Influence of Flood Areas on the Number of Diazotrophic Bacteria from Pasture Grasses

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Abstract Floodplain areas are driven by flood pulse and with consequence, flood gradient are formed, such as, lakes, wetlands and dry areas. And this gradient is one of the most important forcing functions for animal and plant ecology that areas. The aim of this study is testing the hypothesis of the flood gradient in the bacteria diazotrophic association with grasses in their roots and shoots. The study was realized in the Pantanal of the Mato Grosso do Sul, in two periods, rainy (March, 2009) and dry (August, 2010) and the samples of grasses were made in different flood gradients areas: in dry, in seasonally flood and in permanent flooded. The number of bacteria in the roots and stems were counted using the most probable number method using the semi-solid media or semi-specific JNFb and the morphotypes identified using the type strains of *Azospirillum* and *Herbaspirillum*. The results show that the grasses presented no difference in roots and shoots and neither for the periods for bacteria number. The principal difference in number were recorded among genera of grasses, were the in grasses influenced by water of flood, the grasses *Hymenachne amplexicaulis, Axonopus purpusii* presented higher number of bacteria in its tissues and the grass *Mesosetum chaseae* found in the dry area presented lowest value. The morphotype found belong to three genera, *Azospirillum* and *Herbaspirillum* and non-identified genera. The *Azospirillum* and *Herbaspirillum* presented high number in grasses *Hymenachne amplexicaulis* of permanent flood areas and the non-identified genera presented high number in grasses *Axonopus purpusii* and *Mesosetum chaseae* dry and seasonally flood area. We conclude that flood gradient hypothesis is valid for association of diazotrophic bacteria and grasses for Pantanal floodplain in two ways: driven the grasses host species distribution along the gradient and the host driven number and bacteria population composition.

Keywords: *Azospirillum, Herbaspirillum, Brazilian Pantanal, floodplain diazotrophic ecology*

1. Introduction

The ecology of floodplain environments is driving by flood pulse, where the water cover large areas and the organisms are adapted to this pulse [1]. Records of the diazotrophic bacteria association with graminae are scarce in the flooding areas such as the Pantanal region, the biggest floodplain of the word [2]. The diazotrophic bacteria in association with graminae are important for pasture renovation, mainly in extensive cattle farming, that depends highly or totally of the grass for cattle food supply such as Pantanal.

Currently, there are several records of genera of diazotrophic bacteria in associations with grasses [3,4,5]. These bacteria can promote the plants grows by biological nitrogen fixation (BNF) and production of growth phytohormones, such as, gibberellin and indol acetic acid (IAA) becoming important in soil with low concentration of organic matter, increasing yields and enriching nitrogen content of the plants [6].

It were recorded nine genera of diazotrophics in grasses, including *Azospirillum* and *Herbaspirillum* [7], the first being an alpha-proteobacteria and the second a beta-proteobacteria [5]. Both these genera were considered to be *Azospirillum* until 1986 after that they are separated into two, creating the new genus *Herbaspirillum* [8].

These two genres are different as their ecology, where the *Azospirillum* genus, when compared with *Herbaspirillum,* has low specificity to the host plant where colonizing both its external parts as well as their internal tissues [8,9] being facultative endophytic. While *Herbaspirillum* genre has high specificity with the host plant colonizing only

internal tissues, these bacteria are called obligate endophytes, which are bacteria that colonize the internal tissues of plants without causing disease [7,10].

In the wetlands such as mangroves, the mosaic of flooding environments influences the composition of diazotrophics microorganisms [11]. In the Pantanal environment grasses are adapted to different flood gradients [12] forming the mosaic and the bacteria association can be influenced by this.

In this study, we tested the hypothesis of the gradients of flooding, where the gradient drives the population in these environments and how flood gradients affect the populations of diazotrophic and its association with roots and shoots of the grasses adapted to different flood areas.

2. Materials and Methods

2.1. Study Area and Plant Sampling

The study was conducted in the Brazilian Pantanal at sub-region of the Nhecolândia, Mato Grosso do Sul State (Figure 1A). The samples were collected around permanent floodplain lake (coordinates: 18° 58.230' S and 56° 37.359' W). Sampling was carried out in March 2009 (end of the rainy season) and August 2010 (middle of the dry period). Pluviometric data were obtained from the Weather Monitoring Center of Mato Grosso do Sul State (www.agraer.ms.gov.br).

Plants were sampled areas with different flood gradients, such as permanent dry area (DA), seasonally flooded area (SF) and permanently flooded area (FP) (Figure 1B).

The diazotrophic associated with grasses *Mesosetum chaseae* were quantified (sampled in AD area), *Axonopus purpusii* and *Hymenachne amplexicaulis* (sampled in areas SF and FP), these grasses are native and common in Pantanal [13]. Composite samples (shoots and roots) of the three species were collected in triplicate in the pastures around and lakes.

2.2. Counting and Isolation of the Bacteria

The plant samples, distinct for each species, were separated into shoots and roots in the laboratory. The roots were separated from the soil by agitation and washed in running water. Subsequently, shoots and roots cut into fragments and 10g of each plant part were weighed and surfaces superficially disinfected with sodium hypochlorite at 1% (v/v) for one minute, and then immersed in distilled sterile water for one minute. Finally they were transferred to a 0.05 mol L^{-1} phosphate buffer (pH 6.8) solution for remove of hypochlorite residues and, rinsed three times in distilled sterile water. Time spend in surface sterilization with hypochlorite was determinate through preliminary tests. Serial dilutions of the samples $10^{^1}$ to $10^{^5}$ in NaCl 0.85% (w/v) were carried out.

Aliquot of 100 µL from each dilution was inoculated in a semi-solid JNFb medium [14] that contains (g L^{-1}) malic acid, 5.0; K_2HPO_4, 0.6; KH_2PO_4, 1.8;$MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.02; Micronutrient solution (above), 2 mL; bromothymol blue (5g L^{-1} in 0.2 N KOH), 2 mL; FeEDTA (16.4g L^{-1}),4 mL; vitamin solution (biotin, 10 mg; pyridoxal-HCl, 20 mg. Dissolve in hot water bath,

complete to 100 mL by adding distilled water), 1 mL; KOH, 4.5. Add distilled water to bring total solution to 1,000 mL. Adjust the pH to 5.8 with KOH. To semi-solid and solid medium add 1.8 and 17g agar L^{-1}, respectively. This medium is enabling the growth of diazotrophic bacteria, including the genera *Herbaspirillum* and *Azospirillum* [3].The diazotrophic population sizes were estimated using the most probable number (MPN) technique, using the McCrady table for three replications [1].

The isolation process was conducted using the cultures that presented a growth characteristic to diazotrophs [3]. The colonies of bacterial isolates were compared with regard to shape and coloring with the type strains of *Herbaspirillum seropedicae* (BR11175[T]), *H. rubrisubalbicans* (BR11192[T]), *Azospirillum brasilense* (BR11001[T]) and *A. lipoferum* (BR11080[T]) in NFb [16] (3x) (NFb medium containing three times the dye volume) and in BMS medium.

The NFb medium, contains (g L^{-1}): malic acid, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.02; micronutrient solution ($CuSO_4.5H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.12; H_3BO_3, 1.40; $Na_2MoO_4.2H_2O$, 1.0; $MnSO_4$. H_2O, 1.175. Complete volume to 1,000 mL with distilled water), 2 mL; bromothymol blue (5 g L^{-1} in 0.2 N KOH), 2 mL; FeEDTA (solution 16.4 g L^{-1}), 4 mL; vitamin solution (see above), 1 mL; KOH, 4.5 g; distilled water to bring the final volume to 1,000 mL and adjust pH to 6.5. A quantity of 1.6 to 1.80 g agar L^{-1} should be added to prepare the semi-solid medium and 15 g agar L^{-1} for the solid medium. The BMS (Potato – malate – sucrose) or Potato agar medium has the following composition (g L^{-1}): potatoes peeled and sliced, 200; DL-malic acid, 2.5; KOH, 2.0; crystalized cane sugar, 2.5; vitamin solution (see above), 1.0 mL; Micronutrient solution (see above), 2 mL; bromothymol blue (5 g L^{-1} in 0.2 N KOH), 2 drops; agar, 15.0. The potatoes are placed in a gauze bag, boiled in 0.5 l of water for 30 min, and then filtered through cotton, saving the filtrate. The malic acid is dissolved in 50 mL of water and the bromothymol blue added. KOH is added until the malic solution is green (pH 6.8-7.0). This solution, together with the crystalized cane sugar, vitamins and agar, is added to the potato filtrate. The final volume is made up to 1,000 L with distilled water.

For storage, the isolated colonies were inoculated in liquid Dygs medium (Dextrose yeast glucose) [16], and shaken at 150 rpm for 24 h at 30°C. Later, an aliquot of 1.0 mL from the culture was transferred to 1.5 mL microtubes and centrifuged for 15 minutes, adding to the precipitate 500 µL of glycerol 50% and stored at -20 °C.

The cell number estimated by MPN method did not follow a normal distribution, thus, they were transformed in log (x+1) and submitted to non-parametric tests were used, namely Spearman rank correlation and Kruskal-Wallis ANOVA, followed by multiple comparisons and the Mann-Whitney U test.

3. Results and Discussion

The Figure 2A and Figure 2B shows the density of diazotrophic bacteria (log (x+1)) in aerial part (shoots) and radicular system (roots) in the grasses sampled. All grasses presented association with diazotrophic and none

of them presented significant difference between parts. The highest abundances of diazotrophic populations were recorded in the grass species in the permanent and seasonally flooded areas (Figure 2A), where *H. amplexicaulis* and *A. purpusii* contained total numbers of 588 and 522, in log (x+1), diazotrophic bacteria, for the entire plant (roots plus aerial), respectively. The lowest numbers of diazotrophic were recorded (222 in log (x+1)) in *M. chaseae* that occur in the permanent dry area.

The bacteria colonization of roots and shoots depends on plant species as well as edaphoclimatic condition [17,18,19]. In our study the soil is different because the flood area is generally more productive with higher proportion of organic material than dry ones [20,21] and the plant species difference is drive by flood gradient [22]. This gradient separates the grasses species and the species from dry area has low association capacity when compares with flood area species as expected for different cultivate and native species [17,23,24,25].

Seasonality, with 22 mm of cumulative rain in March and zero in August, had no significant influence (tested by Kruskal-Walls) on the total numbers of diazotrophic bacteria, neither in the grasses nor in the environment (flood and dry) (Figure 2B).

This stability numeric of the diazotrophic populations in both sampled period is probably by maintenance of vegetation after flood events where the flood peak occurs in May [26]. This results show that vegetation types is more important in this environment for diazotrophic bacteria than flood events and cattle pastures corroborating with other studies of specificity between plants and diazotrophic bacteria [17].

Three morphotypes colonies were isolated and characterized, and two of these identical morphological characteristics of the type strains of *Azospirillum* and *Herbaspirillum*. The third morphotype showed different characteristics of the strains used. The JNFb medium used allows the growth of other genres of diazotrophics as *Sphingomonas*, *Burkholderia* and other [3,17]. These genera were recorded at different rates in each grass and sampling area. The identified genera *Azospirillum* and *Herbaspirillum* were found in high concentrations in the grass *H. amplexicaulis* (Figure 2C), indicating the high associative capacity of this grass; *Azospirillum* has been recorded in this grass before [27] while *Herbaspirillum* has not [7,28,29].

The non-identified bacteria were found in *H. amplexicaulis* in smaller numbers as compared to *Azospirillum* and *Herbaspirillum*. In contrast, for the grasses *M. chaseae* and *A. purpusii*, the association with the non-identified bacteria was more representative than with the identified ones. The abundances of these non-identified genera were the same in all three host plants, indicating an opportunistic pattern with low specificity and capacity to use many physiological pathways [5,30].

The opposing concentrations of *Herbaspirillum* + *Azospirillum* and the non-identified bacteria in the three grass species studied also indicate that these groups compete for the occupation of the interior grass tissues. Both genera identified in this study present several mechanisms of plant growth promotion (PGP), such as BNF, auxin biosynthesis [31]. The mechanisms used by the other bacteria remain unknown. Nevertheless, the

bacterial isolates are able to overcome the plant constraint mechanisms that avoid the colonization of bacterial isolates that do not present PGP mechanisms [32] indicating that those unidentified bacterial isolates are also able to form beneficial associations with the grasses.
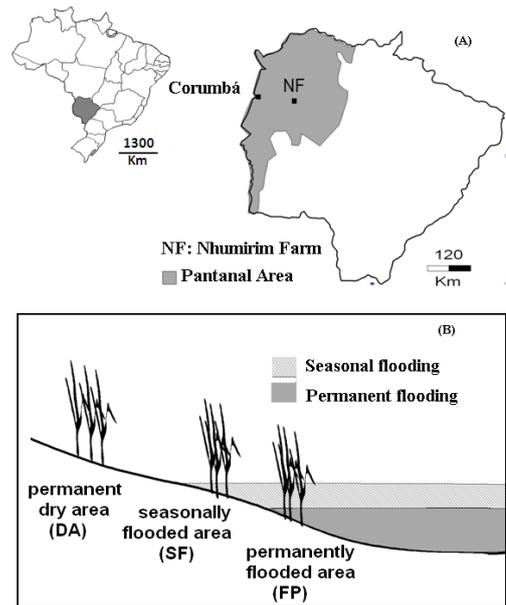

Figure 1. A. Location of Mato Grosso do Sul State in Brazil and the location of Nhumirim Farm and Corumbá city in the state. B. Scheme of the lake flooding, showing the flood gradients
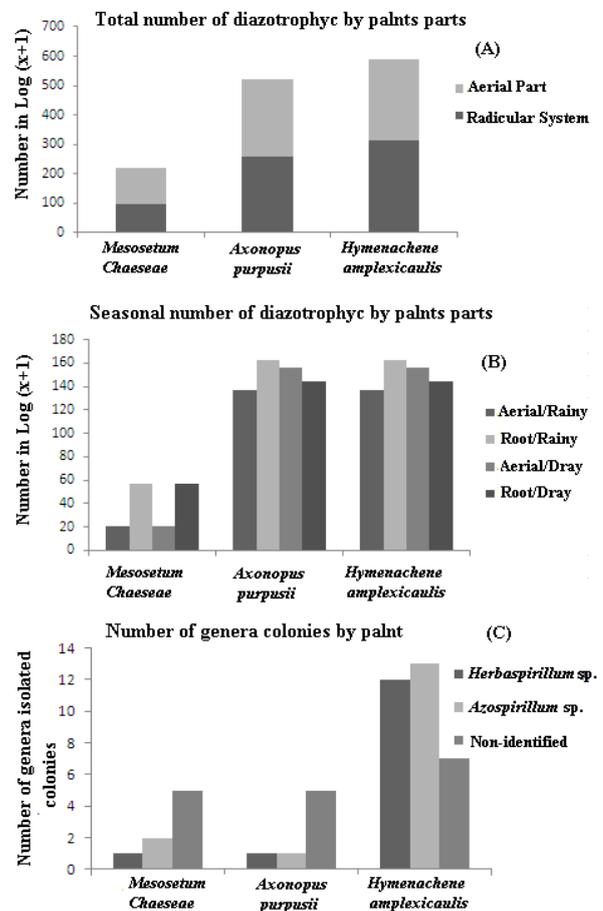

Figure 2. A. Number of total diazotrophic bacteria in roots and arial parts. **B**. Seasonal number of diazotrophic bacteria in roots and arial parts. **C**. Number of isolated colonies of genera in the plants

We conclude that the level of flooding select plant species adapted to these gradients and these plant species leads to associated diazotrophic population them. And the flood gradient hypothesis is valid for association of diazotrophic bacteria and grasses for Patanal floodplain in two ways: driven the grasses host species distribution along the gradient and the host driven number and bacteria population composition.

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