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FUNGICIDE RESISTANCE OF PLANT PATHOGENIC FUNGI AND THEIR CHEMOSENSITIZATION AS A TOOL TO INCREASE ANTI-DISEASE EFFECTS OF TRIAZOLES AND STROBILURINES

(review)

L.A. SHCHERBAKOVA

All-Russian Research Institute of Phytopathology, 5, ul. Institut, pos. Bol'shie Vyazemy, Odintsovskii Region, Moscow Province, 143050 Russia, e-mail larisavniif@yahoo.com
ORCID:
Shcherbakova L.A. orcid.org/0000-0003-0254-379X
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Abstract

The chemical method for plant protection is still the most reliable way to provide the high yield of economically significant crops and ensure its quality. In the world agriculture, at least 150 different fungicidal compounds with different mechanisms of action are now used, and the number of products developed and registered on their basis is much more. Triazoles and strobilurins belong to fungicides, which have expanded the opportunities to control causative agents of the most damaging diseases (D. Fernández-Ortuco et al., 2008). Nevertheless, multiple applications of fungicides during each new growing season are often required to achieve an effective control of fungal and oomycete pathogens. Such extensive applications of fungicides exacerbate negative impact on environment, and promote developing the resistance by these pathogens, representing the most disturbing consequence of fungicidal treatments (J.A. Lucas et al., 2015) that makes them relatively short-lived and eventually uneconomical (K.J. Brent et al., 2007; R.P. Oliver, 2014). Attempts to combat resistant forms of plant pathogenic fungi and oomycetes by increasing the dosage of fungicides and treatment numbers are futile, as they cause accumulation of more and more resistant strains in fungal populations. Therefore, control of these pathogens by minimal effective dosages of fungicides, without any decrease in the fungicidal efficacy, and search for ways to overcome the plant pathogen resistance to fungicides are dominant trends in plant protection for current sustainable agriculture. At the same time, the rejection of modern fungicides with high and medium risk of the resistance, including strobilurins and triazoles, does not seem to be practically rational, since they provide a highly effective control of a wide range of diseases and have several other advantages (A.V. Filippov et al., 2016). Chemosensitization of plant pathogens by natural compounds to increase efficacy of fungicidal treatments is an approach to solving the aforementioned problems. Chemosensitization can be accomplished by combining a commercial fungicide with a certain non- or marginally fungicidal substance at concentrations where, alone, neither compounds would be effective, while after their co-application a synergistic fungicidal effect is achieved, sometimes at a level significantly exceeding that of the fungicide dosages to which resistant strains are insensitive (B.C. Campbell et al., 2012; V.G. Dzhavakhiya et al., 2012). Since biochemical and structural targets of chemosensitizing substances differ from those targeted by fungicides, chemosensitization do not contribute to the selection of resistant pathogenic form, and reduces the toxic impact on the environment by lowering effective dosage levels of toxic fungicides. In this review, the promise of chemosensitization as an antiresistant strategy to improve efficacy of the protective fungicide effect is exemplified by experiments with several economically significant phytopathogenic fungi, which sensitivity to strobilurins and triazoles was demonstrated to enhance significantly by co-application of these fungicides with secondary plant or microbial metabolites and their synthetic analogues. In addition, the problem of the development of resistance in plant pathogenic fungi and the methods for its management are briefly described, information on the types and main mechanisms of resistance, in particular, those responsible for resistance to triazoles and strobilurins as well as data on the mechanisms of action of some chemosensitizers are presented.

Keywords: chemosensitization, plant pathogenic fungi, resistance to fungicides, triazoles, strobilurins, fungicide stress-responsive metabolic pathways, resistance overcoming

In the context of intensively developing horticulture the stability of agricultural crops, especially commercially cultivated, cannot be ensured without taking steps to fight crop disease. Plant pathogenic fungi and oomycetes are some of the most dangerous pathogens [1, 2)]. In the vast majority of cases fungicidal treatment still remains (and for a long time will remain) the most desirable method of combating pathogens that guarantees high yielding crops, preservation and quality of agricultural products.

At the same time, it is a known fact that wide-scale application of fungicides is connected with serious environmental and medical risks, and with pathogen resistance to these antifungal agents. In spite of the fact that integrated plant protection systems aimed at environmentalization of agricultural production is dominated by the tendency of reduced application of fungicides, multiple fungicidal treatment during each season is often required for reliable protection of crops and control of many plant pathogenic fungi and oomycetes. For instance, potato farming in Russia has 9-11 fungicidal treatments per season, and in some European countries — up to 18 sprayings with fungicides per season. Such an intensive application of fungicides, in spite of strict regulations developed with the purpose of risk minimization, increases pesticide load in agrobiocenosis, deteriorates the environmental situation and is accompanied by development of resistance. Arguably, the accumulation of persistent forms of fungi and oomycetes in natural populations is the most formidable undesirable consequence of fungicidal treatment [3], which in many cases makes them ineffective and economically unjustified [5, 6], and sometimes results in removal of entire classes of fungicides from circulation [7].

It has to be pointed out that resistance phenomenon is of general biological nature. Resistance to fungicides is a particular case of natural biological evolution of organisms capable of adapting to changing environmental conditions, which creates problems not only for horticulture. For instance, resistance of bacteria and fungi to medical preparations significantly complicates treatment of patients suffering from bacterial disease and mycosis [8, 9]. In general, resistance of pathogenic microorganisms remains one of the major problems of our age, and reduction of resistance of pathogenic microorganisms in phytopathogenic fungi is a pressing task of agricultural science. In 1994, the Fungicide Resistance Action Committee (FRAC, https://www.frac.info/) was established. This Committee supervises monitoring of resistant mutations in populations of phytopathogens and coordinates the development of anti-resistance defense technologies and gives recommendations for resistance development risk mitigation when using fungicides against pathogens of agricultural plants of economic significance in different countries. EuroBligh (http://agro.au.dk/forskning/inter-nationale-platforme/euroblight/) specifically pursues the issue of resistance of *Phytophthora infestans* and early blight of potato (*Alternaria solani*). Whereas potato is a high demand culture, this network combines not only the scientists, but also agricultural producers interested in obtaining maximum crops via multiple treatments using various fungicides. In Russia, the problem of antibiotic and fungicide resistance is also emphasized. For instance, a suitable strategy has been developed and recently approved (Resolution of the Government of the Russian Federation No. 2045-p dated September 25, 2017) to prevent advancement of resistance of pathogenic organisms, including pathogenic organisms affecting plants.

The problem of pathogenic fungi resistance of plants in agriculture. At least 150 chemical formulations with various mechanisms of action (MA) presently divided into 46 classes are used as fungicide active agents in agriculture worldwide. Furthermore, 12 groups of multisite fungicides are combined in a separate class [3, 4, 10]. The triazoles (class G1) and strobilurins

(class C3) belong to three groups of active agents, commercialization of which in 1980-1990s ensured a breakthrough in the fight against pathogens of the most maleficent plant diseases in many countries [11].

The triazoles or DMI-fungicides suppress synthesis of fungal strains by inhibiting 14α- demethylase (CYP51, *cyp51/erg11* gene), which belongs to cytochrome P450 superfamily. This enzyme is responsible for cleavage of 14α-methyl group of lanosterol, the ergosterine precursor [12] (primary sterol component of fungal membranes, which the plants do not have). The inhibiting effect of this enzyme results in deficit of ergosterine and accumulation of toxic 14-α-methyl sterol, high concentrations of which intensify the oxidative stress, cause damage to the membrane and, consequently, death of fungal cell [13]. The strobilurins or QoI-fungicides act as blocking agents of ubiquinone oxidase (cytochrome bc1, CYTB, *cyt b* gene). For the first time, strobilurins A and B were detected in culture liquid of *Strobilurus tenacellus* basidiomycete, following which their chemical derivatives were obtained with an identical mechanism of action, which are divided into 9 groups [11, 14]. The strobilurins inhibit fungal mitochondrial respiration by connecting to the external bc1 cytochrome site (complex III) and inhibiting the transfer of electrons between cytochromes b and c. As a result, strobilurins suppress ATF synthesis, thus causing deficit of energy in fungal cells, which resulting in their stasis.

Numerous fungicidal agricultural products were developed and registered based on various triazoles and strobilurins and their combinations with active agents from different classes; triazoles are also used in medicine as medicinal products.

Fungicide resistance development. Until the turn of 1970s, reports about reduced efficiency of fungicides after prolonged periods of use due to emergence of resistant phytopathogen forms were relatively rare [15]. Therefore, it seemed that in agriculture the resistance problem does not have practical value and can be solved with the help of tank mixtures, development of new preparative forms and treatment rules based on experience of application of active agent groups [16] predominant in the 1960s. However, as new systemic and contact fungicides emerge, especially with single-site activity type (including DMI- and QoI-fungicides), the number of cases of reduced or lost sensitivity and cross-resistance of different fungi [17-21], including those affecting potato [22], and oomycetes [23, 24] grew significantly [4, 25], and the time required for the emergence of resistant forms in many cases decreased significantly — sometimes to 2 years after the first commercial application [11, 26, 27].

The attempts to counter resistant forms by increasing dosages, usage rates and repetition factor of new fungicide treatment, which were recommended for use in relatively small volumes due to high activity against phytopathogens only aggravated the problem causing accumulation of ever more resistant strains and stimulating their spread in populations [3]. This tendency was first observed in practice when the efficiency of chemical protection is reduced, and later proven experimentally. In particular, the laboratory research validated a positive correlation between concentration increase of triazole fungicide and accumulation of resistant forms, and development of intraspecific cross-resistance of winter wheat leaf blotch pathogenic agent (*Zymoseptoria tritici* = *Mycosphaerella graminicola*) [27]. It was also demonstrated that isolate frequency resistant to this QoI-fungicide [28] increases in case of increasing dosages and azoxystrobin usage rate by the end of the growing season in the population of powdery mildew (*Blumeria graminis* f. sp. *hordei*). Similarly, the sensitivity of *P. infestans* to mancozeb and chlorothalonil gradually decreases in the course of their multiple use, although the risk of resistance development to these multisite fungicides is low [29].

Presently, there have been registered cases of emerging resistance to almost all key classes of fungicides in the most diverse phytopathogen types [10]. In this context, triazoles and strobilurins are not an exception, and according to FRAC rating the risk of resisting them is evaluated correspondingly as average and high. It is believed that fast development of resistance to QoI-fungicides is due to the fact that ubiquinone oxidase is encoded with mitochondrial DNA, which possesses less expressed reparation ability than nuclear DNA [25]. The forms of potato blight pathogens (*A. solani* and *A. alternata*) resistant to azoxystrobin for the first time were identified as early as in 2000 (the preparations on its basis came into use at the end of 1990s). The intraspecific resistance was identified for all active agents of QoI-fungicides. For DMI-fungicides it is usually marked for those active agents that are active against the same pathogen, and are not detected with regard to inhibitors of strain synthesis from different classes.

Primary mechanisms of fungal resistance to fungicides. Resistance types. The molecular research of stable strain genome changes of various fungi and oomycetes enable understanding the resistance mechanisms and showed that in most cases it is caused by gene mutations encoding metabolic targets of fungicides, and is connected with different adaptation mechanisms triggered by chemical stress.

Quality resistance [7] usually developes relatively fast and with a high degree of probability occurs in the first instance typical for single-site fungicides. When it spreads, only fully resistant strains accumulate in populations of pathogens, whereas forms with average resistance are absent (complete loss of fungicide efficiency). However, forms with quality type resistance can be preserved only provided that gene mutations encoding structural and metabolic fungicide targets do not interfere with pathogen viability. In this way, strains resistant to QoI-fungicides cannot be detected in populations of *Rhynchosporium secalis* and *Puccinia* spp. in spite of regular use of QoI-fungicides in the course of many years. Due to special structure of cytochrome b gene, in fungi, the G143A mutation typical for resistance to strobilurins disrupts mRNA processing and expression of cytochrome non-functional protein, as a result of which the mutants with the allel carrying G143A do not survive.

The quantitative resistance can impart resistance to fungicides with different mechanism of action and result in existence of forms with incomplete resistance in populations (reduced fungicide efficiency). This is due to several adaptation mechanisms the effect of which is aimed at maintaining nonlethal concentrations of fungicides in a cell. This is achieved by strengthening the expression of ABC transporter genes (proteins that remove molecules of pharmaceutical and other toxic substances from a cell into extracellular space; in some phytopathogens MFS-transporters are also involved in this process), plasmatic membrane modifications (reduced permeability for fungicides), and synthesis of enzymes that destroy a fungicide or transform its molecules into compounds that are not toxic for the fungus [7, 30-32]. Furthermore, the overexpression of genes encoding biochemical or structural targets of fungicides [33] and use of alternative metabolic pathways by pathogens can also make a contribution to ensuring stability of the qualitative type [34, 35].

Mechanisms of resistance to triazoles and strobilurins. Several mechanisms of phytopathogenic fungi and oomycetes take part in the development of resistance and tolerance to triazoles and strobilurins resulting in both qualitative and quantitative resistance. Primarily, point mutations V136A, Y136F, Y137F, A379G, I381V in *cyp51*(*erg 11*) gene and its promoter and activation of ABC-transporters are responsible for resistance to triazoles. For instance, it has been demonstrated that Y136F, the point mutation in the 136-th

codon resulting in replacement of phenylalanine with tyrosine, causes the development of resistance to triadimenol in powdery mildew pathogens in grapes (*Uncinula necator*) and barley (*Erysiphe graminis* f. sp. *hordei*) [10, 36]. Almost all possible *cyp51* single nucleotide changes were detected in high-, weak and moderately resistance strains of winter wheat leaf blotch pathogen *Z. tritici* (*M. graminicola*) from France and the UK, and some strains of the pathogen with moderate or high resistance contained an insertion in the promoter of this gene or combinations of point mutations [37]. At the same time, it was determined that resistance to DM-inhibitors of field isolates of some phytopathogenic fungi is not always related with amino acid replacements in CYP51 protein [12]. For instance, in mutant phenotype of *Z. tritici* (*M. graminicola*) high multiple resistance to DMI-fungicides, apparently, is also ensured by overexpression of genes of transporter proteins [37].

Resistance to strobilurins is primarily due to two mutations of *cyt b* target gene resulting in glycine replacement with alanine (G143A) and phenylalanine with leucine (F129L), as well as adaptation mechanisms, specifically, overexpression of alternative oxidase functioning in circumvention of respiratory complex III [10, 12]. None of the named mutations causes negative impact on viability of pathogens; therefore, resistant strains with these point replacements are often encountered in populations of different fungi, including potato, rice, barley, wheat and gourd family pathogens [12]. The G143A point mutation has been identified in *A. alternata*, but still not identified in *A. solani* [38]. Quite the opposite, phenotype with F129L mutation are known for both types causing potato blight. The degree of resistance of such mutants *A. alternata* is usually lower than for phenotypes with G143A, but in American, Canadian and Swedish populations of *A. solani* the contribution of isolates with F129L into reduction of efficiency of azoxystrobin usage against early potato blight has been observed [39, 40].

For more detailed information refer to surveys [7, 11, 12, 41, 42], which are specifically dedicated to resistance mechanisms, and to experimental articles analyzing various mutations related with resistance to DM- [37], Qo-inhibitors and some other fungicides [43-48], as well as the role of ABC-transporters in the processes of their detoxication [49, 50]. It has to be pointed out that accumulation of mutations in natural populations occurs gradually. Furthermore, mutants that are tolerant or completely resistant to fungicides often do not display any phenotypical differences or weakening of pathogenicity or other defects [45, 51]; therefore, loss of treatment efficiency due to development of resistance usually becomes noticeable after resistant strains begin to dominate in the population. Similarly, the populations of *P. infestans*, which are fully resistant to fungicides of acrylanine and carbamate group [29] developed in the territory of potato growing regions.

"Best practices" antiresistant strategy. The realization of a problem of a mounting decline of biological and economical effectiveness of fungicidal products resulted in the development of a strategy of fungicide resistance management, which is based on experience of their most efficient and rational practical application (best practices). The fungicide resistance management is a body of rules of using fungicides and methods of cultivating treated cultures aimed at deceleration of selection of stable pathogen forms and mitigation of the risk of spreading resistance [6, 52]. The essential elements of this strategy are the following: firstly, proper selection of fungicides (if possible, priority is given to the so-called low risk fungicides) and their rational usage (combining preparations with single-site and multisite active agents); secondly, treatment with fungicides with varying mechanisms of actions, so that if a pathogen develops resistance to one of them it would become controlled by a partner preparation with different

mechanisms of action, and periodic rotation of preparation with different mechanisms of action. Furthermore, one must take into consideration the prohibitions to combine or consecutively use of several fungicides, regulations for a summary dose and number of treatments, strict compliance with the instruction for a method of using the preparation and not violate the usage rates listed in it. Also, in fungicide resistance management great value is attached to the selection of resistant varieties, crop rotation (preceding crop should not contribute to infection accumulation) and alternative (non-chemical) plant protection methods, as well as agricultural practices adequate to the region of culture growing to avoid insufficient or excessive irrigation and/or fertilization because both of these factors can contribute to the disease development.

Apparently, for a specific combination of crop-disease-geographical region the tactics of resistance management in scope of strategy description and its efficiency can vary significantly. For instance, in Northern Ireland the introduction of fungicide resistance management in potato growing practices resulted in containing the selection of *P. Infestans* strains, which are resistant to phenylamides [53]: their share in pathogen populations in 1999-2001 reached 76%, and after introduction of fungicide resistance management in 2002 it was reduced after 3 years to 22% [54]. In this region phenylamides are successfully used to combat potato blight, and it is assumed that their application against the background of the antiresistant technology will continue [54]. In a similar manner in 2004-2005 they managed to change the tendency of spreading resistance to azoxystrobin. However, in a number of regions of the UK and USA adherence to a rational scheme of preventive or curative spraying against potato early blight and late blight, although successful for the development of disease at the level not yielding economic damage, at the same time stimulated the selection of strains with hyposensitivity to phenylamides of *P. infestans* or strobilurin in *A. solani* [38]. Furthermore, there are voiced concerns that even if the recommendations of fungicide resistance management are adhered to, the risk of resistance development of certain fungi and oomycetes, especially those affecting perennial plants can remain quite high [55].

However, according to FRAC, the abandonment of modern systemic and contact single-site fungicides from high and medium resistance risk groups, including strobilurins and triazoles, is not deemed successful from the practical point of view, because they ensure highly efficient control of a wide range of diseases and have a number of other advantages [29]. For instance, strobilurins preserve their efficiency with regard to winter wheat leaf blotch pathogen (*Z. tritici*) in Italy [56]. The results of analyses made in 2015 under project EUROwheat (http://agro.au.dk/forskning/internationale-platforme/eurowheat/) also demonstrated that triazoles that have been used in the Northern and Central Europe for more than 35 years to fight Septoria spot (*Z. tritici*), yellow (*P. stri-iformis*) and brown (*P. triticina*) rust successfully protect wheat from these diseases, including Septoria spot, in spite of detecting in it a pathogen of six different mutations of 14α-demethylase gene and intraspecific cross-resistance [57].

Under the circumstances the development of additional approaches seems rather promising, which would allow preserving or even strengthening the protective effect of modern fungicidal preparations with the help of environmentally friendly compounds, and thus reduce their selective action in phytopathogen populations facilitating the selection of resistant forms without increasing the dosages or the number of treatments. Chemosensitization of phytopathogenic fungi to agricultural fungicides can be one of such approaches.

Chemosensitization as an advanced antiresistant strategy of increasing the efficiency of protective effect of fungicides. The term

"chemosensitization" is borrowed from medicine where initially it was introduced to designate an approach preventing resistance of cancer cells to radiation and chemotherapeutic anticancer agents with the help of chemosensitizers — chemical compounds or natural substances. Synergism is often observed in their use, due to which the efficiency of medicines and radiation exposure increases [8, 58].

It turned out that key mechanisms determining the resistance of cancer cells [59] are in many respects identical to resistance mechanisms of microorganisms, including fungi, which are pathogenic for humans. Therefore, chemosensitization began to be actively developed in medicine to overcome the resistance of mycosis pathogens and increase of their sensitivity to antimycotic agents with the help of natural and synthetic compounds nontoxic or insufficiently toxic to completely suppress the development of the mentioned pathogens [60]. In the meantime, the chemosensitization approach can be useful not only for improvement of antifungal chemotherapy of human mycosis, but also to fight phytopathogenic fungi. It could become an essential component of antiresistant strategy of protecting cultivated plants with the help of fungicides because it would facilitate weakening or overcoming the resistance of commercial preparations used presently. However, only isolated attempts have been made so far to apply a similar strategy to plant protection and adapt it to overcome resistance of phytopathogenic fungi to commercial fungicides [8, 61, 62]. Whereas the need to reduce unfavorable environmental effects is taken into consideration, natural compounds or their alternatives degradable in the environment are usually used as sensibilizators.

The natural or synthetic compounds functioning as chemosensitizers are either nontoxic for fungi or posses weak fungitoxicity, which is at least an order lower than that of active agent fungicides. Potentially fungitoxic substances are also very effective sensibilizators (for instance, such secondary plant metabolites as thymol or berberine) used in minimal concentrations, which have insignificant inhibiting (subfungicidal) effect on the pathogen. In other words, during chemosensitization the used concentrations of both fungicide and sensibilizator are such that acting alone these agents are inefficient; however, when used collectively they suppress a pathogen, including levels significantly surpassing the effect of fungicide dosages, to which resistant strains are insensitive [8, 61]. This phenomenon is based on the ability of chemosensitizers to cause various stresses, disrupt cellular structures or otherwise weaken phytopathogenic fungi, thus increasing their sensitivity to antifungal preparations and strengthening their effect. The interaction of chemosensitizers with fungicides can be additive, but in most cases, it is synergetic, which allows significantly reducing effective concentrations of active agents (by one or even two orders) [8, 61]. Moreover, as a result of changes of metabolism weakening the pathogen and caused by chemosensitizers even its resistant forms can become more sensitive to fungicide. Subsequently, the suppression of resistant strains and effectively inhibiting their accumulation can be achieved without increasing the recommended dosages of fungicidal preparation, which enables reducing the risk of selecting resistant forms and their spread in populations of phytopathogenic fungi.

A wide-scale screening of natural compounds aimed at identifying their sensibilizating activity to overcome cancer cell, bacteria and fungi resistance to medicines resulted in identifying target activity of a number of secondary plant metabolites and certain microorganisms. Potential chemosensitizers have been identified in plants among phenolic acids, tannins, terpenoids (including mono-, di- and triterpenes, the saponins), steroids, alkaloids, flavonoids, catechines, as well as in some other groups of secondary metabolites [63] and their synthetic

equivalents [61, 64, 65]. Many of these substances are of interest as components of medicines efficient against resistant hospital strains of mycosis pathogens [8, 66]. As for naturally growing fungi, the ability of cinnamic aldehyde, which is almost nontoxic for basidiomycetes, is known to strengthen its activity manifold versus tinder *Laetiporus sulphureus* in combination with vegetable phenols: eugenol, quercetine and catechine [67, 68].

The mechanisms of action of chemosensitizers responsible for amplification of fungicidal effect are not always clear. Nevertheless, the research of synergism of natural and synthetic compounds with medicinal antimycotic agents summarized in a review of Campbell at al. [8] demonstrated that many of these compounds affect the ability of fungi to respond to stress. This stress can be caused both by environmental factors (for instance, UV radiation, salinity, drought, etc.) and by impact of fungicidal preparations. Using molecular genetics methods in these studies allowed identifying links in the systems of fungal protection against stress, which could become biochemical or structural targets of chemosensitizers. In particular, it was determined that sensitivity of pathogenic yeast and aspergilli to antimycotic preparations can be significantly increased in case of exposure to compounds violating the defenses of these fungi against oxidative and osmotic stress [69-71]. It has also been demonstrated that adding the aforementioned cinnamic aldehyde, which causes osmotic stress, to the cultures of four types of xylotroph fungi synergistically amplifies the inhibition of fungal growth by octyl gallate, a plant phenol, which disrupts the cellular membrane structure [72] and causes apoptosis.

By contrast to medical studies, there are few works, in which metabolites of germs and plants are used for sensibilization of phytopathogenic fungi to fungicides in order to increase the efficiency of the latter, including against resistant strains. So far, these experiments [73-75] have not included treatment of plants infected with resistant strains, and with the exception of two reports [62, 76] are performed during growing of phytopathogenic fungi in the culture. Nevertheless, the authors convincingly proved the presence of synergism when using certain concentrations of these metabolites and their synthetic equivalents, and fruitfulness of chemosensitization approach to increase fungicidal effect against phytopathogens was confirmed.

The first detailed studies of sensibilization of fungi that are saprotrophic and potentially pathogenic for plants via secondary metabolites of plant origin were undertaken by employees of a scientific center of the United States Department of Agriculture, USDA in the state of California. These studies were performed using cultures of several aspergilli, which, although are optional human pathogens, have agricultural significance, and *Penicillium expansum* fungus causing mildew of apples. These experiments showed the efficiency of 2,3-dioxybenzaldehyde (2,3-DOBA), 4-oxybenzaldehyde (4-OBA), thymol and 2,5-dihydroxybenzoic acid as sensibilizators *P. expansum*, certain types of *Aspergillus* and toxigenic strains *A. flavus* (69, 77). It was discovered that in vitro usage of 2,3-DOBA or gallic acid along with strobilurin fungicides kresoxim-methyl and fludioxonil results in synergetic increase of their activity against *A. flavus* and *A. parasiticus* (78). When using *Saccaromyces cerevisiae* with point deletions in *sodA* gene encoding mitochondrial superoxide dismutase (Mn-SOD) as mutant models it was determined that salicylic aldehyde, gallic, ascorbic and chlorogenic acids make *A. flavus* more sensitive to kresoxim-methyl because they disrupt the functioning of metabolic fungus systems responsible for protection from oxidative stress caused by this fungicide [79]. The increase of fungicidal activity of fludioxonil towards *A. flavus* was demonstrated for berberine (barbery alkaloid) and certain phenol compound that also affect the antioxidant fungal systems [69]. It was

determined that aldehyde activity of salicylic acid and other benzole analogues was based on inhibiting HOG1-signal system controlling the defense against osmotic stress of *A. flavus* and *P. expansum* [64, 70, 79]. Furthermore, as exemplified by kojic acid produced by many filamentous fungi it was demonstrated that, in spite of the common mechanism of action, some of the sensibilizators can be species- and/or strain-specific [80, 81]. To summarize it was demonstrated that alkylgallates are capable of amplifying the sensitivity to fludioxonil in resistant strains of *P. expansum* [73].

Generally speaking, as a result of studies conducted by aforementioned group of American scientists understanding was gained that presently known sensibilizators attack those paths of fungal metabolism that control their defense response to oxidative stress, and amplify it by provoking generation of reactive oxygen species toxic for fungi and disrupt the integrity of cellular and vacuolar membranes causing osmotic stress and apoptosis. However, the significance of these mechanisms was so far demonstrated only for a small number of fungal pathogens, and many aspects of phytopathogenic chemosensitization to agricultural fungicides remain unstudied [8].

By virtue of studies conducted in the recent years, the list of fungi whose sensitivity to strobilurins and triazoles can be multiplied with the help of chemosensitization by natural metabolites and their analogues includes commercially significant phytopathogens [61, 62, 74-76]. For some of them, the long-term benefits of using this strategy to amplify the protective effect of triazoles were demonstrated on plants, including field conditions [62, 76]. Furthermore, a possibility in principle of overcoming the resistance of natural strains of wheat Septoria spot pathogen to these fungicides [75] was demonstrated.

It was determined that in vitro azoxystrobin (Quadris® KC 25 %, Syngenta AG, Switzerland) in combination with concentrations of thymol (monoterpene phenolic derivative of one of the aromatic compounds of thyme) not toxic for fungi and non-phytotoxic caused a much higher inhibition of growth of *Bipolaris sorokiniana*, *Phoma glomerata*, *A. alternata* and *Parastagonospora nodorum* (= *Stagonospora nodorum*) than in the case of its individual application. The effect of difenoconazole used together with thymol is also significantly amplified with regard to *B. sorokiniana* and *P. nodorum*, whereas fungitoxicity of DIVIDEND® KE 3 % (difenoconazole; Syngenta AG, Switzerland) for *B. sorokiniana* increased tenfold. The application of tebuconazole with 4-OBA, 2,3-BODA or thymol was accompanied by amplification of the inhibiting effect of fungicide on *A. alternata*. The introduction of tebuconazole alone in the growth medium of this fungus (in the form of fungicide Folicur® KE 25 % (Bayer AG, Germany) or only thymol in corresponding 0.5 and 10 ppm concentrations resulted in insignificant suppression of mycelium growth, whereas in case of their combination in the same concentrations the inhibition reached 50%, which exceeded the expectation cumulative effect almost twofold. The same tendency was observed for *Fusarium culmorum* during combination of the same fungicide with 4-OBA [61].

The compounds capable of increasing sensitivity of phytopathogenic fungi to fungicides were detected not only in plants. The examples of using metabolites of microorganisms as sensibilizators can be the experiments where they were used to amplify sensitivity of three wheat pathogens (*P. nodorum*, *B. sorokiniana* and *F. graminearum*) to four DMI-fungicides. For instance, recently we demonstrated the sensibilization of pathogen of common root rot and cereal mottle leaf (*B. sorokiniana*) to tebuconazole with the help of 6-demethylmevinolin (6-DMM), which *P. citrinum* produces. As the triazoles, 6-DMM inhibits strain biosynthesis; however, unlike DMI-fungicides it affects 3-oxy-3-methyl glutaryl-CoA-reductase responsible for one of the earlier stages of formation of steroid com-

pounds. In these experiments a range of combinations of fractions of Folicur® KE 25 % and 6-DMM was determined, which produced the synergism effect indicative of significant increase of sensitivity of pathogen to fungicide. In case of combined application of substances in the most effective combination of fractions of this range, complete suppression of growth of fungal colonies was achieved, whereas sensibilizator and fungicide, taken individually in the same concentrations gave rise only to 10-20% growth inhibition [74].

Furthermore, in exometabolites of *F. sambucinum* isolate, which is non-pathogenic for wheat, sensitizing activity was first identified with regard to *P. nodorum* during in vitro testing along with tebuconazole, which consisted not only in amplification but also in prolonging the fungicidal effect [61]. Subsequently, these metabolites were successfully used in greenhouse studies to amplify sensitivity of this pathogen to Folicur® BT KE 225 (active agents tebuconazole and its derivative triadimefon). The results of subsequent field tests showed that mutual treatment of plants with culture liquid filtrate *F. sambucinum* and this fungicide containing sensibilizators permits the reduction of its dosage as compared with the recommended fivefold without loss of protective potency and fungicidal effect against Septoria spot pathogen [76]. Whereas for wheat the condition of flag-leaf is critical for the crops, it is important that in tests with artificial inoculation of plants the fungicide treatment at the tillering stage along with the culture liquid filtrate better protected the flag-leaf during the entire vegetation period than fungicidal treatment. For plants sprayed with Folicur® BT KE 225 in usual dosage the average affected area of flag leaves was 12-15 smaller than for those that were not treated. If the fungicide dosage was one fifth of the norm, its individual application resulted in a 5-7-fold reduction of the total affected area, whereas after the usage of the same dosage of the preparation in combination with the culture liquid filtrate this figure decreased by almost 40 times. Based on sensibilizators from *F. sambucinum* a composite preparation was developed for plant protection [82].

It has been proven that the other active sensibilizators of microbial origin are cyclic lipopeptides (Iturin A, fengycin and surfactin) of one of the strains (JCK-12) of soil-inhabiting *Bacillus amyloliquefaciens* bacterium. The synergism between the essence containing them and triazoles (difenoconazole and tebuconazole), as well as fungicides with the other mechanisms of action (fludioxonil and benomyl) resulted in noticeable amplification of *F. graminearum* colony growth inhibition. The lipopeptides themselves did not display fungicidal activity until concentrations reached 30 ppm; however, the inhibiting effect of their mixtures with fungicides for fungal conidia germination was of synergetic nature. The greenhouse and field treatment of plants using the preparation based on cultural JCK-12 broth showed that its mutual usage with Almuri® fungicide (active agents difenoconazole and propiconazole; Syngenta, Korea) can significantly improve the efficiency of protecting wheat with this dual formulation [62]. The authors presume that increased sensitivity of the fungus to fungicides under the impact of JCK-12 can be the result of cell wall damage and change of permeability of cell membrane *F. graminearum* due to the mix of bacterial lipopeptides.

In terms of synthetic compounds, we have recently identified certain chemosensitizing activity in vitro in some phosphoanalogues of natural amino acids in tests with *P. glomerata*, *A. alternata* and *F. culmorum* [65]. For example, we have checked several structural analogues of amino acids inhibiting biosynthesis of polyketide mycotoxins, which in some fungi play an important role in pathogenicity. The chemosensibilization effect and synergism with tebuconazole (Folicur® KE 25 %) was identified both for nonfungicidal and for subfingicidal concentrations of these compounds. Whereas the structure of these analogues of

natural substances is known and is not very complex, its gradual modification with simultaneous definition of target activity could result in synthesis of relatively cheap and environmentally friendly preparations to increase fungicidal sensitivity of phytopathogenic fungi. The success of this concept would have become a major confirmation of an assumption about prospects of using synthetic compounds as chemosensitizers [8] commercial interest to which is still held back because of rather high production costs.

Finally, we managed to obtain new experimental evidence of efficiency of natural chemosensitizers against resistant forms of phytopathogens, and, specifically, against natural mutant of *P. nodorum* resistant to Dividend® (3% difenoconazole), sensitivity of which to this triazoles was successfully increased with the help of thymol [75]. When culturing the pathogen in media with sublethal concentrations of difenoconazole, a sector was identified in one of the colonies, which grew more actively than its other sectors. The inhibition of clone growth isolated from this sector in presence of the fungicide turned out almost twice weaker than for wild isolates. PNm1 strain resistant to Dividend® was selected on media with the increasing concentrations of fungicide, and it was determined that its resistance was attributable by the genetic mutation. The application the fungicide together with thymol resulted in statistically significant reduction of mutant strain resistance to the level corresponding to sensitivity of nonresistant parent isolate, whereas growth inhibition after adding difenoconazole together with the chemosensitizer to the medium exceeded the expected additive effect of these substances when used separately [75].

In conclusion of the discussion about resistance of phytopathogenic fungi to fungicides, we would like to point out that unlike medical antimycotic agents that should ensure complete destruction of the pathogen, the methods of plant protection are rather designed to attenuate the pathogen population development as long as possible at the level below the threshold of economic harmfulness. In this regard, the ability of certain chemosensitizers to prolong the fungicidal effect [61] is of particular interest.

Therefore, due to potentiation of fungicides, if they are used in combination with the sensibilizators, pathogens can be reliably controlled with a significant decrease of dosages and expenditure of preparations without loss of their fungicidal effect or even with an increased fungicidal effect. This suggests that using the chemosensitization approach in integrated crop protection could make protective measures more cost efficient and successful, inter alia, against resistant forms of phytopathogenic fungi. An important factor can be the affordability of many sensibilizators of natural origin. Analysis of the research in general shows prospects of development of efficient, environmentally compatible and biodegradable chemosensitizers for use in combination with fungicides.

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