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Genetic diversity and population densities of endophytic Bacillus spp. in yam plants

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ABSTRACT: This study was conducted as a first step in exploiting endophytic bacteria to improve yam production. Little information is available on population densities and genetic diversity of endophytic bacteria in yam cultivated in tropical regions. This study demonstrated that higher densities of total endophytic bacteria and *Bacillus* occur in the interior of roots and decrease as the sampled plant organ departs from soil. *Bacillus* represents approximately 1% of the total bacterial endophytic population. Random Amplified Polymorphic DNA (RAPD) analyses performed with 88 randomly-selected *Bacillus* isolates resulted in 28 groups, and sequence analyses of the 16S region of the ribosomal DNA of 24 isolates representing all major RAPD groups revealed that they could be clustered in 2 clades: *Bacillus cereus* and *B. pumilus*. While *B. cereus* was able to colonize the entire plant, *B. pumilus* remained confined to the rhizophores. Further research should concentrate on the application of these isolates in biotechnological processes, such as biocontrol and yam growth promotion.

Key words: Dioscoriaceae, genetic diversity, population densities, endophytes, bacterial ecology.

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Yam is the common name given to several species of herbaceous vines from the genus *Dioscorea* (Dioscoreaceae). The genus *Discorea* is cultivated in Africa, Tropical America, Asia, and Oceania, being used as a source of fibers, carbohydrates, potassium, manganese, and vitamins B6, E, and C. Additionally, yam contains a substance known as diosgenin, with potential anti-cancer effects (He et al. 2014). Of the approximately 600 existing species, only the following 6 are used as food sources: *Discorea rotundata* (white yam), *D. alata* (water yam), *D. dumentorum* (trifoliate yam), *D. cayenensis* (yellow yam), *D. esculenta* (Chinese yam), and *D. bulbifera* (aerial yam) (Ng and Ng 1994). Other plant species are also referred to as yam, such as *Alocasia*, *Colocasia*, and *Xanthosoma* (Araceae), commonly known as "cará", "taro", "Chinese taro", and "elephant's ear". The yam production worldwide in 2014 was around 68.2 million tons, mainly concentrated in West Africa. Nigeria, Ghana, Ivory Coast, Benin, and Togo are the biggest producers, and Nigeria alone is responsible for 69% of the world's yam production — 5,416,400 ha with a yield of 830.89 $t \cdot ha^{-1}$ (FAO 2014). The Brazilian production of *Discorea* spp. is concentrated in the Northeastern Region, especially in the States of Paraíba, Pernambuco, Alagoas, Bahia, and Maranhão; *Colocasia* spp. is mainly grown in Rio de Janeiro State. Brazil contributes with 0.38% of the world's production with a harvested area of 25,700 ha and yield of 961.09 $t \cdot ha^{-1}$. However, yam cultivation experienced a vertiginous growth in the country between 1961 and 2014, with increases of 270.5; 107.4 and 2,905.9% in harvested area, yield, and produced quantities, respectively (FAO 2014). Yam yields are limited by several factors including pests and diseases as well as the low technological levels adopted by most farmers (Garrido et al. 2003). The use of microorganisms to control pests and diseases and to improve crop growth and yields is favoured by consumers and is predicted to assume a greater importance in the near future. However, little scientific information is available on the beneficial microorganisms associated with yam, especially in tropical areas.

In this study, we assessed the densities and diversity of endophytic *Bacillus* in yam plants as a first step toward their application as agents of biological control and growth promotion.

Yam (*D. rotundata*) plants were collected in the Batatã region, which comprises the municipalities of Cruz das Almas, Maragogipe, and São Felipe, all in Bahia State. Thirteen plants were collected, separated into roots, rhizophores, stems, and leaves, and 1-g samples of each of these parts were weighed. Rhizophore samples were taken from depths of 0 – 1; 1 – 3 and more than 3 cm from the peel, and stem samples were taken at 50 and 100 cm from the rhizophore insertion. Samples were superficially disinfected with 70% alcohol for 1 min as well as 1% sodium hypochlorite for 1 min and washed 3 times in sterile distilled water. The efficiency of the disinfestation was confirmed by plating an aliquot of 100 µL from the water used in the final wash. Only samples without microbial growth were used in further experiments. The samples were crushed in porcelain mortars containing 9 mL of sterile saline solution (0.85% NaCl), and 100-µL aliquots of each dilution prepared in 10X steps were plated onto Trypticase Soy Agar (TSA) medium, consisting of 5 g peptone; 15 g tryptone; 15 g NaCl; 15 g $agar \cdot L^{-1}$ and onto the semi-selective *Bacillus* medium, composed of 1.3 g $(NH_4)_2SO_4$; 0.37 g KH_2PO_4; 0.25 g $MgSO_4 \cdot 7H_2O$; 0.07 g $CaCl_2 \cdot 2H_2O$; 0.02 g $FeCl_3$; 1 g glucose, 1 g yeast extract, and 15 g of $agar \cdot L^{-1}$ to determine the total endophytic bacteria and *Bacillus* spp., respectively. To assess the *Bacillus* populations, tubes containing the serial dilutions underwent a thermal treatment of 80 °C for 10 min (Sneath 1986). Three replicates were prepared for each dilution, and plates were incubated at room temperature. Colony forming units (CFU) were determined 48 h after plating. Means of population densities were compared with the Scott-Knott test at 5% probability with the R software (R Development Core Team 2009). Random colonies were transferred from the semi-selective *Bacillus* medium to fresh plates containing the same medium and were preserved in 20% glycerol at –20 °C for subsequent studies. For genomic DNA extraction, preserved strains were cultivated in semi-selective *Bacillus* medium at 25 °C for 24 h. Two colonies were transferred to sterilized 1.5-mL microcentrifuge tubes containing 200 µL of cell lysis buffer (0.05 M NaOH; 0.25% SDS). The microtubes were heated at 100 °C for 20 min. Tubes containing the samples were centrifuged at 10,000 rpm for 3 min, and the supernatant was diluted 20X in sterile MilliQ water and stored at –20 °C for PCR reactions. A PCR reaction with the primer pair B-K1/F 5'-TCA CCA AGG CRA CGA TGC G-3' and B-K1/R 5'-CGT ATT CAC CGC GGC ATG-3' (Wu et al. 2006) specific to the genus *Bacillus* spp. was performed to confirm the identity of the isolates collected from the semi-selective medium. Random Amplified Polymorphic DNA (RAPD) reactions were performed with primers D7; M12 →

and M13, as described by Keel et al. (1990). The presence or absence of bands was converted into a matrix of binary data (1 or 0 for presence or absence of a particular size band on the gel, respectively), which was used to calculate Jaccard's similarity coefficient. Clustering with the Neighbor-Joining method and bootstrap analysis with 1,000 resampling were performed with the FreeTree program (Hampl et al. 2001), and the dendrogram was edited and viewed in TreeView (Page 1996). Molecular identification of selected isolates was performed by 16S rDNA sequence analysis. Genomic DNA was extracted by the modified CTAB method, as described by Souza et al. (2003). The 16S rDNA gene was amplified in PCR using primers 8fn and 1429r; amplifications, sequencing, and sequence analyses were performed as previously described (Souza et al. 2003). Sequences were deposited in public databases, and the accession numbers are listed in Figure 1b.

The highest population densities of bacteria were found in roots, followed by rhizophores at depths of 0 – 1 cm, stems, and leaves; the lowest values were found in rhizophores at depths higher than 1 cm (Table 1). Population densities in roots (10^7 CFU·g^{-1}) were 5,000X higher than those found in rhizophores at depths greater than 3 cm from the peel (2×10^3 CFU·g^{-1}), which corresponded to the lowest densities. The highest densities of *Bacillus* spp. occurred in roots, followed by stems, leaves, and rhizophores, where they occurred at similar densities. The difference between the highest (roots) and the lowest densities (rhizophores at > 3 cm of the peel) was ~ 300X. *Bacillus* densities decreased as sampling depths increased and represented, in average, 1% of the total endophytic bacteria from different parts of yam plants (Table 1). In absolute numbers, densities of both total bacteria and *Bacillus* were higher in roots than in other plant parts and decreased acropetally (Table 1).

The soil acts as a reservoir of bacteria and, as a consequence, their densities and diversity are higher in this environment as well as in plant organs in contact with soil (Hallmann et al. 1997). Endophytic colonisation by a subset of bacteria is subjected to plant regulation (Hunter et al. 2010), and it appears that this control becomes more stringent as the organ departs from soil, which was also observed for other plant species (Hallmann and Berg 2006). Although many other authors found this reduction in densities and diversity of total bacterial populations in plant organs away from the soil (McInroy and Kloepper 1995; Compant et al. 2011), *Bacillus* seems to lack a clear pattern. For example, in *Trifolium pratense*, several *Bacillus* species were found in higher populations in leaves and stems than in root and nodules (Sturz et al. 1997). The ability of *Bacillus* species to grow anaerobically (Nakano and Zuber 1998) may explain, at least in part, the higher proportions of this genus in deeper layers of the rhizophores.

Among the 88 randomly-selected isolates used in this study, 11 were obtained from roots, 28 from rhizophores, 1 from stems, and 48 from leaves, and all of them were amplified by the *Bacillus*-specific PCR reaction, generating a specific product with 1,114 bp (data not shown). RAPD analysis generated a total of 85 bands, of which 34 were polymorphic. The 88 isolates were clustered in 28 RAPD groups (Figure 1a). The greatest genetic diversity was found in roots and rhizophores with 10 and 15 RAPD groups, respectively (Figure 1a). Despite the fact that a higher number of isolates was obtained from leaves, these isolates showed a low genetic diversity with only 2 RAPD groups. Isolates from different plant parts were not clustered in the same RAPD group (Figure 1a). Sequence analyses of 24 isolates representing all major RAPD groups resulted in 2 clusters: one of them close to the *B. cereus* clade, containing

Table 1. Population densities of total bacteria and *Bacillus* spp. in different parts of yam plants.

Plant part[1]	Total of bacteria (CFU·g^{-1})	*Bacillus* spp. (CFU·g^{-1})	*Bacillus* spp. (%)[2]
Roots	1×10^7a	2×10^4a	0.12
Stems (50 cm)	3×10^5b	9×10^2b	0.26
Stems (100 cm)	2×10^5b	0	0.00
Leaves	1×10^5b	4×10^2b	0.32
Rhizophores (0 – 1 cm)	4×10^5b	3×10^2b	0.05
Rhizophores (1 – 3 cm)	4×10^3c	1×10^2b	3.02
Rhizophores (> 3 cm)	2×10^3c	6×10^1b	3.62

[1]Population densities in each plant part are the result of 13 plant samples. Means followed by the same letter are not significantly different according to the Scott-Knott test (p = 0.05); [2]Percentage of the total endophytic population represented by *Bacillus*.

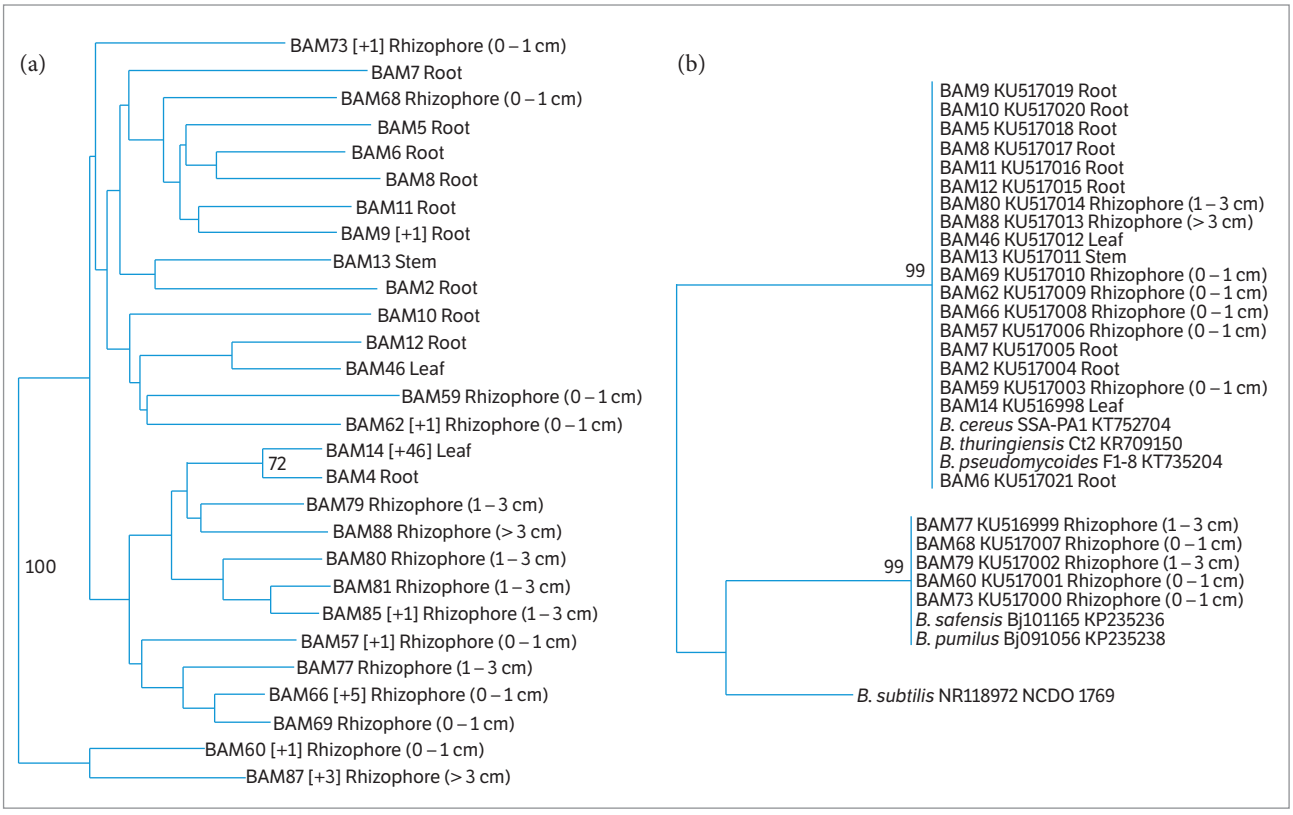

Figure 1. Genetic diversity of the endophytic *Bacillus* spp. population in yam plants. (a) RAPD-based dendrogram of *Bacillus* spp. isolated from yam. Jaccard's similarity coefficient was used to group the isolates with the Neighbor-Joining algorithm. The end groups were defined based on 100% similarity, and the numbers between square brackets represent the additional number of isolates in each group. The scale indicates the percentage of similarity; (b) Phylogenetic tree of *Bacillus* spp. based on the maximum likelihood method with the K2 substitution model. The bootstrap analysis was done with 1,000 replications, and values equal to or greater than 70% are shown. The scale indicates the number of substitutions per site.

19 isolates from all plant parts, and a second group which was close to the *B. pumilus* clade, containing 5 isolates, all of which obtained from rhizophores (Figure 1b). These results indicate that isolates from the *B. cereus* group are competent endophytes in yam, whereas isolates of the *B. pumilus* clade are opportunistic or passenger endophytes (Hardoim et al. 2008).

Species in both *B. cereus* and B. *pumilus* clades were isolated as endophytes of several plant species such as *Solanum tuberosum*, *Solanum lycopersicum*, *Zea maydis*, *Gossypium hirsutum*, *Phaseolus vulgaris*, and others (McInroy and Kloepper 1995; Melnick et al. 2008; López-López et al. 2010). Representatives of both clades were found to be opportunistic human pathogens, but their presence as plant endophytes was already reported by other authors (Bentur et al. 2007; Bottone 2010).

The lack of a clear differentiation between human pathogens from safe environmental strains hampers their use in agriculture as agents of biocontrol and growth promotion. This study had an exploratory nature, and further studies should be conducted to effectively implement the use of these isolates to improve yam production through growth promotion and disease control.

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REFERENCES

Bentur, H. N., Dalzell, A. M. and Riordan, F. A. I. (2007). Central venous catheter infection with *Bacillus pumilus* in an imunocompetent child: a case report. Annals of Clinical Microbiology and Antimicrobials, 6, 12-14. http://dx.doi.org/10.1186/1476-0711-6-12.

Bottone, E. J. (2010). *Bacillus cereus*, a volatile human pathogen. Clinical Microbiology Reviews, 23, 382-398. http://dx.doi.org/10.1128/cmr.00073-09.

Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H. and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: Identification of cultivable bacteria comparison with other plant parts, and visualization of niches of colonization. Microbial Ecology, 62, 188-197. http://dx.doi.org/10.1007/s00248-011-9883-y.

Food and Agriculture Organization (2014); [accessed 2016 Jan 13]. http://faostat3.fao.org/download/Q/QC/E

Garrido, M. D. S., Soares, A. C. F., Mendes, L. D. N. and Perez, J. O. (2003). Novas tecnologias para a produção do inhame (*Dioscorea cayennensis* Lam.) no Estado da Bahia. Revista Bahia Agrícola, 6, 19-22.

Hallmann, J. and Berg, G. (2006). Spectrum and population dynamics of bacterial root endophytes. In B. J. E. Schulz, C. J. C. Boyle and T. N. Sieber (Eds.), Microbial root endophytes (p. 15-21). Berlin: Springer-Verlag.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F. and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology, 43, 895-914. http://dx.doi.org/10.1139/m97-131.

Hampl, V., Pavlicek, A. and Flegr, J. (2001). Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. International Journal of Systematic and Evolutionary Microbiology, 51, 731-735. http://dx.doi.org/10.1099/00207713-51-3-731.

Hardoim, P. R., van Overbeek, L. S. V. and van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology, 16, 463-471. http://dx.doi.org/10.1016/j.tim.2008.07.008.

He, Z., Chen, H., Li, G., Zhu, H., Gao, Y., Zhang, L. and Sun, J. (2014). Diosgenin inhibits the migration of human breast cancer MDA- MB-231 cells by suppressing Va2 activity. Phytomedicine, 21, 871-876. http://dx.doi.org/10.1016/j.phymed.2014.02.002.

Hunter, P. J., Hand, P., Pink, D., Whipps, J. M. and Bending, G. D. B. (2010). Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* species) phyllosphere. Applied Environmental Microbiology, 76, 8117-8125. http://dx.doi.org/10.1128/aem.01321-10.

Keel, C., Wirthner, P. H., Oberhansli, T. H., Voisard, C., Burger, U., Haas, D. and Defago, G. (1990). *Pseudomonads* as antagonists of plants pathogens in the rhizosphere: role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis, 9, 327-341.

López-López, A., Rogel, M. A., Ormeño-Orrilo, E., Martínez-Romero, J. and Martínez-Romero, E. (2010). *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. Systematic and Applied Microbiology, 33, 322-327. http://dx.doi.org/10.1016/j.syapm.2010.07.005.

McInroy, J. A. and Kloepper, J. W. (1995). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Canadian Journal of Microbiology, 41, 895-901. http://dx.doi.org/10.1139/m95-123.

Melnick, R. L., Zidack, N. K., Bailey, B. A., Maximova, S. N., Guiltinan, M. and Backman, P. A. (2008). Bacterial endophytes: *Bacillus* spp. from annual crops as potential biological control agents of black pod rot of cacao. Biological Control, 46, 46-56. http://dx.doi.org/10.1016/j.biocontrol.2008.01.022.

Nakano, M. M. and Zuber, P. (1998). Anaerobic growth of a "strict aerobe" (*Bacillus subtilis)*. Annual Review of Microbiology, 52, 165-190. http://dx.doi.org/10.1146/annurev.micro.52.1.165.

Ng, N. Q. and Ng, S. Y. C. (1994). Approaches for germplasm conservation. In M. O. Akoroda (Ed.), Root crops for food security in Africa (p. 135-140). Kampala: International Society for Tropical Root Crops.

Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences, 12, 357-358. http://dx.doi.org/10.1093/bioinformatics/12.4.357.

R Development Core Team (2009). R: a language and environment for statistical computing. Vienna: R Development Core Team.

Sneath, P. H. A. (1986). Endospore-forming gram-positive rods and cocci. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (Eds.), Bergey's manual of systematic bacteriology. 2. ed. (p. 1104-1139). Baltimore: Williams and Wilkins.

Souza, J. T., Mazzola, M. and Raaijmakers, J. M. (2003). Conservation of the response regulator gene gacA in *Pseudomonas* species. Environmental Microbiology, 5, 1328-1340. http://dx.doi.org/10.1111/j.1462-2920.2003.00438.x.

Sturz, A. V., Christie, B. R., Matheson, B. G. and Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biology and Fertility of Soils, 25, 13-19. http://dx.doi.org/10.1007/s003740050273.

Wu, X. Y., Walker, M. J., Hornitzky, M. and Chin, J. (2006). Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. Journal of Microbiological Methods, 64, 107-119. http://dx.doi.org/10.1016/j.mimet.2005.04.021.