DIAZOTROPHIC BACTERIA IN STAR FLOWERS

BACTÉRIAS DIAZOTRÓFICAS EM SEMPRE-VIVA

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ABSTRACT: This study evaluated the occurrence and density of associative diazotrophic bacteria in the rhizosphere, collar, roots and leaves of *Syngonanthus elegans* (Bong) Ruhland (Goldenfoot) and *Syngonanthus aciphyllus* (Mini-skirt). These species are able to grow in poor soils. Five samples, with 25 plants both of *S. elegans* and *S. aciphyllus,* each were collected in Soberbo stream, Diamantina-MG, Brazil. The occurrence and the density of diazotrophic bacteria were evaluated by the most probable number (MPN) method in semi-selective N-free media NFb, LGI, JNFb, and JMV. In general, for both species, a high density of diazotrophic bacteria was observed in all of the microenvironments evaluated. The density was greater in *S. elegans* than in *S. aciphyllus.* The associative occurrence of diazotrophic bacteria in *S. elegans* and *S. aciphyllus* can contribute to plant adaptation to low fertility soils.

KEYWORDS: *Syngonanthus elegans. Syngonathus aciphyllus.* Associative diazotrophic bactéria, Biological nitrogen fixation.

INTRODUCTION

The municipality of Diamantina, State of Minas Gerais, Brazil, is on the southern strip of the Cordilheira do Espinhaço Meridional, extending from Minas Gerais state to the North of Bahia. It is a region with great diversity of peculiar and contrasting soils and special climatic conditions. Different vegetation forms coexist, representing a mosaic of different plant communities. One of these plant communities is "campos rupestres" where Eriocaulaceae, plants characterized by herbaceous growth and flower-head type inflorescence, can be observed (GIULIETTI; HENSOLD, 1990). Some of them, such as those of the genus *Syngonanthus,* are commonly called star flowers. It is a generic name given to the flowers which after being collected, retain their color and form for a long time, giving them high commercial value.

The production area of star flowers in Minas Gerais state is concentrated in Diamantina region with approximately 57.26 hectares (LANDGRAF; PAIVA, 2009). Great part of this production is being exported to Europe, Asia and North America.

In Brazil, the floriculture sector is one of the best alternatives for those who are looking for agricultural investment. However, it has to be conducted sustainably, avoiding species extinction. This is not observed when dealing with star flowers because their exploitation is done indiscriminately. Inflorescences are removed before seed ripening, without any type of control or cultivation (MENEZES; GIULIETTI, 2000). As a result, several species of star flowers appear in Minas Gerais official endangered species list (COPAM, 1997).

Most species from the Eriocaulaceae family are found in sandy and, or, stony soils, with acid pH, remaining dry for several months of the year (GIULIETTI; HENSOLD, 1990). However, they are subjected to flooding in the rainy period. It is known that some species of this family present xeromorphic adaptations, such as: thick-walled epidermal cells, hypodermis, aquiferous parenchyma, compact mesophyll (SCATENA et al., 2004). These characteristics help them survive in adverse environments. The soils where such plants occur are poor in nutrients because they originate from metarenite rock, where quartz thoroughly prevails. Also, vegetable coverage, that could maintain a thick layer of organic matter in the soil is lacking, thus contributing to low fertility. These conditions are unfavorable and inappropriate for the commercial cultivation of most of domesticated plants, since they demand soil with better fertility. As a result, the capacity of star flowers to develop in these soils can be related to strict mutualistic relationships with local microorganism communities, with peculiar characteristics and well adapted to the environment.

Several important groups of the edaphic microbial community in the rhizosphere, and endophytes play a fundamental role in plant development. These microorganisms promote the

mineralization of organic matter (ALEXANDER, 1961), solubilize sources of insoluble inorganic phosphates (SPERBER, 1957; MARRA et al., 2011), compete with plant pathogens for space and nutrients, thus controlling diseases, act as bioremediators for herbicides (PEREYRA et al., 2009) and heavy metals (MOREIRA et al., 2008) in polluted soils, accelerate and increase seed germination (KUMAR et al., 1999). Among these microorganisms should be pointed out the group of diazotrophic bacteria, which contribute to the biological nitrogen fixation.

Based on these advantages, it would be interesting to gain knowledge about the occurrence of associative diazotrophic bacteria in star flowers in order to better understand how these plants survive and develop in low fertility soils.

Several studies have been reported on *Syngonanthus* spp. with different foci, such as: morphology and anatomy (SCATENA et al., 2004), germination and pollination (NUNES et al., 2008; ORIANE, et al., 2009), pharmaceutics (BATISTA et al., 2004); population ecology (SCHMIDT et al., 2007) and symbiosis with arbuscular mycorrhizal fungi (BORBA; AMORIM, 2007). However, there are no reports about the occurrence of associative diazotrophic bacteria in *Syngonanthus* spp.

Considering the chemical attributes of the soils where star flowers occur and the absence of information about nitrogen-fixing bacteria occurrence associated with this important group of plants, this work was conducted to evaluated the occurrence and density of associative diazotrophic bacteria in star flowers plants (*Syngonanthus* spp.).

MATERIALS AND METHODS

The occurrence and density of nitrogen fixing bacteria in *Syngonanthus elegans* (Bong.) Ruhland and *Syngonanthus aciphyllus* (Bong.) Ruhland star flowers were evaluated in naturally occurring plants collected in a 100 x 100 meter area. It is near Soberbo stream at Campus II of Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), in Diamantina – MG, Brazil. The coordinates are: latitude 18º12´21" S and longitude 43º33´49" W. Ectorhizosphere bacteria, collar bacteria, and endophytic bacteria of the leaves and the roots were evaluated.

Five samples, with 25 plants of *S. elegans and S. aciphyllus*, each, were collected. The plants were collected with the root system and the soil up to 15 cm depth. The samples were stored in plastic bags properly identified and transported in thermal boxes to the Soil Microbiology laboratory at UFVJM. Ten soil sub-samples were collected in order to obtain a compound by sample, to analyze soil chemical attributes.

The plant samples were separated into leaves, collar and roots in the laboratory. The roots were separated from the soil by agitation. Subsequently, roots and leaves were cut into 1 to 2 cm fragments. Plant tissues were processed differently according to the plant microenvironment. Rhizosphere diazotrophic bacteria were evaluated in unwashed 1g samples of root fragments, while diazotrophic bacteria from the collar were evaluated in 10 g samples. Endophytic diazotrophic bacteria evaluation was done in 10 g samples of leaves and 1 g samples of cut and previously washed and superficially disinfected roots. Root sterilization was accomplished in several steps. First, they were put in contact with a 1% solution of chloramine-T ($C_7H_7ClNNaO_2.3H_2O$) for 7 minutes, and then immersed in distilled sterile water for 5 minutes. Finally they were transferred to a 0.05 mol L^{-1} (pH 6.8) phosphate buffer solution for removal of chloramine-T residues and, finally, rinsed five times in distilled sterile water. Time spent in surface sterilization with chloramine-T was determined through preliminary tests. Aliquots of water used in the last rinse were inoculated on Potato Dextrose Agar to evaluate disinfection efficiency.

All of the samples were processed in the following way: they were agitated for 20 minutes at 250 rpm in a 125 mL Erlenmeyer containing 5 g of 5 mm-diameter glass beads and 9 mL of sterile saline solution (0.85% NaCl), for the root samples, or 90 mL for leaves and collars (Sala et al., 2005). After this period, serial dilutions of the samples were done in saline solution (0.85% NaCl), until the dilution 10^{-7}.

For the two species, bacterial density was evaluated by inoculating 0.1 mL of each dilution, in five flasks of each semi-solid media with the following composition: NFb (malic acid 5.0 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2.2H_2O$ 0.01 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, $MnSO_4.H_2O$ 0.00235 g L^{-1}, H_3BO_3 0.0028 g L^{-1}, $CuSO_4.5H_2O$ 8.0 x 10^{-5} g L^{-1}), bromothymol blue (0.5% solution in 0.2 M NAOH) 2 mL, FeEDTA (solution 1.64%) 4 mL, at pH 6.8 (DÖBEREINER et al., 1995), known to favor growth of *Azospirillum lipoferum* and *A. brasilense,* LGI (sacarose 5.0 g L^{-1}, K_2HPO_4 0.2 g L^{-1}, KH_2PO_4 0.6 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, $CaCl_2.2H_2O$ 0.02 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, KOH 4.5 g L^{-1}, bromothymol blue (0.5% solution in 0.2 M NAOH) 2 mL, FeEDTA (solution 1.64%) 4 mL, biotin 1.0 x 10^{-4} g L^{-1} and HCl-pyridoxine 2.0 x 10^{-4} g L^{-1}, pH between 6.0 and

6.2) (DÖBEREINER et al., 1995), known to favor growth of *Azospirillum amazonense*, and JNFb (malic acid 5.0 g L^{-1}, K_2HPO_4 0.6 g L^{-1}, KH_2PO_4 1.8 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, KOH 4.5 g L^{-1}, $CaCl_2.2H_2O$ 0.02 g L^{-1}, $Na_2MoO_4.2H_2O$ 0.002 g L^{-1}, $MnSO_4.H_2O$ 0.00235 g L^{-1}, H_3BO_3 0.0028 g L^{-1}, $CuSO_4.5H_2O$ 8.0 x 10^{-5} g L^{-1}, $ZnSO_4.7H_2O$ 0.002 g L^{-1}, bromothymol blue (0.5% solution in 0.2 M NAOH) 2 mL, FeEDTA (solution 1.64%) 4 mL, biotin 1.0 x 10^{-4} g L^{-1} and HCl-pyridoxine 2.0 x 10^{-4} g L^{-1}, pH 5.8) (BALDANI et al., 1986), known to favor growth of *Herbaspirillum* spp. For *S. aciphyllus,* bacterial occurrence and density in the semi-solid JMV (mannitol 5.0, K_2HPO_4 0.6, KH_2PO_4 1.8, MgSO4.$7H_2O$ 0.2, NaCl 0.1, $CaCl_2.2H_2O$ 0.02, $Na_2MoO_4.2H_2O$ 0.002, $MnSO_4.H_2O$ 0.00235, H_3BO_3 0.0028, $CuSO_4.5H_2O$ 8.0 x 10^{-5}, $ZnSO_4.7H_2O$ 0.002, bromothymol blue (0.5% solution in 0.2 M NAOH) 2 mL, FeEDTA (solution 1.64%) 4 mL, biotin 1.0 x 10^{-4} and HCl-pyridoxine 2.0 x 10^{-4}, pH between 4.2 and 4.5) (BALDANI et al., 1996), known to favor growth of *Burkholderia* spp., was also evaluated. These flasks were incubated for 7 days at 28 ºC. The ones that showed presence of associative diazotrophic bacteria were considered positive (DÖBEREINER et al., 1995). The most probable number (MPN) of diazotrophic bacteria per gram of fresh tissue was estimated by McCrady´s table (McCRADY, 1915).

The NFb, JNFb and LGI culture data were analyzed in a 2 x 4 factorial design, considering two plant species and four plants microenvironments. JMV culture medium was used just for *S. aciphyllus*. The experimental design was completely randomized, with five replicates. The experimental unit was composed of 25 plants. The cell number estimated by the MPN method did not follow a normal distribution, thus, they were transformed in ln (x + 2) and submitted to analysis of variance, and the averages compared by the Tukey test, at 5% probability, using the software Sisvar 5.3 (FERREIRA et al., 2008).

RESULTS AND DISCUSSION

The soil with *Syngonanthus* spp. is generally poor in nutrients and organic matter (Table 1), thus demonstrating that plants need to develop a strategy to survive under these conditions. Diazotrophic bacteria were detected by all media in all samples.

Table 1. Soil characteristics in the collection area of star flowers *Syngonanthus elegans* and *S. aciphyllus* at the Soberbo stream in Diamantina MG – Brazil.

Property	Value	Interpretation class[a]
pH (H_2O)	5.0	Low
P (mg dm^{-3})	1.9	Very low
K ($cmol_c$ dm^{-3})	0.01538	Very low
Ca ($cmol_c$ dm^{-3})	0.4	Very low
Mg ($cmol_c$ dm^{-3})	0.1	Very low
Al ($cmol_c$ dm^{-3})	0.9	Medium
V(%)	27	Low
OM (%)	0.5	Very low
Sand (%)	91	-
Silt (%)	3	-
Clay (%)	6	-

V%= index of base saturation, OM= organic matter, [a]RIBEIRO et al. (1999).

The density, estimated in NFb medium, known to favor growth of *Azospirillum* spp., observed for *S. elegans,* varied from 7.80 x 10^3 to 4.98 x 10^4 cells per gram of plant tissue and for *S. aciphyllus* from 3.60 x 10^3 to 4.50 x 10^4 cells per gram of plant tissue (Table 2). Its presence was detected in all microenvironments in both plant species. The highest density was detected on *S. elegans* collar surface (4.98 x 10^4) and on the rhizosphere of *S. aciphyllus* (4.50 x 10^4), while inside the leaves and roots the lowest densities were detected, independent of the species. These results are in agreement with those reported in the literature where these bacteria are considered rhizospheric, mainly colonizing the elongation areas and the root hairs; they are able to survive in the soil in the form of cysts, but also occur inside the plants, colonizing plant tissues (BALDANI et al., 1997). The endophytic occurrence of *Azospirillum* spp. was also observed in banana and pineapple (WEBER et al.,

1999), sugarcane (REIS-JUNIOR et al., 2000) and in grasses such as carona grass (*Elyonurus muticus*), mimoso grass (*Axonopus purpusii*) and in brachiaria (*Brachiaria humidicola*) (BRASIL et al., 2005).

Table 2. Density of diazotrophic bacteria detected by semi-selective NFb, LGI and JNFb media in different microenvironments of star flowers *Syngonanthus elegans* and *S. aciphyllus*, as estimated by the most probable number.

Plant Species	Rhizospheric		Endophytic	
	Roots	Collar	Leaf	Roots
	MPN x 10^4 g^{-1} of plant tissue			
	NFb[1]			
Syngonathus elegans	2.72 Bb[2]	4.98 Aa	0.67 Ac	0.78 Ac
Syngonathus aciphyllus	4.50 Aa	1.18 Bb	0.36 Ac	0.44 Ac
	LGI[1]			
S. elegans	4.08 Aa	3.00 Ab	0.65 Ac	0.59 Ac
S. aciphyllus	1.28 Ba	1.22 Ba	0.38 Ab	0.28 Ab
	JNFb[1]			
S. elegans	1.42 Bc	3.34 Aa	2.49 Ab	1.18 Ac
S. aciphyllus	2.40 Aa	0.96 Bc	1.66 Bb	0.17 Bd

[1]Culture medium [2]Averages followed by distinct uppercase letters in column and lowercase letters in the line differ by the Tukey test at 5% probability. For statistical analysis data were transformed by ln (x + 2).

The highest density, estimated in LGI medium, known to favor growth of *A. amazonense*, was found in the rhizosphere and in the collar surface of *S. elegans,* varying from 3.00 x 10^4 to 4.08 x 10^4 cells per gram of plant tissue, in comparison with *S. aciphyllus,* which was, on average, 1.25 x 10^4 cells per gram of plant tissue (Table 2). In spite of its presence being detected in all microenvironments evaluated, in both plant species, the lowest densities were detected inside the leaves and roots, independent of the species. These bacteria are found in the ectorhizosphere and the endorhizosphere of grasses (REIS-JUNIOR et al., 2000; BRASIL et al., 2005; MOREIRA et al., 2008) and in species of the Orchidaceae family (LANGE; MOREIRA, 2002).

The density, estimated in JNFb medium, known to favor growth of *Herbaspirillum* spp., found in *S. elegans* as endophytes, varied from 1.18 x 10^4 to 2.49 x 10^4 cells per gram of plant tissue and in *S. aciphyllus* from 0.17 x 10^4 to 1.66 x 10^4 cells per gram of plant tissue (Table 2). It should be highlighted that the highest density found inside the plants occurred in this medium, since this genus is considered an endophyte (OLIVARES et al., 1996). SILVA et al (2003) and OLIVEIRA et al (2009) confirmed the presence of *Herbaspirillum seropedicaea* in sugarcane roots using electron microscopy.

Although DÖBEREINER and DAY (1994) proposed that grasses were the preferential hosts of diazotrophic bacteria, the star flowers studied in this work showed high endophytic diazotrophic bacteria density. It is even higher than that observed by MELLONI et al. (2004) in ryegrass and *Eupathorium* sp. and equivalent to or higher than that observed by REIS-JÚNIOR et al. (2000) in sugarcane.

The highest densities in *S. aciphyllus*, estimated in JMV medium, known to favor growth *Burkholderia* spp., were observed in the rhizosphere, on the collar surface and endophytically in the roots (Figure 1). The diazotrophic capacity of these bacteria was discovered recently (GILLIS et al., 1995) and further studies are ongoing. Some species of nitrogen fixing *Burkholderia* have been described as associative, some as symbiotic, some as severe human pathogens and some as plant growth promoters. It reveals a surprising phenotypic diversity of that genus in the interaction with plants, animals and microorganisms (MOREIRA et al., 2008).

The total density of diazotrophic bacteria found in *S. elegans* (25.90 x 10^4 cells per gram of plant tissue) was greater than that found in *S. aciphyllus* (16.83 x 10^4 cells per gram of plant tissue) (Table 2). However, the total percentage of the bacterial density characterized by the different semi-selective followed the same order for the two plant species: greater density in NFb, followed by JNFb and LGI and even for *S. aciphyllus* a lower density in JMV (Figure 2). Variation in the existent density of diazotrophic bacteria in the same species

and, or in the same genus, in different microenvironments was also observed in cassava, sugarcane, wheat and other plant species (MAGALHÃES; DÖBEREINER, 1984; WEBER et al., 1999; REIS-JUNIOR et al., 2000).

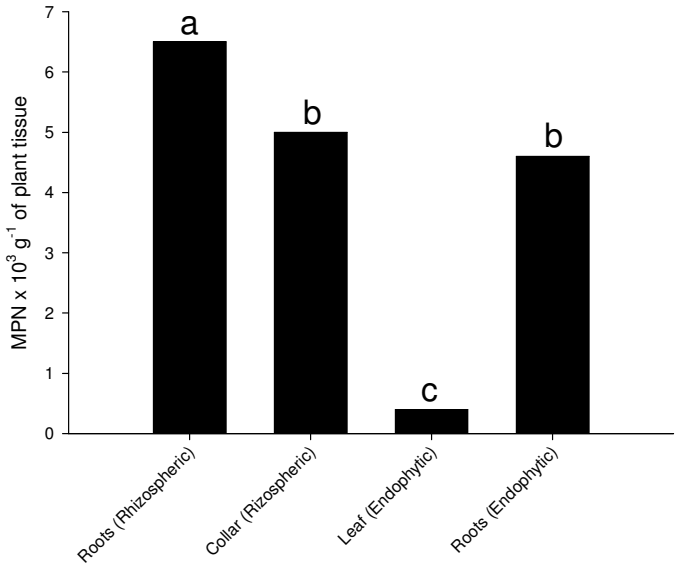

Figure 1. Density of *Burkholderia* spp., associated to different microenvironments in *Syngonanthus aciphyllus*, calculated by most probable number in semi-selective JMV medium. Columns denoted by different letters differ significantly at 5% by the Tukey test. For statistical analysis data were transformed to ln (x + 2).

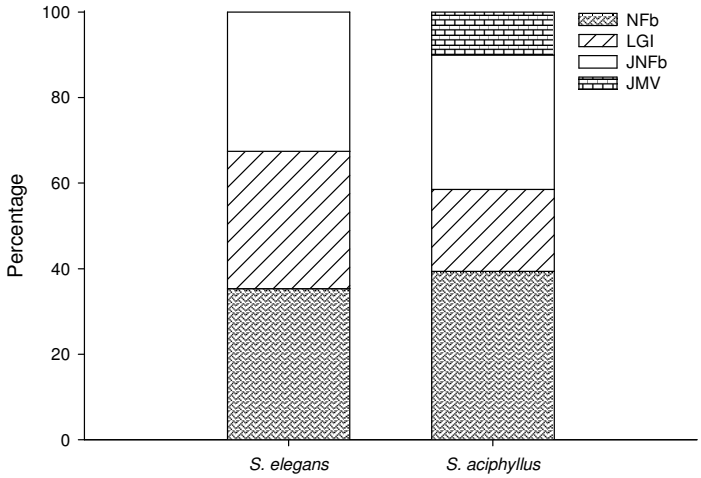

Figure 2. Total percentage of the diazotrophic bacteria density characterized by the semi-selective NFb, LGI, JNFb and JMV medium, obtained from the plant species *S. elegans* and *S. aciphyllus.*

There are no further reports in the literature on the associative occurrence of diazotrophic bacteria in *Syngonanthus* spp., or in other genera of Eriocaulaceae. The relatively high occurrence of diazotrophic bacteria in *S. elegans* and *S. aciphyllus* can contribute to the adaptation of these plants to soil of low fertility.

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RESUMO: O objetivo deste trabalho foi avaliar a ocorrência e densidade de bactérias diazotróficas associativas na rizosfera, colo, raízes e folhas de *Syngonanthus elegans* (Bong) Ruhland (Goldenfoot) e *Syngonanthus* aciphyllus (Mini-saia). Cinco amostras, com 25 plantas de *S. elegans* e *S. aciphyllus* cada foram coletadas próximo a nascente do córrego Soberbo, em Diamantina-MG. A ocorrência e a densidade foram avaliadas pelo número mais provável (NMP) em meios semi-seletivos NFb, LGI, JNFb e JMV. Em geral, nas duas espécies, observou-se uma alta densidade de bactérias diazotróficas em todos os microambientes avaliados. A densidade foi maior em *S. elegans* do que em *S. aciphyllus*. A ocorrência de bactérias diazotróficas associativas em *S. elegans* e *S. aciphyllus* pode contribuir para a adaptação destas plantas em solos de baixa fertilidade.

PALAVRAS-CHAVE: *Syngonanthus elegans*. *Syngonathus aciphyllus*. Bactérias diazotróficas associativas. Fixação biológica de nitrogênio.

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