Evaluation of sensitivity of apple scab pathogen to difenoconazole using the discriminatory dose technique

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Abstract. The most serious disease of the apple tree in all areas of its growth is scab. In the integrated apple tree protection system, the main method is chemical. However, the use of chemical fungicides is characterized by the risk of developing resistance to them by pathogen. The sensitivity of 118 monospore isolates of *Venturia inaequalis* was studied from three orchards of Jeromine, Reinette Simirenko and Gala cultivars, differing in the frequency of application of difenoconazole. Sensitivity was determined using the discriminatory dose technique (0.01 mg/l of active substance) in terms of RG, the relative growth of the mycelium. RG was expressed as the degree of change in mycelium growth in a nutrient medium with fungicide relative to the control variant in percent. All pathogen populations studied differed significantly in mean RG values. Populations treated three times per season with difenoconazole had higher RG values compared to populations treated two times. From Gala orchard, for some isolates, a stimulating effect of a discriminatory dose of difenoconazole on their growth was observed, that is, a hormesis effect was manifested. The proportion of isolates with RG values above the cutoff value, which was 84 and 100 % for the Reinette Simirenko and Gala orchards, may indicate that the pathogen populations studied are resistant, and in these orchards, there may be a decrease in the effectiveness of protection against apple scab. The discriminatory dose technique allowed us to objectively and promptly assess the sensitivity of *V. inaequalis* populations from orchards with varying intensity use of difenoconazole.

1 Introduction

Apple scab is one of the most serious diseases that affect apple trees. The disease is caused by the specialized ascomycete *Venturia inaequalis* (Cooke) G. Winter. Annual control of the development of apple scab in orchards is the main condition for obtaining high-quality products in the growing of industrial fruit. Fetal losses during the epiphytotic development of the disease can reach 80 % [1]. In the integrated system for protecting apple plantations, the main method is chemical. However, the use of systemic fungicides is associated with

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the risk of developing resistance to them in the fungus. These risks are due to the systematic use of fungicides with active ingredients that have the same or similar mechanism of action, leading to rapid selection of resistant forms of the fungus in the population and their accumulation. To prevent these processes, a number of conditions are observed: the use of fungicides with various active substances belonging to different chemical groups, the alternation of such fungicides during the season, etc.

Single-site fungicides that affect only one specific 'target' in the biochemical processes of the pathogen and have therapeutic or 'postinfectious' properties began to be used against the apple scab pathogen in the 1950s and showed high efficiency. However, after ten years of dodin use, resistance to it was recorded [2]. Faster resistance development rates were observed in *V. inaequalis* for methylbenzimidazole carbamates and benzimidazoles [2]. The development of resistance to single-site fungicides, in contrast to multisite fungicides, is due to their narrowly selective mechanism of action. To inhibit the action of a fungicide, a mutation in only one gene is sufficient, while the probability of a mutation occurring is quite high.

Difenoconazole is widely used in the protection of apple scabs. This active ingredient, belonging to the group of sterol demethylation inhibitors, leads to disruption in the formation of fungal membranes. The fungicide acts on a single micromycete enzyme in the pathway for the synthesis of a specific lipid, increasing the risk of developing resistance to it [3]. In the FRAC (Fungicide Resistance Action Committee) system, the risk is characterized as medium. In general, for this chemical class of fungicides, resistance to flutriafol, myclobutanil and several other active substances has been found [2, 3]. Individual evidence of a decrease in *V. inaequalis* sensitivity to it has also been discovered around the world [3, 4], including in Italy [5], Uruguay [6], Morocco [7], and Russia's Krasnodar Territory [8].

To assess sensitivity to a fungicide, it is usually carried out to identify the 'dose effect' on the growth of mycelium of pure pathogen cultures at various concentrations of the active substance (up to 10 concentration options), with a further calculation of 50 % of the effective dose (ED_{50}). However, such a technique is difficult to use for the large-scale screening of pathogenic fungus susceptibility in various orchards. In such cases, the sensitivity assessment is used according to the discriminatory dose of the active substance, which is one of its concentrations [6, 9, 10]. A prompt assessment of the sensitivity of the apple scab pathogen to it is relevant for large arrays of plantations with a wide range of varieties and long-term intensive use of difenoconazole.

The aim of the work is to assess the sensitivity of *V. inaequalis* populations from industrial plantations with a different frequency of application of difenoconazole using the discriminatory dose method.

2 Materials and methods

The studies were carried out in 2020-2021 in the laboratory of biotechnological control of phytopathogens and phytophages of the North Caucasian Federal Scientific Center for Horticulture, Viticulture, Winemaking. The object of study is *V. inaequalis* isolates from various orchards.

We studied 118 monospore isolates of the apple scab pathogen, represented by three populations, differing in the place of selection, the variety of host plants, and the frequency of application of difenoconazole in the overall system of protection against disease (Table 1). All pathogen populations were sampled in the Krasnodar Territory of Russia at two points: two in the Dinskaya region and one in the Krasnoarmeisky region. The distance between these locations was 50 km.

The general scheme of work included the selection of leaves with symptoms, the isolation of monospore isolates of the pathogen, and an experiment on the effect of a discriminant dose of difenoconazole on the growth of *V. inaequalis* mycelium.

Leaves with fresh apple scab lesions were sampled during the growing season and leaf litter with perithecia in March-April. Selection was carried out randomly, while only one affected leaf was taken from one tree. At least 50 symptomatic leaves were collected for each pathogen population.

Table 1. Population features *Venturia inaequalis.*

Population	Place of selection, district	Number of treatments	Number of isolates
Jeromine	Dinskaya	2	35
Reinette Simirenko	Dinskaya	3	49
Gala	Krasnoarmeisky	3	34

The isolation of monospore isolates from a conidial source was carried out from one scab spot of each leaf [11]. At the first stage, the conidia were washed with sterile water. With a sterile glass spatula, the resulting suspension was evenly distributed on the surface of 2 % aqueous agar and incubated for 18 hours at 20 ° C. Subsequently, individual germinated conidia were transferred under the control of a stereomicroscope to Petri dishes on a fresh nutrient medium (PGA, potato glucose agar). The inoculation was incubated in the dark at 20 ° C for one month.

Monospore isolates were isolated from the ascospore source according to the original method [12]. To obtain the ascospore inoculum, a spore trap was used, which was a Petri dish with water agar, which was turned upside down. A predisinfected leaf with apple scab perithecia was placed on the lid of a Petri dish. The spore trap was incubated for 18 hours at 20 ° C. Single germinated ascospores were transferred to PGA and incubated in the dark at 20 ° C for one month.

The sensitivity of the isolates to difenoconazole was evaluated on a potato-glucose agar medium at a discriminatory dose (DD) of the fungicide. The DD concentration of was 0.01 mg/l of the active ingredient (ai), as recommended [13]. The Skor drug, EC (Syngenta LLC, 250 g/l difenoconazole) was used as a source of the active ingredient. After sterilization, the fungicide was added to the nutrient medium at a temperature not exceeding 55 °C. An equivalent volume of sterile water was added to the control instead of the fungicide in PGA. Control and experimental variants were inoculated with a piece of nutrient medium with 6 mm of fungal mycelium, which was cut from the isolate with a cork drill. The Petri dishes with inoculation were left at 20 ° C in a thermostat for month. The experiment was repeated three times.

The effect of the fungicide on the growth of the fungus mycelium was evaluated after a month by measuring the diameter of the culture in two perpendicular directions using a ruler. Sensitivities of isolates were expressed as relative growth (RG; mean diameter of mycelial colonies developing on fungicide-amended medium per diameter on fungicide-free medium × 100). The significance of differences in sensitivity of different populations of *V. inaequalis* was assessed using the Kolmogorov-Smirnov test for two samples.

3 Results and discussion

As a result of the studies, differences in the sensitivity of various populations of orchard scab to difenoconazole were revealed (Table 2). All studied populations of *V. inaequalis* differed significantly from each other in terms of average RG values. The Jeromine cultivar orchard, which had the minimum number of treatments with difenoconazole during the growing season, was characterized by the lowest value of this indicator.

Table 2. Sensitivity of *Venturia inaequalis* populations.

Population	Mean RG *	Range RG	RG ≥ 70, %
Jeromine	62[a]	23-100	37
Reinette Simirenko	81[b]	34-100	84
Gala	97[c]	78-112**	100

* differences between the mean RG values were assessed based on the two-sample Kolmogorov-Smirnov test, letter discrepancies indicate significant differences at $p<0.001$
** RG values greater than 100% are due to hormesis phenomena.

The proportion of isolates with RG greater than 70 % was also the lowest for Jeromine orchard. The difference in the number of isolates with the last population was almost three times. In the population from cv. Gala, the share of such isolates was 100 %; the mean RG was also the largest. In studies by South American scientists, apple scab isolates with RG ≥ 70 in an orchard with fewer difenoconazole treatments (up to four sprays per season) were found in lower numbers compared to another treated orchard (more than five sprays per season). The shares amounted to 39.4 % and 63.6 %, respectively [6]. On the other hand, Pfeufer and Ngugi, using a higher difenoconazole concentration of 0.125 µg/ml as a discriminatory dose, found in a sample of 644 isolates isolated from various cultivated orchards, only 9.0 % of isolates with RG ≥ 75 [3].

In our study, in the cv. orchard Gala, fungus forms were also encountered that had a relative mycelial growth value greater than 100 % (Table 2, the values are marked **), that is, the relative growth of mycelium at the discriminatory dose was higher than in the control. This fact indicates the presence of the phenomenon of hormesis at a given concentration of difenoconazole. Hormesis is the stimulation of the growth of a controlled organism at low concentrations of a fungicide (pesticide) [14]. Moreover, the hormetic effect can manifest itself only in a part of the initial forms of the pathogen and in resistant forms at higher concentrations of the active substance than in the initial ones. Therefore, when studying the stimulating effect of sublethal doses of dimethachlone on the growth rate of the mycelium of the phytopathogen *Sclerotinia sclerotiorum* (Lib.) de Bary on potato dextrose agar, an effect was shown for 100 % of resistant strains and for 24 % of wild strains. The range of concentrations at which the stimulating effect was manifested for the initial strains was 1.0–5.0 µg/mL, for the resistant strains, 1.0–25.0 µg/mL [15]. In our case, the hormetic effect was observed in the population of *V. inaequalis* (Gala) with the highest mean RG value.

The pathogen populations studied by us differed not only in mean RG values but also in structure. Thus, in the RG ranges (Table 2), one can see how the lower limit of values differs: between the populations of Jeromine and Renet Simirenko, on the one hand, and Gala, on the other. For the last sample of isolates, this limit is much higher. The difference in the lower limit of the indicator between the populations of Jeromine and Reinette Simirenko is small. However, the values of the proportion of RG ≥ 70 % are very significant, which may indicate the occurrence in the second plantation of only single individuals with low relative growth values, and the actual population structure is close to the pathogen sample from the Gala orchard. The most obvious change in the population structure of sensitivity to the studied triazole can be seen in the frequency diagram of the occurrence of isolates with different RG values (Fig.).

The figure shows that in populations of the scab pathogen from garden plantations with more frequent use of difenoconazole, the proportion of isolates with high RG values increases during the season (right side of the graph).

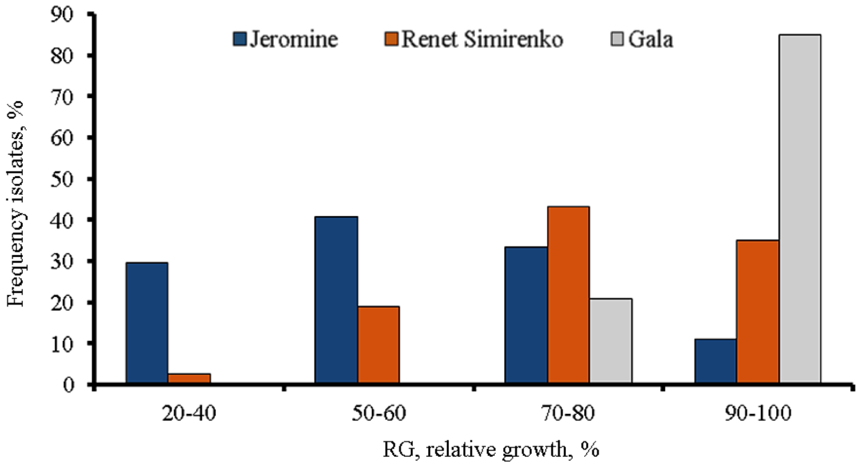

Fig. The frequency of occurrence of *Venturia inaequalis* isolates with different RG values isolated from three orchards: Jeromine, Renet Simirenko and Gala.

The discriminating dose is not in itself a concentration that allows one to cut off sensitive and identify resistant isolates. However, a comparison of the occurrence of isolates with certain RG values in orchards with practical resistance and effective protection allows us to determine a certain threshold value of RG, above which isolates are characterized as resistant [6]. According to the sensitivity studies of *V. inaequalis* to myclobutanil and fenarimol by Köller et al., it is the isolates with RG ≥ 80 % that were identified as resistant to these fungicides. This was confirmed in experiments with infection of apple seedlings in greenhouses: isolates with RG ≥ 80 % were not controlled by the manufacturer's recommended concentrations of these two drugs. The proportion of such isolates in orchards with recorded practical resistance was significantly different from the proportion of isolates in orchards with effective protection against apple scab. At the same time, the authors note that the variation in the proportion of isolates with RG values from 0 to 80 % was wide for different initial populations and did not depend on the history of application of fungicide [16]. Data obtained in our work on the proportion of isolates above the threshold value (RG ≥ 70 %) make it possible to characterize the pathogen populations from Renet Simirenko and Gala stands as resistant. These results require close attention to the studied orchards, as they may show a decrease in the effectiveness of protection against apple scab.

4 Conclusion

Sensitivity to difenoconazole of 118 monospore isolates of *V. inaequalis* from three orchards of varieties Jeromine, Reinette Simirenko and Gala, differing in the number of applications of difenoconazole, was assessed using a discriminatory dose according to RG. All pathogen populations studied differed significantly in mean RG values. Populations treated four times per season with difenoconazole had higher RG values compared to populations treated twice. For some isolates from the Gala variety plantation, a stimulating effect of the discriminatory dose of difenoconazole on their growth was observed, that is, the effect of hormesis was manifested. The proportion of isolates with RG values above the cutoff value, which was 84 and 100% for Renet Simirenko and Gala orchards, may indicate that the studied pathogen populations are resistant and, in these orchards, there may be a decrease in the effectiveness of protection against apple scab. The discriminatory dose technique allowed us to objectively and quickly assess the sensitivity of *V. inaequalis* populations from orchards with different intensity of difenoconazole application, so it can be used for the broad monitoring of pathogen populations in commercial apple plantations.

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References

1. G.V. Yakuba Monografiya. Krasnodar: GNU Severo-Kavkazskogo zonalnogo NII sadovodstva i vinogradarstva, 213, (2013) https://new-disser.ru/_avtoreferats/01000207101.pdf
2. K.D. Cox, *Fungicide Resistance in Plant Pathogens* (Springer, Tokyo) 433–447 (2015) https://doi.org/10.1007/978-4-431-55642-8_27
3. E.E. Pfeufer, H.K. Ngugi Phytopathology, **102(3)**, 272–282 (2012) https://doi.org/10.1094/PHYTO-04-11-0117
4. S.M. Villani, A.R. Biggs, D.R. Cooley, J. J. Raes, & K. D. Cox Plant Dis., **99**, 1526–1536 (2015) https://doi.org/10.1094/PDIS-01-15-0002-RE
5. R. Fiaccadori, American Journal of Plant Sciences, **8 (09)**, 2056–2068 (2017) https://doi.org/10.4236/ajps.2017.89138
6. P. Mondino, L. Casanova, A. Celio, O. Bentancur, C. Leoni, S. Alaniz Journal of Phytopathology, **163 (1)**, 1–10 (2015) https://doi.org/10.1111/jph.12274
7. R. Lahlali, A. Moinina, S. Ezrari, D. Maclean, M. Boulif Notulae Scientia Biologicae, **11(2)**, 249–257 (2019) https://doi.org/10.15835/nsb11210434
8. E.V. Lobodina, I.L. Astapchuk, A.I. Nasonov Scientific Works of the North Caucasian Federal Scientific Center for Horticulture, Viticulture, Winemaking, **26**, 165–169 (2019)
9. W. Köller, D.M. Parker, K.L. Reynolds Plant Dis., **75**, 726–728 (1991) https://www.apsnet.org/publications/PlantDisease/BackIssues/Documents/1991Articles/PlantDisease75n07_726.PDF
10. M. Seyran, T.B. Brenneman, K.L. Stevenson Crop Prot., **29**, 1257–1263 (2010) https://doi.org/10.1016/j.cropro.2010.07.016
11. W. Koller, D.M. Parker, W.W. Turechek Plant Dis., **(88)**, 537–544 (2004) https://doi.org/10.1094/PDIS.2004.88.5.537
12. A.I. Nasonov Mikologiya i fitopatologiya, **53 (1)**, 46–48, (2019) https://doi.org/10.1134/S0026364819010094
13. A.I. Nasonov, G.V. Yakuba, I. L. Astapchuk Mikologiya i fitopatologiya, **55(4)**, 297-308, (2021) https://sciencejournals.ru/view-article/?j=mikfit&y=2021&v=55&n=4&a=MikFit2104010Nasonov
14. E. J. Calabrese Homeopathy, **104(02)**, 69-82 (2015) 10.1016/j.homp.2015.02.007 https://www.thieme-connect.com/products/ejournals/html/10.1016/j.homp.2015.02.007

15. F. Zhou, H.-J. Liang, Y.-L. Di, H. You, F.-X. Zhu Plant Dis., **98**, 1364-1370, (2014) https://apsjournals.apsnet.org/doi/pdfplus/10.1094/PDIS-10-13-1059-RE
16. W. Koller, W.F. Wilcox, J. Barnard Phytopathology, **87**, 184–190, (1997) https://doi.org/10.1094/PHYTO.1997.87.2.184