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BIOLOGICAL BACKGROUND TO USING CHITOSAN INDUCERS TO INCREASE THE EFFICIENCY OF BIOFUNGICIDES

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Abstract

Microbiological preparations form the basis of modern technologies phytosanitary optimization of agroecosystems, therefore, increasing their efficiency in protecting crops from a wide range of plant pathogens is an urgent task of crop production. The All-Russian Research Institute of Plant Protection (VIZR) has developed Vitaplan, a biological product based on the composition of two strains, the *Bacillu subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D with a different composition of active complexes and a mechanism of action that are highly effective against a wide range of plant pathogens. The aim of the study is to substantiate increasing the biological effectiveness of new Vitaplan formulations supplemented with chitosan as an inducers of plant resistance. In this work, for the first time, two new formulations were developed, the Vitaplan, CF + colloidal chitin and the Vitaplan, CF + 0.1 % chitosan salicylate with increased antagonistic and elicitor activity compared to the original biological Vitaplan, CF. The effect of disease resistance inducers, such as colloidal chitin and chitosan salicylate, on the biological activity of Vitaplan has been investigated. The colloidal chitin (1 %) added to the deep culture medium increased the antagonistic activity of *B. subtilis* VKM B-2604D and VKM B-2605D against the tomato bacterium *Clavibacter michiganensis* subsp. Michiganensis (Smith) Davis et al. (strain 101) and the fungus *Alternaria solani* Sorauer, and also ensured effective suppression of the growth of *Cochliobolus sativus* mycelium up to 84.9-88.1 % on day 5 and day 7 of the experiment, which is comparable to the efficiency of Vitaplan CF (80.9-87.5 %, respectively). Chitosan salicylate at a concentration of 0.1 % had a moderate fungistatic activity, with only 36.5-46.0 % suppression of the growth of the *C. sativus* mycelium. The study of the immunomodulatory activity of various Vitaplan CF preparative forms in protecting wheat against the brown spot pathogen *Cochliobolus sativus* (S. Ito & Kurib.) was carried out under various infectious loads. Depending on the infectious load of the pathogen, preliminary spraying of wheat plants with Vitaplan, CF at a dilution of 1:10 followed by infection with the brown spot pathogen reduced the leaf lesion area to 50-80 % compared to 65-100 % in control. Adding 1.0 % colloidal chitin to the culture medium for the producer strains reduced leaf damage to 40-50 %, which indicates a higher immunomodulatory activity of this form of Vitaplan compared to Vitaplan CF at a dilution of 1:10. Chitosan salicylate proved to be an effective inducer of disease resistance, reducing damage to wheat plants by dark brown spots to 10-20 %, depending on the infectious load. The addition of chitosan salicylate at a concentration of 0.1 % to original form Vitaplan, CF at a 1:10 dilution also had a positive effect on the antagonist with a 2.0-2.5-fold increase of its biological activity. The biological effectiveness of the new formulation Vitaplan CF (1:10) + chitosan salicylate (0.1 %) in protecting wheat from brown spot when spraying plants is determined by two mechanisms, i.e., by i) direct pathogen suppression due to antibiotics and enzymes the *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D produce, and ii) through the induction of plant systemic resistance. The chitosan salicylate in the Vitaplan biological increases the inducing activity of this new formulation compared to the original form. Thus, the prospect of combining active selected strains of microbial antagonists of plant pathogens and chitosan complexes to increase the biological efficiency and expand the spectrum of action of drugs has been experimentally confirmed and theoretically substantiated.

Keywords: biological control, *Bacillus subtilis*, Vitaplan formulations, fungistatic activity, antagonistic effect, chitosan, chitin, systemic resistance, *Triticum aestivum* L., *Cochliobolus sativus*

The widespread use of plant protection chemicals and mineral fertilizers formed the base of traditional intensive farming in the 20th century. However, having increased the yield, intensive technologies have led to a phytosanitary deterioration of crops due to appearance of pesticide-resistant populations of pest species, a decrease in product quality, soil degradation and a drop in soil fertility. In this regard, the development of new biological means of protecting agricultural crops from harmful organisms is relevant. Environmentally friendly crop production should ensure a decrease in the chemical load on agroeco-systems, optimization of soil microbial community and restoration of microbiological soil activity.

Microbiological preparations are the main element of technologies for phytosanitary optimization of agroecosystems with relevance to biology. According to numerous studies, bacteria of the genus *Bacillus* Cohn. are one of the most promising for biological control of plant pathogens [1-4].

Biologicals based on *B. subtilis* strains are efficient against plant diseases on the main agricultural crops in Russia and abroad. Bacillus strains suppressed the spread and development of fusarium wilting of maize [5], fusarium ear of wheat [6, 7], fusarium and ophiobolous root rot [8], powdery mildew [9], yellow and brown rust of cereals [10-12]. Biopreparations based on this group of antagonist bacteria significantly reduce the incidence of leaf diseases in rice [13]. In vegetable crops, *B. subtilis* strains are efficient against fusarium and bacteriotic wilting of tomato [14, 15], cucumber root rot [16], and phytophluorosis of pepper [17]. *Bacillus*-based preparations s protect strawberries from rust [18]. A number of reviews focused on obstacles and prospects for the use of *B. subtilis*-based biologicals [19-21].

The effectiveness of controlling the density of populations of plant pathogens when using microbiological plant protection products depends on the biological characteristics of producer strains and is due to a number of factors. These include both successful competition for nutrients and space for soil and rhizosphere colonization [22-23], and the ability of microorganisms to synthesize bioactive compounds (antibiotics, biosurfactants, siderophores, etc.) [24-26]. Production of hydrolytic enzymes (chitinases, glucanases, proteases, and lipases) which destroy the cell walls of plant pathogenic fungi, is of great importance [26]. In addition to direct antagonistic action on the cells of the causative agent, bacilli are able to increase plant disease resistance due to the presence of bacterial determinants (microbe-associated molecular patterns, MAMPs), such as flagellin, lipopolysaccharides (LPS), and other compounds associated with the cell wall of *B. subtilis* [27-29], as well as volatile organic substances [30]. The synthesis of elicitor compounds activates induced resistance of plants. Elicitors induce a nonspecific immune response, but also stimulate the production of plan hormones, i.e., salicylic acid (activator of systemic induced resistance) and jasmonic acid (activator of systemic acquired resistance).

Bacillus strains used for biologicals are capable of a variety of metabolic processes, including production of bioactive substances (BAS) which differ in their chemical nature and mechanisms of action. In this regard, it is very promising to design compositions from different strains or species of microorganisms to provide high activity of various biologicals.

A biological product Vitaplan (developed at the All-Russian Research Institute of Plant Protection. VIZR) is based on two strains, the *B. subtilis* VKM V-2604D and *B. subtilis* VKM V-2605D with different bioactive complexes and mechanisms of action which are highly effective towards a wide range of plant pathogens [4, 31].

Enhancing the ability of bacteria to trigger a cascade of defense reactions and increase the systemic resistance of plants is a way to increase the effect of

biologicals. Natural or synthetic activators of disease resistance in the formulation can strengthen the inducing activity of the producer strain. Natural inducers, e.g., polysaccharides (chitin, chitosan) and salicylic acid (SA) as a signaling molecule of systemic acquired resistance are most preferable. Chitosan and chitosan-based preparations are common in plant protection against diseases as inducers of non-specific resistance [32-34], and SA is a classical inducer of disease resistance which plays a central role in protecting plants from biotrophic pathogens [35].

Biological product Vitaplan is included in the State catalog of pesticides and agrochemicals permitted for use on the territory of the Russian Federation in the form of a wettable powder (WP). When developing new multifunctional formulations, we used the previously obtained data on the high immunomodulatory activity of the conjugate of chitosan with SA (chitosan salicylate) [36].

In this work, for the first time, we have developed two new optimized formulations of Vitaplan, KZh (Vitaplan, KZh + colloidal chitin and Vitaplan, KZh + 0.1% chitosan salicylate) with increased antagonistic and elicitor activity compared to the original form of the biological product. It was found that the chitosan salicylate in the new formulation Vitaplan, KZh increases the inducing activity by 2.0-2.5 times compared to the original formulation.

The purpose of our research was to improve the effectiveness of the multifunctional biological Vitaplan by including chitosan-based disease resistance inducers in the formulation.

Materials and methods. The strains *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D (State collection of microorganisms pathogenic for plants and their pests, Center for collective use of scientific equipment "Innovative technologies for plant protection" VIZR; registered January 28, 1998 at No. 760 in the World Federation for Culture Collections, World Data Center for Microorganisms — WFCC WDCM, Japan) were used. Bacteria were cultured on an artificial nutrient medium (30 g/l corn extract, 15 g/l molasses, pH 7.2; a laboratory shaker, 28 °C, 220 rpm for 72 h, 750 ml flasks, and 100 ml medium volume).

The antagonistic activity against *Alternaria solani* Sorauer and *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davisetal. (strain 101) was assessed for i) Vitaplan, KZh (titer of viable cells 10^{10} CFU/ml), the microbial culture of *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D strains at a 1:1 ratio without additives (control); ii) Vitaplan, KZh (10^{10} CFU/ml) + 1.0% dry chitin; iii) Vitaplan, KZh (10^{10} CFU/ml.) + 1.0% colloidal chitin as calculated per the dry weight of chitin; and iv) Vitaplan, KZh (1010 CFU/ml) + 1.0% colloidal chitosan as calculated per the dry weight of chitosan.

Fungistatic activity against *Cochliobolus sativus* S. Ito & Kurib. Drechsler ex Dastur (=*Bipolaris sorokiniana*, =*Drechslera sorokiniana* Subram et Jain, =*Helminthosporium sativum* Pam.) was evaluated as follows: i) control (water); ii) Vitaplan, KZh (10^{10} CFU/ml); iii) Vitaplan, KZh (diluted 1:10 with distilled water, 10^9 CFU/ml); iv) Vitaplan, KZh (without dilution, 10^{10} CFU/ml) + 1.0% colloidal chitin as calculated per the dry weight of chitin; v) Vitaplan, KZh (diluted 1:10 with distilled water, 10^9 CFU/ml) + 0.1% chitosan salicylate; and vi) 0.1% chitosan salicylate.

To study the inducing activity in the pathosystem of wheat (*Triticum aestivum* L.)—*C. sativus*, the Vitaplan, KZh; Vitaplan KZh + 1% colloidal chitin; and Vitaplan, KZh + 0.1% chitosan salicylate were diluted 10 times with distilled water (as per the norms of using Vitaplan KZh), the titers of all working solutions were 10^9 CFU/ml. The 0.1% chitosan salicylate was used.

For colloidal chitin, 100 kDa chitin was dissolved in concentrated hydrochloric acid followed by precipitation with acetone [38]. For colloidal chitosan,

the method developed by us was applied. Dry chitosan (100 kDa. 1 g) was dissolved with permanent stirring in 100 ml of a 2.5% aqueous solution of lactic acid and neutralized with 1.5% aqueous sodium hydroxide to pH 8.0. The colloidal solution of chitosan was kept in a refrigerator to form a precipitate.

Chitosan (60 kDa) was obtained by the oxidative destruction method [37] from 150 kDa chitosan with 85% deacetylation (Bioprogress, Russia) to synthetize chitosan salicylate containing 25% of ion-bound SA fragments. The bands from the CO_2^- carboxylate group in the IR spectrum (1552.92 cm^{-1} and 1386.12 cm^{-1}) were characteristic of a salt between chitosan and SA. A broad strong band at 3100-2600 cm^{-1} corresponded to stretching vibrations from the NH_3^+ and OH^- functional groups.

The titer of viable cells in the samples was determined by the 10-fold serial dilutions with plating on SPA agar medium and counting colonies. Antibacterial activity against the causative agent of bacterial canker of tomato *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davisetal. (strain 101) and antifungal activity against *Alternaria solani* Sorauer was assessed by the paper disk method by the diameter of the lysis zone of the test pathogens on agar nutrient medium. Czapek agar in Petri dishes was plated with 10^5 CFU/ml suspension of the test culture and placed on the agar surface sterile paper filters (diameter 8 mm) onto which a suspension of a certain concentration of a test formulation was applied with a pipette. Test cultures were grown in a TC-1/80 SPU thermostat (SKTB SPU, Russia) at 22-25 °C for 3-5 days.

The direct fungistatic effect of the studied formulations was assessed in vitro by the method of agar blocks. Czapek agar medium cooled to 40 °C was poured into sterile Petri dishes. After solidification, suspensions of the test formulations (0.2 ml) were evenly applied to the surface of the agar medium. Blocks (6 mm diameter) of 10-day-old cultures of *C. sativus* cut with sterile drill from the 8-10-day mycelial culture on Czapek agar were also placed on the agar medium. Plates with Czapek agar medium with blocks of test culture without specimens of the formulations served as a control. The dishes were incubated in the dark at 25 °C. The diameters of the fungus colonies were measured on days 5 and 7 of co-culture, and the fungistatic effect of the test samples was assessed as per the Abbott formula: $S = (D_c - D_{test})/D_c \times 100\%$, where S is the suppression of the fungal growth compared to control, %; D_c is the diameter of the fungus colony in the control, mm; D_{test} is the diameter of the colony of the fungus in the test treatment, mm.

Experiments to assess the immunomodulatory activity of samples of preparative forms were carried out by the method of detached leaves [39]. Twenty-four hours before inoculation with the pathogen, 7-day-old wheat seedlings of the disease-susceptible cultivar Saratovskaya 29 were sprayed with suspensions of the formulations diluted 1:10. Wheat leaves were infected with a spore suspension of *C. sativus* (4×10^3 and 20×10^3 spores/ml). The degree of leaf infestation was assessed on day 4 as a percentage of the affected leaf area. Control plants were treated with water.

All experiments were performed in three replicates. An analysis of variance (the Statistica 6.0, StatSoft, Inc., USA and Excel 2016 programs) was used to process the data. In the calculations, the methods of parametric statistics based on mean values (*M*) and standard errors of means (±SEM), 95 % confidence intervals, the least significant difference at $p < 0.05$ (LSD_{05}) were applied.

Results. Chitin and chitosan in the medium for submerged cultures of *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D had no negative effect on the cell titer of these strains use to produce the Vitaplan, KZh biological (Table 1). The colloidal chitin in the medium slightly increased the antagonistic activity

of the producer strains towards both test cultures.

Vitaplan, KZh showed high fungistatic properties which did not differ significantly for 10^9 and 10^{10} CFU/ml (74.3 and 80.9% suppression the growth of *C. sativus* mycelium on day 5, and 81.2 and 87.5% suppression on day 7). With the addition of 1.0% colloidal chitin to the growth medium, the biological activity of the culture remained high, the 84.9 and 88.1% on days 5 and 7, respectively. Chitosan salicylate at a concentration of 0.1% had a moderate fungistatic activity, inhibiting the mycelium growth of *C. sativus* by 30.5 and 42.5%. Note that the 0.1% chitosan salicylate in the diluted culture fluid of the producer strains insignificantly decreased the direct inhibitory effect on the mycelial growth of C. *sativus*, to 61.9 and 69.4% (Table 2).

1. Antagonistic activity of *Bacillus subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D in submerged culture added with chitin and chitosan (M±SEM)

Treatment	Zone free from test culture growth, mm	
	for *Alternaria solani*	for *Clavibacter michiganensis* subsp. *michiganensis* 101
Vitaplan, KZh (control)	40.3±1.0	31.9±1.0
Vitaplan, KZh + dry chitin (1.0 %)	37.8±0.5	31.9±1.2
Vitaplan, KZh + colloidal chitin (1.0 %)	43.6±0.8	34.8±1.8
Vitaplan, KZh + colloidal chitosan (1.0 %)	36.0±1.0	31.6±0.5
LSD_{05}	1.1	0.9

N o t e. For all treatments, the titers of the producer strains were 10^{10} CFU/ml.

2. Growth of the test culture of plant pathogen *Cochliobolus sativus* as influenced by various formulations of Vitaplan, KZh (M±SEM)

Treatment	Concentration of additional component, %	Viable cells, CFU/ml	Day 5		Day 7	
			diameter of colony, mm	growth inhibition, %	diameter of colony, mm	growth inhibition, %
Water (control)			52.5±0.5	–	80.0±1.2	–
Vitaplan, KZh		10^{10}	10.0±0.5	80.9	10.0±1.0	87.5
Vitaplan, KZh (diluted 1:10)		10^9	13.5±0.5	74.3	15.0±1.2	81.2
Vitaplan, KZh + colloidal chitin	1,0	10^{10}	8.0±0.2	84.9	9.5±1.0	88.1
Витаплан, КЖ (diluted 1:10) + chitosan salicylate	0,1	10^9	20.0±1.5	61.9	24.5±1.5	69.4
Chitosan salicylate	0,1		36.5±2.2	30.5	46.0±2.0	42.5
LSD_{05}				1.3		0.6

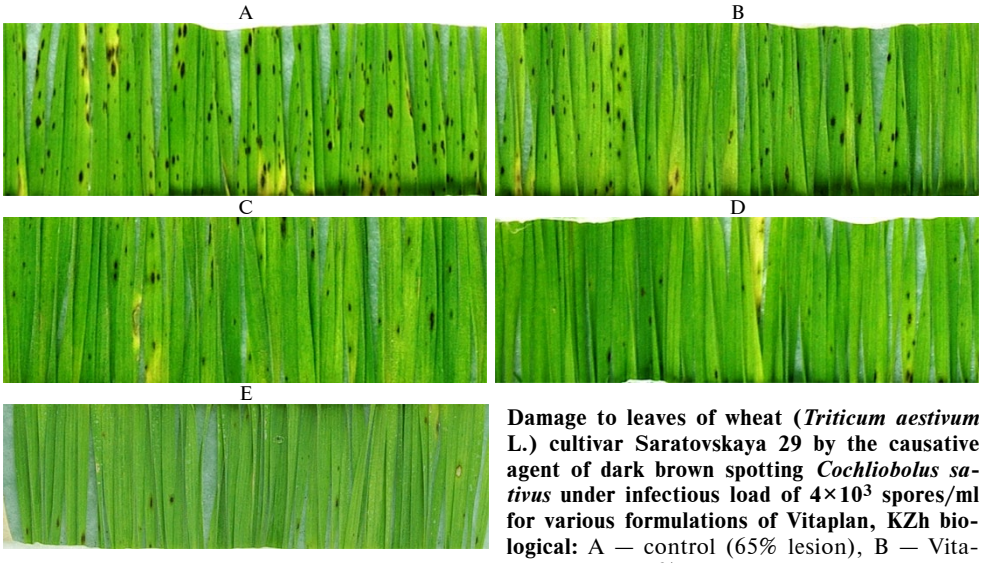
N o t e. Dishes means the absence of growth inhibition.

Upon infecting the leaves of susceptible wheat cultivar Saratovskaya 29 with a suspension of *C. sativus* spores in the control, the signs of the disease appeared as brown spots occupying 65 and 100% of the leaf area under the infectious load 4×10^3 and 20×10^3 spores/ml, respectively (Fig.). Preliminary spraying of plants with Vitaplan, KZh a dilution of 1:10 followed by infection with the causative agent of dark brown spotting reduced the area of leaf damage to 50 and 80%, depending on the infectious load of the pathogen.

A 1.0% concentration of colloidal chitin at in the growth medium for the strains producers reduced leaf damage to 40 and 50% at an infectious load of 4×10^3 and 20×103 spores/ml, which indicates a higher immunomodulatory activity of this formulation compared to Vitaplan, KZh at a dilution of 1:10. Chitosan salicylate showed itself to be an effective inducer of disease resistance, reducing plant damage to 10 and 20%. The 0.1% chitosan salicylate supplementation to Vitaplan, KZh diluted 10-fold also had a positive effect on the the antagonist, increasing its biological activity 2.0-2.5-fold with the leaf damage of 20 and 40% at an infectious load of 4×10^3 and 20×10^3 spores/ml (see Fig.).

Our experiments showed that the effect of Vitaplan formulations against dark brown spot depends on the intensity of damage to wheat plants by the causative agent of the disease *C. sativus*. Vitaplan, KZh at a dilution of 1:10 in

combination with 0.1% chitosan salicylate had the greatest protective effect (see Fig.).



Damage to leaves of wheat (*Triticum aestivum* L.) cultivar Saratovskaya 29 by the causative agent of dark brown spotting *Cochliobolus sativus* under infectious load of 4×10^3 spores/ml for various formulations of Vitaplan, KZh biological: A — control (65% lesion), B — Vitaplan, KZh (50%), C — Vitaplan, KZh + colloidal chitin (40%), D — Vitaplan, KZh + chitosan salicylate (20%), E — chitosan salicylate (10%).

Two mechanisms can underlay the efficiency of a biological product in protecting wheat from dark brown spotting, namely a direct suppression of the causative agent due to the synthesis of antibiotics and enzymes by producer strains and an indirect influence through the induction of systemic resistance. In our opinion, there are significant differences in the mechanisms of wheat protection against dark brown spotting for Vitaplan, KZh and its formulations containing colloidal chitin and 0.1% chitosan salicylate.

Vitaplan, KZh based on *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D strains exhibits suppressive effect due to high fungistatic activity against *C. sativus*, caused by the synthesis of a multicomponent metabolite complex which includes peptide and polyene antibiotics, while the inducing activity of the microbial preparation is, on the contrary, low (see Table 2). Depending on the infectious load, the Vitaplan, KZh formulations supplemented with colloidal chitin and chitosan salicylate reduced the disease sings 1.5-3.0-fold compared to the control. The main protective mechanism providing high biological activity of a formulation combining Vitaplan, KZh (1:10) and chitosan salicylate (0.1%) is induced resistance. Fungistatic activity of this composition was somewhat lower than that of Vitaplan, KZh (1:10). The biological activity of the form combining Vitaplan, KZh (1:10) with colloidal chitin (1.0%), apparently, was due to the combination of the fungicidal activity of the preparation and the induction of plant defense reactions. Thus, the formulations of Vitaplan, KZh that we have developed provided direct suppression of the pathogen and exerted an indirect protective effect through an increase in the disease resistance of wheat plants to dark brown spot.

According to contemporary concepts, microbe-plant interaction in the plant—plant pathogen—plant pathogen antagonist system are complex and multidirectional. For example, *B. subtilis* 26D induced systemic resistance in wheat plants upon infection with *Septoria nodorum* and in potato plants infected with *Phytophthora infestans* oomycete through the accumulation of H_2O_2 and an increase in the transcriptional activity of SC-regulated PR protein genes, in peroxidase activity, and lignin deposition at the sites of infection [40].

Elicitors triggering plant defense mechanisms can be proteins, lipopeptides, polysaccharides, and other compounds associated with the cell wall of *B. subtilis* [41]. Bacterial metabolites possessing the properties of induced resistance include a chain of interrelated defense reactions, e.g., formation of reactive oxygen species, phosphorylation of proteins, and the triggering of the basic mechanisms of plant immunity, which lead to the development of systemic resistance [42-45].

Chitin and chitosan are molecular determinants of many plant pathogenic microorganisms that are recognized by plant protein receptors [46]. Interactions with receptors activate a complex of defense reactions of nonspecific immunity to form a systemic resistance to pathogens. Induced protective reactions include i) generation of reactive oxygen species (ROS), ii) synthesis of callose, iii) strengthening of the cell wall with lignin, iv) development of a hypersensitivity reaction (HS reaction) which causes the death of plant cells and the pathogen in the zone of its introduction, v) induction of genes involved in synthesis of protective proteins, vi) synthesis of 18 classes of pathogen-induced proteins with antimicrobial (thionines, defensins, proteinase inhibitors) and lytic activity (chitinases, glucanases), vii) induction of defense hormones (abscisic acid, jasmonates, salicylic acid), and viii) induction of the phenol-propanoid pathway and an increase in the level of phytoalexins [47].

It is known that a mechanism associated with an increase in the tobacco plant resistance is an increase for SA under the influence of treatment with *B. pumilus* SE34 strain [48]. Since SA is a signaling molecule that triggers a cascade of defense reactions in plants, the exogenous SA together with *Pseudomonas fluorescence* (pf4-92) enhanced the inducing activity of the antagonist in protecting chickpea seedlings from fusarium wilt [49]. The combined use of *P. fluorescence* (SE21 and RD41) and resistance inductors (chitin and salicylic acid) stimulated plant growth and increased the efficiency of biological control of pepper rhizoctoniosis [50]. Several works confirmed that the combination of microbial antagonists and chitosan increase the effectiveness of biologicals in protecting vegetables and strawberries from powdery mildew [51]. Supplementation of the *Bacillus* sp. culture medium with chitin significantly reduced wilting of the cotton [52].

It is obvious that protecting plants from diseases requires new multifunctional microbiological compositions effective against a wide range of plant pathogens. Our previous studies have shown that the combined use of microbial strains and chitosan complexes is effective for protecting wheat from a complex of major diseases, including root rot and leaf spots, as well as for increasing yields [53].

So, we have developed two new formulations, the Vitaplan, KZh + colloidal chitin and Vitaplan, KZh + chitosan salicylate with increased biocidal activity and inducing properties compared to the original formulation Vitaplan, KZh. The Vitaplan, KZh + colloidal chitin formulation exerts high antagonistic activity. Colloidal chitin (1.0%) added to the medium for submerged culture of producer strains ensured both effective suppression of *Cochliobolus sativus*, the causative agent of wheat dark brown spotting (up to 84.9-88.1%), and a 1.5-2.0-fold increased inducing effect compared to the original biological product. Chitosan salicylate (0.1%) added to Vitaplan, KZh increases its biological activity 2.0-2.5-fold. High protective effect of the new optimized formulation of Vitaplan, KZh towards *C. sativus* that we revealed was apparently due to a combined action of metabolites produced by *B. subtilis* strains which suppress or detain growth of the plant pathogen and chitosan-based inducers of disease resistance. Our findings indicate that the combination of active strains of microbial antagonists of plant pathogens and chitosan complexes is promising for increasing the biological efficiency and expanding the spectrum of action of the developed formulations.

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