ISSN 1021-4437, Russian Journal of Plant Physiology, 2018, Vol. 65, No. 3, pp. 412–418. © Pleiades Publishing, Ltd., 2018.
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RESEARCH PAPERS

Effect of Salicylic and Jasmonic Acids on the Content of Hydrogen Peroxide and Transcriptional Activity of the Genes Encoding Defense Proteins in Wheat Plants Infected with *Tilletia caries* (DC.) Tull.

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Received April 7, 2017

Abstract—We investigated the effect of presowing treatment of seeds with salicylic (SA) and jasmonic (JA) acids on the growth of wheat *Triticum aestivum* L. seedlings, generation of hydrogen peroxide (H_2O_2) therein, and transcriptional activity of the genes encoding defense proteins—oxalate oxidase (OxO), peroxidase (PO), and proteinase inhibitor (PI)—upon their inoculation with stinking smut pathogen *Tilletia caries* (DC.) Tull. SA and JA were found to reduce adverse effect of *T. caries* on growth of seedlings and the extent of their infection by the pathogen. Improvement of plant resistance to *T. caries* depended on a stimulatory effect of SA and JA on the formation of H_2O_2 in plant tissues and changes in activity of OxO, PO, and catalase. SA was shown to elevate transcriptional activity of the genes of oxalate oxidase and peroxidase. We detected a considerable stimulatory effect of JA on transcriptional activity of the gene encoding proteinase inhibitor. Revealed differences in the activation of defense proteins pointed to differing mechanisms of SA and JA action on protective potential of wheat plants infected with *T. caries*.

Keywords: Triticum aestivum, *Tilletia caries*, oxidoreductases, proteinase inhibitors, salicylic acid, jasmonic acid, induced resistance

DOI: 10.1134/S1021443718020097

INTRODUCTION

Plant resistance to abiotic and biotic environmental factors, including infection with pathogens, is based on realization of a complex of physiological and biochemical processes occurring at cell, tissue, and organism levels. It is known that protective reaction of plant organisms to a fungal infection in many respects depends on trophic specialization of fungi. A long coevolution of plants and microorganisms resulted in emergence of pathogens with different types of parasitism: biotrophs, necrotrophs, and hemibiotrophs.

Typical representatives of pathogens with biotrophic nutrition are smuts whose control is an important task today [1]. Stinking smut pathogen *Tilletia caries* (DC.) Tull. penetrates plants primarily via coleoptiles several days after germination of infected caryopsis. Prior to the onset of grain ripening, the fungus develops in plant tissues without visible symptoms and is detected only by means of cytological analysis of microscopic sections [2], which complicates investigation of the mechanism of resistance to pathogen and search for inductors of resistance [3].

Induction of defense response in plants involves various signal systems that are triggered by signal molecules. Such a function may be performed by salicylic (SA) and jasmonic (JA) acids, hydrogen peroxide, and some other molecules [4, 5]. In relation to discovery of a signal role of hydrogen peroxide, the enzymes regulating its level in the course of pathogenesis, such as peroxidase (PO) and oxalate oxidase (OxO), have attracted particular attention.

A pathogen's ability to invade plant organisms and develop therein very largely depends on the activity of its extracellular hydrolases, specifically proteinases. In response to the action of proteinases, plants induce the synthesis of proteinaceous inhibitors that suppress the activity of these enzymes [6, 7]. Investigation of the effect of SA and JA on development of a protective reaction of wheat involving proteinase inhibitors (PI) and oxidoreductases (oxalate oxidase, peroxidase, and catalase) upon infection with *Tilletia caries* is of scientific and applied interest.

Abbreviations: BAPNA—N,α-benzoyl-DL-arginine *p*-nitroanilide; IU—inhibitor units; JA—jasmonic acid; OxO—oxalate oxidase; PB—phosphate buffer; PI—proteinase inhibitors; PO—peroxidase; SA—salicylic acid; SB—succinate buffer.

The mechanisms of induction of defense response in plants to SA and JA present in smuts are not known in detail. It was found that presowing treatment of infected cereals with a stress phytohormone JA brought about a considerable activation of proteinase inhibitors; exposure of plants to exogenous influence of another stress phytohormone (salicylic acid) boosted production of ROS [4, 8]. These compounds efficiently combated stinking smut both under controlled conditions and in the field [6, 9, 10].

The aim of this work was to look into the effect of SA and JA on the growth of wheat plants, generation of H_2O_2 therein, and modification of expression of the genes encoding defense proteins (oxalate oxidase, peroxidase, and proteinase inhibitor) upon inoculation with *T. caries*.

MATERIALS AND METHODS

Test subjects. Sprouting seeds of wheat *T. aestivum* L., cv. Zhnitsa, were powdered with dry spores of pathogen *Tilletia caries* (DC.) Tull. on the basis of 1 g per 100 seeds [11]. Infectious material was obtained from the Laboratory of Plant Immunity Biochemistry, Institute of Biochemistry and Genetics, Ufa Scientific Center, Russian Academy of Sciences. SA (Reakhim, Russia) and JA (Reakhim) at appropriate concentrations (0.05 mM and 10^{-7} M, respectively) were used for the soaking of wheat seeds during 3 h. Inoculated seeds were sown in pots that were kept for three days in a moist chamber at a temperature of 10–15°C. Seven days after inoculation, we estimated the extent of seedlings' affection. The fungi were stained according to Gram with aniline gentian violet [12].

Assay of H_2O_2. Seven-day-old seedlings were homogenized in 0.025 M phosphate buffer (PB), pH 6.2, at a ratio of 1 : 3 and centrifuged for 20 min at 10000 *g*. Supernatant was used for the assay of H_2O_2. The content of H_2O_2 was determined at 560 nm using xylenol orange [13]. The reagent contained 0.074% Mohr's salt in 5.81% sulphuric acid and 0.009% xylenol orange in 1.82% sorbitol (at a ratio of 1 : 100). Optical density of reaction products was measured using a Biospec-Mini spectrophotometer (Shimadzu, Japan).

Determination of oxalate oxidase activity. Cytoplasmic fraction of the enzyme was isolated using 0.05 M succinate buffer (SB), pH 3.8. To this end, the seedlings were homogenized in SB at a ratio between the weight of leaf sample and SB volume of 1 : 3. Extract was centrifuged for 20 min at 12000 *g* (Eppendorf, Germany). Reaction mixture designed to determine OxO activity contained 100 μL of SB, 0.0025 M oxalic acid (Reakhim, Russia), 50 μL of enzyme extract and commercial horse-radish peroxidase (Amresco, United States) at a concentration of 15 units/mL, and 0.08% chromogenic substrate *o*-phenylenediamine (OPD) (Reakhim).

Determination of peroxidase activity. In order to isolate cytoplasmic fraction of PO, segments of leaves were homogenized in 0.01 M Na-phosphate buffer (PB), pH 6.2. The ratio between the weight of leaf sample and PB volume was 1 : 3. Extract was centrifuged for 25 min at 12000 *g* (Eppendorf). Supernatant was used for determination of PO activity. PO and OxO activities were determined by means of a micromethod by OPD oxidation at 490 nm using a Benchmark Microplate Reader photometer for immune-enzyme analysis (BioRad) [13].

Determination of catalase activity. Plant tissue was homogenized in 50 mM PB (pH 7.8). The ratio between the weight of a sample and the volume of PB was 1 : 10. After centrifugation at 12000 *g,* supernatant was used for determination of catalase activity. Reaction was initiated by the addition of 0.1 mL of supernatant to 0.2 mL of 0.03% H_2O_2. Control sample contained 0.1 mL of distilled water instead of supernatant. Reaction was terminated in 10 min with 1 mL of 4% ammonium molybdate. Intensity of the resulting color was measured at a wavelength of 410 nm using a Biospec-Mini spectrophotometer.

Determination of activity of proteinases and their inhibitors. Activity of proteinases hydrolyzing N,α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA) was determined according to Erlanger [14]. The amount of enzyme producing 1 μmole of *p*-nitroaniline per min in standard conditions was taken as a unit of enzyme activity (EU).

Activity of trypsin inhibitors was determined according to a method described by Gofman and Vaisblai [15] with modifications. One milliliter of extract and 0.5 mL of enzyme (1 mg/mL) were added to 0.5 mL of 0.05 M Tris-HCl buffer, pH 8.2. Then the mixture was supplemented with 1.0 mL of BAPNA solution (1 mg/mL) and incubated for 10 min in a water bath at 37°C. The reaction was terminated with 0.5 mL of 30% acetic acid. The control mixture lacked the enzyme that was added after the termination of the reaction. Optical density of the obtained solutions was determined at 405 nm using a Biospec-Mini spectrophotometer.

Activity of inhibitor was expressed in inhibitor units. Under standard conditions, the amount of inhibitor necessary for total suppression of a unit of trypsin activity was taken as a unit of inhibitor activity.

Transcriptional activity of the genes of defense proteins. RNA was isolated from plants using TRIzol (Molecular Research Center, Inc., United States). cDNA was obtained on the basis of mRNA of investigated samples by means of reverse transcription using M-MuLV reverse transcriptase according to the manufacturer's protocol. Polymerase chain reaction with reverse transcription proceeded in a TP4-PCR-01 amplifier (Tertsik, Russia). After amplification, DNA fragments were fractionated by means of electrophoresis in 1–2% agarose gel or 7% PAAG. PCR of the

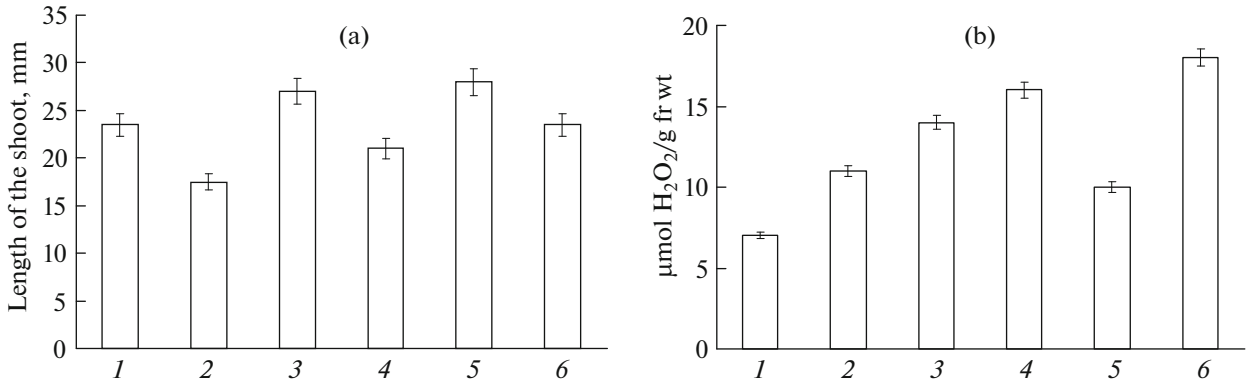

Fig. 1. Effect of SA, JA, and *T. caries* on growth of (a) aboveground part of 7-day-old seedlings of wheat, cv. Zhnitsa and (b) content of H_2O_2 therein. *1*—noninoculated seedlings; *2*—inoculated seedlings; *3*—SA treatment; *4*—SA treatment + inoculation; *5*—JA treatment; *6*—JA treatment + inoculation.

gene encoding constitutively expressed tubulin was used as a positive standard. By means of Primer Select program (DNASTAR), we selected high-specific primers to the gene of oxalate oxidase (GenBank database record number AJ556991), anionic peroxidase (AK333699), proteinase inhibitor (EU 293132.1), and flanking DNA fragments of 410, 157, and 106 bp, respectively. PCR parameters were chosen experimentally. Amino acid and nucleotide sequences were analyzed using a Lasergene program package (DNASTAR Inc, United States).

Statistic treatment of the results. As to biochemical parameters, all the experiments were repeated at least three times; determination of transcriptional activity was repeated at least 15 times. The results were statistically treated using StatSoft programs (Statistica 6.0). Figures show the means and their standard errors.

RESULTS AND DISCUSSION

Effect of SA and JA on Wheat Plants' Growth and Resistance to T. caries

Our experiments showed that presowing treatment of wheat seeds with SA and JA stimulated growth of the aboveground part of seedlings (by 17% and 21% in SA and JA types of treatment, respectively) (Fig. 1a). Inoculation with *T. caries* suppressed seedlings' growth by 25%, which was earlier observed by other researchers [6]. Most probably, this is accounted for by a considerable rise in the level of ABA in susceptible wheat plants infected with stinking smut pathogen [16]. On the one hand, a high level of ABA is plant response to stress and, on the other hand, it causes a suppression of growth processes.

When the plants were pretreated with inductors of resistance prior to inoculation, the growth of seedlings slowed down by approximately 20% as compared with plants pretreated but not infected. At the same time, pretreated and inoculated seedlings grew faster than untreated and infected (in SA type of treatment by 22% and by 33% in JA type of treatment). Thus, pretreatment of wheat seedlings with SA and JA reduced an adverse effect of *T. caries* on their growth. Apparently, this occurs owing to considerable changes in the content of phytohormones [6, 10] and due to a reduction in the number of affected plants (Fig. 2).

Changes in H_2O_2 Content in Wheat Seedlings Treated with SA and JA and Inoculated with T. caries

Some mechanisms of improvement of plant resistance to *T. caries* under the effect of SA and JA could depend on changes in H_2O_2 concentration in plant tissues. Our experiments showed that, in the seedlings pretreated with SA and JA, the level of H_2O_2 was higher than in control plants (Fig. 1b). Inoculation of seedlings brought about a rise in H_2O_2 content therein,

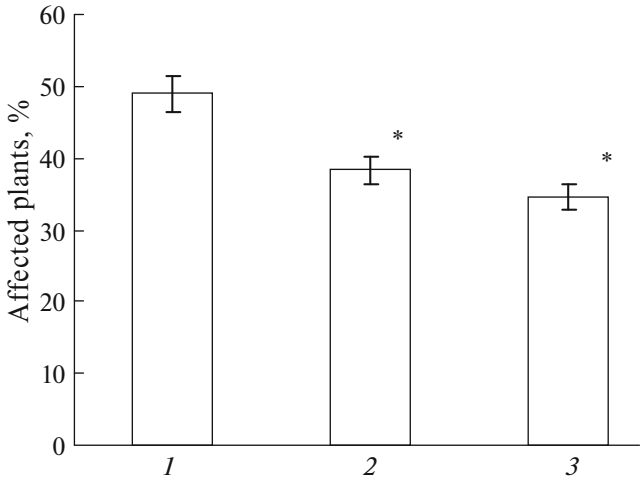

Fig. 2. Effect of presowing treatment of seeds with SA and JA on resistance to pathogen of stinking smut of wheat, cv. Zhnitsa. *1*—untreated seedlings; *2*—SA treatment; *3*—JA treatment. * Differences are reliable at $P < 0.05$ as compared with control material (*1*).

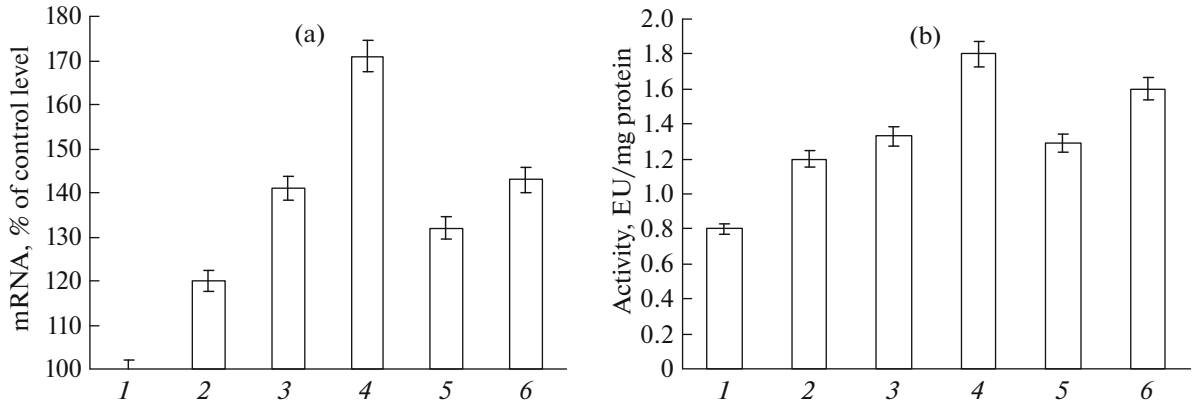

Fig. 3. Accumulation of transcripts of (a) oxalate oxidase gene and (b) activity of the enzyme in the leaves of wheat inoculated with *T. caries* and treated with signal molecules. *1*—control material; *2*—inoculation with *T. caries*; *3*—SA treatment; *4*—SA treatment + *T. caries*; *5*—JA treatment; *6*—JA treatment + *T. caries*.

with accumulation of H_2O_2 in pretreated plants being more pronounced than in untreated plants. More intense generation of ROS in infected cultured plant cells was also observed by other researchers [17]. Similar results were obtained in wheat plants contaminated with powdery mildew pathogen and in numerous plants infected with gray rot pathogen [18, 19]. This is related to the fact that a high content of H_2O_2 in the cell is a sign of supersensitivity [5].

Effect of SA and JA on the Activity of Defense Proteins upon Infection with T. caries

Changes in the content of H_2O_2 in plant tissues in the course of pathogenesis may occur as a result of numerous metabolic processes but largely owing to fluctuation of enzyme activity of the pro/antioxidant system. Along with NADPH-oxidase and amine oxidase, oxalate oxidase is an important component of this system and its activation elevates the content of H_2O_2 in infected plants [20].

Our experiments showed that SA and JA stimulate transcriptional activity of the gene encoding OxO both in robust wheat seedlings and in the seedlings infected with *T. caries*, with the enzyme activity also rising (Fig. 3).

The main function of OxO is participation in degradation of oxalic acid that is a factor of virulence in numerous pathogenic fungi [5]. The enzyme activity is high in the seedlings of monocots but it decreases in the course of their growth and development and is essentially lacking in mature plants [17]. However, the gene encoding OxO may be expressed as a result of infection. This implies participation of oxalate oxidase in the modification of cell wall components in the course of germination and protection of seedlings against phytopathogens. In this relation, OxO is ranked among *germin*-proteins [20]. Its activation in the cells adjacent to contamination zone is probably responsible for the formation of a safety area with a high content of H_2O_2.

In our investigations, pretreatment of plants with SA and JA also induced transcriptional activity of peroxidase gene (Fig. 4). The same as with OxO, the stimulatory effect of SA on PO activity was more pronounced than the effect of JA (Fig. 4).

Peroxidase is a dual-purpose enzyme. Its main functions are protection of plant organism from adverse effect of ROS [21] and direct participation in differentiation of tissues and organs in higher terrestrial plants. These functions of various peroxidases are closely related to catalyzing polymerization of phenolic monomers leading to lignin, suberin, and cutin. At the same time, apoplastic peroxidases may show oxidase activity and generate superoxide radical and hydrogen peroxide upon a change in physiological pH [22].

Signal molecules may influence peroxidase activity in different ways. For instance, exogenous treatment of wheat coleoptiles with H_2O_2 inhibited peroxidase activity therein [19]. A reason for suppression of peroxidase activity caused by H_2O_2 may be its switching over to catalase activity. In particular, such an occurrence was recorded for several forms of apoplastic peroxidases that acted as catalases at high concentrations of H_2O_2 [18, 22].

Our experiments showed that catalase activity in the course of experiment rose by 30% on average in wheat seedlings infected with *T. caries* (Fig. 5). In noninoculated and inoculated seedlings treated with SA and JA, catalase activity was lower than in control material over the whole experiment. It is interesting that, under the effect of SA, catalase activity was inhibited stronger than in the presence of JA, especially in infected wheat seedlings. This is probably accounted for by the fact that one of the mechanisms of development of a system of induced resistance consists in SA-caused inhibition of catalase activity, which promotes accumulation of H_2O_2 in plant tissues [10].

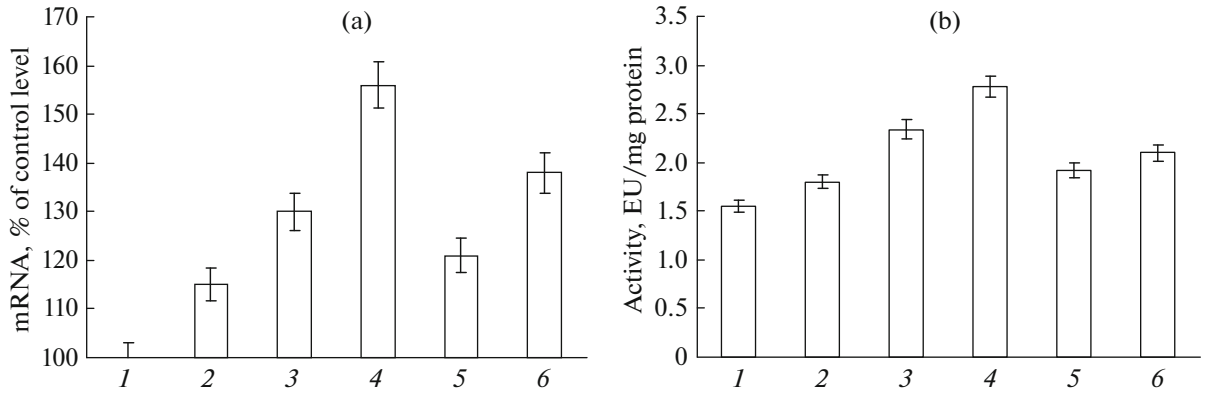

Fig. 4. (a) Accumulation of peroxidase gene transcripts and (b) the enzyme activity in the leaves of wheat inoculated with *T. caries* and treated with signal molecules. *1*—control material; *2*—inoculated with *T. caries*; *3*—SA treatment; *4*—SA treatment + *T. caries*; *5*—JA treatment; *6*—JA treatment + *T. caries*.

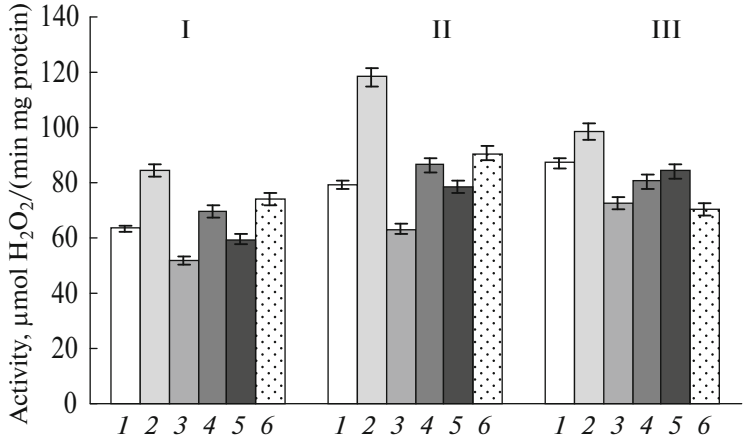

Fig. 5. Effect of SA and JA on catalase activity in the seedlings of wheat inoculated with stinking smut pathogen *T. caries*. *1*—control material; *2*—inoculation with *T. caries*; *3*—SA; *4*—SA + *T. caries*; *5*—JA; *6*—JA + *T. caries*. I—3 days; II—5 days; III—10 days.

The number of immunosuppressants (or pathogenicity factors) of pathogenic organisms that counteract defense reactions of the host plant is high and our knowledge about them is slight. It was found that catalases that quickly decompose H_2O_2 are actively produced by a pathogen during the period of infectivity. As a result, the fungi may counteract the influence of high concentrations of H_2O_2 generated by plant cells for defense and successfully develop in plants. It is interesting that a low concentration of H_2O_2 in the zone of contamination induces catalase activity in pathogenic fungi [19]. One can assume that negative regulation of H_2O_2 level involves compounds excreted by the fungus in the course of its interaction with the host plant.

Effect of SA and JA on the Activity of Proteinases and Their Inhibitors

The main pathogenicity factors of microorganisms are hydrolases and, specifically, proteinases. For instance, fungal proteinases may cleave antibacterial proteins of plants and actively participate in the degradation of cell wall proteins [23]. Suppression of hydrolase activity by specific inhibitors sets up efficient barriers to pathogens' invasion and migration in plants [24].

On the seventh day after inoculation with *T. caries* spores, proteinase activity in wheat coleoptiles slightly decreased (Fig. 6). After pretreatment with signal molecules, hydrolytic activity also decreased, which was probably accounted for by a rise in antihydrolytic activity in plant tissues (Fig. 7b).

As compared with necrotrophs, biotrophic pathogens are less diverse; they accumulate lower concentrations of metabolites toxic for plants and hydrolytic enzymes therein are less active. For instance, in barley powdery mildew pathogen *Erysiphe graminis*, hydrolytic enzymes are only excreted from the tip of infectious hypha and their activity shows only at a distance of 0.1 μm from the hypha. Moreover, the effect of some enzymes detrimental to plant tissue may be nonexistent. For instance, a biotroph *Uromyces fabae* lacked activity of some enzymes participating in degradation of plant cell wall carbohydrates: endopolyga-

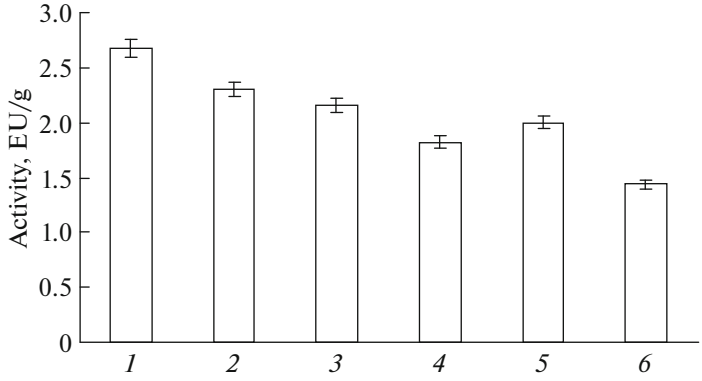

Fig. 6. Proteinase activity in coleoptiles of wheat treated with signal molecules and inoculated with *T. caries*. *1*—control material; *2*—inoculation with *T. caries*; *3*—SA treatment; *4*—SA treatment + *T. caries*; *5*—JA treatment; *6*—JA treatment + *T. caries*.

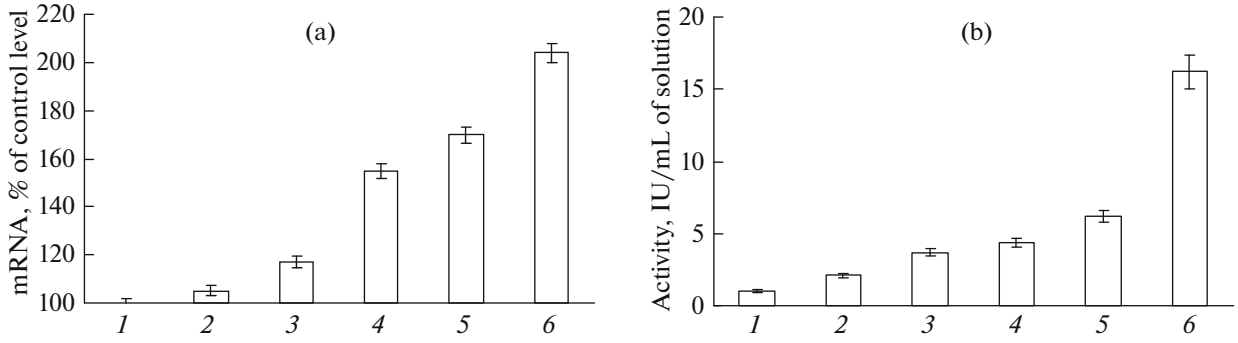

Fig. 7. (a) Accumulation of transcripts of proteinase inhibitor gene and (b) the enzyme activity in the leaves of wheat inoculated with *T. caries* and treated with signal molecules. *1*—control material; *2*—inoculation with *T. caries*; *3*—SA treatment; *4*—SA treatment + *T. caries*; *5*—JA treatment; *6*—JA treatment + *T. caries*. IU—inhibitor units.

lacturonase, endo-pectate lyase, and cellulase. At the same time, a nectrotroph *Verticillium albo-atrum* showed rather high activity of these enzymes [17].

Treatment with SA and JA prior to inoculation reduced hydrolase activity. A decrease in proteolytic activity is most probably related to a stimulatory effect of signal molecules on expression of the gene encoding proteinase inhibitor, with the greatest activation of gene expression occurring in plants pretreated with JA (Fig. 7a).

Thus, presowing treatment of the seeds with SA and JA improved wheat resistance to *T. caries* owing to accumulation of more H_2O_2 and stimulation of activity of defense proteins in plant tissues, with SA activating transcriptional activity of the genes of OxO and PO. On the contrary, treatment of plant material with JA considerably stimulated transcriptional activity of PI gene. Detected differences in activation of individual defense proteins pointed to differing mechanisms of SA and JA action on protective potential of wheat plants infected with *T. caries*.

ACKNOWLEDGMENTS

This work was done using equipment belonging to Biomika Shared Instrumentation Center (Department of Biochemical Research and Nanobiotechnology, Agidel' Regional Shared Instrumentation Center) and KODINK Unique Research Unit.

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Translated by N. Balakshina