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Yield responses of Nepalese spring wheat (*Triticum aestivum* L.) cultivars to inoculation with *Azospirillum* spp. of Nepalese origin

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Abstract

Azosprilla were collected in wheat fields from subtropical and temperate soils of central Nepal at various elevations. Different wheat cultivars responded positively and significantly in grain yield, grain N-yield, and total N-yield in plant shoots to the inoculation with Nepalese isolate *Azospirillum* 10SW. Nepalese wheat cv. Seto responded significantly better with *Azospirillum* 10SW than with the Brasilian isolate *A. lipoferum* Sp 108 st, a strain which was found highly efficient in earlier experiments with German wheat cultivars, especially cv. Turbo. Yield of Turbo was increased by inoculations of both *Azospirillum* strains too, but it showed no significant differences depending from the inoculum used. The higher efficacy of combining *Azospirillum* 10SW and Seto, both collected from the same locality, indicates the possibility of improved associations using traditional cultivars and local bacteria.

Introduction

Azospirilla are the most common N_2-fixing bacteria which are found in all major climatic zones (Döbereiner and Pedrosa, 1987). These bacteria were first isolated by Beijerinck (1925) in the Netherlands. The worldwide interest in these bacteria grew considerably when Döbereiner and Day (1976) isolated azospirilla from the rhizosphere and roots of tropical grasses. Tarand et al. (1978) proposed the name *Azospirillum* as genus and distinguished two species, *A. lipoferum* and *A. brasilense*. More recently, three other species of *Azospirillum* were described: *A. amazonense* (Magalhaes et al., 1983), *A. halopraeferans* (Reinhold et al., 1987) and *A. irakense* (Khammas et al., 1989).

Inoculation of *Azospirillum* spp. can lead to improvements in the yield of various cereals (Baldani et al., 1987; Kapulnik et al., 1987; Mertens and Hess, 1984; Millet et al., 1985; Pacovsky et al., 1985; Sarig et al., 1988). Inoculated plants have also produced higher N-yield in grain in several experiments (Baldani et al., 1987; Kapulnik et al., 1983; Mertens and Hess, 1984). Positive responses to inoculation on cereal productivity depend on plant genotypes (Giller et al., 1986; Jain and Patriquin, 1984; Kapulnik et al., 1987; Millet et al., 1984; Murti and Ladha, 1988), bacterial strains (Baldani et al., 1987; Ferreira et al., 1987; Jain and Patriquin, 1984), soil types (Baldani et al., 1987), and environmental conditions.

The actual mechanism of the *Azospirillum*-plant interaction and its positive effect on plant growth is still in controversy. *Azospirillum* can fix atmospheric nitrogen and also synthesize different plant hormones. Azospirilla stimulate root growth and change root morphology (Kapulnik et al., 1985b; Lin et al., 1983; Martin et al., 1989), enhance uptake of minerals (Kapulnik et al., 1985a; Lin et al., 1983; Murti

and Ladha, 1988), and enhance proton efflux and cell division in roots of different cereals (Bashan, 1990; Bashan et al., 1989; Bashan and Levanony 1989). Which of these effects play the major role in the plant response is still under investigation.

As diazotrophic bacteria are widely distributed, it is possible that many diazotrophs, possibly including the most efficient one, have not yet been identified (Döbereiner, 1988). Bacteria found in poor agricultural fields and the traditional crop genotypes cultivated in these fields may have a special type of reciprocal relation as both bacteria and plants have to survive in the poor soil. Such associations could be more efficient than the association seen between bacteria and the hybrid cultivars of crop plants which are selected for the utilization of nitrogen applied by farmers. Considering this, azospirilla were isolated in the present studies from marginal wheat fields of hilly regions of Nepal, and the associations between these bacteria and Nepalese wheat cultivars were studied.

Materials and methods

Media

Nitrogen-free basic (NFb) medium (modified from Döbereiner and Day, 1976; Neyra et al., 1977; Tyler et al., 1979): A semisolid (0.175%) agar medium was used for the isolation of bacterial strains. One litre of the medium was prepared with: Malic acid 4 g, Na-succinate 2 g, glucose 1 g, KOH 3.7 g, K_2HPO_4 $3H_2O$ 0.5 g, $MgSO_4$ $7H_2O$ 0.2 g, NaCl 0.2 g, $CaCl_2$ 0.02 g, $MnSO_4$ H_2O 0.1 g, Na_2MoO_4 0.002 g, $FeSO_4$ $7H_2O$ 0.2 g, yeast extract 0.2 1g, pH 6.5.

Congo red plate (RC): This medium was used to select *Azospirillum* colony. This medium was a solid agar (1.5%) medium and was prepared as described by Rodriguez-Caceres (1982). The pH was adjusted to 6.5.

Nutrient broth (NB): The medium was prepared with the following components: Nutrient broth (Merck) 8 g L^{-1}, KCl 1 g L^{-1}, $MgSO_4$ $7H_2O$ 0.12 g L^{-1}, $MnCl_2$ $2H_2O$ 1 mg L^{-1}, pH adjusted to 6.5.

Soil and root samples

Soils and wheat root samples were collected from marginal wheat fields of Nepal, where the peasants fertilize their land largely with organic manure and either are not using or using only very low amounts of chemical fertilizer. Samples were collected from the northern part of central Nepal from the altitudes of 900 m to 3700 m. Samples were taken from a depth of 20 cm, were air dried and transported to Germany. Samples were kept at 4°C for several weeks until use.

Isolation

About 0.5 g of soil or root pieces with adhering soil or washed root (washed extensively in sterile water and immersed in 5% NaOCl with 0.25% SDS for 20 min and washed successively five times in sterile water) from each sample were placed separately in 30-mL bottles with 10 mL of semisolid NFb medium. Bottles were incubated at 30°C for 96 h. Nitrogenase activity of each culture that formed a pellicle 1 to 3 mm below the surface of the medium was evaluated by the acetylene reduction method. The aluminium foil caps of the bottles were replaced by sterile rubber stoppers and 0.2 mL of acetylene was injected to each bottle. Ethylene production was measured after 4 h of acetylene injection. Ethylene was measured in a gas chromatograph with a flame ionization detector. One loopfull of culture from each acetylene reduction assay (ARA) positive cultures was inoculated to fresh semisolid NFb medium. ARA was conducted as described above after 48 h of incubation at 30°C. The ARA positive cultures were streaked on RC plates and incubated for five days at 30°C. Single, round, red to scarlet colonies from RC plate were inoculated to fresh semisolid NFb-medium. ARA were conducted after 48 h of incubation. ARA positive cultures were streaked again on RC plate. Pure cultures of bacteria were obtained after streaking several times on NB plate.

Pure cultures were obtained from bacterial cultures showing all of the following characteristics: nitrogenase activity, pellicle formation under the surface of semisolid NFb medium, red

to scarlet colony formation on RC plates, and demonstrated spiral movement.

Preservation of bacteria

Strains were maintained at −80°C in NFb medium plus nitrogen with 20% glycerol.

Physiological and biochemical tests

Physiological tests, like Gram reaction, catalase activity, oxidase activity, urease activity, and H_2S formation were performed as described by Cowan and Steel (1974), Drew (1983) and Süssmuth et al. (1987). Denitrification activity was tested by the methods of Neyra et al. (1977). Tests for biotin requirement were done by the method of Krieg and Döbereiner (1984). Further tests and confirmation of identification were done with the help of the German Collection of Microorganisms (Deutsche Sammlung für Mikroorganismen, DSM), Braunschweig, Germany.

The following characteristics were shared by all isolates: cell form slightly curved or rod; size 2 to 4×1 μm; spiral movement; red colony in congo red plate; presence of single polar flagellum; gram negative; lysis in 3% KOH; no spore formation; show nitrogenase activity; aminopeptidase positive; oxidase positive; catalase positive; acid from fructose (in peptone medium) positive; acid from fructose positive; produce NO_2 from NO_3; anaerobic growth with denitrification; urease positive; H_2S formation positive; hydrolysis of starch positive, gelatin negative, casein negative, esculin positive; growth in N-free medium with single source of carbon: glucose, ketoglutarate, mannitol, and sucrose. All isolates grew well and showed nitrogenase activity in NFb medium with malic acid and Na-succinate as C source.

Utilisation of sole carbon source in NFb medium

Bacteria were grown overnight in NB medium in grooved Erlenmeyer flasks (180 rpm, 30°C). Bacterial cultures were washed two times in a saline solution (5 g NaCl and 0.12 g $MgSO_4$ $7H_2O$ in one litre). Washed bacteria were inoculated in semisolid nitrogen free medium as described by Krieg and Döbereiner (1984). The medium contained 1.75 g agar and 5 g L^{-1} single carbon source of either malate or glucose or fructose or sucrose or Na-succinate. Bacteria were incubated at 30°C for 48 h. An ARA was performed to measure the nitrogenase activity.

Association experiments

Bacteria

In vitro associations were performed with all *Azospirillum* isolates of Nepalese origin, greenhouse experiments only with the best suited of them, *Azospirillum* 10SW, and with *A. lipoferum* Sp 108 st. This strain, isolated in Brazil from maize roots, was obtained by the courtesy of Dr J Döbereiner, EMBRAPA, Seropedica, Rio de Janeiro. It was found to be the best of 11 strains of *A. lipoferum* tested in our institute in the utilization of different C sources in in vitro experiments. Furthermore, Sp 108 st has been used successfully in associations with German spring wheat cultivars Arkas and Turbo under greenhouse and field conditions (Mertens and Hess, 1984; Hess, 1990).

Wheat

Nepalese cultivars of spring wheat (*Triticum aestivum* L.), Seto, Lungle, Dawadi, and RR-21, and the Germans spring wheat cultivar Turbo (cf. above) were selected for association experiments.

Preparation of bacterial inocula

Pure cultures of bacteria were grown overnight in NB medium in grooved Erlenmeyer flasks (180 rpm at 30°C). Cells were harvested in the log phase by centrifugation (5000 *g* for 20 min), washed 2 times with saline (5 g NaCl and 0.12 g $MgSO_4$ $7H_2O$ in one litre water), and resuspended at a concentration of 10^8–10^9 colony forming units per mL. Where necessary, cells were killed by autoclaving.

In vitro experiments

Wheat grains were surface sterilised by treatment with 7% sodium hypochlorite solution for one hour and washed successively with sterile water at least five times. Wheat grains were

germinated in replica plates (5 × 5 chambers). After 3 days, one surface sterile and germinating wheat seedling was transferred to a 100-mL test tube with 20 mL of modified WHK medium (medium for plant and bacteria, Hess and Kiefer, 1981): without sucrose and glucose but with 1 g L^{-1} malic acid and 0.025 g L^{-1} KNO_3, 0.1 g L^{-1} $Ca(NO_3)_2$ and 4 g L^{-1} agar. Test tubes were kept at 30°C and a photoperiod of 14 h light (5000 Lux) was maintained. After 3 days, test tubes were inoculated with bacteria. As controls, medium with only bacteria or with only plants were performed. After 48 h, the mouth of the tubes were closed with sterile rubber stopper and 2 mL of acetylene were added to it. Higher acetylene concentrations were found to cause damage of the wheat plants, indicated by browning of the leaf tips in the second half of the incubation period. The acetylene concentration mentioned (2.7%) was used in all assays, including bacterial controls. Ethylene production was measured every day for eight days. To measure the endogenous ethylene production experiments were also performed on tubes with bacteria, with bacteria and wheat, and only with wheat but without adding acetylene as controls. With bacteria alone, depending from the bacterial strain used ARA values were linear up to 96–120 h, and with associations up to 144 h. All the treatments were done in 12 replications.

Greenhouse experiments

Wheat grains were surface sterilized and germinated 48 h in sterile water on sterile filter paper. Twenty wheat seedlings were planted in an 8-L Kick-Brauckmann pot (Kick and Grosse-Brauckmann, 1961) containing 14 kg of steam-sterilized substrate (100°C for 5 h). The substrate was a mixture of loam, sand, and Einheitserde ED 73 (a mixture of 70% peat and 30% clay used by gardeners) in the ratio of 3:1:1. Initially, it contained 1.8 mg NO_3^--N, 0.12 mg NH_4^+-N, 2 mg P, 7 mg K, and 12 mg Mg 100 g^{-1} soil. After one week, plants were thinned to 15 plants per pot and then the pots were either inoculated with 150 mL of bacterial suspension or inoculated with the same amount of autoclaved bacteria. The inoculation was repeated once after 3 weeks. To avoid cross-contamination of the controls, pots were placed with intervals of 50 cm from each other.

Pots were laid out in split plot design with six replications (blocks), and treatments were in plots. Each plot contained pots planted with different wheat cultivars. Plots and the different cultivars of wheat in plots were randomized completely.

Greenhouse experiments were run from April to September under light and temperature conditions of a temperate region (Central Europe), except that the greenhouses were heated if the ambient temperature sank below 15°C. The highest temperatures measured on several days in July and August reached approximately 30°C. Plants were harvested 13 weeks after germinating. After harvest, dry weights of grain and straw per pot were determined by drying at 80°C for 72 h in an air oven. Percentage of nitrogen in grain and straw were determined using the Kjeldahl method.

Statistical analysis

For each data, mean values per pot were calculated. Data were subjected to analysis of variance, and significance at the 5% level was tested by Tukey's test (Lee, 1975). Statistical analysis was done by using a statistical programme packet: Statistical Analysis System (SAS). In tables, least significant difference (LSD) is given and in graphs either standard errors of the means are indicated by bars or significant differences are shown by using different letters.

Results

Characterization of isolates

Azospirillum were isolated from most of the samples collected from subtropical and lower elevation, temperate regions, but they were found only in one sample out of 5 samples collected from cool temperate regions (altitude 2500–3700 m; the positive sample was collected from Langtang at 3700 m). All the isolates were identified as being in the genus *Azospirillum*. Identification was confirmed by the German Collection of Microorganisms (DSM). *Azospirillum* isolates were placed in 5 groups according to

morphological and biochemical characters (Table 1). The characters of group C correspond to the characters of *A. brasilense* and the characters of group D and E correspond to *A. lipoferum*. The isolates of group A and B shared all characters of group C and D, respectively, but they grew and fixed nitrogen in medium with sucrose as a sole C source. The two species, *A. amazonense* and *A. irakense*, seem to be related to these isolates, as they can also utilize sucrose. But group A and B isolates differ from *A. amazonense* as they can utilize fructose (Magalhaes et al., 1983) and from *A. irakense* since they produce acid from fructose in peptone-based medium (Khammas et al., 1989).

In vitro associations

In the in vitro association experiments with wheat, the *Azospirillum* 10SW showed comparatively higher nitrogenase activity in association than other isolates (results not shown). Therefore, this bacteria was selected for further association experiments. Nitrogenase activities of *Azospirillum* 10SW were always significantly higher in associations than in bacteria growing alone (Fig. 1). In associations ethylene accumulation reached a constant level after 7 to 8 days of acetylene addition, whereas in controls (bacteria alone) ethylene production reached a constant level after 3 to 4 days. Maximum amounts of ethylene were produced in association with wheat cultivar RR-21, which was followed by cultivars Seto, Turbo, Dawadi and Lungle. No ethylene was produced in tubes where bacteria were absent and where acetylene was not added.

Greenhouse experiment I

The experiment was designed to find out the effect of *Azospirillum* 10SW inoculation on the yield responses of 5 different cultivars of wheat. Grain yields of wheat increased significantly (Fig. 2) by 8.8 to 25.2% due to *Azospirillum* inoculation. Cultivar RR-21 showed the highest increase in grain yield. A significant increase in number of grains pot^{-1} (21.16%) was seen only in cv. Seto (Table 2).

Percent nitrogen content of grains of cultivars Seto, Lungle and Turbo increased significantly by inoculation (Fig. 2). The nitrogen yield in grain and total nitrogen yield in plant top (grain and straw) were significantly higher in all inoculated wheat varieties. The cv. RR-21 showed the

Table 1. Grouping of *Azospirillum* isolates based on physiological and biochemical tests

Group	*Azospirillum* isolates	ARA in NFb medium with			Biotin requirement	Acid from glucose	Autotrophic growth	Pleomorphic cells	Clumping of cells
		Sucrose	Fructose	Glucose					
A.	1B, 17B4	+	+	−	−	−	−	−	−
B.	21SWC4	+	+	+	+	+	+	+	+
C.	2B, 3B, 4B, 5B, 10B, 10SWC, 11B, 14SWC3[a], 15B3, 16WB1, 16SWC3, 16SWC4, 18WB4, 18SWC3, 20B, 22WB[a], 23BI[a,b], 23B2[c], 31B3[b] *A. brasilense*	−	+	−	−	−	−	−	−
D.	1SWC, 4BOR, 10SW 19B2[d], 17SWC3	−	+	+	+	+	+	+	+
E.	6B[e], 19SWC3, 30SWC4 *A. lipoferum*	−	+	+	+	−	−	+	+

[a] No ARA in fructose. [b] Low ARA in sucrose. [c] ARA in glucose. [d] No ARA in glucose and fructose. [e] Acid produced from glucose and fructose in peptoe medium.

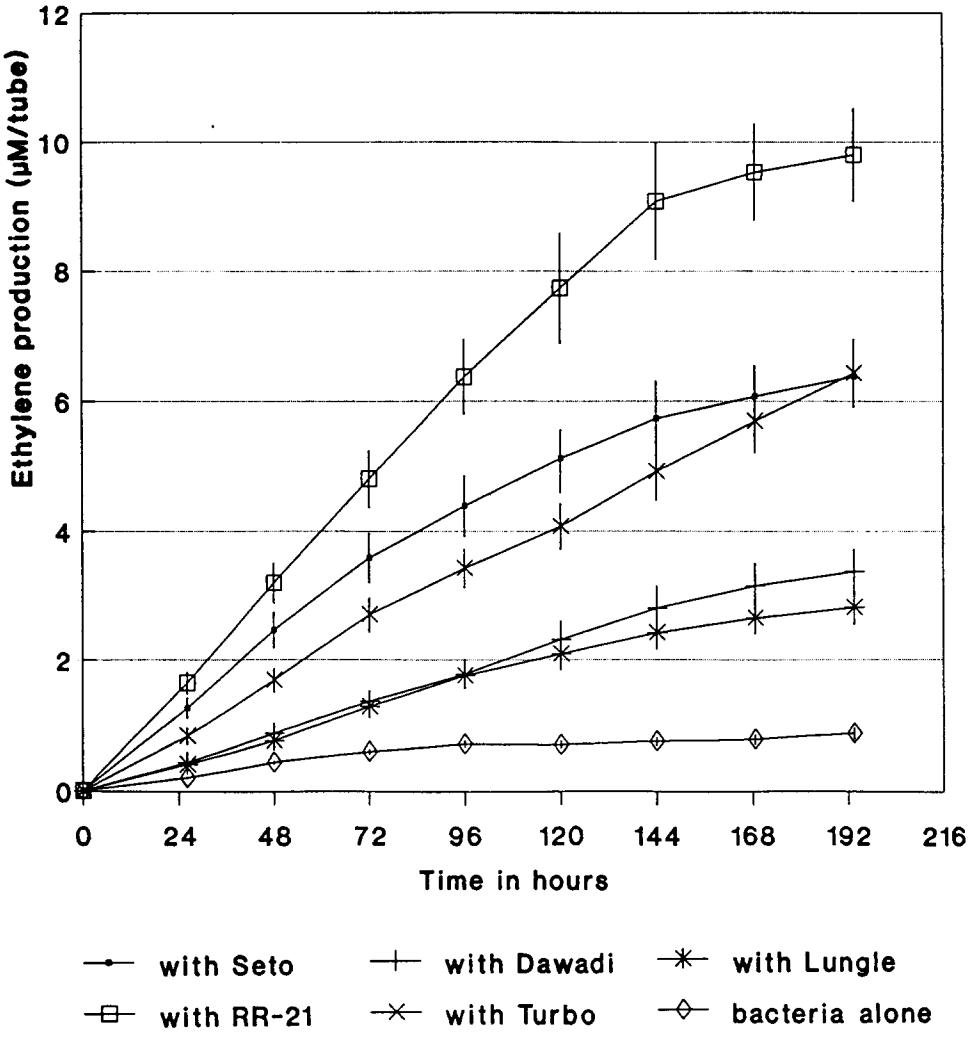

Fig. 1. Time course of nitrogenase activity (μM C_2H_4 $tube^{-1}$) of *Azospirillum* isolates 10SW either growing alone or growing in association with one of the wheat cultivars: Seto, Dawadi, Lungle, RR-21 or Turbo. Vertical *bars* represent standard error of the means. With bacteria alone the bars are within the symbols used.

Table 2. Number of grains/pot for different cultivars of wheat inoculated with *Azospirillum* 10SW (+bac) or autoclaved *Azospirillum* 10SW (control). LSD: Least Significance Difference (Analysis of variance, Tukey's test at the 5% level)

Treatments	Cultivars of wheat				
	Seto	Lungle	Dawadi	RR-21	Turbo
1. + bac.	562.5	447.3	346.3	300.8	414.7
2. control	435.5	425.3	322.3	265.0	403.5
LSD	99.8	57.7	35.4	48.4	79.5

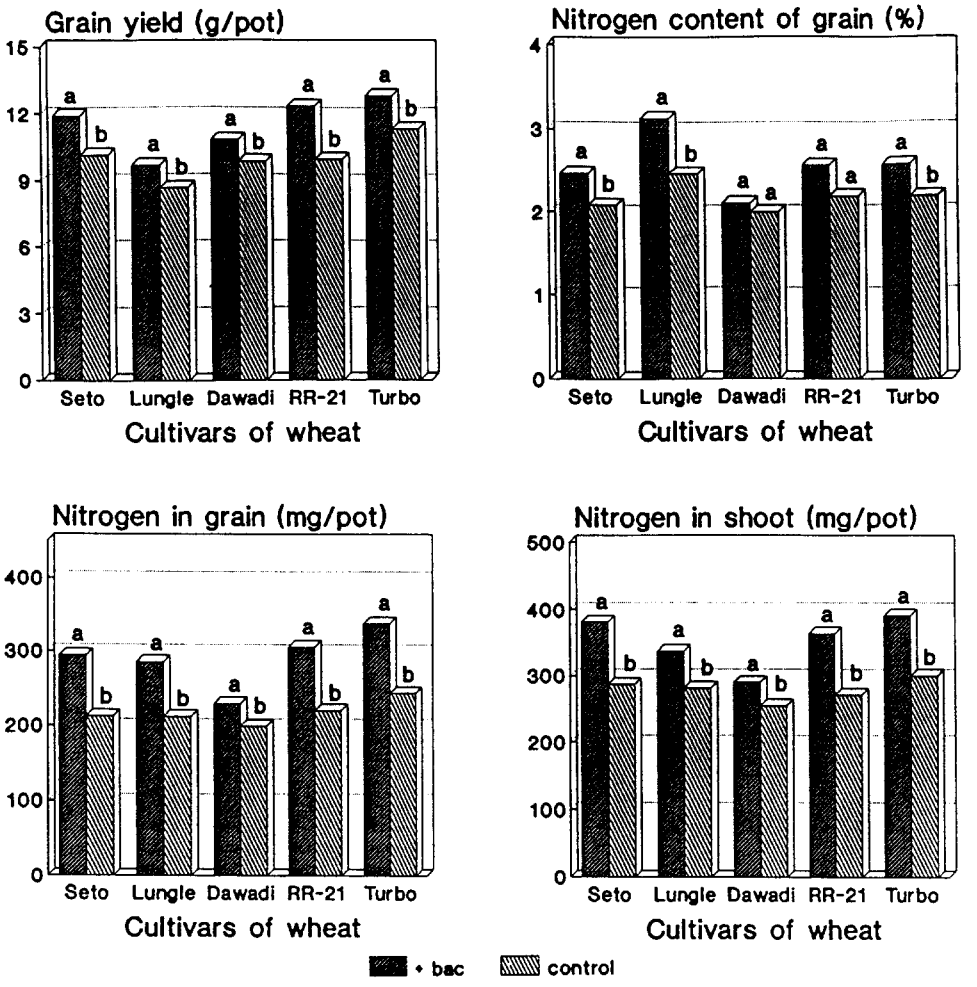

Fig. 2. The effects of *Azospirillum* 10SW inoculation on grain yield, percent N in grain, N-yield in grain, and N-yield in shoot of the wheat cultivars Seto, Lungle, Dawadi, RR-21 and Turbo. + bac: inoculated with *Azospirillum*, control: inoculated with heat killed *Azospirillum*. Means with the same letter within each cultivar are not significantly different at $p < 0.05$.

highest increase of 39.5% in grain nitrogen. The differences in straw yield, percentage of nitrogen in straw, and nitrogen yield in straw between inoculated and control were insignificant (results not shown).

Greenhouse experiment II

In Experiment I of this work, grain yields of wheat increased significantly when inoculated with *Azospirillum* 10SW. On the other hand, an increase in grain yield of wheat inoculated with *A. lipoferum* Sp 108 st was already described (Mertens and Hess, 1984). Therefore, Experiment II was conducted to compare the effects of the *Azospirillum* 10SW and *A. lipoferum* Sp 108 st inoculations in the yields of Seto and Turbo, representatives of the Nepalese and German wheat cultivars, respectively.

Grain yields were significantly increased (Fig. 3) by 19.4% and 12.5% in Seto, or 14.9% and 13.3% in Turbo plants inoculated with 10SW and Sp 108 st, respectively. The number of grains were significantly increased in pots inoculated with *Azospirillum* 10SW or with *A. lipoferum* Sp 108 st in the case of cv. Seto (17.1% and 11%, respectively), but the differences were not significant in cv. Turbo (Table 3).

Both cultivars showed no differences in percentage of N in grain and straw between inocu-

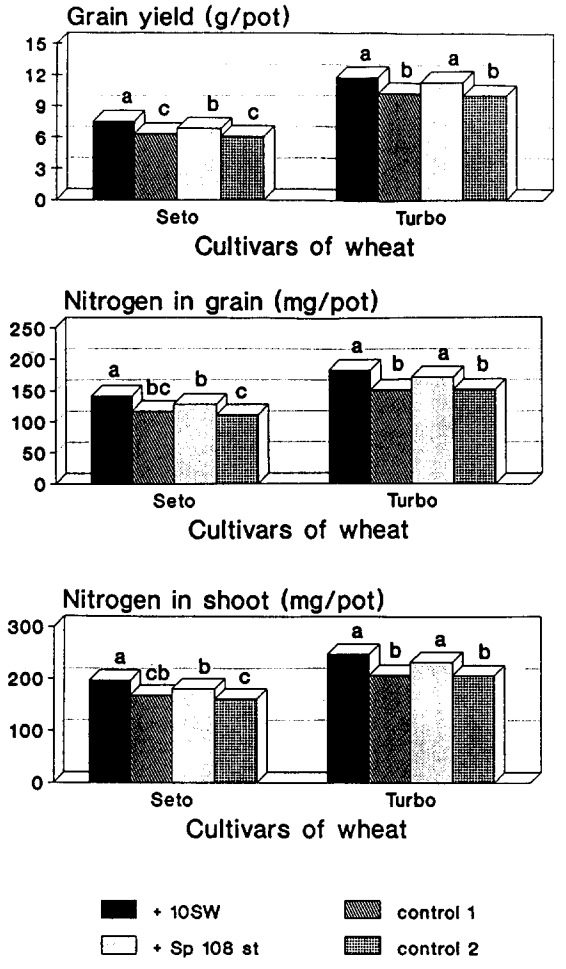

Fig. 3. Grain yield, N-yield in grain, and N-yield in shoot of wheat cultivars Seto and Turbo inoculated with *Azospirillum* 10SW (+10SW), *A. lipoferum* Sp 108 st (+ Sp 108 st), autoclaved *Azospirillum* 10SW (control 1) and autoclaved *A. lipoferum* Sp 108 st (control 2). Means with the same letter within each cultivar are not significantly different at $p < 0.05$.

Table 3. Number of grains/pot of wheat cultivars Seto and Turbo, inoculated with: *Azospirillum* 10SW (+10SW), autoclaved *Azospirillum* 10SW (control 1), *A. lipoferum* Sp 108 st (+Sp 108 st) and autoclaved *A. lipoferum* Sp 108 st (control 2)

Treatments	Seto	Turbo
	No. of grains	
+10SW	360.3	429.8
+Sp 108 st	329.0	427.5
control 1	307.7	397.8
control 2	296.3	397.8
LSD	31.0	36.5

lated and control pots (results not shown), however the yield of N in grain and in shoot were significantly increased in both cultivars by the inoculation of the both *Azospirillum* species (Fig. 3).

The cv. Seto showed comparatively low, but significant increase in grain yield (by 10.3%), nitrogen in grain (by 10.25%), and N-yield in shoot (by 9.2%) in pots inoculated with 10SW in comparison to pots inoculated with Sp 108 st (Fig. 3).

Discussion

Inoculation of *Azospirillum* increased the yield of wheat. The higher grain yields of inoculated pots was achieved by increases in grain number (Kapulnik et al., 1987; Mertens and Hess, 1984; Millet et al., 1985). Increases in total N-yield of shoots of inoculated wheat in the present work were higher than the dry matter increases. This stresses the effect of *Azospirillum* inoculation on N-nutrition of plant. The possible mechanisms of higher N-accumulation may be the transfer of atmospheric nitrogen to the plant through bacterial nitrogen fixation (Kucey, 1988) and/or by improved nitrogen uptake in the inoculated plants (Murty and Ladha, 1988). The induction of nitrogenase activity of *Azospirillum* in association with wheat in the in vitro experiments and the significant increases in percentage of nitrogen in grain in some cultivars due to inoculation, support the hypothesis that biological nitrogen fixation by the *Azospirillum*-root association could be responsible for the observed higher N-input in inoculated plants. On the contrary, N-inputs in inoculated wheat in the second experiment increased without altering the percentage of nitrogen, which stresses the role of factors other than nitrogen fixation for the positive responses (see introduction).

Strains of *Azospirillum* isolated from surface-sterilized roots of the same crop in which they are subsequently inoculated have been termed 'homologous strains' (Döbereiner and Baldani, 1981). Of interest is the comparison between an efficient homologous (with *Azospirillum* 10SW) and a nonhomologous (with *A. lipoferum* Sp 108

st) association (Mertens and Hess, 1984). Inoculations of both *Azospirillum* 10SW or *A. lipoferum* Sp 108 st were able to produce higher yields in both wheat cultivars Seto and Turbo. Significant differences in yield parameters were observed between the plants inoculated with 10SW or Sp 108 st in the case of cv. Seto, whereas the cv. Turbo showed no such differences. *Azospirillum* 10SW is a homologous strain for wheat cv. Seto as it was isolated from the washed roots of this cultivar. Therefore, the association of Seto with this *Azospirillum* strain may be closer than the associations with other azospirilla. The results show the superiority of 10SW over Sp 108 st in bringing yield advantage in wheat, although the Sp 108 st was selected as the best suited bacterial strain for wheat inoculation in our institute. *Azospirillum* 10SW was not only isolated from the roots of Seto, but the bacteria and grain used were collected from the same locality. The results obtained are consistent with an higher efficacy in homologous systems (Baldani et al., 1987; Boddey et al., 1986). The number of *Azospirillum* strains and wheat cultivars tested, however, was too low to allow a final conclusion.

In earlier studies on associations between wheat and *A. lipoferum* yield increases were obtained under the same greenhouse conditions, which had been used in the present investigations. They were paralleled by yield increases under field conditions (Mertens and Hess, 1984). The same has to be supposed for the associations tested presently. Therefore, the Nepalese isolate *Azospirillum* 10SW, which showed the best positive responses in inoculated wheat in the present study, seems to be a suitable inoculant for wheat. The increase of up to 25% in grain yield and up to 39% in total nitrogen in shoot of inoculated wheat obtained in the present experiments is economically quite promising. The better yield responses of Nepalese wheat cv. Seto to Nepalese isolates *Azospirillum* 10SW inoculation compared to the Brazilian isolate *A. lipoferum* Sp 108 st indicate the possibility of improved associations using traditional cultivars and local bacteria. Local isolates should be preferred in the selection of bacteria for inoculation of crop plants, as they are adapted in the environment and can be more competitive than the foreign bacteria. The *Azospirillum* 10SW can be a suitable biofertilizer for wheat cultivation in Nepal.

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