https://doi.org/10.35662/unine-thesis-2755

Improving bio-inoculation for sustainable agriculture

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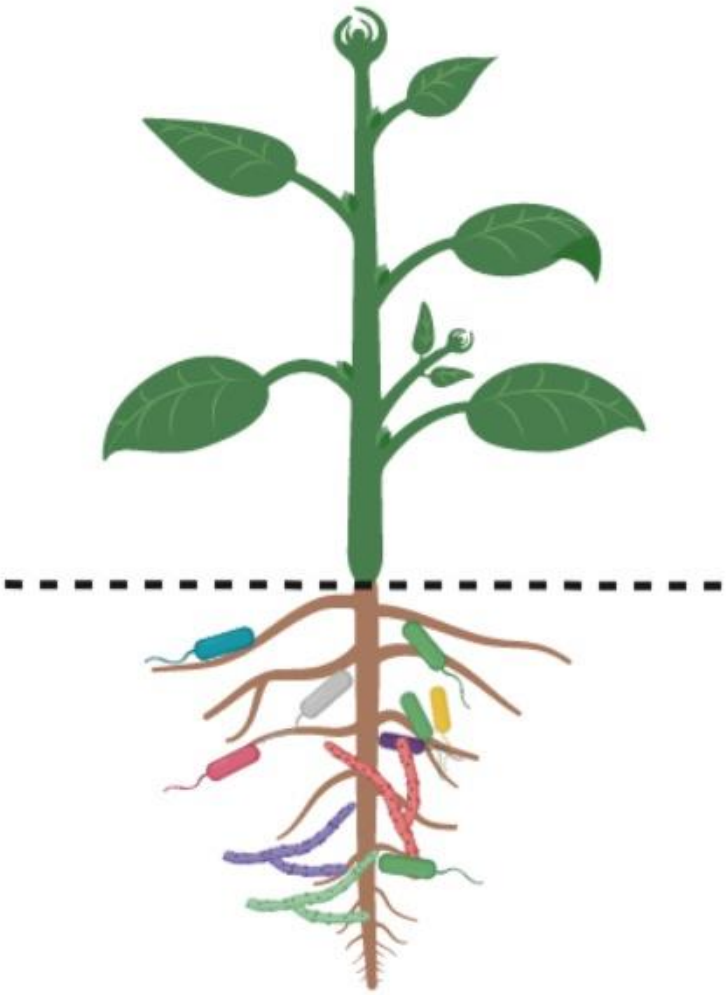
University of Neuchâtel, 2019



Improving bio-inoculation for sustainable agriculture

PhD thesis dissertation submitted to the University of Neuchâtel by

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Neuchâtel, le 17 mai 2019

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Summary

Global environmental pollution is one of the greatest threats to the inhabitants of planet Earth. Among the different types of environmental problems, the present study discusses issues related to the extensive utilization of agrochemicals in conventional agriculture by focusing on plant health and soil microbes as integral parts of terrestrial ecosystems. In conventional agriculture, inappropriate management practices and the use of agrochemicals have led to a negative impact on both soil and water. As a result, the search for sustainable alternatives in agriculture has become a priority. In the mycorrhizosphere, microbes are involved in many beneficial activities supporting the whole soil ecosystem and thus are considered as crucial actors of soil functioning. With such a perspective, an *in vitro* screening was carried out to evaluate the plant growth promoting (PGP) activities of different endospore forming bacteria (EFB), as well as of fungi with different ecological niches. We found that most of the screened EFB possessed one or more PGP traits. The fungal species that were screened were also positive for PGP traits. Moreover, confrontation of these bacteria and fungi resulted in many different interactions (antagonistic, neutral or positive). Interestingly, these findings did not only depend on the competing microbes, but also on the medium used, pointing at the importance of the nutritional context in microbial interactions. After the *in vitro* screening, the PGP effect of three different *Bacillus* strains was assessed *in planta*. Different bacterial inoculation schemes were compared: each strain alone or the three as a consortium and both life forms, vegetative cells and endospores, were compared. Then, the effect of the bio-inoculants was measured at different levels of complexity, from *in vitro* conditions to a field trial. This step-wise approach allowed deciphering whether more complex bio-inoculants (as compared to single strains) were more effective in promoting plant growth and whether this had any impact on the autochthonous microbial communities. The results obtained demonstrated that bacterial consortia were more consistent in promoting plant growth than single strains, even when in contact with the autochthonous soil microbes. Importantly, the bio-inoculated bacteria did not affect the structure of the native microbial communities, as demonstrated by using targeted metagenomics on both fungal and bacterial communities. This thesis also highlighted microbial interactions between members belonging to two kingdoms, i.e.

bacteria and fungi. Both of them play crucial roles in terrestrial ecosystem functioning and are known for their diversified nutritional capabilities. This can lead to all type of interactions, from positive to negative ones. In this thesis, one antagonistic interaction, the mycophageous life-style of bacteria was investigated in detail. In mycophagy, bacteria obtain nutrients from living fungi and exert a negative impact on the fungal partner. The findings of this work suggest that the bacterium *Lysinibacillus sphaericus* 1003 has a preferential mycophagous lifestyle towards the phytopathogenic fungus *Rhizoctonia solani,* while it does not harm the saprophytic *Trichoderma rossicum*. The bacterium can grow at the expense of the living mycelium of *R. solani* and, more importantly, has a negative impact on fungal biomass. In contrast, and even though *L. sphaericus* 1003 grew at the expense of *T. rossicum*, the bacterium has a positive effect on *T. rossicum* biomass. Therefore, this differential behavior towards a plant pathogen and a plant beneficial fungus could constitute an interesting model to better understand the role of mycophagy in soil carbon cycling. Overall, this study brings novel insights in the frame of a sustainable exploitation of agricultural ecosystems by combining different types of bio-inoculation methods. Moreover, aspects related to future developments required in the field of plant growth promotion and biocontrol are discussed at the end of this study.

Résumé

La pollution environnementale globale est l'une des plus grandes menaces pour les habitants de la planète. Parmi les différents types de problèmes environnementaux, la présente étude discute de questions liées à l'utilisation intensive des produits agrochimiques dans l'agriculture conventionnelle, en se focalisant sur la santé des plantes et sur les microorganismes du sol en tant que parties intégrantes des écosystèmes terrestres. En agriculture conventionnelle, des pratiques de gestion inadaptées et l'utilisation de produits agrochimiques ont un impact négatif à la fois sur le sol et sur la qualtié de l'eau. La recherche de solutions alternatives durables est ainsi devenue une priorité. Dans la mycorrhizosphère, les microorganismes sont impliqués dans de nombreux processus biologiques bénéfiques soutenant l'ensemble de l'écosystème sol, et sont ainsi considérés comme des acteurs cruciaux du fonctionnement des écosystèmes.

Dans cette perspective, une évaluation *in vitro* des activités favorisant la croissance des plantes (PGP) chez différentes bactéries endosporulantes (EFB), ainsi que chez des champignons ayant différentes niches écologiques. Nous avons trouvé que la plupart des EFB testées possèdent des traits PGP. C'est également le cas des espèces de champignons testées. De plus, la confrontation entre ces bactéries et champignons a donné lieu à différentes interactions (antagonistes, neutres et positif). De manière intéressante, ces observations n'étaient pas uniquement dépendantes des microbes en concurrence, mais également du milieu de culture utilisé, soulignant l'importance du contexte nutritionnel dans les interactions microbiennes. A la suite de l'évaluation *in vitro*, l'effet PGP sur des plantes de trois différentes souches de Bacillus a été évalué. Différents schémas d'inoculation ont été comparés : chaque souche seule ou sous la forme d'un consortium des trois souches, ainsi que deux types de formes de vie, les cellules végétatives et les endospores.

Ensuite, l'effet des bio-inoculants a été mesuré à différents niveaux de complexité, allant de conditions *in vitro* à un essai en champs. Cette approche par étape a permis de déterminer si des bio-inoculants plus complexes (en comparaison aux souches seules) étaient plus efficaces à stimuler la croissance des plantes, et quel était leur impact sur les communautés microbiennes autochtones. Les résultats obtenus ont démontré que les consortiums étaient plus constants dans la stimulation de la croissance des plantes que les souches seules, même au contact des microbes

autochtones du sol. Fait important, les bactéries bio-inoculées n'ont pas affecté la structure des communautés bactériennes natives, comme démontré par l'utilisation de méthodes métagénomiques ciblant les communautés de champignons et de bactéries.

Cette thèse a aussi mis en évidence les interactions microbiennes entre des membres de deux règnes, i.e. les bactéries et les champignons. Chacun d'eux joue un rôle crucial dans le fonctionnement des écosystèmes terrestres, et sont connus pour la diversité de leurs modes de nutritions. Cela peut conduire tant à des interactions positives, neutres ou négatives. Dans cette thèse, une interaction antagoniste, c'est à dire le mode de vie mycophage des bactéries, a été étudiée en détail. Lors de la mycophagie, les bactéries obtiennent des nutriments directement d'un champignon vivant, exerçant un impact négatif sur ce partenaire fongique. Les découvertes de ce travail suggèrent que la bactérie *Lysinibacillus sphaericus* 1003 a un mode de vie mycophage préférentiel vis-à-vis du champignon phytopathogène *Rhizoctonia solani*, alors qu'il ne nuit pas au champignon saprophyte *Trichoderma rossicum*. La bactérie peut croître aux dépends du mycélium de *R. solani* et, plus important, elle a un impact négatif sur la biomasse fongique. Au contraire, et malgré que *L. sphaericus* 1003 croisse aux dépends de *T. rossicum*, la bactérie a un impact positif sur la biomasse de *T. rossicum*. Par conséquent, ce comportement différent vis-à-vis d'un pathogène de plante (*R. solani*) et d'un champignon bénéfique à la plante (*T. rossicum*) peut constituer un modèle intéressant pour mieux comprendre le rôle de la mycophagie dans le cycle du carbone dans les sols. Globalement, cette étude apporte de nouvelles connaissances et réflexions au domaine de l'agriculture durable puisqu'elle combine différentes approches de bio-inoculation. Finalement, cette étude contient de nouveaux éléments de discussions proposant des voies pour le développement futur de la stimulation de la croissance des plantes et le biocontrôle par l'utilisation de micro-organismes.

Acknowledgments

I gratefully thank my thesis supervisor Prof. Pilar Junier for accepting my PhD proposal and giving me the opportunity to work in her team, for opening doors to the world of Microbes to which I was not very familiar, for her continuous guidance, her endless support and her motivational words that always encouraged me throughout my research work. A special thanks to my co-supervisor Dr. Saskia Bindschedler for her immense guidance, support and patience from the beginning to the end of my work. It has been an honor to be her first PhD student. I acknowledged her contribution and funding (4th year) to make my PhD experience smooth and productive. I am highly indebted to both of them for providing such a healthy work environment, where I stayed focused while doing my work with full freedom; I learned so many things from you both, which helped me in shaping my personality.

I would also like to acknowledge the thesis jury Dr. Klaus Schlaeppi, Prof. Christoph Keel and Prof. Ted Turlings for accepting and evaluating this work.

I am very grateful to the Swiss Government for granting me a 'Swiss Government Excellence Scholarship', without which this work would not have been possible.

I would like to offer my gratitude to all the collaborators and specially Dr. Claire Stanley for the fruitful discussions and for providing material for microfluidics. Special thanks to Christoph Paul for his help, especially his magical tricks to solve problems using R software. Moreover, I would like to thank Dr. Thomas Junier for his kind help in the bioinformatics analysis.

Likewise, sincere wishes are extended to the past and present members of the laboratory of microbiology during these four years: more specifically Nicole Jeanneret for her support & teaching basics of microbiology, in the start of my PhD work, Andrej Al-Dourobi, Priscilla Deschamps, Frédéric Sandoz, Teddy Monrouzeau, Clément Etter and Celine Vallotton for their kind help in this research work. Thanks to Dr. Sevasti Filippidou for her support and motivational words.

Special thanks to Dr. Wafa Kooli for listening same old stories thousand times and thanks for her encouragement and support that I needed in my last months. Thanks to Kasimir Rüegg for all his jokes and his kind and loving attitude, he always reminds me of Dr. Vincent Hervé. Thanks to Andrea Lohberger for staying late in the lab with me and for eating pizza and Indian food, she and

Fabio Palmeri suffered a lot due to my eating habits during our stay in Spain at the FEMS meeting.

Thanks to Aislinn Estoppey for her kind help in using adobe illustrator.

Mariam Afzal, Maimona Hashmi and Tehreem Butt for their continuous support and Taiba Gul for dedicating nice songs for keeping my spirits high.

I would also like to thank my parents and my in-law parents especially late Prof. Asmat Hashmi who encouraged me to pursue this path.

And finally, last but not least Hussain Hashmi and Ihtsham Hashmi who were source of my happiness and my encouragement, have helped me survive with their love, support and patience.

Contents

Glossary

Inhibition: Restricted growth of a microorganism by another microorganism.

Antagonistic interaction: A negative or competitive interaction between two or more microorganisms resulting in the inhibition of one or more of them.

Neutral interaction: No competing or harmful interaction between two or more microorganisms, resulting in the absence of inhibition among the interacting microorganisms.

Synergistic interaction: A beneficial interaction where interacting microbes perform better (more than the additive effects) in the presence of each other (i.e. the whole is more than the sum of the parts) and where neither a competitive interaction, nor inhibition are present.

PGP bacteria: Plant beneficial bacteria having plant growth promoting activities.

PGP fungi: Plant beneficial fungi having plant growth promoting activities.

Rhizosphere: The narrow soil area surrounding the vicinity of the plant root. By definition, the soil that is adhering to the root upon sampling.

Mycorhizosphere: The soil volume influenced by the hyphal network of mycorrhizal fungi linked to plant roots.

Mycophagy: A trophic interaction in which bacteria grow at the expense of living fungal mycelium, but not on fungal exudates.

Chapter 1
Introduction

The introduction of the present PhD thesis highlights the problems of conventional agriculture, as well as the importance of the use of microorganisms (endospore forming *Bacillus* species and fungi of different ecological niches) as an alternative to those practices. It also sheds light on bacterial-fungal interactions.

Limits of conventional agriculture

Global population growth is currently putting a tremendous pressure on agriculture because of the decrease in arable land and the ever-growing demand for reliable food supplies. Additionally, inappropriate agricultural practices, such as the intensive exploitation of soil have considerably reduced soil fertility (Souza et al. 2015). Indeed, competitive food production in conventional agriculture is only maintained through the continuous use of agrochemicals to enhance soil fertility, crop yield, and to control diseases. For instance, chemical fertilizers are being used to fulfill crop requirements in nitrogen and phosphorous (Rosenblueth et al. 2018). Likewise, chemical iron chelators are being used to overcome the low bioavailability of this element in plant-soil systems. While the use of these chemicals might be cheap and effective, they are often not environmentally friendly (Godsey et al. 2003; Radzki et al. 2013). For instance, leaching or runoff of agrochemicals into groundwater, rivers and lakes has created low oxygen zones where survival of aquatic life is at risk (Muhibbullah et al. 2005). Moreover, direct and indirect exposure to hazardous chemicals has deleterious effects on animal health (Muhibbullah et al. 2005).

Another drawback of the use of agrochemical use is that the uptake efficiency by plants decreases over time and non-assimilated nutrients are eventually leached. Regular applications are thus required to counterbalance this, resulting in an increase of production costs for farmers. Moreover, for some nutrients, application (even when regular) does not result in the desirable increase in nutrient bioavailability. For instance, it has been reported that phosphorous fertilizers often precipitate with metal cations in the soil solution and turn into an insoluble form that is no longer bioavailable to plants (Ahemad and Kibret 2014). Therefore, a change in the conventional intensive agricultural practices by innovative ways of conducting a more sustainable food production is a clear need for the future of agriculture.

The use of microorganisms is considered as one of these suitable alternatives. In both managed and natural ecosystems, microorganisms play a key role in supporting plant health and growth (Bhattacharyya and Jha 2012). Due to their importance in ecosystem functioning, microorganisms are expected to provide an ecofriendly and cost-effective alternative to limit the use of agrochemicals globally (Ahemad and Kibret 2014).

Plant growth promoting microorganisms

Microorganisms include bacteria, archaea, algae, fungi, and protozoa. Many of them live in the soil near to the plant roots, i.e. the rhizosphere. These microorganisms often play a central role in plant growth and development as they establish neutral, negative and positive interactions with plants. Neutral interactions are neither harmful nor beneficial. Negative interactions are harmful to plants and are often responsible for plant disease. Such microbes are known as phytopathogens and may produce toxic substances that influence negatively plant growth and health. In contrast to phytopathogens, many beneficial microorganisms are also present in the rhizosphere. They establish positive interactions with plants through the colonization of roots and the promotion of plant growth. In the case of bacteria, they are commonly known as plant growth promoting rhizobacteria (PGPR). PGPR can enhance plant growth either directly or indirectly (Figure 1). Direct mechanisms by which PGPR affect positively plant growth include fixation of atmospheric nitrogen, solubilization of inorganic phosphorus (Zaidi and Khan 2007), production of siderophores (Rajkumar et al. 2010), and production of phytohormones or substances modifying the hormonal plant profile (Hayat et al. 2010). Mechanisms that indirectly result in an increase in plant growth include the inhibition of the growth of phytopathogens (biocontrol) or the promotion of the growth of other beneficial microorganisms. In case of the former, the production of chemicals such as hydrogen cyanide, phenazines, pyrrol-nitrin, tensin, or lytic enzymes, can impair directly the development of pathogens (Bhattacharyya and Jha 2012). Also, root colonized with beneficial or even neutral microbes are otherwise physically unavailable for colonization by pathogens. In the latter, PGPR can enhance beneficial plant-microbe symbioses e.g. stimulation of mycorrhizae development (Glick 2012). Moreover, these plant growths promoting bacteria can also indirectly protect plants by enhancing the ability to defend themselves against phytopathogenic microorganism through induced systemic resistance (Beneduzi et al. 2012).

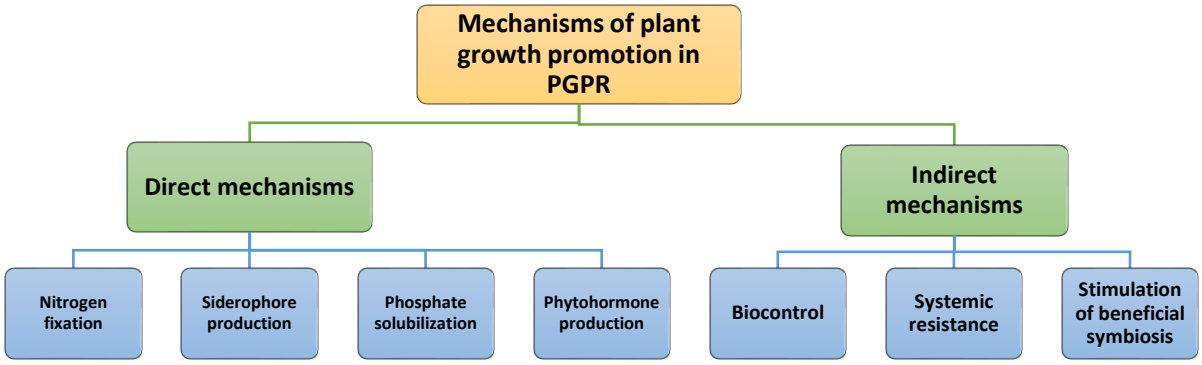

Figure 1: Mechanisms of plant growth promotion in PGPR.

Many studies have shown the potential of inoculation with beneficial microorganisms to improve agricultural yield (Ahemad and Kibret 2014; Bhattacharyya and Jha 2012; Souza et al. 2015). Microbial inoculation helps the plant to cope with both abiotic and biotic stress and it has also been shown to increase systemic protection against phytopathogens (Sarma et al. 2015). In spite of its potential, ensuring the success of bio-inoculation presents many challenges (Ahemad and Kibret 2014; Bhattacharyya and Jha 2012; Souza et al. 2015). One of the critical issues involves the survival of bacteria acclimated under laboratory conditions to the harsh conditions in soils. Inoculated microbes have to compete with autochthonous microbial communities (Souza et al. 2015) and are also vulnerable to the relatively low nutrient availability in natural environments (as compared to laboratory media). This often results in a decline overtime in the numbers of the bio-inoculant in soil (Souza et al. 2015; Trabelsi and Mhamdi 2013). Moreover, the delivery of these microorganisms in an active form is an additional challenge. Application of carrier materials for the protection of bio inoculants, for instance karnolite, peat, or charcoal, is not only environmentally unfriendly (i.e. extensive mining of peat and its adverse effect on climate), but also costly, making this approach not easily applicable in agriculture, especially in developing countries (Arora et al. 2014). These concerns are an incentive to identify not only more efficient ways of inoculating microorganisms into natural environments, but also to ensure their long-term survival. Because of this, *Bacillus* and related endospore-forming bacteria may constitute a suitable model for the development of highly efficient inoculants.

Endospore-forming bacteria, as their name suggest, are a group of bacteria that share the ability to form endospores in response to unfavorable environmental conditions. Endospores are highly resistant dormant cells that promote persistence in soils of bacteria able to form such structures (Radhakrishnan et al. 2017). For these reasons, one of the focus of this PhD thesis is to investigate endospore-forming bacteria, specifically *Bacillus* spp. and related genera, as potential bio-inoculant PGP bacteria.

Bacillus spp. as Plant Growth Promoting Bacteria

There are several examples of PGPR and among those, the genus *Bacillus* is one of the best-studied examples. There are many species of *Bacillus* that are well known for having plant growth promoting activities and that are already used commercially (Kumar et al. 2011). Examples of commercialized *Bacillus*-based biofertilizer products include Serenade®, Quantum-400, Alinit, Kodiak ™, and Rhizovital®42 (Radhakrishnan et al. 2017). *Bacillus* species are known to play a vital role in helping plants to withstand the osmotic stress caused by salt (in water or soil) by limiting the uptake of sodium and chloride ions and by enhancing plant growth and seed germination (Jeschke and Wolf 1988; Qurashi and Sabri 2013). For instance, *Bacillus licheniformis* A2 has been reported as a salt-tolerant PGPR when applied to peanut plants (Goswami et al. 2014). Bio-inoculation with *Bacillus megaterium* and *Pseudomonas aeruginosa* diminished salt-induced cell death in rice (Jha and Subramanian 2015). Likewise, *Bacillus amyloliquefaciens* NBRISN13, another salt-tolerant PGPR, is reported to mitigate the changes in microbial diversity in the rice rhizosphere in response to salt stress (Nautiyal et al. 2013).

Bacillus spp. can also help plants by stimulating growth through increasing nutrient acquisition or phytohormone production (Bhattacharyya and Jha 2012). *Bacillus cereus, Bacillus marisflavi*, *Bacillus megaterium*, *Paenibacillus polymyxa,* and *Paenibacillus massiliensis* were reported to be nitrogen fixing bacteria isolated from the rhizosphere of different plant species (Ding et al. 2005). *Bacillus azotofixans,* isolated from the roots of different poaceae grasses, was also reported to fix atmospheric nitrogen (Seldin et al. 1984). *Bacillus* spp. are also known to produce cytokinin, gibberellins, and indole acetic acid; all of which can directly or indirectly affect plant growth and yield (Arkhipova et al. 2005; Radhakrishnan and Lee 2016).

Furthermore, in the literature, *Bacillus* spp. are reported to produce a wide variety of volatile

organic compounds (VOCs) that play an important role in plant growth promotion. For instance, VOCs produced by *Bacillus subtilis* SYST2 enhanced biomass and photosynthetic activity of tomato plants (Tahir, Gu et al. 2017). In those plants, the concentration of different types of endogenous phytohormones (cytokinin, auxin and gibberellin) was also increased. A commercial plant growth promoting bacterium, *Bacillus subtilis* GB03, which is able to produce VOCs, showed beneficial effects (increased growth promotion) on *Arabidopsis thaliana* (Xie, Zhang et al. 2009). In another study, VOCs produced by *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a possessed biocontrol activities against the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Ryu, Farag et al. 2004). In *Bacillus megaterium* XTBG34, isolated from a tropical botanical garden soil (Xishuangbanna, China), the production of 2-pentylfuran was responsible for triggeering plant growth with a two-fold increase of *Arabidopsis thaliana* biomass (Zou, Li et al. 2010).

The genus *Bacillus* has also been used as a biocontrol agent against plant pathogens (O'Callaghan 2016; Widnyana and Javandira 2016). *Bacillus* spp. can produce a wide range of antiviral, antibacterial and antifungal compounds, which may be important in their interaction with plants and other soil microorganisms. These chemicals have significant commercial potential for increasing agricultural production (Niu et al. 2011). For instance, two antifungal compounds produced by *B. amyloliquefaciens* control the development of the fungal soil-borne plant pathogen *Fusarium oxysporum*. Moreover, it has been reported in many studies that *Bacillus* spp. are effective biocontrol candidates against diverse diseases that are caused by the soil-borne fungus *Rhizoctonia solani*, for instance tomato root rot, damping-off, sheath blight of rice, and potato black scurf (Ben Khedher et al. 2015; Solanki et al. 2012; Yang et al. 2009). In addition to their use as bio-fungicides, *Bacillus* spp. have also been widely used as bio-insecticides in sustainable agriculture. For instance, *Bacillus thuringiensis* has been used as a broad range bio-insecticide that limits the growth of larvae of pest insects by fatally damaging the midgut epithelium (Radhakrishnan et al. 2017). There are many other *Bacillus* species that have also been used in pest management systems, including *B. amlyloliquefaciens*, *B. subtilis*, and *B. cereus* (Gadhave and Gange 2016).

Along with *Bacillus*, there are many other genera within the phylum Firmicutes that are equally important in agroecology. The contribution of other genera of the phylum Firmicutes as beneficial microorganisms with reference to their importance in plant growth promotion (biofertilizers), their role in biocontrol of plant pathogens (biocontrol agent), as well as in the

phytoremediation of heavy metals (metal uptake enhancers) are discussed in detail in this thesis, in chapter 2 (review paper submitted in "Beneficial Microbes in Agro-ecology": Volume I Bacteria").

Plant Growth Promoting Fungi (PGPF)

Besides bacteria, fungi represent a significant portion of the rhizosphere microbiota. Similarly, to bacteria, fungi can establish negative, neutral or positive interactions with plants. Soil-borne phytopathogenic fungi play a negative role in plant growth, enventually causing losses in crop production. For instance, annually, about 20% of wheat production is lost worldwide due to only one phytopathogenic fungus, *R. solani* (Dada 2017). Due to inadequate agricultural practices, these microbes are currently widespread in soil. Enzymes are an important aspect of fungal pathogens. First, most of them are capable of producing cell-wall degrading enzymes that help them penetrating plant cells (Chandrasekaran et al. 2016). Then, plant pathogenic fungi produce a large array of hydrolytic enzymes that help them obtaining nutrients from the cellular content of their host (Chandrasekaran et al. 2016).

In contrast to phytopathogenic fungi, there are many groups of beneficial fungi that play a vital role in sustainable agricultural systems, for instance by improving plant growth through nutrient and water uptake, or by helping plants to tolerate abiotic stresses, as well as through biocontrol activities (Devendra et al. 2016).

One of the best-studied form of beneficial association between plants and fungi are mycorrhizae (Johnson and Gehring 2007). The two main types of mycorrhizal association are Arbuscular mycorrhizae (also known as endomycorrhizae) and ectomycorrhizae. They are observed in a wide variety of plant species, representing approximately 90% of all plant species. Arbuscular mycorrhizal fungi (AMF), belong to the Glomeromycota and form an intracellularly symbiotic relationship with root cells of about 80% of terrestrial plants, including ferns, mosses, gymnosperms, and angiosperms. AMF are most prevalent in temperate and tropical natural and managed ecosystems (Johnson and Gehring 2007). AMF not only help to ameliorate soil properties through aggregation of soil particles with glomalin thereby controlling the degradation of soils, but they also strongly influence soil fertility (Lovelock et al. 2004). For instance an inoculum of an AMF consortium (*Septoglomus constrictum*, *Funneliformis geosporum*, *Glomus fuegianum*, *Rhizophagus irregularis* and *Glomus* sp.) increased the shoot dry weight of forage legume Sulla plants. Moreover, the

phosphorous content was also enhanced by 50% in Sulla plants treated with the AMF consortium, as compared to the un-inoculated plants (Labidi et al. 2015).

The majority of the fungal bio-inoculants that are available in the market consists of AMF fungi formulations. However their performances are often very difficult to monitor (Hart et al., 2018). Moreover, most of the studies assessing the effect of AMF inocula on plant health were performed in sterile controlled conditions that do not mimic the original scenario of a real soil system and thus, result in inconsistent outcomes concerning plant growth promotion. Therefore, there is a clear gap between scientific research and industrial practices when it comes to producing AMF inocula. Mostly, these commercial products are not always tailored to the specific needs of a particular plant system.

In contrast to AMF, Ectomycorrhizal (ECM) associations are those where the fungus colonizes the plant root cells extracellularly. This symbiotic association occurs in different plants, including woody gymnosperm and angiosperms, and play an important role in temperate and boreal forests (Johnson and Gehring 2007). Diverse members of the phyla Ascomycota, Basidiomycota, and Zygomycota (Endogonales) are reported to form ectomycorrhizal association. According to (Mohammadi et al. 2011), about 30% of the microbial biomass in forest soil is comprised of ECM fungi. Plants with ECM associations have greater absorption area for nutrient acquisition by connecting their roots with sometimes extensive mycelial networks and eventually have greater uptake of nutrients and water (Johnson and Gehring 2007). These mycorrhizal association not only help to increase nutrients and water uptake, but also protect the plant from a variety of abiotic and biotic stresses (Johnson and Gehring 2007). Fungi are also reported to produce a broad spectrum of volatile organic compounds (VOCs) that can play an important role in plant-fungal communication (Hung et al., 2013; Ditengou et al., 2015; Lee et al., 2016). Relatively little is known about the role of VOCs emitted by AMF and ECM in plant-microbe interaction. For instance, Ditengou et al., 2015, reported that sesquiterpenes released by *Laccaria bicolor* stimulated lateral roots formation of both, mycorrhizal (*Populus*) and non-mycorrhizal plants (*Arabidopsis thaliana*).

Mycorrhizal fungi can have a positive effect on plant growth by increasing nitrogen uptake in nutrient deficient soils (Domínguez Núñez et al. 2013). ECM fungi provide nitrogen to plants thanks to a complex system of different hydrolytic enzymes for the solubilization of organic nitrogen (Chalot and Brun 1998). In another example, *Laccaria bicolor,* which forms ECM associations with the trembling aspen (*Populus tremuloides* Michx.), contributes to the

uptake of phosphorous and is also reported to reduce oxidative stress due to nutrient deficiency (Desai et al. 2014). In another study, where five different ECM fungi were used as single inocula or as a consortium, it was observed that the consortium of ECM was more efficient and increased the productivity of *Pinus densiflora* by enhancing its biomass and height (Sim and Eom 2006). Vayssières, Pěnčík et al. (2015) reported that the mycelium of *Laccaria bicolor* is capable of producing high amounts of auxin, a plant hormone that can play an important role in the root system of *Populus tremula* and *P. alba.* In another study, it was reported that an inoculum of *L. bicolor* enhanced lateral root formation in poplar and *Arabidopsis* plants (Felten, Kohler et al. 2009).

In addition to mycorrhizal fungi, other fungi, such as saprobic *Trichoderma* spp., are beneficial for plant growth. *Trichoderma* spp. can form mycorrhizal-like associations with plants and help those in the uptake of water and nutrients from soil. Moreover, *Trichoderma* spp. is known to release different types of compounds that can induce systemic resistance in plants as reviewed by (Harman et al. 2004). It is noteworthy to mention that *Trichoderma* spp. have been reported to emit a wide range of VOCs. For instance, *Trichoderma viride* released VOCs that were responsible to enhance total biomass and chlorophyll content in *Arabidopsis thaliana* (Hung et al., 2013). Moreover, another study showed that *Trichoderma* VOCs had biocontrol activities by reducing the disease symptoms caused by the phytopathogenic fungi *Botrytis cinerea* and *Alternaria brassicicola* in *Arabidopsis thaliana* (Kottb et al., 2015). Lee et al., 2016, investigated nine different *Trichoderma* strains that were able to emit volatile metabolites with agricultural significance (for instance, positive effect on plant biomass and size, as well as on chlorophyll concentration of *Arabidopsis* plants). In addition to this, VOCs produced by another strain of *T. viride* (BBA 70239) induced significant enhancement in lateral roots formation and of biomass of tomato plants. There are many commercially available products consisting of *Trichoderma* spp. alone or with other microbes (either bacteria or fungi). They are marketed as plant growth promoter and/or biocontrol agent, for instance**;** ASPERELLO™ T34 Biocontrol®**,** Remedier WP, Trichopel, Vinevax, Esquive®WP, T-Gro, RootShield®WP, Quickroots® and Cérès®. The interspecific interaction of fungi is also important in soil-plant interactions. *Trichoderma* spp. is widely known for its antagonism against other fungi through mycoparasitism. For this reason, it is used as a biocontrol agent of soil-borne phytopathogenic fungi. For instance, *Trichoderma* spp. has the ability to disintegrate the cell wall of the pathogen *R. solani* (Melo and Faull 2000).

The use of beneficial fungi in a dual inoculation with other beneficial fungi, as well as with endospore forming bacteria could create a synergistic association that may result in a significant increase in the growth of plants. For instance, by enhancing root surface area, ultimately enhancing the uptake of minerals and other nutrients and the surface for interaction with beneficial microbes in a positive feed-back loop. Studies involving dual inoculation of mycorrhizal fungi and PGPR are uncommon (Nadeem et al. 2014) and further work is required to screen and select promising bacterial-fungal partners that could be used as potential growth promoting agents.

Bacterial Fungal Interactions

With reference to plants, bacteria and fungi establish synergistic as well as antagonistic interactions and these interactions can promote plant growth and development (Deveau et al. 2018). These interactions are strongly influenced by the co-emergence of bacteria and fungi in terrestrial ecosystems (Boer et al. 2005). On the one side, in terrestrial environments fungi overtook niches commonly associated to bacteria in aquatic ecosystems. On the other side, fungi themselves, created new opportunities for bacterial growth and development, for instance through the consumption of fungal exudates or fungal biomass (Boer et al. 2005). These interactions may be vital in the frame of sustainable agriculture because they exploit biological processes to maintain growth and development of plants. For instance, it has been observed that the synergistic interaction of fluorescent pseudomonads with *Rhizopogon luteolus* and *Laccaria* spp. help the formation of ectomycorrhizal associations with plants (Frey-Klett et al. 2007). Bacilli and Paenibacilli are also reported to stimulate mycorrhizal associations (Bending et al. 2002; Poole et al. 2001). Moreover, mycorrhizal fungi also help bacterial communities in the mycorrhizosphere by stimulating root exudation and by shaping soil structure (Nadeem et al. 2014).

Another example of a positive interaction involving bacteria and fungi, is the dispersal of the former on fungal hyphae in soils, a mechanism called “Fungal highways”. Bacterial dispersal in soils is limited due to the unsaturated heterogeneous nature of the soil matrix. In contrast, thanks to their filamentous growth, fungi can easily disperse and cross air-filled gaps between soil aggregates. The disadvantage generated by limited-dispersal of bacteria in soils can be overcome by their dispersal on fungal hyphae. This general concept, defined as fungal highway, has been shown to be important for soil processes such as biodegradation

(Kohlmeier et al. 2005; Wick et al. 2007) or soil functioning (Bravo et al. 2013; Martin et al. 2012). It was reported by (Bravo et al. 2013) that the hyphae of *Trichoderma* sp. were used by oxalotrophic bacteria for their active dispersal in soil. Thus, it would be a good strategy to combine the plant growth promoting characteristics of *Trichoderma* spp., with their ability to act as a fungal highway in order to use it as a delivery system of PGPR in the field of agriculture.

Besides these synergistic interactions, many types of antagonistic interactions among bacteria and fungi can be harnessed to improve application of PGP microorganisms in sustainable agriculture. For instance, fungi provide nutritional niches for bacterial growth and development (Boer et al. 2005). Bacterial mycophagy is an example of an antagonistic trophic interaction in which bacteria can grow at the expense of fungal mycelium or fungal exudates (Leveau and Preston 2008). Bacteria belonging to *Collimonas* spp. are reported to be a mycophagous bacteria with the ability to obtain nutrients from living fungi by producing chitinases and antibiotics and to grow at the expense of fungi by converting fungal biomass into bacterial biomass (Boer et al. 2005; Leveau et al. 2010). It has also been observed that some *Bacillus* species are able to disintegrate the cell wall of phytopathogenic fungi and can cause complete loss of cytoplasm (Chérif et al. 2002). Additionally, *Bacillus* spp. can produce a wide range of antibacterial and antifungal compounds, which are important in their interaction with plants and other soil microorganisms (Sansinenea and Ortiz 2011). For instance, *Bacillus subtilis* fmbj synthesize bacillomycin D, a toxin with lytic properties that disrupts the cell wall and cell membrane of the phytopathogen *Aspergillus flavus* (Gong et al. 2014). Moreover, *Bacillus cereus* X16 and *B. thuringiensis* 55T also showed fungistatic and fungitoxic inhibitory effect on fungal plant pathogens (Chérif et al. 2002). *Bacillus* sp. has also a detrimental effect on hyphae and led to the reduced production of spores of the fungus *Alternaria* spp. (Sansinenea et al. 2016).

Aims of this PhD thesis

The main focus of this PhD thesis is to highlight the importance of microbes in plant growth and health in the perspective of their exploitation for the sustainability of agriculture. For this reason, an extensive review on other genera of the phylum Firmicutes than *Bacillus* and their importance as biofertilizers, biocontrol agents, and metal uptake enhancers is presented in chapter 2. Then, the first experimental objectives of this PhD work were to characterize a

selection of endospore-forming *Bacillus* spp. and fungi of different ecological niches for physiological traits linked to plant growth promotion, as well as to examine their interactions under different nutrient conditions. The aim of these initial experiments was to understand how complex ecological interactions could help in developing innovative systems to promote plant growth (Chapter 3). Based on the result of the screening for PGP traits, the role of a consortium of three endospore-forming *Bacillus* spp. on plant growth of *Avena sativa* (oat) was evaluated in simple (laboratory conditions) to complex environments (field). In addition, the effect of bio-inoculation in the form of vegetative cells or endospores was compared. This was complemented by the analysis of the effect of bio-inoculation on native soil microbial communities (Chapter 4). This study also investigated mycophagy in a selected endospore-forming bacterial strain interacting with two fungi with contrasting ecology. This was completed by a test on the use of mycophagy as a mechanism of biocontrol of the phytopathogenic fungus *R. solani* (Chapter 5). Studies that were done in collaboration with other research groups are summarized in chapter 6. Finally, the different aspect highlighted in this work and the outlooks that may be envisioned are discussed in chapter 7.

References

Ahemad, Munees and Kibret, Mulugeta (2014), 'Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective', *Journal of King Saud University - Science,* 26 (1), 1-20.

Arkhipova, T. N., et al. (2005), 'Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants', *Plant and Soil,* 272 (1), 201-09.

Arora, Kumar. Naveen., Tiwar, Saskhi., and Singh, Ratan (2014), 'Comparative study of different carriers inoculated with nodule forming and free living plant growth promoting bacteria suitable for sustainable agriculture', *Journal of Plant Pathology & Microbiology,* 05 (02).

Ben Khedher, S., et al. (2015), 'Efficacy of *Bacillus subtilis* V26 as a biological control agent against *Rhizoctonia solani* on potato', *C R Biol,* 338 (12), 784-92.

Bending, Gary D., et al. (2002), 'Characterisation of bacteria from *Pinus sylvestris–Suillus luteus* mycorrhizas and their effects on root–fungus interactions and plant growth', *FEMS Microbiology Ecology,* 39 (3), 219-27.

Beneduzi, Anelise., Ambrosini, A., and Passaglia, L. M.P. (2012), 'Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents', *Genetics and Molecular Biology,* 35 (4), 1044-51.

Bhattacharyya, P. N. and Jha, D. K. (2012), 'Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture', *World Journal of Microbiology and Biotechnology,* 28 (4), 1327-50.

Boer, Wietse de, et al. (2005), 'Living in a fungal world: impact of fungi on soil bacterial niche development', *FEMS Microbiology Reviews,* 29 (4), 795-811.

Bravo, Daniel, et al. (2013), 'Isolation of oxalotrophic bacteria able to disperse on fungal mycelium', *FEMS Microbiology Letters,* 348 (2), 157-66.

Chalot, Michel and Brun, Annick (1998), 'Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas', *FEMS Microbiology Reviews,* 22 (1), 21-44.

Chandrasekaran, Murugesan, et al. (2016), 'Proteases from phytopathogenic fungi and their importance in phytopathogenicity', *Journal of General Plant Pathology,* 82 (5), 233-39.

Chérif, M., et al. (2002), 'Ultrastructure and cytochemistry of *in vitro* interactions of the antagonistic bacteria *Bacillus cereus* x16 and *B. thuringiensis* 55t with *Fusarium roseumvar. Sambucinum'*, *Journal of Plant Pathology & Microbiology,* 84 (2), 83-93.

Dada, Taiwo Esther (2017), 'Epidemiology of Wheat *Rhizoctonia'*, *International Journal of Scientific and Research Publications,* 7 (5).

Desai, Shalaka, Naik, Dhiraj, and Cumming, Jonathan R. (2014), 'The influence of phosphorus availability and *Laccaria bicolor* symbiosis on phosphate acquisition, antioxidant enzyme activity, and rhizospheric carbon flux in *Populus tremuloides'*, *Mycorrhiza,* 24 (5), 369-82.

Deveau, A., et al. (2018), 'Bacterial-fungal interactions: ecology, mechanisms and challenges', *FEMS Microbiol Rev,* 42 (3), 335-52.

Devendra, K. Choudhary, Ajit, Varma, and Narendra, Tuteja (2016), 'Plant-Microbe Interaction: An approach to sustainable agriculture', *Springer Nature*.

Ding, Y., et al. (2005), 'Isolation and identification of nitrogen-fixing Bacilli from plant rhizospheres in Beijing region', *J Appl Microbiol,* 99 (5), 1271-81.

Ditengou, F.A., Müller, A., Rosenkranz, M., Felten, J., Lasok, H., van Doorn, M.M., Legué, V., Palme, K., Schnitzler, J.-P., Polle, A., 2015. Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nature Communications* 6, 6279.

Domínguez Núñez, J.A. Alfonso, et al. (2013), 'Short Communication. Physiological effects of *Rhizopogon Roseolus* on *Pinus halepensis* seedlings', *2013,* 22 (3), 5.

Felten, J., A. Kohler, E. Morin, R. P. Bhalerao, K. Palme, F. Martin, F. A. Ditengou and V. Legué (2009). "The ectomycorrhizal fungus Laccaria bicolor stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signaling." Plant physiology 151(4): 1991-2005.

Frey-Klett, P., Garbaye, J., and Tarkka, M. (2007), 'The mycorrhiza helper bacteria revisited', *New Phytologist,* 176 (1), 22-36.

Gadhave, Kiran R. and Gange, Alan C. (2016), 'Plant-associated *Bacillus* spp. alter life-history traits of the specialist insect *Brevicoryne brassicae* L', *Agricultural and Forest Entomology,* 18 (1), 35-42.

Glick, Bernard R. (2012), 'Plant Growth-Promoting Bacteria: Mechanisms and Applications', *Scientifica,* 2012, 15.

Godsey, Chad B., et al. (2003), 'Correcting Iron Deficiency in Corn with Seed Row–Applied Iron Sulfate, *Agronomy Journal,* 95 (1), 160-66.

Gong, Qingwei, et al. (2014), 'Identification of Bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against *Aspergillus flavus'*, *Food Control,* 36 (1), 8-14.

Goswami, D., et al. (2014), 'Screening of PGPR from saline desert of Kutch: growth promotion in *Arachis hypogea* by *Bacillus licheniformis* A2', *Microbiol Res,* 169 (1), 66-75.

Harman, Gary E., et al. (2004), '*Trichoderma* species — opportunistic, avirulent plant symbionts', *Nature Reviews Microbiology,* 2, 43.

Hart, M.M., Antunes, P.M., Chaudhary, V.B., Abbott, L.K., Field, K., 2018. Fungal inoculants in the field: Is the reward greater than the risk? Functional Ecology 32, 126-135.

Hayat, Qaiser, et al. (2010), 'Effect of exogenous salicylic acid under changing environment: A review', *Environmental and Experimental Botany,* 68 (1), 14-25.

Hung, R., Lee, S., Bennett, J.W., 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. *Fungal Ecology* 6, 19-26.

Jeschke, W.D. and Wolf, O. (1988), 'External potassium supply is not required for root growth in saline conditions: Experiments with *Ricinus communis* L. grown in a reciprocal split root-system', *Journal of experimental Botany,* 39 (206), 1149-67.

Jha, Yachana and Subramanian, R. B. (2015), 'Reduced cell death and improved cell membrane integrity in rice under salinity by root associated bacteria', *Theoretical and Experimental Plant Physiology,* 27 (3), 227-35.

Johnson, Nancy C. and Gehring, Catherine A. (2007), 'CHAPTER 4 - Mycorrhizas: Symbiotic Mediators of Rhizosphere and Ecosystem Processes', in Zoe G. Cardon and Julie L. Whitbeck (eds.), *The Rhizosphere* (Burlington: Academic Press), 73-100.

Kohlmeier, Stefanie, et al. (2005), 'Taking the Fungal Highway: Mobilization of Pollutant-Degrading Bacteria by Fungi', *Environmental Science & Technology,* 39 (12), 4640-46.

Kottb, M., Gigolashvili, T., Großkinsky, D.K., Piechulla, B., 2015. *Trichoderma* volatiles effecting *Arabidopsis:* from inhibition to protection against phytopathogenic fungi. *Frontiers in Microbiology* 6.

Kumar, Ankit, Prakash, Anil, and Johri, B. N. (2011), '*Bacillus* as PGPR in Crop Ecosystem', *Bacteria in Agrobiology: Crop Ecosystems*, 37-59.

Labidi, S., F. B. Jeddi, B. Tisserant, M. Yousfi, M. Sanaa, Y. Dalpé and A. L.-H. Sahraoui (2015). "Field application of mycorrhizal bio-inoculants affects the mineral uptake of a forage legume (*Hedysarum coronarium* L.) on a highly calcareous soil." Mycorrhiza 25(4): 297-309.

Lee, S., Yap, M., Behringer, G., Hung, R., Bennett, J.W., 2016. Volatile organic compounds emitted by *Trichoderma* species mediate plant growth. *Fungal Biology and Biotechnology* 3, 7.

Leveau, J. H. and Preston, G. M. (2008), 'Bacterial mycophagy: definition and diagnosis of a unique bacterial-fungal interaction', *New Phytol,* 177 (4), 859-76.

Leveau, Johan H. J., Uroz, Stéphane, and De Boer, Wietse (2010), 'The bacterial genus *Collimonas:* mycophagy, weathering and other adaptive solutions to life in oligotrophic soil environments', *Environmental Microbiology,* 12 (2), 281-92.

Lovelock, Catherine E., et al. (2004), 'Soil stocks of glomalin produced by arbuscular mycorrhizal fungi across a tropical rain forest landscape', *Journal of Ecology,* 92 (2), 278-87.

Martin, Gaëtan, et al. (2012), 'Fungi, bacteria and soil pH: the oxalate–carbonate pathway as a model for metabolic interaction', *Environmental Microbiology,* 14 (11), 2960-70.

Melo, Itamar Soares de and Faull, Jane L. (2000), 'Parasitism of *Rhizoctonia solani* by strains of *Trichoderma* spp', *Scientia Agricola,* 57, 55-59.

Mohammadi, Khosro., et al. (2011), 'A Review: Beneficial effects of the mycorrhizal fungi for plant growth', *Journal of Applied Environmental and Biological Sciences,* 1 (9), 310-19.

Muhibbullah, M., Salma, M., and Chowdhury, A. (2005), 'Use of agrochemical fertilizers and their impact on soil, Water and Human Health in the Khamargao Village of Mymensingh District, Bangladesh', *Jownal of Agronomy,* 4 (2), 109-15.

Nadeem, Sajid Mahmood, et al. (2014), 'The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments', *Biotechnology Advances,* 32 (2), 429-48.

Nautiyal, C. S., et al. (2013), 'Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress', *Plant Physiol Biochem,* 66, 1-9.

Niu, Dong-Dong. , et al. (2011), 'The Plant Growth–Promoting Rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways', *The American Phytopathological Society,* 24, 533–42.

O'Callaghan, M. (2016), 'Microbial inoculation of seed for improved crop performance: issues and opportunities', *Appl Microbiol Biotechnol,* 100 (13), 5729-46.

Poole, Elizabeth J., et al. (2001), 'Bacteria associated with *Pinus sylvestris–Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro*', *New Phytologist,* 151 (3), 743-51.

Qurashi, A. W. and Sabri, A. N. (2013), 'Osmolyte accumulation in moderately halophilic bacteria improves salt tolerance of chickpea', *Pakistan Journal of Botany,* 45 (3), 1011-16.

Radhakrishnan, R. and Lee, I. J. (2016), 'Gibberellins producing *Bacillus methylotrophicus* KE2 supports plant growth and enhances nutritional metabolites and food values of lettuce', *Plant Physiol Biochem,* 109, 181-89.

Radhakrishnan, R., Hashem, A., and Abd Allah, E. F. (2017), '*Bacillus:* A biological tool for crop improvement through bio-molecular changes in adverse environments', *Front Physiol,*

8, 667.
Radzki, W., et al. (2013), 'Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture', *Antonie van Leeuwenhoek,* 104 (3), 321-30.
Rajkumar, M., et al. (2010), 'Potential of siderophore-producing bacteria for improving heavy metal phytoextraction', *Trends Biotechnol,* 28 (3), 142-9.
Rosenblueth, Mónica, et al. (2018), 'Nitrogen Fixation in Cereals', *Frontiers in Microbiology,* 9 (1794).
Ryu, C.-M., M. A. Farag, C.-H. Hu, M. S. Reddy, J. W. Kloepper and P. W. Paré (2004). "Bacterial volatiles induce systemic resistance in Arabidopsis." Plant Physiology 134(3): 1017.
Sansinenea, E. and Ortiz, A. (2011), 'Secondary metabolites of soil *Bacillus* spp', *Biotechnol Lett,* 33 (8), 1523-38.
Sansinenea, Estibaliz, et al. (2016), 'Cellular damage of plant pathogenic fungi by antifungal compounds produced by *Bacillus* spp. isolates', *Chemistry and Ecology,* 32 (8), 722-32.
Sarma, Birinchi Kumar, et al. (2015), 'Microbial consortium-mediated plant defense against phytopathogens: Readdressing for enhancing efficacy', *Soil Biology and Biochemistry,* 87, 25-33.
Seldin, L., Van Elsas, J. D., and Penido, G. C. (1984), 'B*acillus azotofixans* sp. nov. a nitrogen-fixing species from brazilian soils and grass roots', *International journal of systematic bacteriol*, 451-56.
Sim, M.-Y. and A.-H. Eom (2006). "Effects of ectomycorrhizal fungi on growth of seedlings of *Pinus densiflora*." Mycobiology 34(4): 191-195.
Solanki, Manoj Kumar, et al. (2012), 'Diversity and antagonistic potential of *Bacillus* spp. associated to the rhizosphere of tomato for the management of *Rhizoctonia solani'*, *Biocontrol Science and Technology,* 22 (2), 203-17.
Souza, Rocheli de, Ambrosini, Adriana , and Passaglia, Luciane M.P. (2015), 'Plant growth-promoting bacteria as inoculants in agricultural soils', *Genet Mol Biol,* 38 (4), 401-19.
Tahir, H. A. S., Q. Gu, H. Wu, W. Raza, A. Hanif, L. Wu, M. V. Colman and X. Gao (2017). "Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2." *Frontiers in microbiology* 8: 171-171.
Trabelsi, Darine. and Mhamdi, Ridha. (2013), 'Microbial inoculants and their impact on soil microbial communities: a review', *Biomed Res Int,* 2013, 863240.
Vayssières, A., A. Pěnčík, J. Felten, A. Kohler, K. Ljung, F. Martin and V. Legué (2015). "Development of the *Poplar-Laccaria bicolor* ectomycorrhiza modifies root auxin metabolism, signaling, and response." Plant physiology 169(1): 890-902.
Wick, Lukas Y., et al. (2007), 'Effect of fungal hyphae on the access of bacteria to phenanthrene in soil', *Environmental Science & Technology,* 41 (2), 500-05.
Widnyana, I. Ketut and Javandira, Cokorda (2016), 'Activities *Pseudomonas* spp. and *Bacillus* sp. to stimulate germination and seedling growth of tomato plants', *Agriculture and Agricultural Science Procedia,* 9, 419-23.
Xie, X., H. Zhang and W. P. Pare (2009). "Sustained growth promotion in Arabidopsis with long-term exposure to the beneficial soil bacterium *Bacillus subtilis* (GB03)." *Plant Signaling & Behavior* 4(10): 948-953.
Yang, Dongjing, et al. (2009), 'Activity and efficacy of *Bacillus subtilis* strain NJ-18 against rice sheath blight and *Sclerotinia* stem rot of rape', *Biological Control,* 51 (1), 61-65.
Zaidi, Almas and Khan, Md. Saghir (2007), 'Stimulatory effects of dual inoculation with phosphate solubilising microorganisms and arbuscular mycorrhizal fungus on chickpea', *Australian Journal of Experimental Agriculture,* 47 (8), 1016-22.

Zou, C., Z. Li and D. Yu (2010). "*Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran." *The Journal of Microbiology* 48(4): 460-466.

Chapter 2

The Phylum Firmicutes

Contribution in print for the book: Beneficial Microbes in Agro-Ecology, Volume I: Bacteria.

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Abstract

Among the Phylum of Firmicutes, members of the genus *Bacillus* are probably the most extensively studied as beneficial microorganisms with application in agroecology. However, members of other genera within the Phylum are equally important. This chapter highlights the beneficial role of members of the Phylum Firmicutes, with the exception of *Bacillus* spp., which is not considered. Their importance in plant growth promotion (biofertilizers), their role in biocontrol of plant pathogens (biocontrol agent), as well as in the phytoremediation of heavy metals (metal uptake enhancers) are discussed for individual genera. All these traits are of relevance in the perspective of exploiting these organisms in sustainable agricultural systems.

Key words:

Firmicutes, biofertilizer, biocontrol, phytoremediation, *Lysinibacillus*, *Aneurinibacillus, Brevibacillus, Oceanobacillus, Planococcus, Clostridium, Sporosarcina, Virgibacillus, Terribacillus, Staphylococcus, Jeotgalibacillus, Thalassobacillus, Halobacillus, Exiguobacterium, Piscibacillus, Gracibacillus, Fictibacillus, Viridibacillus.*

Introduction

Among the different bacterial clades inhabiting the rhizosphere of plants, the Firmicutes is one of the most commonly found bacterial phyla. Firmicutes are reported to be abundant in rhizospheric soils, but also in other compartments such as the endorhizosphere of plants

(Kumar et al., 2012). Because of their supposed high culturability, with an estimate of 14% of the bacterial species represented in culture collections (Hugenholtz, 2002), firmicute species are relevant for the development of bio-based approaches applicable to sustainable agriculture. The aim of this review is to provide an extensive summary of the literature concerning the beneficial traits in plant growth promotion reported for members of the Phylum Firmicutes (with the exception of the genus *Bacillus*). In addition, examples of their use as potentially beneficial microorganisms in sustainable agriculture are indicated.

The most traditionally investigated traits in microorganisms with beneficial use in agriculture are those related to assisting the plant in acquiring a resource (e.g. nitrogen, iron, phosphorous or other essential minerals), modulating plant hormonal production or producing direct analogues of plant hormones, and controlling the development of plant pathogens (Glick, 2012; Ahemad and Kibret, 2014). However, nowadays, other soil-related challenges such as land desertification and the increase in soil salt content, as well as contamination by organic (e.g. pesticides) and inorganic pollutants (e.g. metals), could also be tackled by the use of beneficial microorganisms. Indeed, the ability of a specific bacterium to either withstand extreme environmental conditions and/or to detoxify its growth medium are becoming increasingly relevant in terms of its potential use to promote plant growth *in situ* (Shrivastava and Kumar, 2015; Tóth et al., 2016). Therefore, this review provides a summary of the traits related to plant growth promotion, biocontrol, and bioremediation for the genera *Lysinibacillus*, *Aneurinibacillus, Brevibacillus, Oceanobacillus, Planococcus, Clostridium, Sporosarcina, Virgibacillus, Terribacillus, Staphylococcus, Jeotgalibacillus, Thalassobacillus, Halobacillus, Exiguobacterium, Piscibacillus, Gracibacillus, Fictibacillus,* and *Viridibacillus*, all of which belong to the Phylum Firmicutes.

In order to facilitate the access to the material gathered in this review, we provide four tables that summarize the information concerning the plant growth promoting traits of individual species (Table 1); their ability to produce compounds with inhibitory activity and the plant pathogens against which the species have been tested (Table 2); information concerning the potential of the strains in the bioremediation of metal-contaminated soils (Table 3); and finally, *in vivo* tests with different plant models and the reported beneficial effect on plant growth or health (Table 4).

Genus *Lysinibacillus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae

Isolation

Members of the genus *Lysinibacillus* have been isolated from diverse environments and are reported to be potential symbionts of animals and plants, as well as free-living soil microorganisms (Kämpfer et al., 2013; de Fatima Gomes Cavados et al., 2017; Naureen et al., 2017; Martínez and Dussán, 2018).

Identification

Members of *Lysinibacillus* genus are motile, with rod-shaped cells that produce endospores of ellipsoidal or spherical shape. Members are positive for oxidase and catalase tests while negative for indole and H_2S production. The cell wall peptidoglycan type is A4 α (Lys–Asp). The predominate cellular fatty acid is iso-C 15 : 0. The major menaquinone is MK-7, whereas the predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and ninhydrin-positive phosphoglycolipid. The G+C content is 35–38 mol% (Ahmed et al., 2007b).

Beneficial role in Agroecology

Members of the genus *Lysinibacillus* have been long-known as biocontrol agents of insects due to their larvicidal activity against mosquitoes (Lozano and Dussán, 2013). However, *Lysinibacillus* spp. are also important as plant growth promoting bacteria. Naureen et al. (2017) isolated *Lysinibacillus sphaericus* ZA9 from maize *(Zea mays)* rhizospheric soil and showed that this strain was positive for multiple plant growth promoting traits. *L. sphaericus* ZA9 was able to produce Indole 3 Acetic acid (IAA), siderophores, and HCN. Additionally, because of the production of uncharacterized organic acids, this strain was also capable of solubilizing phosphate from sources such as potash and silicates. Moreover, the detection of hydrolytic enzymes such as chitinases, lipases, and proteases led to the screening of its biocontrol activity against different phytopathogenic fungi. *L. sphaericus* ZA9 was able to suppress the growth of several fungi. Moreover, the production of the antifungal compound 2-Penthyl-4-quinolinecarboxylic acid suggests a considerable potential of this strain as a fungal biocontrol agent. Finally, in a greenhouse experiment, *L. sphaericus* ZA9 showed remarkable beneficial effects on vegetable crops (cucumber and tomato) by augmenting seed germination and seedling growth (Naureen et al., 2017).

In another study, an endophytic *L. sphaericus* L1 strain was isolated from the phyllosphere of rice *(Oryza sativa)* (Shabanamol et al., 2017a). This strain was able to produce different kinds of phytohormones (cytokinin, IAA, and gibberellic acid) and to fix atmospheric nitrogen. An *in vitro* confrontation assay showed that *L. sphaericus* L1 inhibited the growth of *Rhizoctonia solani*, the phytopathogenic fungus responsible for rice sheath blight disease. Moreover, the biocontrol activity of this strain was also tested in a leaf detached assay. This assay confirmed the effectiveness of the strain to inhibit the germination of *R. solani* sclerotia. In another greenhouse experiment, the same group found that *L. sphaericus* L1 induced systemic resistance in rice plants against *R. solani*, emphasizing the potential of this strain to be used against sheath blight disease (Shabanamol et al., 2017b).

Inoculation with *L. sphaericus* SNCh5, a strain isolated from spinach *(Spinacia oleracea)* phyllosphere, enhanced seed germination and seedling growth of two important crops: methi (*Trigonella foenum-graecum*) and mung bean (*Vigna radiate*). This strain was positive for diverse plant growth promoting traits, for instance nitrogen fixation, ammonia production (through ammonification of urea), IAA production, phosphate solubilization, all of which directly or indirectly can exert a beneficial effect on plant health (Sharma and Saharan, 2015). There are many other reports showing the potential of *Lysinibacillus* spp. in plant growth promotion. For instance, an inoculant of *Lysinibacillus* sp. 1TH16a (positive for IAA production and phosphate solubilization) was used to improve the growth of tomato (*Solanum lycopersicum*) (Sahu et al., 2018). The endophytic *Lysinibacillus fusiformis* E-I-20, isolated from ginseng stem, was found to be positive for various plant growth promoting traits (IAA production, phosphate solubilization, and siderophore production), suggesting a beneficial role in growth promotion of ginseng (Vendan et al., 2010). Likewise, *L. fusiformis* Lf89, a strain isolated from tomato rhizosphere, showed a beneficial effect on the growth of different plants (*Arabidopsis thaliana*, *Datura stramonium,* and *Datura tatula*) (Rahmoune et al., 2017). Two other strains of *L. fusiformis* (strains PM-5 and PM-24), isolated from soya bean *(Glycine max)* and rice rhizospheric soil, respectively, produced IAA and were able to fix atmospheric nitrogen (Park et al., 2005). Similarly, *Lysinibacillus xylanilyticus*, isolated from rhizospheric soil of *Phyllanthus amarus,* was positive for phosphate solubilization as well as IAA and siderophore production (Kadyan et al., 2013). *Another strain with interest for the control of* foodborne fungal and bacterial pathogens *is Lysinibacillus* sp. JX416856, which was isolated from fruits and vegetables waste. *Lysinibacillus* sp. JX416856 was able to produce a

thermostable bacteriocin with antimicrobial properties (Ahmad et al., 2014).

L. sphaericus has also a considerable potential in the phytoremediation of heavy metals (Martínez and Dussán, 2018). A consortium of four different *L. sphaericus* strains (strain CBAM5, isolated from hydrocarbon contaminated soil; strain III(3)7, isolated from an oak forest soil; and strains OT4b.31 and OT4b.49, both isolated from beetle larvae) improved the growth of jack bean (Canavalia *ensiformis*) planted on a lead-contaminated soil. The bioaccumulation of lead in the roots, enhanced root and shoot growth, and resulted in an increase in the number of leaves. All these beneficial effects were credited to the plant growth promoting activities of the strains in the consortium. These strains were positive for IAA production and nitrogen fixation, two properties that can play a vital role in ameliorating the response to toxic metal stress of the plant. *Lysinibacillus* sp. WB, isolated from industrial waste, is also a promising candidate for the phytoremediation of cadmium by alleviating its toxic effect and facilitating bioaccumulation of Cd in wheat (*Triticum aestivum*) plants (Gusain et al., 2017).

Genus *Aneurinibacillus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae

Isolation

Members of the genus *Aneurinibacillus* have been found in different environments, for instance, soil, marine, or heavy metal contaminated sites (Chauhan et al., 2014; Dey et al., 2016; Balan et al., 2017).

Identification

The genus *Aneurinibacillus* was identified by pyrolysis mass spectrometry, fatty acid methyl ester analysis, sodium dodecylsulfate-polyacryl- amide gel electrophoresis of whole-cell proteins and 99 API Biotype 100 assimilation tests (Heyndrickx et al., 1997). Members of this genus are motile, Gram-positive, rod-shaped, and generally aerobic (Tsubouchi et al., 2015; Subhash et al., 2017).

Beneficial role in Agroecology

Aneurinibacillus aneurinilyticus CKMV1 was isolated from the rhizosphere of the medicinal herb *Valeriana jatamansi* and was reported to be a plant growth promoting rhizobacterium

(Chauhan et al., 2014). In the *in vitro* screening for plant growth promoting traits, this strain was positive for solubilization of phosphate, nitrogen fixation, and also produced siderophores and AAI, all of which presuppose the significance of this strain in agronomical practices. *A. aneurinilyticus* CKMV1 was also able to produce the antimicrobial compound hydrogen cyanide (HCN). Moreover, a confrontation assay with different fungal plant pathogens showed that this strain was capable of inhibiting the pathogens growth and could be used as a disease suppressing agent. Bio-inoculation of tomato seeds with *A. aneurinilyticus* CKMV1 enhanced seed germination, shoot and root length, and also increased plant dry weight. Moreover, an enhancement in the NPK content of tomato plants treated with *A. aneurinilyticus* CKMV1 was also observed, as compared to untreated control plants, suggesting that this bacterium increases plant nutrient uptake (Chauhan et al., 2017). Another strain, *A. aneurinilyticus* BS-1 was found to be a heavy metal tolerant bacterium capable of removing 52% arsenite and 50% arsenate from arsenic-containing media. Therefore, this strain has a great potential to be used in the bioremediation of arsenic. Moreover, this bacterium was also reported to be salt tolerant, withstanding 8-10% sodium chloride concentrations (Dey et al., 2016). Aneurinifactin, a novel lipopeptide biosurfactant with antimicrobial activity was obtained from *A. aneurinilyticus* strain SBP-11, a strain isolated from a marine environment (Balan et al., 2017). Other species in the genus *Aneurinibacillus,* for instance, *A. migulanus* Nagano and *A. migulanus* NCTC 7096, are potential biocontrol agents against a wide range of fungi responsible for serious plant diseases. Both strains had antifungal activity against *Fusarium* spp., *B. cinerea, Rhizoctonia* spp. *Heterobasidion annosum* O2721, and *Phaeolus schweinitzii* P107. Moreover, both strains also inhibited the growth of different species of pathogenic oomycetes for example, *Pythium* spp., *Phytophthora* spp., and *Phytopythium vexans* P0-0/0046. *A. migulanus* Nagano and *A. migulanus* NCTC 7096 also possessed the genomic machinery for the production of different biosynthetic products with potential antimicrobial activity (Alenezi et al., 2016b). Moreover, these strains were also reported to produce biosurfactants that helped to inhibit spore germination of plant phytopathogens by reducing water tension at the surface of the plant, thereby decreasing water availability (Alenezi et al., 2017). In another study, both of these strains were tested against red band needle blight disease in young pine, a disease caused by the fungus *Dothistroma septosporum.* In forest nurseries, this fungal disease is commonly controlled by the application of copper-based chemicals. Among the two strains, *A. migulanus*

Nagano showed a dramatic reduction in infection with *D. septosporum,* resulting in a considerable potential as a biocontrol agent that could be used in forest nurseries of *Pinus contorta* (Alenezi et al., 2016a).

Genus *Brevibacillus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae

Isolation

Members of this genus were isolated from diverse sources, for instance, soil contaminated with heavy metals, marine habitats, natural hot water springs or the rhizosphere of different plants (e.g. *Phyllanthus amarus and Mangifera indica)* (Vivas et al., 2005; Desjardine et al., 2007; Saikia et al., 2011; Zhao et al., 2012; Kadyan et al., 2013).

Identification

Members of the genus *Brevibacillus* are characterized as rod shaped, motile, aerobic bacteria with the production of oval endospores. Members of the genus were identified by fatty acids analysis and polar lipid analysis. Moreover, the major isoprenoid quinone found in the members of this genus was MK-7 (Inan et al., 2012)

Beneficial role in Agroecology

The genus *Brevibacillus* also comprises strains with plant growth promoting traits. *B. brevis,* is a thermotolerant species that can tolerate high temperature fluctuations and could survive up to 52 °C. This species produced the phytohormone IAA and was positive for acetylene reduction up to 43°C (ammonia production was measured up to 46 °C). Moreover, antifungal activity against the fungus *Macrophomina phaseolina* was also attested and an inhibition zone was observed up to 40°C, showing that this strain could be used as a plant growth and biocontrol agent even at high temperatures. Inoculation of cotton seeds with *B. brevis* accelerated seed germination rate and also improved growth and productivity of cotton plants (Nehra et al., 2016). In another study where *B. brevis* IPC11 was used against a bacterial pathogen responsible for canker disease in tomato plants, the inoculant appeared to reduce disease symptoms for up to 54% as compared to control plants. Tomato seeds treated with *B. brevis* IPC11 also showed higher germination rates and bioinoculation resulted in boosted seedling length as compared to untreated seedlings (Girish and Umesha, 2005). Moreover, a

B. brevis strain isolated from soils contaminated with cadmium (Cd) and zinc (Zn) showed a positive interaction with *Glomus mosseae* (an arbuscular–mycorrhizal fungus; AMF) and was reported to be a mycorrhizal helper bacterium that could be used in management of Cd and/or Zn polluted soils. An *In vitro* experiment using media supplemented with different concentrations of Cd and Zn showed that the presence of *B. brevis* accelerated the rate of spore germination and mycelial growth of *G. mosseae*. This beneficial effect was the result of bioaccumulation of Cd by the bacterium, thus reducing the toxicity of the medium. Moreover, this strain was also capable of producing IAA, which may aid in promoting mycelial growth of this AMF (Vivas et al., 2005). In another study where co-inoculation of these two microorganisms (*B. brevis* and *G. mosseae*) was performed to assess the effect of Cd concentrations on the growth of white clover (*Trifolium repens)* in Cd-contaminated soil, co-inoculation reduced the toxicity symptoms associated to heavy metals and, at the same time, accelerated plant growth and development, increased mycorrhization, enhanced uptake of essential nutrients, and reduced Cd uptake in plant biomass (Vivas et al., 2003).

Brevibacillus laterosporus JP44SK51 isolated from the rhizosphere of *Phyllanthus amarus* also displayed plant growth promoting activities, for instance phosphate solubilization, IAA and siderophore production, and was positive for chitinolytic activity (Kadyan et al., 2013). Additionally, in the literature, *B. laterosporus* is reported as a source of diverse bioactive secondary metabolites with antibacterial and antifungal activities, for instance, tauramamide, a lipopeptide produced by *B. laterosporus* PNG276 (isolated from a marine habitat) that showed strong antibiotic activity against a pathogenic *Enterococcus* sp. (Desjardine et al., 2007). Another lipopeptide named BL-A60 and produced by *B. laterosporus* A60 (isolated from rhizospheric soil of a mango -*Mangifera indica*- plant), was tested *in vivo* on leaves of pepper plants infected with a conidial suspension of the oomycete plant pathogen *Phytophthora capsica,* which is responsible of blight and fruit rot in many crops. In this experiment, different concentrations of the lipopeptide BL-A60 were tested and a 100% disease control (no symptoms of infection on leaves and stems of the pepper plant) was noticed at the concentration of 300 µg/ml (Zhao et al., 2012).

B. laterosporus G4 isolated from soil samples, showed nematicidal activity, destroying the cuticle and ultimately digesting the entire body of nematodes. A crude extracellular proteinaceous extract of this bacterium contained a nematotoxic component of hydrolytic nature, which was responsible for the detrimental effect observed and that could potentially

be used in the biological control of pathogenic nematodes (Huang et al., 2005). In another study, *B. laterosporus* showed insecticidal activity against leaf beetle by producing parasporin, a crystalline protein that caused 100% death of the insect (Ghazanchyan et al., 2018). *B. laterosporus* BPM3 isolated from mud of a natural hot water spring, displayed a broad range of *in vitro* antifungal activities towards phytopathogenic fungi (*Magnaporthe grisea, Rhizoctonia oryzae, Fusarium oxysporum* and *Fusarium semitectum*). Moreover, this strain was also used in *in vivo* greenhouse pot experiments to control blast disease of rice caused by *Magnaporthe grisea* and provided 67% disease protection as compared to untreated plants (Saikia et al., 2011).

Genus *Oceanobacillus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae

Isolation

Members of this genus were isolated from marine environments (Antarctic sea water), sediments, hot springs and also from the roots of the Jojoba plants (*Simmondsia chinensis*) (Pakpitcharoen et al., 2008; El-Tarabily and Youssef, 2010; Jadhav et al., 2013; Perez-Rosales et al., 2017).

Identification

Members of this genus are Gram-positive, rod shaped cells, obligately aerobic or facultative anaerobic, motile by means of peritrichously flagella and form ellipsoidal spores (Yumoto et al., 2005). Growth occurs at temperature between 5 and 48 °C and at pH 7-12. Circular, convex and white colonies appeared when inoculated on peptone-yeast extract-glucose medium at 27 °C. Major isoprenoid quinone is menaquinone-7 (MK-7) and the DNA G+C content is 35.8–40.6 mol%. The cellular fatty acid analysis consisted of iso-C(15:0), anteiso-C(15:0), C(16:0) and anteiso-C(17:0). The polar lipids analysis consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and two unidentified lipids and phospholipids. The oxidase and catalase reactions are positive. The cell-wall peptidoglycan is composed of meso-Diaminopimelic acid identified by thin-layer chromatography (Hirota et al., 2013).

Beneficial role in Agroecology

Oceanobacillus picturae, isolated from sediments around the mangrove tree *Avicennia marina*, was capable of producing diverse organic acids and had the ability to solubilize phosphate. In a greenhouse experiment, inoculation of *O. picturae*, together with the addition of an insoluble phosphate source to the substrate, showed that this strain had the ability to improve growth (dry weight and length of the plants) of mangrove tree (El-Tarabily and Youssef, 2010). In another study where the halotolerant *Oceanobacillus* sp., which had 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, was inoculated on wheat, inoculation improved growth in a hydroponic culture containing a NaCl concentration of up to 200 mM (Orhan, 2016). When the enzyme ACC deaminase is synthetized by bacteria in the vicinity of plant roots, it can covert ACC to ammonia and alpha-ketobutyrate, a process that indirectly reduce the amount of ethylene produced by the plant (Glick, 2012; Ahemad and Kibret, 2014). In the same study, another species, *O. oncorhynch*, was positive for the production of IAA (Orhan, 2016). The endophyte *O. kimchi,* isolated from roots of Jojoba plants (*Simmondsia chinensis*), showed diverse plant growth promoting activities, for instance, phosphate solubilization, nitrogen fixation, IAA production, and ACC deaminase activity (Perez-Rosales et al., 2017).

In another study, *O. picturae,* which was isolated from a hot spring, was able to produce high levels of biosurfactants and also caused growth inhibition of a phytopathogenic *Fusarium* species (Pakpitcharoen et al., 2008). A thermostable biosurfactant of glycolipoproteic nature was produced by *Oceanobacillus* sp. BRI 10 (isolated from Antarctic sea water). This thermostable biosurfactant possessed antimicrobial properties (Jadhav et al., 2013). This strain has been reported to have also a considerable potential to be used in bioremediation of hydrocarbon pollution, as it shows excellent biodegradation of crude oil (Jadhav et al., 2013). *O. sojae* also produced a biosurfactant. However, when tested against three phytophatogenic fungi (*Botrytis cinerea* ATCC 46522, *Fusarium pallidoroseum* ATCC 48152, and *Fusarium moniliforme* ATCC 60846), *O. sojae* was unable to suppress fungal growth (de Senna and Lathrop, 2017).

Genus *Planococcus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae

Isolation

The genus *Planococcus* is widely spread in different environments and has been isolated from saline soils, plant rhizosphere, sea water, and from a cyanobacterial and algal mat (Reddy et al., 2002; Romano et al., 2003; Yoon et al., 2003; Siddikee et al., 2010; Rajput et al., 2013).

Identification

Members of this genus are non-spore forming motile or non-motile and Gram-positive, and grow as cocci that can associate in pairs or tetrads. Circular, raised to slightly convex, glistening, smooth, pale yellow in color colonies formed on marine agar after three days incubation at 30 °C. Growth occurs at 4–38 °C and pH range is 7–7.5. The chemotaxonomic characteristics of the members of this genus are the presence of major isoprenoid quinone (menaquinone 7 and 8) and also the presence of phosphatidylethanolamine, phosphatidylglycerol and bisphosphatydylglycerol as cellular polar lipids (Yoon et al., 2010). DNA G+C content is 39.0–51.2 mol% (Nakagawa et al., 1996; Yoon et al., 2010). Major cellular fatty acids are anteiso-C 15 : 0 and anteiso-C 17 : 0. Enzyme activities determined using the API ZYM system (bioMérieux) showed the absence of alkaline phosphatase, lipase (C14), valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase and presence of leucine arylamidase and cystine arylamidase (Yoon et al., 2010).

Beneficial role in Agroecology

Planococcus rifietoensis SAL-15, which was isolated from rhizospheric soil of wheat grown in a saline soil, exhibited various plant growth promoting traits (production of IAA, phosphorous solubilization, and ACC deaminase activity). Inoculation of *P. rifietoensis* SAL-15 improved growth (plant height and biomass) and yield of wheat plants under salt stress, alleviating the deleterious effect of salinity and stimulating plant growth (Rajput et al., 2013). Another halotolerant strain, *P. rifietoensis* RS18, which was isolated from saline soil samples (coastal region of the Yellow Sea), was positive for nitrogen fixation, thiosulfate oxidation, ammonia production, and cellulase production. However, when inoculated on canola seeds (*Brassica*

campestris), this strain failed to improve growth and development of canola plants in salt-stress conditions (Siddikee et al., 2010). *Planococcus citreus* BNE6 and *Planococcus salinarum* BSH13, isolated from wheat rhizosphere, had multiple plant growth promoting characteristics as assessed *in vitro*. *P. citreus* BNE6 solubilized phosphate, potassium and zinc, produced IAA and ammonia, and was also positive for the production of some lytic enzymes such as chitinases, lipases and β-glucanases. The other strain, *P. salinarum* BSH13, was also able to solubilize phosphate, potassium and zinc, and to produce lytic enzymes (protease and lipase) (Verma et al., 2016). Some psychrophilic strains isolated from North Western Indian Himalayas also displayed plant growth promoting traits. *Planococcus antarcticus* AL9 was positive for siderophore, ammonia, and HCN production. *Planococcus donghaensis* AN39 was positive for phosphate solubilization, IAA, siderophore, and ammonia production; while *Planococcus kocurii* AL3 was positive for IAA, siderophore, HCN and ACC deaminase activity (Yadav et al., 2016).

Strains of the genus *Planococcus* also exhibited biocontrol activities against plant pathogenic fungi. For instance, *P. rifitoensis* M2-26 isolated from a shallow saline lake possessed chitinase and glucanase activities and was able to suppress the growth of *B. cinerea*, a fungus responsible for the grey mould disease in strawberries and tomatoes (Sadfi-Zouaoui et al., 2008; Essghaier et al., 2009). Members of *Planococcus* spp. are also reported to be heavy metal tolerant, for instance, *Planococcus* sp. KRPC10YT, isolated from an arsenic contaminated bore-well, was capable of tolerating arsenate (30 mM) and arsenite (20 mM) (Chowdhury et al., 2009). Another strain of *Planococcus* sp. TRC1, isolated from a tannery effluent, showed tolerance to high concentrations of chromium (Cr) and was able to reduce toxicity in the tannery liquid waste by Cr biosorption, showing its potential to be used in bioremediation processes (Behera et al., 2015).

Genus *Clostridium*

Taxonomy

Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae

Isolation

Clostridium is one of the most important genus within the Firmicutes and members of this genus are isolated from different ecological niches (Zeiller et al., 2015) e.g. rhizosphere of pea

(*Pisum sativum*), or leaves of *Miscanthus sinensis* leaves or either isolated from a rice paddy field (Polyanskaya et al., 2002; Ye et al., 2005; Doni et al., 2014).

Identification

Members of *Clostridium* genus are obligate anaerobes (some species can tolerate oxygen) motile or non-motile, spherical or oval endospore-forming bacteria with ability to form up to five endospores per cell (Duda et al., 1987; Zeiller et al., 2015). Members are usually Gram-positive-staining, although in some species cells are noticed as Gram-variable or even Gram-negative-staining. The cell wall of this genus usually contains meso-diaminopimelic acid. Members are able to metabolize alcohols, amino acids, carbohydrates, purines, steroids or other organic compounds. Optimal growth usually occurs at pH 6.5–7 and at temperatures between 30- 37 °C. Some species are thermophilic or psychrophilic, Acidophilic and alkaliphilic. The DNA G+C content is between 23-37 mol% (Lawson and Rainey, 2016).

Beneficial role in Agroecology

A *Clostridium* sp., which was isolated form rhizosphere of pea (*Pisum sativum*), was reported to enhance growth of barley (*Hordeum vulgare)* and cucumber (*Cucumis sativus*) plants. Moreover, it promoted the growth of native bacterial populations induced and hindered fungal growth, possibly due to antifungal activities (Polyanskaya et al., 2002). Two strains of *Clostridium beijerinckii* (strains H110 and TB8) showed strong antifungal activity against *F. oxysporum*, a pathogen causing, among other diseases, spinach wilt disease. The co-incubation of these bacteria in anaerobic media caused damage to the mycelial cell wall by degrading major cell wall components, for instance, chitosan, curdlan and laminarin (Ueki et al., 2017). Inoculation of spores of *Clostridium botulinum* 2301 on clover seedlings showed enhanced plant growth and was effective for the bacterial colonization of the rhizosphere, as well as endophytic colonization (Zeiller et al., 2015). In another study endophytic clostridia were reported to have nitrogen fixing abilities associated to gramineous plants (Minamisawa et al., 2004). A consortium of anaerobic nitrogen fixing bacteria consisting of an endophytic *Clostridium* sp. Kas201 (isolated from *Miscanthus sinensis* leaves) and *Enterobacter* sp. B901-2 (isolated from *Oryza oficinalis* stems) alleviated plant damage and increased tolerance to salinity stress in the grass *Miscanthus sinensis* (Ye et al., 2005). *Clostridium* sp. FWM1, isolated from a rice paddy field, improved growth of rice seedlings, possibly due to plant growth promoting traits, although this was not directly measured (Doni et al., 2014). A consortium of *Bacillus* spp. and *Clostridium* spp. was reported to control the growth of the fungus

Peniophora sacrata, which causes root and stem canker in apple trees (Taylor and Guy, 1981).

Genus *Sporosarcina*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae

Isolation

Members of this genus can be found in sea water, plants, and also in sites contaminated with hydrocarbons (Yoon et al., 2001a; Janarthine and Eganathan, 2012; Cruz-Morales et al., 2016).

Identification

Members of the genus *Sporosarcina* are motile, facultative anaerobic, spore-forming Firmicutes. The cell wall peptidoglycan is A4alpha, based on L-Lys-L-Ala-D-Asp. The major menaquinone is MK-7 and the major cellular fatty acid is ante-C15:0. DNA G+C content is 40 mol% (Yoon et al., 2003).

Beneficial role in Agroecology

Sporosarcina aquimarina SjAM16103, isolated from the pneumatophores of mangrove trees, had multiple plant growth promoting attributes such as siderophore production, phosphate solubilization, IAA production, and nitrogen fixation. Inoculation of this endophytic bacterium in sterile explants accelerated plant growth and development of four different plants (*Bacopa monnieri*, *Eupatorium triplinerve*, *Excoecaria agallocha*, and *Avicennia marina*). Moreover, this experiment demonstrated that *S. aquimarina* SjAM16103 is a non-host specific strain (Janarthine and Eganathan, 2012). Another IAA-producing strain of *S. aquimarina* (strain Q3), which was isolated from soil contaminated with petroleum hydrocarbons, influenced positively seedlings growth of the grass *Axonopus affinis* (Cruz-Morales et al., 2016). *Sporosarcina* sp. BNW4, isolated from wheat rhizosphere, exhibited multiple plant growth promoting characteristics, such as phosphate and zinc solubilization, IAA production, and nitrogen fixation. This strain was also capable of producing lytic enzymes e.g., lipases and β-glucanases (Verma et al., 2016).

As in the case of psychrophilic *Planococcus* spp., psychrophilic *Sporosarcina* spp. have been attributed multiple beneficial plant growth promoting even at low temperatures. This could be used to ameliorate cold stress in plants (Yadav et al., 2016). For instance, *S. aquimarina*

IARI-AL77 was positive for IAA, siderophore, giberrellic acid, and ammonia production, as well as for ACC deaminase activity. *Sporosarcina globispora* IARI-AR111 was positive for IAA and gibberellic acid production, potassium and zinc solubilization, and ACC deaminase activity. Similarly, *Sporosarcina pasteurii* IARI-AR37 was capable of producing IAA and siderophore, and of solubilizing phosphate, potassium and zinc; while *Sporosarcina psychrophila* IARI-AR110 was positive for the production of IAA and ammonia, and for ACC deaminase activity. All of these PGP traits were assessed at three different temperatures: 4, 15 and 30°C in all the above-mentioned strains (Yadav et al., 2016), suggesting that such strains may be used over a wide range of environmental temperatures.

Genus *Virgibacillus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae

Isolation

Members of this genus are found in different habitats, for instance, saline lake, saline or soil deep-sea rock (Essghaier et al., 2009; Sánchez-Porro et al., 2014; Orhan, 2016; Huang et al., 2018)

Identification

Gram-positive, endospore-formers producing spores with different shapes (from spherical to ellipsoidal) (Kämpfer et al., 2011; Sánchez-Porro et al., 2014). Positive for catalase and casein hydrolysis (Heyrman et al., 2003). For optimal growth, salt is required at concentrations ranging from 5 and 10 % with an optimal temperature range at 25–30 °C. Species of this genus can only be distinguished from each other based on the mean percentages of their major fatty acid, anteiso-C 15 : 0. The polar lipid consists of diphosphatidyl glycerol and phosphatidyl glycerol. The major menaquinone is MK-7. DNA G+C content is 36–43 mol% (Heyrman et al., 2003)

Beneficial role in Agroecology

Virgibacillus dokdonensis MCCC 1A00493, isolated from a deep-sea rock, exhibited antibacterial activity against the bacterial plant pathogen *Xanthomonas oryzae* pv. *Oryzae* (Huang et al., 2018). *A* halotorelant *Virgibacillus marismortui*, isolated from a saline lake, had the ability to produce volatile compounds and inhibited the growth of the phytophathogenic

fungus *B. cinerea*. Moreover, the same species was reported to produce enzymes with antifungal activity (chitinase and β-1,3-glucanase) (Essghaier et al., 2009). *V. marismortui* was applied on harvested strawberries and showed considerable suppression of *B. cinerea* growth (Essghaier et al., 2009). Finally, a halotolerant *Virgibacillus picturae* IB, isolated from a saline soil, was positive for ACC deaminase (Orhan, 2016).

Genus *Terribacillus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae

Isolation

Members of this genus have been isolated from saline environment and also from rhizosphere of plants (*Phyllanthus amarus* or *Cryptomeria forunei*) (An et al., 2007; Kadyan et al., 2013; Lu et al., 2015).

Identification

Members of this genus are Gram-positive, aerobic, endospore forming and rod-shaped bacteria (An et al., 2007; Liu et al., 2010). Chemotaxonomic analyses showed that members of this genus are halotorelant (An et al., 2007). Acetoin is produced while H_2S and indole are not produced. Bacterial colonies are circular and convex. The DNA G+C contents varies from 44 to 46 mol%. The major fatty acids are anteiso-C 15 : 0 and anteiso-C 17 : 0. The cell-wall peptidoglycan is composed of meso-diaminopimelic acid. The major menaquinone type is MK-7 (An et al., 2007).

Beneficial role in Agroecology

Terribacillus aidingensis MP602, isolated from an old tree of *Cryptomeria forunei*, tolerated up to 14% salt concentrations and exhibited strong antifungal activities against phytopathogenic fungi such as *B. cinerea*, *M. grisea* and *R. solani* (Lu et al., 2015). In another study, two strains of *Terribacillus halophilus* (strains J16 *and* J31) were reported as potential biocontrol agents for the inhibition of *B. cinerea.* Both of these strains were reported to produce hydrolytic enzymes such as proteases, chitinases and β-1,3-glucanases, which possibly provided the observed biocontrol activity (Essghaier et al., 2009). *Terribacillus saccharophilus* JP44SK46 and *Terribacillus goriensis* JP44SK47, isolated from the rhizosphere of *Phyllanthus amarus,* were positive for phosphate solubilization, IAA, and siderophore

production (Kadyan et al., 2013).

Genus *Staphylococcus*

Taxonomy

Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae

Isolation

Members of this genus have been isolated from saline water (lake) or saline soil (Essghaier et al., 2009; Orhan, 2016).

Identification

Members are identified as Gram-positive coccus that forms cluster, cell wall contain peptidoglycan (A3α l-Lys–Gly5) and teichoic acid. The major cellular fatty acids are i-C15 : 0, ai-C15 : 0, i-C17 : 0 and C20 : 0 (Freney et al., 1999; Taponen et al., 2012). DNA G+C content in a range of 30-40 mol% (Freney et al., 1999).

Beneficial role in Agroecology

Although less work has been done on the genus *Staphylococcus* because members of this group are reported to be potential pathogens posing a serious health risk (Essghaier et al., 2009), there are also reports of beneficial traits for specific species. *Staphylococcus equorum* and *Staphylococcus* sp., isolated from a saline lake, showed biocontrol activity against *B. cinerea* on harvested strawberries. Both of these strains were reported to be halotolerant and grew on high salt concentration (5-20% NaCl) (Essghaier et al., 2009). In another study, *S. equorum* L40 and *S. equorum* L50 were able to suppress *B. cinerea* infection of tomatoes (Sadfi-Zouaoui et al., 2008). *Staphylococcus succinus* EN4, isolated from a saline soil, exhibited plant growth promoting characteristics, such as ammonia production and phosphate solubilization (Orhan, 2016). It has been reported that *S. succinus* EN4 enhanced wheat growth and development in soil with high salt content (200 mM NaCl). This positive response could be attributed to the presence of plant growth promoting properties present in the species (Orhan, 2016).

Other genera

For information concerning the taxonomy, isolation and identification of each genus in this section, please refer to Table 5.

Beneficial role in Agroecology

A member of the genus ***Jeotgalibacillus****, Jeotgalibacillus* sp. JP44SK37, isolated from the rhizosphere of the medicinal herb *Phyllanthus amarus, showed phosphate solubilization and IAA production (Kadyan et al., 2013).* ***Thalassobacillus*** sp. (strain ID) and ***Halobacillus*** sp. (strain IF), both isolated from a saline soil, exhibited only a limited growth promoting activity (AAC deaminase production), but nonetheless, boosted significantly wheat health by alleviating salt stress (200 mM NaCl) (Orhan, 2016). *Halobacillus* sp. ADN1 and *Halobacillus* sp. MAN6, isolated from rhizospheric soil of mangrove, displayed plant growth promoting characteristics in the presence of heavy metals and salinity stress, which could be helpful in the process of heavy metals phytoremediation (Desale et al., 2014). Both of them were able to perform phosphate solubilization and produced IAA, HCN, and siderophores, as well as possessed interesting enzymatic activities (amylase and protease) (Desale et al., 2014). Moreover, both strains showed antifungal activity against *Fusarium* spp. The application of a consortium of two *Halobacillus* spp. (strains ADN1 and MAN6) along with the non-Firmicutes *Halomonas* sp. MAN5 promoted growth of *Sesuvium portulacastrum* (Desale et al., 2014). A member of the genus ***Exiguobacterium****, E. aurantiacum* EN9 was positive for ACC deaminase activity (Orhan, 2016). In another study, ***Piscibacillus*** *salipiscarius* E5, isolated from a saline lake, increased biomass of wheat plants that were exposed to salt stress (200 mM NaCl) by mitigating the deleterious salinity effects. This species exhibited multiple plant growth promoting attributes, including nitrogen fixation, phosphate solubilization, IAA, HCN, and siderophore production (Naili et al., 2018). A halophilic ***Gracilibacillus*** *saliphilus* EA8, isolated from the phyllosphere of *Arthrocnemum macrostachyum*, showed IAA and siderophore production. This species was used in a consortium with other *Bacillus* spp. and promoted seed germination and sodium uptake in plant biomass, which may lead to enhanced phytoextraction during restoration of land damages caused by salinity (Navarro-Torre et al., 2017). ***Fictibacillus*** *barbaricus* TSA50, isolated from spores of the AMF *Rhizophagus intraradices* (now *R. irregularis*), displayed high production of IAA (Battini et al., 2016). Isolates of *Fictibacillus* spp. have been found endophytically in roots and stems of rice (Raweekul et al., 2016). Two strains, *F. phosphorivorans* and *F. arsenicus,* were reported to have nematicidal activity (Zheng et al., 2016). Another study reported that *Fictibacillus* spp. could be important in bioremediation processes in saline areas because of the production of surfactants that have emulsification properties both at high temperature and in saline

environments (de Almeida Couto et al., 2015). Two strains of the genus ***Viridibacillus,*** *V. arenosi* SG15 and *V. arvi* SG26 isolated from a mangrove tree soil were positive for siderophore production and ACC deaminase activity (Lyngwi et al., 2016).

Conclusion

This review demonstrates that members of Phylum Firmicutes are extremely relevant as beneficial microorganisms in agroecology. Different genera and species affiliated to this Phylum have been isolated from diverse environments. These isolates display a plethora of plant beneficial activities, some of which are expressed even under extreme environmental conditions (temperature, salinity, metal contamination). Experiments performed already in a multitude of plant models demonstrate that members of this Phylum can either directly or indirectly contribute to the germination, establishment and growth of plants, even under different biotic (pathogen pressure) and abiotic (temperature, salt, or metal contents) stresses.

Acknowledgments

This research was supported by the U.S. Department of Energy Biological and Environmental Research Division through a Science Focus Area grant to P.J. (Grant number KP1601010) and “Fondation Pierre Mercier pour la Science” grant to S.B.

Table 1. Summary of the plant growth promoting activities reported for different members of the Firmicutes. The genus, species name and strain denomination, when available, are provided. Those species for which a specific strain information is not available are indicated as N.D. (not determined). When available the source of isolation is also indicated. The beneficial activities are divided into three main categories: nutrient acquisition, phytohormone production, and biocontrol (Glick, 2012). For nutrient acquisition the processes more commonly reported are nitrogen fixation (Nfix), phosphate solubilization (Psol), and siderophore production (Iron); in addition, other processes such as ammonia production, or solubilization of other essential nutrients are indicated. In the case of phytohormone modulation, the two most reported are auxin-like indole-3-acetic acid (IAA), as well as other hormones such cytokinins (C) and gibberellins (G), or the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCd). It is important to indicate that for some of the traits, the function could be multiple. For instance, siderophore production can also inhibit pathogen development. Likewise, ammonia production could be seen as indirect evidence of the production of the AAC deaminase.
*= acetylene reduction assay positive above 40°C.

				Plant growth promoting properties							**Reference**
				Nutrient acquisition				**Phytohormones**			
Genus	**species**	**Strain**	**Isolation source**	**Nfix**	**Psol**	**Iron**	**Other**	**IAA**	**ACCd**	**Other**	
Lysinibacillus	*sphaericus*	ZA9	Rhizospheric soil of maize		+	+	Silica solubilization	+			(Naureen et al., 2017)
Lysinibacillus	*sphaericus*	L1	Rice phyllosphere	+				+		C/G	(Shabanamol et al., 2017a; Shabanamol et al., 2017b)
Lysinibacillus	*sphaericus*	SNCh5	Spinach phyllosphere	+	+		Ammonia production	+			(Sharma and Saharan, 2015)
Lysinibacillus	sp.	1TH16a			+			+			(Sahu et al., 2018)
Lysinibacillus	*fusiformis*	E-I-20	Stem of ginseng		+	+	+	+			(Vendan et al., 2010)
Lysinibacillus	*fusiformis*	PM- 24	Rhizosphere of rice	+				+			(Park et al., 2005)
Lysinibacillus	*xylanilyticus*	N.D.	Rhizosphere of *Phyllanthus amarus*		+	+	+	+			(Kadyan et al., 2013)
Lysinibacillus	*sphaericus*	CBAM5	Hydrocarbon contaminated soil	+				+			(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	III(3)7	soil of an oak forest	+				+			(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	OT4b.31	Beetle larvae	+				+			(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	OT4b.49	Beetle larvae	+				+			(Martínez and Dussán, 2018)

Aneurinibacillus	*aneurinilyticus*	CKMV1	Rhizosphere of *Valeriana jatamansi*	+	+	+		+			(Chauhan et al., 2014)
Brevibacillus	*brevis*	SVC(II)14	Rhizosphere of cotton	+			Ammonia production	+			(Nehra et al., 2016)
Brevibacillus	*brevis*	N.D.	Cadmium/Zinc-contaminated soils					+			(Vivas et al., 2003; Vivas et al., 2005)
Brevibacillus	*laterosporus*	JP44SK51	Rhizosphere of *Phyllanthus amarus*		+	+		+			(Kadyan et al., 2013)
Oceanobacillus	*picturae*	N.D.	Rhizospheric soil of mangrove (*Avicennia marina*)		+						(El-Tarabily and Youssef, 2010)
Oceanobacillus	sp.	N.D.	Soil affected with salinity						+		(Orhan, 2016)
Oceanobacillus	*oncorhynch*	N.D.	Soil affected with salinity					+			(Orhan, 2016)
Oceanobacillus	*kimchi*	N.D.	Roots of Jojoba plants (*Simmondsia chinensis*)	+	+			+	+		(Perez-Rosales et al., 2017)
Planococcus	*rifietoensis*	SAL-15	Saline wheat rhizospheric soil		+			+	+		(Rajput et al., 2013)
Planococcus	*rifietoensis*	RS18	Saline soil	+			Thiosulfate oxidation, Ammonia and cellulase production				(Siddikee et al., 2010)
Planococcus	*citreus*	BNE6	Wheat rhizosphere		+		Potassium and zinc solubilization	+			(Verma et al., 2016)
Planococcus	*salinarum*	BSH13	Wheat rhizosphere		+		Potassium and zinc solubilization				(Verma et al., 2016)
Planococcus	*antarcticus*	AL9	Cold desert of Indian Himalayas			+	Ammonia production				(Yadav et al., 2016)
Planococcus	*donghaensis*	AN39	Cold desert of Indian Himalayas		+	+	Ammonia production	+			(Yadav et al., 2016)
Planococcus	*kocurii*	AL3	Cold desert of Indian Himalayas			+		+	+		(Yadav et al., 2016)
Clostridium	sp.	Kas201	Leaves of *Miscanthus sinensis*	+							(Ye et al., 2005)
Sporosarcina	*aquimarina*	SjAM16	Pneumatophores of	+	+	+		+			(Janarthine and

		103	mangroves								Eganathan, 2012)
Sporosarcina	*aquimarina*	Q3	Soil contaminated with petroleum hydrocarbons					+			(Cruz-Morales et al., 2016)
Sporosarcina	sp.	BNW4	Wheat rhizosphere	+	+		Zinc solubilization	+			(Verma et al., 2016)
Sporosarcina	*aquimarina*	IARI-AL77	Cold desert of Indian Himalayas			+	Ammonia production	+	+	G	(Yadav et al., 2016)
Sporosarcina	*globispora*	IARI-AR111	Cold desert of Indian Himalayas				Potassium and zinc solubilization	+	+	G	(Yadav et al., 2016)
Sporosarcina	*pasteurii*	IARI-AR37	Cold desert of Indian Himalayas		+	+	Potassium and zinc solubilization	+			(Yadav et al., 2016)
Sporosarcina	*psychrophila*	IARI-AR110					Ammonia production	+	+		(Yadav et al., 2016)
Virgibacillus	*picturae*	IB	Saline soil						+		(Orhan, 2016)
Terribacillus	*saccharophilus*	JP44SK46	Rhizosphere of *Phyllanthus amarus*		+	+		+			(Kadyan et al., 2013)
Terribacillus	*goriensis*	JP44SK47	Rhizosphere of *Phyllanthus amarus*		+	+		+			(Kadyan et al., 2013)
Staphylococcus	*succinus*	EN4	Saline soil		+		Ammonia production				(Orhan, 2016)
Jeotgalibacillus	sp.	JP44SK37	Rhizosphere of *Phyllanthus amarus*		+			+			(Kadyan et al., 2013)
Thalassobacillus	sp.	ID	Saline soil						+		(Orhan, 2016)
Halobacillus	sp.	IF	Saline soil						+		(Orhan, 2016)
Halobacillus	sp.	ADN1	Rhizospheric soil of mangrove (*Avicennia marina*)		+	+		+			(Desale et al., 2014)
Halobacillus	sp.	MAN6	Rhizospheric soil of mangrove (*Avicennia marina*)		+	+		+			(Desale et al., 2014)
Exiguobacterium	*aurantiacum*	EN9	Soil affected with salinity						+		(Orhan, 2016)
Piscibacillus	*salipiscarius*	E5	Salt lake	+	+	+		+			(Naili et al., 2018)
Gracilibacillus	*saliphilus*	EA8	Phyllosphere of *Arthrocnemum macrostachyum*			+		+			(Navarro-Torre et al., 2017)

Fictibacillus	*barbaricus*	TSA50	Spores of *Rhizophagus intraradices*					+			(Battini et al., 2016)
Viridibacillus	*arenosi*	SG15	Soil of mangrove			+			+		(Lyngwi et al., 2016)
Viridibacillus	*arvi*	SG26	Soil of mangrove			+			+		(Lyngwi et al., 2016)

Table 2. Summary of biocontrol traits reported. The genus, species name and strain denomination, when available, are provided. Those species for which a specific strain information is not available are indicated as N.D. (not determined). When available the source of isolation is also indicated. Hydrogen cyanide (HCN) production or the specific production of bioactive molecules are indicted. Enzymes such as chitinases, cellulases, -1,3 glucanases, proteases, and lipases that can lyse a portion of the cell walls of many pathogenic fungi are indicated as "lytic enzymes" when no specific information can be provided. Finally, the specific phytopathogens against which the species is relevant are indicated.

Genus	species	Strain	Isolation source	HCN	Active compounds	Antifungal/Antibacterial properties against	Reference
Lysinibacillus	*sphaericus*	ZA9	Rhizospheric soil of maize	+	+ (2-Penthyl-4-quinolinecarboxylic acid)	*Alternaria alternata, Curvularia lunata, Aspergillus* sp., *Sclerotinia* sp., *Bipolaris spicifera, Trichophyton* sp.	(Naureen et al., 2017)
Lysinibacillus	*sphaericus*	L1	Rice phyllosphere			*Rhizoctonia solani*	(Shabanamol et al., 2017a; Shabanamol et al., 2017b)
Lysinibacillus	sp.	JX416856	Waste of fruits and vegetable		+ (thermostable bacteriocin)		(Ahmad et al., 2014)
Aneurinibacillus	*aneurinilyticus*	CKMV1	Rhizosphere of *Valeriana jatamansi*	+		*Sclerotium rolfsii, Rhizoctonia solani, Dematophora necatrix, Alternaria spp, Fusarium oxysporum*	(Chauhan et al., 2014)
Aneurinibacillus	*aneurinilyticus*	SBP-11	Marine environment		+ (aneurinifactin)		(Balan et al., 2017)
Aneurinibacillus	*migulanus*	Nagano	Culture collection, University of Aberdeen, United Kingdom		+	*Fusarium* spp., *B. cinerea, Rhizoctonia* spp. *Heterobasidion annosum* O2721, *Phaeolus schweinitzii* P107, and *Dothistroma septosporum*	(Alenezi et al., 2016a; Alenezi et al., 2016b)
Aneurinibacillus	*migulanus*	NCTC 7096	National Collection of Type Cultures (NCTC, Porton Down, Salisbury, UK		+	*Fusarium* spp., *B. cinerea, Rhizoctonia* spp. *Heterobasidion annosum* O2721, and *Phaeolus schweinitzii* P107	(Alenezi et al., 2016b)
Brevibacillus	*brevis*	SVC(II)14	Rhizosphere of cotton			*Macrophomina phaseolina*	(Nehra et al., 2016)
Brevibacillus	*brevis*	IPC11			+ (antibacterial)		(Girish and Umesha, 2005)
Brevibacillus	*laterosporus*	JP44SK51	Rhizosphere of *Phyllanthus amarus*		+ (chitinases)		(Kadyan et al., 2013)

Brevibacillus	*laterosporus*	PNG276	Marine habitat		+ (tauramamide)	*Enterococcus* spp.	(Desjardine et al., 2007)
Brevibacillus	*laterosporus*	A60	Soil of a mango plant		+ (lipopolipeptide BL-A60)	*Phytophthora capsica* (Oomycete)	(Zhao et al., 2012)
Brevibacillus	*laterosporus*	G4	Soil		+ (nematocidal protein)		(Huang et al., 2005)
Brevibacillus	*laterosporus*	BPM3	Mud of a natural hot water spring			*Magnaporthe grisea, Rhizoctonia oryzae, Fusarium oxysporum* and *Fusarium semitectum*	(Saikia et al., 2011)
Oceanobacillus	*picturae*	N.D.	Hot spring		+ (biosurfactant)	*Fusarium* sp.	(Pakpitcharoen et al., 2008)
Oceanobacillus	sp.	BRI 10	Antarctic sea water		+ (thermostable biosurfactant)		(Jadhav et al., 2013)
Oceanobacillus	*sojae*	N.D.			+ (biosurfactant)	*Botrytis cinerea* ATCC 46522 *Fusarium pallidoroseum* ATCC 48152 and *Fusarium moniliforme* ATCC 60846	(de Senna and Lathrop, 2017)
Planococcus	*rifietoensis*	M2-26	Shallow salt lake		+ (chitinase and glucanase)	*Botrytis cinerea*	(Sadfi-Zouaoui et al., 2008; Essghaier et al., 2009)
Planococcus	*citreus*	BNE6	Wheat rhizosphere		+ (lytic enzymes)		(Verma et al., 2016)
Planococcus	*salinarum*	BSH13	Wheat rhizosphere		+ (lytic enzymes)		(Verma et al., 2016)
Planococcus	*antarcticus*	AL9	Cold desert of Indian Himalayas	+			(Yadav et al., 2016)
Planococcus	*kocurii*	AL3	Cold desert of Indian Himalayas	+			(Yadav et al., 2016)
Clostridium	*beijerinckii*	H110	Soil treated with wheat bran			*Fusarium oxysporum*	(Ueki et al., 2017)
Clostridium	*beijerinckii*	TB8	Soil treated with plant material (*Brassica juncea)*			*Fusarium oxysporum*	(Ueki et al., 2017)
Sporosarcina	sp.	BNW4	Wheat rhizosphere		+ (lytic enzymes)		(Verma et al., 2016)
Virgibacillus	*dokdonensis*	MCCC 1A00493	Deep-sea rock		+ (antibacterial activity)	*Xanthomonas oryzae* pv. *Oryzae*	(Huang et al., 2018)
Virgibacillus	*marismortui*	M3-23	Salt lake		+ (volatiles)	*Botrytis cinerea*	(Essghaier et al., 2009)
Terribacillus	*aidingensis*	MP602	Old tree of *Cryptomeria forunei*		+	*Botrytis cinerea*, *Magnaporthe grisea, Rhizoctonia solani*	(Lu et al., 2015)

Terribacillus	*halophilus*	J16			+ (lytic enzymes)	*Botrytis cinerea*	(Essghaier et al., 2009)
Terribacillus	*halophilus*	J31			+ (lytic enzymes)	*Botrytis cinerea*	(Essghaier et al., 2009)
Staphylococcus	*equorum*	N.D.	Salt lake			*Botrytis cinerea*	(Essghaier et al., 2009)
Staphylococcus	sp.	N.D.	Salt lake			*Botrytis cinerea*	(Essghaier et al., 2009)
Staphylococcus	*equorum*	L40				*Botrytis cinerea*	(Sadfi-Zouaoui et al., 2008)
Staphylococcus	*equorum*	L50				*Botrytis cinerea*	(Sadfi-Zouaoui et al., 2008)
Halobacillus	sp.	ADN1	Rhizospheric soil of mangrove (*Avicennia marina*)	+		*Fusarium* spp.	(Desale et al., 2014)
Halobacillus	sp.	MAN6	Rhizospheric soil of mangrove (*Avicennia marina*)	+		*Fusarium* spp.	(Desale et al., 2014)
Piscibacillus	*salipiscarius*	E5	Salt lake	+			(Naili et al., 2018)

Table 3. Summary of species with the potential for metal bioremediation. The genus, species name and strain denomination, when available, are provided. Those species for which a specific strain information is not available are indicated as N.D. (not determined). When available the source of isolation is also indicated. When the metal bioremediated is know, this is indicated within brackets.

Genus	**species**	**Strain**	**Isolation source**	**Metal bioremediation**	**Reference**
Lysinibacillus	*sphaericus*	CBAM5	Hydrocarbon contaminated soil	+ (lead)	(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	III(3)7	soil of an oak forest	+ (lead)	(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	OT4b.31	Beetle larvae	+ (lead)	(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	OT4b.49	Beetle larvae	+ (lead)	(Martínez and Dussán, 2018)
Lysinibacillus	sp.	WB	Industrial waste	+ (cadmium)	(Gusain et al., 2017)
Aneurinibacillus	*aneurinilyticus*	BS-1		+ (arsenite and arsenate)	(Dey et al., 2016)
Planococcus	sp.	KRPC10YT	Arsenic contaminated bore-well	+ (arsenite and arsenate)	(Chowdhury et al., 2009)
Planococcus	sp.	TRC1	Tannery effluent	+ (chromium)	(Behera et al., 2015)
Halobacillus	sp.	ADN1	Rhizospheric soil of mangrove (*Avicennia marina*)	+	(Desale et al., 2014)
Halobacillus	sp.	MAN6	Rhizospheric soil of mangrove (*Avicennia marina*)	+	(Desale et al., 2014)

Table 4. Summary of the *in planta* experiments performed with selected species and beneficial effect on plant reported. The genus, species name and strain denomination, when available, are provided. Those species for which a specific strain information is not available are indicated as N.D. (not determined). When available the source of isolation is also indicated.

Genus	**species**	**Strain**	**Isolation source**	***In vivo* plant tests**	**Effect**	**Reference**
Lysinibacillus	*sphaericus*	ZA9	Rhizospheric soil of maize	Cucumber, Tomato	Seedling vigor and germination of seeds and promoted the shoot length	(Naureen et al., 2017)
Lysinibacillus	*sphaericus*	L1	Rice phyllosphere	Rice	Increase seed germination and seedling growth	(Shabanamol et al., 2017a; Shabanamol et al., 2017b)
Lysinibacillus	*sphaericus*	SNCh5	Spinach phyllosphere	Methi (*Trigonella foenum-graecum*) and mung bean (*Vigna radiate*)	Enhanced seed germination and growth of the seedling	(Sharma and Saharan, 2015)
Lysinibacillus	sp.	1TH16a	Culture collection, India	Tomato		(Sahu et al., 2018)
Lysinibacillus	*fusiformis*	Lf89	Rhizosphere of tomato	*Arabidopsis thaliana*, *Datura stramonium* and *Datura tatula*		(Rahmoune et al., 2017)
Lysinibacillus	*sphaericus*	CBAM5	Hydrocarbon contaminated soil	Jack bean (*Canavalia ensiformis*)	Enhanced root and shoot length and increased number of leaves	(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	III(3)7	soil of an oak forest	Jack bean (*Canavalia ensiformis*)	Enhanced root and shoot length and increased number of leaves	(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	OT4b.31	Beetle larvae	Jack bean (*Canavalia ensiformis*)	Enhanced root and shoot length and increased number of leaves	(Martínez and Dussán, 2018)
Lysinibacillus	*sphaericus*	OT4b.49	Beetle larvae	Jack bean (*Canavalia ensiformis*)	Enhanced root and shoot length and increased number of leaves	(Martínez and Dussán, 2018)
Lysinibacillus	sp.	WB	Industrial waste	Wheat (*Triticum aestivum*)		(Gusain et al., 2017)
Aneurinibacillus	*aneurinilyticus*	CKMV1	Rhizosphere of *Valeriana jatamansi*	Tomato	Enhanced seed germination, shoot and length, shoot and root dry weight	(Chauhan et al., 2014)
Aneurinibacillus	*migulanus*	Nagano	Culture collection, University of Aberdeen, United Kingdom	*Pinus contorta*	Reduction in infection with *D. septosporum*	(Alenezi et al., 2016a; Alenezi et al., 2016b)
Brevibacillus	*brevis*	N.D.	Rhizospheric soil of cotton	Cotton seeds	Accelerated seed germination rate and improved growth and productivity of cotton plants	(Nehra et al., 2016)

Brevibacillus	*brevis*	IPC11	Culture collection, University of Mysore, India	Tomato	Reduce canker disease symptoms, improved seed germination rates, and boosted seedling length.	(Girish and Umesha, 2005)
Brevibacillus	*brevis*	N.D.	Cadmium/Zinc-contaminated soils	White clover (*Trifolium repens)*	Mycorrhizal helper bacterium of *Glomus mosseae.* Reduced heavy metal toxicity symptoms and promoted growth	(Vivas et al., 2003; Vivas et al., 2005)
Brevibacillus	*laterosporus*	A60	Soil of a mango plant	Leaves of pepper plants	No symptoms of infection at 300 µg/ml	(Zhao et al., 2012)
Brevibacillus	*laterosporus*	BPM3	Mud of a natural hot water spring	Rice	Provided 67% disease protection of blast disease	(Saikia et al., 2011)
Oceanobacillus	*picturae*	N.D.	Rhizospheric soil of mangrove (*Avicennia marina*)	Mangrove (*Avicennia marina*)	Increased dry weight and length of the plants	(El-Tarabily and Youssef, 2010)
Oceanobacillus	sp.	EN8	Saline soil	Wheat (*Triticum aestivum*)	Improved growth at high salt concentration (200 mM NaCl)	(Orhan, 2016)
Planococcus	*rifietoensis*	SAL-15	Saline wheat rhizospheric soil	Wheat (*Triticum aestivum*)	Improved growth and yield under salt stress	(Rajput et al., 2013)
Planococcus	*rifietoensis*	RS18	Saline soil	Canola seeds (*Brassica campestris*)	Failed to improve growth and development under salt stress	(Siddikee et al., 2010)
Planococcus	*rifietoensis*	M2-26	Shallow salt lake	Strawberries and tomatoes	Suppress the growth of the fungus responsible of grey mould disease	(Sadfi-Zouaoui et al., 2008; Essghaier et al., 2009)
Clostridium	*botulinum*	2301	Culture collection of Switzerland, Zurich University of Applied Sciences	White clover (*Trifolium repens)*	Enhanced plant growth and colonization of rhizosphere and endophytic colonization	(Zeiller et al., 2015)
Clostridium	sp.	Kas201	Leaves of *Miscanthus sinensis*	Grass (*Miscanthus sinensis*)	Alleviated plant damage and increased tolerance to salinity stress	(Ye et al., 2005)
Clostridium	sp.	FWM1	Rice paddy field	Rice	Improved growth of seedling	(Doni et al., 2014)
Sporosarcina	*aquimarina*	SjAM16103	Pneumatophores of mangroves	*Bacopa monnieri*, *Eupatorium triplinerve*, *Excoecaria agallocha*, and *Avicennia marina*	Accelerated plant growth and development	(Janarthine and Eganathan, 2012)
Sporosarcina	*aquimarina*	Q3	Soil contaminated with petroleum hydrocarbons	Grass seedlings (*Axonopus affinis*)	Improved growth of seedling	(Cruz-Morales et al., 2016)
Virgibacillus	*marismortui*	N.D.	Salt lake	Strawberry (harvested fruit)	Considerable suppression of *B. cinerea* growth	(Essghaier et

						al., 2009)
Staphylococcus	*equorum*	N.D.	Salt lake	Strawberry (harvested fruit)	Considerable suppression of *B. cinerea* growth	(Essghaier et al., 2009)
Staphylococcus	sp.	N.D.	Salt lake	Strawberry (harvested fruit)	Considerable suppression of *B. cinerea* growth	(Essghaier et al., 2009)
Staphylococcus	*equorum*	L40	Salt lake	Tomato	Suppression of fungal infection	(Sadfi-Zouaoui et al., 2008)
Staphylococcus	*equorum*	L50	Salt lake	Tomato	Suppression of fungal infection	(Sadfi-Zouaoui et al., 2008)
Staphylococcus	*succinus*	EN4	Saline soil	Wheat	Enhanced growth and development under salt stress	(Orhan, 2016)
Thalassobacillus	sp.	ID	Saline soil	Wheat	Enhanced growth and development under salt stress	(Orhan, 2016)
Halobacillus	sp.	IF	Saline soil	Wheat	Enhanced growth and development under salt stress	(Orhan, 2016)
Halobacillus	sp.	ADN1	Rhizospheric soil of mangrove (*Avicennia marina*)	*Sesuvium portulacastrum*	Enhanced growth	(Desale et al., 2014)
Halobacillus	sp.	MAN6	Rhizospheric soil of mangrove (*Avicennia marina*)	*Sesuvium portulacastrum*	Enhanced growth	(Desale et al., 2014)
Piscibacillus	*salipiscarius*	E5	Salt lake	Wheat	Increased biomass under salt stress	(Naili et al., 2018)
Gracilibacillus	*saliphilus*	EA8	Phyllosphere of *Arthrocnemum macrostachyum*	*Arthrocnemum macrostachyum*	Promotion of seed germination and sodium uptake in biomass	(Navarro-Torre et al., 2017)

Table 5. Taxonomy, isolation and identification for genera indicated in the section "Other genera".

Genus	Taxonomy	Isolation	Identification	References
Jeotgalibacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae	Members of this genus are widely spread and isolated from sandy beaches, salty environments and also from the rhizosphere of the herb *Phyllanthus amarus*	Gram-positive, rod shaped, anaerobe, moderately halophilic, round endospore forming bacteria. Positive for aesculin, Tween 80, gelatin and casein, hydrolysis. The cell-wall peptidoglycan type is A1α directly linked through L-lysine. The major menaquinones are MK-7 and MK-8. The predominant cellular fatty acid is iso-C 15:0. The DNA G+C content is 43-44 mol%. The major cellular polar lipids are diphosphatidylglycerol, phosphatidylglycerol and sulfoquinovosyl diacylglycerol	(Yoon et al., 2001b; Yaakop et al., 2015) *(Kadyan et al., 2013)*
Thalassobacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	Isolated from saline soil	Gram-positive, motile, aerobic, moderately halophilic endospore-forming bacteria. The cell-wall peptidoglycan type is Orn– D-Asp. Positive for Catalase activity, whereas negative for oxidase and urease. Positive for hydrolyzing Gelatin and Tween 80 while negative for Starch and casein. The predominant fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. Major menaquinone is MK-7. DNA G+C content is 42·4 mol%	(García et al., 2005)
Halobacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	Isolated from marine sediment and from rhizosphere of mangroves.	Gram-variable, cocci or oval cells. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid or L-Orn– D-Asp. Neither able to hydrolyze Tween 20, 40 and 60, hypoxanthine and xanthine, nor able to produce H_2S. The predominant isoprenoid quinone found is MK-7. The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. DNA G+C content of 42.1 mol%.	(Yoon et al., 2007)
Exiguobacterium	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	Isolated from mud of the Yellow sea	Members are Gram-variable, alkaliphilic, peritrichous flagellum, facultatively anaerobic, rod or cocci shaped bacteria. Colonies are circular, glossy, white colored after incubation for 2 days on Marine agar medium at 30 °C. The cell wall peptidoglycan types are L-Lys–Gly. The predominant menaquinone is MK-7. The cellular fatty acids are iso-C17:0, anteiso-C13:0, iso-C13:0 and iso-C15:0. The DNA G+C content is 48.0-48.6 mol%. The polar lipid analysis revealed the presence of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylserine phosphatidylethanolamine and phosphatidylinositol	(Kim et al., 2005)
Piscibacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	Mostly isolated from saline environment.	Endospore-forming, moderately halophilic, Gram-positive, aerobic and motile bacteria. The cell-wall peptidoglycan type is meso-diaminopimelic acid. The predominant menaquinone is MK-7. The predominant fatty acids profile mainly consisted of iso-C (15:0) and anteiso-C(15:0). The predominant polar lipids are phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content is 36.7 mol%	(Tanasupawat et al., 2007; Amoozegar et al., 2009)
Gracilibacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	Isolated from fermented *Polygonum indigo* liquor samples, contaminated soil with boron	Members are Gram-positive staining, facultatively moderately alkaliphilic and anaerobic, halophilic and rod shaped motile (by means of peritrichous flagella) cells. Growth temperatures ranges between 13 and 48 °C and at pH	(Ahmed et al., 2007a; Hirota et al., 2014) (Navarro-Torre et al.,

		minerals or phyllosphere of *Arthrocnemum macrostachyum*	range 7-10. The major isoprenoid quinone is MK-7. In the fatty acid profile, the major fatty acids are iso-C15 : 0, anteiso-C15 : 0 and anteiso-C17 : 0. DNA G+C content is 41.3 mol%	2017)
Fictibacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	Isolated endophytically in roots and stems of rice. Also isolated from wastewater treatment plant.	Aerobic or facultatively anaerobic, Gram-positive or Gram-variable stain, endospore forming bacteria are present in this genus. Bacterial colonies are circular or slightly irregular, smooth, glossy and almost opaque on high nutrient content media. The major quinone system consists of menaquinone MK-7. The polar lipid consisted of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Cell wall peptidoglycan is meso-diaminopimelic acid. DNA G+C content varies from 35.0 mol%-42 mol%	(Glaeser et al., 2013) Raweekul et al., 2016)
Viridibacillus	Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae	Isolated from forest soil, river sediment or rhizosphere of mangroves.	Motile, rod, Gram-positive or Gram-variable endospore-forming bacteria. Cell wall peptidoglycan is of A4 type containing either L-Lys– D-Glu or L-Lys– D-Asp. Positive for catalase and for gelatin hydrolysis, whereas negative oxidase and for starch hydrolysis. The major polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Moreover, unknown aminophospholipid and phospholipids and polar lipids are also present. DNA G+C content ranges from 35 to 40.4mol%	(Albert et al., 2007) (Lyngwi et al., 2016)

References

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University - Science* 26**,** 1-20.

Ahmad, V., Muhammad Zafar Iqbal, A.N., Haseeb, M., and Khan, M.S. (2014). Antimicrobial potential of bacteriocin producing *Lysinibacillus* jx416856 against foodborne bacterial and fungal pathogens, isolated from fruits and vegetable waste. *Anaerobe* 27**,** 87-95.

Ahmed, I., Yokota, A., and Fujiwara, T. (2007a). *Gracilibacillus boraciitolerans* sp. nov., a highly boron-tolerant and moderately halotolerant bacterium isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 796-802.

Ahmed, I., Yokota, A., Yamazoe, A., and Fujiwara, T. (2007b). Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 1117-1125.

Albert, R.A., Archambault, J., Lempa, M., Hurst, B., Richardson, C., Gruenloh, S., Duran, M., Worliczek, H.L., Huber, B.E., Rosselló-Mora, R., Schumann, P., and Busse, H.-J. (2007). Proposal of *Viridibacillus* gen. nov. and reclassification of *Bacillus arvi, Bacillus arenosi* and *Bacillus neidei* as *Viridibacillus arvi* gen. nov., comb. nov., *Viridibacillus arenosi* comb. nov. and *Viridibacillus neidei* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 2729-2737.

Alenezi, F.N., Fraser, S., Bełka, M., Doğmuş, T.H., Heckova, Z., Oskay, F., Belbahri, L., and Woodward, S. (2016a). Biological control of *Dothistroma* needle blight on pine with *Aneurinibacillus migulanus. Forest Pathology* 46**,** 555-558.

Alenezi, F.N., Rekik, I., Belka, M., Ibrahim, A.F., Luptakova, L., Jaspars, M., Woodward, S., and Belbahri, L. (2016b). Strain-level diversity of secondary metabolism in the biocontrol species *Aneurinibacillus migulanus. Microbiol Res* 182**,** 116-124.

Alenezi, F.N., Rekik, I., Chenari Bouket, A., Luptakova, L., Weitz, H.J., Rateb, M.E., Jaspars, M., Woodward, S., and Belbahri, L. (2017). Increased biological activity of *Aneurinibacillus migulanus* strains correlates with the production of new gramicidin secondary metabolites. *Front Microbiol* 8**,** 517.

Amoozegar, M.A., Sánchez-Porro, C., Rohban, R., Hajighasemi, M., and Ventosa, A. (2009). *Piscibacillus halophilus* sp. nov., a moderately halophilic bacterium from a hypersaline Iranian lake. *International Journal of Systematic and Evolutionary Microbiology* 59**,** 3095-3099.

An, S.-Y., Asahara, M., Goto, K., Kasai, H., and Yokota, A. (2007). *Terribacillus saccharophilus* gen. nov., sp. nov. and *Terribacillus halophilus* sp. nov., spore-forming bacteria isolated from field soil in Japan. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 51-55.

Balan, S.S., Kumar, C.G., and Jayalakshmi, S. (2017). Aneurinifactin, a new lipopeptide biosurfactant produced by a marine *Aneurinibacillus aneurinilyticus* SBP-11 isolated from Gulf of Mannar: Purification, characterization and its biological evaluation. *Microbiol Res* 194**,** 1-9.

Battini, F., Cristani, C., Giovannetti, M., and Agnolucci, M. (2016). Multifunctionality and diversity of culturable bacterial communities strictly associated with spores of the plant beneficial symbiont Rhizophagus intraradices. *Microbiological Research* 183**,** 68-79.

Behera, M., Dhali, D., Chityala, S., Mandal, T., Bhattacharya, P., and Mandal, D.D. (2015). Evaluation of performance of *Planococcus* sp. TRC1 an indigenous bacterial isolate monoculture as bioremediator for tannery effluent. *Desalination and Water Treatment* 57**,** 1-12.

Chauhan, A., Balgir, P.P., and Shirkot, C.K. (2014). Characterization of *Aneurinibacillus aneurinilyticus* Strain CKMV1 as a Plant Growth Promoting Rhizobacteria. *International Journal of Agriculture, Environment and Biotechnology* 7**,** 37-45.

Chauhan, A., Guleria, S., Balgir, P.P., Walia, A., Mahajan, R., Mehta, P., and Shirkot, C.K. (2017). Tricalcium phosphate solubilization and nitrogen fixation by newly isolated *Aneurinibacillus aneurinilyticus*

CKMV1 from rhizosphere of *Valeriana jatamansi* and its growth promotional effect. *Braz J Microbiol* 48**,** 294-304.

Chowdhury, R., Sen, A.K., Karak, P., Chatterjee, R., Giri, A.K., and Chaudhuri, K. (2009). Isolation and characterization of an arsenic-resistant bacterium from a bore-well in West Bengal, India. *Annals of Microbiology* 59**,** 253-258.

Cruz-Morales, N.K., Rodríguez-Tovar, A.V., Guerrero-Zúñiga, L.A., and Rodríguez-Dorantes, A. (2016). Plant growth promoting characterization of soil bacteria isolated from petroleum contaminated soil. *International Journal of Environmental & Agriculture Research* 2.

De Almeida Couto, C.R., Alvarez, V.M., Marques, J.M., De Azevedo Jurelevicius, D., and Seldin, L. (2015). Exploiting the aerobic endospore-forming bacterial diversity in saline and hypersaline environments for biosurfactant production. *BMC microbiology* 15**,** 240-240.

De Fatima Gomes Cavados, C., Pires, E.S., Chaves, J.Q., Alvarez, D.N., Benites Gil, H., Braz Ribeiro De Oliveira, I., De Barros Pinto Viviani Cunha, A., and Pereira Da Cunha De Araujo-Coutinho, C.J. (2017). Isolation and genetic characterization of *Lysinibacillus sphaericus* strains found in mosquito larvae (Diptera: Culicidae). *Res Rep Trop Med* 8**,** 17-20.

De Senna, A., and Lathrop, A. (2017). Antifungal screening of bioprotective Isolates against *Botrytis cinerea, Fusarium pallidoroseum* and *Fusarium moniliforme. Fermentation* 3.

Desale, P., Patel, B., Singh, S., Malhotra, A., and Nawani, N. (2014). Plant growth promoting properties of *Halobacillus* sp. and *Halomonas* sp. in presence of salinity and heavy metals. *Journal of Basic Microbiology* 54**,** 781-791.

Desjardine, K., Pereira, A., Wright, H., Matainaho, T., Kelly, M., and Andersen, R.J. (2007). Tauramamide, a Lipopeptide Antibiotic Produced in Culture by *Brevibacillus laterosporus* Isolated from a Marine Habitat: Structure Elucidation and Synthesis. *Journal of Natural Products* 70**,** 1850-1853.

Dey, U., Chatterjee, S., and Mondal, N.K. (2016). Isolation and characterization of arsenic-resistant bacteria and possible application in bioremediation. *Biotechnol Rep (Amst)* 10**,** 1-7.

Doni, F., Anizan, I., Che Radziah, C.M.Z., Ahmed, W.N.W., Ashari, A., Suryadi, E., and Yusoff, W.M.W. (2014). Enhanced rice seedling growth by *Clostridium* and *Pseudomonas. Biotechnology* 13**,** 186-189.

Duda, V.I., Lebedinsky, A.V., Mushegjan, M.S., and Mitjushina, L.L. (1987). A new anaerobic bacterium, forming up to five endospores per cell —*Anaerobacter polyendosporus* gen. et spec. nov. *Archives of Microbiology* 148**,** 121-127.

El-Tarabily, K.A., and Youssef, T. (2010). Enhancement of morphological, anatomical and physiological characteristics of seedlings of the mangrove *Avicennia marina* inoculated with a native phosphate-solubilizing isolate of *Oceanobacillus picturae* under greenhouse conditions. *Plant and Soil* 332**,** 147-162.

Essghaier, B., Fardeau, M.L., Cayol, J.L., Hajlaoui, M.R., Boudabous, A., Jijakli, H., and Sadfi-Zouaoui, N. (2009). Biological control of grey mould in strawberry fruits by halophilic bacteria. *Journal of Applied Microbiology* 106**,** 833-846.

Freney, J., Kloos, W.E., Hajek, V., Webster, J.A., Bes, M., Brun, Y., and Vernozy-Rozand, C. (1999). Recommended minimal standards for description of new staphylococcal species. *International Journal of Systematic Bacteriology* 49**,** 489-502.

García, M.T., Gallego, V., Ventosa, A., and Mellado, E. (2005). *Thalassobacillus devorans* gen. nov., sp. nov., a moderately halophilic, phenol-degrading, Gram-positive bacterium. *International Journal of Systematic and Evolutionary Microbiology* 55**,** 1789-1795.

Ghazanchyan, N.L., Kinosyan, M.H., Tadevosyan, P.E., Khachaturyan, N.S., and Afrikian, E.G. (2018). *Brevibacillus laterosporus* as perspective source of new bioinsecticides. *Annals of Agrarian Science* 16**,** 413-415.

Girish, N., and Umesha, S. (2005). Effect of plant growth promoting rhizobacteria on bacterial canker of tomato. *Archives Of Phytopathology And Plant Protection* 38**,** 235-243.

Glaeser, S.P., Dott, W., Busse, H.-J., and Kämpfer, P. (2013). *Fictibacillus phosphorivorans* gen. nov., sp. nov. and proposal to reclassify *Bacillus arsenicus,Bacillus barbaricus,Bacillus macauensis,Bacillus nanhaiensis,Bacillus rigui,Bacillus solisalsi* and *Bacillus gelatini* in the genus *Fictibacillus. International Journal of Systematic and Evolutionary Microbiology* 63**,** 2934-2944.

Glick, B.R. (2012). Plant Growth-Promoting Bacteria: Mechanisms and Applications. *Scientifica* 2012**,** 15.

Gusain, P., Paliwal, R., and Singh, V. (2017). Rhizoremediation of cadmium-contaminated soil associated with hydroxamate siderophores isolated from Cd-resistant plant growth-promoting *Dietzia maris* and *Lysinibacillus strains. Int J Phytoremediation* 19**,** 290-299.

Heyndrickx, M., Lebbe, L., Vancanneyt, M., Kersters, K., De Vos, P., Logan, N.A., Forsyth, G., Nazli, S., Ali, N., and Berkeley, R.C.W. (1997). A polyphasic reassessment of the genus *Aneurinibacillus,* reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer et al. 1996) as *Aneurinibacillus thermoaerophilus* comb. nov., and emended descriptions of *A. aneurinilyticus corrig*., *A. migulanus*, and *A. thermoaerophilus. International journal of systematic bacteriolo* 47**,** 808-817.

Heyrman, J., Logan, N.A., Busse, H.-J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., Swings, J., and De Vos, P. (2003). *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus. International Journal of Systematic and Evolutionary Microbiology* 53**,** 501-511.

Hirota, K., Hanaoka, Y., Nodasaka, Y., and Yumoto, I. (2013). *Oceanobacillus polygoni* sp. nov., a facultatively alkaliphile isolated from indigo fermentation fluid. *International Journal of Systematic and Evolutionary Microbiology* 63**,** 3307-3312.

Hirota, K., Hanaoka, Y., Nodasaka, Y., and Yumoto, I. (2014). *Gracilibacillus alcaliphilus* sp. nov., a facultative alkaliphile isolated from indigo fermentation liquor for dyeing. *International Journal of Systematic and Evolutionary Microbiology* 64**,** 3174-3180.

Huang, D., Shao, Z.Z., Yu, Y., Cai, M.M., Zheng, L.Y., Li, G.Y., Yu, Z.N., Yi, X.F., Zhang, J.B., and Hao, F.H. (2018). Identification, Characteristics and Mechanism of 1-Deoxy-N-acetylglucosamine from Deep-Sea *Virgibacillus dokdonensis* MCCC 1A00493. *Mar Drugs* 16.

Huang, X., Tian, B., Niu, Q., Yang, J., Zhang, L., and Zhang, K. (2005). An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. *Res Microbiol* 156**,** 719-727.

Hugenholtz, P. (2002). Exploring prokaryotic diversity in the genomic era. *Genome Biology*.

Inan, K., Canakci, S., Belduz, A.O., and Sahin, F. (2012). *Brevibacillus aydinogluensis* sp. nov., a moderately thermophilic bacterium isolated from Karakoc hot spring. *International Journal of Systematic and Evolutionary Microbiology* 62**,** 849-855.

Jadhav, V.V., Yadav, A., Shouche, Y.S., Aphale, S., Moghe, A., Pillai, S., Arora, A., and Bhadekar, R.K. (2013). Studies on biosurfactant from *Oceanobacillus* sp. BRI 10 isolated from Antarctic sea water. *Desalination* 318**,** 64-71.

Janarthine, S.R., and Eganathan, P. (2012). Plant growth promoting of endophytic *Sporosarcina aquimarina* SjAM16103 Isolated from the Pneumatophores of *Avicennia marina* L. *Int J Microbiol* 2012**,** 532060.

Kadyan, S., Panghal, M., Kumar, S., Singh, K., and Yadav, J.P. (2013). Assessment of functional and genetic diversity of aerobic endospore forming Bacilli from rhizospheric soil of *Phyllanthus amarus* L. *World Journal of Microbiology and Biotechnology* 29**,** 1597-1610.

Kämpfer, P., Arun, A.B., Busse, H.-J., Langer, S., Young, C.-C., Chen, W.-M., Syed, A.A., and Rekha, P.D. (2011). *Virgibacillus soli* sp. nov., isolated from mountain soil. *International Journal of Systematic and Evolutionary Microbiology* 61**,** 275-280.

Kämpfer, P., Martin, K., and Glaeser, S.P. (2013). *Lysinibacillus contaminans* sp. nov., isolated from surface water. *International Journal of Systematic and Evolutionary Microbiology* 63**,** 3148-3153.

Kim, I.-G., Lee, M.-H., Jung, S.-Y., Song, J.J., Oh, T.-K., and Yoon, J.-H. (2005*). Exiguobacterium aestuarii* sp. nov. and *Exiguobacterium marinum* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology* 55**,** 885-889.

Kumar, G., Kanaujia, N., and Bafana, A. (2012). Functional and phylogenetic diversity of root-associated bacteria of *Ajuga bracteosa* in Kangra valley. *Microbiological Research* 167**,** 220-225.

Lawson, P.A., and Rainey, F.A. (2016). Proposal to restrict the genus *Clostridium Prazmowski* to *Clostridium butyricum* and related species. *International Journal of Systematic and Evolutionary Microbiology* 66**,** 1009-1016.

Liu, W., Jiang, L., Guo, C., and Yang, S.S. (2010). *Terribacillus aidingensis* sp. nov., a moderately halophilic bacterium. *International Journal of Systematic and Evolutionary Microbiology* 60**,** 2940-2945.

Lozano, L.C., and Dussán, J. (2013). Metal tolerance and larvicidal activity of *Lysinibacillus sphaericus*. *World Journal of Microbiology and Biotechnology* 29**,** 1383-1389.

Lu, P., Lei, M., Xiao, F., Zhang, L., and Wang, Y. (2015). Complete Genome Sequence of *Terribacillus aidingensis* Strain MP602, a Moderately Halophilic Bacterium Isolated from *Cryptomeria fortunei* in Tianmu Mountain in China. *Genome Announc* 3.

Lyngwi, N.A., Nongkhlaw, M., Kalita, D., and Joshi, S.R. (2016). Bioprospecting of plant growth promoting Bacilli and related genera prevalent in soils of Pristine sacred groves: biochemical and molecular approach. *PLoS One* 11**,** e0152951.

Martínez, S.A., and Dussán, J. (2018). *Lysinibacillus sphaericus* plant growth promoter bacteria and lead phytoremediation enhancer with *Canavalia ensiformis*. *Environmental Progress & Sustainable Energy* 37**,** 276-282.

Minamisawa, K., Nishioka, K., Miyaki, T., Ye, B., Miyamoto, T., You, M., Saito, A., Saito, M., Barraquio, W.L., Teaumroong, N., Sein, T., and Sato, T. (2004). Anaerobic nitrogen-fixing consortia consisting of *Clostridia* Isolated from Gramineous Plants. *Applied and Environmental Microbiology* 70**,** 3096-3102.

Naili, F., Neifar, M., Mohamed, D., Cherif, H., Bejaoui, B., Aroua, M., Bejaoui, Z., Abassi, M., Mguiz, K., Chouchane, H., Ouzari, H., and Cherif, A. (2018). Optimization of the effect of PGPR–based biofertlizer on wheat growth and yield. *Biometrics & Biostatistics International Journal* 7.

Nakagawa, Y., Sakane, T., and Yokota, A. (1996). Emendation of the Genus *Planococcus* and Transfer of *Flavobacterium okeanokoites* Zobell and Upham 1944 to the Genus *Planococcus* as *Planococcus okeanokoites* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 46**,** 866-870.

Naureen, Z., Rehman, N.U., Hussain, H., Hussain, J., Gilani, S.A., Al Housni, S.K., Mabood, F., Khan, A.L., Farooq, S., Abbas, G., and Harrasi, A.A. (2017). Exploring the Potentials of *Lysinibacillus sphaericus* ZA9 for plant growth promotion and biocontrol activities against phytopathogenic fungi. *Frontiers in microbiology* 8**,** 1477-1477.

Navarro-Torre, S., Barcia-Piedras, J.M., Mateos-Naranjo, E., Redondo-Gómez, S., Camacho, M., Caviedes, M.A., Pajuelo, E., and Rodríguez-Llorente, I.D. (2017). Assessing the role of endophytic bacteria in the halophyte *Arthrocnemum macrostachyum* salt tolerance. *Plant Biology* 19**,** 249-256.

Nehra, V., Saharan, B.S., and Choudhary, M. (2016). Evaluation of *Brevibacillus brevis* as a potential plant growth promoting rhizobacteria for cotton (*Gossypium hirsutum*) crop. *Springerplus* 5**,** 948.

Orhan, F. (2016). Alleviation of salt stress by halotolerant and halophilic plant growth-promoting bacteria in wheat (*Triticum aestivum*). *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]* 47**,** 621-627.

Pakpitcharoen, A., Potivejkul, K., Kanjanavas, P., Areekit, S., and Chansiri, K. (2008). Biodiversity of thermotolerant *Bacillus* sp. producing biosurfactants, biocatalysts, and antimicrobial agents. *ScienceAsia* 34**,** 424–431.

Park, M., Kim, C., Yang, J., Lee, H., Shin, W., Kim, S., and Sa, T. (2005). Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiological Research* 160**,** 127-133.

Perez-Rosales, E., Alcaraz-Meléndez, L., Puente, E.M., Vázquez-Juárez, R., Quiroz-Guzmán, E., Zenteno-Savín, T., and Morales-Bojórquez, E. (2017). Isolation and characterization of endophytic bacteria associated with roots of jojoba (*Simmondsia chinensis* Schneid). *Current science* 112**,** 396-401.

Polyanskaya, L.M., Vedina, O.T., Lysak, L.V., and Zvyagintsev, D.G. (2002). The Growth-promoting Effect of *Beijerinckia mobilis* and *Clostridium* sp. Cultures on Some Agricultural Crops. *Microbiology* 71**,** 109-115.

Rahmoune, B., Morsli, A., Khelifi-Slaoui, M., Khelifi, L., Strueh, A., Erban, A., Kopka, J., Prell, J., and Van, T.J. (2017). Isolation and characterization of three new PGPR and their effects on the growth of *Arabidopsis* and *Datura* plants. *Journal of Plant Interactions* 12**,** 1-6.

Rajput, L., Imran, A., Mubeen, F., and Hafeez, Y.F. (2013). Salt-tolerant PGPR strain *Planococcus rifietoensis* promotes the growth and yield of wheat (*Triticum aestivum* L.) Cultivated in saline soil. *Pakistan Journal of Botany* 45**,** 1955-1962.

Raweekul, W., Wuttitummaporn, S., Sodchuen, W., and Kittiwongwattana, C. (2016). Plant Growth Promotion by Endophytic Bacteria Isolated from Rice (Oryza sativa). *Thammasat International Journal of Science and Technology* 21.

Reddy, G., Prakash, J., Vairamani, M., Prabhakar, S., Matsumoto, G., and Shivaji, S. (2002). *Planococcus antarcticus* and *Planococcus psychrophilus* spp. nov. isolated from cyanobacterial mat samples collected from ponds in Antarctica. *Extremophiles* 6**,** 253-261.

Romano, I., Giordano, A., Lama, L., Nicolaus, B., and Gambacorta, A. (2003). *Planococcus rifietensis* sp. nov, Isolated from Algal Mat Collected from a Sulfurous Spring in Campania (Italy). *Systematic and Applied Microbiology* 26**,** 357-366.

Sadfi-Zouaoui, N., Essghaier, B., Hajlaoui, M.R., Fardeau, M.L., Cayaol, J.L., Ollivier, B., and Boudabous, A. (2008). Ability of moderately halophilic bacteria to control grey mould disease on tomato fruits. *Journal of Phytopathology* 156**,** 42-52.

Sahu, P.K., Shivaprakash, M.K., Mallesha, B.C., Subbarayappa, C.T., and Brahmaprakash, G.P. (2018). Effect of Bacterial Endophytes *Lysinibacillus* sp. on Plant Growth and Fruit Yield of Tomato (*Solanum lycopersicum)*. *International Journal of Current Microbiology and Applied Sciences* 7**,** 3399-3408.

Saikia, R., Gogoi, D.K., Mazumder, S., Yadav, A., Sarma, R.K., Bora, T.C., and Gogoi, B.K. (2011). *Brevibacillus laterosporus* strain BPM3, a potential biocontrol agent isolated from a natural hot water spring of Assam, India. *Microbiol Res* 166**,** 216-225.

Sánchez-Porro, C., De La Haba, R.R., and Ventosa, A. (2014). "The Genus *Virgibacillus*," in *The Prokaryotes: Firmicutes and Tenericutes,* eds. E. Rosenberg, E.F. Delong, S. Lory, E. Stackebrandt & F. Thompson. (Berlin, Heidelberg: Springer Berlin Heidelberg), 455-465.

Shabanamol, S., Divya, K., George, T.K., Rishad, K.S., Sreekumar, T.S., and Jisha, M.S. (2017a). Characterization and in planta nitrogen fixation of plant growth promoting endophytic diazotrophic *Lysinibacillus sphaericus* isolated from rice (*Oryza sativa*). *Physiological and Molecular Plant Pathology* 102**,** 46-54.

Shabanamol, S., Sreekumar, J., and Jisha, M.S. (2017b). Bioprospecting endophytic diazotrophic *Lysinibacillus sphaericus* as biocontrol agents of rice sheath blight disease. *3 Biotech* 7**,** 337.

Sharma, N., and Saharan, B.S. (2015). Role of *Lysinibacillus sphaericus* SNCh5 Bacterial Strain as Bio-inoculant for Agriculture Practice. *International Journal of Current Microbiology and Applied sciences* 4**,** 484-499.

Shrivastava, P., and Kumar, R. (2015). Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi journal of biological sciences* 22**,** 123-131.

Siddikee, M.A., Chauhan, P.S., Anandham, R., Han, G.-H., and Sa, T. (2010). Isolation, Characterization, and Use for Plant Growth Promotion Under Salt Stress, of ACC Deaminase-Producing Halotolerant Bacteria Derived from Coastal Soil. *Journal of Microbiology and Biotechnology* 20**,** 1577–1584.

Subhash, Y., Kim, H.-J., and Lee, S.-S. (2017). *Aneurinibacillus sediminis* sp. nov., isolated from lagoon sediments. *International Journal of Systematic and Evolutionary Microbiology* 67**,** 2544-2548.

Tanasupawat, S., Namwong, S., Kudo, T., and Itoh, T. (2007). *Piscibacillus salipiscarius* gen. nov., sp. nov., a moderately halophilic bacterium from fermented fish (pla-ra) in Thailand. *International Journal of Systematic and Evolutionary Microbiology* 57**,** 1413-1417.

Taponen, S., Supré, K., Piessens, V., Van Coillie, E., De Vliegher, S., and Koort, J.M.K. (2012). *Staphylococcus agnetis* sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. *International Journal of Systematic and Evolutionary Microbiology* 62**,** 61-65.

Taylor, J.B., and Guy, E.M. (1981). Biological control of root-infecting basidiomycetes by species of *Bacillus* and *Clostridium*. *New Phytologist* 87**,** 729-732.

Tóth, G., Hermann, T., Da Silva, M.R., and Montanarella, L. (2016). Heavy metals in agricultural soils of the European Union with implications for food safety. *Environment International* 88**,** 299-309.

Tsubouchi, T., Mori, K., Miyamoto, N., Fujiwara, Y., Kawato, M., Shimane, Y., Usui, K., Tokuda, M., Uemura, M., Tame, A., Uematsu, K., Maruyama, T., and Hatada, Y. (2015). *Aneurinibacillus tyrosinisolvens* sp. nov., a tyrosine-dissolving bacterium isolated from organics- and methane-rich seafloor sediment. *International Journal of Systematic and Evolutionary Microbiology* 65**,** 1999-2005.

Ueki, A., Takehara, T., Ishioka, G., Kaku, N., and Ueki, K. (2017). Degradation of the fungal cell wall by Clostridial strains isolated from soil subjected to biological soil disinfestation and biocontrol of *Fusarium* wilt disease of spinach. *Appl Microbiol Biotechnol* 101**,** 8267-8277.

Vendan, R.T., Yu, Y.J., Lee, S.H., and Rhee, Y.H. (2010). Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. *The Journal of Microbiology* 48**,** 559-565.

Verma, P., Yadav, A.N., Khannam, K.S., Kumar, S., Saxena, A.K., and Suman, A. (2016). Molecular diversity and multifarious plant growth promoting attributes of Bacilli associated with wheat (*Triticum aestivum* L.) rhizosphere from six diverse agro-ecological zones of India. *Journal of Basic Microbiology* 56**,** 44-58.

Vivas, A., Barea, J.M., and Azcon, R. (2005). *Brevibacillus brevis* isolated from cadmium- or zinc-contaminated soils improves in vitro spore germination and growth of *Glomus mosseae* under high Cd or Zn concentrations. *Microb Ecol* 49**,** 416-424.

Vivas, A., Vörös, I., Biró, B., Campos, E., Barea, J.M., and Azcón, R. (2003). Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus (*G. mosseae*) and *Brevibacillus* sp. isolated from cadmium polluted soil under increasing cadmium levels. *Environmental Pollution* 126**,** 179-189.

Yaakop, A.S., Chan, K.-G., Ee, R., Kahar, U.M., Kon, W.C., and Goh, K.M. (2015). Isolation of *Jeotgalibacillus malaysiensis* sp. nov. from a sandy beach, and emended description of the genus *Jeotgalibacillus. International Journal of Systematic and Evolutionary Microbiology* 65**,** 2215-2221.

Yadav, A.N., Sachan, S.G., Verma, P., and Saxena, A.K. (2016). Bioprospecting of plant growth promoting psychrotrophic Bacilli from the cold desert of north western Indian Himalayas. *Indian Journal of Experimental Biology* 54**,** 142-150.

Ye, B., Saito, A., and Minamisawa, K. (2005). Effect of Inoculation with anaerobic nitrogen-fixing *Consortium* on Salt Tolerance of *Miscanthus sinensis*. *Soil Science & Plant Nutrition* 51**,** 243-249.

Yoon, J.-H., Kang, S.-J., Jung, Y.-T., and Oh, T.-K. (2007). *Halobacillus campisalis* sp. nov., containing meso-diaminopimelic acid in the cell-wall peptidoglycan, and emended description of the genus *Halobacillus. International Journal of Systematic and Evolutionary Microbiology* 57**,** 2021-2025.

Yoon, J.-H., Kang, S.-J., Lee, S.-Y., Oh, K.-H., and Oh, T.-K. (2010). *Planococcus salinarum* sp. nov., isolated from a marine solar saltern, and emended description of the genus *Planococcus. International Journal of Systematic and Evolutionary Microbiology* 60**,** 754-758.

Yoon, J.H., Lee, K.C., Weiss, N., Kho, Y.H., Kang, K.H., and Park, Y.H. (2001a). *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., *International Journal of Systematic and Evolutionary Microbiology* 51**,** 1079-1086.

Yoon, J.H., Weiss, N., Kang, K.H., Oh, T.K., and Park, Y.H. (2003). *Planococcus maritimus* sp. nov., isolated from sea water of a tidal flat in Korea. *Int J Syst Evol Microbiol* 53**,** 2013-2017.

Yoon, J.H., Weiss, N., Lee, K.C., Lee, I.S., Kang, K.H., and Park, Y.H. (2001b). *Jeotgalibacillus alimentarius* gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of *Bacillus marinus* Rüger 1983 . as *Mrinibacillus marinus* gen nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 51**,** 2087-2093.

Yumoto, I., Hirota, K., Nodasaka, Y., and Nakajima, K. (2005). *Oceanobacillus oncorhynchi* sp. nov., a halotolerant obligate alkaliphile isolated from the skin of a rainbow trout (*Oncorhynchus mykiss*), and emended description of the genus *Oceanobacillus*. *International Journal of Systematic and Evolutionary Microbiology* 55**,** 1521-1524.

Zeiller, M., Rothballer, M., Iwobi, A.N., Bohnel, H., Gessler, F., Hartmann, A., and Schmid, M. (2015). Systemic colonization of clover (*Trifolium repens*) by *Clostridium botulinum* strain 2301. *Front Microbiol* 6**,** 1207.

Zhao, J., Guo, L., Zeng, H., Yang, X., Yuan, J., Shi, H., Xiong, Y., Chen, M., Han, L., and Qiu, D. (2012). Purification and characterization of a novel antimicrobial peptide from *Brevibacillus laterosporus* strain A60. *Peptides* 33**,** 206-211.

Zheng, Z., Zheng, J., Zhang, Z., Peng, D., and Sun, M. (2016). Nematicidal spore-forming Bacilli share similar virulence factors and mechanisms. *Scientific Reports* 6**,** 31341.

Chapter 3

Screening of plant growth promoting activities in selected bacterial and fungal strains and their possible use in sustainable agriculture

Abstract

In conventional agriculture, inappropriate management practices and the use of agrochemicals has led to a negative impact on both soils and water. For these reasons, the search for sustainable alternatives in agriculture has become a priority. In the mycorrhizosphere, microbes are considered as crucial actors of soil functioning, as they are involved in many beneficial activities supporting the whole soil ecosystem. For instance, the bio-inoculation of microbes with plant growth promoting activities represent a potential ecofriendly strategy to reduce the usage of agrochemicals. With such a perspective, an *in vitro* screening was carried out to evaluate the plant growth promoting activities of different endospore forming bacteria as well as of fungi with different ecological niches. This study also aimed at investigating both, bacterial-fungal and fungal-fungal interactions with reference to the possible use of interacting microorganisms for bio-inoculation. Most of the screened endospore forming bacteria possessed one or more plant growth promoting traits. The fungal species that were screened in this study were also positive for plant growth promoting traits. In the confrontation assays between bacteria and fungi, we found bacteria that were able to inhibit the growth of a plant pathogenic fungus (*Rhizoctonia solani*), while at the same time displaying synergistic interactions with a plant growth promoting fungus (*Trichoderma rossicum*). Interestingly, we also noticed that these findings did not only depend on the competing microbes, but also on the medium used, pointing at the importance of nutritional context in microbial interactions. Moreover, in the confrontation assays between fungal species of different ecological niches, we found a promising fungal combination consisting of the ectomycorrhizal fungus *Laccaria bicolor* and the saprobic fungus *T. rossicum* for the inhibition of the phytopathogenic fungus *R. solani*. In the future, the efficiency of this fungal consortium using *L. bicolor* in its ectomycorrhizal form could be evaluated as a promising bi-fungal

bio-inoculant to control this fungal pathogen in an agro forested system.

Introduction

The main nutrients required to sustain plant growth are nitrogen (N), phosphorous (P), potassium (K), and iron (Fe). N is an essential mineral nutrient for plant growth and N deficiency in soil adversely affects plant development. For this reason, inorganic N fertilizers have long been used to overcome soil N limitation (Rosenblueth et al. 2018). In soil, N can be present either linked to organic compounds (from urea or simple amino-acids to complex proteins), or in an inorganic form (nitrate, nitrite, and ammonium). Plants usually uptake inorganic N as well as amino-acids (Harrison et al. 2007). A large proportion of this bioavailable N (30 to 50% of the total N) is generated thanks to the contribution of atmospheric N-fixing bacteria (Vadakattu and Paterson 2006). Bacteria commonly known as rhizobia (for instance those related to the genus *Rhizobium*) are the most extensively studied symbiotic N-fixing bacteria and are well-known to increase plant productivity through this activity. Rhizobia form a very intricate symbiosis by colonizing the roots of leguminous plants and by forming a N-fixing organ within the plant root (nodule) (Ahemad and Kibret 2014). Rhizobial bio-inoculants constitute a biological alternative to compensate soil N deficiency. However, their long-term survival in the soils is a major concern and their effect in soil N content, and conversely on plant growth, is inconsistent (Igiehon and Babalola 2018).

Many other bacterial species isolated from the rhizosphere are also reported to be able to fix N. Examples of N-fixing bacteria include *Bacillus cereus, Bacillus marisflavi*, *Bacillus megaterium*, *Bacillus azotofixans, Paenibacillus polymyxa,* and *Paenibacillus massiliensis* (Ding et al. 2005; Seldin et al. 1984). Recently, bioinoculation with a genetically modified strain of *Pseudomonas protegens* Pf-5, which contains the genes encoding the enzymatic complex responsible for nitrogen fixation (*nif* genes), was reported to promote the growth of cereal crops (maize and wheat) thanks to the increased rate of N fixation (Fox et al. 2016). Regarding fungi, there are no conclusive studies reporting the ability of fungi to fix atmospheric N (Allison et al. 1934). However, this has largely been considered not to be the case. Nonetheless, the co-inoculation of arbuscular mycorrhizal fungi (AMF) with N-fixing bacteria has been shown to produce a positive effect on the growth of *Piptadenia gonoacantha* (Junior et al. 2017). In this study, AMF were supposedly to

promote plant growth through enhanced P uptake. However, AMF were also required for nodule formation, thus indirectly helping the process of N-fixation (Junior et al. 2017).

Organic N (Norg) represents also a large fraction of the N present in soil (Schulten and Schnitzer 1997). This fraction plays a vital role in the field of agriculture. Throughout the proteolytic activities of microorganisms, proteins present in organic matter can be used as a N source for plants in agricultural systems (Chalot and Brun 1998). Both AMF and Ectomycorrhizal fungi (EcM) are able to solubilize N present in organic polymers and transfer it to plants (Hodge et al. 2001). EcM possess complex systems of different proteolytic enzymes for the solubilization of Norg and their ability to transfer N to plants has been well-documented (Chalot and Brun 1998).

P is also essential for the development of plants. After N, P is often considered as the main limiting factor for plant growth. P comprises 0.2 - 0.8% of the total dry weight of a plant and its deficiency may lead to reduction in plant growth (Sharma et al. 2013). Similar to N, P is present in two different forms in soil, either as inorganic (apatite minerals, rock phosphate or phosphorite, as well as various phosphate salts with e.g. Calcium, Manganese, Aluminum, and Iron (Ahemad and Kibret 2014; Alori et al. 2017)) or as organic P (animal and plant residues and humic substances) (Ahemad and Kibret 2014). Despite the fact that large reservoirs of P are present in soils, most of this P is present in an insoluble form and thus only 0.1% of the total P is bioavailable to plants. Moreover, plants uptake P only in two soluble forms: monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{-2}) phosphate anions (Ahemad and Kibret 2014). However, P-solubilizing microorganisms can help plants to uptake non-bioavailable P forms by converting non-bioavailable P into bioavailable P (Bhattacharyya and Jha 2012). It has been shown that P-solubilizing microorganisms not only enhance plant growth by increasing phosphate uptake, but also present other beneficial effects, such as improving the bioavailability of other micronutrients (e.g. iron and zinc), which are also essential for the growth and development of plants (Alori et al. 2017). In a study by (Seshachala and Tallapragada 2012), *Bacillus subtilis* and *Aspergillus niger,* isolated from the rhizosphere of black pepper, were able to solubilize P from different non-bioavailable sources such as rock phosphate, tri calcium phosphate and potassium dihydrogen phosphate. Some other examples of soil bacterial genera with species having the ability to solubilize P are *Burkholderia*, *Enterobacter, Paenibacillus*, *Rhodococcus, Serratia, Bradyrhizobium, Salmonella, Thiobacillu, Pseudomonas,* and

Agrobacterium. Not only bacteria but also a substantial number of fungal (or fungal-like) species that belong to different ecological niches have been reported to solubilize phosphate. Arbuscular mycorrhizal fungi, such as *Glomus* spp., are well-known for their ability to enhance P bioavailability to plants (Smith and Read 2008). Additionally, strains of *Alternaria, Aspergillus, Cephalosporium, Cladosporium, Fusarium, Penicillium, Pythium, Rhizoctonia, Rhizopus, Saccharomyces, Sclerotium,* and *Trichoderma* (reviewed in (Alori et al. 2017)) also show this ability. Due to the production of diverse organic acids and because of their hyphal growth, which facilitates colonization in soil, fungi are likely more important actors in solubilizing P than bacteria (Sharma et al. 2013). Among the different organic acids reported to be produced by microbes (for instance, malic, lactic, citric, oxalic, fumaric, tartaric, acetic, and glutamic acid, among others) gluconic acid is the most relevant for the release of bioavailable phosphate for plant uptake (Alori et al. 2017).

Iron is an essential micronutrient for proper growth and development of plants. It is the fourth most abundant element in the Earth crust and is mostly present as insoluble Fe oxides or hydroxides. For this reason, Fe is poorly bioavailable to most living organisms and plants are not the exception. To overcome Fe deficiency in plant-soil agroecosystems, chemical Fe-chelators are being used. They are cheap and effective, but do not represent an environmentally friendly solution (Godsey et al. 2003; Radzki et al. 2013). The application of plant growth promoting (PGP) microbial inoculants, which are capable of biosynthesizing siderophores (low molecular weight iron chelating compound) is an alternative that represents an environmentally friendly way to improve Fe uptake by plants.

Besides enhancing nutrient bioavailability and uptake, bacteria and fungi are also capable of producing substances regulating plant development, which may directly or indirectly promote plant growth. These phytohormones can, for instance, alter root architecture or increase root surface area, which eventually contributes to nutrient acquisition (Bhattacharyya and Jha 2012). Phytohormones produced by rhizobacteria have also been shown to help to alleviate abiotic stress in plants (Egamberdieva et al. 2017). Moreover, plant beneficial bacteria may also have biocontrol activity against plant pathogens, whether these are fungi or insects, for instance. It has been reported that *Bacillus* spp. are able to produce different kinds of antimicrobial products,

which may reduce the dependency on agrochemical products to protect crops from plant pathogens (Shafi et al. 2017). Therefore, bio-inoculation of plant growth-promoting (PGP) bacteria and fungi with abilities of competitive rhizosphere colonization, plant growth stimulation, and biocontrol are being used for the sustainability of agriculture (Bhattacharyya & Jha, 2012). Many studies have shown the potential of bio-inoculation to improve agricultural yield (Ahemad and Kibret 2014; Bhattacharyya and Jha 2012; Souza et al. 2015). However, bio-inoculation of PGP microorganisms also presents many challenges. One of the critical issues that need to be addressed for application in the field is the survival of bacterial and fungal strains acclimated under laboratory conditions to the harsh conditions in soils. Bio-inoculants are also vulnerable to the scarce nutrient availability in soil, as compared to rich nutrient conditions usually provided by growth media, and ultimately a decline in soil in the number of inoculated microbes is frequently reported (Souza et al. 2015; Trabelsi and Mhamdi 2013). Moreover, the delivery of these microorganisms in an active form is an additional challenge. Application of carrier materials for the protection of bio-inoculants (for instance karnolite, peat, or charcoal) has proven to be environmentally unfriendly, as well as costly, making this approach inapplicable as a general practice in agriculture (Arora et al. 2014). These concerns are the incentive to identify effective bacterial and/or fungal inoculants to be applied in bio-inoculation technologies.

In this study, we aimed at characterizing PGP traits of different *Bacillus* strains, as well as of fungal species from different ecological niches, to explore the possibility of using combinations of different organisms to enhance the bio-fertilization and/or biocontrol activity of bio-inoculants. The rational for this is that in natural environments, microorganisms interact in many ways and this leads to numerous beneficial activities supporting the entire soil ecosystem functioning. Therefore, we first evaluated PGP traits of the organisms alone and then screened both, bacterial-fungal and fungal-fungal interactions to find promising synergistic or antagonistic partners, which may be further used in the field of bio-inoculants for sustainable agriculture.

Materials and Methods

Selection of microorganisms

For the screening of bacterial and fungal strains, we selected 15 mesophilic bacterial strains and five fungal species of different ecological niches. All microbial strains come from the collection of the laboratory of microbiology and their source of isolation is described in Table 1, respectively. Most *Bacillus* strains were isolated either from soil or from geothermal environments (Table 1). *Laccaria bicolor* and *Boletus edulis* were used as model EcM fungi. We selected EcM fungi rather than AMF fungi in our experimental plan, as they are easier to grow *in vitro,* and this makes the evaluation of PGP traits and confrontation assays with other microbes easier to investigate. The other fungal (or fungi-like) strains were: *Pythium ultimum* (an oomycete) and *Rhizoctonia solani*, which were used as model phytopathogenic fungi; and *Trichoderma rossicum,* which was used as a model saprophytic fungus. *Trichoderma* fungi are also well-known for their ability to promote plant growth through various processes (Harman et al. 2004).

In vitro screening of bacterial and fungal strains for plant growth promoting (PGP) activities

Dinitrogen fixation, proteolysis, siderophore production, auxin-like phytohormone production and phosphate solubilization were the PGP traits, which were assessed on bacterial strains while PGP traits assessed for fungal strains were proteolysis, siderophore production and phosphate solubilization.

Dinitrogen fixation assay

A loop of an overnight bacterial culture on nutrient agar (nutrient broth, 8 g/L; Biolife italiana; agar 15 g/L; Biolife italiana) medium (NA) was streaked onto a nitrogen-free medium (Döbereiner 1980). The inoculated culture medium was incubated at 30°C for 48-72 hours. Bacterial inoculum was passed three times on the N-free medium and the capacity to fix atmospheric N_2 was assessed by the growth of bacterial strains on the third-generation plates. *Enterobacter cloacae* and *Escherichia coli* were used as positive and negative controls, respectively.

Table 1: Bacterial and fungal strains and their origin

Bacterial strains		
Number	**Name**	**Origin / Source of isolation**
1	*Bacillus polymyxa* 25	Soil of the botanical garden, Neuchâtel (CH)
2	*Bacillus subtilis* 1055	Merrel syrup (probiotics)
3	*Bacillus thurigiensis* 1310	Lirima, Atacama (CL)
4	*Bacillus pumilus* 2	Drinking water bottle, Vichy (F)
5	*Bacillus cereus* 88	Soil of the botanical garden, Neuchâtel (CH)
6	*Bacillus thuringiensis* 1311	Soil, Lirima, Atacama (CL)
7	*Bacillus subtilis* 16	Lab contamination, Laboratory of Microbiology, Neuchâtel (CH)
8	*Bacillus thuringiensis* 1070	Unknown origin, DSMZ collection no. 350 (D)
9	*Bacillus thuringiensis* 1321	Soil, Neuchâtel canton (CH)
10	*Bacillus thuringiensis* 1312	Soil, Lirima, Atacama (CL)
11	*Bacillus licheniformis* 1062	Soil, Bern (CH)
12	*Bacillus macerans* 1004	Unknown origin, Beckenham (UK)
13	*Bacillus weihenstephanensis* 1324	Soil, Krinides, Kavala (GR)
14	*Bacillus thuringiensis* 1318	Soil, Krinides, Kavala (GR)
15	*Lysinibacillus sphaericus* 1003	Wellcome Collection of Bacteria, Wellcome Research Laboratories, Beckenham, Kent (UK)
Fungal strains		
Number	**Name**	**Origin / Source of isolation**
1	*Boletus edulis*	WSL research institute, Birmensdorf (CH)
2	*Laccaria bicolor*	WSL research institute, Birmensdorf (CH)
3	*Trichoderma rossicum*	Soil, Cameroon (Bravo et al. 2013)
4	*Rhizoctonia solani*	ND
5	*Pythium ultimum*	Defago group, UNIZH (CH)

Proteolysis (Casein solubilization)

A modified protocol proposed by (Frazier and Rupp 1930) was used to assess casein solubilization. This assay was used as a proxy for the ability of the selected microorganisms to solubilize organic nitrogen. The medium was composed of powder milk (50 g/L; Rapilait, Migros), malt extract (12 g/L; SIOS Home Brewing and agar (15 g/L; Biolife italiana). For bacteria, a loop of an overnight culture on NA was streaked onto the casein-containing medium. The plates were incubated for 48-72 hours at 30°C. For fungi, a 5mm^2 plug was taken from a 4 days old culture prepared on a malt extract (12 g/L; SIOS Home Brewing) and agar (15 g/L; Biolife italiana) (MA) medium and

placed in the center of the casein solubilizing medium. The plates were incubated in the dark at room temperature and results were noted after 6 days. The appearance of a transparent halo around a bacterial or a fungal colony indicated that the strain was able to solubilize casein.

Siderophore production assay

A siderophore production test was performed to check for the production of this chelator by the bacterial and fungal strains. In this test, chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) form a complex with ferric iron resulting in a blue coloration of the medium (CAS medium). When a strong iron chelator, such as a siderophore, removes iron from the dye complex, the color of the medium turns to yellow (Schwyn and Neilands 1987). A loop of an overnight bacterial culture on NA was inoculated onto the CAS medium and the plates were incubated at 30°C for 48-72 hours. *Pseudomonas fluorescens* and *Escherichia coli* were used as positive and negative controls, respectively. The positive control has the ability to turn the blue color of the medium into yellow, as a result or Fe chelation. For fungi, a 5 mm^2 plug was taken from a 4 days old culture on MA and placed in the center of a CAS medium. The plates were incubated in the dark at room temperature and results were noted after 6 days.

Auxin-like phytohormone production assay

To check for the production of auxin-like compounds in the *Bacillus* strains a modified version of the protocol proposed by (Bric et al. 1991) was used. In this assay, Angle medium (Angle et al. 1991) was supplemented with 5 mM Tryptophane as a precursor for auxin biosynthesis. The test was performed in 96 well microplates with 270 µL of medium per well. A loop of an overnight bacterial culture from NA medium was inoculated in the three microplate wells for each corresponding bacterial strain. The microplate was then incubated at 25°C for 72 hours in the dark. After this time, one drop of Salkowski's reagent was added to each well and placed in dark for 45 minutes. A pink to red coloration indicates the production of auxin-like compounds. *Enterobacter cloacae* was used as a positive control while *Pseudomonas fluorescens* was used as a negative control in this assay. This trait was not assessed for fungal strains.

Phosphate solubilization assay

Solubilization of calcium phosphate using National Botanical Research Institute's Phosphate growth medium (NBRIP) plates were used to test the ability of bacterial and fungal strains to solubilize P from $Ca_3(PO_4)_2$ (Nautiyal 1999). A loop of an overnight bacterial culture on NA was streaked on NBRIP plates. The plates were incubated at 30°C for 48-72 hours. *Enterobacter cloacae* and *Azospirillum brasilense* were used as positive and negative controls, respectively. The capacity to solubilize phosphate was assessed by the presence of a clearing halo in the NBRIP plate around the growth of the bacterial colonies. For fungi, a $5mm^2$ plug was taken from a 4 days old culture on MA and placed in the center of the medium. The plates were incubated in the dark at room temperature and results were noted after 6 days. The assessment of a positive result was made in the same way as for the bacterial strains.

In vitro screening of microbial interactions

Simple bacterial-fungal confrontation assays (one to one) were carried out in order to assess the type of interaction (such as neutral, antagonistic or positive interactions for instance) that could be established between two strains. For selected couples, further experiments were run to assess the mechanisms involved (exudates, volatiles). Fungal-fungal confrontations assays were also performed, involving two or three partners. Confrontation assays were performed in several media with contrasting nutrient content (Table 2). All media were autoclaved at 121°C for 20 minutes and poured into Petri dishes under a laminar flow hood. When necessary, filter-sterilized antimicrobial compounds were added to autoclaved media cooled down to approximately 55°C.

Table 2: Medium composition with carbon to nitrogen ratio (C: N).

Medium	Composition	C: N	Favor microbial growth	Reference
Nutrient agar (NA)	Nutrient broth (8 g/L; Biolife italiana) and agar (15 g/L; Biolife italiana).	4-7	Bacteria	Mwangi et al. 2012
Potato dextrose agar (PDA)	Potato dextrose powder (39 g/L; BioLife) and agar (15 g/L; Biolife italiana).	10	Fungi	Mwangi et al. 2012
Skimmed milk malt agar medium (SM-MA)	Powder milk (50 g/L; Rapilait, Migros), Malt extract (12 g/L; SIOS Home Brewing and agar (15 g/L; Biolife italiana).	4.47	Both (bacteria and fungi)	Lohberger et al. 2019
Antifungal medium	Cycloheximide (1g/L) (Sigma-Aldrich), Nutrient broth (8 g/900mL; Biolife italiana) and agar (15 g/L; Biolife italiana).		Bacteria	
Antibacterial medium	Streptomycin sulfate (0.064 g/L) (AppliChem), Tetracycline (0.008g/L) (Fluka, Biochemika), Malt extract (12 g/L; SIOS Home Brewing and agar (15 g/L; Biolife italiana).		Fungi	

Bacterial-fungal confrontation assays

For bacterial-fungal confrontation, one bacterial and one fungal strain were co-cultured in three different media: nutrient agar (NA), potato dextrose agar (PDA), and skimmed milk malt agar (SM-MA) (Table 2). Initially, confrontation assays were carried out with five bacterial strains selected based on their contrasting PGP activities: three bacterial strains positive for four PGP activities (*Bacillus thuringiensis* 1310, *Bacillus thuringiensis* 1312 and *Bacillus licheniformis* 1062) and two with no, or only one, PGP activity (*Bacillus macerans* 1004 and *Lysinibacillus sphaericus* 1003). Based on the results of this first experiment, another set of confrontation assay was performed using only NA medium and confronting the 15 bacterial strains screened with two fungal strains: *T. rossicum* and *R. solani.* The experimental design of the confrontation assays consisted in the inoculation of the fungus in the middle of the culture medium using a 5 mm^2 plug taken from 4-day old cultures grown on MA. Two days after fungal inoculation, two lines of bacteria from an overnight bacterial culture in NA were streaked at a distance of 2 cm from the apical margin of the fungal colony, on both sides of the colony (Figure 1).

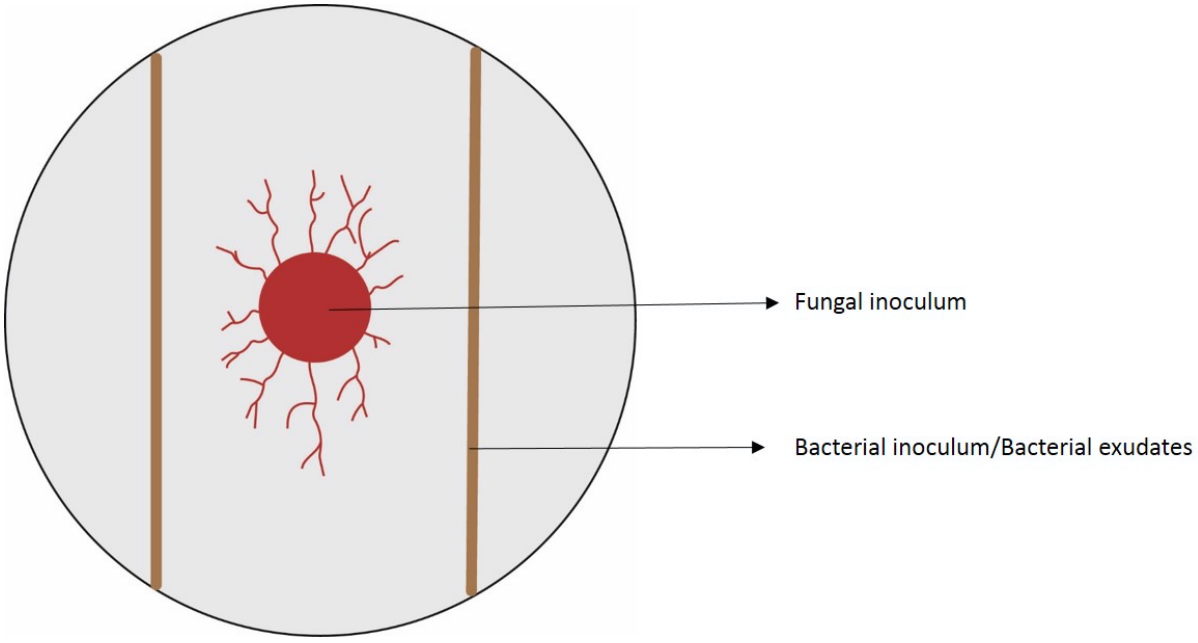

Figure 1: Schematic representation of the experimental design used in the bacterial-fungal confrontation assays. The same scheme was used to assess the effect of bacterial exudates on the growth of selected fungi.

After incubation the following parameters were noted: 1) growth of the two organisms and visual aspect of the colonies; 2) the ability of both organisms to re-grow after confrontation (i.e. viability) in media with a biocide to avoid the growth of the confrontation partner (see below); and 3) the ability of bacteria to disperse on the fungal network (fungal-highway like dispersal).

To check for the viability and dispersal of bacteria after the confrontation, NA was supplemented with cycloheximide (an antifungal compound) (Table 2) to suppress fungal growth. The same was done to assess the viability of fungi with MA supplemented with tetracycline and streptomycin sulfate (antibacterial compounds) to suppress bacterial growth. The ability of bacteria to disperse on fungal hyphae using the fungal highway mechanism was measured using two approaches. First, in the confrontation assays, agar plugs were cut from inner and outer parts of the fungal colonized agar and then placed on an antifungal medium where only bacteria could grow (Figure 2). In case of *R. solani*, the two plugs were obtained from the inner part of the fungal colony that was away from the bacterial inoculum (highlighted in circle in Figure 2) and from another part of fungal colony that was close to the bacterial inoculum. In case of *T. rossicum*, the two plugs were obtained from the inner part of the fungal colony that was away from the bacterial inoculum and from an area in the fungal mycelium that crossed the bacterial inoculum as explained in Figure 2. The presence of bacterial colonies on the antifungal medium was assessed after one to two days. Second, the specific dispersal of bacteria via fungal highways using *T. rossicum* network was examined using the gap-in-medium method (Bravo et al. 2013) by using NA medium. The distance between the two agar surfaces was 0.5 cm. To inoculate the fungal strains, a 5 mm^2 plug was taken from 4-day old cultures grown on MA. *R. solani* was inoculated on one side and *T. rossicum* on the other side to observe the transfer of bacteria from one fungus to another. After one day, a loop of an overnight bacterial culture on NA was streaked on the compartment where *T. rossicum* was inoculated, i.e. direct inoculation of bacteria on its hyphal network (Figure 3). These plates were incubated at room temperature for one week. To verify whether the inoculated bacteria dispersed via fungal highways, an agar plug was sampled from the *R. solani* compartment and inoculated on an antifungal medium. Results were noted after one to two days.

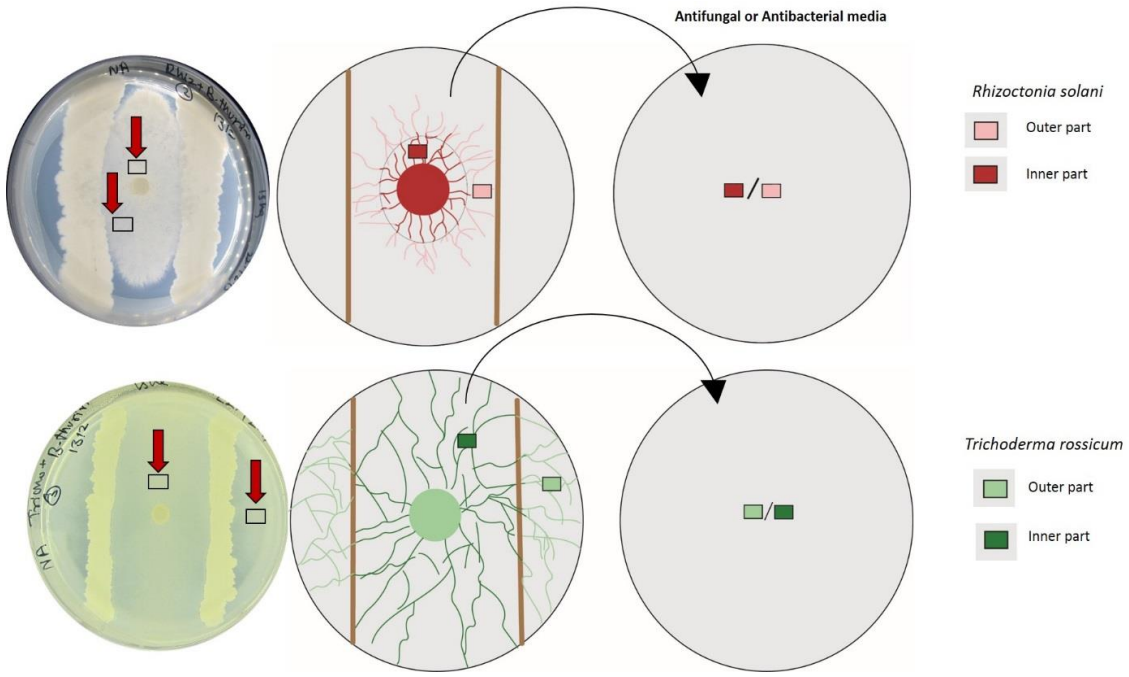

Figure 2: Experimental design used to evaluate the viability of bacteria after confrontation with the fungus as well as bacterial dispersal on the fungal network via fungal highways. The viability of the fungus after confrontation with the bacteria was measured by regrowth using antibacterial media. This was performed with samples taken from two different places of the fungal network: the inner part = away from the bacterial inoculum and the outer part = in contact with the bacterial inoculum).

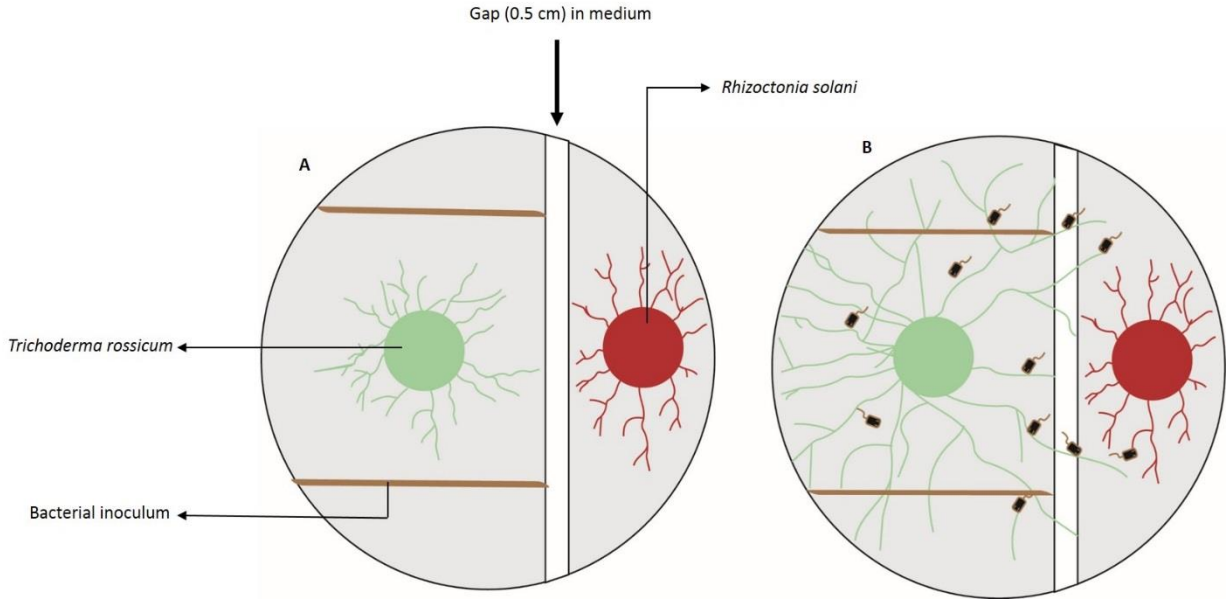

Figure 3: Schematic representation of the gap-in-medium method used for determining bacterial dispersal towards *R. solani* using fungal highways of *T. rossicum*. (A) At the time of bacterial inoculation; (B) after few days of bacterial inoculation.

Effect of bacterial exudates on fungal growth

In order to investigate the growth inhibition of *R. solani,* the effect of bacterial exudates on fungal growth was assessed. In this experiment, the effect of bacterial exudates on *T. rossicum* was not assessed as the growth of *T. rossicum* was not affected or not inhibited in NA. Exudates of all of the 15 bacterial strains were screened against the phytophathogenic fungus *R. solani.* Bacteria were incubated in 10 mL nutrient broth (8 g/L; Biolife italiana; NB) at 30°C on an orbital shaker (150 rpm). After an overnight incubation, the supernatant of each bacterial culture was filtered through a 0.22 um membrane filter in order to obtain a sterile spent medium containing bacterial exudates. This filtered solution was then used for the assay. For *R. solani* inoculation, a 5 mm^2 plug was taken from a 4-day old fungal culture and inoculated on NA medium. After one day, 0.5 mL of the bacterial supernatant was streaked out 2 cm away from the fungal inoculum on both sides of the inoculum using the same scheme as for the confrontation assays (Figure 1). The only difference was that instead of alive bacteria, bacterial exudates were used. The Petri dishes were incubated at room temperature and results were recorded after one week.

Effect of bacterial volatiles on fungal growth

In order to assess whether volatiles produced by bacteria were responsible for the inhibitory effect observed on the growth of *R. solani*, a Petri dish with two compartments was used as shown in Figure 4. In this experiment, the effect of volatiles on *T. rossicum* growth was not assessed as the growth of *T. rossicum* was neither affected nor inhibited with most of the bacterial strains. All of the 15 bacterial strains were screened against *R. solani*. In one compartment, the fungus was inoculated on a NA medium using a 5 mm^2 fungal plug from a 4-day old culture. In the second compartment, a small Petri dish was placed and contained a NA medium inoculated with a loop of an overnight bacterial culture on NA. The Petri dishes were incubated at room temperature and results were recorded after one week.

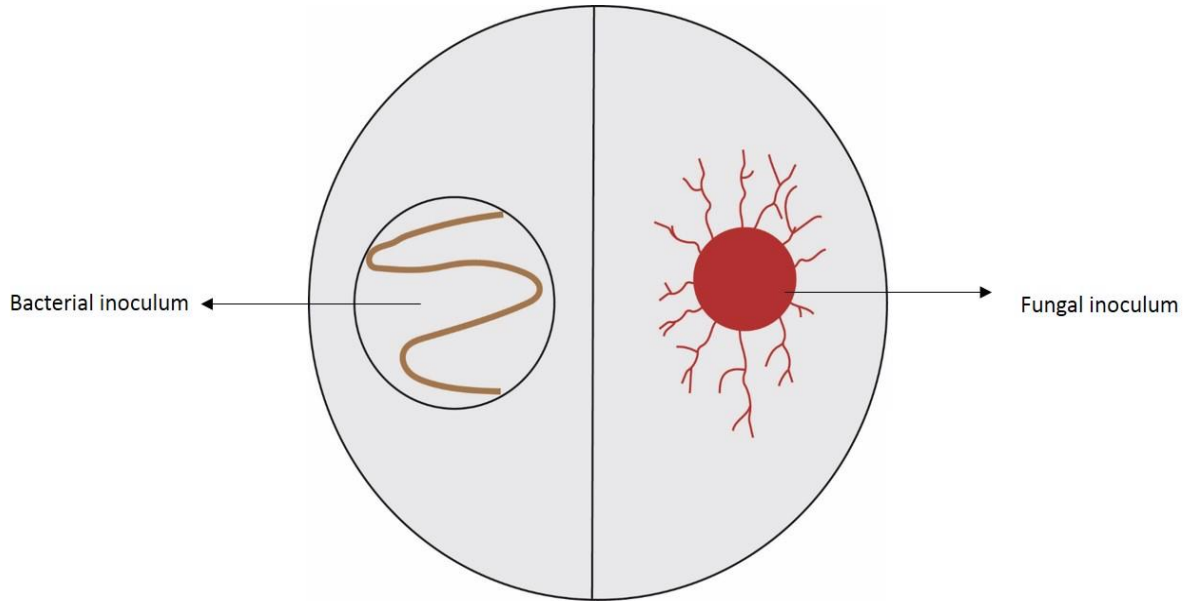

Figure 4: Schematic representation of the experimental design used for assessing the effect of volatile compounds produced by bacterial strains on *R. solani* growth.

Fungal-fungal interactions

Fungal-fungal interactions were assessed in two different media, PDA and SM-MA, with the five selected fungal strains mentioned previously. The aim of this experiment was to select promising candidates that could be used in the agricultural practices for the control of phytopathogenic fungi. Fungal-fungal confrontation experiments were carried out with two fungal partners (bi-partite interactions) and also with combinations of three different fungal partners (tri-partite interactions) (Table 3; Figure 5). For the phytopathogenic fungi and the saprophytic fungus (which are fast-growers) 4-day old cultures were used. For the ectomycorrhizal fungi, 10-day old cultures were used as they had a slower growth. In the confrontation assays, to avoid slow-growers being out-competed too quickly by fast-growing fungi, a sequential inoculation strategy was applied. The slow-growing fungus was inoculated first, followed by fast-growing fungus. For instance, for the couple *L. bicolor* + *T. rossicum*, *L. bicolor* (slow-grower) was inoculated first and *T. rossicum* (fast-grower) was inoculated one week later. Results were recorded one week after the last fungal inoculation.

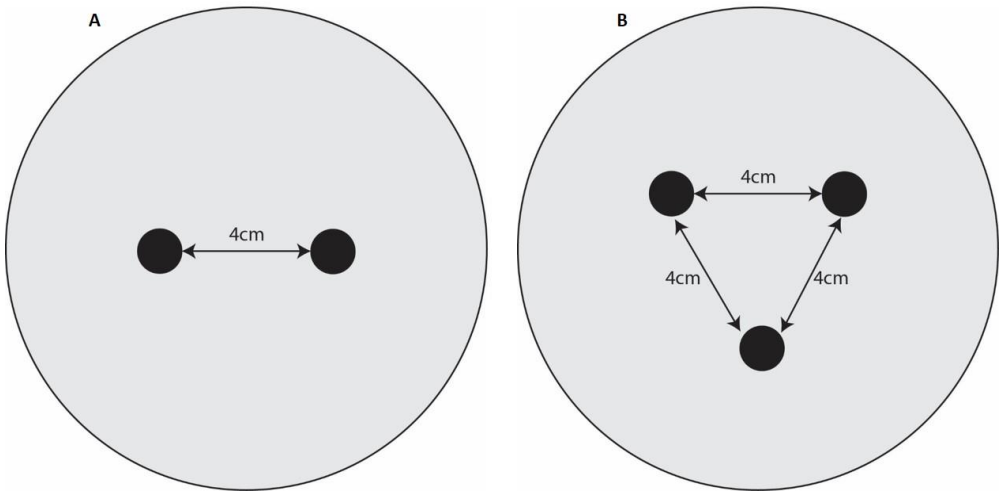

Figure 5: Schematic representation of the experimental set-up used in (A) bi-partite fungal interactions and (B) tri-partite fungal interactions.

Table 3: Combinations of fungal strains for bi- and tri-partite confrontations

Fungal combinations for bi-partite confrontations
T. rossicum + R. solani
T. rossicum + P. ultimum
T. rossicum + L. bicolor
T. rossicum + B. edulis
R. solani + L. bicolor
R. solani + B. edulis
P. ultimum + L. bicolor
P. ultimum + B. edulis
Fungal combinations for tri-partite confrontations
T. rossicum + L. bicolor + R. solani
T. rossicum + L. bicolor + P. ultimum
T. rossicum + B. edulis + R. solani
T. rossicum + B. edulis + P. ultimum

Results

Bacterial PGP traits

The screening of bacterial strains for PGP activities was carried out using different physiological assays and the results are summarized in Table 4. Out of the 15 selected bacterial strains, 10 strains were able to grow on N-free medium, showing their ability to fix atmospheric N_2. All bacterial strains, except for *Bacillus macerans* 1004, were positive for casein solubilization and were thus able to solubilize and acquire N from an organic N source. Moreover, 11 bacterial strains were found to produce siderophores as observed by the formation of an orange/yellow halo around the colonies. Out of the 15 bacterial strains, only 4 were able to produce auxin-like phytohormones. Finally, as none of the strains was positive for phosphate solubilization, none of the strains was positive for all the tested PGP traits. Overall, out of the 15 selected bacterial strains, only 1 strain, *B. macerans* 1004, did not show any PGP trait and another strain, *Lysinibacillus sphaericus* 1003, was positive for only one trait (proteolysis). Most of the bacterial strains exhibited more than one characteristic, with 3 strains showing 4 PGP traits, 6 strains with 3 PGP traits, and 4 strains with 2 PGP traits. The results of the bacterial screening performed in this part of this chapter was published in a review article within the topic “Biotechnological applications of spore-forming bacteria, Plant growth promotion” by (Paul et al. 2018).

Table 4: Screening of bacterial strains for PGP traits. + stands for a positive activity; - stands for a negative activity.

Number	Bacterial strains	Nitrogen fixation	Proteolysis	Siderophore production	Auxin-like phytohormone production	Phosphate solubilization
1	*Bacillus thuringiensis* 1312	+	+	+	+	-
2	*Bacillus thuringiensis* 1310	+	+	+	+	-
3	*Bacillus thuringiensis* 1318	+	+	+	-	-
4	*Bacillus thuringiensis* 1070	-	+	+	+	-
5	*Bacillus thuringiensis* 1311	+	+	+	-	-
6	*Bacillus thuringiensis* 1321	+	+	+	-	-
7	*Bacillus subtilis* 1055	+	+	+	-	-
8	*Bacillus cereus* 88	-	+	+	-	-
9	*Bacillus licheniformis* 1062	+	+	+	+	-
10	*Bacillus subtilis* 16	-	+	+	-	-
11	*Lysinibacillus sphaericus* 1003	-	+	-	-	-
12	*Bacillus polymyxa* 25	+	+	-	-	-
13	*Bacillus weihenstephanensis* 1324	+	+	+	-	-
14	*Bacillus pumilus* 2	+	+	-	-	-
15	*Bacillus macerans* 1004	-	-	-	-	-

Fungal PGP activities

Five fungal species of different ecological niches were also screened for some PGP traits and the results are shown in Table 5. All fungal strains, except for the fungus-like Oomycete *Pythium ultimum*, were able to solubilize casein and to produce siderophores. Finally, *Laccaria bicolor* was the only fungus that had the ability to solubilize inorganic phosphorous and was thus the only species that had all three PGP traits tested.

Table 5: Screening of fungi of different ecological niches for plant growth promoting activities.
Legends: + stands for a positive activity; - stands for a negative activity.

Name	Proteolysis	Siderophore production	Phosphate solubilization
Boletus edulis	+	+	-
Laccaria bicolor	+	+	++
Trichoderma rossicum	+	+	-
Rhizoctonia solani	+	++	-
Pythium ultimum	-	-	-

Bacterial-fungal confrontation assays

Interactions and type of medium

Five bacterial strains were selected for further confrontation assays. *B. licheniformis* 1062, *B. thuringiensis* 1310, and *B. thuringiensis* 1312 were selected because they were positive for 4 out of 5 PGP traits tested. The other two strains were selected because they were negative to all the traits (*B. macerans* 1004) or positive to one trait only (*L. sphaericus* 1003). The first set of bacterial-fungal confrontation assays showed that these strains established neutral and antagonistic interactions with the selected fungal strains. Importantly, it appeared that bacterial-fungal interactions were strongly influenced by the medium in which the confrontations took place (Table 6). *B. macerans* 1004, which had none of the PGP traits assessed, was unable to inhibit the growth of any of the fungi on all of the media tested.

Table 6: Summary of the bacterial fungal interactions on different media. Bacterial-fungal confrontations were performed with: two EcM fungi (*Boletus edulis* and *Laccaria bicolor*), one saprophytic fungus (*Trichoderma rossicum*), and two plant pathogenic fungus or fungus-like oomycetes (*Rhizoctonia solani* and *Pythium ultimum*) confronted to five different bacterial strains with: plant growth promoting activities (*Bacillus licheniformis* 1062, *Bacillus thuringiensis* 1312 and *Bacillus thuringiensis* 1310) or no plant growth promoting activities (*Bacillus macerans* 1004 and *Lysinibacillus sphaericus* 1003). Three different media were used: skimmed milk malt agar (SM-MA), potato dextrose agar (PDA), and nutrient agar (NA). The effect of bacteria on fungal growth were summarized by using the following color code: blue for no fungal growth inhibition by bacteria; grey for a neutral interaction between fungi and bacteria; orange for an inhibition of fungal growth by bacteria; and yellow for a strong inhibition of fungal growth by bacteria.

Legends	No inhibition	Neutral	slight inhibition	Strong inhibition

Bacterial strains	Fungal strains	Three different media		
		SM-MA	PDA	NA
Bacillus licheniformis 1062	*Boletus edulis*			
	Laccaria bicolor			
	Trichoderma rossicum			
	Rhizoctonia solani			
	Pythium ultimum			
Bacillus thuringiensis 1312	*Boletus edulis*			
	Laccaria bicolor			
	Trichoderma rossicum			
	Rhizoctonia solani			
	Pythium ultimum			
Bacillus thuringiensis 1310	*Boletus edulis*			
	Laccaria bicolor			
	Trichoderma rossicum			
	Rhizoctonia solani			
	Pythium ultimum			
Bacillus macerans 1004	*Boletus edulis*			
	Laccaria bicolor			
	Trichoderma rossicum			
	Rhizoctonia solani			
	Pythium ultimum			
Lysinibacillus sphaericus 1003	*Boletus edulis*			
	Laccaria bicolor			
	Trichoderma rossicum			
	Rhizoctonia solani			
	Pythium ultimum			

Table 7: Summary of the bacterial fungal interactions on different media. Bacterial-fungal confrontations were performed with: two EcM fungi (*Boletus edulis* and *Laccaria bicolor*), one saprophytic fungus (*Trichoderma rossicum*), and two plant pathogenic fungus or fungus-like oomycetes (*Rhizoctonia solani* and *Pythium ultimum*) confronted to five different bacterial strains with: plant growth promoting activities (*Bacillus licheniformis* 1062, *Bacillus thuringiensis* 1312 and *Bacillus thuringiensis* 1310) or no plant growth promoting activities (*Bacillus macerans* 1004 and *Lysinibacillus sphaericus* 1003). Three different media were used: skimmed milk malt agar (SM-MA), potato dextrose agar (PDA), and nutrient agar (NA). The disoersal of the bacteria through fungal highways were summarized by using the following codes:

Conditions	**Code**
No bacterial dispersal on fungal network	0
Bacterial dispersal on part of the fungal network	1
Bacterial dispersal on the whole fungus network	2

Bacterial strains	**Fungal strains**	**Three different media**		
		SM-MA	**PDA**	**NA**
Bacillus licheniformis 1062	*Boletus edulis*	2	0	1
	Laccaria bicolor	2	2	2
	Trichoderma rossicum	2	2	2
	Rhizoctonia solani	2	2	2
	Pythium ultimum	2	2	2
Bacillus thuringiensis 1312	*Boletus edulis*	1	1	1
	Laccaria bicolor	2	0	2
	Trichoderma rossicum	2	2	2
	Rhizoctonia solani	2	2	2
	Pythium ultimum	2	2	2
Bacillus thuringiensis 1310	*Boletus edulis*	2	1	1
	Laccaria bicolor	1	1	1
	Trichoderma rossicum	2	2	2
	Rhizoctonia solani	2	2	2
	Pythium ultimum	2	2	2
Bacillus macerans 1004	*Boletus edulis*	2	0	1
	Laccaria bicolor	2	0	2
	Trichoderma rossicum	2	2	2
	Rhizoctonia solani	2	2	2
	Pythium ultimum	2	2	2
Lysinibacillus sphaericus 1003	*Boletus edulis*	2	1	2
	Laccaria bicolor	2	1	2
	Trichoderma rossicum	2	2	2
	Rhizoctonia solani	2	2	2
	Pythium ultimum	2	2	2

The growth of the EcM fungus *B. edulis* was slightly inhibited by 4 out of the 5 bacterial strains on SM-MA medium. On PDA medium, only *B. licheniformis* 1062, was able to inhibit the growth of

this fungus. On NA medium, 3 bacterial strains strongly inhibited *B. edulis* growth. Inhibition of the other EcM fungus, *L. bicolor,* was also observed with 2 bacteria on SM-MA medium, while on NA 4 bacteria showed a strong inhibition towards this fungus. On PDA none of the bacteria inhibited *L. bicolor*. On SM-MA medium the growth of the plant pathogen *R. solani* was inhibited by *B. licheniformis* 1062, *B. thuringiensis* 1310, and *B. thuringiensis* 1312. *L. sphaericus* 1003 also inhibited *R. solani,* but the inhibition was not as prominent as compared to the other 3 bacteria. On PDA, *L. sphaericus* 1003 failed to inhibit *R. solani* growth, while the other 3 strains were still able to inhibit the growth of this fungus (Figure 6). All bacterial strains, except *B. macerans* 1004, strongly inhibited the growth of *R. solani* on NA medium. None of the bacterial strains were able to inhibit the growth of *P. ultimum* and *T. rossicum*, and this was valid for all three media used in the confrontation assays.

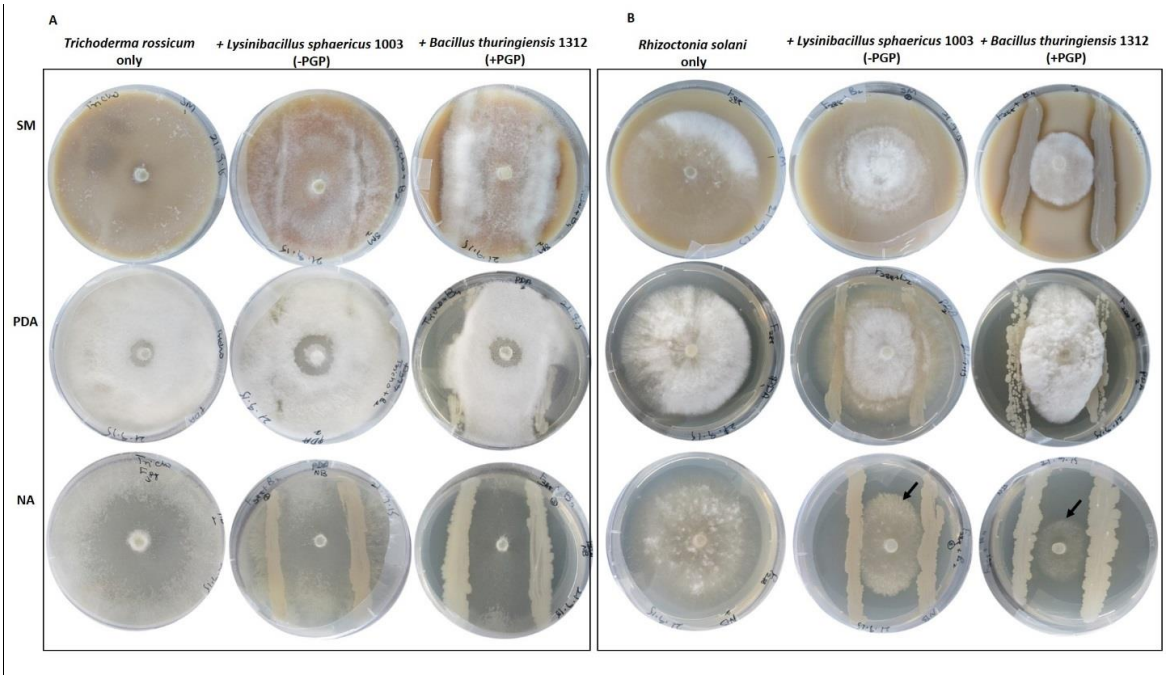

Figure 6: Example of bacterial-fungal confrontations on three different media: skimmed milk malt agar (SM-MA), potato dextrose agar (PDA) and nutrient agar (NA) with: a bacterial strain negative for plant growth promoting activities (-PGP, *Lysinibacillus sphaericus* 1003) and another one positive for plant growth promoting activities (+PGP, *Bacillus thuringiensis* 1312). (A) The growth of *Trichoderma rossicum* was not inhibited with any of the two bacterial strains on the three different media. (B) The growth of *Rhizoctonia solani* was strongly inhibited on NA medium (arrow) by both -PGP and +PGP bacteria, as compared to *R. solani* growing alone.

Fungal highways

The investigation of active dispersal of bacteria on fungal networks through fungal highways is summarized in Table 7. No bacterial dispersal on *B. edulis* hyphal network was observed on PDA, however dispersal was effective on part of the hyphal network on the other two media. Regarding *L. bicolor*, it was noticed that bacteria with several PGP traits such as *B. licheniformis* 1062 and *B. thuringiensis* 1310 were able to disperse on fungal hyphae on both, NA and SM-MA media (Table 7). On PDA medium, the dispersal of bacteria was restricted to a small area where both organisms overlapped, and bacterial dispersal seemed to be prevented by the fungus. Interestingly, and contrary to the 2 EcM fungi, all five bacteria were able to spread all over the hyphal network of *T. rossicum*, *P. ultimum*, and *R. solani* in all three media. In addition, bacteria did not produce an inhibition zone with these fungi and they were able to colonize the entire fungal network. Nevertheless, the re-growth of *R. solani* was restricted in most of the cases (*B. thuringiensis* 1312, *B. thuringiensis* 1310, *B. licheniformis* 1062 and *Lysinibacillus sphaericus* 1003), with the exception of *B. macerans* 1004, which was unable to inhibit the growth of the fungus. To summarize, fungal highways were observed in all cases with both *R. solani* and *T. rossicum*, but restricted growth of the first fungus only.

Further confrontation assays

A second set of confrontation assays was carried out in order to determine whether this selective mode of action was present in the 15 selected *Bacillus* spp. The viability of *R. solani* and *T. rossicum* after confrontation as well as dispersal of bacteria via fungal highways was assessed with all 15 bacterial strains on NA medium only. 12 out of the 15 bacterial strains were unable to inhibit the growth of *T. rossicum* (Figure 7).

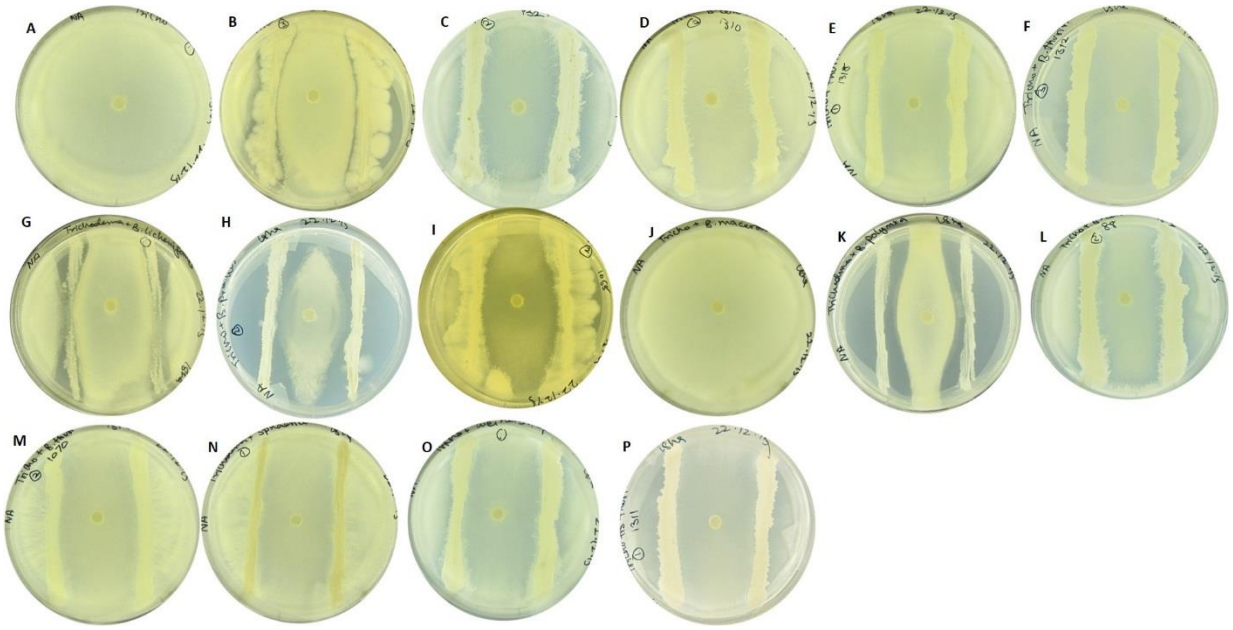

Figure 7: Confrontation assays on NA media of *Trichoderma rossicum* co-cultured with 15 bacterial strains: (A) *T. rossicum* without bacteria (control), (B) *Bacillus subtilis* 16, (C) *B. thuringiensis* 1321, (D) *B. thuringiensis* 1310, (E) *B. thuringiensis* 1318, (F) *B. thuringiensis* 1312, (G) *B. licheniformis* 1062, (H) *B. pumilus* 2, (I) *B. subtilis* 1055, (J) *B. macerans* 1004, (K) *B. polymyxa* 25, (L) *B. cereus* 88, (M) *B. thuringiensis* 1070, (N) *Lysinibacillus sphaericus* 1003, (O) *B. weihenstephanensis* 1324 and (P) *B. thuringiensis* 1311.

Interestingly, some of the bacterial strains used the hyphal network to disperse in both the inner and outer parts of the mycelial network (Figure 2, Table 8). In other cases, only one side of the fungal colony was colonized by bacteria. For instance, *B. subtilis* 1055 colonized only the outer parts of *T. rossicum* hyphal network (Table 8). Confrontation of *T. rossicum* with *B. thuringiensis* 1321, *B. thuringiensis* 1318, or *B. weihenstephanensis* 1324 showed that these strains were not able to disperse using its hyphal network. With the plant pathogenic fungus *R. solani*, the outer part of the fungal colony (which was close to the bacterial inoculum) was colonized by all of the 15 bacterial strains and these latter spread over this part of the fungal network. On the contrary, the inner part of the fungal colony was only partially colonized by dispersing bacteria, this was the case of *B. thuringiensis* 1311 and *B. thuringiensis* 1070. *B. subtilis* 1055, *B. pumilus* 2, *B. thuringiensis* 1318 and *B. weihenstephanensis* 1324 did not colonize the mycelial network (Table 8). Besides bacterial dispersal on the fungal network, the viability of both fungi after bacterial dispersal was also determined. *T. rossicum* was able to re-grow in all of the combinations, except

in confrontation with *B. pumilus* 2 (Table 8). Regarding *R. solani*, the results were the opposite: most of the *Bacillus* spp. tested strongly inhibited growth, with the exception of *B. macerans* 1004 (Figure 8).

Table 8: Summary of fungal growth inhibition by bacteria, presence of fungal highways, and fungal viability for *Rhizoctonia solani* or *Trichoderma rossicum* in co-cultures with the 15 bacterial strains. All experiments were carried out in triplicates.
Legend: 3/3 indicates that the related property is present in all three replicates; 2/3 indicates that the related property is present in two replicates out of three; 1/3 indicates that the related property is present in one replicate out of three; 0/3: indicates that the related property is absent in all three replicates.

Bacterial strains	*Rhizoctonia solani*					*Trichoderma rossicum*				
	Fungal inhibition	Presence of fungal highways		Survival of fungus		Fungal inhibition	Presence of fungal highways		Survival of fungus	
		Inner parts	Outer parts	Inner parts	Outer parts		Inner parts	Outer parts	Inner parts	Outer parts
B. polymyxa 25	3/3	1/3	3/3	3/3	0/3	2/3	2/3	2/3	3/3	3/3
B. subtilis 1055	3/3	0/3	3/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3
B. thuringiensis 1310	3/3	3/3	3/3	0/3	0/3	0/3	1/3	3/3	3/3	3/3
B. pumilus 2	3/3	0/3	3/3	2/3	1/3	2/2	2/2	-	2/2	0/2
B. cereus 88	3/3	3/3	3/3	2/3	1/3	0/3	1/3	2/3	3/3	3/3
B. thuringiensis 1311	3/3	1/3	3/3	1/3	1/3	0/3	0/3	3/3	3/3	3/3
B. subtilis 16	3/3	3/3	3/3	2/3	2/3	3/3	3/3	-	3/3	-
Lysinibacillus sphaericus 1003	2/3	3/3	3/3	2/3	1/3	0/3	3/3	3/3	3/3	3/3
B. thuringiensis 1070	3/3	2/3	3/3	2/3	2/3	0/3	2/3	3/3	3/3	3/3
B. thuringiensis 1321	3/3	3/3	3/3	2/3	2/3	0/3	0/3	0/3	3/3	3/3
B. thuringiensis 1312	3/3	3/3	3/3	3/3	2/3	0/3	0/3	2/3	3/3	3/3
B. licheniformis 1062	3/3	3/3	3/3	3/3	3/3	0/3	3/3	0/3	3/3	3/3
B. macerans 1004	0/3	3/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	3/3
B. weihenstephanensis 1324	3/3	0/3	3/3	3/3	3/3	0/3	0/3	0/3	3/3	3/3
B. thuringiensis 1318	3/3	0/3	3/3	3/3	3/3	0/3	0/3	0/3	3/3	3/3

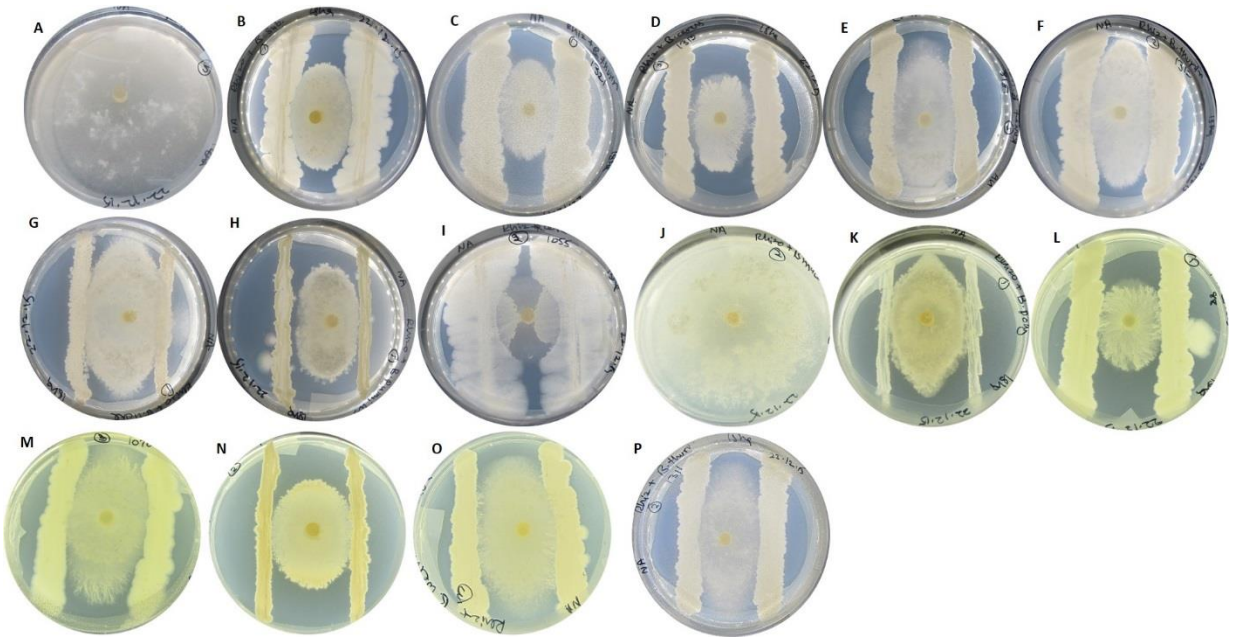

Figure 8: Confrontation assays on NA media of *Rhizoctonia solani* with 15 bacterial strains: (A) *R. solani* without bacteria (control), (B) *Bacillus subtilis* 16, (C) *B. thuringiensis* 1321, (D) *B. thuringiensis* 1310, (E) *B. thuringiensis* 1318, (F) *B. thuringiensis* 1312, (G) *B. licheniformis* 1062, (H) *B. pumilus* 2, (I) *B. subtilis* 1055, (J) *B. macerans* 1004, (K) *B. polymyxa* 25, (L) *B. cereus* 88, (M) *B. thuringiensis* 1070, (N) *Lysinibacillus sphaericus* 1003, (O) *B. weihenstephanensis* 1324 and (P) *B. thuringiensis* 1311.

Although in most of the cases, the bacterial strains did not produce an inhibition zone on the edge of the fungal colony, when comparing fungal growth with control cultures of *R. solani* only, bacterial inoculation restricted the area colonized by the fungus (Figure 8). Additionally, *R. solani* hyphae that were in physical contact with bacteria were unable to re-grow. For instance, *R. solani* was completely unable to re-grow after confrontation with *B. subtilis* 1055 and *B. cereus* 1310. However, with *B. polymyxa*, *B. pumilus* 2, *B. cereus* 88, and *B. thuringiensis* 1311, the inner part of the fungal colony that were not colonized by bacteria were able to re-grow, while the outer parts, which were fully colonized by bacteria, were unable to re-grow (Table 8).

The dispersal of bacteria via fungal highways was also assessed with the "gap-in-medium" method by using *T. rossicum* hyphae as a dispersal network (Figure 3). Out of the 15 initial bacterial strains, only 11 were screened. *B. thuringiensis* 1321, *B. thuringiensis* 1318 and *B. weihenstephanensis* 1324 were eliminated for this test as they were unable to disperse on *T. rossicum* hyphal network (Table 8). *B. polymyxa* 25, *B. subtilis* 16, *B. cereus* 88, *B. licheniformis* 1062, *L. sphaericus* 1003, *B. pumilus* 2, *B. thuringiensis* 1310, *B. thuringiensis* 1311, *B.*

thuringiensis 1070, *B. subtilis* 1055, *B. thuringiensis* 1311, and *B. thuringiensis* 1312 were able to use the fungal hyphae of *T. rossicum* to reach the other compartment colonized by *R. solani,* and together with *T. rossicum,* inhibited the growth of *R. solani* (Figure 9).

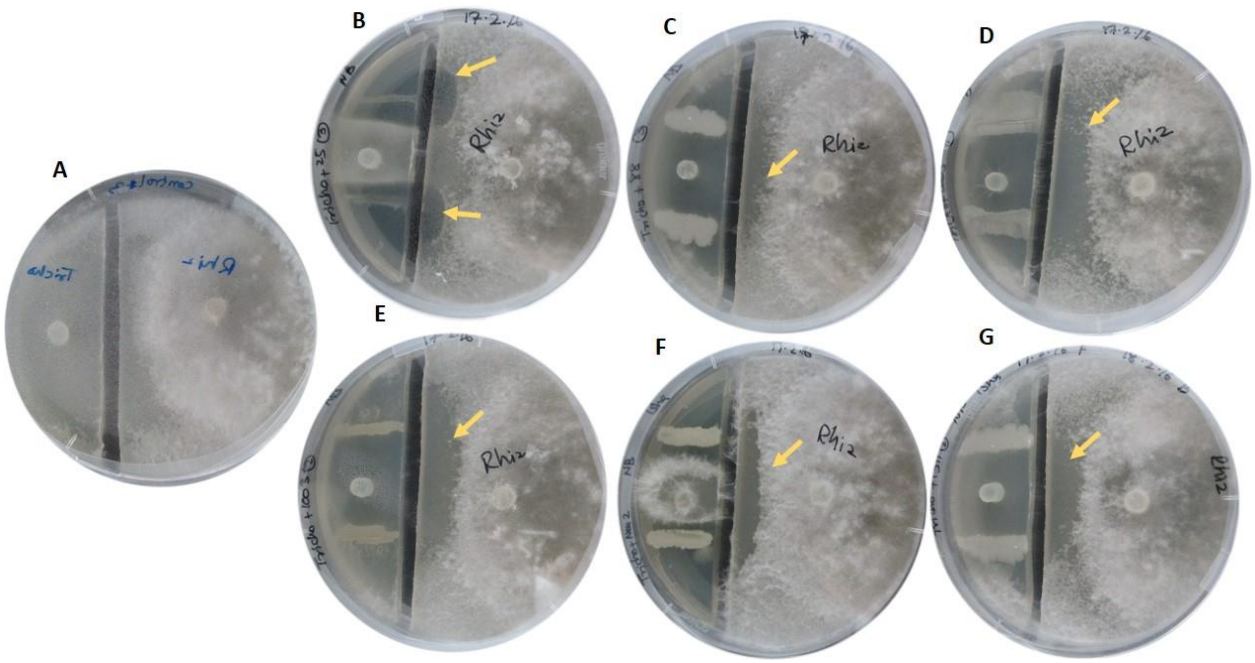

Figure 9: Growth inhibition of *Rhizoctonia solani* in the "gap-in-medium" experiment which used *Trichoderma rossicum* and bacterial strain able to disperse on its mycelial network through fungal highways (NA media). The gap between the two agar surfaces had a width of 0.5 cm. (A) Control without bacteria in the compartment of *T. rossicum*. With bacteria, growth of *R. solani* is inhibited (yellow arrows) by (B) *Bacillus polymyxa* 25, (C) *B. cereus* 88, (D) *B. licheniformis* 1062, (E) *L. sphaericus* 1003, (F) *B. pumilus* 2*,* and (G) *B. thuringiensis* 1311.

The growth inhibition of *R. solani* by bacteria was further investigated by using bacterial exudates. It was observed that none of the exudates produced by the 15 bacterial strains were able to inhibit the growth of *R. solani* (Figure 10).

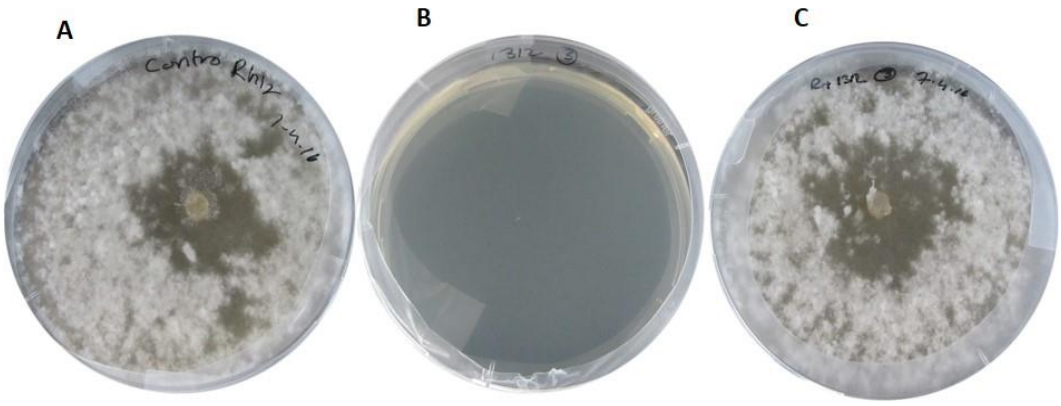

Figure 10: Example of the effect of bacterial exudates on the growth of *Rhizoctonia solani* on NA medium; (A) *R. solani* only (fungal control), (B) Exudates of *Bacillus thuringiensis* 1312 showing no presence of bacterial cells (bacterial control), (C) *R. solani* with bacterial exudates of *Bacillus thuringiensis* 1312.

Finally, the role of bacterial volatiles in the growth inhibition of *R. solani* was also assessed by using two compartment plates. We found that the volatiles produced by the 15 bacterial strains were not responsible for the growth inhibition (Figure 11).

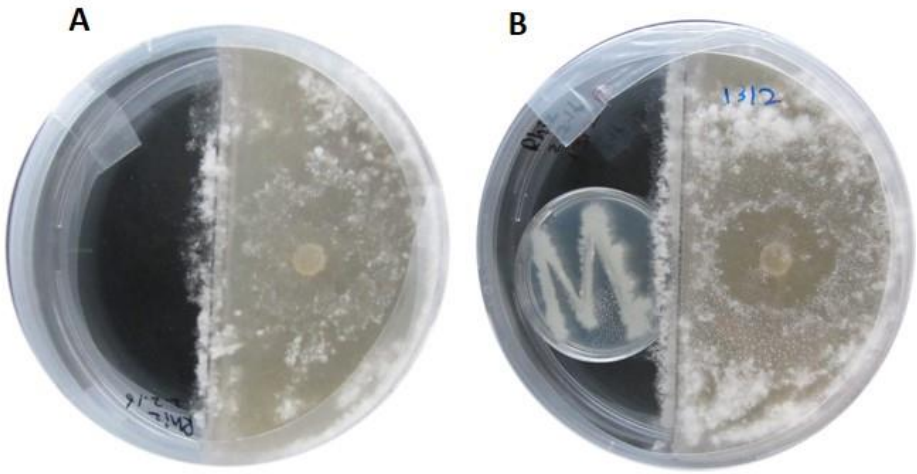

Figure 11: Example of the analysis used for the detection of volatiles with an inhibitory effect; (A) *Rhizoctonia solani* (Control), (B) Effect of *Bacillus thuringiensis* 1312 on the growth of *R. solani.* Test performed in NA medium.

Fungal-fungal interactions

In addition to bacterial-fungal interactions, fungal-fungal interactions also play a vital role in the mycorrhizosphere. For this reason, we examined which type of interactions are established

between the fungal species of different niches that were selected in this study. Only qualitative data (observations and images) were recorded for this experiment. In bipartite interactions (involving two fungal partners), neutral interactions between *T. rossicum* and both, *L. bicolor* and *B. edulis* occurred on SM-MA medium, while on PDA medium antagonistic interactions were noticed (Table 9).

Table 9: Bi-partite fungal confrontation assays using two different media.
Legends: + stands for an inhibition of the fungus in the second column by the fungus in the first column; **-** stands for no inhibition of the fungus in the second column by the fungus in the first column.

Fungal partners		**Skimmed milk malt agar (SM-MA)**	**Potato dextrose agar (PDA)**
L. bicolor	*T. rossicum*	-	+
	R. solani	+	+
	P. ultimum	-	+
B. edulis	*T. rossicum*	-	-
	R. solani	+	+
	P. ultimum	-	+
T. rossicum	*R. solani*	+	+
	P. ultimum	-	-

Furthermore, in bipartite interactions, *B. edulis* was able to inhibit the growth of *P. ultimum* on PDA medium, while this was not the case on SM-MA medium (Figure 12).

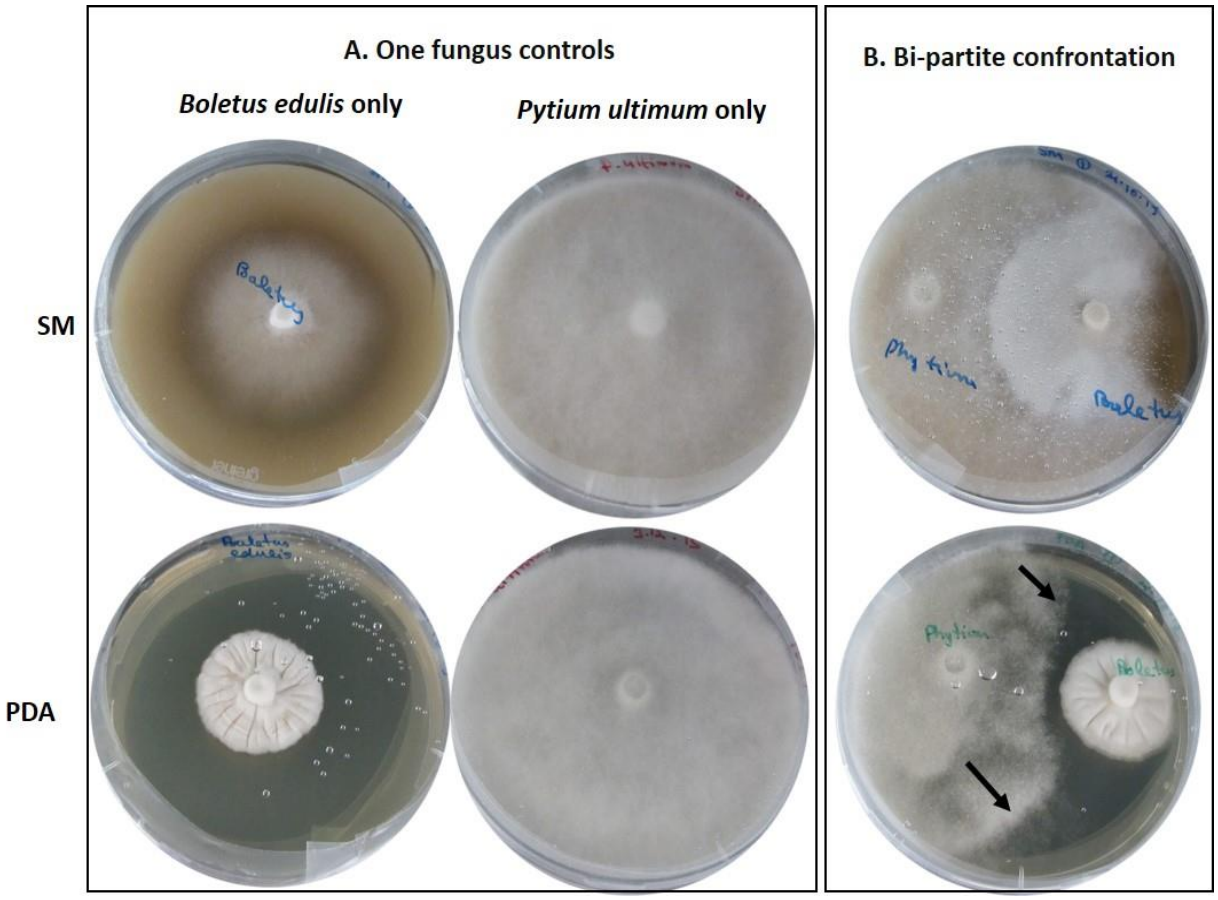

Figure 12: Bi-partite fungal confrontation assays involving *Boletus edulis* and *Pythium ultimum* on two different media: skimmed milk malt agar (SM-MA) and potato dextrose agar (PDA). (A) *B. edulis* and *P. ultimum* control on SM-MA and PDA media; (B) *P. ultimum* confronted with *B. edulis* on SM-MA medium and showing no inhibition between both organisms (upper figure) and *P. ultimum* confronted with *B. edulis* on PDA, black arrows show the inhibition zone between both fungi (lower figure).

In tri-partite interactions, it was observed that *L. bicolor* along with *T. rossicum* strongly limited the growth of the phytopathogenic fungus *R. solani* on SM-MA medium (Figure 13). Additionally, a slight inhibition of *R. solani* by both fungi was also noticed on PDA (Figure 14). Moreover, the tri-partite interaction with the other EcM fungus, *B. edulis, T. rossicum*, and the phytopathogenic fungus-like oomycete (*P. ultimum*) showed that the growth of *P. ultimum* was not inhibited at all on both media (SM-MA and PDA). However, with the other phytopathogenic fungus *R. solani*, there was a slight inhibition on both SM-MA and PDA media (Table 10).

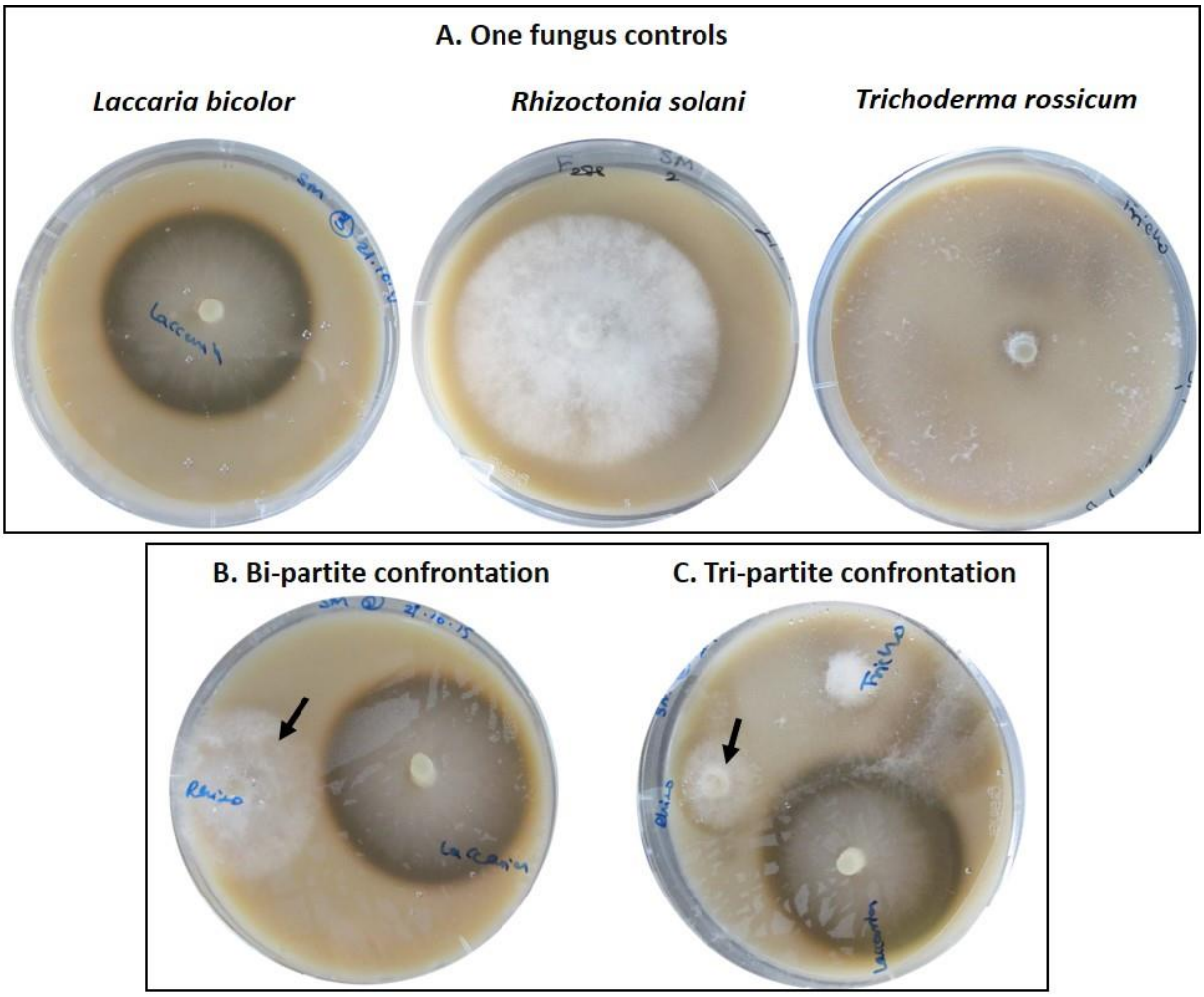

Figure 13: Bi- and tri-partite fungal confrontation assay on skimmed milk malt agar (SM-MA) medium with *Laccaria bicolor, Rhizoctonia solani,* and *Trichoderma rossicum*. (A) *L. bicolor, R. solani* and *T. rossicum* controls; (B) *R. solani* confronted to *L. bicolor,* the black arrow shows the inhibition of *R. solani* growth by *L. bicolor*; and (C) confrontation of *R. solani, L. bicolor*, and *T. rossicum,* the black arrow points to the strong inhibition of *R. solani* growth with the presence of *T. rossicum.*

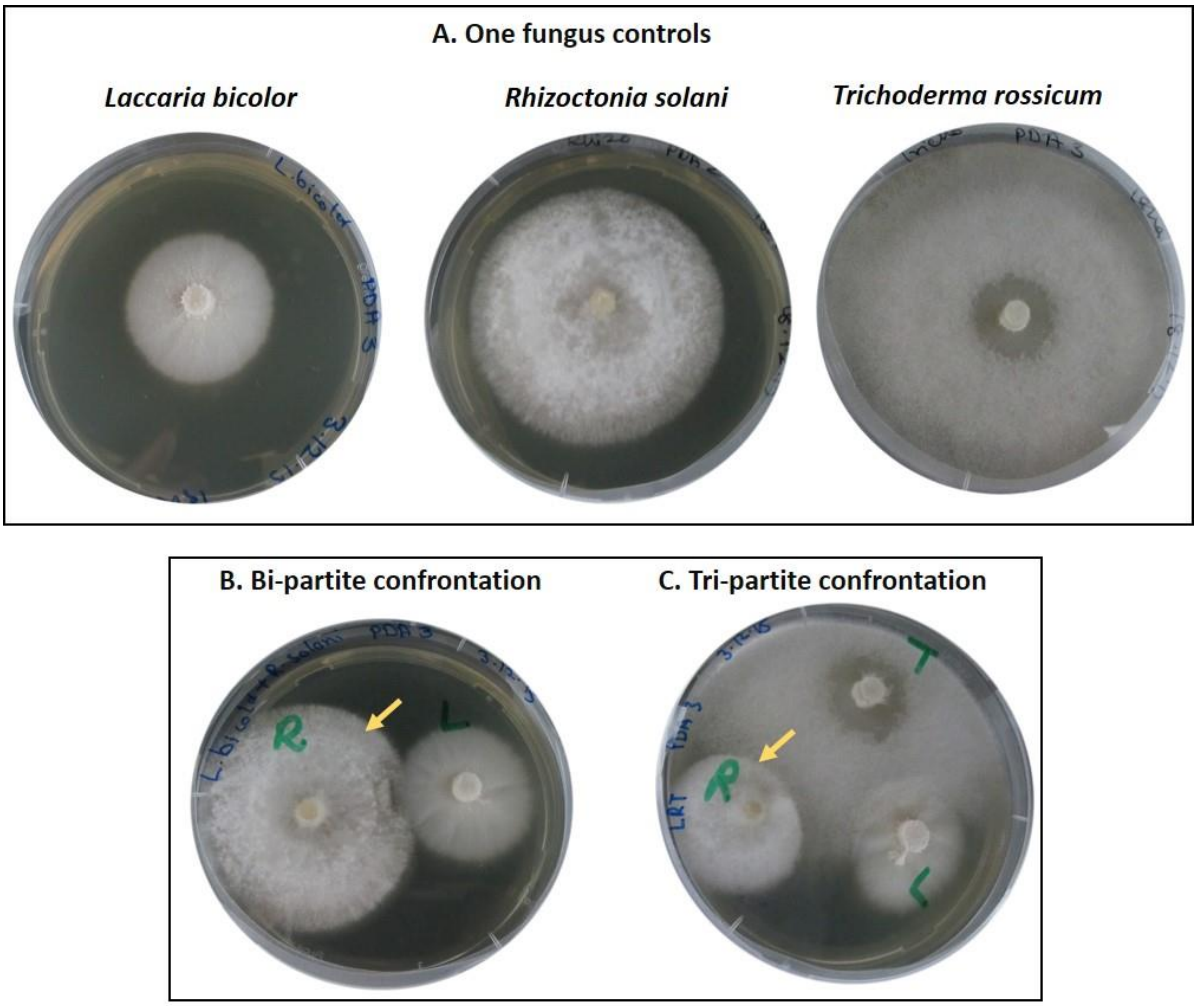

Figure 14: Bi- and tri-partite fungal confrontation assay on PDA medium with *Laccaria bicolor, Rhizoctonia solani,* and *Trichoderma rossicum*. (A) *L. bicolor, R. solani* and *T. rossicum* controls; (B) *R. solani* confronted to *L. bicolor,* the yellow arrow shows the inhibition of *R. solani* growth by *L. bicolor*; and (C) confrontation of *R. solani, L. bicolor*, and *T. rossicum,* the yellow arrow points to the strong inhibition of *R. solani* growth in the presence of *T. rossicum.*

Table 10: Tri-partite fungal confrontation assays using two different media.
Legends: ++ stands for a strong inhibition of the fungus in the second column by the two fungi in the first column; + stands for an inhibition of the fungus in the second column by the two fungi in the first column; - stands for no inhibition of fungus in the second column by the two fungi in the first column.

Fungal partners		Skimmed milk malt agar (SM-MA)	Potato dextrose agar (PDA)
L. bicolor+ T. rossicum	*R. solani*	++	+
	P. ultimum	+	+
B. edulis+ T. rossicum	*R. solani*	+	+
	P. ultimum	-	-

Discussion

Screening of PGP activities

The following plant growth promoting (PGP) traits were assessed in selected bacterial and fungal strains: organic N solubilization, siderophore production, and phosphate solubilization. In addition to this, bacteria were also tested for atmospheric N_2 fixation and auxin-like phytohormones production. Most of the bacterial strains were able to fix N_2, as well as to solubilize organic nitrogen (Norg) through proteolysis and Fe through siderophore production. Additionally, few of them were able to produce auxin-like phytohormones. In the literature, *Bacillus* spp. have been reported to produce different plant hormones, thus helping plants either directly or indirectly by regulating their growth. None of the bacterial strains were able to form a halo around their colonies in NBRIP media, indicating their inability to solubilize phosphate from this medium. However, it has been reported that such a result should not be considered as a sole reliable test for a potential to solubilize P and further experiments should be performed to discard the ability of the strains to solubilize P. For instance, other tests for the solubilization of phosphate can be run in liquid cultures or the production of organic acids could be assessed directly (Nautiyal 1999; Sharma et al. 2013).

Besides bacteria, five fungal or fungal-like species (including a filamentous oomycete, which is ecologically akin to filamentous fungi) of different ecological niches were also screened for PGP traits. All fungi, except the oomycete *P. ultimum*, presented at least two out of the three PGP traits assessed. Both ectomycorrhizal (EcM) fungi had the capacity to utilize Norg. This ability is

well-described in EcM and previous reports showed that EcM possess hydrolytic enzymes as part of their molecular machinery, making them capable of degrading peptide macromolecules. For this reason, EcM have been reported to be effective in mobilizing Norg from organic matter in forest ecosystems (Chalot and Brun 1998). The ability of mobilizing Norg is important for the interaction of EcM fungi with plants. Plants that establish a symbiotic interaction with EcM fungi can obtain N and P from the organic sources exploited by the fungus (Nasholm et al. 2013). Similarly, plants benefit from mycorrhizal associations by accessing insoluble P sources of both inorganic and organic origin. Accordingly, the EcM *L. bicolor* was the only fungus able to solubilize the inorganic calcium phosphate source provided. This fungus is well-known for its ability to acquire nutrient from various sources (Desai et al. 2014; Martin et al. 2008). *B. edulis* is also capable of acquiring P from diverse sources, but this aspect has been less studied than in the case of *L. bicolor*. However, in a recent study with *B. edulis*, it has been demonstrated that P transport seemed to be enhanced in conditions of low P availability (Wang et al. 2014). So, it might be possible that the P content in the test medium was too high to trigger P solubilization. In addition to this, testing EcM fungi in saprophytic conditions probably leads to biases as compared to their metabolic potential when in symbiotic association with plants.

It was observed that not only fungi that traditionally belong to PGP organisms possessed PGP traits. Indeed, *R. solani* a well-known soil borne plant pathogen, was also able to perform proteolysis, which shows its versatile mode of living as a fungus that needs to pass part of its life cycle as a saprophytic microorganism. Additionally, *R. solani* also produced siderophores, a property that is not surprising given the critical role of Fe acquisition for any organism. In addition to this, Fe acquisition is known to be linked to fungal pathogenesis in humans (Hogan et al. 1996), and likely in plant pathogenesis as well. For instance, mutants of the plant pathogenic fungus *Magnaporthe grisea* (responsible for the rice blast disease), lacking the ability to synthesize the siderophore ferricrocin (involved in intracellular Fe storage), were unable to develop disease symptoms in rice, suggesting that siderophores are important for fungal pathogenicity (Expert et al. 2012).

The results of the PGP screening demonstrated that most strains presented at least one PGP trait (Table 1 and 2). But as discussed earlier, this should be considered only as a potential PGP activity.

Indeed, known PGP organisms may respond negatively to a test, while a pathogenic organism may respond positively. For this reason, after *in vitro* screening, further *in planta* experiments are required. In addition to this, PGP traits are often assessed first under ideal laboratory conditions and these conditions are always very different from the natural environment.

Microbial interactions

In terrestrial ecosystems, bacteria and fungi not only interact with plants but also among themselves and their interaction can have significant impact on plant growth. In the one-to-one trials confronting five bacterial strains with contrasting PGP abilities with fungi of different ecological niches, either neutral or antagonistic interactions were established. However, the type of interaction depended on the type of medium, i.e. on the trophic conditions. For instance, in PDA medium, we observed that the two EcM fungi were not inhibited (neutral interaction) by the five bacterial strains selected. On the other hand, the same fungi were slightly inhibited by bacteria in NA and SM-MA media. Therefore, the type of nutrient or the relative quantity of one nutrient over another (such as the C/N ratio) might affect the outcome of the interaction between PGP microbes in the mycorrhizosphere. In a previous study, isolates of *Bacillus* positively affected the development of the EcM fungus *Suillus luteus* and also increased the growth of *Pinus sylvestris*, thus establishing a positive interaction with both, the plant and the EcM fungus (Bending et al. 2002).

In our screening, with the saprophytic fungus *T. rossicum*, most of the selected bacterial strains established a neutral interaction and did not inhibit fungal growth. In addition to this, most bacteria assessed were able to use its network for dispersal. The combined use of *Bacillus* spp. together with *Trichoderma* spp. has been widely investigated as an alternative to agrochemicals for the biocontrol of fungal phytopathogens. Abeysinghe (2009) have investigated the use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 as a combined biocontrol agent against the dumping off disease caused by *R. solani*. They found that bacteria did not inhibit the growth of *T. harzianum* RU01 and that the lowest severity of fungal disease was observed when seeds were coated with *B. subtilis* CA32 and *T. harzianum* RU01 was applied directly to the soil. However, the outcome of the bio-inoculation was not the same when the treatment was applied

the other way around (seeds coated with a conidial suspension of *T. harzianum* RU01 and the bacterial suspension applied directly in the soil). For the second application, disease severity was not reduced as much as in the previous treatment and the effect was the same as for the application containing *T*. *harzianum* RU01 alone. One of the reasons explaining these results could be the limited bacterial dispersal in the soil environment. Indeed, the dispersal of bacteria is limited in soils, due to its unsaturated heterogeneous nature. In such environments, bacteria cannot disperse as efficiently as fungi, which can disperse more readily thanks to their filamentous growth. This disadvantage can be overcome by their dispersal on fungal hyphae, a mechanism coined fungal highways (Dechesne et al. 2010; Kohlmeier et al. 2005). In the study of Abeysinghe (2009), they did not consider the possible dispersal of bacteria via fungal hyphae, nor did they discuss the viability of both fungal strains after interacting with *B. subtilis* CA32. Bravo et al. (2013) observed that the hyphae of *T. rossicum* were used by oxalotrophic bacteria for their active dispersal in soils. In the present study, the tests performed with the same strain showed that indeed most of the *Bacillus* spp. were able to disperse on *T. rossicum* hyphal network grown in NA. Interestingly, it was noticed that the bacteria used fungal highways in many different ways. In some cases, bacteria dispersed all over the fungal network colonizing the medium surface, while in other cases bacteria were only present on one side (either the inner or the outer part as defined in Figure 2 and Table 8) of the Petri dish. Additionally, the viability of the fungi was also determined, and we found that the viability of *P. ultimum* and *T. rossicum* was not affected in confrontation with any of the 15 bacteria. However, with *R. solani,* it was observed that in some cases, the fungus was unable to re-grow after interacting with the bacteria. Indeed, it has been reported that some *Bacillus* species are able to inhibit the growth of phytopathogenic fungi and cause cellular damage (Chérif et al. 2002).

In the current study, besides bacterial dispersal on fungal hyphae and fungal viability upon interaction with bacteria, we also examined the effect of bacterial exudates and volatiles on the growth of *R. solani.* For instance, it is well-known that some *Bacillus* spp. can inhibit the growth of phytopathogens by producing different kinds of anti-fungal volatiles, causing damage to hyphae (Chaurasia et al. 2005; Vespermann et al. 2007). We found that the exudates of the 15 bacteria were not involved in *R. solani* growth inhibition, similarly, our experiments showed that

none of the bacterial strains that inhibited *R. solani* in the confrontation assays did this through volatiles. Therefore, we concluded that the *Bacillus* strains tested might either lack the ability to produce anti-fungal volatiles or use another mechanism to inhibit the fungus.

Along with bacterial-fungal interactions, fungal-fungal interactions also play a vital role in the mycorrhizosphere. For this reason, we examined fungal species of different ecological niches and their interactions to find promising fungal associations that could be used in the frame of sustainable agricultural approaches. In fungal-fungal interaction trials, it was observed that *L. bicolor* along with *T. rossicum* could strongly limit the growth of the phytopathogenic fungus *R. solani*. This *in vitro* result was investigated further in a Master thesis (Kolly 2018) by performing *in planta* experiments using three different cultivars of wheat. This work highlighted that the role of *T. rossicum* as a biocontrol agent was unclear as plants treated with this fungus still showed necrosis symptoms due to *R. solani*. However, to a lesser extent than control plants without *T. rossicum*. Therefore, *T. rossicum* alone does not appear as a good target for bio-inoculation. Moreover, he found that the combined treatment consisting of *L. bicolor* and *T. rossicum* had no significant effect on any of the wheat cultivars. It is worth to mention that in the *in planta* experiments, *L. bicolor* was not used in its ectomycorrhizal form but in its saprophytic lifestyle and thus was probably less competitive in terms of soil colonization and resource acquisition. This provides an interesting outlook to perform further experiments using this fungus in association with plants and to assess its role as a biocontrol of phytopathogenic fungus (*R. solani*). However, further *in planta* studies need to be performed.

Also, it is worth mentioning that the findings of the confrontation assays not only depended on the competing microbes but also on the nutrients and the medium used. For instance, *B. edulis* was able to inhibit the growth of *P. ultimum* on PDA medium, while this was not the case on SM-MA medium. This is something that should be investigated further, as trophic conditions in soils are highly dynamic, both in space and time. Additionally, in all of the screening experiments, either for PGP traits, bacterial-fungal or fungal-fungal confrontations, only qualitative data were recorded. Quantitative studies, both at a cellular level and with plants need to be performed to verify the result obtained with Petri dishes at other relevant scales.

Conclusion

This part of the PhD research led to interesting observations. First, it was concluded that several of the *Bacillus* and fungal strains possessed PGP traits that could be harnessed for their use as bio-fertilizers. Three of these strains are further investigated in Chapter 4 for this aspect. In the bacteria-fungal confrontation assays, promising candidates have been found and for this reason, the interactions between *L. sphaericus* 1003, *R. solani*, and *T. rossicum* were further investigated in Chapter 5. Moreover, *T. rossicum* also established positive interactions with EcM fungi. This interaction with *L. bicolor* was further assessed *in vivo* with wheat plants and led to interesting results (Kolly 2018). As both types of fungi are known for having a significant role in plant health, EcM along with *T. rossicum* (or other *Trichoderma* species) could be used as a promising combination with PGP bacteria to develop new approaches to boost plant health and growth promotion.

References

Abeysinghe, S. (2009), ' Effect of Combined Use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on Biological Control of *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum*.', *Plant Pathology Journal,* 8, 9-16.

Ahemad, Munees and Kibret, Mulugeta (2014), 'Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective', *Journal of King Saud University - Science,* 26 (1), 1-20.

Allison, Franklin. E., Hoover, Sam. R., and Morris, Herman. J. (1934), 'Nitrogen fixation studies with fungi and actinomyces', *Journal of agricultural Research,* 49, 1115-23.

Alori, E. T., Glick, B. R., and Babalola, O. O. (2017), 'Microbial phosphorus solubilization and its potential for use in sustainable agriculture', *Front Microbiol,* 8, 971.

Angle, J. Scott , Mcgrath, Stephen P. , and Chaney, Rufus L. (1991), 'New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution', *Applied and environmental microbiology,* 57 (2), 3674-76.

Arora, Kumar. Naveen., Tiwar, Saskhi., and Singh, Ratan (2014), 'Comparative study of different carriers inoculated with nodule forming and free living plant growth promoting bacteria suitable for sustainable agriculture', *Journal of Plant Pathology & Microbiology,* 05 (02).

Bending, Gary D., et al. (2002), 'Characterisation of bacteria from *Pinus sylvestris–Suillus luteus* mycorrhizas and their effects on root–fungus interactions and plant growth', *FEMS Microbiology Ecology,* 39 (3), 219-27.

Bhattacharyya, P. N. and Jha, D. K. (2012), 'Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture', *World J Microbiol Biotechnol,* 28 (4), 1327-50.

Bravo, Daniel, et al. (2013), 'Isolation of oxalotrophic bacteria able to disperse on fungal mycelium', *FEMS Microbiology Letters,* 348 (2), 157-66.

Bric, John M. , Bostock, Richard M. , and Silverstone, Sara E. (1991), 'Rapid *in Situ* assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane', *Applied and environmental microbiology, ,* 57 (2), 535-38.

Chalot, Michel and Brun, Annick (1998), 'Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas', *FEMS Microbiology Reviews,* 22 (1), 21-44.

Chaurasia, Bhaskar, et al. (2005), 'Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi *in vitro*', *Microbiological Research,* 160 (1), 75-81.

Chérif, M., et al. (2002), 'Ultrastructure and cytochemistry of *in vitro* interactions of the antagonistic bacteria *Bacillus cereus* x16 and *B. thuringiensis* 55t with *Fusarium roseumvar*. sambucinum', *Journal of Plant Pathology & Microbiology,* 84 (2), 83-93.

Dechesne, Arnaud, et al. (2010), 'Hydration-controlled bacterial motility and dispersal on surfaces', *Proceedings of the National Academy of Sciences,* 107 (32), 14369.

Desai, Shalaka, Naik, Dhiraj, and Cumming, Jonathan R. (2014), 'The influence of phosphorus availability and *Laccaria bicolor* symbiosis on phosphate acquisition, antioxidant enzyme activity, and rhizospheric carbon flux in *Populus tremuloides*', *Mycorrhiza,* 24 (5), 369-82.

Ding, Y., et al. (2005), 'Isolation and identification of nitrogen-fixing Bacilli from plant rhizospheres in Beijing region', *J Appl Microbiol,* 99 (5), 1271-81.

Döbereiner, J. (1980), 'Forage grasses and grain crops: In methods for evaluating biological

nitrogen fixation', *New York: John Wiley & Sons Ltd.*, pp. 535-55.
Egamberdieva, D., et al. (2017), 'Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness', *Front Microbiol,* 8, 2104.
Expert, Dominique, Franza, Thierry, and Dellagi, Alia (2012), 'Iron in Plant–Pathogen Interactions', in Dominique Expert and Mark R. O'Brian (eds.), *Molecular Aspects of Iron Metabolism in Pathogenic and Symbiotic Plant-Microbe Associations* (Dordrecht: Springer Netherlands), 7-39.
Fox, A. R., et al. (2016), 'Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X940', *Environ Microbiol,* 18 (10), 3522-34.
Frazier, William C. and Rupp, Philip (1930), 'Studies on the proteolytic bacteria of milk. Action of proteolytic bacteria on milk serum', *Journal of Bacteriology*.
Godsey, Chad B., et al. (2003), 'Correcting iron deficiency in corn with seed row–applied iron sulfate Kansas Agric. Exp. Stn. Contrib. no. 02-110-J', *Agronomy Journal,* 95, 160-66.
Harman, Gary E., et al. (2004), '*Trichoderma* species — opportunistic, avirulent plant symbionts', *Nature Reviews Microbiology,* 2, 43.
Harrison, Kathryn A., Bol, Roland, and Bardgett, Richard D. (2007), 'Preferences for different nitrogen forms by coexisting plant species and soil microbes', *Ecology,* 88 (4), 989-99.
Hodge, Angela, Campbell, Colin D., and Fitter, Alastair H. (2001), 'An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material', *Nature,* 413, 297.
Hogan, L. H., Klein, B. S., and Levitz, S. M. (1996), 'Virulence factors of medically important fungi', *Clinical Microbiology Reviews,* 9 (4), 469.
Igiehon, N. O. and Babalola, O. O. (2018), 'Rhizosphere microbiome modulators: contributions of nitrogen fixing bacteria towards sustainable agriculture', *Int J Environ Res Public Health,* 15 (4).
Junior, J. Q., et al. (2017), 'Nitrogen-fixing bacteria and arbuscular mycorrhizal fungi in Piptadenia gonoacantha (Mart.) Macbr', *Braz J Microbiol,* 48 (1), 95-100.
Kohlmeier, Stefanie, et al. (2005), 'Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi', *Environmental Science & Technology,* 39 (12), 4640-46.
Kolly, Thomas. (2018), 'Biocontrol of *Rhizoctonia solani* by two soil-borne fungi (*Trichoderma rossicum* and *Laccaria bicolor)*: *in vivo* trials on wheat.', *Master thesis, University of Neuchâtel*.
Lohberger, Andrea., et al. (2019), 'Effect of organic carbon and nitrogen on the interactions of soil fungi and bacteria dispersing on fungal mycelium ', *Frontiers in microbiology*.
Martin, F., et al. (2008), 'The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis', *Nature,* 452, 88.
Mwangi, Erastus S.K., Gatebe, Erastus G. , and Ndung'u, Mary W. (2012), 'Impact of nutritional (c: n ratio and source) on growth, oxalate accumulation, and culture ph by *Sclerotinia Sclerotiorum*.', *Journal of Biology, Agriculture and Healthcare,* 2 (10).
Nasholm, T., et al. (2013), 'Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests?', *New Phytol,* 198 (1), 214-21.
Nautiyal, C. Shekhar (1999), 'An efficient microbiological growth medium for screening phosphate solubilizing microorganisms', *FEMS Microbiology Letters,* 170 (1), 265-70.

Paul, Christophe, et al. (2018), 'Bacterial spores, from ecology to biotechnology', *Advances in Applied Microbiology* (Academic Press).

Radzki, W., et al. (2013), 'Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture', *Antonie Van Leeuwenhoek,* 104 (3), 321-30.

Rosenblueth, M., et al. (2018), 'Nitrogen Fixation in Cereals', *Front Microbiol,* 9, 1794.

Schulten, H.-R. and Schnitzer, M. (1997), 'The chemistry of soil organic nitrogen: a review', *Biology and Fertility of Soils,* 26 (1), 1-15.

Schwyn, Bernhard and Neilands, B (1987), 'Universal Chemical Assay for the Detection and Determination of Siderophores', *Analytical biochemistry,* 160.

Seldin, L., Van Elsas, J. D., and Penido, G. C. (1984), '*Bacillus azotofixans* sp. nov. a Nitrogen-Fixing Species from Brazilian Soils and Grass Roots', *International journal of systematic bacteriol*, 451-56.

Seshachala, Usha and Tallapragada, Padmavathi (2012), 'Phosphate Solubilizers from the Rhizosphere of *Piper nigrum* L. in Karnataka, India', *Chilean journal of agricultural research,* 72, 397-403.

Shafi, Jamil., Hui, Tian., and Ji, Mingshan. (2017)*, 'Bacillus s*pecies as versatile weapons for plant pathogens a review', *Biotechnology & Biotechnological Equipment,* 31 (3), 446-59.

Sharma, Seema B., et al. (2013), 'Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils', *SpringerPlus,* 2 (1), 587.

Smith, Sally. E. and Read, David . J (2008), 'Mycorrhizal Symbiosis', *Academic Press,* 3rd Edition.

Souza, Rocheli de, Ambrosini, Adriana , and Passaglia, Luciane M.P. (2015), 'Plant growth-promoting bacteria as inoculants in agricultural soils', *Genet Mol Biol,* 38 (4), 401-19.

Trabelsi, Darine. and Mhamdi, Ridha. (2013), 'Microbial inoculants and their impact on soil microbial communities: a review', *Biomed Res Int,* 2013, 863240.

Vadakattu, G. and Paterson, J. (2006), 'Free-living bacteria lift soil nitrogen supply', *Farming Ahead,* 169.

Vespermann, Anja, Kai, Marco, and Piechulla, Birgit (2007), 'Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*', *Applied and environmental microbiology,* 73 (17), 5639-41.

Wang, Junling, et al. (2014), 'Molecular cloning and functional analysis of a H+-dependent phosphate transporter gene from the ectomycorrhizal fungus *Boletus edulis* in southwest China', *Fungal Biology,* 118 (5), 453-61.

Chapter 4

Comparison of the plant growth promotion performance of a consortium of Bacilli inoculated as endospores or as vegetative cells

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Status: under review in FEMS microbiology ecology

Keywords

Bacterial consortia, *Bacillus thuringiensis*, *Bacillus licheniformis*, *Avena sativa*, plant growth promotion, native microbial communities

Abstract

The effect of three plant growth-promoting *Bacillus* strains inoculated either alone or as a consortium was tested on oat (*Avena sativa)* growth. The bio-inoculants were applied as vegetative cells or endospores at low cell densities on the seeds and their effect were tested in *in vitro* sterile conditions, pot experiments and a field trial. The *in vitro* seed germination assay showed that both individual inocula and consortia had a positive effect on seed germination. Greenhouse pot experiments with sterile and non-sterile soil showed that consortia increased the total dry biomass of oat plants as compared to single strain bio-inoculation and un-inoculated controls. However, the positive impact on plant growth was less prominent when the bio-inoculated strains had to compete with native soil microbes. Finally, the field experiment demonstrated that the vegetative cells consortium was more efficient in promoting oat growth than the endospore consortium and the un-inoculated control. Moreover, the consortia successfully colonized the roots and the rhizosphere of oat plants, without modifying the overall structure of the autochthonous soil microbial communities.

Introduction

Global population growth is putting a tremendous pressure on agriculture because of the decrease in arable land and the concomitant increase in the demand for a reliable food supply. Additionally, inappropriate agricultural techniques, such as intensive exploitation of soil, have considerably reduced soil fertility (Souza et al. 2015). In conventional agriculture, competitive food production is maintained through the continuous use of agrochemicals to enhance soil fertility, crop yield, and to control diseases. However, this has resulted in leaching or runoff of agrochemicals into groundwater, rivers and lakes, creating low oxygen zones where survival of aquatic life is at risk (Muhibbullah et al. 2005). Moreover, direct and indirect exposure to hazardous chemicals has deleterious effects on health (Muhibbullah et al. 2005). Another drawback of agrochemical use is that uptake efficiency by plants decreases over time, requiring regular applications along with increased production costs for farmers. Moreover, for some nutrients, application does not result in higher bioavailability of nutrients. For instance, it has been reported that phosphorous fertilizers precipitate with metal cations and turn into an

insoluble form that is no longer bioavailable to plants (Ahemad and Kibret 2014). Therefore, a change in these practices and innovative ways of conducting sustainable food production are a clear need for the future of agriculture.

Many studies have shown the potential of inoculation with plant growth-promoting (PGP) microorganisms to improve agricultural yield (Ahemad and Kibret 2014; Bhattacharyya and Jha 2012; Souza et al. 2015). More recently, bacterial consortia (mixtures of PGP bacterial strains) have shown to have higher performance as compared to the effect of individual species inoculation (Baez-Rogelio et al. 2017). For instance, a consortium of PGP bacteria induced the production of defense compounds (such as proline, ascorbate peroxidase or catalase) in response to the biotic stress caused by the phytopathogenic fungus *Fusarium oxysporum* (Akhtar et al. 2016). Bacterial consortia were also shown to elicit plant drought tolerance (Wang et al. 2012). In beans, a consortium of *Rhizobium tropici* and two strains of *Paenibacillus polymyxa* increased growth and helped to alleviate the effect of abiotic drought stress as compared to single strain inoculation (Figueiredo et al. 2008).

Bio-inoculation of PGP microorganisms also presents many challenges. One of the critical issues that need to be addressed for application in the field is the survival of bacteria acclimated under laboratory conditions to the harsh conditions in soils. In order to persist in soils, inoculated microorganisms have to compete with autochthonous microbial communities (Souza et al. 2015). Bio-inoculants are also vulnerable to the scarce nutrient availability in soil, as compared to rich nutrient conditions usually provided by growth media, and ultimately a decline in soil in the number of inoculated bacteria is frequently reported (Souza et al. 2015; Trabelsi and Mhamdi 2013). Moreover, the delivery of these microorganisms in an active form is an additional challenge. Application of carrier materials for the protection of bio-inoculants (for instance karnolite, peat, or charcoal) has proven to be environmentally unfriendly, as well as costly, making this approach inapplicable as a general practice in agriculture (Arora et al. 2014). These concerns are the incentive to identify effective bacterial inoculants to be applied in bio-inoculation technology.

Among the currently used PGP bacteria, *Bacillus* is one of the best-studied examples. There are many species of *Bacillus* that are well known as plant growth-promoters (Kumar et al. 2011). One

of the most important characteristics of this genus is that they form endospores, which are a resistant structure that fosters survival. This property can be harnessed in bio-inoculation technologies, as it results in long shelf-life of the product before application. Moreover, *Bacillus* spp., followed by *Enterobacter* spp. and *Pseuodomonas* spp., are the most abundant bacteria with PGP traits in the rhizosphere of plants grown in arid conditions because of their high tolerance to extreme environmental conditions (El-Sayed et al. 2014). *Bacillus* spp. are also used in phytoremediation technologies, increasing biomass production and multi-metal accumulation in plants (Chibuike and Obiora 2014). *Bacillus* spp. can help plants by stimulating their growth through increasing nutrient acquisition (e.g. phosphate solubilization, atmospheric nitrogen fixation, phytohormone and siderophore production (Bhattacharyya and Jha 2012), but also acting as biocontrol agents against various pests (O'Callaghan 2016; Widnyana and Javandira 2016). *Bacillus* spp. can produce a wide range of antiviral, antibacterial and antifungal compounds, which may be important in their interaction with plants and other soil microorganisms. In addition, it has been reported that many species of *Bacillus* induce systemic resistance in plants against a broad spectrum of phytopathogens (Nui et al. 2011)

In this study, a consortium consisting of three *Bacillus* strains was used to promote the growth of oat (*Avena sativa*) plants. The strains were initially tested for physiological traits linked to plant growth-promoting activities. Then, their ability to promote plant growth was assessed in experiments at four levels of complexity (i.e. *in vitro* seed germination under sterile conditions, greenhouse pot experiments with sterile substrate and non-sterile soil, and finally in a field trial). We hypothesized that based on the complementarity of the plant growth-promoting traits of the strains, the consortium will provide a more robust effect on plant growth, as compared to single strain inoculation. This should represent a real advantage when going from controlled laboratory to field conditions, where the inoculated PGP bacteria will compete with the autochthonous microbial community. Additionally, we also compared two forms of bacterial inocula: endospores and vegetative cells, to assess whether dormancy alters the functionality of the consortium. Finally, we investigated the effect of bio-inoculation on native bacterial and fungal communities in the field assay. We hypothesized that a consortium applied directly onto the seeds at low cell densities (ca. 10^3 cells per oat seed), should not affect the indigenous soil microbial communities.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains *B. thuringiensis* 1312 (BT1) and *B. thuringiensis* 1310 (BT2) were isolated from soils of the Atacama Desert, Chile, during a sampling campaign carried out by the laboratory of microbiology, University of Neuchâtel in 2011. *B. licheniformis* (BL) was isolated from soil at Agroscope Liebefeld, Bern, Switzerland. The strains were cryopreserved in 60% glycerol and when required for the experiment, the strains were pre-cultured in nutrient agar (NA) media.

Physiological characterization of bacterial strains

Dinitrogen fixation assay

A loop of an overnight bacterial culture on NA was streaked onto a nitrogen-free medium (Döbereiner 1980), which was then incubated at 30°C for 48-72 h. Bacteria were cultured over three generations on the nitrogen-free medium and the capacity to fix N_2 was assessed by the growth of bacterial strains in the third-generation plates.

Casein solubilization (Proteolysis)

Casein solubilization was assessed based on a modified protocol from (Frazier and Rupp 1930). This assay was used as a proxy for the ability of the selected *Bacillus* strains to solubilize organic nitrogen from protein origin. The medium was composed of 5% skimmed milk, 1.2% Malt extract powder and 1.5% agar. A loop of an overnight grown bacterial culture on NA was streaked onto the casein-solubilizing medium. The plates were incubated for 48-72 h at 30°C. A transparent halo observed around a bacterial colony indicated that the strain was able to solubilize casein.

Siderophore production assay

To assess the ability of the three bacterial strains to chelate iron, a siderophore production test was performed as described in (Schwyn and Neilands 1987). Briefly, the initial medium has a blue coloration resulting from the complexation of ferric iron with chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA). When a strong iron chelator (such as a siderophore) removes iron from the dye complex, the color of the medium turns to yellow. A loop of an overnight bacterial culture on NA was inoculated onto the medium. The plates were

incubated at 30°C and a change of color was assessed after 48-72 h.

Auxin-like phytohormone production assay

To check for the production of auxin-like compounds, a modified version of the protocol proposed by (Bric et al. 1991) was used. In this assay, Angle medium (Angle et al. 1991) was supplemented with 5 mM Tryptophane, as a precursor for auxin biosynthesis. The test was performed in 96-well microplates with 270 µL of medium per well. A loop of an overnight bacterial culture from NA medium was inoculated in the three microplate wells for each corresponding bacterial strain. The microplate was then incubated at 25°C for 72 h in the dark. After 72 h, one drop of Salkowski's reagent was added to each well and placed in dark for 45 min. A pink to red coloration indicates the production of auxin-like compounds.

Bacterial compatibility

Compatibility of each strains to constitute a consortium was tested by growing them on a favorable medium (NA). Each strain was inoculated at equidistance (2 cm) in the Petri dish and incubated at 30°C for 72 h. Signs for any antagonistic interaction among strains were visually assessed.

Preparation of bacterial inoculants

Seed bio-inoculation with the three selected bacterial strains was performed using either vegetative cells or endospores, and both either as individual cells or as a consortium.

Vegetative cells treatment

Pure cultures of the three selected bacterial strains on NA were used to individually inoculate 50 mL of nutrient broth (NB) medium which was further incubated overnight at 30°C and 150 rpm (HT, Infors AG). An overnight bacterial culture of each strain was transferred into a sterile falcon tube and centrifuged (Sigma 2-16PK) at 2150 g for 3 min. The resulting pellet was washed five times with sterile physiological water. After this, the optical density of each bacterial suspension was measured at 550 nm (spectrophotometer, Genesys 10S UV-VIS) and adjusted to reach a final concentration of 10^6 colony forming units CFU mL^{-1}. The resulting suspension of vegetative cells

were used for experiments with plants (*in vitro* assay of seed germination, greenhouse pot experiments and field experiment). This resulted in four different treatments for experiments with vegetative cells: VBT1= *B. thuringiensis* 1312, VBT2= *B. thuringiensis* 1310, VBL= *B. licheniformis* and vegetative cells consortium= VM.

Endospore treatment

A synthetic medium was used to induce sporulation of the bacterial strains (sporulation medium) (Donnellan et al. 1964). Each bacterial strain was refreshed in 50 mL NB. Overnight bacterial cultures were centrifuged (Sigma 2-16PK) at 7673 g for 3 min. The resulting pellet was washed five times with physiological water. At the end, the pellet of each bacterial strain was suspended in 100 mL of sporulation medium and incubated for 3 weeks at 30°C and 200 rpm. After 3 weeks, endospore formation was verified by phase contrast microscopy. Vegetative cells observed in the suspension were killed by a heat shock at 70°C for 15 min for *B. thuringiensis* 1312 (BT1) and *B. thuringiensis* 1310 (BT2) and at 80°C for 10 min for *B. licheniformis* (BL). Afterwards, endospores of each bacterial strain were collected by centrifugation at 7673 g for 10 min. The resulting pellet was suspended in 2 mL of sterile distilled water. The quantities of spores were estimated using the Neubauer Chamber cell counting method and dilutions were made and adjusted to reach a final concentration of 10^5 spores mL^{-1}. The endospore suspensions were then used in the *in vitro* assay of seed germination, greenhouse pot experiments, as well as in the field experiment. This resulted in four different treatments for experiments with endospores: SBT1= *B. thuringiensis* 1312, SBT2= *B. thuringiensis* 1310, SBL= *B. licheniformis,* and endospore consortium= SM.

Seed inoculation with bacterial inoculants

Oat seeds (*Avena sativa*) were soaked in 5% sodium hypochlorite (NaClO) solution for 2 min and then thoroughly rinsed five times with sterile distilled water. Sterilized seeds were incubated for 30 min and placed on a shaker at 150 rpm in the eight respective bacterial treatment suspensions (i.e. VBT1, VBT2, VBL, VM, SBT1, SBT2, SBL and SM) under sterile conditions in order to allow for the cells or spores to adhere to the seeds. Control treatment consisted in surface sterilized seeds treated in the same way, but using sterile distilled water instead of bacterial suspensions.

Bacterial adhesion onto seeds

To assess bacterial adhesion onto seeds (each of the eight different treatments and the control), three seeds were randomly sampled in order to count bacterial cells or endospores adhering at their surface by flow cytometry. Seeds of each individual treatment were placed in sterile Eppendorf tubes containing 2 mL of hexa-meta-phosphate $(NaPO_3)_6$ and shaken vigorously with a vortex mixer for few sec. For the vegetative cell treatment, 10 µL of SYBR Green was added to fluorescently label the cells. This treatment was not applied for endospore treatments because SYBR Green is unable to stain dormant cells (Zheng et al. 2017).

In addition to flow cytometry, scanning electron microscopy (SEM) was used to assess adhesion of vegetative cells or endospores onto the seeds. Un-inoculated control seeds and seeds treated with both consortia (vegetative cells or endospores) were prefixed in 2.5% glutaraldehyde for 1 h followed by washing twice with sterile distilled water. Secondary fixation was carried out by using 4% osmium tetroxide (OsO_4) for 1 h, followed by two washing steps with sterile distilled water. Then, stepwise dehydration in graded alcohol was carried out by incubating samples for 15 min at each of the following ethanol concentrations (50%, 75%, 90% and 100%). After this, the samples were dipped in tetramethysilane ($C_4H_{12}Si$; TMS) for 20 min with a second step of 1 h until complete TMS evaporation. The samples were mounted onto SEM stubs covered with graphite and coated with gold with a sputter coater (Bal-Tec sputter coater SCD 005). The seeds were then examined using high-vacuum SEM (Philips ESEM XL30 FEG) at an acceleration of 10 KeV and a working distance of about 8.6 mm.

In vitro seed germination assay

In order to determine the effect of the different bacterial treatments on oat seed germination an *in vitro* assay was performed. Five seeds inoculated with each of the eight bacterial treatments were grown aseptically on sterile filter paper. Filter papers were kept moist with sterile distilled water and incubated at 22°C in the dark. Un-inoculated seeds were used as control. Experiments were replicated 5 times such as 25 seeds were eventually assessed for each treatment. After 10 days, the number of germinating seeds was counted, and percentage of seed germination was calculated based on the number of seeds that germinated over the total number of seeds placed in Petri dishes.

Greenhouse pot experiments

To study the effect of each of the eight bacterial treatments (i.e. VBT1, VBT2, VBL, VM, SBT1, SBT2, SBL and SM) on growth of oat plants in soil, pot experiments were conducted in a greenhouse located at the University of Neuchâtel, Switzerland. Two different experiments were carried out, either with a sterile substrate or a non-sterile soil, in order to compare plant growth-promoting activities of the different type of inoculation (single inocula versus consortium as well as vegetative cells versus endospores). This approach was used to observe how the inoculants perform in a simple (sterile substrate) to a more complex (non-sterile soil) environment. The sterile substrate was composed of 25% peat, 25% Seramis® Pflanz-granulat (clay granules) and 50% sand (mixed carbonate and silicate). A thin layer of this substrate was sterilized by five successive autoclave cycles. Sterile substrate was then added in sterile pots (122 g in 130 mL pots). For the non-sterile treatment, soil was collected from a grassland nearby the University of Neuchâtel, Switzerland and corresponded to a calcaric Cambisol (WRB 2015). Pots corresponding to nine treatments (eight bacterial treatments and a control un-inoculated treatment) were arranged randomly with seven and ten replicates for the sterile artificial and non-sterile soil, respectively. Pots were irrigated regularly with 0.5 X concentrated Murashige and Skoog (MS) solution for the sterile artificial soil and tap water for the non-sterile natural soil. Plants from each individual treatment were harvested after 45 days. Plant consisting of shoots, roots, flowers, and seeds were dried at 60°C for 72 h and their total dry weight determined by using a fine-scale balance.

Field experiment

To study the effect of both consortia, composed of either vegetative cells or endospores, on oat plants growth, a field experiment was conducted at a designated research field of the University of Neuchâtel, Switzerland. This was carried out in collaboration with the GRAMU association (Groupe d'Aménagement de l'Unine) involved in various projects related to sustainable agriculture and permaculture. Based on the results of the greenhouse pot experiments, we selected only three treatments for the field experiment, i.e. un-inoculated control, vegetative cells consortium (VM) and endospores consortium (SM). These three treatments were applied randomly in the field and three replicates were made for each treatment (Figure S1). The total

area of the field was 5 x 1.35 m, which was subdivided into nine plots to get three replicates of each treatment. Each plot consisted of a length of 40 cm with a consecutive gap of 18 cm in which no treatment was applied. Seed inoculated with bacteria were sprinkled in each sub-divided plot. Plots were irrigated regularly and weeds that grew in the plots were manually removed. Plants (consisting of shoots, roots and seeds) were harvested after 85 days and dried at 60°C for 72 h. The total dry weight of the plants was measured using a fine-scale balance and the number of seeds per plant counted. Physicochemical analyses (pH, water content and water holding capacity, concentrations in bioavailable and total phosphorus, nitrates, ammonium, organic carbon as well as calcium carbonate) were carried out on the soil of the field experiment to characterize its fertility. This was carried out before and after the experiment for each of the three treatments (Control, VM and SM; Table S1).

DNA extraction

In order to study the effect of both consortia on native bacterial and fungal communities of the field experiment, different soil samples were compared. To this end, bulk soil before sowing and after harvesting, rhizospheric soil that was adhering to oat roots, as well as oat roots samples were collected for molecular biology analyses. Roots were washed with hexa-metaphosphate ($(NaPO_3)_6$) to remove rhizospheric soil. DNA was obtained from 3 g of soil and 0.5 g of root samples. Fungal DNA was obtained by following the protocol of the FastDNA®SPIN kit for soil (MP Biomedicals, USA; direct DNA extraction). Bacterial DNA was obtained using an indirect DNA extraction method previously developed to enhance the extraction of resistant structures such as endospores (Wunderlin et al., 2013). This method consists in an initial pre-extraction from the environmental samples (indirect method) followed by a modified DNA extraction protocol with the FastDNA®SPIN kit for soil (MP Biomedicals, USA). 2 x 10 mL of $(NaPO_3)_6$ (1%) was used instead of 2 x 15mL for the initial pre-extraction step. DNA concentrations were measured with a Qubit Fluorometer using the dsDNA HS Assay Kit (Invitrogen, California). The concentration of all samples was adjusted to 2 ng μL^{-1} by diluting with double distilled sterile water.

Microbial community analyses

Amplicon sequencing was performed on an Illumina MiSeq platform, using the services of Fasteris SA (Switzerland). For the bacterial community analysis, the regions V3-V4 of the 16S rRNA gene were targeted using universal primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). For the fungal community analysis, the 18S rRNA gene was targeted using the primer pair AMV4.5NF (5'-AAGCTCGTAGTTGAATTTCG-3') and AMDGR (5'-CCCAACTATCCCTATTAATCAT-3'), which have been specifically designed for the analysis of Arbuscular mycorrhizal fungal communities (AMF; Glomeromycota) (Sato et al., 2005). The Illumina reads were processed with Mothur (Schloss et al., 2009) following the MiSeq standard procedure (Kozich et al., 2013), and using the SILVA NR v123 (16S rRNA) and SILVA NR v132 (18S rRNA) reference databases (Quast et al., 2013) for the alignment of amplicons and the taxonomic assignment of representative OTUs. Chimeras and singletons were removed prior to OTUs clustering. For the 16S rRNA sequencing, average neighbor clustering of the 535919 retained sequences (79430 unique sequences) at 97% identity led to the identification of 6475 OTUs. For the 18S rRNA sequencing, clustering using the opticlust algorithm (Westcott & Schloss, 2017) of the 7978155 retained sequences (54754 unique sequences) at 97% identity led to the identification of 11466 OTUs.

Although the primers used for analyzing the fungal community were specifically designed for the analysis of AMF (Sato et al. 2005), sequences assigned to other Divisions were also found to be highly represented, mostly in the Basidiomycota (Figure S1). This contrasts with a previous study evaluating the potential of using these primers for targeting the AMF community in root samples, which showed a high specificity of these primers (Van Geel et al., 2014). The differences in the soil compartments analyzed (bulk soil, rhizosphere soil and roots in the present study) and the use of different sequencing technologies with different sequencing depths (Miseq VS 454 pyrosequencing) might partly explain these results. Sequences were submitted to GenBank under the Bioproject accession numbers PRJNA472865 for bacterial communities and XXX (under process) for fungal communities.

Statistical analysis

All statistical analyses were computed using R (version 3.4.3) (R Core Team 2014). Plant data from the greenhouse pots and field experiments were analyzed for statistical significance ($p < 0.05$) using one-way ANOVA (analysis of variance) followed by a Tukey's post hoc test. Comparisons of *in vitro* seed germination were analyzed with binomial test followed by Tukey's post hoc test. Bacterial and fungal community analyses were performed using *phyloseq* and *vegan* packages (McMurdie and Holmes 2013; Oksanen et al. 2017). Bacterial and fungal communities were analyzed by principal coordinates analysis (PCoA), based on Bray-Curtis dissimilarity and Hellinger transformation of the OTU table. OTUs accounting for less than 4 reads in the whole dataset were removed prior to the analysis.

Results

Screening of plant growth-promoting activities of the three bacterial strains

The screening for plant growth-promoting (PGP) traits of the three bacterial strains showed that the three strains were positive for all tested traits. All three strains were able to grow on nitrogen-free medium, showing their ability to fix atmospheric nitrogen. They were also able to utilize organic nitrogen, as demonstrated by their ability to solubilize casein. Moreover, all of them produced siderophores and auxin-like phytohormone compounds. Furthermore, the three stains were able to grow as a consortium as it was observed that the individual growth of each strain was not affected when combined as a co-culture (Figure S2).

Assessment of bacterial inoculants adhesion onto seeds

The attachment of bacterial cells onto the seeds was quantitatively measured by flow cytometry. The number of cells attached per seed was in the range of 10^3 cells/seed (Figure 1A). A slightly higher number of cells was observed on seeds treated with vegetative cells as compared to endospores, with the exception of the endospore consortium. The number of cells in seeds treated with the vegetative cells (VM) or endospores (SM) consortia was also assessed qualitatively by scanning electron microscopy (SEM) observation (Figure 1B and 1C). The number of bacterial cells attached to the seeds appeared higher for the treatment with vegetative cells compared to the seeds treated with endospores (Table S2). No bacterial cells were observed on

the untreated control seeds.

In vitro seed germination assay

The *in vitro* seed germination assay showed that all three bacterial strains enhanced seed germination. The individual vegetative cells inoculum of *B. thuringiensis* 1312 (VBT1) had the highest effect on seed germination (92%), which was significantly (adj $p < 0.05$) different from the control (52% of seed germination) and other treatments with vegetative cells (Figure 2A; Table S3). The vegetative cells consortium (VM) also induced higher germination than the untreated control (72% seed germination), although this difference was not statistically significant. In seeds treated with endospores, a trend of an increase in seed germination was observed in all treatments. However, only the treatment with *B. thuringiensis* 1310 (SBT2; 92%) and the consortium (96%) were significantly different from the control (Figure 2B; Table S3). Overall, the percentage of seed germinating increased with both individual and consortia inoculations as compared to the un-inoculated control treatment.

Greenhouse pot experiments

The effect of individual strains on the total dry weight of oat plants was not significantly different from the untreated control treatment, whether the bacteria were individually delivered as vegetative cells or as endospores. This result was consistent in experiments using a sterile substrate (Figure 3A) and a non-sterile soil (Figure 3B). In contrast, inoculation with the consortia of the three selected *Bacillus* strains had a positive effect on plant growth, and this was the case for both the vegetative cells and endospores consortia. However, this effect was less prominent in the experiments with non-sterile soil as compared to the sterile substrate (Figure 3). Inoculation with consortia showed a significant increase (adj $p<0.05$; Table S3) in total dry weight of oat plants compared to un-inoculated control plants. No significant difference was observed when comparing the type of consortium (vegetative cells or endospores; Figure 3; Table S3).

Field experiment

Given the results obtained in the greenhouse experiments, only consortia were evaluated in the field experiment. The plants inoculated with the vegetative cells consortium (VM) showed a

significant increase in total dry weight compared to the un-inoculated control (Figure 4A). The number of seeds produced was also significantly enhanced in the plants treated with the VM consortium compared to the un-inoculated control (Figure 4B). Inoculation with the vegetative cells consortium (VM) did not show a significantly higher beneficial effect on plant growth-promotion compared to the endospores consortium (SM).

Microbial community analyses

The effect of bio-inoculation with both consortia on the autochthonous bacterial and fungal communities in different soil compartments (pre-sowing and post-harvest bulk soil, rhizospheric soil and roots) was evaluated. Principal coordinate analyses (PCoA) computed from the bacterial communities (Figure 5) exhibited four significant axes according to the Kaiser–Guttman criterion, while the broken stick model retained only the first axis, indicating that axis 2 of the PCoA must be interpreted with caution. Axis 1 of the PCoA, explaining 34.4% of the variance, clearly separated two groups of samples: root from soil (Figure 5). Based on the analysis, bacterial communities associated with oat plant roots were clearly different from those in the rhizospheric and in the bulk soils (pre-sowing and post-harvest). Samples from the pre-sown bulk soil grouped together, indicating that the community composition was homogenous at the beginning of the experiment. Although there was some variability between samples from the rhizospheric and the post-harvest bulk soils (illustrated by their arrangement along the axis 2), there was no clear separation between samples from both soil compartments. Comparing the three treatments (i.e. untreated control, inoculation with vegetative cells (VM) or endospore (SM) consortia), no prominent difference was observed regardless of the soil compartment considered. The relative abundance of OTUs assigned to *Bacillus* spp. was also analyzed (Figure 6). OTUs affiliated to *Bacillus* spp. represented only a minor fraction of the community (3-6%). The highest relative abundance was observed in the case of the rhizosphere of VM-treated plants (Figure 6). A BLAST search with the OTUs corresponding to *Bacillus*, showed that all of them were between 96-100% identical to *B. thuringiensis* or *B. licheniformis* (Table S5).

Analysis of the fungal communities yielded a similar trend to the one observed for the bacterial communities. Only the first axis was significant based on the broken stick model, compared to 11

axes determined using the Kaiser-Guttman criterion. The axis 1 of the PCoA, explaining 22.7% of the variance, separated the two same groups of community components: root-associated fungi versus soil-associated fungi (including rhizospheric soil; Figure 7). Similar to the bacterial communities, fungal communities of the pre-sown bulk soil appeared homogenous. Samples from the rhizospheric and post-harvest bulk soils did not show clear separation according to the soil compartment. Although PCoA tended to group samples belonging to the same soil compartment, some samples fell out of these groups. Moreover, no clear distinction was made based on the different treatments (i.e. untreated control, inoculation with vegetative cells (VM) or endospore (SM) consortia).

Discussion

In this study, a consortium of three *Bacillus* strains showed plant growth-promoting activities *in vitro* and were as well effective in growth promotion of oat plants in pots (sterile substrate and non-sterile soils) and in the field. Inoculation of oat seeds with single strains or consortia of three bacteria increased germination rate as compared to un-inoculated seeds. Therefore, direct seed coating seems to have beneficial effects on seed germination. This could be due to the ability of the selected strains to produce auxin-like hormones. There are many reports showing that *Bacillus* spp. are able to enhance plant growth by producing different plant growth hormones such as gibberellins, indole acetic acid (IAA) and cytokinins (Arkhipova et al. 2005; Radhakrishnan and Lee 2016). It has also been reported that some *Bacillus* spp. were able to produce more than one growth hormone, with a beneficial effect on the overall physiology of plants (Kumar et al. 2011).

In the current study, we choose seed inoculation over foliar spray because the seed inoculation introduces the required inoculants directly in the spermosphere and then into the soil at the vicinity of the emerging roots. When the roots grow, the beneficial bacteria are already present to stimulate plant growth. It has been reported that bio-inoculation of seeds reduces seed-borne diseases caused by soil phytopathogens (O'Callaghan 2016). Furthermore, according to the findings of Ciccillo et al. (2002), the application method and density of the bacterial inoculants play a vital role on plant growth promotion. For instance, different concentrations (10^6, 10^7 and

10^8 CFU g^{-1} soil) of the plant growth promoting strain *Burkholderia ambifaria* MCI 7 incorporated into the soil had a negative effect on plant growth, with the highest reduction in plant biomass observed at 10^8 CFU g^{-1} soil. Conversely, in the same study, the authors observed that when the same plant growth promoting strain was directly applied onto seeds at similar concentrations (10^6, 10^7 and 10^8 CFU per seed), a significant positive effect on the growth of maize plants was observed. However, differences in the bacterial inoculum concentration was not a significant factor when comparing the effect on plant biomass (Ciccillo et al. 2002). In our study, we found that oat seeds carried approximately 10^3 cells of bio-inoculated bacteria, which represents a rather low cell density. Nonetheless, even at such a density, a positive effect towards oat growth was observed. This result is in accordance with a previous study where an inoculum of *Bacillus amyloliquefaciens* BNM122B was used for the seed coating of soybean against the plant pathogen *Rhizoctonia solani* (Correa et al. 2009). In that experiment, the initial concentration of the bacterial suspension was 10^8 CFU mL^{-1} and the final concentration of bacterial cells attached per seed was 10^4 CFU, showing that even a low concentrated bacterial inoculum can lead to the desired outcome.

One of the most significant results obtained in the present study is that consortia had a more robust effect on oat biomass than inoculation with individual strains. Similar observations were also obtained in other studies dealing with combined bioinoculants. For instance, a consortium of two *Bacillus* strains and *Enterobacter cloacae* increased tomato seedling growth and helped in disease suppression (Abdeljalil and Renault 2016). Moreover, it has been reported that inoculation with a consortium of *Rhizobium tropici* and two different strains of *Paenibacillus polymyxa* elicits plant growth in presence of an abiotic stress (drought), in contrast to inoculation with *Rhizobium tropici* alone (Figueiredo et al. 2008). Finally, (Jetiyanon 2007) showed that a consortium of *Bacillus* spp. alleviated biotic stress caused by phytopathogens by producing defense-related enzymes.

In our study, we found that the bacterial inoculum applied in the form of vegetative cells had a higher effect in terms of total dry weight and grain yield of oat plants, as compared to the inoculum containing endospores, even though the effect of bio-inoculation with endospore was still superior to the results from the un-treated control plants. In a study comparing inoculation

with vegetative cells and endospores of *Bacillus subtilis* EA-CB0575 (at concentration of 1×10^7 and 1×10^8 CFU mL^{-1}, respectively), both the vegetative cells and endospores, enhanced total dry weight of banana plants as compared to un-treated controls (Posada et al. 2016). This suggest that both cellular forms can be equally used for bio-inoculation technology.

Besides assessing the beneficial effect of bio-inoculation on plant growth, another aspect often neglected in studies dealing with the use of bio-inoculants is the impact of this practice on the indigenous soil microflora (Korir et al. 2017; Kuan et al. 2016; Moustaine et al. 2017; Noumavo et al. 2013; Orhan et al. 2006; Widnyana and Javandira 2016). Investigating whether the applied consortium has an impact on the structure of the native microbial community is crucial for allowing further usage of the inoculum in replacement of agrochemicals in sustainable agricultural ecosystems. It is important to consider that bio-inoculants could potentially have a major impact on the long-term functional capabilities of the resident microbial communities by changing their overall structure (Trabelsi and Mhamdi 2013). Several studies have reported that bacterial inoculants alter the bacterial community composition after inoculation (García de Salamone et al. 2012; Schwieger and Tebbe 2000; Trabelsi et al. 2012). Although a previous study has shown that the plant growth promoting rhizobacteria *Bacillus* sp. SUT1 and *Pseudomonas* sp. SUT19 did not alter the native fungal communities of Chinese broccoli (Piromyou et al. 2013), more recently, it was reported that inoculation of three *Bacillus* strains (*B*. *cereus*, *B*. *subtilis*, and *B*. *amyloliquefaciens*) changed the endosphere bacterial communities of broccoli (Gadhave et al. 2018). In some cases, drastic shifts were observed, for instance, two biocontrol agents *Pseudomonas corrugate* IDV1 and *Pseudomonas putida* RA2, changed the composition of the autochthonous bacterial community from a Gram-positive- to a Gram-negative-dominated population in the rhizosphere of maize (Kozdrój et al. 2004). Such effects should not be ignored. One of the main factors explaining drastic changes in the resident bacterial community could be the high cell densities (up to 10^9 CFU mL^{-1}) used in bio-inoculation experiments (Kozdrój et al. 2004). In our study we found that using a low bacterial cell density (10^3 cells per seeds) for the direct application of the inoculant onto seeds effectively increased the growth of oat plants, with no drastic effect on the structure of the native bacterial and fungal communities (Figure 5 and 7). The *Bacillus* spp. fraction remained a minor fraction of the bacterial community even after the

experiment and some of these were likely the bio-inoculated strains as suggested by the outcome of the BLAST search. This again demonstrates that the bio-inoculated strains were able to colonize the rhizosphere of oat plants, but not to outcompete native bacteria.

Noteworthy, the bacterial and fungal communities associated to the roots were found to be different from the communities of the other soil compartments (bulk and rhizospheric soils), showing that oat roots had a unique root-associated community. This is in accordance with previous studies investigating the differences in the microbiome of roots and other soil compartments. This was true for the bacterial microbiomes of the roots and the surrounding soil of maize (Niu et al. 2017). Similar observations regarding fungi were reported by (Urbina et al. 2018) in a study comparing roots, rhizospheric and bulk soil fungal communities. Finally, both fungal and bacterial communities associated to *Populus* differed between roots and rhizospheric soil (Gottel et al. 2011).

Conclusion

In summary, a consortium of three *Bacillus* strains with plant growth promoting activities positively affected oat seed germination and plant growth. This effect was less prominent in non-sterile soil as compared to a sterile artificial substrate, highlighting the importance of the competition with the native microbial communities and demonstrating that consortia are more robust than single strain inocula. In sterile conditions (*in vitro* seed germination and pot experiment with sterile substrate) both vegetative cells and endospores performed similarly. Conversely, in non-sterile conditions (pot experiment with non-sterile soil and field trial), vegetative cells showed slightly higher performances in promoting plant growth. Moreover, we also highlighted that direct seed inoculation with consortia at low cell density did not lead to an alteration of the indigenous bacterial and fungal communities. Indeed, it appeared that the bio-inoculated strains actually established in the rhizosphere of oat plants, but without a major effect on native microbial communities. This is of particular importance since a change in the indigenous communities induced by the inoculation could lead to unpredictable and undesirable effects on plant and ecosystem health. Therefore, we conclude that a consortium of three different *Bacillus* strains could be used as a low-cost and effective alternative for improving growth of oat plants.

Finally, even though vegetative cells performed slightly better than endospores, both type of cells may be used to promote plant growth. This study may be helpful in the field of sustainable agriculture and could lead to more effective bacterial inoculant for promoting plant growth and health. Indeed, this study helps expanding our knowledge on microbial consortium and how they interact with plants and soil native microbial communities. This promotes a deeper and more comprehensive understanding of the factors required for the development and the formulation of next-generation bacterial inoculants to be used in the frame of a sustainable exploitation of agricultural ecosystems.

Acknowledgements

This work was supported by: Swiss Government Excellence Scholarships for Foreign Scholars (number 2014.0865 to IH), University of Neuchâtel SNF overheads (grant number 11.1 to SB), the "Fondation Pierre Mercier pour la Science" to SB, and the U.S. Department of Energy Biological and Environmental Research Division through a Science Focus Area grant (Grant number KP1601010 to PJ).

Competing interest statement

The authors declare no competing interests.

Figures

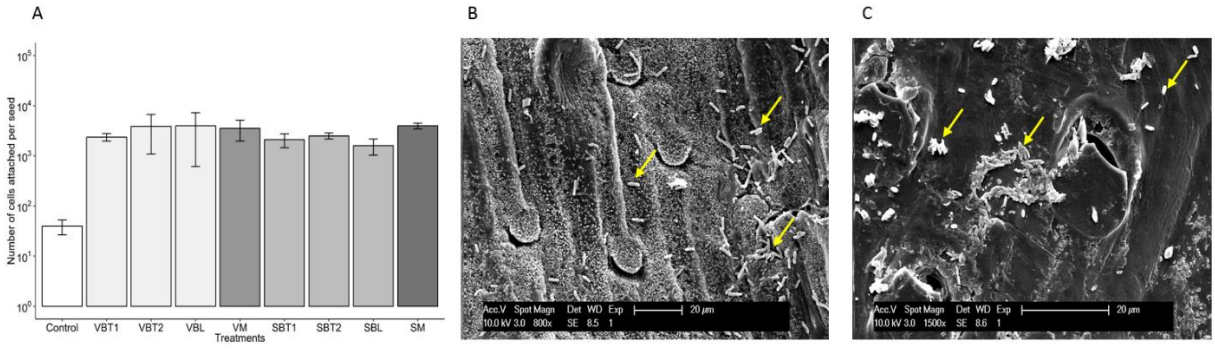

Figure 1: Assessment of the number of cells or spores attaching to *A. sativa* seeds after bio-inoculation. (A) Number of vegetative cells or endospores attached per seed measured by flow cytometry (Control = Un-inoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312; BT2 = *B. thuringiensis* 1310; BL= *B. licheniformis*; Consortium= M). (B-C) Scanning electron microscopy images of seeds bio-inoculated with either (B) the consortium of vegetative cells or (C) the consortium of endospores. Arrows show bacterial cells or spores attached onto the seeds

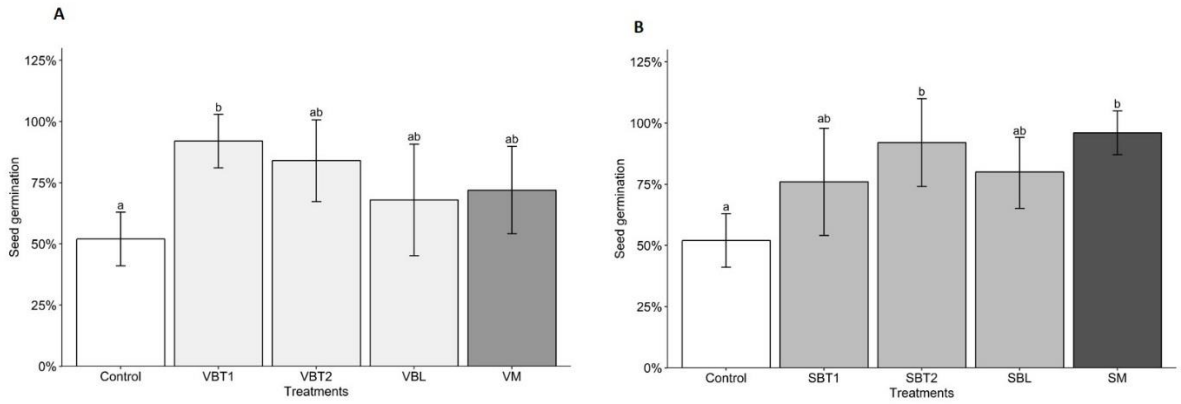

Figure 2: Percentage of *A. sativa* seed germination comparing the effect of seeds treated with individual inocula and consortia of both (A) vegetative cells and (B) endospores. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's post hoc test (n=5). Control = Un-inoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312; BT2 = *B. thuringiensis* 1310; BL= *B. licheniformis*; consortium= M.

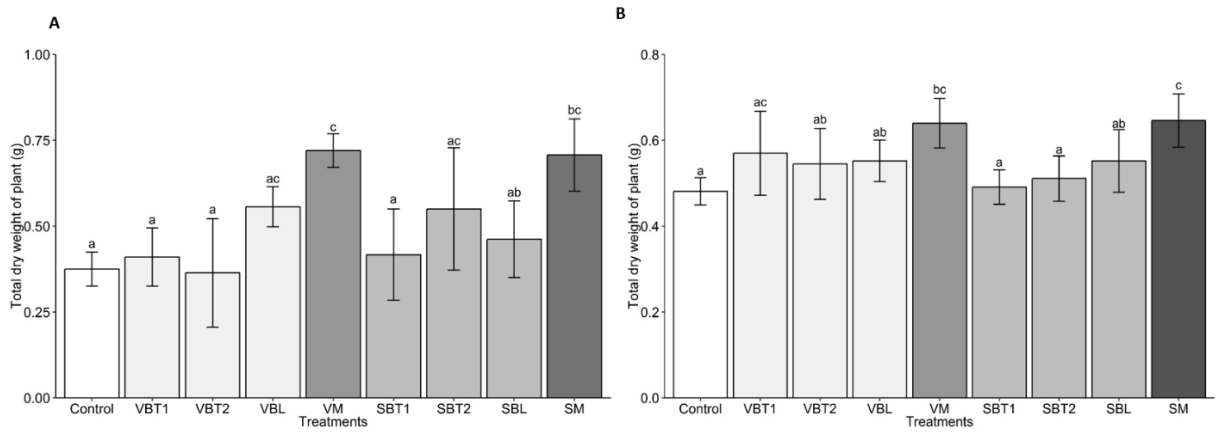

Figure 3: Effects of individual inocula and both consortia (vegetative cells and endospores) on total dry weight of *A. sativa* grown in pots using (A) a sterilized substrate (n=3 to 7) and (B) a non-sterilized soil (n=8 to 10). Error bars represent standard deviation of the mean. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's post hoc test. Control = Un-inoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312; BT2 = *B. thuringiensis* 1310; BL= *B. licheniformis*; consortium= M.

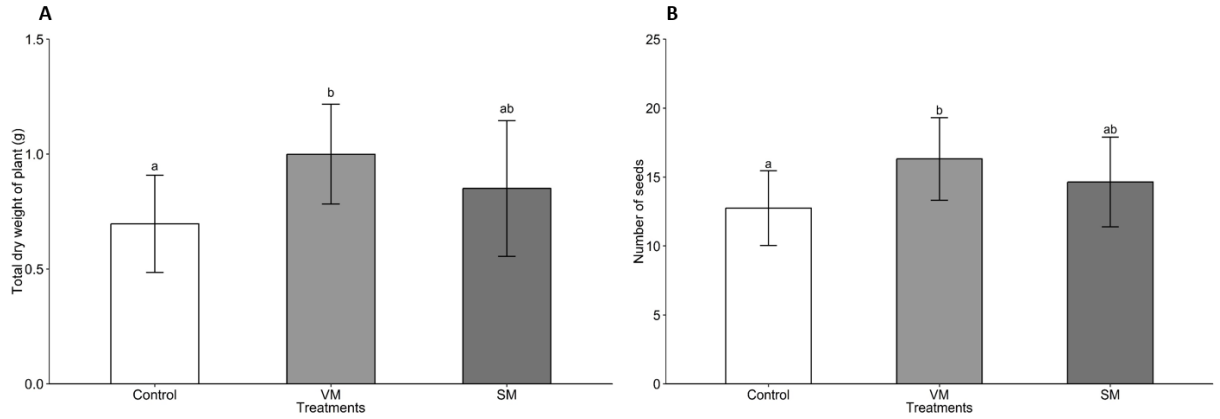

Figure 4: Plant growth promoting effects of both consortia (vegetative cells and endospores) on *A. sativa* plants grown in the field. (A) Total dry weight of oat plants and (B) total number of seeds produced per oat plant. Error bars represent standard deviation of the mean. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's post hoc test (n=28). Control = Un-inoculated; VM = Consortium of vegetative cells; SM = Consortium of endospores.

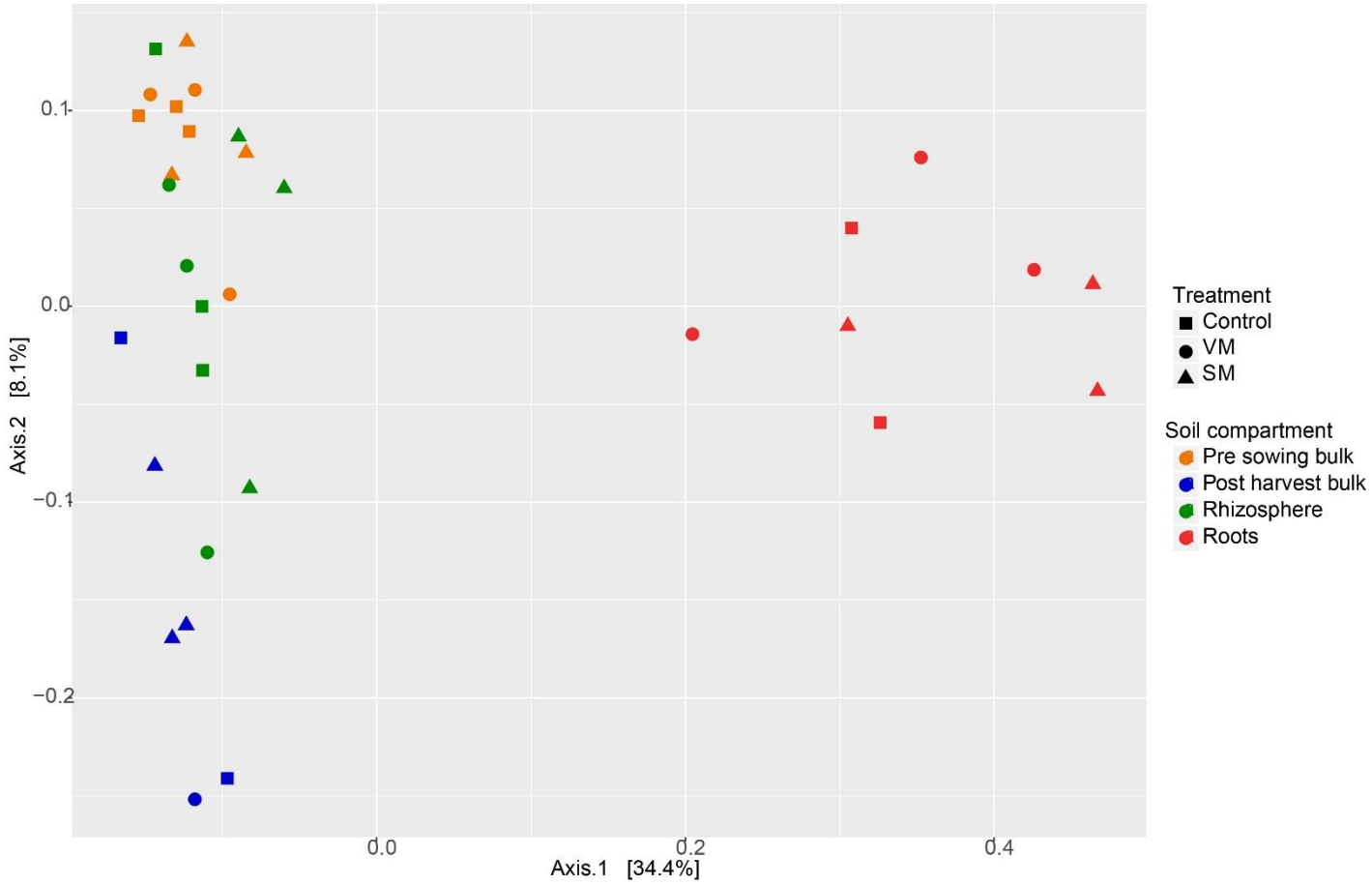

Figure 5: Principal coordinate analysis (PCoA) based on Bray Curtis dissimilarity of bacterial community composition in bulk soils (pre-sowing and post-harvest), rhizospheric soil, and roots. Percentage of the variation between the samples explained by each axis is indicated on the respective axis. The shape of the symbol represents the different treatments (Control= Un-inoculated; VM = Consortium of vegetative cells; SM = Consortium of endospores). Colors represent the different soil compartments.

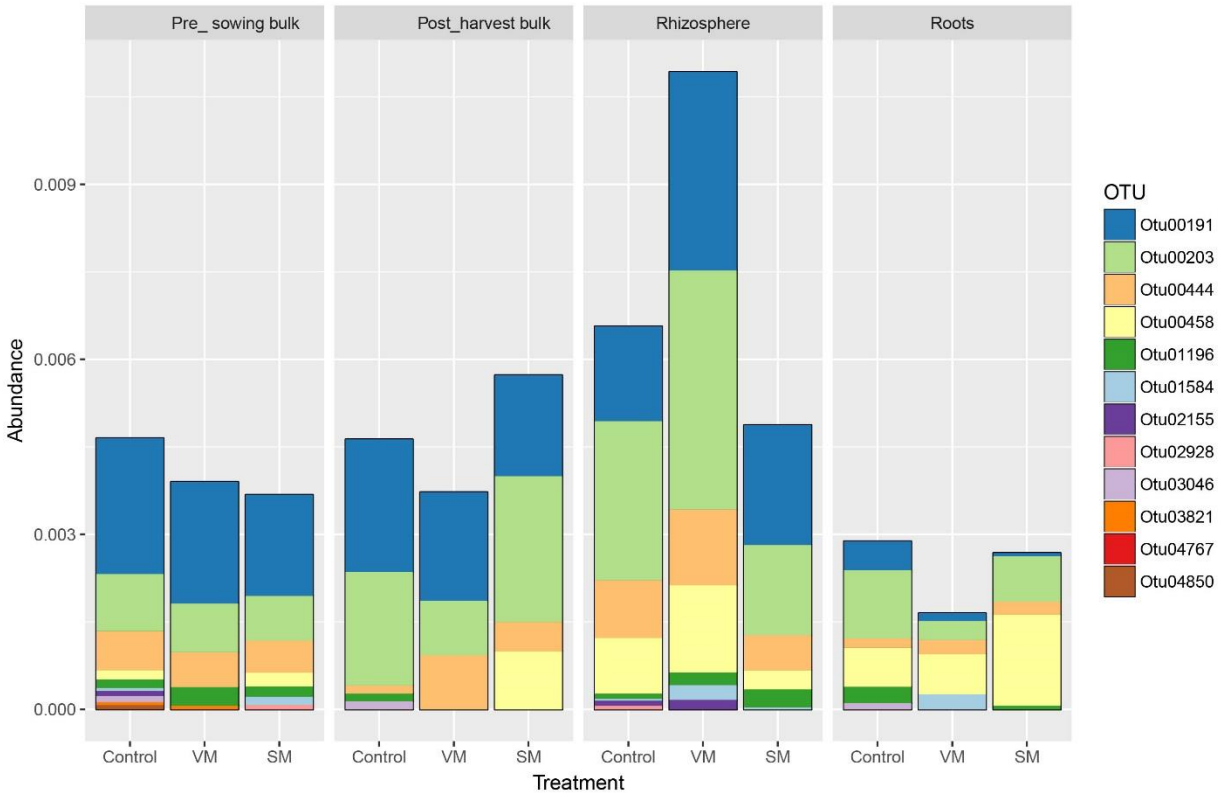

Figure 6: Relative abundance of the 12 OTUs assigned to *Bacillus* spp. in pre-sowing bulk soil (bulk soil before the experiment), post-harvest bulk soil (bulk soil after the experiment), rhizospheric soil, and roots. The different colors represent the different OTUs.

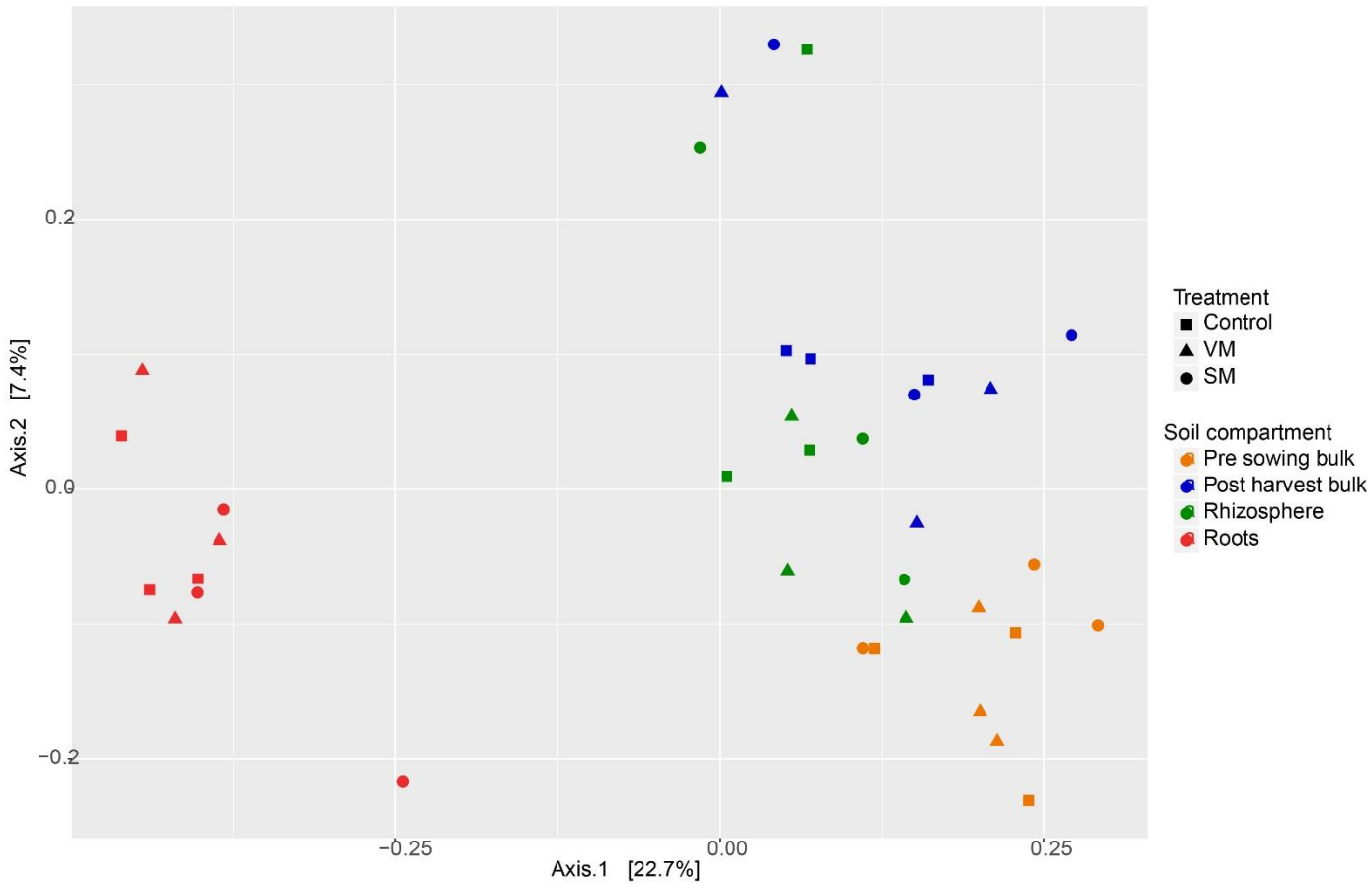

Figure 7: Principal coordinate analysis (PCoA) based on Bray Curtis dissimilarity of fungal community composition in bulk soils (pre-sowing and post-harvest), rhizospheric soil, and roots. Percentage of the variation between the samples explained by each axis is indicated on the respective axis. The shape of the symbol represents the different treatments (Control= Un-inoculated; VM = Consortium of vegetative cells; SM = Consortium of endospores) and the colors represent the different soil compartments.

References

Abdeljalil, Nada Ouhaibi Ben and Renault, David (2016), 'Evaluation of the effectiveness of tomato-associated rhizobacteria applied singly or as three-strain consortium for biosuppression of *Sclerotinia* Stem Rot in Tomato', *Journal of Microbial & Biochemical Technology,* 8 (4).

Ahemad, Munees and Kibret, Mulugeta (2014), 'Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective', *Journal of King Saud University - Science,* 26 (1), 1-20.

Akhtar, Naseem, et al. (2016), 'Effect of consortium of plant growth promoting and compost inhabiting bacteria on physicochemical changes and defense response of maize in fungus infested soil', *The Pakistan Journal of Agricultural Sciences,* 53 (01), 59-68.

Angle, J. Scott , Mcgrath, Stephen P. , and Chaney, Rufus L. (1991), 'new culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution', *Applied and environmental microbiology,,* 57 (2), 3674-76.

Arkhipova, T. N., et al. (2005), 'Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants', *Plant and Soil,* 272 (1-2), 201-09.

Arora, Kumar. Naveen., Tiwar, Saskhi., and Singh, Ratan (2014), 'comparative study of different carriers inoculated with nodule forming and free living plant growth promoting bacteria suitable for sustainable agriculture', *Journal of Plant Pathology & Microbiology,* 05 (02).

Baez-Rogelio, A., et al. (2017), 'Next generation of microbial inoculants for agriculture and bioremediation', *Microb Biotechnol,* 10 (1), 19-21.

Bhattacharyya, P. N. and Jha, D. K. (2012), 'Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture', *World J Microbiol Biotechnol,* 28 (4), 1327-50.

Bric, John M. , Bostock, Richard M. , and Silverstone, Sara E. (1991), 'Rapid *in situ* assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane', *Applied and environmental microbiology, ,* 57 (2), 535-38.

Chibuike, G. U. and Obiora, S. C. (2014), 'Heavy metal polluted soils: effect on plants and bioremediation methods', *Applied and Environmental Soil Science,* 2014, 1-12.

Ciccillo, Fabio, et al. (2002), 'Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizospheric bacterial diversity', *Environmental Microbiology,* 4 (4), 238–45.

Correa, Olga S., et al. (2009), *'Bacillus amyloliquefaciens* BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities', *Applied Soil Ecology,* 41 (2), 185-94.

Döbereiner, J. (1980), 'Forage grasses and grain crops: in methods for evaluating biological nitrogen fixation', *New York: John Wiley & Sons Ltd.*, pp. 535-55.

Donnellan, J. Edward , Nags, Ella H. , and Levinson, Hillel S. (1964), 'Chemically defined, synthetic media for sporulation and for germination and growth of *Bacillus subtilis'*, *Journal of bacteriology,* 87, 332-36.

El-Sayed, W. S., et al. (2014), *'In vitro* antagonistic activity, plant growth promoting traits and phylogenetic affiliation of rhizobacteria associated with wild plants grown in arid soil', *Front Microbiol,* 5, 651.

Figueiredo, Márcia V. B., et al. (2008), 'Alleviation of drought stress in the common bean (Phaseolus vulgaris L.) by co-inoculation with *Paenibacillus polymyxa* and *Rhizobium tropici', Applied Soil Ecology,* 40 (1), 182-88.

Frazier, William C. and Rupp, Philip (1930), 'Studies on the proteolytic bacteria of milk v. action of proteolytic bacteria on milk serum', *Journal of Bacteriology*.

Gadhave, K. R., et al. (2018), 'soil inoculation with bacillus spp. modifies root endophytic bacterial diversity, evenness, and community composition in a context-specific manner', *Microb Ecol*.

García de Salamone, Inés E., et al. (2012), 'Inoculation of paddy rice with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact of plant genotypes on rhizosphere microbial communities and field crop production', *Applied Soil Ecology,* 61, 196-204.

Gottel, Neil. R., et al. (2011), 'Distinct microbial communities within the endosphere and rhizosphere roots across contrasting soil types', *Applied and Environmental Microbiology,* 77 (17), 5934.

Jetiyanon, K. (2007), 'Defensive-related enzyme response in plants treated with a mixture of *Bacillus* strains (IN937a and IN937b) against different pathogens', *Biological Control,* 42 (2), 178-85.

Korir, H., et al. (2017), 'Co-inoculation effect of *rhizobia* and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil', *Front Plant Sci,* 8, 141.

Kozdrój, J., Trevors, J. T., and van Elsas, J. D. (2004), 'Influence of introduced potential biocontrol agents on maize seedling growth and bacterial community structure in the rhizosphere', *Soil Biology and Biochemistry,* 36 (11), 1775-84.

Kuan, K. B., et al. (2016), 'Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions', *PLoS One,* 11 (3), e0152478.

Kumar, Ankit, Prakash, Anil, and Johri, B. N. (2011), '*Bacillus* as PGPR in Crop Ecosystem', *Bacteria in Agrobiology: Crop Ecosystems*, 37-59.

McMurdie, P. J. and Holmes, S. (2013), 'phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data', *PLoS One,* 8 (4), e61217.

Moustaine, M., et al. (2017), 'Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth in tomato (*Solanum Lycopersicum* L.) and characterization for direct PGP abilities in Morocco', *International Journal of Environment, Agriculture and Biotechnology,* 2 (2), 590-96.

Muhibbullah, M., Salma, M., and Chowdhury, A. (2005), 'Use of agrochemical fertilizers and their impact on soil, water and human health in the Khamargao village of Mymensingh district, bangladesh', *Jownal of Agronomy,* 4 (2), 109-15.

Niu, B., et al. (2017), 'Simplified and representative bacterial community of maize roots', *Proc Natl Acad Sci U S A,* 114 (12), E2450-E59.

Noumavo, Pacôme A., et al. (2013), 'Effect of different plant growth promoting rhizobacteria on maize seed germination and seedling development', *American Journal of Plant Sciences,* 04 (05), 1013-21.

Nui, Dong-Dong, et al. (2011), 'The plant growth–promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating

salicylate- and jasmonate/ethylene-dependent signaling pathways', *Molecular Plant-Microbe Interactions,* 24 (5), 533-42.

O'Callaghan, M. (2016), 'Microbial inoculation of seed for improved crop performance: issues and opportunities', *Appl Microbiol Biotechnol,* 100 (13), 5729-46.

Oksanen, Jari , et al. (2017), 'vegan: Community Ecology Package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. Version 2.4-4. URL https://CRAN.R-project.org/package=vegan'.

Orhan, Emine, et al. (2006), 'Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry', *Scientia Horticulturae,* 111 (1), 38-43.

Piromyou, P., et al. (2013), 'Indigenous microbial community structure in rhizosphere of Chinese Kale as affected by plant growth-promoting rhizobacteria inoculation', *Pedosphere,* 23 (5), 577-92.

Posada, Luisa F., et al. (2016), 'Bioprospecting of aerobic endospore-forming bacteria with biotechnological potential for growth promotion of banana plants', *Scientia Horticulturae,* 212, 81-90.

R Core Team (2014), 'R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.'.

Radhakrishnan, R. and Lee, I. J. (2016), 'Gibberellins producing *Bacillus methylotrophicus* KE2 supports plant growth and enhances nutritional metabolites and food values of lettuce', *Plant Physiol Biochem,* 109, 181-89.

Sato, Kouichi, et al. (2005), 'A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis', *Grassland Science,* 51 (2), 179-81.

Schwieger, Frank and Tebbe, Christoph C. (2000), 'American Society for Microbiology', *Applied and environmental microbiology,* 66 (8), 3556–65.

Schwyn, Bernhard and Neilands, B (1987), 'Universal Chemical Assay for the Detection and Determination of Siderophores', *Analytical biochemistry,* 160.

Souza, Rocheli de, Ambrosini, Adriana , and Passaglia, Luciane M.P. (2015), 'Plant growth-promoting bacteria as inoculants in agricultural soils', *Genet Mol Biol,* 38 (4), 401-19.

Trabelsi, Darine and Mhamdi, Ridha (2013), 'Microbial inoculants and their impact on soil microbial communities: A Review', *BioMed Research International,* 2013, 11.

Trabelsi, Darine, et al. (2012), 'Appraisal of the crop-rotation effect of rhizobial inoculation on potato cropping systems in relation to soil bacterial communities', *Soil Biology and Biochemistry,* 54, 1-6.

Urbina, Hector, et al. (2018), 'Specificity in *Arabidopsis thaliana* recruitment of root fungal communities from soil and rhizosphere', *Fungal Biology,* 122 (4), 231-40.

Van Geel, Marteen, Busschaert, Pieter, Honnay Olivier, Lievens Bart. (2014), 'Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal (AMF) communities using 454 pyrosequencing'. *Journal of microbiology method*, 106, 93-100.

Wang, C. J., et al. (2012), 'Induction of drought tolerance in cucumber plants by a consortium of three plant growth-promoting rhizobacterium strains', *PLoS One,* 7 (12), e52565.

Widnyana, I. Ketut and Javandira, Cokorda (2016), 'Activities *Pseudomonas* spp. and *Bacillus* sp.

to stimulate germination and seedling growth of tomato plants', *Agriculture and Agricultural Science Procedia,* 9, 419-23.

WRB (2015), 'World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.'.

Zheng, X. L., Xiong, Z. Q., and Wu, J. Q. (2017), 'The use of a simple flow cytometry method for rapid detection of spores in probiotic *Bacillus licheniformis*-containing tablets', *Food Sci Biotechnol,* 26 (1), 167-71.

Supplementary information

Soil samples were collected before (pre-sowing bulk soil) and after (post-harvest bulk soil) the field experiment, air-dried at 40°C for three days, and sieved at 2 mm. This soil was used for the characterization of the following parameters: pH H_2O, water holding capacity (WHC), residual humidity (Hr %), bioavailable phosphorous (P), total phosphorous (TP), and total carbon, hydrogen and nitrogen (CHN). CHN results were used to calculate H/C and C/Ntot ratios. Fresh soil samples were collected and directly processed for the determination of NH_4^+, NO_3^{2-} and dry matter content.

Table S1: Summary of physicochemical and trophic analyses of bulk soils before sowing (pre-sowing) and after harvesting (post-harvest). For post-harvest soils, the characterization was performed for each of the three different treatments: un-inoculated, consortium of vegetative cells, and consortium of endospores Legend: ± Standard error; nd not determined

Parameters	**Control (untreated)**		**Vegetative cells treatment**		**Endospores treatment**	
	Pre-sowing	Post-harvest	Pre-sowing	Post-harvest	Pre-sowing	Post-harvest
pH	7.78±0.01	7.83±0.04	7.78±0.03	7.81±0.02	7.76±0.05	7.87±0.00
CEC (cmolc/kg)	18.40±1.26	16.34±0.73	21.07±1.66	16.28±0.57	12.80±0.56	14.23±0.25
Bioavailable P (mg/g)	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.03±0.00	0.03±0.00
Total P (mg/g)	0.35±0.08	0.94±0.05	0.40±0.01	0.89±0.00	0.35±0.04	0.84±0.05
Nitrates (ug/g)	5.03±0.33	19.70±0.63	3.36±0.74	47.66±4.60	3.47±1.13	26.36±1.95
Ammonium (ug/g)	0.60±0.01	0.87±0.02	0.95±0.15	0.98±0.04	0.55±0.37	1.03±0.03
H/C ratio	0.21±0.02	nd	0.32±0.10	nd	0.24±0.06	nd
C org/Ntot ratio	0.21±0.02	nd	0.32±0.10	nd	0.24±0.06	nd
Organic C (%)	4.11±0.75	nd	3.33±0.30	nd	3.43±0.55	nd
CaCO3 %	26.57±6.91	nd	37.40±1.42	nd	16.79±7.67	nd
Dry matter content %	75.45±1.44	73.21±0.78	78.81±1.57	74.20±1.00	76.22±0.41	72.17±1.83
Water content %	24.55±1.44	26.79±0.78	21.19±1.57	25.80±1.00	22.14±1.68	27.83±1.83
Water holding capacity %	72.66±2.43	90.21±4.88	63.25±5.08	85.98±2.73	65.89±2.04	94.51±8.79
Hr %	1.98±0.16	1.69±0.06	1.91±0.11	1.70±0.00	1.91±0.10	1.59±0.10

Table S2: Number of bacterial cells counted on seeds bio-inoculated with the consortium of vegetative cells or with the consortium endospores by using SEM images.

Vegetative cells treatment		**Endospores treatment**	
Area (μm^2)	**No. of seeds counted**	**Area** (μm^2)	**No. of seeds counted**
5	21	5	-
	22		-
20	40	20	6
	22		7
	79		15
	54		20
	85		45
50	26	50	47
	47		31
100	46		13
	80		17
	80	100	-
	98		-
mean	54		22
Standard deviation	28		15

Table S3: Comparison of the adjusted and unadjusted *p*-values calculated for the effect of bio-inoculation on seed germination. Pair-wise comparisons were performed by using Tukey's post hoc test. Control = Un-inoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312, BT2 = *B. thuringiensis* 1310, BL= *B. licheniformis*, Consortium= M. Statistically significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

In vitro seed germination (inoculation with vegetative cells)		
Treatments	Adjusted *p*	Unadjusted *p*
VBT2 - Control	0.1308	0.01968 *
VBL - Control	0.7758	0.25074
VBT1 - Control	0.0378 *	0.00486 **
VM - Control	0.5928	0.14892
VBL - VBT2	0.6831	0.1924
VBT1 - VBT2	0.9108	0.39252
VM - VBT2	0.8457	0.31084
VBT1 - VBL	0.2696	0.04768 *
VM - VBL	0.998	0.75775
VM – VBT1	0.4028	0.08200

In vitro seed germination (inoculation with endospores)		
Treatments	Adjusted *p*	Unadjusted *p*
SBT2 - Control	0.0364 *	0.00486 **
SBL - Control	0.2351	0.04141 *
SBT1 - Control	0.3938	0.08167
SM - Control	0.0352 *	0.00472 **
SBL - SBT2	0.7489	0.2358
SBT1 - SBT2	0.5632	0.13977
SM - SBT2	0.976	0.55899
SBT1 - SBL	0.9969	0.73309
SM - SBL	0.4976	0.1149
SM – SBT1	0.357	0.07128

Table S3: Comparison of the adjusted and unadjusted *p*-values calculated for the effect of bio-inoculation on plant growth in sterile substrate and non-sterile soil. Pair-wise comparisons were performed by using Tukey's post hoc test. For the codes see Table S2. Statistically significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

Sterile substrate			Non-sterile soil	
Treatments	Adjusted *p*	Unadjusted *p*	Adjusted *p*	Unadjusted *p*
SBL-Control	0.7998464	0.118597	0.268579	0.01625 *
SBT1-Control	0.9605671	0.254777	0.999994	0.73243
SBT2-Control	0.1693687	0.009428 **	0.980791	0.303
SM-Control	0.0049604**	0.000177 ***	7.5E-06***	2.17e-07 ***
VBL-Control	0.1655501	0.009159 **	0.277243	0.01698 *
VBT1-Control	0.9865647	0.339177	0.101436	0.00469 **
VBT2-Control	0.9998812	0.623465	0.483592	0.03949 *
VM-Control	0.0019175**	6.52e-05 ***	5.62E-05***	1.66e-06 ***
SBT1-SBL	0.9992971	0.532881	0.432502	0.03286 *
SBT2-SBL	0.9430808	0.224627	0.869035	0.14804
SM-SBL	0.0964267	0.004718 **	0.032705*	0.00127 **
VBL-SBL	0.9295788	0.206893	1	0.96322
VBT1-SBL	0.9992344	0.528059	0.999531	0.54697
VBT2-SBL	0.9328409	0.210858	1	0.81461
VM-SBL	0.0431831*	0.001859 **	0.091829	0.00417 **
SBT2-SBT1	0.5254979	0.048283 *	0.998457	0.47802
SM-SBT1	0.0134429*	0.000513 ***	1.65E-05***	5.527 4.78e-07 ***
VBL-SBT1	0.5136917	0.046406 *	0.44003	0.03379 *
VBT1-SBT 1	1	0.925896	0.182402	0.00971 **
VBT2-SBT1	0.9976509	0.460505	0.671991	0.07357
VM-SBT1	0.0039433**	0.000139 ***	0.000123***	3.68e-06 ***
SM-SBT2	0.5110636	0.045996 *	0.000257***	7.78e-06 ***
VBL-SBT2	1	0.922093	0.865859	0.14606
VBT1-SBT2	0.6594216	0.074488	0.559575	0.05107
VBT2-SBT2	0.2168987	0.012985 *	0.965349	0.25676
VM-SBT2	0.321237	0.022277 *	0.001437**	4.56e-05 ***
VBL-SM	0.6049031	0.062574	0.047465*	0.00194 **
VBT1-SM	0.0332918*	0.001388 **	0.223998	0.01270 *
VBT2-SM	0.0044478**	0.000157 ***	0.028914*	0.00111 **
VM-SM	1	0.879135	1	0.8407
VBT1-VBL	0.6391813	0.069827	0.999778	0.58607
VBT2-VBL	0.2151671	0.012848 *	0.999999	0.78525
VM-VBL	0.4165941	0.032885 *	0.120399	0.00577 **
VBT2-VBT1	0.9996879	0.576505	0.996622	0.42784
VM-VBT1	0.0137809*	0.000527 ***	0.396414	0.02865 *
VM-VBT2	0.001332**	4.46e-05 ***	0.076982	0.00338 **

Table S4: Comparison of the adjusted and unadjusted *p*-values calculated for the effect of bio-inoculation on plant growth in the field experiment. Pair-wise comparisons were performed by using Tukey's post hoc test. For the codes see Table S2. Statistically significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05.

	Total plant weight (dry)		Number of seeds	
Treatments	Adjusted *p*	Unadjusted *p*	Adjusted *p*	Unadjusted *p*
SM-Control	0.0540668	0.021 *	0.0526359	0.0204 *
VM-Control	0.0000393***	4.638 1.33e-05 ***	0.0000758***	4.464 2.57e-05 ***
VM-SM	0.0638396	0.025 *	0.096547	0.0390 *

Table S5: Comparison of the 16S rRNA sequences of the 12 OTUs corresponding to *Bacillus* spp. to the Genbank database and showing the ten first identical strains (94-100%).

OTU	Strain name	Identity %	Accession number
Otu00191	*Bacillus licheniformis*	99%	MF319819.1
	Bacillus licheniformis	99%	MF319812.1
	Bacillus licheniformis	99%	MF319807.1
	Bacillus thuringiensis	97%	KP342157.1
	Bacillus thuringiensis	97%	KJ009514.1
	Bacillus thuringiensis	97%	KJ000207.1
	Bacillus licheniformis	97%	KR999920.1
	Bacillus thuringiensis	97%	JN036714.1
	Bacillus licheniformis	97%	HQ238934.1
	Bacillus thuringiensis	97%	GU434216.1
Otu00203	*Bacillus thuringiensis*	100%	KT597529.1
	Bacillus thuringiensis	100%	KU362281.1
	Bacillus thuringiensis	100%	KP966473.1
	Bacillus thuringiensis	100%	KJ831618.1
	Bacillus thuringiensis	100%	KF836519.1
	Bacillus thuringiensis	100%	KF982022.1
	Bacillus thuringiensis	100%	KF317874.1
	Bacillus thuringiensis	100%	JN036709.1
	Bacillus thuringiensis	99%	MG709212.1
	Bacillus licheniformis	99%	FJ607346.1
Otu00444	*Bacillus licheniformis*	96%	FJ607346.1
	Bacillus thuringiensis	96%	KJ000207.1
	Bacillus licheniformis	96%	EU221362.1
	Bacillus thuringiensis	96%	JQ904721.1
	Bacillus thuringiensis	96%	KT597529.1
	Bacillus thuringiensis	96%	KU362281.1
	Bacillus licheniformis	96%	KR999920.1
	Bacillus thuringiensis	96%	KP966473.1
	Bacillus licheniformis	96%	KM462853.1
	Bacillus thuringiensis	96%	KJ831618.1
Otu00458	*Bacillus thuringiensis*	100%	KX507085.1
	Bacillus thuringiensis	100%	CP022345.1
	Bacillus thuringiensis	100%	KY863510.1
	Bacillus thuringiensis	100%	KX417256.1
	Bacillus thuringiensis	100%	KX652268.1
	Bacillus thuringiensis	100%	KX652267.1
	Bacillus thuringiensis	100%	CP020723.1

	Bacillus thuringiensis	100%	MF193910.1
	Bacillus thuringiensis	100%	MF193494.1
	Bacillus thuringiensis	100%	CP019230.1
Otu01196	*Bacillus licheniformis*	98%	EU221362.1
	Bacillus licheniformis	98%	KM462853.1
	Bacillus licheniformis	98%	HQ234338.1
	Bacillus licheniformis	98%	FJ607346.1
	Bacillus thuringiensis	97%	KT597529.1
	Bacillus thuringiensis	97%	KU362281.1
	Bacillus thuringiensis	97%	KP966473.1
	Bacillus thuringiensis	97%	KJ831618.1
	Bacillus thuringiensis	97%	KF836531.1
	Bacillus thuringiensis	97%	KF836519.1
Otu01584	*Bacillus thuringiensis*	99%	KY818698.1
	Bacillus thuringiensis	99%	JN036712.1
	Bacillus licheniformis	99%	HQ238934.1
	Bacillus licheniformis	99%	KR999920.1
	Bacillus thuringiensis	99%	KF204583.1
	Bacillus thuringiensis	99%	JN036714.1
	Bacillus thuringiensis	99%	GU434216.1
	Bacillus licheniformis	99%	MH613231.1
	Bacillus licheniformis	99%	GU592215.1
	Bacillus licheniformis	99%	KF803996.1
Otu02155	*Bacillus licheniformis*	97%	FJ607346.1
	Bacillus licheniformis	96%	EU221362.1
	Bacillus thuringiensis	96%	JQ904721.1
	Bacillus thuringiensis	96%	KT597529.1
	Bacillus thuringiensis	96%	KU362281.1
	Bacillus thuringiensis	96%	KP966473.1
	Bacillus licheniformis	96%	KM462853.1
	Bacillus thuringiensis	96%	KJ831618.1
	Bacillus thuringiensis	96%	KF836531.1
	Bacillus thuringiensis	96%	KF836519.1
Otu02928	*Bacillus thuringiensis*	99%	HF566172.1
	Bacillus licheniformis	99%	JX281693.1
	Bacillus thuringiensis	99%	JN036715.1
	Bacillus thuringiensis	99%	JN036711.1
	Bacillus licheniformis	99%	KY652112.1
	Bacillus licheniformis	99%	MF592453.1
	Bacillus thuringiensis	99%	KT725787.1
	Bacillus licheniformis	99%	KP686131.1

	Bacillus thuringiensis	99%	JN036713.1
	Bacillus thuringiensis	98%	KX856311.1
Otu03046	*Bacillus licheniformis*	100%	MF429159.1
	Bacillus licheniformis	100%	CP021677.1
	Bacillus licheniformis	100%	CP021669.1
	Bacillus licheniformis	100%	KY120749.1
	Bacillus licheniformis	100%	KX270723.1
	Bacillus licheniformis	100%	KX146965.1
	Bacillus licheniformis	100%	KY970108.1
	Bacillus licheniformis	100%	KY970104.1
	Bacillus licheniformis	100%	KY819038.1
	Bacillus licheniformis	100%	CP014794.1
Otu03821	*Bacillus thuringiensis*	94%	KJ000207.1
	Bacillus thuringiensis	94%	KP342157.1
	Bacillus thuringiensis	94%	KJ009514.1
	Bacillus thuringiensis	94%	KT597529.1
	Bacillus thuringiensis	94%	KU362281.1
	Bacillus thuringiensis	94%	KP966473.1
	Bacillus licheniformis	94%	KF879225.1
	Bacillus thuringiensis	94%	KJ831618.1
	Bacillus thuringiensis	94%	KF836531.1
	Bacillus thuringiensis	94%	KF836519.1
Otu04767	*Bacillus licheniformis*	99%	EU834235.1
	Bacillus licheniformis	94%	LN995478.1
	Bacillus licheniformis	94%	LN995452.1
	Bacillus licheniformis	94%	MF620064.1
	Bacillus licheniformis	94%	KY234256.1
	Bacillus licheniformis	94%	KU955686.1
	Bacillus licheniformis	94%	KT027672.1
	Bacillus licheniformis	94%	KT027661.1
	Bacillus licheniformis	94%	KP255998.1
	Bacillus licheniformis	94%	KJ722443.1
Otu04850	*Bacillus licheniformis*	96%	EU221362.1
	Bacillus thuringiensis	96%	JQ904721.1
	Bacillus licheniformis	96%	KR999920.1
	Bacillus licheniformis	96%	KM462853.1
	Bacillus thuringiensis	96%	JN036714.1
	Bacillus licheniformis	96%	HQ238934.1
	Bacillus licheniformis	96%	HQ234338.1
	Bacillus thuringiensis	96%	GU434216.1
	Bacillus licheniformis	96%	FJ607346.1

	Bacillus licheniformis	96%	GU592215.1

Supplementary Figures:

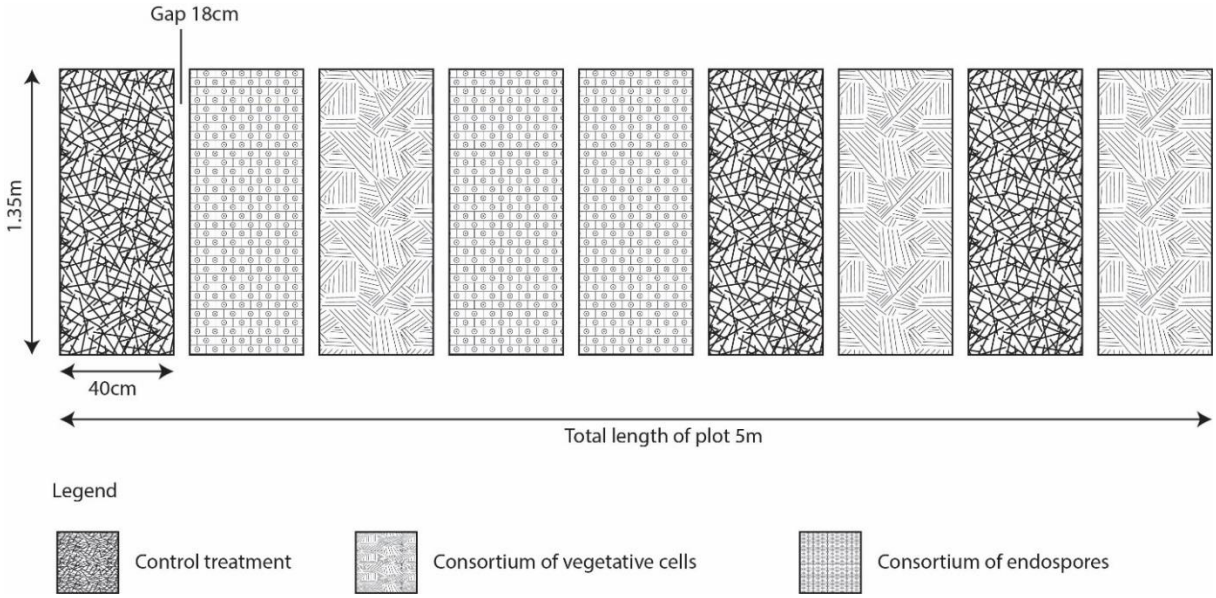

Figure S1: Experimental design of the field experiment showing the repartition of the three treatments: un-inoculated control, consortium of either vegetative cells (VM) or endospores (SM).

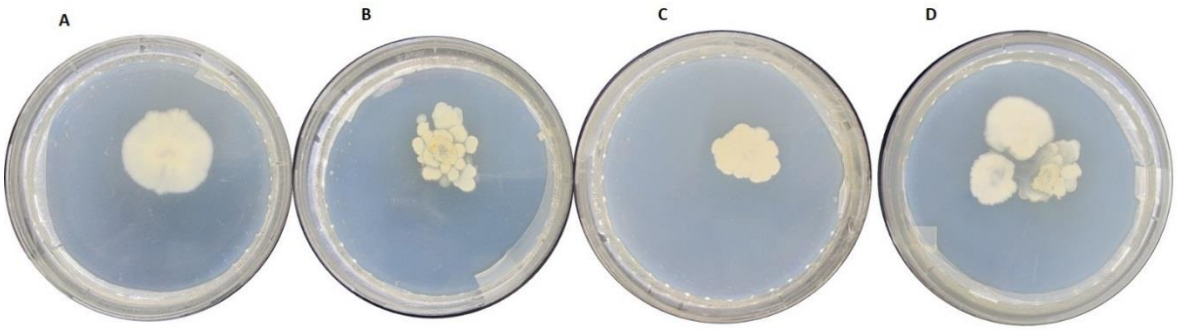

Figure S2: Compatibility of the three *Bacillus* strains growing on NA. (A-C) controls with one strain only. (A) *Bacillus thuringiensis* 1312 (BT1), (B) *Bacillus licheniformis* (BL), (C) *Bacillus thuringiensis* 1310 (BT2), and (D) Consortium of the three strains growing together and showing no inhibition between each other.

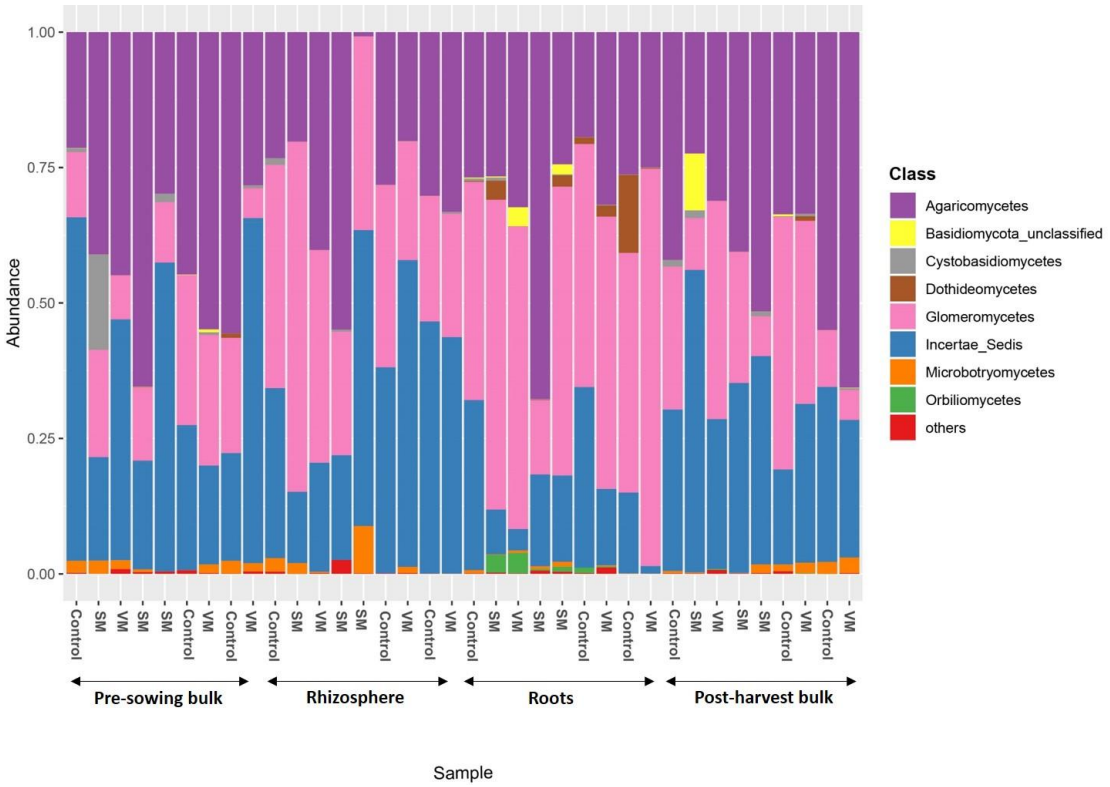

Figure S3: fungal OTUs relative abundances observed among all samples (pre-sowing bulk soil, post-harvest bulk soil, rhizospheric soil and roots). Control = Un-inoculated; VM = Consortium of vegetative cells and SM = Consortium of endospores. All treatments were done in triplicates and are shown in the plot as they were applied in the field.

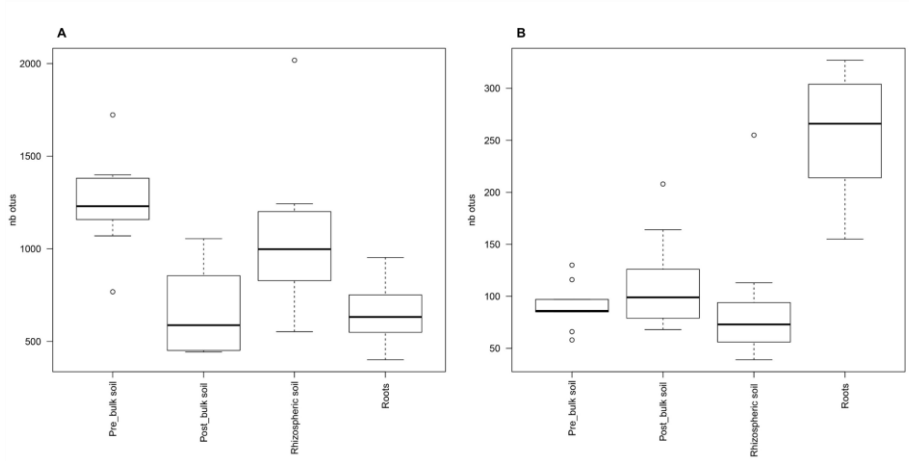

Figure S4: Number of OTUs per sample (pre-sowing bulk soil, post-harvest bulk soil, rhizospheric soil and roots) observed for (A) bacterial communities and (B) fungal communities.

Chapter 5

Differential mycophagy of *Lysinibacillus sphaericus* towards fungi with two contrasting ecologies

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Abstract

Bacteria and fungi play crucial roles in ecosystem functioning in terrestrial environments. Both are known for their diversified nutritional capabilities, leading to positive and negative interactions. Mycophagy is a type of antagonistic interaction in which bacteria obtain nutrients from living fungi, producing a negative impact on the fungal partner. Growth tests with various Bacilli strains using living fungal biomass or fungal exudates as sole carbon source allowed the identification of a mycophagus lifestyle in the bacterium *Lysinibacillus sphaericus* 1003. This bacterium grew using the living mycelium of the soil-borne phytopathogenic fungus *Rhizoctonia solani* as sole carbon source. Ergosterol measurements showed that in the presence of the bacterium, fungal biomass decreased. *L. sphaericus* growth was also assessed on the saprophytic fungus *Trichoderma rossicum*. While the bactericum grew at the expense of fungal mycelim, ergosterol measurements showed that for *T. rossicum,* the interaction led to an increase in fungal biomass. Observations at a cellular scale using microfluidics revealed hyphal deformation and bacterial penetration inside the hyphae of *R. solani*. In contrast, no phenotypic changes were observed for *T. rossicum*, despite the fact that numerous bacteria attached to its mycelium. Additional confrontation assays were performed in complex media. The bacterium was capable

of inhibiting the growth of *R. solani* in a medium favoring bacterial growth, but not in two media in which the fungus developed at a faster rate. Conversely, the bacterium never inhibited the growth of *T. rossicum* in any of the media tested. This was observed despite the fact that the bacterium used the mycelium of both fungi to disperse. However, hyphae of *R. solani* in direct contact with bacteria were adversely damaged and were unable to re-grow. Genome analysis of the bacterium revealed the presence of genes potentially causing damages to the fungal cell wall, as well as other genetic elements that might contribute to mycophagy. Due to its differential behavior, *L. sphaericus* constitute an interesting model to better understand the role of mycophagy in soil carbon cycling.

Introduction

Soil is a hub for bacterial-fungal interactions (BFI), where the entire spectrum of interaction types, from mutualism to antagonism, can be found. BFI are not only important for ecosystem functioning (Deveau et al. 2018), but at an organismal level, could be an approach to find sustainable alternatives to fight phytopathogenic fungi (Whipps 2001). This is normally achieved by selecting either bacteria or fungi with an antagonistic effect on a selected pathogen (i.e. biocontrol agents) (Knudsen and Dandurand 2014; Whipps 2001). However, given the complexity of microbial communities in soils, a biocontrol agent will certainly encounter not only the targeted phytopathogen, but also plant beneficial organisms that should not be antagonized. Synergistic and antagonistic interactions between bacteria and fungi also affect plant growth and development. For instance, it has been observed that the synergistic interaction of fluorescent pseudomonads with *Rhizopogon luteolus* and *Laccaria* spp. contribute to the formation of ectomycorrhizal associations with plants (Frey-Klett et al. 2007). Bacilli and Paenibacilli are also reported to stimulate mycorrhizal associations (Bending et al. 2002; Poole et al. 2001). Moreover, mycorrhizal fungi also have a positive effect on bacterial communities in the mycorrhizosphere by stimulating and modyfing root exudation, as well as by stabilizing soil structure (Nadeem et al. 2014).

Besides synergistic interactions, many types of antagonistic interactions have been reported. Given the fact that fungi provide nutritional niches for bacterial growth and development, several

negative interactions involve bacteria using fungi as a nutritional resource (Boer et al. 2005). Bacterial mycophagy is an example of an antagonistic trophic interaction in which bacteria can grow at the expense of fungal mycelium (both living and dead) or fungal exudates (Leveau and Preston 2008). Although there are several types of bacterial mycophagy (Leveau and Preston 2008), in this study mycophagy will be defined as bacterial growth at the expense of living fungal mycelium with a negative impact on fungal growth as a proxy to fitness. We chose this definition to highlight the difference to bacteria that use fungal exudates or dead hyphal biomass for growth, with a neutral effect on the fungal partner.

Rhizospheric soil appears to be a potential hotspot for bacteria possessing mycophagous traits. A microcosm study performed using bacterial communities extracted from the rhizosphere of *Carex arenaria* and *Festuca rubra* showed the widespread ability of rhizospheric bacteria to attach and to feed on living hyphae of the saprotrophs *Trichoderma harzianum* and *Mucor hiemalis.* However, the identity of the fungus had a strong effect on the composition of the potentially mycophagous communities (Rudnick et al. 2015b). A bait experiment using mycelium of phytopathogenic and saprophytic fungi also allowed the enrichment of a large diversity of rhizospheric mycophagous bacteria (Ballhausen and Wietse 2016). This shows that not only saprophytic fungi, but also phytopathogenic fungi, favor the adherence and growth of mycophagous bacteria. The apparently widespread existence of a mycophagous lifestyle in rhizospheric bacteria suggest the possibility of this nutritional strategy as a natural mechanism for the biocontrol of soil-borne fungal plant pathogens. Therefore, investigating the plasticity of mycophagy of potential biocontrol agents and different partners is required.

Bacillus spp. is one of the best-studied genera for fungal biocontrol. This is explained by the diverse spectrum of antagonistic interactions that members of this genus establish with fungi. *Bacillus* spp. can produce a wide range of antibacterial and antifungal compounds, which are important in their interaction with plants and other soil microorganisms (E. Sansinenea and Ortiz 2011). For instance, *Bacillus subtilis* fmbj synthesizes bacillomycin D, a toxin with lytic properties that disrupts the cell wall and cell membrane of the saprophytic and opportunistic pathogen *Aspergillus flavus* (Gong et al. 2014; Scully and Bidochka 2005). *Bacillus cereus* X16 and *B. thuringiensis* 55T also showed fungistatic and fungitoxic inhibitory effects (damaging of the cell

wall and loss of protoplast) on *Fusarium roseum* var. *sambucinum* (Chérif et al. 2002). In another study, 71 *Bacillus* spp. confronted to 5 different plant pathogenic fungi (*Alternaria* sp., *Fusarium oxysporum*, *Fusarium avenaceum*, *Fusarium equiseti* and *Bipolaris* sp.) showed antifungal activities. A detrimental effect on hyphae and the reduced production of fungal spores by *Alternaria* sp. was observed for the strain with the highest percentage of fungal growth inhibition (Estibaliz Sansinenea et al. 2016). The development of a mycophagous lifestyle might be one of the emerging behaviors in response to an intimate and negative relationship of Bacilli with fungi. In this study, various representatives of Bacilli were investigated to assess their growth at the expense of fungal mycelium. This led to the selection of the bacterium *Lysinibacillus sphaericus* 1003. *L. sphaericus* is a well-known insect biocontrol agent with larvicidal activity against mosquitos (Lozano and Dussan 2013). More recently, the potential of *L. sphaericus* for plant growth promotion was investigated. *L. sphaericus* ZA9 displayed a remarkable beneficial effect on vegetable crops (cucumber and tomato) by increasing seed germination and seedling growth (Naureen et al. 2017). In addition, endophytic *L. sphaericus* have been shown to promote plant growth and to control rice sheath blight disease caused by *Rhizoctonia solani* (Shabanamol et al. 2017). This suggest that *L. sphaericus* might establish complex interactions with different microorganism in the mycorrhizosphere. However, a mycophagous lifestyle in this species has not yet been reported. This study shows that mycophagy in *L. sphaericus* 1003 is a plastic behavior in response to specific fungal partners and nutritional conditions.

Materials and methods

Model organisms and growth medium

In this study two fungal species were used. *R. solani* (Basidiomycota), is a well-known soil borne phytopathogenic fungus of many economically important crops (e.g. root rot, damping off) (Ajayi-Oyetunde and Bradley 2018). *Trichoderma rossicum* (Ascomycota), belongs to a genus which contains commonly known saprophytic, mycoparasitic, plant growth promoting, and biocontrol fungi (Mukherjee et al. 2012; Zhang et al. 2016). In addition to these two fungal strains, eight bacterial strains were investigated. All microbial strains are routinely conserved in the strain collection of the Laboratory of Microbiology of the University of Neuchâtel. Their identity and

origin are presented in Table S1. The two fungal strains are maintained on malt agar (MA) medium (Malt extract 12 g/L; SIOS Home Brewing and agar 15 g/L; Biolife italiana) and stored at 4°C. Regaular growth was done on MA at room temperature. Bacterial cultures were cryopreserved in 60% glycerol and stored at -80°C. For regular bacterial growth, nutrient agar (NA) medium (8 g/L; Biolife italiana and agar 15 g/L; Biolife italiana) was used.

In vitro assessment of mycophagy using living mycelium of *R. solani* as sole carbon source

To investigate whether any of the eight bacterial strains had a mycophageous lifestyle, they were grown on the mycelium of *R. solani* as a sole carbon source. The experimental design was based on the system previously established by (Ballhausen et al. 2015), with minor changes (Figure 1A). Briefly, the experimental device consisted of a 90 mm Petri dish, which was filled with highly purified water agar (WA) medium (15 g/L; Merck KGaA, Darmstadt, Germany). On the middle of the agar surface, a 35 mm Petri dish filled with 3.5 ml of 2X concentrated (24 g/L) MA medium was placed to supply nutrients for fungal colonization of the nutrient-free WA medium. A 2 mm^2 fungal plug was placed on the surface of the 2X MA medium. The inoculum was taken from the apical region of a 4-day old fungal culture. Once, the entire WA medium was colonized by fungal mycelium (after 15 days), the 35 mm MA-containing Petri dish was removed and the bacterial inoculum was introduced directly on the fungal mycelium. To prepare the bacterial inoculum, an overnight bacterial culture in nutrient broth (NB) (8 g/L; Biolife italiana) medium was transferred into a sterile falcon tube and centrifuged at 1856 g (Sigma 2-16PK) for 5 min. The supernatant was removed, and the cell pellet was washed with 10 mL physiological water (9 g/L NaCl; Sigma-Aldrich). The washing step was repeated four times to completely remove all potential nutrients from NB medium. After this thorough washing, the pellet was suspended in 10 mL physiological water and dilutions were made to obtain a cell suspension with a cell density ranging from 7 to 45 cells/µL. 100 uL of this bacterial suspension was introduced in the nutrient-free WA medium, with or without fungal mycelium. To control the number of viable cells that were introduced, colony forming unit (CFU) were enumerated by plating the same amount of bacterial suspension (100 uL) on a NA medium and colonies counted after 1 to 2 days. After 17 days of incubation, the surface of the WA medium was washed with 2 mL of phosphate buffer solution (137mM NaCl,

10mM Na_2HPO_4, 2.7mM KCl, 1.8mM KH_2PO_4, pH 7.4). This suspension was collected in a sterile Eppendorf tube and bacterial CFU was counted as described before and compared to the initial value. The experiment was performed in triplicates.

In vitro assessment of mycophagy with two different fungal species

Based on the result obtained from the first experiment, only one bacterial strain (*Lysinibacillus sphaericus*) was selected for additional experiments. The same method was applied to compare mycophagy in *L. sphaericus* using *T. rossicum* in addition to *R. solani.* The only modification to the experimental design indicated above consisted on a longer incubation period (24 days).

Quantification of active fungal biomass

Ergosterol content was quantified as a proxy of active fungal biomass. For this, the Petri dishes used in the 24-day mycophagy assay were stored at -20°C for approximately 1 h and then freeze-dried for 48h at -50°C. The freeze-dried samples were stored in a desiccator and protected from light, until further processing. The samples were then crushed with liquid nitrogen by using a pestle and a mortar. Ergosterol was extracted using the following procedure adapted from (Barajas-Aceves et al. 2002). Briefly, 15 mL of methanol at 5°C was added. The samples were homogenized using an ultrasound probe (Branson Sonifier 450) for 3 min (80% of the nominal converter amplitude and 20 kHz connector) on an ice bath. The samples were further incubated on an ice bath for 15 min. After that, the samples were transferred to a 50 mL falcon tube and centrifuged for 15 min at 11092 g (Sigma 2-16PK) at 4°C. The supernatant was transferred to an Erlenmeyer containing 2 g of KOH. The pellet was washed with 10 mL MeOH and centrifuged again for 15 min at 11092 g (Sigma 2-16PK). The supernanat was added in the Erlenmeyer containing the first supernatant and 2 g KOH. 5 mL of absolute ethanol was added to the supernatant and the resulting solution was placed in a water bath at 80-85°C for 30 min under constant shaking. After cooling, 10 mL Hexane were added and the samples were placed on an orbital shaker for 20 min, after which the hexane phase was removed and transferred to a clean glass vial. This step was repeated twice and the successive hexane phases were pooled. This solution was then dried under a constant N_2 flux at a temperature of 38°C using a heater along with a sample concentrator (Techne, Dri-Block® DB.3D). The dried samples were then dissolved in

5 mL of 80% methanol by using an ultrasound bath. Prior to HPLC-MS measurements, 1 mL of solution was centrifuged for 5 min at 17968 g (Sigma 2-16PK) at 4°C and the supernatant was transferred to a HPLC vial. Ergosterol was quantified by Ultra High-Performance Liquid Chromatography (UHPLC) (Ultimate 3000 Dionex, Thermo Fisher Scientific) coupled with tandem mass spectrometry (4000 QTRAP, ABSciex). The system was controlled by Analyst 1.5.1 software. A 5 µL aliquot of each sample was injected onto an ACQUITY UPLC® BEH C18 column (2.1 x 100mm, 1.7µm) (Water). The flow rate and the temperature of the column were kept at 0.45 mL/min and 30°C, respectively. The mobile phase A consisted of MilliQ water and the mobile phase B of methanol (ULC-MS grade, Biosolve). The following gradient was used: 70-100% B for 2 min, holding at 100% for 6 min followed by re-equilibration at 70% B for 2 min. Mass spectrometry detection was achieved using the multiple reaction monitoring (MRM) mode in APCI positive ionization, with the transition 379.5/69.4. The selected precursor ion at m/z 379.5 was the [M+H-H2O]+ fragment of the protonated molecule. Declustering potential (DP) was set to 91 V, collision energy (CE) to 60 V and collision cell exit potential (CXP) to 5 V. Ion source parameters were as follows: nebulizer current of 5 µA, source temperature of 375°C, nebulizing gas (GS1) of 45 psi, drying gas (GS2) of 15 psi and curtain gas (CUR) of 15 psi. Quantification was done by external calibration using a linear regression from five calibration points (5 to 500 ng/mL).

Assessment of bacterial growth on fungal exudates

To observe if *L. sphaericus* 1003 could grow at the expense of fungal exudates only, both fungal strains were grown on WA medium as explained above for the mycophagy experiment. When the mycelium had fully covered the medium, each fungus (together with the WA medium) was transferred into a falcon tube and 25 mL of sterile physiological water were added. This falcon tube was placed in the cold room (4°C) overnight. The resulting suspension was filtered using a sterile membrane filter (Syringe filters ClearLine®, pore size 0.2 µm). The chemical composition of the fungal exudates was determined using LiquiTOC. Bacterial growth at the expense of fungal exudates was monitored in 96 well plates under agitation by measuring the optical density at 550 nm with a spectrophotometer (SpectraMax® i3X, Molecular Devices). The bacterial inoculum was added at an optical density of 0.02 (1.4 X 10^6). As a control, bacterial growth was monitored in NB medium.

Observation of mycophagous bacterial-fungal interactions at a single cell level

A bacterial-fungal interaction microfluidic device was used to observe the interaction between the bacterium *L. sphaericus* 1003 and either one of the two fungi (*R. solani* or *T. rossicum*) at a cellular level. The design and manufacturing of the device is explained elsewhere (Stanley et al. 2014). For the experiment, sterile distilled water was used as growth medium. Both fungal strains were refreshed in 10X diluted NA medium. The NA medium was prepared at a low concentration so that the fungus did not colonize too rapidly the microfluidic device. When the channels were filled with fungal hyphae, 10 uL of bacterial inoculum containing 10^4 cells were added in the bacterial inlet. The microscopic analyses were performed using two inverted microscopes (Nikon and Olympus IX81) 4 days after bacterial inoculation.

In-vitro bacterial-fungal confrontation assay

In order to test the influence of nutritional conditions on the interaction between *L. sphaericus* 1003 and *R. solani* or *T. rossicum*, *in vitro* confrontation assays were performed using three different media: NA (8 g/L of nutrient broth, Biolife italiana; 15 g/L agar, Biolife italiana), potato dextrose agar (PDA; 39 g/L; BioLife) and Skimmed milk malt agar (SM-MA; 50 g/L of powder milk, Rapilait, Migros; 12 g/L Malt extract, SIOS Home Brewing; and 15 g/L agar, Biolife italiana). These three media have contrasting nutrient composition, in particular regarding their C/N ratio. The experimental design of the confrontation assays consisted of the inoculation of the fungus in the middle of the culture medium by transferring a 5 mm^2 plug taken from a 4-day old fungal culture grown on MA media. Two days after fungal inoculation, two lines of bacteria were streaked at two opposing sides of the fungal colony at a distance of 2 cm from the apical margin (Figure S1). Petri dishes were placed in the dark and results of fungal inhibition by the bacterium were noted after 7 days. After incubation the following parameters were scored: first, growth of the two organisms and visual aspect of the colonies; second, the ability of both organisms to re-grow after confrontation (i.e. viability) in media with a biocide to avoid the growth of the confrontation partner.

To check for the viability and dispersal of bacteria after the confrontation, NA was supplemented with 0.1% cycloheximide (1g/L; Sigma-Aldrich), an antifungal compound, to suppress fungal growth and allow only bacterial grow. The same was done to assess the viability of fungi with MA

supplemented with tetracycline (0.008g/L, Fluka, Biochemika) and streptomycin sulfate (0.064 g/L, AppliChem), which are antibacterial compounds used to suppress bacterial growth and thus allow only fungal growth. Agar plugs from the confrontation assays were cut from different places of the fungal colonized agar and then placed on either of the antimicrobial media. The presence of bacterial or fungal colonies on the respective antimicrobial media was assessed after one to two days.

Genomic DNA extraction and sequencing

Genomic DNA extraction of *L. sphaericus* 1003 was carried out from an overnight culture in NB medium. Genomic DNA was extracted using the Genomic-tip 20/G kit (QIAGEN, Hilden, Germany). Genome sequencing was performed using a Pac Bio SMRT (Single Molecule Real Time) Sequencing platform (Pacific Biosciences, California) and the genome assembled with HGAP (Hierarchical Genome Assembly Process) (Chin et al. 2013). Genome annotation of *L. sphaericus* was performed using the Rapid Annotation Search Tool (RAST) (Aziz et al. 2008). After the annotation, the result was analyzed using SEED viewer (http://www.theseed.org). The complete genome sequence of this bacterium was submitted to the NCBI GenBank and can be retrieved under the accession number XXX.

Comparative genomic analysis

The comparative genomics program EDGAR (Blom et al. 2016) was used to compare the genomic content of *L. sphaericus* 1003 with the genomic content of other 14 *L. sphaericus* strains. The genome assemblies of the other *L. sphaericus* strains were recovered from NCBI. The list of genomes of *L. sphaericus* with their respective accession assemble number and the source of isolation are presented as supplementary information (Table S2). EDGAR was used to calculate the pan genome, core genome, singleton and Average Nucleotide Identity (ANI) similarity. Moreover, to identify gene clusters potentially involved in the production of secondary metabolites, the online web-based server antiSMASH version 3.0 (Weber et al. 2015) was used to predict secondary metabolite biosynthesis gene clusters.

Statistical analysis

All statistical analyses were computed using R (version 3.5.1) (R Core Team 2014). Data of mycophagy experiments were first log transformed and ANOVA (analysis of variance) followed by a Tukey's post hoc test was applied ($p < 0.05$). For ergosterol measurments, data were not log transformed.

Results

Mycophagous lifestyle of the selected Bacilli strains in interaction with two different fungi

The potential of eight Bacilli strains (Table S1) to grow at the expense of living fungal mycelium was tested by incubating the bacterial strains with the mycelium of *R. solani* as sole carbon and nutrient source (Figure 1A). In this experiment four different type of interactions were observed. In the first one (double negative), the bacterium *Bacillus thuringiensis* 1310 did not develop in the control medium (WA only) or in the presence of fungal mycelium (Figure S2A). In the second type of interaction (*Bacillus subtilis* 1055 and *Bacillus thuringiensis* 1311), bacterial growth in presence of the fungus did not differ from the growth on WA (Figure S2B-C). In the third type of interaction (four Bacilli strains), bacteria grew better in WA, while in the presence of the fungus, the concentration of bacteria decreased during the experiment (Figure S2D-G). This suggested that bacteria were potentially used as a nutritional resource by the fungus. In the fourth type of interaction, the bacterium *L. sphaericus* 1003 grew significantly better in the presence of fungal mycelium, as compared to the control (Figure S2H). The results obtained with *L. sphaericus* 1003 were confirmed and further investigated using not only *R. solani*, but also *T. rossicum* as fungal partner. After 25 days of incubation, a significant increase in bacterial biomass was observed in presence of living fungal mycelium. Cell density changed from 6 CFU to 1.28×10^5 and 6.1×10^3 CFU, in the plates containing mycelium of *R. solani* or *T. rossicum*, respectively (Figure 1B). Fungal biomass was also quantified by measuring ergosterol as a proxy to active fungal biomass. This measurement was made in order to assess the potential detrimental effect of mycophagy on the fungal partner. In the plates with *R. solani* and *L. sphaericus* 1003, ergosterol content decreased as compared to the control with fungal mycelium only (Figure 1C). In contrast, for *T. rossicum*, fungal biomass increased in the presence of bacteria as compared to the control (Figure 1C).

However, in both cases, the difference in ergosterol content in the presence or absence of bacteria was statistically non-significant due to the large variation observed for one of the three replicates. In order to confirm that bacterial grew at the expense of living fungal hyphae rather than on fungal exudates, an additional test was performed to evaluate the growth of bacteria using fungal exudates as a carbon source. The exudates from both *R. solani* and *T. rossicum* provided a similar amount of organic carbon, around 200-250 mg/mL of exudate suspension, and had a very high C/N ratio (Figure S3). Growth of *L. sphaericus* 1003 on fungal exudates was observed neither for *R. solani*, nor for *T. rossicum* (Figure 1D and 1E).

Observation of mycophagous bacterial-fungal interactions at a cellular level

In order to assess the effect of mycophagy on the growth and morphology of fungal hyphae, a microfluidic device was used to observe the interaction of *L. sphaericus* 1003 with both fungi. Bacterial development alongside fungal growth caused a morphological change in *R. solani* hyphae (Figure S5). While cytoplasmic content was evident in contrast phase images in the control without bacteria, in the device co-colonized by bacteria, part of the mycelium appeared to be empty (Figure 2). In addition, visible deformation in the hyphal tips was noticed, and bacteria were detected inside deformed fungal tips (Supplementary Video). In the case of *T. rossicum*, no visible changes in hyphal morphology were observed (Figure S5). Moreover, numerous bacteria appeared attached to the hyphae, but this did not seem to affect mycelial development or apparent viability (Figure 2).

Mycophagous interaction using different media

In order to test if the interaction between *L. sphaericus* 1003 and *R. solani* or *T. rossicum* change in response to the nutritional conditions of the medium, confrontation experiments were performed using three different media with contrasting nutrient composition and C/N ratio. Bacterial-fungal interactions were strongly influenced by the medium in which the confrontations took place. *L. sphaericus* 1003 inhibited the growth of *R. solani* only on NA medium. In the other two media (PDA and SM-MA), fungal growth was not inhibited (Figure 3A). On the other hand, *L. sphaericus* 1003 did not inhibit *T. rossicum* on any of the three media (Figure 3B). Moreover, *L. sphaericus* 1003 was able to disperse on mycelial network (fungal-highway like dispersal

(Kohlmeier et al. 2005)) of both, *R. solani* and *T. rossicum*. Besides investigating bacterial dispersal on the fungal network, the viability of both fungi after the confrontation with *L. sphaericus* 1003 was also determined. *A*reas of *R. solani* mycelium that were in physical contact with *L. sphaericus* 1003 were unable to re-grow, despite the fact that the bacterium did not produce an inhibition zone. On the other hand, *T. rossicum* was able to re-grow after confrontation with the bacterium in all conditions.

Genomic insights into mycophagy in *L. sphaericus* 1003

The full genome sequence of *L. sphaericus* 1003 was obtained in order to identify putative genomic determinants related to a mycophagus lifestyle. This genome was compared with 14 genomes of other *L. sphaericus* strains. The selected strains are generally classified as pathogenic to insects due larvicidal activity against mostly mosquito or as non-pathogenic (Table S2). A heat map based on the average nucleotide identity (ANI) matrix obtained for the 14 *L. sphaericus* strains is shown in Figure S6. According to the definition of species based on ANI measurements (ANI threshold 95-96% identity) (Kim et al. 2014), the 14 genomes of *L. sphaericus* should be separated into two distinct genomic species. The strains previously reported to have a larvicidal effect on mosquito (*L. sphaericus* OT4b.49, *L. sphaericus* CBAM5, *L. sphaericus* 2362, *L. sphaericus* C3-41, *L. sphaericus* III (3)7, *L. sphaericus* OT4b.25, *L. sphaericus* KU skv2 1, *L. sphaericus* SSII-1, *L. sphaericus* 2297, and *L. sphaericus* LP1-G) were clustered together into one group. The other strains (*L. sphaericus* 1003, *L. sphaericus* FSL, *L. sphaericus* A1, *L. sphaericus* DSM 28, and *L. sphaericus* KCTC 3346) were placed into a second one. The genome of *L. sphaericus* 1003 showed highest ANI with the genomes of *L. sphaericus* DSM 28 and *L. sphaericus* KCTC 3346 (99.12% and 99.21%, respectively). These type strains were reported to be non-pathogenic against mosquitos (Jeong et al. 2013). *L. sphaericus* 1003 has also 96.16% ANI with *L. sphaericus* A1 (isolated from municipal solid waste and used for lignin degradation) and 96.19% ANI with *L. sphaericus* FSL (isolated from raw milk). None of the closest relatives of *L. sphaericus* 1003 has been reported as pathogenic against mosquito, and thus a larvicidal biocontrol effect for *L. sphaericus* 1003 appears unlikely.

To identify its unique features, the *L. sphaericus* 1003 genome was compared to the genome of *L. sphaericus* DSM 28 (=KCTC 3346) using the comparative genomics software EDGAR. A total of 519 singletons were found (Table S3). Many of the singletons corresponded to hypothetical proteins and phage-like elements. In addition, a chitinase (EC 3.2.1.14), lipoprotein, peptidase, Type IV and V secretory pathways were also observed within the singletons of *L. sphaericus* 1003, all of which were absent in *L. sphaericus* DSM 28 (=KCTC 3346). The presence of the aforementioned putative functions might be important in antifungal activities in the case of *L. sphaericus* 1003, as *L. sphaericus* DSM 28 (=KCTC 3346) did not display any antifungal activity against the plant pathogenic fungi *Botrytis cinerea* and *R. solani* (Jeong et al. 2013).

Additional genome mining was performed for the identification of genes responsible for production of putative enzymes that might be helpful in the mycophagous lifestyle of *L. sphaericus* 1003 (Table S4.). We searched for genes responsible for putative enzymes involved in mycophagous traits previously reported for *Collimonas* spp., which is the best-studied model of bacterial mycophagy so far (Song et al. 2015). Chitinases, enzymes that hydrolyse chitin, have been documented in *Collimonas fungivorans* Ter331 (Fritsche et al. 2008). In the genome of *L. sphaericus* 1003, three putative genes responsible for the degradation of chitin were found. Phospholipases and proteases, which are responsible for hydrolyzing phospholipids and peptide bonds of proteins, respectively, were found to be present in mycophagous *Collimonas* species (Song et al. 2015). *L. sphaericus* 1003 harbored genes encoding for phospholipases, lysophospholipases and a peptidase. In bacteria, the secretory machinery is an important virulence factor. In the Gram-negative *Collimonas,* Type II, III and VI secretion systems can be important in pathogenicity against fungi (Song et al. 2015). These secretion systems can be used to deliver deleterious proteins or for inserting membrane disrupting proteins in the fungal host (Leveau et al. 2010; Song et al. 2015). In the genome of *L. sphaericus* 1003, putative genes for Type II, IV and V secretion systems were also present. In addition, chemotaxis might play a crucial role in the recognition, physical attachment or migration towards fungal hyphae (Haq et al. 2016; Rudnick et al. 2015a). A set of more than 80 genes were present for flagellar synthesis and chemotactic activity in *L. sphaericus* 1003.

Bacillus spp. is known to produce a plethora of secondary metabolites (E. Sansinenea and Ortiz

2011). Secondary metabolites were also reported in the mycophagous *C. fungivorans* Ter331, which harbored a gene cluster encoding a compound with antifungal properties known as Collimomycin (Fritsche et al. 2014). We analyzed the genome of *L. sphaericus* 1003 to identify potential secondary metabolites with antimicrobial properties by using the Anti SMASH web tool (Weber et al. 2015) and found 10 clusters in the genome, albeit with low gene cluster similarity to known secondary metabolite clusters (Supplementary Table S5).

Discussion

Based on the definition of mycophagy used in this study, the results demonstrate that *L. sphaericus* 1003 displays a mycophagus lifestyle. Our findings suggest that *L. sphaericus* 1003 has a preferential mycophagous lifestyle towards the phytopathogenic fungus *R. solani,* but not in the case of *T. rossicum*. We showed that this bacterium can grow at the expense of the living fungal mycelium of *R. solani* and, more importantly, had a negative impact on fungal biomass. In contrast and even though *L. sphaericus* 1003 grew at the expense of *T. rossicum*, we found a positive effect on fungal biomass as compared to the fungus only controls. However, the type of interaction depended on the type of medium, i.e. on the nutrient conditions. For instance, an antagonistic interaction with *R. solani* was observed only on WA and NA medium. Observations made with microfluidics highlighted the importance of mycophagy on physical disturbance at a cellular scale. Hyphal deformation was observed in the interaction of *L. sphaericus* 1003 with *R. solani*. In contrast, hyphal deformation, growth inhibition, or a fungistatic activity were not observed in the interaction with *T. rossicum*. According to Leaveau et al. (2010), bacteria might gain entrance to the fungal hyphae at their growing tip. In a previous study, the deformation of *Aspergillus niger* hypha was observed in confrontation experiments with *C. fungivorans* (Mela et al. 2011). The ability of *L. sphaericus* 1003 to enter the mycelium of *R. solani* is noteworthy (supplementary video). However, the mechanism used by *L. sphaericus* 1003 to penetrate hyphae is still unknown. It is important to highlight the fact that the viability of the fungus was also investigated and that the areas of *R. solani* mycelial network that were colonized by bacteria were unable to re-grow. It has already been reported that some *Bacillus* species are able to inhibit the growth of phytopathogenic fungi by causing damage at a cellular level (Cheriff et al., (2002).

The bacterium *L. sphaericus* has been investigated in the past for its insect larvicidal activity, plant-growth promoting activities, and its capability to remediate heavy metals. However, to the best of our knowledge, a mycophagous lifestyle has not yet been reported or investigated in detail. However, there is evidence of the presence of *L. sphaericus* in an ecosystem in which fungal biomass might constitute an important nutritional resource. Indeed *Lysinibacillus* spp. were part of the gut bacterial communities of mites, whose diet changed from normal rearing (diet composed of mixtures of wheat germs, flat dried oat and dried yeast extract in a 10:10:1 ratio), to a fungal mycelium only diet. In this study, it was suggested that *Lysinibacillus* spp. might contribute to the hydrolysis of fungal mycelium in the mite gut (Hubert et al. 2012).

Demonstrating a mycophagus lifestyle in *L. sphaericus* 1003 offers the possibility to learn more about mycophagy as a nutritional strategy. Mycophagy has been extensively studied in the bacterial genus *Collimonas*, which was originally isolated from a sand dune (De Boer et al. 2001). *Collimonas* spp. extract nutrients from living fungal mycelium, or fungal exudates, using diverse mechanisms including chitinolytic enzymes and antibiotics (De Boer et al., 2005; Leveau et al., 2010). Similar mechanisms could potentially be found in *L. sphaericus*. This was assessed by a comparative genomic analysis of *L. sphaericus* 1003 and other 14 strains classified in the same species. This comparative analysis suggested the separation of *L. sphaericus* into various species based on DNA homology, as proposed previously (Jeong et al. 2013). The idea of separating the strains with a mosquitocidal activity into a new species has been suggested in the past (Xu et al. 2015), and it is supported by the analysis performed here. Moreover, genomes of mosquito-pathogenic strains were reported to be highly conserved, while profound heterogeneity was observed within the genomes of non-pathogenic strains. This is despite the fact that genes responsible for pathogenicity against mosquitoes were reported to be acquired through horizontal gene transfer (Xu et al. 2015).

Among the key genomic functions that can contribute to mycophagy, five putative chitinases were found in the annotation of *L. sphaericus* 1003, one of which, corresponded to a singleton (CDS 2839) that was not detected in the genome of the closely related strain *L. sphaericus* DSM 28. Chitinases hydrolyze chitin, which is a component of the fungal cell wall. Although chitinases have been reported for *C. fungivorans* Ter331, a mutant of *C. fungivorans* Ter331 without

chitinolytic activity is still mycophagous, suggesting that mycophagy is the cumulative result of many functions and that chitinolysis play only a marginal role (Fritsche et al. 2008). Accordingly, the genome mining of *L. sphaericus* 1003 indicates the presence of other metabolites and enzymes potentially involved in mycophagy. Lipases, lysophospholipases and patatin-like phospholipases, alone or in combination, have been reported as virulence factors in many bacterial species (Jaeger et al. 1994) (Flores-Diaz et al. 2016). *The genomic analysis of L. sphaericus* 1003 *also r*evealed gene clusters responsible for non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) encoding specialized functions for the biosynthesis of antimicrobial secondary metabolites including a bacteriocin and siderophores. Bacteriocin is known to have antifungal properties (Arun and Sai Jeevana Madhuri 2015; Gerbaldo et al. 2012) and a previous study has shown that a bacteriocin produced by *L. sphaericus* has great potential as a fungistatic against different fungi including *Trichoderma viridae* (Ahmad et al. 2014). In a previous study by (Bakthavatchalu Sasirekha and Srividya 2016) a siderophore produced by *Pseudomonas aeruginosa* FP6 was able to suppress the growth of *R. solani*. Moreover, the siderophore bacillibactin produced by *Bacillus amyloliquefaciens* SQR9 was also reported to have antifungal properties (Li et al. 2014).

Interestinly, with the saprophytic fungus *T. rossicum*, *L. sphaericus* 1003 established a synergistic interaction on all media by not inhibiting fungal growth and even stimulating fungal growth in WA. In addition to this, the viability of *T. rossicum* was also determined and we found that it was not affected by the presence of bacteria. Combinations of Bacilli and *Trichoderma* spp. have been used in the past to stimulate plant growth. Potentially the same could be achieved by using mycophagus *L. sphaericus* 1003 in combination with *T. rossicum*. However, in a previous study in which Collimonas bacteria were co-cultured with *Trichoderma harzianum* in a sand microsocom containing the fungus *Absidia* sp., the biomass of *T. harzianum* was reduced. It was suggested that this negative effect could be due to competition between *Collimonas* spp. and *T. harzianum*, both mycophagous microorganisms, for the consumption of *Absidia* sp. hyphae. Moreover, in the sand microcosm where only collimonads and *Absidia* sp. were growing, it was noticed that the growth of *Absidia* sp. was not reduced (Hoppener-Ogawa et al. 2009). In another study with the mycophagous *C. fungivorans*, no antagonistic effect in the *in vitro* confrontation assay was shown,

but when applied in a greenhouse pot experiment with tomato plants, *C. fungivorans* inhibited the growth of *Fusarium oxysporum* (Kamilova et al. 2007). The contrasting results between *in vitro* testing and *in vivo* application, highlight the difficulties of translating results obtained in the laboratory into soil. Moreover, the study confronting C. *fungivorans* and *F. oxysporum* also points out at a critical element to take into account: the mode of application of the bacterial inoculum (Kamilova et al. 2007). For instance, (Abeysinghe 2009), combined *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 as a biocontrol strategy of *R. solani*. They found that disease severity was lower when seeds were coated with *B. subtilis* CA32 and *T. harzianum* RU01 was applied directly to the soil. However, the outcome was not the same when bio-inoculation was applied the other way around (seeds coated with a conidial suspension of *T. harzianum* RU01 and bacterial suspension applied directly in the soil). In the second mode of application, disease severity was not reduced as much as in the previous treatment and the effect was the same as with the application of *T. harzianum* RU01 alone.

Conclusion

We demonstrated that *L. sphaericus* 1003 established specific interactions with two fungi of different ecologies. While it was able to grow at the expense of the living mycelium of both fungi, it affected negatively the growth only of the soil-borne plant pathogen *R. solani*. Understanding the underlying mechanisms of recognition and exploitation of living fungal mycelium are important future directions of research in mycophagy. Differences in the selection of a particular fungal partner could be due to the specific chemical composition of the cell wall or to specific chemical signals or receptors that allow for the interaction. However, this needs to be demonstrated experimentally. More generally, mycophagous interactions in the mycorrhizosphere and their relevance in carbon transfer from plants to the soil trophic food chain is a poorly studied component of the soil carbon cycle (Ballhausen and Wietse 2016). It has been reported that a large amount of organic carbon in the form of root exudates can be uptaken by saprophytic fungi, which are often considered to be the primary consumers of plant exudates. However, by exploiting directly fungal biomass, mycophagous bacteria might enhance the redistribution of this carbon to other components of the trophic chain. Therefore, such a lifestyle

may be an important aspect to understand to overall soil C turnover.

Conflict of Interest Statement

Authors declare no conflict of interest.

Acknowledgments

This work was supported by: Swiss Government Excellence Scholarships for Foreign Scholars (number 2014.0865 to IH), the “Fondation Pierre Mercier pour la Science” to SB, and the U.S. Department of Energy Biological and Environmental Research Division through a Science Focus Area grant (Grant number KP1601010 to PJ).

For the microscopy analysis, I would like to thanks Clément Duckert, laboratory of soil biodiversity, University of Neuchâtel for his help in utilization of inverted microscope. I would also like to acknowledge Dr. Claire Stanely, Agroscope, for providing microfluidics material and for her help in image analysis. I would also like to thank Slobodeanu Radu, University of Neuchâtel, for his guidance in statistical analysis.

Figures

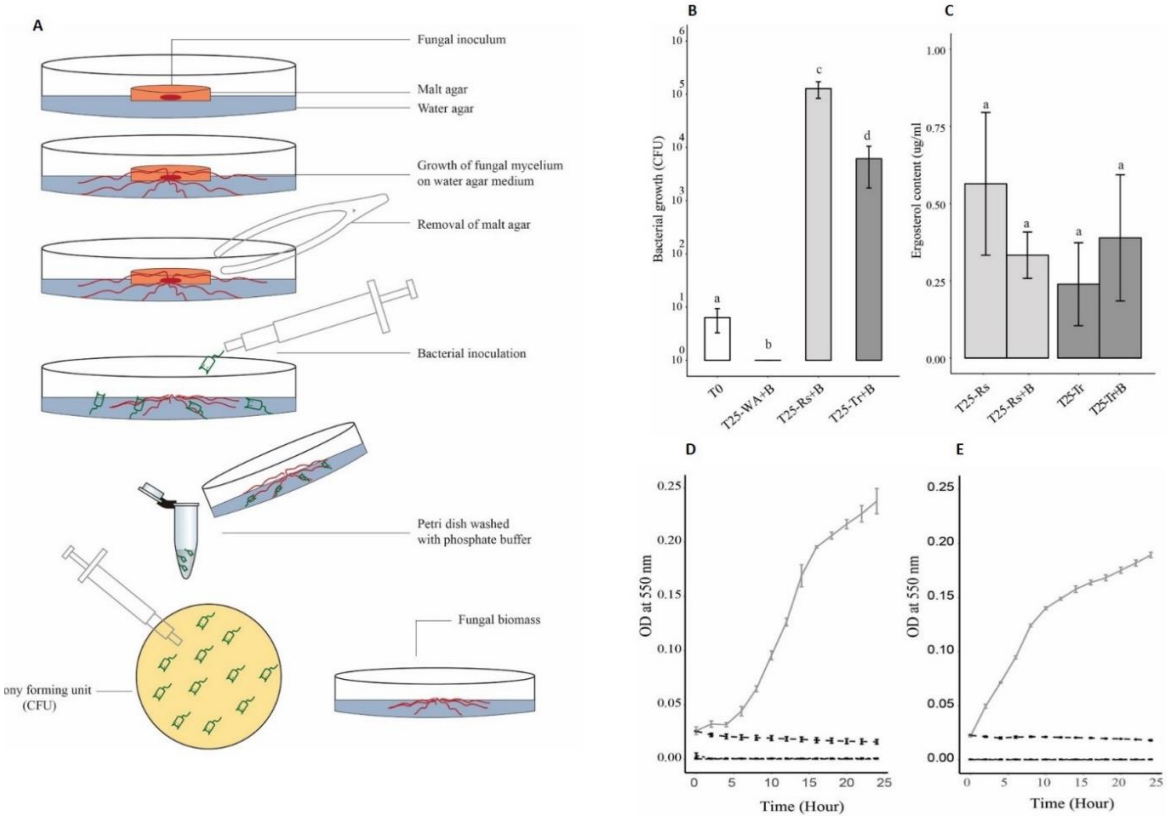


Figure 1: Investigation of a mycophagous lifestyle in *Lysinibacillus sphaericus* 1003. (A) Schematic representation of the microcosm experiment to measure the growth of bacteria at the expense of living fungal mycelium. The experiment was performed in a 90 mm Petri dish containing water-agar medium on which living fungal mycelium was grown by placing a 35 mm Petri dish containing a Malt extract and agar medium. (B) Measurements of bacterial biomass (colony forming unit -CFU- counting) comparing the number of cells at T0 (day of inoculation) and after 25 days (T25). A statistically significant (*p* value < 0.05) increase in bacterial biomass was obtained for both fungi. T25-Wa+B= Bacteria alone on water agar medium. T25-Rs+B= Bacteria grown on *Rhizoctonia solani* mycelium. T25-Tr+B= Bacteria grown on *Trichoderma rossicum* mycelium. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's post hoc test. (C) Egrosterol contents as a proxy of living fungal mycelium at the same time points indicated for bacterial growth (T0 and T25). The difference in ergosterol contents after 25 days were not statistically significant *(p*-value> 0.05; Anova test followed by TukeyHSD test). T25-Rs = Egrosterol content of *R. solani* grown alone. T25-Rs+B= Ergosterol content of *R. solani* grown with bacteria. T25-Tr= Ergosterol content of *T. rossicum* grown alone. T25-Tr+B= Ergosterol content of *T. rossicum* grown with bacteria. (D) No growth of *Lysinibacillus sphaericus* 1003 was observed on fungal exudates of *R. solani* and (E) *T. rosscium.* Legends:

Bacterial growth on nutrient broth medium

Control of nutrient broth medium (without bacteria)

Bacterial growth on the exudates of *R. solani/T. rossicum*

Control of fungal exudates of *R. solani/T. rossicum* (without bacteria)

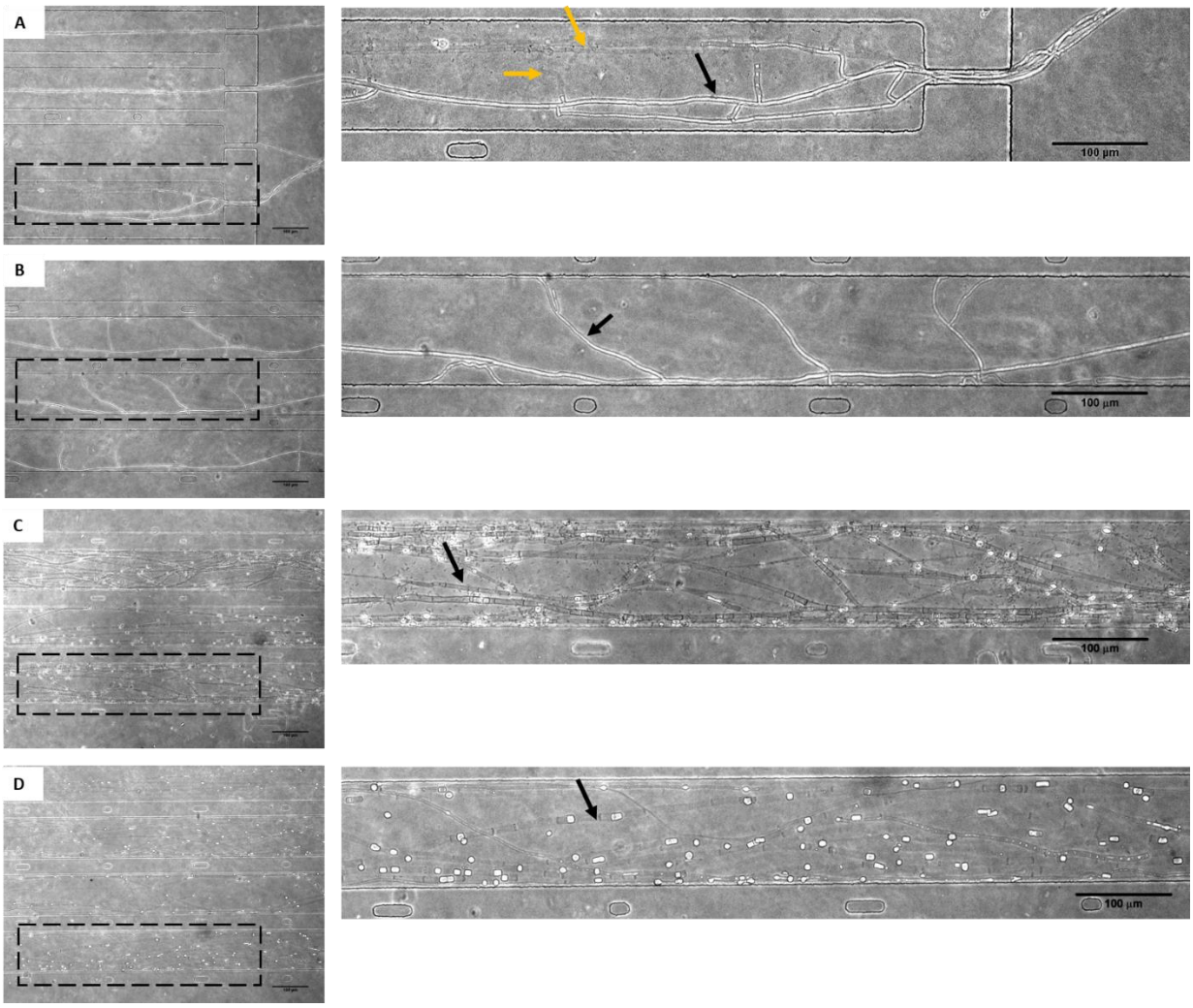


Figure 2: Microscopic observation of microfluidic devices in which *Rhizoctonia solani* and *Tricoderma rossicum* were grown in the presence or absence of *Lysinibacillus sphaericus* 1003. An overview at 10X is shown on the left and close-ups of the section indicated within the left panels are shown on the right. (A) Hyphae of *R. solani* in the presence of *L. sphaericus* 1003 showing loss of hyphal integrity (yellow arrow) as compared to other hyphae (black arrow) in the same microfluidics channel. (B) Hyphae of *R. solani* grown alone showing no signs of loss of hyphal integrity. (C) Mycelium of *T. rossicum* in the presence of bacteria showing no morphological changes despite the fact that numerous bacteria were attached to its mycelium. (D) Mycelium of *T. rossicum* growing alone. Black arrows in B, C and D images indicate no difference in hyphal deformation. Scale bars in all images= 100 um.

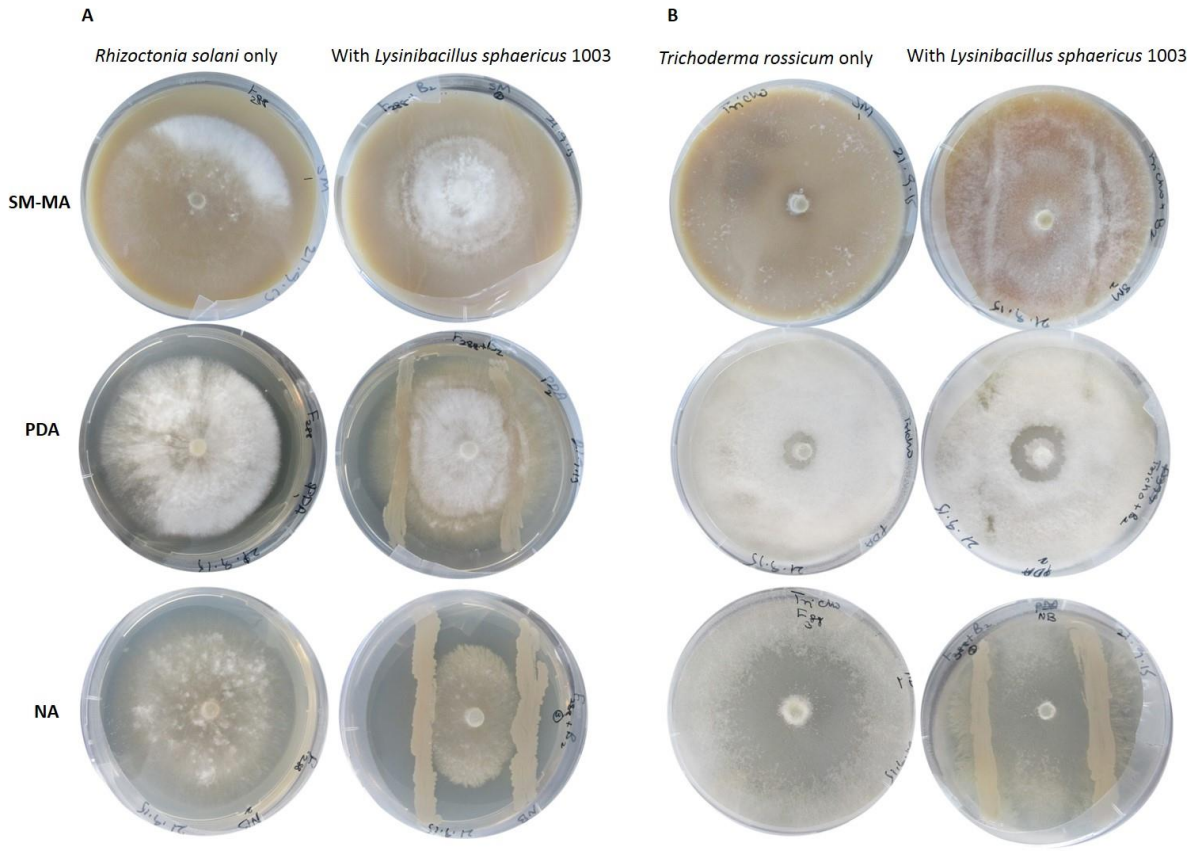


Figure 3: *In-vitro* bacterial-fungal confrontation assays using three different media (SM-MA: skimmed milk and malt agar; PDA: potato dextrose agar; NA: nutrient agar) and showing the inhibition of (A) *Rhizoctonia solani* only in nutrient agar (NA) medium, as compared to control. (B) For *Trichoderma rossicum*, no inhibition was observed. C/N ratio for the media used corresponded to: NA= 4-7 (Mwangi et al. 2012); PDA=10 (Mwangi et al. 2012); and SM-MA=4.47 (Lohberger et al. 2019).

References

Abeysinghe, S. (2009), ' Effect of Combined Use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on Biological Control of *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum*.', *Plant Pathology Journal,* 8, 9-16.

Ahmad, V., et al. (2014), 'Antimicrobial potential of bacteriocin producing *Lysinibacillus* jx416856 against foodborne bacterial and fungal pathogens, isolated from fruits and vegetable waste', *Anaerobe,* 27, 87-95.

Ajayi-Oyetunde, O. O. and Bradley, C. A (2018), *'Rhizoctonia solani:* taxonomy, population biology and management of *Rhizoctonia* seedling disease of soybean', *Plant Pathology,* 67, 3-17.

Arun, B. and Sai Jeevana Madhuri, G. (2015), 'Antifungal activity of bacteriocin produced by lactic acid bacteria from fermented green gram', *International Journal of Science and Research*.

Aziz, Ramy K., et al. (2008), 'The RAST Server: rapid annotations using subsystems technology', *BMC genomics,* 9, 75-75.

Bakthavatchalu Sasirekha and Srividya, Shivakumar (2016), 'Siderophore production by *Pseudomonas aeruginosa* FP6, a biocontrol strain for *Rhizoctonia solani* and *Colletotrichum gloeosporioides* causing diseases in chilli', *Agriculture and Natural Resources,* 50, 250e56.

Ballhausen, M. B. and Wietse, de Boer. (2016), 'The sapro-rhizosphere: Carbon flow from saprotrophic fungi into fungus-feeding bacteria', *Soil Biology and Biochemistry,* 102, 14-17.

Ballhausen, M. B., et al. (2015), 'Methods for baiting and enriching fungus-feeding (mycophagous) rhizosphere bacteria', *Front Microbiol,* 6, 1416.

Barajas-Aceves, M., et al. (2002), 'Effect of pollutants on the ergosterol content as indicator of fungal biomass', *Journal of Microbiological Methods,* 50, 227–36.

Bending, Gary D., et al. (2002), 'Characterisation of bacteria from *Pinus sylvestris–Suillus luteus* mycorrhizas and their effects on root–fungus interactions and plant growth', *FEMS Microbiology Ecology,* 39 (3), 219-27.

Blom, J., et al. (2016), 'EDGAR 2.0: an enhanced software platform for comparative gene content analyses', *Nucleic Acids Res,* 44 (W1), W22-8.

Boer, Wd, et al. (2005), 'Living in a fungal world: impact of fungi on soil bacterial niche development', *FEMS Microbiol Rev,* 29 (4), 795-811.

Chérif, M, et al. (2002), ' Ultrastructure and cytochemistry of *in vitro* interactions of the antagonistic bacteria *Bacillus cereus* x16 and *B. thuringiensis* 55t with *Fusarium roseum* var. sambucinum. Journal of Plant Pathology, 84(2), 83-93. ', *Journal of Plant Pathology,* 84 (2), 83-93.

Chin, Chen-Shan, et al. (2013), 'Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data', *Nature Methods,* 10, 563.

De Boer, W., et al. (2001), 'Growth of chitinolytic dune soil beta-subclass Proteobacteria in response to invading fungal hyphae', *Appl Environ Microbiol,* 67 (8), 3358-62.

Deveau, A., et al. (2018), 'Bacterial-fungal interactions: ecology, mechanisms and challenges', *FEMS Microbiol Rev,* 42 (3), 335-52.

Flores-Diaz, M., et al. (2016), 'Bacterial Sphingomyelinases and Phospholipases as Virulence Factors', *Microbiol Mol Biol Rev,* 80 (3), 597-628.

Frey-Klett, P., Garbaye, J., and Tarkka, M. (2007), 'The mycorrhiza helper bacteria revisited', *New Phytologist,* 176 (1), 22-36.

Fritsche, K., et al. (2008), 'Identification and characterization of genes underlying chitinolysis in *Collimonas fungivorans* Ter331', *FEMS Microbiol Ecol,* 66 (1), 123-35.

Fritsche, K., et al. (2014), 'Biosynthetic genes and activity spectrum of antifungal polyynes from *Collimonas fungivorans* Ter331', *Environ Microbiol,* 16 (5), 1334-45.

Gerbaldo, G. A., et al. (2012), 'Antifungal activity of two *Lactobacillus* strains with potential probiotic properties', *FEMS Microbiol Lett,* 332 (1), 27-33.

Gong, Qingwei, et al. (2014), 'Identification of bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against *Aspergillus flavus*', *Food Control,* 36 (1), 8-14.

Haq, I. U., et al. (2016), 'Chemotaxis and adherence to fungal surfaces are key components of the behavioral response of *Burkholderia terrae* BS001 to two selected soil fungi', *FEMS Microbiol Ecol,* 92 (11).

Hoppener-Ogawa, S., et al. (2009), 'Mycophagous growth of *Collimonas* bacteria in natural soils, impact on fungal biomass turnover and interactions with mycophagous *Trichoderma* fungi', *ISME J,* 3 (2), 190-8.

Hubert, J., et al. (2012), 'Shift of bacterial community in synanthropic mite T*yrophagus putrescentiae* induced by *Fusarium* fungal diet', *PLoS One,* 7 (10), e48429.

Jaeger, Karl-Erich., et al. (1994), 'Bacterial lipases', *FEMS Microbiology Reviews,* 15, 29-63.

Jeong, H., et al. (2013), 'Genome Sequence of *Lysinibacillus sphaericus* Strain KCTC 3346T', *Genome Announc,* 1 (4).

Kamilova, F., Leveau, J. H., and Lugtenberg, B. (2007), '*Collimonas fungivorans*, an unpredicted in vitro but efficient *in vivo* biocontrol agent for the suppression of tomato foot and root rot', *Environ Microbiol,* 9 (6), 1597-603.

Kim, M., et al. (2014), 'Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes', *Int J Syst Evol Microbiol,* 64 (Pt 2), 346-51.

Knudsen, Guy R. and Dandurand, Louise-Marie C. (2014), 'Ecological complexity and the success of fungal biological control agents', *Advances in Agriculture,* 2014, 1-11.

Kohlmeier, Stefanie, et al. (2005), 'Taking the Fungal Highway: Mobilization of Pollutant-Degrading Bacteria by Fungi', *Environmental Science & Technology,* 39 (12), 4640-46.

Leveau, J. H. and Preston, G. M. (2008), 'Bacterial mycophagy: definition and diagnosis of a unique bacterial-fungal interaction', *New Phytol,* 177 (4), 859-76.

Leveau, J. H., Uroz, S., and de Boer, W. (2010), 'The bacterial genus *Collimonas*: mycophagy, weathering and other adaptive solutions to life in oligotrophic soil environments', *Environ Microbiol,* 12 (2), 281-92.

Li, B., et al. (2014), 'Responses of beneficial *Bacillus amyloliquefaciens* SQR9 to different soilborne fungal pathogens through the alteration of antifungal compounds production', *Front Microbiol,* 5, 636.

Lohberger, Andrea., et al. (2019), 'Effect of organic carbon and nitrogen on the interactions of soil fungi and bacteria dispersing on fungal mycelium ', *Frontiers in microbiology*.

Lozano, L. C. and Dussan, J. (2013), 'Metal tolerance and larvicidal activity of *Lysinibacillus sphaericus*', *World J Microbiol Biotechnol,* 29 (8), 1383-9.

Mela, F., et al. (2011), 'Dual transcriptional profiling of a bacterial/fungal confrontation:

Collimonas fungivorans versus *Aspergillus niger'*, *ISME J,* 5 (9), 1494-504.
Mukherjee, M., et al. (2012), '*Trichoderma*-plant-pathogen interactions: advances in genetics of biological control', *Indian J Microbiol,* 52 (4), 522-9.
Mwangi, Erastus S.K., Gatebe, Erastus G. , and Ndung'u, Mary W. (2012), 'Impact of nutritional (c: n ratio and source) on growth, oxalate accumulation, and culture pH by *Sclerotinia Sclerotiorum*.', *Journal of Biology, Agriculture and Healthcare,* 2 (10).
Nadeem, S. M., et al. (2014), 'The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments', *Biotechnol Adv,* 32 (2), 429-48.
Naureen, Z., et al. (2017), 'Exploring the Potentials of *Lysinibacillus sphaericus* ZA9 for Plant growth promotion and biocontrol activities against phytopathogenic fungi', *Front Microbiol,* 8, 1477.
Poole, Elizabeth J., et al. (2001), 'Bacteria associated with *Pinus sylvestris–Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro'*, *New Phytologist,* 151 (3), 743-51.
R Core Team (2014), 'R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.'.
Rudnick, M. B., van Veen, J. A., and de Boer, W. (2015a), 'Oxalic acid: a signal molecule for fungus-feeding bacteria of the genus Collimonas?', *Environ Microbiol Rep,* 7 (5), 709-14.
--- (2015b), 'Baiting of rhizosphere bacteria with hyphae of common soil fungi reveals a diverse group of potentially mycophagous secondary consumers', *Soil Biology and Biochemistry,* 88, 73-82.
Sansinenea, E. and Ortiz, A. (2011), 'Secondary metabolites of soil *Bacillus* spp', *Biotechnol Lett,* 33 (8), 1523-38.
Sansinenea, Estibaliz, et al. (2016), 'Cellular damage of plant pathogenic fungi by antifungal compounds produced by *Bacillus* spp. isolates', *Chemistry and Ecology,* 32 (8), 722-32.
Scully, Lisa R. and Bidochka, Michael J. (2005), 'Serial passage of the opportunistic pathogen *Aspergillus flavus* through an insect host yields decreased saprobic capacity', *Canadian Journal of Microbiology,* 51 (2), 185-89.
Shabanamol, S., Sreekumar, J., and Jisha, M. S. (2017), 'Bioprospecting endophytic diazotrophic *Lysinibacillus sphaericus* as biocontrol agents of rice sheath blight disease', *3 Biotech,* 7 (5), 337.
Song, C., et al. (2015), 'Exploring the genomic traits of fungus-feeding bacterial genus *Collimonas'*, *BMC Genomics,* 16, 1103.
Stanley, C. E., et al. (2014), 'Probing bacterial-fungal interactions at the single cell level', *Integr Biol (Camb),* 6 (10), 935-45.
Weber, T., et al. (2015), 'antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters', *Nucleic Acids Res,* 43 (W1), W237-43.
Whipps, John M. (2001), 'Microbial interactions and biocontrol in the rhizosphere', *Journal of Experimental Botany,* 52 (suppl_1), 487-511.
Xu, K., et al. (2015), 'Genome comparison provides molecular insights into the phylogeny of the reassigned new genus *Lysinibacillus*', *BMC Genomics,* 16, 140.
Zhang, S., Gan, Y., and Xu, B. (2016), 'Application of plant-growth-promoting fungi *Trichoderma longibrachiatum* T6 enhances tolerance of wheat to salt stress through improvement of

antioxidative defense system and gene expression', *Front Plant Sci,* 7, 1405.

Supplementary information

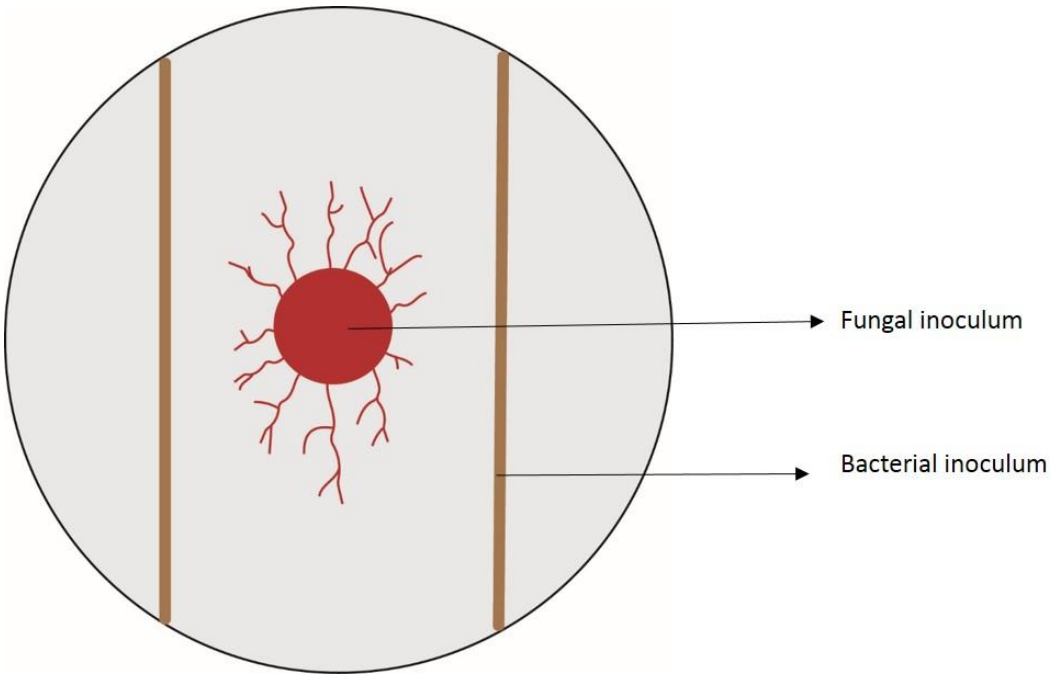


Figure S1: Scheme summarizing the design of the bacterial-fungal confrontation assays.

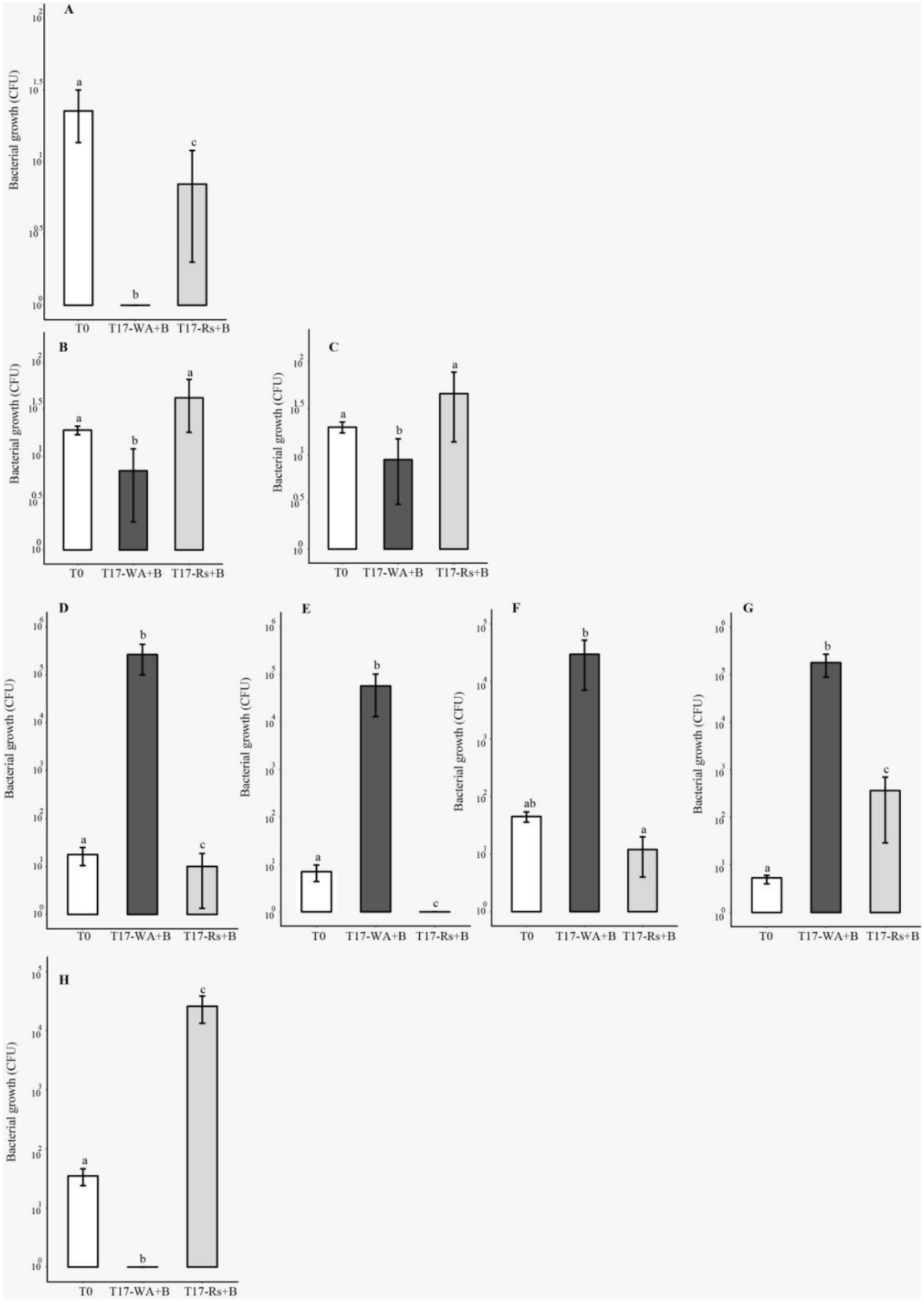

A
B
C
D
E
F
G
H
Bacterial growth (CFU)
T0
T17-WA+B
T17-Rs+B

Figure S2: Panel highlighting the four different group of interactions observed in the mycophagy experiment with the plant pathogenic fungus *R. solani*. T0= Day 0, corresponding to the time of bacterial inoculation. T17- WA+B = Growth of bacteria on water agar medium 17 days after bacterial inoculation. T17-Rs+B= Growth of bacteria on the mycelium of *Rhizoctonia solani* as a sole C source 17 days after bacterial inoculation. (A) First group consisting of *Bacillus thuringiensis* 1310. The number of bacterial cells decreased between T0 and T17, both in the control medium and in the presence of the fungus, (B-C) Second group consisting of (B) *Bacillus subtilis* 1055 and (C) *Bacillus thuringiensis* 1311. The number of bacterial cells did not drastically change between T0 and T17 and between fungal mycelia and the water agar medium. (D-F) Third group consisting of (D) *Bacillus cereus* 88, (E) *Bacillus subtilis* 16, (F) *Bacillus thuringiensis* 1312, and (G) *Bacillus polymyxa* 25. The number of bacterial cells increased between T0 and T17 in water agar (T17-WA+B), but not with the fungal mycelium (T17- Rs+B). (H) Fourth group consisting of *Lysinibacillus sphaericus* 1003). The number of bacterial cells increased in the presence of *R. solani* fungal mycelium as a sole C source. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's post hoc test.

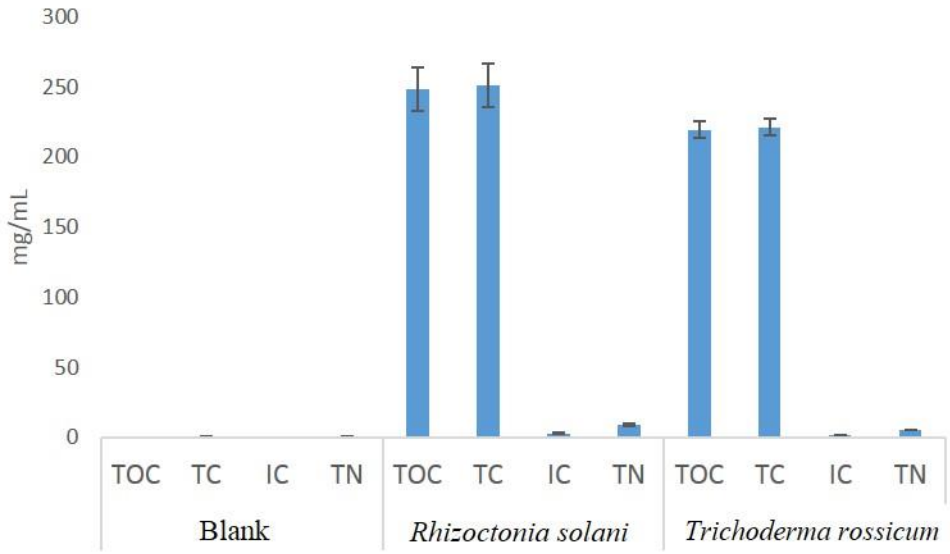


Figure S3: Carbon and nitrogen contents of the fungal exudates of *Rhizoctonia solani* and *Trichoderma rossicum*, measured by liquiTOC. TOC=Total organic carbon, TC= Total carbon, IC= Inorganic carbon, TN= Total nitrogen.

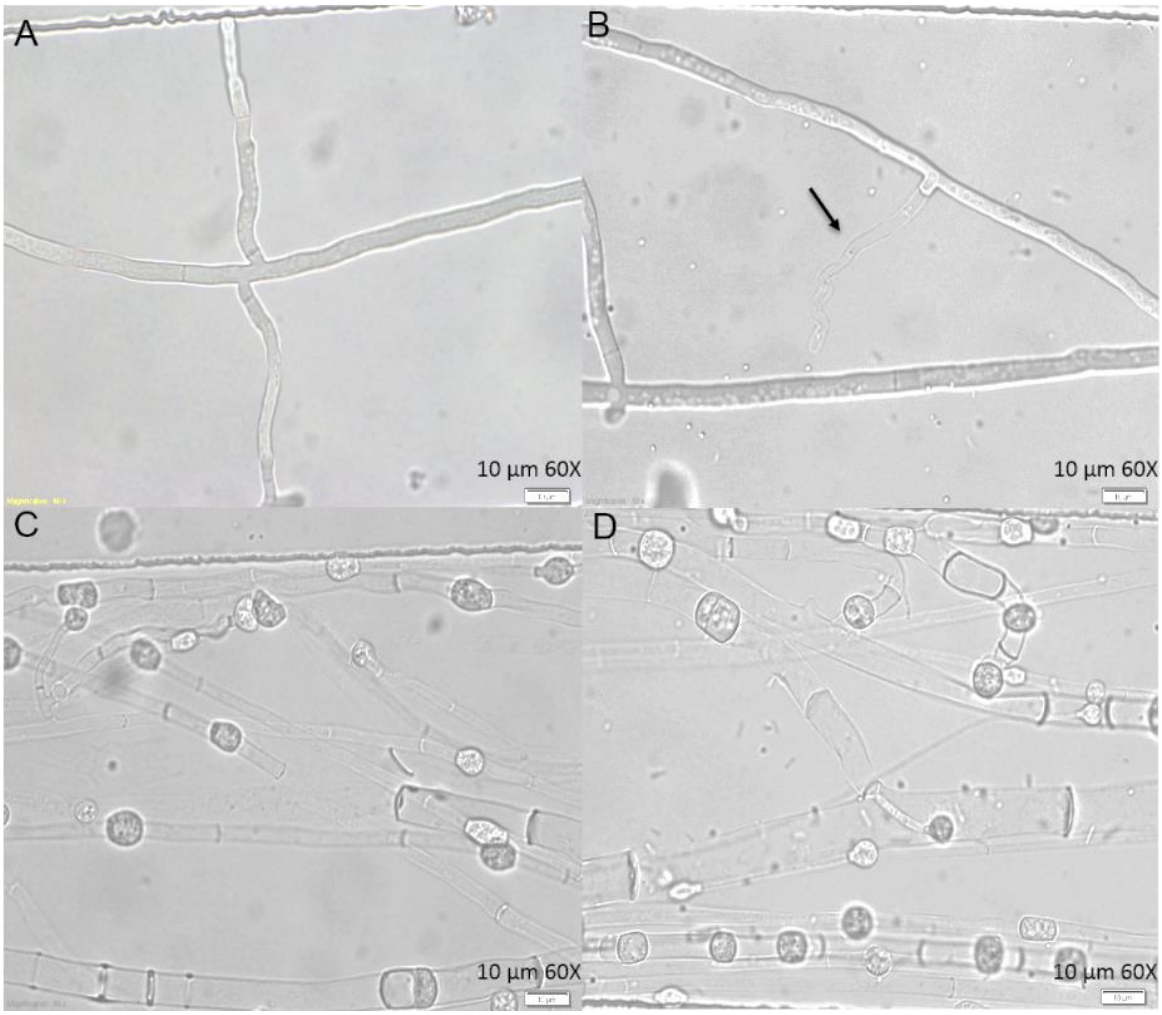


Figure S5: Inverted microscopy images of *Rhizoctonia solani* (A) without and (B) with *Lysinibacillus sphaericus* 1003 and showing hyphal deformation only with the bacterium (black arrow); *T. rossicum* (C) without and (D) with *Lysinibacillus sphaericus* and showing no difference in the morphology of hyphal cells in both conditions. Scale bars =10 um.

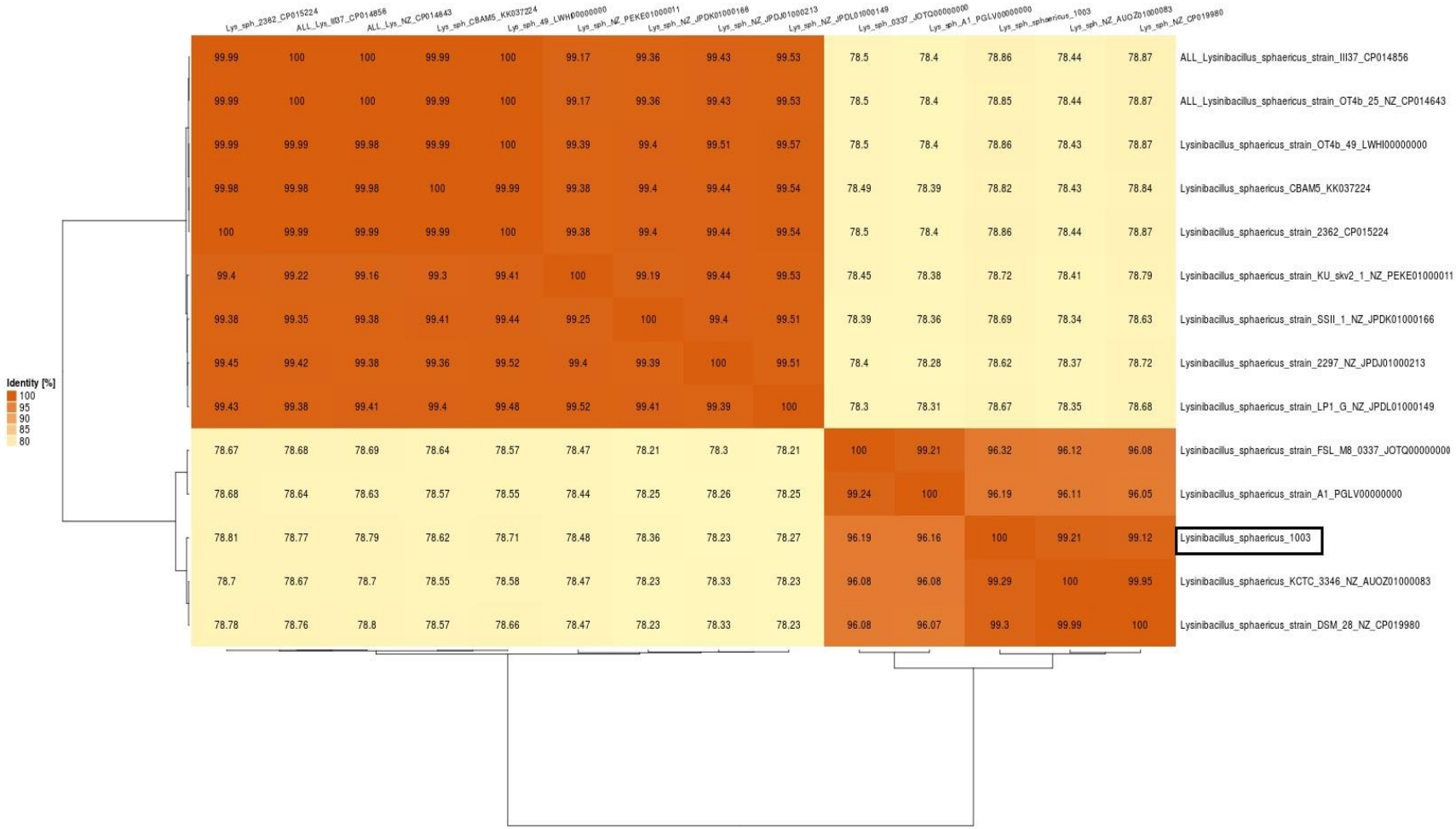


Figure S6: Heat map representation of the average nucleotide identity (ANI) between *Lysinibacillus sphaericus* 1003 and 13 other *Lysinibacillus sphaericus* using Edgar software and highlighting high (dark orange) to low similarities (light orange).

Table S1: Microbial strains and their source of isolation, B stands for bacterial and F for fungal strains.

Type of organism	Microbial strains	Origin/Source of isolation
B	*Bacillus thurigiensis* 1310	Lirima, Atacama, Chile
B	*Bacillus subtilis* 1055	Merrel syrup (probiotics)
B	*Bacillus cereus* 88	Soil from the botanical garden, Neuchâtel, Switzerland
B	*Bacillus thuringiensis* 1311	Soil, Lirima, Atacama, Chile
B	*Bacillus subtilis* 16	Lab contamination, laboratory of microbiology, University of Neuchâtel.
B	*Bacillus thuringiensis* 1312	Soil, Lirima, Atacama, Chile
B	*Bacillus polymyxa 25*	Soil, Canton of Neuchâtel, Switzerland
B	*Lysinibacillus sphaericus* 1003	Wellcome Collection of Bacteria, Wellcome Research Laboratories, Beckenham, Kent, UK
F	*Rhizoctonia Solani*	Origin unknown, laboratory of microbiology, Universits of Neuchâtel
F	*Trichoderma rossicum*	Soil, Cameroon (Bravo et al. 2013)

Table S2: List of the 14 *Lysinibacillus* sp. strains used for comparative genomic analyses and showing their NCBI assembly number, isolation source, ecology, and associated reference.

No.	Name	Assembly	Isolation source	Location/ Country	Pathogenic/ Nonpathogenic strains	Ecology	Reference
1	*Lysinibacillus sphaericus* C3-41	GCA_000017965.1	Mosquito breeding site	China	Pathogenic	This strain is entomopathogenic, mesophilic spore forming bacteria that has been used as a biocontrol of mosquito. It has inabilty to utilize most of the sugars except N-acetylglucosamine. In contrast, protein-metabolizing systems were abundantly present.	(Hu et al. 2008)
2	*Lysinibacillus sphaericus* KCTC 3346	GCF_000427235.1	NA	NA	Non-pathogenic	This strain encodes genes responsible for insecticidal toxins. It also contains nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS) gene cluster but demonstrated no antifungal activity against plant pathogenic fungus i.e., Rhizoctonia solani, Botrytis cinerea. This strain also showed no plant growth-promoting activity against Arabidopsis thaliana.	(Jeong et al. 2013)
3	*Lysinibacillus sphaericus* strain DSM 28	GCF_002982115.1	Mosquito	France			(Jeong et al. 2013)
4	*Lysinibacillus sphaericus* strain 2297	GCF_000733505.1	Dead mosquito larve	Ceylon, Sri Lanka	Pathogenic	This strain has mosquitocidal activity	(Xu et al. 2015)
5	*Lysinibacillus sphaericus* strain A1	GCF_002923795.1	Municipal solid waste soil	Woodley Reading, United Kingdom		It is a lignin-degrading bacterium which also boost the production of methane gas.	(Rashid et al. 2017)
6	*Lysinibacillus sphaericus* strain OT4b.25	GCF_001581875.1	Coleopteran larvae	Cundinamarca, Colombia	Pathogenic	This strain showed larvicidal activity in both vegetative and spores form, also showed tolerance to lower concentration of arsenate and chromimum	(Lozano et al. 2011; Lozano and Dussan 2013; Rey et al. 2016)
7	Lysinibacillus sphaericus, strain: SSII-1	GCF_000733525.1	Dead mosquito larve	India	Pathogenic	This strain has mosquitocidal activity	(Xu et al. 2015)
8	*Lysinibacillus sphaericus* strain: III(3)7	GCA_001598075.1	Soil of Oak forest	Colombia	Pathogenic	This strain is only pathogenic in the vegetative state and showed high tolerance towards lead, arsenate and chromium	(Lozano and Dussan 2013)

9	*Lysinibacillus sphaericus* strain: CBAM5	GCA_000568835.1	Hydrocarbon contaminated soil, Savanna	Colombia		This strain is pathogenic in vegetative and sporulated cultures. It showed high tolerance to 200 mM of arsenic and also carry arsenate reductase gene, also found two chitin-binding proteins and also chitin deacetylase enzyme,	(Lozano and Dussan 2013; Peña-Montenegro et al. 2015)
10	*Lysinibacillus sphaericus* strain: OT4b.49	GCA_001623495.1	beetle larvae	Savanna, Colombia	Pathogenic	This strain is pathogenic in vegetative and sporulated cultures, showed highest tolerance to lead, arsenate and chromium	(Lozano and Dussan 2013)
11	*Lysinibacillus sphaericus* strain: LP1-G	GCA_000733575.1	Culex quinquefasciatus	Singapore	Pathogenic	This strain has mosquitocidal activity	(Xu et al. 2015)
12	*Lysinibacillus sphaericus* strain: 2362	GCA_001629735.1	Blackfly	Nigeria	Pathogenic	This entomopathogenic strain encodes genes responsible for mosquitocidal toxin proteins found.it also showed tolerance to metal.	(Hernandez-Santana et al. 2016)
13	*Lysinibacillus sphaericus strain: FSL M8-0337*	GCA_001728805.1	Raw milk	New York, USA			(Hernandez-Santana et al. 2016)
14	Lysinibacillus sphaericus strain: KU skv2 1	GCF_002751965.1	Microbial mat on an anthropogenic pipe	Russia			

Table S3: List of 519 singletons found in *L. sphaericus* 1003 genome when compared with the one of *Lysinibacillus sphaericus* KCTC-3346 (=DSM 28).

Link for the data

https://github.com/ishahashmi/Mycophagy_Table-S3-and-Table-S4.git

Table S4: Comparison of putative enzymes that might play role in a mycophagous lifestyle in *Lysinibacillus sphaericus* 1003, *L. sphaericus* KCTC-3346, and *L. sphaericus* DSM 28. Legends color indicate the corresponding putative enzymes present in each three genomes: chitinase ( ), peptidase/protease ( ), lysophospholipase ( ), siderophore ( ), chemotaxis ( ), flagellar ( ), and some putative enzymes, which were absent or present with different names ( ).

Link for the data

https://github.com/ishahashmi/Mycophagy_Table-S3-and-Table-S4.git

Table S5: Biosynthetic gene cluster found in the genome of *L. sphaericus* 1003 and corresponding to Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) encoding secondary metabolites predicted by antiSMASH. The location, percentage of homology with known biosynthetic gene cluster, and MIBiG (Minimum Information about a Biosynthetic Gene cluster) reference number are indicated.

Number	Function	Secondary metabolite	Cluster category	Location	Most similar known cluster	MIBiG Biosynthetic gene cluster-ID
1	Antifungal/ Antibacterial		Type I PKS-NRPS	31619 - 100971	Zwittermycin A biosynthetic gene cluster (11% of genes show similarity)	BGC0001059:
2		Terpene	Putative cluster of unknown type	201526-214088	Carotenoid_biosynthetic_gene_cluster (33% of genes show similarity)	BGC0000645_c1
3		siderophore	Siderophore	264879-282131		
4	Antifungal	Unknown	Putative cluster of unknown type	362228-367161	Plipastatin_biosynthetic_gene_cluster (23% of genes show similarity)	BGC0000407_c1
5		Unknown	Type III PKS	202419-243675		
6	Antibacterial	Unknown	other	31152-74328	Kijanimicin_biosynthetic_gene_cluster (4% of genes show similarity)	BGC0000082_c1
7	Antifungal	Unknown	Putative cluster of unknown type	146165-154777	Acinetobactin_biosynthetic_gene_cluster (8% of genes show similarity)	BGC0000294_c1
8	Antibacterial	Unknown	Putative cluster of unknown type	42315-52563	Nosiheptide_biosynthetic_gene_cluster (15% of genes show similarity)	BGC0000610_c1
9		Unknown	Putative cluster of unknown type	68997-75924	Butirosin_biosynthetic_gene_cluster (7% of genes show similarity)	BGC0000693_c1
10	Antifungal	Bacteriocin	Bacteriocin	595997 - 606233		

References

Bravo, D., et al. (2013), 'Isolation of oxalotrophic bacteria able to disperse on fungal mycelium', *FEMS Microbiol Lett,* 348 (2), 157-66.

Hernandez-Santana, A., Gomez-Garzon, C., and Dussan, J. (2016), 'Complete Genome Sequence of *Lysinibacillus sphaericus* WHO Reference Strain 2362', *Genome Announc,* 4 (3).

Hu, X., et al. (2008), 'Complete genome sequence of the mosquitocidal bacterium *Bacillus sphaericus* C3-41 and comparison with those of closely related *Bacillus* species', *J Bacteriol,* 190 (8), 2892-902.

Jeong, H., et al. (2013), 'Genome Sequence of *Lysinibacillus sphaericus* Strain KCTC 3346T', *Genome Announc,* 1 (4).

Lozano, L. C. and Dussan, J. (2013), 'Metal tolerance and larvicidal activity of L*ysinibacillus sphaericus*', *World J Microbiol Biotechnol,* 29 (8), 1383-9.

Lozano, L. C., Ayala, J. A., and Dussan, J. (2011), '*Lysinibacillus sphaericus* S-layer protein toxicity against *Culex quinquefasciatus*', *Biotechnol Lett,* 33 (10), 2037-41.

Peña-Montenegro, Tito David, Lozano, Lucia, and Dussán, J.enny (2015), 'Genome sequence and description of the mosquitocidal and heavy metal tolerant strain *Lysinibacillus sphaericus* CBAM5. ', *Standards in genomic sciences,* 10 (2).

Rashid, G. M., et al. (2017), 'Delignification and enhanced gas release from soil containing lignocellulose by treatment with bacterial lignin degraders', *J Appl Microbiol*.

Rey, A., Silva-Quintero, L., and Dussan, J. (2016), 'Complete Genome Sequence of the Larvicidal Bacterium *Lysinibacillus sphaericus* Strain OT4b.25', *Genome Announc,* 4 (3).

Xu, K., et al. (2015), 'Genome comparison provides molecular insights into the phylogeny of the reassigned new genus *Lysinibacillus*', *BMC Genomics,* 16, 140.

Chapter 6

Collaborative studies

In addition to the chapters presented in this thesis, this chapter briefly presents collaboration projects that are linked to this PhD thesis. This includes the setting-up of microfluidics to investigate fungal-fungal interactions, the comparison of DNA sequencing results from the consortium field trial from chapter 4 obtained with two type of soil microbial biomass DNA extraction methods, and the *in planta* assay with the mycophagous bacteria characterized in chapter 5.

Investigation of fungal-fungal interactions at a cellular level

Collaboration with the research group of Dr. Claire Stanley (Agroscope) and Professor Anil Wipat (Newcastle University)

In order to increase our understanding of fungal-fungal interactions (FFI) at a cellular level, a collaboration with Dr. Claire Stanley (Agroscope) was established for the design and evaluation of a novel microfluidic device, the FFI device (Figure 1). The overall design of the device was conducted by Dr. Stanley. My contribution to this project consisted in the selection of an interesting fungal-fungal couple that could be used to test the effectiveness of the FFI device. The selected couple was *Trichoderma rossicum* and *Rhizoctonia solani*. In addition, we took the opportunity of the positive interaction leading to the dispersal of bacteria on the mycelium of *T. rossicum* (fungal highways (Bravo et al. 2013; Kohlmeier et al. 2005), to demonstrate the importance of this dispersal mechanism in unsaturated systems. We aimed at observing the dispersal of selected bacteria through fungal highways of *T. rossicum* and the effect of this mechanism on the phytopathogenic fungus *R. solani*. For this, the microfluidic device was used "unsaturated", that is without any liquid medium added to the microfluidic channels, to mimic an unsaturated environment. For this experiment, an inoculum consisting of *T. rossicum* and selected bacteria was placed on one side of the device, while on the other side, a plug of *R. solani* was placed. Two types of bacterial inocula were tested: the first one consisted in the three *Bacillus*

strains acting as a plant growth promoting consortium investigated in Chapter 4 (*Bacillus thuringiensis* 1312, *B. thuringiensis* 1310, and *B. licheniformis*); the second one consisted in *Lysinibacillus sphaericus*, the strain identified in Chapter 5 as possessing a differential mycophagous lifestyle towards *R. solani* and *T. rossicum*.

In this experiment, it was observed that both fungi were able to colonize the unsaturated FFI device (Figure 2A and B). Water condensation caused by fungal hyphae colonizing the device was noticed in the channels (Figure 2C). This was likely the result of water production during fungal respiration. Due to the high density of the fungal mycelial network (Figure 2D), the observation of bacterial growth along fungal hyphae was impaired. It was also difficult to distinguish both fungi, as their mycelia appeared similar. To overcome this problem, another plant pathogenic fungus, a tagged *Fusarium graminearum* (PH1 dsRED), will be used in future experiments currently performed by Dr. Stanley. In addition to this, a second collaboration was established with the group of Prof. Anil Wipat (University of Newcastle) with the aim to generate fluorescently tagged bacteria from the *Bacillus* consortium, in order to help in the visualization of the three bacteria in *in vitro* experiments and to investigate their distribution on fungal hyphae in the devices. Indeed, fluorescent strains will make easier to differentiate the three bacteria from the consortium in the interaction devices in the presence of dense mycelium.

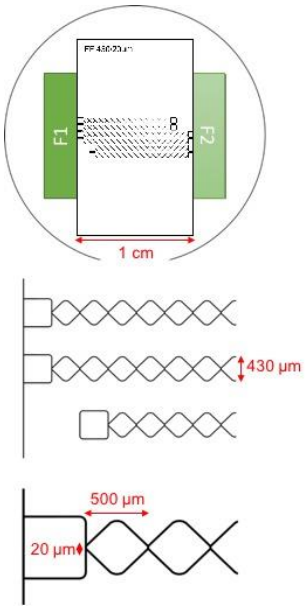


Figure 1: Design of the Fungal-fungal interaction device showing channels and their dimensions. F1 stands for one fungus and F2 stands for the second fungus.

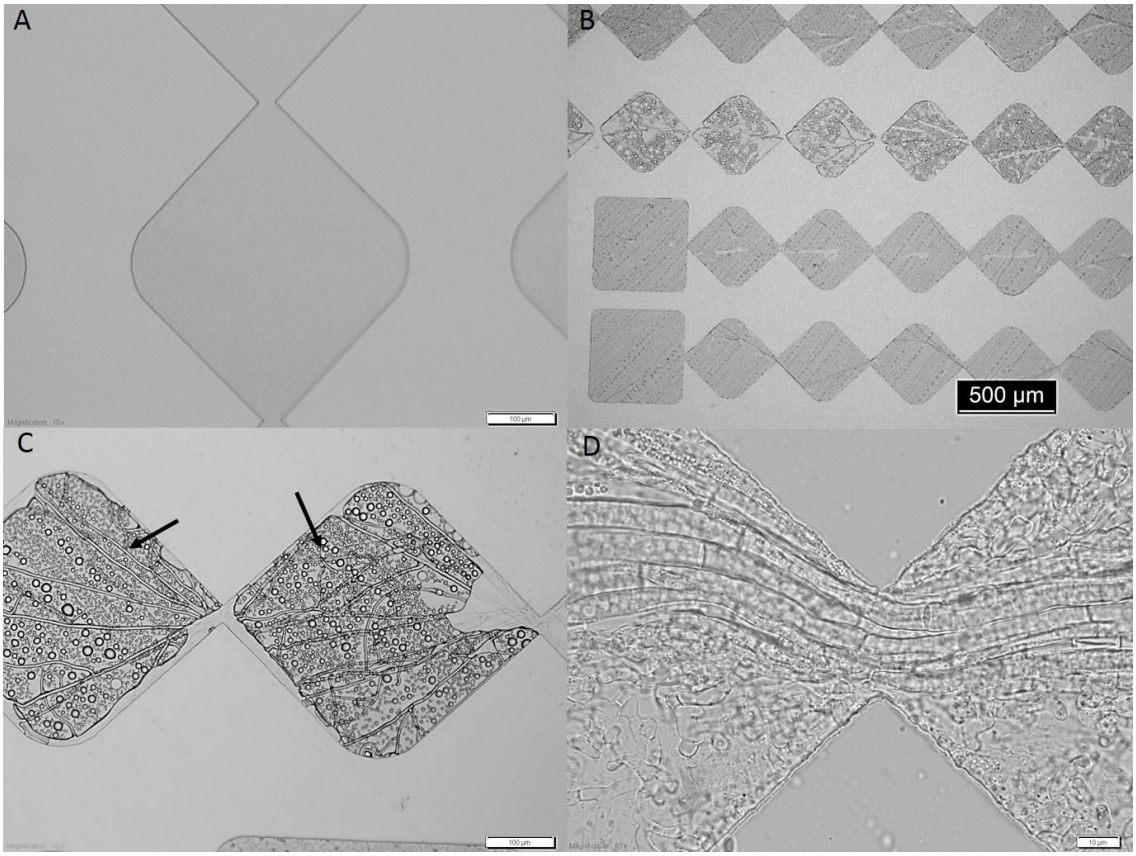


Figure 2: Interaction of *T. rossicum* and *R. solani* in an unsaturated microfluidic device after five days of co-inoculation. (A) View of an empty observation channel of the microfluidic device (bar = 100 µm) prior to fungal colonization. (B) Overview of the fungus (*R. solani*) growing in the microfluidic device (bar = 500 µm). (C) Condensed liquid phase present in the channels of the device due to fungal growth (bar = 100 µm). (D) Observation channel where interaction between both fungi can be observed. In this interaction channel it is impossible to distinguish the hyphae of either fungus (bar = 10 µm).

As perspective of these collaborations, we expect:

1- To repeat the experiments using the tagged bacterial strains, something that will help to observe bacterial dispersal and distribution of the strains on the mycelial network of both fungi.

2- To repeat the experiment using tagged fungal strains to distinguish between the two strains competing in the device.

Comparison of different extraction methods to assess the diversity of bacterial and fungal communities

Collaboration with Christophe Paul (University of Neuchâtel)

During the analysis of the effect of bio-inoculation of a bacterial consortium on autochthonous soil microbial communities (Chapter 4), some of the methodological questions that needed to be addressed were the selection of the best method for DNA extraction. Besides this, the selection of fungal markers to use as target to investigate the diversity of the whole fungal community (both higher and lower fungi) also represents a critical step. Regarding DNA extraction, the research conducted at the laboratory on the diversity of endospore-forming bacteria has demonstrated that most commonly used DNA extraction methods fail in the detection of this group (Filippidou et al. 2015; Wunderlin et al. 2013). An indirect DNA extraction method consisting in a treatment to separate the biomass followed by successive bead-beating steps to access the DNA in resistant cells such as endospores was developed for sediments. Such an approach showed a superior performance for bacterial diversity studies as compared to DNA extraction following the manufacturer's guidelines (Wunderlin et al., 2013). However, the effect of this indirect DNA extraction method on the assessment of fungal diversity was not investigated previously. Fungi, similarly to bacteria, produce spores, which harbor a thicker cell wall and therefore are likely to resist to traditional DNA extraction methods. Therefore, both methods, the direct and the indirect DNA extractions were applied to all the samples of the *Bacillus* consortium study (Chapter 4). We observed that the DNA extraction method clearly affected the structure of both, bacterial and fungal communities (Figure 3 and 6, respectively). In the case of bacterial communities, both DNA extraction methods clearly showed that the root compartment was distinct from other samples (rhizospheric and bulk soils). However, in the indirect method a higher dispersal of the samples in the PCoA plot could be observed for the root fraction (Figure 3). By using the indirect method, we highlighted more diverse bacterial communities in roots samples, while with the direct method, the roots samples were clustered together. The indirect method was basically used to access DNA of bacterial cells that are hard structures (such as endospores), making their DNA extraction difficult. Bacterial communities differed when comparing both extraction methods as a result of the extra DNA that was accessed thanks to the indirect method

(Figure 3). Moreover, the most abundant bacterial classes found by both extraction methods are shown in Figure 4. It is noteworthy to mention an increase in Bacilli can be observed for the indirect method in the root system, in particular in the treatment with vegetative cells. Analysis of the overlap between the two methods showed that about 25% of the OTUs are unique to either systems (Figure 5A). However, when OTUs that were only present at a minimum of 10 occurrences were considered, the fraction of unique OTUs to each system droped considerably (Figure 5B).

For the fungal community analysis, the 18S rRNA gene was targeted using the primer pair AMV4.5NF and AMDGR, which have been specifically designed for the analysis of Arbuscular mycorrhizal fungal communities (AMF; Glomeromycota) (Sato et al., 2005). However, sequences assigned to other Divisions were also found to be highly represented in our community analysis, highlighting the issues of the specificity of the primers (Figure 7). Moreover, regarding fungal communities, the PCoA obtained using the results obtained with the indirect DNA extraction method did not show a clear pattern of differentiation between soil compartments (Figure 6). Despite the fact that the root samples were still partly differentiated from the other soil samples, this distinction is not as clearly observed as for the bacterial communities in the indirect DNA extraction or with the results of the direct DNA extraction with fungi. Likewise, when analyzing the overlap of the communities (Figure 5C-D), the fraction of unique OTUs was of 50% for the direct DNA extraction method and of 30% in the indirect DNA extraction method, suggesting that either methods accessesed a very different community. A hypothesis to explain these results is that the indirect method of extraction may target predominantly fungal spores (hard structures), while with the direct DNA extraction, mycelial cells are mainly extracted. This is supported by the analysis of the composition of the communities at the class level, which shows large differences among both extraction methods (Figure 7).

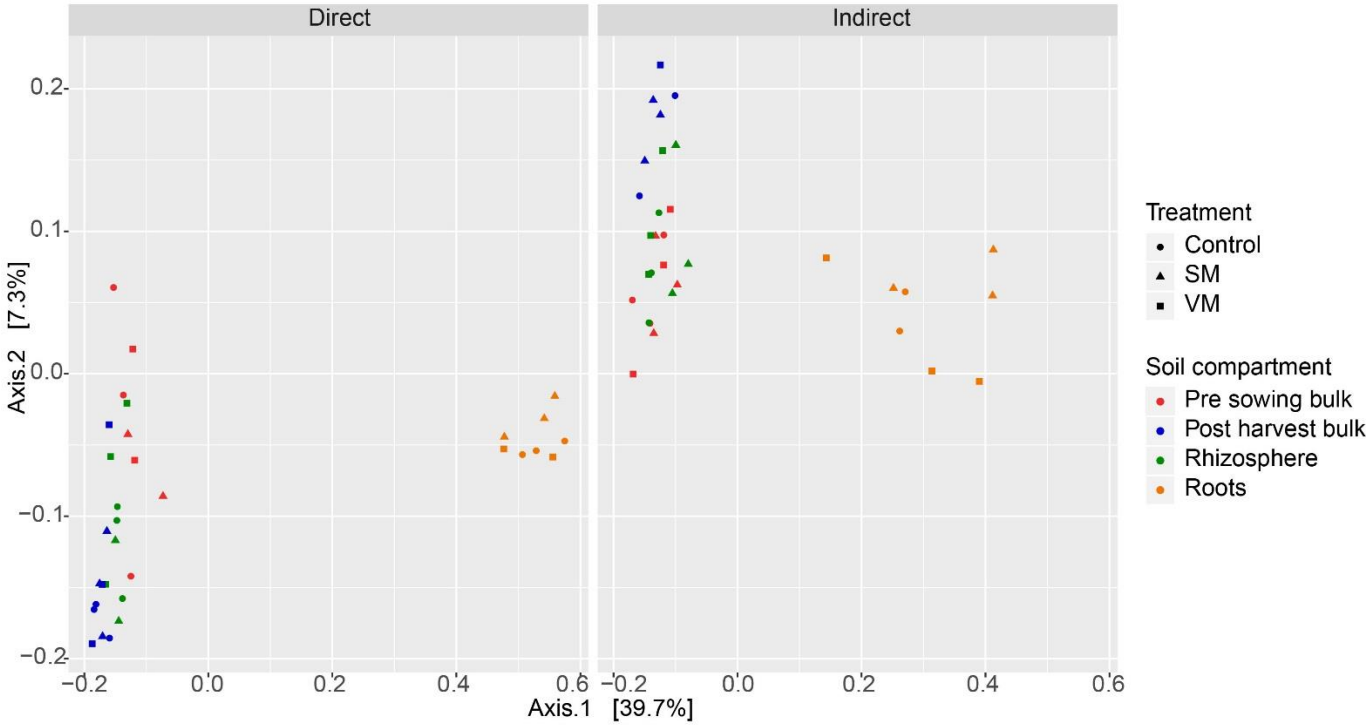


Figure 3: PCoA of bacterial communities associated to oat plant roots, as well as rhizospheric soil and bulk soils, before (pre-sowing) and after (post-harvest) the experiment. The PCoA based on the communities obtained by two different DNA extraction methods (direct and indirect) is shown in each panel.

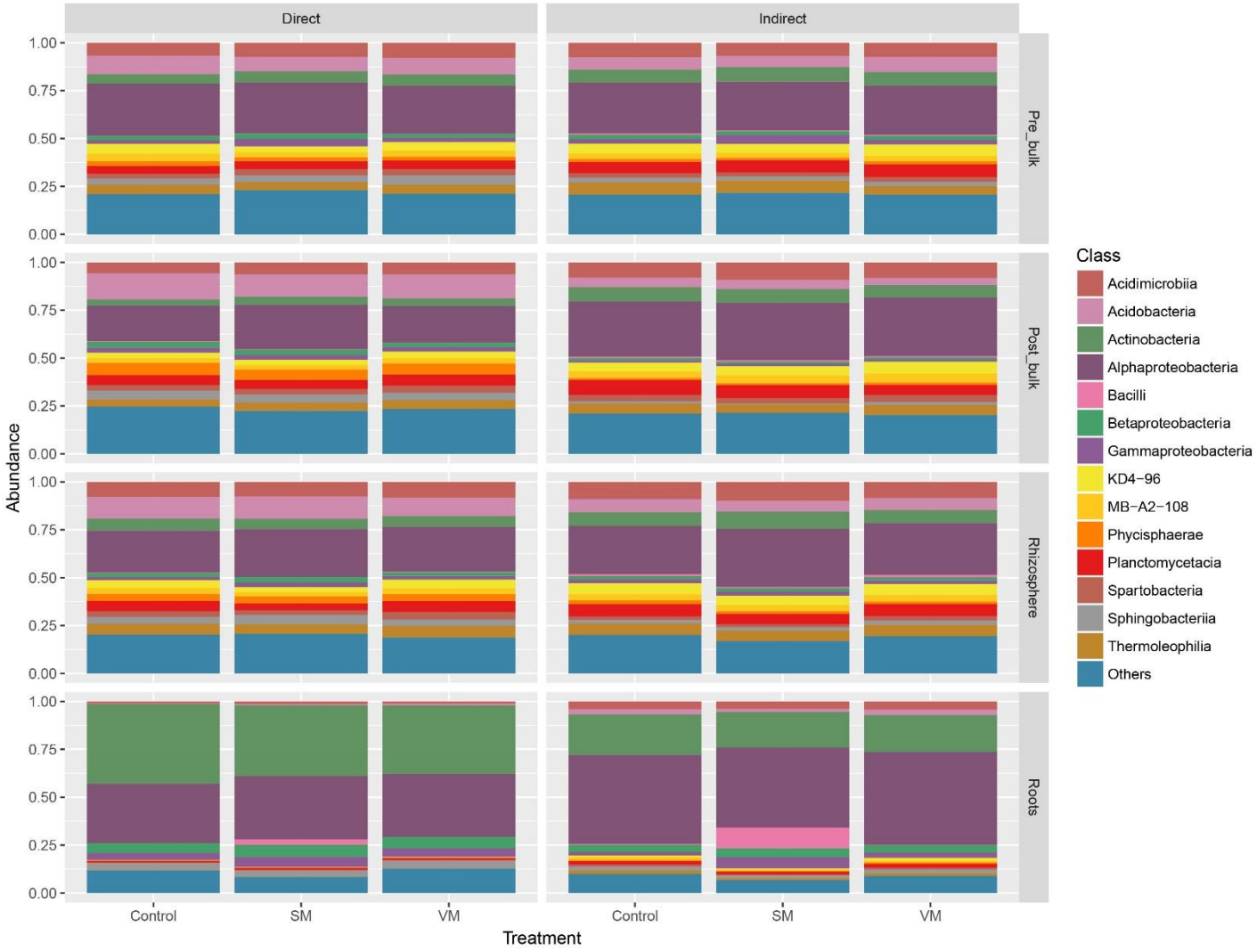


Figure 4: Most abundant classes of bacteria found when comparing direct and indirect DNA extraction methods applied to the soil samples from Chapter 4.

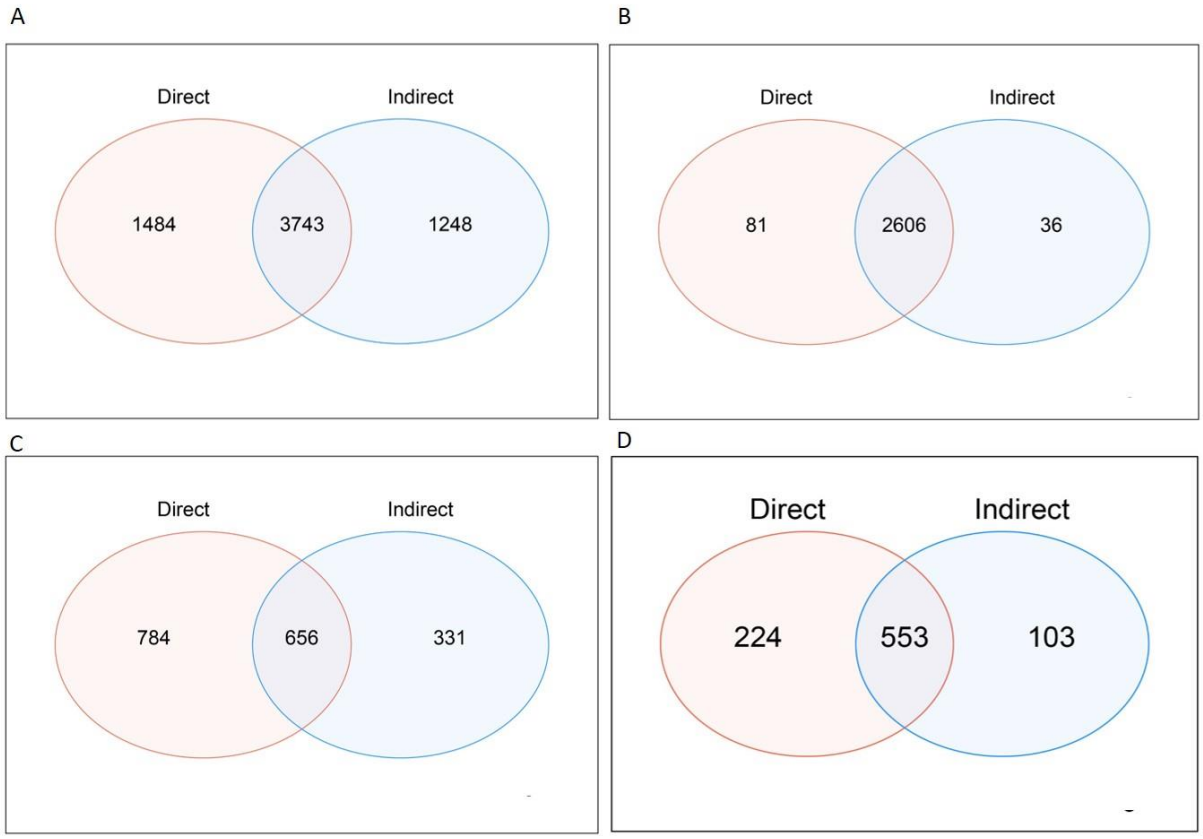


Figure 5: Venn diagram of the OTUs obtained through direct and indirect DNA extraction methods. (A) Venn diagram of all of the bacterial OTUs found in either DNA extraction methods (B) Venn diagram of the bacterial OTUs which were present at a minimum of 10 occurrences, (C) Venn diagram of all of the fungal OTUs found in either DNA extraction methods, and (D) Venn diagram of the fungal OTUs which were present at a minimum of 10 occurrences.

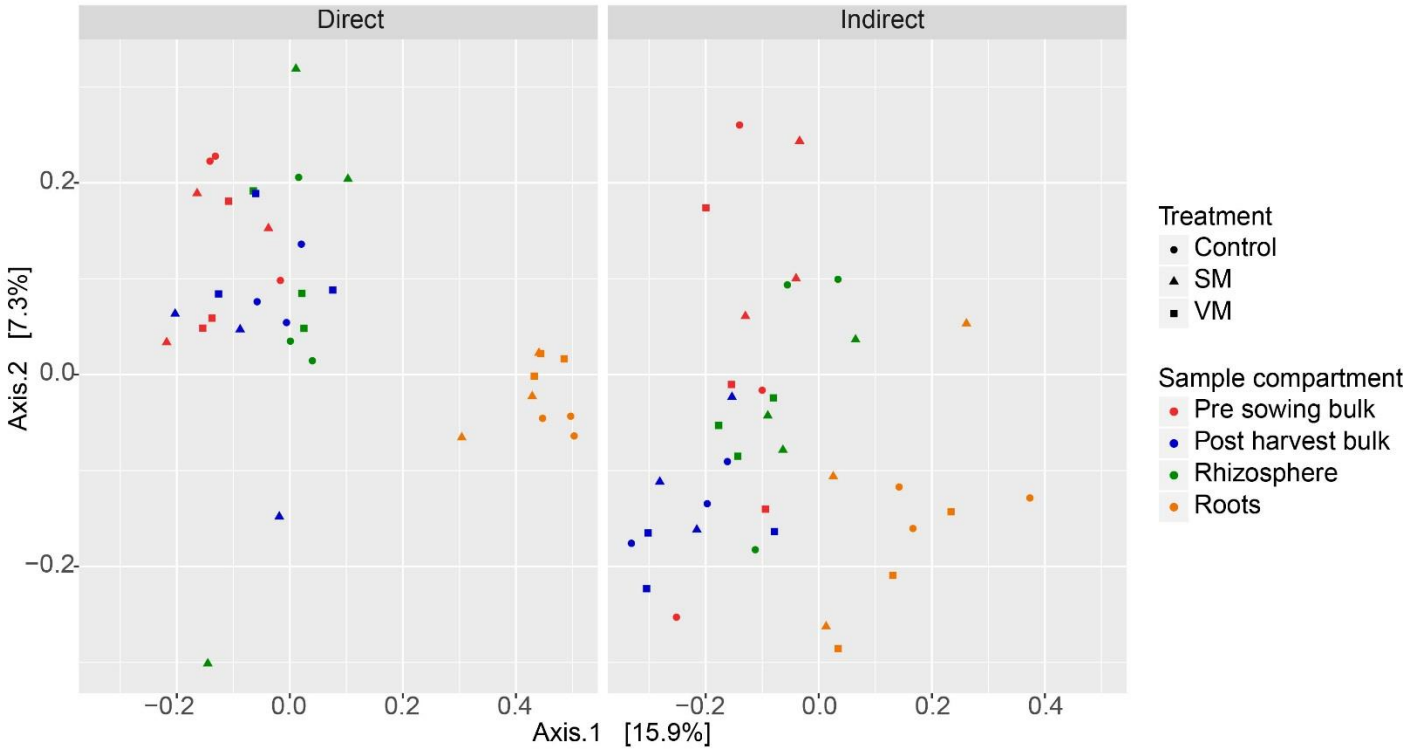


Figure 6: PCoA of fungal communities associated to oat plant roots, as well as rhizospheric soil and bulk soils, before (pre-sowing) and after (post-harvest) the experiment and extracted by two different DNA extraction methods, a direct and an indirect extraction (see text for details).

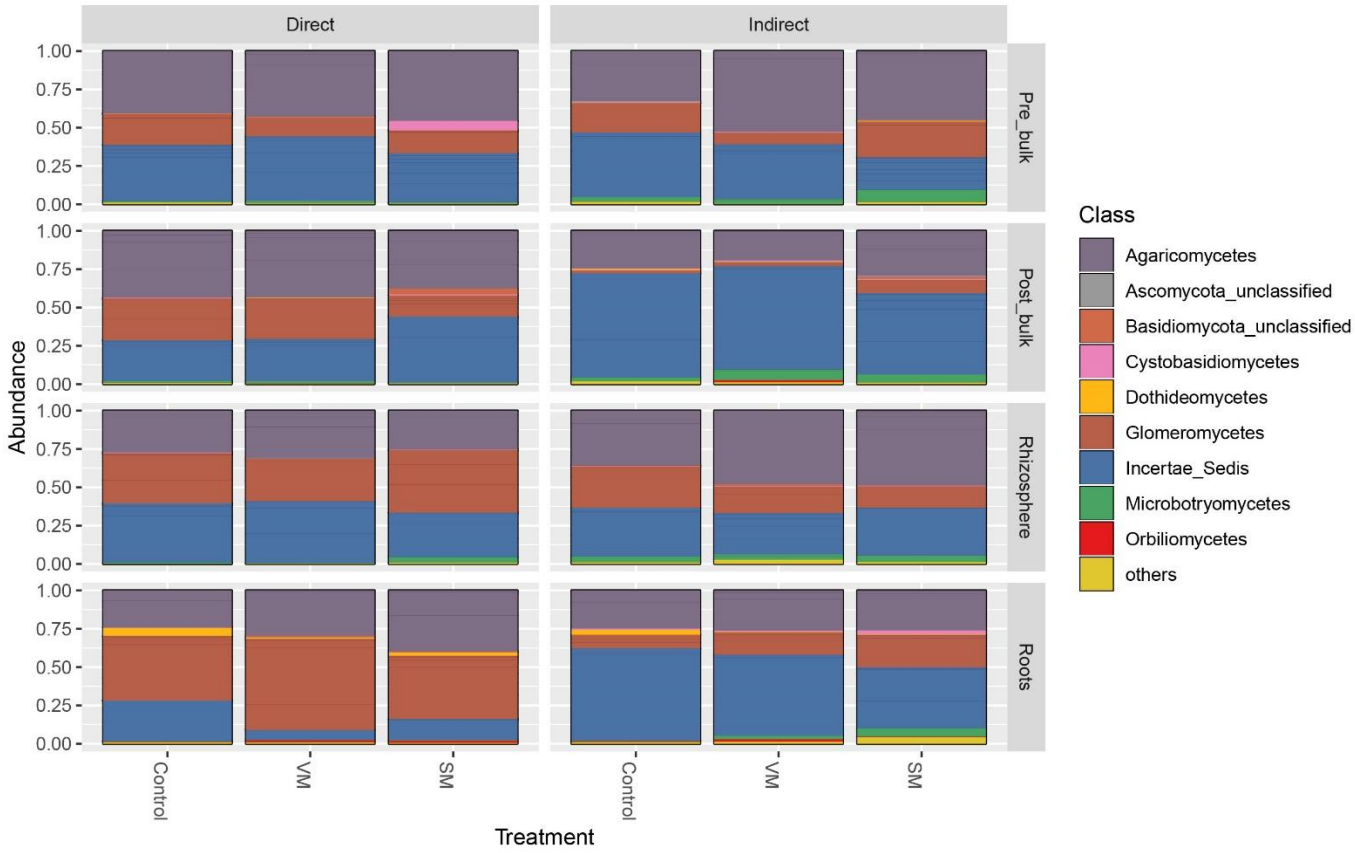


Figure 7: Most abundant classes of fungi found when comparing direct and indirect DNA extraction methods applied to the soil samples from Chapter 4.

Evaluation of *L. sphaericus* 1003 as a biocontrol agent using an *in planta* greenhouse pot experiment

Collaboration with Clément Etter (Master thesis in Biology 2018, University of Neuchâtel)

In order to investigate the effect of co-inoculation of *L. sphaericus* 1003 and *T. rossicum* for the biological control of *R. solani* infecting plants, an *in planta* greenhouse pot experiment was performed by (Etter 2018). Lettuce (*Lactuca sativa*) plants were used as a crop model to investigate the potential of *L. sphaercius* 1003 to act as a biocontrol agent against *R. solani* without a negative impact on the saprotroph *T. rossicum*, as demonstrated in chapter 5. For this, a greenhouse pot experiment was conducted at the University of Neuchâtel. Four treatments were compared: healthy control plants without *R. solani*, plants infected with *R. solani,* healthy plants co-inoculated with *T. rossicum* and *L. sphaericus*, plants infected with *R. solani* and co-inoculated with *T. rossicum* and *L. sphaericus*. Ten replicates were made for each treatment. 700 mL pots were filled with autoclaved substrate (Potting soil Capito, Landi Schweiz AG, Dotzigen, Switzerland). Five seeds of lettuce var. red salad bowl (Samen Mauser AG, Winthertur, Switzerland) were sown and covered with a thin layer of around 0.05 cm of 2 mm sieved substrate. The healthy treatments consisted in only autoclaved substrate without any inoculum of *R. solani.* In the treatments with *R. solani*, each pot contained 2 g of a *R. solani* inoculum that was previously grown on soaked and sterilized millet seeds. The inoculum of *R. solani* was prepared using 100 g of dry millet seeds that were rinsed 5 times with distilled water and autoclaved 2 times. First, *R. solani* was grown on MA medium for 3 days and then 25 agar plugs were taken from the MA colonized medium. The plugs were then added into the autoclaved millet seeds along with 30 mL distilled water. Inoculated millet seeds were incubated for 14 days in the dark at room temperature (23°C). For the co-inoculation treatment of *T. rossium* and *L. sphaericus* 1003, *L. sphaericus* 1003 was grown overnight in 15 mL NB medium and centrifuged at 5867 g (Sigma 2-16PK) for 8 min. The supernatant was discarded, and the pellet was washed with 10 mL of physiological water. The washing step was repeated twice. At the end, the bacterial pellet was suspended in 10 mL physiological water. Spores of *T. rossicum* were collected by adding 10 mL of 0.016% Tween 80 (0.16g/L) on the surface of a one-month old *T. rossicum* culture grown on a MA medium. The Petri dish was gently shaken for 2 min and the suspension retrieved in a 15 mL falcon

tube. The number of spores in the recovered suspension was assessed by direct counting using a Neubauer chamber. For the preparation of the co-inoculation solution, 4 mL of the bacterial suspension and 3 mL of *T. rossicum* spore suspension were mixed together and adjusted to 60 mL with sterile tap water. The final concentration of vegetative cells of *L. sphaericus* 1003 and *T. rossicum* spores in the solution were 10^6 cells/mL and 10^4 spores/mL, respectively. The co-inoculation treatment was carried out by adding 3 mL of microbial solution containing vegetative cells of *L. sphaericus* and spores of *T. rossicum* in pots. Pots were irrigated daily with tap water to maintain the substrate moisted and randomized regularly to change the place of pots. After 10 days, lettuce seedlings were picked to keep only one plant per pot. The experiment lasted for 50 days and shoots and washed roots were dried at 60°C for 3 days to determine their dry weight using a fine scale balance. Dry weight of roots and shoots were measured as plant parameters.

The co-inoculation of *L. sphaericus* 1003 and *T. rossicum* failed to protect lettuce plants against *R. solani*. Co-inoculation of bacteria and both fungi caused a 37% reduction of root biomass as compared to control plants; however, this difference was not statistically significant (*p* value>0.05; one-way ANOVA followed by Tukey post hoc test). Similar results were found for the shoot biomass. *R. solani* significantly reduced shoot biomass in all treatments (*p* value <0.05), and the co-inoculation had no significant effect on shoot biomass, either with or without *R. solani*. Noteworthy, no leaf necrosis caused by *R. solani* was observed in any of the treatments, suggesting that the treatment with the fungal pathogen might not have been optimal (Figure 8).

This experiment showed that despite the antagonistic and mycophagous behavior of *L. sphaericus* 1003, a positive effect on lettuce growth infected or not with *R. solani* was not observed. It is worth mentioning that in this *in planta* trial, we used spores of *T. rossicum* which might not have germinated. As a result, bacteria were likely not able to disperse through fungal highways and for this reason, was not affective in the biocontrol of *R. solani*. Addtionally, it might be the way of inoculation of *L. sphaericus* 1003 and *T. rosscium* that could be optimized. Indeed, the method of inoculation also play important role in the inhibition of phytopathogenic fungi or in the protection of plants against plant pathogens. (Abeysinghe 2009), investigated the use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 as a combined biocontrol strategy against the damping off disease caused by *R. solani*. They found that disease severity was lower when seeds were

coated with *B. subtilis* CA32 and *T. harzianum* RU01 was applied directly to the soil. However, the outcome of the bio-inoculation was not the same when the treatment was applied the other way around that is seeds coated with a conidial suspension of *T. harzianum* RU01 and bacterial suspension applied in the soil. In this second mode of application of exactly the same microbes, disease severity was not reduced in a similar way and the effect was the same as with the application of *T. harzianum* RU01 alone. This highlights the importance of the method of inoculation for an effective biocontrol activity. In this study, we applied the bio-inoculants (*L. sphaeriucs* 1003 in vegetative cells and *T. rossicum* in spore form) together in the soil and this may not have been the ideal way of inoculation. For instance, T. rossicum spores may have failed to germinate and thus bacterial colonization of plant roots through fungal highways may have been reduced.

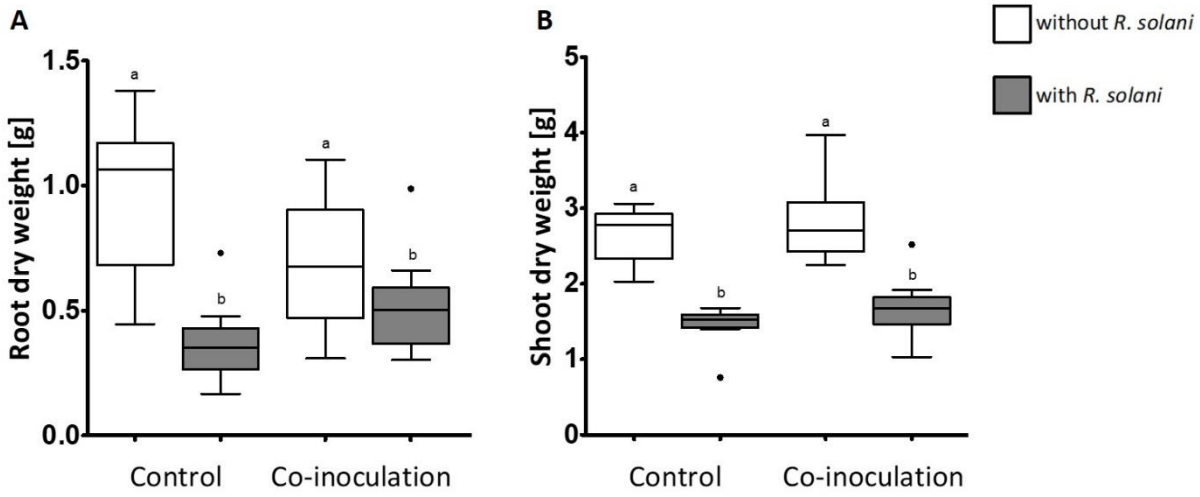


Figure 8: Effect of the co-inoculation of *Lysinibacillus sphaericus* 1003 and *Trichoderma rossicum* on the growth of lettuce plants infected or not with *Rhizoctonia solani*. Dry root (A) and shoot (B) weight were measured after 50 days of inoculation in substrate.

References

Abeysinghe, S. (2009), ' Effect of Combined Use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on Biological Control of *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum.*', *Plant Pathology Journal,* 8, 9-16.

Bravo, Daniel, et al. (2013), 'Isolation of oxalotrophic bacteria able to disperse on fungal mycelium', *FEMS Microbiology Letters,* 348 (2), 157-66.

Etter, Clément . (2018), 'Evaluation of a microbial consortium encapsulated in agar beads for the biocontrol of the fungal pathogen *Rhizoctonia solani* in lettuce', *Master thesis in Biology , University of Neuchâtel*.

Filippidou, Sevasti, et al. (2015), 'Under-detection of endospore-forming Firmicutes in metagenomic data', *Computational and structural biotechnology journal,* 13, 299-306.

Kohlmeier, Stefanie, et al. (2005), 'Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi', *Environmental Science & Technology,* 39 (12), 4640-46.

Wunderlin, T., et al. (2013), 'Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming Firmicutes', *Environ Microbiol Rep,* 5 (6), 911-24.

Chapter 7

General discussion and perspectives

The general motivation to develop this thesis was to propose an alternative to the numerous problems surrounding traditional agriculture (i.e. inappropriate agricultural practices and soil degradation, among others). The use of microorganisms is considered as one of these suitable alternatives. However, and despite the potential identified in various studies (Ahemad and Kibret 2014; Bhattacharyya and Jha 2012; Souza et al. 2015), ensuring the success of bio-inoculation still presents many challenges (Ahemad and Kibret 2014; Bhattacharyya and Jha 2012; Souza et al. 2015). Among those, the survival of bio-inoculants in soils, their delivery in an active form, and their competition with autochthonous microbial communities, are some of the challenges that need to be addressed urgently (Souza et al. 2015; Trabelsi and Mhamdi 2013) These concerns are an incentive to identify not only more efficient ways of inoculating microorganisms into natural environments, but also to ensure their long-term survival. Therefore, we started our study by screening the potential of selected endospore forming bacterial strains and fungi of different ecological niche for plant growth promoting activities. We chose endospore-forming bacteria (specifically *Bacillus* spp.), as their name suggest, as a group of bacteria that share the ability to form endospores in response to unfavorable environmental conditions. Endospores are highly resistant dormant cells that promote persistence in soils (Radhakrishnan et al. 2017), and could thus, promote persistence of the bio-inoculant. We found that the majority of the bacterial strains tested possessed one or more plant growth promoting traits. We also screened fungi with contrasting lifestyles in order to confront them further with bacteria. The aim was then to highlight bacterial-fungal association that were either antagonistic, neutral or synergistic and that could be used as combined bio-inoculants.

Bio-inoculation strategies

Based on the screening results (chapter 3), three different bacterial strains were selected based on the consideration of their PGP activities. Individual inocula, as well as inoculation as a

consortium of the three bacterial strains were applied using oat as a plant model (chapter 4). The bio-inoculants were applied as vegetative cells or endospores on the seeds and their effect were tested in *in vitro* sterile conditions, pot experiments and a field trial. One of the most important results obtained in the present study is that consortia had a more robust positive effect on oat biomass than inoculation with individual strains. More specifically, the bacterial inoculum applied in the form of vegetative cells had a higher effect in terms of total dry weight and grain yield of oat plants, as compared to the inoculum containing endospores, even though the effect of bio-inoculation with endospore was still superior to the results from the un-treated control plants. Inoculation of oat seeds with single strains or consortia of three bacteria increased germination rate as compared to un-inoculated seeds. Therefore, direct seed coating seems to have beneficial effects on seed germination. In the current study, we choose seed inoculation over foliar spray because seed inoculation introduces the required inoculants directly in the spermosphere and then into the soil at the vicinity of the emerging roots. It has been reported that bio-inoculation of seeds reduces seed-borne diseases caused by soil phytopathogens (O'Callaghan 2016). Furthermore, the application method and density of the bacterial inoculants play a vital role on plant growth promotion (Ciccillo et al. 2002). The positive effect of bacterial inoculant on the growth of plant has extensively been studied, but the importance of a reduced cell number during bio-inoculation and its effect on plant growth is rarely discussed. In this study, the bio-inoculants were applied at low cell densities on the seeds as we found that oat seeds carried approximately 10^3 cells of bio-inoculated bacteria. With such a density we showed a positive effect on both seed germination and plant growth.

Impact of bio-inoculation on autochthonous microbes

Besides assessing the beneficial effect of bio-inoculation on plant growth, another aspect often neglected in studies dealing with the use of bio-inoculants is the impact of this practice on the indigenous soil microflora (Korir et al. 2017; Kuan et al. 2016; Moustaine et al. 2017; Noumavo et al. 2013; Orhan et al. 2006; Widnyana and Javandira 2016). Investigating whether the applied consortium has an impact on the structure of the native microbial community is crucial for allowing further usage of the inoculum in replacement of agrochemicals in sustainable

agricultural ecosystems. We chose the use of a consortium in the present study because a consortium resembles best the complexity of the real soil environment where the functionality of the ecosystem is dependent on the cumulative result of many partners. Application of a bio-inoculant with more than one strain is not a new strategy and has been applied for more than a decade (Wang et al. 2018). However, only a few studies focused on their effect on native microbial communities (Trabelsi and Mhamdi 2013). This is of importance since a change in the indigenous communities induced by inoculation could lead to unpredictable and undesirable effects on both, plant and ecosystem health. It is important to consider that bio-inoculants could potentially have a major impact on the long-term functional capabilities of the resident microbial communities by changing their overall structure (Trabelsi and Mhamdi 2013). Several studies have reported that bacterial inoculants alter the bacterial community composition after inoculation (García de Salamone et al. 2012; Schwieger and Tebbe 2000; Trabelsi et al. 2012). Such effects should not be ignored. We observed that the consortium used here positively affected oat plant growth. This effect was less prominent in non-sterile soil as compared to a sterile artificial substrate, highlighting the importance of the competition with the native microbial communities and demonstrating that consortia are more robust than single strain inocula. We also observed that the consortia successfully colonized the roots and the rhizosphere of oat plants, without modifying the overall structure of the autochthonous soil microbial communities. We found that the *Bacillus* spp. remained a minor fraction (3-6%) of the bacterial community even after the experiment and some of these were likely the bio-inoculated strains as suggested by the outcome of a BLAST search. This demonstrates that the bio-inoculated strains were able to colonize the rhizosphere of oat plants, but not to outcompete native bacteria. However, in order to confirm colonization and to establish the abundance of individual species, specific primers to quantify the applied inoculant through quantitative PCR needs to be developed in the future experiment. This is now possible as we have already sequenced the full genome of these strains. Likewise, in the future one could consider observing the interaction of the inoculated strains with roots in a microfluidic device, in order to increase our understanding of the dynamics of plant-microbe-microbe interactions at a cellular level.

Other factors influencing microbial inoculants

Furthermore, in our study, we did not check the effect of different species ratio on the performance of the bacterial inoculant and its effect on native microbial communities. In the study reported by (Wang et al. 2018) they investigated the effect of the same multi species microbial inoculant (*Ensifer* sp. NYM3, *Acinetobacter* sp. P16 and *Flavobacterium* sp. KYM3) but at two different ratios (2:1:2 vs. 1:10:1). They found that despite the fact that both microbial inoculants enhanced the growth of cucumber, a difference in the ratio of the three different species significantly affected the indigenous bacterial communities. Moreover, in the same study, they found that different ratios have a significant effect on soil properties (urease activity, phosphatase activity, pH, total nitrogen, invertase activity), which also explained the differences observed for the bacterial communities. In our study, we did not observe a drastic change in the soil physicochemical properties after bacterial inoculation, which might explain the lack of an effect on soil microbial communities. To verify this, experiments using different soil properties (or soil type) and with different ratio of the bacterial communities should be performed.

Moreover, in the literature, the plant species as well as different stages of development also play a significant role in shaping microbial communities (Bailey et al. 2008; Wieland et al. 2001). The current study was performed with only one plant species and the effect of the consortium on plant growth and on the soil microbial communities were measured at a single time point (after 85 days of the experiment). However, no data was recorded at different time points/different stages of plant growth. In future studies, the plant factor and the different developmental stages of the corresponding plant species should be considered. This will shed light onto the effectivity and robustness of our consortium on different plants and its role in shaping the microbial community overtime. Moreover, with reference to food production, plant nutritional content is also an important factor and in the current study, we did not test the influence of the bio-inoculant on nutrient uptake and final nutrient content in oat plants. This factor needs to be considered in the future.

Additionally, above and below ground biotic and abiotic factors could play a crucial role in the functioning of the applied bio-inoculant. This is even more relevant in the current context of climate change, which may impact beneficial associations between microbes and plants.

Therefore, additional experiments with our consortia should aim at analyzing the abiotic factors shaping the microbial communities in association to the plant or that can alter the outcome of interactions between plants and microbes. It may be that our inoculated bacterial strains could impact differently plants in stress condition. The impact of elevated atmospheric carbon dioxide (CO_2) levels, higher temperatures and drought stress had thoroughly been reviewed by (Compant et al. 2010). In this review, the authors summarized 135 studies investigating the aforementioned stress factors and their effect on PGP microorganisms with reference to plant growth promotion. In this summary the authors found that both PGP bacteria and fungi displayed a positive effect on plants and mitigated drought stress, while for elevated CO_2, ectomycorrhizal and arbuscular mycorrhizal fungi had a positive effect on plant growth, while the effect of PGP bacteria was variable. Moreover, the effect of an increase in temperature induced diverse responses (positive, neutral or negative) on PGP bacteria and fungi.

Noteworthy, the bacterial and fungal communities associated to the roots were found to be different from the communities of the other soil compartments (bulk and rhizospheric soils), showing that oat roots had a unique root-associated community. This is in accordance with previous studies investigating the differences in the microbiome of roots and other soil compartments. This was true for the bacterial microbiomes of the roots and the surrounding soil of maize (Niu et al. 2017). Roots of the plants work as a hot spot, which continuously excrete compounds (known as root exudates), for instance, amino acids, organic acids, different kinds of lipids, proteins that play key role for the growth of microbial population (Bailey et al. 2008).

It is also important to mention that there is a need to investigate the plant-beneficial effects of the bio-inoculated strains via transcriptomics or similar technologies. This will allow to better pinpoint the molecular determinants/mechanisms that might be involved in plant growth promotion.

At the moment, it is unknown which genomic traits of the bacterial consortium were responsible for colonizing the rhizosphere of oat plants and also which gene network was responsible for the increased total dry weight and grain yield of oat plants. Also, examining the variation in genomic machinery of the three bacterial strains making the consortium and ultimately their individual contribution on plant growth is something that should be accomplished in the future.

Additionnaly, studying the changes in gene expression of the applied consortium confronted to different host would be useful. This will give new insights into the complex interactions involving plants and bio-inoculated bacteria.

Overall, our understanding of the variety of factors that could modify the plant growth promoting effect of bio-inoculants (summarized in Figure 1) is still in its infancy and this is surely a research area to investigate in the near future.

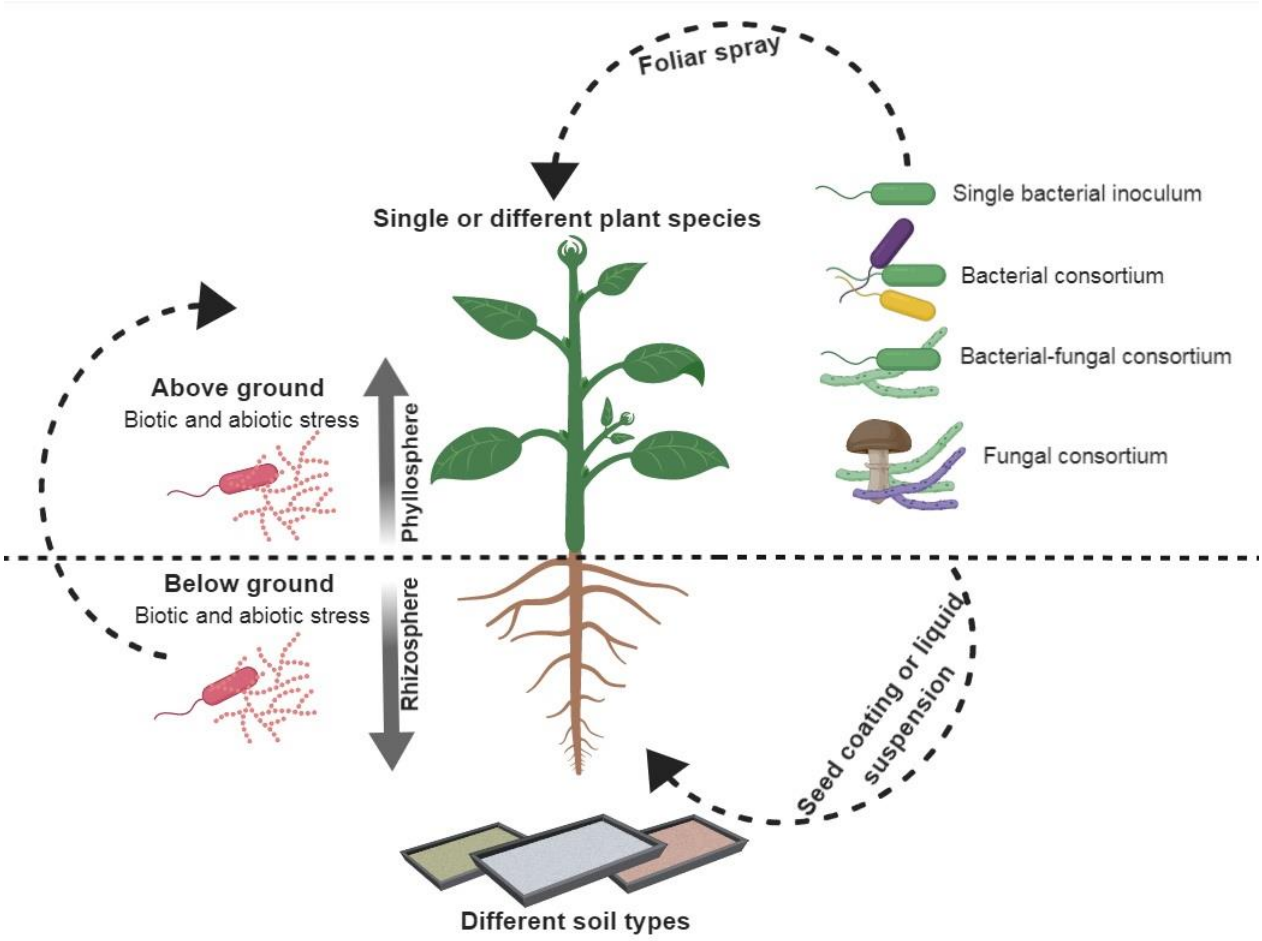


Figure 1: Scheme showing the different factors that could influence the effect on plant health and growth of plant growth promoting microorganisms.

Bio-inoculant formulation and delivery strategies

Along with *Bacillus*, there are many other genera within the phylum Firmicutes that are equally

important in agroecology and therefore there are still many organisms that may be investigated. In the frame of this study, a review article summing up the contribution of other genera of the phylum Firmicutes as plant beneficial microbes was written (Chapter 2). The aim was to emphasize the importance of these bacteria as beneficial microorganisms with reference to plant growth promotion (biofertilizers), their role in biocontrol of plant pathogens (biocontrol agent), as well as in the phytoremediation of heavy metals (metal uptake enhancers).

Besides bacteria, fungi also represent a significant portion of the rhizosphere microbiota. For this reason, the rhizosphere is sometimes referred to as the mycorrhizosphere to include this fraction (Timonen and Marschner 2006). With reference to plant-soil ecosystems, bacteria and fungi establish synergistic as well as antagonistic interactions and these interactions can promote plant growth and development (Deveau et al. 2018). This PhD thesis was built around this assumption and this is summarized in Figure 2. To explore the possibility of using combinations of different microorganisms to enhance bio-fertilization and/or biocontrol activity of bio-inoculants, we screened bacterial and fungal interactions by using different *Bacillus* strains and fungal strains from different ecological niches. The rational for this is that in natural environments, microorganisms interact in many ways and this leads to emerging properties supporting the functioning of the whole soil ecosystem. An interesting example of a synergistic interaction involving bacteria and fungi that was highlighted in this thesis was the dispersal of bacteria on fungal hyphae in soils, a mechanism called “Fungal highways” (Kohlmeier et al. 2005). Bacterial dispersal in soils is limited due to the unsaturated heterogeneous nature of the soil matrix. In contrast, thanks to their filamentous growth, fungi can easily disperse and cross air-filled gaps between soil aggregates. The disadvantage generated by limited-dispersal of bacteria in soils can be overcome by their dispersal on fungal hyphae. This general concept, defined as fungal highway, has been shown to be important for soil processes such as biodegradation (Kohlmeier et al. 2005; Wick et al. 2007) or soil functioning (Bravo et al. 2013; Martin et al. 2012). As mentioned above, we choose seed inoculation over foliar spray because the seed inoculation introduces the required inoculants directly in the spermosphere and then into the soil at the vicinity of the emerging roots. However, seed coating cannot always be used as a bio-inoculation strategy, because of limitation on the side of crop growers (for instance, the small size of the seed) and in

those cases, foliar spray or suspension of bio-inoculant might be a better option. In this case, the dispersal of bacteria in the soil will be limited and there is a risk that the bio-inoculant fails to reach plant roots, and will not provide a beneficial effect. This might explain already some of the inconsistent results of bio-inoculant currently applied in the field. It was reported by (Bravo et al. 2013) that the hyphae of *Trichoderma* sp. were used by oxalotrophic bacteria for their active dispersal in soil. Thus, it would be a good strategy to combine the plant growth promoting characteristics of *Trichoderma* spp., with their ability to act as a fungal highway in order to use it as a delivery system of PGPR in the field of agriculture.

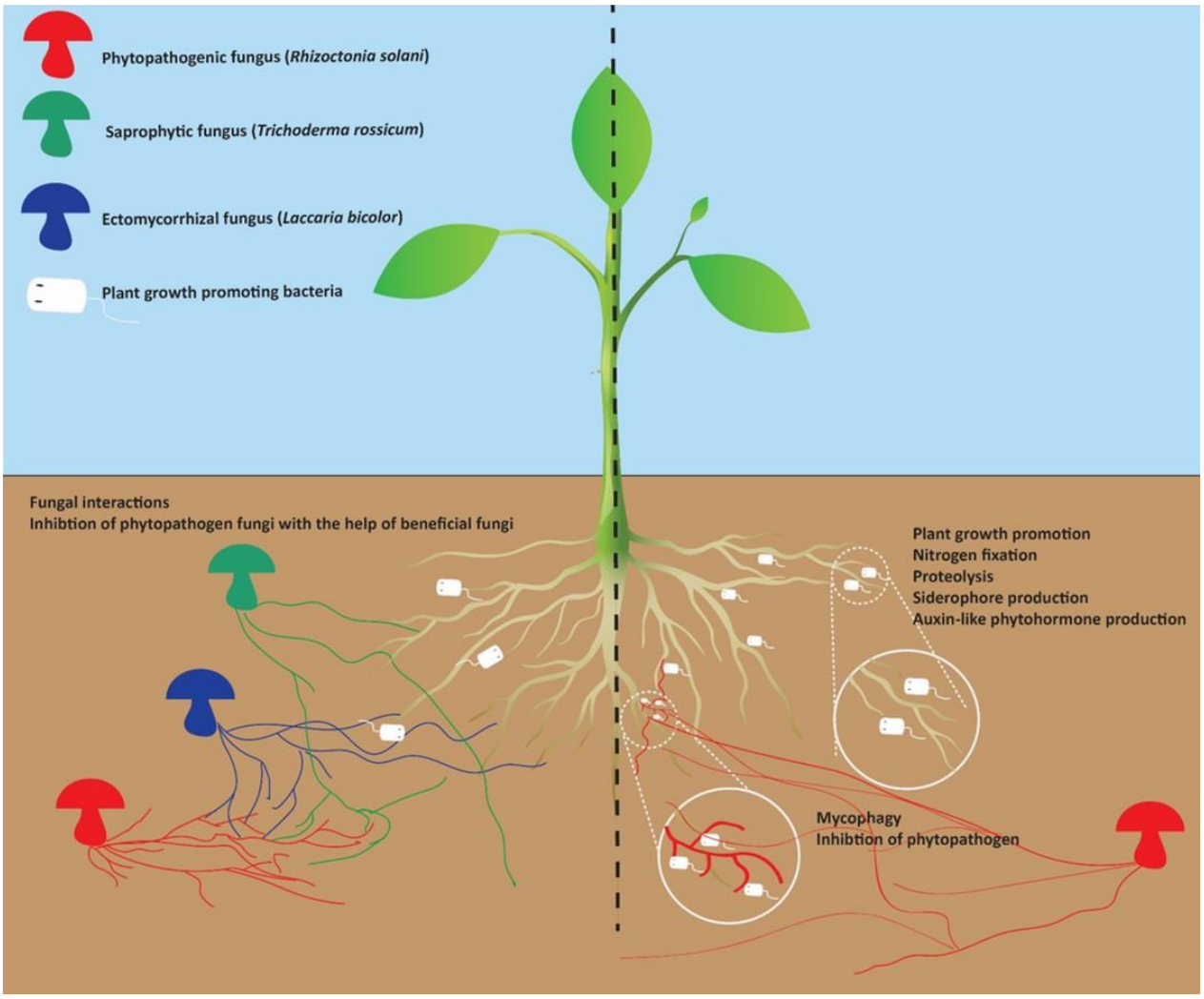


Figure 2: Pictorial summary of the whole study.

Besides these benefical interactions, many types of antagonistic interactions among bacteria and

fungi can be harnessed to improve application of PGP microorganisms in sustainable agriculture. For instance, fungi are not only important for dispersal but also provide nutritional niches for bacterial growth and development (Boer et al. 2005). Bacterial mycophagy is an example of an antagonistic trophic interaction in which bacteria can grow at the expense of fungal mycelium or fungal exudates (Leveau and Preston 2008). This nutritional strategy has been extensively studied in the bacterial genus *Collimonas* (De Boer et al. 2001). In this thesis, mycophagy was also investigated in a selected endospore-forming bacterial strain interacting with two fungi with contrasting ecology. To the best of our knowledge, a mycophagous lifestyle for the endospore forming *L. sphaericus* was reported for the first time in the current thesis. Our findings suggest that *L. sphaericus* 1003 has a preferential mycophagous lifestyle towards the phytopathogenic fungus *R. solani* as compared to *T. rossicum*. We showed that this bacterium can grow at the expense of the living fungal mycelium of *R. solani* and, more importantly, had a negative impact on fungal biomass. Observations made with microfluidic devices highlighted the importance of mycophagy on physical disturbance at the hyphal scale. The genome mining of *L. sphaericus* 1003 indicated the presence of metabolites and enzymes potentially involved in mycophagy. However, the exact differences in gene expression in the selective mycophahous lifestyle towards both fungi are unknown, and experiments designed at RNA sequencing are currently on going to investigate this in detail.

In the case of the interaction with *R. solani*, it is important to highlight the fact that the viability of this fungus upon interaction with *L. sphaericus* 1003 was also investigated and that it was found that parts of *R. solani* mycelial network that were fully colonized by bacteria were unable to re-grow. *Interestingly,* it was also found that *L. sphaericus* 1003 established a synergistic interaction on all media with *T. rossicum* by not inhibiting fungal growth. Moreover, the bacterium was able to use the fungal network of both fungi for its dispersal. Therefore, an *in planta* experiment (as explained previously in collaboration chapter 6) to evaluate the effect of the differential mycophagy of *L. sphaericus* (synergistic effect with *T. rossicum* and versus biocontrol effect against the plant pathogen *R. solani*) as a biocontrol strategy against *R. solani* was carried out (Etter 2018). This *in planta* experiment showed that despite the antagonistic and mycophagous behavior of *L. sphaericus* 1003, a positive effect on lettuce growth infected or not with *R. solani*

was not observed. It is worth mentioning that in the *in planta* test, we used spores of *T. rossicum* which might not have germinated, rendering the bacterial-fungal consortium ineffective. This points to the importance of the method of bio-inoculation for an effective protection of plants against their pathogens. For instance, (Abeysinghe 2009) investigated the use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 as a combined biocontrol strategy against the damping off disease caused by *R. solani*. They observed that disease severity was lower when seeds were coated with *B. subtilis* CA32 and *T. harzianum* RU01 was applied directly to the soil. However, the outcome of the bio-inoculation experiment was not the same when the treatment was applied the other way around, which is seeds coated with a conidial suspension of *T. harzianum* RU01 and bacterial suspension applied in the soil. In this second mode of application, disease severity was not reduced as much as in the previous treatment and the effect was the same as with the application of *T. harzianum* RU01 alone. This study highlights the importance of the method of inoculation to control the plant disease.

Investigating fungal-fungal interactions

Along with bacterial-fungal interactions, fungal-fungal interactions also play a vital role in the mycorrhizosphere. For this reason, in this thesis, we also investigated fungal strains of different niches and their interactions, so that we could identify promising fungal associations that could be used in the frame of sustainable agricultural approaches. In fungal-fungal interaction trials, it was observed that the ectomycorrhizal fungus *L. bicolor,* along with the saprophytic fungus *T. rossicum,* could strongly limit the growth of the phytopathogenic fungus *R. solani*. This work was followed up by a Master thesis (Kolly 2018) who investigated further this specific *in vitro* result by performing an *in planta* experiment using three different cultivars of wheat. He found that the combined treatment consisting of both *L. bicolor* and *T. rossicum* had no significant effect on any of the three wheat cultivars. This was likely due to the fact that *L. bicolor* was not grown in its ectomycorrhizal lifestyle, but in a saprophytic one, which might affect its competitive ability and thus its biocontrol activity. Nevertheless, using *L. bicolor* in its ectomycorrhizal form along with the saprophytic *T. rossicum* could constitute a promising biocontrol strategy in the future that could be used in an agro-forested context for instance. As mentioned earlier in this thesis, the

application of agrochemicals, and more specifically chemical fungicides that are being used in agricultural lands to control fungal pests, is no longer an entirely acceptable practice. This is due to their negative impact on the whole ecosystem. Dual inoculation of the ECM *L. bicolor,* along with *T. rossicum,* could provide an efficient combined fungal bio-inoculant able to compete with currently existing agrochemicals used for fighting plant diseases caused by *R. solani*. This fungal consortium would not only help by inhibiting the fungal pathogen, but through the ectomycorrhizal association, could also help in the uptake of nutrients and water by the target plant. To the best of my knowledge, there is no commercial fungal product combining the ectomycorrhizal fungus *L. bicolor* and *T. rossicum* for the inhibition of plant pathologies caused by *R. solani*. However, in order to understand how this fungal consortium could act as a more effective biocontrol agent, *in planta* studies with *L. bicolor* in symbiotic association with a host tree need to be performed to exploit the power of fungal microbes for sustainable agriculture and food security. This fungal consortium could be tailored to an agroforested cropping system including, for instance, wheat and poplar. In Switzerland, only one third of the area is considered as amenable to agriculture and this area is decreasing with time. Between 1985 and 2009, almost 850 Km^2 of the Swiss agricultural land had been lost (Sereke et al., 2015), together with a considerable decrease in the number of trees grown in these lands. In 1951, approximately 14 million trees were present and this number has been reduced to only 2.9 million trees in 2001 (Sereke et al., 2014). One of the main reasons of this loss is the unprofitability or unproductivity of agroforested systems that are not designed appropriately. In this context, a fungal consortium composed of *L. bicolor* along with *T. rossicum* could be used in a mixed cropping system, including trees and economical important crops, to control fungal diseases more efficiently.

Factors affecting microbial interactions

It is worth mentioning that in this study, one of the interesting findings of the confrontation assays mentioned earlier in chapter 3 and also in chapter 5 was the influence of the trophic factor on microbial interactions. We observed that either antagonistic, neutral or synergistic interactions not only depended on the competing microbes, but also on the media in which these interactions were taking place. For instance, on a PDA medium that has a C/N ratio of 10 (Mwangi et al. 2012),

bacteria were not able to inhibit the growth of *R. solani*; while on a NA medium (C/N ratio of 4-7), the same microorganisms were able to inhibit the growth of *R. solani*. Such type of interactions was not only observed when microbes of two distant taxonomic relationships were competing (e.g bacteria or fungi), but also for microbes that are somehow related. For instance, *B. edulis* was able to inhibit the growth of *P. ultimum* only on PDA medium, but not on SM-MA medium, where the C/N ratio was 4.47 (Lohberger et al. 2019). These observations highlight the importance of nutrient quality and quantity, and considering the soil heterogeneity in terms of nutrient distribution, these *in vitro* screening results are significant for the transition to application.

However, in all of the screening experiments, either for PGP traits, bacterial-fungal or fungal-fungal confrontations, only qualitative data were recorded. Therefore, quantitative studies, both at a cellular level and also, as mentioned earlier, in association with plants need to be performed to verify the result obtained with Petri dishes at other relevant scales. With the collaboration of Dr. Claire Stanley group, we aimed to use fungal-fungal microfluidics device to observe these fungal-fungal interactions at a cellular level with interesting fungal partners. This is definitely an aspect that deserves further attention in order to understand the mechanisms underlying microbial interactions.

Overall, the results obtained in this study may be helpful in the field of sustainable agriculture and could lead to more effective microbial inoculants for promoting plant growth and health. Indeed, this study helps expanding our knowledge on microbial consortia, whether these are composed of bacteria, fungi, or both, and how they interact with plants and soil native microbial communities. This promotes a deeper and more comprehensive understanding of the factors required for the development and the formulation of next-generation microbial inoculants to be used in the frame of a sustainable exploitation of agricultural ecosystems.

References

Abeysinghe, S. (2009), ' Effect of Combined Use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on Biological Control of *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum*.', *Plant Pathology Journal,* 8, 9-16.

Ahemad, Munees and Kibret, Mulugeta (2014), 'Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective', *Journal of King Saud University - Science,* 26 (1), 1-20.

Bailey, Mark J., et al. (2008), 'Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops', *FEMS Microbiology Ecology,* 65 (2), 193-201.

Bhattacharyya, P. N. and Jha, D. K. (2012), 'Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture', *World Journal of Microbiology and Biotechnology,* 28 (4), 1327-50.

Boer, Wietse de, et al. (2005), 'Living in a fungal world: impact of fungi on soil bacterial niche development', *FEMS Microbiology Reviews,* 29 (4), 795-811.

Bravo, Daniel, et al. (2013), 'Isolation of oxalotrophic bacteria able to disperse on fungal mycelium', *FEMS Microbiology Letters,* 348 (2), 157-66.

Ciccillo, Fabio, et al. (2002), 'Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizospheric bacterial diversity', *Environmental Microbiology,* 4 (4), 238–45.

Compant, Stéphane, Van Der Heijden, Marcel G.A., and Sessitsch, Angela (2010), 'Climate change effects on beneficial plant–microorganism interactions', *FEMS Microbiology Ecology,* 73 (2), 197-214.

De Boer, W., et al. (2001), 'Growth of chitinolytic dune soil beta-subclass Proteobacteria in response to invading fungal hyphae', *Appl Environ Microbiol,* 67 (8), 3358-62.

Deveau, A., et al. (2018), 'Bacterial-fungal interactions: ecology, mechanisms and challenges', *FEMS Microbiol Rev,* 42 (3), 335-52.

Etter, Clément . (2018), 'Evaluation of a microbial consortium encapsulated in agar beads for the biocontrol of the fungal pathogen *Rhizoctonia solani* in lettuce', *Master thesis in Biology , University of Neuchâtel*.

García de Salamone, Inés E., et al. (2012), 'Inoculation of paddy rice with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact of plant genotypes on rhizosphere microbial communities and field crop production', *Applied Soil Ecology,* 61, 196-204.

Kohlmeier, Stefanie, et al. (2005), 'Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi', *Environmental Science & Technology,* 39 (12), 4640-46.

Kolly, Thomas. (2018), 'Biocontrol of Rhizoctonia solani by two soil-borne fungi (*Trichoderma rossicum* and *Laccaria bicolor*): *in vivo* trials on wheat.', *Master thesis, University of Neuchâtel*.

Korir, H., et al. (2017), 'Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil', *Front Plant Sci,* 8, 141.

Kuan, K. B., et al. (2016), 'Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse

conditions', *PLoS One,* 11 (3), e0152478.
Leveau, J. H. and Preston, G. M. (2008), 'Bacterial mycophagy: definition and diagnosis of a unique bacterial-fungal interaction', *New Phytol,* 177 (4), 859-76.
Martin, Gaëtan, et al. (2012), 'Fungi, bacteria and soil pH: the oxalate–carbonate pathway as a model for metabolic interaction', *Environmental Microbiology,* 14 (11), 2960-70.
Moustaine, M., et al. (2017), 'Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth in tomato (*Solanum Lycopersicum* L.) and characterization for direct PGP abilities in Morocco', *International Journal of Environment, Agriculture and Biotechnology,* 2 (2), 590-96.
Niu, B., et al. (2017), 'Simplified and representative bacterial community of maize roots', *Proc Natl Acad Sci U S A,* 114 (12), E2450-E59.
Noumavo, Pacôme A., et al. (2013), 'Effect of different plant growth promoting rhizobacteria on maize seed germination and seedling development', *American Journal of Plant Sciences,* 04 (05), 1013-21.
O'Callaghan, M. (2016), 'Microbial inoculation of seed for improved crop performance: issues and opportunities', *Appl Microbiol Biotechnol,* 100 (13), 5729-46.
Orhan, Emine, et al. (2006), 'Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry', *Scientia Horticulturae,* 111 (1), 38-43.
Radhakrishnan, R., Hashem, A., and Abd Allah, E. F. (2017), '*Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments', *Front Physiol,* 8, 667.
Schwieger, Frank and Tebbe, Christoph C. (2000), ' Effect of Field Inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*)—linking of 16s rrna gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria', *Applied and environmental microbiology,* 66 (8), 3556–65.
Sereke, F., Graves, A.R., Dux, D., Palma, J.H.N., Herzog, F., 2014. Innovative agroecosystem goods and services: key profitability drivers in Swiss agroforestry. Agronomy for Sustainable Development 35, 759-770.
Souza, Rocheli de, Ambrosini, Adriana , and Passaglia, Luciane M.P. (2015), 'Plant growth-promoting bacteria as inoculants in agricultural soils', *Genet Mol Biol,* 38 (4), 401-19.
Timonen, Sari and Marschner, Petra (2006), 'Mycorrhizosphere Concept', in K. G. Mukerji, C. Manoharachary, and Jagjit Singh (eds.), *Microbial Activity in the Rhizoshere* (Berlin, Heidelberg: Springer Berlin Heidelberg), 155-72.
Trabelsi, Darine and Mhamdi, Ridha (2013), 'microbial inoculants and their impact on soil microbial communities: A Review', *BioMed Research International,* 2013, 11.
Trabelsi, Darine, et al. (2012), 'Appraisal of the crop-rotation effect of rhizobial inoculation on potato cropping systems in relation to soil bacterial communities', *Soil Biology and Biochemistry,* 54, 1-6.
Wang, Jingjing, et al. (2018), 'Traits-based integration of multi-species inoculants facilitates shifts of indigenous soil bacterial community', *Frontiers in microbiology,* 9, 1692-92.
Wick, Lukas Y., et al. (2007), 'Effect of Fungal Hyphae on the Access of Bacteria to Phenanthrene in Soil', *Environmental Science & Technology,* 41 (2), 500-05.

Widnyana, I. Ketut and Javandira, Cokorda (2016), 'Activities *Pseudomonas* spp. and *Bacillus* sp. to stimulate germination and seedling growth of tomato plants', *Agriculture and Agricultural Science Procedia,* 9, 419-23.

Wieland, Gabriele, Neumann, Regine, and Backhaus, Horst (2001), 'Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development', *Applied and Environmental Microbiology,* 67 (12), 5849

Annex

Publications

Presentations at national and international conferences

Curriculum vitae

Publications

Christophe Paul, Sevasti Filippidou, Isha Jamil, Wafa Kooli, Geoffrey L. House, Aislinn Estoppey, Mathilda Hayoz, Thomas Juniera, Fabio Palmieri, Tina Wunderlin, Anael Lehmann, Saskia Bindschedler, Torsten Vennemann, Patrick S. Chain and Pilar Junier (2018), 'Bacterial spores, from ecology to biotechnology', Advances in Applied Microbiology (Academic Press).

Isha Hashmi, Christophe Paul, Andrej Al-Dourobi, Frederic Sandoz, Priscilla Deschamps, Thomas Junier, Pilar Junier and Saskia Bindschedler. Comparison of the plant growth promotion performance of a consortium of Bacilli inoculated as endospores or as vegetative cells. FEMS microbiology ecology, Status: under review.

Isha Hashmi, Saskia Bindschedler, Pilar Junier. The Phylum Firmicutes, Beneficial Microbes in Agro-Ecology, Volume I: Bacteria. Status: in press

Isha Hashmi, Claire Stanley, Clement Etter, Jochen Blom, Valeria Verrone, Thomas Junier, Anil Wipat, Saskia Bindschedler, Pilar Junier. Differential mycophagy of *Lysinibacillus sphaericus* towards fungi with two contrasting ecologies. Status in preparation.

Participation at national and international conferences

Oral presentations

7th Swiss Microbial Ecology Meeting (SME), Lausanne, Switzerland, 2019

Effect of a mixed consortium of three *Bacillus* strains on plant growth promotion of oat and its consequence on native microbial communities.

Annual PhD students meeting - Intellectual Property: from academia to industry, Neuchâtel, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

17th International Symposium on Microbial Ecology, Leipzig, Germany, 2018 (short talk)

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

Annual congress of the Swiss Society for Microbiology (SSM) Lausanne, Switzerland, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

Zürich Mycology Symposium 2018, Agroscope Reckenholz, Jaunary, Switzerland, 2018

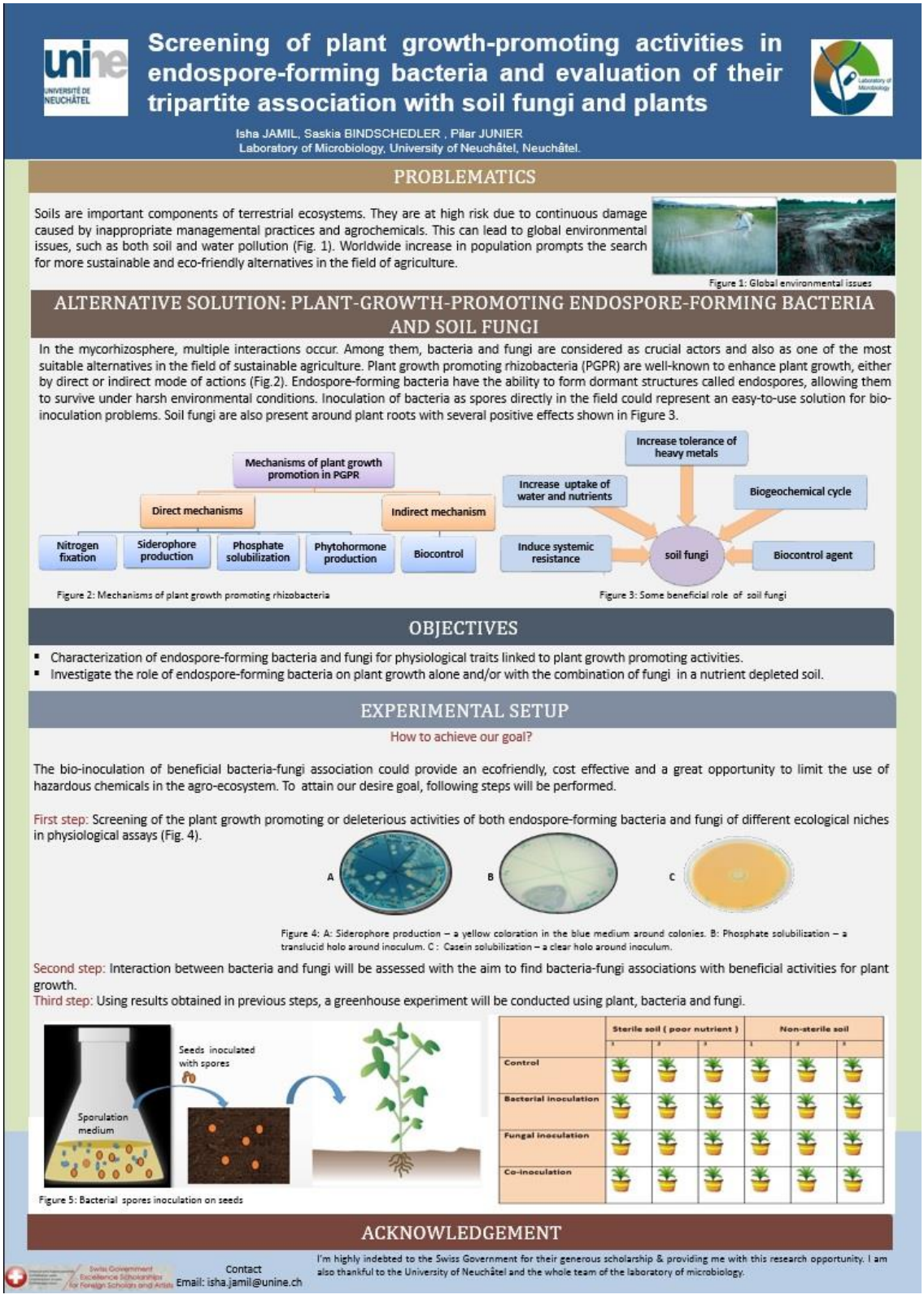
Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

Annual Ph.D. students meeting 2015 - Novel Techniques in Science, Neuchâtel, Switzerland ()

Screening of plant growth promoting activities in endospore forming bacteria and evaluation of their tripartite association with soil fungi and plant.

Poster presentations

Annual Ph.D. students meeting 2015 - Novel Techniques in Science, Neuchâtel, Switzerland



6th Swiss Microbial Ecology Meeting, Ascona, Switzerland, 2015

Screening of plant growth-promoting activities in endospore-forming bacteria

Isha JAMIL[1], Saskia BINDSCHEDLER[1], Pilar JUNIER[1]
[1] Laboratory of Microbiology, University of Neuchâtel, Neuchâtel.

WHY USING MICROBES TO SUSTAIN PLANT GROWTH?

Soil degradation

- Inappropriate management practices in agriculture
- Use of agrochemicals (Pesticides, Fertilizers)
- Soil and water pollution
- Worldwide increase in population

Figure 1: Global environmental issues

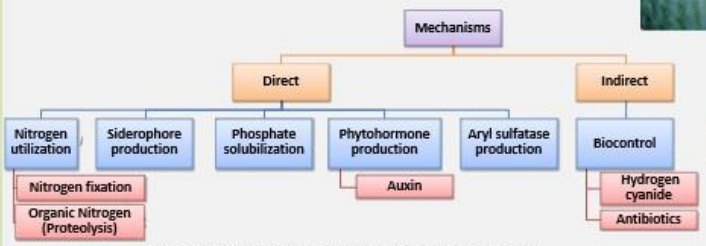


Figure 2: Mechanisms of plant growth promoting rhizobacteria (PGPR)

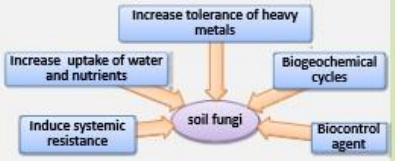


Figure 3: Examples of beneficial roles of soil fungi

Objective of this study

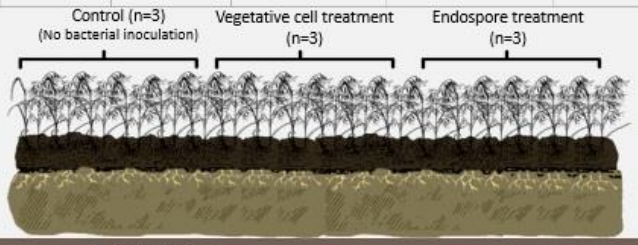
To investigate the role of inoculated endospore-forming bacteria (EFB) either as vegetative cells or as endospores on plant growth in the field.

EXPERIMENTAL SETUP

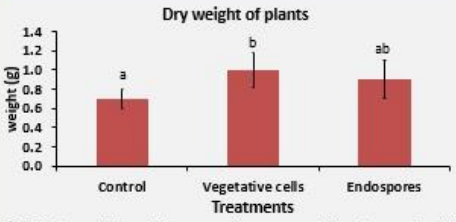
Screening of EFB

Bacterial strains	Nitrogen fixation	Proteolysis	Siderophore production	Phosphate solubilization	Auxin production
Bacillus licheniformis	✓	✓	✓	x	✓
Bacillus cereus	✓	✓	✓	x	✓
Bacillus thuringiensis	✓	✓	✓	x	✓

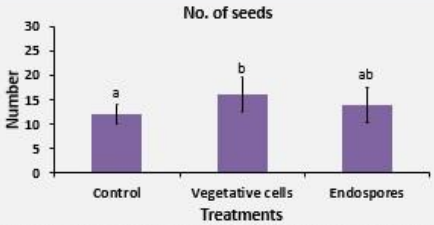
A **field experiment** was carried out with *Avena sativa* (oat) using a mixed inoculant of the three EFB strains, which were previously screened for PGPR activities. The inocula were delivered in the form of vegetative cells or endospores.



RESULTS



➢ Bio-inoculation influences plant growth by increasing biomass. The highest biomass was observed in treatment with vegetative cells, followed by the treatment with endospores.



➢ The greater quantity of seeds was observed in plants treated with vegetative cells as compared to other treatments.

Conclusion: Results so far suggest that bio-inoculation with EFB, either as vegetative cells or endospores has a positive effect on plant fitness. Moreover, the inoculation of bacteria in the form endospore as a delivery system seems to be an effective way to supply PGPR in the soil as survival of inoculated bacteria is often a critical task. In future, this could provide an ecofriendly and cost effective alternative to limit the use of agrochemicals.

Outlook: Analysis of physicochemical parameters, assessment of fungal communities and the survival of bio-inoculated EFB in soil are in progress.

ACKNOWLEDGEMENT

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Swiss Government Excellence Scholarships for Foreign Scholars and Artists

Screening of plant growth-promoting activities in endospore-forming bacteria

Isha JAMIL[1], Saskia BINDSCHEDLER[1], Pilar JUNIER[1]
[1] Laboratory of Microbiology, University of Neuchâtel, Neuchâtel.

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- Inappropriate management practices in agriculture
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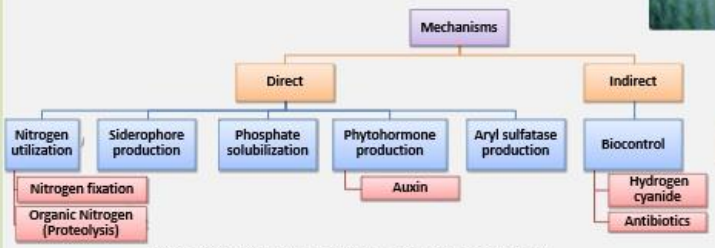

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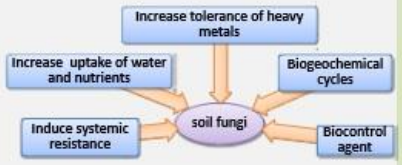

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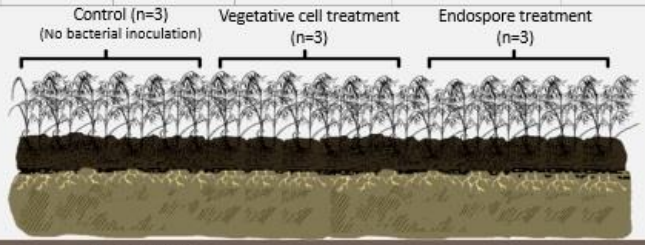
To investigate the role of inoculated endospore-forming bacteria (EFB) either as vegetative cells or as endospores on plant growth in the field.

EXPERIMENTAL SETUP

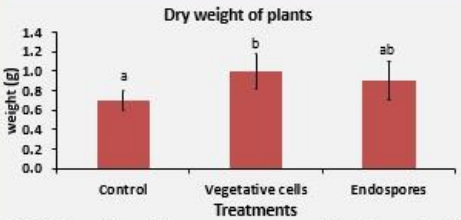
Screening of EFB

Bacterial strains	Nitrogen fixation	Proteolysis	Siderophore production	Phosphate solubilization	Auxin production
Bacillus licheniformis	✓	✓	✓	x	✓
Bacillus cereus	✓	✓	✓	x	✓
Bacillus thuringiensis	✓	✓	✓	x	✓

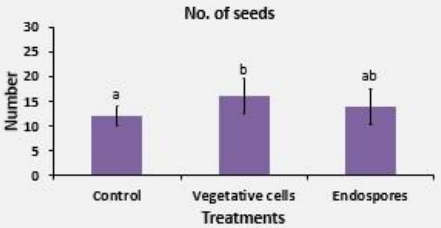
A **field experiment** was carried out with *Avena sativa* (oat) using a mixed inoculant of the three EFB strains, which were previously screened for PGPR activities. The inocula were delivered in the form of vegetative cells or endospores.



RESULTS



➢ Bio-inoculation influences plant growth by increasing biomass. The highest biomass was observed in treatment with vegetative cells, followed by the treatment with endospores.



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Outlook: Analysis of physicochemical parameters, assessment of fungal communities and the survival of bio-inoculated EFB in soil are in progress.

ACKNOWLEDGEMENT

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Bio-inoculation of plant growth promoting bacteria as endospore: a promising strategy towards sustainable agriculture

Isha JAMIL[1], Pilar JUNIER[1], Saskia BINDSCHEDLER[1]
[1]Laboratory of Microbiology, University of Neuchâtel, Neuchâtel.

WHY USING MICROBES TO SUSTAIN PLANT GROWTH?

Soil degradation

- Inappropriate management practices in agriculture
- Use of agrochemicals (Pesticides, Fertilizers)
- Soil and water pollution

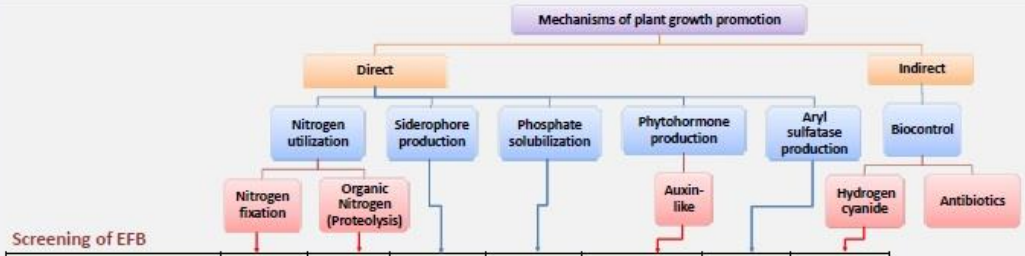
Figure 1: Global environmental issues

Potential solution: Inoculation of microorganisms as substitute of agrochemicals could represent an alternative solution to make agriculture more sustainable and ecofriendly.

Objective of this study

To investigate the effect of inoculated endospore-forming bacteria (EFB) with plant growth-promoting traits, either as vegetative cells or as endospores, on plant growth in the field.

EXPERIMENTAL SETUP

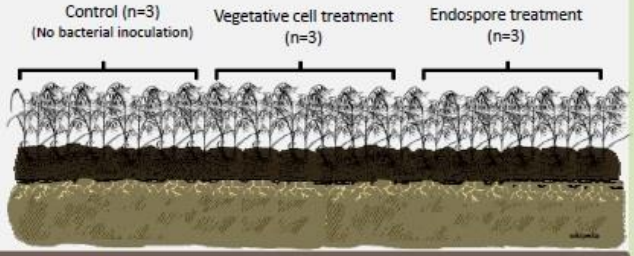


Screening of EFB

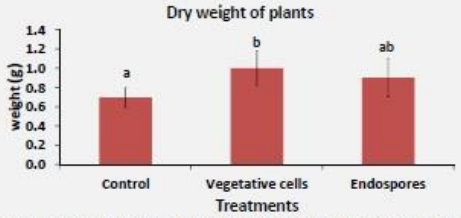
Bacillus licheniformis	✓	✓	✓	×	✓	×	×
Bacillus thuringiensis 1310	✓	✓	✓	×	✓	×	×
Bacillus thuringiensis 1312	✓	✓	✓	×	✓	×	×

A **field experiment** was carried out with *Avena sativa* (oat) using a mixed inoculant of the three EFB strains, which were previously screened for plant growth promotion activities. The inocula were delivered in the form of vegetative cells or endospores onto the seeds.

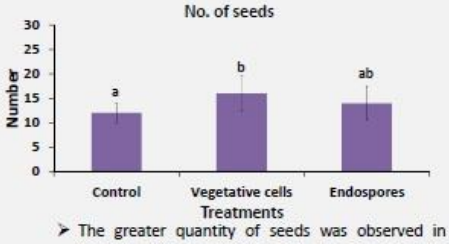
Scanning electron microscopy images of untreated oat seeds (a), compared to seeds coated with EFB vegetative cells (b) or endospores (c).



RESULTS



- Bio-inoculation influences plant growth by increasing biomass. The highest biomass was observed in the treatment with vegetative cells, followed by the treatment with endospores.



- The greater quantity of seeds was observed in plants treated with vegetative cells as compared to other treatments.

Conclusion: The results obtained so far suggest that bio-inoculation with EFB, either as vegetative cells or endospores, has a positive effect on plant growth and fitness. Moreover, as the survival of inoculated bacteria is often a critical issue, the inoculation of bacteria in the form of endospores as a delivery system seems to be an effective way to supply bioinoculants in the soil. In future, this could provide an ecofriendly alternative to limit the use of agrochemicals.

Outlook: Soil physicochemical analysis, assessment of bacterial and fungal communities and the survival of bio-inoculated EFB in soil are in progress.

ACKNOWLEDGMENTS

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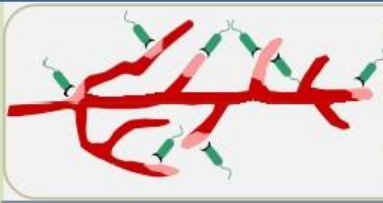
Swiss Government Excellence Scholarships for Foreign Scholars and Artists
FNSNF Swiss National Science Foundation

7th Congress of European Microbiologists (*FEMS*), Spain, 2017

Mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani*

Isha JAMIL, Saskia BINDSCHEDLER, Pilar JUNIER
Laboratory of Microbiology, University of Neuchâtel, Neuchâtel.

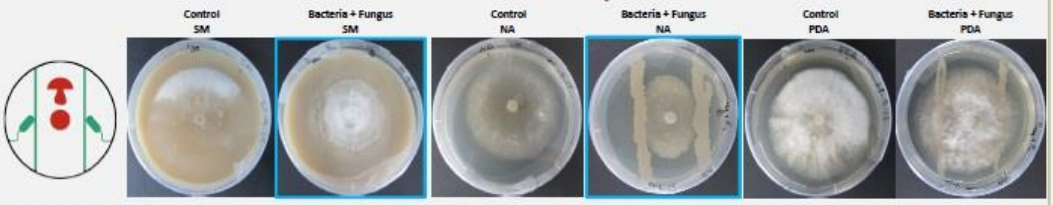


Background: Bacteria are well known for their diverse nutritional capabilities, which can lead to positive or negative interactions with other organisms. Mycophagy represents a series of phenotypic behaviors allowing bacteria to obtain nutrients from living fungi with a negative impact on the fungal partner[1].

Objective: To investigate the interaction between *Lysinibacillus sphaericus* and the phytopathogenic fungus *Rizoctonia solani* in order to attest an unexplored mycophagous lifestyle in the former.

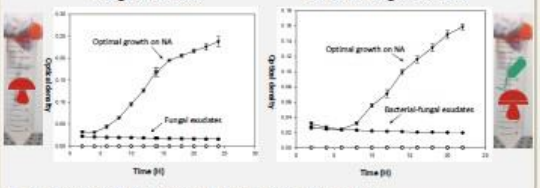
RESULTS

Confrontation assays



- Three types of media were used: Skimmed milk agar (SM), Nutrient agar (NA) and Potato dextrose agar (PDA).
- An antagonistic interaction was observed between *L. sphaericus* and *R. solani* on SM and NA, but not on PDA.
- On SM and NA the fungus was colonized by bacteria and was unable to re-grow.

Can bacteria grow on fungal and bacterial-fungal exudates?

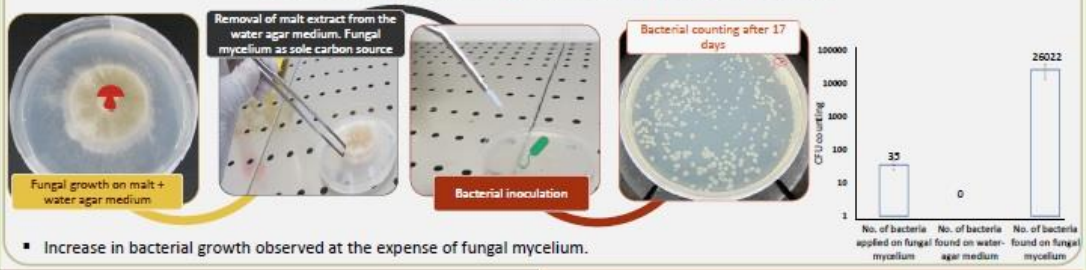


- *L. sphaericus* showed no growth on both exudates.

How do the interaction at the cellular level look?

- Microscopic observation in microfluidic devices revealed the deformation of the fungal cell wall (arrow) in presence of bacteria.

Can bacteria grow at the expense of living fungal mycelium?



- Increase in bacterial growth observed at the expense of fungal mycelium.

CONCLUSION

- *L. sphaericus* inhibited fungal growth and exploited fungal hyphae for its dispersal.
- Fungal tissues that were in direct contact with bacteria were unable to re-grow.
- Bacterial growth occurred at the expense of fungal mycelium confirming that living mycelium is required for bacterial growth.

OUTLOOK

- This yet unexplored mycophagous lifestyle of *L. sphaericus* could be exploited for the control of this phytopathogen in sustainable agriculture.

1) Leveau, J.H. and G.M. Preston, *Bacterial mycophagy: definition and diagnosis of a unique bacterial–fungal interaction.* New Phytologist, 2008. 177(4): p. 859-876.

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Acknowledgments
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Bio-inoculation of plant growth promoting bacteria as consortium: a promising strategy towards sustainable agriculture

Isha JAMIL[1], Pilar JUNIER[1], Saskia BINDSCHEDLER[1]
[1] Laboratory of Microbiology, University of Neuchâtel, Switzerland

WHY USING MICROBES IN SUSTAINABLE AGRICULTURE?

Inoculation of microorganisms as substitute of agrochemicals could represent an alternative solution to make agriculture more sustainable and ecofriendly.

Objective of this study

To investigate the effect of individual or mixed inocula (consortium) of endospore-forming bacteria (EFB) for plant growth promotion.

Variables:

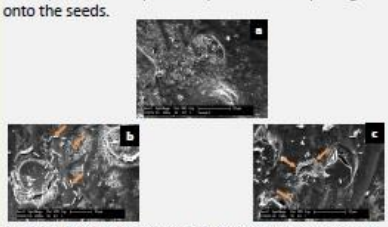
1. Vegetative cells VS endospores
2. Simple VS complex environment
   - Sterile soil
   - Non-sterile soil
   - Field

Screening of EFB

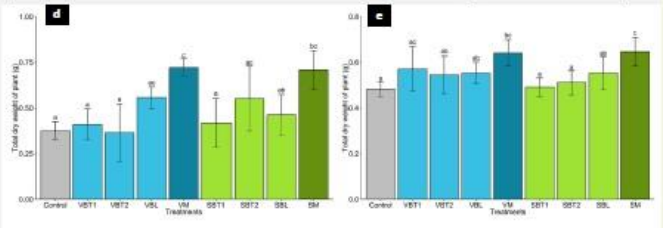
Name	Dinitrogen fixation	Casein solubilization	Siderophore production	Phytohormone production (Auxin-like)
Bacillus thuringiensis (BT1)	✓	✓	✓	✓
Bacillus thuringiensis (BT2)	✓	✓	✓	✓
Bacillus licheniformis (BL)	✓	✓	✓	✓

POT EXPERIMENT

Pot experiments were performed in sterile and non-sterile soil with *Avena sativa* (oat) using individual or mixed inocula (consortium) of the three EFB strains, which were previously screened for plant growth promotion activities. The inocula were delivered in the form of vegetative cells or endospores onto the seeds.



Scanning electron microscopy images of untreated oat seeds **(a)**, compared to seeds coated with EFB vegetative cells **(b)** or endospores **(c)**.



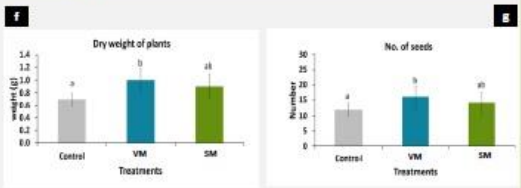
Effects of individual and mixed inocula on total dry weight of *A. sativa* using sterile soil **(d)** and non sterile soil **(e)**. Different lowercase letters denote significant differences (Tukey's HSD $P < 0.05$).

Result: Total dry weight of plants were increased in mixed inocula of vegetative cells and endospores.
Consortium of vegetative cells and endospores have better results as compared to individual inoculum in both sterile and non-sterile soil.

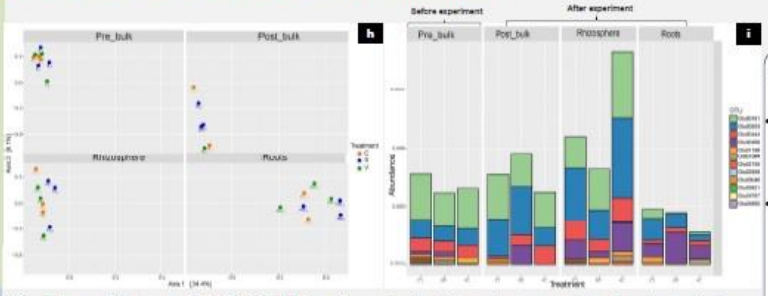
FIELD EXPERIMENT

A field experiment was carried out with *Avena sativa* (oat) using only a mixed consortium of the three EFB strains, based on the results of the pot experiments.

Result: Bio-inoculation influences positively plant growth and fitness by increasing biomass **(f)** and quantity of seeds **(g)** produced by plants treated with mixed consortium of vegetative cells and endospores.



Effect of consortium on the native bacterial communities



Principal coordinates analysis (PCoA) **(h)** based on bacterial OTU relative abundances in the field experiment of *A. sativa*.

Result: Consortium inoculation does not affect the structure of the indigenous microbial communities, which is important with regard to the safety of the introduction of microbes in the environment.

Stacked bar charts **(i)** showing the relative abundance of all the *Bacillus* OTUs found across four different samples.

Result: The relative abundance of *Bacillus* were higher in the rhizospheric soil of *A. sativa* treated with the mixed inocula of vegetative cells

Conclusion

- Consortium of three EFB are more robust than the single strain inoculums in the complex environment (non-sterile soil).
- The survival of inoculated bacteria is often a critical issue, the inoculation of EFB either vegetative or endospores as a delivery system seems to be an effective way to supply bioinoculants in the soil.
- In future, this could provide an ecofriendly alternative to limit the use of agrochemicals.

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Biology 18 meeting, Neuchâtel, 2018

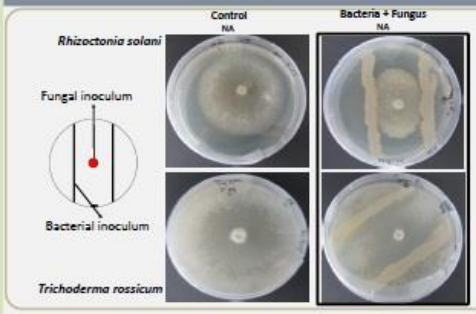
Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani*

Isha JAMIL[1], Clément ETTER[1], Claire STANLEY[2], Saskia BINDSCHEDLER[1], Pilar JUNIER[1]
[1] Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, Switzerland
[2] Agroscope, Reckenholz, Switzerland

Background: Bacteria are well known for their diverse nutritional capabilities, which can lead to positive or negative interactions with other organisms. **Mycophagy** represents a series of phenotypic behaviors allowing bacteria to obtain nutrients from living fungi with a negative impact on the fungal partner[1].

Objective: To assess the mycophagous lifestyle of *Lysinibacillus sphaericus* in interaction with two fungi: *Rizoctonia solani* (phytopathogen) and *Trichoderma rossicum* (saprotroph and mycopathogen).

RESULTS

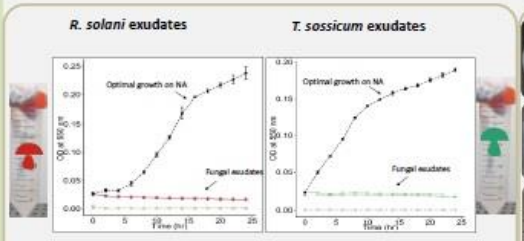


Confrontation assays

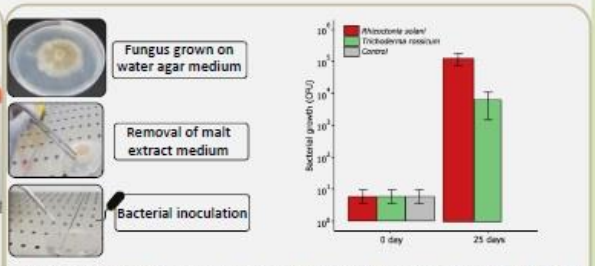
- Influence of trophic conditions on bacterial fungal interaction were assessed with three different media: Skimmed milk agar, Potato dextrose agar and Nutrient agar (NA).
- An antagonistic interaction was only observed on NA medium and in the confrontation with *R. solani*, but not with *T. rossicum*.
- *L. sphaericus* was able to colonize and disperse on the fungal mycelium of both fungal species.
- Inhibition of fungal growth was not the result of the production of bacterial volatiles or exudates with inhibitory activity.

Bacterial mycophagy

No: if bacteria grow passively on fungal exudates — **Yes: if bacteria grow at the expense of living fungal mycelium**

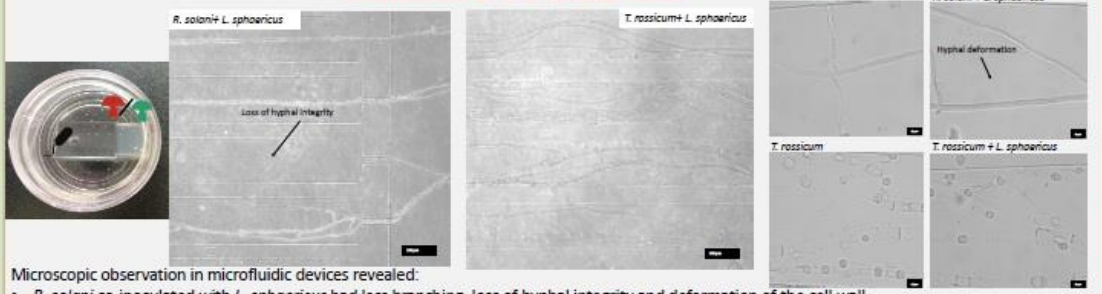


L. sphaericus does not grow on exudates of both fungi



Increase in bacterial biomass at the expense of fungal mycelium of both fungi

Interaction at the cellular level



Microscopic observation in microfluidic devices revealed:

- *R. solani* co-inoculated with *L. sphaericus* had less branching, loss of hyphal integrity and deformation of the cell wall.
- No difference on hyphal aspect was observed with *T. rossicum* in presence or absence of bacteria.

CONCLUSION

- *L. sphaericus* inhibited fungal growth and exploited fungal hyphae for its dispersal.
- Hyphal deformation was observed only for *R. solani* in the presence of *L. sphaericus*.
- Bacterial growth occurred at the expense of living fungal mycelium of both fungi; conformation of a mycophagous lifestyle.
- Variability in the mycophagous lifestyle of *L. sphaericus* towards different fungi may be important for determining ecological niche differentiation.

1) Leveau, J.H. and G.M. Preston, New Phytologist, 2008. 177(4): p. 859-876.

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Acknowledgments
Swiss Government Excellence Scholarships for Foreign Scholars and Artists

17th International Symposium on Microbial Ecology (ISME17), Germany, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani*

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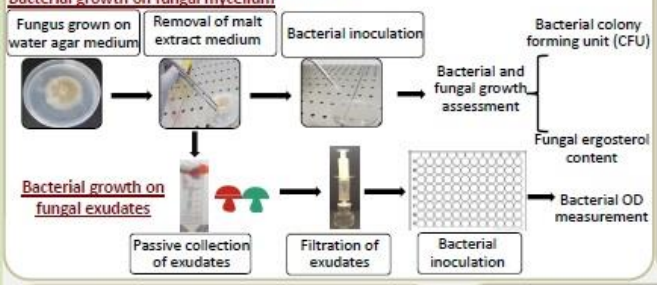
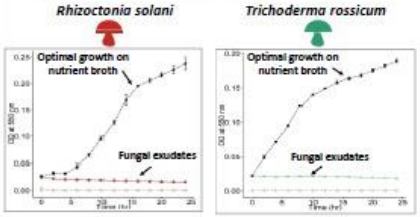
Background: Bacteria are well known for their diverse nutritional capabilities, which can lead to positive or negative interactions with other organisms.
Mycophagy represents a series of phenotypic behaviors allowing bacteria to obtain nutrients from living fungi with a negative impact on the fungal partner[1].

Objective: To assess the mycophagous lifestyle of *Lysinibacillus sphaericus* in interaction with two fungi: *Rhizoctonia solani* (phytopathogen) and *Trichoderma rossicum* (saprotroph and mycopathogen).

Methods

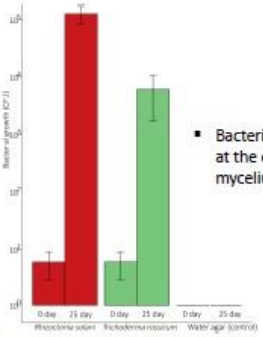


Bacterial growth on fungal exudates



- *Lysinibacillus sphaericus* does not grow passively by consuming exudates of either fungal species.

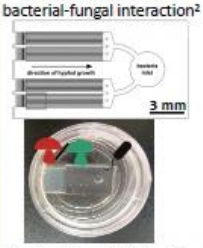
Bacterial growth on fungal mycelium

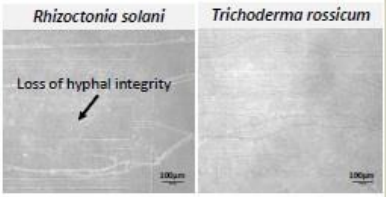


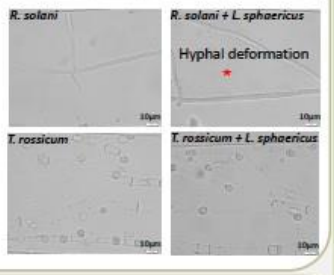
- Bacterial growth occurred at the expense of fungal mycelium of both fungi.

Interaction at the cellular level

Microfluidic device for bacterial-fungal interaction[2]



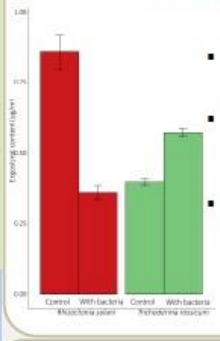




The hyphae of *Rhizoctonia solani* in presence of the bacterium:
- Branch less.
- Lose integrity (arrow).
- Deform (cell wall; star).

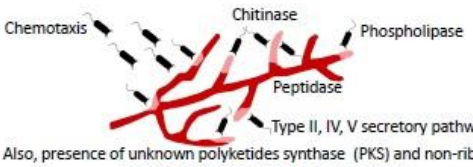
The hyphae of *Trichoderma rossicum* in presence of the bacterium:
- Display no morphological difference.

Effect of bacterial growth on fungal fitness



- Ergosterol content in *R. solani* decreases when *L. sphaericus* grew at its expense.
- Ergosterol content in *T. rossicum* increases when *L. sphaericus* grew at its expense.
- Mycophagus lifestyle only for the interaction with *R. solani*.

Putative genomic determinants for exploiting living fungal mycelium



Also, presence of unknown polyketides synthase (PKS) and non-ribosomal peptide synthetase (NRPS) gene clusters.

Conclusion

Variability in the mycophagous lifestyle of *Lysinibacillus sphaericus* towards different fungi may play a crucial role for determining ecological niche differentiation in soils and the mycorhizosphere

1) Leveau, J.H. and G.M. Preston, New Phytologist, 2008. 177(4): p. 859-876.
2) Stanley, C.E., Stockli, M., van Swaay, D., Sabotic, J., Kallio, P.T., Kunzler, M., deMello, A.J., Aebi, M., 2014. Integr Biol (Camb) 6, 935-945.

Curriculum Vitae

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EDUCATION AND TRAINING

11/2014–04/2019	PhD University of Neuchâtel Improving bio-inoculation for sustainable agriculture Supervisors: Prof. Pilar Junier and Dr. Saskia Bindschedler
08/2009–06/2011	Master of Science (Plant science/Botany) University of the Punjab, Lahore, Pakistan Role of some plant growth regulators and autochthonous fungi in enhancement of metal uptake in some selected plants.
09/2005–06/2009	Bachelor of Science (Plant science/Botany) University of the Punjab, Lahore, Pakistan

Awards and

- Travel grant from Interuniversity Doctoral Program in Organismal

achievements

Biology, Neuchâtel, Switzerland

- Swiss Government Excellence Scholarship for Foreign Scholars for Ph.D.
- Pakistan Higher Education Commission Scholarship for Master studies

Conferences

Oral presentation

- 7th Swiss Microbial Ecology Meeting (SME), Lausanne, Switzerland, 2019

Effect of a mixed consortium of three *Bacillus* strains on plant growth promotion of oat and its consequence on native microbial communities.

- Annual PhD students meeting - Intellectual Property: from academia to industry, Neuchâtel, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

- 17th International Symposium on Microbial Ecology, Leipzig, Germany, 2018 (short oral presentation of poster)

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

- Annual congress of the Swiss Society for Microbiology (SSM) Lausanne, Switzerland, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

- Zürich Mycology Symposium 2018, Agroscope Reckenholz, Switzerland, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

- Annual Ph.D. students meeting 2015 - Novel Techniques in Science, Neuchâtel, Switzerland (short oral presentation of poster)

Screening of plant growth promoting activities in endospore forming bacteria and evaluation of their tripartite association with soil fungi and plant.

Poster presentation

- 17th International Symposium on Microbial Ecology, Leipzig, Germany, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

- Biology 18 meeting, Neuchâtel, 2018

Targeted mycophagous lifestyle of *Lysinibacillus sphaericus* against the phytopathogenic fungus *Rhizoctonia solani.*

- International Symposium on Microbe-assisted Crop Production (miCROPe), Vienna, 2017

Bio-inoculation of plant growth promoting bacteria as consortium: a promising strategy towards sustainable agriculture.

- 7th Congress of European Microbiologists (FEMS), Spain, 2017

Mycophagous lifestyle of *Lysinibacillus sphaericus* against phytopathogenic fungus *Rhizoctonia solani*

- 74th Annual Swiss Society for Microbiology Meeting, Berne, Switzerland, 2016

Bio-inoculation of plant growth promoting bacteria as consortium: a promising strategy towards sustainable agriculture.

- MiCroP Workshop, Denmark, 2016

Screening of plant growth promoting activities in endospore-forming bacteria

- 6th Swiss Microbial Ecology Meeting, Ascona, Switzerland, 2015

Screening of plant growth promoting activities in endospore-forming

bacteria

- Annual Ph.D. students meeting 2015 - Novel Techniques in Science, Neuchâtel, Switzerland

Screening of plant growth promoting activities in endospore forming bacteria and evaluation of their tripartite association with soil fungi and plant.

Teaching and supervision

- Practical teaching in molecular biology
- Scientific project supervision (APP) (2015-2018), Master students and Internee