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In situ Localization and PGPR-Effect of *Azospirillum brasilense* Strains Colonizing Roots of Different Wheat Varieties

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Abstract

Azospirillum brasilense strains are considered as Plant Growth Promoting Rhizobacteria (PGPR) because of their beneficial effects on plant development after inoculation. Two strains of *A. brasilense*, Sp245 and Sp7, were examined for their endophytic potential on German, Brazilian and Israeli wheat cultivars. Plate count and Most Probable Number (MPN) methods were applied for quantification, as well as the fluorescent *in situ* hybridization (FISH) technique in combination with confocal laser scanning microscopy for the species specific detection and localization of the two *Azospirillum* strains in roots. Additionally, a plasmid bearing a constitutively expressed *gfp* gene was transformed into both strains, which enables visualization of the bacteria omitting the fixation process during the FISH protocol. The microscopic techniques showed that the potential of strain Sp245 to grow in the roots of all analyzed wheat varieties as an endophyte was greater than of Sp7, but overall cell densities were rather low under the applied experimental conditions. A plant growth promoting effect was clearly visible in all examined inoculated plants, irrespective of the *A. brasilense* strain used as inoculum.

Keywords: *Azospirillum brasilense*, PGPR, localization, quantification, FISH, *gfp*, wheat colonization

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1. Introduction

Species of the genus *Azospirillum* were found living in close association with roots of various plants in temperate to tropical climates all over the world (Krieg and Döbereiner, 1986). They are nitrogen fixing rhizosphere bacteria (Döbereiner, 1992) belonging to the phylogenetic group of α-proteobacteria (Kirchhof and Hartmann, 1992). The scientific interest in these bacteria is mainly based on their ability to enhance growth and yield of agronomically important crops, such as wheat and maize (Dobbelaere et al., 2001), which is why they are considered as Plant Growth Promoting Rhizobacteria (PGPR). This stimulation effect was first thought to be due to the ability of *Azospirillum* spp. to fix atmospheric nitrogen and thereby supplying the host plant with otherwise limited nitrogen compounds (Döbereiner and Day, 1976).

But later studies proofed that the beneficial effect of *Azospirillum brasilense* inoculation on plant prosperity was rather an attribute to morphological changes of the roots, resulting in a greater root surface area, which allowed them to take up more water and nutrients (Okon and Kapulnik, 1986). In fact, the production of considerable amounts of the auxin indole-3-acetic acid by the bacteria (Hartmann et al., 1983) seems to contribute at least partially to this stimulatory effect (Dobbelaere et al., 1999), by increasing root hair density (Hadas and Okon, 1987) and number of lateral roots (Barbieri et al., 1986). Usually *Azospirillum* strains colonize the root externally (Bashan et al., 1989), living embedded in the mucigel layer (Murthy and Ladha, 1987) in variable numbers (Drozdowicz and Ferreira, 1987). The colonization density depends on the host plant and the bacterial strain (O'Hara et al., 1983).

Additionally to these externally associated root colonizers, it has been shown that some *Azospirillum* species have mechanisms not only to colonize injured cortex cells but also the interior of intact roots (Umalia-Garcia et al., 1980). It was suggested that this localization within the host plant could be a way to increase efficiency of exchanging beneficial factors and substrates (Patriquin et al., 1983) and to escape the competition with other rhizosphere bacteria (Hartmann, 1989). *A. brasilense* strain Sp245 was demonstrated to be able to penetrate the outer root layers and to establish itself in intercellular spaces of the root cortex of a Brazilian wheat variety (Schloter and Hartmann, 1998). It is not clear so far, if there is a high specificity in this kind of association, or if this ability of strain Sp245 is detectable in various wheat host cultivars.

Therefore, the aim of this work was to show whether this peculiar colonization behavior of *A. brasilense* strain Sp245 can also be observed in different wheat varieties and if this internal colonization has any impact on the plant growth promoting effect. For this reason the colonization potential of two *A. brasilense* strains, Sp7 and Sp245, on roots of three different wheat

cultivars (Naxos, Atir, and PF839197) was studied. Quantification of colonizing bacteria was carried out by plate count and Most Probable Number (MPN) methods.

Furthermore the localization and specific detection of the two strains on cellular level using a confocal laser scanning microscope (CLSM) was accomplished by marking individual cells by fluorescent *in situ* hybridization (FISH) with fluorescence-labeled oligonucleotide probes or by *gfp* (green fluorescent protein) labeling. The FISH technique has been successfully applied for the localization of *A. brasilense* on roots (Assmus et al., 1995), but until recently available probes were only partially suited for whole cell *in situ* hybridization, or were of limited specificity. Using a new set of 16S rRNA targeted probes, developed by Stoffels et al. (2001), it is now possible to perform highly specific identifications of *A. brasilense* on roots grown in a monoxenic system as well as in soil. The final approach was to tag *A. brasilense* with a *gfp* bearing plasmid and to inoculate the wheat seeds with those marked strains, because of the possibility to detect Gfp-fluorescing cells using a CLSM without any further treatment of the colonized root. The examination of a totally intact, neither chemically nor physically treated root, is a major premise for the final microscopic proof of endophytic colonization. The pBBR-Vector harboring this constitutively expressed *gfp* gene was chosen because it had been shown to replicate in several other soil bacteria (Kovach et al., 1995), e.g. *Acetobacter xylinum, Pseudomonas fluorescens, P. putida, Sinorhizobium meliloti, Rhizobium leguminosarum*. This and its reported stability in these organisms without antibiotic pressure made it well suited for our application.

2. Materials and Methods

Bacterial strains and culture conditions

Azospirillum brasilense Sp7, which was originally isolated from a *Digitaria* rhizosphere (Tarrand et al., 1978), was provided by the German Type Culture Collection (DSMZ, Braunschweig, Germany). The strain *A. brasilense* Sp245 originates from inner root tissue of wheat (Baldani et al., 1987) and was obtained from the culture collection of the Centro National de Agrobiologia (CNPAB, EMBRAPA) in Seropedica, Rio de Janeiro, Brazil. *A. brasilense* was grown at 37°C either on NB (nutrient broth No. 4, Fluka, Buchs, Switzerland) as complex medium or MMAB (Vanstockem et al., 1987) as mineral salt medium. *Escherichia coli* strain HB101 with plasmid pRK600 (Figurski et al., 1979) was grown on Luria-Bertani medium (Bertani, 1951, modified) containing 10 µg/ml chloramphenicol. *E. coli* strain MT102 harboring plasmid pBAH7 (B. Huber, Institute of Microbiology, Technical University of

Munich, unpublished) was cultivated on LB medium supplemented with 50 µg/ml kanamycin.

Wheat cultivars and growth conditions

Three different summer wheat cultivars (*Triticum aestivum*) were used: Naxos (Germany, supplied by the IPK, Gatersleben), Atir (Israel, provided by E. Jurkevitch, The Hebrew University of Jerusalem, Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot), and PF839197 (Brazil, obtained from Michael Mackay, Australian Agricultural Collection). For non-monoxenic cultivation sandy soil from a tilled horizon of an agricultural field near Neumarkt, Germany was used. The soil was sieved (<2 mm) and mixed with 7 g $CaCO_3$ per kg of soil to raise the pH from about 5 to 6. Single plants were grown in 400 cm^3 pots under greenhouse conditions with a temperature of 15 to 25°C during day and 10 to 15°C during night. For watering deionized H_2O was used. The monoxenic cultivation was performed in sealed glass tubes (3 cm width, 50 cm length) filled with 100 g of autoclaved quartz sand and 10 ml of sterile Hoagland's solution (Terry, 1980, modified). Component quantities in deionized water were as follows: 136 mg l^{-1} KH_2PO_4, 174 mg l^{-1} K_2HPO_4, 20 mg l^{-1} $MgSO_4$, 10 mg l^{-1} $CaCl_2$, 1 mg l^{-1} $FeCl_3 \times 6\ H_2O$, 5 mg l^{-1} $Na_2MoO_4 \times 2\ H_2O$, 1 ml l^{-1} trace element solution (dissolved in deionized water: 100 mg l^{-1} $ZnSO_4 \times 7\ H_2O$, 30 mg l^{-1} $MnCl_2 \times 4\ H_2O$, 300 mg l^{-1} H_3BO_3, 200 mg l^{-1} $CoCl_2 \times 6\ H_2O$, 10 mg l^{-1} $CuCl_2 \times 2H_2O$, 20 mg l^{-1} $NiCl_2 \times 6\ H_2O$). Growth conditions were the same as in the soil system.

Inoculation with bacterial strains

For the soil system seeds were germinated on moist paper tissue in Petri dishes at 30°C in the dark for 3 to 4 days. Seeds for the monoxenic system were treated with 1% (v/v) Tween 20 (Sigma, Steinheim, Germany) in sterile deionized water for 2 minutes, surface sterilized with 70% ethanol (5 min) and washed three times with sterile deionized water. Subsequently, seeds were incubated in sodium hypochlorite solution (6–14% Cl active, Riedel-de Haën, Seelze, Germany) for 20 minutes and washed five times with sterile deionized water. After incubating the seeds for 3 to 4 days at 30°C in the dark on NB plates, only those seeds were picked for incubation, which showed no visible contamination. Inoculation was performed when roots were between 1 to 2 cm in length using the seed inoculation method described by Schloter and Hartmann (1998) with slight modifications: Overnight cultures of *A. brasilense* Sp7 or Sp245 were washed twice with 1xPBS and diluted to a concentration of 10^8 cells per ml 1xPBS. The seeds were incubated in this bacterial suspension for one hour before planting.

Root harvesting and quantification of bacterial cells

After 4 to 7 weeks, roots were harvested by taking the whole plant out of the pot containing soil or out of the glass tube with quartz sand, and rinsing off the adhering material with sterile 1xPBS. Prior to the determination of root and shoot fresh weight, exogenous water was removed with a sterilized paper tissue. As there were always three plants of each cultivar with the same inoculum, these parallel samples were pooled to obtain sufficient root material (>200 mg fresh weight). Two third of the material was dried for two days at 70°C to obtain dry weight. The remaining root material was split into equal amounts and in order to detect endophytic colonizers one half was surface sterilized (Baldani et al., 1986, modified) for 10 minutes with 1% chloramine T (Sigma, Steinheim, Germany). The other half remained untreated. Subsequently, the roots were ground thoroughly in 10 ml 1xPBS per g root fresh weight. Dilution series were prepared and plated in triplicates on NB and MMAB Agar plates and inoculated in semisolid NFb medium (Döbereiner, 1995). The colonies were counted after two days incubation at 37°C. The MPN method facilitated calculation of bacterial quantity from the numbers of inoculated glass vials with semisolid NFb in which *A. brasilense* had formed a pellicle. For the soil system the plate counts were corrected by subtracting the number of colonies growing on MMAB Agar from the uninoculated control. These bacteria were considered contaminants able to grow on MMAB. Concerning the use of semisolid NFb this was not necessary as there were no contaminants visible in the vials with root suspension from the control plants.

Tagging of A. brasilense with a gfp containing plasmid

The plasmid pBAH7, constructed from a pBBR1MCS-2 vector (Kovach et al., 1995) with a kanamycin resistance cassette and a costitutively expressed *gfp* (modified form mut3) gene (Cormack et al., 1996) under control of a $P_{A1/04/03}$ promoter (Andersen et al., 1998), was transferred to *A. brasilense* strains Sp7 and Sp245 with a conjugative transfer by triparental mating (Kristensen et al., 1995, modified). Therefore 5 ml of an overnight culture of the *A. brasilense* acceptor strain grown on NB medium were sucked onto a poly-ether-sulfon filter (pore size 0.22 µm, Millipore Corp., Bedford, USA) by a vacuum pump and preincubated for 2 hours at 30°C (Vanstockem et al., 1987). *E. coli* strain HB101 containing the helper plasmid pRK600 as well as the donor strain *E. coli* MT102 bearing pBAH7 were incubated under selective conditions overnight. The two strains were washed with NB medium, mixed in a 1:1 ratio and incubated at room temperature for 30 minutes before sucking them onto the filter carrying the preincubated acceptor strain. With this procedure a donor / helper / acceptor strain ratio of about 1:1:5 was obtained. After the filter had been incubated at

30°C overnight, the bacterial mixture was resuspended with sterile PBS and 10–50 µl were plated onto MMAB Agar plates supplemented with 50 µg/ml kanamycin and 6 µg/ml nalidixic acid for selection of the plasmid bearing *A. brasilense* transconjugants.

Fluorescent in situ hybridization of A. brasilense on roots

From tip, root hair zone, and basal part 1 cm pieces of the freshly harvested roots were taken and placed between two small blocks of styrofoam. Radial and longitudinal slices of about 50–100 µm thickness were prepared with a razor blade by cutting the roots fixed between the styrofoam blocks. These slices were transferred to a glass slide and were immobilized by incubation at 70°C for 30 minutes. After this heat immobilization the slices were fixed with 4% paraformaldehyde for 2 hours at room temperature (Amann et al., 1990, modified). Hybridization with fluorochrome (FITC, Cy3, Cy5) labeled oligonucleotide probes followed the protocols described by Manz et al. (1992) and Amann et al. (1992). Two rRNA-targeted oligonucleotide probes were used, which were synthesized and fluorescently labeled by ThermoHybaid (Ulm, Germany). EUB338-I (Amann et al., 1990), EUB338-II and III (Daims et al., 1999) were used as equimolar mixture and are specific for the domain Bacteria at varying formamide concentrations. The 16S rRNA targeted probe Abras1420 (5'-CCACCTTCGGGTAAAGCCA-3', Stoffels et al., 2001) in combination with a competitor (5'-CACCTTCGGGTAAAACCA-3') enables specific identification of the species *A. brasilense* at a formamide concentration of 45% in the hybridization buffer. Fluorescing cells were detected by a confocal laser scanning microscope (LSM510, Zeiss, Oberkochen, Germany). An Argon Ion Laser supplied the wavelength of 488 nm for excitation of FITC, and two Helium Neon Lasers provided excitation wavelengths of 543 nm and 633 nm for Cy3 and Cy5, respectively. The Cy5 fluorescent dye emits in the far-red spectrum of light but a blue color is assigned for illustration, whereas Cy3 and FITC are shown in the color of their fluorescence, red and green. For every single hybridization the probes EUB338-mix and Abras1420 were used in combination. As the two probes labeled with different fluorescent dyes (EUB338-mix with FITC or Cy3, Abras1420 with Cy3 or Cy 5) were both supposed to bind to *A. brasilense*, only such signals were regarded as positive, which showed a combination of both colors. Thus, after combining the three single color images to one RGB (red green blue)-picture, *A. brasilense* cells were identified by their magenta staining (EUB338-mix with Cy3 and Abras1420 with Cy5), or alternatively by yellow (EUB338-mix with FITC and Abras1420 with Cy3). The remaining third color channel would therefore only show autofluorescence by the root or other particles.

An optical sectioning of the observed 50–100 µm root slice could be achieved by moving the focus position deeper into the root in 1 µm steps producing z-stacks of individual pictures from the same xy-area with a penetration depth of about 30 µm. The resulting set of pictures could be combined to a three dimensional image with the help of the LSM510 software package provided by Zeiss.

Detection of gfp tagged cells

For the visualization of the *gfp* tagged *A. brasilense* cells, freshly harvested root parts were selected as with the FISH method described above, but remained intact and were placed as a whole on a glass slide. They were embedded in Citifluor (Citifluor Ltd., Canterburry, UK) and Gfp derived fluorescence was detected using the CLSM. The same set of three lasers was used, with Gfp fluorescing at an excitation wavelength of 488 nm. The fluorescence detectable in the samples when excited at 543 nm and 633 nm was unspecific autofluorescence, which was mostly too week to be still visible in the combined RGB-images. Three dimensional scans were performed on the intact root as explained above.

3. Results

Quantification of root colonization by A. brasilense in the monoxenic system

Seedlings of the wheat cultivars Naxos, Atir and PF839197 were inoculated with *A. brasilense* Sp7 or Sp245 using the seed inoculation technique. Together with uninoculated control plants they were grown under greenhouse conditions on quartz sand with Hoagland's solution for 4 weeks. After this period, no significant stimulatory effect of the *Azospirillum* inoculum was visible. Roots were harvested, and colony forming units (CFU) of *Azospirillum* were determined before and after surface sterilization. In each experiment, root material of three separate plants was pooled. The cell counts obtained from MMAB and NB plates (Fig. 1) were between 2×10^7 and 2×10^8 CFU per g root fresh weight without surface sterilization. The highest numbers were observed on the Brazilian wheat cultivar (PF839197) inoculated with strain Sp245 and counts were lowest on Atir inoculated with Sp245 as well as Sp7. After treatment with chloramine T bacterial cell numbers decreased to between 5×10^5 and 2×10^4 CFU per g root fresh weight. In cultivars Naxos and Atir the numbers of Sp7 were higher than of Sp245 after surface sterilization. Only in the Brazilian wheat variety, strain Sp245 was found in equally high numbers as strain Sp7 (see Fig. 1).

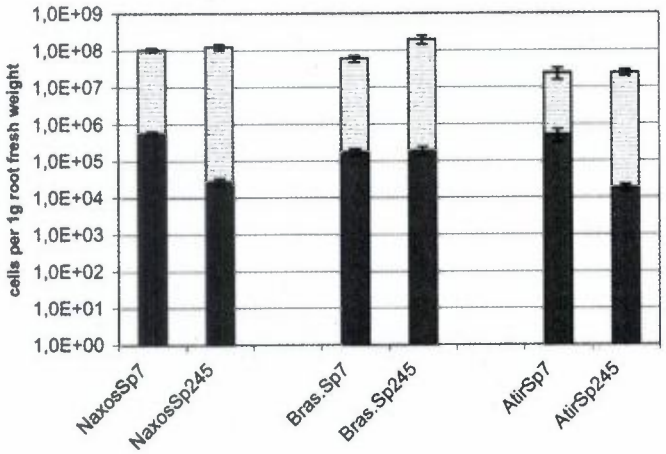

Figure 1. Cell numbers of root associated *Azospirillum brasilense* strains Sp7 and Sp245 on 1g root fresh weight of monoxenically grown Naxos, Brazilian (PF839197), or Atir wheat cultivars before (total columns) and after (filled columns) surface sterilization. The seedlings were inoculated at a root length of 1–2 cm with an overnight culture of bacteria washed twice with 1xPBS and diluted to a cell density of 10^8 cells per ml 1xPBS.

On NFb semisolid medium, bacterial numbers with and without chloramine T treatment were both 30-50% lower than on NB and MMAB Agar plates (data not shown), but otherwise matched the colony counts determined on plates.

Quantification of root colonization by A. brasilense in the soil system

Three seeds of each cultivar were inoculated with either strain Sp245 or Sp7. Not inoculated control plants were raised in parallel. Wheat plants were grown for 4 and 7 weeks in the greenhouse. A part of the roots was ground and the resulting suspension plated on MMAB Agar or inoculated to NFb containing vials (see material and methods for details). The resulting cell counts from MMAB plates after 4 weeks were for both strains at about 1×10^4 CFU per gram root fresh weight and this number did not change significantly after 7 weeks (data not shown). The colonizing numbers obtained from the NFb vials by MPN calculations were again about 30% lower than the plate counts. Applying these methods, the determination of reliable cell counts after surface sterilization was not possible, because cell numbers were below the detection limit of about 1×10^3 CFU per gram root fresh weight.

Detection of the PGPR-effect

The shoots and the rest of the root material were air dried to obtain dry weight per single plant. After 4 weeks no significant increase in root dry weight due to inoculation was observed in any of the three cultivars, and only a marginal increase of shoot dry weight was found in the inoculated plants. In contrast, compared to the uninoculated controls a reduction of root dry weight in the Brazilian cultivar inoculated with both strains and in the variety Atir inoculated with Sp7 was determined. After 7 weeks, there was a clearly detectable growth promoting effect of *A. brasilense* inoculation in the Naxos and the Brazilian wheat cultivar. The strongest response to inoculation was triggered in the Brazilian wheat cultivar, where the increase of root dry weight went up to 50% in the Sp7 inoculated plants. In the Naxos variety the most pronounced growth stimulation effect was also to be seen in the Sp7 inoculated plants, with a 20% increase in root dry weight. While root weight of the variety Atir was not stimulated due to inoculation (Fig. 2A), shoot dry weight of Atir showed an increase of about 25% in inoculated plants.

The overall greatest shoot weights were found in the Naxos variety, which was also the only one where the Sp245 inoculated plants displayed a significantly higher shoot weight than the Sp7 inoculated ones. The Sp245 colonized plants exhibited a 30% weight gain compared to the control. In the Brazilian wheat variety, plants inoculated with Sp7 showed the highest shoot weight, which was almost 60% over the control (Fig. 2B).

Detection of root colonizing A. brasilense with fluorescent in situ hybridization

Since the plate count methods were not able to give detailed information on root colonization by the *A. brasilense* strains Sp7 and Sp245, a microscopic approach was applied. As Abras1420 is a species specific oligonucleotide probe for *A. brasilense*, it was well suited for identifying the inoculated bacteria of interest by FISH analysis (Stoffels et al., 2001). Strain Sp7 could be found in great numbers colonizing the surfaces of main roots, side roots, and root hairs in the monoxenic system (Fig. 3C, D).

In all three cultivars bacterial density was highest on the older basal root parts with cell numbers decreasing slightly toward the root's apical end. Strain Sp245 colonized the root surface similar to Sp7, but was also detected in low numbers within the outer cortex cells. *A. brasilense* Sp245 was able to colonize the intercellular spaces (apoplast), as well as the inside of root cells. Whether these were living cortex cells or already dead ones could not be determined. We were able to detect these endophytic bacteria in all three cultivars in about every 8th examined root slice.

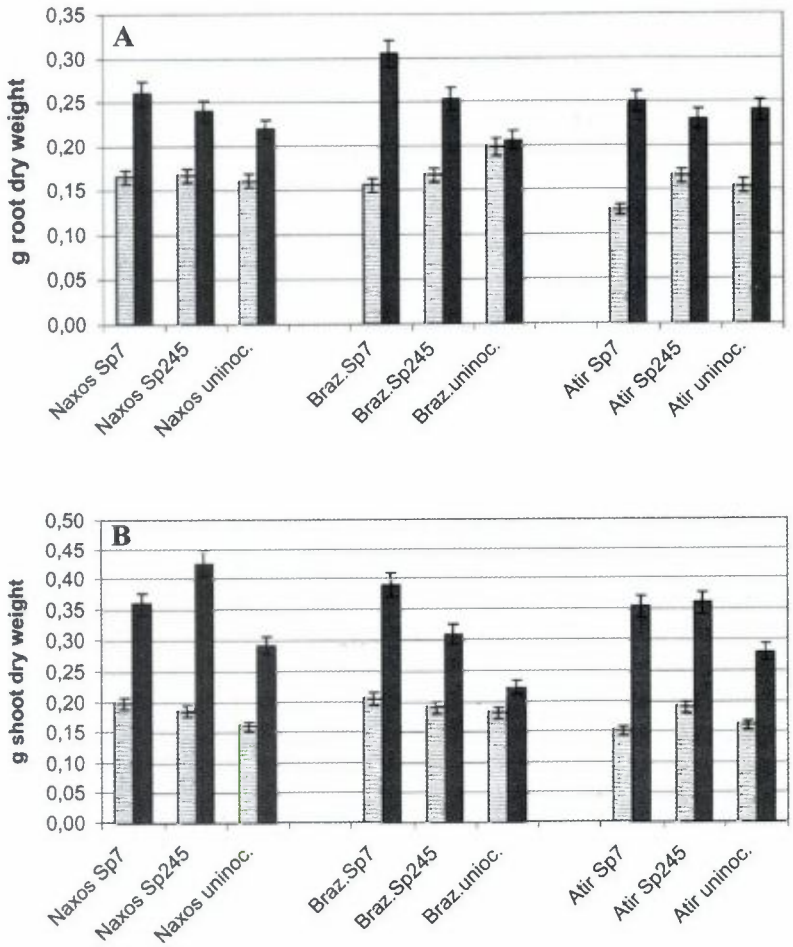

Figure 2. Root (A) and shoot (B) dry weight per plant of *Azospirillum brasilense* Sp7 or Sp245 inoculated, as well as uninoculated wheat varieties Naxos, Brazilian (PF839197), and Atir after growing on soil for 4 (striped columns) and 7 weeks (filled columns).

In soil-grown roots of the three wheat varieties the root colonization by strain Sp7 and Sp245 was considerably lower than in the monoxenic system. *A. brasilense* had to compete with various other root colonizers, which occur in greater numbers than *Azospirillum* itself. These other rhizosphere bacteria were detected by the Cy3 labeled probe EUB338-mix and are therefore depicted in red (Fig. 3A, B). Accordingly, cells of strain Sp245 colonizing the interior of

the roots were found less frequently. Only about every 20th slice revealed positive hybridization signals within the cortex layer. In contrast, strain Sp7 was only detected on the root surface throughout all of the scanned samples.

Visualization of the gfp tagged cells

In order to enable visualization of *A. brasilense* cells in intact root tissue, which was neither cut nor exposed to the chemical and physical stress during fixation and FISH procedure, both strains were tagged with a *gfp* marker gene. The plasmid pBAH7 containing this molecular marker was tested for its stability within the cell without antibiotic pressure. After growth for more than one hundred generations on NB Agar plates, about 50% of the cells were still fluorescent. The germinated seeds of all three wheat cultivars were inoculated with the *gfp* tagged *A. brasilense* Sp7 or Sp245 and grown in a monoxenic system for 4 weeks at greenhouse conditions. After harvest parts of intact roots were examined by confocal microscopy for endophytic colonization. With the help of the CLSM it was possible to reach up to 40 µm into the root cortex. The *A. brasilense* strains were distributed quite equally on the surface area and root hairs, as was already discovered with the FISH technique. Again the cell densities were found to diminish slightly from basal to apical end. A clear difference was observed in the colonization behavior of the two *A. brasilense* strains. Strain Sp245 was found quite frequently in between or even within the outer cortex cells (Fig. 4A, C), whereas this endophytic root colonization could not be attributed to strain Sp7 (Fig. 4B, D). Though these cortex colonizing Sp245 cells were easier to locate within the root tissue than with FISH due to their gfp marker, still the overall numbers of endophytic bacteria detected were rather low. Since structurally unaffected root parts were used, it could be finally proofed that, although in small numbers, *A. brasilense* strain Sp245 is able to colonize the outer cortex cells of all three wheat cultivars endophytically.

4. Discussion

The stimulatory effect of *A. brasilense* inoculation on wheat shoot and root development and yield has been documented several times in various previous studies (Boddey et al., 1986; Mertens and Hess, 1984; Millet et al., 1985; Dobbelaere et al., 2001). Consistently, a pronounced growth stimulation effect was found in all three examined wheat cultivars after 7 weeks of growth on a German sandy soil when inoculated with *A. brasilense* strains. This effect was not so clear after 4 weeks of growth, possibly because stored nutrients in the seed dominated the early plant development. Seven weeks after inoculation the

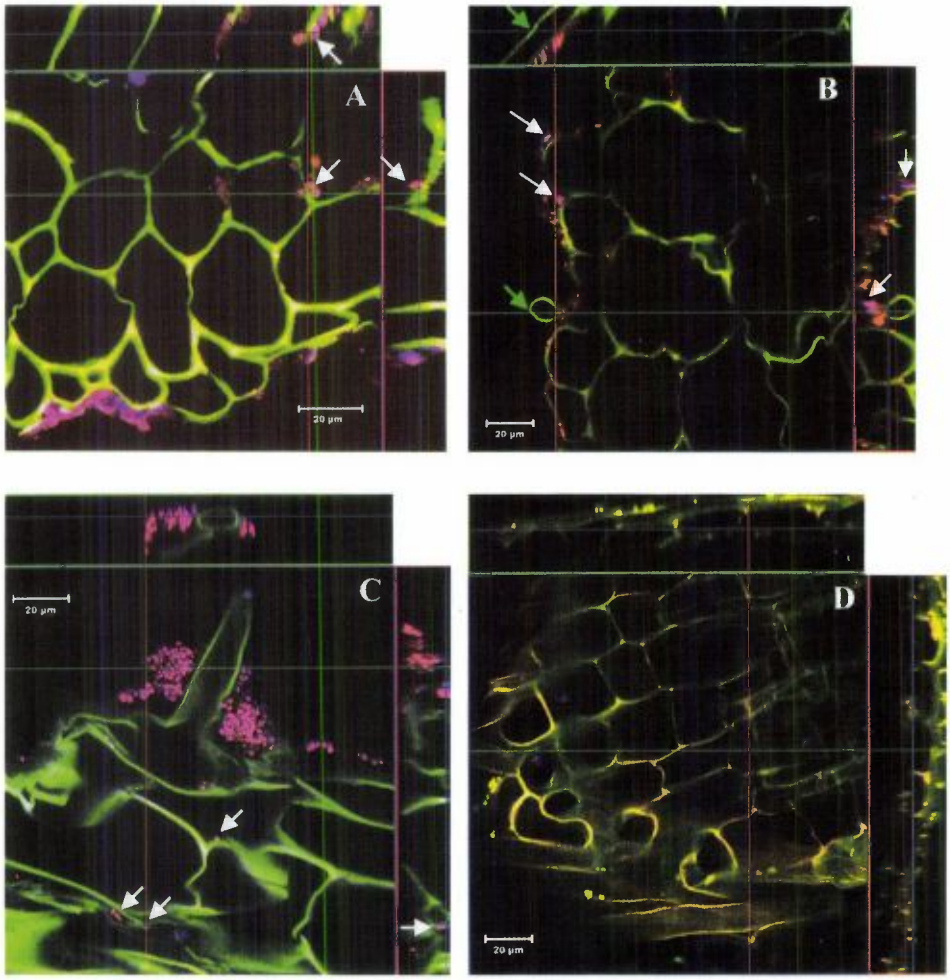

Figure 3. *In situ* detection of *Azospirillum brasilense* strains Sp7 and Sp245 by FISH analysis and CLSM in root slices. All pictures were taken from roots, which were harvested after 4 weeks of growth. The pictures show orthogonal views of a three dimensional confocal image created from a z-stack of xy-scans. The top view, framed in blue, gives one picture from the middle of this z-stack. The red and green lines represent vertical optical cuts through the stack, which result in the side view images framed in red and green, respectively. In these side views the blue line marks the vertical position, where the top view image is located within the z-stack.

(A) Radial slice from the root hair zone of the Brazilian wheat PF839197 grown in soil and inoculated with *A. brasilense* strain Sp245. *A. brasilense* is identified by the color magenta, which is the combination of both the fluorescent signal of EUB338-mix labeled with Cy3 (red) and Abras1420 labeled with Cy5 (blue). Numerous Sp245 cells are visible on the root surface. Arrows indicate positive hybridization signals within the root cortex.

Brazilian wheat cultivar showed the strongest response to *A. brasilense* colonization, which indicates that, although both *A. brasilense* strains colonize all three cultivars to the same extent, the Brazilian wheat cultivar may be able to gain greater benefit from its partnership with *Azospirillum*. Possibly, this is due to an adaptation process of the plant to a common bacterium in tropical soils, such as *A. brasilense* (Döbereiner et al., 1976). Although the cultivar Naxos had the largest shoot weight, the difference of inoculated and not inoculated plants as a measure of the growth promoting effect of *A. brasilense* was much smaller than in the Brazilian wheat cultivar. The fact that the wheat variety Atir did not respond as well to inoculation, may be explained by a lack of adaptation to the soil conditions and the inoculum used.

Another aim of this study was to determine whether *A. brasilense* Sp245 was able to colonize the root interior effectively and to compare its colonization behavior to strain Sp7. Schloter and Hartmann (1998) reported that strain Sp245 was able to colonize the Brazilian wheat cultivar PF879197 grown in Loess soil endophytically, whereas strain Sp7 could not.

In this study Sp7 or Sp245 inoculated seeds were grown in a monoxenic system, which was used because of higher colonization numbers compared to inoculated roots grown in soil. The detected amount of Azospirilli colonizing the root surface of monoxenically grown plants did neither depend on the inoculated

Figure 3. *Continued.*

(B) Radial slice from the root hair zone of the Brazilian cultivar PF839197 grown in soil, inoculated with *A. brasilense* strain Sp7. Several cells (magenta, marked by white arrows) are visible on the root surface, but none in the interior. Additionally some other colonizing bacteria are shown in red (EUB338-mix Cy3). The green line indicates an optical sectioning through a root hair (green arrow), which in the green framed side view is shown to be colonized by several *A. brasilense*.

(C) Longitudinal slice from the root hair zone of a monoxenically grown Naxos wheat inoculated with *A. brasilense* strain Sp245. Arrows indicate endophytic colonization. Hybridization was performed with EUB338-mix Cy3 and Abras1420 Cy5. The green framed side view shows an optical cut through the massively surface colonized root hair.

(D) Longitudinal slice from the root hair zone of a monoxenically grown Naxos wheat root inoculated with *A. brasilense* strain Sp7. Hybridization was performed with EUB338-mix FITC and Abras1420 Cy3, which results in a yellow signal. Clearly, the Sp7 cells on the surface of the root and the absence of colonizing bacteria in the inside of the cortex are noticeable in the side views. The top view shows the not colonized interior of some root cells and *A. brasilense* Sp7 attached to root hairs on the cell surface.

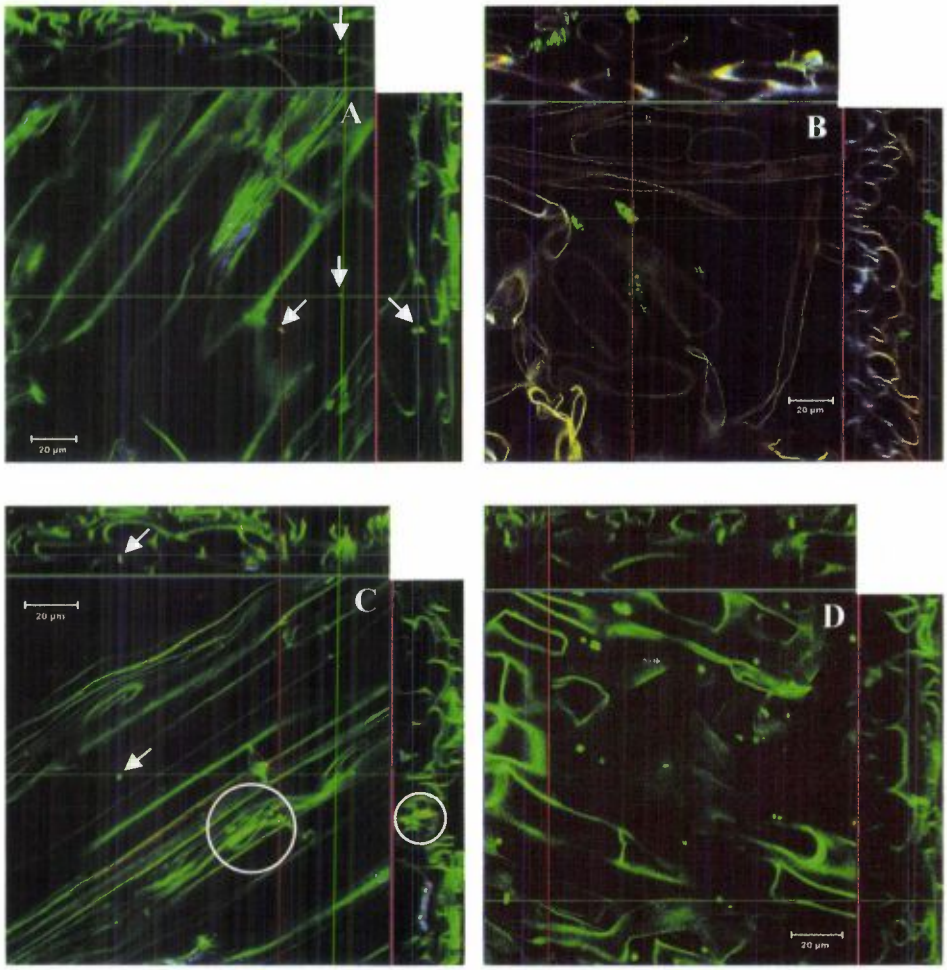

Figure 4. Localization of Gfp-marked *Azospirillum brasilense* strains Sp7 and Sp245 colonizing uncut roots of 4 weeks old, monoxenically grown wheat plants using optical sectioning by CLSM. For explanation of orthogonal view see Fig. 3.
(A) Basal part of an Atir wheat root inoculated with *A. brasilense* Sp245. Several Gfp-fluorescing Sp245 cells (green) are visible on the surface of the root but some are also revealed in a cortex cell (white arrows). The same individual cells can be seen in top and side views.
(B) Basal part of an Atir wheat root inoculated with *A. brasilense* Sp7. Gfp-fluorescing Sp7 cells are shown in top and side views, colonizing the surface of a root hair in great numbers. None are detectable inside the root.
(C) Root hair zone of a Naxos root, inoculated with gfp tagged *A. brasilense* strain Sp245. The white arrow marks an *A. brasilense* endophyte both in top and side view. The white circle encloses some cells, which could be easily mistaken for endophytic colonizers, as the top view shows a picture of the z-stack, which is from a position already quite deep in the root interior.

strain nor the cultivar used. The conditions of surface sterilization with chloramine T (Baldani et al., 1986) had been optimized in terms of chloramine T concentration and incubation time to provide a pronounced inactivation of surface colonizing strain Sp7 by a factor between 10^{-2} and 10^{-3}. After this treatment it is most likely that not only endophytes remain, but also other closely associated cells, which are embedded in the mucigel of the rhizoplane or colonize folds in the root surface and otherwise protected locations. Using this chloramine T treatment and plating techniques a clear difference of root colonization between strain Sp7 and Sp245 could not be demonstrated in our study. Schloter and Hartmann (1998), who showed a more extensive endophytic colonization of wheat roots by strain Sp245 under similar greenhouse conditions, used different soil and a wheat variety, which had been used for the original isolation of strain Sp245.

The application of microscopic *in situ* detection techniques made the demonstration of endophytic colonization of wheat roots by Sp245 possible. A 16S rRNA-targeted oligonucleotide probe for a species specific identification of *A. brasilense* by FISH (Stoffels et al., 2001) was used. All root material exhibited strong autofluorescence especially after fixing and dehydrating the samples. When observing thicker root samples (>10 μm) additional unfocused light comes from beneath and above the focal plane, which adds up to a quite intense background fluorescence, frequently masking the specific signals by the fluorescently labeled target cells.

Therefore, a confocal laser scanning microscope (CLSM) had to be applied to circumvent these obstacles (Hartmann et al., 1998). By producing three dimensional images from a z-stack of optical sections an exact determination of the position of colonizing cells in the tissue down to a depth of 40 μm was possible. Because of the low accessibility of deeper root tissues for the probes it was necessary to prepare radial or longitudinal slices not exceeding a thickness of about 100 μm. Cells which were transferred during the cutting process from

Figure 4. *Continued.*

(C) But the red framed side view reveals, that in fact the bacteria are attached to the root surface, but at the lowest point of a root surface depression.

(D) Root hair zone of a Naxos wheat, which was inoculated with *gfp* tagged strain Sp7. The blue framed top view window shows a picture out of the z-stack from the root surface area, which is heavily colonized by strain Sp7. Both side views demonstrate that this colonization is limited to the surface of the root and root hairs, and no *A. brasilense* Sp7 cells are present in the root interior.

their original position to a location within the tissue, were most likely washed away during the following fixation, dehydration, hybridization, and washing steps. The complete absence of Sp7 cells inside the roots supports this conclusion. In contrast to strain Sp7, strain Sp245 was repeatedly found in the inter- and intracellular spaces of the root cortex of all three wheat cultivars. As the overall number of colonizing *A. brasilense* was higher on roots from monoxenic systems, it was reasonable to find Sp245 more often within the root tissue of monoxenically grown wheat compared to soil grown plants.

To resolve all doubts in proving the endophytic localization of strain Sp245, the gfp bearing plasmid pBAH7 was transferred to both *A. brasilense* strains. The *gfp* tagged bacteria could be detected on the root surface and interior without any further manipulation or chemical treatment of the sample. By producing three dimensional images with the CLSM an unequivocal demonstration of endophytic colonization of strain Sp245 in the root cortex was possible (see Fig. 4C). For further studies on the localization of *A. brasilense* Sp245 within the root cell an analysis with an electron microscope will be necessary. However, these findings are in agreement with many previous studies (Baldani et al., 1987; Schloter et al., 1994; Assmus et al., 1995; Schloter and Hartmann, 1998), which all suggested that Sp245 is able to penetrate root tissue. The observation of different colonization efficiency and the endophytic colonization of whole cells and root hairs may be attributed to different wheat cultivars and growth conditions used. This endophytic behavior did not correspond to the observed increase in plant growth promotion. Therefore, endophytic colonization, at least in such low numbers, is apparently not an important determinant of the observed beneficial effect of the inoculum.

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