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Development of a biofertilizer based on filamentous nitrogen-fixing cyanobacteria for rice crops in Chile

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Abstract The purpose of this study was to develop a biofertilizer based on filamentous nitrogen-fixing cyanobacteria selected from rice fields and to generate a technological package compatible with its use for the rice crop in Chile. Thirty-four Chilean rice fields, located between Maule and BioBío regions, were sampled during the 1998/1999 and 1999/2000 growing seasons. A total of 9 species and 3 varieties of cyanobacteria were found, and the nitrogen fixation rate under laboratory conditions was determined for 6 of them. Only 4 were used for the small-scale production of a biofertilizer, which was assayed in field trials. To check the efficiency of the biofertilizer during the rice crop, the nitrogen fixation rates in soil samples were estimated. Additionally, the biofertilizer application efficiency was tested in combination with nitrogen synthetic fertilizer, in rates that were previously established in field trials. Biofertilization allowed a decrease of up to 50% in the use of nitrogen synthetic fertilizer (50 kg N ha^{-1}), resulting in the same grain yield (7.4 t ha^{-1}) and quality in relation to the fertilized control. The use of biofertilizers based on local strains of cyanobacteria shows promise to increase nitrogen use efficiency in rice.

Keywords Biofertilizer · Cyanobacteria · Decrease use synthetic fertilizer · Nitrogen-fixation · Rice crop · Yield and industrial quality of rice grain

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Introduction

It is known that cyanobacteria supply more nitrogen in wetland rice fields in tropical regions (Singh 1985, 1988) than in dryland fields (Yamaguchi 1979), and that this is attributable to the unique characteristics of wetland rice fields: along with water, there is a natural supply of plant nutrients, especially N, which encourages general plant growth, and the soil pH is more neutral. Additionally, there is considerable literature indicating that, in rice-flooded soils of temperate regions, cyanobacteria increase soil nitrogen through nitrogen fixation (Vaishampayan et al. 2001), such as, for example, in Japan and in most Southeast Asian countries (Watanabe 1973). However, this behavior is not shown in all rice soils in temperate regions, since the environmental conditions such as moisture, soil pH (Granhall 1975), combined nitrogen levels (Roger and Kulasooriya 1980; Howarth et al. 1988) and temperature are factors that can play an ecologically determinant role on the abundance of cyanobacteria and on nitrogen fixation.

The inoculation of rice soils of the West Pacific area with two cyanobacteria species, *Tolypothrix tenuis* and *Aulosira fertillisima*, has had differential effects on the nitrogen supply mainly due to differences in the pH of the inoculated soils (Watanabe 1973). On the other hand, the best results with algalization have been obtained when the inoculants contained mixtures of algae from at least one of the local stocks (Venkataraman 1981) and where the inoculants are added in a proportion equivalent or superior to the applied nitrogen synthetic fertilizer (Fernández-Valiente et al. 1996; Quesada et al. 1991, 1997).

In South America, Brazil and Argentina have been among the first countries to perform research on this topic (de Halperin et al. 1992). In Argentina, this agronomic practice has been applied with very encouraging results (Prosperi et al. 1996).

In Chile, several studies of response to N fertilizer additions were performed from the end of the 1960s up to the 1980s and they demonstrated that rice yields in control (nitrogen-free) treatments were higher than those that could be supported by the naturally poor soil fertility (Rojas and Alvarado 1982). These results indicated that an important proportion of the available nitrogen in the soil came from biological nitrogen fixation.

A few studies have have been performed to enrich Chilean rice soils with nitrogen, using symbiotic and free-living nitrogen-fixing organisms, such as the fern *Azolla* and cyanobacteria. The first study was performed using *Azolla* and the results indicated that, for the same nitrogen rate, the use of *Azolla* produced equal or superior yields in comparison to urea, indicating that it would be possible to change or complement the nitrogen synthetic fertilization with this fern. The study also demonstrated that the incorporation of the fern into the soil before the rice crop was more effective than simultaneously growing rice and *Azolla*. Nevertheless, there was a limiting factor in the use of this fern arising from the coincident life cycle of the fern and rice plant, which did not allow the production of enough fresh biomass of the fern to be applied as a biofertilizer during the rice plant's current life cycle (Vidal 1991).

The second study was on the introduction of the commercial biofertilizer, MICROP-BG, produced in the United States. This biofertilizer was imported to Chile and tested at the field level during two growing seasons without positive effects on rice yield or quality (Ortega et al. 1991). The causes of this Product's lack of effect were that it contained cyanobacteria species unadapted to the edapho-climatic conditions of the rice soils in Chile. More specifically, the product also contained a low percentage of filamentous *Tolypothrix tenuis* and a high quantity of diatoms and sand. However, in the same studies, several indigenous species of cyanobacteria were found in the flooded water.

Considering the lack of knowledge on indigenous filamentous nitrogen-fixing cyanobacteria in Chilean rice soils, the high nitrogen rates traditionally applied in rice crops (50–100 kg N ha^{-1}), and the potential ecological damage produced by excessive nitrogen use in terrestrial and aquatic ecosystems, we began evaluating algalization as a new agronomic practice in Chile. The objectives of the present study were: (1) to identify the filamentous heterocystous nitrogen-fixing cyanobacteria that are part of the indigenous algal flora in Chilean rice soils, (2) to isolate the algae, maintain uni-algal cultures and multiply them on a small-scale, (3) to determine the nitrogen fixation rates of the isolated algae, (4) to develop a biofertilizer using some of these algae for field trials in actual rice crops in Chile, (5) to estimate the nitrogen fixation in soil samples during the rice growing period, and (6) to evaluate the biofertilizer's efficiency in combination with nitrogen synthetic fertilizer in terms of rice grain yield and industrial quality (in comparison to a control) under an integrated rice crop management system. This new agronomic practice could favor a more organic rice production minimizing the environmental impacts, and therefore being more attractive for farmers and consumers.

Materials and methods

The study area included 34 sites located between 34°30′52″– 36°36′40″S and 71°18′46′–72°33′36″W, an area that corresponds to the O'Higgins, Maule and BioBìo regions of Chile (Table 1, Fig. 1). Site elevation varied from 100 to 400 m asl. Soil texture was mainly clay to clay loam. Maximum air temperatures during the rice growing period varied from 29 to 38°C (November to March) and water temperature ranged from 22 to 28°C. The pH of the soil slurry varied between 5.5 and 7.0 but, as expected, the predominant soil's pH was close to neutrality.

Collection of algal material

Three water samples were obtained from each sampling location using 500-mL glass or plastic flasks. They were randomly collected near the levees of each field. One of the samples from each location was fixed in 4% formalin and used for taxonomic determination. The two remaining samples were used for isolating, establishing and maintain-

Table 1 Geographic location of the sampled locations

Location	Geographic position	Regions[a]
1	36°22′46″S, 71°57′50″W	VIII
2	36°28′50″S, 72°33′36″W	VIII
3	36°17′16″S, 72°07′41″W	VII
4	36°07′25″S, 72°28′50″W	VII
5	36°28′50″S, 72°33′36″W	VII
6	36°04′35″S, 72°00′07″W	VII
7	36°04′33″S, 72°00′07″W	VII
8	36°05′24″S, 71°56′22″W	VII
9	36°07′00″S, 71°54′11″W	VII
10	36°02′51″S, 71°52′38″W	VII
11	36°01′34″S, 71°53′24″W	VII
12	35°58′59″S, 71°50′17″W	VII
13	35°53′16″S, 71°42′11″W	VII
14	34°31′41″S, 71°21′07″W	VI
15	34°30′52″S, 71°19′38″W	VI
16	34°33′41″S, 71°23′39″W	VI
17	34°44′45″S, 71°20′14″W	VI
18	34°46′34″S, 71°18′46″W	VI
19	35°23′25″S, 71°23′14″W	VII
20	35°22′58″S, 71°21′02″W	VII
21	35°24′56″S, 71°24′05″W	VII
22	35°49′42″S, 71°41′42″W	VII
23	35°48′23″S, 71°44′20″W	VII
24	35°47′04″S, 71°45′22″W	VII
25	35°44′06″S, 71°45′04″W	VII
26	35°46′58″S, 71°46′09″W	VII
27	35°46′58″S, 71°48′26″W	VII
28	35°41′42″S, 71°41′07″W	VII
29	35°45′38″S, 71°40′38″W	VII
30	35°45′25″S, 71°25′05″W	VII
31	35°45′01″S, 71°24′56″W	VII
32	35°55′09″S, 71°39′24″W	VII
33	35°55′09″S, 71°51′44″W	VII
34	36°36′40″S, 72°06′22″W	VIII

[a] VI O'Higgins, VII Maule, VIII BioBìo

ing unialgal cultures and small-scale mass culture in order to produce the biofertilizer. In addition, soil samples were also taken for a similar purpose.

Taxonomic determination was by light microscopy and based on Desikachary (1959) and Geitler (1932). The vegetative and reproductive characters used in the taxonomic determination were: shape, color, and size of the thallus; breadth and length of trichomes; shape, size, and color of vegetative cells, heterocysts and akinetes; as well as texture, color and ornamentation of cell walls of the akinetes.

Isolation

The flasks containing the collected samples were poured into Petri dishes and then carefully screened under a stereomicroscope in order to identify each nitrogen-fixing algae species and further isolate them. Different techniques were used depending on the type of algae being isolated. In some cases, a successive washing technique was employed: the algae were successively washed and transferred from one watch glass to another using a sterilized micropipette at each step. Before washing, a certain amount of algae previously identified under the microscope was accumulated in a watch glass. In other cases, the dilution was carried out in test tubes. The algal samples were diluted at least 5–10 times in distilled water in test tubes and then planted on Petri dishes. Of the two isolation methods used, the micropipette-washing method was the most efficient for obtaining unialgal cultures.

Culture media and maintenance of unialgal cultures

To grow the algae, four culture media were tested: Chu 10, Water-soil, liquid Watanabe, and agar Watanabe (Stein 1973). Of these media, only two (liquid Watanabe and agar Watanabe) were used for both maintaining algae and for mass culturing them.

Unialgal cultures were maintained as follows: Watanabe liquid medium, 14 h light: 10 h dark photoperiod, culture chamber temperature 25°C, and a light intensity of 25 µmol photons m^{-2} s^{-1}, first in Petri dishes and then in 500-mL Erlenmeyer flasks.

Mass culture of the algae

To obtain the required amount of algal mass, the algae had first to be homogenized with a porcelain Potter. Using a Pasteur pipette, some drops of the homogenized algal solution were plated on Petri dishes containing Watanabe agar medium. The Petri dishes were put in a culture chamber at 25°C under a constant light and 18 µmol photons m^{-2} s^{-1} irradiance. The cultures were transferred to 400-ml Erlenmeyer flasks containing Watanabe liquid medium under the same conditions and produced 0.425 g L^{-1} after a 1 month growth period. The process was continued until the amount of algal material required for pot assays and for the subsequent elaboration of biofertilizer for field experiments had been reached (Venkataraman 1981; Vonshak 1985).

Determination of the nitrogen fixation rates under laboratory conditions

Nitrogen fixation rate was determined by the acetylene reduction technique (Hardy et al. 1973). The algae samples used had been maintained on Watanabe liquid culture medium. A total of 25 mL of the culture was transferred to a 450-mL sealed glass flask, reducing the air volume to

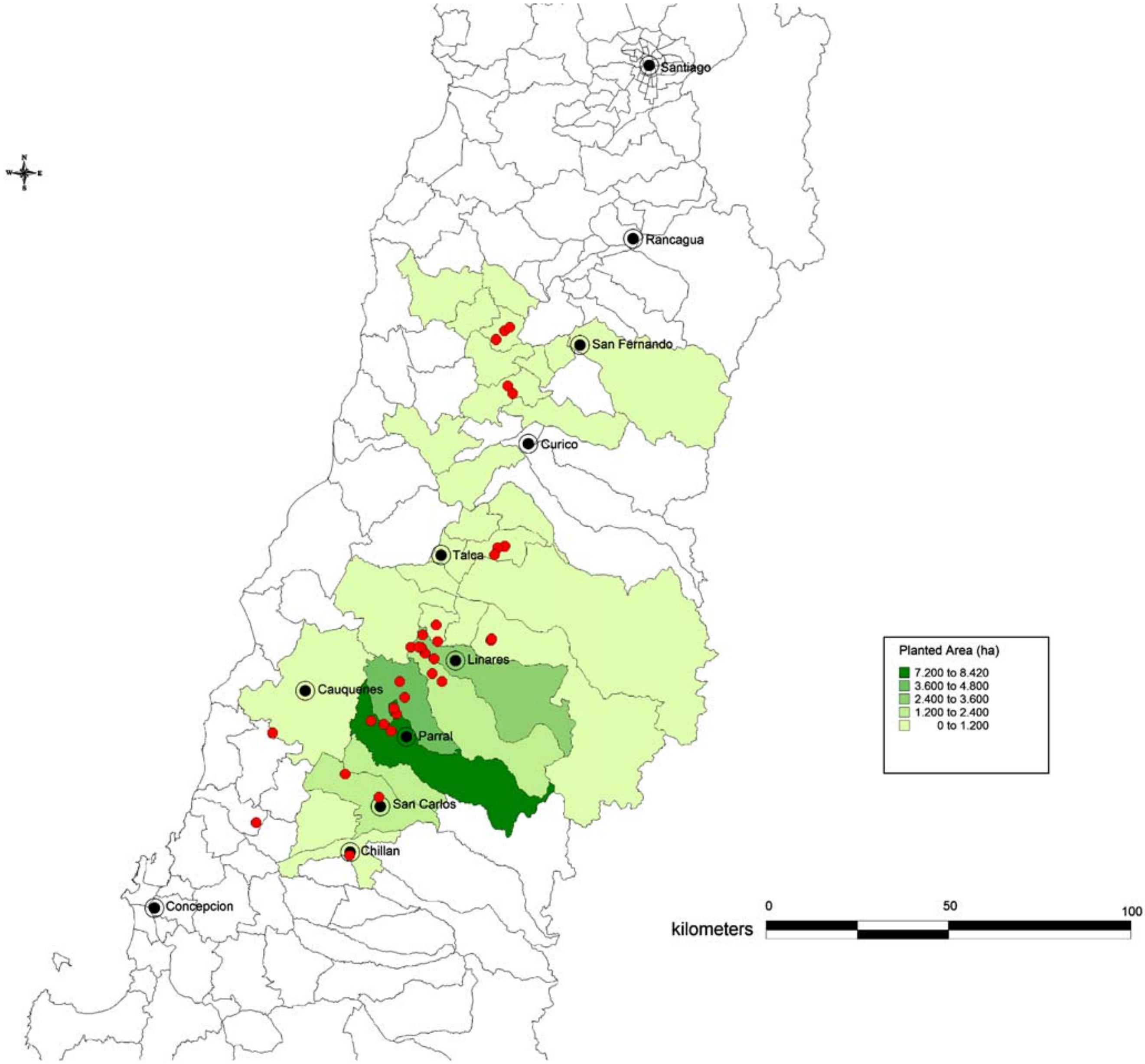

Fig. 1 Geographic distribution of collection locations for blue-green algae within the rice area of Chile

425 mL. Then, 10% of the air volume was extracted (i.e., 42.5 mL) and replaced by 42.5 mL acetylene. Nine samples (including a control sample without algae) in triplicate were used. The sample flasks were stored in a hermetically sealed box and incubated for 3 h in the dark. At the end of the incubation period, air was extracted from each sample with a double needle and transferred to a labelled 10-mL Venojet tube. Ethylene concentration in the Venojet tubes was measured in a Perkin Elmer gas chromatograph, model 8600, equipped with a flame ionisation detector and a Poropak N packed column. Injection temperature was 75°C. Sample concentration was established by comparing it with peak heights of ethylene curves. Ethylene reduction activity was expressed as μmoles C_2H_2 h^{-1}.

Biofertilizer preparation

Four nitrogen-fixing species were used in the preparation of the biofertilizer (Table 2). The mass-cultured species were: *Anabaena iyengarii* var. *tenuis, Nostoc commune, N. linckia* and *Nostoc* sp. 1. The selected strains came from different locations within the rice area (Fig. 2). The algae were harvested by filtration on Whatman filter paper, frozen and lyophilized, and then pulverized in a porcelain mortar. The powder was weighed and mixed in known proportions according to the following criteria: (1) high nitrogen fixation rate of the algae, (2) biomass availability following cultivation under laboratory conditions, and (3) tolerance to desiccation, hot and cold temperatures and previously

Table 2 Taxa and proportions of algae used in the biofertilizer

Algae compounding the biofertilizer	Proportion
Nostoc commune	45%
Anabaena iyengarii var *tenuis*	25%
Nostoc linckia	25%
Nostoc sp. 1	5%

evaluated high viability (data not shown). Besides the algae, no other inert or organic materials were added to the biofertilizer formulation.

Field trials

Three different field experiments were performed:

(1) Estimation of the nitrogen fixation rates in soil samples during a rice growing period.

The nitrogen fixation rate was evaluated on soil samples and taken as an indirect measure of biofertilizer activity in a field trial. Different nitrogen rates (0, 45, and 90 kg N ha^{-1}, as urea) and biofertilizer (0, 30, 60 and 120 g ha^{-1}) were mixed. The low biofertilizer rates used are due to the fact that they corresponded to pure algae strains with no inert

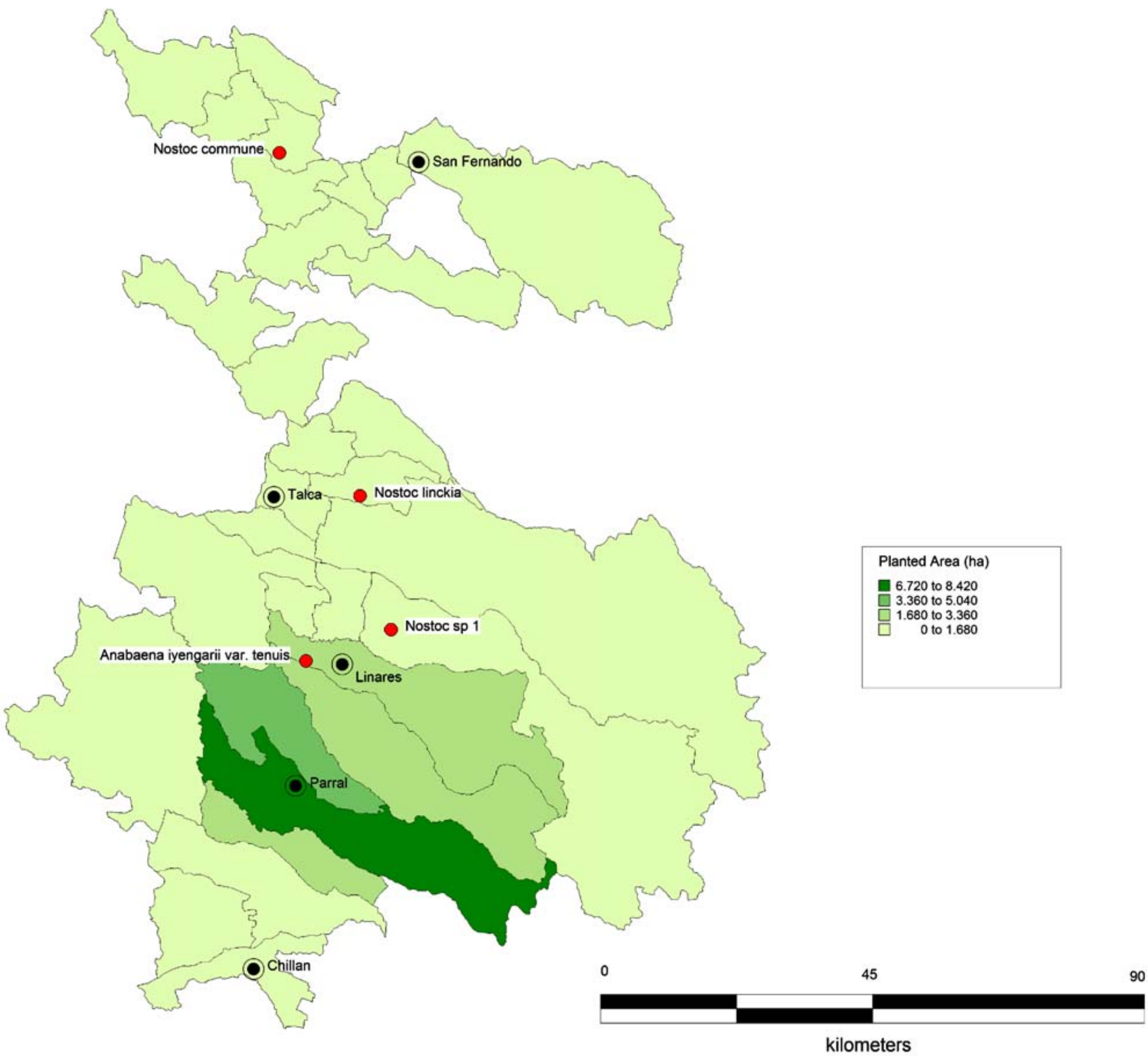

Fig. 2 Geographic locations within the rice area for selected strains of blue-green algae composing the evaluated biofertilizer

carrier in their formulation. Sampling for estimating nitrogen fixation was performed in November 2001, January 2002, and March 2002. In this trial, 14 soil cores (2 cm diam. × 7 cm long) were obtained from each treatment, and placed in a 1-L glass flask. The soil samples were incubated for 3 h, using the acetylene reduction technique of Hardy et al. (1973). A sample without soil was used as control. The ethylene concentration was determined by gas chromatography as above

(2) Effect of nitrogen fertilizer and and biofertilizer rates on grain yield and quality.

In order to establish the most suitable combination between nitrogen fertilizer and biofertilizer rates under an integrated rice crop management system, a field experiment was performed during the 2001/2002 growing season. Different rate combinations of nitrogen (0, 45, 90 kg N ha^{-1}, as Urea) and biofertilizer (0, 30, 60, 120 g ha^{-1}) were evaluated in a complete randomized design with three replicates. The experimental unit had a size of 15 m^2. Rice was seeded under standard management. At harvest, each experimental unit was sampled, using seven 0.25-m^2 quadrats. Weight of grain was determined for each quadrat, corrected to 14% moisture, and extrapolated to obtain yield per hectare. Rice grain quality was evaluated by determining industrial yield, which corresponds to the percent whole grain (in weight basis) after dehulling.

(3) Integrated rice crop management at the field level.

In planning this field validation trial, the results obtained in the first and second field experiments were considered as well as the different recommendations proposed by Watanabe (1973) for inoculation with cyanobacteria in the West Pacific area. Considerations such as the prevailing agro-climatic conditions during the cereal cultivation period, which in Chile takes place between November and March (end of spring and summer), when the highest air temperatures are registered (28–38°C), were taken into account. Rice crop is grown using water from melting snow coming from the high Andes Mountains, which means it travels a distance of approximately 100 km before arriving to the Central Valley where rice is grown. The water temperature in the rice fields reaches between 22 and 28°C in January. The soil was flooded only up to a depth of 30 cm; soil pH was adjusted by adding lime; the use of nitrogen synthetic fertilizer was reduced by 50%, and was incorporated at the mudding time; the soil inoculation was done with a mixture of algae that had shown significant nitrogen-fixation rates as determined in laboratory conditions. All the algae came from local stocks, were tolerant to desiccation and temperature changes, and presented high viability, similar to that shown by *Nostoc commune* in previous studies (Trainor 1985; Potts and Bowman 1985; Whitton 1987; Lin et al. 2004). Additional evidence of these strains' viability is indicated by its survival in spite of having been dried for 3 years by lyophilization.

Details of the experiment are as follows:

The objective of the experiment was to evaluate a biofertilizer made of selected local strains at the commercial scale. The trial was performed during the 2001/2002 growing season in the rice area of Chile, close to the city of Parral (36.05°S, 71.93°W). Two treatments were evaluated: (1) control, only with synthetic N fertilizer (urea), and (2) biofertilizer + synthetic N fertilizer at a reduced rate.

For treatment 1 (control), an area of 1,500 m^2 was used where 100 kg ha^{-1} of nitrogen as Urea (46% N) was applied in two splits: 50% at the time of sowing and 50% at the beginning of tillering. Additionally, 60 kg P_2O_5 ha^{-1} (Triple super-phosphate), 60 kg K_2O ha^{-1} (Potassium chloride), 2,000 kg $CaCO_3$ ha^{-1}, 20 kg Zinc Sulphate ha^{-1}, 20 kg Boronatrocalcite ha^{-1}, and 20 kg $CuSO_4$ ha^{-1}, were also applied at the time of sowing.

For treatment 2, the area used was 1,400 m^2. The base fertilization was the same as treatment 1 except that the nitrogen rate was reduced to 50 kg N ha^{-1} (110 kg urea ha^{-1}), which was applied at the time of sowing. After 15 days from planting, the biofertilizer was applied in a rate equivalent to 60 g ha^{-1}. Application was by spraying the products over the flooding water using a manual sprayer with a water volume of 200 lha^{-1}. As mentioned before, the low rate used is due to the fact that the biofertilizer corresponded to a mixture of pure strains and did not have any carrier (clay or talcum) in its formulation.

The rice was sown during the second week of November 2001. In both treatments, weed control was achieved chemically with the application of Molinate at a rate of 3 lha^{-1}, 20 days after sowing, and MCPA at tillering (40 days post-sowing) at a rate of 1 lha^{-1} (Alvarado and Hernaíz 1995). Finally, seven samples were randomly taken to measure grain yield and industrial quality in each treatment. Sample size was 0.25 m^2 and was independent of the number of ears contained in this area. The mean differences were then tested for significance using a *t* test.

Results

In the plankton and benthos samples from the Chilean rice fields, the following algae species and varieties were identified: *Anabaena fertilissima* C..B. Rao, *A. iyengarii* Bharad. var. *tenuis* C.B Rao, *A. iyengarii* var. *unispora* Rama N.Singh, *Aphanizomenon* cf. *holsaticum* P. Richt., *Cylindrospermum gorakpurense* Rama N. Singh, *C. muscicola* Kütz. ex. Bornet et Flahault. var. *longispora* S.C. Dixit, *Gloeotrichia natans* Rabenh. ex Bornet et Flahault., *Nostoc commune* Vaucher ex. Bornet et Flahault., *N. ellipsosporum* (Desm.) Rabenh., *N. linckia* Bornet ex Bornet et Flahault, *N. spongiaeforme C.* Agardh.var. *tenue* C.B. Rao and *Nostoc* sp.1. (Pereira et al. 2005). Also, there were representatives of Oscillatoriaceae (*Oscillatoria, Spirulina*) and Chroococcaeae (*Aphanothece, Chroococcus*), though these were not identified to the species level.

Geographic distribution of filamentous heterocystous nitrogen-fixing cyanobacteria in the study area

The indigenous filamentous heterocystous cyanobacteria in the Chilean rice soils examined were represented by 12 taxa: 9 species and 3 varieties. Among them, *Gloeotrichia natans* stands out in terms of its frequency and abundance, having the widest distribution in the study area (present in 15 of the 34 sites studied). Other species that showed wide distributions were: *Cylindrospermum muscicola* var. *longispora*, *Anabaena iyengarii* var. *unispora* and *Nostoc spongiaeforme* var. *tenue*. Also there were taxa of narrow distribution,such as: *Anabaena fertilissima, Aphanizomenon* cf. *holsaticum* and *Nostoc ellipsosporum*.

Nitrogen fixation rates

Among the species collected within the study area, *N. commune* had the highest nitrogen fixation rate (Table 3). This was eight times greater than the lowest fixation rate, exhibited by *Nostoc* sp. 1. It was followed in decreasing order by: *A. fertilissima, G. natans. N. ellipsosporum, A. iyengarii var. tenuis, N.linckia, Nostoc* sp. 1 and *Microchaete tenera* Thuret ex Bornet. The last one was isolated from an Argentinean rice soil (Table 3).

Table 3 Nitrogen fixation rates of selected algae species found within the study area

Species and varieties	Location	Nitrogen fixation µmoles C_2H_2 h^{-1}
Nostoc commune	16	4.54 a
Anabaena fertilissima	15	1.55 b
Gloeotrichia natans	31	0.85 b
Nostoc ellipsosporum	15	0.76 b
Anabaena iyengarii var *tenuis*	22	0.66 b
Nostoc linckia	21	0.64 b
Nostoc sp. 1	31	0.55 b
Microchaete tenera	Argentinian rice soil	0.48 b

Distinct letters indicate statistical difference ($p<0.05$)

Field experiments

Biofertilizer nitrogen fixation efficiency under field conditions

The amount of fixed nitrogen was very variable, depending on the sampling date and the treatment applied. The best nitrogen fixation rates were observed in January 2002 in line with the highest temperatures and solar radiation (Fig. 3).

The best treatments, in relation to nitrogen fixation rates, were treatment 10 (application of 120 g biofertilizer ha^{-1} and no N fertilizer), treatment 6 (30 g biofertilizer ha^{-1} and 90 kg N ha^{-1}), and treatment 4 (30 g biofertilizer ha^{-1} without N fertilizer).

Biofertilizer effect on rice grain yield and quality

In general, the rice yields obtained were modest, for the potential of the area, the best being 5.1 t ha^{-1}. These results were probably due to a late infestation of Cyperaceae weeds that escaped the traditional weed control. Under these conditions, no significant differences in grain yield among the evaluated treatments were observed (Table 4).

Regarding rice quality, the results obtained indicated that different rates of synthetic nitrogen fertilizer and biofertilizer did not have a major influence on the industrial yield of the rice grain (Tables 5, 6). Still, it could be seen that a high nitrogen rate (90 kg ha^{-1}) in combination with medium and high rates of biofertilizer (60 and 120 g ha^{-1}) increased the percent of whole grain.

Biofertilizer efficiency under an integrated rice crop management

No statistically significant differences were found between treatments. However, high yields were obtained, a situation that was favored by a low weed pressure in the conditions of this field trial. The industrial quality of the rice harvested was also similar between treatments.

The use of the biofertilizer allowed a 50% decrease in the use of synthetic nitrogen fertilizer (from 100 kg N ha^{-1} to 50 kg N ha^{-1}) with similar results with respect to grain yield, 7.43 t ha^{-1} versus 7.27 t ha^{-1}, and with a similar industrial yield (54.4% vs 54.2% with respect to the control).

Discussion

The algal species *Anabaena iyengarii* var. *tenuis*, *Nostoc commune, Nostoc linckia* and *Nostoc* sp.1 proved to be good options for the formulation of a biofertilizer. Never-

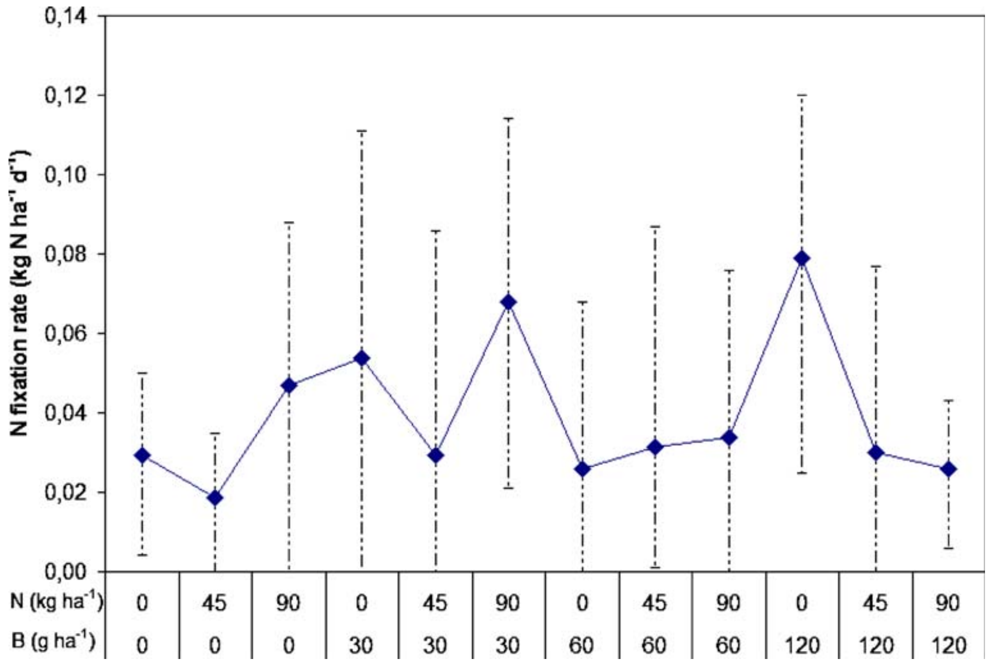

Fig. 3 Nitrogen fixation rates in intact soil cores coming from a rice crop fertilized with different combinations of biofertilizer and nitrogen rates. *Bars* indicate minimum and maximum N fixation rates obtained at different sampling dates. Parral 2001/2002

theless, other species such as *Gloeotrichia natans*, which also have high N fixation rates under laboratory conditions, should be considered. These species were not included in the biofertilizer preparation because they could not be maintained in culture for a prolonged time. These species require high phosphate concentrations for growth and development (Bisoyi and Singh 1988), conditions that were not present in our culture medium, but could be present in phosphorus fertilized rice fields.

Of the four culture media used in the maintenance and mass culture of the algae, the most efficient ones were liquid Watanabe and agar Watanabe. The first was more efficient for culture maintenance, while the second was better for mass culturing the algae. Since neither medium contained free nitrogen, the algae grew and developed without major problems. Chu 10 medium is also nitrogen-free, but the pH attained in the culture did not facilitate normal growth and development of the collected algae. The presence of free nitrogen in the soil–water medium negatively affected growth of the nitrogen-fixing algae since it affected heterocyst formation, and consequently the nitrogen fixation rate.

Table 4 Effect of nitrogen and biofertilizer rate on rice grain yield

N rate (kg ha^{-1})	Grain yield (t ha^{-1})			
	Biofertilizer rate (g ha^{-1})			
	0	30	60	120
0	3.5 n.s.	5.1	4.2	4.3
45	3.8	4.7	4.4	3.3
90	4.2	4.2	4.3	3.3

Parral 2001/2002

n.s. Non-significant *F* test ($p>0.05$)

The results of this study confirm the suggestions of several earlier authors on the incorporation and use of indigenous species in biofertilizer preparations (Watanabe 1973; Venkataraman 1981). Additionally, the suggested reduction in the use of synthetic nitrogen fertilizers (Watanabe 1973; Howarth et al. 1988) for the Chilean rice crop is demonstrated in the field trials conducted as part of this study.

In European countries, such as Spain, algalization has also been performed and the results have shown that a reasonable production of biological nitrogen fixation in combination with inorganic N fertilization allowed the reduction of up to 50% in N fertilizers, without significant loss of productivity and with benefits to the ecosystem (Fernández-Valiente et al. 1996; Quesada et al. 1991, 1997). The results of the present study are in line with those obtained in studies in Spain with respect to rice grain yield and industrial quality. However, there are several differences between these and our study: (1) the species used in the inoculation in Spain do not correspond with the ones utilised in the present study, and (2) the basal phosphorous rate applied to the soils were different (100 and 60 kg P_2O_5 ha^{-1} for Spanish and Chilean rice studies, respectively), and the depth of flooding water used was also different.

The developed biofertilizer has yet to be adopted for commercial purposes due to the lack of a commercial partner to exploit this product in Chile, although we hope to continue with the development of a pilot photobioreactor and accessories in order to optimize the design and the

Table 5 Effect of nitrogen and biofertilizer rate on industrial yield of rice grain

N rate (kg ha^{-1})	% entire grain				
	Biofertilizer rate (g ha^{-1})				
	0	30	60	120	Average
0	50.7 a	53.7 abc	52.7 abc	55.0 abc	53.0
45	54.7 abc	55.3 bc	51.7 ab	51.7 ab	53.4
90	55.3 bc	51.0 ab	53.3 abc	56.3 c	54.0
Average	53.6	53.3	52.6	54.3	53.5

Parral 2001/2002
Distinct letters indicate statistical difference ($p<0.05$)
n.s. Non-significant *F* test ($p>0.05$)

environmental parameters for an industrial plant. This development would allow the large-scale production of this biofertilizer for commercial purposes.

The results obtained in relation to grain yield and industrial quality (whole grain) under an integrated rice crop management system, using a combination of biofertilizer and synthetic nitrogen fertilizer, indicate clear economical and environmental advantages of adopting this agronomic practice (algalization). However, there are same factors that still need to be evaluated, such as grazer action and herbicide use, factors that are known to play an important role in the presence and abundance of cyanobacteria in soils. In other words, there could be a preferential consumption by the grazers and/or high sensitivity of some cyanobacteria species in relation to the herbicides used.

In future investigations, the biofertilizers developed in this study should be evaluated in rice crops for at least three consecutive years in the same plots without a fallow period. The actual agronomic practice for the rice crops in Chile is to let the soil rest once every 2–3 years since levee construction is costly in time and money, which means that the soil is available mainly for rice. Also, the grain yield and industrial quality over time should be evaluated in order to estimate the efficiency of continuous application of the biofertilizer. With this information, the time between inoculations required to obtain a profitable rice crop can be identified, and the potential for a more sustainable and ecologically friendly production system can be determined.

It would be interesting also to intensify the phytochemical and ecological studies over the selected strains in order to have more information about the presence of macro- and micro-elements, along with other substances such as amino acids, phytohormones and vitamins (Venkataraman and Neelakantan 1967; Gupta and Shukla 1969). In this way, it would be possible to improve the commercial attractiveness of the nitrogen-fixing cyanobacteria that could be used in combination with other kinds of algae, especially marine ones, since many of these are already being used as green manure in Chile with encouraging results (e.g., to grow certain types vegetables such as tomatoes, potatoes and grapes). However, until now, only empirical results of their application are known, while there is a lack of scientific knowledge of the Chilean algal flora that would validate the results observed. Such approaches will allow the development of a more organic form of agriculture and also contribute to decreasing the potential negative ecological impacts of agriculture, such as contamination of soils, water and air produced by the indiscriminate use of synthetic nitrogen fertilizers in Chilean rice crops.

There are also cyanobacteria strains that can establish symbioses with the roots of rice plants (Nilsson et al. 2002). Therefore, the results of this study could open a new line of research in Chile, assaying some of the strains identified with high nitrogen fixation rate, which can establish symbioses with some varieties of rice cultivated in Chile.

In conclusion: (1) the number of taxa of filamentous heterocystous nitrogen-fixing cyanobacteria in Chilean rice soils examined is relatively poor, 9 species and 3 varieties corresponding to only 8% of the species reported from Asian rice soils, where the abundance of cyanobacteria reaches up to 90% or more of the total algal flora of the soil; (2) the filamentous heterocystous cyanobacteria utilized in the development of the biofertilizer were selected from collected strains that presented high potential nitrogen fixation rate and high regeneration capacity even when subjected to a drastic desiccation method, such as lyophi-

Table 6 Comparison between fertilization programs in rice with and without biofertilizer application

Treatments	Conditions of the treatments	Yield grain (t ha^{-1})	Industrial quality (% entire grain)	Estimated cost of nitrogen fertilization (US$ ha^{-1})
1	Control farmer	7.27.	54.2.	31,111
2	50% of N + biofertilizer	7.43	54.4	26,363
	Difference	−0.16 n.s.	−0.2 n.s.	4,748
	C.V. (%)	11.8	4.2	

Parral 2001/2002
Distinct letters indicate statistical differences ($p<0.05$)
n.s. Non-significant *F* test ($p>0.05$)

lization; (3) for the species used in the biofertilizer developed in this study, *Nostoc commune* is the species that presented the highest N fixation and survival rates in spite of the drastic desiccation system utilized; (4) estimates of the nitrogen fixation in the soil samples during the rice growth indicated that the application of different biofertilizer rates, along with reduced nitrogen rates, contributed to a significant improvement of nitrogen levels in the soil; and (5) the use of the biofertilizer allowed a 50% decrease in the use of synthetic nitrogen fertilizer with similar results with respect to grain yield and industrial quality in comparison with the control.

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