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Relationship between Root Colonization and Initial Adsorption of *Azospirillum* to Plant Roots

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Abstract. Four strains of *Azospirillum* were ranked according to numbers of cells adsorbed on the roots of seedlings in liquid medium, and the rankings were evaluated for their usefulness in predicting success of colonization of the roots of pot-grown plants.

Different rankings were observed on different parts of the roots and on different host plants. Rhizosphere colonization results for rice were similar to those for clover and showed little difference between bacterial strains. The population densities were approximately equal to those of the most dense strains in the wheat rhizosphere, whereas the highest concentrations in the root interior of clover and rice were only about one-tenth of those in wheat.

Rankings of initial adsorptive ability on various parts of the roots showed potential for predicting the best strains for colonizing the root interiors of wheat and clover. On wheat, the two strains (Cd and SpBr14) which showed best initial adsorption to the root cap were best at colonizing the endorhizosphere of pot-grown plants. For rice, strains Cd and SpBr14 gave lowest and highest values, respectively, both for adsorption to the terminal 2 cm of roots and for subsequent colonization of the root interior. Data on initial adsorption were of no value in predicting the relative success of strains in colonizing the root surface of any host plants or the interior of clover roots.

Introduction

Members of the nitrogen-fixing genus *Azospirillum* are widespread in soils and are found in high numbers in association with the roots of many plants, particularly grasses. Inoculation of cereal and forage grasses with *Azospirillum* has resulted in yield increases in many field experiments [5, 9, 10, 13, 18, 19, 22], though the response is probably not entirely due to nitrogen fixation [3, 15,

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25]. On the other hand, a smaller but significant number of experiments failed to find any beneficial effect of inoculation [5, 22, 24].

In some cases, this may have been due to failure to use the correct strain of inoculant for the particular crop being studied. Different cultivars of cereal plants were found to differ in their response to inoculation with given strains of bacteria [11, 15, 19, 20], while different *Azospirillum* strains varied in their effect on plant growth [2, 9]. It has been proposed [2, 11] that only homologous strains, isolated from the root interior (endorhizosphere) of the same species of host plant, are capable of stimulating plant growth. Nitrogen fixation by inoculated roots is related to the numbers of azospirilla in symbiosis within the root, rather than to numbers in the rhizosphere [2]. Heterologous strains were considered unable to establish symbiosis within the root, even though they may attain high numbers in the rhizosphere [11]. A more general rule for preferential colonization of the endorhizosphere, in which plants with a C3 photosynthetic pathway are colonized by *Azospirillum brasilense* and C4 plants by *A. lipoferum,* has been proposed [1] in Brazil. This rule is not universal since preferential colonization by *A. brasilense* was not observed in field grown wheat in eastern Australia [16].

In view of the importance of correctly matching bacterial strain with host plant, a simple assay method which enables rapid prediction of the ability of different *Azospirillum* strains to colonize plant roots would be very useful. Several studies on the initial interactions between the roots of seedlings and azospirilla in liquid medium have given encouraging results. For example, the pattern of initial adsorption of bacterial cells to different parts of the root was affected by differences in plant species and bacterial strain [11] and by the presence or absence of combined nitrogen [23] in a way analogous to the effect of these factors on nodulation and nitrogen fixation in the *Rhizobium*-legume interaction.

In the present study, we attempted to correlate the success of different strains of *Azospirillum* in colonizing the rhizosphere and root interior of three host plants with initial adsorptive ability of the bacterial cells on plant roots in liquid medium.

Materials and Methods

Bacteria

Azospirillum brasilense Sp7 [17] was supplied by the Department of Microbiology, University of Queensland, St. Lucia, Qld, Australia. *A. brasilense* strain Cd [7] and *A. lipoferum* strain Sp59b [17] were supplied by the American Type Culture Collection as ATCC 29710 and ATCC 29707, respectively, and *A. brasilense* strain SpBr14 [17] was supplied by N.R. Krieg, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA. Stock cultures were grown at 30° on agar slants of peptone yeast extract (PYE) agar (per liter: peptone, 10.0 g; yeast extract, 5.0 g; NaCl, 5.0 g; agar, 15.0 g; pH 7.2) or tryptone soy broth (TSB) solidified with 1.5% agar (TSB (per litre): tryptone, 15.0 g; soya peptone, 5.0 g; NaCl, 5.0 g; pH 7.3) and were stored up to 20 days at 4°C. Before use, they were tested for contamination by streaking on PYE agar and by microscopic examination of a wet mount.

Preparation of Inoculum

An isolated colony on a TSB agar plate was inoculated into TSB and incubated with gentle shaking at 37°C. The bacteria were harvested by centrifugation at early stationary phase (36 hours) and resuspended in potassium phosphate buffer (pH 7.0, 0.2 M) to give a total count of 10^9 cells/ml.

Plants

Seeds of wheat (*Triticum aestivum* L.), cv. 'Festiguay,' subterranean clover (*Trifolium subterraneum* L.), cv. 'Mara,' and rice (*Oryza sativa* L.), cv. 'Pelde' were surface sterilized by immersion in 95% ethanol for 1 min, followed by 20 min in approx. 1% NaOCl (⅕ strength household bleach containing approx. 5% NaOCl). After rinsing six times with sterilized distilled water, the sterilized seeds were transferred to Petri dishes of water agar (1% agar in distilled water), which were incubated in an inverted position for 3–4 days at room temperature to allow germination without root growth into the agar.

The husks (palea and lemma) were removed from the rice seeds before sterilization since they protected some contaminating microorganisms, but the wheat and clover seeds were sterilized as supplied. The three to four-day-old sterile seedlings were used in setting up pot experiments, or they were transferred to slopes of FS (Fåhraeus seedling solution [8], minus the trace element solution, ⅛ strength) solidifed with 1.5% agar, and incubated for two more days in the glasshouse (21°C day/ 18°C night) before being used in the adsorption experiments.

Bacterial Adsorption to Roots

A six-day-old seedling was placed on a sterile microscope slide and 0.1 ml of bacterial inoculum was added to the roots. After incubation at 35°C for 1 hour, the roots were gently rinsed with FS to remove the excess bacteria and the numbers of adsorbed bacteria were determined by phase contrast microscopy or by viable count. For microscopy, the roots were covered with a cover slip, and the numbers of azospirilla adsorbed to the upper surface of the root epidermis or the root cap or to the entire surface of the root hairs were counted at 400× magnification. Each result is the mean of observations of at least 10 roots.

Viable counts were obtained by homogenizing the terminal 2 cm of the roots of 5 plants in 10 ml of phosphate buffer and inoculating appropriate dilutions onto plates of Congo red agar [21], using either the spread plate method, or the drop method of Miles and Misra [14]. Homogenization, using a Sorvall Omnimixer (Ivan Sorvall Inc, Newton, CT), for a period of 40 sec gave highest recovery of bacteria. Plates were incubated 2–4 days at 35°C, before the typical dark red *Azospirillum* colonies were counted. The concentration of viable cells in the inoculum for each experiment was determined in the same way. Results were corrected for differing proportions of viable cells in the inoculum by dividing the number of adsorbed bacteria by the concentration of viable bacteria/ml in the inoculum.

Pot Experiments

Three-day-old sterile seedlings were soaked in bacterial inoculum (10^9 cells/ml) for one hour at 35°C, before being transplanted into sterile sand-vermiculite mixture (1:1, v/v; Kenyan exfoliated vermiculite, average particle diameter >4 mm) in small plastic pots (8 cm diameter × 9 cm height). The pots were not protected from contamination by airborne organisms, but cross-contamination was minimized by having a distance of at least 50 cm between different bacterial treatments and by standing the pots in trays and watering them by sub-irrigation with sterile FS (once weekly) or

sterile distilled water (twice weekly). These precautions have been found to be effective in preventing cross-contamination between the different *Azospirillum* treatments.

Pots were grown under glass house conditions (21°C day/18°C night) for up to four weeks with different pots being sampled at weekly intervals. Plant roots were dug out of each pot and shaken gently to remove most of the adhering sand and vermiculite. Roots were then cut at the soil line and weighed. Rhizosphere bacteria were counted by shaking the roots for one minute in 10 ml phosphate buffer using a Vortex mixer to remove all bacteria on the root surface, then preparing ten fold dilutions in buffer and inoculating 0.1 ml aliquots into triplicate tubes of Nfb medium [12].

To determine the population of bacteria within the root interior, the bacteria adhering to the root surface were removed by surface sterilization before homogenizing the root. The root surface was sterilized by immersion in 1% ethanol (1 min) followed by approx. 1% NaOCl for an appropriate period of time. It was then washed at least 6 times with sterile distilled water. Surface sterilized roots were ground for 1 min in a small volume of phosphate buffer with a sterile mortar and pestle, after which the volume was made up to 10 ml, and dilutions were prepared and inoculated into tubes of Nfb medium.

All tubes were incubated four days at 35°C and examined for presence of a rising pellicle and development of an alkaline reaction (indicator in the medium changes from green to blue), which are positive evidence for the presence of *Azospirillum*. Numbers of *Azospirillum* in the rhizosphere or in the root interior were calculated from Most Probable Number tables [6].

The three host plants were tested on different occasions. For each strain of bacteria, the pots for determining counts from the different root zones (root interior and root surface) at different times-of-sampling were completely randomized, but were kept separate from the pots containing other strains. There were 3 or 4 replicates of each treatment. Within each combination of host plant/time-of-sampling/root zone, the concentrations of different bacterial strains were compared by analysis of variance of log transformed data.

Results

Surface Sterilization of Roots

In order to determine the minimum time required to kill all azospirilla on the root surface, roots of sterile six-day-old wheat seedlings were incubated for one hour with *A. brasilense* strain SpBr14 (10^9 cells/ml) before being immersed in 95% ethanol for one minute followed by dilute bleach (approx. 1% NaOCl) for varying periods of time. After rinsing in sterile distilled water, the roots were macerated with a sterile mortar and pestle, and dilutions were inoculated into triplicate tubes of Nfb medium.

Treatment with NaOCl for 15 min was sufficient to entirely inactivate the rhizoplane population (Table 1) and this result was confirmed by incubating roots in TSB after varying lengths of sterilization. It was decided to use a 15 min sterilization with NaOCl for all plant roots, since roots up to 4 weeks of age were soft and quite similar to the roots of six-day-old seedlings.

Adsorption to Roots of Six-Day-Old Seedlings

The different strains of *Azospirillum* differed in their adsorption to different parts of the root. There were also differences in the patterns of adsorption for the three different host plants (Fig. 1A). On wheat, the viable count on the

Table 1. Surface sterilization of inoculated roots

Treatment	*Azospirillum* cells recovered (per root piece)[a]
Unwashed	1.5×10^8
Washed[b]	4.0×10^6
95% Ethanol (1 min), then NaOCl for:	
1 min	9.0×10^5
2 min	4.0×10^5
4 min	4.0×10^4
8 min	4.0×10^2
15 min	—[c]

[a] Most Probable Number. Root pieces were approx. 2 cm long
[b] Washed 5 times with sterile distilled water
[c] No growth detected. MPN <4 cells per root piece

terminal 2 cm of root gave results which agreed in part with the microscopic counts of the root cap, in that, by both methods, strain Cd gave highest or equal highest numbers and Sp59b gave lowest numbers. However, strain Sp59b adsorbed best of all strains to the root epidermis and was equally as good as Cd on the root hairs. No single strain consistently gave poor adsorption at all sites.

On rice, strain SpBr14 gave highest numbers of adsorbed bacteria on the whole root (viable count) and on the root cap and the root hairs. Strain Cd adsorbed significantly better than SpBr14 on the root epidermis and was equally numerous on the other parts of the root, but was significantly under-represented in viable counts of the whole root. Strain Sp7 adsorbed worst of all strains at all 3 sites determined microscopically, but this was not reflected in the viable counts.

With clover the results were somewhat different, with strains Sp7 and Sp59b showing good attachment to the root cap and the terminal 2 cm of root. Strain SpBr14 adsorbed best to the surface of the epidermis and the root hairs though it was among the most poorly adsorbed on the root cap and over the terminal 2 cm.

Colonization of Roots of Pot-Grown Plants

Comparison of *Azospirillum* numbers at different times after inoculation showed that all strains attained maximum concentrations both inside and outside the root at about 3 weeks after transplanting (Table 2). Therefore colonization on the different hosts was compared at a standard time of 3 weeks (Fig. 1B).

On wheat, strain Sp7 colonized the rhizosphere best of all strains. Strains Cd and SpBr14 were not significantly different from one another, but were better than strain Sp59b. On the other hand, strain Cd was significantly better at colonizing the interior of the wheat root than SpBr14, which was much better than the other strains.

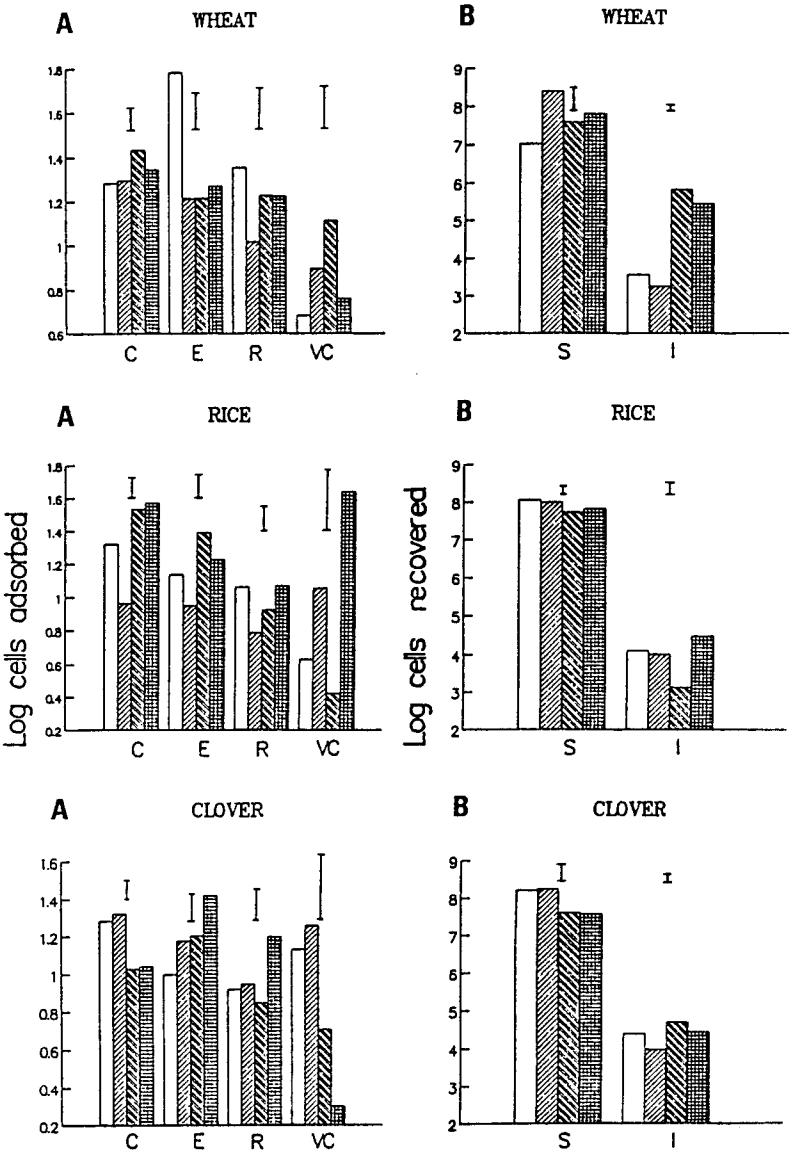

Fig. 1. Numbers of *Azospirillum* cells adsorbed onto root parts after 1 hour or recovered from roots after 3 weeks. *A. lipoferum* strain Sp59b (□), *A. brasilense* strains Sp7 (▨), Cd (▩) and SpBr14 (▤). Vertical bars are Least Significant Differences ($P = 0.05$) between strains. A) Initial adsorption of *Azospirillum* strains on roots. Numbers per root cap (C) or per 200 μm of root epidermis (E) or root hair (R) were determined by microscopic examination. Numbers per terminal 2 cm of root were determined by viable count (VC) on Congo red agar [21] and are expressed as X/I $\times$ 10^5, where X = numbers recovered per 2 cm of root, and I = count per ml of the viable bacteria in the inoculum. B) Colonization of root surface (S) and interior (I) by *Azospirillum* strains 3 weeks after inoculation (Most Probable Number per g fresh weight of root).

Table 2. Colonization of roots after different times

Host plant	Strains	Log_{10} of MPN/g root at different times after inoculation						
		Root surface				Root interior[a]		
		1 wk	2 wk	3 wk	4 wk	2 wk	3 wk	4 wk
Wheat	Sp59b	6.3	6.6	7.0	5.2	5.4	3.6	3.6
	Cd	8.3	6.8	7.6	5.9	4.0	5.9	4.4
	Sp7	8.8	7.1	8.4	5.5	4.0	3.3	ND[c]
	SpBr14	7.9	7.3	7.8	6.0	3.3	5.5	3.8
	L.S.D.[b] ($P = 0.05$)	0.7	0.3	0.5	0.7	0.2	0.2	0.1
Rice	Sp59b	7.3	7.4	8.1	6.1	2.3	4.1	4.1
	Cd	7.1	7.2	7.7	5.8	1.8	3.1	2.9
	Sp7	7.1	7.1	8.0	5.8	2.4	4.0	3.8
	SpBr14	8.3	7.3	7.8	6.3	3.4	4.5	4.5
	L.S.D. ($P = 0.05$)	0.2	0.3	0.2	0.1	0.4	0.3	0.2
Clover	Sp59b	7.5	ND	8.0	6.1	3.3	4.4	2.3
	Cd	6.5	6.2	7.6	5.9	2.9	4.7	3.4
	Sp7	9.8	7.8	8.0	5.9	2.6	3.9	2.6
	SpBr14	6.5	7.0	7.6	6.1	2.4	4.4	2.9
	L.S.D. ($P = 0.05$)	0.8	1.1	0.4	0.2	0.2	0.2	0.4

[a] not sampled in week 1
[b] Least Significant Difference ($P = 0.05$) between strains
[c] ND, not determined

Clover and rice plants were similar, in that all four bacterial strains colonized the rhizosphere approximately equally, with strains Sp59b and Sp7 attaining slightly higher counts than the other strains. Strain SpBr14 colonized the interior of rice roots in slightly higher numbers than Sp59b and Sp7. Strain Cd, although giving lowest numbers within rice roots, was better than other strains at colonizing the clover root interior, by a small but significant margin. The highest concentrations within the roots of clover and rice plants were only about one-tenth of those in wheat roots.

Discussion

Azospirilla adsorbed on the roots of six-day-old seedlings were counted by microscopy of three sites on the roots and by a viable count of the terminal 2 cm of the roots. In addition to differences in efficiency of adsorption between bacterial strains, there were differences between different host plants, and even between different parts of the roots of a single host plant, when bacterial strains were ranked in order of numbers adsorbed. Our results confirm and extend previous findings that adsorption of azospirilla is affected by bacterial strain, host plant, and part of the root involved [4, 11, 23], using different host plants and bacterial strains.

It was expected that the rankings from viable counts would closely agree with those from microscopic counts of the root epidermis, but this was not the case on any plant. The rankings of strains on the root caps agreed fairly well with

the rankings obtained from viable counts on the terminal 2 cm of the roots for wheat and clover, but the ranking for viable counts on the terminal 2 cm of rice roots did not correlate with any rankings obtained by microscopic means. The lack of agreement between rankings by viable count and rankings by direct count of the root epidermis would suggest that cells of some bacterial strains were more easily missed in the microscopic count, perhaps being obscured by clumps of bacteria or granular material produced by the roots [23]. The viable counts data were obtained at different times from the total counts and it is possible, though unlikely, that plants and/or bacterial cultures could have had different physiological and surface properties in the two groups of experiments, despite all attempts to standardize conditions. With wheat and other grasses, it has been shown that *Azospirillum* cells from different phases of the growth cycle have different adsorptive abilities; the optimum culture age for adsorption is variously described as the logarithmic phase [4] or two-days-old [23].

Pot experiments were conducted to provide information on colonization of the root surface and interior over a longer period of time. With few exceptions, optimal colonization of roots occurred at about three weeks for most bacteria-plant combinations, and the concentration of bacteria decreased after this time. This is probably an artefact caused by the small size of the pot; by three weeks the roots had reached the bottom of the pot and were starting to coil around inside its base. As the pots were standing in irrigation water, the lower roots would be under water for much of the time.

It has been postulated by Jain and Patriquin [11] that azospirillum strains which attain high numbers in the rhizosphere are poor colonizers of the root interior, and vice versa, but our results do not suggest that this is a general phenomenon. We confirmed their observation that strain Sp7 was a good colonizer of the wheat rhizosphere but not of the wheat root interior. However, the two best strains at colonizing the root interior, Cd and SpBr14, were intermediate in their ability to colonize the rhizosphere. *A. lipoferum* strain Sp59b was poor at colonizing both the rhizosphere and root interior of wheat, despite having been originally isolated from wheat roots.

On clover, the differences in colonization ability between strains were small, but the best colonizer of the root interior (strain Cd) was the poorest colonizer of the rhizosphere, and the poorest colonizer of the endorhizosphere (strain Sp7) gave equal highest numbers on the root surface, in accord with Jain and Patriquin's [11] proposal. By way of contrast, the ranking of strains colonizing the root interior of rice was not the reverse of that obtained for the rhizosphere: strain Cd gave lowest numbers in both sites.

Cd is the most widely used inoculant strain for wheat, and our results show that it is more efficient than other strains in colonizing the root interior of wheat and, to a lesser extent, clover. It did not establish as well as the other strains in the roots of rice and may be of less benefit to this plant. Although Cd is thought to be a mutant of Sp7, obtained by passage through a grass host [7], it behaves quite differently from its putative parent in its interaction with plant roots.

None of the estimates of initial adsorption to roots was of any value for predicting the best strains for colonizing the wheat rhizosphere or for showing that all strains would be almost equally as efficient at colonizing the rhizospheres

of rice and clover. The bacterial adsorption data were also of no use in predicting the best or worst strains at colonizing the root interior of clover.

However, several measures of initial adsorption showed potential for determining the best strains at colonizing the endorhizospheres of wheat or rice. High initial counts of strains Cd and SpBr14 on the root cap of wheat may be correlated with the ranking of these strains as the best colonizers of the root interior, but results from the root hairs or from viable counts of the terminal portion of root were only partially successful in predicting relative colonizing ability in the wheat endorhizosphere. For rice plants, viable counts of the terminal 2 cm of the root seem to be most useful; they successfully predicted that strains SpBr14 and Cd would be best and worst, respectively, at colonizing the root interior.

It does not appear that a quick adsorption assay will be of any help in screening *Azospirillum* strains for their likely root colonizing ability on clover, and more work needs to be done with wheat and rice before such an assay could be considered reliable.

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