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Detection of Colonization by *Pseudomonas* PsIA12 of Inoculated Roots of *Lupinus albus* and *Pisum sativum* in Greenhouse Experiments with Immunological Techniques

W. WIEHE[1*], M. SCHLOTER[2], A. HARTMANN[2], and G. HÖFLICH[3]
[1]*BTU Cottbus, Fakultät Umweltwissenschaften und Verfahrenstechnik, Zentrales Analytisches Labor, Postfach 101344, 03013 Cottbus, Germany, Tel. and Fax. +49-355-692840, E-mail. wiwo@Umwelt.TU-Cottbus.de;*
[2]*GSF–Forschungszentrum für Umwelt und Gesundheit GmbH, Institut für Bodenökologie, Ingolstädter Landstr. 1, 85758 Oberschleissheim, Germany; and*
[3]*Zentrum für Agrarlandschafts- und Landnutzungsforschung e.V. (ZALF), Institut für Mikrobielle Ökologie und Bodenbiologie, Eberswalder Str. 84, 15374 Müncheberg, Germany*

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Abstract

The plant-growth-promoting, non-diazotrophic strain *Pseudomonas* PsIA12, isolated from wheat rhizosphere, was used as inoculum for the legumes *Lupinus albus* and *Pisum sativum*. Root colonization of 8 week-old plants, under non-sterile greenhouse conditions, was assessed in both legumes by a strain-specific polyclonal antiserum and a sensitive chemoluminescence immunoassay. Although the autochtone bacterial colonization of the rhizoplane as well as of the root interior was similar in both plants, the roots of *Lupinus albus* were colonized by *Pseudomonas* PsIA12 more intensively than the roots of *Pisum sativum*. In the roots of *Lupinus albus*, the introduced strain contributed 50%, in pea roots only about 1% to the total bacterial population. Using the immunogold labelling technique, microcolonies of the introduced strain were detected in the rhizoplane and in the inner root tissue of *Lupinus albus*.

Keywords: *Pseudomonas*, rhizosphere, plant-growth-promotion, immunohistology, quantitative immunoassay, polyclonal antisera

*The author to whom correspondence should be sent.

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1. Introduction

In the last decades many attempts have been made to use bacteria with a plant growth promoting potential to improve crop growth (Elsas and Heijnen, 1990). There is still growing interest in the application of plant beneficial bacteria to soil, mainly as biofertilizer or biopesticides (Rodgers, 1993). The survival of inoculated plant-growth-promoting rhizobacteria (PGPR) in the plant rhizosphere is in most cases a precondition for a potential plant stimulation effect during the vegetation time or at least during young plant development (Höflich et al., 1995). Potential mechanisms of plant-growth-promotion, such as production of growth-stimulating phytohormones, mobilization of phosphorus, antagonism against soil-born plant pathogens or nitrogen fixation (Kloepper et al., 1988; Jagnow et al., 1991; Höflich et al., 1994), require apparently a contact of bacteria with active root surfaces or sites.

Studies have shown that establishment of the released bacteria and plant growth stimulation is possible in laboratory experiments with an axenic system, but the results in greenhouse and field trials have been in many cases variable (Burr and Caesar, 1984). The different climatic conditions in a natural system and the natural microflora may be an important cause for the differences between axenic experiments and many greenhouse and field studies (Richards, 1987; Kluepfel, 1993). Furthermore, studies indicate that certain interactions are to some extent possible with roots of certain plant cultivars and specific bacterial strains also in nonsymbiotic systems (Schloter et al., 1994). In addition, the mode of inoculation (soil- or seed-inoculation) plays an important role in the survival of released bacteria in the rhizosphere in greenhouse or field experiments (Elsas and Heijnen, 1990).

In contrast to the specific interactions of symbiotic and most non-symbiotic bacterial strains which show plant-growth-promotion with certain plant cultivars, fluorescent *Pseudomonas* strain PsIA12, isolated from the rhizosphere of wheat, stimulated the growth of different crops in greenhouse (Höflich et al., 1995) and field experiments (Wiehe and Höflich, 1995). Plant growth stimulation of *Pseudomonas* PsIA12 is mainly due to phytohormone production and antagonistic activities (Höflich et al., 1994). The plant growth promoting potential of certain associative *Pseudomonas* strains is mentioned also in other publications (deWeger et al., 1987; deFreitas et al., 1992 a, b). The mode of root colonization of *Pseudomonas* PsIA12 was studied by electron microscopy in different crops using axenic hydroponic cultures (Wiehe et al., 1994; Höflich et al., 1995). The survival of the strain was examined by a rifampicin-resistant mutant in greenhouse (Höflich et al., 1995) and field experiments (Wiehe and Höflich, 1995). It was shown previously that the population dynamic of the plant-growth-promoting *Pseudomonas* strain PsIA12 (Höflich, 1992) depends on

the activity of the plant root system and on the plant species (Wiehe and Höflich, 1995). During vegetative plant development the strain was able to develop high colonization numbers in the root system of lupin, to a lower extent in pea, but no establishment of this strain was found in maize (Wiehe and Höflich, 1995). It is known from axenic culture experiments that this strain had a closer contact to the lupin root surface and inner root tissues as compared with pea, wheat and maize (Wiehe et al., 1994; Höflich et al., 1995).

Although the use of antibiotic-resistant mutants is a common technique to selectively detect bacteria in complex environments (Kloepper and Beauchamps 1992; Compeau et al., 1988; Glandorf et al., 1992; deFreitas et al., 1992 a,b), so far there is no direct proof of the suitability and potential of the original isolated strain *Pseudomonas fluorescens* PsIA12. The production of additional protein(s) in an ecosystem of limited nutrients (Kloepper and Beauchamp, 1988), the stability of the mutation including marker transfer to other microorganisms (Jakeman et al., 1993) and a common natural resistance against antibiotics in the rhizosphere (Gilbert et al., 1993) are often discussed as disadvantages of antibiotic resistant mutants as compared to the wild type. Furthermore, an *in situ* localization of antibiotic mutants in natural habitats is not possible. The aim of this study was to follow the fate of the wild type strain *Pseudomonas* PsIA12 in the rhizosphere of two different legumes (*Lupinus albus* and *Pisum sativum*) in a greenhouse experiment using immunological techniques, which provide a good tool for the localization and quantification of the original strains in non-sterile systems (Schloter et al., 1994; Reinhold and Hurek, 1989).

2. Materials and Methods

Bacterial strains

The bacterial strain *Pseudomonas* PsIA12 was isolated from wheat rhizosphere (Höflich, 1992) and characterized as *Pseudomonas fluorescens* by its membrane fatty acid pattern (Liste, 1993) and by a 500 bp long variable region of its 23S-rRNA sequence (Kirchhof, unpublished). All other bacterial strains were obtained from the German Collection of Microorganisms (Braunschweig, Germany). All strains were grown overnight in LB-medium (Miller, 1972). The determination of the viable number of soil bacteria was carried out on R_2A-medium (Reasoner and Geldreich, 1985).

Plant cultivation and bacterial inoculation

Lupinus albus cv. Lublanc and *Pisum sativum* cv. Grapis were used for pot

experiments on loamy sand (for soil parameters see Höflich et al., 1995). The plants were grown for 8 weeks under the following greenhouse conditions: soil humidity 40–60% water capacity, 15–22°C during the day, 8–12°C at night. Plants were inoculated with *Pseudomonas* strain PsIA12 by seed inoculation (log 8 CFU/seed) directly before sowing.

Extraction of bacteria from root material

Washed root material (1 g) was mixed with 10 ml of sterile 0.1% sodium-cholate solution and disrupted with an ultrasonic desintegrator (50 watts; 7 minutes). The addition of 0.25 g of polyethylenglycol (Boehringer, Mannheim, Germany) and 0,2 g of chelating resin (Sigma, Munich, Germany) followed and the resulting solution was incubated for 2 hours at 4°C. The suspension was filtered through a 5 µm filter (Millipore, Frankfurt, Germany), centrifugated (5,000 x g; 10 minutes) and resuspended in carbonate buffer (pH 9.6). To determine the number of bacteria in the root tissue, the root was incubated for 5 minutes in 1% chloramine T solution and washed overnight in PBS. The bacteria were extracted as described above.

Production and purification of the polyclonal antiserum

The polyclonal antiserum pAk 337 (raised in 6-month old female *New Zealand* rabbits) was produced by Scholz et al. (1991). The serum was cleaned using a protein A column (Biorad, Munich, Germany) and purified from unspecific antibodies using an affinity chromatography column (Biorad) (Harlow and Lane, 1988) and *Pseudomonas fluorescens* DSM 50001 as antigen.

Immunoassay

All immunoassays were performed in 96-well PVC microtiter plates (Flow, Meckenheim, Germany) according to Schloter et al. (1992) with an antirabbit-peroxidase coupled secondary antibody (Amersham, Braunschweig, Germany) and ABTS (Boehringer, Mannheim, Germany) as substrate. The quantitative immunoassay was performed in 96-well white colored PE microtiter plates (Merlin, Hamburg, Germany) with an antirabbit-peroxidase coupled secondary antibody and luminol (Amersham) as substrate according to Schloter et al. (1992).

Characterization of the antigenic determinant

Total proteins and lipopolysaccharides were isolated from an overnight

culture of *Pseudomonas* PsIA12. 2-D-gels were performed according to Schloter et al. (1994) as isoelectric focusing gels (ampholytes pH 3–10) (Sigma), in the first dimension and SDS-polyacrylamide-pore-gradient gels (10–22%) with 4% stacking gels in the second dimension. 1-D-gels were performed as SDS-polyacrylamide-pore-gradient gels (10–22%) with 4% stacking gels (Laemmli et al., 1970). The gels were transferred by electroblotting onto nitrocellulose membranes (Biorad) for western blotting or stained with Coomassie brilliant blue R250 (Serva, Heidelberg, Germany) followed by silver nitrate staining (Heukeshoven and Dernick, 1983). Immunodetection on the blotted membranes was performed in combination with an antirabbit-peroxidase coupled secondary antibody with 4-chloro-1-naphtol as substrate to develop the blots (Harlow and Lane, 1988).

Electron microscopy

Bacterial suspensions of an overnight culture and root segments of the lateral 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 LR white resin (The Resin Company, London, Great Britain) with polymerization at 60°C for 24 hours. *In situ* localization studies were performed on ultrathin sections treated with the polyclonal antiserum and a secondary antirabbit antibody, coupled to gold particles (5 nm) (Amersham) (James et al., 1991). The specimens were examined in a transmission electron microscope (Zeiss EM 9).

3. Results

Characterization of the polyclonal antiserum

Cross-reactions of the antiserum. Four different antisera were raised against *Pseudomonas* PsIA12 and purified by protein A treatment. The serum showing the lowest cross-reactivity as determined with ELISA was selected for further purification steps. The cross-reactions of this antiserum (pAk 337) are shown in Table 1. The serum showed high cross-reactivity (more than 20% compared to the immunogen) in ELISA tests with whole cells of *Pseudomonas fluorescens* DSM 50001, *Pseudomonas aeruginosa* DSM 50001 and *E. coli* DSM 423 as antigens. To remove unspecific antibodies from the serum, affinity purification with *Pseudomonas fluorescens* as antigen was used for a further cleaning step. The cross-reactions of the affinity purified antiserum were reduced to less than 10% compared to the immunogen (Table 1). To determine the cross-check of affinity

purified pAk 337 *in situ*, bacteria were isolated from the rhizosphere of non-inoculated *Pisum sativum* and *Lupinus albus* roots, diluted stepwise and cultivated for 2 days on R_2A-medium. R_2A plates with about 20–40 grown colonies were blotted on a nitrocellulose membrane and treated with the affinity purified pAk 337. Immunodetection on the blotted membranes was performed in combination with an antirabbit-peroxidase coupled secondary antibody and with 4-chloro-1-naphtol as substrate to develop the blots. The results of all blots were negative. The affinity purified pAk 337 was used for further experiments.

Table 1. Cross-reaction of protein A-treated antiserum pAk 337 (pAK 337-1) and the affinity purified-antiserum pAK 337 (pAK 337-2) in ELISA using whole cells of different bacteria as antigens (signal strength in ELISA in %, compared to the immunogen).

Bacterial strain	pAK 337-1	pAK 337-2
Pseudomonas PSIA12 (immunogen)	100	100
Agrobacterium tumefaciens DSM 30205	<10	<10
Alcaligenes eutrophus DSM 516	<10	<10
Arthrobacter citreus DSM 20133	<10	<10
Azetobacter pasteurianus DSM 3509	<10	<10
Azospirillum brasilense sp7 DSM 1690	<10	<10
Bacillus polymyxa DSM 365	<10	<10
E. coli K12 DSM 423	25	<10
Klebsiella pneumoniae DSM 30104	10	<10
Ochrobactrum anthropi LMG 2136	10	<10
Paracoccus denitrificans DSM 1408	<10	<10
Pseudomonas fluorescens DSM 50001	45	<10
Pseudomonas fluorescens DSM 6147	30	<10
Pseudomonas aeruginosa DSM 50071	25	<10
Pseudomonas alcaligenes DSM 50342	10	<10
Pseudomonas cepacia DSM 50180	15	<10
Pseudomonas corrugata DSM 7228	10	<10
Pseudomonas diminuta DSM 1635	10	<10
Pseudomonas plantarii DSM 7128	15	<10
Pseudomonas putida DSM 291	10	<10
Rhizobium meliloti DSM 1021	10	<10

Characterization of the antigenic determinant. To localize the antigenic determinants on the cell surface of *Pseudomonas* PsIA12 of the protein A- and affinity purified serum immunogold treated ultrathin cuts of an overnight culture of *Pseudomonas* PsIA12 were used. Fig. 1 shows a TEM micrograph of bacteria with gold-coupled pAk 337.

To describe the antigenic determinant in more detail, extracts of total protein and lipopolysaccharides were separated on 2-D-gels (total proteins) or 1-D-gels (lipopolysaccharides), blotted onto a nitrocellulose membrane and incubated with pAk 337. Fig. 2a shows a 2-D-gel of a total protein extract of *Pseudomonas* PsIA12. The corresponding western-blot with pAk 337 is shown in Fig. 2b. Fig. 3a shows a 1-D-gel of a lipopolysaccharide extract of *Pseudomonas* PsIA12. The corresponding western-blot with pAk 337 is shown in Fig. 3b. PAk 337 gave a signal with a 30 kD protein with an isoelectric point of pH 8.5 and with low molecular weight lipopolysaccharides.

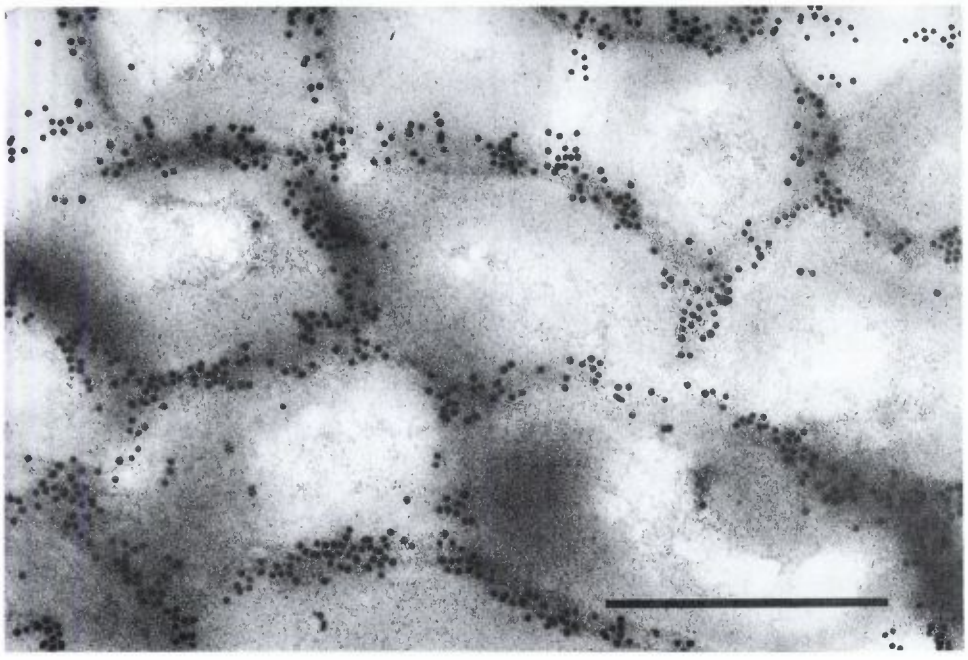

Figure 1. Localization of the pAk epitopes by immunogold labelling of *Pseudomonas* PsIA12 cells and TEM; bar = 0.5 µm.

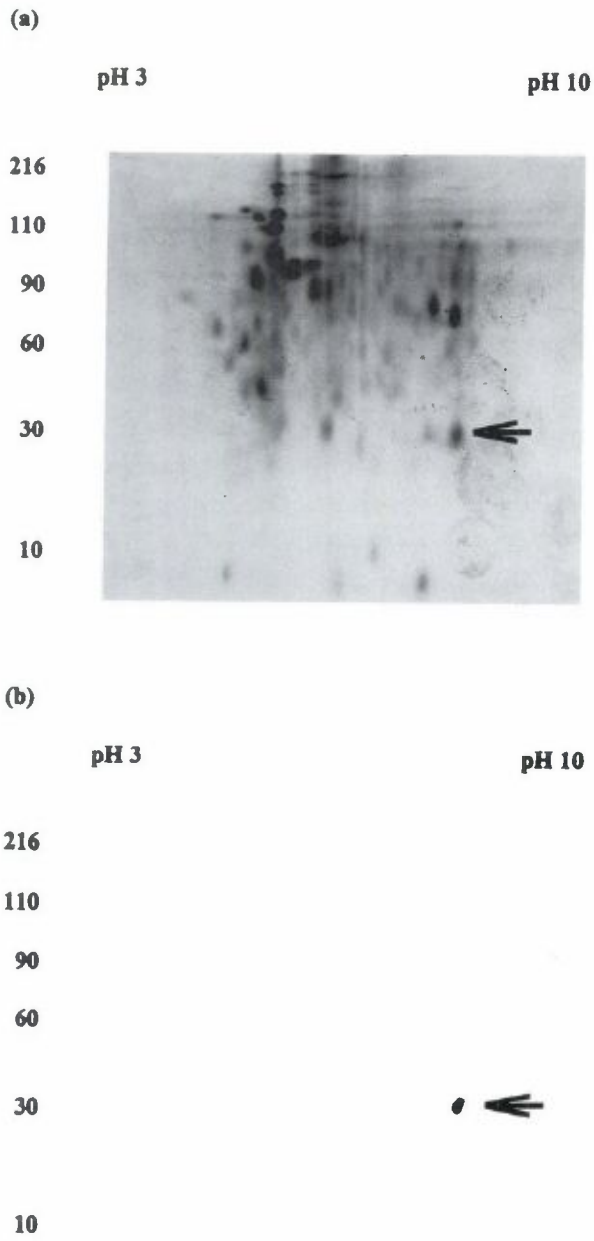

Figure 2. Biochemical characterization of antigenic epitopes of the *Pseudomonas* PsIA12 specific pAk: (a) 2-D-fingerprint of a total protein extract *Pseudomonas* PsIA12. The gel was stained with silver. The estimated molecular masses of the standard proteins are shown. (b) Western-blot of (a) with pAk 337. For detection 4-chloro-1-naphtol was used (→).

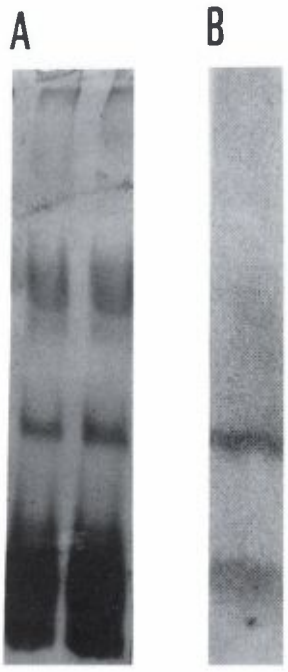

Figure 3. Biochemical characterization of antigenic epitopes of the *Pseudomonas* PsIA12 specific pAk: (a) 1-D-fingerprint of the lipopolysaccharides of *Pseudomonas* PsIA12. The gel was stained with silver. (b) Western-blot of (a) with pAk 337 stained with 4-chloro-1-naphtol.

Validation of the antiserum for a quantitative immunoassay. The validation of the antiserum for a quantitative immunoassay is shown in Fig. 4. A quantification of at least log 4 bacteria/ml is possible using an overnight culture of *Pseudomonas* PsIA12 (respectively *Pseudomonas fluorescens* as control), a peroxidase-coupled secondary antibody, luminol as substrate and a luminometric detection system. In order to use the antiserum for a direct quantification of *Pseudomonas* PsIA12 from the rhizosphere, the numbers of antigens/cell surface has to be constant under laboratory conditions as well as in the rhizosphere since this technique compares the signal of a known bacterial number from an overnight culture with the signal of the bacteria from a root extract. Therefore, log 8 cells of *Pseudomonas* PsIA12 were introduced into a sterile soil with a sterile *Lupinus albus* or respectively a *Pisum sativum* seedling. The bacteria were reextracted and quantified after 3 weeks by a plate counting procedure and an immunoassay. Both methods gave the same number of bacteria/g dried root surface or inner

root tissue (Fig. 5). Furthermore, the isolated bacteria were embedded in resin, ultrathin sections were prepared and treated with the antiserum coupled to gold particles. The number of antigenic determinants per cell surface of the isolated bacteria and bacteria from an overnight culture were identical (data not shown).

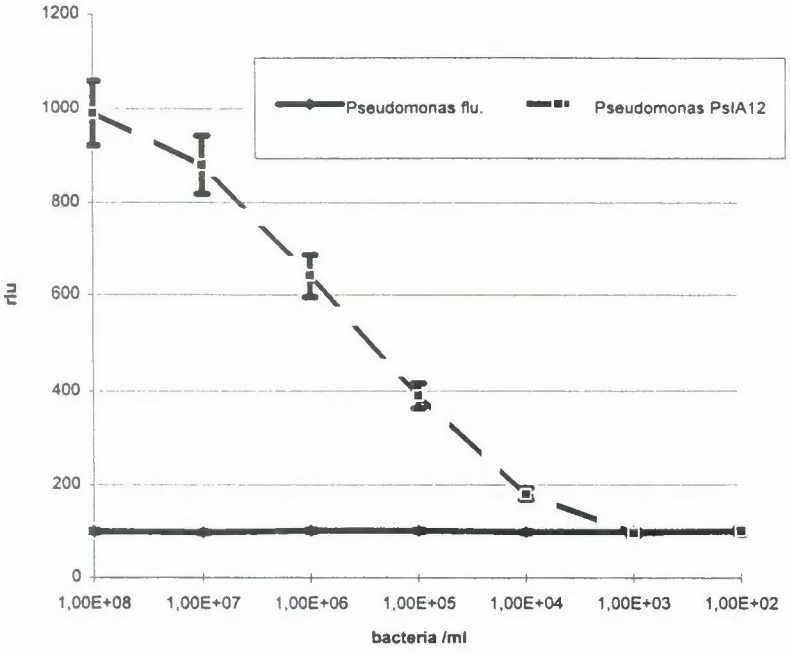

Figure 4. Validation of pAk 337 using an antirabbit-peroxidase coupled secondary antibody and chemoluminescence for the quantification of *Pseudomonas* PsIA12: Dilutions of *Pseudomonas* PsIA12 (----) or *Pseudomonas fluorescens* DSM 50001 (——) were subjected to the quantitative chemoluminsecence immunoassay (7 parallels/dilution). The light counts were measured in a microtiter-plate luminometer (rlu = relative light units).

Interactions between plant roots and introduced bacteria

Root colonization with Pseudomonas PsIA12 and interrelationships with the total bacterial population. Root colonization with the inoculated strain was detected by ELISA in roots and surface sterilized roots of both plant species. Colonization was higher in lupine than in pea roots (Table 2). The colonization of interior root spaces, shown as values of the surface sterilized roots, was remarkably higher in

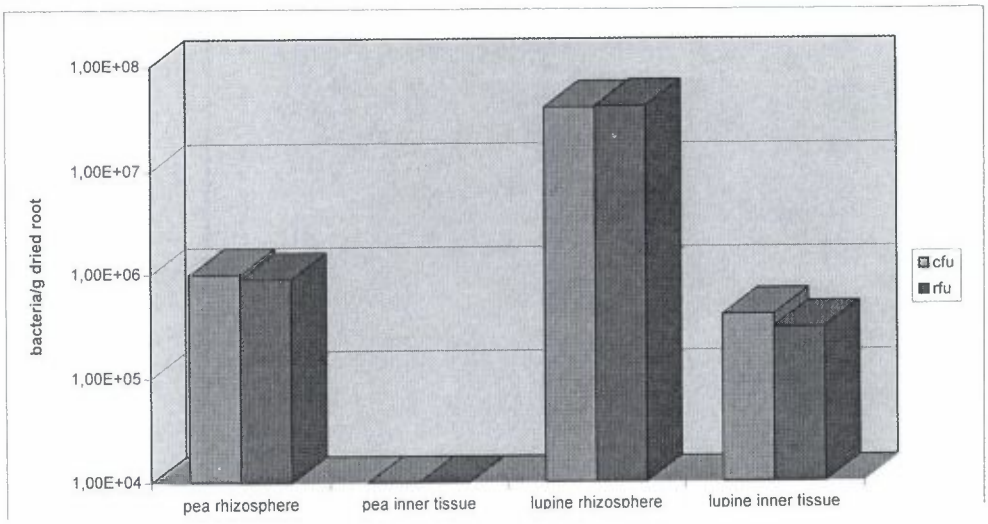

Figure 5. Comparison of colony counts and quantitative chemoluminscence immunoassay in an axenic system with inoculated pea and lupine plants. 3 weeks after inoculation roots were washed (rhizosphere fraction) or surface sterilized (inner tissue fraction). The bacteria were extracted and subjected in parallel to a plate counting-procedure on R_2A-agar plates (CFU) (7 parallel plates/dilution) and to a quantitative chemoluminsecence immunoassay (rlu) (7 parallels/plant). The standard deviation in both systems was less than 10%.

Table 2. Root colonization with *Pseudomonas* PsIA12 and autochtone bacteria on 8 weeks plants; greenhouse experiment with loamy sand.

Plant	Inoculation	Root colonization (CFU/g root) PsIA12[1]		Total bacteria[2]	
		Root	Surface sterilized root	Root	Surface sterilized root
Lupine	–	<4.0	<4.0	7.6	6.6
	+	7.7	6.5	7.9	6.8
Pea	–	<4.0	<4.0	7.7	6.3
	+	5.9	3.8	7.8	6.6

[1]Detection of PsIA12 with CIA (log counts/g dried root);
[2]CFU on R_2A-agar (log CFU /g dried root).

Table 3. Relationships of colonization between different root fractions, inoculated strain, and total bacteria.

Plant	Inoculation	Contribution to inner root on whole root colonization (%)		Contribution of PsIA12 to total bacteria (%)	
		PsIA12	Total bacteria	Whole root	Surface sterilized root
Lupine	–	–	4.0	–	–
	+	6.3	7.9	63	50
Pea	–	–	4.0	–	–
	+	0.8	6.3	1.3	0.2

lupine roots as compared with the pea roots. This is noteworthy as the total bacterial population is similar in both plant species with a mean value of log 7.8 cfu/g root and about tenfold lower in the inner root tissues. In lupines, the inoculated strain contributed 63% to the total root bacterial colonization, respectively to 50% of the inner root colonization. In pea, however, the inoculated strain contributed only 1.3% to the total root and 0.2% to the inner root colonization (Table 3).

In situ localization of Pseudomonas PsIA12 on plant roots. It was possible to detect immunogold labelled bacteria only in lupine root segments. These bacterial cells showed a feature of typical rods (Fig. 6a) and differed from other not marked bacterial forms (Fig. 6b). Marked cells were detected only on the root surface, in lysed epidermal and cortex cells, and formed mostly microcolonies. Gold particles were bound to the surface of the bacterial cell as observed with cells of an overnight culture *in vitro* (Fig. 1). In contrast, in ultrathin sections of inoculated pea roots no bacteria labeled with immunogold could be detected. This result is not surprising as compared to the quantitative data shown above, as the detection limit of root colonization with bacteria in electron microscopy is very high due to the low thickness of ultrathin sections (~ 60 nm). We estimate the detection limit at about log 5 cfu/cm root (~ log 6 cfu/g root). That means that a single detected bacteria represents a high colonization status.

4. Discussion

To use immunological methods for the localization and quantification of

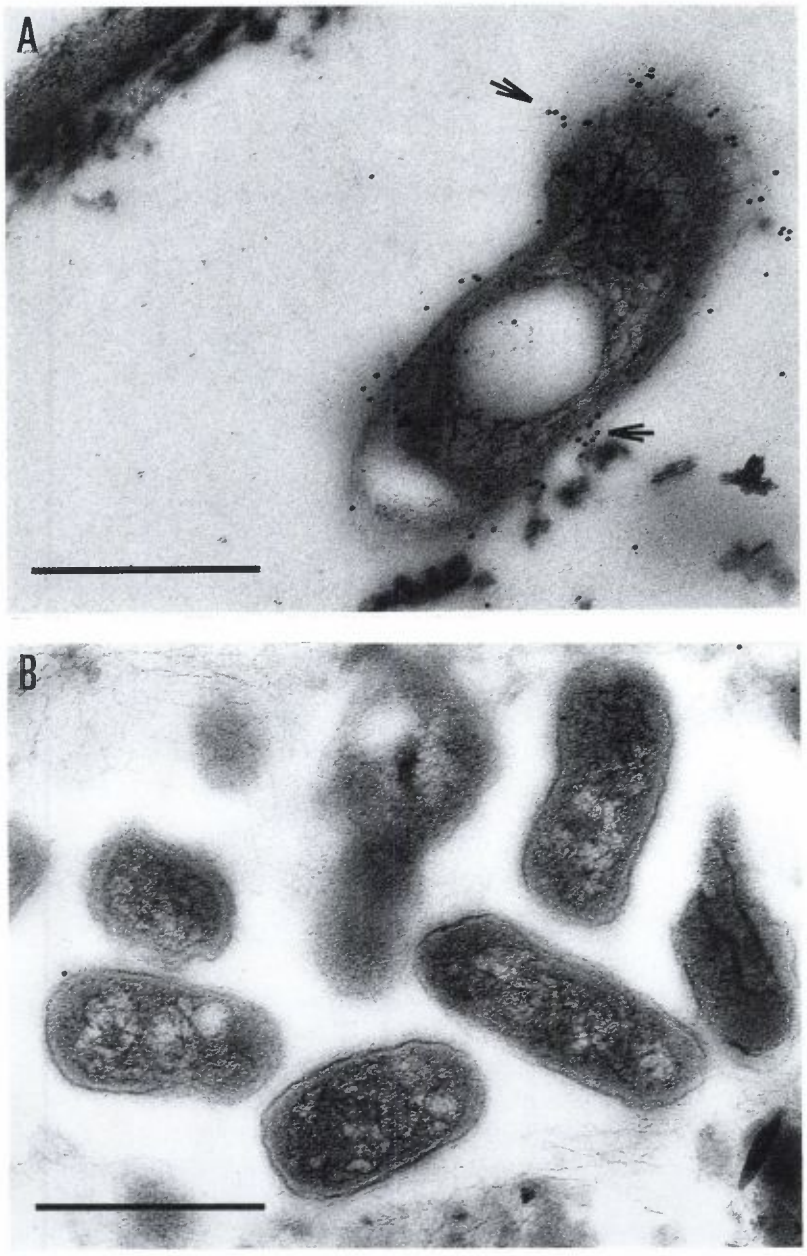

Figure 6. *In situ* localization of *Pseudomonas* PsIA12 in the rhizosphere of inoculated lupine plants with immunogold labeled pAk 337 and TEM. (a) immunogold labeled *Pseudomonas* PsIA12 cells in an autolysed cortex cell of lupine (b) non-marked, unknown bacteria of the autochtone microflora on the lupine root surface; bar = 0.5 µm.

bacteria in complex habitats the antibodies must comply with four quality criteria: (1) localization of the antigenic determinant on the cell surface, (2) no cross-reactions with other strains, (3) stability of the antigenic determinant *in situ* and (4) high affinity for the antigen (Schloter et al., 1995). After several purification steps the antibody pAK 337 showed no cross-reaction in ELISA with the other bacteria tested. The affinity of the pAk 337 was relatively high (data not shown). Using immunogold technique it could be shown that the antigenic determinant is localized on the cell surface and is stable after releasing and recovering the bacteria from the rhizosphere. The biochemical characterization of the antigenic determinant showed that the antiserum is not monospecific and therefore the sensitivity of the quantitative immunoassay is reduced. Similar results were obtained also by other groups (Jakeman et al., 1993).

Nevertheless, an *in situ* detection with immunological methods of the inoculated strain *Pseudomonas* PsIA12 was possible not only on the root surface, but also in the inner root tissues, after 8 weeks of plant growth. It could be demonstrated that the colonization capacity of the endorhizosphere by PsIA12 was higher in lupine than in pea. This corresponds well to the above described results under axenic hydroponic conditions (Wiehe et al., 1994), or to those with a rifampicin-resistant mutant (Höflich et al., 1995; Wiehe and Höflich, 1995). In contrast, the natural bacterial flora was able to colonize the root surface and the inner root tissues in both plant species to the same extent. Plant-specific bacterial interactions are prominent in symbiotic systems (*Rhizobium, Frankia*) (Werner, 1989), while in the case of *Pseudomonas* PsIA12 the associative mode of bacteria-root interaction seems less specific. Colonization differences between pea and lupine may be the result of qualitative/quantitative differences in exudation, especially of citric acid in lupine (Dinkelaker et al., 1989), of different root structure/architecture and dynamics (eg. flat rooting pea *versus* deep rooting lupin) as well as of differences in plant-pathogen defence mechanisms. Different modes of root interior colonization such as, passive migration via lesions caused by pathogens, deleterious microorganisms, mode of lateral root development or active invasion by cellulytic/pectinolytic enzymes are still in discussion (see critical discussion of the term endorhizosphere in Kloepper et al., 1992).

The results in this paper describe only the status in 8 weeks old plants. In our case the introduced strain tends to stimulate lupine plants more than pea, but no clear relationships between colonization mode extent and stimulation effect could be found yet.

The selected strain PsIA12 possesses different characteristics (bacterium with a r-metabolism, antagonism against soil born root pathogenes, production of siderophores), which render this strain aggressive and highly competitive with the native, plant-specific bacterial flora.

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