Brazilian Journal of Microbiology (2012): 653-660
ISSN 1517-8382

INTERSPECIFIC VARIATION OF THE BACTERIAL COMMUNITY STRUCTURE IN THE PHYLLOSPHERE OF THE THREE MAJOR PLANT COMPONENTS OF MANGROVE FORESTS

Armando Cavalcante Franco Dias[1,2], Rodrigo Gouveia Taketani[2*], Fernando Dini Andreote[3], Danice Mazzer Luvizotto[3], João Luis da Silva[2], Rosely dos Santos Nascimento[2], Itamar Soares de Melo[2]

[1]Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, SP, Brasil; [2]Laboratório de Microbiologia Ambiental, Empresa Brasileira de Pesquisa Agropecuária, Jaguariúna, SP, Brasil; [3]Departamento de Ciência do Solo, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, SP, Brasil.

Submitted: June 16, 2011; Returned to authors for corrections: August 18, 2011; Approved: June 07, 2012.

ABSTRACT

Mangrove forests encompass a group of trees species that inhabit the intertidal zones, where soil is characterized by the high salinity and low availability of oxygen. The phyllosphere of these trees represent the habitat provided on the aboveground parts of plants, supporting in a global scale, a large and complex microbial community. The structure of phyllosphere communities reflects immigration, survival and growth of microbial colonizers, which is influenced by numerous environmental factors in addition to leaf physical and chemical properties. Here, a combination of culture-base methods with PCR-DGGE was applied to test whether local or plant specific factors shape the bacterial community of the phyllosphere from three plant species (*Avicenia shaueriana*, *Laguncularia racemosa* and *Rhizophora mangle*), found in two mangroves. The number of bacteria in the phyllosphere of these plants varied between 3.62 x 10^4 in *A. schaeriana* and 6.26 x 10^3 in *R. mangle*. The results obtained by PCR-DGGE and isolation approaches were congruent and demonstrated that each plant species harbor specific bacterial communities in their leaves surfaces. Moreover, the ordination of environmental factors (mangrove and plant species), by redundancy analysis (RDA), also indicated that the selection exerted by plant species is higher than mangrove location on bacterial communities at phyllosphere.

Key words culture-independent profiling, plant genotype, surface leaves.

INTRODUCTION

Mangrove forests are important coastal ecosystems found in transition zones between marine and freshwater ecosystems such as estuaries, bays and lagoons (16). Despite their spread, mangroves are at danger of extinction due to human action and climate change (17). However, the threat against mangroves worldwide does not affect only this environment, but can result in effects at nearby environments, due to the role of mangroves as sinkholes and sources of organic matter to neighboring environments (1, 9, 11). One of the main characteristic of mangroves is their location, which have selected over time the

***Corresponding Author. Mailing address:** Laboratório de Microbiologia Ambiental – CNPMA - Embrapa Meio Ambiente Rodovia SP 340 - Km 127,5 Caixa Postal 69 Jaguariúna - SP - Brasil - CEP: 13820-000.; Tel.: (19) 3311.2700.; E-mail: rgtaketani@gmail.com

residing biota, which should be adapted to the high variability in the salinity, periods of flood, and the consequent low availability of oxygen (10, 18, 28). These characteristics lead to distinct structure of the mangrove forest in comparison to other tropical forests (14). In Brazilian southern mangroves, the most dominant plant species are *Laguncularia racemosa*, *Avicennia schaueriana* and *Rhizophora mangle*, which present adaptation to these environments; such as, specialized roots and salt toleration systems (10, 25).

The particularities of this environment have lead to the hypothesis that not only the macroorganisms that inhabit this environment are specific, but the microorganisms should also have been selected over the time. Therefore, plant associated microorganisms must have been specially selected due to their close interaction, possibly leading to a co-evolution of mangrove plants over a long time span (13).

The phyllosphere is the microbial habitat found on the surface of leaves, constituting one of the largest environment in Earth and presenting a wide fluctuation on the environmental parameters (e.g. humidity, temperature, UV exposure) (22). Regardless of these conditions, microbes find in the leaf surface a suitable place to thrive and flourish. The association between plants and microorganisms in leaves surfaces vary from plant pathogens to biocontrol agents, also including all sorts of neutral associations (3). Hence, the relationships between plant and phyllosphere microbes present a high interspecies variation (24), can act for conservation of species diversity (21) and possibly support the plant and microbial adaptation to mangroves conditions (26).

Therefore, this study was designed to evaluate two different hypothesis concerning phyllosphere communities. [1] Mangrove trees phyllosphere communities are shaped by local environmental factors; [2] phyllosphere communities are selected by plant factors. To test these hypothesis the three major plant components of mangrove forests were sampled in two different mangrove forests and were sampled and their bacterial communities were evaluated by culture dependent and independent methods.

MATERIALS AND METHODS

Sampling of mangrove phyllosphere

Samples were collected in two distinct mangrove located in São Paulo State (Brazil). The first one is located in the city of Bertioga (23°53'80''S and 46º12'46''W), with close contact with urban area and therefore subjected to pollution of air and water. The second mangrove was sampled in a protected area near the city of Cananéia (22°42'01''S, 46º58'58''W); all samples were collected in the summer. For the purpose of this study, leaves from the three dominant plant species found in this mangrove (*L. racemosa, A. schaueriana* and *R. mangle*) were sampled. Leaves were collected from four individuals of each mangrove tree species, stored in sterile plastic bags (~10 x 20 cm) and carried to the lab. In total, four replicates were collected from each sampling site. Additional cares were taken, in order to avoid damaged leaves, and also to select samples from around the canopy at the same height on each species, as recommended in previous studies (19).

Bacterial isolation form mangrove phyllosphere

In the lab, the sampled leaves were cut in circular fragments using a sterile paper puncher with 16mm diameter. To obtain the cells adhered to the leaf surface, 30 disks of 28 cm^2 were submitted deep into 100 ml of saline solution (0.9% NaCl) and submitted to sonication (10 min at 100 watts and 30 kHz), and shaken for 20 min at 150 rpm. Four replications from each point were used for this analysis. Cells suspensions were then serially diluted Nutrient Agar (Difco™) for plate counting of total epiphytic bacteria. For the purpose of this study the CFU counts were calculated per disk surface area and both sides of the leaf are considered on the area calculation.

Scanning electron microscopy (SEM) of mangrove leaves

Material for SEM examination was obtained from disks that were cut using a sterile paper puncher with 16 mm

diameter. Disks were fixed for 2h at 28°C under vacuum of 760mmHg and using glutaraldheide 2,5% and cacodilate buffer 0,2M pH 7.2. After it, samples were washed in the same buffer and dehydrateded in crescent ethanolic series (10, 25, 40, 60, 75, 85, 95, 100%) with 15 min per change (ethanol in water). A final dehydratation step was performed in acetone, prior to drying at critical point. Samples were then stacked in stubs and covered with a thin gold layer. Observations were made in SEM of high-resolution model Leo 982 GEMINIDMS (Zeiss and Leica, Germany).

DNA Extraction from bacterial cells suspensions

Aliquots for DNA extraction were obtained from the cells suspension prepared for bacterial cultivation. For each sample, the liquid containing the cell suspension was transferred to a 50 ml sterile disposable tube and centrifuged at 7,000 x g for 20 min. The supernatant was carefully removed, and the total genomic DNA was extracted from the pellet using a phenol chloroform method. Briefly, the pellet was suspended in 500 µL of TE (Tris-HCl pH 7.8 10 mM, EDTA 1 mM), and amended with 10 µL 10% sodium-dodecyl-sulfate and 0.1 g glass beads (0.1 mm) (Sigma, Washington DC, USA). The mixture was subjected to a bead beater (BioSpec, Bartlesville, OK, USA) for 1 min (5000 rev/min), after that total DNA extraction was conducted as described in Ausubel *et al.* (4). In total, four samples from each tree species, in each mangrove, were used in this analysis. The quality and integrity of extracted DNA was then checked in agarose electrophoresis gel and the quantity was measured on spectrophotometer.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis based on bacterial 16S rRNA gene

PCR amplification of the V6-V8 region of the 16S rRNA gene was performed using primers U968-GC and 1401R as described previously (15). DGGE run protocol was 6% acrylamide, 45-65% denaturant gradient for 16h under 100V. After the run the gels were stained with silver nitrate (5) and photographed using a light box. The gel image was then analyzed in Bionumerics (Applied Maths, Belgium) and band matching data exported to allow further investigation. The ordination analysis was carried out using the CANOCO 4.5 (29) software, were the DGGE band patterns were correlated with the environmental variables (plant species and mangrove location). The analysis was carried out as previously described by Andreote *et al.* (2), leading to the use of redundancy analysis (RDA) as the best model in this case. Additionally, the separation of samples was also analyzed by the application of similarity analysis (ANOSIM) at Primer-e 6.0 (6).

RESULTS AND DISCUSSION

The present study has shown that mangrove tree phyllosphere is an environment capable of hosting a diverse bacterial community. Although other microbes might be present at this niche, the phyllosphere is widely dominated by bacteria, already pointed as dominant organisms in this environment (3, 22).

Abundance of bacteria in distinct mangrove phyllospheres

The plate count assessments of bacteria inhabiting the phyllosphere was carried out to evaluate differences in the magnitude of the bacterial abundance found in the leaf surface of distinct mangrove trees and mangroves (figure 1).

Concerning the differences among plant species, a remark should be made for the values found in *A. shaueriana* that presented the highest numbers of CFUs/cm^2. The number of colonies observed in the surface of these trees decreased between *A. shaueriana, L. racemosa* and *R. mangle*. The average bacterial counts of *R. mangle* presented the lowest numbers among the samples plants and the most stable between the three plants, even between the different mangrove forests.

Due to the salt exudation by the leafs of *A. schaueriana*, we expected to see a negative effect of salt accumulation on the

overall community. Recently, the observation of a strong correlation between plant genera and the phyllosphere community has lead to the idea relation between these microorganisms and plants might be have been selected during the evolution of both (24), which would explain the resistance to salt observed on the bacteria inhabiting the *A. schaueriana* leafs.

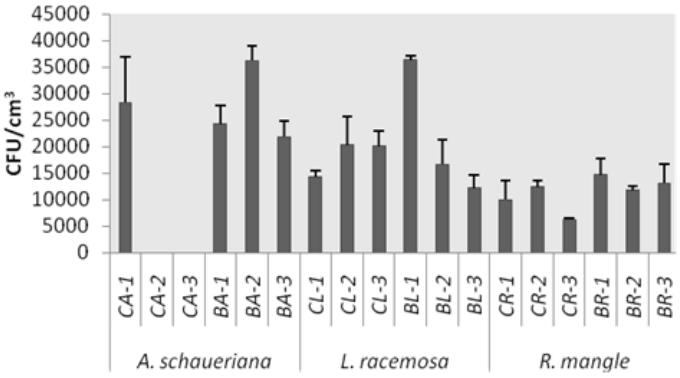

Figure 1. Density of cultivable bacterial community on the phyllosphere tree mangrove species estimated by serial dilution plating method. CFU – colony forming units. Sample codes are as follows: CA - *Avicennia schaueriana* from Cananéia mangrove; BA – *A. schaueriana* from Bertioga; CL - *Laguncularia racemosa* from Cananéia; BL – *L. racemosa* from Bertioga; CR - *Rhizophora mangle* from Cananéia; BR - *R. mangle* from Bertioga; numbers following the letters indicate different replicates. Bars represent mean values of CFU.cm^{-3} and error bars represent standard errors of the means (n = 3).

SEM of bacterial community in mangrove phyllosphere by SEM

Samples of *A. schaueriana* and *L. racemosa* leaves were scanned by SEM to observe the incidence of microorganisms on the phyllosphere (Figure 2). Both plants, *A. schaueriana* (Figure 2A) and *L. racemosa* (Figure 2B), have shown the high incidence of microbial cells, with a particular occurrence of these organisms in the region nearby the stomata. Such accumulation of bacteria in this leaf region might indicate the use of the stomata as an entrance for bacteria in the host plant, as previously described (30).

Additionally, some particularities of these leaves surfaces could be observed, with a remarkable occurrence of salt granules on leaf surface of *A. schaueriana*, as indicated by the figure 1A. Such salt accumulation is related to the ability of this plant species in resisting the salty condition in mangrove soils (7). However, nothing is completely known about the effects of the salt accumulation in leaves over the microbial communities in the phyllosphere.

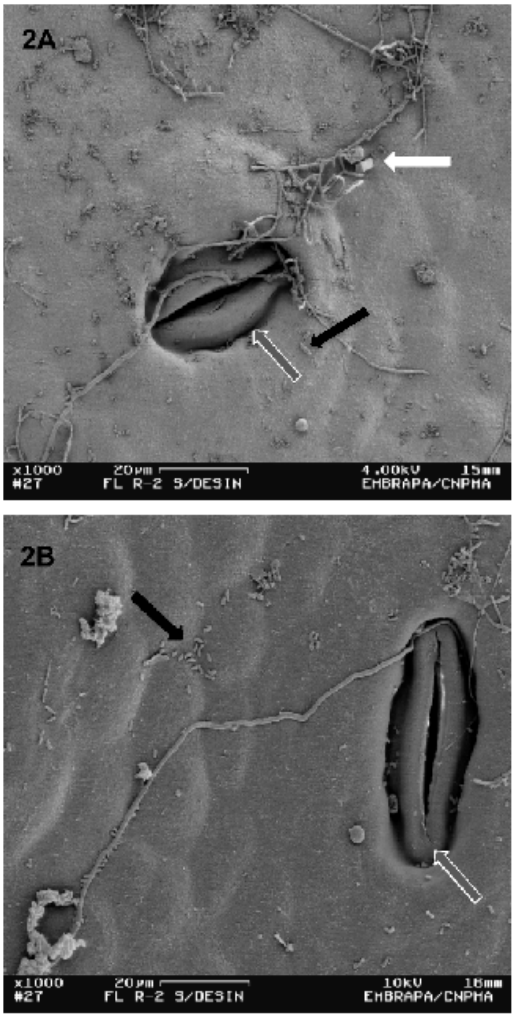

Figure 2. Scanning electron microscopy 1.000x (bar of 20µm) of Leaves of *Avicennia schaueriana* (2A) and *Laguncularia racemosa* (2B). White arrow indicates a granule of salt, the black arrow showing bacteria and gray arrow the stomata.

Culture-independent assessment of bacterial community in mangrove phyllosphere

In order test the interspecific and spatial variations of the bacterial community inhabiting the leaves surfaces, the samples from *A. shaueriana*, *R. mangle* and *L. racemosa* from both mangroves were subjected to the culture-independent approach, PCR-DGGE (Figure 3).

Based on visual inspection of the DGGE fingerprints, the majority of the bands observed were specific to each plant, indicating that there is some degree of selection exert by the plant on the bacterial community living in the phyllosphere (Figure 3). Also, in each of the plant species, it could be observed that a large portion of the taxa (i.e. bands) found in the community of one mangrove could also be detected on the other (Figure 3), supporting the later inference, and also including the low effect of geographical distance on the composition of such communities.

However, in order to prove these observations statistically, an ordination analysis was applied, correlating the DGGE patterns with the environmental variables. Due to the low value of the first axis data distribution (1.459), the ordination method used was the redundancy analysis (RDA), which has ordinate the environmental variables in the following order of importance for the structuring of bacterial communities in the phyllosphere of mangroves: plant species > mangrove location. This ordination is also possible to be observed in the RDA plot (Figure 4), where the variance represented in the first axis indicates a strong effect of plant species in the community composition, mostly by the separation of *R. mangle* samples. In the second axis it is indicated the spatial effect on the community composition, although small overlap was observed for samples from distinct mangroves (Figure 4). These observations were corroborated by the Monte Carlo test (Table 1). However, the effect of the observed environmental variables could only explain a small part of the variation, as observed by the sum of λA.

According to the analysis of similarities (ANOSIM) the RDA dispersion pattern according to tree species was significant (Global R=0.452, p=0.01). Despite the partial overlap indicated by the R value, the most intense effect was observed between *L. racemosa* and *R. mangle* (R=0.685, p=0.001), while the other pairwise test indicate a larger overlap despite the significant difference (Table 2). Additionally, the ANOSIM test indicates a significant effect of sampled mangrove (p=0.011) with a large superimposition (Global R=0.182).

In summary, the analysis of PCR-DGGE does indicate that each tree species harbor a distinct bacterial community, corroborating the results from previous studies that have shown pronounced plant interspecies variation (20, 23, 31, 32). Furthermore, although this issue was not addressed in the present work, it is expected that these communities must be composed by populations belonging to lineages of *Bacteroidetes, Actinobacteria* and *Proteobacteria*, commonly described as major groups living in the phyllosphere of distinct plant species (8, 20, 24, 32).

Also, considering the patterns from same species in distinct mangroves, the DGGE analysis of *A. schaueriana* samples showed that a strong resemblance in both mangroves, i.e. had a large portion of its bands in common (with similar relative abundance). In a similar analysis for the remaining trees, it is observed that fewer bands were present in both mangroves and with pronounced differences in relative intensities.

Still, up to now, it is not known if the development of phyllosphere communities is regulated by local environmental conditions (12, 22, 24) or by plant genotype (23). This study assessed the bacterial diversity in mangroves phyllosphere, where the environmental conditions are nearly the same, and the variations observed might be related to plant genotypes and physiology. In this context, our results indicated that the microbial community present on the phyllosphere of mangrove trees might be regulated by a large variety of stimuli with strong effect of plant species in the selection of these communities.

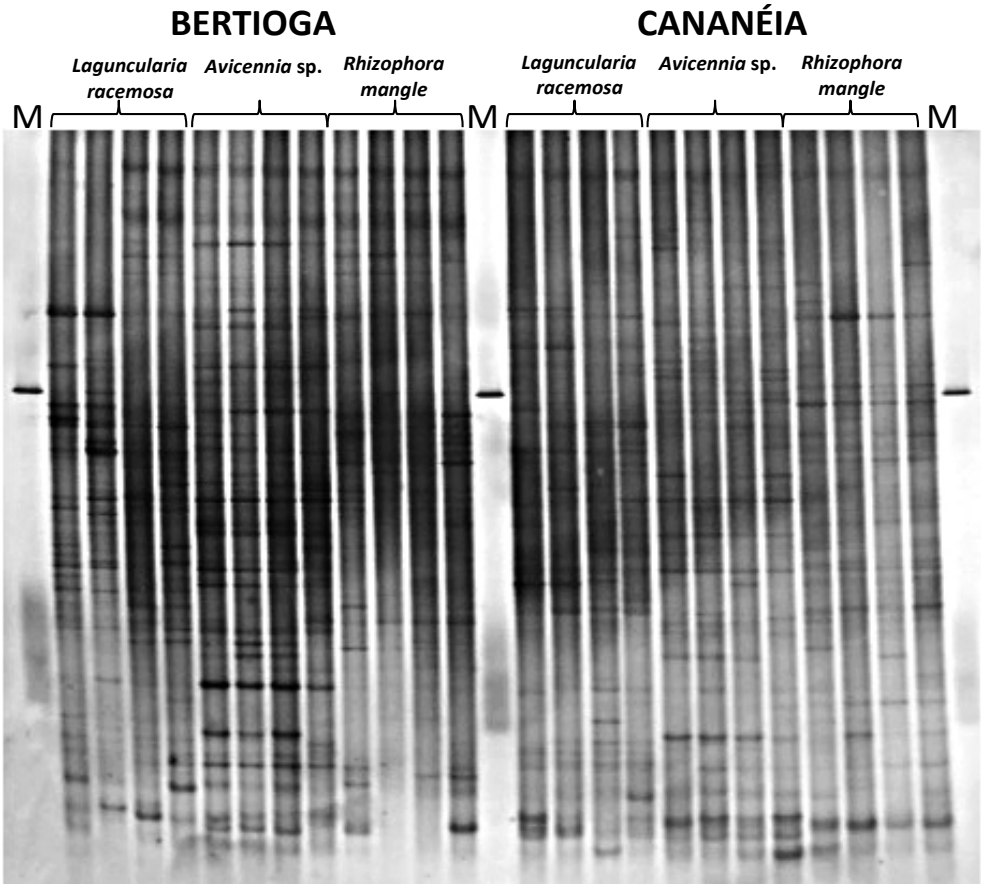

Figure 3. Denaturing gradient gel electrophoresis (DGGE) analysis of bacterial communities in surface species tree leaves mangrove in different localities (Bertioga and Cananéia). Each vertical lane represents the bacterial community present in one replicate (4 replicates per treatment). M – Marker.

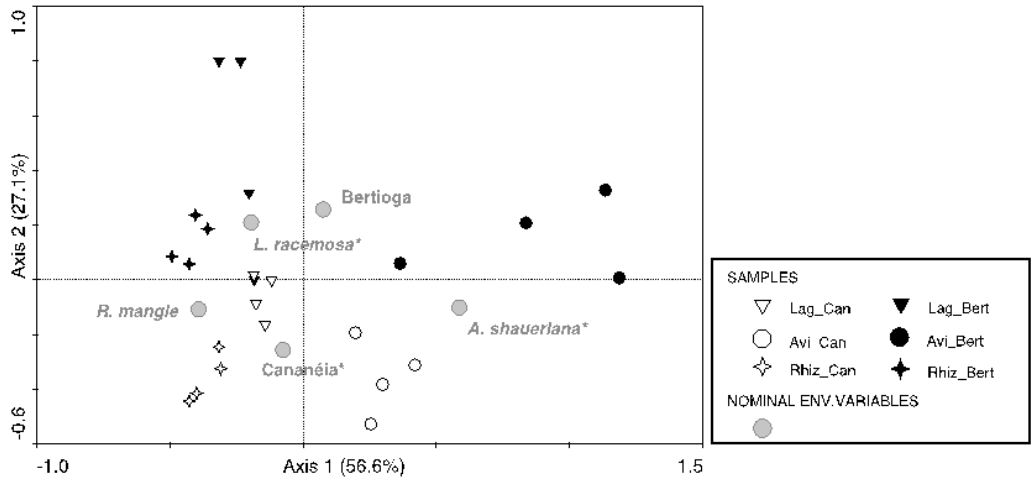

Figure 4. Redundancy Analysis (RDA) performed on the DGGE patterns obtained for bacteria 16S of the mangrove trees and locations. Gray dots represent the centroid of each environmental characteristic. Sample codes are as follows: Lag - *Laguncularia racemosa*. Avi - *Avicennia schaueriana*, Rhiz - *Rhizophora mangle*, Can – Cananéia mangrove, Bert – Bertioga mangrove. The significance of the correlations between species and environmental data was evaluated according to a Monte Carlo permutation test and is indicated as follows: * $P<0.05$.

Table 1. Significant conditional effects of RDA.

Variable	Conditional Effects		
	λA	***P***	**F**
A. schaueriana	0.18	0.002	4.74
Cananéia	0.08	0.002	2.37
L. racemosa	0.06	0.022	1.9

Table 2. Results of ANOSIM test comparing the community composition between species.

	R statistic	***P***
L.racemosa vs *A. schaueriana*	0.354	0.001
L. racemosa vs *R. Mangle*	0.358	0.001
R. mangle vs *A. schaueriana*	0.685	0.001
Cananéia vs *Bertioga*	0.182	0.011

ACKNOWLEDGEMENTS

This study was supported by a grant from the State of São Paulo Research Foundation (FAPESP/BIOTA 2004/13910-6). R.G. Taketani was a recipient of a postdoctoral grant from FAPESP (2010/50799-7). Also, A.C.F. Dias (2008/54013-8) received a graduate fellowship. We also thank the support from the Oceanographic Institute (IO, USP, São Paulo), especially Dr. Ricardo P. Menghini for their support in mangrove expeditions and samplings.

REFERENCES

1. Alongi, D.M.; Boto, K.G.; Tirendi, F. (1989) Effect of exported mangrove litter on bacterial productivity 11 and dissolved organic carbon fluxes in adjacent tropical nearshore sediments. *Mar. Ecol. Prog. Ser*. 56 (1), 133–144
2. Andreote, F.D.; Azevedo, J.L.; Araújo, W.L. (2009) Assessing the diversity of bacterial communities associated with plants. *Braz. J. Microbiol*. 40, 417–432
3. Andrews, J.H.; Harris, R.F. (2000) The ecology and biogeography of microorganisms on plant surfaces. *Annu. Rev. Phytopathol*. 38, 145–180
4. Ausubel, F.M.; Brent, R., Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A.; Struhl, K. (1995) *Short protocols in molecular biology*, 3rd. ed. John Wiley And Sons, New York, USA.
5. Blum, H.; Beier, H.; Gross, H. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrilamyde gels. *Electrophoresis* 8, 93–99
6. Clarke, K. (1993) Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol*. 18 (1), 117–143
7. Clough, B.F. (1984) Growth and salt balance of the mangroves Avicennia marina (Forsk.) Vierh. and Rhizophora stylosa Griff. in relation to salinity. *Aust. J. Plant. Physiol.* 11, 419–30
8. Delmotte, N.; Knief, C.; Chaffron, S.; Innerebner, G.; Roschitzki, B.; Schlapbac, R.; von Mering, C.; Vorholt, J.A. (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc. Natl. Acad. Sci. USA*. 106 (38), 16428–16433
9. Dias, A.C.F.; Andreote, F.D.; Dini-Andreote, F.; Lacava, P.T.; Sá, A.L.B.; Melo, I.S.; Azevedo, J.L.; Araújo, W.L. (2009) Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment. *W. J. Microbiol. Biotechnol*. 25 (7), 1305–1311
10. Dias, A.C.F.; Andreote, F.D.; Rigonato, J.; Fiore, M.F.; Melo, I.S.; Araújo, W.L. (2010) The bacterial diversity in a Brazilian non-disturbed mangrove sediment. *Antonie van Leeuwenhoek* 98 (4), 541–551
11. Ferreira, T.O.; Otero, X.L.; Souza Jr, V.S.; Vidal-Torrado, P.; Macías, F.; Firme, L.P. (2010) Spatial patterns of soil attributes and components in a mangrove system in Southeast Brazil (São Paulo). *J. Soils. Sediments*. 10, 995–1006
12. Fierer, N.; Hamady, M.; Lauber, C.; Knight, R. (2008) The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc. Natl. Acad. Sci. USA.* 105 (6), 17994–17999
13. Flores-Mireles, A.L.; Winans, S.C.; Holguin, G. (2007) Molecular Characterization of Diazotrophic and Denitrifying Bacteria Associated with Mangrove Roots. *Appl Environ. Microbiol*. 11, 7308–7321
14. Gomes, N.C.M.; Borges, L.R.; Paranhos, R.; Pinto, F.N.; Mendonça-

Hagler, L.C.S.; Smalla, K. (2008) Exploring the diversity of bacterial communities in sediments of urban mangrove forests. *FEMS Microbiol. Ecol*. 66 (1), 96–109

15. Heuer, H.; Kroppenstedt, R.M.; Lottmann, J.; Berg, G.; Smalla, K. (2002) Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere relative to communities are negligible relative to natural factors. *Appl. Environ. Microbiol.* 68 (3), 1325–1335
16. Holguin, G.; Bashan, Y.; Mendoza-salgado, R.; Amador, E.; Toledo, G.; Vázquez, P.; Amador, A. (1999) A La microbiología de los manglares. Bosques en la frontera entre el mar y la tierra. *Ciencia y Desarrollo* 26–35
17. Holguin, A.G.; Gonzalez-Zamorano, B.P.; Bashan, A.L.E.; Mendoza, A.R.; Amador, A.E.; Bashan, A.Y. (2006) Mangrove health in an arid environment encroached by urban development—a case study. *Sci. Total. Environ*. 363 (1), 260–274
18. Kathiresan, K.; Bingham, B.L. (2001) Biology of mangrove and mangrove ecosystems. *Adv. Mar. Biol*. 40, 81–251
19. Kinkel, L. (1997) Microbial population dynamics on leaves. *Annu. Rev. Phytopathol*. 35, 327–347
20. Lambais, M.R.; Crowley, D.E.; Cury, J.C.; Büll, R.C.; Rodrigues, R.R. (2006) Bacterial diversity in tree canopies of the Atlantic forest. *Science* 312 (5782), 1917
21. Lankau, R.A.; Strauss, S.Y. (2007) Mutual Feedbacks Maintain Both Genetic and Species Diversity in a Plant Community. *Science* 317, 1561–1563
22. Lindow, S.E.; Brandl, M.T. (2003) Microbiology of the phyllosphere. *Appl. Environ. Microbiol*. 69 (4), 1875–1883
23. Redford, A.J.; Bowers, R.M.; Knight, R.; Linhart, Y.; Fierer, N. (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves *Environ. Microbiol*. 12 (11), 2885–2893
24. Redford, A.J.; Fierer, N. (2009) Bacterial succession on the leaf surface: a novel system for studying successional dynamics. *Microb. Ecol*. 58,189–198
25. Schaeffer-Novelli, Y.; Cintron-Molero, G.; Adaime, R.R.; Camargo, T.M. (1990) Variability of mangrove ecosystems along the Brazilian coast. *Estuaries*. 13, 204–218
26. Shan, L.; RenChao, Z.; SuiSui, D.; SuHua, S. (2008) Adaptation to salinity in mangroves: Implication on the evolution of salt-tolerance. *Chinese Sci. Bull*. 53, 1708–1715
27. Sinsabaugh, R.L.; Follstad, S.J.J. (2010) Integrating resource utilization and temperature in metabolic scaling of riverine bacterial production. *Ecology* 91 (5), 1455–1465
28. Taketani, R.G.; Yoshiura, C.A.; Dias, A.C.F.; Andreote, F.D.; Tsai, S.M. (2010) Diversity and identification of methanogenic archaea and sulphate-reducing bacteria in sediments from a pristine tropical mangrove. *Antonie van Leeuwenhoek* 97 (4), 401–411
29. Ter Braak, C.J.F.; Smilauer, P. (2002) CANOCO reference manual and CanoDraw for Windows User's guide: Software for Canonical Community Ordination (version 4.5). Microcomputer Power, Ithaca NY. pp 352
30. Van Vuurde, J.W.L.; Van Henten, C. (1983) Immunosorbent immunofluorescence microscopy (ISIF) and immunosorbent dilution plating (ISDP): new methods for the detection of plant pathogenic bacteria. *Seed Scie. Technol*. 11, 523–533
31. Whipps, J.M.; Hand, P.; Pink, D.; Bending, G.D. (2008) Phyllosphere microbiology with special reference to diver diversity and plant genotype. *J. Appl. Microbiol*. 105 (6), 1744–1755
32. Yang. C.H.; Crowley, D.E.; Borneman, J.; Keen, N.T. (2001) Microbial phyllosphere populations are more complex than previously realized. *Proc. Natl. Acad. Sci. USA* 98 (7), 3889–3894.