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Change in the Ratio Between the Activities of Different Types of Proteases and Their Inhibitors in Plant Roots Exposed to *Azospirillum* Lectins

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Abstract *Azospirillum brasilense*, which has the potential to stimulate plant growth, belongs to the group of plant-growth-promoting rhizobacteria. The surface lectins of *A. brasilense* strains Sp7 and Sp245 can bind specific carbohydrates and ensure adhesion of the bacteria to the root surface. We investigated possible effects of the Sp7 and Sp245 lectins on the activities of proteases and their inhibitors in wheat seedling roots. Spectrophotometry was used to determine the protease and trypsin inhibitor activities in the cells of wheat-root seedlings. The Sp7 lectin decreased the activities of acidic (pH 3.5), neutral (pH 6.8), and alkaline (pH 7.8) proteases, whereas the Sp245 lectin activated all enzymes but acidic proteases, whose activity remained unchanged. Both lectins changed the ratio between the activities of the three types of proteases in germinating seeds. The trypsin-inhibiting activity in wheat seedling roots increased in the presence of either lectin. The results suggest that the range of effects of *Azospirillum* lectins on host plant metabolism is wider then previously thought. Together with the already existing evidence, they allow correction of the current views about the mechanisms that govern the interaction between the plant and the bacterium involved in an associative relationship.

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Introduction

The associative N_2-fixing bacteria *Azospirillum* are classified as plant-growth-promoting rhizobacteria. Their growth-stimulatory effect has been associated with their ability to fix N_2, synthesize phytohormones, solubilize phosphates, improve plant water and mineral status, produce compounds that increase membrane activity and proliferation of root tissues, decrease stressor effects on plants, and control numerous phytopathogens. The mechanisms of the plant-mediated biocontrol effect include the bacterial ability to induce resistance-enhancing defense responses in plants (Bashan and de-Bashan 2010).

Although research in this area is actively working, an open question remains on which of the above plant-beneficial factors have priority over the others. Current views hold that lectins, molecules of a protein nature, are involved in the establishment of N_2-fixing systems, as well as of any other intercellular biological interactions. It has long been thought that when carbohydrates interact with proteins in the formation of N_2-fixing associations and symbioses, plant lectins act as recognizing molecules (Antonyuk and Evseeva 2006). However, new data for the lectins of N_2-fixing bacteria have emerged suggesting an active role for bacterial lectins in plant–bacterium relationships (Castellanos and others 1998; Nikitina and others 2005).

Nikitina and others (2005) demonstrated that the surface lectins of *Azospirillum* have a role in bacterial adhesion to roots. From the surface of two *A. brasilense* strains, Sp7 and Sp245, which differ in their mode of

plant colonization, lectins were isolated that were found to be glycoproteins with different molecular masses and carbohydrate specificities (Nikitina and others 2005; Shelud'ko and others 2009). These lectins are polyfunctional; apart from functioning as adhesins, they can influence plant cell metabolism by promoting seed germination (Nikitina and others 2005) and expressing mitogenic and enzyme-modifying activities toward the plant cell (Alen'kina and others 2006). They also can alter the plant cell content of stress metabolites, which indicates that they can induce adaptation processes in wheat seedling roots (Alen'kina and others 2006, 2010, 2013, 2014; Alen'kina and Nikitina 2010).

Many physiological and biochemical processes help plants to counter and adapt to unfavorable external factors, and most of these processes are directly or indirectly involved in the development of resistance. Proteases are significant components of plant defense mechanisms, because (i) they can release elicitors from a pathogen's cells, which subsequently spread in and are recognized by plants; (ii) they provide the cell with monomeric substrates for the synthesis of stress (shock) proteins, which are important for plant resistance; and (iii) they can activate the specific binding of elicitors owing to the formation of signal components during proteolysis (Pesquet 2012).

The activity of various classes of plant proteases increases under biotic and abiotic conditions. Proteolytic activity is influenced by many factors, including inhibitory proteins, which are widespread in living organisms and are being increasingly studied. Protease inhibitors are a group of plant proteins with the common ability to form stereochemical protein–protein complexes with proteolytic enzymes, resulting in competitive inhibition of protease catalytic activity. These inhibitors play a large role in plant defense against adverse factors (Domash and others 2008).

Here, we examine the possible effect of the *A. brasilense* Sp7 and Sp245 lectins on the activity of proteases and their inhibitors in roots of wheat seedlings.

Materials and Methods

Strains and Growth Conditions

Azospirillum brasilense Sp7 (an epiphytic strain) was obtained from the culture collection of the Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow. *A. brasilense* Sp245 (an endophytic strain) was from the Collection of Rhizosphere Microorganisms (this institute). The cultures were grown in a synthetic medium at 37 °C for 18 h (Alen'kina and others 2006).

Lectin Isolation

Lectins were isolated from the surface of Sp7 and Sp245 and were purified by gel filtration on a 30×2.2-cm column of Sephadex G-75 (particle diameter, 40–120 μm). The emergence of protein fractions was followed at 278 nm with a Uvicord SII apparatus (LKB, Sweden). The eluents were 0.1-M CH_3COOH (pH 4.8) and 0.05-M phosphate-buffered saline (PBS; pH 7.0) containing 0.15 M NaCl. The flow rate was 1.5 ml min^{-1} (Alen'kina and others 2006). The lectin nature of the purified material was confirmed by hemagglutination assay as described by Lakhtin (1989). Fifty-microliter portions of successive twofold dilutions of lectin solutions were added to the wells of a microtitration plate, with PBS as a control. Washed trypsin-treated rabbit erythrocytes were added at a concentration of 2% in PBS and were incubated at room temperature for 2 h. The minimum lectin concentration that gave hemagglutination was recorded as the hemagglutination titer.

Animal care and handling were in accordance with the Guide for the Care and Use of Laboratory Animals, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the legislation of the Russian Federation. The use of the animals was also approved by the institution where the experiments were done.

Seed Sterilization, Obtainment of Seedling Roots, and Root Pretreatment with Lectins

Seeds of *Triticum aestivum* L. cv. Saratovskaya 29 [Agricultural Research Institute for South-East Region (ARISER), Saratov, Russia] were surface sterilized in 70% v/v ethanol for 1 min and were washed five times with sterile water. For seedling roots, seeds were grown aseptically in Petri dishes on sterile distilled water and incubated in the dark at 25 °C. Seedlings used for experiments were 4 days old.

Seedling roots were placed in solutions each containing 5–40 μg ml^{-1} of either Sp7 or Sp245 lectin. After incubation with the lectins (concentration, 5–40 μg ml^{-1}; maximum incubation time, 1 h), the roots were homogenized in 0.15 M PBS (pH 7.8). The homogenate was centrifuged at 7000×*g* for 10 min, and the supernatant liquid was used to determine proteolytic and inhibitor activities.

Assay for Proteolytic Activity

This was done by the method of Anson as described in Gilmanov and others (1981). The substrate for acidic proteases was 0.5% hemoglobin (Sigma, USA) diluted with citrate–phosphate buffer (pH 4.5). The assays for neutral and alkaline proteases used 0.25% casein (Sigma) diluted with citrate–phosphate buffer (pH 6.8 and 7.8). The incubation

media contained 0.1 ml of the homogenate, 0.1 ml of either hemoglobin or casein, and 0.1 ml of the buffer. The enzyme activity was determined spectrophotometrically at 280 nm in a 1-cm path cuvette.

For inhibitor studies, the following compounds (Sigma) were added to the incubation media: *p*-chloromercuribenzoic acid (PCMB), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) (all at a final concentration of 1×10^{-3} M), and pepstatin (2×10^{-5} M). The samples were left to stand at 37 °C for 1 h, after which residual activity was determined. Residual activity is expressed as the percentage of the enzyme activity in the absence of the inhibitors (control).

Assay for Trypsin Inhibitor Activity

Trypsin inhibitor activity was assayed by determining residual trypsin activity by the method of Kakade and others (1974) as modified by Benken (1982). With casein as the substrate, the reaction mixture contained 0.1 ml of the homogenate, 0.1 ml of trypsin (1 mg ml^{-1} in 50-mM Tris–HCl, pH 8.0, containing 20 mM $CaCl_2$ and 0.001 N HCl) and 0.4 ml of 2% casein. The mixture was left to stand for 20 min, after which it was precipitated with 0.3 ml of 5% trichloroacetic acid, and the absorbance was measured at 280 nm. The activity is expressed in milligrams of pure trypsin bound by the inhibitor per gram of root wet weight.

Statistics

The figures show arithmetic means ± standard error (SE) of three independent experiments, done in five biological replications. Significant differences between control and treated plants were determined by Student's *t* test. Differences were considered significant at $p<0.05$.

Results and Discussion

The influence of the *A. brasilense* Sp7 and Sp245 lectins on proteolysis in wheat seedling roots was examined at several pH values. This made it possible to explore the effects of the lectins on protease activity. The Sp7 lectin caused the activity to drop at all pH values tested, and this effect was greatest in acidic and alkaline proteases (Fig. 1). The most effective inhibitory concentrations of the Sp7 lectin were 10 and 20 µg ml^{-1} at all pH values tested. In acidic proteases, inhibition was greatest after 15 min of incubation; in neutral proteases, the activity was the same after all incubation periods; and in alkaline proteases, inhibition was greatest after 15 and 30 min of exposure to the lectin (Fig. 1).

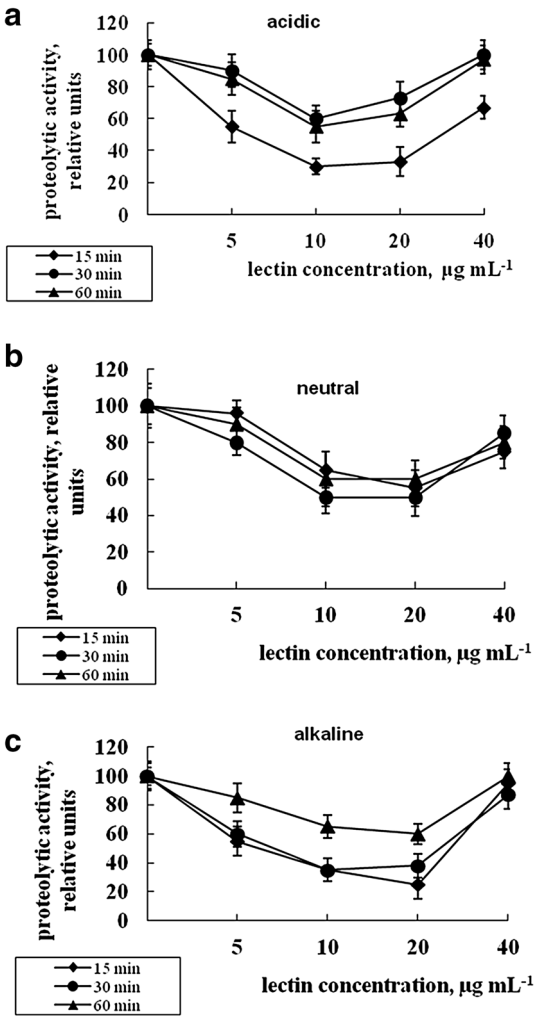

Fig. 1 Activities of acidic, neutral, and alkaline proteases in the presence of the *A. brasilense* Sp7 lectin. Control (100%): 7 µM tyrosine min^{-1} g wet $weight^{-1}$ for acidic proteases, 10 µM tyrosine min^{-1} g wet $weight^{-1}$ for neutral proteases, and 0.5 µM tyrosine min^{-1} g wet $weight^{-1}$ for alkaline proteases. 1–3, period of lectin–root incubation (15, 30, and 60 min, respectively). Results are expressed as mean ± SE ($n=3$). All differences significant ($p<0.05$)

The Sp245 lectin produced an opposite effect. After incubation for 15 min, the activities of neutral and alkaline proteases increased, peaking after 30 min of incubation. This increase was more substantial in neutral proteases. In both cases, the most effective lectin concentration was 10 µg ml^{-1}. The activity of acidic proteases was unaffected (Fig. 2).

The breakdown of proteins in germinating seeds involves enzymes belonging to all four mechanistic classes of proteases aspartic proteases, serine proteases, cysteine proteases, and metalloproteases. To elucidate how much the lectins were involved in the activity changes observed in these protease types at the pH values tested, we did an independent series of experiments that used protease

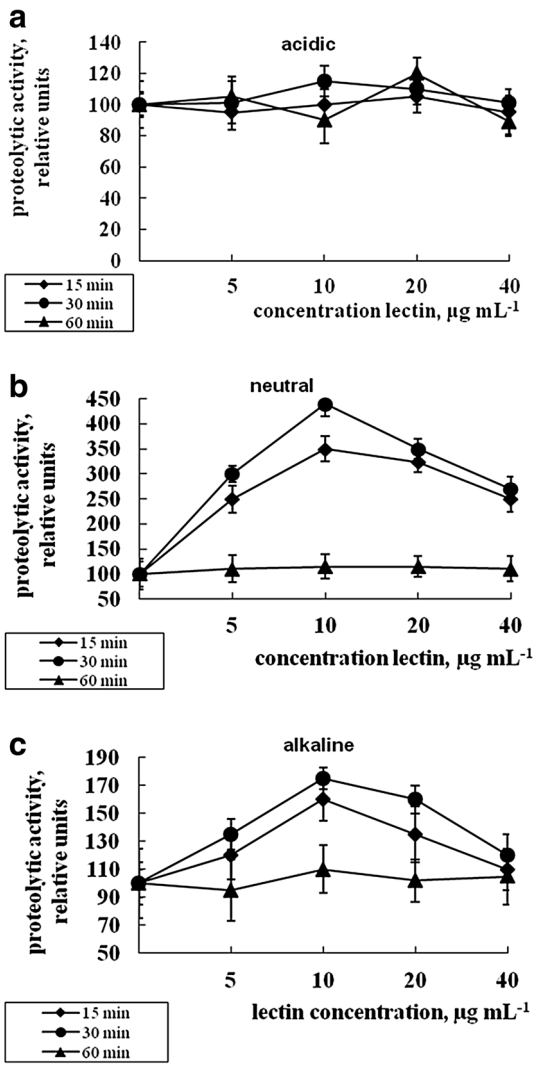

Fig. 2 Activities of acidic, neutral, and alkaline proteases in the presence of the *A. brasilense* Sp245 lectin. Control (100%): 7-µM tyrosine min^{-1} g wet $weight^{-1}$ for acidic proteases, 10-µM tyrosine min^{-1} g wet $weight^{-1}$ for neutral proteases, and 0.5-µM tyrosine min^{-1} g wet $weight^{-1}$ for alkaline proteases. 1–3, period of lectin–root incubation (15, 30, and 60 min, respectively). Results are expressed as mean ± SE ($n = 3$). All differences significant ($p < 0.05$)

Table 1 Activities of acidic, neutral, and alkaline proteases in wheat seedling roots exposed to the *A. brasilense* Sp7 and Sp245 lectins

Proteases	Control	*A. brasilense* Sp7	*A. brasilense* Sp245
	µM tyrosine min^{-1} g wet $weight^{-1}$		
Acidic	7 ± 0.2	2.1 ± 0.1	n.f
Neutral	10 ± 1.0	5 ± 0.1	44 ± 2.3
Alkaline	0.5 ± 0.1	0.13 ± 0.05	0.88 ± 0.2

n.f. not found

Results are expressed as mean ± SE ($n = 3$). All differences are significant ($p < 0.05$). Control: roots (100%)

inhibitors, the peak inhibition values for the Sp7 lectin, and the peak inhibition values for the Sp245 lectin.

With the Sp7 lectin, the lectin concentrations used were 10 µg ml^{-1} (acidic and neutral proteases) and 20 µg ml^{-1} (alkaline proteases), and the incubation times were 15 min (acidic), 30 min (neutral), and 15 min (alkaline). With the Sp245 lectin, the lectin concentration was 10 µg ml^{-1} and the incubation time was 30 min for both neutral and alkaline proteases. Table 1 shows the protease activity values taken as 100% in calculating the percent inhibition of protease activity.

The major contribution to the decrease in acidic protease activity obtained with the Sp7 lectin was made by aspartic proteases (a 50% decrease in the presence of pepstatin; Fig. 3a). The lectin effect on cysteine protease activity was much smaller, because the activity decrease under the effect of PCMB, an inhibitor of cysteine proteases, was lower than that in the former case. Adding EDTA brought about no changes in activity, which shows that the lectin did not affect metalloprotease activity at a given pH value. It should be noted that aspartic and cysteine proteases contribute substantially to the cleavage of reserve proteins when cereal crop grains germinate at acidic pH values (Aleksandrova and others 1999).

At pH 6.8, after exposure to the Sp7 lectin, protease activity was inhibited to a greater extent at the cost of the activities of serine proteases, aspartic proteases, and metalloproteases (by 33, 83, and 67%, respectively), and to a lesser extent at the cost of cysteine proteases (a mere 17%). Exposure to the Sp245 lectin had more of an effect on metalloprotease activity (70% inhibition), and had less of an effect on aspartic, serine, and cysteine proteases (44, 37, and 50%, respectively; Fig. 3b).

As shown in Fig. 3c, neither of the lectins affected cysteine proteases at the alkaline pH value, but both produced an effect on metalloproteases, because the presence of EDTA decreased the activity substantially (by 67% with *A. brasilense* Sp7 and by 60% with *A. brasilense* Sp245). The lectins also affected serine proteases; adding PMSF inhibited the activity by 50% with the Sp7 lectin and by 39% with the Sp245 lectin. Possibly, the lectin-induced change in alkaline protease activity occurs largely through the influence on the activities of serine proteases and metalloproteases.

Regulation of proteolytic enzyme activity in plant cells involves inhibitory proteins which have the common property of being able to reversibly bind enzymes, forming inactive complexes. Plants synthesize various enzyme inhibitors, the most widespread of which are serine protease inhibitors, in particular the trypsin inhibitor. Protease inhibitors are intensely elaborated in plants suffering from mechanical damage or pathogen attack (Joanitti and others 2006).

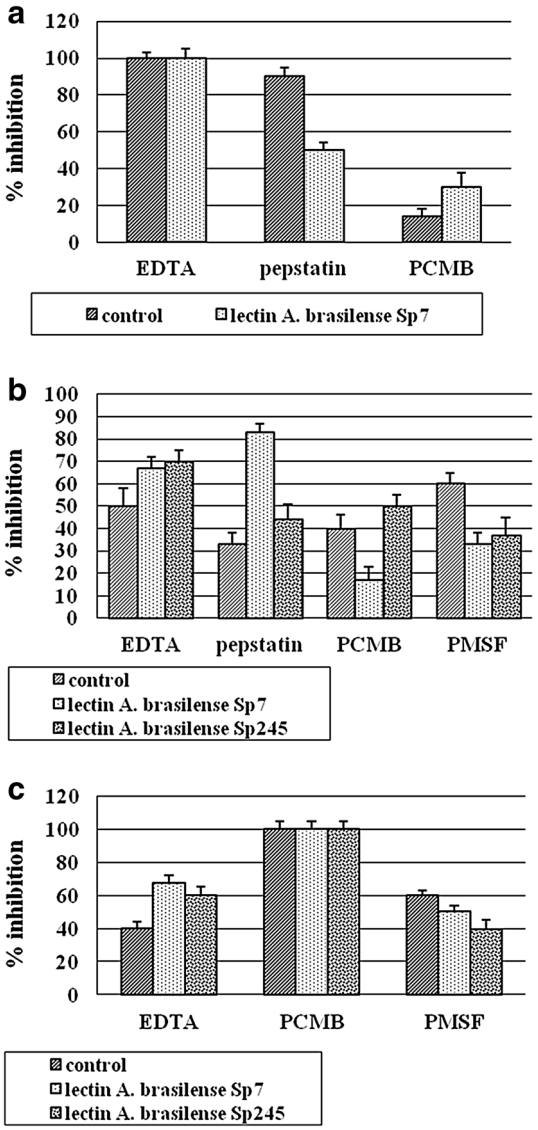

Fig. 3 Inhibitor effects on the activities of **a** acidic, **b** neutral, and **c** alkaline proteases in wheat seedling roots exposed to the *A. brasilense* Sp7 and Sp245 lectins. Values are expressed as mean ± SE of three independent experiments. All differences are significant ($p < 0.05$)

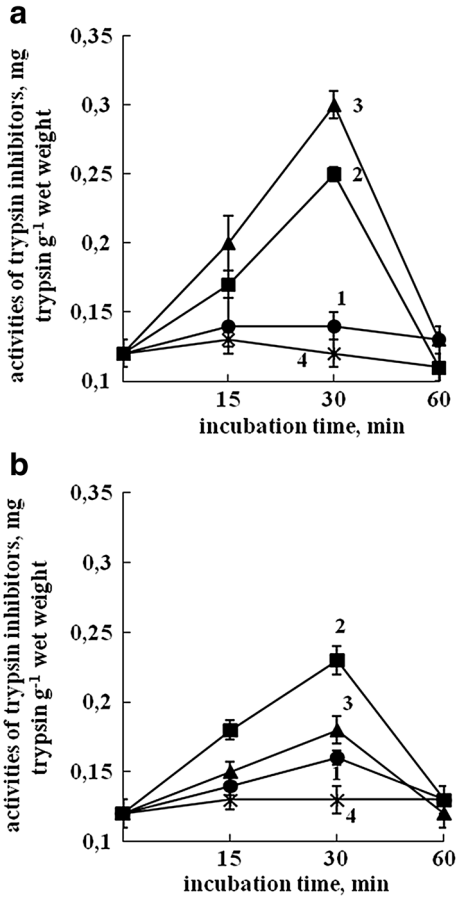

Fig. 4 Trypsin inhibitor activity in wheat seedling roots exposed to the **a** *A. brasilense* Sp7 and **b** *A. brasilense* Sp245 lectins. Control: roots (0.12 mg trypsin g wet weight^{-1}). 1–4, roots + lectins at a concentration of 5 (1), 10 (2), 20 (3), and 40 (4) μg ml^{-1}. Values are expressed as mean ± SE of three independent experiments. All differences are significant ($p < 0.05$)

Both lectins had similar effects on trypsin inhibitor activity in seedling roots (Fig. 4). As early as 15 min after the roots had been exposed to the lectins, the trypsin inhibitor activity increased. On subsequent exposure, the activity increased further, had peaked by 30 min, and then decreased gradually. For all lectin concentrations tested, the trypsin inhibitor activity went through similar dynamics of change, but the peak values were attained with 20 μg ml^{-1} of the Sp7 lectin (Fig. 4a) and with 10 μg ml^{-1} of the Sp245 lectin (Fig. 4b). That the lectins with different carbohydrate specificities produced the same effect indicates once again that their ability to bind specific carbohydrates is not of basic importance for their biological effects. The noted distinctions in the concentrations leading to the maximal effect could be explained by the different numbers of binding sites on the lectin molecules that are necessary for the effect to manifest itself.

In summary, exposure of wheat-root seedlings to the Sp245 lectin considerably increased the protease and trypsin inhibitor activities. The increased protease activity may have been an indication of degradative processes just beginning to develop and was directed to blocking these processes to ensure that the disrupted or damaged structures and functions were restored. With the Sp7 lectin, the enhancement of trypsin inhibitor activity, which occurred with a concomitant decrease in protease activity, was due to the ability of the lectin to reversibly bind enzymes and convert them into an inactive state (Valuev and Mosolov 2002). The Sp7 lectin inhibited proteolytic enzyme activity at all pH values tested by us, whereas the Sp245 lectin

enhanced proteolysis only at neutral and alkaline pH values. Both lectins changed the ratio between the activities of the different types of proteases. The greatest changes induced by the Sp7 lectin occurred at the cost of decreased activities of acidic aspartic proteases and serine proteases and of alkaline aspartic proteases and metalloproteases. The activating effect of the Sp245 lectin was due to neutral aspartic proteases, neutral cysteine proteases, and neutral and alkaline metalloproteases.

A possible reason for the different functional activities of the lectins may be their different carbohydrate specificities, structural differences (Nikitina and others 2005; Shelud'ko and others 2009), and, as a consequence, differences in the interaction with the plant cell surface, which is crucial for the "switch-on" of the subsequent steps. Considering that an active role of *Azospirillum* proteolytic enzymes in bacterial penetration of inner root tissue was shown previously (Chernyshova and others 2005), all the above differences may be related to the possible involvement of the Sp245 lectin in this process.

As pointed out by other researchers (Habib and others 2007; Shan and others 2008), changes in the activities of proteases and their inhibitors in plant tissues are a nonspecific response of plants to biotic and abiotic stress. From this, it follows that changing the balance between the activities of proteases and their inhibitors in plant tissues is a mechanism of improving plant resistance.

Together with the previous data showing that *Azospirillum* lectins can have inductive effects on the plant cell signal systems (Alen'kina and others 2014), the current data suggest that the Sp7 and Sp245 lectins can induce the development of responses in plants that, in combination with the plant-growth-promoting effect of the bacteria, contribute to plant resistance and performance.

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