Microb Releases (1993) 1:243–251

Microbial Releases

Inoculation of maize varieties with salt-tolerant mutants of *Azospirillum brasilense* and VAM fungi in saline calcareous soil

R. Rai[1], **P. G. Hunt**[2]

[1] Department of Microbiology, N. D. University of Agriculture and Technology Kumarganj, 224229, Faizabad, U. P., India
[2] Coastal Plains Soil and Water Conservation Research Center, P.O. Box 3039, Florence, SC 29502-3039, USA

Abstract. *Azospirillum brasilense* and VAM fungi have been shown to benefit plant growth and seed yield of numerous crops, but their combined impact on maize in calcareous soils has not been investigated. Five nitrosoguanidine-induced salt-resistant mutant strains of *A. brasilense* were isolated. Three mutant strains exhibited significantly greater growth, nitrogenase activity, and total auxin production than the parent strain in a nitrogen-free medium containing 650 mEq of sodium salts. The content and concentrations of root exudates and extracts of maize varieties were different, and chemotaxis ratios for the mutant strains in response to root exudates and extracts were variable and strain-specific. Inoculation of salt-resistant mutants of *A. brasilense* and *Glomus albidus* led to significant increases in both populations in and on the maize roots. Associative nitrogen fixation along with maize mineral nutrient content and growth were also increased in both sterilized and nonsterilized saline calcareous soils. Salt-resistant strain SR 4, *G. albidus*, and the Hemant variety of maize were the most effective combination for grain yield in saline calcareous soil under field conditions.

Key words: *Azospirillum brasilense* – Chemotaxis – Associative nitrogen fixation – *Glomus albidus* – Salt tolerance – Plant growth promotion – Nutrient uptake

Introduction

Saline-affected areas in India and elsewhere are increasing each year, and elevated soil salinity can have a detrimental effect on both plants and microorganisms (Rai 1985a; Rai 1991). One approach to ameliorate limiting soil environments is plant inoculation with associative nitrogen-fixing bacteria of the genus *Azospirillum*. Such inoculations have given increased grain and straw yields particularly under low soil nitrogen conditions (Barber et al. 1980; Kapulnik et al. 1981; Rai et al. 1984; Rai 1985b). However, there is little information about the influence of various salts on the growth, auxin production, and associative nitrogen fixation by *Azospirillum brasilense* with maize varieties in saline calcareous soil.

Azospirillum species may elicit host growth response by producing growth-promoting substances (Okon and Kapulnik 1986; Tien et al. 1979) and enhancing mineral uptake by the roots (Lin et al. 1983; Rai 1988). Significant increases in root elongation and root surface area concomitant with enhanced mineral uptake were reported in wheat after inoculation with a mixture of *A. brasilense* strains (Kapulnik et al. 1985a, b). Mineral uptake can also be increased by the endomycorrhizal symbionts, and vesicular arbuscular mycorrhizal (VAM) fungi root infection is known to improve the growth and yield of many agriculturally important crops grown in harsh soil environments (Mosse 1973; Rai 1988; Subba Rao et al. 1985a). The tripartite systems of plants – *Azospirillum* – VAM fungi are particularly complex associations (Pacovsky et al. 1985). However, dual inoculation with VAM fungi and associative nitrogen-fixing bacteria could be expected to provide sufficient nitrogen and mineral nutrients, especially phosphorus, to enhance growth and yield of maize in saline calcareous soils.

The objectives of this research were to assess: (1) the interrelationship between root exudates and *A. brasilense* strain-specific chemotaxis; (2) the role of associative nitrogen fixation in saline calcareous soils; (3) the role of *A. brasilense* and *Glomus albidus* in enhancing mineral uptake and seed yield of maize.

Materials and methods

Organisms

A. brasilense strain RAU 59 was isolated from the roots of the maize variety Lakshmi grown in saline calcareous soil as described by Caceres (1982) and was characterized using the *A. brasilense* strain Sp7 as reference strain (Dobereiner and Day 1976).

Correspondence to: R. Rai

Growth conditions

A. brasilense RAU 59 was grown in liquid nitrogen-free medium of Dobereiner and Day (1976). The medium contained 5.0 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 0.1 g NaCl, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.002 g Na_2MoO_4, 4.7 g KOH, pH 7.0. Three salts (NaCl, Na_2SO_4, and $NaHCO_3$) were added to this medium separately, before sterilization, to give final concentrations of 0–300 mEq Cl^-, SO_4^{--}, and HCO_3^-. Concentrations of 10, 25, 50, 100, 150, and 200 mEq were prepared for Cl^-, SO_4^{--}, and HCO_3^-. Additional concentrations of 250 mEq for Cl^- and SO_4^{--} and 300 mEq for Cl^- were prepared, and all concentrations were replicated five times. *A. brasilense* was grown in microaerobic conditions for 10 days at 28 ± 1° C. For microaerobic conditions, the PO_2 was maintained at 0.02–0.03 atm. The dissolved oxygen tension (DOT) of the medium was maintained initially by a mixture of 2.0% (v/v) O_2 in N_2, which was sparged through the medium. The DOT was measured with an immersed galvanic-type oxygen electrode. Growth (optical density at 560 nm), nitrogenase activity (acetylene reduction assay), and total nitrogen were determined as described by Rai (1985b).

Induction of mutants

A fresh culture of *A. brasilense* RAU 59 grown in nitrogen-free medium was washed and suspended in phosphate-buffered saline, pH 7.2. A culture suspension (2 ml) containing 5.7×10^6 cells/ml was treated with 30 µg/ml of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) for 20 min at 35° C, with another 2 ml of the culture kept as untreated control. Both cultures were washed separately and diluted in 10 ml of nitrogen-free medium, grown at 28 ± 1° C in microaerobic conditions for 24 h, washed and resuspended in 2 ml of nitrogen-free medium and 0.2 ml (1.7×10^7 CFU), and plated on agar medium containing 650 mEq of salts (300, 200, and 150 mEq of Cl^-, SO_4^{--} and HCO_3^- respectively). The cultures were further incubated at 29 ± 1° C for a period of 15 days in microaerobic conditions. Five clones of mutagenized culture were isolated and designated as SR 1, SR 2, SR 3, SR 4, and SR 5. These mutants were grown with the 650 mEq salt combination or without salts for ten subcultures, and they all proved to be stable mutants.

Growth, nitrogenase activity, total indoles and IAA production of salt-resistant mutant strains

All the mutant strains and parental strain RAU 59 were grown for 7 days in nitrogen-free medium containing 650 mEq of three salts of sodium in microaerobic conditions and growth (*D* at 560 nm) was observed. Nitrogenase activity, nitrogen fixation/mg protein, and nitrogen fixed/g lactate consumed were determined as described by Volpon et al. (1981). Total indoles and indole acetic acid (IAA) were determined in culture filtrates by a modified calorimetric method (Gordon and Paleg 1957), e.g., cultures grown in liquid lactate medium supplemented with 500 ppm tryptophane were filtered and culture filtrates (2 ml) were added to 8 ml solution of 0.5 M $FeCl_3$ + 5% $HClO_4$ for 30 min (100 ml 5% $HClO_4$ + 2 ml 0.5 M $FeCl_3$) to develop the color. After 30 min optical density was measured at 540 nm on a Spectronic 20.

Determination of growth of strains in response to root extracts and exudates of maize varieties

Maize varieties Lakshmi and Hemant were grown aseptically on moist wool over distilled water in 25-ml universal bottles for 10 days in a light chamber. The exudates were collected (Rao and Venkateswarlu 1985) and the same roots were crushed with a pestle and mortar and extracted in boiling water. The hot water extract was filtered and extracts were filter-sterilized and added to tubes containing sodium lactate medium supplemented at different concentrations (0.4, 0.8, and 1.0 ml) by keeping the final volume of medium constant at 5 ml. Bacterial strains were inoculated and growth was determined after 5 days of incubation at 28 ± 1° C in microaerobic conditions.

Chemotaxis to root exudates, root extracts, amino acids, sugars, and organic acids

Cultures growing exponentially were centrifuged (20 ml) at 8000*g* at 10° C for 10 min and the pellet was suspended in chemotaxis buffer (pH 7, potassium phosphate buffer) to a density of 6×10^6 cells/ml and chemotaxis was assessed by the blindwell chamber method as used by Rai (1987). A chemotaxis ratio was obtained by dividing the number of bacteria passing into test solution (root extracts and exudates) by the number passing into the control solution. The number of bacteria which passed through the membrane was assessed with a hemocytometer. Ring formation on the modified minimal medium containing 0.25% agar to which a single substance like amino acid, sugar, organic acid, root extracts, or exudates had been added was evidence that the substance was chemotactic (Armitage et al. 1977). Alanine, aspartic acid, glutamic acid, histidine, proline, sucrose, fructose, malic acid, citric acid, and fumaric acid were used as single components in the blindwell chamber assay.

Composition of root exudates and extracts of maize varieties

Root exudates and extracts of the maize varieties were obtained from 10-day-old seedlings grown on moist cotton wool over distilled water in 25-ml universal bottles as described previously. The cationic, neutral, and anionic components of the root exudates and extracts were determined (Gaworzewska and Carlile 1982). Amino acids were determined using an automatic amino acid analyzer. Sugars and organic acids were assessed and identified respectively by two-dimensional paper chromatography (Rai and Strobel 1966; Somogyi 1952).

Pot experiment

The two varieties of winter maize were planted in a pot experiment in October. Each 35-cm-diameter pot contained 15 kg sterilized saline calcareous soil. Ten replicates were used for each treatment in completely randomized design. Seeds were surface-sterilized and inoculated with 1-ml culture suspensions of *A. brasilense* prepared in sterilized water. Two plants were maintained in each pot. The survival of mutants and parental strains on the seeds was examined after 10 days using dilution and plate-counting technique. In all cases the survival was assessed at more than 10^6–10^7 viable cells per seed.

G. albidus was inoculated by use of a layering method (Hall 1978). The inoculum (200 mg/pot) contained finely chopped segments of maize variety Hemant root that were heavily infected with hyphae and chlamydospore of *G. albidus*. Inoculum was added in layers at a depth of 4–5 cm in mycorrhizal treated pots. Maize seeds were sown just above the inoculum layer. Sterilized (autoclaved) inoculum of the mutant strains *A. brasilense* RAU 59 and 200 mg/pot root materials of maize grown without *G. albidus* were inoculated in control treatments.

Fertilizers were applied uniformly to the pots at the rate of 80 kg N, 60 kg P_2O_5 and 40 kg K_2O/ha in the form of urea, single superphosphate and potassium chloride. Micronutrients such as Zn and Fe were applied in the form of SO_4 at the rate of 12 kg/ha. The entire quantity of zinc, phosphorus, potash, iron and half the

nitrogen were applied at planting; the remaining nitrogen was applied 35 days later.

The experimental saline calcareous soil was sandy loam. It had a pH of 8.6, free $CaCO_3$ of 32.7%, electrical conductivity of 6.95 mm mho/cm (soil:water, 1:2) at 25° C, organic carbon content of 0.213%, and a cation exchange capacity of 11.92 mEq/g. Available P_2O_5 and K were 21 and 65.9 mg/kg soil, respectively. DTPA-extractable Fe, Mn, Cu, and Zn were 6.15, 3.75, 0.93, and 0.78 mg/kg, respectively (Jackson 1978).

Fifty days after sowing, plants were taken from each treatment and nitrogenase activity of the excised fresh roots (3–5 cm long) was determined by acetylene reduction and gas chromatography (De-Polli et al. 1982). Fresh excised roots (1 g) were immersed in sterilized distilled water contained in glass nursing bottles (300 ml). Before assay, the water was removed completely from the bottles by air displacement and the bottles sealed tightly. For the low O_2 treatment, the bottles were capped and 5% air added (equivalent to 1% O_2). No pre-incubation time was allowed in either case before the start of the acetylene reduction assays. Acetylene (10% v/v) was injected into each assay vial. Samples were taken at time intervals for gas analysis. Ethylene production was determined by gas chromatography equipped with a H_2-air flame-ionization detector and a stainless steel column packed with Poropack N (192 × 0.3 cm). The column was operated at 100° C and N_2 was used as carrier gas (flow rate, 28 ml/min).

Plant height, plant and root dry weight (on an oven dry basis at 60° C) were measured. Mineral contents (K, P, Fe, and Zn) of plants (stem + leaf) were determined by the methods of Jackson (1978).

Determination of most probable number of A. brasilense strains in different root zones and mycorrhizal infection percentage

The number of RAU 59 and salt-tolerant mutant strains in different root zones (endo- and exorhizospheres) was determined 50 days after sowing by the most probable number (MPN) method using two pots in each treatment. Roots were washed thoroughly in sterile water. The roots were then weighed on a per plant basis and the nonsurface-sterilized populations were counted (Rao and Venkateswarlu 1985). The same roots were then surface-sterilized with 0.1% mercuric chloride for 10 min, followed by 1% chloroformin-T for 1 h, followed by six washings in sterile water. Surface-sterilized roots were crushed aseptically in a sterile mortar and pestle with a pinch of acid-washed sand and suspended in a diluent blank. All of the samples were serially diluted by tenfold series and analyzed for salt-tolerant and RAU-59 strains by the MPN method using semi-solid nitrogen-free medium containing 650 mEq of sodium salts (300, 200, and 150 mEq of Cl^-, SO_4^{--}, and HCO_3^-, respectively). The percentage mycorrhizal infection in root samples was determined following the method of Bierman and Linderman (1981). The proportion of root-containing vesicular arbuscules or hyphae of *G. albidus* was estimated to the nearest 10%.

Field experiment

On the basis of the performance of RAU-59 and salt-tolerant strains of *A. brasilense* relative to mineral ion uptake by roots, associative nitrogen fixation, and other growth-attributing characters of maize in the pot culture experiment, two salt-tolerant strains SR 1 and 4 were found suitable for field evaluation. VAM endophytes of *G. albidus* were applied uniformally in the furrow before seeds were sown in October. Surface-sterilized seeds of both maize varieties were inoculated with SR 1 or 4. Seeds were inoculated (10^8 cells/seed) early in the morning, kept for 2 h in a cool place, and sown in 80 × 30 cm spacing. A split-plot design with three replicates was used. Plot size was 4 × 3 m. Maize varieties comprised main plots and *A. brasilense* and *G. albidus* the subplots. All the chemical fertilizers were applied to plots at the same rate as in the pot culture experiment. Normal agricultural operations were performed when required. The soil of the experimental plots was saline calcareous. Its pH, free $CaCO_3$, and electrical conductivity were 8.5, 31.2%, 6.7 mm moh/cm (soil:water, 1:2) at 27° C.

Sixty days after sowing, 15 plants of each variety were taken from each treatment. Nitrogenase activity of excised roots, plant height, plant and root dry weight, mineral contents of plant, population of *A. brasilense* strains in and on the root surface, and percentage of mycorrhizal infection in root samples were determined as described previously. After maturity, maize was harvested for dry matter and grain yield.

Results and discussion

Growth and nitrogen fixation of *A. brasilense* RAU 59 (parental) declined in media with 25 mEq or greater sodium salts of Cl, SO_4, and HCO_3. The most effective inhibitor was HCO_3; concentrations of 100 mEq inhibited the growth by 95%. It appears that similar to most *Rhizobium* strains, *A. brasilense* is also sensitive to higher concentrations of salts (Rai 1983a, b). However, SR strains were able to grow, fix nitrogen, and produce IAA in media containing 650 mEq of these sodium salts. Strain SR4 of *A. brasilense* had the greatest growth, nitrogenase activity, nitrogen fixed/g lactate consumed, total indoles, and IAA production (Table 1). Mutant

Table 1. Growth, nitrogen fixation, production of indole acetic acid (IAA) and indoles by salt-tolerant strains and *Azospirillum brasilense* RAU 59 after 7 days incubation in saline media. Values are the means of seven replicates

Strains	Growth (optical density at 560 nm)	Nitrogenase activity (nmol C_2H_4/mg protein/h)	Total N (μg N/ml)	Nitrogen fixation (μmol/mg) protein	Nitrogen fixed/g lactate consumed (mg)	Total indoles (μg/ml)	IAA (μg/ml)
RAU 59	0.085	—	—	—	—	—	—
SR 1	0.755	199.5	48.12	3.92	61.21	1.85	1.21
SR 2	0.631	163.2	35.75	2.75	49.79	1.39	1.15
SR 3	0.701	154.8	42.93	3.78	56.82	1.69	1.33
SR 4	0.859	221.7	69.76	4.48	76.39	2.35	1.79
SR 5	0.521	108.3	27.28	2.06	34.44	2.21	1.69
LSD (*P* 0.05)	0.019	9.5	6.26	0.42	7.49	0.02	0.01

Table 2. Chemotaxis ratios for salt-tolerant and parental strains in response to 2 mM concentration of amino acids, sugars, and organic acids. Values are the mean of five replicates. Chemotaxis ratios above 2.0 are significant at the 1% level

Substrate	RAU 59	SR 1	SR 2	SR 3	SR 4	SR 5	SE
Amino acids							
Alanine	1.6	2.7	2.1	1.8	3.2	1.9	0.02
Aspartic acid	2.3	3.8	2.5	1.9	4.2	2.4	0.01
Glutamic acid	1.9	3.1	2.3	2.0	3.7	2.2	0.05
Histidine	1.3	2.7	1.6	1.8	3.1	1.8	0.01
Proline	1.7	2.9	1.3	1.5	3.5	1.2	0.02
Sugars							
Sucrose	2.6	3.9	2.1	1.9	4.6	2.3	0.03
Fructose	2.9	4.6	3.2	2.5	5.8	2.6	0.02
Organic acids							
Malic acid	3.8	6.2	4.2	3.1	7.7	3.8	0.05
Citric acid	2.4	5.4	2.3	1.9	6.8	2.7	0.04
Fumaric acid	1.5	2.7	1.7	2.1	3.8	1.3	0.03

SR5 had the lowest growth and nitrogen fixation, but it was second to SR4 for hormone production in liquid medium. Thus, at least one mutant strain showed promise for growth in the rhizosphere of maize roots in saline soil conditions. All the mutants are stable because they have been maintained on nitrogen-free medium without higher concentrations of salts for several subcultures.

Root exudates and extracts of both the maize varieties stimulated the growth of the salt-resistant strains as measured by the optical density of liquid media containing root extracts and exudates (Table 3). This is in agreement with Rao and Venkateswarlu (1985), who found that the root exudates of pearl millet enhanced the growth and multiplication of *Azospirillum*. Maize root extracts were found to be more effective than exudates for stimulation of the growth for all strains, and Hemant gave better growth of all strains than Lakshmi. Strain SR 4 showed maximum response to root exudates and extracts of both varieties. The present results support the view that the ability to respond to gradients of exudates and extracts components may explain the attraction of salt-resistant mutants to the rhizosphere, the surface of roots, and the root interior.

Strain RAU 59 had significant chemotaxis ratios to aspartic acid, sucrose, fructose, malic acid, and citric acid, but SR1 and SR4 were more responsive (Table 2). They had significant positive chemotaxis responses to all the selected amino acids, sugars, and organic acids. These chemicals were among those measured in the root exudates and extracts of the maize seedling.

Analyses of the cationic, anionic, and neutral components of the low-molecular-weight fractions of root exudates and extracts of maize varieties demonstrated the presence of 15 amino acids, six sugars, and six organic acids (Table 4). Greater weights of amino acids, sugars, and organic acids were found in the root extracts than in the root exudates of both varieties. Hemant had greater total amounts than Lakshmi in both extracts and exudates. Aspartic acid, fructose, sucrose, malic acid, and citric acid were present in the greatest concentration in exudates and extracts of both the varieties. However, varietal differences did occur. Hemant produced more alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, cysteine, leucine, phenylalanine, serine, threonine, proline, sugars, and organic acids. Lakshmi produced more isoleucine, lysine, and valine.

Chemotaxis ratios for the SR and RAU 59 strains to root exudates and extracts of maize seedling roots obtained 5 and 10 days after germination are presented in Table 5. Chemotaxis ratios of SR1 and SR4 were significantly greater than the ratio obtained for other strains. Root exudates and extracts of Hemant had a greater chemotaxis effect than those of Lakshmi. It is possible that the ability to respond to a wide range of readily diffusing components (amino acids, sugars, and organic acids) may facilitate bacterial entry into root hairs, their rhizosphere, and approach to the root surface. It seems that chemotaxis is a major factor in bacterial-host-plant specificity, and motility is guided by the chemotaxis to a particular component.

Pot experiments with maize, A. brasilense, and G. albidus

Inoculation with the SR strains led to a significant increase in their endo- and exo-rhizosphere population on

Table 3. Effect of root exudates and extracts of maize varieties on growth of salt-resistant mutant strains after 5 days' incubation

Strains	Bacterial growth (optical density at 560 nm)							
	Root exudates of Lakshmi (ml)		Root extracts of Lakshmi (ml)		Root exudates of Hemant (ml)		Root extracts of Hemant (ml)	
	0	1.0	0	1.0	0	1.0	0	1.0
RAU 59	0.31	0.43	0.26	0.46	0.30	0.50	0.29	0.46
SR 1	0.32	0.48	0.30	0.51	0.31	0.51	0.31	0.54
SR 2	0.30	0.43	0.30	0.45	0.30	0.49	0.30	0.50
SR 3	0.30	0.41	0.30	0.41	0.30	0.42	0.30	0.49
SR 4	0.42	0.51	0.42	0.52	0.42	0.51	0.42	0.53
SR 5	0.21	0.28	0.21	0.29	0.21	0.24	0.21	0.24
LSD (*P* 0.05)	0.04	0.07	0.03	0.05	0.03	0.04	0.06	0.09

Table 4. Composition of root exudates and extracts (μg/plant) of maize varieties grown for 10 days. Values are the mean of three replicates

Substrate	Maize varieties			
	Hemant		Lakshmi	
	Root exudates	Root extracts	Root exudates	Root extracts
1	2	3	4	5
Amino acids				
Alanine	5.0	6.6	4.4	5.8
Arginine	5.7	6.5	4.2	6.3
Aspartic acid	7.5	8.6	5.5	6.7
Glutamic acid	5.5	6.2	3.7	4.8
Glycine	3.5	4.7	3.0	3.9
Histidine	6.5	7.9	4.8	5.6
Cysteine	2.7	3.5	1.4	2.2
Isoleucine	2.9	3.3	2.4	5.2
Leucine	5.5	6.7	4.8	5.7
Lysine	3.9	4.4	4.5	5.3
Phenylalanine	3.2	3.7	1.6	2.2
Serine	5.2	6.4	4.8	5.9
Threonine	5.3	7.2	3.9	4.7
Valine	0.8	1.3	1.5	2.6
Proline	2.5	6.2	3.2	4.6
Total	65.7	83.2	54.7	71.5
Sugars				
Glucose	2.8	3.4	2.5	2.9
Sucrose	7.9	9.4	7.2	8.6
Fructose	8.2	9.5	7.9	9.1
Maltose	0.9	1.2	0.5	0.8
Ribose	1.7	2.6	1.2	2.1
Xylose	6.2	9.5	5.6	7.2
Total	27.7	35.6	24.9	30.7
Organic acids				
Citric acid	11.3	13.2	9.7	10.1
Gluconic acid	3.9	4.6	2.2	3.7
Succinic acid	1.6	2.1	1.2	1.8
Malic acid	13.6	15.2	11.7	13.2
Fumaric acid	4.5	5.7	3.3	4.6
Isocitrate	2.8	3.5	1.9	2.6
Total	37.7	44.3	30.0	36.0
Total contents of root exudates and extracts (μg/plant)	131.1	163.1	109.6	138.2

Table 5. Chemotaxis ratios of salt-resistant mutant strains in response to root exudates and root extracts of maize varieties after 5 and 10 days' germination

Days after germination	Chemotaxis ratios of strain	Chemotaxis ratios			
		Root exudates		Root extracts	
		Lakshmi	Hemant	Lakshmi	Hemant
5	RAU 59	1.26	1.65	1.03	1.25
	SR 1	1.92	2.06	2.12	2.23
	SR 2	1.65	1.79	1.78	1.92
	SR 3	1.85	1.97	2.05	2.17
	SR 4	2.13	2.26	2.28	2.36
	SR 5	1.31	1.07	1.49	1.38
10	RAU 59	1.59	1.78	1.75	2.05
	SR 1	1.79	1.96	2.02	2.11
	SR 2	1.95	2.07	1.62	1.85
	SR 3	2.06	2.15	2.16	2.23
	SR 4	2.25	2.29	2.33	2.39
	SR 5	1.76	1.45	1.89	1.63
LSD (P 0.05)		0.88	0.10	0.28	0.89

both varieties (Table 6). Hemant exhibited a higher bacterial population than Lakshmi, and a greater percentage of the population was on the root surface. The infection percentage of *G. albidus* was also increased by inoculation, and application of *G. albidus* increased both the endo- and exo-rhizosphere populations of *A. brasilense* for both maize varieties. The interaction among *G. albidus*, SR strains, and varieties was significant at the 0.05 level. Co-inoculation of Hemant with SR4 and *G. albidus* resulted in the highest population of bacteria and infection percentage of *G. albidus*.

All SR strains had significantly more nitrogenase activity than parental strain RAU 59, and all had higher nitrogenase activity with *G. albidus* (Table 6). SR4 and SR1 had the highest activity, and activity was generally higher with the Hemant than Lakshmi.

Inoculation increased the uptake of K, P, Fe, and Zn by both maize varieties relative to maize that was uninoculated or inoculated with parental strain RAU 59 (Table 7). However, co-inoculation of SR strains with *G. albidus* resulted in greater mineral nutrient uptake by both maize varieties than inoculation of SR strains alone. Salt-tolerant strains SR4 and SR1 were found to be more effective than other mutant strains. The impact of SR strains on nutrient uptake by plants was significantly different for specific nutrients and varietal combinations. Hemant, SR4, and *G. albidus* resulted in maximum uptake of mineral nutrients.

Plant height and dry weight of both varieties were substantially increased by inoculation with SR strains; SR4 and SR1 were most effective (Table 8). Hemant was more responsive to co-inoculants than Lakshmi. However, maximum increases were found when the SR strains were co-inoculated with VAM fungi (Table 8). Interaction among SR strain, variety, and *G. albidus* was significant. SR4, Hemant, and *G. albidus* resulted in maximum plant growth, plant height, root volume, and plant dry weight for both varieties.

These data demonstrate for the first time that inoculation of salt-resistant mutants with or without *G. albidus* in saline calcareous soils produced a significant increase in the uptake of mineral nutrients over uninoculated controls. This increase may be related to the production of hormones by *Azospirillum* strains (Tien et al. 1979) such as auxin, gibberellin, and cytokinins. Thus, the response of *Azospirillum* may have resulted from several factors. *Azospirillum* which lives on or in the roots may be responsible for the production of hormones affecting the plant growth by stimulating root growth proliferation of maize. Increased root surface might have resulted in enhanced mineral uptake by in-

Table 6. Nitrogenase activity, most probable number (MPN) of *A. brasilense* and VAM-fungal infection on roots of maize varieties after 50-day growth period (pot experiment). ND, Not detectable; SR, salt resistant

Strain	Lakshmi				Hemant			
	Nitrogenase activity (nmol C_2H_4/g fresh roots/h)	MPN of *Azospirillum* strain ($\times 10^4$)		*G. albidus* infection (%)	Nitrogenase activity (nmol C_2H_4/g fresh roots/g)	MPN of strains ($\times 10^4$)		*G. albidus* infection (%)
		Washed and fresh crushed roots/g	Surface sterilized and crushed roots/g			Washed and crushed roots/g	Surface sterilized and crushed roots/g	
Uninoculated control	ND	ND	ND	ND	ND	ND	ND	ND
Inoculated *G. albidus*	0.5	0.0	0.0	60.5	0.7	0.0	0.0	65.9
Inoculated RAU 59	2.9	1.7	0.2	0.0	1.0	0.0	0.0	0.0
Inoculated SR 1	62.5	19.3	11.1	0.0	68.7	21.1	12.2	0.0
Inoculated SR 2	44.6	13.9	7.3	0.0	35.4	15.0	9.0	0.0
Inoculated SR 3	51.2	15.7	9.3	0.0	57.8	14.2	8.1	0.0
Inoculated SR 4	78.2	22.7	13.2	0.0	89.6	26.2	17.4	0.0
Inoculated SR 5	37.0	9.2	3.0	0.0	41.2	11.3	5.8	0.0
Inoculated RAU 59 + *G. albidus*	3.1	1.9	0.4	61.3	3.6	1.8	0.4	66.7
Inoculated SR 1 + *G. albidus*	69.8	22.3	13.7	68.2	75.6	24.9	16.3	76.4
Inoculated SR 2 + *G. albidus*	48.4	14.3	7.9	67.4	39.4	16.9	10.2	72.4
Inoculated SR 3 + *G. albidus*	55.7	17.3	10.3	70.1	63.2	16.4	10.4	73.0
Inoculated SR 4 + *G. albidus*	81.4	24.2	15.0	73.4	95.3	30.2	19.1	77.0
Inoculated SR 5 + *G. albidus*	39.2	10.1	4.9	68.5	45.8	12.8	7.9	69.1
LSD (*P* 0.05)								
Strain	0.93	0.85	0.79	0.99	0.78	0.67	0.98	0.76
Variety	1.65	1.98	1.67	2.34	1.95	1.86	1.85	1.78
Strain × variety	2.72	2.43	2.95	2.99	2.84	2.94	2.79	2.89

Table 7. Effect of salt-resistant mutant strains, RAU 59 and *G. albidus* on K, P, Fe, and Zn contents of maize in pot culture experiment after 50-day growth period

Strain	Maize genotypes							
	Lakshmi				Hemant			
	K (%)	P (%)	Fe (μg/g dry wt. of plant)	Zn (μg/g dry wt. of plant)	K (%)	P (%)	Fe (μg/g dry wt. of plant)	Zn (μg/g dry wt. of plant)
Uninoculated control	0.21	0.32	78.5	21.7	0.26	0.37	86.2	28.5
Inoculated *G. albidus*	0.36	0.44	97.8	31.8	0.37	0.53	99.3	39.1
Inoculated RAU 59	0.22	0.34	77.7	21.9	0.28	0.36	78.5	29.6
Inoculated SR 1	0.34	0.47	103.5	33.0	0.34	0.49	92.5	35.7
Inoculated SR 2	0.26	0.42	94.2	25.5	0.29	0.41	88.7	30.5
Inoculated SR 3	0.29	0.44	92.5	27.9	0.31	0.44	98.8	31.3
Inoculated SR 4	0.37	0.51	110.2	35.3	0.44	0.55	115.9	37.2
Inoculated SR 5	0.27	0.34	85.5	25.9	0.30	0.41	82.6	28.5
Inoculated RAU 59 + *G. albidus*	0.39	0.46	102.6	33.5	0.40	0.56	106.8	42.8
Inoculated SR 1 + *G. albidus*	0.45	0.57	118.7	39.0	0.48	0.63	120.3	45.1
Inoculated SR 2 + *G. albidus*	0.41	0.51	108.5	33.5	0.39	0.60	109.2	41.0
Inoculated SR 3 + *G. albidus*	0.39	0.47	105.2	32.9	0.38	0.58	104.5	40.0
Inoculated SR 4 + *G. albidus*	0.51	0.66	125.7	42.3	0.57	0.72	137.6	49.6
Inoculated SR 5 + *G. albidus*	0.37	0.46	99.3	33.2	0.38	0.57	102.1	41.2
LSD (*P* 0.05)								
Strain	0.08	0.10	2.92	0.86	0.16	0.17	1.08	1.34
Variety	0.17	0.18	4.80	1.68	0.28	0.86	2.82	2.86
Strain × variety	0.25	0.39	6.86	2.86	0.96	1.72	4.95	3.99

Table 8. Effect of salt-resistant mutants, RAU 59, and *G. albidus* on growth-attributing characters of maize in saline calcareous soil after 50-day growth period (pot experiment)

Strain	Lakshmi			Hemant		
	Plant height (cm)	Plant dry wt. (g)	Root dry wt. (g)	Plant height (cm)	Plant dry wt. (g)	Root dry wt. (g)
Uninoculated control	29.6	55.2	10.9	33.7	68.0	12.8
Inoculated *G. albidus*	36.7	69.6	14.2	39.5	81.3	16.5
Inoculated RAU 59	30.1	56.0	11.1	33.9	69.9	13.0
Inoculated SR 1	37.2	75.6	15.3	41.7	86.3	17.6
Inoculated SR 2	32.9	70.9	13.2	36.9	78.8	14.2
Inoculated SR 3	36.7	73.8	12.2	38.8	81.5	15.7
Inoculated SR 4	43.2	90.5	16.3	47.5	98.7	17.7
Inoculated SR 5	31.3	69.0	11.3	35.8	70.5	12.9
Inoculated RAU 59 + *G. albidus*	42.5	73.9	16.3	42.4	86.7	14.7
Inoculated SR 1 + *G. albidus*	45.0	76.7	17.5	49.8	97.2	21.6
Inoculated SR 2 + *G. albidus*	36.3	73.0	15.2	41.8	85.9	17.8
Inoculated SR 3 + *G. albidus*	41.9	72.3	15.6	43.2	91.5	18.5
Inoculated SR 4 + *G. albidus*	51.5	97.3	22.4	55.8	108.7	24.6
Inoculated SR 5 + *G. albidus*	37.0	73.5	14.9	41.0	82.5	17.0
LSD (*P* 0.05)						
Strain	0.97	0.95	0.78	0.83	1.03	0.24
Variety	1.63	1.85	1.39	1.59	1.85	0.59
Strain × variety	2.72	2.86	2.76	2.24	2.96	1.15

Table 9. Nitrogenase activity, most probable number (MPN) of *A. brasilense* strains and VAM fungi infection of maize varieties after 60 days' growth (field experiment)

Strain	Lakshmi				Hemant			
	Nitrogenase activity (nmol C_2H_4/g fresh roots/h)	MPN of *Azospirillum* strain ($\times 10^4$)		*G. albidus* infection (%)	Nitrogenase activity (nmol C_2H_4/g fresh roots/g)	MPN of *Azospirillum* strains ($\times 10^4$)		*G. albidus* infection (%)
		Washed and fresh crushed roots/g	Surface sterilized and crushed roots/g			Washed and crushed roots/g	Surface sterilized and crushed roots/g	
Uninoculated control	22.9	6.15	2.8	9.5	26.2	7.2	3.0	11.2
Inoculated *G. albidus*	26.5	6.9	3.1	56.3	30.1	7.9	3.3	61.6
Inoculated SR 1	53.3	15.2	9.9	7.2	63.7	19.9	10.1	9.2
Inoculated SR 4	65.8	18.3	10.3	8.3	85.9	21.5	14.3	10.3
Inoculated SR 1 + *G. albidus*	61.8	18.9	11.3	62.2	73.5	21.6	14.8	71.0
Inoculated SR 4 + *G. albidus*	75.2	19.7	12.7	70.9	92.5	25.0	16.2	72.9
LSD (*P* 0.05)								
Strain	0.92	0.75	0.11	1.10	0.87	0.08	0.15	0.79
Variety	1.82	0.97	0.62	2.08	1.54	0.17	0.96	1.26
Strain × variety	2.33	1.52	0.96	2.97	1.96	0.47	1.23	2.38

oculated plants and this mechanism is affected by the interaction between specific strain and specific host variety. These observations support the findings of Lin et al. (1983) that *A. brasilense* inoculation can improve the ion uptake of plants and improve the availability and efficiency of use of applied mineral nutrients.

Field experiments

Data from the pot study showed SR1 and SR4 to be most effective on maize in sterile, saline calcareous soil for root colonization, nitrogenase activity, plant height, and dry matter production. Therefore, SR4 and SR1 were selected for the field experiment in saline calcareous soil. Both strains showed significantly more nitrogenase activity and exo- and endo-rhizosphere populations on maize roots than the uninoculated control with or without *G. albidus* (Table 9). The infection percentage of *G. albidus* varied with varieties, and maximum infection percentage was recorded from the roots of Hemant. Hemant, SR4, and *G. albidus* was the best combination. An ineffective low population of indigenous strains of *A. brasilense* and *G. albidus* was de-

Table 10. Effect of *A. brasilense* strains (salt-tolerant) and *G. albidus* on K, P, Fe, and Zn contents of plants of maize in saline calcareous soil (field experiment) after 60 days' growth

Strain	Lakshmi				Hemant			
	K (%)	P (%)	Fe (μg/g dry wt. of plant)	Zn (μg/g dry wt. of plant)	K (%)	P (%)	Fe (μg/g dry wt. of plant)	Zn (μg/g dry wt. of plant)
Uninoculated control	0.24	0.35	72.6	22.6	0.29	0.39	88.33	27.2
Inoculated *G. albidus*	0.42	0.46	99.9	33.3	0.41	0.54	103.5	37.5
Inoculated SR 1	0.38	0.47	106.2	35.2	0.36	0.52	97.3	36.9
Inoculated SR 4	0.42	0.56	115.5	37.8	0.45	0.56	117.0	39.5
Inoculated SR 1 + *G. albidus*	0.44	0.62	121.2	40.2	0.50	0.64	121.6	43.2
Inoculated SR 4 + *G. albidus*	0.47	0.68	126.0	41.7	0.60	0.73	132.2	45.5
LSD (*P* 0.05)								
Strain	0.35	0.09	1.96	0.98	0.06	0.09	1.37	0.93
Variety	0.97	0.17	2.75	1.29	0.14	0.12	2.52	1.56
Strain × variety	1.26	0.69	4.08	1.89	0.40	0.63	3.16	2.38

Table 11. Effect of *A. brasilense* strains (salt-tolerant) and *G. albidus* on grain and dry matter yield of maize in saline calcareous soil (field experiment)

Strain	Lakshmi		Hemant	
	Grain yield (t/ha)	Dry matter yield (t/ha)	Grain yield (t/ha)	Dry matter yield (t/ha)
Uninoculated control	4.9	9.6	5.0	10.2
Inoculated *G. albidus*	5.1	11.2	5.4	12.8
Inoculated SR 1	5.2	12.1	5.7	13.4
Inoculated SR 4	5.4	12.3	5.9	13.8
Inoculated SR 1 + *G. albidus*	5.8	13.0	6.2	14.1
Inoculated SR 4 + *G. albidus*	6.1	13.3	6.4	14.4
LSD (*P* 0.05)				
Strain	0.39	0.15	0.75	0.91
Variety	0.98	0.76	1.13	1.32
Strain × variety	1.25	0.95	1.76	1.69

tected in roots of both varieties in uninoculated treatments.

The nutrient content of both maize varieties increased significantly with inoculation of strain SR4 and SR1 (Table 10). Application of *G. albidus* either alone or in combination with SR4 or SR1 also increased nutrient uptake. *G. albidus* inoculation lowered the shoot to root ratio in both the field and pot studies. It is of interest that it caused relatively more root growth than the SR 1 and SR 4 mutant strains. Maximum nutrient uptake was determined in plants of the variety Hemant; and the interaction among strains, varieties, and *G. albidus* was significant. The most effective combination for nutrient uptake was SR4, *G. albidus*, and Hemant; this combination was also the highest producer of grain and straw (Table 11).

The beneficial effect of *G. albidus* on plant growth as well as grain and straw yield of maize varieties was likely a result of its ability to increase mineral nutrient uptake (Bierman and Linderman 1981; Rai 1988). This conclusion is in agreement with other reports. VAM fungi were found to support the uptake of mineral nutrients such as P, K, Cu, Zn, and Fe more effectively than root hairs on a nonmycorrhizal maize plant (Hall 1978). Increased uptake of Zn from Zn-deficient soils in mycorrhizal maize, potato, and wheat plants was further supported by several workers (Nielsen and Jensen 1983; Sanders and Tinker 1981; Subba Rao et al. 1985a, b).

The most effective combination for grain and straw yield was SR4, *G. albidus*, and Hemant. This is consistent with the laboratory and greenhouse findings and indicates that the SR4, *G. albidus*, and Hemant have the potential to improve the straw and grain yield of maize in areas suffering from saline calcareous soils. Nevertheless, the system needs further refinement and testing in multiple weather patterns before it can be recommended as a production practice.

Acknowledgements. We are grateful to Shree V. Prasad, K. K. Giri, A. Rai, and P. Mahto (T.C.A., Dholi, Muzaffarpur, Bihar), for technical assistance. This research has been financed in part by a grant from the U.S. Department of Agriculture under CARP-PL-480 project.

References

Armitage JP, Josey D, Smith DG (1977) A simple, quantitative method for measuring chemotaxis and motility in bacteria. J Gen Microbiol 102:199–201

Barber LE, Russel SA, Evanse HJ (1980) Inoculation of millet with *Azospirillum*. Plant Soil 52:49–57

Bierman B, Linderman RG (1981) Quantifying vesicular-mycorrhizae: a proposed method towards standardization. New Phytol 87:63–67

Caceres EAR (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 51:990–991

De-Polli H, Boyer CD, Neyra CA (1982) Nitrogenase activity associated with roots and stems of field-grown corn (*Zea may* L.) plants. Plant Physiol 70:1609–1613

Dobereiner J, Day JM (1976) Associative symbiosis in tropical grass: characterization of microorganisms, and dinitrogen fixing sites. In: Newton WE, Nyman CJ (eds) 1st International Symposium on N-fixation. Washington State University Press Pullman, pp 518–538

Gaworzewska ET, Carlile MD (1982) Positive chemotaxis of *Rh.*

zobium leguminosarum and other bacteria towards root exudates from legumes and other plants. J Gen Microbiol 128:1179–1188

Gordon SA, Paleg LG (1957) Quantitative measurement of indole acetic acid. Physiol Plant 10:37–48

Hall IR (1978) Effect of vesicular-arbuscular mycorrhizas on two varieties of maize and one of sweetcorn. NZ J Agric Res 21:517–519

Jackson ML (1978) Soil chemical analysis. Prentice-Hall, New Delhi

Kapulnik Y, Kigel J, Okon Y, Nur I, Henis Y (1981) Effect of *Azospirillum* inoculation on some growth parameters and N content of wheat, sorghum and panicum. Plant Soil 61:65–70

Kapulnik Y, Feldman M, Okon Y, Henis Y (1985a) Contribution of nitrogen fixed by *Azospirillum* to the N nutrition of spring wheat in Israel. Soil Biol Biochem 17:509–516

Kapulnik Y, Okon Y, Henis Y (1985b) Changes in root morphology of wheat caused by *Azospirillum* inoculation. Can J Microbiol 31:881–887

Lin W, Okon Y, Hardy RWF (1983) Enhanced mineral uptake by *Zea mays* and *Sorghum biocolor* roots inoculated with *Azospirillum brasilense*. Appl Environ Microbiol 45:1775–1779

Mosse B (1973) Plant growth responses to vesicular-arbuscular mycorrhiza. IV. In soil given additional phosphate. New Phytol 72:127–136

Nielsen JD, Jensen A (1983) Influence of vesicular-arbuscular mycorrhizal fungi on growth and uptake of various nutrients as well as uptake ratio of fertilizer P for lucerne (*Medicago sativa*). Plant Soil 70:165–172

Okon Y, Kapulnik Y (1986) Field inoculation studies with *Azospirillum* in Israel. Plant Soil 90:3–16

Pacovsky RS, Fuller C, Paul EA (1985) Influence of soil on interactions between endomycorrhizal and *Azospirillum* in soybean. Soil Biol Biochem 17:525–531

Rai R (1983a) Efficiency of associative N_2-fixation by streptomycin-resistant mutants of *Azospirillum brasilense* with genotypes of chickpea *Rhizobium* strains. J Agric Sci Camb 100:75–80

Rai R (1983b) The salt tolerance of *Rhizobium* strains and lentil genotypes and the effect of salinity on aspects of symbiotic N-fixation. J Agric Sci Camb 100:81–86

Rai R (1985a) Studies on nitrogen fixation by antibiotic-resistant mutants of *Azospirillum brasilense* and their interaction with cheena (*Panicum milliaceum* L.) genotypes in calcareous soil. J Agric Sci Camb 105:261–270

Rai R (1985b) Studies on nitrogen fixation by machete-resistant mutants strains of *Azospirillum brasilense*: their associative N_2-fixation and yield response on rice to root inoculation in calcareous soil. J Gen Appl Microbiol 31:1–16

Rai R (1987) Chemotaxis of salt-tolerant and sensitive *Rhizobium* strains to root exudates of lentil (*Lense culinaris* L.) genotypes and symbiotic N-fixation, proline content and grain yield in saline calcareous soil. J Agric Sci Camb 108:25–37

Rai R (1988) Interaction response of *Glomus albidus* and *Cicer Rhizobium* strains on iron uptake and symbiotic N_2 fixation in calcareous soil. J Plant Nutr 11:863–869

Rai R (1991) Strain-specific salt tolerance and chemotaxis of *Azospirillum brasilense* and their associative N_2-fixation with finger millet in saline calcareous soil. Plant Soil 137:55–59

Rai PV, Strobel GA (1966) Chemotaxis of Zoospores of *Aphanomyces cochliodes* to sugar beet seedlings. Phytopathology 56:1365–1368

Rai R, Prasad V, Shukla IC (1984) Interaction between finger millet (*Eleusine coracana*) genotypes and drug-resistant mutants of *Azospirillum brasilense* in calcareous soil. J Agric Sci Camb 102:521–529

Rai R, Nasar SKT, Singh SJ, Prasad V (1985) Interaction between *Rhizobium* strains and lentil (*Lens culinaris* Linn.) genotypes under salt stress. J Agric Sci Camb 104:199–205

Rao AV, Venkateswarlu B (1985) Most probable number of *Azospirillum* associated with the roots of inoculated pearl millet. Plant Soil 88:153–158

Sanders FE, Tinker PB (1981) Mechanism of absorption of phosphate from soil by endogenous mycorrhizas. Nature 33:278–279

Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19–23

Subba Rao NS, Tilak KVBR, Singh CS (1985a) Effect of combined inoculation of *Azospirillum brasilense* and vesicular mycorrhizae on pearl millet. Plant Soil 84:283–286

Subba Rao NS, Tilak KVBR, Singh CS (1985b) Synergistic effect of vesicular-arbuscular mycorrhizas and *Azospirillum brasilense* on the growth of barley in pot. Soil Biol Biochem 17:118–121

Tien TM, Gaskin MH, Hubbell DH (1979) Plant growth substance produced by *Azospirillum brasilense* and their effect on the growth of pearl millet. Appl Environ Microbiol 37:1012–1024

Volpon AGT, De-Polli H, Dobereiner J (1981) Physiology of nitrogen fixation in *Azospirillum lipoferum* Br 17 (AICC 29709). Arch Microbiol 128:371–375