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Characterization and activity of endophytic bacteria from 'Prata Anã' banana crop (*Musa* sp., AAB)[1]

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ABSTRACT

The objective of this study was to characterize banana tree endophytic bacteria at genus and species level and to determine the metabolic reactions associated with the nitrogen transformations. The identification at genus and species levels was performed using the partial sequencing of the rDNA 16S region. The assimbyotic nitrogen fixation, the reduction of nitrate and the production of urease were in vitro evaluated. The DNA of the bacterial isolates was also amplified to verify the presence of the *nif*H, *nir*K and *nir*S regions. Biochemical tests were performed in a complete randomized design; the treatments consisted of 39 bacterial isolates with three replications. Sequence analysis enabled the identification of four genera: *Bacillus*, *Rhizobium*, *Klebsiella* and *Enterobacter*. The *Bacillus* genus occurred more frequently, nine species were identified. By evaluating the results of biochemical tests, it was observed that three isolates showed multiple abilities: growth in NFb medium, nitrate reduction and production of urease. The isolates belong to the genus *Bacillus* and of the species *subtilis*, *thuringienses* and *amyloliquefaciens*. Approximately 12.5% of the isolates amplified the region corresponding to the *nif*H gene, 7.5% amplified gene *nir*K and 3.9% amplified the *nir*S gene. Endophytic bacteria evaluated in the present study showed *in vitro* activity for urease, nitrate reductase enzymes, however, relevant nitrogenase activity was not observed.

Keywords: *Bacillus;* diversity; *nifH*; nitrate reductase; urease

RESUMO

Caracterização e atividade de bactérias endofíticas de bananeira 'Prata Anã' (*Musa* sp., AAB)

O presente estudo teve como objetivo caracterizar as bactérias endofíticas de bananeira em nível de gênero e espécie e determinar as reações metabólicas associadas às transformações do nitrogênio. A identificação em níveis de gênero e espécie foi realizada por meio do sequenciamento parcial da região rDNA 16S. Foram avaliadas *in vitro* a capacidade de fixação assimbiótica de nitrogênio, a redução do nitrato e a produção de urease. O DNA dos isolados bacterianos também foi amplificado para a verificação da presença das regiões gênicas *nif*H, *nir*K e *nir*S. Os testes bioquímicos foram realizados em delineamento inteiramente casualizado, os tratamentos foram constituídos de 39 isolados bacterianos com três repetições. A análise das sequências possibilitou identificar quatro gêneros, *Bacillus*, *Rhizobium, Klebsiela* e *Enterobacter.* O gênero *Bacillus* ocorreu com maior frequência, nove espécies foram identificadas. Avaliando-se conjuntamente os resultados dos testes bioquímicos observou-se que três isolados apresentaram habilidades múltiplas: crescimento em meio NFb, redução de nitrato e produção de urease. Os isolados pertencem ao gênero *Bacillus* e das espécies *subtilis, thuringienses* e *amyloliquefaciens*. Em torno de 12% dos isolados amplificaram a região correspondente ao gene *nifH*, 7,5% o gene *nirK*, 3,9% o gene *nirS*. As bactérias endofíticas avaliadas no presente estudo indicaram atividade *in vitro* para as enzimas urease e nitrato redutase, não apresentando, entretanto, atividade relevante da nitrogenase.

Palavras-chave: *Bacillus;* diversidade*; nif*H; urease; nitrato redutase.

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INTRODUCTION

Among the endophytic microorganisms, plant growth promoting bacteria (PGPB) are considered a group of high biotechnological potential. They play important roles, among them: biological nitrogen fixation (BNF), nutrient solubilization, production of phytohormones, biological control of pathogens and alleviation of biotic and abiotic stresses (Liaqat & Eltem, 2016). Although studies involving PGPB are relatively advanced in some annual species, studies on this bacterial group in perennial species, fruit in particular, are scarce and requires further effort on the part of the scientific community.

Banana (*Musa* sp.) is a fruit tree widely grown in different regions of the world and plays an important role in the economy of the major producing countries. Its production requires a considerable use of agrochemicals, which contributes to the cost of production and increase environmental liabilities. The manipulation of endophytic microorganisms as an alternative to conventional practices emerges as an alternative to guarantee sustainable crop production (Karthik *et al*., 2017).

The bioprospecting, manipulation and characterization of new species is considered a fundamental step to the better understanding of these biological processes. Among the tools used, genotype identification via sequencing of the 16S rRNA region and studies of its metabolic functions are widely used (Haidar *et al.*, 2018; Khamwan *et al.*, 2018; Rajamanickam *et al.*, 2018).

According to Moreira *et al*. (2010), associative diazotrophic bacteria may contribute to plant growth, not only by BNF, but also by other processes. Studies have shown that the efficiency of nitrogen uptake of plants metabolized by microorganisms is directly related to the sources of nitrogen available in the environment.

The understanding of nitrogen bacterial metabolism and the major pathways involved in this reaction may help to optimize the *in vivo* use of these microorganisms. Much attention has been given to the biological fixation of nitrogen, but studies related to the denitrification process are reduced, being necessary for a broader understanding of the processes of nitrogen transformation.

Hence, the objective of this study was to characterize banana tree endophytic bacteria at the genus and species level and to determine the metabolic reactions associated with the nitrogen transformations.

MATERIAL AND METHODS

This study was carried out in the laboratories at the Universidade Estadual de Montes Claros (UNIMONTES), Janaúba Campus, northern region of the State of Minas Gerais, from March 2013 to August 2014. The bacteria used in this study were isolated by Souza *et al*. (2013). For the extraction of DNA, 39 isolates were used. Each isolate was grown in TSB liquid medium (Triptic Soy Broth) for 24 h at 37 °C, under constant stirring at 180 rpm. The extraction of genomic DNA from the bacteria was carried out with the aid of extraction Kit of Miniprep HiPura Bacterial Genomic DNA manufactured by HIMEDIA.

The isolates were identified by partial sequencing of the 16S rDNA region. The 16S region was amplified using primers 27F (5'AGAGTTTGATC(AC)TGGCTCAG3') and 1492R (5'ACGG(CT)TACCTTGTTACGACTT-3') by means of the PCR technique. The amplification reaction consisted of mixing 2.0 µl of dNTPs (2.0 mM each), 2.5 µl of 10X buffer solution, 0.75 µl of $MgCl_2$ (50 mM), 2.5 µl of each primer (5 mM), 0.3 µl of Taq DNA polymerase (5 U μL^{-1}), 3 ì l of DNA (50ng), completing the final volume of 25 µl with ultra pure water. Then, the material was placed in a thermocycler and submitted to amplification: one denaturation step (94° C for 3 min), followed by 30 intermediate cycles (94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 1 min) and a final extension step (72 °C for 7 min). After amplification, the fragments were analyzed by 1.2% agarose gel electrophoresis and photographed in a digital system. Choice and the amplification process were performed as described by Souza *et al*. (2013).

Sequencing of the samples was performed by Hellixxa company. Sequencing data were obtained using the Sanger method. The sequences were compared with those found in the NCBI database (Nacional Center for Biotechnology Information) (www.ncbi.nlm.nih.gov), by BLAST (Basic Local Alignment Search Tool) software for nucleotides.

By comparing the obtained sequences with the sequences deposited in the GenBank public database through the BLAST software (NCBI www.ncbi.nih.gov), the isolates were identified at the species level when the similarity values found ranged from 98% and 100%, and at the gender level, when they ranged from 92% to 98% (Souza *et al*., 2013).

In the biochemical tests, the treatments were qualitative, consisting of 39 bacterial endophytic isolates. For each test, three replicates and a completely randomized design were used. Firstly, the isolates were sorted to determine which of them had the ability to grow in semi-solid, nitrogen-free NFb medium and form aerotaxis film.

Following bacterial growth, a bacterial suspension was prepared with 0.85% saline solution under aseptic conditions. It was inoculated 250 µL of that bacterial suspension adjusted to DO of 0.5 of absorbance at 540 nm in spectrophotometer into test tubes containing 10 mL of semisolid NFb medium. The test tubes were incubated at 28 °C for 10 days in BOD, when bacterial growth was observed.

After the initial screening, the isolates that showed the capacity to grow and to form aerotaxis film in NFb medium were characterized as positive and selected for the trial of nitrogenase activity in gas chromatography. The trial for nitrogenase activity was carried out by the reduction of acetylene (ARA). To carry out the trail, the isolates were grown in BDA medium (200 g of potato, 20 g of agar and 20 g of dextrose) and with the aid of a previously sterilized wooden toothpick, a colony of each isolate was withdrawn and introduced into the NFb semi-solid medium and incubated in an oven at 30 °C. The flasks were capped with rubber, acetylene was added to a final concentration of 12% (v/v) and the ethylene production was determined after 24 h on a gas phase chromatograph (Shimadzu GC-14A). Pure ethylene was used as standard.

The biochemical nitrate reduction test was performed according to Araújo *et al*. (2002). A distinct dark red or pink color indicated the reduction of nitrate in the medium. The non-change of color of the medium indicated the negative reaction.

The urease test was performed according to Maringoni (2010). The color change of the medium from intense red to purplish characterized the positive reaction for urea hydrolysis, while the negative reaction was characterized when the medium remained with no change in the color.

Following the biochemical tests, two pairs of primers were tested: F1acd (TA(C/T) CAC CC(C/G) GA(A/G) CCG) and R4cd (CCGT TGAACT T(G/A)C CGT(C/G)G) referring to *nir*S gene; F1acu (ATC ATGGT(C/G) CTG CCG CG) and R3cu (GCC TCG ATC AG(A/G) TTG TGG TT) referring to *nir*K gene, both related to the enzyme nitrite reductase. The amplification conditions were according to Throbäck *et al*. (2004). The amplification products were observed on 1.2% agarose gel and fragments of approximately 780 bp and 450 bp were expected for the *nir*S and *nir*K gene, respectively. The 100 bp-DNA molecular weight marker was used to serve as a parameter for estimating the size of the amplified fragments. The analysis was performed by analyzing the presence or absence of the band that characterized the gene.

DNA amplification of the bacterial isolates to verify the presence of the *nif*H gene region was performed according to Teixeira *et al*. (2007). The amplification products were observed on 1.2% agarose gel and fragments of approximately 270 bp were expected for the *nif*H gene. A 100pb-DNA molecular weight marker was used to serve as a parameter for estimating the size of the amplified fragments. The analysis was made by evaluating the presence or absence of the band that characterized the gene.

After isolates were identified, the frequency of genera and species was measured. The evaluations related to the biochemical tests, ability of aerotaxis film formation, nitrate reduction and urease production were performed visually. The achieved qualitative data were submitted to frequency distribution analysis. The frequency distribution was also applied to the molecular data.

RESULTS AND DISCUSSION

By comparing the sequences deposited in GenBank, similarity values were found between them, which ranged from 92% to 99%. After partial sequencing of the 16S rDNA region of the 39 bacterial isolates, it was possible to distinguish them in four different genera: *Bacillus*, *Rhizobium*, *Klebsiela* and *Enterobacter* (Table 1).

The genus *Bacillus* occurred most frequently (92.5%). In relation to the other genera, there was only one representative of each genus (2.5%). Nine species of the genus *Bacillus* were identified: *B. subtilis* (11 isolates), *B. pumilus* (10 isolates), *B. safensis* (5 isolates), *B. altitudinus* (3 isolates), *B. thuringiensis* (2 isolates), *B. cereus* (2 isolates), *B. amyloliquefaciens* (1 isolate), *B. axarquienses* (1 isolate) and *B. megaterium* isolate (1 isolate). The *B. subtilis* and *B. pumilus* predominated over the others, representing 27.5% and 25% of the identified isolates, respectively (Table 1).

Different authors have reported the incidence of those genera and species associated to banana crop in several parts in the world, such as India (Karthik *et al*., 2017; Rajamanickam *et al*., 2018), the Dominican Republic (Marcano *et al*., 2016), Kenya (Ngamau *et al*., 2012) and Brazil (Souza *et al*., 2013). The frequency of these endophytes may be associated with the conditions of the environment and the management of the banana crop. Factors such as the farming system, the use or not of agrochemicals, plant genotype, phenological stage and soil type somewhat influence the diversity and richness of bacterial populations, being responsible for the differences and similarities between the species found in the different areas of collection (Souza *et al*., 2015).

The great predominance of the genus *Bacillus* sp. associated with 'Prata Anã' banana tree had been previously reported. Souza *et al*. (2013) described the endophytic coexistence of twelve different species of *Bacillus* in banana roots. The results of both studies demonstrate that the cultivar of the banana tree Prata Anã, triploid, belonging to the AAB genomic group, grown in the semi-arid regions of the states of Minas Gerais and Bahia, has a narrow association with non-diazotrophic endophytic bacteria of the genus *Bacillus* sp.

The species *B. subtilis* and *B. pumilus* predominated over the others, representing over 50% of the isolates. According to Bulgarelli *et al*. (2012), soil shelters inoculum of microbial communities, however, the roots determine

quantitatively the bacterial species that will be established endophytically, owning receptors for conserved bacterial structures, such as flagellin 16. The greatest proportion of the mentioned genera may indicate that they establish endophytically more easily or rapidly in the roots of banana cultivar Prata Anã. From a biotechnological point of view, this is an ability to be considered, once, when choosing a possible isolate to compose a bioinoculant, species that are competitive, adapted and capable of being easily established in the agricultural environments are needed.

Experiments on competition with endophytes have already shown that some colonizers are more aggressive and faster than others (Beneduzi *et al*., 2012). Jaizme-Vega *et al*. (2004), when working with the inoculation of *Bacillus* sp. in two banana cultivars, showed that, depending on the cultivar, some differences in time and magnitude of response to bacterial inoculation are detected. This can

Table 1: Identification of the endophytic bacteria of 'Prata Anã' banana roots based on the identity of the partial sequence of the 16S rDNA gene

Isolate	E [(1)]	ID [(2)]	OMR [(3)]	NPB[(4)]	GenBank access number
6	0.0	98%	*Bacillus pumilus*	970	KX189587
13	0.0	96%	*Bacillus subtilis*	900	KX189588
18	0.0	93%	*Bacillus axarquiensis*	430	KX189589
20	0.0	99%	*Bacillus thuringienses*	930	KX189590
22	$2e^{-12}$	92%	*Klebsiela pneumonia*	780	KX189591
29	0.0	98%	*Bacillus pumilus*	970	KX189592
31	0.0	98%	*Bacillus subtilis*	960	KX189593
36	0.0	98%	*Bacillus megaterium*	940	KX189594
43	0.0	96%	*Bacillus pumilus*	960	KX189595
59	0.0	96%	*Bacillus pumilus*	960	KX189596
61	0.0	97%	*Bacillus pumilus*	970	KX189597
78	0.0	96%	*Bacillus pumilus*	970	KX189598
80	0.0	92%	*Bacillus thuringiensis*	770	KX189599
81	0.0	98%	*Bacillus altitudinis*	960	KX189600
86	0.0	98%	*Bacillus subtilis*	970	KX189601
93	0.0	97%	*Bacillus subtilis*	960	KX189602
97	0.0	97%	*Bacillus subtilis*	960	KX189603
100	0.0	97%	*Enterobacter sp.*	970	KX189604
102	0.0	93%	*Bacillus sp.*	880	KX189605
105	0.0	99%	*Bacillus thuringiensis*	960	KX189606
112	0.0	96%	*Bacillus subtilis*	970	KX189607
115	$2e^{-12}$	92%	*Rhizobium sp.*	600	KX189608
119	0.0	94%	*Bacillus safensis*	960	KX189609
123	0.0	97%	*Bacillus subtilis*	960	KX189610
130	0.0	96%	*Bacillus altitudinis*	950	KX189611
131	0.0	98%	*Bacillus cereus*	980	KX189612
135	0.0	97%	*Bacillus pumilus*	960	KX189613
137	0.0	98%	*Bacillus safensis*	960	KX189614
142	0.0	97%	*Bacillus subtilis*	970	KX189615
156	0.0	96%	*Bacillus subtilis*	980	KX189616
163	0.0	98%	*Bacillus safensis*	980	KX189617
170	0.0	97%	*Bacillus safensis*	900	KX189618
171	0.0	99%	*Bacillus amyloquefaciens*	950	KX189619
172	0.0	93%	*Bacillus subtilis*	960	KX189620
173	0.0	98%	*Bacillus altitudinis*	710	KX189621
177	0.0	98%	*Bacillus pumilus*	950	KX189622
179	0.0	95%	*Bacillus pumilus*	970	KX189623
189	0.0	98%	*Bacillus pumilus*	970	KX189624
192	0.0	95%	*Bacillus cereus*	900	KX189625

[(1)] **E value:** probability of randomily finding the same alignment between two species. [(2)] **Identity**: percentage of identity between the sequence of the banana isolate and the related organism. [(3)] **OMR**: organism that has the sequence with which the partial sequence of the 16S rDNA gene of the banana isolate showed the greatest homology. [(4)] **NPB**:Base pair numbers.

be explained by the genetic and physiological differences between the genotypes of *Musa* sp., which are likely to occur in the root system and/or in the composition of the produced radicular exudate. Root exudates may influence bacterial cultures on the ability of colonization, which may be affected by affinity for exudate compounds. Such affinity may determine the rate and location of colonization (Karthik *et al.* (2017).

By considering the biochemical evaluations of aerotaxis film formation in Nfb medium and the acetylene reduction technique, it can be suggested that the bacteria under study present low biological nitrogen fixation capacity, confirmed by the slow growth in nitrogen free medium and by the inability to reduce of acetylene in gas chromatography. Therefore, they cannot be described as diazotrophic endophytic bacteria in 'Prata Anã' banana (Table 2).

The genus *Rhizobium*, widely described as nitrogen fixing, did not demonstrate the same ability in the present work. Loss in the ability of nodulating and binding N_2 in several strains is caused when the symbiotic plasmid, pSym, which contains structural genes in the nitrogenase, *nif*, or *nod*D nodulation genes is rearranged genomically by a recombination process losing its function (Toledo, 2008).

The reduction of nitrate to nitrite was performed by 90% of the bacterial isolates. Only the isolates 36, 61,102 and 119 of the species *B. megaterium*, *B. pumilus*, *Bacillus* sp. and *B. safensis*, respectively, did not present the same capacity (Table 2).

Mohanty *et al*. (2016), also obtained positive responses to nitrate reduction when working with bacteria isolated from *Jatropha curcas* L. plants. These results demonstrate that the isolates of endophytic bacteria characterized in the present work present the capacity to metabolize nitrate.

Most nitrate positive isolates did not show amplification of the *nir*K and *nir*S primers (Table 2), demonstrating that they do not have genes associated with denitrification enzymes. The reduced number of denitrifying bacteria found in this work may be associated with the characteristic environmental conditions of the properties where the isolation was performed. According to Lin *et al*. (2018), environmental conditions and fertilization management affect the abundance and diversity of the denitrifying communities associated with the plant. Hence, it is believed that the *nar*G and *nap*A genes (Lin *et al*., 2018) are involved in the assimilatory route of nitrate and should be targeted in further studies aiming at a better understanding of this system.

Bacteria that belong to the genera *Bacillus*, *Enterobacter* and *Klebsiela* may be commonly associated with dissimilatory reduction, which means that some of the nitrogen used in their metabolism may be extruded in the form of the ammonium ion (Moreira & Siqueira, 2006).

In monocotyledonous roots, the endophytic bacteria are present in the apoplast, its principal niche. Therefore, the extrusion of ammonium in the plant interior can generate its rapid conversion into amino acid, through the action of the enzyme glutamine synthetase, which acts to avoid a possible toxicity caused by its accumulation in the cell (Gyaneshwar *et al*., 2001).

The reduction of nitrate is a critical feature of these endophytes, since the time for the plants to absorb the readily available nitrogen, before part of it or even its total content is converted to nitrous oxide and, consequently, in atmospheric N_2, will be short. Considering these results and evaluating the biotechnological potential of the characterized isolates, it is recommended as a better way to use them, through the microbiolization of the seedlings. The application of the bacterial solution in micropropagated seedlings in the acclimatization phase and not the application of the solution of bacteria directly to the soil, as this practice may result in an increased loss of N.

Urease production was verified in 30% of the isolates studied (Table 2). Bacteria of the genera *Bacillus*, *Klebsiela* and *Enterobacter* were able to hydrolyze urea, thus indicating the action of the enzyme urease. Considering only the genus *Bacillus*, six distinct species were urease positive, where *B. subtilis* species stood out. The other isolates were identified as negative urease, since they did not present intense pink coloration but an intense yellow color, which is probably the result of the release of acidic metabolites in the medium.

Urease catalyzes urea hydrolysis to unstable carbamic acid. The positive test for 30% of the isolates showed that they have the potential to break urea in simpler forms that may be readily available to the host plant. However, if this urea is hydrolyzed very quickly, losses resulting from ammonia volatilization can be observed. This indicates that in the *in vivo* use of the bacterial isolates, the application of fertilizers in the form of urea should be split as well as their incorporation the soil/substrate in order to avoid losses by volatilization.

Among the isolates evaluated, 17.5% amplified *nif*H gene: B *subtilis* isolates 13, 86, 93 and 112; *B pumilus*, isolate 61; *B. sapensis*, isolate 137 and *B. altitudinus*, isolate 173. For the *nir*K gene, 7.5% of the isolates amplified this region, especially the isolates 22, 61 and 137 of the species *K. pneumonia*, *B. pumilus* and *B. safenis*, respectively. Moreover, in relation to the *nir*S gene, only the isolated 18 (*B. axarquienses*) amplified for this region. The other isolates did not amplify in any of the reactions performed (Table 2).

Table 2: Biochemical tests for nitrogen fixation with film formation in NFb semi-solid medium, acetylene reduction, nitrate test and urease activity, and DNA amplification results of bacterial isolates to verify the presence of the *nif*H, *nir*S and *nir*K gene regions in 39 isolates of endophytic bacteria isolated from banana roots

Isolate	MRO [1]	FFNM [2]	ARA [3]	RN [4]	UP [5]	*nifH*	*nir*S	*nirK*
6	*B. pumilus*	-	-	+	-	-	-	-
13	*B. subtilis*	+	-	+	+	+	-	-
18	*B. axarquiensis*	-	-	+	-	-	+	-
20	*B. safensis*	+	-	+	-	-	-	-
22	*K. pneumonia*	-	-	+	+	-	-	+
29	*B. pumilus*	+	-	+	-	-	-	-
31	*B. subtilis*	-	-	+	-	-	-	-
36	*B. megaterium*	+	-	-	-	-	-	-
43	*B. pumilus*	-	-	+	-	-	-	-
59	*B. pumilus*	-	-	+	-	-	-	-
61	*B. pumilus*	+	-	-	-	+	-	+
78	*B. pumilus*	-	-	+	-	-	-	-
80	*B. thuringiensis*	-	-	+	-	-	-	-
81	*B. altitudinus*	-	-	+	-	-	-	-
86	*B. subtilis*	-	-	+	-	+	-	-
93	*B. subtilis*	-	-	+	+	+	-	-
97	*B. subtilis*	-	-	+	+	-	-	-
100	*Enterobacter sp.*	-	-	+	+	-	-	-
102	*Bacillus sp.*	-	-	-	-	-	-	-
105	*B. thuringiensis*	+	-	+	+	-	-	-
112	*B. subtilis*	+	-	+	-	-	-	-
115	*Rhizobium sp.*	+	-	+	-	-	-	-
119	*B. safensis*	-	-	-	+	-	-	-
123	*B. subtilis*	-	-	+	-	-	-	-
130	*B. altitudinis*	-	-	+	-	-	-	-
131	*B. cereus*	-	-	+	-	-	-	-
135	*B. pumilus*	-	-	+	-	-	-	-
137	*B. safensis*	-	-	+	-	-	-	-
142	*B. subtilis*	-	-	+	-	-	-	-
156	*B. subtilis*	-	-	+	-	-	-	-
163	*B. safensis*	-	-	+	+	-	-	-
170	*B. safensis*	-	-	+	-	-	-	-
171	*B.amyloliquefacies*	+	-	+	+	-	-	-
172	*B. subtilis*	-	-	+	-	-	-	-
173	*B. altitudinis*	-	-	+	-	-	-	-
177	*B. pumilus*	-	-	+	-	-	-	-
179	*B. pumilus*	-	-	+	+	-	-	-
189	*B. pumilus*	-	-	+	+	-	-	-
192	*B. cereus*	-	-	+	-	-	-	-

[1]**MRO:** the most related organism.[2] **FFNM:** film formation in NFb medium. [3]**ARA:** Acetylene reduction. [4] **RN:** Nitrate reduction. [5] **UP:** Urease production. + positive for the reaction; - negative for the reaction.

That isolates 22, 61 and 137 of the species *K. pneumoniae*, *B. pumilus* and *B. safensis*, respectively, amplified for the *nir*K gene. The isolates 18 and 193 corresponding to *B. axarquienses* and *B. aerophilus*, respectively, have amplified for the *nir*S gene, demonstrated that these isolates probably possess the enzymatic set that can propitiate the respiratory denitrification, or the denitrification itself. It is noteworthy that under appropriate conditions and from specific studies, these isolates could be tested for their capacity as bioremediators of contaminated wastewater and organic pollutants.

The results of amplification of the *nif*H gene, combined with the results obtained for growth in NFb medium and reduction of acetylene, indicate that despite presenting *nif*H gene, some of the characterized isolates do not behave as such, demonstrating a low potential for biological fixation of nitrogen.

Besides the issues associated with the increment in growth and production via minimization of nitrogen

losses to plants, the results of the present work open a new perspective of scientific research, concentrating efforts on the characterization and application of the isolates characterized as denitrifiers in the degradation of organic pollutants and wastewater contaminated with nitrites.

The described genera have demonstrated other potentials in diverse crops. Species of the genera *Bacillus*, *Enterobacter*, *Klebsiela* and *Rhizobium* have been described as plant growth promoters (Orozco-Mosqueda *et al*., 2018), acting as producers of phytohormones, solubilizers of inorganic and organic phosphates of low solubility and disease suppressors.

CONCLUSIONS

Analysis of the nucleotide partial sequences of the 16S rDNA region allows separating the 40 isolates in four different genera.

The genus *Bacillus* is that with the greatest frequency among the studied bacteria.

Although the endophytic bacteria studied in the present work do not present the capacity for the fixation of nitrogen, they do have positive activity for the enzymes nitrate reductase and urease.

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