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Research Article

Diazotrophic Bacterial Community of Degraded Pastures

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Pasture degradation can cause changes in diazotrophic bacterial communities. Thus, this study aimed to evaluate the culturable and total diazotrophic bacterial community, associated with regions of the rhizosphere and roots of *Brachiaria decumbens* Stapf. pastures in different stages of degradation. Samples of roots and rhizospheric soil were collected from slightly, partially, and highly degraded pastures. McCrady's table was used to obtain the Most Probable Number (MPN) of bacteria per gram of sample, in order to determine population density and calculate the Shannon-Weaver diversity index. The diversity of total diazotrophic bacterial community was determined by the technique of Denaturing Gradient Gel Electrophoresis (DGGE) of the *nif*H gene, while the diversity of the culturable diazotrophic bacteria was determined by the Polymerase Chain Reaction (BOX-PCR) technique. The increase in the degradation stage of the *B. decumbens* Stapf. pasture did not reduce the population density of the cultivated diazotrophic bacterial community, suggesting that the degradation at any degree of severity was highly harmful to the bacteria. The structure of the total diazotrophic bacterial community associated with *B. decumbens* Stapf. was altered by the pasture degradation stage, suggesting a high adaptive capacity of the bacteria to altered environments.

1. Introduction

In Brazil, there are approximately 180 million hectares of pastures and the great majority have species from the genus *Brachiaria* spp. [1]. Diagnoses performed in the country found that 70% of the pastures are at some level of degradation [2]. Various factors contribute to the degradation of these pastures, such as inadequate management of the cultivation area, use of forage species little adapted to the edaphoclimatic conditions, and reduction of soil fertility, due to erosion, fixation of nutrients by the soil, and extraction for conversion into products of animal origin [3].

The use of new technologies in the crop-livestock production systems has acquired relevance, for contributing with more-sustainable systems, causing the management to allow lower degradation of the pastures and impacts on the soil and minimizing the damage on the beneficial microbiota associated with the plants [4, 5]. These microorganisms, especially diazotrophic bacteria, have the capacity to fix atmospheric N and solubilize inorganic phosphate, reestablishing soil fertility and reducing the negative impacts caused by the use of chemical fertilizers, besides being related to the promotion of plant growth [6, 7].

Diazotrophic bacteria can be associated with many plants [8], providing them with a high degree of resistance to different types of stress [9]. Although the importance of these bacteria in cultivation systems of forage grasses is frequently acknowledged [1], the interactions between these

microorganisms and these plants under different management regimes are not yet well understood [10]. In spite of that, the comparative study of the functional beta-diversity of bacterial communities in contrasting environments has been used as a parameter to indicate stability of biotic and abiotic phenomena in ecosystems [11, 12].

Therefore, this study aimed to evaluate the density and diversity of culturable and total diazotrophic bacterial community, present in rhizospheric and root-endophytic niches in *B. decumbens* Stapf. pastures in different stages of degradation.

2. Materials and Methods

2.1. Characterization of the Sites of Collection of Plant and Rhizospheric Soil Samples and Pasture Management. Plant and rhizospheric soil samples were collected from pastures in three degradation stages (highly, partially, and slightly degraded). The pastures were of *B. decumbens* Stapf. cultivated at the Riacho do Papagaio Farm, in the municipality of São João, Pernambuco state, Brazil (08°52′30″S and 036°22′00″W). The municipality is located at an altitude of 716 m [13].

According to the data of the Water and Climate Agency of Pernambuco [14], the mean annual rainfall is 780 mm and the rainiest four months are from May to August. The mean annual temperature is 21.1°C and the climate is classified as Aw, according to Beltrão et al. [13].

The soil of the pasture cultivation area was classified as typic eutrophic Regolithic Neosol [15, 16]. Ten samples of soil were randomly collected in the layer of 0.0–0.2 m in each area of degraded pasture and transformed into a sample composed of area for soil physical and chemical characterization (Table 1).

The chemical characterization evaluated pH (H_2O), Ca^{2+}, Mg^{2+}, K^+, Na^+, Al^{3+}, (H + Al), P, and TOC (total organic carbon). Ca^{2+}, Mg^{2+}, and Al^{3+} were extracted with 1.0 mol L^{-1} KCl and dosed through titration. P, K^+, and Na^+ were extracted with Mehlich-1; P was dosed through spectrophotometry and K^+ and Na^+ through flame photometry. Potential acidity (H + Al) was extracted with 0.5 mol L^{-1} calcium acetate and dosed through titration. The TOC was determined through wet combustion using potassium dichromate and dosed through titration. All analyses were performed according to the methodologies described by Donagema et al. [17].

The results of these chemical analyses were used to calculate base saturation (V), Al saturation (m), effective cation exchange capacity ($CEC_{effective}$), and potential cation exchange capacity ($CEC_{potential}$). The soil was physically characterized through granulometric analysis, defining its textural class [17].

Based on the criteria for pasture degradation established by Stoddart et al. [18] and in the application time of the N, three areas were selected for the collection of plant and rhizospheric soil samples to evaluate the bacterial community: highly degraded pasture, with 30 years of formation without fertilization (P1), geographic coordinates 08°49′11″S and 036°23′38″W; partially degraded pasture, with 10 years of formation and six years without fertilization (P2), geographic coordinates 08°48′35″S and 036°24′27″W; and slightly degraded pasture, with six years of formation and three years without fertilization (P3), geographic coordinates 08°49′00″S and 036°23′38″W.

When the pastures were fertilized, 100 kg ha^{-1} of N was applied broadcast, annually split along the rainy period (May to August). The N source was urea and P and K were not applied. Ten plant samples were collected in each area (P1, P2, and P3).

Sampling was randomly performed in each area of degraded pasture. The samples of rhizospheric soil were collected at the same sites where the plant material was collected in the layer of 0.0–0.2 m in each area. After root collection the excess soil was withdrawn through circular movements and in laboratory the remaining soil in direct contact with the roots was collected (rhizospheric soil).

2.2. Population Density of the Cultivated Diazotrophic Bacterial Community. Rhizospheric and root-endophytic diazotrophic bacteria were isolated according to Döbereiner et al. [19] and Kuklinsky-Sobral et al. [20].

In order to isolate bacteria present in the rhizosphere, 5 g of rhizospheric soil was placed in containers with 5 g of glass beads (0.1 cm) and 50 mL of phosphate-buffered saline (PBS), containing 1.44 g L^{-1} of Na_2HPO_4; 0.24 g L^{-1} of KH_2PO_4; 0.20 g L^{-1} of KCl; and 8.00 g L^{-1} of NaCl, buffered at pH 7.4. These containers were maintained under agitation (90 rpm) at ambient temperature for 40 min. Then, serial dilutions (10^{-4}, 10^{-5}, and 10^{-6}) were performed in the same PBS buffer, and aliquots of these solutions were inoculated in selective semisolid NFb medium [5 g L^{-1} of malic acid; 0.5 g L^{-1} of K_2HPO_4; 0.2 g L^{-1} of $MgSO_4{\cdot}7H_2O$; 0.1 g L^{-1} of NaCl; 0.01 g L^{-1} of $CaCl_2{\cdot}2H_2O$; 4 mL L^{-1} of Fe·EDTA (1.64% solution); 2 mL L^{-1} of bromothymol blue (0.5%); 2 mL L^{-1} of solution of micronutrients (0.2 g L^{-1} of $Na_2MoO_4{\cdot}2H_2O$; 0.235 g L^{-1} of $MnSO_4{\cdot}H_2O$; 0.28 g L^{-1} of H_3BO_3; 0.008 g L^{-1} of $CuSO_4{\cdot}5H_2O$); 1.75 g L^{-1} of agar, buffered at pH 6.8], added to 50 μg mL^{-1} of the fungicide Cercobin 700 (thiophanate methyl), per sample.

For the isolation of root-endophytic bacteria, the shoots of the plant samples were disregarded and the roots were washed in distilled water. Approximately 3 to 5 g of roots were subjected to the process of superficial disinfection (1 min in 70% alcohol; 3 min in Na hypochlorite; 30 sec in alcohol; and two washings in sterile distilled water). The disinfection efficiency was evaluated in control samples. Then, the protocol described above of inoculation used for rhizospheric soil bacteria was used, with serial dilutions (10^{-3}, 10^{-4}, and 10^{-5}).

The population density of the cultivated diazotrophic bacterial community was determined using the McCrady's table, obtaining the Most Probable Number (MPN) of bacteria per gram of sample, according to Döbereiner et al. [19].

After isolation in semisolid NFb medium, 90 bacteria were subcultured and isolated in solid NFb medium, 30 of each pasture (P1, P2, and P3) and 15 of each niche (rhizospheric soil and root-endophytic). The selection of the

Table 1: Soil chemical and physical attributes in the layer of 0.0–0.2 m of pastures cultivated in different degradation stages for the collection of plant and rhizospheric soil samples to characterize the diazotrophic bacterial community.

Attribute	Pasture		
	Highly degraded	Partially degraded	Slightly degraded
pH water (1 : 2.5)	6.02	5.05	6.01
Ca^{2+} ($cmol_c\ dm^{-3}$)	0.45	0.40	0.50
Mg^{2+} ($cmol_c\ dm^{-3}$)	0.15	0.10	0.20
K^{+} ($cmol_c\ dm^{-3}$)	0.12	0.18	0.12
Na^{+} ($cmol_c\ dm^{-3}$)	0.02	0.02	0.03
Al^{3+} ($cmol_c\ dm^{-3}$)	0.10	0.15	0.10
(H + Al) ($cmol_c\ dm^{-3}$)	0.40	0.25	0.35
P ($mg\ dm^{-3}$)	15.44	52.63	31.79
TOC ($g\ kg^{-1}$)[1]	1.20	0.70	0.60
$CEC_{effective}$ ($cmol_c\ dm^{-3}$)[2]	0.84	0.85	0.95
$CEC_{potential}$ ($cmol_c\ dm^{-3}$)[3]	1.14	0.91	1.20
V (%)[4]	64.91	76.92	70.83
m (%)[5]	11.90	17.65	10.53
Total sand ($g\ kg^{-1}$)	92.13	91.82	91.94
Coarse sand ($g\ kg^{-1}$)	76.45	75.86	74.92
Fine sand ($g\ kg^{-1}$)	15.68	15.96	17.02
Silt ($g\ kg^{-1}$)	4.41	5.40	3.24
Clay ($g\ kg^{-1}$)	3.45	2.77	4.83
Textural class	Sandy	Sandy	Sandy

[1]Total organic carbon. [2]Effective cation exchange capacity. [3]Potential cation exchange capacity. [4]Base saturation. [5]Al saturation.

bacterial isolates was performed based on the morphologic variability of the colonies. The selected bacterial isolates were evaluated for growth in a culture medium free from N source at 28°C for eight days [19]. Again positive growth was considered as the isolate with characteristic halo of growth observed inside the culture medium. The test was performed in triplicate and repeated for two times to better confirm the results.

The use of the semisolid NFb medium favored the selection of diazotrophic bacteria because they moved to a region where the oxygen diffusion rate was in equilibrium with the respiration rate, forming a bacterial growth film. Nitrogenase was activated when excess oxygen was removed by higher bacterial growth. Therefore, the bacterial isolate that was capable of forming growth-characteristic film on semisolid NFb medium was considered diazotrophic [19]. No other techniques were used to validate the diazotrophic character of the bacteria, such as acetylene reduction activity (ARA), or amplification of the *nif* H gene.

2.3. Genetic Variability of the Cultivated Diazotrophic Bacterial Community. The 90 bacterial isolates were evaluated for genetic variability through the *Polymerase Chain Reaction* (BOX-PCR) technique. Bacterial DNA was extracted using Genomic DNA Purification Kit (Fermentas). After extraction of the bacterial DNA, PCR reaction was performed with the primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′), with final volume of 25 μL, containing 1 μL of total DNA (10 ng quantified in nanodrop spectrophotometer); 1 μM of the primer; 1 mM of each dNTPs; 1x of DMSO (dimethyl sulfoxide); 1x of the enzyme buffer, *Taq Buffer*; 3.5 mM of $MgCl_2$; and 0.08 U of the enzyme Taq DNA polymerase. The amplification started with 95°C for 2 min; 35 cycles at 94°C for 2 min; 92°C for 30 sec; 50°C for 1 min; 65°C for 1 min; and one final step of 65°C for 10 min. The PCR product was evaluated through 1.5% agarose gel electrophoresis in a 1x TAE buffer for 4 h and dyed with *blue green loading*. The molecular marker used to compare size/weight of the bands was 1 kb.

BOX-PCR matrix was made manually, observing the presence and absence of the bands in the gel, obtaining the binary matrix of the genetic profile.

2.4. Diversity of the Total Diazotrophic Bacterial Community. The diazotrophic bacterial diversity was analyzed by the technique of *Denaturing Gradient Gel Electrophoresis* (DGGE) of the gene *nif* H. The DNA of the rhizospheric soil and roots was extracted and the integrity and quality of the DNAs were verified through 1% agarose gel electrophoresis, in a 1x TAE. The *nif* H gene was amplified using the primers FGPH19 (5′-TACGGCAARGGTGGNATH-3′) and PolR (5′-ATSGCCATCATYTCRCCG-3′), with final volume of 25 μL containing 10 ng of total DNA of soil or root; 1 μM of each primer; 1 mM of each dNTPs; 1x of the enzyme buffer, *Taq Buffer*; 3.5 mM of $MgCl_2$: 0.05 μ of BSA *(Bovine Serum Albumin)*: and 0.08 U of the enzyme Taq DNA polymerase.

The amplification cycles were 5 min at 94°C; 30 cycles of 1 min at 94°C; 1 min at 56°C; 2 min at 72°C; and one final step of 30 min at 72°C.

The generated amplicons were used in a second reaction for the *nif*H gene, using the primers PolF-GC (5′-GCC-CGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGC-CCCTGCGAYCCSAARGCBGACTC-3′) and AQER (5′-ACGATGTAGATYTC CTG-3′). The amplification occurred in 50 μL containing 10 ng of DNA of the first reaction; 1 μM of each primer; 1 mM of each dNTPs; 1x of the enzyme buffer, *Taq Buffer*; 3.5 mM of $MgCl_2$; and 0.08 U of the enzyme Taq DNA polymerase. The amplification cycles were 5 min at 94°C; 30 cycles of 1 min at 94°C; 1 min at 48°C; 2 min at 72°C; and one final step of 30 min at 72°C. The utilized gels were of 6% polyacrylamide, with denaturing gradient of 40 to 65% and a 1x TAE, dyed with SYBR-gold.

The analyses through DGGE were performed in the Ingeny PhorU System (Ingeny, Goes, Netherlands). For the analysis, 6% polyacrylamide gel (w/v) was prepared with denaturing gradient of 40 to 65% for the PCR product of the *nifH* gene. The gel was subjected to electrophoresis for 16 h run at 75 Volts, at temperature of 60°C. After the electrophoresis run, the gels were dyed with SYBR-gold (Invitrogen, Breda, Netherlands) and a 1x TAE at the proportion of 1 : 10,000 for 30 min and photodocumented.

The amplicons profiles obtained from the PCR-DGGE were analyzed and compared using the program Image Quant Software (Molecular Dynamics, Sunnyvale, CA, USA), generating a matrix of presence and absence of bands.

2.5. Shannon-Weaver Diversity Index. Based on the genetic matrix, the Shannon-Weaver diversity index (H') was calculated according to the following expression: $H' = \sum P_i \times \ln P_i$, in which $\sum P_i = n_i/N$, where n_i is the number of genetically different isolates; N is the total number of isolates; and ln is the natural logarithm [21]. This index was calculated for both cultivated diazotrophic bacterial community and total diazotrophic bacterial community.

2.6. Statistical Procedures. The sampling sufficiency in each pasture area was statistically determined based on the coefficient of variation (CV), mean angular error ($f = 50\%$), and the tabulated value of the Student's t-distribution with a 0.05 significance level ($p \leq 0.05$) and ($n - 1$) degrees of freedom [22, 23].

Thus, the data of bacterial density and the Shannon-Weaver diversity indices were analyzed as a factorial arrangement (3×2), consisting of three stages of pasture degradation, two niches of colonization, and 10 replicates. The data of density were transformed to $\log_{10} x$ and the means were subjected to analysis of variance by the Fisher-Snedecor's F statistics. When the main factors and/or the interactions were significant, the Scott-Knott test ($p \leq 0.05$) was applied for the comparison of means.

The diversities of diazotrophic bacterial communities evaluated through the DGGE and BOX-PCR techniques were analyzed through multivariate analysis and the variation between communities was determined by the analysis of similarity, which identifies significant differences based on algorithms of mean distances between the bacterial groups, generating R correlation [24].

Table 2: Population density of the cultivated diazotrophic bacterial community in different niches of plant/bacteria association in *B. decumbens* Stapf. pasture in different degradation stages.

Pasture degradation stage	Niche of plant/bacteria association		Mean
	Rhizospheric soil	Root-endophytic	
	CFU/g ($\log_{10}$)		
Highly degraded	6.70^{Aa}	5.91^{Aa}	6.31
Partially degraded	7.10^{Aa}	5.92^{Ba}	6.51
Slightly degraded	6.31^{Aa}	6.15^{Aa}	6.23
Mean	6.70	5.99	
	F test ($p \leq 0.05$)		
Degradation stage	0.582^{ns}		
Niche of association	10.482^{**}		
Stage × association	1.783^{*}		
CV (%)[1]	7.34		

[1]Coefficient of variation (CV) = 100 × standard deviation/average. Means followed by the same letter, uppercase in the row and lowercase in the column, do not differ statistically by the Scott-Knott test ($p \leq 0.05$). *,**Significant at 5 and 1% probability by F test, respectively. ^{ns}Not significant.

3. Results and Discussion

3.1. Cultivated (Not Validated) Diazotrophic Bacterial Community. The population density of the cultivated diazotrophic bacterial community varied with the niche of plant/bacteria association and was only higher in the rhizospheric soil, when the pasture was under the partially degraded condition (Table 2).

Population density of the bacterial community did not differ between niches, when the pasture was slightly degraded (Table 2). As the pasture degraded, the rhizospheric soil niche showed more diazotrophic bacteria, while there was a reduction in the root-endophytic niche, probably due to the deficient nutrition of the degraded pasture, which did not meet the needs of these microorganisms. The rhizosphere is the region of release of root exudates and concentration of organic matter, which make this niche an environment of microbial interaction and selection [25, 26].

Additionally, it should be pointed out that, during the collection of the plant material (root) and rhizospheric soil, the highly and partially degraded pastures had been at rest for six months, while the slightly degraded pasture had been at rest for only one month. This favored a greater deposition of organic matter on the soil (Table 1) and consequently greater bacterial density (Table 2). According to Rasche et al. [27], the organic matter is an important source of energy for the growth of soil microorganisms.

When the pasture was even more degraded, the soil also lost its capacity of nutrition, for both pasture and microorganisms, especially because of the accentuated reduction

Table 3: *R* values of the analysis of similarity of the diversity of the cultivated diazotrophic bacterial community determined through *Polymerase Chain Reaction* (BOX-PCR) in different niches of plant/bacteria association in *B. decumbens* Stapf. pasture in different stages of degradation.

Degradation stage and association region	P1-R[1]	P2-R[2]	P3-R[3]	P1-E[4]	P2-E[5]	P3-E[6]
P1-R		—	—	—	—	—
P2-R	0.113		—	—	—	—
P3-R	0.109	0.076		—	—	—
P1-E	0.241	0.230	0.210		—	—
P2-E	0.089	0.154	0.054	0.192		—
P3-E	0.062	0.141	0.214	0.144	0.113	

[1]P1-R = highly degraded pasture and rhizospheric soil niche; [2]P2-R = partially degraded pasture and rhizospheric soil niche; [3]P3-R = slightly degraded pasture and rhizospheric soil niche; [4]P1-E = highly degraded pasture and root-endophytic niche; [5]P2-E = partially degraded pasture and root-endophytic niche; [6]P3-E = slightly degraded pasture and root-endophytic niche.

Table 4: Shannon-Weaver diversity index of the cultivated diazotrophic bacterial community in different niches of plant/bacteria association in *B. decumbens* Stapf. pasture in different stages of degradation.

Pasture degradation stage	Niche of plant/bacteria association		Mean
	Rhizospheric soil	Root-endophytic	
		H'	
Highly degraded	2.30^{Aa}	1.75^{Bb}	2.03
Partially degraded	1.98^{Ab}	2.35^{Aa}	2.16
Slightly degraded	2.47^{Aa}	2.02^{Ab}	2.24
Mean	2.25	2.04	
	F test ($p \leq 0.05$)		
Degradation stage	1.260^{ns}		
Niche of association	3.400^{ns}		
Stage × association	6.736^{**}		
CV (%)[1]	24.92		

[1]Coeffcient of variation (CV) = 100 × standard deviation/average. Means followed by the same letter, uppercase in the row and lowercase in the column, do not differ statistically by the Scott-Knott test ($p \leq 0.05$). **Significant at 1% probability by F test. ^{ns}Not significant.

in P contents (Table 1), causing the difference between the rhizospheric and root-endophytic potentially diazotrophic bacterial communities to no longer exist (Table 2).

Impacts on the community of microorganisms associated with the plant and present in the soil are expected after the use of chemical fertilizers [28, 29] and changes in the management [10].

The population density of the cultivated diazotrophic bacterial community did not change with the degradation stage of the pasture in each niche individually. Neither the bacterial population of the rhizospheric-soil niche nor the root-endophytic population was altered with the degradation of the pastures (Table 2). This suggests that, in any degradation stage of the pasture, there was a reduction in the population density, regardless of the severity of the degradation. The severity did not intensify the reduction in the bacterial population, because the population was already reduced in the beginning of the degradation and, subsequently, it did not change with the increase in degradation.

The multivariate analysis of the data of genetic diversity of the cultivated diazotrophic bacterial community determined through BOX-PCR did not indicate the formation of independent groups between the different stages of pasture degradation and the niches of plant/bacteria association, with overlapping between the bacterial groups (Figure 1). This was referenced by the *R* value of the analysis of similarity, which was lower than 0.25 for all intersections of the groups (Table 3).

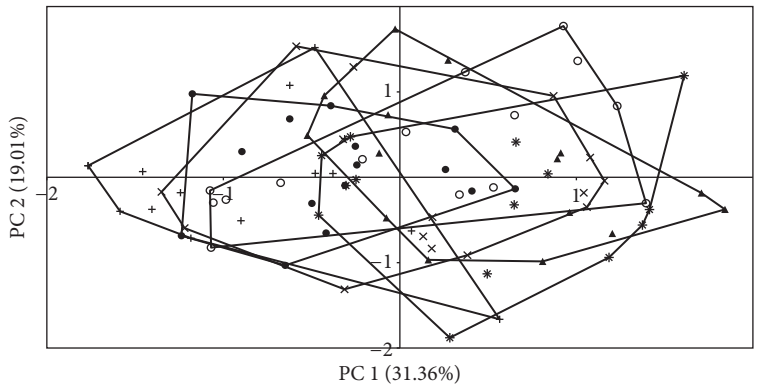
The isolates of the cultivated diazotrophic bacterial community did not change with pasture degradation, and the bacteria that colonized the rhizospheric soil were not different from those that endophytically colonized the roots. The greater specificity of this community did not allow an adaptation of the bacteria to the different stages of pasture degradation. The population density probably decreased, but the structure of the community remained unchanged, justifying the overlapping of the data of diversity of the groups.

The Shannon-Weaver diversity index of the cultivated diazotrophic bacterial community varied with the degradation stage of the *B. decumbens* Stapf. pasture and depended on the niche of plant/bacteria association (Table 4). For the isolates of rhizospheric soil and root-endophytic bacteria, the diversity in the slightly degraded pasture was similar to that of the highly degraded pasture. The diversity index of the rhizospheric bacterial community was higher than that of the root-endophytic bacterial community, only in the highly degraded pasture (Table 4).

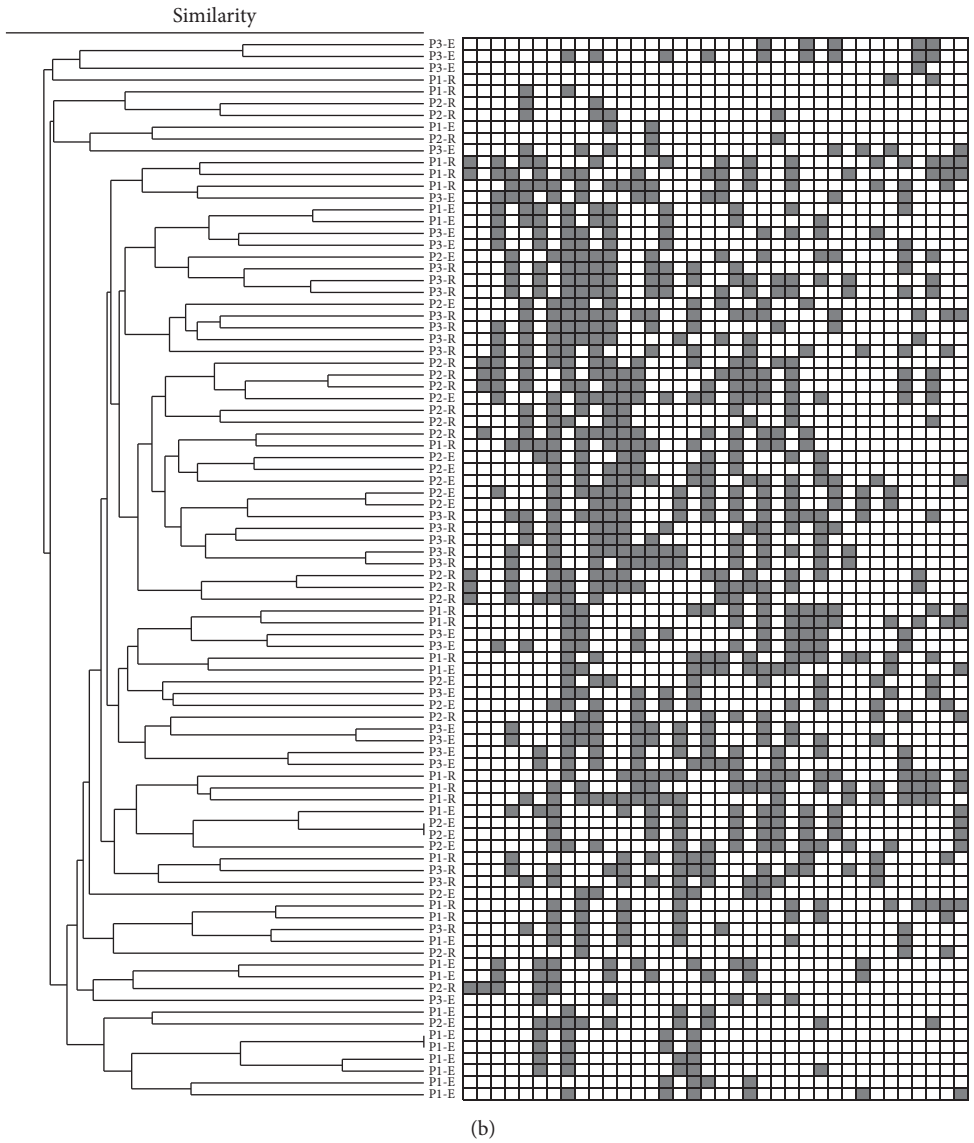
The low N content of tropical soils has been the main limiting factor for growth and development of forage grasses in the tropics [1, 3]. The association between diazotrophic bacteria and forage grasses can be able to overcome part of this limitation to plant development [30]. However, natural and anthropic imbalances can influence the bacterial community, interfering with its biochemical activity [31] and genetic diversity [27].

The diazotrophic community can be altered, especially by soil characteristics, by exudation patterns of the plants [32], by type of vegetation, among plant genotypes, by phenological stage, and by niche of colonization [6, 10, 26, 33], besides the action of animals on the vegetation, during grazing [34]. However, the association of these bacteria with forage grasses constitutes an important resource to be used in the recovery of areas with soil and grasses in various stages of degradation [7, 30].

The density and diversity of diazotrophic bacteria in association with forage grasses have been reported in the



(a)



(b)

Figure 1: Principal component (PC) analysis (a) and dendogram of the binary matrix (b) of the diversity of cultivated potentially diazotrophic bacteria determined through *Polymerase Chain Reaction* (BOX-PCR) in different niches of plant/bacteria association in *B. decumbens* Stapf. pasture in different stages of degradation. × represents highly degraded pasture and rhizospheric soil niche; + represents highly degraded pasture and root-endophytic niche; ○ represents partially degraded pasture and rhizospheric soil niche; ∗ represents partially degraded pasture and root-endophytic niche; ▲ represents slightly degraded pasture and rhizospheric soil niche; and ● represents slightly degraded pasture and root-endophytic niche.

Table 5: Values of R of the analysis of similarity of the culture-independent total diazotrophic bacterial community determined through *Denaturing Gradient Gel Electrophoresis* (DGGE) of the *nif* H gene in different regions of the plant/bacteria association in *B. decumbens* Stapf. pasture in different stages of degradation.

Degradation stage and association region	P1-R[1]	P2-R[2]	P3-R[3]	P1-E[4]	P2-E[5]	P3-E[6]
P1-R		—	—	—	—	—
P2-R	0.748		—	—	—	—
P3-R	0.944	0.796		—	—	—
P1-E	0.759	0.791	1.000		—	—
P2-E	0.833	0.741	1.000	0.815		—
P3-E	0.163	0.796	0.926	0.963	0.889	

[1]P1-R = highly degraded pasture and rhizospheric soil niche; [2]P2-R = partially degraded pasture and rhizospheric soil niche; [3]P3-R = slightly degraded pasture and rhizospheric soil niche; [4]P1-E = highly degraded pasture and root-endophytic niche; [5]P2-E = partially degraded pasture and root-endophytic niche; [6]P3-E = slightly degraded pasture and root-endophytic niche.

literature [35, 36], but the management of cultivation of these grasses and the niche of colonization can alter this community [12, 30]. Therefore, due to the capacity to colonize intra- and intercellular spaces, the endophytic microorganisms have a close relationship with their host [10], which contributed to reducing their diversity.

The advance of the knowledge about the microbial diversity was provided by the evolution of the methodologies of molecular biology applied to the study of the environment [37]. The study of bacterial diversity can be performed by the BOX-PCR technique, which uses the DNA of bacteria cultured *in vitro*. However, bacterial culture provides limited information, because culture medium does not accurately reproduce the different ecological niches in the laboratory environment [38, 39].

The PCR-DGGE technique evaluates the predominant microbial communities in the soil or that associated with the plant [40]. This technique can be used to evaluate changes in microbial community structure in response to changes in environmental and management parameters [41].

3.2. Total Diazotrophic Bacterial Community. The multivariate analysis of the data of genetic diversity of the total diazotrophic bacterial community determined through DGGE indicated the formation of independent groups between the different pasture degradation stages and plant/bacteria niches, except for the diversity of the total diazotrophic bacterial community of the rhizosphere of the highly degraded pasture, which grouped with the root-endophytic bacterial community of the slightly degraded pasture (Figure 2).

This grouping was referenced by the R value of the analysis of similarity, which was 0.163 for the group of the highly degraded pasture and rhizospheric soil niche with the group of the slightly degraded pasture and root-endophytic niche (Table 5). According to Clarke and Gorley [24], values of $R > 0.75$ determine different groups. For $0.5 < R < 0.75$, the groups are also different, but with overlaps, while values of $R < 0.25$ indicate overlapping of the groups.

The total diazotrophic bacterial community was altered by the pasture degradation stage and niche of bacterial colonization. The bacterial community adapted to the more degraded environment. In addition, the bacteria that colonized the rhizospheric soil were different from those that endophytically colonized the roots (Figure 2).

On the other hand, a relevant result was that bacteria that colonized the root-endophytic niche in the slightly degraded pasture was the same as rhizospheric soil when the pasture degraded, justifying the overlapping of these two groups (Figure 2 and Table 5).

The diazotrophic bacterial communities associated with *B. decumbens* Stapf. in different degradation stages and regions of plant/bacteria association were positively altered to adapt to soil conditions, plant nutritional requirements, changes in the exudation pattern of the plant, and pasture diversity, which increases in degraded areas to promote the permanence of the grass under stress conditions, in agreement with the results reported by Bacon and Hinton [9].

The Shannon-Weaver diversity index numerically indicates which is the biological diversity of a sample [42]. This index of the total diazotrophic bacterial community varied with the degradation stage of the *B. decumbens* Stapf. pasture and depended on the region of plant/bacteria association (Table 6). For the bacterial isolates of the rhizospheric soil, the diversity was significantly higher in the slightly degraded pasture. However, the diversity of root-endophytic bacterial isolates increased with the increase in the pasture degradation stage (Table 6).

In the rhizosphere, the more fertile the environment, the greater the diversity of microorganisms. As the fertility of the environment decreases, with the degradation of the pastures, for example, the diversity tends to decrease, because the region becomes selective, leaving only a small part of microorganisms more resistant to unfavorable conditions of the environment [6, 26].

The PCR-DGGE technique used in this study aimed to evaluate bacterial diversity in general. DGGE gel band sequencing was not performed to not restrict groups, providing generic evaluation of the diazotrophic community. These restrict groups can be due the limitations of the PCR-DGGE technique; for example, long sequences are not

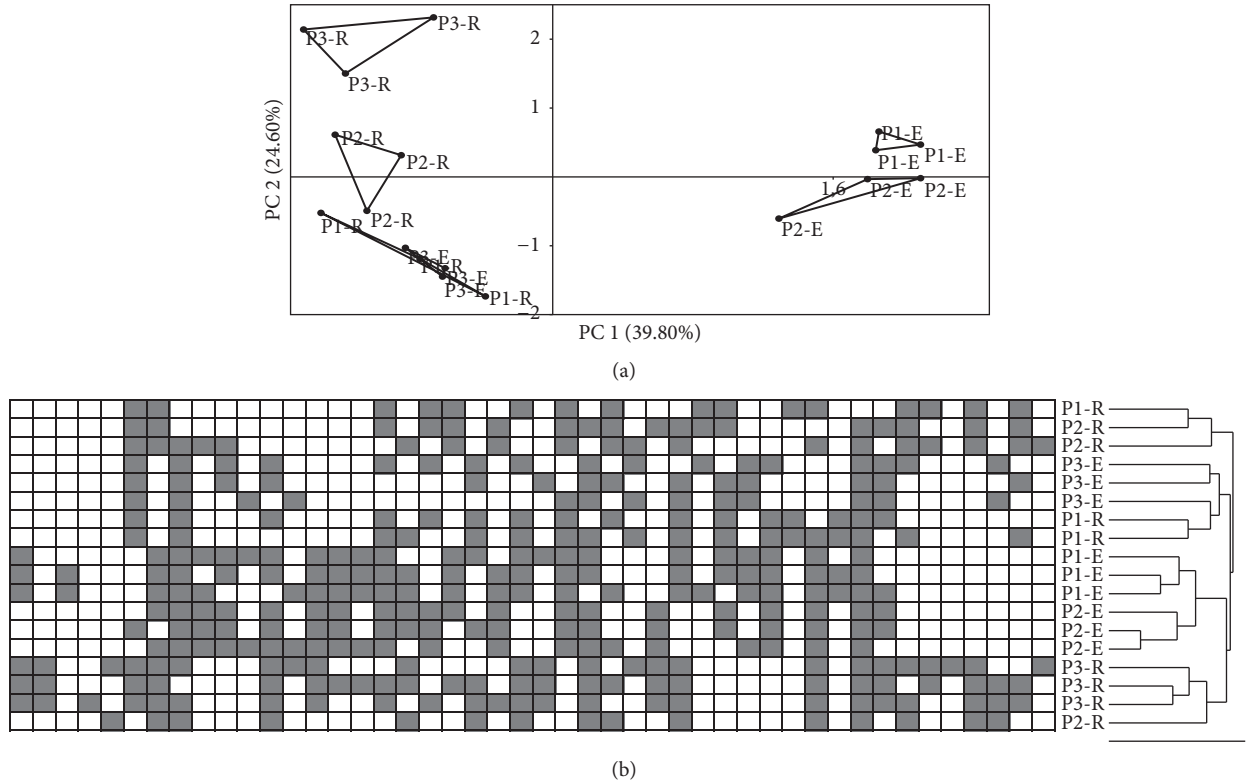


Figure 2: Principal component (PC) analysis (a) and dendogram of the binary matrix (b) of the total diazotrophic bacterial community determined through *Denaturing Gradient Gel Electrophoresis* (DGGE) of the *nif*H gene in different regions of the plant/bacteria association in *B. decumbens* Stapf. pasture in different stages of degradation. P1-R represents the highly degraded pasture and rhizospheric soil niche; P2-R represents the partially degraded pasture and rhizospheric soil niche; P3-R represents the slightly degraded pasture and rhizospheric soil niche; P1-E represents the highly degraded pasture and root-endophytic niche; P2-E represents the partially degraded pasture and root-endophytic niche; and P3-E represents the slightly degraded pasture and root-endophytic niche.

Table 6: Shannon-Weaver diversity index of the total diazotrophic bacterial community in different niches of plant/bacteria association in B. decumbens Stapf. pasture in different stages of degradation.

Pasture degradation stage	Niche of plant/bacteria association		Mean
	Rhizospheric soil	Root-endophytic	
	Shannon-Weaver diversity index		
	H'		
Highly degraded	2.81^{Bb}	3.15^{Aa}	2.98
Partially degraded	2.85^{Bb}	3.01^{Aa}	2.93
Slightly degraded	3.19^{Aa}	2.68^{Bb}	2.94
Mean	2.95	2.95	
	F test ($p \leq 0.05$)		
Degradation stage	0.625^{ns}		
Niche of association	0.007^{ns}		
Stage × association	40.479^{***}		
CV (%)[1]	2.93		

[1]Coeffcient of variation (CV) = 100 × Standard deviation/Average; Means followed by the same letter, uppercase in the row and lowercase in the column, do not differ statistically by the Scott-Knott test ($p \leq 0.05$); ***Significant at 0,1% probability by F test. ^{ns}Not significant.

recommended, because the PCR product can not exceed 500 bp, which may limit the information for phylogenetic inferences [43]; bacterial DNA sequences can migrate to other bands [44]; the DGGE technique compares only the most abundant members of the communities, generally restricting to the 50 most frequent organisms in the group, which represents an important limitation [45].

4. Conclusions

The increase in the degradation stage of *B. decumbens* Stapf. pasture did not reduce the population density of the cultivated diazotrophic bacterial community, suggesting that the degradation was not in any degree of severity harmful to the bacteria. The population density of the cultivated diazotrophic bacterial community was higher in the rhizospheric soil niche, compared with those that endophytically colonized the roots. The structure of the culture-independent total diazotrophic bacterial community associated with *B. decumbens* Stapf. was altered by the pasture degradation stage, suggesting a high adaptive capacity of the bacteria to inhospitable environments. The cultivated diazotrophic bacterial community was not influenced by the degradation stage of *B. decumbens* Stapf. pasture, regardless of the niche of plant/bacteria association.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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