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The interaction of *Vatairea macrocarca* and *Rhizobium tropici*: net H^+ efflux stimulus and alteration of extracellular Na^+ concentration

Cosme R. Martínez [a,b,*], Christopher P. Chanway [c], Alessandro V.P. Albertini [b], Márcia V.B. Figueiredo [d], Alexandre H. Sampaio [e], Rolando R. Castellon [e], Benildo S. Cavada [e], José L. Lima-Filho [b]

[a] *Instituto Tecnológico do Estado de Pernambuco, Recife-Brasil*
[b] *Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235 Cidade Univesitaria, CEP 50, 670-901 Recife-PE, Brasil*
[c] *Department of Forest Sciences/University of British Columbia, Vancouver-Canada*
[d] *Empresa Pernambucana de Pesquisa Agropecuária, Recife-Brasil*
[e] *Departamento de Bioquímica-Universidade Federal do Ceará, Fortaleza-Brasil*

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Abstract

We studied the effect of a lectin isolated from seeds of the legume *Vatairea macrocarpa* on net H^+ efflux in *Rhizobium tropici*, a bacterium capable of nodulating legume *Phaseolus vulgaris*. *V. macrocarpa* lectin (VML) was observed to temporarily stimulate the specific net H^+ efflux in *R. tropici*. When VML was present at 32 $\mu g\,ml^{-1}$, with or without 2 μM galactose (Gal), a specific net efflux >2.4 pM $H^+(min)^{-1}$ mg dry biomass^{-1} was induced. There was no detectable net H^+ efflux when bovine serum albumin (16 $\mu g\,ml^{-1}$) was tested. Addition of 16 $\mu g\,VML\,ml^{-1}$ resulted in a 700% increase of the extracellular Na^+ concentration. The soluble proteins in the supernatant containing VML extract indicate a maximum immobilization of ±10 $\mu g\,VML\,ml^{-1}$, with a minimum of 36,600 dimers or 8500 larger aggregates of VML binding in each bacterium. Our data suggest that VML activates *Rhizobium* as a bioenergetic substrate molecule, resulting in potential alterations of the external bacterial membrane.

Keywords: Ion transport; *Rhizobium*; Lectin; *Vatairea macrocarpa*

1. Introduction

Lectins are proteins or glycoproteins that bind reversibly and specifically to mono and oligosaccharides, without enzymatic modifications [1]. *Vatairea macrocarpa* lectin (VML) is a lectin (Gal/GalNac specific) isolated from seeds of the legume *V. macrocarpa* [2]. At neutral pH, VML is predominantly a dimeric (70 kDa) protein, although tetramers (115 kDa) and larger aggregates (300 kDa) are present [2]. VML has been shown to bind specifically to the bacterial surfaces of *Rhizobium tropici* CIAT899 and *R. etli* USDA 9032 [3]. These two strains have been reported to induce nodule formation and fix atmospheric nitrogen in the roots of the legume *Phaseolus vulgaris* [4]. *Rhizobium*

* Corresponding author. Tel.: +55-81-21268484; fax: +55-81-21268485.
E-mail addresses: cosme@lika.ufpe.br, zeluiz@lika.ufpe.br (C.R. Martínez).

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cells bind to the surface of root hairs and penetrate them via infection threads to reach emergent nodules in the host [5]. The presence of lectins in the bacterial infection process led to the hypothesis that these proteins could be mediators of highly specific legume×root nodule bacterium associations [6,7]. However, many questions regarding the specific role of lectins in legume×bacteria interactions remain.

Until now, only a few lectins have been isolated and characterized from roots of leguminous plants. There is some information regarding the relationship between the *P. vulgaris* lectin (PHA) and *Rhizobium* [7]. Pre-incubation of *R. etli* with PHA has been shown to increase significantly the number of infection threads [8] and functional nodules in bean plant roots [9]. Moreover, pre-incubation of *Bradyrhizobium* with soybean seed lectin led to increase the number of bacteria absorbed on the root surface [10].

Lectins have been shown to be involved other facets of bacterial ecology. For example, the hemolinphatic lectin of the crab *Scylla serrata* (GalNac specific) exhibited antimicrobial activity by inhibiting respiration in *Bacillus cereus* and *Escherichia coli* [11]. On the other hand, the ConA lectin (Man/Glc specific) has been shown to stimulate O_2 demand in *B. cereus* [12]. The lectins from the leguminous plants, mainly VML, have also shown to stimulate respiration in *R. tropici* and *R. etli* [13].

Rhizobium and relatives are Gram-negative bacteria that have an energized internal membrane due to electron transport systems, which generate an H^+ electrochemical gradient that drives membrane-associated ATPases [14]. The energy of this gradient is invested in the synthesis of ATP, the energy-rich compound involved in several critical metabolic functions. These include active transport, flagellar movement and the generation of electrochemical gradients of Na^+ and K^+ – both considered to be critical in the bacterial homeostasis of pH, osmoregulation and in the maintenance process of non-toxic solute levels [15,16]. The present work was undertaken to study the VML effects on energy generation in *Rhizobium*.

2. Materials and methods

2.1. Bacteria, lectins and reagents

Rhizobium tropici-CIAT899 was obtained from the USADA/ARS/Beltsville *Rhizobium Germplasm* Collection. VML lectin was provided by BioMol-Lab/Departamento de Bioquímica/UFC-Fortaleza-Brasil. 2-[*N*-morpholino]ethanesulfonic, salt sodium (MES), galactose, bovine serum albumin (BSA) and other reagents were purchased from Sigma–Aldrich Chemicals Inc. (St. Louis, MO, USA).

2.2. Growth conditions, total cells and bacterial biomass

Rhizobium starter cultures with 10^6 bacteria ml^{-1} were established on YM medium [1] (150 rpm at 30 °C for 24 h) and stored at 4 °C. Bacterial biomass was obtained from cultures at the end of the exponential phase initiated by an inoculum of 2×10^3 bacteria ml^{-1}. Bacterial numbers were determined using a Neubauer chamber. The dry biomass ($mg\,ml^{-1}$) was determined gravimetrically from standard bacterial suspensions dried at 100 °C for 6 h.

2.3. Bacterial suspensions and lectin solution

Rhizobium cells (24 h culture) were centrifuged (25,000 g for 15 min, at 25 °C), re-suspended in a MES-S buffer [2] and centrifuged again (7000*g* for 10 min at 4 °C). These steps were repeated twice. The bacterial suspension was homogenized using a monofilament nylon screen (Schweiz., Seidengazefabrik AG Thal) with 16 μm mesh-opening. The screen was also used to obtain a standardized bacterial suspension in MES-S buffer (an $OD_{595}{}^{1cm} = 1.5$ was used as a reference to adjust the bacterial density). Lectin solutions ($0.002\ g\,ml^{-1}$) in MES-S or S buffer [3] were incubated at 43 °C (ultrasonic cleaner) and at room temperature for 30 min each. Lectin solutions were then centrifuged at 22,000*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.

2.4. Bioreactor and evaluation of hydrogen activity

An open system or ''bioreactor'', in polypropylene, was used to monitor the H^+ activity in the bacterial suspensions. The temperature (30 ± 0.1 °C), agitation (170 rpm) and air injection ($18\ ml\,min^{-1}$) were controlled (Fig. 1). A glass electrode specific for H^+ (∅ = 10 mm), attached to a pH meter (pH meter, Model F8L, Horiba, Tokyo), and a glass thermometer were incorporated into the bioreactor. The data were registered continuously on a chart recorder. The refrigerated bacterial suspension was re-suspended, incubated (30 °C for 5 min) and transferred (7 ml) to the bioreactor. After 45–60 min, the H^+ activity was stationary, and the lectin solution (0.5 ml) was added. H^+ activity was then monitored for 20 min. At the end of this period, the reaction mixture was drained and the residues removed by washing.

[1] YM medium: 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 Yeast extract l^{-1}; pH 6.8.

[2] MES-S buffer: 10 mM MES, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 3 mM NaCl.

[3] S buffer: 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 3 mM NaCl.

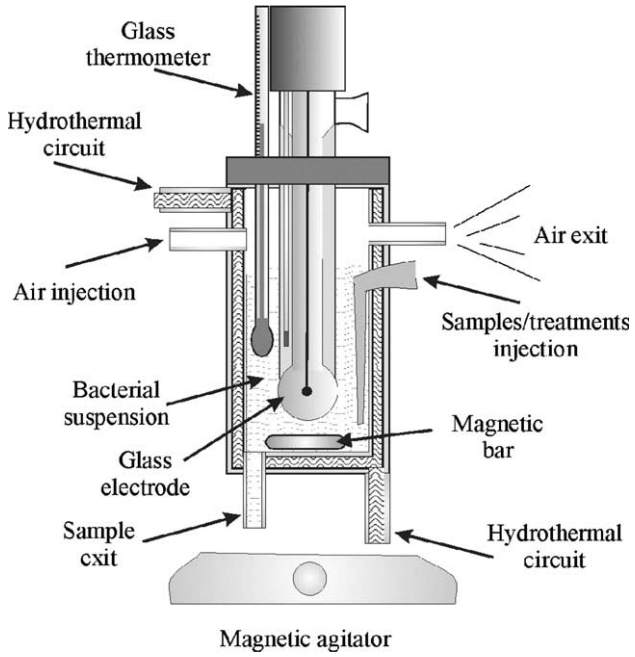

Fig. 1. Bioreactor used to monitor hydrogen activity: open system with glass electrode and controlled air flux, agitation and temperature.

2.5. H^+ efflux detection of *Rhizobium sp.* (in MES-S buffers) stimulated by VML

An experiment to determine the MES-S buffer concentration that allows the detection of a lectin-dependent bacterial H^+ efflux was performed as follows: bacterial suspensions (3.13 mg dry biomass ml^{-1}) in MES-S buffer were diluted with bacterial suspensions (3.13 mg dry biomass ml^{-1}) in the S buffer, respectively, to 1:10 proportion, to obtain 'test bacterial suspensions'. The test bacterial suspensions and lectin solutions (prepared in S buffer), were adjusted (KOH) to pH 6.7 and stored under refrigeration. The experimental procedure and H^+ activity monitoring were performed as described above. The experiment was repeated twice.

2.6. Extracellular concentrations of soluble protein, Na^+, K^+, and net H^+ efflux

The study was carried out using the following treatments: (a) VML at concentrations of 8; 16 and 32 $\mu g\,ml^{-1}$; (b) VML at concentrations of 8; 16 and 32 $\mu g\,ml^{-1}$ with 2 μM Gal; (c) 2 μM Gal; (d) BSA (16 $\mu g\,ml^{-1}$), non-lectin control; and; (e) absolute control (without VML lectin, Gal and BSA). In this experiment, the CIAT899 strain was used at concentration of 3.13 $mg\,dry\,biomass\,ml^{-1}$ and the MES-S buffer at 1 zM (zepto Molar). After the incubation with VML, the bacterial suspensions were then used for Na^+ and K^+ determination by flame photometry analyses in a photometer (Digimed, model DM-61) and protein determination. A control (C_{lb}) to test for potentially available bacterial proteins was also included. The bacterial suspensions were processed in a similar way as absolute control, i.e., sonicated (three pulses of 50 W) for one minute at 4 °C, immediately centrifuged and the supernatant stored at −20 °C.

2.7. Data and statistical analyses

Time-dependent quadratic adjustment of variation in H^+ activity was performed to calculate the H^+ efflux. The specific net H^+ efflux of the bacterial suspensions, based on dry biomass, was obtained by absolute control estimate correction. The variance was analysed using Statistic soft (Statsoft Inc.,Tulsa) and the effects evaluated by *F*-test. The standard error ($P < 0.05$) was estimated and the comparison of treatment means was determined using Tukey's HSD.

3. Results and discussion

The variation of H^+ activity induced by VML (10 $\mu g\,ml^{-1}$) in CIAT899 suspensions showed four distinct H^+ production phases: (1) initial lag production phase; (2) maximum production phase; (3) equilibrium phase; and (4) final H^+ demand phase (Fig. 2(a)). Each phase

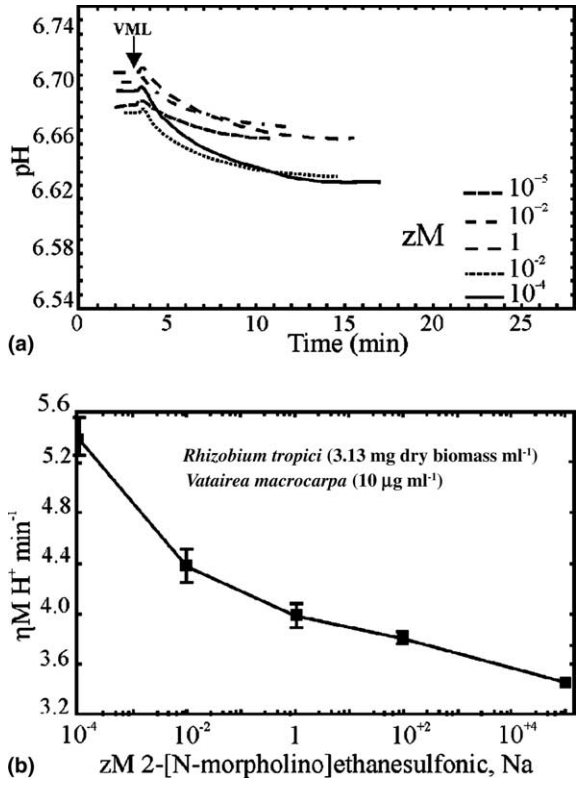

Fig. 2. Time-dependent H^+ activity (a) and H^+ efflux (b) in *R. tropici*-CIAT899 suspension in response to the *V. macrocarpa* lectin (10 $\mu g\,ml^{-1}$): influence of the MES-S buffer concentration.

was clearly associated with a transition period. In the first phase (<1 min after VML administration), a decrease in the H^+ activity was observed that might be associated with the induction of the cellular transport. The simultaneous absorption of H^+ with VML, and with Na^+, K^+ and Ca^{2+} ions (co-administered with the treatments), may explain these observations. Co-absorption could be satisfying the requirements of the various permeases and of the bacterial ionic homeostasis [15]. Phase 2 appears to be the opposite situation where a quick increase of the H^+ activity in the CIAT899 suspensions was observed. This is likely a consequence of H^+ production or H^+ efflux dependent on bacterial VML-catabolism. In phase 3, the H^+ production is in equilibrium with the bacterial demand. Here, the decrease in the substrate availability that is also associated with the induction of other processes that require H^+, could be contributing with these effects. However, in phase 4 – ±10 min after VML administration – a slow decrease of H^+ activity was observed (data not shown). Here, the lack of substrate, the residual metabolic H^+ demand, the ionic homeostasis and the buffer effect of the solution can be determinant factors for the observed outcome. Similar H^+ activity was observed for treatments with Gal and BSA. The O_2 demand increase model in CIAT899, under VML, BSA and Gal treatments [13], appeared to be synchronized with the H^+ activity model presented here. In this context, when considering phases 2 and 3 of this model, an estimation of net H^+ efflux in the bacterial suspensions may be obtained.

For the bacterial density we utilized (3.13 mg ml^{-1}), 10 μg VML ml^{-1} induced an efflux of 3.4 to 5.4 ηM H^+ min^{-1}, in MES concentrations of 10^5–10^{-4} zM, respectively. No linear correlation was observed between H^+ efflux and MES concentration (Fig. 2(b)). At higher MES concentrations (>10^5 zM), the H^+ efflux induced by VML could not be estimated. Consequently, 1 zM MES concentration was selected as an additional experimental control.

Fig. 3(a) and (b), show the specific net H^+ efflux and extracellular ions concentration in bacterial suspensions associated with the VML, VML + Gal, Gal and BSA treatments. The increase of VML concentrations significantly stimulated the net H^+ efflux whether or not co-administered with 2 μM Gal. VML, added at a concentration of 32 μg ml^{-1}, with or without Gal, induced higher net H^+ efflux (>2.4 pM H^+ (min)$^{-1}$ mg dry biomass^{-1}) than did 8 μg of VML (±0.6 pM H^+ (min)$^{-1}$ mg dry biomass^{-1}). Treatment using BSA (16 μg ml^{-1}) did not stimulate the net H^+ efflux in *Rhizobium* suspensions, while treatment with 2 μM Gal had a small stimulatory effect on the net H^+ efflux (±0.36 pM H^+ (min)$^{-1}$ mg dry biomass^{-1}) that was not significantly different from the BSA treatment. Furthermore, co-administration of VML with Gal exhibited no significant increase in the net H^+ efflux compared to treatments with

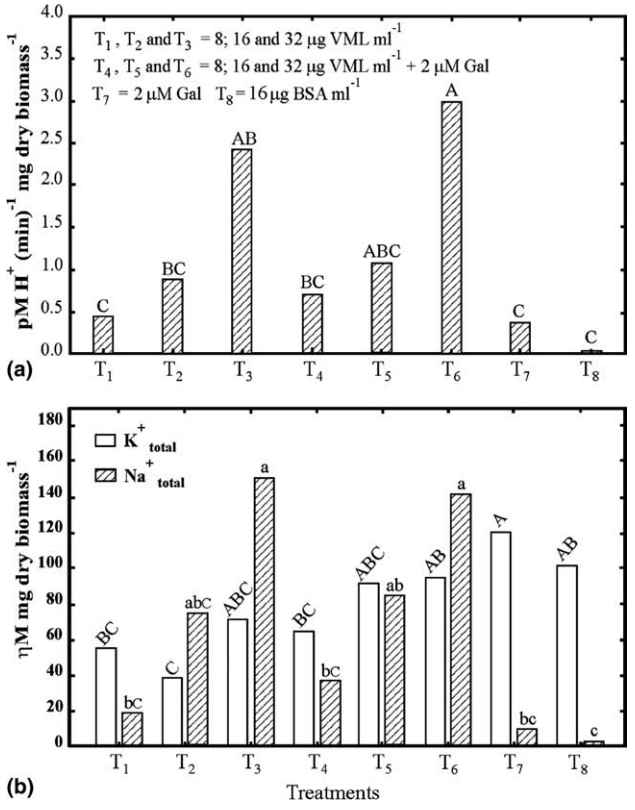

Fig. 3. Specific net H^+ efflux (a) and extracellular concentrations of Na^+ and K^+ (b) in *R. tropici*-CIAT899 suspension: influence of *V. macrocarpa* VML lectin. Standard errors (SE) to $P < 0.05$ were 1.89 pM H^+ (min)$^{-1}$ mg dry biomass^{-1}, 49.7 ηM K^+ and 80.3 ηM Na^+, both based on mg of dry biomass. The treatment's averages (two repetitions) with the same letters do not differ significantly according to Tukey's HSD test.

VML alone. This is likely due to the fact that the free Gal fraction may contribute to the metabolism associated with the net H^+ efflux. The comparison between VML and BSA results suggest that net H^+ efflux on *Rhizobium* is specifically stimulated by the lectin.

Fig. 3(b) shows the specific concentrations of Na^+ and K^+ in the extracellular environment (15 min after the administration of the treatments). The results show great similarity to the observed treatment effect in specific net H^+ efflux. The increase of VML concentration significantly improved extracellular Na^+ concentration whether or not co-administered with 2 μM Gal. The treatments with 32 μg ml^{-1} of VML with or without Gal induced levels of ±140 ηM Na^+ mg dry biomass^{-1}. However, the treatment with 8 μg VML ml^{-1} showed concentration of ±36 ηM Na^+ mg dry biomass^{-1}.

As expected, the treatment with BSA and Gal did not show any increase in the level of extracellular Na^+. The treatment with BSA showed the smallest levels of Na^+ (±5 ηM Na^+ mg dry biomass^{-1}), while treatment with 2 μM Gal (similar to BSA) did not stimulate any increase of extracellular Na^+ concentration. The extracellular concentration of K^+ was not altered by different

concentrations of VML whether or not co-administered with 2 μM Gal. The treatment with 2 μM Gal and BSA significantly increased extracellular K^+ concentration in comparison to treatment with 8 μg VML ml^{-1} (with or without Gal). In general, VML induces specific net H^+ efflux and the liberation of cytoplasmatic Na^+ in the CIAT899 strain. It is likely that the lectin makes available enough metabolic energy to promote a greater Na^+ efflux and, consequently facilitates energy storage (in the Na^+ gradient) in order to promote the secondary transport of other metabolites [14,15]. For the Gal level used, the total available metabolic energy is just enough to induce the release of cytoplasmatic K^+ (K^+/H^+ antiporter) to reach the bacterial pH-homeostasis. BSA is a source of amine, and the internal pH of the bacterium *Vibrio alginolyticus* was alkalinized, and cellular K^+ was released simultaneously under the effect of the membrane-permeable amine added to an alkaline pH [17].

The available extracellular protein indicates that Gal (2 μM) promotes the decrease of protein concentration in relation to the control (Fig. 4). The reabsorption of transportable extracellular polypeptides of bacterial origin by the nitrogen demand of *Rhizobium* is probably induced by the observed Gal-dependent metabolic stimulus. The co-administration of VML + Gal yielded similar available protein levels to those obtained with VML treatments. However, the protein levels associated to 8 μg ml^{-1} of VML with 2 μM Gal, had a similar effect to the Gal treatment. Incremental increases in the levels of VML with/without Gal, resulted in significant increases in the extracellular protein concentrations. This represents approximately 12%, 34% and 69% of administrated protein concentration indicating a saturation effect in the binding process of VML with the *Rhizobium* surface. Apparently, ±10 μg ml^{-1} was the maximum immobilized quantity in 3.13 mg dry biomass ml^{-1} (2.34×10^9 bacteria ml^{-1}) for the CIAT899 strain. When comparing VML with BSA effects at 16 μg ml^{-1}, the extracellular available protein was 34% and 75% of administered protein, respectively. At the maximum immobilized quantity of VML indicated here, we can make an approximation of at least 36,600 dimer or 8500 larger aggregates of VML, interacting in each CIAT899 bacterium. Soybean seed lectin stimulates adsorption of *Bradyrhyzobium japonicum* to its host roots with 480,000 monomeric (30 kDa) protein per bacteria [10]. The sonicated bacterial cells (C_{LB}) indicated that the potentials of bacterial hydrosoluble proteins (15.9 μg ml^{-1}) are significantly superior to those registered in the supernatants of the treatments with 8 μg VML ml^{-1} (with/without Gal).

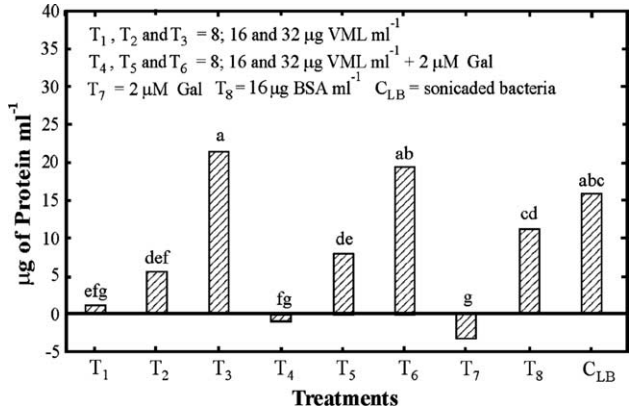

Fig. 4. Extracellular concentrations of soluble protein in *R. tropici*-CIAT899 suspension: influence of *V. macrocarpa* VML lectin. Standard error (SE) to $P < 0.05$ was 7.14 μg ml^{-1}. The treatment's averages (two repetitions) with the same letters do not differ significantly according to Tukey's HSD test.

Considering the approximations presented above and the results in the induced O_2 demand by VML [13], it can be concluded that there is evidence that VML is, in part, a substrate molecule for *Rhizobium.* Furthermore, if the lectin represents a special function to activate and to select a specific *Rhizobium*, it could become much more competitive in the symbiotic process than other non-specific *Rhizobium* strains. It is probable that the binding between VML and the surface *Rhizobium* glyco-receptors, with distinguished affinity and specificity, could create conditions to become an external membrane protein, as suggested for other lectins [18]. The VML hydrophobic domain could confer additional determinant specificity. Since VML is incorporated into the external membrane, periplasmic proteolytic enzymes may have access to it, thus liberating transportable polypeptide fragments, which are consequently assimilated by bacteria, that can be used as substrate for synthesis or for *Rhizobium* activation. It is probable that the lectin residual fragments in the external membrane may imitate bacterial membrane proteins [6] especially those with carbohydrate domain recognition, consequently improving the symbiotic *Rhizobium* behavior. The results clearly show that the lectin receptor present in the *Rhizobium* has galactose or its epimers in the receptor structure, which may lead to greater specificity in its interaction with its legume host.

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References

[1] Vijayan, M. and Chandra, N. (1999) Lectins. Curr. Opin. Struct. Biol 9, 707–714.

[2] Cavada, B.S., Santos, C.F., Grangeiro, T.B., Nunes, E.P., Sales, P.V. and Ramos, R.L., et al. (1998) Purification and characterization of a lectin from seeds of *Vatairea macrocarpa* Duke. Phytochemistry 49, 675–680.
[3] Martínez, C.R., Netto, A.M., Figueiredo, M.V.B., Cavada, B.S. and Lima-Filho, J L. (2004) Kinetic sedimentation of *Rhizobium*-aggregates produced by leguminous lectins. World J. Microbiol. Biotechnol., in press.
[4] Learemans, T. and Vanderleyden, J. (1998) Review: Infection and nodulation signalling in rhizobium *Phaseolus vulgaris* symbiosis. World J. Microbiol. Biotechnol. 14, 787–808.
[5] Cohn, J., Day, R.B. and Stacey, G. (1998) Legume nodule organogenesis. Trends Plant Sci. 3, 105–110.
[6] Kijne, J.W., Bauchrowitz, M.A. and Diaz, C.L. (1997) Root lectins and rhizobia. Plant Physiol. 115, 869–873.
[7] Hirsch, A.M. (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. Curr. Opin. Plant. Biol. 2, 320–326.
[8] Brelles-Mariño, G., Costa, G.A. and Boiardi, J.L. (1996) Enhancement of infection thread formation by *Rhizobium etli* incubated with bean seed lectin. Microbiol. Res. 151, 243–246.
[9] Mestrallet, M.G., Defilpo, S.S. and Abril, A. (1999) Effect of the addition of specific lectin on the symbiosis of *Rhizobium leguminosarum* and *Phaseolus vulgaris*. Rev. Argent Microbiol. 31, 72–77.
[10] Lodeiro, A.R., López-Gracía, S.L., Vázquez, T.E.E. and Favelukes, G. (2000) Stimulation of adhesiveness, infectivity, and competitiveness for nodulation of *Bradyrhizobium japonicum* by its pretreatment with soybean seed lectin. FEMS Microbiol. Lett. 188, 177–184.
[11] Majumder, M., Chattopadhyay, T., Guha, A.K. and Chatterjee, B.P. (1997) Inhibition of bacterial respiration by a low-molecular weight lectin from *Scylla serrata* crab hemolymph. Indian J. Biochem. Biophys. 34, 87–89.
[12] Lau, T.M. and Chan, K.y. (1984) Phyiological responses of *Bacillus* species to cocanavalinv A: 2 Effect on growth, oxygen uptake, enzyme activities and intracellular cyclic guanosine 3′, 5′-monophosphate level of *B. cereus* ATCC 14579. Microbios 39, 137–150.
[13] Martínez, C.R., Albertini, A.V.P., Figueiredo, M.V.B., Silva, V.L., Sampaio, A.H. and Cavada, B.S., et al. (2004) Respiratory stimulus in *Rhizobium* sp. by legume lectins. World J. Microbiol. Biotechnol. 20, 77–83.
[14] Udvardi, M.K. and Day, D.A. (1997) Metabolite transport across symbiotic membranes of legume nodules. Annu. Rev. Physiolog. Plant Mol. Biol. 48, 493–523.
[15] Hediger, M.A. (1994) Structure, function and evolution of solute transporters in prokaryotes and eukaryotes. J. Exp. Biol. 196, 15–49.
[16] Lolkema, J.S., Speelmans, G. and Konings, W.N. (1994) Na^+-coupled versus H^+-coupled energy transduction in bacteria. Biochim. Biophys. Acta 1187, 211–215.
[17] Nakamura, T., Kawasaki, S. and Unemoto, T. (1992) Roles of K^+ and Na^+ in pH homeostasis and growth of the marine bacterium *Vibrio alginolyticus*. J. Gen. Microbiol. 138, 1271–1276.
[18] Booij, P., Demel, R.A., dePater, B.S. and Kijne, J.W. (1996) Insertion of pea lectin into a phospholipid monolayer. Plant Mol. Biol. 31, 169–173.