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Endophytic and Surface Colonization of Wheat Roots (*Triticum aestivum*) by Different *Azospirillum brasilense* Strains Studied with Strain-Specific Monoclonal Antibodies

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Abstract

Strain-specific monoclonal antibodies against the *Azospirillum brasilense* strains Sp7, Sp245 and Wa5 were used to study the colonization of two wheat cultivars under axenic conditions and in soil microcosms. The wheat cultivars were colonized with different efficiency by the *A. brasilense* strains used. The rhizoplane of wheat cultivar PF878197 (a cultivar from the south of Brazil) was colonized in high numbers by all *A. brasilense* strains four weeks after inoculation. *A. brasilense* Sp245 showed the highest colonizing potential. This strain could also be detected in the inner root tissue and it formed microcolonies in intercellular spaces. The other strains formed exclusively microcolonies on the root surface. All strains colonized mostly the root tip in high numbers. *A. brasilense* Sp245 could also be detected in other parts of the root. In a time course experiment – from the appearance of cotyledons to flowering – the numbers of *Azospirillum* colonizing the root were measured. While the colonization of the total root by *A. brasilense* Sp7 and Wa5 dropped continuously, the numbers of *A. brasilense* Sp245 inside the roots were constant over the whole experiment. Coinoculation of all three *A. brasilense* strains

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resulted in a similar colonization pattern, but strain Wa5 was further decreased on the roots. As a control, *Escherichia coli* K12 did not colonize wheat roots.

Keywords: *Azospirillum brasilense* strains, PGPR, inoculation, monoclonal antibodies, immunological techniques, quantification, localization

1. Introduction

In recent years, there has been increasing interest in the occurrence and colonization potential of bacteria inside plant roots (Döbereiner and Baldani, 1995; McInroy and Kloepper, 1995; Triplett, 1996). In the endorhizosphere, bacteria escape the harsh competition for nutrients in the rhizosphere soil, but have to cope with the special and selective requirements of the plant interior. Endophytic diazotrophic bacteria are thought to be good candidates for a productive association with the plant, possibly also in respect to nitrogen fixation and nitrogen nutrition of the plant. An ever increasing variety of diazotrophic bacteria have been isolated from many plants including *Gramineae* in recent years. These bacteria belong to genera *Azospirillum* (Krieg and Döbereiner, 1984), *Herbaspirillum* (Baldani et al., 1996; Kirchhof et al., 1997), *Burkholderia* (Baldani, V.L.D., personal communication; Hartmann et al., 1995), *Acetobacter diazotrophicus* (Gillis et al., 1989), and *Azoarcus* (Reinhold-Hurek et al., 1993). In addition, certain *Rhizobium leguminosarum* bv. *trifolii* strains were demonstrated to colonize the interior of gramineous plants (Hoeflich et al., 1995; Schloter et al., 1997a; Yanni et al., 1997). In several different plants, certain members of these diazotrophic species occur in rather high numbers (10^5 to 10^7 bacteria per g root wet weight) inside roots, stems and even leaves (Olivares et al., 1996). Especially in sugar cane, which harbours diazotrophic bacteria of the genera *Acetobacter*, *Herbaspirillum*, *Burkholderia* and *Azospirillum* endophytically, a very substantial amount of nitrogen fixation has been demonstrated (Urquiaga et al., 1992).

Bacteria of the genus *Azospirillum* are ubiquituously occurring in the root zone of different plants including *Gramineae*. It has been shown by different research groups, that *A. brasilense* strains have the potential to promote plant growth (Okon, 1987). Several mechanisms of plant growth stimulation are discussed, which propose the involvement of phytohormones (Tien et al., 1979; Hartmann et al., 1983), improved supply with limiting nutrients and water (Okon, 1988), enhanced nitrate reductase activity and nitrogen fixation (Ferreira et al., 1987). Some *Azospirillum* strains have specific mechanisms to interact with roots and colonize even the root interior, while others colonize the mucigel layer or injured root cortex cells. Already Patriquin and Döbereiner

(1978) found tetrazolium reducing bacteria in the root interior of maize and other grasses, but a specific identification was not possible. Umalia-Garcia et al. (1980) studied in detail the colonization of different *A. brasilense* strains with pearl millet and guinea grass in hydroponic systems and found also limited colonization of intercellular spaces. Patriquin et al. (1983) suggested that localization of *Azospirillum* in the inner regions of the root could be expected to facilitate efficient exchange of substrates, products and effectors between the bacteria and the host.

To use beneficial bacteria as a potential inoculum, we need a better understanding of the process of root colonization by different bacterial strains. However, bacteria added to the rhizosphere not necessarily will survive, grow and remain metabolically active after inoculation. Survival of the inoculated bacteria is rarely specifically monitored. Most investigators, who attempted to quantify the inoculated organism in soil grown plants used enrichment culture methods (MPN counts) or selective plate counts when antibiotic resistant mutants have been used for inoculation. Especially in a normal soil situation with a complex microbial community, very specific identification techniques are required.

Strain specific monoclonal antibodies and immunological techniques can help to follow the fate of the inoculants specifically. Immunological techniques are known to be very sensitive and specific and cultivation of the bacteria is not necessary (Schloter et al., 1995). In a previous study, a differential colonization of soil grown wheat by *A. brasilense* strains was demonstrated (Schloter et al., 1994). In this communication, we compare the colonization of the roots of two different wheat cultivars by three *A. brasilense* strains. While *A. brasilense* Sp245 has originally been isolated from surface sterilized wheat roots, the strains Sp7 and Wa3 are rhizosphere soil isolates. As a negative control for rhizosphere colonization, inoculations were performed with *Escherichia coli* K12.

In a series of experiments, we studied the influence of the autochtone soil microflora (1), the effect of different wheat cultivars (2), the difference between soil- and seed- inoculations technique (3) and the root areas, which are heavily colonized by the different *A. brasilense* strains (4). Furthermore we performed time course experiments over a vegetation period, to follow the fate of the inocula in different root compartments and zones.

2. Material and Methods

Bacterial strains

The bacterial strain *Azospirillum brasilense* Sp7 was obtained from the

German Type Culture Collection (DSMZ, Braunschweig, Germany) and was originally isolated from the rhizosphere of *Digitaria* (Döbereiner et al., 1978). The bacterial strain *A. brasilense* Sp245 was obtained from the culture collection at the Centro de Agrobiologia, (EMBRAPA, Seropedica Brazil) and was isolated from an inner root tissue of wheat (Baldani et al., 1987). *A. brasilense* Wa5 was kindly provided by C. Christiansen-Weniger and has been isolated from the rhizosphere of a greenhouse grown spring wheat by Christiansen-Weniger (1988). All strains were characterized as *A. brasilense* by physiological properties (Baldani et al., 1987; Christiansen-Weniger, 1988) and 23S-rRNA sequence (Kirchhof and Hartmann, 1992). *E. coli* K12 was obtained from the German Type Culture Collection. Nutrient broth-medium (Merck, Darmstadt, Germany) was used to grow the bacteria.

Characterization of the monoclonal antibodies

The important characteristics of the monoclonal antibodies used are listed in Table 1. The development and validation of these antibodies has been described previously in detail (Schloter et al., 1994; Schloter and Hartmann, 1996; Schloter et al., 1997b; Obst et al., 1989).

Table 1. Properties of the monoclonal antibodies (mabs) used

Name of mab	Cross reactivity	ag determinant	Stability of ag	No. of ag/cell	Affinity
A. brasilense Sp7					
Mic 3-8/1	Strain specific	85 kDa (pI 9.0) OMP	Stable	800	High
A. brasilense Wa5					
Bo-33	Strain specific	LPS	Stable	1,500	High
A. brasilense Sp245					
Mipe 3-2.7	Strain specific	30 kDa (pI 3.0) OMP	Stable	800	High
E. coli K12					
ECA	Genus specific	LPS	Stable	n.d.	High

ag = antigenic epitope; OMP = outer membrane protein; LPS = lipopolysaccharides.

Soils

For non-axenic plant cultivation, soil from the tilled horizon of a cambisol

derived from loess was used. The soil was stored in plastic bags at 4°C without drying. The soil was sieved (<2 mm) before use. For axenic plant cultivation, quartz sand (1–5 mm) was autoclaved for 60 min at 121°C.

Plant cultivation

Seeds of wheat cultivar PF879197 (Brazil) or BR16 (Germany) were grown in pots, which had a volume of approximately 100 cm^3 (1 plant/pot). Plants were grown under greenhouse conditions with a soil humidity of 40–60% water capacity and a temperature of 15–22°C during day and 8–12°C at night. The illumination, supplied by three lamps (Osram, Germany; type HQI-E 250) for 12 hours a day, achieved 30 Wm^{-2} at the top of the soil surface. For watering, Whites plant salts solution (Sigma, Germany) were used.

In the axenic experiments, wheat cultivar PF879197 was surface sterilized by treatment with hypochloric acid (Baldani et al., 1986).

Inoculation

Seed inoculation

Seeds were incubated with a bacterial suspension for 30 min just before planting. Bacterial overnight cultures were washed twice in PBS and diluted in 0.9% NaCl to a density of 10^5 cells/ml of each strain. For coinoculation all three *A. brasilense* strains were mixed with an individual density of 10^5 cells/ml.

Soil inoculation

One day after the cotyledons appeared, the inoculation with 10^5 bacteria of each strain (overnight culture), using all three A. brasilense strains and *E. coli* K12 in different combinations, was carried out by pouring 20 ml of bacterial suspension uniformly over the soil surface. Before inoculation the bacteria were washed twice in PBS.

Preparation of the root material for quantification

Rhizosphere soil and roots were separated by shaking the root in PBS for 10 min at 4°C. Washed roots (1 g) were mixed with 10 ml of sterile 0.1% sodium cholate solution and macerated in a waring blender (Schloter et al., 1997a) at low speed for 5 min. Afterwards 0.25 g of polyethylenglycol PEG1500 (Boehringer, Mannheim, Germany) and 0.2 g of chelating resin (Sigma, Germany) was added and incubated on a stirring apparatus for 2 h at 4°C, followed by an ultrasonic treatment (50 W) for 7 min. The bacteria were

separated from larger soil particles by filtering the suspension through a 5 μm filter (Millipor, Germany). The extracted bacteria were centrifuged (5000 × g) for 10 min and fixed in a 4% paraformaldehyde solution at 4°C overnight to reduce natural peroxidase activity by the root material, followed by a centrifugation step and resuspension in carbonate buffer (50 mM, pH 9.6). To determine the number of bacteria in the root tissue, the root was incubated 5 min in 1% chloramin T solution (Baldani et al., 1986) and washed overnight in PBS at 4°C.

For some experiments plant roots were divided into segments before macerating: the upper root (3 cm from stem base); the root tip (3 cm from the root tip) and the central part.

Strain specific quantification by immunoassay

Quantitative chemoluminescence immunoassays were performed in 96-well white coloured polyethylene microtiter plates (Merlin, Germany) with a peroxidase-coupled secondary antibody and luminol (Amersham, Germany) as substrate according to Schloter et al. (1992). Before the use of the ECA monoclonal antibody, the samples were treated in boiling water for two minutes (Obst et al., 1989).

Quantification of the total number of bacteria

For determination of the total microflora, staining with DAPI was used (Assmus et al., 1995). To prevent fading an antifading reagent containing 100 mg paraphenylenediamin in 10 ml phosphate buffered saline (pH 9) and 90 ml glycerine was used. The bacteria were immobilized on a nitrocellulose membrane (0.2 μm; Millipore, Germany). An epifluorescence microscope (Axiplan, Carl Zeiss, Germany) was used to count the bacteria. The instrument was equipped with UV excitation (360 nm). Objective lenses 40×/1.3, 63×/1.4 and 100×/1.4 were used.

Statistical analysis

All colonization experiments were carried out with 5 different pots, each containing 1 plant. Assuming a normal distribution and homogeneous variances, the mean values were tested by the Students-Newman-Keuls test.

In situ localization by immunogold staining

Root segments of the root hair zone were fixed overnight with 3%

paraformaldehyde and 0.1% glutaraldehyde, buffered in PBS pH 7.4. After washing with 50 mM NH_4Cl in PBS the samples were dehydrated with ethanol up to 80% and embedded in L.R. white resin (The Resin company, Great Britain) with polymerization at 60°C for 24 h. Ultrathin sections (70 nm) were prepared. For a strain-specific staining of the *A. brasilense* strains Sp7 and Sp245, monoclonal antibodies and a secondary antibody, which was coupled with gold particles (5 nm) (Amersham) was used (James et al., 1991). The specimens were examined in a transmission electron microscope (EM10, Carl Zeiss, Jena, Germany).

3. Results

Influence of inoculum application

Non axenically grown seedlings of the wheat cultivar PF879197 were inoculated with *A. brasilense* Sp7, Sp245, Wa5 or *E. coli* K12 using either soil or seed inoculation techniques. The plants were grown in loess soil. The colonization of the whole root system by the *A. brasilense* strains or *E. coli* was determined by ELISA four weeks after inoculation using the strain-specific monoclonal antibodies. Using both inoculation techniques, all *A. brasilense* strains were able to form microcolonies in the rhizosphere of the inoculated plants (Table 2). In contrast, *E. coli* could not colonize the rhizosphere of wheat neither using seed- or soil- inoculation techniques. It could be clearly demonstrated, that all *A. brasilense* strains could establish in the rhizosphere of wheat in significantly higher numbers after seed inoculation than after soil inoculation. The number of established Azospirilla was depending on the strain used for inoculation. The highest numbers were achieved using *A. brasilense* Sp245 as inoculum. In contrast, *A. brasilense* Wa5 was detected only in relatively low numbers.

Colonization of two different wheat cultivars

Soil grown seedlings of the wheat cultivars BR16 and PF879197 were inoculated with *A. brasilense* Sp7, Sp245, Wa5 or *E. coli* K12 using the seed inoculation technique. Four weeks after inoculation, the colonization of the root system by the different *A. brasilense* strains was determined by ELISA using the corresponding strain-specific monoclonal antibodies. The total bacterial population was determined as DAPI counts. The Brazilian wheat cultivar PF879197 was colonized in significantly higher numbers as compared to the German wheat cultivar BR16 (Table 3). Not only the inoculated *Azospirillum* strains could be detected in higher numbers, but also the autochtone microflora.

Overall, the root colonization of wheat cultivar BR16 was 100-times lower as compared to the cultivar PF879197.

Table 2. Colonization of 4 weeks old wheat plant roots (cultivar PF879197) by *A. brasilense* Sp7, *A. brasilense* Wa5, *A. brasilense* Sp245 and *E. coli* K12 using soil- and seed inoculation techniques in a greenhouse experiment (loess soil) (mean values from different plants). Standard deviation between different plants with the same treatment was less than 10%. Assuming normal distribution and homogeneous variances the mean values of soil inoculated/seed inoculated plants were tested by the Student-Newman-Keuls test (** = highly significant; * = significant; n.s. = not significant). Values are given as "log bacteria/g dried root".

Inoculum	Inoculation technique		Significance
	Seed inoculation	Soil inoculation	
A. brasilense Sp7	6.30	5.40	*
A. brasilense Wa5	6.00	5.00	**
A. brasilense Sp245	7.20	6.40	**
E. coli K12	<3.0	<3.0	n.s.

Table 3. Colonization of roots of 4 weeks old wheat cultivars PF879197 and BR16 by *A. brasilense* Sp7, *A. brasilense* Wa5, and *A. brasilense* Sp245 in a greenhouse experiment in loess soil, using seed inoculation technique. Total bacterial counts (in brackets) were measured as DAPI count. (mean value from five different plants). Standard deviation between different plants with the same treatment was less than 10%. Assuming normal distribution and homogeneous variances the mean values of PF879197/BR16 were tested by the Student-Newman-Keuls test (** = highly significant; * = significant; n.s. = not significant). Values are printed as "log bacteria/g dried root".

Inoculum	Wheat cultivar		Significance
	PF879197	BR16	
A. brasilense Sp7	6.3 (8.3)	4.3 (6.6)	**
A. brasilense Wa5	6.0 (8.4)	4.3 (6.4)	**
A. brasilense Sp245	7.2 (8.4)	4.2 (6.2)	**

Influence of the autochtone soil microflora

The influence of the autochtone microflora on the establishment of the inocula was estimated by comparing a loess and an axenic quartz sand system. Non sterile and sterile wheat seeds, respectively. of cultivar PF879197 were inoculated with *A. brasilense* Sp7, Sp245, or Wa5 using the seed inoculation technique. After four weeks, the colonization of the whole developed root system by the corresponding *A. brasilense* strains was determined using the ELISA-technique. The total number of bacteria was determined as DAPI counts. All *A. brasilense* strains were detected in significantly higher numbers in the axenic plant system without soil microflora. *A. brasilense* Wa5 and Sp7 were the strains with the lowest competitive potential, since their numbers were about two orders of magnitude lower in the non-axenic soil system as compared to the axenic system. In contrast, root colonization by *A. brasilense* Sp245 was only reduced by about one order of magnitude by the competitive soil microflora in the rhizosphere of the wheat cultivar PF879197 (Table 4).

Table 4. Colonization of 4 weeks-old wheat plant roots (cultivar PF879197) using seed inoculation technique in a non axenic and an axenic system by *A. brasilense* Sp7, *A. brasilense* Wa5, and *A. brasilense* Sp245 (loess soil). Total bacterial counts (in brackets) were measured as DAPI counts (mean values from five different plants). Standard deviation between different plants with the same treatment less than 10%. Assuming normal distribution and homogeneous variances the mean values of axenic / loess system were tested by the Student-Newman-Keuls test (** = highly significant; * = significant; n.s. = not significant). Values are printed as "log bacteria/g dried root".

Inoculum	Plant cultivation system		Significance
	Axenic system	Loess soil	
A. brasilense Sp7	6.9	6.3 (8.3)	**
A. brasilense Wa5	7.1	6.0 (8.4)	**
A. brasilense Sp245	7.7	7.2 (8.4)	**

Colonization of different root parts and the inner root tissues

Four weeks after inoculation of non sterile seeds of wheat cultivar PF879197 with *A. brasilense* Sp7, Sp245, or Wa5, the colonization of different root segments of the root system was determined by ELISA using the corresponding

strain-specific monoclonal antibodies (Fig. 1). All *A. brasilense* strains were detected mainly in the root tip zone. In the central and upper parts only very low numbers of the strains Sp7 and Wa5 were found. In contrast, the numbers for the strain Sp245 were significantly higher in the central and in the upper root parts. To determine the endophytic root colonization, four week old roots of wheat plants, which were inoculated with the strains Sp7, Sp245 or Wa5 by the seed inoculation technique and grown in loess soil, were surface sterilized by chloramine T treatment. *A. brasilense* Sp245 could be quantified by ELISA in surface-sterilized roots (Fig. 2). About 10% of the detected Sp245 cells were in the inner tissue. The strains Sp7 and Wa5 were not able to penetrate into the inner root tissue and were therefore removed by the surface sterilization treatment.

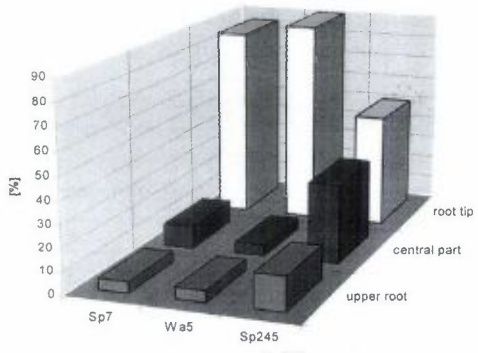

Fig. 1.

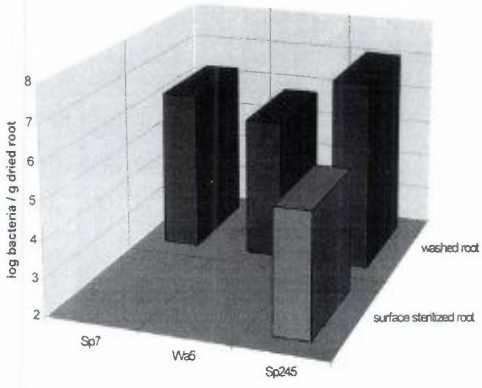

Fig. 2.

Figure 1. Colonization of different parts of the root (root tip; central part; upper root) of 4 weeks-old wheat plants (cultivar PF879197) using seed inoculation technique by *A. brasilense* Sp7, *A. brasilense* Wa5, *A. brasilense* Sp245 in a greenhouse experiment (loess soil) (mean value from five different plants). Standard deviation between different plants with the same treatment was less than 10%. Values are printed as "log bacteria/g dried root".

Figure 2. Colonization of the inner root tissue of 4 weeks-old wheat plants (cultivar PF879197) using seed inoculation technique by *A. brasilense* Sp7, *A. brasilense* Wa5, *A. brasilense* Sp245 in a greenhouse experiment (loess soil) (mean value from five different plants). Standard deviation between different plants with the same treatment was less than 10%. Values are printed as "log bacteria/g dried root".

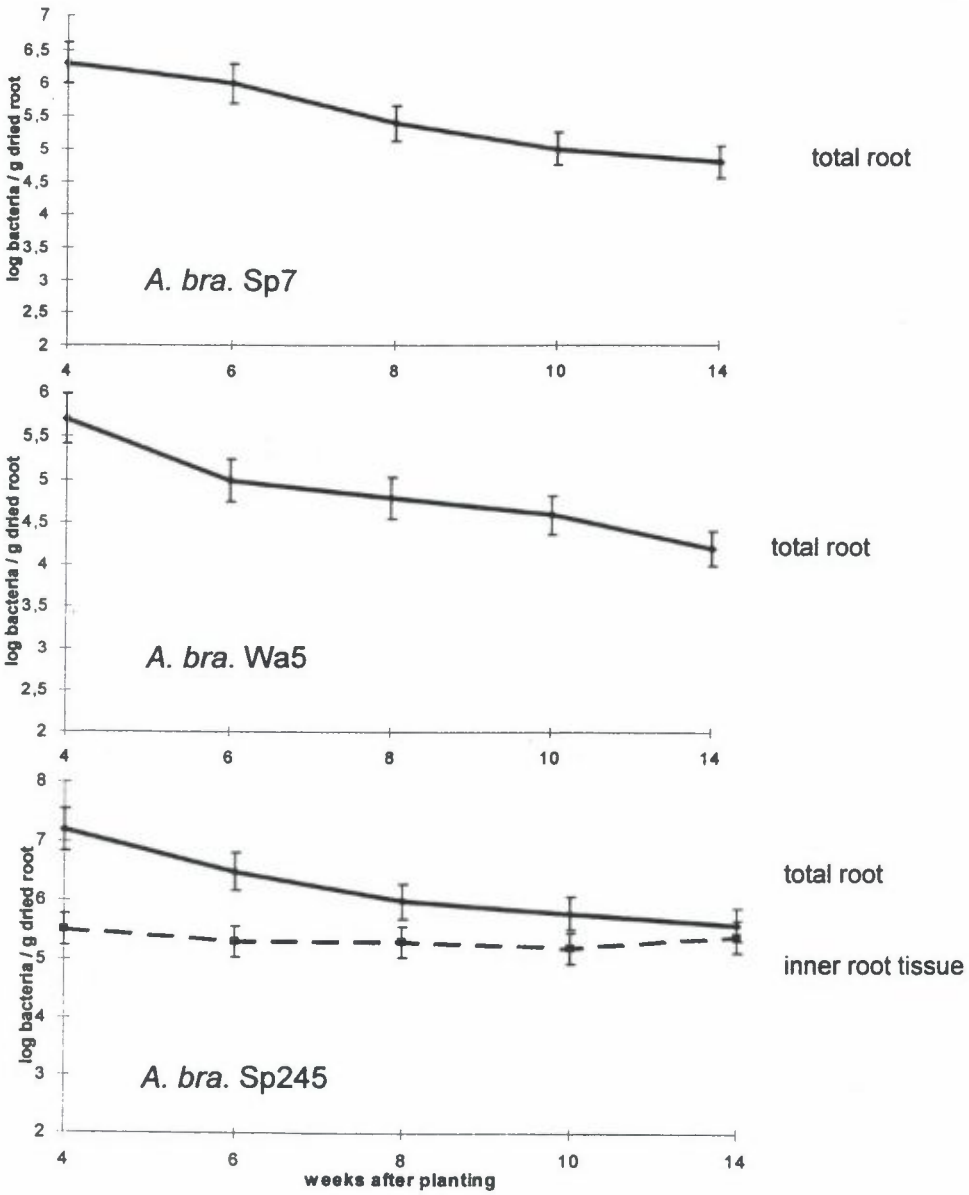

Figure 3. Colonization of the 4, 6, 8, 10 and 14 weeks old wheat root plants (cultivar PF879197) (rhizoplane; inner root tissue) using seed inoculation technique by *A. brasilense* Sp7, *A. brasilense* Wa5, *A. brasilense* Sp245 in a greenhouse experiment (loess soil) (mean value from five different plants; standard deviation between different plants with the same treatment less than 10 %). Values are printed as "log bacteria/g dried root". The numbers of *A. brasilense* Sp7 and *A. brasilense* Wa5 in the inner root tissue were below the detection limit of the antibodies.

Time course experiments

To characterize the root colonization of different *A. brasilense* strains in more detail, a time course study was performed with inoculated wheat roots over a growth period until flowering started (14 weeks after planting). Seeds of cultivar PF 879197 were inoculated with the strains Sp7, Sp245, or Wa5 using the seed inoculation technique and the plants were grown in loess soil. The colonization of the rhizoplane and the inner root tissue by the *A. brasilense* strains was determined using ELISA and the strain-specific monoclonal antibodies 4, 6, 8, 10 and 14 weeks after inoculation. All three strains were still present 14 weeks after planting in the rhizosphere of wheat (Fig. 3). The highest numbers were obtained 4 weeks after inoculation for all three strains. Compared to the autochtone microflora, the numbers of *Azospirillum* were between 1–5% (data not shown). Fourteen weeks after planting, the number of Azospirilla was significantly reduced compared to 4 weeks after planting. Mainly *A. brasilense* Wa5 dropped during that time from 10^6 to 10^4 bacteria/g dried root. The strain, which showed the highest colonization during the whole experiment, was *A. brasilense* Sp245. Interestingly, although the total number of the strain Sp245 dropped over the 14 weeks period, the number of bacteria in the root interior kept constant. At the end of the experiment, almost all of the bacteria of this strain were detected inside the root. The other strains were not able to colonize the inner root tissue at any time of plant development.

To study the influence of coinoculation over the growth period, a second time course experiment was performed as described above using all three *A. brasilense* strains together as inoculum (Fig. 4). The colonization pattern was similar as compared to the inoculation with the single strains (Fig. 3). However, the colonization of total roots by *A. brasilense* Wa5 was further decreased as in single inoculation.

In situ localization with immunogold labeling

To verify the high numbers of *A. brasilense* Sp245 in the inner root tissue, ultrathin sections of the inoculated wheat roots were treated with immunogold coupled antibodies. Fig. 5 shows ultrathin sections of a 10 weeks old wheat root. A high number of specifically labeled *A. brasilense* Sp245 cells was located in the inner root tissue. The bacteria formed microcolonies in intercellular spaces of the central root cylinder. In contrast, *A. brasilense* Sp7 (Fig. 6) and *A. brasilense* Wa5 (not shown) were preferentially located in the rhizoplane in close contact to the root. Almost no penetration of these strains

into the inner root tissue was observed. Marked cells were detected only on the root surface, and in lyzed epidermal and cortex cells.

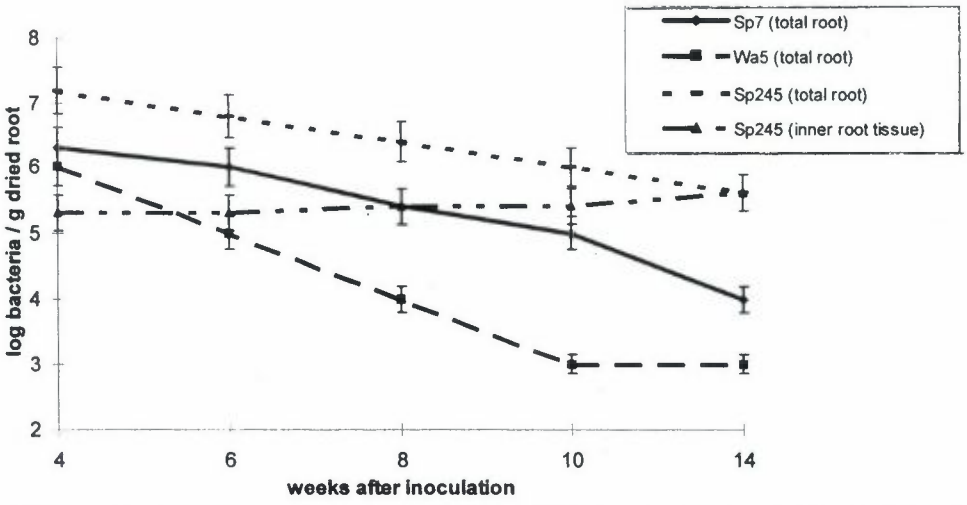

Figure 4. Colonization of 4, 6, 8, 10, and 14 weeks old wheat root plants (cultivar PF879197) (rhizoplane; inner root tissue) using seed inoculation technique after coinoculation by *A. brasilense* Sp7, *A. brasilense* Wa5, *A. brasilense* Sp245 in a greenhouse experiment (loess soil) (mean value from five different plants; standard deviation between different plants with the same treatment less than 10%). Values are printed as "log bacteria/g dried root". The numbers of *A. brasilense* Sp7 and *A. brasilense* Wa5 in the inner root tissue were below the detection limit of the antibodies

4. Discussion

Immunological techniques provide a good tool to follow the fate of microrganisms in complex environments. The antibodies used must meet at least four quality criteria: (i) no cross reaction with other bacteria, (ii) stability of the antigenic epitope in situ, (iii) high affinity to the antigen, (iv) localization of the antigen on the cell surface (Schloter et al., 1995). The monoclonal antibodies for *A. brasilense* Sp7, Wa5, Sp245 and *E. coli* fulfilled all these criteria.

Using these strain-specific monoclonal antibodies the colonization of inoculated wheat plant roots by *A. brasilense* Sp7, Wa5, Sp245 and *E. coli* K12 was followed quantitatively (ELISA) and qualitatively (TEM). All *A. brasilense* strains were able to colonize wheat roots after inoculation and established a population over the whole vegetation period. The number of *A.*

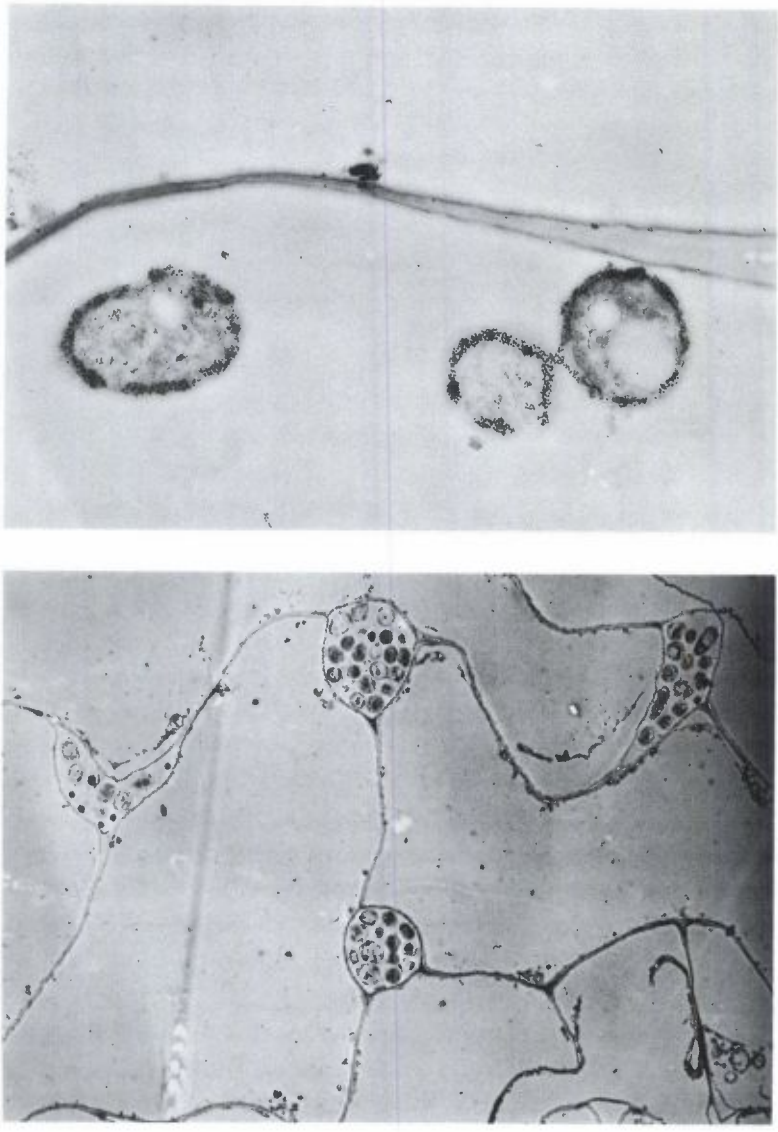

Figure 5. Microcolonies of *A. brasilense* Sp245 in intercellular spaces of 10 weeks old wheat roots (cultivar PF879197) using immunogold labelled mab Mipe 3-2.7 and TEM (seed inoculation technique; loess soil). (a) Overview over central root (5000 × magnification). (b) Labelled bacteria in intercellular spaces (25000 × magnification).

brasilense compared to the autochtone microflora was in the percentage range. However, it could be demonstrated that there are strain specific differences

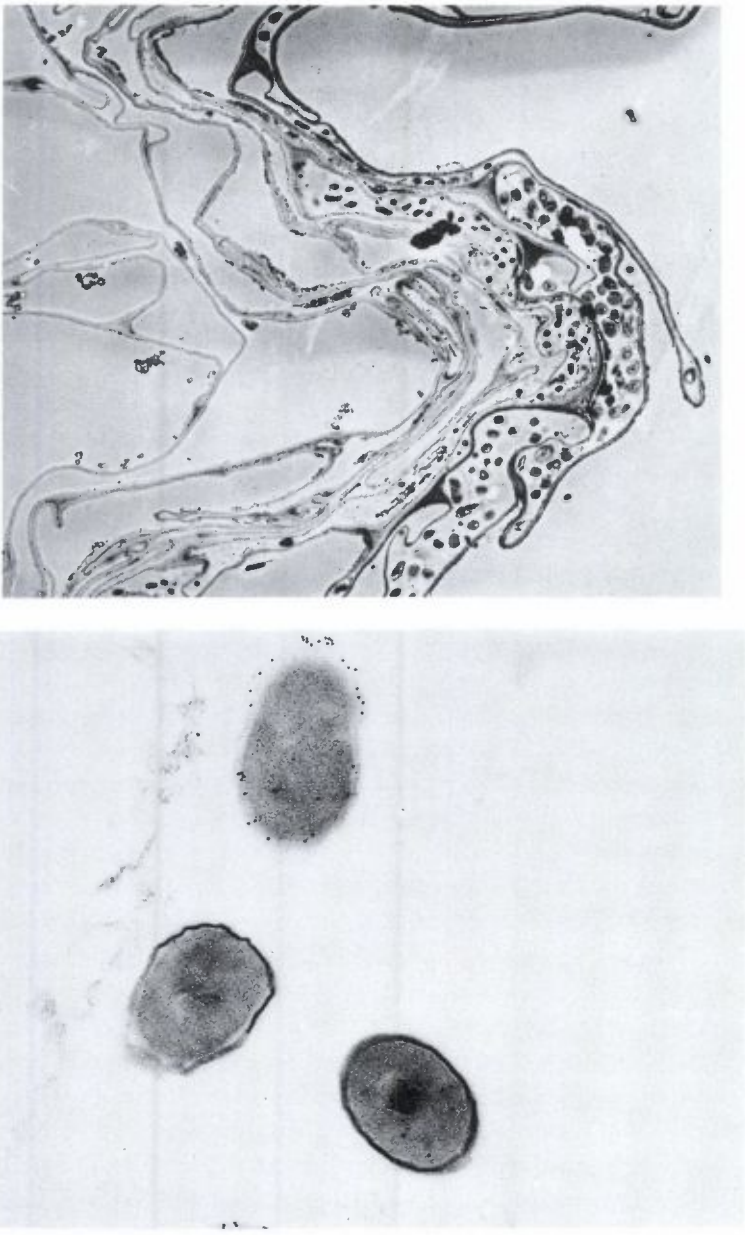

Figure 6. Microcolonies of *A. brasilense* Sp7 on the rhizoplane of 10 weeks old wheat roots (cultivar PF879197) using immunogold labelled mab Mic 3-8.1 and TEM (seed inoculation technique; loess soil). (a) Overview over rhizoplane (root tip zone) (5000 × magnification). (b) Labelled and non labelled bacteria on the rhizoplane (25000 × magnification).

among *A. brasilense* strains in colonising the examined wheat cultivars' roots. *A. brasilense* Sp245 showed the highest and most persisting colonising potential for the Brazilian cultivar. This strain could also be detected in the inner root tissue and formed microcolonies in intercellular spaces. This corroborates a previous study, which showed a differential localization of Sp245 and Sp7 in wheat plants in Brazil (Schloter et al., 1994). A colonization of the inner root tissue by the strain Sp245 was already suggested by Baldani et al. (1986). In their experiments, strain Sp245 was localized in the root interior when antibiotic resistant mutants of this strain were used as inoculum. This colonization of the root interior is remarkable, because only close contact of bacteria with the plant root surface or inner root tissue provides reproducible plant growth stimulating effects. So far it is not known if *A. brasilense* Sp245 is able to invade the plant shoot, as has been shown for other plant endophytic bacteria like *Herbaspirillum seropedicae* or *Acetobacter diazotrophicus* (Döbereiner et al., 1995). The physiological basis for the observed invasiveness of the strain *A. brasilense* Sp245 is not known. Pectinolytic- or other polymer degrading enzymes, which may be specifically induced by components of root exudates (Umalia-Garcia et al., 1980), may facilitate the colonization of the root interior. On the other hand, the colonization of the root interior may be a statistical phenomenon, as the strain Sp245 was also found in the highest numbers on the root surface. De Troch et al. (1992) found characteristic differences in the EPS and LPS composition of Sp245 and Sp7, which may contribute to the different colonization pattern observed. Further physiological and genetic studies are needed to understand the mechanisms of root colonization by *A. brasilense* Sp245. The other strains formed exclusively microcolonies on the root surface or colonized dead cells of the root cortex. An interaction between *A. brasilense* Sp7 and the mucigel layer was also found by Bashan et al. (1989). Levanony and Bashan (1989) demonstrated the colonization of dead root cortex cells by *A. brasilense* Cd. Interestingly, the colonization pattern was found similar in the coinoculation experiment with all three *A. brasilense* strains as compared to single inoculation. However, strain Wa5 disappeared faster. This corroborates findings with dual inoculation of *A. brasilense* Sp7 and Wa5, that strain Sp7 successfully outcompetes Wa5 (Assmus et al., 1997). Thompson et al. (1990) could show, that inoculation of an *Arthrobacter* strain to soil 21 days before adding a *Flavobacterium* strain significantly reduced the survival of *Flavobacterium* compared to the case when the two strains were co-inoculated or the *Flavobacterium* strain was the sole inoculum. In contrast to the *A. brasilense* strains used, *E. coli* K12 could not colonize wheat roots. Four weeks after inoculation, the *E. coli* numbers were below the detection limit of the antibody (less than 10^3 bacteria/g). The results are not surprising as *E. coli* K12 is a

bacterium isolated from the mammalian intestine and cannot compete with the autochtone microflora of the rhizosphere.

Previous inoculation trials described the colonization results of the rhizosphere by *A. brasilense* strains as not very consistent (Bashan et al., 1986). Our results demonstrate a different performance of *A. brasilense* strains upon inoculation of the Brazilian wheat cultivar: the often used strain Sp7 did not perform very strongly. However, strain Sp245 performed well. From earlier results it is known, that there are strain specific differences in the mode of root colonization between strains of *A. brasilense*, which depend on inoculum size and plant growth system. Using a high inoculum Umali-Garcia et al. (1978) localized *A. brasilense* Sp7 in the root hair region. In contrast, Levanony and Bashan (1989) detected *A. brasilense* Cd, which is closely related to Sp7, on the whole root surface.

Not only the presence or absence of a certain strain is important for plant growth stimulation but also its in situ activity. The combination of serological and molecular approaches, reviewed by Kirchhof et al. (1997), is able to get closer insight to rhizosphere bacterial populations and their in situ activities. Assmus et al. (1995) could demonstrate, by using species-specific 23S rRNA directed oligonucleotide probes for *A. brasilense*, that after inoculation of axenically and soil grown wheat seedlings with *A. brasilense* Sp245, most of the bacteria living in the rhizosphere as well as inside cortical root cells and root hairs harbour a high ribosome content. Since the oligonucleotide probes are species-specific, an application of these techniques to follow the fate of a particular strain in a natural environment is not possible. However, a combination of immunological and molecular methods (Assmus et al., 1997) is able to reveal more about the activity of a particular strain used for inoculation in a complex environment.

This work shows that environmental factors could heavily influence the colonization of wheat roots by *A. brasilense* strains. The presence of the autochtonous microflora exerted the strongest influence on the establishment of the *A. brasilense* strains. In axenic systems, the number of colonising Azospirilla was much higher as compared to the natural soil system. This confirms earlier results by Fallik et al. (1988), who showed a drop of rhizosphere colonization by inoculated *A. brasilense* Sp7 in soils with high organic content and a highly active microflora It was shown, that efficient colonization could be achieved by the seed inoculation technique. The close contact to the roots makes colonization easier and more effective. In contrast, using soil inoculation techniques, strains used for inoculation have to move actively towards the root and have to compete with the autochtone soil microflora. Similar results were obtained by Kragelund and Nybroe (1996) for *Pseudomonas fluorescens* and *Alcaligenes eutrophus* strains.

Interestingly, significant differences in the colonization behaviour among the two wheat cultivars used were found. The wheat cultivar BR16 was colonized only in very low numbers after seed inoculation as compared to the Brazilian wheat cultivar PF879197. This work also clearly indicates, that there should be a more intensive selection of bacterial strains used for inoculation. The selection of the plant cultivar and environmental factors govern plant microbe interactions and plant growth stimulating effects. The endophytic potential of *A. brasilense* Sp245 – possibly as a result of not yet understood root-bacterium interactions – should be studied in more detail.

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REFERENCES

Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J.R., and Hartmann, A. 1995. In situ localisation of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Applied and Environmental Microbiology* **61**: 1013–1019.

Assmus, B., Schloter, M., Kirchhof, G., Hutzler, P., and Hartmann, A. 1997. Improved in situ tracking of rhizosphere bacteria using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. *Microbial Ecology* **33**: 32–40.

Baldani, V.B., Alvarez, I., Baldani, I., and Döbereiner, J. 1986. Establishment of inoculated *Azospirillum* spp. In the rhizosphere and in the roots of field grown wheat and sorghum. *Plant and Soil* **90**: 35–46.

Baldani, V.L., Baldani, J., and Döbereiner, J. 1987. Inoculation of field grown wheat with *Azospirillum brasilense* spp. *Biology and Fertility of Soils* **4**: 37–40.

Baldani, J. I., Pot, B., Kirchhof, G., Falsen, E., Baldani, V.L.D., Olivares, F.L., Hoste, B., Kersters, K., Hartmann, A., Gillis, M., and Döbereiner, J. 1996. Emended description of *Herbaspirillum*, inclusion of [*Pseudomonas*] *rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov., and classification of a group of clinical isolates (EF group I) as *Herbaspirillum* species 3. *International Journal of Systematic Bacteriology* **46**: .802–810.

Bashan, Y. 1986. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biology and Biochemistry* **18**: 297–301.

Bashan, Y., Levanony, H., and Whitmoyer, R. 1989. Pleomorphic root surface colonization of non-ceral crop plants inoculated with *Azospirillum brasilense* Cd. *Canadian Journal of Botany* **123**: 209–223.

Christiansen-Weniger, C. 1988. An influence of plant growth substances on growth and nitrogenase activity from *Azospirillum brasilense*. In: *Azospirillum IV*, Klingmüller, W., ed. Springer-Verlag, New York. pp. 141–149.

De Troch, P., Philip-Hollingsworth, S., Orgambide, G., Dazzo, F.B., and Vanderleyden, J. 1992. Analysis of extracellular polysaccharides isolated from *Azospirillum brasilense* wild type and mutant strains. *Symbiosis* **13**: 229–241.

Döbereiner, J. and Baldani, V.L.D. 1995. Endophytic diazotrophs other than rhizobia. In: *Azospirillum VI and Related Microorganisms*. I. Fendrik, M. del Gallo, J. Vanderleyden, and M. de Zamarocy, eds. Springer-Verlag, Berlin, pp. 1–6.

Fallik, E., Okon, Y., and Fischer, M. 1988. Growth response of maize roots to *Azospirillum* inoculation: effects of soil organic matter content, number of rhizosphere bacteria and timing of inoculation. *Soil Biology and Biochemistry* **20**: 45–49.

Ferreira, M., Fernandes, M., and Döbereiner, J. 1987. Role of *Azospirillum brasilense* nitrate reductase in nitrate assimilation by wheat plants. *Biology and Fertility of Soils* **4**: 47–53.

Gillis, M., Kersters, K., Hoste, B., Janssens, D., Kroppenstedt, R.M., Stephan, M.P., Teixeira, K.R.S., Döbereiner, J., and de Ley, J. 1989. *Acetobacter diazotrophicus* sp. nov. a nitrogen-fixing acetic acid bacterium associated with sugarcane. *International Journal of Systematic Bacteriology* **39**: 361–364.

Hartmann, A., Singh, M., and Klingmüller, W. 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indolacetic acid. *Canadian Journal of Microbiology* **29**: 916–923.

Hartmann, A., Baldani, J.I., Kirchhof, G., Assmus, B., Hutzler, P., Springer, N., Ludwig, W., Baldani, V.L.D., and Döbereiner, J. 1995. Taxonomic and ecological studies of diazotrophic rhizosphere bacteria using phylogenetic probes. In: *Azospirillum VI and Related Microorganisms*. I. Fendrik, M. del Gallo, J. Vanderleyden and M, de Zamarocy, eds. Springer-Verlag, Berlin, pp. 415–427.

Hoeflich, G., Wiehe, W., and Hecht-Buchholz, C. 1995. Rhizosphere colonisation of different crops with growth promoting *Pseudomonas* and *Rhizobium* bacteria. *Microbial Research* **150**: 139–147.

James, E., Sprent, J., Minchin, F and Brewin, N. 1991. Intracellular location of glycoprotein in soyabean nodules: effect of altered rhizosphere oxygen concentration. *Plant Cell and Environment* **14**: 467–476.

Kirchhof, G. and Hartmann, A. 1992. Development of gene-probes for *Azospirillum* based on 23S-rRNA sequences. *Symbiosis* **13**: 27–35.

Kirchhof, G., Reis, V.M., Baldani, J.I., Eckert, B., Döbereiner J., and Hartmann, A. 1997. Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant and Soil* **194**: 45–55.

Kirchhof, G., Schloter, M., Assmus, B., and Hartmann, A. 1997. Molecular microbial ecology approaches applied to diazotrophs associated with non-legumes. *Soil Biology and Biochemistry* **29**: 853–862.

Kragelund, L. and Nybroe, O. 1996. Competition between *Pseudomonas fluorescens* AgI and *Alcaligenes eutrophus* JMP134 (pJP4) during colonization of barley roots. *FEMS Microbial Ecology* **29**: 41–51.

Krieg, N.R. and Döbereiner, J. 1984. Genus *Azospirillum*. In: *Bergeys Manual of Systematic Bacteriology*. N.R. Krieg and J.G. Holt, eds. Williams and Wilkins, Baltimore, pp. 94–104.

Levanony, H. and Bashan, Y. 1989. Enhancement of cell division in root tips and growth of the elongation zone in wheat roots induced by *Azospirillum brasilense* Cd. *Canadian Journal of Botany* **67**: 56–61.

McInroy, J.A. and Kloepper, J.W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and Soil* **173**: 337–342.

Obst, U., Hübner, L., Wcker, M., and Bittersuermann, D. 1989. Immunological method using monoclonal antibodies to detect *Enterobacteriaceae* in drinking water. *Aqua* **28**: 136–142.

Okon, Y., Fallik, E., Sarig, S. Yahalom, E., and Tal, S. 1988. Plant growth promoting effects of *Azospirillum*. In: *Nitrogen Fixation: Hundred Years After*, Bothe, H., deBruijn, F. and Newton, F., eds. Gustav Fischer, New York. pp. 741–746.

Olivares, F.L., Baldani, V.L.D., Reis, V.M., Baldani, J.I., and Döbereiner, J. 1996. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of *Gramineae*. *Biology and Fertility of Soils* **21**: 197–200.

Patriquin, D.G. and Döbereiner, J. 1978. Light microscopy observations of tetrazolium reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology* **24**: 734–742.

Patriquin, D.G., Döbereiner, J., and Jain, D.K. 1983. Sites and process of the association between diazotrophs and grasses. *Canadian Journal of Microbiology* **29**: 900–915.

Reinhold-Hurek, B., Hurek, T., Gillis, M., Hoste, B., Vancanneyt, M., Kersters, K., and de Ley J. 1993. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. *International Journal of Systematic Bacteriology* **43**: 574–584.

Schloter, M., Bode, W., Hartmann, A., and Beese, F. 1992. Sensitive chemoluminescence-based immunological quantification of bacteria in soil extracts with monoclonal antibodies. *Soil Biology and Biochemistry* **24**: 399–403.

Schloter, M., Kirchhof, G., Heinzmann, U., Döbereiner, J., and Hartmann, A. 1994. Immunological studies of the wheat root colonization by *Azospirillum brasilense* strains Sp7 and Sp245 using strain-specific monoclonal antibodies. In: *Nitrogen-Fixation with Non-Legumes*. N.A. Hegazi, M. Fayez, and M. Monib, eds. The American University in Cairo Press, Cairo, pp. 291–297.

Schloter, M., Assmus, B., and Hartmann, A. 1995. The use of immunological methods to detect and identify bacteria in the environment. *Biotechnology Advances* **13**: 75–90.

Schloter, M., Moens, S., Croes, C., Reidel, G., Esquenet, M., DeMot, R., Hartmann, A., and Michiels, K. 1994. Characterization of cell surface components of *Azospirillum brasilense* Sp7 as antigenic determinants for strain-specific monoclonal antibodies. *Microbiology* **140**: 823–828.

Schloter, M. and Hartmann, A. 1996. Production and characterization of strain-specific monoclonal antibodies against outer membrane components of *Azospirillum brasilense* Sp245. *Hybridoma* **15**: 225–231.

Schloter, M., Wiehe, W., Assmus, B., Steindl, H., Becke, H., Hoeflich, G., and Hartmann, A. 1997a. Root colonization of different plants by plant-growth-promoting *Rhizobium leguminosarum* bv. *trifolii* R39 studied with monospecific polyclonal antisera. *Applied and Environmental Microbiology* **63**: 2038–2046.

Schloter, M., Bode, W., and Hartmann, A. 1997b. Characterization and application of a strain-specific monoclonal antibody against the rhizosphere bacterium *Azospirillum brasilense* Wa5. *Hybridoma* **16**: 183–187.

Tien, T.M., Gaskins, M.H., and Hubbell, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on growth of pearl millet. *Applied and Environmental Microbiology* **37**: 1016–1020.

Thompson, L.P. Cook, K.A., Lethbridge, G., and Burns, R.G. 1990. Survival of two ecologically distinct bacteria (*Flavobacterium* and *Arthrobacter*) in unplanted and rhizosphere soil. *Soil Biology and Biochemistry* **22**: 1029–1037.

Triplett, E.C. 1996. Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots. *Plant and Soil* **186**: 29–38.

Umali- Garcia, M., Hubell, D.H., Gaskins, M., and Dazzo, F. 1980. Association of *Azospirillum* with grass roots. *Applied and Environmental Microbiology* **39**: 219–226.

Urquiaga, S., Cruz, K.H.S., and Boddey, R.M. 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. *American Soil Science Society* **56**: 105–114.

Yanni, Y.G., Rizk, R.Y. Corich, V., Squartini, A., Ninke, K., Philip-Hollingsworth, S., Orgambide, G., DeBruijn, F., Stoltzfus, J., Buckley, D., Schmidt, T.M., Mateos, P.F., Ladha, J.K., and Dazzo, F. 1997. Natural endophtic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. *Plant and Soil* **194**: 67–81.