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Cadaverine production by *Azospirillum brasilense* and its possible role in plant growth promotion and osmotic stress mitigation

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ABSTRACT

Polyamines are considered as plant growth regulating compounds; among them, cadaverine has been correlated with root growth promotion or osmotic stress mitigation in some plant species. The purpose of this study was to evaluate the capacity of bacterial *Azospirillum brasilense* Az39 strain to produce cadaverine in chemically defined medium and inoculated plants, and to correlate this capacity with root growth promotion or osmotic stress mitigation in hydroponics conditions. To evaluate cadaverine production in chemically defined medium *A. brasilense* Az39 was cultivated aerobically at 30 °C and 80 rpm in NFb medium or NFb-*l* supplemented with the precursor L-lysine. To evaluate the bacterial cadaverine production and growth promotion in plants, rice (*Oryza sativa* L.) cv. El Paso 144 seedlings were inoculated and hydroponically cultured under optimal conditions in growth chamber. In both, cadaverine was identified and quantified by dansyl-derivative method using a fluorescence-HPLC system, and lysine decarboxylase (LDC) activity was determined by $^{14}CO_2$ production in a closed tube system fed with [^{14}C]-lysine. To evaluate the possible role of bacterial cadaverine in osmotic stress conditions, abscisic acid (ABA) production was analyzed in rice seedlings hydroponically cultured under 0 (no stress), −0.47 (stress) or −0.82 (severe stress) MPa osmotic potential generated by mannitol, with the addition of 1 nM or 1 μM cadaverine or *A. brasilense* Az39 inoculation. Our results indicate that *A. brasilense* Az39 promoted root growth and helped mitigate osmotic stress in rice seedlings, due in part to cadaverine production.

1. Introduction

The capacity of species of the bacterial genus *Azospirillum* to promote plant growth, and consequently enhance crop productivity, has been the subject of numerous research reports worldwide for the past 30 years [1,2]. These bacteria originally attracted the attention of researchers because of their ability to fix atmospheric nitrogen, in a symbiotic

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relationship with plant tissues. Using a plant–diazobacteria interaction model, there were many attempts to generalize the plant promotion response, with highly variable experimental conditions [3]. Twenty years of studies on effects of *Azospirillum* sp. inoculation in a variety of crops were evaluated by Okon and Labandera-González [4], who concluded that success of field and greenhouse experiments was in a range of 60–70%, and yield increase ranged from 5–30%. Since the bacterial contribution to plant growth based on biological nitrogen fixation is typically 5–18% [5], it was necessary to consider promotion mechanisms other than nitrogen fixation to explain the more strongly positive results and high variability of results obtained in the inoculation experiments.

Based on a definition proposed in 1978 by Kloepper and Schroth [6] for "plant growth promoting rhizobacteria" (PGPR), there was an attempt to elucidate the physiological capabilities of *Azospirillum* sp. by a new approach. This led to a functional "rediscovery" of these bacteria, based on the search for new mechanisms involved in positive response by plants to inoculation. From 1990 to the present, many reports have focused on the ability of *Azospirillum* sp. to promote plant growth and increase agricultural productivity through certain mechanisms that act in an additive way, in combination with biological nitrogen fixation, to enhance the overall response of the plant to inoculation [2]. Only a few studies have addressed biocontrol or indirect mechanisms to limit the proliferation of pathogens, e.g., bacteriocines or catecholamine biosynthesis [7,8]. Most attention has been paid to direct mechanisms, in which growth promotion depends exclusively on (i) bacterial nutrient contribution, e.g., nitrate reductase activity [9]; phosphate solubilization [10]; iron chelation [8]; or even amino acid biosynthesis [11], or (ii) production and release of molecules that directly affect plant growth, e.g., phytohormones (indole-3 acetic acid (IAA); zeatine (Z); gibberellic acid (GA3); abscisic acid (ABA) [12]), or the plant growth regulator, ethylene [13].

Our group, since 1990, has studied the growth promotion and regulatory mechanisms present in *Azospirillum* sp., particularly strain Az39 which is widely used for inoculation of corn and wheat in Argentina [12]. We found that, in addition to biological nitrogen fixation and phytohormone biosynthesis, these bacteria in chemically defined media produce polyamines, including spermidine and spermine. Production of putrescine was reported by Thuler et al. [11]. Production of cadaverine was reported for some α-proteobacteria of the *Rhizobiaceae* family [14,15]. In a recent study, cadaverine and many other molecules were identified by gas chromatography-mass spectrometry (GC–MS) in *Azospirillum brasilense* and representative strains from three other proteobacteria subdivisions [16].

Polyamines are low molecular weight organic polymers. Like phytohormones, they are present and display biological activity in such processes as plant growth, development, and stress mitigation. Thus, they are considered multifunctional regulators of plant physiological processes [17]. Cadaverine (1,5-diaminopentane) is of particular interest because it has been correlated with adventitious root formation in pine [18] and root development in soybean seedlings [19]. Cadaverine is also involved in response to osmotic stress in turnip (*Brassica napus* L.) leaf discs [20], and (along with other exogenous polyamines) in closure of stomata guard cells of *Vicia faba* seedlings subjected to abiotic stress [21]. In view of the potential capacity of *Azospirillum* sp. to produce this diamine in symbiotic association with plants, we hypothesize that bacterial cadaverine participates in physiological processes such as growth promotion and abiotic stress mitigation in inoculated plants. The goals of the present study were to (i) evaluate cadaverine production by *A. brasilense* strain Az39 in chemically defined media or hydroponically grown rice (*O. sativa* L.) seedlings, and (ii) correlate bacterial cadaverine production with growth promotion and osmotic stress mitigation in plants.

2. Materials and methods

2.1. *Azospirillum strain*

A. brasilense strain Az39 [41], which is recommended for corn and wheat inoculation in Argentina, was provided by the Strains Collection Laboratory of the Agricultural Zoology and Microbiology Institute–Agricultural Technology National Institute (IMYZA–INTA), Castelar, Argentina.

2.2. *Culture media*

Bacteria were cultured in the nitrogen free, NFb medium [22] or in NFB-l, an alternative formulation with addition of the precursor L-lysine (0.1 $g\,l^{-1}$), since cadaverine is produced by L-lysine decarboxylation through the action of lysine decarboxylase (LDC), (L-lysine carboxylyase, EC 4.1.1.18), a pyridoxal-phosphate dependent enzyme [23].

2.3. *Bacterial culture conditions*

A. brasilense Az39 was cultured in 250 ml flasks containing 100 ml NFb or NFb-l medium at 30 °C and 80 rpm shaking until exponential late growth phase (24 h), DO_{600} nearly 1.28, equivalent to 1.15 and 1.18×10^8 colony forming units (cfu) ml^{-1} in NFb–agar. Cadaverine production in exponential growth phase was estimated according to Perrig et al. [12], and aliquots of biological material were taken for subsequent assays.

2.4. *LDC activity and Cad production in chemically defined media*

LDC activity was measured using radiolabeled substrates by the method of Tabor et al. [24], with modification. 20 ml tubes containing 5 ml NFb medium, plus 50 μCi L-[$^{14}C_{(U)}$] lysine and 10 μM L-lysine, were inoculated with 500 μl pure *A. brasilense* Az39 culture (DO_{600} nearly 1.2) and incubated for 2, 8, 12, and 24 h at 30 °C and 80 rpm shaking. $^{14}CO_2$ released by bacterial LDC activity was trapped in paper discs soaked in 2 N KOH, and radioactivity was determined using 200 μl scintillation fluid in a Beckman LS 5000 TD scintillation counter. Protein content was determined by Bradford's method [25], using bovine serum albumin as reference standard. Bacterial growth was determined by DO_{600} as described previously. For free

cadaverine production, bacterial culture was centrifuged at 8000 rpm for 20 min at 4 °C, and supernatant was acidified with perchloric acid to obtain 5% v/v final solution. The supernatant was derivatized using the dansylation method of Smith and Meeuse [26]. Cadaverine was separated and identified by TLC with both chloroform–triethylamine (9:1) and *n*-hexane-ethyl acetate (2:1) solvent systems, by comparing the R_f values of dansylated standard. Silica plates were observed under UV light, and selected bands were scraped off the plates and eluted with 1 ml methanol–toluene (9:1) solution. Fluorescence was measured by spectrofluorometry using excitation wavelength 415 nm and emission wavelength 510 nm.

2.5. Rice inoculation assay

Rice seeds (*O. sativa* L.) cv. El Paso 144 were pre-germinated for 48 h in Petri dishes containing sterile water saturated filter paper at 28 °C in the dark. Five seedlings were selected by homogeneous growth, sown in plastic pots (2 L) filled with sterile perlite: sand mixture (1:1) and saturated with Hoagland solution (25% v/v) [27]. 48 h later, seedlings were inoculated at the root level with 100 μl exponential growth culture of *A. brasilense* Az39. The pots were then transferred to a plant growth chamber with controlled photoperiod 16 h light at 28 °C/8 h in dark at 20 °C, and kept at field capacity by periodic capillary irrigation with Hoagland's solution. The following growth and biochemical parameters were evaluated on day 30 after seeds were sown: (a) root and shoot fresh weight (FW); (b) root and shoot dry weight (DW); (c) root and shoot length (*L*); (d) bacterial colonization in roots and shoots as described by Döbereiner [28].

2.5.1. Cad production and LDC activity in rice plants

Plant material was ground to a fine powder with liquid N_2 using mortar and pestle, and then homogenized (500 mg FW ml^{-1}). For free cadaverine analysis, the material was homogenized in 5% v/v perchloric acid (300 mg FW ml^{-1}) and incubated at 4 °C for 24 h. 1,7-diaminoheptane was added to the supernatant as internal standard. Dansylated polyamines were obtained and analyzed by reversed phase HPLC as we described previously [29]. For LDC activity analysis, plant material was homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 20 mM ascorbic acid, 5 mM EDTA, 10 mM PMFS, 10 mM PLP, 1 mM 2-mercaptoethanol, 0.1% PEG, and PVP. LDC activity was assayed by 45 min incubation at 37 °C, using 50 μCi L-[$^{14}C_{(U)}$] lysine with 10 μM lysine. Reaction was stopped by the addition of 10% v/v perchloric acid, and released $^{14}CO_2$ was trapped in paper discs soaked in 2 N KOH. Radioactivity and protein content were determined as described above, in chemically defined media.

2.6. Bacterial inoculation and exogenous application of cadaverine in rice plants under osmotic stress conditions

Rice (*O. sativa* L.) cv. El Paso 144 seeds were surface sterilized with ethanol (70% v/v) solution for 20 s, treated with sodium hypochlorite (2% v/v) solution for 20 min, and washed with sterile distilled water. Five seeds were sown in 250 ml sealed glass beakers containing 50 ml Hoagland's solution (25% v/v), modified by the addition of agar (0.8% w/v), and supplemented with mannitol to generate three physiologically distinct conditions based on osmotic potential: 0 MPa (no stress); −0.47 MPa (stress), and −0.82 MPa (severe stress). Seeds were sown and incubated at 30 °C, with continuous fluorescent light (20 μmol m^{-2} s^{-1}) and saturated humidity. After 48 h, seedlings were (i) treated with 100 μl of 1 nM or 1 μM exogenous cadaverine solution placed carefully on the leaf surface using a microsyringe, or (ii) inoculated with 100 μl exponential growth culture (~1.2×10^8 cfu ml^{-1}) of *A. brasilense* Az39, placed aseptically on the root surface using an automatic micropipette. The following growth and physiological parameters were evaluated on day 7 after treatment: (a) root and shoot fresh weight (FW); (b) root and shoot dry weight (DW); (c) endogenous ABA (μg g FW^{-1}).

2.6.1. Abscisic acid identification and quantification

Plant material (500 mg fr wt^{-1}) was ground to a fine powder with liquid N_2 using mortar and pestle, homogenized in 500 ml methanol: water (4:1), and kept at 4 °C overnight. After filtration, the solid residue was extracted again and filtered. Filtrates were combined, and methanol was evaporated under reduced atmospheric pressure. The aqueous phase was adjusted to pH 2.5 with acetic acid (1% v/v), added with 100 ng of $^2H_6$–ABA (provided by Prof. Richard P. Pharis, Univerisity of Calgary, Canada) as deuterated internal standard, and kept at 4 °C for 2 h. Samples were partitioned four times with the same volume of acetic acid/saturated ethyl acetate (1% v/v). After the last partition, acidic ethyl acetate was evaporated to dryness at 36 °C. Dried samples were diluted in 100 μl acetic acid:methanol:water (1:30:70) and injected into a reversed phase C_{18} HPLC column (μBondapack, 300 × 3.9 mm, Waters Associates, Milford, MA) in a Konik 500 (Konic Instruments) system coupled to a diode-array spectrometer UV–vis Konik 3000. For each sample, elution was performed at 1 ml min^{-1} flow rate, and fractions eluting at the retention time (RT) corresponding to each pure standard were collected. ABA was identified and quantified by gas chromatography–mass spectrometry with selective ion monitoring (GC–MS-SIM). UV-absorbing fractions at 262 nm were grouped and methylated with ethereal diazomethane and silylated with 1:1 pyridine: BSTFA [bis-(trimethylsilyl)-trifluoroacetamide] plus (1% v/v) trimethyl-chlorosilane [Fluka Chemika, Switzerland] to obtain methyl-trimethylsilyl derivates. Aliquots of each sample were injected directly into a DB1-15 N (15 m × 0.25 mm, 0.25 μM methyl silicone) capillary column (J&W Scientific Inc.) fitted in a Hewlett–Packard 5890 Series II GC with a capillary direct interface to a 5970B Mass Selective Detector. The GC temperature program was 60–195 °C at 20 °C min^{-1}, then 4 °C min^{-1} to 260 °C. Carrier gas (He) flow rate was 1 ml min^{-1}, interface temperature was 280 °C, and data acquisition was controlled by a HP 300 Series computer. The amount of free ABA was calculated by comparison of peak areas for ions at mass/charge (*m*/*z*) 196 (molecular ion for [$^2H_6$]ABAMeTMSi) and the ion at *m*/*z* 190 (molecular ion for [^{1}H]ABAMeTMSi) at the corresponding time [30].

2.7. Statistical analysis

Experiments were carried out in triplicate. Values shown represent mean ± standard error of mean (SEM). Experimental

data were analyzed for variance by ANOVA followed by Tukey's *post hoc* analysis at $p < 0.05$. Analyses were performed with Statistical Package PRISM V 4.0 for Windows.

3. Results

3.1. *Cad production and LDC activity of* A. brasilense *in chemically defined media*

This experiment was designed to evaluate the capacity of A. *brasilense* Az39 to produce cadaverine and to express LDC activity in chemically defined media, in relation to bacterial growth curve phase. Cadaverine production in NFb and NFb-*l* media was observed for A. *brasilense* Az39 strain; however, NFb-*l* diamine concentration was significantly higher in medium added with L-lysine precursor, in which bacteria produced 34.54 ± 0.76 nmol ml^{-1}, as compared to 8.04 ± 1.35 nmol ml^{-1} in standard medium. L-lysine addition to NFb-*l* nearly quadrupled cadaverine biosynthesis, but had no significant effect on biomass or cell production (*see* Section 2.3). This finding indicates that metabolite production is independent of bacterial growth when precursor is added into the culture medium. Cadaverine accumulation and LDC activity were evaluated in NFb standard medium for four bacterial growth phases: lag, exponential, late exponential and early senescent (Table 1). In the absence of precursor, cadaverine production increased jointly with biomass, and bacterial cell number reached maximal value, ~9.2 nmol ml^{-1} in senescent phase, starting from late exponential growth, which could be due simply to metabolite accumulation in culture supernatant. Minimal, non-significant LDC activity was observed in control samples without inoculation; this was attributed to experimental error. In contrast, LDC activity reached its maximum value, ~1.45 nmol $^{14}CO_2$ mg protein h^{-1}, in exponential late growth, indicating a correlation of cadaverine accumulation with bacterial growth.

3.2. *Bacterial growth promotion, cadaverine production, and LDC activity in rice*

This experiment was designed to evaluate the capacity of A. *brasilense* Az39 to colonize and promote the growth of rice seedlings (*O. sativa* L.) cv. El Paso 144, and to correlate this effect with bacterial production of cadaverine, and LDC activity. The promoting effect of Az39 on rice growth in hydroponic culture is shown in Fig. 1. Length (*L*), fresh weight (FW), and dry weight (DW) of inoculated plants were compared with those of non-inoculated plants. Table 2 shows cadaverine production by Az39 associated with roots and shoots of rice plants. Cadaverine accumulation was significantly higher in inoculated roots (0.352 nmol ml^{-1}) and diamine concentration was twice that found in inoculated shoots of the same plants (0.151 nmol ml^{-1}). Cadaverine concentration in roots increased jointly with Az39 colonization (6.7×10^4 cfu g fr wt^{-1}), compared to shoots. In contrast, LDC activity was higher in shoots of inoculated plants (39.612 pmol $^{14}CO_2$ mg protein h^{-1}), and reached values four times those found in roots (10.451 pmol $^{14}CO_2$ mg protein h^{-1}). Similarly to the experiments described in Section 3.1 above, minimal, non-significant LDC activity was detected in non-inoculated control plants.

3.3. *Bacterial inoculation and exogenous cadaverine application in rice plants under osmotic stress conditions*

This experiment was designed to compare the ability of A. *brasilense* Az39 inoculation or exogenous cadaverine addition to promote root growth, or mitigate the inhibitory effect of −0.47 (stress) and −0.82 MPa (severe stress) osmotic potential solutions in early stages of rice development, with reference to previous studies [18–21]. Stress inhibition was too severe in seedlings subjected to severe stress, so we only considered seedlings subjected to stress condition, in which all parameters evaluated were significantly reduced compared to optimal culture conditions. Az39 inoculation and exogenous cadaverine application both promoted early growth of roots and shoots in rice seedlings, and to some extent mitigated the inhibitory effect of osmotic stress condition (Fig. 2). Exogenous diamine application to seedlings showed a significant growth promoting effect, in terms of FW and DW of both, root and shoot fractions, under optimal condition or osmotic stress. The effect of cadaverine on plant growth was not dose-dependent, since seedlings showed similar response to treatment with 1 nM and 1 μM concentrations. Inoculation with Az39 caused a growth promoting effect higher than that

Table 1 – Bacterial growth, cadaverine production, and LDC activity of *Azospirillum brasilense* Az39 in a chemically defined medium (NFb)

Time (h)	Bacterial growth phase	Biomass production (DO_{560})	Cells number (cfu ml^{-1})	Cadaverine production (nmol ml^{-1})	L-lysine decarboxylase activity (nmol $^{14}CO_2$ mg protein h^{-1})
Control	Non-inoculated	nd	nd	nd	ns
2	Lag	0.12 ± 0.02	2.25×10^7	2.17 ± 0.27	0.867 ± 0.021
8	Exponential	0.65 ± 0.08	5.18×10^7	3.04 ± 0.40	1.381 ± 0.084
12	Exponential late	1.15 ± 0.10	1.04×10^8	5.45 ± 0.12	1.456 ± 0.012
24	Senescent	1.27 ± 0.07	1.18×10^8	9.18 ± 0.24	1.047 ± 0.025

Note: nd: not determinated; ns: not significant.
Note: Experiments were conducted in triplicate. Values shown are mean ± standard error of mean (SEM).

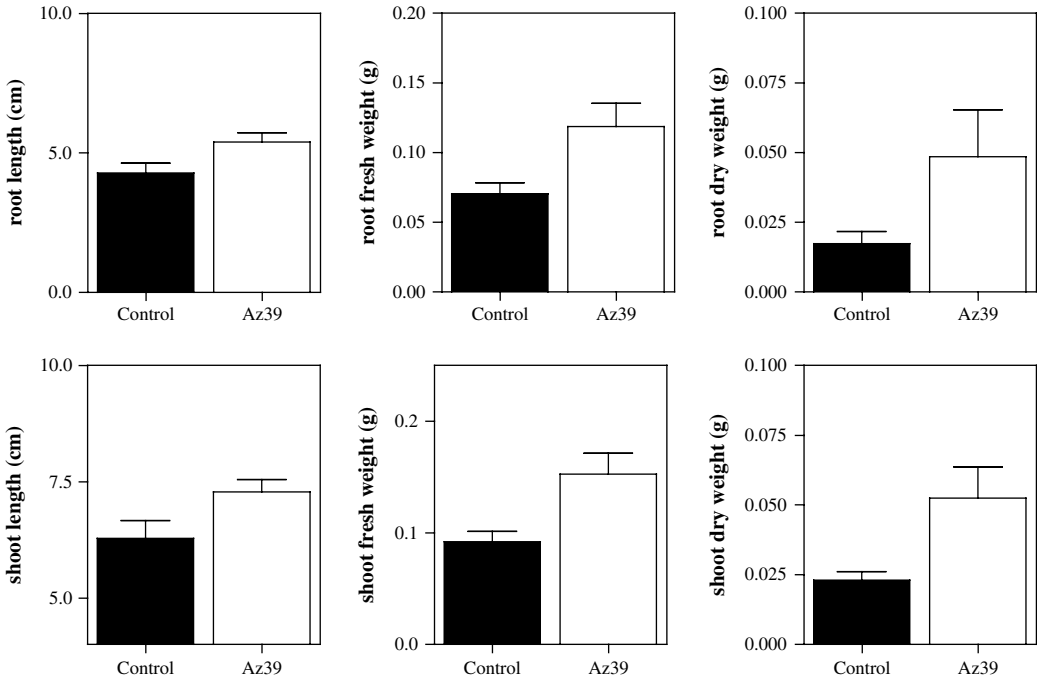

Fig. 1 – Plant growth promoting assay for rice seedlings (*O. sativa* L.) cv. El paso 144 non-inoculated (white bars) or inoculated with *A. brasilense* Az39 (black bars). Data were analyzed by ANOVA and *post hoc* Tukey test at $p < 0.05$.

of exogenous cadaverine in root and shoot fractions under both optimal and osmotic stress conditions.

Endogenous ABA production by rice seedlings treated with exogenous cadaverine, or inoculated with Az39, and cultured in hydroponic medium under optimal or stress conditions, is shown in Table 3. ABA production was significantly higher in rice seedlings subjected to osmotic stress, compared to optimal culture conditions. ABA is considered a physiological stress marker in higher plants; its biosynthesis has been correlated with plant responses to various unfavorable conditions, such as drought or salinity [31]. Both inoculation and exogenous diamine addition reversed this physiological condition, and phytohormone concentrations were nearly 75% lower than in controls. Differences between inoculated seedlings and those treated with exogenous cadaverine were not significant. Unexpected high ABA levels were found in control seedlings under optimal conditions, which could be attributed to a mild photoxidative stress generated by continuous light exposure.

4. Discussion

A. brasilense Az39 strain was shown to possess LDC activity and produce cadaverine in chemically defined media, as well as in symbiotic association with plants. Since cadaverine has the capacity to promote plant root growth [18,19] and to mitigate (at least to some extent) osmotic stress [20,21], we hypothesize that cadaverine production is a novel bacterial mechanism involved in plant growth promotion and/or regulation of plant response to osmotic stress. In chemically defined media, Az39 produced maximal cadaverine concentration in the presence of its immediate precursor L-lysine; this production was not correlated with change in bacterial

Table 2 – Bacterial colonization, cadaverine production, and LDC activity of *A. brasilense* Az39 in rice (*O. sativa* L.) cv. El Paso 144 plants cultured 30 days after inoculation under hydroponic conditions

Treatment	Plant fraction	Cells number (cfu g fr wt^{-1})	Cadaverine production (nmol g fr wt^{-1})	L-lysine decarboxylase activity (pmol $^{14}CO_2$ mg protein h^{-1})
Control plants	Root	nd	nd	ns
	Shoot	nd	nd	ns
Inoculated with Az39	Root	6.7×10^4	0.352 ± 0.010	10.451 ± 0.353
	Shoot	4.3×10^3	0.151 ± 0.012	39.612 ± 0.284

Note: nd: not determinated; ns: not significant.
Note: Experiments were conducted in triplicate. Values shown are mean ± standard error of mean (SEM).
Note: "control seedlings" were treated with 100 μl buffer per root and "inoculated with Az39" were treated with 100 μl Az39 per root.

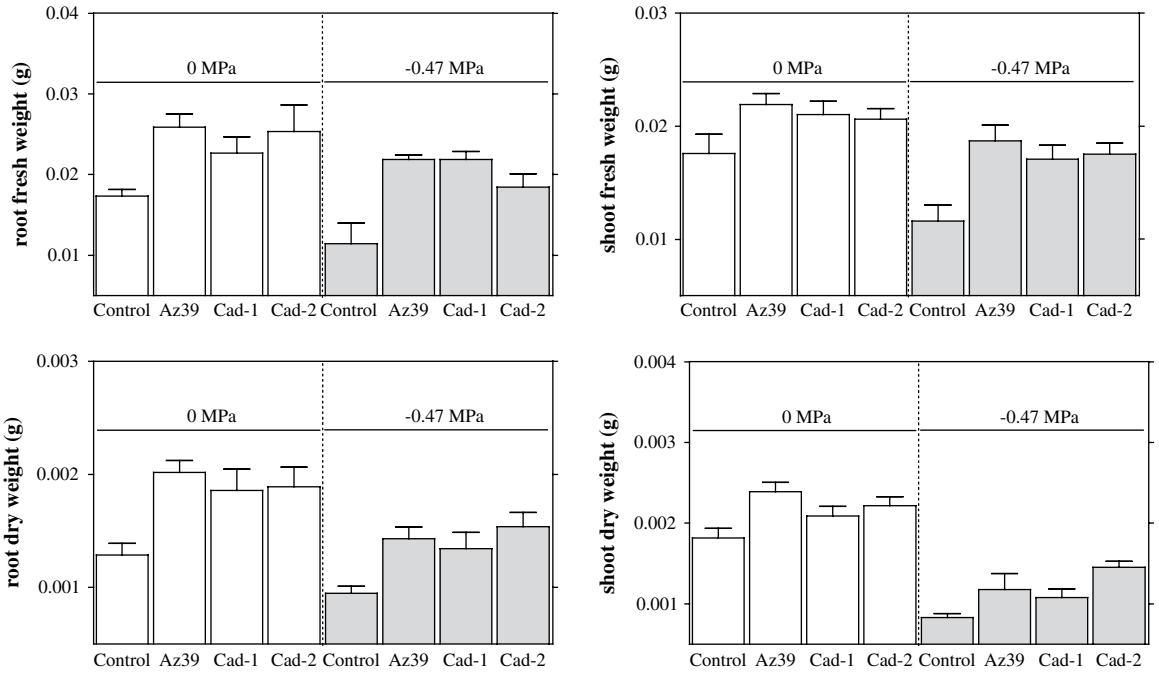

Fig. 2 – Bioassay of rice seedlings (*O. sativa* L.) cv. El paso 144, under optimal (0 MPa, white bars) and osmotic stress (−0.47 MPa, gray bars) conditions. Seedlings were inoculated with 100 μl exponential growth (~1.2×10^8 cfu ml^{-1}) *A. brasilense* Az39 culture medium or buffer solution (control), or treated with 100 μl of 1 nM (Cad-1) or 1 μM (Cad-2) exogenous cadaverine. Data were analyzed by ANOVA and *post hoc* Tukey test at $p < 0.05$.

biomass. In contrast, cadaverine production without the precursor was correlated with bacterial growth. This phenomenon appears to be a simple substrate-induction process, and could be explained from a nutritional or co-evolutionary point of view. From a nutritional perspective, we propose that the maximal capacity of the bacteria to metabolize L-lysine is reached as a result of preferential reduction of the inorganic nitrogen source (NH_4Cl) in the surrounding environment, and possible use of alternative nitrogen sources to sustain exponential growth. Results for kinetics of cadaverine production in the absence of L-lysine (Table 1) were similar, i.e., accumulation of the molecule paralleled microbial growth, with maximal value at the senescent stage. Maximal induction of LDC activity was observed in late exponential growth phase, when the bacterial population would begin to perceive a nutritional stress condition. From a co-evolutionary perspective, L-lysine and other organic compounds could be considered attractive signals for chemotactic movement of bacteria toward the rhizosphere. Alternatively, the amino acid could be considered as an immediate precursor for biosynthesis of active molecules with the ability to induce plant growth, as proposed previously for the tryptophan-indole-3 acetic acid model. L-lysine present in exudates of wild rice (*Zizania aquatica* L.) was previously reported as a preferential source of nitrogen for *Azospirillum* sp. under rhizospheric nutritional deficiency condition [32]. Another group described the ability of rice exudates (*O. sativa* L.) to induce low chemotactic response in *A. brasilense* 6-81 strain, and detected L-lysine production in such exudates [33].

Table 3 – Abscisic acid (ABA) production in rice (*O. sativa* L.) seedlings, under optimal and osmotic stress conditions. Seedlings were inoculated with *A. brasilense* Az39 or treated with exogenous cadaverine (1 nM or/and 1 μM). Data were analyzed by ANOVA and *post hoc* Tukey test at $p < 0.05$

Optimal (0 MPa)		Osmotic stress (−0.47 MPa)	
Treatment	ABA production (pg g fr wt^{-1})	Treatment	ABA production (pg g fr wt^{-1})
Control	162.85 ± 2.63	Control	218.23 ± 10.22
Az39	38.83 ± 9.04	Az39	116.83 ± 18.88
1 nM Cad	32.97 ± 8.67	1 nM Cad	127.29 ± 8.34
1 μM Cad	12.94 ± 17.95	1 μM Cad	132.31 ± 2.55

Note: nd: not determinated; ns: not significant.
Note: ABA determinations were conducted in triplicate. Values shown are mean ± standard error of mean (SEM).

Regarding the capacity of cadaverine to promote root development, Gamarnik and Frydman [19] found that the molecule is involved in cell division and proliferation in germinating soybean (*Glycine max* L.) seedlings, as indicated by high concentration and LDC activity measured in embryonic axis during germination. Exogenous addition of cadaverine in seeds may have similar effects. In the *Azospirillum*-rice model, promotion of root growth may be due in part to bacterial cadaverine accumulation in culture medium (Table 1), or colonization conditions (Table 2). Our previous study [12] indicates that inoculants are complex biological formulations

that combine two elements: cultured microorganisms and compounds secreted under controlled conditions. Bacterial production of cadaverine and others phytohormones as GA_3, Z, and IAA and their release into culture medium could have an ''initial impact'' on the seed during inoculation. A subsequent ''late impact'' could be due to biosynthesis of these molecules during endophytic colonization. Because we do not have an *Azospirillum* sp. mutant strain completely deficient in biosynthesis of phytohormones such as IAA (only a hypoproducer is available), GA_3, or Z, it is not yet possible to isolate the effect of cadaverine vs. these other bacterial regulatory compounds in the plant. However, the present study shows that application of exogenous cadaverine at a concentration similar to that produced by bacteria in chemically defined medium (Table 3) promotes root growth in rice seedlings. This could help explain the initial promoting effect observed in seeds or seedlings inoculated with *Azospirillum* sp. and its medium. The ''late'' promoting effect is supported by the observation that free cadaverine and LDC activity were detected in *Azospirillum* colonized plants, but not in control plants, after 30 days (Table 2).

Regarding osmotic stress, many previous reports indicate the capacity of *Azospirillum* sp. to enhance plant growth under abiotic stress conditions [34,35]. In view of this capacity, we [36] suggested a reclassification of the Plant Growth Promoting Bacteria (PGPB) group proposed by Bashan and Holguin [1], and addition of a new functional group termed Plant Stress Homeostasis-regulating Rhizobacteria (PSHR). PSHR would include bacteria with the capacity to promote plant growth under abiotic stress conditions by various mechanisms such as biosynthesis of ABA [37] or jasmonic acid (JA) [38], ACC-deaminase activity [39], and cadaverine production. Kuznetsov et al. [40] showed that cadaverine accumulation mediated by NaCl plays a protective role in the adaptation of *Mesembryanthemum crystallinum* L. to salt stress. Liu et al. [21] proposed that all natural polyamines, including cadaverine, strongly inhibit opening and closing of stomata by regulating potassium channels in guard cells, an important effect under abiotic stress condition. The present study shows that *A. brasilense* Az39 inoculation or exogenous cadaverine addition mitigates osmotic stress in rice seedlings (Fig. 2), based on improved water status (Fig. 2) and decreased production of ABA (Table 3) in inoculated seedlings. Cadaverine clearly should not be considered as the sole or major causative agent in physiological processes (e.g., nitrogen fixation, phytohormone production) observed in plants inoculated with *Azospirillum* sp., but rather as one of many complex factors under the ''additive hypothesis'' of Bashan and Holguin [1].

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