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PBS and $MgSO_4$ Differentially Affect the Response of Maize (*Zea Mays* L. Mill cv. B73) Seedlings to *Azospirillum brasilense* Sp7

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The effect of *Azospirillum brasilense* Sp7 (Sp7) on maize (*Zea mays*, Mill cv. B73) seedlings was compared when using two common carriers to deliver Sp7 to the seed: phosphate buffered saline (PBS) and magnesium sulfate ($MgSO_4$). Seedling height, leaf chlorophyll levels, and root growth parameters were analyzed with WinRHIZO® at an early vegetative stage of plant development. Scanning and transmission electron microscope (SEM & TEM) analysis showed that carrier components do not effect bacterial binding to plant roots. $MgSO_4$+Sp7 caused a significant increase in the abundance of thick lateral roots, but stunted plant height, compared to other treatment groups, while relative chlorophyll contents (SPAD) significantly increased in seedlings inoculated with PBS+Sp7, revealing that the two inoculation carriers differentially affect the Sp7-associated plants.

Keywords: PGPR, cereal crops, nutrients, root morphology

Abbreviations: PBS – phosphate buffered saline; Sp7 – *Azospirillum brasilense* Sp7; PGPR – plant-growth promoting rhizobacteria; JA – jasmonic acid; ET – ethylene; nfb – nitrogen-free broth; OAB – Okon, Albrecht and Burris; tyg – tryptone yeast extract and glucose; LB – Lauria-Bertani; cr – congo-red

Introduction

Plant-growth promoting rhizobacteria (PGPRs) are biofertilizers which increase crop growth rates and yields (Barassi et al. 2007). The effectivity of PGPRs on plant growth is highly dependent on the efficiency of their application, which includes being delivered to the rhizosphere in a viable form, persisting in the soil environment and colonizing host plant root systems. Selection of a suitable carrier for the rhizobacteria is a crucial component to this success because it provides a microenvironment keeping the bacterial cells alive and active (Reddy and Saravan 2013). There is no universal carrier for PGPRs, and choosing the proper formula has been identified as a strain-specific process (Rivera et al. 2014). Unfortunately, there is little literature that makes scientific distinction between the carriers/buffers or classifies their efficiency by bacterial strain.

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Azospirillum brasilense is a well-studied PGPR that has many positive effects on its plant hosts and that is commonly used in commercial farming operations. Some of the benefits include: nitrogen fixation (Bashan and Holguin 1996), inciting root hair proliferation and increasing plant growth, facilitating water and nutrient uptake (Barassi et al. 2007), influencing phytohormone production (Perrig et al. 2007), and reducing biotic and abiotic stresses (Bashan and Holguin 1997; Lugtenberg and Kamilova 2009). To reduce stress, *A. brasilense* provokes the plant defense mechanism induced systemic resistance (ISR) (Alström 1991), which consists of a complex network of molecular signalling cascades that are stress- and host species-dependent. This system relies primarily on jasmonic acid (JA) and ethylene (ET) phytohormone signal transduction pathways to 'prime', or sensitize distal plant parts for enhanced pathogen defense (Van Peer et al. 1991). In general, priming is rather elusive because it involves only a few perceivable changes, such as enhanced callose deposition (Ton et al. 2005) and rapid stomatal closure (Kumar et al. 2012). However, studying ISR in combination with pathogenesis magnifies the effects of the resistance, causing marked increases in plant JA and ET levels (Verhagen et al. 2004; Wang et al. 2005).

The interaction with abiotic stress factors also influence how *A. brasilense* induces ISR. For one, *A. brasilense* has been extensively studied for mitigating salinity stress in the rhizosphere because it accumulates amino acids such as proline and glutamate, which act as osmoprotectants (Bashan and Holguin 1997; Casanovas et al. 2002, 2003). Furthermore, the exopolysaccharides produced by *A. brasilense* restrict sodium (Na^{+1}) influx into roots (Ashraf et al. 2004). Studies addressing the efficacy of *A. brasilense* in the control of salinity use pure phosphate or Lauria-Bertani (LB) buffers, likely because both magnesium (Mg^{+2}) and Na^{+1} ions (found in phosphate buffered saline (PBS) and magnesium sulfate ($MgSO_4$) carriers, respectively) can disrupt the binding of rhizobacteria to roots (Gafny et al. 1985), though it is not known exactly to what extent.

A number of solid, liquid, oil and gel-based formulas have been developed for the delivery of cultured *Azospirillum* spp. colonies to the rhizosphere or to seeds. Liquid inoculants are the most economical for small-scale use (Mugilan et al. 2011), and some of the most commonly used formulas are PBS (Cohen et al. 2009), magnesium sulfate ($MgSO_4$) (Mangmang et al. 2015), 0.9% sodium chloride (NaCl) (Bashan and Levanony 1985), 66 mM phosphate buffer (Creus et al. 1997) and LB-broth (Khalid et al. 2017). The majority of research focusing on *A. brasilense* Sp7 (Sp7) uses PBS as the carrier, though $MgSO_4$ has appeared in more contemporary work. It is not known how the various chemical components in these inoculants effect the activity of *A. brasilense* in the rhizosphere, influence plant growth, and ultimately, if and how they regulate ISR.

The aims of our study are to investigate the effects of PBS and $MgSO_4$ carriers on the phenotype of Sp7-inoculated maize seedlings and determine if Sp7 binding and activity is modulated by the two common carriers. Based on previous research, we hypothesize that inoculation carrier components will distinctly alter the way in which Sp7 interacts with seedlings, affecting plant growth overall.

Material and Methods

Experimental design and general information

The experiment consisted of 4 seed treatments (1) PBS, (2) PBS + Sp7, (3) $MgSO_4$, (4) $MgSO_4$ + Sp7 with ten biological replicas each, and arranged in a completely randomized design. Maize seeds were sown in 0.3 L pots filled with autoclaved commercial substrate (Traysubstrat®, Klasmann-Deilmann, Gmbh, Geeste, Germany) characterized by having an extra fine structure and pH of 6, which was sufficient for this study since ranges for N-fixation by *Azospirillum* spp. are between 5.5 and 9 (Day and Döbereiner 1976). Both inoculated and non-inoculated (control) maize seeds were grown in a greenhouse on the Escola Tecnica Superior d'Enginyeria Agraria (ETSEA) Campus at the University of Lleida, in Catalonia, Spain. They were maintained at 70–80% humidity, 25–30 °C, and protected from insect attack by an anti-aphid mesh. Automatic drip irrigation without nutrient amendment was administered in the morning and evening everyday for an hour.

Imbibition curve preparation

A water imbibition curve was defined for maize. Initially, groups of ten maize (*Zea mays* L. Mill cv. B73) seeds (with five replicates) were disinfected with 70% ethanol (EtOH) for 2 min. Seeds were then disinfected with 4% sodium hypochlorite (NaClO) for 15 min (under constant agitation) and subsequently rinsed six times with sterile distilled water (SDW). Finally, seeds were dried on sterile paper.

The seeds, prepared as above, were weighed and moved to 50 mL Falcon tubes containing SDW and maintained at 30 °C for 30 min. The seeds were removed from the SDW and briefly allowed to dry on sterile distilled paper. Group seed weight was recorded. This process was repeated until the seeds stopped water uptake. A linear regression model was calculated from the curve and it was estimated that to imbibe 70% of its potential, the seeds should be left for 156 min to absorb 0.05 mL.

Azospirillum brasilense *Sp7 inoculum preparation and inoculation*

The *Azospirillum brasilense* Sp7 strain was kindly provided by the Colección Española de Cultivos Tipo (acc. CECT 590) at the Polytechnic University of Valencia (Spain). The material arrived freeze-dried and was cultivated in Petri® dishes containing sterile nitrogen free broth medium (Nfb) supplemented with 15 mL/L of 1 : 400 aqueous solution of Congo-red (CR) (Bashan et al. 1993) for 48 hours.

To prepare the Sp7 suspension, OAB liquid media [per liter for Solution A: (g/L) DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; NH_4Cl, 1; yeast extract, 0.1; $FeCl_3$, 0.01; (mg/1) $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3, 2.8; $Cu(NO_3)_2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24 – per 100 mL for Solution B: (g/L) K_2HPO_4, 6; KH_2PO_4, 4 – Solution A + B post-autoclaving, pH 6.8] was prepared as described by Okon et al. (1977), inoculated with a single Sp7 colony selected from the CR medium and

incubated at 32 °C in constant agitation (8.96 g) for 48 hours. The bacterial suspension was allowed to reach its late-log growth phase with an absorbance of 0.399 nm at OD_{600} (using an Amersham Biosciences Ultraspec 3100 Pro spectrophotometer); a concentration yielding approximately 1.16×10^8 cfu·mL^{-1} based on a previously established bacterial curve.

All seeds were disinfected with 4% NaClO for 15 min (under constant agitation) and washed six times with SDW in the laminar flow hood. They were laid out to dry on sterile autoclaved paper until all water had evaporated. Seeds were then inoculated with the rhizobacteria via soaking at approximately 1.16×10^8 cfu·mL^{-1} per seed. To do so, 10 mL of the bacterial suspension was aliquoted for each inoculated seed group of ten and pelleted by centrifuging at 448 g for 10 min. When PBS was used as inoculation carrier, the supernatant was poured off and the bacterial pellet was washed twice with 1 × PBS adjusted to a pH of 7.4 (per L for 10 × PBS: 80 g NaCl, 2 g KH_2PO_4, 29 g Na_2HPO_4 $12H_2O$, 2 g KCl, 2 g NaN_3). Then 0.056 mL 1X PBS was added to the tube together with ten sterile maize seeds and vortexed. In the case of $MgSO_4$, the bacterial pellet was washed twice with autoclaved 30 mM $MgSO_4$, and then resuspended in the same volume of carrier solution as for PBS (Mangmang et al. 2015). For control groups, ten sterile seeds were placed in tubes with carrier only. All treatments and controls were left in horizontal agitation at 8.96 g to imbibe as previously described.

Plant measurements

Maize leaf arch height and chlorophyll contents (SPAD 502Plus, Konica Minolta) were recorded four weeks after planting. Arch height was measured from the soil surface to the arch of the uppermost leaf that was at least 50% emerged from the whorl.

Root structure analysis

Plant roots from all treatments were washed, extended on glass and photographed. Photographs were scanned and analyzed with WinRHIZO® (version 2009; Regent Instruments Inc., Quebec, ON, Canada).

Transmission electron microscopy (TEM)

To visualize the *Azospirillum* polar flagella, a drop of bacterial suspension in OAB was applied onto a Formvar©-covered electron microscope 200 mesh copper grid for 1 min. The liquid was absorbed by filter paper, then a drop of the staining solution (1% uranyl acetate in water) was applied to the grid and allowed to absorb for 30 s. After drying, the specimen was ready to be introduced into the electron beam of an EM 910 Transmission Electron Microscope (Zeiss, Oberkochen, Germany). A minimum of two grids per treatment were analysed (Borisov et al. 2009).

Scanning electron microscopy (SEM)

To visualize the biofilm formed by bacterial root colonization with scanning electron microscopy (SEM), root pieces were excised for both treatment groups and controls, and fixed in 3% (v/v) glutaraldehyde solution buffered with 0.1M phosphate buffer (pH 7.4) for 3 hours at room temperature. They were then postfixed in 1% (v/v) osmium tetraoxide in the same buffer. Specimens were washed three times in sterile distilled water and treated with aqueous solution of uranyl acetate 2% (w/v) for 40 min. After fixation, samples were dehydrated through a graded ethanol series (30–100%) followed with acetone (100%), critical-point dried, mounted on aluminum stubs, coated with gold and examined with a Jeold JSM-6300 scanning electron microscope (Guerrero-Molina et al. 2012).

Statistical analysis

Statistical analyses were conducted for all the obtained data for each treatment group by using a one-way ANOVA test and Tukey's HSD test. Analyses were processed with JMP® 12.1 (SAS Institute Inc.), while software and graphics were created with Microsoft Excel 2016. The F-ratio of 0.05 for a One-way ANOVA was used to evaluate the difference between averages.

Results

Chlorophyll contents (SPAD) was significantly higher in treatments with PBS + Sp7 ($p < 0.05$) than other treatment groups (Fig. 1). Scanned images of the washed roots reflected qualitative differences between root systems (Fig. 2).

WinRHIZO® analysis evidenced the efficacy of Sp7, detailing value increases in all root parameters over treatments without Sp7 (Tables 1 and 2). Then, subjecting the

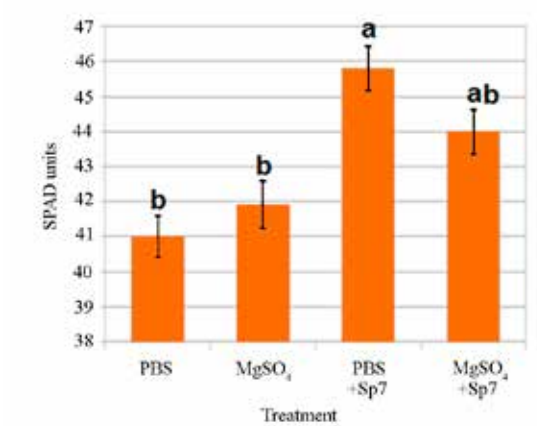

Figure 1. Relative chlorophyll contents of maize seedlings subjected to four inoculation treatments. Statistical differences ($p < 0.05$) are denoted by different letters and error bars represent a 5% standard error (SE)

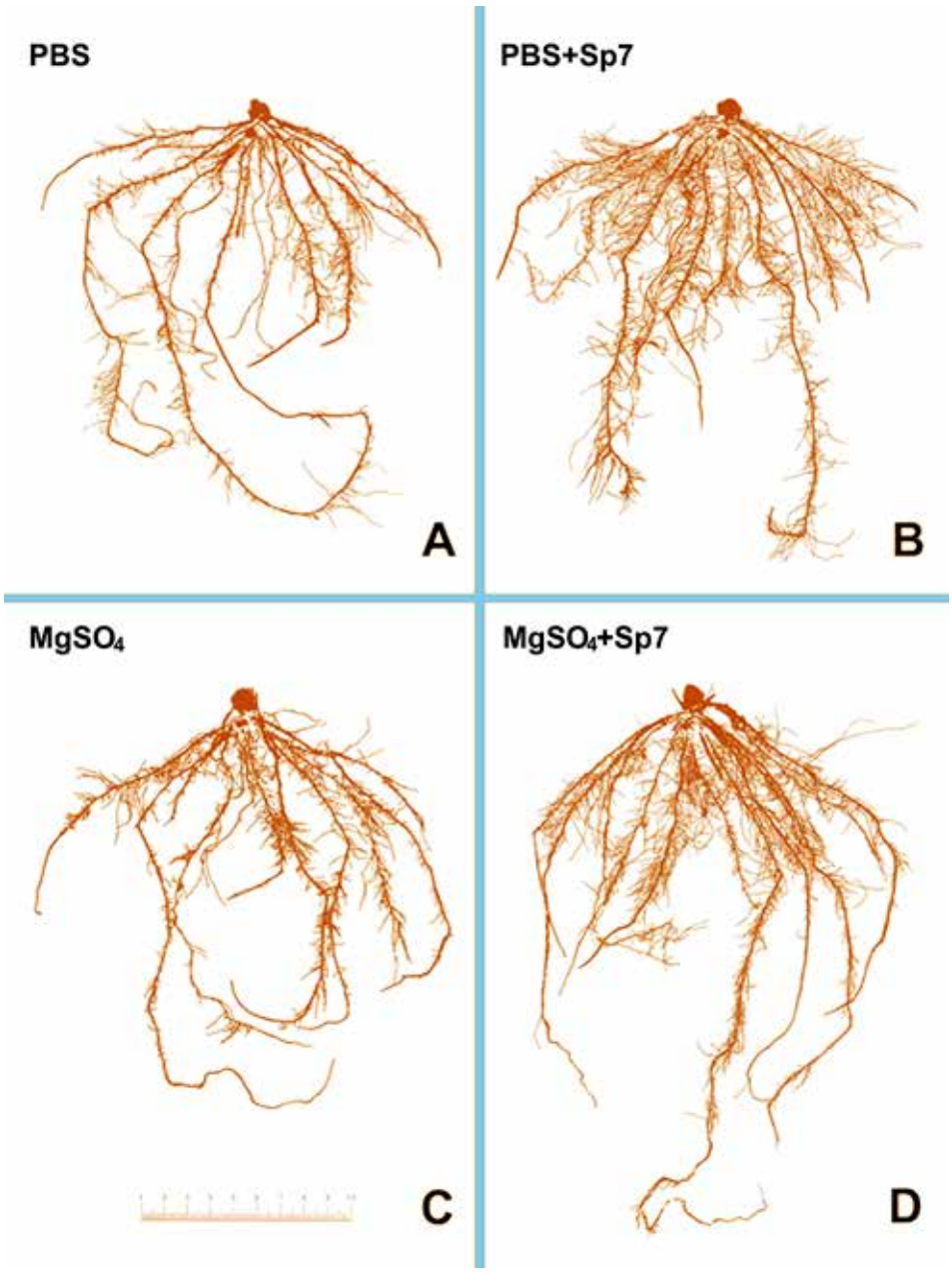

Figure 2. Representative images of maize seedling root systems with four inoculation treatments: PBS, $MgSO_4$, PBS + Sp7 and $MgSO_4$ + Sp7. Images are the most representative example from sample groups of five (per treatment). C – bar = 10 cm

Table 1. WinRHIZO® root values for four inoculation treatments in maize seedlings

	PBS	$MgSO_4$	PBS + Sp7	$MgSO_4$ + Sp7
Total length (cm)	245.50	265.06	336.23	346.81
Average diameter (cm)	0.76	0.49	0.80	0.86
Total surface area (cm^2)	61.10	68.79	85.06	92.36
Total projected area (cm^2)	19.41	21.88	28.73	29.39
Total volume (cm^3)	1.23	1.42	1.71	1.96

Table 2. Total root area (cm^2) in function of diameter class (mm) for four inoculation treatments in maize seedlings

Root diameter class (mm)	Inoculation treatment and total root area (cm^2) in function of root diameter class			
	PBS	$MgSO_4$	PBS + Sp7	$MgSO_4$ + Sp7
0.1–0.5	133	144	198	196
0.5–1.0	71	73.8	77	91.8
1.5–2.0	8.76	9.25	10.40	14.40
2.0–2.5	3.0	4.3	3.62	6.0*
2.5–3.0	5.34	2.0	1.44	2.95
3.0–3.5	0.6	0.86	2.75	1.80
3.5–4.0	0.32	0.61	0.55	1.12
4.0–4.5	0.17	0.02	0.32	0.68
4.5–5.0	0.11	0.69	0.32	1.07*

Data was subjected to a one-way ANOVA test and values with an asterisk (*) denote significant differences between treatments ($p < 0.05$).

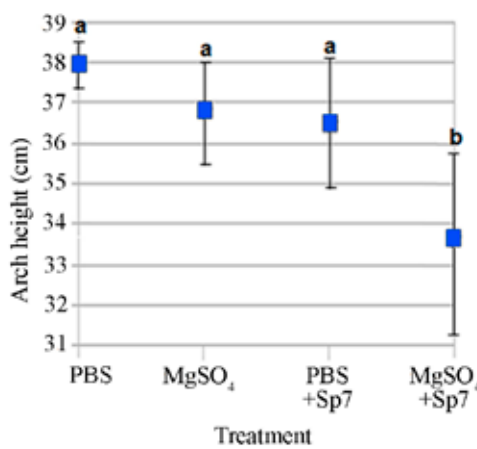

Figure 3. Average highest leaf arch height (cm). Statistical differences ($p < 0.05$) are denoted by different letters and error bars represent a 5% SE

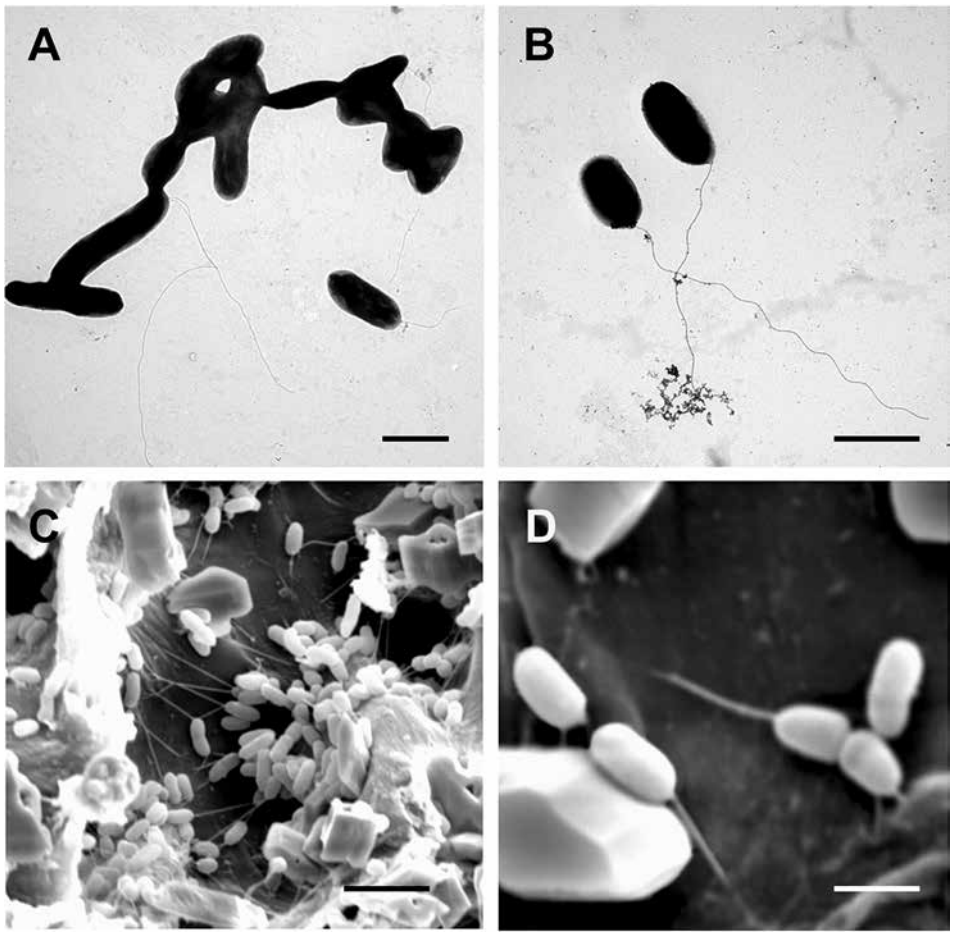

Figure 4. Electron microscopy (EM) images of *Azospirillum brasilense* (Sp7). A–B: Transmission electron microscopy (TEM) images of Sp7; C–D: Scanning electron micrographs (SEM) of Sp7-treated maize roots (bars: A, B = 2.5 μm; C = 2 μm; D = 6 μm)

WinRHIZO® data to a one-way ANOVA statistical analysis revealed the abundance of certain root diameters to be significant between treatment groups. $MgSO_4$ + Sp7 inoculated seedlings had significantly more abundance of roots measuring 2.0–2.5 mm and 4.5–5.0 mm than other treatment groups (Table 2).

Plant arch height (Fig. 3) was significantly shorter for $MgSO_4$ + Sp7 seedlings compared to other groups. This finding was disproportional to increased root hair proliferation in $MgSO_4$ + Sp7 seedlings (Table 1).

TEM examination of root microorganisms confirmed that the size of Sp7 bacterial cells is variable in general. There were some spiral shaped cells (typical to the *Azospirillum* genus) (Fig. 4A), but the most abundant Sp7 phenotype was smaller (1.2 × 2–3 μm)

exhibiting a polar flagella (Fig. 4B). All inoculated plants revealed different degrees of bacterial colonization and biofilm formation on the root surfaces, but from these figures, there was not a clear pattern based on carrier (Fig. 4C). The higher magnification in Fig. 4D shows Sp7 adherence to the root.

Discussion

Azospirillum brasilense Sp7 inoculation to the rhizosphere or to seeds in a liquid carrier has repeatedly been shown to enhance plant growth (Bashan et al. 2006; Fukami et al. 2016). Our work confirms these findings, adding that the specific elements present in the carriers can modulate the influence that Sp7 has over plant growth. When Sp7 is inoculated with the two carriers tested, PBS or $MgSO_4$, plant root diameter, height and leaf chlorophyll contents are affected in different ways, but bacterial root binding is not disrupted in either case, excluding the possibility that these differences are attributed to variations in root binding.

Sp7 priming typically enhances plant growth overall, both above and below ground (Correa et al. 2007). In this study, plants inoculated with $MgSO_4$ + Sp7 had a higher frequency of thick root hairs, but plant height was stunted relative to other treatment groups. Stunting is most likely attributed to distress caused by the $MgSO_4$ carrier-Sp7 combination, based on a similar study that reported stunting alongside pathogenesis-related (PR) protein induction and activation of systemic acquired resistance (SAR) in PGPR-inoculated plants held under certain conditions (Maurhofer et al. 1994). On the other hand, a higher frequency of thicker root hairs has several possible explanations based on literature: (1) Sp7 potentiates the interaction of the carrier elements with early Mg-responsive genes in root hairs (Guo et al. 2016); (2) Mg^{+2} in the carrier improves N_2 fixation by Sp7, thereby increasing plant root growth (Peng et al. 2018); (3) high Mg-supply decreases starch and sucrose accumulation in leaves, but increases root concentrations, enhancing carbohydrate import into associated rhizobia (Peng et al. 2018). Further study is required to elucidate the molecular intricacies of our findings.

The PBS + Sp7 combination also induces a stress-like reaction in the plant. Results showed that inoculation with PBS + Sp7 increased plant chlorophyll contents, reflecting C gain in the C-assimilation process through photosynthesis, which is often a byproduct of stress (Hernández-González et al. 2010). Previous work carried out with a similar PGPR, *Bacillus thuringiensis*, revealed that the bacteria was instrumental in reducing salt-stress in the plant by targeting the up-regulation of chloroplast proteins (Subramanian et al. 2016). We may be observing a similar reaction to the NaCl present in the PBS buffer, which Sp7 counterbalances by increasing chloroplast activity.

In conclusion, we have found that the two common Sp7 carriers tested interact with the plant to stimulate minor stress reactions, which in turn, are mitigated by Sp7. Considering the carriers tested, PBS + Sp7 induces an increase in plant growth that is positive, and that coincides more closely with broadly reported results. Most importantly, this study provides preliminary evidence that the carrier used for inoculating Sp7 is a factor that exerts influence over the plant phenotype.

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