Brazilian Journal of Microbiology (2001) 32:201-205
ISSN 1517-8382

HERBASPIRILLUM-LIKE BACTERIA IN BANANA PLANTS

Olmar B. Weber[1]*; Leonardo M. Cruz[2]; José I. Baldani[3]; Johanna Döbereiner[4]

[1]Embrapa – Centro Nacional de Pesquisa de Agroindústria Tropical, Fortaleza, CE, Brasil. [2]Departamento de Bioquímica - Universidade Federal do Paraná, Curitiba, PR, Brasil. [3]Embrapa – Centro Nacional de Pesquisa de Agrobiologia, Seropédica, RJ, Brasil. [4]In memoriam

Submitted: December 11, 2000; Returned to authors for corrections: March 12, 2001; Approved: September 03, 2001

SHORT COMMUNICATION

ABSTRACT

Diazotrophic bacteria isolated from banana plants were characterized by morphological and physiological aspects. Three different groups of these plant-bacteria could be established. Two of them showed similarity to species of the *Herbaspirillum* genus. The third one was different because used only a few carbon substrates and produced water diffusible compounds that fluoresced under UV light. All three bacterial groups were thin rods with mono or bipolar flagella, presented negative reaction in Gram stain, showed catalase activity, were able to reduce nitrate and grew better in semi-solid JNFb medium at 31ºC. The nitrogenase activity was detected in semi-solid N-free JNFb medium and expressed higher values when pH ranged from 6.5 to 7.0 (groups I and II) and 6.0 to 6.5 (group III). The diazotrophs isolated from banana plants were distinct from species of *Herbaspirillum* previously identified in gramineous plants.

Key words: *Herbaspirillum, Herbaspirillum*-like, *Musa* spp., microbial ecology

Over the last decades there has been a great interest in nitrogen fixing-bacteria associated to non-legumes, principally gramineous plants. The bacterial genera *Azospirillum* (10,27), *Herbaspirillum* (2,3,13,23), *Burkholderia* (4,16) and *Gluconoacetobacter* (8) were identified in these plants. In addition, diazotrophs also occurred in sweet potato (17), cassava (5), coffee (19), pineapple (27,29) and banana plants (29).

The banana fruit crop is widely cultivated in tropical areas where high dosages of fertilizers are commonly applied. The biological nitrogen fixation could be an alternative for this crop system, once the plants are able to establish association with *Herbaspirillum*-like and *Burkholderia* related bacteria (29), which are not well known yet. This work aimed physiological and morphological characterization of the *Herbaspirillum*-like bacteria isolated from banana plants.

Among more than twenty-five strains of *Herbaspirillum*-like bacteria previously obtained (29), the representative diazotrophs from root samples of banana Caipira (BA12), stems of cultivars Butuhan (BA10) and Caipira (BA14, BA22), and leafs of cultivars Butuhan (BA11), Caipira (BA13), Prata Anã (BA22) and Maçã (BA87) were included in this study. All isolates were activated in semi-solid NFb medium (11) and grown on solid NFb and 79 + potato agar media (4) during three days at 30ºC. Controls (*Bacillus* sp. and *Escherichia coli*), grown on the last medium, and diazotrophs were evaluated for Gram stain, catalase and oxidase reactions (26). The nitrate reduction (7) with nitrite accumulation (21) of diazotrophs was evaluated after two days incubation in liquid DYGS medium (25) modified by adding 1 g/L of malate and pH adjusted to 6.5. All isolates and a strain of *Pseudomonas fluorescens* were also evaluated for fluorescence of siderophores on King B medium under UV light after two days incubation at 30ºC (20).

Nitrogenase activity of diazotrophic bacteria isolated from banana and type strains *Herbaspirillum seropedicae* Z67^T and *Herbaspirillum rubrisubalbicans* M4^T was evaluated in JNFb

* Corresponding author. Mailing address: Rua Dra. Sara Mesquita, 2270, Planalto Pici, 60511-110, Fortaleza, CE, Brasil, Fax: (+55 85) 299-1833, E-mail: weber@cnpat.embrapa.br

medium, at different pH and temperature of incubation (3). The inocula were prepared in liquid DYGS medium incubated for 24 h at 30ºC. Adjustment of pH (5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) was made by adding sterile 5% H_2SO_4 or 10% KOH to sterile semi-solid JNFb medium. Three vials containing 5 mL of this medium were inoculated with 15 µL aliquots of bacterial suspensions and incubated at 30ºC during 52 h. In the temperature experiment, the pH of semi-solid JNFb medium was adjusted to 6.0 and incubated for 48 h at 28, 31, 34, 37 and 41ºC. Nitrogenase activity in both experiments was determined by the C_2H_2 reduction method, after 1 h of incubation at 30ºC.

The cell morphology of isolates BA10, BA12 and BA22 grown on 79 + potato agar medium during two days at 30ºC was observed under transmission electron microscopy (TEM) ZEISS M-900 operated at 80 kW. The cells were fixed on silver 200 Hex Mesh grids coated with Formvar and stained with 5% uranil acetate solution (22).

The capability of isolates BA10, BA11, BA12, BA13, BA22, BA23 and Z67^T to survive in soil was also evaluated. All strains were grown in liquid DYGS medium for 24 h at 30ºC, centrifuged for 20min at 4.000 rpm and suspended in saline solution to 1.0 OD at 600 nm. Aliquots (1 mL) of bacterial suspensions were inoculated into 10 g samples of red-yellow podzolic soil (typic Hapludult). The soil samples were moistened to 70% field capacity and incubated at 30ºC. Three samples of each soil treatment were harvested at different times (1 h and 8, 16 and 24 days) and submitted to estimation of MPN diazotrophs, according to Döbereiner *et al*. (11).

On solid NFb medium, the banana plant *Herbaspirillum*-like bacteria formed colonies with blue centers. In semi-solid N-free JNFb medium, they formed veil-like membranes near the surface. All isolates were Gram negative, catalase and oxidase positive and were able to reduce nitrate in liquid DYGS medium similarly to *H. seropedicae* (3) and *H. rubrisubalbicans* (2,23).

The three bacterial groups were able to form good pellicles in semi-solid media containing D-mannitol, D-sorbitol, glycerol, citrate, α-ketoglutarate, succinate, fumarate and malate as sole carbon source. The isolates of group I were able to use N-acetyl-glucosamine, those of group II used meso-erythritol + NH_4, and those of group III were unable to use these carbon sources in semi-solid media (Table 1). The characteristic observed in isolates of group I was similar to that described for *H. seropedicae* (3), while those of group II was similar to *H. rubrisubalbicans* (2). However, differently from these species of *Herbaspirillum*, the two first groups were able to use L-tartrate as sole carbon source. The groups I and II had the same pattern as *H. rubrisubalbicans* in ARDRA analysis and showed phylogenetic relationship among the *Herbaspirillum* cluster (9). Both bacterial groups failed to react with species-specific oligonucleotide probes (29) and may represent new *Herbaspirillum* species.

The isolates from group III were able to produce blue fluorescent compounds on King B medium under UV light (Table 1), which has never been observed with diazotrophs before. The pigment production has been detected in plant pathogenic *Pseudomonas* species (18), *Burkholderia cepacia* (14) and

Table 1. Phenotypic differences of *Herbaspirillum*-like bacteria from banana plants and species of *Herbaspirillum* genus.

Characteristic	Groups of diazotrophs			*Herbaspirillum*	
	I	II	III	Z67^T	M4^T
Rod shape [a]	Curved	Curved	Straight	Curved	Curved
Size µm on batata+79) medium [a]	2.0 x 0.6 to 0.7	1.9 x 0.7 to 0.8	1.8 x 0.6 to 0.8	Un	Un
Flagellum	0 to 3	0 to 3	0 to 1	Un	Un
mono,	+	+	+		
bipolar [a]	+	+	-		
Fluorescence on King B medium [b]	-	-	+	-	-
pH in JNFb semi-solid medium [c]	6.5 to 7.0	6.0 to 7.0	6.0 to 6.5	6.0 to 7.0	6.0 to 7.0
Soil Surviving for 24 days [d]	Poor	Nd	Nd	Poor	Un
Use of carbon source in semi-solid media [c]					
D-Glucose	+	+	-	+	+
L-arabinose	+	+	-	+	+
Rhamnose	-	-	-	+	-
L-tartrate	+	+	-	-	-
Meso-erythritol + NH_4	-	+	-	-	+
N-acetylglucosamine	+	-	-	+	-

After Weber *et al.* (29); [a] Strains BA10 (group I), BA12 (group II) e BA22 (group III); [b] Strains BA10 e BA11 (group I), BA12, BA13 e BA14 (group II) and BA22, BA23 e BA87 (group III) and *Pseudomonas fluorescens*; [c] All fruit crop isolates; [d] Strains BA10 e BA11 (group I), BA12, BA13 (group II) and BA22, BA23 (group III); Characteristics undetermined (Un) or no-detected (Nd).

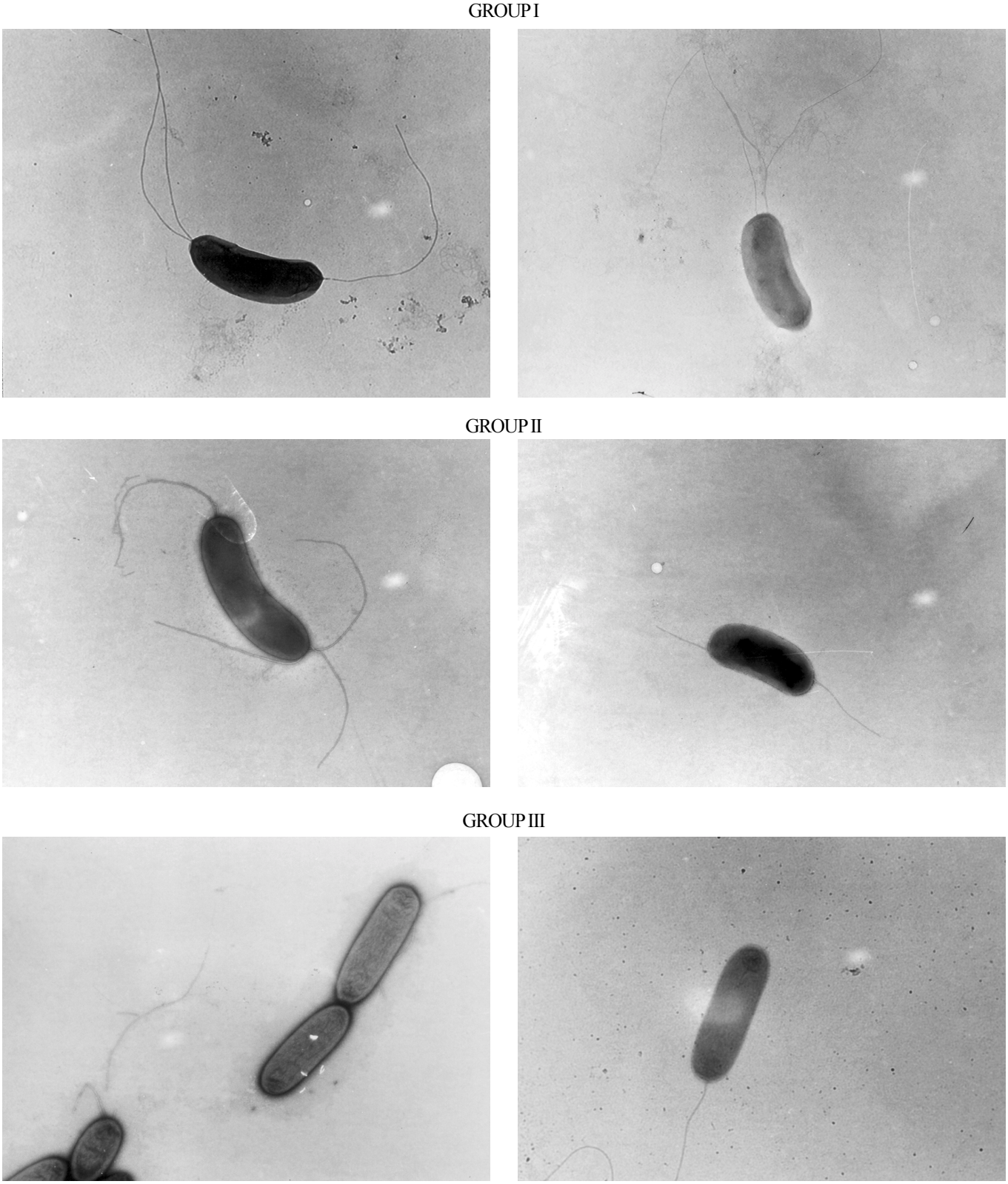

Figure 1. Negative staining of *Herbaspirillum*-like bacteria from banana plants. Young cells from isolates: BA10 (group I), BA12 (group II) and BA22 (group III) on 79 + potato medium. Bar with <==> 1.1 µm.

members of *Comamonadaceae* family (30) without nitrogenase activity. The phylogenetic relationship of ARDRA types from the bacterial group III with the *Comamonadaceae* was observed (9). The pigment production was a characteristic described for *Hidrogenophaga, Xilophilus and Brachymonas* genera (30).

The bacterial group III may represent a new genus of diazotrophic bacteria. The representative isolate (BA22) from the group was a straight rod (1.8 µm long and 0.6 to 0.8 µm wide) with only one polar flagellum (Table 1 and Fig. 1), distinguishing it from the known species of *Herbaspirillum* genus identified in gramineous plants. Short rods with one polar flagellum were observed in N_2-fixing *Pseudomonas* sp. isolated from rhizosphere of *Oryza sativa* (6) and described for *Brachymonas*, *Hidrogenophaga* and *Xilophilus* genera (30). However, these bacteria fermented sucrose, except the *Brachymonas* genus, which occurred in activated sludge (30). Another dinitrogen-fixing *Pseudomonas* sp., isolated from *Deschampsia caespitosa*, was a slightly curved rod and presented tuff polar flagella (15).

The bacterial groups I and II presented curved rod-shaped cells (Table 1 and Fig. 1) and were mobile in water, with increased movement next to air bubbles. The isolate BA10 (representative from group I) measured 2.0 µm by 0.6 to 0.7 µm and presented one to three polar flagella or 1+1 and 1+2 flagella in both cell poles, similarly to *H. seropedicae* (3). The isolate BA12 (representative of group II) presented 1.9 µm by 0.7 to 0.8 µm, with flagella similar to isolate BA10. Bipolar flagella had never been observed in strains of *H. rubrisubalbicans* (23).

Isolates from groups I and II presented higher nitrogenase activity in N-free JNFb medium when the pH ranged from 6.5 to 7.0 (Fig. 2) and the temperature was 31ºC (Fig. 3). No nitrogenase activity was detected in semi-solid JNFb medium at 41ºC. The optimal pH value for *H. seropedicae* ranged from 5.0 to 8.0 in malate nitrogen-free semi-solid medium, at 34ºC (3). Concerning the bacterial group III, we detected higher nitrogenase activity when the pH ranged from 6,0 to 6.5 (Fig. 2) and temperature of 31ºC (Fig. 3). The dinitrogen-fixing *Pseudomonas* sp. from rice plants grew better on semi-solid NFb medium with 0.5% glucose and 0.01% yeast extract, with pH ranging from 6.5 to 7.0 and temperature ranging from 30 to 35ºC (28).

The three bacterial groups survived poorly in a red-yellow podzolic soil. After three weeks, bacteria from groups II and III were no more detectable in the soil while less than 10^1 bacteria/g was observed for bacteria from group I. The poor surviving capability was also observed for bacteria of *Herbaspirillum* genus (23) and for *Burkholderia brasilensis* (4) and could be an indication of endophytic diazotrophs, as related by Döbereiner (12) and Baldani *et al*. (1). Further, the populations (10^2 to 10^3 bacteria per gram) of native *Azospirillum brasilense* and *Azospirillum lipoferum* present in the control soil showed no decrease during the incubation time. This genus of diazotrophic bacteria has been originally isolated from rhizosphere of gramineous plant (12) and behaves as facultative endophytic bacterium (1).

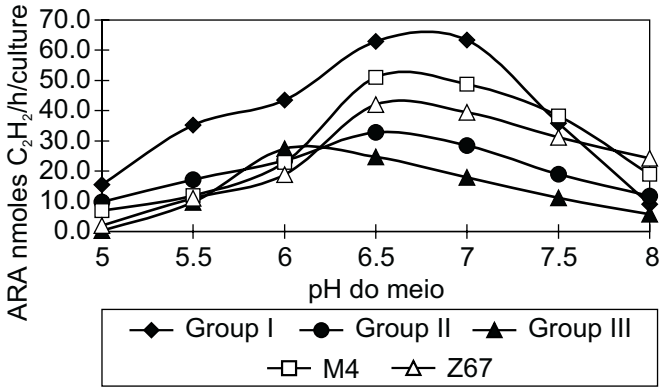

Figure 2. Effect of initial pH on the nitrogenase activities of log-phase cultures of *Herbaspirillum*-like bacteria from banana plants and type strains $Z67^T$ and $M4^T$. Average of isolates BA10 and BA11 (group I), BA12, BA13 and BA14 (group II) and BA22, BA23 and BA87 (group III).

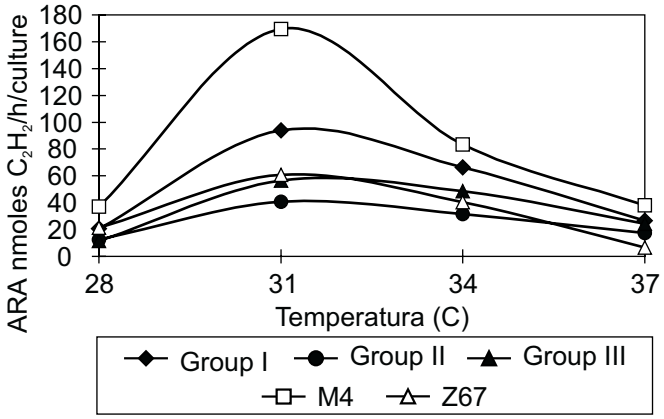

Figure 3. Effect of incubation temperature on the nitrogenase activities of log-phase cultures of *Herbaspirillum*-like bacteria from banana plants and type strains $Z67^T$ and $M4^T$. Average of isolates BA10 and BA11 (group I), BA12, BA13 and BA14 (group II) and BA22, BA23 and BA87 (group III).

ACKNOWLEDGEMENTS

The authors would like to express deep appreciation to Dr. Francisco Freire of Embrapa Tropical Agroindustry, for critically reviewing the manuscript.

RESUMO

Bactérias do tipo *Herbaspirillum* em bananeira

Bactérias diazotróficas do tipo *Herbaspirillum* isoladas de bananeiras foram avaliadas pelas características morfológicas

e fisiológicas de crescimento. Três grupos de bactérias foram estabelecidos, sendo dois relacionados às espécies de *Herbaspirillum* e diferentemente o terceiro grupo apresentou habilidade em crescer com poucos substratos orgânicos e produziu substância fluorescente em meio B de King. As bactérias dos três grupos eram bastonetes com flagelos mono ou bipolares, apresentaram reação negativa na coloração de Gram, expressaram atividade de catalase e oxidade, foram capazes de reduzir o nitrato e cresceram melhor em meio JNFb semi-sólido incubado a 31ºC. A atividade da nitrogenase, medida através da atividade de redução de acetileno, foi máxima em meio JNFb semi-sólido, após o ajuste de pH na faixa de 6,0 a 7,0 (grupos I e II) e 5,5 a 6,5 (grupo III). As bactérias diazotróficas associadas às bananeiras são diferentes das espécies de *Herbaspirillum* anteriormente identificadas em gramíneas.

Palavras-chave: *Herbaspirillum,* bactérias tipo *Herbaspirillum*, *Musa* spp., ecologia microbiana

REFERENCES

1. Baldani, J.I.; Caruso, L.; Baldani, V.L.D.; Goi, S.R.; Döbereiner, J. Recent advances in BNF with non-legume plants. *Soil Biol. Biochem*., 29:911-922, 1997.
2. Baldani, J.I.; Pot, B.; Kirchhof, G.; Falsen, E.; Baldani, V.L.D.; Olivares, F.L.; Hoste, Kersters, K.; Hartmann, A.; Gillis, M.; Döbereiner, J. Emended description of *Herbaspirillum*; inclusion of *[Pseudomonas] rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. *Int. J. Sys. Bacteriol*., 46:802-810, 1996.
3. Baldani, J.I.; Baldani, V.L.D.; Seldin, L.; Döbereiner, J. Characterization *of Herbaspirillum seropedicae* gen. nov., a root-associated nitrogen-fixing bacterium. *Int. J. Syst. Bacteriol*., 36:86-93, 1986.
4. Baldani, V.L.D. *Efeito da inoculação de Herbaspirillum spp. no processo de colonização e infecção de plantas de arroz, e ocorrência e caracterização parcial de uma nova bactéria diazotrófica*. UFRRJ, Seropédica, 1996. 238p. (D.Sc. thesis).
5. Balota, E.L. *Interação de bactérias diazotróficas e fungos micorrízicos arbusculares na cultura da mandioca (Manihot esculenta Crants)*. UFRRJ, Itaguaí, 1994. 281p. (D.Sc. thesis).
6. Barraquio, W.L.; Ladha, J.K.; Watanabe, I. Isolation and identification of N_2-fixing *Pseudomonas* associated with wetland rice. *Can. J. Microbiol*., 29:867-873, 1983.
7. Cataldo, D.A.; Haroon, M.; Shrader, L.E.; Youngs, V.L. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicilic acid. *Com. Soil Sci. Pl. An*., 6:71-80, 1975.
8. Cavalcante, V.A.; Dobereiner, J. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant and Soil*, 108:23-31, 1988.
9. Cruz, L.M.; Souza, E.M.; Weber, O.B.; Baldani, J.I.; Döbereiner, J.; Pedrosa, F.O. 16S rDNA characterization of new nitrogen-fixing bacteria from Banana (*Musa* spp.) and pineapple (*Ananas comosus* (L) Merril). *Appl. Environ. Microbiol*., 67:2375-2379, 2001.
10. Döbereiner J. Fixação de nitrogênio em gramíneas. *Rev. brasi. Ci. Solo*, 1:1-54, 1977.
11. Döbereiner J.; Baldani V.L.D.; Baldani J.I. *Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas*. Embrapa-SPI, Brasília, 1995. 60p.
12. Döbereiner, J. History and new perspectives of diazotrophs in association with non-leguminous plants. *Symbiosis*, 13:1-13, 1992.
13. Gillis, M.; Döbereiner, J.; Pot, B.; Good, M.; Falsen, E.; Hoste, B.; Reinhold, B.; Kersters, K. Taxonomic relationships between (*Pseudomonas) rubrisubalbicans*, some clinical isolates (EF group I), *Herbaspirillum seropedicae* and (*Aquaspirillum*) *autotrophicum*. *Dev. Plant and Soil Sci*., 48:293-294, 1991.
14. Gillis. M.; Van, T.V.; Bardin, R.; Goor, M.; Hebbar, P.; Willems, A.; Segers, P.; Kersters, K.; Heulin, T.; Fernandez, M.P. Polyphasic taxonomy in genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp: nov. for N_2-fixing isolates from rice in Vietnam. *Int. J. Syst. Bacteriol*., 45:274-289, 1995.
15. Haahtela, K.; Helander, I.; Nurmiaho-Lassila, E.L.; Sundman, V. Morphological and physiological characteristics and lipopolyssaccharide composition of N_2-fixing (C_2H_2-reducing) root-associated *Pseudomonas* sp. *Can. J. Microbiol*., 29:874-880, 1983.
16. Hartmann, A.; Baldani, J.I.; Kirchhof, G.; Assmus, B.; Hutzler, P.; Springer, N.; Ludwig, G.W.; Baldani, V.L.; Döbereiner, J. Taxonomic and ecological studies of diazotrophic rhizosphere bacteria phylogenetic probes. *In*: Fendrik, I.; Del Gallo, M.; Vanderleyden, J.; Zamaroczy, M. (Eds). *Azospirillum VI and related microorganisms: genetics, physiology and ecology*. Springer Verlag, New York, 1995. p.416-427.
17. Hill, W.A.; Bacon Hill, P.; Crossman, S.M.; Stevens, C. Characterization of N_2-fixing bacteria associated with sweet potato roots. *Can. J. Microbiol*., 29:860-862, 1983.
18. Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T.; Williams, S.T. *Bergey's manual of determinative bacteriology*, 9.ed. Williams & Williams, Baltimore, 1994. 787p.
19. Jiménez Salgado, T.; Fuentes Ramires, L.E.; Tapia Hernandes, A.; Mascarua Esperanza, M.A.; Martinez Romero, E.; Caballero Mellado, O. *Coffea arabica* L., a new host plant for *Acetobacter diazotrophicus* and isolation of the nitrogen-fixing-acetobacteria. *Appl. Environ. Microbiol.*, 63:3676-3683, 1998.
20. King, E.O.; Ward, M.K.; Raney, D.E. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Cl. Med*., 44:301-307, 1954.
21. Neyra, C.A.; Döbereiner, J.; Lalande, R.; Knowles, R. Denitrification by N_2-fixing *Spirillum lipoferum*. *Can. J. Microbiol*., 23:300-305, 1976.
22. Olivares, F.L. *Taxonomia, ecologia e mecanismos envolvidos na infecção e colonização de plantas de cana-de-açúcar (Saccharum sp. híbrido) por bactérias endofíticas do gênero Herbaspirillum*. UFRRJ, Seropédica, 1997. 344p. (D.Sc. thesis).
23. Olivares, F.L.; Baldani, V.L.D.; Reis, V.M.; Baldani J.I.; Döbereiner, J. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of Gramineae. *Biol. Fertil. Soils.*, 21:197-200, 1996.
24. Patriquim, D.G.; Döbereiner, J. Light microscopy observation of tetrazolium reducing bacteria in endorhizosphere of maise and other grasses in Brazil. *Can. J. Microbiol*., 24:734-742, 1978.
25. Rodrigues Neto, J.; Malavolta Jr., V.A.; Victor, O. Meio simples para isolamento e cultivo de *Xanthomonas campestris* pv. citri Tipo B. *Sum. Phytol*., 2:16-16, 1986.
26. Smibert, R.; Krieg, N.R. Genaral characterization. *In*: Gerhard, P.; Murray, R.G.E.; Costilow, R.N.; Nester, E.W.; Wood, W.A.; Krieg, N.R.; Phillips, G.B. (ed.). *Manual of methods for general bacteriology*. Am. Soc. Microbiol, Washington, 1981. p409-443.
27. Tapia-Hernandez, A.; Bustillos-Cristales, M.R.; Jimenez-Salgado, T.; Caballero-Mellado, J.; Fuentes-Ramírez, L.E. Natural endophytic occurrence of *Acetobacter diazotrophicus* in pineapple plants. *Microbial Ecol*., 39:49-55, 2000.
28. Watanabe, I.; So, R.; Ladha, J.K. A new nitrogen-fixing species of pseudomonad: *Pseudomonas diazotrophicus* sp. nov. isolated from the root of wetland rice. *Can. J. Microbiol*., 33:670-678, 1987.
29. Weber, O.B.; Baldani, V.L.D.; Teixeira, K.R.S.; Kirchhof, G.; Baldani, J.I.; Döbereiner, J. Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. *Plant Soil*., 210:103-213, 1999.
30. Wen, A.; Fegan, M.; Hayward, C.; Chakraborty, S.; Sly, L.I. Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka *et al*. 1987) gen. Nov., comb.nov. *Int. J Sys. Bacteriol*., 49:567-576, 1999.