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Azospirillum sp. Promotes Root Hair Development in Tomato Plants through a Mechanism that Involves Ethylene

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Abstract

Tomato seeds were inoculated with the plant growth–promoting rhizobacteria *Azospirillum brasilense FT326*, and changes in parameters associated with plant growth were evaluated 15 days after inoculation. *Azospirilla* were localized on roots and within xylematic tissue. An increase in shoot and root fresh weight, main root hair length, and root surface indicated that inoculation with *A. brasilense FT 326* resulted in plant growth improvement. The levels of indole-3-acetic acid (IAA) and ethylene, two of the phytohormones related to plant growth, were higher in inoculated plants. Exogenously supplied ethylene mimicked the effect of inoculation, and the addition of an inhibitor of its synthesis or of its physiological activity completely blocked *A. brasilense* growth promotion. Based on our results, we propose that the process of growth promotion triggered by *A. brasilense* inoculation involves a signaling pathway that has ethylene as a central, positive regulator.

Key words: ACS; auxins; *Azospirillum* sp.; ethylene; IAA; *Lycopersicon esculentum*; plant growth promotion.

Introduction

In natural conditions plants continually interact with soil microorganisms. This interaction exists primarily at the root level and may be harmful, neutral, or beneficial. Regarding the latter, bacteria belonging to the *Azospirillum* genus have been studied as plant growth–promoting rhizobacteria (PGPR; Glick and others 1999). Conceptually, PGPR can affect plant growth and development either directly or indirectly. On the one hand, rhizobacteria may decrease or prevent some effects of phytopathogenic organisms through the production of antibiotics. On the other hand, bacteria either

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directly provide the plant with different compounds or facilitate their incorporation, like nitrogen fixation or phosphorus solubilization. Production and metabolism of phytohormones like auxins, cytokinins, and gibberellins (Bottini and others 2004; Cassán and others 2001) are among the mechanisms used by PGPR to promote plant growth. Inoculation with *Azospirillum* sp. mainly changes growth or morphology of roots, by increasing the number of lateral roots and root hairs; the enlargement of the root surface results in better nutrient uptake and an improved water status that may be the main factor enhancing plant growth (Bottini and others 2004; Lin and others 1983).

Azospirillum sp. are widespread colonizer bacteria that have been isolated from the root surface or the rhizosphere of a wide variety of plant species, mainly gramineous ones, like *Pennisetum purpureum*, *Digitaria decumbens* (Umali-García and others 1980), wheat (Jain and Patriquin 1984), and corn (Gafny and others 1986). However, plants of other families including cotton and tomato can be inoculated (Levanony and Bashan 1991). In particular, inoculated tomato plants (*Lycopersicon esculentum* L.) have shown an increase in dry weights, stem circumference, number of leaves, shoot height, and density and length of root hairs (Bashan and others 1989*a*, 1989*b*; Bashan 1998). Colonization takes place mainly in areas of cell elongation and at the base of radical hairs (Levanony and Bashan 1991).

It is well known that bacteria of the genus *Azospirillum* synthesize auxins, especially indole-3-acetic acid (IAA) (Crozier and others 1988), and a variety of other auxins like indole-3-pyruvic acid, indole-3-butyric acid, and so on (Crozier and others 1988; Costacurta and others 1994; Martínez-Morales and others 2003). These bacterial compounds contribute to the plant auxin ''pool'' in such a way that the effect of *Azospirillum* inoculation can be mimicked by exogenous auxin application (Glick and others 1999). Production of auxins by azospirilla is thus related to the rapid establishment of a bigger root system that stimulates the general growth of the host plant.

On the other hand, ethylene and the regulation of its biosynthesis provide a clear example of how the predominance of one signal over another could promote or inhibit plant growth. As Alonso and Ecker (2001) pointed out, this hormone has multiple effects over plant development, including stress responses, fruit ripening, senescence, development of root hairs, and differentiation of adventitious roots. Ethylene is synthesized from methionine through a pathway that involves two key enzymes: 1-aminocyclopropane-1-carboxylate synthase (ACS, E.C.4.4.1.14) and 1-aminocyclopropane-1-carboxylate oxidase (ACC-O, E.C.1.14.17.4). The first enzyme controls the limiting step of ethylene synthesis, and some genes that codify its synthesis are transcriptionally activated by IAA (Kende 1993). Consequently, it is possible that high concentrations of bacterial IAA could induce the expression of ACS, increasing the synthesis of ethylene and thereby causing root growth. The increase in root volume and surface would be the result of greater development in the length of the primary root, production of adventitious roots, and increasing number and length of root hairs. Nevertheless, the simultaneous existence of several rhizobacterial mechanisms capable of inducing physiological changes in the plant (including production of other phytohormones like gibberellins) makes it difficult to understand the expression of a single character. In this sense, regarding root growth stimulation by *Azospirillum* sp., it is difficult to determine whether the development of roots was a direct consequence of IAA directly, or by IAA-induced plant ethylene, or both, as the increase in the root number on the cuttings also correlates with an increase in ethylene production. Our hypothesis maintains that part of the growth-promoting effect in plant roots inoculated with *Azospirillum* sp. is the result of bacterial modulation of the plant biosynthesis of ethylene via IAA. The objective of the present work was to find direct evidence of this regulation in the *Azospirillum*–tomato plant system as one of the bacterial mechanisms to promote plant growth.

Materials and Methods

Bacterial Strain and Cultivation

The bacterial strain used was *Azospirillum brasilense FT 326*, obtained from EMBRAPA, (Seropédica, Brazil). Bacteria were cultured in nitrogen-free base (NFb) broth medium supplemented with NH_4Cl (1g l^{-1}), with continuous agitation (150 rpm) for 24 h at 33°C. Cells were harvested by centrifugation at 5000 rpm for 15 min, and washed twice with the same volume of 0.9% NaCl. Finally, cells were resuspended and stored in the same solution.

Plant Growth Conditions and Inoculation

Tomato plants (*Lycopersicon esculentum*, cv. Liso Marglove) were used throughout the study. Before being sown, seeds were superficially disinfected by soaking them for 15 min in a 30% sodium hypochlorite solution containing 0.1% Triton X-100, followed by two washes with sterile water. The seeds

were aseptically transferred to 170 ml flasks (three seeds per flask) containing agarized Hoagland medium and maintained at 25 ± 2°C in a Sanyo MRL-350 culture chamber with a 12 h photoperiod. Once roots emerged, seedlings were inoculated with 50 µl of the bacterial suspension containing 2×10^5 colony-forming units (CFU), and incubation continued over a 15-day period. Plant material was kept under sterile conditions, ensuring proper gas diffusion.

Treatments

Exogenous ethylene was supplied to developing seeds by incorporating 50 µM of the ethylene-releasing compound 2-chloroethylphosphonic acid (ethephon) into the growth medium. The effect of 1-methylcyclopropene (1-MCP), an ethylene antagonist, was analyzed to investigate the involvement of ethylene in the *Azospirillum* growth-promotion mechanism. Flasks containing 24 h inoculated seedlings were sealed, and a solution of 1-MCP was injected into the head space. The amount of 1-MCP injected was calculated to yield a gas phase concentration of 125 nl l^{-1}. After 7 days at 30°C, a second dose of 1-MCP was injected, and incubation was continued for an additional 8-day period. Controls were run under the same conditions but in the absence of 1-MCP.

To test the effect of 1-MCP on bacterial growth, *Azospirillum* was inoculated in 10 ml flasks containing NFb semisolid medium, and 1-MCP was injected in the same way as for plant treatment. The effect of 1-MCP on bacterial growth was assessed after 48 h of culture.

Plant Growth–promoting Capacity of *A. brasilense FT 326*

After incubation under the conditions indicated for each experiment, the following parameters were analyzed to evaluate plant growth promotion: (1) fresh weight (FW) in shoots and roots, (2) shoot height, (3) main root length, and (4) root surface. The latter was measured as described by Ansari and others (1995).

Histological Preparations for Microscopic Observation

Plants randomly withdrawn from each treatment were fixed in a solution containing formaldehyde, ethanol, and acetic acid (Cutler 1978) until they were used for microscopic observation. For the low-magnification observation, terminal fragments of roots were stained with methylene blue. Segments of entire roots and histological cuts of stems and roots were observed with electronic sweeping environment microscopy (ESEM). Using ESEM allowed us to locate the microorganism in different tissues and also to determine the average root hair's length using the Image Tool 3.0 software.

Biochemical Determinations

The amount of IAA in shoots and roots of inoculated and control plants was measured under the conditions specified below. Tissues from five plants were homogenized with liquid nitrogen and the resulting powder suspended in 200 ml of a methanol:water solution (4:1 v/v) containing 0.5 % polyvinylpyrrolidone (PVP) and kept at 4°C overnight. After filtration, the solid residue was extracted again and filtered. Filtrates were combined and methanol evaporated under reduced pressure. The aqueous phase was adjusted to pH 2.5 with acetic acid and partitioned four times with the same volume of 1% [v/v] acetic acid–saturated ethyl acetate. The acidic ethyl acetate extract was evaporated and the residue suspended in acetic acid:methanol:water (1:10:89), filtered through a 0.45 µm nitrocellulose membrane and injected into a high-performance liquid chromatography (HPLC) system (KNK-500, Konic Inc., Barcelona) with a C18 reverse phase column (µBondapack, 300 3 3.9 mm, Waters Associates, Milford, MA). Elution was at 2 ml min^{-1} using a gradient of 10% to 73% (v/v) methanol in 1% (v/v) acetic acid over 30 min. The fractions corresponding with the retention times of the internal standard [$^2H_6$]-IAA were collected, evaporated, and assessed by capillary GC-SIM-MS as described by Chen and others (1988). Determination of IAA from bacteria was performed according to the procedure described by Crozier and others (1988). Briefly, 30 ml of a fresh culture of *A. brasilense* (10^7 CFU ml^{-1}) in NFb broth (tryptophan-free) were sonicated (2 × 15 s, output 40 W) and the extract centrifuged at 2000 × *g* for 20 min. The supernatant was adjusted to pH 2.5. Partitioning and processing of samples and IAA measurement by GC-SIM-MS were performed as described above.

Ethylene production from plants grown in the conditions described, and from bacteria, was measured. Shoot segments (five fragments from each plant, each about 1 cm long) were transferred to 10 ml flasks containing 1 ml of a 50 mM sodium phosphate buffer (pH 6.8). The flasks were hermetically sealed with a rubber stopper, and samples were incubated at 30°C for 4 h. The same procedure was used to measure ethylene released for each root of the same plants. For the bacteria-produced ethylene measurement, 0.1 ml of NFb broth culture

(10^7 CFU ml^{-1}) was aseptically injected into 5 ml of semisolid NFb medium contained in a 10 ml flask. After they were sealed, flasks were incubated in darkness for 24 h at 30°C; 1 ml of the head space gas of all samples was withdrawn, and ethylene was quantified in a gas chromatograph (Hewlett Packard 5890 Series II) fitted with a flame ionization detector (FID) and a stainless-steel Porapak N column (3.2 mm × 2 m; 80/100 mesh). The injector, oven, and detector temperatures were 110°C, 90°C, and 250°C, respectively; N_2 was used as the carrier gas; linear gas velocity was 4.5 cm s^{-1}. Finally, the fresh weight of shoot segments was determined.

1-Aminocyclopropane-1-carboxylate Synthase Activity Measurement

Shoots and roots from 15 plants withdrawn from each treatment were separately ground in liquid nitrogen, and the resulting powder was suspended in 500 mM potassium phosphate buffer (pH 8.5) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% 2-mercaptoethanol, and 10 μM pyridoxal phosphate. The homogenate was filtered through cheese cloth and centrifuged at 12,000 rpm for 15 minutes. The supernatant was centrifuged at 36,000 × *g* for 1 h, and the resulting supernatant was used as a source of enzyme. The enzymatic activity was measured as described by Yip and others (1991). Briefly, the assay was performed in 500 μl final volume of a mixture containing 50 mM phosphate buffer (pH 8.5), 10 μM pyridoxal phosphate, and 200 μM S-adenosyl-L-methionine. After incubation for 15 min at 30°C, the ACC formed was converted to ethylene and then measured by gas chromatography (Lizada and Yang 1979).

Western Blot Analysis

Proteins from the shoot and root 36,000 × *g* supernatants were resolved by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970) and transferred to nitrocellulose membranes by semi-dry electroblotting. The blots were blocked with 5% nonfat dried milk and incubated overnight with a polyclonal anti *L. esculentum* ACS2 antibody (Rottmann and others 1991). The bounded antibody was detected with anti-rabbit IgG conjugated to alkaline phosphatase.

Protein Determination

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Endophytism Evaluation

The method described by Döbereiner and others (1995) was applied to evaluate the number of bacteria inside shoots and roots. Plants were sampled 15 days after inoculation (15 DAI). Tissue samples from shoots and roots (1 g) were surface-sterilized by immersion in 1% Chloramine-T for 15 min and washed twice for 10 min with sterile water and once with sterile saline. Tissue was then macerated in saline and the number of CFU was determined by serial dilution plating on Congo Red nutrient agar (Rodríguez-Cáceres 1982). Controls were performed with uninoculated plants.

Experimental Design and Statistical Analysis

A complete hazard block design was used (DBCA), each block consisting of a series of equally independent treatments (with 10 individuals each). When the plants were cultured in the presence of 1-MCP, the experimental design used was a factorial with two factors and two levels each. The results were analyzed using the statistical software InfoStat.

Results

Fresh weights of roots and shoots, shoot heights, and main root lengths in plants inoculated with *A. brasilense* were assessed at 15 days after inoculation (DAI). As shown in Figure 1, the fresh weights of shoot and root tissue from inoculated plants were about 4% and 30% higher, respectively, than comparable weights of control plants. A small but statistically significant (about 20%) increase in shoot height was also observed. No significant difference was observed in main root length of inoculated plants.

A direct relationship between the growth-promoting capability of *Azospirillum* and bacterial auxin secretion has been suggested by several authors (Glick and others 1999; Patten and Glick 2002). We evaluated the level of the IAA pool (for example, IAA synthesized by the plant plus IAA contributed by the bacteria) in shoot and root tissues from plants at 15 DAI. The results, summarized in Table 1, show that the amount of IAA in shoots and roots increased upon *Azospirillum* inoculation (7-and 19-fold increase, respectively). To confirm that *A. brasilense FT 326* is indeed an IAA producer, the amount of phytohormone generated by the bacteria was evaluated. Cells harvested from a 30 ml NFb broth culture (10^7 CFU ml^{-1}) rendered 66.13 μg l^{-1} of IAA. Taken together, these results indicate that

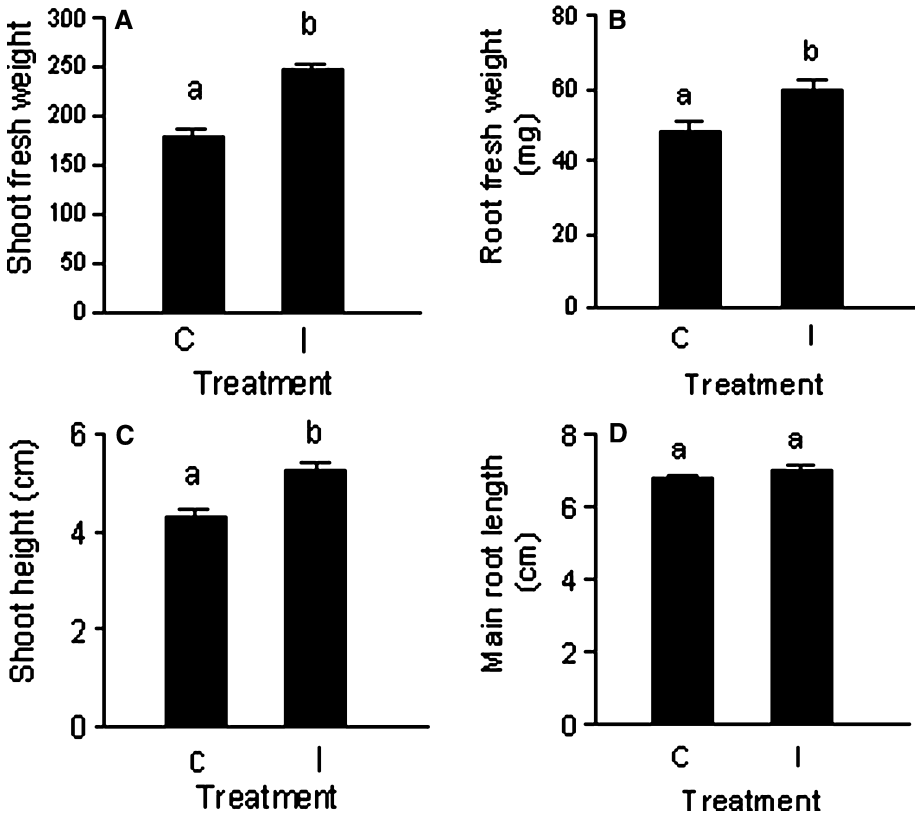

Figure 1. Effect of *A. brasilense FT 326* inoculation on tomato shoot fresh weight (**A**), root fresh weight (**B**), shoot height (**C**), and main root length (**D**). Control plants, C; inoculated plants, I. Error bars represent means ± SE (n = 20). Different letters for each parameter mean significant differences for $p \leq 0.05$ (Tuckey test).

Table 1. IAA Content in Tomato Plants Inoculated with *A. brasilense FT 326*

	IAA μg g DW^{-1}	
Treatment	Shoot	Root
Control	0.95	1.01
A. *brasilense FT 326*	7.06	18.96

Note: Results represent the mean of two experiment with n = 5.
IAA levels were determined in shoots and roots of tomato plants at 15 days of incubation (DAI).

even though we cannot discriminate between IAA generated by the plant and that contributed by *Azospirillum*, the available phytohormone is notably higher in the inoculated plants.

Because IAA is a positive regulator of ethylene synthesis (Abeles and others 1992; Kende 1993), and because this phytohormone is also thought to be involved in mediating the PGPR interaction with plants (Glick and others 1999; Mayak and others 1999; Alonso and Ecker 2001), we assessed the endogenous production of ethylene in *Azospirillum*-inoculated tomato plants. Data in Figure 2 show that inoculation promoted a 40% increase in ethylene production in shoots; ethylene released by roots was below the detection level of the analytical method. To further investigate the biochemical aspects of the process, we measured the activity of ACS, the enzyme responsible for the synthesis of the ethylene precursor ACC. As shown in Figure 3A, a rise in the activity of ACS paralleled the increase in ethylene levels in shoot and root tissues (27% and 100%, respectively). In the case of shoots, an increase in the amount of the enzymatic protein was also detected (Figure 3B). The scarce amount of protein in the root extract hampered the immunoblot analysis in the fraction.

If the rise in the ethylene level is responsible for the changes observed in inoculated tomato plants, an exogenous supply of the hormone should create the same effect. To test this hypothesis, the ethylene-releasing compound ethephon was added to the culture medium and its effect was compared with that of the *Azospirillum* inoculation. Ethephon was assayed at 1, 10, 50, and 100 μM. Because 50 and 100 μM concentrations yielded the same effect, 50 μM ethephon was routinely used. As shown in Table 2, last row, the root surface increased 40% and 16% over controls upon inoculation and ethephon treatment, respectively. No significant differences were evident between ethephon treatments and controls in the rest of the parameters evaluated.

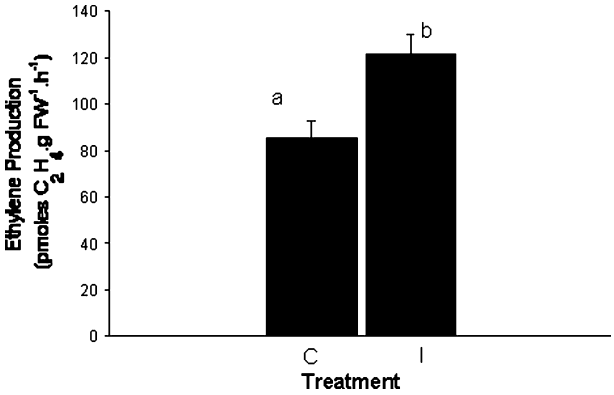

Figure 2. Ethylene production in tomato plants. The amount of ethylene released by shoots from control and inoculated plants was measured as stated in *Materials and Methods*. Control plants, C; inoculated plants, I. The results are the mean ± SE of three separate experiments, with $n = 20$. Different letters mean significant differences for $p \leq 0.05$ (Tuckey test).

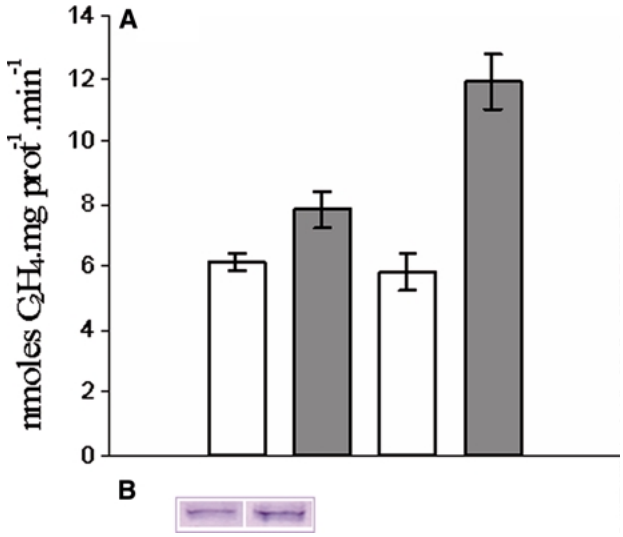

Figure 3. 1-Aminocyclopropane-1-carboxylate synthase (ACS) in tomato plants. **A**. ACS activity measured in the S-100 fractions from shoots and roots as described in *Materials and Methods*. White bars, control plants; gray bars, inoculated plants. **B**. Immunoblot of the shoot S-100 fraction reacted with the Le-ACS2 antibody. Error bars represent means ± SE ($n = 3$).

These results are in line with those from the optic and ESEM observation shown in Figure 4A and 4B. It is noteworthy that, besides depicting a higher amount of hairs over the control, inoculated and ethephon-treated plants also show a different distribution of the hairs along the root (Figure 4A). In the microphotograph shown in Figure 4B, an amplified view of fragments from base to tip is shown; it is evident that the root hairs in ethephon-treated plants concentrated in a zone close to the tip and are absolutely absent in the rest of the root. In the control plants, a lower density of hair development was observed, localized about 2 mm above the tip. The presence of bacteria on the surface of inoculated plants was also detected (see Figure 4B, arrows). Hair length was also measured in samples from the two treatments and from the control plants. As shown in Figure 5, inoculated and ethephon-treated plant root hairs are longer than those in the control.

Viability of bacteria in internal tissues of inoculated plants was confirmed; values estimated were 2.5 10^4 CFU g^{-1} FW for shoots and 3 10^6 CFU g^{-1} FW for roots. No bacteria could be isolated from uninoculated control plants. In addition, as shown in Figure 6, *Azospirilla* were also localized within the xylematic tissue of inoculated plants at 15 DAI.

To establish whether ethylene mediates the signaling pathway leading to *Azospirillum* promotion of tomato plant growth, inoculation experiments were carried out in the presence of 1-MCP, which blocks ethylene binding to its receptor (Sisler and others 1999). The results are summarized in Table 3. As shown, the effect of inoculation is mainly evident on root FW and root surface area (around 98% and 68% enhancement respectively) and, in both, the presence of the ethylene antagonist had a blocking effect. Figure 7 clearly illustrates the effect of 1-MCP on the *Azospirillum*-promoted root hair development. It is also evident from the analysis of data in Table 3 that shoot FW and root FW are lower than those reported in Table 2. We hypothesize that the condition in which the plants were grown in this experiment (sealed flasks) imposes a stressful situation that could be responsible for those differences.

The possible influence of 1-MCP on bacterial growth was assessed as stated in *Materials and Methods*, and no effect of the drug was detected (data not shown). The fact that the blocking of ethylene binding to the receptor abolished the capability of *Azospirillum* to improve tomato plant growth strongly suggests that the process depends on signal pathway(s) involving ethylene.

Discussion

In this article we have addressed the effect of *A. brasilense* inoculation on tomato plant growth promotion. Our results indicate that inoculation of tomato seedlings with *A. brasilense FT 326* promotes development of plants, as revealed by increased

Table 2. Effect of Ethephon Treatment of Tomato Seedlings on Development of Plants

	Control	Inoculated	Ethephon treated treated
Shoot FW (mg)	179.1 ± 9.2[b]	247.5 ± 6.1[a]	188.7 ± 15.6[b]
Root FW (mg)	48.15 ± 2.7[b]	59.72 ± 2.6[a]	50.35 ± 3.2[b]
Shoot height (cm)	4.3 ± 0.2[b]	5.3 ± 0.1[a]	4.4 ± 0.1[b]
Main root length (cm)	6.77 ± 0.1[a]	6.95 ± 0.2[a]	6.6 ± 0.1[a]
Root surface (cm^2)	10.7 ± 0.3[c]	15.2 ± 0.3[a]	12.5 ± 0.5[b]

Note: Numbers represent means ± SE (n =12). Repetition of the same superscript letter within a row indicates no significant differences between treatments for $p \leq 0.05$ (t-test). 50 μM ethephon was incorporated into the culture medium, and the effects were evaluated at 15 DAI.
FW: fresh weight.

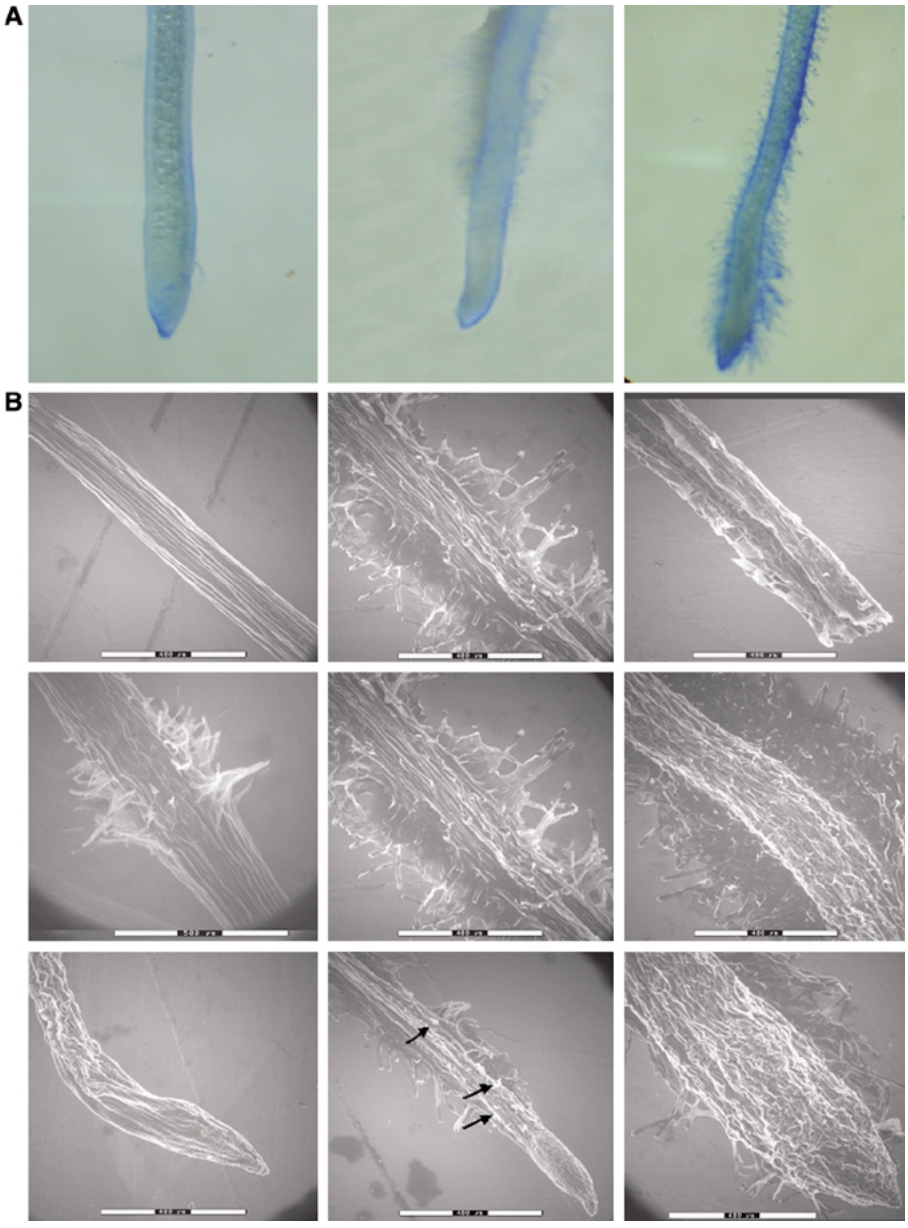

Figure 4. Root hairs of 15-day tomato plants. **A**. Light micrographs of root tips stained with methylene blue. (left) control plants; (center) *Azospirillum*-inoculated plants; (right) ethephon-treated plants. Magnification 20×. **B**. Electronic sweeping environment microscopy (ESEM) showing, from top to bottom, basal segments, medium segments, and tips of 15-day tomato roots. (left) Control plants; (center) *Azospirillum*-inoculated plants; (right) ethephon-treated plants. **A**. magnification 115×.

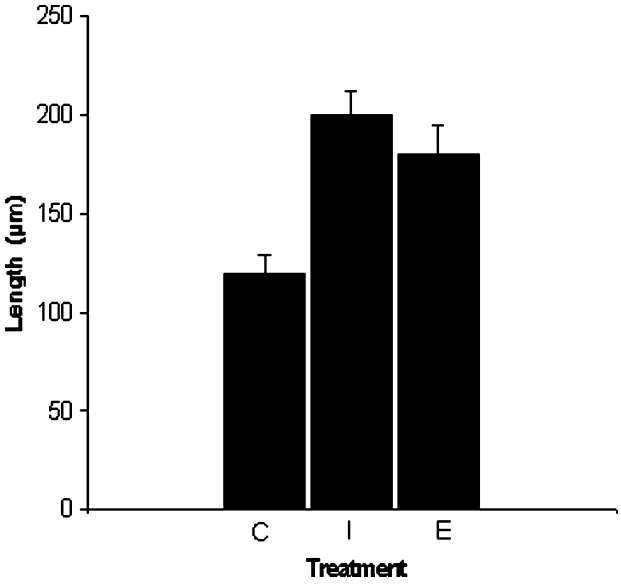

Figure 5. Effect of *A. brasilense FT 326* inoculation and ethephon on hair length of tomato plants. Hair length was measured in samples of plants shown in Figure 4. C, control plants; I, inoculated plants; E, ethephon-treated plants. Error bars represent means ± SE (n = 15). Root hair length was determined using the Image-Tool 3.0 program.

shoot and root biomass, shoot height, and total root surface. These results agree with those previously reported by Bashan (1998).

To investigate further the biochemical mechanism at play in the plant–bacteria interaction we assessed the levels of IAA and ethylene, two hormones seemingly active in growth promotion, especially in root development (Nordstrom and Eliasson 1984; Riov and Yang 1989). The IAA content in plants increased strongly in response to inoculation. Because *A. brasilense FT 326* is an IAA producer, the measured hormone level is the pool contributed to in unknown proportions by the bacteria and the plant. Such a rise in the hormone level is expected to have important consequences on plant physiology. On the one hand, IAA can trigger, *per se*, biochemical signals leading to plant growth improvement, and, on the other, it can stimulate ethylene production through the activation of the ACS (Abeles and others 1992; Kende 1993). Our results have shown that, in fact, the ethylene level is higher in inoculated plants and, in addition, exogenously supplied ethylene mimicked inoculation by increasing root surface area and root hair length (Figure 2, Table 2, and Figure 5). We have also shown that ACS activity increased in shoot and root tissues upon inoculation, the change being more relevant in root tissues. Nevertheless, an increase in ACS expression could be detected in shoot tissue. Taken together, the results in Figure 3 strongly suggest that ACS expression and/or activity are modulated in the process.

The optic and ESEM observations of inoculated and ethephon-treated plants have also shown that, in addition to the change in hair density, the distribution of root hairs along the main root appeared to be altered in the latter. It should be noted that in this case, the rise in ethylene production is dissociated from the increase in IAA concentration; because auxins also seem to have a role in the mechanisms leading to root hair development, it is possible that the alteration in the IAA/ethylene balance is responsible for the abnormal location of root hairs.

Experiments carried out in the presence of 1-MCP, an inhibitor of ethylene physiological activity, showed that ethylene is a positive regulator of hair root development and consequently of root surface increase.

The involvement of auxins and ethylene as important regulators of plant growth and development has been widely studied (Glick and others 1999). As for root elongation, elevated ethylene concentrations have been proven inhibitory (Jackson 1991). *Arabidopsis* has served as a useful model for biochemical, genetic, and physiological studies concerning the positive role of both phytohormones on root hair development (Pitts and others 1998; Tanimoto and others 1995).

The relevance of auxins and ethylene as mediators of the PGPR-plant interaction has also been the focus of many studies (Glick and others 1994*a*, 1994*b*; Glick 1995; Xie and others 1996; Patten and Glick 2002). Based on the results of several studies on the mechanisms of interaction between *Pseudomonas* sp. and agronomics crops, a model has been proposed in which the main role of the bacteria is to modulate the ethylene level in the plant to relieve the inhibitory effect of a high hormone level (Glick and others 1998). The current concentration of ethylene in the plant would be the result of the balance between its synthesis, stimulated by a higher level of IAA promoted by the PGPR and its sequestration through the activity of the bacterial ACC deaminase. According to this model, the capability of bacteria to act as a PGPR, as far as root length is concerned, would depend on the presence of ACC deaminase in the microorganism. Because *Azospirillum* sp. lacks ACC deaminase (Kende 1993), this model seems, at first glance, inappropriate in the discussion of the results in this work.

We found neither elongation nor inhibition of the tomato plant root. In our case the effects of

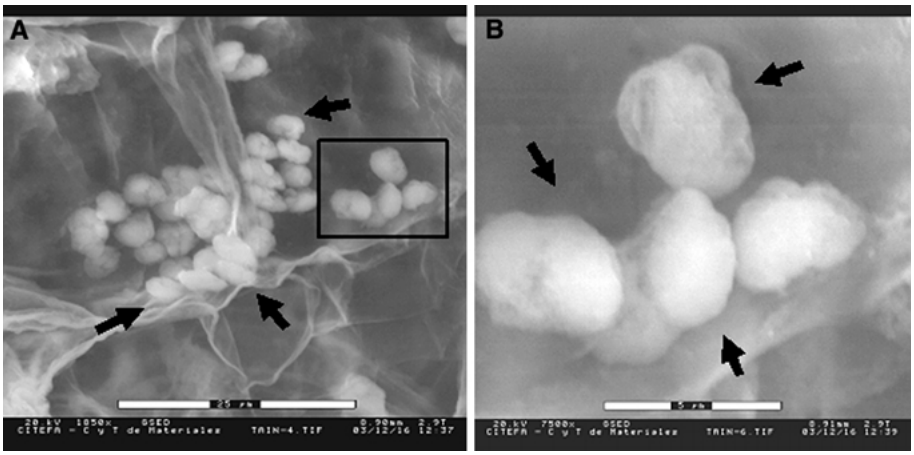

Figure 6. Electronic sweeping environment microscopy (ESEM) image of shoots. **A**. Image of tomato plant shoot at 15 DAI. **B**. Amplified view of boxed area in **A**. Arrows point to the presence of bacteria inside the tissue. Magnification 2500×.

Table 3. Effect of 1-MCP on the Growth Parameters of Tomato Plants Inoculated with *A.brasilense FT 326*

	Treatment	None	+1-MCP
Shoot FW (mg)	C	96.68 ± 6.5[a]	91.08 ± 6.0[a]
	I	92.0 ± 7.0[a]	97.5 ± 12.1[a]
Root FW (mg)	C	21.17 ± 1.69[b]	20.66 ± 2.29[b]
	I	42.03 ± 6.06[a]	29.33 ± 3.69[a,b]
Shoot Height (cm)	C	4.91 ± 0.14[a]	4.58 ± 0.18[a]
	I	4.71 ± 0.16[a]	4.51 ± 0.26[a]
Main Root Length (cm)	C	8.95 ± 0.20[a]	9.01 ± 0.26[a]
	I	8.32 ± 0.28[a]	8.55 ± 0.27[a]
Root Surface (cm^2)	C	15.40 ± 0.99[c]	18.85 ± 0.92[b,c]
	I	26.61 ± 1.0[a]	22.27 ± 0.85[b]

Note: Numbers represent means ± SE (n = 12). Different superscript letters within a row indicate mean significant differences for $p \leq 0.05$ (Tukey test).
C: control; I: inoculated.

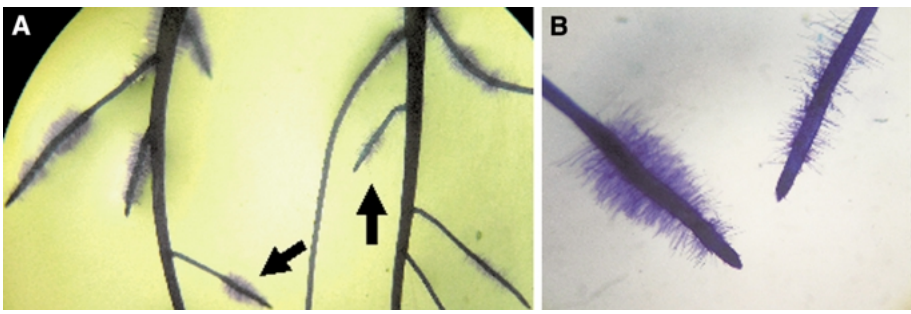

Figure 7. Blocking effect of 1-MCP on *Azospirillum* promoted root hair development. **A**. Roots of tomato plants at 15 DAI in the absence (left) and in the presence (right) of 1-MCP. Magnification 10 ×. **B**, Amplified view of the root tips shown by arrows in **A**. Magnification 40×.

inoculation with *A. brasilense* were mainly detected in root hair density and shoot development of plants and undoubtedly correlated with a rise in ethylene level. It could be hypothesized that, for tomato plants, inoculation with *A. brasilense FT 326* provides an ethylene concentration sufficient to promote root hair development but not to inhibit main root development. This hypothesis would explain why even though *Azospirillum* lacks ACC deaminase activity, it behaves as a PGPR.

Although more studies are necessary to gain insight into the biochemical mechanisms involved in the tomato-*Azospirillum* interaction, our results provide strong evidence that ethylene is an intermediate in a signaling pathway leading to improved root hair density in tomato plants inoculated with *A. brasilense*. To our knowledge, this is the first report on ethylene positively regulating root hair development in response to PGPR inoculation. Because strong root systems play an essential role in

nutrient uptake improving mineral nutrition of plants, *Azospirillum* inoculation may be an important tool to be used as a suitable biofertilizer.

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