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REGULAR ARTICLE

Signal effects of the lectin from the associative nitrogen-fixing bacterium *Azospirillum brasilense* Sp7 in bacterial–plant root interactions

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Abstract
Background and aims Azospirillum brasilense, which has the potential to stimulate plant growth, belongs to the group of plant growth-promoting bacteria. The lectin found on the surface of *A. brasilense* strain Sp7 has the ability to bind specific carbohydrates and ensures adhesion of the bacteria to the root surface. The aim of this work was to investigate possible inductive effects of the Sp7 lectin on the plant cell signal systems.
Methods Enzyme-linked immunosorbent assay, spectrophotometry, and thin-layer and gas–liquid chromatography were used to determine the content of signal intermediates in the cells of wheat root seedlings. Laser scanning confocal microscopy was used to examine the localization of fluorescently labeled lectin on the plant cell.
Results The Sp7 lectin acted on the signal system components in wheat seedling roots by regulating the contents of cAMP, nitric oxide, diacylglycerol, and salicylic acid, as well as by modifying the activities of superoxide dismutase and lipoxygenase. The revealed cell membrane localization of the lectin is of deciding importance for its signal function.
Conclusions The results of the study suggest that the *A. brasilense* Sp7 lectin acts as a signal molecule involved in the interaction of growth-promoting rhizobacteria with plant roots.

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Introduction

The free-living bacteria *Azospirillum* live in close association with plant roots and are some of the best characterized plant growth-promoting rhizobacteria (PGPR). Plants obtain direct benefit from the ability of these bacteria to fix N_2 (Baldani and Baldani 2005), produce phytohormones (Tsavkelova et al. 2006),

solubilize phosphates (Rodriguez et al. 2004), improve plant water and mineral status (Ogut and Er 2006), produce compounds to increase membrane activity (Alen'kina et al. 2006) and proliferation of the root system tissues (Nikitina et al. 2004), decrease stressor effects on plants (Bashan et al. 2004), and control numerous phytopathogens (Dadon et al. 2004).

Many azospirilla are unable to enter plant cells, and this presupposes that these bacteria can form signal molecules that cross the plant cell wall and are recognized by the plant membrane receptors. This interaction can initiate a chain of events resulting in altered metabolism of the inoculated plant and in proliferation of roots. Since plant membranes are extremely sensitive to any change, their response may serve as a precise indicator of *Azospirillum* activity at the cellular level (Bashan et al. 2004; Bashan and de-Bashan 2010).

The binding of wheat germ agglutinin (WGA) to cell receptors of *A. brasilense* Sp245 alters bacterial cell metabolism, promoting N_2 fixation, excretion of ammonium ions, and synthesis of indole-3-acetic acid (IAA). It also alters the relative proportion of acidic phospholipids of the membrane; that is, WGA may function as a signal molecule in the *Azospirillum*–plant association (Antonyuk and Evseeva 2006).

Some *Azospirillum* strains are known to produce several lectins in vitro (Castellanos et al. 1998), and Nikitina et al. (1996) speculated a role for *Azospirillum* cell surface lectins in bacterial adhesion to roots. Alen'kina et al. (1998) isolated the surface lectin of *A. brasilense* Sp7 and found it to be a 36-kDa glycoprotein with specificity for L-fucose (1.87 mM) and D-galactose (20 mM). The lectin affected α-glucosidase, β-glucosidase, and β-galactosidase activities in the membrane and apoplast fractions of wheat seedling roots (Alen'kina et al. 2006). Lectins have also been found to induce changes in the mitotic state of growing onion plant cells (Nikitina et al. 2004).

In this context, we sought here to investigate how plants would respond to the effect of the *A. brasilense* Sp7 lectin and to prove that the lectin has a signal function.

Materials and methods

Strain and growth conditions

Azospirillum brasilense Sp7 was obtained from the culture collection of Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow. The culture was grown in the synthetic medium described by Sadasivan and Neyra (1985) at 37ºC for 18 h.

Lectin isolation

The lectin was isolated from the Sp7 cell surface by the method of Echdat et al. (1978) and was purified by gel filtration on a 30×2.2-cm column of Sephadex G-75 (40–120 μm particle diameter). 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}. 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 a lectin solution were added to the wells of a microtitration plate, with PBS serving 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 concentration of the lectin solution that gave hemagglutination was recorded as the hemagglutination titer.

Seed sterilization, obtainment of seedling roots, and root pretreatment with lectin

Seeds of *Triticum aestivum* L. "Saratovskaya 29" (All-Russia Science Research Institute of Agriculture in the South-East, 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. The roots of 4-day-old seedlings were held in a solution containing 5 to 40 μg mL^{-1} of lectin and, in a separate series of experiments, in a lectin solution containing 0.1 mM $CaCl_2$. After that, the content of signal intermediates was determined, with non-lectin-treated root samples as controls.

Protein assay

Protein was estimated by the Bradford method (1976).

cAMP assay

The seedling roots were fixed in liquid nitrogen and then homogenized in an isolation buffer consisting of 50 mM

Tris–HCl (pH 7.4), 0.1 mM theophylline, 1 mM dithiothreitol, and 0.5 mg mL^{-1} polyvinylpyrrolidone. The mixture was filtered and centrifuged at 10,000×*g* for 40 min. Enzyme-linked immunosorbent assay (ELISA) was conducted in 96-well polystyrene plates (SPL Life Sciences, Korea). Each well received 50 μl of successive twofold dilutions of the samples, and the samples were immobilized by drying in a flow of air at room temperature. The primary antibodies were rabbit anti-cyclic adenosine monophosphate (cAMP) antibodies (0.1 mg mL^{-1}; Sigma, USA), and the secondary antibodies were peroxidase-labeled goat antirabbit antibodies (2 μg mL^{-1}). The ELISA result was presented as the percent difference between the absorbance (*A*) values obtained for the experimental and control roots.

Nitric oxide assay

Nitric oxide (NO) content was determined by measuring the level of nitrite (NO^{-}_{2}) accumulated in the root homogenate, by using the Griess reagent consisting of equal volumes of 0.3 % sulfanilic acid and 0.5 % α-naphthylamine. After 10 min of contact, the absorbance at 540 nm (A_{540}) was measured (Schulz et al. 1999).

Citrulline assay

Citrulline was determined by thin-layer chromatography (TLC) on silica gel 60A (Merck, Germany), with *n*-butanol–acetic acid–water (4:1:1 *v*/*v*) as the solvent system. The chromatograms were stained with a ninhydrin solution (Darbre 1989), and citrulline was identified with pure commercial citrulline (Sigma, USA). The spots were scraped off and eluted, and citrulline was quantified at 570 nm.

Diacylglycerol assay

Lipid extracts of wheat seedling roots were obtained by the methods of Folch et al. (1957) and Blight and Dyer (1959). The lipid components were identified by TLC on silica gel, with hexane–diethyl ether–acetic acid (55:45:1 *v*/*v*) as the solvent system, as well as by qualitative reactions and by comparison of the chromatographic mobility of the samples with that of standards (Keyts 1975). The amount of diacylglycerol (DAG) was determined by gas–liquid chromatography on a GH-2010 gas chromatograph (Shimadzu, Japan) fitted with an Equity-1 capillary column (30 m length, 0.32 mm inside diameter; Supelco, USA). The flow rate of the helium carrier gas was 34 mL min^{-1}, and the oven and detector temperatures were 270 °C. Methylation was done according to Christie (1993). DAG was identified by comparing its retention time with that of the standard.

Lipoxygenase assay

The activity of lipoxygenase (EC 1.13.11.12) in the root homogenates was measured spectrophotometrically, with linoleic acid as a standard (Axelrod et al. 1981).

Salicylic acid assay

For determination of free and bound salicylic acid (SA), 1 g of roots was thoroughly washed with distilled water and was fixed with hot 96 % ethanol. The extract was divided into two parts to obtain the free and the bound form (Palva et al. 1994). SA was determined on a GH-2010 gas chromatograph equipped with an Equity-1 column at 200 °C.

Phenylalanine ammonia lyase assay

Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5) was extracted from roots with 0.1 M borate buffer (pH 8.8) at 4 °C for 30 min, with a root:buffer ratio of 1:17. The reaction mixture, consisting of 0.1 mL of root extract and 0.4 mL of borate buffer (pH 8.8) with 12 mM L-phenylalanine, was incubated at 37 °C for 1 h. Enzyme activity was measured spectrophotometrically by the change in absorbance at 290 nm (A_{290}) and was expressed in absorbance units (ΔE g^{-1} of root wet weight) (Zucker 1969).

Superoxide dismutase assay

For determining superoxide dismutase (SOD) (EC 1.15.1.11) activity, roots were homogenized in 0.15M PBS (pH 7.8). The homogenate was centrifuged at 7,000×*g* for 15 min, and the enzyme activity was determined by the inhibition of the reduction rate for tetrazolium nitroblue in a nonenzymatic system containing phenazine methosulfate and NADH (Alscher et al. 2002).

Tetramethylrhodamine isothiocyanate labeling of the lectin and the determination of lectin localization on wheat root cells

Fluorescent labeling of the A. brasilense Sp7 lectin

For labeling, 50 μL of TRITC (1 mg mL^{-1} in dimethylsulfoxide) was mixed with 1 mL of a lectin solution (2 mg of dry sample in 1 mL of 0.1 M sodium bicarbonate buffer, pH 9.0). The reaction was run at 4 ºC for 3 h in the dark, after which the labeled lectin was separated from unreacted fluorochrome by gel filtration on a column (NAP 5; Sigma, USA) of Sephadex G-25 in 20 mM sodium bicarbonate.

In a preliminary test of the specificity of the labeled preparation, a dot assay using rabbit erythrocyte ghosts was conducted. The ghosts were prepared by osmotic hemolysis in 0.015 M sodium chloride, resuspended in the same solution, and washed three times with physiological saline. Finally, the ghosts were sedimented by centrifugation at 3,000×*g* for 10 min, and the supernatant liquid was discarded.

The immunodot reaction was run on 1.5-μm-pore-size nitrocellulose membranes (Synpor, Czech Republic). One-microliter drops of twofold dilutions of the erythrocyte ghosts were spotted onto a membrane in the centers of drawn 5-mm squares, dried, and fixed in a desiccator at 60 °C for 15 min. For preventing nonspecific adsorption of the label on the sample and carrier, the membrane was incubated in a solution of PBS (pH 7.2), 0.2 % BSA, and 0.02 % Tween 20 at room temperature for 15 min. Next, the membrane was incubated at room temperature for 30 min in a solution of a labeled lectin or of a lectin pretreated with the specific hapten L-fucose (1.87 mM). Finally, the membrane was washed with PBS (pH 7.2) containing 0.02 % Tween 20 and was visualized with a Leica LMD 7000 microscope (Carl Zeiss, Germany) set to the fluorescence mode (dichroic cube I3).

Microscopy

Root segments were washed with PBS (pH 7.0), mounted on a glass slide, and, on application of 50 μL of labeled lectin, held in the dark for 30 min. After being washed with PBS three times for 10 min each, the preparations were examined with a Leica TCS SP5 laser scanning confocal microscope (Carl Zeiss, Germany). For additional labeling, the fluorescent dyes rhodamine and FM 1-43 (Hanton and Brandizzi 2006) were used.

Statistics

All experiments were performed in triplicate, and the results were statistically analyzed and presented as mean ± standard error (SE). Significant differences between control and treated plants were determined by Student's *t* test. Differences were considered significant at $p<0.05$.

Results

Although *Azospirillum* imparts an evident growth-promoting effect on a variety of plants, very little is known about the signaling events in the early interaction between bacteria and plant cells. It has been reported that *A. brasilense* Sp7 induced the generation of reactive oxygen species (H_2O_2) in *Arabidopsis* interacting with *Azospirillum*, both at the early and at the later stages of interaction (Ahmed 2010). The effects of *Azospirillum lipoferum* and *A. brasilense* on plant antioxidant enzymes, including catalase, peroxidase, and superoxide dismutase, have been investigated (Baniaghil et al. 2013), and *A. brasilense* has been shown to promote the accumulation of SA in plant roots either locally or systemically (Bashan and de-Bashan 2002a; Ramos Solano et al. 2008).

Some molecules responsible for the eliciting activity of PGPR strains have been characterized and may be cell surface components (Coventry and Dubery 2001; Meziane et al. 2005; Reitz et al. 2002). In this context, the lectins of *Azospirillum* are of much research interest. Earlier work showed that *Azospirillum* lectins are involved in bacterial adhesion to plant roots through their ability to bind carbohydrates (Nikitina et al. 1996). Further study of the lectins' physiological functions showed that in addition to expressing adhesive properties, they can regulate seed germination ability in a concentration-dependent manner and that this lectin action is related to a change in the mitotic state of plant cells (Nikitina et al. 2004). Lectins exhibit enzyme-modifying activity toward homologous hydrolytic enzymes (Chernyshova et al. 2005) and plant cell enzymes (Alen'kina et al. 2006). With this in mind, we now proposed that *Azospirillum* lectins might have a role in the functioning of the plant signal systems.

Lectin effect on the root content of cAMP

An important role in the functional and structural responses of plant cells to external abiotic and biotic influences is played by the adenylate cyclase signal system. One component of this system is cAMP, generated from ATP by adenylate cyclase. The concentration of cAMP in plant samples may vary between lowest possible (femtomoles) and quite high (tens of micromoles) values (Lomovatskaya et al. 2008). Such a scatter depends on external medium factors, which have a substantial effect on the content of this secondary messenger.

An ELISA study of the effect of the *A. brasilense* Sp7 lectin on the quantity of cAMP in wheat root homogenates demonstrated that after 15 min of incubation, all lectin concentrations tested decreased the cell quantity of cAMP. The decrease was the greater the higher the lectin concentration was. After 30 min of incubation, the content of cAMP increased but still was lower than the control (roots, 100 %). After 60 min of incubation, the cAMP content was greater than the control with 5, 10, and 20 μg mL^{-1} of lectin, but lower than the control with 40 μg mL^{-1} (Table 1).

Calcium ions are effective modulators of adenylate cyclase activity (Cali et al. 1994; Willoughby and Cooper 2006). In this study, adding Ca^{2+} ions to the lectin-containing incubation solution increased cAMP content in all treatments as compared with the control (untreated roots). This effect was the greater the more inhibitory was the lectin action. When the lectin had an activating effect, no influence of Ca^{2+} ions was recorded (Table 1).

Lectin effect on SOD activity

The synthesis of hydrogen peroxide is one of the quickest plant cell responses to inducing factors, and a large role in it is played by special enzyme systems. Active oxygen species function mainly within the NADPH-oxidase signal system. SOD is one of the most important enzymes in the antioxidant defense of plants, which catalyzes the conversion of the superoxide radical to hydrogen peroxide. SOD activity has been observed to increase under different effects (Babithaa et al. 2002; Kuzniak and Sklodowska 2004). In this study, after 2 h of root incubation with Sp7 lectin, SOD activity increased at all lectin concentrations tested. The largest (and almost identical) increases were found with 20 and 40 μg mL^{-1} of lectin (Fig. 1).

Lectin effect on the root content of NO

NO is an important participant in signal transduction and regulator of physiological processes in the plant cell. It is involved in the regulation of the plant cell cycle (Wilson et al. 2008), plant differentiation and morphogenesis (Simpson 2005), and the establishment of symbiotic relations between legumes and rhizobia (Glyan'ko and Vasil'eva 2010). The content of NO increased with all lectin concentrations used, but 40 μg mL^{-1} was found to be the most effective. The effect appeared after 1 h, peaked at 3 h, and then decreased to the control value (Fig. 2).

Many investigators believe that plants can have several sources of NO formation and that only some of them can be regulated via signal pathways (Flores et al. 2008; Glyan'ko et al. 2009). One of such pathways

Table 1 Changes in the content of cAMP in wheat seedling roots incubated with the *A. brasilense* Sp7 lectin

Treatment	15 min		30 min		60 min	
	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}
A. brasilense Sp7 lectin						
5 μg mL^{-1}	87±2	137±4	95±2	101±4	143±6	145±4
10 μg mL^{-1}	82±2	137±4	91±2	101±4	141±6	145±4
20 μg mL^{-1}	75±3	150±4	86±3	104±4	125±4	128±3
40 μg mL^{-1}	70±2	179±2	75±1	110±4	73±2	127±2

Results are means ± SE (n=3). All differences significant (p<0.05). Control, roots (100 %) (cAMP content, 0.3 μM) and roots ± $CaCl_2$ (100 %) (cAMP content, 0.34 μM)

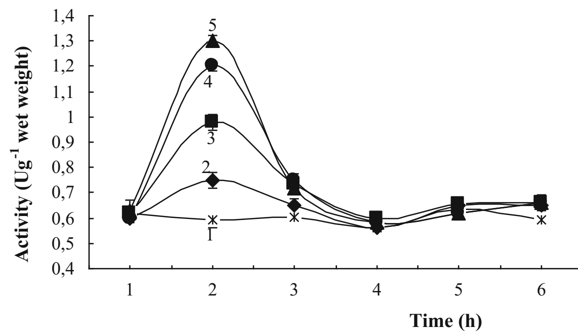


Fig. 1 Effect of the *A. brasilense* Sp7 lectin on SOD activity in wheat roots. *1* control (roots), *2–5* roots + lectin at 5 (*2*), 10 (*3*), 20 (*4*), and 40 (*5*) μg mL^{-1}. Results are means ± SE (n=3). All differences significant (p<0.05)

is α-arginine + O_2 + NADPH → α-citrulline + NO, a reaction catalyzed by NO synthase. To prove that the lectin could induce this pathway of NO formation, we determined the quantity of citrulline in the roots incubated with 40 μg mL^{-1} of lectin as the most effective concentration for NO synthesis. The results showed that the lectin caused an increase in the citrulline quantity during the first hours of coincubation, with a peak at 3 h (Fig. 3).

The finding that root incubation with Sp7 lectin led to a simultaneous increase in the root contents of NO and citrulline permits the conclusion that the lectin can activate the NO signal system of plants.

Lectin effect on the root content of DAG

In plants, phospholipase C is localized in the plasma membrane and is a key enzyme of the inositol cycle. Its functioning gives rise to two intracellular messengers—the water-soluble inositol-1,4,5-triphosphate (IP3) and the lipid-soluble DAG. The Sp7 lectin induced DAG synthesis in seedling roots only when used at 40 μg mL^{-1}. The induction occurred after 3 min of coincubation, with a peak after 40 min. By 60 min of coincubation, synthesis had decreased sharply, with the amount of DAG declining to the control value. As Ca is the major activator among the ions able to affect the activity of phospholipase C (Novotnà et al. 2000), the induction was enhanced when Ca was added to the root incubation medium (Fig. 4).

Lectin effect on lipoxygenase activity

One of the mechanisms responsible for the formation of signal products of lipid transformation is the lipoxygenase signal system, the starting enzyme of which is lipoxygenase. Determination of lipoxygenase activity in lectin-incubated roots showed that there was a sharp rise in activity—by 30 % after a 30-min incubation and by 50 % after a 60-min incubation. Extending the incubation time caused the enzyme activity to decline to the control value. Only 5 μg mL^{-1} of lectin had inducing activity (Fig. 5).

Lectin effect on the root content of SA

In plants, SA is present both in free form and in bound forms, of which SA 2-O-β-D-glucoside is the most abundant. It should be stressed that SA participates in resistance induction only in its free form. Bound forms of SA have no such property; instead, they act as a kind of reserve that ensures SA storage in tissues (Raskin 1992).

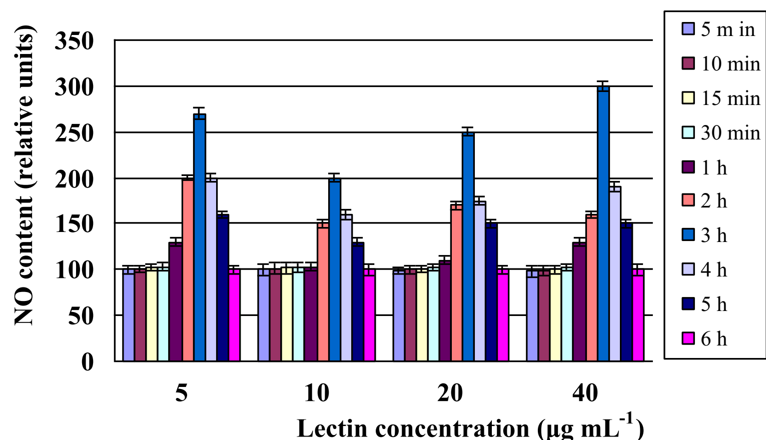


Fig. 2 NO content in lectin-incubated wheat roots. Control, roots 100 %; NO content, 0.8 μM g^{-1} roots. Results are means ± SE (n=3). All differences significant (p<0.05)

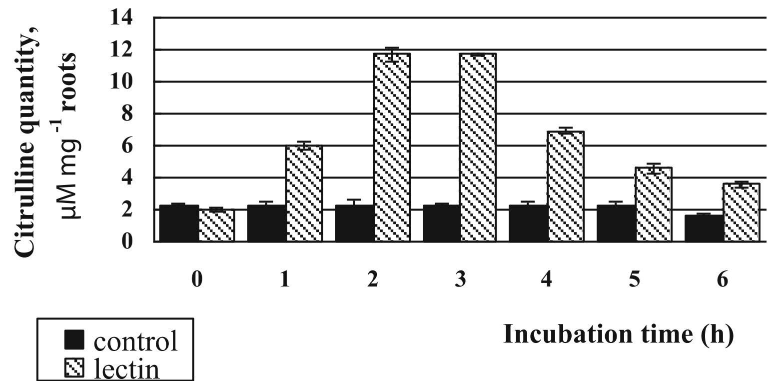


Fig. 3 Citrulline content in lectin-incubated wheat roots. Control, roots. Values are the means ± SE of three independent experiments. All differences significant ($p<0.05$)

Various biogenic factors may increase the plant tissue content of SA by several tens of times (Vasyukova and Ozeretskovskaya 2007). The considerable attention given to SA is primarily due to its being involved in plant defense reactions against pathogens. Thus, the infection of tobacco leaves by tobacco mosaic virus was reported to increase the content of SA by 180 times (Malamy et al. 1990). Such effects in response to infection or elicitor treatment have been recorded with many plant species (Wang and Li 2006; Catinot et al. 2008).

The change in the SA content of lectin-incubated roots indicated a noticeable effect of the lectin. In our experiments, we determined the amounts of free and conjugated SA, as the two forms easily pass into each other but differ in their biochemical and physiological activities (Tarchevsky et al. 1999). The results showed that the lectin changed the content of SA only after 1 h of incubation with roots and that as the lectin concentration increased, the amount of free SA increased and that of conjugated SA decreased. As seen in Fig. 6, the amounts of the formed free SA and the hydrolyzed bound SA were different. The question arises, did SA accumulation result only from hydrolysis of the conjugates, or was it also synthesized de novo? To answer this question, we determined the activity of phenylalanine ammonia lyase (PAL), an enzyme responsible for the synthesis of SA. As shown in Fig. 6 and in Table 2, the lectin did induce the activity of PAL, but there was no correlation between the change in the content of free SA and the activity of PAL.

Localization of the *A. brasilense* Sp7 lectin on the cells of wheat seedling roots

Studies on the plant cell localization of *Azospirillum* lectins are of particular interest, as they provide insights into the possible mechanism of lectin action on cellular metabolism. *A. brasilense* preferentially colonizes the root tip and root hairs (Bashan and Levanony 1989; Levanony et al. 1989); therefore, we examined the localization of the Sp7 lectin in these very root zones. Using

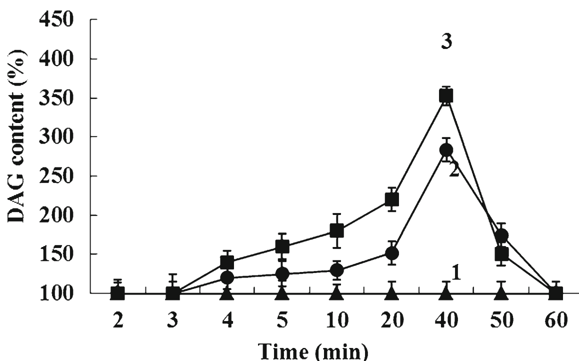


Fig. 4 DAG content in lectin-incubated wheat roots. *1* controls [roots, roots + $CaCl_2$ (100 %); DAG content, 20 nM g^{-1} roots], *3* lectin + roots, *4* lectin + roots + $CaCl_2$. Lectin concentration, 40 μg mL^{-1}. Values are the means ± SE of three independent experiments. All differences significant ($p<0.05$)

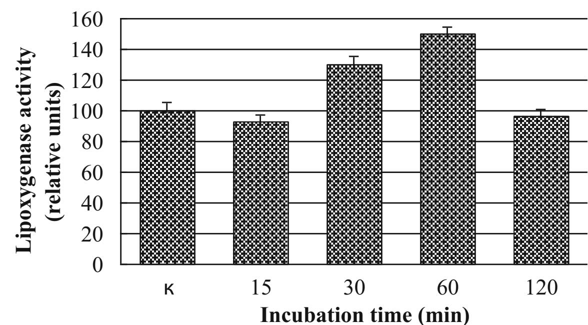


Fig. 5 Effect of the *A. brasilense* Sp7 lectin on the lipoxygenase activity of wheat roots. Control (roots, 100 %; lipoxygenase activity, 30 U g^{-1} root wet weight). Lectin concentration, 5 μg mL^{-1}. Results are means ± SE (n=3). All differences significant ($p<0.05$)

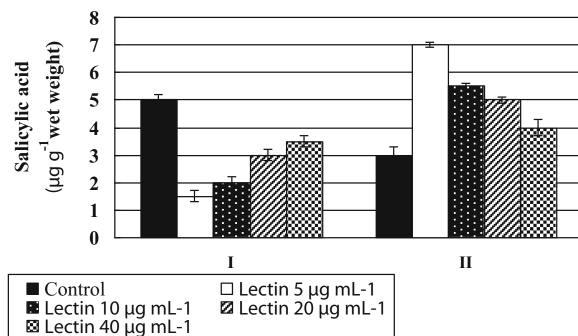


Fig. 6 The content of conjugated (*1*) and free (*2*) SA in the control and lectin-incubated roots. Incubation time, 1 h. Results are means ± SE (n=3). All differences significant (p<0.05)

fluorescence microscopy and tetramethylrhodamine isothiocyanate (TRITC)-labeled Sp7 lectin, we demonstrated that the lectin was present only on the cell surface of wheat roots. Figure 7a and b shows clearly that the labeled lectin was distributed along the perimeter of the sheath cell and the root hair cell. Additional staining of root cells with the fluorescent dye FM 1-43, used to visualize plasma membranes, showed that the lectin was present exclusively on the plasma membrane, but not on the cell wall. In the set of optical sections of the root sheath cell shown in Fig. 7a, the red color corresponds to TRITC fluorescence and can be visualized on the outside of the plasma membrane, whereas the green color, corresponding to fluorescence from the lipophilic dye FM 1-43, is revealed on the inside of the cytoplasmic membrane.

To test whether the binding sites for the lectin could be localized intracellularly, we used the mitochondrial dye rhodamine 123. The middle optical section of the root hair shown in Fig. 7b demonstrates lectin localization on the cell surface (red color). The yellow color of the intracellular matrix is due to the red and green (fluorescence of rhodamine 123) colors being mixed. The intracellular matrix was not revealed on preparations stained only with TRITC–lectin.

This report is the first to present data on the localization of an *Azospirillum* lectin on the plant cell. The character of lectin distribution on the plasma membrane revealed by fluorescence, together with the results of the other experiments, indicates that the reception of the lectin signal occurs primarily on the cell surface.

Table 2 PAL activity in lectin-incubated roots

Treatment	PAL activity (%)
Control	100±3
Lectin at 5 μg mL^{-1}	115±5
Lectin at 10 μg mL^{-1}	105±4
Lectin at 20 μg mL^{-1}	110±6
Lectin at 40 μg mL^{-1}	120±3

Results are means ± SE (n=3). All differences significant (p<0.05). Control, roots (100 %) (PAL activity, 0.8±0.2 U g^{-1} root wet weight)

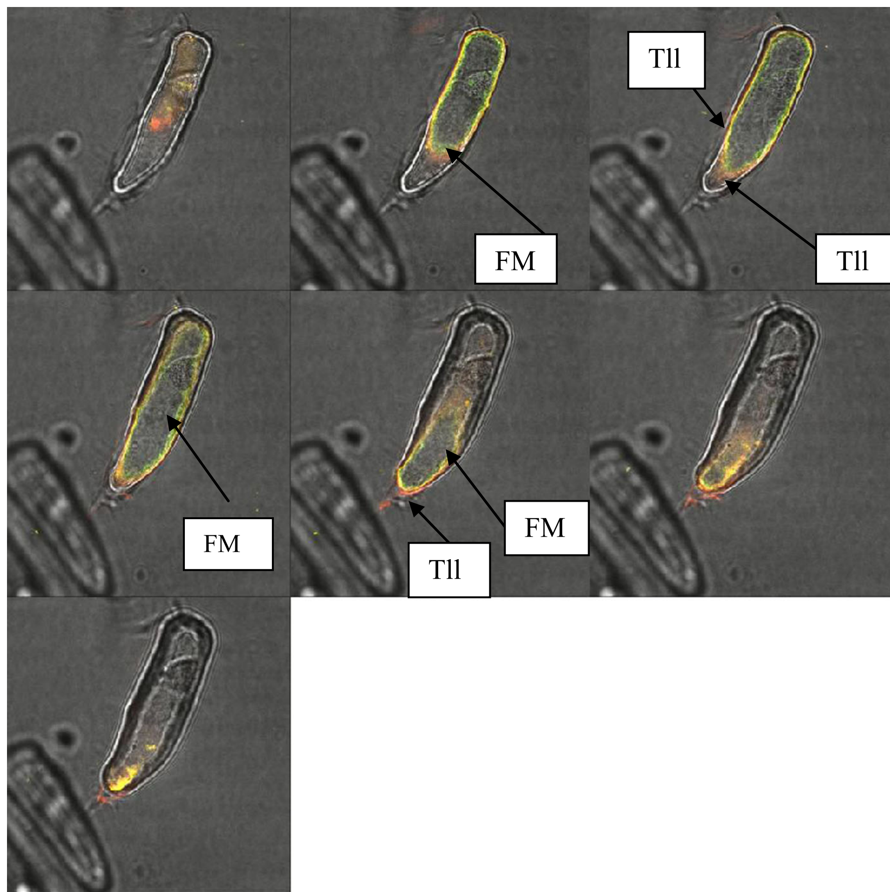
Discussion

In this study, we have demonstrated that the *A. brasilense* Sp7 lectin can induce the adenylate cyclase, NO synthase, NADPH oxidase, Ca phosphoinositol, and lipoxygenase signal systems of wheat roots during recognition early in the establishment of a plant–bacterial association.

The induction of the adenylate cyclase signal pathway, which occurred 15 min into lectin incubation with seedling roots, was one of the early plant cell responses to the lectin effect. One can conclude that the Sp7 lectin can both elicit and suppress cAMP in the plant cell. This signal system plays an important role in the functional and structural responses of plant cells to many extrinsic abiotic and biotic factors (Lomovatskaya et al. 2008).

Several authors have shown that the plant perception and transduction of signals from metabolites of fungal and bacterial pathogens involve receptor–G protein complexes (Kawakita and Doke 1994; Zhu et al. 2009). The activation or inhibition of adenylate cyclase always occurs through the corresponding ligand–receptor interactions and various types of G proteins, which are known to be either stimulatory (G_s) or inhibitory (G_i). After binding to the ligand, the receptor undergoes conformational changes, resulting in the same changes in the G protein (Chen and Iyengar 1993; Sunahara and Taussig 2002).

The most probable explanation for the lectin effect is that the lectin acts dose-dependently on the receptors associated with the G_i and G_s proteins. Adding Ca^{2+} to the incubation medium changed the interaction of the lectin with these receptors, resulting in changes in adenylate cyclase activity and, correspondingly, in cAMP content. Support for such conclusions can be inferred from the earlier data that the Sp7 lectin can have dose-dependent effects (Nikitina et al. 2004) and that the ions



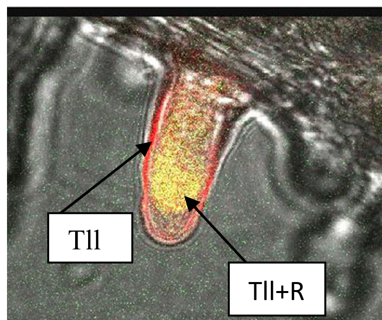


Fig. 7 Localization of the *A. brasilense* Sp7 lectin on the plant cell. **a** Cells of the root sheath + lectin−TRITC + FM. **b** root hair + lectin−TRITC + FM + rhodamine. Fluorescence was laser excited at 488 nm. Reception at 555–618 nm (*red*) for TRITC, 615–630 nm (*green*) for FM 1-43, and 505–540 nm (*green*) for rhodamine. *Tll* TRITC-labeled lectin, *R* rhodamine

of bivalent metals, including Ca, can modulate the biological activity of many lectins (Imberty et al. 2004; Bulgakov et al. 2007).

One of the most important signal systems in plants is the lipoxygenase system. Previous research from other authors has indicated that rhizosphere bacteria,

including PGPR (among them azospirilla), activate lipoxygenase metabolism in the plant cell (Choudhary et al. 2007; Beneduzi et al. 2012). In this study, root incubation with Sp7 lectin for 30 min induced the lipoxygenase signal pathway, as evidenced by an increase in lipoxygenase activity.

A 40-min incubation resulted in an increase in the DAG quantity owing to the activation of phospholipase C, which is localized in the plasma membrane and is a key enzyme of the phosphoinositide cycle. The functioning of phospholipase C gives rise to two intracellular messengers—the water-soluble inositol-1,4,5-triphosphate (IP3) and the lipid-soluble DAG. IP3 mobilizes Ca^{2+} from the endoplasmic reticulum, increasing the concentration of free Ca^{2+} ions in the cytosol, and DAG, which remains in the membrane, activates Ca^{2+}-sensitive phospholipid-dependent protein kinase (Krasilnikov 2000).

Root incubation with Sp7 lectin for 1 h increased the content of NO, which participates in the NO signal system and regulates physiological processes in the plant cell. NO is involved in the regulation of the plant cell cycle (Wilson et al. 2008), plant differentiation and morphogenesis (Simpson 2005), and the establishment of symbiotic relations between legumes and rhizobia (Glyan'ko and Vasil'eva 2010). Creus et al. (2005) reported that NO is involved in the lateral root formation induced by *A. brasilense* Sp245 in tomato plants.

That the incubation of roots with Sp7 lectin led to a simultaneous increase in the root contents of NO and citrulline permits the conclusion that the lectin can activate the NO signal system of plants. It is known that an increase in NO concentration activates guanylate cyclase. The resulting cGMP activates protein kinase, which opens the Ca channels of the intracellular Ca repositories; this brings about an increase in the cytosolic Ca concentration, activation of Ca-dependent protein kinases, phosphorylation of the protein factor of transcription regulation, and the beginning of synthesis of specific proteins (Dyakov et al. 2001).

Root incubation with Sp7 lectin for 1 h increased the content of SA, a stress metabolite that combines the properties of a signal intermediate with those of a phytohormone. Although most previous studies on SA have been focused on interactions between plants and virulent or avirulent pathogens (Bari and Jones 2009; Delaney et al. 1994; Tarchevsky et al. 2010), it has been demonstrated that some PGPB, including *A. brasilense* (Bashan and de-Bashan 2002b; Ramos Solano et al. 2008), can stimulate plants to accumulate SA either locally in roots (Chen et al. 1999) or systemically in leaves (De Meyer et al. 1999; Zhang et al. 2002). Our present results permit the conclusion that the *A. brasilense* Sp7 lectin can induce SA-mediated signaling in plant cells. We infer that the Sp7 lectin induces two routes of SA formation: the release from the conjugated form through an increase in β-glucosidase activity (Alen'kina et al. 2006) and the activation of PAL, which is responsible for SA synthesis. The effects of SA under biotic stress are largely determined by its influence on the activities of the enzymes involved in the regulation of the prooxidant/antioxidant equilibrium, in particular catalase, NADPH oxidase, peroxidase (Geetha and Shetty 2002), and SOD (Rao et al. 1997).

Of particular interest is the synthesis of hydrogen peroxide, which is one of the quickest plant cell responses to inductive factors. SOD is one of the most important enzymes in the antioxidant defense of plants, which catalyzes the conversion of the superoxide radical to hydrogen peroxide (Kuzniak and Sklodowska 2004). In this study, SOD activity increased after 2 h of root exposure to Sp7 lectin.

Finally, the revealed membrane localization of Sp7 lectin on the plant cell is of deciding importance for its signal function.

We propose that *Azospirillum* lectins may act at the initial stages of plant–bacterial interaction by ensuring a strategy of interaction related to the induction of plant defense responses. This is something similar to what is observed in nodule and phytopathogenic bacteria, despite the outcomes of these interactions being substantially different. In plants, a physiological response to various pathogens includes a diversity of defense reactions to the danger of infection (Dmitriev 2003). The legume–*Rhizobium* interaction also leads to the induction of defense mechanisms in the host plant, which is accompanied by the generation of active oxygen species and NO, enhancement of the activities of oxidative enzymes (peroxidase, catalase, SOD), accumulation of phenolic compounds, and enhancement of antioxidase defense (Glyan'ko et al. 2007). Our results could be of practical significance, as pretreatment with growth-promoting antistress inducers contributes to plant resistance and productivity. Our results are also of considerable interest for understanding the biological role of lectins in bacterial–plant relationships during the formation of nitrogen-fixing associations.

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