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Dynamics of a Transposon Tn5 Mutant of *Azospirillum brasilense* in Soil and Rhizosphere of Spring Wheat

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Abstract

Azospirillum brasilense was marked by insertion of transposon Tn5 into its genome. The Tn5 insertion did not interfere with physiological characteristics as nitrogen fixation, auxine production and nitrate reduction nor with the growth rate of the bacterium. The detection limit of the technique was as low as approx. 25 cells per gram dry soil.

Upon introduction of the Tn5 marked *A. brasilense* to an unplanted, sterilized soil, the number of cells remained constant over a period of 100 days at approximately 10^7 cells per gram dry soil. In a non-sterilized soil the population decreased during the same period from 10^4 to 10^3 cells per gram soil. Within 45 days after introduction *A. brasilense::Tn5* was found in roots and rhizosphere soil of a sterile-grown spring wheat in amounts of approximately 10^6 cells per gram dry root and 10^6 cells per gram dry soil, respectively. The number in a similar non-sterile grown plant was low at approximately 10^3 cells per gram dry root and 10^4 cells per gram dry rhizosphere soil. In all cases the number of *A. brasilense::Tn5* cells was 10–100 times higher in the soil fraction close to the roots than in the root-free soil. *A. brasilense::Tn5* could not be isolated from inner root tissue after root surface sterilization.

Significant differences in later root colonization were not found when *A. brasilense::Tn5* was applied to the plant either by seedling inoculation with

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a cell suspension or by bacterial seed coating. *A. brasilense::Tn5*, when introduced locally by seed coating, was able to develop with the elongating root. In plants grown under non-sterile conditions the colonization of the developing root system appears to be much lower than in sterile-grown plants. Competition between *A. brasilense::Tn5* and a *Pseudomonas fluorescens* strain as a competing introduced rhizosphere bacterium was found not to have an effect.

Keywords: *Azospirillum brasilense*, rhizosphere, spring wheat, Transposon, Tn5

1. Introduction

Azospirillum spp. are known to influence growth and nutrient uptake of the host plant (O'Hara et al., 1987; Warenbourg et al., 1987). Increases in root dry matter production, nitrogen content and plant yield due to inoculation by *Azospirillum* have been reported by Baldani et al. (1983), Schank et al. (1981), Watanabe and Lin (1984) and others. The mechanisms of this growth-stimulating activity of *Azospirillum* are still under debate. Improved N-supply by N_2 fixation, the production of plant growth-stimulating substances (Tien et al, 1979; Harari et al., 1988) and bacterial nitrate reductase activity (Scott et al., 1979) have been mentioned in this respect. Several attempts have been made to make use of these potentially beneficial characteristics of *Azospirillum* by field inoculation of crops (Kapulnik et al., 1981; Reijnders and Vlassak, 1982).

Irrespective of the mechanisms involved, a vital factor for successful application is that *Azospirillum* is able to establish in soils and in the rhizosphere in sufficient numbers, and that the introduced cells are able to compete with the native bacterial population in a natural environment. Such survival studies require a bacterial marker which does not interfere with essential physiological characteristics. Spontaneous drug-resistant *Azospirillum spp.* have been used before to trace introduced *Azospirillum* in a natural environment (Nayak et al., 1986). In our experiments we marked *Azospirillum brasilense* by a transposon (Tn5), inserted into its genome. This marked *A. brasilense::Tn5* was used to study its potential survival capacities either under sterile or under non-sterile conditions. The suitability of Tn5 transposon mutants for such studies was shown successfully by van Elsas et al. (1986) and Fredrickson et al. (1989). The main objective of our work was to quantify the dynamics of introduced *A. brasilense::Tn5* in a wheat-root environment.

2. Materials and Methods

Bacterial strains

As wild type *Azospirillum brasilense* we used strain Wa5, originating from the rhizosphere of greenhouse-grown spring wheat (Christiansen-Weniger, 1988). Tn5 transposon insertion into *A. brasilense* was carried out as described by Singh and Klingmüller (1986). The Tn5 transposon contains genes coding for resistance to kanamycin (Kn) and streptomycin (Sm). The bacteria were screened for spontaneous resistance to rifampicin (50 μg ml^{-1}). Rifampicin-resistant *A. brasilense* was mated overnight at 37°C on a nitrocellulose filter in coculture with *Escherichia coli* MV12, containing the transposon Tn5 on the suicide plasmid pGS9 (Selvaraj and Iyer, 1983; a gift from W. Klingmüller, Bayreuth). Mated bacteria were resuspended in 0.8% NaCl solution and plated out on selective Luria broth ager (50 μg rifampicin ml^{-1}, 80 μg kanamycin ml^{-1}). Transconjugant *A. brasilense* were picked up as small white rifampicin- and kanamycin-resistant colonies. Selected Tn5 mutant *A. brasilense* were tested for their growth, nitrogenase activity, auxin production and nitrate reductase activity to see whether the transposon insertion had damaged these key characteristics of the bacterium.

Pseudomonas fluorescens strain R2f was used for competition studies. This strain, an isolate from the rhizosphere of grass, contained the plasmid RP4 (Van Elsas et al., 1988). The bacterium was resistant to kanamycin and tetracyclin.

Biochemical assays

Bacterial growth (OD 540) was determined in a 50 ml-batch (30°C, 350 rpm) of minimal NFB medium (Okon et al., 1977), supplemented with 10 mM NH_4Cl.

Acetylene reduction was measured under oxygen-limited conditions in a semi-solid (0.2% agar) NFB medium with 10% v/v acetylene added to the head space.

Total bacterial protein was analysed after cell lysis in 1 N NaOH at 60°C by the procedure of Lowry et al. (1951) using bovine serum albumine as standard.

Auxin formation was tested by incubating *A. brasilense* for 48 hr in a batch of NFB minimal medium containing 10 mM NH_4Cl and 100 mg DL-tryptophan per litre (30°C, 350 rpm). Produced indole-acetic-acid (IAA) in the supernatant was determined by the Salkofski color reaction after centrifugation (Tang and Bonner, 1946).

Nitrate reductase activity was determined in semi-solid (0.2% agar) NFB medium containing 8 mM NH_4NO_3 (Nicholas and Nason, 1957).

Soils and plants

The soil used was a loamy sand (0.3 mg N kg^{-1}, 3.5% organic matter, 15% moisture). Seventy mg P, 98 mg K, 50 mg N per kg soil and trace elements according to the Hoagland nutrient solution (Hoagland and Broyer, 1936) were added. The soil was sterilized by exposing soil samples to 4 megarad γ-radiation. For incubation studies 2-liter pots with 1 kg fresh soil were used, covered with plastic lids to avoid contamination. Sterilized water was added daily to adjust the moisture content.

To be able to follow root colonization over different distances from the point of inoculation, plants were grown for 2 weeks in narrow soil columns (5 cm diameter and 50 cm length), both under axenic and natural conditions. As the columns could be opened without disturbing the root environment, separation of root and soil samples over the entire length of the column was possible.

The spring wheat used was *Triticum aestivum* var. "Ralle" (received from the Foundation for Agricultural Plant Breeding SVP, Wageningen). Seeds were surface-sterilized with 1.5% Na-hypochlorite (90 min) and pregerminated on tryptone soya agar (TSA, oxoid) to check for sterility. Seeds with a bacterial coating were pregerminated in long tubes on sterilized or non-sterilized soil. After 7 days the seedlings were transferred to the growing pots, 3 plants each. One plant was transferred to each soil column. Plants were sealed around the stem with a cotton plug. Growth conditions were: 12 hr day at 20°C and 12 hr night at 12°Cwith a relative humidity of 70%.

Bacterial inoculations

Bacteria were grown in a batch (350 rpm) at 37°C in Luria Broth (LB) until the end of the logarithmic growth phase. The culture was centrifuged and resuspended in a 0.85% NaCl solution. Final cell density was 1.4×10^8 CFU (colony forming units) ml^{-1}. Five mls of the cell suspension were injected in the centre of each pot in case of unplanted soils and for seedling inoculation. The final bacterial density was 7.9×10^5 CFU per gram dry soil. Controls were treated with an autoclaved bacterial suspension. For bacterial coating seeds were treated with a solution of sterile gum arabic (40%), supplemented with a bacterial culture to a final cell density of 1.3×10^7 CFU ml^{-1}. Surface-sterilized, as well as untreated seeds, were dipped into this mixture and transferred to germination tubes. The average number of bacteria on the seeds was 2×10^6 CFU per seed. Treatments were carried out with 3 replicate plants each; in column experiments with 2 replicate columns each.

Sampling and determination of bacterial numbers

Soil samples were taken from 2 l pots with sampling tubes of 1 cm diameter and 10 cm length of 3 cm distance from the point of inoculation; material was collected over the full depth of the pot. Samples were shaken intensively in 95 ml 1% Na-Pyrophosphate solution; 15 g gravel was added to disrupt soil aggregates. The suspensions were plated out after subsequent tenfold dilution steps.

To determine the numbers of root colonizing bacteria, root samples were divided into 3 different fractions. The first fraction consisted of rhizosphere soil, which remained on the roots after shaking and which was released by careful washing in 95 ml 1% Na-pyrophosphate solution. The second fraction was the washed root itself, and the third fraction was formed by the roots after surface-sterilization with 1% chloramine T solution (15 min Patriquin and Döbereiner, 1978). For counting bacteria, root material was macerated in homogenizing tubes. Bacteria were determined in these rhizosphere fractions and also in root-free bulk soil. Since an accurate separation of replicate plant roots was impossible in pot experiments (roots were intertwining) an average sample of all 3 replicate plants was taken.

Soils and roots were dried in 80°C for dry matter analysis.

The bacterial suspensions were plated on the following media:

- for total bacteria: tryptone soya agar, TSA (Oxoid);
- for *A. brasilense::Tn5*: NFB minimal malate medium with kanamycin (50 mg l^{-1}), rifampicin (50 mg l^{-1}) and kongo-red added to a final concentration of 4 g l^{-1} (Bashan and Levanony, 1985);
- for *P. fluorescens R2f*: Kings B medium (20 g l^{-1} proteose peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 15 ml glycerol, pH 7.3) with kanamycin 50 mg l^{-1} and tetracycline 30 mg l^{-1}.

All media were supplemented with cycloheximide (50 mg l^{-1}) and benomyl (30 mg l^{-1}) to suppress fungal growth.

Double-resistant *A. brasilense* or *P. fluorescens spp.* were not detected in the natural soils as evidenced by plating on selective Kings B and NFB media.

3. Results and Discussion

Neither the occurrence of a spontaneous rifampicin-resistance nor the transposon Tn5 mutagenesis caused a serious decrease in the growth rate of *Azospirillum brasilense*. Key characteristics such as N_2 fixation, auxin production and nitrate reductase activity were not significantly affected.

When plated out on congo-red containing NFB medium, *A. brasilense* formed typical small red colonies. Together with the double resistance to kanamycin and rifampicin, a powerful marker combination is formed, which allows efficient detection at a detection limit as low as 25 cells per gram dry soil. Bentjen and coworkers (1989) reported that plate counting in case of a Tn5 containing *A. lipoferum* was less effective than MPN counting by DNA-hybridization. However, in the cited paper the authors used only a single drug resistance (Tn5 bound kanamycin resistance) and observed that in this case indigenous kanamycin resistant bacteria overgrew *Azospirillum* on selective plates, in particular at low *Azospirillum* cell densities. Encystation of *A. brasilense* (Papen and Werner, 1982; Sadasivan and Neyra, 1985) may cause an underestimation of real *A. brasilense* populations. Colony development from cysts on minimal agar plates is till now a matter of debate.

Upon introduction into a sterilized, unplanted soil the number of *A. brasilense::Tn5* cells increased within 20 day after incubation from approximately 10^6 CFU per gram oil to 10^8 CFU and remained at that level until day 100 (Fig. 1). These numbers were similar to those observed for *Rhizobium leguminosarum* biovar trifolii and *Pseudomonas fluorescens* R2f in

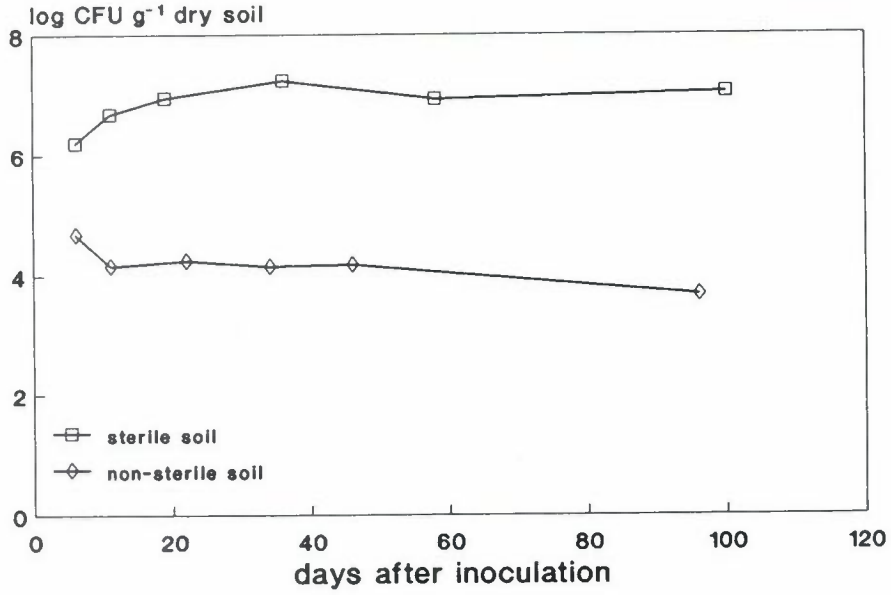

Figure 1. Survival of *A. brasilense::Tn5* in a sterilized and in a non-sterilized soil; inoculation by 5 ml bacterial suspension (1.4×10^8 CFU ml^{-1}) to 1 kg fresh soil.

the same soil (van Elsas et al., 1989; Postma et al., 1990). The observed dynamics of an introduced *A. brasilense::Tn5* population in a sterilized soil were similar to data reported by Steinberg et al. (1989); total cell numbers, however, were approximately 100 times lower in our study. This may be attributed to the fact that in the study of Steinberg et al. (1989), bacterial dynamics were studied in small microcosms of 8–10 g soil to which the bacteria were introduced by spreading over the complete samples, followed by intensive mixing. In our experiments a relatively small amount of inoculation suspension (5 ml) was added to a large amount of soil (1 kg) so that the bacteria may have been clustered through the lack of any vector for bacterial movement over larger distances. Thus full exploitation of the available substrate was not achieved (Postma and Van Veen, 1989).

The survival of *A. brasilense::Tn5* in a non-sterilized soil was low. 10^3 CFU were counted on day 6 and this number decreased slowly to 10^2 CFU on day 100 (Fig. 1). These results correspond with those of Harris et al. (1989) for the survival of introduced *A. brasilense* under field conditions. These results show that genetically modified *A. brasilense::Tn5* survives for more than 3 months in a natural environment, which may have consequences for the assessment of the risks involved in the use of genetically modified microorganisms in soil.

When introduced to wheat plants by coating of surface-sterilized seeds, *A. brasilense::Tn5* was observed in the root-free soil fraction with cell numbers above the detection limit only after 14 days, once they slightly increased in sterilized soils (Figs. 2 and 3). On washed roots grown under non-sterile conditions the number of *A. brasilense::Tn5* cells decreased from 10^4 CFU per gram root on day 7 after planting, to 10^3 CFU on day 35. In the rhizosphere soil, the number of *A. brasilense::Tn5* remained stable over the entire period of plant growth at 10^4 CFU per gram dry soil as compared to only 10^2 in the root-free soil fraction (Fig. 2). With sterile-grown plants, the number of *A. brasilense::Tn5* increased in the root-free soil within 35 days to 10^5 CFU per gram soil. The amounts of *A. brasilense::Tn5* in the root and in rhizosphere soil were stable at 10^5 CFU per gram dry root or 10^6 CFU per gram rhizosphere soil, respectively (Fig. 3).

In sterile as well as in non-sterile grown plants, the cell densities of *A. brasilense::Tn5* were 10–100 times higher in the rhizosphere soil than in corresponding root-free soil (Tables 1 and 2).

Large numbers of *A. brasilense::Tn5* (10^4 CFU to 10^5 CFU per gram root, Tables 1 and 2) were also found on the roots after careful washing. *Azospirillum* was mentioned to be bound to roots by fibrillar material (Whallon et al., 1985; Murty and Ladha, 1987), as well as to colonize inside intercellular spaces of the root cortex (Bashan and Levanony, 1988). This means that the plant root

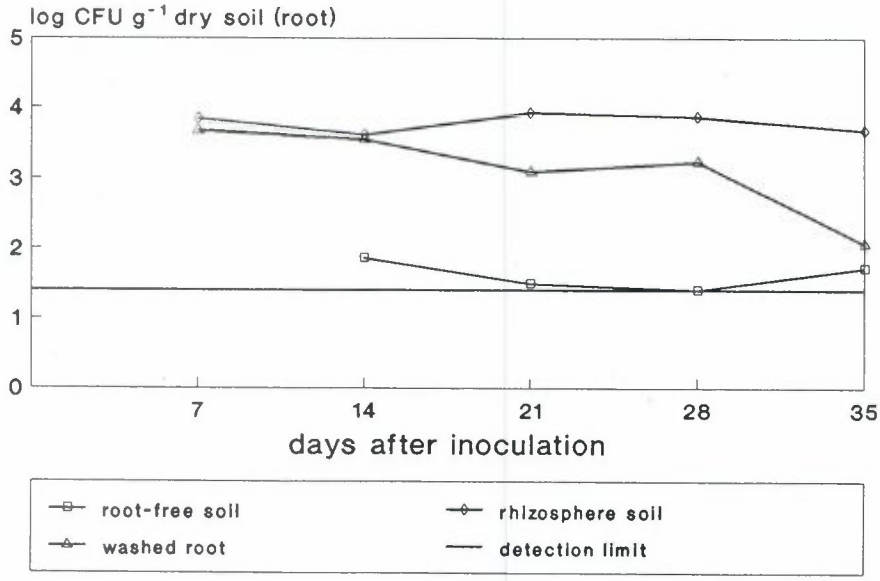

Figure 2. Survival of *A. brasilense::Tn5* in the rhizosphere of non-sterile-grown summer wheat; inoculation by bacterial seed coating (2×10^6 CFU seed^{-1}).

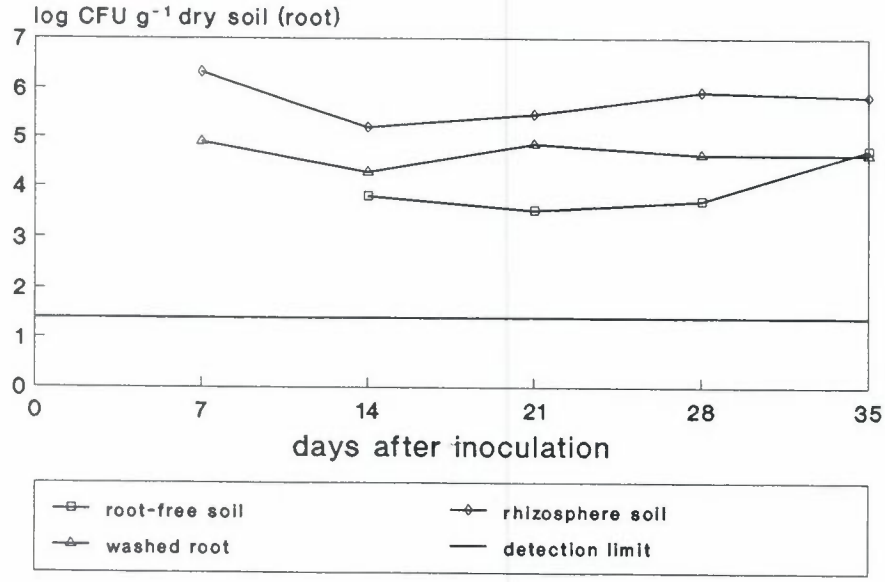

Figure 3. Survival of *A. brasilense::Tn5* in the rhizosphere of sterile-grown summer wheat; inoculation by bacterial seed coating (2×10^6 CFU seed^{-1}).

Table 1. Number of *A. brasilense::Tn5* cells from soil (log CFU g^{-1} dry soil) and roots (log CFU g^{-1} dry root) of sterile-grown plants (35 days after inoculation)

	Seedling inoculation	Coating of surface-sterilised seed	Coating of untreated seed
Root-free soil	4.94	4.69	4.72
Rhizosphere soil	5.90	6.43	5.85
Washed root	4.38	5.19	4.41
Surface-sterile root	[0][1]	[0]	[0]

[1] arithmetic zero, below detection limit (1.4 log CFU g^{-1} dry root)

Table 2. Number of *A. brasilense::Tn5* cells from soil (log CFU g^{-1} dry soil) and roots (log CFU g^{-1} dry root) of nonsterile grown plants (35 days after inoculation)

		Seedling inoculation	Coating of surface-sterilised seed	Coating of untreated seed
Root-free soil	TP[1]	7.61	7.13	7.26
	AZO[2]	2.06 (0.01)[3]	2.88 (0.25)	1.82 (< 0.01)
Rhizosphere soil	TP	8.11	8.08	8.18
	AZO	4.50 (0.25)	4.7 (0.42)	3.71 (0.03)
Washed root	TP	7.34	7.02	7.38
	AZO	3.57 (0.17)	3.62 (0.40)	2.04 (< 0.01)
Surface-sterile root	TP	5.23	5.33	5.62
	AZO	[0][4]	[0]	[0]

[1] TP: total bacterial population
[2] AZO: *A. brasilense::Tn5*
[3] relative densities of *A. brasilense::Tn5* are given in parentheses as ‰ of the total number of bacteria
[4] arithmetic zero, below detection limit (1.4 log CFU g^{-1} dry root)

forms a suitable colonization niche for introduced *A. brasilense*. Moreover, *Azospirillum* is chemotactically attracted by organic acids, sugars and amino acids exuded by roots (Barak et al., 1983; Heinrich and Hess, 1985; Reinhold et al., 1985). Active migration of *A. brasilense* in soils towards an attractant is reported by Bashan (1986). This binding to roots, however, is not an essential process for the survival of introduced *Azospirillum::Tn5* as can be concluded from the large numbers of bacteria in the adhering soil. Furthermore, no introduced *A. brasilense::Tn5* was reisolated form surface-sterilized roots, although 10^5 total bacterial CFU, which is approximately 1% of the total root

population, was counted inside the roots after surface-sterilization with 1% chloramine T (Table 2). This contradicts the hypothesis of Patriquin and Döbereiner (1978) that *Azospirillum* settles at high cell densities inside the xylem system of the host plant.

Among the different application methods, the highest number of *A. brasilense::Tn5* cells was observed in the root-free soil following seedling inoculation. Seed coating (using surface-sterilized seeds) caused better *A. brasilense::Tn5* root colonization (Tables 1 and 2), although the initial numbers of introduced *A. brasilense::Tn5* cells were approximately 100 times lower in case of seed coating than upon seedling inoculation. Coating of non-surface-sterilized seeds caused lower numbers of *A. brasilense::Tn5* cells in all three root fractions than coating of surface-sterilized seeds (Table 1). As the percentage of *A. brasilense::Tn5* relative to the total bacteria population was very low (maximum 0.042% of total number of bacteria, Table 2), competition with the natural flora might be an important aspect. However, *Pseudomonas fluorescens* R2f, when introduced together with *A. brasilense::Tn5* as a second rhizosphere bacterium, did not affect the root colonization of *A. brasilense::Tn5*. Again, no differences in later rhizosphere colonization occurred between seedling inoculation and coating of surface-sterilized seeds (Table 3).

This indicates that *P. fluorescens* R2f and *A. brasilense::Tn5* may occupy different niches on the host root. Yet, when *P. fluorescens* R2f was inoculated in a later stage of plant growth (day 15) smaller amounts of soil and root colonization were observed (Table 3). Van Elsas et al. (1989, 1991b) reported recently a plasmid loss of RP4 from *P. fluorescens* R2f when bacteria were introduced to a loamy sand. This led to an underestimation of the counted bacterial population.

A. brasilense::Tn5 when introduced by seed coating grew along the developing root and established a population over the total rhizosphere (Fig. 4). Under axenic conditions, the numbers of *A. brasilense::Tn5* at distances 25 to 37.5 cm from the seed were of the same order of magnitude as the numbers close to the seeds. This was true for all three rhizosphere fractions (Fig. 4). With plants grown under non-sterile conditions, the number of *A. brasilense::Tn5* decreased with the distance from the place of inoculation, but root and rhizosphere were still colonized. At a distance of 33 to 50 cm from the seed, introduced *A. brasilense::Tn5* could not be detected in the root-free soils (Fig. 5). When treated with *A. brasilense::Tn5*, plants growing in sterilized soil developed a significantly higher root biomass than plants in non-sterilized soil; the phenotype of a sterile-grown root showed increased branching and higher lateral root development than roots in a natural environment (Fig. 6). This may be explained by the fact that associated *Azospirillum* stimulates root growth

Table 3. Number of *A. brasilense::Tn5* cells from soil (log CFU g^{-1} dry soil) and roots (log CFU g^{-1} dry root) of sterile-grown plants inoculated together with *Pseudomonas fluorescens R2f* (35 days after inoculation)

		No PS	PS, seedling inoculation	PS, late inoculation (day 15)
		A. brasilense introduced by seedling inoculation		
Rootfree soil	PS[1]		6.92	5.24
	AZO[2]	4.94	4.97	4.63
Rhizosphere soil	PS		7.58	6.01
	AZO	5.9	6.42	5.98
Washed root	PS		6.26	4.98
	AZO	4.38	4.94	4.23
		A. brasilense introduced by seed coating		
Rootfree soil	PS		6.52	5.76
	AZO	4.69	5.08	5.25
Rhizosphere soil	PS		8.04	6.48
	AZO	6.43	5.90	6.14
Washed root	PS		6.71	4.83
	AZO	5.19	4.90	4.55

[1] PS: *P. fluorescens R2f*
[2] AZO: *A. brasilense::Tn5*

and morphology, probably due to the production of plant growth substances such as indole-acetic-acid (IAA) (Barbieri et al., 1986; Harari et al., 1988).

Thus, although roots provided a suitable niche for *Azospirillum::Tn5*, and movement together with the developing root system is possible, competition, predation and other interactions with the soil fauna and flora prevent the bacterium to fully develop over the root system. This clearly also affects the influence of *Azospirillum::Tn5* on root development.

Our experiments demonstrate the establishment of *Azospirillum::Tn5* in the soil and in the wheat rhizosphere; cell densities, however, were negligibly low. This makes it unlikely that under temperate field conditions *Azospirillum* inoculation will have a considerable effect on the host plant. The Tn5 transposon insertion in combination with congo red staining and growth on nitrogen-free medium was found to be a reliable marker for tracing introduced *Azospirillum* in natural environments.

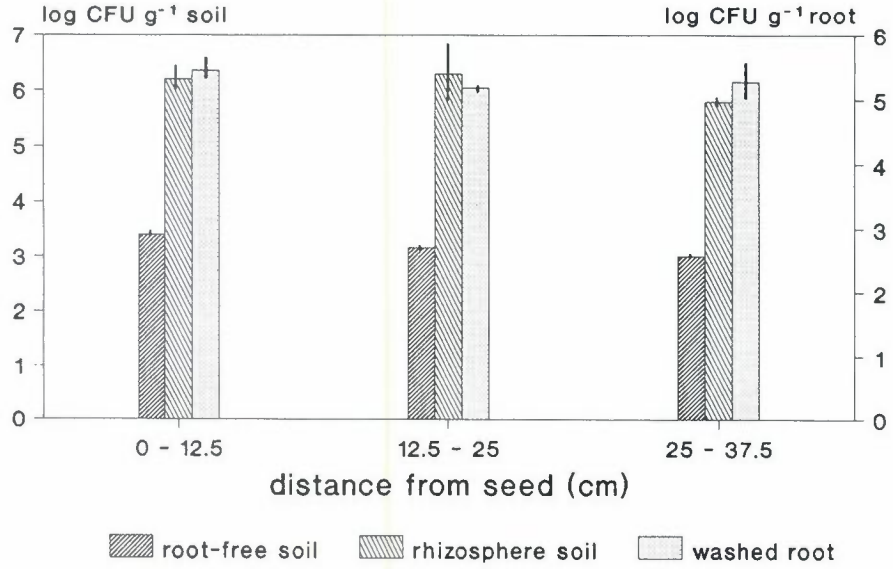

Figure 4. Survival of *A. brasilense::Tn5* in the rhizosphere of sterile-grown summer wheat at different distances from the point of inoculation; inoculation by bacterial seed coating (2×10^6 CFU seed^{-1}); harvest 14 days after planting. Small bars indicate the standard deviation of 2 replicate columns.

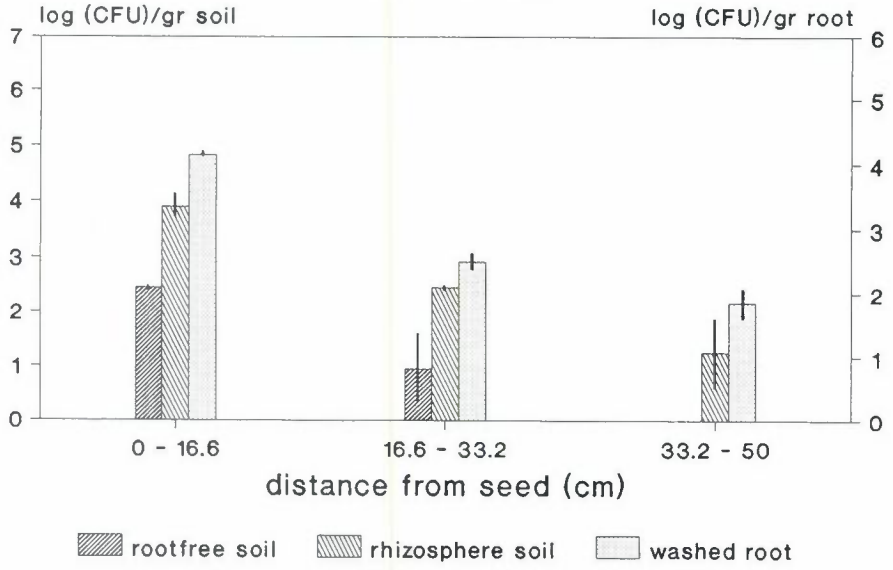

Figure 5. Survival of *A. brasilense::Tn5* in the rhizosphere of non-sterile grown summer wheat at different distances from the point of inoculation; inoculation by bacterial seed coating (2×10^6 CFU seed^{-1}); harvest 14 days after planting. Small bars indicate the standard deviation of the 2 replicate columns.

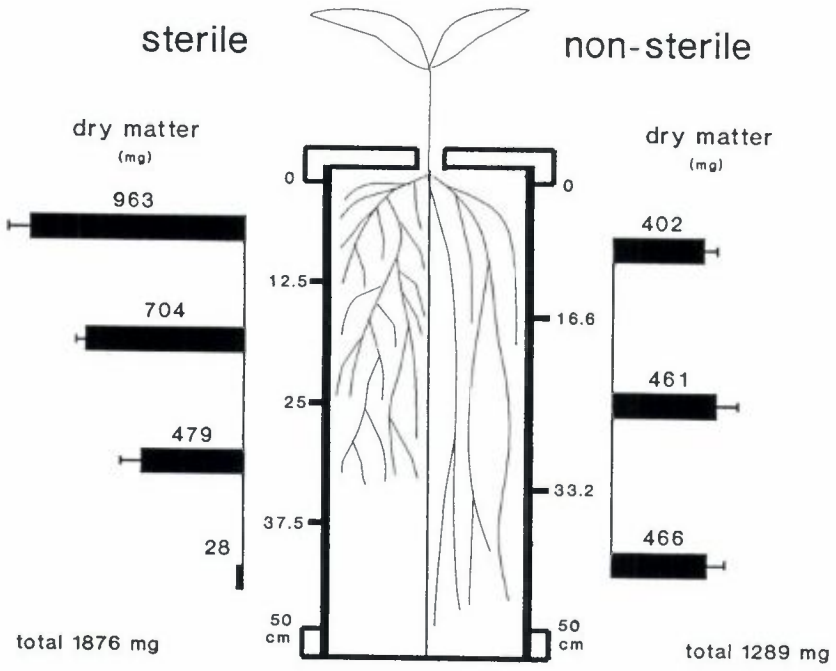

Figure 6. Root dry matter and root morphology of *A. brasilense::Tn5* inoculated summer wheat grown on sterilized and non-sterilized soil; inoculation by bacterial seed coating (2×10^6 CFU seed^{-1}); harvest 14 days after planting. Small bars indicate the standard deviation of 2 replicate columns.

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