World J Microbiol Biotechnol (2007) 23:237–242
DOI 10.1007/s11274-006-9219-0

ORIGINAL PAPER

Cyanobacterial inoculation and nitrogen fertilization in rice

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Received: 7 April 2006 / Accepted: 10 June 2006 / Published online: 14 July 2006

Abstract During three rice-growing seasons in Uruguay, field experiments were conducted to study the contribution of cyanobacterial inoculation and chemical N fertilization to rice production. Neither grain yield nor fertilizer recovery by the plant were affected by inoculation with native cyanobacterial isolates. A low fertilizer use efficiency (around 20%) was observed when labelled $(NH_4)_2SO_4$ was applied at sowing. Recovery of applied ^{15}N by the soil–plant system was 50%. Inoculation did not modify ^{15}N uptake by the plant when the fertilizer was three-split applied either. The total N-fertilizer recovery was higher when the fertilizer was split than when applied in a single dose. Plant N-fertilizer uptake was higher when the fertilizer was applied at tillering. Uptake of ^{15}N from cyanobacteria by rice was studied in a greenhouse pots experiment without chemical nitrogen addition. Recovery of ^{15}N from labelled cyanobacteria by rice in greenhouse growth conditions was similar to that of partial recovery of $(NH_4)_2SO_4$ applied at sowing in the field. Cyanobacterial N mineralization under controlled conditions was fast as cyanobacterial N was detected in plants after 25 days. Moreover 40 days after inoculation non-planted and inoculated soil had more inorganic N than the non-inoculated one.

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Keywords Fertilizer N utilization · heterocystous cyanobacteria · mineralization · ^{15}N · ricefield

Introduction

Rice is mainly grown under irrigated conditions where nitrogen fertilizer efficiency is low due to large N losses from flooded soils (De Datta and Buresh 1989). Soil N pool in ricefields is principally maintained by fertilizer N and biological nitrogen fixation (Kundu and Ladha 1995). Among nitrogen-fixers in rice fields, cyanobacteria are important contributors to N_2 fixation (Roger and Ladha 1992). Cyanobacterial trophic independence makes them suitable for being used as biofertilizers (Irisarri 2006). Most published data of inoculation with cyanobacteria refer to tropical ricefields, which are different in characteristics and agricultural management from temperate ones. Biological N fixation is far more diverse and complex in the tropics than under temperate conditions (Balandreau and Roger 1996). Assays of cyanobacterial inoculation in temperate climates were performed in the USA (Reynaud and Metting 1988), Spain (Fernández-Valiente et al. 1996), Italy and Argentine (Zaccaro 2000).

The average heterocystous cyanobacterial density in Uruguayan temperate ricefields is lower than those reported for other ricefields (Irisarri et al. 2001). Therefore inoculation with native cyanobacterial isolates appeared as a possible supplementary nitrogen input to this ecosystem. Inoculation may decrease the necessary time for cyanobacteria to divide and efficiently colonize the soil (Balandreau and Roger 1996). Nitrogen fixed by cyanobacteria may become available to rice plants only after its release into their

surroundings, by mineralization of their intracellular content and/or as extracellular products (Sinha and Häder 1996). So, it was supposed that a successful set-up of desired cyanobacteria in the ricefields would be a source of slow release of nitrogen according to plant demands. Quantitative information regarding transfer of cyanobacterial nitrogen to rice is scarce (Ghosh and Saha 1997).

Nitrogen taken up by the crop growing on a soil that has been fertilized with N comes partly from the fertilizer and partly from soil, mineralized from organic matter. When the fertilizer is added in a labelled form (^{15}N) the amount of N in the crop originating from the soil, which includes that from cyanobacteria and from the fertilizer, can be determined.

An assay with labelled N-fertilizer and cyanobacterial inoculation was proposed to quantify the N provided to the rice plant by the fertilizer and one with labelled cyanobacteria to estimate the time required for their mineralization.

The aim of this study was to quantify how much nitrogen from fertilizer was incorporated into rice and to establish if cyanobacterial inoculation contributed to nitrogen nutrition and rice yield during the crop cycle.

Materials and methods

Field experiments

Field assays were conducted during three consecutive crop seasons at Estación Experimental Paso de la Laguna—INIA Treinta y Tres at the east of Uruguay. The soil was, according to FAO taxonomy, Albic Natraqualf. Main properties of the soil for the three growth seasons are described in Table 1. The mean 5-month daily precipitation for the 3-year growing period of rice culture (2000–2003) was 7.3 mm and the mean temperature was 21°C. Rice was sown in dry soil and flooding was established four weeks after.

Experiments were established in a completely random design with three replicates. Basal P fertilization and herbicide application were similar for all treatments.

Table 1 Soil properties for the three rice growing periods (October–March for the South Hemisphere)

Year	pH (H_2O)	OM%	P Bray ppm	K meq/100 g
2000–2001	5.4	2.71–3.26	6.4–7.1	0.22–0.23
2001–2002	5.4–5.5	2.41–2.81	5.6–6.5	0.22–0.23
2002–2003	5.1–5.2	2.64–3.21	7.3–9.1	0.27–0.29

For the first two crop seasons, when the labelled fertilizer was applied at sowing, the rice variety was El Paso 144 and for the last crop season, when the fertilizer was split applied, rice variety was INIA Tacuarí.

During 2000–2001 and 2001–2002, plots were fertilized with ^{15}N-labelled ammonium sulfate (ISOTEC Inc.) in solution at a rate of 10 Kg N ha^{-1} (30 at.% excess ^{15}N) and inoculated with cyanobacteria 40 days after, at tillering. Non-inoculated ^{15}N-fertilized plots were used as controls. Experimental plots of 6 m^2 were used for yield evaluation and 1 m^2 microplots for ^{15}N assays.

The inoculant was prepared culturing three Uruguayan heterocystous ricefield cyanobacterial isolates in BGII medium, *Nostoc* BI42, *Anabaena* BI46 and *Calothrix* BI 22, and the mixture final concentration applied at flooding was 2×10^6 c.f.u. m^{-2}.

Soil (upper 15 cm) and aboveground plant samples were collected at harvest, the grain and straw of rice plants were separated and were analysed for total nitrogen by Kjeldahl digestion and ^{15}N with a mass spectrometer (IRMS Micromass Isochrom) at SIDI-Laboratorio de Isótopos Estables, UAM, Spain. Fertilizer use efficiency (FUE) was calculated according to IAEA (2001).

In the crop season 2002–2003 total N rate (30 kg N/ha) was split at three different moments, rice emergence, tillering and panicle initiation. In the factorial experiment (3 fertilization dates × 2 inoculation) only the time of the labelled fertilizer application (10 kg N ha^{-1} as 5 atom % excess ^{15}N-ammonium sulfate) changed, thus the effect of timing was measured in the absence of any plant-fertilizer interaction. Plants samples were collected at tillering, panicle initiation and harvest when the labelled fertilizer was applied at emergence, tillering and panicle initiation, respectively. Partial fertilizer use efficiency was calculated for each application time and total FUE as the sum of the three partial FUE.

Data were analysed with SAS software. Analysis of variance (ANOVA) was used to determine the significance of main effects and their interactions.

Mineralization assay

An experiment with rice sown in plastic boxes and inoculated with ^{15}N-labelled cyanobacteria was conducted in greenhouse. Isolates of *Nostoc* BI42, *Anabaena* BI46 and *Calothrix* BI 22 were grown in BGII with ^{15}N-ammonium sulfate as nitrogen source and the suspension was centrifuged and washed with H_2O in order to eliminate ^{15}N excess. The inoculant was added

simultaneously with flooding at a concentration of 0.9 Kg N ha^{-1} (9×10^7 c.f.u. m^{-2}) and 38 at.% excess ^{15}N. Plant samples were collected at 25, 55 and 100 days after inoculation and were analysed for total nitrogen and ^{15}N content.

Results and discussion

Fertilizer nitrogen recovery

Nitrogen incorporated to rice comes both from fertilizer and soil organic matter mineralization. The use of a ^{15}N-labelled fertilizer allows discrimination of how much nitrogen derives from fertilizer.

The percentage of ^{15}N recovery by plants without inoculation (Table 2) ranged from 14% to 22% depending on the year. N-fertilizer recovery by plants was low but similar to that reported for other ricefields (De Datta et al. 1987; Sheehy et al. 2004; Fernandez-Valiente et al. 2000).

The total ^{15}N recovered from the soil–plant system for the crop season 2000–2001 was 43% (Table 2). Applied N loss in rice paddy soils is mainly attributed to coupled nitrification–denitrification and NH_3 volatilization. Volatilization has been reported as the main source of N loss in rice fields (Cassman et al. 1998) but in Uruguayan culture conditions, nitrification may be considerable before flooding (Tarlera et al. 2006).

When the fertilizer was applied at a dose of 10 Kg N ha^{-1}, at least 98% of the plant N-uptake came from the soil. In spite of this, the soil N content (0.16%) did not decrease after harvest, meaning that biological nitrogen fixation contributed to the nitrogen budget of this ecosystem.

Spliting the N rate has been suggested as the best choice to optimize fertilizer efficiency (Stevens et al. 2001) so a three-way split timing of N fertilizer was assayed. The partial fertilizer use efficiency of the 10 Kg of labelled N ha^{-1} for the three application dates varied from 7% at panicle initiation to 21% at tillering (Table 3). Significant differences were found in plant N derived from fertilizer among the three application dates ($p < 0.01$). In agreement with other reports (Norman et al. 1992; Wilson et al. 1989) the highest N-fertilizer recovery was found at tillering (Fig. 1). The plant N derived from fertilizer was low (0.24%) when the fertilizer was added at panicle initiation. The plant N-content evolution along the crop cycle when the fertilizer was split applied, shows that most of the nitrogen uptake occurred previous to panicle initiation (Fig. 2).

The total N-fertilizer recovery in plants was higher when the fertilizer was split, 40%, (Table 3) than when applied in a single dose, 14% and 22% (Table 2). More effective crop utilization of nitrogen and reduced ^{15}N-losses with the three split, may explain the increase in N recovered by the aboveground biomass (grain and straw).

Table 2 Productivity, plant nitrogen, fertilizer use efficiency and N recovery by plants and soil

Crop	Treatment	Grain Yield (Kg ha^{-1})	Plant N* (Kg ha^{-1})	Plant Ndff (Kg ha^{-1})	% Ndff	FUE%	Soil Ndff (Kg ha^{-1})	%^{15}N Soil + Plant recovery
2000–2001	Inoculated	7276 ± 921	127.5	2.2	1.7	21.8	4.2	64.1
	Non-inoculated	7858 ± 219	135.8	2.1	1.6	21.5	2.2	43.4
2001–2002	Inoculated	6401 ± 381	135.3	1.7	1.2	17.2	nd	nd
	Non-inoculated	6250 ± 226	127.0	1.4	1.0	14.0	nd	nd

Ndff, N derived from fertilizer; nd, not determined; Values of grain yield, plant N, Ndff and FUE for each crop were not significantly different at $p < 0.05$; Soil Ndff values (2000–2001) were only significantly different at $p < 0.08$; *grain + straw

Table 3 Effect of inoculation and fertilizer timing on partial plant N recovery

Treatment	$(^{15}NH_4)_2SO_4$ Application	Plant N(Kg ha^{-1})	Straw Ndff (Kg ha^{-1})	Grain Ndff (Kg ha^{-1})	Plant % Ndff	Partial FUE%
Inoculated	Emergence	195.7	0.73	1.29	1.00[b]	20.1
	Tillering	183.3	1.21	1.50	1.51[a]	27.1
	Panicle initiation	156.2	0.14	0.24	0.24[c]	3.8
Non-inoculated	Emergence	162.9	0.40	0.70	0.65[b]	11.1
	Tillering	184.3	0.85	1.28	1.15[a]	21.3
	Panicle initiation	200.5	0.15	0.57	0.37[c]	7.2

Interaction between inoculation and date of N fertilizer application was non-significant; Means in the same column followed by different letters were significantly different at $p < 0.01$

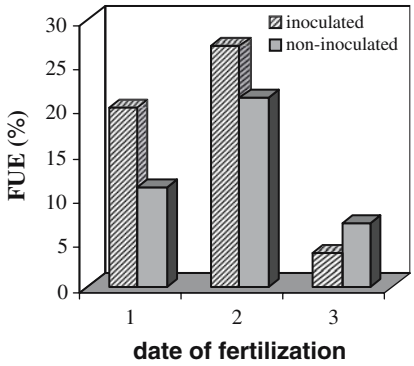

Fig. 1 Partial fertilizer use efficiency when the labelled fertilizer was applied at: (1) rice emergence, (2) tillering and (3) panicle initiation

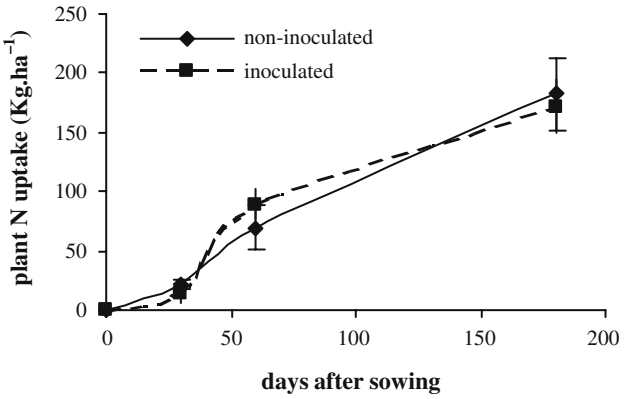

Fig. 2 Plant nitrogen uptake since sowing to harvest for the inoculated and non-inoculated treatments. The data are mean of three replicates and the bars correspond to the standard deviations

Effect of inoculation on yield response and nitrogen uptake

Inoculation of cyanobacteria did not cause a positive grain yield response compared with the non-inoculated controls (Table 2) like in many other cases (Roger et al. 1993; Watanabe 1986). However, yield increase after inoculation was reported by Balandreau and Roger (1996) and by Ghosh and Saha (1997).

No effects on yield could be explained by several causes. The number of heterocystous cyanobacteria in soil was not higher in the inoculated plots than in the non-inoculated ones immediately after inoculation although it increased at the end of the crop cycle (data not shown). The initially slightly acidic pH of the soil could affect cyanobacterial growth besides favour eucaryotic algal development. Likewise light could be considered a limitant to cyanobacterial N_2 fixation mainly at the end of the cycle.

Reddy and Roger (1988) showed that inoculated strains did multiply but rarely dominated the native population of cyanobacteria. The fate of cyanobacteria in the ricefield ecosystem depends on their ability to grow, colonize and survive in the soil (Tomaselli and Giovannetti 1993). Because knowledge of the factors that allow cyanobacteria to establish and bloom in ricefields is limited, inoculation is conducted on a trial-and-error basis. The inoculant in this work was produced in the laboratory growing the three most abundant field isolates without a chance to compete with algae and grazers present in the ricefield, which would affect inoculant survival.

Plant nitrogen derived from fertilizer was not different between the inoculated plots and the non-inoculated ones (Table 2). This does not necessarily mean that there was no biological nitrogen fixation but that inoculation could not increase the nitrogen currently fixed and mineralized in this flooded soil.

For the crop season 2000–2001, the N derived from fertilizer in soil at harvest was different with $p < 0.08$ between treatments. More N-fertilizer was recovered by the soil than by the plant in the inoculated plots (Table 2). Since there was no difference in the plant nitrogen derived from fertilizer for both treatments (Table 2), the explanation of this result may be an increase in microbial N-immobilization due to inoculation. The photosynthetic biomass has been reported as decreasing N losses by transitory immobilization as organic N in soil (Roger 1996).

Since no effect of inoculation could be detected when all the fertilizer was applied in a single dose, a three-way split timing of N fertilizer was assayed. Inoculation did not increase yield when the fertilizer was split (data not shown). Partial plant Ndff for each fertilization time was not different for the inoculated treatment also (Table 3).

Although the cyanobacterial isolates used for the inoculant were good fixers under laboratory conditions, the failure of inoculation may be explained by the amount of inoculant applied and by field conditions that limit N_2 fixation.

Cyanobacterial nitrogen mineralization

The biofertilizer N efficiency measured at different growth stages of rice ranged from 5%, 25 days after inoculation, to 12% at harvest (Table 4). Nitrogen from cyanobacteria could be detected in plants as early as 25 days after inoculation.

At harvest the fertilizer use efficiency of cyanobacterial N was similar to the partial one of chemical fertilizer applied at emergence in the field experiment (Tables 3 and 4). The applied biomass of cyanobacteria

Table 4 Cyanobacterial N recovery efficiency

Days after inoculation	Growth stage of rice	g Ndff (Cyanobacteria)[a]	FUE %
25	Floral Primordia	45 ± 9	5.0
55	Flowering	60 ± 16	6,7
100	Harvest	105 ± 22	11,7

[a] Values are means of four replicates ± standard error

started decomposition within a few days of inoculant application. This is evidenced by the assimilation of ^{15}N by the rice plant within 25 days (Table 4) and by ^{15}N inorganic accumulation in soils (Fig. 3). The cyanobacterial mineralization rate was not therefore the limitative to N plant uptake from cyanobacteria, although this N may not come from N_2-fixation. It should be regarded that the greenhouse assay had no N chemical addition, had more cyanobacteria in the inoculant, more cyanobacterial blooms and no aquatic weeds.

In pots without plants, with and without inoculation, soil inorganic N content was higher in the inoculated pots after 5 weeks (Fig. 3). The increase in inorganic N in soil due to inoculation indicated a release of N of cyanobacterial cells, but most nitrogen should have been mineralized after the maximal nitrogen-requiring stage of rice.

However, the variation in the magnitude of the increase at different periods might be the result of many interacting processes including mineralization-immobilization and losses through various means (Ghosh and Saha 1997). Depending on field conditions, cyanobacteria may have a role in assimilating nitrogen and protecting it from being lost as well as being a major driving force for NH_3 volatilization through diurnal increases in pH. Cyanobacteria may also cause

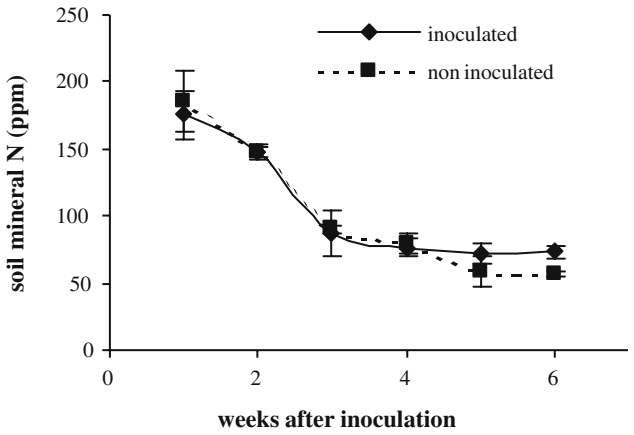

Fig. 3 Soil mineral nitrogen (nitrate plus ammonium) evolution after cyanobacterial inoculation. The data are mean of three replicates and the bars correspond to the standard deviations

N loss through stimulation of nitrification–denitrification processes as they affect the depth of the aerobic soil layer through their O_2 input, but more data are required for confirm this assertion (Mandal et al. 1999).

Conclusions

Rice inoculation at flooding with heterocyst cyanobacteria isolated from Uruguayan ricefields, increases neither rice yield nor the plant N fertilizer recovery, meaning that the inoculant nitrogen fixation was not vital to plant nutrition under these conditions. N-fertilizer recovery by plant and soil system is not different with inoculation.

N use efficiency when fertilizer was applied at sowing is around 20% for these culture conditions, meaning that most of the N uptake comes from the soil. The total N-fertilizer recovery was higher when the fertilizer was split than when applied in a single dose. Plant N-fertilizer uptake was higher when the fertilizer was applied at tillering. Cyanobacterial mineralization under controlled conditions was fast; as cyanobacterial N was detected in plants after 25 days. Moreover 40 days after inoculation non-planted and inoculated soil has more inorganic N than the non-inoculated one.

Soil inorganic N increase occurred five weeks after inoculation coinciding with panicle initiation, date with the lowest plant fertilizer recovery and so, cyanobacterial mineralization occurs after the more N-demanding plant stages. For considering cyanobacterial inoculation in organic rice culture without chemical fertilizer addition, conditions to improve inoculant survival and N_2 fixation must be studied.

Acknowledgements This work was supported by IFS (Grant C-2747) and INIA-FPTA No. 108. We thank V. Berriel for total N analysis and A. Bereta for statistical analysis.

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