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Influence of Azospirillum inoculation on the mineral uptake and growth of rice under hydroponic conditions

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Abstract

Seedlings of rice (*Oryza sativa* L. var. IR42) were inoculated with nitrogen-fixing *Azospirillum lipoferum* (strain 34H) by immersing the roots in the inoculum for 6 h. The plants were grown in the prescence of NH_4^+-N for 47 days in a hydroponic system under greenhouse conditions. Inoculation significantly enhanced PO_4-ion uptake of the plants in 4 of the 7 samplings tested, while the uptake of NH_4-ion was significantly increased in two samplings and was decreased in one sampling. Inoculation reduced root length significantly and caused significant increases in shoot fresh and dry weights. Root surface area was not affected by inoculation. Bacterial population counts suggested that *A. lipoferum* survived on the roots till the end of the experiment.

Introduction

Several reports indicate significant increases in plant growth, total N, and yield of wheat and various grasses following inoculation with N_2-fixing Azospirillum species (Boddey and Döbereiner, 1982; Patriquin *et al.* 1983). However, recent reports suggest that N_2 fixation is not the sole cause of growth responses in Azospirillum-inoculated plants (Kapulnik *et al.*, 1985a; Smith *et al.* 1984). Positive host responses were observed at early growth stages when N_2-fixing activity was very low (Kapulnik *et al.*, 1985c), in the presence of high levels of N fertilizers (Reynders and Vlassak, 1982; Kapulnik *et al.*, 1983), and at temperatures that were nonconducive for Azospirrilum to fix nitrogen (Kapulnik *et al.*, 1985b). It has been suggested that these bacteria elicit host growth responses by producing growth-promoting substances (Okon and Kapulnik., 1986; Tien *et al*, 1986) and enhancing mineral uptake by the roots (Kapulnik *et al.*, 1985b; Lin *et al.*, 1983). Significant increases in root elongation and root surface area concomitant with enhanced mineral uptake were also reported in wheat after inoculation with a mixture of *A. brasilense* strains (Kapulnik *et al.*, 1985b; 1985c).

Literature on the above subject is, however, limited and confined mostly to wheat and *A. brasilense* associations. In the present study, *A. lipoferum* strain 34H, an N_2-fixing isolate from the rhizosphere of wetland rice (Ladha *et al.*, 1982) was used. Previous inoculation of rice with this organism in pot culture experiments using ^{15}N and acetylene reduction techniques indicated that the increased yields obtained were not due to N_2 fixation (Nayak *et al*, 1986; Watanabe and Lin, 1984). We have, therefore, undertaken the present investigation, using a hydroponic system to check whether inoculation of rice with this organism (in the presense of combined N) affects mineral uptake, such as NH_4- and PO_4-ions, and overall vegetative growth of the plants.

Materials and methods

Bacterium

Azospirillum lipoferum strain 34H was earlier isolated from rice roots (Ladha *et al.*, 1982). A spontaneous mutant strain was selected that was resistant to streptomycin (300 $\mu g\,ml^{-1}$) with unimpaired nitrogenase activity.

Plant material

Rice (*Oryza sativa* L.) seeds of cultivar IR42 were obtained from the germplasm unit of the International Rice Research Institute (IRRI), Philippines.

Preparation of bacterial inoculum

Azospirillum was grown in 250-ml Erlenmeyer flasks containing 100 ml Difco Bacto nutrient broth with streptomycin sulfate (Calbiochem, California) at a concentration of 300 $\mu g\,ml^{-1}$. Flasks were incubated at 30°C for 30 h on a rotary shaker (120 rev. min^{-1}). Actively growing cells were then washed three times with sterile phosphate-buffered saline (100 mM phosphate buffer; 0.85% NaCl; pH 7.0) by centrifugation (10 min, 15000 × g). The washed cells were resuspended in the same buffered saline as described earlier to a final concentration of about 10^8 colony-forming units (cfu)/ml and used for plant inoculation.

Germination and inoculation of seedlings

Seeds were surface-disinfected with 70% (v/v) ethanol (5 min) followed by Saniclor (5% sodium hypochlorite) bleach (20 min), washed and germinated in demineralized water on a nylon mesh in a greenhouse (26–31°C day and 22–27°C night, 11 h light photoperiod, 70% relative humidity).

Seedlings at the two leaf stage (12-day-old) were inoculated by soaking the roots, previously washed with deionized water, in the bacterial suspension for 6 h at room temperature (25 ± 1°C). The seedlings with inoculum were shaken manually at frequent intervals to facilitate contact of bacteria with the seedling roots. After the incubation period, the excess inoculum from the roots was allowed to drain off for 5 min. Control plants were treated similarly by using autoclaved cells of the same bacterial suspension. The seedlings were transferred to N-free nutrient solution (described below) and returned to the greenhouse. After 24 h, the seedlings were transferred to a nutrient solution with NH_4^+-N. Other measurements were started 5 d after inoculation to allow the plants to acclimatize to these conditions.

Hydroponic growth conditions and mineral uptake experiments

The plant nutrient solution used was a bicarbonate-based, ammonium-containing medium as reported by Higuchi and Murayama (1982) with some modifications as follows: NH_4HCO_3, 1.0; $(NH_4)_2SO_4$, 0.05; Na_2HPO_4, 0.03; $KHCO_3$, 0.2; $MgSO_4$, 0.04; $CaCl_2$, 0.02; Fe-EDTA, 0.04 m*M* and microelements (Yoshida *et al.*, 1976). The concentration of nutrients in the solution was doubled when the seedlings were 24 days old. The pH of the solution was adjusted to 5.5 by bubbling CO_2 gas. In each culture tube (2.5 cm uniform dia. × 20 cm height) was 70 ml nutrient solution, and one seedling supported by a piece of flexible sponge. Tubes were covered with black paper to prevent the exposure of roots to light. The solution in the tubes was changed every other day. The pH remained stable during this period.

Mineral uptake was measured at weekly intervals over a period of six weeks after inoculation. The experiments were carried out only up to 47 days after inoculation (including 5 d acclimatization period) as the nutrient uptake was most active at earlier stages of rice growth. Each assay was performed by placing the root system of each plant (control or inoculated) for 3 h in 70 ml of fresh nutrient solution. At the end of the assay period, the solutions were decanted separately from the tubes without disturbing the root systems and the concentrations of NH_4- and PO_4-ions left behind were measured (as described in the next section) by taking 10 ml aliquots from these solutions. After each sampling, the tubes were refilled with fresh nutrient solution.

Determination of NH_4- and PO_4-ions in the plant nutrient solution.

Ammoniacal N was determined by the phenolhypochlorite method (Liddicoat *et al.*, 1975). Analysis of PO_4-ions was carried out by an automated acid molybdate method using the Technicon Autoanalyzer.

Enumeration of bacteria

The entire root system was ground (mortar and pestle) into sterile water and the population of total aerobic heterotrophic bacteria and the putative Azospirillum were estimated by the standard serial dilution plating. Bacteria were counted on nutrient agar medium with streptomycin (for putative Azospirillum) and also without streptomycin (for total aerobic heterotrophs). Azospirillum was identified by its colony morphology using Congo red medium and also by fluorescent antibody (FA) technique (Ladha *et al.*, 1982; 1987).

Plant growth measurements

The plant growth measurements were taken once every two weeks until termination. Total surface area of roots developed in the nutrient solution was estimated from washed and air-dried (30 min at room temperature) samples by a gravimetric method (Carley and Watson, 1966) using an aqueous solution of calcium nitrate. The shoot and root portions were separated, and the growth measurements were taken. Shoot and root dry weights were that of oven-dried matter at 80°C for 24 h.

Data analysis

Culture tubes were fully randomized with eight replicate plants per treatment per sampling. Student's *t* test was applied to determine the significance of the data.

Results

There was an increase in the uptake of NH_4-ion in the inoculated rice over the controls on 0, 7, 21, 28 and 42 days after inoculation (Table 1). This increase was, however significant only on day 0 (day 0 starts 5 days after the acclimatization period) and 7 days after inoculation. A decrease in the uptake process was noticed both in controls and inoculated plants on 21 and 28 days after inoculation. It was not apparent whether this decrease was due to sudden fluctuations in light and temperature. The inoculated plants showed a significant decrease in NH_4-ion uptake on the day 35 when compared with the controls.

In the case of PO_4-ion uptake, the inoculated plants showed enhanced uptake over the controls from 7 days after the inoculation until the termination of the experiment (Table 1). The increase was significant from 7 to 28 days after inoculation. A gradual drop in the uptake process was evident from 28 days after inoculation till the end of the experiment both in control and the inoculated plants.

Inoculation did not bring about significant effects on root and shoot parameters of rice until 28 days after inoculation (data not shown). Plants showed response to the inoculation after 42 days (Table 2). Inoculation reduced the root length of

Table 1. Effect of *Azospirillum lipoferum* inoculation on the rate of NH_4- and PO_4-ion uptake ($\mu g.plant^{-1} h^{-1}$) by rice during growth in a hydroponic system

Ion	Treatment	Days after inoculation						
		0	7	14	21	28	35	42
NH_4	Control[a]	120 ± 13	393 ± 23	660 ± 33	348 ± 5	485 ± 66	699 ± 46	900 ± 62
	Inoculated[a]	181[b] ± 20	472[b] ± 17	633 ± 21	384 ± 25	523 ± 62	551[b] ± 48	950 ± 27
PO_4	Control[a]	3 ± 0.4	9 ± 0.6	30 ± 1.2	24 ± 1.2	60 ± 1.3	63 ± 2.4	51 ± 1.2
	Inoculated[a]	3 ± 0.4	12[b] ± 0.4	39[b] ± 2.4	39[b] ± 0.4	78[b] ± 3.7	72 ± 4.9	57 ± 3.7

Note: The day 0 starts 5 days after the acclimatization period.
[a]Values are the mean of 8 replicates ± standard error.
[b]Significantly different from the control at the 5% level.

Table 2 . Effect of *Azospirillum lipoferum* on root and shoot development of rice in a hydroponic system, 42 d after inoculation

Treatment	Root				Shoot			
	Length (cm. plant^{-1})	Surface area (g $Ca(NO_3)_2$. (plant^{-1})	Fresh wt. (g.plant^{-1})	Dry wt. (g.plant^{-1})	Leaf no. 2 length (cm.plant^{-1})	Shoot length cm.plant^{-1})	Fresh wt. (g.plant^{-1})	Dry wt. (g.plant^{-1})
Control[a]	26.60 ±0.49	2.36 ±0.22	1.93 ±0.06	0.33 ±0.01	26.00 ±6.75	41.60 ±0.64	1.90 ±0.06	0.47 ±0.01
Inoculated[a]	24.90[b] ±0.57	2.75 ±0.22	1.95 ±0.14	0.34 ±0.01	23.00 ±8.16	42.00 ±0.53	2.11 ±0.05	0.51[b] ±0.01

Note: Leaf number 2 is from the top.
[a] Values are the mean of eight replicates ± standard error.
[b] Significantly different from the control at the 5% level.

rice significantly, while it did not show significant effects on other root parameters such as surface area and fresh and dry weights. Inoculation, however, increased the shoot fresh and dry matter significantly at this stage when compared with controls (Table 2). The length of leaf number 2 and the length of shoot did not differ significantly from controls.

Putative *A. lipoferum* could be recovered from the inoculated root samples up to 42 days after inoculation. The numbers per gram dry root ranged (from 0 towards 42 days after inoculation) 218×10^7 to 24×10^7 in the plates where no streptomycin was added and 29×10^7 to 6×10^7 in those plates with streptomycin. Several representative colonies tested from these plates were positive for Azospirillum on the Congo red medium and also to FA test. The control samples did not show any Azospirillum colonies in streptomycin-containing plates.

The total aerobic heterotrophic bacteria per gram dry root ranged from 335×10^7 to 1×10^7 in the plates where no streptomycin was added and 74×10^7 to 1×10^7 in those plates where streptomycin was added. The numbers in the controls did not differ markedly from the inoculated plants except with a slight increase in the latter where no streptomycin was added, at the 42 d after inoculation stage.

Discussion

It has been reported that, inoculation of corn seeds with *A. brasilense* enhanced the uptake of NO_3-, H_2PO_4-, and K-ions by root segments (Lin *et al.*, 1983). Similar improved nutrient utilization upon inoculation was also observed in sorghum plants (Lin *et al.*, 1983).

Total shoot and root dry matter, plant height and grain yield of wheat increased significantly after inoculation with a mixture of Azospirillum strains in hydroponic systems (Kapulnik *et al.*, 1985b). The benefits obtained were attributed to enhancement of root development and the amount of NO_3-ion taken up by the roots in those studies. Furthermore, the rate of NO_3-ion uptake by wheat inoculated with Azospirillum was shown to have increased because of a general increase in root surface area, and not because of a specific uptake rate (Kapulnik *et al.*, 1985b). However, under the conditions employed in our studies, there was enhanced NH_4- and PO_4-ion uptake by rice in the inoculated plants but without concomitant increase in the surface area of the roots.

Azospirillum inoculation increased root lengths of wheat in seven cultivars tested, but the root surface area increased only in three cultivars (Kapulnik *et al.*, 1985c). Reynders and Vlassak (1982) observed a decrease in the root mass of wheat after Azospirillum inoculation. The effects seem to vary according to the age of the culture and the bacterial concentration (Okon and Kapulnik, 1986). Kapulnik *et al.*, (1985c) have reported that wheat inoculated with 10^5 to 10^6 cfu ml^{-1} of *A. brasilense* strains caused the largest root elongation and total surface area while 10^8 to 10^9 cfu inhibited root development. The concentration of *A. lipoferum* used *i.e.* 10^8 cfu ml^{-1} phosphate buffer, significantly reduced the root length of rice in the present investigation (Table 2). This observation corroborates the findings of Kapulnik *et al.* (1985c).

A rapid decline of inoculated Azospirillum from

rhizospheres of various plants has been reported Nayak *et al.*, 1986; Smith *et al.*, 1984). The closed nature of the system used and a possible lower competition for other nutrients from other microflora might be the reasons for better survival of the organism in the present study.

That the enhanced uptake in the inoculated plants might be due to the utilization of minerals by the inoculated organism is ruled out, because there is an increased plant biomass *i.e.* shoot fresh and dry matter, linked with enhanced mineral uptake in the inoculated plants. That the increase in shoot biomass could be the result of possible bacterial N_2 fixation under the conditions employed is also not likely as the concentration of combined N used was high enough (about 28 mg $N l^{-1}$) to inhibit N_2 fixation. Several reports claim that combined N inhibits N_2 fixation (Watanabe and Cabrera, 1979; Rao *et al.*, 1981). Furthermore, while the enhancement of biological N_2 fixation activity in associative symbiotic systems has been reported mainly at flowering stage of the plants (Watanabe and Lin, 1984), the present experiments were carried out with plants at the vegetative phase.

Conclusions

The present investigation lends further support to the view that inoculation with diazotrophs promotes plant growth, through processes other than N_2 fixation, apparently by enhancing plant mineral uptake. Earlier report on wheat and *A. brasilense* associations indicated a positive correlation between root development, especially at the surface area, and enhanced mineral uptake (Kapulnik *et al.*, 1985b). Our studies with the rice and *A. lipoferum* association on the other hand, showed enhanced mineral uptake without significant increases in root parameters. If growth was simply increased, however, the larger plants would obviously take up more nutrients, which may be an effect rather than a cause (Tinker, 1984). Furthermore, during plant development many processes seem to be controlled by a balance between the amounts of various growth regulators rather than by an absolute amount of individual compounds (Van Andel and Fuchs, 1972). *Azospirillum* spp. were shown to produce auxins, gibberellin — and cytokinin-like substances in culture which was presumed to affect the root growth of inoculated plants (Tien *et al.*, 1979). Putting these points together, it appears that, an internal shift in the balance of plant growth-promoting substances within the plant in response to inoculation, may control the promotion of the root and/or shoot development. However, more work on these lines is essential to elucidate the actual mechanism underlying the enhanced plant growth following inoculation.

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