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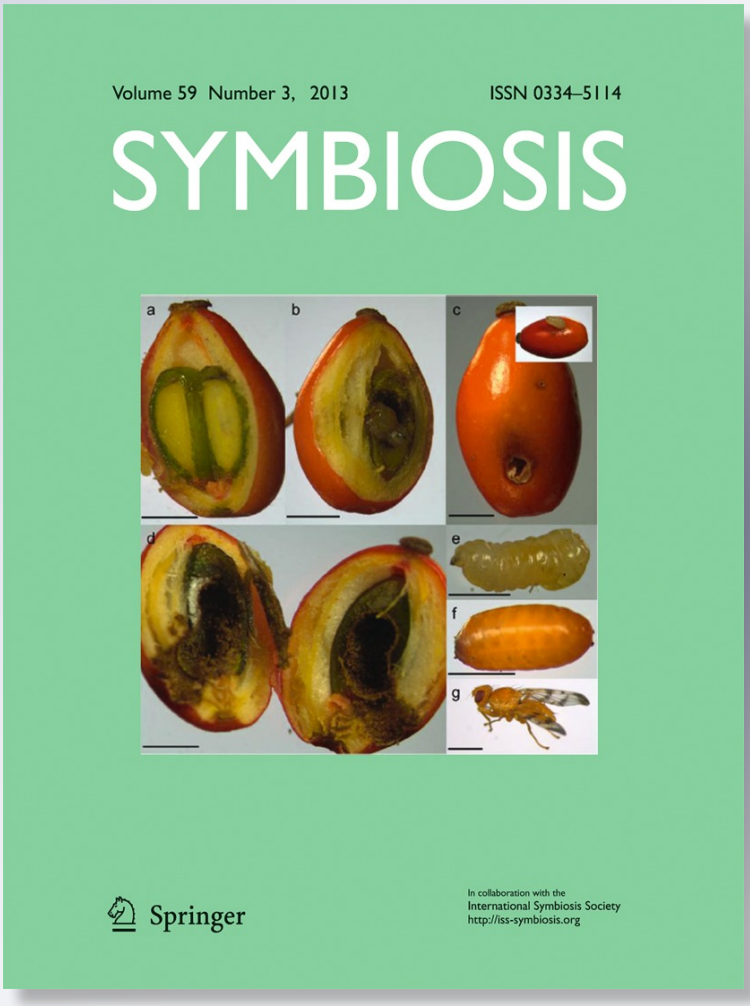

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Abstract Combined forms of nitrogen negatively influence rhizobia-legume symbiosis. The effects of combined nitrogen are known for nodulation and dinitrogen (N_2) fixation, but little is known about the effect on preinfection events. Here, we studied the effects of combined nitrogen on the adhesion of *Rhizobium etli* to common bean (*Phaseolus vulgaris* L.) roots. When potassium nitrate (KNO_3) or sodium glutamate was added to an incubation mixture of rhizobia and plants that were previously grown in nitrogen-free solution, rhizobial adhesion to roots was stimulated. However, the rhizobial adhesion to bean roots that were previously grown with 10 mM KNO_3 was reduced by half. A fraction of the bean root exudates, which is thermolabile and has molecular mass larger than 12 kDa stimulated rhizobial adhesion, but this stimulatory activity was lost in root exudates obtained with 10 mM KNO_3. Thus, the inhibition of symbiosis in response to combined nitrogen may be controlled by the plant at the preinfection stage as well.

Keywords Nitrate · Root exudate · Adhesion · Rhizobium

1 Introduction

Legume-*Rhizobium* symbiosis for dinitrogen (N_2) fixation is a complex process achieved through a series of events controlled by the bacteria and the plant (Patriarca et al. 2004). This process is initiated when soil rhizobia colonize the rhizosphere and adhere to root surfaces, mainly root hairs. The adhesion of rhizobia to root surfaces occurs in two steps. In the first step, which is completed in a few hours, single rhizobial cells adhere reversibly to the roots; however, in the second step, which requires more than 12 h, a cap of rhizobia is formed on the previously adhered cells and adhesion becomes irreversible (Dazzo et al. 1984; Smit et al. 1987). The reversible adhesion step is dependent on various bacterial cell surface components, such as surface and extracellular polysaccharides (Gay-Fraret et al. 2012; Laus et al. 2006; Pérez-Giménez et al. 2009; Williams et al. 2008), bacterial adhesins (Ho et al. 1990; Mongiardini et al. 2008; Smit et al. 1989) and cellulase (Robledo et al. 2008), environmental factors such as divalent cations and pH (Caetano-Anollés et al. 1989; Lodeiro et al. 1995; Mongiardini et al. 2008; Smit et al. 1987), or root-exuded compounds and lectins (Dazzo and Hubbell 1975; Díaz et al. 1989; Laus et al. 2006; Lodeiro et al. 2000; van Rhijn et al. 1998; Wall and Favelukes 1991). The irreversible adhesion step requires the synthesis of cellulose fibrils by the rhizobia, which firmly anchor the bacterial cells to the root surface (Ausmees et al. 1999; Dazzo et al. 1984; Robledo et al. 2012; Smit et al. 1987). Although rhizobial adhesion is clearly essential for the initiation of root infection, the bacterial surface and extracellular polysaccharides as well as the legume lectins are the only factors affecting adhesion that have also been demonstrated to be required for nodulation (Díaz et al. 1989; Laus et al. 2004; Noel et al. 2000; Quelas et al. 2010; Stacey et al. 1991; van Rhijn et al. 1998; van Workum et al. 1998).

During rhizosphere colonization and the adhesion of rhizobia to the roots, an exchange of molecular signals between both symbiotic partners occurs. The roots release specific flavonoids in their exudates, thus inducing the rhizobia to synthesize a lipochitooligosaccharide recognition signal (*nod* factor) that is required for root hair infection and nodule development (reviewed by Oldroyd et al. 2011). Interestingly, the core structure of the *nod* factor also

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stimulates rhizobial adhesion to roots and biofilm formation on inert surfaces, likely through a pathway different than that of infection (Fujishige et al. 2008).

Finally, rhizobia invade and colonize the root nodules, where they differentiate into bacteroids, the bacterial state able to fix N_2. Thus, the plant partner provides not only a protected environment to the bacteria but also dicarboxylic acids as carbon and energy sources, while the rhizobia reduce atmospheric N_2 to ammonia, which is used by the plant.

In addition to obtaining nitrogen from the air due to rhizobial N_2 fixation, legume plants are also able to satisfy their nitrogen needs with combined nitrogen (mainly ammonia or nitrate) obtained from the soil solution. Because symbiotic N_2 fixation requires additional energetic expenditure, legumes inhibit symbiosis if combined nitrogen sources are available in the soil (Schubert 1995). The inhibition of the symbiotic process by combined nitrogen has been demonstrated at two levels: the local inhibition of root infection and nodulation and the systemic inhibition of nodule development and N_2 fixation. This regulatory circuit is related to the autoregulation of nodulation, through which the plant regulates its total number of nodules to balance the cost of nodule function and maintenance and the satisfaction of the nitrogen needs of the plant by nodule activity (Mortier et al. 2012). At the molecular level, this process is regulated by a cycle of kinase/phosphatase activity within the root cells (Reid et al. 2011).

Although the inhibitory effects of combined nitrogen were also described for the expression of genes encoding *nod* factor biosynthesis (Dusha et al. 1999), little is known about the possible effects of combined nitrogen on the preinfection steps of *Rhizobium*-legume symbiosis. Therefore, to gain insight into the effects of combined nitrogen at this stage, we tested whether its presence may affect the adhesion of *Rhizobium etli* to common bean (*Phaseolus vulgaris* L.) roots. The study examined two scenarios: the effect of combined nitrogen during the rhizobia-plant incubation or the effect of the addition of combined nitrogen to the plant growth medium prior to contact with rhizobia. With this approach, different effects of combined nitrogen on adhesion were evaluated, specifically, the differential influence on rhizobial adhesion of root exudates obtained from roots grown with or without combined nitrogen.

2 Materials and methods

2.1 Plants and rhizobial strain

Rhizobium etli LP 1003 S (resistant to streptomycin, Lodeiro et al. 1995) was grown in yeast extract-mannitol broth (YMB) or yeast extract-mannitol agar 15 $g.l^{-1}$ (YMA, Vincent 1970) at 28 °C. For adhesion experiments, liquid cultures were grown to the late exponential phase (Lodeiro et al. 1995). Streptomycin and cycloheximide were used at a concentration of 100 $mg.l^{-1}$.

Seeds of the common bean Dor 41 (provided by IMYZA, INTA-Castelar, Argentina) were surface-sterilized with 20 %v/v commercial bleach (5 % NaClO) and germinated in water-agar or Fåhraeus-agar 15 $g.l^{-1}$ for 4 days at 28 °C, as described previously (Lodeiro et al. 1995).

2.2 Root exudates

One-day-old germinated roots (approximately 1 cm in length) were aseptically passed through sterile stainless steel mesh and dipped in sterile Fåhraeus solution (Fåhraeus 1957) with or without 10 mM potassium nitrate (KNO_3). The roots were maintained in these solutions in the dark at 28 °C for three days (Wall and Favelukes 1991). Then, plants were removed and the root exudates were collected. Root exudates were dialyzed with a membrane with a 12-kDa cutoff against Fåhraeus solution without KNO_3 in a 1:100 volume ratio, with at least three changes of the dialyzing solution. After dialysis, the root exudates were filter-sterilized through a 0.2-μm pore diameter polycarbonate membrane and used immediately.

To evaluate the thermal stability of the root exudate activity, the root exudates were obtained in N-free Fåhraeus solution, filter-sterilized, and then heated at 100 °C for 30 min before use.

2.3 Electrophoresis of root exudate proteins

A total of 500 ml of root exudate was prepared from 200 g of bean seedlings in Fåhraeus solution either with or without KNO_3 and was dialyzed as described above. Root exudates were precipitated overnight with 80 % saturated ammonium sulfate at 4 °C, centrifuged at 9,000 x g and resuspended in 5 ml of double-distilled water. The suspension was dialyzed again and precipitated with four volumes of acetone at −20 °C. After resuspension in 100 μl of double-distilled water, the sample was diluted 1:2 and a 2.5-μl aliquot was loaded into a 12.5 % polyacrylamide gel with SDS (SDS-PAGE), which was run at 100 V and silver stained as previously described (Althabegoiti et al. 2008).

2.4 Adhesion experiments

To measure adhesion of rhizobia to bean roots, a previously described method was used (Caetano-Anollés and Favelukes 1986; Lodeiro et al. 1995). In brief, 10 four-day-old seedlings were incubated in 50 ml of Fåhraeus solution (pH=7.0) containing a $1:10^5$ dilution of YMB-grown rhizobia. The

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exact numbers of colony-forming units (CFU) present in the suspensions were quantified by plate count in each experiment and are indicated below. The plant-rhizobia incubation was performed for the indicated times at 28 °C with rotary shaking at 50 rpm. After incubation, rootlets with adsorbed rhizobia were washed four times, each time by shaking with N-free Fåhraeus solution for 1 min at 120 rpm. The rootlets were distributed at the bottom of Petri dishes and overlaid with molten (45 °C) YMA supplemented with streptomycin and cycloheximide both for selection of the assayed strain and to avoid root growth after the adhesion experiment. After incubating the plate at 28 °C, rhizobia remaining adsorbed on the embedded root surfaces developed microcolonies, which were counted along the visible surface of each primary root under a dissecting microscope at 25x magnification. Total counts of visible microcolonies on primary roots, expressed as the percentage of the total number of CFU present in the original inoculum, represented the adhesion index, *%A* (Lodeiro et al. 1995).

To determine whether the presence of KNO_3 during early growth of common bean roots modifies adhesion, the plants were permitted to germinate in water-agar or Fåhraeus-agar, each either with or without the addition of 10 mM KNO_3. Then, adhesion of *R. etli* LP 1003-S to these roots in N-free Fåhraeus solution was assayed as described above.

To study the effects of root exudates on adhesion, the YMB-grown rhizobia were diluted $1{:}10^5$ in 25 ml of root exudates (test rhizobia) or in 25 ml of Fåhraeus solution (control rhizobia) and preincubated for 2 h at 28 °C with shaking at 50 rpm in the absence of plants. After the pretreatment period, 25 ml of either fresh Fåhraeus solution or root exudate was added to the test and control rhizobia, respectively, to equalize the composition of both incubation mixtures (Wall and Favelukes 1991). Immediately, 10 four-day-old bean plants were added to each rhizobia suspension. Incubation was continued at 28 °C and 50 rpm for an additional 30 min, a period that was long enough to observe *R. etli* adhesion to bean roots (Lodeiro et al. 1995) but brief enough to minimize the effects of exudates released by the newly added roots. Finally, the bean roots were washed and included in the YMA petri dishes to obtain *%A* values, as described above.

2.5 Statistical analysis

The results presented here are representative of at least two independent experiments. The statistical analysis was performed by analysis of variance (ANOVA) of total CFU adhered to each plant. Confidence intervals of *%A* values (95 %) were calculated as previously described (Caetano-Anollés and Favelukes 1986).

3 Results

3.1 Effects of combined nitrogen in the plant-rhizobia incubation mixture

Ten bean seedlings and $5.75 \pm 0.71 \times 10^4$ *R etli* LP 1003-S CFU were incubated together during 4 h in 50 ml of Fåhraeus solution with 0, 2, or 10 mM KNO_3 as an inorganic nitrogen source, each one in the presence of 0, 4, or 20 mM sodium glutamate as an organic nitrogen source. The number of viable rhizobia doubled during the incubation period, but no significant differences were observed among the final viable bacteria numbers obtained with the different incubation mixtures (data not shown). When a single source of combined nitrogen was present, the adhesion index (*%A*) increased from 58 % to 145 % with respect to incubation without combined nitrogen. However, the increase of KNO_3 concentration had little effect in the presence of sodium glutamate, especially at the highest sodium glutamate concentration (20 mM), where no effects of KNO_3 were observed on adhesion (Fig 1a). The ANOVA with a 3×3 factorial design performed on these data indicated a significant interaction between both nitrogen sources, as well as significant effects of KNO_3. However, there were no significant differences in *%A* in response to sodium glutamate (Fig 1b). Therefore, subsequent experiments were performed using KNO_3 as the nitrogen source.

3.2 Effects of combined nitrogen on the plant

To determine whether the roots play a passive or active role during rhizobial adhesion, the plants were killed by incubating them overnight in Fåhraeus solution with 100 $mg.l^{-1}$ cycloheximide, while a control group was kept alive and treated similarly with Fåhraeus solution without antibiotics. The plants treated with cycloheximide did not react to triphenyl tetrazolium compared to the control plants. In addition, the root surface morphology looked unaltered under the light microscope. The adhesion index of *R. etli* LP 1003-S for the cycloheximide-treated roots was 0.57 %± 0.06 compared with 1.85 %±0.13 obtained with the control roots, thus suggesting the existence of root activity during rhizobial adhesion. To examine whether this root activity is affected by combined nitrogen, the adhesion of *R. etli* LP 1003-S to bean roots grown with or without 10 mM KNO_3 was measured. The *%A* value was significantly lower in KNO_3-grown roots than in nitrogen-free roots (Table 1). This effect was stronger in the roots germinated in Fåhraeus-agar, for which only 52.6 % adhesion to the KNO_3-grown roots in relation to the nitrogen-free roots was observed; the roots in water-agar had 64.6 % adhesion

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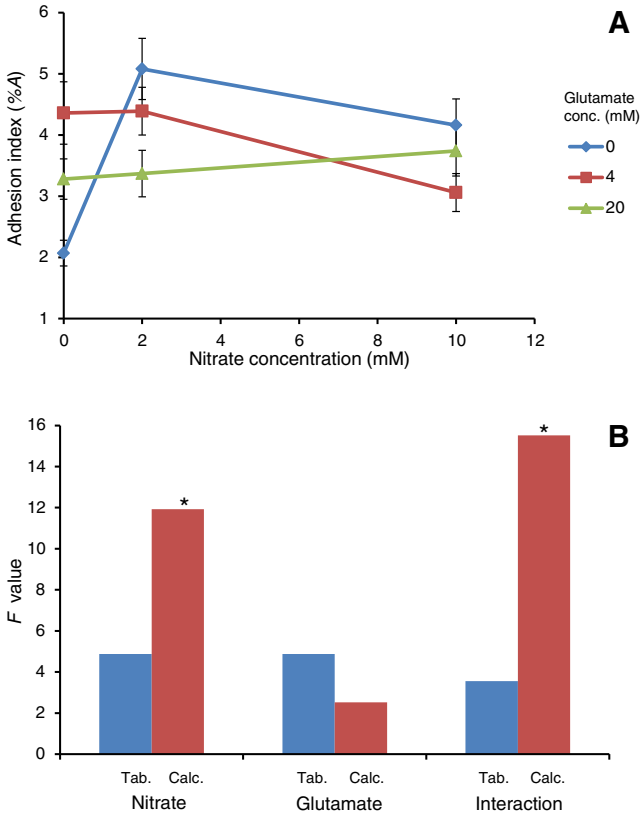

Fig. 1 Effect of combined nitrogen on the adhesion of *R. etli* 1003 S to common bean roots grown in nitrogen-free Fåhraeus solution. **a** *%A* values (± confidence intervals with $p<0.05$) at different concentrations of KNO_3 with Na glutamate at 0 mM (*diamonds*), 4 mM (*squares*), or 20 mM (*triangles*). **b** Tabulated (*Tab.*, left bar, $p<0.01$) or calculated (*Calc.*, right bar) *F* values from the factorial analysis of variance to assess the significance of KNO_3 or sodium glutamate (individual effects, 2 degrees of freedom), or their interaction (4 degrees of freedom) on the adhesion of *R. etli* to bean roots. *Statistically significant differences

to the KNO_3-grown roots in relation to the nitrogen-free roots.

To see whether the inhibitory effect of KNO_3 could have some relation to the compounds released in the bean root exudates, $3.71 \pm 0.59 \times 10^3$ CFU ml^{-1} of *R. etli* 1003-S were preincubated with root exudates obtained with or without KNO_3 and were then incubated for a short time with bean roots in the absence of a nitrogen source. The results of these experiments are summarized in Table 2. The root exudates obtained in N-free Fåhraeus solution significantly stimulated rhizobial adhesion to the bean roots. In contrast, the root exudates obtained in Fåhraeus solution with 10 mM KNO_3 did not stimulate adhesion. These results indicated that a high-molecular weight compound, larger than 12 kDa, present in the root exudate might be able to stimulate adhesion of rhizobia to the root surface. The compound might be thermally unstable, as indicated by the loss of activity after heating the root exudate to 100 °C for 30 min (Table 2).

Table 1 Effect of the addition of 10 mM KNO_3 during bean root growth on the adhesion of *R. etli* 1003 S. *%A* values (± confidence intervals with $p<0.05$)

KNO_3 (mM) during root growth	Root growth medium	*%A*	N inhibition (%)
0	water-agar	5.90 ± 0.77	—
10	water-agar	3.81 ± 0.64	35.4
0	Fåhraeus-agar	6.37 ± 0.82	—
10	Fåhraeus-agar	3.35 ± 0.34	47.4

In each condition, $7.02 \pm 0.97 \times 10^4$ rhizobial CFU were incubated for 4 h in nitrogen-free Fåhraeus solution with 10 bean plants grown for 4 days in the growth medium indicated

The macromolecular nature and thermal instability of the active root-exuded compound suggested that it might be a protein. Therefore, SDS-PAGE was used to examine possible differences in the protein profiles of root exudates obtained either with or without 10 mM KNO_3. The root exudate samples obtained after ammonium sulfate precipitation and dialysis were viscous and dark, which prohibited protein quantification by colorimetric methods. Absorbance at 280 nm from a 1:10 dilution obtained readings of approximately 1.0. However, when this material was separated by SDS-PAGE, the bands were not observed after coomassie blue staining, which indicated that most of the UV-absorbing material was not protein. Bands were observed after silver staining, although there was a smear that prevented optimal definition (Fig. 2). Nevertheless, a pair of bands was observed above the 29-kDa marker, from which the band of higher mobility (36 kDa) was more intense in the root exudates obtained with 10 mM KNO_3 than in the N-free root exudates, compared with the band of lower mobility (pointed by arrows in Fig. 2).

4 Discussion

The results presented here show two different effects of combined nitrogen on the first, reversible step of adhesion of *R. etli* to bean roots. Firstly, adhesion was stimulated when either sodium glutamate or KNO_3 was added for the short time during which the rhizobia were incubated together with plants grown in nitrogen-free medium. Secondly, adhesion was inhibited when the rhizobia were incubated with plants that were previously grown in media containing KNO_3. These results are in agreement with the previous observations that the inhibition of symbiosis in the presence of combined nitrogen is exerted mainly by the plant during infection and nodule functioning (Mortier et al. 2012; Reid et al. 2011; Schubert 1995), but suggest that in the common bean this inhibition might also take place earlier, at the preinfection stage.

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Table 2 Stimulation of adhesion of *R. etli* 1003 S by pretreatment with bean root exudates obtained in Fåhraeus solution either with or without the addition of 10 mM KNO_3. In all cases, the bean root exudates were extensively dialyzed against Fåhraeus solution without KNO_3 and were filter-sterilized before use. Adhesion index (*%A*) ± confidence intervals with $p<0.05$ are shown

Previous root exudates treatment	Preincubation media[a]	Media added for incubation[b]	*%A*
None	Fåhraeus solution	Root exudate	0.37 ± 0.04
None	Root exudate	Fåhraeus solution	0.54 ± 0.06
Addition of 10 mM KNO_3 during root exudation	Root exudate (obtained with KNO_3)	Fåhraeus solution	0.38 ± 0.04
Heating at 100 °C 30 min after dialysis	Root exudate (heated)	Fåhraeus solution	0.37 ± 0.04

[a] Preincubation of rhizobia was performed for 2 h at 28 °C and 50 rpm in 25 ml

[b] 25 ml of the indicated medium was added to the preincubated rhizobia. Immediately, 10 four-day-old bean plants were added, and the incubation was continued for 30 min at 50 rpm and 28 °C. Then, the plants with adhered rhizobia were washed and *%A* was determined

The combined nitrogen present during the root-rhizobia incubation might have stimulated adhesion through a nutritional or energetic effect on the rhizobia. Nitrate and glutamate may be used as nitrogen sources by *R. etli*; additionally, glutamate may serve as carbon source. However, nitrate is not used as electron acceptor by *R. etli* CFN42 (Bueno et al. 2005; Calderón-Flores et al. 2005). Because the Fåhraeus solution has no nitrogen and the only carbon it contains is the 20 μM ferric citrate (Fåhraeus 1957), the simultaneous presence of sodium glutamate and KNO_3 might have provided extra carbon and nitrogen sources. This effect may have led to a different metabolic state of the rhizobial cells compared to those incubated in the presence of each nutrient alone, as indicated by the significant interaction observed between these two compounds. However, the effects of these nutrients on bacterial growth during this short incubation period were small, considering that the numbers of CFU at the beginning and at the end of the experiment were similar regardless of the presence of one or both nitrogen sources. Furthermore, the observed stimulation of adhesion by each combined nitrogen source tested individually indicates that in this short incubation period, the production of *nod* factor, whose core structure enhances adhesion (Fujishige et al. 2008), was either not completely inhibited by the presence of combined nitrogen (Dusha et al. 1999) or had not yet reached an appreciable rate in response to the flavonoids that could have been released by the roots.

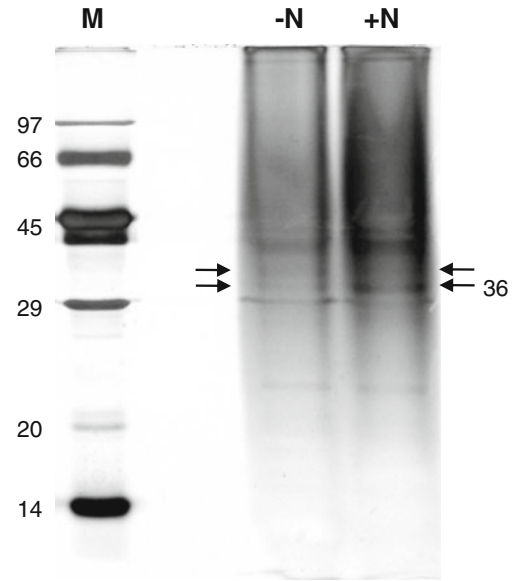

Fig. 2 Polypeptides from bean root exudates obtained in N-free Fåhraeus solution (−N) or Fåhraeus solution supplemented with 10 mM KNO_3 (+N). Molecular mass markers are shown on the left (lane M), and their values are in kDa. The pair of bands mentioned in the text, and the differential 36-kDa band are indicated by arrows

The inhibition of adhesion of rhizobia to nitrate-grown roots seems to be mediated, at least in part, by a compound released in the root exudates. Plant root exudates contain many substances, including sugars, organic acids, amino acids and phenolic compounds, which can be used by the bacteria as nutrients or chemoattractants and may induce the expression of several genes. Among the genes induced are those involved in the biosynthesis of *nod* factors (Badri and Vivanco 2009). However, these small molecules should have been removed from the root exudates after the dialysis process. In addition, the active compound was thermolabile after treatment at 100 °C for 30 min, further suggesting that it is not a molecule such as those mentioned above. Because the properties observed for the active compound correspond with the protein properties, the protein profiles of bean root exudates obtained in either the presence or absence of KNO_3 were compared. A differential band in the root exudates obtained in the absence of KNO_3, to which the stimulatory activity might be attributed, was not found. Instead, the only difference was a 36-kDa band present in higher amount in the root exudates obtained with KNO_3. Therefore, if this polypeptide modifies adhesion, it should be an inhibitor of the stimulatory activity, although future studies are required to ascertain whether this polypeptide has any role.

Taken together, these results indicate that the inhibition of symbiosis by combined nitrogen could be a multi-step process

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controlled mainly by the plant and taking place at several levels, including the preinfection and infection stages.

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