

# INOCULATION AND ISOLATION OF PLANT GROWTH-PROMOTING BACTERIA IN MAIZE GROWN IN VITÓRIA DA CONQUISTA, BAHIA, BRAZIL

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## ABSTRACT

Maize is among the most important crops in the world. This plant species can be colonized by diazotrophic bacteria able to convert atmospheric N into ammonium under natural conditions. This study aimed to investigate the effect of inoculation of the diazotrophic bacterium *Herbaspirillum seropedicae* (ZAE94) and isolate new strains of plant growth-promoting bacteria in maize grown in Vitória da Conquista, Bahia, Brazil. The study was conducted in a greenhouse at the Experimental Area of the Universidade Estadual do Sudoeste da Bahia. Inoculation was performed with peat substrate, with and without inoculation containing strain ZAE94 of *H. seropedicae* and four rates of N, in the form of ammonium sulfate (0, 60, 100, and 140 kg ha<sup>-1</sup> N). After 45 days, plant height, dry matter accumulation in shoots, percentage of N, and total N (N<sub>Total</sub>) were evaluated. The bacteria were isolated from root and shoot fragments of the absolute control; the technique of the most probable number and identification of bacteria were used. The new isolates were physiologically characterized for production of indole acetic acid (IAA) and nitrogenase activity. We obtained 30 isolates from maize plants. Inoculation with strain ZAE94 promoted an increase of 14.3 % in shoot dry mass and of 44.3 % in N<sub>Total</sub> when associated with the rate 60 kg ha<sup>-1</sup> N. The strains N11 and N13 performed best with regard to IAA production and J06, J08, J10, and N15 stood out in acetylene reduction activity, demonstrating potential for inoculation of maize.

**Keywords:** diazotrophic bacteria, *Herbaspirillum* spp., *Zea mays*.

Received for publication on December 6, 2013 and approved on October 7, 2014.

DOI: 10.1590/01000683rbc20150725

## RESUMO: INOCULAÇÃO E ISOLAMENTO DE BACTÉRIAS PROMOTORAS DO CRESCIMENTO VEGETAL EM MILHO CULTIVADO EM VITÓRIA DA CONQUISTA, BAHIA

O milho é uma das culturas agrícolas mais importantes do mundo. Essa espécie vegetal pode ser colonizada por bactérias diazotróficas capazes de reduzir N atmosférico a amônio em condições naturais. Objetivaram-se estudar o efeito da inoculação da bactéria diazotrófica *Herbaspirillum seropedicae* (ZAE94) e isolar novas estirpes de bactérias promotoras do crescimento vegetal na cultura do milho cultivada em Vitória da Conquista, BA. O estudo foi conduzido em casa de vegetação na Área Experimental da Universidade Estadual do Sudoeste da Bahia. A inoculação foi realizada com veículo turfoso com presença e ausência de inoculação contendo a estirpe ZAE94 de *H. seropedicae* e quatro doses de N, na forma de sulfato de amônio (0, 60, 100 e 140 kg ha<sup>-1</sup> N). Após 45 dias, avaliaram-se altura de plantas, produção de matéria seca da parte aérea, o percentual de N e N total. O isolamento foi realizado a partir de fragmentos das raízes e parte aérea da testemunha absoluta, e utilizaram-se a técnica do número mais provável e a identificação das bactérias. Os novos isolados foram caracterizados fisiologicamente quanto à produção de ácido indol acético (AIA) e atividade da nitrogenase (ARA). Foram obtidos 30 isolados de plantas de milho. A inoculação da estirpe ZAE94 promoveu aumentos de 14,3 % na produção de matéria seca da parte aérea e 44,3 % do N total, quando associado à dose 60 kg ha<sup>-1</sup> de N. Dentre as estirpes isoladas, as N11 e N13 se destacaram quanto à produção de AIA; e as J06, J08, J10 e N15, para a ARA, apresentando potencial para inoculação na cultura milho.

Palavras-chave: bactérias diazotróficas, *Herbaspirillum* spp., *Zea mays*.

## INTRODUCTION

Maize (*Zea mays* L.) is among the most important crops in the world. In Brazil, this grain is significant both in the economic and the social fields, being used as food and feed, aside from serving as raw material in a diverse food processing procedures.

Of the whole national maize production, about 70 to 80 % of the grain yield is used in the feed industry (Kwiatkowski and Clemente, 2009). The South, Southeast, and Central-West regions account for 90 % of the Brazilian production, with the second largest cultivated area, behind only soybean (CONAB, 2013).

Nitrogen (N) is among the major cell constituents and a crucial element for all living organisms (Soratto et al., 2010). Nitrogen availability is a limiting factor in agricultural productivity. It is one of the nutrients absorbed in greater quantity by crops. In maize, it influences the grain yield directly (Amado et al., 2002).

Although N represents around 78 % of all atmospheric gases, plants cannot use it directly as a nutrient, due to the triple covalent bond between the two atoms of atmospheric N<sub>2</sub> to produce ammonium (Hungria et al., 2001).

Among the natural processes contributing to N supply of the soil environment, biological N fixation is the most important. This reaction occurs by the action of diazotrophic bacteria that have the nitrogenase enzyme, able to break the triple covalent bond between the two atoms of atmospheric N and reduce them to ammonium, under natural

conditions (Reis and Teixeira, 2005). The use of diazotrophic bacteria seem to be a promising strategy to reduce dependence on synthetic N fertilizers (Conceição et al., 2009). The species *Herbaspirillum seropedicae*, described by Baldani et al. (1986), comprises endophytic diazotrophic bacteria, mandatory, gram-negative, microaerobic, which are able to use various C sources.

Studies have demonstrated a positive effect of inoculation with diazotrophic bacteria in several commercially important crops (Baldani et al., 2000; Viana, 2012), e.g., rice (Cavallet et al., 2000; Alves, 2007), maize (Sala et al., 2008) and wheat.

According to Moreira et al. (2010), the contribution of biologically fixed N on Poaceas is estimated at 25 to 50 kg ha<sup>-1</sup> yr<sup>-1</sup>, equivalent to the average supply of 17 % of the demand required by these crops. Therefore, since major global territories are used for the cultivation of various cereals, indispensable to human and animal diet, the supply of 17 % of the crop requirement by biological N fixation (BNF) would represent gains both in economic and environmental terms.

The significance of this study for maize, as well as the other Poaceas in Northeastern Brazil, is due to great economic and social relevance of this cereal, since it is widely grown by family farmers. Thus, the studies focused on BNF may represent a strategy to reduce the use of synthetic N fertilizers, thus decreasing economic costs and environmental impacts. Given the above, the isolation and characterization of diazotrophic bacteria found under the local edaphoclimatic conditions, may contribute to greater gains in BNF, since the local

isolates are already adapted to the conditions and must therefore be evaluated under field condition, both in maize and the other Poaceas.

This article aimed to investigate the effect of inoculation of the diazotrophic bacterium *H. seropedicae* (ZAE94) and isolate new strains of plant growth-promoting bacteria in maize grown in Vitória da Conquista, Bahia, Brazil.

## MATERIAL AND METHODS

### Effect of inoculation of the diazotrophic bacterium *H. seropedicae*

The experiment was conducted in a greenhouse in the *campus* of the Universidade Estadual do Sudoeste da Bahia (UESB), in Vitória da Conquista (lat 14° 51' S, long 40° 50' W, on average 928 m asl), with a total annual rainfall of 750 mm (Brasil, 1992).

The soil used in the experiment was taken from the 0-20 cm layer, horizon A of a Yellow Latosol (Oxisol). The soil chemical analysis showed the following results: pH (H<sub>2</sub>O) = 5.4; P = 2 mg dm<sup>-3</sup>; K<sup>+</sup> = 0.10 cmol<sub>c</sub> dm<sup>-3</sup>; organic matter (OM) = 22 g dm<sup>-3</sup>. Corrections were made according to the Soil Fertility Commission of the State of Minas Gerais (Alvarez V and Ribeiro, 1999). All treatments received rates of P and K according to the crop requirements, considering the soil OM content.

The experimental units consisted of pots containing 9.0 dm<sup>3</sup> soil. The experimental design was completely randomized in a 2 × 4 factorial scheme, with and without inoculant of strain ZAE94 and four N rates (0, 60, 100, and 140 kg ha<sup>-1</sup> N) in the form of ammonium sulfate with five replications.

Inoculation took place with peat substrate containing strain ZAE94 (BR 11417) of *H. seropedicae* with an established population of 10<sup>9</sup> cells g<sup>-1</sup> of inoculant for inoculation treatments, at a proportion of 250 g of inoculant per 10 kg of maize seed. Four seeds of the maize hybrid AG 1051 were sown per pot. The distribution of N fertilization was divided in three times, i.e. 20 % at sowing, 20 % 15 days after planting (DAP), and 60 % 20 DAP. Thinning was done 18 DAP, keeping two plants per pot.

Data were collected 45 DAP, and the agronomic parameters evaluated were plant height, shoot dry weight, nitrogen percentage (N%), and total nitrogen (NTotal), the latter determined by Kjeldahl's digestion, as described by Nogueira and Souza (2005).

The data were subjected to homogeneity and normality tests and variance analysis; mean values were compared by Tukey's test at 5 % significance, using software Sisvar version 4.0 (Ferreira, 2000).

Linear and quadratic regressions were performed to compare N rates.

### Isolation and morphological and physiological characterization of diazotrophic bacteria

The diazotrophic bacteria were isolated from maize plants, absolute control, grown in pots in a greenhouse for 45 days. Then, the plant material was sent to the laboratory of grasses of the Empresa Brasileira de Pesquisa Agropecuária - Centro Nacional de Pesquisa em Agrobiologia (Embrapa Agrobiologia), in Seropédica, Rio de Janeiro, Brazil, for isolation.

Isolation and counting were performed as proposed by Döbereiner et al. (1995), in samples of roots and shoots (leaf and stem) of maize plants, using 5 g of each fragment. The plant material was added to 45 mL of saline solution and ground in a blender. Serial dilutions of 10<sup>-3</sup> to 10<sup>-7</sup> were made and inoculated in semisolid, N-free culture media (JNFb for *Herbaspirillum seropedicae*, NFb for *Azospirillum spp.*, JMV for *Burkholderia*, and LGI for *A. amazonense*).

Diazotrophic bacteria populations were counted based on the most probable number (MPN), using McCrady's table for three replication per dilution (Döbereiner et al., 1995). After purification, the isolates were morphological characterized in specific solid media. Characteristics such as size, shape, edge, surface, elevation, transparency, and chrome genesis of the colonies (Döbereiner et al., 1995) were observed. Strains of *H. seropedicae* (ZAE94) and *A. brasilense* (Sp245), from the collection of diazotrophic bacteria cultures of Embrapa Agrobiologia, were used as growth patterns, being grown and characterized under the same conditions.

For the physiological characterization, based on the production of indole acetic acid (IAA), the microplate method was used as described by Sarwar and Kremer (1995). Pure isolate colonies were replicated in tubes containing DYGS medium, where they were grown at 30 °C for 24 h under constant agitation (150 rpm). The concentration of indole compounds was determined by a calibration curve prepared with serial dilutions of IAA patterns (0-50 µg mL<sup>-1</sup>). The proteins were quantified according to Bradford (1976).

The analysis of the nitrogenase enzyme activity was evaluated indirectly by acetylene reduction activity (ARA), according to the method described by Boddey (1987). Pure colonies of isolates were cultured in DYGS medium under constant agitation for 24 h. The protein content was determined by the method described by Bradford (1976). Protein concentration was estimated using a standard curve obtained through known quantities of bovine serum albumin (0.25 at 1.5 µg of BSA).

The data were subjected to homogeneity and normality tests and variance analysis; means were

compared by Scott-Knott's test at 5 % of significance, using software Sisvar version 4.0 (Ferreira, 2000).

## RESULTS AND DISCUSSION

For N fertilization, no significant effects were observed on the variable shoot dry weight (SDW), since the accumulation observed was due to inoculation with diazotrophic bacteria. Strain ZAE94 of *H. seropedicae* induced a significant increase (14.33 %) in the accumulation of SDW in hybrid AG1051, compared to the non-inoculated treatment (Table 1). In general, dry matter production was highest in the treatments with diazotrophic bacteria inoculation. This may be due to the ability of these bacteria to promote plant growth through the synthesis of phytohormones, stimulating the early plant growth. These microorganisms can cause morphological and physiological changes in root structure, contributing to increased water and nutrient uptake. According to Bastian et al. (1998), the species *H. seropedicae* can promote plant growth in Poaceas.

Similar results were obtained by Quadros (2009), who observed increases of 19.71 % in maize shoot dry weight, in the hybrids AS 1575 and SHS 5050 inoculated with diazotrophic bacteria. Ramos et al. (2010) found that 30 DAP, maize plants inoculated with *Azospirillum lipoferum* (strain BR 11084) and fertilized with 30 kg N showed significant differences in SDW when compared to the control treatment. On the other hand, Reis Junior et al. (2008) found no significant effects of *A. amazonense* inoculation on the SDW of maize plants.

With regard to nitrogen percentage (N%) in the SDW of the maize hybrid AG 1051, significant increases due to N fertilization were only observed at the rate of 100 kg ha<sup>-1</sup> N. A quadratic regression model was fit for N%, which showed that the highest N content (2.79 %) can be obtained by fertilization with 109 kg ha<sup>-1</sup> N (Figure 1a). No significant effect of inoculation was observed on N% in maize shoots (Table 1). Different results were obtained by Sabino (2003), who found increases of up to 34 % in N% of shoots of rice cultivar IR42 inoculated with strain ZAE94 and supplemented with 40 kg N.

However, Dotto et al. (2010) observed that bacteria inoculation with genus *Herbaspirillum* induced no increase in N content in maize plants.

Regarding NTotal in plant tissue, inoculation with strain ZAE94 associated with the 60 kg N rate promoted increases of 44.32 % over the non-inoculated control. In turn, in treatments supplemented with 100 and 140 kg N, although statistically different, inoculation induced no marked increases (Figure 1b). In both treatments, a quadratic adjustment was observed. These results reveal that the accumulation in NTotal observed in maize shoots resulted from an increased shoot dry mass, induced by inoculation with strain ZAE94.

Sabino (2003) observed that ZAE94 inoculation, along with 80 kg ha<sup>-1</sup> N, promoted significant increases in NTotal accumulation in shoots over the control treatment. Guimarães et al. (2003) also observed that ZAE94 inoculation provided increases of 86 % in NTotal in rice shoots, although these values were not significant. Ferreira et al. (2010), testing the wheat variety BRS 296 inoculated with strain Sp245 along with a 40 kg ha<sup>-1</sup> N rate also observed significant differences in NTotal of shoots over the non-inoculated control (average increase 23.2 %).

In general, the results suggested that the significant effect of inoculation on the variables under study may have been a result of the action of N-fixing diazotrophic bacteria that are also plant growth promoters.

Another important factor is the specificity of the diazotrophic bacterium used, considering that it was isolated when endophytically colonizing roots of maize, sorghum, and rice (Baldani et al., 1986). Recent studies demonstrated that the species *H. seropedicae* provided significant results in several commercially relevant agricultural crops (Reis et al., 2005).

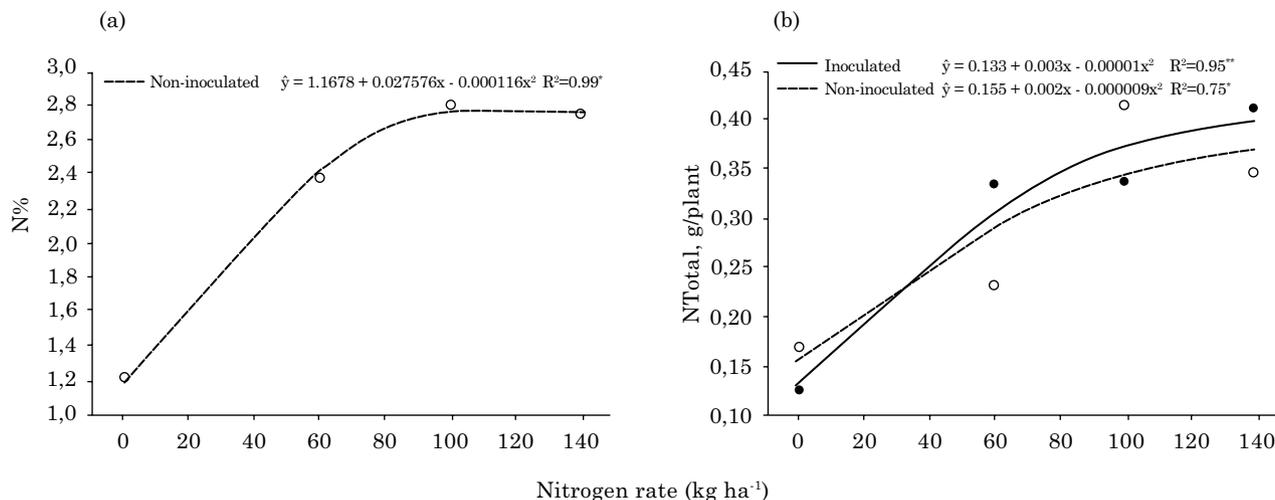
### Isolation and count of diazotrophic bacteria

The result of isolation and count by means of the NMP technique showed that there were variations in the occurrence of diazotrophic bacteria in the root and shoot fragments of maize plants. Of a total of 30 isolates, 80 % were found in roots and 20 % in shoots. Analyses by semi-specific media and

**Table 1. Height, dry mass, percentage N (N%), and total N (NTotal) of shoots of maize plants inoculated or not with the diazotrophic bacterium *H. seropedicae* (strain ZAE94) grown in Vitória da Conquista, average of five replications**

Treatment	Height	Dry matter	N%	NTotal
	cm	g	dag kg <sup>-1</sup>	g/plant
Strain ZAE 94 (inoculated)	36.27 A	13.48 A	2.14 B	30.29 A
Control (uninoculated)	32.54 A	11.79 B	2.40 A	29.07 A

Mean values followed by the same letter in the column do not differ by Tukey's test at 5 % of significance.



**Figure 1. Nitrogen percentage (N%) (a) and nitrogen total (NTotal) (b) in maize shoots, with and without inoculation (ZAE94) and different N rates, in Vitória da Conquista, average of five replications.**

morphological characterization suggested that 50 % of the isolates are similar to bacteria from genera or species of *Herbaspirillum* and 50 % of *Azospirillum*. However, for confirmation at the level of genus or even species to which the isolates belong, analyses by molecular techniques are required.

The results corroborate previous studies that reported the occurrence of diazotrophic bacteria in crops such as maize (Roesch et al., 2007; Gomes et al., 2010), rice (Sabino, 2003; Viana, 2012), and sugarcane (Antonio, 2010), in which an increased number of diazotrophic bacteria was found in roots. According to Dobbelaere et al. (2003), the root region, for being a nutrient-rich environment, favors the activity and growth of microorganisms, which will use the organic compounds released by root exudation and deposition, as energy and carbon sources.

It was not possible to identify any isolate similar to that of the species *A. amazonense* by morphological characterization, although the culture medium LGI (indicated for the species) showed the occurrence of diazotrophic bacteria in roots and shoots.

These results suggest that isolates were possibly not reactivated, due to the loss of bacteria viability in the period they remained in stock or maybe the culture medium recommended exclusively for isolation of this species failed to make a detection possible, since the culture media are regarded as semi-specific.

Sala et al. (2005) studied the diversity of diazotrophic bacteria in wheat plants but detected no microorganisms in culture medium LGI-P, indicated to isolate *G. diazotrophicus* species, in spite of the characteristic layers observed in the culture medium.

Under the same test conditions, no occurrence of diazotrophic bacteria was observed in culture medium JMV, indicated for bacteria of the genus *Burkholderia*.

All 30 isolates obtained were able to produce indole compounds in L-tryptophan supplemented culture medium. In general, the production of IAA by isolates similar to the genera *Herbaspirillum* and *Azospirillum* spp. ranged from 0.269 to 15.649  $\mu\text{g mg}^{-1}$  of protein (Table 2).

In all isolates, IAA was detected, however, the production of those similar to the genus *Herbaspirillum* spp. was lower than of those similar to *Azospirillum* spp. These results corroborate those of Radwan et al. (2004), who found that isolates of *Azospirillum* produce more indole compounds than those of *Herbaspirillum*.

Among isolates similar to those of genus *Herbaspirillum* spp., IAA production ranged from 0.269 to 11.246  $\mu\text{g mg}^{-1}$  of protein and of those similar to genus *Azospirillum* spp. from 1.234 to 15.649  $\mu\text{g mg}^{-1}$  of protein. These results were similar to those found by Pedraza et al. (2004), who, by evaluating the ability of five isolates of *Azospirillum* spp., observed an IAA production from 2.680 to 38.286  $\mu\text{g mg}^{-1}$  of protein, and Bergamaschi et al. (2007), who reported values from 0.70 to 10.70  $\mu\text{g mg}^{-1}$  of protein for diazotrophic bacteria isolated from sorghum (Table 2).

The strains N11 and N13 stood out regarding the production of IAA, with values statistically equal to the standard strain of *H. seropedicae* (ZAE94) and higher than the other strains. The biosynthesis of IAA is part of the metabolism of various species of bacteria associated with plants (Patten and

**Table 2. Acetylene reduction activity (ARA), synthesis of indole acetic acid (IAA) by native strains isolated from maize plants AG 1051 and standard bacteria**

Isolate	ARA	AIA	Plant component	Culture medium
	mmol h <sup>-1</sup> mg <sup>-1</sup> of protein	ug mg <sup>-1</sup> of protein		
UESBN1	1.7084 c	5.3923 d	Root	NFb
UESBN2	2.1438 c	5.5390 d	Root	NFb
UESBN3	1.3153 c	6.5483 c	Root	NFb
UESBN4	2.1017 b	2.9423 e	Root	NFb
UESBN5	0.0428 c	1.2343 e	Shoot	NFb
UESBN6	Nd	1.9773 e	Shoot	NFb
UESBN7	2.8579 b	7.0870 c	Root	NFb
UESBN8	2.6939 b	6.7760 c	Shoot	NFb
UESBN9	2.7339 b	7.3426 c	Shoot	NFb
UESBN10	1.5782 c	4.5596 d	Shoot	NFb
UESBN11	Nd	15.6496 d	Shoot	NFb
UESBN12	0.9145 c	11.7833 b	Root	NFb
UESBN13	2.3515 b	14.2116 a	Root	NFb
UESBN14	0.3577 c	9.5226 b	Root	NFb
UESBN15	4.3543 a	3.6013 e	Root	NFb
UESBJ1	0.8599 c	5.9120 d	Root	JNFb
UESBJ2	0.8894 c	6.5533 c	Root	JNFb
UESBJ3	Nd	0.2686 e	Root	JNFb
UESBJ4	0.8643 c	8.6676 c	Shoot	JNFb
UESBJ5	0.7939 c	5.3703 d	Root	JNFb
UESBJ6	1.2948 c	5.8583 d	Root	JNFb
UESBJ7	0.8713 c	6.9230 c	Root	JNFb
UESBJ8	1.0889 c	9.7990 b	Root	JNFb
UESBJ9	0.7362 c	11.2463 b	Root	JNFb
UESBJ10	1.0767 c	6.4453 c	Root	JNFb
UESBJ11	0.9493 c	8.4286 c	Root	JNFb
UESBJ12	0.8922 c	5.8163 d	Root	JNFb
UESBJ13	0.7470 c	4.3853 d	Root	JNFb
UESBJ14	0.6195 c	4.9546 d	Root	JNFb
UESBJ15	0.7288 c	7.7493 c	Root	JNFb
Sp245	0.6431 c	-	Standard	NFb
ZAE94	-	15.7853 a	Standard	JNFb

Same letters in the same column do not differ significantly from each other by the Scott-Knott test at 5 % significance. Nd: not detected.

Glick, 1996), providing these microorganisms with the ability to stimulate and promote plant growth.

The nitrogenase enzyme activity, responsible for BNF, may be indirectly measured by the acetylene reduction technique. Therefore, out of the 30 native isolates, 28 isolates obtained detectable ARA from 0.013 to 4.354 nmol C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> mg<sup>-1</sup> of protein. Under the test conditions, strains similar to the genus *Herbaspirillum* spp. obtained ARA between

0.6196 and 1.2949 2949 nmol C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> mg<sup>-1</sup> of protein. Those similar to the genus *Azospirillum* showed variation between 0.013 and 4.354 nmol C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> mg<sup>-1</sup> of protein. For ARA production, the isolates that stood out were J06, J08, and J10, morphologically characterized as similar to the genus *Herbaspirillum* spp. and N15, morphologically characterized as belonging to the genus *Azospirillum* spp. However, N15 obtained

the highest value of ARA production under *in vitro* conditions, demonstrating potential to be used in inoculation tests (Table 2).

In the literature, there are mixed results regarding the ability to reduce acetylene to ethylene between strains of a same species. Rodrigues et al. (2006) observed a large variation in the detection of ARA in bacteria from the genera *Herbaspirillum* and *Burkholderia*, isolated from rice plants. VIANA (2012) evaluated isolates similar to *Herbaspirillum* for ARA, detected values with amplitude from 16.559 to 199.717 nmol C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> mg<sup>-1</sup> of protein. Pedrinho (2009), studying the ability to reduce acetylene in bacteria isolated from maize plants, obtained ARA values between 0.1 and 0.64 nmol C<sub>2</sub>H<sub>4</sub> culture<sup>-1</sup> h<sup>-1</sup>, of bacteria from the genera *Pseudomonas sp.*, *Herbaspirillum sp.*, *Burkholderia sp.*, and *Sphingomonas sp.*

The bacteria isolated in this study, for being native, may have more interactions with maize plants grown *in situ*, allowing greater contribution of BNF to the crop. According to Baldani and Baldani (2005), studies show that strains isolated from a species are more likely to reestablish on roots when inoculated on the same plant species, and are called homologous strains.

## CONCLUSIONS

Inoculation with strain ZAE94 promoted, under greenhouse conditions, increases of 14.33 % in shoot dry weight and, when inoculation was supplemented with 60 kg N, increases of 44.32 % in NTotal of maize plants.

The diazotrophic bacteria ZAE94 is promising for testing under local edaphoclimatic conditions.

It was possible to obtain 30 isolates under the local edaphoclimatic conditions.

The new isolates J10, N13, and N15, derived under the local conditions, proved promising for further studies, where their effectiveness must be tested in field experiments.

## ACKNOWLEDGEMENTS

The authors thank the field board of directors and all its contributors, for their assistance during the experiment, the Foundation for Research Support of the State of Bahia (FAPESB), for granting a scholarship, and the Southwest Bahia State University (UESB), for its collaboration in carrying out this research.

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