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ARABIDOPSIS THALIANA AS A MODEL SYSTEM FOR THE STUDY OF THE EFFECT OF INOCULATION BY *AZOSPIRILLUM BRASILENSE* Sp-245 ON ROOT HAIR GROWTH

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Summary-Seedlings of *Arabidopsis thaliana,* Columbia ecotype, were used *in vitro* to analyze root hair growth response to inoculation by the plant-growth-promoting rhizobacterium, *Azospirillum brasilense* Sp-245. Root hair length was measured at physiologically-identical stages of root growth. In seedlings inoculated with *A. brasilense,* root hairs were more than twice the length of those of the non-inoculated control. This effect was consistent, reproducible and independent of N or C sources in the growth medium (although root growth was significantly suppressed in C-starved seedlings). Root hair growth promotion was similar in media with or without 1 % sucrose. Although root hair length of non-inoculated seedlings growing in the presence of various amounts of potassium nitrate (0-20 mM) was affected, it was significantly lower than that caused by inoculation with *A. brasilense.* We propose that *A. brasilense* inoculation affects the size of individual root hairs of *Arabidopsis* plants *in vitro.*

INTRODUCTION

Free-living associative diazotroph bacteria have been considered possible agents for the improvement of plant growth. However, the mechanisms involved have yet to be clarified in many cases (Döbereiner, 1989; Michiels *et al.*, 1989; Bashan and Levanony, 1990). One of these bacteria, *Azospirillum,* can associate and enhance plant growth of Poaceae (Gramineae) and also Solanaceae (Hadas and Okon, 1987; Bashan *et al.,* 1989a, b), Fabaceae (Bashan *et al.*, 1990; Gamo and Alm, 1991), Cucurbitaceae (Gamo, 1991), Cactaceae (Mascarua-Esparza *et al.*, 1988; Puente and Bashan, 1993), Chenopodiaceae (Saha *et al.,* 1985; Gamo and Ahn, 1991), Convolvulaceae (Crossman and Hill, 1987), Fagaceae (Zaady *et al.,* 1993) and Brassicaceae (Saha *et al.*, 1985; Rao *et al.*, 1990; Gamo and Alm, 1991). Since it appears that the plant-*Azospirillum* association is not bacteria-plant specific (Bashan *et al.*, 1989a), it should be possible to develop a simple model for research into mechanisms involved in positive plant responses to inoculation. One such model system is the plant *Arabidopsis thaliana* (Brassicaceae) which has advantages such as small size, simple structure, simple genetic organization and extensive literature defining many aspects of the plant growth and morphogenesis of this plant under experimental conditions (Bowman *et al.,* 1988; Schiefelbein and Benfey, 1991).

Two components in the medium for growing *Arabidopsis in vitro* can affect the establishment of a plant-bacteria association, namely N and C sources. The N concentration in the media affects the N metabolism of *Azospirillum* (Pedrosa, 1988) as well as plant growth. Glucose or sucrose are normally used as a C source for *Arabidopsis* cultivated *in vitro* (Wilson *et al.*, 1990; Meinke, 1992). *Azospirillum* does not grow on media containing these compounds as the sole C source (Pedrosa, 1988), however actively-growing roots in these media excrete various C sources in root exudates metabolized by rhizosphere bacteria (Curl and Truelove, 1986) which may affect the functioning and efficiency of the root-bacteria association. Consequently, it is important to find C and N combinations which do not interfere with the association and are optimal for plant response *in vitro*.

Our objectives were: (i) To develop a simple system for seed inoculation and the subsequent observation and measurement of root and root hair growth of *Arabidopsis* seedlings without damaging the plant root. (ii) To measure the root hair growth as a response to inoculation with *Azospirillum brasilense.* (iii) To determine whether the N and C contents of the media have any effect on this response.

MATERIALS AND METHODS

Plant material *and growth* conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh., Columbia ecotype (Col-0) were surface sterilized for 10 min

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in a solution containing commercial bleach (5.25% of sodium hypochlorite) and 1 µl ml^{-1} of 20% Triton-X-100 ml^{-1}, then thoroughly washed with sterile distilled water. A special seed sterility test was not made because when the suspension of homogenized inoculated roots in PBS (see the subsection Root *colonization)* was plated on nutrient agar (Difco), there was no contamination. Furthermore, during the incubation of seeds and seedlings on plant nutrient media, no contamination was recorded either with or without sucrose. Seeds were suspended in 0.05% agarose (Litex, Denmark), and a drop of this suspension containing 12-15 seeds was placed in a plastic Petri plate (90 mm dia) containing 5 ml of agar medium. Under aseptic conditions with a microbiological loop, the seeds were carefully arranged in a line without damaging the agar surface. Plates were sealed with parafilm and kept in a controlled growth chamber at 23±0.5°C under constant light of 55 µE m^{-2} s^{-1}.

The plant nutrient medium described by Wilson *et al.* (1990) was used with minor modifications. The medium contained 2.5 mM KH_2PO_4, 2 mM $MgSO_4.7H_2O$, 2 mM $CaCl_2$, 50 µM Fe-EDTA, 70 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM $NaMoO_4$, 10 µM NaCl, 0.01 µM $CoC1_2$ and 8 g 1^{1} of "plant-cell-culture-tested-agar" (Sigma). Concentrations of KNO_3 and sucrose in the medium varied and are indicated for each experiment. Medium pH was adjusted to 5.6 with KOH. Unless otherwise indicated, all chemicals were of analytical grade from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Bacterial culture and plant inoculation

A. brasilense Sp-245 was grown in nutrient broth (8 g 1^{-1}) (Merck) under rotary agitation (180 rev min^{-1}) for 16 h at 30°C, then 1.8 ml of bacterial suspension were transferred into sterile vials, mixed with 0.2 ml glycerol (final concentration 10%, v/v) and stored in the freezer at -40°C. For each experiment, 2 ml of this stock culture were transferred into 125 ml Erlenmeyer flasks containing 25 ml of nutrient broth (8 g 1^{-1}) and cultivated for 23 h under the same conditions as the original culture. After washing twice in 60 mm phosphate buffer (pH 7.0), bacteria were resuspended in the liquid plant nutrient medium without KNO_3 and sucrose (pH 5.6) and their concentration was adjusted to densities ranging from 2 x 10^6 to 2 x 10^7 cfu ml^{-1}. The same medium, but without the bacteria, was used as control.

Plated seeds were left on the agar surface for at least 20 min, then inoculated with 150 µl of bacterial suspension which were spread over the seeds with a pipette. Then, the Petri plates were sealed with Parafilm and left for 24 h in a horizontal position before being positioned vertically, where they remained for the duration of the experiments. All experiments were carried out under sterile conditions.

Bacteria surrounding seeds and seedling roots were routinely inspected by light microscopy and were found to be motile and alive for at least 10 days, usually located in close proximity to the root surface (data not shown).

Observations and measurements of root and root hair lengths

The term "root hair growth" in the text only refers to an irreversible increase in the length of a root hair after the beginning of its emergence.

An established method for the analysis of plant-bacteria associations is the Fahraeus technique, where seedlings grow between two slide assemblies (Fahraeus, 1957; Umali-Garcia *et al.*, 1980). The major drawbacks of this system are (i) root hairs can be easily damaged during the transfer of seedlings to the assemblies and (ii) sterile material is contaminated after one observation. For morphological observations of root response to inoculation, it seemed more appropriate to plate seeds on an agar surface and observe inoculated seedlings through the agar layer using an inverted microscope, without any mechanical interference and without contamination. Therefore, all of our measurements were made on seedling roots growing on the surface of an agar layer in Petri plates. Two days after the start of seed imbibition (ASI), 7-10 seedlings per plate with approximately equal root lengths of 1-2 mm were selected in all treatments, numbered and used for measurements. Root hair lengths were measured through the agar layer with an ocular-micrometer under an inverted D microscope (Zeiss, Germany) at x 100 magnification. Root lengths at day 2 ASI were measured in the same way. After day 2, root lengths were measured under a stereoscopic microscope with a millimeter scale.

To estimate the root hair length, we used two methods: an individual root hair method and a random group method. The individual root hair method consisted of the following. At the beginning of day 3 ASI, the youngest root hair was measured. Then, 24 and 48 h later, we measured the root hair which was located in the same position and at the same distance from the basal root border (determined by the location of the most basal root hairs). Preliminary tests confirmed that the youngest root hairs (25-30 µm in size) were located above the elongating zone. This method was also applied to the measure-ments of the most basal (oldest) root hairs. Using this approach, only one root hair from each of the 7-10 roots in each treatment was measured in each experiment.

The other method of measuring root hair growth was a random group method. Root hairs which began their emergence at day 2 ASI were the most basal root hairs. Root hairs completed their growth within 24 h (see the Results section). So, root hairs which began their emergence, for example, at day 3 were measured at day 4 or at day 7, at a distance from the radicle-hypocotyl border equal to the root length at day 3. Using this random group method at a particular

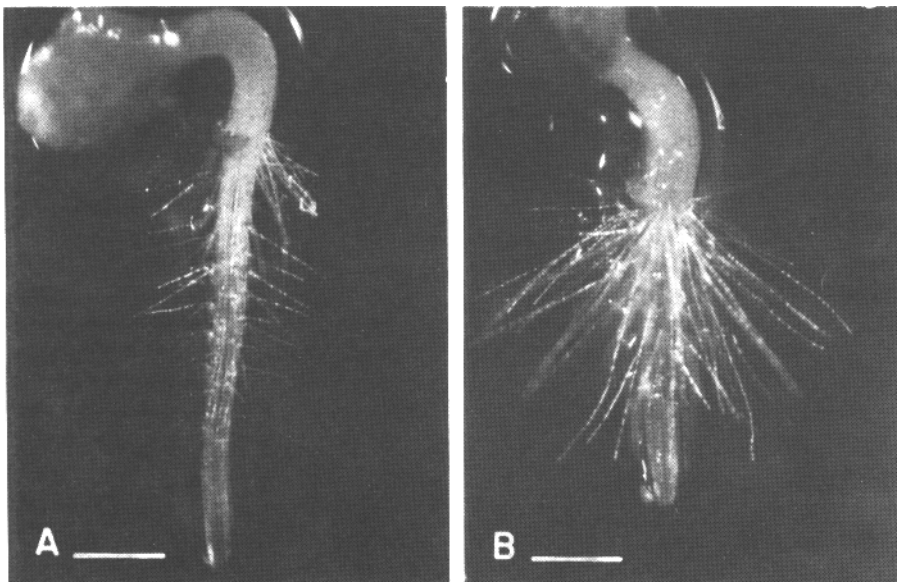

Fig. 1. Non-inoculated (A) and inoculated (2 x 10^7 cfu ml^{-1}) seedlings of *A. thaliana*, growing on medium with 5 mM KNO_3, without sucrose, 45 h ASI. Bar= 400 µm.

distance from the root base, 5 sequential root hairs were measured in each of the 5 roots in each treatment, at each time of initiation in each experiment.

Root colonization

After inoculation of *Arabidopsis* seed at an inoculum concentration of 2.3 x 10^1 cfu ml^{-1} (which corresponds to 3.45 x 10^6 cfu 15 $seeds^{-1}$) and incubation under identical conditions of root hair promotion experiments, the roots of 5 seedlings from each plate (in triplicate) were homogenized in 1 ml PBS by a Teflon-glass mechanical homogenizer on ice. The slurry underwent light sonication (ultrasonic homogenizer 4710, Cole Palmer Instruments, U.S.A.) for 3 min at 20 W on ice to detach *A. brasilense* cells from root debris. This sonication is harmless to *Azospirillum* cells (Bashan and Levanony, 1989). Then, the slurry was decimally diluted in PBS and the bacteria were counted by a conventional plate count method on nutrient agar after 48 h at 30°C. Results are presented as cfu mm^{-1} root because the miniature dimensions of *Arabidopsis* seedling roots did not allow precise dry weight determinations.

Statistical analysis

Each experiment was repeated at least four times. Results were analyzed using one-way ANOVA (LSD test at $P \leq 0.01$ and $P \leq 0.05$) and Student's *t*-test at $P \leq 0.05$.

RESULTS

Azospirillum and root hair growth

At day 2 ASI, differences in root hair length between non-inoculated (control) and inoculated seedlings of *Arabidopsis, growing* on medium containing 0, 0.5 or 5 mm of KNO_3 without sucrose, were observed. Root hairs in inoculated seedlings were much longer than in non-inoculated seedlings (Fig. 1). Root hair growth response was more consistent at the inoculation level 2 x 10^7 cfu ml^{-1} than at 2 x 10^6 cfu ml^{-1}. In the following experiments, the inoculation at this higher level was analyzed. Root hairs in both non-inoculated and inoculated seedlings completed their growth within the first 24 h after emergence. No further growth was observed when evaluated later by the individual root hair method (Fig. 3).

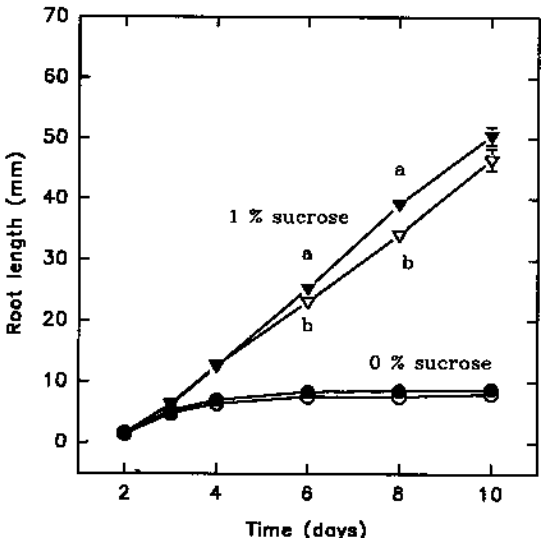

Fig. 2. *Arabidopsis* root growth in non-inoculated (control, ○ and ▽) and inoculated (2 x 10^7 cfu ml^{-1}, ● and ▼) seedlings, growing on media without sucrose or with 1% sucrose, both with 0.5 mm KNO_3. Combined data of two experiments. Mean±SE (n=15). Where the SE is not indicated, it was smaller than the symbol size. Points denoted by different letters at the same day are statistically different at $P \leq 0.05$ in Student's *t*-test.

Effect of sucrose on the promotion of root hair growth by A. brasilense

Root growth in seedlings growing in media with 0.5 mm KNO_3without sucrose ceased at day 4 (Fig. 2) with no difference in root length between non-inoculated and inoculated seedlings ($P \leq 0.05$, Student's *t*-test). However, in media with 1 % sucrose, roots grew throughout the experiment (10 days); roots of inoculated seedlings were significantly longer than those of the control at days 6 and 8 ASI ($P \leq 0.05$ Student's t-test). Root growth curves are presented in Fig. 2.

Final root hair length in inoculated seedlings growing in media without sucrose ranged from 2.13- to 2.89-fold greater than non-inoculated seedlings

Table 1. Final root hair length and root colonization of *Arabidopsis* roots inoculated by *A. brasilense* Sp-245 growing on media at two concentrations of KNO_3 without sucrose (controls were not inoculated)

KNO_3 (mM)	Day*	Control (μm)	Inoculation (μm)	Proportional increase	Colonization level (x 10^3 cfu (mm root length)$^{-1}$)
0.5	2	277±12a†	624±25b	2.25	9.0±1.7
	3	269±18a	574±33b	2.13	6.1+0.8
5	2	258±12x	647±30y	2.51	4.4±0.2
	3	179±13x	518±28y	2.89	10.5±1.3

*Day from the start of seed imbibition at which emergence of measured root hairs began. Measurements were done at day 4.

†Mean ± SE (n=50). Means at the same concentration of KNO_3 denoted by different letters are significantly different at P<0.01 in one-way ANOVA. Data from two of six independent representative experiments. Colonization data from 3 determinations per value, 5 roots per determination.

Table 2. Final root hair length (,um) *of Arabidopsis* seedlings roots growing in medium supplemented with sucrose (1%) and KNO_3 (0.5 mM) and inoculated with *A. brasilense* Sp-245

Days*	Non-inoculated (mm)	Inoculated (μm)	Proportional increase
2	548±20a†	890±33b	1.62
4	530±36a	1038±47c	1.96
7	576±39a	1160±78c	2.01

*Days from the start of seed imbibition at which emergence of measured root hairs began. Measurements were performed at day 8.

†Means denoted by different letters differ significantly at $P<0.05$ in one-way ANOVA. Means±SE (n=50). Data from two experiments.

(Table 1 and Fig. 3). The improvement of root hair growth was consistent and was observed for the first root hairs initiated at day 2 ASI and for root hairs initiated at day 3 ASI. A similar phenomenon was observed in seedlings growing in medium supplemented with 1 % sucrose (Table 2). This effect was also consistent. Root hairs which began their emergence immediately after seed germination at day 2 ASI, and root hairs that emerged later at days 4 and 7 ASI responded similarly to inoculation. However, the effect was more pronounced for root hairs that emerged at days 4 and 7 ASI. The distribution of root hairs along the roots was similar in inoculated and non-inoculated seedlings (data not shown).

Effects of KNO_3 on root hair growth and colonization

The measurement of final root hair lengths by the random group method demonstrated that in seedlings growing on media with 0.5 or 5 mM KNO_3 without sucrose, root hairs which began their emergence both at days 2 and 3 ASI were significantly longer when inoculated by *Azospirillum* (Table 1). However, root hairs which began their emergence at day 3 had a tendency to be shorter than at day 2 because root growth had slowed down. The concentration of N did not affect root hair growth promotion induced by *Azospirillum* (Table 1). The colonization of *Arabidopsis* roots by *A. brasilense* was relatively low. From ≈3 x 10^6 bacteria applied to the seeds only ≈(6-9)x10^3 cfu were attached to 1 mm of roots or ≈1.6 x 10^5 per 15 seedlings in the plate (on average). At low nitrate concentration, the bacteria survived from day 2 to day 3 but did not multiply. In medium containing 5 mM nitrate, the bacteria multiplied on roots, but the numbers were also low (Table 1).

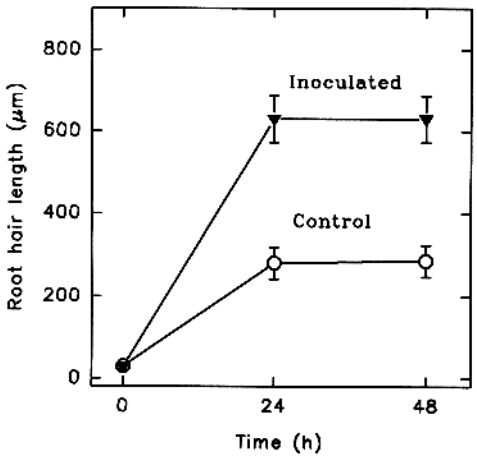

Fig. 3. Root hair growth in control (○) and inoculated (2 x 10^7 cfu ml^{-1}, ▼) seedling *of Arabidopsis* growing in media without sucrose. Measurements by individual root hair method. Combined data of two experiments. Mean ± SE (n =15).

To further evaluate the role of N alone in root hair growth, the final size of root hairs was recorded when seedlings grew in media with various concentrations of KNO_3 supplemented with 1 % sucrose. The concentration of nitrate affected the final size of the root hairs but at a low magnitude (Table 3); the mean varied less than 32% (from a minimum of 253 μm at 0 mM KNO_3 to a maximum of 334 μm at 5 mM KNO_3) compared with an approximately 1.5- to 2.5-fold increase in the cases of bacteria inoculation (compare Tables 1 and 2 to Table 3).

The nitrate concentration in the medium had no significant effect on root growth until day 4 ASI. At day 6, root growth only decreased slightly in media without N, and by day 10 significant effects on root length were recorded (Fig. 4).

DISCUSSION

The individual root hair method showed that *Azospirillum* promoted root hair growth by at least 2-fold (Fig. 3). Since root hairs reached their final length within 24 h after emergence, root hairs which began emergence at day n were measured by the random group method at day n + 1. In all cases, results obtained by the individual root hair method were confirmed by the random group method (Tables 1 and 2).

The role of plant growth hormones in root hair formation is known (Cormack, 1949). *Azospirillum* has the ability to synthesize auxins (Tien *et al.*, 1979; Jain and Patriquin, 1985; Horemans *et al.*, 1986; Zimmer et al., 1988, 1991; Fallik *et al.*, 1989), gibberellins (Bottini *et al.*, 1989) and cytokinins (Tien et al., 1979; Horemans *et al.*, 1986) which could modify root developmental and growth processes, including root hair morphogenesis. It is possible that one or more of these hormones are involved in increases in root hair growth in *Arabidopsis.*

Nitrate concentration in the medium slightly increased root hair growth, but the effect of *Azospirillum* was considerably stronger (compare Tables 1-3). It is possible that some features of bacterial N metabolism are involved in plant growth response. Bacterial nitrate reductase activity may be one such property of *Azospirillum* (Ferreira *et al.*, 1987). Nitrite formed by nitrate respiration of *Azospirillum* partially replaces auxin activity (Zimmer *et al.*, 1988) and is a possible factor involved in the promotion of root hair growth.

Table 3. Final root hair length (μm) in *Arabidopsis* seedlings growing at various concentrations of KNO_3 in medium supplemented with 1% sucrose

KNO_3 (mM)						
0	0.5	1	2	5	10	20
253± 12a	319± 18cde	358± 15e	291 ± 14abc	334± 15de	300± 18bcd	276± 13ab

Mean ± SE (n =50). Means followed by different letters differ significantly at $P \leq 0.05$ in one-way ANOVA. Measurements of the length of root hairs which began their emergence at day 3 were done by the random group method at day 7 ASI.

Root hair growth and root growth are coordinated in plants. For example, in plants with "cytokinin root syndrome" (Su and Howell, 1992), root growth is inhibited, but root hair growth is promoted. Thus, to evaluate root hair growth it is necessary to take into account the whole root growth response. In addition, it has been reported that at high inoculation rates, *Azospirillum* can inhibit root growth (Bashan, 1986; Okon and Kapulnik, 1986; Morgenstern and Okon, 1987; Yahalom *et al.*, 1991) In our experiments, *Azospirillum* inoculation levels of 2 x 10^7 cfu ml^{-1} did not inhibit root growth compared to non-inoculated seedlings (Fig. 2). Thus, promotion of root hair growth induced by inoculation was not a compensative reaction to slow root growth.

It is improbable that Ca^{2+} ions, an essential factor of root hair growth in Arabidopsis (Schiefelbein *et al.*, 1992), were responsible for increased root hair growth in inoculated seedlings. The concentration of Ca^{2+} (2 mM) was equal in the control treatment and bacterial suspensions used for inoculation, and this Ca^{2+} concentration is the optimal one for root hair growth in *Arabidopsis* (same ecotype) (Schiefelbein *et al.*, 1992).

In some species, *Azospirillum* inoculation resulted in denser root hair formation (Tien *et al.*, 1979; Jain and Patriquin, 1985; Hadas and Okon, 1987; Morgenstern and Okon, 1987; Barbieri *et al.*, 1991). The density of root hairs is controlled by differentiation processes in root cells which are independent of root hair growth processes in root hair producing (trichoblast) cells (Cormack, 1962). Furthermore, denser root hair formation can be the result of retarded cell elongation in inoculated seedlings. In our research with *Arabidopsis*, we observed no apparent differences in root hair distribution, even though root hair growth was affected.

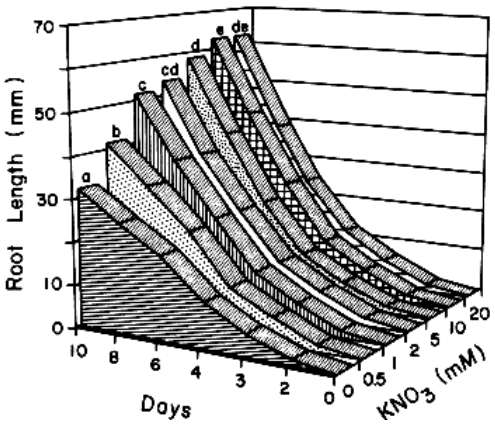

Fig. 4. Arabidopsis root growth at the various concentration of KNO_3. Means of 17-20 roots at each point. Points on the graph denoted by different letters differ significantly at $P \leq 0.05$ in one-way ANOVA.

There are indications that the effect of *Azospirillum* on root hair may be a general, non-specific phenomenon. In wheat inoculated by *A. brasilense*, root hair length increased in root segments 1.5-2.5 cm from the root cap, but not in segments 4-5 cm from the cap (Kapulnik et al., 1985). This would indicate one of two possibilities: (i) the bacteria induced faster root hair growth or (ii) the distance between root tip and the zone of root hair emergence was shorter in *Azospirillum*-treated seedlings. The latter phenomenon did appear in a sorghum hybrid inoculated by *Azospirillum* which might have caused observed differences in the root hair lengths located at constant distances from root tip (Morgenstern and Okon, 1987). Increase in root hair length also occurred in sugarcane inoculated with *A. brasilense* (Patriquin et al., 1983) and in winter wheat inoculated with *Burkholderia cepacia* (basonym *Pseudomonas cepacia*) (de Freitas and Germida, 1990). In cases of inoculation, measurements of root-hair size at a constant distance from root tip may be misleading since non-fully developed root hairs are also measured. In our study we measured the final size of the root hairs and, therefore, our results can not be compared to these previous studies.

This research demonstrated that *Arabidopsis* seedlings provide a simple and efficient model for the study of plant root *Azospirillum* interactions. This bacterium increased root hair growth by more than double as compared to the non-inoculated control. This effect was consistent and reproducible, and was not related to nitrate or sucrose in the medium. Increases in root surface area caused by root hair lengthening may lead to an increase in mineral uptake which could explain the plant-growth-promoting effect of *Azospirillum* inoculation (Murty and Ladha, 1988; Bashan et al., 1990).

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