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Respiratory stimulus in *Rhizobium* sp. by legume lectins

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Summary

High molecular weight lectins (>100 kDa) from seeds of the legumes *Canavalia brasiliensis* (CnBr), *Cratylia floribunda* (CFL), *Phaseolus vulgaris* (PHA) and *Vatairea macrocarpa* (VML), temporarily stimulate the respiration of *Rhizobium tropici*-CIAT899 and *R. etli*-CFN42. These stimulants were significant ($P < 0.05$) in bacterial suspensions (>2.85 mg dry biomass ml^{-1}), having at least 6200 molecules of lectins per bacteria. The VML (20 μg ml^{-1}), induced specific O_2 demand of 2.3–2.5 ηM O_2 min^{-1} mg dry biomass^{-1}, in CFN42 and CIAT899, respectively. However, CnBr, CFL and PHA induced smaller demands of O_2 (5×), in both strains. The order of affinities of the lectins was approximately VML > PHA > CFL > CnBr, with regard to respiratory stimuli in CIAT899 strain. The co-administration of 10 μg VML ml^{-1} and 9.8 ηM galactose, in CIAT899 suspensions, reduced the respiratory stimuli significantly in relation to the treatment with VML alone. These respiratory stimuli, induced by the lectins, increase the significance of the interaction lectin × *Rhizobium* in terms of bacterial physiology. Its understanding could be important in relation to bacterial symbiotic behaviour.

Introduction

Rhizobium tropici and *Rhizobium etli* are gram-negative bacteria which induce nodules and fix atmospheric nitrogen in *Phaseolus vulgaris* [L.] bean plants (Learemans & Vanderleyden 1998). The nodulation is a multistep process, characterized by the recognition of molecular signals between specific partners. The *Rhizobium* interacts with the surface of the root hairs and invades them to reach the emergent nodules in the host plant (Cohn *et al.* 1998; Learemans & Vanderleyden 1998). In this process flavonoids of the host are recognized by the bacteria which present diffused lipochitin oligosaccharides signals (Nod factors) and surface lipopolysaccharides, both with specific structure and size (Downie & Walker 1999). There is considerable evidence in the literature (Hirsch 1999) that these rhizobial polysaccharides bind to lectins present in the surface of the root hair. These lectins have motivated claims that they may be mediators of that symbiotic specificity (Brewin & Kardansky 1997; Kijne *et al.* 1997; Hirsch 1999). Direct and indirect elements support this suggestion. Among direct elements in the literature we have: (1) the aggregation of *Rhizobium* in the rhizosphere; (2) *Rhizobium* adheres to the surface of the root hairs; and the recognition by the lectin of both the (3) lipo-chitin oligosaccharides and (4) lipopolysaccharides of the *Rhizobium* (Kijne *et al.* 1997; Hirsch 1999). In relation to the indirect elements (in the plant) we have: (1) cellular division stimulation; a participation in the (2) protein storing system and (3) in protein vesicle trafficking; and (4) cytokinesis (Brewin & Kardansky 1997). Despite the efforts, there is still the need to better understand the role of lectins as adherent molecules between the *Rhizobium* and the host plant, or as more active molecules in the symbiotic specificity with the transmission of signals between partners (Hirsch 1999).

Lectins are proteins with varied structures that bind reversibly and specifically to mono- and oligosaccharides, without enzymatic modifications. With respect to legume lectins, the majority are tetramers or dimers. Their basic units (monomers) are made up of

polypeptides with regions of β sheet and α helix. The monomer shows two hydrophobic domains with steric importance. The carbohydrate-binding site occurs in four folding regions found in the extremity of the basic unit. The general aspects of this unit are well conserved in the legume lectins (Loris *et al.* 1998; Vijayan & Chandra 1999).

Few lectins have been characterized from legume roots and there is little information about the possible relationship between the *Phaseolus vulgaris* lectin (PHA) and *Rhizobium* (Kijne *et al.* 1997). However, information about lectin effects on *Rhizobium* symbiotic activity is being made available. In this sense, the *Rhizobium etli* incubation, treated with PHA, significantly improved the number of infection threads (Brelles-Mariño *et al.* 1996) and of functional nodules (Mestrellet *et al.* 1999) in bean roots. Lodeiro *et al.* (2000), pre-incubated *Bradyrhizobium* with soybean lectins and found stimulation of bacterial absorption to the host's root surface.

The effects of legume and other types of lectins in bacterial physiology have been evaluated in other systems. Antonyuk *et al.* (1997), indicated that wheat lectin (Man/Glc specific) stimulated N_2 fixation, ammonium excretion, glutamine synthetase activity and indolacetic acid production in *Azospirillum brasiliensis* culture. Majumder *et al.* (1997), showed that GalNAc-specific haemolymph lectin from crab (*Scylla serrata*) had inhibitory activity on the endogenous respiration and on the exogenous glucose oxidation of *Bacillus cereus* and *Escherichia coli*. On the other hand, Lau & Chan (1984), obtained a stimulation in the O_2 demand of *B. cereus* in response to the ConA lectin (Man/Glc-specific). The present investigation studies the influence of the Gal- and Glc-specific legume lectins on the respiratory activity of *Rhizobium* sp.

Materials and methods

Bacteria, lectins and reagents

Rhizobium tropici-CIAT899 and *R. etli*-CFN42 were provided by USDA/ARS/Beltsville *Rhizobium* Germplasm Collection. The legume lectins: *Canavalia brasiliensis* (CnBr), *Cratylia floribunda* (CFL) (Glc-specific) and *Vatairea macrocarpa* (VML) (Gal-specific) were provided by BioMol-Lab./Departamento de Bioquímica/UFC-Fortaleza-Brasil. PHA (*Phaseolus vulgaris*) lectin, MES (2-[*N*-morpholino]ethanesulphonic, sodium) buffer, glucose, galactose and other reagents utilized were acquired from Sigma-Aldrich Chemicals Inc. (St. Louis, MO, USA). Bovine serum albumin (BSA), was obtained from Becton, Dickinson and Co.

Growth conditions, total cells and bacterial biomass

Growth medium 79 [3.4 mM NaCl; 2.9 mM K_2HPO_4; 6.6 mM $MgSO_4$; 10^{-2} mM $FeCl_3$; 55 mM mannitol; 1 g. l^{-1} yeast extract – pH 6.8] was used in the culture of *Rhizobium*. The inocula ($\pm 10^6$ bacteria ml^{-1}) were established (20 ml 79 medium; 150 rev min^{-1}; at 30 °C for 24 h) and stored at 4 °C. The *Rhizobium* biomass was obtained from cultures at the end of the exponential phase (24 h), from an inoculum of 2×10^3 bacteria ml^{-1}. The counting of total cells in bacterial suspension, were carried out in a Neubauer camera (Hirschmann Techcolor-0.10 mm; 0.0025 mm^2). The dry biomasses (mg ml^{-1}) were determined gravimetrically from standard bacterial suspensions, dried at 100 °C for 6 h.

Bacterial suspensions and lectin solutions

Rhizobium cells (24 h culture) were centrifuged ($25{,}000 \times g$ for 15 min at 25 °C), re-suspended in MES-S buffer (10 mM MES, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 3 mM NaCl) and centrifuged again ($7000 \times g$ for 10 min at 4 °C). In addition, three cycles of centrifugation/re-suspension were carried out to obtain a dense bacterial suspension. This bacterial suspension was homogenized in a screen nylon monofilament (Schweiz., Seidengazefabrik AG Thal) with 16 μm mesh-opening and used to obtain standardized bacterial suspensions in MES-S buffer. ${OD_{595}}^{1\,cm} = 1.5$ was used as reference to adjust the bacterial density. The suspensions were divided (7.5 ml $tube^{-1}$) and stored under refrigeration. Lectin solutions (0.002 g ml^{-1}) in MES-S buffer, were incubated at 43 °C (ultrasonic cleaner) at room temperature for 30 min each. At the end, the lectin solutions were centrifuged ($22{,}000 \times g$ for 5 min at room temperature) and the pellets discarded. Protein determinations in the supernatant were carried out according by the Bradford method, using BSA as reference. Standard lectin solutions (in relation to dissolved protein) were established by dilution in MES-S buffer. The monosaccharide solutions were prepared in MES-S buffer.

Bioreactor and evaluation of oxygen activity

An open system or 'bioreactor', in polypropylene (Figure 1), was used to monitor the dissolved O_2 activity in bacterial suspensions. Temperature (30 ± 0.1 °C), agitation (170 rev min^{-1}) and air injection (18 ml min^{-1}) were controlled by: (1) a hydrothermal circuit; (2) an air injection/agitation device and (3) O_2 activity and temperature detectors. A Clark's electrode was used, with an incorporated thermo-sensor, connected to the oxymeter (Model 58-Yellow Springs Instrument Co., Ohio). The bioreactor was adjusted with nitrogen and air fluxes individually, 0 and 100% O_2 saturation, respectively, in MES-S buffer, under working conditions. The data was registered continuously on a chart recorder.

The refrigerated bacterial suspension was re-suspended, incubated (30 °C for 5 min), re-suspended again and transferred (7 ml) to the bioreactor. After 45–60 min (the O_2 activity was stationary), 0.5 ml of solution treatment was added, and the O_2 activity monitored for

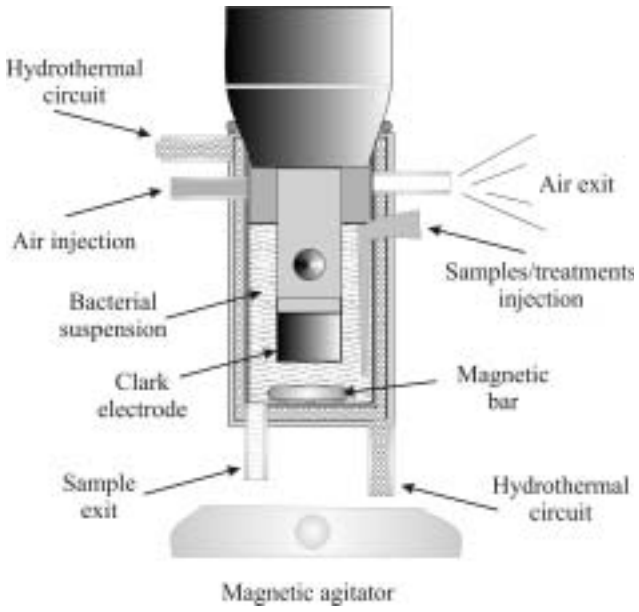

Figure 1. Bioreactor used to monitor dissolved oxygen activity: open system with Clark's electrode and controlled air flux, agitation and temperature.

20 min. At the end of this period, the reaction mixture was drained and the residues removed by three washings (distilled water–detergent solution–distilled water).

R. tropici respiratory activity: interaction bacterial density × lectin concentration

Four experiments were carried out, one experiment per lectin (with three repetitions). The bacterial and lectin factors were tested at four levels. Reference treatments (relative control (5 ηM mannitol) and absolute control (without lectin and without mannitol)) for the four bacterial densities were included. In this experiment, the *R. tropici*-CIAT899 strain was used as reference.

Lectin influence on the respiratory activity of **Rhizobium** *sp.*

Two-factor experiments were carried out to test lectin influence over *Rhizobium* respiration, one experiment for each *Rhizobium* sp. with two repetitions. The evaluated treatments were: (a) lectins: VML, PHA, CnBr and CFL (20 μg lectin ml^{-1}); and (b) lectins plus the hapten-monosaccharides: VML + Gal, PHA + Gal, CnBr + Glc and CFL + Glc (20 μg lectin ml^{-1} + 2.5 ηM monosaccharides). Reference **treatments** (a) monosaccharides (2.5 ηM Gal or Glc); (b) **reference BSA protein**: (20 μg ml^{-1}) and; (c) absolute control (without lectins, without monosaccharides and without BSA) – were included. In the experiments involving *R. tropici*-CIAT899 and *R. etli*-CFN42, a bacterial concentration of 3.13 and 2.80 mg dry biomass ml^{-1}, respectively, was used for each.

Respiratory activity of the R. tropici: influence of the hapten-monosaccharides

Two experiments were carried out to verify the influence of hapten-monosaccharides on lectin dependent *Rhizobium* respiration, one experiment for each lectin (CnBr and VML), with two repetitions. The evaluated treatments were: lectin fixed at concentrations of (10 μg ml^{-1}) with hapten-monosaccharides concentration of 0.6–10^4 ηM. Reference treatments: (a) lectin (10 μg ml^{-1}), (b) monosaccharides (625–10^4 ηM) and (c) absolute control (without lectin and without monosaccharides) – were included. In the experiments, the bacterial density of 3.13 mg dry biomass ml^{-1}, of the *R. tropici*-CIAT899 strain, was tested.

Data and statistical analysis

Quadratic adjustment of time-dependent O_2 activity variations, under treatment influence, were carried out and the estimated specific O_2 demand calculated. The bacterial suspensions' specific O_2 demands, correlated with the treatments, were corrected by the subtraction of the estimate in the absolute control. An analysis of variance for specific O_2 demand (ηM O_2 min^{-1} mg dry biomass^{-1}) was carried out and treatment effects were evaluated using the F statistic ($P < 0.05$). These variance analyses, were carried out with the software Statistic (Statsoft, Inc., Tulsa-OK). The standard error SE ($P < 0.05$), was estimated and the comparison of the treatment averages carried out by a Tukey test.

Results and discussion

Figure 2 shows the behaviour of dissolved O_2 activity with respect to CIAT899 suspensions, under the influence of bacterial density, lectin concentration, and mannitol. References A and B in Figure 2 are from different lectin experiments. The O_2 activities in the bacterial suspensions were inversely related to the bacterial biomass. An activity of ± 6 and ± 3.8 mg O_2 l^{-1} was observed, respectively for 2.74 and 3.22 mg dry biomass ml^{-1}, in both experiments. In general, the O_2 activity registered after the application of the treatments, showed an increase followed by four different phases of O_2 demand: (1) initial small stationary demand; (2) maximum demand, (3) final stationary demand and (4) decrease in the O_2 demand, with their respective transition periods. Phase 4 was omitted in Figure 2. A similar behaviour was observed in the bacterial suspensions that received mannitol. In relation to the activity increase before phase 1, it is probably due to: (a) co-administration of O_2 with the treatment, (b) temporary increase of turbulence in the bacterial suspension, during treatment administration or (c) both. The predominance and intensity of the phases mentioned are related to the concentration and type of lectin, as well as to the bacterial density. In this sense,

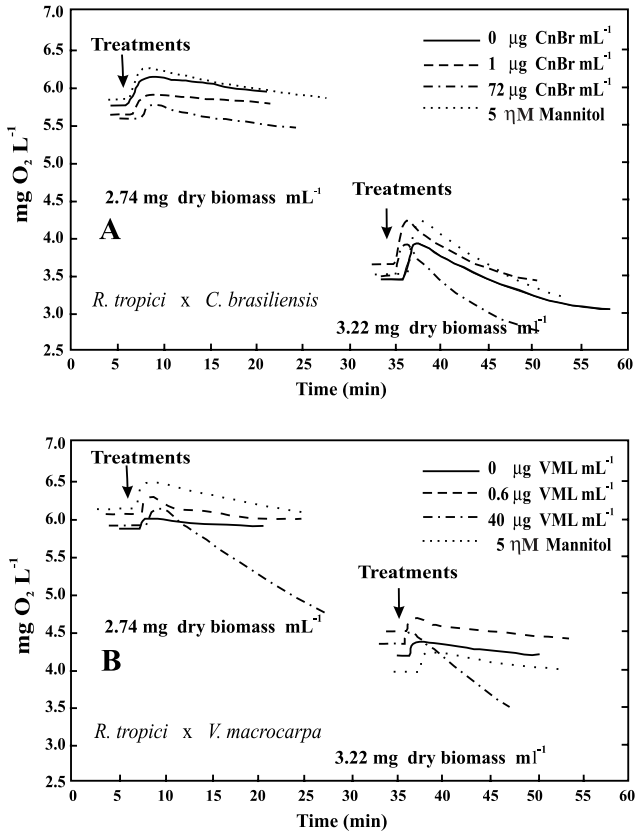

Figure 2. Time-dependent dissolved O_2 activity in *Rhizobium tropici*-CIAT899 suspension: bacterial density and lectin concentration influences. The *Canavalia brasiliensis*-CnBr (A) and *Vatairea macrocarpa*-VML (B) were glucose- and galactose-specific, respectively.

when considering the O_2 activity in phases 2 and 3, for a quadratic adjustment, estimates of O_2 maximum demands were better approximated for the treatments. The specific O_2 demands for *Rhizobium* (dry biomass-based) were calculated from this specific viewpoint.

The variance analyses of this data showed that the *R. tropici* CIAT899 specific O_2 demands were influenced by the interaction of bacterial densities and lectin concentrations. Figure 3 shows the results of the four experiments. In general the CIAT899 respiration is stimulated differently. Strong significant stimuli (**>1 ηM O_2 min^{-1} mg dry biomass^{-1}**) were observed in the experiment with VML (Figure 3D). On the other hand, the CnBr, CFL and PHA lectins, showed relatively little respiratory stimuli (**<0.5 ηM O_2 min^{-1} mg dry biomass^{-1}**), although significant differences were detected (Figure 3 A–C). The experiment with CnBr lectin (Figure 3A), bacterial density of ≥3.13 mg dry biomass ml^{-1}, made possible the detection of significant respiratory stimuli for concentrations ≥18 μg CnBr ml^{-1}, when compared to the stimulus induced by 1 μg CnBr ml^{-1}. In relation to the experiment with CFL lectin (Figure 3B), bacterial density of ≥2.85 mg dry biomass ml^{-1}, showed significant respiratory stimuli for 24 μg CFL ml^{-1} and the **5 ηM mannitol**, having 0.4 μg CFL ml^{-1} as a reference treatment. The PHA lectin showed small respiratory stimuli. In these results, lectin concentrations of the order of 1000× smaller than those used by Lau & Chan (1984), permitted the detection of respiratory stimuli. It is relevant that the stimulants in the present investigation have at least 6200 lectin molecules per bacterial cell involved. In experiments with soybean seed lectin (SBL), Lodeiro *et al.* (2000) observed stimulant effects in the adsorption of *B. japonicum* to its host roots with as few as 100,000 SBL molecules per bacterium.

It is important to highlight that the specific O_2 demands associated with 5 ηM mannitol were reproduced in all four experiments. This favours extrapolations among the experiments. It can be inferred that there is a positive relationship between the bacterial density and the specific O_2 demand induced by the mannitol and the lectins evaluated. In addition, when considering the treatments of the lower bacterial densities and lectin concentrations, which reach significant stimuli in the respiration, a series of lectin affinities (VML > PHA > CFL > CnBr) can be defined for *R. tropici*.

The statistical significance obtained with the higher bacterial density indicated that it is propitious to detect substances with physiological significance, at least in the conditions adopted in this investigation (open system). This can be justified by the quick replacement of the dissolved O_2 consumed (in low density suspensions) by the atmospheric O_2, which provides lower specific O_2 demand estimates. On the other hand, in high density bacterial suspensions, the dissolved O_2 consumption can exceed the capacity of diffusion/dissolution of the atmospheric O_2 in the reaction medium, making possible better estimates of specific O_2 demands – supposing that in both situations, the same concentrations of test-substances were used. In this way, high density bacterial suspensions are more suitable to obtain better estimates of specific O_2 demand, making possible better contrasts among treatments. This fact, associated with the saturation effect showed by the VML lectin, in high bacterial density, was used as a criterion for the selection of the 3.13 mg ml^{-1} and the 20 μg lectin ml^{-1}, to compare the lectins in terms of weight-equivalence.

Figure 4 shows the respiratory stimuli in the CFN42 and CIAT899 strains under the influence of the lectin's weight-equivalence, co-administered with (2.5 ηM) and without each respective hapten-monosaccharide. In general, both strains showed specific O_2 demands around **2.6–0.2 ηM O_2 min^{-1} mg dry biomass^{-1}**, with a significant difference among the treatments. For the experiment with the CFN42 strain (Figure 4A), the treatments with VML and VML + Gal were significantly higher. On the other hand, for PHA, CnBr, CFL and CnBr + Glc there was no difference in relation to BSA (non-lectin control). The treatments Glc, Gal, PHA + Gal and CFL + Glc with intermediary respiratory stimulus were significantly different from the VML, VML + Gal and BSA treatments. In relation to the experiment with CIAT899 strain (Figure 4B), the lectins VML and VML + Gal were higher than the other treatments. The treatments Gal, PHA, CnBr,

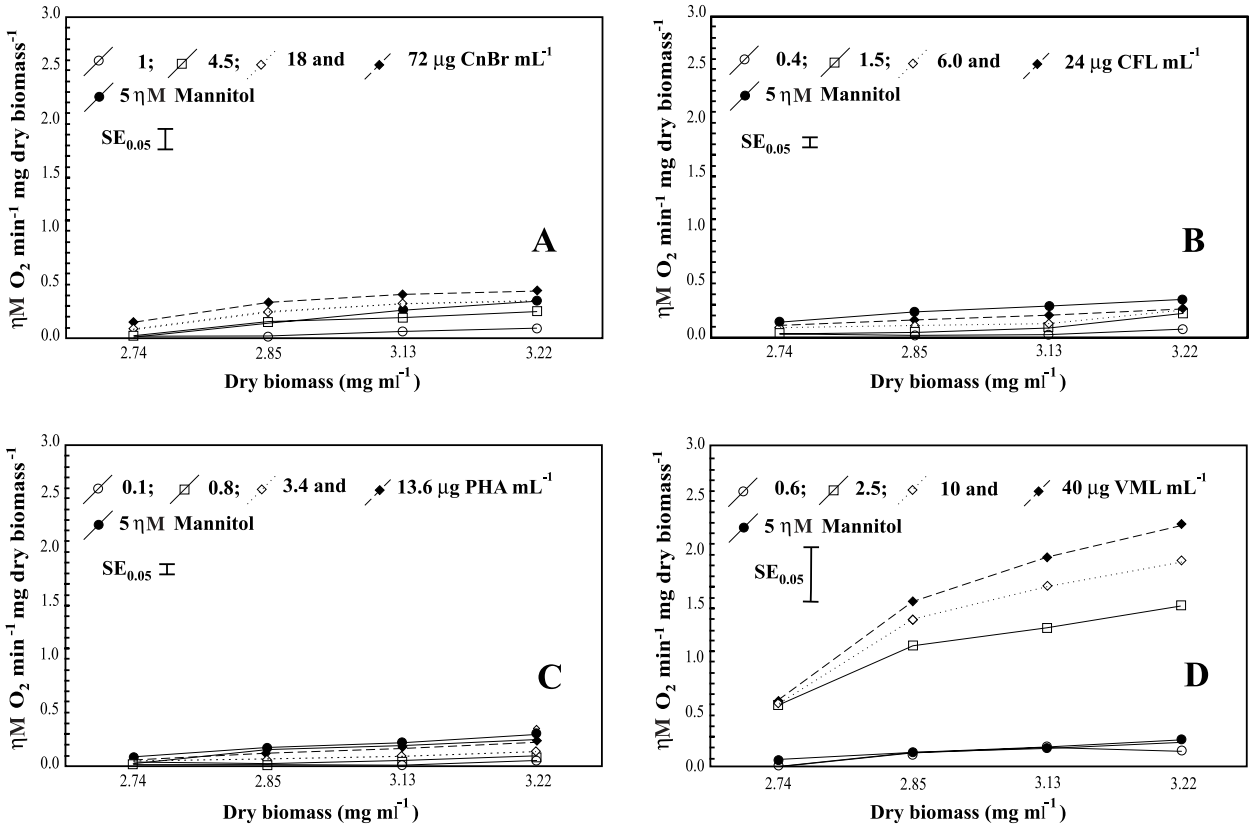

Figure 3. Dependent specific O_2 demand of the bacterial density and lectin concentration in *Rhizobium tropici*-CIAT899 suspension: influence of different lectins. (A) *Canavalia brasiliensis*-CnBr, (B) *Cratylia floribunda*-CFL, (C) *Phaseolus vulgaris*-PHA and (D) *Vatairea macrocarpa*-VML. Average of three repetitions and standard error (SE) to $P < 0.05$.

CnBr + Glc, Glc, CFL and CFL + Glc, did not differ from BSA, while PHA + Gal, with intermediary values, presented differences from the treatments VML, VML + Gal and BSA. These results produce additional support for the fact that the VML lectin strongly stimulates respiration in both *Rhizobium* strains. Moreover, it indicates that the co-administration of lectins, with their respective hapten-monosaccharides, does not appear to have any additive effect in the specific O_2 demand, except for the treatments with PHA and PHA + Gal in the CIAT899 strain. Considering the behaviour of lectin cellular aggregation by sugar inhibition, it is probable that the formation of lectin–monosaccharide complexes can make unavailable the lectin and monosaccharide fractions for the bacterial surface receptors, impeding the additive effect in specific O_2 demand. The intensity of this effect will depend on the surface receptor competitiveness for monosaccharides and binding lectin sites, where affinity and molecular concentration of both are important. The CIAT899 and CFN42 surfaces showed high relative affinity by PHA(VML) and PHA lectins, respectively (Martínez 2002).

The specific O_2 demands of CIAT899 strains under the influence the CnBr and VML lectins, co-administered with different concentrations of hapten-monosaccharides are shown in Figure 5. In both experiments (CnBr and VML), sugar-dependent respiratory stimuli were detectable for **≥625 ηM of Glc** and Gal (control treatments). The specific O_2 demand induced by Glc was higher to that of Gal for 10^4 ηM. The respiratory stimuli induced by both lectins were lower than those induced by the co-administrated lectins with hapten-monosaccharides for the concentrations >625 ηM of Glc and Gal. The co-adm inistration of CnBr lectin with ≤156 ηM Glc, did not show any difference to the CnBr without Glc treatment (Figure 5B).

Respiratory stimuli related to the co-administration of the VML lectin with ≤625 ηM Gal, did not differ from those induced by VML without Gal treatment (Figure 5A). On the other hand, VML + 9.8 ηM Gal was the exception, presenting an apparent inhibition of the specific O_2 bacterial demand induced by the VML lectin. Lau & Chan (1984) found similar results in experiments with *B. cereus* suspension, where Concanavalin A lectin and Methyl-D-mannopyranoside were co-administered. This Gal concentration (9.8 ηM) probably represents a critical level, where the specific O_2 demand associated with the VML lectin concentration was minimized by the block of lectin and bacterial receptor interaction. With lower Gal concentrations, the specific O_2 demands were predominantly determined by the VML lectin. Specific O_2 demands of higher Gal concentrations were probably induced by the action of partially blocked lectins, and by free Gal simultaneously. The saturated monosaccharide–lectin complexes can coexist without

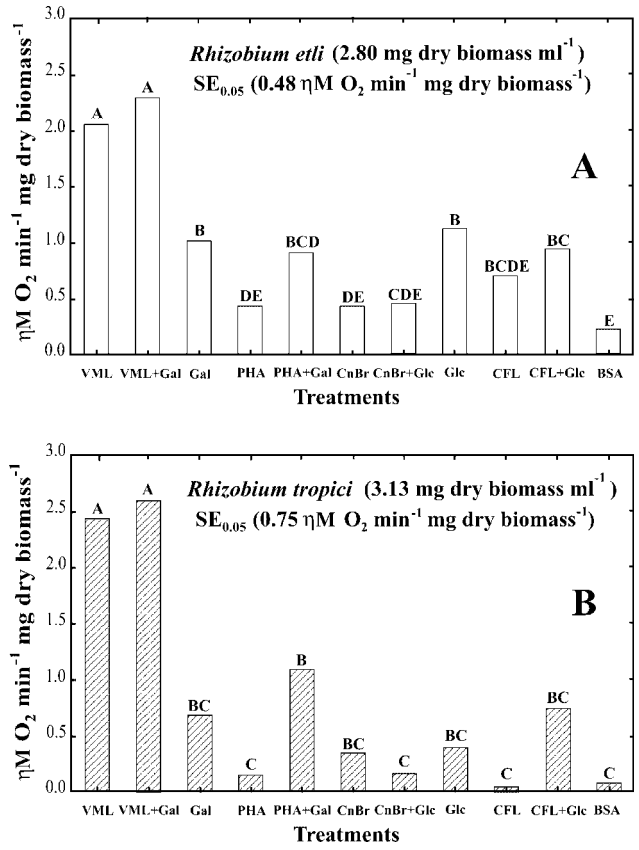

Figure 4. Lectin-dependent specific O_2 demand in *Rhizobium* sp. suspension: hapten-monosaccharide influence. The *Vatairea macrocarpa*-VML and *Phaseolus vulgaris*-PHA are Gal specific. The *Canavalia brasiliensis*-CnBr and *Cratylia floribunda*-CFL are Glc-specific. Bovine serum albumin (BSA) is a non-lectin control. (A) *Rhizobium etli*-CFN42 and (B) *R. tropici*-CIAT899. The treatment averages (two repetitions) with the same letters do not differ significantly ($P < 0.05$).

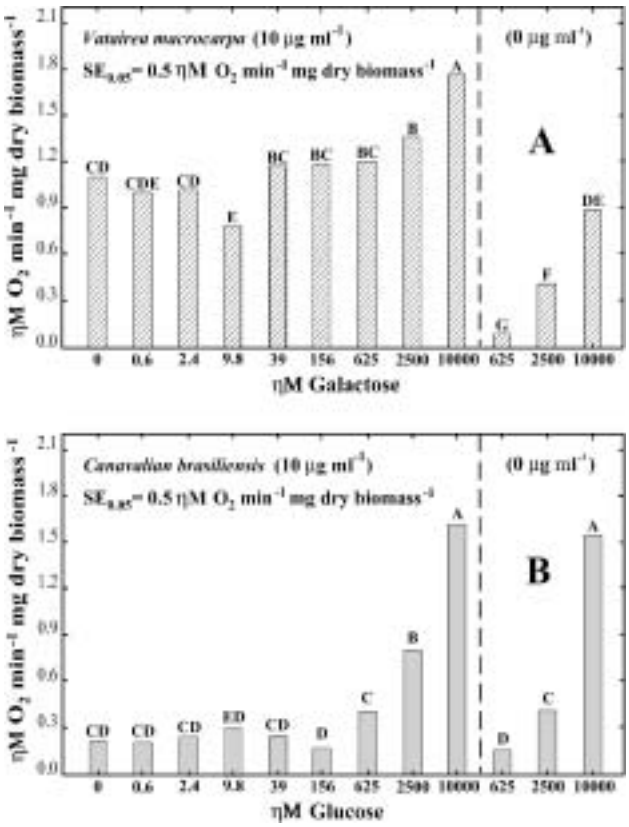

Figure 5. Lectin-dependent specific O_2 demand in *Rhizobium tropici* suspension: stimuli dependent on hapten-monosaccharide doses. (A) *Vatairea macrocarpa*-VML and (B) *Canavalia brasiliensis*-CnBr. The treatment averages (two repetitions) with the same letters do not differ significantly ($P < 0.05$).

interacting with the bacterial surfaces, unless the lectin hydrophobic domain interacts with the external membrane of the bacterium, modifying its membrane permeability. In Booij *et al*. (1996), a pea lectin was inserted in mono lipidic layers, independently of being bound or not bound to the specific sugar. This makes possible direct interaction of lectins with the *Rhizobium* membrane during the infection process of the root hairs (Booij *et al*. 1996; Brewin & Kardansky 1997; Kijne *et al*. 1997). According to Kijne *et al*. (1997), this property will allow lectins to imitate external membrane proteins and, consequently, influence the performance of *Rhizobium*.

Conclusions

The results indicate that legume lectins stimulate the respiration of *Rhizobium* sp. We suggest that this stimulating activity involves the interaction of these lectins with the external membrane of the bacteria. The mechanism involved in this interaction could have symbiotic significance.

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