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Detection of inoculated plant growth-promoting rhizobacteria in the rhizosphere of rice

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Abstract

A mixture of five plant growth-promoting rhizobacterial strains belonging to the genera *Azospirillum, Azoarcus, Pseudomonas* and *Zoogloea* was used to inoculate rice seedlings growing in microplots. The population of these bacteria, as well as the indigenous bacterial population in the rice rhizosphere, was determined at 4-week intervals throughout the growth season. In rhizosphere soil a continuous increase in total bacterial population (CCM-plate counts) in both inoculated and non-inoculated plots was noted until 3 months after transplantation, while the maximum population of diazotrophic bacteria (ARA-based MPN counts) was observed 1 month after transplantation. A larger number of bacteria was found associated with plant roots (10^7/g) as compared to those present in rhizosphere soil (10^6/g). The total bacterial population (plate counts) in plant roots showed a continuous increase until harvest, while the population of diazotrophs (MPN counts) started declining 1 month after transplantation. Considerably higher levels of nitrogenase activity (ARA) were noted in the roots of inoculated plants as compared to those of non-inoculated plants, where only a small number of diazotrophic bacteria were detected. The population of one *Azospirillum* strain (N-4) used as inoculum was determined by using selective media containing antibiotics, morphological characteristics, reaction with fluorescent antibodies and ARA of re-isolates. The maximum number of cells (1.4×10^5/g) of this strain were detected in the roots 1-month after seedling transplantation from the nursery, constituting about 0.4% of the total bacterial population and 2% of the diazotrophic population.

Introduction

Nitrogen fixation carried out by associative and free-living microorganisms in the rhizosphere of plants has been recognized to play an important role in

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nitrogen nutrition of plants (Boddey *et al.*, 1996). Diazotrophs belonging to diverse bacterial genera such as *Azospirillum, Azotobacter, Acetobacter, Alicaligens, Bacillus, Enterobacter, Herbaspirillum, Klebsiella* and *Pseudomonas* appear to be frequent colonizers of important cereal crops including rice, wheat, sugar cane and maize (Baldani *et al.*, 1986: Roger and Watanabe, 1986; Cavalcante and Dobereiner, 1988; Berge *et al.*, 1991; You *et al.*, 1991; Malik *et al.*, 1993). Beneficial effects of these plant growth-promoting rhizobacteria (PGPR) have been attributed to biological nitrogen fixation and production of phytohormones that promote root development and proliferation, resulting in efficient uptake of water and nutrients (Tien *et al.*, 1979; Hartmann *et al.*, 1983; Haahtela *et al.*, 1990). Interest in the beneficial rhizobacteria associated with cereals has increased recently due to their potential use as biofertilizers (Okon and Labandera-Gonzalez, 1994; Bashan and Levanony, 1990). Application of bacterial inoculants as biofertilizers has resulted in improved growth and increased yield of cereal crops (Kapulnik *et al.*, 1981; Boddey *et al.*, 1986; Pereira *et al.*, 1988; Kennedy and Tchan, 1992). A number of immunological and DNA-based techniques are being developed and used to study root colonization by the inoculated bacteria and their survival in the rhizosphere (Levanony and Bashan, 1989; Hartmann *et al.*, 1993; Schloter *et al.*, 1993; Vermeiren *et al.*, 1996).

Here we report on the detection of inoculated PGPR and indigenous bacteria present in the rice rhizosphere during different stages of growth. In addition, beneficial effects of PGPR inoculation on growth and yield of rice are given.

Materials and methods

Inoculum preparation

In the present study, a mixture of five PGPR strains (Malik *et al.*, 1994) was used as inoculum for rice. *Azospirillum lipoferum* (strain N-4), *Azospirillum brasilense* (strain WB-3), *Azoarcus* (strain K1), *Pseudomonas* (96–51) and *Zoogloea* strain (Ky-1) were grown in Luria Bertani (LB) medium with shaking at 30°C. After 24 h growth, bacterial cells were harvested by centrifugation at 10 000 rpm for 5 min. Pellets were washed and resuspended in 0.85% saline to get approximately 1×10^9 cells/ml.

Inoculation of rice seedlings

Inoculum was prepared by mixing equal volumes of the cultures of five PGPR strains (10^9 cells/ml). Roots of the rice seedlings (variety NIAB-6) grown in the nursery were kept for ½ h in the inoculum. The seedlings were transplanted in cemented plots (1.5×1.5×0.6m) filled with saline soil (Ece=4.87 mS/cm, pH=7.8, K=0.15 mEq/L, Na=66 mEq/L, Ca=4.1 mEq/L; Ali *et al.*, 1995). In each plot, seedlings were transplanted in eight rows, with eight hills in each row separated

by 20 cm. Two seedlings were planted on each hill. Ammonium sulphate was applied in solution form (3–5 cm below the soil surface) 7 days after transplantation in all plots. For both treatments (un-inoculated and inoculated) five replicates were used.

ARA of roots

For estimation of ARA, root samples were taken at 4-week intervals throughout the growth season. Roots were thoroughly washed with sterile water and excess water was removed with blotting paper. The root samples were incubated at 30°C with 10% C_2H_2 in vacutainers. The ARA was carried out on a gas chromatograph (Gasukuro kogyo Model 370) using Porapak N column.

Enumeration of bacteria from roots and soil

From each plot a 1 g soil sample (obtained by mixing 10 random samples) was shaken with 9 ml of saline for 30 min at 200 rpm. Serial dilutions were prepared from this suspension and spread on combined carbon medium (CCM plates; Rennie, 1981). These plates were incubated at 30°C for 48 h. The number of bacterial colonies was recorded with the help of a colony counter. For MPN counts (Alexander, 1965), dilutions were added in vials containing semi-solid CCM. These vials were incubated for 72–96 h at 30°C and then subjected to ARA. Vials showing positive results were used to calculate MPN counts.

Root samples were washed thoroughly with sterile water. One gram roots (fresh weight) were homogenized with a pestle and mortar in 9 ml saline and serial dilutions were prepared. These dilutions were porcessed as described above for plate counts and MPN counts.

For selective retrieval of *Azospirillum* strain N-4, antibiotic gentamycin (100 µl/ml) was added in plates containing LB. Serial dilutions of the crushed root suspension were prepared and two dilutions (10^{-2} and 10^{-3}) were spread on plates in triplicate. Ten colonies, resembling N-4 in their colony morphology, from each of these plates were randomly selected for detailed studies to confirm as being *Azospirillum* N-4. The colonies were used to inoculate vials containing semi-solid CCM, to observe formation of characteristic sub-surface pellicle, typical *Azospirillum*-like cell morphology and motility, ARA and reaction with specific fluorescent antibodies.

Plant biomass and grain yield

Plants were harvested after 3 months at the completion of the rice growth season. For estimation of plant biomass and grain yield, whole plants, along with the roots, were harvested from an inner 1 m^2 area, leaving one row of plants near the

borders of plots. Dry weight of the roots, aerial parts and grains was measured by keeping plant material at 70°C until no change in weight was noted. For statistical analysis the *t*-test was applied.

Results and discussion

In the microplots the bacterial population was determined at 4-week intervals for 3 months after transplantation of the seedlings. In the rhizosphere of both inoculated and non-inoculated plants considerably lower numbers of bacteria were recorded as compared to plant roots (Figures 1–4). At the nursery stage the total number of bacteria (plate counts; indigenous population) colonizing roots was approximately 1×10^8 cells/g of root (Figure 1). The presence of a relatively high number of bacteria in roots is most probably the result of accumulation of root exudates from densely populated rice seedlings at the nursery stage. The PGPR are known to be attracted to root exudates of a number of plants (De Troch and Vanderleyden, 1996). From this observation it can be concluded that application of inoculum at the nursery stage is not useful as the bacterial population is already high. Application of inoculum at the time of transplantation in the field, where the population of indigenous rhizobacteria is low, seems more adequate.

After seedling transplantation the bacterial population started building up again and reached its maximum after 12 weeks. However, more diazotrophic bacteria (ARA-based MPN counts) were detected in roots 1 month after transplantation, and this number started decreasing until crop harvest (Figure 2). In the roots, 1 month after transplantation, the nitrogen-fixing bacteria comprised about 30% of the total bacterial population. At the time of harvesting (3 months after transplantation), the proportion of nitrogen fixers declined to only less than 1% of the total bacterial population in inoculated plots. This decline in MPN counts at later stages of plant growth indicates that nitrogen fixers are out-competed by non-fixers. The total bacterial population, as well as

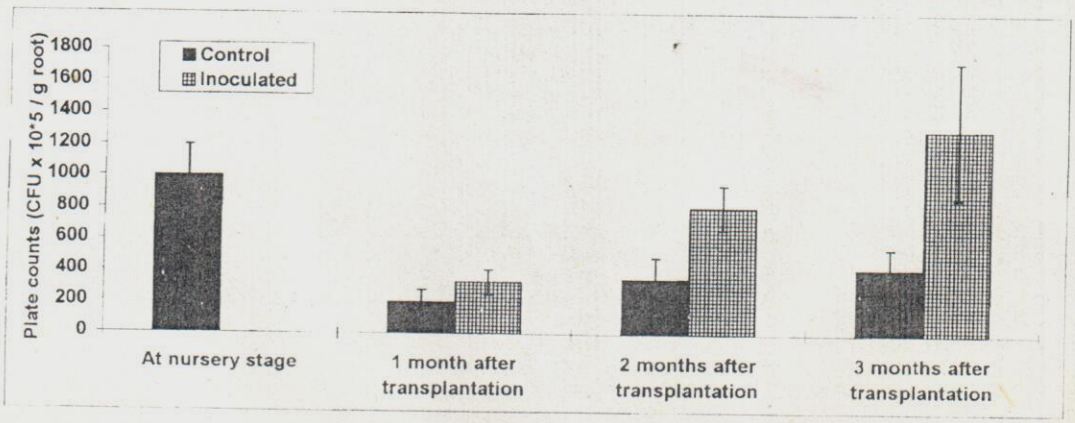

Figure 1. Detection of bacterial population associated with the roots of rice. Plate counts on combined carbon medium (CCM) were used to determine the bacterial population.

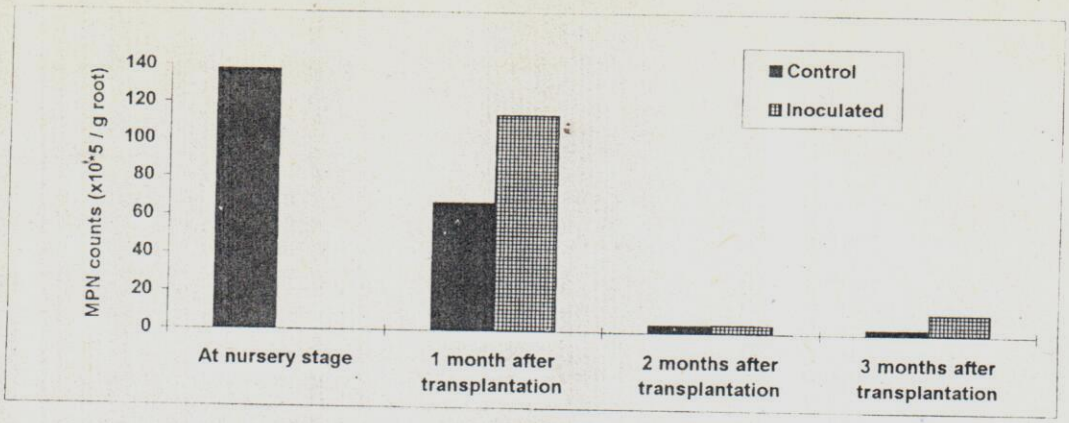

Figure 2. Detection of nitrogen-fixing bacteria in roots of rice. ARA-based MPN counts were used to determine the bacterial population.

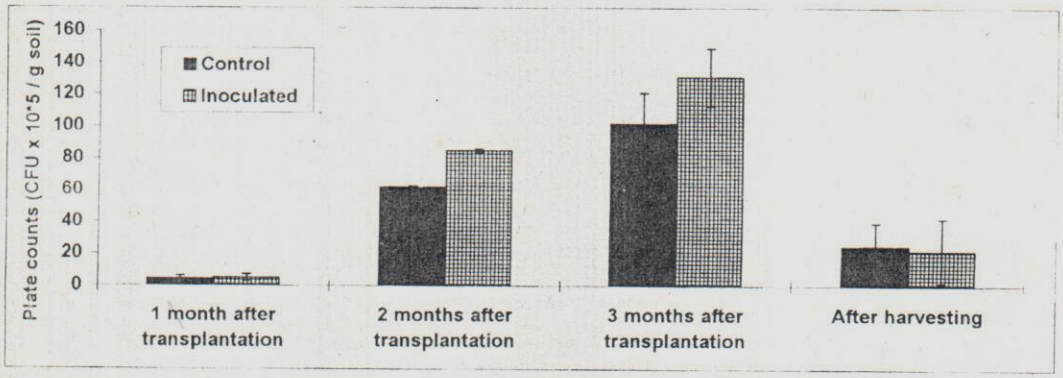

Figure 3. Detection of bacterial population in the rhizosphere soil of rice. Plate counts on combined carbon medium (CCM) were used to determine the bacterial population.

diazotrophs, were found in much greater numbers in inoculated plots as compared to non-inoculated plots (Figures 1 and 2). In the rhizosphere soil the same trend was observed (Figures 3 and 4). Total bacterial population (CCM plate counts) gradually increased 1 month after transplantation, but the diazotrophic population (ARA-based MPN counts) started decreasing with the age of the crop. A similar decrease in bacterial population at harvest in rice rhizosphere has been reported by Van Holm *et al.* (1993) where *Azorhizobium caulinodans* was used as inoculum.

Nitrogenase activity was detected in roots of both inoculated and non-inoculated plots. This ARA activity of non-inoculated roots reflects the presence of an indigenous diazotrophic population. Maximum nitrogenase activity in association with plant roots was detected 1 month after transplantation of the seedlings from the nursery (Figure 5). This high activity level coincides with the maximum number of diazotrophic bacteria found associated with the plant roots at this stage of plant growth.

The population of one inoculated strain (*Azospirillum* N-4) was determined by identifying these bacteria initially on the basis of their colony morphology,

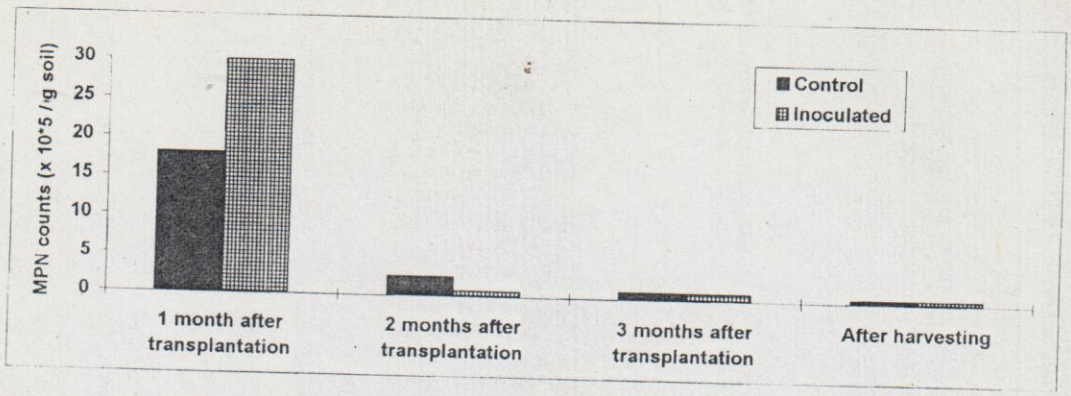

Figure 4. Detection of nitrogen-fixing bacteria in the rhizosphere soil of rice. ARA-based MPN counts were used to determine the bacterial population.

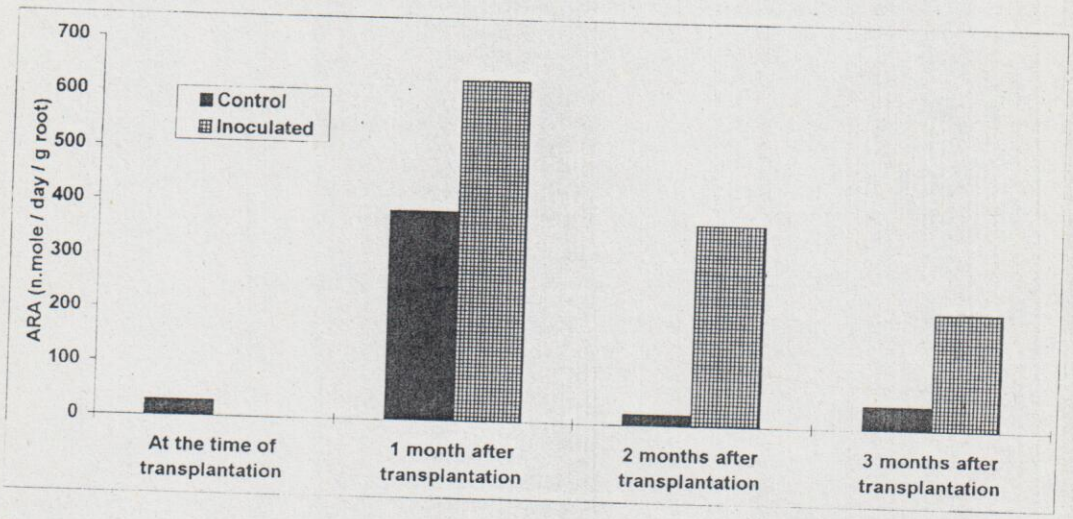

Figure 5. Nitrogenase activity estimated in rice roots.

and then on the basis of their cell morphology, ARA and by using specific fluorescent antibodies. One month after inoculation, approximately 1.4×10^5 cells of *Azospirillum* strain N-4 per gram of root were found. In a study carried out by Yanni *et al.* (1996), 1.1×10^6 cells of *Rhizobium leguminosarum* bv. *trifolii* were detected when used to inoculate rice plants. It was estimated that among the bacteria associated with roots (both originating from inoculations and the indigenous population), strain N-4 comprised about 0.4% of the total bacterial population and about 2% of the total nitrogen-fixing population when peak nitrogenase activity was recorded in roots (Figure 6). In a study carried out by Schloter *et al.* (1993), in which *Azospirillum brasilense* was used as inoculum for wheat, the inoculated strain comprised about 0.1% of the total bacterial community.

A beneficial effect of bacterial inoculation was observed on plant biomass and grain yield (Table 1). In inoculated plots an approximately 12% increase in both grain yield and straw weight was noted (Table 1). The beneficial effect of bacterial inoculation on total plant biomass and root weight was significant as a

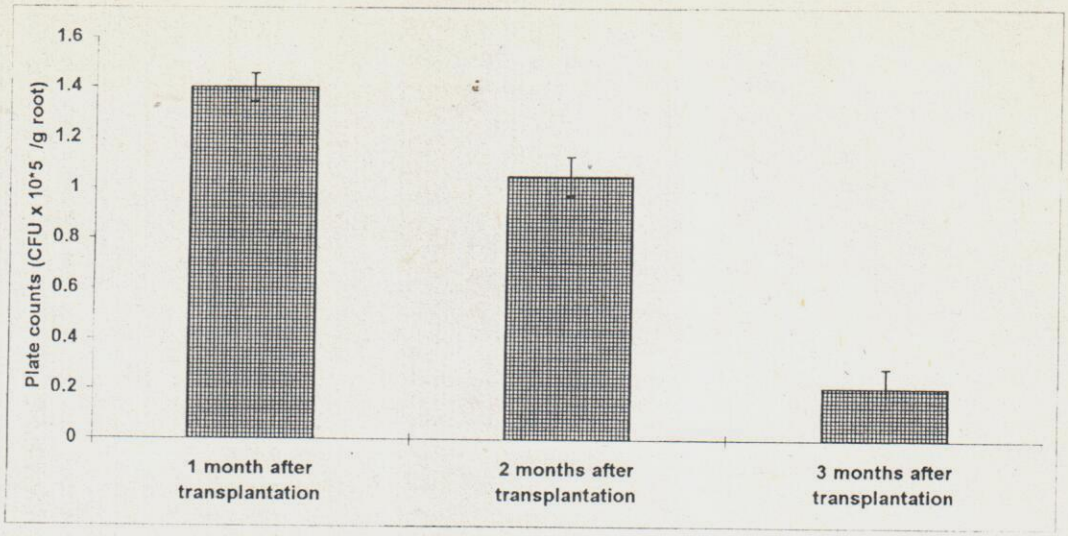

Figure 6. Detection of *Azospirillum* strain N-4 used as inoculum for rice.

41% increase in root weight and a 18.7% increase in plant biomass was recorded. Similar beneficial effects of PGPR inoculations on rice have been reported (Ali *et al.*, 1995) where use of inoculum along with a low input of chemical fertilizer-N was found useful for increasing rice biomass, N uptake and fertilizer-N recovery in rice grown in pots containing saline soils.

The results of the present investigation clearly showed that the inoculated bacterial strains survived in the rhizosphere especially during early stages of plant growth and, as a result of these inoculations, plant growth and yield was improved. On the basis of these results this mixture of bacterial strains may be recommended to form an excellent active ingredient of a commercial biofertilizer for rice.

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Table 1. Effect of PGPR inoculations on plant biomass of rice

Treatments	Straw weight (g/m^2)	Root weight (g/m^2)	Grain yield (g/m^2)	Total plant biomass (g/m^2)
30 kg N/ha	296	206	266	767
30 kg n/ha+inoculum	$332^{n.s.}$	291*	$297^{n.s.}$	911*
	(12)	(41)	(11.7)	(18.7)

* Significant difference between control and inoculated at the 5% level, n.s. = non significant. Figures in parentheses show percentage increase over control.

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