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Araştırma Makalesi (Research Article)

Evaluation of the Fungicide Resistance of Gray Mold (*Botrytis cinerea*) in Tomatoes to Boscalid and Pyraclostrobin in Greenhouse Areas of Turkey

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Abstract: *Botrytis cinerea*, a polyphagous pathogen, can infect all of the aboveground parts of tomato plants and cause significant yield and quality losses. Fungicides are commonly used for the control of this pathogen. Currently, resistance to fungicides, which provide the effective and fast control of pathogens, is an important problem. In this study, resistance of *B. cinerea* isolates obtained from tomato greenhouses in Antalya province against Signum® (boscalid + pyraclostrobin) and Cantus® (boscalid) fungicides were evaluated under *in vitro* conditions. Mycelium growth tests conducted with different fungicide concentrations and EC_{50} values were calculated. While EC_{50} values of isolates sensitive to boscalid varied between 0.7 and 8.6 µg/ml, EC_{50} values of isolates sensitive to boscalid + pyraclostrobin were found to be between 0.1 and 1.9 µg/ml. Conidial germination tests were carried out in a 2% water agar (WA) medium. It was determined that isolates 61, 69, 72, and 81 were resistant to both fungicides, while isolates 57 and 97 were sensitive to boscalid and resistant to boscalid + pyraclostrobin. It has been determined that 20% of the isolates were resistant to both fungicides. Isolates resistant to boscalid but sensitive to boscalid+pyraclostrobin were not found. With this current *in vitro* study, the first data on the resistance formation against boscalid and boscalid + pyraclostrobin active ingredients in *B. cinerea* populations in Antalya province were presented. There is a need to develop integrated control programs that can be used in the control of the pathogen.

Türkiye'de Domates Seralarında Kurşuni Küf (*Botrytis cinerea*) Hastalığının Boscalid ve Pyraclostrobin'e Karşı Fungisit Dayanıklılığının Değerlendirilmesi

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Anahtar kelimeler
Antalya,
Boscalid,
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Pyraclostrobin
Domates.

Öz: Polifag bir patojen olan *Botrytis cinerea*, domatesin toprak üstü kısımlarını enfekte edebilmekte ve önemli verim ve kalite kayıplarına neden olmaktadır. Fungisitler bu patojenin kontrolünde yaygın olarak kullanılmaktadır. Günümüzde patojenlerin etkili ve hızlı kontrolünü sağlayan fungisitlere karşı dayanıklılık oluşumu önemli bir sorundur. Bu çalışmada Antalya ili domates seralarından elde edilen *B. cinerea* izolatlarının Signum® (boscalid + pyraclostrobin) ve Cantus® (boscalid) fungisitlerine karşı dayanıklılığı *in vitro* koşullarda değerlendirilmiştir. Farklı fungisit konsantrasyonları kullanılarak gerçekleştirilen misel gelişim testleri ile EC_{50} değerleri hesaplanmıştır. Boscalid'e hassas izolatların EC_{50} değerleri 0,7 ile 8,6 µg/ml arasında değişirken, boscalid + pyraclostrobin etken maddelerine karşı hassas izolatların EC_{50} değerleri 0,1 ile 1,9 µg/ml arasında bulunmuştur. Konidi çimlenme testleri % 2'lik su agarı (WA) ortamında gerçekleştirilmiştir. 61, 69, 72 ve 81 numaralı izolatların her iki fungisite

dayanıklı olduğu, 57 ve 97 numaralı izolatların boscalide hassas iken boscalid + pyraclostrobin'e dayanıklı olduğu belirlenmiştir. İzolatların %20'sinin her iki fungiside dayanıklı olduğu tespit edilmiştir. Boscalid'e dayanıklı, ancak boscalid + pyraclostrobin'e hassas izolatlar bulunamamıştır. Bu çalışmada Antalya ilindeki *B. cinerea* populasyonlarında boscalid ve boscalid + pyraclostrobin etken maddelerine karşı dayanıklılık oluşumuna ilişkin ilk veriler sunulmuştur. Patojenin mücadelesinde entegre mücadele programlarının geliştirilmesine ihtiyaç duyulmaktadır.

1. Introduction

Tomato is one of the most produced vegetables in the world, which is highly valuable in fresh markets and used as an important component in the making of various industrial products (FAO, 2020). Turkey is an important tomato producer country in the world. In 2019, 12 841 990 tons of tomatoes are produced in Turkey. Thirty-nine percent of the tomato production in Turkey was carried out in Antalya province for both domestic consumption and export. In Antalya province, 2 421 247 tons in plastic tunnels and greenhouses and 2 528 905 tons from the field are produced (Tuik, 2019). *Botrytis cinerea*, a polyphagous pathogen, can infect all of the aboveground parts of tomato plants and cause significant yield and quality losses. *B. cinerea*, which is a fungus prone to fungicide resistance, has been found to be resistant to most fungicides (Kim and Xiao, 2010; Yin et al., 2011; Weber, 2011; Liu et al., 2016). Site-specific fungicides are mainly used in the chemical control of gray mold disease (Konstantinou et al., 2015). Boscalid and pyraclostrobin active ingredients are included in this group of fungicides. Together with the use of these fungicides, which spread systemically within the plant, the development of resistance in pathogen populations and a decrease in fungicide activity were observed (Hahn, 2014). These fungicides cause death in the fungus by preventing mitochondrial respiration and by preventing energy production in the cell (Fernández-Ortuño et al., 2008). Mutations that cause resistance in fungi against these fungicides have been identified (De Miccolis Angelini et al., 2010; Leroux et al., 2010; Veloukas et al., 2014).

Currently, mixtures of QoIs and SDHIs fungicides are among the recommended fungicides for the control of *B. cinerea* in Turkey (Bkü Veri Tabanı, 2021).

It is stated that there is a high risk of resistance to the active ingredient pyraclostrobin in the QoIs group, and the risk of fungicide resistance to the boscalid active ingredient in the SDHIs group is medium-high (FRAC, 2020). Resistance in *B. cinerea* has been reported against fungicides containing boscalid (Yin et al., 2011; Veloukas et al., 2011; Konstantinou et al., 2015; Kanetis et al., 2017) and boscalid + pyraclostrobin active ingredients (Kim and Xiao, 2010; Fernández-Ortuño et al., 2012, 2014;). It is stated that if fungicide-resistant populations become dominant due to selection pressure, the control of the disease will be difficult (Kim and Xiao, 2010). Therefore, it is important to evaluate the resistance status of fungus populations.

In Turkey, there is no research on the fungicide resistance in *B. cinerea* populations in greenhouse tomato production areas. Determining the effects of commonly used fungicides against this important pathogen of tomato will contribute to the development of programs for controlling the pathogen. Fungicides with boscalid + pyraclostrobin active ingredients are widely used to control *B. cinerea* in the greenhouse areas of the Antalya province of Turkey. This study was conducted to determine fungicide resistance under *in vitro* conditions against fungicides with the active ingredients boscalid and boscalid + pyraclostrobin.

2. Materials and Methods

2.1. Survey study in tomato greenhouses and isolation of *Botrytis cinerea* isolates

A survey study was carried out in tomato greenhouses of Antalya province of Turkey in December 2018. Leaf, stem, and fruit samples that were infected with *Botrytis cinerea* from different districts of Antalya were taken. Diseased plant samples were brought to the laboratory and the parts containing diseased and healthy tissues were cut with a scalpel and kept in a 1% NaOCI solution for 1 minute. Then the tissue pieces dried between sterile paper towels and they were transferred to Potato

Dextrose Agar (PDA) medium. Isolates were purified by taking the hyphal tips under a stereomicroscope and then transferred to agar slants and stored at +4 ºC. The isolates used in this current experiment were selected from the isolates representing different districts and greenhouses and they were previously phenotypically characterized (Gül and Karakaya, 2020).

2.2. Fungicides

Cantus® (50%) with the boscalid active ingredient and Signum® with boscalid 26.7% + pyraclostrobin 6.7% active ingredients (BASF) were used in mycelium growth and conidial germination tests. The fungicides used in the tests were obtained from BASF Central Anatolia, Turkey branch. The stock solutions of the fungicides were prepared by dissolving them in water. The SHAM stock solution (100 mg/ml) was prepared by dissolving in methanol for boscalid + pyraclostrobin active ingredients (Kim and Xiao, 2010). Stock solutions were stored for up to one week at +4 ˚C. Different concentrations were prepared by adding to the agar media from these stock solutions.

2.3. Mycelium growth tests

Twenty isolates presented in Table 1 were used in mycelium growth tests. Zero, 0.01, 0.05, 0.1, 0.5, 5, 25 µg/ml doses of Cantus® fungicide, and 0, 0.01, 0.05, 0.1, 0.5, 1, 10, 50 µg/ml doses of Signum® fungicide were added to the PDA medium. In the test conducted with the Signum® fungicide, to prevent alternative oxidase breathing, 1 ml of SHAM stock solution was added to 1 liter of PDA medium to the control and different fungicide concentrations. From the isolates grown for 2-3 days in the dark at 20° C in PDA medium, 6 mm diameter discs were taken from the tips of the actively growing colonies using a sterile cork borer and transferred to the Petri dishes containing different fungicide concentrations. There were 3 replications. The test was repeated twice. Petri dishes were incubated in the dark at 20°C for 3 days and the colony diameters were measured in two directions (Kim and Xiao, 2010).

2.4. Calculation of EC_{50} values and statistical analysis

Percentage inhibition values were calculated by measuring the mycelium growth diameters of fungal isolates at different fungicide doses and control Petri dishes. Regression analysis was performed in GraphPad Prism 8 statistics program (GraphPad Software, San Diego, CA) using percent inhibition values obtained from mycelium growth tests and logarithmic values of fungicide doses, and EC_{50} values were calculated. In order to determine whether there was a significant difference among the EC_{50} values of resistant and sensitive isolates, all isolates were subjected to a *t*-test.

2.5. Conidial germination tests

Two percent WA was used in conidial germination tests of active ingredients boscalid and boscalid + pyraclostrobin. Conidial germination tests were performed as 5 µg/ml and 100 µg/ml doses in boscalid + pyraclostrobin active ingredients and 5 µg/ml dose in boscalid active ingredient using 3 replications (Kim and Xiao, 2010; Yin et al., 2011). SHAM was added to media containing the boscalid + pyraclostrobin and their controls.

Isolates numbered as 2, 26, 43, 57, 72, 61, 69, 81, 95, 97, 103, and 143 which were thought to be resistant and sensitive according to EC_{50} values, were included in the conidial germination test. These isolates used in conidial germination tests were grown in PDA medium for 10 days. To prepare the conidial suspension, 2 ml of sterile distilled water was poured into the Petri dishes, and conidia were harvested by using a sterile scalpel. Then, the density of the spore suspension, which was passed through a sterile cheesecloth, was adjusted to $1x\ 10^5$ conidia/ml by using the hemocytometer. Twenty µl of the spore suspension of each isolate was transferred to the WA medium to which fungicide was added. After the incubation of Petri dishes at 20°C for 12 hours, spore germination was examined under a stereomicroscope.

3. Results

Regression equations obtained as a result of mycelium growth tests, their EC_{50} values and the isolates found to be resistant and sensitive are presented in Table 1. While EC_{50} values of isolates sensitive to boscalid active ingredient varied between 0.7 and 8.6 µg/ml, EC_{50} values of isolates sensitive to boscalid + pyraclostrobin active ingredients were found to be between 0.1 and 1.9 µg/ml. It was determined that isolates 61, 69, 72, and 81 were resistant to both fungicides, while isolates 57 and 97 were sensitive to boscalid and resistant to boscalid + pyraclostrobin. As a result of the *t*-test, the difference between the EC_{50} values of the sensitive and resistant isolates to Cantus® (boscalid 50%) and Signum® (boscalid 26.7% + pyraclostrobin 6.7%) fungicides were found statistically significant ($P<0.05$).

According to EC_{50} values, sensitive and resistant isolates were selected and conidial germination tests were carried out. While conidial germination was not observed in sensitive isolates at the dose of 5 µg/ml in boscalid active ingredient, it was determined in resistant isolates under a stereomicroscope. In boscalid + pyraclostrobin active ingredients, there was no conidial germination in sensitive isolates at the 5 µg/ml and 100 µg/ml fungicide doses whereas conidial germination was observed in resistant isolates at both concentrations.

Resistance to boscalid was determined in S3, S5, M1, and M6 phenotypic groups, and resistance against boscalid + pyraclostrobin active ingredients occurred in phenotypic groups of S2, S3, S5, M1, and M6. Fungicide resistance was determined in both sclerotial and mycelial type phenotypes. No relationship could be inferred between fungicide resistance and phenotypic groups of isolates (Table 1).

Table 1. Some information about *Botrytis cinerea* isolates and their reactions to active ingredients boscalid and boscalid + pyraclostrobin

Isolate no	Districts of Antalya province where the isolates were obtained	Phenotypes of the isolates (Gül and Karakaya 2020)	Plant parts from which *B. cinerea* was obtained	Regression equations (boscalid)	EC_{50} values µg/ml (boscalid)	Resistance/Susceptibility to boscalid	Regression equations (boscalid + pyraclostrobin)	EC_{50} values µg/ml (boscalid + pyraclostrobin)	Resistance/Susceptibility to boscalid + pyraclostrobin
2	Serik	S4	Leaf	Y = 11x+49	1.4	S	Y=19x+62	0.2	S
8	Serik	M1	Leaf	Y = 14x+45	2.5	S	Y=20x+66	0.3	S
11	Serik	S5	Leaf stalk	Y= 17x+41	2.9	S	Y=40x+84	0.2	S
26	Serik	S2	Leaf	Y = 15x+51	0.9	S	Y=22x+66	0.2	S
38	Serik	S3	Leaf	Y = 14x+44	2.8	S	Y=24x+69	0.2	S
43	Serik	S1	Leaf	Y = 17x+43	2.6	S	Y=23x+73	0.1	S
57	Serik	M6	Leaf stalk	Y = 9x+45	6.7	S	Y=10x+29	49	R
59	Serik	S4	Leaf	Y = 12x+47	2	S	Y=10x+52	0.6	S
61	Finike	S3	Stem	Y = 10x+34	25	R	Y= 10x+19	27	R
69	Finike	S5	Leaf	Y = 11x+33	29	R	Y=9x+20	30	R
72	Finike	M1	Fruit	Y = 10x+35	19	R	Y=4x+24	12	R
74	Finike	S1	Leaf	Y = 17x+40	4.2	S	Y=15x+59	0.3	S
81	Kepez	M6	Leaf	Y = 11x+32	30	R	Y=6x+20	44	R
92	Kepez	M1	Leaf stalk	Y = 17x+47	1.5	S	Y=17x+45	1.9	S
95	Kepez	M3	Leaf	Y = 12x+41	0.7	S	Y=17x+42	1.8	S
97	Kepez	S2	Leaf stalk	Y = 9x+46	2.9	S	Y=12x+32	15	R
98	Kepez	S4	Leaf stalk	Y = 14x+41	4.4	S	Y=26x+54	0.6	S
102	Aksu	S2	Leaf stalk	Y = 11x+45	3.1	S	Y=26x+61	0.4	S
103	Aksu	S3	Leaf stalk	Y= 11x+46	2.5	S	Y=30x+71	0.2	S
143	Kumluca	S3	Leaf	Y = 15x+37	8.6	S	Y=15x+51	0.9	S

* The isolates selected for the conidial germination tests are written in bold. S: Sensitive, R: Resistant.

4. Discussion and Conclusion

In the Aksu and Kumluca districts of Antalya province resistance to boscalid + pyraclostrobin was not found. Resistance to boscalid was not detected in Serik, Aksu, and Kumluca districts. A lower number of isolates showed resistance to boscalid. Isolates resistant to both fungicides have been found in the Finike and Kepez districts of Antalya (Table 1). It has been determined that 20% of the isolates were resistant to both fungicides. While the rate of isolates resistant to boscalid was 20%, the rate of those resistant to boscalid + pyraclostrobin was determined to be 30%.

It is stated that differential doses can be used as a reliable method in determining the resistance against boscalid and boscalid + pyraclostrobin active ingredients **(**Kim and Xiao, 2010; Yin et al., 2011). In our current study, similar to previous findings, it appears that conidia germination tests are compatible with mycelium growth tests and can be used as a practical way to detect resistant isolates. In order to calculate the EC_{50} values of isolates by mycelium or conidia germination tests, *in vitro* tests should be established using different doses of fungicides.

While resistant to boscalid, isolates sensitive to boscalid + pyraclostrobin were not found in our current study. Similar results were obtained in a previous study by Fernández-Ortuño et al. (2012). The high EC_{50} values of the isolates against boscalid + pyraclostrobin indicate a high risk of pathogen resistance to this fungicide (Table 1). Therefore, it may be advisable to use boscalid active ingredient alone in its chemical control to delay the formation of fungicide resistance in the pathogen (Fernández-Ortuño et al., 2012). Fungicides with boscalid active ingredients are not recommended alone in the control against *B. cinerea* in Turkey (Bkü Veri Tabanı, 2021).

Resistance to a fungicide with the active ingredients boscalid + pyraclostrobin has been reported in stored apples (Kim and Xiao, 2010), strawberry fields (Fernández-Ortuño et al., 2012; 2014), and kiwifruit (Bardas et al., 2010). In tomato, it was reported that the EC_{50} values of *B. cinerea* isolates against Signum® were 0.014-0.48 µg/ml and there was no significant resistance (Rodríguez et al., 2014). The EC_{50} values obtained in our current study show that resistance against this fungicide is significant (Table 1).

To our knowledge, fungicide resistance against boscalid and pyraclostrobin active ingredients of *B. cinerea* has been reported for the first time in greenhouse tomato areas in Turkey. In our future studies, molecular characterization of fungicide resistance is planned.

It appears that the use of fungicides with active ingredients of boscalid + pyraclostrobin in tomato greenhouses will lead to increased resistance in the *B. cinerea* population. For this reason, other active ingredients should be used alternately to prevent fungicide resistance. However, in order to create chemical control programs in tomato greenhouses, multiple fungicide resistance of *B. cinerea* populations should also be evaluated. Moreover, there is a need to develop integrated control programs that can be used in the control of the pathogen.

With this current *in vitro* study, the first data on the resistance formation against boscalid and boscalid + pyraclostrobin active ingredients in *B. cinerea* populations in Antalya province were presented. This resistance should also be determined molecularly by using a large number of isolates. It is necessary to implement measures to decrease fungicide resistance to reduce the damage caused by gray mold disease and methods that could be alternatives to chemical control should be developed (Hahn, 2014).

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