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Sensitivity stability and fitness of *Botrytis cinerea* isolates to captan

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The present study investigated the sensitivity and stability of *Botrytis cinerea* isolates collected from vineyards to captan. To determine the stability of sensitivity to captan, *B. cinerea* isolates on fungicide-free potato dextrose agar (PDA) was evaluated after 15 culture cycles. In some isolates with reduced sensitivity to captan, EC_{50} value of the isolates did not change compared to that of isolates that were initially sensitive to captan; suggesting that the decrease/increase in the sensitivity to fungicide may be stable. Isolates sensitive to captan adapted to increasing doses of captan by decreasing their sensitivity, and this adaptation remained stable in the fungicide-free medium. The fitness components included mycelial growth rate, sporulation, and virulence of the isolates. There were significant differences between isolates sensitive to captan and those with decreased sensitivity to captan, in terms of mycelial growth rate, sporulation, and virulence. The growth rate of isolates with decreasing sensitivity to captan was as high as the growth rate of those sensitive to captan. However, isolates with decreased sensitivity to captan showed higher virulence than those sensitive to captan, and the difference between these isolates was significant. Sporulation was dependent on the performance of the individual isolates.

Key words: Gray mould, sensitivity, captan, fungicide, virulence.

INTRODUCTION

Botrytis cinerea Pers.: Fr. (Teleomorph: *Botryotinia fuckeliana*) is known to cause important economic losses in vineyards found in Turkey (Delen et al., 2000; Özer et al., 2004; Köycü et al., 2005; Köycü 2007; Köycü et al., 2012). This is similar to that observed in many countries (Leroux, 2004). The use of fungicides and cultural control measures is important in the fight against this pathogen (Courderchet, 2003). However, rapid development of resistance to single-site fungicides used in chemicalcontrol of *B. cinerea* has increased the importance of implementing effective chemical control measures in vineyards (Courderchet, 2003; Leroux, 2004). Therefore, every country has implemented an effective chemical control program by determining the resistance profile of these pathogens against available fungicides (Leroux et al., 1999; Koplay et al., 2004;

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Köycü, 2012). The use of single-site fungicides, such as benzimidazoles and dicarboxamides, in combination with multi-site fungicides, such as chlorothalonil and dichlofluanid, and captan on vegetables has been suggested to be an effective control measure for preventing rapid development of resistance to single-site fungicides (Elad et al., 1995). Although no instances of decreased sensitivity to captan in fungal pathogens have been encountered, there have been reports of decreased sensitivity to captan among *B. cinerea* isolates recovered from ornamental plants and strawberries (Lorenzini 1983; Dianezet al., 2002; Walter et al., 2007). Studies in Turkey have reported decreased sensitivity of *B. cinerea* isolates to captan in greenhouses and vineyards (Delen et al., 1999; Koplay et al., 2004; Köycü, 2007). In particular, the activity of captan against *B. cinerea*in vineyards varies from 39 to 57% (Delen et al., 2000; Köycü, 2007). In other countries, captan is licensed for use against *B. cinerea* on vegetables and fruits. In Turkey, captan has been used under license since 1970 against *B. cinerea* on vegetables as well as against downy mildew (*Plasmopara viticola*) and dead-arm (*Phomopsis viticola*) in vineyards (Tosun and Onan, 2014). Captan, which is a multi-site fungicide belonging to trichloromethyl thiocarboxides, influences cell metabolism by preventing inorganic phosphates from being assimilated in the fungal cell; resulting in a fungitoxic effect by interacting with thiols in the cell membrane structure of the fungus. These reactions also involve thiol-containing enzymes, leading to the deterioration of the cell membrane structure and inhibition of oxidative phosphorylation in the cell (Delen, 2016).

The emergence of isolates resistant to fungicides within the fungal population largely depends on the fitness of isolates with resistance (Wang and Coley-Smith, 1986; Raposo et al., 2000; Leroux, 2004). The decrease in the ability to fitness in subpopulations resistant to fungicides is important for preventing development of fungicide resistance in the field. Although captan has not been licensed for use as a multi-site fungicide against *B. cinerea* in vineyards, determining the stability of *B. cinerea* sensitivity to captan is important for the continuity of reactivity of single-site fungicides against this pathogen as well as for the success of chemical control measures and cross-sensitivity of this pathogen to fungicides. Therefore, the purposes of the present study are (a) to determine the stability in sensitivity of *B. cinerea* isolates subcultures to fungicide captan and (b) to determine fitness of isolates in terms of sporulation, mycelial growth, and virulence in subcultures.

MATERIALS AND METHODS

Selection of Isolates

B. cinerea isolates were recovered from table and wine grape vineyards in Trakya Region and cultivated in a potato dextrose agar (PDA, Merck/Turkey). A discriminatory dose of 30 µg/ml captan was used (Köycü, 2007) to identify isolates with sensitivity and those with decreased sensitivity to captan (Best Captan 50 WP, Agrobest, Turkey). The spores of each isolate were inoculated on PDA plus fungicide prepared at a dose of captan 30 µg/ml. After inoculation of spores, isolates showing colony development and those showing no colony development were classified as decreased sensitivity to captan and sensitive to captan, respectively. The fungicide stock solution was prepared in ethanol. A total of 29 single-spores isolates were evaluated in terms of mycelial growth after amendment with captan at concentrations of 10, 15, 30, 100, 150, and 300 µg/ml to determine their EC_{50} (dose inhibited 50% mycelial growth) and Minimal Inhibition Concentration (MIC, µg/ml) on PDA plates. Mycelial plugs (4 mm in diameter) were cut from actively growing margins of 72-h-old colonies on PDA plates; three replications for each isolate were tested on PDA for fungicide amendment. Based on the evaluation, seven isolates with the highest EC_{50} value were selected as isolates with decreased sensitivity (R: main isolates), and three isolates with the lowest EC_{50} value were selected as isolates with sensitivity (S: main isolates). Isolates were stored at +4°C in PDA slants until they were used in the experiment.

Sensitivity levels

To determine whether decreased captan sensitivity among *B. cinerea* isolates was stable, seven *B. cinerea* isolates with resistance were transferred on the fungicide-free medium after completion of colony development for 2 weeks in captan-free PDA (Bardas et al., 2007). The isolates were re-cultured 15 times. The subcultures were named as Re isolates. Colony development of three S isolates that were selected as captan-sensitive isolates was performed on a PDA plate at 10-µg/ml. These isolates were developed with continuous dose escalation up to a captan dose value of 440 µg/ml in PDA plate and were named as Sy subcultures. These isolates obtained with high captan doses were inoculated again on a fungicide-free PDA as many times as the number of dose escalations. The resulting pathogen subcultures were named Ss isolates. The R, Re, S, Sy, and Ss isolates obtained from the test results were evaluated with respect to mycelial growth at doses of 3, 10, 15, 30, 100, 150, and 300 µg/ml, and their EC_{50}value were obtained by Log-probit analysis. FC was calculated by comparing the EC_{50} value of the R and S isolates to those of the Re, Sy and Ss isolates. The experiment was conducted with five replications using a randomized complete block design. Both experiments were repeated twice.

Fitness of Isolates

The fitness components, including sporulation, mycelial growth rates, and virulence, were evaluated to determine fitness and competitive ability of *B. cinerea* R, Re, S, Sy, Ss isolates (Dekker, 1982). Sporulation and mycelial growth rates of the isolates were evaluated *in vitro* in a PDA, and the virulence was assessed on leaves of Emir (white type) wine grapes, which were previously determined to be sensitive to *B. cinerea* (Köycü et al., 2005).

For evaluating mycelial growth rates, each isolate was placed on a 4 mm mycelial plug after 72 h of incubation at 23°C. For evaluating sporulation, the isolates were then cultivated on PDA at 23°C for 10 days with a 12-h photoperiod, and spore concentration measured in a hemocytometer.

For virulence assessments, medium-sized leaves of Emir were washed with tap water and were then kept in 1% sodium

hypochlorite (NaOCl) for 5 min; a repeated rinsing with sterile distilled water followed this. A fourfold of sterile and dry paper was placed in plastic containers. Two-centimeter-wide wood chips pre-sterilized in an autoclave were placed in the plastic containers to prevent direct contact of the leaves with the drying papers. The leaves were laid on top of the drying paper, with only the stems touching the moistened drying paper. Two leaves were used at every repetition. The leaves were battered using a fine-tip sterile injector, so that only epidermal tissues were punctured. After the *B. cinerea* isolates were cultivated on PDA at 23°C in darkness, they were inoculated with 1-cm-diameter agar plugs taken from the sides of 4-day-old colonies. Three discs were placed on each leaf (Vallejo, 2003). Then, the plastic containers were placed in transparent polyethylene plastic bags at 23°C and were allowed to incubate for 4 days with a 12-h photoperiod. The data were obtained by measuring lesion diameters. The experiment was performed with three replications, using a randomized complete block design.

Statistical analysis

Analysis of variance was conducted using SPSS (version 22; IBM Corp., Armonk, NY) for sensitivity levels of the isolates and all data from fitness tests. Means were separated using the Duncan Multiple Comparison Test (P≤0.05).

RESULTS AND DISCUSSION

Sensitivity levels

Numeric distribution of EC_{50} and MIC values for 29 isolates tested for their *in vitro* sensitivity to captan is provided. Two of the isolates had EC_{50} values of 10 to 20 µg ml^{-1}; one isolate had values of 21 to 25 µg ml^{-1}; eight isolates had values of 26 to 30 µg ml^{-1}; 11 isolates had values of 31 to 40 µg ml^{-1}; and six isolates had values of 41 to 50 µg ml^{-1}. However, only one isolate was found to have an EC_{50} value of >50 µg ml^{-1}. The MIC values of the isolates varied between 30 and 300 µg ml^{-1}. One isolate had an MIC value of 30 µg ml^{-1}, 10 isolates had an MIC value of 100 µg ml^{-1}, four isolates had a MIC value of 150 µg ml^{-1}, and 14 isolates had a MIC value of 300 µg ml^{-1}.

Captan-sensitive and those with decreased sensitivity were selected based on their EC_{50} values. Three *B. cinerea* isolates (4d, 15a, and 17b) with an EC_{50} value of <25 µg ml^{-1} were regarded to be sensitive (S) and seven *B. cinerea* isolates (12a, 12b, 12c, 22b, 49a, 49b, and 57b) with an EC_{50} value of >40 were regarded to have decreased sensitivity (R), and Re, Sy, and Ss subcultures of these isolates were obtained. These isolates were included in the experiment to evaluate the stability of sensitivity to fungicide. FC varied between 1.09 and 4.73 within the group of R and Re isolates (Table 1).

FC1 and FC2 changed 0.67 and 1.60 within the group S, Sy and Ss subcultures. FC1 of three S main isolates and those of Sy and Ss subcultures of the same isolates were less than, but there was an increase in EC_{50} values of their Ss subcultures, except for one isolate. Although the EC_{50} value of the S isolate 15a was 10 µg ml^{-1}l, the EC_{50} value of its subcultures, Sy and Ss, was 15 µg/ml (Table 2).

B. cinerea can lead to the development of resistance to single-site fungicides because of its heterokaryotic structure (Leroux, 2004), and isolates resistant to fungicide may also have a high level of fitness and become competitive with isolates with sensitivity and therefore, lead to an increased number of isolates that is resistance in nature (Dekker, 1982). In contrast, it is more difficult for pathogens to acquire resistance to multi-site inhibiting fungicides. Therefore, chemical control programs have been based on the use of a mixture of single-site and multi-site inhibiting fungicides to avoid development of resistance in *B. cinerea* strains. Regarding studies on *B. cinerea* isolates recovered from ornamental plants, like strawberry, eggplant, pepper and tomato, researchers have found that captan may become ineffective against the pathogen when it is intensely used in areas severely affected by the disease as the isolates develop resistance to captan (Lorenzini, 1983; Delenet al., 1999; Sarıbıyık and Benlioğlu, 2004; Walter et al., 2007). Glutathione levels were higher in isolates with decreased sensitivity than in wild-type isolates (Barak and Edgington, 1984). Studies have reported variable sensitivity profiles against captan for *B. cinerea* isolates obtained from vineyards in Turkey. The EC_{50} values of *B. cinerea* isolates in the Aegean region were reported to vary between 4 and 24 µg/ml (Koplay, 2003), whereas EC_{50} values of isolates in the Trakya region were found to be between 5 and 100 µg ml^{-1} (Köycü, 2007). The decrease in fungicide sensitivity occurs by sporulation and transfer of resistance to subsequent generations; therefore, the problem of fungicide resistance emerges in the vineyards. The current research concludes that resistance to captan might be stable based on the findings that there is a slight decrease in EC_{50} values of the isolate 12b (R) and Re subculture. Similarly there was an increase in EC_{50} values of Sy isolate subcultured from the isolate 15a (S) due to exposure to high doses of captan, and increased EC_{50} values remained stable after transfer of this isolate to a fungicide-free PDA. In fact, researchers suggest that there is an accelerated decrease in the sensitivity of isolates to captan because of continuous application of captan on vegetables, and this sensitivity reduction is stable in most isolates (Delen et al., 2000). Captan can prevent spore germination in isolates obtained from vineyards at a dose of 100 µg ml^{-1} (Köycü, 2007), and may become ineffective against the pathogen when it is applied >12 times a year, particularly in strawberry fields (Walter et al., 2007); thus results revealed that sensitivity to captanin field conditions has increased over time.

Researchers have found a significant decrease in cross-reactivity of *B. cinerea* isolates recovered from greenhouses to captan in comparison to thiram, and to

Table 1. Changes in EC_{50} values of *B. cinerea* isolates with decreased sensitivity to captan.

Isolate No.	EC_{50} (µg ml^{-1})[A]		FC[B]
	R	Re	
12[a]	46	29	1.59
12[b]	46	42	1.09
12[c]	52	11	4.73
22[b]	40	31	1.29
49[a]	42	30	1.40
49[b]	46	14	3.28
57[b]	42	13	3.23

A: Effective concentration at 50% (EC_{50}) values in µg ml^{-1}. B: FC: Factor of change R/Re. R: Main isolate with decreased sensitivity to captan.
Re: Subculture isolates with decreased sensitivity to captan and then cultivated in a fungicide-free medium 15 times.

Table 2. Changes in EC_{50} values of *B. cinerea* isolates sensitive to captan.

Isolate No.	EC_{50} (µg ml^{-1})[A]			FC1[B]	FC2[C]
	S	Sy	Ss		
4[d]	16	18	10	0.89	1.60
15[a]	10	15	15	0.67	0.67
17[b]	16	18	10	0.89	1.60

A: Effective concentration at 50%(EC_{50}) values in µg ml^{-1}. B: FC1: Factor of change 1: S/Sy. C: FC2: Factor of change 2: S/Ss.
S: Isolates sensitive to captan. Sy: Subculture isolates cultivated in a medium with a high dose of fungicide. Ss: Subculture isolates cultivated in a medium with a high dose of fungicide and then cultivated in a fungicide-free medium.

captan in comparison to Mancozeb (Delen et al., 1999, 2000; Leroux, 2004). The results of this study suggest that low FC for a few isolates [12b (R) and three S] might increase the number of isolates with resistance in the field conditions; leading to decreased sensitivity to captan currently licensed for use against *B. cinerea* in the vineyards, which may cause development of cross-resistance. Moreover, determining the presence of *B. cinerea* isolates with high EC_{50}values among those obtained from vineyards (Koplay et al., 2004; Köycü, 2007) reveals that isolates with resistance can compete with sensitive isolates in the field. Indeed, the fact that Köycü (2007), detected EC_{50} values as high as 100 µg ml^{-1} for some *B. cinerea* isolates obtained from vineyards in this region indicates that isolates resistant to fungicides may have a high level of fitness and therefore, can compete with isolates sensitive to captan. Similarly, this study suggested that the R and Re isolates could compete with the S, Sy, and Ss isolates with respect to growth rate and virulence as in the case of isolates 12a, 12c, 49b, and 57b R. If the reduction in sensitivity to captan occurs because of adaptation of the pathogen to captan, then the reduction in the fungicidal sensitivity may not be a problem in field conditions. Indeed, Sarıbıyık and Benlioğlu (2004) reported no significant difference in decreased sensitivity to captan among *B. cinerea* isolates recovered from strawberry parcels after application of captan 10 times, and they, therefore, supported the notion that a reduction in fungicidal sensitivity may not emerge as a problem in field conditions. However, decreased sensitivity to fungicide may be regarded to be stable because in the present study, decreased sensitivity in isolates 12b (R) and 15a (S) persisted in the subcultures of these isolates. With some exceptions, it is known that an increase in fungicide doses also increases the resistance in pathogens (Dekker, 1982). In addition, isolate 15a (S) sensitive to captan showed adaption to escalating doses of captan by increasing EC_{50} values and a subculture of this isolate sensitive to captan on a fungicide-free medium resulted in no change in EC_{50} values; a finding that might be indicative of a shift toward fungicide-resistance over time.

Fitness of Isolates

In the fitness tests of R and S *B. cinerea* isolates, isolates 12a and 4d were not selected. For all isolates tested, the fitness parameters were significantly ($P \leq 0.01$) different between the main isolates and subcultures of those

Table 3. Fitness components of *B. cinerea* isolates.

Isolate No.	Fitness components		
	Growth rate[A]	Sporulation[B]	Virulence[C]
12[b] R	8.50*[a]	4.33[e]	3.32[ab]
12[b] Re	6.90[d]	0.67[e]	2.73[bc]
12[c] R	8.50[a]	12.67[de]	3.24[ab]
12[c] Re	7.67[abcd]	0.67[e]	2.68[bc]
22[b] R	7.53[bcd]	5.67[e]	3.29[ab]
22[b] Re	4.23[e]	1.33[e]	3.97[a]
57[b] R	7.40[bcd]	12.33[de]	3.11[ab]
57[b] Re	7.50[bcd]	0.67[e]	3.54[ab]
49[a] R	8.50[a]	32.00[bc]	3.27[ab]
49[a] Re	8.50[a]	11.33[de]	2.83[bc]
15[a] S	8.13[abc]	31.67[bc]	2.72[bc]
15[a] Sy	2.90[f]	1.00[e]	2.75[bc]
15a Ss	4.17[e]	0.33[e]	2.18[c]
17[b] S	8.23[ab]	22.33 [cd]	2.92[bc]
17b Sy	8.00[abc]	55.00[a]	3.07[b]
17[b] Ss	7.33[cd]	42.33[b]	2.98[bc]

R: Main isolate with decreased sensitivity to captan. Re: Subculture isolate with decreased sensitivity to captan. S: Isolates sensitive to captan

Sy: Subculture isolates cultivated in a medium with a high dose of fungicide. Ss: Subculture isolates cultivated in a medium with a high dose of fungicide and then cultivated in a fungicide-free medium. A: Growth rate: Average growth rate measured after 3 days in PDA. B: Spore yield $\times 10^6$ ml^{-1}

C: Diameter of lesions on vine leaves (cm). *Each value is the average of three repetitions. The values indicated by different letters in the same column are significantly different from each other according to the Duncan multiple comparison test (P≤0.05).

isolates (Table 3).

Comparison of mycelial growth rates of S isolates showed that there were differences in growth rates between the main cultures and subcultures of some isolates, whereas no differences were observed in other isolates. The mycelial growth rates of S isolates were as high as those of R isolates. There was a significant (P ≤ 0.05) difference between the growth rates of R and Re subcultures of the isolate 22b and the growth rate in the Re subculture was decreased. However, no significant differences were found between the mycelial growth rates of R and Re subcultures of isolates 57b and 49a. In S isolates, mycelial growth rates of Sy and Ss subcultures of the S isolate 15a were lower than those of the main isolate. In contrast, the growth rate of the S isolate 17b was found to be close to those of the Sy and Ss subcultures.

The evaluation of sporulation showed significant differences between R and S isolates and their subcultures in terms of sporulation. Within R and S isolates, spore production was found to be high, except for 17b S, Sy, and Ss, and spore production of Re subcultures of the same isolates were found to be lower. No significant differences were found in the spore production of Re subcultures of the R isolates 12b and 22b, whereas there was a significant difference between the R and Re isolates of 49a (P ≤ 0.05). Sporulation of the S isolate of 15a was found to be quite high; whereas that of the Sy and Ss subcultures of the same isolate was found to be very low, with no significant difference between them. However, sporulation was found to be higher in the Sy subculture of isolate 17b and showed a slight decrease in the Ss subculture.

About virulence, there was a significant (P ≤ 0.05) difference between the virulence of the R and S isolates and their subcultures. There was a decrease in the virulence of subcultures of other R isolates. In S isolates, the virulence did not change in the Sy isolate of 15a subjected to high doses; whereas the virulence was greater in the Sy isolate of 17b in comparison with that of the main isolate. However, it was determined that the virulence of Ss isolates of both S isolates decreased again. Therefore, we can suggest that isolates with sensitivity also show a tendency toward developing resistance over time, and isolates sensitive to fungicides can be the basis of emerging resistance to captan used against *B. cinerea* in field conditions as much as their counterparts that are resistant to captan with high fitness parameters. These results are important as they show whether a successful chemical control program against

the pathogen could prove to be sustainable, and it is likely that isolates resistant to multi-site fungicides may develop over time. Therefore, captan should be revalued and recommended to be used as a mixture with other single-site fungicides to prevent the development of resistance against it in *B. cinerea* in vineyards.

Conclusion

B. cinerea is high-risk pathogen for fungicide resistance due to its high genetic variability. However, it is very difficult for *B. cinerea* to resist multi-site fungicides. For this reason, it is recommended to use single-site fungicides in a mixture in the control programs. Stability of resistance development for multi-site fungicides in *B. cinerea* isolates and fitness of resistant isolates is important both for reorganization and the recommendations of the single-site fungicides and the multi-site fungicides mixtures in the fungicide application programs and to prediction regarding the risk for resistance evolution.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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