ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 5, pp. 630–635. © Pleiades Publishing, Ltd., 2015.
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EXPERIMENTAL ARTICLES

Effect of Azospirillum Lectins on the Activity of Proteolytic Enzymes and Their Inhibitors in Wheat Seedling Roots

S. A. Alen'kina[1] and V. E. Nikitina

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia

Received April 13, 2015

Abstract—The lectins of associative nitrogen-fixing strains *Azospirillum brasilense* Sp7 and Sp245 were shown to exert a multidirectional effect on the activity of acidic (pH 3.5), neutral (pH 6.8), and alkaline (pH 7.8) proteinases. The lectin of the epiphytic *A. brasilense* Sp7 decreased proteolytic activity at all pH values, whereas the lectin of the endophytic *A. brasilense* Sp245 activated neutral and alkaline proteinases, while not affecting the alkaline ones. Experiments with protease inhibitors made it possible to conclude that the lectins of the studied *A. brasilense* strains alter the ratio between the activities of different protease types in germinating seeds. The activity of trypsin inhibitors in wheat seedling roots was found to increase in the presence of the lectins. Our results indicate a broader spectrum of effects of azospirilla lectins on the host plant organism.

Keywords: rhizosphere, associative nitrogen fixation, azospirilla, lectins, wheat seedling roots, proteinases, trypsin inhibitors

DOI: 10.1134/S0026261715050021

Associative nitrogen-fixing bacteria of the genus *Azospirillum* belong to the group of plant growth-promoting rhizobacteria (PGPR) which stimulate plant growth. This effect is considered to be associated with such features as nitrogen fixation, production of phytohormones, phosphate solubilization, improvement of water and mineral balance, and biosynthesis of a number of compounds which increase the membrane activity and proliferation of the root system tissues, as well as with their ability to decrease the stress impact on the host plant and to control numerous phytopathogens (Baldani and Baldani, 2005; Bashan et al., 2004; Alen'kina et al., 2006). Azospirilla are also able to induce defense reactions which increase plant resistance (Bhattacharyya and Jha, 2012). However, in spite of intensive studies, priority of some of the mentioned factors in the favorable effect of nitrogen-fixing bacteria on plant growth and productivity remains unclear.

According to modern concepts, formation of the nitrogen-fixing systems, much like any other biological intercellular interactions, involves the functioning of protein molecules, lectins. It has been considered for a long time that plant lectins fulfill a role of recognizing molecules in the system of carbohydrate–protein interactions during formation of the nitrogen-fixing associations and symbioses (Antonyuk and Evseeva, 2005). However, new information on the lectins of nitrogen-fixing bacteria caused some changes in the understanding of lectin–carbohydrate interactions involved in formation of the nitrogen-fixing associations, with more attention paid to the role of bacterial lectins in these processes (Castellanos et al., 1998; Nikitina et al., 2005).

Nikitina et al. (1996) revealed the presence of lectins on the cell surface of azospirilla, which were involved in adhesion of these bacteria to roots. Lectins with different molecular masses and carbohydrate specificity were isolated from the cell surfaces of two strains of associative nitrogen-fixing bacteria, *A. brasilense* Sp7 and *A. brasilense* Sp245, differing in the way of plant colonization (Nikitina et al., 2005; Shelud'ko et al., 2009). It was shown that lectins of azospirilla were multifunctional molecules; in addition to adhesion function, they also affected the metabolism of plant cells and stimulated seed germination (Nikitina et al., 2004), showed mitogenic and the enzyme-modifying activities (Alen'kina et al., 2006; Chernyshova et al. 2005), and changed the content of the stress metabolites in plant cells. These data indicated the lectin ability to induce the adaptation processes in the wheat seedling roots (Alen'kina et al., 2014).

Numerous physiological and biochemical processes are known to be involved in the defense-adaptive reactions of plants in response to impact of unfavorable environmental factors (Titov et al., 2005; Trunova, 2007).

[1] Corresponding author; e-mail: alenkina@ibppm.sgu.ru

Proteolytic enzymes play an important role in metabolism of a living organism and its protection against damage; they control the concentrations of proteins and peptides and take part in modification and degradation of biopolymers, which don't fulfill (or fulfill insufficiently) their necessary functions. Moreover, proteolytic enzymes provide the cells with monomeric substrates for the synthesis of the stress (shock) proteins, which are important factors of cell resistance (Tarchevskii, 2011).

Numerous studies showed that inhibitors of proteolytic enzymes had an important role in plant protection against various unfavorable factors (Lawrence and Koundal, 2002; Frolova and Titov, 2008; Domash et al., 2008).

The goal of the present work was to study the effect of lectins from *A. brasilense* Sp7 and *A. brasilense* Sp245 on the activities of proteases and their inhibitors in wheat seedling roots.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The study was carried out with two strains of nitrogen-fixing associative bacteria of the genus *Azospirillum*, the epiphytic strain *A. brasilense* Sp7 obtained from the culture collection of the Winogradsky Institute of Microbiology, Russian Academy of Sciences, and the endophytic strain *A. brasilense* Sp245 obtained from the culture collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences.

Obtaining of lectin preparations. Isolation of lectins from the cell surface and their purification were performed according to the earlier described methods (Nikitina et al., 1996).

Sterilization of seeds, obtaining of seedling roots, and pretreatment of roots with lectin preparations. Seeds of wheat *Triticum aestivum* L., variety Saratovskaya 29 (Research Institute of Agriculture of the South-East, Russian Academy of Agricultural Sciences, Saratov) were subjected to surface-sterilization with 70 % ethanol (vol/vol) for 1 min and washed with sterile water. To obtain seedling roots, the seeds were grown aseptically in petri dishes with sterile distilled water.

The roots were treated with lectin preparations (5–40 μg/mL) for the maximal period of 1 h, homogenized in 0.15 M phosphate buffer (pH 7.8), and centrifuged at 7000 *g*; the supernatant was used for analyses of inhibitors and proteolytic activity.

Protein assay. Protein amount was determined by the Bradford method (Bradford, 1976).

Analysis of proteolytic activity. Proteolytic activity was determined by the Anson method (Gil'manov et al., 1981). To assay acidic proteinases, a 0.5% solution of hemoglobin (Sigma, United States) in citrate–phosphate buffer (pH 4.5) was used as a substrate. To determine proteolytic activities of neutral and alkaline proteinases, a 0.25% solution of casein (Sigma, United States) in citrate–phosphate buffer (pH 6.8 and 7.8, respectively) was used. The incubation medium contained 0.1 mL of the root homogenate, 0.1 mL of the substrate, and 0.1 mL of the relevant buffer.

To study the effect of the protease inhibitors on enzyme activity, the incubation medium was supplemented with *p*-chloromercuribenzoic acid (PCMB, Sigma, United States), ethylenediaminetetraacetic acid (EDTA, Sigma, United States), or phenylmethyl sulfonylfluoride (PMSF, Sigma, United States) in final concentration of 1×10^{-3} M, and pepstatin (2×10^{-5} M) (Sigma, United States). After incubation of the reaction mixture for 1 h at 37°C, residual activity was analyzed. Residual activity was expressed as a percentage of the control (enzyme activity in the absence of inhibitors was taken as 100%).

Assay of activity of the trypsin inhibitors. The inhibitor activity was determined from repression of trypsin activity (Ermakov et al., 1987). Crystalline trypsin (Serva, Germany) was used. Trypsin-inhibiting activity was expressed as mg of pure trypsin inactivated by the inhibitor per 1 g of wet roots.

Statistical analysis of results. The data were statistically processed by the Student method. The figures show the arithmetical mean values of three independent experiments carried out in five biological replicates and standard deviations at a significance level $P < 0.05$.

RESULTS AND DISCUSSION

The effects of lectins from *A. brasilense* Sp7 and *A. brasilense* Sp245 on proteolysis in the wheat seedling roots were investigated at several pH values, making it possible to elucidate the effects of lectins on the activities of different proteinases. As seen from Fig. 1, lectin from *A. brasilense* Sp7 inhibited proteinase activities at all pH values studied. The inhibitory effect of lectin was the most pronounced against acidic and alkaline enzymes. Lectin concentrations of 10 and 20 μg/mL showed the maximum inhibitory effect at all pH values. The optimum time of incubation with lectin was 15 min for inhibition of acidic proteinases and 15 or 30 min in the case of alkaline proteinases, whereas inhibition of neutral proteinases was independent of the incubation time (Fig. 1).

On the contrary, in the case of lectin from *A. brasilense* Sp245, the activities of neutral and alkaline proteinases increased after 15-min incubation and reached the maximum after 30 min; activities of neutral proteinases increased to a greater extent. In both cases, the most effective lectin concentration was 10 μg/mL. At the same time, activities of acidic proteinases were not changed in the presence of lectin (Fig 2).

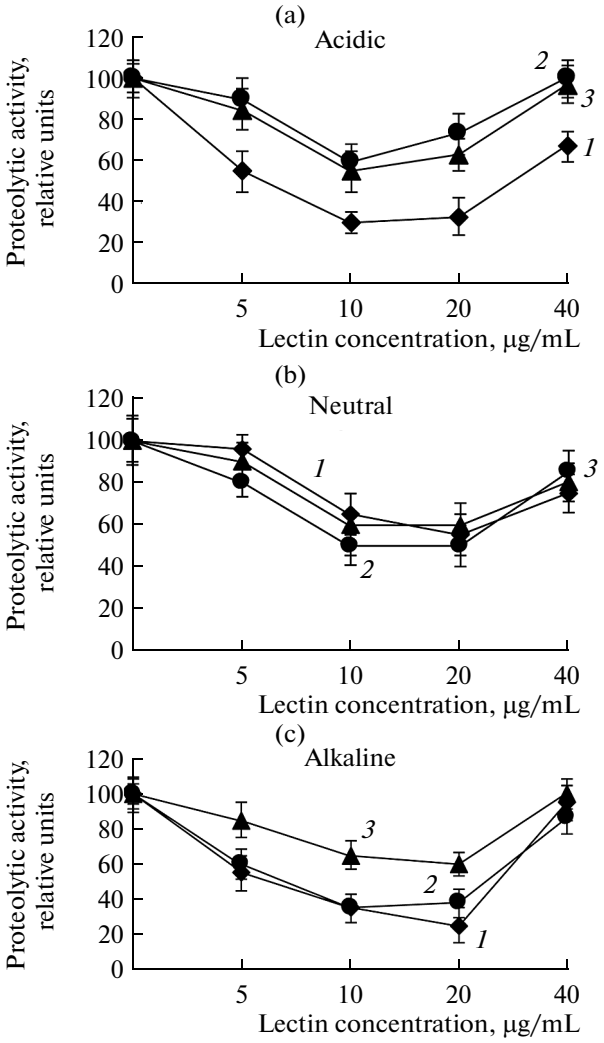

Fig. 1. Changes in activities of acidic (a), neutral (b), and alkaline (c) proteinases in the presence of lectin from *A. brasilense* Sp7. Control (100% activity) for acidic, neutral, and alkaline proteinases was 7, 10, and 0.5 μmol Tyr min^{-1} g wet $mass^{-1}$, respectively. Time of root incubation with lectin was 15 (*1*), 30 (*2*), and 60 min (*3*).

It is known that enzymes involved in the protein degradation during seed germination belong to all four mechanistic classes of proteinases: serine, aspartate, cysteine, and metalloproteinases. To elucidate the effect of lectins on activities of these types of proteases at the studied pH values, a series of experiments with the use of protease inhibitors was carried out. The variants in which lectin from *A. brasilense* Sp7 showed the maximum inhibitory effect and lectin from *A. brasilense* Sp245 exhibited the maximum stimulatory effect on enzyme activities were used. In the case of the lectin from *A. brasilense* Sp7, we used the variants with lectin concentration of 10 μg/mL and incubation for 15 min for acidic or 30 min for neutral proteases. The lectin concentration of 20 μg/mL and incubation for 15 min was used for alkaline proteases. In the case of the lectin from *A. brasilense* Sp245, the concentration of 10 μg/mL and incubation for 30 min were used for both neutral and alkaline proteases. The table shows absolute values of protease activities, which were taken as 100% when the percentage of their inhibition was calculated.

As seen from Fig. 3, the inhibitory effect of the lectin from *A. brasilense* Sp7 on acidic proteinases was the most pronounced in the case of aspartate proteinases, with their activity in the presence of pepstatin decreased by 50%. The lectin showed a lower effect on cysteine proteinases; in the presence of the inhibitor PCMB, activities of these enzymes were decreased to a lesser extent than in the case of aspartate proteinases. The enzyme activities were not changed in the presence of EDTA, which indicated that the lectin showed no effect on the activities of metalloproteases at the given pH value. It should be noted that aspartate and cysteine proteases play the most important role in the degradation of storage proteins during germination of the cereals caryopsis at acidic pH (Aleksandrova et al., 1999; Dunaevskii et al., 1990).

At pH 6.8, inhibition of protease activity after treatment of the root with the lectin from *A. brasilense* Sp7 occurred mainly due to lowering activities of serine, aspartate, and metalloproteases (by 33, 83, and 67%, respectively), whereas the inhibition of cysteine proteases was as low as 17% (Fig. 2b). The lectin from *A. brasilense* Sp245 inhibited mainly metalloproteases (by 70%) and inhibited aspartate, serine, and cysteine proteases to a lesser extent (by 44, 37, and 50%, respectively) (Fig. 3).

As seen from Fig. 3c, both lectins showed no effect on cysteine proteases at alkaline pH but considerably affected metalloproteases, with their activities in the presence of EDTA decreased by 67 and 60% after treatment with lectins from *A. brasilense* Sp7 and *A. brasilense* Sp245, respectively. It was found that lectins affected serine proteases; after addition of PMSF, enzyme activities decreased by 50 and 29% in the presence of lectins from *A. brasilense* Sp7 and *A. brasilense* Sp245, respectively. The effect of lectins on alkaline proteases was most pronounced in the case of serine- and metalloproteases.

One of the ways to control the activities of proteolytic enzymes in plant cells involves proteinaceous inhibitors, which are capable of reversible binding of enzymes into inactive complexes (Mosolov, 1971; Joanitti et al., 2006). Plants produce various enzyme inhibitors, among which inhibitors of serine proteinases, including trypsin inhibitors, are the most abundant. Intense synthesis of proteinase inhibitors in plants is evoked by mechanical damage, various pathogens, etc. (Mosolov, Valueva, 2005, 2006).

It was found that both lectins showed similar effects on the activities of trypsin inhibitors in the seedling roots (Fig. 4). After treatment of the seedling roots with lectins from strains Sp7 and Sp245, activities of trypsin inhibitors increased after 15 min, reached the

Activities of acidic, neutral, and alkaline proteases in the wheat seedling roots after incubation with lectins from *A. brasilense* Sp7 and Sp245

Proteases	Control	*A. brasilense* Sp7	*A. brasilense* Sp245
	μmol Tyr min^{-1} g wet $mass^{-1}$		
Acidic	7 ± 0.2	2.1 ±0.1	–
Neutral	10 ± 1	5 ± 0.1	44 ± 2.3
Alkaline	0.5 ± 0.1	0.13 ± 0.05	0.88 ± 0.2

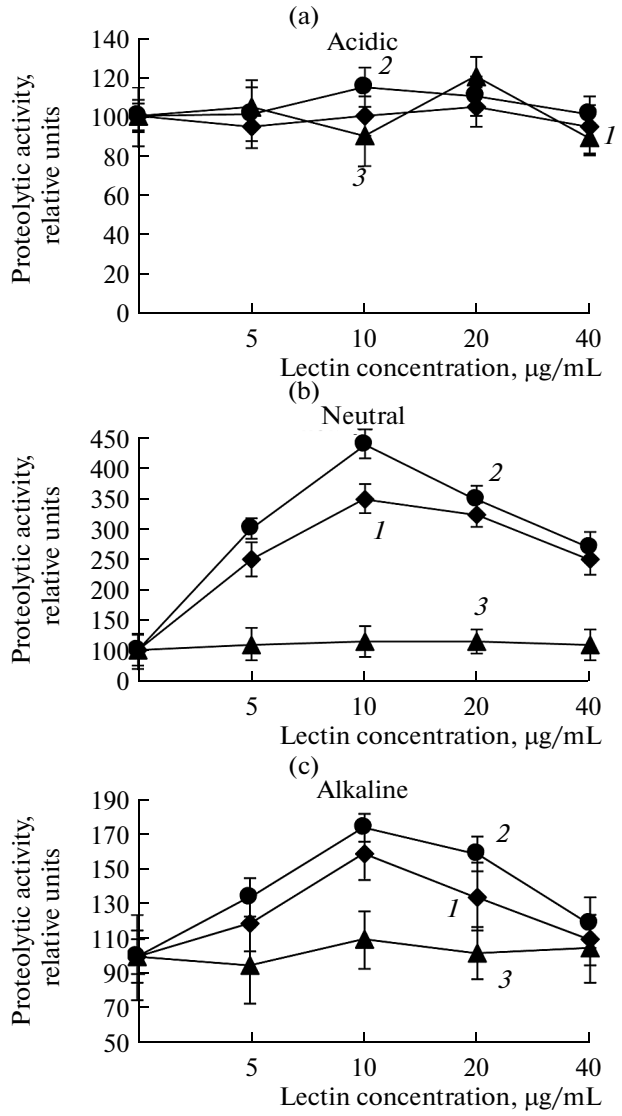

Fig. 2. Changes in activities of acidic (a), neutral (b), and alkaline (c) proteinases in the presence of lectin from *A. brasilense* Sp245. Control (100% activity) for acidic, neutral, and alkaline proteinases was 7, 10, and 0.5 μmol Tyr min^{-1} g wet $mass^{-1}$, respectively. Time of root incubation with lectin was 15 (*1*), 30 (*2*), and 60 min (*3*).

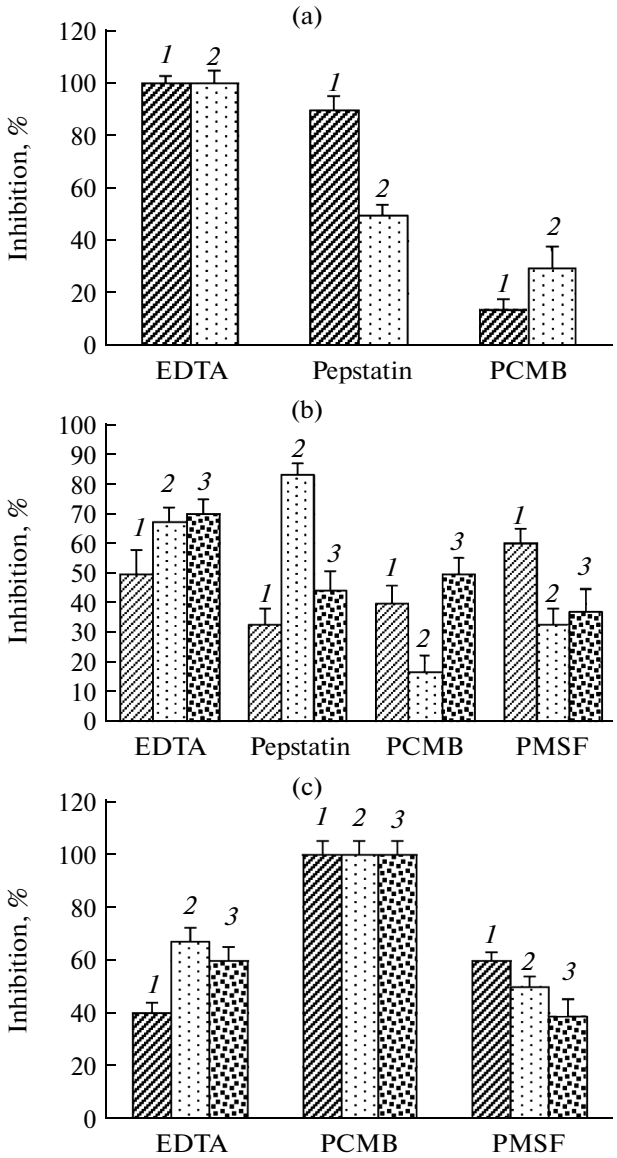

Fig. 3. Effects of inhibitors on activity of acidic (a), neutral (b), and alkaline (c) proteases in wheat seedling roots in the control (*1*) and under the action of lectins from *A. brasilense* Sp7 (*2*) and Sp245 (*3*).

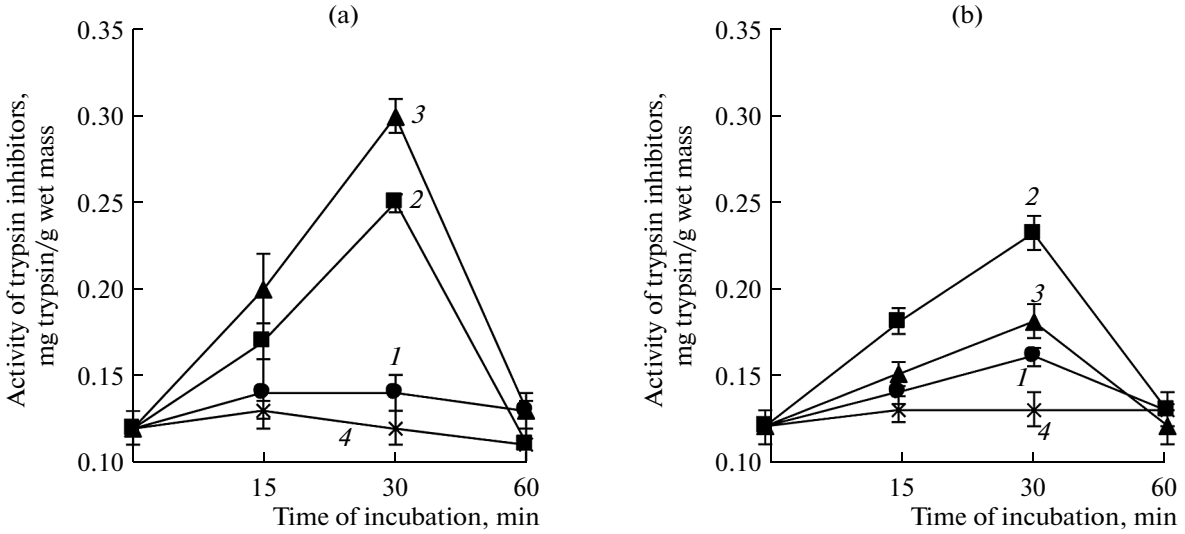

Fig. 4. Activities of trypsin inhibitors in wheat seedling roots after the action of lectins from *A. brasilense* Sp7 (a) and *A. brasilense* Sp245 (b). In the control, roots were treated with trypsin (0.12 mg/g wet mass). In experimental variants, the roots were treated with lectins in concentrations (μg/mL): 5 (*1*), 10 (*2*). 20 (*3*), and 40 (*4*).

maximum by 30 min, and then gradually decreased. It should be emphasized that the activities of trypsin inhibitors were changed in a similar manner at all lectin concentrations studied but reached the maximum at 20 μg/mL in the presence of the lectin from *A. brasilense* Sp7 (Fig. 4a) and at 10 μg/mL in the case of the lectin from *A. brasilense* Sp245 (Fig. 4b). The fact that lectins, which are different in their carbohydrate specificity, show similar effects indicates that the lectin capacity for binding certain carbohydrates is not principal for their biological activity. The finding that maximal activities of lectins depended on their concentrations may probably be explained by different numbers of binding sites in the lectin molecules.

Thus, it was revealed that treatment of wheat seedling roots with lectins from *A. brasilense* Sp245 considerably increased the activities of proteolytic enzymes and trypsin inhibitors. At the same time, in the case of the lectin from *A. brasilense* Sp7, an increase in activities of trypsin inhibitors was accompanied by a decrease in proteinase activities due to the inhibitor capability for reversible binding of the enzymes and their transformation into an inactive state (Valueva and Mosolov, 2002).

The lectin from *A. brasilense* Sp7 inhibited activities of proteolytic enzymes at all the studied pH values, whereas the lectin from *A. brasilense* Sp245 increased proteolysis only at neutral and alkaline pH. Lectins could change the proportions between activities of proteinases belonging to different types. The lectin from *A. brasilense* Sp7 decreased mainly the activities of aspartate and cysteine acidic proteinases, as well as of alkaline aspartate proteases and metalloproteases. The lectin from *A. brasilense* Sp245 activated neutral aspartate, cysteine, and metalloproteases, as well as alkaline metalloproteases.

According to the literature data (Ryan, 1992; Kladnitskaya et al., 1996), a change in activities of proteinases and their inhibitors in plant tissues is a nonspecific response reaction of plants to the impact of biotic and abiotic stress factors. It can be concluded that an artificial change in the balance between the activities of proteinases and their inhibitors in plant tissues is a mechanism increasing plant resistance. The preparations which induce prolonged synthesis of proteinase inhibitors in plants seem to be the most promising for application. Changes in activities of proteinases and trypsin inhibitors in plants under the action of lectins—which were accompanied, on the one hand, by increased accumulation of inactive and damaged proteins and peptides and, on the other hand, by increased requirement for low-molecular compounds for synthesis of the stress proteins (Tarchevskii, 2001)—appeared to be directed at formation of plant resistance. Therefore, it is reasonable to suggest that lectins of azospirilla which have several "channels" of their effect on the cells (Alen'kina et al., 2006, 2014) can affect plant resistance, in particular, by changing the activities of proteolytic enzymes and their inhibitors.

A possible reason for different functional activities of lectins can be a difference in their carbohydrate specificity and protein structure (Nikitina et al., 2005; Shelud'ko et al., 2009) and, as a consequence, their different interactions with the plant cell surface, which is a key factor for realization of the subsequent stages. Different functioning of the lectins studied may be also associated with possible involvement of the lectin from *A. brasilense* Sp245 in penetration of bacteria into the root tissues; it has been earlier shown that proteolytic enzymes of azospirilla play an important role in this process (Chernyshova et al., 2005).

In view of the foregoing, it is reasonable to conclude that lectins can affect plant resistance by changing the activities of proteolytic enzymes and their inhibitors. Our results demonstrate that effects of lectins of azospirilla on the host-plant metabolism are more extensive than it has been earlier considered; this finding, together with the literature data, makes it possible to formulate a comprehensive view on the bacteria–plant interactions at the molecular level.

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Translated by E. Dedyukhina