Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2023, Vol. 7 (3), 280-297
https://doi.org/10.29329/ijiaar.2023.602.3

Original article

Determination of the Effectiveness of Some Fungicides on Botrytis cinerea, the Causative Agent of Grapevine Gray Mold Disease

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Abstract

In this study, the effectiveness of Fenhexamid, Captan, Cyprodinil, Pyrimethanil and Hymexazol on *Botrytis cinerea* isolates obtained from vineyards were determined. For this purpose, the efficacy of fungicides at 0.01, 0.05, 0.05, 0.1, 0.5, 1, 5, 10, 25 and 50µg/mL concentrations of each fungicide on mycelium development of Botrytis cinerea and on grape berries were investigated. PDA media containing different doses of fungicides were used to determine their efficacy on mycelium growth. In order to determine their efficacy on grape berries, they were wounded with a needle and treated with fungicides in two different ways, before and after infection. As a result of the experiment, Fenhexamide inhibited mycelium growth 100% at 0.5ppm, while the other fungicides hymexazole, cyprodinil and pyrimethanil inhibited 100% at 25ppm. Captan reached 100% inhibition rate only at 50ppm. In grape berries trials, fenhexamide and captan were more effective after infection, pyrimethanil was more effective when applied before infection, but hymexazole and cyprodinil had the same effect when applied before or after infection.

Keywords: Grape, Botrytis Cinerea, Chemical Control, Resistance, Fungicides.

Received: 08 September 2022 * **Accepted:** 30 September 2023 * **DOI:** https://doi.org/10.29329/ijiaar.2023.602.3

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INTRODUCTION

The vine, a member of the order Rhamnales, is one of the oldest and most widely cultivated agricultural crops in the world (Winkler et al, 1974). The homeland of viticulture is the region including Anatolia and present-day southern Russia. The history of viticulture dates back to 5000 BC. The grapevine has many varieties compared to other plants. It is known that there are more than 10.000 grape varieties in the world and since Türkiye is the homeland of viticulture, it is home to more than 1.200 grape varieties (Çelik et al, 1998). Only 50-60 of these varieties grown in Türkiye have economic importance and have been widely cultivated (Ateş and Karabat, 2016).

Türkiye ranks fifth after Spain, China, France and Italy in terms of vineyard area and sixth after China, Italy, Spain, France and the USA in terms of production, and the total vineyard area is 400,998 ha and the production amount is 4,208,908 tons (FAO, 2020). Considering the production quantities, grapes are among the most produced fruits in the world in fruit production. Grape, which is one of the most produced and consumed fruits in the world, is produced 78 million tons on 7 million hectares annually. China ranks first with approximately 15 million tons on 768 thousand hectares and Italy ranks second with approximately 8.5 million tons on 704 thousand hectares. Türkiye ranks 6th with a production area of approximately 401 thousand hectares and a production of approximately 4.5 million tons (FAO, 2020).

Grape has many fungal diseases cause serious problems during its cultivation. Important fungal diseases such as powdery mildew (*Uncinula necator*), *Botrytis cinerea*, *Plasmopara viticola*, anthracnose (*Elsinoe ampelina*), black rot (*Guignardia bidwelli*) and bitter rot (*Greeneri auvicola*) are among the most important pathogens that cause serious problems in the plant (Jermini and Gessler, 1996; Schilder et al, 2005). Bunch rot or Gray mold disease caused by *B. cinerea* Pers. also known as gray rot, is known as one of the most important diseases of grapevine in viticulture areas worldwide (Elmer and Michailides, 2007; Komarek et al, 2010; Wu et al, 2010; Aminifard and Mohammadi, 2012; Mundy et al, 2014). The gray mold agent *B. cinerea* causes serious yield loss before and after harvest in more than 200 plant varieties such as grapes, strawberries, tomatoes, etc. (Shao et al, 2015). In vineyards, bunch rot disease (*B. cinerea*), which causes economic damage in every growing period in table and wine grape varieties, causes serious losses. Botrytis bunch rot has been reported to cause an average of 20% crop loss per year (Genescope, 2002).

In economically important crops, unconscious and uncontrolled chemical applications are carried out intensively due to the lack of sufficient knowledge of our producers about plant protection and the concern of crop loss (Delen et al, 2006). In addition to the negative effects of unconscious pesticide use in the control of diseases and pests on human and environmental health, the risk of pathogens developing resistance to pesticides is another negative factor. The emergence of resistance to fungicides poses the most important problem in the success of chemical control. The emergence of resistance is related to the

mechanisms of action of the fungicides used. Contact fungicides that inactivate the fungus by interfering with more than one vital function of the fungus are called multi-site inhibitors, while systemically acting fungicides that act by inhibiting specific life events of the fungus are called single-site inhibitors. Due to their advantages, the intensive and sequential use of systemic fungicides (single site inhibitors) effective in specific areas has led to the emergence of the problem of fungicide resistance (Demirci, 1996). Fungicide resistance is the stable and hereditary adaptation of a fungus to fungicides and the formation of new races as a result of reduced susceptibility to the chemicals (Delp and Dekker, 1985). Resistance is when a pathogen is less affected by a fungicide, i.e. its sensitivity is reduced. This is genetically governed and is usually irreversible. Single-site fungicides have a much higher risk of inducing resistance in the fungal organism than multi-site fungicides. Pathogens to which the fungicide is effective are susceptible, while pathogens to which it is not effective are naturally or inherited resistant. Resistance usually manifests itself as complete or near complete failure of disease control (Georgopoulos, 1982).

Despite the use of biological control methods, the most common method used to control *B. cinerea* is the use of chemicals. Some fungicides such as anilinopyrimides, dicarboximides, hydroxyanilides, phenylpyrroles and succinatedehydrogenase inhibitors are widely used in Türkiye and in the world. However, the sudden emergence of fungicide resistance in *B. cinerea* makes the control very difficult. To this day, there have been many reports of fungicide resistance of *B. cinerea* in different crops from Türkiye and different parts of the world (Angelini et al, 2014; Fernandez-Ortuno et al, 2015; Li et al, 2014; Panebianco et al, 2015; Saito et al, 2014; Veloukas et al, 2014; Walker et al, 2013; Weber, 2010; Yin et al, 2012). As reported in many studies, *B. cinerea* is a plant pathogenic fungus with a high potential to develop fungicide resistance. Therefore, in order to minimize this risk, fungicide resistance studies should be routinely repeated in the same region and in many different hosts. The results obtained in this context can be used to prepare an effective chemical control program for the control of gray mold disease. The aim of this study was to determine the efficacy of different fungicides in the control of *B. cinerea*, the causal agent of lead mildew disease in vineyard.

MATERIALS and METHODS

Fungal Isolate

One highly virulent *B. cinerea* isolate, which was identified and used in previous studies and kept as a stock culture in the Mycology laboratory of the Department of Plant Protection, Mustafa Kemal University, Hatay, Türkiye was used in this study.

Pathogenicity Test

In order to determine the pathogenic properties of the obtained *B. cinerea* isolate, pathogenicity test was performed. The pathogenicity test was performed according to the method proposed by Saito

et al (2019). According to this method, single spore isolate of *B. cinerea* were grown on PDA medium for 7 days and then mycelium and spores were scraped with sterile distilled water and a spatula. Mycelial residues were then removed by passing through 4 layers of cheesecloth and then the spore concentration was adjusted to 10^7 using a hemocytometer (Thoma slide). Healthy leaves of grapevine plants were collected and sterilized from the surface by first immersing them in 2% sodium hypochlorite solution and waiting for 2 minutes, then immersed in sterile pure water, rinsed, and transferred to sterile blotting papers to remove excess water. Then, inoculation points were determined on the leaves and small wounds were made on these marked areas with a needle. Using a micropipette, 20 microliters of spore suspension of the isolate was taken and inoculated into the marked areas on the grape leaves. Disease symptoms in the inoculation points on the leaves were observed after 7 days and recorded.

Effects of Fungicides on Mycelium Development of B. cinerea

Five fungicides produced by different companies and widely used in Türkiye were used in the trials: (Cyprodinil; Safa Tarım (Carpaz 50 WG), Pyrimethanil; Safa Tarım (Milis 30 SC), Captan; Safa Tarım (Safa Captan 50 WP), Hymexazol; Hektaş (Sound 360 SL) (100% solution in acetone) and Fenhexamid; Bayer CropScience (Teldor SC 500), Research Triangle Park, NC, USA, (100% solution in ethanol), Fenhexamid was prepared as a solution in ethanol and the other fungicides as a solution in acetone. Stock solutions of each fungicide were prepared at a concentration of 10,000 ppm and diluted to the respective dose before use.

In order to determine the efficacy of fungicides on mycelium development of the fungal disease agent *B. cinerea*, fungicides were added to petri dishes containing PDA medium at different concentrations. For this purpose, pre-prepared stock solutions were used and all fungicides were adjusted to 100 ml PDA at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 µg/mL. In addition, fungicide-free PDA media were used as control. The 6 mm diameter disks containing mycelium of *B. cinerea* isolate were inverted into the center of the petri dishes and allowed to grow in an incubator set at 25°C. After 5 days of incubation, colony diameters were measured and recorded. Each experiment was repeated at least twice.

Effects of Fungicides on B. cinerea in Grape Berries

Grape berries of Pafı grape variety were used to determine the effects of fungicides on *B. cinerea* in fruit tests. For this purpose, grape berries were cut from the clusters together with their stems and then washed and dried and small wounds were made on the dried grape berries with a pin. Stock solutions of fungicides were prepared at concentrations of 0.5, 1.0, 5.0, 10.0, 25.0 µg/mL. First, grape berries were coated with fungicides by dipping method and after 24 hours, the spore suspension of the pathogen was sprayed on the berries with a sprayer. Thus, the protective efficacy of the fungicides was determined. In the other method, grape berries were first sprayed with the spore suspension of the

pathogen and then dipped in different concentrations of fungicides by dipping method after 24 hours (Wang et al, 2018) and the therapeutic properties of the fungicides were investigated (Hill et al, 2010). At the end of the incubation period of about 7 days, the effects of the pathogen on grape berries were recorded and each experiment was repeated at least twice. A scale of 0-5 was used to assess the severity of the disease in grape berries. A value of 0 indicates that there is no sign of infection in the wounds opened, and values from 1 to 5 indicate that there is infection and graded accordingly. A value of 1 indicates that the infection has just started and is very mild, while a value of 5 indicates that the infection is very severe.

Statistical Analysis

Statistical analysis of the data was determined by analysis of variance using the SPSS 19 package program and differences between means were determined and analyzed with the Duncan multiple comparison test.

RESULTS and DISCUSSION

Pathogenicity Studies

As a result of the pathogenicity test carried out on healthy leaves of grapevine plants, it was observed that the leaf treated with *B. cinerea* isolate first yellowed and then necrotic area was formed on the inoculation point. In the next stage, the leaf rotted completely, and a layer of lead colored mycelium was formed on it. Pieces taken from these symptomatic tissues of leaves were sterilized and transferred in PDA medium and *B. cinerea* was re-isolated. The leaf treated with pure water only and left as a control did not show any symptoms (Figure 1). Therefore, this *B. cinerea* isolate used in this experiment was found to be pathogenic.

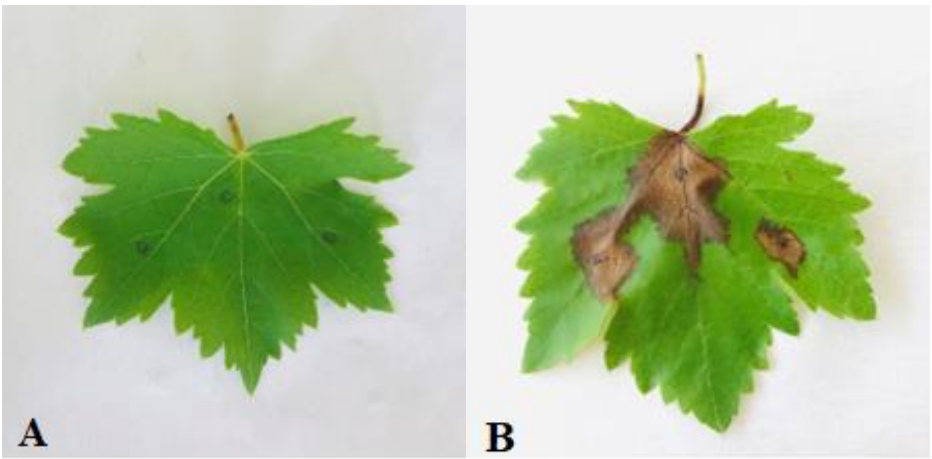

Figure 1. Pathogenicity on grapevine leaves. A: Control, B: Leaf treated with spore suspension

Effects of Fungicides on Mycelium Growth of B. cinerea

Fenhexamide

Mycelium length and inhibition percentage of fenhexamide at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 µg/mL. were determined. At dose 0.01, mycelium length 34 mm inhibition percentage was 62%, at 0.05 mycelium length 17 mm inhibition percentage was 80% and at 0.1 mycelium length 12 mm inhibition percentage was 88%, while no mycelial growth was observed at other doses (Table 1).

Table 1. Effects of Fenhexamide on B. cinerea mycelium and inhibition percentages

Dose	Colony diameter	Inhitibiton (%)
Control	90	0
0.01	34d*	62
0.05	17c	80
0.1	12b	88
0.5	0a	100
1	0a	100
5	0a	100
10	0a	100
25	0a	100
50	0a	100

*The values shown with different letters were found to be statistically different from each other as a result of Duncan multiple comparison test.

Captan

Colony diameters of 90 mm were measured at 0.01, 0.05, 0.1, 0.5 and 1ppm doses of Captan and the doses did not inhibit the development of pathogen mycelium. At 5.0ppm, the colony diameter was 76mm and the inhibition percentage was 15%, at 10.0ppm the mycelium diameter was 48mm and the inhibition percentage was 43%, at 25.0ppm the mycelium diameter was 12mm and the inhibition percentage was 87%, and at 50.0ppm no mycelium development was observed (Table 2).

Table 2. Effects of captan on B. cinerea mycelium and inhibition percentages

Dose	Colony diameter	Inhitibiton (%)
Control	90	0
0.01	90f*	0
0.05	90f	0
0.1	90f	0
0.5	90f	0
1	85g	0.3
5	76d	15
10	48c	43
25	12b	87
50	0a	100

*The values shown with different letters were found to be statistically different from each other as a result of Duncan multiple comparison test.

Hymexazole

Mycelium lengths of 90 mm were measured at 0.01, 0.05, 0.1 and 0.5 doses of Hymexazol and the doses did not inhibit the development of pathogen mycelium. At dose 1.0, mycelium length was 76mm with an inhibition percentage of 15%, at dose 5 mycelium diameter was 56mm with an inhibition percentage of 37%, at dose 10.0 mycelium diameter was 48 mm with an inhibition percentage of 43%. At 25.0 and at 50.0ppm dose, no mycelium development was observed (Table 3).

Table 3. Effects of Hymexazol on B. cinerea mycelium and inhibition percentages

Dose	Colony diameter	Inhitibiton (%)
Control	90	0
0.01	90d*	0
0.05	90d	0
0.1	90d	0
0.5	90d	0
1	76c	15
5	56b	37
10	48b	43
25	0a	100
50	0a	100

*The values shown with different letters were found to be statistically different from each other as a result of Duncan multiple comparison test.

Cyprodinil

Mycelium lengths of 90 mm and 83 mm were measured at 0.01 and 0.05 doses of Cyprodinil, and the doses did not inhibit the development of pathogen mycelium. At 0.1 dose, mycelium length was 72 mm and inhibition percentage was 10%, at 0.5 mycelium diameter was 43 mm and inhibition percentage was 52%, at 1.0 mycelium diameter was 24 mm and inhibition percentage was 73%, at 5.0 mycelium diameter was 10mm and inhibition percentage was 89% and at 10.0 mycelium diameter was 8 mm and

inhibition percentage was 91%. At 25.0 and 50.0 doses, no mycelium development was observed and these doses of cyprodinil inhibited mycelium development of the pathogen by 100% (Table 4).

Table 4. Effects of Cyprodinil on B. cinerea mycelium and inhibition percentages

Dose	Colony diameter	Inhitibiton (%)
Control	90	0
0.01	90g*	0
0.05	83f	7
0.1	72e	10
0.5	43d	52
1	24c	73
5	10b	89
10	8b	91
25	0a	100
50	0a	100

*The values shown with different letters were found to be statistically different from each other as a result of Duncan multiple comparison test.

Pyrimethanil

At 0.01 dose of pyrimethanil, mycelium length was measured 90 mm and this dose did not inhibit pathogen mycelium development. At 0.05 dose of the fungicide, mycelium length was 65 mm with an inhibition percentage of 27%, at 0.1 mycelium diameter was 54 mm with an inhibition percentage of 40%, at 0.5 mycelium diameter was 46 mm with an inhibition percentage of 48%, at 1 mycelium diameter was 43 mm with an inhibition percentage of 52%, at 5.0 mycelium diameter was 9 mm with an inhibition percentage of 90% and at 10.0 mycelium diameter was 8 mm with an inhibition percentage of 91%. At 25.0 and 50.0 doses, no mycelium development was observed and these doses of pyrimethanil inhibited mycelium development of the pathogen by 100% (Table 5).

Table 5. Effects of Pyrimethanil on B. cinerea mycelium and inhibition percentages

Dose	Colony diameter	Inhitibiton (%)
Control	90	0
0.01	90f*	0
0.05	65e	27
0.1	54d	40
0.5	46c	48
1	43c	52
5	9b	90
10	8b	91
25	0a	100
50	0a	100

*The values shown with different letters were found to be statistically different from each other as a result of Duncan multiple comparison test.

Effects of Fungicides on B. cinerea in Grape Berries

Fenhexamide

In fungicide applications before infection, the infection was very severe at dose 0.5 and the infection was milder at other doses. In fungicide applications after infection, very severe disease symptoms observed at 0.5, severe infection was observed at 1.0 dose and very mild or no infection was observed at other doses (Figure 2-3, Table 6).

Figure 2. Grape berries treated with Fenhexamid before infection. K indicates control and different numbers indicate dose in ppm.

Figure 3. Fenhexamide-treated grape berries after infection. K indicates control and different numbers indicate dose in ppm.

Table 6. Efficacy of fenhexamide on B. cinerea in grape berries

Dose	Pre-infecition application	Post-infection application
Control	5	5
0.5	4b*	4c
1	1a	3b
5	1a	0a
10	0a	0a
25	0a	0a

*The values shown with different letters were found statistically different from each other as a result of Duncan multiple comparison test.

Captan

In fungicide applications before infection, infection was severe at doses 0.5 and 1.0, moderate at dose 5.0 and no infection was observed at other doses. In fungicide applications after infection, the infection was very severe at 0.5 and none at the other doses (Figure 4-5, Table 7).

Figure 4. Grape berries treated with Captan before infection. K indicates control and different numbers indicate dose in ppm.

Figure 5. Grape berries treated with Captan after infection. K indicates control and different numbers indicate dose in ppm.

Table 7. Efficacy of Captan on B. cinerea in grape berries

Dose	Pre-infecition application	Post-infection application
Control	5	5
0.5	4c*	4b
1	3b	0a
5	2a	0a
10	0a	0a
25	0a	0a

*The values shown with different letters were found statistically different from each other as a result of Duncan multiple comparison test.

Hymexazole

In fungicide applications before infection, infection was severe at doses 0.5 and 1.0, moderate at doses 5.0 and 10.0, and no infection was observed at dose 25.0. In fungicide applications after infection, infection was severe at doses 0.5, 1.0 and 5.0, and very mild or no infection was observed at doses 10.0 and 25.0 (Figure 6-7, Table 8).

Figure 6. Grape berries treated with Hymexazole before infection. K indicates control and different numbers indicate dose in ppm.

Figure 7. Grape berries treated with Hymexazole after infection. K indicates control and different numbers indicate dose in ppm.

Table 8. Efficacy of Hymexazole on B. cinerea in grape berries

Dose	Pre-infecition application	Post-infection application
Control	5	5
0.5	4cd*	4c
1	3bc	3bc
5	2b	3bc
10	2b	1ab
25	0a	0a

*The values shown with different letters were found statistically different from each other as a result of Duncan multiple comparison test.

Cyprodinil

When the disease symptoms in grape berries treated with Cyprodinil were evaluated, the infection was severe at dose 0.5, mild at dose 1.0, and no infection was observed at doses 5.0, 10.0 and 25.0 (Figure 8-9, Table 9).

Figure 8. Grape berries treated with Cyprodinil before infection. K indicates control and different numbers indicate dose in ppm.

Figure 9. Grape berries treated with Cyprodinil after infection. K indicates control and different numbers indicate dose in ppm.

Table 9. Efficacy of Cyprodinil on B. cinerea in grape berries

Dose	Pre-infecition application	Post-infection application
Control	5	5
0.5	4c*	3c
1	2b	1b
5	0a	0a
10	0a	0a
25	0a	0a

*The values shown with different letters were found statistically different from each other as a result of Duncan multiple comparison test.

Pyrimethanil

In pyrimethanil, in fungicide applications before infection, moderate disease symptom was observed at dose 0.5, very mild infection was observed at dose 1.0 and no infection was observed at other doses. In fungicide applications after infection, infection was severe at dose 0.5, mild at doses 1.0 and 5.0, and no infection was observed at other doses (Figure 10-11, Table 10).

Figure 10. Grape berries treated with Pyrimethanil before infection. K indicates control and different numbers indicate dose in ppm.

Figure 11. Grape berries treated with Pyrimethanil after infection. K indicates control and different numbers indicate dose in ppm.

Table 10. Efficacy of Pyrimethanil on B. cinerea in grape berries

Dose	Pre-infecition application	Post-infection application
Control	5	5
0.5	3b*	4c
1	1a	2b
5	0a	1a
10	0a	0a
25	0a	0a

*The values shown with different letters were found statistically different from each other as a result of Duncan multiple comparison test.

When similar studies were compared with our study (Alzohairy at al, 2021; Angelini et al, 2014; Fernandez-Ortuno et al, 2015; Li et al, 2014; Panebianco et al, 2015; Saito et al, 2014; Veloukas et al, 2014; Walker et al, 2013; Weber, 2010; Yin et al, 2012), it was seen that fenhexamide gave positive results when used as a preventive and therapeutic agent at low doses, while cyprodinil and pyrimethanil gave good results when used at higher doses. In our study, unexpectedly, hymexazole did not work well. Fenhexamide inhibited mycelium growth 100% at 0.5ppm, while the other fungicides hymexazole, cyprodinil and pyrimethanil inhibited mycelium growth 100% at 25ppm. Captan reached 100% inhibition rate only at 50 ppm. Fenhexamid and captan at 10ppm, hymexazole at 25ppm, cyprodinil and pyrimethanil at 5ppm were effective on pathogen development before infection. After infection, fenhexamide and cyprodinil at 5ppm, captan at 1 ppm, hymexazol at 25ppm and pyrimethanil at 10ppm were effective. When all the fungicides used were evaluated, fenhexamide and captan were more

effective after infection, pyrimethanil was more effective when applied before infection, but hymexazole and cyprodinil had the same effect when applied before or after infection.

Conclusion

Considering the effects of fungicides on both the environment and the economy, it would be better to choose the fungicide with the lowest effective dose. For this reason, it is recommended to use fenhexamid, which is effective even at very low doses, among fenhexamid and pyrimethanil, which are licensed in vineyards. However, it is recommended to keep in mind that B. cinerea rapidly develops resistance to fenhexamid, which is widespread all over the world, and to use fenhexamid only once a season and to consider cross-resistance when rotating fungicides. In fungicides such as captan, hymexazol and cyprodinil, which are not licensed for *B. cinerea* in vineyards, cyprodinil was found to be effective at lower doses than other fungicides. Therefore, it is recommended to carry out studies on the use of cyprodinil in vineyards in licensing studies.

Conflict of Interest

There is no conflict of interest between the authors of the article.

Acknowledgement

This study was financially supported by Hatay Mustafa Kemal University Scientific Research Projects Commission in the category of Graduate Project (Project Number: 21.YL.019).

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