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Phenotypic variation in *Azospirillum brasilense* Sp7 does not influence plant growth promotion effects

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ABSTRACT

The *Azospirillum* genus comprises free-living, plant growth-promoting, nitrogen-fixing bacteria found in the rhizosphere of plant roots. Azospirilla are able to promote plant growth mainly through improvement of root development. Bacterial surface components, such as extracellular polysaccharides and proteins, are involved in root colonization. Phase variation – or phenotypic variation – is one of the mechanisms by which microorganisms adapt to environmental changes. This phenomenon is characterized by the presence of a sub-population of the bacteria presenting a different phenotype relative to the major population. In this study we characterized phenotypic variation of *Azospirillum brasilense* Sp7. When plated on solid media, some *A. brasilense* colonies were shown to possess a much more mucoid morphology, producing 7.5–8 times more exopolysaccharide with different monosaccharide composition than the parental strain Sp7. The rate of appearance of this kind of variant colonies was 1 in 5000, in agreement with the accepted rate for the phase/phenotypic variation phenomenon. The variants were significantly more resistant to heat and UV-exposure than the parental strain and displayed genomic changes as seen by the different band patterns following ERIC-PCR, BOX-PCR and RAPD analyses. In plant inoculation experiments under greenhouse conditions, with maize, wheat, soybean and peanuts, the EPS overproducing variants performed as similar as the parental strain. Therefore, EPS overproduction did not confer an apparent advantage to *A. brasilense* in terms of induction of plant growth promotion.

1. Introduction

Bacteria of the genus *Azospirillum*, belonging to the class alphaproteobacteria, are free-living, plant growth-promoting, nitrogen-fixing bacteria found in the rhizosphere of plant roots (Baldani et al., 2005; Wisniewski-Dyé et al., 2011). Azospirilla are able to promote plant growth through improvement of root development (Spaepen et al., 2009). Evidence suggests that production of phytohormones by the bacteria plays an important role in plant growth promotion (Spaepen et al., 2009). The beneficial effects result in increased crop yield, in many plants of agronomic importance (Bashan and de Bashan, 2010; Fibach-Paldi et al., 2012).

Bacterial surface components, such as extracellular polysaccharides and proteins, are involved in root colonization (Burdman et al., 2000a). Extracellular polysaccharides secreted by *Azospirillum* comprises lipopolysaccharides (LPS) and capsular polysaccharides (CPS), which form an adherent cohesive layer on the cell surface, and exopolysaccharides (EPS) that form an extracellular matrix that has little or no association with the cells. EPS properties contribute to cell protection against environmental stresses, attachment to surfaces, nutrient gathering and cell antigenicity. The relative monosaccharide composition and the molecular weight of the EPS and CPS in *Azospirillum* vary within species and strains, growth conditions and physiological states (Burdman et al., 2000b; Bahat-Samet et al., 2004).

Phase variation – or phenotypic variation – is one of the mechanisms by which microorganisms adapt to environmental changes. This phenomenon is characterized by the presence of a sub-population presenting a different phenotype from the major population. This sub-population appears at a relatively high ratio, more than 10^{-5} (as compared with less than 10^{-6}, as for spontaneous mutations), but during appropriate conditions can become

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dominant. Phase variation has been described for many different bacterial genera belonging to diverse taxonomic groups and displaying different ecological behaviors (*eg.* pathogens, saprophytes and symbionts). Phase variation can be associated with changes in various phenotypes, such as motility, synthesis of pili, and expression of capsule and production of antifungal metabolites. The molecular mechanisms associated with phase variation are diverse and include genetic and epigenetic changes, such as DNA inversions, duplications and deletions, transpositions, homologous recombinations, slipped-strand mispairings as well as differential methylation patterns (Wisniewski-Dyé and Vial, 2008). In phase variation, the expression of a given factor is either ON or OFF and the changes (variations) are usually reversible (i.e. ON ↔ OFF), resulting in a heterogeneous population (Henderson et al., 1999).

Several studies with some *Azospirillum* species and strains have identified phenotypic variants. First studies reported frequently occurring spontaneous mutants in *Azospirillum brasilense* Sp7 with increased resistance to stress conditions like salt stress and iron acquisition (Hartmann et al., 1992) as well as the stable variant $4V_I$ of *Azospirillum lipoferum* 4B, which lost the ability to swim and had altered carbohydrate utilization abilities (Alexandre and Bally, 1999). In another report, an *A. brasilense* WN1 stable variant was reported to be non-motile and to produce translucent colonies in contrast to the parental strain. In this study, a plasmid pattern change was observed: while the wild-type cells harbors five plasmids, the variants carried only four of them, with the disappearance of a 260-kb replicon (Vial et al., 2006). A recent study has described several phenotypic variants of *A. brasilense* Sp7 that were obtained after exposure to prolonged starvation or after re-isolation from maize roots. Some of the variants were found to produce significantly more EPS, and of different monosaccharide composition, and displayed DNA rearrangements compared to the parental strain (Lerner et al., 2010).

In the present study we further examined physiological aspects of phenotypic variation in *A. brasilense* Sp7. The most frequent variants of these strains were found to overproduce EPS. Three representative EPS variants were randomly selected for more detailed characterization. Plant growth promotion of the variants was assessed with four plant species in the greenhouse, and it was demonstrated that the variants in general do not differ in their plant growth promotion effects as compared with the parental strain.

2. Materials and methods

2.1. Media and bacterial growth conditions

A. brasilense Sp7 and variants of this strains that were obtained in this study were routinely grown at 30 °C in fructose minimal medium, with high carbon to nitrogen ratio (C:N) (Burdman et al., 1998) containing (g l^{-1}): D-fructose (6.67), $MgSO_4$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), K_2HPO_4 (6.0), KH_2PO_4 (4.0), yeast extract (Difco) (0.1), NH_4Cl (0.214) and microelements as described (Okon et al., 1977). For preparation of solid fructose minimal medium, agar (Difco) was amended at 15 g l^{-1}. Cell growth was determined spectrophotometrically at OD_{590}, by dilution plating on D-fructose minimal medium or by drying the bacteria in an oven at 80 °C for 48 h, and measuring the dry cell weight.

2.2. Assessment of phenotypic variation in A. brasilense Sp7 and characterization of variants

To assess the rate of appearance of phenotypic variants, a single colony of the parental strain was used to inoculate 5 ml of D-fructose minimal medium. After overnight incubation at 30 °C with shaking (200 rpm), a 10^{-5} dilution was plated onto fructose minimal medium plates. In all experiments the morphology of the colonies was examined after 3 days of incubation. At least 10,000 colonies were screened in each experiment. The stability of the variants was tested by transferring individual colonies to new minimal medium plates incubated at 30 °C.

2.3. Polymerase chain reaction (PCR)

PCR was performed to confirm that colonies showing varied morphology were *A. brasilense* phenotypic variants rather than contaminations. Total DNA was isolated using the Wizard Genomic DNA Purification kit (Promega). All suspected variants were tested for the *A. brasilense ipdC* gene, encoding indole-pyruvate decarboxylase, a key enzyme in indole-3-acetic acid (IAA) synthesis. This gene, and the corresponding PCR primers, has been suggested as a reliable and specific indicator for *A. brasilense* species (Shime-Hattori et al., 2011). Each 25 μl PCR reaction contained 12.5 μl of Taq Master MIX (Lambda Biotech), $MgCl_2$ (1 mM), BSA (0.04 μg μl^{-1}), and 1.6 μM of each *ipdC* primer: A32f (forward), 5′-ACCCCTCCACAATTTCCGGCGCAT-3′, and A42r (reverse), 5′-CGCCACCCCTAGAGTGGAGCTGTA-3′ (Shime-Hattori et al., 2011). PCR amplifications were performed in an automated thermal cycler (Eppendorf Mastercycler Gradient Machine) with an initial denaturation (94 °C, 2 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s) and extension (72 °C, 1 min), and a single final extension (72 °C, 5 min). PCR products were then electrophoresed directly on 1% agarose gels, visualized by ethidium bromide staining, excised from the gel with the HiYield Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience) and sequenced at Hy Labs Co.

2.4. DNA fingerprinting analyses

Phenotypic variants were compared with the parental strain by repetitive-PCR [Enterobacterial Repetitive Intergenic Consensus (ERIC)- and BOX-PCR] and random amplified polymorphic DNA (RAPD) analysis. Repetitive-PCR reactions were performed as described by Louws et al. (1994). Reaction mixtures (25 μl) contained 12.5 μl of Taq Master mix (Lambda Biotech), $MgCl_2$ (3 mM), BSA (0.02 μg ul^{-1}), 2 μM of each primer [ERICIR, 5′-GTAAGCTCCTGGGGATTCAC-3′, and ERIC2, 5′-AAGTAAGTGACTGGGGTGAGCG-3′ for ERIC-PCR; BOXA1R 5′-CTACGGCAAGGCGACGCTGACG-3′ for BOX-PCR) and 100 ng of template (genomic) DNA. PCR amplifications were performed in an automated thermal cycler with an initial denaturation (95 °C, 7 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (42 and 53 °C for ERIC- and BOX-PCR, respectively, 1 min) and extension (65 °C, 8 min) with a single final extension (65 °C, 16 min). Samples of 8 μl from each reaction were separated by gel electrophoresis on 1.5% agarose gels at 60 V for 3 h, and the gels were stained with ethidium bromide. RAPD analysis was performed as described by Vial et al. (2006). Briefly, reaction mixtures (25 μl) contained 12.5 μl Taq master mix (Lambda Biotech), primer F1253 (5′-GTTTCCGCCC-3′) (2 μM), BSA (0.02 μg ul^{-1}), $MgCl_2$ (2 mM) and bacterial DNA (~100 ng). Amplification conditions were: initial denaturation (95 °C, 5 min) followed by 35 cycles of denaturation (95 °C, 45 s), annealing (36 °C, 1 min) and extension (72 °C, 2 min) with a final extension at 72 °C for 7 min and 60 °C for 10 min. PCR products were analyzed as described for repetitive-PCR above.

2.5. Extraction of EPS and determination of EPS monosaccharide composition

EPS were extracted from D-fructose minimal medium liquid cultures, by fractionation with cold ethanol as described by del

Gallo et al. (1989) and modifications by Lerner et al. (2009a). Briefly, following growth, bacterial cultures were centrifuged at 7000 *g* for 10 min at 10 °C. The supernatant was collected and left to stand in three volumes of cold ethanol for 72 h at 4 °C, resulting in EPS precipitation. After ethanol evaporation, precipitated polysaccharides were suspended in and dialyzed against distilled water for 72 h at 4 °C, using a dialysis membrane with a molecular mass cutoff of 12–14 kDa, and lyophilized. Absence of proteins in the extracted EPS was confirmed by the Bradford method, following manufacturer conditions (BioRad). Evaluation of the sugar amount was done by weighing or by the Anthrone method using glucose as a standard (Dische, 1962). For determination of EPS monosaccharide composition, EPS was hydrolyzed following lyophilization according to Lerner et al. (2009a). Briefly, sugar composition of modified alditols was determined in Hewlett Packard HP 5890 Series II gas chromatograph, equipped with a DB-225 capillary column (30 m × 0.25 mm) from J&W Scientific and a flame ionization detector (FID) at 250 °C. Samples (1 μl) of mixed alditols were separated at 220 °C with helium as the carrier gas.

2.6. Assessment of stress endurance, biofilm formation and aggregation

Bacteria were grown in D-fructose minimal liquid medium. The resulting cultures were used to assess bacterial survival under UV and heat stresses. In all experiments, the initial cell concentration was of approximately 5×10^8 CFU ml^{-1} ($OD_{590} \sim 0.8$). In heat resistance experiments, 10 ml of cultures were incubated in a water bath at 55 °C for 60 min. Bacterial viability was determined every 15 min. In experiments performed to assess resistance to UV radiation, 20 ml of cells in 90-mm Petri dishes were exposed to UV radiation (254 nm) using a VL-6LC ultraviolet lamp (Vilber-Lourmat) for 120 s, and bacterial viability was determined every 30 s. The percentage of viable cells was determined following dilution plating at the beginning and during the course of the experiment. In addition, the *A. brasilense* Sp7 variants were assessed for biofilm formation ability using the microplate method according to Lerner et al. (2010) and for cell aggregation according to Burdman et al. (1998).

2.7. Plant growth promotion experiments

Peanut (*Arachis hypogaea* cv. Granoleico), soybean (*Glycine max* L. cv. Don Mario), wheat (*Triticum aestivum* L cv Baguette 9) and maize [*Zea mays* L. var. Mass 484 (Morgan)] seeds were surface-sterilized with 10% H_2O_2 for 15 min and rinsed five times with sterilized distilled water, then germinated in darkness at room temperature on sterile water-agar Petri dishes (0.8% agar) for 2 days. Pre-germinated seeds were transferred aseptically to 500 ml pots (two seeds per pot) containing sterilized and nitrogen-free sand, and immediately inoculated with *A. brasilense* strain Sp7 or tested variants at 1×10^6 CFU ml^{-1}. Experiments with peanuts and soybean included inoculation and co-inoculation (with *A. brasilense*) treatments with *Bradyrhizobium* sp. SEMIA6144 (MIRCEN/FEPAGRO, Brazil) or *Bradyrhizobium japonicum* E109 [Instituto de Microbiologia y Zoologia Agrícola (IMYZA), Instituto Nacional de Tecnologia Agropecuaria (INTA), Castelar, Argentina] at 1×10^5 CFU ml^{-1}, respectively. Forty eight hours after sowing, seedlings were thinned down to uniformity to one per pot and were arranged in a complete randomized block design with five replicates per treatment. Pots were placed in a growth chamber at 24 °C, with illumination for 16 h day^{-1}. Plants were cultured with modified Hoagland nitrogen-free nutrient solution (Dardanelli et al., 2008). The following parameters were evaluated: shoot and root dry weights (after incubation at 65 °C for 72 h), total nitrogen by Kjeldahl method and in the case of legumes, number of nodules per plant and nodule dry weight. For determination of nodulation parameters, plants were removed from the pots, the roots were thoroughly rinsed with water, blotted dry on filter paper, and nodules were picked. All experiments were repeated three times. Results (presented in Tables 3–6) are the averages of three experiments, each treatment in five replicates.

Table 1
Exopolysaccharide (EPS) production, cell aggregation and biofilm formation by *Azospirillum brasilense* Sp7 (parental colony) and its phenotypic variants.

Strain	EPS/Cell dry weight (g g^{-1})	Cell aggregation (%)	Biofilm formation ($OD_{590\ nm}$)
Sp7 (parental strain)	0.20 b	5.0 c	0.47 b
Variant phv1	1.50 a	18.0 b	0.88 a
Variant phv2	1.60 a	18.5 b	0.15 c
Variant phv3	1.60 a	27.0 a	0.70 a

Values represent averages from three independent experiments each with five replicates for each parameter. Different letters in each column indicate significant differences ($p = 0.05$).

2.8. Statistical analyses

Experiments were performed using a completely randomized design and statistical analyses were done by Analysis of Variance (ANOVA). Multiple comparisons of means were done by the L.S.D. method. All hypotheses were tested at the 95% confidence level. Analyses were performed using Statistix 8.0 software (NH Analytical Software).

3. Results

3.1. Determination of the rate of appearance of phenotypic variants of A. brasilense Sp7 and characterization of selected variants

To isolate phenotypic variants of *A. brasilense* Sp7, cells from a late logarithmic culture in D-fructose minimal liquid medium were plated on D-fructose minimal solid medium and more than 10,000 colonies were screened in two independent experiments. The Sp7 parental strain forms white, slightly mucoid colonies (Fig. 1). A main type of phenotypic variants was identified that formed exceedingly mucoid colonies. The frequency of appearance of this type of variants was 1:5000; thus the frequency obtained is in accordance with the definition of phase/phenotypic variation rather than spontaneous mutation (Wisniewski-Dyé and Vial, 2008). It was not possible to distinguish between the highly mucoid variants by the naked eye; therefore, for further characterization, highly mucoid variants were randomly selected from three different colonies displaying the same morphology, and were named phv1, phv2 and phv3 (the latter is shown in Fig. 1).

3.2. DNA fingerprinting analysis of A. brasilense variants

To verify that the different morphological colonies are indeed variants of *A. brasilense* Sp7 (and not contaminations), we screened

Table 2
Monosaccharide composition of EPS of *A. brasilense* Sp7 and phenotypic variants.

Sugar	Sp7	phv1	phv2	phv3
Rhamnose	17.6 ± 0.5	32.1 ± 2.2	3.5 ± 2.5	9.9 ± 1.7
Xylose	1.7 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.7 ± 0.4
Mannose	25.5 ± 0.5	8.4 ± 1.1	7.4 ± 1.6	6.1 ± 1.4
Galactose	24 ± 0.1	1.5 ± 1.1	56.2 ± 4.8	44.2 ± 4.4
Glucose	12.7 ± 1.0	57.9 ± 5.6	32.7 ± 4.1	33.1 ± 1.5

EPS were purified after 72 h of growth in D-fructose minimal medium. Results represent average ± standard deviation (SD) of the relative presence of each sugar (percentage) from two independent experiments.

Table 3
Effect of inoculation with *A. brasilense* Sp7 or its phenotypic variants on shoot length, shoot dry weight, shoot nitrogen accumulation, root length and root dry weight of *Zea mays* var. Mass 484.

Treatments	Shoot length (cm)	Shoot dry weight (g plant^{-1})	Shoot N accumulation mg N plant^{-1} (% increase[a])	Root length (cm)	Root dry weight (g plant^{-1})
Not inoculated	13.2 ± 1.4b	0.19 ± 0.02b	9.7 ± 0.5c	14.46 ± 1.00c	0.21 ± 0.01b
Sp7	17.1 ± 1.3a	0.27 ± 0.04a	16.2 ± 1.0a (66.5)	21.12 ± 1.38b	0.27 ± 0.03a
phv1	16.3 ± 0.5a	0.21 ± 0.03ab	12.1 ± 0.7b (24.2)	26.60 ± 1.00a	0.24 ± 0.02ab
phv2	16.7 ± 1.0a	0.26 ± 0.03a	14.7 ± 0.9 ab (51.6)	24.44 ± 2.43a	0.24 ± 0.02ab
phv3	15.0 ± 1.0a	0.24 ± 0.02a	14.9 ± 0.5 ab (53.7)	21.28 ± 0.80b	0.26 ± 0.02a

Each value is the average ± SD of three independent experiments (5 replicates per treatment per experiment) replicates. Significant differences ($p = 0.05$) between values within a column are indicated by different letters.
[a] % of increase in N content of shoots of inoculated plants relative to non-inoculated plants.

them for the presence of the *ipdC* gene using unique primers that have been proposed to be specific for *A. brasilense* (Shime-Hattori et al., 2011). All tested colonies were *ipdC* positive (not shown), thus confirming that these are indeed phenotypic variants of *A. brasilense* Sp7.

Since phenotypic variation is often associated with genomic DNA rearrangements (Kivisaar, 2003; Van der Woude and Baumler, 2004; Wisniewski-Dyé and Vial, 2008), ERIC- and BOX-PCR were performed using genomic DNA extracted from *A. brasilense* Sp7 and from selected variants as templates. As can be seen, ERIC-PCR (Fig. 2A) revealed pattern changes in all variants relative to the parental strain, with more substantial differences being observed for variants phv2 and phv3. Also, all variants differed each from the other in their profiles (Fig. 2A). BOX-PCR (Fig. 2B) did not reveal apparent differences between variant phv1 and the parental strain, but the patterns were clearly different when comparing the parental strain with variants phv2 and phv3, and also here, the fingerprinting profile of the three variants were substantially different (Fig. 2B). A similar picture was obtained in RAPD assays (Fig. 2C). Overall, these results support that phenotypic variation in *A. brasilense* Sp7 is associated with different types of genetic rearrangements, which can be relatively slight (as in variant phv1) or pronounced (as in phv2 and phv3).

3.3. EPS production, cell aggregation and biofilm formation by Sp7 variants

The exceeding mucoidity of the variant colonies (Fig. 1) was suspected to be due to overproduction of EPS. Therefore, EPS from parental strain and the variants were purified after 48 h of growth in D-fructose liquid minimal medium and were quantified. As can be seen in Table 1, the three tested variants produced 7.5–8 times more EPS per cell dry weight as compared with the parental strain Sp7 and these differences were statistically significant ($p = 0.05$). Under tested conditions, variants phv1 and phv3 formed significantly ($p = 0.05$) more biofilm than the parental strain and all variants showed significantly ($p = 0.05$) higher levels of cell aggregation than the parental strain (Table 1). In contrast to variants phv1 and phv3, variant phv2 produced significantly ($p = 0.05$) less biofilm than strain Sp7 (Table 1).

3.4. Monosaccharide composition of EPS extracted from Sp7 variants

To gain more insight into EPS properties of the variants relative to the parental strain, EPS were extracted after growth for 72 h in D-fructose minimal medium and their monosaccharide composition was analyzed. Results, representing average percentages of the relative presence of each monosaccharide from two independent experiments, are shown in Table 2. The three phenotypic variants differed in their EPS composition, among them and relative to the parental strain. EPS produced by *A. brasilense* Sp7 had higher xylose and mannose contents as compared with all phenotypic variants, and contained more rhamnose than variants phv2 and phv3. EPS produced by variants phv2 and phv3 were rich in glucose and galactose relative to the parental strain. EPS from variant phv1 also had a high content of glucose, but in contrast to the other variants, had a reduced concentration of galactose (Table 2). In addition, EPS of variant phv1 had the highest concentration of rhamnose among all tested strains (Table 2).

3.5. Resistance of Sp7 phenotypic variants to heat and UV

EPS contribute to the structural integrity of the cell envelope (Luderitz et al., 1982) and to protection against different stresses such as osmotic shock, oxidative stress, desiccation, heat, UV radiation and antibiotics (Mao et al., 2001; Wang et al., 2007; Lerner et al., 2009a,b, 2010). Since the Sp7 selected variants showed significantly more EPS relative to the parental strain, and different EPS monosaccharide composition, they were assessed for their ability to survive under heat and UV-radiation. Fig. 3 shows that the three variants were more resistant to both stresses than the parental strain, as seen by the moderate slopes of their mortality

Table 4
Effect of inoculation with *A. brasilense* Sp7 or its phenotypic variants on shoot length, shoot dry weight, shoot nitrogen accumulation, root length and root dry weight of *Triticum aestivum* cv. Baguette 9.

Treatments	Shoot length (cm)	Shoot dry weight (g plant^{-1})	Shoot N accumulation mg N plant^{-1} (%increase[a])	Root length (cm)	Root dry weight (g plant^{-1})
Not inoculated	8.8 ± 1.4c	0.045 ± 0.003b	15.2 ± 1.0b	12.9 ± 1.5c	0.15 ± 0.03b
Sp7	22.6 ± 3.9b	0.068 ± 0.009a	20.1 ± 1.3a (32.4)	16.9 ± 1.9b	0.20 ± 0.02a
phv1	27.1 ± 3.5a	0.070 ± 0.013a	20.5 ± 1.35a (35.0)	19.4 ± 2.7a	0.23 ± 0.03a
phv2	29.2 ± 3.3a	0.070 ± 0.009a	16.7 ± 0.5b (10.20)	20.9 ± 2.0a	0.16 ± 0.01b
phv3	22.2 ± 2.5b	0.071 ± 0.005a	20.7 ± 1.1a (36.0)	19.4 ± 1.4a	0.25 ± 0.03a

Each value is the average ± SD of three independent experiments (5 replicates per treatment per experiment) replicates. Significant differences ($p = 0.05$) between values within a column are indicated by different letters.
[a] % of increase in N content of shoots of inoculated plants relative to non-inoculated plants.

Table 5
Effect of inoculation with *A. brasilense* Sp7 or its phenotypic variants on shoot length, shoot dry weight, shoot nitrogen accumulation, root length and root dry weight of *Glycine max* L. cv. Don Mario.

Treatments	Shoot length (cm)	Shoot dry weight) (g plant^{-1})	Shoot N accumulation mg N plant^{-1} (% increase[a])	Root length (cm)	Root dry weight (g plant^{-1})
Not inoculated	29.9 ± 1.0b	0.67 ± 0.010c	17.4 ± 0.7a	15.2 ± 0.6b	0.25 ± 0.008b
Sp7	33.3 ± 1.5a	0.76 ± 0.005a	18.2 ± 0.8a (4.8)	18.0 ± 0.4a	0.29 ± 0.007a
phv1	34.8 ± 1.5a	0.7 ± 0.006b	19.2 ± 0.7a (10.3)	19.5 ± 1.2a	0.28 ± 0.004a
phv2	30.5 ± 0.5b	0.74 ± 0.009b	17.5 ± 0.8a (0.6)	16.9 ± 0.6b	0.29 ± 0.007a
phv3	32.3 ± 1.4a	0.78 ± 0.011a	17.3 ± 0.8a (−0.6)	18.1 ± 0.7a	0.28 ± 0.005a

Each value is the average ± SD of three independent experiments (5 replicates per treatment per experiment) replicates. Significant differences ($p = 0.05$) between values within a column are indicated by different letters.
[a] % of increase in N content of shoots of inoculated plants relative to non-inoculated plants.

curves. Variant phv2 showed clearly highest resistance to both stresses, as compared with the other variants.

3.6. Plant growth promotion by Sp7 phenotypic variants

To assess whether phenotypic variation in *A. brasilense* Sp7 alters plant growth promotion traits of the bacterium, we performed several inoculation experiments with cereal and legume plants. In maize inoculation experiments, both the parental strain and the tested phenotypic variants significantly ($p = 0.05$) promoted root and plant growth as well as accumulation of nitrogen content, as compared with non-inoculated controls (Table 3). In general, no significant differences were observed between parental strain and variants for most plant growth parameters. In wheat, *A. brasilense* Sp7 and the phenotypic variants significantly ($p = 0.05$) increased to different extents plant growth parameters above non-inoculated controls (Table 4). Moreover, in wheat experiments, variants phv1 and phv2 showed slightly but significantly ($p = 0.05$) higher promotion of shoot and root length than the parental strain. Variant phv3 also showed significantly ($p = 0.05$) higher promotion of root length relative to the parental strain; however, plants inoculated with this variant showed significantly ($p = 0.05$) reduced shoot length than plants inoculated with the other variants (Table 4).

In comparison with the results with cereal plants, inoculation of soybeans and peanuts with *A. brasilense* Sp7 or the phenotypic variants alone (without the specific *Bradyrhizobium*) did only slightly benefit legume growth parameters (Tables 5 and 6). Nevertheless, significant differences among *Azospirillum* inoculated and non-inoculated plants were detected for most parameters in both legumes. In soybeans inoculated with effective *Bradyrhizobium*, co-inoculation with *A. brasilense* or the phenotypic variants generally (and sometimes significantly) improved plant growth parameters above inoculation with *Bradyrhizobium* alone, and all co-inoculation treatments lead to a significant ($p = 0.05$) increase in nodulation parameters (nodule numbers and dry weight per plant), relative to inoculation with *Bradyrhizobium* (Table S1). Co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* (parental strain or phenotypic variants) also increased nodulation parameters and nitrogen content of peanut shoots above inoculation with *Bradyrhizobium* alone; however, not in all cases the differences were statistically significant ($p = 0.05$) (Table S2). Shoot and root dry weight of peanuts was also improved above inoculation with *Bradyrhizobium* alone by co-inoculation with *Bradyrhizobium* and *A. brasilense* strains (Table S2).

4. Discussion

In the present study we further characterized the phenomenon of phenotypic variation in *A. brasilense* Sp7. When plated on solid media, some colonies were characterized by possessing a much more mucoid morphology than the typical colonies of the parental strain (Fig. 1). The rate of appearance of mucoid variant colonies was 1 in 5,000, in agreement with the accepted rate for the phase/phenotypic variation phenomenon (Vial et al., 2006; Wisniewski-Dyé and Vial, 2008). These variants seem to be highly stable since during the course of this study, we were not able to observe a reverse phenotype (namely, reversion to the parental strain phenotype). However, it is important to clarify that in this study the variants were routinely grown in fructose minimal medium. We cannot discard the possibility that under other growth conditions, phenotypic reversion may occur, thus this question should be carefully assessed in the future.

The analyzed variants produced 7.5–8 times more EPS, with different monosaccharide composition, than parental strain Sp7. EPS have an important role in protecting cells against environmental stresses such as fluctuations in temperature, salinity, osmolarity, oxygen and nutrient concentrations that may occur in the rhizosphere and in the soil (Lerner et al., 2010). Here we found that the EPS-overproducing variants were significantly more resistant to heat and UV-exposure than the parental strain, suggesting that overproduction of EPS via phenotypic variation is an adaptive behavior of *A. brasilense*. These findings are in agreement with results reported by Lerner et al. (2010), following characterization of

Table 6
Effect of inoculation with *A. brasilense* Sp7 or its phenotypic variants on shoot length, shoot dry weight, shoot nitrogen accumulation, root length and root dry weight of *Arachis hypogaea* cv. Granoleico.

Treatments	Shoot length (cm)	Shoot dry weight (g plant^{-1})	Shoot N accumulation mg N plant^{-1} (% increase[a])	Root length (cm)	Root dry weight (g plant^{-1})
Not inoculated	15.8 ± 0.5b	0.18 ± 0.02c	22.3 ± 0.9b	8.9 ± 1.3b	0.21 ± 0.01c
Sp7	16.5 ± 0.7b	0.28 ± 0.01b	25.0 ± 0.8a (12.4)	12.2 ± 1.2a	0.27 ± 0.02a
phv1	21.3 ± 1.6a	0.30 ± 0.02b	24.3 ± 1.5a (9.0)	10.2 ± 0.8a	0.25 ± 0.03a
phv2	19.3 ± 0.7a	0.34 ± 0.01a	25.5 ± 1.4a (14.7)	11.6 ± 0.6a	0.28 ± 0.01a
phv3	19.4 ± 1.1a	0.30 ± 0.01b	24.7 ± 1.2a (10.9)	11.8 ± 0.5a	0.24 ± 0.001b

Each value is the average ± SD of three independent experiments (5 replicates per treatment per experiment) replicates. Significant differences ($p = 0.05$) between values within a column are indicated by different letters.
[a] % of increase in N content of shoots of inoculated plants relative to non-inoculated plants.

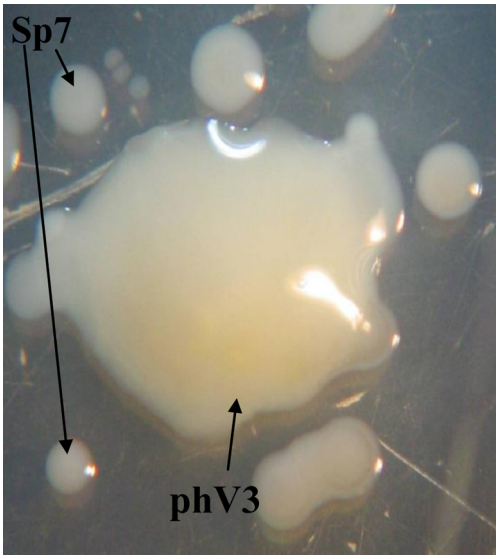

Fig. 1. *Azospirillum brasilense* Sp7 and one of its phenotypic variants. The parental strain (Sp7) forms slightly mucoid colonies. Phenotypic variant phv3 represents a type of phenotypic variants of strain Sp7 that form exceedingly mucoid colonies as compared with the parental strain.

other variants that were selected after stress or after reisolation from maize roots. Results from the present work also suggest that EPS overproducing variants are present in the population irrespective of culture conditions. It can be hypothesized that even if the variant subpopulation is small under normal conditions, its existence is significant because it enables the strain to survive stress conditions. On the other hand, since EPS overproduction is expensive in terms of carbon utilization, it is limited to a minor subpopulation, which might expand in case of developing stress conditions in the culture.

Biofilm formation by the phenotypic variants was higher than by the parental strain with exception of variant phv2. This variant showed a substantially lower concentration of rhamnose in its EPS relative to the parental strain and the other variants (Table 2), which could affect adhesive properties of this variant. Mutation in a rhamnose biosynthetic gene cluster in *A. brasilense* Cd affected rhamnose production, altered the LPS structure and reduced maize root attachment and colonization (Jofre et al., 2004). In inoculation experiments in natural soil it is difficult to assess the colonization of the introduced bacterium, also because we lack a specific marker or selective medium for the isolation and identification of the phenotypic variants. Assessment of colonization under gnotobiotic conditions could provide some information, but it would be less relevant for rhizosphere colonization in natural soil.

Phenotypic variation is often associated with DNA rearrangements (Kivisaar, 2003; Van der Woude and Baumler, 2004; Wisniewski-Dyé and Vial, 2008). To assess whether the variants characterized in this study have DNA rearrangements relative to the parental strain, we used repetitive-PCR and random amplification of polymorphic DNA (RAPD) techniques. Repetitive-PCR techniques like ERIC- and BOX-PCR are based on the amplification of bacterial DNA using primers designed from entire conserved central core inverted repeats. RAPD uses arbitrary, short primers (8–12 nucleotides) to randomly DNA fragments (Vial et al., 2006). PCR amplifications of bacterial genomic DNA by repetitive-PCR and RAPD result in different banding patterns for different genomes (Louws et al., 1994; Vial et al., 2006; Wisniewski-Dyé and Vial, 2008; Lerner et al., 2010). Using these techniques we found that the Sp7 phenotypic variants indeed displayed genomic changes as seen by the different band patterns following ERIC-, BOX-PCR and RAPD analyses. In the future we plan to sequence the genome of selected variants and perform genome comparative analyses with the parental strain, in order to gain insights into genetic alterations associated with phenotypic variation in *A. brasilense* Sp7.

Investigation of the traits that contribute to bacterial survival of plant growth promoting bacteria under adverse conditions during storage, inoculation and colonization of plants and seeds is of high economic importance (Fibach-Paldi et al., 2012). Cell surface components such as EPS provide enhanced resistance to diverse stress conditions (Lerner et al., 2009b,c). Therefore, it is reasonable to speculate that utilization of EPS overproducing variants of *A. brasilense* could result in better performance than use of parental strains, in terms of bacterial survival, plant colonization and plant growth promotion effects.

In the present study we compared the EPS overproducing variants of *A. brasilense* Sp7 with the parental strain for their ability to exert plant growth promotion in two grain grasses (maize and wheat) and two legumes (soybean and peanuts). Results from these experiments corroborated the extensively reported plant growth promotion effects of *A. brasilense* on both cereals and legumes, as

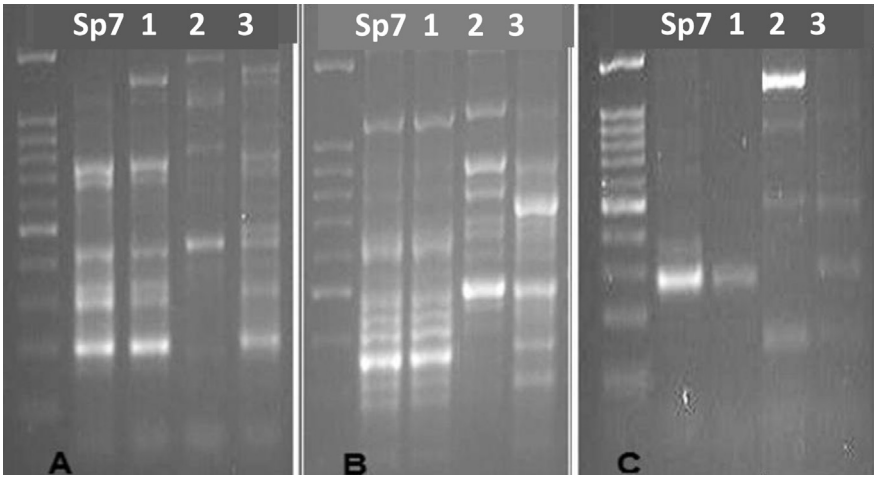

Fig. 2. Repetitive-PCR and RAPD of *A. brasilense* Sp7 and selected phenotypic variants. A, ERIC-PCR; B, BOX-PCR; C, RAPD. Lanes: Sp7, parental strains; 1, 2 and 3 are phv1, phv2 and phv3, respectively.

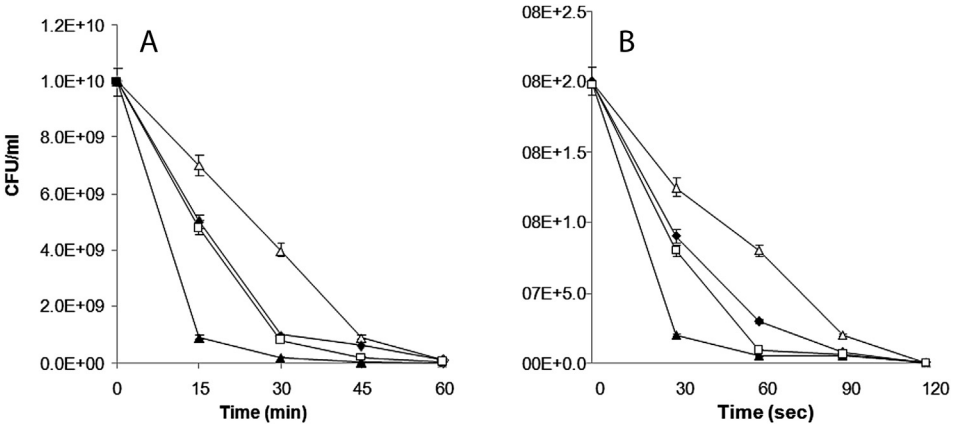

Fig. 3. Survival of *A. brasilense* Sp7 and its phenotypic variants following exposure to heat (55 °C; A) and UV-radiation (B). For each stress, data represent average ± SD of one experiment (5 replicates per treatment) out of three with similar results. Sp7, ▲; phv1, □; phv2, △; phv3, ◆.

well as the positive effects of *A. brasilense* inoculation on legume root nodulation by specific bradyrhizobia (Dardanelli et al., 2008; Bashan and de Bashan, 2010; Hungria et al., 2010; Star et al., 2012, Fibach-Paldi et al., 2012; Helman et al., 2011). Increments were generally observed in root shoot and nitrogen content, possibly due to enhanced nitrogen uptake from the soil (Fibach-Paldi et al., 2012). In the case of legumes the increases in nitrogen content could be derived from both enhanced nitrogen uptake by the roots and biological nitrogen fixation by the nodules (Spaepen et al., 2009). An important conclusion from the plant inoculation experiments reported in this work is that overall, the EPS over-producing variants performed as similar as the parental strain in both grasses and legumes. Therefore, at least under tested, green-house conditions, EPS overproduction did not confer an advantage to *A. brasilense* in terms of induction of plant growth promotion. On the other hand, we were concerned that the phenomenon of phenotypic variation, taking place at high frequency in *A. brasilense*, could affect the consistent performance of commercial inoculants. Based on the plant promotion effects observed in this work, this concern seems to be less important for consideration, although it would still be important to verify this issue by commercial-scale, field experiments. Moreover, due to the observed increased resistance of *A. brasilense* variants to several stresses in this and in other studies (Lerner et al., 2010), in the future it will be important to assess whether the phenotypic variants possess increased shelf-life in inoculants relative to parental strains.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.09.008.

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