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Effect of mineral fertilizer, pig manure, and *Azospirillum rugosum* on growth and nutrient contents of *Lactuca sativa* L.

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Abstract Benefits from the application of plant growth-promoting bacteria in agriculture largely depend on the complex interactions between several factors including the nature of fertilizers selected. This study was designed to determine the fine tuning between the inoculated bacteria and different fertilizers and their effect on the growth of lettuce plants (*Lactuca sativa* L.). Plant growth promotion by a novel species of the genus *Azospirillum*, namely *A. rugosum* IMMIB AFH-6, was tested by biochemical, bioassay, and greenhouse studies. The treatments used in the greenhouse study were; unfertilized control (Blank), half recommended dose of chemical fertilizer (1/2CF), full recommended dose of chemical fertilizer (1CF), pig manure fertilizer (PMF), pig manure fertilizer+half recommended dose of chemical fertilizer (PMF+1/2CF), and pig manure fertilizer+full recommended dose of chemical fertilizer (PMF+1CF). All these treatments when inoculated with *A. rugosum* IMMIB AFH-6 inoculation were, respectively, In-Blank, In-1/2CF, In-1CF, In-PMF, In-PMF+1/2CF, and In-PMF+1CF. Significant increase in plant biomass and shoot N, P, Ca, and Fe was shown in the In-Blank treatment. Plant growth in soil amended with PMF and *A. rugosum* IMMIB AFH-6 was significantly lower than in soil treated with the chemical fertilizer, but inoculation combined with chemical fertilizer significantly elevated the plant biomass. The In-PMF+1/2CF treatment showed the highest yield. *A. rugosum* IMMIB AFH-6 facilitated the accumulation of trace minerals in higher concentrations when PMF was combined with 1CF. To examine the benefits of inoculation by *A. rugosum* IMMIB AFH-6, we have proposed a new type of data analysis which considers both biomass and nutrient content of plants. This new type of analysis has shown the importance of the mineral content of plant.

Keywords *Azospirillum* · Plant growth promotion · Chemical fertilizer · Organic fertilizer · N₂ fixation · Mineral accumulation

Introduction

Inoculation of soil with plant growth-promoting bacteria (PGPB) is used to increase yield, germination rate, root growth, mineral content, protein content, stress tolerance of plants, and as biocontrol agents (Glick 2004; Bashan and de-Bashan 2005). Use of free-living plant growth-promoting rhizobacteria as inoculants for variety of crops is important and useful in crop productivity and environmental restoration (Vessey 2003). *Azospirillum*, a widely used soil inoculant, is free-living and can promote the growth and yield of numerous agronomically and ecologically important plant species (Bashan et al. 2004). This well-studied PGPB is capable of indole-3-acetic acid (IAA) and gibberllin production, non-symbiotic nitrogen fixation, and can increase the plant mineral uptake and plant growth by increasing the chlorophyll content of plant; in addition, they can colonize plant root (Seshadri et al. 2000; Thuler et al. 2003; Bashan et al. 2006). Determining the fine tuning between inoculated bacteria, different fertilizers, and their effect on plant response is important for assessing the efficiency of the PGPB.

Intensive animal farm operation, in particular, the swine, produces a considerable amount of manure as daily waste,

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which cannot be used as a fresh material due to its odor, presence of volatile phytotoxic compounds, and pathogens. The benefits of inoculation on plant growth depend on the type of fertilizers used. The aim of our research was to characterize a recently described novel species of *Azospirillum* for plant growth-promoting traits to study its effect on plant growth when inoculated with different fertilizers.

Materials and methods

We have used a bacterial strain IMMIB AFH-6 named *Azospirillum rugosum* and isolated from a soil in Taiwan (Young et al. 2008). The DDBJ/EMBL/GenBank accession number for the 16S rRNA gene sequence of the strain IMMIB AFH-6 is AM419042.

Pig manure-based organic fertilizer (PMF) developed in the Chung Hsing University, Taiwan, was used in this study. The EC, pH, total solids, ash content, and chemical analyses of the PMF were carried out as described by Thompson et al. (2002). Microbial respiration was measured using a NaOH trap method (Zibilske 1994) modified according to Thompson et al. (2002). Average daily CO_2–C evolved per gram of total solids (TS) of organic fertilizer was calculated on the basis of 7-day data. Properties of the pig manure fertilizer are reported in Table 1.

Bacterial growth condition

The strain *A. rugosum* IMMIB AFH-6 was grown on Dobereiner nitrogen-free medium (Dobereiner et al. 1976)

Table 1 Physico-chemical characteristics of the organic fertilizer (PMF) used in the study

Parameter	
pH (1:5 *v/v*)	7.1
EC (μS cm^{-1}) (1:5 *v/v*)	2,510
CO_2 evolution rate (mg CO_2–C g^{-1} TS d^{-1})	6.1
Organic matter (%)	72.0
Humic acid (%)	9.2
Total N (%)	2.4
P_2O_5 (%)	8.5
C/N	17.5
K_2O (%)	1.5
CaO (%)	4.3
Mg (%)	1.5
Fe (%)	1.3
Mn (%)	0.05
S (%)	0.49
Cu (mg kg^{-1})	174.5
Zn (mg kg^{-1})	1,088.1

at 30°C on a shaker at 150 rpm. After 48 h, cells were harvested by centrifugation (6,000 $\times g$ for 10 min). Cell pellet was washed twice with sterile water. Washed cells were re-suspended in sterile water and used as inoculum for both in vitro seed germination bioassay and greenhouse pot inoculation. Bacterial concentration was determined by dilution plating on Congo red plates (Rodrıguez-Caceres 1982).

Identification of plant growth-promoting traits

A. rugosum IMMIB AFH-6 was tested for nitrogen-fixing activity by acetylene reduction assay (Holguin et al. 1992). The organism was cultivated at 30°C in semisolid nitrogen-free media in culture tubes sealed with a rubber stopper, and 10% of the tube atmosphere was replaced by acetylene after 48 h when the cell density was 4×10^8 CFU ml^{-1}. Culture tubes without acetylene were used as controls. Ethylene production was detected after 24 h of incubation at 30°C using a gas chromatograph (Hitachi 163, Japan) equipped with flame ionization detector. Rate of C_2H_2 reduction by *A. rugosum* IMMIB AFH-6 per milliliter of culture was calculated from the total C_2H_4 produced. Mineral phosphate solubilization (MPS) activity was tested on tricalcium phosphate agar plates (Nautiyal 1999). Inoculated plates were incubated at 30°C for 48–72 h. Clear zone around the colonies was considered as positive for MPS activity. IAA was quantified by colorimetric analysis (Gordon and Weber 1951). In brief, bacteria were grown for 72 h in nutrient broth supplemented with L-tryptophan (500 mg l^{-1}) as IAA precursor. Supernatants were obtained after centrifugation of cell cultures at 10,000 $\times g$ for 5 min. Two milliliters of Salkowski reagent (in perchloric acid) were added to 1 ml of a culture supernatant in a glass test tube and incubated at room temperature for 30 min. The optical density was quantified using a UV–visible spectrophotometer (Hitachi U-3010) at 530 nm. The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase was assayed by measuring the amount of a-ketobutyrate produced from cleavage of ACC (Penrose and Glick 2003). Siderophore production was detected in chrome azurol S agar plates (Milagres et al. 1999). Nitrate reduction was tested by API-20NE test strips (bioMerieux) according to the methods outlined by the manufacturer.

In vitro seed germination bioassay

Preliminary evaluation of the plant growth promotion by the isolate, *A. rugosum* IMMIB AFH-6, was carried out using seed germination bioassay. Lettuce (*Lactuca sativa* L. cv. Taiwan sword leaf) and Radish (*Raphanus sativus* L. cv. *longipinnatus*) seeds were purchased from Known-You

seed company (Taiwan). The seeds were surface sterilized with 1% sodium hypochlorite solution for 30 min and washed several times with sterile water. Twelve seeds of lettuce or radish were arranged randomly over two sheets of 70-mm sterile filter papers (Advantec, Toyo, Roshi Kaisha Ltd., Japan) soaked with 2 ml of *A. rugosum* IMMIB AFH-6 cell suspension (10^8 CFU ml^{-1}) and placed in a petriplate. Control treatment received 2 ml of sterile water. Each treatment was replicated four times. The plates were incubated at 28°C under dark condition. Number of seeds germinated and length of root and shoot were recorded after 72 h. Germination rate in the inoculated treatments was calculated as the percentage of the seeds germinated under control conditions. Root and shoot elongation rates were similarly calculated.

Pot experiment in greenhouse: experiment design and treatment conditions

Pot experiment in greenhouse was performed at the Department of Soil and Environmental Science, National Chung Hsing University under greenhouse conditions to investigate the interaction of mineral, organic, and a combination of both fertilizers with *A. rugosum* IMMIB AFH-6 inoculation on lettuce (*L. sativa* L. cv. Taiwan sword leaf) yield. A pig manure fertilizer free of pathogens and recommended dose of N, P, and K as chemical fertilizer were used as suggested by the Department of Agriculture & Forest, Taiwan Province Government. Nitrogen, P, and K were applied at a rate of 100, 50, and 90 kg ha^{-1} as urea, single super phosphate, and potassium chloride, respectively. A $3 \times 2 \times 2$ factorial arrangement of treatments was employed. Hence, 12 treatments represent three levels (i.e., zero, half, and full recommended dose of NPK) of chemical fertilizer (CF), with (5 g kg^{-1}) or without organic fertilizer (PMF) and with or without inoculation. Each treatment was replicated four times. The treatments and their abbreviations (in parentheses) are as follows: no fertilizers and no inoculation (Blank); inoculation and no fertilizers (In-Blank); pig manure fertilizer (PMF); pig manure fertilizer and inoculation (In-PMF); half dose of chemical fertilizer (1/2CF); half dose of chemical fertilizer and inoculation (In-1/2CF); pig manure fertilizer, half dose of chemical fertilizer (PMF+1/2CF); inoculation, pig manure fertilizer, and half dose of chemical fertilizer (In-PMF+1/2CF); full dose of chemical fertilizer (1CF); inoculation and full dose of chemical fertilizer (In-1CF); pig manure fertilizer and full dose of chemical fertilizer (PMF+1CF); inoculation, pig manure fertilizer, and full dose of chemical fertilizer (In-PMF+1CF).

Soil sample, collected from the top layer (0–15 cm) of an experimental farm located in Guoshing, Taiwan, was used. The main soil properties were: pH, 7.5; EC, 800 μS cm^{-1};

soil organic C, 1.68%; total N, 1.4 g kg^{-1}; Bray-1 P, Mehlich's K, Ca, Mn, Fe, Zn, and Cu were respectively 11.0, 7.0, 339.0, 5.47, 22.0, 0.35, and 0.08 mg kg^{-1}. Experiments were conducted in 48 pots (10 cm height and 7 cm diameter on the top and 5 cm in the bottom) containing 1 kg of air-dried and sieved (4 mm mesh) soil. Soil moisture was maintained at 25% during the experiment and for this reason pots were watered regularly and uniformly. Fertilizers were applied prior to seeding. Five pre-soaked seeds per pot were sown 0.5 cm below the soil surface. *A. rugosum* IMMIB AFH-6 cell suspension in 10 ml sterile water (10^8 CFU ml^{-1}) was added to the soil immediately after seeds were sown. Seven days after sowing, the excess seedlings were thinned to only two plants per pot. Greenhouse conditions during the experiment were as follows: photoperiod 13 h; average relative humidity 78%; and average temperatures 25.7°C day/16.5°C night. Pest infestation monitored throughout the experiment gave a negative response. The plants were harvested on the 40th day after sowing.

Plant analyses

Soon after the harvest, shoot samples were washed and dried at 65°C, cooled to room temperature, and weighed. The dried samples were powdered and sieved (0.5 mm) before the chemical analyses. The plant N content was measured by Kjeldahl method. The contents of P, K, Ca, Mg, Fe, Mn, Cu, and Zn were measured by inductively coupled plasma–atomic emission spectrometry (ICP–AES) with a sequential Jobin Yvon JY 138 Ultrace spectrometer (Faithfull 2002).

Microbiological analyses of soil

Rhizosphere soil and root samples (three sub-samples each) were collected aseptically immediately after harvest from each pot for quantifying the rhizosphere and root colonizing (rhizoplane) bacteria. Pre-weighed soil and root samples ($n=3$) were mixed in sterile water (1:10 *w/v*). Samples were then serially diluted and plated on tryptic soy agar medium (Difco). Cell counts were presented as log CFU g^{-1} rhizosphere soil d.w. and log CFU g^{-1} root d.w. (rhizoplane bacteria). Estimation of *A. rugosum* IMMIB AFH-6 was made from about 1 g dry weight equivalent soil including the roots randomly collected from all the pots (three sub-samples each). Soil and roots were suspended in 10 ml sterile water, homogenized, serially diluted, and plated on Nfb medium with 2% (*w/v*) agar. To confirm, representatives of *A. rugosum* IMMIB AFH-6-like colonies were sub-cultured and analyzed by 16S rRNA sequencing. Cell counts were represented as log CFU g^{-1} soil d.w.

Soil organic carbon analysis

Total soil organic carbon content was analyzed by the loss on ignition method (Ben-Dor and Banin 1989). Briefly, a weighed amount of oven-dried (105°C 24 h) soil sample was placed in a high-form porcelain crucible and set in a muffle furnace (±5°C precision) for combustion at 400°C for 4 h. Organic carbon was determined by the mass difference.

Statistical analysis

Mean and standard deviation values of data were calculated from at least four replicates. One-way analysis of variance (ANOVA) was used for the seed germination bioassay and soil organic carbon data. Data on the plant growth parameters (greenhouse study) were analyzed by three-way ANOVA. Interaction effects for treatment factors, namely chemical fertilizer, organic fertilizer, and inoculation rate, were analyzed. Data on root colonization and rhizosphere bacteria were log transformed prior to statistical analyses. All the statistical analyses were performed using software package STATISTICA (Stat Soft Inc. 1998). A p value <0.01 was considered as significant throughout unless specified.

Results

Plant growth promotion by *A. rugosum* IMMIB AFH-6 and its characteristics

A. rugosum IMMIB AFH-6 showed plant growth-promoting traits similar to other representatives of the genus *Azospirillum*. The isolate was able to produce IAA (7.1± 1.2 μg ml^{-1}) when the media was supplemented with tryptophan (500 mg l^{-1}). The isolate showed free-living nitrogen fixation (93.1±24.7 nmol C_2H_2 h^{-1} ml^{-1} culture (~10^8 CFU)), solubilized mineral P, and showed nitrate reductase activity (API-20 NE), but it did not show ACC deaminase activity and siderophore production.

Root elongation, shoot elongation, and germination rates were significantly ($p<0.01$) greater in both verities of seeds after inoculation with *A. rugosum* IMMIB AFH-6 than in the non-inoculated control (Fig. 1).
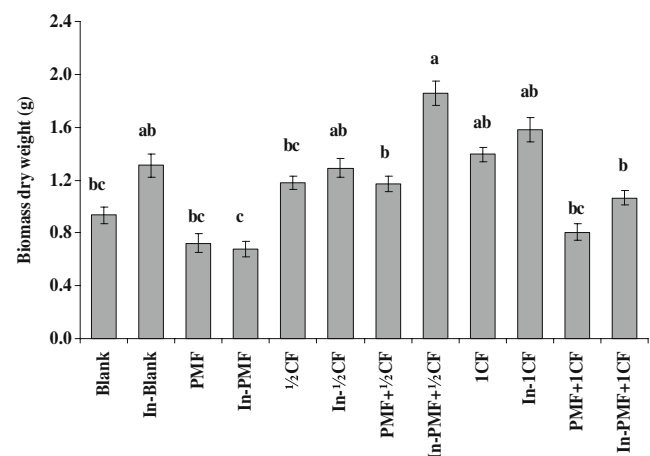
Source of variation	d.f.	F	P-level
CF	2	123.14	<0.0001
PMF	1	184.00	<0.001
In	1	648.53	<0.001
CF x PMF	2	137.58	<0.00001
CF x In	2	67.09	<0.001
PMF x In	1	2.54	ns
CF x PMF x In	2	75.20	<0.001

Fig. 2 Effect of interaction between different fertilizers and *A. rugosum* IMMIB AFH-6 inoculation on shoot yield. Standard deviation (n=4) is presented as *bars*. *Columns in the chart with different lowercase letters are significantly different at $p<0.01$.* The nested table illustrates variation in the interaction between chemical fertilizer (CF), organic fertilizer (PMF), and inoculation (In) as tested by three-way ANOVA. ns: p value not significant. Treatments are: Blank, no fertilizer; In-Blank, inoculation and no fertilizers; PMF, pig manure fertilizer; In-PMF, inoculation and PMF; 1/2CF, half dose of chemical fertilizer; In-1/2CF, inoculation and 1/2CF; PMF + 1/2CF, pig manure fertilizer and half dose of chemical fertilizer; In-PMF + 1/2CF, inoculation and PMF+1/2CF, 1CF, full dose of chemical fertilizer; In-1CF, inoculation and 1CF; PMF+1CF, pig manure fertilizer and full dose of chemical fertilizer; In-PMF+1CF, inoculation and PMF+1CF
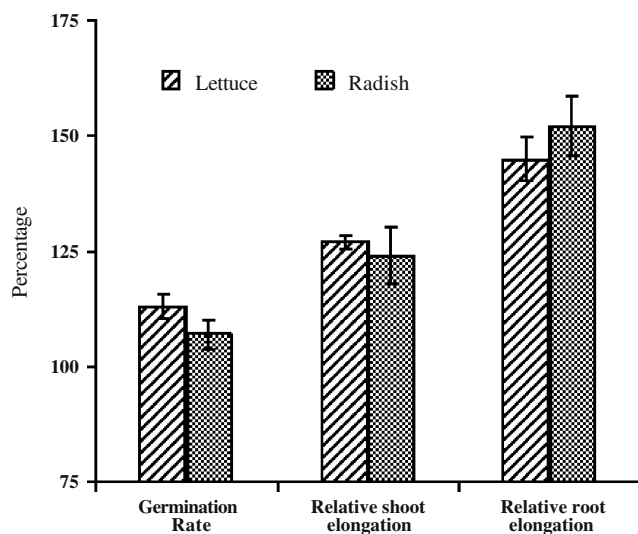
Fig. 1 Germination rate and relative shoot and root elongation of radish and lettuce seeds germinated in vitro in presence of *Azospirillum rugosum* IMMIB AFH-6 ($1×10^8$ CFU ml^{-1}). Standard deviation (n=4) is presented as *bars*. Germination rate = $\frac{\text{Number of seeds germinated in inoculated}}{\text{Number of seeds germinated in control}} \times 100$, Relative shoot (or root) elongation = $\frac{\text{Average shoot (or root) length in inoculated}}{\text{Average shoot (or root) length in control}} \times 100$

Yield and nutrient contents of lettuce

Dry matter yield and mineral nutrient levels of lettuce plants as affected by different fertilizers and inoculation are shown in Fig. 2 and Tables 2 and 3. Plant response to increasing chemical fertilizer was significant with some exceptions between 1CF and 1/2CF. The yield and the N and P contents showed significant increase by increasing the chemical fertilizer dose. Significant increase in the P, Ca, and Fe contents in most of the treatments with PMF was observed. But significant decrease in the yield was recorded in PMF and PMF+1CF treatments compared to other treatments.

The interaction between inoculation and chemical fertilizer was highly significant. The plant yield of the inoculated treatments was significantly higher than their respective non-inoculated treatments. Increase in the yield was by 41% in the In-Blank, by 14% in the In-1CF, and by 9% in the In-1/2CF treatments. Responses of most of the studied parameters (yield and plant contents of N, P, Ca, Mg, and Fe) were positive when inoculation was used. Interaction between chemical fertilizer, organic fertilizer, and inoculation showed significant effects on yield and nutrient contents of plant. The yield increased significantly in the In-PMF+1/2CF compared to all other treatments. Inoculation increased the N levels significantly in In-PMF+1/2CF and In-PMF+1CF treatments compared to their respective non-inoculated treatments, but none of the combined treatments showed N levels similar to that of 1CF. Zinc concentrations in all inoculated treatments were significantly higher compared to the respective non-inoculated treatments.

Bacterial counts in rhizosphere soil

Bacterial counts of soil sampled immediately after harvest showed that *A. rugosum* IMMIB AFH-6 in the inoculated treatments ranged from 4.1 to 6.0 log CFU g^{-1} soil (Table 4). None of the non-inoculated soils showed the presence of *Azospirillum*. Bacterial counts of rhizosphere soil were significantly higher in In-Blank, PMF+1/2CF and 1/2CF than the other treatments. Only in the PMF the bacterial counts were lower than 8.0 log CFU g^{-1} soil d.w.

Soil organic carbon

The initial organic carbon content of soil was 1.68%. After the plant harvest, the PMF-fertilized soils showed signifi-

Table 2 Effect of inoculation and fertilization on macronutrient contents of the lettuce plants grown under greenhouse conditions

Treatments	N (g kg^{-1} plant dry biomass)	P (g kg^{-1} plant dry biomass)	K (g kg^{-1} plant dry biomass)	Ca (g kg^{-1} plant dry biomass)
Blank	20.9±0.62	2.7±0.02	52.2±4.78	8.2±0.33
In-Blank	27.1±0.31	3.1±0.03	48.9±5.91	9.0±0.01
PMF	17.2±4.91	3.9±0.08	51.2±3.57	9.3±0.25
In-PMF	17.0±0.73	4.4±0.01	55.2±0.58	9.4±0.17
1/2CF	33.9±4.72	3.2±0.12	55.0±3.98	9.4±0.31
In-1/2CF	33.4±1.21	3.1±0.21	52.3±1.36	9.4±0.21
PMF+1/2CF	16.5±0.26	4.3±0.14	53.3±1.52	9.7±0.51
In-PMF+1/2CF	20.7±3.42	3.7±0.03	54.1±5.21	9.2±0.45
1CF	34.3±1.46	3.2±0.08	54.7±1.12	8.9±1.0
In-1CF	29.2±3.92	3.2±0.22	52.4±0.56	9.1±0.22
PMF+1CF	17.6±0.83	3.7±0.11	52.7±1.03	9.3±1.14
In-PMF+1CF	19.0±2.78	3.6±0.35	53.14±1.61	8.8±0.37
Source of variation[a]	*F* (*p*-level)	*F* (*p*-level)	*F* (*p*-level)	*F* (*p*-level)
CF (d.f. 2)	14.65 (<0.01)	1.30 (ns)	23.43 (<0.01)	4.24 (ns)
PMF (d.f. 1)	237.48 (<0.001)	327.97 (<0.001)	43.21 (<0.01)	20.87 (<0.05)
In (d.f. 1)	4.50 (ns)	0.58 (ns)	2.50 (ns)	0.002 (ns)
CF×PMF (d.f. 2)	94.56 (<0.001)	8.14 (<0.05)	11.11 (<0.01)	8.90 (<0.05)
CF×In (d.f. 2)	1.81 (ns)	4.05 (ns)	1.22 (ns)	14.70 (<0.01)
PMF×In (d.f. 1)	0.88 (ns)	2.12 (ns)	48.45 (<0.01)	8.81 (ns)
CF×PMF×In (d.f. 2)	7.93 (<0.02)	1.04 (ns)	12.38 (<0.01)	0.10 (ns)

Values are mean±SD (*n*=4). Treatments are: *Blank*, no fertilizer; *In-Blank*, inoculation and no fertilizers; *PMF*, pig manure fertilizer; *In-PMF*, inoculation and PMF; *1/2CF*, half dose of chemical fertilizer; *In-1/2CF*, inoculation and 1/2CF; *PMF+ 1/2CF*, pig manure fertilizer and half dose of chemical fertilizer; *In-PMF+ 1/2CF*, inoculation and PMF+1/2CF; *1CF*, full dose of chemical fertilizer; *In-1CF*, inoculation and 1CF; *PMF+ 1CF*, pig manure fertilizer and full dose of chemical fertilizer; *In-PMF+ 1CF*, inoculation and PMF+1CF
[a] Interaction between chemical fertilizer (CF), organic fertilizer (PMF), and inoculation (In) are tested by three-way ANOVA. *ns*: *p* value not significant

Table 3 Effect of inoculation and fertilization on micro nutrient content of the lettuce plants grown under greenhouse conditions

Treatments	Mg (μg g^{-1} plant dry biomass)	Fe (μg g^{-1} plant dry biomass)	Zn (μg g^{-1} plant dry biomass)	Cu (μg g^{-1} plant dry biomass)
Blank	3.2±0.01	51.3±8.82	86.4±21.18	8.3±0.01
In-Blank	4.0±0.22	172.5±17.67	97.6±1.32	8.3±0.01
PMF	3.2±0.14	177.5±66.72	32.6±15.85	7.6±0.36
In-PMF	3.2±0.03	95.0±31.83	63.9±14.08	7.6±0.36
1/2 CF	4.0±0.67	155.0±17.69	90.9±3.53	5.8±0.02
In-1/2CF	4.3±0.32	190.0±98.02	148.9±49.45	5.8±0.01
PMF+1/2CF	3.5±0.09	167.5±59.13	83.9±48.52	7.6±0.18
In-PMF+1/2CF	3.6±0.22	111.3±15.89	125.1±5.34	8.3±0.01
1CF	3.3±0.28	127.5±10.56	73.5±52.20	8.0±0.18
In-1CF	3.7±0.21	117.5±42.24	146.4±54.28	8.3±0.02
PMF+1CF	2.9±0.72	141.3±40.67	90.7±56.89	3.9±0.36
In-PMF+1CF	3.3±0.36	305.0±96.78	158.9±41.32	6.4±0.40
Source of variation[a]	F (p-level)	F (p-level)	F (p-level)	F (p-level)
CF (d.f. 2)	91.8 (<0.001)	6.0 (<0.05)	21.9 (<0.01)	18.7 (<0.01)
PMF (d.f. 1)	45.9 (<0.01)	15.6 (<0.05)	0.87 (ns)	516 (<0.001)
In (d.f. 1)	14.79 (<0.05)	3.5 (ns)	90.7 (<0.01)	41.4 (<0.01)
CF×PMF (d.f. 2)	3.14 (ns)	6.2 (<0.05)	5.8 (<0.05)	8.8 (<0.05)
CF×In (d.f. 2)	0.16 (ns)	12.4 (<0.01)	2.4 (ns)	87.1 (<0.001)
PMF×In (d.f. 1)	203.5 (<0.001)	2.0 (ns)	0.1 (ns)	10.1 (ns)
CF×PMF×In (d.f. 2)	4.93 (ns)	45.0 (<0.001)	0.3 (ns)	36.7 (0.001)

Values are mean (SD); $n=4$. Treatments are: *Blank*, no fertilizer; *In-Blank*, inoculation and no fertilizers; *PMF*, pig manure fertilizer; *In-PMF*, inoculation and PMF; *1/2CF*, half dose of chemical fertilizer; *In-1/2CF*, inoculation and 1/2CF; *PMF+ 1/2CF*, pig manure fertilizer and half dose of chemical fertilizer; *In-PMF+ 1/2CF*, inoculation and PMF+1/2CF; *1CF*, full dose of chemical fertilizer; *In-1CF*, inoculation and 1CF; *PMF+ 1CF*, pig manure fertilizer and full dose of chemical fertilizer; *In-PMF+ 1CF*, inoculation and PMF+1CF
[a] Interaction between chemical fertilizer (CF), organic fertilizer (PMF), and inoculation (In) are tested by three-way ANOVA. *ns*: p value not significant

cantly the highest levels of soil organic C (Fig. 3). Soil organic matter contents did not show any significant differences among non-PMF treatments.

Discussion

Inoculation with *A. rugosum* IMMIB AFH-6 promoted plant growth as revealed by an increase in the root and shoot lengths (in the seed germination bioassay) shoot biomass and mineral accumulation (greenhouse experiment). Plant growth promotion by *Azospirillum* probably depended on the production of phytohormones such as IAA, N fixation, and stimulation of plant nutrient uptake (Bashan 1999; Bashan et al. 2004). Promotion of early growth in the seedlings (both lettuce and radish) was mainly caused by the phytohormone IAA. Biochemical analyses showed that plant growth-promoting traits of *A. rugosum* IMMIB AFH-6 were comparable to those of *Azospirillum* species.

The yield decline with organic fertilizer may be attributed to several adverse effects of organic manure. According to Cooperband et al. (2003), the benefits of organic fertilizer on plant growth depend mainly on the quality of the used fertilizer. The use of fresh manure, although economical with some benefits on plant yield and soil health, is not suggested mainly due to the presence of pathogens with possible pathogen colonization of soil after application (Vanotti et al. 2005). Release of nutrients from the composts and processed organic manures are generally slower than nutrient release from mineral fertilizer (Adegbidi et al. 2003) and this can affect plant growth. In addition, the high EC of the pig manure, due to the high salt content, can negatively affect plant growth via imbalanced cation exchange (Lee et al. 2000). Another reason for the reduced plant growth with the organic fertilizer could be the presence of phytotoxic compounds due to the improper stabilization of organic matter (Wu and Ma 2002; Wang et al. 2004). However, the use of by-products of pig farming in agriculture is useful since it can increase the soil organic matter content. Organic compounds of soil can chelate cations linked to phosphates thus preventing their precipitation as for example calcium phosphates (Alvarez et al. 2004). This can explain the increased accumulation of P, Ca, and Fe in the plants treated with PMF.

Plant growth promotion by diazotrophs is due to the enhancement of plant nutrient uptake by altering the root morphology and by stimulating the ion uptake systems

Table 4 Effect of fertilization on bacterial counts of the rhizosphere soil and rhizoplane

Treatments	Rhizosphere bacteria (log CFU g^{-1} soil d.w.)	Rhizoplane bacteria (log CFU g^{-1} root d.w.)	*A. rugosum* IMMIB AFH-6 (log CFU g^{-1} soil d.w.)
Blank	8.09	7.86	Nil
In-Blank	11.40	8.56	5.60
PMF	7.61	7.84	Nil
In-PMF	8.96	8.36	4.10
1/2CF	10.77	10.81	Nil
In-1/2CF	8.90	7.19	5.40
1CF	9.39	8.74	Nil
In-1CF	8.10	8.30	5.30
PMF+1/2CF	10.85	12.11	Nil
In-PMF+1/2CF	8.62	7.82	6.00
PMF+1CF	9.09	8.86	Nil
In-PMF+1CF	8.03	8.05	4.80
Source of variation[a]	*F* (*p*-level)	*F* (*p*-level)	*F* (*p*-level)
CF (d.f. 2)	41.1 (<0.001)	16.14 (<0.01)	1.46 (ns)
PMF (d.f. 1)	81.6 (<0.01)	5.06 (ns)	6.9 (ns)
In (d.f. 1)	3.1 (ns)	278.1 (<0.001)	–
CF×PMF (d.f. 2)	5.6 (<0.05)	11.72 (<0.01)	6.65 (<0.05)
CF×In (d.f. 2)	814 (<0.001)	74.3 (<0.001)	–
PMF×In (d.f. 1)	4.9 (ns)	8.1 (ns)	–
CF×PMF×In (d.f. 2)	21.34 (<0.01)	0.12 (ns)	–

Treatments are: *Blank*, no fertilizer; *In-Blank*, inoculation and no fertilizers; *PMF*, pig manure fertilizer; *In-PMF*, inoculation and PMF; *1/2CF*, half dose of chemical fertilizer; *In-1/2CF*, inoculation and 1/2CF; *PMF+1/2CF*, pig manure fertilizer and half dose of chemical fertilizer; *In-PMF+ 1/2CF*, inoculation and PMF+1/2CF; *1CF*, full dose of chemical fertilizer; *In-1CF*, inoculation and 1CF; *PMF+1CF*, pig manure fertilizer and full dose of chemical fertilizer; *In-PMF+1CF*, inoculation and PMF+1CF

[a] Interaction between chemical fertilizer (CF), organic fertilizer (PMF), and inoculation (In) are tested by three-way ANOVA. *ns*: *p* value not significant
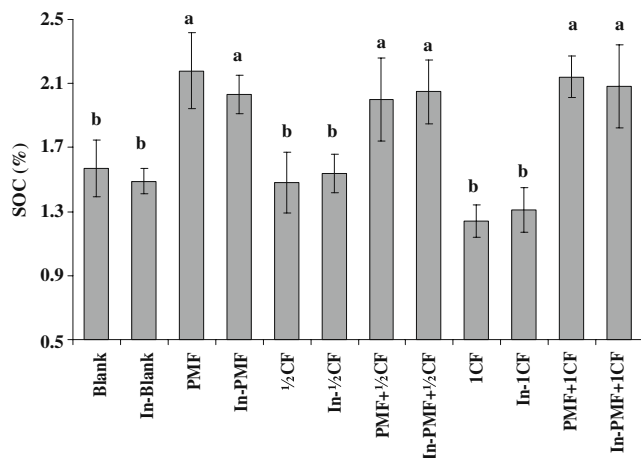

Fig. 3 Soil organic carbon content after the addition of organic fertilizer. *Columns in the chart with different lowercase letters* are significantly different at *p*<0.01. Treatments are: Blank, no fertilizer; In-Blank, inoculation and no fertilizers; PMF, pig manure fertilizer; In-PMF, inoculation and PMF; 1/2CF, half dose of chemical fertilizer; In-1/2CF, inoculation and 1/2CF; PMF+1/2CF, pig manure fertilizer and half dose of chemical fertilizer; In-PMF+1/2CF, inoculation and PMF+1/ 2CF; 1CF, full dose of chemical fertilizer; In-1CF, inoculation and 1CF; PMF+1CF, pig manure fertilizer and full dose of chemical fertilizer; In-PMF+1CF, inoculation and PMF+1CF

(Bloemberg and Lugtenberg 2001; Mantelin and Touraine 2004). It has been shown that, in addition to IAA, *Azospirillum* can produce nitric oxide (NO), which has a direct role in the lateral root development of plant (Creus et al. 2005). We have observed that, with the exception of the In-PMF treatment, all inoculation with *A. rugosum* IMMIB AFH-6 increased plant yield and the plant content of one or more nutrients among N, P, K, Fe, Ca, Mg, Zn, and Cu, thus confirming what was reported by Bashan et al. (1990) and Okon and Vanderleyden (1997) with *Azospirillum*-inoculated plants. Inoculation with *Azospirillum* can lower the pH of the rhizosphere soil by affecting the proton and organic acid extrusion of plants and can increase the availability of phosphorus and iron to plants (Carrillo et al. 2002). Lower doses of N fertilizer can increase the microbial protease activities with an efficient turnover of applied organic N (Schloter et al. 2003). Kamnev et al. (2005, 2006) observed accumulation of trace metals in *Azospirillum brasilense* cells when cultures were treated with trace metals. Stimulation of plant nutrient uptake by *Azospirillum* may be a possible reason for the higher concentration of minerals in the inoculated plants. These results show that plant growth promotion might be caused

by the cumulative effects of more than one factor as observed in earlier studies (Lippmann et al. 1995). An additive hypothesis has been already proposed as a possible mechanism of plant growth promotion by *Azospirillum* (Bashan and Levanony 1990; Bashan et al. 2004).

Inconsistent results of plant growth and yield were evidenced when *Azospirillum* was used under different conditions (Bashan et al. 1989; Okon and Labandera-Gonzalez 1994). In this study, *A. rugosum* IMMIB AFH-6 inoculation improved the nutrient accumulation with fertilizer application and the highest yield was observed in the In-1/2CF+PMF treatment. Under well-fertilized conditions, the yield increase was not directly related to overall accumulation of nutrients in the plant tissue. It is possible that the concentration of certain nutrient ions in tissues will down-regulate the absorption of others as it occurs for NO_3^-. Indeed, external presence of NO_3^- stimulated the lateral root elongation (Zhang et al. 1999), while the accumulation of NO_3^- in shoots led to the reduction in the number of lateral roots and stunting their development at some stage before or after emergence (Tranbarger et al. 2003). Comparatively lower concentrations of N content in some of the inoculated treatments can be attributed to the nitrate reductase activity of the *A. rugosum* IMMIB AFH-6 and to the decreased N fixation due to nitrogenase inhibition by higher concentration of readily available NO_3^-–N (Nelson 1987). Nitrite (NO_2^-) can catalyze the degradation of IAA, blocking the IAA-mediated plant growth promotion by *Azospirillum* (Tanner and Anderson 1964). Certain PGPB species with nitrate reductase activity can use NO_3^- as an N source and may decrease NO_3^- concentration at the root surface (Larcher et al. 2003). Nitric oxide produced by *Azospirillum* under aerobic conditions at lower concentrations will have direct benefit on the plant (Molina-Fevero et al. 2007). But, exogenous presence of higher concentration of NO will diminish the primary root growth (Correa-Aragunde et al. 2004). All the abovementioned hypotheses should be verified by further research.

Inoculation with *A. rugosum* IMMIB AFH-6 increased the shoot biomass in almost all the cases but overall concentration of the mineral per unit varied. If the plant response is calculated as a factor of increase in total biomass, from our data, the 41% percent increase in plant biomass in the inoculated unfertilized pots (In-Blank) may be due to 30%, 13%, 9%, 27%, 237%, and 13% increase (compared to the respective non-inoculated treatment) in N, P, Ca, Mg, Fe, and Zn contents, respectively. Contrarily, the higher increase by 60% in the biomass of the In-PMF+1/2CF treatment compared with PMF+1/2CF treatment was not due to the increase in all nutrient concentrations but only to the increase of N and Zn (25% and 49%, respectively) compared with their respective concentration of the non-inoculated plants. This comparison can underestimate the overall benefits of inoculation. For this reason, we propose a new method of data analysis wherein the inoculation effect can be assessed. The relative nutrient accumulation rate can be calculated by the following relationship:

$$\frac{\text{Biomass in inoculated}}{\text{Biomass in non}-\text{inoculated}} \times \frac{\text{Mineral content in inoculated}}{\text{Mineral content in non}-\text{inoculated}} \times 100$$

From this relationship, we could observe the direct role of inoculation on the overall nutrient accumulation rate with respect to their non-inoculated treatments (Table 5). The highest value was observed in the In-Blank, In-PMF+1/2CF and In-PMF+1CF treatments. Inoculation facilitated in the plant yield and mineral content by some selected mechanisms or by a cascade of mechanisms operating simultaneously under suitable conditions. Mantelin and Touraine (2004) reported that plant response to inoculation can be the additive effect of biotic and abiotic factors present in the rhizosphere. Inoculation of unfertilized pots with *A. rugosum* IMMIB AFH-6 stimulated the plant growth and mineral uptake very efficiently thus emphasizing the direct role of *Azospirillum* on nutrient accumulation. Dobbelaere et al. (2002) observed similar plant response to inoculation with *A. brasilense* and *A. irakense* under low N

Table 5 The relative nutrient accumulation rates

Treatments with inoculation	N %[a]	P %[a]	K %[a]	Ca %[a]	Mg %[a]	Fe %[a]	Zn %[a]	Cu %[a]
In-Blank	183	159	132	154	178	474	159	141
In-PMF	93	104	102	95	92	51	185	94
In-1/2CF	108	106	104	109	117	134	179	117
In-PMF+1/2CF	200	138	162	151	163	106	237	159
In-1CF	97	111	109	116	128	105	226	134
In-PMF+1CF	143	128	133	126	152	285	231	206

Treatments are: *In-Blank*, inoculation and no fertilizers; *In-PMF*, inoculation and pig manure fertilizer; *In-1/2CF*, inoculation and half dose of chemical fertilizer; *In-PMF+1/2CF*, inoculation and pig manure fertilizer and half dose of chemical fertilizer; *In-1CF*, inoculation and full dose of chemical fertilizer; *In-PMF+1CF*, inoculation and pig manure fertilizer and full dose of chemical fertilizer

[a] The relative nutrient accumulation rate was calculated by the following relationship: $\frac{\text{Biomass in inoculated}}{\text{Biomass in non}-\text{inoculated}} \times \frac{\text{Mineral content in inoculated}}{\text{Mineral content in non}-\text{inoculated}} \times 100$

supply. Fertilization provides the inoculated bacteria with easily available mineral nutrition reducing its dependency on plant root exudates (Singh et al. 2004). But under suitable fertilizer conditions, as in the case of In-PMF+1/2CF and In-PMF+1CF, significant inoculation effect was obtained due to the multiple mechanisms operating favorably as hypothesized earlier by Bashan et al. (2004, 2006).

In conclusion, understanding the interactions of different fertilizers in the rhizosphere helps to resolve the inconsistency in plant growth and nutrient accumulation in response to the inoculated PGPB. *A. rugosum* IMMIB AFH-6 proved to be a potential plant growth-promoting bacteria in vitro and in greenhouse under different fertilizer conditions. The new method of data analysis suggested here for evaluating the efficiency of the PGPB highlighted the benefits of inoculation in terms of plant yield and nutrient accumulation and will certainly be useful in further studies.

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