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Chitosan-Based Innovative Spring Wheat Protection Agents Obtained Using Mechanochemical Methods

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Abstract

The mechanochemical method of preparation supramolecular complexes of tebuconazole (TBC) was used to obtain new complex agents of plant protection from pests and diseases of cereal crops. The use of chitosan, as well as licorice root extract, in these processes, made it possible to obtain preparations with improved physicochemical and biological parameters. Under field conditions, the high biological efficiency of the composition of tebuconazole with chitosan against Puccinia recondita, Septoria nodorum and Blumeria graminis was shown with a single treatment at the beginning of heading of spring wheat plants, which led to an increase in grain yield by 0.55 t/ha, while the dose of tebuconazole fungicide was reduced by a factor of 5.5. The possibility of making similar spring wheat protection products based on the combination of chitosan with licorice root extract without the inclusion of tebuconazole is demonstrated. This increases the grain productivity of wheat by 0.54-0.6 t/ha.

Keywords: chitosan, tebuconazole, licorice root extract, soft spring wheat, leaf diseases, thrips, productivity

INTRODUCTION

The application of chitosan-based aminopolysaccharides in agriculture is based on its ability to enhance the stability of plants against hazardous biological objects of viral, bacterial and fungal nature, as well as to strengthen the resistance to insect pests. Chitosan in complex with organic acids is able to limit the growth of fusarial fungi, intensify the dissolution of phosphorous compounds in soil making them better available for plants [1]. Chitosan, as the active substance, is permitted by the Agency on Environmental Protection for application with the majority of crops including cereals, both to treat the seeds and to spray the plants [2]. It is assumed that chitosan is able to stimulate the production of the protective metabolites in plants – the active forms of oxygen and phytoalexins [3]. Chitosan and preparations based on it are not toxic for humans and for the environment, they do not cause stability in pathogenic microorganisms. Possessing fungicidal [4], antibacterial [5] properties, chitosan is able to induce nonspecific versatile disease-resistance and may be used to protect plants from the diseases of different etiologies. Good potential of the use of polyfunctional chitosan-containing compositions for the protection of wheat from powdery mildew, brown rust and leaf blotch was demonstrated [6]. It was established that the antifungal activity of chitosan depends on its molecular mass and on the kind of pathogen. Chitosan preparations with the molecular mass from 6.5 to 150 kJ improve the stability of wheat to brown rust, which results in the suppression of the survived pustules on leaves by 79.5–89.0 % [7]. It was revealed that chitosan promotes a decrease in the density of the nematode population [8]. The effect of chitosan on insects is extremely insufficiently investigated. However, there are some data on the application of Artafidin, a chitosan preparation, against various green-lice species on orchard crops, so that its efficiency was 96.5–100, on melons and gourds – 97.4–98.0, on vegetables – 100 %. The preparation demonstrated high selective activity with respect to beneficial fauna. The formation of resistance to the preparation was not observed during its tests against Aphis pomi Deg. in a perennial apple station. Artafidin demonstrated 100 % efficiency against Dermacentor marginatus Sulzer ticks [9].

Enhancement of the biological activity of preparations is achieved due to the formation of supramolecular structures – inclusion complexes of chitosan with other biologically active substances, the introduction of biologically active additives (the dry extract of licorice roots), enhancement of solubility and bioavailability, and improvement of the methods of application. A preparation belonging to the new generation of natural elicitors was developed on the basis of nanometer-sized chitosan particles: 1 ml of this preparation contains more than $14.4 \cdot 10^{13}$ molecules of low-molecular chitosan and is 600 times more efficient than usual (non-standardized) chitosan [2, 7].

The goal of the investigation was to evaluate the efficiency of the compositions of commercial chitosan (industrially manufactured in the RF) with the dry extract of licorice roots and fungicidal agent tebuconazole for the protection of spring soft wheat from leaf diseases, to reveal their effect on the production process and on the safety of grain crops.

EXPERIMENTAL

Materials

The compositions under investigation included tebuconazole (TBC) (Shenzhen Sunrising Industry Co., Ltd., Chinese People's Republic), with the major substance content >98.0 %; auxiliary substances: the dry extract of the roots of Clycyrrhiza uralense Fisch. (LC Visterra, Russia) [10], with the mass fraction of glycyrrhisic acid 20–25 %; foodstuff low-molecular water-soluble chitosan, TU 9289-067-00472124–03 (LC Bioprogress, Russia).

Preparation of compositions

The joint mechanochemical treatment (MCT) of TBC and auxiliary substances with the mass ratio of TBC/auxiliary substance = 1 : 5, 1 : 10 was carried out under the conditions described previously [11]. The treatment was carried out for 24 h with sampling every 2 h.

Optimal time of MCT was chosen using the criteria of maximal solubility of TBC compositions in water, provided that its concentration is conserved at a level not lower than 98 % of the initial one.

The best composition and the conditions of its preparation were chosen relying on the set of characteristics (the maximal increase in solubility and the stability of TBC) for subsequent physicochemical and biological studies: TBC/chitosan, mass ratio 1 : 10, MCT time in a roller mill (RM) 24 h, an increase in TBC water solubility by a factor of 1.2. For biological studies, the samples were prepared: chitosan (MCT time 24 h); chitosan/extract of licorice roots (mass ratio 1 : 5, MCT time 24 h).

Physicochemical studies

The prepared compositions were analyzed by means of high-performance liquid chromatography (HPLC), gel-permeation chromatography, dynamic NMR 1H spectroscopy, granulometric, X-ray diffraction (XRD) analysis and thermal analysis according to the procedures described in [10–13]. The formation of inclusion complexes TBC/chitosan in aqueous solutions (D_2O) was confirmed by means of NMR spectroscopy with the help of an Avance III 500 spectrometer (Bruker, Germany) at the frequency of 500 MHz at 30 °C. Measurement of the diffusion coefficients of chitosan was carried out by means of NMR using a special pulse sequence with the magnetic field gradient.

Biological studies

To reach the formulated goal two field experiments were laid at the experimental field of the SibRIA&Ch SFRCA RAS in the central forest-steppe Ob agricultural landscape district of the

Novosibirsk Region with the spring soft wheat of Novosibirskaya 31 variety, sown over the fallow. Sowing was carried out on the 21st of May, 2019, with the seed application rate of 6 mln of germinable seeds per hectare. Each of the two experiments included three versions. The first experiment: 1 – reference, involving no treatment with the preparations; 2 – treatment with Folikur fungicide, KE (TBC, 250 g/L) with the standard application rate of 1 L/ha; 3 – treatment with the composition TBC/chitosan (1 : 10) with standard application rate 0.5 kg/ha. The second experiment: 1 – reference without treatment with preparations; 2 – treatment with chitosan with the standard application rate of 0.5 kg/ha; 3 – treatment with the composition chitosan/extract of licorice roots (1 : 5) with standard application rate 0.5 kg/ha. The treatment of the crops with the preparations was carried out at the start of heading with a manual sprayer with the standard application rate of the working liquid 300 L/ha. The efficiency of chitosan and its compositions with the extract of licorice roots and fungicide TBC against the leaf diseases of soft spring wheat (septoriosis – Septoria nodorum Berk., Septoria tritici Rob. et Desm. [14]; brown leaf rust – Puccinia recondita Rob. et Desm. (Peterson scale); powdery mildew – pathogenic organism: Blumeria graminis (DC) Speer. (a synonym: Erysiphe graminis DC) f. tritici Em. Marchal of the Erysiphales order [15]) was evaluated at the phase of milky ripeness of the crops, the occupancy of heads with the larvae of wheat thrips was determined visually checking the samples of heads [16]. The parameters of head structure (the size of sampling $n = 50$; the area of the allotment 10 m^2) were recorded at the stage of waxy ripeness of grains [17]. Harvesting was carried out directly by combine. The crop capacity was brought to the standard humidity and purity. Mathematical processing of the data was carried out with the help of the applied software package SNEDECOR [18] and Statistica 7.0.

RESULTS AND DISCUSSION

Physicochemical studies

Solid phases of the compositions. The diffraction patterns of initial TBC contain characteristic reflections of the crystal phase of TBC (Fig. 1, *a*). One can see in the diffraction patterns of the mechanically activated mixture TBC/chitosan that the result of MCT is TBC amorphization. The data of differential scanning calorimetry (DSC) (see Fig. 1, *b*) of initial TBC and the composition TBC/chitosan also provide evidence of the loss of sample crystallinity as a result of MCT. A typical endothermic peak of the melting of the crystal phase of TBC is observed in the thermogram of tebuconazole. The area of this peak decreases after MCT with chitosan. The peak related to melting shifts to lower temperature, maybe due to the partial loss of crystallinity and the accumulation of lattice defects. The thermograms of auxiliary substances do not exhibit clear effects of phase transitions, which points to the amorphous state.

According to the data obtained by means of SEM (Fig. 2), MCT involves the destruction of crystal TBC and chitosan particles, with subse-

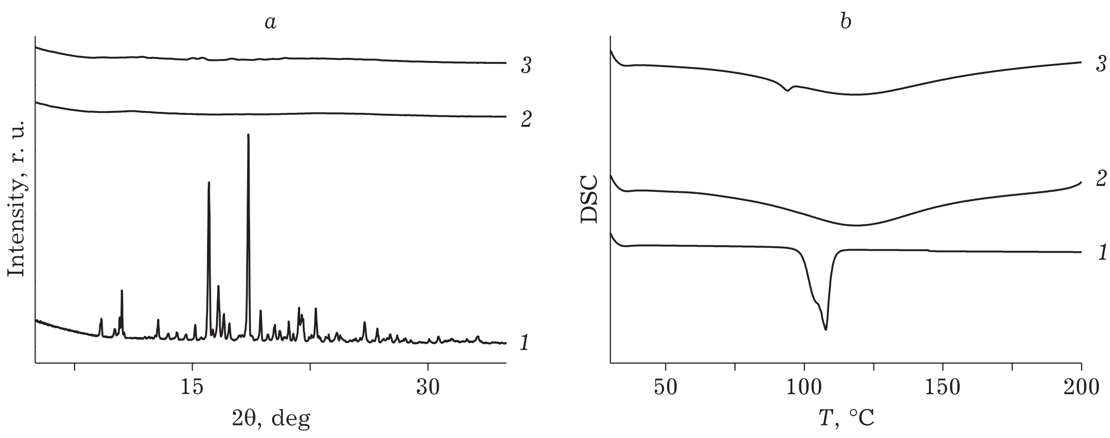

Fig. 1. Diffraction patterns (*a*) and DSC thermograms (*b*) of TBC (*1*), chitosan (*2*) and TBC/chitosan composition (1 : 10) after MCT in RM for 24 h (*3*).

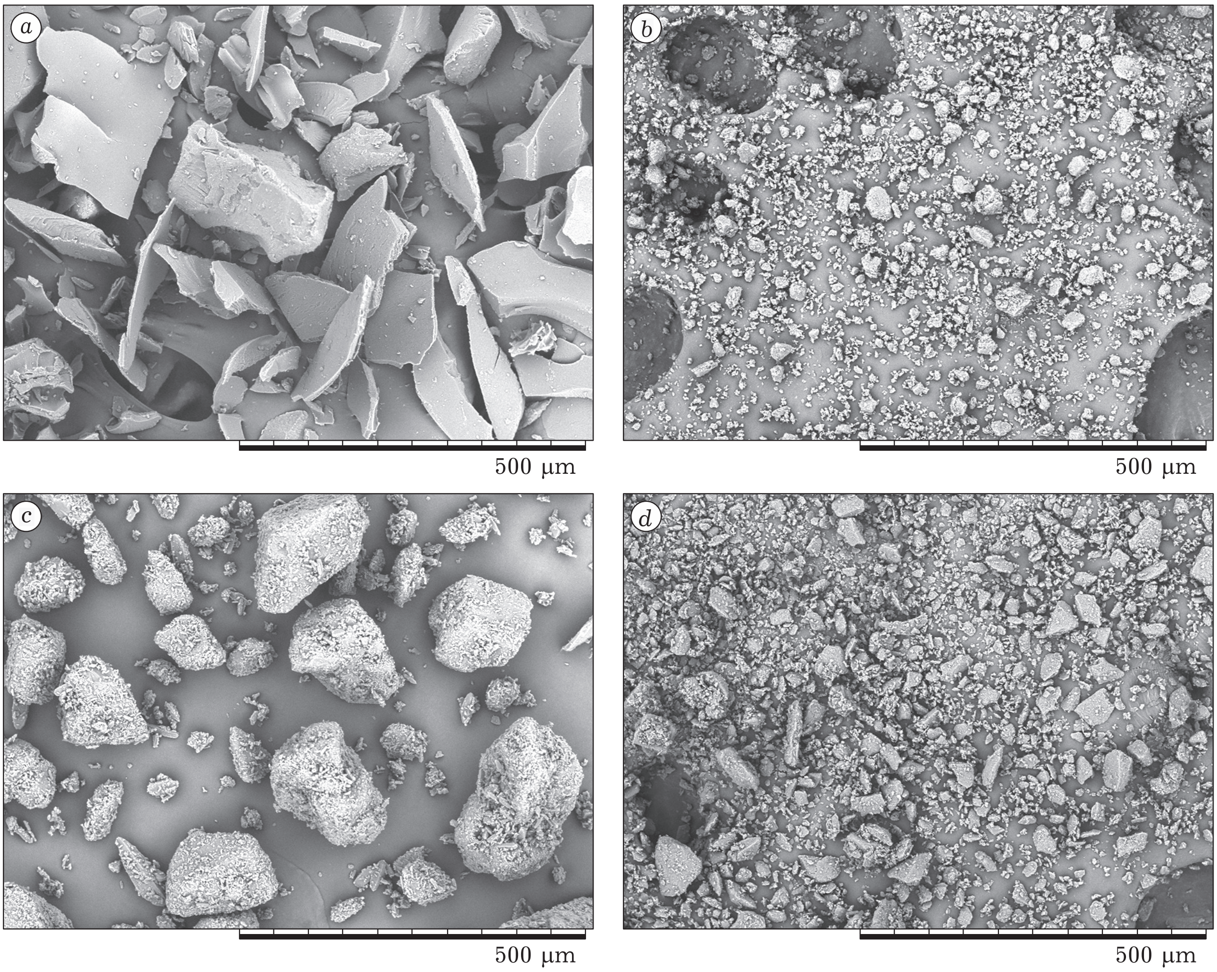

Fig. 2. Microphotographs of initial chitosan (*a*), chitosan treated in RM for 24 h (*b*), initial TBC (*c*), composition TBC/chitosan (1 : 10) treated in RM for 24 h (*d*).

quent formation of polydisperse powders composed mainly of the particles with irregular shapes 5–20 μm in size, as well as their aggregates.

Water solutions of compositions. The data on the granulometric analysis of TBC suspensions and its compositions with chitosan are shown in Fig. 3. One can see that the TBC substance (see Fig. 3, *a*) is a polydisperse powder with particle sizes 1–261 μm. The size distribution contains a single mode with the maximum near 60 μm, with 80 % of sample mass being within the particle size range 20–135 μm.

Chitosan substance, insoluble in water under the existing conditions, treated in the VM (see Fig. 3, *b*) for 24 h, is a polydisperse powder with particle size 3–262 μm. The size distribution contains a single mode with a maximum near 70 μm, and 90 % of sample mass is within the particle size range 24–112 μm. B As a result of joint MCT, the particles with the mixed composition are likely to be formed; size distribution contains a single mode, too, with a maximum near 66 μm, and 80 % of sample mass is within the particle size range 32–160 μm (see Fig. 3, *c*).

To study the molecular mass properties (MMP) of chitosan, gel filtration chromatography was used (Fig. 4). The chromatograms of chitosan before and after MCT contain the peaks of high-molecular compounds with the calculated masses (M_w) 1175 and 392 kDa. For mechanically treated chitosan, a hardly detectable shift of MMP is observed, which points to almost complete absence of its mechanical destruction.

NMR ^{1}H spectroscopy of TBC and its compositions with auxiliary substances. Analysis of NMR spectra of initial chitosan and chitosan treated in RM revealed the absence of changes, which points to the stability of chitosan molecules during MCT. More detailed investigation of chitosan samples was carried out with the application of dynamic NMR: measurement of the times of

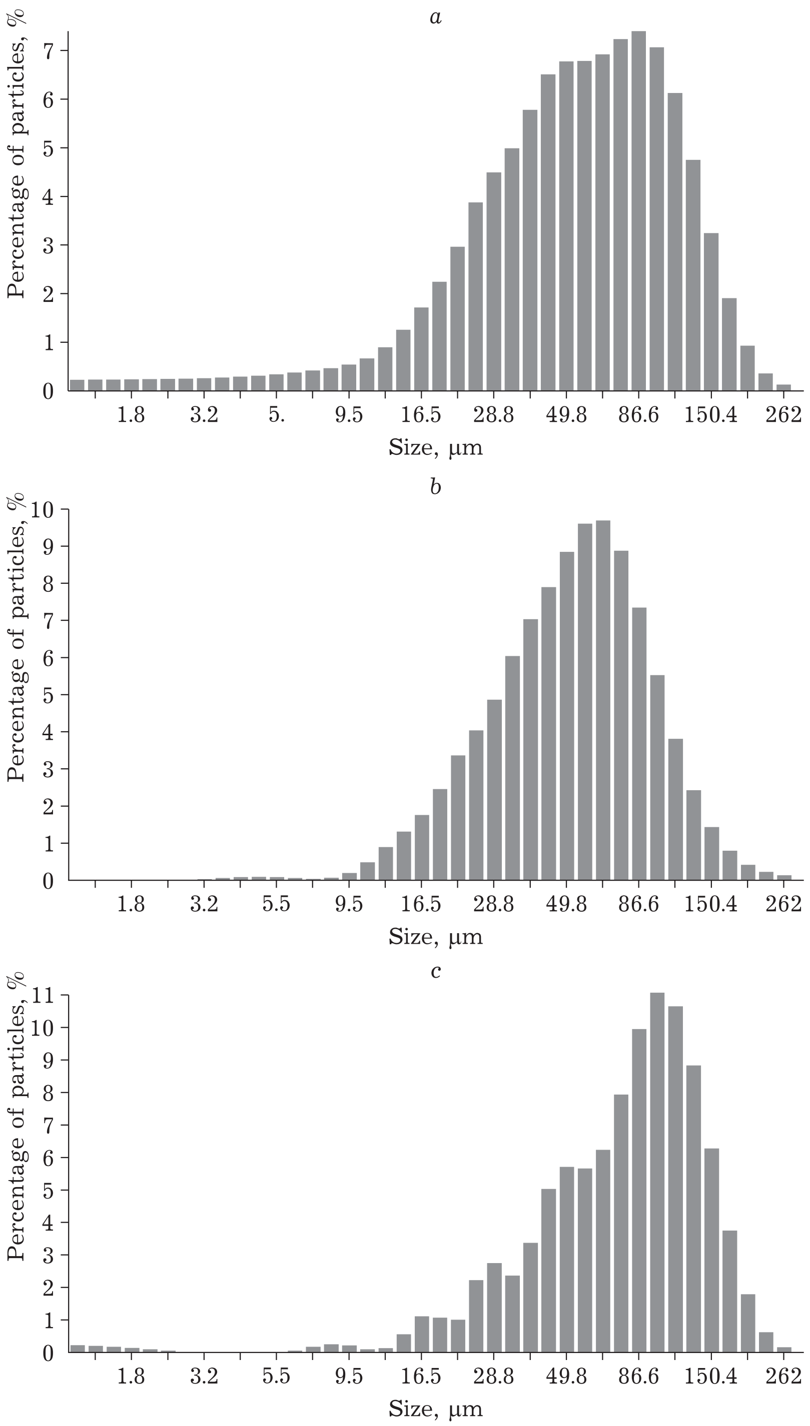

Fig. 3. Granulometric composition of TBC suspension (*a*), chitosan (MCT for 24 h) (*b*), TBC/chitosan composition (1 : 10) (MCT for 24 h) in water (*c*).

spin-spin relaxation of the protons of chitosan and diffusion coefficients of the molecules. NMR relaxation was also used to prove the formation of intermolecular complexes of TBC with chitosan. It is known that the times of spin-spin (T_2) relaxation are very sensitive to intermolecular interaction and to the diffusion mobility of molecules [19]. This is explained by the change in the time of the rotational reorientation of molecules due to the deceleration of diffusion. In the general case, bi- or monoexponential kinetics of the echo signal decay may be observed in the experiment, depending on the rate of "exchange" between the complex and the solution. Analysis

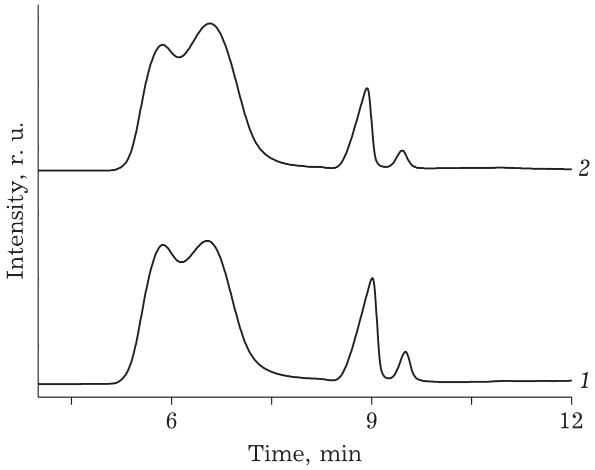

Fig. 4. Gel chromatograms of the 0.2 mass % solution of chitosan before (*1*) and after MCT for 24 h (*2*).

of the results obtained for chitosan solutions showed that all the kinetics of NMR signal decay in relaxation experiments are multiexponential but can be fitted well with two exponents with times about 3 and 30 ms, which may be a consequence of a broad size distribution of the polymer. Treatment in the RM causes an increase in the fraction of more mobile phase (30 ms) by 10 %. Measurement of diffusion coefficient (D) of chitosan is in qualitative agreement with relaxation measurements:

$D_{in} = (2.6 \pm 0.1) \cdot 10^{-11}$ m^2/s

$D_{RM} = (3.2 \pm 0.2) \cdot 10^{-11}$ m^2/s

that is, treatment in the RM causes a slight increase in the percentage of a low-molecular fraction. The observed effect requires a more thorough investigation.

The addition of glycyrrhizic acid (GA), the major component of the extract of licorice roots, leads to gelation of the solution and to the formation of a precipitate; a decrease in the intensity of chitosan lines in the NMR spectra is observed. It may be assumed that the anion of GA (a deprotonated form of GA) interacts with positively charged amino groups of chitosan forming high-molecular insoluble polymer particles. NMR signals from chitosan and GA are not observed in the spectra of the aqueous suspensions of the composition chitosan/extract of licorice roots (1 : 5).

We also measured relaxation times of aromatic protons in TBC in the pure form and in composition TBC/chitosan (1 : 10) to get a confirmation of the formation of intermolecular complexes after the dissolution of the mechanochemically prepared composition in water. The kinetics of NMR signal drop are shown in Fig. 5 along with the

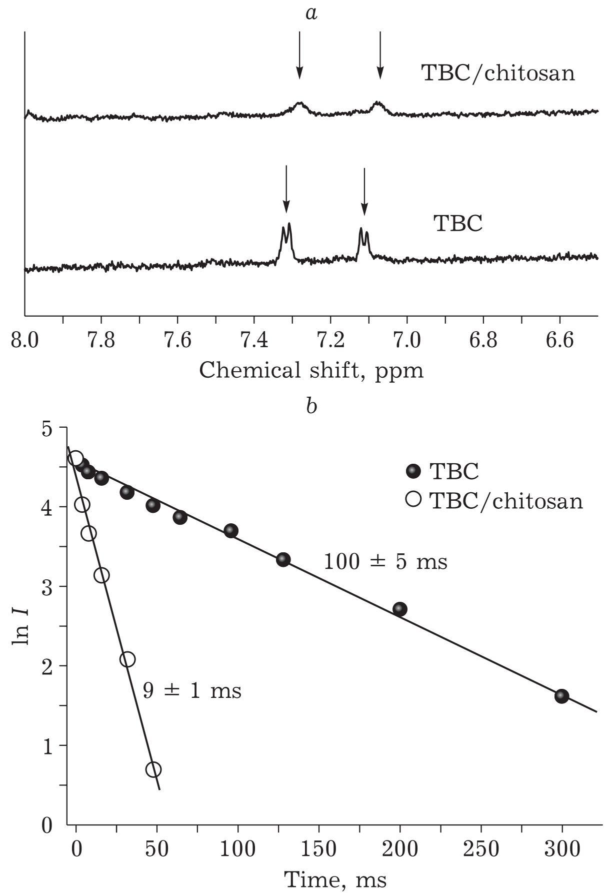

Fig. 5. *a* –NMR [1]H spectra of TBC and TBC/chitosan composition in D_2O. Arrows indicate the signals that were used to analyze relaxation times; *b* – kinetics of the drop of echo signal (logarithmic scale) and the times of spin-spin relaxation of aromatic protons in TBC and TBC/chitosan composition.

times of proton relaxation in TBC in the free form and in composition with chitosan, calculated from these dependencies. One can see that the kinetics of the decay of NMR signal from TBC protons becomes substantially shorter in the solution of the composition with chitosan in comparison with a pure aqueous solution of TBC, which points to a decrease in the mobility of TBC molecule and serves as a direct confirmation of the formation of intermolecular complexes.

Biological tests. Before the heading phase, sole mycelial pouches of powdery mildew were recorded in the top level of Novosibirskaya 31 wheat. By the phase of milky ripeness of grains, the infectious background increased in the top level of plants: the intensity of the affection of the top leaf area in 100 % of plants by powdery mildew, septoriosis and brown rust reached 16.83, 19.11 and 35.30 %, respectively (Table 1).

TABLE 1

Effect of preparations under investigation on the development and incidence of the main diseases of leaves in soft spring wheat Novosibirskaya 31, start of milky-wax ripeness of grains

Composition of preparations	Index of disease development, %			Incidence of disease, %		
	Powdry mildew	Septoriosis	Brown rust	Powdry mildew	Septoriosis	Brown rust
Reference (without treatment)	16.83	19.11	35.30	100	100	100
Folikur, 1 L/ha (0.25 kg/ha with respect to TBC)	0.03	0.06	0.02	3	6	2
Chitosan, 0.5 kg/ha	24.85	20.47	37.10	100	100	100
Chitosan/extract of licorice roots (1 : 5), 0.5 kg/g	16.69	16.54	33.10	96	99	97
TBC/chitosan (1 : 10), 0.5 kg/ha (45.5 g/ha with respect to TBC)	3.38	2.29	2.41	68	66	45

TABLE 2

Effect of the studied preparations on the structure of productivity and the crop capacity of soft spring wheat Novosibirskaya 31

Composition of preparations	Structure of main head				Mass of 1000 grains, g	Crop capacity, t/ha
	Length, cm	Number of cones, ps.	Number of grains, ps.	Grain mass, g		
Reference (without treatment)	9.30 ± 0.14	16.20 ± 0.24	33.50 ± 1.06	1.14 ± 0.04	32.60	3.16
Folikur 1 L/ha (0.25 kg/ha with respect to TBC)	10.40 ± 0.11	16.62 ± 0.15	40.20 ± 1.01	1.47 ± 0.04	36.50	3.90
TBC/chitosan (1 : 10), 0.5 kg/ha (45.5 g/ha with respect to TBC)	10.49 ± 0.09	16.38 ± 0.15	39.93 ± 1.08	1.59 ± 0.03	38.20	3.71
LCD_{05}					0.23	0.06
Reference (without treatment)	10.45 ± 0.16	16.56 ± 0.17	39.86 ± 0.95	1.40 ± 0.04	33.80	2.34
Chitosan	10.49 ± 0.12	16.56 ± 0.17	39.88 ± 0.97	1.43 ± 0.03	34.00	2.88
Chitosan/extract of licorice roots (1 : 5)	10.50 ± 0.07	16.30 ± 0.20	39.90 ± 0.83	1.48 ± 0.03	35.60	2.94
LSD_{05}					0.27	0.09

Note. LSD_{05} is the lowest substantial difference with a 5 % significance level.

Treatment with chitosan promoted some intensification of top leaf colonization with all the three diseases, to a higher extent (by a factor of 1.5 with respect to the reference) with powdery mildew, to a lower extent with brown rust and septoriosis. The development of the latter two diseases was somewhat decelerated (by a factor of 1.2 and 1.1, respectively) by the treatment with a complex of chitosan/the extract of licorice roots. The composition tebuconazole/chitosan was effective against the set of leaf diseases. The biological efficiency of this composition against brown leaf rust (which dominated in the pathogenic complex) reached 93.2, septoriosis – 88.0, powdery mildew – 79.9 % (Folikur, 1 L/ha – 100, 99.6 and 99.8 %, respectively). It was established in the course of field observations that the treatment of wheat with the preparations with chitosan affected the number of the generative organ pests – wheat thrips individuals. For the number of larvae 78.8–84.2 sp/head, the biological efficiency of chitosan was 16.5, chitosan/extract of licorice roots – 27.4, while TBC/chitosan – 36.5 %. So, the use of chitosan-containing preparations may be promising to decrease the number and hazard of this pest. The selection of components or dosage of the preparation may allow reliable control not only for the diseases but also for the number of wheat thrips.

In addition to the high phytosanitary effect, TBC/chitosan (1 : 10) also demonstrated growth-stimulating action, which was manifested as a 27.9 % increase (Folikur, 1 L/ha – by 20.0 %; reference – by 12.2±0.07 cm^2) of the area of the top leaf of the leading shoot, which is known to make the substantial contribution into the formation of grain productivity. With the use of the composition TBC/chitosan (1 : 10), the mass of grains in the major head increased by 39.5 %, while the application of Folikur caused an increase in the parameter by 30 %, and the mass of 1000 grains increased in the former case by 5.6 g and in the latter case by 3.9 g (Table 2). Rather good results were obtained also for the treatment of wheat with the complex chitosan/extract of licorice roots: the mass of grains in the main head increased by 6 %, and the mass of 1000 grains increased by 1.8 g.

The yield of wheat grains with respect to the reference increased by 23.4 % with the application of Folikur, while for the composition TBC/chitosan it increased by 17.4 % (Snedecor affection degree = 99.1 %). The complex TBC/chitosan is slightly behind the commercial preparation, but the amount of the active substance in it is 5.5 times smaller. In the second experiment, the application of chitosan provided an increase in grain productivity by 23 %, while chitosan/extract of licorice roots by 25.6 % (Snedecor affection degree = 98.5 %). Though the efficiency of the developed preparations is somewhat lower than that of the standard fungicide TBC, the results provide evidence of the good potential of the application of chitosan, either in complex with substantially decreased doses of the above-indicated chemical fungicide or with the extract of licorice roots for spring wheat as fungicides or inductors of protective mechanisms. It is important that this is the way to enhance the ecological safety of the developed preparations. To increase the efficiency of the complexes, it is necessary to continue the studies in the direction of optimization of the ratio of components, and more precise determination of the rates of application of the obtained preparations.

CONCLUSION

Under the conditions of the forest-steppe zone of West Siberia, a single treatment of soft spring wheat at the start of heading with a new mechanochemically synthesized fungicidal composition tebuconazole/chitosan with the mass ratio of 1 : 10 (the time of mechanochemical treatment in RM is 24 h) restrains the development of phytopathogens Puccinia recondita, Septoria nodorum, Blumeria graminis by 93.2, 88.0, 79.9 %, respectively; it stimulates the growth of the top leaf by 27 %; affects the population of heads with wheat thrips and increases the grain productivity by 0.55 t/ha. This is lower than the value obtained with the application of commercial fungicide by 0.19 t/ha, however, the dose of tebuconazole in the developed preparation is 5.5 times lower.

The complex chitosan/extract of licorice roots with the mass ratio 1 : 5 (the time of mechanochemical treatment in RM is 24 h) does not control the development of powdery mildew and only slightly decreases the development of brown rust and septoriosis (by a factor of 1.2 and 1.1, respectively). However, its application increases the mass of grains in the main head (by 1.8 g, or 6 %) and the mass of 1000 grains, which promotes an increase in grain productivity of wheat by 0.6 t/ha, or 25.6 %.

The treatment of wheat with chitosan promotes colonization of the top leaf with the pathogenic organism causing powdery mildew Blumeria graminis (by a factor of 1.5), does not restrain the development of septoriosis and brown rust. At the same time, the obtained increase in grain yield (0.54 t/ha, or 23 %) provides evidence of the possible stimulation of protective mechanisms of plants after the treatment with chitosan, which has a positive effect on the grain yield of spring soft wheat.

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