Plant and Soil 90, 35–46 (1986). Ms. NF 03

Establishment of inoculated *Azospirillum* spp. in the rhizosphere and in roots of field grown wheat and sorghum

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Key words *Azospirillum* Establishment in roots Inoculation N_2 fixation

Summary Four field experiments were carried out with wheat or sorghum in different regions of Brazil. The aim was to study the establishment of inoculated *Azospirillum* strains, marked with resistance to various antibiotics, in the rhizosphere and in roots. The levels of the various antibiotics were chosen according to the resistance of the indigenous *Azospirillum* population. *Azospirillum brasilense* strains Sp 107 and Sp 245 could be established in all three wheat experiments and predominated within the *Azospirillum* population in washed, and especially in surface sterilized, roots. Strains Sp 7 and Cd established poorly in wheat roots. *Azospirillum lipoferum* Sp S82 represented 72% of the root isolates from sorghum inoculated with this strain. This strain and natural *Azospirillum* infection became concentrated in the upper parts of the root system. Improved methods for root surface sterilization in which the absence of *Azospirillum* on the root surface was established by pre-incubating roots with paraffin-capped ends in NFb medium confirmed the establishment of inoculated *Azospirillum* strains within sorghum roots in the field.

Introduction

An important pre-requisite for field inoculation responses with selected or genetically manipulated *Azospirillum* strains is their establishment and multiplication in the rhizosphere or in roots, where strong competition with indigenous strains and other microorganisms is to be expected. Most data showing *Azospirillum* inoculation effects on cereal yields are from Israel[17,20,27] where this organism seems not to be generally present in high numbers in soil. There, and in recent experiments in Florida[28], strains Sp 7 or Cd were used. In India where local isolates were used, 14 out of 56 field experiments with various cereals showed significant yield increases[29]. Under Egyptian conditions a mixed inoculum of five local *A. lipoferum* strains almost doubled plant dry weights and increased nitrogenase activity (C_2H_2) significantly[14]. Few data which compare the effects of inoculation with *Azospirillum* strains of various origins are, however, available although it seems "logical to speculate that (bacterial) activity within and around roots may be associated with a hierarchy of requirements for specificity"[15]. The question of specificity has been much discussed in relation to associative nitrogen fixation and experimental evidence so far indicates affinities of certain strains for their original host or group of hosts[4,26]

rather than absolute specificities. Reynders and Vlassak[25] showed higher increases in wheat yield when they used strain Sp Br 14 isolated from the wheat rhizosphere than with strain S 631 isolated from maize. In Brazil, strains Sp 107st and Sp 245, both isolated from surface sterilized wheat roots[3], and strain Sp 242st isolated from surface sterilized maize roots[10,13], when tested under field conditions, gave consistent increases of total plant N of their respective hosts while strain Sp 7 and several others, had no effect. Strains Sp 107st and Sp 245 also caused characteristic root hair deformations in wheat seedlings which were not observed with Sp 7 or Sp 242st[22].

Additional evidence of 'specificity' of *Azospirillum* strains was recently presented in relation to chemotaxis of various organic acids[23] and to root tip exudates[19] and in relation to attachment to roots[16].

No data on the establishment and multiplication of inoculated *Azospirillum* under field conditions are available. Albrecht *et al.*[1] and Smith *et al.*[28] reported the failure of *A. brasilense* strains 13tSR2 and CdSR (resistant to streptomycin and rifampicin) to become established in the rhizosphere soil of maize, sorghum and millet in Florida. In the present paper the establishment of inoculated *Azospirillum* strains on the root surface and within roots of field-grown wheat and sorghum, were studied.

Materials and methods

Plants from four field experiments were used, three with wheat and one with sorghum.

Exp. 1 was planted in 1983 in a 'Terra Roxa Estruturada' soil, Parana, the major wheat growing region of Brazil. The wheat cultivar used was Anahuac. Three inoculants were used. (*A. brasilense* strains Cd (ATCC 29710), Sp 107st and Sp 245). Details of the strains are given in Table 1. Controls received heat killed cells. The inoculants were grown for 24 h in aerated 3 l bottles with NFb medium[8] containing 1 g.l^{-1} NH_4Cl. Each culture (1, 1 l) was mixed with 6 kg of peat granules and 10 g applied to the plant holes in each 1 m of row. Plots were 10 × 6 m in size (10 m rows) and the fertilizer used was 80 kg P.ha^{-1}, 50 kg K.ha^{-1} and two levels of nitrogen (15 and 60 kg N.ha^{-1}). There were 6 complete randomized blocks.

Exp. 2 was planted in 1983 in a red-yellow podzolic soil in Seropedica, Rio de Janeiro (km 47). Treatments consisted of eight wheat cultivars (Lagoa Vermelha, Cotiporan, Anahuac, Cocoraque, BH 1146, IAC. 5, Alondra, El Pato) and two inoculation treatments (Sp 245 and uninoculated control). Details of inoculation were as described in Exp. 1. Plots were 3 × 2 m in size (3 m rows) and were fertilized with 30 kg N.ha^{-1}, 70 kg P.ha^{-1} and 66 kg K.ha^{-1}.

Exp. 3 was planted in 1984 in red-yellow podzolic soil in Seropedica, Rio de Janeiro (km 47) and inoculation was as described for Exp. 1 but with different *Azospirillum* strains (*A. brasilense* Sp 7 nad st, Sp 245 spec, Sp 245 spec nr$^-$ which was a nitrate reductase negative mutant of Sp 245[9], Sp 246 spec and *A. amazonense* Am Ytr st; Table 1). The wheat cultivar used was Cocoraque and plot size and fertilizer treatment were as described for Exp. 2.

Exp. 4 was planted in 1984 in a red-yellow podzolic soil at km 47. The sorghum cultivar used was CSH-5 obtained from ICRISAT, India. Two double marked strains were used as inoculant, *A. lipoferum* Sp S82 chl st and Sp 262 chl kan (Table 1). The inoculants were grown for two days on a shaker in 125 ml Erlenmeyer flasks with 70 ml NFb medium containing 1 g.l^{-1} of yeast extract. Each culture was mixed with 330 g neutralized peat granules (1–2 mm)

Table 1. Origin and characteristics of *Azospirillum* strains

Strain No.	Characteristics[a]	Origin
Sp 7 nad st (Sp FP_2)	*A. brasilense* (ATCC 29145) resistant to 15 $\mu g.ml^{-1}$ nalidixic acid and 200 $\mu g.ml^{-1}$ streptomycin	*Digitaria* rhizosphere soil, plants grown in the field, Rio de Janeiro.
Cd	*A. brasilense,* (ATCC 29710) red pigmented	Isolated from pots with *Cynodon* inoculated with Sp 7^{12} (possibly derived from Sp 7 but not confirmed)
Sp 107 st	*A. brasilense* resistant to 150 $\mu g.ml^{-1}$ streptomycin	Chloramine-t treated (15 min) wheat roots grown in pots with soil, Rio de Janeiro
Sp 245	*A. brasilense* resistant to 20 $\mu g.ml^{-1}$ spectinomycin	Chloramine-t treated (5 min) wheat roots grown in the field in a soil planted to wheat for many years in the wheat region, South Brazil
Sp 245 spec	Spontaneous mutant of Sp 245 resistant to 60 $\mu g.ml^{-1}$ spectinomycin	
Sp 245 spec nr^-	Nitrate reductase negative mutant obtained by anaerobic growth in chlorate medium containing NO_2^- as e^- acceptor	
Sp 246 spec.	*A. brasilense* resistant to 60 $\mu g.ml^{-1}$ spectinomycin	Washed wheat roots grown in the field in Parana, the wheat region, South Brazil
Sp 262 chl kan	*A. brasilense* resistant to 25 $\mu g.ml^{-1}$ chloramphenicol and 10 $\mu g.ml^{-1}$ kanamycin	Chloramine-t treated (15 min). *Setaria* roots grown in pots with vermiculite-soil mixture, Rio de Janeiro
Sp S82 chl st	*A. lipoferum* resistant to 10 $\mu g.ml^{-1}$ chloramphenicol and 100 $\mu g.ml^{-1}$ streptomycin	Chloramine-t treated (30 min) sorghum roots grown in the field, Rio de Janeiro
Am Ytr st	*A. amazonense* resistant to 20 $\mu g.ml^{-1}$ streptomycin	Washed wheat roots, grown in the field, Rio de Janeiro

[a] Strains without antibiotic designation were resistant upon isolation. Strains with antibiotic designations are spontaneous mutants obtained in the laboratory (see Materials and methods).

and 15 g applied to the plant holes in each 1 m of row. Plots were 5 × 2 m in size (5 m rows) and a randomized complete block design with 4 replicates was used. The basic fertilizer treatment consisted of one ton of calcitic limestone, 35 kg P, 33 kg K and 40 kg fritted trace elements (FTE Br 12).ha^{-1}. A top dressing of 20 kg N.ha^{-1} was applied 40 days after planting.

Identification of strains and MPN counts

To estimate the antibiotic resistance of the indigenous *Azospirillum* population, pooled soil samples were taken from each block (replicate) in each experiment and used to inoculate (with one loop of soil) 4 vials with semisolid NFb medium containing increasing concentrations of the various antibiotics to be used to label the strains. No *Azospirillum* growth occurred with 100 $\mu g\,ml^{-1}$ streptomycin, 20 $\mu g\,ml^{-1}$ spectinomycin, 10 $\mu g\,ml^{-1}$ chloramphenicol or 10 $\mu g\,ml^{-1}$ kanamycin in any experiment. Spontaneous mutants resistant to at least the indicated levels of these antibiotics were then obtained by the following method: 1 ml of cell suspensions of *A. brasilense* strains Sp 107, Sp 245 and Sp 246 with 10^7-10^9 cells ml^{-1} were spread on potato agar plates[8] containing increasing concentrations of one of the various antibiotics. Single mutant colonies from these plates were streaked out on potato agar containing the same concentration and on plates with higher concentrations of the same antibiotic. Once the resistance level to one antibiotic was established, the mutants were grown up and submitted to a second antibiotic in the same way, for double marking. Before use as field inoculants the antibiotic resistance levels of all strains where rechecked by streaking out on potato agar plates containing both antibiotics. The chloramphenicol resistant mutants of *A. brasilense* Sp 262 and *A. lipoferum* Sp S82 were obtained by successive passages in liquid NFb medium containing 0.5 $g\,l^{-1}$ yeast extract and increasing concentrations of chloramphenicol. Cells from the highest concentrations which still showed growth were then subjected in the same way to kanamycin (Sp 262) or streptomycin (Sp S82). For confirmation the strains were then streaked out on potato agar plates containing both antibiotics. The nad st mutant of strain Sp 7 (ATCC 29145) was obtained by courtesy of Fabio Pedrosa.

For *Azospirillum* counts, samples of rhizosphere soil (soil from between roots), or washed or surface-sterilized roots (1% chloramine-t) were crushed and homogenized in a blender and serial dilutions prepared in saline solution (salts of NFb medium). Three serum vials each with 5 ml semisolid NFb medium were inoculated with 0.1 ml of each dilution. For *A. amazonense* counts and isolation, vials with LGI medium[18] were used.

In experiments 1 and 2 the three highest positive dilution cultures were replicated into NFb vials containing the various antibiotics. Vials from plants inoculated with strain Cd were streaked out on potato agar and strain Cd identified by its red pigment. Samples from non-inoculated control plots were treated in exactly the same manner as samples from inoculated plants except that identification tests for all strains used in the same experiment were applied.

In Exp. 3, instead of serial dilutions, crushed, 1 cm long, root pieces were used to prepare enrichment cultures. These were streaked out on plates with NFb medium (+ 20 mg yeast extract.l^{-1}) and single colonies inoculated into vials with semisolid NFb medium. All vials with a typical *Azospirillum* pellicle were then replicated into the respective antibiotic-containing NFb vials and the % of resistant *Azospirillum* isolates determined. In contrast to the procedure with MPN vials described in experiments 1 and 2, where theoretically within the dilution flasks any number between one and 10 inoculated bacteria could have been present together with 0 to 9 other *Azospirillum* cells, this enrichment culture method tested individual isolates. It must be presumed however that in the enrichment medium, the inoculated *Azospirillum* strains grow with growth rates similar to those of the *Azospirillum* strains from soil. Neither method is perfect but the good agreement between the two and the pronounced reproducible differences between treatments are good evidence that the results are meaningful.

In Exp. 4 roots were carefully extracted from the field and all intact roots either washed or washed and surface sterilized in 1% chloramine-t for the times stated. Both ends of the washed roots were dipped into molten paraffin wax to seal them. The surface-sterilized roots were dipped into ethanol then flamed, and also capped with paraffin wax on both ends. Root-surface bacteria were obtained by placing the intact, washed, paraffin-capped roots into large test tubes containing 80 ml semisolid NFb medium. To obtain *Azospirillum* from the inside of roots, the capped sterilized intact roots were kept for three days in large test tubes with NFb and all tubes with *Azospirillum* growth discarded. Roots from tubes showing no *Azospirillum* growth were either crushed within the tubes or cut aseptically into 1 cm long pieces and crushed into small NFb vials. Identification of antibiotic resistance of single colony isolates was made as described in Exp. 3.

Results

Wheat experiments

In Exp. 1, carried out in Parana, South Brazil, total N incorporation into the straw and grain was increased significantly by 28 and 17%, respectively, with strain Sp 245 in the plots with 15 kg N ha^{-1}, but there was no inoculation response at the higher fertilizer application (60 kg N ha^{-1}) (Table 2). It can not be established at present, however, whether

Table 2. Effect of inoculation with various *A. brasilense* strains on total N incorporation of field-grown wheat at two levels of fertilizer nitrogen

	Straw		Grain	
Inoculant strain	15 kg N*	60 kg N	15 kg N	60 kg N
Non-inoculated	16.0	20.9	40.9	48.6
Cd	14.9	19.1	41.4	46.7
Sp 107 st	17.9	18.9	44.6	43.6
Sp 245	20.5	20.4	48.0	47.9
D.M.S. $p = 0.05$	3.7		4.9	

*Fertilizer nitrogen given at rates of 15 and 60 kg N.ha^{-1}. Results given as kg N.ha^{-1}.

these effects were derived from associative N_2 fixation or from more efficient use of small amounts of combined N at relatively low levels of fertilizer application as suggested by the data of Sarig *et al.*[27] for sorghum and Boddey *et al.*[5] for wheat. The highest efficiency of strain Sp 245 followed by that of strain Sp 107st is in accordance with results obtained in previous field experiments with wheat[3] where a significant correlation ($r = 0.92^{**}$) was also obtained between the numbers of *Azospirillum* cells inside wheat roots and the total plant N.

For this reason we attempted in this experiment to identify the inoculated strains in the rhizosphere soil and in roots by their antibiotic resistance or red pigment (Table 3). The results indicate differences in the ability of different strains to colonize roots: whereas in the rhizosphere soil each inoculated strain represented about half of the *Azospirillum* population, strains Sp 245 and Sp 107st predominated in both the surface and internal parts of the roots. Practically no cross-contamination with any of the inoculated strains was observed in the control plots. Also, very few indigenous *Azospirillum* strains with antibiotic resistance or red colonies which could be mistaken for the inoculated strains, were encountered (Table 3).

Results confirming the establishment of strain Sp 245 within roots of 8 wheat cultivars are presented in Table 4. As in experiment 1, few spectinomycin-resistant azospirilla occurred in the control plots while

Table 3. Establishment of antibiotic resistant *Azospirillum brasilense* strains in soil and roots of field grown wheat

Inoculant strain	Soil	Washed roots	Surface sterilized roots[a]
Non-inoculated	1[b]	0	5
Cd	61 ± 10	29 ± 8	11 ± 5
Sp 107 st	54 ± 7	67 ± 5	82 ± 3
Sp 245	44 ± 12	62 ± 7	76 ± 5

[a] Roots exposed 15 min to 1% chloramine-t

[b] Values give percentage of cultures identified as inoculated strain. Percentage calculated from 18 cultures obtained from the highest positive dilutions (MPN counts) of soil or root homogenates. Values are means of 2 harvests and 2 fertilizer levels with standard deviations of means.

Table 4. Establishment of *A. brasilense* strain Sp 245 in 8 field grown wheat cultivars

	log no.g^{-1} fresh wt		% inoculant strain[a]	
	Control	Inoc	Control	Inoc.
Rhizosphere soil	5.30 ± 0.04	5.71 ± 0.15	2.0 ± 1.3	51 ± 6
Washed roots	6.40 ± 0.22	7.12 ± 0.16	2.0 ± 1.3	72 ± 5
Surface ster. roots	3.16 ± 0.18	3.74 ± 0.19	0	84 ± 3

[a] % of the highest positive dilution vials containing spectinomycin (20 μg.ml^{-1}) resistant *Azospirillum*. Values are means of 12 vials each of 8 cultivars with standard deviations of means.

strain Sp 245 could be identified in 72 and 84% of the highest dilution vials from washed and surface-sterilized roots, respectively (Table 4).

In order to compare the establishment of various other *Azospirillum* strains in washed and surface sterilized wheat roots, the third field experiment was performed in 1984, at km 47. There the actual percentage of cells of the inoculated strains within the total *Azospirillum* population was estimated (Table 5). Again strain Sp 245 spec showed preferential establishment. A nitrate reductase negative mutant (Sp 245 spec nr$^-$)[9] became established on washed roots but was absent within roots. Sp 246 spec, a strain isolated from washed roots, was predominant on washed roots but not in surface-sterilized roots (Table 5).

Strain Sp 7 nad st and also one *A. amazonense* strain (Am Yt st) were less readily isolated from the inside of roots.

Maize and sorghum experiments

In a preliminary experiment with maize in a red-yellow latosol in the cerrado region near Brasilia significant N yield increase had been observed in the summer of 1983 with two of five *A. lipoferum* strains but attempts to identify inoculant strains in a second experiment in winter 1984 failed almost completely (J.F.W. van Bülow and J.I. Baldini, personal communication).

Table 5. Establishment of various *Azospirillum* strains in field grown wheat

Strains[a]	Washed roots		Surface sterilized roots	
	No.	% of isolates inoc. strain[b]	No.	% of isolates inoc. strain
Control[c] S	16	0	12	0
Sp 245 spec	16	100	12	67
Sp 245 spec nr⁻	16	81	8	0
Sp 246 spec	16	94	11	27
Sp 7 nad st	16	50	8	0
Am Y Tr st	16	56	9	11

[a] Origin and antibiotic resistance level see Table 1.
[b] Enrichment cultures of surface sterilized crushed root pieces used for single colony isolations where then identified by their antibiotic marker.
[c] Control isolates tested for all antibiotics.

Attempts were made to improve the methodology to evaluate establishment of inoculated strains within maize roots. The time necessary for the complete elimination of *Azospirillum* from the root surface varies with plant species, age and several other factors. For maize roots at flowering stage, 60 min. exposure to chloramine-t was necessary[2]. Even then, after crushing the roots, *Azospirillum* was recovered. Similar observations were reported by Patriquin and Döbereiner[21].

In Exp. 4 the data from surface sterilized roots capped with paraffin wax at both ends (Table 6) show that 68–78% of the sorghum roots after 15 min. sterilization did not contain *Azospirillum* on the surface. When crushed, 100 and 73% of these roots, respectively, showed growth of the two inoculated strains (Table 6).

Using this method, we examined in more detail the root system of several sorghum plants inoculated with strain Sp S82 chl st (Exp. 4). Initial observations were made to determine the localization of the inoculated strains. For this, crushed 1 cm pieces of surface sterile roots (*Azospirillum* free) were incubated in semisolid NFb vials containing the respective antibiotics. Fig. 1 is a schematic representation of the presence of strain Sp S82 chl st along the root system. For a second evaluation root pieces from washed and surface-sterilized (*Azospirillum*-free) roots were crushed and incubated in NFb medium without antibiotics and then used for single colony isolations which only then were identified by their antibiotic resistance. The distribution of *Azospirillum* was similar to that displayed schematically in Fig. 1 and is quantified in Table 7.

Two observations arise from Fig. 1 and Table 7: (a) with few exceptions all infections were continuous and (b) infections were more frequent in the upper part of the root system than in the extremities.

Table 6. Establishment of *A. lipoferum* within sorghum roots

Strain	Origin	Washed roots		Roots exposed to chloramine-t			
				7 min		15 min	
		No.	% ster. on surf.	No.	% ster. on surf.[b]	No.	% ster. on surf.[b]
Sp S82 chl st	Sorghum roots	42	0	42	19	41	68
Sp 262 chl kan	Setaria roots	36	12	48	48	47	78
		No.	% roots inoc. str[a].	No.	% roots inoc. str.	No.	% roots inoc. str.
Sp S82 chl st	Sorghum roots	42	19	8	87	28	100
Sp 262 chl kan	Setaria roots	36	48	23	65	37	73

[a] % roots containing the inoculated strain as verified by growth of crushed roots in semisolid NFb medium with antibiotics (see Table 1).
[b] *Azospirillum*-free as verified after 3 days pre-incubation of paraffin capped roots in NFb medium.

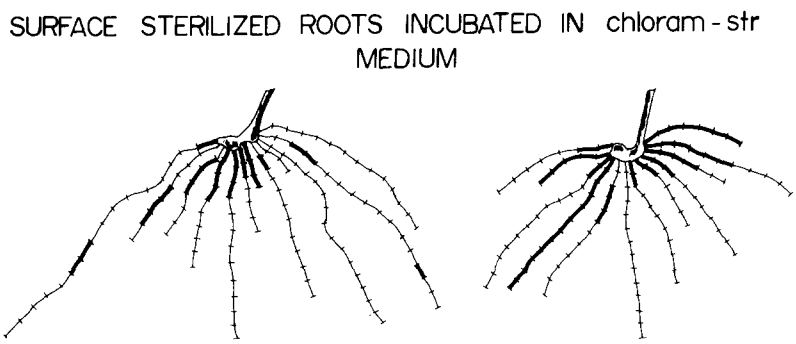

Fig. 1. Localization of *A. lipoferum* strain Sp S82 chl st in field grown sorghum roots. Sorghum roots inoculated with *A. lipoferum* (strain Sp S82 chl st) were surface sterilized (15 min.) capped with paraffin wax on both ends and pre-incubated in NFb medium. Roots which showed no *Azospirillum* growth were cut aseptically into small pieces from root tip to top, numbered and crushed into small NFb vials containing chloramphenicol and streptomycin. Root pieces marked black on the figure indicate growth in this medium.

An evaluation of the proportion of the inoculated strain by identification of single colony isolates according to their antibiotic resistance (Table 1) is also given in Table 7. As in the case of wheat (Table 5), the majority of the *Azospirillum* population in washed and surface-sterilized roots consisted of the inoculated strain (*A. lipoferum* Sp S82 chl st).

Table 7. Distribution of *Azospirillum* spp. in field grown sorghum root systems and identification of the inoculated strain

	Washed roots	Surface sterilized roots[a]
No. of roots examined	28	37
No. of *Azospirillum* negative roots	0	5
% upper root pieces with *Azospirillum*[b]	93	72
% lower root pieces with *Azospirillum*	71	38
% of positive root pieces containing Sp S82 chl st	62	75
% isolates identified as Sp S82 chl st	72	72

[a] 15 min. in chloramine-t 1%, *Azospirillum* free surface
[b] 5 upper root pieces approximately 1 cm each.

Discussion

The two methods used in this paper for identification of inoculated strains both based on spontaneous antibiotic-resistant mutants have proved convenient and satisfactory. In no case was there any significant occurrence of *Azospirillum* with antibiotic resistance in the uninoculated control plots which could compromise the results. The same was true for the red colonies of strain Cd. All marker characters were stable during the experiment which is best seen by the large proportion of strains which could be identified after re-isolation. Results with the two methods were comparable and therefore the much easier identification in MPN vials (Exps. 1 and 2) should be preferred even though it does not permit the expression of data in percentages of individual isolates. In most cases comparisons between treatments seem more important than exact percentages.

Diazotrophic rhizocoenoses as defined during the First International Symposium on Associative N_2 Fixation[31] require bilateral interactions of both partners. Host-bacterial associations have been most extensively studied and defined by plant pathologists. Holl[15] and Wilson[32] considered such associations 'beneficial' plant diseases. Within these lines the alternatives of virulence/avirulence and specificity should be expected[15]. True specificity so far has been shown only for two associations of grasses with N_2 fixing bacteria, that of a *Bacillus* sp. with certain wheat lines[24] and that of *Azotobacter paspali* with one cultivar of *Paspalum notatum*[7]. Other more or less restricted associations may therefore be better defined as host plant affinities (P. Vose personal communication). The term host plant specificity in earlier work[4,26] has been used in comparison with the legume – *Rhizobium* symbiosis where the term specificity is used in broader terms than in plant pathology.

As in the *Rhizobium* – legume symbiosis, the success of inoculation practices in cereals under field conditions will depend on two pre-requisites: (a) the selection of strains which are superior to those already present in the soil, and (b) the establishment and multiplication of the selected strains in the rhizophere and in roots. Non-specific legumes like *Phaseolus atropurpureus* and *Glycine wightii* which are nodulated by the 'cowpea miscellany' formed by *Bradyrhizobium* produced, in Australia, less than 5% of the nodules with the inoculated strain[6]. With more specific legumes, like soybeans, 80–100% of the nodules can be formed by the inoculated strain when this species is planted for the first time[30]. During consecutive plantings establishment of the inoculated strain will be dependent on its 'competitiveness' which, in turn, depends on soil, climatic factors and plant cultivar.

With all these problems in mind it seems surprising that selected *Azospirillum* strains could be established on and in roots in four of five field experiments although the organism occurred in these soils in much higher numbers ($> 10^4\ g^{-1}$) than *Rhizobium* is usually found. The failure to establish inoculated *Azospirillum* in maize experiments in Brasilia (data not shown) indicates the need for selection of distinct strains for these regions.

The fact that selected strains can become predominant on and in field-grown cereal roots opens a large field of possibilities for genetic manipulation of associative N_2 fixation. As a first step it should now be possible, by the use of *nif* negative, nitrate reductase negative and hormone deficient mutants to identify the role of *Azospirillum* in promoting plant responses. In the experiments described here and also in a previous experiment[3] the inoculation of wheat with strains Sp 107st and Sp 245 gave proportionally larger increases in total plant N than in dry matter yields and therefore indicates effects on nitrogen nutrition. In a third experiment carried out in lysimeters with the same strains Boddey *et al.*[5] using ^{15}N labelled fertilizer showed that strains Sp 107st and Sp 245 did not fix N_2 but in fact contributed most by a higher recovery of nitrogen fertilizer. In this experiment C_2H_2 reduction activity was negligible and strain Sp7 showed similar effects to the other two strains.

The preferential establishment of *Azospirillum* within the upper parts of the roots (Fig. 1 and Table 7) and the continuous nature of the infection of the roots seems to indicate longitudinal proliferation within the sorghum roots as observed previously for maize[21]. The upper 4 cm of *Digitaria* roots were shown to have the highest nitrogenase activities[11] and this was interpreted as a requirement of the bacteria for medium-aged roots with many laterals which favour infection.

It is evident that the results of this paper open more questions than are answered showing the urgent need for better understanding of the mechanism of *Azospirillum*/Gramineae associations.

Acknowledgements

Financial support for this research was provided by the U.S. National Academy of Sciences/ National Research Council by means of a grant from the U.S. Agency for International Development.

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