Participation of abscisic acid and gibberellins produced by endophytic *Azospirillum* in the alleviation of drought effects in maize

Ana C. Cohen, Claudia N. Travaglia, Rubén Bottini, and Patricia N. Piccoli

Abstract: *Azospirillum* spp. are plant growth promoting bacteria (PGPB) that enhance growth by several mechanisms, including the production of phytohormones such as abscisic acid (ABA), indole-3-acetic acid (IAA), and gibberellins (GAs). Their presence may also alleviate plant water stress. In the present paper, the effects of *Azospirillum lipoferum* in maize (*Zea mays* L.) plants treated with inhibitors of ABA and GA synthesis, fluridone (F) and prohexadione-Ca (P), respectively, and either submitted to drought stress or provided sufficient water, were analysed. Fluridone diminished the growth of plants that had been well watered, in a manner similar to drought, but inoculation with *Azospirillum* completely reversed this effect. The relative water content of the F-treated and drought-stressed plants was significantly lower (even though drought-stressed plants had been allowed to recover for one week), and this effect was completely neutralized by *Azospirillum*. These results were correlated with ABA levels assessed by GC-EIMS. Growth was diminished in drought-submitted plants treated with P, alone or combined with F, even though ABA levels were enhanced, suggesting that GAs produced by the bacterium are also important in stress alleviation. The results suggest that both ABA and GAs contribute to water-stress alleviation of plants by *Azospirillum*.

Key words: abscisic acid, *Azospirillum lipoferum*, drought, gibberellin, maize, PGPB.

Résumé : Les *Azospirillum* spp. constituent des bactéries qui stimulent la croissance des plantes (PGPB) via plusieurs mécanismes incluant la production de phytohormones telles que l'acide abscissique (ABA), l'acide indole-3 acétique (IAA) et les gibbérellines (Gas). Leur présence peut également réduire le stress hydrique. Les auteurs analysent ici les effets du *A. lipoferum* chez des plants de maïs (*Zea mays* L.) traités avec l'inhibiteur de la synthèse de l'ABA et des GAs, le fluridone (F) et le prohexadione de calcium (P) respectivement, en les soumettant soit à un stress hydrique ou à un approvisionnement suffisant en eau. Le Fluridone diminue la croissance des plantes bien approvisionnées en eau tout comme la sécheresse et l'inoculation avec *l'Azospirillum* renverse complètement cet effet. La teneur relative en eau des plantes traitées au F et stressées par la sécheresse est significativement plus faible (bien qu'on ait laissé les plantes stressées par la sécheresse recouvrir pendant une semaine), et cet effet a été complètement neutralisé par l'*Azospirillum*. Ces résultats montrent une corrélation avec les teneurs en ABA, telles que mesurées au GC-EIMS. La croissance diminue chez les plantes soumises à la sécheresse et traitées avec le P, seul ou combiné avec le F, bien que les teneurs en ABA soient stimulées, ce qui suggère que les GAs produits par la bactérie jouent également un rôle important dans l'évitement de la sécheresse. Ces résultats suggèrent que l'ABA aussi bien que les GAs participent à l'évitement de la sécheresse chez les plantes par l'*Azospirillum*.

Mots-clés : acide abscissique, *Azospirillum lipoferum*, sécheresse, gibbérellines, maïs, PGPB.

[Traduit par la Rédaction]

Introduction

Several bacterial genera, called plant growth promoting bacteria (PGPB, Bashan and Holguin 1998), stimulate plant growth and yield in cereals, legumes, and other crops (Okon and Labanderas-González 1994; Glick et al. 2001). Some strains of *Azospirillum lipoferum* and *Azospirillum brasilense* colonize the interior of wheat (Bashan et al. 2004), maize (Lucangeli and Bottini 1996), and rice (Cassán et al. 2001*a*, 2001*b*), and therefore are endophytic. One of the major effects attributed to *Azospirillum* spp. is the augmentation of the physiologically active root surface (Sarig et al. 1988; Steenhoudt and Vanderleyden 2000), and among the mechanisms proposed to explain these beneficial effects is the production of phytohormones by the bacteria (Okon and Labanderas-González 1994; Bloemberg and Lugtenberg 2001; Persello-Cartieaux et al. 2003; Bottini et al. 2004), especially indole-3-acetic acid (IAA, Costacurta and Vanderleyden 1995; Patten and Glick 1996) and gibberellins (GAs, Bottini et al. 1989, 2004; Fulchieri et al. 1993). However, other mechanisms may be considered, such as the effect of nitric oxide, nitrite, enhanced mineral and water uptake,

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Fig. 1. Mean plant length (from the shoot crown to the extreme of the longest leaf) of (A) 45-day-old, well-watered unstressed maize plants, or (B) maize plants submitted to drought treatment (two periods up to development of permanent wilting point). C, control (noninoculated) plants sprayed with water; F, plants sprayed with 60 μmol·L^{-1} fluridone; P, plants sprayed with 800 μmol·L^{-1} prohexadione-Ca^{++}; F + P, plants indicated as FP on graphs were sprayed with both 60 μmol·L^{-1} of fluridone + 800 μmol·L^{-1} of prohexadione-Ca^{++}. Gray bars, control plants; black bars, plants inoculated with *Azospirillum lipoferum* USA 59b (see Materials and methods for details). Lines above bars indicate the standard error of the mean. Different letters indicate a significant difference at $P < 0.05$ according to Fisher's least significant difference test.

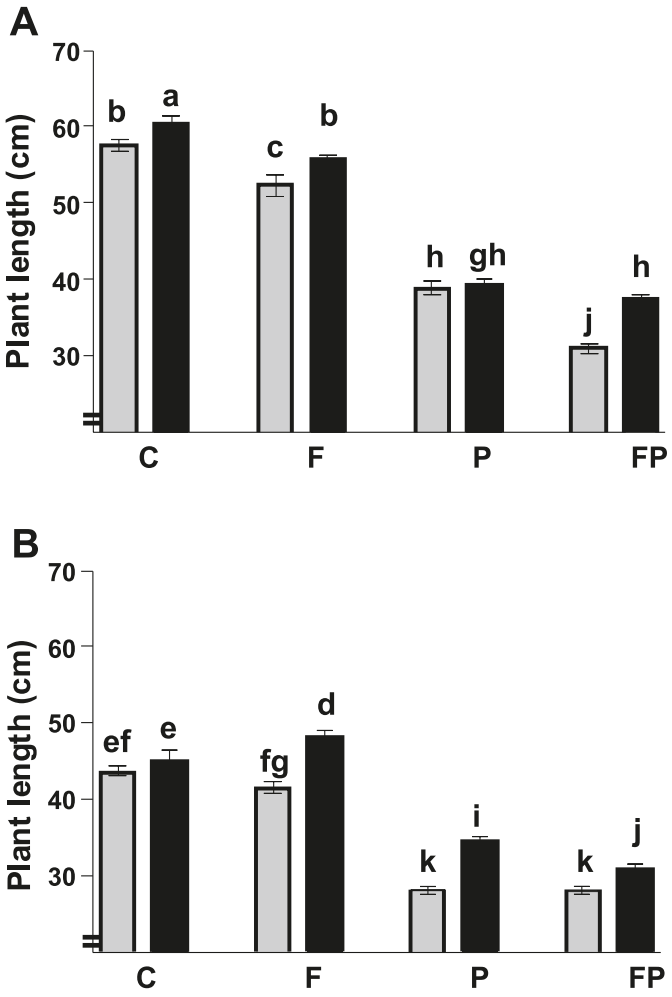
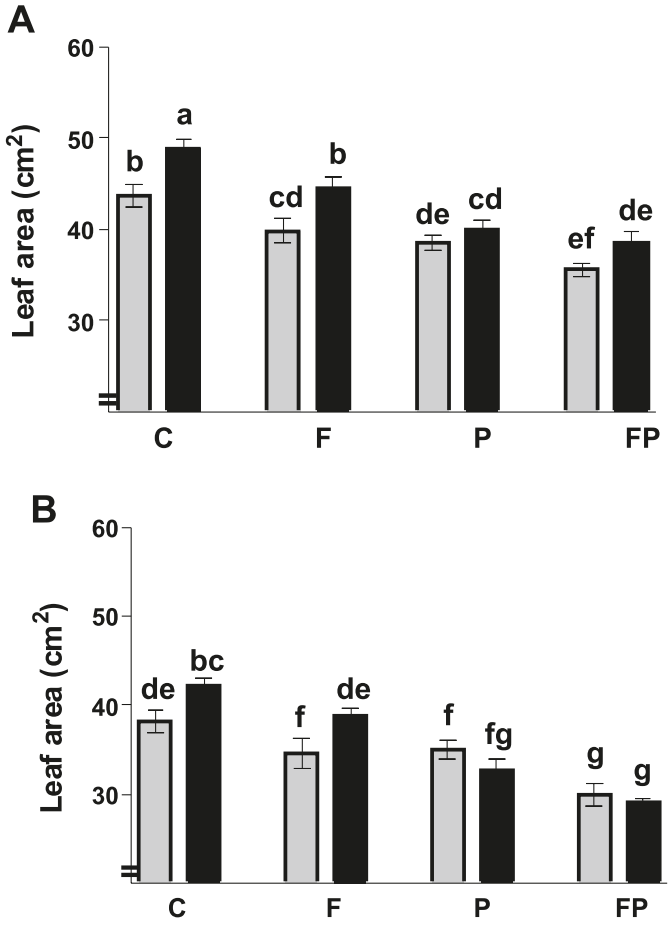


Fig. 2. Mean leaf area of the fourth fully expanded leaves of (A) 45-day-old, well-watered unstressed maize plants, or (B) maize plants submitted to drought treatment (two periods up to development of permanent wilting point). C, control (noninoculated) plants sprayed with water; F, plants sprayed with 60 μmol·L^{-1} of fluridone. P, plants sprayed with 800 μmol·L^{-1} of prohexadione-Ca^{++}; F + P indicated as FP on graphs indicates plants sprayed with both 60 μmol·L^{-1} of fluridone + 800 μmol·L^{-1} of prohexadione-Ca^{++}. Gray bars, control plants; black bars, plants inoculated with *Azospirillum lipoferum* USA 59b (see Materials and methods for details). Lines above bars indicate the standard error of the mean. Different letters indicate a significant difference at $P < 0.05$ according to Fisher's least significant difference test.

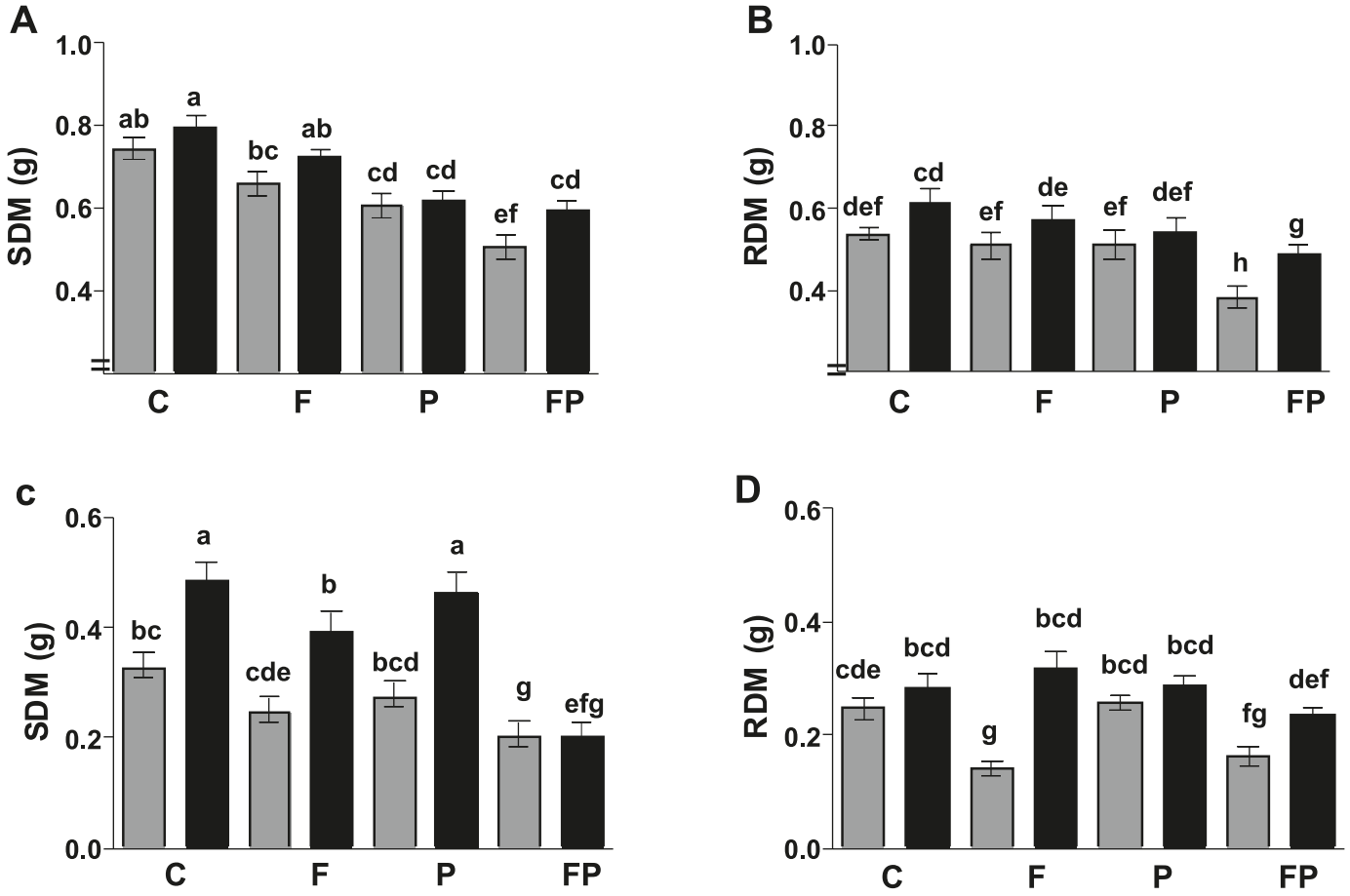


augmented proton extrusion, and nitrogen fixation (Bashan et al. 2004); some of them may be the consequence of root growth stimulation by phytohormones (Bottini et al. 2004).

Drought is one of the main adverse environmental conditions that reduce crop yield worldwide, since biomass production by plants is regulated by water availability. Although plant responses to water deficits are complex and involve coordination in gene expression and its integration with hormones, it has been claimed that the main reaction is the increase in abscisic acid (ABA) biosynthesis and (or) a decrease in ABA breakdown (Bray 2002). Abscisic acid is a phytohormone implicated in mediating stomatal closure (Mansfield et al. 1990; Mac Robbie 1997) and regulating aspects of plant growth and development in the absence of stress (Cheng et al. 2002). Abscisic acid is ubiquitous and produced by higher plants, algae, and fungi (Zeevaart 1999), and it has been recently characterized by full scan mass spectrometry as a by-product of chemically defined cultures of *A. brasilense* Sp 245 (Cohen et al. 2008).

Abscisic acid and GAs have antagonistic roles in many processes. In *Arabidopsis* seedlings, ABA antagonizes growth promotion by GAs (Nemhauser et al. 2006), and plants carrying mutations in the *DELLA* genes (that encode negative regulators of GAs) exhibit altered response to exogenous ABA (Achard et al. 2006). It has been also claimed that drought resistance is acquired by inhibiting GA biosynthesis (Vettakkorumakankav et al. 1999). However, the literature is controversial on this subject, since stress al-

Fig. 3. Mean dry mass of (A) the aerial part of shoots (SDM) and (C) roots (RDM) of 45-day-old, well-watered unstressed maize plants or (B and D) submitted to two drought treatments up to the development of permanent wilting point. C, control plants (noninoculated) sprayed with water; F, plants sprayed with 60 μmol·L^{-1} of fluridone; P, plants sprayed with 800 μmol·L^{-1} of prohexadione-Ca^{++}; F + P plants sprayed with both 60 μmol·L^{-1} of fluridone + 800 μmol·L^{-1} of prohexadione-Ca^{++}. Gray bars, control plants; black bars, plants inoculated with *A. lipoferum* USA 59b. Lines above bars indicate the standard error of the mean. Different letters indicate a significant difference at $P < 0.05$ according to Fisher's least significant difference test.



leviation by *Azospirillum* spp. has also been attributed, at least in part, to GA production (Creus et al. 1997).

Based on the above information, we hypothesized that alleviation of water stress in maize plants by endophytic *Azospirillum* is partially due to bacterially produced ABA. Additionally, GAs produced by the bacteria may hamper ABA effects or, conversely, these GAs may be necessary for an adequate plant response to the stress. The effects of inoculation with *A. lipoferum* was therefore studied in water-stressed maize plants in which ABA and GA synthesis were diminished by inhibitors of their own biosynthetic pathways.

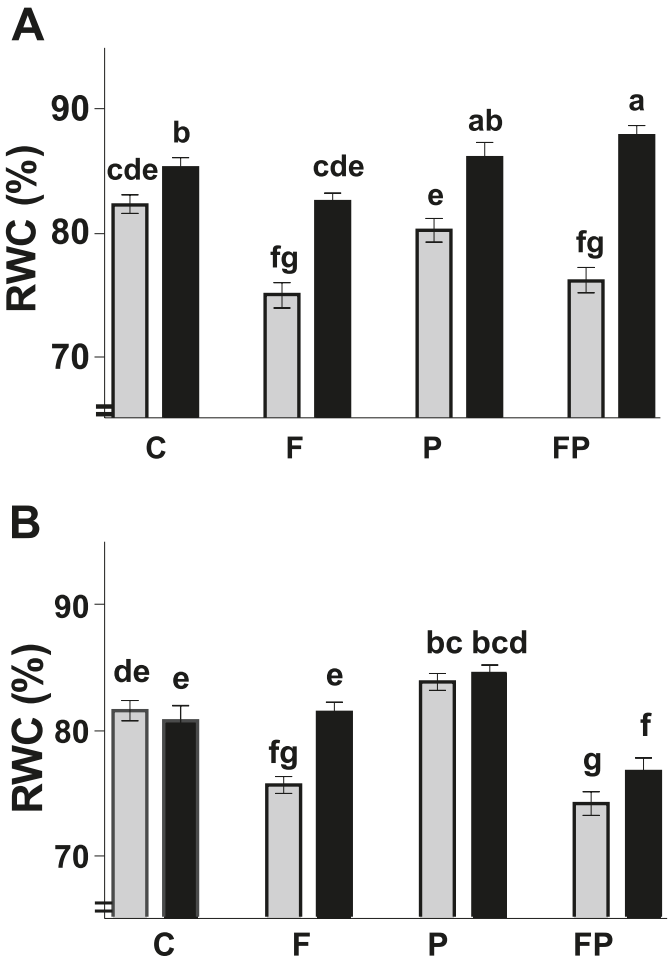
Material and methods

Bacterial cultures, plant material, treatments, and growth conditions

For inoculation purposes, *A. lipoferum* strain USA 59b (ATCC 29707, J. Döbereiner, EMBRAPA, Seropédica, Brasil) was grown in 500 mL flasks with 125 mL of NFb medium, as previously described in Bottini et al. (1989) and Piccoli and Bottini (1994*a*, 1994*b*) with malic acid (5 g·L^{-1}) and NH_4Cl (1.25 g·L^{-1}) as the source for C and N, respectively. The bacteria were cultured in an orbital shaker (80 r·m^{-1}) at 32 °C until reaching an OD_{540} of 1.0 corresponding to a concentration of ca. 10^9 colony forming units (CFU)·mL^{-1} as assessed by growth in NFb agar plates. Bacteria were harvested by centrifugation at 8000*g* for 10 min at 4 °C and the pellet washed with sterile phosphate buffered saline (PBS; KH_2PO_4 6.85 g·L^{-1}, K_2HPO_4 0.5 g·L^{-1}, $MgSO_4$ 0.24 g·L^{-1}, NaCl 8.1 g·L^{-1}, pH = 6.1), centrifuged again, and the pellet resuspended and diluted in the same PBS buffer to obtain a titer of 10^7 CFU·mL^{-1} for further inoculation.

Greenhouse experiments were conducted with seedlings of the maize hybrid Dekalb 696 (AgroUcacha, Río Cuarto, Argentina). The seeds were surface disinfected by soaking in 2% $NaClO_4$ for 5 min and then washed extensively with sterile distilled water. The seeds were imbibed overnight at 4 °C, and then allowed to germinate in Petri dishes on two sterile wet layers of filter paper (Whatman Ashless 41) for 72 h at 25 °C, in darkness. The emerged seedlings were either inoculated with *A. lipoferum* strain USA 59b (10^7 CFU·mL^{-1}) in PBS medium, or PBS alone (controls), and grown for another 24 h at 25 °C. After that, the seedlings were aseptically transferred to 4.5 L plastic pots filled with river sand – garden clay soil (1:1, *v*/*v*), previously sterilized for 10 h at

Fig. 4. Mean relative water content (RWC) in leaves of 45-day-old maize plants, well-watered unstressed plants (A), or submitted to drought treatment (B, two periods up to development of permanent wilting point). C, control (noninoculated) plants sprayed with aqueous solution; F, plants sprayed with 60 μmol·L^{-1} of fluridone; P, plants sprayed with 800 μmol·L^{-1} of prohexadione-Ca^{++}; F + P plants sprayed with both 60 μmol·L^{-1} of fluridone + 800 μmol·L^{-1} of prohexadione-Ca^{++}. Gray bars, control plants; black bars, plants inoculated with *Azospirillum lipoferum* USA 59b (see Materials and methods for details). Lines above bars indicate the standard error of the mean. Different letters indicate a significant difference at $P < 0.05$ according to Fisher's least significant difference test.



180 °C. The plants were watered every 2 d with sterile distilled water, and once a week with sterile Hoagland solution (1/2 strength), to keep the soil water status close to field capacity. For the drought treatment, irrigation was withheld at day 10 after planting for 7 d until there were visible symptoms of the permanent wilting point, and then the plants were rewatered and allowed to recover for 7 d; after another 7 d without irrigation (up to permanent wilting point) plants were normally watered until the experiment ended (another 7 d). The drought treatment, therefore, consisted of two periods of drought. The reason why plants were allowed to recover from desiccation is because we wanted to assess midterm changes/responses in plant length, leaf area, dry mass (DM), and relative water content (RWC), and not the immediate obvious variations produced by the transient lack of turgidity.

All plants were sprayed twice, once at day 10 after planting and once again when the plants had recovered from the first drought treatment, with aqueous solutions of prohexadione-Ca (P, inhibitor of GA biosynthesis, BASF, Linburgerof, Germany), fluridone (F, inhibitor of ABA biosynthesis, as Sonar 42% active ingredient, Dow Chemicals, Buenos Aires, Argentina), alone or as a mixture of both regulators, or with sterile distilled water. Prohexadione-Ca and F were dissolved separately in 96% ethanol, and then diluted to a final concentration of 800 μmol·L^{-1} and 60 μmol·L^{-1}, respectively. The solutions contained 0.005% Tween-20 and 0.2% ethanol. Control plants were treated with sterile distilled water plus 0.005% Tween-20 and 0.2% ethanol.

Pots were arranged in a complete randomized design with 30 replicates for each treatment, with one plant per pot (a total of 480 plants), and surrounded with extra maize pots as borders. Thus, the 16 variants were (*i*) control plants; (*ii*) *A. lipoferum*; (*iii*) 60 μmol·L^{-1} F; (*iv*) 60 μmol·L^{-1} F + *A. lipoferum*; (*v*) 800 μmol·L^{-1} P; (*vi*) 800 μmol·L^{-1} P + *A. lipoferum*; (*vii*) 60 μmol·L^{-1} F + 800 μmol·L^{-1} P; (*viii*) 60 μmol·L^{-1} F + 800 μmol·L^{-1} P + *A. lipoferum*; (*ix*) drought; (*x*) drought + *A. lipoferum*; (*xi*) drought + 60 μmol·L^{-1} F; (*xii*) drought + 60 μmol·L^{-1} F + *A. lipoferum*; (*xiii*) drought + 800 μmol·L^{-1} P; (*xiv*) drought + 800 μmol·L^{-1} P + *A. lipoferum*; (*xv*) drought + 60 μmol·L^{-1} F + 800 μmol·L^{-1} P; (*xvi*) drought + 60 μmol·L^{-1} F + 800 μmol·L^{-1} P + *A. lipoferum*.

Average environmental conditions throughout the 45 d experiment were as follows: day–night cycles of 32–12 °C and RH of 49%–87%, with a PAR of ca. 1800 μmol·cm^{-2}·s^{-1} at noon. The experiment was repeated three times, one experiment per year for three consecutive years, with the results showing the same tendency across the years. However, the results presented are for the last year's experiment since the average results for the three years gave a high SE.

Growth parameters

After 45 d the plants were carefully removed from the sand–soil mixture. The roots were excised and gently washed with tap water to eliminate sand and clay particles and dried with a paper towel to remove excess water. Plant length (the maximum plant length assessed from the shoot crown to the tip of the first fully elongated leaf), leaf area, shoot dry mass (SDM), root dry mass (RDM), number of bacteria in roots and shoots, and leaf relative water content (RWC), were measured. The RWC was calculated according to the method of Kramer (1974) using the following formula: $\mathrm{RWC} = [(F_{wt} - D_{wt})/(\mathrm{SAT}_{wt} - D_{wt})] \times 100$; where F_{wt} is the fresh mass, registered for each leaf; then the saturated leaf mass (SAT_{wt}) was measured after a period of 12 h immersion in distilled water; finally the dry leaf mass (D_{wt}) was obtained after oven-drying the leaves in a ventilated oven at 80 °C until constant mass was reached. Leaf area was quantified measuring the length and width of five leaves separately and calculated as the product of the length and width divided by 2 and multiplied by a correction factor (Pearce et al. 1975, with modifications). The dry mass of the leaf tissue was determined at 80 °C until constant weight values were obtained. The aerial parts of three plants of average size and mass from each treatment were immedi-

Table 1. ABA levels ($ng \cdot g^{-1}$ ± SD of two biological replicates) assessed by GC-MS-SIM with $^2H_6$-ABA as internal standard (see Materials and methods for details) of 45-day-old maize plants treated with water (C), fluridone (F), prohexadione-Ca (P), or a mixture of F + P.

Treatment	C	F	P	F + P
Ww	1996±76ef	1297±156g	2655±137bcd	2866±421bc
S	1846±94f	1061±107g	2895±103bc	3857±660a
I	3013±18b	2280±131def	2457±47cde	2301±751def
I + S	1920±283f	1317±144g	2427±99cde	2819±100bc

Note: Ww, well-watered unstressed plants; S, plants submitted to drought treatment (two periods up to permanent wilting point); I, well-watered unstressed plants inoculated with *A. lipoferum*; S + I, plants submitted to drought treatment and inoculated with *A. lipoferum*. Values followed by the same letter are not significantly different.

ately frozen in liquid nitrogen and stored at –35 °C for ABA and GA analysis.

Bacterial counts in stems and roots

Bacterial counting for roots and aerial parts was performed following the standard plate-counting method described earlier (Piccoli and Bottini 1994*a*, 1994*b*). The root and aerial tissues from both inoculated and noninoculated plants were surface-sterilized by soaking 3 min in 1% $NaClO_4$ and then washed with sterile distilled water to eliminate traces of $NaClO_4$. Then they were soaked with PBS and ground in a sterile mortar and pestle, and resuspended in enough PBS to give a serial dilution of 10^{-1} to 10^{-9}. The number of typical colonies was counted after 5 d of incubation at 30 °C by plating each dilution in NFb medium.

Abscisic acid quantification

The equivalent of 1 g FM of freeze dried aerial parts for each sample was homogenized in a mortar and pestle with liquid nitrogen and extracted with 50 mL of methanol – H_2O – acetic acid (80:19:1) at 4 °C. After 24 h, 20 ng of hexa-deuterated ([$^2H_6$])-ABA (courtesy of J.D. Cohen, University of Minnesota, Minn.) were added for further ABA quantification. Then the sample was filtered through Whatman No. 1 filter paper, and the methanol was evaporated under vacuum at 35 °C. The aqueous residue was adjusted to pH 3.0 and partitioned four times with equal volumes of ethyl acetate saturated with 1% acetic acid. After solvent evaporation, the residue was dissolved in 1 mL of methanol – H_2O – acetic acid (89:10:1), filtered through a 0.45 μm-pore filter and injected in an HPLC (KONIK Model KNK-500, KONIK, Barcelona, Spain) with a C18 reverse phase (μ-Bondapack 3.9 × 300 mm, Waters Associates, www.waters.com) column. The elution was performed at a flow rate of 2 $mL \cdot min^{-1}$ using the following gradient: from 0 to 10 min with 10% methanol in 1% acetic acid; from 10 to 40 min with 10% to 73% methanol in 1% acetic acid; from 40 to 50 min with 73% methanol in 1% acetic acid; from 50 to 60 min with 100% methanol. Fractions with HPLC retention times similar to pure ABA were collected and, after solvent evaporation under vacuum and at room temperature, they were transformed to their methyl-ester (Me) derivatives with 10–20 μL methanol plus 50–100 μL of fresh CH_2N_2 and left for 30 min at room temperature. After solvents had been eliminated under N_2, the samples were dissolved in 5 μL of hexane, and 1 μL was injected in splitless mode in a capillary gas chromatography – electron impact mass spectrometry – selected ion monitoring (GC-EIMS-SIM) system (Hewlett Packard 5890 Series II GC with a capillary direct interface to a 5970B Mass Selective Detector). The GC column was an HP-1 (cross-linked methyl silicone capillary column, 25 m length, 0.22 mm internal diameter, 0.25 μm film thickness) eluted with He (1 $mL \cdot min^{-1}$). The GC temperature program was 100–260 °C at 20 $°C \cdot min^{-1}$, then 10 min at 260 °C. The amount of ABA was calculated by comparison of the peak areas of the two major characteristic ions for the methyl derivative of the deuterated internal standard ([$^2H_6$]-ABAMe, 194–166) versus its nonlabelled counterpart (ABAMe, 190–162); measurements for each treatment were done in duplicate.

Experimental design and statistical analysis

The experimental design consisted in 16 treatments with 30 replicates (one plant per pot). Three plants were employed for hormone analysis and the remaining 27 used for the other determinations. Statistical analysis was performed with the one-way ANOVA and Fisher's multiple tests to discriminate between the averages by the minimum difference with a significance level of $P \leq 0.05$ (Statgraphics).

Results

Inoculation of *Azospirillum lipoferum* USA 59b in maize plants

Bacterial counting assessed as $CFU \cdot mL^{-1}$ reached values of 3.5×10^6 in roots and of 2.3×10^4 in the aerial parts of the inoculated maize plants versus control, noninoculated plants where no bacteria were found, showing that maize plants were well colonized. That is, 45 d post infection *A. lipoferum* had entered and colonized the plants, not only at root level, but also in stems and leaves, although in the latter, bacterial numbers were two orders of magnitude lower, as has been previously found (Cassán et al. 2001*a*, 2001*b*).

Figure 1 shows plant length of 45-day-old maize plants in either well watered (A) or drought (B) treatments. Plant length was reduced ca. 25% by drought, and *Azospirillum* inoculation could not reverse this situation. Both, F and P were effective in reducing plant length of either well watered or droughted plants, but this effect was stronger with P. When both regulators were applied together, the dwarfing effect was additive, especially under well-watered conditions. *Azospirillum* reversed the effect of F, with a length similar to the control in well-watered plants, or even pro-

moted shoot length above controls in plants under drought. *Azospirillum* in general promoted plant length, with statistical significant mean values in F and F + P for well-watered plants, and in F, P, and F + P treatments under drought.

Leaf area was also diminished by drought. Only results of the fourth blade are shown (Figs. 2A and 2B). Inoculation with *A. lipoferum* augmented leaf area in both well watered and drought situations. Both inhibitors of GA and ABA synthesis reduced leaf area, especially when applied together. *Azospirillum* reversed the F-inhibitory effects in both situations. However, the bacteria were not able to counteract the P or F + P effects on leaf area in both well watered and drought conditions.

Both, SDM and RDM were affected by drought treatments (Figs. 3A, 3B, 3C, and 3D). The pattern of SDM response was similar under both watered treatments; the plants treated with F + P had the lowest SDM, which was partially restored by *Azospirillum*, and the bacterium was able to stimulate SDM for all treatments (Figs. 3A and 3B). The F treatment had a detrimental effect for RDM for plants in the drought treatment, either drought alone or drought combined with P, and such effects were completely reversed by *Azospirillum* (Figs. 3C and 3D). However, in well-watered plants, F was not as effective at inhibiting growth as it was at reducing RDM. Again, *Azospirillum* increased RDM in all situations, except for the treatment with F + P under well-watered conditions.

As expected, the RWC of the aerial parts in well-watered plants was similar to the plants submitted to drought (Figs. 4A and 4B), since the stressed plants had a whole week to recover turgidity. The RWC was only affected by F, alone or combined with P, and this effect was completely neutralized by *Azospirillum*, with the exception of the F + P treatment under drought in which reversion was only partial although significant.

The results obtained suggested that reversion of F and P effects by *Azospirillum* on the various parameters measured (Figs. 1–4) may be explained by ABA synthesis by endophytic bacteria. Table 1 shows the results of ABA determinations by GC-EIMS analysis. The ABA levels were similar for both well watered and drought-stressed plants. However, inoculated well watered plants had higher levels of ABA implying that *Azospirillum* is responsible for the increase, although this effect was not noticeable in inoculated drought-stressed plants. As expected, F decreased ABA concentration in all treatments, but again the inoculated well-watered plants had higher levels. Both, inoculated and non-inoculated P-treated plants showed higher ABA levels as compared to F-treated and control plants under both watered conditions. In the F + P treatment ABA levels were also higher as compared with control and F-treated plants.

Discussion

As expected, growth was reduced in drought-stressed maize plants, as assessed by plant length, leaf area, SDW and RDW. Reduction in ABA levels by F affected growth in well-watered plants to a level found in drought-stressed ones. It is known that F has the ability to inhibit the phytoene desaturase activity, a crucial step in the pathway of β-carotene biosynthesis, and consequently reduces ABA biosynthesis (Crozier et al. 2000). The implication of our results is that F-treated plants, which were as short as those submitted to a period of water stress, had partially blocked the pathway for ABA synthesis. Therefore they did not control water loss efficiently, which in turn reduced cell turgidity. Eventually, less turgidity reduced growth and as a consequence also reduced SDW and RDW. Actually, F restrained turgidity in plants grown under both watered conditions, even though the drought-treated were allowed to recover for one week. This implies that well-watered plants were at some point suffering water stress because of poor stomatal control. Since it has been demonstrated that ABA promotes shoot length and leaf area in water-stressed *Ilex paraguariensis* plants (Sansberro et al. 2004), and that turgidity is essential for cell expansion (Acevedo et al. 1971), therefore F may affect cell elongation and in consequence general growth. Endophytic *Azospirillum* was able to restore growth parameters at the level of control unstressed plants. These results suggest that *Azospirillum* might supply the plant with ABA as to cover the deficit produced by F. There was also an additive effect of F with the drought treatment, but again *Azospirillum* reversed (although only partially) such effects. Moreover, the bacterium enhanced growth parameters in well-watered nontreated plants. Fluridone also affected the RWC even in well-watered plants, and there was a reversion of this effect by *Azospirillum*. Taken together, the results suggest that ABA produced by the bacterium may account, at least partially, for the amelioration of growth parameters either in drought-stressed and F-treated plants. The ABA levels assessed by GC-EIMS were enhanced by inoculation with the bacterium in correlation with the reversion by *Azospirillum* of the F-induced growth restraint. This finding suggests that ABA biosynthesis in the bacterium is not significantly affected by F in the *in* vivo plant system. It is important to observe, however, that *Azospirillum* did not enhance ABA levels in the drought-treated plants. This apparent inconsistency may be explained by the fact that in our case ABA was assessed from both shoots and leaves, and the differences in ABA concentration could exist in specific tissues (Christmann et al. 2007). Additionally, in our experiments the drought-treated plants were allowed to recover from the stress and thus the ABA levels may be lowered (if previously raised) as the tissues were recovering turgidity. Likewise, we have recently demonstrated that *Azospirillum* cultured in chemically defined medium produces ABA and inoculation with the bacterium enhanced ABA content in *Arabidopsis thaliana* (Cohen et al. 2008). Additionally, the enhancement of ABA levels in P-treated plants may be the consequence of the GA inhibition that favoured ABA synthesis, since both substances have a common biosynthetic route (Crozier et al. 2000).

A similar pattern as above was observed when P, alone or in combination with F, was used. *Azospirillum* has the capacity to synthesize and to metabolize GAs both in vitro (Bottini et al. 1989; Piccoli and Bottini 1994*a*, 1994*b*; Piccoli et al. 1996, 1997) and in planta (Cassán et al. 2001*a*, 2001*b*; Bottini et al. 2004 and references cited therein). Therefore, part of the reversion found may be a consequence of GA production by bacteria. As well, GA produced by *Azospirillum* partially mitigated the drought effects as has been previously found (Creus et al. 1997; Jacoud et al.

1998). The maize plants stunted by P (this growth regulator diminishes the levels of GAs bioactive on shoot elongation, Rademacher 2000) under both water conditions, did not show a significant reversion by *Azospirillum*, as has been previously reported (Lucangeli and Bottini 1996). In effect, the inhibition by P of *Azospirillum* production of "bioactive" GAs (e.g., GA_1 and GA_3) has been previously demonstrated in vivo (Cassán et al. 2001*b*), since the chemical was able to block the conversion of $^2H_2$-GA_{20} to $^2H_2$-GA_1 in rice *dy* mutant plants (where GA3ox activity is blocked) inoculated with the bacterium.

As well, ABA may have effects on carbohydrate transport and accumulation as found in wheat (Travaglia et al. 2007). It is clear that SDW and RDW of maize plants submitted or not to water stress, and (or) treated with F, were improved by inoculation with *Azospirillum*. In addition, the finding that both inhibitors of GA and ABA synthesis diminished leaf surface, especially when applied together, implies that ABA is also essential for normal expansion growth, probably through the control of stomatal water loss. The promoting effect of *Azospirillum*, which was most noticeable in control well-watered plants, may be partially due to endophytic production of both hormones. The results presented here and in a previous paper (Cohen et al. 2008) demonstrate that ABA levels are increased by inoculation with the bacteria, and it is well known that plant GA levels are also increased by endophytic *Azospirillum* sp. (Fulchieri et al. 1993; Lucangeli and Bottini 1997; Cassán et al. 2001*a*, 2001*b*). One possible observation however, is that both inhibitors used, F and P, may affect the activity of different enzymes apart from the specific action over carotene desaturase and GA3ox, respectively.

In conclusion, the results obtained indicate that among the mechanisms involved in water stress alleviation of plants by *Azospirillum* is the production of stress-type hormones such as ABA (Cohen et al. 2008) along with growth promoters such as auxins (Costacurta and Vanderleyden 1995; Patten and Glick 1996) and GAs (Bottini et al. 2004 and references cited therein). In fact, Christmann et al. (2007) demonstrated that, even though the signal root-to-shoot is of hydraulic nature, ABA plays a crucial role in adjustment of plants to abiotic stress conditions by mediating stomatal closure. Therefore, the implication is that ABA concentration augmented by the bacterium accounts, at least partially, for the improved plant's ability to deal with the stress.

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