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EXPERIMENTAL ARTICLES

Comparative Assessment of Inductive Effects of *Azospirillum* Lectins with Different Antigenic Properties on the Signal Systems of Wheat Seedling Roots

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Abstract—The lectins of associative nitrogen-fixing bacteria *Azospirillum brasilense* Sp7 and its mutant *A. brasilense* Sp7.2.3 were shown to have different effects on the components of the wheat seedling root signal system, namely to regulate the levels of cAMP, nitric oxide, diacylglycerol, and salicylic acid, as well as to induce the activities of superoxide dismutase and lipoxygenase. Our results make it possible to consider azospirilla lectins as inducers of the signal systems in wheat seedling roots, since they cause development of several flows of primary signals. These data are of general biological importance, since lectins are present in all living organisms and most of the functions of lectins remain insufficiently understood.

Keywords: rhizosphere, associative nitrogen fixation, *Azospirillum* lectins, wheat seedling roots, signal molecules

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Associative bacteria of the genus *Azospirillum* hold a prominent place among the microorganisms that are potentially able to stimulate plant growth and development. Plants benefit directly from the ability of these microorganisms to fix nitrogen, produce phytohormones, solubilize phosphates, improve water and mineral status, and produce a number of compounds increasing the membrane activity and proliferation of root system tissues, as well as decreasing the effect of stress factors on plants, and controlling multiple plant pathogens [1–3]. One of the mechanisms of plant-mediated biocontrol effect of the microorganisms is their ability to induce defensive reactions aimed to improve plant stability. Salicylic acid, bacterial lipopolysaccharides, and siderophores may be the signaling molecules triggering the cascade of protective reactions.

Since many of azospirilla are unable into penetrate plant cells, these bacteria are able to produce the signal molecules that penetrate through the plant cell wall and are recognized by the membrane receptors of the plant. This interaction initiates a chain of events leading to changes in the plant metabolism. Plant response at the cellular level may be an indicator of plant–bacteria interactions mediated by bacterial molecular signals. Binding of *A. brasilense* Sp245 cell receptors with wheat germ agglutinin (WGA) is known to induce changes in metabolism of the bacterial cell: it increases nitrogen fixation, excretion of ammonium ions, and synthesis of indole-3-acetic acid (IAA) and changes the ratio between acidic phospholipids of the membrane. WGA may function as a signaling molecule in the *Azospirillum–plant* association [4].

At the same time, some *Azospirillum* strains are known to be capable of production of various lectins in vitro [5]. Nikitina et al. [6] demonstrated the presence of lectins involved in bacterial adhesion to roots on the surface of azospirilla cells. A lectin of glycoprotein nature with molecular mass of 36 kDa and specificity to L-fucose (1.87 mM) and D-galactose (20 mM) was isolated from the surface of *A. brasilense* Sp7. The lectin of a mutant strain *A. brasilense* Sp7.2.3 had molecular mass and carbohydrate specificity identical to those of the parent strain lectin, but differed by its antigenic properties [7]. These proteins were demonstrated to exhibit varying functional activity. Lectins affected the activity of α-, β-glucosidase, and β-galactosidases in the membrane and the apoplast fraction of wheat germ roots with varying efficiency [2].

The goal of the present work was to reveal the signaling functions of the lectins of *A. brasilense* Sp7 and Sp7.2.3 (in comparison with each other) in response reactions of plants.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Two strains of nitrogen-fixing associative bacteria of the genis *Azospirillum*, *A. brasilense* Sp7, obtained from

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the Winogradsky Institute of Microbiology, Russian Academy of Sciences (Moscow, Russia), and its mutant deficient of lectin activity, *A. brasilense* Sp7.2.3 [7], were the subjects of the work.

Lectin isolation from cell surface was performed using a previously described technique [7].

Sterilization of seeds, preparation of wheat seedling roots, and pre-treatment of roots with lectins. Seeds of the *Triticum aestivum* L. wheat of the Saratovskaya 29 breed (Research Institute of Agriculture of the South-East, Russian Academy of Agricultural Sciences, Saratov, Russia) were surface-sterilized with 70% (vol/vol) ethanol for 1 min and washed with sterile water. To obtain the roots of the seedlings, the seeds were grown under aseptic conditions in petri dishes on sterile distilled water.

Protein content was determined according to Bradford [8].

Nitric oxide (NO) content determination. The amount of NO was determined by an increase in the content of its metabolite $\left(NO_2^-\right)$ in root homogenates determined with the Griess reagent, comprising equal volumes of 0.3% sulfanilic acid and 0.5% α-naphthylamine. After 10-min contact, optical density was determined at 540 nm [9].

Citrulline content was determined by thin-layer chromatography (TLC). Homogenates of seedling roots were subjected to TLC on silica gel 60A (Merck, Germany) plates in the solvent system containing *n*-butanol, acetic acid, and water (4 : 1 : 1 vol/vol). The plates were developed with ninhydrin solution [10] and citrulline was identified using a pure commercial compound as a reference. Spots were cut out, eluted from the sorbent, and citrulline content was determined quantitatively at 570 nm.

Determination of diacylglycerol (DAG). To obtain lipid extracts, wheat seedling roots were treated according to Folch [11] and Bligh and Dyer [12]. Lipid components were identified by TLC in hexane–diethyl ether–acetic acid (55 : 45 : 1 vol/vol), qualitative reactions, and comparison of chromatographic mobility of the spots with reference compounds [13]. The amount of DAG was determined by gas–liquid chromatography. Methylation was performed according to [14]. Chromatographic separation was carried out on a Shimatzu GH-2010 (Japan) gas chromatograph using an Equity-1 (Supelco, United States) capillary column 30 m long and 0.32 mm in diameter; helium flow rate was set to 34 mL/min. Temperatures of the evaporator and detector were 270°C. DAG was identified by the retention time upon comparison with the standard.

Determination of lipoxygenase activity. Lipoxygenase (EC 1.13.11.12) was determined by spectrophotometry using linoleic acid as a substrate [15].

Determination of salicylic acid (SA). To determine the amounts of free and bound SA, the roots (1 g) were thoroughly washed with distilled water and fixed with 96% hot ethanol. The roots were homogenized, and SA was extracted with boiling 80% ethanol. The extract was divided into two parts to obtain free and bound form of SA [16]. Determination of SA was performed on a Shimatzu GH-2010 (Japan) gas chromatograph using the Equity-1 (Supelco) column at 200°C.

Determination of phenylalanine ammonia-lyase (PAL) activity. PAL (EC 4.3.1.5) was extracted from the roots with 0.1 M borate buffer, pH 8.8, at 4°C for 30 min at mass-to-volume ratio of 1 : 17. The reaction mixture contained 0.1 mL enzyme preparation and 0.4 mL 12 mM L-phenylalanine solution in borate buffer, pH 8.8. The reaction mixture was incubated for 1 h at 37°C. Enzyme activity was determined spectrophotometrically by monitoring the optical density at 290 nm. Activity of PAL was expressed in units of optical density (ΔE/g wet weight) [17].

Determination of superoxide dismutase (SOD) activity. To determine SOD (EC 1.15.1.11) activity, the roots were homogenized in 0.15 M phosphate buffer (pH 7.8). The homogenate was centrifuged for 15 min at 7000 *g*. Enzyme activity was determined by the rate of inhibition of nitro blue tetrazolium (NBT) reduction in a non-enzymatic system of phenazine methosulfate and NADH [18].

TRITC-labeled lectins and determination of lectin localization on the cells of wheat seedling roots. A fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) [19] was used to label bacterial lectins. To verify the specificity of the preparation, preliminary dot analysis on rabbit erythrocyte ghosts was performed. Rabbit erythrocyte ghosts were prepared by osmotic hemolysis in 0.015 M sodium chloride solutions. The ghosts were resuspended in the same solution and washed with saline three times, which was followed by centrifugation for 10 min at 3000 rpm and separation of supernatant.

Immunodot reaction was performed on nitrocellulose membranes (pore diameter 1.5 μm, Synpor). One microliter of erythrocyte membranes (a series of two-fold dilutions was used in the work) was applied to the membrane lined in 5 × 5 mm squares, dried, and fixed in a drying chamber at 60°C for 15 min. To prevent nonspecific adsorption of the label on either the sample or carrier surface, the membrane was incubated in a solution of the following composition: phosphate buffer, pH 7.2, 0.2% BSA, and 0.02% Tween-20, for 15 min at room temperature. The membrane was incubated in solutions of labeled lectins or lectins pretreated with a specific hapten (1.87 mM L-fucose) for 30 min at room temperature; they were then washed with phosphate buffer (pH 7.2) with 0.02% Tween-20. Dots were visualized under a Leica LMD 7000 microscope in the dichroic mirror filter fluorescence mode.

Sample preparation for microscopy. Segments of wheat seedling roots were washed with phosphate buffer (pH 7.0), placed on a microscope slide, supplemented with 50 μL labeled lectins, and incubated for

PAL activity in seedling roots after incubation with lectins of *A. brasilanse* strains Sp7 and Sp7.2.3

Treatment	PAL activity, %
Water (control)	100 ± 3
A. brasilense Sp7 lectin	
5 μg/mL	115 ± 5
10 μg/mL	105 ± 4
20 μg/mL	110 ± 6
40 μg/mL	120 ± 3
A. brasilense Sp7.2.3 lectin	
5 μg/mL	150 ± 4
10 μg/mL	210 ± 3
20 μg/mL	110 ± 9
40 μg/mL	105 ± 3

30 min in the dark. After triple washing with phosphate buffer (each for 10 min), the preparations were studied under a Leica TCS SP5 (Zeiss, Germany) laser scanning confocal microscope. For additional staining of the preparations, fluorescent dyes rhodamine 123, and FM 1-43 [20] were used. For computer analysis, the images were divided into channels (R, red, G, green, and B, blue) using the ImageJ software.

Statistical treatment of the results. The experiments were performed in triplicate biologically and in five repeats, analytically. Digital material was treated statistically with Microsoft Excel software. The tables in the text present the arithmetic means of all determinations and their standard deviations.

RESULTS

Effect of lectins on SOD activity in plant cells. For all concentrations of lectins under study, increase in SOD activity was noted after 2 h incubation with wheat seedling roots; the effect was most pronounced at concentrations of 20 and 40 μg/mL for the parent strain and at 40 μg/mL for the mutant one. The level of stimulating effect for the lectin of the mutant strain was considerably lower than for that of the parent strain (Fig. 1).

Changes in nitric oxide (NO) content in plant cells under the effect of lectins. It was shown that preincubation with lectins of both the parent and mutant strain led to an increase in NO content in the seedling roots at all lectin concentrations under study. In both cases, the most efficient concentration was 40 μg/mL. For both lectins the effect was noted after 1 h, reached its maximum after 3 h, and then decreased to the control level. At the same time, the parent strain lectin exhibited higher effector activity than the other lectin under study (Fig. 2).

In plants, there may be several sources of NO formation and only some of them can be regulated through the signaling pathways [21]. One of the pathways is the reaction catalyzed by nitric oxide synthase according to the following scheme: α-arginine + O_2 → α-citrulline + nitric oxide. To verify the hypothesis that lectins are able to induce NO formation via this reaction, we determined citrulline content in the roots after exposure to lectin solutions at the most efficient concentration (40 μg/mL). It was demonstrated that citrulline content in plant cells increased, following the same trend as nitric oxide (Fig. 3). The fact that incubation with lectins led to a simultaneous increase in NO and citrulline content in the roots allows for the conclusion that lectins are able to activate the NO signaling system in plants.

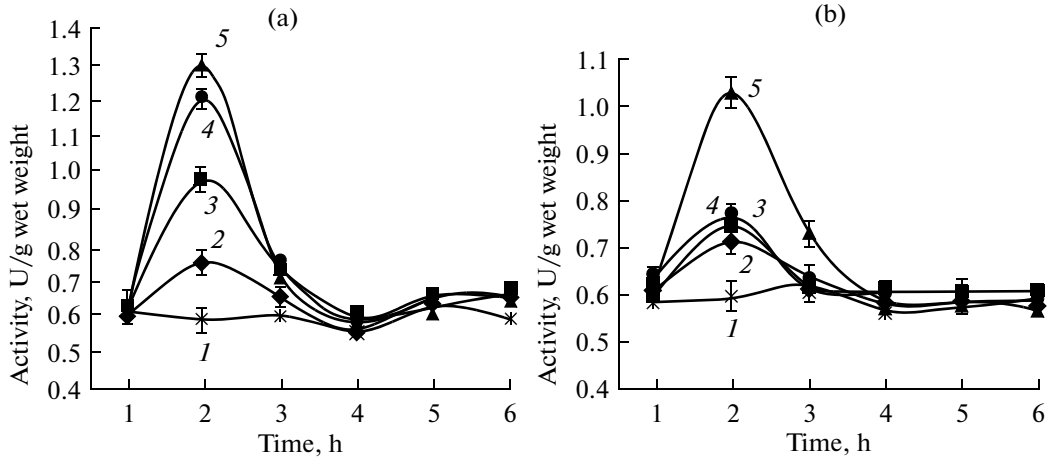

Fig. 1. Effect of *A. brasilense* Sp7 (a) and Sp7.2.3 (b) lectins on the activity of superoxide dismutase of wheat seedling roots: (*1*), control roots; (*2*–*5*), roots + lectins at concentrations of 5 (*2*), 10 (*3*), 10 (*4*), and 40 (*5*) μg/mL.

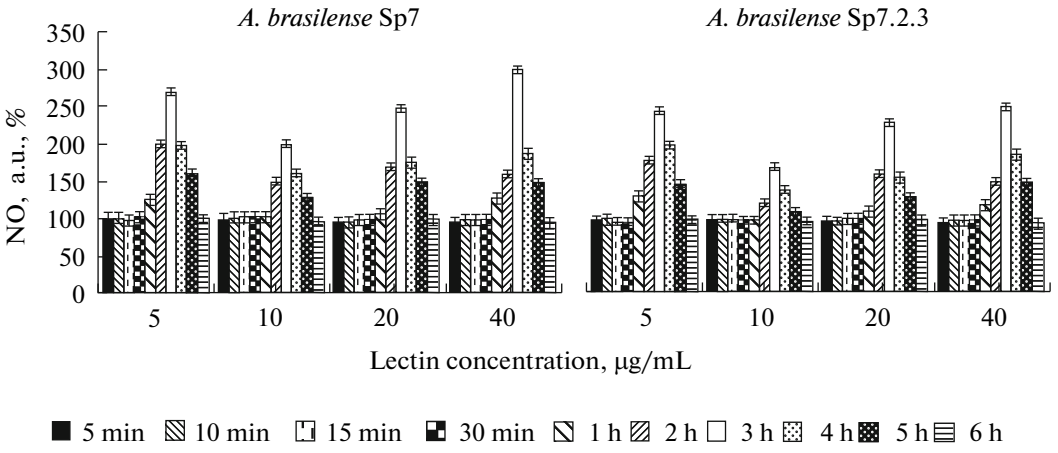

Fig. 2. Nitric oxide (NO) content in wheat seedling roots after incubation with lectins of *A. brasilense* Sp7 and Sp7.2.3.

Effect of lectin on diacylglycerol content. The lectin of *A. brasilense* Sp7 induced DAG synthesis in the seedling roots already after 3 min of co-incubation only at one of the concentrations checked, 40 μg/mL. The maximum values were registered after 40-min incubation, then a sharp decrease in synthesis occurred and by 60 min, DAG content returned to the control level. Lectin of the mutant strain did not exhibit induction activity at any of the concentrations used (Fig. 4). Among ions, calcium is the main activator, capable of influencing phospholipase C activity [22]. Upon introduction in the incubation medium with roots and lectins, 1 mM $CaCl_2$ solution enhanced the effect produced by the parent strain lectin and DAG synthesis was induced by the mutant strain lectin.

Determination of lipoxygenase activity in plant cells after incubation with lectins. One of the mechanisms for formation of the signal products of lipid transformation is the lipoxygenase signaling system, the starting enzyme of which is lipoxygenase. Determination of the enzyme activity after incubation with both lectins revealed the activating effect at concentration of 5 μg/mL. After 30 and 60 min of root incubation with the parent strain lectin, enzyme activity increased by 30 and 50%, respectively, while in the roots treated with the mutant strain lectin, it increased by 25 and 34%. Upon increase in the duration of root treatment with the lectins, enzyme activity decreased to the control level (Fig. 5).

Salicylic acid content in plant cells treated with lectins. During the experiments, amounts of the free and conjugated forms were determined. The results demonstrated that SA content changed after only an hour of root incubation with the lectins. Both lectins in all studied concentrations caused an increase in the content of free SA and a decrease in the conjugated form. The maximum of increase in the free SA content was observed at lectin concentrations of 5 and 10 μg/mL for the parent and mutant strain, respectively. For the lectins of the parent strain, a decrease of the effect was observed with increased lectin concentration. As for the bound SA, for both strains, an increase in lectin concentration resulted in a decrease of their effect. Therefore, the mutant strain lectin was more efficient in increase of the free SA content and less efficient in affecting the conjugated SA compared to the parent strain lectin (Fig. 6).

Formation of free SA may result not only from conjugate hydrolysis, but from *de novo* synthesis as well. To determine which forms were detected, we measured the activity of phenylalanine ammonia-lyase, the enzyme responsible for SA synthesis. Both the parent and mutant strain lectins were found to induce PAL activity, but the mutant strain lectin exhibited higher activity, especially at the concentration of 10 μg/mL. As follows from Fig. 6 and the table, treatment with the mutant strain lectin resulted in distinct correlation between the changes in free SA and PAL activity in the roots, which was not the case for the parent strain lectin. These results evidence that the lectins increase the

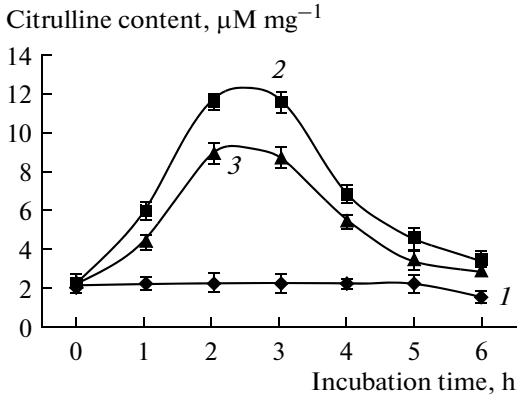

Fig. 3. Citrulline content in wheat seedling roots after incubation with lectins of *A. brasilense* Sp7 and Sp7.2.3 at concentration of 40 μg/mL.

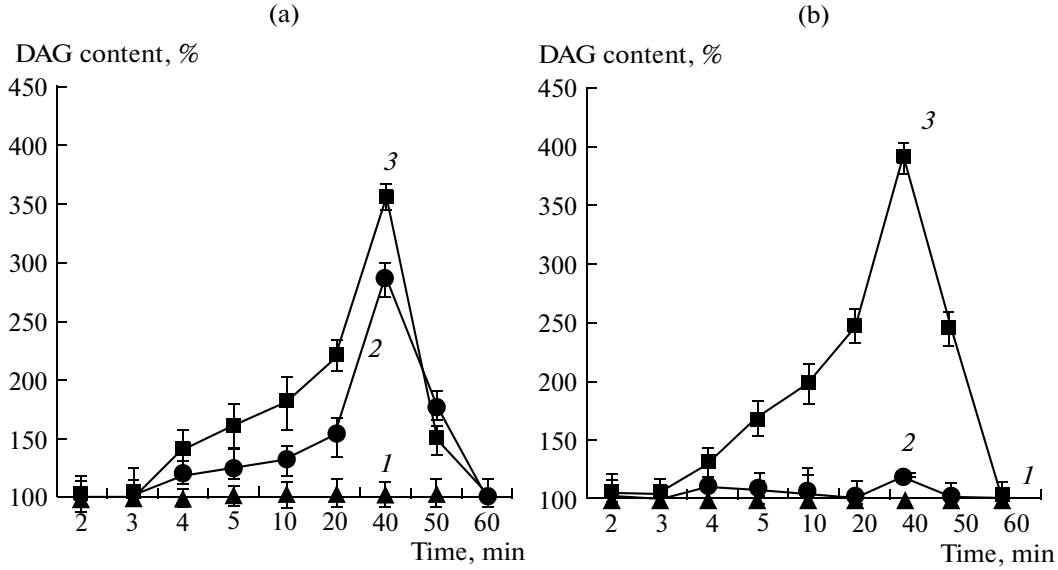

Fig. 4. DAG content in wheat seedling roots after incubation with lectins of *A. brasilense* Sp7 (a) and Sp7.2.3 (b): control (*1*); lectins + roots (*2*); lectins + roots + $CaCl_2$ (*3*). Lectin concentration, 40 μg/mL.

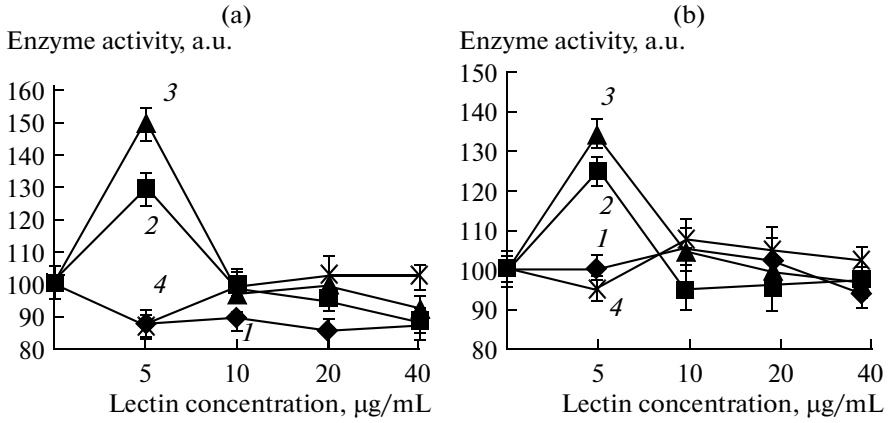

Fig. 5. Effect of *A. brasilense* Sp7 (a) and Sp7.2.3 (b) lectins on lipoxygenase activity in wheat seedling roots: (*1*), control roots, 100%; (*2–4*), roots + lectins, incubation time of 30 (*2*), 60 (*3*), and 120 (*4*) min.

activity of β-glucosidase, which transforms conjugated SA into free one, and activate PAL, the enzyme responsible for SA synthesis, which confirms our previous data [2]. However, the levels of lectin involvement in the former and the latter processes were different. The parent strain lectin regulated more the activity of β-glucosidase, while the mutant strain, that of PAL.

Determination of lectin localization on wheat seedling root cells. Application of fluorescence microscopy with TRITC-labeled lectins demonstrated that lectins of both strains were associated with wheat seedling root cell surface in a similar manner and were distributed over the perimeter of the cap and root hair cells. Additional staining of root cells with the fluorescent dye FM 1-43, which is used to visualize plasma membranes, demonstrated that lectins were detected exclusively at the outer side of the plasma membrane, and not at the cell wall (Fig. 7a).

To test the possibility of intracellular localization of lectin-binding sites, experiments on staining the preparations with a mitochondrial stain rhodamine 123 were performed. Medial optical section of a root hair presented on Fig. 7b demonstrates localization of the mutant strain lectin at the cell surface. Yellow color of the intracellular matrix was caused by the merging of red and green (rhodamine 123 fluorescence) colors. It should be noted that in preparations stained only with TRITC-labeled lectins, intracellular matrix was not colored.

The data on azospirilla lectin localization on plant cells evidence that signal reception occurs mainly on cell surface.

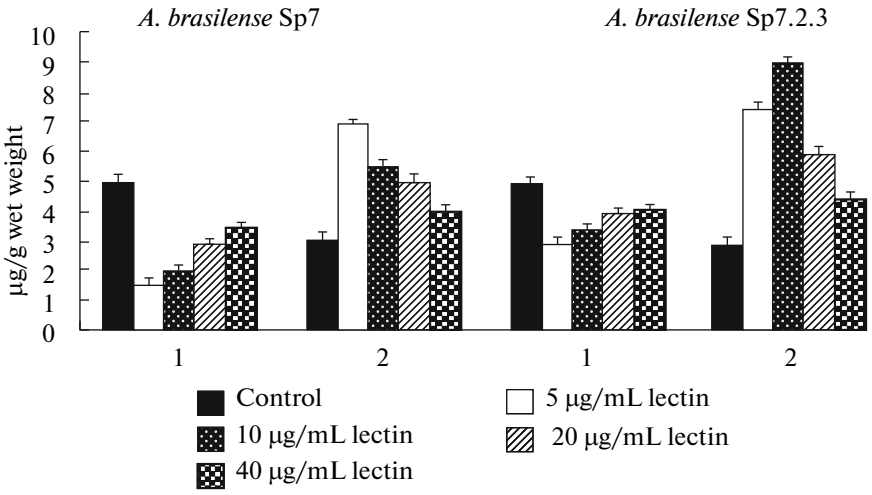

Fig. 6. Content of conjugated (1) and free (2) SA forms in the roots of wheat seedlings in the control sample and upon treatment with the *A. brasilense* Sp7 and *A. brasilense* Sp7.2.3 lectins.

DISCUSSION

Thus, summarizing our previous results and the data obtained in the present work demonstrates the ability of lectins of *A. brasilense* Sp7 and *A. brasilense* Sp7.2.3 to induce, with varying efficacy, adenylate cyclase, NO synthase, NADPH oxidase, Ca-phosphoinositol, and lipoxygenase signaling systems in wheat seedling roots in the process of recognition at the initial stages of development of plant–bacteria associations.

One of the early responses of plant cells to the effect of lectins is induction of the adenylate cyclase signaling pathway occurring after 15 min of co-incubation of lectins with wheat seedling roots. Earlier, we demonstrated that the lectins of *A. brasilense* Sp7 and Sp7.2.3 with identical tendencies but different efficiencies induced the generation of cAMP, one of the components of the adenylate cyclase signaling pathway in plant cells [23]. This signaling system is known to play an important role in functional and structural responses of plant cells to the effects of multiple external biotic and abiotic factors [24].

After 30 min of lectin effect on the roots, induction of the lipoxygenase signaling pathway occurred, which was evidenced by an increase in lipoxygenase activity.

In plants, phospholipase C is localized in the plasma membrane and is one of the key enzymes of the phosphoinositide cycle. As a result of its activity, two intracellular messengers are formed, water-soluble inositol-1,4,5-triphosphate (IP3) and lipid-soluble DAG. IP3 mobilizes Ca^{2+} from the endoplasmic reticulum, increasing free Ca^{2+} concentration in the cytosol, and DAG remaining in the membrane activates a Ca^{2+}-sensitive phospholipid-dependent protein kinase [25]. After 40 min of incubation of lectins with the roots, increase in DAG content occurred, which was the result of phospholipase C activation.

After an hour of root treatment with lectins, the amount of nitrogen monoxide (NO), a participant of the NO signaling system and a regulator of the physiological processes in plant cells, increased. NO was shown to be involved in regulation of cell cycle of a plant cell [26], processes of differentiation and morphogenesis in plants [27], and formation of symbiotic relationships with rhizobia [28]. The fact that incubation of the roots with lectins led to a simultaneous rise of nitric oxide and citrulline in the roots is an additional proof that lectins are able to activate the NO signaling system in plants.

After a 1-h treatment with lectins, an increase in the amount of salicylic acid (a stress metabolite combining the properties of a signal intermediate and a phytohormone) occurred in the roots. Realization of the effects of SA in biotic stresses is considered to be caused, to a large extent, by the effect on activity of the enzymes involved in regulation of the pro-/antioxidative equilibrium, in particular, of catalase, NAPH oxidase, peroxidase [29], and superoxide dismutase [30].

Synthesis of hydrogen peroxide, one of the most rapid responses of plant cells to inducing stimuli, is of particular interest. Reactive oxygen species function mainly within the NADPH oxidase signaling system. SOD, which catalyzes the reaction of superoxide radical reduction to hydrogen peroxide, is the most important enzyme of the antioxidant protection in plants [31]. After two hours of treatment with the lectins, an increase in SOD activity occurred.

Finally, the detected membrane localization of the lectins of the strain under study in plant cell has a determining effect on the fulfillment of signaling functions by the lectins.

Our results demonstrated that in the case of both the parent and the mutant strains, lectins induced identical effects but possessed different regulatory activity. This agrees well with the previously obtained

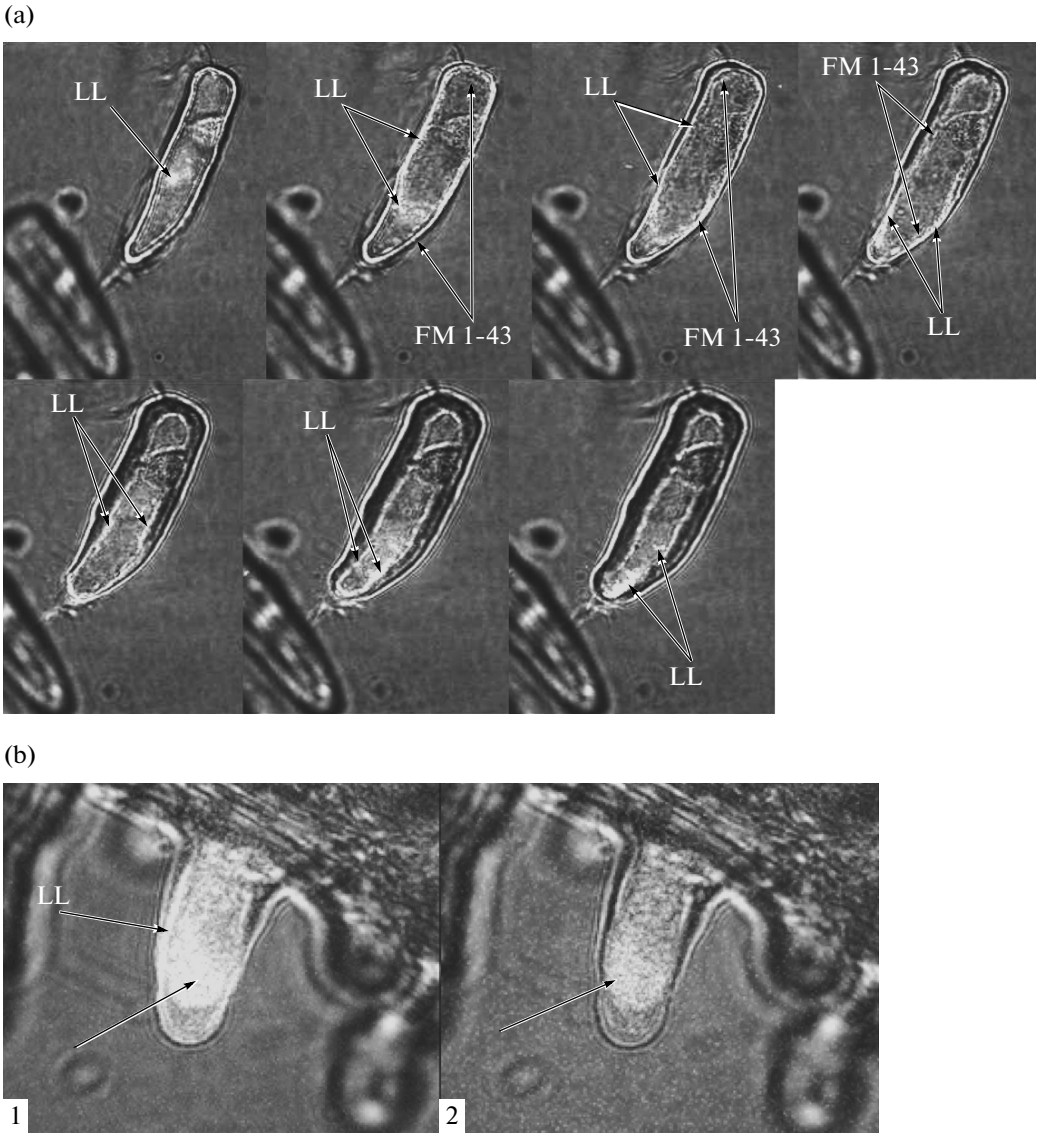

Fig. 7. Localization of the *A. brasilense* Sp7 and *A. brasilense* Sp7.2.3 lectins on plant cell. Cells of root cap + TRITC-labeled lectin of Sp7 + FM (a); root hair + TRITC-labeled lectin of Sp7.2.3 + rhodamine (b). Red (1) and green (2) channels of the images are presented. Areas stained with TRITC and rhodamine are indicated with arrows. Light gray colors correspond to more intense red (a) or green (b) fluorescence. Fluorescence was excited with a 488 nm laser, and emission was registered at 555–618 nm for TRITC, 615–630 nm for FM1-43, and 505–540 nm for rhodamine. LL, labeled lectin.

data [2, 7] and is another confirmation of the fact that the lectins of these two strains, while possessing structural differences and similar carbohydrate specificity, have different functional activity.

The lectins of azospirilla probably provide for the strategy of bacterial interaction with plants similar to that adopted by root nodule bacteria and plant pathogens at the initial stages, which is associated with the induction of protective reactions of the plant, despite the fact that the results of these interactions are considerably different. Physiological response of plants to various pathogens includes a series of protective reactions to the danger of infection [32]. Leguminous–rhizobial interaction also leads to induction of protective mechanisms in the host plant cells, accompanied by generation of reactive oxygen species, nitric oxide, enhanced activity of oxidative enzymes (peroxidase, catalase, superoxide dismutase), accumulation of phenolic compounds, as well as an increase in the level of antioxidant protection [21]. The results obtained may be of practical importance, since pretreatment with such inducers characterized by an anti-stress effect, in combination with growth stimulation, promotes the development of resistance and productivity in plants.

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