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Full Length Research Paper

Sensitivity of *Botrytis cinerea* isolates against some fungicides used in vineyards

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During August 2004 and 2005, isolates of *Botrytis cinerea* were collected from table and wine grapes in the Trakya region, Turkey. They were tested for sensitivity to cyprodinil+fludioxonil, fenhexamid, procymidone, pyrimethanil and tebuconazole under laboratory conditions. Fungicide sensitivity tests showed that *B. cinerea* isolates appeared more sensitive to cyprodinil+fludioxonil than to fenhexamid, imazalil, procymidone, pyrimethanil and tebuconazole. Efficacy tests *in vivo* showed that cyprodinil+fludioxonil and tebuconazole were the most effective fungicides (100%) on isolates both resistant and sensitive to these fungicides. No lesion on berries inoculated was observed on the fungicide applied and inoculated fruits. However, imazalil was ineffective, even in commercial concentrations, in controlling gray mold in fruit infection. In addition, fitness of fungicide-resistant isolates compared with that of sensitive isolates was determined through mycelial growth, sporulation and virulence. Analysis of data also indicated significant differences (P=0.05) between fungicide-R and -S isolates in fitness characteristics between the fungicide-R/S isolates.

Key words: *Botryotinia fuckeliana*, sensitivity, efficacy, fungicides, fitness.

INTRODUCTION

Botrytis cinerea Pers ex. Fr. (anamorph of *Botryotinia fuckeliana* (de Bary) Whetz), occurring in a wide range of plants, is a causal agent of severe pre- and post-harvest disease of the grapevine industry (Jarvis, 1980; Holz et al., 2003; Droby and Lichter, 2004; Delen et al., 2004). It causes severe yield losses in grape, especially in coastal regions of Turkey (Burçak, 1998; Koplay, 2003; Özer et al., 2004; Köycü et al., 2005). Current disease management strategies aim to reduce the initial inoculum source of *B. cinerea*, preventing flower infection by fungicide applications (Holz et al., 2003; Walter et al., 2005; Zitter and Wilcox, 2006). Chemical control of the pathogen is impeded by development of resistance to many botryticides due to high genetic variability among the pathogen population. Due to this genetic characteristic of the pathogen, over the last 20 years, resistance of *B. cinerea* to anilinopyrimidine, benzimidazole, dicarboximide hydroxyanilide and phenylpyrrole has been reported in different countries soon after their introduction for the control of gray mold disease (Gullino et al., 1989; Latorre et al., 1994; Hilber and Hilber-Bodmer, 1998; Ziogas and Klamarakis, 2001; Baroffio et al., 2003; Leroux, 2004; Guido et al., 2007); therefore, authorities and Fungicide Resistance Action Committee (FRAC) have restricted numbers of applications per season of each family of botryticides (Rosslenbroich and Stuebler, 2000; Leroux, 2004).

Monitoring sensitivity of isolates in the presence of fungicides is necessary to develop a better chemical control strategy. Therefore, surveys have been performed in the essential grape growing regions of Turkey. One of the earliest surveys that was done in the Aegean and Marmara regions of Turkey, showed that most isolates sampled were sensitive to benzimidazole (e.g. carbendazim) and dicarboximide (e.g. vinclozolin, iprodione, procymidone) fungicides (Erkan et al., 1997). In the following years, isolates of *B. cinerea* resistant to

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carbendazim were found in the Aegean region (Burçak, 1998). Recently a few isolates exhibited reduced sensitivity to fenhexamid, a hydroxyanilide derivative (Koplay, 2003). Trakya is a region in north-west Turkey where table and especially wine grapes are cultivated in about 6800 ha. This region is humid due to coastal region. *B. cinerea* causes of economic losses in this region. High disease severity (60%) occurs in some wine grape varieties in this region and its control relies on chemical applications (Köycü et al., 2005). Growers in this location apply different groups of fungicides such as pyrimethanil and cyprodinil from anilinopyrimidines, fludioxonil from phenylpyrroles, fenhexamid from hydroxyanilides, procymidone from dicarboximides and imazalil from imidazoles against the gray mold disease. The use of imazalil, procymidone, pyrimethanil, tebuconazole in Trakya region began in 1996. Cyprodinil+fludioxonil and fenhexamid have been using since 1998 and 2001, respectively.

Although these fungicides have medium or low resistance risks of *B. cinerea* (Hilber and Hilber-Bodmer, 1998; Leroux, 2004; Guido et al., 2007), sensitivity of the pathogen in the Trakya region vineyards of Turkey has not been reported previously. Characterizing the extent of the fungicide resistance problem within this region would provide growers with information that could potentially improve their gray mold chemical control program. The aims of this study were to determine sensitivity of *B. cinerea* isolates to fungicides of the different groups used in vineyards in the Trakya region, to investigate efficacy of fungicides on field isolates on grape berries and to examine fitness characters of field isolates.

MATERIALS AND METHODS

Fungicides

Commercial formulation fungicides were used: cyprodinil+fludioxonil (Switch 62.5 WG, Syngenta Crop Protection AG), fenhexamid (Teldor 50 SC, Bayer Crop Science AG) imazalil (Magnate 50 EC, Hektaş Crop Science AG), procymidone (Sumisclex 50 SC, Sumitomo Chemical Corporation), pyrimethanil (Mythos 30 SC, Bayer Crop Science AG) and tebuconazole (Folicur 25 WP, Bayer Crop Science AG). Stock solutions of fungicides were prepared in ethanol, with exceptions of fenhexamid, procymidone and pyrimethanil which were dissolved in sterilized distilled water. After dilutions of the fungicides in ethanol or distilled water, small amounts of these fungicide solutions were added to autoclaved molten agar media to obtain desired concentrations. The final ethanol or sterile distilled water concentration never exceeded 1% (v/v) in treated and control samples.

Isolates and media

B. cinerea isolates were collected from table and wine grape cultivars in the Trakya region/Turkey. Sampling was carried out in August 2004 and 2005 from vineyards where fungicides were intensively sprayed and were transferred to the laboratory in individual polyethylene bags in coolers. Isolations for each isolate of *B. cinerea* were made from berries using minimal medium (MM), containing per litre: 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $FeCl_3$, 15 g L-asparagine, 1 g yeast extract, 20 g glucose, 20 g agar and 1 L distilled water (Delen, et al., 1984).

Fungicide sensitivity tests

70 isolates were selected for sensitivity treatment. The sensitivity of isolates was tested on MM amended with 0 (control) and various concentrations from 0.01 to 10 µg/ml of fungicides. All fungicide concentrations are expressed as active substance (µg/ml). Agar disks were cut from actively growing mycelial plugs (4 mm in diameter) obtained from three-day-old cultures and placed with the surface mycelium face down on Petri dishes containing fungicides or un-amended control. Colony diameters were measured after incubation for four days at 23°C in the dark. Three replicates were used per treatment. To evaluate the effectiveness of fungicides EC_{50} values (µg/ml), the effective concentration for a 50% reduction of mycelial growth were determined in the presence of fungicides. This experiment was repeated twice.

Effectiveness of fungicides on selected isolates

The two isolate for each fungicide were selected. Selection of isolates was defined according to their EC_{50} values (µg/ml) as follows: Isolate with the highest EC_{50} value was considered with the symbol (fungicide-R); isolate with the less EC_{50} value was considered with the symbol (fungicide-S). Fungicide-R/S field isolates of *B. cinerea* were tested on the white wine grape cultivar *Emir* (*Vitis vinifera* cv. Emir) accordance with the method of Latorre et al. (2002) and Koplay (2003). This cultivar was determined to be sensitive to *B. cinerea* in a previous study by Köycü et al. (2005). Fruits were sterilized for 4 to 5 min by immersing in sterile water with 1% NaOCl. Fungicides were used at the commercially recommended dosage, half and quarter of that dosage. The fungicides as aqueous suspensions of each dosage were sprayed with a hand-sprayer until runoff on fruits punctured with a needle. Fruits were then inoculated with 20 µL of a conidial suspension of *B. cinerea* adjusted to 1.5×10^6 conidia/ml. Control fruits were sprayed with sterile distilled water. Treatments were replicated three times using 10 fruit as the experimental unit. Inoculated fruits were placed on sterile long and narrow wood strips in sterile plastic boxes containing wetted Whatman paper; these were enclosed in polyethylene bags at 90 to 95% relative humidity to stimulate disease development. All boxes were incubated for four days at 23°C with a cycle of 12 h light/12 h darkness. This experiment was repeated twice. Diameter of growing lesions on fruits was measured and the efficacy of fungicide was calculated according to Abbott's formula = 100*[Untreated control grapes (A)-treated grapes/A] (Abbott, 1925).

Fitness parameters of selected isolates

Each fungicide-R/S isolate of *B. cinerea* was tested for mycelial growth rate, conidial production on MM media and virulence of isolates on grape leaves. The two isolates were tested for each fungicide. Three mycelial MM-plugs (1 cm in diameter) were transferred to the centre of MM-plates for mycelial growth measurements and incubated at 23°C in dark. The colony diameter of each isolate was measured approximately with 24 h intervals to calculate growth rate in cm/day. To determine conidial production, each isolate was grown in 9 cm Petri dishes on MM at 23°C. Three agar disks (1cm in diameter) were randomly taken from each fungicide-R/S isolate after seven to 10 days of incubation. These disks were placed in Eppendorf tubes containing 0.01% Tween 20 solution and centrifuged for 5 min at 12000 g. The number of conidia/ml was determined under a light microscope using a hemacytometer.

Virulence of isolates was tested on vine leaves according to the method described by Vallejo et al. (2003). Leaves were sterilized for 5 min with sterile water. They were punctured with a needle and a 1-cm mycelial plug from the margin of a colony on MM was placed upon the wound. The inoculated leaves were incubated as described above. Controls received MM plugs without any fungus. Treatments were replicated three times using two leave for each replication. Four days after inoculation, diameters of lesions on leaves were measured. This experiment was repeated twice.

Data analysis

The EC_{50} value (µg/ml) of each isolate was calculated from data subjected to probit-analysis. Mean values of efficacy of each fungicide concentration on resistant/sensitive isolates were analyzed by Duncan's multiple range test (P = 0.05). In the fitness experiments of selected isolates, differences were calculated by Student's t test.

RESULTS

Sensitivity of the isolates

The ranges of EC_{50} values for the isolates varied between the different fungicides. Results of the sensitivity tests showed that *B. cinerea* isolates appeared more sensitive to cyprodinil+fludioxonil than to fenhexamid, imazalil, procymidone, pyrimethanil, and tebuconazole (Figure 1). 10 of the isolates had EC_{50} ≤0.01 µg/ml and EC_{50} value 60 isolates were determined between 0.01<-≤0.1 µg/ml for cyprodinil+fludioxonil fungicide. The EC_{50} values of isolates for fenhexamid ranged from <0.01 to 3 µg/ml. The EC_{50} values of 64 isolates tested were determined as ≥0.1 µg/ml for this fungicide. The EC_{50} values of isolates ranged from 0.03 to 1 µg/ml for procymidone, pyrimethanil and tebuconazole. Among the isolates tested, five isolates for pyrimethanil and tebuconazole, one isolate for procymidone were EC_{50} values ≥1 µg/ml and 69 isolate had EC_{50} 0.03≤ - ≤0.1µg/ml. 25 of the collected isolates for imazalil had EC_{50} values ≥3 µg/ml.

Effectiveness of fungicides to selected isolates

Effectiveness studies showed that fungicide-R/S isolates of *B. cinerea* maintained their ability to cause infection on fruit as indicated in Table 1. Efficacy tests showed that cyprodinil+fludioxonil and tebuconazole were the most effective fungicides (100%) to R and S isolates. No lesion was observed on berries inoculated both with R and S isolates on the fungicide applied and inoculated fruits. Effectiveness of procymidone and pyrimethanil to fungicide-R isolates were found between 67 to 100% and 80 to 94% respectively, however, all concentrations of procymidone and pyrimethanil were effective at a high rate (>90%) on fungicide-S isolates. Although all concentrations of fenhexamid reduced more than 90% the growth of the fungicide-S isolate on fruits, effectiveness of the same fungicide concentrations to fungicide-R isolate were rather low (between 22.10 to 50.53%). In contrast, imazalil did not show enough effectiveness to both fungicide-R and S isolates.

Fitness of isolates

Individual data for mycelial growth, sporulation on agar media and lesion growth (virulence) on leaves showed wide variability among isolates (Figure 2). Analysis of data also indicated significant differences (P = 0.05) between fungicide-R and -S isolates in fitness characterristics. Mycelial growth, sporulation and virulence of tebuconazole-R isolates were significantly high compared to tebuconazole-S isolate. Virulence of cyprodinil+fludioxonil-R, imazalil-R and procymidone-R isolates was significantly higher than that of isolates sensitive to these fungicides; however mycelial sensitivity of *B. cinerea* isolates growth was low compared to S isolates. Conversely, with the exception of procymidone-R, these isolates had higher sporulation ability than the sensitive isolates, although differences were not significant. Pyrimethanil-R/S isolates showed no significant differences for sporulation and virulence. Moreover, fenhexamid-R/S isolates appeared to be equally fit for all parameters tested in this study.

DISCUSSION

During the last decade, chemical control of gray mold on grape vines has been based upon the use of cyprodinil+fludioxonil, fenhexamid, imazalil, pyrimethanil, procymidone and tebuconazole in the Trakya region of Turkey. Therefore, *B. cinerea* isolates were collected from this area and surveyed for their sensitivity to these fungicides. Multidrug sensitivity, the effectiveness of fungicides and fitness of *B. cinerea* isolates were also monitored.

Resistance of *B. cinerea* to anilinopyrimidine (cyprodinil and pyrimethanil) is well known. Resistance to anilinopyrimidine has monogenic inheritance (Hilber and Hilber-Bodmer, 1998; Chapeland et al., 1999). Sensitivity to anilinopyrimidines in field isolates of *B. cinerea* was reduced and their efficacy lessened in control of gray mold due to their intensive use. Therefore, resistance of *B. cinerea* to anilinopyrimidine has occurred rapidly in vineyards (Forster and Staub, 1996; Latorre et al., 2002; Baroffio et al., 2003; Leroux, 2004). More than 50% resistance to anilinopyrimidines (cyprodinil and pyrimethanil) has been reported (Esterio et al., 2010). In addition, pyrimethanil-resistant isolates were detected at high frequency (12 to 93%) in fields (Rotolo et al., 2010). Laboratory tests have shown that resistance to phenylpyrrole (fludioxonil) may occur after chemical mutagenesis and that mutant strains were highly resistant to fludioxonil, whereas fludioxonil-R isolates were rarely determined in the field (Baroffio et al., 2003). The

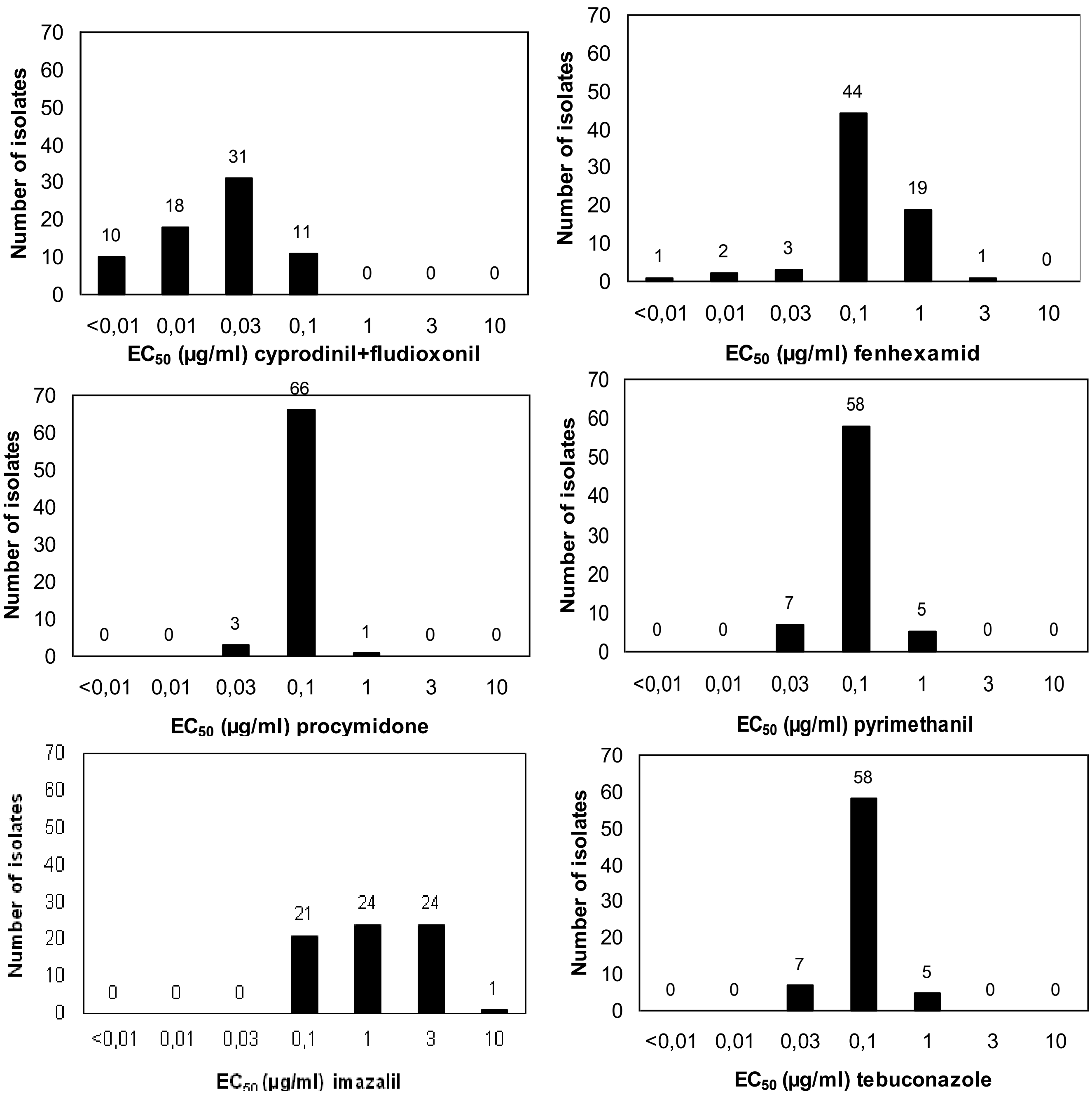

Figure 1. Frequency distribution of EC_{50} values (µg/ml) for cyprodinil+fludioxonil, fenhexamid, imazalil, pyrimethanil, procymidone and tebuconazole determined with *Botrytis cinerea* isolates.

anilinopyrimidine derivatives cyprodinil and pyrimethanil have been used since 1997 and 1996, respectively, in Turkey; but cyprodinil is used as a mixture with fludioxonil in vineyards.

Previous reports demonstrated that decreasing efficacy of cyprodinil in vineyards coincided with a high level of cyprodinil-R isolates in laboratory tests (Forster and Staub, 1996; Latorre et al., 2002).

In this study, most of the isolates were sensitive (EC_{50}≤0.03 µg/ml) to cyprodinil+fludioxonil. When cyprodinil and fludioxonil were applied as a mixture in vineyards, the mixture tested showed much better disease control (57 to 83%) (Forster and Staub, 1996). The current research also showed that all applied concentrations of cyprodinil+fludioxonil, significantly reduced infection on fruit by *B. cinerea.* A previous study reported that isolates of *B. cinerea* were EC_{50} values <1 µg/ml for pyrimethanil in the Aegean Region of Turkey (Koplay, 2003). In our

Table 1. Effectiveness of fungicides against sensitive and resistant isolates of *Botrytis cinerea* on fruits.

Fungicide	Phenotype*	EC_{50} (µg/ml)	Concentration (µg a.i /ml)**	Lesion growth (cm)***	Percent inhibition (%)****
Cyprodinil+fludioxonil	R	0.13	312.5	0^{o}	100
			156.25	0^{o}	100
			78.125	0^{o}	100
			0	0.95^{fg}	-
	S	<0.01	312.5	0^{o}	100
			156.25	0^{o}	100
			78.125	0^{o}	100
			0	1.31^{de}	-
Fenhexamid	R	3.00	500	$0.47^{h\text{-}l}$	50.53
			250	$0.54^{h\imath}$	43.16
			125	0.74^{gh}	22.10
			0	0.95^{fg}	-
	S	<0.01	500	0^{o}	100
			250	0.06^{no}	97.47
			125	$0.21^{k\text{-}o}$	91.14
			0	2.37^{a}	-
Imazalil	R	10.00	150	1.40^{d}	45.52
			75	1.97^{b}	23.35
			37.5	1.88^{b}	26.85
			0	2.57^{a}	-
	S	0.12	150	$0.42^{\imath\text{-}m}$	67.94
			75	$0.41^{\imath\text{-}m}$	68.70
			37.5	1.28^{de}	2.29
			0	1.31^{de}	-
Procymidone	R	1.00	325	0^{o}	100
			162.5	$0.18^{l\text{-}o}$	88.68
			81.25	$0.51^{h\text{-}k}$	67.92
			0	1.59^{cd}	-
	S	0.05	325	0^{o}	100
			162.5	0^{o}	100
			81.25	0^{o}	100
			0	1.31^{de}	-
Pyrimethanil	R	1.40	300	0.09^{no}	
			150	$0.19^{l\text{-}o}$	94.77
			75	$0.33^{\imath\text{-}n}$	88.95
			0	1.72^{bc}	80.81
	S	0.04	300	0^{o}	100
			150	0^{o}	100
			75	$0.13^{m\text{-}o}$	92.78
			0	1.80^{bc}	-

study, five field isolates of *B. cinerea* were found to be >1 µg/ml for pyrimethanil in the Trakya region.

However, the efficacy of applied concentrations of pyrimethanil was also high (>80%) against a fungicide-R isolate on fruit.

Fenhexamid, a hydroxyanilide derivative, is one of the most recently introduced botryticides. High mutation frequency of highly fenhexamid-resistant mutants was

Table 1. Contd.

Fungicide	Phenotype*	EC50 (µg/ml)	Concentration (µg a.i /ml)**	Lesion growth (cm)***	Percent inhibition (%)****
Tebuconazole	R	2.00	100	0^{o}	100
			50	0^{o}	100
			25	0^{o}	100
			0	0.35^{l-n}	-
	S	0.02	100	0^{o}	100
			50	0^{o}	100
			25	0^{o}	100
			0	1.04^{ef}	-

*R, Isolate with highest EC_{50} value; S, isolate with lowest EC_{50} value. **Quarter, half and recommended commercial dose of fungicides respectively.***Values within columns followed by different letters are significantly different according to Duncan's multiple range test (P = 0.05). ****Efficacy data calculated according to Abbott's formula with respect to percent inhibition.

reported, indicating a mutation frequency of 0.9×10^{-5} (Ziogas et al., 2003). However, fenhexamid was found to be highly effective (72%) in inhibiting disease development in vineyards (Baroffio et al., 2003). In Switzerland vineyard monitoring, a steady increase of the resistant (EC_{50} ≥0.1µg/ml) subpopulation was found from 1999 to 2001 (Baroffio et al., 2003). More also, reduced sensitivity of *B. cinerea* isolates to fenhexamid has been recently reported in the Chilean and South Italy (Esterio et al., 2010; Rotolo et al., 2010). Among field isolates surveyed from vineyards in the Aegean region/Turkey, only two isolate was determined as EC_{50} value >0.1 µg/ml (Koplay, 2003). In populations of the Trakya region, sensitivity of *B. cinerea* to fenhexamid showed fluctuation compared with other fungicides, but 64 of *B. cinerea* isolates surveyed from this location were found to be EC_{50} values ≥0.1 µg/ml for fenhexamid. All concentrations of fenhexamid were less effective (<51%) on fruit infection by fenhexamid-R isolates, with the inherent resistance risk to fenhexamid. Therefore, we considered that there might be genetic differentiation of these isolates.

Resistance to dicarboximide occurred in laboratory tests, and a reduction in sensitivity to dicarboximides occurred heavily in the field (Gullino et al., 1989; Latorre et al., 1994). Conversely, *B. cinerea* isolates were found to be sensitive to procymidone in this study with a high frequency. Procymidone inhibited fruit infection by fungicide-R/S isolates. Tebuconazole, a triazole derivative tested for resistance management on *B. cinerea* for the first time, has been registered for use against powdery mildew in vineyards in Turkey. In this study, 65 isolates of *B. cinerea* showed sensitivity (EC_{50} value ≤1 µg/ml) to tebuconazole. This fungicide controlled fruit infection by *B. cinerea* at the same rate as cyprodinil+fludioxonil. Therefore, the spray program with tebuconazole may be used to reduce the initial inoculum source in early stage and fruit infection as an alternative to cyprodinil+fludioxonil. Furthermore, isolates of *B. cinerea* were found to be sensitive to imazalil (EC_{50} values <1 µg/ml) in the Aegean region of Turkey (Burçak, 1998; Koplay, 2003). In this research, sensitivity of field isolates to imazalil was reduced. 25 isolates were determined to this fungicide with EC_{50} value ≥3 µg/ml. In addition, imazalil was ineffective, even in commercial concentrations, in controlling gray mold in fruit infection.

Fitness of isolates resistant to fungicides is an important parameter in disease management. If fitness of fungicide resistant isolates in the population is lower than fitness of sensitive isolates in the field, resistant isolates would decline in the absence of fungicide. Furthermore, fungicide applications such as use of mixtures, reduction in the application dose or in the number of spray applications influence the rate of variation of fungicide resistant isolates (Pringle and Taylor, 2002; Bardas et al., 2008). To achieve an effective disease management program, researches have compared fitness of resistant strains with that of sensitive strains. In most reports, development of resistance was associated with little difference in fitness of resistant isolates (Ziagos and Kalamarakis, 2001; Moyano et al., 2004). Fitness of anilinopyrimidine-R/S isolates surveyed from vineyards was evaluated in the absence of fungicides. In one of the first published reports regarding use of an anilinopyrimidine and phenylpyrrole mixture against gray mold, it was found that a certain proportion of the less sensitive strains of *B. cinerea* was fit enough to survive over winter (Forster and Staub, 1996). Similarly, growth rate, sclerotia production and osmotic sensitivity between cyprodinil-R/S isolates of *B. cinerea* isolated from table grapes were not significant (Latorre et al., 2002). The sporulation capacity and lesion growth rate of pyrimethanil-R/S isolates of *B. cinerea* obtained from vegetable crops were also not found to be significantly different (Moyano et al., 2004). However, this study showed that cyprodinil+fludioxonil-R/S and pyrimethanil-R/S isolates were significantly different in mycelial growth or virulence. These fungicides-R isolates were more virulent than sensitive isolates. Fenhexamid-resistant isolates showed lower spore

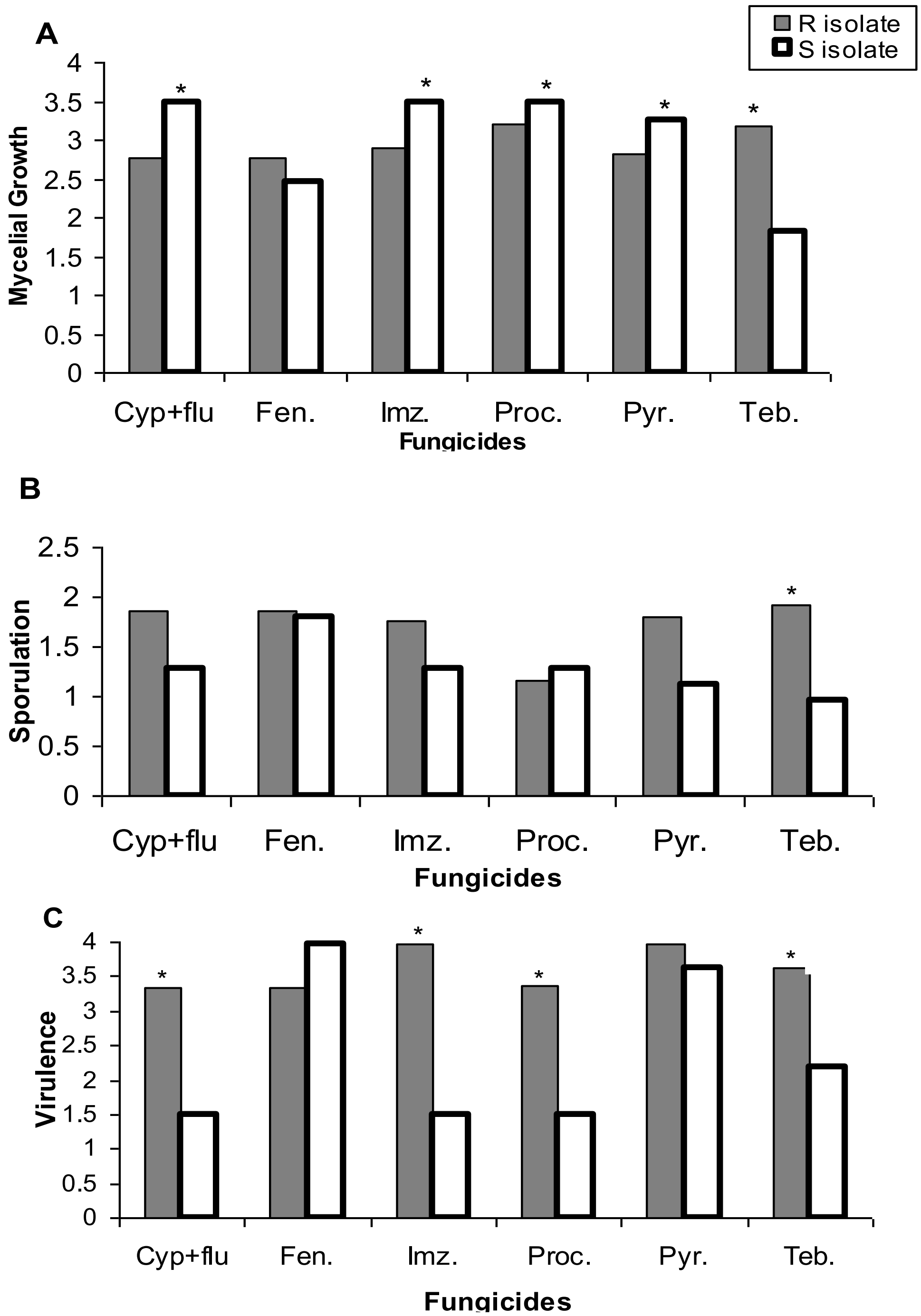

Figure 2. A, Mean values of mycelial growth rate (cm); B, sporulation (mean number ($\times10^6$) of conidia per ml); C, lesion development (mean colony diameter (cm)) after 4 days of incubation on leaves for fungicide-R/S phenotypes of *Botrytis cinerea* collected from vineyards in northwest Turkey in 2004 to 2005. Cyp+flu, Cyprodinil+fludioxonil; Fen., fenhexamid; Imz., imazalil; Proc., procymidone; Pyr., pyrimethanil; Teb., tebuconazole. Phenotype: R, isolate with highest EC_{50} value or S, isolate with lowest EC_{50} value. Data are means of three replications, *P=0.05 versus sensitive (S) isolate according to student t-test.

production, more sensitive to frost and less survival in the field than sensitive isolates (Delen, 2008). In this study, fenhexamid-R/S isolates appeared to be equally fit. The procymidone-R isolate was more virulent than the procymidone-S isolate, while the tebuconazole-R isolate was more fit than the tebuconazole-S isolate.

Based on results presented herein, sensitivity, efficacy, and fitness data have demonstrated that the number of applications per growing season should be considered carefully in an anti-resistance strategy. Limitation of the number of treatments will therefore slow the selection process for fungicide resistant isolates of *B. cinerea* in vineyards.

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