with fungicides from other chemical groups, fungitoxicity studies were undertaken with representative fludioxonil (B/FLD), iprodione (B/$IPR_{osm/s}$-1), cyprodinil (B/CPL-27) and fenhexamid (B/FNH-21) resistant strains of *B. cinerea*. As shown in Table 2, the mutation(s) for resistance to fludioxonil reduced the sensitivity to dicarboximides (iprodione, procymidone and chlozolinate) and to aromatic hydrocarbons (quintozene, chloroneb, tecnazene and tolclofos-methyl). A mutant (B/$IPR_{osm/s}$-1), isolated from iprodione-selection medium, showed more than a 3000-fold decreased sensitivity to fludioxonil and

*Table 1.* Comparison of *Botrytis cinerea* isolates resistant to fludioxonil with their parental wild-type strain with respect to 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] | Sclerotial production[h] |
|---|---|---|---|---|---|---|
| wt-$B_1$ | | 48a[i] | 99a[i] | 8.4a[i] | 87.8a[i] | 97.6a[i] |
| B/$FLD_{osm/s}$-19 | 4960 ± 19.18 | 24cd | 17b | 3.5c | 68.2b | 36.1d |
| B/$FLD_{osm/s}$-21 | 5030 ± 25.67 | 23d | 13bc | 1.7d | 30.3d | 38.4d |
| B/$FLD_{osm/s}$-26 | 4910 ± 13.92 | 20d | 11c | 1.9d | 25.9d | 40.2d |
| B/$FLD_{osm/s}$-42 | 2980 ± 14.96 | 25c | 20b | 2.4cd | 37.7d | 39.2d |
| B/$FLD_{osm/r}$-5 | 5080 ± 18.26 | 36b | 100a | 7.9ab | 87.4a | 88.9b |
| B/$FLD_{osm/r}$-17 | 3000 ± 11.78 | 29bc | 96a | 6.5b | 61.6c | 70.8c |
| B/$FLD_{osm/r}$-22 | 3840 ± 22.36 | 26c | 95a | 5.9b | 59.7c | 76.4c |
| B/$FLD_{osm/r}$-24 | 4370 ± 16.98 | 36b | 98a | 8.6a | 86.7a | 96.4a |
| B/$FLD_{osm/r}$-32 | 5020 ± 14.04 | 34b | 100a | 8.2a | 84.3a | 98.6a |
| B/$FLD_{osm/r}$-33 | 4780 ± 20.33 | 33b | 99a | 7.2ab | 80.7ab | 95.8a |

[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 incubation ($n = 3$).
[e]Growth (as % of control) in presence of KCl (25 mg ml$^{-1}$) after 4 days inoculation ($n = 3$).
[f]Mean number ($\times 10^6$) of conidia per cm$^2$ of colony after 20 days incubation ($n = 3$).
[g]Percentage of germinated conidia after 6 h incubation ($n = 100$).
[h]Mean dry weight of sclerotia (mg) per plate after 10 days incubation ($n = 3$).
[i]Within columns, values followed by the same letter do not differ significantly according to Dunnett's multiple range test ($P = 0.05$).

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

| Fungicide | Wild-type $EC_{50}$[b] ($\mu g\ ml^{-1}$) (mean ± SE[c]) | Resistance factor[a] based on $EC_{50}$[b] (mean ± SE[c]) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $B/FLD_{osm/s}$-19[d] | $B/FLD_{osm/s}$-21[d] | $B/FLD_{osm/s}$-26[d] | $B/FLD_{osm/r}$-5[d] | $B/FLD_{osm/r}$-24[d] | $B/FLD_{osm/r}$-32[d] | $B/IPR_{osm/s}$-1[d] | B/CPL-27[d] | B/FNH-21[d] |
| Fludioxonil | 0.005 ± 0.0006 | 4960 ± 19.18 | 5030 ± 25.67 | 4910 ± 13.92 | 5080 ± 18.26 | 4370 ± 16.98 | 5020 ± 14.04 | 3200 ± 43.28 | 1.4 ± 0.23 | 0.6 ± 0.08 |
| Iprodione | 0.25 ± 0.065 | 100 ± 12.47 | 110 ± 12.28 | 100 ± 9.36 | 125 ± 7.89 | 120 ± 13.26 | 117 ± 11.66 | 400 ± 16.68 | 1.3 ± 0.41 | 0.3 ± 0.01 |
| Procymidone | 2.5 ± 0.13 | 27 ± 5.62 | 25 ± 4.28 | 30 ± 6.92 | 28 ± 5.02 | 32 ± 8.86 | 27 ± 2.18 | 45 ± 8.34 | 1.0 ± 0.03 | – |
| Chlozolinate | 4.78 ± 1.62 | 42 ± 9.37 | 45 ± 8.23 | 40 ± 6.17 | 48 ± 11.73 | 46 ± 5.23 | 42 ± 6.54 | 45 ± 7.53 | 1.2 ± 0.07 | – |
| Quintozene (PCNB) | 0.25 ± 0.04 | 100 ± 8.23 | 98 ± 5.13 | 104 ± 10.03 | 100 ± 8.37 | 106 ± 11.03 | 100 ± 9.77 | 100 ± 14.62 | – | – |
| Chloroneb | 0.65 ± 0.12 | 130 ± 16.37 | 135 ± 12.56 | 128 ± 11.17 | 148 ± 16.72 | 140 ± 13.18 | 133 ± 9.18 | 125 ± 11.31 | 1.1 ± 0.11 | – |
| Tecnazene | 0.58 ± 0.02 | 79 ± 14.31 | 85 ± 9.23 | 74 ± 11.03 | 78 ± 7.34 | 84 ± 10.85 | 80 ± 13.67 | 80 ± 23.17 | – | – |
| Tolclofos-methyl | 0.14 ± 0.04 | 724 ± 30.23 | 750 ± 23.78 | 675 ± 31.13 | 720 ± 12.17 | 680 ± 23.58 | 748 ± 30.06 | 720 ± 23.97 | 1.2 ± 0.34 | – |
| Benomyl | 0.04 ± 0.005 | 1.4 ± 0.31 | 1.1 ± 0.12 | 1.3 ± 0.27 | 1.3 ± 0.06 | 1.0 ± 0.02 | 1.4 ± 0.31 | 1.0 ± 0.16 | 1.0 ± 0.16 | 1.2 ± 0.15 |
| Carbendazim + Diethofencarb | 0.18 ± 0.03 | 1.2 ± 0.12 | 1.6 ± 0.13 | 1.1 ± 0.22 | 1.3 ± 0.43 | 1.6 ± 0.06 | 1.0 ± 0.04 | 1.0 ± 0.28 | | – – |
| Cyprodinil | 0.05 ± 0.008 | 1.1 ± 0.43 | 1.0 ± 0.07 | 1.2 ± 0.36 | 1.6 ± 0.54 | 1.2 ± 0.12 | 1.7 ± 0.29 | –[e] | 125 ± 12.18 | 1.2 ± 0.23 |
| Fluazinam | 0.025 ± 0.004 | 1.3 ± 0.26 | 1.5 ± 0.72 | 1.1 ± 0.07 | 1.4 ± 0.07 | 1.0 ± 0.23 | 1.3 ± 0.12 | – | 1.2 ± 0.06 | 1.6 ± 0.34 |
| Fenhexamid | 0.19 ± 0.027 | 1.2 ± 0.28 | 1.3 ± 0.18 | 1.2 ± 0.42 | 1.4 ± 0.06 | 1.4 ± 0.21 | 1.3 ± 0.35 | – | 1.1 ± 0.22 | 547 ± 19.34 |
| Chlorothalonil | 0.06 ± 0.002 | 1.0 ± 0.02 | 1.2 ± 0.13 | 1.0 ± 0.05 | 1.9 ± 0.53 | 2.1 ± 0.52 | 2.3 ± 0.78 | – | 1.3 ± 0.23 | – |
| Triadimenol | 2.53 ± 0.19 | 1.1 ± 0.04 | 1.1 ± 0.17 | 1.3 ± 0.19 | 1.1 ± 0.03 | 1.0 ± 0.21 | 1.1 ± 0.41 | – | 1.0 ± 0.12 | – |
| Fenpropimorph | 0.18 ± 0.013 | 1.2 ± 0.18 | 1.0 ± 0.03 | 2.14 ± 0.72 | 1.9 ± 0.33 | 1.9 ± 0.38 | 1.7 ± 0.93 | 1.1 ± 0.16 | – | 2.3 ± 0.34 |

[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]B/FLD mutant strains resistant to fludioxonil; B/IPR-1 mutant strain resistant to iprodione; B/CPL-27 mutant strain resistant to cyprodinil; B/FNH-21 mutant strain resistant to fenhexamid.
[e]Not tested.

at least 45- to 720-fold resistance to the aromatic hydrocarbon and dicarboximide fungicides (Table 2). Contrary to that, both mutant strains (B/FLD and B/IPR) were as sensitive as the wild-type to the benzimidazoles (benomyl), benzimidazoles + phenylcarbamates (carbendazim and diethofencarb), anilinopyrimidines (cyprodinil), phenylpyridinamines (fluazinam), hydroxyanilides (fenhexamid), sterol biosynthesis inhibiting fungicides (triadimenol and fenpropimorph) and to chlorothalonil. Moreover, fungitoxicity tests with B/CPL-27 mutant strain of *B. cinerea* showed that the mutations for resistance to anilinopyrimidines did not affect the sensitivity of the mutant to phenylpyrroles and aromatic hydrocarbon and dicarboximide fungicides. In contrast, the mutation(s) for resistance to fenhexamid were responsible for a slightly increased sensitivity to fludioxonil and iprodione (Rf: 0.3–0.6 based on $EC_{50}$ values) in all six mutant strains tested, 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 of *B. cinerea* to fenhexamid (Table 2).

A similar pattern of cross-resistance of phenylpyrroles with fungicides of other chemical groups was also found after testing fungicide sensitivity of fludioxonil-resistant mutant isolates of *U. maydis* (results not shown).

### *Stability and competitive ability* in vitro *of fludioxonil-resistant strains of* B. cinerea

Growth of mutant strains in fungicide-free medium resulted in a rapid decrease in fludioxonil resistance, particularly in osmotic-sensitive phenotypes. The resistance to fludioxonil was reduced by up to 80% after seven transfers of both phenotypes in fludioxonil-free medium. However, a rapid recovery of the initial level of resistance was observed when the mutants were subcultured (up to three transfers) on medium containing 10 $\mu$g ml$^{-1}$ fludioxonil (Figure 1). *In vitro* studies showed that the mutation(s) for resistance to fludioxonil had significant adverse effects on the ability of mutants to compete with the wild-type

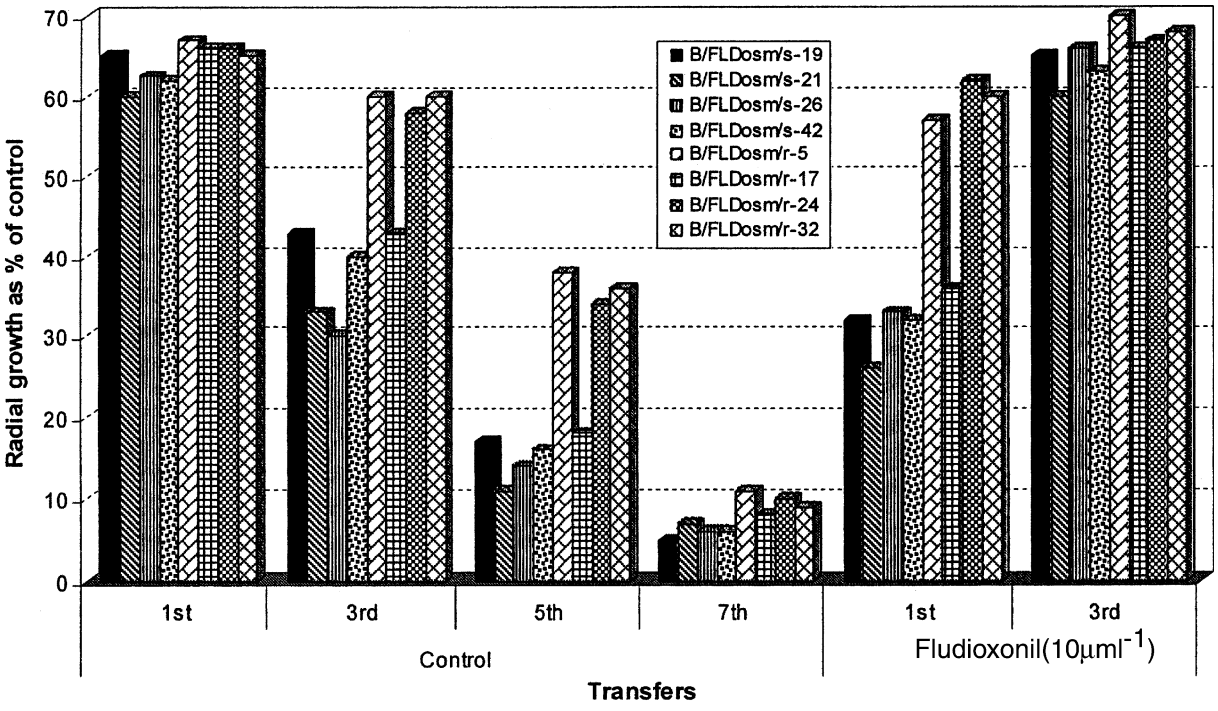


*Figure 1.* Growth of eight representative fludioxonil-resistant isolates of *Botrytis cinerea* at 10 $\mu$g ml$^{-1}$ fludioxonil, after subculturing on PDA medium with or without fludioxonil (10 $\mu$g ml$^{-1}$). Measurements were made after 4 days at 22 °C.

strain (Figure 2). After three subcultures in fungicide-free medium, a rapid decline in the frequency of all osmotic-sensitive isolates was observed, indicating that the resistant isolates were weak competitors against the wild-type (fludioxonil-sensitive) strain, presumably, due to their reduced growth rate, sporulation and/or spore germination (Table 1). The competitiveness of most osmotic-resistant mutant isolates was higher than the osmotic-sensitive ones, and the proportion of resistant isolates remained stable after three transfers in fungicide-free medium. However, after subculturing five times, a significant reduction in the proportion of all osmotic-resistant isolates was observed.

*Pathogenicity, expression of resistance and competitiveness* in planta *of fludioxonil-resistant strains of* B. cinerea

Pathogenicity studies showed that none of the fludioxonil-resistant strains of *B. cinerea* tested lost their ability to cause infection on cotyledons of cucumber plants. However, all osmotic sensitive isolates had a significantly reduced infection ability (60–70%) compared with the wild-type. On the contrary, only a slight reduction (10–20%) in pathogenicity was observed in the case of osmotic-resistant isolates (Table 3).

The results of preventive applications of fludioxonil against the wild-type and the representative mutant strains of *B. cinerea* that were resistant to fludioxonil (B/FLD), iprodione (B/IPR$_{osm/s}$-1), fenhexamid (B/FNH-21) and cyprodinil (B/CPL-27) are shown in Table 3. Fludioxonil was found to be highly effective against the wild-type and the mutant strains of *B. cinerea* which were resistant to the anilinopyrimidines and to the hydroxyanilides. Moreover, as in the case of *in vitro* tests, fludioxonil and iprodione were slightly more effective against grey mould caused by a fenhexamid-resistant mutant compared with the wild-type strain of the pathogen. The fludioxonil- and iprodione-resistant strains could not be controlled by preventive applications even at high concentrations of the commercial formulations of Saphire and Rovral (Table 3). However, preventive applications of the commercial products Teldor 50 WP (fenhexamid), Chorus 75 WP (cyprodinil), Benlate 50 WP (benomyl) and Sumico 50 WP (carbenda-

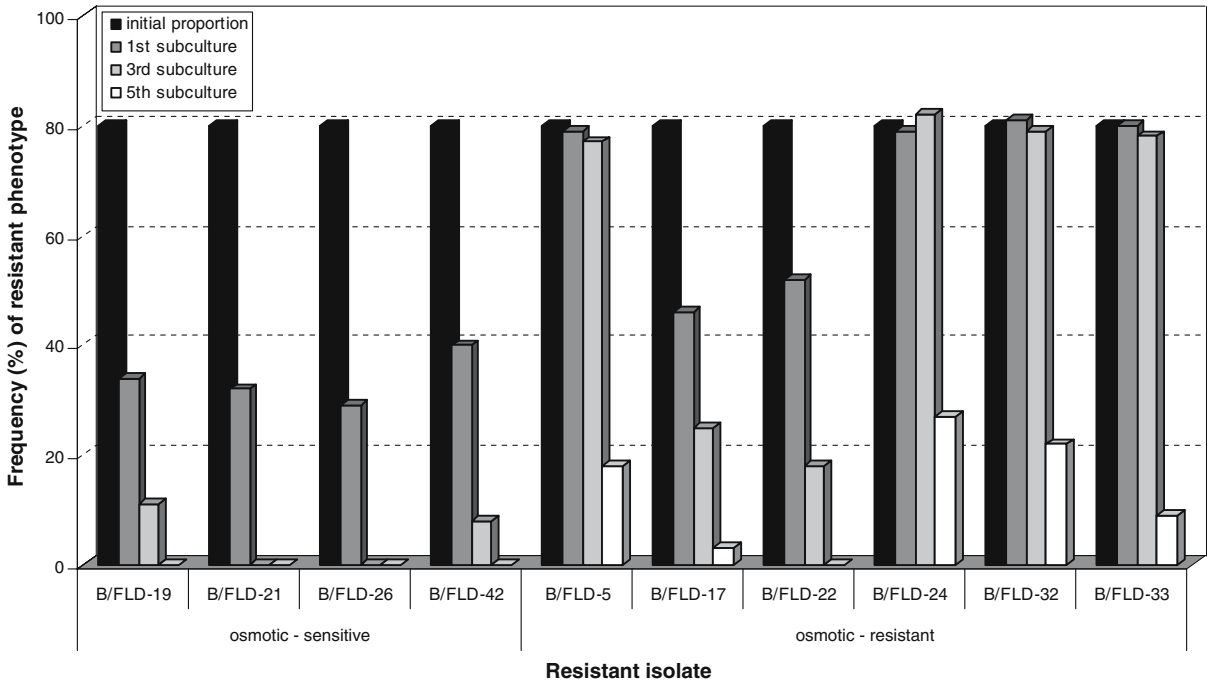


*Figure 2. In vitro* competition between fludioxonil-resistant isolates and the wild-type strain of *Botrytis cinerea*, co-inoculated on PDA medium at the initial ratio 80:20 respectively. Measurements were made after 10 days at 22 °C.

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

| Fungicide concentration ($\mu$g a.i. ml$^{-1}$) | Infection of cotyledons (% of control) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | wt-$B_1$ | B/$FLD_{osm/s}$-19[a] | B/$FLD_{osm/s}$-21[a] | B/$FLD_{osm/s}$-26[a] | B/$FLD_{osm/r}$-5[a] | B/$FLD_{osm/r}$-17[a] | B/$FLD_{osm/r}$-24[a] | B/$FLD_{osm/r}$-32[a] | B/$IPR_{osm/s}$-1[a] | B/CPL-27[a] | B/FNH-21[a] |
| Control | 100 (59a[b])[c] | 100 (21cd)[c] | 100 (14d)[c] | 100 (17d)[c] | 100 (50ab)[c] | 100 (54a)[c] | 100 (44b)[c] | 100 (52ab)[c] | 100 (30c)[c] | 100 (29c)[c] | 100 (42b)[c] |
| Saphire 50 WP[d] | | | | | | | | | | | |
| 1 | 28b | 100a | 100a | 100a | 100a | 100a | 100a | 100a | 100a | 30b | 17c |
| 5 | 6b | 100a | 97a | 96a | 100a | 100a | 94ab | 100a | 94ab | 8b | 3c |
| 10 | 3d | 90ab | 84bc | 87b | 94a | 97a | 81bc | 92ab | 78c | 0d | 1d |
| 100 | 0d | 82ab | 78b | 74b | 88a | 86a | 72bc | 83ab | 66c | 0d | 0d |
| Rovral 50 WP[d] | | | | | | | | | | | |
| 100 | 24b | 94ab | 96a | 90ab | 99a | 100a | 100a | 102a | 100a | 26b | 22b |
| 200 | 0c | 88a | 81b | 82ab | 86ab | 89a | 80b | 91a | 79b | 0c | 0c |
| Teldor 50 WP[d] | | | | | | | | | | | |
| 2.5 | 36b | 36b | 34b | 35b | 32bc | 38b | 34b | 37b | –[e] | 33bc | 97a |
| 25 | 27b | 26b | 28b | 24bc | 19c | 26b | 23bc | 28b | – | 29b | 88a |
| 100 | 15b | 17b | 14b | 13b | 5c | 17b | 16b | 11bc | – | 8c | 76a |
| 750 | 1b | 2b | 0c | 1b | 2b | 2b | 3b | 5b | – | 0c | 70a |
| 1000 | 0b | 0b | 0b | 0b | 0b | 0b | 0b | 0b | – | 0b | 67a |
| Chorus 75 WP[d] | | | | | | | | | | | |
| 100 | 23b | 20b | 22b | 18bc | 23b | 19b | 20b | 22b | 19b | 87a | 21b |
| 250 | 0b | 0b | 0b | 3b | 0b | 0b | 2b | 0b | 0b | 8a | 0b |
| Benlate 50 WP[d] | | | | | | | | | | | |
| 50 | 0a | 0a | 2a | 0a | 1a | 0a | 0a | 4a | 0a | 0a | – |
| Sumico 50 WP[d] | | | | | | | | | | | |
| 100 | 0a | 0a | 0a | 0a | 0a | 0a | 0a | 0a | 0a | 0a | – |

[a]B/FLD mutant strains resistant to fludioxonil; B/$IPR_{osm/s}$-1 mutant strain resistant to iprodione; B/CPL-27 mutant strain resistant to cyprodinil; B/FNH-21 mutant strain resistant to fenhexamid.
[b]Within rows, values followed by the same letter do not differ significantly according to Dunnett's multiple range test ($P = 0.05$).
[c]Value in parenthesis equals the sum of indices of 16 cotyledons for each control.
[d]Commercial formulations: Saphire (fludioxonil); Rovral (iprodione); Teldor (fenhexamid); Chorus (cyprodinil); Benlate (benomyl); Sumico (carbendazim + diethofnecarb).
[e]Not tested.

zim + diethofencarb), at the fungicide concentration of 750, 250, 50 and 100 $\mu$g a.i. ml$^{-1}$, respectively, resulted in complete inhibition of lesion development on cucumber seedlings by fludioxonil- and iprodione-resistant strains.

Studies to verify the competitive ability of fludioxonil-resistant mutants of *B. cinerea in planta*, showed a rapid reduction in the frequency of resistant isolates of both phenotypic classes (osmotic sensitive and resistant), indicating that all fludioxonil-resistant isolates are weak competitors of the wild-type strain *in planta* (Figure 3). However, a relatively higher competitiveness was recognized for the osmotic-resistant isolates. After one disease cycle the proportion of resistant isolates was almost 0% in all B/FLD$_{osm/s}$/wild-type pairs, while in the B/FLD$_{osm/r}$/wild-type pairs the proportion of resistant isolates ranged from 20 to 40% (Figure 3).

*Selection and characterization of fludioxonil-resistant isolates of* U. maydis

Mutants of *U. maydis* resistant to fludioxonil were isolated at a high mutation frequency of $2.8 \times 10^{-3}$ after UV-light irradiation (97% lethality) and selection on UCM agar medium containing 75 $\mu$g ml$^{-1}$ fludioxonil. Depending on the parent sensitive isolate 201 or 501, they were designated as U/FLD-20.. or U/FLD-50.., respectively. Testing for their sensitivity to KCl (25 mg ml$^{-1}$) on UCM agar medium resulted in the identification of two fludioxonil-resistant phenotypes with differing osmotic sensitivity. As in *B. cinerea*, most fludioxonil-resistant isolates (approximately 95% of mutants) were found to be more sensitive (U/FLD$_{osm/s}$) to high osmotic pressure than the wild-type strains. The remaining fludioxonil-resistant isolates (approx. 5% of total isolates), which appeared in the first 5–6 days of incubation, had a similar tolerance to high osmolarity (U/FLD$_{osm/r}$) as the wild-type. From this initial screening, a random sample of six U/FLD$_{osm/r}$ and six U/FLD$_{osm/s}$ mutants were chosen for further studies. Fungitoxicity tests of fludioxonil in liquid culture with the resistant strains showed a dose-dependent decrease of growth of wild-type and both classes of mutant isolates. A Rf of 400 and 840–900 was calculated based on MIC or EC$_{50}$ values, respectively.

Comparison of growth rates in liquid culture without fungicide by measuring the optical density or dry weight increases showed that the growth

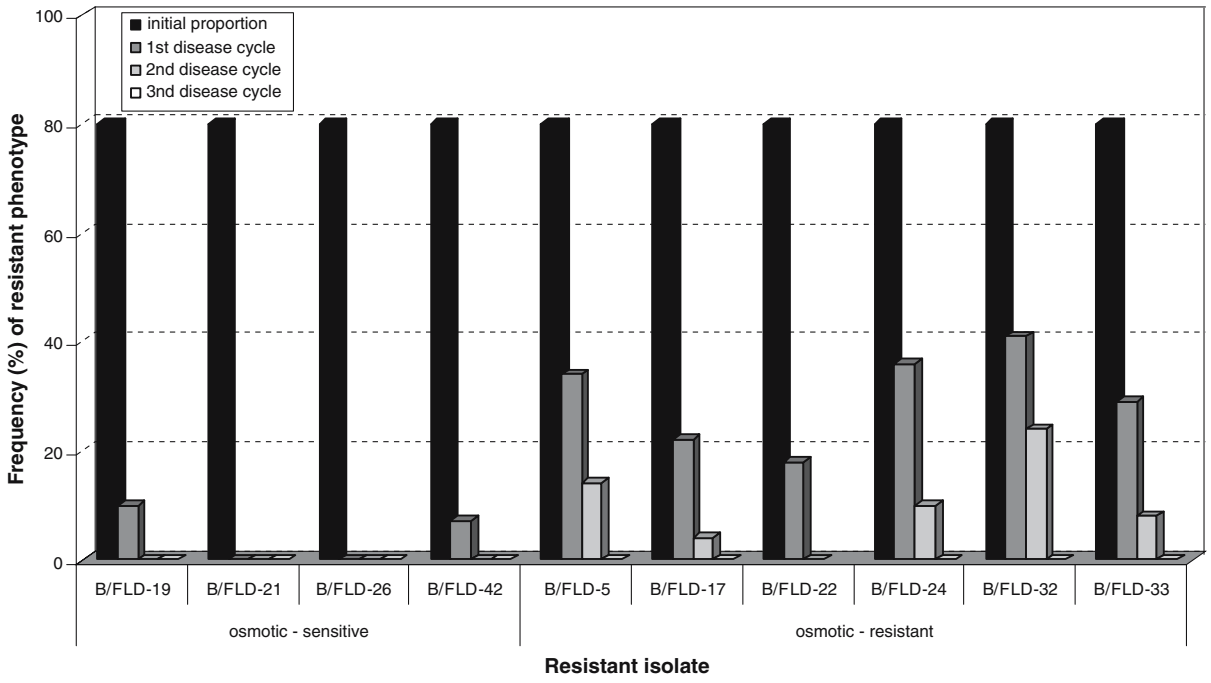


*Figure 3. In planta* competition between fludioxonil-resistant isolates and the wild-type strain of *Botrytis cinerea*, co-inoculated on cucumber seedlings at the initial ratio 80:20 respectively. Measurements were made after 10 days incubation.

rates of the twelve mutant isolates were all similar to those of the wild-type strains, indicating that the mutations for resistance to fludioxonil had no apparent effect on the saprophytic fitness determining characteristics of *U. maydis*. The doubling time for the growth of the wild-type was approximately 3 h, whereas five resistant isolates (U/FLD-5001, U/FLD-5015, U/FLD-2012, U/FLD-2010 and U/FLD-2026) had faster growth rates with doubling times of 2.6–2.4 h. Two other isolates, U/FLD-5013 and U/FLD-2003, showed a growth rate similar to that of the wild-types (doubling time 2.9–3.1 h). The growth of the remaining five mutant isolates was slower (doubling time 3.5–4.2 h).

*Genetic control of resistance to fludioxonil in U. maydis*

*Crosses between wild-type and fludioxonil-resistant isolates*

Each of the 12 fludioxonil-resistant isolates of *U. maydis* was crossed first with the compatible wild-type strain and approximately 100 random progeny from each of these R × S crosses were tested for sensitivity to fludioxonil at: (a) the MIC for the sensitive parent (0.25 $\mu$g ml$^{-1}$); and (b) a non-inhibitory concentration for the resistant parent (50 $\mu$g ml$^{-1}$). As shown in Table 4, in all cases the ratio of resistant/sensitive (R/S) progeny was exactly the same at both fludioxonil concentrations, and this segregation was not significantly different from a Mendelian 1:1 ratio, as measured by the Chi-square tests ($P = 0.05$). This indicates that each of these mutant isolates was the result of a mutation of a single chromosomal gene for resistance to fludioxonil. The effect of gene mutation(s) on the response of mutant strains to high osmotic pressure was determined by measuring the ratio of resistant/sensitive progeny on medium containing 25 mg ml$^{-1}$ KCl. As shown in Table 4, a 1:1 progeny segregation was also observed regarding the sensitivity to high osmolarity in all crosses between wild-type and U/FLD$_{osm/s}$ isolates. All the fungicide-resistant progeny were more sensitive than the wild-type, indicating that these gene mutation(s) for resistance to fludioxonil are pleiotropic, having significantly adverse effects on the response of mutants to high osmotic pressure. The absence of osmotic sensitive recombinants, in crosses between U/FLD$_{osm/r}$ isolates with the compatible wild-type strain, indicates that these mutation(s) for resistance to fludioxonil have no pleiotropic effect on the response of mutants to high osmotic pressure.

*Crosses between fludioxonil-resistant isolates*

To identify the number of mutated chromosomal genes, the allelism test was carried out in all possible (depending on mating type) R × R crosses. A random sample of approximately 100 progeny from each cross was tested for resistance to fludioxonil at: (a) the MIC for the wild-type strains (0.25 $\mu$g ml$^{-1}$), in order to examine if the mutant genes are mutations of the same locus; and (b) a non-inhibitory concentration for the resistant parent (50 $\mu$g ml$^{-1}$) (Table 4). The two mutants mated were assumed to be allelic if no sensitive progeny were produced. Otherwise, they should carry resistance genes at different loci. In this way, three chromosomal loci (*U*/*fld*-1, *U*/*fld*-2 and *U*/*fld*-3) for resistance to fludioxonil were identified. Among the 12 single gene mutants studied, six were identified as carrying the *U*/*fld*-1, three the *U*/*fld*-2 and three the *U*/*fld*-3 mutations (Table 4). The large number of recombinants with wild-type sensitivity to fludioxonil, with a R/S segregation not significantly different from the Mendelian 3:1 ratio, shows that the three *U*/*fld* genes are unlinked, segregating independently of each other during meiosis. Moreover, the large number of recombinants with wild-type resistance to osmotic pressure, in crosses between mutants carrying the *U*/*fld*$_{osm/r}$-1 gene mutation with mutants carrying the *U*/*fld*$_{osm/s}$-2 or *U*/*fld*$_{osm/s}$-3 mutations, indicate an epistatic effect of osmotic resistant gene (*U*/*fld*$_{osm/r}$-1) on the osmotic sensitive ones (*U*/*fld*$_{osm/s}$-2, 3), which results in osmotic resistant progeny carrying both type of mutations (double mutants).

In an attempt to recognize inter-allelic interactions when two non-allelic genes are present in the same haploid nucleus, progeny from crosses between non-allelic compatible mutants were tested for sensitivity at three concentrations of fludioxonil: (a) the MIC for the wild-types (0.25 $\mu$g ml$^{-1}$); (b) the non-inhibitory (50 $\mu$g ml$^{-1}$); and (c) the MIC (100 $\mu$g ml$^{-1}$) for the resistant parents. As shown by the examples given in Table 5, the segregation ratio was approximately 3R:1S at the first and second concentration and all progeny were sensitive at the third fludioxonil concentration.

*Table 4*. Osmotic and fludioxonil sensitivity of progeny from crosses involving wild-type and fludioxonil-resistant isolates of *Ustilago maydis*

| Cross | Number of progeny tested | Ratio of resistant/sensitive (R/S) progeny at the indicated KCl and fludioxonil concentrations | | | $x^2$ for fungicide sensitivity[a] | Mutated loci |
|---|---|---|---|---|---|---|
| | | KCl (mg ml$^{-1}$) | Fludioxonil ($\mu$g ml$^{-1}$) | | | |
| | | 25 | 0.25 | 50 | | |
| $S^b \times R^c$ | | | | | | |
| 201 × U/FLD-5015$_{osm/r}$ | 92 | 92/0 | 48/44 | 48/44 | 0.174a | |
| 201 × U/FLD-5013$_{osm/r}$ | 100 | 100/0 | 54/46 | 54/46 | 0.640a | |
| 201 × U/FLD-5001$_{osm/r}$ | 110 | 110/0 | 61/49 | 61/49 | 1.309a | |
| 201 × U/FLD-5008$_{osm/s}$ | 104 | 59/45 | 45/59 | 45/59 | 1.885a | |
| 201 × U/FLD-5006$_{osm/s}$ | 100 | 47/53 | 53/47 | 53/47 | 0.360a | |
| 201 × U/FLD-5061$_{osm/s}$ | 98 | 54/44 | 44/54 | 44/54 | 1.020a | |
| 501 × U/FLD-2012$_{osm/r}$ | 87 | 87/0 | 48/39 | 48/39 | 0.931a | |
| 501 × U/FLD-2010$_{osm/s}$ | 96 | 51/45 | 45/51 | 45/51 | 0.375a | |
| 501 × U/FLD-2003$_{osm/r}$ | 112 | 112/0 | 61/51 | 61/51 | 0.893a | |
| 501 × U/FLD-2007$_{osm/s}$ | 100 | 44/56 | 56/44 | 56/44 | 1.440a | |
| 501 × U/FLD-2009$_{osm/s}$ | 90 | 43/47 | 47/43 | 47/43 | 0.178a | |
| 501 × U/FLD-2026$_{osm/r}$ | 84 | 84/0 | 40/44 | 40/44 | 0.190a | |
| $R \times R$ | | | | | | |
| U/FLD-5015$_{osm/r}$ × U/FLD-2012$_{osm/r}$ | 100 | 100/0 | 100/0 | 100/0 | – | *U*/*fld*-1 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2003$_{osm/r}$ | 102 | 102/0 | 102/0 | 102/0 | – | *U*/*fld*-1 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2026$_{osm/r}$ | 89 | 89/0 | 89/0 | 89/0 | – | *U*/*fld*-1 |
| U/FLD-5013$_{osm/r}$ × U/FLD-2012$_{osm/r}$ | 110 | 110/0 | 110/0 | 110/0 | – | *U*/*fld*-1 |
| U/FLD-5001$_{osm/r}$ × U/FLD-2012$_{osm/r}$ | 81 | 81/0 | 81/0 | 81/0 | – | *U*/*fld*-1 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2010$_{osm/s}$ | 115 | 80/35 | 82/33 | 82/33 | 0.838b | *U*/*fld*-1 × *U*/*fld*-2 |
| U/FLD-5013$_{osm/r}$ × U/FLD-2010$_{osm/s}$ | 100 | 76/24 | 74/26 | 74/26 | 0.053b | *U*/*fld*-1 × *U*/*fld*-2 |
| U/FLD-5001$_{osm/r}$ × U/FLD-2010$_{osm/s}$ | 94 | 70/24 | 66/28 | 66/28 | 1.148b | *U*/*fld*-1 × *U*/*fld*-2 |
| U/FLD-5006$_{osm/s}$ × U/FLD-2010$_{osm/s}$ | 76 | 0/76 | 76/0 | 76/0 | – | *U*/*fld*-2 |
| U/FLD-5061$_{osm/s}$ × U/FLD-2010$_{osm/s}$ | 100 | 0/100 | 100/0 | 100/0 | – | *U*/*fld*-2 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2007$_{osm/s}$ | 114 | 79/35 | 82/32 | 82/32 | 0.573b | *U*/*fld*-1 × *U*/*fld*-3 |
| U/FLD-5006$_{osm/s}$ × U/FLD-2007$_{osm/s}$ | 111 | 30/81 | 81/30 | 81/30 | 0.243b | *U*/*fld*-2 × *U*/*fld*-3 |
| U/FLD-5008$_{osm/s}$ × U/FLD-2007$_{osm/s}$ | 106 | 0/106 | 106/0 | 106/0 | – | *U*/*fld*-3 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2009$_{osm/s}$ | 98 | 73/25 | 71/27 | 71/27 | 0.341b | *U*/*fld*-1 × *U*/*fld*-3 |
| U/FLD-5006$_{osm/s}$ × U/FLD-2009$_{osm/s}$ | 100 | 28/72 | 72/28 | 72/28 | 0.480b | *U*/*fld*-2 × *U*/*fld*-3 |
| U/FLD-5008$_{osm/s}$ × U/FLD-2009$_{osm/s}$ | 104 | 0/104 | 104/0 | 104/0 | – | *U*/*fld*-3 |

[a]Expected value of $x^2$ for a 1:1 (a) or a 3:1 (b) segregation ratio for 1 degree of freedom is $< 3.841$ at $P = 0.05$ level of probability.
[b]S: sensitive (wild-type) isolates.
[c]R: fludioxonil-resistant mutant isolates.

The absence of resistant progeny at the highest concentration indicates that there is no additive effect between these non-allelic genes.

*Pathogenicity of fludioxonil-resistant strains of U. maydis*

The effect of *U*/*fld* mutations for resistance to fludioxonil on the phytopathogenic fitness parameters of *U. maydis* was determined by comparing the number of infected corn seedlings and the rate of disease development after inoculation with the following crosses; mutant × wild-type, mutant × mutant and wild-type × wild-type. All seedlings were infected by the crosses and there was no significant difference between the crosses for each pathogenic characteristic measured (results not shown).

## Discussion

Mutants of *B. cinerea* highly resistant to fludioxonil, with or without sensitivity to high osmotic

*Table 5*. Osmotic and fludioxonil sensitivity of progeny from crosses involving fludioxonil-resistant isolates of *Ustilago maydis* carrying the *U*/*fld*-1, *U*/*fld*-2 and *U*/*fld*-3 mutations

| Cross | Number of progeny tested | Ratio of resistant/sensitive (R/S) progeny at the indicated fludioxonil concentrations ($\mu$g ml$^{-1}$) | | | Linkage between mutated genes | |
|---|---|---|---|---|---|---|
| | | 0.25 | 50 | 100 | % recombination | $x^2$ values (3:1)[a] |
| *U*/*fld*-1 × *U*/*fld*-2 | | | | | | |
| U/FLD-2012$_{osm/r}$ × U/FLD-5006$_{osm/s}$ | 110 | 79/31 | 79/31 | 0/110 | 28.18 | 0.594 |
| U/FLD-2003$_{osm/r}$ × U/FLD-5061$_{osm/s}$ | 115 | 86/29 | 86/29 | 0/115 | 25.22 | 0.003 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2010$_{osm/s}$ | 102 | 73/29 | 73/29 | 0/102 | 28.43 | 0.641 |
| *U*/*fld*-1 × *U*/*fpl*-3 | | | | | | |
| U/FLD-2012$_{osm/r}$ × U/FLD-5008$_{osm/s}$ | 115 | 84/31 | 84/31 | 0/115 | 29.96 | 0.235 |
| U/FLD-2003$_{osm/r}$ × U/FLD-5008$_{osm/s}$ | 84 | 62/22 | 62/22 | 0/84 | 26.19 | 0.063 |
| U/FLD-5015$_{osm/r}$ × U/FLD-2007$_{osm/s}$ | 98 | 71/27 | 71/27 | 0/98 | 27.55 | 0.341 |
| *U*/*fld*-2 × *U*/*fld*-3 | | | | | | |
| U/FLD-2010$_{osm/s}$ × U/FLD-5008$_{osm/s}$ | 112 | 81/31 | 81/31 | 0/112 | 27.68 | 0.429 |
| U/FLD-5006$_{osm/s}$ × U/FLD-2007$_{osm/s}$ | 73 | 54/19 | 54/19 | 0/73 | 26.03 | 0.041 |
| U/FLD-5061$_{osm/s}$ × U/FLD-2009$_{osm/s}$ | 104 | 75/29 | 75/29 | 0/104 | 27.88 | 0.462 |

[a]Expected value of $x^2$ for a 3:1 ratio for 1 degree of freedom is $<3.841$ at $P = 0.05$ level of probability.

pressure, were isolated at high mutation frequencies, after chemical mutagenesis and selection on medium containing fludioxonil. A study of the sensitivity of mutant strains to fungicides from other chemical groups showed that the mutation(s) for resistance to phenylpyrroles also reduced the sensitivity of mutant strains to the aromatic hydrocarbon and dicarboximide fungicides, but not to the benzimidazoles, hydroxyanilides, anilinopyrimidines, phenylpyridinamines or the sterol biosynthesis inhibiting fungicides. Laboratory resistance to phenylpyrroles has been reported for *B. cinerea* (Leroux et al., 1992; Faretra and Pollastro, 1993; Hilber et al., 1994; Ziogas and Kalamarakis, 2001), but problems with practical resistance have not yet been reported (Leroux et al., 1999). Monitoring studies with field isolates of *B. cinerea* revealed that most dicarboximide-resistant strains lacked resistance or were weakly resistant to phenylpyrroles (Hilber et al., 1995; Leroux et al., 1999). However, one strain of *B. cinerea* with low to moderate resistance to fludioxonil was isolated after several years of monitoring in France (Vignutelli et al., 2002).

Fitness of mutant strains is a very important parameter regarding the risk for practical resistance development. Results from studies to verify the overall cost of resistant mutations on fitness are informative in determining the subsequent response of pathogen populations in the field (Brent and Hollomon, 1998). In the present work, study of fitness determining characteristics in wild-type and representative fludioxonil-resistant mutants of *B. cinerea*, showed that the mutation(s) leading to fludioxonil resistance carry some fitness penalties. All osmotic-sensitive isolates presented a significant reduction in the saprophytic fitness parameters, such as growth rate, sporulation, conidial germination and sclerotial production. Failures or severe reductions in fitness parameters indicate that the osmosensitive fludioxonil-resistant mutations can be induced in the laboratory, but should not cause a serious practical problem. However, the saprophytic fitness penalties were less in osmotic-resistant isolates. Most of the fitness characteristics were slightly affected or unaffected in these mutant strains. Pathogenicity tests showed that none of the fludioxonil-resistant strains of *B. cinerea* tested lost their ability to cause infection on cucumber plants. However, all osmotic-sensitive isolates presented a highly reduced infection ability compared with the wild-type strain, indicating that this type of mutation causes adverse effects on the virulence of the mutant strains. However, only a slight reduction in the pathogenicity of osmotic-resistant isolates was observed. A highly decreased osmotic stress tolerance of laboratory phenylpyrrole-resistant

strains of *B. cinerea* in comparison with their sensitive parental strains and in parallel with a low pathogenicity were also found by other researchers (Leroux et al., 1992; Hilber et al., 1994). The osmosensitivity question has been discussed in the past as being important to the survival of dicarboximide-resistant mutants of *B. cinerea* in field populations. An increase of osmotic sensitivity of resistant strains of *B. cinerea* was frequently quoted as a possible explanation for their low fitness and pathogenicity. However, studies with field isolates of *B. cinerea* showed that dicarboximide resistance and osmotic sensitivity are not necessarily correlated (Lorenz and Pommer, 1985).

Experiments on the stability of fludioxonil-resistant phenotypes of *B. cinerea*, showed a significant reduction of resistance in both phenotypic classes when the mutants were grown on medium without fludioxonil, indicating intracellular selection of wild-type nuclei in multinuclear fungal cells of *B. cinerea*. However, the high level of resistance to fludioxonil returned rapidly when the mutant strains were subcultured on to fludioxonil containing medium again, indicating intracellular selection of mutated nuclei. Similar results were also found in the past with dicarboximide-resistant field isolates of *B. cinerea* that have not been cultivated on nutrient media containing fungicides for any length of time. The proportion of resistant conidia decreased rapidly, and such isolates regained complete sensitivity (Lorenz and Eichhorn, 1982; Lorenz and Pommer, 1982). However, the experiments with osmotic-resistant isolates showed that the resistant phenotype was much more stable compared with the osmotic-sensitive ones. A partial stability of resistant phenotype was also observed in isolates of *B. cinerea* from vine plots in which no applications of dicarboximides had been carried out for four years, yet part of the population was found to retain the dicarboximide-resistance (Lorenz and Pommer, 1985).

Competition experiments, using mixed inocula of spores from sensitive and resistant strains of *B. cinerea*, showed that *in vitro* all resistant strains were less competitive than the wild-type parent strain. However, the osmotic-resistant isolates were more competitive than the osmotic-sensitive ones. Furthermore, *in planta* tests on cucumber seedlings showed that all mutant strains could not compete with the wild-type strain and they were suppressed by the sensitive isolate after a few disease cycles. Obviously, the penalties in the ecological fitness characteristics greatly reduced the competitive ability of all mutant strains *in planta*. However, beyond the genetic variability caused by mutations and sexual reproduction, *B. cinerea* shows a genetic flexibility that may be caused by the selection of different alleles within the heterokaryon. High genetic variability and flexibility, high reproducibility, wide host range, the ability of saprophytic growth and the epistatic effect of osmotic-resistant genes on the osmotic-sensitive ones creates an increased inherent risk for resistance to phenylpyrroles.

As well as mutations, gene recombination also provides an opportunity to introduce novel genotypes into a population, and thus the investigation of genetic control of resistance to fungicides gives useful information for the evaluation of resistance risk. In this study, the phytopathogenic basidiomycete *U. maydis* was used as a model to help further understand the nature and genetical basis for resistance to phenylpyrroles. Genetic analysis of progeny from crosses with fludioxonil-resistant mutants of *U. maydis* showed that the two fludioxonil-resistant phenotypes, with differing sensitivity to high osmolarity, were due to three unlinked chromosomal genes, *U|fld*-1, *U|fld*-2 and *U|fld*-3, from which only the *U|fld*-1 mutation is responsible for an osmotic sensitivity similar to the wild-type. No increase in resistance to fludioxonil was observed in haploid isolates which carried two of the non-allelic genes in the same nucleus, indicating that there is no inter-allelic interaction between these non-allelic genes. Moreover, in all crosses tested, no segregation between phenylpyrrole and dicarboximide resistance was observed. An interesting aspect in our work with *U. maydis*, is the absence of significant pleiotropic effects of *U|fld* mutations on pathogenicity to maize seedlings. Comparisons of the virulence, rate of disease development and teliospore production revealed that *U|fld* mutations for resistance to fludioxonil did not affect the pathogenicity of the dikaryotic phase. It appears that the mechanism of aromatic hydrocarbon and dicarboximide fungicides and phenylpyrrole-resistance was not linked genetically to the phytopathogenic fitness characteristics of *U. maydis*. Similar results were also obtained with laboratory mutants of *U. maydis* resistant to aromatic hydrocarbon and dicarboximide fungicides by Orth et al. (1994). Genetic analysis revealed the

presence of three genes (*adr*-1, *adr*-2 and *adr*-3) for resistance to aromatic hydrocarbon and dicarboximide fungicides (Orth et al., 1994). Subsequent studies showed that a serine (threonine) protein kinase encoded by the *adr*-1 gene exhibits a high degree of identity to the catalytic subunit of a cAMP-dependent protein kinase (PKA) (Orth et al., 1995). However, more detailed studies found that the *adr*-encoded enzyme does not appear to be a direct target of vinclozolin inhibition (Ramesh et al., 2001). Further molecular studies (Ramesh et al., 2001) with *U. maydis* mutants resistant to vinclozolin and chloroneb revealed a mutation at the *ubc*-1 gene, which encodes the regulatory subunit of the PKA. The above data indicate that the cAMP-dependent protein kinase signalling pathway is involved in resistance to aromatic hydrocarbon and dicarboximide fungicides at least in *U. maydis*.

A similar genetic control of resistance to aromatic hydrocarbon and dicarboximide fungicides and phenylpyrroles was also observed in the filamentous fungi *B. cinerea* and *Neurospora crassa*. Genetic studies conducted by Faretra and Pollastro (1993) showed that the polymorphic *Daf* gene mutations (*Daf 1*, *Daf 2*) were responsible for the high resistance of laboratory mutant strains of *Botryotinia fuckeliana* to phenylpyrroles and aromatic hydrocarbon and dicarboximide fungicides and the osmotic sensitivity was coded only by *Daf 1 HR* alleles. The phenotype of high resistance cosegregated with a higher osmotic sensitivity in the progeny of *B. fuckeliana* crosses. A study of field isolates of *B. cinerea* showed the presence of *Daf 1 LR* alleles, which coded a low resistance to dicarboximides, without modification on phenylpyrrole sensitivity. Biochemical studies conducted by Steel (1996) showed that field dicarboximide resistance in *B. cinerea* is positively correlated with enhanced oxidative protecting enzymes, but no such correlation was found with fludioxonil. The results of the above studies indicate that the mechanism of low to medium field resistance to dicarboximides, which is coded by *Daf 1 LR* allele does not apply to phenylpyrroles and at the biochemical level there are differences between phenylpyrrole and dicarboximide resistance. The above conclusion is strongly supported by the genetic studies conducted by Vignutelli et al. (2002), which showed an independent segregation of field resistance to fludioxonil and vinclozolin. Molecular studies with laboratory and field strains of *B. cinerea* resistant to dicarboximides revealed point mutations resulting in amino acid changes in a two-component histidine protein kinase gene (Leroux et al., 2002; Cui et al., 2002; Oshima et al., 2002). Studies with *N. crassa* revealed the presence of several osmotic sensitivity genes (*os*-1, *os*-2, *os*-4 and *os*-5), which confer resistance to aromatic hydrocarbon and dicarboximide fungicides and phenylpyrroles (Grindle and Temple, 1985; Fujimura et al., 2000). Molecular studies showed that the *os* genes encode enzymes of the osmosensing histidine kinase pathway (Schumacher et al., 1997; Ochiai et al., 2001; Cui et al., 2002; Zhang et al., 2002). Furthermore, a protein kinase (PK-III), possibly involved in the regulation of the glycerol synthesis in *N. crassa*, was found to be inhibited by phenylpyrroles, whereas vinclozolin was without effect (Pillonel and Meyer, 1997).

The above mentioned molecular and biochemical data indicate a differential interaction of dicarboximides and phenylpyrroles with the protein kinases, which are involved in the osmosensing signal transduction pathway(s), and apparently there are differences between filamentous and yeast-like fungal species. Future molecular studies with fludioxonil-resistant mutants will provide more data to elucidate the nature of mutations in *B. cinerea* and *U. maydis*, and will help to verify the involvement of protein kinases in dicarboximide and phenylpyrrole fungicidal action and fungal resistance to these fungicides.

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