Activities of Hydrolases and Their Protein Inhibitors in Wheat Leaves Treated with Salicylic and Jasmonic Acids and Infected with *Septoria nodorum* Strains Differing in Aggressiveness

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Abstract—The effect of infection with fungus *Septoria nodorum* strains differing in aggressiveness on the activity of hydrolytic enzymes (amylases and proteinases) and their protein inhibitors in the leaves of *Triticum aestivum* L. pretreated with salicylic and jasmonic acids was studied. It was shown that aggressive *Septoria nodorum* strains accelerated development, caused intensive necrotic reaction on the wheat leaves, increased the production of amylases and proteinases, and decreased the activity of their protein inhibitors. Salicylic and jasmonic acids altered the activity of the "hydrolase-inhibitor" complex, which indicated the possibility of regulation of the activity (content) of this group of compounds via exogenous effects, including the treatment of plants with inducers of resistance of various natures.

INTRODUCTION

Agricultural plants are attacked throughout ontogenesis by pathogenic microorganisms, which cause significant damage to the yield and quality of crop production. The ability of pathogens to penetrate and propagate in plant tissues is largely determined by the activity of their hydrolytic enzymes [1−3]. The interest in hydrolytic enzymes secreted by microorganisms is due to a number of reasons, one of the most important of which is the initiation and development of the pathological process in plant tissues. Carbohydrases (EC 3.2.1) and proteinases (EC 3.4.21-24), which are involved in the degradation of components of plant cell walls [1, 3, 4] and represent the main physical barrier to microorganism penetration, are the enzymes most widely and diversely present in pathogenic microorganisms. An effective mechanism preventing phytopathogen penetration of the plant and propagation involves the suppression of hydrolase activity by specific protein inhibitors [5−7]. These inhibitors deactivate the foreign enzymes of the penetrating microorganism and increase plant resistance by reducing the intensity of degradation by their own enzymes [8, 9].

Since the morphogenetic features of partners determine the development of plant resistance to pathogens, it is necessary to study the role of hydrolytic enzymes and their inhibitors in the formation of relationships in the "plant−host−pathogen" system, taking into account the characteristics of the causative agent of the disease. It is known that, under natural conditions, the population of the causative agent of Septoria leaf blotch, *Septoria nodorum* Berk, consists of a mixture of fungal strains with various aggressiveness, and the efficiency of protective measures depends on the ratio of aggressive and nonaggressive forms [10, 11].

Salicylic (**SA**) and jasmonic (**JA**) acids are widely used in practical crop farming, since they positively affect plant resistance to pathogens [12−14]. However, the mechanisms of the formation of protective plant response induced by SA and JA have not been studied sufficiently. The study of these mechanisms is especially important due to the need to develop environmentally safe and resource-conserving technologies for agricultural production.

The goal of this study was a comparative analysis of changes in the activity of hydrolytic enzymes and their protein inhibitors in wheat plants treated with JA and SA and infected with strains of the causative agent of Septoria leaf blotch differing in their aggressiveness.

MATERIALS AND METHODS

Object of study. The experiments were performed with leaf sections of the common wheat *Triticum aestivum* L. cultivar Zhnitsa. Seeds were germinated on filter paper at room temperature after treatment (3 h) with solutions of 10^{-6} M SA (Reachim, Russia) or 10^{-7} M JA (Reachim, Russia). The first completely unfolded leaves of seven-day-old seedlings were cut and placed on filter paper in a moist chamber. The cuts were covered with cotton wool wetted with benzimidazole (40 mg/L) [15]. Some of the leaves were inoculated with suspensions of pycnospores of *S. nodorum* strains differing in aggressiveness (10^6 spores/mL) provided by employees of the V.F. Kuprevich Institute of Experimental Botany, National Academy of Sciences, Belarus. Preliminary experiments demonstrated that the 9MN strain was highly virulent and the 4VD strain was low-virulent toward spring soft wheat cultivars Bashkirskaya 24, Saratovskaya 29, Zhnitsa, and Omskaya 9. The inoculated leaves were incubated in the dark for 24 h at room temperature and then transferred to a light pad with a photoperiod of 16 h and an illumination of 12−16 thousand lux. The intensity of symptom expression was assessed 72 h after inoculation. Uninfected and infected plant leaves not treated with SA and JA were used as a control.

Preparation of protein extracts. The plant leaves were washed, dried on filter paper, weighed, and ground in a mortar in 0.05 M Na-phosphate buffer (**PB**), pH 6.2, (1 part plant material per 5 volumes of PB). The extraction was carried out for 30 min at 4°C, the homogenate was centrifuged at 15 000 *g*, and the supernatant was collected to isolate the cytoplasmic protein fraction.

Protein determination. The determination was performed according to Bradford colorimetric method [16]. The protein concentration was determined based on a calibration curve plotted for bovine serum albumin in the range from 1.0 to 0.1 mg/mL.

Proteinase activities. The activity of proteinases hydrolyzing N, α-benzoyl-DL-arginine p-nitroanilide (**BAPNA**) was determined according to the Erlanger method [17].

The amount of enzyme catalyzing the formation of 1 μM of p-nitroanilide per 1 min under standard conditions was taken as one unit (**U**) of activity.

Activities of proteinase inhibitors. The activity of trypsin inhibitors was determined according to the Gofman−Vaisblai method [18] with some modifications. Extract (1.0 mL) and enzyme (0.5 mL, 1 mg/mL) were added to 0.5 mL of 0.05 M Tris-HCl buffer, pH 8.2. BAPNA solution was then added (1.0 mL, 1.0 mg/mL), and the mixture was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 mL of 30% acetic acid. The mixture without enzyme, which was added after the reaction was stopped, was used as the control. The optical density of the resulting solutions was determined with a Bios-pek-Mini spectrophotometer (Shimadzu, Japan) at 405 nm.

The activity of the inhibitor was expressed in inhibitory units (**IU**s). The amount of the inhibitor required for the inhibition of one unit of trypsin activity by 100% under standard conditions was taken as one unit of inhibitory activity.

Amylases and their protein inhibitor activities. Amylase activity was determined by a method based on the use of colored substrate (1% starch) of the enzyme immobilized in 1% gel agarose. The gel was incubated with a solution containing 1 mg/mL of the enzyme for 12 h, and the enzyme activity was determined based on the size of part of the hydrolyzed colored substrate [19]. The amount of the enzyme hydrolyzing the substrate at 1 mm^2 of gel was taken as one milliunit (**mU**) of activity.

The amount of inhibitor suppressing 1 mU of enzyme activity was taken as one milliunit (**mIU**) of inhibitor activity.

Inhibitor activities were determined according to the formula

$$A_i = A_e - A_{e+i},$$

where A_e is the enzyme activity and A_{e+i} is the enzyme activity with inhibitors.

Expression activity of the proteinase inhibitor gene. The expression activity of the proteinase inhibitor (**PI**) gene was evaluated 24, 48 and 72 h after infection. The gene activity was evaluated simultaneously.

RNA from plants was isolated by the phenol-detergent method [20]. The reverse transcription reaction performed with M-MuLV reverse transcriptase according to the company protocol (Fermentas, Lithuania) was used to produce cDNA from the mRNA of the studied plants. Polymerase chain reaction (**RT-PCR**) was carried out in a TP4-PCR-01 type thermocycler (Tertsik, Russia). After amplification, the DNA fragments were fractionated by electrophoresis in 1−2% agarose gel or 7% PAGE in a S2 electrophoretic chamber (Helicon, Russia). PCR of the gene encoding constitutively expressed tubulin was used as a positive control. Highly specific primers for the PI gene (EU 293132.1) and flanking DNA fragments of 121 bp respectively were selected with the Primer Select program (DNAStar). The PCR conditions were selected experimentally. Computer analysis of the amino acid and nucleotide sequences was carried out with the Lasergene computer software package (DNASTAR, Inc, United States).

Statistical data processing. The experiments were performed in at least three biological replications for analysis of the biochemical parameters and at least 15 replications for analysis of the expression. Statistical analysis of the results was performed with the Stat-Soft computer program (Statistica 6.0).
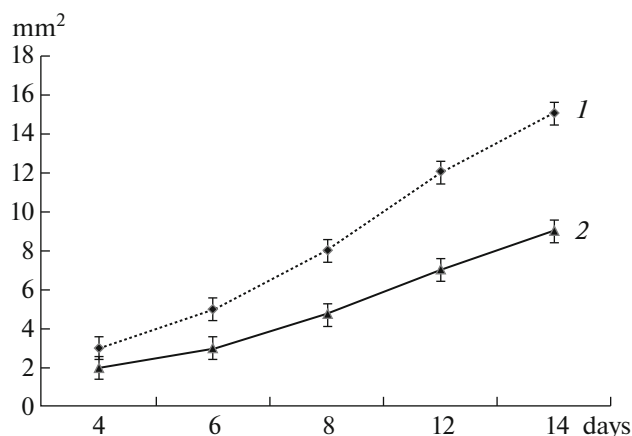
Fig. 1. Dependency of the change in the size of infectious spot (mm^2) on the time of development of highly aggressive (*1*) and weakly aggressive (*2*) strains of the causative agent of Septoria leaf blotch, *S. nodorum*.

RESULTS AND DISCUSSION

Development of *S. nodorum* strains differing in aggressiveness on wheat leaves. Observation of the development of the causative agent of Septoria leaf blotch on wheat leaves made it possible to reveal differences in the dependence of the degree of damage to plant tissues on the aggressiveness of the fungal strains (Fig. 1). Thus, 4 days after inoculation with a highly aggressive strain, the size of the infectious spot was 3.11 ± 0.02 mm^2, while the size of the spot was 1.92 ± 0.01 mm^2 after inoculation with a weakly aggressive strain. After 12 days, the degree of infectivity of plant tissues with a highly aggressive strain was twice that of a weakly aggressive strain.

It can be assumed that the more successful development of highly aggressive *S. nodorum* strains on wheat leaves was associated with the intensive secretion of hydrolytic enzymes. It is known that phytopathogenic microorganisms start to secrete hydrolases, causing the destruction of molecular complexes of the plant cell wall, after initial contact with plants. In cases when hydrolases are involved in pathogenesis, their functions can be quite diverse: from assistance with microorganism penetration of the plant and irreversible inactivation of plant protective compounds to involvement in the transformations of pathogen compounds [2, 21]. Protein inhibitor synthesis is induced in plants in response to the aggressive action of microorganism hydrolases, and the action of such inhibitors can suppress hydrolase activities [5, 6, 9].

Activity of amylases and proteinases in wheat leaves infected with *S. nodorum* treated with SA and JA. It was shown that infection with *S. nodorum* strains with different aggressiveness was accompanied with increased amylase (Figs. 2a and 2b, columns 2) and proteinase activities (Figs. 3a and 3b, columns 2) in plant tissues.
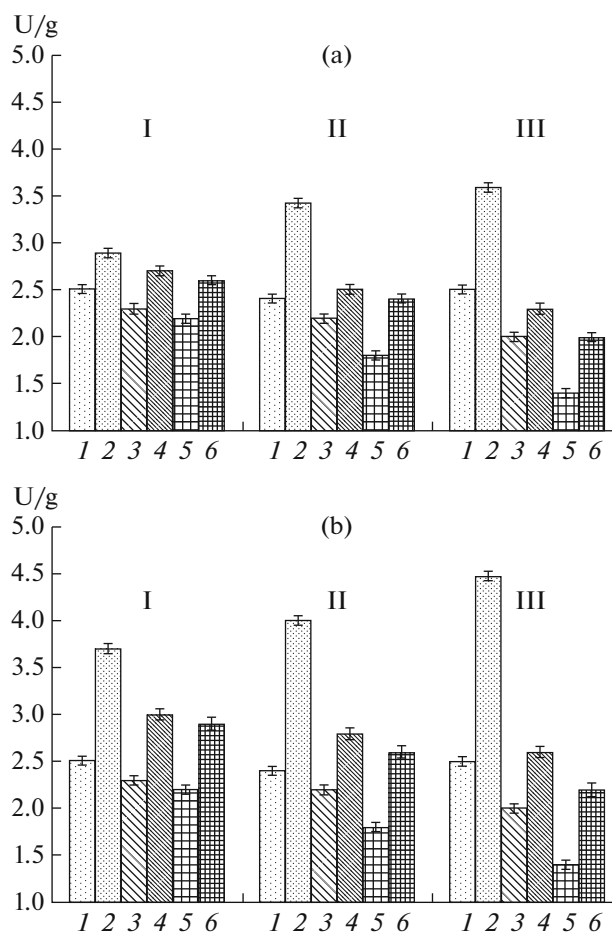

Fig. 2. Amylase activities in wheat leaves of Zhnitsa cultivar in control (*1*) and after treatment with SA (*3*) or JA (*5*) infected with *S. nodorum* strains with low (a) and high (b) aggressiveness before (*2*) and after treatment with SA (*4*) and JA (*6*) 24 (I), 48 (II), and 72 (III) h after inoculation.

At the same time, hydrolase activities increased during the development of the infectious process. The highest increase in hydrolase activities was found for infection with a highly aggressive strain. Thus, 3 days after infection with the 9MH strain, the amylase and proteinase activities in plant tissues increased by 1.8 times in comparison with the uninfected control (Fig. 3b, column 2).

It is known that intensification of biopolymer hydrolysis in plants is one of the nonspecific reactions caused by biotic and abiotic stressors [12]. It can be assumed that, in plants infected with a highly aggressive *S. nodorum* strain, high hydrolytic activity was exhibited by plant enzymes, the synthesis of which was induced by the development of the pathogen and extracellular fungal hydrolases.

Proteinases and amylases were found in a wide range of phytopathogens. Thus, it was shown in the fungi of the genus *Fusarium* (*F. sporotrichioides* and *F. heterosporum*) that the presence of trypsin-like
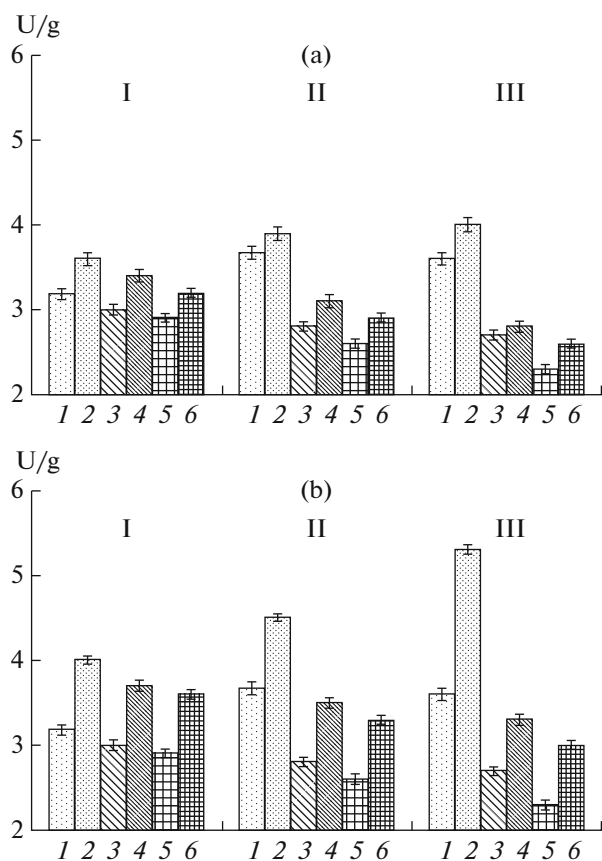
Fig. 3. Proteinase activities in wheat leaves of Zhnitsa cultivar in control (*1*) and after treatment with SA (*3*) and JA (*5*) infected with *S. nodorum* strains with low (a) and high (b) aggressiveness before (*2*) and after treatment with SA (*4*) and JA (*6*) 24 (I), 48 (II), and 72 (III) h after inoculation.

Fig. 4. Activities of amylase inhibitors in wheat leaves of Zhnitsa cultivar in control (*1*) and after treatment with SA (*3*) and JA (*5*) infected with *S. nodorum* strains with low (a) and high (b) aggressiveness before (*2*) and after treatment with SA (*4*) and JA (*6*) 24 (I), 48 (II), and 72 (III) h after inoculation.

activity might indicate the fungal pathogenicity and that the value of this activity might indicate the degree of pathogenicity. Some pathogens are able to transfer proteins through membranes and cell walls directly to the apoplast or to the cytoplasm of the plant cell [1, 5]. Proteins and effectors, which are products of avirulence genes (**Avr**), provoke a protective reaction in plants containing the corresponding resistance genes (**R**) and promote the development of diseases in plants without R genes [3]. The direct correlation between the activity of extracellular proteinases of microorganisms and the intensity of disease in plants observed in a number of cases [2] indicates the important role of pathogen proteases in penetration via the protective barriers of the host plant and active propagation in plant tissues.

The role of amylases in the processes of plant pathogenesis has not been adequately studied. However, their wide prevalence in fungi suggests that amylases are important participants of their metabolism, providing successful development in plant tissues. Indeed, it was shown that oomycete *Phyto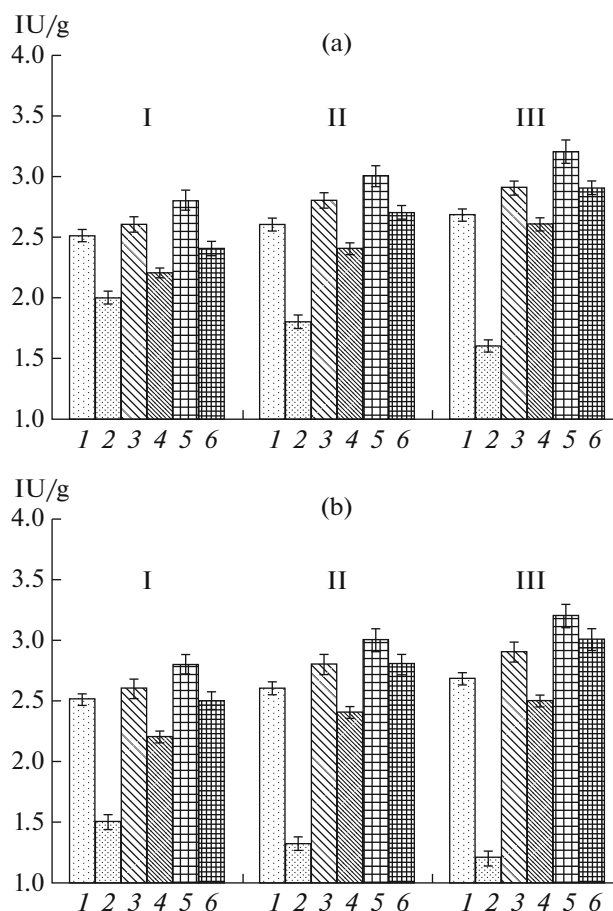phthora infestans* does not contain amylase but produces compounds inducing the biosynthesis of these enzymes in infected potato tubers [6].

It turned out that treatment with SA and JA caused a decrease in hydrolase activities (Figs. 2a, 3a and 3b, columns 3–6), both in healthy and in infected plants. This indicated the induction of protective plant mechanisms by SA and JA.

Effect of SA and JA treatment on the activity of hydrolase inhibitors in wheat leaves infected with *S. nodorum*. Infection of the wheat leaves changed the activity level of hydrolase inhibitors. In this case, the activity of amylase inhibitors decreased (Fig. 4), and this decrease was especially significant in plants infected with a highly aggressive *S. nodorum* strain (Fig. 4b, column 2). Thus, after 72 h, the activity of amylase inhibitors in infected leaves decreased by more than two times in comparison with the control (Fig. 4b, column 2 in III). It can be assumed that this was due to the formation of inactive complexes between enzyme and inhibitor molecules [5].
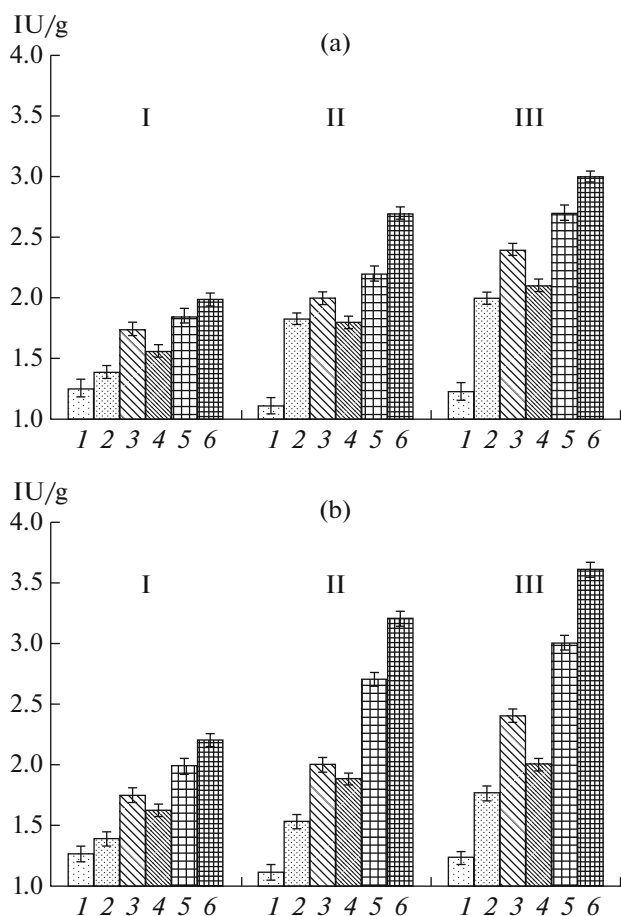
Fig. 5. Activities of proteinase inhibitors in wheat leaves of Zhnitsa cultivar in control (*1*) and after treatment with SA (*3*) and JA (*5*) infected with *S. nodorum* strains with low (a) and high (b) aggressiveness before (*2*) and after treatment with SA (*4*) and JA (*6*) 24 (I), 48 (II) and 72 (III) h after inoculation.

It is known that inhibitors of protein nature selectively interact with amylases [7]. Among them there are bifunctional inhibitors capable of simultaneously interacting with α-amylase and protease [14].

It turned out that the activity of proteinase inhibitors increased in infected tissues (Fig. 5). The maximum antiproteinase activity was observed in plants within 72 h after infection with a weakly aggressive strain (Fig. 5a, column 2).

In the previous study [22], it was shown that an important factor providing high aggressiveness of the fungus *S. nodorum* is the degree of expression activity of the fungal catalase gene. Now, it has been proved that reactive oxygen species (**ROS**), including hydrogen peroxide (H$_2$O$_2$), play an important role in the relationship between plants and pathogens [23, 24]. Thus, a sharp and multifold increase in the ROS content (oxidative burst) during infection induces in plants a cascade of protective reactions, including the synthesis of protective proteins [22, 25], and their low

concentration promotes the pathogen growth [26]. It can be assumed that the differences in the dynamics of proteinase inhibitor activity in wheat leaves infected with *S. nodorum* strains with different degrees of aggressiveness were due to different catalase activity in plant tissues.

Pretreatment with signaling molecules promoted an increase in the activity of hydrolase inhibitors upon infection with *S. nodorum* strains differing in aggressiveness (Figs. 4, 5), and JA had the most significant stimulating effect.

It was shown that SA induces the synthesis of NBS-LRR proteins [23], which are involved in the functioning of many signaling pathways in pea roots. One of their functions involves participation in the recognition of effector proteins excreted by pathogens. Effector proteins trigger signaling pathways, resulting in the synthesis of protective proteins and the induction of phytoimmunity [12].

JA is an important signaling molecule involved in the activation of protective responses upon mechanical damage (wound stress) to plant tissues and pathogen attack [14, 23]. It was shown that the treatment of plants with methyl jasmonate resulted in an increased content of two isoforms of proteinase inhibitors [27].

Change in the transcriptional activity of the proteinase inhibitor gene under the effect of SA and JA and infection with *S. nodorum*. One mechanism of increased proteinase inhibitor activity is associated with an increased expression of their genes. It was shown that the expression of EU 293132.1 gene, which encodes the proteinase inhibitor, increased as a result of infection with *S. nodorum* after treatment with JA and SA (Fig. 6). SA had a more effective stimulating effect on the expression of this gene in healthy plants than in plants infected with both weakly and highly aggressive strains. The expression level of the EU 293132.1 gene was maximal in JA-treated plants in comparison with all other variants of the experiment.

A high activity of proteinase inhibitors in tissues is one of the factors of high plant resistance to fungal and bacterial invasions [1, 3, 8]. SA and JA are able to increase the level of formation of H$_2$O$_2$ [2, 14], thus inducing the synthesis of protective proteins. Studies using reconstructed signaling systems in *Argobidopsis thaliana* L. protoplasts demonstrated that H$_2$O$_2$ via MAP-kinase (mitogen-activated protein kinases) cascade regulates the expression of protective protein genes [22].

CONCLUSIONS

Thus, it was shown that aggressive strains of *S. nodorum* accelerated development and intensive necrotic reaction on wheat leaves and exhibited the ability to suppress the development of plant protective reactions, increasing the production of amylases and proteinases while reducing the activity of their protein
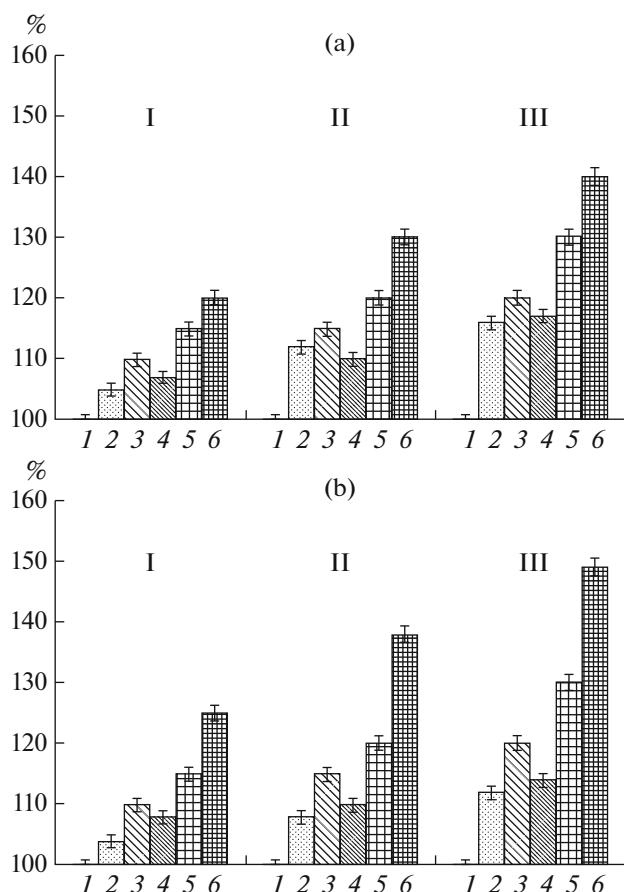
Fig. 6. Level of transcripts (% of control) of the proteinase inhibitor EU 293132.1 gene in wheat leaves in control (*1*) and after treatment with SA (*3*) and JA (*5*) infected with *S. nodorum* strains with low (a) and high (b) aggressiveness before (*2*) and after treatment with SA (*4*) and JA (*6*) 24 (I), 48 (II), and 72 (III) h after inoculation.

inhibitors. The ability of SA and JA to change the activity of the "hydrolase-inhibitor" complex indicated the possibility of regulation of the activity (content) of this group of compounds by exogenous effects, including plant treatment with inducers of resistance of various natures [2, 4, 9].

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