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Azospirillum brasilense ameliorates the response of *Arabidopsis thaliana* to drought mainly via enhancement of ABA levels

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Production of phytohormones is one of the main mechanisms to explain the beneficial effects of plant growth-promoting rhizobacteria (PGPR) such as *Azospirillum* sp. The PGPRs induce plant growth and development, and reduce stress susceptibility. However, little is known regarding the stress-related phytohormone abscisic acid (ABA) produced by bacteria. We investigated the effects of *Azospirillum brasilense* Sp 245 strain on *Arabidopsis thaliana* Col-0 and *aba*2-1 mutant plants, evaluating the morphophysiological and biochemical responses when watered and in drought. We used an in vitro-grown system to study changes in the root volume and architecture after inoculation with *Azospirillum* in *Arabidopsis* wild-type Col-0 and on the mutant *aba*2-1, during early growth. To examine *Arabidopsis* development and reproductive success as affected by the bacteria, ABA and drought, a pot experiment using *Arabidopsis* Col-0 plants was also carried out. *Azospirillum brasilense* augmented plant biomass, altered root architecture by increasing lateral roots number, stimulated photosynthetic and photoprotective pigments and retarded water loss in correlation with incremented ABA levels. As well, inoculation improved plants seed yield, plants survival, proline levels and relative leaf water content; it also decreased stomatal conductance, malondialdehyde and relative soil water content in plants submitted to drought. *Arabidopsis* inoculation with *A. brasilense* improved plants performance, especially in drought.

Introduction

It is well known that plant growth-promoting rhizobacteria (PGPR) are rhizosphere-inhabiting microorganisms that are applied to many agronomic important crop plants to obtain beneficial effects, such as germination enhancement, increased nutrient availability, improved plant growth and crop yield and reduced susceptibility

Abbreviations – ABA, abscisic acid; CFU, colony-forming units; Chl, chlorophyll; D, drought treatment; DW, dry weight; FW, fresh weight; GAs, gibberellins; GC-EIMS, capillary gas chromatography-electron impact mass spectrometry; gs, stomatal conductance; I, inoculated; IAA, indole-3-acetic acid; LA, leaf area; LR, lateral root; MDA, malondialdehyde; MS, Murashige and Skoog; PBS, phosphate-buffered saline; PCA, principal component analysis; PGPR, plant growth-promoting rhizobacteria; ROS, reactive oxygen species; RLWC, relative leaf water content; RSWC, relative soil water content; SE, standard error; sp. (spp.), species; TPC, total phenolic compound; W, daily watered.

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to diseases (Kloepper et al. 1991, Zhang et al. 2011). *Azospirillum* sp. is one of the best-studied PGPR for improving plant growth of different species in various environments (Bashan and de-Bashan 2010). However, there are no reports on the inoculations with *Azospirillum brasilense* in *Arabidopsis thaliana* plants throughout the whole plant cycle in drought conditions.

The major visual effects of inoculations with *Azospirillum* sp. are morphological root changes, such as promotion of root elongation (Levanony and Bashan 1989, Dobbelaere et al. 1999), development of lateral and adventitious roots (Creus et al. 2005, Molina-Favero et al. 2008) and lengthening and branching of root hairs (Fulchieri et al. 1993). These root changes increase the volume of soil explored and consequently the uptake of water and nutrients. It has also been proven that some PGPR increase the plant resistance to environmental threats such as drought, salinity and heavy metals (Mayak et al. 2004, Creus et al. 2005, Cohen et al. 2009, Dodd et al. 2010).

Results of several studies indicate that the way *Azospirillum* sp. improves plant growth is by the production of phytohormones by itself and by inducing synthesis of phytohormones by the plant tissues (Costacurta and Vanderleyden 1995, Bottini et al. 2004, Spaepen et al. 2007, Bashan and de-Bashan 2010), mainly indole-3-acetic acid (IAA; Crozier et al. 1988) and gibberellins (GAs; Bottini et al. 1989, 2004, Fulchieri et al. 1993). In chemically defined growth cultures *A. brasilense* Sp 245 produced abscisic acid (ABA) and the production increased when NaCl was added to the culture medium. Also, *A. brasilense* enhanced ABA levels in *Arabidopsis* seedlings (Cohen et al. 2008).

Drought is one of the main environmental stresses reducing plant productivity worldwide (Boyer 1982). Under water stress plants increase ABA biosynthesis and/or decrease its catabolism (Bray 2002). In drought conditions, ABA is the signal that induces different adaptive responses, mainly closure of stomata to avoid water loss (Zhang and Outlaw 2001). ABA plays a role in mediating root branching, thereby improving the plant water uptake capacity (De Smet et al. 2006). Tardieu et al. (2010) proposed that ABA induces leaf growth by augmenting water movement in the plant because of increased tissue hydraulic conductivity. In tomato, ABA overproduction enhanced transpiration efficiency and root hydraulic conductivity, thereby affecting leaf expansion through improvements in water status (Thompson et al. 2007). Also, ABA increases sugar transport and promotes carbon allocation toward sink organs involved in plant survival (roots and fruits; Moreno et al. 2011). Similarly, maize plants inoculated with *Azospirillum lipoferum* increase ABA levels and reverse the effects of inhibitors of ABA and GA synthesis (fluridone and prohexadione-Ca, respectively) and augment the plant tolerance to drought (Cohen et al. 2009).

Drought induces oxidative stress that caused metabolic damage, increased lipid peroxidation, resulting in greater membrane injury. Proline is a cytosol-compatible solute involved in osmotic regulation (Voetberg and Sharp 1991), with possible function as a drought injury sensor. It is also involved in stress tolerance mechanisms (like protection of proteins and membrane structures; Yancey et al. 1982) by scavenging reactive oxygen species (ROS; Smirnoff and Cumbes 1989) and by regulating the cellular redox status (Hare et al. 1998). Proline accumulation is partially regulated by ABA, but ABA applied in the absence of stress is insufficient to induce high levels of proline (Sharma and Verslues 2010). Malondialdehyde (MDA) is a cytotoxic product of lipid peroxidation, and is used as an indicator of free radical production. Hence, quantification of tissue MDA content indicates degree of macromolecules destruction, which results in the loss of cell function and (eventually) death (Zhao et al. 1992).

ABA increases leaf carotenoid content and distribution of carbohydrate in grains of wheat and soybean (Travaglia et al. 2007, 2010), and also augments yield in field-grown wheat under mild drought (Travaglia et al. 2010). Besides, the inoculation of wheat seedlings with *A. brasilense* Cd increases the quantity of photosynthetic and photoprotective pigments significantly (Bashan et al. 2006).

Although reports on the effects of phytohormones produced by PGPR on plants are abundant, information regarding the mechanisms involved in plants inoculated with *Azospirillum* sp. under drought conditions and reports on plant growth throughout the whole plant cycle are scarce. In this work, an in vitro-grown system was used to study changes in the root volume and architecture after inoculation with *Azospirillum* in *Arabidopsis* Col-0 (wild type) and on the *aba*2-1 mutant, defective in ABA biosynthesis, during early growth. A pot experiment using *Arabidopsis* Col-0 plants was used to examine *Arabidopsis* development and reproductive success as affected by *Azospirillum*, ABA and drought.

Materials and methods

Plant material and in vitro growth conditions

Seeds of *A. thaliana* wild-type Columbia (Col-0 ecotype) and the *aba*2-1 mutant were surface sterilized (by soaking 10 min in 75% ethanol plus 0.01% Triton X-100; Sigma-Aldrich, St. Louis, MO), then washed briefly two times in 100% ethanol and placed on Petri dishes containing half-strength MS (Murashige and Skoog 1962)

medium with 0.1% (w/v) agar and 1% (w/v) sucrose. The seeds were vernalized 3 days at 4°C in darkness, and then were located in a growth chamber with a photoperiod of 16 h of cool white light (100 μmol m^{-2} s^{-1}) at a temperature of 22 ± 2°C. After 2 days, the plates with seedlings were placed vertically to allow root growth along the agar surface and to permit hypocotyls to grow. The *aba*2-1 mutant (defective in ABA biosynthesis and with Col-0 background; Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH) was utilized to assess the ability of the bacteria to overcome the mutant reduced ABA levels (Schwartz et al. 1997, González-Guzmán et al. 2002). It is known that the *aba*2-1 mutant responds to exogenous ABA (Verslues and Bray 2006).

Bacterial cultures and plant inoculation

Azospirillum brasilense Sp 245 (gift of Dr Raúl Pedraza, Universidad de Tucumán, Argentina) was grown in 500-ml flasks with 125 ml of nitrogen-free (NFb) medium, as previously described by Piccoli and Bottini (1994), with malic acid (5 g l^{-1}) and NH_4Cl (1.25 g l^{-1}) as sources of C and N, respectively. The bacteria were cultured in an orbital shaker (120 rpm) at 32°C until reaching a concentration of ca. 10^9 colony-forming units (CFU) ml^{-1}. Bacteria were harvested by centrifugation at 8000 *g* for 10 min at 4°C and the pellets were washed with sterile phosphate-buffered saline (PBS; Cohen et al. 2009), centrifuged again and diluted to 10^6 CFU ml^{-1} of PBS buffer for further inoculation. Seven-day-old *Arabidopsis* Col-0 and *aba*2-1 mutant seedlings, cultured in MS plate as previously described, were inoculated in the root with 10 μl of PBS containing 10^6 CFU ml^{-1} of *Azospirillum* (Col-0 + I and *aba*2-1 + I) or with 10 μl of PBS (Col-0 and *aba*2-1). Plates were sealed with low-density polyethylene, arranged in a completely randomized design and maintained in the growth conditions described above. At day 23 after inoculation, the main root length, and the leaf area (LA) starting from the oldest pairs of leaves (leaf one) and followed by the second and third pairs of leaves were measured (photographic images using UTHSCSA Image Tool version 3.0, University of Texas, Health Science Center, San Antonio, TX). Also, the rosettes fresh weight (FW) and rosettes ABA concentration were measured. The bacteria were counted in roots and rosettes of the *Arabidopsis* 23 days post-inoculation to control the treatment effectiveness and the *Azospirillum* colonization capacity. It was performed following the standard plate-counting method (Salomon et al. 2013). The roots and rosettes were surface-sterilized by soaking in 1% commercial bleach for 3 min and then washed with sterile distilled water. Then, the tissues were soaked with PBS buffer and ground to powder in a sterile mortar and pestle, and resuspended in PBS to give a serial dilution (10^{-1} to 10^{-9} CFU ml^{-1}). Finally, CFU were counted after 5 days of incubation at 30°C by plating each dilution in a selective NFb medium, and the shapes and colors of the colonies were used to corroborate that they correspond to *A. brasilense* Sp 245.

ABA in *Arabidopsis* rosettes

ABA was quantified by capillary gas chromatography with electron impact mass spectrometry (GC-EIMS; Clarus 500, PerkinElmer, Shelton, CT), following the technique described by González et al. (2012) with modifications (Salomon et al. 2013). A total of 100 mg FW rosettes of 30-day-old Col-0 and *aba*2-1 plants, inoculated and non-inoculated with *Azospirillum*, were homogenized in a mortar and pestle with liquid N_2 and extracted overnight with 1 ml of methanol:twice-distilled water:acetic acid (80:19:1) at 4°C. Then 50 ng of [$^2H_6$]-ABA (a gift from Prof. R. P. Pharis, University of Calgary, Canada) dissolved in 5 μl of methanol was added and allowed 1 h for equilibration of the isotopes. Finally, 2 ml of 80% aqueous methanol in 1% acetic acid was added, and the sample was evaporated with vacuum at 35°C. Further purification and derivatization were performed prior to quantification of ABA.

Water loss rate determination

For rapid dehydration assays, rosettes of 30-day-old Col-0 and Col-0 + I, growing in Petri dishes as previously described, were severed from the root system and weighed immediately and at regular progressive intervals of time during 42 min. Water loss rate was estimated as the percentage of FW water loss rate relative to the initial tissue FW according to Salomon et al. (2013).

Experiments with pot-grown plants under stress

Seeds of Col-0 were surface disinfected (as described for in vitro-grown experiments), and placed in 180-ml plastic pots containing five parts of peat moss (Premix 6, Sunshine; Sun Gro Horticulture Canada Ltd., Vancouver, Canada) and two parts of perlite No. 4. Then, 12 pots were put in each tray and located in a growth chamber with a 16/8 h photoperiod (100 μmol m^{-2} s^{-1}), 22 ± 2°C and a relative humidity ~50%. After germination, a solution of 1 g l^{-1} Hakaphos® Base 7-12-40 (Compo Agricultura, Barcelona, Spain) was used for watering (600 ml by tray). Then, the plants were watered in the trays with 240 ml sterile distilled water every 7 days to keep

the soil water status close to field capacity. The plants with two fully expanded leaves were inoculated with 1 ml of PBS containing 10^6 CFU ml^{-1} of *Azospirillum* (I) or with 1 ml of PBS (both on the soil surface). After that, some 30-day-old plants were daily watered with 120 ml of water (W, the same way as described before) and the other submitted to drought (D) conditions, resulting in the following treatments: (1) W, (2) W + I, (3) D and (4) D + I. The treatments were arranged in a completely randomized design. The D treatment consisted of two periods of water restriction performed by stopping watering until visible symptoms of temporary wilting point (flaccid leaves, ca. 10 days after water was withheld) and two periods of recovering (ca. 48 h each one). After the second re-watered period, the diameter of the rosettes, the total number of inflorescences and flowers per plant were determined (54 days from germination). At that time the dry weight (DW) of rosettes and inflorescences and the rosettes photosynthetic and photoprotective pigments and ABA levels (as described for in vitro-grown experiments) were measured. After 60 days, the percentage of plant survival (initial number of plants was considered to be 100%) and the total seeds production per plant (yield) were evaluated.

Photosynthetic and photoprotective pigments

Determinations were done spectrophotometrically as described by Berli et al. (2010), using rosettes samples (54 days). Total chlorophyll (Chl; Chl *a* + Chl *b*) and carotenoid levels were measured from 250 mg FW samples, and total phenolic compounds (TPCs) and anthocyanin levels were determined using 500 mg FW samples.

Determination of stomatal conductance, relative soil water content, lipid peroxidation and proline concentration

For these determinations, 40-day-old Col-0 plants inoculated with *Azospirillum* (Col-0 + I) and non-inoculated (Col-0), obtained as previously described, were used. Half of the Col-0 plants were treated with 100 μ*M* ABA (±-S-*cis*, *trans*-abscisic acid; Kelinon Agrochemical Co., Beijing, China; Col-0 + ABA), resulting in the following treatments: (1) Col-0, (2) Col-0 + I and (3) Col-0 + ABA. The plants were maintained without watering and the stomatal conductance (gs; mmol H_2O m^{-2} s^{-1}) of fully expanded leaves (sum of adaxial and abaxial leaf surfaces) was assessed at the beginning of the experiment, using a portable steady-state porometer (SC-1; Decagon Devices, Pullman, WA). Following the first gs measurement, 10 μ*M* ABA was sprayed to all the plants to reduce gs differences between treatments. Then, gs values were registered at 1, 3, 5, 7 and 9 days from water shortage. Relative leaf water content (RLWC) was measured according to Cohen et al. (2009), and relative soil water content (RSWC) in the pots was estimated according to González et al. (2012). To evaluate oxidative damage as lipid peroxidation, MDA content was measured following the procedure described by Beligni and Lamattina (2002) and modified by Berli et al. (2010). Proline content was determined essentially as described by Bates et al. (1973) and modified by Berli et al. (2013).

Statistical analysis

Statistical analyses were performed using the software Statgraphics Centurion xv version 15.0.10 (Statpoint Technologies Inc., Warrenton, VA). One-way ANOVA and Fisher's least significant difference test were used to evaluate the effect of *Azospirillum* inoculation ($P \leq 0.05$). Multifactorial ANOVA was used to analyze the effect of *Arabidopsis* genotypes, *Azospirillum* inoculation, water status, leaf ontogeny and their interactions. Data are reported as a mean of independent replicated assays with their standard error (SE). Principal component analysis (PCA) was performed using the InfoStat software (InfoStat version 2011v.; Grupo InfoStat, Córdoba, Argentina), and the results of this analysis are presented as biplot graphs.

Results

Azospirillum inoculation enhanced LA and main root length in *Arabidopsis* plants, in concordance with increased ABA levels in rosettes

Bacterial counting showed that 23 days post-inoculation *Azospirillum* had entered and colonized the plants, not only at root level but also in the rosettes, although in the latter bacterial numbers were two orders of magnitude lower. That is, in 30-day-old *Arabidopsis* Col-0 and *aba*2-1 plants values were, respectively, $2.5 \times 10^6 \pm 0.2 \times 10^6$ and $2.0 \times 10^6 \pm 0.3 \times 10^6$ in roots, and $1.6 \times 10^4 \pm 0.1 \times 10^4$ and $1.9 \times 10^4 \pm 0.3 \times 10^4$ CFU ml^{-1} in the rosettes. No significant differences in CFU ml^{-1} in Col-0 and *aba*2-1-inoculated plants were observed. *Azospirillum* were not detected from extracts of non-inoculated Col-0 and *aba*2-1 plants.

Inoculations with *Azospirillum* increased LA in both Col-0 and *aba*2-1 plants (Fig. 1A and Fig. S1, Supporting Information). The LA of Col-0 and *aba*2-1 plants grown on agar plates for 30 days in the non-inoculated treatments was similar, while the inoculation with *Azospirillum* stimulated LA in all leaves, but specially in the third pair of leaves of Col-0 plants (Fig. 1A). Also, rosettes FW were increased by the bacteria in Col-0 and

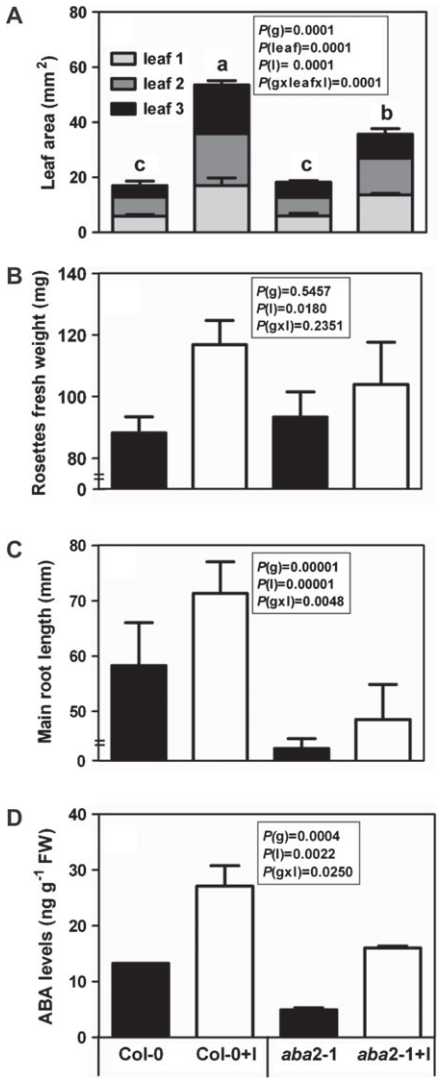

Fig. 1. (A) LA (mm^2), (B) rosettes FW (mg), (C) main root length (mm) and (D) ABA levels (ng g^{-1} FW, assessed by GC-EIMS with $^2H_6$-ABA as internal standard) in rosettes of *Arabidopsis thaliana* Col-0 and *aba*2-1 of 30-day-old plants non-inoculated (Col-0, *aba*2-1) and inoculated with *Azospirillum brasilense* Sp 245 (Col-0 + I, *aba*2-1 + I), grown on agar plates. *P*(g), effect of Col-0 and *aba*2-1 genotype; *P*(leaf), leaf ontogeny effect; *P*(I), *Azospirillum* effect; *P*(g × leaf × I), genotype × leaf ontogeny effect × *Azospirillum* interaction effect; *P*(g × I), genotype × *Azospirillum* interaction effect. Values are means ± SE (n = 20 and n = 3 for ABA levels). Different letters indicate significant differences ($P \leq 0.05$).

*aba*2-1 plants, without differences between genotypes (Fig. 1B). The main root length was also promoted by *Azospirillum* in both Col-0 and *aba*2-1 plants (Fig. 1C), although the roots of *aba*2-1 grew less than those of Col-0 in both control and inoculated ones (significant genotype effect). Inoculated Col-0 and *aba*2-1 plants had a larger number of lateral roots (LRs) than the non-inoculated modifying the root architecture (Fig. S1),

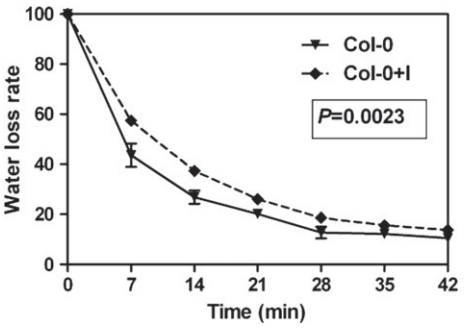

Fig. 2. Water loss rate of 30-day-old *Arabidopsis thaliana* non-inoculated (Col-0, continuous line) and inoculated with *Azospirillum brasilense* Sp 245 (Col-0 + I, dashed line). The plants were grown on the surface of agar plates, then the rosette was severed and the water loss rate was calculated based on differences in FW. Values are means ± SE (n = 20).

while Col-0 plants had fewer LR than *aba*2-1 mutants. The number of LR was augmented by *Azospirillum* in Col-0 and *aba*2-1 plants (Fig. S1), and the root FW was also increased by the bacteria (1.24 mg root $plant^{-1}$) when compared with the control (0.86 mg root $plant^{-1}$). In 50-day-old Col-0 plants cultivated in pots, the inoculation with *Azospirillum* also increased the root growth (Fig. S2). Fig. 1D shows the ABA levels in Col-0 and *aba*2-1 rosettes of plants grown 30 days on agar plates; inoculation with *Azospirillum* increased the plants ABA levels in both genotypes, but more markedly in *aba*2-1 (interaction effect). The non-inoculated *aba*2-1 treatment had 63% less ABA when compared with non-inoculated Col-0 plants, while inoculated *aba*2-1 plants showed higher values than non-inoculated Col-0.

Azospirillum inoculation retarded water loss in *Arabidopsis*

Bacterial inoculation retarded water loss of Col-0 rosettes that had been detached from their root system when compared with non-inoculated plants (Fig. 2), and they kept turgor (less dehydration) at 42 min (Fig. S3). In pots, Col-0 + I anticipated closure of stomata thereby preventing water losses, which was confirmed by measurement of gs (Fig. 4A). Also, Col-0 closed their stomata after ABA application (Col-0 + ABA).

Azospirillum inoculation ameliorated the response of *Arabidopsis* to drought involving several morphophysiological and biochemical changes, including rises in pigments, ABA, lipid peroxidation and proline

In 54-day-old Col-0 pot-grown plants photosynthetic pigments (total Chl and carotenoids) were augmented

Table 1. Total Chl (μg cm^{-2} leaf), carotenoids (μg cm^{-2} leaf), TPCs (OD_{305} cm^{-2} leaf), anthocyanins (OD_{546} cm^{-2} leaf) and ABA levels (ng g^{-1} FW) in 54-day-old *Arabidopsis thaliana* Col-0 pot-grown plants. Treatments: W and D, inoculated with *Azospirillum brasilense* Sp 245 (W + I) and (D + I). Values are means ± SE (n = 12) in photosynthetic and photoprotective pigments, and (n = 3) in ABA determination. *P*(W), water effect; *P*(I), *Azospirillum* effect; *P*(W × I), water × *Azospirillum* interaction effect. $P < 0.05$ shown in bold.

Treatments	Chl (μg cm^{-2} leaf)	Carotenoids (μg cm^{-2} leaf)	TPCs (OD_{305} cm^{-2} leaf)	Anthocyanins (OD_{546} cm^{-2} leaf)	ABA (ng g^{-1} FW)
W	2.10 ± 0.26	61.91 ± 8.54	0.08 ± 0.080	0.12 ± 0.022	141 ± 2.82
W + I	2.49 ± 0.33	75.44 ± 12.39	0.06 ± 0.015	0.11 ± 0.008	211 ± 4.94
D	2.17 ± 0.24	56.59 ± 4.64	0.09 ± 0.018	0.13 ± 0.004	874 ± 36.06
D + I	2.40 ± 0.45	66.51 ± 14.07	0.19 ± 0.058	0.19 ± 0.045	2598 ± 22.62
P(W)	0.9069	0.0667	**0.0001**	**0.0001**	**0.0001**
P(I)	**0.0091**	**0.0040**	**0.0008**	**0.0445**	**0.0001**
P(W × I)	0.4459	0.6328	**0.0001**	**0.002**	**0.0001**

Table 2. Growth and development parameters of 54-day-old *Arabidopsis thaliana* Col-0 pot-grown plants. Treatments: W and D, inoculated with *Azospirillum brasilense* Sp 245 (W + I) and (D + I). Values are means ± SE (n = 12). *P*(W), water effect; *P*(I), *Azospirillum* effect; *P*(W × I), water × *Azospirillum* interaction effect. $P < 0.05$ shown in bold.

	Ø Rosette (cm)	N° inflorescence	N° flowers	Rosettes DW (mg $plant^{-1}$)	Inflorescence DW (mg $plant^{-1}$)	Seed yield (mg $plant^{-1}$)	Plant survival (%)
W	13.75 ± 1.03	5.62 ± 0.74	27.50 ± 1.07	0.61 ± 0.12	0.22 ± 0.03	23.11 ± 10.45	93.22 ± 2.19
W + I	15.78 ± 1.44	6.12 ± 0.99	39.50 ± 1.60	0.61 ± 0.07	0.36 ± 0.13	42.20 ± 13.68	99.50 ± 0.51
D	13.12 ± 1.84	4.87 ± 1.25	1.06 ± 0.61	0.29 ± 0.16	0.22 ± 0.09	6.56 ± 1.19	35.50 ± 4.93
D + I	14.13 ± 2.12	4.88 ± 0.64	9.13 ± 0.99	0.54 ± 0.12	0.19 ± 0.04	28.12 ± 1.69	82.12 ± 2.19
P(W)	0.0634	**0.0004**	**0.0001**	**0.0006**	**0.0292**	**0.0280**	**0.0002**
P(I)	**0.0150**	0.9545	**0.0009**	0.1328	0.1522	**0.0058**	**0.0006**
P(W × I)	0.3912	0.6489	**0.0121**	0.2010	**0.0299**	0.8525	**0.0017**

by *Azospirillum*, but only the combination of D + I promoted an increment in photoprotective compounds (TPCs and anthocyanins, Table 1).

The inoculated pot-grown plants had larger rosettes than the controls under both water statuses. It was also observed that *Azospirillum* inoculation anticipated the flowering phenological stage with respect to the non-inoculated ones under both W and D conditions. There were no differences in the number of inflorescences at 54 days in W and W + I treatments; in plants submitted to drought (D and D + I) the number of inflorescences decreased (Table 2). There was an interactive effect between D and I in the total number of flowers per plant, D reduced markedly the number of flowers, but bacteria alleviated in part the effect of D (Table 2).

The DW of rosettes and inflorescences was affected by D, and the inflorescences DW was increased by inoculation with bacteria, but only when the plants were watered (Table 2). Drought markedly reduced the seed production per plant, but inoculation with *Azospirillum* increased yield under both water conditions, reversing the negative D effects (D + I induced an increase of fourfold compared to D, Table 2). After 60 days, the inoculated and non-inoculated plants did not differ in the percentage of plant survival when plants were watered. Meanwhile, when plants were submitted to D, the inoculation with *Azospirillum* increased their survival rate (Table 2).

In the plant-pot experiment foliar ABA concentrations were assessed at day 54 (Table 1), right after the D-treated plants had recovered from the wilting and where gs (Fig. 4A) and proline (Fig. 6) accumulation were significantly different (at 41–50 days). As it was expected, D induced an increase of sixfold in the ABA levels of Col-0 plants (D, 874 ng g^{-1} FW) when compared to those measured under W conditions (W, 141 ng g^{-1} FW; Table 2). In both water statuses, bacterial inoculation augmented 1.5- to 3-fold the ABA levels (211 ng g^{-1} FW and 2598 ng g^{-1} FW for W + I and D + I, respectively, Table 1). There was an interaction between water and *Azospirillum* inoculation (Table 1), in which D + I showed the highest ABA levels.

Fig. 3 shows the biplot graph for 54-day-old Col-0 plants cultivated in pots. The matrix for the analysis consisted of four cases corresponding to the combination of the two irrigation conditions (W and D) and two treatments (non-inoculated and inoculated), and 10 variables (different parameters evaluated). PC1 explained 62.6% of the variance and separated D from W + I. The Chl and carotenoids levels, the rosettes size and DW, the number of inflorescences, the inflorescences DW, the seed production and the percentage of plant survival were associated with W + I treatment. PC2 explained 25.6% of the

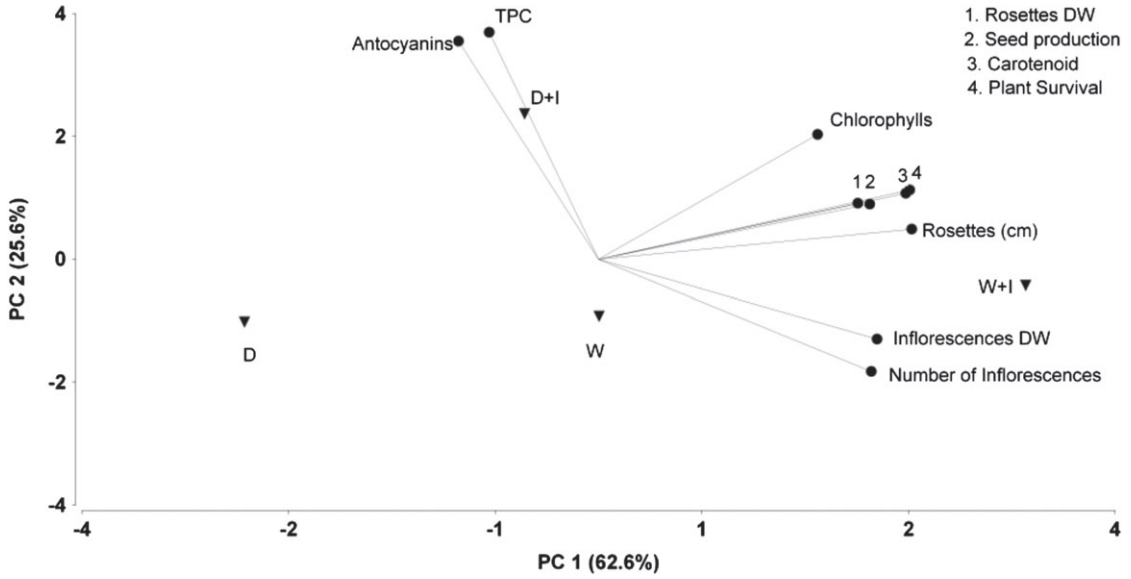

Fig. 3. Biplot display of PCA of the parameters analyzed in 54-day-old *Arabidopsis thaliana* Col-0 pot-grown plants. Treatments: W and D, inoculated with *Azospirillum brasilense* Sp 245 (W + I) and (D + I); variables: TPCs, carotenoids and anthocyanins levels, rosettes DW and diameter, seed production, plant survival (%), inflorescences number and DW, number of flowers (n = 12).

variance and separated D + I from the other treatments. Anthocyanins and TPC levels were associated with treatment D + I.

The gs differed since the beginning of water shortage, being reduced in treatments with application of ABA (Col-0 + ABA) or inoculated with *Azospirillum* (Col-0 + I). On day 1 after applying 10 μ*M* ABA, no differences between Col-0 and Col-0 + I were found; then, at day 3 they started to differentiate in the gs values, where Col-0 + I plants kept lower gs than Col-0, and finally, at day 7 gs decreased in all treatments. Even though on days 7 and 9 no differences were observed between Col-0 + I and Col-0 + ABA plants (Fig. 4A); at soil level, the RSWC was minor in Col-0 + I (31.9 ± 6.4%) than Col-0 (36.7 ± 9.3%) and Col-0 + ABA (49.3 ± 4.9%, Fig. 5). Non-inoculated plants wilted earlier than Col-0 + I or Col-0 + ABA (Fig. 4B). Moreover, Col-0 + ABA showed on leaves the highest percentage of RLWC (76.9 ± 5.7%) without significant differences with Col-0 + I (71.7 ± 6.6%), whereas that in Col-0 plants were 58.3 ± 5.9% (Fig. 5). However, the MDA levels in Col-0 plants were the highest (2.30 ± 0.23 n*M* g^{-1} leaf), while in Col-0 + I and Col-0 + ABA they were 1.75 ± 0.28 and 1.63 ± 0.25 n*M* g^{-1} leaf, respectively (Fig. 5). Either Col-0 + I or Col-0 + ABA plants mildly increased the levels of proline compared to Col-0 at time 0. From the cessation of irrigation proline accumulated steadily in all the treatments, the greatest accumulation was observed in Col-0 + I plants at the final sampling date (Fig. 6).

Discussion

We found that *A. brasilense* Sp 245 induced different positive effects on *A. thaliana* plants (Col-0 and *aba*2-1). A single event of inoculation in seedlings had effects on growth parameters during the early (in vitro) and late (pot-grown) stages of the plant development, and inoculation also improved tolerance to drought. As far as we know, this is the first report on the effects of *Azospirillum* in *aba*2-1 mutants. The bacteria colonized both the roots and the rosettes of Col-0 as well as *aba*2-1 *Arabidopsis* plants with similar CFU ml^{-1}, although as in previous works (Cassán et al. 2001a, Cassán et al. 2001b, Cohen et al. 2009) the CFU ml^{-1} of *A. brasilense* Sp 245 was again two orders of magnitude superior in roots than in rosettes.

Azospirillum increased the main root length and LR number in both Col-0 and *aba*2-1 plants. It is known that many signals affect root architecture and branching, and plant growth regulators influence LR initiation and growth (Ivanchenko et al. 2008, Negi et al. 2008, Nibau et al. 2008). De Smet et al. (2006) observed that *Arabidopsis* seedlings grown on a medium containing exogenous ABA did not form visible LR clearly. In this study, it is worth noting that roots of *aba*2-1 plants had a larger number and longer LR than Col-0, something previously found by Deak and Malamy (2005). Shkolnik-Inbar and Bar-Zvi1 (2010) observed that the transcription factor ABI4 (ABA-INSENSITIVE 4) mediates ABA and cytokinin inhibition of LR formation via

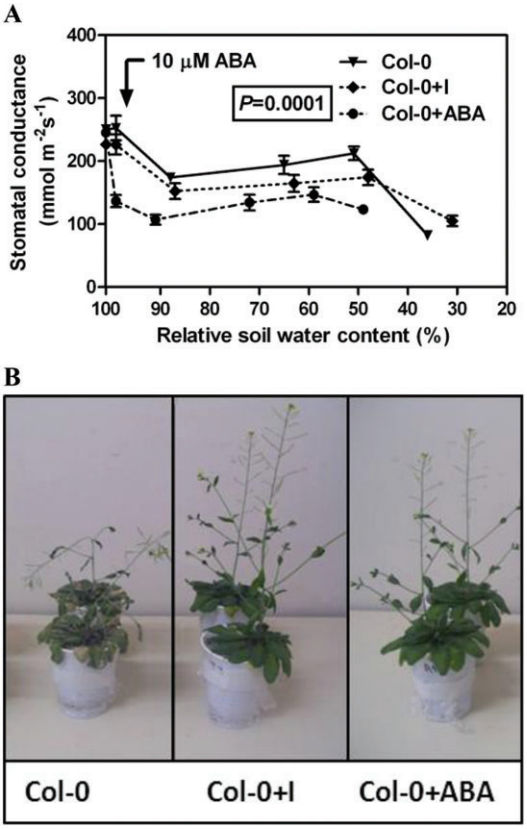

Fig. 4. (A) gs (mmol m^{-2} s^{-1}) under drought conditions as a function of RSWC of the 40-day-old *Arabidopsis thaliana* Col-0 pot-grown plants (control, Col-0), inoculated with *Azospirillum brasilense* Sp 245 (Col-0 + I) and treated with 100 μ*M* ABA (Col-0 + ABA); gs was measured in the middle of the photoperiod (12 h). Each point corresponds to 0 (100% RSWC), 1, 3, 5, 7 and 9 days since the start of the water shortage treatment in 40-day-old Col-0, Col-0 + I and Col-0 + ABA. Values are means ± SE (n = 12). (B) Representative images for each treatment at 9 days after water shortage.

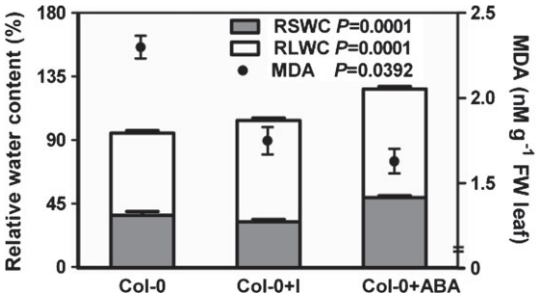

Fig. 5. RSWC, RLWC and MDA (n*M* g^{-1} FW leaf) content in *Arabidopsis thaliana* Col-0 pot-grown plants (control, Col-0), inoculated with *Azospirillum brasilense* Sp 245 (Col-0 + I) and treated with 100 μ*M* ABA (Col-0 + ABA). Determinations were at 7 days after water shortage. Values are means ± SE (n = 12).

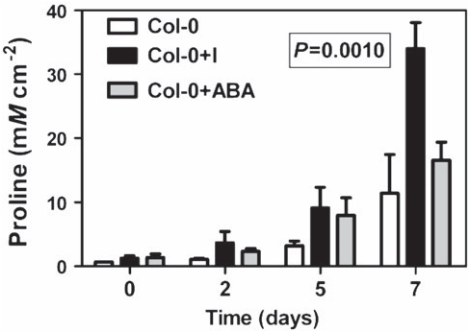

Fig. 6. Proline content (m*M* cm^{-2}) of the 40-day-old *Arabidopsis thaliana* Col-0 pot-grown plants (control, Col-0), inoculated with *Azospirillum brasilense* Sp 245 (Col-0 + I) and treated with 100 μ*M* ABA (Col-0 + ABA). Determinations were at 0, 2, 5 and 7 days since the start of the water shortage treatment. Values are means ± SE (n = 6).

reduction of polar auxin transport and that the resulting decrease in root auxin leads to a reduction in LR development. In preliminary experiments, we observed that fluridone, an inhibitor of carotenoid biosynthesis (and by extension of ABA synthesis), further increased the LR length and number. In this work, we also found that *A. brasilense* inoculation of *aba*2-1 mutants increased those parameters, suggesting that the ABA-signaling pathway participates in this response, though ABA levels in roots were below detection levels. In this respect, the effect of the bacterium-produced ABA-lessening LR may be counteracted by IAA and gibberellin A3 produced by the same microorganism in the plant interaction (Crozier et al. 1988, Bottini et al. 1989), thus taking part in the signaling cascade that changes the root architecture (phytohormone balance). That is, the bacteria increased the number of LR and roots FW in Col-0 and *aba*2-1 plants, suggesting that the effects may be mediated by IAA and GAs, which is confirmed by the fact that *Azospirillum* reversed the phenotype in dwarf mutant rice (Cassán et al. 2001a, Cassán et al. 2001b).

Shoot development, particularly leaf expansion, requires normal levels of endogenous ABA in watered *Arabidopsis* plants. This is caused partly by suppressing ethylene synthesis (and perhaps also sensitivity) and partly by another mechanism that is independent of ethylene (LeNoble et al. 2004).

In the rosettes, we found that *Azospirillum* increased LA and FW as a consequence of root branching that improves the area active in water and nutrient uptake, although the effect may be mere consequence of stimulated growth by hormones. These results are consistent with those found in maize plants inoculated with *A. lipoferum* USA 5b that augmented LA in both watered and water stressed (Cohen et al. 2009). Zhang et al. (2008) reported that *Bacillus subtilis* strain GB03 can stimulate growth of *Arabidopsis* by the emission of

volatile organic compounds and can increase photosynthesis through modulation of ABA signaling in *Arabidopsis*. Xie et al. (2009) and Bresson et al. (2013) found that GB03 resulted in a delayed flowering in *Arabidopsis*. In contrast, we observed with *Azospirillum* a shortened vegetative phase with a different experimental scheme. Recently, Poupin et al. (2013) reported similar results with *Burkholderia phytofirmans* PsJN in *Arabidopsis* plants that presented bigger rosette areas and early flowering times. As can be seen flowering time may be depending more on the bacterium strain itself than on the assembly PGPR–plant variety, probably because of differential modulation in the plant hormonal homeostasis.

Understanding the mechanisms behind PGPR–plant interactions, it is important to improve strategies for the use of these beneficial bacteria in agriculture. Here, we showed that *Azospirillum* would affect the whole life cycle of a plant, accelerating its growth rate and shortening its vegetative period, both effects relevant for most crops. Also, *Azospirillum* increased the different parameters evaluated as both vegetative (rosettes size and DW, carotenoid, Chl, TPC and anthocyanins levels) and reproductive stages (number of inflorescences and flowers, inflorescences DW and seed production).

ABA has been shown to be essential in plant responses to drought. The mutant *aba*2-1 had only 37% of the total ABA measured in the Col-0, but when inoculated, it had higher ABA levels than Col-0. These results confirm that endophytic *A. brasilense* produces ABA per se and/or increases the plant biosynthesis of ABA in both Col-0 and *aba*2-1, suggesting that *Azospirillum* has the enzyme involved in this reaction. The association of plant/bacteria determines higher ABA levels than the sum of plant plus bacteria alone as it was previously shown by Cohen et al. (2008) and more recently by Salomon et al. (2013). These higher ABA levels may prepare the plant to cope better with unfavorable environmental conditions. Plants inoculated with *Azospirillum* were greener than non-inoculated plants, with increases in both photosynthetic and photoprotective pigments. This increment in Chl and, consequently, enhanced photosynthesis, is a well-known response of plants to inoculation with several PGPR (Deka and Dileep 2002, Bashan et al. 2006). Carotenoids were elevated by ABA treatments in field-grown grapevines (Berli et al. 2010) and wheat (Travaglia et al. 2007, 2010), so the implication is that *A. brasilense* could be increased pigment levels in *Arabidopsis* involved in ABA production. Also, TPC and anthocyanins were strongly associated with D + I because these compounds are related with stress conditions (Berli et al. 2010, 2011). The photoprotective role of anthocyanins can be due to either radiation filtering and/or to ROS quenching through the powerful antioxidative capacity (Sperdouli and Moustakas 2012). Additionally, phenolic compounds may also enhance protection against oxidative stress, as they possess chemical structures capable of scavenging free radicals (Blokhina et al. 2002, Berli et al. 2010). In this work, we observed an increased lipid peroxidation in Col-0 plants reflected by MDA content, whereas Col-0 + I or Col-0 + ABA showed less damage, indicating that these plants are protected against the adverse effects of oxidative stress and demonstrating the efficiency of the *Azospirillum* and ABA to induce antioxidative defense mechanisms.

Another consequence of ABA increase in inoculated *Arabidopsis* is water economy. During drought, ABA induces stomatal closure to minimize water loss through transpiration. *Azospirillum* inoculation delayed water losses after cutting rosettes by controlling stomatal closure through increased ABA levels. This result agrees with previous work (Salomon et al. 2013) in which we reported that in in vitro grape plants inoculated with *P. fluorescens* and *B. licheniformis*, ABA content increased (compared to controls) 70- and 40-fold and retarded water losses 4 and 10%, respectively. The gs value is a crucial characteristic that determines plant water status. Although the inoculated plants had greater LA, the gs decreased in these plants and reached the wilting point later than the Col-0, most probably (based on results from determinations at days 30 and 54) because they had more ABA than the non-inoculated. As expected, D caused a marked increase in ABA levels when compared to those watered; however, *Azospirillum* increased the ABA levels under W and D conditions. The highest RLWC found in Col-0 + I plants confirms once again how inoculated plants were able to control water loss, because Col-0 plants had a very low percentage of RLWC. The root system and LA of pot-grown Col-0 + I plants were higher than Col-0. It favored exploration of the whole soil volume of the pot, so suggesting an increased ability to obtain water from the soil under water stress according to the determinations of the RSWC being that all plants were irrigated with the same volume of water. Such ability may be probably related to the presence of aquaporins, as it is known that *Azospirillum*-inoculated barley seedlings stimulate the expression of PIP2;1 (plasma membrane intrinsic protein 2-1), a gene involved in the synthesis of aquaporin (Zawoznik et al. 2011). All these differences in the physiologic response of inoculated plants to drought are in part explained by a better control of stomata closure mediated by ABA, although a more developed root system induced by bacterial IAA and GA production may not be excluded.

The plants inoculated with *Azospirillum,* in plate as well as pots, were less affected than non-inoculated submitted to drought. Another effect of inoculation was the increase of proline levels since irrigation was suspended, and the highest value was recorded at day 7 since water was withheld and before entering wilt. This osmolyte contributes to osmotic adjustment during stress allowing the plant to obtain water even with very low soil water potentials, and it protects the structure of membranes during extreme dehydration (Meloni et al. 2001). Col-0 + ABA plants had higher proline levels than Col-0 until day 5 probably because ABA acts as stress signal. Also, under drought stress, wheat plants inoculated with *Azospirillum* showed an enhanced osmotic adjustment that maintains cell turgor, preventing degenerative processes (Creus et al. 2004). Enhanced proline synthesis in stressed plants was reported in other PGPR such as *Burkholderia* sp., *Arthrobacter* sp. and *Bacillus* sp. (Dodd and Pérez-Alfocea 2012).

In summary, *A. brasilense* stimulated *Arabidopsis* general growth, LR number, proline levels, improved survival, seed yield and RLWC, and decreased gs and RSWC in plants submitted or not to drought. *Azospirillum* also augmented photosynthetic and photoprotective pigments and retarded water losses in correlation with augmented ABA levels. In short, inoculation of *Arabidopsis* with *A. brasilense* enhanced plant biomass and seed yield of watered plants, but it especially had impact on plants submitted to water restrictions, where the harmful effects of drought were ameliorated by inoculation. The parallel enhancements in root surface and ABA levels induced by the bacteria are correlated with a higher sensitivity of inoculated plants to close stomata when experiencing water deficit. However, the increase in photosynthetic and photoprotective compounds and the decrease in MDA levels suggest that *Azospirillum* enhances plant tolerance to drought and seed yield by additional biochemical mechanisms that include phytohormones production, comprising ABA and also osmoprotector compounds such as proline.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 *Arabidopsis thaliana* Col-0 and *aba*2-1, non-inoculated and inoculated plants with *Azospirillum brasilense* Sp 245.

Fig. S2 Roots from 50-day-old *Arabidopsis thaliana* Col-0, inoculated and treated with ABA plants.

Fig. S3 Non-inoculated and inoculated rosettes 42 min after being detached from their root system.

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