Brazilian Journal of Botany (2018) 41:579–587
https://doi.org/10.1007/s40415-018-0489-1

ORIGINAL ARTICLE

Regulation by *Azospirillum* lectins of the activity of antioxidant enzymes in wheat seedling roots under short-term stresses

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Received: 6 March 2018 / Accepted: 13 July 2018 / Published online: 31 July 2018

Abstract
Azospirillum brasilense Tarrand, which has the potential to stimulate plant growth, belongs to plant-growth-promoting bacteria. Many species of azospirilla colonize the rhizosphere, the portion of soil attached to the root surface. Some species can also enter the host root system and enhance their beneficial effects with an endophytic lifestyle. Depending on the specific agroecological situation, the positive effect of *Azospirillum* on plants may be due to different mechanisms. Azospirilla can assist in mitigation of many kinds of abiotic stress. Although they can affect antioxidant enzyme activity in abiotically stressed plants, the underlying mechanisms are not fully understood. The surface lectins of *A. brasilense* strains Sp7 and Sp245 differ in carbohydrate specificity and in the mode of plant root colonization. They promote plant growth and enzyme activity, and they also can alter the plant cell content of stress metabolites, which attests that they can induce adaptation processes in wheat seedling roots. Here we comparatively investigated the ability of the Sp7 and Sp245 lectins (concentration, 5–40 μg ml^{-1}) to regulate the activities of antioxidant enzymes in roots of 4-day-old seedlings of wheat subjected to hypothermic (5 °C) and hyperthermic (42 °C) stress. Both lectins increased peroxidase and superoxide dismutase activities and decreased catalase activity, but the effects lasted for different times and the concentrations involved were also different. We conclude that the *Azospirillum* lectins are involved in adaptational changes in wheat seedling roots and that this involvement promotes the normal course of metabolism and ensures regulation of the plant–*Azospirillum* interaction in a wider range of soil and climatic factors.

Keywords Abiotic stresses · Associative nitrogen fixation · *Azospirillum* lectins · Catalase superoxide dismutase · Peroxidase · Plants

1 Introduction

Adverse climatic conditions, creating abiotic stresses, are among the principal factors limiting agricultural productivity (Padgham 2009; Grayson 2013). Extreme temperatures conditions can severely affect plants. Therefore, study of the mechanisms governing tolerance and adaptation in higher plants is of great scientific and practical significance. Because plants lack behavioral mechanisms of defense against unfavorable factors, the major adaptive changes occur primarily at a biochemical level (Tarchevskii 2001). A group of nonspecific responses to adverse exposures have been found recently, including (1) changes in cell membrane permeability and in intracellular pH and (2) accumulation of protective substances such as stress proteins, lipids, and soluble carbohydrates (Hasanuzzaman et al. 2013). One of the earliest effects is oxidative stress caused by the accumulation of reactive oxygen species (ROS). To protect themselves from this stress, plants have developed enzymatic antioxidative systems consisting of superoxide dismutase, catalase, and peroxidases (Almeselmani et al. 2006; Nagesh Babu and Devraj 2008).

The role of microorganisms, with their potential metabolic and genetic capabilities, in alleviating abiotic stress in plants has been studied intensely in the past few decades (Nadeem et al. 2007; Turner et al. 2013; Gopalakrishnan et al. 2015; Souza et al. 2015). Many researchers have

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argued that plant-growth-promoting rhizobacteria (PGPR) can reduce the consequences of abiotic stress in plants (El-Komy et al. 2003; Pereyra et al. 2006; Arzanesh et al. 2009). These PGPR include *Pseudomonas* (Ali et al. 2009; Sorty et al. 2016), *Azotobacter* (Sahoo et al. 2014), *Azospirillum* (Creus et al. 2004; Omar et al. 2009), *Rhizobium* (Remans et al. 2008; Sorty et al. 2016), *Bacillus* (Vardharajula et al. 2011; Sorty et al. 2016), *Enterobacter* (Nadeem et al. 2007; Sorty et al. 2016), *Bradyrhizobium* (Panlada et al. 2013), *Methylobacterium* (Madhaiyan et al. 2007; Meena et al. 2012), and *Burkholderia* (Oliveira et al. 2009).

The application of PGPR to abiotically stressed plants significantly increases the content of defense-related enzymes such as superoxide dismutase, peroxidase, catalase, polyphenol oxidase, phenylalanine ammonia-lyase, and lipoxygenase (Liang et al. 2011; Chakraborty et al. 2015). However, few data are available about the mechanisms of the bacteria-mediated antioxidative protection of plants.

Azospirillum spp. are the most studied PGPR and are a common model for research on plant–bacterial interactions. These bacteria take advantage of many plant-growth-promoting mechanisms (Bashan et al. 2014) and have been used as inoculants in crop production, initially with cereals but later with other plants. They stimulate plant growth through fixation of N_2, synthesis of phytohormones, solubilization of phosphates, improvement of plant water and mineral status, production of compounds that increase membrane activity and proliferation of root tissues, and decrease in stressor influence (Bashan et al. 2004; Baldani and Baldani 2005; Alen'kina et al. 2006). Although research in this area is active, an open question remains on which of the above factors, explaining the benefit of N_2-fixing bacteria to plant growth and performance, has priority over the others.

Azospirillum can colonize roots externally and/or internally, or it can colonize the stem as an endophyte, as seen in rice (*Oryza sativa* L.), with some strains doing both (Ramos et al. 2002; Xie and Yokota 2005). By use of fluorescently labeled probes and monoclonal antibodies, Assmus et al. (1995) and Schloter et al. (1997) detected *Azospirillum* in both the plant interior and the rhizosphere. Specifically, *A. brasilense* Tarrand Sp245 (Tarrand et al. 1978) was found in the root xylem, whereas Sp7 (Tarrand et al. 1978) was detected only on the root surface (Schloter et al. 1997). Endophytic bacteria are of particular research interest, because they can lead a mutualistic life in the plant tissue interior. This ability permits them to depend less on extrinsic environmental factors, as compared with other microorganisms, and to manifest a complex of economically useful properties. When inside the plant tissue, endophytes contribute to sustained plant defense against environmental stress.

Among the high molecular weight and specific substances implicated in interorganismal communication, an important part is played by lectins, glycoproteins that bind strictly specified carbohydrate groups on the surface of a target cell. There is ample evidence that plant lectins are implicated in bacterial colonization of plants and in the restructuring of the metabolism of the bacterial symbiont. Plant lectins act as adaptogens for plants; for example, wheat germ agglutinin changes antioxidant enzyme activity in seeds and broadens plant adaptability (Kruhova et al. 1999). Less is known about the role of bacterial lectins, which nonetheless are involved in the important "molecular dialog" during the development of a symbiosis (Nikitina et al. 1996; Castellanos et al. 1998).

Previously, we have reported the isolation of surface lectins from two *A. brasilense* strains, Sp7 (epiphyte) and Sp245 (endophyte), differing in the mode of plant colonization. The lectins have been found to be glycoproteins with different molecular masses and carbohydrate specificities (Nikitina et al. 2005; Shelud'ko et al. 2009). The 36-kDa Sp7 lectin was specific for L-fucose (1.87 mM) and D-galactose (20 mM). The Sp245 lectin had an affinity for the bacterium's own polysaccharide, an acidic D-rhamnan, and had a molecular mass of 67 kDa.

Both Sp7 and Sp245 lectins are polyfunctional. Apart from functioning as adhesins, they can influence plant cell metabolism by promoting seed germination (Nikitina et al. 2004) and by expressing mitogenic and enzyme-modifying activities toward the plant cell (Chernyshova et al. 2005; Alen'kina et al. 2006; Alen'kina and Nikitina 2015, 2017). In addition, they can alter the plant cell content of stress metabolites (Alen'kina et al. 2014). Finally, lectin activity in *Azospirillum* can be promoted by adverse effects and even by stress, possibly also owing to the adaptogenic function of lectins (Nikitina et al. 2005).

Here we comparatively evaluated the ability of the lectins from *A. brasilense* Sp7 and Sp245 to regulate the activities of peroxidase, catalase, and superoxide dismutase in roots of wheat seedlings subjected to short-term hypothermic and hyperthermic stress.

2 Materials and methods

Strains and growth conditions – *Azospirillum brasilense* Sp7 (epiphytic strain) was obtained from the culture collection of Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow. *A. brasilense* Sp245 (IBPPM 219; endophytic strain) was from the IBPPM RAS Collection of Rhizosphere Microorganisms (http://collection.ibppm.ru). The cultures were grown in the minimal

salts medium described by Sadasivan and Neyra (1985) at 37 °C for 18 h.

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 et al. 2006). To confirm the lectin nature of the purified material, we conducted a 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 hemagglutination titer was the minimum lectin concentration that gave hemagglutination.

Animals were cared for and handled in compliance 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.

Protein assay – Protein was estimated by the Bradford method (1976).

Seedling growth and stress treatments – Seeds of wheat (*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 were incubated in the dark at 25 °C. Seedlings used for experiments were 4 days old. In all experimental treatments, the initial and experimental seedlings were adjusted for physiological age. A root length of 25–30 mm was the criterion that the initial plant material was homogeneous.

For stress experiments, roots were simultaneously exposed for 2 h to either Sp7 or Sp245 lectin (concentration, 5–40 μg ml^{-1}) and temperatures of 42 and 5 °C. The roots were then 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 enzyme activities. Seedlings grown at 25 °C were the control group.

Peroxidase assay – Peroxidase (EC 1.11.1.7.) was assayed by Khairullin et al.'s (2001) micromethod, based on the oxidation of *o*-phenylenediamine (OPD). A 50-μl portion of supernatant liquid prediluted 20-fold with PBS (pH 5.6) and 25 μl of OPD solution (concentration, 0.5 mg ml^{-1}) were added to each well of a flat-bottomed immunoassay plate (Nunc, USA). Two min after 25 μl of 0.43 mM H_2O_2 was added, color development was stopped with 50 μl of 4 N H_2SO_4. The absorption of the samples was measured at 492 nm with an AIF-Ts-01S ELISA reader (ZAO ILIP, St. Petersburg, Russia). Peroxidase activity was expressed as absorption units per g of root wet weight and, for comparative purposes, as relative units.

Catalase assay – Catalase (EC 1.11.1.6) activity was assayed as described by Aebi (1984). The decrease in H_2O_2 was measured at 240 nm, and the activity was calculated as units (μM H_2O_2 consumed per min) per g of root weight (extinction coefficient, 39.4 mM^{-1} cm^{-1}). For comparative purposes, it was also expressed as relative units.

Superoxide dismutase assay – The activity of superoxide dismutase (SOD; EC 1.15.1.11) was assayed by the inhibition of the reduction rate for tetrazolium nitroblue in a nonenzymatic system containing phenazine methosulfate and NADH (Alscher et al. 2002). The absorbance of formazan (oxidation product of tetrazolium nitroblue) was measured at 560 nm and was used to calculate the enzyme activity. The results are presented as relative units.

Statistics – The analysis was run with the AGROS program package for statistical and biometrical–genetic analysis in plant breeding and selection (version 2.09; Department of Statistical Analysis, Russian Academy of Agricultural Sciences). Least significant differences (LSD0.05) were determined at a significance level of $P = 0.05$. Values followed by different letters (a, b, c, d) differ significantly at $P \leq 0.05$, according to Duncan's multiple range test. The figures show arithmetic mean ± standard error (SE) of three independent experiments, done in five biological replications.

3 Results

Lectin effects on antioxidant enzyme activity in wheat seedling roots exposed to hypothermic and hyperthermic stress were investigated with three enzymes. These included (1) SOD, catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen and (2) peroxidase and catalase, which degrade hydrogen peroxide.

Both Sp7 and Sp245 lectins increased peroxidase activity in roots exposed to hypothermic and hyperthermic stress. The picture was the same under both types of stress. With all four concentrations of the Sp7 lectin, the activity

rose after 30 min of incubation, peaking at 20 μg ml^{-1}, and then declined gradually to the control value. With the Sp245 lectin, peroxidase activity increased after 60 min of incubation and the increase was proportional to the lectin concentration (Fig. 1).

Both lectins also enhanced the activity of SOD in the stressed roots (Fig. 2). Under hypothermic conditions, the activity of SOD increased with all concentrations of either lectin after the roots were exposed for 1 h. The most effective lectin concentrations were 20 μg ml^{-1} (Sp7 lectin) and 10 μg ml^{-1} (Sp245 lectin). The same picture emerged under hyperthermic conditions: the enzyme activity rose after the roots were incubated with the lectins for 1 h. The most effective lectin concentrations were 10 μg ml^{-1} (Sp7 lectin) and 5 μg ml^{-1} (Sp245 lectin).

Under hypothermic conditions, both Sp7 and Sp245 lectins decreased root catalase activity. The inhibition peaked as early as 15 min after exposure. Thirty min into exposure, the inhibition slowly decreased, and by 1 h of incubation, it was back to the control value. The effect was maximal with 5 μg ml^{-1} of either lectin (Fig. 3). The same was observed when the stress was changed to hyperthermic: catalase activity declined with both lectins, and the effect was maximal with 5 μg ml^{-1} (Fig. 3).

The Sp7 and Sp245 lectins regulated the enzyme activities differently. Under both types of stress, the Sp245 lectin promoted peroxidase and SOD activities more than did the Sp7 lectin.

As noted above, both lectins decreased catalase activity in the temperature-stressed roots. Under both types of stress, the inhibition achieved with the Sp245 lectin was greater than that attained with the Sp7 lectin.

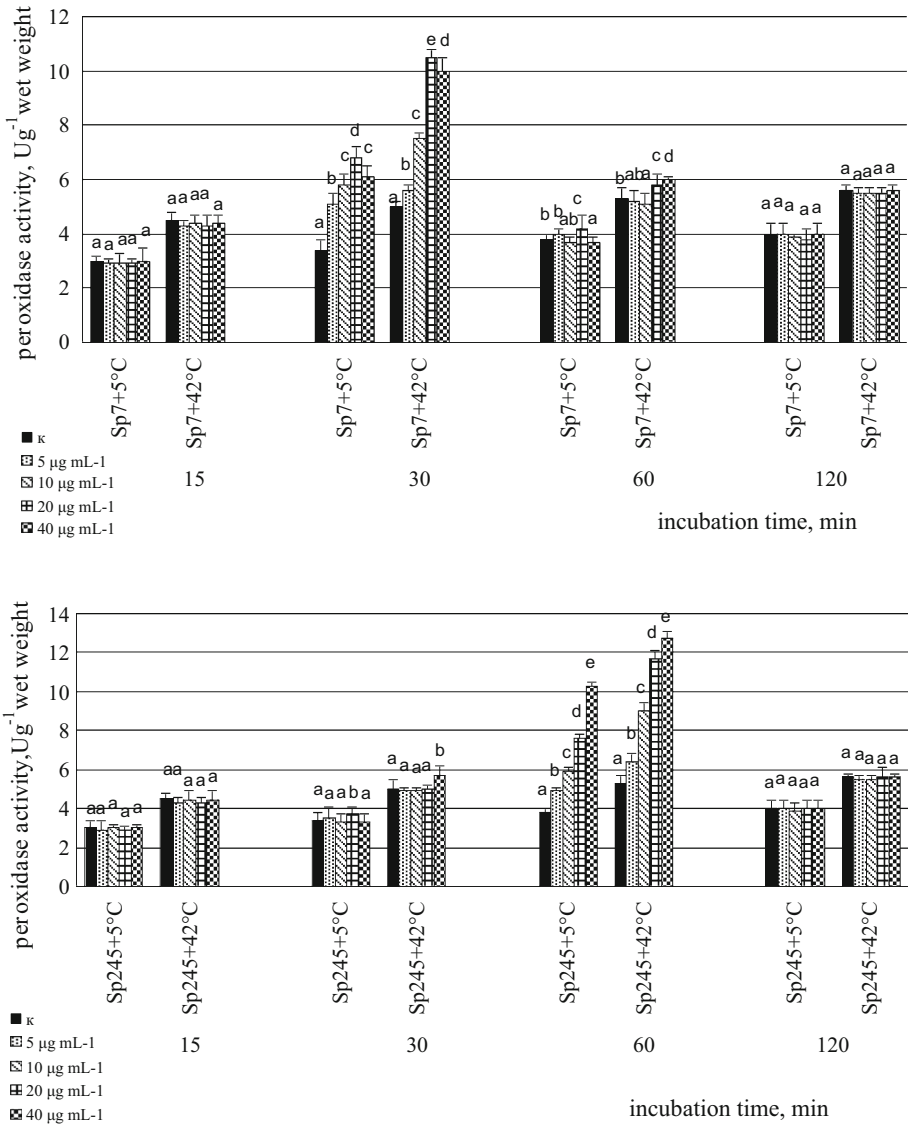

Fig. 1 Effect of the *Azospirillum brasilense* Sp7 and Sp245 lectins on the activities of peroxidase in wheat seedling roots exposed to 5 and 42 °C. Results are expressed as mean ± SE ($n = 5$). Mean separation among treatments was done by Duncan test at $P \leq 0.05$. Mean values followed by different letters are significantly different

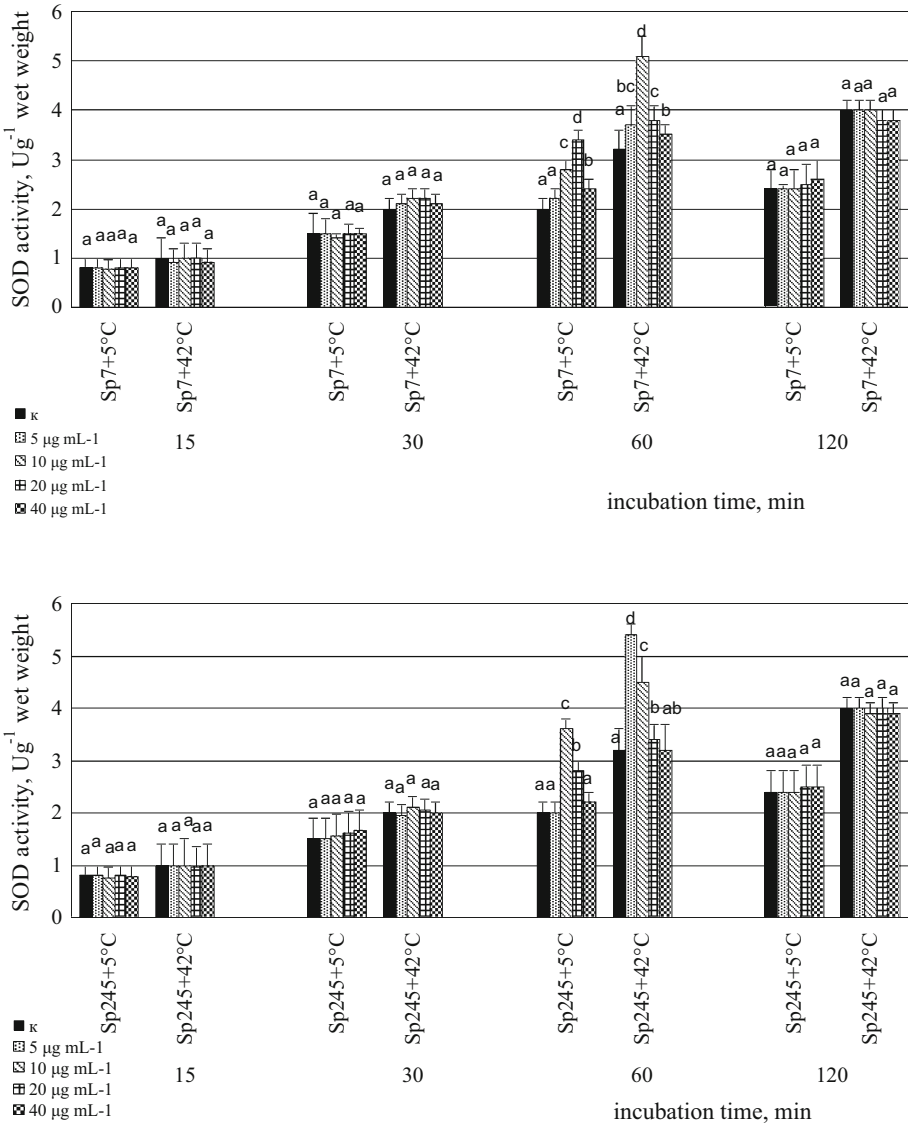

Fig. 2 Effect of the *Azospirillum brasilense* Sp7 and Sp245 lectins on the activities of SOD in wheat seedling roots exposed to 5 and 42 °C. Results are expressed as mean ± SE (n = 3). Mean separation among treatments was done by Duncan test at $P \leq 0.05$. Mean values followed by different letters are significantly different

4 Discussion

Temperatures stress is a serious problem in agriculture and a critical factor for plant survival. Both high and low temperatures affect plant metabolism: high temperature disrupts the quaternary structure of protein complexes, whereas low temperature greatly decreases plant performance (Timperio et al. 2008; Zhestkova et al. 2009).

For most wheat cultivars, the highest germination temperature is, on average, 38° and the best germination temperature lies between 20 and 32 °C. Temperatures beyond these limits are considered unfavorable and have adverse effects on plants, including decreased yields and grain quality.

PGPR have been used mostly to promote plant growth, because they can stimulate plants through different means, including production of plant growth regulators and fixation of N_2 (Bashan et al. 2014). Studies have reported additional beneficial effects of PGPR on plants through their ability to improve tolerance for abiotic (including temperature) stress (El-Komy et al. 2003; Pereyra et al. 2006; Arzanesh et al. 2009).

Several abiotic stresses are related to the accumulation of ROS in plant cells. Reactions of these compounds with proteins, membrane lipids, and DNA may cause severe oxidative damage. Avoiding oxidative stress is necessary for plant survival under temperature stress. ROS are removed by several enzymes such as catalase, peroxidases, and SOD, which together form the plant antioxidant system. However, few data are available about the mechanisms of the bacteria-mediated antioxidative protection of plants (Reddy et al. 2004).

Temperature stress affects the growth of plants throughout their ontogeny, although the threshold level

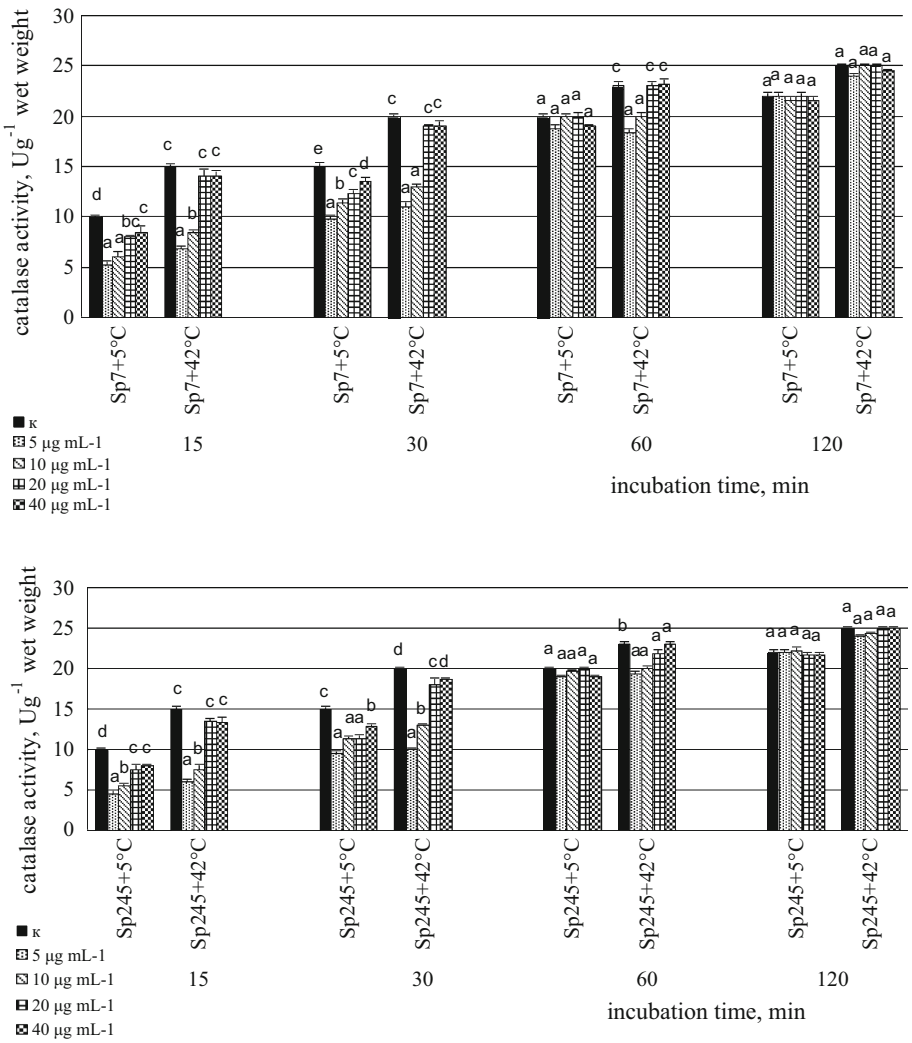

Fig. 3 Effect of the *Azospirillum brasilense* Sp7 and Sp245 lectins on the activities of catalase in wheat seedling roots exposed to 5 and 42 °C. Results are expressed as mean ± SE (n = 3). Mean separation among treatments was done by Duncan test at $P \leq 0.05$. Mean values followed by different letters are significantly different

varies considerably at different developmental stages. For instance, during seed germination, high and low temperatures may slow down or totally inhibit germination, depending on the plant species and the stress intensity. The events determining the entire course of resistance development occur in this very period of adaptation to unfavorable factors.

The large body of experimental data in the literature indicates that lectins are polyfunctional proteins. In addition to being able to reversibly and specifically bind to target cells, they can express biological activity. This means that low concentrations of lectins can induce cellular responses (Messina et al. 1987; Antonyuk et al. 1993). This ability was confirmed in our previous work on the effect of *Azospirillum* lectins on seed germination (Nikitina et al. 2004), mitogenic and enzyme-modifying activities (Chernyshova et al. 2005; Alen'kina et al. 2006; Alen'kina and Nikitina 2015, 2017), and alteration of the plant cell content of stress metabolites (Alen'kina et al. 2014). The lectin effects found in this work were recorded in the same concentration range as used in those previous studies.

Our research has shown that both Sp7 and Sp245 lectins substantially modified the enzyme activities as early as several minutes into stress. Both lectins increased peroxidase and SOD activities but decreased catalase activity in the stressed roots. In all cases, the two lectins regulated the enzyme activities differently, a finding in good agreement with our earlier results (Alen'kina et al. 2006, 2010, 2014; Alen'kina and Nikitina 2015, 2017). These differences may have been caused by the differences in structure and in carbohydrate specificity (Nikitina et al. 2005; Shelud'ko et al. 2009), resulting in differences in the interaction with the plant cell surface, which are of deciding importance for the "switch on" of the subsequent stages.

The differences in the concentration at which the lectins were effective may have been due to the action of adverse temperatures on lectin binding to the root receptors. Our data attest to the complex character of growth regulation, which is reflected in the complex concentration effects.

Concentration dependences may be conducive to high physiological heterogeneity even when concentrations vary slightly for natural reasons. In view of this, concentration dependence studies are important for understanding the processes occurring during plant adaptation to environmental conditions and for the correct application of lectins as plant growth regulators.

Our data confirm the results of Arzanesh et al. (2009) and Baniaghil et al. (2013) that azospirilla can increase the activities of plant peroxidase and SOD at different abiotic stresses. The decrease in catalase activity could have been due to the effect of salicylic acid, whose synthesis is induced by *Azospirillum* lectins (Scandalios 2005).

Together with our earlier data, the findings of this study indicate that the *Azospirillum* lectins are implicated in plant adaptation and can induce plant defense mechanisms. These lectin properties, in combination with the growth-promoting activity of *Azospirillum* bacteria, conduce to plant resistance to adverse factors and to increased plant productivity.

Acknowledgements We thank Dmitry N. Tychinin (this institute) for the English version of this manuscript.

Author contributions VN is guarantor of integrity of entire study; SA and VN were involved in study concepts; NR was involved in study design, experimental device and data acquisition ; SA was involved in literature research, data analysis, statistical analysis, manuscript preparation, and manuscript editing ; VN reviewed the manuscript.

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