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# The fate of marker *Azospirillum lipoferum* inoculated into rice and its effect on growth, yield and $N_2$ fixation of plants studied by acetylene reduction, $^{15}N_2$ feeding and $^{15}N$ dilution techniques

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**Summary.** A spontaneous mutant of *Azospirillum lipoferum*, resistant to streptomycin and rifampicin, was inoculated into the soil immediately before and 10 days after transplanting of rice (*Oryza sativa* L.). Two rice varieties with high and low nitrogen-fixing supporting traits, Hua-chou-chi-mo-mor (Hua) and OS4, were used for the plant bacterial interaction study. The effect of inoculation on growth and grain and dry matter yields was evaluated in relation to nitrogen fixation, by in situ acetylene reduction assay, $^{15}N_2$ feeding and $^{15}N$ dilution techniques. A survey of the population of marker bacteria at maximum tillering, booting and heading revealed poor effectivety. The population of native *Azospirillum* followed no definite pattern. Acetylene-reducing activity (ARA) did not differ due to inoculation at two early stages but decreased in the inoculated plants at heading. In contrast, inoculation increased tiller number, plant height of Hua and early reproductive growth of both varieties. Grain yield of both varieties significantly increased along with the dry matter. Total N also increased in inoculated plants, which was less compared with dry matter increase. $^{15}N_2$ feeding of OS4 at heading showed more $^{15}N_2$ incorporation in the control than in the inoculated plants. The ARA, $^{15}N$ and N balance studies did not provide clear evidence that the promotion of growth and nitrogen uptake was due to higher $N_2$ fixation.

**Key words:** *Azospirillum lipoferum* inoculation – Rice yield – Acetylene reduction assay – $^{15}N$ feeding and dilution techniques

The beneficial effects of inoculating $N_2$-fixing bacteria on plant growth, dry weight, total nitrogen content and yield of various cereal crops and grasses have been demonstrated by many scientists from different parts of the world. In wetland rice, some benefit of inoculation has been reported (Subba Rao et al. 1979; Rao et al. 1983; Rao and Rao 1983; Watanabe and Lin 1984). It is not known whether the increased yield is due to $N_2$ fixation or improved mineral nutrition (Okon 1982; Lin et al. 1983), and/or to plant-growth promoting substances (Tien et al. 1979). A more detailed study by Okon (1984) showed that, in many cases, higher crop yield was due to more rapid mineral N uptake, dry matter and water accumulation, and not to $N_2$ fixation. Baldani et al. (1984), using antibiotic-resistant *Azospirillum*, traced the establishment of inoculated bacteria in the rhizosphere and roots of field-grown sorghum, maize and wheat. Similarly, Smith et al. (1984) reported rapid decline of the inoculated organisms from the rhizosphere of various grasses. Such studies tracing the establishment of inoculated organisms are very important but are lacking in wetland rice. We, therefore, investigated the fate of *Azospirillum lipoferum*, resistant to streptomycin and rifampicin, following inoculation into the rhizosphere of rice. We used in

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situ acetylene reduction assay, $^{15}N_2$ feeding and $^{15}N$ dilution techniques to determine whether inoculation stimulates associative $N_2$ fixation.

## Materials and methods

*Soil treatment.* Maahas clay soil (Aquic Tropudalf, pH, 6.7; organic carbon, 12.4 g/kg; total N, 1.5 g/kg; Cation Exchange Capacity (CEC), 335 mEq/kg; Olsen P, 13.5 mg/kg; free $Fe_2O_3$, 29 g/kg) collected from the International Rice Research Institute (IRRI) experimental farm, Laguna, Philippines, was used. The wet soil was mixed thoroughly and passed througha 2-mm sieve and 8-kg dry weight equivalent amounts of soil were put into 15-liter Wagner pots. Dried and finely ground straw (24 g) was incorporated into each pot 2 weeks before transplanting and pots were flooded. The treatments for the measurement of acetylene reduction activity (ARA) and $^{15}N$ dilution were: Hua-chou-chi-mo-mor (Hua) and OS4, uninoculated (U) and inoculated (I). Hua and OS4 were reported to be high and low in stimulating $N_2$ fixation respectively (IRRI, unpublished). There were five replications for the ARA and four for $^{15}N$ dilution measurements. For $^{15}N_2$ feeding, the treatments were: (1) uninoculated OS4 (control), and (2) inoculated OS4, with three replications each. All treatments were randomized in separate blocks in the greenhouse. Each pot received 160 mg $P_2O_5$, 106 mg $K_2O$ and 180 mg N as ammonium sulphate, 1 day before transplanting. $^{15}N$-labelled ammonium sulphate (18 atom% ecxess) was added, instead of unlabeled ammonium sulphate, to the pots used for $^{15}N$ dilution study.

Two 15-day-old seedlings were transplanted per pot. The pots were covered with black cloth to inhibit photodependent $N_2$ fixation, and watered with deionized water until harvesting.

*Preparation of inoculum and inoculation. Azospirillum lipoferum* 34H (Ladha et al. 1982) was used in the study. Spontaneous mutant, resistant to 400 μg/ml streptomycin (stremptomycin sulphate, Calbiochem Behring Corp.) and 200 μg/ml rifampicin (Sigma), was obtained using standard microbiological procedure. The mutant strain is referred to as 34H Str Rif$^r$ in the text for convenience. The nitrogenase activity and antibiotic marker properties were thoroughly checked before being inoculated into the rice rhizosphere.

The 34H Str Rif$^r$ strain was cultivated in Erlenmeyer flasks containg 40 ml nutrient broth (enriched with 10 g/litre yeast extract) for 18–20 h with continuous shaking at ambient temperature (25–30°C). The cells were harvested by centrifugation at about 5000 × g for 15 min and washed 3 times and resuspended in 30 ml 0.01 *M* phosphate-buffered saline pH 7.0 (PBS). The optical density (450 nm) of the resulting cell suspension was adjusted to $1.2 \times 10^9$ cells/ml. Five millilitres of the resultant bacterial suspension mixed with 10 ml sterile PBS was added aseptically to each flask containing 50 g peat soil and mixed (so that the final moisture became 40% of the water-holding capacity). The peat soil, collected locally from Calauan, Laguna, which contained 38% organic matter, was neutralized and sterilized by autoclaving beforehand (Smith et al. 1984). The flasks were covered with cotton plugs and stored in the incubator for 1 month at 30°C. Equal amounts of heat-killed bacterial cells were added to peat soil and stored similarly for the uninoculated control. The viable counts were made in Congo red medium (Caceres 1982) containing 400 μg/ml streptomycin and 200 μg/ml rifampicin.

Before use, the number of surviving bacteria in the bacterial inoculum was checked by selecting three flasks randomly. For inoculation, 100 g/pot peat-based inoculum (containing $5 \times 10^8$ cells) was added and mixed into the soil. Uninoculated treatments received equal amounts of peat soil with heat-killed bacterial cells. The viable counts of the inoculated bacteria were made from composite soil samples just after inoculation. A second inoculation with strain 34H Str Rif$^r$ was made 10 days after transplanting. Ten millilitres of live and heat-killed inoculum ($10 \times 10^6$ cells/ml) was applied to each pot of inoculated and uninoculated treatments respectively, using sterile pipettes.

*Acetylene reduction assay and bacterial enumeration.* In situ acetylene reduction assays (ARassay) were conducted at maximum tillering (40 DAT), booting (50 DAT) and heading (70 DAT) of the rice plants. Plants were cut 20 cm above the soil level and the floodwater was removed with a hand suction pump without disturbing the surface soil. The surface soil and basalportion of the rice shoot were covered with aluminium soil. Plastic bag (45 × 85 cm) covers were then tightly attached to the pot and the air was evacuated. The bag was filled with a known volume of a gas mixture containing 25% acetylene, 74.99% air and 0.1% propane by volume through the gas port. Pots were placed outdoors; zero time and final samples were collected after 30 min and 6 h respectively. The 6-h incubation period did not significantly affect the population of total heterotrophs and $N_2$ fixers (Barraquio et al. 1985). The analysis of gases and the amount of ethylene produced was calculated as described by Barraquio et al. (1985).

After the ARassay, the same plants were used to count bacteria as described by Watanabe et al. (1979). Total aerobic heterotrophs were counted in tryptic soy agar (TSA) plates, and for percentage of $N_2$ fixers about 100 colonies per treatment were picked up and tested for ARA in semisolid glucose-yeast extract medium. To count the population of putative native azospirilla and inoculated *A. lipoferum*, Congo red plates with and without antibiotics (400 μg/ml streptomycin and 200 μg/ml rifampicin) were used and colonies were tested for ARA in malate-yeast extract semisolid medium.

*$^{15}N_2$ feeding.* For $^{15}N_2$ incorporation, plants were cut 20 cm above the soil to minimize the quantity of $^{15}N_2$. The soil surface and the exposed stems were covered with aluminium foil to inhibit the photodependent $N_2$ fixation. The cut rice hill in the pot was covered with 12.5-liter glass jars (20 cm diameter, 49 cm high) by pushing it into the soil to a depth of 15 cm. The jar enclosing the rice hill was completely filled with deionized distilled water and the mouth tightly plugged with a rubber stopper and modelling clay. Fifteen liters of gas mixture containing 75% $^{15}N_2$ (98% $^{15}N$ enrichment, supplied by Monsanto Research Corp., US Dept. of Energy) and 25% air by volume was prepared in a plastic bag. The $^{15}N_2$ gas was cleaned before use as described by Ohyama and Kumazawa (1978). The glass tubes attached to the rubber plugs were sealed with serum stoppers. Into each jar, 2.5 liters gas mixture was introduced through the glass tube outlet to replace the water. The rice hills were incubated for 72 h, uprooted and washed carefully with running water; roots (including aerial roots) and shoots were separated. The soil was divided into two fractions. The washings of the root were considered as a fraction I and the rest of the rhizosphere soil as fraction II.

*Dry matter and yield measurements.* Plant height and tiller number were recorded until maturity at 15-day intervals. Grain and straw yields were recorded at 14% w/w moisture content. Submerged plant parts were divided into roots and stubble plus submerged leaf sheath, and their dry matter was recorded.

*Total nitrogen and $^{15}N$ analysis.* Total nitrogen content of dried powdered grains, straw, roots and stubble plus submerged leaf sheath was determined by the micro-Kjeldahl technique, and the $^{15}N$ by the emission spectrometric method as modified by Yoneyama and Kumazawa (1974) using the JASCO N–150 $^{15}N$ analyzer. Macro-Kjeldahl analysis of soil was done for total nitrogen that included nitrate and nitrite nitrogen (Bremner and Shaw 1958) and $^{15}N$ by mass spectrometer (VG micromass 622). The $^{15}N_2$ gas content from each jar was measured at zero time (1/2 h after stabilization) and 72 h by emission spectrometry using the JASCO N–150 $^{15}N$ analyzer.

## Results and discussion

*Establishment of inoculated A. lipoferum strain 34H Str Rif$^r$.* The inoculated bacteria could easily be traced because of the antibiotic marker. After 4 days of plating on Congo red medium with streptomycin and rifampicin, small, round, flat, brownish red (rifampicin imparts its own colour to the Congo red medium) colonies appeared. The colonies were further tested by a fluorescent antibody (FA) technique, and showed cross-reaction with the FA of *A. lipoferum* prepared previously (Ladha et al. 1982). They also exhibited $C_2H_2$ reduction activity, when inoculated to semisolid malate medium. The plant parts and soil from the pots of the uninoculated treatment were checked for indigenous antibiotics-resistant *Azospirillum* in Congo red plates with antibiotics at $10^1$ and higher dilutions. No growth was found. The results suggest that inoculated bacteria could survive in the soil and around the roots and stem of both varieties (Table 1). There was no difference in the counts of inoculated bacteria between the two varieties except in the roots of Hua, which harboured more inoculat-

Table 1. Survival of 34H Str. Rif$^r$ in soil and plant parts after inoculation of two rice varieties

| | Population ($10^2$/g dry weight)[a] | | | | | |
|---|---|---|---|---|---|---|
| | OS4 | | | Hua-chou-chi-mor | | |
| Location | 40 DAT | 50 DAT | 70 DAT | 40 DAT | 50 DAT | 70 DAT |
| Rhizosphere soil | 8 | 5 | 0 | 50 | 20 | 0 |
| Root | 120 | 280 | 40 | 530 | 360 | 110 |
| Stem | 57 | 17 | 0 | 40 | 38 | 0 |

DAT, Days after transplanting

[a] No 34 H Str. Rif$^r$ was detected in the control (uninoculated treatment)

ed azospirilla than OS4 roots. Inoculated bacteria established, but in low numbers. Furthermore the numbers declined progressively, resulting in their absence in the soil and stem of both varieties at the heading stage. The cause of such decline, whether antagonism, soil character or lack of support in the form of root exudation by the plants, is not known. Poor survival of inoculated bacteria in the rhizosphere was reported in greenhouse and field trials by Gaskins et al. (1984), Smith et al. (1978) and Smith et al. (1984). In a previous experiment we found that when rice seedlings were dipped in bacterial suspension (*A. lipoferum* 34H Str Rif$^r$) for 6 h and transplanted in pots, 34H Str Rif$^r$ failed to establish and disappeared after 1 month in the rice root and rhizospheric soil (unpublished). In this study we tried to reduce the rapid decline by growing the bacteria for 1 month beforehand in peat soil rich in organic matter, and with enough moisture (40%), suitable temperature and pH. Before inoculation the soil was amended with rice straw as an additional energy source and fertilized moderately to give a basal dose of essential minerals. The first inoculation through peat established $8.5 \times 10^4$ viable counts/g soil immediately after the inoculation. To ascertain the establishment, a second inoculation was given 10 days after transplanting. Two inoculations were used by Watanabe and Lin (1984). Crossman and Hill (1984) used multiple inoculations, i.e., 2, 4 and 6 weeks after transplanting.

*Enumeration of azospirilla, aerobic heterotrophs and percentage of $N_2$-fixing bacteria.* Before inoculation, initial counts of putative native azospirilla (malate-utilizing $N_2$-fixers), total heterotrophs and percentage of $N_2$ fixers in the soil were $2.4 \times 10^5$, $22 \times 10^6$ (per gram dry weight) and 8%, respectively. These populations were estimated in inoculated and control plants of both varieties at 40, 50 and 60 DAT (Table 2). Compared with the initial population, the number of putative azospirilla did not change much in inoculated and uninoculated treatments of both varieties. Inoculation of *Azospirillum* (34H Str Rif$^r$) did not seem to affect the total number of azospirilla.

The total heterotrophs in the root and stem of both varieties and treatments were 10- to 100 fold higher than in the soil. This could indicate strong competition for carbon substrate and its role in limiting the number of organisms in the soil.

The percentage of $N_2$ fixers was always larger in the roots at all stages of rice plant growth, indicating that free-living $N_2$ fixers are definitely associated with roots. The percentage of $N_2$ fixers increased gradually, with the growth of the rice plant, to 85% at heading. A similar trend was observed by Barraquio et al. (1982).

*Effect of inoculation on growth and yield.* At 20 DAT there was no difference in either tiller number or plant height between the treatments and the varieties. However, at 40 DAT, inoculated Hua plants showed a significant difference in tiller number over uninoculated Hua plants (Table 3). In OS4 the inoculated and uninoculated plants showed no difference in tiller number. Plant height of OS4 in both treatments did not differ at any stage. On the other hand, inoculated Hua plants at 100 DAT were significantly taller than uninoculated plants.

**Table 2.** Population of azospirilla, heterotrophs and percentage of $N_2$ fixers at three growth stages of control and inoculated treatments of two rice varieties

| Treatment | Location | Population ($10^5$/g dry weight) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | OS4 | | | | | | Hua-chou-chi-mo-mor | | | | | |
| | | 40 DAT | | 50 DAT | | 70 DAT | | 40 DAT | | 50 DAT | | 70 DAT | |
| | | a | b | a | b | a | b | a | b | a | b | a | b |
| Control | Soil | 1 | 110 (1) | 1.3 | 1000 (6) | 25 | 600 (10) | 0.8 | 340 (1) | 4.6 | 300 (5) | 1.9 | 4800 (11) |
| | Root | 37 | 6900 (24) | 45 | 2200 (47) | 63 | 5400 (59) | 26 | 6400 (13) | 21 | 4100 (38) | 30 | 17 000 (70) |
| | Stem | 43 | 2700 (2) | 26 | 890 (9) | 35 | 1500 (14) | 10 | 4100 (2) | 3.9 | 1800 (8) | 9 | 1200 (15) |
| Inoculated | Soil | 5 | 470 (1) | 1.9 | 190 (9) | 0.4 | 300 (11) | 2.4 | 130 (2) | 1.3 | 280 (4) | 0.6 | 400 (22) |
| | Root | 33 | 16 000 (32) | 28 | 1900 (54) | 15 | 2000 (66) | 28 | 5800 (22) | 46 | 1700 (56) | 48 | 1300 (85) |
| | Stem | 5.1 | 4900 (6) | 17 | 580 (9) | 6 | 900 (10) | 18 | 3700 (8) | 4.2 | 1800 (9) | 7 | 1000 (19) |

Figures in parenthesis indicate % $N_2$ fixers
a: putative native azospirilla; b: total heterotrophs

**Table 3.** Effect of inoculation on some morphological traits of rice

| Variety | Treatment | 20 DAT | | 40 DAT | | 60 DAT | | 100 DAT | |
|---|---|---|---|---|---|---|---|---|---|
| | | a | b | a | b | a | b | a | b |
| OS4 | Control | 51 | 2 | 101 | 8 | 131 | 9 | 159 | 9 |
| | Inoculated | 46 | 2 | 98 | 6 | 127 | 9 | 162 | 9 |
| Hua-chou-chi-mo-mor | Control | 54 | 2 | 105 | 7 | 149 | 15 | 155 | 16 |
| | Inoculated | 62 | 3 | 116 | 10[a] | 151 | 17 | 162[a] | 18 |

DAT, days after transplanting; a, plant height (cm); b, tiller number/plant

[a]Significantly different from uninoculated plant at the 5% level ($n = 4$)

**Table 4.** Dry matter yield responses to inoculation

| Variety | Treatment | Dry weight of different parts (g/pot) | | | | Total Dry weight (g/pot) | Total N (mg/pot) |
|---|---|---|---|---|---|---|---|
| | | Root | Straw | Submerged culm and leaf sheath | Grain | | |
| OS4 | Control | 5.5 | 36.3 | 5.8 | 35.7 | 83.4 | 639 |
| | Inoculated | 8.0* | 40.6* | 8.0* | 39.5** | 96.2* | 765.0* |
| Hua-chou-chi-mo-mor | Control | 2.6 | 32.0 | 3.5 | 40.4 | 78.5 | 718 |
| | Inoculated | 4.7*** | 39.8** | 4.3 | 47.8** | 96.6** | 770 |

*, **, *** Significantly different from the uninoculated treatment at 5%, 1% and 0.1% respectively ($n = 4$)

Inoculation enhanced flowering and grain formation of both varieties. Inoculation significantly increased dry weight of root, shoot and grain of both varieties except the submerged culm-leaf sheath of Hua and shoot of OS4. In Hua, dry weight increases were 80.8% in roots, 24.3% in leaves and 22.8% in submerged culm and leaf sheath. In OS4 the increases were 45.4%, 11.9% and 37.9%, respectively. Inoculation increased the grain yield of Hua and OS4 by 18.3% and 10.6% respectively. Total nitrogen uptake also increased in both varieties: 19.7% (significant at the 5% level) in OSA and 7.2% i Hua (Table 4).

There was no significant difference in percentage N between treatments. The results indicate that, in Hua, total dry matter increase was 23% against the total N increase of 7.2%. On the other hand, dry matter increase in OS4 was 15.3% against total N increase of 19.7%. Cohen et al. (1980) and Kapulnik et al. (1981a) in greenhouse and field trials with maize, *Setaria* and some summer cereal crops also obtained growth increases relatively higher than the total nitrogen content of the plants.

*Acetylene reduction assay.* To assess the effect of inoculation on both rice varieties a time course in situ ARassay was conducted. The results are shown in Table 5. There was no significant difference in ARA between inoculated and control treatments except at 70 DAT in OS4, where the inoculated treatment had a significantly lower ARA than the control. There was a definite increasing rate of ARA of both inoculated and uninoculated plants from maximum tillering to heading. The ARA of Hua was higher than OS4 at heading. Yoshida and Ancajas (1973) and Watanabe et al. (1979) reported the peak of rice plant associated ARA at heading stage. The same trend was noticed in the present study. In three extensive field trials with inoculation of *Azospirillum brasilense* to *Sorghum* and *Pennisetum* species, Smith et al. (1984) found no effect of inoculation on ARA; rather, in one case ARA negatively correlated with yield. On the other hand, Watanabe and Lin (1984) reported that of three growth stages studied, only at the early flowering stage was the ARA of inoculated plants higher than that of uninoculated plants. With

**Table 5.** Effect of inoculation on plant-associated acetylene reduction (ARA) of two rice varieties

| Variety | Treatment | ARA ($\mu$mol $C_2H_4$/pot/6 h) | | |
|---|---|---|---|---|
| | | 40 DAT | 50 DAT | 70 DAT |
| OS4 | Control | 1.3 | 1.17 | 5.7[a] |
| | Inoculated | 1.6 | 2.35 | 1.45 |
| Hua-chou-chi-mo-mor | Control | 1.3 | 1.67 | 13.6 |
| | Inoculated | 1.65 | 2.76 | 10.0 |
| | SE | 0.56 | 0.76 | 10.3 |

Mean of five replicates ± SD

[a] Significantly different from inoculated treatment at the 5% level

other crops Okon et al. (1983), Kapulnik et al. (1981b), Cohen et al. (1980) and Hegazi et al. (1983) obtained a considerable increase in ARA due to inoculation. This could also be because of different bacterial strains were used in their study.

*$^{15}N_2$ fixation and incorporation into the plant tissue.* Rice plant exhibits maximum $N_2$-fixing activity at heading. $^{15}N_2$ feeding was, therefore, done at heading of OS4 only. The results are shown in Table 6. Although there was clear evidence of $N_2$ fixation and N incorporation into soil and plant parts, there was no significant difference between inoculated and uninoculated plants. About 90% of the fixed $N_2$ remained in the soil. The plants used for the $^{15}N$-feeding experiment had been cut which might have limited the translocation of fixed nitrogen. Yoshida and Yoneyama (1980) and Eskew et al. (1981) also reported that most of the fixed N remained in the soil and a small fraction of the fixed N was found in the roots and leaves. In a similar study on *Setaria italica* (Okon et al. 1983) the rhizosphere soil was pulsed for 72 h with $^{15}N_2$; although found measurable, only a very small portion of bacterial fixed N was incorporated into the plant. The level of $^{15}N_2$ incorporation a 3-day incubation period accounted for microgram quantities only. In this study fixed $^{15}N_2$ was higher in the submerged portion of leaf sheath and stem than in the root. Ito et al. (1980) and Gowda and Watanabe (1985), using whole plant and excised plant, respectively, also found higher $^{15}N_2$ incorporation in the outer leaf sheath than in the root. Ito et al. (1980) attributed this to two factors: (1) the outer leaf sheath functions like a sink to which nitrogenous materials from the root are transported and (2) it is also a good $N_2$ fixation site because of the decomposed and dead plant tissues which serve as a good energy source for heterotrophic $N_2$ fixation.

*$^{15}N$ dilution.* The atom % excesses of $^{15}N$ in various plant parts and entire plants are shown in Table 7. On the total plant basis the inoculated OS4 and Hua plants showed higher atom % $^{15}N$ excess than the uninoculated ones. This indicated no enhanced fixation due to inoculation, which would have been shown by the dilution of $^{15}N$ in the inoculated plants. Dry matter weight, grain yield (Table 4) and uptake of soil and fertilizer nitrogen were increased (Table 8). The data showed that inoculated plants recovered more labelled fertilizer nitrogen than uninoculated

**Table 6.** $^{15}N_2$ [a] fixation (in situ) and incorporation into OS4 plant parts and soil

| Plant and soil portion sampled/treatment | Dry wt. (g/pot) | Total N (mg/pot) | $^{15}N$ [b] atom (% excess) | $^{15}N_2$ fixed (µg/pot) |
|---|---|---|---|---|
| Root (C) | 3.61 | 29.12 | 0.0427 ± 0.015 | 29.3 |
| Root (I) | 3.47 | 22.93 | 0.026 ± 0.004 | 11.4 |
| Basal shoot and submerged leaf sheath (C) | 6.41 | 33.89 | 0.105* ± 0.01 | 67.7 |
| Basal shoot and submerged leaf sheat (I) | 8.31 | 44.07 | 0.0762 ± 0.01 | 64.1 |
| Soil fraction I (C) | 25.0 | 36.42 | 0.120* ± 0.068 | 83.3 |
| Soil fraction I (I) | 25.0 | 33.46 | 0.0379 ± 0.01 | 24.2 |
| Soil fraction II (C) | 8000 | 12 000.00 | 0.0071 ± 0.0014 | 1600.0 |
| Soil fraction II (I) | 8000 | 11 900.00 | 0.0062 ± 0.0014 | 1400.0 |

C: control; I: inoculated

[a] Source = 52.42%

[b] Mean of three replicates ± SD

*Significantly different over the inoculated treatment at the 5% level

**Table 7.** Distribution of $^{15}N$ atom % excess in various plant parts at harvest

| Variety | Treatment | Grain | Straw | Root | Root stubble and leaf sheath | Total plant |
|---|---|---|---|---|---|---|
| OS4 | Control | 2.587 | 2.617 | 1.892 | 2.331 | 2.542 |
| | Inoculated | 2.569 | 2.735 | 1.789 | 2.363 | 2.560 |
| Hua-chou-chi-mo-mor | Control | 2.622* | 2.573 | 2.001 | 2.317 | 2.582* |
| | Inoculated | 2.747 | 2.702 | 1.807* | 2.203* | 2.681 |
| SE | | 0.05 | 0.073 | 0.128 | 0.052 | 0.057 |

* Significantly different at the 5% level

**Table 8.** Atom % excess of $^{15}N$ in plant and nitrogen derived from a labelled source and soil and/or air

| Variety | Treatment | Nitrogen (mg/pot) | |
|---|---|---|---|
| | | Labelled N[a] | From soil and/or air |
| OS4 | Control | 87.7 | 551.0 |
| | Inoculated | 106.0* | 659.0* |
| Hua-chou-chi mo-mor | Control | 100.0 | 518.0 |
| | Inoculated | 112.0 | 662.0 |
| SE | | 8.37 | 96.8 |

[a] Atom % excess of $^{15}N$ source was 18.48%
* Significantly different from the control (uninoculated) treatment at the 5% level

plants. Inoculation caused 4.8% more nitrogen in Hua and 11.5% more in OS4. N balance data also showed a negative balance in most of the pots (data not shown). However, one crop is not enough to detect the contribution of heterotrophic nitrogen fixation (Ventura and Watanabe 1982).

The nitrogen 15 dilution and $^{15}N_2$ incorporation results seem to suggest that increased dry matter production and nitrogen uptake due to inoculation are not caused by enhanced biological $N_2$ fixation. However, such a conclusion should be considered with caution (Witty 1983). For the $^{15}N$ dilution method it is implicited that the enrichment of plant available soil N remains constant with time or that the control and inoculated plants have similar nitrogen uptake patterns; otherwise highly misleading results will be obtained (Witty 1983). In this study, inoculation might have stimulated the earlier plant growth and nitrogen uptake, resulting in changed $^{15}N$ uptake of inoculated and uninoculated plants, because the $^{15}N/^{14}N$ ratio in soil ammonium-N is higher at earlier growth stages.

It can be concluded that the inoculated organisms had poor survival, declining rapidly from the soil and plant parts. Increased yield and total nitrogen in both varieties due to inoculation could not be attributed to $N_2$ fixation based on time course ARA and $^{15}N_2$ feeding results. Nitrogen 15 dilution results should be considered with caution. It is not clear whether this N acquisition and growth stimulation by inoculation was due to production of a growth factor or to removal of inhibitory action by chemicals or pathogens and/or to enhanced mineral uptake.

The possibilities that the sterilization of peat by autoclaving might have caused toxicity into the soil and to the rice plant and that such toxicity might have been reversed by inoculated organisms in the inoculated treatment need further study.

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## References

Baldani VLD, De-Polli H, Alvarez MA, Baldani JI, Dobereiner J (1984) Establishment of inoculated *Azospirillum* spp. in the rhizosphere and roots of field grown cereals. Proceedings of 3rd Int. Symp. on $N_2$ fixation, Helsinki, 2–8 Sept 1984 (Abstract)

Barraquio WL, de Guzman MR, Barrion M, Watanabe I (1982) Population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland and dryland rice. Appl Environ Microbiol 43:124–128

Barraquio WL, Daroy MLG, Tirol AC, Ladha JK, Watanabe I (1985) Laboratory acetylene reduction assay for relative measurement of $N_2$ fixing activities associated with field grown wetland rice plants. Plant and Soil (in press)

Bremner JM, Shaw K (1958) Denitrification in soil: 1. Method of investigation. J Agric Sci 51:22–39

Caceres EAR (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 44:990–991

Cohen E, Okon Y, Kigel J, Nur I, Henis Y (1980) Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen-fixing *Azospirillum* spp. Plant Physiol 66:746–749

Crossman SM, Hill WA (1984) Response of centennial sweet potato to inoculation with *Azospirillum*. The 3rd Int. Symp. on $N_2$ fixation, Helsinki, 2–8 Sept 1984 (Abstract)

Eskew DL Eaglesham ARJ, App AA (1981) Heterotrophic $^{15}N_2$ fixation and distribution of newly fixed N in a rice flooded soils system. Plant Physiol 68:48–52

Gaskins MH, Alberecht SL, Milan JR (1984) Survival of root-associated bacteria in the rhizosphere. In: Veeger C, Newton WE (eds) Advances in nitrogen fixation research. Martinus Nijhoff/ Dr. W. Junk Publshers, The Hague, Boston, Lancaster, pp. 52

Gowda TKS, Watanabe I (1985) Stimulating effect of hydrogen on $N_2$-fixation in association with field-grown wetland rice. Can J Microbiol 31:503–507

Hegazi NA, Monib M, Amer HA, Shokar ES (1983) Response of maize plants to inoculation with azospirilla and (or) straw amendment in Egypt. Can J Microbiol 29:888–894

Ito O, Cabrera D, Watanabe I (1980) Fixation of dinitrogen-15 associated with rice plants. Appl Environ Microbiol 39:554–558

Kapulnik Y, Okon Y, Kigel J, Nur I, Henis Y (1981a) Effects of temperature, nitrogen fertilization, and plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (Strain Cd). Plant Physiol 68:340–343

Kapulnik Y, Sarig S, Nur I, Okon Y, Kigel J, Henis Y (1981b) Yield increases in summer cereal crops in Israeli fields inoculated with *Azospirillum*. Expt Agric 17:179–187

Ladha JK, Barraquio WL, Watanabe I (1982) Immunological techniques to identify *Azospirillum* associated with wetland rice. Can J Microbiol 28:478–485

Lin W, Okon Y, Hardy RWF (1983) Enhanced mineral uptake by *Zea mays* and *sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Appl Environ Microbiol 45:1775–1779

Ohyama T, Kumazawa K (1978) A simple preparation method of $^{15}N_2$ for water experiment. J Sci Soil Manure Jpn 49:424–425

Okon Y (1982) *Azospirillum*: physiological properties, mode of association with roots and its application for the benefit of cereal and forage grass crops. Isr J Bot 31:214–220

Okon Y (1984) Development and function of *Azospirillum*-inoculated roots. Proceedings of 3rd Int Symp on $N_2$ fixation, Helsinki, 2–8 Sept 1984 (Abstract)

Okon Y, Heytler PG, Hardy RWF (1983) $N_2$ fixation by *Azospirillum brasilense* and its incorporation into host *Setaria italica*. Appl Environ Microbiol 46:694–697

Rao JLN, Rajamamohan Rao VR (1983) Nitrogenase activity in the rice rhizosphere soil as affected by *Azospirillum* inoculation and fertilizer nitrogen under upland conditions. Curr Sci 52-:686–688

Rao VR, Nayak DN, Charyulu PBBN, Adhay TK (1983) Yield responses of rice to root inoculation with *Azospirillum*. J Agric Sci Camb 100:689–691

Smith RL, Schank SC, Bouton HH, Quesenberry KH (1978) Yield increases of tropical grasses after inoculation with *Spirillum lipoferum*. In: Granhall U (ed) Environmental role $N_2$-fixing blue-green algae and asymbiotic bacteria, vol 26. Ecol Bull Swedish Natural Science Research Council, Liber Tryck, Stockholm, pp 380–385

Smith RL, Schank SC, Milan JR, Baldensperger AA (1984) Response of Sorghum and *pennisetum* species to the $N_2$-fixing bacterium *Azospirillum brasilense*. Appl Environ Microbiol 47:1331–1336

Subba Rao NS, Tilak KVBR, Singh CS, Kumari L (1979) Response of a few species of graminaceous plants to inoculation with *Azospirillum brasilense*. Curr Sci 48:133–134

Tien TM, Gaskins MH, Hubbell DH (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L) Appl Environ Microbiol 37:1016–1024

Ventura W, Watanabe I (1982) $^{15}N$ dilution technique of assessing nitrogen fixation in association with rice. Philipp J Crop Sci 7:44–50

Watanabe I, Lin C (1984) Response of wetland rice to inoculation with *Azospirillum lipoferum* and *Pseudomonas* sp. Soil Sci Plant Nutr 30:117–124

Watanabe I, Barraquio WL, De Guzman M, Cabrera DA (1979) Nitrogen fixing (acetylene reduction) activity and population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland rice. Appl Environ Microbiol 37:813–819

Witty JF (1983) Estimating $N_2$ fixation in the field using $^{15}N$-labelled fertiliser: some problems and solutions. Soil Biol Biochem 15:631–639

Yoneyama T, Kumazawa K (1974) A simple determination of $^{15}N$ abudance in plant powder sample. J Sci Soil Manure (Japan) 45:480–482

Yoshida T, Ancajas RR (1973) Nitrogen-fixing activity in upland and flooded rice fields. Soil Sci Soc Am Proc 37:45–46

Yoshida T, Yoneyama T (1980) Atmospheric dinitrogen fixation in the flooded rice rhizosphere determined by the N-15 isotope technique. Soil Sci Plant Nutr 26:551–589

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