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NOVEL HYBRID MODULATORS OF PLANT IMMUNE RESPONSES BASED ON CHITOSAN AND BIOACTIVE ANTI-OXIDANTS AND PRO-OXIDANTS

E.V. POPOVA ✉, N.S. DOMNINA, C.V. SOKORNOVA, N.M. KOVALENKO, S.L. TYUTEREV

All-Russian Research Institute of Plant Protection, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail elzavpopova@mail.ru (✉ corresponding author), ninadomnina@mail.ru, mymryk@gmail.com, nadyakov@mail.ru, mail@vizr.spb.ru
Popova E.V. orcid.org/0000-0003-3165-6777
Domnina N.S. orcid.org/0000-0002-7124-899X
Sokornova C.V. orcid.org/0000-0001-6718-4818
Kovalenko N.M. orcid.org/0000-0001-9577-8816
Tyuterev S.L. orcid.org/0000-0003-2397-9656
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Abstract

During the infection of plants with pathogens, the equilibrium between oxidative processes and antioxidant activity shifts toward an increase in the formation of ROS, which affects the course of the development of the disease and manifests itself differently in phytopathosystems of various types of parasitism. So, to protect plants from biotrophic pathogens, ROS generation is necessary, but the resulting high level of ROS does not protect, but, on the contrary, favors the pathological process caused by necrotrophs. Substances that can control not only ROS generation, but also their utilization, can become effective immunomodulators to protect plants when infected with pathogens of different lifestyles. For the first time, chitosan-based hybrid immunomodulators (Chit-Van+SA) were created, in the structure of which there are fragments of biologically active substances that have antioxidant and prooxidant effects. The basis of such systems is chitosan (Chit), containing covalently attached vanillin (Chit-Van), which reduces the ROS level by stimulating cellular antioxidant activity (antioxidant effect), and salicylic acid (Chit+SA), which is associated with a labile ionic bond, generating ROS (prooxidant act). It was shown that (Chit+SA) increases the wheat resistance to *Puccinia recondita* Roberge ex Desmaz f. sp. *tritici* biotroph and *Cochliobolus sativus* Drechs hemibiotroph, while (Chit-Van) effectively induces resistance only to *C. sativus* hemibiotroph. The effectiveness (Chit+CA) as an inducer of resistance is 1.2-2.0 times higher than the inducing effect of chitosan itself (Chit). It was found that hybrid immunomodulators (Chit-Van+SA) with respect to both the biotroph (brown rust) and the hemibiotroph (brown spot) with a molar ratio of vanillin and salicylic acid in them in the range 1:1-1:2 show a high immunomodulating activity, regardless of the molecular weight of chitosan, which is expressed in a decrease in the area of leaf damage to 5-10 % of the control. The results obtained indicate that the biological effectiveness of the hybrid polymer system (Chit-Van+SA) as an immunomodulator correlates with the content of SA in them, which confirms the participation of the signal salicylate system in the induction of resistance. It is established that specific enzymatic antioxidants superoxide dismutase, catalase and peroxidase, which regulate the relationship between oxidative processes and antioxidant activity, play an important role in the development of induced resistance when using the created immunoinductors. In general, the results of studying the effect of the hybrid immunomodulators (Chit-Van+SA) on the activity of antioxidant enzymes in the model system wheat—*C. sativus* suggest that the increase in plant resistance to pathogens is realized through the control of the intensity of redox processes in plants, which is caused by the presence in the structure of hybrid immunomodulators of a certain pro- and antioxidant balance.

Keywords: *Puccinia recondita, Cochliobolus sativus*, chitosan, induced resistance, salicylic acid, vanillin, wheat, dark brown spotting, brown rust

Increasing grain production of spring soft wheat remains an urgent task, since this crop occupies up to 70% of the grain area. Every year, in different regions of Russia, an unfavorable phytosanitary situation is noted on grain crops, which negatively affects the quantity and quality of the crop. The most common and harmful leaf diseases of common wheat (*Triticum aestivum* L.) include brown rust (pathogen *Puccinia recondita* Roberge ex Desmaz f. sp. *tritici*) and brown spot (pathogen *Cochliobolus sativus* Drechs) [1, 2]. To protect plants from diseases, chemical methods are mainly used, among which treatment with fungicides prevails, which are highly effective, but at the same time have a negative impact on the environment [3]. In this regard, the development of agricultural technologies using the method of induced resistance, based on the activation of innate defense mechanisms of plants and the formation of resistance to pathogens in plants, is of particular relevance. The inducers of plant disease resistance created on the basis of these principles are an ecologically safe alternative to fungicides used both abroad and in Russia [4-6].

The development of molecular biology methods has led to a more complete understanding of the mechanisms of plant defense against pathogens and the identification of the role of various signaling systems in the development of defense reactions leading to a coordinated biochemical and physiological defense response to infection with a pathogen [4, 7-10]. The ability to control the pathological process is associated with the ability of inducers to direct metabolism in a direction unfavorable for the pathogen [11-13]. In this case, the formation of a protective response is due to the expression of genes under the action of signaling molecules [10, 14-16] and exogenous biologically active substances (BAS) – immunomodulators [7, 11].

Numerous studies have convincingly shown that in response to the action of factors of different natures, the pro/antioxidant balance is shifted, acting on which, with the help of BAS, it is possible to activate the genetic potential of protective reactions and form an immune response. In this regard, the hypothesis has recently become increasingly popular, according to which the adaptation of plants to the action of stressors of various natures largely depends on the functioning of the antioxidant system. It is known that infection of plants at the initial stage is accompanied by a sharp increase in the intensity of oxidative processes, expressed in the generation of reactive oxygen species (ROS) (oxidative burst), which include a chain of subsequent protective reactions [16-19]. ROS include superoxide radical anion, hydroxyl radical, hydrogen peroxide (H_2O_2), and nitric oxide. ROS play a dual role in the interaction of the host with the pathogen. First, they have a direct antimicrobial effect, strengthen the barrier properties of cell walls through the polymerization of phenolic compounds [20, 21]. Second, ROS, as signaling molecules, are involved in the formation of acquired systemic resistance to pathogens, regulating gene expression, the activity of defense systems [22, 23] and including the expression of genes of pathogenesis-related proteins, the synthesis of phytoalexins, and the triggering of the hypersensitivity response. It should be noted that among ROS, the role of hydrogen peroxide, the longest-lived transmembrane signal in plant cells, has been most studied [18, 19].

According to the currently available data [23-27], plant protection from biotrophic pathogens is aimed at generating ROS, while a high ROS content favors the pathological process caused by necrotrophs. In the case of hemibiotrophic pathogens characterized by a combined feeding strategy, information on the role of ROS is often contradictory and depends on the host-pathogen system [25, 26, 28]. It is assumed that the pathogen passes through the biotrophic and necrotrophic phases of development at different concentrations of hydrogen peroxide [26]. The important role of the balance between the formation and degradation of ROS (for example, H_2O_2) in the formation of plant defense reactions and their survival under stresses of various natures has been reported in many publications [26, 28, 29].

It is believed that maintaining the concentration of ROS already formed in the cell at a sufficient level is possible due to the regulation of the activity of antioxidant enzymes [30-32]. Note that studies aimed at identifying the role of antioxidant enzymes in the formation of plant resistance to stress factors are being carried out quite intensively both in Russia and abroad [30, 31, 33]. It is known that under a strong infectious load, its own antioxidants cannot neutralize the overproduction of ROS, which leads to cell death and the development of necrosis. Therefore, an increase in the antioxidant activity of plants should increase their tolerance to the development of necrosis caused by pathogens or abiotic stresses [31-33]. Thus, by influencing in a certain way the induction of ROS and antioxidants, it is possible to regulate the susceptibility and resistance of plants to pathogens. To control these processes, it is advisable to use immunomodulators capable of controlling not only the generation of ROS but also their utilization. Despite the fact that intensive research on the development of effective immunomodulators is actively continuing all over the world, there is no information about the creation of inductors with the help of BAS of different natures (prooxidant and antioxidant) with the directed regulation of the formation of induced plant immunity, in particular when wheat is infected with pathogens with different types of nutrition (necrotrophic and hemibiotrophic). In this regard, it is important to obtain new inductors capable of regulating the relationship between oxidative processes and antioxidant activity in plant tissues when wheat is infected with pathogens with different trophicity.

We were the first to create hybrid immunomodulators based on chitosan, the structure of which contains fragments of BAS (vanillin and salicylic acid SA) that have anti- and prooxidant effects. The resulting hybrid immunomodulators are effective against pathogens of dark brown spot and brown rust of wheat (hemibiotroph and biotroph, respectively). It was shown that their modulating effect was manifested through the effect on the activity of the main antioxidant enzymes of plants – superoxide dismutase (SOD), catalase (CAT), and peroxidase (PO) during the development of induced resistance.

The purpose of this study was to develop immunomodulators – chitosan derivatives with prooxidant and antioxidant activity and to assess their effect on wheat resistance to brown spot (hemibiotroph *Cochliobolus sativus* Drechs) and to leaf rust (biotroph *Puccinia recondita* Roberge ex Desmaz f. sp. *tritici*).

Materials and methods. To create immunomodulators, chitosans with a molecular weight of 6.5, 60, and 100 kDa (Chit) were obtained by the method of oxidative destruction [34] from chitosan with a molecular weight of 150 kDa and a degree of deacetylation of 85% (Bioprogress, Russia). Chitosan derivatives containing 25 wt% of covalently bound vanillin fragments (Van) – (Chit-Van) were synthesized through a Schiff base followed by reduction with an excess of sodium borohydride [35]. The amount of introduced vanillin fragments was determined by UV spectra (spectrophotometer UV-2600, Shimadzu, Japan) at λ = 280 nm [35]. The ionic nature of the bond between chitosan and SA in chitosan derivatives (Chit+SA) (salt formation) was confirmed on the basis of IR spectra (Spectrum BX spectrometer, Perkin Elmer, Inc., USA) by the presence of characteristic bands 1552.92 and 1386.12 cm^{-1} from the COO– carboxylate group, as well as a wide band in the region of 3100-2600 cm^{-1}, reflecting stretching vibrations from the NH_3^+ and OH functional groups [36].

Chitosan, containing simultaneously covalently attached vanillin and ionically bound SA, is a hybrid system (Chit-Van+SA). The synthesis of such systems was carried out by introducing SA into (Chit-Van) containing 25 wt% of vanillin, varying the amount of SA from 1:0.5 to 1:2 with respect to the attached vanillin.

The inducing activity of the test compounds was assessed by the detached leaves method [37]. Seven-day-old wheat seedlings (*Triticum aestivum* L.) of the susceptible cultivar Saratovskaya 29 were sprayed with 0.1% (in terms of chitosan) solutions of immunomodulators 24 h before inoculation with the pathogen − hemibiotroph *C. sativus* (4000 spores/ml) or biotroph *P. recondita* (2000 pustules/ml). In each variant of the experiment, 100 seedlings were used. Leaf infection was assessed on the 4th day after infection with *C. sativus* and on the 7th day after infection with *P. recondita* according to the intensity of the disease development (the degree of damage to the leaf area) according to the description [37]. In the control, plants were treated with water. The infection of wheat leaves in the form of brown spots during infection with *C. sativus* and uredopustules during infection with *P.* recondita was taken as 100% in the control. The immunomodulatory activity of the test substances was assessed as a percentage as the extent of damage of plant leaves by the pathogen in relation to the control.

The effect of hybrid systems (Chit-Van+SA) (1:2) and SA on enzyme activity was assessed by analyzing samples of wheat leaves taken before infection and on days 1, 2, 3, and 4 after *C. sativus* infestation. Variants included plants without infection and treatments (control) and plants treated 24 h before infection with *C. sativus* with 0.1% solution (Chit-Van+SA) or 2 mM SA solution (experiment). To determine the enzyme activity, a weighed portion of 20 leaves was ground in 0.05 M phosphate buffer (pH 6.2) at a ratio of 1:5, extracted for 30 min at 4 °C, then centrifuged for 10 min at 8000 g (Eppendorf 5415R microcentrifuge, Eppendorf, USA).

CAT activity in wheat leaves was determined spectrophotometrically [38] on days 1, 2, 3, and 4 after infection with *C. sativus*, PO activity − colorimetrically according to Boyarkin [39]. SOD activity was assessed by a method based on measuring the inhibition of photochemical reduction of nitro blue tetrazolium [40].

All experiments were performed in 3 biological and 3 chemical replicates.

Analysis of variance was used for data processing (Statistica 6.0 (StatSoft, Inc., USA) and Excel 2016. In the calculations, the methods of parametric statistics were used (based on the mean *M* and their standard errors ±SEM, 95% confidence intervals, the least significant difference (LSD) at $p < 0.05$).

Results. Analysis of the literature data suggests that the effectiveness of disease resistance inducers is determined by their ability to change the conditions of pathogen development in a plant at the stage of its introduction by regulating redox processes, which are the main ones in the dynamics of pathogenesis. In accordance with this approach, the created inducers of disease resistance should be compounds with multidirectional functions (hybrid) and contain two active centers in the structure: one will facilitate the generation of ROS, the other, on the contrary, will inactivate them. In addition, it is necessary to know the mechanism of the introduction and spread of the pathogen in plants. For example, for a biotroph, it is necessary to limit the time of its propagation, while for a hemibiotroph, it is desirable to increase the duration of residence in living plant tissues. In this regard, when interacting with a pathogen, the time order of the release of the administered BAS from the immunomodulator must be observed.

To create innovative hybrid immunomodulators, the authors have chosen the natural polysaccharide chitosan. Chitosan and preparations based on it are widely used in plant protection against diseases as inducers of nonspecific resistance [41-45]. In addition, chitosan attracts the attention of researchers by the presence of reactive functional groups, which make it possible to carry out various chemical modifications that enhance or impart new biological properties to this polymer [42, 46]. A classic inducer of disease resistance, SA, which plays a central role in plant protection from biotrophic pathogens, was used as a BAS with prooxidant properties [14, 47, 48]. Among the BAS with antioxidant properties (usually phenolic compounds of natural and synthetic origin, for example, lilac, cinnamic acids, vanillin, etc.), vanillin was chosen.

Thus, the hybrid immunomodulator is chitosan, into the structure of which SA, which provides prooxidant activity, and vanillin, which has antioxidant properties, have been introduced. Due to the presence of a labile bond between chitosan and SA (Chit+SA), SA, an intense inducer of ROS generation, will be released first from the hybrid system [14, 15, 49, 50]. This effect is necessary at the initial stage of infection, when an increased concentration of ROS will promote necrosis and thereby limit the development of the biotroph. In the case of hemibiotrophic pathogens, for which the process of necrosis is favorable, sporulation requires, on the contrary, a decrease in the ROS content due to the work of the antioxidant system, which will lead to an increase in the duration of the stay of *C. sativus* necrotroph in living tissues at the biotrophic stage of its development. It is in this later process that the exogenous antioxidant vanillin, bound to chitosan by a strong covalent bond and gradually released under the action of enzymes, should be included.

When creating hybrid immunomodulators, the authors took into account the previously established [51] concentration dependencies of the inducing activity of SA and vanillin in relation to the hemibiotrophic pathogen *C. sativus*.

1. Wheat resistance to brown spot (*Cochliobolus sativus* Drechs) **and brown rust** (*Puccinia recondita* Roberge ex Desmaz f. Sp. *tritici*) **when exposed to low molecular weight** (6.5 kDa) **chitosan and its derivatives based on bioactive substances** (BAS) (method of separated leaves, cv. Saratovskaya 29)

Treatment	BAS, mM	Van:SA, mg/mg	Leaf damage, % of control	
			brown spot (LSD_{05} = 4.5)	brown rust (LSD_{05} = 9.0)
Control			100[a]	100[a]
Chit			25[a]	25[a]
Ван	1.5		60[a]	100[a]
СК	0.5		40[a]	45[a]
СК	1.0		50[a]	35[a]
СК	2.0		45[a]	30[a]
Chit-Van	1.5		15[a]	50[a]
Chit+SA	0.5		20[a]	10[a]
Chit-Van+SA	2.2	1:0.5	20[a]	40[a]
Chit-Van+SA	3.0	1:1	10[a]	15[a]
Chit-Van+SA	4.5	1:2	5[a]	10[a]

N o t e. The molecular weight of the initial Chitosan (Chit) is 6.5 kDa, BAS: vanillin (Van) and salicylic acid (SA). The concentration of the used sample solutions is 0.1% (based on chitosan).
Δ[a] — significant differences in the treatments compared to the control.

Table 1 shows that the original chitosan with a molecular weight of 6.5 kDa has good inducing activity, reducing the infection of wheat plants with leaf rust and brown spot to 25% in relation to the control. The presence of ionic-bound SA in chitosan increases its efficiency as an inducer in comparison with the initial chitosan, which is consistent with the literature data [20]. Treatment of wheat with a derivative (Chit-Van) reduced the infestation of leaves with brown spot by more than 6 times compared with the control. In the experiment with brown rust, the infection of leaves in wheat plants treated with the same derivative decreased by 2 times in comparison with the control. The results of studying three-component hybrid immunomodulators (Chit-Van+SA) showed that their inducing activity depended on the molar ratio of vanillin and SA. The highest inducing activity was observed for samples containing vanillin and SA in a molar ratio of 1:1 or 1:2. A further increase in the proportion of SA in the molar ratio to vanillin (1:3) in the hybrid derivative led to its phytotoxicity. The sample (Chit-Van+SA) containing vanillin and SA in a molar ratio of 1:0.5 was even less effective as an immunomodulator than the original chitosan. The results obtained indicate that the SA concentration determines the biological activity of the hybrid system as an immunomodulator.

There is no unambiguously accepted opinion in the literature on a possible correlation between the molecular weight of chitosan and its inducing activity. The range of molecular weight values exhibiting high activity as an inducer of disease resistance is quite large, from 2 kDa to 300 kDa [4, 52]. In this regard, the authors carried out a comparative study of the inducing activity of hybrid immunomodulators obtained on the basis of chitosan with different molecular weights. Table 2 shows data on the assessment of the effect of chitosans with different molecular weights and their derivatives containing vanillin and/or SA on the resistance of wheat to brown spot and leaf rust.

2. Brown spot (*Cochliobolus sativus* Drechs) **and brown rust** (*Puccinia recondita* Roberge ex Desmaz f. sp. *tritici*) **infestations** (% of control) **of wheat** (*Triticum aestivum* L.) **leaves when exposed to chitosan with different molecular weights and its derivatives based on bioactive substances** (method of separated leaves, cultivar Saratovskaya 29)

Treatment	Chit 6.5 kDa		Chit 60 kDa		Chit 100 kDa	
	1	2	1	2	1	2
Control	100	100	100	100	100	100
Chit	30	20	25	20	20	15
Chit+SA	20	10	20	10	15	5
Chit-Van	15	40	15	25	10	35
Chit-Van+SA (Van:SA 1:2)	5	10	5	5	< 5	5

N o t e. Chit — Chitosan, Van — vanillin, SA — salicylic acid; 1 — brown spot (LSD_{05} = 5.0), 2 — brown rust (LSD_{05} = 8.0). The concentration of the used sample solutions is 0.1% (based on chitosan).).

Among chitosans with different molecular weights, chitosan with a molecular weight of 100 kDa had the highest efficiency as an inducer of disease resistance. In this variant of the experiment, the area of damage to the leaves of wheat *C. sativus* was only 20%, *P. recondita* – 15%.

Regardless of the molecular weight, chitosan containing SA (Chit+SA) in all variants of the experiment showed a higher immunomodulatory activity in comparison with unmodified chitosan, reducing the area of damage to leaves of *C. sativus* to 15-20%, and *P. Recondita* – to 5-10 % relative to the control. The inducing activity of chitosans with different molecular weights, containing only vanillin (Chit-Van), was significantly lower than that of unmodified chitosans in protecting wheat from brown rust and, on the contrary, higher in the case of brown spot. An innovative immunomodulator (Chit-Van+SA), regardless of the molecular weight of chitosan, effectively increased the resistance of wheat plants to leaf rust and dark brown spot, reducing the area of leaf damage to 5-10% of the control.

Thus, it has been experimentally confirmed that the high immunomodulatory activity of hybrid systems with a molar ratio of vanillin and SA in the range of 1:2 does not depend on the molecular weight of chitosan.

As noted above, antioxidant enzymes, primarily CAT, PO, and SOD, play an important role in the regulation of the amount of ROS in the cells of plants infected with the pathogen [28-31]. In order to assess the participation of antioxidant enzymes in the formation of induced resistance to brown spot in wheat seedlings under the action of a hybrid immunomodulator (Chit-Van+SA) containing vanillin and SA in a molar ratio of 1:2, the authors studied the CAT, PO, and SOD activity in the model system wheat—hemibiotroph of *C. sativus* when treated with this drug.

Analysis of the dynamics of changes in the activity of these enzymes in infected wheat seedlings showed that the process of infection of wheat plants with the causative agent of brown spot *C. sativus* was accompanied by an increase in the activity of all antioxidant enzymes relative to control uninfected plants throughout the experiment. By the time of the strong development of the disease (day 4), when the entire leaf tissue was necrotic, their activity gradually decreased (Fig. 1, 2).

The experimental results revealed temporary differences in the increase in the activity of antioxidant enzymes during the development of the disease (see Fig. 1). On the 1st day, a sharp increase in the activity of PO, CAT and SOD was observed in the leaves of wheat infected with the pathogen, with a gradual decrease in their activity on day 4. The maximum increase in PO activity was observed on the 3rd day after infection, when clear signs of the disease appeared in the form of brown spots. As is known, the initial stage of plant infection is accompanied by the activation of the ROS generation system, and, first of all, a superoxide anion radical is generated, which is reduced by SOD to hydrogen peroxide. This enzyme forms the front line of defense against ROS, catalyzing dismutation of the superoxide radical anion to H_2O_2, which leads to a significant increase in the activity of the enzyme. Hydrogen peroxide, being a CAT substrate, in turn induces an increase in the activity of this enzyme, which contributes to its increase on day 1 of infection. On day 2 of the development of the disease, the continuing increase in SOD activity led to further accumulation of hydrogen peroxide and was already accompanied by a significant increase in PO activity (day 3), which, along with CAT, was included in the control over the amount of hydrogen peroxide formed.

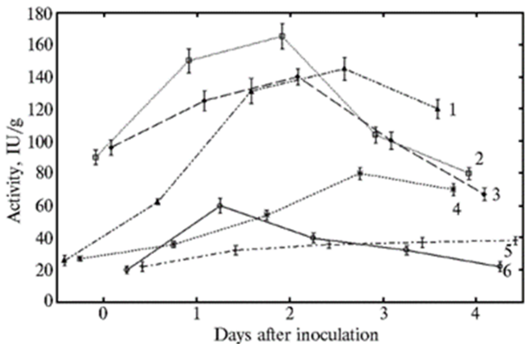


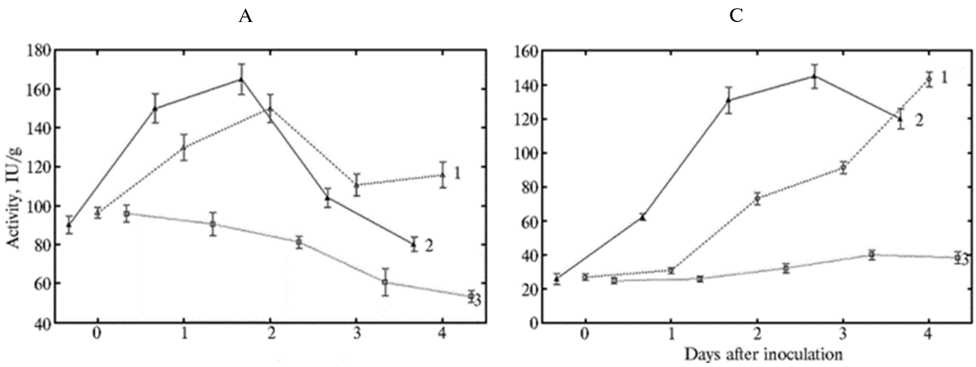
Fig. 1. Activity of antioxidant enzymes in wheat (*Triticum aestivum* L.) leaves infected with *Cochliobolus sativus* Drechs, when exposed to a hybrid immunomodulator based on chitosan (Chit) **in combination with vanillin** (Van, covalent bond) **and salicylic acid** (SA, ionic bond)**:** 1 — peroxidase, 2 — superoxide dismutase, 6 — catalase (controls without Chit-Van+SA treatment); 3 — superoxide dismutase, 4 — peroxidase, 5 — catalase (with Chit-Van+SA treatment). *M*±SEM, the differences are statistically significant at $p < 0.05$ (lab test, cv. Saratovskaya 29).

Thus, an increase in the activity of SOD, CAT, and PO, observed in the initial asymptomatic biotrophic period (1-2 days) after infection of *C. sativusplants*, indicates a sharp increase in the amount of ROS and the involvement of antioxidant enzymes in the regulation of ROS accumulation in response to the introduction of a pathogen.

A gradual decrease in the activity of antioxidant enzymes with the development of the disease (4th day) and the transition of the pathogen to the necrotrophic phase led to an increase in oxidative processes, which in intensity exceeded the level of activity of the plant antioxidant system. As a result, intensive necrosis was initiated in the plant, which created a favorable habitat for the pathogen *C. sativus*. The development of the disease during this period is visually manifested in the form of brown spots on the leaves. The consequence of this is an increase in the susceptibility of plants to brown spot, which corresponds to the literature data [53].

Treatment of wheat plants with a hybrid system (Chit-Van+SA) upon infection with *C. sativus* hemibiotroph decreased the activity of antioxidant enzymes, although the dynamics of its change remained the same as in control (untreated) infected plants. Thus, although the CAT activity increased on day 1 of infection, it was significantly lower than in the infected control plants (see Fig. 1). The PO activity throughout the entire experiment was also significantly lower than that of the infected control plants. It should be noted that (Chit-Van+SA) insignificantly reduces the SOD activity at the first time (from the 1st to the 3rd day), while the dynamics of changes in activity remain the same as in infected plants. The latter means that due to increased SOD activity, superoxide anion-radicals are inactivated and peroxide accumulates in tissues within 1-2 days after infection. In turn, a decrease in CAT and PO activity in plants treated with (Chit-Van+SA) also leads to an increase in the amount of peroxide required to neutralize the phytopathogen in tissues and to restrain its spread during the biotrophic stage. Defense reactions are switched on, leading to the development of induced resistance to brown spot of wheat plants, which is manifested in a decrease in the development of the disease to 5% of the control.

It should be noted that the peculiarity of the created hybrid system is the presence in the working solution of the immunomodulator of SA, which is easily cleaved from chitosan, which suggests its participation in the manifestation of the inducing activity of immunomodulators at the initial stage of plant infection.



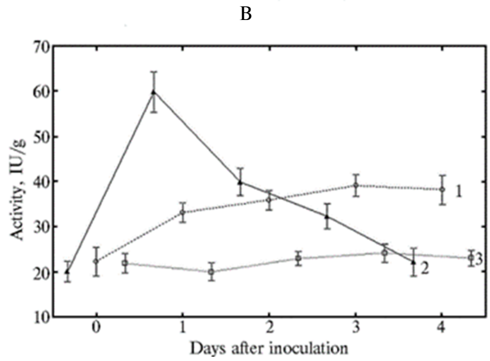


Fig. 2. Activity of superoxide dismutase (A), **catalase** (B), **and peroxidase** (C) **in leaves of wheat (*Triticum aestivum* L.) infected with *Cochliobolus sativus* Drechs when exposed to salicylic acid:** 1 — infected treated leaves, 2 — infected untreated leaves, 3 — uninfected untreated leaves (controls). *M*±SEM, the differences are statistically significant at $p < 0.05$ (lab test, cv. Saratovskaya 29).

To assess the role of SA in the inducing activity of a hybrid immunomodulator, we studied its effect on the activity of antioxidant enzymes upon infection of wheat with *C. sativus* hemibiotroph (see Fig. 2). It was found that the activity of SOD under the action of SA, although it decreased, was insignificant and remained high on the first day of the experiment. Similarly (as in the variant with wheat pretreatment Chit-Van+SA), in plants treated with SA and infected with the causative agent of brown spot, the activity of CAT (day 1) and PO (day 3) decreased 2-fold. That is, there is an analogy in the dynamics of changes in the activity of enzymes in plants treated with a hybrid immunomodulator and SA, upon infection with *C. sativus hemibiotroph*. The revealed analogy in the change in the activity of antioxidant enzymes (SOD, CAT, PO) in wheat leaves pretreated with SA and (Chit-Van+SA) indicates the participation of SA as a resistance inducer in the defense reactions of wheat plants activated by (Chit-Van+SA). Hence, it follows that SA, by reducing the activity of antioxidant enzymes, stimulates the accumulation of H_2O_2 in an amount sufficient to activate the protective mechanisms of induced immunity, which is confirmed by the literature data [11, 54]. The observed inducing effect of SA is consistent with the data of phytopathological analysis, which showed that the effect of exogenous SA was manifested in the suppression of disease symptoms for hemibiotroph *C. sativus* up to 45% of the control (see Table 1). According to the literature, the inducing effect of exogenous SA in increasing plant resistance to phytopathogens is due to its ability to inhibit CAT, an enzyme that detoxifies hydrogen peroxide, which leads to the accumulation of ROS [14, 15, 30, 54]. The latter are considered as important signaling mediators in the formation of plant resistance. Taking into account these data, it can be assumed that the high inducing activity of hybrid systems (Chit-Van+SA), in which the Van:SA ratio is 1:1 or 1:2, is associated with the ability of exogenous SA to be first released from the polymer system and to induce the generation of H_2O_2 to an amount that leads to the activation of a whole spectrum of plant defense reactions that inhibit the development of pathogens. This increases the duration of the asymptomatic biotrophic stage of the development of the hemibiotroph and significantly slows down the development of the disease (the lesion is up to 5-10% of the control). The increased content of peroxide during this period also contributes to the formation of resistance to biotroph, and the development of *P. recondita* decreases to 10-15% of the control (see Table 1).

Treatment of plants with an immunomodulator (Chit-Van+SA) (Van:SA 1:0.5) with a smaller amount of exogenous SA (see Table 1) induces the appearance of a smaller amount of ROS. The exogenous antioxidant vanillin in the hybrid (Chit-Van+SA) also reduces the concentration of ROS. In this case, the amount of H_2O_2 decreases to a value that does not allow the development of resistance to the *P. recondita biotroph*, but is sufficient to induce resistance to the *C. sativus* hemibiotroph during the asymptomatic biotrophic period of the pathogen development. The results obtained indicate that the biological effectiveness of a hybrid derivative based on chitosan (Chit-Van+SA) as an immunomodulator correlates with the content of SA in them, which confirms the participation of the signaling salicylate system in the induction of resistance, which is consistent with the data of Sari and Etebarian [55], who, using the example of wheat lesions by the ascomycete *Gaeumannomyces graminis*, revealed the dependence of the effectiveness of SA as an inducer of resistance on concentration.

Thus, the research results suggest that the mechanism of action of new hybrid immunomodulators with covalently attached vanillin and ion-bound SA in the chitosan structure, leading to an increase in the resistance of wheat plants to pathogens with different nutritional strategies, is realized through the control of the activity of antioxidant enzymes (in particular, catalase CAT, peroxidase PO, and superoxide dismutase SOD), regulating the intensity of oxidative stress induced by the introduction of the pathogen. The high inducing effect of the created hybrid systems is apparently associated with the ability of exogenous SA, which is first released from the polymer system, to inhibit CAT and PO. As a result, cells accumulate hydrogen peroxide, which can enhance the expression of genes that determine protection against pathogens. Innovative hybrid immunomodulators as resistance inducers may be of practical interest in protecting plants from pathogens.

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