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Research article

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Abstract – Field-grown wheat, *Triticum aestivum* L. cv. ProINTA Isla Verde, was inoculated with two *Bacillus* species, *B. simplex* and *B. firmus*, isolated from wheat roots, in four experiments during three years at two sites, Buenos Aires and Castelar, Argentina. Plants were inoculated with a liquid inoculant twenty-two days after sowing, or seeds were pelleted with inoculated peat prior to sowing, according to the experiment. In all experiments a set of plots was left unfertilized, and another set was fertilized with 200 kg N/ha in a split fertilization, 100 kg at sowing and 100 kg at the booting stage. Inoculation affected the results in all experiments, augmenting biomass production by up to 77% in some experiments, through an increase in the number of ears per plot close to 50%. However, as the number of grains per ear was decreased by inoculation by more than 40%, grain yield was not consistently augmented. Inoculation always produced an increase in nitrogen accumulation by the crop, with the inoculated plants showing more than twice the N content of those not inoculated. The most remarkable result due to inoculation was the consistent increase from 9.4% to 15.6% dry matter (DM), with or without N fertilization. Our findings show that inoculation with adequate rhizobacteria consistently increases wheat grain quality and the efficiency of use of the applied fertilizer, with the potential benefit of reducing losses to the environment.

wheat / *Bacillus* / biofertilization / grain protein concentration

1. INTRODUCTION

Grain protein concentration is one of the major determinants of wheat baking and nutritional quality, and many efforts have been made to improve this character through breeding and management (Triboi and Triboi-Blondel, 2002). The application of chemical nitrogen fertilizers is the most effective practice for increasing wheat grain protein concentration. However, the efficiency of nitrogen fertilizer utilization is low, generating losses that produce adverse environmental effects (Freney, 1997; Yadav et al., 1997; Grignani and Zavattaro, 2000).

Colonization of plant roots by bacteria has been observed for a long time, but only lately has its importance for plant growth and development become clear (Glick, 1995). Since the discovery of plant-growth-promoting rhizobacteria (PGPR) there have been many attempts to improve plant growth through inoculation with rhizospheric bacteria, with variable results. Many rhizobacteria have the capacity to fix atmospheric nitrogen (Dobbelaere et al., 2003), and although it has been reported that in most cases this amount of nitrogen is negligible for plant demand, they can promote plant growth through the production of plant growth regulators that affect plant development (de Freitas, 2000; Dobbelaere et al., 2003). Several of these bacteria have been described as increasing plant nutrient uptake and concentration (Okon and Labandera-Gonzalez, 1994; Dobbelaere et al., 2003).

In wheat several rhizobacteria have been reported as improving grain yield, grain protein concentration or both (Rai and Gaur, 1982; Reynders and Vlassak, 1982; Mertens and Hess, 1984; Millet et al., 1985; Boddey et al., 1986; de Freitas and Germida, 1992; Saubidet et al., 2002). However, inconsistent results, with large variation and little reproducibility, have prevented the widespread use of biofertilizers (Okon and Labandera-Gonzalez, 1994; de Freitas, 2000; Vessey, 2003). In the present paper we describe a consistent increase in grain protein concentration and nitrogen content in field-grown wheat as a response to inoculation with *Bacillus* strains isolated from wheat roots, despite the variable effects observed on biomass and grain yield.

2. MATERIALS AND METHODS

Four experiments were performed in different years and localities. Three experiments during the growing seasons of 2000, 2001 and 2002 were located at the experimental field of the Faculty of Agronomy, University of Buenos Aires, Buenos Aires, Argentina, on a Typical Argiudol with an organic matter content of 2.5% and a total nitrogen content of 0.14%, previously cultured with maize for several years. Every year the experiments were moved to a new site within the field. During 2002 a fourth experiment was carried out at the experimental

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plots of the Institute of Biological Resources (CIRN - INTA), Castelar, Argentina, on a Typical Argiudol, with an organic matter content of 3.94% and a total nitrogen content of 0.2%.

In all experiments wheat seeds (*Triticum aestivum* L. cv. ProINTA Isla Verde) were sown in 2-m^2 plots, at a density of 150 plants/m^2, in a completely randomized design, with five replicates per treatment. Fertilizer treatments consisted of no fertilization, or 200 kg N/ha applied as urea, 100 kg at sowing and 100 kg at the booting stage.

Strains of *Bacillus simplex* BS BNM-10 and *Bacillus firmus* BF BNM-4, isolated from the wheat rhizosphere and characterized by 16S rRNA gene alignment were provided by the Banco Nacional de Microorganismos, Facultad de Agronomía UBA, Argentina, and were selected according to previous results from inoculation into wheat plants in liquid culture and in pots with soil.

Bacterial cells were grown for two days in medium containing L-malic acid 5.0 g/L, K_2HPO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.2 g/L, NaCl 0.1 g/L, $CaCl_2$ 0.02 g/L, Bromothymol blue 0.001%, FeEDTA 0.066%, Biotin 0.01 mg/L; Pyridoxin 0.02 mg/L, KOH 4.0 g/L, NH_4Cl 1.0 g/L and 2.0 mL/L of a trace elements solution pH 6.8. In the 2000 and 2001 experiments, twenty-two days after sowing each plot was inoculated with 2.0 L of medium containing 1×10^7 cfu/mL^{-1}, or dead cells (autoclaved) as a control (uninoculated treatment), and immediately after, 1.0 L of demineralized water. In the 2002 experiments, seeds were inoculated at sowing with a peat-based inoculant. Sterile 60 mesh-ground peat was inoculated with the mentioned growth media with 10^9 cell/mL of either strain. The culture (110 mL) was injected aseptically into sealed polyethylene bags containing 150 g (wet weight) of sterilized peat and mixed manually. Before sowing seeds were pelleted in the inoculated peat, 0.04 g of peat for every 100 g of seeds.

At the booting stage 1 linear meter was sampled from every plot, while at maturity 1.0 m^2 of each parcel was sampled. Plant material was dried at 60 °C for 48 h, weighed and ground. The harvest index was calculated as the ratio of grain yield to total aerial biomass. Total nitrogen and protein concentration were analyzed with a micro-Kjeldahl procedure after digestion in concentrated H_2SO_4 (Barneix et al., 1992). Analysis of variance was performed with the Statgraphics ® software.

3. RESULTS AND DISCUSSION

3.1. Plant growth and nitrogen accumulation at the booting stage

Inoculation with rhizobacteria induced many changes in the crop, with the strain *B. simplex* BS BNM-10 producing better results than *B. firmus* BF BNM-4 during 2000 and 2001. For this reason, during the 2002 experiments only the *B. simplex* strain was used.

At the booting stage the inoculation effects on aboveground biomass production varied among the experiments, showing an increase as a response to the inoculation with rhizobacteria strain BS BNM-10 only in 2001 in the fertilized treatment (Tab. I), and to nitrogen fertilization only at the Buenos Aires site during 2001 and 2002 (Tab. I). However, in every experiment the inoculated plants presented a higher total nitrogen concentration than the uninoculated ones in both nitrogen treatments, with strain BS BNM-10 causing a more pronounced effect than BF BNM-4 (Tab. II). The total nitrogen content in the biomass also showed an increase due to inoculation in every experiment, except at the Buenos Aires site in 2002 (Tab. II).

Table I. Total aboveground biomass (g/m^2) of wheat plants inoculated with rhizobacteria at the booting stage, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

	Year	2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha				
None	0	46.5	26.7	14.1	36.5
BS BNM-4	0	39.9	31.7	ND	ND
BS BNM-10	0	41.2	30.4	11.5	39.3
None	200	37.3	42.0	69.5	38.5
BS BNM-4	200	47.3	38.2	ND	ND
BS BNM-10	200	51.5	70.0	61.5	41.5
Strain		NS	***	NS	NS
Fertilizer		NS	***	***	NS
Interaction		NS	***	NS	NS
LSD $P < 0.05$		-	11.0	48.7	-

Diverse *Bacillus* strains have been reported to produce different effects on plant growth, from biocontrol agents (Glick, 1995) to enhancing grain yield in cereals (de Freitas, 2000) and legumes (Estevez de Jensén et al., 2002). The present results show that although the crop growth varied among the experiments, several effects of the inoculation of wheat plants with these rhizobacteria could be consistently observed. Both inoculation methods applied presented similar results, but the inoculation with peat was cheaper and more convenient for the inoculation of a large number of seeds, while bacteria counts showed that root colonization was similar in both methods (data not shown).

3.2. Plant growth and grain yield at maturity

At maturity, the addition of nitrogen fertilizer showed a significant response in every experiment in most of the measured parameters in both inoculation treatments, and in every experiment. The inoculated plants showed a bigger aboveground biomass (Tab. III) and higher number of ears per plot than the non-inoculated ones in every experiment where measured, and in both nitrogen treatments (Tab. IV). An increase in the number of tillers in wheat after inoculation with rhizobacteria was also observed by Khalid et al. (2004).

However, grain yield was only affected by inoculation in both experiments in 2002, where inoculation with rhizobacteria strain BS BNM-10 increased yield in all nitrogen treatments

Table II. Total N concentration (% DM) and total N content (g/m^2) in aboveground biomass in wheat plants inoculated with rhizobacteria at the booting stage, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference. DM: dry matter.

		N concentration (%DM)				N content (g/m^2)			
	Year	2000	2001	2002		2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha								
None	0	1.19	1.39	1.26	1.47	0.55	0.37	0.18	0.71
BS BNM-4	0	1.48	1.61	ND	ND	0.59	0.49	ND	ND
BS BNM-10	0	2.02	1.88	1.39	1.97	0.84	0.72	0.16	0.95
None	200	2.21	1.53	1.90	2.37	0.83	0.49	1.31	1.14
BS BNM-4	200	2.78	1.76	ND	ND	1.31	0.74	ND	ND
BS BNM-10	200	3.43	2.19	2.50	3.75	1.74	1.53	1.54	1.80
Strain		***	***	***	***	***	***	NS	***
Fertilizer		***	***	***	***	**	***	***	***
Interaction		***	***	***	***	NS	***	NS	***
LSD $P < 0.05$		0.188	0.076	0.120	0.331	0.726	0.206	0.94	0.159

Table III. Total aerial biomass (g/m^2) in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

	Year	2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha				
None	0	ND	351.4	302.8	522.75
BS BNM-4	0	ND	461.2	ND	ND
BS BNM-10	0	ND	621.7	441.5	642.3
None	200	ND	586.1	902.6	624.0
BS BNM-4	200	ND	759.6	ND	ND
BS BNM-10	200	ND	912.4	1,067.2	780.3
Strain			***	***	***
Fertilizer			***	***	***
Interaction			NS	NS	NS
LSD $P < 0.05$			63.3	115.4	78.9

(Tab. V), with a decrease in grain number per ear in every year measured (Tab. IV). The individual grain weight was augmented with fertilizer addition in both 2002 experiments (Tab. VI), while the harvest index was mostly unaffected by the nitrogen or inoculation treatments, showing a decrease due to inoculation with strain BS BNM-10 only in the 2001 experiment (Tab. VII).

Grain yield is determined by the amount of ears per m^2, grains per ear and individual grain weight (Petr et al., 1988; Fischer, 1984). The culture can compensate for the ratio of these yield components, mainly through individual grain weight, to adjust the available photosynthates to the available sinks (Petr et al., 1988). The increase in the number of ears per plot did not increase grain yield because the inoculation treatments decreased the number of grains per ear (Tab. IV), and although there was an increase in individual grain weight (Tab. VI), this compensation was not enough to increase yield.

It is not clear at this point through which mechanism the bacteria in the rhizosphere can affect the plant yield components. Plant development is under hormonal control, and the effect of rhizobacteria on plant growth is known to be exerted through the production of plant growth regulators (Dobbelaere et al., 2003).

3.3. Plant growth and nitrogen accumulation at maturity

All parameters of nitrogen utilization were affected by inoculation in every experiment, and in both nitrogen regimes. The inoculated plants accumulated more nitrogen in the aerial biomass (Tab. VII) and in the grain (Tab. VIII), with strain BS BNM-10 showing a greater effect than BF BNM-4. The higher nitrogen content in the biomass was a consequence of the increase in biomass production, but also due to an increment in grain and in straw total nitrogen concentration (Tabs. VIII and X). It has been repeatedly reported that rhizospheric bacteria can increase the plant nitrogen content and concentration (Okon and Labandera-Gonzalez, 1994; Dobbelaere et. al., 2003). However, the way in which this increase is achieved is not completely clear. It has been mentioned that bacterial inoculation can increase root growth, improving soil exploration

Table IV. Number of ears per m^2 and grains per ear in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

		Number of ears per m^2				Grains per ear			
	Year	2000	2001	2002		2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha								
None	0	ND	131.2	73.75	213.5	ND	24.7	62.22	41.5
BS BNM-4	0	ND	175.8	ND	ND	ND	21.5	ND	ND
BS BNM-10	0	ND	234	108.5	251.5	ND	18.3	40.78	26.4
None	200	ND	202.4	221.2	235.8	ND	26.6	35.5	39.1
BS BNM-4	200	ND	232.0	ND	ND	ND	24.4	ND	ND
BS BNM-10	200	ND	275.8	256.8	302.0	ND	19.1	40.7	24.3
Strain			***	***	***	-	**	*	***
Fertilizer			***	***	**	-	NS	**	NS
Interaction			*	NS	NS	-	NS	**	NS
LSD $P < 0.05$		-	20.7	23.8	38.5	-	7.79	16.8	11.4

Table V. Grain yield (g/m^2) in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

	Year	2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha				
None	0	92.7	102.3	93.5	168.8
BS BNM-4	0	86.5	115.6	ND	ND
BS BNM-10	0	88.9	128.9	154.8	223.3
None	200	264.5	163.4	319.4	204.3
BS BNM-4	200	224.7	172.0	ND	ND
BS BNM-10	200	282.2	162.3	389.2	260.8
Strain		NS	NS	***	***
Fertilizer		***	***	***	**
Interaction		NS	NS	NS	NS
LSD $P < 0.05$		59.8	40.7	46.5	43.3

and nutrient uptake (Okon and Labandera-Gonzalez, 1994). However, it has also been reported that some rhizobacteria can increment specific nitrogen uptake by the root (Saubidet et al., 2002; Dobbelaere et al., 2003).

Grain protein concentration was also augmented in every experiment, with increases from 9.4% to 15.6% DM when inoculated with strain BS BNM-10 (Tab. IX), while the nitrogen concentration remaining in the straw was also higher in the inoculated plants (Tab. X). The effect on the nitrogen harvest index, on the other hand, was variable, for in 2001 it was decreased by inoculation, while in 2002 at the Buenos Aires site it was slightly increased only in the nitrogen-fertilized treatment, and at the Castelar site showed no difference between inoculated and non-inoculated plants (Tab. X).

Plant breeding efforts to increase grain protein concentration have been slow and not very successful. The actually available high-grain protein concentration cultivars accumulate at most a 1–2% higher concentration than the low-protein lines (Johnson et al., 1979; Joppa and Cantrell, 1990; Triboi and Triboi-Blondel, 2002). Compared with these values, the increments in grain protein concentration in these experiments due to inoculation with rhizobacteria are remarkably high. Consequently, this methodology represents an alternative agronomical practice for increasing wheat grain quality.

4. CONCLUSION

The present results show that inoculation with the *B. simplex* strain BS BNM-10 can be a viable and cheap method for consistently augmenting wheat grain protein concentration, and eventually, grain yield. Inoculation also produced a higher nitrogen accumulation by the plant, increasing the efficiency of use of the applied fertilizer, with the potential benefit of reducing losses to the environment.

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Table VI. Grains per m^2 and individual grain weight (mg) in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

		Grains/m^2				Grain weight (mg)			
	Year	2000	2001	2002		2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha								
None	0	ND	3227.1	4532.8	8853.7	ND	31.8	20.5	19.3
BS BNM-4	0	ND	3809.1	ND	ND	ND	30.7	ND	ND
BS BNM-10	0	ND	4270.7	4392.8	6624.4	ND	30.2	41.2	33.8
None	200	ND	5380.4	7830.4	9191.8	ND	30.4	32.5	22.3
BS BNM-4	200	ND	5640.2	ND	ND	ND	30.5	ND	ND
BS BNM-10	200	ND	5228.0	10417.1	7309.5	ND	31.7	40.0	35.8
Strain		-	NS	NS	**	-	NS	*	***
Fertilizer		-	***	***	NS	-	NS	***	***
Interaction		-	NS	NS	NS	-	NS	**	NS
LSD $P < 0.05$		-	1540.5	3140.8	2413.4	-	-	6.64	3.39

Table VII. Harvest Index and total N content (g/m^2) in aerial biomass in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

		Harvest Index				N content (g/m^2)			
	Year	2000	2001	2002		2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha								
None	0	ND	29.6	30.7	32.3	ND	3.26	1.67	3.36
BS BNM-4	0	ND	24.9	ND	ND	ND	5.42	ND	ND
BS BNM-10	0	ND	20.7	35.5	34.7	ND	9.01	3.26	6.16
None	200	ND	28.0	35.1	33.1	ND	6.48	10.14	5.72
BS BNM-4	200	ND	22.7	ND	ND	ND	10.50	ND	ND
BS BNM-10	200	ND	17.8	36.5	33.5	ND	15.30	15.3	6.16
Strain		-	***	NS	NS	-	***	***	***
Fertilizer		-	NS	NS	NS	-	***	***	***
Interaction		-	NS	NS	NS	-	***	***	*
LSD $P < 0.05$		-	7.98	-	-	-	0.81	1.55	0.86

Table VIII. Total grain N content (g/m^2) in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

	Year	2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Cautelar
Strain	Fertilizer kg N/ha				
None	0	2.67	1.75	1.15	1.79
BS BNM-4	0	1.55	2.31	ND	ND
BS BNM-10	0	2.01	3.00	2.26	3.78
None	200	4.34	3.11	8.00	3.10
BS BNM-4	200	5.00	3.73	ND	
BS BNM-10	200	7.67	4.4	11.3	5.15
Strain	-	***	***	***	***
Fertilizer	-	***	***	***	***
Interaction	-	**	NS	***	NS
LSD $P < 0.05$	-	1.05	0.9	1.40	0.87

Table IX. Grain protein concentration (%DM) in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant. Bs. As.= Buenos Aires. LSD: least significant difference.

	Year	2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha				
None	0	8.27	9.83	7.09	6.70
BS BNM-4	0	10.3	11.4	ND	ND
BS BNM-10	0	12.9	13.3	8.38	9.68
None	200	9.4	10.87	14.35	8.69
BS BNM-4	200	12.8	12.5	ND	ND
BS BNM-10	200	15.6	15.5	16.7	11.3
Strain		***	***	***	***
Fertilizer		***	***	***	***
Interaction		***	***	NS	NS
LSD $P < 0.05$		0.713	0.538	1.21	0.86

Table X. Straw N concentration (%DM) and Nitrogen Harvest Index in wheat plants inoculated with rhizobacteria at maturity, in different years and locations. Asterisks indicate significant differences within a column. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: not determined. NS: not significant Bs. As.= Buenos Aires. LSD: least significant difference.

		Straw N concentration (%DM)				Nitrogen Harvest Index			
	Year	2000	2001	2002		2000	2001	2002	
	Site	Bs. As.	Bs. As.	Bs. As.	Castelar	Bs. As.	Bs. As.	Bs. As.	Castelar
Strain	Fertilizer kg N/ha								
None	0	0.317	0.431	0.291	0.582	ND	54.8	68.7	ND
BS BNM-4	0	0.364	0.577	ND	ND	ND	42.4	ND	61.2
BS BNM-10	0	0.427	0.805	0.413	0.82	ND	33.1	69.4	54.2
None	200	0.348	0.550	0.459	0.858	ND	47.9	78.9	ND
BS BNM-4	200	0.506	0.778	ND	ND	ND	35.7	ND	54.5
BS BNM-10	200	0.571	0.979	0.726	1.12	ND	28.8	74.1	58.5
Strain		***	***	***	***	-	***	*	NS
Fertilizer		***	***	***	***	-	*	***	*
Interaction		***	NS	*	NS	-	NS	**	NS
LSD $P < 0.05$		0.027	0.066	0.092	0.098	-	13.1	3.64	8.24

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