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Acetylation Degree of Chitin in the Protective Response of Wheat Plants

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Abstract—Influences on the acetylation degree of chitin manifested by proteins from cultural filtrates of strains of the fungus *Septoria nodorum* different in aggressiveness and of extracts from leaves of the susceptible (*Triticum aestivum*) and resistant (*Triticum timopheevii*) wheat plants infected with these strains were studied. Chitin deacetylase was found among the extracellular proteins of the fungus. Its activity was higher in the aggressive strain of the fungus than in the non-aggressive one, and this suggested that this enzyme could play an important role in the further formation of compatible relationship of the pathogens with the plants. Protein extracts from the susceptible wheat seedlings infected with the septoriosis agent also contained a component decreasing the acetylation degree of chitin. Protein extracts from the resistant wheat seedlings increased the chitin acetylation degree. It is supposed that this can be a pattern of the plant counteracting the action of chitin deacetylases of the pathogen.

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Key words: wheat (*Triticum aestivum*, *Triticum timopheevii*), septoriosis agent (*Septoria nodorum*), chitin, chitosan, acetylation degree

There are various mechanisms of the functioning of the plant defense system. The same population of host plants can differently react to infection with aggressive and non-aggressive strains of a pathogen. Thus, the non-aggressive form of the fungus *Septoria nodorum* induced in wheat plants an accumulation of reactive oxygen species (ROS) in the infected zone, whereas aggressive forms of the fungus inhibited generation of ROS due to production of catalases in some cases [1]. Such examples of disengagement of the plant defense system with involvement of pathogen components decreasing the ROS level in the infected zone is one of the most debatable points in the current concept of the development of aggressiveness mechanisms in pathogens of plants [2] and animals [3].

Chitin and glucans are the main structural biopolymers of the cell wall of fungal pathogens, and their presence is an important taxonomic characteristic of representatives of the kingdom Mycota [4]. The acetylation degree of chitin of pathogenic fungi, including wheat parasites, has been shown to actively influence the defense functions of plant cells [5-7]. During the infection process acetylated chitin is rapidly deacetylated in the infection structures due to production by the pathogen of chitin deacetylases (CDA) [5].

Although fungal CDA are considered to determine the aggressiveness of fungi in both plants [8, 9] and animals [10, 11], until now the acetylation degree of chitin is not reliably proved to be important for plant resistance or pathogen aggressiveness. As written in work [12], up to now acetylation of polysaccharides in plants has not been shown to have a clear function. However, highly acetylated chitooligosaccharides [13] and also oligopeptides [14] increased plant resistance to pathogenic fungi more efficiently than poorly acetylated compounds.

Thus it was supposed that pathogens producing CDA could decrease the acetylation degree of chitin of their own cell walls in infected tissues of plants. This promotes development of resistance of the pathogen cell walls to hydrolysis by plant chitinases [15] and lysozyme [11] and also decreases the ROS concentration and accumulation of chitin-specific oxidoreductases in the infected zone [16]. As a result, the efficiency of the subsequent protective lignification of the host's cell walls is suppressed. On the other hand, plants are likely to have a mechanism pre-

Abbreviations: CDA, chitin deacetylases; ROS, reactive oxygen species.

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venting the deacetylation of chitin or other polysaccharide biopolymers, which may determine the course of the infection.

The purpose of the present work was to study the influence on acetylation degree of chitin and chitosan of culture filtrate from strains of the fungus *S. nodorum* Berk. different in aggressiveness and of protein extract from the pathogen-infected wheat plants with different resistance to septoriosis.

MATERIALS AND METHODS

Experiments were performed on seedlings of the susceptible to the septoriosis agent *S. nodorum* Berk. wheat *Triticum aestivum* L. (Zhnitsa cultivar.) and of the resistant wheat *Triticum timopheevii* (Zhuk.) Zhuk. (specimen k-58666 from the collection of the Vavilov Institute of Plant Industry). The mycelium and spores of strains of the chitin-containing fungus *S. nodorum* [17] were kindly presented by workers of the Kuprevich Institute of Experimental Botany, National Academy of Sciences of Belarus.

Wheat seeds were sterilized for 3 min with 70% ethanol, washed in sterile water, and grown on a damp filtering paper at room temperature. Wheat seedlings were grown at room temperature (20-22°C) in an area illuminated for 16-h period with 12,000-16,000 lx (LD-4 and LB-40 lamps). Fully unfolded first leaves of 7-day-old seedlings were cut off and placed into Petri dishes on humid cotton supplemented with benzimidazole (40 mg/liter). Some of the leaves were inoculated with a suspension of pycniospores of the non-aggressive (4VD) and of the aggressive (9MN) strains of the fungus *S. nodorum* (10^6 spores/ml) [18].

To isolate the soluble protein fraction, the control and pathogen-infected wheat leaves were homogenized in 0.01 M Na-phosphate buffer (PB) (pH 6.2) supplemented with 1 mM PMSF, 0.1 μM pepstatin A, polyvinylpyrrolidone (360 Da, 3% of wet weight of the leaves) and incubated at 4°C for 1 h. The ratio of the plant sample weight to the PB volume was 1 : 3 (g/ml). Then the homogenate was centrifuged for 25 min at 14,000*g* in a 5415 K centrifuge (Eppendorf, Germany). The subsequent procedures were performed using the supernatant.

To prepare the culture medium, mycelia of the fungus *S. nodorum* strains different in the aggressiveness were grown for 20 days in a liquid nutrient Czapek medium containing 0.01% KH_2PO_4, 0.02% $NaNO_3$, 0.005% $MgSO_4$, 0.005% KCl, and 0.0001% $FeSO_4$. Glucose (0.5%) was used as a source of carbohydrates on cultivation of the fungi in the culture medium.

Crab chitin (Sonat, Russia) was ground in a mill and sifted. To remove mineral admixtures, the fraction containing particles 125-200 μm in size was suspended in 2 M HCl at room temperature and 2 h later washed in distilled water. The chitin was purified from admixtures by heating in a water bath to 96°C with 1 M NaOH for 30 min using 5-6 changes of the alkaline solution. Then the sorbent was washed in water to neutral value of pH, treated twice with 96% ethanol, and the precipitate was dried. The resulting chitin has ash level lower than 0.1% and acetylation degree ~85%. Chitosan acquired from Sonat had acetylation degree ~3.5%, according to the producer's certificate and analysis data.

Culture filtrates of the fungus *S. nodorum* strains and protein extracts isolated from wheat seedlings were added to chitin or to chitosan at the ratio of 0.1 : 1 (g/ml), and the mixtures were incubated for 24 h at room temperature. Then chitin and chitosan were deproteinated by treatment with 0.1 M NaOH for 1 h, neutralized to pH 5.5, and dried under air flow. The acetylation degree of the biopolymers was determined by titration using an EKSPERT-123456 chemical multimeter (Ekoniks-Ekspert, Russia) and a BAT-15.2MP automated titration block (RUP Gomel factory of measuring instruments, Russia). To the sample of biopolymers under analysis, NaOH solution was added. The acetylation degree (%AD) of chitin and chitosan was calculated using the formula [19]:

$$\%AD = 100 - (0.161 \cdot V_{NaOH} \cdot N_{NaOH}/m),$$

where V_{NaOH} is the titrant volume in ml, N_{NaOH} is molarity of the NaOH solution, and m is the sample weight in grams.

Moreover, acetyl groups in chitosan before and after treatment by the protein extracts from wheat were determined using an AM-300 NMR spectrometer (Bruker, USA) at the working frequencies 300 and 75.47 MHz. Tetramethylsilane dissolved in 1 M HCl in deuterium water was used as an internal standard.

The experiments were performed in three biological repetitions and in each variant 8-10 leaves were fixed. In figures the mean values and standard errors are shown.

RESULTS AND DISCUSSION

The plant response to infection by strains of the pathogenic fungus *S. nodorum* was studied. The pathogen strains were earlier characterized by cultural–morphological parameters and also by aggressiveness toward the wheat cultivar Zhnitsa [1].

Signs of septoriosis appeared as brown spots on the fourth day after the leaves were inoculated with pycniospores of the *S. nodorum* strains. The rates of development of the disease caused by the aggressive and non-aggressive strains of the fungus were different (Fig. 1). On the leaves infected by strain 9MN the light-brown zone of necrosis rapidly increased; on the leaves infected by strain 4VD the disease developed more slowly and the zone of

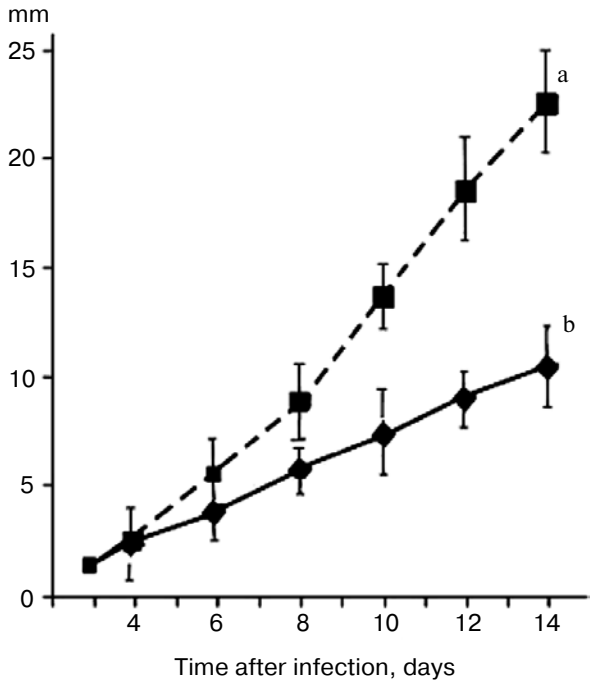

Fig. 1. Development of septoriosis on wheat leaves (mm) infected with the fungus *S. nodorum* strains with different aggressiveness. a) Aggressive strain 9MN; b) non-aggressive strain 4VD.

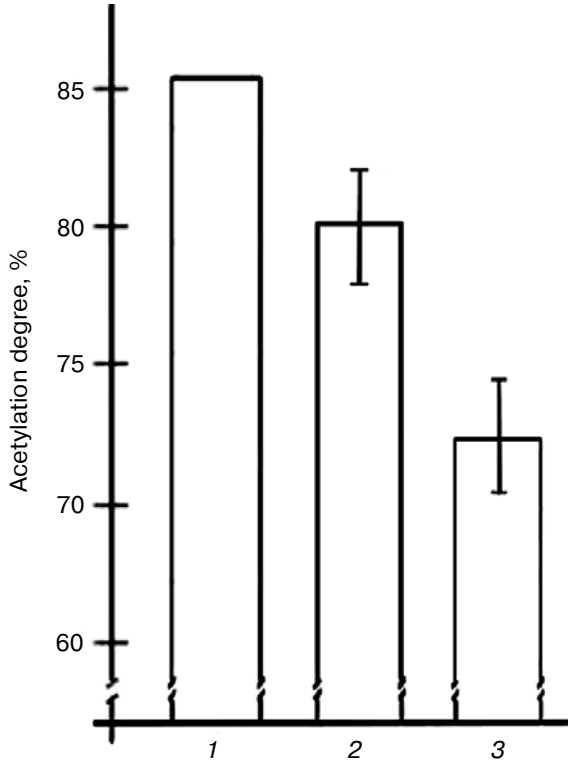

Fig. 2. Changes in the acetylation degree of chitin under the influence of culture filtrates from 20-day-old strains of *S. nodorum* grown in liquid Czapek medium. *1*) Initial chitin (85%); *2*) chitin after treatment by filtrate from non-aggressive fungus strain 4VD; *3*) chitin treated by filtrate from aggressive fungus strain 9MN.

necrosis had an intense brown color. By the eighth day after the inoculation the septoriosis spots on the leaves infected with the 9MN strain were twofold larger than the spots on the leaves infected with the non-aggressive strain. Moreover, the 9MN strain was characterized by a shorter latent period and by formation of picnides with spores as early as by the seventh day of the experiment, whereas with the 4VD strain it occurred only 14 days after infection. Thus, the disease progressed on the wheat leaves infected with the aggressive 9MN strain twofold more intensively than on the leaves infected with a non-aggressive strain 4VD.

Data presented in Fig. 2 show that the culture filtrate from the fungus *S. nodorum* actively influenced the acetylation degree of chitin, and this influence was more pronounced in the case of proteins isolated from the aggressive strain of the fungus. Thus, the acetylation degree of chitin before the experiment was ~85%, and after the treatment with the culture filtrate from the non-aggressive strain 4VD it was 81% and under the influence of the 9MN strain it decreased to 73%.

Because the plant response to a pathogen-caused infection depends on the plant resistance, it was interesting to analyze the ability to acetylate chitosan inherent in proteins from wheat species with different in resistance to the pathogen. Figure 3 shows the acetylation degree of chitosan under the influence of protein extracts from seedlings of the resistant wheat *T. timopheevii* and of the susceptible wheat *T. aestivum* L. Zhnitsa cultivar non-

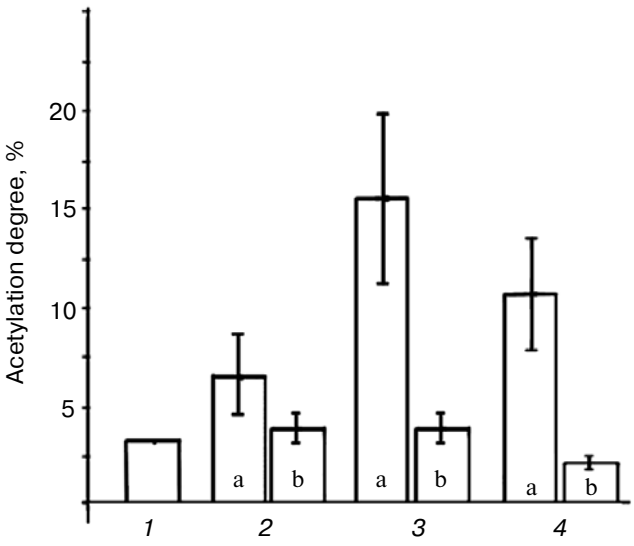

Fig. 3. Influences of extracts from seedlings of *T. timopheevii* (a) and *T. aestivum* (b) on the acetylation degree of chitosan. *1*) Initial chitosan (3.5%); *2*) chitosan treated with protein extract from non-infected wheat seedlings; *3*) chitosan treated with protein extract from wheat seedlings infected with the non-aggressive strain 4VD of the fungus *S. nodorum*; *4*) chitosan treated with protein extract from wheat seedlings infected with the aggressive strain 9MN of the fungus *S. nodorum*.

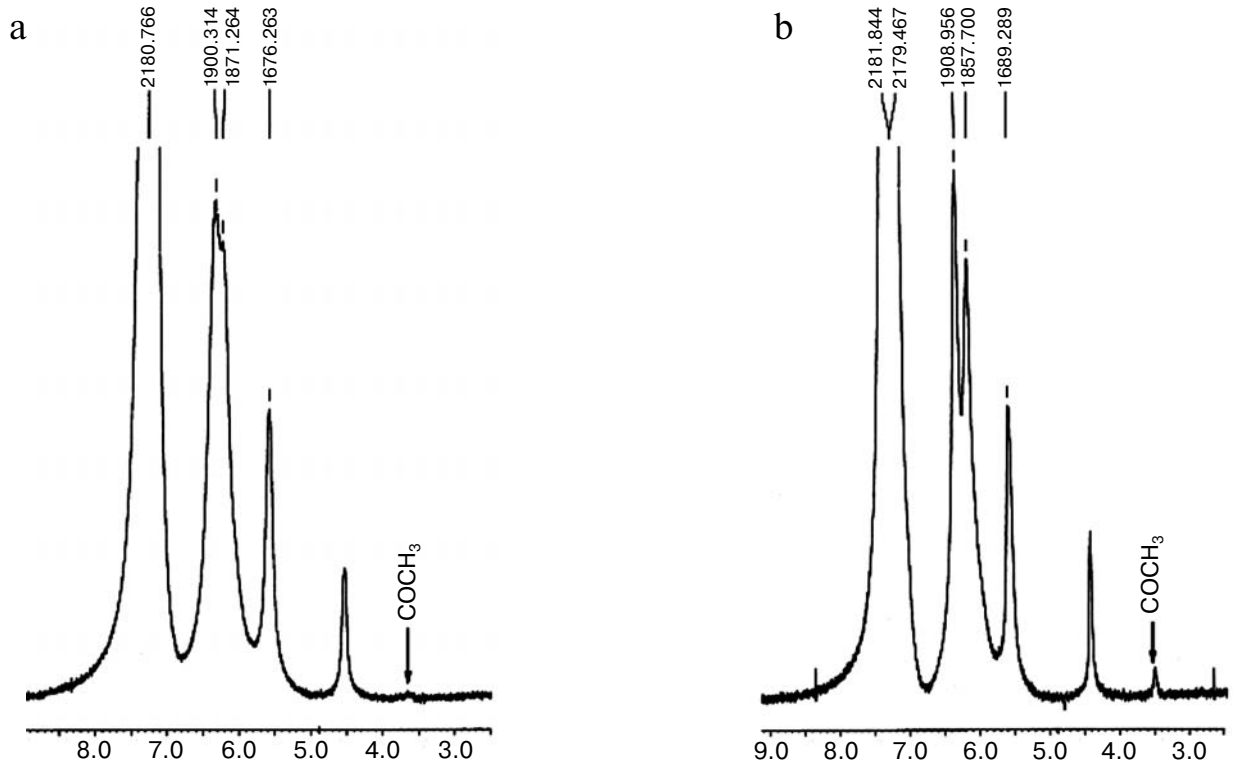

Fig. 4. ^{1}H-NMR spectra of chitosan before (a) and after (b) interaction with extract from seedlings of the resistant wheat *T. timopheevii*.

infected and infected with different strains of the fungus *S. nodorum*. The treatment with the protein extracts changed the chitosan titration profile, and the protein extract isolated from the control and especially from the *S. nodorum*-infected leaves of the wheat *T. timopheevii* acetylated chitosan more rapidly than the protein extract from seedlings of the susceptible wheat. Note that in the case of the susceptible wheat the acetylation degree of the studied biopolymer was also decreased, similarly to the case of the treatment of chitin with the culture filtrate (Fig. 2). An additional determination of the acetylation degree using ^{1}H-NMR spectrometry (Fig. 4) revealed the appearance in the biopolymer spectrum of a peak that according to the literature data [19] corresponds to the acetyl group.

Thus, we found that resistant forms of plants contain substances possessing high chitosan-acetylating activity, which counteracted CDA produced by the aggressive pathogens. Consequently, both the pathogen and the plant during their interaction can regulate the acetylation degree of polysaccharides, and this correlates with the regulation of the local generation of ROS in the infected zone [1].

An increase in the acetylation degree of chitosan under the influence of soluble proteins from seedlings of the wheat *T. timopheevii*, which is highly resistant to various pathogens including the fungus *S. nodorum*, confirmed our hypothesis that acetylation of chitin of the cell walls of fungi should play an important role in the regulation of activities of enzymes involved in ROS production in the pathogen-occupied zone. Another independent mechanism regulating some components of the pathogen activity may be an activation of fungal catalase and CDA followed by a decrease in the ROS level in the infected zone and suppression of sorption on chitin of defense proteins, such as oxalate oxidase and peroxidase with pI ~3.5 [16].

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