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Field Inoculation of Wheat *(Triticum aestivum)* With *Azospirillum brasilense* Under Temperate Climate

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Abstract

Inoculation of wheat with various strains of *Azospirillum* caused significant increases over controls in grain yield, ranging from 23% to 63% in 1986 and from 29% to 43% in 1987. The yield increases obtained depended upon the strain utilized in non N fertilized soil. In 1987, significant increases in grain yield and in N, P, K yield in grain over controls were obtained in all inoculated treatments fertilized with 0, 60, 90 or 120 kg N/ha. The reported yield increases were due to processes other than nitrogen fixation. Results may be of importance in developing countries where the use of fertilizers is low.

1. Introduction

Inoculation of cereals (wheat, maize, sorghum) with *Azospirillum*, both in greenhouse and in field conditions, have been performed in several countries. Often plant growth, dry matter and crop yield were significantly increased by such inoculation (Sarig et al., 1984; Mertens and Hess, 1984; Subba Rao et al., 1985; Warembourg et al., 1987). In some experiments, variable responses have been observed (Reynders and Vlassak, 1982; Smith et al., 1984; Wani et

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al., 1985; Kapulnik et al., 1987). These have been attributed to the ability of the host plant genotype to support associative symbiosis (Avivi and Feldman, 1982; Wani et al., 1985), to the use of inefficient strains (Reynders and Vlassak, 1982), to the lack of affinity of certain strains to their original host (Freitas et al., 1982; Baldani et al., 1986), and to improper colonization of roots by *Azospirillum* (Fallik et al., 1988). However, at present there is no definitive explanation. In most inoculation experiments, *Azospirillum brasilense* strains Sp 7 or Cd (Boddey et al., 1986; Baldani et al., 1987) have been used.

On the other hand, it has been proposed that a more extensive evaluation with additional cultivars in more locations would be justified (Kapulnik et al., 1987). It has also been suggested that many more strains of various origins will have to be tested for each cereal (Baldani et al., 1987). In this work, results of *Azospirillum* inoculation on wheat growing under field conditions in a temperature region of México during two consecutive years (1986 and 1987) are presented. Local *Azospirillum* strains and a single wheat cultivar commonly used in México were used. *Azospirillum brasilense* strains Sp 7 and Cd were also used for comparison.

2. Materials and Methods

The experiments were carried out during the rainy season (May–October) of 1986 and 1987 in two different places in the Ejido San José Teacalco, Tlaxcala State, México (Lat N 19°19′38″, Long W 98°03′36″; altitude 2600 m above sea level). During the months of the inoculation trials mean rainfall and mean temperature were 860 mm and 15.6°C in 1986, and 910 mm and 15°C in 1987 respectively. The soil was sandy loam both in 1986 and in 1987, pH 5.7 and 5.4; total N 0.1% and 0.09%; organic matter 1.4% and 1.1%, respectively.

Bacterial cultivars

In the first year, 1986, five *Azospirillum brasilense* strains (UAP 04, UAP 26, UAP 55, Sp 7 and Cd) were used separately as inoculum. These strains were the most effective among 20 strains isolated from different plants which previously were screened in wheat pot experiments. Strain UAP 04 was isolated from Cactaceous plants (Mascarúa-Esparza et al., 1988). Strain UAP 26 was isolated from maize rhizosphere and UAP 55 from *Brachiaria mutica* rhizosphere (López-Reyes et al., 1989). *A. brasilense* Cd was kindly provided by Dr. Y. Okon, while strain Sp 7 (ATCC 29145) was from the American Type Culture Collection.

In the second year, 1987, only the most effective two strains from the 1986 inoculation trials (UAP 55 and Cd) were used as inoculum.

Inoculation

Each strain of *A. brasilense* was grown separately in 500 ml flasks containing 350 ml of liquid nitrogen-free malate medium (Döbereiner et al., 1976) supplemented with 0.04% NH_4Cl and incubated at 32°C for 48 hr in a rotary shaking incubator (Lab-line instruments, Inc. model 3597) at 220 rpm. The culture (350 ml) was mixed with 500 g of finely-sieved sterilized peat, previously adjusted to pH 6.8 with $CaCO_3$. The inoculant was incubated for 7 days at 32°C in clean polyethylene bags under aseptic conditions. At the time of field inoculation, the populations of *Azospirillum* ranged from $3 - 5 \times 10^8$ colony forming units (CFU)/g peat, as determined by plating on congo red medium (Rodriguez Cáceres, 1982). For control treatments, the peat received a mixture culture heat-killed (121°C, 20 min) strains.

Minutes before sowing, the seeds were pelleted in a shadow in the field. An aqueous solution of arabic gum (20%) was used as adhesive. Polyethylene bags containing seeds were added to the sticker (40 ml/kg seeds) and mixed. Then the inoculant (15 g/kg seeds) was added and mixed uniformly. Control treatments were prepared in the same manner. All treatments were shown immediately.

Experimental design

Seeds of *Triticum aestivum* var. Cleopatra 74 were obtained from Productora Nacional de Semillas, México. Seeds were sown on 27 May 1986 (Experiment I) and on 24 May 1987 (Experiment II). Broadcast sowing was made at the rate of 120 kg seeds/ha.

Experiment I

The plot size was 10 m^2 (5×2 m), each treatment was replicated 4 times in completely randomized blocks. To reduce the possibility of cross-contamination, spaces of 1.5 m between adjacent plots were provided. Urea nitrogen fertilizer was applied over inoculated and control plots at rates of 0, 60 and 90 kg N/ha. Rates of 50 kg P_2O_5/ha as triple superphosphate was applied. Potassium was not applied because sufficient amounts existed in the soil. Half the urea and the total superphosphate were broadcasted 25 days after sowing, and at 45 days, a complementary amount of urea was applied.

Experiment II

The plot size was 40 m^2 (8 × 5 m). Each treatment was replicated 5 times. Nitrogen fertilizer was applied at rates of 0, 60, 90, and 120 kg N/ha. Other experimental design characteristics were similar to those in the experiment of 1986.

Harvest and analyses

Grain was harvested in plants covering an area of 4 m^2 (1×4 m in 1986) and of 28 m^2 (7×4 m in 1987) in the center of the plots for evaluation of field grain yields. After recorded grain yields, grain samples of each plot were dried at 70°C for 3 days, then were ground to pass through a 40-mesh screen. Total nitrogen content was determined by the Kjeldhal method. Potassium and phosphorus content were determined in harvested grains in experiment II. Potassium was determined by flame photometry; Phosphorus by the vanadomolybdate-nitric acid method.

3. Results

Experiment I

Azospirillum inoculation of wheat at the fertilization rate of 0 kg N/ha significantly increased the grain yield over uninoculated control (Table 1). The yield increase ranged from 23–63%, depending on the strain inoculated. Grain yield in inoculated treatments with strains UAP 55 and Cd alone without N fertilization were statistically similar to the grain yield obtained with an application of 60 kg N/ha. Nitrogen concentration (%) in grain from all inoculated treatments without N fertilization were higher (statistically significant) than the respective uninocualted control. Consequently, nitrogen yields (N total/ ha) were higher in inoculated treatments in respect to uninoculated control at the rate of 0 kg N/ha. Moreover, there were no statistically significant differences in nitrogen percentage from inoculated treatments relative to the control of 60 kg N/ha (Table 1). On the other hand, grain and nitrogen yields (kg/ha) in all inoculated treatments plus 60 or 90 kg N/ha did not differ statistically in respect to controls, except in inoculated treatment with strain UAP 04 and fertilized with 60 kg N, where a smaller yield was obtained compared to the control (Table 2).

Table 1. Effect of inoculation with different *A. brasilense* strains on grain yield and on N content of wheat (*Triticum aestivum*) var. Cleopatra 74

Treatment	Grain yield (kg/ha)[1]	Nitrogen in grain (%)[2]	N yield in grain (kg/ha)
Inoculated			
UAP 04	1564 136 g	2.31 0.15 c, d	36.12 3.69 f
Sp 7	1745 132 f, g	2.24 0.14 d	39.08 2.70 e
UAP 26	1825 167 e, f	2.30 0.03 c, d	41.97 3.32 d, e
Cd	1934 177 d, e, f	2.34 0.00 b, c, d	45.25 4.15 c, d
UAP 55	2080 186 c, d	2.36 0.06 a, b, c	49.08 2.95 b
Controls			
00 kg N/ha	1273 197 h	2.14 0.03 e	27.24 4.39 g
60 kg N/ha	2118 175 b, c, d	2.29 0.04 c, d	48.50 3.15 b, c
90 kg N/ha	2310 184 a, b	2.41 0.26 a, b	55.67 6.29 a

[1] Based on yields from plants in 4 m^2 harvested from each plot.
[2] Data represent the mean values of three samples for each from four plots.
Data with deviation of the mean followed by the same letter within each column do not differ at $P = 0.05$, using Student's t test.

Experiment II

Azospirillum inoculation at all fertilization levels (0, 60, 90 and 120 kg N/ha), significantly increased grain yield over their respective uninocualted controls at the same fertilization rates. The higher response to inoculation with either of the two strains of *A. brasilense* was observed at lower rates of nitrogen fertilizer than at the higher ones (Table 3). Strain UAP 55 in combination with application of 0, 60, 90 and 120 kg N/ha increased the grain yield 43%, 15%, and 11% respectively, compared with the respective uninoculated controls. Increases in yield (kg/ha) of nitrogen phosphorus and potassium in grain were obtained generally in inoculated treatments over uninoculated controls. Inoculation response was variable depending upon the strain utilized and by the rate of nitrogen fertilizer applied. The higher increases in N, P, and K were recorded in inoculated treatments at the rate of 0 kg N/ha. When strain UAP 55 was inoculated alone without nitrogen fertilization, N, P and K yields (kg/ha) in grain were increased 55%, 54% and 51% respectively, as compared to uninocualted control (Table 3). Nitrogen percent (data not shown) did not differ between inoculated treatments and respective controls. Nitrogen yield

Table 2. Effect of N fertilization and *A. brasilense* inoculation on grain yield and on N content of wheat (*Triticum aestivum*) var. Cleopatra 74

Treatment	N level (kg/ha)	Grain yield (kg/ha)[1]	Nitrogen in grain (%)[2]	N yield in grain (kg/ha)
Control	60	2118 175 a	2.29 0.04 a	48.50 3.15 a, b, c
UAP 55	60	2132 141 a	2.33 0.06 a	49.67 4.55 a
Cd	60	2107 178 a	2.32 0.15 a	48.88 5.53 a, b
Sp 7	60	2061 185 a	2.24 0.08 a	46.16 4.66 b, c
UAP 26	60	2012 141 a	2.32 0.03 a	46.68 3.98 b, c
UAP 04	60	1853 79 b	2.31 0.15 a	42.80 3.09 c
Control	90	2310 184 a, b	2.41 0.26 a	55.67 6.29 a, b
UAP 55	90	2361 121 a	2.44 0.04 a	57.61 2.38 a, b
Cd	90	2341 288 a	2.49 0.10 a	58.29 5.31 a
UAP 26	90	2263 58 a, b	2.44 0.10 a	55.22 2.96 a, b
Sp 7	90	2172 178 a, b	2.46 0.10 a	53.43 2.26 a, b
UAP 04	90	2121 144 b	2.45 0.13 a	51.96 3.80 b

[1] Based on yields from plants in 4 m^2 harvested from each plot
[2] Data represent the mean values of three samples for each from four plots.
Data with standard deviation of the means on each N level followed by the same letter within each column do not differ at $P = 0.05$, using Student's t test.

(kg/ha) in grain was significantly higher in all inoculated treatments than in fertilized controls, except with strain Cd with 120 kg N/ha (Table 3).

4. Discussion

Inoculation of wheat with each of the *Azospirillum brasilense* strains and without N fertilization, under temperature field conditions, in both experiments (years 1986 and 1987) caused consistent significant increases in the grain yield and total nitrogen, when compared to uninoculated controls. The degree of response differed significantly depending upon the strain used. For example, strain UAP 04, nitrate reductase positive (Nir^+) was the strain of *A. brasilense* that showed the lowest response at all three levels of nitrogen fertilizer applied in experiment I, while strain UAP 55, Nir^-, showed the higher grain yield increase. Reduction in yield has been attributed to the loss of soil NO_3^- by denitrification capacity of strain Sp 7 (Wani et al., 1985), but this observation has not been substantiated in many other field experiments.

Table 3. Effect of N fertilization and *A. brasilense* inoculation on grain yield and on N, P and K content of wheat (*Triticum aestivum*) var. Cleopatra 74

Treatment	N Level (kg/ha)	Grain yield[1] (kg/ha)	N yield in grain[2] (kg/ha)	P yield in grain[2] (kg/ha)	K yield in grain[2] (kg/ha)
Control	00	1205 85 c	25.79 3.12 c	3.78 0.29 b	3.72 0.23 c
Cd	00	1560 125 b	34.96 3.37 b	5.59 0.89 a	4.99 0.64 b
UAP 55	00	1720 116 a	39.95 3.78 a	5.83 0.44 a	5.62 0.22 a
Control	60	1875 55 b	42.61 1.79 c	6.19 0.12 c	5.70 0.25 b
Cd	60	2243 127 a	53.33 7.07 a	8.03 0.70 a	7.69 0.47 a
UAP 55	60	2165 202 a	48.96 5.06 b	6.90 0.57 b	7.34 0.70 a
Control	90	2214 89 c	49.81 2.09 c	7.64 0.47 a	6.91 0.36 b
Cd	90	2310 109 b	54.29 3.19 b	7.85 0.58 a	8.01 0.34 a
UAP 55	90	2450 179 a	59.71 4.67 a	7.89 1.04 a	8.16 0.67 a
Control	120	2591 110 b	66.53 6.27 b	8.21 0.36 c	7.64 0.15 c
Cd	120	2926 164 a	67.28 3.55 a, b	9.74 0.61 a	9.54 0.59 b
UAP 55	120	2885 178 a	70.68 4.42 a	9.05 0.26 b	9.75 0.79 a

[1] Based on yields from plants in 28 m^2 harvested from each plot
[2] Data represent the mean values of three samples for each from five plots.
Data with standard deviation of the means on each N level followed by the same letter within each column do not differ at $P = 0.05$, using Student's t test.

It is possible that the different responses in grain yield following inoculation with *A. brasilense* depend on the affinity between strains and plant as reported (Saric et al., 1987), but not by denitrification capacity of strain UAP 04, because strain Cd is also Nir^+ (Danneberg et al., 1986). In both experiments of this work strain Cd similarly enhanced grain yield as strain UAP 55. These results, together with previous reports (Baldani et al., 1987; O'Hara et al., 1987; Paredes-Cardona et al., 1988), emphasize the important of selecting the best associations of cultivars and *Azospirillum* strains for inoculation practices.

In experiment II, increases in grain yield in inoculated wheat treatments was recorded at four levels of N fertilization (0, 60, 90, and 120 kg N/ha) (Table 3). Increases in total nitrogen, phosphorus and potassium were also observed at different levels of nitrogen fertilization. Therefore, the present results suggest that the beneficial inoculation effects may be due to increased uptake of minerals from the soil (Lin et al., 1983; Sarig et al., 1984; Pacovsky et al., 1985), possibly as a result of an increase in proton efflux through membranes of root cells (Bashan et al., 1989), or attributed to plant hormones produced by *Azospirillum* that increase root development (Tien et al., 1979; Barbieri et al., 1986; Fallik et al., 1989).

We discard the possibility that the reported yield increases can be due to a nitrogen fixation effect, because the mean temperature from the study region during the months of the inoculation trials was 15°C. Only very low nitrogenase activity of *Azospirillum* is observed below 24°C, and none below 17°C (Day and Döbereiner, 1976). Furthermore, high nitrogen levels repress nitrogenase activity (Neyra and Döbereiner, 1977) and in experiment II, statistically significant differences in grain yield were recorded in inoculated treatments with 90 or 120 kg N/ha.

Increases in total nitrogen, phosphorus and potassium content have also been reported (Sarig et al., 1984; Kapulnik et al., 1987) with other cultivars under different climatic conditions.

On the other hand, it is difficult to explain the different response to inoculation between experiment I (1986) and experiment II (1987), particularly on inoculated treatments plus nitrogen fertilizer, although experimental and environmental conditions were very similar in both years. One explanation may be different degrees of root colonization in 1986 due to suboptimal conditions on the day of inoculation.

Another explanation may be the lower organic matter content in soil in experiment II (1.1%) than in experiment I (1.4%), because the competitive ability of *Azospirillum* diminishes markedly once the organic matter exceeds 1.0% (Fallik et al., 1988). Lack of consistent inoculation responses have been recorded on several crops inoculated with *A. brasilense* (Smith et al., 1984; Kapulnik et al., 1987; Bashan et al., 1988), but the causes of inconsistency to inoculation have so far not been explained.

In conclusion, the present studies have shown that: (a) the degree of response differs significantly depending upon the strain inoculated; (b) *Azospirillum* inoculated plants better assimilate mineral ions (N, P, K) from soil or applied fertilizers than uninoculated plants, thus consequently leading to significant increases in the grain yield of wheat var. Cleopatra growing in the field in temperate climate. This fact may be of importance for developing countries where the use of fertilizers is low.

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