# A calcium-dependent bacterial surface protein is involved in the attachment of rhizobia to peanut roots

**Marta Dardanelli, Jorge Angelini, and Adriana Fabra**

**Abstract:** As part of a project to characterize molecules involved in the crack-entry infection process leading to nodule development, a microscopic assay was used to visualize the attachment of cells of *Bradyrhizobium* sp. strains SEMIA 6144 and TAL 1000 (labelled by introducing a plasmid expressing constitutively the green fluorescent protein GFP-S65T) to *Arachis hypogaea* L. (peanut). Qualitative and quantitative results revealed that attachment was strongly dependent on the growth phase of the bacteria. Optimal attachment occurred when bacteria were at the late log or early stationary phase. Cell surface proteins from the *Bradyrhizobium* sp. strains inhibited the attachment when supplied prior to the attachment assay. Root incubation with a 14-kDa protein (eluted from sodium dodecyl sulphate – gel electrophoresis of the cell surface fraction) prior to the attachment assay resulted in a strong decrease of attachment. The adhesin appeared to be a calcium-binding protein, since cells treated with EDTA were found to be able to bind to adhesin-treated peanut roots. Since this protein has properties identical to those reported for rhicadhesin, we propose that this adhesin is also involved in the attachment process of rhizobia to root legumes that are infected by the crack-entry process.

*Key words:* peanut, crack entry, rhizobia, attachment, adhesin.

**Résumé :** Dans le cadre d'un projet visant à caractériser les molécules impliquées dans le processus d'infection par entrée par les fissures, menant au développement de nodules, une analyse microscopique a été effectuée afin de visualiser l'attachement de cellules de *Bradyrhizobium* sp. SEMIA 6144 et TAL 1000 (marquées grâce à l'introduction d'un plasmide exprimant constitutivement la protéine fluorescente verte GFP-S65T) à *Arachis hypogaea* L. (arachide). Les résultats qualitatifs et quantitatifs on révélé que l'attachement était fortement dépendant de la phase de croissance de la bactérie. L'attachement était optimal lorsque les bactéries étaient dans leurs phases logarithmique tardive ou stationnaire précoce. Les protéines de la surface cellulaire de ces souches ont inhibé l'attachement lorsqu'elles étaient fournies avant l'analyse d'attachement. Une incubation préalable de racines avec une protéine de 14 kDa (éluée d'un gel d'électrophorèse – SDS de la fraction de la surface cellulaire) a entraîné une forte diminution de l'attachement. Cette adhésine semble être une protéine se liant au calcium puisque des cellules traitées au EDTA ont pu se lier à des racines d'arachides traitées à l'adhésine. Comme cette protéine a des caractéristiques identiques à celles de la rhicadhésine, nous proposons que cette adhésine est également impliquée dans le processus d'attachement de rhizobiums aux racines de légumineuses qui sont infectées par le processus d'entrée par les fissures.

*Mots clés :* arachide, entrée par les fissures, rhizobiums, attachement, adhésine.

[Traduit par la Rédaction]

## Introduction

Attachment of *Rhizobiaceae* nodule bacteria to the roots of their host plants is considered the first step in the infection process leading to a nitrogen-fixing symbiosis. Root nodule bacteria can then enter the roots in different ways: infection thread formation between cells of intact epidermises or "crack" entry. In the former, rhizobia first attach to the root hair and then induce deformation and curling of the root hair, followed by infection thread formation. Concurrent with infection, root cortical cells dedifferentiate and start dividing. This results in the formation of a nodule primordium from which the nodule will develop (Kijne 1992). Besides the more widely studied formation of infection threads through root hairs, the rhizobia may enter through cracks in the epidermis.

*Arachis hypogaea* L. (peanut) differs from other herbaceous legumes in that although root-hair deformation was observed when roots were inoculated with rhizobia (Hadri et al. 1999), any colonization of root-hair cells was apparently

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**M. Dardanelli.** Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, 5800 Río Cuarto (Córdoba), Argentina.
**J. Angelini and A. Fabra.**[1] Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, 5800 Río Cuarto (Córdoba), Argentina.

[1]Corresponding author (e-mail: afabra@exa.unrc.edu.ar).

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abortive. Instead, infection through the epidermis involves intercellular penetration (crack entry). In the presence of rhizobia, cell divisions are induced in the cortex of emerging lateral roots. Growth of the young roots causes separation of cortical and epidermal cells and enables entry and intercellular spread of rhizobia. Thus, there are no cell-to-cell infection threads (Boogerd and van Rossum 1997).

For leguminous plants infected by infection thread formation, a two-step model was proposed for the process of rhizobial attachment to plant root hairs. A bacterial protein called rhicadhesin is involved in the first step of the attachment process (direct attachment of rhizobia to the surface of the root-hair cells). The mechanism of the second step depends on the growth conditions of the rhizobia, and it results in an accumulation of the bacteria at the tip of the root hair, which leads to cap formation. In the case of carbon-limited bacteria, rhizobial cellulose fibrils are involved in the second step of attachment, whereas in the case of manganese-limited rhizobia, host plant lectins are also involved (Smit et al. 1989*a*, 1989*b*).

Rhicadhesin was found to be involved in the attachment of several rhizobial strains (*Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *phaseoli*, *Sinorhizobium meliloti*, *Rhizobium lupini*, *Phyllobacterium rubiacearum*, *Phyllobacterium myrsinacearum*, and *Bradyrhizobium japonicum*) to *Pisum sativum* root hairs, a legume that is infected by the formation of infection threads. The ability of *R. leguminosarum* 248 to attach to developing root-hair tips of several leguminous plants (*Vicia sativa*, *Phaseolus vulgaris*, *Macroptilium* spp., *Trifolium repens*, and *Medicago* spp.) was also tested, and it was found that attachment is one of the prerequisites for root-hair curling (Smit et al. 1989*a*). For *B. japonicum* (as well as for many bacterium–plant interactions), only the first attachment step was reported to be essential in the root infection by thread formation, leading to nodule formation (Smit et al. 1991).

Rhicadhesin is characterized by the following properties. (*i*) It is sensitive to boiling and to protease treatment. (*ii*) It is released from cells growing under calcium ion ($Ca^{2+}$) limitation. (*iii*) Its isoelectric point is 5.1 and its molecular mass is about 14 000 Da. (*iv*) Its affinity for $Ca^{2+}$ is high. Calcium ions are not involved in binding rhicadhesin to the plant cell surface but appear to be important for anchoring the adhesin to the bacterial cell surface (Smit et al. 1991).

Although there is a vast amount of knowledge about the steps that allow nodule formation by the infection threads, hardly anything is known about the molecular nature of the bacterial factors involved in the crack infection process. As part of a project meant to characterize the molecules involved in peanut nodulation, we initiated the present study.

## Materials and methods

### Bacteria and culture conditions

Two peanut nodulating and nitrogen-fixing reference strains, *Bradyrhizobium* sp. strains SEMIA 6144 and TAL 1000 (provided by the UNESCO Microbial Resources Centre (Mircen) (Porto Alegre, Brazil) and the University of Hawaii Nitrogen Fixation by Tropical Agricultural Legumes (NifTAL) Centre (Paia, Hawaii), respectively), were used as peanut inoculant. These strains were transformed (Gage et al. 1996) and contained the plasmid pTB93F that encoded for the green fluorescence protein (chloramphenicol resistant, spectinomycin resistant).

The composition of the medium yeast extract – mannitol (YEM) has been previously described (Vincent 1970). For attachment assays, bacteria were cultivated in 500-mL Erlenmeyer flasks containing 100 mL of YEM medium on a rotatory shaker (180 r/min) at 28 °C. Cells were harvested in the late logarithmic phase of growth ($OD_{620}$ = 0.7), unless stated otherwise.

For adhesin isolation, bacteria were grown in 1000-mL Erlenmeyer flasks containing 250 mL of YEM medium until they reached an $OD_{620}$ of 0.7.

### Plant culture conditions

Seeds of *Arachis hypogaea* L. 'Tegua' were washed with tap water and surface sterilized by immersion in ethanol for 30 s and in 15% $H_2O_2$ for 15 min. After five washes with sterilized water, they were incubated at 28 °C on one layer of Whatman No. 1 filter paper and moist cotton in Petri dishes until the radicle reached 1.5–2.0 cm. Seedlings were transferred to pots containing sterilized sand – vermiculite (1:1) and placed in a growth chamber at 28 °C (16 h) and 18 °C (8 h) under a photosynthetically active radiation of 124 μE $m^{-2}$ $s^{-1}$ provided by fluorescent tubes. They were watered regularly with sterilized tap water and once a week with Jensen medium. Seedlings were harvested after 4 weeks.

### Isolation of crude adhesin fraction

For standard isolation of crude adhesin fraction, we followed the method described by Smit et al. (1989*a*), with minimal modifications. *Bradyrhizobium* sp. strains SEMIA 6144 and TAL 1000 cells were grown in 250 mL of YEM medium (Vincent 1970) and harvested by centrifugation (10 000*g*) at $A_{620}$ = 0.7, since we found that the attachment ability of these rhizobia is better at this $A_{620}$ value. The pellets were washed three times with 25 mmol/$m^3$ phosphate buffer (pH 7.5) and suspended in 2.5 mL of phosphate buffer. Then, cells were sheared for 5 min with a sonicator (Sonics & Materials Inc., Danbury, Conn., U.S.A.) at a setting of 5.

The suspension was centrifuged at 12 000*g* for 10 min at 4 °C, and the resulting supernatant fluid was centrifuged at 100 000*g* for 2 h at 4 °C. The supernatant obtained after ultracentrifugation was used as the crude adhesin fraction. This method enables bacterial surface proteins to efficiently detach from the cells by shearing forces without killing the cells and also enables the precipitation of the flagellum-containing fraction by ultracentrifugation (Smit et al. 1989*a*). The crude adhesin fraction remained active for at least 6 months when stored at –20 °C.

### Bacterial attachment assay

The attachment assay used was described by Smit et al. (1986). After absorbance at 620 nm of the bacterial culture was determined, it was centrifuged at 10 000*g* for 5 min. The pellet was washed three times with 25 mmol/$m^3$ phosphate buffer (pH 7.5) and suspended in the same solution to give a bacterial concentration of $2 \times 10^8$ rhizobial cells/mL. Five young peanut roots with emerging lateral roots (approximately 2 cm long) were immersed in 1 mL of bacterial sus-

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pension for 2 h at room temperature under gentle agitation. Then, roots were washed 10 times in phosphate buffer to remove free and weakly attached bacteria. Bacteria attached to each root were visualized by fluorescence microscopy (×400 magnification) with a Zeiss Axiophot microscope (Carl Zeiss, Argentina S.A.) equipped with an epifluorescent system and the filter combination 450 and 490 nm. Seven replications of this experiment were done.

For the quantitative assays, we incubated the roots, as described above. After roots were washed 10 times in phosphate buffer, the number of bound bacteria was determined by resuspending roots in 200 μL phosphate buffer, grinding them in a sterilized mortar, and plating different dilutions of the grindate on YEM agar, supplemented with chloramphenicol (10 μg/mL) and spectinomycin (100 μg/mL). Plates were incubated at 28 °C, and the number of viable cells was determined. Three replicates of this experiment were done.

**Attachment inhibition by the presence of an adhesin**

Smit et al. (1989*a*) experimentally defined an adhesin as a surface component of the bacteria able to competitively inhibit the attachment of *Rhizobium* spp. cells to root hairs when supplied prior to or during an attachment assay. To test whether the crude adhesin fraction had an inhibitory effect on the attachment of bacteria, we incubated five peanut roots with 100 μL of the crude fraction (300 μg protein) in 1 mL of 25 mmol/$m^3$ phosphate buffer (pH 7.5) for 60 min at room temperature on a rotatory shaker (20 r/min). Controls were incubated for 60 min in buffer only. After this incubation, the roots were washed five times in phosphate buffer and incubated with bacteria for the attachment assay, as described above. To determine the adequate amount of crude fraction able to inhibit the bacterial attachment, we added different protein concentrations (150, 300, 450, and 600 μg/mL) of crude adhesin preparation to the peanut roots. Seven replications of this experiment were done. Quantitative determinations were done as described for bacterial attachment assays.

**SDS – polyacrylamide gel electrophoresis, silver stain, and recovery from gels**

Sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis was performed as described by Smit et al. (1989*a*). The marker protein used was a 14-kDa lysozyme (1 mg/mL). A 300-μg amount of the crude adhesin fraction and 10 μg of lysozyme were applied to the slots. Silver staining of the gel was performed by the method described by Ausubel et al. (1997).

For the isolation of the 14-kDa fraction, we followed the method described by Smith et al. (1989*a*). After electrophoresis of an unstained SDS – polyacrylamide gel, the representative area of the 14-kDa fraction was cut from the gel. A portion of the gel that did not have the sample loaded was used as a control. The gel pieces were incubated with 500 μL of 100 mmol/$m^3$ Tris–hydrochloride buffer (pH 7.5) for 30 min at room temperature, under gentle agitation. This eluted fraction (150 μL) was tested for attachment-inhibiting activity. Three replications of this experiment were done. It was determined by the methods of de Rudder et al. (1997) and Trevelyan and Harrison (1952) that this fraction does not contain detectable lipids or carbohydrates, respectively.

**Determination of calcium-binding activity of the adhesin**

To determine whether the adhesin activity of the cell surface preparation is $Ca^{2+}$ dependent, we grew cells in YEM medium with added 100 mmol/$m^3$ Tris–hydrochloride in 1 mmol/$m^3$ EDTA (pH 7.5). This culture condition had no effect on bacterial viability. Cells were harvested and used to determine (by the attachment assay described above) their attachment to peanut roots previously incubated with adhesin obtained from rhizobia growing in YEM medium without EDTA. It was assumed that previous binding of this adhesin to a root receptor would allow normal binding of adhesin-detached bacteria, in a later attachment assay. Three replications of this experiment were done. Quantitative determinations were done as described for bacterial attachment assays.

**Determination of the effect of protease on the attachment-inhibiting activity of adhesin**

To determine whether treatment of the cell surface preparation with proteolytic enzymes results in loss of attachment-inhibiting activity of the adhesin, we incubated the crude adhesin fraction and the 14-kDa fraction with proteinase K (1 mg/mL) at 37 °C for 60 min before the attachment assay. As it has been reported that protease is not easily removed from the cell surface preparation after the treatment, the roots used in the control experiments were incubated for 60 min at room temperature with proteinase K before incubation with bacteria (Smit et al. 1989*a*).

**Protein determination**

Protein was determined by the method of Bradford (1976), using bovine serum albumin (1 mg/mL, Sigma Chemical Co., St. Louis, Mo.) as the standard.

## Results and discussion

**Conditions for attachment to peanut roots**

Two techniques, microscopy and numerical study, were used to examine the role of peanut rhizobia adhesin in the root attachment process. As was pointed out (Matthysse and McMahan 2001), microscopic assays necessitate the use of large numbers of bacteria (more than one million cells per millilitre) to obtain reliable results. Caetano-Anolles and Favelukes (1986) developed an interesting assay that uses a low number of bacteria, better representing the amount of rhizobia naturally encountered in many soils. Although it is very useful in discovering the specific phenomena involved during the early interaction between rhizobia and roots, disadvantages of this method have also been pointed out (Matthysse and Kijne 1998).

For many rhizobia, it has been found that although the primary target sites for infection are young, growing root hairs, they are not the exclusive loci for rhizobial attachment. In legumes, such as peanut, that are infected by crack entry, rhizobia induce cell divisions in the cortex of an emerging lateral root and penetrate through separations between cortical and epidermal cells. In this work, young peanut lateral roots were chosen to study rhizobial attachment.

The attachment ability of peanut microsymbionts was investigated as a function of the rhizobia growth stage. Bacterial suspensions containing $2 \times 10^8$ rhizobial cells/mL from

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rhizobia cultures at $OD_{620}$ of 0.5, 0.7, 1, and 1.5 were tested. It was found that the attachment ability was a function of bacteria growth state. We ranked attachment into the following three classes (Fig. 1) by screening the attached bacteria distributed along the surface of at least 35 lateral roots: class 1, few attached bacteria (bacteria at early logarithmic phase); class 2, the root surface almost covered with bacteria (bacteria at late logarithmic phase); and class 3, many attached bacteria on the root surface, some of them forming aggregates (bacteria at early stationary phase). The degree of attachment did not change on the same root segment. Rhizobia harvested during or after the late logarithmic phase of growth had better attachment activity than those harvested at the early logarithmic phase (Fig. 1). Lodeiro et al. (1995) and Smit et al. (1986) obtained similar results for *R. leguminosarum* root attachment. Kijne et al. (1988) reported that for *R. leguminosarum* cells, nutrient limitation in the YEM culture medium (producing the growth interruptions and the autoagglutination observed) always coincided with optimal attachment. In their study, it was demonstrated that the availability of manganese ions is the growth-limiting factor in YEM medium. Although cell agglutination was not observed in our study, a high level of bacterial adhesion was found with high density cultures.

It is interesting to note that unlike the results reported for *R. leguminosarum* attachment to pea roots (Smit et al. 1986), we have never observed roots without attached bacteria (class 1, as defined by these authors). It is also interesting that under identical attachment conditions, 100% of the roots showed the same attachment class, and there was no variation in the attachment rate among the roots examined. In addition, attached bacteria forming a caplike aggregate on top of the root (classes 3 and 4, as defined by Smit et al. 1986) have never been observed in our experiments.

According to the model of Smit et al. (1986, 1989*b*), classes 1 and 2 attachment to peanut roots we report here correlate with the first step of the attachment process. The second step is characterized by the observation of peanut rhizobia aggregates on already attached bacteria but without cap formation. The poor class 4 attachment (cap formation) to pea roots reported for *B. japonicum* and *R. lupini* compared with that reported for *R. leguminosarum* has been related to differences in the production of cellulose fibrils (Smit et al. 1986).

### Attachment-inhibiting activity of the cell surface preparation

The method we used to obtain the soluble surface proteins allowed precipitation of the flagella-containing fraction by ultracentrifugation (Smit et al. 1989*b*). However, it has been reported that flagella and the O-antigenic side chain of lipopolysaccharide are not involved in rhizobia attachment (Smit et al. 1989*b*). These authors also determined that shearing of the rhizobia for 5 min is an efficient way to detach cell surface adhesions without killing the cells.

The supernatant obtained after ultracentrifugation was found to possess attachment inhibiting activity when it was incubated with peanut roots prior to root incubation with rhizobia, since cell binding appeared to be reduced compared with that of the control. A shift from attachment class 2 (control) to class 1 (preincubated roots) was observed in all the lateral roots examined (data not shown). Quantitative analysis showed a significant reduction in the number of bacteria attached to roots. These results indicate that this fraction

**Fig. 1.** Attachment of *Bradyrhizobium* sp. strain TAL 1000 to peanut lateral roots as a function of the bacterial growth stage. (A) Class 1. (B) Class 2. (C) Class 3. (For a description of classes see Results.) Similar results were obtained in *Bradyrhizobium* sp. strain SEMIA 6144. Thirty-five roots were examined for each OD. Magnification: ×400. Conditions for assay are described in Materials and methods.

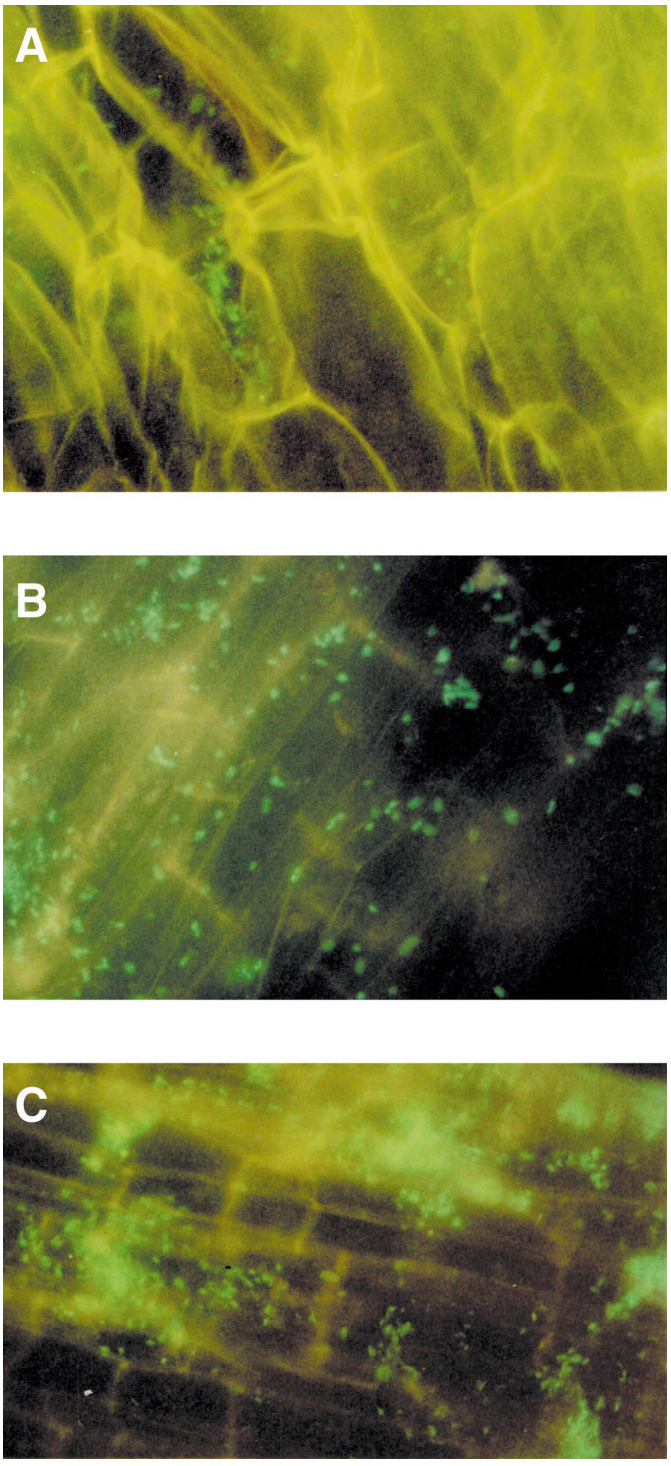


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**Table 1.** Rhizobia attachment following preincubation of peanut root with a total bacterial crude adhesin fraction or a 14-kDa protein fraction.

| | Relative attachment (% of control) | |
|---|---|---|
| | *Bradyrhizobium* sp. strain TAL 1000 | *Bradyrhizobium* sp. strain SEMIA 6144 |
| **Addition of different protein concentrations from crude adhesin fraction (μg/mL)[a]** | | |
| 0 | 100 | 100 |
| 150 | 12.43±0.77 | 20.71±1.34 |
| 300 | 3.20±0.06 | 5.35±0.11 |
| 450 | 1.23±0.06 | 2.06±0.11 |
| 600 | 0.18±0.01 | 0.29±0.01 |
| **Addition of the 14-kDa protein fraction (150 μL)** | 27.07±0.96 | 29.72±0.58 |

**Note:** Data is the mean ± standard error of three experiments.
[a]Crude adhesin fraction (100 μL) was added 60 min prior to the addition of the bacteria.

contained adhesins, which were active when the cell surface preparation (300 μg/mL protein concentration) was incubated with peanut roots before the attachment assay (Table 1).

To test the optimal amount of crude adhesin fraction able to inhibit rhizobia attachment when incubated with peanut roots, we assayed 100 μL of crude fraction containing 150, 300, 450, or 600 μg/mL of protein. It was observed that adhesin inhibited attachment of peanut rhizobia in a dose-dependent way. These results are in agreement with those obtained by Smit et al. (1989*a*) for *R. leguminosarum* bv. *viciae*.

### Attachment-inhibiting activity of a 14-kDa protein in the crude adhesin fraction

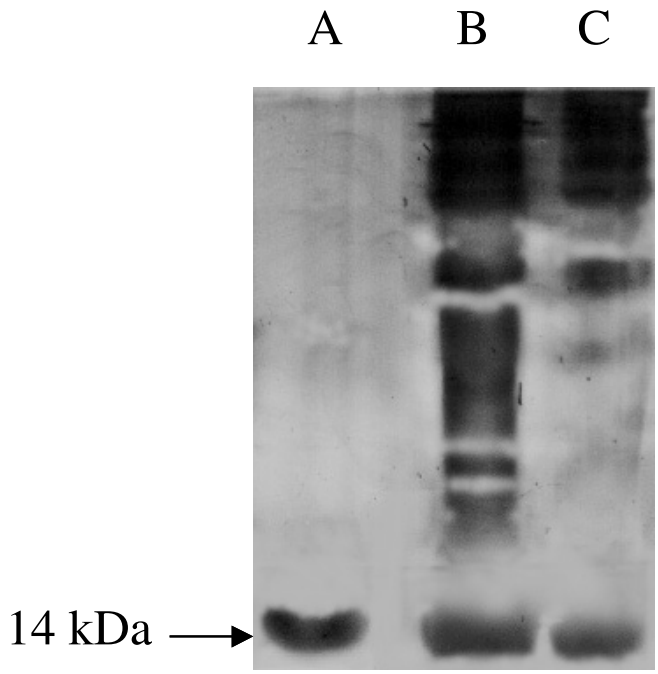
SDS – polyacrylamide gel electrophoresis revealed the presence of a protein band at a position of about 14 kDa (Fig. 2). As was reported by Smit et al. (1989*a*), the fixation with glutaraldehyde resulted in visualization of protein bands on gels. This property appears to be a common feature for most $Ca^{2+}$-binding proteins.

When the 14-kDa eluted band was tested for its attachment-inhibiting activity, attachment activity was found to shift from attachment class 2 to class 1. The eluted band corresponding to the lysozyme (14 kDa) was used as a control and, under this condition, class 2 attachment was observed in all the examined roots (data not shown). As observed by fluorescence microscopy, quantitative assays revealed that the 14-kDa fraction inhibited the binding of bacteria to peanut root. Compared with the control binding, there was a significant reduction in the number of viable bound bacteria under this condition (Table 1). These results suggest that this 14-kDa protein from the crude adhesin preparation has attachment-inhibiting ability.

### Properties of the attachment-inhibiting molecule

It has been demonstrated that rhicadhesin is a proteinaceous molecule (Swart et al. 1993; Smit et al. 1989*a*). To test this property for the adhesin isolated in this study, we treated the crude adhesin preparation and the 14 kDa fraction eluted from the SDS – electrophoresis gel with proteinase for 60 min at 37 °C prior to incubation with the roots. Under these conditions, a significant amount of rhizobia were found attached to peanut root (class 2), indicating that the attachment-inhibiting activity of the adhesin was affected by the proteinase treatment (Fig. 3). These results suggest that proteinaceous molecules are involved in the attachment of rhizobia to peanut roots.

**Fig. 2.** Sodium dodecyl sulphate – polyacrylamide gel electrophoresis of crude adhesin fractions isolated from *Bradyrhizobium* sp. Lane A, molecular mass marker (lysozyme). Lane B, *Bradyrhizobium* sp. strain TAL 1000. Lane C, *Bradyrhizobium* sp. strain SEMIA 6144.




It has been reported that the binding of rhicadhesin to *Rhizobium* spp. and *Agrobacterium* spp. cells is mediated by $Ca^{2+}$. It was found that the reduction in the attachment ability of *R. leguminosarum* 248 to pea root-hair surfaces, as a result of $Ca^{2+}$ limitation, was not due to the loss of flagella or motility (Smit et al. 1989*b*). It seems that under low $Ca^{2+}$ conditions, rhicadhesin was released from the bacterial surface into the growth medium (Smit et al. 1991). Calcium ions are not involved in the binding of rhicadhesin to the plant surface but seem to be involved in the anchoring of adhesin to the bacterial cell surface (Smit et al. 1991).

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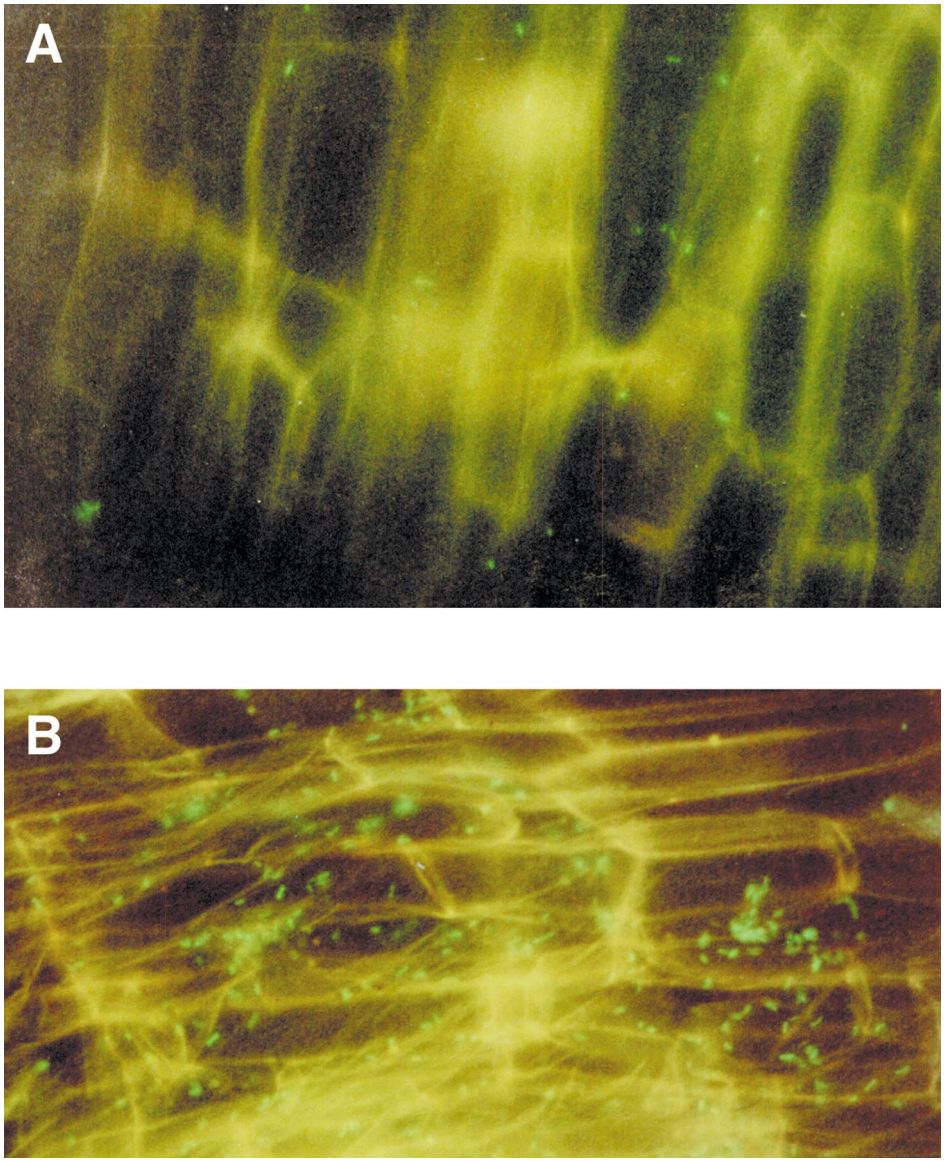
**Fig. 3.** Influence of the proteinase K treatment on the attachment–inhibition activity of the 14-kDa protein of *Bradyrhizobium* sp. strain TAL 1000. (A) Control. (B) The 14-kDa protein treated with proteolytic enzyme. Similar results were obtained with *Bradyrhizobium* sp. strain SEMIA 6144. Fifteen roots were examined for each OD. Magnification: ×400. Conditions for assay are described in Materials and methods.




To know whether the adhesin present in the cell surface preparation is $Ca^{2+}$ dependent, cells grown in YEM medium with the addition of EDTA were used to determine their ability to bind to adhesin-treated peanut roots. It was assumed that previous binding of the adhesin to a root receptor would allow, in a following attachment assay, a normal binding of adhesin-detached bacteria. A high level of attached viable cells was found (46% and 76% of the control for strains TAL 1000 and SEMIA 6144, respectively), indicating that adhesin bound to bacteria could be released into the EDTA-supplemented medium, probably enabling the binding of the cells to root-bound adhesin. The fact that the number of viable cells bound to peanut root under this condition did not reach the control level might be related to the EDTA concentration used or to deficiencies in the growth of the bacteria caused by the calcium-limiting growth conditions. These results are in agreement with results reported by Smit et al. (1989*a*, 1989*b*, 1991) in *R. leguminosarum*.

## Conclusions

It has been demonstrated for leguminous plants infected by means of an infection thread that rhizobia attachment is very helpful for the optimal induction of *nod* genes, the consequent delivery of the rhizobial Nod factors, as well as for root-hair curling. So far, three components have been identified that play a role in the attachment of *Rhizobium* spp. bacteria to pea root-hair tips: rhicadhesin, cellulose fibrils, and plant lectin. Although the involvement of rhicadhesin in the attachment process has been studied for different rhizobia–legume interactions, there are no indications about the role

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of this molecule in the attachment of bacteria to legumes, such as peanut, infected by the "crack" infection process.

Partial characterization of the adhesin detached from the *Bradyrhizobium (Arachis hypogaea)* sp. revealed that it is a soluble surface component. Treatment of adhesin with proteinase K completely abolished the ability of the preparation to inhibit rhizobia attachment to peanut root. Furthermore, cells treated with EDTA were found to be able to bind to adhesin-treated peanut roots, suggesting that adhesin was not anchored to the cell surface because of the low calcium concentration. This result clearly demonstrates that calcium is essential for the attachment ability in these cells. Moreover, as was demonstrated for other rhizobia strains, a 14-kDa protein from the crude adhesin preparation seems to play a role in the attachment process. Taken together, these results indicate that the attachment of *Bradyrhizobium* sp. to peanut roots is mediated by a $Ca^{2+}$-dependent, cell-surface-located, water-soluble, 14-kDa adhesin, likely rhicadhesin.

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## References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. Short protocols in molecular biology. 3rd ed. John Wiley & Sons Inc., New York. pp. 10–36.

Boogerd, F., and van Rossum, D. 1997. Nodulation of groundnut by *Bradyrhizobium*: a simple infection process by crack entry. FEMS Microbiol. Rev. **21**: 5–27.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.

Caetano-Anolles, G., and Favelukes, G. 1986. Quantitation of adsorption of rhizobia in low number to small legume roots. Appl. Environ. Microbiol. **52**: 371–376.

de Rudder, K., Thomas-Oates, J., and Geiger, O. 1997. *Rhizobium meliloti* mutants deficient in phospholipids *N*-methyltransferase still contain phosphatidylcholine. J. Bacteriol. **179**: 6921–6928.

Gage, D.J., Bobo, T., and Long, S.R. 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J. Bacteriol. **178**: 7159–7166.

Hadri, A., Spaink, H., Bisseling, T., and Brewin, N. 1999. Diversity of root nodulation and rhizobial infection processes. *In* The *Rhizobiaceae*. *Edited by* H. Spaink, A. Kondorosi, and P. Hooykaas. Kluwer Academic Publishers, Dordrecht, Netherlands. pp. 347–360.

Kijne, J. 1992. The *Rhizobium* infection process. *In* Biological nitrogen fixation. *Edited by* G. Stacey, R. Burris, and H. Evans. Chapman and Hall Inc., New York. pp. 349–398.

Kijne, J., Smit, G., Díaz, C., and Lugtenberg, B. 1988. Lectin enhanced accumulation of manganese-limited *Rhizobium leguminosarum* cells on pea root hair tips. J. Bacteriol. **170**: 2994–3000.

Matthysse, A., and Kijne, J. 1998. Attachment of *Rhizobiaceae* to plant cells. *In* The *Rhizobiaceae*. *Edited by* H.P. Spaink, A. Kondorosi, and P. Hooykaas. Kluwer Academic Publishers, Dordrecht, Netherlands.

Matthysse, A., and McMahan, S. 2001. The effect of the *Agrobacterium tumefaciens attR* mutation on attachment and root colonization differs between legumes and other dicots. Appl. Environ. Microbiol. **67**: 1070–1075.

Smit, G., Kijne, J., and Lugtenberg, B. 1986. Correlation between extracellular fibrils and attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. **168**: 821–827.

Smit, G., Logman, T., Boerrigter, M., Kijne, J., and Lugtenberg, B. 1989*a*. Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* $Ca^{2+}$-dependent adhesin, which mediates the first step in attachment of cells of the Family *Rhizobiaceae* to plant root hair tips. J. Bacteriol. **171**: 4054–4062.

Smit, G., Kijne, J., and Lugtenberg, B. 1989*b*. Roles of flagella, lipopolysaccharide, and a $Ca^{2+}$-dependent cell surface protein in attachment of *Rhizobium leguminosarum* biovar *viciae* to pea root hair tips. J. Bacteriol. **171**: 569–572.

Smit, G., Tubbing, D., Kijne, J., and Lugtenberg, B. 1991. Role of $Ca^{2+}$ in the activity of rhicadhesin from *Rhizobium leguminosarum* biovar *viciae* which mediates the first step in attachment of *Rhizobiaceae* cells to plant root hair tips. Arch. Microbiol. **155**: 278–283.

Swart, S., Smit, G., Lugtenberg, B., and Kijne, J. 1993. Restoration of attachment, virulence and nodulation of *Agrobacterium tumefaciens chvB* mutants by rhicadhesin. Mol. Microbiol. **10**: 597–605.

Trevelyan, W., and Harrison, J. 1952. Studies on yeast metabolism. Fractionation and microdetermination of cell carbohydrates. Biochem. J. **50**: 298–303.

Vincent, J.M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publications, Oxford.

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