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ORIGINAL PAPER

Azospirillum spp. from native forage grasses in Brazilian Pantanal floodplain: biodiversity and plant growth promotion potential

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Abstract A sustainable alternative to improve yield and the nutritive value of forage is the use of plant growth-promoting bacteria (PGPB) that release nutrients, synthesize plant hormones and protect against phytopathogens (among other mechanisms). *Azospirillum* genus is considered an important PGPB, due to the beneficial effects observed when inoculated in several plants. The aim of this study was to evaluate the diversity of new *Azospirillum* isolates and select bacteria according to the plant growth promotion ability in three forage species from the Brazilian Pantanal floodplain: *Axonopus purpusii, Hymenachne amplexicaulis* and *Mesosetum chaseae*. The identification of bacterial isolates was performed using specific primers for *Azospirillum* in PCR reactions and partial sequencing of the 16S rRNA and *nif*H genes. The isolates were evaluated in vitro considering biological nitrogen fixation (BNF) and indole-3-acetic acid (IAA) production. Based on the results of BNF and IAA, selected isolates and two reference strains were tested by inoculation. At 31 days after planting the plant height, shoot dry matter, shoot protein content and root volume were evaluated. All isolates were able to fix nitrogen and produce IAA, with values ranging from 25.86 to 51.26 mg N mL^{-1} and 107–1038 µmol L^{-1}, respectively. The inoculation of *H. amplexicaulis* and *A. purpusii* increased root volume and shoot dry matter. There were positive effects of *Azospirillum* inoculation on *Mesosetum chaseae* regarding plant height, shoot dry matter and root volume. Isolates MAY1, MAY3 and MAY12 were considered promising for subsequent inoculation studies in field conditions.

Keywords *Azospirillum brasilense* · Biological nitrogen fixation · Diazotrophic bacteria · Indole-3-acetic acid · Inoculant

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Introduction

The Brazilian Pantanal is a continuous sedimentary plain, formed 1000 years ago with the uplift of the Andes. It is a part of the Paraguay River Basin, covering the Brazilian states of Mato Grosso (35%) and Mato Grosso do Sul (65%), with an approximate area of 140,000 km^2 and altitude from 80 to 170 m (Silva and Abdon 1998). One of the main economic activities in Pantanal is cattle ranching, which has been the dominant economic activity and land use in the region for the past two centuries (Seidl et al. 2001). However, the native forage occurring in the large fields presented relatively low values of zootechnical index, due to low productivity and quality of the forage, which is used as pastures (Santos et al. 2011). Furthermore, cattle

access to forage depends on forage adaptation to flooding (Santos et al. 2011). Among the main native forage consumed by cattle in Pantanal, grasses species such as *Axonopus purpusii, Hymenachne amplexicaulis* and *Mesosetum chaseae* are considered "preferred" by livestock and classified as species that present regular level of consumption (Santos et al. 2002). *Axonopus purpusii* has C4 photosynthetic pathway, is resistant to temporary water submersion but dies when submitted to prolonged flooding (Allem and Valls 1987), being considered "preferred" and a key species for management plans of native pastures (Santos et al. 2002). The forage *H. amplexicaulis* is a semi aquatic plant with C3 photosynthetic pathway, native from Central and South America (Silva et al. 2012), occurring in soil flooded for long time period (Agriculture and Resource Management Council of Australia and New Zealand 2000). The species has high digestibility due to the lower presence of fibrovascular structures than other forage species (Santos et al. 2002). *Mesosetum chaseae* is a perennial Poaceae with a C4 photosynthetic pathway, drought tolerant and widely distributed in Pantanal region. This plant has high productivity, cattle acceptability, being a species with potential for cultivation in higher and not flooded areas, with low fertility (Santos et al. 2011).

In Brazil, the main studies on isolation and identification of diazotrophic bacteria in forage were developed between the 1960s and 1980s. These researches observed considerable contribution of the biological nitrogen fixation (BNF). Among these studies, Boddey et al. (1983), using the N^{15} isotopic dilution technique, demonstrated a BNF contribution of 10% for the plant N (20 kg ha^{-1} $year^{-1}$) in *Paspalum notatum* cv. *batatais*. In 1986, Boddey and Victoria (1986), using the same technique, observed for *Urochloa humidicola* and *U. decumbens*, 30–40% of increase for the accumulated N using BNF. Another study with these same species showed a BNF contribution of up to 42% for the plant N (Silva et al. 2010). Lately, Morais et al. (2012) studying the contribution of BNF in different *Pennisetum purpureum* genotypes, observed a BNF contribution of 18–70% of the N accumulated by plants, corresponding to 36–132 kg N ha^{-1}, respectively. In this context, *Azospirillum* spp. is one of the most studied diazotrophic bacteria which can be associated with several plant families (Radwan et al. 2005), presenting a wide distribution, being found in soil, roots, leaves and plant stems (Baldani et al. 2014).

Besides BNF, many effects of *Azospirillum* spp. in plants are assigned to the production of phytohormones and plant growth-promoting substances by bacteria, auxin, cytokinins and gibberellins (Cavallet et al. 2000; Reis Junior et al. 2004). Bashan and Holgin (1997) reported that phytohormones, mainly the indole-3-acetic acid (IAA), produced and excreted by *Azospirillum*, play an essential role in promoting plant growth, due to higher production of dry matter and nutrient accumulation observed in plants inoculated with these bacteria. Increase in root development by *Azospirillum* results in an improvement of water and nutrients absorption, thereby increasing the capacity of plant to produce and withstand environmental stresses, such as drought, flood and salinity, resulting in more vigorous and productive plants (Bashan and Holguin 1997; Dobbelaere et al. 2001; Bashan et al. 2004). Barassi et al. (2008) also reported the improvement in photosynthetic parameters of leafs, including increases in chlorophyll content and stomatal conductance, in addition to the production of biomass and plant height. Due to these evidences, more studies regarding BNF and effects of IAA production by diazotrophic bacteria in forage are necessary to understand the role of these microorganisms in promoting plant grow in the particular environment of the Pantanal. Reis Junior et al. (2002) affirm the need of research regarding BNF and diversity of diazotrophic microorganisms in grasses aiming to obtaining strains with plant growth promotion potential. The genus *Azospirillum* was first defined by Tarrand et al. (1978) after the characterization of *Azospirillum brasilense* and *A. lipoferum*. Currently, there are 17 species described and the most studied is *A. brasilense*, mostly the strain Sp7, which is used in inoculation experiments. This genus exhibits a versatile carbon and nitrogen metabolism, which is an adaptive feature to survive in different environments (Steenhoudt and Vanderleyden 2000).

For the management of forage grasses native from Pantanal, the use of agricultural inputs, particularly nitrogen fertilizers, is nonexistent. The BNF and IAA production by diazotrophic microorganisms associated with plants roots can improve its yield and nutritional value, association that may contribute to the socio-economic development and the maintenance of this agroecosystem. In Pantanal, diazotrophic bacteria from the genera *Azospirillum, Herbaspirillum* and *Burkholderia* were isolated from native forage used for cattle feed, such as *Axonopus purpusii, Elyonurus muticus, Hymenachne amplexicaulis, Mesosetum chaseae*, and *Urochloa humidicola* (Brasil et al. 2005). Therefore, the aim of this study was to identify and evaluate the diversity of *Azospirillum* bacteria isolated from Pantanal native forage grasses, and to evaluate the inoculation effects of *Azospirillum* isolates on *Axonopus purpusii, Hymenachne amplexicaulis* and *Mesosetum chaseae*.

Materials and methods

Origin of isolates and microbial growth conditions

Putative diazotrophic bacteria were isolated from roots of three Pantanal native forage: *M. chaseae, A. purpusii* and

H. amplexicaulis (Table 1), from high (free flooding), intermediate (seasonal flooding) and low (intense flooding) altitudinal gradient, respectively. The plants were sampled in March and July of 2008 and in March and August of 2010 at Nhumirim Farm (18°59′S and 56°39′W; 4130 hectares), which belongs to the Embrapa Pantanal, and is located in the Nhecolândia sub-region of Pantanal, Corumbá city, Mato Grosso do Sul State, Brazil. For isolation of bacteria, the technical protocols of Baldani et al. (2014) were followed. The bacteria isolates grew in Dygs medium (Rodrigues Neto et al. 1986) for 24 h, under agitation at 100 rpm and 30 °C. Aliquots 20 µL were transferred from the culture to NFb semi-solid medium. After grown for 5 days at 30 °C, the isolates were cultivated in potato solid medium (Baldani et al. 2014) to verify contaminants. This procedure were realized to obtain a pure and viable isolates to continue the experiments.

Genetic identification of bacteria isolates

Specific primers for Azospirillum

The DNA of each bacterial isolate and both type strains (Table 1) were extracted using the Pure Link Genomic DNA Mini Kit (Invitrogen). The PCR reactions were conducted according to Shime-Hattori et al. (2011) using the following primers: Az16-A (5′GCGGTAATACGA AGGGGGCK forward sequence) and (5′CTTGTCACC GGCAGTTCCACCAG reverse sequence), with a product size of 646 bp. Two strains (*A. brasilense*—Sp7 and *A. lipoferum*—Sp59) were used as positive controls and DNA from *Bradyrhizobium* sp., *Ralstonia* sp., *Burkholderia* sp. and *Chryseobacterium indologenes* were used as negative controls.

Partial sequencing of 16S rRNA and nifH genes

The 16S rRNA and *nifH* genes were amplified by PCR in a reaction volume of 25 µL under the following conditions: 40 ng of genomic DNA, 10× of Taq DNA polymerase buffer, 1.5 mmol L^{-1} of $MgCl_2$, 0.2 mmol L^{-1} dNTP, 5.0 pmol L^{-1} of each primer, 1U of Taq DNA polymerase. The thermocycling conditions were: initial denaturation at 93 °C for 45 s; 35 cycles of denaturing at 93 °C for 45 s, 1 min annealing at 62 °C for 16S rRNA gene or 45 s at 55 °C for *nifH* gene, extension at 72 °C for 2 min; and final extension at 72 °C for 5 min. The primers used for 16S rRNA gene were Y1f and Y3r or Y1f and 16S805r (Young et al. 1991; Cruz et al. 2001; Soares-Ramos et al. 2003). PolF and PolR primers (Poly et al. 2001) were used for n*ifH* gene. The PCR products were submitted to enzymatic purification using the Thermo Scientific commercial kit.

Table 1 Origin of diazotrophic bacteria isolated from native forage from Pantanal and origin of type strains of *Azospirillum brasilense* and *A. lipoferum*

Plants species	Diazotrophic isolate	
Hymenachne amplexicaulis	HAI1, MAY1, MAY4, MAY6, MAY8, MAY10, MAY12, MAY14, MAY17, MAY19, MAY22, MAY23, MAY24, MAY25, MAY26, MAY27, MAY30	
Mesosetum chaseae	MAY2, MAY5, MAY7, MAY9, MAY11, MAY15, MAY16, MAY18, MAY20, MAY21, MAY28, MAY29, MAY31	
Axonopous purpusii	AZM8, MAY3, MAY13	
Type strain	Specie	References
Sp7 (BR11001T)[a]	*A. brasilense*	*Digitaria decumbens* (Tarrand et al. 1978)
Sp59 (BR11080T)[a]	*A. lipoferum*	*Triticum aestivum* (Tarrand et al. 1978)

[a]From culture collection of Diazotrophic bacteria Johanna Dobereiner

Sequencing of amplified fragments were carried out using the BigDye® kit Terminator v3.1 Cycle Sequencing in the automatic sequencer ABI PRISM 3500 Genetic Analyser (Applied Biosystems). The sequences were analyzed using BioEdit software version 7.1.9 (Hall 1999) to verify sequences quality and chimeric origins. The sequences were verified and assembled using the CLC Genomics workbench 65.1 (CLC bio, Aarhus, Denmark) software and compared with Genbank sequences using BLASTn (http://blast.ncbi.nlm.nih.gov/). All sequences were deposited in GenBank (access number KX618442 to KX618472 for 16S rRNA and KX881787 to KX881817 for *nif*H).

The new sequences were combined with obtained sequences of 16S rRNA and *nif*H genes from Ribosomal Database Project (http://rdp.cme.msu.edu) and GenBank. The multiple sequence alignments were performed using MUSCLE. The phylogenetic tree was built using the Maximum-Likelihood method and Junkes–Cantor model with 1000 *bootstrap* replicates with the aid of MEGA 6.0 software (Tamura et al. 2013).

Evaluation of BNF and production of IAA

The putative nitrogen fixation capacity was evaluated according to the method of Kuss et al. (2007) with the following modifications: a culture medium without inoculation was used as control and the pure cultures were inoculated in Dygs medium for 24 h at 30 °C. After growth the optical density was adjusted to 0.5 in a spectrophotometer at 600 nm. An amount of 600 µL from culture were inoculated in bottles with 10 mL of NFb semi-solid medium and incubated at 30 °C for 5 days. After the bacteria growing, the bottles were frozen until the evaluations.

To lyse cells, the frozen flasks were heated in microwave for 15 s; 4.0 mL of each solution was added to digestion tubes containing five glass beads, 330 mg of catalytic mix (10.0 g of $NaSO_4$:0.5 g of $CuSO_4{\cdot}5H_2O$) and 2.0 mL of H_2SO_4. The tubes were heated in digesters blocks for 2 h at 180 °C with a gradual increasing in temperature until 400 °C, which was maintained until the mix achieved a green color. We removed the tubes from digesters blocks and when the temperature reduced to 40 °C, 2.0 mL of deionized water was added. For distillation, 6.2 mL NaOH 40% was added in each tube. This solution was then transferred to Erlenmeyer flasks containing 1.24 mL of boric acid 2% solution with mixed indicators (70 mg of methyl red and 100 mg of bromocresol green, both dissolved in 200 mL methanol). The titration of the samples was carried out with HCl 0.01 M. To calculate the N content, the following formula was used: % N_2 in the sample = (Sample titer − Blank titer/Sample wt. in g × 1000) × Molarity of HCl × 14 × 100. The results were presented in µg mL^{-1} and were submitted to variance analysis and the average compared according to Scott–Knott's test at 5% of probability.

To confirm the BNF capacity of all bacterial isolates, a quantitative evaluation were realized regarding the nitrogenase activity in semi-solid medium using the Acetylene Reduction Assay (ARA) (Boddey 1987). The bacteria were grown in NFb semi-solid medium as described above. The vials were closed with rubber taps and 5 mL of air were taken from the vials and replaced by 5 mL of acetylene. The vials were incubated in the dark for 30 min when an aliquot of the air were taken for the quantification of ethylene by gas chromatography in a Perkin-Elmer GC Auto System. The bacterial isolates that presented the characteristic peak when compared with the injection of pure ethylene were considered as positive (+) in the ARA.

The production of IAA was analyzed according the colorimetric method described by Sarwar and Kremer (1995) with modifications reported by Reis Junior et al. (2004). The pure bacteria cultures were inoculated in NFb medium containing 0.1% of NH_4Cl (without yeast extract, vitamin and bromothymol blue) and incubated for 24 h at 30 °C. After sufficient time has elapsed for bacterial growth, the optical density was adjusted to 0.5 in a spectrophotometer at 600 nm, 2.0 mL of the suspension added in 28 mL NFb medium with 100 µg mL^{-1} of filtrated tryptophan. After the optical density (500 nm) of the cultures was adjusted to approximately 10^8 cells mL^{-1}. Posteriorly, 1.0 mL of these cultures was centrifuged at 8000 g for 15 min; 150 µL was added to polystyrene plates containing 100 µL of Salkowisk reagent (1.0 mL $FeCl_3{\cdot}6H_2O$ 0.5 M in 50 mL $HClO_4$ 35%) and incubated in dark at room temperature for 30 min. Positive results were considered when the solution presented a pinkish color and only positive cultures were used to analyze the absorbance in spectrophotometer at 492 nm. The IAA concentration was estimated using a standard curve with known IAA concentration (0, 25, 50, 100, 200, 500, 1000 µM), according the equation $y = 910.75x - 0.0975$ ($R^2 = 0.998$). The results were submitted to variance analysis and the average comparison was realized using the Scott–Knott's test at 5% of probability.

Effects of *Azospirillum* spp. inoculation on growing forage

To evaluate the effect of *Azospirillum* inoculation, a pot experiment was conducted in a greenhouse using soil and seedlings from three forage species, *M. chaseae, A. purpussi, H. amplexicaulis*, collected in the Nhumirim Farm, Pantanal region, Brazil.

The soil was classified as a hydromorphic Ultisol, with the following chemical characteristics: pH 5.3, 6.7 mg L^{-1} P, 0.045 $cmmol_c/dm^{-3}$ K, 0.29 Ca, 0.07 $cmmol_c/dm^{-3}$ Mg, 0.625 CTC, 0.425 $cmmol_c/dm^{-3}$ SB, and 22.07%

V. The quantified micronutrients were 2.23 mg L^{-1} Cu, 319.76 mg L^{-1} Fe, 5.9 mg L^{-1} Mn, and 2.7 mg L^{-1} Zn. The granulometry was 32.22% of coarse sand, 61.23% of fine sand, 93.45% of total sand, 5.03% of silt and 1.52% of clay. In this soil, the diazotrophic bacteria was also counted previously using NFb medium and Most Probable Number (MPN) tables, according to Baldani et al. (2014) and around 3.0×10^3 cells g^{-1} of soil was observed.

Pots with 8.0 L of capacity were filled with six kilograms of sieved soil. For the inoculant production, the *Azospirillum* isolates were cultivated in Dygs medium for 24 h at 30 °C and 150 rpm of agitation. Before inoculation, the seedlings were washed in running water and pruned (roots and stem) to obtain uniform height and volume. A 1.0 mL suspension containing 10^9 cells mL^{-1} was inoculated in the roots of seedlings of each selected forage species. The experiment was conducted in a completely randomized block design, with four repetitions and eight treatments; using one as control, without inoculation, and two *Azospirillum* type strains, Sp7 and Sp59, with other five treatments using different isolates, which were selected based on their total nitrogen and indole-3-acetic acid production.

Plant growth after 31 days of inoculation was evaluated according to plant height, shoot dry matter, total nitrogen, shoot protein content and root volume. For *H. amplexicauilis*, the height from the stem base to the taller apical bud was measured. For *M. chaseae and A. purpusii*, the height from the start of the stalk until the taller leaf were measured. The root volume was measured by immersing samples in containers of 300 mL capacity; the volume of water displacement was registered in cm^3. The leaves, roots and stems from each treatment/plant were packed into paper bags and dried at 70 °C for 72 h. Materials were then weighed to determine dry mass. The shoot protein content was calculated from the total nitrogen accumulated in the shoots, assessed by semi-micro Kjedahl method (Liao 1981). The variance analysis of each parameter and when the variance was significant, the Scott–Knott's test at 5% probability was performed aiming the averages comparison.

Results

Identification of isolates

Specific primers for *Azospirillum* were effective for preliminary identification of the isolates, but the isolates AZM8 and HAI1 did not present amplification. There was amplification for the type strains, Sp7 and Sp59, but not for bacteria from other genus and species used as negative controls, *Bradyrhizobium* sp., *Ralstonia* sp., *Burkholderia* sp., *Chryseobacterium indologenes*. Most of the isolates presented amplification products with the same size as of type strains. These results are consistent with morphological observations (data not shown). The sequencing of 16S rDNA and *nifH* genes corroborates with the results for specific primers and showed that most of the isolates are closely related with *A. brasilense* and *A. formosense* (Fig. 1a, b).

Evaluation of BNF and IAA production

Variation was observed for values of total N in culture, from 25.86 to 51.26 μg g^{-1} (Table 2). From the thirty analyzed isolates, MAY3 and MAY15 had higher values of total accumulated N, including when compared to with type strains (Table 2). All isolates were able to produce IAA, which varied from 107 to 1038 μg mL^{-1}. The higher values were observed for MAY1, MAY3 and MAY12 (Table 2). The isolate MAY25 did not grow in the selective culture medium; therefore, it was not evaluated for these two parameters. The ARA showed that all bacterial isolates presented the nitrogenase activity due to the presence of a single and clear ethylene peak in the chromatogram.

Inoculation effects of *Azospirillum* spp. on forage

Regarding the *M. chaseae* species, and analyzing the parameters separately, the results showed that, for plant height, MAY12 isolate was not statistically different when compared to MAY1, MAY3 and MAY8 isolates, which presented higher values than MAY11, Sp7, Sp59 and control. For root volume, isolates MAY1, MAY3, MAY12, and the type strain Sp7 presented no statistical differences, but they differ from control, isolates MAY8, MAY11, and the Sp59 strain. For shoot dry weight, isolate MAY12 was statistically different when compared to others (Fig. 2).

Considering all parameters together, plants of *M. chaesea* inoculated with MAY12 presented higher height, root volume and shoot dry weight when compared to control and the type strains. Inoculation in *A. purpusii* showed no significant results for plant height and root volume, but for shoot dry weight, MAY12 isolate presented significantly differences when compared to other treatments. In *H. amplexicaulis*, plant height was not different among treatments. For root volume, the treatments MAY1, MAY3 and MAY11 were better than other treatments, without statistical differences among them. For shoot dry weight, the isolates MAY1, MAY3, MAY12 and the type strain Sp7 presented the better results for this species. There was no significant effect of inoculation on the percentage of shoot protein content in the plants' aerial parts.

Fig. 1 Phylogenetic tree of 31 isolates from native forage from Pantanal using the maximum-likelihood method and Jukes-Cantor model, based on their *nif*H (**a**) and 16S rRNA (**b**) sequences. Numbers indicate the results of the bootstrap analysis with 1000 replicates (bootstrap values above 50% are not shown)

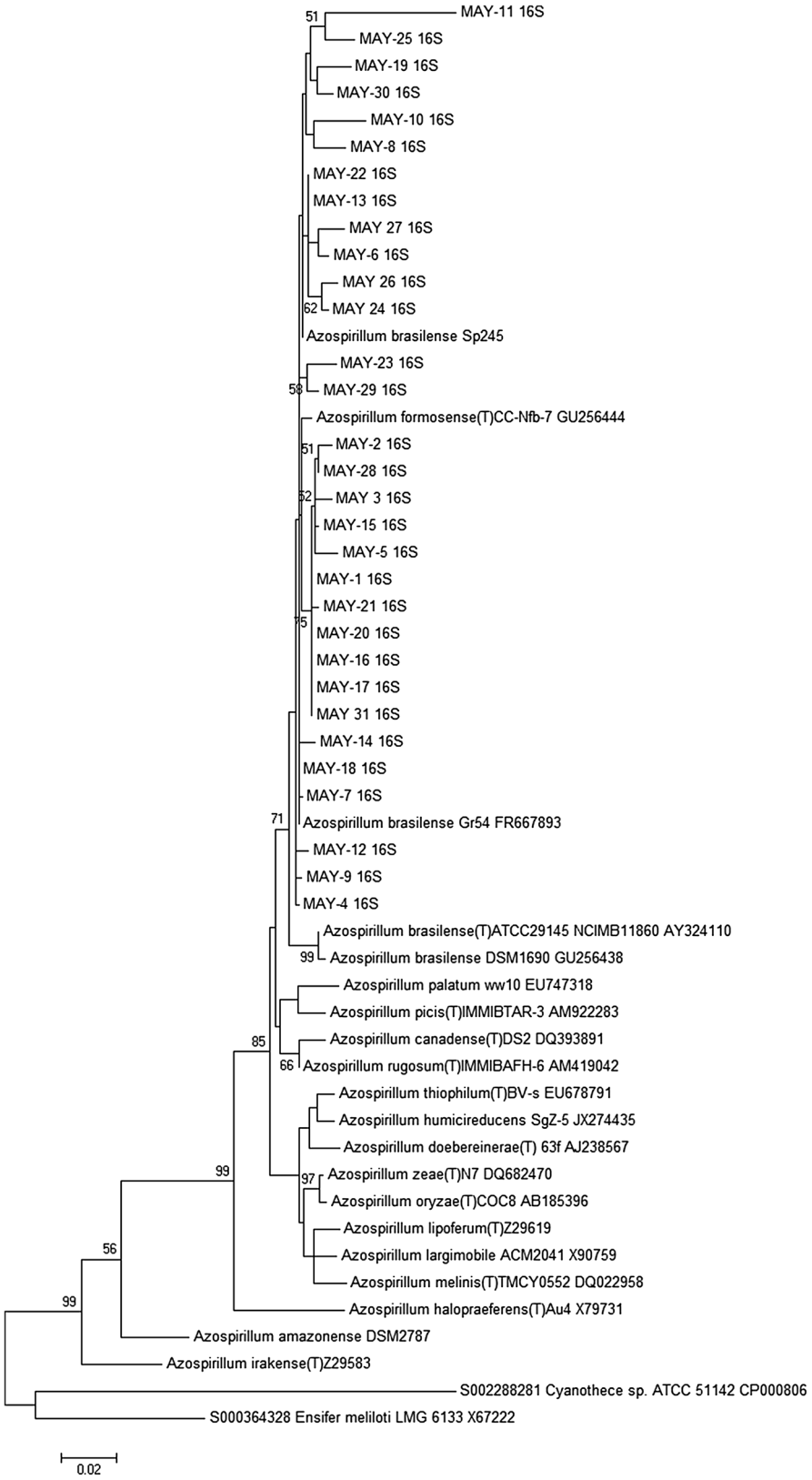

Fig. 1 (continued)

Table 2 Total nitrogen (N) and production of indole-3-acetic acid (IAA) from different diazotrophic isolates of *Azospirillum* spp.

Isolate	N[1] (μg mL^{-1})	IAA[2] (μg mL^{-1})
Sp7	**41.38 b**	**214.70 e**
Sp59	**44.53 b**	**141.72 e**
MAY1	**43.68 b**	**1038.61 a**
MAY2	36.62 c	384.24 d
MAY3	**51.26 a***	**963.27 a**
MAY4	36.15 c	381.80 d
MAY5	34.33 c	575.95 c
MAY6	36.10 c	232.25 e
MAY7	43.26 b	603.72 c
MAY8	**25.86 e**	**267.34 e**
MAY9	36.39 c	712.56 b
MAY10	48.14 a	414.51 d
MAY11	**37.50 c**	**451.63 d**
MAY12	**48.55 a**	**1027.68 a**
MAY13	34.62 c	281.70 e
MAY14	36.62 c	136.66 e
MAY15	50.78 a	181.19 e
MAY16	43.20 b	802.13 b
MAY17	46.67 a	501.65 d
MAY18	41.62 b	246.21 e
MAY19	31.33 d	631.51 c
MAY20	49.14 a	333.69 d
MAY21	37.03 c	107.02 e
MAY22	40.09 c	223.87 e
MAY23	34.92 c	296.06 e
MAY24	39.44 c	359.89 d
MAY26	42.56 b	397.76 d
MAY27	36.86 c	754.46 b
MAY28	45.85 b	151.65 e
MAY29	44.62 b	501.27 d
MAY30	38.62 c	311.46 e
MAY31	36.68 c	445.21 d
Mean	40.28	457.23
CV (%)	6.80	17.50

Type strains and isolates marked in bold were used in inoculation treatments

*Averages with the same letter in the same variable were not different by the Skott–Knott range mean test ($p < 0.05$)

[1]Semisolid NFb medium

[2]Liquid NFb medium supplemented with L-tryptophan

Discussion

Most of the isolates are closely related to *A. brasilense* and *A. formosense*, which was probably due to the culture medium used for isolation. Besides, it is known that these two species are related concerning the two genic sequences used. The isolates characterized in our study presented BNF and IAA production, showing a potential for plant growing experiments. Regarding the inoculation experiments, the isolate MAY12 showed the best results in terms of shoot dry weight for all forage species, especially for *M. chaseae* and *A. purpusii*. Therefore, MAY12 isolate seems to be very interesting for plant growth promotion experiments due to the increasing on shoot dry weight, indicating higher sequestered carbon and forage mass production, fact essential for livestock production.

Identification of *Azospirillum* spp.

Currently, an important step in classification of bacteria isolates is the use of molecular techniques and genetic information associated with phenotypic characteristics revealing details about the isolates and enabling polyphasic approaches (Reis et al. 2010). In our approach, after the use of the selective culture medium (for *A. brasilense* and *A. lipoferum*), the specific primers identified most of the isolates as *Azospirillum* spp. (91.2%). The use of specific primers simplifies the identification and detection of isolates inside rizosphere population and can be used for population ecology studies of a genus (Shime-Hatori et al. 2011). However, for species identification it is necessary to use different genetic techniques such as gene sequencing.

All isolates were closely related to *Azospirillum brasilense* or *A. formosense*, regarding 16S and *nifH* genes. However, each gene tree presented different topographies (Fig. 1a, b). According to the *A. formosense* description (Lin et al. 2012), the 16S and *nifH* genes in this species are highly similar to those of *A. brasilense*. Moreover, Liu et al. (2012) described that, despite the partial similarity of these genes, there is not a reasonable level of evolutionary synchronism between them, and *nifH* gene are usually used to define different genus. Besides, Gaby and Buckley (2014) found that due to the genetic divergence of *nifH* and 16S rRNA genes, they generally produce different results, for example the strains that have high similarity for 16S rRNA gene but moderate similarity for *nifH* gene. According to Wisniewski-Dyé et al. (2012), half of the *Azospirillum* genome comprises genes from horizontal transfer, including genes related to defense mechanisms, transport, adaptation, and amino acids metabolism, carbohydrates, inorganic ions and secondary metabolites. Also, the genus presented close phylogenetic characteristics when compared to bacteria adapted to aquatic environment, suggesting that the ancestor of these bacteria have already been adapted to these conditions (Wisniewski-Dyé et al. 2012). This can explain the observed divergences on the phylogenetic trees of the present study.

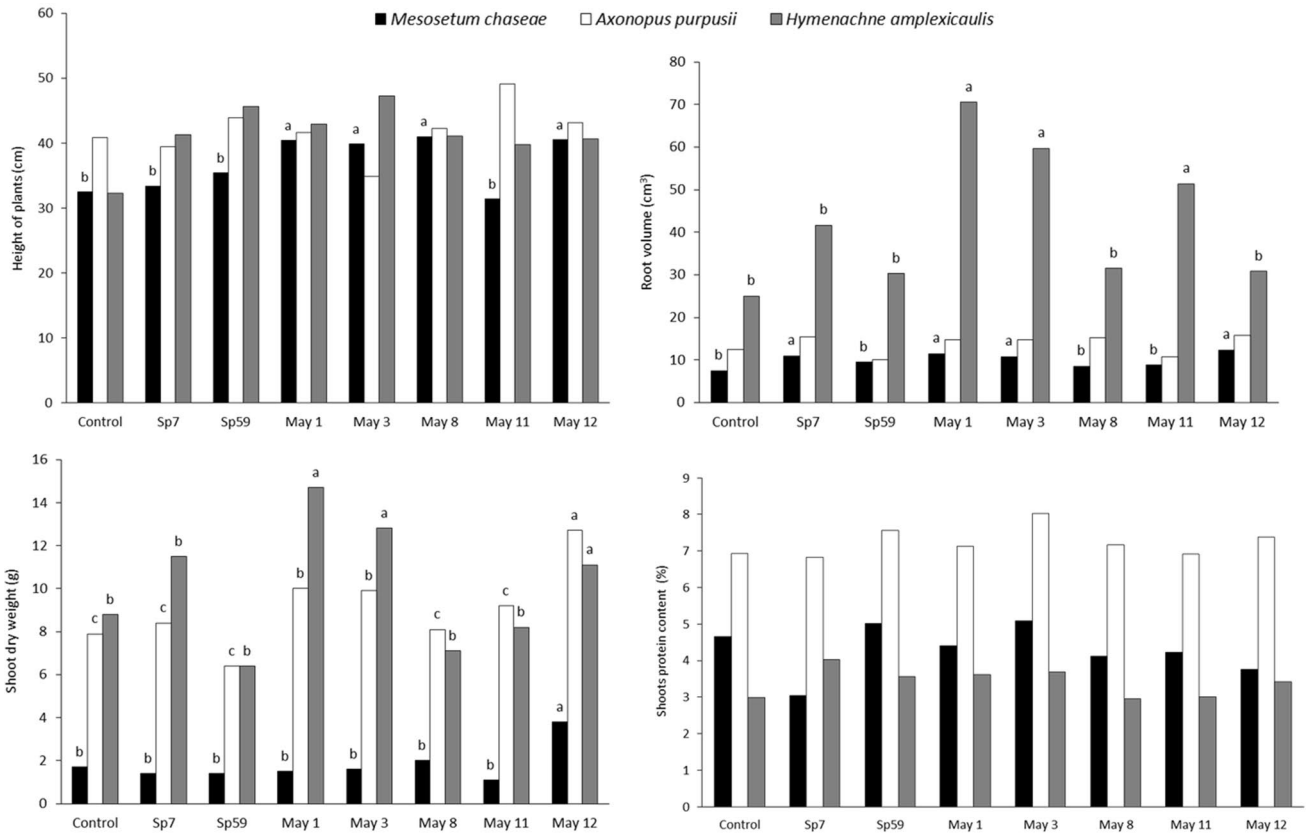

Fig. 2 Means for height, root volume, shoot dry weight and shoots protein content for each *Mesosetum chaseae, Axonopus purpusii* and *Hymenachne amplexicaulis* plant inoculated with five native *Azospirillum* sp. and two reference strains of *Azospirillum* (*A. brasilense* Sp7 and A. *lipoferum* Sp59). Means with the *same letter* in each bar, within the same plant species are not different according to the Scott–Knott mean range test ($p < 0.05$). *Bars without letters* were not statistically different

BNF and IAA production

Variation was observed for values of total N in culture medium, from 25.86 to 51.26 µg mL^{-1} (Table 2). Many studies have shown evidences of variations in the nitrogenase activity. Santos et al. (2013) analyzed 25 *Azospirillum* isolates (from forage) for BNF by acetylene reduction technique and observed results of 26–256 nmol C_2H_4 h^{-1}. Han and New (1998) observed for wheat isolates a variation from 0 to 154.9 nmol of C_2H_4 h^{-1}.

In the present study, among the 30 analyzed isolate, MAY3 and MAY15 presented higher values of N content in the culture medium (NFb medium) also compared with the strain types (Table 2). The values presented in our results are higher than other studies that used the same technique. Cardoso et al. (2010) reported values of N fixed by *Azospirillum* in NFb and LGI medium ranging from 5.18 to 22.8 µg mL^{-1}. Kuss et al. (2007) obtained total N values from 5.56 to 12.99 µg mL^{-1}, also in NFb medium for *Azospirillum* isolated from rice. However, the authors found for the type strains Sp7 and Sp59, values of BNF of 41.09 and 46.82 µg mL^{-1}, respectively, similar to the present study (Table 2). Moreover, Fernandes-Junior et al. (2015) observed values of total N in culture medium, from 3.5 to 42 µg mL^{-1}, and for *Azospirillum brasilense* strain Ab-V5, the BNF was 35.0 µg mL^{-1}. Kanimozhi and Panneerselvam (2010) found the maximum nitrogen fixing value of 15.6 µg mL^{-1} for *Azospirillum* isolated from Indian soil and for *A. brasilense* the values founded was only 3.3 µg mL^{-1}. Although, *Azospirillum* spp. have the ability to fix nitrogen, freely in the soil or in association with plant roots, the role of BNF in promoting plant growth have not been well elucidated and reported levels of nitrogen fixation provide little or no contribution to the plant's overall nitrogen demand (Pankievicz et al. 2015). However, field studies with wild grass species suggested that BNF can provide 30% or more of the plant's nitrogen demand (Boddey and Victoria 1986; Morais et al. 2012), attesting the promise of this approach.

On average, the concentration of IAA (mean = 457.23 µg mL^{-1}) produced by isolates was higher than that observed for type strains Sp7 and Sp59 (Table 2), and higher than the values reported by other studies for *Azospirillum*. For example, Radwan et al. (2005) observed for *Azospirillum* strains a production from 300 to 500 µM

of IAA, and Mascarua-Esparza et al. (1988) detected values from 205 to 428 µM for *A. brasilense* and 28.54–97.03 µM for *A. lipoferum*. Crozier et al. (1988), evaluating IAA production in the same species, reported values from 7.99 to 140.97 µM for *A. brasilense* and 0–85.9 µM for *A. lipoferum*. Reis Junior et al. (2004) showed that *A. amazonense*, associated with *Urocloa* spp., produced from 35 to 110 µM of IAA. Cardoso et al. (2010) found that *Azospirillum* strains isolated from rice produced from 5.67 to 119.72 µg IAA mL^{-1}. In a recent study conducted by Silva et al. (2013), isolates from *Urochloa brizanta* roots produced IAA from 0.39 to 195 µg IAA mL^{-1}. The type strain Sp7 used in the present study produced higher amounts of IAA than the strain Sp59 (Table 2), corroborating with results observed by Radwan et al. (2005). Is remarkably that our isolates produced higher amounts of IAA, from 107.02 to 1027.70 µg IAA mL^{-1} (Table 2). These results are important since the IAA production by diazotrophic bacteria may positively influence plant grow (Bashan et al. 2014; Yasmin et al. 2007; Vasconcellos et al. 2010).

The main effect of IAA is to promote root and shoot growing, throughout cell division and differentiation on meristematic tissues, which depends directly on hormone concentration. Also, according to Pedraza and Diaz Ricci (2012), the quantity of IAA produced by bacteria strains depends on several factors, for example, the bacteria species and the culture medium conditions (presence or absence of the L-tryptophan precursor, oxidation, pH and bacterial growth stage), that can influence the production of the vegetal hormone. The isolates of our study are from native forage from different elevation gradient in Pantanal, where the soil presents low fertility under constant hydric stress due to full river system from dry soil to flooded soil (Brasil et al. 2016). These conditions may favor genetic adaptation of the isolates, which had high capacity of in vitro production of IAA. According to Ona et al. (2005), IAA is produced under stress conditions, such as low carbon and oxygen concentration, developing different mechanism for adaptation in these bacteria (Scholoter et al. 2000).

Plant growth promotion by *Azospirillum* sp. inoculation

According to the presented results, no differences in shoot protein content considering all inoculation treatments (Fig. 2) was observed. This result can be due to the short time of inoculation that shoots protein content was evaluated: 30 days; Brasil et al. (2005) reported significant levels of N content after 90 days of inoculation. Likewise, according to Pankievicz et al. (2015), the level of nitrogen fixation would provide little or no contribution to the plant's overall nitrogen demand. Regarding plant growth, an increasing between 25 and 60% (data not shown) in shoot dry weight was observed, for isolates MAY1, MAY3 and MAY12 (Fig. 2). These results corroborate with data from Lana et al. (2012), which showed that *Azospirillum brasilense* inoculation in maize, in absence of nitrogen fertilization, provided an increase of 7.2% in production of aerial part dry biomass. Ramos et al. (2010) observed an increase of 48% in aerial part dry matter production and 27% in dry matter production of roots in plants inoculated with *Azospirillum*, compared to control. Brasil et al. (2005), studying native forage species from Pantanal, also found higher production (between 45%) of shoot dry weight in treatments with *Azospirillum* inoculation.

Forage species *H. amplexicaulis* and *M. chaseae* presented positive responses to inoculation in relation to root volume, ranging from 50% to more than 100% of increase, when inoculated with MAY1, MAY3 and MAY11. For *M. chaseae* the percentage of increase in root volume was approximately 64%, indicating that IAA produced by inoculated bacteria induces root development. Reis Junior et al. (2008) observed increased production of dry root (14%) in the treatment inoculated with *A. amazonense* compared with the non-inoculated control in two maize genotypes. Quadros (2009) also obtained larger root volume in treatments with maize inoculation. Similar results were obtained by Didonet et al. (2003) in ten rice genotypes under inoculation with *Azospirillum spp*. These authors reported an increase in root length when compared to inoculated plants with the control treatment, and showed that *A. brasilense* induced higher responses in the development of seedlings than *A. lipoferum*. According to Hungria et al. (2010), the root volume may indicate whether there has been an increase in its growth. The root development increases the area for water and nutrients absorption, consequently increasing plant production capacity and contributing to environmental stresses tolerance, such as salinity, flooding and drought.

Effects of inoculation on plant height were only observed for *M. chaseae*. Similar results were found by Guimarães et al. (2011) in a study with *U. brizantha* inoculated with nine *Azospirillum* isolates, where higher plants were obtained using AZ02, when compared to control. Ramos et al. (2010) evaluated the growth of corn plants inoculated with *Azospirillum lipoferum* (BR strain 11084), 30 days after sowing, they observed an increase in plant height. However, the results found by Cavallet et al. (2000) using the commercial product "Graminante", based on *Azospirillum brasilense*, showed no effects of inoculation on corn plants height.

In terms of protein content, the results of the present study were similar to those reported by Ficagna and Gai (2012) for Tifon 85 forage samples inoculated with *A. brasilense* at 20, 40 and 60 days. Those authors reported that protein content did not vary significantly among inoculated treatments when compared with the nitrogen

fertilizer treatment. In these cases, the use of bacteria can represent an alternative to nitrogen fertilizers. In another study, the inoculation of *A. brasilense* in seeds of *U. brizantha* resulted in an increase of 5% in mass production (Silva et al. 2013). However, there was no effect of inoculation on nitrogen content or protein accumulation, similar to the presented results. Although the isolates used in the inoculation experiments did not provide an increase in N and protein content, the contribution to plant growth was clear, especially for isolates MAY1, MAY3 and MAY12, which may be due to high IAA production. Therefore, these three strains can be considered to have potential for field experiments designed to evaluate the enhancement in plant growth.

The selection of microorganisms with features that increase plant growth is one way to improve high-yield pastures and ecosystem sustainability (Roesch et al. 2007). In this context, the selection of strains with high BNF and IAA production, which are adapted to local conditions, is essential to ensure the growth increment on native forage associated with bacteria.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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