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Stylosanthes spp. from Amazon savanna harbour diverse and potentially effective rhizobia

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ABSTRACT

The *Stylosanthes* genus stands out among the forage legume species well-adapted to the soil and climatic conditions of tropical regions. This legume establishes a symbiotic relationship with nitrogen-fixing bacteria, which can be used to improve the quality of pastures. However, there are few studies about the diversity and symbiotic efficiency of nitrogen-fixing bacteria in *Stylosanthes* plants. Therefore, the objective was to isolate, characterize and evaluate the symbiotic efficiency of nitrogen-fixing bacteria in symbiosis with *Stylosanthes* in savanna areas in Roraima, Brazil. Plants with nodules were collected at 6 sites, including cultivated and native areas of the savanna. Shoots of *Stylosanthes* with flowers were collected to prepare exsiccatae for species identification. The nodules were surface-sterilized with 5% sodium hypochlorite after the roots were washed, and isolation and morphological characterization were performed in 79 culture medium. The isolates were selected according to their morphology, the representativeness of each collection site and the associated *Stylosanthes* species. Representative isolates were tested for nodulation in cowpea plants, and the isolates able to nodulate the gene 16S rRNA was sequenced. The symbiotic efficiency was evaluated in *Stylosanthes capitata* cv. Lavradeiro in greenhouse conditions for 65 days. A total of 504 nodules were collected, from which 258 pure isolates were obtained from 5 species of *Stylosanthes* (*S. capitata*,*S. scabra*, *S. gracilis*, *S. humilis* and *S. angustifolia*). The isolates showed significant phenotypic diversity, and 89 isolates were selected. Thirty-one isolates were confirmed to be nodulating bacteria in cowpea plants. These isolates were identified as α-Proteobacteria, with *Bradyrhizobium*, *Rhizobium* and *Mesorhizobium* genera, and β-Proteobacteria, with *Burkholderia* and *Herbaspirillum* genera. When evaluated in *S. capitata*, treatment with two *Bradyrhizobium* isolates, ERR 1178 and ERR 942, resulted in significant production of plant biomass production and N accumulation. These results indicated that a large diversity of α-Proteobacteria and β-Proteobacteria are able to nodulate *Stylosanthes* spp. and some isolates have potential for testing under field conditions.

1. Introduction

The *Stylosanthes* genus includes approximately 50 species and subspecies native to the tropical and subtropical areas of Asia, Africa and the Americas, particularly South America (Quecini et al., 2002), including annual, biennial and perennial herbs and subshrubs (Lewis et al., 2005; Calles and Schultze-Kraft, 2010a). *Stylosanthes* has a wide distribution in Brazil, and about 30 species occur in the country (Flora do Brasil, 2016). These legumes are able to fix high levels of nitrogen in symbiosis with nitrogen-fixing bacteria (rhizobia), and several species are acidic and low-fertility soils tolerant (Schultze-Kraft and Giacometti, 1978; Calles and Schultze-Kraft, 2010b). Studies about symbiotic efficiency conducted in Australia with bacteria obtained from Brazil, Venezuela, Colombia and Australia reported the predominance of symbiotic bacteria from the *Bradyrhizobium* genus (Date, 2010; Date and Eagles, 2010).

The Amazon region contains a great diversity of plant species, and a wide variety of nitrogen-fixing bacteria have also been documented (Lima et al., 2005; Lima et al., 2009; Guimarães et al.,

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2012; Da Silva et al., 2014). Roraima, which belongs to the Legal Amazon states in Brazil, has different biomes. These biomes include a savanna region with climate and vegetation characteristics very different from those of other areas in the Amazon region. These savanna areas are locally known as “lavrado” and they are similar to the Cerrado in Central Brazil but with peculiar soil and vegetation characteristics. Low soil fertility and low carbon levels are common in these savanna regions due to low primary plant production, especially grasses, which are subject to constant fires associated with intense laminar soil erosion (Melo et al., 2003). Seven species in the *Stylosanthes* genus have been recorded in the Roraima savanna areas: *S. capitata*, *S. angustifolia*, *S. gracilis*, *S. viscosa*, *S. humilis*, *S. guianensis* and *S. scabra* (Medeiros and Flores, 2014).

In Brazil, two bacterial strains are currently recommended by the Ministry of Agriculture, Livestock and Food Supply (Ministério da Agricultura Pecuária e Abastecimento, MAPA) for inoculation of *Stylosanthes*. Both of these strains, BR 446 and BR 502, belong to the *Bradyrhizobium* genus (Brazil, 2011; Delamuta et al., 2016). However, there is a lack of published information about the symbiotic efficiency of BR 446 and BR 502 (Santos et al., 2007; Nunes, 2013).

Despite the widespread occurrence of *Stylosanthes* species in Brazil, little is known about the diversity of nitrogen-fixing bacteria associated with the root nodules of this genus, and the symbiotic efficiency remains unrevealed. Only a few studies about their efficiency exist, but they were carried out in Australia (Date, 2010; Date and Eagles, 2010). Thus, searching for and evaluating new bacterial strains is necessary because of the high diversity of species within the *Stylosanthes* genus in Brazil.

The objective of this work was to isolate, characterize and evaluate the symbiotic efficiency of nitrogen-fixing bacteria in symbiosis with *Stylosanthes* in savanna areas in Roraima, Brazil.

2. Material and methods

2.1. Origin of isolates

Forty-five isolates were previously isolated from Roraima savanna soil samples using *Stylosanthes* as a trap plant. The isolates were deposited in the culture collection at Embrapa Roraima, albeit without information concerning the collection site. The new collections for this study were performed at six different sites distributed in the municipalities of Boa Vista and Bonfim. The collections at Boa Vista were performed at the Água Boa Experimental Field (Campo Experimental Água Boa) of Embrapa Roraima. This region contained crop-livestock-forestry integration areas, planted pasture and native savanna. In contrast, the collections at Bonfim were performed at a private farm nearly to agricultural crops (Table 1). The soils were classified as Yellow latosols (Hapludox) (Soil Survey Staff, 2010).

2.2. Collection and identification of Stylosanthes **in the Roraima savanna areas**

Shoots of *Stylosanthes* plants with flowers were collected to prepare exsiccatae. Ten plants were collected at each collection site. The plants were then deposited in the Museu Integrado de Roraima (MIRR) for botanical identification as described by Medeiros and Flores (2014).

Roots were collected using a straight shovel at a depth of 30 cm and a distance of 20 cm from the plant stem. These samples were placed in plastic bags with damp paper and taken to the Soil Microbiology Laboratory of Embrapa Roraima. There, the roots were washed before nodulation observation, nodule collection and bacterial isolation.

Ten simple soil samples were collected from each site at a depth of 0 – 20 cm. Samples were collected 5 m equidistant from one another to form a composite sample. Chemical and particle size analysis was performed according to the methods described by Embrapa (1997).

2.3. Isolation and purification of bacteria from the nodules of Stylosanthes

The roots of plants used for the collection of nodules were washed to remove excess soil. This washing was followed by the manual removal of nodules for the isolation of bacteria. Then, the nodules were surface disinfected using 70% alcohol for 30 s, 5% sodium hypochlorite for 5 min and 10 successive washes in sterile

Table 1
Origin, chemistry and particle size analysis of soil samples and the identification of isolates obtained from *Stylosanthes* of Roraima savanna areas in Brazil.

Municipality/Location	Site characteristics	Identification	Coordinates	Chemical characteristics							Particle size			Species	Isolates obtained No.
				pH H_2O	Ca	Mg	K	Al	P	OM[a]	Clay	Silt	Sand		
					($cmol_c\,dm^{-3}$)				($mg\,dm^{-3}$)	(%)	($g\,kg^{-1}$)				
Boa Vista/Água Boa Experimental Field of Embrapa Roraima (CEAB)	Crop-livestock-forestry integration – CLF	P1	N02°39′49.8″ W60°50′55.8″	5.4	0.32	0.10	0.03	0.25	4.42	1.08	173	16.5	810	*S. capitata*	99
														S. scabra *S. gracilis*	
	Planted pasture	P2	N02°39′53.0″ W60°50′37.1″	4.8	0.26	0.16	0.03	0.4	3.13	–	–	–	–	*S. capitata*	16
	Native savanna	P3	N 02°40′10.7″ W60°50′55.8″	4.1	0.3	0.2	0.01	0.8	3.00	1.10	200	40	760	*S. gracilis*	37
Bonfim/Private farm	Near soybean crop	P4	N03°17′20.5″ W60°20′45.6″	6.8	0.81	0.24	0.14	0.01	11.54	2.61	124	68	808	*S. humilis*	32
	Near soybean crop	P5	N03°17′32.8″ W60°21′26.7″	5.0	0.39	0.12	0.04	0.24	15.02	4.96	109	148	743	*S. angustifolia*	35
	Rice field in fallow	P6	N03°16′03.8″ W60°16′18.5″	5.4	0.24	0.07	0.10	0.17	22.84	9.72	111	127	762	*S. angustifolia*	39

[a] Organic matter.