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

Esse trabalho foi desenvolvido nos seguintes locais:

Laboratório de Genética Molecular Vegetal, Departamento de Genética, Instituto de Biociências, UFRGS, Porto Alegre, Brasil, sob a orientação da Profa. Dra. Luciane Passaglia;

Genetic of Prokaryotes Laboratory, Faculty of Biology, University of Bielefeld, Bielefeld, Germany, sob a orientação do Prof. Dr. Volker F. Wendisch.

Microbial Ecology Group, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands; sob a orientação do Prof. Dr. Jan Dirk van Elsas

Esse trabalho foi financiado pelas seguintes agências de fomento:

Conselho nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil e German Academic Exchange Service (DAAD), Alemanha

Esse trabalho é dedicado aos meus pais, Guaracy and Nara,
cujo suporte foi fundamental para todas
as etapas da minha vida.

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. I've 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

Sumário

Estrutura da Tese

A introdução mostra uma breve descrição do papel das bactérias no desenvolvimento de plantas e ressalta a importância dos temas dessa tese.

O Capítulo 1 mostra o efeito da fertilização em bactérias 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 se propõe a testar o modelo proposto em um experimento de casa de vegetação e apresenta uma grande coleção de interações e correlações.

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

As considerações finais mostram um quaddro final 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 este 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: 1–9.

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: 233–266. 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: 1761–1778. 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 (1998–2013). Plant Soil 378: 1–33. 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: 648–656. doi:10.3390/agronomy3040648.

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

Griffiths BS, Philippot L (2012) Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev: 1–18. 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: 1221–1230. doi:10.1038/ismej.2008.80.

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

Koonin E V (2014) The origins of cellular life. Antonie Van Leeuwenhoek 106: 27–41. 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: 709–742.

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: 41–54. 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: 707–716.

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: 305–339. 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: 1–9.

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: 233–266. 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: 1761–1778. 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 (1998–2013). Plant Soil 378: 1–33. 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: 648–656. doi:10.3390/agronomy3040648.

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

Griffiths BS, Philippot L (2012) Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev: 1–18. 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: 1221–1230. doi:10.1038/ismej.2008.80.

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

Koonin E V (2014) The origins of cellular life. Antonie Van Leeuwenhoek 106: 27–41. 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: 709–742.

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: 41–54. 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: 707–716.

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: 305–339. 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.

Chapter 1 - How fertilization affects the selection of plant growth promoting rhizobacteria by host plants

Pedro B. da Costa and Luciane M. P. Passaglia

Published as book chapter 95, in: Biological Nitrogen Fixation, vol 2, 1º edition. Edited by Brujin FJ, John Wiley & Sons Inc, New York. 1260 p. 2015.

Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[1]Corresponding author: LMP Passaglia
Email: luciane.passaglia@ufrgs.br, Telephone: +55(51)33089813, Fax: +55(51)33087311

Keywords: plant growth promoting rhizobacteria, inoculants, fertilization level.

Introduction

Currently, 40% of the world population is fed by the increased crop productivity that results from the use of fertilizers (UNEP, 2007). However, fertilizer production is dependent on limited fossil fuels, the use of which is harmful to vital natural resources. This dependence creates a completely unsustainable situation that will prevent humanity from maintaining its current food production system: it is estimated food production worldwide will either change or collapse within a few years. Obviously, this issue requires the attention of the scientific community.

Fertilizers supply plants with important nutrients, allowing growth and productivity that is much greater than those obtained with unfertilized plants. Chemical fertilizers were first used in the mid-19th century in Europe, when low levels of phosphates in the soil endangered continuous food production in industrialized countries (Dawson and Hilton, 2011; Neset and Cordell, 2011). Traditional organic fertilization did not recycle enough phosphate to replace what was removed during increasingly large harvests in lands that had been cultivated for centuries. The discovery of rock phosphate as an amendment to crops in 1840 allowed the continuity of the

agricultural system, avoiding a food production crisis for 1.5 billion humans on Earth (Dawson and Hilton, 2011). However, by the start of the 20th century, the lack of biologically available nitrogen for crops presented a new challenge for scientists and farmers. The Haber-Bosch process, developed in 1909, enabled ammonia to be synthesized from atmospheric nitrogen and to be used on crop production (as well as in explosives and ammunition production, as World War I was about to occur). In subsequent years, man-made reactive nitrogen greatly increased food production, and the inventors of the ammonia synthesis process received two Nobel prizes, one in 1918 and another 1931. After the food shortages caused by World War II, there was intense investment in crop production policies, especially in enhancing plant responsiveness to fertilizers, greatly increasing the productivity and profitability of farming systems (Dawson and Hilton, 2011). This new technological package (heavily dependent on fertilizers, pesticides, herbicides, and fertilizer-responsive high-yield plant varieties) was adopted by several developing countries, giving rise to the Green Revolution (Evenson and Gollin, 2003). These developments greatly improved food security and prevented starvation for millions of people, but they have come with a steep cost to nature.

The excessive use of pesticides has damaged many ecosystems and caused health problems for human workers, and the high-yield varieties of rice, wheat, corn, and soybeans developed in Europe and the USA have resulted in a decreased use of native varieties and crops. Furthermore, local farming has become overshadowed by industrialized farming. Agriculture has grown completely dependent on the use of chemical NPK fertilizers to ensure food production at the required levels. However, the production of fertilizers is dependent on non-renewable resources. Despite manifold improvements, the Haber-Bosch process still used today consumes natural gas to break down the strong triple bond of atmospheric dinitrogen and to supply free H to form 2 molecules of NH_3 out of N_2 and CH_4, consuming about of 1.1% of the entire world's energy production (Dawson and Hilton, 2011; FAO, 2011). There is no other feasible source of reactive nitrogen at an industrial scale: it cannot be harvested, extracted or mined. Its only source is chemical synthesis, and the only economically viable fuel for its production is natural gas. Reactive nitrogen can also be produced through water hydrolysis, but such an approach is not yet cost-effective.

Nevertheless, nitrogen is not the only finite source that humanity utilizes to produce food: the reserves of rock phosphate, the main source of P for fertilizers, are

also limited and non-renewable. The largest reserves of rock phosphate are found in only five countries, and 77-85% of the phosphate supply is found only in Morocco (which holds a significant part of this reserve in a conflict zone). Approximately 90% of all phosphate mined on earth is used for fertilizer production, but only approximately 20% of the phosphate removed from the reserves ever reaches the consumers' tables, illustrating the remarkable inefficiency of the entire production process. The peak of rock phosphate production might be reached as soon as 2033 – from this point on, prices should increase while supply decreases (Dawson and Hilton, 2011).

Potassium is the least troublesome of the NPK nutrients. It is also mined from underground, but salty lakes (Römheld and Kirkby, 2010) or even ocean waters could be used as a secondary and renewable source of potassium because it is found at naturally high concentrations in salty bodies water (400 mg L^{-1} of K against 0.06 mg L^{-1} of P) (Dawson and Hilton, 2011). Additionally, high concentrations of potassium in soils and water are not harmful to human health, nor do they threaten wildlife (Ramasamy et al., 2005); in addition, they usually do not create detectable environmental damage.

Fertilizer applied to crops can also run off because significant portions of the introduced nutrients are not taken up by crops and escape to the environment, causing damage. Depending on the soil characteristics, 0.4 to 90% of the applied fertilizers might not be taken up by crops, with losses of 50% being very common (Adesemoye and Kloepper, 2009; Simpson et al., 2011). This means that millions of tons of expensively processed nutrients are washed away by rain or volatized to the atmosphere, causing damaging nutrient imbalances around the world. Biologically active nitrogen and phosphate can reach water bodies, creating disturbances in nutrient cycling and availability and causing eutrophication in rivers, lakes and coastal waters. An excess of P causes algal blooms of cyanobacteria (which are often toxic) because P is the major nutrient that limits their development. In P-rich conditions, a few cyanobacteria outcompete other plankton species and drastically change the biological component of water bodies. Eutrophication has a negative impact on public health, the economy and the environment, including water hypoxia (consumption of dissolved oxygen by bacterial decomposition, which leads to fish death by suffocation), changes in trophic webs, biodiversity loss, blocking of sunlight from the water column, toxic algae blooms, changes in water quality for human consumption and other such effects (Carpenter et al., 1998; Lau and Lane, 2002). Many coastal waters are in severe danger,

as this type of damage is difficult to control because it is non-punctual (unlike sewer water from cities, which has a punctual geographical origin) and is difficult to counter because it affects entire environments.

While P causes severe damage to water bodies, reactive nitrogen could be volatized to the atmosphere by microbial nitrification and denitrification, causing damage on a global scale. These volatized gases (especially N_2O) might return to the soil in regions far away from farming centers, creating nutrient unbalances, altered growth rates and biodiversity changes (UNEP, 2007). Approximately 75% of all anthropically generated N_2O comes from agriculture. N_2O is a very dangerous greenhouse gas because it is very stable (it survives in the atmosphere for approximately 100 years) and very potent (1 g of N_2O has the global warming potential of 300 g of CO_2) (UNEP, 2007). Another problem that agriculture faces is the reduction in farming lands, meaning there is the need for even greater productivity. The world's best areas for high crop productivity due to good soil conditions were historically next to cities, but such farms are quickly being replaced by urban expansion. Even if the best agricultural practices are used on the most fertile lands, the financial gain from farming cannot compete with the financial gain from turning these lands into high-profile residential areas (Singh et al., 2011).

The production and release of artificially produced reactive nitrogen compared to the amount of nitrogen fixed naturally by diazotrophs gives us a picture of the scale of the problems generated by these processes. The continual increase in the use of fertilizers worldwide has resulted in a release of reactive nitrogen that is now equal to the amount of naturally fixed nitrogen worldwide, seriously threatening the natural cycling of this nutrient. Approximately 100 million tons of both man-made and natural reactive nitrogen are produced every year, effectively doubling the amount of nitrogen available to the biosphere. This is one of the most disrupted natural cycles man has interfered with, and this disruption will lay waste to precious water resources sooner rather than later if left unchecked. (FAO, 2011; UNEP, 2007)

Thus, humanity faces a truly unsustainable situation: it is completely dependent on an environmentally dangerous (with respect to both use and production), limited natural resource, the supply of which is expected to decline sharply in less than 50 years. This situation cannot continue for an indefinite time. Thankfully, there are many alternatives to this traditional system, including improved techniques and materials for fertilizer use and production. One very promising technology is the utilization of plant

growth-promoting rhizobacteria (PGPR), which can easily reduce up to 50% of the use of fertilizers on a crop without any loss in productivity (Baldani et al., 1986; Alves et al., 2003; Hayat et al., 2010; Good and Beatty, 2011; Miransare, 2011)

The effect of fertilizers on the use of PGPR

Fertilizers reduce nitrogen fixation by PGPR

The use of fertilizers influences the relationship between PGPR and their host plants. Nitrogen fixation by diazotrophs may be reduced or even halted by the use of nitrogen fertilizers in soils because the presence of reactive nitrogen in the soil acts as a repressor for the nitrogen fixation mechanism. Because the whole cascade is energetically expensive for the bacteria, they will not activate their nitrogen fixation mechanism if it is much easier to simply take the reactive nitrogen from the soil. It has been widely reported that nitrogen fertilization reduces the number and activity of root nodules in legumes and affects the diversity of *nifH* genes in microbial populations (Kolb and Martin, 1988; Muthukumarasamy et al., 2006; Prakamhang, 2009). This issue has already been extensively discussed and is not the focus of this review.

Fertilizers interfere with plant selection of PGPR with respect to two traits simultaneously: phosphate solubilization and auxin production

Previous work from our group (Costa et al., 2012) demonstrated an interesting correlation between phosphate solubilization and the indolic compound production abilities of bacteria isolated from different NPK fertilization conditions. Root-associated diazotrophs isolated from rice plants under unfertilized conditions have shown higher phosphate solubilization activity and lower indolic compound production, while root-associated bacteria isolated from rice plants under light fertilization conditions (50% fertilizer dose) have shown higher indolic compound production and lower phosphate solubilization activity. Additionally, the indolic compound production of rhizospheric bacteria isolated from rice plants grown under unfertilized conditions was higher than the indolic compound production displayed by root-associated bacteria, and the phosphate solubilization ability was higher in rhizospheric bacteria isolated from rice plants under light fertilization conditions compared to the root-associated bacteria. Plants were effectively hosting bacteria based on their PGP characteristics: nutrient solubilization over hormone production in poor nutrient conditions and hormone production over nutrient solubilization in moderate nutrient conditions. Rhizospheric and root-associated bacterial populations isolated from full nutrient conditions (100%

fertilizer dose) were very similar with respect to their phosphate solubilization activities and indolic compound production, suggesting that at such fertilization levels, these two PGP traits were not important factors for plant-driven bacterial selection. We also found an inverse correlation between these two PGP traits: good phosphate solubilizers were not good indolic compound producers, and good indolic compound producers were not good phosphate solubilizers. Still, the isolates could present an intermediary phenotype or poor expression of both PGP traits. There appears to be no naturally occurring good phosphate solubilizers that are also good indolic compound producers. These findings have a series of implications.

First, plants select bacteria according to their specific PGP traits in response to the specific nutrient availability. It was not of benefit to the plant to host better hormone producers (found in the rhizosphere) in a situation where nutrients were more limiting. Once nutrients were more readily available, the plant left the best phosphate solubilizers on the rhizosphere and kept the best hormone producers inside their tissues. This result could mean that nutrient solubilizers are better PGPR in nutrient-poor conditions and that hormone producers are better PGPR in nutrient moderate conditions. When we tested some selected strains in a field experiment, the good phosphate solubilizers (originally isolated from unfertilized conditions) could only enhance plant growth under unfertilized conditions, while the good growth hormone producers (originally isolated from light fertilization conditions) only improved plant growth under light fertilization conditions (Costa et al., 2012). Under heavy fertilization conditions, these two PGP traits were not driving factors for plant growth. It is interesting to note that the isolates promoted growth only in the environments they were isolated from and that PGPR prospection usually takes bacteria from conventional crop fields (under 100% fertilizer) and tests them for optimal use at the 50% fertilizer level. These findings help us to better select PGPR candidates according to the target field conditions as well as help us to direct the PGPR bioprospection according to the PGP trait of interest.

Results similar to those described above have already been reported. Poonguzhali et al. (2006) studied the effects of organic and chemical fertilization on rhizospheric and endophytic isolates of Chinese cabbage. While the focus of their work was on Principal component analysis (PCA) grouping of taxonomic and substrate utilization characteristics of the microbiote, a factorial analysis considering the interaction effect between the niche and the fertilization condition was missing. Using the data available from this paper, we performed a two-way ANOVA analysis of the

indolic acetic acid (IAA) production ability of the isolates in relation to niche and fertilization interaction effects. There was a significant interaction between these factors ($p=0.04$), indicating that the IAA production ability was higher for the endophytic isolates than for the rhizospheric isolates when they were subjected to chemically fertilized conditions, but in the unfertilized condition, the rhizospheric isolates were the best indolic compound producers. Dias et al. (2008) also studied the IAA production and phosphate solubilization abilities of endophytic bacteria isolated from strawberry plants. He reported that the best indolic compound producers were not the best phosphate solubilizers. These studies agreed with our proposed model for IAA production. It has already been reported that the application of phosphate fertilizer at many different levels may increase the general bacterial biomass, including phosphate solubilizers (Hu et al., 2009; Chu et al., 2007; Gu et al., 2009). Additionally, phosphate fertilization may reduce the phosphate solubilization activity and potential of the microorganisms (Hu et al., 2009) because they no longer need to expend so much energy to solubilize a more readily available resource.

Effects of fertilization on siderophore production

Although the effects of different nitrogen fertilizers on rhizosphere pH is well documented (Kurek and Ściseł, 2003), there is little work regarding the effects of fertilization on siderophore production, even if pH affects iron bioavailability directly. A previous work (Costa et al., 2012) showed that rhizospheric bacteria isolated from a heavy fertilization condition presented higher siderophore solubilization indexes (a ratio of halo and colony size on a plate trial) than endophytic bacteria isolated from the same condition, while both rhizospheric and endophytic bacteria isolated from light fertilization and unfertilized conditions presented similar siderophore solubilization indices. Another report showed that there were no differences in siderophore production due to the addition of straw or manure to crop soil when compared to an untreated control (Rodgers-Gray and Shaw, 2001). A previous work also suggested that there was a slight correlation between siderophore production and phosphate solubilization (Costa et al., 2012) that needed further confirmation. Perhaps the increase in siderophore production due to fertilization is linked to the higher bacterial biomass and diversity caused by the use of fertilizers, as siderophore production is related to bacterial competition and exclusion due to iron nutrition limitation (Weyens et al., 2010).

Fertilizers reduce the activity of ACC-deaminase by bacterial isolates

Some bacteria may produce the enzyme 1-aminocylopropane-1-carboxylate (ACC)-deaminase, which hydrolyzes ACC to α-ketobutyrate and ammonia. ACC is the immediate precursor of ethylene, an important plant hormone that regulates growth according to its concentration and the plant's physiological condition (Arshad and Frankenberger, 2002). Because high levels of ethylene are expected to inhibit root development, bacteria that prevent its synthesis by hydrolyzing the immediate precursor ACC might help plant root development and growth, as has been shown in several studies (Long et al, 2008; Babalola, 2010; Miransari, 2011). Because ethylene production is sensitive to the nutrient status around the roots (Abeles et al., 1992) and increases under nutrient-deficient stress conditions (Glick et al., 1998; Jalili et al., 2009), one might expect that its production relates closely to fertilizer application, ultimately leading to shifts in the rhizobacteria community structure or function.

There is evidence suggesting that the use of fertilizers reduces the effectiveness of ACC-deaminase activity. Shaharoona et al. (2006) tested three strains belonging to the *Pseudomonas* genus that were selectively isolated from the maize rhizosphere for ACC production in field assays under different fertilization conditions. All treatments received P and K at 100 and 50 kg ha^{-1}, respectively, but N was added at 175 kg ha^{-1} only for some treatments. While two strains were able to induce plant growth even in the presence of the N fertilizer, their effectiveness was greater in the absence of the N fertilizer compared to the control. Furthermore, the third isolate tested promoted plant growth only in the absence of the N fertilizer. In their subsequent work (Shaharoona et al., 2008), two isolates of *Pseudomonas fluorescens* that presented ACC-deaminase activity were tested in greenhouse and field trials. In greenhouse trials, both isolates were able to increase plant root weight and biomass in the presence of 0, 25, 50, 75 and 100% NPK fertilizer of 120-100-60 kg ha^{-1}, respectively, but the efficiency of the inoculation clearly decreased with increasing fertilizer levels. This pattern was repeated in the field trials, as several yield measurements were improved by both isolates under the different fertilization conditions, but their growth efficiency compared to the non-inoculated control was greater under lower fertilization conditions. There were significant negative correlations between the inoculation efficiency and the increasing fertilizer application for various yield parameters in both greenhouse and field trials. In Zabihi et al. (2010), four *Pseudomonas* strains presenting ACC-deaminase activity were tested on wheat plants in greenhouse and field trials under different P fertilization conditions of 0, 25 and 50 kg ha^{-1}. While the 25 kg ha^{-1} dose of P fertilizer generally

presented the best productivity with respect to yield, in some cases, the highest PGPR activity was achieved at zero P fertilization and with decreasing efficiency as the fertilization levels increased.

These reports suggest that ACC-deaminase becomes less important as the plant liberates less ethylene in response to stressful nutritional conditions. In fully fertilized conditions, less ethylene is produced, and ACC-deaminase no longer functions to elongate root length. This PGP trait activity ceases to be a very important trait of the rhizhosphere community under such conditions. It is tempting to speculate that the number, activity and niche occupation of ACC-deaminasing bacteria under different fertilization conditions might change.

Effects of fertilization on bacterial motility

There is no information about the effects of fertilizer on bacterial motility, even though motility and bacterial dispersion are important factors for successful root colonization (Babalola, 2010; Turnbull et al., 2001b, Kristin and Miranda, 2013). Bacterial mobility is also related to nutrient availability, as nutrient limitation increases motility, and high nutrient concentrations reduce swimming speed and the selective advantages for higher motility (Mitchell and Kogure, 2006; Matz and Jürgens, 2003). A study by Turnbull et al. (2001a) showed that mobile *Pseudomonas* strains attached to roots more successfully than non-mobile strains in low-nutrient media, while high-nutrient media reduced general attachment by mobile and non-mobile strains to an equal extent. Thus, less mobile bacteria could survive in crop soils due to fertilization-induced increased nutrient availability, which ultimately might hinder plant-bacteria interactions. The exact impact of this possible interaction should be looked into, as nutrient excesses might generate “lazy” bacteria that are less suitable for use as PGPR.

Effects of fertilization on antagonism

Antagonism effects vary greatly because each single plant-antagonist-pathogen relationship is different. Several biotic and abiotic characteristics that influence rhizhosphere composition might affect antagonistic relationships as well. For example, organic agriculture and the use of organic composts contribute to the improvement of soil quality, increase microbial richness and evenness, promote a decrease in pathogenic damage and prevent diseases in the soil (Chaparro et al., 2012; Wu et al., 2008; Bulluck III et al., 2002). However, the type of chemical or organic fertilizer as well as the type of compost used might greatly influence the effectiveness of antagonism (Kurek and Ściseł, 2003; Wu et al., 2008; Hoitink and Fahy, 1986). The enhancement of

antagonism is more likely to occur with the use of organic fertilizers than chemical fertilizers, but this enhancement is still subject to many potentially disruptive biotic and abiotic factors.

Fertilizers affect the bulk soil, rhizospheric and endophytic bacterial populations in different ways

It has been established that plants select certain types of bacteria to execute specific functions (Hartmann et al., 2008). One can see this by looking at shifts in the associated microbiome following environmental changes or comparing the bacteria found in the different niches of the rhizobiome: endophytic and rhizoplane bacteria, rhizospheric bacteria and bulk soil bacteria. These groups of bacteria represent different communities that are subjected to different selective pressures and challenges. As the physical distance from the roots increases, the total bacterial population decreases in number but increases in diversity. Bulk soil works as a "storage area" of very different bacteria, and a subset of the diversity found in bulk soil will be selected by plants to join the rhizosphere, creating the "biased rhizosphere". In the same manner, endophytic bacteria are a subset of the more diverse rhizospheric bacteria. Plants are expected to greatly control which bacteria will be found in which niche (Hartmann et al., 2008).

There are many reports that show differences between the taxa or function of endophytic and rhizospheric bacteria. Seghers et al. (2004) used a DGGE approach with maize samples to show that type I metanotrophs were present in both endophytic and rhizospheric communities, while type II methanotrophs were present only in the rhizospheric community. Additionally, the metanotroph diversity was higher after organic fertilization compared to chemical fertilization. Wang et al. (2009) detected significant differences between root-associated and rhizopheric ammonia-oxidant bacteria from rice under different N fertilization conditions. Most of the root-associated and rhizhospheric samples clustered differently in a PCA when T-RFLP patterns were compared. These authors observed a trend wherein the relative abundance of *Nitrosomonas* increased relative to *Nitrosospira* with the increase of N fertilization right after the application of fertilizers. Pariona-Llanos et al. (2010) showed that organic fertilization resulted in greater endophytic bacterial numbers than chemical or no-fertilization conditions in sugarcane. Two enzymes related to active penetration of plant cells were also screened, and it has been found that pectinase activity was more present after chemical fertilization, while endoglucanase activity was more present after organic fertilization. Unfertilized conditions had lower occurrence of both traits. Ferrara et al.

(2011) studied endophythic and rhizospheric enterobacteria of the *Enterobacter* and *Klebsiella* genera isolated from sugarcane in commercial fields and compared some PGP traits. They found that amino acid release was greater in endophytic isolates, while the antagonism and nitrogen fixation was greater in rhizospheric isolates, but IAA production was similar in both niches. Many PGP traits can be differentially selected by plants under different conditions; thus, there are no "typical" endophytic or rhizospheric bacteria (besides the symbiotic legume-rhizobium), but instead, plants select bacteria according to their current needs.

Conclusions

As we can see, fertilization works differently on PGPR from different niches and on several different traits of the community. Community genera composition, bacterial concentration, enzyme production, antagonism, nitrogen fixation, nutrient solubilization, genetic and functional diversity of rhizospheric and endophytic bacteria were all affected differently by fertilization. Fertilization affects many important PGP traits, community structure and the efficiency of PGPR inoculation. Simple experiments with double-cross comparisons of endophytic and rhizospheric communities of fertilized and unfertilized conditions may still reveal many of the plants' preferred PGP traits. The discovery of the selected attributes may greatly increase inoculation efficiency because researchers might be able to inoculate crops with the "more favored" PGPR under field conditions.

Endophytic and rhizospheric bacteria under fertilized and unfertilized conditions can be statistically compared in many ways. For continuous PGP traits, a two-way factorial ANOVA with a simple main effect analysis and a post-hoc test can reveal interesting interaction effects. However, one must not forget to confirm the homogeneity of variances before testing: IAA production levels frequently violate this assumption and should be treated with a non-parametric Kruskal-wallis test followed by Dunn's post-hoc test. Discrete traits can be easily compared with the chi-square statistic, with multiple layers and residue analysis used to single out what conditions and traits are occurring outside expected values. Finally, plot multivariate statistics such as CatPCA or MCA can greatly facilitate simultaneous visualization of the multiple correlations between PGP traits, bacterial taxa, and environmental conditions, as long as numeric, ordinal, nominal and supplementary variables are treated as such. For more information on multivariate statistics for microbial ecology, see Ramette (2007).

Many research institutes worldwide have standing long-term trials involving different fertilization conditions at experimental stations, which are perfect for identifying these interactions and greatly facilitate studies that take this type of statistical approach. A single practiced microbiologist who knows what comparisons must be made can isolate, screen and analyze the PGP traits of hundreds of isolates in just six months, which also facilitates the studies that take this type of approach. Given the relative ease and plentitude of opportunities to run this comparison, the intense biological interaction of PGPR with fertilizers, and the importance of this issue with respect to resource conservation and economic sustainability, additional research on this topic that take this approach are greatly encouraged.

Acknowledgements

This work was financed by a grant and fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and the Instituto Nacional de Ciência e Tecnologia (INCT) da Fixação Biológica do Nitrogênio (Brazil).

References

Abeles FB, Morgan PW, Saltveit ME Jr (1992) The biosynthesis of ethylene. In: Ethylene in plant biology. San Diego, Academic Press. p. 26–55.

Adesemoye AO, Kloepper JW (2009) Plant-microbes interactions in enhanced fertilizer-use efficiency. Appl. Microbiol. Biotechnol. 85:1-12.

Alves BJR, Boddey RM, Urquiaga S (2003) The success of BNF in soybean in Brazil. Plant Soil 252: 1-9.

Arshad M, Frankenberger WT Jr (2002) Ethylene: agricultural sources and applications. New York, Kluwer. p. 342.

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

Baldani JI, Baldani VLD, Seldin L, Dobereiner J (1986) A root- associated nitrogen-fixing bacterium. Int. J. Syst. Bacteriol. 36:86-93.

Bulluck III LR, Brosius M, Evanylo GK, Ristaino JB (2002) Organic and synthetic fertility amendments influence soil microbial, physical and chemical properties on organic and conventional farms. Appl. Soil Ecol. 19:147–160.

Carpenter SR, Caraco NF, Correl DL, Howarth RW, Sharpley AN, Smith VH (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol. Appl. 8:559-568.

Chaparro JM, Sheflin AM, Manter DK, Vivanco JM (2012) Manipulating the soil microbiome to increase soil health and plant fertility. Biol. Fert. Soils. 48:489–499.

Chu H, Lin X, Fujii T, Morimoto S, Yagi K, et al (2007) Soil microbial biomass, dehydrogenase activity, bacterial community structure in response to long-term fertilizer management. Soil Biol. Biochem. 39:2971–2976.

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.

Dawson CJ, Hilton J (2011) Fertilizer availability in a resource-limited world: Production and recycling of nitrogen and phosphorus. Food Policy 36:S14-S22.

Dias ACF, Costa FEC, Andreote FD, Lacava PT, Teixeira M, et al. (2008) Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J. Microbiol. Biotechnol. 25:189–195.

Evenson RE, Gollin D (2003) Assessing the impact of the green revolution, 1960 to 2000. Science 300:758-62.

FAO (2011) "Energy-smart" food for people and climate: Issue paper. Rome, Food and Agriculture Organization of the United Nations. p. 78.

Römheld V, Kirkby E (2010) Research on potassium in agriculture: needs and prospects. Plant Soil 335:155-180.

Glick B, Penrose D, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190:63-8.

Good AG, Beatty PH (2011) Fertilizing Nature: A Tragedy of Excess in the Commons. PLoS Biol 9: e1001124.

Gu Y, Zhang X, Tu S, Lindström K (2009) Soil microbial biomass, crop yields, and bacterial community structure as affected by long-term fertilizer treatments under wheat-rice cropping. Europ. J. Soil Biol. 45:239–246.

Hartmann A, Schmid M, Tuinen D Van, Berg G (2008) Plant-driven selection of microbes. Plant Soil 321:235–257.

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.

Hoitink HAJ, Fahy PC (1986) Basis for the control of soilborne plant pathogens with composts: Ann. Rev. Phytopathol. 24:93-114

Hu J, Lin X, Wang J, Chu H, Yin R, Zhang J (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.

Jalili F, Khavazi K, Pazira E, Nejati A, Asadi Rahmani H, et al. (2009) Isolation and characterization of ACC deaminase producing fluorescent pseudomonads, to alleviate salinity stress on canola (*Brassica napus* L.) growth. J. Plant Physiol. 166:667-674.

Kolb W, Martin P (1988) Influence of nitrogen on the number of N-fixing and total bacteria in the rhizosphere. Soil Biol. Biochem. 2:211-22.

Kristin A, Miranda H (2013) The root microbiota—a fingerprint in the soil? Plant and Soil, in press (available at http://link.springer.com/article/10.1007%2Fs11104-013-1647-7)

Kurek E, Jaroszuk-Ściseł J (2003) Rye (*Secale cereale*) growth promotion by Pseudomonas fluorescens strains and their interactions with *Fusarium culmorum* under various soil conditions. Biol. Control 26:48–56.

Lau SSS, Lane SN (2002) Biological and chemical factors influencing shallow lake eutrophication: a long-term study. Sci. Total Environ. 288:167-181.

Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PloS one 3: e2702.

Matz C, Jürgens K (2003) Interaction of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. Microb. Ecol. 45:384–398.

Miransari, M (2011) Soil microbes and plant fertilization. Appl. Microbiol. Biotechnol. 92:875-85.

Mitchell JG, Kogure K (2006) Bacterial motility: links to the environment and a driving force for microbial physics. FEMS Microbiol. Ecol. 55:3–16.

Muthukumarasamy R, Kang UG, Park KD, Jeon W-T, Park CY, et al. (2007) Enumeration, isolation and identification of diazotrophs from Korean wetland rice varieties grown with long-term application of N and compost and their short-term inoculation effect on rice plants. J. Appl. Microbiol. 102:981–991.

Neset TSS, Cordell D (2011) Global phosphorus scarcity: identifying synergies for a sustainable future. J. Sci. Food Agricul. 92:2-6.

Pariona-Llanos R, Ferrara FIS, Gonzales HHS, Barbosa HR (2010) Influence of organic fertilization on the number of culturable diazotrophic endophytic bacteria isolated from sugarcane. Europ. J. Soil Biol. 46:387–393.

Poonguzhali S, Madhaiyan M, Sa T (2006) Cultivation-dependent characterization of rhizobacterial communities from field grown Chinese cabbage *Brassica campestris* ssp *pekinensis* and screening of traits for potential plant growth promotion. Plant Soil 286:167–180.

Prakamhang J, Minamisawa K, Teamtaisong K, Boonkerd N, Teaumroong N (2009) The communities of endophytic diazotrophic bacteria in cultivated rice (Oryza sativa L.). Appl. Soil Ecol. 42:141–149.

Ramasamy C, Ramanathan S, Dhakshinamoorthy M (2005) Perspectives of Agricultural Research and Development. Coimbatore, Tamil Nadu Agricultural University. p. 685.

Ramette A (2007) Multivariate analyses in microbial ecology. FEMS Microbial. Ecol. 62: 142–160.

Rodgers-Gray BS, Shaw MW (2001) The effect of incorporating straw or manure into the soil on the natural microflora of winter wheat. Plant Pathol. 50:537-545.

Ferrara FIS, Oliveira ZM, Gonzales HHS, Floh EIS, Barbosa HR (2011) Endophytic and rhizospheric enterobacteria isolated from sugar cane have different potentials for producing plant growth-promoting substances. Plant Soil 353:409–417.

Seghers D, Wittebolle L, Top EM, Siciliano SD, et al. (2004) Impact of Agricultural Practices on the Zea Impact of Agricultural Practices on the *Zea mays* L . Endophytic Community. Appl. Environ. Microbiol. 70:1475-1482

Shaharoona B, Arshad M, Zahir AZ, Khalid A (2006) Performance of *Pseudomonas* spp. containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. Soil Biol. Biochem. 38:2971–2975.

Shaharoona B, Naveed M, Arshad M, Zahir ZA (2008) Fertilizer-dependent efficiency of Pseudomonads for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). Appl. Microbial. Biotechnol. 79:147–155.

Simpson RJ, Oberson A, Culvenor RA, Ryan MH, Veneklaas EJ, Lambers H, et al. (2011) Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems. Plant Soil 342: 89-120.

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.

Turnbull GA , Morgan JAW, Whipps JM, Saunders JR (2001a) The role of motility in the in vitro attachment of *Pseudomonas putida* PaW8 to wheat roots. FEMS Microbial. Ecol. 35:57–65.

Turnbull GA, Morgan JAW, Whipps JM, Saunders JR (2001b) The role of bacterial motility in the survival and spread of *Pseudomonas fluorescens* in soil and in the attachment and colonisation of wheat roots. FEMS microbial. Ecol. 36:21–31.

UNEP, WHRC (2007) Reactive Nitrogen in the Environment: Too Much or Too Little of a Good Thing. Paris, United Nations Environment Programme. p. 56.

Wang Y, Ke X, Wu L, Lu Y (2009) Community composition of ammonia-oxidizing bacteria and archaea in rice field soil as affected by nitrogen fertilization. System. Appl. Microbiol. 32:27–36.

Weyens N, Monchy S, Vangronsveld J, Taghavi S, and van der Lelie D (2010) Plant microbe partnerships. In Timmis KN, Editor. Handbook of Hydrocarbon and Lipid Microbiology. Berlin Heidelberg, Springer-Verlag. p. 2545-2574

Wu H, Yang X, Fan J, Miao W, Ling N, et al. (2008) Suppression of Fusarium wilt of watermelon by a bio-organic fertilizer containing combinations of antagonistic microorganisms. BioControl 54:287–300.

Zabihi HR, Savaghebi GR, Khavazi K, Ganjali A., Miransari M (2010) Pseudomonas bacteria and phosphorous fertilization, affecting wheat (*Triticum aestivum* L.) yield and P uptake under greenhouse and field conditions. Acta Physiol. Plant. 33:145–152.

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

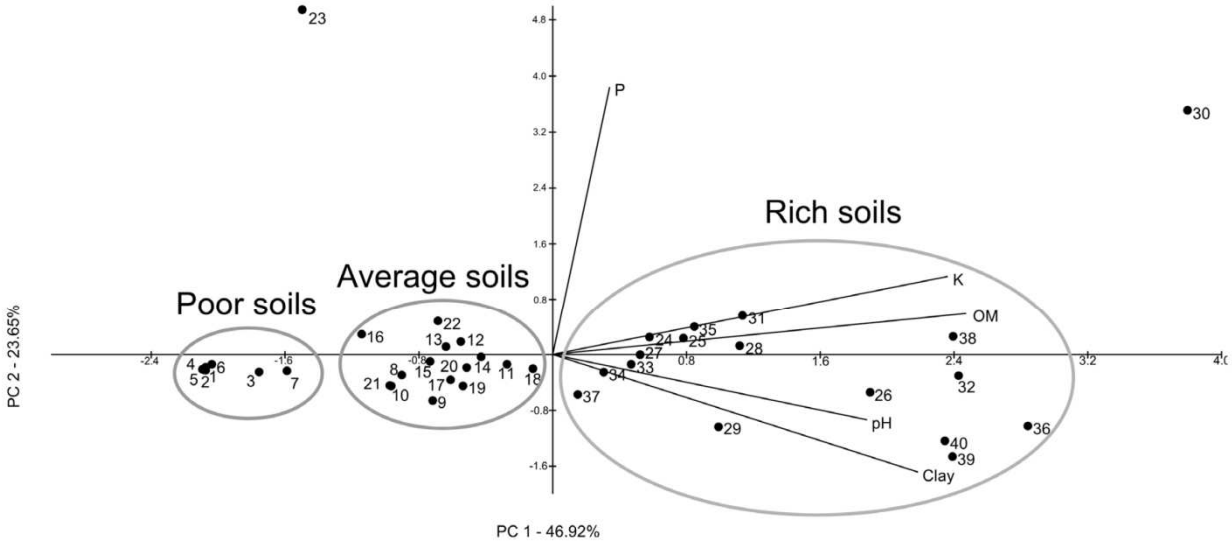


Fig. 1. PCA analysis of the soil characteristics from the 40 soils samples (numbered black circles) that were used for bacterial isolation. The percentages show how much variation is explained by each principal component. The soils with higher pH, organic matter (OM), potassium (K), phosphorus (P), and 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.

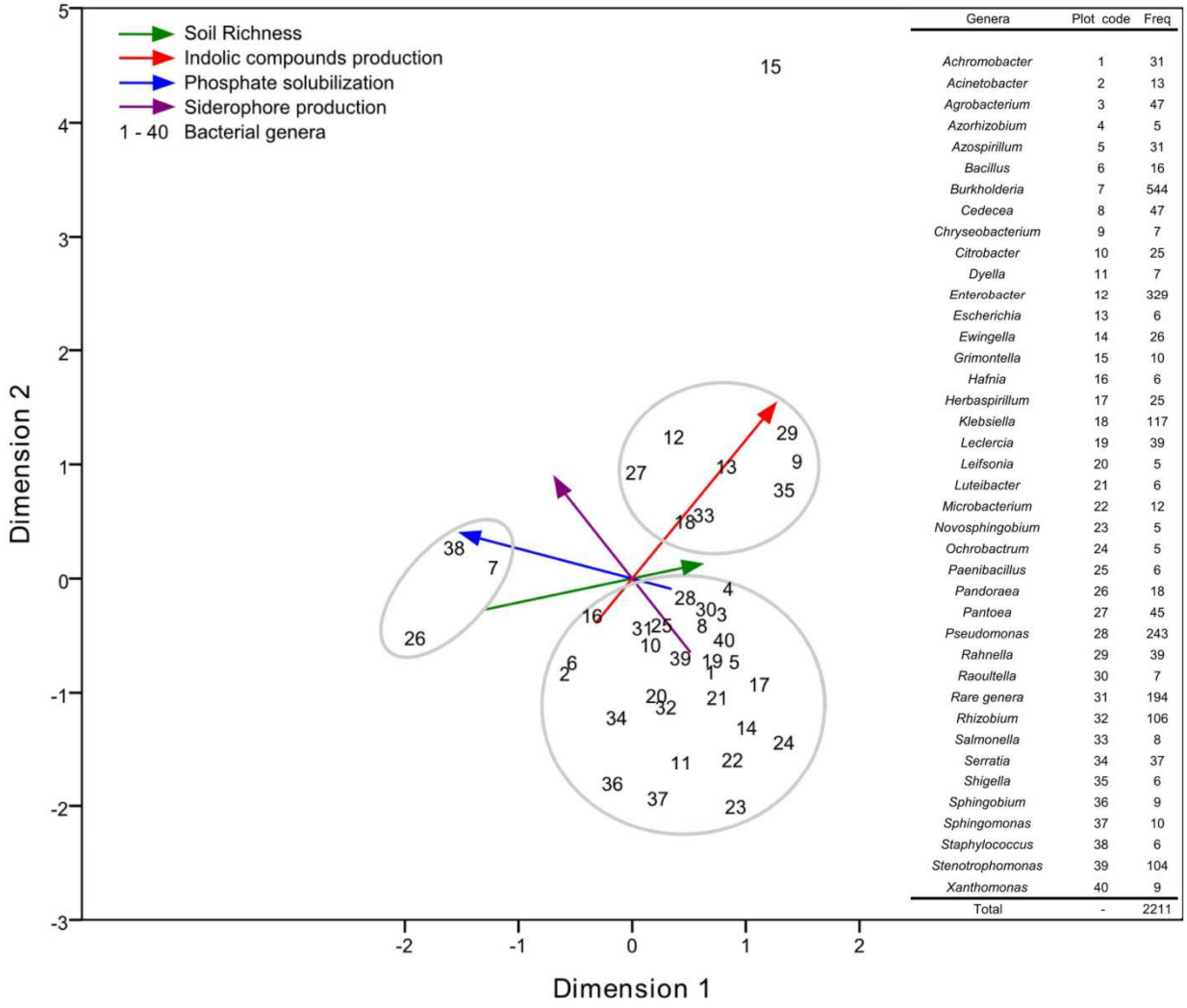
doi:10.1371/journal.pone.0116020.g001

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

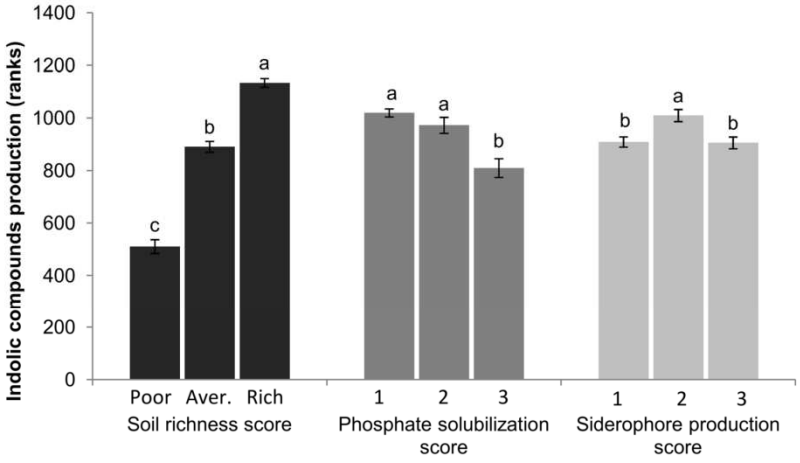


Fig. 3. Indolic compound production ability of the isolates (average rank ±1 SE) according to the soil nutrient conditions and TCP solubilization and siderophores production abilities. The phosphate solubilization and siderophores production scores are 1= no halo, 2= small or average halo, and 3= large 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

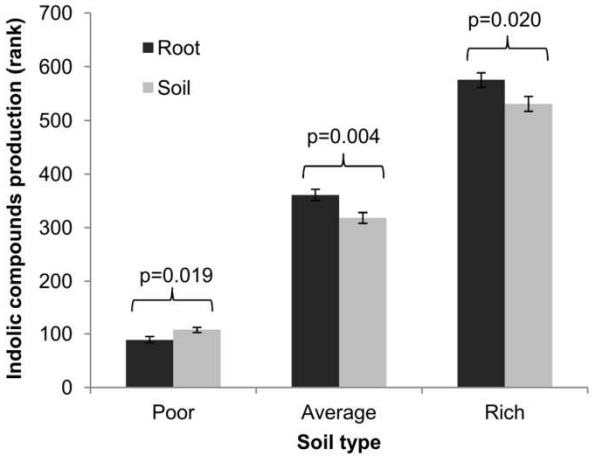


Fig. 5. Niche effect on ICs production (average ±1 SE) between endophytic (root) and rhizospheric (soil) isolates under each soil condition. The best ICs producers shift their colonization site according to soil richness. Sample sizes and p values are presented on S3 Table.

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					
													Poor		Average		Rich	
	1	2	3	1	2	3	1	2	3	Poor	Aver	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.

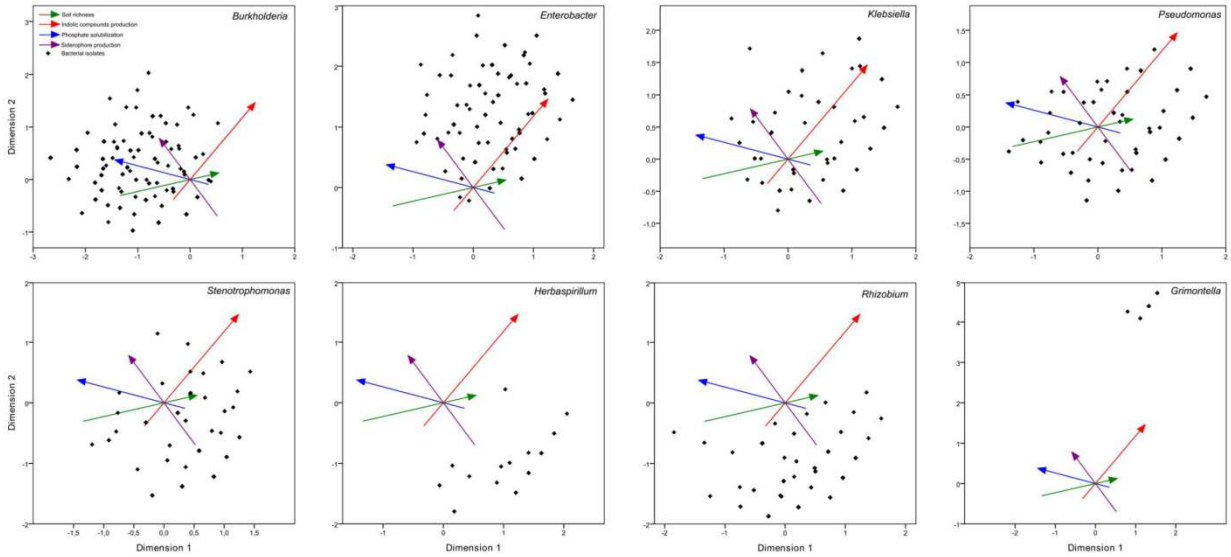


Fig. 9. CatPCA analysis of 2,211 bacterial isolates (the legend and interpretation are similar to those of Fig. 2). The genera *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, *Herbaspirilum*, *Rhizobium*, and *Grimontella* are represented one at a time. Each black dot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

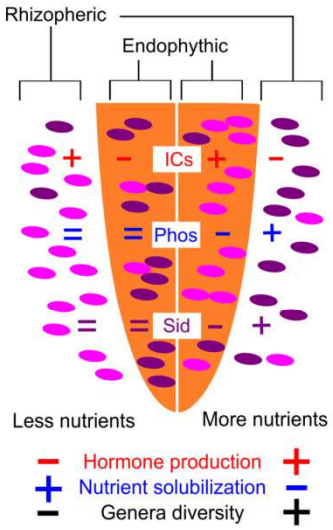


Fig. 10. A model to explain the distribution of bacteria displaying different plant growth promotion traits. In soils with fewer nutrients, plants leave the best growth hormone producers in the rhizosphere, while both endophytic and rhizospheric bacteria are good nutrient solubilizers. In soils with 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 $\pm$ 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.

Chapter 3 - Diversity gradient microcosm as an approach to test a model based on plant-bacteria interaction

Pedro Beschoren da Costa[a, b], Jan Dirk van Elsas[b], Cyrus Mallon[b], and Luciane Maria Pereira Passaglia[a, *]

Manuscript in preparation to be submitted to the Environmental Microbiology journal.

[a] Federal University of Rio Grande do Sul, Institute of Biosciences, Department of Genetics, Av. Bento Gonçalves, 9500, Postal box 15.053, CEP 91501-970,
Porto Alegre, RS, Brazil

[b] University of Groningen, Faculty of Mathematics and Natural Sciences, Groningen Institute for Evolutionary Life Sciences, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

[*]Corresponding author at Departamento de Genética, Instituto de Biociências, UFRGS. Av. Bento Gonçalves, 9500, Caixa Postal 15.053, Prédio 43312, sala 207b, Porto Alegre, RS, CEP 91501-970, Brazil. Tel.: +55 51 3308 9813; fax +55 51 3308 7311. E-mail address: luciane.passaglia@ufrgs.br (L. M. P. Passaglia)

Abstract

Management of the soil microbial community to increase crop productivity is one of the main challenges of modern agriculture. Bacterial inoculants can help the achievement of this goal, but are not always efficient due to the complex plant-bacteria-environment interactions. In this work, we test a model that intent to explain the relationship between plant growth promoting bacteria (PGPB) and their host plants in contrasting nutrient conditions, over a manipulated soil diversity gradient. Rice plants were single or co-inoculated with two selected strains belonging to *Burkholderia* and *Enterobacter* genera in rich clay and poor sandy soils. Before planting, both soil types were sterilized and inoculated with a natural soil solution, with three increasing dilutions that reduced diversity for each microcosm. Inoculant survival, plant growth promotion (PGP) of plants, strain colonization, production of indolic compounds (ICs) and P solubilization abilities from both rhizospheric and endophytic bacterial

communities were analyzed. Several proposals of the model were experimentally confirmed, such as the best PGP efficiency of *Burkholderia* in poor soils and of *Enterobacter* in rich soils, the highest bacterial ICs production on rich soils and the highest bacterial P solubilization on poor soils, and also that these two PGP traits were inversely correlated. Multiple interactions among bacterial survival and colonization with soil type, diversity level and plant growth promotion were observed. We conclude that PGPB efficiency depends largely on initial microbial community composition, and that the proposed model is very useful for PGPB testing and bioprospection.

Key words: Dilution-to-extinction gradient, P solubilization, indolic compounds, inoculant colonization, inoculant survival.

Introduction

Plants and bacteria interact in many different ways, and manipulation of the microbial communities can largely affect plant biomass (Babalola, 2010; Berg et al., 2013). Some of these interactions can be used in sustainable agriculture to improve food production while diminishing chemical inputs (Ahemad and Kibret, 2014; Adesemoye and Kloepper, 2009). Manipulation of local microbial communities through the inoculation of selected strains has been already used on industrial scale for years, with clear improvements on crop productivity in a cost-effective manner. Bacterial inoculants for plants, however, are not 100% effective in many ocasions. Many environmental variables, most notably the native microbial community, can greatly affect the effectiveness of plant growth promoting bacteria (PGPB). Plant-bacteria-environment interactions are complex, but understanding of rhizosphere ecology will be a key element in future agriculture (Singh, 2015).

A previous work from our group (Costa et al., 2014) proposed a theoretical model to explain some plant-bacteria interactions, but this model still had to be tested. The model predicts that plants on rich nutrient conditions will favor their interactions with bacteria that are able to produce high amounts of growth hormones, to better increase plant biomass by improving the use of the available nutrients. Meanwhile, plants on nutrient poor conditions will favor their interactions with bacteria able to solubilize nutrients, such as phosphorus and iron, to help them to survive in an environment with limited availability of nutrients. According to the model, bacteria belonging to the *Burkholderia* genus would be a typical PGPB in soils with low

nutrients availability, while those belonging to the *Enterobacter* genus would be typical PGPB in soils with high nutrients availability. The model simplifies PGPB effectiveness to a dualistic scenario, but lacks information regarding bacterial diversity levels. However, it is well know that the native bacterial community plays a major role on PGPB effectiveness (Owen et al., 2015). One way to experimentally manipulate the native microbial community is through a dilution-to-extinction microcosm approach, creating a controlled diversity gradient that could affect the plant-bacteria interactions. This method has already been used to generate diversity gradients several times (van Elsas et al., 2012; Malon et al., 2015; Franklin and Mills 2006; Wertz et al., 2006), keeping the differential diversity levels for months (Bonkowski and Roy, 2005).

In this work we aim to: (I) test the hypothesis predicted in the model (Costa et al., 2014), by inoculating rice plants with *Enterobacter* and *Burkholderia* strains in soils rich and poor in nutrients; (II) associate inoculant survival in these soils and the colonization of endosphere and rhizosphere of rice plants with the effective PGPB action of the inoculant; and (III) evaluate the effect of a soil diversity gradient in the survival-colonization associations.

Methods

Strains and selective media

The two strains used in this work where isolated and tested in different fertilization conditions: *Burkholderia vietnamiensis* 45 was isolated from a zero fertilizer condition and *Enterobacter asburiae* 68 was isolated from a light fertilization condition (60 kg of N, 20 kg of P_2O_5, and 60 kg of K_2O ha^{-1} $year^{-1}$). Both strains were isolated from rice fields cultivated in the IRGA (Instituto Riograndense do Arroz) research station located in Cachoeirinha, Brazil. The *Enterobacter* strain is reported to produce almost three times more indolic compounds than the *Burkholderia* strain, and the *Burkholderia* strain had the siderophore and phosphate solubilization indexes ten times higher than the *Enterobacter* (Costa et al., 2012). These strains had already been tested in greenhouse and field assays, and had shown potential as effective PGPB (Costa et al., 2012).

The two strains had their 16S rDNA gene completely sequenced. DNA extraction from 1.5 mL of LB liquid (Sigma) cultures was performed with the Ultraclean Microbial DNA extraction Kit (MOBIO). Polymerase Chain Reaction (PCR) was performed with the following reaction: 5.0 μL of PCR buffer 10 X (Roche), 0.8 μL

of $MgCl_2$ (50 mM), 1.0 µL of DMSO 100%, 0.5 µL of BSA (20 mg ml^{-1}), 1.0 µL of dNTP mix (10 nm, Roche), 1 µL of primer B8F (10 µM, Edwards et al., 1989), 1 µL of primer 1492R (10 µM, Lane, 1991), 0.2 µL of Taq Polimerase (Roche), 1 µL of DNA template (5.0 ng μL^{-1}) and 38.5 µL of PCR-grade water. Initial denaturation was made by 95ºC for 5 min, followed by 35 cycles of 95ºC for 1 min, 52ºC for 30 s, and 72ºC for 2 min, and then a final extension at 72ºC for 7 min. Sequences where compared to the GenBank database and had identities of 99% with corresponding species (accession numbers are: XXXXX for *E. asburiae* 68 and YYYYY for *B. vietnamiensis* 45).

The natural resistance of *Burkholderia* strain to different antibiotics was evaluated on solid LB medium (Sigma). A LB medium supplemented with 50 µg mL^{-1} of spectinomycin and 25 µg mL^{-1} of gentamycin was chosen as the most suppressive condition that allowed a good development of this strain. For the *Enterobacter* strain, spontaneous mutants were generated by plating 150 µL of high density cell suspension on LB medium supplemented with rifampicin at 10, 20, 50 and 100 µg mL^{-1}. Plates were incubated at 37ºC for 7 days. Single colonies that acquired antibiotic resistance were streaked to purity and grown on liquid LB medium with the original amount of rifampicin. Fully grown mutant cultures were transferred to LB without antibiotic to compare their growth rates with the wild type strain. One *Enterobacter* strain naturally resistant to rifampicin at 100 µg ml^{-1} and with no observed growth impairment was used for the soil inoculations. Mutations for rifampicin resistance, generally mediated by a mutation in the β subunit of RNA polymerase, are rare in soil bacteria (Compeau et al., 1988). Both selective media also received cycloheximide at 50 µg mL^{-1} to suppress fungi growth. Both strains were stored in glycerol 50% at -20ºC.

Microcosm set up

The soil considered “poor” was taken from a sandy section with no vegetal cover within a forest patch in Noordlaren (53°06'59.5"N 6°38'48.8"E), The Netherlands. Its organic matter content was 0.95%±0.12, and pH 5.18±0.14. The soil considered “rich” was taken from a clay soil pasture covered by grass in Steenharst (53°15'43.1"N 6°10'18.9"E), The Netherlands. Its organic matter content was 6.40%±0.90, and pH 6.7±0.22. To determine organic matter and pH, both soils were grinded and dried (72 h at 60ºC). Organic matter was calculated from dry soil weight loss after burning at 550ºC for 5 h [(initial weight – final weight)/ initial weight*100]. pH was measured in a 1:4.5 soil to water solution after 30 min of mixing and 1 h of decantation.

Natural soils were autoclaved twice, in autoclavable bags with no more than 8 kg of soil each. Soils were cooled in open bags overnight before the second time autoclaving, and then a 10^1 solution of this soil was plated on LB medium to check for sterility. Approximately 300 g of autoclaved soil were used to fill each 0.3 L plastic pots. The pots from each treatment were put in separate plastic trays, distributed at random in the greenhouse and filled with distilled water up to 3-7 cm, watering the pots from the bottom. Then, sterile soils were immediately inoculated with serial dilutions of 10^1, 10^3 and 10^6 from the natural soil, creating the microcosm diversity gradients. The serial dilutions started with 150 g of either rich or poor natural soils in 1,350 mL of sterile 0.85% NaCl (saline solution). Each pot received 30 mL of the serial dilution on July, 21st, 2015, and the microcosms were left to maturate for 3 months. Temperature was kept at 28ºC, photoperiod at 14 h/day.

Seedling planting and bacterial inoculation

Before strain inoculation, microcosm soil samples (15 g) were taken to determine pre-planting CFUs and for chemical soil analysis. Two mL of inoculant cultures grown in liquid LB medium with OD_{600nm} 0.982±0.04 (9.39±0.34 log CFU mL^{-1}) were added to each treatment. Non-inoculated controls received 2 mL of sterile LB medium. Water levels on the trays were raised to the same height as the soil on the pots to simulate the flooding conditions. Rice seeds (*Oriza sativa* IRGA-409 variety) were pre-germinated in Petry dishes with wet paper filter for 2 days in the dark prior planting. Seedlings were also exposed to the liquid inoculant for about 30 min prior planting. Three seedlings were planted per pot, buried about 2.5 cm deep in the soil, and plants were tinned to 1 plant per pot 10 days after planting. The rice seeds used in this experiment were developed by the same research agency from where the strains were isolated (IRGA, Brazil), and were gently conceded by IRRI (International Rice Research Institute, Philippines). The experiment was set on 12, 17, and 20 of October, 2015 for the 10^1, 10^3, and 10^6 treatments, respectively. Plant biomasses were measured as shoot length 15 days post-inoculation, shoot and root lengths at harvest (30 days after inoculation), and shoot dry weight at harvest (30 days after inoculation).

Pots received 50 mL of 1 X Hoagland solution at planting, and every 10 days thereafter. Hoagland solution was composed of 8 mL of solution A (KNO_3 82.15 g L^{-1}, and $Ca(NO_3)_2.4H_2O$ 118.08 g L^{-1}), 8 mL of solution B (KH_2PO_4 8.5 g L^{-1} , $MgSO_4.7H_2O$ 61.62 g L^{-1}), 1 mL of solution C (H_3BO_3 0.284 g L^{-1}, $MnCl_2.4H_2O$ 0.099 g L^{-1}, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.124 g L^{-1}, $CuSO_4.5H_2O$ 0.050 g L^{-1}, and $ZnSO_4.4H_2O$

0.055 g L^{-1}), and 1 mL of solution D (Fe-EDTA 18.8 g L^{-1}), and filled with sterile distilled water to 1 L.

The four factors in the experimental design were abbreviated according to soil types (P for poor, R for rich) followed by bacterial inoculation (C for control, colored in black; B for *Burkholderia*, colored in blue; E for *Enterobacter*, colored in red; M for mixed strain inoculation, colored in green in the figures), noted with the microcosm dilution in superscript (^{1}, ^{3}, and ^{6} for 10^1, 10^3, and 10^6 dilutions, respectively). Abbreviations like PB^3 means poor soil inoculated with *Burkholderia* strain on the 10^3 microcosm dilution. Rhizospheric and endophytic samples were abbreviated as Rhizo and Endo, respectively, when necessary.

Microcosm sampling and CFU determination

All samples from the microcosms were composed of three independent composite samples, one of each composed from three independent pots. To determine inoculant survival, top soil samples were collected with a sterile loop from the first 2 to 3 cm of soil at days 1, 5, 15 and 30 after planting. Rhizospheric samples were obtained from the soil that was immediately attached to plant roots, and putative endophytic isolates were obtained from surface-sterilized plant roots. Rhizospheric and endophytic samples were taken only at harvest, 30 days after planting. Root sterilization was performed by root washing in 70% ethanol for 1 min followed by a sodium hypochlorite 4.0% wash for 2 min, followed by 5 washings in sterile distilled water and then shred with disinfected scissors. For all CFU counts approximately 1 g of fresh soil or shred roots was added to 9 mL of sterile saline solution in sterile 15 mL falcon tubes. The tubes containing rhizospheric soil were agitated in a vortex for 30 s and left in a shaker at 28ºC and 180 rpm for 1 h prior plating for CFU counting. Shred roots where left in the shaker overnight at the same conditions. All further serial dilutions were made with sterile saline solution, and plates received 200 µL of the serial dilutions. Inoculated plates grown for 48 h at 28ºC prior colonies counting.

Phosphate solubilization and indolic compound assays

An indicator medium based on tri-calcium phosphate (TCP) was used for the phosphate solubilization assay (Ambrosini et al., 2012). Autoclaved solutions of 10% K_2HPO_4 and 10% $CaCl_2$ were added to GY medium (10 g L^{-1} glucose, 2 g L^{-1} yeast extract, 15 g L^{-1} agar) at 50 and 100 ml L^{-1}, respectively. The TCP medium was inoculated with colonies randomly picked from the LB medium plates (without

antibiotics) that were used to estimate CFUs counting from rhizosphere and endosphere populations. About 42 colonies were picked per experimental condition. Results were recorded as colonies with no halo, with small halos (diameter between 0.1 to 6 mm) or with large halos (diameter above 6 mm). Inoculated plates were grown for 48 h at 28ºC prior evaluation. Using this approach we added the bias of cultivability from LB and GY media, and also of the phosphate source used (Bashan et al., 2012).

The indolic compounds (ICs) detection assay with the Salkowski reagent was based on the PC method (Glickmann and Dessaux, 1995), using the King B medium supplemented with tryptophan and 50 μg mL^{-1} of cycloheximide to suppress fungi growth. Two-hundred μL of this medium in a sterile 96 well plate was inoculated with 10 μL of the serial dilutions (from 10^2 to 10^5) that were also used for CFUs counting of rhizospheric and endospheric populations. Each sample was inoculated in triplicate with two different dilutions. Standard curves for each plate used 0, 1, 5, 10, 15, 20, 30, 50, 75 and 100 μg mL^{-1} of 3-Indoleacetic acid (Sigma) as a standard. Inoculated plates were incubated for 48 h at 28ºC prior evaluation. The OD_{600} for each plate was determined, prior the supernatant collection, for normalization of the ICs production by bacterial biomass. Using this approach we added the bias of cultivability and of the medium's capacity to represent ICs production.

Statistical analysis

Plant biomass measures were evaluated with a two-way ANOVA. Separate tests were made for plants cultivated in rich and poor soils. Shoot dry weight in rich soils had to be transformed to the forth root due to unequal variances.

For inoculant survival, we applied the log-transformed CFU data from the different time points to a non-linear regression (Gauss–Newton method). The expression used was $C_t = N_o \times e^{(-m \times t)}$, where C_t is the log CFU g fresh $weight^{-1}$ at time *t* (days), *No* is the initial log CFU g fresh $weight^{-1}$ on day 1, and *m* is a slope parameter (Semenov et al., 2007). The slope parameter is calculated by an iterative process to minimize the residual square mean, and was given a starting value of 1. Pseudo-r values were checked to verify fit of expected to observed values. The equations were used to determine how many days it would take for the *Burkholderia* and *Enterobacter* strains to reach, respectively, 5.5 and 4.2 log CFU g fresh $weight^{-1}$. These values are the detection limit, based on background antibiotic resistance of non-inoculated controls 30 days after planting. The equation output was tested on a two-way ANOVA, transformed

to ln due to unequal variances. Separate tests were run for each soil type and the two different inoculants.

To determine colonization of top soil, rhizospheric and endospheric environments, CFU data from selective media were normalized by the total CFU in LB medium without antibiotic for each sample (selective media CFU/ LB CFU*100). Due to the high variance that could not be fixed by transformations and multiple factors that could not be properly analyzed with non-parametric tests, analysis on jitter plots was the best way to evaluate the data (Cumming et al., 2007).

For the P solubilization data, we used the chi-square statistic to obtain the exact p value. Analysis of adjusted standardized residuals was used to detect significant associations between halo size and experimental factors (niche, inoculation, soil type, and microcosm dilution), that were reported on a heat map.

Production of ICs was evaluated with a two-way ANOVA having niche (rhizospheric or endophytic) and soil type as factors, with separate tests for soil type, inoculation and microcosm dilution. Data were normalized by biomass (ICs production/OD_{600}) in each well of the plate.

To correlate the different variables measured, we used a Categorical Principal Component Analysis (CatPCA), an iterative process that evaluates non-linear correlations on ordinal and nominal variables. Inoculation treatment was split by diversity level and considered as a multiple nominal variable, while all other variables were considered ordinal. For the CatPCA, P solubilization was split by halo size (only small or large) and transformed to percentages of the total number of colonies evaluated.

All transformed averages and confidence intervals were back-transformed to report results. The statistical term “interaction” was often used, meaning that the effect of one factor of the two-way ANOVA depended on the second factor (such as the effect of inoculation depending on diversity level). Tests and plots were made using the software SPSS (IBM corp. 2010) and PAST (Hammer et al., 2001).

Results

Aiming to test the proposed model, in this work we evaluate if: (I) bacteria on rich soils were able to produce higher amounts of indolic compounds (ICs) than those in poor soils, while bacteria on poor soils were better phosphate solubilizers than those on rich soils; (II) if the endophytic bacteria would produce higher amounts of ICs than the

rhizospheric bacteria on rich soils, while in poor soils rhizospheric bacteria would produce higher amounts of ICs than the endophytic bacteria; (III) if phosphate solubilizers would be more frequent in the rhizosphere than in the endosphere on rich soils, while on poor soils they would be in similar frequencies; (IV) *Burkholderia* would be mostly a rhizospheric bacterium that better exerted its PGP effects in poor soils, while *Enterobacter* would be mostly an endophytic bacterium that better displayed its PGP effects in poor soils; and finally (V) if the bacterial ICs production ability increased, as bacterial phosphate solubilization ability decreased and vice-versa. To achieve all these objectives, 792 seedlings were planted in 264 pots distributed in 24 trays. All plants had both their rhizospheric and endophytic bacterial populations sampled. Survival curves and colonization percentages were based on 648 and 612 plated dilutions, respectively. P solubilization was based on 2,058 randomly picked colonies, and ICs production was based on 432 plate well inoculations.

Plant biomass

As could be expected, rich soils developed larger plants than poor soils. In both soil types, the effect of the inoculant on plant biomass was dependent on the diversity level (interactions at $p<0.05$, $n = 99$ for poor soils; $n =132$ for rich soils)

In the poor soils, interactions were significant for shoot length and shoot dry weight, marginally significant for shoot length 15 days post-inoculation ($p=0.056$), but root length had only a diversity effect (Figure 1). There were no statistically significant differences between treatments and control, but the PB^3 condition consistently presented higher averages for all plant biomass measurements, always higher than the PC^3 condition. The *Burkholderia* strain in the PB^3 condition was then considered to be an effective plant growth promoter. In this perspective, the *Burkholderia* inoculation presented better results than the *Enterobacter* inoculation on poor soils, as suggested by the model. It is interesting to note, however, that the *Burkholderia* strain in the PB^1 and PB^6 conditions did not behave as a PGPB, and, sometimes, plants in those conditions were significantly smaller than plants in the PB^3 condition.

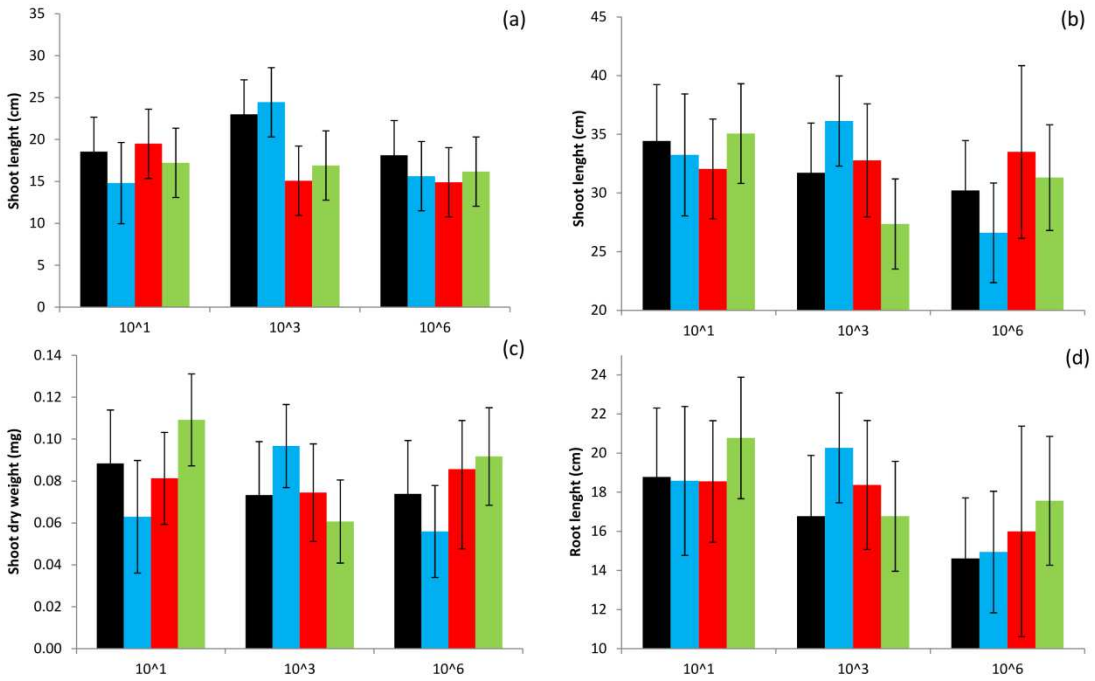


Figure 1: Plant biomass (average ±CI 95%) in poor soils, with different inoculation treatments and according to the microcosm diversity gradient. (a) Shoot length after 15 days (b) Shoot length after 30 days, (c) Shoot dry weight after 30 days, (d) Root length after 30 days. Red = *Enterobacter* inoculation, Blue = *Burkholderia* inoculation, Green = Mixed strain inoculation, Black = non-inoculated control

For the rich soils, interactions were significant for shoot length, root length, shoot dry weight, and shoot length 15 days post-inoculation (Figure 2). Both RE^6 and RM^6 conditions presented significantly larger plants than RC^6 and RB^6 conditions. In this perspective, the *Enterobacter* strain behaved better as a PGPB than the *Burkholderia* strain on rich soils, as was suggested by the model. It is interesting to note the effects of *Enterobacter* inoculation according to the diversity gradient, compared to the control: RE^1 condition reduced plant biomass, while RE^3 condition had no significant effect, but the *Enterobacter* strain in the RE^6 condition was an effective PGPB. Similarly, plants that were inoculated with a mix of both strains in the 10^3 microcosm had their biomasses reduced, but in the 10^6 microcosm the mix of strains displayed an effective PGPB effect.

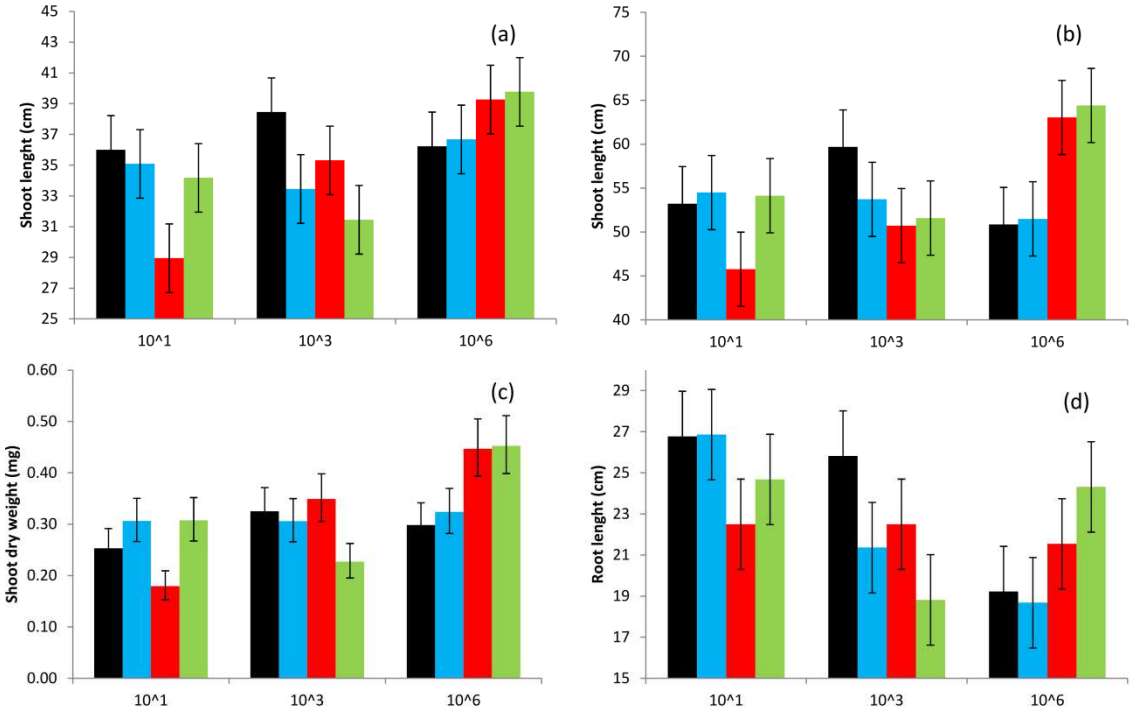


Figure 2: Plant biomass (average ±CI 95%) in rich soils, with different inoculation treatments and according to the microcosm diversity gradient. (a) Shoot length after 15 days, (b) Shoot length after 30 days, (c) Shoot dry weight after 30 days , (d) Root length after 30 days. Red = *Enterobacter* inoculation, Blue = *Burkholderia* inoculation, Green = Mixed strain inoculation, Black = non-inoculated control

Inoculant survival

All pots received approximately 9.5 log CFU as inoculant, and 24 h after inoculation we detected an average ± SD of 7.5±0.6 log CFU g^{-1} fresh top soil. The specific CFU count 24 h after inoculation of each treatment was used as the first time point to calculate the survival curves. In poor soils (n=18 survival curves), mixed strain inoculation affected strain survival depending on the diversity level (interaction $p<0.05$), but in rich soils (n=18 survival curves) strain survival was affected only by diversity. This means that diversity played a major role on inoculant survival, but this role was affected by mixed strain inoculation in poor soils. Raw CFU values are presented in Figure S1.

As can be observed in Figure 3a, the survival of *Burkholderia* strain in rich soils clearly decreased as diversity decreased, either when single or mixed with the *Enterobacter* strain. Mixed strain inoculation seemed to decrease the *Burkholderia* survival compared to the single strain inoculation, but this effect was not statistically

significant (p= 0.098). The survival of *Burkholderia* strain in rich soils was also better than on poor soils. In the poor soil condition, the survival of this strain was less affected by the diversity gradient, since survival only decreased in the 10^6 microcosm. However, the PM^6 condition had an effect on *Burkholderia* survival, increasing it instead of reducing it, such as was observed in RM^6, RB^6, and PB^6 conditions.

The survival of *Enterobacter* strain in rich soils (Figure 3b) presented a fluctuation according to the microcosm diversity gradient, as in the 10^3 microcosm it clearly showed the highest survival rates. To contrary to what was observed for the *Burkholderia*, mixed strain inoculation seemed to increase the *Enterobacter* survival compared to the single strain inoculation, but this effect was also not statistically significant (p= 0.058). On poor soils, single strain inoculation survival was very similar to rich soils, with the highest survival at the 10^3 microcosm. However, the PM^6 condition had an effect on *Enterobacter* survival, increasing it instead of reducing it, such as was observed in RM^6, RB^6, and PB^6 conditions.

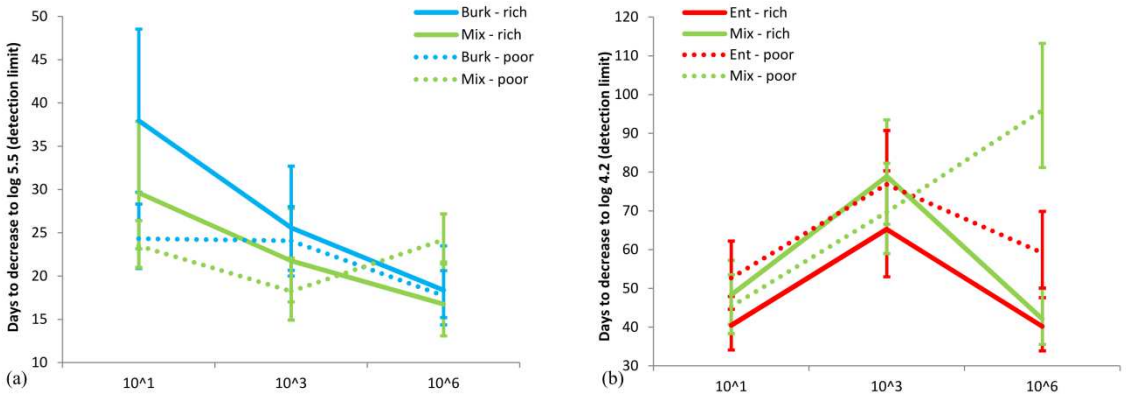


Figure 3: Inoculant survival, as in days (average ±CI 95%) necessary to decrease until the detection limit (5.5 log CFU g^{-1} for *Burkholderia* and 4.2 log CFU g^{-1} for *Enterobacter*), and according to the microcosm diversity gradient. (a) *Burkholderia*, (b) *Enterobacter*. Red = *Enterobacter* (Ent) inoculation, Blue = *Burkholderia* (Burk) inoculation, Green = Mixed (Mix) strain inoculation, full lines = rich soils, dashed lines = poor soils

Niche colonization by the inoculant

Burkholderia colonization was higher on top soil and rhizosphere than on endosphere (Figure 4a-b). Raw CFU values are presented in Figure S2. Colonization was not especially higher either when *Burkholderia* was an effective PGPB or when it was reducing biomass (Figures 1 and 2), except for endophytic colonization in RM^6

condition. On rich soils, lower survival (Figure 3a) correlated to lower top soil and rhizospheric colonizations (Figure 4a). On poor soils, this correlation was not observed. *Enterobacter* colonization was higher on the endospheric than on top soil and rhizospheric (Figure 4c-d) environments. In rich soils, *Enterobacter* endophytic colonization could reach up to 99% on some samples. Colonization did not seem especially higher either when this bacterium was acting as an effective PGPB or when plants inoculated with this strain presented a reduction on their biomass (Figure 1 and 2), but *Enterobacter* colonization correlated with survival. On rich soils, higher *Enterobacter* survival (Figure 3b) was correlated with lower endophytic colonization, and higher rhizospheric and top soil colonization (Figure 4c). On poor soils, higher *Enterobacter* survival (Figure 3b) was correlated to higher endophytic colonization, and lower rhizospheric and top soil colonizations (Figure 4d). All together, these data suggested that inoculant colonization was correlated to bacterial survival, but the occupied niche would depend on soil type and strain.

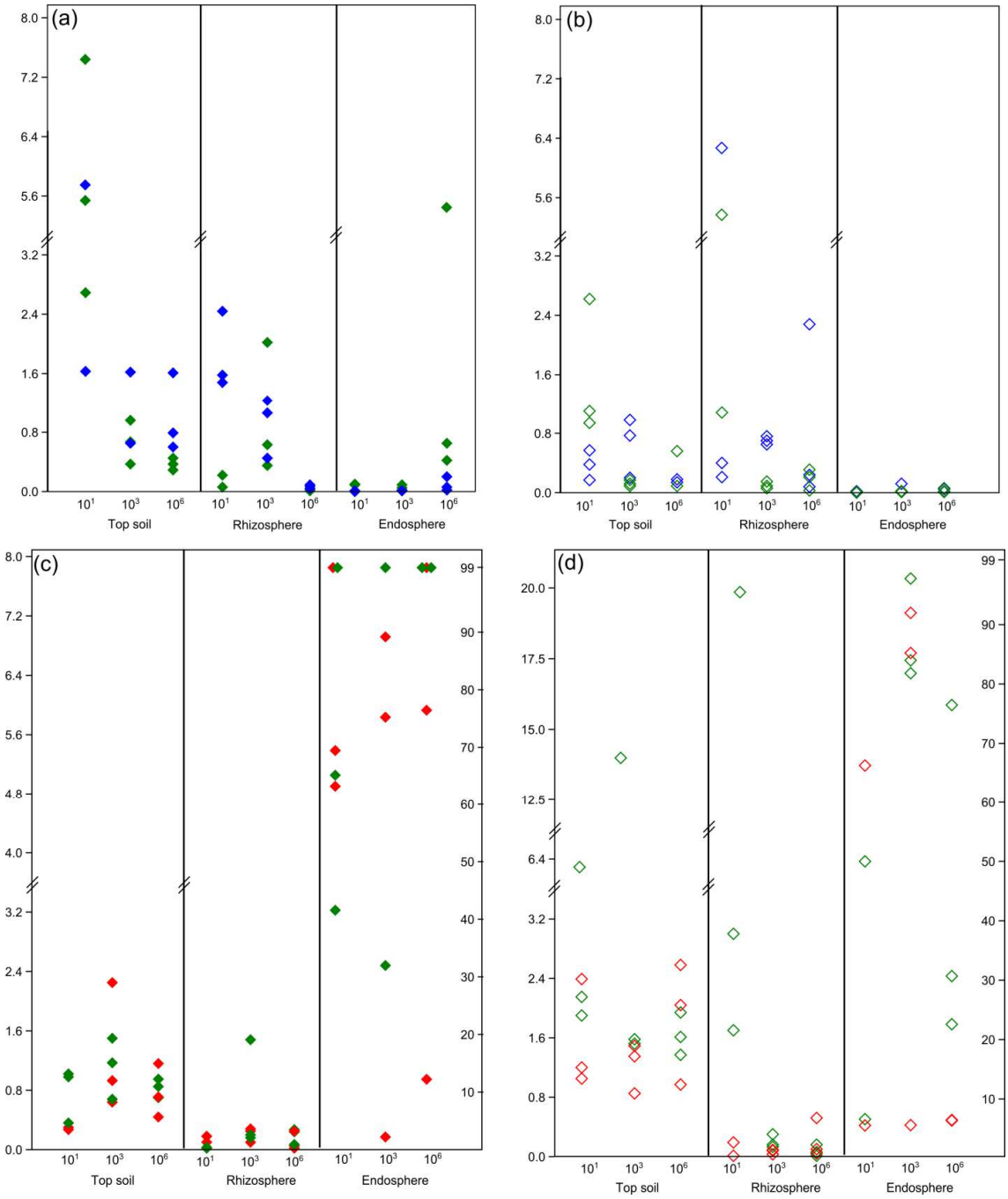


Figure 4: Inoculant colonization percentages in top soil, rhizosphere and endosphere and according to the microcosm diversity gradient, with one point for each sample.

(a) *Burkholderia* in rich soils, (b) *Burkholderia* in poor soils, (c) *Enterobacter* in rich soils and (d) *Enterobacter* in poor soils. Note that there is another scale for *Enterobacter* endophytic colonization. Red = *Enterobacter* inoculation, Blue = *Burkholderia* inoculation, Green = Mixed strain inoculation, full diamonds = rich soils, empty diamonds = poor soils

Phosphate solubilization ability of randomly selected cultivable bacteria

The cultivable bacterial community ability to solubilize tri-calcium phosphate was largely affected by soil type, niche, diversity level, and inoculation treatment. The heat map in Figure 5 shows how much the observed values deviated from the expected ones, which assume that there would be no association between halo sizes and factors of the experimental design. Sums of the adjusted residuals help us to evaluate the factors that most affected the bacterial P solubilization ability, as table cells with lower adjusted residuals represented counts closer to the expected values. A treatment with high frequency of non-solubilizers or low frequency of normal solubilizers was considered to present low P solubilization activity, while a treatment that had low frequency of non-solubilizers and showed an excess of normal and/or good solubilizers was considered to have high P solubilization ability.

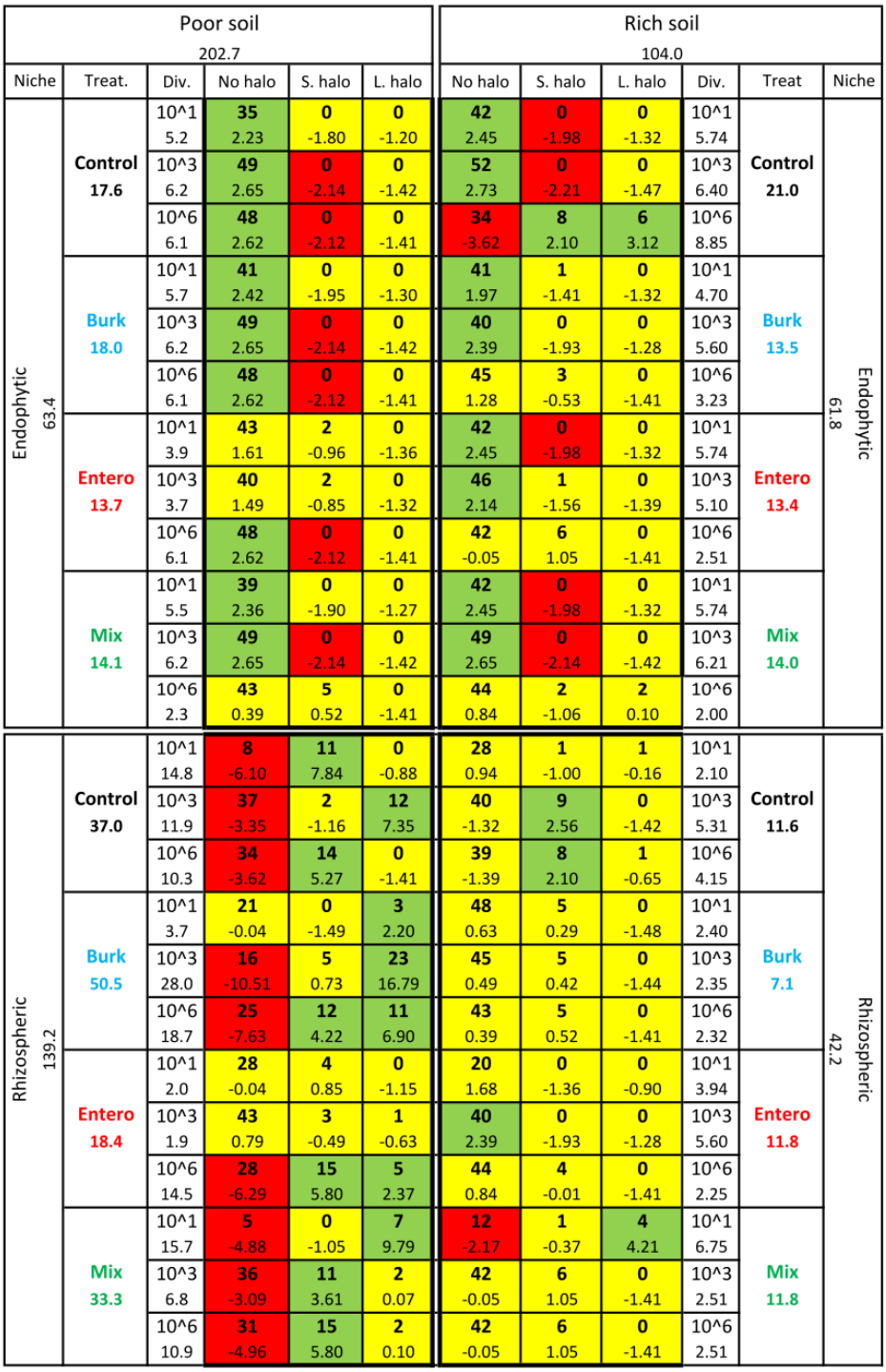
Firstly, it could be observed that poor soils presented a higher number of colonies with P solubilization ability than rich soils, especially the solubilizers with larger halos ("good" solubilizers). Looking at the sum of adjusted residuals from soils, it was possible to observe that the poor soil condition affected the bacterial P solubilization ability more than the rich soil condition (sums of 202.7 and 104.0, respectively).

Secondly, it could be observed that bacterial P solubilization ability was higher on the rhizosphere than on the endosphere, on both soil types. Sum of adjusted residuals showed that the endophytic niche affected P solubilization at a similar level in the different soils (sums of 63.4 and 61.8, for poor and rich soil conditions, respectively), while the rhizospheric niche effect was strikingly different between both soils (sums of 139.2 and 42.2, for poor and rich soil conditions, respectively). This means that P solubilization was affected by both soil type and niche, but the difference between the soils was only clear on the rhizospheric environment (Figure 5).

Regarding bacterial inoculation, P solubilization ability was most affected in the *Burkholderia* single strain treatment, increasing it on the rhizosphere of plants cultivated in poor soil condition. This was the only treatment that had an excessive number of good P solubilizers on all three diversity levels, and it deviated from expected values much more than any other treatment (sum of 50.5). It is worth noting that the rhizospheric P solubilization ability increased by *Burkholderia* inoculation was not observed in rich soils, and was independent of *Burkholderia* colonization (Figure 4a-b). The *Enterobacter* treatment, on the other hand, caused the smallest changes on the bacterial P solubilization ability, having the lowest frequency of either normal or

good solubilizers. The mixed strain treatment presented P solubilization ability between those of *Burkholderia* and *Enterobacter* single strain inoculations. Control conditions had P solubilization ability similar to treatments in poor soils, but not in rich soils. RC^6 condition was the only one with an excessive number of P solubilizers in the endosphere, while RC^3 and RC^6 conditions were the only ones that presented an excessive number of strains presenting small halo of P solubilization in the rhizosphere.

Inoculation only increased bacterial P solubilization ability on poor soils, where according to our model this trait was supposed to be more important. Our model expected, however, that P solubilization ability would be similar among rhizospheric and endophytic communities in poor soil condition. It is interesting to notice that PB^3 condition, which was the condition that exhibited an effective PGP effect in poor soils, presented the highest number of strains with large halos of P solubilization in the whole experiment. However, the effective PGP treatments in rich soils (RE^6 and RM^6) did not present any expressive P solubilization ability, unlike the endophytic bacteria on RC^6.



Poor soil 202.7						Rich soil 104.0					
Niche	Treat.	Div.	No halo	S. halo	L. halo	No halo	S. halo	L. halo	Div.	Treat	Niche
Endophytic 63.4		10^1	35	0	0	42	0	0	10^1		Endophytic 61.8
		5.2	2.23	-1.80	-1.20	2.45	-1.98	-1.32	5.74		
	Control 17.6	10^3	49	0	0	52	0	0	10^3	Control 21.0	
		6.2	2.65	-2.14	-1.42	2.73	-2.21	-1.47	6.40		
		10^6	48	0	0	34	8	6	10^6		
		6.1	2.62	-2.12	-1.41	-3.62	2.10	3.12	8.85		
		10^1	41	0	0	41	1	0	10^1		
		5.7	2.42	-1.95	-1.30	1.97	-1.41	-1.32	4.70		
	Burk 18.0	10^3	49	0	0	40	0	0	10^3	Burk 13.5	
		6.2	2.65	-2.14	-1.42	2.39	-1.93	-1.28	5.60		
		10^6	48	0	0	45	3	0	10^6		
		6.1	2.62	-2.12	-1.41	1.28	-0.53	-1.41	3.23		
		10^1	43	2	0	42	0	0	10^1		
		3.9	1.61	-0.96	-1.36	2.45	-1.98	-1.32	5.74		
	Entero 13.7	10^3	40	2	0	46	1	0	10^3	Entero 13.4	
		3.7	1.49	-0.85	-1.32	2.14	-1.56	-1.39	5.10		
		10^6	48	0	0	42	6	0	10^6		
		6.1	2.62	-2.12	-1.41	-0.05	1.05	-1.41	2.51		
		10^1	39	0	0	42	0	0	10^1		
		5.5	2.36	-1.90	-1.27	2.45	-1.98	-1.32	5.74		
	Mix 14.1	10^3	49	0	0	49	0	0	10^3	Mix 14.0	
		6.2	2.65	-2.14	-1.42	2.65	-2.14	-1.42	6.21		
		10^6	43	5	0	44	2	2	10^6		
		2.3	0.39	0.52	-1.41	0.84	-1.06	0.10	2.00		
Rhizospheric 139.2		10^1	8	11	0	28	1	1	10^1		Rhizospheric 42.2
		14.8	-6.10	7.84	-0.88	0.94	-1.00	-0.16	2.10		
	Control 37.0	10^3	37	2	12	40	9	0	10^3	Control 11.6	
		11.9	-3.35	-1.16	7.35	-1.32	2.56	-1.42	5.31		
		10^6	34	14	0	39	8	1	10^6		
		10.3	-3.62	5.27	-1.41	-1.39	2.10	-0.65	4.15		
		10^1	21	0	3	48	5	0	10^1		
		3.7	-0.04	-1.49	2.20	0.63	0.29	-1.48	2.40		
	Burk 50.5	10^3	16	5	23	45	5	0	10^3	Burk 7.1	
		28.0	-10.51	0.73	16.79	0.49	0.42	-1.44	2.35		
		10^6	25	12	11	43	5	0	10^6		
		18.7	-7.63	4.22	6.90	0.39	0.52	-1.41	2.32		
		10^1	28	4	0	20	0	0	10^1		
		2.0	-0.04	0.85	-1.15	1.68	-1.36	-0.90	3.94		
	Entero 18.4	10^3	43	3	1	40	0	0	10^3	Entero 11.8	
		1.9	0.79	-0.49	-0.63	2.39	-1.93	-1.28	5.60		
		10^6	28	15	5	44	4	0	10^6		
		14.5	-6.29	5.80	2.37	0.84	-0.01	-1.41	2.25		
		10^1	5	0	7	12	1	4	10^1		
		15.7	-4.88	-1.05	9.79	-2.17	-0.37	4.21	6.75		
	Mix 33.3	10^3	36	11	2	42	6	0	10^3	Mix 11.8	
		6.8	-3.09	3.61	0.07	-0.05	1.05	-1.41	2.51		
		10^6	31	15	2	42	6	0	10^6		
		10.9	-4.96	5.80	0.10	-0.05	1.05	-1.41	2.51		

Figure 5: Heat map of associations of TCP solubilization in the whole experiment. The number on the top of each table cell shows the total number of colonies with each type of halo in TCP media, and the number on the bottom of each table cell shows the adjusted residual values, based on column and line totals. Numbers below the diversity (Div,) levels, inoculation treatment (Treat.), niche and soil type (poor or rich) show the total sum of adjusted residuals, which represent how much each factor affects the distribution of P solubilizing bacteria. Red cells = less colonies than expected under

those conditions, green cells = excessive number of colonies under those conditions, and yellow cells = no significant differences between the observed and expected values (less then 1.96 adjusted residuals). Only some table cells containing zero colonies with small halos are highlighted because of differences in line totals (as is the case of endophytic PB^1 and PB^3). S = small halo; L = large halo

Bacterial community ICs production ability

Figure 6 shows the production of ICs by bacterial communities in the course of the experiment. The serial dilutions used for CFU counting were used as inoculums for the indicator media. Statistical tests were performed between rhizospheric and endophytic samples of poor and rich soils, with separate tests for different microcosm dilutions and inoculations (n = 42 to 62, depending on outliers and detection limits). There were significant interactions between soil and niche at the 10^1 and 10^3 microcosms on different treatments, but not on the 10^6 microcosms.

Production of ICs dropped with the diversity reduction, likely due to the extinction of species by progressive dilution. This reduction occurred at both soil types, and was bigger for endophytic bacteria. On rich soils, however, this drop was less intense for the mixed treatment, and was not present in the control, where ICs productivity fluctuated. Inoculated treatments seemed to produce lower amounts of indolic compounds than the control, especially on rich soils.

In addition to the ICs production drop according to the diversity gradient, it could also be observed that rich soils communities produced more ICs than poor soils communities, and endophytic bacteria produced higher amounts of ICs than rhizospheric bacteria. This pattern was slightly different on the 10^6 microcosm, and it was also different on the control treatment at the 10^1 microcosm. Indeed, our model expected that bacterial ICs production ability would be higher in rich soils than in poor soils, but the effect of the niche did not support the model. We expected that the endophytic bacterial communities would produce higher amounts of ICs than the rhizospheric bacterial communities in rich soils, and that the rhizospheric bacterial communities would produce higher amounts of ICs than endophytic bacterial communities in poor soils.

Higher production of ICs did not explain the effective PGP effects of the strains. Production of ICs by PB^3 treatment was statistically similar to PC^3 treatment at both niches, while for RM^6 treatment this trait was similar to RC^6 treatment at the

endosphere, but lower on the rhizosphere. For RE[6] treatment, the production of ICs was actually lower than the control at both niches.

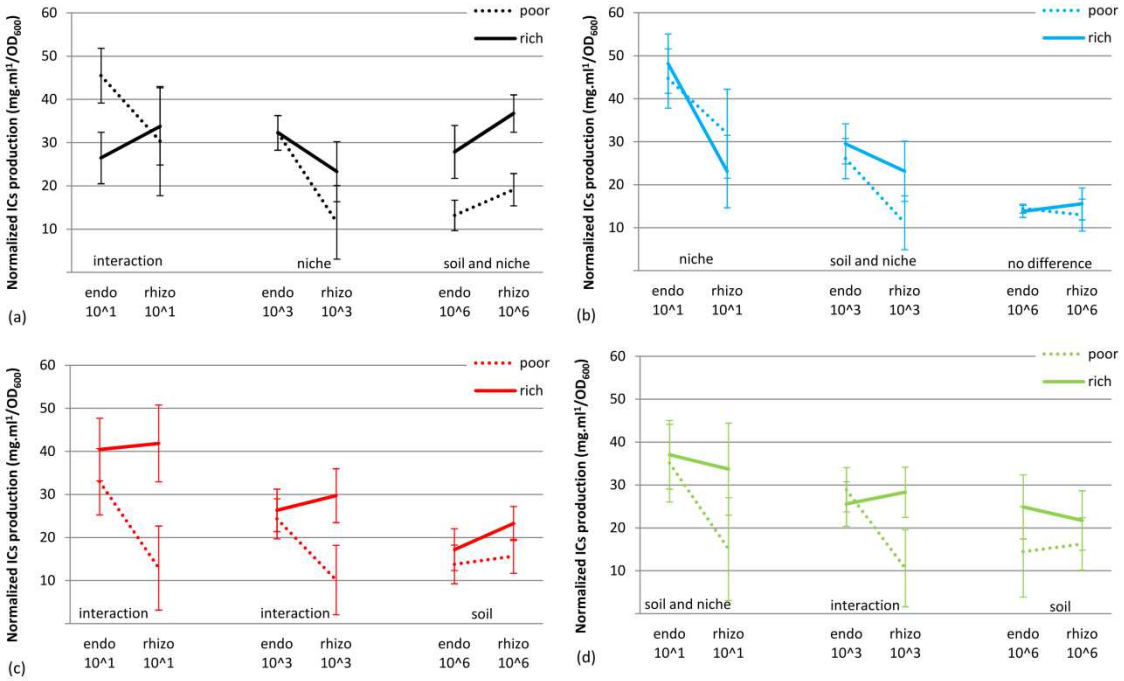


Figure 6: Indolic compounds production by endophytic (endo) and rhizospheric (rhizo) populations in poor and rich soils (average ±CI 95%), according to the microcosm diversity gradient and separated by inoculation treatment. Factors that significantly affected ICs production are shown above the niches, with interaction meaning that the effect of the niche depends on the soil type. (a) Control, (b) *Burkholderia*, (c) *Enterobacter* (d) Mixed strain inoculation; full lines = rich soils, dotted lines = poor soils

Comparisons between the phosphate solubilization and ICs production abilities

Neither ICs production nor P solubilization abilities fully followed the model at the niche level, but when these traits were simultaneously analyzed the predicted correlation was observed. It was expected that bacterial communities with high phosphate solubilization ability will not simultaneously present a high ICs production ability, and vice versa. This correlation can be clearly observed when we merged the inoculation treatments and focalized on the diversity levels, keeping in mind that when the ICs production ability shifted in one direction, the P solubilization ability shifted to the other direction.

On the 10^1 microcosm, the bacterial ICs production was lower in the rhizosphere than in the endosphere, especially for poor soils (Figure 7, left), while P solubilization

ability was higher in the rhizosphere than in the endosphere, especially for poor soils (Figure 8, left). This pattern was repeated on the 10^3 microcosm. In rich soils, bacterial ICs production (Figure 7, center) and P solubilization (Figure 8, center) were quite similar across niches. In the poor soils, however, difference on ICs production due to niche was very high (Figure 7, center), and so was the difference in proportion of large halo P solubilizers due to niche (Figure 8, center). The 10^3 microcosm showed that when differences in ICs productions were smaller, differences in P solubilization were also smaller, and when differences in ICs production were bigger, differences in P solubilization were also bigger. The 10^6 microcosm showed a different behavior on the pattern for both traits. While on rich soils the bacterial ICs production was slightly smaller for endophtytic bacteria (Figure 7, right), P solubilization was actually higher for endophtytic bacteria (Figure 8, right). On poor soils, ICs production was also slightly smaller for the endophytic bacteria, while P solubilization ability compared to the other poor soil microcosms was the lowest for rhizospheric bacteria and the highest for endophytic bacteria.

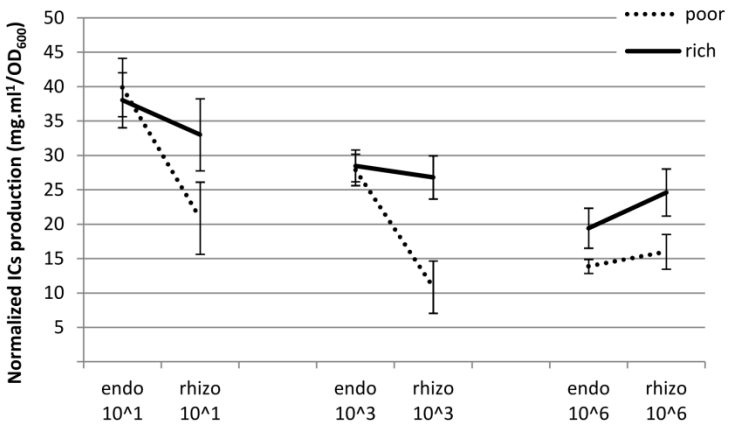


Figure 7: Indolic compounds production by endophytic (endo) and rhizospheric (rhizo) populations in poor and rich soils (average ±CI 95%), according to the diversity gradient with 10^1 microcosm (left), 10^3 microcosm (middle) and 10^6 microcosm (right). Full lines = rich soils, dotted lines = poor soils

Div.	Soil	Niche	No halo	Small halo	Large Halo	Total
10^1	Rich	Endo	167 3.8	1 -2.7	0 -2.7	168 9.2
		Rhizo	108 -3.8	7 2.7	5 2.7	120 9.2
		Total	275	8	5	288
	Poor	Endo	158 6.6	2 -4.7	0 -4.4	160 14.7
		Rhizo	62 -6.6	15 4.7	10 4.4	87 14.7
		Total	220	17	10	247

Div.	Soil	Niche	No halo	Small halo	Large Halo	Total
10^3	Rich	Endo	187 4.3	1 -4.3		188 8.6
		Rhizo	167 -4.3	20 4.3		187 8.6
		Total	354	21		375
	Poor	Endo	187 7.9	2 -4.1	0 -6.5	189 18.5
		Rhizo	132 -7.9	21 4.1	38 6.5	191 18.5
		Total	319	23	38	380

Div.	Soil	Niche	No halo	Small halo	Large Halo	Total
10^6	Rich	Endo	166 -.3	18 -.8	8 2.4	192 3.5
		Rhizo	168 .3	23 .8	1 -2.4	192 3.5
		Total	334	41	9	384
	Poor	Endo	187 8.7	5 -7.1	0 -4.3	192 20.1
		Rhizo	118 -8.7	56 7.1	18 4.3	192 20.1
		Total	305	61	18	384

Figure 8: Heat maps of associations of TCP solubilization ability of endophytic (Endo) and rhizospheric (Rhizo) communities, and according to the soils and the microcosm diversity (Div.) gradients. 10^1 microcosm (left), 10^3 microcosm (middle) and 10^6 microcosm (right). The numbers on top of each cell show the total number of colonies with each type of halo in TCP medium, and the numbers on the bottom of each cell show the adjusted residual values. Red cells = less colonies than expected under those conditions, green cells = excessive number of colonies than expected under those conditions, and yellow cells = no significant differences between the observed and expected values (less than 1.96 adjusted residuals)

Correlations of all variables – Categorical Principal Component Analysis

Multivariate statistics can help to find patters in complex datasets, by reducing dimensionality. To correlate all of our variables at once, a Categorical Principal Component Analysis (CatPCA) was used. The CatPCA allows the exploration of non-linear correlations, through ordinal and nominal variables.

Figure 9a shows the CatPCA for poor soils. It can be observed that plant biomasses increased on positive values of the first dimension, with root length slightly apart from the shoot measures and closer to higher values of ICs production. The shoot biomass measures were close to higher *Burkholderia* survival and colonization, and also to the occurrence of strains displaying large halos of P solubilization in the rhizosphere. These correlations might suggest that shoot biomass increased because of an improved P solubilization ability, caused by *Burkholderia* inoculation, as seen on Figure 5. As microcosm dilution increased (that is, as diversity decreased) ICs production decreased, as explored in Figures 6 and 7, and strains displaying small halos of P solubilization were more frequent, as shown on Figure 8. Finally, it can be seen that *Enterobacter* survival, while correlated to endophytic colonization, was opposed to *Enterobacter* colonization of the top soil and rhizosphere.

Figure 9b shows the CatPCA for rich soils. It can be observed that root length was quite separated from the shoot biomass parameters. It was more highly correlated to ICs production and good phosphate solubilization on the rhizosphere, while shoot biomass was closely correlated to higher endophytic colonization by both strains. Strain survival and colonization of the rhizosphere and top soil were opposite to plant growth and endophytic colonization. Shoot biomass was also closely related to RE^6 and RM^6 treatments - the effective PGPB in rich soils. Endophytic P solubilization was almost exclusively associated to RC^6 condition, as seen on Figure 5, and it was opposite to root length. The microcosm dilution vector was directly opposite to large halos in rhizospheric P solubilization, meaning that as diversity dropped the good P solubilizers in the rhizosphere were less present, which is also seen on Figure 8.

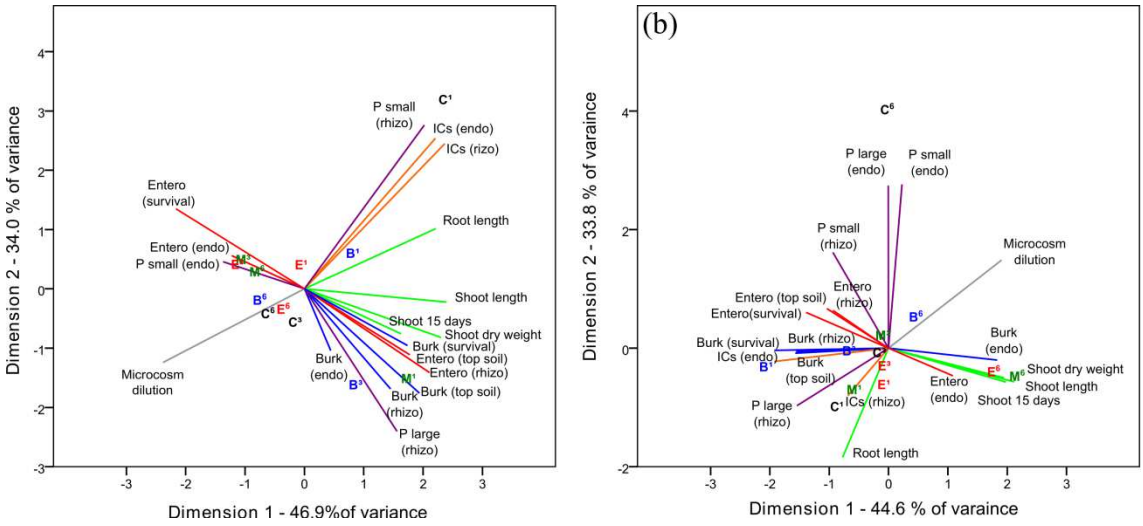


Figure 9: Categorical Principal Component Analysis (CaPCA) of all variables evaluated in poor (a) and rich (b) soils. Each line represents a variable that increases in value from the center, and longer lines explain more variance. Each letter represents the centroid of triplicates from each strain inoculation and microcosm dilution treatments. Axis show how much of the variance of the transformed variables is explained. Green lines = plant biomass measurements, Blue lines = *Burkholderia* (Burk) colonization and survival, Red lines = *Enterobacter* (Entero) colonization and survival, Purple lines = P solubilization, Orange lines = ICs production, Grey line = microcosm dilution. Black letters = Control, Blue letters = *Burkholderia* (B), Red letters = *Enterobacter* (E), Green letters = Mixed (M) strain inoculation

Discussion

After the analyses of all results we concluded that the model had several of its hypothesis confirmed, although it failed for others. We were able to show that the *Burkholderia* strain was a better PGPB in poor soils, while the *Enterobacter* strain was a better PGPB in rich soils. We were also able to show that ICs production and bacterial P solubilization abilities are inversely correlated. We confirmed that bacterial communities on rich soils were able to produce higher amounts of ICs than those on poor soils, and that bacterial communities on poor soils displayed better P solubilization ability than those on rich soils. However, the occupation of these traits by niche did not follow the model's prediction. In addition, we were able to observe that colonization and survival were correlated to the analyzed PGP traits and effective PGP effect in different ways, depending on soil, niche, and diversity. As far as we know, this is the first dilution-to-extinction microcosm experiment that test different PGPB, although some works explore plant reactions to bacterial diversity manipulations (Bonkowski and Roy, 2005; Tkacz et al., 2015; Becker et al., 2012). Simultaneous correlations of bacterial survival, colonization, P solubilization and ICs production abilities after inoculation were difficult to find in the literature.

Enterobacter has a diversity-dependent PGP effect: plant biomass reduction on high diversity and plant biomass increase in low diversity

Enterobacter asburiae has already been reported to produce quorum sensing molecules (Lau et al., 2013), to degrade pesticides, to solubilize P, to produce indolic compounds, (Abraham and Silambarasan 2015), to alleviate heavy metal toxicity in soybean (Kang et al., 2015), and to promote plant growth in mung bean (Zhao et al., 2011). It has already been isolated from lettuce (Lau et al., 2013) and from different soils (Abraham and Silambarasan, 2015; Zhao et al. 2011). Here we show that an *Enterobacter asburiae* strain can either promote or reduce rice growth, largely colonizes the plant endosphere, and had its survival and plant interactions affected when it was co-inoculated with a *Burkholderia* strain.

Enterobacter inoculation on rich soils demonstrated a clear diversity-dependent PGP effect: plants in the RE^1 condition presented biomasses lower than the control, those in the RE^3 condition presented biomasses similar to the control, and those in the RE^6 condition presented biomasses higher than the control (Figure 2). There are many *Enterobacter* strains that can be pathogenic to plants (Toth et al., 2006), just as there are many that are efficient PGPB (Singh et al., 2011). The same PGPB strain can show both behaviors, depending on the environmental conditions. Chanway et al. (2000) showed

that three different *Pseudomonas* inoculants improved spruce seedlings biomass in one location, but reduced plant biomass in another. In a previous work (Costa et al., 2012) the same *Burkholderia* strain used here increased plant biomass on a zero fertilization condition, but reduced plant biomass in a light fertilization condition. Although plant biomass is known to increase with increasing bacterial diversity (Weidner et al., 2015) we could not find other works testing a PGPB over a diversity gradient, so we do not know how common is this diversity-dependent PGPB effect. It is wrong, however, to consider that PGPB in general reduce plant biomass in high bacterial diversities, as there is much evidence showing that PGPB can improve plant biomass in natural soils (Berg et al., 2013).

Niche occupation theory could explain this diversity-dependent PGPB effect by considering that an invasion of *Enterobacter* under more intense competition (more diversity and less free niches) could trigger more selfish responses towards the plant interaction. For example, the strain could be colonizing the plant and consuming the root exudates for energy without providing benefits to the plant. In a condition under lower competition (less diversity and more free niches) *Enterobacter* could trigger more beneficial responses towards the plant interaction, in a positive feedback provided by lower stress. Drastic changes in the behavior of a strain, including display of pathogeny, could happen due to quorum sensing mechanisms (Bodman et al., 2003), which are sensible to strain survival (van Elsas et al., 2007). However, data on strain survival, our best measure of strain stress in the microcosms, does not agree with this. *Enterobacter* survival on RE^{6} condition is the same as on RE^{1} condition (Figure 3b), instead of having a higher survival, which would be characteristic of a lower stress. Likewise, *Enterobacter* survival on poor soils was almost identical to that on rich soils, but this diversity-dependent biomass effect was not present on poor soils (Figure 1). Furthermore, field trials in natural soils (without manipulation of diversity) using the same *Enterobacter* strain used in this work have already been effective (Costa et al., 2012). An alternative explanation to the diversity-dependent biomass effect is that an antagonist to *Enterobacter* positive PGP effect (and not necessarily antagonist to its survival) was extinguished on more diluted microcosms, which then allowed *Enterobacter* to display a positive PGP effect. Becker et al. (2012) showed that beneficial biocontrol effects can be suppressed in excessively antagonistic communities. These authors created communities with one to eight *Pseudomonas fluorescens* strains antagonistic to an oomycete causing root rot on alfalfa plants. The strains were also

antagonistic to each other, and protection to root rot and bacterial biomass decreased as more strains were added to the community.

Data on *Enterobacter* IC's production (Figure 6) might suggest that ICs production was too high on higher diversities, causing an indole-3-acetic-acid mediated pathogenic effect (Patten and Glick, 1996; Hardoim et al., 2008; Long et al., 2008). However, although ICs production in the RE^1 condition has higher than in the RC^1 condition, it was similar in RM^1 and RB^1 conditions, which did not present any biomass reduction effect. Excessive endophytic colonization (Figure 4c) is unlikely to be responsible for the effect either, because although RE^1 condition colonization was very high, RE^6 condition colonization was higher than in RE^3 condition, and *Enterobacter* also colonizes at high rates in RM^6 condition. Unsurprisingly, a multitude of factors should be responsible for this diversity dependent effect, but we present clear evidence that PGPB efficiency is strongly dependent on the native microbial community, as already widely reported (Owen et al., 2015; Berg et al., 2013).

For the mixed strain treatment, the reduction on biomass effect appeared only on the 10^3 microcosm, unlike the linear diversity-dependent effect of the single strain *Enterobacter* inoculation (Figure 2). This showed that the addition of *Burkholderia* interfered with *Enterobacter* plant interactions. Co-inoculation was expected to interfere on plant-bacteria interactions, but also to improve the PGP effect (Aung et al., 2013; Jarak et al., 2012). Differential survival is unlikely to explain the plant biomass reduction effect, because although the highest *Enterobacter* survival occurred on RM^3 condition, RE^3 condition also had a high survival (Figure 3), and no negative plant biomass effect. Plant biomass reduction did not occur when *Burkholderia* was inoculated as a single strain, either at higher or lower survival rates, so *Burkholderia* should not be responsible for this effect. Colonization and ICs production were not too high or too low to explain the reduction on plant biomass. Apparently, species interactions between the native community and the invading inoculant at the 10^3 microcosm were stressful to the plant.

Effective PGP effect by Burkholderia on poor soils depends on the improvement of P solubilization ability of the bacterial community

Burkholderia vietnamensis is a known PGPB, able to resist to several kinds of stresses, to solubilize P (Park et al., 2010), to fix atmospheric nitrogen, to produce ICs, and to produce quorum sensing molecules (Suárez-Moreno et al., 2012). It has already been isolated from sugarcane (Govindarajan et al., 2006), maize (Suárez-Moreno et al.,

2012), and rice (Govindarajan et al., 2008; Van et al., 2000), and it is also able to improve plant growth of these crops. It colonizes both endosphere and rhizosphere, but its co-inoculation with other strains might be not as efficient as single strain inoculation (Govindarajan et al., 2006). Here we showed that a *Burkholderia vietnamensis* strain was able to improve rice growth, favored rhizospheric colonization, and had its survival and plant interactions affected by the co-inoculation with an *Enterobacter* strain. We also showed that its PGP effect may depend on the alteration of the microbial community, as discussed below.

Plants in the PB^3 condition were considered to show an effective PGP effect, for presenting overall higher averages on plant biomass than those in the PC^3 condition (Figure 1). Figure 9a suggested that shoot biomass was correlated to *Burkholderia* colonization, survival and to the occurrence of colonies with large P solubilization halos. Figure 5 confirmed that the PB^3 condition had the highest P solubilization values. Thus, it seems that *Burkholderia* invasion on poor soils improved P solubilization, and this improved plant biomass. This result agrees strongly with the model (Costa et al., 2014) and many other reports that demonstrated that bacterial P solubilization is higher on poor soils (Wakenlin et al., 2009; Jakobsen et al., 2005; Hu et al., 2009; Matsuoka et al., 2013), and that poorer nutrient conditions optimize the plant-bacteria interactions (Güneş et al., 2014; Costa and Passaglia, 2015; Bonkowski and Roy, 2005; Tkacz et al., 2015; Lapsansky et al., 2016).

The logical conclusion would be that *Burkholderia*, a good phosphate solubilizer, was largely present on PB^3 condition, and thus improved P solubilization. But data regarding strain colonization (Figure 4b) showed that this was not true. It can be observed that the *Burkholderia* strain on PB^3 condition colonized approximately 0.7% of the rhizosphere, while the large halo P solubilizers composed approximately 47% of the total rhizospheric LB colonies screened in the indicator medium (Figure 5). This means that the P solubilization in PB^3 condition was not improved because of the rhizospheric abundance of the inoculated *Burkholderia* itself, but instead that the invasion of this strain altered the microbial community and this alteration improved the general P solubilization ability of the bacterial community. Large halo P solubilizers compose 16 and 23% of the screened rhizospheric colonies in PB^1 and PB^6 conditions, respectively, while the rhizosphere colonization of the *Burkholderia* strain was of 2.5 and 0.8% respectively. Besides showing that this colonization-solubilization correlation was non-linear, these proportions showed that the improvement on P solubilization

ability was dependent of the starting microbial community. If *Burkholderia* invasion cannot stimulate the recruitment of P solubilizers from the initial community, P solubilization will not improve. This behavior was observed in rich soils, which have less P solubilizers but similar *Burkholderia* colonization. If *Burkholderia* was improving P solubilization because of its own abundance, rhizospheric colonization in RB^{1}, RB^{3} and RB^{6} conditions, of 1.8, 0.9 and 0.1 %, respectively (Figure 4a), would not have a complete absence of large halo P solubilizers (Figure 5). Likewise, survival of *Burkholderia* (Figure 3) did not explain the improvement on P solubilization: on rich soils, *Burkholderia* survival decreased while P solubilization was stable, and on poor soils, *Burkholderia* survival was stable as P solubilization fluctuated.

This independence of P solubilization improvement in the rhizosphere in relation to strain colonization and survival help us to explain why bacterial inoculation sometimes is effective and sometimes is not, even if the inoculant is present on the soil and colonizes the plant. If an inoculant invasion cannot impact the population in a way useful to the plant, the PGP effect not might occur. In soil systems, sub communities of different bacteria may cooperate for better survival or to degrade a substrate in syntrophy, especially in poor nutrient conditions (Zelezniak et al., 2015). The invasion of the *Burkholderia* strain might be responsible for organizing such sub communities with improved P solubilization ability, even if the strain itself is not so present in the rhizosphere. It has already been proposed that the alteration of the rhizosphere community could be a PGPB trait (Barea et al., 2005); as such alterations can be correlated to effective PGPB effect (Ramos et al., 2003). Addition of microorganisms in degraded soils might break the dormancy of resident bacteria, recovering lost or attenuated functions on degraded soils (Seneviratne and Kulasooriya, 2013). Recovery or improvement of P solubilization in our experiment could be occurring in this fashion.

Biotic and abiotic factors affecting survival of the inoculant

Survival of the bacterial strains was expected to increase as diversity lowers, but this was not observed. For the *Burkholderia* strain in rich soils (Figure 3a), two hypothesis can explain its lower survival on lower diversity microcosms. One is that an antagonist to the inoculated strain, highly abundant on the natural soil, became increasingly more dominant on the microcosm as the more rare species where extinguished by dilution. It is possible that a native *Burkholderia,* which can be very common in soil (Ahemad and Kibret, 2014; Kunito et al., 2011) actually increased a specific competition or direct antagonism to the inoculated strain. Bacteria of

Burkholderia genus have large genomes (Parke and Gurian-sherman, 2001) and flexible metabolisms capable of exploring a variety of niches (Coenye and Vandamme, 2003), making it a good competitor. *Burkholderia* strains can be antagonistic to other *Burkholderia* strains as well (Lin et al., 2011; Marshall et al., 2010), and the native strain should be more adapted to the local conditions than the invasive inoculated strain. The other hypothesis would be that the inoculated *Burkholderia* needed a basic sub community network providing specific ecological functions or cooperative metabolism to effectively survive. Occurrence of such sub communities with such effect is common in soils (Zelezniak et al., 2015). Once such sub community is weakened by diversity loss, *Burkholderia* survival could drop as well. It should be noted that such functional loss should be quite specific, as general functional traits (ammonia oxidizers, denitrifiers, and heterotrophs) are not affected in a dilution-to-extinction approach (Wertz et al., 2006).

On poor soils, the diversity effect on *Burkholderia* survival was not as clear. Although there was a reduction on survival in the lowest diversity microcosm, there was no difference on survival between PB^1 and PB^3 conditions (Figure 3a). It can be observed that the highest survival on poor soils was statistically similar to the lowest survival on rich soils. Since poor soils are expected to have less diversity than rich soils, the 10^1 microcosm on poor soil might have already compromised the sub community that does not allow higher *Burkholderia* survival, and such sub community became more compromised only on the 10^6 microcosm. The alternative, that specific antagonists prevent the *Burkholderia* strain survival, can also be in effect. *Burkholderia* are typical for poor soil conditions (Parke and Gurian-Sherman, 2001; Granada et al., 2013), and could have large populations on the natural soil. Furthermore, the natural poor soil used in the experiment (sandy forest soil with no vegetal cover) had more stressful abiotic conditions than the soil from where the *Burkholderia* strain utilized in this work was originally isolated (farm clay soil with no chemical fertilizers). In addition, conditions with less nutrients are less invasible (Kassen et al., 2000), and this could affect the inoculant survival. Thus, the native microbial community, which was already living in harsh conditions, might be competing very strongly with the invading *Burkholderia* from the start.

The *Enterobacter* strain made use of the free new niches available to increase its survival from the 10^1 to the 10^3 microcosm in both soils (Figure 3), as was expected (van Elsas et al., 2012; Mallon et al., 2015). However, its survival did not keep

increasing from the 10^3 to the 10^6 microcosm, and instead dropped to the same levels of the 10^1 microcosm. This effect was consistent as it occurred in both soil types. It could have been caused by specific interactions between the native community and the inoculant. Perhaps a minimal sub community necessary for *Enterobacter* survival was compromised on the 10^6 microcosm, or an antagonist present in the soil overcame a threshold, and could then suppress the *Enterobacter*.

K and r strategies can explain survival changes in mixed co-inoculation

The mixed inoculant was expected affect survival, as both strains could compete for the same niches and nutrients of the top soil sampled to determine the survival curves. In the rich soils, mixed inoculation was improving *Enterobacter* survival while reducing *Burkholderia* survival, if we consider single strain inoculation as a reference (Figure 3). It could be considered that *Enterobacter* was selfishly exploiting the presence of *Burkholderia*. According to the model, *Enterobacter* was expected to be more fit than *Burkholderia* in rich conditions, and it might be quicker to make use of the impact caused by invasion (such as suppression of an antagonist or competitor to *Enterobacter*), even if the impact was actually caused by *Burkholderia*. Considering the two-sided nature of the model and the behaviors of *Burkholderia* and *Enterobacter*, K-/r- strategies concepts could be applied here. The *Enterobacter* strain could be described as a quick-growing, generic and opportunistic r strategist adapted to rich soils and easily accessible substrates, with high invasion potential. The *Burkholderia* strain could be described as a slow growing, specialist K strategist resistant to stress, with lower colonization ability. The K-/r- strategies concepts have already been applied to the rhizosphere (van Elsas et al., 2007), considering that r strategists would be quite typical for the rhizosphere of younger plants and K strategists more typical to older plants (Garbeva et al., 2004). Haichar et al. (2008) studied root colonization in different plants by different taxa and described *Enterobacter* as a generalist and *Burkholderia* as a specialist, supporting our own classification. The K-/r- concept has long been applied on invasion ecology, with r strategists as a more typically successful invader than K strategists (Facon et al., 2006). While it could be argued that the *Burkholderia* strain used in this work should have a better survival on poor soils to be considered a K strategist, it must be considered that this *Burkholderia* was more outside of its original environment than the *Enterobacter* strain, and that the poor soils had a local community adapted to its specific conditions.

The PM^6 condition improved the survival of both strains, but especially the *Enterobacter*, allowing it to further exploited free niches. The mixed co-inoculation could make up for diversity or ecological functions loss, such as restoration of productivity by inoculation of biofilmed biofertilizers on degraded soils (Seneviratne and Kulasooriya, 2013), although the addition of only two strains might not generate consistent functional recovery. Considering *Enterobater* as an r strategist, it could be expected that it made better use of changing conditions and free niches than the K strategist *Burkholderia* (Facon et al., 2006).

Colonization correlates to survival in different ways, depending on plant selection

The favoring of niches for colonization shown by the strains – rhizospheric for *Burkholderia* and endophytic for *Enterobacter* (Figure 4) was already expected. According to the model, *Enterobacter* isolates are supposed to be mostly endophytic, while *Burkholderia* isolates are supposed to be mostly rhizospheric (Costa et al., 2014). Furthermore, the *Burkholderia* strain used in this work was isolated from the rhizosphere, while the *Enterobacter* strain was isolated from the endosphere (Costa et al., 2012). Nonetheless, it was surprising to see the extent of *Enterobacter* colonization of the root tissues, although it was already known that the endosphere can hold a large bacterial population (Dong et al., 2003; Hardoim et al., 2008). This favoring of niches was also observed by Dong et al. (2003), where the best endosphere colonizer was the worst rhizosphere colonizer, and the best rhizosphere colonizer was the worst endosphere colonizer.

Enterobacter endophytic colonization presented an interaction effect between soil and survival. Endophytic colonization by *Enterobacter* was positively correlated with its survival on poor soils (Figure 9a), but on rich soils this correlation was negative (Figure 9b). This can be explained partly by the model. On rich soils, the plant is more receptive to *Enterobacter* and its PGP traits (Costa et al., 2014), and could use its exudates to attract *Enterobacter*-types. Plants use complex communication and protection systems to attract and manipulate bacteria, and are often colonized for only a selected portion of the total soil community (Hartmann et al., 2008). When strain survival on the top soil was lower while the plant actively offered a safe niche plenty of nutrients, *Enterobacter* would be more intensively attracted to the roots, leading to high colonization rates, in a “colonize-or-die” scenario. On higher strain survival, *Enterobacter* made better use of nutrients available in the top soil, and colonization was not as high because the strain was not so attracted to root exudates. Bacterial motility is

an important trait for rhizosphere competence (Hardoim et al., 2008), and bacteria might not be as mobile on high nutrient conditions (Mitchell and Kogure, 2006; Matz and Jürgens, 2003; Turnbull et al., 2001). If there was a lower pressure for *Enterobacter* to swarm to the plant roots due to easier access to local nutrients, colonization could be expected to drop, in a "colonize-after-lunch" scenario.

On poor soils, the plant was not as receptive to *Enterobacter*-types as it was on rich soils, since endophytic colonization on poor soils was not as high as on rich soils (Figure 4). This was expected by the model (Costa et al., 2014), although endophytic ICs producers were selected in poor soils (Figure 6c), contrary to what the model expected. When *Enterobacter* had a lower survival rate in poor soils (Figure 3b), endophytic colonization was lower (Figure 4d), suggesting that it was a less fit PGPB with lower rhizosphere competence in that situation. When *Enterobacter* had a higher survival rate in poor soils, it had higher numbers and could have more attempts to infect the roots, colonizing the endosphere more efficiently even if the plant was not as receptive as in rich soils. As in sand soils root exudates are the dominant carbon source (Tkacz et al., 2015), a "colonize-after-lunch" scenario should not be expected. *Burkholderia* colonization behaved similarly in rich soils, where the plant was not as receptive to *Burkholderia*-types, since higher survival in this soil type was positively correlated to higher rhizospheric colonization (Figure 9b). *Burkholderia* in rich soils could be in a "colonize-or-die" scenario because it was less fit to invade the soil and make use of organic matter. These two scenarios might help to explain other situations where colonization is not correlated to survival (Becker et al., 2012). It could be expected that the PM^6 condition should have higher *Enterobacter* colonization if survival correlated directly with colonization, but as the presence of the *Burkholderia* strain affected the interactions of *Enterobacter* with the plant and with the environment, it likely affected colonization rates.

Figure 9b suggested that endophytic colonization was correlated to higher plant biomass on rich soils. It is possible that RE^6 and RM^6 conditions were promoting plant growth by mechanisms that were not analyzed here, since Figure 9b also showed that shoot biomass was not correlated to bacterial P solubilization or ICs production. PGPB traits such as nitrogen fixation, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, motility, chemotaxis, solubilization of other nutrients, and production of other plant hormones might be responsible for the effective PGP effect observed in rich soils. Bacterial metabolism and strategies such as carbon utilization profiles, quorum sensing,

antibiotic production, antagonism, and competitive ability could also be affecting bacterial survival, colonization and PGP effectiveness.

Figure 9a suggested a correlation between *Burkholderia* colonization and plant biomass increase. But this was not clear when we observed colonization (Figure 4b) and plant biomass (Figure 1) individually. It is often assumed that effective plant growth promotion requires extensive colonization of the roots, but it might not always be the case. Chanway et al. (2000) showed that on the three locations where a PGPB improved plant biomass the most, it had the lowest colonization, while on the three locations where it reduced plant biomass the most, it had the highest colonization. In Liu et al. (1995) it was shown that the colonization of two PGPB was not correlated to induced systemic resistance in cucumber. Considering the effect of *Burkholderia* on P solubilization and plant biomass (explored above) we are cautious with the correlation showed in Figure 9a.

The best P solubilizers were not the best ICs producers, and the best ICs producers were not the best P solubilizers

At least four previous works had already detected the inverse correlation between P solubilization and ICs production bacterial abilities (Costa et al 2012, Costa et al 2014, Passos et al., 2014, Moreira et al., 2016). This correlation can also be directly observed on works from other researchers (Otieno et al., 2013; Dias et al., 2008; Poonguzhali et al., 2006), even if the correlation itself was not described. Other works may show that ICs production of bacteria from stressed conditions is lower than from optimal conditions (Ilyas et al., 2012). Those similar outcomes from independent works reduced the chances of artifacts by a methodological bias in the present work.

We now consider that this inverse correlation was confirmed. The best P solubilizers are not the best ICs producers, and the best ICs producers are not the best P solubilizers. This is very important because: (I) P solubilization and IAA production are some of the key components of PGPB activity, measured and reviewed simultaneously in hundreds of papers; (II) these traits are sensible to the nutrients available in soil, which is of tremendous importance to agriculture. Here we confirm that an IC producer is effective on rich soils but ineffective in poor soils, and a P solubilizer is effective on poor soils but ineffective in rich soils.

ICs production reduces with diversity and root length

On both soils, root length reduced as diversity reduced (Figures 1c and 2c). This reduction on root length due to the reduction on diversity could likely be explained by

the drop on ICs production (Figure 6). This was reinforced by the observed negative correlation of ICs production and microcosm dilution in both niches (Figure 9). The decrease in ICs production due to diversity reduction likely happened to the extinction by dilution of bacteria more able to produce ICs. In the mixed inoculation treatment, this drop on ICs productivity was not so steep, likely due to the interaction between both strains, since the co-cultive may improve the display of PGP traits such as indole acetic acid production (Jha and Saraf 2012). Bacterial ICs production is well known to increase plant biomass (Spaepen et al., 2007), so it is not surprising to observe that the lower production of ICs was correlated to smaller root lengths. Detection of this well-known correlation in the present work suggests that the other associations described here might also be consistent.

Endophytic production of ICs on poor soils reduced as diversity dropped, until it reached the same level as rhizospheric ICs production (Figure 7). This decrease can explain a bias in our model. Our original samples of poor soils used to describe the model might have already been in a "low diversity" setting, like those in the 10^6 microcosm. The detected increase in P solubilization of endophytes in the model might be simply a side effect of the reduction of ICs producers in the environment, since both traits are very clearly correlated.

This may also explain why better ICs producers in the endosphere of rich soils could not be found. Apparently, in rich soils the plant is lacking a diverse pool of candidates for endophytic colonization, which would increase ICs production. On rich soils, ICs production in the different niches was already similar at the 10^1 microcosm, and even more similar at the 10^3 microcosm (Figure 7). Perhaps the initial microcosm dilution on rich soils was enough to remove a large portion of ICs producers available, so that the plant could not select the best ICs producers from the endosphere. If this is true, this would mean that the best ICs producers compose the more rare parts of the microbial community in soil that is selected by plants.

Conclusions

The starting microbial community is a key factor on plant inoculation aiming to improve crop biomass. The diversity gradient largely affected plant biomass, inoculant survival and colonization, bacterial ICs production and bacterial P solubilization. Co-inoculation of *Burkholderia* and *Enterobacter* affected the strains interaction with the plant and with the environment, which was not always beneficial to the plant. The

challenge on sustainable food production in the near future will be to take the most out of plant-bacteria interactions, which will happen by identifying understanding key correlations in the plant-bacteria-environment interactions. The use of the right inoculants on the right conditions so that a defined function is optimally filled for greater crop productivity might seem distant, but there will be many opportunities of application for this in the future. The advancement in efficiency and reduction of cost of next generation sequencing (NGS) might fuel the next green revolution, as the Haber-Bosch process fueled the first. As food prices increase and NGS costs decrease, farmers within 20 or 30 years will use NGS to evaluate microbial management before planting crops, such as today they take measurements of pH and NPK content to define several farming practices.

Parts of the model were confirmed with the experiment. *Burkholderia* and *Enterobacter* promoted plant growth only on the soils they were expected to be a better PGPB. ICs production and P solubilization are inverse-correlated, higher on soils they were expected to be higher, and lower on soils they were expected to be lower. Complementing the model, we now consider *Enterobacter*-types as r strategists while *Burkholderia*-types would be K strategists. However, production of ICs by niche did not follow the model. The initial diversity on 10^1 microcosms might have been too low on rich soils and too high on poor soils, compared to the soils used to describe the model, to fully reproduce the expected results. P solubilization by niche in poor soils also did not follow the model, perhaps because our initial description was too influenced by the correlation between ICs production and P solubilization. An experiment with natural soils presenting different diversity levels may be able to settle this niche occupation issue. We conclude that even if not 100% accurate, the model is very useful to guide PGPB testing and prospection.

Acknowledgements

This work was financed by a Grant and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil).

References

Abraham J, Silambarasan S (2015) Plant growth promoting bacteria *Enterobacter asburiae* JAS5 and *Enterobacter cloacae* JAS7 in mineralization of endosulfan. Appl Biochem Biotechnol 175: 3336–3348. doi:10.1007/s12010-015-1504-7.

Adesemoye AO, Kloepper JW (2009) Plant-microbes interactions in enhanced fertilizer-use efficiency. Appl Microbiol Biotechnol 85: 1–12. doi:10.1007/s00253-009-2196-0.

Ahemad M, Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J King Saud Univ - Sci 26: 1–20. doi:10.1016/j.jksus.2013.05.001.

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: 245–26. doi:10.1007/s11104-011-1079-1.

Aung TT, Buranabanyat B, Piromyou P, Longtonglang A (2013) Enhanced soybean biomass by co-inoculation of *Bradyrhizobium japonicum* and plant growth promoting rhizobacteria and its effects on microbial community structures. African J Microbiol Res 7: 3858–3873. doi:10.5897/AJMR2013.5917.

Babalola OO (2010) Beneficial bacteria of agricultural importance. Biotechnol Lett 32: 1559–1570. doi:10.1007/s10529-010-0347-0.

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

Bashan Y, Kamnev A, 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. doi:10.1007/s00374-012-0737-7.

Becker J, Eisenhauer N, Scheu S, Jousset A (2012) Increasing antagonistic interactions cause bacterial communities to collapse at high diversity. Ecol Lett 15: 468–474. doi:10.1111/j.1461-0248.2012.01759.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: 648–656. doi:10.3390/agronomy3040648.

Bodman SB, Bauer WD, Coplin DL (2003) Quorum sensing in plant-pathogenic bacteria. Annu Rev Phytopathol 41: 455–482. doi:10.1146/annurev.phyto.41.052002.095652.

Bonkowski M, Roy J (2005) Soil microbial diversity and soil functioning affect competition among grasses in experimental microcosms. Oecologia 143: 232–240. doi:10.1007/s00442-004-1790-1.

Chanway CP, Shishido M, Nairn J, Jungwirth S (2000) Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. For Ecol Manage 133: 81–88.

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

Compeau G, Al-achi BJ, Platsouka E, Levy SB (1988) Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl Environ Microbiol 54: 2432–2438.

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. doi:10.1007/s11104-012-1513-z.

Costa PB, Granada CE, Ambrosini A, Moreira F, Souza R, et al. (2014) A model to explain plant growth promotion traits : a multivariate analysis of 2,211 bacterial isolates. PLoS One 9: 1–25. doi:10.1371/journal.pone.0116020.

Costa PB, Passaglia LMP (2015) Chapter 95: How fertilization affects the selection of plant growth promoting rhizobacteria by host plants. In: Biological Nitrogen Fixation, vol 2, 1º edition. Edited by Burjin FJ, John Wiley & Sons Inc, New York. 1260 p.

Cumming G, Fidler F, Vaux DL (2007) Error bars in experimental biology. J Cell Biol 177: 7–11. doi:10.1083/jcb.200611141.

Dias ACF, Costa FEC, Andreote FD, Lacava PT, Teixeira M, et al. (2008) Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J Microbiol Biotechnol 25: 189–195. doi:10.1007/s11274-008-9878-0.

Dong Y, Iniguez AL, Ahmer BMM, Triplett EW (2003) Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula*. Appl Environ Microbiol 69: 1783–1790. doi:10.1128/AEM.69.3.1783.

Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res; 17(19):7843-53.*

Facon B, Genton BJ, Shykoff J, Jarne P, Estoup A, et al. (2006) A general eco-evolutionary framework for understanding bioinvasions. Trends Ecol Evol 21: 130–135. doi:10.1016/j.tree.2005.10.012.

Franklin RB, Mills AL (2006) Structural and functional responses of a sewage microbial community to dilution-induced reductions in diversity. Microb Ecol 52: 280–288. doi:10.1007/s00248-006-9033-0.

Garbeva P, van Veen J, van Elsas JD (2004) Microbial diversity in soil: selection microbial populations by plant and soil type and implications for disease suppressiveness. Annu Rev Phytopathol 42: 243–270. doi:10.1146/annurev.phyto.42.012604.135455.

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.

Govindarajan M, Balandreau J, Kwon S-W, Weon H-Y, Lakshminarasimhan C (2008) Effects of the inoculation of *Burkholderia vietnamensis* and related endophytic diazotrophic bacteria on grain yield of rice. Microb Ecol 55: 21–37. doi:10.1007/s00248-007-9247-9.

Govindarajan M, Balandreau J, Muthukumarasamy R, Revathi G, Lakshminarasimhan C (2006) Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*. Plant Soil 280: 239–252. doi:10.1007/s11104-005-3223-2.

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. doi:10.1007/s11104-013-1796-8.

Güneş A, Turan M, Güllüce M, Şahin F (2014) Nutritional content analysis of plant growth-promoting rhizobacteria species. Eur J Soil Biol 60: 88–97. doi:10.1016/j.ejsobi.2013.10.010.

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: 1221–1230. doi:10.1038/ismej.2008.80.

Hammer Ø, Harper DAT, Ryan PD (2001). PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4(1): 9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hardoim PR, Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16: 463–471. doi:10.1016/j.tim.2008.07.008.

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

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-termP-deficiency fertilization in a sandy loam soil. Pedobiologia 53: 49–58.

IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.

Ilyas N, Bano A, Iqbal S, Raja NI (2012) Physiological, biochemical and molecular characterization of *Azospirillum* spp. isolated from maize under water stress. Pak J Bot 2: 71–80.

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.

Jarak M, Mrkovački N, Bjelić D, Jošić D, Hajnal-jafari T, et al. (2012) Effects of plant growth promoting rhizobacteria on maize in greenhouse and field trial. African J Microbiol Res 6: 5683–5690. doi:10.5897/AJMR12.759.

Jha CK, Saraf M (2012) Evaluation of multispecies plant-growth-promoting consortia for the growth promotion of *Jatropha curcas* L. J Plant Growth Regul 31: 588–598. doi:10.1007/s00344-012-9269-5.

Kang S-M, Radhakrishnan R, You Y-H, Khan L, Lee K-E, et al. (2015) *Enterobacter asburiae* KE17 association regulates physiological changes and mitigates the toxic effects of heavy metals in soybean. Plant Biol 17: 1013–1022. doi:10.1111/plb.12341.

Kassen R, Buckling A, Bell G, Rainey P (2000) Diversity peaks at intermediate productivity in a laboratory microcosm. Nature 406: 508-512.

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

Lane DJ (1991) 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics. Stackebrandt E, Goodfellow M, editor. Chichester, United Kingdom: John Wiley and Sons; 1991. 16S/23S rRNA sequencing; pp. 115–175

Lapsansky ER, Milroy AM, Andales MJ, Vivanco JM (2016) Soil memory as a potential mechanism for encouraging sustainable plant health and productivity. Curr Opin Biotechnol 38: 137–142. doi:10.1016/j.copbio.2016.01.014.

Lau YY, Sulaiman J, Chen JW, Yin W-F, Chan K-G (2013) Quorum sensing activity of *Enterobacter asburiae* isolated from lettuce leaves. Sensors 13: 14189–14199. doi:10.3390/s131014189.

Lin H-H, Chen Y-S, Li Y-C, Tseng I-L, Hsieh T-H, et al. (2011) *Burkholderia multivorans* acts as an antagonist against the growth of *Burkholderia pseudomallei* in soil. Microbiol Immunol 55: 616–624. doi:10.1111/j.1348-0421.2011.00365.x.

Liu L, Kloepper JW, Tuzun S (1995) Induction of systemic resistance in cucumber by plant growth-promoting rhizobacteria: duration of protection and effect of host resistance on protection and root colonization. Phytopathology 85: 1064–1068.

Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS One 3: e2702. doi:10.1371/journal.pone.0002702.

Mallon CA, Poly F, Roux X, Marring I, van Elsas J Dirk, et al. (2015) Resource pulses can alleviate the biodiversity – invasion relationship in soil microbial communities. Ecology 96: 915–926.

Marshall K, Shakya S, Greenhill AR, Padilla G, Baker A, et al. (2010) Antibiosis of *Burkholderia ubonensis* against *Burkholderia pseudomallei*, the causative agent for melioidosis. Southeast Asian J Trop Med Public Health 41: 904–912.

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.

Matz C, Jürgens K (2003) Interaction of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. Microb Ecol 45: 384–398. doi:10.1007/s00248-003-2000-0.

Mitchell JG, Kogure K (2006) Bacterial motility: links to the environment and a driving force for microbial physics. FEMS Microbiol Ecol 55: 3–16. doi:10.1111/j.1574-6941.2005.00003.x.

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

Otieno BN, Culhane J, Germaine KJ, Brazil D, Ryan D, et al. (2013) Screening of large collections of plant associated bacteria for effective plant growth promotion and colonization. Asp Appl Biol 120: 23–28.

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

Park K-H, Lee O-M, Jung H-I, Jeong J-H, Jeon Y-D, et al. (2010) Rapid solubilization of insoluble phosphate by a novel environmental stress-tolerant *Burkholderia vietnamiensis* M6 isolated from ginseng rhizospheric soil. Appl Microbiol Biotechnol 86: 947–955. doi:10.1007/s00253-009-2388-7.

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

Passos J, 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 Mol Biol 572: 560–572.

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 220: 207–220.

Poonguzhali S, Madhaiyan MST (2006) Cultivation-dependent characterization of rhizobacterial communities from field grown Chinese cabbage *Brassica campestris* ssp *pekinensis* and screening of traits for potential plant growth promotion. Plant Soil 286: 167–180. doi:10.1007/s11104-006-9035-1.

Ramos B, Garcı JAL, Barrientos LF (2003) Alterations in the rhizobacterial community associated with European alder growth when inoculated with PGPR strain *Bacillus licheniformis*. 49: 61–68.

Semenov AV, van Bruggen AHC, van Overbeek L, Termorshuizen AJ, Semenov AM (2007) Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar *Typhimurium* in cow manure. FEMS Microbiol Ecol 60: 419–428. doi:10.1111/j.1574-6941.2007.00306.x.

Seneviratne G, Kulasooriya S (2013) Reinstating soil microbial diversity in agroecosystems: The need of the hour for sustainability and health. Agric Ecosyst Environ 164: 181–182. doi:10.1016/j.agee.2012.10.002.

Singh JS (2015) Microbes: The chief ecological engineers in reinstating equilibrium in degraded ecosystems. Agric Ecosyst Environ 203: 80–82. doi:10.1016/j.agee.2015.01.026.

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. doi:10.1016/j.agee.2011.01.017.

Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 31: 425–448. doi:10.1111/j.1574-6976.2007.00072.x.

Suárez-Moreno ZR, Caballero-Mellado J, Coutinho BG, Mendonça-Previato L, James EK, et al. (2012) Common features of environmental and potentially beneficial plant-associated *Burkholderia*. Microb Ecol 63: 249–266. doi:10.1007/s00248-011-9929-1.

Tkacz A, Cheema J, Chandra G, Grant A, Poole PS (2015) Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. ISME J 9: 2349–2359. doi:10.1038/ismej.2015.41.

Toth IK, Pritchard L, Birch PRJ (2006) Comparative genomics reveals what makes an enterobacterial plant pathogen. Annu Rev Phytopathol 44: 305–336. doi:10.1146/annurev.phyto.44.070505.143444.

Turnbull GA., Morgan JAW, Whipps JM, Saunders JR (2001) The role of bacterial motility in the survival and spread of *Pseudomonas fluorescens* in soil and in the attachment and colonization of wheat roots. FEMS Microbiol Ecol 36: 21–31.

van Elsas JD, Chiurazzi M, Mallon CA, Elhottova D, Kristufek V, et al. (2012) Microbial diversity determines the invasion of soil by a bacterial pathogen. Proc Natl Acad Sci U S A 109: 1159–1164. doi:10.1073/pnas.1109326109.

van Elsas JD, Torsvik V, Hartmann A, Ovreas L, Janson JK (2007) Chapter 4: The Bacteria and Archaea in soil. In: Modern Soil Microbiology, 2º edition. Edited by van Elsas JD, Trevors JT, Janson JK. CRC, New York, 2007. 646p

Van VT, Berge O, Kê SN, Balandreau J, Heulin T, et al. (2000) Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and

late yield components in low fertility sulphate acid soils of Vietnam. Plant Soil 218: 273–284. doi: 10.1023/A:1014986916913.

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.

Weidner S, Koller R, Latz E, Kowalchuk G, Bonkowski M, et al. (2015) Bacterial diversity amplifies nutrient-based plant-soil feedbacks. Funct Ecol 29: 1341–1349. doi:10.1111/1365-2435.12445.

Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, et al. (2006) Maintenance of soil functioning following erosion of microbial diversity. Environ Microbiol 8: 2162–2169. doi:10.1111/j.1462-2920.2006.01098.x.

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

Zhao H, Yan H, Zhou S, Xue Y, Zhang C (2011) The growth promotion of mung bean (*Phaseolus radiatus*) by *Enterobacter asburiae* HPP16 in acidic soils. African J Biotechnol 10: 13802–13814. doi:10.5897/AJB11.2028.

Supplementary material

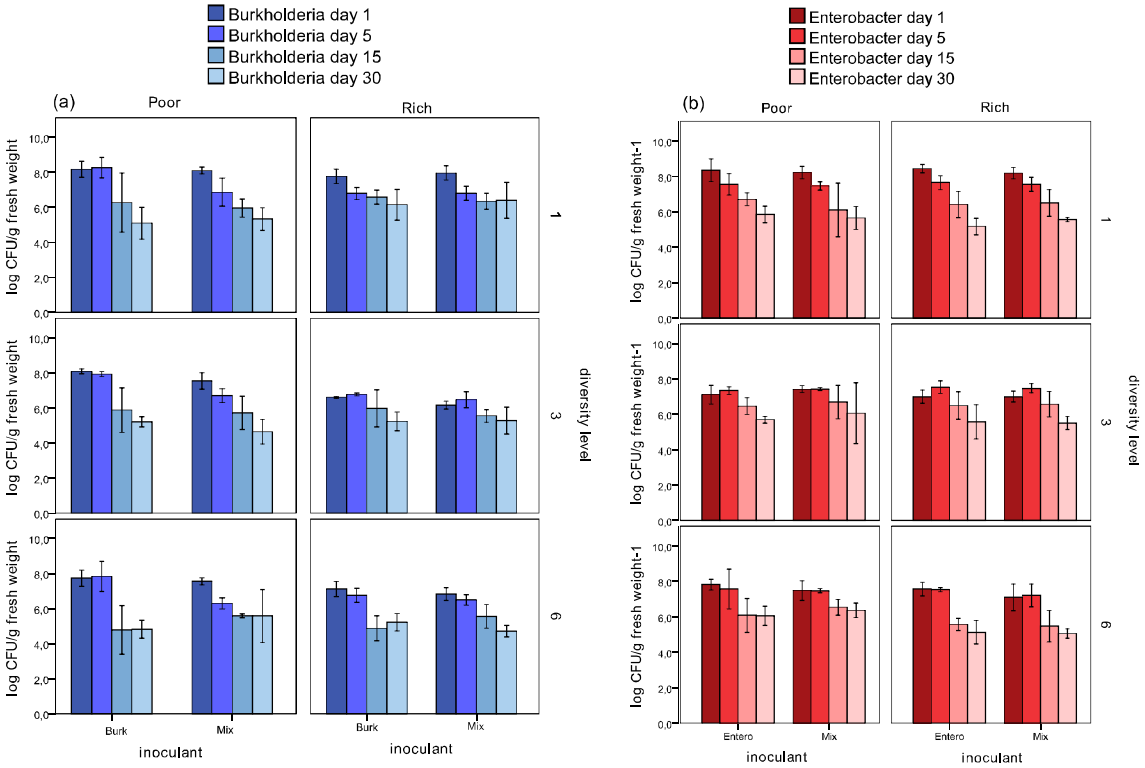


Figure S1: Log CFU g fresh weight^{-1} from top soil used to determine the survival curves for (a) *Burkholderia* and (b) *Enterobacter*, split by soil type and diversity level. Burk = single strain *Burkholderia* inoculation, Entero = single strain *Enterobacter* inoculation, Mix = co-inoculation of mixed strains. Diversity level 1, 3 and 6 refers to dilutions of 10^1 microcosm (top), 10^3 microcosm (middle) and 10^6 microcosm (bottom)

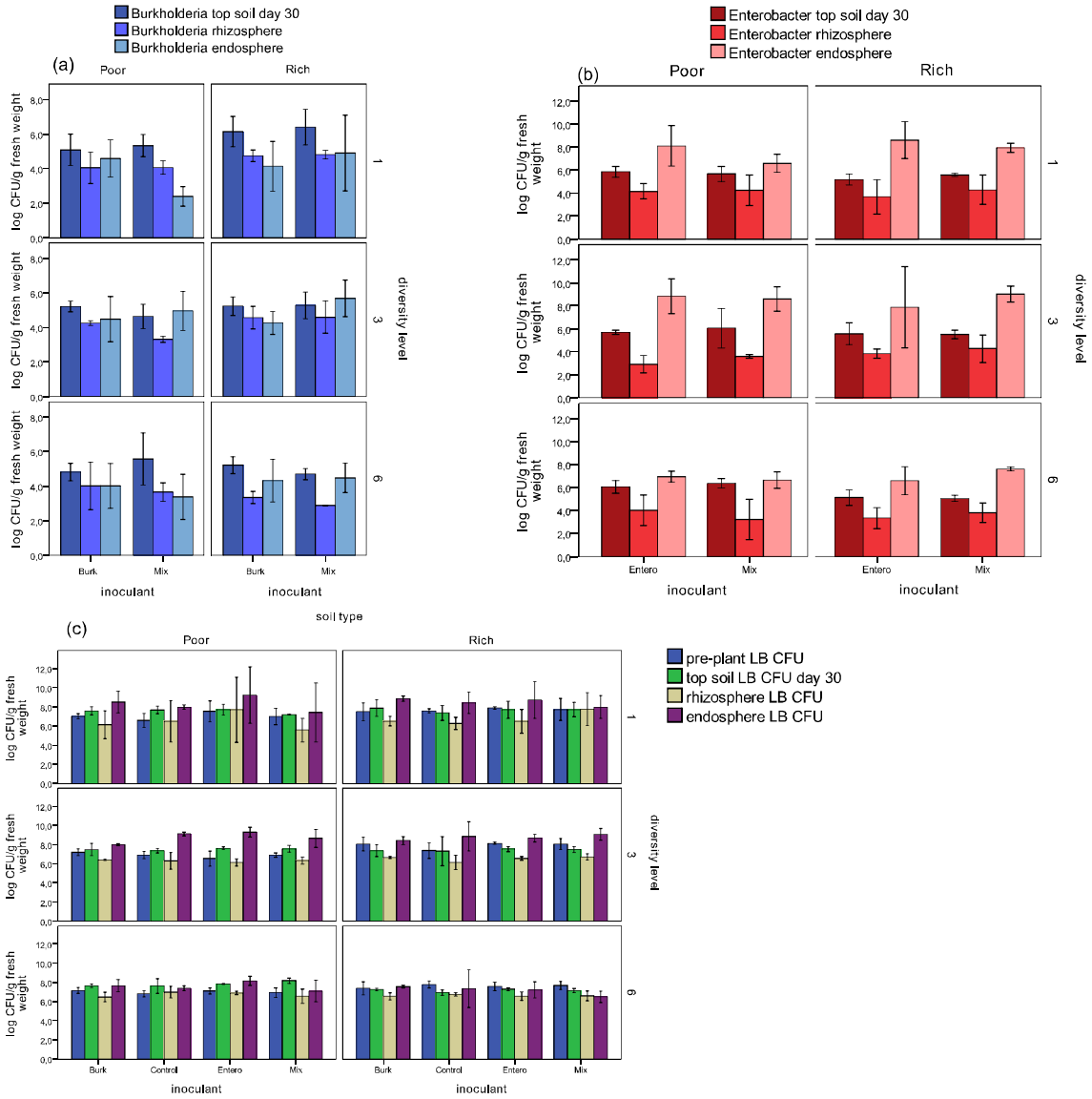


Figure S2: Log CFU g fresh weight^{-1} from top soil used to determine the populations of (a) *Burkholderia*, (b) *Enterobacter*, and (c) total LB population, split by soil type and diversity level. Burk = single strain *Burkholderia* inoculation, Entero = single strain *Enterobacter* inoculation, Mix = co-inoculation of mixed strains. Diversity level 1, 3 and 6 refers to dilutions of 10^1 microcosm (top), 10^3 microcosm (middle) and 10^6 microcosm (bottom)

Chapter 4 - Invasion ecology applied to inoculation of plant growth promoting bacteria through a novel SIMPER-PCA approach

Pedro Beschoren da Costa[a], Samanta Bolzan de Campos[b], Volker F. Wendisch[b], André Luis Pereira Dresseno[c], Paul Dirksen[d], Alexander Sczyrba[d], Andreas Albersmeier[e], and Luciane Maria Pereira Passaglia[a, *]

Manuscript to be submitted to the Soil Biology and Biochemistry journal.

[a]Federal University of Rio Grande do Sul, Institute of Biosciences, Department of Genetics, Av. Bento Gonçalves, 9500, Postal box 15.053, CEP 91501-970,
Porto Alegre, RS, Brazil
[b]Genetics of Prokaryotes, Faculty of Biology & Center for Biotechnology, Bielefeld University, Universiteatsstr. 25, 33615, Bielefeld, Germany
[c]Federal University of Rio Grande do Sul, Institute of Biosciences, Department of Ecology, Av. Bento Gonçalves, 9500, Postal box 15.053, CEP 91501-970,
Porto Alegre, RS, Brazil
[d]Computational Metagenomics, Center for Biotechnology (CeBiTec), Bielefeld University, Sequenz 1, 33615, Bielefeld, Germany
[e]Technology Platform Genomics, Center for Biotechnology (CeBiTec), Bielefeld University, Sequenz 1, 33615, Bielefeld, Germany

[*]Corresponding author at Departamento de Genética, Instituto de Biociências, UFRGS. Av. Bento Gonçalves, 9500, Caixa Postal 15.053, Prédio 43312, sala 207b, Porto Alegre, RS, CEP 91501-970, Brazil. Tel.: +55 51 3308 9813; fax +55 51 3308 7311. E-mail address: luciane.passaglia@ufrgs.br (L. M. P. Passaglia)

Abstract

Plant growth promoting bacteria (PGPB) have already been used on crops at industrial scale for years. Yet, inoculants that are efficient in some locations may not be efficient in others that present physical and chemical characteristics apparently similar. This shows that the understanding of plant-bacteria interactions, and how to manipulate them to fulfill agricultural goals, is not an easy task. Classical invasion ecology theory can be readily applied to PGPB inoculation, helping to identify natural patterns that can

be used to optimize PGPB efficiency. In this work, the hypothesis that the most invasive inoculant will be the most efficient PGPB was tested. Using next generation sequencing technology targeting the 16S rDNA gene in metagenomics samples, we analyzed the rhizosphere of maize plants inoculated with four PGP strains cultivated in three different locations. The microbial communities of inoculated plants were compared to the non-inoculated controls, in order to estimate the inoculant invasion ability. We also evaluate the bacterial diversity and soil nutrient contents from pre-planting conditions, to test if an environment with higher nutrient per diversity ratio would be easier to invade. Using a novel SIMPER-PCA approach that uses controls as standards, we could observe that the most efficient inoculant was neither the most invasive nor the one that caused unique impacts in the community. Nonetheless, the location with a higher nutrient per diversity ratio presented the most efficient PGPB results. We concluded that the native community strongly affects PGPB efficiency, and discussed alternatives to manipulate the nutrient per diversity ratio aiming for a better PGPB application. We also showed details of the SIMPER-PCA approach, which facilitates the detection of microbial community dissimilarities in relation to a defined standard, and can be very useful for next generation sequencing data and PGPB research.

Key words: Next generation sequencing, metagenomics, PGPB, rhizosphere, maize.

Introduction

Bacterial inoculants have already been successfully used to improved crop production at industrial scale for many years (Alves et al., 2003, Castro-Sowinski et al., 2007). However, as plant-bacteria-environment interactions are far from being completely understood, inoculation might not be fully efficient at all times (Berg et al., 2013, Owen et al., 2015). Even commercialized plant growth promoting bacteria (PGPB) might fail to improve crop yield (Otieno et al., 2013; Owen et al., 2015), in part due to interactions with the local microbial community that probably will be competing with the inoculant for nutrients and niches (Bashan et al., 2013). One form of overcoming this issue is the biotechnological intensive construction of very effective and competitive strains (Germaine et al., 2013) and the other is the elucidation and manipulation of ecological interactions in the rhizosphere to maximize PGPB efficiency (Castro-Sowinski et al., 2007).

Classical invasion ecology has already been applied to bacteria (van Elsas et al., 2012), but picturing the plant inoculant itself as an invader to the local community had not been considered until recently (Ambrosini et al., 2015). An environment with a high amount of nutrients but low bacterial diversity would have free niches, and would be easier to invade than an environment with a low amount nutrients and high diversity because it would have filled niches (Davis and Pelsor, 2001). An efficient invader must display a series of traits, such as high dispersal, high reproduction rate, and will cause heavy impact on the environment (Parker et al., 1999; Mallon and van Elsas, 2015). Although dispersal and growth rates of inoculants might be difficult to measure on field; their impact on the community is not, thanks to the advancements of Next Generation Sequencing (NGS).

Here, we tested the hypothesis that a bacterial inoculant with the best invasion ability will be the best PGPB. We consider that the best invader will cause more impact on the microbial community it invades, and use control-treatment dissimilarities from NGS as a proxy for the impact caused by the invader on this community. In addition, we also tested the hypothesis that the environment with the highest availability of nutrients per diversity ratio will be easier to invade than an environment with a lower availability of nutrients per diversity ratio. Finally, we presented a novel approach to analyze metagenomic NGS results, which focus on dissimilarities to a standard and can be useful on PGPB research.

Material and Methods

Field trials design, sampling and soil chemical analysis

Maize (*Zea mays*) plants (hybrid variety 30F53H, Pioneer) were subjected to a PGPB trial in three different locations in Paraná state, Brazil. The field crops were settled in Londrina (L) (23° 17′ 34″ S, 51° 10′ 24″ W), Marechal Cândido Godoi (M) (24° 33′ 24″ S, 54° 3′ 24″ W), and Ponta Grossa (P) (25° 5′ 40″ S, 50° 9′ 48″ W). All of these locations have climate classified as humid subtropical (Köppen climate classification Cfa). Inoculation treatments were as follow: (1) a non-inoculated control, (2) *Azospirillum brasilense* Ab-V5 (Hungria et al., 2010), (3) *Achromobacter* sp. VC36, (Arruda et al., 2013) (4) *Pseudomonas* sp. 4311, and (5) *Pseudomonas* sp. 4312 (André Oliveira, personal communication). When condition “L3” is cited, we mean *Achromobacter* sp. VC36 inoculation on Londrina location. Bacteria were grown in liquid LB medium at 28°C under agitation (200 rpm) for 16 h. Twenty mL of inoculants

cultures containing 10^9 cells mL^{-1} were used per kg of seeds. The seeds were exposed to the inoculant for 30 min at room temperature, and let dry for additional 30 min. All treatments and controls received 30 kg $hectare^{-1}$ of N fertilizer, and randomized blocks were composed of 5 lines of 10 m (length) x 3.2 m (width) with 0.8 m spacing between each line. Fields were sown between November 2012 and January 2013. All of the strains were isolated from maize in previous works. The *A. brasilense* Ab-V5 strain is a well-know PGP bacterium that display a high N_2-fixing ability *in vitro* and had increased the contents of some nutrients in the leaves and grains of maize and wheat, as P, K and Cu, and increased the N contents in the leaves of these crops (Hungria et al., 2010). The *Achromobacter* sp. VC36 strain is able to produce indolic compounds and had displayed positive effects on shoot and root weight and nutrient uptake of maize plants (Arruda et al., 2013). The two *Pseudomonas* strains have 97% and 95% similarity with *P. koreensis* (for 4311 and 4312, respectively). Both produce indolic compounds but only strain 4312 is able to solubilize P (André Oliveira, personal communication).

Ten days after plant emergence the rhizospheric soil directly attached to the plant roots was scraped from three independent plants and pooled to compose a composite sample. Two independent composite samples (a and b) were used per treatment. Two additional independent composite samples consisting of bulk soil were taken immediately before planting to characterize the pre-planting conditions. The bulk soil samples (treatment 0) were also used for soil chemical analysis, using standard methods (Sparks et al., 1996).

DNA extraction, amplification and sequencing

Rhizospheric and bulk soil DNAs were extracted from 0.3 g of each soil sample using the Nucleo Spin Soil™ kit (Macherey-Nagel). DNA amplification of the V4 region of the bacterial 16S rRNA was performed using the primers F515 and R806 (Caporaso et al. 2011) and purified using MinElute (Qiagen). The protocol for barcoded Illumina pyrosequencing was described by Caporaso et al. (2011). TruSeq DNA protocol (Illumina) was followed for library preparation, starting with the end repair of the fragments. Libraries were sequenced on the MiSeq sequencer (Illumina) with a read length of 2 x 300 nt. MiSeq Control Software v 2.3.0 was used for sequencing and MiSeq Reporter Software v 2.3.32 for demultiplexing and generation of FASTQ files.

Bioinformatics analyses

All sequence analyses were done using QIIME 1.8.0. Read quality control was performed with the FastQC tool, and merging of sequences was performed with the

pairedendoverlap tool, available at the Bioinformatics Resource Facility of the CeBiTec computer cluster (Bielefeld, Germany). QIIME defaults were used for filtering of raw and merged Illumina data, OTUs picking and clustering at 97% identity. Representative sequence alignments were performed with the PyNAST tool, and the greengenes database was used for comparisons through the RDP classifier. The final OTU table was generated in the BIOM format.

Statistical analysis

Firstly, reads were cut off at 200 OTU occurrences minimum. Read counts were transformed to relative abundances for normalization, and then transformed to the square-root. The Bray-Curtis distance, widely used on ecological studies (Rees et al., 2004) was used for the ordination method NMDS (Non-Metric Multidimensional Scaling) and for the SIMPER (SIMilarity PERcentage) tests. The Bray-Curtis distance is appropriate for metagenomic data because there is less bias introduced by shared absences of amplicons than in a Euclidian distance (Mills et al., 2006). The SIMPER test produces direct comparisons of 2 or more groups of samples, returning the dissimilarities each taxa is responsible for as percentages of total dissimilarity. Here, SIMPER comparisons were made with the 2 independent samples from the inoculated treatments and the appropriate non-inoculated control from each location. The total SIMPER difference on the treatment-control pairs was used as a proxy for invasion. All the taxa dissimilarity from the SIMPER test was signal-transformed to show if each treatment had more or less of a particular OTU than the appropriate control. The signal-transformed, Bray-Curtis based SIMPER dissimilaries were then processed in a within-group PCA (Principal Component Analysis), grouping per location. Within-group PCA minimizes the differences between groups, and was used to reduce the clustering by location effect, highlighting the control-treatment differences. All tests were performed on Paleontological Statistics (PAST) software (Hammer et al., 2001).

Data from nutrient analysis (clay content, P, K, Organic matter, Ca, Mg, S, B, and Mn) were normalized by using relative percentages. Simpson, Shannon and Eveness indexes from the pre-planting conditions were also normalized with relative percentages, in the different taxonomic levels (Phyla, Class, Order, Family, Genera, and OTU). The relative nutrient content was then divided by the relative diversity indexes. This nutrient per diversity ratio was then directly compared to SIMPER values normalized by relative percentage. We normalized the SIMPER values because they increase as diversity level decreases, and also for consistency with the normalized

diversity and nutrient ratios. We could not run statistical tests on this data because of low sample size and non-independent data points between taxa levels.

Results

Sequencing

Sequencing of amplicons from the V4 region of the bacterial 16S rRNA of the 36 samples with the MiSeq platform returned 12.1 million reads, with 5.6 million of unique sequences clustered in 1.3 million OTUs. There were about 844 thousand singleton sequences, composing 15.1% of sequences and 64% of OTUs. Reads assembling was of 95±4%, except for samples P2b and M3b that resulted in only 37% and 50% assembled reads, respectively. The average number of valid reads was 313,984, ranging from 49,623 (P2b) to 1,711,823 (M5a). Two samples (P4a and M0b) were entirely removed from analysis because of a large dominance (60 and 40%, respectively) of *Shewanellaceae* sp., a marine bacterium associated to fish spoilage (Satomi, 2014). The presence of *Shewanellaceae* was inconsistent with the replicates, and the samples were standing as clear outliers on all tests even when all the *Shewanellaceae* OTUs were removed (data not shown). After cut-offs, a total of 6,457,333 reads distributed in 3,179 OTUS was available for analysis. Total reads for each taxa level can be found in Supplementary Table 1.

The most common Phyla found was Proteobacteria (39.32% of reads), followed by Actinobacteria (30.69%), Firmicutes (12.83%), and Acidobacteria (5.55%). All other 24 phyla (11.59%) had less than 5% of reads each (Figure 1). The three different sampling locations could easily be discriminated at the phyla level, with Londrina showing a higher number of Actinobacteria, Ponta Grossa showing more Proteobacteria, and Marechal Cândido Godoi presenting more Firmicutes representatives (Figure 1).

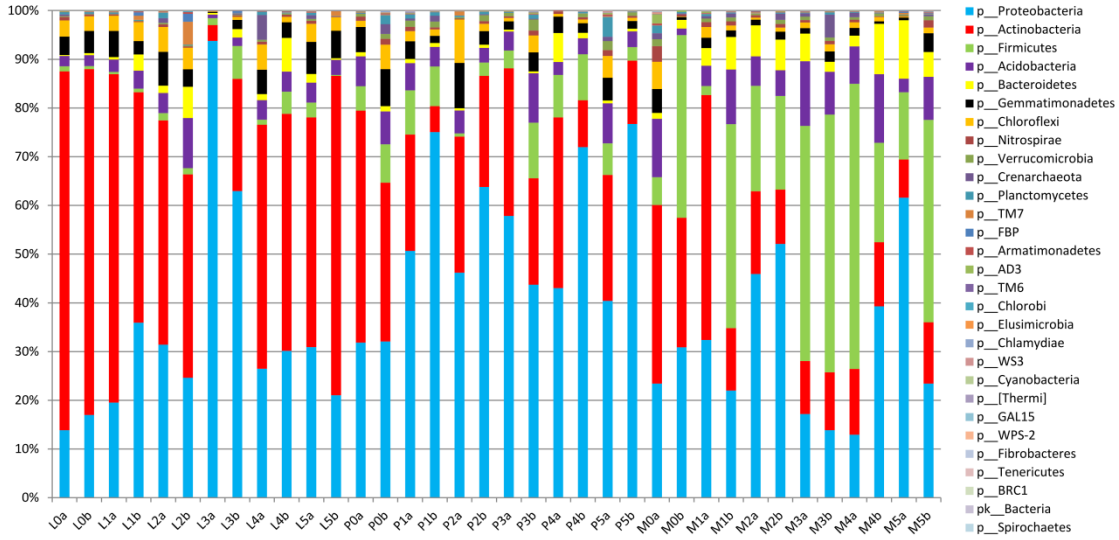


Figure 1: Relative Phyla (p_) distribution per sample. Phyla legend is in the order of the most common to the most rare. L = Londrina, P = Ponta Grossa, M = Marechal Cândido Godoi, 0 = pre-planting, 1 = non-inoculated control, 2 = *Azospirilum brasilisense* Ab-V5 , 3= *Achromobacter* sp. VC36, 4= *Pseudomonas* sp. 4311, 5 = *Pseudomonas* sp. 4312, a = first replicate, b = second replicate

Multivariate ordination and SIMPER tests

The NMDS plot at the OTU level (Figure 2) showed a clear clustering according to location, but no patterns due to the inoculation treatments were observed. Thus, the effect of inoculation on community composition was much smaller than the effect of local conditions.

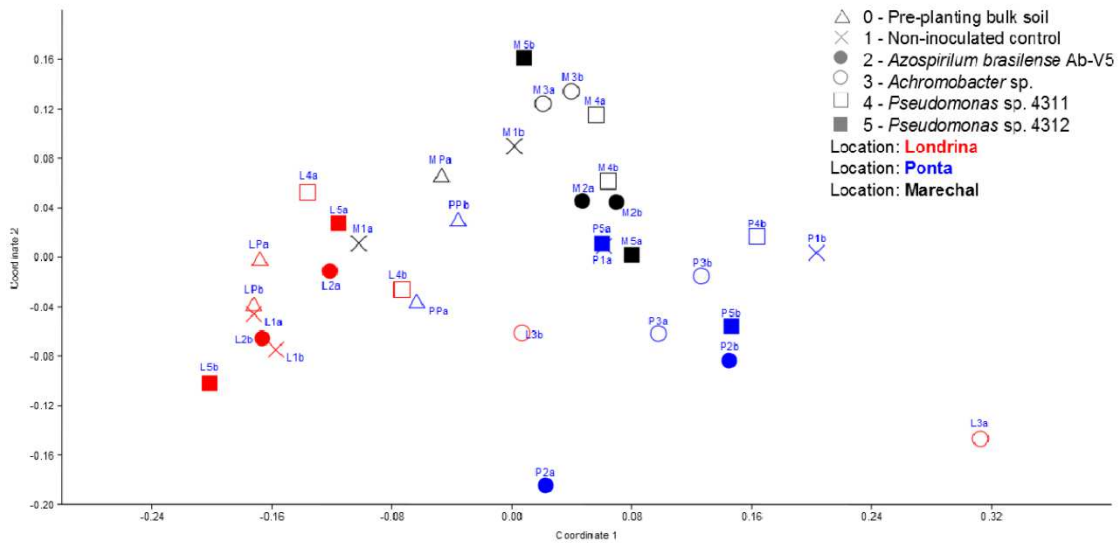


Figure 2. Non-Metrical Multidimensional Scaling (NMDS) of OTUs based on Bray-Curtis distance. The three different sampling locations clustered together while the five different inoculation treatments did not group. Different colors show samples from different locations, with red = Londrina, Blue = Ponta Grossa, Black = Marechal Cândido Godoi. Empty triangle = bulk soil from pre-planting condition, X = non-inoculated control, full circle= *Azospirillum brasilense* Ab-V5, empty circle = *Achromobacter* sp. VC36, empty square = *Pseudomonas* sp. 4311 and full square = *Pseudomonas* sp. 4312. a = first replicate, b = second replicate

Using the SIMPER test we quantified the differences on community composition in each treatment-control comparison, easily sorting taxas that would be the most dissimilar (Clarke, 1993). The SIMPER value dissimilarity, a percentage of the differences in taxa composition from the treatment-control pair, was used as an indicative of the impact of invasion, and thus the invasion ability of the inoculant. Condition L3 (*Achromobacter* sp. VC36 inoculation on Londrina location) presented the highest SIMPER value at both Phyla (34.68%) and OTU level (59.89%), while condition P4 presented the lowest SIMPER value at Phyla level (11.26%) and condition M4 the lowest SIMPER value at OUT level (26.96%) (Figure 3). Inoculant 3 (*Achromobacter* sp. VC36), however, did not presented the highest SIMPER values at Ponta Grossa or Marechal Cândido Godoi locations, meaning that none of the four bacterial inoculants presented an overall higher SIMPER value - and thus higher invasion ability in all locations. Many of the OTUs that were the most highly dissimilar

in the control-treatment pairs belonging to the *Bacillus*, *Burkholderia*, *Pseudomonas*, and *Pseudonocardia* genera, and also to the *Enterobacteriaceae* family (Table 1).

Treatment	Taxonomic level					
	Phyla	Class	Order	Family	Genera	Otu
L2	15.50	18.15	20.23	20.56	21.43	27.76
L3	34.69	38.19	41.61	44.31	45.47	59.89
L4	15.13	19.79	22.08	24.19	25.83	36.87
L5	12.28	17.20	19.25	20.29	21.72	29.91
P2	18.11	24.09	27.72	29.37	31.01	40.16
P3	14.39	19.76	21.20	21.88	23.43	33.09
P4	11.26	17.22	18.48	18.96	20.11	38.04
P5	15.81	19.76	21.54	21.60	22.64	42.06
M2	18.15	24.34	25.12	26.50	27.58	42.76
M3	18.20	18.64	19.68	21.03	22.35	35.91
M4	19.58	24.20	25.30	26.01	27.10	26.96
M5	22.15	25.80	26.70	27.78	28.69	32.07

Figure 3: SIMPER percentages from the treatment-control pairs, at each taxonomic level. Color gradient shows lower values in red, higher values in green. L = Londrina, P = Ponta Grossa, M = Marechal Cândido Godoi; 2 = *Azospirillum brasilense* Ab-V5, 3 = *Achromobacter* sp. VC36, 4 = *Pseudomonas* sp. 4311 , and 5 = *Pseudomonas* sp. 4312

Table 1: SIMPER percentages from the treatment-control pairs, at OTU level. Av. dissim. = taxa average dissimilatity; contrib. % = contribution of that taxa to total dissimilarity; Cumul. % = cumulative total dissimilarity; Mean abundances = normalized and transformed counts of each taxa for control and treatments; p_ = Phyla; c_ = Class; o_ = Order; f_ = Family; g_ = genera.

	Londrina			Mean abundance		Marechal			Mean abundance		Ponta			Mean abundance	
	Taxon	Av. dissim	Contrib. %	Cumul %	control	treatment	Taxon	Av. dissim	Contrib. %	Cumul %	control	treatment	Taxon	Av. dissim	Contrib. %	Cumul %	control	treatment
2 - Azospirillum brasilense	k_Bacteria	0.155	0.558	0.558	0.504	1.050	g__Burkholderia	0.533	1.328	1.328	1.150	4.220	g__Pseudomonas	0.408	0.955	0.955	2.970	1.590
	g__Pseudonocardia	0.140	0.504	1.062	2.660	2.840	*Bacillus flexus*	0.297	0.740	2.068	2.180	2.650	c__Betaproteobacteria	0.408	0.954	1.909	0.645	2.420
	f__Comamonadaceae	0.128	0.461	1.522	2.560	2.160	f__Xanthomonadaceae	0.257	0.640	2.707	0.223	1.660	g__Pseudonocardia	0.385	0.900	2.809	1.110	2.690
	f__Comamonadaceae	0.123	0.442	1.964	1.300	0.835	g__Chryseobacterium	0.247	0.616	3.323	0.138	1.540	f__Enterobacteriaceae	0.382	0.894	3.702	2.700	1.290
	f__Xanthomonadaceae	0.116	0.417	2.381	0.713	0.128	g__Bacillus	0.224	0.557	3.880	1.620	1.710	f__Micrococcaceae	0.287	0.670	4.372	1.710	1.910
	f__Oxalobacteraceae	0.093	0.336	2.717	0.357	0.954	c__[Chloracidobacteria]	0.201	0.500	4.380	1.180	1.150	g__Burkholderia	0.269	0.630	5.002	3.980	3.070
	c__Chloroflexi	0.092	0.330	3.047	0.609	0.063	g__Cupriavidus	0.196	0.488	4.869	0.176	1.300	c__Ktedonobacteria	0.248	0.579	5.582	0.537	1.580
	o__Acidimicrobiales	0.090	0.326	3.372	0.835	0.454	g__Paenibacillus	0.174	0.433	5.301	1.060	0.580	*Bacillus flexus*	0.237	0.555	6.137	1.280	0.185
	g__Sphingomonas	0.090	0.323	3.695	0.123	0.652	f__Enterobacteriaceae	0.153	0.382	5.683	0.236	1.140	g__Bacillus	0.223	0.521	6.657	1.450	0.494
	g__Cellulomonas	0.087	0.314	4.009	0.620	0.313	g__Bacillus	0.153	0.380	6.063	1.100	1.430	g__Cupriavidus	0.173	0.404	7.062	0.281	1.050
3 - Achromobacter sp.	g__Burkholderia	0.970	1.619	1.619	0.423	5.020	*Bacillus flexus*	0.320	0.967	0.967	2.180	3.750	f__Enterobacteriaceae	0.524	1.458	1.458	2.700	0.327
	f__Enterobacteriaceae	0.529	0.883	2.502	0.224	2.510	g__Bacillus	0.241	0.728	1.694	1.620	3.050	g__Pseudomonas	0.385	1.073	2.531	2.970	1.380
	Salinispora tropica	0.442	0.737	3.240	0.271	2.490	c__[Chloracidobacteria]	0.228	0.689	2.384	1.180	2.360	f__Micrococcaceae	0.317	0.882	3.412	1.710	3.150
	f__Comamonadaceae	0.410	0.685	3.924	2.560	0.674	g__Bacillus	0.217	0.656	3.040	1.100	2.350	g__Burkholderia	0.253	0.703	4.115	3.980	4.750
	g__Pseudonocardia	0.408	0.681	4.605	2.660	0.786	g__Paenibacillus	0.180	0.544	3.584	1.060	2.080	g__Bacillus	0.164	0.457	4.573	1.450	0.804
	g__Burkholderia	0.388	0.647	5.252	0.041	1.740	g__Bacillus	0.168	0.507	4.091	0.680	1.660	f__Micrococcaceae	0.157	0.437	5.010	0.189	0.920
	f__Enterobacteriaceae	0.336	0.560	5.812	0.038	1.510	g__Pseudonocardia	0.141	0.427	4.518	1.800	0.959	f__Oxalobacteraceae	0.150	0.417	5.427	1.090	0.404
	g__Burkholderia	0.333	0.556	6.369	0.017	1.470	g__Bacillus	0.121	0.365	4.883	0.761	1.480	g__Pseudomonas	0.146	0.408	5.835	0.682	0.863
	f__Solirubrobacteraceae	0.295	0.493	6.861	1.820	0.438	g__Ammoniphilus	0.113	0.340	5.224	0.046	0.689	*Bacillus flexus*	0.134	0.374	6.209	1.280	0.875
	Acinetobacter rhizosphaerae	0.259	0.433	7.294	0.082	1.200	*Candidatus nitrososphaera*	0.105	0.318	5.541	0.549	1.010	g__Bacillus	0.109	0.304	6.513	1.410	1.240
4 - Pseudomonas sp. 4311	f__Comamonadaceae	0.204	0.552	0.552	2.560	1.380	*Bacillus flexus*	0.333	0.876	0.876	2.180	3.360	f__Enterobacteriaceae	0.640	2.374	2.374	2.700	5.370
	f__Micrococcaceae	0.194	0.525	1.077	1.370	2.330	g__Bacillus	0.259	0.682	1.558	1.620	2.240	g__Burkholderia	0.368	1.365	3.738	3.980	2.510
	g__Burkholderia	0.162	0.439	1.515	0.423	1.390	g__Burkholderia	0.259	0.681	2.239	1.150	2.690	g__Pseudomonas	0.357	1.323	5.062	2.970	4.510
	Candidatus Nitrososphaera	0.155	0.421	1.936	0.106	0.908	g__Bacillus	0.249	0.655	2.894	1.100	2.190	*Bacillus flexus*	0.157	0.583	5.644	1.280	0.930
	Methylobacterium adhaesivum	0.149	0.404	2.341	0.351	0.994	c__[Chloracidobacteria]	0.201	0.529	3.422	1.180	0.884	g__Pseudomonas	0.132	0.490	6.134	0.682	0.587
	f__Comamonadaceae	0.127	0.345	2.685	1.300	0.626	g__Paenibacillus	0.169	0.445	3.867	1.060	0.903	f__Oxalobacteraceae	0.130	0.484	6.618	1.090	0.591
	Candidatus Nitrososphaera	0.126	0.342	3.028	0.085	0.795	g__Bacillus	0.168	0.443	4.310	0.680	1.680	f__Micrococcaceae	0.114	0.424	7.041	1.710	1.910
	f__Xanthomonadaceae	0.122	0.332	3.360	0.713	0.149	f__Acidobacteriaceae	0.141	0.370	4.680	0.260	1.080	g__Pseudonocardia	0.112	0.415	7.456	1.110	0.753
	g__Kaistobacter	0.118	0.319	3.678	0.298	0.967	g__Pseudonocardia	0.131	0.345	5.025	1.800	1.060	g__Bacillus	0.110	0.406	7.863	1.450	1.690
	g__Pseudonocardia	0.106	0.288	3.966	2.660	2.040	f__Chitinophagaceae	0.115	0.303	5.328	0.498	1.150	g__Rhodoplanes	0.106	0.392	8.254	1.470	1.180
5 - Pseudomonas sp. 4312	f__Comamonadaceae	0.155	0.518	0.518	2.560	1.780	g__Burkholderia	0.420	0.999	0.999	1.150	3.390	f__Enterobacteriaceae	0.395	1.231	1.231	2.700	0.969
	f__Comamonadaceae	0.124	0.414	0.932	1.300	0.795	*Bacillus flexus*	0.310	0.736	1.735	2.180	2.690	g__Pseudomonas	0.306	0.955	2.186	2.970	3.150
	f__Xanthomonadaceae	0.118	0.393	1.325	0.713	0.165	g__Bacillus	0.227	0.540	2.275	1.620	2.050	f__Micrococcaceae	0.197	0.615	2.801	1.710	2.370
	f__Oxalobacteraceae	0.103	0.345	1.669	0.357	0.839	c__[Chloracidobacteria]	0.206	0.490	2.765	1.180	1.200	g__Burkholderia	0.194	0.606	3.407	3.980	4.020
	g__Pseudonocardia	0.101	0.339	2.008	2.660	2.350	g__Paenibacillus	0.198	0.472	3.236	1.060	1.610	g__Pseudomonas	0.172	0.536	3.943	0.380	0.928
	c__Chloroflexi	0.089	0.297	2.305	0.609	0.091	g__Bacillus	0.160	0.381	3.617	1.100	1.040	*Bacillus flexus*	0.167	0.522	4.465	1.280	0.733
	g__Cellulomonas	0.088	0.296	2.601	0.620	0.317	g__Bacillus	0.155	0.369	3.987	0.680	1.560	g__Bacillus	0.156	0.487	4.951	1.450	0.867
	g__Kaistobacter	0.088	0.295	2.896	0.298	0.681	g__Pseudonocardia	0.155	0.367	4.354	1.800	0.901	g__Burkholderia	0.145	0.452	5.403	0.411	0.820
	g__Bacillus flexus	0.088	0.293	3.189	0.235	0.693	f__Paenibacillaceae	0.137	0.324	4.678	0.318	0.887	g__Pseudomonas	0.128	0.398	5.801	0.682	0.258
	f__Gaiellaceae	0.082	0.274	3.463	0.846	0.880	g__Bacillus	0.126	0.299	4.978	0.324	0.749	g__Pseudomonas	0.124	0.387	6.189	0.767	0.821

Multivariate ordination on SIMPER tests

The SIMPER-PCA plot (Figure 4) does not show the composition of the communities such as the NMDS, but instead it shows the differences each inoculant has to its appropriate controls. In this plot, objects (data points) that cluster together present a similar set of differences in relation to the control, while an object that does not belong to the cluster has a different set of differences in relation to the control. The longer lines (colored red) indicate taxas that explain more variance on the treatment-control dataset. The longer lines (colored red) indicate taxas that explain more variance on the treatment-control dataset. Objects plotted in the direction of the lines presented more representatives of that particular taxa than the control, and those in the opposite direction of the line presented less representatives of that taxa than the control.

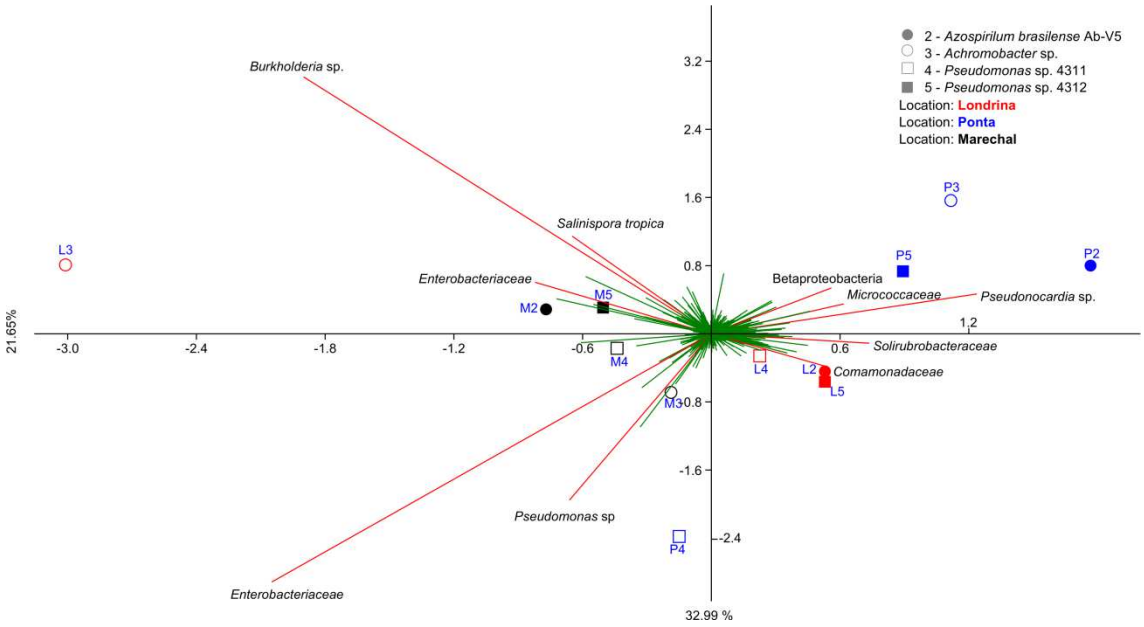


Figure 4: SIMPER-PCA approach based on OTU taxa level showing differences in treatment-control pairs. In this plot, a cluster of samples present a similar set of differences compared to the control, while a sample far from the cluster have a different set of differences to the control. Each line represents an OUT, with red lines explaining more variance than the green lines. Different colors show samples from different locations, with red = Londrina, Blue = Ponta Grossa, Black = Marechal Cândido Godoi; full circle= *Azospirillum brasilense* Ab-V5, empty circle = *Achromobacter* sp. VC36, empty square = *Pseudomonas* sp. 4311, and full square = *Pseudomonas* sp. 4312

As can be observed, Figure 4 also shows the SIMPER-PCA results at OTU level. Conditions L2, L4 and L5 from Londrina location clustered very closely, showing that most differences to control were associated to the presence of Comamonadaceae family

OUT representatives. Condition L3, however, had dissimilar dissimilarities to the control at Londrina, mostly associated to the presence of *Burkholderia* and Enterobactereaceae OTU representatives. Conditions P2, P3 and P5 from Ponta Grossa location also clustered together, with differences more associated to the presence of Betaproteobacteria, *Pseudonocardia* and *Micrococcaceae* OUT representatives, and the absence of Enterobacteriaceae representatives. Condition P4 (*Pseudomonas sp*. 4331), however, also had a different set of differences, more highly associated to the presence of a *Pseudomonas* sp. OTU. In the sense of impacting the environment in a different manner than other inoculants, condition P4 is similar to L3, but with a lower SIMPER value (38.03%). Although data points from Marechal Cândido Godoi location were not very spread, conditions M2 and M5 were separated from conditions M3 and M4 by the 2º Principal Component (PC).

Analysis of pre-planting conditions

Table 2 shows the soil chemical and nutrient analysis for each soil sampling at pre-planting condition, and Figure 5 shows the diversity indexes for those pre-planting conditions. The normalized nutrients per diversity indexes, compared to normalized SIMPER values, are shown on Figure 6. Londrina was the location with the highest relative nutrient per diversity ratio, and was the one with the highest relative invasion value. The Marechal Cândido Godoi location presented a slightly higher nutrient per diversity ratio and SIMPER values than the Ponta Grossa location. Together, this result supports the hypothesis that an environment with free niches would be easier to invade; although the relationship would better fit by a non-linear correlation. The SIMPER analysis was also used on pre-planting conditions at OUT level (Table S2), using Londrina as a standard compared to Marechal Cândido Godoi and Ponta Grossa locations (39.05% and 38.43% dissimilarity, respectively). The 20 most dissimilar OTUs from both comparisons showed that Londrina had more *Actinobacteria* representatives, especially from the *Thermoleophilia* Class, and less representatives of the Bacillaceae family.

Table 2: Soil chemichal analysis from pre-planting conditions on the different locations

Location	pH	Clay	Organic Matter	P	K	Ca	Mg	S	Zn	B	Mn
		%		($mg.dm^{-3}$)		($nmol_c\ dm^{-3}$)					
Londrina	5.7	5.9	3.3	6.4	339	6.7	2	11	3.2	0.5	30
Marechal	5.4	6	3.2	11	173	5	2.6	25	0.9	0.7	25
Ponta	6.1	2.2	7.2	7.9	292	5.3	3.6	15	4.1	0.4	7

Taxa level	Location	Simpson	Shannon	Eveness
Phyla	L0	0.230298	0.220176	0.180859
	M0	0.40249	0.424558	0.474808
	P0	0.367212	0.355265	0.344333
Class	L0	0.298026	0.262097	0.177328
	M0	0.357482	0.394538	0.481367
	P0	0.344492	0.343365	0.341305
Order	L0	0.312499	0.269693	0.165552
	M0	0.347999	0.383845	0.482377
	P0	0.339503	0.346462	0.352071
Family	L0	0.323838	0.291027	0.178379
	M0	0.338738	0.360263	0.439643
	P0	0.337423	0.34871	0.381977
Genera	L0	0.316487	0.277232	0.151065
	M0	0.342266	0.366123	0.450038
	P0	0.341248	0.356645	0.398897
Otu	L0	0.332812	0.325137	0.263144
	M0	0.334376	0.344951	0.399536
	P0	0.332812	0.329911	0.33732

Figure 5: Diversity indexes from pre-planting conditions of the three locations, sorted by taxa level. Color gradient shows lower values in red, higher values in green. L0 = Londrinha, M0 = Marechal Cândido Godoi, P0 = Ponta Grossa

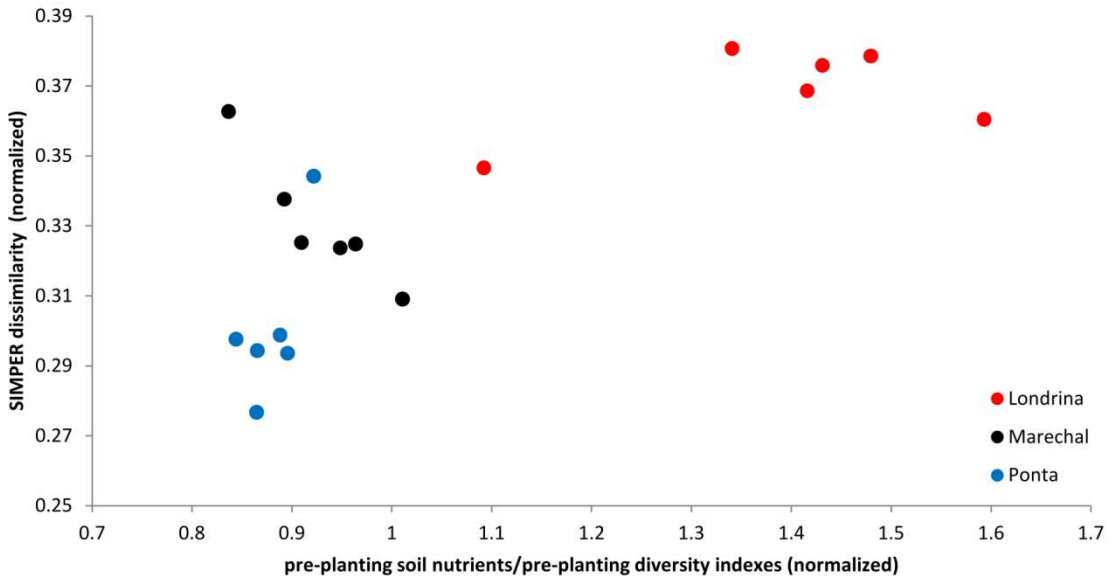


Figure 6: Nutrient per diversity ratios of pre-planting conditions, compared to SIMPER values of control-treatments pairs after inoculation. Each point represents one taxa level for each of the locations. All values were transformed to relative percentages, as in a constrained analysis

Discussion

The SIMPER-PCA approach: visualizing control-treatment dissimilarities

The clustering of the OTUs by location observed in NMDS (Figure 2) was largely expected, as geographic distance is one of the key limiting factors for bacterial composition in different environments (Hanson et al., 2012), and was evidently more important than the effect of the inoculation treatment on soil, otherwise data points would cluster by treatment. The observed phyla distribution (Figure 1) is typical for rhizospheric soil (Berg et al., 2013). Although we cannot take more conclusions from these plots with our experimental design, the SIMPER results (Table 1) provide an extensive description of the treatment-control differences, that we aimed to correlate to PGPB effectiveness. Several works already provided interesting analysis exploring both the tabled SIMPER results and ordination methods or permutation tests (Thomas et al., 2012; Stevens and Olson, 2013; Wilkins et al., 2013; Wang et al., 2014). Yet, to the best of our knowledge, this is the first attempt to visualize SIMPER results on ordination methods.

The SIMPER-PCA approach facilitated the interpretation of the SIMPER results, and allowed new hypothesis that were not evident on NMDS to be formulated. This approach is interesting because it can visualize the difference related to a defined standard (such as the control) and could be applied to a large number of reports that rely on cluster analysis from ordination methods. To apply this approach, three issues must be addressed: (I) the SIMPER test is based on the Bray-Curtis distance, so for consistency is appropriate to use this distance on other tests (such as ANOSIM, PERMANOVA or NMDS) (Wang et al., 2014); (II) Taxa frequency should be square root transformed to adjust for weights of dominant taxa (Clarke and Warwick, 2001); and (III) The SIMPER dissimilarities per taxa must be signal-transformed according to total abundances, as explained below.

The SIMPER test output from the PAST software (Table 1) shows what are the differences of each taxa between compared datasets, and also shows the average abundances of each taxa for each dataset. The average dissimilarity output does not show directly if one taxa is more present on dataset 1 (control) or on dataset 2 (treatment). If the collective SIMPER dissimilarities are directly applied on a PCA, it would be impossible to tell if one condition had more or less of a certain taxa than the control, confounding taxa excesses with taxa absences. An example can be found at Table 1, on the average abundances of *Paenibacillus* OTU from samples M2, M3 and

the appropriate controls for Marechal Cândido Godoi location. Compared to control, M2 has about half the abundance of this OUT (0.580), while M3 has about twice the abundance of this OUT (2.080). Since both have a abundance difference of about 50% in relation to the control, the average dissimilarity of this same OTU on M2 and M3 samples is almost the same (0.174 and 0.180, respectively). If this dissimilarity of almost equal value is imputed on a PCA analysis, conditions M2 and M3 would be almost on the same spot in the ordination plot, ignoring their differences on this OTU. In fact, conditions M2 and M3 should be in opposite directions on the plot, reflecting either the excess or the lack of this OTU. In addition, as all other OTUs are added in the ordination plot, there will be almost no taxa associated to negative values of the first principal component, making visualization of the plot less clear and the arrangements resembling artifacts (Figure S1). However, if the average dissimilarity is multiplied by -1 when the control average abundance is higher than the treatment average abundance, the signal shift will differentiate the two OTUs exemplified above, letting conditions M2 and M3 separate when plotted on the ordination method. This signal shift will also transform the dataset to a normal distribution (Figure S2). This transformation is appropriate as the variance-covariance matrix on which the PCA is based performs better with normally distributed data, since the PCA assumes linear correlations.

The SIMPER data could be used on other ordination methods. Categorical Principal Component Analysis (CatPCA) can evaluate non-linear correlations with ordinal and nominal data (Linting et al., 2007); NMDS is free from assumptions of normality; and Canonical Correspondence Analysis (CCA) can include explanatory environmental variables (Ramette, 2007) – or even use the SIMPER data as the explanatory variables. Applying our approach, the control samples will not appear on the SIMPER-PCA plot, as they were used as the standard. Additional information on the control treatments that could be added on the plot, such as plant biomass or nutrient concentration, does not have to be discarded. Biomass data from treatments could be transformed to percentages or fold differences to control, before input in the ordination method. This could correlate higher biomass to higher differences to a certain taxa. Appropriate sample sizes should be kept in mind: in this work, we could not use the SIMPER-PCA approach on the pre-planting conditions (Table S2) as we only had two points to plot on the PCA.

Impact on community does not predict PGPB effect

The impact of inoculation on the rhizosphere native bacterial community has already been studied for some time (Gilbert et al., 1996; Ambrosini et al., 2015). This impact, and also PGPB effectiveness, is known to depend on many factors (Castro-Sowinski et al., 2007; Owen et al., 2015). Evaluation of invasion ability as observable community impact has also already been considered (Parker et al., 1999), but we could not find reports that use SIMPER differences to measure invasion ability.

Based on the SIMPER-PCA approach (Figure 4), it can be observed that no single inoculant induced the same set of differences in the different locations, which would characterize a consistent invasion effect. The impact of the inoculant on the environment was strongly dependent on the location. Also based on the SIMPER-PCA approach, we raised the hypothesis that treatment L3 would have the highest agronomical productivity, since it was the treatment with the highest SIMPER value and also had a different set of differences from the other inoculants at the same location. Since treatment P4 also had a different set of differences to control when compared to other inoculation treatments at the same location, we raised another hypothesis that treatment P4 would also have a higher productivity, at least compared to treatments in the same location. Finally, since the samples from Marechal Cândido Godoi location were divided by the 2º PC, we raised the third hypothesis that this division would be also noticed on agronomical productivity.

None of the above hypothesis was held by field data. Field results showed that L4 (*Pseudomonas* sp. 4311 strain inoculated in Londrina location) was the one that presented the best PGP results, as maize plants inoculated with *Pseudomonas* sp. 4311 strain presented 37% higher productivity then the control plants (Carolina Galvão, personal communication), while all other treatments were statistically similar to each other and to the respective controls. This shows that the condition with the highest SIMPER values (L3) was not the one that presented the best productivity, and plants inoculated with strains that presented a different set of differences (L3 and P4) did not present the most productive results. PGPB invasion ability, as measured here, is not a predictor of inoculant efficiency. However, as *Rhizobium* inoculants impact the environment more than free-living inoculants (Ambrosini et al., 2015), just as they are also more efficient PGPB than free living inoculants due to legume symbiosis, the best invader might be the best inoculant in their particular case.

Treatment L4, that had a distinguishable PGP effect, had no distinguishable features in any of the analysis made. With the SIMPER approach on pre-planting soils

using Londrina as a standard (Table S2), we could argue that the higher abundance of *Actinobacteria* from the Thermoleophilia Class facilitated the display of plant growth promotion by L4. Alternatively (or in addition to) it was the lower abundance of *Bacillales* that facilitated the plant growth effect, as it might have been acting as a specific antagonist. Both *Actinobacteria* and *Bacillus* can act as antagonist in soils and produce antibiotics (van Elsas et al., 2012), so they have the potential to largely influence rhizosphere interactions. These synergic or antagonistic effects to inoculant 4 (*Pseudomonas* sp. 4311) could be tested in greenhouse trials, by enriching or suppressing these taxa.

Free niches facilitate invasion and could help PGPB efficiency

Our sampling sizes were limited, diversity and nutrient availability were not manipulated to better test the hypothesis, and we used an oversimplification to measure invasion and invasibility (Davis and Pelsor, 2001). Also, it has been proposed that is not diversity itself that can increase resistance to invasion, but, instead, the ability of the communities to exploit available resources (Malon et al., 2015). Yet, the nutrient per diversity ratio could still be useful when choosing soils, strains and crops for inoculation. The Londrina location was the one with the highest nutrient per diversity ratio, SIMPER values (Figure 6), and inoculation efficiency. Perhaps the L4 treatment was efficient because the *Pseudomonas* sp. 4331 strain could better survive or colonize the plant due to lower competition (Compant et al., 2010), supporting our hypothesis. Alternatively (or in addition to), L4 had a more favorable bacterial community to coexist with, such as one with less specific antagonists.

While it is already well known that bacterial inoculation can help crop production, there could be other forms of manipulating the microbial community and the environment to maximize beneficial plant-bacteria interactions. This would be very different from traditional crop research in the post-war period (Dawson and Hilton J, 2011), where plant-fertilizer interactions were optimized in lieu of plant-bacteria interactions (Philippot et al., 2013), that might not be very effective under intense fertilization (Costa and Passaglia, 2015). In fact, some level of stress to plants optimizes their beneficial interactions with microorganisms (Güneş et al., 2014).

Experiments that manipulate and evaluate the bacterial community and the availability of nutrients from pre-planting conditions could correlate those factors to an effective PGPB effect. Fumigating soils can temporarily suppress the native community and facilitate microbial invasions (Liu et al., 2015; Yakabe et al., 2010), but there are

many important ecological services provided by soil bacteria that could be disrupted by fumigation (Griffiths et al., 2000). Fumigation is chemically intensive, non-specific, and not ecologically friendly (Liu et al., 2015). For this, fumigating soils should not be the first option to alter the nutrient per diversity ratio.

Addition of nutrients to soil to improve plant growth is a method used for centuries, but we might consider to "fertilizing" the bacteria instead of the plants to improve inoculation efficiency. If at the time of inoculation soils also receive nutrients usable by bacteria, this would increase the nutrient per diversity ratio, and could improve inoculant survival and colonization. Soil bacteria are often limited by carbon, a resource liberated by plant roots through exudation that affects some bacteria more than others (Castro-Sowinski et al., 2007). This pool of resources in soil is subject of competition between the inoculant and the native community. Addition of carbon sources at inoculation might make the native community less attracted to the exudates and more attracted to the added carbon sources, liberating rhizosphere niches and making the colonization easier for the inoculant. It has already been shown that resource pulses can increase an invader's persistence in soil (Malon et al., 2015; van Elsas et al., 2007), and this effect has also been observed on plant invasions under fluctuating resources availability (Davis et al., 2000). Baby and Manibhushanrao (1993) also demonstrated that the simultaneous addition of fungal antagonists and organic amendments can increase plant protection, plant biomass, and inoculant CFUs. In this scenario, the inoculant would also be drawn to the added carbon source. It is well known that the carrier for the inoculant can affect PGPB effectiveness, and they can be used to provide nutrients and niches to the inoculant (Bashan et al., 2002). These additions aim specifically to increase the survival of the inoculant, and may include sugar, glycerol, gum arabic or others (Bashan et al., 2013).

If a carbon source that could be used by the native community but not by the inoculant is added to the soil in the moment of inoculation, then an effective "diversion" might happen. The native rhizospheric community could swarm to the added nutrient, freeing the root niche for the inoculant, which is not attracted by the added nutrient. r-strategists, which are typical for the rhizosphere of young roots and quickly consume easily degradable substrates (van Elsas et al., 2007), might be especially affected by this approach. The carbon source could be specifically chosen based on the inoculant and the native communities' metabolism capabilities or the inoculant could have a suppressive mutation on an easily degradable source, like glucose. There are several

factors that could interfere in this (like motility, chemotaxis, substrate use, plant development, competition, plant exudates, and soil type), but positive results could be useful to the inoculants companies.

There are many forms of manipulating the environment and the associated microbial community, including non-intentional ones, which could use bacterial inoculations to improve crop production or prevent environmental damage. Undesired effects of pesticide application like the loss of nitrifiers (Jacobsen and Hjelmsø, 2014) could be minimized or avoided if nitrifiers are inoculated afterwards. Inconsistencies in pathogen suppression by fumigation can be due to the re-colonization of the pathogen after the diversity loss (Yakabe et al., 2010). A spore-forming biocontrol agent, like *Bacillus* strains, could survive fumigation effects, and prevent pathogen re-colonization. The impacts actually caused by farming practices could even be an opportunity in favor of the farmer. Glyphosate application might increase *Burkholderiales* in soil (Lancaster et al., 2010), and reduce the number of carbon substrates explored by the community when mixed with 2,4-D (Lupwayi et al., 2009). This could actually help in *Burkholderia* inoculation that could be used as a biocontrol or pesticide-degrading agent. Application of different kinds of biochar or NPK fertilizers can also affect local communities (Sun et al., 2016; Shang and Yi, 2015), creating a window of opportunity for bacterial inoculation. If farmers consider who are the actual residents in the local soils and know how they may react to inputs, microbiome manipulation by strain inoculation could counter impacts before they happen, or make use of such impacts to further optimize desired plant-bacteria interactions.

Conclusions

With the results obtained in this work, the hypothesis that the best invader would be the best PGPB was rejected. Inoculant invasion ability, measured as dissimilarity of the inoculated treatments compared to the control, was not associated to an effective PGPB effect. The location with higher nutrients per diversity ratio had higher invasion, possibly because it had more free niches and lower competition. This location was also the only one with positive PGPB effect. Even as invasion ability of the inoculant itself was not associated to positive PGPB effect, an environment with higher invasibility might improve PGPB effectiveness. We conclude that interactions with the initial native bacterial communities, including competition for available resources, are a key factor for PGPB effectiveness.

We urge that more attention is given to microbial communities before planting, especially with NGS tools. If the microbial community is to be manipulated by inoculation, its structure cannot be ignored. Considering that NGS costs are reducing drastically over time, and food prices and the need for food production increase over time, NGS might be a standard tool for common farmers within less than 30 years. Just like today farmers take pH measurements of their soils to calculate the right inputs for optimal productivity, they could use NGS at pre-planting stages or before addition of inputs to calculate the proper microbial management strategy. For example, a soil with many antagonists to a *Pseudomonas* PGP effect should not be inoculated with a *Pseudomonas* PGPB. This is a long term goal and requires much research, but as long as there is enough food to feed scientists, NGS research on plant-soil interactions will not stop.

Acknowledgements

We deeply thank Dr. André Luiz Martinez de Oliveira from Estate University of Londrina, Dr. Carolina Weigert Galvão from Estate University of Ponta Grossa, Dr. Vandeir Francisco Guimarães, from Estate University of West Paraná by performing all the field experiments and sending us the samples for all analysis. This work was financed by a grant and fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Deutscher Akademischer Austauschdienst (DAAD/Germany) and Instituto Nacional de Ciência e Tecnologia (INCT) da Fixação Biológica do Nitrogênio (Brazil).

References

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

Ambrosini A, de Souza R, Passaglia LMP (2015) Ecological role of bacterial inoculants and their potential impact on soil microbial diversity. Plant Soil 400: 193–207. doi:10.1007/s11104-015-2727-7.

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. doi:10.1016/j.apsoil.2012.09.001.

Baby UI, Manibhushanrao K (1993) Control of rice sheath blight through the integration of fungal antagonists and organic amendments. Trop Agric 70:240–44.

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

Bashan Y, Hernandez J-P, Leyva L, Bacilio M (2002) Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. Biol Fertil Soils 35: 359–368. doi:10.1007/s00374-002-0481-5.

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: 648–656. doi:10.3390/agronomy3040648.

Caporaso JG, Lauber CL, Walters WA, Berg-lyons D, Lozupone CA, et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. PNAS 108: 4516–4522. doi:10.1073/pnas.1000080107

Castro-Sowinski S, Herschkovitz Y, Okon Y, Jurkevitch E (2007) Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. FEMS Microbiol Lett 276: 1–11. doi:10.1111/j.1574-6968.2007.00878.x.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18: 117–143.

Clarke KR, Warwick, RM (2001) Change in marine communities: An approach to statistical analysis and interpretation. (2nd ed.) PRIMER-E, Plymouth, 172 pp .

Compant S, Clément C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42: 669–678. doi:10.1016/j.soilbio.2009.11.024.

Costa PB, Passaglia LMP (2015) Chapter 95: How fertilization affects the selection of plant growth promoting rhizobacteria by host plants. In: Biological Nitrogen Fixation, vol 2. Frans J. de Bruijn (editor), Wiley-Blackwell; 1º edition, July 2015. 1260p.

Davis MA, Grime JP, Thompson KEN (2000) Fluctuating resources in plant communities : a general theory of invasibility. J Ecol 88: 528–534.

Davis MA, Pelsor M (2001) Experimental support for a resource-based mechanistic model of invasibility. Ecol Lett 4: 421–428.

Dawson CJ, Hilton J (2011) Fertiliser availability in a resource-limited world: Production and recycling of nitrogen and phosphorus. Food Policy 36: S14–S22. doi:10.1016/j.foodpol.2010.11.012.

Germaine KJ, McGuiness M, Dowling DN (2013) Chapter 8: Engineering of microbes for plant and soil systems. In: Applications of Microbial Engineering. Gupta VK, Schmoll M, Maki M, Tuohy M, Mazutti MA (editors), CRC Press; 1º edition, June 2013. 504p.

Gilbert GS, Clayton MK, Handlesman J, Parke J. (1996) Use of cluster and discriminant analysis to compare rhizosphere bacterial communities following biological perturbation. Microb Ecol 32: 123–147.

Griffiths BS, Ritz K, Bardgett RD, Cook R, Christensen S, et al. (2000) Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions : an examination of the biodiversity – ecosystem function relationship. OIKOS 90: 279–294.

Güneş A, Turan M, Güllüce M, Şahin F (2014) Nutritional content analysis of plant growth-promoting rhizobacteria species. Eur J Soil Biol 60: 88–97. doi:10.1016/j.ejsobi.2013.10.010.

Hammer Ø, Harper DAT, Ryan PD (2001). PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4(1): 9pp.

Hanson C a, Fuhrman J a, Horner-Devine MC, Martiny JBH (2012) Beyond biogeographic patterns: processes shaping the microbial landscape. Nat Rev Microbiol 10: 497–506. doi:10.1038/nrmicro2795.

Hungria M, Campo RJ, Souza EM, Pedrosa FO (2010) Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. Plant Soil 331: 413–425. doi:10.1007/s11104-009-0262-0.

Jacobsen CS, Hjelmsø MH (2014) Agricultural soils, pesticides and microbial diversity. Curr Opin Biotechnol 27: 15–20. doi:10.1016/j.copbio.2013.09.003.

Lancaster SH, Hollister EB, Senseman S a, Gentry TJ (2010) Effects of repeated glyphosate applications on soil microbial community composition and the mineralization of glyphosate. Pest Manag Sci 66: 59–64. doi:10.1002/ps.1831.

Linting M, Meulman JJ, Groenen PJF, Van der Kooij AJ (2007) Nonlinear principal components analysis: Introduction and application. Psychological Methods. 12(3):336-58.

Liu X, Cheng X, Wang H, Wang K, Qiao K (2015) Effect of fumigation with 1,3-dichloropropene on soil bacterial communities. Chemosphere 139: 379–385. doi:10.1016/j.chemosphere.2015.07.034.

Lupwayi NZ, Harker KN, Clayton GW, O'Donovan JT, Blackshaw RE (2009) Soil microbial response to herbicides applied to glyphosate-resistant canola. Agric Ecosyst Environ 129: 171–176. doi:10.1016/j.agee.2008.08.007.

Mallon CA, Elsas JD Van (2015) Microbial Invasions : The Process, Patterns, and Mechanisms. Trends Microbiol 23: 719–729.

Mallon CA, Poly F, Roux X, Marring I, van Elsas JD, et al. (2015) Resource pulses can alleviate the biodiversity – invasion relationship in soil microbial communities. Ecology 96: 915–926.

Mills DK, Entry JA, Voss JD, Gillevet PM, Mathee K (2006) An assessment of the hypervariable domains of the16S rRNA genes for their value in determining microbial community diversity : the paradox of traditional ecological indices. 57: 496–503. doi:10.1111/j.1574-6941.2006.00135.x.

Otieno BN, Culhane J, Germaine KJ, Brazil D, Ryan D, et al. (2013) Screening of large collections of plant associated bacteria for effective plant growth promotion and colonisation. Asp Appl Biol 120: 23–28.

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: 41–54. doi:10.1016/j.apsoil.2014.09.012.

Parker IM, Simberloff D, Lonsdale WM, Goodell K, Wonham M, et al. (1999) Impact : toward a framework for understanding the ecological effects of invaders: 3–19.

Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH (2013) Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev Microbiol 11: 789–799. doi:10.1038/nrmicro3109.

Ramette A (2007) Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 62: 142–160. doi:10.1111/j.1574-6941.2007.00375.x.

Rees GN, Baldwin DS, Watson GO, Perryman S (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms : application of multivariate statistics. Antonie Van Leeuwenhoek 86: 339–347.

Satomi M. (2014) Chapter 31: The Family Shewanellaceae in: The Prokaryotes: Gammaproteobacteria. Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F. Springer, 4º edition. 409 p.

Shang S, Yi Y (2015) A greenhouse assay on the effect of applied urea amount on the rhizospheric soil bacterial communities. Indian J Microbiol 55: 406–414. doi:10.1007/s12088-015-0551-7.

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.

Stevens JL, Olson JB (2013) Invasive lionfish harbor a different external bacterial community than native Bahamian fishes. Coral Reefs 32: 1113–1121. doi:10.1007/s00338-013-1072-7.

Sun D, Hale L, Crowley D (2016) Nutrient supplementation of pinewood biochar for use as a bacterial inoculum carrier. Biol Fertil Soils. doi:10.1007/s00374-016-1093-9.

Thomas T, Gilbert J, Meyer F (2012) Metagenomics - a guide from sampling to data analysis. Microb Inform Exp 2: 3. doi:10.1186/2042-5783-2-3.

van Elsas JD, Chiurazzi M, Mallon C a, Elhottova D, Kristufek V, et al. (2012) Microbial diversity determines the invasion of soil by a bacterial pathogen. PNAS 109: 1159–1164. doi:10.1073/pnas.1109326109.

van Elsas JD, Torsvik V, Hartmann A, Ovreas L, Janson JK (2007) Chapter 4: The Bacteria and Archaea in soil. In: Modern Soil Microbiology. Edited by van Elsas JD, Trevors JT, Janson JK. CRC, 2º edition, 2007. 646p

Wang J, Linnenbrink M, Künzel S, Fernandes R, Nadeau M, et al. (2014) Dietary history contributes to enterotype-like clustering and functional metagenomic content in the intestinal microbiome of wild mice. PNAS 1–8. doi:10.1073/pnas.1402342111.

Wilkins D, Lauro FM, Williams TJ, Demaere MZ, Brown M V, et al. (2013) Biogeographic partitioning of Southern Ocean microorganisms revealed by metagenomics. Environ Microbiol 15: 1318–1333. doi:10.1111/1462-2920.12035.

Yakabe LE, Parker SR, Kluepfel DA (2010) Effect of pre-plant soil fumigants on *Agrobacterium tumefaciens*, pythiaceous species, and subsequent soil recolonization by *A. tumefaciens*. Crop Prot 29: 583–590. doi:10.1016/j.cropro.2010.01.001.

Supplementary material

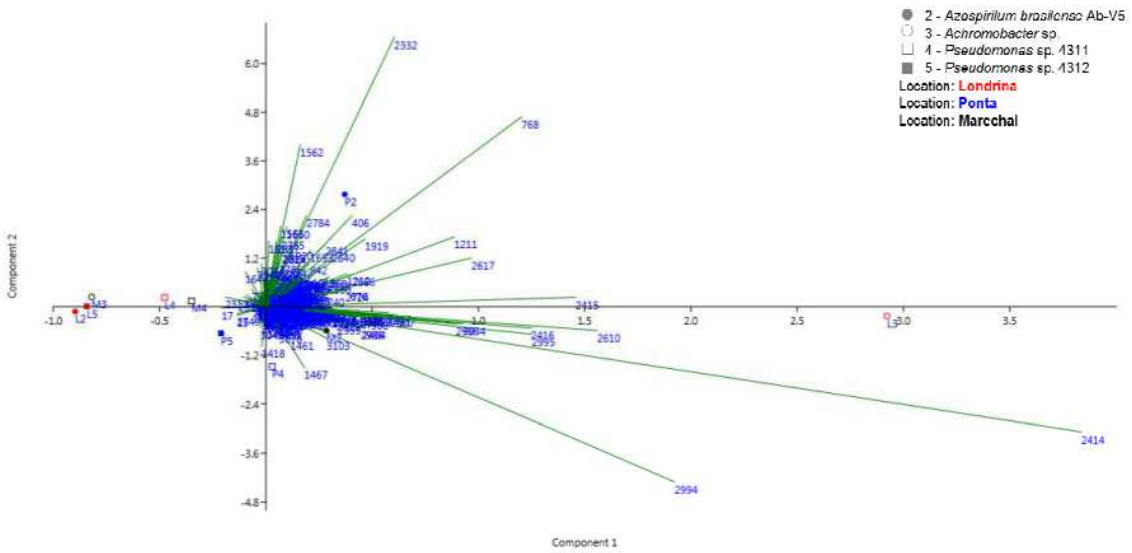


Figure S1: Exemple of the SIMPER-PCA aproach at OTU level without transforming the signal as suggested in the paper. Diferent numbers represent different OTUs

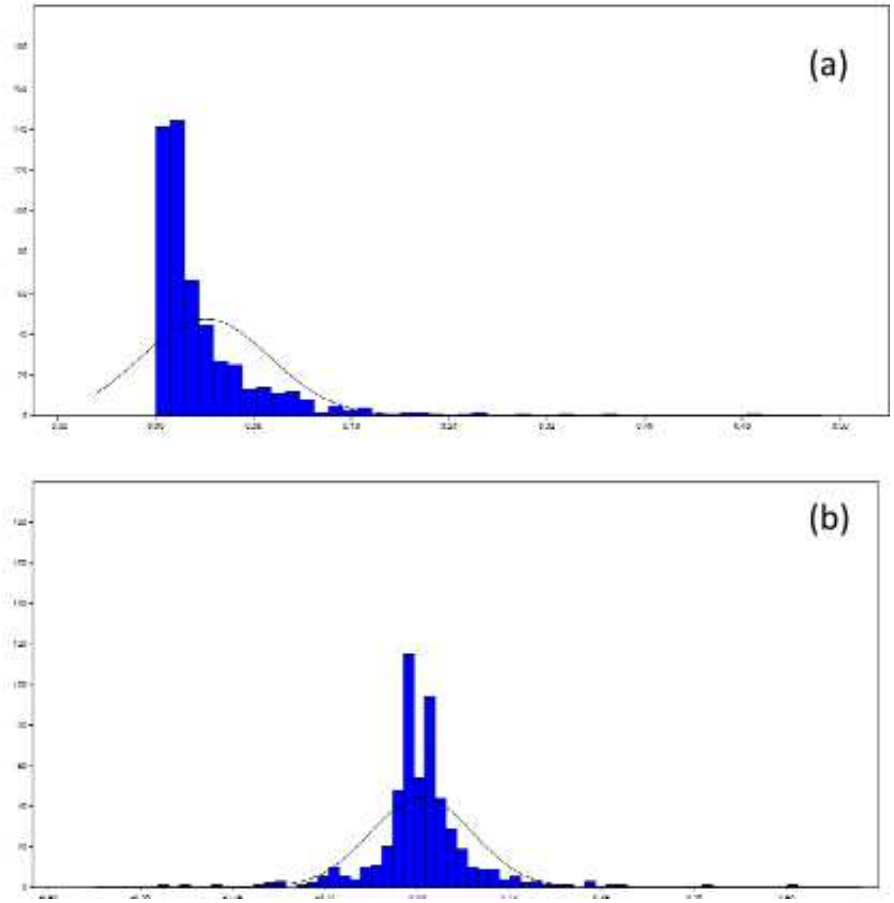


Figure S2: Histograms for frequencies of the average dissimilarities calculated from SIMPER tests as given by the PAST output (a) and with the signal modification (b). The curved line shows the teoretical normal distribution for the dataset

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 – 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 – plant age – 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 don't 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 – 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 – idade da planta – 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: 585–603. 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: 339–358. 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 systems : Diversity and antagonistic activity against *Colletotrichum gloeosporioides*. Genet Mol Biol 572: 560–572.

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: 111–121.

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: 191–197. 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 – Brazilian-Argentine Biotechnology Center "Microorganism-plant-soil interactions: biotechnological innovations for a sustainable agriculture, biocontrol and bioinoculation." From August 5 to 16 (2013), in Buenos Aires, Argentina.

Honorous mention - Prof. Milton Krieger Prize – 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 – 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* – 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: 585–603. 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: 339–358. 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 systems : Diversity and antagonistic activity against *Colletotrichum gloeosporioides*. Genet Mol Biol 572: 560–572.

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: 111–121.

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: 191–197. 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 – Centro brasileiro-Argentino de Biotecnologia. “Interações Microorganismo-planta-solo: Inovações biotecnológicas para uma agricultura sustentável, biocontrole e bioinoculação” de 5 a 16 de Agosto (2013), em Buenos Aires, Argentina.

Menção honrosa – Prêmio Prof. Milton Krieger – 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 – 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* – 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.