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Alternative Mechanism for the Evaluation of Indole-3-Acetic Acid (IAA) Production by *Azospirillum brasilense* Strains and Its Effects on the Germination and Growth of Maize Seedlings

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We evaluated the production of indole-3-acetic acid (IAA) by *Azospirillum brasilense* strains *in vitro* (cell culture supernatants) and *in vivo* (stems and roots of maize seedlings) to clarify the role of this phytohormone as a signaling and effector molecule in the symbiotic interaction between maize and *A. brasilense*. The three strains all showed IAA production when cultured in NFb medium supplemented with 100 μg/ml L-tryptophan. The level of IAA production was 41.5 μg/ml for Yu62, 12.9 μg/ml for Az39, and 0.15 μg/ml for *ipdC*. The release of IAA into culture medium by the bacteria appeared to be the main activator of the early growth promotion observed in the inoculated maize seedlings. The application of supernatants with different IAA contents caused significant differences in the seedling growth. This observation provides the basis for novel technological tools for effective quality control procedures on inoculants. The approach described can be incorporated into different inoculation methods, including line sowing, downspout, and foliar techniques, and increase the sustainability of symbiotic plant-bacteria systems.

Keywords: *A. brasilense*, plant growth-promoting activity, indole 3-acetic acid, inoculation, maize roots

Introduction

The evaluation of 20 years of data on inoculation practices using *Azospirillum* spp. indicated that 60–70% of field experiments were successful, with yield increases ranging from 5 to 30% (Okon and Labandera-Gonzalez, 1994). The promoting effect of inoculation with this bacterium occurs at early growth stages, during the first few days after root colonization (Fallik *et al*., 1994).

In Argentina, *Azospirillum brasilense* Az39 is the most widely used strain for inoculant formulation. During the past few decades, it has been applied to the cultivation of maize (Gaudin *et al*., 1994), wheat (Weyers and Paterson, 2001), forage grasses (Zimmer and Bothe, 1988), and other crops. Increases in wheat grain production in the semiarid region of Argentina by inoculation with *A. brasilense* have been reported (Thuar *et al*., 2005).

The extensive commercial exploitation of *Azospirillum* spp. strains as inoculants will not be possible until we can better elucidate their association with host plants. We do not yet clearly understand the roles of many key components of the bacterium-plant system. Studies along this line have been focused on the thorough characterization of potential plant growth-promoting strains and on their interactions with different plant species (Bashan *et al*., 2004). Janistyn (1973) found that inoculation with *Azospirillum* spp. had no major impact on plant growth in sterile soil as compared with non-sterile soil. They also showed that the overdosing of an *Azospirillum* strain in sterile soil inhibited plant growth because of the overproduction of hormones by the bacterium. Such hormones can accumulate in the soil and cause the suppression of seedling growth (James, 2000). In another study, the inoculation of maize with *Azospirillum* spp. had a growth-promoting effect in the early growth stages during the first few days after root colonization (Döbereiner, 1989). A dose-response curve for plant root development with increasing concentrations of an *Azospirillum* strain was similar to the curve obtained with increasing concentrations of indole-3-acetic acid (IAA) (Hernández *et al*., 1996), suggesting the importance of this phytohormone.

Several studies have shown that the biosynthesis of IAA by *Azospirillum* spp., *per se*, does not lead to plant growth promotion. An "additive hypothesis" was suggested to explain the increases of plant growth and yield observed following inoculation with an *Azospirillum* strain. According to this hypothesis, plant growth promotion is the result of different mechanisms (e.g., biosynthesis of phytohormones, nitrogen fixation) that operate simultaneously or in succession (Bashan and Levanony, 1990). The stimulatory effect of *Azospirillum* spp. on root development in maize and wheat is well documented (Hasenstein and Evans, 1986). Morphological root changes have been frequently observed following inoculation with various *Azospirillum* strains. Such changes have been attributed to the production of plant-growth promoting substances, particularly auxins, by the bacteria (Canny and McCully, 1988; Díaz Vargas *et al*., 2001; Spaepen *et al*., 2008). Döbbelaere *et al*. (2001), using a plate assay, showed that the inoculation of wheat with *A. brasi-*

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lense wild-type strain Sp245 significantly increased root hair formation. In contrast, a Sp245 mutant strain with reduced production of IAA had no such effect, indicating the important role of IAA production by *A. brasilense* in altering wheat root morphology.

The early stimulation of seedling growth following inoculation is an important consideration in the development of inoculation strategies. Mechanisms of enhanced resistance to environmental stresses, e.g., cell wall reinforcement, root hair formation, and differentiation of vascular tissues, are essential in allowing the seedling to successfully establish the plant. Nutrient uptake and distribution to various organs in maize have been found to vary depending on soil fertility, the application of chemical fertilizers, the growth stage of the plant, and environmental conditions (Gaudin *et al*., 1994), as well as on the application of *A. brasilense* (James, 2000; Ona *et al*., 2005). However, the most prominent changes after inoculation could be observed in changes of root morphology directly stimulating plant growth. We will therefore discuss these mechanisms with reference to effects of each on root architecture.

As explained in our previous report (Masciarelli *et al*., 2009), inoculant is a complex biological formulation that combines two elements: cultured microorganisms and the compounds that are secreted into their growth medium under controlled conditions. Inoculants should not be regarded as carriers of microorganisms, but as complexes that result from the biotransformation of components added to the growth medium into various metabolites by the bacteria.

The auxins indole-3-acetic acid (IAA) quantitatively seems to be the most important of the three phytohormones. Bacterial phytohormone production is assumed to induce changes in root morphology after *Azospirillum* inoculation. The work with *Azospirillum* mutants altered in indole-3-acetic acid production endorses this hypothesis (Spaepen *et al*., 2008).

The hypothesis tested in the present study was that early stimulation of maize seedling growth is determined mainly by the content of IAA in the culture supernatant of an *Azospirillum* strain rather than by the IAA produced by bacteria that colonize the plant. We compared the effects of inoculating pre-germinated seeds with (i) aqueous cell suspensions, (ii) culture supernatants, (iii) cell pellets from the commercially available strain Az39, IAA-overproducing strain Yu62, and mutant strain Sp245 (*ipdC*$^-$).

Materials and Methods

Strains and culture conditions

The *A. brasilense* strains used were: Az39 (kindly provided by Dr. Alejandro Perticari, Institute of Agricultural Microbiology and Zoology, INTA-Castelar, Argentina); Yu62 (from Dr. Stijn Spaepen, Center of Microbiology and Plant Genetics, Heverlee, Belgium), and Sp245 (*ipdC*$^-$) (from Dr. San Feng Chen, Laboratory of Microbiology and Agro-Applications of Life Sciences, Beijing, China). Each of the strains was grown on Petri dishes containing K-malate agar (Döbereiner, 1989) and inoculated into NFb liquid medium with and without the addition of L-tryptophan (100 μg/ml). Bacteria were cultured for 48 h at 30°C on an orbital shaker (100 rpm) until they reached exponential phase. The purity of the cultured strains was confirmed by Gram staining and motility assay.

Preparation of bacterial inocula

Bacteria were cultured for 24 h at 30°C in NFb medium; from this culture, a pre-inoculum (10 ml) was taken and used to inoculate flasks containing 100 ml NFb medium. These cultures were incubated until reaching OD_{595nm}=1, which corresponds to approximately 1×10^8 colony-forming units (CFU/ml). To obtain cell pellets and supernatants, 50 ml of a given bacterial culture was centrifuged (8,000 rpm) for 15 min at 4°C. The cell pellets were resuspended in 50 ml of fresh NFb medium.

Plant material

Maize (*Zea mays* L.) seeds (Dekalb, DK190 MG RR2/08, lot IE1011986; AgroUcacha, Río Cuarto, Córdoba, Argentina) were surface disinfected by soaking in 70% ethanol for 3 min and then in 2% sodium hypochlorite solution for 15 min, thoroughly rinsed 10 times with sterile distilled water, and placed into test pots for inoculation tests.

Inoculation tests and greenhouse experiments

Maize seeds were inoculated in separate experiments with 1 ml of aqueous cell suspension (10^7 CFU/seed) (Rodriguez Caceres *et al*., 1996), resuspended cell pellet (10^7 CFU/seed), or culture supernatant from strains Az39, Yu62, and Sp245 and placed into 300 cm^3 pots (5 seeds/pot) containing sterile vermiculite. Fresh uninoculated NFb medium was used as a control. The pots were incubated in a growth chamber under controlled conditions: 16 h light at 25°C/ 60% RH and 8 h darkness at 18°C/ 40% RH for 14 days, with light intensity 11500 Lux ≈ 155 μE/m^2seg.

Dosages of inocula from the supernatants were based on the content of IAA/ml of culture. Pots were capillary-irrigated with 25% Hoagland nutrient solution. Each treatment consisted of 5 replicates and the experiments were repeated 3 times.

Determination of IAA

The remaining 50 ml from the liquid cultures of each strain and NFb medium (control) were subjected to quantification of IAA after addition of 50 ng internal standard ($^2H_5$-IAA, Olchemlm Ltd., Czech Republic). Plant material consisting of 0.15–0.2 g lyophilized roots or coleoptiles was added with 3 ml extraction solvent [MeOH:H_2O:AcH (80:19:1)] and 50 ng internal standard and left to stabilize for 30 min at 4°C. The suspensions were then filtered through membranes (0.45 μm) and the aqueous fractions were evaporated. The samples were partitioned 3 times with ethyl acetate at pH 3, and each organic fraction was collected and evaporated to dryness. The samples were then resuspended in methanol and passed through a pre-purification Sephadex DEAE 25 column. The active fractions (with eluted IAA) containing the deuterated internal standard were evaporated to dryness and resuspended in vials with 100 μl methanol. The vials

were placed in the autosampler of an Alliance 2695 liquid chromatograph (Waters Inc., USA) and 10 µl of the suspension was injected. The chromatographic conditions were constant: an isocratic solvent gradient of acetonitrile:water (65:35) and a flow rate of 0.2 ml/min. The sample was further analyzed using a Quattro UltimaTM Mass Spectrometer (Micromass, UK). IAA identification was performed by comparing the retention times of the peaks in the sample with those of the pure standard, and quantification was performed using the MRM (Multiple Reaction Monitoring) mode, following the 174/179 molecular masses and 130/135 transition masses, which corresponded to endogenous/deuterated, respectively. The MRM mode was required because several compounds might present the same nominal molecular mass. Thus, the combination of parent mass and unique fragment ions was used to selectively monitor each of the standards in culture media and in crude plant extracts. Data were acquired and analyzed using MassLynx^{TM} 4.1 and QuanLynx^{TM} 4.1 (Micromass) software. For quantification, values were obtained from a calibration curve previously constructed using IAA pure standard (Sigma, USA).

Determination of the dose-response effect of inoculation

The procedure was the same as described for the inoculation test. The dose was on the order of 10^7 CFU/seed as suggested by Rodríguez Cáceres *et al.* (1996). All applications contained the same volume of inoculums (1 ml) of aqueous cell suspension (10^7 CFU/seed) or supernatant (1 µg IAA/ml), respectively. The physiological parameters were assessed as described above.

Determination of biometrics

At 14 days after inoculation, the following growth parameters were evaluated: number of established seedlings, shoot and root length, diameter of coleoptiles, fresh and dry root weight, root morphology, and root volume. Root volume was determined as described by Díaz Vargas *et al.* (2001); i.e., as the difference in water level before vs. after placing intact roots in a graduated column containing distilled water. Shoot and root dry weights were determined after drying the plant material in an oven for 96 h at 60°C. For the microscopic analysis of root cell morphology, three seedlings per treatment were randomly selected and their main roots were cut 1.0 to 1.5 cm above the apex. Root segments were taken from the same region for root hair observation. Root fixations, embedding, cross-sectioning, and staining were performed as described by Reinoso *et al.* (2004).

Colonization assay for determination of endophytism

To determine whether the mutant *ipdC*$^-$ strain was defective in the ability to colonize roots, maize seeds were inoculated

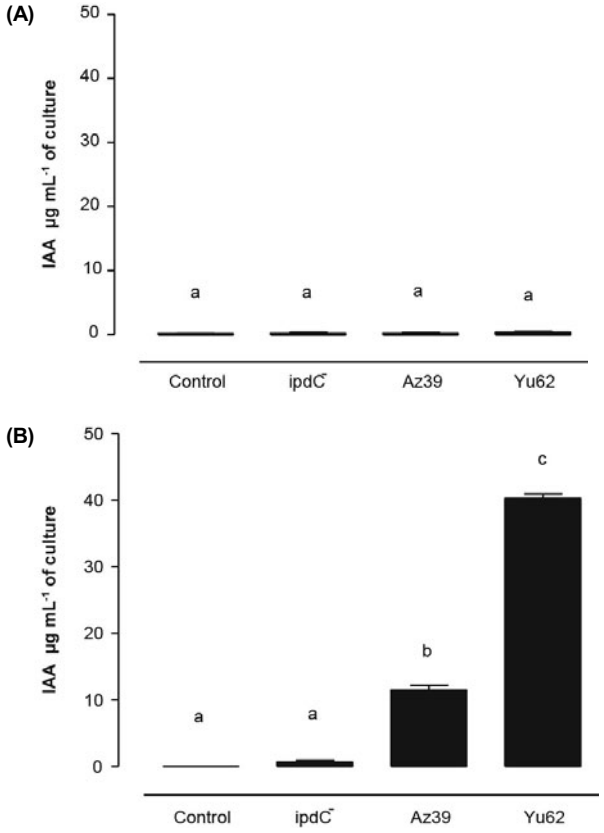

Fig. 1. Production of IAA in NFb-containing media. (A) medium without L-tryptophan. (B) medium added with 100 µg/ml L-tryptophan.

with either Az39, *ipdC*$^-$, or a 1:1 mixture of the two strains. The bacteria and seeds were prepared, inoculated, and incubated as described above for the greenhouse experiments. Fresh roots (1 g for each treatment) were surface disinfected with sodium hypochlorite by dipping and removing (3 times), washed several times with distilled water, dipped in ethanol (70%), and briefly flamed. The roots were then placed in a sterile mortar, macerated with saline buffer (25 mM NaH_2PO_4, 25 mM Na_2HPO_4; pH 7.0), shaken vigorously for 30 min, and a 1 ml aliquot was taken to start serial dilutions (1:10). A 100 µl aliquot from each dilution was applied on a Petri dish containing NFb medium to determine total bacterial counts and placed in an oven for 48 h at 28°C. For the subsequent identification and differentiation of strains, *ipdC*$^-$ strain resistant to kanamycin (50 µg/ml) was used.

Mineral nutrient content

The contents of mineral nutrients in roots were determined 14 days after inoculation. The contents of K^+, Na^{++}, and

Table 1. Bacterial growth measured at $OD_{595\ nm}$, viability counts (CFU/ml), and pH of NFb with and without added tryptophan

Treatment	Without tryptophan			With tryptophan	
	OD	CFU/ml	pH	OD	CFU/ml
ipdC$^-$	0.82 ± 0.01	1.00^8 ± 0.1	8.2/8.1	0.75 ± 0.01	1.20^8 ± 0.1
Az39	0.74 ± 0.01	1.00^8 ± 0.1	8.2/7.5	0.64 ± 0.01	1.80^8 ± 0.1
Yu62	0.98 ± 0.01	1.00^8 ± 0.1	8.1/7.0	0.78 ± 0.01	1.75^8 ± 0.1

Ca^{++} were assessed by photometry, flame ionization, and atomic absorption spectrometry, respectively, as described by Jackson *et al.* (1986). For this purpose, roots (200 mg dry weight) were homogenized in a mortar with liquid nitrogen, and the material was placed in digestion tubes containing 3 ml pure nitric acid. The tubes were placed in a sand bath for 3 h at 180°C, cooled, and centrifuged at 5,000 rpm for 10 min. The clear supernatant was separated for the determination of K^{+} and Na^{++} contents using flame adjusting equipment. Lanthanum chloride (5 ml) was added to 2 ml extract to determine Ca^{++} content by absorption at 422 nm in an atomic absorption spectrophotometer. For quantification, values were obtained from calibration curves constructed previously with known concentrations of K^{+}, Na^{+}, and Ca^{++}. All measurements were expressed in mg/g distilled water.

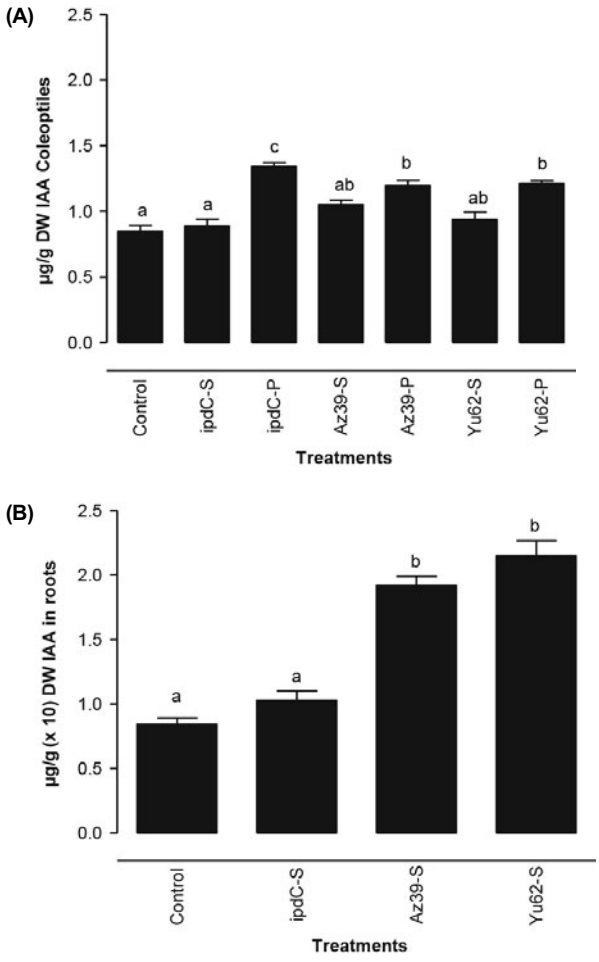

Fig. 2. IAA content in coleoptiles or roots of maize seedlings. (A) IAA content in coleoptiles of maize seedlings after treatment of pre-germinated seeds with culture supernatants or resuspended cell pellets. Data are expressed as µg IAA/g distilled water. Data differ significantly ($P<0.05$ and $R^2=0.958$, n=3). (B) IAA content in roots of maize seedlings after treatment of pre-germinated seeds with culture supernatants. Data are expressed as µg IAA/g (×10) distilled water. Data differ significantly ($P<0.0001$ and $R^2=0.9579$, n=3).

Table 2. Correlation of three inoculant strains on CFU/ml and IAA content (µg/ml)

Treatment	NFb medium without Trp		NFb medium with Trp	
	CFU/ml	IAA (µg/ml)	CFU/ml	IAA (µg/ml - M)
Control	0	0	0	0
ipdC-	$10^7 \pm 0.1$	0	$10^7 \pm 0.1$	$0.015 - 8.6^{-8}$
Az39	$10^7 \pm 0.1$	0	$10^7 \pm 0.1$	$12.9 - 7.4^{-6}$
Yu62	$10^7 \pm 0.1$	0	$10^7 \pm 0.1$	$41.5 - 2.4^{-5}$

Statistical analysis

For statistical analyses, the data and/or variances were normalized and then subjected to Analysis of Variance (ANOVA). When ANOVA indicated a treatment effect, Tukey's Multiple Comparisons Test was applied for comparisons between the means. Prism 2.0 software was used for these analyses.

Results

Bacterial viability and production of IAA profile

Typical production profiles for each strain, adjusted to the same CFU or viability (1×10^8 CFU/ml of culture), are shown in Fig. 1. The addition of Trp to the medium resulted in a lower biomass for all three strains, with OD being the lowest for Az39 (Table 1). The largest CFU were obtained in Trp-supplemented, with Az39 reaching the greatest viability. The pH was increased only slightly in *ipdC*⁻ under this condition.

In NFb medium, with or without the addition of L-tryptophan, none of the three inoculants strains had significant effects on biomass production or viability. The addition of L-tryptophan decreased the growth rate of all three strains but increased cell concentration (Table 1). We also observed an increase in the duration of the logarithmic phase, which tended to increase cell concentration (data not shown). Plants with a higher IAA content in the coleoptiles were treated with resuspended cell pellets of the three strains (Fig. 2A). No significant differences in IAA content were observed among the plants inoculated with supernatants of Az39; the values were slightly higher than those of controls. The plants inoculated with supernatants or aqueous cell suspension of Yu62 had IAA levels lower than those of plants inoculated with washed cells or pellets. For all three strains, treatment with pellets gave IAA content higher than treatments with supernatants. In similar experiments with roots, IAA content was highest for treatment with supernatants of Yu62 and Az39 (Fig. 2B).

In the present study, treatment of pre-germinated seeds with culture supernatant of strain Az39 or Yu62 did not induce higher levels of IAA in coleoptiles (Fig. 2A). In contrast, treatment with cell pellets from each of the strains did increase IAA levels in coleoptiles, suggesting that the pres-

Table 3. Inoculation parameter and colonization ability (11 days after inoculation) of strains Sp245 and Az39

Inoculum density (10^7)		Inocula relationship	Population recovered		Recovered value
Az39	Sp245		Az39 (10^4)	Sp245 (10^2)	
1.8 ± 0.1		1 – 0	5.4 ± 0.1		1 – 0
	1.2 ± 0.1	0 – 1		1.1 ± 0.1	0 – 1
1.8 ± 0.1	1.2 ± 0.1	1 – 0.7	3.2 ± 0.1	5 ± 0.1	1 – 0.015

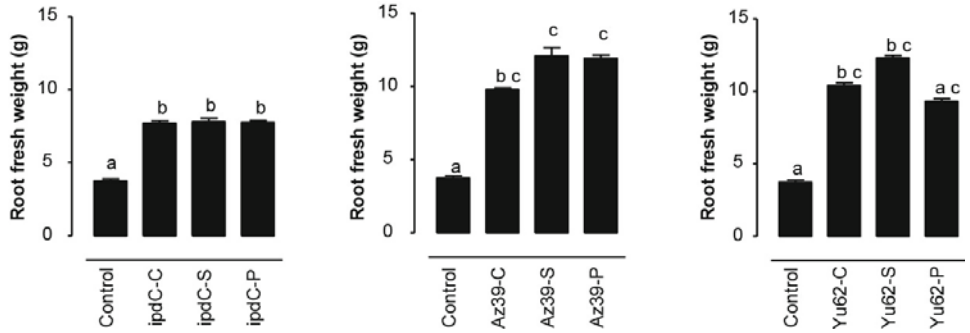

Fig. 3. Root fresh weight (g) of maize plants treated with the three inoculants strains grown in culture medium supplemented with L-tryptophan. Data differ significantly ($P<0.05$ and $R^2=0.98$, n=3).

ence of the bacteria is necessary for this. Plants previously treated with a pellet from *ipdC*⁻ had higher IAA content. There were no significant differences in IAA content between plants treated with pellets or supernatants from Az39 and Yu62. IAA content of plants treated with pellets from these strains was higher than that of controls. IAA accumulated in the root system of plants inoculated with supernatants from Az39 or Yu62 (Fig. 2B) but not with pellets from these strains (data not shown).

Correlation between cfu/IAA and colonization

Correlation of the three inoculants strains on viability (CFU/ml) and IAA content (µg/ml) are shown in Table 2. For each strain, the inoculants volume per seedling was the same (~100 µl).

The degree of effective colonization (endophytic) of Az39 and *ipdC*⁻ in maize roots at 11 days after inoculation is shown in Table 3.

Biometric parameters in plants

When Trp was added to the medium, we observed dramatic increases in the root fresh weight: 100% for *ipdC*⁻; 160% and 220% for cell suspension and supernatant of Az39; 190, 220, and 150% for cell suspension, supernatant, and pellet of Yu62, respectively (Fig. 3). Similarly to the pattern of response for root fresh weight, a significant difference in root dry weight was observed for Yu62 vs. Az39 (Fig. 4). No significant differences in IAA accumulation were observed between treatments with vs. without addition of L-tryptophan (data not shown). The most significant increases in root fresh weight were observed for plants treated with cell suspensions, supernatants, or pellets from strains grown in medium supplemented with L-tryptophan (Fig. 3), as compared with strains grown in media without L-tryptophan or in fresh NFb medium. We observed differential responses in plants inoculated with the three strains. Yu62, as compared with Az39, did not produce a substantial change in root fresh weight.

We observed significant increases in root fresh and dry weights, and a correlation between these parameters, in plants treated with Az39 and Yu62 grown in culture media supplemented with L-tryptophan (Figs. 3 and 4). Supernatants from Az39 and Yu62 also caused the greatest increases in root growth.

Ion levels in seedlings and root morphology

Also a slight increase in the root tissue incorporation of K^+ with aqueous cell suspensions of all three strains and with supernatants of Az39 and Yu62, but not with pellets of Yu62 (Table 4). There was a decrease of root Na^+ level for supernatants and pellets of Az39 and *ipdC*⁻ but not for Yu62. Root Ca^{++} level showed a significant increase in plants treated with suspensions or supernatants of Az39 and Yu62.

Ion concentrations were affected by inoculation with pellets from all three strains or with supernatants from Yu62 or Az39 (Table 4). Ca^{++} content was higher in plants inoculated with Yu62 and Az39 in any of their forms, whereas treatment with *ipdC*⁻ caused no significant change in this parameter.

The effects of various inoculation treatments on the formation of absorbent root hairs are shown in the left-hand images in Fig. 5. Treatment with supernatant of Az39 gave a better root hair distribution. Supernatant of Yu62 had an effect similar to that of 10^{-4} M IAA, with condensation and thickening of the hairs. Lateral roots are formed near the apical meristem, with a deep origin from pericycle cells.

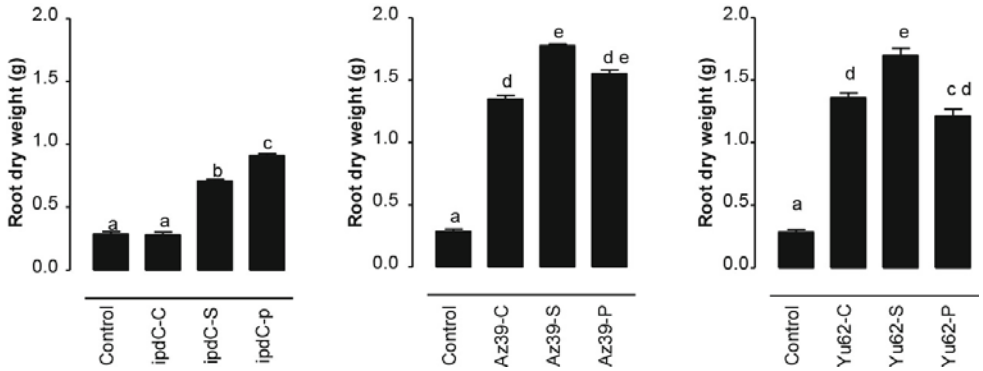

Fig. 4. Root dry weight (g) of maize plants treated with the three inoculants strains grown in culture medium supplemented with L-tryptophan. Data differ significantly ($P<0.05$ and $R^2=0.954$, n=3).

Table 4. Ion levels in root tissues. Data differ significantly ($P<0.05$, $R^2=0.952$, n=3)

Treatment	Ion levels (mEq/g distilled water)		
	K^+	Na^+	Ca^{++}
Control	0.27 ± 0.01b	0.97 ± 0.01d	0.032 ± 0.01a
ipdC⁻ C	0.21 ± 0.01a	0.52 ± 0.01a	0.030 ± 0.01a
ipdC⁻ S	0.19 ± 0.01a	0.57 ± 0.01b	0.033 ± 0.01a
ipdC⁻ P	0.19 ± 0.01a	0.59 ± 0.01b	0.029 ± 0.01a
Az39 C	0.36 ± 0.01c	0.71 ± 0.01c	0.059 ± 0.01c
Az39 S	0.34 ± 0.01c	0.71 ± 0.01c	0.069 ± 0.01d
Az39 P	0.33 ± 0.01c	0.63 ± 0.01ab	0.042 ± 0.01b
Yu62 C	0.33 ± 0.01c	1.15 ± 0.01e	0.087 ± 0.01e
Yu62 S	0.33 ± 0.01c	1.03 ± 0.01e	0.083 ± 0.01e
Yu62 P	0.29 ± 0.01bc	0.91 ± 0.01cd	0.044 ± 0.01b

C, cell suspension; S, supernatant; P, pellet.

Discussion

As expect and consistent with the results of previous studies, *Azospirillum* in minimal medium without the addition of L-tryptophan (Trp; the precursor of IAA biosynthesis) was not capable of producing IAA. Media not inoculated with NFb were used as negative controls. Strain Yu62 showed the greatest ability to produce IAA in medium supplemented with Trp (41.5 µg IAA/ml culture). Strain Az39 produced 12.9 and *ipdC*⁻ produced 0.15 µg IAA/ml culture.

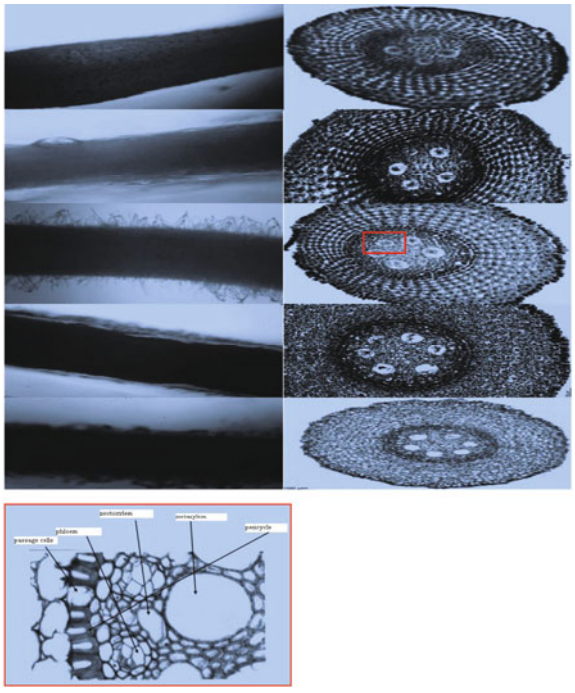

Fig. 5. Effect of inoculation on root morphology. Seedlings were inoculated with (top to bottom) uninoculated NFb medium (control), supernatants of strains *ipdC*⁻, Az39, and Yu62, and 10^{-4} M IAA. Left: effect of inoculation on root hair formation. Right: effect of inoculation on root morphology and structure (optical microscope; 10× magnification). The image at bottom is an enlargement, with labeling, of the area indicated by red outline in the Az39 photograph.

Thus, the production by Yu62 was 70% and 99% greater than that by Az39 and *ipdC*⁻, respectively.

An increase in the duration of the logarithmic phase is consistent with that of Ona *et al.* (2005). The pH was increased slightly in culture media in which the mutant strain *ipdC*⁻ was grown, presumably because of its reduced capacity for producing IAA. Gaudin *et al.* (1994) showed that tryptophan and other amino acids could stimulate bacterial growth by providing nitrogen. The production of such metabolites by plants can therefore promote bacterial growth. Studies of *Escherichia coli* and some species of *Pseudomonas* and *Bacillus* showed that tryptophan can be a ready source of carbon and nitrogen (Zimmer *et al.*, 1991).

To evaluate the regulation of IAA content, we quantified this compound in coleoptiles from plants inoculated separately with fresh uninoculated medium and with supernatant or resuspended cell pellets from each of the three strains. Bacterial IAA interacts with a plant through supplementation of the plant's endogenous "pool" of IAA and can potentially modify various anatomical, biochemical, and physiological characteristics of the plant (Cassán *et al.*, 2009). Regulatory processes (biosynthesis, conjugation, hydrolysis of conjugates, transport, and catabolism) or their homeostasis allow the plant to regulate its level of free IAA according to its current metabolic needs. Those needs depend on several factors that affect the external and internal environment of the plant, including hormonal contributions from bacteria (Sgroy *et al.*, 2009). Because the roots are sensitive to this hormone, this accumulation resulted in increases in growth parameters.

In the present study, IAA was identified and quantified in the supernatants from the three strains grown in NFb medium supplemented with L-tryptophan as described by Zakharova *et al.* (1999). IAA production levels were consistent with those observed in other studies (Perrig *et al.*, 2007; Malhotra and Srivastava, 2008; Spaepen *et al.*, 2008; Masciarelli *et al.*, 2009). *Azospirillum* strains lacked the capacity to produce IAA when grown in minimal medium without addition of L-tryptophan, as also observed in previous studies (Zimmer *et al.*, 1991).

The root colonization ability was affected by inoculation, particularly by the strain with reduced capacity to produce IAA (*ipdC*⁻). The population size achieved by *ipdC*⁻ at 11 days after inoculation was much lower than that achieved by Az39. The colonization ability of *ipdC*⁻ was increased by 1.5% when it was co-inoculated with Az39.

The ion content was modified by the contribution of IAA in the supernatants. These findings indicate a relationship between Ca^{++} and IAA levels. Similarly, Janistyn (1973) showed that IAA increased the flow of Ca^{++} into maize roots. Hasenstein and Evans (1986) suggested that Ca^{++} is important for the activity of IAA in roots because it prevents premature lignification. Votrubová and Votruba (1986) tested two concentrations of IAA (10^{-8} M and 10^{-5} M) and found a direct relationship with growth, water absorption, and incorporation and accumulation of cations. Ca^{++} plays an important role as second messenger in the response of roots to gravitropism under the control of IAA (Lee and Evans, 1985) and is involved in the transport and secretion of this phytohormone (Dela Fuente, 1984).

The uptake of K^+, Na^+, and Ca^{++} occurs through ion channels in the plasma membrane of cells. Certain plant hormones, including IAA, are well known to induce activation of plasma membrane ATPases and a consequent hyperpolarization of the cell membrane. This electrical signaling in plants is essential for the generation of a wide variety of cellular responses based on changes in ion flux, including the efflux of Cl^- and K^+ and the influx of Ca^{++}. K^+, Na^+, and Ca^{++} levels may thus regulate a wide variety of physiological responses in plants, including elongation, respiration, water uptake, phloem unloading, activation of proteinase inhibitor genes, and gas exchange (Bandurski, 1980, 1984; Canny and McCully, 1988; Weyers and Paterson, 2001). Inoculation with *Azospirillum* is repeatedly attributed with increased mineral uptake by the plant. In addition to N availability both in the form of nitrate and ammonium, the colonization is demonstrated to improve the uptake of other mineral nutrients such as Rb^+, $Fe^{2+/3+}$, K^+, H_2PO_4 etc. (Lin *et al.*, 1983).

Root morphology and absorbent hairs showed changes according to the amount of exogenous IAA applied. The right-hand images show cross sections of the roots under various treatments. Inoculation with supernatants of Az39 and Yu62, or with IAA, resulted in a greater cell volume in the endodermis and a bulkier and less compacted pericycle. Treatment with Yu62 supernatant or with IAA also caused increased size of metaxylem cells and loss of the pericycle.

The observed increase in the distribution of root hairs was consistent with the increase in IAA concentration. Cortex cells were larger in mature roots from plants inoculated with Az39 or Yu62, or treated with IAA. We observed a parenchymatous pericycle surrounding the vascular tissue and a higher number of metaxylem vessels as compared with roots treated with supernatants, which had a lower IAA content (Fig. 5). In mature roots, the cortex cells were significantly larger in size and most endodermal cells were thicker.

In agreement with our results, the major mechanism of plant growth promotion by *Penicillium radicum* was suggested to be related to the production of plant growth-promoting metabolites such as auxins (Anstis, 2004), which increase the length and density of fine roots, and enhance the plant's ability to capture available nutrients regardless of increased nutrient availability *per se*. Inoculation with *Penicillium bilaiae* also enhanced the production of root hairs and lateral roots (Gulden and Vessey, 2000; Vessey and Heisinger, 2001) in field peas, and plant growth was promoted by *Penicillium citrinum* through auxin and gibberellins production (Khan *et al.*, 2008). Thus, the synthesis of phytohormones that stimulate root growth may provide an indirect mechanism where by rhizosphere microorganisms facilitate the acquisition of water and mineral nutrients and consequent plant growth (Richardson *et al.*, 2009a).

Conclusions

The present findings indicate that the ability of *Azospirillum* spp. to promote the root growth of maize seedlings depends on the synthesis of IAA and that this hormone is a component of the culture supernatant when NFb medium is supplemented with L-tryptophan. Thus, the presence of the bacteria *per se* is not sufficient to promote the early growth of maize seedlings; IAA produced by the bacteria and released to the medium (supernatant) is required. Bacterial IAA is utilized by the plant mainly for root development and to generate a rich microcosm around the roots which attract more bacteria.

Kloepper and Schroth (1978) proposed the term "plant growth-promoting rhizobacteria" (PGPR) as bacterial species associated with the plant rhizospheres that affect plant growth by producing and releasing secondary metabolites. Bashan and Holguin (1998) proposed two new terms for general scientific use; "biocontrol plant growth-promoting bacteria" (Biocontrol-PGPB) and "plant growth-promoting bacteria" (PGPB) to encompass all the plant beneficial bacteria according to their particular role.

Nevertheless, in this study we found that the effect of a bacterial strain plus its culture supernatant is not synergistic. This observation has important technological implications for inoculants formulation because different concentrations of growth regulators are produced by different strains and under different culture conditions. In view of the diversity of existing inoculants formulations, we propose that phytohormone production (IAA, gibberellins, cytokinins, abscisic acid, jasmonic acid, salicylic acid, and polyamines) should be analyzed in culture supernatants as quality control of inoculants, proposing terminology: quality control of biofertilizers on plant growth regulators (PGRs-QCBio). The presence of the microorganism *per se* does not guarantee the desired plant growth promoting effect. Thus, we propose that an interesting technological implication of our findings consist on the possibility of manipulating and generating better-defined environments for bacterial growth and metabolites production according to their specific requirements. The approach described here provides new useful and promising insights for the inoculants industry.

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