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ORIGINAL PAPER

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Can *Azospirillum* strains capable of growing at a sub-optimal temperature perform better in field-grown-wheat rhizosphere

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Abstract Two *Azospirillum brasilense* strains, CDJA and A40, capable of growing and producing plant growth-promoting (PGP) substances at the sub-optimal temperature (SOT) of 22°C, were tested for their ability to survive, colonize and enhance wheat growth and yield under field conditions upon inoculation. The response was compared with that of *A. brasilense* strain, A9, impaired in growth and PGP activities at SOT (22°C) but otherwise comparable to CDJA and A40 at 37°C. A field experiment was carried out in a split-plot design with four levels of N as main plots and three strains and an uninoculated control as subplots. A differential response in the establishment of the strains and in plant growth and yield was obtained, due to the categories of strains, particularly at lower levels of N (0 kg and 40 kg N ha^{-1}). The results clearly demonstrated that strains capable of growing and producing PGP substances at SOT are better inocula for wheat.

Keywords *Azospirillum* · Temperature · Inoculation response

Introduction

Azospirillum inoculation of plants has been tested worldwide in view of their contribution to plant productivity by their ability to fix N_2, their phytohormone production, siderophore production, enhancement of plant mineral uptake, and NO_3^- production (Bashan and Holguin 1997). Despite many successful experiments using *Azospirillum* spp. as plant inocula, their commercial application on a large scale has lagged behind, and the main obstacle to their use is the unpredictability and inconsistency of field results (Okon and Labandera-Gonzalez 1994). The results of field experiments with winter crops are more inconsistent than those with crops grown under a high temperature or tropical conditions (Sumner 1990; Wani 1990; Pandey et al. 1998), as an optimum temperature (e.g. 37°C) is required for growth and the production of beneficial plant growth-promoting (PGP) substances (Tripathi and Klingmuller 1992). *Azospirillum* survives and establishes poorly in the rhizosphere of crops grown in winter due to the prevailing low temperature (18–25°C) (Harris et al. 1989; Wani 1990; Pandey et al. 1998; Kaushik et al. 2001). The low or non-significant effect of *Azospirillum* inoculation in winter crops discourages the large-scale use of *Azospirillum* as a biofertilizer for these as compared to its use in summer crops.

In earlier studies, we identified two *Azospirillum brasilense* strains capable of growing and stimulating plant growth at a sub-optimal temperature (SOT) in microcosms (Kaushik et al. 2001). Their establishment and ability to improve wheat growth were tested using Tn*5*-*lacZ* insertion mutants, otherwise isogenic to wild type strains under glasshouse conditions (Kaushik et al. 2000).

In the present study these *Azospirillum* strains were used to study the survival and colonization of inocula in the wheat endorhizosphere in the field, in order to test the hypothesis that the poor effects of *Azospirillum* are due to the poor survival of inocula in winter.

Materials and methods

Bacterial cultures and growth media

Two *A. brasilense* strains, CDJA and A40, capable of growing and producing PGP substances at SOT (22°C) and one strain, A9, sensitive to SOT but otherwise efficient at the optimum temperature of 37°C, were obtained from the culture collections of the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi. Their intrinsic antibiotic resistant patterns are given in Table 1. These strains were grown and maintained on N-containing sodium malate medium (Bulow and Dobereiner 1975).

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Table 1 Intrinsic antibiotic resistance pattern of the selected *Azospirillum brasilense* strains. *S* Sensitive

Strain	Concentration of antibiotics (µg ml^{-1})
CDJA	Nalidixic acid (100), ampicillin (50), chloramphenicol (10), kanamycin (s), streptomycin (s), tetracycline (s)
A40	Chloramphenicol (10), streptomycin (25), tetracycline (15), nalidixic acid (50), ampicillin (s), kanamycin (s)
A9	Tetracycline (15), nalidixic acid (50), ampicillin (10), chloramphenicol (100), kanamycin (s), streptomycin (s)

Plant and chemical fertilizers

Seeds of wheat cultivar HD2285 were obtained from the Division of Genetics, IARI, New Delhi and fertilized with commercially available urea (46% N) and single super phosphate (16% P_2O_5) as per the rates mentioned below.

Field studies

A field experiment was conducted during the winter of 1997–1998 at the experimental farm, IARI, New Delhi. The soil was a sandy loam (pH 8.1; 683.2 kg N ha^{-1}; 25.2 kg P ha^{-1}; 628 kg K ha^{-1}; organic C 0.48%). The soil temperature of the field was recorded daily at a depth of 10 cm from the date of sowing till harvest of the crop.

The field was divided into 48 plots of 15 m^2 and the experiment was laid in a split-plot design. P as single super phosphate (60 kg ha^{-1}) was applied at a basal dose during field preparation. Four urea-N levels (0, 40, 80, 120 kg N ha^{-1}) were taken as main blocks in three replicates. Three *Azospirillum* inocula (CDJA, A40 and A9) along with one uninoculated control were taken as sub plots in each main block. N was applied in two split doses, one after 21 days of germination and the other one at the tillering stage. A pre-sowing irrigation was given 10 days before field preparation.

A carrier-based inoculum of each strain was freshly prepared using sterilized charcoal:soil (3:1) as the carrier following the method described by Jauhri et al. (1979). Forty millilitres of the broth culture (ca. 10^9 cells ml^{-1}) was mixed with 100 g sterilized carrier material under aseptic conditions. The inoculum was packed in pre-sterilized polypropylene bags and opened at the time of usage. The inoculum maintains a population of 10^8 cells ml^{-1} for a period of 3 months when stored at 4°C. Each inoculum carried 2.8–3.7×10^8 cells g^{-1} enumerated by the dilution plate method. Half an hour before sowing, wheat seeds were coated with different carrier-based inocula (as per treatments) by using a 10% sucrose solution as an adhesive. Coated seeds were air-dried for 15 min under shade and sown immediately. Seeds for the uninoculated control were also coated in a similar manner with the sterilized carrier. The seeds were air-dried for 10 min under shade before sowing.

After 30 and 60 days of seedling emergence, five plants were uprooted from each plot and shoot length was measured. The nitrogenase activity per gram root was measured by the acetylene reduction assay as per the method described by Saxena et al. (1996). The endorhizospheric count of *Azospirillum* was estimated following dilution plating as described by Bulow and Dobereiner (1975) using Congo red medium (Rodriguez-Caceres 1982) with the selection pressure of appropriate antibiotics as given in Table 1 for different *Azospirillum* strains. For the strain CDJA, Congo red medium was supplemented with nalidixic acid at 100 µg ml^{-1} and ampicillin at 50 µg ml^{-1}. For the strain A40, the medium was supplemented with streptomycin at 25 µg ml^{-1} and tetracycline at 15 µg ml^{-1}. For the strain A9, the medium was supplemented with tetracycline at 15 µg ml^{-1} and chloramphenicol at 100 µg ml^{-1}. The distinctive scarlet red colonies appearing on the selective Congo red medium plates were counted as those of the inocula.

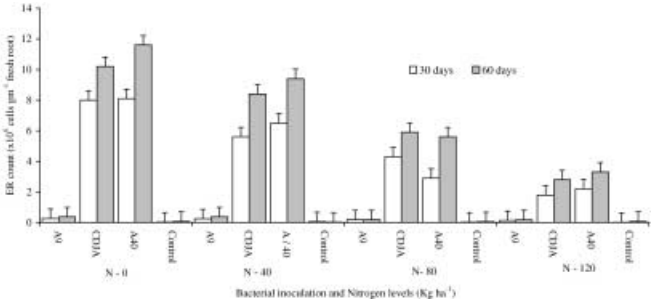

Fig. 1 Endorhizospheric (*ER*) counts of strains of *Azospirillum brasilense* after 30 and 60 days of germination at different N levels. Least significant difference at *P*=0.05. ANOVA for 30-day incubation: N levels, 0.55; interaction, 1.14. ANOVA for 60-day incubation: N levels, 0.68; interaction, 1.24

All the antibiotic plates and the plain plates (without antibiotics) were also used to enumerate the native *Azospirillum* population with the same antibiotic profile as that of inocula strains in the uninoculated control.

The shoot portion was oven dried at 80°C and the dry weight was recorded. The total N content in dried shoots was determined by the Kjeldhal method as described by Page et al. (1982). After harvesting, the grain and straw yield along with their N contents were recorded.

The data were analysed statistically as described by Fisher (1958) using a split-plot design. The SEM and critical difference at the 5% significance level were calculated.

Results

Seeds of wheat coated with the carrier-based inocula of *Azospirillum* strains had a population count of 2.0–2.5×10^8 cells g^{-1} seed. The endorhizospheric count (EC) of inocula strains in the respective plots 30 and 60 days after germination is shown in Fig. 1. The EC after 60 days was significantly higher at all N levels than after 30 days of germination. A significant reduction in the EC was observed with an increase in the N level; the EC of strain A9 was significantly lower than that of the strains capable of growing at SOT (CDJA and A40) at all the levels of N and sampling stages. Strain A9 could maintain a lower population, i.e.10^4 cells g^{-1} root, after 30 and 60 days of germination in contrast to 10^6–10^7 cells g^{-1} root exhibited by CDJA and A40. In control plots (no inoculation) the *Azospirillum* count on antibiotic plates was zero, thereby indicating the absence of a native population with the same antibiotic profile as that of inocula strains. However, on plain plates the native *Azospirillum* population was in the range of 2–8×10^4 cells g^{-1} root in all the plots at 30 and 60 days after germination.

The effect of *A. brasilense* on different plant parameters after 30 and 60 days of germination is shown in Table 2. Inoculation with strain CDJA and A40 produced significantly higher plant height and shoot biomass than inoculation with A9, at the low N levels of 0 and 40 kg N ha^{-1}. At higher N levels (80 kg N ha^{-1} and 120 kg N ha^{-1}) no beneficial effect of inoculation was observed and all the treatments were statistically at a par. Inocula-

Table 2 Wheat growth as influenced by *Azospirillum* inoculations and N levels after 30 and 60 days of germination. *LSD* Least significant difference

N level (kg ha^{-1})	*Azospirillum* strain	Growth parameters							
		Plant height (cm)		Shoot dry wt. (g)		Total shoot N (mg)		ARA (nmol C_2H_4 h^{-1} gm^{-1} root)	
		30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
0	A9	27.7	63.0	0.34	6.5	13.9	181.3	49.4	38.4
	CDJA	34.0	72.0	0.61	11.2	38.2	670.1	109.6	122.3
	A40	32.0	69.7	0.55	11.3	35.9	673.5	80.5	95.6
	No inoculation	20.7	47.0	0.28	5.6	3.1	129.6	1.5	10.2
40	A9	29.7	66.7	0.41	10.7	21.2	412.8	36.6	28.3
	CDJA	33.7	77.8	0.58	18.8	37.8	1,135.6	95.9	106.4
	A40	32.0	76.7	0.55	18.4	40.9	1,124.9	73.2	92.0
	No inoculation	29.0	63.2	0.40	11.2	12.1	463.5	1.0	4.5
80	A9	33.0	68.9	0.50	12.2	28.3	733.9	24.0	17.0
	CDJA	35.0	77.8	0.60	17.5	40.0	1,100.1	82.6	82.2
	A40	29.3	77.3	0.55	17.6	39.2	1,133.3	55.7	59.6
	No inoculation	31.0	67.3	0.47	11.9	19.3	596.2	0.5	5.1
120	A9	31.3	78.2	0.55	16.7	38.8	1,065.8	7.7	15.5
	CDJA	33.7	83.7	0.53	17.7	39.0	1,175.5	37.1	43.2
	A40	32.0	82.2	0.53	18.1	32.0	1,239.8	29.2	37.4
	No inoculation	30.7	75.7	0.53	15.2	28.2	1,040.1	0.52	4.6
LSD	N levels	1.41	2.2	0.05	1.2	5.9	97.54	3.86	5.39
(*P*=0.05)	Strain	1.40	2.7	0.04	1.04	4.9	96.46	6.11	5.03
	Interaction	2.8	5.4	0.08	2.08	8.4	192.9	12.23	10.06

Table 3 Grain yield, straw yield and total shoot and grain N at harvest as influenced by *Azospirillum* inoculations and N levels. *NS* Not significant

N level (kg ha^{-1})	*Azospirillum* inoculations	Growth parameters			
		Grain yield (t ha^{-1})	Shoot dry weight (t ha^{-1})	Total grain N (mg gm^{-1} dry weight)	Total shoot N (mg gm^{-1} dry weight)
0	A9	2.40	9.5	13.91	4.79
	CDJA	4.20	14.3	16.94	8.06
	A40	4.30	14	16.09	6.98
	No inoculation	1.90	7.3	12.06	2.49
40	A9	3.90	12.7	15.44	5.99
	CDJA	5.86	22.7	19.37	10.07
	A40	5.53	17.7	18.42	9.80
	No inoculation	3.43	12.3	14.24	4.77
80	A9	5.57	16.7	17.34	9.16
	CDJA	5.50	19.3	19.58	9.87
	A40	5.73	20	19.02	9.96
	No inoculation	4.43	14.3	16.02	8.08
120	A9	5.30	17.7	18.04	8.55
	CDJA	5.73	21.7	19.46	10.19
	A40	5.83	21.3	19.05	9.48
	No inoculation	5.27	18.7	15.92	8.25
LSD	N levels	0.49	0.21	0.61	0.42
(*P*=0.05)	Strain	0.34	0.15	0.69	0.42
	Interaction	0.68	0.30	NS	0.84

tion with CDJA and A40 resulted in significantly higher total shoot N and root nitrogenase activity compared to inoculation with A9. Root nitrogenase activity decreased with an increase in N level and increased 30–60 days after germination.

The grain yield showed variations both with the *Azospirillum* inocula and N levels (Table 3). Strains CDJA and A40 produced significantly higher grain yields than strain A9 and the uninoculated control at all four N levels, except at 80 kg N ha^{-1}. In general, no significant increase in grain yield could be achieved by increasing the level of N beyond 40 kg ha^{-1}. Among the treatments, inoculation with CDJA at 40 kg N ha^{-1} was optimum and gave a maximum grain yield of 5.86 t ha^{-1}. A similar trend was observed in the case of straw yield. Grain and shoot N increases effected by inoculation with A9 in all main plots were significantly lower than those achieved with the other inocula.

Discussion

According to Harris et al. (1989), the poor and unpredictable performance of *Azospirillum* inoculation in winter crops is mainly due to its poor establishment and survival. In agreement with his finding, the effect of strain A9 (sensitive to SOT) on wheat was poor at lower N levels of 40 kg N ha^{-1} as compared to inoculation with CDJA and A40 (strains capable of growing at SOT). Skvortsov et al. (1995) reported that attachment of *Azospirillum* to the root hair zone is dependent on bacterial proteins and extracellular surface polysaccharides. The low growth and metabolic activity of A9 at a prevailing low temperature (Kaushik et al. 2001) could have resulted in the low production of such proteins and polysaccharides, thereby restricting its ability to colonize the wheat endorhizosphere in high numbers.

It has been reported earlier that available soil N affects the response of plants to inoculation; a good response to inoculation has been obtained at intermediate initial levels of N fertilizer, i.e. in the range of 10–80 kg N ha^{-1} (Tilak and Subba Rao 1987). A higher mineral N application drastically reduced inoculation responses (Wani 1990). In agreement with this, the inoculation response in the present study decreased with an increase in N level, and at 120 kg N ha^{-1} the increase over the uninoculated control was only 10%, as compared to 125%, 74% and 30% at 0, 40 and 80 kg N ha^{-1}, respectively. This decrease in the inoculation response can be attributed to the fact that that higher concentration of readily available NO_3^--N inhibits nitrogenase activity, thereby affecting N fixation (Nelson 1987). Moreover, NO_3^--N can cause the catalytic destruction of indoleacetic acid (Tanner and Anderson 1964) thereby blocking the growth promotion of the plant by *Azospirillum* through the production of growth-promoting hormones, chiefly indoleacetic acid.

From sowing till harvesting of the crop, the maximum soil temperature remained below 25°C for most of the time. During the entire growth period, except for the 87th day and at the time of harvesting, the minimum temperature never exceeded 20°C. The temperature data give credence to the hypothesis that only those *Azospirillum* strains which are capable of growing at SOT (CDJA and A40) can perform well with wheat and other winter crops (Kaushik et al. 2001) as they can fix N, produce growth-promoting substances, colonize roots and survive optimally at temperatures below 25°C.

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