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Attachment, colonization and proliferation of *Azospirillum brasilense* and *Enterobacter* spp. on root surface of grasses

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Root colonization studies, employing immunofluorescence and using locally isolated strains, showed that *Enterbacter* sp. QH7 and *Enterobacter agglomerans* AX12 attached more readily to the roots of most plants compared with *Azospirillum brasilense* JM82. Heat treatment of either root or inoculum significantly decreased the adsorption of bacteria to the root surface. Kallar grass and rice root exudates sustained the growth of *A. brasilense* JM82, *Enterobacter* sp. QH7 and *E. agglomerans* AX12 in Hoagland and Fahraeus medium. All the strains colonized kallar grass and rice roots in an axenic culture system. However, in studies involving mixed cultures, *A. brasilense* JM82 was inhibited by *Enterobacter* sp. QH7 in kallar grass rhizosphere and the simultaneous presence of *Enterobacter* sp. QH7 and *E. agglomerans* AX12 suppressed the growth of *A. brasilense* JM82 in rice rhizosphere. The bacterial colonization pattern changed from dispersed to aggregated within 3 days of inoculation. The colonization sites corresponded mainly to the areas where root mucigel was present. The area around the point of emergence of lateral roots usually showed maximum colonization.

Key words: *Atriplex amnicola*, *Azospirillum brasilense*, colonization, diazotrophs, *Enterobacter agglomerans*, *Enterobacter* sp., *Phalarics minor*, *Suaeda fructicosa*.

The population of bacteria is greater in soil immediately around the root (rhizophere) than in soil further from the root (Newman 1985). Healthy roots exude a wide range of soluble organic substrates, including sugars, organic acids and amino acids (Rovira 1969), that become substrates for the microorganisms. Generally, a variety of microorganisms are present on the root surface (Bowen & Rovira 1976). However, N_2-fixing bacteria form an integral part of the bacterial population of rhizospheric organisms because of their property of N_2-fixation (Shimshick & Herbert 1979).

Kallar grass (*Leptochloa fusca* L. Kunth) is a salt-tolerant grass which can grow in very low fertility and saline environments. Root-associated N_2-fixation has been reported in this grass (Malik *et al.* 1980) and a variety of diazotrophs have been isolated from its roots and identified (Bilal & Malik 1987; Reinhold *et al.* 1987; Zafar *et al.* 1987). The use of the ^{15}N-isotope dilution technique has demonstrated agronomically significant inputs due to associative N_2-fixation in this grass (Malik *et al.* 1987; Malik & Bilal 1989).

The objectives of this study were to elucidate the specificity, attachment, survival, proliferation and colonization of some diazotrophs in the rhizosphere of various plants, with special emphasis on kallar grass. Studies were carried out using pure and mixed cultures under axenic conditions of plant growth, either in a hydroponic culture system or on Fahraeus slides. Specific fluorescent antibody stains, prepared for each strain, were used in these studies.

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Materials and Methods

Bacterial Strains

Azospirillum brasilense JM82 was obtained from Dr Hubbell, University of Florida, USA. The other diazotrophic strains, *Enterobacter* sp. QH7 and *E. agglomerans* AX12, were isolated from the roots of *Atriplex* spp., Kallar grass, wheat and rice (Bilal *et al.* 1990; Malik *et al.* 1991).

Inoculum Preparation

All the strains were grown in 250-ml Erlenmeyer flasks containing 100 ml of modified Okon's medium (Okon *et al.* 1977). The cultures at the end of growth were harvested by centrifugation and

resuspended in phosphate buffer (pH 7) to give 10^8 cells/ml. In some experiments, a mixture of two or more strains (in equal volume) was used while in others the suspension was heat-treated. For heat-treatment, the cultures were immersed in a boiling-water bath for 1 h and were used when cool.

Preparation of Fluorescent Antibody Stains

Strain-specific fluorescent antibody (FA) stains were prepared as detailed by Somasegaran & Hoben (1985) with some modifications. All strains were grown on the N-free medium of Okon *et al.* (1977) except that 1.0 g NH_4Cl and 5.0 g sucrose were added per litre. Antibodies were prepared against all the three strains in young adult female rabbits by multiple injection of heat-killed whole cell suspensions (Freund's incomplete adjuvant and washed cells; 1:1 v/v). Injections were administered subcutaneously, intravenously and intramuscularly.

After 25 to 30 days, blood samples were taken from the marginal ear vein for titre determination by the tube agglutination method (Somasegaran & Hoben 1985). The animals with appropriate titre (> 1280) were then bled by cardiac puncture. Blood was permitted to clot and the antiserum was decanted. Separation of immunoglobulins and conjugation with fluorescein isothiocyanate, Isomer I (BBL, Cockesville, MD), was carried out in carbonate/bicarbonate buffer instead of phosphate. The unconjugated dye was separated on a Sephadex G-25 column using phosphate buffered saline (PBS) as eluant. The quality and specificity of the fluorescent antibody (FA) was determined using the controls described by Schmidt (1974). Pre-immune serum, processed in the say way, was used as control.

Root Staining and Observation

Root pieces or the entire root length were covered with gelatine/rhodamine/isothiocyanate conjugate to minimize non-specific binding, as described by Bohlool & Schmidt (1968). The roots were subsequently covered with a few drops of specific FA stain and incubated for 30 min in a moist chamber. Excess FA stain was removed by washing in PBS. The roots were then counterstained to suppress their autofluorescence, using 0.01% (w/v) Crystal Violet solution for 1 to 2 min. Excess crystal violet stain was removed by washing in PBS (usually overnight). The roots were then observed after mounting in PBS glycerol mounting fluid using a Zeiss microscope with epifluorescence ultraviolet lighting from a mercury vapour light source and an FITC filter pack system. Controls against non-specific reactions were run using pre-immune serum FA.

Seed Germination

Kallar grass (*Leptochloa fusca*), rice (*Oryza sativa*), *Atriplex amnicola*, *Phalaris minor* and *Suaeda fruticosa* were surface-disinfected with 5% (w/v) sodium hypochlorite for 30 min and 0.1% (w/v) $HgCl_2$ for 30 s and then usually germinated on water agar plates.

Determination of Adsorption/Attachment

For the adsorption assay, seeds of *A. amnicola*, *P. minor*, *S. fruticosa*, *L. fusca* and *O. sativa* were germinated in moist autoclaved sand. Seedlings, 2 to 3 cm in length, were washed with tap water to remove sand particles. The root samples were then placed in Petri plates containing 5 ml of bacterial suspension. Sets of roots in triplicate were taken after 5, 10, 30 and 90 min and washed in PBS to remove unadsorbed bacteria. Numbers of adsorbed bacteria per microscopic field were rated as follows: +4, abundant (more than 100 bacteria/field); +3, 50 to 100 bacteria/field; +2, 10 to 50 bacteria/field; +1, five to 10 bacterial/field and 0, none.

Site and Pattern of Colonization

Long, glass tube assemblies (4 cm internal diameter × 44 cm length) were used when plants were grown for more than 2 weeks under aseptic condition. The tubes contained a 3.9 cm diameter Perspex disc with two holes, supported on a glass rod. The rod, along with the disc, was removable. The rod was taken out and Kallar grass seedlings were anchored through the disc holes. The anchored seedlings on the disc were then transferred to the tubes containing 30 ml, N-free, half-strength Hoagland nutrient solution. The tubes were incubated at 30°C (day) and 26°C (night) with a day length of 16 h and light intensity of 400 $\mu E.m^{-2}.s$. One week after establishment of the plants, 0.1 ml (1.5×10^8 to 2×10^8 cells/ml) inoculum of *A. brasilense* JM82, *Enterobacter* sp. QH7 or *E. agglomerans* AX12 was added to each tube. Three tubes were used for every treatment. Samples were taken after 3 weeks.

In another experiment, for detailed study of the phenomenon, *A. brasilense* JM82 was inoculated onto Kallar grass in a similar manner. Seminal root samples were taken at different intervals over the next 9 days. The length of the seminal root and the number of lateral roots on it were recorded. The roots were stained with FA and the colonization pattern on the main and lateral roots was observed. The observed fields were then grouped according to the number of bacteria per field. Inoculated unplanted and uninoculated plant roots, stained with a mixture of FA, were used as controls.

Proliferation and Extent of Colonization

To study the proliferation of inoculated diazotrophic bacteria in the plant rhizosphere, the Fahraeus system as described by Somesagaran & Hoben (1985) was used. For this purpose, 48-h-old Kallar grass seedlings were soaked in inoculum (1.5×10^8 to 2×10^8 cells/ml) of *Enterobacter* sp. QH7, *E. agglomerans* AX12, or *A. brasilense* JM82 for 30 min, gently washed in PBS and then transferred to Fahraeus slides. Incubation conditions were the same as those for the long glass assemblies. Fahraeus slides were observed after various intervals over the next week. Roots were removed from the agar and observed after staining. The agar on the slides was dried in an oven at 70°C and stained with respective FA. The stained root imprint on the agar was observed to determine the gradient, both laterally and vertically, of bacteria around the root. Uninoculated plant root slides, stained in a similar manner with a mixture of FA stains, were used as controls.

Competition

Kallar grass and rice were used to study probable competition among the different diazotrophs, *A. brasilense* JM82, *E. agglomerans* AX12 and *Enterobacter* sp. QH7, to colonize grass roots. Seedling roots were soaked in a mixed inoculum of two or three strains for 30 min, transferred to a Fahraeus slide after gentle washing in PBS and then observed 6 days after inoculation.

Results

Attachment

Enterobacter sp. QH7, *E. agglomerans* AX12 and *A. brasilense* JM82 adhered to all the plant roots included in the study (Table 1). *Enterobacter* sp. QH7 and *E. agglomerans* AX12 attached more readily than *A. brasilense* JM82 during the first 10 min. *Azospirillum brasilense* JM82, on the other hand, showed an increased attachment with increase in time of soaking. A difference in the ability of bacteria to adsorb to different plant roots was also observed. For example, *A. brasilense* JM82 attached more readily to *S. fruticosa* roots

Table 1. Attachment of *Enterobacter* sp. QH7, *E. agglomerans* AX12 and *A. brasilense* JM82 to the roots of various plants after 5, 10, 30 and 90 min*.

Plant	*Enterobacter* sp. QH7				*E. agglomerans* AX12				*A. brasilense* JM82			
	5	10	30	90	5	10	30	90	5	10	30	90
A. amnicola	+3	+4	+4	+4	+3	+4	+4	+4	+2	+3	+3	+4
Rice	+3	+4	+4	+4	+4	+4	+4	+4	+1	+1	+3	+4
Kallar grass	+3	+4	+4	+4	+1	+3	+3	+4	+1	+2	+3	+3
P. minor	+3	+4	+4	+4	+3	+3	+4	+4	+2	+3	+4	+4
S. fructicosa	+1	+2	+3	+4	+3	+3	+4	+4	+3	+3	+4	+4

* The times are those for which roots were soaked in inoculum before staining and observation.
+4—abundant bacteria/field (more than 100); +3—comparatively less abundant (50 to 100/field); +2—countable (10 to 50/field); +1—5 to 10/field.

than to the roots of other plants (Table 1), whereas *Enterobacter* sp. QH7 attached readily to all the plant roots studied except to those of *S. fructicosa*, where the attachment was slow but increased with time. The attachment of *E. agglomerans* AX12 was equally good for all the plants studied.

Effect of Heating on Attachment

When either the bacterial inoculum or the roots were heat-treated prior to the adsorption assay, the attachment profile changed (Table 2). When roots were heated, *Enterobacter* sp. QH7 showed poor adsorption to all the plant roots, whereas *E. agglomerans* AX12 and *A. brasilense* JM82 showed better attachment to Kallar grass and rice than *Atriplex*. Conversely, when heated culture and unheated roots were used, *Enterobacter* sp. QH7 attached to *Atriplex* roots better than to rice and Kallar grass, whereas both *E. agglomerans* AX12 and *A. brasilense* JM82 showed moderate attachment (Table 2).

Site and Pattern of Colonization

Enterobacter sp. QH7, *E. agglomerans* AX12 and *A. brasilense* JM82 were abundant on kallar grass roots when studied in glass tube assemblies (Figure 1). Detailed observations regarding colonization by *A. brasilense* JM82 are presented in Table 3. On the first day after inoculation, nearly 90% of the root length was colonized but only five to 10 bacteria per field could be observed. On days 2 and 3, the bacteria showed a tendency to produce microcolonies; 12% to 20% of the observed fields contained more than 50 bacteria per field while 12% and 27% were uncolonized after 2 and 3 days, respectively. By day 6 50% of the fields were free of bacteria. The bacterial colonization pattern changed from scattered to aggregated within 9 days. However, maximum colonization was observed on day 3 and this was consistent with the results for other strains (data not shown).

The roots stained immediately after removal from the Fahraeus slide assemblies after 24 h showed that bacteria were present, not only on the root surface but formed a

Table 2. Effect of heating of bacteria and/or plant roots on their attachment to each other.

Bacteria	Unheated seedlings			Heated seedlings*		
	Kallar grass	Rice	*Atriplex*	Kallar grass	Rice	*Atriplex*
Unheated						
Enterobacter sp. QH7	+4	+4	+4	+1	+1	+1
E. agglomerans AX12	+4	+4	+4	+3	+1	+1
A. brasilense JM82	+3	+4	+4	+2	+2	+1
Heated*						
Enterobacter sp. QH7	+1	+2	+4	0	0	0
E. agglomerans AX12	+2	+2	+2	+1	0	0
A. brasilense JM82	+1	+2	+1	+1	0	0

* Boiled in a water bath for 1 h.
+4—abundant (>100 bacteria/field); +3—less abundant (50 to 100/field); +2—countable (10 to 50/field); +1—5 to 10/field; 0—none.

Figure 1. Colonization of kallar grass root by various diazotrophic strains grown in long glass tube assemblies for 3 weeks. Microphotographs show stained (a) *Enterobacter* sp. QH7 and (b) *E. agglomerans* AX12 colonizing the root hair on day 6. Scale bar—10 μm.

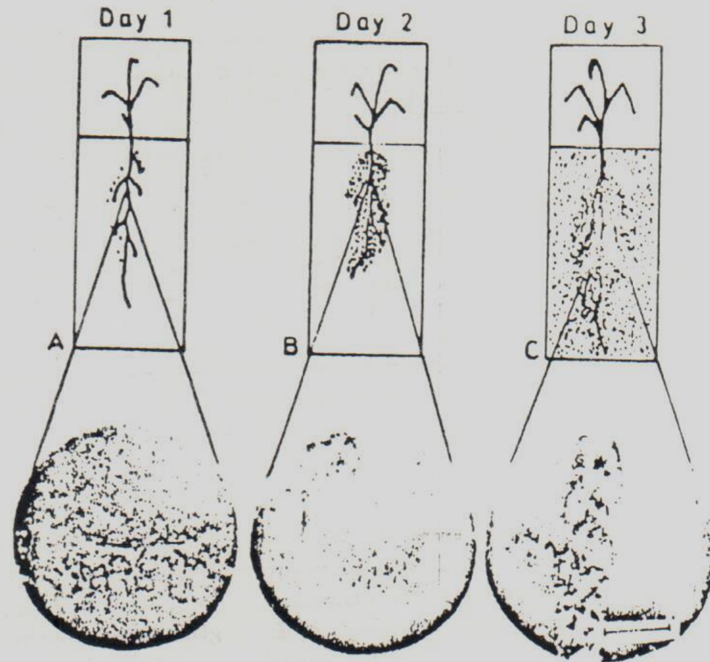

Figure 2. Schematic diagram of the pattern of colonization and proliferation of *Enterobacter* sp. QH7 in Fahraeus slide assemblies, showing (A) the bacteria attached to the root surface, (B) the observed gradient of proliferating bacteria indicating the diffusion of root exudates in agar and (C) the entire slide covered with bacteria. The diagram is based on microscopic observation made after specific FA staining of the roots and Fahraeus slide agar. Scale bar—20 μm.

Figure 3. Proliferation of diazotrophs utilizing kallar grass root exudates in the Fahraeus slide system. The microphotograph shows a typical concentration gradient of stained cells away from the root on day 2 of *E. agglomerans* AX12 growth. Similar observations were also made with the other two bacterial strains. Scale bar—10 μm.

gradient, 7 to 10 mm thick extending from the lateral roots. The root tip and the adjoining regions on the slide were devoid of bacteria on the first day. On day 2, the bacterial proliferation showed an increase as measured by lateral distance on the slide (Table 4). However, the bacteria were found all around the tip region as well, and extended vertically up to 17 mm from the tip (Figure 2). The entire slide was covered with bacteria on day 3, and the results were consistent for all the diazotrophs studied. Figure 3 shows an example of the gradient of bacteria (*E. agglomerans* AX12) which originated from the root. Similar observations were made for all the strains studied.

Specificity/Competition

When used as axenic/pure culture inocula, all the three bacterial strains colonized kallar grass and rice roots. However, when a mixture of two or more strains was used, the colonization pattern changed and the results were different for rice and kallar grass (Table 5). When inoculated together, *A. brasilense* JM82 and *Enterobacter* sp. QH7 colonized rice roots simultaneously, whereas *A. brasilense* JM82 was a poor competitor for kallar grass roots in the presence of *Enterobacter* sp. QH7. In the other treatment, where *E. agglomerans* AX12 plus *A. brasilense* JM82 inocula were given, both the strains were found on both types of plant roots. In the third treatment (*E. agglomerans* AX12 plus *A. brasilense* JM82 plus *Enterobacter* sp. QH7), all three strains colonized kallar grass roots simultaneously, while *A. brasilense* JM82 failed to co-exist with *Enterobacter* sp. QH7 and *E. agglomerans* AX12 on the rice roots.

Table 3. Colonization of kallar grass roots by *A. brasilense* JM82 studied in long glass tube assemblies using specific FA stain.

Day	No. observed fields	Length observed (mm)	Length of main root (mm)	No. of laterals	Bacteria/field (% distribution)			
					0	5 to 10	20 to 50	> 50
1	450	126	25	10	10	80	10	0
2	477	134	14	15	12	55	25	12
3	851	238	34	15	27	44	10	20
6	1072	300	46	21	48	33	14	5
9	1118	313	32	19	50	36	13	3

Table 4. Proliferation of inoculated bacteria on kallar grass root exudates studied in Fahraeus slide system using FA staining.

Days after inoculation	Strain*	Thickness of bacterial gradient (mm)	
		Lateral†	Vertical‡
1	QH7	7 to 9	0
	JM82	8 to 10	0
	AX12	7 to 9	0
2	QH7	15	16
	JM82	16	17
	AX12	15	16
6	QH7	15	10
	JM82	16	10
	AX12	15	10

* QH7- *Enterobacter* sp., JM82—*A. brasilense*: AX12—*E. agglomerans*.

† Distance measured laterally along the width of the slide.

‡ Distance from the root tip along the length of the slide.

Discussion

Attachment of bacteria to roots is an important prelude to long-term colonization. There are reports that infective *Rhizobium* strains adsorb in higher numbers to the roots than non-infective strains (Dazzo *et al.* 1976). Similarly, more cells of *A. brasilense* sp. 7 than of *A. brasilense* sp 245 attached to wheat roots and attachment of the former increased with time in contrast to the latter. In the present study, the adsorption assay with two *Enterobacter*, strains QH7 and AX12, indicated that both adsorbed quickly to the roots of all the plants studied except those of *S. fructicosa*, where adsorption increased with time. Adsorption assays with *A. brasilense* JM82 also showed a similar trend (Table 1).

Evidence is available that pili and fimbriae mediate the attachment of bacteria to plant roots (Danguid 1959; Haahtela *et al.* 1985; Korhonen *et al.* 1983, 1986). Cellulose fibrils and Ca^{2+}-dependant adhesin, a cell-surface protein, are also implicated (Smit *et al.* 1987). If these structures and molecules are non-specific and responsible for attachment, then there should be no difference in adsorption of bacteria on different plant roots. However, during the present studies, a large difference was observed in adsorption of *Enterobacter* sp. QH7, *E. agglomerans* AX12 and *A. brasilense* JM82 to the different plant roots (Table 1).

There are some reports that plant roots harbour receptor sites (Korhonen *et al.* 1986). Moreover, specificity in the bacterial inhibition of haemagglutination by certain

Table 5. Competition among various diazotrophic strains to colonize Kallar grass and rice (*Oryza sativa*) roots studied in the Fahraeus slide system using strain-specific fluorescent antibody (FA) stains.

Inoculum	*A. brasilense* JM82		*Enterobacter* sp QH7		*E. agglomerans* AX-12	
	Kallar grass	Rice	Kallar grass	Rice	Kallar grass	Rice
JM82 + QH7	0	+4	+4	+2	HET	HET
JM82 + AX12	+4	+4	HET	HET	+4	+4
JM82 + QH7 + AX12	+2	0	+4	+4	+4	+4

HET—heterologous FA; +4—abundant homologous bacteria; +2—less abundant; 0—no homologous cell observed.

carbohydrates (Isaacson 1985) may suggest lectin-like sites on pili which may be involved in the attachment to the root surface. Some studies appear to disprove this lectin theory (Badenoch-Jones *et al.* 1985). However, work reported by Gafny *et al.* (1986) indicated the participation of corn root protein in binding of *Azospirillum*.

During the present investigation, when heat-treatment was given either to bacteria or to the roots, adsorption decreased, maximum adsorption was observed with untreated roots and bacteria (Table 2). Data presented revealed that factors responsible for adsorption are present on the bacteria and are heat labile. Heat-treatment to seedlings also reduced attachment, indicating some role for the plants and this was supported by the adsorption of heat-treated bacteria only to untreated seedlings and not to treated ones. Plant and bacteria both have a necessary role in the maximal adsorption.

Colonization was mostly on the root hair, but colonization of the main root surface was also observed. The proliferation of diazotrophs in the glass tubes and Fahraeus slides showed that all three strains tested (AX12, QH7 and JM82) were utilizing kallar grass root exudates. *Azospirillum brasilense* JM82 colonized the kallar grass roots in higher numbers than *E. agglomerans* AX12. A patchy pattern of colonization was observed with all the diazotrophs assayed and was probably due to the distribution of receptor structures on the plant root surface (Korhonen *et al.* 1986), relating to sites of greater root exudation. The change in root colonization pattern from scattered to microcolony formation and the observation of bacteria first in 90% and later in only 50% of microscope fields suggests that the diazotrophs utilize root exudate rather than root lysate (Newman 1985). If lysate were used colonization would have increased with increased root cortical senescence.

Root mucilage is rich in polysaccharides which also provide carbon substrates. Colonization of the lateral root mucigel was due to this substrate (Figure 1). Similar observations have also been made by Schank *et al.* (1979) for tropical grasses and Umali-Garcia *et al.* (1980) for pearl millet and guinea grass. However, the studies indicated no apparent specificity of colonization, since the isolates used to inoculate kallar grass were isolated from other grasses. Lack of specificity of colonization in grass bacteria has been observed previously (Haahtela *et al.* 1986). The difference in colonization by different bacteria might be related to the site of exudation and availability of root lysate (in some cases). Although much work related to re-inoculation of diazotrophs has been done on grass rhizosphere, our knowledge about colonization of roots is still not complete. Information is available about the role of motility, chemotaxis, carbohydrate utilization and the presence of binding and receptor structures but little is known about the mechanism of initial attachment of bacteria to the root surface. There are probably specific sites on the root where colonization is initiated. Understanding this phenomenon will have practical implication for developing inoculants of N_2-fixing, root-colonizing bacteria for different plants.

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