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Original article

FITNESS OF VENTURIA INAEQUALIS ISOLATES WITH RESISTANCE TO MULTIPLE CHEMICAL CLASSES OF FUNGICIDES IN VITRO

A.I. Nasonov, G.V. Yakuba, M.V. Bardak, N.A. Marchenko

Abstract

Background. The development of fungicide resistance in the apple scab pathogen suggests the development of measures to manage the development of resistance that can functionally delay the emergence of resistant populations of the pathogen. Knowledge of the pathogen's fitness is essential to applying this approach and assessing how successful it will be, since resistance may come with a fitness cost.

Purpose. To evaluate the fitness of sensitive *Venturia inaequalis* isolates and isolates with multiple fungicide resistances to osmotic and oxidative stress in vitro.

Methods. The size of isolates from four groups (a group with baseline sensitivity and three groups with different multiple resistances to fungicides) was evaluated on potato-glucose agar with the addition of various concentrations of NaCl (2, 3, 4, and 6%) and H_2O_2 (2, 5, and 10 mM).

Results. The inhibition of mycelium growth in variants with the addition of NaCl relative to the control occurred on average by about 10% with an increase in concentration of 1%. In all variants of the experiment, the average size of isolates in the initial group was smaller than in the pathogen groups with resistance to fungicides. Oxidative stress has the following effect on the average size of the isolates: in the control, it was 12.2 mm; at a concentration of H_2O_2, it was 2 mM–6.7 mm; and at 5 mM–1.3 mm. There was a tendency for the isolates to have a higher average size in the group with simultaneous resistance to two fungicides, compared with other groups at concentrations of H_2O_2 of 2 mM and 5 mM.

Conclusion. The absence of significant lower values of the average size of isolates in groups with multiple resistances to fungicides in comparison with the baseline group under in vitro stress conditions shows the absence of fitness cost.

Keywords: apple scab; fitness cost; resistance; fungicide; DMI, anilinopyrimidines; SDHI; osmotic stress; oxidation stress

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Научная статья

ПРИСПОСОБЛЕННОСТЬ ИЗОЛЯТОВ VENTURIA INAEQUALIS С УСТОЙЧИВОСТЬЮ К НЕСКОЛЬКИМ ХИМИЧЕСКИМ КЛАССАМ ФУНГИЦИДОВ IN VITRO

А.И. Насонов, Г.В. Якуба, М.В. Бардак, Н.А. Марченко

Аннотация

Обоснование. Развитие устойчивости к фунгицидам у возбудителя парши яблони предполагает разработку мер по управлению её развитием, которые могут функционально отсрочить появление резистентных популяций патогена. Знание о приспособленности патогена необходимо для применения этой подхода и оценки того, насколько успешным он будет, поскольку резистентность может сопровождаться «платой» за приспособленность.

Цель. Оценить приспособленность чувствительных изолятов *Venturia inaequalis* и изолятов со множественной устойчивостью к фунгицидам к осмотическому и оксидативному стрессу *in vitro*.

Материалы и методы. Оценивали размер изолятов четырех групп (группа с исходной чувствительностью и 3 группы с различной множественной устойчивостью к фунгицидам) на картофельно-глюкозном агаре с добавлением различных концентраций NaCl (2, 3, 4 и 6 %) и H_2O_2 (2, 5 и 10 мМ).

Результаты. Торможение роста мицелия в вариантах с добавлением NaCl относительно контроля происходило в среднем примерно на 10 % при увеличении концентрации на 1 %. В всех вариантах опыта средний размер изолятов в исходной группе был меньше, чем в группах патогена с устойчивостью к фунгицидам. Оксидативный стресс оказывал следующее влияние на средний размер изолятов: в контроле он был 12,2 мм; при концентрации H_2O_2 2 мМ - 6,7 мм; 5 мМ - 1,3 мм. Была отмечена тенденция более высокого среднего размера изолятов в группе с одновременной устойчивостью к двум фунгицидам, в сравнение с другими группами при концентрации H_2O_2 2 мМ и 5 мМ.

Заключение. Отсутствие значимых более низких значений среднего размера изолятов в группах с множественной устойчивостью к фунгицидам в сравнение с исходной группой в условиях стрессов *in vitro* показывает отсутствие «платы» за приспособленность.

Ключевые слова: парша яблони; «плата» за приспособленность; резистентность; фунгициды; триазолы; анилинопиримидины; SDHI; осмотический стресс; оксидативный стресс

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Introduction

Among the fungal diseases of the apple tree, scab plays a leading role in terms of economic importance. It is caused by the obligate parasite *Venturia inaequalis* (Cooke) G. Winter. An integrated protection system in orchards against this ascomycete requires the use of chemical fungicides. It is known that the use of fungicides with a single point of effect on the metabolism of a phytopathogenic fungus can V. inaequalis resistance has been revealed, lead to the development of resistance to them [18]. A wide spread of which refers to fungicides from the chemical class of benzimidazoles, which led to a ban on their use against scab in many countries, including Russia [7; 25]. There is also a decrease in the sensitivity of pathogen populations to other fungicides from chemical groups: Sterol Demethylation Inhibitors (DMI), Quinone outside inhibitor (QoI), Anilinopyrimidines (AP), and Succinate Dehydrogenase Inhibitors (SDHI) fungicides [3-5; 12; 18; 22]. Reducing the effectiveness of existing and future active substances contributes, on the one hand, to the creation of new effective compounds by manufacturing companies [1], and on the other hand, to the search for ways to reduce the development of resistance to fungicides [29].

The development of resistance to fungicides in the field is determined by many factors, such as selection pressures, the susceptibility of the host plant, the rate of consumption of the fungicide, the intervals between applications, the resistance of the fungicide to environmental conditions, and the method of its application. Selection pressure, expressed in the toxic effect of the active substance, is one of the main factors leading to the selection of fungus forms with reduced sensitivity to the fungicide. The stability of a sign of resistance to a fungicide, in turn, is determined by whether it is a genetic load in the absence of exposure to a fungicide. If such a sign leads to a decrease in the fitness of the

pathogen in non-selective conditions, we are dealing with the so-called fitness cost [20]. The identification of such effects is of great practical importance for the creation of anti-resistance programs, as it allows you to regulate the rate and time of onset of resistance in the pathogen population.

The cost of fitness can be studied relating to of predicted fitness by evaluating various indicators of isolates in the laboratory: mycelium growth, germination, and spore formation. The actual fitness of a pathogenic microorganism can be studied *in vivo* in competitive exclusion experiments [9, 11]. Many studies aimed at identifying fitness disorders in fungicide-resistant fungi have contradictory results [20]. While some studies have demonstrated that resistant isolates' fitness decreases in both *in vitro* and *in vivo* trials [20], other investigations have demonstrated that the fitness cost in such populations is negligible or nonexistent [11-13; 15; 17; 26].

Materials and methods

Isolate selection. About 45 isolates were selected from the working collection of single-spore cultures of *Venturia inaequalis* at the Laboratory for Biotechnological Control of Phytopathogens and Phytophages. The isolates differed in sensitivity to various fungicides and were collected either in industrial orchard plantations of domestic apple trees or natural eastern apple trees in various regions of the Krasnodar Territory (Table 1). Sensitivity assessment of isolate pathogens was carried out earlier [3-5; 7]. The preparation of single-spore isolates was carried out using the technique described earlier [2]. The isolates were divided into four groups depending on their sensitivity to four chemical groups of fungicides (benzimidazoles, Qol, DMI, and AP) in vitro. The "baseline" group included isolates with baseline sensitivity, that is, isolates of the pathogen that never interacted with fungicides and were collected in the natural ecosystems of the oriental apple tree. The remaining groups were characterized by multiple resistance to two ("Resist2"), three ("Resist3"), and four ("Resist4") active substances of fungicides (Table 1).

When selecting the studied isolates into one or another resistance group, the following assumptions were used: isolates pathogen with EC_{50} values for difenoconazole (the chemical group of active substances, DMI) > 0.1 mg/l, pyrimethanil (anilinopyrimidines) > 0.1 mg/l, carbendazim (benzimidazoles), and trifloxystrobin (Qol) were considered resistant isolates at 100 mg/l.

Maintaining pure cultures. The cultures were inoculated in tubes with a beveled potato glucose agar medium (PGA) and incubated for 20 days at 20 °C in the dark. After the culture, it was placed in the refrigerator at 4 °C, stored for 6 months, and replanted.

Table 1.

Characteristics of groups of single-spore isolates of Venturia inaequalis

Group	Fungicide resistance	Host plant species	Place of selection,
Baseline	absent	*Malus orientalis* Uglitzk.	Seversky district
Resist2	Benzimidazoles and Qol*	*Malus x domestica* Borkh.	Krasnoarmejskij, Vyselkovskij district
Resist3	Benzimidazoles, Qol and DMI**		
Resist4	Benzimidazoles, Qol, DMI and AP***		

Note: * Qol - Quinone outside inhibitor; ** DMI - Sterol Demethylation Inhibitors; ***AP – Anilinopyrimidines

Mycelial growth. Agar blocks containing mycelium from single-spore cultures were transferred to a PGA medium in plastic Petri dishes with a diameter of 90 mm using cork borer and incubated at 20 °C for 30 days. Experimental Petri dishes were seeded with 5 mm-diameter agar blocks using cork borer and incubated at 18 °C for 30 days [6]. After that, the diameter of each culture was measured with a ruler in two mutually perpendicular directions, and an average value was obtained. The size of the isolates was taken into account without a seed block. Each isolate was sown four times.

Osmotic sensitivity. The effect of salt stress conditions on mycelium growth was assessed by adding sodium chloride solution to the PGA medium to a final concentration of 2, 3, 4, and 6%.

Oxidative stress. To simulate oxidative stress, a solution of H_2O_2 (technical hydrogen peroxide 37%, grade A) was added to experimental cups with a PGA medium to a final concentration of 2, 5, and 10 mM. An equivalent amount of water was added to the control versions of the PGA medium.

Statistical analysis. Experiments were carried out for each isolate in fourfold repetition. The data is presented as the average values of the values for each group with their standard errors. Due to the fact that the normal distribution of the values of the size of the isolates was not observed for all groups, the nonparametric Kruskal-Wallis test was used for statistical verification of the data. A posteriori Dunn's test was performed to identify differences between individual groups. To reduce the probability of a first-kind error in multiple comparisons, the Bonferroni correction was used. Statistical data processing was performed using the software PAST [19]. The differences between the average values of the groups were considered significant at $p < 0.05$.

Results

Evaluation of mycelium growth of the entire sample of isolates at different concentrations of NaCl

All the studied salt concentrations led to a significant inhibition of the growth of the mycelium of pathogen isolates on the PGA medium (Figure 1). Meanwhile, at a concentration of NaCl in the nutrient medium of 6%, there was no growth in all isolates (data are not shown).

The average values of the size of the isolates in the control were 11.5 mm at a NaCl concentration of 2%–3.7 mm, 3%–1.9 mm, and 4%–0.9 mm. Inhibition of mycelium growth in the salt-added experimental variants relative to the control occurred on average by about 10% with an increase in NaCl concentration of 1%, which amounted to 68%, 84%, and 92%, respectively.

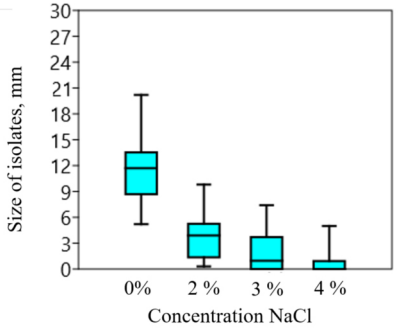


Fig. 1. Diagram of the range of values of the size of *Venturia inaequalis* isolates at different concentrations of NaCl

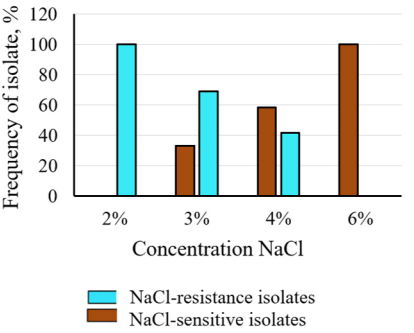


Fig. 2. Distribution of *Venturia inaequalis* isolates with different resistance to salt stress, at different concentrations of NaCl

In addition to differences in the average size of the isolates, there were also differences in the ratio of isolates capable of growing at a specific salt concentration (salt-resistant) and non-growing (salt-sensitive, Figure 2).

Thus, at 2% NaCl, all isolates were able to grow, whereas at concentrations of 3 and 4%, the proportion of such isolates was 69 and 42%, respectively. All the studied isolates were sensitive to a NaCl concentration of 6%.

It was also noted that the values of mycelium growth of isolates at salt concentrations of 2% and 3% correlated significantly with mycelium growth without NaCl, with r values of about 0.4, which corresponds to the average correlation strength. Thus, the reduction in the size of the isolates under the influence of the stress factor occurred monotonously, and the isolates that had

large sizes relative to other isolates in the control retained their position relative to the same isolates in salt-added variants.

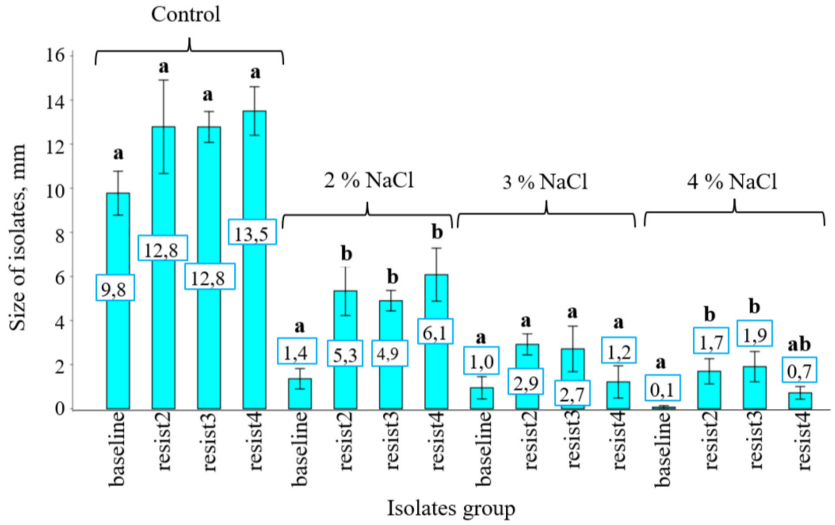
Table 2.

Correlation between different experimental options according to the level of mycelial growth *Venturia inaequalis*

	Control	2 % NaCl	3 % NaCl	4 % NaCl
Control		0,022*	0,048	0,109
2 % NaCl	0,465**		0,022	0,003
3 % NaCl	0,408	0,464		0,001
4 % NaCl	0,336	0,587	0,673	

Note: * in the upper right corner are the significance values of *p*; **in the lower left corner of the table are the correlation values of *r*

Evaluation of mycelium growth in groups of pathogen isolates at different concentrations of NaCl



Note: Different letters show the presence of significant differences between the average values of the diameter of the isolates in the groups within each experiment according to the data of the a posteriori test of multiple comparisons of Dann, taking into account the Bonferroni correction at $p < 0.05$.

Fig. 3. Variations in the average values of mycelium growth of *Venturia inaequalis* isolates at different concentrations of NaCl in groups differing in multiple resistance to fungicides, mm

In the control variant, as well as at all concentrations of NaCl, the average size of isolates in the initial group was smaller than in other pathogen groups (Figure 3). In experiments with salt concentrations of 2% and 4%, these differences were statistically significant.

The qualitative composition of the isolates also differed in terms of resistance to salt stress. At NaCl concentrations of 3% and 4%, the number of non-stress-tolerant isolates in the original group was greater than in the others (Figure 4). The data obtained show that there is no fitness cost in groups of isolates with multiple resistance to fungicides.

Evaluation of the mycelium growth of the entire sample of isolates at different concentrations of hydrogen peroxide

The addition of H_2O_2 to the nutrient medium led to a significant decrease in the growth of the mycelium of pathogen isolates (Figure 5). At the maximum concentration of hydrogen peroxide in the nutrient medium, at 10 mM, growth was absent in all isolates. The average values of the size of the isolates in the control were 11.5 mm at a concentration of H_2O_2 of 2 mM–6.7 mm and 5 mM–1.3 mm. The inhibition of mycelium growth in the experimental variant with a concentration of hydrogen peroxide of 2 mM relative to the control was 50%, and at a concentration of 5 mM, it was 60%.

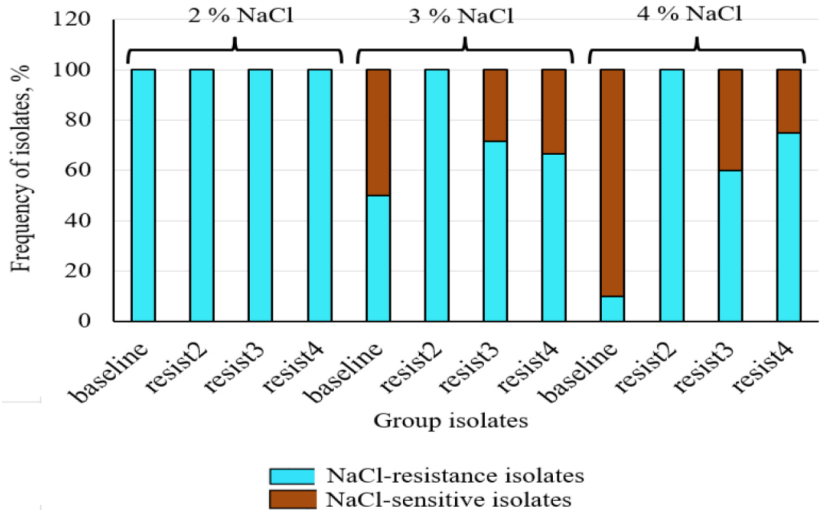


Fig. 4. Distribution of *Venturia inaequalis* isolates in groups characterized by varying degrees of resistance to salt stress at different concentrations of NaCl

There were also differences in the ratio of isolates growing (resistant) at a specific concentration of H_2O_2 to non-growing (sensitive) isolates. Already at the first concentration, 12% of the isolates showed no signs of growth; at 5 mM H_2O_2, the proportion of such isolates reached 72%; at a concentration of 10 mM, 100% of the isolates were sensitive to oxidative stress and showed no signs of mycelium growth in the nutrient medium.

An evaluation of the relationship between the experimental variants showed that the sizes of isolates at H_2O_2 concentrations of 2 mM and 5 mM correlated with each other at an acceptable level of significance (Table 3).

Evaluation of mycelium growth in groups of pathogen isolates at different concentrations of H_2O_2

A comparison of the growth of mycelium isolates in different groups showed no significant differences between them. At the same time, it is possible to note the tendency for a higher average size of isolates in the Resist2 group in comparison with other groups, both in the variant with the addition of H_2O_2 of 2 mM and the addition of 5 mM (Figure 7).

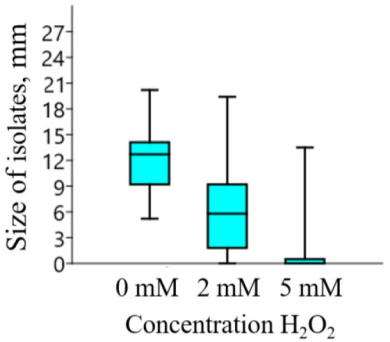


Fig. 5. Diagram of the range of values of the size of *Venturia inaequalis* isolates at different concentrations of H_2O_2

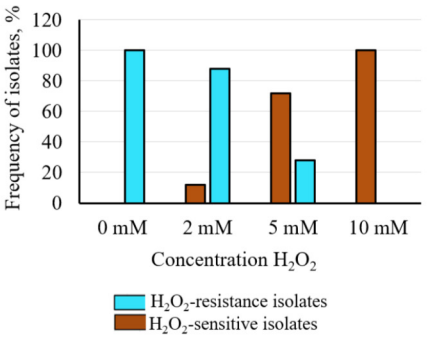


Fig. 6. Distribution of *Venturia inaequalis* isolates with different resistance to oxidative stress, at different concentrations of $H_{2O}2$

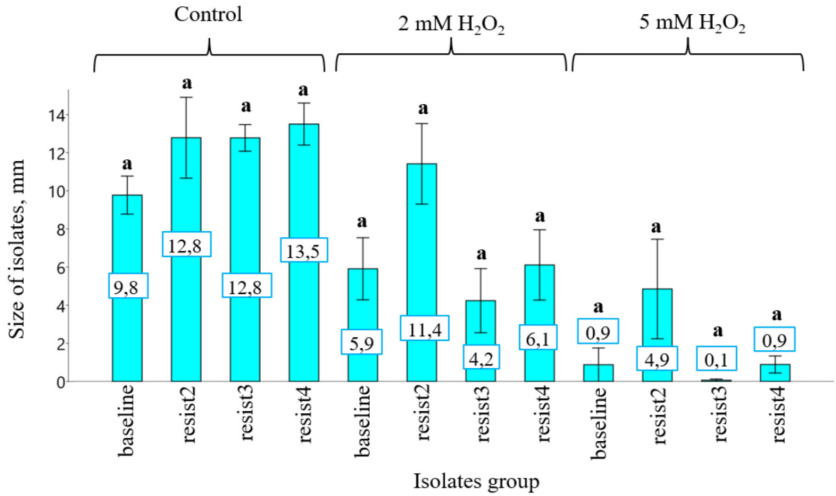
The same trend is confirmed by data on the distribution of isolates sensitive to and resistant to oxidative stress in these two experiments. Thus, at a concentration of hydrogen peroxide of 2 mM, all isolates in the Resist2 group were resistant to oxidative stress, and at a concentration of 5 mM, the proportion of such isolates in this group was higher than in the rest (Figure 8).

Table 3.

Correlation between different variants of the experience in terms of mycelium growth *Venturia inaequalis*

	Control	2 mM $H_{2O}2$	5 mM H_2O_2
Control		0,817*	0,194
2 mM H_2O_2	-0,044**		0,018
5 mM H_2O_2	0,244	0,428	

Note: * in the upper right corner are the significance values of *p*; **in the lower left corner of the table are the correlation values of *r*



Note: One and the same letter shows the absence of significant differences between the average values of the diameter of the isolates in the groups within each experiment according to the data of the a posteriori test of multiple comparisons of Dunn, taking into account the Bonferroni correction at $p < 0.05$.

Fig. 7. Variations in the average values of mycelium growth of *Venturia inaequalis* isolates at different concentrations of H_2O_2 in groups differing in multiple resistance to fungicides, mm

The same trend in the qualitative structure of these groups was observed in the experiment with osmotic stress. The Resist 2 group at NaCl concentrations of 3 and 4% consisted of salt-stress-resistant isolates. This group of isolates is characterized by resistance to two chemical groups of fungicides: benzimidazoles and Qol, which are characterized by the development of resistance by qualitative type, while isolates from the Resist3 and Resist4 groups, in addition, were resistant to fungicides with a quantitative type of resistance development.

The absence of significant differences between the size of isolates in the baseline group and the group with resistance to fungicides indicates the absence of fitness costs.

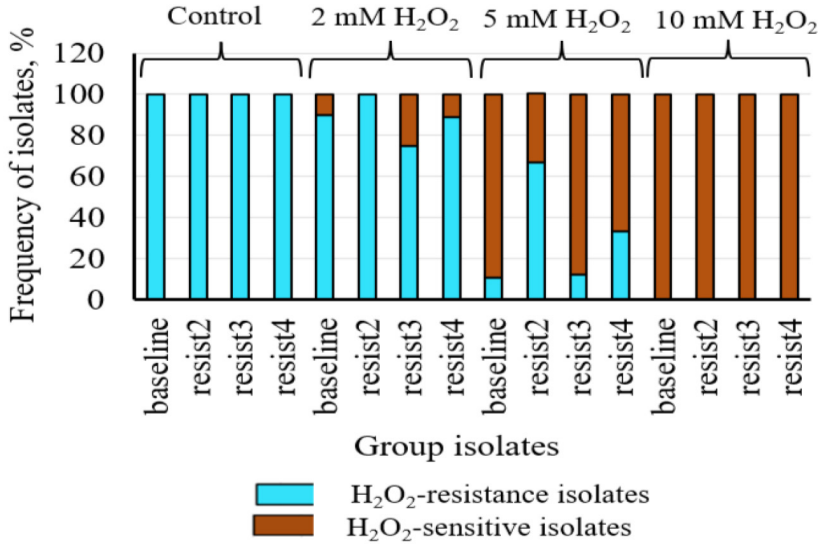


Fig. 8. Distribution of *Venturia inaequalis* isolates in groups characterized by varying degrees of resistance to oxidative stress at different concentrations of H_2O_2

Discussion

This is the first study of the fitness of the in vitro to osmotic and oxidative stress of *Venturia inaequalis* isolates resistant to fungicides from several chemical classes. Given the recent empirical confirmation of fungicide resistance in *V. inaequalis* populations in Russia [3-5; 7], we must implement fungicide-resistant management measures that can effectively delay the emergence of new resistance. Knowledge of fitness is essential to successfully applying these tactics and assessing how successful they will be in postponing the selection of resistant strains, as resistance may be accompanied by fitness penalties that affect the likelihood of resistance accumulation in the absence of selection pressure [24]. In the present study, the fitness of the apple scab pathogen was assessed by measuring in vitro parameters from the point of view of "predicted fitness" [9].

Our work showed that baseline and fungicide-resistant *V. inaequalis* isolates did not differ significantly in vitro mycelial expansion in response to osmotic and oxidative stress, suggesting the absence of any fitness impairment under

these in vitro parameters. Likewise, some studies assessing fungicide-resistant isolates of different pathogens of the diseases have not shown significant variations in predicted fitness metrics [6; 11-13; 15; 20; 21; 30]. Thus, American scientists have shown the absence of a negative effect on the growth of isolates with multiple resistances to fungicides at 19 °C on PGA [20]. Similar data were obtained by Russian researchers when assessing the suitability of V. inaequalis with multiple resistances to fungicides in vitro [6]. This study assessed the effects of different temperatures and agar concentrations on the growth of isolates; none of these factors showed the superiority of the original isolates over the resistant isolates, except a temperature of 27 °C.

There are no studies evaluating the fitness of the scab pathogen to osmotic and oxidative stress; however, there are numerous studies performed on other phytopathogenic fungi [10; 11; 13-16; 21; 23; 26; 28]. Many studies have shown that high resistance to fludioxonil, belonging to the chemical class of phenylpyrroles, is associated with increased osmotic sensitivity [14; 16; 23; 26; 28]. It is assumed that fludioxonil mimics the osmotic stress signal by binding to a special receptor, causing inadequate activation, which leads to excessive accumulation of glycerol, ion fluxes, and high turgor pressure in cells [14]. For other fungicides, there was no association between reduced growth of isolates on media with the addition of salt or hydrogen peroxide and resistance to fungicides [10; 15; 21; 27; 30]. For example, of the six osmotic stress agents tested, all isolates of *Alternaria alternata* with dicarboxymide resistance had increased tolerance only to D-sorbitol; however, no significant differences between sensitive and resistant isolates were observed in response to the addition of 0.6 M KCl, 0.6 M NaCl, 32 mM H_2O_2, SDS at a concentration of 0.05 mg/ml, or Congo red [30]. The analysis of three *A. alternata* isolates having resistance to SDHI, QoI, and MBC fungicides simultaneously showed no signs of decreased fitness in response to osmotic stress. In the case of oxidative stress, one of the three isolates with multiple resistances to these fungicides showed reduced mycelium growth rates on a medium with the addition of paraquat. This resistance isolate was distinguished from all others by the presence of a mutation in the D subunit of the SDH enzyme, which determines resistance to SDHI fungicides [15]. Reactive oxygen species (ROS) in small concentrations play the role of regulatory molecules for various cellular functions. However, an increase in the processes of free radical oxidation and a violation of the balance between pro- and antioxidants lead to an increase in the concentration of ROS, which contribute to oxidative damage to macromolecules, activation of proteolytic enzymes, and other

events leading to a violation of the regulation of cell proliferation and apoptosis [8]. Hydrogen peroxide and paraquat are the most common agents for modeling the process of oxidative stress.

The absence of fitness cost in vitro under osmotic stress has been shown in various studies for *Botrytis cinerea* isolates that cause diseases of vegetable crops and apple trees and are resistant to anilinopyrimidines, QoI (pyraclostrobin), and SDHI (boscalid) fungicides [10, 21]. Similar results for the object closest to the one we studied were obtained on isolates of *Venturia effusa*, a pecan scab pathogen resistant to fentin hydroxide and tebuconazole [27].

Conclusion

For the first time, the fitness of isolates with multiple resistances to fungicides under osmotic and oxidative stress was evaluated in vitro for *Venturia inaequalis*. Exposure to various types of stress had a significant effect on the growth of the pathogen mycelium in the laboratory. At the same time, *V. inaequalis* isolates with multiple resistances to fungicides did not significantly differ in mycelium growth from isolates with basic sensitivity or had higher values. The results obtained show that there is no fitness cost for resistant isolates. The results obtained show the absence of fitness cost in resistant isolates. It can be assumed that resistant isolates can successfully compete with sensitive isolates in the orchard in the absence of a selective factor (fungicide), for example, when rotating a fungicide with one mechanism of action to another. In this case, there may be a further accumulation of resistant isolates in the population and an increase in its resistance. Thus, the currently used anti-resistance approaches based on the rotation of active substances with different mechanisms of action and the use of blended drugs can reduce the selection pressure on the sign of resistance and, consequently, reduce the rate of resistance development. However, such anti-resistance programs do not contribute to restoring the sensitivity of the population or reducing its resistance level. Therefore, when developing protection systems, it is necessary to focus only on the fact of the presence of resistance to chemical fungicides in this population.

Meanwhile, the data obtained by us indicate the absence of fitness cost in the conditions of assessing the “predicted fitness,” which was obtained in laboratory conditions, whereas the most accurate assessment is the “real fitness” in the interaction of the pathogen with the host plant. It seems promising to study the effect of the fitness cost in vivo experiments with an assessment of the pathogenesis features in *V. inaequalis* isolates resistant and sensitive to various fungicides.

Conflict of interest information. The authors of this study declare that they have no conflict of interest.

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