Universidade Federal do Rio Grande do Sul

Instituto de Biociências

Programa de Pós-Graduação em Genética e Biologia Molecular

Um modelo para inoculantes bacterianos promotores de crescimento vegetal baseado na ecologia da rizosfera

Pedro Beschoren da Costa

Tese apresentada ao PPGBM/UFRGS

como parte dos requesitos para a obtenção

do título de Doutor em Ciências

Orientadora: Luciane Maria Pereira Passaglia

Porto Alegre, maio, 2016

This work was performed in:

Vegetal Molecular Genetics Laboratory, Biosciences Institute, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, under supervision of Prof. Dr. Luciane Passaglia;
Genetic of Prokaryotes Laboratory, Faculty of Biology, University of Bielefeld, Bielefeld, Germany; under supervision of Prof. Dr. Volker F. Wendisch.
Microbial Ecology Group, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands; under supervision of Prof. Dr. Jan Dirk van Elsas

This work was funded by:

National Council of Scientific and Technological Development (CNPq), Brazil
German Academic Exchange Service (DAAD), Germany

This work is dedicated to my parents, Guaracy and Nara,
whose support was fundamental at all stages
of my life.

Acknowledgements

I thank my supervisor, Luciane Passaglia, for her support all over the years, since my first internship in 2006. More importantly than helping to evaluate experiments, results, and pointing out mistakes, she offered me many opportunities and trusted my work.

I thank my colleagues Fernanda Moreira, Camille Granada, João Passos, Adriana Ambrosini, Rocheli Souza and Letícia Arruda for granting me access to the data they generated in their own thesis and dissertations. This was essential for me to develop the model that is the center of this thesis.

I thank Volker F. Wendisch for receiving me in his laboratory in Bielefeld for a 5 month internship. Professional and personal growth in that period was exceptionally high. I also thank Samanta Campos for her warm welcome and scientific assistance in Germany.

I thank Jan Dirk van Elsas for receiving me in his laboratory in Groningen for a 1 year internship, even without ever hearing of my work or my name beforehand. My work there was extremely challenging, but helped measure my potential as an independent scientist.

I thank Anelise Beneduzi and Luciano Kayser from Fepagro, Hironori Tanigushi from Bielefeld University, Cyrus Mallon and Stephanie Jurburg from Groningen University for help in many scientific discussions. I also thank all lab technicians from the 3 labs.

I thank all my other colleagues in these 3 labs, especially: Evelise Bach, Gabriela Fernandes, Gisele Passaia, Rafael Arenhart, Lauro Neto, Ronei Machado, and Fernanda Lazzaroto from Porto Alegre; Luciana Fernandes, Fernando Perez, Marta Irla, Jaide Jensen João Jorge and Sabine Heider from Bielefeld; Rachel Miranda, Pu Yang, Miao Wang, Pillar Puentes and Maryam de Mares from Groningen. Daily lab work and weekendly celebrations were great with you around.

I thank my foreign friends, especially Dorota Bayer, Simone Leidelmeijer, Tim Schut, Heidi Käkelä, Jana Gieselmann, Leyre Pescador, Stefania Colella and Bert. I thank very much the opportunity of meeting and hanging out with you

I thank my good old friends, especially Lindsey Cassini, André Dresseno, Felícia Fisher, Tiago Degrandi, Gustavo Reolon, Eduardo Bittencourt, Richar Menna, Sérgio Menna, Amanda Stoffel, and Milton Fonseca. Iôve been very far for too long, but thankfully I could always count on you.

I thank the CNPq, DAAD and other funding agencies for supporting my work. I had outstanding professional opportunities during my PhD, only possible by public funding of science.

Finally, I thank my parents, Guaracy and Nara, for supporting me at all times. I could never seize the opportunities I was offered if they had not granted me conditions to do so.

Pedro Beschoren da Costa

Summary

Thesis outline

The introduction shows a brief picture of the bacterial role in plant development, and points the importance of the subjects of this thesis;

Chapter 1 shows the effect of fertilization on plant growth-promoting bacteria (PGPB) action and also on the environment, and it is used as part of the introduction for this thesis;

Chapter 2 presents a model that was based on previous data from several works from our group, and it was the basis for Chapter 3;

Chapter 3 intended to test the proposed model under a greenhouse microcosm experiment, and presents a large collection of interactions and correlations;

Chapter 4 presents a novel hypothesis and a novel statistical method for PGPB research. It also discusses proposals for future research in the PGPB field;

The concluding remarks show the final picture we have for the model and some future directions for the research field.

In the Appendage 1 is shown a brief list of other works and achievements obtained during this PhD work.

Estrutura da Tese

A introdução mostra uma breve descrição do papel bacteriano no desenvolvimento de plantas, e aponta a importância dos temas dessa tese

O Capítulo 1 mostra o efeito da fertilização em bacterias promotoras de crescimento vegetal (*plant growth-promoting bacteria*, PGPB) e também no ambiente, e é usado como parte da introdução desta tese

O capítulo 2 apresenta o modelo que foi baseado em dados anteriores de diversos trabalhos de nosso grupo, e é a base para o capítulo 3

O Capítulo 3 pretendia testar o modelo proposto em um experimento de casa de vegetação, e apresenta uma grande coleção de interações e correlaçãoes

O Capítulo 4 apresenta uma nova hipótese e um novo método estatístico para pesquisa em PGPB. Ele também discute propostas para future pesquisa no campo de PGPB

As considerações finais mostram um quaddro dinal que temos para o modelo e algumas direções futures para o campo de pesquisa

No Apêndice I é mostrada uma breve lista de outros trabalhos e destaques obtidos durante ester trabalho de doutorado

Abstract

Soil bacteria greatly interact with plants, and are key components for plant health and vigor. The natural associations and interactions of plant and bacteria can be manipulated by addition of bacterial inoculants, which are of great interest to agriculture. However, even highly efficient bacterial strains extensively tested under diverse conditions might fail to act as plant growth promoting bacteria (PGPB) at full efficiency and at all times. This happens because of the multiple, complex interactions between the plant, the inoculant, the bacterial community and the environment. This thesis aims to detect patterns in plant-bacteria interactions, so that the right inoculant is added at the right conditions. Using data over 2200 strains isolated in several projects from our research group, we raised a model that proposes to explain which bacterial traits would be selected by the plant in nutrient poor or nutrient rich conditions. Our model says that plants will favor P solubilizers, like *Burkholderia*, in nutrient poor conditions and plant hormone producers, like *Enterobacter*, in nutrient rich conditions. This model was then tested in a diversity gradient microcosm, inoculating *Burkholderia* and *Enterobacter*, as single strains and also co-inoculated. We tested these strains in rice plants, using rich clay soils and poor sandy soils, under a dilution-to-extinction diversity gradient. Results show that, as the model suggested, *Burkholderia* was a better PGPB in poor soils and *Enterobacter* was a better PGPB in rich soils, and that P solubilization and production of plant hormones by the bacterial communities are indeed inversely correlated. Some of the predictions of the model were not confirmed, specifically on the display of each trait in the rhizosphere and endosphere niches. The diversity gradient shows that PGPB efficiency, strain survival, and strain niche colonization largely depend on the initial microbial community. On the last chapter of this thesis, we used a novel statistical methodology when analyzing the impact of bacterial inoculation on rhizosphere communities under the assumptions of invasion ecology. Although some of our hypothesis did not hold in this case, they are still interesting to consider, and the novel methodology can be very useful for PGPB research as it facilitates comparisons of next generation sequencing results of a test group and a standard, like a non-inoculated control. We conclude this thesis stating that our model and the statistical methodology presented can be very useful for PGPB research and application.

Resumo

Bactérias de solo interagem com plantas e são componentes chave para saúde e vigor vegetal. As associações e interações naturais de plantas e bactérias podem ser manipuladas pela adição de inoculantes bacterianos, que são de grande interesse para a agricultura. Porém, mesmo linhagens bacterianas eficientes e extensivamente testadas em diferentes condições podem falhar em agir como bactérias promotoras de crescimento vegetal (*plant growth promoting bacteria*, PGPB) em total eficiência e em todos os casos. Isso ocorre devido às numerosas, e complexas, interações entre a planta, o inoculante, a comunidade bacteriana e o ambiente. Esta tese buscou detectar padrões em interações planta-bactéria, para que o inoculante certo seja adicionado nas condições certas. Usando dados de mais de 2200 estirpes isoladas em diversos projetos de nosso grupo, propusemos um modelo que busca explicar quais características bacterianas seriam selecionadas pela planta em condições pobres ou ricas em nutrientes. O modelo propõe que plantas irão favorecer solubilizadores de fosfato (P), como *Burkholderia*, em condições pobres em nutrientes, e produtores de hormônio vegetal, como *Enterobacter*, em condições ricas em nutrientes. Este modelo foi, então, testado em um microcosmo, inoculando *Burkholderia* e *Enterobacter*, isoladas e em co-inoculação. Testamos estas estirpes em plantas de arroz, usando solos argilosos ricos e arenosos pobres, em um gradiente de diversidade de diluição-até-extinção. Os resultados mostraram que, como sugerido pelo modelo, *Burkholderia* era uma melhor PGPB em solos pobres, *Enterobacter* era uma melhor PGPB em solos ricos, e que a solubilização de P e produção de hormônios vegetais são inversamente correlacionadas. Algumas das previsões do modelo não foram confirmadas, especificamente na quantificação de cada característica nos nichos rizosférico e endofítico. O gradiente de diversidade mostrou que eficiência de PGPB, sobrevivência das linhagens e colonização dos nichos são bastante dependentes da comunidade microbiana inicial. No ultimo capítulo desta tese, uma nova metodologia estatística foi testada, enquanto analisou-se o impacto da inoculação bacteriana nas comunidades rizosféricas em relação a teorias da ecologia de invasão. Apesar de algumas de nossas hipóteses terem sido rejeitadas, elas ainda assim são consideravelmente interessantes, e a nova metodologia pode ser muito útil para pesquisa em PGPB, pois ela facilita comparações de resultados de sequenciamento de nova geração. Nós concluímos esta tese afirmando que o modelo e a metodologia estatística apresentada podem ser muito úteis para pesquisa e aplicação de PGPB.

Introduction and General Objectives

Natural soils have an outstanding diversity, much higher than any other environment in our planet. A single gram of soil can have up to 10^9 prokaryotic cells (Griffiths and Philippot, 2012), 2,000 to 18,000 genomes (Daniel, 2005), summing 300 to 3,000kg of biomass per ha (Ranjard and Richaume, 2001). Soils receive diverse kinds of substrates, nutrients, contaminants, and are subjected to many types of stresses. This creates a very complex, open system that operates with networks and communities, where competition, antagonism, cooperation, and symbiosis are all occurring within one cubic millimeter (Zelezniak et al., 2015). Bacteria blur our defined lines of organism and species, when it develops in microbial mats, lichens and other complex communities, where specific components have specific functions. Bacteria struggle for survival on Earth was the first to start since and least 3.5 billion years ago (Koonin, 2014), so it not surprising they can take so many different roles and forms all over the planet. A single example can demonstrate the ability of bacteria to cooperate with other organisms. The development of endosymbiosis, which eventually turned into modern mitochondria and chloroplast, shaped the biosphere as we know it. This event, that independently occurred at least 7 times for chloroplasts (Koonin et al., 2001), generated organisms that were metabolically very efficient, and alone it is enough evidence that bacteria can develop highly efficient, intimate cooperation with other organisms. Being so present in the biosphere, interacting with other organisms at such a basal level, and taking part in all biogeochemical cycles, bacteria influence life on earth with more than by-products to be explored.

Plants had to coexist with bacteria since the start of their evolutive history. Today, we see that both plants and bacteria have multiple kinds of interactions as they share the same space (Barea et al., 2005). It is very clear that the bacteria in soil can affect plant health and development, not only through pathogens and disease as it was thought when bacteria living inside the roots were first discovered. Through the action of carbon substrates and signaling molecules liberated by the roots (exudates) plants are capable of influencing the composition and function of the microbial communities around its roots (Bais et al., 2006). These exudates can take around 20% of the plants photosynthetic products (Haichar et al., 2008), and compose about 40% of the available carbon input in soils (Richardson et al., 2009). Using exudates to select parts of the microbial community to colonize the plant rhizosphere and endosphere, plants populate their roots with the most appropriate partners they can find. Bacteria, in their turn,

compete very strongly with each other to colonize these niches, in a fierce struggle for life while under the influence of the plant host. This creates a rhizosphere effect, effectively generating a gradient of diversity in soil: bulk soil has fewer nutrients and more diversity, the rhizosphere has much more carbon substrates but less diversity, and the endosphere has more nutrients, less competition, and less diversity, but its entry is tightly regulated by the plant (Hatmann et al., 2008). As they feed from the exudates, beneficial bacteria will help plants by solubilizing nutrients, producing plant hormones, and prevent colonization of pathogens or other deleterious microorganisms. Details on the mechanisms used by bacteria to help the plant are presented in Chapter 1.

As they are so important for plant health, it is not surprising that such bacteria are important for agriculture today. Improvement of food production has always been a pursue for humans. It is clear, however, that the modern methods we use to produce our food, and the production of chemicals necessary for food production, are not sustainable. Environmental concerns about production and use of fertilizers are also presented on Chapter 1. It is enough to say for now that sustainable production of food is one of the most critical issues to ensure continuity of modern society in the future. The plant growth promoting bacteria (PGPB) enter modern agriculture exactly on this point, for being a renewable, cheap resource that can help in shifting the way we produce food into forms that are less dependent of finite resources (Berg et al., 2013).

PGPB have been in fact been used at industrial scale for years, saving billions of dollars in fertilizers (Alves et al., 2003). The commercialization of inoculants as formulated biofertilizers reached about U$ 440 million in 2012 and is expected to increase 10% per year (Owen et al., 2015). Even so, plant-bacteria-environment interactions are so complex due to biological diversity in soil that a selected strain that is effective in one location might not be effective on another. Issues like soil type, pH, crop varieties, farming practices, chemical amendment, formulation of the inoculant, previous soil use and also the interaction with the native microbial community might reduce PGPB activity or even deny it (Bashan et al., 2013). The interaction of inoculated PGPB with the native community is one of the most difficult issues to solve, because of the sheer diversity found in soil systems. Until recently, it was difficult to study this specific issue, since most approaches were based on culture-dependent methods, which are often reported to represent only about 1% of the total diversity in soil (Daniel, 2005). The development of metagenomics greatly improved our ability to detect bacteria in soil, and the evolution of the next generation sequencing (NGS)

technology and bioinformatics have already changed the field completely. Processing information and generating useful knowledge out of databases is one of the new challenges that the field will have to address. But it must be kept in mind that even the most recent methods will have their limitations and bias, from which that 1% of the cultivable population can help us detect and correct.

But even with extensive testing of strains and variables, and using the most advanced technology available, there may always be unpredicted interactions happening in real-world applications. That is why it is important to study patterns and correlation on functional groups and ecological networks that can be generalized and applied to PGPB testing. Understanding how the plant-soil system will react to the addition of different types of inoculants can help us to choose the best candidates for different conditions. This is the main objective of this thesis: to detect correlations between what the plants selects from the microbial community in different conditions and how different bacteria can contribute to it, anticipating experimental results. Using the data available in our laboratory, we managed to construct a simplified model that polarizes plant-bacteria interactions to nutrient-rich and nutrient-poor conditions. This model is presented in Chapter 2, and describes some bacterial traits that would be selected by the plant in these polarized conditions. On Chapter 3, this model is tested on a greenhouse assay, also challenged over a diversity gradient, as we apply classical invasion ecology theories to PGPB inoculation. Finally, on Chapter 4 we show a statistical approach that can be useful on PGPB inoculation research that uses NGS, as it easily highlight differences in community composition to a standard, such as a negative or positive controls.

References

Alves BJR, Boddey RM, Urquiaga S (2003) The success of BNF in soybean in Brazil. Plant Soil: 1ï 9.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57: 233ï 266. doi:10.1146/annurev.arplant.57.032905.105159.

Barea J-M, Pozo MJ, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56: 1761ï 1778. doi:10.1093/jxb/eri197.

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P (2013) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998ï 2013). Plant Soil 378: 1ï 33. doi:10.1007/s11104-013-1956-x.

Berg G, Zachow C, Müller H, Philipps J, Tilcher R (2013) Next-Generation Bio-Products Sowing the Seeds of Success for Sustainable Agriculture. Agronomy 3: 648ï 656. doi:10.3390/agronomy3040648.

Daniel R (2005) The metagenomics of soil. Nat Rev Microbiol 3: 470ï 478. doi:10.1038/nrmicro1160.

Griffiths BS, Philippot L (2012) Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev: 1ï 18. doi:10.1111/j.1574-6976.2012.00343.x.

Haichar FEZ, Marol C, Berge O, Rangel-Castro JI, Prosser JI, et al. (2008) Plant host habitat and root exudates shape soil bacterial community structure. ISME J 2: 1221ï 1230. doi:10.1038/ismej.2008.80.

Hartmann A, Schmid M, Tuinen D Van, Berg G (2008) Plant-driven selection of microbes. Plant Soil 321: 235ï 257. doi:10.1007/s11104-008-9814-y.

Koonin E V (2014) The origins of cellular life. Antonie Van Leeuwenhoek 106: 27ï 41. doi:10.1007/s10482-014-0169-5.

Koonin E V, Makarova KS, Aravind L (2001) Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55: 709ï 742.

Owen D, Williams a. P, Griffith GW, Withers PJ a. (2015) Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. Appl Soil Ecol 86: 41ï 54. doi:10.1016/j.apsoil.2014.09.012.

Ranjard L, Richaume a (2001) Quantitative and qualitative microscale distribution of bacteria in soil. Res Microbiol 152: 707ï 716.

Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 305ï 339. doi:10.1007/s11104-009-9895-2.

Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, et al. (2015) Metabolic dependencies drive species co-occurrence in diverse microbial communities. Proc Natl Acad Sci U S A 112: E7156. doi:10.1073/pnas.1522642113.

Introdução e Objetivos Gerais

Solos naturais tem uma diversidade excepcional, muito maior do que qualquer outro ambiente em nosso planeta. Uma única grama de solo pode ter até 10^9 células procarióticas (Griffiths and Philippot, 2012), 2,000 a 18,000 genomas (Daniel, 2005), somando 300 a 3.000kg de biomassa por hectare (Ranjard and Richaume, 2001). Solos recebem diversos tipos de substratos, nutrientes, contaminantes e são sujeitos a muitos tipos de estresse. Isso cria um sistema muito complex e aberto, que opera com redes e comunidades, onde competição, antagonismo, cooperação e simbiose estão todos ocorrendo em um milímetro cúbico (Zelezniak et al., 2015). Bactérias desafiam nossas definições de organismo e spécie, ao sedesenvolverem em biofilmes, liquens, e outras comunidades complexas, onde components específicos tem funções específicas. A luta pela sobrevivência das bactérias foi a primeira a começar na Terra, desde pelo menos 3.5 bilhões de anos atrás (Koonin, 2014), então não é surpreendente que elas possam tomar tantas formas e funções diferentes por todo o planeta. Um único exemplo pode demonstrar a abilidade de bactérias em cooperar com outros microorganismos. O desenvolvimento da endosimbiose, que eventualmente culminou em mitocôndrias e cloroplastos modernos, formou a biosfera como a conhecemos. Este evento, que ocorreu independentemente ao menos 7 vezes para cloroplastos (Koonin et al., 2001) gerou organismos que eram metabolicamente muito eficientes, e sozinho é evidencia que bactérias podem desenvolver cooperações íntimas, muito eficientes, com outros organismos. Sendo tão presentes na biosfera, interagindo com outros organismos em nível tão basal, e fazendo parte em todos os ciclos biogequímicos, bactérias influenciam a vida na terra com mais do que subprodutos a serem explorados.

Plantas tiveram que coexistir com bactérias desde o início de sua história evolutiva. Hoje, vemos que tanto plantas como bactérias tem muitos tipos de interação pois dividem o mesmo espaço (Bareae et al., 2005). É muito claro que bactérias no solo podem afetar saúde e desenvolvimento das plantas, não apenas através de patógenos e doenças como foi primeiramente considerado quando bactérias vivendo dentro de raízes de plantas foram inicialmente descobertas. Através da ação de substratos carbonicos e moléculas sinalizadoras liberadas pelas raízes (exudatos) plantas são capazes de influenciar a composição e função das comunidades bacterianas ao redor de suas raízes.

Estes exudatos podem conter cerca de 20% dos produtos fotosintéticos das plantas (Haichar et al., 2008), e compor cerca de 40% das fontes de carbono disponível no solo (Richardson et al., 2009). Usando exudatos para selecionar partes da

comunidade microbiana para colonizar a rizosfera e endosfera da planta, elas povoam suas raízes com os parceiros mais apropriados que puderem encontrar. Bactérias, por sua vez, competem para colonizar esses nichos, em uma feroz luta pela vida enquanto sob influência do hospedeiro vegetal. Isso cria o efeito rizosférico, gerando um gradiente de diversidade no solo: solo livre tem menos nutrientes e mais diversidade, a rizosfera tem mais substratos carbônicos mas menos diversidade, e a endosfera tem mais nutrients, menos competição e diversidade, mas sua entrada é altamente regulada pela planta (Hatmann et al., 2008). Enquanto se alimentam dos exudatos, bactérias benéficas vão ajudar plantas ao solubilizar nutrients, produzindo hormônios vegetais, e evitando colonização de patógenos ou outros microorganismos deletérios. Detalhes dos mecanismos usados por bactérias para ajudar as plantas são apresentadas no Capítulo 1.

Como são tão importantes para a saúde vegetal, não é surpreendente que tais bactérias sejam importantes para a agricultura hoje. Melhoramento da produção de alimentos sempre foi um objetivo humano. Está claro, porém, que os métodos modernos usados para produzir nossa comida, e a produção de químicos necessários para tal, não são sustentáveis. Considerações ambientais sobre a produção e uso de fertilizantes são apresentadas no Capítulo 1. Aqui é suficiente dizer que a produção sustentável de alimentos é um dos problemas mais críticos para garantir a sociedade moderna no futuro. As bactérias promotoras de crescimento vegetal (*plant growth promoting bacteria*, PGPB) entram na agricultura moderna exatamente neste ponto, por serem um recurso renovável e barato que pode ajudar em mudar o método com que produzimos comida para formas que são menos dependents em recursos finitos (Berg et al., 2013).

PGPB já tem sido utilizada em escala industrial por anos, economizando bilhões de dólares em fertilizantes (Alves et al., 2003). A comercialização de inoculantes como biofertilizantes formulados alcançou cerca de U$440 milhões em 2012 e é esperado em crescer 10% ao ano (Owen et al., 2015). Mesmo assim, interações planta-bactéria-ambiente são tão complexas devido a diversidade biológica no solo que uma linhagem selecionada que é eficiente em um lugar pode não ser eficiente em outro. Parâmetros como tipo de solo, pH, variedades de semente, práticas agrícolas, adição de químicos, formulação do inoculante, usos anteriores do solo e também a interação com a microbiota naiva podem reduzir ou mesmo negar a ação de PGPB (Bashan et al., 2013). A interação de PGPB inoculadas com a comunidade nativa é um dos problemas mais difíceis de resolver, devido a grande diversidade encontrada em sistemas de solo. Até recentemente, era difícil estudar este ponto específico já que a maioria das abordagens

eram baseadas em métodos dependents de cultivo bacteriano, que são frequentemente reportados como representando apenas 1% da diversidade total do solo (Daniel, 2005) O desenvolvimento da metagenômica melhorou muito nossa habilidade em detectar bactérias no solo, e a evolução da tecnologia de sequenciamento de nova geração (Next generation sequencing, NGS) e ferramentas de bioinformatica já mudaram o campo completamente. Processando informação e gerando conhecimento útil apartir de bancos de dados é um dos novos desafios que o compo terá que enfretar. Mas é preciso manter em mente que até o mais recente dos métodos terão suas limitações e viézes, dos quais aquele 1% da população cultivável pode nos ajudar a detector e corrigir.

Mas mesmo com extensivos testes de linhagens e variáveis, e usando a tecnologia mais avançada disponível, sempre poderão haver interações imprevistas ocorrendo em situações de campo. Por isso que é importante estudar padrões e correlações em grupos funcionais e redes ecológicas que podem ser generalisadas e aplicadas a testes com PGPB. Entendendo como o sistema solo-planta vai reagir a adição de diferentes tipos de inoculantes pode nos ajudar a escolher os melhores condidatos para diferentes condições. Este é o principal objetivo desta tese: detectar correlações entre o que a planta seleciona da comunidade microbiana em diferentes condições e como diferentes bactérias podem contribuir nisso, antecipando resultados experimentais. Usando dados disponíveis em nosso laboratório, nós conseguimos construir um modelo simplificado que polariza interações planta-bactéria em condições ricas e pobres em nutrients. Este modelo é apresentado no Capítulo 2, e descreve algumas características bacterianas que seriam selecionadas pela planta nessas condições polarizadas. No capítulo 3, este modelo é testado em casa de vegetação, também sob um gradiente de diversidade, enquanto aplicamos teorias da ecologia de invasão a inoculação de PGPB. Por fim, no Capítulo 4, nos mostramos uma abordagem estatística que pode ser útil na pesquisa de inoculação de PGPB que usa NGS, pois a abordagem facilmente destaca diferenças na composição da comunidade com um padrão, como controles positivos ou negativos.

Referências

Alves BJR, Boddey RM, Urquiaga S (2003) The success of BNF in soybean in Brazil. Plant Soil: 1ï 9.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57: 233ï 266. doi:10.1146/annurev.arplant.57.032905.105159.

Barea J-M, Pozo MJ, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56: 1761ï 1778. doi:10.1093/jxb/eri197.

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P (2013) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998ï 2013). Plant Soil 378: 1ï 33. doi:10.1007/s11104-013-1956-x.

Berg G, Zachow C, Müller H, Philipps J, Tilcher R (2013) Next-Generation Bio-Products Sowing the Seeds of Success for Sustainable Agriculture. Agronomy 3: 648ï 656. doi:10.3390/agronomy3040648.

Daniel R (2005) The metagenomics of soil. Nat Rev Microbiol 3: 470ï 478. doi:10.1038/nrmicro1160.

Griffiths BS, Philippot L (2012) Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev: 1ï 18. doi:10.1111/j.1574-6976.2012.00343.x.

Haichar FEZ, Marol C, Berge O, Rangel-Castro JI, Prosser JI, et al. (2008) Plant host habitat and root exudates shape soil bacterial community structure. ISME J 2: 1221ï 1230. doi:10.1038/ismej.2008.80.

Hartmann A, Schmid M, Tuinen D Van, Berg G (2008) Plant-driven selection of microbes. Plant Soil 321: 235ï 257. doi:10.1007/s11104-008-9814-y.

Koonin E V (2014) The origins of cellular life. Antonie Van Leeuwenhoek 106: 27ï 41. doi:10.1007/s10482-014-0169-5.

Koonin E V, Makarova KS, Aravind L (2001) Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55: 709ï 742.

Owen D, Williams a. P, Griffith GW, Withers PJ a. (2015) Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. Appl Soil Ecol 86: 41ï 54. doi:10.1016/j.apsoil.2014.09.012.

Ranjard L, Richaume a (2001) Quantitative and qualitative microscale distribution of bacteria in soil. Res Microbiol 152: 707ï 716.

Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 305ï 339. doi:10.1007/s11104-009-9895-2.

Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, et al. (2015) Metabolic dependencies drive species co-occurrence in diverse microbial communities. Proc Natl Acad Sci U S A 112: E7156. doi:10.1073/pnas.1522642113.

PLOS | ONE

RESEARCH ARTICLE

A Model to Explain Plant Growth Promotion Traits: A Multivariate Analysis of 2,211 Bacterial Isolates

Pedro Beschoren da Costa[1], Camille E. Granada[2], Adriana Ambrosini[1], Fernanda Moreira[1], Rocheli de Souza[1], João Frederico M. dos Passos[1,3], Letícia Arruda[1], Luciane M. P. Passaglia[1]*

1. Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, **2.** UNIVATES, Lajeado, RS, Brazil, **3.** Empresa de Pesquisa e Extensão Agropecuária de Santa Catarina (EPAGRI), Lages, SC, Brazil

*luciane.passaglia@ufrgs.br

OPEN ACCESS

Citation: Costa PBd, Granada CE, Ambrosini A, Moreira F, de Souza R, et al. (2014) A Model to Explain Plant Growth Promotion Traits: A Multivariate Analysis of 2,211 Bacterial Isolates. PLoS ONE 9(12): e116020. doi:10.1371/journal.pone.0116020

Editor: Daniele Daffonchio, University of Milan, Italy

Received: July 14, 2014

Accepted: December 1, 2014

Published: December 26, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work was financed by a grant and fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil) and Instituto Nacional de Ciência e Tecnologia (INCT) da Fixação Biológica do Nitrogênio (Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Plant growth-promoting bacteria can greatly assist sustainable farming by improving plant health and biomass while reducing fertilizer use. The plant-microorganism-environment interaction is an open and complex system, and despite the active research in the area, patterns in root ecology are elusive. Here, we simultaneously analyzed the plant growth-promoting bacteria datasets from seven independent studies that shared a methodology for bioprospection and phenotype screening. The soil richness of the isolate's origin was classified by a Principal Component Analysis. A Categorical Principal Component Analysis was used to classify the soil richness according to isolate's indolic compound production, siderophores production and phosphate solubilization abilities, and bacterial genera composition. Multiple patterns and relationships were found and verified with nonparametric hypothesis testing. Including niche colonization in the analysis, we proposed a model to explain the expression of bacterial plant growth-promoting traits according to the soil nutritional status. Our model shows that plants favor interaction with growth hormone producers under rich nutrient conditions but favor nutrient solubilizers under poor conditions. We also performed several comparisons among the different genera, highlighting interesting ecological interactions and limitations. Our model could be used to direct plant growth-promoting bacteria bioprospection and metagenomic sampling.

Introduction

Plant growth-promoting bacteria (PGPB) are microorganisms that are naturally found inside and around plant roots. These microorganisms participate in complex ecological interactions in the rhizosphere, where they can influence the health, growth and stress response of their host plants [1]. PGPB can be used as inoculants for crop plants aiming at sustainable food production. In some cases, the use of these bacteria can reduce chemical fertilizer usage up to 50% [2], which represents a huge benefit to the environment because chemical fertilizers are polluting agents based on finite resources [3]. In fact, research on PGPB has been increasing for years [4], and the use of these bacteria might be the future of modern agriculture [5], either for a biotechnologically intensive or a natural and organic-based approach.

While there are many reports of the successful prospection and use of PGPB, most of the actual interactions that occur in the rhizosphere are unknown, as the soil-plant-microorganism interface is a very complex open system [6]. Because there are many factors affecting rhizosphere dynamics, multivariate statistics for microbial ecology have become a very important tool for understanding the general outcomes that elude univariate statistics and linear relationships [7]. Unfortunately, these methods are not widely used by microbiologists, and even classical statistical tests are absent from several reports. Many papers on PGPB – which are expensive and difficult to obtain – are underexploited. In addition, new molecular biology technologies, such as deep sequencing and microarrays, generate large datasets, requiring advanced statistical analysis [8].

Having at our disposal data from seven different studies that shared a common methodology for bioprospection, we created a databank of 2,211 putative diazotrophic PGPB that were isolated from different crops. We discovered interesting patterns in the soil-plant-microorganism interface that were not clear in the independent studies upon which this paper was based. We propose a model that suggests that plants permit an endophytic relationship with associated bacteria based on the plant nutritional needs and on the bacterial plant growth-promoting abilities. According to this model, nutrient-solubilizing bacteria are favored under nutrient-poor conditions, while hormone-producing bacteria are favored under nutrient-rich conditions. These findings can be used to direct the bioprospection of PGPB, genes or metagenomes, and the methodology that is used in these analyses can be replicated by microbiology researchers who have access to a large collection of isolates.

Materials and Methods

Dataset compilation

To create the dataset that was used in this work, bacterial collections from six published papers and one personal communication from our group were pooled. These works, although performed independently from each other, shared similar methodology, focusing on the isolation and characterization of PGPB for

biotechnological applications. Bacterial isolates were obtained from the rhizospheric soils or roots of rice collected in Cachoeirinha (29°56′51.9′′S, 51°06′46.3′′W) for reference [9]; Aceguá (31°45′11′′S, 54°3′22′′W), Arroio Grande (32°14′19′′S, 53°5′27′′W), Cachoeirinha (29°56′51.9′′S, 51°06′46.3′′W), Santa Vitória do Palmar (33°31′08′′S, 53°22′04′′W), Uruguaiana (29°45′18′′S, 57°05′16′′W), and Viamão (30°04′51′′S, 51°01′22′′W) for reference [10]; wheat collected in: São Borja (28°39′39′′S, 56°00′14′′W), Júlio de Castilhos (29°13′37′′S, 53°40′54′′W), Vacaria (28°30′43′′S, 50°56′02′W), Campina das Missões (27°59′20′′S, 54°50′22′′W), Guarani das Missões (28°08′27′′S, 54°33′29′′W), and Boa Vista do Cadeado (28°35′06′′S, 53°47′57′′W) (Moreira, personal communication); maize collected in: Júlio de Castilhos (29°13′37′′S, 53°40′54′′W), Porto Alegre (30°1′40′′S, 51°13′43′′W), Rio Grande (32°04′54′′S, 52°09′48′′W), Vacaria (28°30′43′′S, 50°56′02′W) and Veranópolis (28°54′3′′S, 51°33′10′′W) for reference [11]; sunflower collected in: Encruzilhada do Sul (30°32′38′′S, 52°31′19′′W)], São Borja (28°39′39′′S, 56°00′14′′W), São Gabriel (30°20′0′′S, 54°19′12′′W)], Vacaria (28°30′43′′S, 50°56′02′′W)], and Viamão (30°04′51′′S, 51°01′22′′W) for reference [12]; apple trees collected in São Joaquim (28°17′36′′S, 49°56′1′′W) for reference [13]; and *Lupinus albescens* grown in arenized and non-arenized areas located between the latitudes of 29°00′S to 31°00′S and longitudes of 54°30′W to 58°45′W for reference [14]. No specific permissions were required for all of these locations and the field studies did not involve endangered or protected species. The analyzed soil chemical characteristics were the pH, clay, organic matter, phosphorous (P) and potassium (K) contents [15]. The characteristics that were considered for the isolates were niche colonization (rhizospheric or endophytic), the amount of indolic compounds (ICs) produced, the halo sizes of bacterial colonies in plate assays for tricalcium phosphate (TCP) the solubilization and siderophores production abilities, the bacterial genera, and the sample origin of the isolate. Nitrogen fixation potential was not quantified for the majoritiy of the isolates, so this PGP trait could not partake in our model. The isolation was performed according to Döbereiner [16]. Isolated diazotrophs are considered putative as some bacteria might survive selective isolation by using cellular N reserves, or scavenging very low N content from the original soil solution. The full dataset for this work is presented in S1 and S2 Tables.

In all of the analyzed studies, rhizospheric isolates were obtained from the soil that was immediately attached to plant roots, and putative endophytic isolates were obtained from surface-sterilized plant roots. Root sterilization was performed in 70% ethanol for 2 minutes and sodium 4.0% hypochlorite for 2 minutes, followed by several water washings. While our surface sterilization procedure might allow the survival of bacteria protected in root crevices or by biofilm, such bacteria nevertheless would have a more intimate colonization of the plant compared to the rhizospheric bacteria. Furthermore, these occasional survivors should not outnumber endophythic bacteria to the point of compromising the results. The halo size of the bacterial colonies in plate assays for TCP solubilization [17] and siderophores production [18] was classified as 1= no

halo, 2= small or average halo size (ranging from 0.1 to 0.6 mm), and 3= large halo size (larger than 0.6 mm). The halo size of positive siderophores producers was not registered by one of the authors [11]; therefore, we could only consider the halo size of the non-producing isolates from this dataset in our analysis. Thus, 99 positive siderophores isolates were not analyzed and were considered as missing data regarding their siderophores production ability. Indolic compounds production was determined after 72 h of incubation in King B medium that was supplemented with tryptophan using the Salkowski reagent [19]. The values were reported as micrograms of ICs per milliliter (μg of ICs ml^{-1}). The isolates were identified at the genus level by PCR-RFLP and the partial sequencing of the 16 S rRNA gene using the procedures described by Ambrosini *et al.* [12]. In this study, the bacterial genus was considered only if it contained at least 5 isolates. The genera that contained 4 or less isolates or isolates that were not identified at the genus level were pooled as the "rare" portion of the microbiota. This rare portion was composed of 134 unidentified isolates and 57 isolates belonging to 40 genera, as shown in S1 Table.

Statistical analysis

To classify the different soils samples into poor, average or rich categories, the soil chemical characteristics (pH, organic matter, clay, K and P contents) were analyzed by a Principal Component Analysis (PCA). Afterwards, we tested the PCA soil classification with ANOVA (log-transformed pH, organic matter, clay, and K contents) and Kruskal-Wallis (P contents). The multivariate analysis of the bacterial isolate characteristics was performed by a Categorical Principal Component Analysis (CatPCA).

To associate the categorical data (halo sizes for siderophores production and TCP solubilization abilities, soil richness, and genera), we used the chi-square statistic obtaining the exact p value. When necessary, a Monte Carlo simulation was used to estimate a p value window (the upper and lower borders were always $0.001>p>0.0001$). An adjusted standardized residual analysis was used to detect significant individual associations that were reported on a heat map. Comparisons of the ICs production levels according to the soil condition, TCP solubilization and siderophores production were performed with the Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons, which considers different sample sizes [20]. The comparison of ICs production according to the colonization niche was performed with the Mann-Whitney pair wise comparison. In these analyses, non-ICs producers were not included. As the variance was too high to return meaningful results when comparing the ICs production across genera, we categorized ICs production as low (0–10), average (11–80) and high (80 or more) μg of ICs ml^{-1} and analyzed it as in phosphate solubilization and siderophores production. The differences were considered significant at $p<0.05$, and to correct for global type I error, we determined a False Discovery Rate of 10% [21]. All hypotheses tests (with sample sizes, p values, degrees of freedom,

and false discovery rate) are shown on S3 Table. Additional information on the statistical methods is presented as Supplementary Material (S1 Text).

Results

Our dataset was composed of 2,211 bacterial isolates classified in 80 genera, with 1,061 endophytic and 1,150 rhizospheric isolates. There were 634 TCP solubilizers, 1,358 siderophores producers, and 1,977 IC producers. These isolates were obtained from 40 different soil samples from seven different plants plus two natural grasslands.

Multivariate plotting and analysis

Soil PCA

The PCA analysis of the soil chemical characteristics allowed us to separate the soil samples into three clusters (Fig. 1). The evaluated characteristics – whose higher values are associated with productive, healthy and rich soils [22] - were plotted on the positive values of the first principal component, with the P contents more associated to the second principal component due to two soils with very high P contents (soils 23 and 30). We considered that these three clusters allowed us to classify the soils from which the bacteria were isolated as poor, average and rich, thereby both grouping and dividing an otherwise very heterogeneous sample origin dataset with mixed plants and farming managements. Soil 23 was considered an average soil, and soil 30 was considered a rich soil. Most of the soils from the poor conditions were from an arenized area that was not used for crop production and that lacks vegetal cover other than *Lupinus* sp., a leguminous plant (Granada *et al.*, 2013). We also performed supervised statistics to test these classifications. For all soil characteristics, richer soils had higher values than poor soils. Average soils presented intermediate values for all soil characteristics, but were statistically similar to rich soils for P contents and pH, and statistically similar to poor soils for clay contents (S1 Fig.).

Analysis of the isolates by CatPCA

In the CatPCA analysis (Fig. 2), the soil richness increases towards the positive values of the first dimension (X axis), while the TCP solubilization ability increases with the negative values of the same dimension. Because the vectors (lines) increase in opposite directions, we could say that the best TCP solubilizers would be found in the poorer soils. The ICs production ability of bacterial isolates increases towards the positive values of both dimensions. This result suggests that the ICs production ability of the isolates increases as the soil richness increases and should decrease as the phosphate solubilization ability of the isolates increases. The siderophores production vector is plotted close to the phosphate solubilization vector, suggesting that these vectors could be associated as well. Finally, the different bacterial genera were separated into three different clusters: one associated with high ICs production, another associated with poor soils and

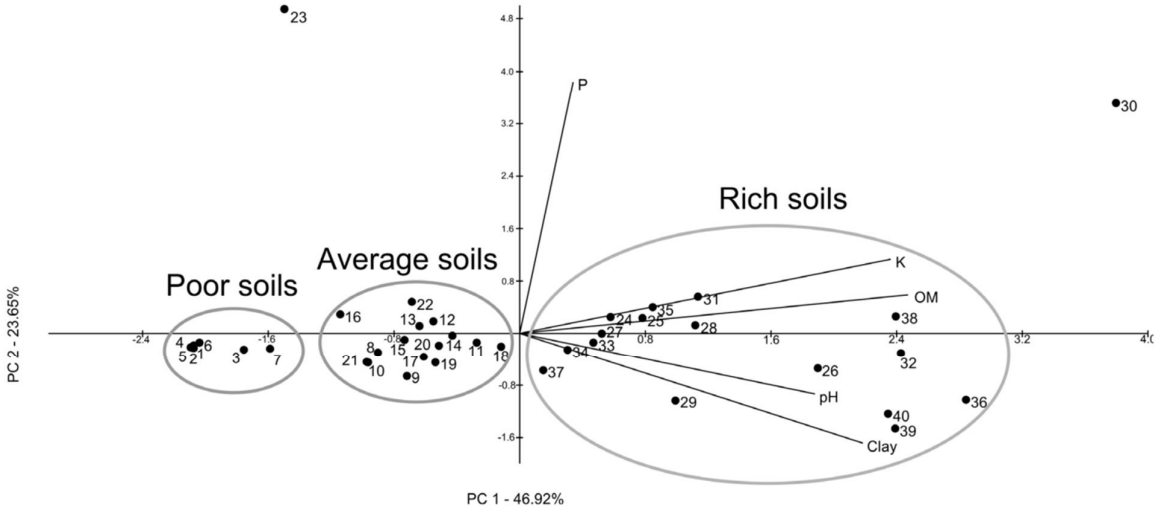


Fig. 1. PCA analysis of the soil characteristics from the 40 soils samples (numbered black circles) that were used for bacterial isolation. The percentages show how much variation is explained by each principal component. The soils with higher pH, organic matter (OM), potassium (K), phosphorus (P), and clay (Clay) contents are plotted to the right. There are three clusters along the first principal component (PC1) that grouped the soils by overall richness. Based on these clusters, all 40 of the soil samples were classified according to their overall soil richness: poor, average or rich. The appropriate soil richness was attributed to each bacterial isolate (according to its origin) before further analysis. Supervised statistics of these data on S1 Fig.

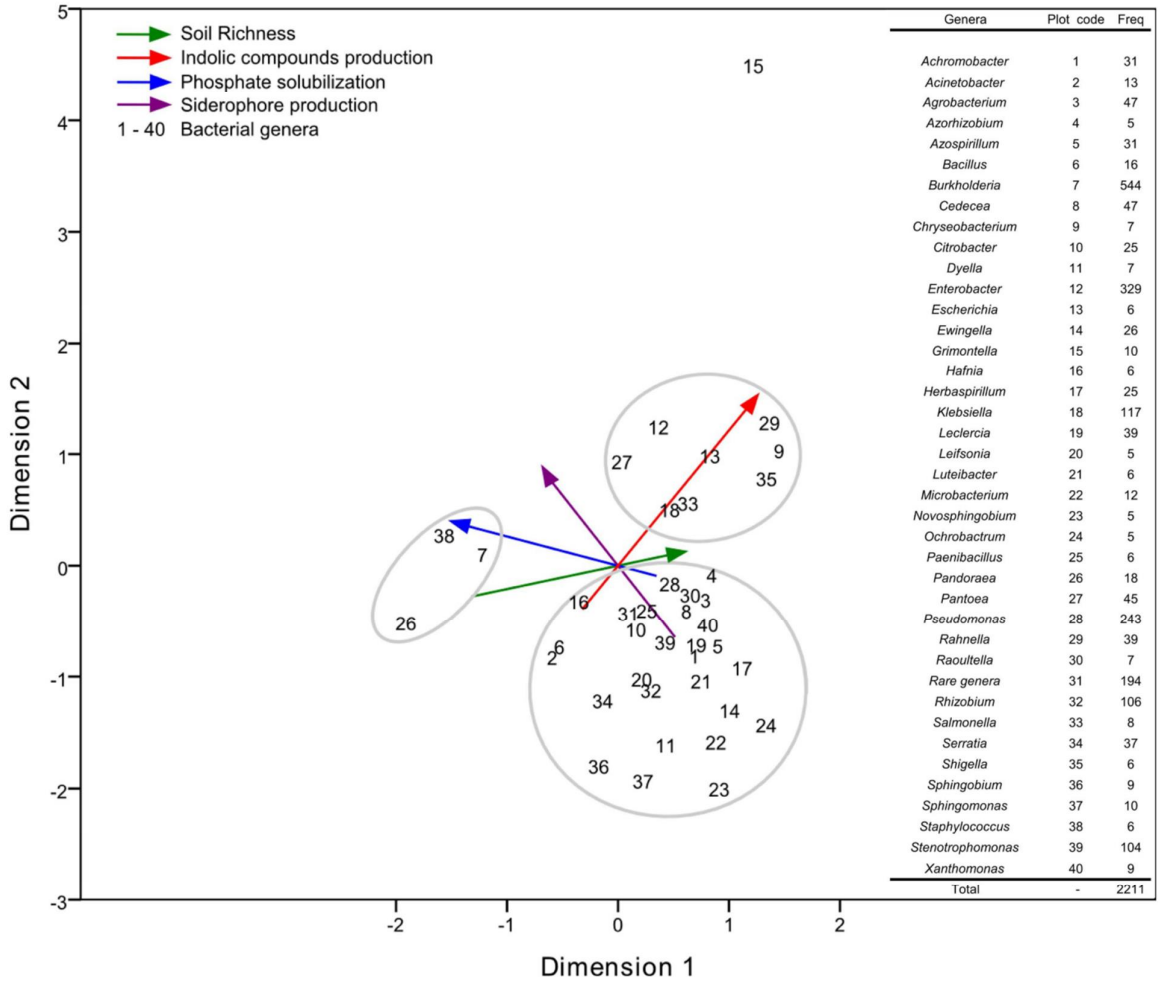
doi:10.1371/journal.pone.0116020.g001

high phosphate solubilization, and a larger cluster that does not seem to be associated with a high expression of any of the evaluated PGP traits.

Hypothesis testing

PGP traits and the environment

The multiple associations between the PGP traits, bacterial genera and environment were further verified by hypothesis testing. The ICs production ability of the bacterial isolates increases as the soil richness increases (Fig. 3). However, the ICs production ability of the best TCP solubilizers is lower than the ICs production ability of those isolates that did not present a good TCP solubilization capacity. Similarly, the best siderophores producers were not the best ICs producers. The association heat map (Fig. 4 and S2 Fig.) shows that higher TCP solubilization ability of the bacterial isolates was associated with poor soils, and that the richer soils were associated with isolates that presented a lower TCP solubilization ability. Similar associations occurred with siderophores production: the isolates with a strong ability to produce siderophores were associated with poor soils, while those with weak siderophores production abilities were associated with richer soils. Finally, we showed that siderophores production and TCP solubilization abilities have some degree of correlation: there is an excessive number of isolates that were level 1 TCP solubilizers and level 1 siderophores producers, or were level 3 TCP solubilizers and level 3 siderophores producers. At the same time, there was a reduced number of isolates that were



Genera	Plot code	Freq
Achromobacter	1	31
Acinetobacter	2	13
Agrobacterium	3	47
Azorhizobium	4	5
Azospirillum	5	31
Bacillus	6	16
Burkholderia	7	544
Cedecea	8	47
Chryseobacterium	9	7
Citrobacter	10	25
Dyella	11	7
Enterobacter	12	329
Escherichia	13	6
Ewingella	14	26
Grimontella	15	10
Hafnia	16	6
Herbaspirillum	17	25
Klebsiella	18	117
Leclercia	19	39
Leifsonia	20	5
Luteibacter	21	6
Microbacterium	22	12
Novosphingobium	23	5
Ochrobactrum	24	5
Paenibacillus	25	6
Pandoraea	26	18
Pantoea	27	45
Pseudomonas	28	243
Rahnella	29	39
Raoultella	30	7
Rare genera	31	194
Rhizobium	32	106
Salmonella	33	8
Serratia	34	37
Shigella	35	6
Sphingobium	36	9
Sphingomonas	37	10
Staphylococcus	38	6
Stenotrophomonas	39	104
Xanthomonas	40	9
Total	-	2211

Fig. 2. CatPCA analysis of 2,211 bacterial isolates. The indolic compounds production, TCP solubilization, siderophores production and soil richness are shown as colored vectors, with arrows indicating the vector's direction in the plot. The black numbers show the average position of each bacterial genus. In the right column are shown the bacterial genera, the number they represent in the plot (Plot code), and their frequency in the dataset (Freq). Cronbach's alpha value was 0.774.

doi:10.1371/journal.pone.0116020.g002

level 3 TCP solubilizers and level 1 siderophores producers or that were level 1 TCP solubilizers and level 3 siderophores producers. This observation indicates, for example, that the simultaneous high expression of these two PGP traits in the same bacterium occurs with a greater frequency than expected.

Niche effect on the PGP traits

The niche effect – which considers the occurrence of certain bacteria within the plant roots (endophytic) or around the rhizosphere – could not be accurately verified by the CatPCA (see S1 Text). As shown in Fig. 5, the ICs production

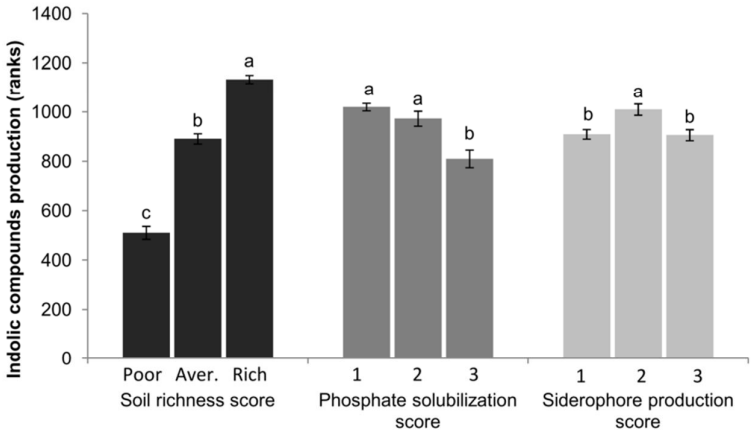


Fig. 3. Indolic compound production ability of the isolates (average rank ±1 SE) according to the soil nutrient conditions and TCP solubilization and siderophores production abilities. The phosphate solubilization and siderophores production scores are 1= no halo, 2= small or average halo, and 3= large halo. The soil richness score is according to the PCA analysis (Fig. 1). Different letters show significant differences. Sample sizes and p values are presented on S3 Table.

doi:10.1371/journal.pone.0116020.g003

ability was different between the endophytic and rhizospheric isolates: the best ICs producers were found in the rhizospheric soils of plants that were cultivated in poor soils or were isolated from the roots of plants that were cultivated in average or rich soils. The niche effects on TCP solubilization and siderophores production are shown in a heat map in Fig. 6 and S3 Fig. Apparently, the endophytic and rhizospheric bacterial populations presenting these two PGP traits behaved in a similar manner in poor soils, as these tests were non-significant. However, in average and rich soils, there were more level 3 TCP solubilizers and more level 3 siderophores producers in the rhizospheric soils than there were inside the plant.

Bacterial genus association with the PGP traits and the environment

The bacterial genus association with all of the PGP traits, the environment, and the niche effects can be seen on the heat map in Fig. 7 and S4 Fig. Few bacterial

Soil	Phos 1	Phos 2	Phos 3
Poor	133	49	39
Average	532	155	125
Rich	912	178	88

Soil	Sid 1	Sid 2	Sid 3
Poor	69	61	91
Average	250	178	343
Rich	435	383	302

Phos	Sid 1	Sid 2	Sid 3
1	611	468	471
2	104	101	127
3	39	53	138

Fig. 4. Heat map associations of the TCP solubilization (left) and siderophores production (middle) abilities of bacterial isolates with soil conditions and with each other (right). Phos = TCP solubilization, and Sid = siderophores production. 1= no halo, 2= small or average halo, and 3= large halo. The red cells = less isolates than expected under those conditions, the green cells = excessive number of isolates under those conditions, and the yellow cells = no significant differences between the observed and expected values. Percentages and residuals are shown in S2 Fig. Sample sizes and p values are presented on S3 Table.

doi:10.1371/journal.pone.0116020.g004

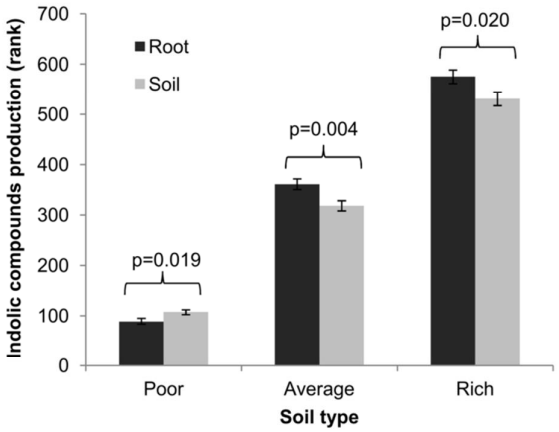


Fig. 5. Niche effect on ICs production (average ±1 SE) between endophytic (root) and rhizospheric (soil) isolates under each soil condition. The best ICs producers shift their colonization site according to soil richness. Sample sizes and p values are presented on S3 Table.

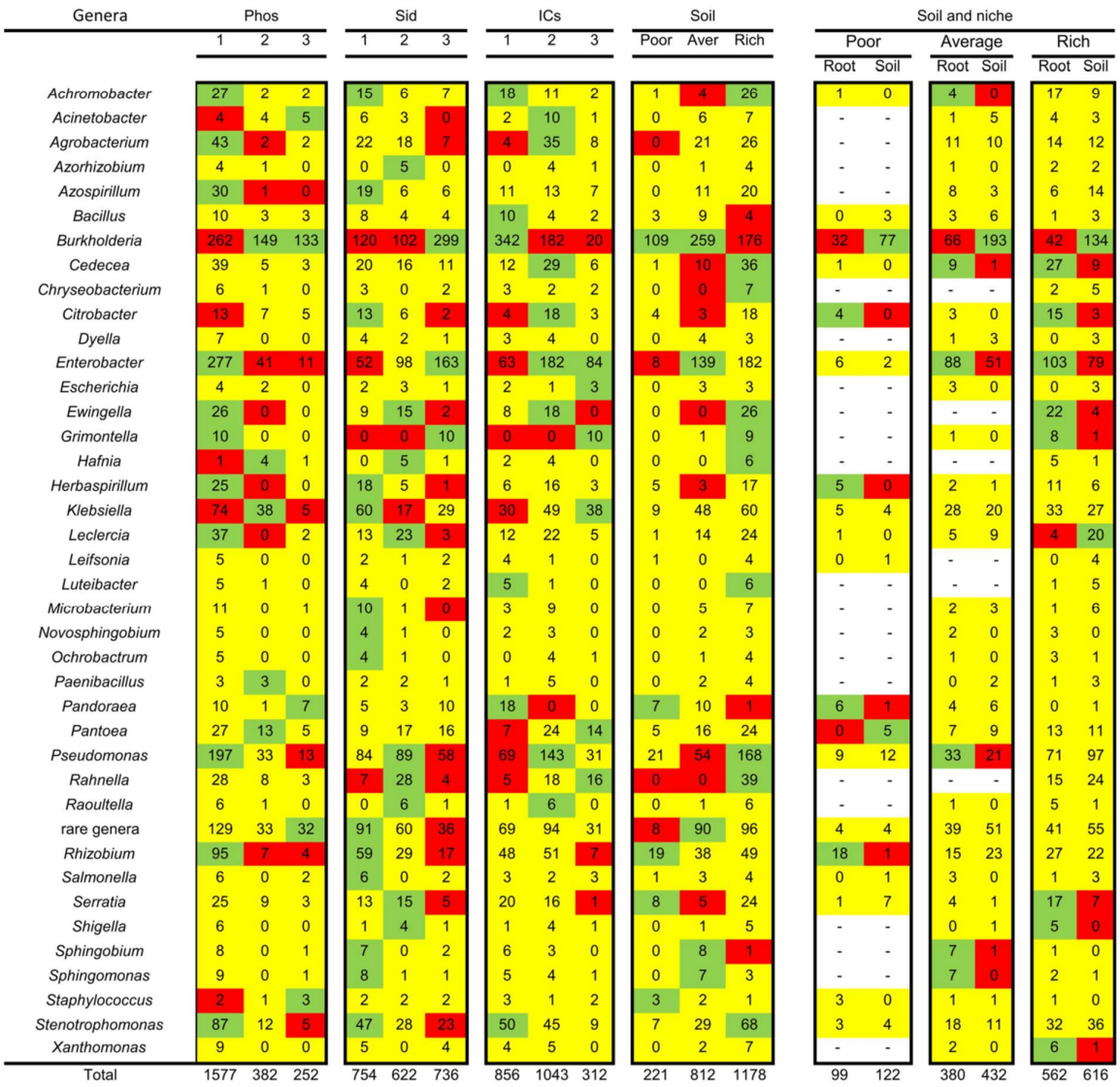
doi:10.1371/journal.pone.0116020.g005

genera (*Burkholderia, Acinetobacter, Hafnia, Pandoreae,* and *Staphylococcus*) presented strains that were associated with a high TCP solubilization ability, while others (*Achromobacter, Agrobacterium, Azospirillum, Enterobacter, Ewingella, Grimontella, Herbaspirillum, Leclercia, Pseudomonas, Rhizobium,* and *Stenotrophomonas*) presented strains that were associated with the non-solubilization ability. Only 29% of the isolates and 77% of the genera presented strains that were able to solubilize TCP. Most of the genera with strains that were associated with high ICs production belonged to the *Enterobactereace* family

Soil	Niche	Phos			Sid		
		1	2	3	1	2	3
Poor	Root	60	22	17	31	21	47
	Soil	73	27	22	38	40	44
Average	Root	263	73	44	127	97	137
	Soil	269	82	81	123	81	206
Rich	Root	448	91	23	202	208	125
	Soil	464	87	65	233	175	177

Fig. 6. Heat map associations of the TCP solubilization and siderophores production abilities of endophytic (root) and rhizospheric (soil) isolates under each individual soil condition. Phos = TCP solubilization, and Sid = siderophores production. 1= no halo, 2= small or average halo, and 3= large halo. The red cells = less isolates than expected under those conditions, the green cells = excessive number of isolates under those conditions, and the yellow cells = no significant differences between the observed and expected values. Percentages and residuals are shown in S3 Fig. Sample sizes and p values are presented on S3 Table.

doi:10.1371/journal.pone.0116020.g006



Genera	Phos			Sid			ICs			Soil			Soil and niche					
	1	2	3	1	2	3	1	2	3	Poor	Aver	Rich	Poor		Average		Rich	
													Root	Soil	Root	Soil	Root	Soil
Achromobacter	27	2	2	15	6	7	18	11	2	1	4	26	1	0	4	0	17	9
Acinetobacter	4	4	5	6	3	0	2	10	1	0	6	7	-	-	1	5	4	3
Agrobacterium	43	2	2	22	18	7	4	35	8	0	21	26	-	-	11	10	14	12
Azorhizobium	4	1	0	0	5	0	0	4	1	0	1	4	-	-	1	0	2	2
Azospirillum	30	1	0	19	6	6	11	13	7	0	11	20	-	-	8	3	6	14
Bacillus	10	3	3	8	4	4	10	4	2	3	9	4	0	3	3	6	1	3
Burkholderia	262	149	133	120	102	299	342	182	20	109	259	176	32	77	66	193	42	134
Cedecea	39	5	3	20	16	11	12	29	6	1	10	36	1	0	9	1	27	9
Chryseobacterium	6	1	0	3	0	2	3	2	2	0	0	7	-	-	-	-	2	5
Citrobacter	13	7	5	13	6	2	4	18	3	4	3	18	4	0	3	0	15	3
Dyella	7	0	0	4	2	1	3	4	0	0	4	3	-	-	1	3	0	3
Enterobacter	277	41	11	52	98	163	63	182	84	8	139	182	6	2	88	51	103	79
Escherichia	4	2	0	2	3	1	2	1	3	0	3	3	-	-	3	0	0	3
Ewingella	26	0	0	9	15	2	8	18	0	0	0	26	-	-	-	-	22	4
Grimontella	10	0	0	0	0	10	0	0	10	0	1	9	-	-	1	0	8	1
Hafnia	1	4	1	0	5	1	2	4	0	0	0	6	-	-	-	-	5	1
Herbaspirillum	25	0	0	18	5	1	6	16	3	5	3	17	5	0	2	1	11	6
Klebsiella	74	38	5	60	17	29	30	49	38	9	48	60	5	4	28	20	33	27
Leclercia	37	0	2	13	23	3	12	22	5	1	14	24	1	0	5	9	4	20
Leifsonia	5	0	0	2	1	2	4	1	0	1	0	4	0	1	-	-	0	4
Luteibacter	5	1	0	4	0	2	5	1	0	0	0	6	-	-	-	-	1	5
Microbacterium	11	0	1	10	1	0	3	9	0	0	5	7	-	-	2	3	1	6
Novosphingobium	5	0	0	4	1	0	2	3	0	0	2	3	-	-	2	0	3	0
Ochrobactrum	5	0	0	4	1	0	0	4	1	0	1	4	-	-	1	0	3	1
Paenibacillus	3	3	0	2	2	1	1	5	0	0	2	4	-	-	0	2	1	3
Pandoraea	10	1	7	5	3	10	18	0	0	7	10	1	6	1	4	6	0	1
Pantoea	27	13	5	9	17	16	7	24	14	5	16	24	0	5	7	9	13	11
Pseudomonas	197	33	13	84	89	58	69	143	31	21	54	168	9	12	33	21	71	97
Rahnella	28	8	3	7	28	4	5	18	16	0	0	39	-	-	-	-	15	24
Raoultella	6	1	0	0	6	1	1	6	0	0	1	6	-	-	1	0	5	1
rare genera	129	33	32	91	60	36	69	94	31	8	90	96	4	4	39	51	41	55
Rhizobium	95	7	4	59	29	17	48	51	7	19	38	49	18	1	15	23	27	22
Salmonella	6	0	2	6	0	2	3	2	3	1	3	4	0	1	3	0	1	3
Serratia	25	9	3	13	15	5	20	16	1	8	5	24	1	7	4	1	17	7
Shigella	6	0	0	1	4	1	1	4	1	0	1	5	-	-	0	1	5	0
Sphingobium	8	0	1	7	0	2	6	3	0	0	8	1	-	-	7	1	1	0
Sphingomonas	9	0	1	8	1	1	5	4	1	0	7	3	-	-	7	0	2	1
Staphylococcus	2	1	3	2	2	2	3	1	2	3	2	1	3	0	1	1	1	0
Stenotrophomonas	87	12	5	47	28	23	50	45	9	7	29	68	3	4	18	11	32	36
Xanthomonas	9	0	0	5	0	4	4	5	0	0	2	7	-	-	2	0	6	1
Total	1577	382	252	754	622	736	856	1043	312	221	812	1178	99	122	380	432	562	616

Fig. 7. Heat map associations of bacterial genera and PGP traits (left), soil richness (middle), and occurrence of putative endophytic (Root) and rhizospheric (Soil) bacteria under each soil richness condition (right). Phos = TCP solubilization, Sid = siderophores production, with 1= no halo, 2= small or average halo, and 3= large halo. ICs = Indolic compounds production, with 1= low (0–10 μg of ICs ml^{-1}), 2= average (11–80 μg of ICs ml^{-1}) and 3= high (80 or> μg of ICs ml^{-1}). The red cells = less isolates than expected under those conditions, the green cells = excessive number of isolates under those conditions, and the yellow cells = no significant differences between the observed and expected values. "–" = an association could not be calculated due to the lack of cases (no expected total marginal values). Percentages and residuals are shown in S4 Fig. Sample sizes and p values are presented on S3 Table.

doi:10.1371/journal.pone.0116020.g007

(*Enterobacter, Escherichia, Grimontella, Klebsiella, Pantoea,* and *Rahnella*), and the most commonly isolated bacterial genus in soil samples, *Burkholderia*, presented strains that were greatly associated with a very low production of ICs. Sixty-one

percent (61%) of the isolates and 95% of the genera presented strains that could produce ICs above a residual level (>10 μg of ICs ml^{-1}). For siderophores production, few bacterial genera presented strains that were associated with high production (*Burkholderia*, *Enterobacter*, and *Grimontella*), while others (*Klebsiella*, *Stenotrophomonas, Rhizobium, Herbaspirillum*, and *Citrobacter*) presented strains that were associated with a low production of siderophores. Sixty-four percent (64%) of all of the isolates and 100% of all of the bacterial genera presented strains that were able to produce siderophores. Approximately one-third of all of the isolated bacterial genera presented at least one positive association with a PGP trait at a high level. The associations between the genera and soil conditions indicate that many genera were more associated with richer conditions. Only few genera, such as *Burkholderia*, *Pandoreae, Rhizobium, Serratia*, and *Staphylococcus*, were associated with poor soils.

Niche effect on the selection of bacterial genera according to the environment

Some bacterial genera might be associated to a colonization niche on some soil richness conditions, but not on others. (Fig. 7 and S4 Fig., right). The strains belonging to the *Burkholderia* genus were found predominantly in the rhizospheric soil samples despite soil richness, whereas the strains belonging to the *Enterobacter* genus were found mostly inside the plant roots (endophytes). The strains belonging to the *Rhizobium, Herbaspirillum*, and *Pandoreae* genera displayed an endophytic behavior only in the samples that were obtained from poor soils. While strains belonging to both the *Rahnella* and *Grimontella* genera were associated with richer soils and presented high levels of ICs production, only those strains belonging to the *Grimontella* genus were more often found inside the plant roots (endophytic). The strains belonging to the *Sphingobium* and *Sphingomonas* genera presented similar PGP traits and behaved endophytically in average soils, which both are associated to. The strains belonging to the *Klebsiella* genus, despite being found very often and presenting a high PGP trait shift (see below), were not associated with any soil condition or colonization niche.

Bacterial genera PGP trait shift

The genera presented on Fig. 8 have shifted the occurrence of some PGP abilities according to the soil richness. Fig. 8 and S5 Fig. show independent chi-square tests for each genus that presented a significant deviation from the expected values due to the soil condition on at least one PGP trait. A PGP trait increases under a given soil condition if the number of level 3 producers is larger than expected and/or the number of level 1 producers is lower than expected. Likewise, a PGP trait decreases when the opposite occurs.

For example, the strains belonging to the *Raoultella*, *Azospirillum* and *Rhizobium* genera had an increase in ICs production in rich soils and presented a decrease in siderophores production. In average soils, however, there was a decrease in ICs production and an increase in siderophores production. However, the TCP solubilization ability was unchanged. The strains belonging to the *Pseudomonas* and *Cedecea* genera had an increase in ICs production in rich soils,

Genera	Soil	Phos			Sid			ICs		
		1	2	3	1	2	3	1	2	3
Azorhizobium	Average	0	1	-	-	1	-	-	0	1
	Rich	4	0	-	-	4	-	-	4	0
Azospirillum	Average	11	0	-	3	3	5	9	0	2
	Rich	19	1	-	16	3	1	2	13	5
Burkholderia	Poor	45	39	25	16	28	65	98	10	1
	Average	141	48	70	52	45	153	173	75	11
	Rich	76	62	38	52	29	81	71	97	8
Cedecea	Poor	1	0	0	0	1	0	1	0	0
	Average	5	2	3	5	2	3	8	2	0
	Rich	33	3	0	15	13	8	3	27	6
Dyella	Average	4	-	-	2	1	1	3	1	-
	Rich	3	-	-	2	1	0	0	3	-
Enterobacter	Poor	8	0	0	1	2	5	7	1	0
	Average	120	16	3	12	35	91	31	61	47
	Rich	149	25	8	39	61	67	25	120	37
Herbaspirillum	Poor	5	-	-	2	3	0	4	1	0
	Average	3	-	-	2	1	0	1	2	0
	Rich	17	-	-	14	1	1	1	13	3
Klebsiella	Poor	9	0	0	2	2	5	6	3	0
	Average	12	31	5	34	3	2	15	19	14
	Rich	53	7	0	24	12	22	9	27	24
Leclercia	Poor	1	-	0	0	1	0	1	0	0
	Average	14	-	0	3	8	3	11	3	0
	Rich	22	-	2	10	14	0	0	19	5
Ochrobactrum	Average	1	-	-	1	0	-	-	0	1
	Rich	4	-	-	3	1	-	-	4	0
Pantoea	Poor	3	0	2	1	2	2	1	4	0
	Average	6	8	2	2	6	8	4	5	7
	Rich	18	5	1	6	9	6	2	15	7
Pseudomonas	Poor	15	4	2	5	14	2	20	1	0
	Average	35	13	6	15	10	21	26	27	1
	Rich	147	16	5	64	65	35	23	115	30
Raoultella	Average	1	0	-	-	0	1	1	0	-
	Rich	5	1	-	-	6	0	0	6	-
Rare genera	Poor	4	1	3	5	1	2	8	0	0
	Average	49	23	18	37	29	18	30	41	19
	Rich	76	9	11	49	30	16	31	53	12
Rhizobium	Poor	14	3	2	14	2	3	12	6	1
	Average	37	0	1	18	8	11	30	8	0
	Rich	44	4	1	27	19	3	6	37	6
Stenotrophomonas	Poor	7	0	0	5	1	1	7	0	0
	Average	23	3	3	18	5	5	19	7	3
	Rich	57	9	2	24	22	17	24	38	6

Fig. 8. PGP traits of some bacterial strains shifted due to the soil richness. Only those bacterial genera that significantly changed their PGP traits are shown. Each box is a separate chi-square test, with non-significant tests shown entirely in yellow. Phos = TCP solubilization, and Sid = siderophores production, with 1= no halo, 2= small or average halo, and 3= large halo. ICs = Indolic compounds production, with 1= low (0–10 μg of ICs ml^{-1}), 2= average (11–80 μg of ICs ml^{-1}) and 3= high (80 or> μg of ICs ml^{-1}). The red cells = less isolates than expected under those conditions, the green cells = excessive number of isolates under those conditions, and the yellow cells = no significant differences between the observed and expected values. "–" = an association could not be calculated due to a lack of cases (no expected total marginal values). Percentages and residuals are shown in S5 Fig. Sample sizes and p values are presented on S3 Table.

doi:10.1371/journal.pone.0116020.g008

while they decreased their TCP solubilization ability. For average soils, however, there was a decrease in ICs production and an increase in phosphate solubilization. For strains belonging to the *Burkholderia, Klebsiella, Leclercia*, *Stenotrophomonas*, *Herbaspirillum*, and *Dyella*, genera there was an increase in ICs production in richer soils and a decrease in poor and average soils. Of these isolates, however, only the *Burkholderia* isolates showed a decrease in siderophores production in richer soils, and only *Klebsiella* isolates showed a decrease in TCP solubilization in richer soils. We note that the strains belonging to the *Burkholderia*, *Klebsiella* and *Pseudomonas* genera were the most variable in their PGP abilities in response to the soil conditions, as all of their three PGP traits that were evaluated in this study changed according to the environmental conditions.

Approximately one-third of the studied bacterial genera presented PGP trait shifting, and in most cases PGP trait shifting follows our model (23 of the 29 cases). Exceptions were found in the siderophores production of strains belonging to *Klebsiella* genus, where the strains with a high production of siderophores were associated with rich soils, and for the ICs production levels of strains belonging to the *Azorhizobium* and *Ochrobactrum* genera, where the strains with lower ICs production levels were associated with richer soils. To better visualize the PGP trait variability of *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, *Herbaspirilum*, *Rhizobium*, and *Grimontella* genera we created additional CatPCA plots (Fig. 9). In Fig. 9 a single genus is visually displayed, showing all isolates from that genus. All the other 39 genera were visually suppressed, but still take part in the mathematical construction of the plot.

Discussion

Multivariate methods are very useful in microbial ecology. These methods permit a massive reduction in complexity while simultaneously exploring several research questions. Despite some limitations, such as the use of cultivable bacteria, the halo size and ICs quantification through spectrometric analyses, our study presents the largest databank of bacterial isolates displaying different plant growth-promoting abilities that we are aware of.

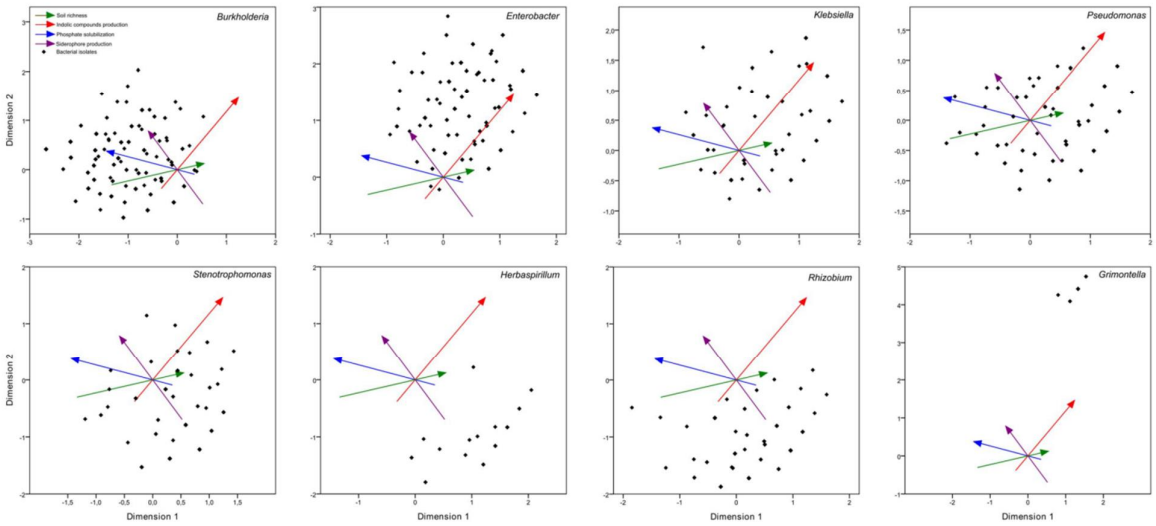


Fig. 9. CatPCA analysis of 2,211 bacterial isolates (the legend and interpretation are similar to those of Fig. 2). The genera *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, *Herbaspirilum*, *Rhizobium*, and *Grimontella* are represented one at a time. Each black dot represents an isolate, but isolates with the same characteristics are stacked on the same dot.

doi:10.1371/journal.pone.0116020.g009

A model to explain the occurrence of PGP traits

The CatPCA analysis demonstrated that the bacterial ICs production levels increase as the soil richness increases. Meanwhile, the bacterial TCP solubilization ability increases as the soil richness decreases, and the bacterial siderophores production seems to be correlated with the TCP solubilization ability (Fig. 2). The interaction between the bacterial ICs production and TCP solubilization abilities with increasing nutrient levels has already been proposed in a previous work [9] but was here confirmed with a sample that was 12-fold larger in size (Fig. 3). While the TCP solubilization ability was expected to be higher or more important in the soils with lower P contents [23–26], the association of higher levels of ICs production with richer soils (Fig. 3) was never suggested by other authors. The association of siderophores production with soil richness was also not reported elsewhere.

We detected a decrease in siderophores production by bacterial isolates in richer soils (Fig. 4). Although siderophores-producing bacteria can be often found in Fe-limited soils [26] the iron concentrations in the sampled soils were not measured in this study. Nevertheless, siderophores have functions other than iron homeostasis. Siderophores may bind to more than 16 different metal ions, either for nourishment or to avoid metal toxicity [27, 28], in addition to being able to reach an optimum production in nutrient scarcity conditions [29]. In a diazotrophic *Azotobacter vinelandii* strain, siderophores were produced to capture Mo and V metals for nourishment [30] even in the presence of Fe [31]. As organic matter and clay act as ligands for metals, affecting their availability [32], bacterial

siderophores production could be associated with these variables. Acidic sandy soils with low organic matter content, such as those from the poor soil conditions described here, are more susceptible to heavy metal toxicity [33] and could have favored bacterial strains that displayed greater siderophores production for toxicity alleviation. Thus, the increased number of bacterial strains that presented larger halo sizes for siderophores production in poor soils may be related to both general metal acquisitions for nutrients and toxicity alleviation. Furthermore, siderophores action could liberate usable molecules that are attached to binding metals, such as $FePO_4$, which potentially acts as a source of P [34]. We found a correlation between the halo sizes of bacterial colonies for siderophores production and TCP solubilization on indicator media (Fig. 4), which might be caused by the Ca binding by siderophores. The occurrence of this correlation in nature is currently being further investigated with an updated phosphate solubilization assay [35]. As 100% of the studied genera presented strains displaying the ability to produce siderophores (Fig. 7), we confirm that this PGP trait is widespread in rhizospheric bacteria [36], similarly to the ability to produce ICs [37, 1]

The inverse correlation between ICs production and TCP solubilization is not deterministic or prohibitive: 23 strains in our database (5 of them belonging to the *Burkholderia* genus) produced more than 80 μg of ICs ml^{-1} and simultaneously showed large halos on TCP medium (S1 Table). Chaiharn and Lumyong [38] isolated 216 bacterial strains where the best ICs producer was also the best phosphate solubilizer, while Bianco and Defez [39] showed that a genetically engineered *Sinorhizobium* strain that overproduced ICs improved its phosphate solubilization ability. While a single strain could enhance plant growth simultaneously via these two mechanisms, our results suggest that the average phosphate solubilization and average ICs production of diazotrophs in soil are under the proposed interaction: the best ICs producers are not the best TCP solubilizers. It seems that the driving mechanism behind this correlation is ecological and not molecular and it is better visualized in a soil richness gradient [9]. Spaepen and Vanderleyden [40] reviewed the molecular aspects of ICs production, and the only reported environmental constrain was that carbon limitation is required for ICs biosynthesis in *Azospirillum brasilense* [41].

Plants have a great effect on the microbial species that surround their roots due to the action of exudates [42]. The rhizosphere is a complex and competitive environment where the bacterial colonization of the interior of the roots is under higher control of the plant and provides more benefits for the bacteria [6, 43]. ICs production by bacteria is also greatly controlled by plants. Not only do plants actively exude tryptophan [37], a necessary amino acid in the tryptophan-dependent indolic acetic acid production pathway, but they might even induce the expression of tryptophan permease genes in bacteria [44]. Rhizospheric bacteria produce more ICs than do bulk soil bacteria [45], but in this study, we expand this effect to endophytic bacteria (Fig. 5) and determine the conditions of its occurrence, corroborating our hypothesis that in richer soils, the best IC producers are endophytic, while the best nutrient solubilizers are not (Fig. 6), due

to active plant influence and selection. This suggests that the plant permits interaction with endophytic or rhizospheric bacteria displaying different PGP abilities according to its nutritional status [6, 9]. It is important to notice that plants have limited space and resources and cannot, therefore, select both good ICs producers and good nutrient solubilizers when these groups are composed of different bacteria. This finding addresses a critical research need raised by Gray and Smith [43], as it demonstrates that differences between ePGPB and iPGPB in relation to indolic acetic acid production may be found across a soil richness gradient. Nutrient solubilizers do not necessarily have to live in the rhizosphere to aid nutrient acquisition by plants. Bacteria may act as phosphate solubilizers and metal chelators endophytically [46-48]. Thus, endophytic nutrient-solubilizing bacteria from poor soils may act on nutrient acquisition directly, perhaps more actively than rhizospheric bacteria that are closer to the soil nutrients themselves.

We could also identify three clusters of bacterial genera scattered on the CatPCA plot: one small group of genera that were associated with nutrient solubilization, another group associated with phytohormone production, and a larger third group that could be associated with other, non-screened PGP traits (Fig. 2). We suggest that good nutrient solubilizers are more common under limited nutrient conditions in which the plant would benefit most from bacteria that help its nourishment. Additionally, good growth hormone producers are more common under nutrient-rich conditions in which the plants are not starving and may use bacterial secondary metabolites for improved shoot and root growth. This situation is reinforced by the observation that TCP solubilization and growth hormone production are inversely related. The large cluster with other PGPB include nitrogen fixers, such as strains belonging to the *Herbaspirillum* and *Rhizobium* genera (Fig. 9), and should include bacteria with other PGP traits that were not tested in this study (for example, nitrogen fixation, ACC deaminase activity, and disease resistance) as well as soil bacteria that do not act as PGPB. Despite the large number of papers evaluating soil bacteria functional groups [49–51] or reviews regarding simultaneous ICs production and nutrient solubilization [1, 4, 52–56], the clustering and interactions of hormone producers and nutrient solubilizers was never suggested before.

Highlights of specific genera that are associated with the PGP traits, niche and environment

Several interesting associations can be found in the heat maps in Figs. 7 and 8. Some of these associations are described below, and can also be noticed on Fig. 9. We believe that our highlights could help direct bioprospection, suggest specific research questions, and illustrate the behavior of some bacterial genera in the plant-soil interface.

Strains from the *Burkholderia* genus are a dominant component of many soil ecosystems [57]. These strains are often found in adverse or unprovided environments, such as in Al-toxic soils [58] or forest to grassland vegetation shift where the soil organic matter content sharply decreases [59]. This genus has

strains that were previously characterized as mostly external to the root tissue [43] and very capable of solubilizing nutrients [48, 56, 60, 61]. *Burkholderia* strains present exceptional metabolic and functional diversity [62], possibly provided by their genomes of 4–9 Mb [57]. Here, we demonstrate that *Burkholderia* is a very common genus living mostly outside the root tissue and that is more associated with poor soils and acts as a good nutrient solubilizer, in addition to having a wide versatility and environmental adaptability – all of which agrees with current knowledge. However, Park and Gurian-Sherman [57] stated that the role of siderophores production by *Burkholderia* in root colonization has not been investigated. Here we provide evidence that shows that the siderophores production potential by *Burkholderia* strains decreases as the soil richness increases (Fig. 8). Also, in rich soils, the best siderophores producers were found in the rhizosphere rather than inside plant tissues – a tendency that disappears under poor soil conditions (Fig. 6). Furthermore, when we consider only *Burkholderia* isolates in an analysis that is similar to the one presented in Fig. 6, we could observe that, in poor soils, the best siderophores producers are actually more often found inside the plant than in the rhizosphere (S6 Fig.). We also depict *Burkholderia* strains as poor indolic compound producers that are more often found in the rhizosphere than inside the plant despite soil richness conditions (Fig. 7). This behavior of the *Burkholderia* genus was not previously described [1, 5, 43, 63].

The *Enterobactericeae* family is well known for widespread IC production [64, 65], and several studies have used *Enterobacter* strains to assay the indole-3-acetic acid production pathways [37, 40]. Still, the only report suggesting that enterobacteria produce more ICs than do other taxa of soil bacteria was from our group (Moreira, personal communication). Although there are many reports demonstrating the efficient endophytic colonization of strains belonging to the *Enterobacter* genus, it was never before reported that this genus might be found more often inside the plant tissues than in the rhizosphere in average or rich soils but not in poor soils. As enterobacteria follow an r-strategy for rapid growth and the quick use of resources [64], the low occurrence of these bacteria in poor soils with less resources is understandable. Additionally, although *Enterobacter* strains are known for displaying P solubilization ability [54, 55], it was not reported that they might solubilize less phosphate than do several other soil bacterial genera (Fig. 7).

It is interesting to notice the differences between the *Burkholderia* and *Enterobacter* genera (Fig. 9). Their PGP traits are almost opposed to each other but both are associated with high siderophores production. Their favored environment and niche are directly opposed as well. It seems that these genera follow distinct strategies for survival and plant interaction, and both are successful. A comparative genomic analysis of these genera could return interesting results for soil bacteria life strategies.

Information concerning the *Grimontella* genus is scarce. Its occurrence in plants is restricted to a previous study [12] of sunflower. This genus stands out among the *Enterobacteriaceae* cluster in Fig. 2 because none of the 10 isolates belonging to

this genus produced low amounts of ICs (Fig. 9). Similarly to *Enterobacter*, strains from the *Grimontella* genus are good siderophores producers that live endophytically in rich soils (Fig. 7). Further investigation of this genus might reveal it as a very useful biotechnological agent that has, so far, been largely ignored. To find more *Grimontella* strains in the environment, we suggest sampling surface-sterilized sunflower roots from rich soil conditions using an *Enterobacteriaceae*-friendly culture medium. Additionally, deeper investigation of the similarities between *Enterobacter* and *Grimontella* could provide valuable scientific insights. It might be interesting to note, as well, that strains from the *Grimontella* and *Rahnella* genera behaved very similarly, yet only the strains belonging to the *Grimontella* genus were mostly endophytic.

The strains from the *Herbaspirillum* genus presented low scores of PGP traits, and behaved more endophytically in poor soils (Fig. 7). It is possible that this behavior is a response to fertilization: in N-rich soils, plants no longer require bacterial strains to fix nitrogen, and there is a reduced need for endophytic diazotroph colonization [66]. The strains from the *Rhizobium* genus behaved similarly, except that these strains were more frequent in poor soils (both are shown in Fig. 9). This observation has important crop management implications, as it indicates that farmers have nitrogen fixers in their soils but prevent them from being useful due to the addition of N fertilizers. It is interesting to notice that none of *Herbaspirillum* isolates that were analyzed in this work were able to solubilize phosphates (Fig. 7), reinforcing the finding of Estrada *et al.* [61], who first identified a phosphate-solubilizing *Herbaspirillum* strain.

Pandoreae strains were previously isolated from contaminated soils [67, 68] and plant rhizospheres [69, 70], and strains from this genus are promising in biodegradation applications [71]. In this study, we found that strains from *Pandoreae,* similarly to strains from *Rhizobium* and *Herbaspirillum* genera, are associated with poor soils, where they showed endophytic behavior (Fig. 7). However, much unlike *Rhizobium* and *Herbaspirillum* strains, these strains were found in the nutrient-solubilizing cluster of Fig. 2, as they were good phosphate solubilizers but were completely unable of producing ICs, one of the most widespread and important PGP traits of soil bacteria. It is possible that the adaptations of these strains to adverse conditions instead of growth hormone production play a key role in their association with plants. Bioprospectors interested in *Pandoreae* biodegradation could consider endophytic bacteria and soil richness conditions in their sampling strategy.

Although bacteria from the *Klebsiella* genus are known for producing ICs [38], fixing nitrogen [72], solubilizing phosphate [55], producing siderophores [56], and actively colonizing the plant rhizosphere [1], there are no reviews regarding their general role in the rhizosphere. Here we illustrate the *Klebsiella* genus as very common in soil and also very adaptable and versatile, with an overall high IC production and a mix of nutrient-solubilizing abilities (Figs. 7 and 9). Strains from this genus were not associated with any environment or colonization niche, although it has already been reported that Klebsiella would be more often found as a rhizospheric than as a endophythic bacteria [73]. *Klebsiella* followed the

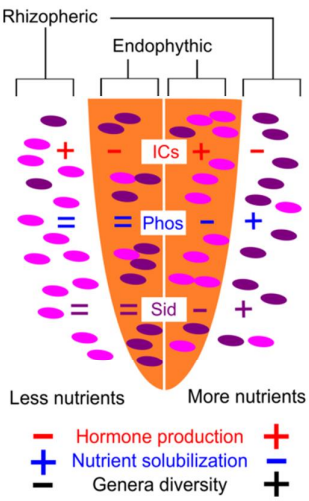


Fig. 10. A model to explain the distribution of bacteria displaying different plant growth promotion traits. In soils with fewer nutrients, plants leave the best growth hormone producers in the rhizosphere, while both endophytic and rhizospheric bacteria are good nutrient solubilizers. In soils with more nutrients, the best growth hormone producers are found inside plant roots, but the endophytic bacteria are poor nutrient solubilizers, with the best solubilizers found in the rhizosphere. In addition, genera diversity and growth hormone producers are more abundant in soils with more nutrients, while phosphate solubilizers and siderophores producers are more abundant in soils with fewer nutrients. Siderophores producers and phosphate solubilizers seem to co-occur, while indolic compound producers are clearly opposed to phosphate solubilizers. Plants seem to select bacterial PGP traits according to their nutritional needs: nutrient solubilizers under poor conditions and growth hormone producers under rich conditions.

doi:10.1371/journal.pone.0116020.g010

model for IC production but was the only genus that behaved against the model concerning siderophores production in rich soils and phosphate solubilization in poor soils (Fig. 8). It becomes clear to us that the ecological significance of *Klebsiella* in soils is largely underestimated.

Most of the PGP trait-shifting bacterial genera presented on Fig. 8 followed our model. In richer soils, the ICs production levels increased as the phosphate solubilization and siderophores production abilities decreased, but in poorer soils, the ICs production decreased as the nutrient solubilization increased.

Based on our data, we updated a previously proposed model that is explained in Fig. 10 and described above. This model helps direct bioprospection for PGPB so that the bacteria (or genes) displaying a trait of interest can be more easily found in the soil and root samples, considering the soil richness and niche occupation by these bacteria. We also found several interesting PGPB-niche-environment interactions at the genus level that could aid PGPB bioprospection by using appropriate selective medium or molecular markers or by directing research questions.

Conclusions

We propose a model for the occurrence of some plant growth-promoting traits in plant-associated bacteria. This model praises that plants will favor their association with endophytic bacteria according to the nutrient status of the soil, permitting an association with nutrient solubilizers under nutrient-poor conditions or selecting growth hormone producers under nutrient-rich conditions. We also suggest several associations at the genus level, demonstrating where some genera are more likely to be located and which phenotypic traits they should be displaying. This model could be used for directed PGPB bioprospection, so that target PGP traits or bacterial genera can be screened in the right niche and under the right conditions, which is important both for cultivation-dependent and -independent methods, as both are time-consuming and expensive and, therefore, should not blindly sample plants and roots.

Supporting Information

S1 Fig. Soil chemical characteristics according to PCA cluster classification. (a) Log-transformed values (average ±1 SE) of Potassium (K), Clay content, Organic matter, and pH for poor, average and rich soils. (b) Rank values (average ±1 SE) of Phosphate (P) content for poor, average and rich soils. Different letters show significant differences.
doi:10.1371/journal.pone.0116020.s001 (TIF)

S2 Fig. Heat map associations of the TCP solubilization (left) and siderophores production (middle) abilities of bacterial isolates with soil conditions and with each other (right), displayed in percentages (a) and adjusted residuals (b). The legend and interpretation are similar to those of Fig. 4.
doi:10.1371/journal.pone.0116020.s002 (TIF)

S3 Fig. Heat map associations of the TCP solubilization and siderophores production abilities of endophytic (root) and rhizospheric (soil) isolates under each individual soil condition, displayed in percentages (a) and adjusted residuals (b). The legend and interpretation are similar to those of Fig. 6.
doi:10.1371/journal.pone.0116020.s003 (TIF)

S4 Fig. Heat map associations of bacterial genera and PGP traits (left), soil richness (middle), and occurrence of putative endophytic (Root) and rhizospheric (Soil) bacteria under each soil richness condition (right), displayed in percentages (a) and adjusted residuals (b). The legend and interpretation are similar to those of Fig. 7.
doi:10.1371/journal.pone.0116020.s004 (TIF)

S5 Fig. PGP traits of some bacterial strains shifted due to the soil richness. Only those bacterial genera that significantly changed their PGP traits are shown. Each box is a separate chi-square test, displayed in percentages (a) and adjusted residuals (b). The legend and interpretation are similar to those of Fig. 8.
doi:10.1371/journal.pone.0116020.s005 (TIF)

5. **Singh JS, Pandey VC, Singh DP** (2011) Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. Agric Ecosyst Environ 140: 339–353.
6. **Hartmann A, Schmid M, Tuinen D Van, Berg G** (2008) Plant-driven selection of microbes. Plant Soil 321: 235–257.
7. **Ramette A** (2007) Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 62: 142–160.
8. **Ramette A, Boetius A** (2009) Crystal ball - 2009. Environ Microbiol Rep 1: 3–26.
9. **Costa PB, Beneduzi A, Souza R, Schoenfeld R, Vargas LK, et al.** (2012) The effects of different fertilization conditions on bacterial plant growth promoting traits: guidelines for directed bacterial prospection and testing. Plant Soil 368: 267–280.
10. **Souza R, Beneduzi A, Ambrosini A, Costa PB, Meyer J, et al.** (2012) The effect of plant growth-promoting rhizobacteria on the growth of rice (Oryza sativa L.) cropped in southern Brazilian fields. Plant Soil 366: 585–603.
11. **Arruda L, Beneduzi A, Martins A, Lisboa B, Lopes C, et al.** (2013) Screening of rhizobacteria isolated from maize (*Zea mays* L.) in Rio Grande do Sul State (South Brazil) and analysis of their potential to improve plant growth. Appl Soil Ecol 63: 15–22.
12. **Ambrosini A, Beneduzi A, Stefanski T, Pinheiro FG, Vargas LK, et al.** (2012) Screening of plant growth promoting Rhizobacteria isolated from sunflower (*Helianthus annuus* L.). Plant Soil 356: 245–264.
13. **Passos JFM, Costa PB, Costa MD, Zaffari GR, Nava G, et al.** (2014) Cultivable bacteria isolated from apple trees cultivated under different crop systems: Diversity and antagonistic activity against *Colletotrichum gloeosporioides*. Genet Molec Biol, In press.
14. **Granada C, Costa PB, Lisboa BB, Vargas LK, Passaglia LMP** (2013) Comparison among bacterial communities present in arenized and adjacent areas subjected to different soil management regimes. Plant Soil 373: 339–358.
15. **Sparks DL, Page AL, Helmke PA, Loeppert RH** (1996) Methods of soil analysis: Part 3. Chemical methods and processes. Madison: Soil Science Society of America. 1264 p.
16. **Dobereiner J** (1988) Isolation and identification of root associated diazotrophs. Plant Soil 110: 207–212.
17. **Sylvester-Bradley R, Asakawa N, La Torraca S, Magalhães FMM, Oliveira L, Pereira RM** (1982) Levantamento quantitativo de microrganismos solubilizadores de fosfatos na rizosfera de gramíneas e leguminosas forrageiras na Amazônia. Acta Amaz. 12: 15–22.
18. **Schwyn B, Neilands JB** (1987) Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160: 47–56.
19. **Glickmann E, Dessaux Y** (1995) A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61: 793–796.
20. **Zar JH** (2010) Biostatistical Analysis. Upper Saddle River: Pearson Prentice-Hall. 944 p.
21. **Benjamini Y, Hochberg Y** (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc 57: 289–300.
22. **Marinari S, Mancinelli R, Campiglia E, Grego S** (2006) Chemical and biological indicators of soil quality in organic and conventional farming systems in Central Italy. Ecol Indic 6: 701–711.
23. **Wakelin Sa, Gregg AL, Simpson RJ, Li GD, Riley IT, et al.** (2009) Pasture management clearly affects soil microbial community structure and N-cycling bacteria. Pedobiologia 52: 237–251.
24. **Jakobsen I, Legget ME**, **Richardson** (2005) Rhizosphere microorganisms and plant phosphorus uptake. in: Sims JT, Sharpley AN, editors. Phosphorus: Agriculture and the Environment. Madison: Soil Science Society of America. pp. 437–494.
25. **Hu J, Lin X, Wang J, Chu H, Yin R, et al.** (2009) Population size and specific potential of P-mineralizing and -solubilizing bacteria under long-term P-deficiency fertilization in a sandy loam soil. Pedobiologia 53: 49–58.
26. **Matsuoka H, Akiyama M, Kobayashi K, Yamaji K** (2013) Fe and P solubilization under limiting conditions by bacteria isolated from *Carex kobomugi* roots at the Hasaki coast. Curr Microbiol 66: 314–321.

S6 Fig. Heat map associations of the TCP solubilization and siderophores production abilities of endophytic (root) and rhizospheric (soil) isolates of the *Burkholderia* genus under each individual soil condition (the legend and interpretation are similar to those of Fig. 6). Only the *Burkholderia* isolates are displayed here.
doi:10.1371/journal.pone.0116020.s006 (TIF)

S1 Table. Full information of each isolate used in this study. Includes quantification of plant growth promoting traits, colonization niche, bacterial genera, soil richness, isolate geographical origin, code on PCA plot, and associated plants.
doi:10.1371/journal.pone.0116020.s007 (XLSX)

S2 Table. Chemical characteristics of all soils analyzed in this study.
doi:10.1371/journal.pone.0116020.s008 (XLSX)

S3 Table. Details of all statistical tests used in this study. Includes p values, sample sizes, false discovery rate, degrees of freedom, names of the tests, and the figures where they are shown in the paper.
doi:10.1371/journal.pone.0116020.s009 (XLSX)

S1 Text. Additional information on statistical methodology, showing how the tests used in the paper were calculated and interpreted.
doi:10.1371/journal.pone.0116020.s010 (DOCX)

Acknowledgments

We would like to thank the Brazilian Microbiology Society for granting this work a best poster prize in the 27th Brazilian Microbiology Congress, and the Brazilian Genetics Society for granting a Honorius Mention in the 59th Brazilian Genetic Congress. We also thank Anelise Beneduzi for valuable scientific comments.

Author Contributions

Conceived and designed the experiments: PC CG AA FM RS JFP LA LP. Performed the experiments: PC CG AA FM RS JFP LA. Analyzed the data: PC LP. Contributed reagents/materials/analysis tools: LP. Wrote the paper: PC LP.

References

1. **Hayat R, Ali S, Amara U, Khalid R, Ahmed I** (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60: 579–598.
2. **Simpson RJ, Oberson A, Culvenor RA, Ryan MH, Veneklaas EJ, et al.** (2011) Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems. Plant Soil 349: 89–120.
3. **Good AG, Beatty PH** (2011) Fertilizing Nature: A Tragedy of Excess in the Commons. PLoS Biol 9: e1001124.
4. **Adesemoye AO, Kloepper JW** (2009) Plant-microbes interactions in enhanced fertilizer-use efficiency. Appl Microbiol Biotechnol 85: 1–12.

27. **Hernlem BJ, Vane LM, Sayles GD** (1999) The application of siderophores for metal recovery and waste remediation: examination of correlations for prediction of metal affinities. Water Res 33: 951–960.

28. **Schalk IJ, Hannauer M, Braud A** (2011) New roles for bacterial siderophores in metal transport and tolerance. Environ Microbiol 13: 2844–2854.

29. **Rajkumar M, Ae N, Prasad MNV, Freitas H** (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol 28: 142–149.

30. **Bellenger JP, Wichard T, Kustka AB, Kraepiel AML** (2008). Uptake of molybdenum and vanadium by a nitrogen-fixing soil bacterium using siderophores. Nature Geosci 1: 243–246

31. **Kraepiel a ML, Bellenger JP, Wichard T, Morel FMM** (2009) Multiple roles of siderophores in free-living nitrogen-fixing bacteria. Biometals 22: 573–581.

32. **Neubauer U, Nowack B, Furrer G, Schulin R** (2000) Heavy Metal Sorption on Clay Minerals Affected by the Siderophore Desferrioxamine B. Environ Sci Technol 34: 2749–2755.

33. **Baath E** (1989) Effects of heavy metals in soil on microbial processes and populations (a review). Water, Air Soil Pollut 47: 335–379.

34. **Jayachandran K, Hetrick BAD, Schwab AP** (1989) Mycorrhizal Mediation of Phosphorus Availability: Synthetic Iron Chelate Effects on Phosphorus Solubilization. Soil Sci Soc Am J 53: 1701–1706

35. **Bashan Y, Kamnev AA, de-Bashan LE** (2012) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. Biol Fertil Soils 49: 465–479.

36. **Guerinot ML** (1994) Microbial iron transport. Annu Rev Microbiol 48: 743–772.

37. **Spaepen S, Vanderleyden J, Remans R** (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 31: 425–448.

38. **Chaiharn M, Lumyong S** (2011) Screening and optimization of indole-3-acetic acid production and phosphate solubilization from rhizobacteria aimed at improving plant growth. Curr Microbiol 62: 173–181.

39. **Bianco C, Defez R** (2010) Improvement of phosphate solubilization and *Medicago* plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. Appl Environ Microbiol 76: 4626–4632.

40. **Spaepen S, Vanderleyden J** (2011) Auxin and plant-microbe interactions. Cold Spring Harb Perspect Biol 3: a001438

41. **Ona O, Van Impe J, Prinsen E, Vanderleyden J** (2005) Growth and indole-3-acetic acid biosynthesis of *Azospirillum brasilense* Sp245 is environmentally controlled. FEMS Microbiol Lett 246: 125–132.

42. **Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM** (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57: 233–266.

43. **Gray EJ, Smith DL** (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol Biochem 37: 395–412.

44. **Mark GL, Dow JM, Kiely PD, Higgins H, Haynes J, et al.** (2005) Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. Proc Natl Acad Sci U S A 102: 17454–17459.

45. **Khalid A, Tahir S, Arshad M, Zahir ZA** (2004) Relative efficiency of rhizobacteria for auxin biosynthesis in rhizosphere and non-rhizosphere soils. Soil Res 42: 921–926.

46. **Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, et al.** (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ Microbiol 6: 1244–1251.

47. **Rajkumar M, Ae N, Freitas H** (2009) Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77: 153–160.

48. **Young LS, Hameed A, Peng SY, Shan YH, Wu SP** (2013) Endophytic establishment of the soil isolate *Burkholderia* sp. CC-Al74 enhances growth and P-utilization rate in maize (*Zea mays* L.). Appl Soil Ecol 66: 40–47.

49. **Emmerling C, Schloter M, Hartmann A, Kandeler E, Trier U, et al.** (2002) Functional diversity of soil organisms ± a review of recent research activities in Germany. J Plant Nutr Soil Sci 165: 408–420.

50. **Wakelin SA, Warren RA, Harvey PR, Ryder MH** (2004) Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. Biol Fertil Soils 40: 36–43.

51. **He Z, Xu M, Deng Y, Kang S, Kellogg L, et al.** (2010) Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO2. Ecol Lett 13: 564–575.

52. **Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C** (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 305–339.

53. **Weyens N, van der Lelie D, Taghavi S, Newman L, Vangronsveld J** (2009) Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol 27: 591–598.

54. **Babalola OO** (2010) Beneficial bacteria of agricultural importance. Biotechnol Lett 32: 1559–1570.

55. **Martínez-Viveros O, Jorquera M, Crowley D, Gajardo G, Mora M** (2010) Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J Soil Sci Plant Nutr 10: 293–319.

56. **Ahemad M, Kibret M** (2014) Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J King Saud Univ - Sci 26: 1–20.

57. **Parke JL, Gurian-sherman D** (2001) Diversity of the *Burkholderia cepacia* complex and inplications for risk assessment of biological control strains. Annu Rev Phytopathol: 225–258.

58. **Kunito T, Owaki M, Ihyo Y, Sumi H, Toda H, et al.** (2011) Genera *Burkholderia* and *Lipomyces* are predominant aluminum-resistant microorganisms isolated from acidic forest soils using cycloheximide-amended growth media. Ann Microbiol 62: 1339–1344.

59. **Nüsslein K, Tiedje JM** (1999) Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Appl Environ Microbiol 65: 3622–3626.

60. **Lin TF, Huang HI, Shen FT, Young CC** (2006) The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-AI74. Bioresour Technol 97: 957–960.

61. **Estrada GA, Baldani VLD, Oliveira DM, Urquiaga S, Baldani JI** (2012) Selection of phosphate-solubilizing diazotrophic *Herbaspirillum* and *Burkholderia* strains and their effect on rice crop yield and nutrient uptake. Plant Soil 369: 115–129.

62. **Coenye T, Vandamme P** (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ Microbiol 5: 719–729.

63. **Miransari M** (2011) Soil microbes and plant fertilization. Appl Microbiol Biotechnol 92: 875–885.

64. **Barraquio WL, Segubre EM, Gonzalez MAS, Verma SC, James EK, et al.** (2000) Diazotrophic enterobacteria: What is their role in the rhizosphere of rice? in: Ladha JK, Reddy PM, editors. The quest for nitrogen fixation in rice. Makati City: International Rice Research Institute. pp. 93–118

65. **Sergeeva E, Hirkala DLM, Nelson LM** (2007) Production of indole-3-acetic acid, aromatic amino acid aminotransferase activities and plant growth promotion by *Pantoea agglomerans* rhizosphere isolates. Plant Soil 297: 1–13.

66. **Boddey RM, Urquiaga S, Alves BJR, Reis V** (2003) Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. Plant Soil 252: 139–149.

67. **Chen CY, Kuo JT, Cheng CY, Huang YT, Ho IH, et al.** (2009) Biological decolorization of dye solution containing malachite green by *Pandoraea pulmonicola* YC32 using a batch and continuous system. J Hazard Mater 172: 1439–1445.

68. **Han-Jen RE, Wai-Fong Y, Kok-Gan C** (2013) *Pandoraea* sp. RB-44, a novel quorum sensing soil bacterium. Sensors 13: 14121–14132.

69. **Kidd PS, Prieto-Fernández A, Monterroso C, Acea MJ** (2007) Rhizosphere microbial community and hexachlorocyclohexane degradative potential in contrasting plant species. Plant Soil 302: 233–247.

70. **Anandham R, Indiragandhi P, Madhaiyan M, Ryu KY, Jee HJ, et al.** (2008) Chemolithoautotrophic oxidation of thiosulfate and phylogenetic distribution of sulfur oxidation gene (soxB) in rhizobacteria isolated from crop plants. Res Microbiol 159: 579–589.

71. **Baptista IIR, Zhou NY, Emanuelsson EAC, Peeva LG, Leak DJ, et al.** (2008) Evidence of Species Succession During Chlorobenzene Biodegradation. Biotechnol Bioeng 99: 68–74.

1

Final remarks

The Objective of this thesis was to increase the scientific understanding of natural plant-bacteria interactions, and how we can use PGPB inoculation to exploit them, improving crop growth. A model to describe some PGPB interactions was raised and tested, confirming several points of the model. In addition, a novel methodology that can help in PGPB microbial community analysis is presented, while applying classical invasion ecology to PGPB inoculation. As for new insights to be added to the published model, incorporation of the K-/r- strategies may be very helpful, since they also try to simplify very complex strategies in a two-sided scheme. While ICs production and P solubilization did not fill the niches under each soil as expected, this could be due to the difference in the experimental designs of Chapter 2 and Chapter 3. A final experiment, aiming at this discrepancy and considering the previous experimental designs, should be able to solve this issue. Obviously, the model can be further expanded by evaluating other traits, like those discussed in Chapter 1. Even expanded, it must be kept in mind that the model is based on averages and correlations ï it would be wrong to propose that *Burkholderia* cannot produce ICs and be an effective PGPB in normal field conditions.

We consider that the thesis was successful on its proposal, because large datasets were thoroughly investigated under the scientific method to generate knowledge that can be applied to sustainable food production. Chapters 3 and 4 were not submitted to scientific publishing yet, and it is worth noting a few points about them.

The data presented on Chapter 3 is only about half of the data generated in that experiment. There was one additional variable ï plant age ï that could not be properly analyzed in time for this thesis presentation. There are additional 216 large pots (9 per experimental condition) were the rice plants developed for 60 days before harvesting, for a total of 480 pots and 300 kg of soil in the microcosm. All this additional plants had rhizospheric and endophytic populations sampled to determine colonization rates, P solubilization, and ICs production. We do not have a survival curve for the 60 day experiment, or shoot length after 15 days. We also have sampled soils from all pots just before planting for a chemical analysis, but the final results are not yet available. In addition to this, we also extracted metagenomics soil DNA from all 480 pots just before rice planting and inoculation. Soils were pooled for composite samples for each trio of pots, like what was done for all rhizospheric and endophytic community sampling. This means that we still have 172 soil metagenomes with 1,777,279 total sequences waiting for analysis. These data will be very important to pinpoint taxa that may be associated

to the PGP effect, strain survival, and colonization. To analyze these data, we will use the methodology described in Chapter 4.

We donôt have access to the full description of the field results from Chapter 4 since the paper that describes the field trials was not published yet. Still, Chapter 4 should be submitted to publishing as soon as we incorporate the feedback from the thesis committee. Chapter 3 still needs some few months of work because of the NGS data, the 60 day experiment, and soil chemical analysis. In addition, the CatPCA analysis from Chapter 3 can still be further optimized to explain more variance if we transform some of our variables to non-monotonic spindle multiple nominal analysis level. This makes the fullest use of non-linear relationships, but interpretation of results and detection of errors can be quite difficult.

Future research in the area must keep in mind that NGS will be a common tool for farmers, given enough time. Costs for NGS should continue to drop and costs of food should continue to rise. Given that the effectiveness of PGPB is dependent on the initial microbial community that is going to be interacting with the plant and the inoculant, farmers will use targeted microbial management once it becomes profitable for them. This evidently needs much research beyond PGPB interactions, but it is very interesting to have a defined, long-term goal in mind. The model itself, as it is, can be useful in future research too. Not only the experimental design and statistical methodology can be replicated with other datasets, the model can be used for targeted bioprospection of strains and genes of interest, especially if contrasting differences are to be explored. Other important traits in the rhizosphere might also follow a two sided, K-/r- like differences that can be very important when choosing strains to fill selected functions in specific conditions. Direct application of the model can also be used at field scale. While most industrial farming today takes place in rich soils, food production in poor soils might be a necessary alterative if environmental degradation is not quickly reverted. It that case, every piece of information will be necessary to ensure food production.

Considerações finais

O objetivo desta tese foi de aumentar o entendimento cientifico de interações naturais entre plantas e bacterias, e como podemos usar a inoculação de PGPB para explora-las, melhorando a produção agrícola. Um modelo para descrever algumas interações PGPB foi levantado e testado, confirmando diversos pontos do modelo. Além disso, uma nova metodologia estatística que pode ajudar em análises de comunidades microbianas é apresentada, enquanto aplica ecologia da invasão clássica à inoculação de PGPB. Quanto a novas considerações para o modelo publicado, incorporação das estratégias K-/r- pode ser muito útil, pois elas também simplificam estratégias muito complexas em um esquema de dois lados. Apesar da produção de ICs e solubilização de P não ocorrerem nos nichoes como esperado, isso pode ter sido devido a diferenças no desenho experimental dos Capítulos 2 e 3. Um experimento final, objetivando essa discrepância e considerando os desenhos experimentais anteriores, deve ser capaz de resolver esse ponto. Obviamente, o modelo pode ser expandido avaliando outras características, como aquelas discutidas no Capítulo 1. Mesmo expandido, deve ser mantido em mente que o modelo é baseado em médias e correlações ï seria errado propor que *Burkholderia* não pode produzir ICs e ser uma PGPB eficiente em condições de campo normais.

Nós consideramos que a tese foi bem sucedida em sua proposta, pois grandes conjuntos de dados foram criteriosamente investigados sob o método científico para gerar conhecimento que pode ser aplicado a produção de alimentos sustentável. Os Capítulos 3 e 4 ainda não foram submetidos a publicação científica, e é valido destacar alguns pontos sobre eles.

Os dados apresentados no capítulo 3 são apenas cerca de metade dos dados gerados no experimento. Havia mais uma variável ï idade da planta ï que não pode ser propriamente analisada a tempo da apresentação dessa tese. Existem mais 216 potes grandes (9 por condição experimental) onde plantas de arroz se desenvolveram por 60 dias antes da retirada do experimento, para um total de 480 potes e 300kg de solo no microcosmo. Todas essas plantas adicionais tiveram populações rizosféricas e endofíticas amostradas para determinar-se taxas de colonização, solubilização de P e produção de compostos indólicos. Não dispomos de uma curva de sobrevivência ou altura das plantas após 15 dias. Também amostramos solos de todos os potes antes do plantio para análise química, mas os dados finais ainda não estão disponíveis. Além disso, também extraímos DNA metagenômico dos solos de todos os 480 potes logo

antes do plantio e inoculação do arroz. Solos foram agrupados para formar amostras compostas a cada três potes, como foi feito para toda amostragem rizosférica e endofítitca. Isso significa que ainda temos 172 metagenomas de solo com 1.777.279 sequências para análise. Estes dados serão muito importantes para destacar taxas que podem estar associados a um efeito de PGP, sobrevivência do inoculante, e colonização. Para analisar esses dados, utilizaremos a metodologia descrita no Capítulo 4.

Não temos acesso a descrição completa dos resultados de campo do Capítulo 4, pois o artigo que descreve os ensaios de campo ainda não foi publicado. Mesmo assim, o Capítulo 4 deve ser submetido a publicação assim que incorporarmos os comentários da banca avaliadora da tese. O Capítulo 3 ainda precisa de alguns meses de trabalho devido aos dados de sequenciamento de nova geração, o experimento de 60 dias, e a análise química do solo. Além disso, a análise com CatPCA do Capítulo 3 pode ser otimizada para explicar mais variância, se transformarmos algumas da variáveis para o nível de análise não-monotônica nominal múltipla. Isso explora melhor relações não-lineares, mas a interpretação dos resultados e detecção de erros pode ser bastante difícil.

Pesquisa futura na area precisa considerer que NGS será uma ferramenta comum para fazendeiros, dado tempo o bastante. Custos para NGS devem continuar baixando, e custos de alimentos devem continuar subindo. Dado que a eficiência de PGPB é dependente da comunidade microbiana inicial que irá interagir com a planta e o inoculante, fazendeiros irão usar manejo microbiano uma vez que isso seja lucrativo para eles. Isso evidentemente requer muita pesquisa além das interações de PGPB, mas é interessante ter um objetivo de longo prazo definido em mente. O modelo em sí, como está, pode ser util em pesquisa no futuro também. Não apenas o desenho experimental e metodologia estatística podem ser replicadas com outros bancos de dados, o modelo pode ser usado para bioprospecção de linhagens e genes de interesse específicos, especialmente se diferenças contrastantes serão exploradas. Outros traços importantes na rizosfera podem seguir um esquema de dois lados, como estratégias K-/r-, que podem ser muito importantes ao escolher linhagens para preencher funções em condições específicas. Aplicação direta do modelo também pode ser feita em escala de campo. Enquanto maior parte da agricultura industrial hoje toma lugar em solos ricos, a produção de alimentos em solos pobres pode ser uma alternative necessária se degradação ambiental não for rapidamente revertida. Em tal caso, cada evidência científica será necessária para garantir a produção de alimentos.

Appendage I

Additional papers published during this thesis

The author of this thesis is very thankful to the several authors that allowed him to make scientific contributions on their papers. These were of great importance for the author to develop his expertise in statistics and scientific writing during his PhD. The published papers are:

Souza R, Beneduzi A, Ambrosini A, Costa PB, Meyer J, et al. (2012) The effect of plant growth-promoting rhizobacteria on the growth of rice (*Oryza sativa* L.) cropped in southern Brazilian fields. Plant Soil 366: 585ï 603. doi:10.1007/s11104-012-1430-1.

Granada C, Costa PB, Lisboa BB, Vargas LK, Passaglia LMP (2013) Comparison among bacterial communities present in arenized and adjacent areas subjected to different soil management regimes. Plant Soil 373: 339ï 358. doi:10.1007/s11104-013-1796-8.

Passos J, Costa PB, Costa MD, Zaffari GR, Nava G, et al. (2014) Cultivable bacteria isolated from apple trees cultivated under different crop systemsز Diversity and antagonistic activity against *Colletotrichum gloeosporioides*. Genet Mol Biol 572: 560ï 572.

Moreira FS, Costa PB, Souza R De, Beneduzi A, Lisboa BB, et al. (2016) Functional abilities of cultivable plant growth promoting bacteria associated with wheat (*Triticum aestivum* L .) crops. Genet Mol Biol 121: 111ï 121.

Campos SB, Lisboa BB, Camargo FAO, Bayer C, Costa PB, et al. (2016) Soil suppressiveness and its relations with the microbial community in a Brazilian subtropical agroecosystem under different management systems. Soil Biol Biochem 96: 191ï 197. doi:10.1016/j.soilbio.2016.02.010.

Grants and awards received during this thesis

Participation in the course funded by the Brazilian Ministry of Science and Technology through the CBAB ï Brazilian-Argentine Biotechnology Center ñMicroorganism-plant-soil interactions: biotechnological innovations for a sustainable agriculture, biocontrol and bioinoculation.ò From August 5 to 16 (2013), in Buenos Aires, Argentina.

Honorous mention - Prof. Milton Krieger Prize ï 59th Brazilian Genetics Congress - "Occurence of plant growth promoting traits: a metanalysis of 2200 bacterial isolates", by the Brazilian Genetics Society.

Best Poster on Soil Microbiology ï 27th Brazilian Microbiology Congress - "Multivariate analysis of 2200 bacterial isolates from plant roots", by the Brazilian Microbiology Society.

Awarded a DAAD scholarship (*Deutscher Akademischer Austauschdienst* - German Academic Exchange Service), for a research stay of 5 months in the Bielefeld Univesity, Bielefeld, Germany. Duration: April 2014 to August 2014.

Awarded a CsF scholarship *(Ciência sem fronteiras* ï Science without borders), for a research stay of 1 year at the Groningen Univesity, Groningen, the Netherlands. Duration: April 2015 to March 2016.

Apéndice I

Artigos adicionais publicados durante esta tese

O autor desta tese é muito grato aos diversos autores que permitiram que ele fizesse contribuições científicas em seus artigos. Estas foram de grande importância para o autor desenvolver sua perícia em estatística e escrita científica durante seu doutorado. Os artigos publicados são:

Souza R, Beneduzi A, Ambrosini A, Costa PB, Meyer J, et al. (2012) The effect of plant growth-promoting rhizobacteria on the growth of rice (*Oryza sativa* L.) cropped in southern Brazilian fields. Plant Soil 366: 585ï 603. doi:10.1007/s11104-012-1430-1.

Granada C, Costa PB, Lisboa BB, Vargas LK, Passaglia LMP (2013) Comparison among bacterial communities present in arenized and adjacent areas subjected to different soil management regimes. Plant Soil 373: 339ï 358. doi:10.1007/s11104-013-1796-8.

Passos J, Costa PB, Costa MD, Zaffari GR, Nava G, et al. (2014) Cultivable bacteria isolated from apple trees cultivated under different crop systemsز Diversity and antagonistic activity against *Colletotrichum gloeosporioides*. Genet Mol Biol 572: 560ï 572.

Moreira FS, Costa PB, Souza R De, Beneduzi A, Lisboa BB, et al. (2016) Functional abilities of cultivable plant growth promoting bacteria associated with wheat (*Triticum aestivum* L .) crops. Genet Mol Biol 121: 111ï 121.

Campos SB, Lisboa BB, Camargo FAO, Bayer C, Costa PB, et al. (2016) Soil suppressiveness and its relations with the microbial community in a Brazilian subtropical agroecosystem under different management systems. Soil Biol Biochem 96: 191ï 197. doi:10.1016/j.soilbio.2016.02.010.

Prêmios e financiamentos recebidos durante esta tese

Participação no curso financiado pelo Ministério da Ciência e Tecnologia através do CBAB ï Centro brasileiro-Argentino de Biotecnologia. ñInterações Microorganismo-planta-solo: Inovações biotecnológicas para uma agricultura sustentável, biocontrole e bioinoculaçãoò de 5 a 16 de Agosto (2013), em Buenos Aires, Argentina.

Menção honrosa ï Prêmio Prof. Milton Krieger ï 59º Congresso Brasileiro de genetic - "Occurence of plant growth promoting traits: a metanalysis of 2200 bacterial isolates", pela Sociedade Brasileira de Genética

Melhor Poster em Microbiologia do Solo ï 27º Congresso Brasileiro de Microbiologia - "Multivariate analysis of 2200 bacterial isolates from plant roots", pela Sociedade Brasileira de Microbiologia

Beneficiado com uma bolsa DAAD (*Deutscher Akademischer Austauschdienst* ï Serviço Alemão de Intercâmbio Academico), para uma estadia de pesquisa de 5 meses na Universidade de Bielefeld, Bielefeld, Alemanha, de Abril a Agosto de 2014

Beneficiado com uma Bolsa CsF (Ciência sem Fronteiras) para uma estadia de pesquisa de 1 ano na Universidade de Groningen, Groningen, Países Baixos, de Abril de 2015 a Março de 2016.