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RESEARCH ARTICLE

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Binding of soluble glycoproteins from sugarcane juice to cells of *Acetobacter diazotrophicus*

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Summary Sugarcane produces two different pools of glycoproteins containing a heterofructan as glycidic moiety, tentatively defined as high-molecular mass (HMMG) and mid-molecular mass (MMMG) glycoproteins. Both kinds of glycoproteins can be recovered in sugarcane juice. Fluorescein-labelled glycoproteins are able to bind to *Acetobacter diazotrophicus* cells, a natural endophyte of sugarcane. This property implies the aggregation of bacterial cells in liquid culture after addition of HMMG or MMMG. Anionic glycoproteins seem to be responsible for the binding activity whereas cationic fraction is not retained on the surface of *A. diazotrophicus*. Bound HMMG is competitively desorbed by sucrose whereas MMMG is desorbed by glucosamine or fructose. On this basis, a hypothesis about the discriminatory ability of sugarcane to choose the compatible endophyte from several possible ones is proposed.

Key words *Acetobacter diazotrophicus* · Cell recognition · Glycoproteins · Sugarcane · Desorption

Introduction

Acetobacter diazotrophicus is a N_2-fixing, aerobic, Gram-negative bacterium found as an endophyte in roots, stems, and leaves of sugarcane [7] as well as in *Pennissetum purpureum*, *Ipomoea batatas* [33] and *Coffea arabica* [15]. Bacteria were present in the intercellular apoplast of sugarcane stems [9], and they have been also detected in the xylem vessels at the base of the stem [14]. Moreover, Bellone et al. [5] reported the occurrence of intracellular bacteria in sugarcane root tips.

The mechanism by which bacteria enter sugarcane seedlings has not been yet established conclusively. *A. diazotrophicus* was not found in Brazilian soils where sugarcane was cultured [4] whereas Li and MacRae [23] reported that the bacterium was present in Australian sugarcane rhizosphere. James et al. [14] suggested that *A. diazotrophicus* colonized the root surface only after bacterial consumption of sucrose in the habitat. Then, bacteria would enter the root apoplast via lateral roots junctions and the loose cells of the root cap. Ashbolt and Inkerman [3] showed the occurrence of *A. diazotrophicus* in the mouth stylus of the pink mealybug, *Saccharococcus sacchari*, an insect that feeds on the meristematic tissue between the leaf sheath and the stem, the leaf sheath pocket. Other *Acetobacter* spp. can be isolated from mealybugs as well as other bacteria also present in the leaf sheath pocket, such as *Leuconostoc mesenteroides* and *Erwinia amylovora*, although these last microorganisms do not enter the host cells. Thus, the insect may be involved in transferring bacteria from plant to plant [13].

However, a new question arises from this hypothesis: Does the host plant—sugarcane—have any mechanism to discriminate between different bacterial species and, then, to favour the colonization by a nitrogen-fixing endophyte? In fact, immunogenic properties on the extracellular matrix surrounding the bacteria have been proposed [14]. This extracellular matrix is synthesized by exocellular enzymes, mainly a levansucrase described in 1996 [2]. Recognition capability is supported by the fact that plants detect the entry of a pathogen in early stages of infection and, consequently, elicit a defense response consisting of the production of phenolics to inhibit the growth of bacteria [36] and fungi [35]. However, phenolics production should not be enhanced when the intruder is a potentially symbiotic endophyte.

An invading agent generally enhaces the production of released exopolysaccharides [22]. However, recognition of a compatible endophyte usually involves selective glycoproteins that specifically bind to a bacterial cell wall ligand [8]. Several glycoproteins are produced by sugarcane stalks [19] as a response to mechanical injuries [40] and both upright and post-collection

impairments [27]. Their glycidic moiety is composed by fructose and galactitol [18] and their molecular mass is higher than 9 kDa [28]. Fructose units polymerize by the formation of β-1,2 linkages whereas galactitol is introduced in the fructan sequence by means of an ether bond [18]. We report herein conclusive evidence of the ability of some sugarcane glycoproteins to bind to the cell wall of *A. diazotrophicus* as the first step in the biological discrimination of a compatible, symbiotic endophyte, therefore resembling a mechanism of specific tolerance such as those found in the immune system of higher eukaryotics.

Materials and methods

Plant material and glycoprotein preparation Stalks from 12 month-old plants of *Saccharum officinarum*, var. Jaronu 60–5, field-grown, were mechanically crushed immediately after having been cut and the crude juice was centrifuged at 5000 ×*g* for 15 min at 4ºC. The pellet was discarded and the supernatant was filtered through filter paper. This centrifuged juice was then filtered through a Sephadex G-10 column (15 × 2.5 cm) embedded in distilled water. Elution was also carried out with distilled water. Fractions (1.0 ml) 1 to 27 were discarded. Fractions 28 to 35 were collected and considered as a mixture of soluble polysaccharides and glycoproteins, MMMG (mid-molecular mass glycoproteins) and HMMG (high-molecular mass glycoproteins). Fractions 36 to 57, mainly composed by sucrose, were also discarded. After this, a mixture of fractions 27 to 35 was filtered through a Sephadex G-50 column (30 × 2.5 cm) pre-equilibrated as described above. Fractions 49 to 85 contained HMMG whereas MMMG eluted in fractions 56 to 157 [20]. Eluted fractions were monitored for carbohydrates according to Dubois et al. [10] and for protein according to Lowry et al. [26].

Alternatively, a sample of centrifuged juice was precipitated with acetone to reach a final concentration of 80 per cent (v/v). The mixture was maintained for 1 h at 0ºC and then centrifuged at 15,000 ×*g* for 20 min at 2ºC. The supernatant was discarded and the precipitate was dried in air flow and redissolved in distilled water to obtain a final concentration of 0.5 mg/ml.

Labelling of glycoprotein for fluorescence analysis Aliquots of 3.0 ml of HMMG, MMMG, and acetone-precipitated proteins were mixed with 3.0 ml 10 mM fluorescein isothiocyanate for 1 h at room temperature with vigorous shaking. After this, mixtures were filtered through a Sephadex G-15 column (12 × 1.0 cm) and eluted with distilled water to remove the excess of fluorescein [30]. The void volume, containing labelled, fluorescent macromolecules, was collected and used for binding experiments.

Bacterial strains and growth conditions *Acetobacter diazotrophicus*, strain AP5, isolated from sugarcane, was maintained in the N-poor solid medium [7], which contains 1.5% agar, 10% sucrose and has a pH of 5.5. Culture was maintained for 5 days at 30ºC, and the formation of acid in parallel to the culture growth was tested by adding bromothymol blue to the medium [9, 39]. This indicator is green at pH 5.5 and turns yellow at pH 5.0. *Leuconostoc mesenteroides*, CECT (Colección Española de Cultivos Tipo) 394, was cultured in conventional peptone-agar medium. The sizes of the inocula used for binding, and of those used for cytoagglutination assays were measured by nephelometry following the absorbance changes at 600 nm.

Binding assays About 35 mg of bacteria in dry weight were resuspended in 3.0 ml of labelled glycoproteins and maintained for 1 h at 30ºC in a shaking bath at 12 oscillations/min. Then, they were collected by centrifugation at 12,000 ×*g* for 10 min at 2ºC and the intensity of the fluorescence emission from supernatants was measured, using an excitation light of 450 nm wavelength. Bound glycoproteins were then desorbed from the bacterial pellet by addition of 3.0 ml of different 50 mM sugars and aminosugars solutions, and 5 mg/ml fetuin, a glycoprotein containing about 9 per cent of sialic acids [38], sometimes used as ligand of several lectin-like glycoproteins [11]. After 1 h of shaking, bacteria were removed again by centrifugation, and fluorescence of the supernatants was measured in the conditions specified above.

After this, solutions containing non-adsorbed glycoproteins were mixed with 0.15 g of bioskin and maintained for 1 h at room temperature. Bioskin is a natural matrix composed by glucosamine and N-acetylglucosamine and produced by a mixed culture of *Acetobacter xylinum*, *Saccharomyces cerevisiae* and *S. pombe* [37]. This product behaves as a polycationic matrix at pH 7.0 and can be used to retain several polyanionic proteins by ionic adsorption [21]. In addition, it can be used also as a support for affinity chromatography on the basis of its aminosugar composition. Bioskin-adsorbed glycoproteins were then desorbed by adding 3.0 ml of 50 mM sugar solutions. Desorption was monitored by measuring absorbance at 280 nm of the washing solutions.

Results

Isolation of HMMG and MMMG fractions and coupling to fluorescein To study glycoproteins occurring in sugarcane which could bind to *A. diazotrophicus*, we filtered centrifuged juice through a Sephadex G-10 column. Elution profile is shown in Fig. 1. A mixture of MMMG and HMMG (8.0 ml, 41.5 mg carbohydrates) was recovered from fraction 28 to 35. The pattern of protein elution was always bimaximal. The main protein peak eluted from fraction 15 to 23, in which no polysaccharides were detected, whereas the second peak eluted from fraction 29 to 43, thus coeluting with the maximum amount of carbohydrates (Fig. 1). Fractions 28 to 35 were mixed and an aliquot of this mixture (3.0 ml) was then loaded onto

a Sephadex G-50 column (Fig. 2). About 70 per cent of total carbohydrates loaded onto the column was recovered, consisting on 0.135 mg of HMMG and 9.66 mg of MMMG. The highest amount of non-glycosylated protein eluted between HMMG and MMMG fractions, from fraction 75 to 109 in which there were negligible amounts of polysaccharides (Fig. 2).

Due to its glycoproteic nature, both HMMG and MMMG showed intrinsic fluorescence. The fluorescence emission spectrum of HMMG under 280 nm light excitation showed a main maximum at 347 nm, a sencondary maximum at 310 nm and a shoulder at 400 nm, whereas MMMG presented two clearcut maxima at 310 nm and 350 nm and a shoulder at 410 nm. No fluorescence emission occurred at 500–535 nm. HMMG and MMMG were then labelled with fluorescein as described under Materials and methods. Labelled fractions showed a new emission maximum, located around 505 nm. Amplification revealed the maximum emission peaks for HMMG and MMMG to be at 510 and 509 nm, respectively (Fig. 3A).

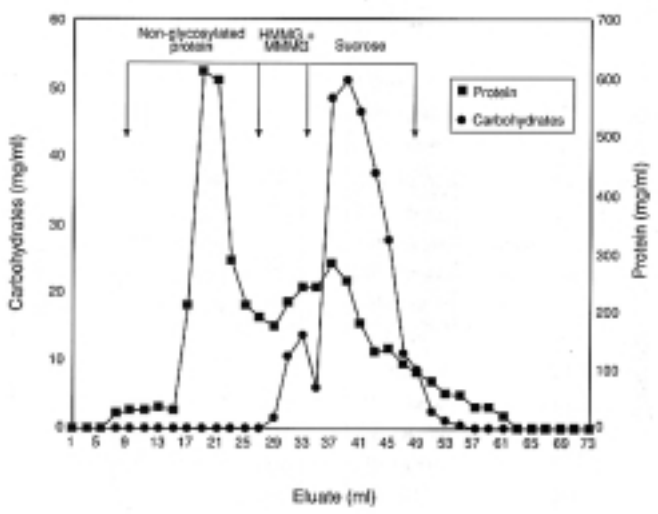

Fig.1 Filtration diagram of centrifuged sugarcane juice through a column of Sephadex G-10. Eluates were monitored for carbohydrate and protein content

Binding of HMMG and MMMG glycoproteins to *A. diazotrophicus* cell wall To ascertain the ability of glycoproteins from the HMMG and MMMG fractions from sugarcane juice to bind to *A. diazotrophicus*, we performed binding assays in which cells were incubated with fluorescein-coupled HMMG (Fl-HMMG) or MMMG (Fl-MMMG). After 1 h incubation, cells were centrifuged and fluorescence emission of the supernatant was measured. The maximum of emission had decreased to less than 25 per cent of its initial value (Fig. 3B). Thus, more than 75 per cent of the labelled compound was bound to the cells (Fig. 4). As a specificity control, the binding of fluorescein-coupled glycoproteins to *Leuconostoc mesenteroides* was measured, showing less than 25 per cent of binding. The lack of free fluorescein in the samples was assessed through adsorption of the labelled glycoprotein on calcium phosphate gel (75 mg dry gel per mg protein). After adsorption, the gel was removed by centrifugation and the supernatant measured for fluorescence emission. No emission was detected, thus confirming the absence of free fluorescein.

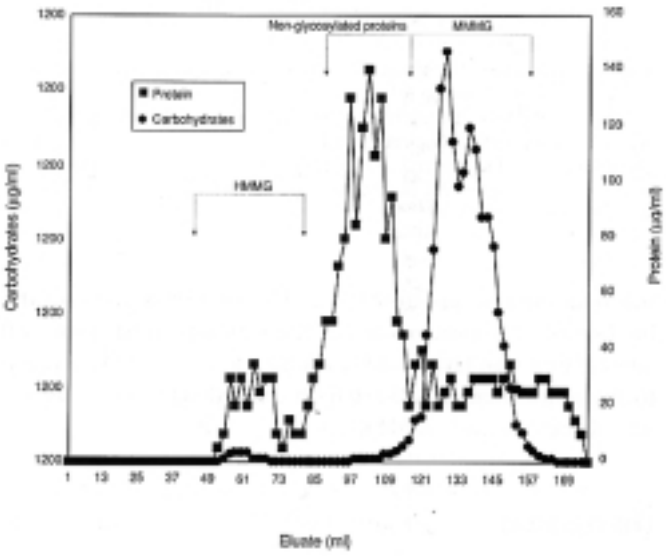

Fig. 2 Filtration diagram through a Sephadex G-50 column of a mixture of HMMG and MMMG contained in fractions 27 to 35 from Sephadex G-10. Eluates were monitored for carbohydrate and protein content

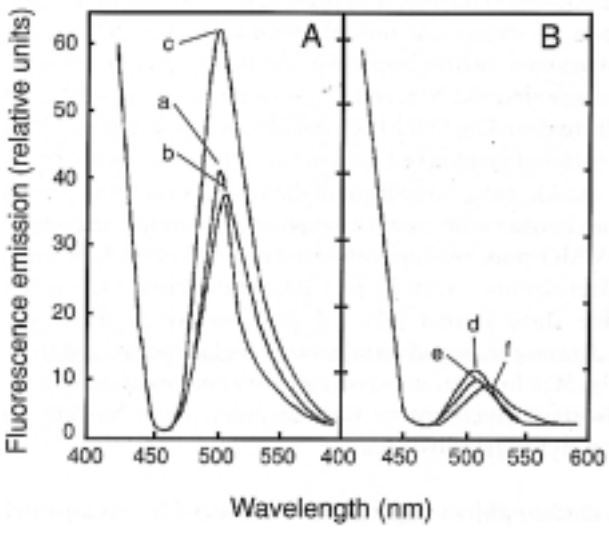

Fig. 3 Fluorescence emission spectra of fluorescein-labelled HMMG, MMMG and acetone precipitated protein before (A) and after (B) 1 h of contact with 35 mg dry weight of *Acetobacter diazotrophicus* cells. a, Fl–acetone-precipitated proteins (507 nm); b, Fl–MMMG (509 nm); c, Fl–HMMG (510 nm); d, same as b; e, Fl–HMMG (512 nm); f, Fl–Acetone-precipitated protein (515 nm)

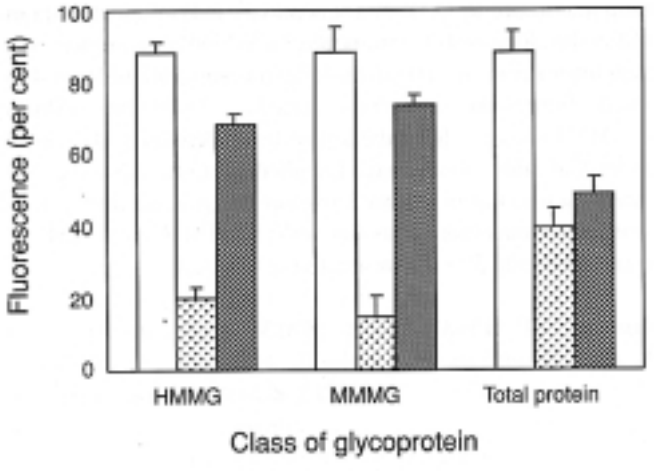

Fig. 4 Percent of total (white), non-adsorbed (gray) and adsorbed (black) fluorescence on *Acetobacter diazotrophicus* cells after 1 h of contact with HMMG, MMMG and acetone-precipitated proteins. Experiments were performed in triplicate. Vertical bars indicate standard error

Desorption of bound glycoproteins to *A. diazotrophicus* To investigate the nature of the carbohydrate moiety related to the binding of HMMG and MMMG to *A. diazotrophicus*, we performed competitive desorption assays. HMMG and MMMG were bound to the cells as described above and incubated in the presence of different sugars, aminosugars and fetuin. Sucrose was the most efficient sugar in removing HMMG from the bacterial cell wall (Fig. 5A), whereas MMMG was mainly desorbed with glucosamine or fructose (Fig. 5B). After removing the bacteria by centrifugation, the supernatants were adsorbed on bioskin at pH 7.0 and desorbed with identical series of sugars. At this pH value, bioskin behaves as a polycationic matrix because of the many $-NH_3^+$ groups from glucosamine and N-acetyl-D-glucosamine components. After selective binding to the bacterial cell envelope, a large fraction of bacterial-unadsorbed, cationic glycoprotein was not retained by bioskin but a component of the HMMG pool was desorbed from bioskin with fructose whereas a different component of MMMG pool was desorbed from bioskin with N-acetyl-galactosamine. Acetone-precipitated protein from sugarcane juice showed that 50% of proteins were adsorbed on *A. diazotrophicus* and desorbed with D-glucosamine and sucrose (Fig. 5C). In addition to polycationic components, only a non-adsorbed glycoprotein was desorbed from bioskin with N-acetyl-D-galactosamine.

***A. diazotrophicus* aggregation is induced by glycoproteins of both HMMG and MMMG fractions** To measure the agglutinating capability of HMMG and MMMG, we incubated bacterial cells dispersed in 5.0 mM acetate buffer, pH 5.0, with HMMG, and measured MMMG fractions and the absorbance at 600 nm. The results showed that cell aggregation, which produced a decrease of the absorbance, started after 5 min and

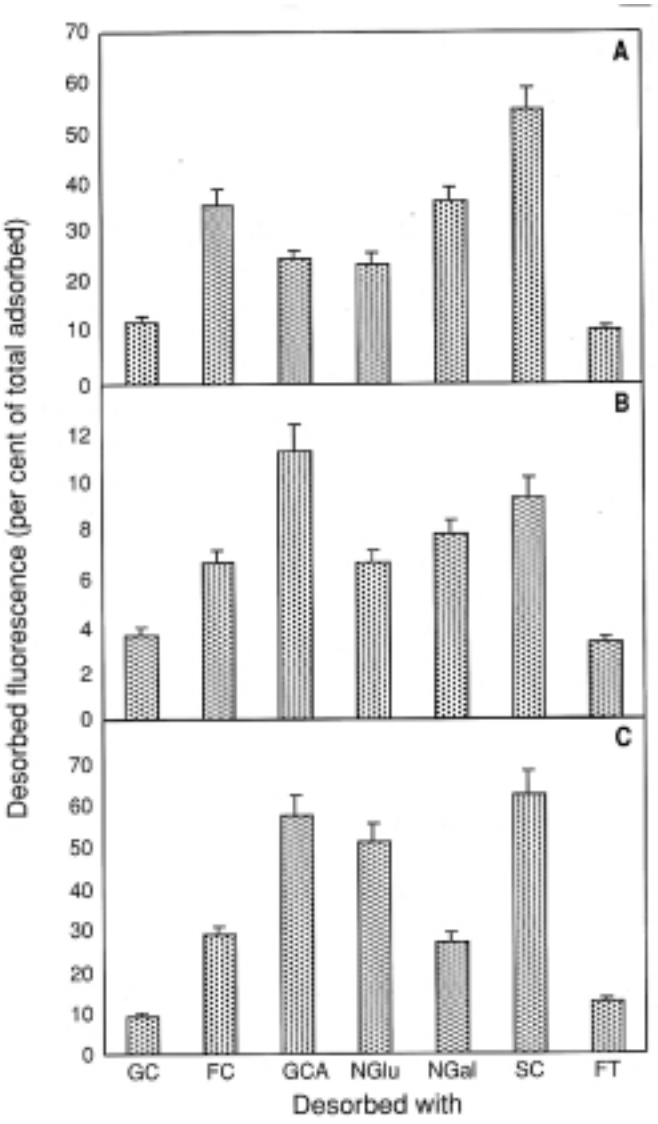

Fig. 5 Desorption by 50 mM sugars and aminosugars of different fluorescent fractions absorbed on *Acetobacter diazotrophicus* cells. Fractions: (A) Fluorescent HMMG; (B) fluorescent MMMG; and (C) fluorescent acetone-precipitated proteins. Desorbed with: GC, glucose; FC, fructose; GCA, glucosamine; NGlu, N-acetyl glucosamine; NGal, N-acetyl galactosamine; SC, sucrose; FT, fetuin. Measurements were performed in triplicate. Vertical bars indicate standard error

reached a maximum at 30 min. The role of sucrose in the binding of glycoproteins to the cell wall and in bacterial cell aggregation was further assessed by adding 100 mM sucrose to the agglutinated cells, which caused immediate disaggregation.

Discussion

Plants have mechanisms to discriminate potential pathogens from symbionts [8]. These mechanisms have been postulated to act through lectin-like glycoproteins. We herein provide

evidence of a selective binding of some glycoproteins produced by sugarcane and extracted from sugarcane juice to *A. diazotrophicus*, the natural endosymbiont of sugarcane plants, whereas they do not bind efficiently to *L. mesenteroides*, an epiphytic bacterium which lives on leaf surfaces. This phenomenon closely resembles the selectivity of other plant lectins, which can discriminate between compatible and incompatible endophytes [29, 31, 32]. Moreover, sugarcane glycoproteins have been demonstrated to exert a cyto-agglutinating effect on *A. diazotrophicus*, a property of plant lectins which behave as phytohaemagglutinins [25, 41] or phycoagglutinins [30]. Bacterial adhesion is undoubtedly produced by HMMG and MMMG, the glycosidic moiety of which is mainly composed by fructose units linked by β-(1, 2) bonds, since sucrose reverses cytoagglutination.

Both HMMG and MMMG have been previously defined as heterogeneous fractions composed by several cationic and anionic glycoproteins [20]. Since cationic glycoproteins are not largely bound to *Acetobacter* cells, the biological activity of these proteins, their binding capability, seems to be restricted to anionic HMMG and MMMG. The pattern of binding behaviour of total, acetone-precipitated protein to *A. diazotrophicus* (Fig. 4) and their desorption (Fig. 5C) seems to be similar to that observed for MMMG (Fig. 5B) rather than for HMMG (Fig. 5A) since the amount of MMMG in sugarcane juice is about 70 times higher than that of HMMG. Moreover, the quantitative adsorption of total protein is low, possibly due to the occurrence of high amounts of non-glycosylated proteins in this fraction (Fig. 2).

HMMG largely contains β-1,2 fructan domains separated by galactitol units in ether linkage [18]. Thus, a large desorption of HMMG may have been produced by sucrose (α-D-glucopyranosyl-(1,2)-O-β-D-fructofuranoside) and secondarily by fructose. This is the first time that a cluster consisting of dimeric fructose with β-1,2 linkages has been described as an active center of a plant lectin-like glycoprotein, but the specificity of some lectins towards a disaccharide with a specific linkage is well known. For example, the lectin of *Erythrina corallodendron* [24] and *E. crystagalli* [12] selectively binds to β-D-galactose-(1,4)-D-N-acetylglucosamine, that of *Euonymus europaeus* [34] to α-D-galactose-(1,3)-D-galactose, and the lectin of *Sambucus nigra* to β-D-galactose-(1,4)-D-glucose [6]. Desorption of MMMG differs significantly since it is produced by glucosamine and, secondarily, by sucrose (Fig. 5A). This can be explained on the basis of the relative length of the homofructan segments in the glycidic moiety of these glycoproteins. The homofructans segments of HMMG are ...–galactitol-fructose(fructose)$_n$-fructose-galactitol-...., where n>4, and, as a consequence, completely hydrolyzed by β-fructosidase [1,20], whereas those of MMMG are ....–galactitol-(fructose)$_3$-galactitol-.... or....–galactitol-(fructose)$_4$-galactitol-.... and, thus, β-fructosidase only produces about 50% of free fructose [16, 20]. This implies that HMMG contain many consecutive β-(1,2) linkages, able to bind the ligand; thus, the probability that they be desorbed by sucrose is high (Fig. 5A). However, the number of active bonds for the binding of MMMG is low, probably because of the steric difficulty to access to the ligand site.

Both HMMG and MMMG are produced by sugarcane stalks as a response to mechanical injuries [28, 40] or attack of pathogens [17]. So, it is feasible that these glycoproteins be produced after the insertion of the mouth stylus of *Saccharococcus sacchari* into the storage cells of parenchymatous tissues of sugarcane stems [3]. If that were so, they would appear simultaneously to the introduction of the potential endophyte, *A. diazotrophicus*, in the host tissues. This hypothesis can explain the discriminatory ability of sugarcane to choose the compatible endophyte from several possible ones.

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