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In vitro and *in vivo* activity of cyprodinil and pyrimethanil on *Botrytis cinerea* isolates resistant to other botryticides and selection for resistance to pyrimethanil in a greenhouse population in Greece

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Abstract

No cross-resistance was observed between pyrimethanil or cyprodinil and the fungicides benomyl, iprodione or carbendazim + diethofencarb. *In vitro*, both anilinopyrimidine fungicides were effective against strains of *Botrytis cinerea* resistant to benzimidazoles and/or dicarboximides and against a wild type strain insensitive to diethofencarb (EC_{50} values ranged between 0.03–0.19 and 0.006–0.054 $\mu g\,ml^{-1}$ for pyrimethanil and cyprodinil, respectively). Preventive applications of anilinopyrimidines completely protected young cucumber plants and fruits that were inoculated with all strains of *B. cinerea*. The effectiveness of pyrimethanil against grey mould was studied in greenhouse grown tomatoes in relation to (a) the type of infection and the progress of the disease on different plant parts and (b) the response of the naturally occurring *B. cinerea* population to the selection pressure caused by eight successive applications of this fungicide. Pyrimethanil effectively controlled grey mould on leaves, fruits and stems but did not significantly reduce the number of dead plants and fruits with 'ghost spot' symptoms. The selection pressure caused by the consecutive applications of pyrimethanil resulted in reduction of its effectiveness on leaves that became apparent after the sixth application. This was correlated with a shift of the *B. cinerea* population (not previously exposed to anilinopyrimidines) towards reduced sensitivity, probably due to the development of a low level of resistance ($R_L = 7.7$). Pyrimethanil delayed the onset of the disease but it did not reduce the infection rate.

Introduction

Crop production in greenhouses has developed considerably during the latter part of the 20th century in Greece and several other countries. *Botrytis cinerea* Pers.:Fr., the causal agent of grey mould, is an ubiquitous pathogen which causes severe losses in vegetables and many other crops under a variety of growing conditions (Jarvis, 1992; Elad et al., 1996). In Greece, the mild environmental conditions along with the simple structures of the greenhouses make grey mould one of the most important diseases of vegetables grown under cover. In particular, tomatoes, a crop of high economic importance in Greece and many other Mediterranean countries, are prone to infection in unheated plastic greenhouses during the winter months, resulting in high yield losses (Elad et al., 1996).

So far, the management of *Botrytis*-incited disease has been principally based on chemical control. The wide and repeated use of selective fungicides such as benzimidazoles initially and later dicarboximides has resulted in the selection and predominance of strains of *B. cinerea* resistant to each of the above mentioned groups of fungicides in Greece and in many other countries (Malathrakis, 1979; Gullino and Garibaldi, 1981; Katan, 1982;

Panagiotakou and Malathrakis, 1983). Strains exhibiting double resistance to benzimidazoles and dicarboximides have also been detected in several countries (Gullino and Garibaldi, 1986; Moorman and Lease, 1992). During 1984, the fungicidal mixture of carbendazim and diethofencarb was introduced in an attempt to exploit the negatively correlated cross-resistance phenomenon between *N*-phenylcarbamates and benzimidazoles (Kato et al., 1984). The intensive commercial use of this mixture against *B. cinerea* in several countries such as Israel, France, Spain and Greece, resulted shortly after its introduction, in the selection of strains resistant to benzimidazoles and diethofencarb (Katan et al., 1989; Leroux and Gredt, 1989; Raposo et al., 1994; Laskaris et al., 1996). The threat of double resistance to benzimidazoles and dicarboximides and the marginal disease control with the mixture of carbendazim and diethofencarb (Elad et al., 1992) have intensified the research for effective fungicides with an alternative mode of action. During the last decade, new chemical groups of fungicides, effective against *B. cinerea*, such as anilinopyrimidines, phenylpyridinamines, phenylpyrroles and hydroxyanilides have been developed (Gehman et al., 1990; Anema and Bouwman, 1992; Newman et al., 1992; Heye et al., 1994; Masner et al., 1994; Kuck et al., 1997).

Anilinopyrimidines, such as pyrimethanil, cyprodinil and mepanipyrim, have been introduced in Greece (1998) and other European countries. Pyrimethanil and mepanipyrim are effective against diseases caused by *Botrytis* in a variety of crops and by *Venturia* spp in pome fruits, while cyprodinil is active against cereal pathogens such as *Pseudocercosporella herpotrichoides*, *Pyrenophora teres* and *Erysiphe graminis* (Neumann et al., 1992; Heye et al., 1994; Masner et al., 1994). Anilinopyrimidine fungicides are closely related compounds, showing a common novel mode of action. They interfere with the biosynthesis of methionine and other aminoacids and inhibit the secretion of hydrolytic enzymes, such as pectinases, cellulases and proteases, associated with pathogenesis of *B. cinerea* (Masner et al., 1994; Miura et al., 1994; Milling and Richardson, 1995), but their primary site of action is still unknown (Chapeland et al., 1999). Cross-resistance between members of anilinopyrimidines has been demonstrated (Hilber and Schüepp, 1996), while no reports about cross-resistance between anilinopyrimidines and benzimidazoles and dicarboximides are available (Leroux and Montcomble, 1994; Forster and Staub, 1996).

The present work aimed to study: (i) the *in vitro* and *in vivo* activity of the anilinopyrimidine fungicides cyprodinil and pyrimethanil, against Greek strains of *B. cinerea* resistant to benzimidazoles, dicarboximides and to a benzimidazole–diethofencarb mixture and the cross-resistance relationships, (ii) the effectiveness of pyrimethanil against grey mould infections in greenhouse grown tomatoes and (iii) the influence of repeated applications of this fungicide, during the same cultural period, on disease progress and on the sensitivity of *B. cinerea* population.

Materials and methods

In vitro *and* in vivo *sensitivity tests*

Isolates, growth media and culture conditions. Five isolates of *B. cinerea*, one wild type and four with reduced sensitivity to benzimidazoles and/or dicarboximides and to a carbendazim–diethofencarb mixture, were used as representatives of numerous similar isolates obtained during 1994, from vegetable crops grown under cover in Greece. All isolates were maintained on Malt Extract Agar (MEA, Oxoid, UK) at 4 °C in the dark. Single hyphal tip transfers were made once a month. Sporulation of *B. cinerea* strains was obtained on a V_8-juice agar medium containing 348 ml V_8 juice and 20 g agar per litre, after incubation for 7 days at 20 °C, in the dark. For the *in vitro* sensitivity tests, a glucose agar medium (gl–agar) containing glucose ($10\,g\,l^{-1}$) and agar ($12.5\,g\,l^{-1}$) was used for all fungicides tested. For anilinopyrimidines, a synthetic minimum medium that contained L-asparagine plus agar (asp–agar) was additionally used (Birchmore and Forster, 1996).

Fungicides. The *in vitro* sensitivity tests were conducted using technical grade cyprodinil (Syngenta Crop Protection GmbH, Basel, Switzerland), pyrimethanil (Aventis Crop Science GmbH, Frankfurt, Germany), iprodione (Aventis Crop Science GmbH, Frankfurt, Germany) and benomyl (DuPont de Nemours & Co., Wilmington, DE, USA) and the wettable powder formulation 'Sumico' (Sumitomo Chemical Co. Ltd, Osaka, Japan) containing $250\,g\,kg^{-1}$ carbendazim and $250\,g\,kg^{-1}$ diethofencarb. Stock solutions of cyprodinil, pyrimethanil, iprodione and benomyl were made in acetone, whereas carbendazim plus diethofencard were dissolved in ethanol. The concentration of the solvents never

exceeded 1% (v/v) in the growth medium and the control. All fungicide concentrations are expressed as $\mu g\,ml^{-1}$ active substance. In the *in vivo* sensitivity tests, aqueous suspensions of the commercial formulations 'Chorus' 50 WG ($500\,g\,kg^{-1}$ cyprodinil), 'Scala' 40 SC ($400\,ml\,l^{-1}$ pyrimethanil), 'Rovral' 50 WP ($500\,g\,kg^{-1}$ iprodione) 'Benlate' 50 WP ($500\,g\,kg^{-1}$ benomyl) and 'Sumico' 25 + 25 WP ($250 + 250\,g\,kg^{-1}$ carbedazim + diethofencarb) were used at the recommended rates.

In vitro *and* in vivo *antifungal assays.* The mycelial growth test described by Hilber and Schüepp (1996) was used in the *in vitro* antifungal assays. Triplicate Petri dishes containing asp–agar or gl–agar media were amended with a range of concentrations of the fungicides pyrimethanil, cyprodinil, iprodione, benomyl and carbendazim + diethofencarb. Three days after inoculation of the dishes with 17 h young mycelium, the diameters of the fungal colonies were measured. The data obtained were subjected to Probit Analysis and the EC_{50} values for each fungicide and isolate were calculated. Cucumber plants (four per treatment) were sprayed up to run-off with an aqueous suspension of the formulated fungicides at the recommended rates and were inoculated with a conidial suspension (1×10^6 conidia ml^{-1}) of the appropriate isolate. In mature cucumber fruits (six replicates per treatment and strain) application of the fungicides and inoculation (mycelial plugs) were carried out according to the method described by Forster and Müller (1996). The inoculated plants and fruits were incubated in a moist chamber at $20 \pm 1\,^{\circ}C$ in the dark for 3 days. Disease severity on leaves was assessed as percentage area infected by *B. cinerea* and was expressed as percent of the untreated control. On fruits, disease was assessed by measuring the diameter of the rotted area around individual holes and expressed as percent of the untreated controls.

Greenhouse trial

From autumn 1996 to spring 1997, a trial was conducted in the region of Heraklion, Crete, in order to examine the effectiveness of pyrimethanil against the different types of infections (on leaves, stems and fruits) caused by *B. cinerea* in greenhouse grown tomatoes and to assess the disease progress and the influence of selection pressure caused by repeated pyrimethanil applications on the sensitivity of the *B. cinerea* population.

Cultural practices. Tomato seeds (*Lycopersicon esculentum* Mill.- cv. 'Stressa', Bruisma Ltd., The Netherlands) were sown in plastic trays containing peat compost (Levington, Fisons Ltd., UK) with fertilizers as a nutrient source and were maintained in a glasshouse nursery. The seedlings were transferred to a polyethylene greenhouse four weeks after emergence, when three leaves were fully expanded. The crop was maintained and fertilized according to the system adopted locally.

Experimental design. The experimental design was a 'complete randomized block', with six replicates and three treatments. Each plot consisted of twelve tomato plants arranged in a double row 50 cm apart and 55 cm between rows. Plots could be separated by moveable plastic screens to avoid drifting of the applied fungicides during spraying.

Treatments. Treatments started in January (9/1/97) when high numbers of *Botrytis* conidia were present in the environment of the greenhouse as monitored using spore traps with a selective medium described by Edwards (1993). The fungicides applied were: pyrimethanil (as Scala 40 SC) at the concentration of $0.8\,ml\,a.i.\,l^{-1}$ and a tank mixture of iprodione + dichlofluanid (as Rovral 50 WP + Euparen 50 WP) at the concentration of $0.5 + 1\,g\,a.i.\,l^{-1}$. Control plants were sprayed with tap water. Eight applications were carried out at 10-day intervals, up to run-off, using knapsack sprayers (one for each treatment) at 3 atm pressure.

Disease assessment. Disease assessment was carried out every 7–12 days from mid February to mid April. Records were taken of: (a) mean number of lesions per leaf in each plant, (b) number of lesions on stems, (c) number of infected fruits, dead plants and fruits with 'ghost spot' symptoms. Data obtained were subjected to appropriate statistical analysis depending on the aim and objectives of the study as described in the relevant section below.

Sensitivity of B. cinerea *population to fungicides.* Before applications were started, a bioassay was conducted to determine the initial sensitivity of the *B. cinerea* population in the greenhouse to pyrimethanil and the commonly used fungicides iprodione, dichlofluanid, the mixture carbendazim plus diethofencarb and benomyl. For this purpose, when the first symptoms of *B. cinerea* were observed in the

tomato crop, samples were randomly collected from the greenhouse, aqueous conidial suspensions were prepared and then plated on Petri dishes containing asp–agar or gl–agar amended with pyrimethanil or the other fungicides (technical grade) respectively at various concentrations. Triplicate Petri dishes were used for each concentration of each fungicide, the dishes were then incubated at 20 °C for 16 or 24 h in the dark and conidial germination or germ tube elongation were measured. The percentage of germinated conidia and the length of germ tubes (only in the case of pyrimethanil) were estimated and recorded by counting a total of 60 conidia per replicate under the microscope, using a micrometer. The length of the germ tubes was assessed only for pyrimethanil because spore germination tests might give false results with anilinopyrimidines (Leroux and Gredt, 1996). The bioassay was repeated at the end of the greenhouse trial. The data obtained from both bioassays were used to estimate the respective EC_{50} values.

Statistical analysis

The EC_{50} value (concentration causing 50% reduction in mycelial growth, germination of conidia or germ tube elongation) was estimated for each fungicide and each *Botrytis cinerea* strain of the *in vitro* results subjected to probit analysis (statistical software SPSS 9.0). The effectiveness of the treatments in the greenhouse was analysed using the area under disease progress curve (AUDPC) approach. The AUDPC was calculated only for disease incidence (in terms of infected sites) on leaves (AUCleav), stems (AUCsles) and the percentage of dead plants/plot (AUCdpl) according to the formula reported by Campbell and Madden (1990). The differences between treatments in cumulative number of diseased fruits and total number of fruits with 'ghost spots' symptoms/plant, were examined by analysis of variance (ANOVA) and means were compared by Duncan's test at $P \leq 0.05$. The values of the number of infected fruits/plant were transformed (square root) for further analysis. Epidemiological tools such as logistic models describing disease progress on leaves were used in order to estimate the impact of pyrimethanil on grey mould progress (Campbell and Madden, 1990). Since disease was not recorded in terms of severity (max 100%), the incorporation of a maximum y, a new parameter called the asymptote K, was exemplified into the logistic model of the controls. Linearized logistic models were used with the assumption that the maximum number of infected spots on leaves/plant for each plot was 62. Logit transformation $[\ln(y/K - y)]$ of y was carried out per replicate (plot) and linear regression analysis was performed to the transformed data. The value of y_o and r_L was estimated with the use of the software (SPSS 9.0). Criteria of selecting the best fitting model included: examination of observed and predicted disease numbers of infected sites *versus* time, coefficients of determination (R^2) values for each model, standard errors of the parameter estimates, F statistic testing the significance of the regression model ($P \leq 0.05$) and Durbin–Watson test. The values of y_o (= logit of disease incidence [y] at time 0) and r_L were used for further analysis of variance (ANOVA) of disease progress in fungicide treated plots in comparison to the control.

Results

In vitro *and* in vivo *activity and cross-resistance*

The phenotypes of resistant *B. cinerea* strains were characterized according to EC_{50} values obtained (data not shown) for each fungicide and isolate on gl–agar medium: Ben^{HR} (mean EC_{50} values for benomyl: 55.7 $\mu g\,ml^{-1}$), $Ben^{HR}Dic^{MR}$ (mean EC_{50} 78.4 and 1.35 $\mu g\,ml^{-1}$ for benomyl and iprodione, respectively), Dic^{MR} (mean EC_{50}: 1.78 $\mu g\,ml^{-1}$ for iprodione) and $Ben^{MR}Dic^{MR}Pcm^{R}$ (mean EC_{50}: 1.96 $\mu g\,ml^{-1}$ for the mixture carbentazim + diethofencarb). The anilinopyrimidine fungicides cyprodinil and pyrimethanil, inhibited mycelial growth of the sensitive and the aforementioned resistant strains of *B. cinerea* at low concentrations. The estimated EC_{50} values for all strains tested on asp–agar medium varied from 0.005 to 0.01 $\mu g\,ml^{-1}$ for cyprodinil and from 0.005 to 0.04 $\mu g\,ml^{-1}$ for pyrimethanil (Table 1). Rather higher EC_{50} values were obtained on gl–agar medium (data not shown).

In the *in vivo* tests, only cyprodinil and pyrimethanil applied preventively completely controlled infections caused by all *B. cinerea* strains tested on cucumber seedlings and on mature fruits, whilst the effectiveness of the reference fungicides was related to the phenotype of the resistant strains (data not shown).

Effectiveness of pyrimethanil against grey mould in tomatoes

The effect of pyrimethanil and the reference mixture (iprodione + dichlofluanid) on disease progress on

Table 1. In vitro effect of cyprodinil and pyrimethanil on mycelial growth of *B. cinerea* strains sensitive or resistant to benzimidazoles, dicarboximides and/or *N*-phenylcarbamates (asparagine–agar medium)

Strain	Phenotype[a]	EC_{50} values in $\mu g\,ml^{-1}$	
		Cyprodinil	Pyrimethanil
2	Wt	0.00498 (0.00257, 0.00831)	0.00543 (0.00242, 0.00977)
3	Ben^{HR}	0.00662 (0.00391, 0.01068)	0.01452 (0.00695, 0.03414)
5	$Ben^{HR}Dic^{MR}$	0.00707 (0.00440, 0.01141)	0.00993 (0.00505, 0.1935)
8	$Ben^{MR}Dic^{MR}Pcm^{R}$	0.01 (0.00607, 0.01683)	0.03108 (0.01820, 0.04580)
14	Dic^{MR}	0.00809 (0.00524, 0.01244)	0.04350 (0.03153, 0.05704)

[a]Wt, wild type; Ben^{HR} and Ben^{MR}, highly or moderately resistant to benzimidazoles; Dic^{MR}, moderately resistant to dicarboximides; Pcm^{R}, resistant to phenylcarbamates.
Numbers in parenthesis indicate 95% confidence limits determined by probit analysis.

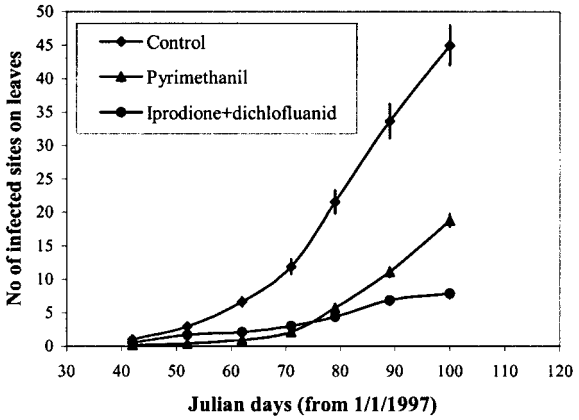

Figure 1. Progress of grey mould on leaves (infected sites/plant), treated with pyrimethanil and the mixture iprodione + dichlofluanid, in greeenhouse grown tomatoes. Assessments were caried out from 11/2/1997 (day 42) to 10/4/1997 (day 100).

leaves and other plant parts of tomatoes is presented in Figures 1 and 2 and Table 2.

Grey mould progress on tomato leaves after repeated applications of pyrimethanil, is shown in Figure 1. The increase of number of *B. cinerea* lesions on leaves became apparent after day 72 (sixth application) in comparison to the reference mixture, when the disease progress curve of pyrimethanil crossed that of the mixture. Despite the decline in effectiveness of pyrimethanil, a significant reduction of infected sites on leaves/plant was obtained in pyrimethanil and iprodione + dichlofluanid treated plots in comparison to the control (Table 2). Both treatments reduced ($P \leq 0.05$) the number of stem lesions in comparison to the control (Table 2). Disease incidence on stems (Figure 2A) in the mixture treated plots started to increase at the very early stage of *Botrytis* epidemic (after first assessment, third application), while in the pyrimethanil treated plots no stem lesions were observed until the third assessment (fifth application). The rates of increase (slopes of the lines between two successive assessments) seemed to differ between the two fungicide(s) treatments at the end of the growing season (Figure 2A). A similar disease pattern was observed in the case where dead plants (Figure 2B) were assessed (shifted time-wise – ca. 20 days later), in the fungicides treated plots. The number of dead plants in these plots was reduced in comparison to the untreated control but the difference was not statistically significant at $P \leq 0.05$ in the overall analysis (Table 2) where the integral of disease progress was taken into account. The cumulative number of infected fruits per plant was reduced in the fungicide treated plots (Table 2 and Figure 2C). Pyrimethanil failed to reduce the total number of tomato fruits/plant with 'ghost spots' symptoms in comparison to the reference mixture iprodione + dichlofluanid (Table 2). The percentage of fruits per plant with 'ghost spots' was found to be 18.53% and 19.60% in control and pyrimethanil treated plots respectively, compared to 3% in the plots treated with the mixture (last assessment, Figure 2D).

Impact of repeated applications of pyrimethanil on the sensitivity of B. cinerea *population in the greenhouse trial*

The mean EC_{50} values of *B. cinerea* population prevailing in the greenhouse, prior to the applications, were as follows: pyrimethanil 0.01314 (0.00632–0.02803) $\mu g\,ml^{-1}$, benomyl 130.54 $\mu g\,ml^{-1}$, iprodione 4.37 (3.89–4.89) $\mu g\,ml^{-1}$, the mixture of carbendazim + diethofencarb 9.86 (4.76–13.20) $\mu g\,ml^{-1}$ and dichlofluanid $<0.0625\,\mu g\,ml^{-1}$. Therefore, *B. cinerea* population in the greenhouse before the applications could be characterized as highly resistant to benzimidazoles, moderately resistant to dicarboximides, resistant to phenylcarbamates and

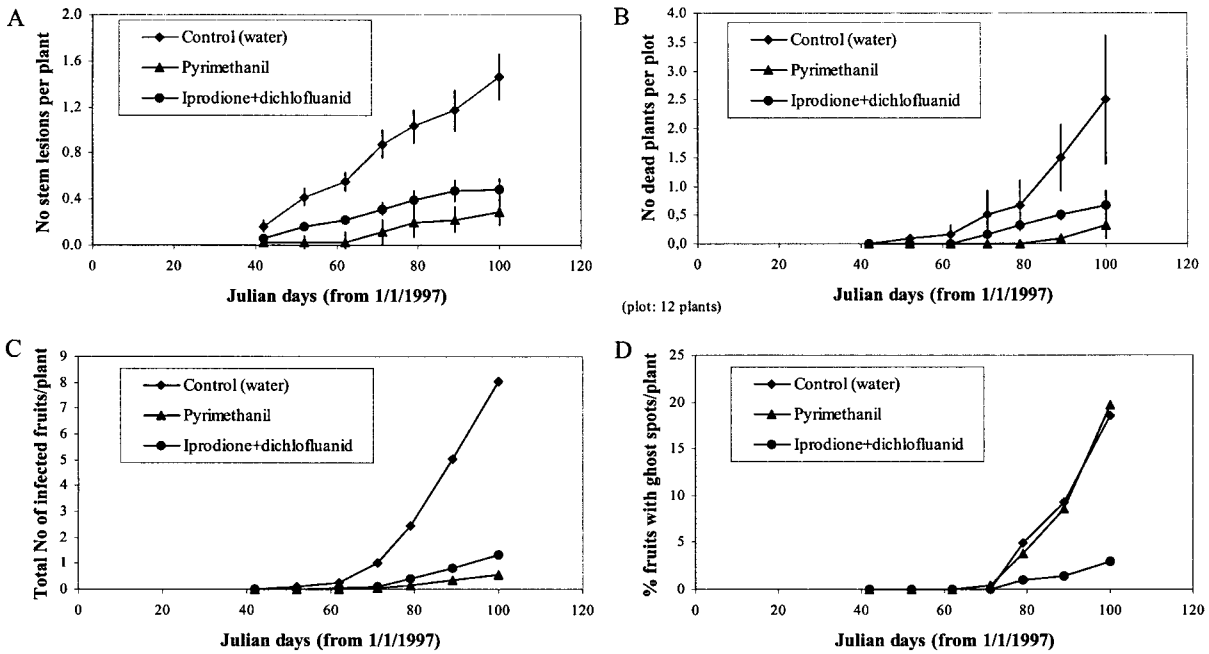

Figure 2. Infected sites on stems (stem lesions/plant) (A), number of dead plants per plot (B), total number of infected fruits by *B. cinerea* per plant (C) and percentage of fruits with ghost spots (at the time of assessment) (D), in greeenhouse grown tomatoes treated with pyrimethanil and the mixture iprodione+dichlofluanid. Assessments were caried out from 11/2/1997 (day 42) to 10/4/1997 (day 100).

Table 2. Effectiveness of pyrimethanil and iprodione + dichlofluanid against grey mould incidence on different parts of tomato plants grown under cover

Treatment	AUCleav	Diseased fruits (No plant^{-1})	AUCsles (plant^{-1})	AUCdepl (plot^{-1})	Fruits with ghost spots (Total no plant^{-1})
Control (water)	1212.70a	8.04a	44.63a	18.30a	5.26a
Iprodione + dichlofluanid	277.446b	0.55b	6.83b	11.10a	0.81b
Pyrimethanil	387.05b	1.32b	17.26b	2.96a	4.72a

Different letters in columns denote statistically significant differences at $P \leq 0.05$.
AUCleav: area under curve of infected sites on leaves per plant (lesionsdays).
AUCsles: area under curve of on infected sites on stems (lesionsdays).
AUCdepl: area under curve of percent dead plants per plots.

sensitive to dichlofluanid and pyrimethanil (phenotype $Ben^{HR}Dic^{MR}Pcm^{R}Dichl^{S}Ani^{S}$).

Results at the end of the experiment showed that the sensitivity of *B. cinerea* population to pyrimethanil was reduced (EC_{50} value 0.1011 (0.0365–0.368) $\mu g\,ml^{-1}$). The R_L (resistance level) value, estimated as the ratio of the EC_{50} value after eight applications of pyrimethanil/EC_{50} value before any application, was found to be 7.7. The EC_{50} value of dichlofluanid did not change much, 0.072 (0.06–0.09) $\mu g\,ml^{-1}$, while that of iprodione slightly increased (EC_{50} value 6.31 (5.75–6.92) $\mu g\,ml^{-1}$).

In the greenhouse, the effectiveness of pyrimethanil on leaves started to decline after day 72 (sixth application) in comparison to the reference mixture (Figure 1). This reduction in effectiveness, which was accompanied by reduced sensitivity of the population of *B. cinerea* to pyrimethanil *in vitro*, was further studied using linearized forms of the logistic model. The impact of repeated applications of pyrimethanil on the parameters of disease progress (y_o and r_L) are presented in Table 3. The infection rate of *B. cinerea* r_L (slope of the linear regression line) in pyrimethanil treated plants was equal to that of the control while

Table 3. Final disease incidence (y_i) of grey mould caused by *B. cinerea*, slope (r_L) and intercept (y_o) for regression of logit transformation of the number of infected sites on leaves over time (greenhouse experiment)

Treatments	y_i	r_L	SE	y_o	SE
Control	44.95	0.09518a	0.00125	−6.6298a	0.619302
Iprodione + dichlofluanid	7.91	0.05016b	0.00683	−8.1540a	0.613195
Pyrimethanil	18.75	0.09396a	0.00466	−10.0747b	0.533293

that of the mixture iprodione + dichlofluanid was significantly lower. Disease onset, as indicated by y_o values, was significantly affected only by pyrimethanil ($P \leq 0.05$).

Discussion

Botrytis cinerea is a classical 'high-risk' pathogen for development of resistance to selective fungicides, due to its short generation time and abundance of sporulation (Jarvis, 1992). The control of grey mould has recently been problematic in Greece and many other countries due to the presence of *B. cinerea* strains with double resistance to commonly used botryticides such as benzimidazoles and dicarboximides and with wild type insensitivity to diethofencarb (Elad et al., 1992; Pappas 1997). Highly resistant strains were found only in the case of benzimidazoles, and these strains, such as strain 3 of this study, exhibit phytopathogenic fitness parameters similar to those of the sensitive strains (Georgopoulos, 1987; Kalamarakis et al., 2000).

In the present study, in the *in vitro* bioassays, cyprodinil and pyrimethanil, were active in both asp–agar and gl–agar media against mycelial growth of all strains of *B. cinerea* sensitive and resistant to benzimidazoles and/or dicarboximides and to the mixture of carbentazim+diethofencarb. On asp–agar, EC_{50} values varied from 0.005 to 0.01 $\mu g\,ml^{-1}$ for cyprodinil and from 0.005 to 0.04 $\mu g\,ml^{-1}$ for pyrimethanil (Table 1). These values, according to Hilber and Schüepp (1996), were in the sensitive range to anilinopyrimidines. The absence of cross-resistance between cyprodinil and pyrimethanil on the one hand and benzimidazoles or dicarboximides on the other (Table 1) was in agreement with published reports (Leroux and Montcomble, 1994; Forster and Staub, 1996; Petsikos-Panayotarou et al., 2001). These results were confirmed *in vivo* (data not shown). On cucumber seedlings or mature fruits inoculated either with a conidial suspension or with young mycelium (17 h) both anilinopyrimidine fungicides effectively controlled *B. cinerea* strains independently of being sensitive or resistant to other botryticides. When older mycelium (3 days) was used as inoculum, in *in vivo* studies carried out during 1996, the effectiveness of cyprodinil and pyrimethanil was not evident in all cases (unpublished data).

The effectiveness of anilinopyrimidines in relation to the type of infection caused by *B. cinerea* on different tomato plant parts was a major concern in Greece and other Mediterranean countries where leaf, stem and fruit infections by *B. cinerea* result in high yield losses (Meneses et al., 1994; Elad et al., 1996). So far, there have been only a few reports, mainly from Israel, concerning the use of anilinopyrimidines in spraying programmes in tomatoes in unheated greenhouses (Shtienberg and Elad, 1997; Shtienberg et al., 1998). In the greenhouse trial (Table 2), pyrimethanil controlled stem, leaf and fruit infections caused by *B. cinerea* (phenotype $Ben^{HR}Dic^{MR}Pcm^{R}Dichl^{S}Ani^{S}$) but it did not reduce the number of fruits with 'ghost spots' symptoms. Although all fungicides reduced the number of infected sites on stems and leaves, they did not reduce the number of dead plants per plot in comparison to the controls, despite the lower but not statistically significant values of the AUCdpl. These results are mostly related to the specific characteristics of *Botrytis* epidemiology in tomatoes in Greece and other Mediterranean countries. In the present study, it was observed that the number of stem lesions at the beginning of the growing season was extremely low, thus the number of lost plants was limited. However, the number of dead plants started to increase during the last three weeks of the experiment. This was probably due to (a) the progression of the direct infection of stems which untimely girdle the stem and killed the plant parts above (which occurred mainly at the early stages of the epidemic) and (b) the gradual progression of the pathogen along infected petioles (macroscopic observation). The short duration of *Botrytis* epidemic in Crete (ca. 2 months) could explain why the leaf infections, even when progressed to the stems, did not cause significant plant death (last assessment – 20.8% dead plant in control plots). In Israel, delayed stem infections (6–8 weeks after leaf infection) resulted in significant plant losses due to the much longer period (4 months)

of disease assessment in the greenhouses (Shtienberg et al., 1998).

The total number of foliar fungicide applications (as single treatments, in alternation or in mixtures) in greenhouses of South Europe and Israel (Elad et al., 1996) is high, reaching a maximum of 8–10 applications during the period of grey mould outbreaks. In such a crop-pathogen system, repeated applications of fungicides may favour the built up of fungicide resistant *Botrytis* populations and the strong selection pressure may influence the performance of site specific fungicides such as anilinopyrimidines. The greenhouse trial showed that selection pressure caused by the repeated applications of pyrimethanil in full rate resulted in declining effectiveness of this fungicide against *B. cinerea*, which was more characteristic on the leaves (overlapping cycles of the pathogen). This became obvious after the sixth application (fourth assessment) when the disease progress curve of pyrimethanil crossed that of the mixture iprodione + dichlofluanid (Figure 1). The infection rate in the pyrimethanil treated plants was statistically higher (data not shown) than that of the reference mixture between the last two assessments.

When further analysing the data of disease incidence on leaves, with the use of linearized forms of the logistic model, pyrimethanil caused a statistically significant reduction of the initial level of disease (y_o) in comparison to the control but it did not affect the infection rate (r_L) (Table 3). By contrast, the applications of the mixture did not significantly reduce the y_o value of the disease but resulted mainly in significantly lower r_L values (Table 3). These results indicate that pyrimethanil had an effect on disease progress similar to 'vertical' or 'race specific' disease resistance. Van der Plank was the first interpreting the horizontal and vertical resistance of the host against pathogen races, using the logistic model. He showed that 'vertical resistance' lowered the initial level of the disease and the 'horizontal' reduced the infection rate. This model of horizontal and vertical resistance can be adapted also for fungicides (Waggoner and Aylor, 2000). Zadoks (1982) stated that the effect of site-specific fungicides such as pyrimethanil is equivalent to 'vertical' resistance.

The results from the greenhouse experiment support those obtained from the laboratory tests indicating that the sensitivity of *B. cinerea* population to pyrimethanil decreased after eight successive applications ($R_L = 7.7$). These results describe a shift of *Botrytis* population towards reduced sensitivity, possibly due to a 'low' level of resistance development. This shift was correlated with a declining disease reduction by pyrimethanil from 86% to 58% (average value 68.09%, as estimated from the values of AUDPCs). The system of eight consecutive sprays (at 10 day intervals) for pyrimethanil was chosen in the present study in order to (a) maintain selection pressure over each pathogen cycle and (b) evaluate the possibility of resistance development. However, eight consecutive treatments is by no means a common practice for anilinopyrimidines, where a limitation in the number of applications up to three per season is recommended by FRAC as antiresistance strategy.

The greenhouse results are similar to published data from long-term strategy trials in Swiss vineyards in which a reduced effectiveness of anilinopyrimidines (after four applications per season) was correlated with the appearance of resistant strains of *B. cinerea* in the field (Forster and Staub, 1996). In a monitoring survey conducted in France (Leroux et al., 2000), the majority of dicarboximide resistant strains of *B. cinerea* isolated from vineyards were of low resistance ($2.5 < R_L < 10$, at the germ tube elongation stage, *in vitro* test) to anilinopyrimidines (types AniR2 and AniR3) and only a small proportion exhibited high levels ($10 < R_L < 300$) of resistance (type AniR1). However, Leroux et al. (2000) did not observe any reduction in efficacy of anilinopyrimidines. This was possibly due to the restricted number of recommended applications of anilinopyrimidines in French vineyards.

The possibility of resistance development of *Botrytis* to anilinopyrimidines in the field is supported by genetic studies (Chapeland et al., 1999), which indicated that one major gene was probably involved in the AniR1 phenotype. Furthermore, investigations carried out by Hilber and Hilber-Bodmer (1998) in Swiss vineyards showed that resistance of *B. cinerea* to cyprodinil was monogenic and resistant strains were detected after 2–6 consecutive applications of cyprodinil.

Taking into account all our results from the laboratory and greenhouse experiments as well as data from field experiments conducted in other countries, a considerable inherent risk of resistance to anilinopyrimidines has to be recognized in *B. cinerea*. Although, antiresistant strategies already exist for anilinopyrimidines (limitation in the number of applications or use of mixtures with other botryticides), additional field data on the phytopathogenic fitness of resistant strains, will be needed to provide useful information about the risk of field failures in *B. cinerea* control programmes.

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