Environmental Microbiology

Tripartite symbiosis of Sophora tomentosa, rhizobia and arbuscular mycorhizal fungi

Maira Akemi Toma\textsuperscript{a}, Teotonio Soares de Carvalho\textsuperscript{a}, Amanda Azarias Guimarães\textsuperscript{a}, Elaine Martins da Costa\textsuperscript{b}, Jacqueline Savana da Silva\textsuperscript{a}, Fatima Maria de Souza Moreira\textsuperscript{a,\textsuperscript{b}}

\textsuperscript{a} Universidade Federal de Lavras (UFLA), Departamento de Ciência do Solo (DCS), Lavras, MG, Brazil
\textsuperscript{b} Universidade Federal de Mato Grosso do Sul (UFMS) – Campus de Chapadão do Sul, Chapadão do Sul, MS, Brazil

\begin{abstract}
Sophora tomentosa is a pantropical legume species with potential for recovery of areas degraded by salinization, and for stabilization of sand dunes. However, few studies on this species have been carried out, and none regarding its symbiotic relationship with beneficial soil microorganisms. Therefore, this study aimed to evaluate the diversity of nitrogen-fixing bacteria isolated from nodules of Sophora tomentosa, and to analyze the occurrence of colonization of arbuscular mycorrhizal fungi on the roots of this legume in seafront soil. Thus, seeds, root nodules, and soil from the rhizosphere of Sophora tomentosa were collected. From the soil samples, trap cultures with this species were established to extract spores and to evaluate arbuscular mycorrhizal fungi colonization in legume roots, as well as to capture rhizobia. Rhizobia strains were isolated from nodules collected in the field or from the trap cultures. Representative isolates of the groups obtained in the similarity dendrogram, based on phenotypic characteristics, had their 16S rRNA genes sequenced. The legume species showed nodules with indeterminate growth, and reddish color, distributed throughout the root. Fifty-one strains of these nodules were isolated, of which 21 were classified in the genus Bacillus, Brevibacillus, Paenibacillus, Rhizobium and especially Sinorhizobium. Strains closely related to Sinorhizobium adhaerens were the predominant bacteria in nodules. The other genera found, with the exception of Rhizobium, are probably endophytic bacteria in the nodules. Arbuscular mycorrhizal fungi was observed colonizing the roots, but arbuscular mycorrhizal fungi spores were not found in the trap cultures. Therefore Sophora tomentosa is associated with both arbuscular mycorrhizal fungi and nodulating nitrogen-fixing bacteria.
\end{abstract}

© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

* Corresponding author.
E-mail: fmoreira@dcs.ufla.br (F.M. Souza Moreira).
http://dx.doi.org/10.1016/j.bjm.2017.03.007
1517-8382/© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Introduction

Due to their ability to establish symbiosis with nodulating nitrogen-fixing bacteria (NNFB) and arbuscular mycorrhizal fungi (AMF), some species of the Fabaceae family are able to grow in unfavorable environments. Generally, individuals of this family are used as pioneers in soil conservation and to recover degraded areas, since they enrich the soil with organic matter with high nitrogen, and consequently allow the establishment of other plants in the ecological succession process. According to Nobrega et al., soil salinization is one of the most serious forms of soil degradation. Areas of saline soils are found in the shores of oceans, lakes, and in arid and semi-arid agricultural areas, due to intensive use of irrigation with water of high salts content, and more recently, in areas of industrial landfill. Plant species adapted to high salt concentrations are able to develop morphophysiological mechanisms that make it possible for them to grow and develop in these conditions. In this context, studying species able to settle in salty conditions and understanding their mechanisms and adaptations have been a challenge for agricultural scientists and for those interested in recovering degraded areas.

The legume Sophora tomentosa L., locally known in Rio de Janeiro as “feijão da praia”, “comandaiba” or “rosario”, is likely one of those species adapted to saline environments because they are usually found in seafront soils subjected to intermittent salination from seawater. In Brazil, it occurs naturally in sandbanks from the northeast to the south of the country, and its natural occurrence has been reported in the Pacific Islands, Oceania, East Asia and Mexico. However, Peña et al. claim that this species has been found in the coast of all tropical regions of the world, characterizing it as a species of pantropical distribution. It is a shrub plant, about 3 m high, with fast growth and high aggressiveness, forming large clumps and offering good coverage to the ground. Its roots are abundant, with a volume about three times greater than the shoot. The fructification is practically continuous, and it blooms throughout the year, with sporadic interruptions. Its capacity of nodulation was observed when it was inoculated with Sinorhizobium fredii and Sinorhizobium adhaerens under controlled conditions, but only the second formed nitrogen-fixing nODULES.

Despite its potential for recovery of degraded and salinized areas, and also for containment of dunes, few studies on this species are found in the literature. Even less works regarding their symbiotic relationships with beneficial microorganisms, such as rhizobia and mycorrhizal fungi, especially at natural conditions are found. Therefore, this study aimed to evaluate the diversity of nodulating nitrogen-fixing bacteria from nodules of S. tomentosa roots, and to analyze the occurrence of colonization of arbuscular mycorrhizal fungi in the roots of this legume, with native inocula from a Spodosol.

Material and methods

Characterization of the sampling area

The Municipal Ecological Park Prainha (MEPP), located in the western part of Rio de Janeiro (RJ), between the coordinates 23°01'52"–23°02'30" S and 43°30'00"–43°30'38" W, has Aw climate (tropical with rains in summer), according to the Köppen classification. The average annual rainfall is between 1200 and 2000 mm, and the average temperature varies from 18 °C in winter to 32 °C in summer. According to Veloso et al., the native vegetation is classified as Sub-mountain Dense Rain Forest (on the slopes) and Pioneer Formations (sandbank and wetlands). S. tomentosa is found on the sandy beach, together with heliophytic and halophytes species, such as “capim-de-praia” (Sporobolus viriginicus – Graminae), “feijão de praia” (Carnavália rosea – Fabaceae), locally scarce “guriri” (Allagoptera arenaria – Palmae), and “abaneiro” (Clusia fluminensis – Gutiferae). Wetlands soils were classified as Sodosols, according to the Brazilian Soil Classification System.

Collection of nodules and seeds of S. tomentosa and soil sampling in the field

Soil samples were collected for chemical and physical analysis, according to the methods compiled by Embrapa, which was carried out in the laboratories of the Federal University of Lavras.

Nodules (Supplementary Material – Figure S2) and seeds of S. tomentosa were collected when plants were at flowering and seed production stages. Roots of three plants were sampled, and all the nodules were collected. These nodules were packed in Nasco® sterile plastic bags and then dried in glass tubes with cotton and silica gel. Seeds were stored in paper bags and preserved in cold chamber at 4 °C. To produce soil inoculum, 5 composite samples of rhizosphere soil of S. tomentosa were collected, which were stored in Nasco® sterile plastic bags at 4 °C, until spores extraction. Part of these soil samples were used to capture rhizobia.

Installation of trap culture to capture rhizobia and to evaluate the colonization of AMF and spores extraction under controlled conditions

S. tomentosa seeds, from the Municipal Ecological Park Prainha were scarified in concentrated sulfuric acid, and the surface was disinfected by immersion in 1% sodium hypochlorite (v/v) for 3 min, followed by successive washes with sterile distilled water. Seeds were germinated in Petri dishes with filter paper and moistened cotton, and put in an oven at 28 °C. After the development of the radicles, three seedlings were transferred to 500 mL plastic pots with substrate, composed of 150 mL soil inoculum and 350 mL sterile sand and vermiculite in a 2:1 ratio, respectively. In order to capture native AMF, Brachiaria decumbens were grown together with S. tomentosa in the same pot. For this, forage seeds were scarified in concentrated sulfuric acid for 1 min, and planted directly into plastic pots, with density of four seeds per plot. Pots were kept in a greenhouse of the Department of Soil Science of the Federal University of Lavras for six months, and plants were regularly irrigated with a modified Hoagland solution, as described by Guimarães et al. After this period, three plants of S. tomentosa were collected for analysis of root nodules and presence of AMF structures. For analysis of root colonization by AMF, it was used the coloring method of root fragments. After dyeing the roots, they were transferred to Petri dishes and were visualized.
in a stereoscopic microscope with 10 to 40 times magnification. Manual cuts were carried out in some nodules in order to capture images, as well as fungal structures within S. tomentosa roots, using the optical microscope Olympus model BX40.

AMF spores extraction was carried out by the technique of wet sieving and centrifugation in sucrose.

Isolation and characterization of bacterial strains

NNFB were isolated from nodules collected in the field and from trap plants. Nodules were first immersed in ethyl alcohol (95%) for 30 s in order to break surface tension. After that, nodules were immersed in hydrogen peroxide (H₂O₂) (30%) for 3 min to disinfect their surface, and then, they were washed six times with sterile distilled water to remove H₂O₂ excess. After that, they were crushed in Petri dishes containing culture medium 79 using a sterilized tweezer. Petri dishes were incubated at 28 °C until the appearance of isolated colonies. All identified colony morphotypes were picked and incubated at 28 °C repeatedly to obtain pure cultures. Subsequently, phenotypic characterization of strains was carried out. The analyzed characteristics were: time to the appearance of isolated colonies (1–3 days – fast growth; 4 days – intermediate growth; 6 days or more days – slow growth), colony diameter (mm), changes in culture medium pH (acidification, neutralization, or alkaline zation), shape (circular or irregular), colony elevation, border appearance, color (yellow, orange, white, beige, or salmon), and the consistency of the exopolysaccharides produced (gummy, aqueous, or dry), and absorption of the culture medium indicator.

From the data of phenotypic characterization, a hierarchical group analysis was carried out using the coefficient of Gower as dissimilarity metric and Ward algorithm for grouping in R. To define homogeneous groups on dendrogram, the criterion of Mojena was used.

16S rRNA gene sequencing

For the 16S rRNA gene sequencing, representative isolates from each group formed in the dendrogram of phenotypic characteristics were selected. Genomic DNA extraction, amplification and sequencing of 16S rRNA gene were carried out following the methodology described by Guimarães et al.

The quality of the sequences was verified by BioNumerics 7.6 (Applied Maths, Saint-Martens-Latem, Belgium). The resulting sequences, with exception of RIOP243I and RIOP243, were assembled into contigs and compared with those available in the GenBank (National Center for Biotechnology Information, NCBI), using Basic Local Alignment Search Tool (BLAST). The sequence alignment was performed with 1115 base pairs using ClustalW. A phylogenetic tree was generated using MEGA version 6.0 with default parameters, Kimura 2-parameter distance model, and Neighbor-Joining algorithm. Statistic support for the tree was evaluated by bootstrap confidence analysis with 1000 samplings. Sequences of the isolates of S. tomentosa and at least two closely-related type/reference strains available in GenBank were included, with the purpose of evaluating whether these strains would form separate clusters with known species.

The sequences presented in this work were deposited in GenBank under accession numbers KY820810 to KY820830.

Authentication experiments

Two experiments were performed aiming to authenticate the isolated bacterial strains in S. tomentosa growing in Leonard vessels filled with sand and vermiculite 1:1 and Hoagland’s nutrient solution, but both of them were unsuccessful due to the poor growth of S. tomentosa in axenic conditions even after 6 months of cultivation.

Results

Soil analysis

Chemical and physical analysis of soil samples from the rhizosphere of S. tomentosa is found in Table 1.

Morphology of S. tomentosa nodules and AMF colonization

After six months of cultivation, using S. tomentosa as trap plant for NNFB capture, it was observed that plants grew very slowly, reaching only about 10 cm, and did not develop the root system well (Fig. 1A). However, plants were nodulated in this period, and nodules with indeterminate growth and

| Table 1 – Chemical and physical analysis of Spodosol from Sephora tomentosa rhizosphere collected in the Municipal Ecological Park Praia, RJ. |
|---------------------------------|----------|-------------|--------|---------|-------|-----|---------|--------|
| pH in H₂O | P | K | Na | Ca | Mg | Al | H + Al | SB | cec | CEC | E.C. | PST |
| mg dm⁻³ | cmolₐ dm⁻³ | mS cm⁻¹ | % | mg L⁻¹ | mg dm⁻³ | % |
| 7.9 | 31.44 | 36.51 | 56.20 | 0.54 | 0.23 | 0.00 | 0.53 | 0.86 | 0.86 | 1.39 | 0.56 | 17 |
| m | V | O.M. | Pₓₑₜ | Zn | Fe | Mn | Cu | B | S | Sand | Silt | Clay | 0.00 | 62.13 | 0.09 | 56.81 | 6.99 | 64.75 | 10.19 | 0.99 | 0.03 | 4.35 | 100 | 0 | 0 |

SB: Sum of bases (Ca²⁺ + Mg²⁺ + K⁺); cec: cation exchange capacity in natural pH; CEC: cation exchange capacity at pH = 7.0; E.C.: electrical conductivity; PST: percent of sodium saturation; m: aluminum saturation; V: base saturation at pH = 7.0; O.M.: organic matter.
redish color inside (Fig. 1B) occurred throughout the root. The observed nodules had length ranging from 1 to 6 mm, and contained one or two apical meristems, suberized surface and darkening when senescent (Fig. 1B and C).

From the analysis of root colonization by AMF, it was observed the presence of typical structures, such as coenocytic hyphae, vesicles and intra radicular spores in the roots (Fig. 1D and E). However, only two spores were found in all extractions carried out from the field samples and trap cultures.

**Capture, isolation and phenotypic characterization of strains**

A total of 20 nodules were selected from the trap cultures (12) and the field (8). As a result, 36 bacterial strains were isolated from the nodules collected in the field, and 15 from the trap culture (Fig. 2). According to the phenotypic characteristics of the strains, five groups were separated according to the homogeneity criterion of Mojena. Of these groups, 21 representatives strains were chosen randomly and had their DNAs sequenced, being 2 strains from the first group, 2 from the second, 13 from the third, 2 from the fourth and 2 from the fifth group. These 21 strains showed rapid growth; 16 strains acidified the culture medium, 3 did not change the pH, and 2 alkalized the culture medium (Table 2).

**Genetic diversity of isolates from Sophora tomentosa**

From the 16S rRNA gene sequence and comparisons with sequences available in the GenBank, 11 known bacterial species were most closely related to the isolates from nodules of S. tomentosa (Table 2). These results are presented in the phylogenetic tree constructed with 16S rRNA gene sequences (Fig. 3). Among the strains, seven formed a homogeneous cluster with *S. adhaerens*. Besides, they were well separated from the next phylogenetically closest *Sinorhizobium* species (*S. morelense*), suggesting their classification as *Sinorhizobium adherens* (RIOP231, RIOP243II, RIOP245II, RIOP245II22, RIOP245II11, RIOP245II22, and RIOP245IIII). The strain SIIP3N1 grouped with three known species within *Sinorhizobium* sp;
Fig. 3 - Phylogenetic relationships based on 16S rRNA sequences among strains isolated from Sophora tomentosa nodules and type/reference strains. Phylogeny was determined by the Neighbor-Joining method with 1115 base pairs. Bootstrap values were based on 1000 trials.
as such, our data only support its classification at the genus level. Four strains formed a separate cluster with *Rhizobium* pusense (RIOP321, RIOP322, RIOP323, RIOP324, RIOP325) and did not group with any other known species within the *Rhizobium* genus, supporting their classification as *R. pusense*. RIOP346 was identified as *Bacillus sp.*, whereas RIOP251I1 and RIOP251I1 were closely and exclusively associated with *Bacillus aryabhattai* (RIOP251I1 and RIOP251I1). RIOP243IV and SIIP1N1 were classified as *Brevibacillus sp.*; and RIOP344, as *Paenibacillus kribensis*.

The group of gram-negative bacteria (*Rhizobium sp.* and *Sinorhizobium sp.*) make up about 68% (13 of 19) of the strains and gram-positive (*Bacillus sp.*, *Brevibacillus sp.* and *Paenibacillus sp.*) the other 32% (Fig. 3).

**Discussion**

**Soil analysis**

According to the Manual of Liming and Fertilization of the state of Rio de Janeiro for N2-fixing legume trees and shrubs, the levels of P and K were classified as appropriate and low, respectively. The highest concentration of P can be related to the influence of seawater in the soil, enriching it with this nutrient in readily available form to plants, or the presence of marine molluscs shells composing sand texture, which are rich in calcium phosphate. Similar results were also find by Lourenço Junior and Cuzzuel, studying the influence of the soil on chemical composition of *Passiflora mucronata* and *Canavalia rosea* in a similar ecosystems.

Although the soil analysis does not indicate saline conditions in the soil at the time of sampling, these ecosystems are subject to intermittent influence of seawater and occasional salinization. However, because of the sandy texture, the ions are easily leached with percolating water after heavy rainfalls, common in the area.

**Morphology of S. tomentosa nodules and AMF colonization**

Transverse and longitudinal sections of the nodules showed reddish color inside, indicating the presence of Leghemoglobin (Fig. 1C) and nitrogen-fixing activity. Nodules that do not internally have this substance express whitish shades and demonstrate the inability of microsymbionts to fix nitrogen.

Despite the observation of arbuscular mycorrhizal fungi colonization in the roots of *S. tomentosa*, few spores were found in soils in all extractions carried out from the field samples and trap cultures. In soil samples from the field, results may be related to the sandy texture and to the frequent soil water saturation. Moreover, the high amount of water in this environment can be a limiting factor for sporulation.
Diversity of bacterial strains isolated from S. tomentosa

In the group of gram-negative bacteria, two distinct groups were found. The first composed by Sinorhizobium spp; the second, with Rhizobium spp. The isolates grouped with S. adhaerens type strain (Fig. 3) presented 100% similarity with this species (Table 2) and did not group with any other species of this genus, suggesting that these strains belong to S. adhaerens. A similar result was also found by Hung et al. in Taiwan, who identified this species as the unique symbiont of S. tomentosa. This interesting relationship between S. tomentosa and S. adhaerens found in distant locations of the globe can be an indicative of a specific symbiotic association, and their co-occurrence in distinct continents suggest that the bacteria is dispersed with S. tomentosa seeds and fruits, through the seawater. The bacteria could infect the seeds right after they fall on the soil, and then been carried to long distances by the ocean, setting a symbiotic association in different places around the world.

In the literature, the nomenclature for S. adhaerens is not well defined. According to Willems et al., it should be classified within the Sinorhizobium genus, since it is widely known as a plant symbiont, but also due to the etymology of the word, and to the great number of nitrogen-fixing representatives within the genus Sinorhizobium, when compared to the Ensifer genus. In a more recent research, Ormeño-Orrillo et al. demonstrated that Ensifer adhaerens species showed enough phylogenetic difference to allocate itself in a separate group from the other species of this genus, as evidenced by Willems et al.

In the second group of gram negative bacteria, Rhizobium spp, it is clear that five strains (RIOP321, RIOP322, RIOP323, RIOP324 and RIOP325) formed a subgroup 100% similar (Table 2) to R. pusense (Fig. 3). There is little information on R. pusense, and it was described by Panday et al. for isolates from the rhizosphere of Cicer arietinum L.

Gram-positive bacteria, such as species of the genera Bacillus, Paenibacillus (syn. Bacillus), and Brevibacillus were also found among the sequences obtained. Although bacteria of these genera are not known to nodulate legumes, they have been commonly isolated from nodules of various legume species and considered to be endophytic.

The genus Bacillus is a phenotypically large and diverse group of Gram-positive or Gram-variable, spore-forming bacteria. In this group, representatives adapted to different environmental conditions are found. Due to advances in molecular biology, this genus has been subjected to considerable reclassification, with high phylogenetic heterogeneity. In our work, the strain RIOP346 presented 100% similarity to two species of Bacillus spp (Bacillus thuringiensis and Bacillus cereus) (Fig. 3). Thus, it was grouped into Bacillus spp. On the other hand, RIOP2511I and RIOP253II, formed one group with Bacillus aryabhattai (100% similar) and no other group, supporting its classification as B. aryabhattai. The RIOP344 strain is 99% similar to Paenibacillus kribensis. This species is a potassium and phosphorus solubilizing bacteria, as well as nitrogen-fixing, which is widely used in agricultural production in China. The Paenibacillus genus was created by Ash et al. to accommodate a group of the genus Bacillus, and its name derived from the latin term “paene”, meaning almost, or almost Bacillus.

Brevibacillus genus is derived from the reclassification of the strains initially allocated as Bacillus brevis, and currently includes 20 species. The strain SIIP1NI1 was grouped together with Brevibacillus spp., but showed 99% similarity to Brevibacillus nirificans and Brevibacillus choshinensis. The strain RIOP243IV was 100% similar to Brevibacillus brevis and Brevibacillus formosus. Therefore, in both situations it was not possible to reliably classify them at species level; so they were identified as Brevibacillus sp.

Thus, our study indicates that S. tomentosa nodules present several endophytic species, and suggests that Sinorhizobium/Ensifer adhaerens establishes symbiosis with S. tomentosa in natural conditions, confirming previous reports in the literature.

Conclusions

S. tomentosa legume establishes symbiosis with nitrogen-fixing bacteria, most likely with Sinorhizobium/Ensifer adhaerens species, and with mycorrhizal fungi under natural conditions of seashore in Brazil.

Bacterial species of the genera Bacillus, Brevibacillus, and Paenibacillus were found within S. tomentosa nodules as endophytic species.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

The authors thank CNPq, CAPES and FAPEMIG funding agencies for the master’s, doctoral and post-doctoral scholarships, and CNPq for the research productivity fellowship granted to FMS Moreira.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2017.03.007.

References

1. Nobrega RSA, Motta JS, Lacerda AM, et al. Tolerance of diazotrophic symbiotic bacteria to salinity = Tolerância de bactérias diazotróficas simbióticas à salinidade in vitro. Ciência Agrotecnol. 2004;28(4):899–905.
2. Santos EC, Goi SR, Neto J J. Utilization of Sophora tomentosa L. Sub espécie litoral (schrad) Yakove to recovery degraded areas with industrial saline residue = Proposta de utilização de Sophora tomentosa L. Subespécie litoral (Schrad) Yakove para recuperação de áreas com resíduo industrial salino. Floresta e Ambiente. 2001;8(1):216–218.
3. Huang J, Redmann RE. Responses of growth, morphology and anatomy to salinity and calcium supply in cultivated and wild barley. Can J Bot. 1995;73(12):1859–1866.
4. Degano CAM. Morphology and anatomy of Tessaria absinthioides (Hook. et Arn.) DC under salinity conditions = Respostas morfológicas y anatómicas de Tessaria absinthioides (Hook. et Arn) DC a la salinidad. Rev Bras Bot. 1999;22(3):1–13.

5. Ahlgren CL. Survivorship of Sophora tomentosa on the reef islands of Mo’orea. In: French Polynesia UCB Moorea Class: Biology and Geomorphology of Tropical Islands 2009; 2009. Available from: <https://escholarship.org/uc/item/7tg1m3wr>. Accessed 12.08.15.

6. Sykes WR, Godley EJ. Transoceanic dispersal in Sophora and other genera. Nature. 1968;218:495–496.

7. Hurr KA, Lockhart PJ, Heenan PB, et al. Evidence for the recent dispersal of Sophora (Leguminosae) around the Southern Oceans: molecular data. J Biogeogr. 1999;26(3):565–577.

8. Peña RC, Iuitarriaga L, Montenegro G, et al. Phylogenetic and biogeographic aspects of Sophora Sect. Edwardsia (Papilionaceae). Pacif Sci. 2000;54(2):159–167.

9. Nogueira EML, Arruda VLV. Frutification and damage to fruits and seeds of Sophora tomentosa L. on a sandbank of Joaquina beach, Florianópolis, SC = Frutificação e danos em frutos e sementes de Sophora tomentosa L. (Leguminosae, Papilionoideae) em restinga da praia da Joaquina, Florianópolis, SC. Revista Biotemas. 2006;19(4):41–48.

10. Zamith RL, Scarno FR. Seedling production of Restinga species of Rio de Janeiro Municipality, RJ, Brazil = Produção de mudas de espécies das restingas do município do Rio de Janeiro, RJ, Brasil. Acta Bot Bras. 2004;18(1):161–176.

11. Krishnan HB, Pueppke SG. Genetic characterization of a mutant of Sinorhizobium fredii strain USDA208 with enhanced competitive ability for nodulation of soybean, Glycine max (L) Merr. FEMS Microbiol Lett. 1998;165(1):215–220.

12. Hung M, Bhagwath AA, Shen FT, et al. Indigenous rhizobia associated with native shrubby legumes in Taiwan. Pedobiologia. 2005;49(6):577–584.

13. Veloso PH, Rangel-Filho ALR, Lima JCA. Classification of Brazilian Vegetation Adapted to a Universal System = Classificação da Vegetação Brasileira Adaptada a um Sistema Universal. Rio de Janeiro: IBGE; 1991:124.

14. Empresa Brasileira de Pesquisa Agropecuária. Manual Soil Analysis Methods = Manual de Métodos de Análise de Solos. 2nd ed. Rio de Janeiro: Embrapa-CNPS; 1997:212.

15. Hoagland DR, Arnon DT. The Water Culture Method for Growing Plants Without Soil. Berkeley: University of California; 1950:32.

16. Guimarães AA, Jaramillo PDM, Nóbrega RSA, et al. Genetic and symbiotic diversity of nitrogen fixing bacteria isolated from agricultural soils in the western Amazon by using cowpea as the trap plant. Appl Environ Microbiol. 2012;78(18):6726–6733.

17. Koske R, Gemma J. A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res. 1989;92(4):486–505.

18. Gerdemann JW, Nicolson TH. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Trans Br Mycol Soc. 1963;46(2):235–246.

19. Jenkins W. A rapid centrifugal-flotation technique for separating nematodes from soil. Plant Dis Rep. 1964;48(9):692.

20. Fred EB, Waksman SA. Laboratory Manual of General Microbiology – With Special Reference to the Microorganisms of the Soil. New York: McGraw-Hill Book Company; 1928:145.

21. Gower JC. A general coefficient of similarity and some of its properties. Biometrics. 1971;27(4):857–874.

22. Ward JH. Hierarchical grouping to optimize an objective function. J Am Stat Assoc. 1963;58(301):236–244.

23. R Development Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2013.

24. Mojena R. Hierarchical grouping methods and stopping rules: an evaluation. Comput J. 1977;20(4):359–363.

25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–2729.

26. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16:111–120.

27. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406–425.

28. Empresa Brasileira de Pesquisa Agropecuária. Liming and Fertilization Manual of the State of Rio de Janeiro = Manual de Calagem e Adubação do Estado do Rio de Janeiro. Rio de Janeiro: Embrapa; 2013:430.

29. Hay JD, Lacerda LD. In: Lacerda LD, et al., eds. Nutrient Cycling in the Seafront Ecosystem = Ciclagens de Nutrientes no Ecossistema Restinga. Restingas: origens estruturas e processos; 1984:459–475.

30. Lourenço Junior J, Cuzzulio GRF. Characterization of soils of two restinga formations and their influence on the chemical composition of Passiflora mucronata Lam (Passifloraceae) and Canavalia rosea (Sw.) DC (Fabaceae) = Caracterização de solos de duas formações de restinga e sua influência na constituição química foliar de Passiflora mucronata Lam. (Passifloraceae) e Canavalia rosea (Sw.) DC (Fabaceae). Acta Bot Bras. 2009;23(1):230–246.

31. Moreira FMS, Siqueira JO. Soil Microbiology and Biochemistry = Microbiologia e Bioquímica do Solo. 2 ed. atual. e ampl Lavras: UFLA; 2006:729.

32. Gomide PHO, Silva MLN, Soares CRFS, et al. Arbuscular mycorrhizal fungi in vegetation types in the Pantanal of Nhecolândia, Mato Grosso do Sul, Brazil = Fungos micorrízicos arbusculares em fitofisionomias do pantanal da Nhecolândia. Mato Grosso do Sul Revista Brasileira de Ciência do Solo. 2014;38(4):1114–1127.

33. Willems A, Fernández-López M, Muñoz-Adelantado E, et al. Description of new Ensifer strains from nodules and proposal to transfer Ensifer adhaerens Casida 1982 to Sinorhizobium as Sinorhizobium adhaerens comb. nov. Request for an opinion. Int J Syst Evol Microbiol. 2003;53:1207–1217.

34. Ormeño-Orrillo E, Servín-Garcidueñas LE, Rogel MA, et al. Taxonomy of rhizobia and agrobacteria from the Rhizobiaceae family in light of genomics. Syst Appl Microbiol. 2015;38(4):287–329.

35. Panday D, Schumann P, Das SK. Rhizobium puseus sp. nov., isolated from the rhizosphere of chickpea (Cicer arietinum L.). Int J Syst Evol Microbiol. 2011;61:2632–2639.

36. De Lajudie P, Willems A, Nick G, et al. Agrobacterium bv. 1 strains isolated from nodules of tropical legumes. Syst Appl Microbiol. 1999;22(1):119–132.

37. Muresu R, Polone E, Sulas L, et al. Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes. FEMS Microbiol Ecol. 2008;63(3):383–400.

38. Li JH, Wang ET, Chen W, et al. Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. Soil Biol Biochem. 2008;40(1):238–246.

39. Lima AS, Nóbrega RSA, Barberi A, et al. Nitrogen-fixing bacteria communities occurring in soils under different uses in the Western Amazon region as indicated by nodulation of siratro (Macroptilium atropurpureum). Plant Soil. 2009;319(1):127–145.

40. Costa EM, Nóbrega RSA, Carvalho F, et al. Plant growth promotion and genetic diversity of bacteria isolated from cowpea nodules = Promoção do crescimento vegetal e
diversidade genética de bactérias isoladas de nódulos de feijão-caupi. Pesq Agropec Bras. 2013;48(9):1275–1284.

41. Claus D, Berkeley CW. The genus Bacillus. In: Bergey’s Manual of Systematic Bacteriology. 2nd ed. Baltimore: Williams and Wilkins; 1986:1105.

42. Nazina TN, Tourova TP, Poltaraus AB, et al. Taxonomic study of aerobic thermophilic bacilli: descriptions of Geobacillus subterraneus gen. nov., sp. nov. and Geobacillus uzenensis sp. nov. from petroleum reservoirs and transfer of Bacillus stearotherophilus, B. thermocatenulatus, B. thermoleovorans, B. kaustophilus, B. thermodenitrificans to Geobacillus as the new combinations G. stearotherophilus, G. th. Int J Syst Evol Microbiol. 2001;51(2):433–446.

43. Panda AK, Bisht SS, DeMondal S, et al. Brevibacillus as a biological tool: a short review. Antonie Van Leeuwenhoek. 2014;105(4):623–639.

44. Zhang DL, Zuo JL. Contrast analysis on determination of inorganic element K with flare photometer and sodium tetrphenylboron. Jingxi Forestry Sci Technol. 2000;5:26–28.

45. Ash C, Priest FG, Collins MD. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus Paenibacillus. Antonie Van Leeuwenhoek. 1993;64(3):253–260.

46. Lai S, Tabacchioni S. Ecology and biotechnological potential of Paenibacillus polymyxa: a minireview. Indian J Microbiol. 2009;49(1):2–10.

47. Edwards SG, Seddon B. Mode of antagonism of Brevibacillus brevis against Botrytis cinerea in vitro. J Appl Microbiol. 2001;91(4):652–659.