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# Characterization of forest soil bacteria isolated at the microhabitat level and of their interactions with Poplar

Milena Gonzalo

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UNIVERSITÉ  
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# Thèse

Présentée pour l'obtention du titre de  
Docteur de l'Université de Lorraine

En Biologie et écologie des forêts et agrosystèmes

**Milena GONZALO**

**Caractérisation des bactéries du sol forestier isolées au niveau des  
microhabitats et de leurs interactions avec le peuplier.**

**Characterization of forest soil bacteria isolated at the microhabitat level and of  
their interactions with Poplar**

Soutenance publique prévue le 17 décembre 2019 devant la commission d'examen:

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## Contexte général

Les forêts forment des écosystèmes très complexes qui se caractérisent par une biodiversité très riche à la fois en surface et sous terre. Au niveau des sols forestiers, on trouve des communautés riches et complexes de microorganismes, en particulier bactéries et champignons, occupant divers micro-habitats et jouant un rôle essentiel dans le cycle des nutriments et la minéralisation des composés organiques. De nombreux facteurs biotiques et abiotiques ont été décrits comme étant les moteurs de la diversité et du fonctionnement microbien du sol, mais le sol demeure une boîte noire pour plusieurs raisons. L'utilisation massive du séquençage haut débit de l'ADN a permis d'obtenir des descriptions très avancées des compositions des communautés microbiennes dans les sols dans des conditions données. De plus, le séquençage des ARN via la métatranscriptomique a ouvert la voie à l'analyse des microorganismes actifs. Cependant, notre connaissance des fonctions exprimées par ces micro-organismes *in situ* et de l'impact de ces activités sur le fonctionnement des communautés est encore parcellaire. De plus, peu d'études réalisées à ce jour prennent en compte les interactions multiples qui se produisent entre les microorganismes dans les milieux naturels et comment ces interactions influencent le fonctionnement de ces communautés et leurs interactions avec les plantes. Bien que l'on suppose que les microbes consacrent d'énormes ressources aux interactions microbiennes, les métabolites et les acteurs moléculaires impliqués dans ces échanges métaboliques, ou dialogues, sont encore mal caractérisés. Ces derniers joueraient un rôle important dans la structuration des communautés microbiennes du sol, la résilience de ces communautés face aux perturbations externes et le développement des plantes. Dans ce contexte, il semble important d'améliorer notre connaissance du comportement des microorganismes lorsque ceux-ci se développent au sein de communautés microbiennes plus ou moins complexes.

## Objectifs de la thèse de doctorat

Ce travail de doctorat s'inscrit dans le cadre de l'étude de l'impact des dialogues moléculaires entre les communautés microbiennes des sols forestiers et les arbres. Les principaux objectifs de ce projet visent à i) déchiffrer les dialogues entre les souches bactériennes isolées à partir d'un même micro-habitat, ii) comprendre comment ces dialogues moléculaires influencent le développement des arbres, et iii) comprendre comment l'arbre affecte les isolats bactériens et leurs interactions. Pour ce faire, nous avons choisi d'étudier le comportement de bactéries cultivables que j'ai isolées de micro-habitats. L'hypothèse sous-jacente était que des bactéries isolées d'un même micro-habitat avaient une forte probabilité d'interagir naturellement et donc que le produit des interactions obtenus serait

réel et non pas une curiosité provoquée par les conditions expérimentales en laboratoire. Par conséquent, mes objectifs de travail étaient i) d'isoler des bactéries à partir de micro-habitats (grains de sol), ii) de caractériser ces bactéries (sur le plan taxonomique et fonctionnel), iii) d'évaluer les interactions entre les bactéries issues d'un même grain, iv) de déterminer si ces bactéries influencent la croissance de la plante (vitroplants de peuplier) et réciproquement, et v) d'identifier les molécules potentiellement impliquées dans les dialogues moléculaires par une approche génomique.

## Principaux résultats obtenus

### *1. Taxonomie des communautés microbiennes issues de grains de sols*

Les sols sont des structures tridimensionnelles complexes composées d'un mélange de particules différentes tailles qui s'agrègent et sont séparés par des pores. Ces agrégats sont une combinaison de minéraux dérivés de roches altérées par les intempéries et les micro-organismes, de matières organiques, de matières modérément décomposées, d'agents liants (par exemple des réseaux d'hyphes fongiques, des racines, des exopolysaccharides microbiens) et de communautés microbiennes (Rashid *et al.* 2016, Rillig *et al.* 2017). Cette organisation structurelle, les propriétés physico-chimiques et les interactions biotiques avec les multiples macro-organismes (plantes, vers, insectes...) façonnent une mosaïque complexe de microenvironnements qui représentent différents habitats pour le microbiome du sol (Ranjard et Richaume 2001). Chaque agrégat individuel fournit des conditions environnementales uniques et compartimentées, de sorte que les bactéries consomment probablement des ressources communes et sont potentiellement impliquées dans un grand nombre d'interactions possibles.

Dans ce contexte, nous avons étudié les communautés bactériennes et fongiques associées à différents micro-habitats du sol d'une hêtraie et chênaie à Champenoux, France (site expérimental du réseau Matière Organique du Sol – MOS). Nous avons dans un premier temps isolé des bactéries partageant les mêmes micro-habitats, un grain de sol. Plusieurs micro-habitats ont été considérés : le sol nu, la rhizosphère, la mycorrhizosphère, le bois mort et la racine nécrosée. Pour mieux appréhender l'environnement biotique dont sont issues ces bactéries, nous avons analysé la communauté microbienne (bactérienne et fongique) de ces micro-environnements par une approche de séquençage haut-débit d'amplicons des marqueurs 16S et ITS. Ces informations nous ont permis d'avoir un aperçu de la représentativité de notre collection des souches et de la diversité des micro-organismes à l'échelle d'un micro-habitat.

Au total, 1247 colonies bactériennes ont été obtenues à partir des différents grains de sol récoltés. Nous avons conservées uniquement les colonies qui présentaient un phénotype unique (couleur,

forme de la colonie, sporulation pour les Actinomycetes). Ainsi, 122 colonies ont été conservées et caractérisées d'un point de vue taxinomique. Ces colonies appartenaient à cinq phyla: les Firmicutes, les Actinobactéries, les Protéobactéries et les Bactéroidetes. La plus grande diversité a été obtenue à partir de bois en décomposition. La comparaison avec les données de séquençage au débit de marqueurs microbiens a montré que les bactéries cultivables isolées appartiennent à la fraction rare des communautés de chaque grain. Plus de 6000 unités taxinomiques opérationnelles bactériennes (OTUs) ont été détectées par séquençage mais les OTUs les plus abondantes correspondaient à des bactéries non-cultivables.

## ***2. Caractérisation de la diversité fonctionnelle des souches bactériennes et des interactions biotiques***

Comme au niveau de chaque grain de sol les bactéries sont soumises aux mêmes conditions biotiques et abiotiques, on s'attend à ce qu'elles utilisent des ressources communes comme le carbone, l'azote, le phosphore et le fer. Nous avons donc déterminé si les bactéries pouvaient entrer en compétition pour les mêmes ressources ou s'il y a des espèces spécialisées à l'intérieur d'un même grain de sol. Dans l'ensemble, les bactéries présentaient une grande diversité dans leurs capacités potentielles à acquérir des nutriments. La ressource la plus communément utilisées était le fer via la production de sidérophores, le phosphore par les phosphatases acides et l'azote organique par les protéases. Par ailleurs, nous avons évalué la capacité des bactéries isolées à produire certaines molécules de quorum sensing, à provoquer un phénomène de quorum quenching, et à synthétiser des molécules antimicrobiennes afin de déterminer les mécanismes potentiellement impliqués dans la compétition entre ces bactéries. Enfin, la capacité à produire des auxines a également été recherchée afin de mieux comprendre les interactions avec les plantes. Dans l'ensemble, peu de souches bactériennes disposaient de la capacité à produire de telles molécules.

Enfin, afin d'obtenir un aperçu des différents types d'interactions (positives, négatives, neutres) pouvant se produire entre bactéries issues d'un même grain et de leur fréquence, nous avons étudié le comportement de ces bactéries lorsqu'elles étaient confrontées par paire en boîte de Petri. Parmi les interactions possibles, la compétition (soit pour les nutriments, soit pour l'espace) peut être considérée comme une interférence due à la production de composés antagonistes envers d'autres organismes et, si elle est mortelle, peut être considérée comme une exclusion éliminant entièrement l'autre organisme (Hibbing *et al.* 2010). Une autre forme de compétition est l'exploitation où un organisme épuise les nutriments, empêchant les autres d'y accéder (Stubbendieck et Straight, 2016). La coopération peut être considérée comme le fait de bénéficier à autrui par le biais de modification bénéfiques de l'environnement ou la production de biens publics (c'est-à-dire toute ressource

produite par un individu disponible pour autrui) (Damore et Gore, 2012). Ce trait pourrait ainsi faciliter la croissance au sein de populations de bactéries proches (sélection des parents) (Damore et Gore, 2012). Cependant, si d'autres peuvent bénéficier de ces biens publics sans en payer le coût (sans le caractère coopératif), alors ces organismes seraient considérés comme des tricheurs (Hibbing *et al.* 2010).

L'ensemble de ces interactions peut être médié par de petites molécules agissant comme des signaux ou en étant directement actifs (Shank et Kolter 2009). Ces molécules peuvent agir à différents niveaux: régulation de voies métaboliques (métabolisme primaire et secondaire), altération de la morphologie et/ou du développement (croissance, germination, sporulation, biofilm). Ces molécules peuvent être solubles et agir à courte distance tandis que d'autres, volatiles peuvent agir à plus longue distance.

Pour étudier ces différentes interactions, les souches bactériennes ont été inoculées par paires soit de manière simultanée, soit en décalage. Dans le second cas, les deux partenaires bactériens étaient inoculés sur boîte de Petri à 48h d'intervalle. Nous avons observé que la plupart des bactéries n'affectaient pas les autres et peu de bactéries interagissaient les unes avec les autres, dans ces conditions. La plupart des interactions visualisées étaient antagonistes et de faible intensité. L'intensité de l'antagonisme variaient en fonction du type d'inoculation (simultané ou décalé): l'inoculation simultanée donnait lieu essentiellement à des inhibitions de faible intensité alors que l'inoculation décalée conduisait à un plus grand nombre d'inhibitions de forte intensité. Peu d'interactions bénéfiques se sont produites et celles-ci ont été observées uniquement lors d'inoculations décalées.

L'objectif suivant était de déterminer si les bactéries et leurs interactions pouvaient influencer le développement de boutures de peupliers *in vitro*. En effet, dans les sols, les molécules produites par les microbes ou les racines lors d'interactions, peuvent moduler de nombreux phénotypes à la fois chez les micro-organismes et chez les plantes. Ainsi, la pathogénicité, la formation de symbioses, la compétition pour les nutriments... peuvent être modulées par les activités des racines tandis que les molécules produites par les micro-organismes peuvent influencer le développement du système racinaire. L'utilisation d'un dispositif simplifié *in vitro* de confrontation bactéries-plante peut permettre de disséquer les mécanismes complexes d'interactions entre les différents partenaires. Dans ce contexte, nous avons utilisé *Populus tremula x alba*, clone de l'INRA 717-1B4 (ci-après dénommé *Populus* ou peuplier) comme système modèle. Le peuplier est utilisé comme arbre modèle dans de nombreux laboratoires en raison de sa facilité de manipulation et de propagation végétative. Nous avons évalué l'effet de l'inoculation de 35 souches bactériennes, inoculées seules ou en

mélanges à proximité des racines de peuplier, sur la morphologie du système racinaire, la biomasse aérienne et racinaire et la physiologie (photosynthèse et production d'anthocyanes). Très peu d'effets statistiquement significatifs ont pu être mesurés. En effet, une très forte variabilité de la réponse phénotypique du peuplier à la présence de bactéries a été observée. Quinze pourcent des souches avaient un effet négatif reproductible à l'encontre du peuplier et une souche du genre *Streptomyces* induisait des nécroses au niveau des racines. A l'inverse aucune souche ne stimulait de manière reproductible la croissance des peupliers *in vitro*.

### ***3. Dialogues moléculaires entre des souches bactériennes issues du sol nu et les racines de peuplier***

Sur la base d'expériences précédentes, nous avons choisi de nous focaliser plus particulièrement sur 4 souches issues du sol nu : une souche de *Bacillus* sp. Bs2, une souche de *Pseudomonas* sp. Bs17 et deux souches de *Streptomyces* (Bs9 et Bs14). Ces différentes souches montraient un comportement différent vis à vis du peuplier lorsqu'elles étaient inoculées seules ou en combinaison. Les différents isolats ont été inoculés en gouttes seules ou en combinaisons binaires et tripartites avec et sans peuplier. De manière générale, toutes les bactéries avaient un effet sur l'architecture du système racinaire mais n'avait d'effet significatif sur la croissance globale du peuplier. De manière intéressante, la souche *Streptomyces* Bs9 avait un effet inhibiteur très intense sur le développement racinaires: les racines étaient plus courtes que le témoin, contenait une teneur élevée en anthocyanine et une nécrose était clairement visible à l'apex des racines après 21 jours d'incubation. A l'inverse, les trois autres souches bactériennes stimulaient la formation de racines latérales. La souche *Streptomyces* Bs14 est celle qui avait le plus fort effet stimulateur. Lorsque des combinaisons binaires ont été réalisées, l'effet nécrotique de *Streptomyces* Bs9 a disparu. Malgré l'inhibition de croissance de *Streptomyces* Bs9 induit par les autres souches, le système racinaire est resté significativement plus court avec une teneur élevée en anthocyanine. La souche *Streptomyces* Bs14 perdait quant elle son activité bénéfique vis à vis de la formation de racines secondaires lorsqu'elle était cultivée en combinaison avec les autres souches. Il est intéressant de noter que bien que les combinaisons binaires n'aient pas totalement supprimé l'effet néfaste de *Streptomyces* Bs9, la combinaison tripartite a permis d'améliorer la croissance des peupliers, tout en réduisant la teneur en anthocyanine et supprimant l'apparition de nécrose racinaire.

Parallèlement à cette étude de l'effet des bactéries sur le développement du peuplier, nous avons cherché à savoir si le peuplier avait un effet sur le développement des colonies et les interactions entre les différentes souches. Pour ce faire, nous avons utilisé le même cadre expérimental que celui décrit précédemment et nous avons mesuré la taille de la colonie. Dans l'ensemble, la taille de la

colonie de *Streptomyces* Bs14 était significativement plus grande en présence du peuplier et quelques soient les combinaisons d'interactions bactériennes. De plus, certaines interactions bactériennes ont été affectées par le peuplier.

Afin d'identifier les molécules impliquées dans ces différentes interactions, nous avons séquencé les quatre génomes bactériens et nous les avons analysés. Après assemblage, une annotation automatique a été réalisée à l'aide du pipeline RAST. Les données préliminaires indiquent que la taille des génomes et leurs teneurs en G-C correspondaient à celles des souches apparentées. Les pipelines antiSMASH et BAGEL ont ensuite été utilisés pour rechercher des clusters de gènes impliqués dans la biosynthèse de métabolites spécialisés. Un nombre élevé de clusters a ainsi pu être identifié, toutefois la plupart des gènes de ces clusters avaient un faible taux de similarité avec des gènes connus. D'autres analyses complémentaires seront donc nécessaires pour pouvoir identifier les composés impliqués dans les interactions. Des analyses métabolomiques par LC-MS et imagerie par spectrométrie de masse (IMS) ont été initiées à cette fin mais des optimisations techniques sont encore nécessaires avant de pouvoir obtenir des résultats tangibles.

Au final, cette étude apporte de nouveaux éclairages sur les interactions entre bactéries issues du sol et leurs effets potentiels sur le développement racinaire. Nous avons observé que les isolats bactériens provenant d'un micro-habitat commun peuvent avoir divers types et degrés d'interactions. Bien que l'on s'attende à ce que les bactéries soient en compétition constante dans les sols, les inhibitions locales étaient faibles. Il est possible que les composés antimicrobiens régulent la composition des communautés microbiennes. De même, les études préliminaires réalisées en présence de peuplier indiquent l'existence d'une grande diversité de comportements lorsque les bactéries se trouvent en culture pure. De plus, nous avons démontré que différents mélanges de composition bactérienne peuvent modifier les interactions individuelles et leur impact sur le développement du peuplier. Il est possible qu'il y ait une action synergique du consortium bactérien jouant un rôle important dans le biocontrôle des agents pathogènes dans les plantes.



# **General Introduction**

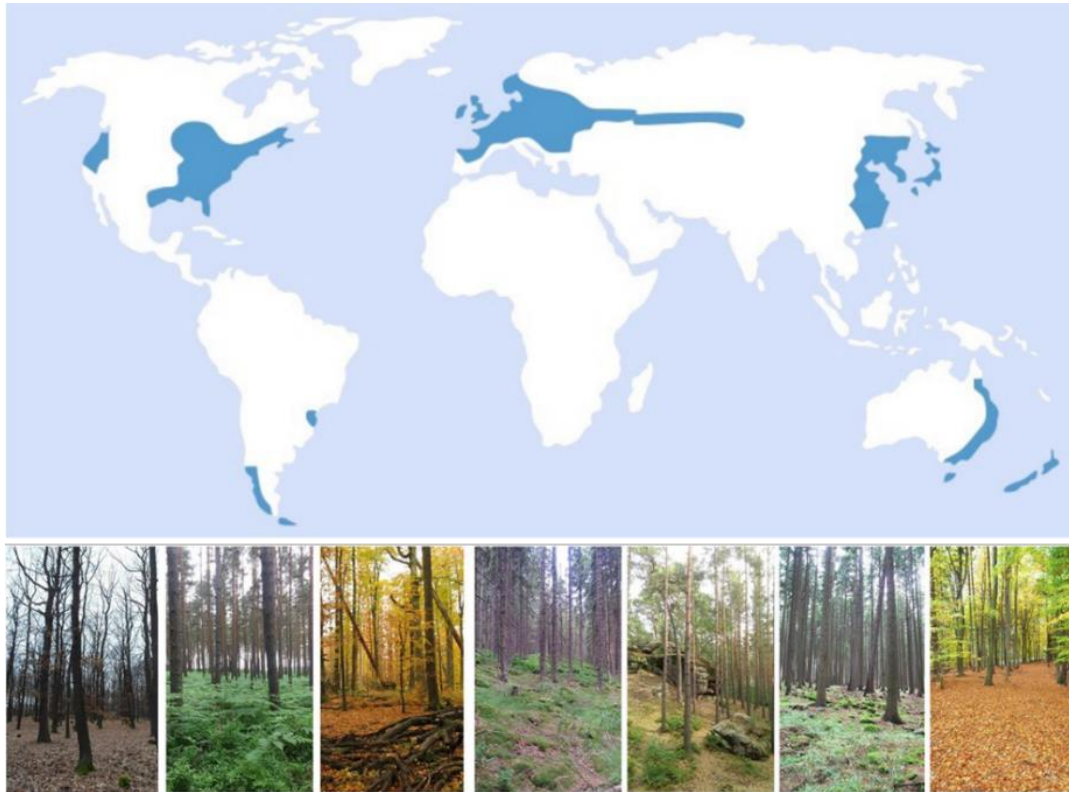
## General Introduction

### 1. Temperate Forest Ecosystems

Forests ecosystems cover around 40 million km<sup>2</sup> of terrestrial surface on Earth, fixing and cycling carbon, regulating hydrologic flows, conserving nutrients and providing structure for biodiversity to live in (Baldrian *et al.* 2017). Due to the large area of forests covering the total global land, the processes occurring in these ecosystems are crucial (Lladó *et al.* 2017). For instance, they represent the main carbon (C) sink (around 40% of the global soil carbon pool, influencing geochemical cycles and the climate) (Crowther *et al.* 2015). While trees capture carbon from the atmosphere, soil microorganisms contribute greatly to the carbon balance in these ecosystems (Lladó *et al.* 2017). Therefore, studying the ecology of forests is essential to understand nutrient cycles and predict the effect of the climate changes.

Temperate forests grow in a climate characterized by warm summers, cold winters and hold deciduous and coniferous trees, including, but not limited to, oak (*Quercus*), poplar (*Populus*), beech (*Fagus*), pine (*Pinus*), spruce (*Picea*) (Figure 1) (Lladó *et al.* 2017). Forest trees influence soil microbial communities through the secretion of metabolites from roots in the form of soluble exudates (i.e. sugars, organic acids, amino acids) and volatile organic compounds which are capable of diffusing into the soil environment as long distance messages (Schmidt *et al.* 2019). In addition, forest trees contribute to soil properties in terms of nutrients and pH by providing litter, dead wood and roots. These carbon, nitrogen and phosphorus stocks are complex and recalcitrant biomass, thus, soils in forests are considered nutrient deficient compared to agricultural lands (Lladó *et al.* 2017). Furthermore, these soils consist of horizons with a vertical gradient of nutrients. In essence, the top organic horizon involves litter, organic matter, and humus. Hence, it is considered as the richest in terms of organic nutrients, but these are hardly bioavailable. In between horizons, there are moderately decomposed organic layers and washed minerals. Lastly, the subsoils, the mineral horizon, has lost nutrients and is rich in bedrock weathered minerals (Uroz *et al.* 2013A).

Overall, heterogeneous distribution of nutrients in soil builds multiple habitats for the microbial component (Ranjard and Richaume, 2001). In addition, the microbiome interacts and contributes to soil functioning, tree nutrition and the dynamics within forest ecosystems (Haas *et al.* 2018).



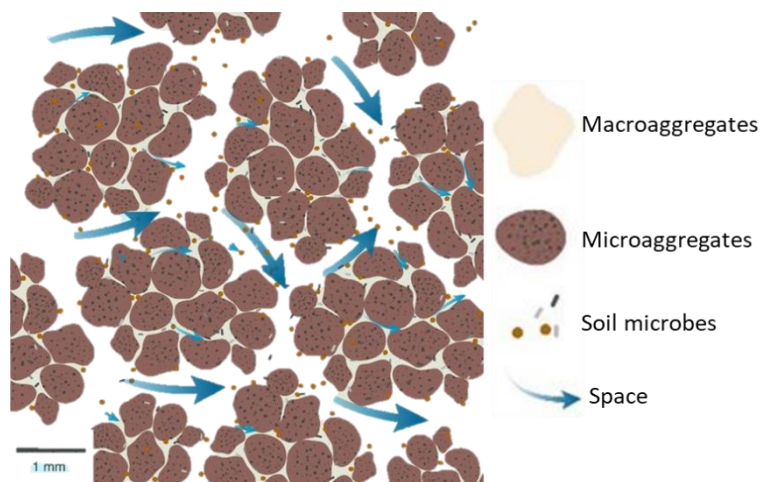
**Figure 1.** Distribution of temperate forests within the biosphere. The bottom images show forest ecosystems dominated by *Quercus*, *Pinus*, *Fagus*, and *Picea* trees, taken from Lladó *et al.* 2017.

## 2. Soil structure

Soils are complex three-dimensional structures composed of i) a mixture of soil particles that aggregate and ii) pore spaces (Figure 2) (Wilpiszkeski *et al.* 2019). Soil particles, defined by The United States Department of Agriculture (USDA) soil taxonomy, involve clay particles with diameters of less than 0.002 mm, silt particles with diameters between 0.002 mm and 0.05 mm and the largest particles are sand particles being larger than 0.05 mm in diameter (Foth 1990).

The assembly of soil particles, a combination of minerals derived from weathered rocks, organic matter, moderately decomposed matter, binding agents (e.g. fungal hyphal networks, roots, microbial exopolysaccharides) and microbial communities (Rashid *et al.* 2016, Rillig *et al.* 2017) form soil aggregates.

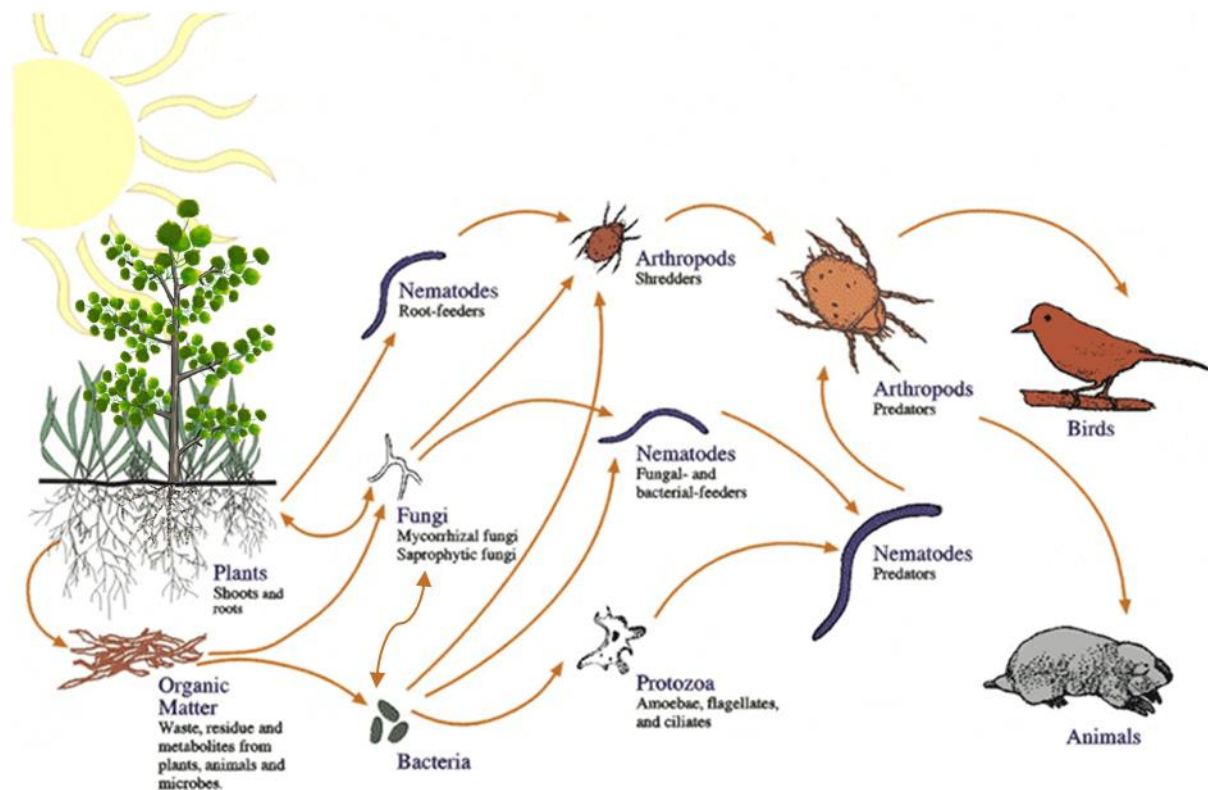
These aggregates of soil can assemble, compact or disintegrate by i) water/desiccation events, ii) mechanical events such as roots passing, animals or even anthropogenic effects. The texture of soil (i.e. relative content in sand, silt and clay) combined with these aggregations' events are key components of the soil structure. Such structural organization shapes complex mosaic of microenvironments that represent different habitats for the biotic component (Ranjard and Richaume 2001). Moreover, Rillig and collaborators suggest that each individual aggregate provides unique and compartmentalized environmental conditions, considering them as microbial incubators (Rillig *et al.* 2017). Hence, the understanding of microbial functions in soil requires the study microbial habitats at the microscale level.



**Figure 2.** Schematic representation of soil macro- and microaggregates. Space between aggregates would allow the flow of water, gases and microbes. Figure adapted from Wilpiszkeski *et al.* 2019.

### 3. Trophic levels and why microbes are essential for nutrient cycling

Soils hold a vast amount of biodiversity that is in charge of multiple essential functions. The soil biota includes bacteria, fungi, protozoa, invertebrates like nematodes and arthropods, mammals and plants (Tugel *et al.* 2000), where all contribute to the soil food web and the global biogeochemistry (Crowther *et al.* 2015). The first trophic level consists of photosynthetic organisms (mainly plants, lichens, moss, algae and some bacteria) which use the energy of the sun to fix carbon dioxide from the atmosphere. When dead, these become the organic matter for the decomposers, the second trophic level, involving mainly bacteria and fungi. These microbes are crucial since they convert and metabolize recalcitrant forms of soil-borne biomass to liberate bioavailable nutrients and elements (Jacoby *et al.* 2017). The third trophic level consist of arthropods, nematodes, annelids and protozoa which shred bigger particles into smaller, and feed from bacterial and fungal communities, controlling their population and processing organic nutrients (Crowther *et al.* 2015). Later, birds and mammals participate in this cycle by consuming the latter ones. Eventually, members of soil biota become dead matter and get recycled (Figure 3). Collectively, they all contribute in processing soil nutrient cycles (Tugel *et al.* 2000), yet, the conversion from complex recalcitrant biomass to available nutrients made by specialized bacterial and fungal enzymatic machineries is a pivotal step in the nutrient cycle.



**Figure 3.** Schematic representation of the trophic levels involved in the nutrient cycle in soils. Figure adapted from Tugel *et al.* 2000.

#### **4. Drivers of microbial distribution in forest soils**

The assembly of soil microbial communities (archaea, bacteria, fungi and viruses) is driven by edaphic conditions, biotic factors including plant-specific features and abiotic factors and their functional ability to adapt (Bulgarelli *et al.* 2013) (Figure 4). For instance, root-derived specialized metabolites that promote or inhibit microbial growth are involved in this selective process creating a horizontal gradient (i.e. distance from tree roots). Microbial communities can be associated with different habitats: inside root tissues (endosphere), in the root surface (rhizoplane) and in the soil directly connected to the roots (rhizosphere). Furthermore, mutualistic associations between fungi and roots (mycorrhizae) significantly change the chemical, physical and microbiological composition of soil due to hyphal exudation (Grayston and Jones, 1996). Thus, certain microbial communities will be determined by soil patches colonized by mycorrhizal fungi (mycorrhizosphere). Lastly, there are microbial communities associated with the surrounding soil defined by the lack of vegetation (bulk soil). These different habitats can be themselves influenced by extrinsic factors such as pH (Lauber *et al.* 2008, Rousk *et al.* 2010), organic matter availability (Maillard *et al.* 2019) or intrinsic factors such as tree species (Urbanová *et al.* 2015).

Microbial communities are also impacted with the gradient of C matter between horizons of forest soils. For instance, studies have reported distribution of bacterial and fungal communities from soil horizons displaying nutrient stratification (Šnajdr *et al.* 2008), mineral surface preferences (Nicolitch *et al.* 2016), or variations of extracellular enzymatic activities (Zifcakova *et al.* 2016).

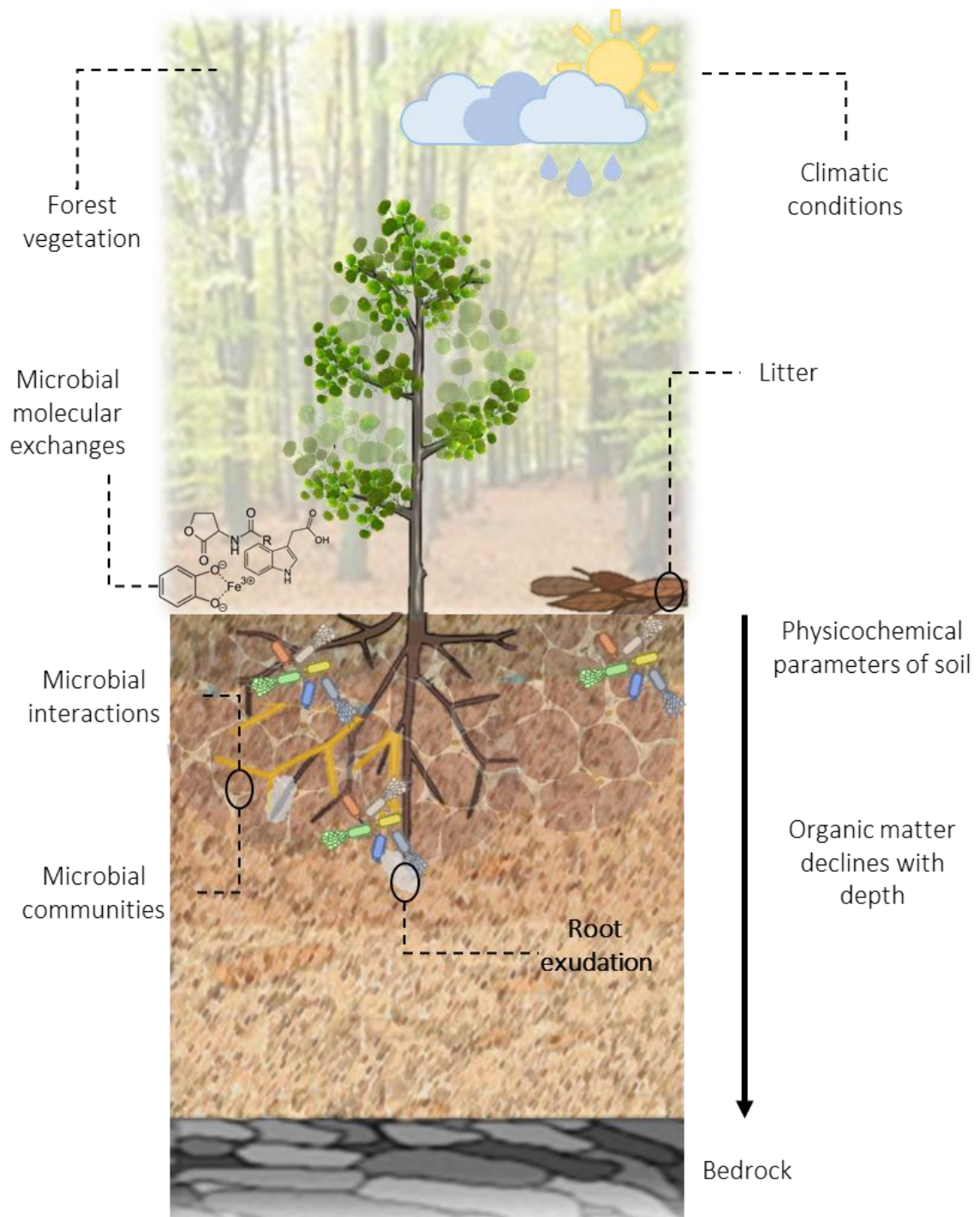
Multiple parameters of the climate change (e.g. atmospheric CO<sub>2</sub> concentrations, warming temperatures, and precipitation regimes) have a known impact on soil microbial communities (Castro *et al.* 2010). The reduction of precipitation and decrease in soil water availability is crucial for soil microbial survival (Felsmann *et al.* 2015). Prolonged drought periods can lead to reduced microbial growth as well as to changes in the microbial community structure (Felsmann *et al.* 2015). Previous studies have found soil microbial communities that are resistant to frequent soil drying (Griffiths and Philippot, 2013) and that drought may only affect specific microbial groups (Bouskill *et al.* 2013). In response, soil microbes, either die, resist or adapt to water availability. For instance, bacteria can lower soil water potentials in their cell walls to prevent water losses (Guhr *et al.* 2015). Moreover, certain bacteria can tolerate drought by forming specialized dormant and resistant structures (i.e. spores and endospores), although this trait is restricted to few genera (certain Actinobacteria, and *Bacillus* and *Clostridium*). Besides bacteria, availability of water can also affect the activities of enzymes that are secreted in soil, leading to decreasing microbial function and decomposition (Wei *et al.* 2018).

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Furthermore, seasonal variations (e.g. soil moisture, temperature, plant activity) add a temporal dynamic to the spatial heterogeneity of soil (Zifcakova *et al.* 2017). For instance, during autumn seasons, litter starts to fall forming a layer of organic matter that modifies the chemical structure of soil in terms of nutrient levels and pH, and in consequence influence soil microbial communities (Urbanová *et al.* 2015, Maillard *et al.* 2019). Moreover, carbohydrate-active enzymes (CAZymes), in charge of decomposing polysaccharides and lignins, were found to be active in the warm season rather than the cold season in temperate spruce forest (Baldrian *et al.* 2012), suggesting seasonal variations drive microbial community structure and functioning (Zhang *et al.* 2014).

Additionally, microbial strategies allow microbial successions and spatial patches over specific events (Kuziyakov and Blagodatskaya, 2015, Buée *et al.* 2009B). These strategies are based on the functionally dominant physiological traits to obtain resources (Ho *et al.* 2017), involving r-strategists analogs to copiotrophs which can grow fast on high and easily available resources, while K-strategists (oligotrophs) grow slowly but more efficiently on nutrient exhausted areas (Dorodnikov *et al.* 2009, Ho *et al.* 2017). At elevated temperature, the growth and enzyme production of certain microbes are stimulated, influencing soil decomposer communities (Crowther *et al.* 2015). Another example involves the increased precipitations which mobilize nutrients favoring fast-growing r-strategists (Kuziyakov and Blagodatskaya, 2015).

Moreover, microbes occupying the same habitat are likely consuming common resources leading to a high amount of possible interactions including competition, cooperation, and signaling, which are critical to growth and fitness and thus affect the structure of soil microbial communities (Schlatter *et al.* 2015, Simon *et al.* 2017). Competition (either by nutrients or space) can be seen as interference due to the production of antagonistic compounds towards other organisms and if lethal can be seen as exclusion removing entirely the other organism (Hibbing *et al.* 2010). Another form of competition is exploitation where one organism depletes nutrients preventing others from accessing them (Stubbendieck and Straight, 2016). By contrast, cooperation can be seen as the act of benefitting others via the release of public goods (i.e. any resource produced by one individual available for others) (Damore and Gore, 2012). This trait is thought to facilitate the growth within populations (kin selection) (Damore and Gore, 2012). However, if others can benefit from these public goods without paying the cost (lacking the cooperative trait), then these organisms would be considered as cheaters (Hibbing *et al.* 2010). In addition, small molecules act as signals between microbes of different species modulating interspecies interactions (Shank and Kolter 2009). Hence, studying microbes at the microscale would lead to elucidate more accurately microbial processes in soil considering the soil origin conditions.



**Figure 4.** Schematic representation of environmental drivers of the soil microbiome in forest soils. Forest vegetation, climatic conditions and physicochemical parameters of soils are few of the factors that determine microbial communities in soil. Microbial interactions, where molecular exchanges take place, contribute as an



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additional factor. Moreover, trees can also communicate through root exudates which attract soil-borne bacteria and may stimulate or inhibit their growth.

### 4.1 Fungal communities

Fungal communities are very well studied in temperate forest soils (Buée *et al.* 2009A). Fungi can grow in two distinct forms: as planktonic individual cells (yeasts) and as hyphal structures (filamentous fungi) (van der Wal *et al.* 2013). The later allows exploration of soils by spreading and bridging the soil pores and penetrating solid materials and plant tissues (de Boer *et al.* 2005). Additionally, the hyphal form allows fungi to translocate nutrients into exhausted soils, having a major impact in tree growth (de Boer *et al.* 2005). Fungi exhibit different lifestyles. For instance, saprophytic fungi serve as decomposers of recalcitrant substrates (Buée *et al.* 2009A) using extracellular hydrolytic and oxidative enzymes (Crowther *et al.* 2015). Pathogenic fungi feed on living organisms, damaging and eventually killing the host (Bonfante and Genre, 2010). Other fungi, endophytic fungi, colonize the inside of plant structures (leaves, stems, roots) as neutral, commensal or beneficial microorganisms. Lastly, mycorrhizal fungi create a symbiotic relationship with roots (Danielsen *et al.* 2012). The latter associate with 90% of plant species having a broad influence on forest ecosystems, and they form two main groups depending on the colonization type: if the fungus develops a hartig net colonizing intercellular spaces (ectomycorrhiza; Figure 5) or if it develops inside the cells (endomycorrhiza) (Bonfante and Genre, 2010). The latter are further divided into orchid (colonizing plants from the Orchidaceae family), ericoid (colonizing plants from the Ericaceae family) and arbuscular mycorrhizae (forming arbuscules in the cortical cells of vascular plant roots). In forests, mycorrhizal associations are crucial for trees since they uptake N and P from the forest soil and trade them against plant C ensuring plant growth. Additionally, they enhance plant resistance to abiotic and biotic stresses (Smith and Read, 2010).



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**Figure 5.** Ectomycorrhizae of *Lactarius* fungus on roots of oak (*Quercus robur*) in symbiosis with. Image taken from this study.

Fungi and bacteria are strongly interconnected and should not be viewed as separate entities (Lladó et al 2017). For example, the fungal hyphae which serves as highways for bacteria to disperse through soil (Simon et al. 2017), and, in return, bacteria may contribute to enhance their proteolytic enzymes, increasing biomass for both partners (Lohberger et al. 2019).

However, in this PhD work, we will focus on bacteria and the effects of diverse specialized metabolites produced during bacterial interactions (i.e. cooperation and competition). We further discuss how these bacterial interactions represent an impact on plant growth and health and conversely.

### 4.2 Bacterial communities

Bacterial communities in forest soils are likewise major components to the functioning of this ecosystem (Lladó et al. 2017). Together with fungi, they are involved in the decomposition of organic matter and degradation of complex, simple and soluble substrates as described (Buée et al. 2009B). Additionally, bacteria are responsible for N fixation (Reed et al. 2011), weather minerals (Uroz et al. 2011), nutrient mobilization (Nicolitch et al. 2016), and production a wide variety of bioactive compounds (Uroz et al. 2009A, Aigle et al. 2014).

Certain bacteria are beneficial to plants by acting with different modes: (i) those that form a symbiotic relationship forming specialized structures on host plant roots (e.g. nodules to fix nitrogen) (Reed et al. 2011), (ii) those that colonize the inner tissues of the plant without being pathogenic (endophytes)(Ryan et al. 2008), and (iii) those that colonize the rhizosphere (soil aggregates directly connected to the roots) and/or rhizoplane (root surfaces) (Glick et al., 1999).

While numerous soil bacteria may act as plant growth promoters, not all bacterial strains belonging to the same species have identical metabolic capabilities (Antony-Babu et al. 2017). Thus, characterization of native bacteria through cultivable approaches would help identify species that can promote development of forest trees.

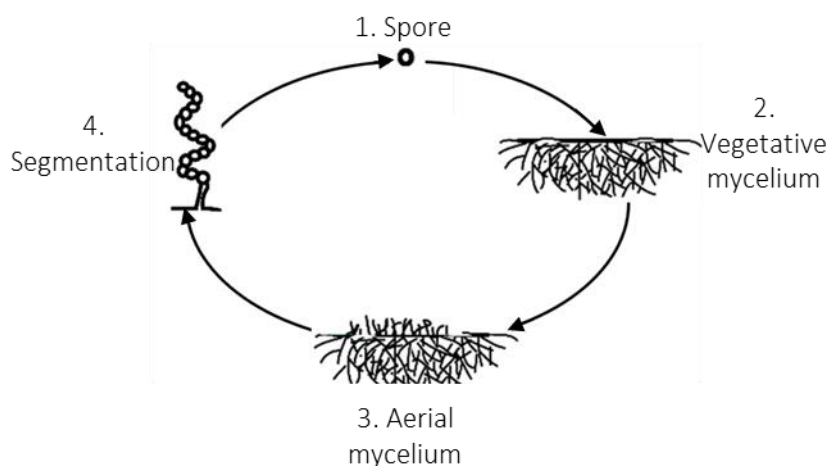
#### 4.2.1 *Streptomyces* consideration

*Streptomyces*, from the phylum Actinobacteria, are Gram-positive filamentous bacteria ubiquitous in soils, sediments and seawater (Labeda et al. 2012). In forest soils, they have been found colonizing the mineral horizon (Colin et al. 2017), the rhizosphere and mycorrhizosphere (Rozycki and Strzelczyk, 1986) and the endosphere in the case of *Quercus* (Lasa et al. 2019) and *Populus* (Klingeman et al. 2015). *Streptomyces* play a significant role in soil processes, notably in nutrient cycle converting

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cellulose and hemicellulose (Bontemps *et al.* 2013), in bioremediation (Bhatti *et al.* 2017) and in the production of important antibiotic metabolites (Aigle *et al.* 2014). While *Streptomyces* is a well-studied genus regarding its potential to produce diversified metabolites with biological activity (Olanrewaju and Babalola 2019), biotic contributions to their natural habitat including interactions with other bacteria and forest trees remain underexplored. Few plant diseases caused by *Streptomyces* are reported in the literature (Bignell *et al.* 2013). While plant growth promoting traits have been reported either by direct mechanisms involving nutrient enhancement, production of plant hormones, and biocontrol of phytopathogens, and indirectly via inducing systemic resistance (Viaene *et al.* 2016). Therefore, there is a motivation in this study to have a specialized isolation method to ensure the selection of this genus.

Importantly, *Streptomyces* exhibit a multicellular and filamentous organization, morphogenesis, and regulated flux of secondary metabolites depending the morphological state (Figure 6) (Grasso *et al.* 2015).



**Figure 6.** *Streptomyces* growth cycle.

A spore (1) produces one or more germ tubes that elongate by apical growth, and initiate side branches with new hyphal tips, allowing colony biomass to increase almost exponentially forming the vegetative mycelium (2). Likely in response to a nutrient limitation, the vegetative mycelium produces branches extending vertically into air spaces (3). After elongating for a while, distal regions of these aerial hyphae divide by sporulation septa into segments that become spores again (4). Figure adapted from Grasso *et al.* 2015.

## **5. Important nutritional functions for survival in forest soils**

### **5.1 Carbon sources**

Since soils contain large pools of complex carbon compounds such as cellulose, hemicellulose and lignin, secretion of a variety of extracellular enzymes are vital functional abilities to survive. From the recalcitrant lignocellulose, fungal saprophytes produce a wide range of extracellular enzymes such as laccases and peroxidases that degrade lignin (Janusz *et al.* 2017). Then other inhabitant species harboring glycosyl hydrolases such as cellulases and hemicellulases can degrade the cellulose and the hemicellulose. These enzymes differ in their mechanisms of action by cleaving internal bonds or acting on reducing or non-reducing ends releasing glucose molecules (Lopez-Mondejar *et al.* 2016). Both, fungi and bacteria, are capable to produce the later enzymes likely acting synergistically to decompose plant biomass (Lopez-Mondejar *et al.* 2016).

Tree roots exude primary metabolites such as sugars, amino acids, and carboxylic acids representing carbon and nitrogen substrates for microbial growth (Hu *et al.* 2018). The main sugars include monosaccharides such as fructose, mannose, and glucose, disaccharides (maltose), five carbon sugars (arabinose), and oligosaccharides that can act as a food and energy source for the microbes (Olanrewaju *et al.* 2019). The type of exudates released into soil depend on the plant species, health, age (Pausch and Kuzyakov 2018) and they regulate the microbial community of the rhizosphere.

### **5.2 Nitrogen sources**

Nitrogen is a limiting element for plant growth, and mainly enters to soil ecosystems through fixation (Lladó *et al.* 2017). Fixation of atmospheric nitrogen consist of the reduction of N<sub>2</sub> to ammonia by the nitrogenase enzyme (Orr *et al.* 2011). Then fixed inorganic nitrogen compounds participate in the biosynthesis of amino acids and proteins pathways. Bacteria able to fix nitrogen involve free-living cyanobacteria, certain *Pseudomonas*, *Frankia*, and *Azotobacter* (Orr *et al.* 2011). Importantly, in forests, most nitrogen is provided to trees by mycorrhizal fungi, and synergy between symbionts have been reported (Quintino de Olivera *et al.* 2017).

Besides plant proteins and root exudates, fungal biomass can also contribute to nitrogen supplies. Their cell wall main component – chitin – can be degraded by proteolytic exoenzymes and chitinases produced by bacteria as a nutritional strategy or as an antagonistic interaction (Lladó *et al.* 2017). As not all fungi act beneficial for the plants, certain bacteria can act as natural biocontrol agents, regulating the damage by interfering in the growth of a phytopathogenic fungus (Brzezinska *et al.* 2014). Chitin is also found in the exoskeleton of arthropods and nematodes, thus, probably microorganisms harboring chitinases can regulate not only fungal, but also, insect community dynamics.

### **5.3 Phosphorus sources**

A source often low or insoluble in forest soils is phosphorus. This limiting macronutrient is vital for plant growth. On crop plantations, it is common to use P-fertilizers to circumvent the deficiency, however this is not the case for forest ecosystems. Instead, in forests, almost all plants and trees live in association with mycorrhizal fungi to trade nutrients (Danielsen *et al.* 2012). In addition, they have extended their root system, and exude organic acids that i) increase P availability or ii) recruits phosphate solubilizing microorganisms (Richardson *et al.* 2002). Indeed, some bacteria can diffuse, transform, mobilize and transport P for the plant to acquire (Shen *et al.* 2011). Fungi and bacteria, both exhibit abilities for solubilization and mineralization of insoluble soil phosphorus ability. The principal mechanism is the production of organic acids which acidifies the environment releasing P ions (Alori *et al.* 2017). Moreover, certain microorganisms display extracellular enzymes that catalyze the hydrolysis of organic P-containing substrates releasing inorganic P like phosphatases (Alori *et al.* 2017). Additionally, among phosphatases, there are different types depending on the pH of mode of action (e.g. alkaline phosphatases and acid phosphatases) or accordingly to the organic form (e.g. phytases hydrolyze phytate, a predominant organic phosphorus in soils, Singh and Satyanarayana, 2011).

### **5.4 Iron sources**

Iron is poorly available in forest soils, yet it is essential for life of all forms, with few exceptions (Andrews *et al.* 2003). Its solubility increases with low pH, hence, plants and microbes can acidify the soil in order to uptake this mineral (Jin *et al.* 2014). Soil acidification can be reached by releasing protons (H<sup>+</sup>) (e.g. via nitrification (Van Miegroet and Cole, 1984)) and low molecular weight organic chelating compounds, such as citric acid and oxalic acid (Jin *et al.* 2014).

Siderophores are secondary metabolites that play a role in iron homeostasis in microorganisms. They solubilize iron from insoluble ferric hydroxides and make it available for the uptake. Thus, synthesis of these compounds is essential for the survival and proper development of many microorganisms. It has been reported that the presence of siderophores can even promote the development of unculturable bacteria (D'Onofrio *et al.* 2010). On the other side, it has been described that siderophores are able to uptake different metal ions such as Al<sup>+3</sup>, Zn<sup>+2</sup>, Cu<sup>+2</sup> and Mn<sup>+2</sup>, but this process depends on their binding affinity (Johnstone and Nolan, 2016). Since these metals are of great importance for all biotic life, there is strong competition for them in forest soils.

## **6. Underground chemical communication**

Signaling molecules are molecules that influence the behavior of the producing organism, but also, of others (Ryan and Dow, 2008). They regulate processes such as metabolic pathways (primary and secondary metabolism), and participate in morphological and developmental pathways (growth, germination, sporulation, biofilm) (Shank and Kolter, 2009). However, in nature, microorganisms grow in polymicrobial communities. Hence, they may act within and between species influencing the development of others and modulating their interactions (Ryan and Dow, 2008). Indeed, in soils, natural encounters between plants and microbes such as: i) root-root, ii) microbes-microbes, iii) root-microbes, form complex interactions (i.e. competition, cooperation, antagonism) where signals are exchanged and detected. Hence signals in soils are directed i) between microbes, ii) from plants to microorganisms, and iii) from microorganisms to plants. Among interspecies and interkingdom interactions, signals may modulate pathogenic and symbiosis behaviors, competition for space, competition for nutrients, cooperation through public goods, direct or indirect antagonism, direct or indirect beneficial interactions, and loops driven by specific species-to-species interactions, where plants alter the soil system inducing new specialized metabolites, and thus different scenarios.

### **6.1 Root-derived molecules**

In soils, complex plant – soil microbes feedback occur, in which specialized metabolites can act as signals (Schlatter *et al.* 2015). Under abiotic stress conditions (e.g. water limitation, nutrient depletion), plants are more dependent on associated microbes that can enhance their ability to combat stress (Khan *et al.* 2016). Studies have found that plants exude specific metabolite cocktails (soluble specialized metabolites or volatile organic compounds) that may repel or attract specific microbes (Massalha *et al.* 2017). Root exudates, specifically allelochemicals, play a role in the interactions with other plants (Massalha *et al.* 2017). These molecules involve benzene-derived compounds, phenolics, hydroxamic acids, and terpenes play a signaling role in plant recognition and cause competitive root proliferation (Semchenko *et al.* 2014). However, if soil microbes transform these molecules, the biological property gets modified, changing the message and the possible interactions (Jilani *et al.* 2008).

Among other root-derived molecules, strigolactones are a group of sesquiterpene lactones, which act as signals triggering hyphal branching and inducing symbiosis between roots and arbuscular mycorrhizal fungi (Akiyama and Hayashi, 2006). Interestingly, while these molecules are produced upon phosphate deficiency to communicate with arbuscular mycorrhizal fungi, parasitic weeds can also detect them and colonize plant roots (Akiyama and Hayashi, 2006). Thus, same molecule can be involved in different types of interactions.

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Roots exude flavonoids that attract nitrogen fixing bacteria and induce root nodulation and other determinants to develop the symbiosis (Hassan and Mathesius, 2012). Additionally, the same molecule may also interfere in quorum sensing acting as a mimic signal (Bauer and Mathesius, 2004). For instance, one study found that flavonoids induced the *nod* gene involved in the nitrogen fixation, possibly to enhance symbiotic behaviors of rhizobia (Perez-Montano *et al.* 2011). Another study showed that flavonoids inhibit quorum sensing in *Escherichia coli*, *Vibrio fischeri* and *Pseudomonas aeruginosa*, possibly as there is no beneficial or negative interaction with these bacteria (Hassan and Mathesius, 2012).

While certain molecules can participate in different interactions, there are some host signals that act selectively towards one beneficial organism to colonize the host. For instance, one study showed that malic acid secreted from the roots of *Arabidopsis* recruited specifically a beneficial *Bacillus subtilis*, but not other *Bacillus* species (Rudrappa *et al.* 2008). This suggests that each beneficial interaction may be elicited by a specific signal and a specific host.

## 6.2 Bacteria-derived molecules

### 6.2.1 Phytohormones

In return, plant-associated microbes assist plants through the production of bacterial phytohormones, which act as growth regulators altering plant metabolism, morphology and stimulating plant defense. Their biological activities influence processes such as germination, early seedling growth and bacterial colonization of plants. Among phytohormones, auxin (indole-3-acetic acid) is one of the most reviewed. The effect of this indole hormone is concentration-dependent causing cell division, elongation and differentiation, affecting mainly root and shoot growth (Spaepen and Vandereven, 2011). Moreover, it has been shown that auxins promote heavy metal tolerance in plants and enhance mineral phytoextraction (Egamberdieva *et al.* 2017). Interestingly, a study reported that auxin stimulates Hartig net development of ectomycorrhizal fungi suggesting a possible cell wall loosening to allow the fungus to enter the plant (Felten *et al.* 2009).

Few studies have reported the effect auxin has on bacteria. Bacterial biosynthesis of this phytohormone can impact gene expression in some bacteria, specially upregulating its own pathway (Koul *et al.* 2014). In this sense, this signal would be beneficial to colonize rhizosphere environments. Moreover, Lee and collaborators found that indole induces biofilm production in *Pseudomonas fluorescens* and influence its virulence factors (Lee *et al.* 2007).

### 6.2.2 Quorum sensing signals

Quorum sensing signals allow bacteria to communicate through different types of molecules within and between species in a cell-population density dependent manner (Miller and Bassler, 2001, Uroz *et al.* 2009A). These signals (extracellular chemicals called autoinducers) regulate the expression of genes that are involved in virulence, motility patterns, biofilm formation, extracellular enzymes and antibiotic resistance (McBride and Strickland, 2019, Uroz *et al.* 2009A).

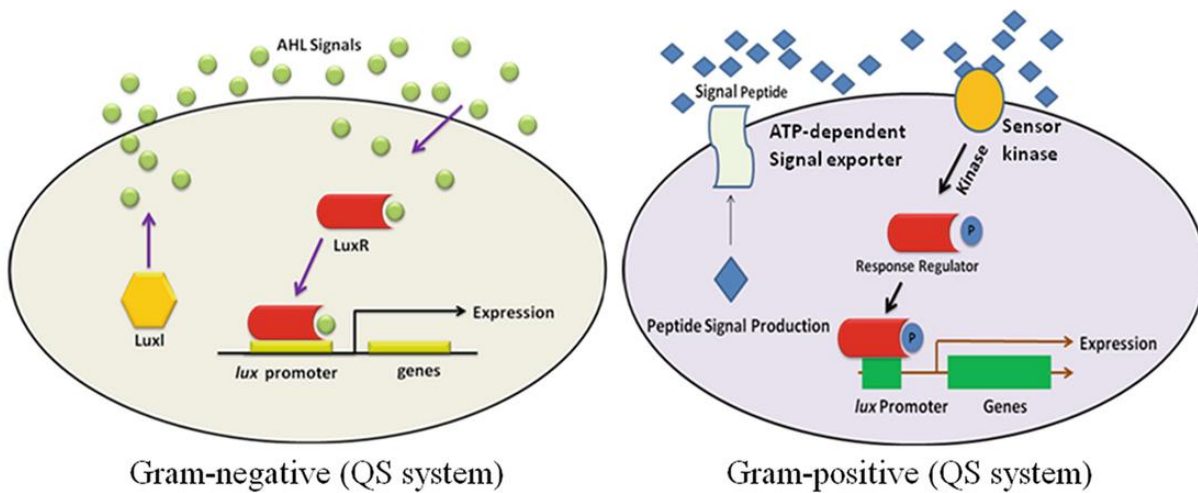
Quorum sensing systems for Gram negative bacteria consist of LuxI/LuxR homologues, similar to those first described in *Vibrio fischeri* circuit (Miller and Bassler, 2001). LuxI-like gene encodes for an acylated homo serine lactone (HSL) molecule that is constitutively produced (i.e. autoinducers). When these autoinducers have reached a concentration threshold correlated with the cell density, LuxR-like proteins can recognize them and bind them. When LuxR-HSL complex is formed, gene transcription is activated, thus it enables to control certain processes (Figure 7, left). Importantly, HSL molecules can hold different acyl chain in terms of carbon and this will depend on the synthase enzyme (Churchill and Chen, 2011).

The majority of Gram positive QS systems employ secreted peptides. These act as autoinducers that bind to signaling receptors on the surface of the receptor bacteria. When these peptides have reached a threshold level, two component sensor kinases detect these signals. Then a phosphorylation/dephosphorylation cascade activates a regulator protein that binds to the DNA and alters the transcription of target genes (Figure 7, right) (Miller and Bassler, 2001).

In the particular case of *Streptomyces*, gamma-butyrolactones (GBL) act as autoinducers and other signal molecules like PI factor [2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol] and AHFCAs (2-alkyl-4-hydroxymethylfuran-3-carboxylic acids), have been described to play similar functions (Du *et al.*, 2011). This system consists of a GBL synthase and a cognate receptor, similar to Gram-negative bacteria systems: at threshold concentrations the diffusible GBL binds to the intracellular receptor and activates expression of the transcriptional activator which in-turn regulates multiple phenotypes (Polkade *et al.* 2016).



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**Figure 7.** Quorum sensing systems. Left: in Gram-negative bacteria. Right: in Gram-positive bacteria. Figure from Ganesh and Raj, 2018.

While quorum sensing is a valuable trait, the ability to interfere with these signals, also termed quorum quenching (QQ), is also a strategy to survive in a selective pressure. Although QQ feature does not kill bacteria, it affects the expression of specific functions among which virulence (Uroz *et al.* 2009A). Besides abiotic factors that affect QS signals like temperature and pH, biotic activities from other microorganisms can also interrupt communication. Among the QQ mechanisms, the best studied system is against AHLs molecules. Enzymatic mechanisms described already involve lactone hydrolysis, amidohydrolysis and oxidoreduction. Isolation and identification of microbes responsible for the degradation of these QS molecules may help control expression of pathogenicity in infected plants (Uroz *et al.* 2003).

Not only bacteria can quench microbial communication, but plants also contribute to deliver (or not) the message. Some plants have been demonstrated to produce AHL analogs which bind to the receptors of plant-associated bacteria (Bauer and Mathesius, 2004). These AHL mimics have been found in higher plants such as *Medicago truncatula*, rice, tomato and pea, either stimulating or inhibiting the QS system, by interfering with AHL synthesis, transport and receptor (Sayre *et al.* 2004).

### 6.2.3 Antibiotics as signal molecules

Antibiotics are naturally occurring organic molecules of low molecular mass (< 3000 Da) with the ability to inhibit the growth living organisms (Ryan and Dow, 2008). Besides acting as microbial weapons, antibiotics, at subinhibitory concentrations, can act as interspecies signaling molecules regulating the homeostasis of microbial communities (Davies, 1990, 2007; Davies *et al.*, 2006; Seshasayee *et al.*, 2006; Yim *et al.*, 2006, 2007). It has been demonstrated that at subinhibitory concentrations, antibiotics regulate the transcription of a large number of genes in different bacteria (Goh *et al.* 2002). For instance, previous studies have shown an increase of the expression of genes

encoding for bacterial determinants that influence interaction with host cells (Linares et al., 2006; Marret et al., 2007). Moreover, antibiotics can induce biofilm formation (Hoffman et al., 2005), among others.

### 6.3 Effect of signal molecules on inhibitory compounds

Importantly, signal molecules may alter the synthesis of specialized metabolites with antagonistic properties, which have major implications in microbial interactions. While some these specialized metabolites are released in the soil environment in a constitutive manner, others are induced by environmental cues and signaling molecules (Massalha *et al.* 2017). Most of these molecules have been characterized through cultivable approaches. However, recent advances in mining of genome data have revealed the presence of thousands of biosynthetic gene clusters for specialized metabolites in bacteria, particularly in Actinomycetes. These clusters are often not expressed in regular laboratory growth conditions (Vicente *et al.* 2018). Among these cryptic metabolic pathways, polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS) and ribosomally synthesized and post-translationally modified peptide (RiPP) pathways are major biosynthetic systems. These may code for biologically active natural products such as antimicrobial agents, cytotoxic and antiviral compounds. Antibiotics can work through a wide array of mechanisms, and depending on the molecule and the target, they will either inhibit the growth of other microorganisms or kill them. Moreover, some molecules can have multiple effects: for example, lipopeptides from *Bacillus subtilis* acting as surfactants have shown antibacterial and antifungal properties (Zhuang *et al.* 2013). Overall, these specialized metabolites can act as biocontrol agents to regulate soil microbial communities structure.

### 7. Tree microbiome

Forest trees prevent soil erosion, support microbial, animal, and plant diversity, play key roles in nutrient and water cycling, and mitigate the effects of climate change acting as carbon dioxide sinks. Thus, the health of forests is of great significance. Soil and plant-associated microorganisms are crucial to sustain the fitness and development of trees (Mercado-Blanco *et al.* 2018). These microorganisms are present in nearly all tissues, both inside and outside their surfaces (Lasa *et al.* 2019). Soil influenced by roots, the rhizosphere, is a niche of great microbial diversity strongly determined by plant metabolism mainly through roots exudates (Lasa *et al.* 2019). These root-associated microorganisms, enhance water and nutrient acquisition, improve resilience against abiotic constraints and protect against pathogens (Cregger *et al.* 2018).

Thanks to the recent ‘-omics’ technologies, it is now possible to explore in depth microbial community structure and their functioning (Lebeis 2015). Distinct microbiome composition of trees rhizosphere and root endosphere across environmental gradients (Gottel *et al.* 2011) and between tree genotypes or species (Bonito *et al.* 2014) has been demonstrated. Thus, each plant microbiome type differs with its surrounding environment contributing to plant health (Lebeis 2015).

### 8. *Populus* as a model system

*Populus tremula x alba*, clone INRA 717-1B4, studied in this project, is a model tree used in the Unit Interactions Arbre-Microorganismes (IAM) to dissect tree-microbe interactions (Figure 8). INRA 717-1B4 is an interspecific hybrid female clone from the cross between a female *Populus tremula* tree and a male *Populus alba* tree (Mader *et al.* 2016). This hybrid was part of a breeding program initiated by Dr. Michel Lemoine in the 1960's (Lemoine 1973, PhD thesis). This woody species clone is a well-established model tree in the world due to its ease to manipulate and propagate vegetatively, to interact with other organisms, its relatively small genome size and the possibility to make transgenic lines allowing for discovering gene expression and function (Brunner *et al.* 2004). Previous co-inoculation studies manipulating poplar plantlets have allowed to dissect the molecular mechanisms of the dialog during the establishment of the symbiosis between the ECM fungus (*Laccaria bicolor*) and roots; including the promotion of plant growth *in vivo* and *in vitro* (Muller *et al.* 2013), the stimulation of lateral root formation through auxin signalling (Felten *et al.* 2009) and the production of small secreted protein to alter root cell functioning (Plett *et al.* 2014). *Populus* has also served as an ideal model to examine how microbial communities are associated to different niches (Cregger *et al.*

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2018). With the purpose to analyze a simplified network of interactions connecting the tree roots to the soil associated bacteria, we selected this *in vitro* tree model.



**Figure 8.** *Populus tremula x alba* clone INRA 717-1B4 clone. Left: grown *in vitro* on modified Murashige and Skoog agar. Bar measures 1 cm. Right: grown in green houses. Image taken from [urgi.versailles.inra.fr](http://urgi.versailles.inra.fr)

## 9. Objectives of the thesis

Overall, although some biotic and abiotic forces driving soil microbial diversity and functioning have been identified, soil remains a black box for two main reasons. First, our knowledge of the functions which are expressed by these microorganisms *in situ* as well as the impact of these activities on the functioning of the communities is still fragmented. Second, only few studies performed so far considered the multiple interactions which occur between microorganisms in natural environments and how these interactions impact the functioning of these communities and their interactions with plants. Although it is hypothesized that microbes dedicate enormous resources to microbial interactions, chemicals and molecular actors involved in these metabolic exchanges are still poorly characterized.

These metabolic exchanges within the rhizosphere are expected to play important roles in the structuration of soil microbial communities, resilience of these communities in response to external perturbations and plant development. In this context, the goals of this PhD project were to i) decipher the dialogue between bacterial partners isolated from the same micro-niche, ii) understand how this molecular dialog influence tree development, and iii) understand how the tree affect the bacterial partners and their interactions.

Culture-dependent approaches allows the analysis of bacterial physiology traits, functional abilities, behaviors, genetics and overall ecology. Thus, to acquire the bacterial partners, an isolation method of bacteria will be used with different grains of soil, to ensure that bacterial isolates were sharing the same habitat and thus potentially naturally interacting. In addition, a culture-independent approach

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will be used to determine the initial microbial content of the grains (cultivable and non-cultivable) as it is known that culture-dependent approaches are restricted.

Lastly, an *in vitro* setting designed previously for the growth of poplar will be used as a nutritional based to perform the different combinations to decipher the impact between bacteria and between bacteria and tree. *Populus tremula x alba*, clone INRA 717-1B4 was chosen since it is a well-established model tree due to its ease to manipulate, to propagate vegetatively and to interact with other organisms.

### Objectives in detail

1. Isolate and characterize bacterial communities via culture-dependent approach using one-grain soil samples from different habitats.
2. Characterize bacterial and fungal communities through culture-independent approach (DNA sequencing).
3. Characterize potential functional abilities of a library of bacterial isolates using enzymatic tests and antimicrobial potentials.
4. Determine the effects bacteria on other bacteria
5. Evaluate the potential biotic interactions by:
  - a. Pairwise bacterium – *Populus* interaction
  - b. Bacterial consortium and *Populus* interaction
6. Investigate if bacterial influence growth and physiology of *Populus* and if poplar influences bacterial behavior.
7. Identify specialized metabolites involved in the molecular dialog between bacteria and between bacteria and poplar through genome mining and metabolomics.

## *Structure*

### **Structure of the thesis**

This manuscript is composed of 3 chapters. The first chapter introduces the microbial communities living in grains of soil through culture-dependent and independent approaches. The second chapter describes the functional diversity of the bacterial collection and the biotic interactions: antimicrobial production, bacterial quorum sensing and quenching, bacterium – bacterium interactions, and bacterial effects on *Populus*. The last chapter focuses on four bacterial strains isolated from a grain of bulk soil. These strains were tested for their capacity to influence *Populus*'s growth and physiology, and how *Populus* can affect colony size. Moreover, the genome of these strains was studied to predict possible biosynthetic gene clusters that might be involved in the molecular dialogue between bacteria and with plants.

Chapter I: **Taxonomy of microbial communities and cultivable bacterial species from grains of soil**

Chapter II: **Potential functional diversity and biotic interactions**

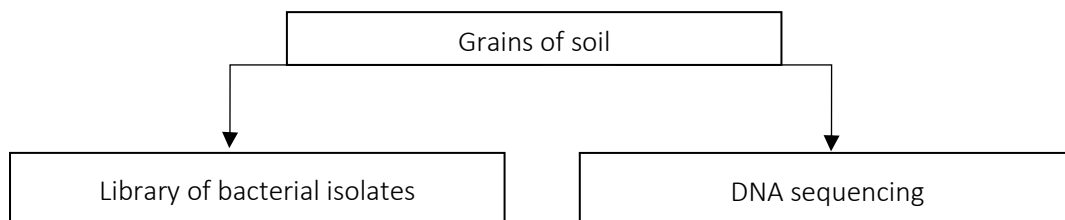
Chapter III: **Potential dialogues between bulk soil bacteria and *Populus***

# Chapter I

## **Taxonomy of microbial communities and cultivable bacterial species from grains of soil**

## Context

Soils are among the most biodiverse microbial habitats on Earth harboring bacteria and fungi that interact and contribute to the ecosystem. These interactions influence their local environment and neighbor microorganisms, through the consumption of nutrients, accumulation of byproducts, production of signal molecules and metabolite exchanges. Since our main goal is to acquire insights into the impact of the molecular dialogues between bacteria and between bacteria and roots of forest trees, we decided to study soil at the microscale: a grain of soil. In order to study bacterial interactions, we need to work with cultivable bacterial strains that are sharing the same ecological niche and are likely interacting in their natural environment. Here, we characterize the cultivable bacterial content of several grains of soil. Then, to evaluate how representative the bacterial collection is, we studied the initial (alive and dead) bacterial communities of grains of soil by high throughput amplicon sequencing. Since bacteria are not alone in soil and as a complementary point of view, fungal communities were also characterized in parallel by the same non-cultivable approach. This chapter will be divided in three sections: i) the characterization of the local environment: tree and ectomycorrhiza involved, ii) the composition of libraries of bacterial isolates and iii) the initial microbial content of grains of soil (bacteria and fungi).





## Introduction

Forests are main carbon sinks where soil borne microbes, bacteria and fungi, play critical roles breaking down organic matter, decomposing dead plant residues, and cycling carbon, nitrogen, phosphorus (Zifcakova *et al.* 2015, Lladó *et al.* 2017). The complex network of interactions occurring underground is an underexplored world at the molecular level. For these reasons, the study of forest ecology, specifically, microbial communities' functions, is key to understand how this ecosystem works (Zifcakova *et al.* 2015, Lladó *et al.* 2016). The development of DNA sequencing tools has proved the microbial diversity in diverse ecosystems (Janssen 2006, Hugerth and Andersson, 2017). It is now well known that soils are among the most biodiverse microbial habitats on Earth (Vos *et al.* 2013, Ladau *et al.* 2019). However, a complete picture is still poorly accomplished (Lladó *et al.* 2016), accounting for efficiencies of DNA extraction which varies from cell to cell (Frostegard *et al.* 1999), effect of amplified region choice (Bukin *et al.* 2019), sample size (Penton *et al.* 2016) and soil sampling strategy (Vos *et al.* 2013). For the moment, libraries of DNA (16srRNA genes and ITS regions) allow a survey of the global soil microbial community structure and composition (Janssen 2006, Hugerth and Andersson, 2017) and ecological interpretations.

Culture-dependent approaches are also very important and are mainly used to characterize bacterial and fungal physiology, functional abilities, behaviors, genetics and overall ecology (Vartoukian *et al.* 2010, Hugerth and Andersson, 2017). However, our ability to cultivate soil microorganisms in laboratory conditions is still poorly achieved. One of the major obstacles is to mimic the physical and nutritional heterogeneity and complexity of soil. Indeed, previous studies have discussed about the '1% culturability paradigm' (Martiny 2019). Several reports have made experimental efforts into finding key components to cultivate bacterial species from soil dominant phyla (Balestra *et al.* 1997, Davis *et al.* 2005, Lladó *et al.* 2016), however, the proportion of cultivated isolates compared to the phlotypes found in DNA sequencing methods is still very low. Consequently, current literature of microbial roles in soil is attributed to genomics of the cultivated ones and metagenomes obtained from environmental samples (Hugerth and Andersson, 2017). Based on these genomes, predictive gene databases are starting to provide new insights of microbial functions (e.g. PICRUSt for bacteria (Langille *et al.* 2013), FUNguild for fungi (Nguyen *et al.* 2016)). Although DNA sequencing tools are useful to estimate microbial composition, it is likely biased since the taxa detected is not necessarily the metabolically active one since sequencing methods do not distinguish active, dead, dormant cells, and spores (Vos *et al.* 2013, Lasa *et al.* 2019).

Combining molecular, cultivable and gene prediction approaches have allowed to roughly estimate the bacterial diversity and interpret dynamics in soil (Balestra *et al.* 1997, Lasa *et al.* 2019). Yet,

defining a scale is important in any ecological question (Studdienbeck *et al.* 2016, Ladau *et al.* 2019). The characterization of bacterial communities from an ecosystem and of their role in the environment has been performed using large soil samples (e.g. forest soils: Lladó *et al.* 2016, Uroz *et al.* 2013B). DNA analysis studies with the purpose to describe the effect of specific environmental factors, for example rhizosphere effect (Buée *et al.* 2009B), pH (Lauber *et al.* 2009), trees species (Urbanová *et al.* 2015), or organic matter removal (Maillard *et al.* 2019), also have targeted large composite samples of soil with defined parameters. In addition, libraries of bacterial strains isolated from soil are also made generally from large composite samples of soil with the purpose of covering the natural diversity of strains as best as possible.

In this study, our main aim was to acquire insights into the influence of molecular dialogues between bacteria with forest trees. As soils are made of multiple microhabitats that are not necessarily interconnected (soil aggregates) (Carson *et al.* 2010, Penton *et al.* 2016, Fierer 2017, Rillig *et al.* 2017), large samples of soil likely leads to the creation of libraries of strains that may never encounter and are thus not good targets to analyze microbial interactions (Stubbendieck *et al.* 2016, Carson *et al.* 2010). By analyzing bigger scales, we may be diluting the potential interactions occurring in soil (Fierer 2017). Bacteria that live in soil may be located too far apart to directly exchange molecules, therefore, the suitable functional scale to study microbial dialogues is at the microscale (Fierer 2017, Grundmann and Normand, 2000). Here, inter-cell distances shorten and allow microbes to meet other microbial cells and interact physically or through the diffusion of active compounds (Raynaud and Nunan, 2014). According to Nunan (2017), bacteria in soil interact with around 11 ( $\pm 4$ ) other species for low bacterial density samples and 97 ( $\pm 24$ ) species in high bacterial density (Nunan, 2017). However, due to abiotic and biotic heterogeneity in soil, we might expect a wide variety of different scenarios.

For these reasons, we hypothesized that the isolation of strains at the microscale would allow capturing bacteria that share same biotic and abiotic conditions and are likely interacting together. In this context, our aims were (i) to isolate and characterize cultivable bacterial strains since bacteria are the main drivers of the nutrient cycle, with special attention to isolate *Streptomyces* since it is an important bacterial genus for the production of bioactive compounds and (ii) to characterize initial microbial communities through DNA sequencing approach at a microscale from different grains of soil to have a reference of the natural living and dead inhabitants (bacteria and fungi). To do this, we performed 16s and ITS DNA sequencing analyses and isolated a library of bacterial strains from isolated grains of soil, a microscale where bacteria likely shared the same environmental conditions.

## Materials & Methods

### *Site description and samples collection*

This study was carried out at one experimental site of the “Matières Organiques des Sols” (MOS) network (<https://www6.nancy.inra.fr/bef/Moyens-analytiques/MOS>). Here, the geological material belongs to Pliensbachian marl, humus type was considered as Eumull/mesomull, the soil texture was classified as clay-loam and the average pH was 5.0 (Akroume *et al.* 2016). Soil samples were collected in a mixed forest with mainly oaks (*Quercus robur*) and beeches (*Fagus sylvatica*) in Champenoux (coordinates Lambert93: dir N 6852020.9 dir E 945689.4 280) in autumn 2016, period of the highest microbial activity (Urbanová *et al.* 2015). Litter was removed next to the roots of an oak and a square of 8 cms<sup>3</sup> of soil was extracted with a shovel and taken to the laboratory in an icebox. From this square of soil, three roots were spotted under the loop where grains of soil from rhizosphere (soil adherent to the root), mycorrhizosphere (soil adherent to a mycorrhiza), dead root (visually) and wood decay were detached and surrounding bulk soil grains were isolated 5 cms away from any root. Each grain weighted around 5-10 mg.

### *Taxonomic identification of root and mycorrhiza*

To identify the tree species to which belonged the root from which were isolated the grains of soil, DNA extraction from the root was performed using REDExtract-N-Amp Plant PCR Kit using manufacturer recommendations (Sigma-Aldrich/Merck). The Internal Transcribed Spacer 2 (ITS) was amplified by PCR with the primers ITS3 (5'- GCATCGATGAAGAACGCAGC-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') as described (White *et al.* 1990, Yao *et al.* 2010). Band with a size corresponding to the expected size of plant ITS2 (approx. 400 bp) was extracted from 1% agarose electrophoresis gel, purified with QIAquick PCR purification kit according to manufacturer recommendations (Qiagen) and sequenced using Sanger sequencing method (Eurofins, Germany). Taxonomy affiliation was determined by BLASTn on All plant EST database. To identify the mycorrhizae fungus, same procedure as root was done, but PCR amplification was done from primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990, Gardes and Bruns, 1993), with an expected band size ranging from 450 to 700 bp.

### *Cultivation of bacterial isolates (Bulk soil, rhizosphere, and mycorrhizosphere grains)*

A total of eleven grains of soil were studied: bulk soil (n=3), rhizosphere (n=3), mycorrhizosphere (n=2), dead root (n=2) and wood decay (n=1). Each grain of soil was resuspended in 0.9% NaCl and mixed with a vortex (n=11). Each solution was divided in two subsamples that were treated

### *Taxonomy of microbial communities and cultivable bacterial species from grains of soil*

independently. First subsamples were used to select specifically for sporulating actinomycetes, since they are important active members of soil and rhizosphere communities (Viaene *et al.* 2016) and only spores can be kept over long period of time in laboratory collections. To do so, a heat treatment (50°C for 1 hour) was performed as described (Bontemps *et al.* 2013) and then serial dilutions were inoculated on SIM agar (1 g.L<sup>-1</sup> starch, 0.4 g.L<sup>-1</sup> casein, 0.1 g.L<sup>-1</sup> CaCO<sub>3</sub>, 0.25 g.L<sup>-1</sup> KNO<sub>3</sub>, 0.1 g.L<sup>-1</sup> MgSO<sub>4</sub>, 0.2 g.L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 20 g.L<sup>-1</sup> agar, pH 7.0). Only sporulating *Streptomyces*-like strains were selected from SIM plates and spread on soy flour medium (SFM) (20 g.L<sup>-1</sup> soy flour, 20 g.L<sup>-1</sup> mannitol and 20 g.L<sup>-1</sup> agar, pH 6.6) and grown for 7 days at 25°C (Kieser *et al.* 2000) before collecting the lawn of spores using sterile water. The spore solution was vortexed, filtered using cotton, centrifuged (2400 g, 10 min, 4°C) resuspended in 20% glycerol, and stored at -80°C.

Second subsamples were used to isolate a broad range of strains using 1/10 strength tryptic soy agar (TSA) as a non-selective medium with low level of nutrients (Uroz *et al.* 2013B) (3 g.L<sup>-1</sup> tryptic soy broth from Difco and 15 g.L<sup>-1</sup> of agar, pH 7.3). Plating on 1/10 TSA was made without pre-treatment. All agar plates were incubated at 25°C for 10 days. One colony per morphotype was picked on Petri dish plates to obtain the broadest diversity of strains. Initial plates were further incubated for 8 additional days to recover slow growers. Each isolate from 1/10 TSA plate was re-streaked on 1/10 TSA 3 times for purification purposes. All the bacterial strains were cryopreserved at -80 °C in 20% glycerol.

### *Taxonomic identification of bacterial isolates*

For non-actinomycete bacteria, DNA was extracted using Wizard genomic DNA purification kit (Promega, France). The 16S rRNA gene sequence of these bacterial strains was amplified by PCR with the primers 16S\_FD1 (5'-AGAGTTTGATCCTGGCTCAG) and 16S\_rP2 (5'-AAGGAGGTGATCCAGCC) primers as described by Weisburg *et al.* 1991. For *Streptomyces*-like isolates, 16S rRNA gene PCR amplification was done using 100% DMSO-cell suspension mix (Van Dessel *et al.* 2003) and the StrepB forward (5'-ACAAGCCCTGGAAACGGGGT) and StrepF reverse (5'- ACGTGTGCAGCCCAAGACA) primers as described (Rintala *et al.* 2001). PCR products were then sequenced by Sanger sequencing method (Eurofins, Germany). Forward and reverse sequences were assembled using CLC Main Workbench (CLC bio) and taxonomical affiliations of bacterial isolates were determined using Ribosomal Database Project (RDP) classifier (Wang *et al.* 2007). MEGA7 software was used to aligned nucleotides using the MUSCLE algorithm and to build a phylogenetic tree based on neighbor-joining method corrected with Kimura's 2 parameter distance and 100 bootstrap correction (Kimura *et al.* 1980, Kumar *et al.* 2016).

## *Taxonomy of microbial communities and cultivable bacterial species from grains of soil*

### *Composition of bacterial and fungal communities from all grains of soil by Meta-barcoding Illumina sequencing*

Illumina Miseq sequencing of soil 16S rRNA and ITS was used to characterize the initial composition of the bacterial and fungal communities of each grain of soil, respectively. DNA was extracted using FastDNA Spin Kit for soil (MP Biomedicals). To ensure DNA from vegetative cells and spores, extraction protocol was modified to include three sequential bead-beating steps as described (Wunderlin *et al.* 2013). To obtain bacterial communities, the V4 hypervariable region of the 16S SSU rRNA was amplified in triplicates using specific primers forward 515F (5'-GTGCCAGCMGCCGCGGTAA) and reverse 806R (5'-GGACTACHVGGGTWTCTAAT) as described (Schenkel *et al.* 2019). To obtain fungal communities, we amplified in triplicates the internal transcribed spacer 2 (ITS2) region of fungal rDNA using ITS86F (5'- TTCAAAGATTCGATGATTCAG) (Vancov and Keen, 2009) and ITS4 (5'- TCCTCCGCTTATTGATATGC) (White *et al.* 1990) primers. PCR product size and concentration were verified using an agarose gel electrophoresis (1%) with an expected size of around 291 bp for V4 and 400 bp for ITS2, and then pooled. PCR products without addition of microbial DNA (negative control) and mock communities of known bacterial and fungal compositions were added as quality controls. Samples of 50 µL (30 ng DNA per µL) were sent for tagging and MiSeq Illumina Next Generation Sequencing (GeT PlaGe INRA sequencing platform, Toulouse, France). Resulting bacterial sequences were further processed with FROGS (Find Rapidly OTU with Galaxy Solution; Escudie *et al.* 2017) based on Galaxy metagenomic analysis platform (Afgan *et al.* 2016) as described in (Schenkel *et al.* 2019). Resulting fungal sequences were further processed according standard operation procedure with INRA pipeline as described (Perez-Izquierdo *et al.* 2017). Bacterial OTUs were affiliated with RDP and SILVA databases and fungal OTUs with UNITE database.

For both fungal and bacterial data, per-sample rarefaction curves were calculated to assess sampling completeness, using function *rarecurve()* in package *vegan* v3.5-1 (Oksanen *et al.* 2015) in R (version 3.4.3) (R Core Team, 2017). Based on these, subsequent analyses of diversity and community structure were performed on datasets where samples had been rarefied with the *phyloseq* (McMurdie and Holmes 2017) package to achieve equal read numbers according to the minimum number of total reads in any sample (21,166 bacteria, 2,357 fungi).

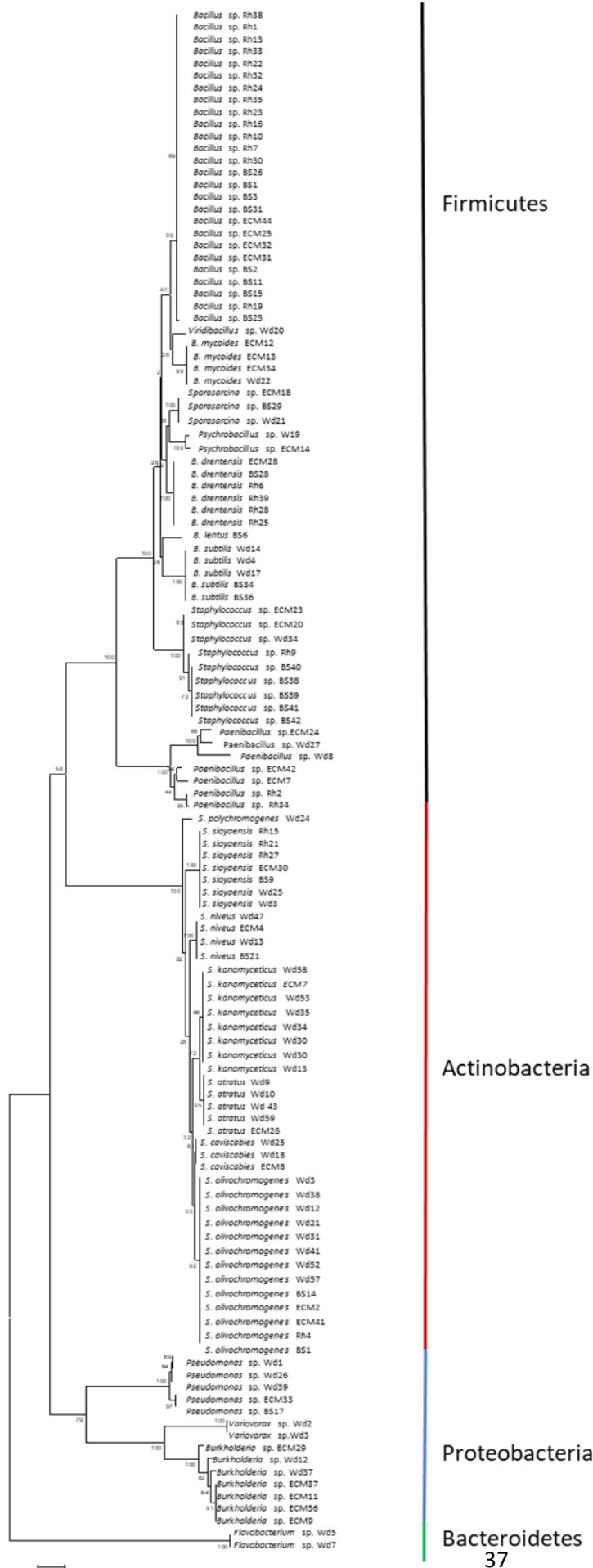
## Results

### **1.1 Molecular identification of root and ectomycorrhiza**

Root sequences analysis confirmed that grains of soil were taken from an oak tree (*Quercus robur*) and ectomycorrhizal sequence analysis indicated that the “ectomycorrhizosphere” grains of soil were taken from an oak tree root colonized by *Lactarius sp.* (data not shown).

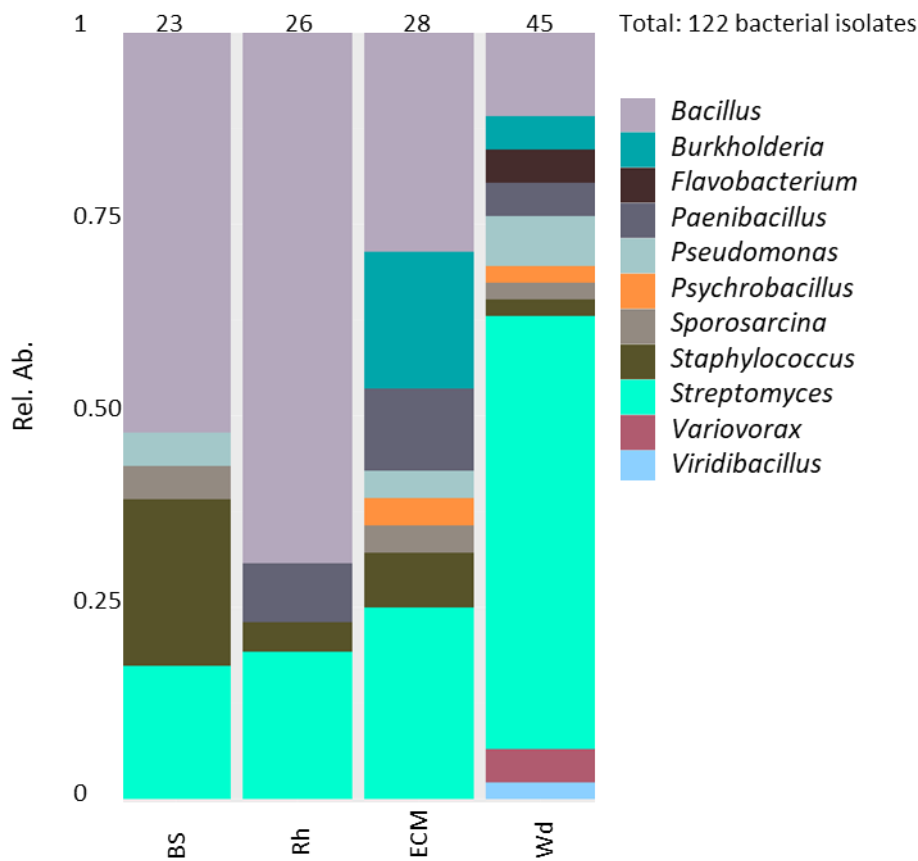
### **1.2 Library of bacterial isolates coming from bulk soil, rhizosphere, mycorrhizosphere and wood decay**

A total of 1247 bacterial colonies were grown from grains of soil dilutions coming from bulk, rhizosphere, mycorrhizosphere and wood decay, but only 122 strains were isolated after 18 days of incubation. These bacterial isolates were picked based on different phenotypes (i.e. color, shape, sporulation). Molecular identification using 16S rRNA gene sequences showed that these 122 strains were affiliated to 11 different genera belonging to 4 phyla (Figure 9). The library was comprised by bacteria from the phyla Firmicutes (53%), Actinobacteria (34%), Proteobacteria (11%) and Bacteroidetes (2%). The largest diversity was found among Firmicutes as they regrouped strains belonging to *Bacillus* (66.2%), *Staphylococcus* (13.8%), *Paenibacillus* (10.8%), *Sporosarcina* (4.6%), *Psychrobacillus* (3.1%), and *Viridibacillus* (1.5%). Actinomycete strains belonged only to *Streptomyces* genus, but this is likely due to a methodology biased (See Materials and Methods, SIM agar), and they regrouped in 7 different species (Figure 9). Proteobacteria consisted of *Burkholderia* (50%), *Pseudomonas* (35.7%) and *Variovorax* (14.3%), and Bacteroidetes was composed by *Flavobacterium* strains only (Figures 9 and 10). The highest bacterial diversity was obtained in wood decay grain accounting for strains belonging to 11 different genera followed by mycorrhizosphere grain with 8 different genera. *Bacillus*, *Staphylococcus* and *Streptomyces* were found in all the grains. *Burkholderia* and *Psychrobacillus* were isolated from mycorrhizosphere and wood decay grains only, *Paenibacillus* was found in all grains except bulk soil, *Pseudomonas* and *Sporosarcina* were found in all grains except for rhizosphere and *Flavobacterium*, *Variovorax* and *Viridibacillus* were found only in wood decay grain in these screening conditions (Figure 10).



**Figure 9.** Taxonomical classification of all phenotypically looking different isolates (n=122) was built based on 16S rRNA sequences using Neighbor-Joining method. The numbers of each branch nodes are bootstrap values from 100 re-samplings. Horizontal branch lengths are proportional to evolutionary distance according to Kimura's 2 parameter distance. Grains of soil belong to bulk soil (Bs), rhizosphere (Rh), mycorrhizosphere (ECM) and wood decay (Wd).

## Taxonomy of microbial communities and cultivable bacterial species from grains of soil



**Figure 10.** Cultivable bacterial composition from grains of soil: bulk soil (Bs, n=3), rhizosphere (Rh, n=3), mycorrhizosphere (ECM, n=2) and wood decay (Wd, n=1).

### 1.3 Initial microbial communities

#### 1.3.1 Initial bacterial communities from grains of soil

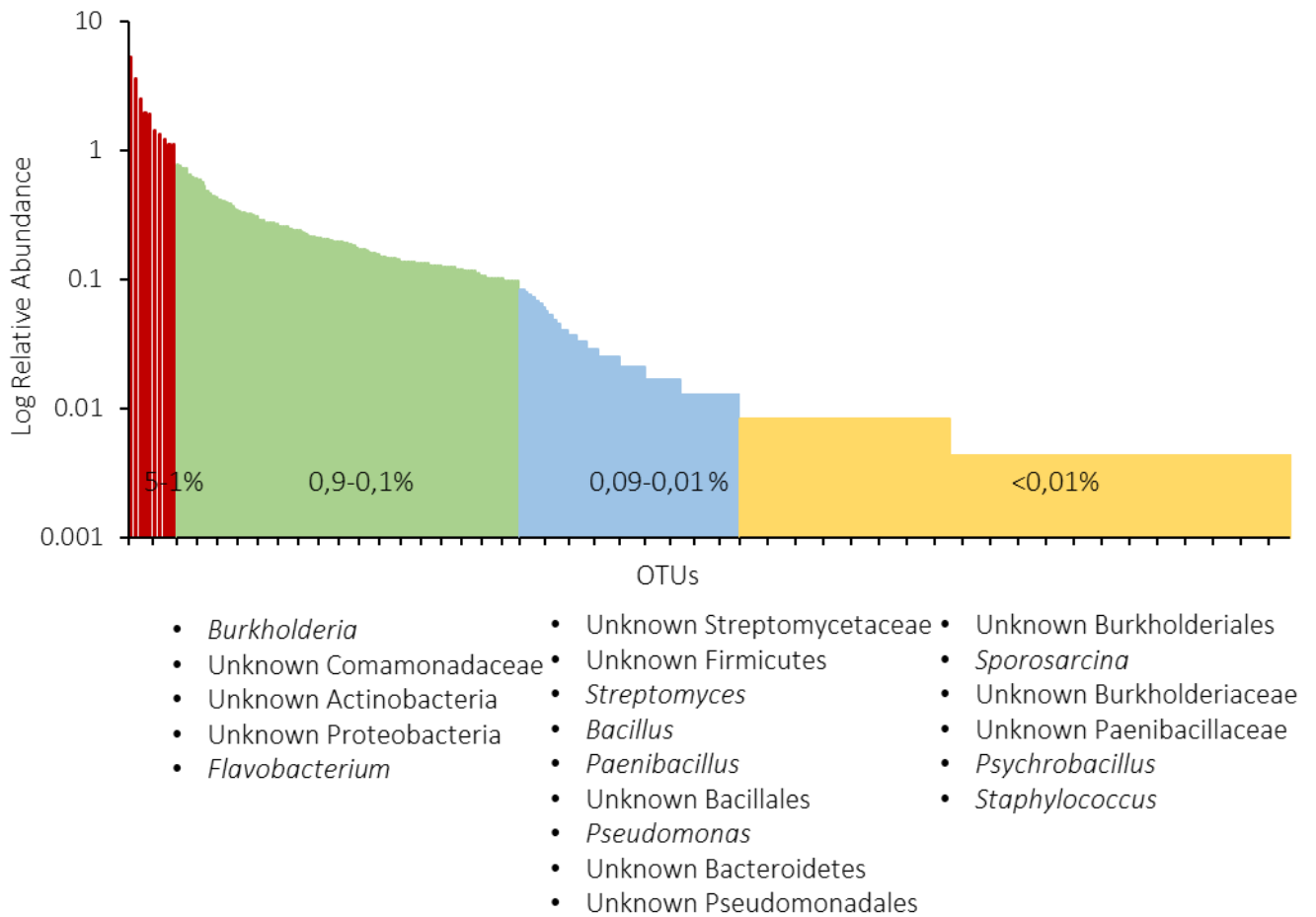
In order to review the differences between the cultivable bacterial library compared to the initial bacterial community, we performed amplicon DNA sequencing via Illumina Miseq platform from grains of soil with the V4 16S rRNA region. The lack of samples was due to amplification failures, making impossible to do statistical analyses on them. Nevertheless, a total of 451,737 16S rRNA gene reads were obtained. After dereplicating (grouping sequences per sample), clustering (grouping sequences by identity), chimera deletion (cleaning sequences that are chimera), and contaminants removal, 427,331 16S rRNA gene sequences were clustered in 7,434 Operational Taxonomic Units (OTUs). To be able to compare samples, sequences were randomly rarefied at the lowest number of sequences (21,166). In total, Illumina Miseq sequencing analysis from bacterial data contained 6,633 OTUs (Table 1), where 32% of OTUs have significant hits to the RDP database with a bootstrap higher than 0.7, and 68% remained unclassified at the genus level. At the family level, 32% remained unknown, 10% at the order level, 8% at the class level and 2% at the phylum level.



**Table 1.** Quality of bacterial and fungal DNA sequences data retrieved by Illumina Miseq platform.

Sequences	Bacteria (16S rRNA gene)	Fungi (ITS region)
Processed sequences	427,331	50,422
Total of sequences after subsampling	275,158	25,927
Processed OTUs	6,633	234
Average Total of sequences per sample	21,166	2,357

Amplicon DNA sequencing analysis via Illumina Miseq platform detected a total of 588 bacterial genera belonging to 30 phyla (one unidentified) from the dataset. Moreover, few genera (21) were highly abundant (Relative abundance >1%, Figure 11 - red), 267 genera were moderately abundant (Rel ab. 1-0.01%, Figure 11 – green and blue) and 300 were considered as rare (Rel. Ab. <0.01%, Figure 11 - yellow). Among the most abundant genera, none of them were able to be cultivated in these laboratory conditions (Figure 11, red), but all the isolates that we obtained were retrieved in the DNA sequencing data within the moderately and rare taxa (Rel. Ab. <1%) (Figure 11, Table 2). These cultivable bacteria belonged to only 4 phyla, 3 highly abundant (Rel. Ab. >1%) (Proteobacteria, Actinobacteria and Bacteroidetes), and one scarce (Firmicutes) (Rel. Ab. <0.1%), according to amplicon sequencing data. The ratio of cultivable bacterial sequences compared to the total of sequences found in each phylum showed that around 50% of Firmicutes could be isolated belonging all to Bacillales order. For the other three phyla, less than 4% from each taxa could be recovered in the isolation conditions applied. Among Firmicutes, *Bacillus* and *Paenibacillus* were highly abundant (Rel ab. 0.9-0.01%) compared to *Sporosarcina*, *Psychrobacillus* and *Staphylococcus* (Rel ab. <0.01%). DNA sequencing data also showed the presence of cultivable bacterial OTUs in grains that do not have these bacteria isolated in the library (Table 2, e.g. *Burkholderia* OTUs were present in all grains, but in the library of isolates was only found in ectomycorrhizosphere and wood decay grains), likely missed by eye. In addition, *Staphylococcus* OTUs were only found in one rhizosphere grain, but in the library, they were present in all the microhabitats (Table 2, Figure 11). Based on these data, DNA sequencing showed a higher bacterial diversity compared to the cultivable bacteria.



**Figure 11.** Relative abundance of OTUs in logarithmic scale (10), with Rel. Ab.>1% in red, 1%>Rel. Ab.>0.1% in green, 0.1%>Rel. Ab. >0.01% in blue and Ab. lower than 0.01% in yellow. All grains had the same trend. Bacterial isolates from the library found in the bacterial DNA sequencing analysis in the Rel. Ab. < 1% are indicated under each percentage compartment.

## Taxonomy of microbial communities and cultivable bacterial species from grains of soil

**Table 2.** Relative abundances of DNA sequences retrieved with Illumina Miseq platform that were found in the cultivable approach. Grains of soil belong to bulk soil (Bs, n=3), rhizosphere (Rh, n=3), mycorrhizosphere (ECM, n=2), wood decay (Wd, n=1) and dead root (Dr, n=2).

Phylum	Genus	Bs1	Bs2	Bs3	Rh1	Rh2	Rh3	ECM2	ECM3	Wd	Dr2.1	Dr2.2
Actinobacteria	Unknown genus: Actinobacteria	0.104	0.146	1.257	0.198	0.090	0.236	0.217	0.298	0.066	0.104	0.633
Actinobacteria	<i>Streptomyces</i>	0.165	0.071	0.014	0.061	0.061	0.019	0.052	0.189	0.151	0.057	0.052
Actinobacteria	Unknown genus: Streptomycetaceae	0.279	0.137	0.113	0.142	0.094	0.057	0.028	0.128	0.028	0.038	0.080
Bacteroidetes	<i>Flavobacterium</i>	0.019	0.009	0	0	0	0	0.024	0.123	0.283	0	0
Firmicutes	Unknown genus: Firmicutes	0.047	0.071	0.085	0.189	0.170	0.076	0.165	0.151	0.000	0.128	0.090
Firmicutes	<i>Paenibacillus</i>	0.038	0.033	0.014	0.019	0.028	0.028	0.047	0.080	0.014	0.076	0.028
Firmicutes	<i>Bacillus</i>	0.038	0.028	0.005	0.014	0.019	0.028	0.028	0.014	0.028	0.047	0.028
Firmicutes	<i>Staphylococcus</i>	0	0	0	0	0.005	0	0	0	0	0	0
Firmicutes	Unknown genus: Bacillales	0	0	0	0	0	0	0	0.005	0	0	0
Firmicutes	<i>Psychrobacillus</i>	0	0	0	0	0	0	0	0.005	0	0	0
Firmicutes	Unknown genus: Paenibacillaceae	0	0.009	0	0	0	0	0	0	0.005	0	0
Proteobacteria	<i>Burkholderia Paraburkholderia</i>	0.298	0.194	0.019	0.345	0.137	0.066	0.132	2.873	0.099	1.280	0.661
Proteobacteria	Unknown genus: Proteobacteria	0	0	0.005	0	0	0	0	0.005	0.090	0	0.005
Proteobacteria	<i>Pseudomonas</i>	0.024	0	0	0	0	0	0	0	0.009	0	0.005
Proteobacteria	Unknown genus: Burkholderiales	0	0	0	0	0.014	0	0	0	0.028	0.005	0

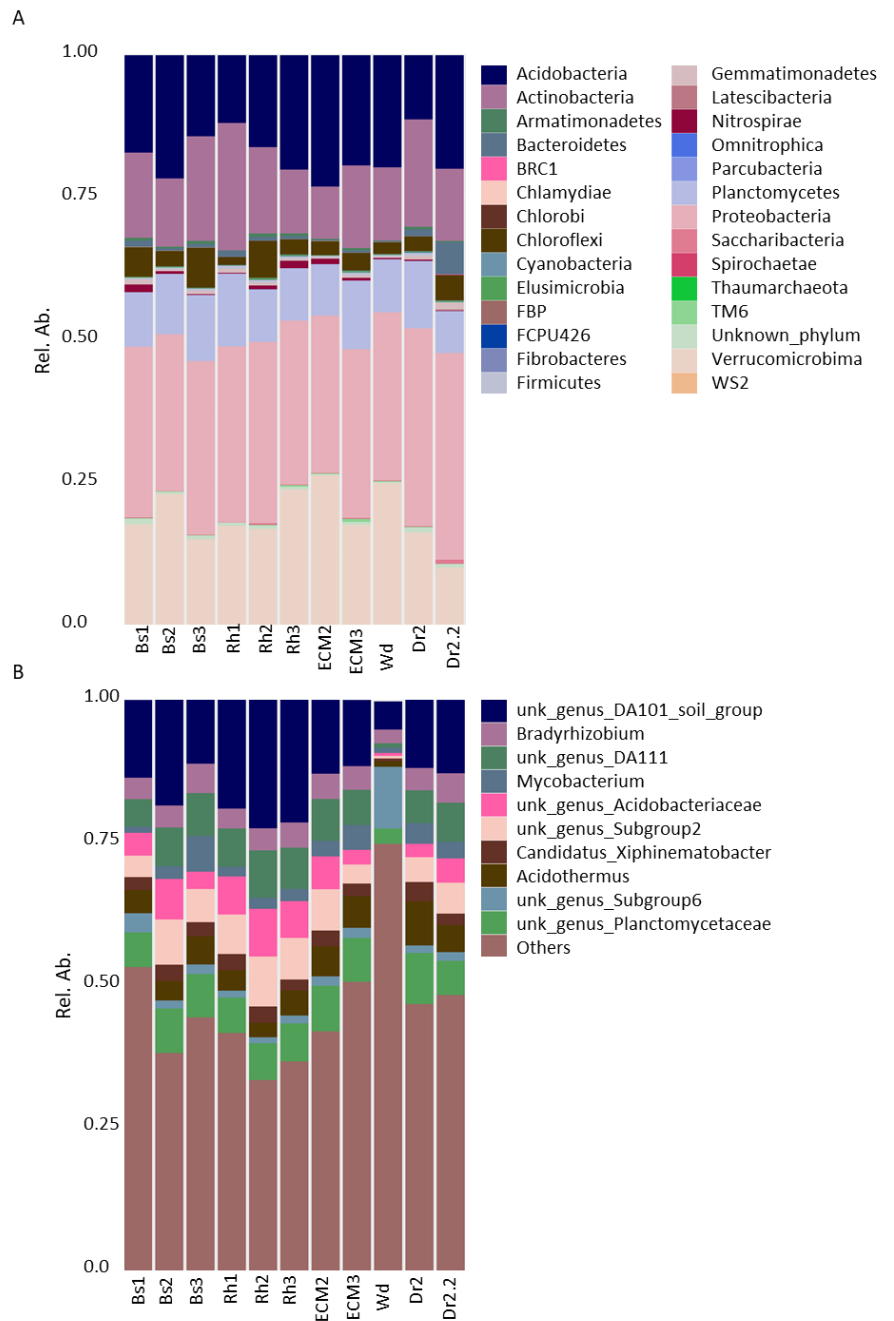
This high-throughput DNA sequencing method provides an opportunity to further unravel bacterial diversity at a scale of a grain. We observed that bacterial communities exhibited similar taxonomic distribution within the different grains (Figure 12). Overall, the different microhabitats shared the most abundant genera of bacteria but the relative abundance of sequences found in each grain varied. On average, these grains of soil were dominated by: Proteobacteria (accounting for  $30.6 \pm 2.6\%$ ), Verrucomicrobia ( $19.2 \pm 4.7\%$ ), Acidobacteria ( $17.6 \pm 3.7\%$ ), Actinobacteria ( $14.7 \pm 3.7\%$ ), Planctomycetes ( $10.2 \pm 1.5\%$ ), Chloroflexi ( $3.6 \pm 1.8\%$ ), and Bacteroidetes ( $1.1 \pm 1.5$ ). At a genus level, 20 different bacterial genera belonging to 5 bacterial phyla dominated these grains of soil (>1% Rel. Ab.). From these dominant bacterial genera, only 8 were identified at a genus level (Figure 12).

Grains comprised different levels of the richness (number of OTUs) that were unique to each of them and the composition of the rare taxa. In more details, the three grains coming from bulk soil shared a total 2855 bacterial OTUs which 355 were unique to this compartment and  $45.2 \pm 6.2\%$  belonged to the rare taxa (<1%). The three rhizosphere grains shared a total of 1944 bacterial OTUs which 212 were unique to this compartment and  $37.2 \pm 3.4\%$  belonged to the rare taxa. Grains from ectomycorrhizosphere (n=2) shared a total of 2155 bacterial OTUs which 224 were unique to this

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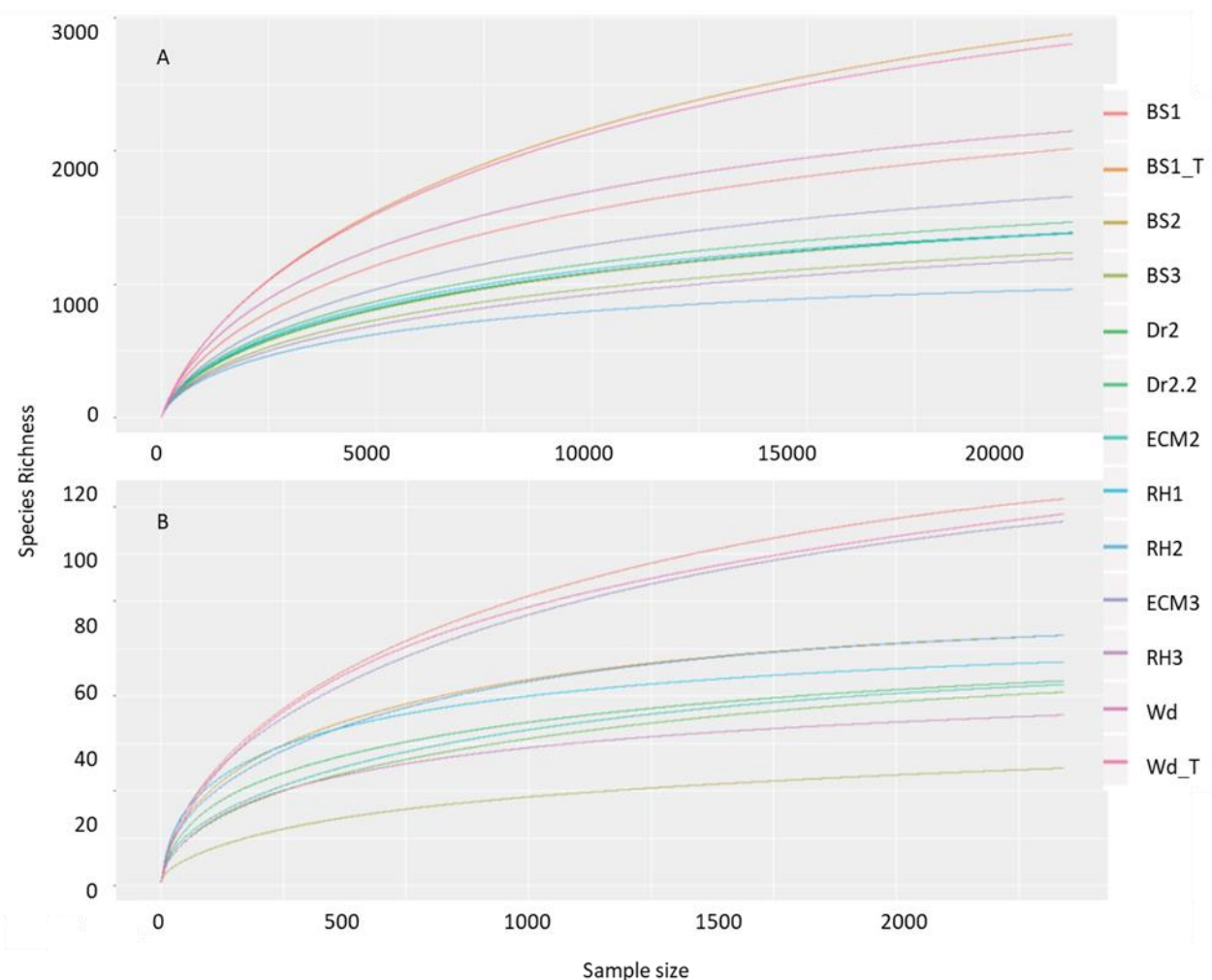
compartment and  $46.1 \pm 4.7\%$  belonged to the rare taxa. Grains coming from dead root ( $n=2$ ) shared a total of 2110 bacterial OTUs which 200 were unique to this compartment and  $46.6 \pm 1.5\%$  belonged to the rare taxa. Lastly, wood decay grain had the highest amount of OTUs including a total of 2206 bacterial OTUs, which 357 were unique to this compartment, and 65.5% were considered rare taxa.

**Figure 12.** Bacterial community composition at phylum level (A) and genus level (B) retrieved by Illumina Miseq platform sequencing the V4 region of the 16S rRNA gene. Grains of soil belong to bulk soil (Bs), rhizosphere (Rh), mycorrhizosphere (ECM), wood decay (Wd) and dead root (Dr).



### 1.3.2 Initial fungal communities from grains of soil

Since bacteria do not live independently and frequently interact with other fungi since they share same microhabitats (Frey-Klett *et al.* 2011), we considered important to determine the fungal assembly in parallel. We analyzed the initial fungal community of the same grains to have a glimpse of the microbial communities co-occurring at this microscale by sequencing the ITS2 region. Raw data comprised a total of 50,422 ITS region reads clustered in 294 OTUs. After data were processed as mentioned before, we obtained 234 OTUs where each sample had 2,357 ITS region sequences (Table 1). Here, 56% fungal OTUs had significant hits to the UNITE database and 42% remained unclassified at the genus level. At the family level, 35% remained unknown, 22% at the order level and 23% at the class level. Although the number of generated sequences and OTUs for both (bacteria and fungi) were different, they both reached the depth of saturation in rarefied curves (Figure 13).



**Figure 13.** Species richness based on DNA sequences using Illumina Miseq platform A. V4 region from the 16S rRNA gene (Bacterial sequences were rarefied at 21,166 sequences per sample), B. ITS2 region (Fungal sequences were rarefied at 2,357 sequences per sample). Grains of soil belong to bulk soil (Bs), rhizosphere (Rh), mycorrhizosphere (ECM), wood decay (Wd) and dead root (Dr).

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DNA sequencing analysis by Illumina Miseq detected a total of 6 fungal phyla (one unidentified) from the dataset. On average, the dominant phyla across all grains were 4 fungal phyla: Basidiomycota (accounting for  $45.5 \pm 15.3\%$ ), Ascomycota ( $37.2 \pm 12.4\%$ ), Zygomycota ( $14.1 \pm 12.8\%$ ) and Rozellomycota ( $1.9 \pm 2.1\%$ ). At a genus level, 12 different fungal genera belonging to 4 fungal phyla dominated these grains of soil ( $>1\%$  Rel. Ab). From these 12 fungal genera, 10 were identified at a genus level.

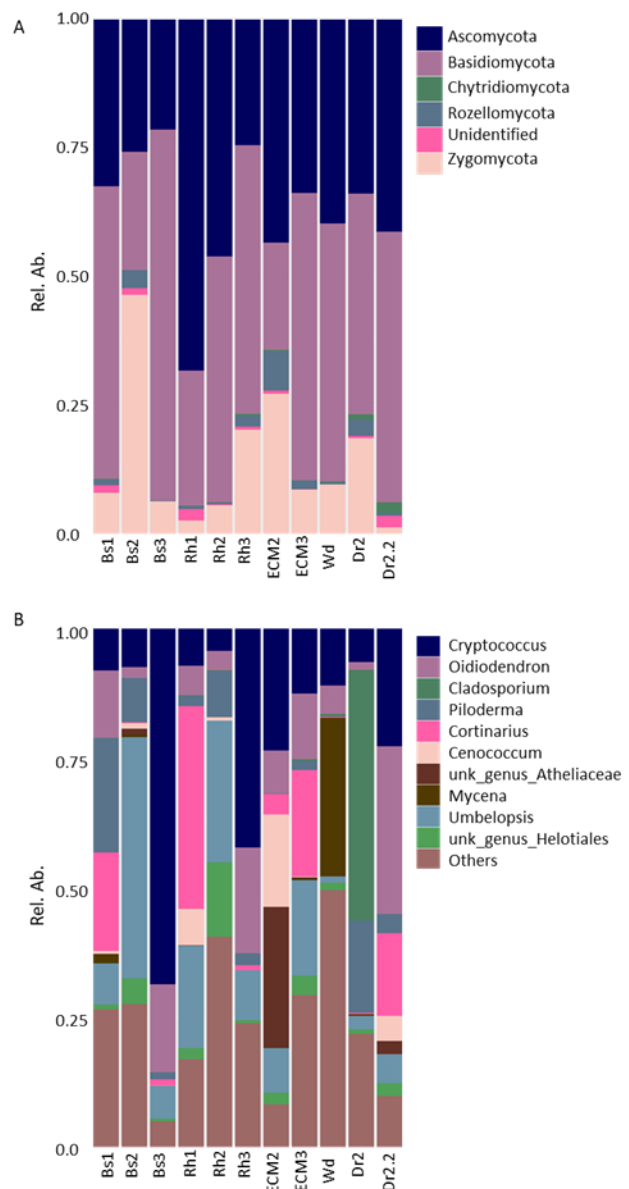
The fungal communities of the grains of soils exhibited distinct taxonomic distribution within different microhabitats (Figure 14). In more details, three grains coming from bulk soil shared a total 146 fungal OTUs which 84 were unique to this compartment and  $12.7 \pm 6.9\%$  belonged to least abundant taxa (Rel ab.  $<1\%$ ). On average the most dominant were the yeasts *Cryptococcus* ( $28.1 \pm 28.7\%$ ), followed by *Umbelopsis* ( $20.3 \pm 18.6\%$ ) and the filamentous fungus *Oidiodendron* ( $10.7 \pm 6.3\%$ ). Three grains coming from rhizosphere shared a total 115 OTUs which 66 were unique to this compartment and  $44.6 \pm 3.3\%$  belonged to least abundant taxa (Rel ab.  $<1\%$ ). On average, the most dominant were *Umbelopsis* ( $56.6 \pm 7.2\%$ ), *Cryptococcus* ( $53.7 \pm 17.2\%$ ), and lastly *Cortinarius* ( $40.1 \pm 18.2\%$ ), yet this last one was only present in two grains. Mycorrhizosphere grains (two) comprised 121 fungal OTUs which 77 were exclusive for this habitat and more than  $13.4 \pm 8.3\%$  represented least abundant taxa. At the genus level, *Cryptococcus* relative abundance was  $18 \pm 5.5\%$ , followed by an unidentified genus belonging to the Atheliaceae family with  $13.7 \pm 13.5\%$  and *Umbelopsis* was  $13.5 \pm 4.9\%$ . Dead root grains (2) shared a total of 89 OTUs which 50 were unique to this condition and  $10 \pm 1.3\%$  belonged to least abundant taxa. The relative abundances of the most dominant genera were *Cladosporium* ( $24.2 \pm 24.2\%$ ), *Oidiodendron* ( $16.9 \pm 15.4\%$ ) and *Cryptococcus* ( $14.6 \pm 8.1\%$ ). Finally, wood decay grain had the highest amount of fungal OTUs (118) which 23 were unique and more than  $23.4\%$  belonged to least abundant taxa. The most abundant fungal genera were *Mycena* (29.9%) followed by *Penicillium* (15.8%) and *Cryptococcus* (11%).

**Figure 14.** Fungal community composition at phylum level (A) and genus level (B) retrieved by Illumina Miseq platform sequencing the ITS2 region. Grains of soil belong to bulk soil (Bs), rhizosphere (Rh), mycorrhizosphere (ECM), wood decay (Wd) and dead root (Dr).

### 1.3.3 Core microbiomes across grains of soil

The membership core is recognized as the common organisms associated with a habitat (Shade and Handelsman 2011). Here, we defined the core microbiome by OTUs present in all the grains from all the habitats: bulk soil, rhizosphere, mycorrhizosphere, wood decay and dead root samples (n=11). The fungal core comprised 11 out of 234 OTUs belonging to 10 out of 121 genera, which were shared in all soil grains, accounting for relative abundances from 0.04-27% (Table 3 & 4). Among most abundant and widespread fungi were *Cryptococcus* (Basidiomycota), *Umbelopsis* (Zygomycota) and *Oidiodendron* (Ascomycota) (Table 3).

The bacterial core comprised 157 out of 6,633 OTUs belonging to 121 out of 588 genera which are shared in all grains accounting for relative abundances from 0.03-5%. Several bacterial groups were present in all grains: DA101 soil group family (Verrucomicrobia), *Bradyrhizobium* (Alphaproteobacteria), DA111 (Alphaproteobacteria), *Variibacter* (Alphaproteobacteria) and *Mycobacterium* (Actinobacteria) (Table 4).



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**Table 3.** First ten OTUs from the fungal membership core from all grains of soil: bulk soil (Bs, n=3), rhizosphere (Rh, n=3), mycorrhizosphere (ECM, n=3), wood decay (Wd, n=1) and dead root (Dr, n=2).

Fungal OTUs	Genus	Bs (%)	Rh (%)	ECM (%)	Wd (%)	Dr (%)
OTU1	<i>Cryptococcus</i>	0.312 ± 0.285	0.251 ± 0.202	0.364 ± 0.14	0.511	0.262 ± 0.065
OTU5	<i>Umbelopsis</i>	0.255 ± 0.224	0.315 ± 0.143	0.256 ± 0.072	0.056	0.080 ± 0.001
OTU3	<i>Oidiodendron</i>	0.138 ± 0.08	0.146 ± 0.084	0.202 ± 0.025	0.254	0.256 ± 0.21
OTU4	<i>Piloderma</i>	0.159 ± 0.146	0.065 ± 0.039	0.016 ± 0.015	0.008	0.298 ± 0.244
OTU13	<i>Helotiales sp.</i>	0.027 ± 0.024	0.084 ± 0.08	0.058 ± 0.01	0.066	0.032 ± 0.004
OTU14	<i>Ascomycota sp.</i>	0.061 ± 0.04	0.021 ± 0.004	0.026 ± 0.007	0.006	0.017 ± 0.005
OTU18	<i>Rozellomycota sp.</i>	0.021 ± 0.017	0.052 ± 0.04	0.047 ± 0.011	0.024	0.010 ± 0.006
OTU17	<i>Eurotiomycetes sp.</i>	0.015 ± 0.008	0.026 ± 0.028	0.016 ± 0.005	0.070	0.037 ± 0.027
OTU21	<i>Laccaria</i>	0.009 ± 0.005	0.024 ± 0.016	0.010 ± 0.009	0.004	0.005 ± 0.003
OTU31	<i>Agaricales sp.</i>	0.003 ± 0.003	0.015 ± 0.011	0.004 ± 0.001	0.002	0.004 ± 0.001



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**Table 4.** First ten OTUs belonging to the bacterial membership core of all grains of soil: bulk soil (Bs, n=3), rhizosphere (Rh, n=3), mycorrhizosphere (ECM, n=2), wood decay (Wd, n=1) and dead root (Dr, n=2).

Bacterial OTUs	Genus	Bs (%)	Rh (%)	ECM (%)	Wd (%)	Dr (%)
OTU1	Unknown genus:DA101 soil group	0.059 ± 0.006	0.066 ± 0.008	0.054 ± 0.001	0.015	0.057 ± 0.003
OTU2	<i>Bradyrhizobium</i>	0.042 ± 0.006	0.038 ± 0.003	0.042 ± 0.001	0.023	0.045 ± 0.006
OTU3	Unknown genus:DA101 soil group	0.037 ± 0.013	0.046 ± 0.018	0.033 ± 0.007	0.005	0.029 ± 0
OTU7	Unknown genus:DA111	0.023 ± 0.008	0.024 ± 0.004	0.026 ± 0.001	0.002	0.025 ± 0.001
OTU5	Unknown genus:DA101 soil group	0.018 ± 0.006	0.022 ± 0.005	0.014 ± 0	0.016	0.017 ± 0.002
OTU4	<i>Variibacter</i>	0.021 ± 0.007	0.023 ± 0.006	0.019 ± 0.002	0.003	0.018 ± 0.002
OTU6	Unknown genus:DA101 soil group	0.016 ± 0.007	0.027 ± 0.014	0.011 ± 0.004	0.001	0.010 ± 0.004
OTU23	<i>Mycobacterium</i>	0.015 ± 0.009	0.014 ± 0.006	0.017 ± 0.005	0.005	0.016 ± 0.001
OTU8	<i>Mycobacterium</i>	0.016 ± 0.013	0.011 ± 0.005	0.016 ± 0.002	0.003	0.015 ± 0.002
OTU10	Unknown genus: Xanthomonadales	0.012 ± 0.001	0.012 ± 0.004	0.013 ± 0.003	0.003	0.015 ± 0.002

## Discussion

Soil borne microbes play an essential role in forest ecosystems providing soil services involving nutrient cycling and sustaining plant growth (Lladó *et al.* 2017). Abiotic conditions have been extensively studied to determine microbial communities' structure (Fierer and Jackson 2006), however, molecules produced during bacterial interactions involved in the structuration of microbial communities and plant development are still underexplored. To obtain insights in the potential interactions between bacteria and bacteria - plants, we need to study soil at a very fine scale. Such scale is within micrometers, involving grains of soil which are considered as unique compartments of biotic and abiotic conditions where significant cell-to-cell interactions occur (Raynaud and Nunan 2014, Rillig *et al.* 2017). As our goal was to obtain bacteria that are likely interacting, we isolated and characterized cultivable bacterial communities from grains of soil collected from different origins to later perform the interactions. In parallel, we analyzed in depth DNA Metabarcoding data using Illumina Miseq platform to see how representative these bacterial libraries were, and to get a first glimpse of the community composition between grains of soil. Although we are aware about the absence of statistical analysis, we would like to provide an overall context.

Our library resulted in low cultivable bacterial diversity of well-known cultured representatives from Actinobacteria, Firmicutes and Proteobacteria phyla consistent with previous studies (Davis *et al.* 2004). Type of media used and short incubation time (18 days) favoured fast aerobic growers, spreading phenotype, with a wide pH tolerance range, resulting in a low bacterial diversity compared to the original community from the grains of soil (Vartoukian *et al.* 2010, Hartman *et al.* 2017). Previous reports have used same media to isolate bacterial strains from forest soil habitats obtaining a much larger taxonomic diversity of strains (Uroz *et al.* 2013B, Colin *et al.* 2017), yet using large composite of samples, suggesting that the low diversity obtained could also be related to the microscale chosen, to the selection by phenotype or other experimental conditions. Moreover, soil sample size influences the retrieved cultivable bacterial community structure (Ellingsoe and Johnsen 2002). In addition, we noticed the presence of cultivable bacterial OTUs that were isolated from some grains but not from others (e.g. *Burkholderia*, *Paenibacillus* Table 2). Culturing limitations could be also associated to the biotic interdependencies between microbes (Hugerth and Andersson, 2017), where some organisms depend on others for specific trade-offs to live, e.g. siderophore production (D'Onofrio *et al.* 2010).

DNA sequencing analysis of the microbiome of each grain of soil showed that the cultivable bacterial isolates were among the rare taxa from the community. Rare microorganisms can be also very active and play important roles within microbial communities (Uroz *et al.* 2009B, 2014, Lasa *et al.* 2019).

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Indeed, Lasa and collaborators reported the total diversity and the potentially active bacterial communities from oak soil and data suggested most abundant taxa were not necessarily the most active while rare taxa may influence the rhizosphere ecology in oak forests more than expected (Lasa *et al.* 2019). In addition, DNA metabarcoding analysis potentially provide a biased view of the real composition of the bacterial community as it captures active microorganisms as well as dead and spore dormant cells (Lasa *et al.* 2019). In this regard, reads corresponding to the genera isolated in this study were retrieved in meta transcriptomic analysis performed on nearby soils indicating that the microorganisms we isolated were likely to be active members of the community (Maillard, Auer, Buée *et al.* unpublished).

Most abundant OTUs, according to DNA sequencing analyses, were affiliated to taxa that are not cultivable yet (Janssen 2006, Vartoukian *et al.* 2010), limiting our chances to isolate them. Moreover, these were composed by a relatively small number of bacterial taxa (21) compared to the least abundant taxa (567). This trend has been also found in a metabarcoding analysis of the rhizosphere of *Quercus pyrenaica* (Lasa *et al.* 2019). Interestingly, most abundant OTUs belong to the membership core microbiome. The fungal composed mainly by *Cryptococcus*, *Umbelopsis* and *Oidiodendron* (Table 3) and the bacterial composed mainly by DA101 soil group, *Bradyrhizobium* and *Variibacter* (Table 4), were part of the membership core soil microbiome of these 11 grains. Although our data may not be statistically representative, this bacterial core composition has been reported previously in an East Europe soil study (Pershina *et al.* 2018). Particularly, Alphaproteobacteria, is well documented as abundant bacteria in acidic soils (Uroz *et al.* 2010, Lladó *et al.* 2017). A 454-pyrosequencing study also revealed *Cryptococcus* as abundant and ubiquitous in all forest samples (Buée *et al.* 2009A). These results suggest that most abundant bacteria are likely inhabiting across temperate forests (Buée *et al.* 2009A, Uroz *et al.* 2010, Shade and Handelsman 2011) while the remaining microbial composition is likely to be specifically defined by biotic and abiotic pressures.

The composition of dominant bacterial taxa was similar among all grains of soil, but dominant fungal composition was different. This result suggests bacteria and fungi respond differently to the scale chosen, likely due to microbiomes diversity depend on the scale and the size of the organism in question (Fierer 2017) and the exploration mode. In a study comparing bacterial and fungal communities from 0.125 to 4 grams of soil samples using automated ribosomal intergenic space analysis (ARISA) found the same trend (Ranjard *et al.* 2003). Under 1 gram of soil, bacterial ARISA profiles showed few variations within replicates and between samples. They could recover the same bacterial community composition in any sample size. In terms of fungal profile, they found numerous differences within and between samples of different sizes, suggesting that there is a higher heterogeneous distribution of fungal species in soil compared to bacteria (Ranjard *et al.* 2003). In

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another study working on soil samples between 0.01 – 10 grams and using denaturing gradient gel electrophoresis (DGGE), variations in bacterial communities were larger in smaller samples (Ellingsøe and Johnsen 2002). A more recent study comparing a range of soil sample sizes from 0.25 to 100 grams and using 16S and 28S rRNA gene sequencing analysis, suggests an appropriate assessment of bacterial and fungal community structure using 10 g of soil (Penton *et al.* 2016). In addition, 10 g of soil presented the least variability within replicates compared to small size samples, suggesting small size samples represent microsites within the habitats (Penton *et al.* 2016). They also found significantly different fungal dominant taxa among sample sizes, suggesting spatial clusters of fungal patches (Penton *et al.* 2016). Differences between studies are likely attributed to the molecular techniques used, the scale and type of soil.

Organic matter heterogeneity in soil can contribute to this microbial composition at a microscale, since it likely distributes microbes unevenly in time and space (horizontally and vertically), involving colonization strategies and degradation capacities of easy and complex substrates (Uroz *et al.* 2013A, Vos *et al.* 2013, Horton and Bruns 2001, Baldrian 2017). This spatial heterogeneity is mainly due to environmental factors that create distinct spatial niches in soil including vegetation composition (Urbanová *et al.* 2015), pH (Lauber *et al.* 2009, Rousk *et al.* 2010), organic matter (Maillard *et al.* 2019). In addition, the distribution of microorganisms depends on their ability to disperse in soil (Buée *et al.* 2009A). Many soil fungi are filamentous, and a single individual can explore several meters while bacteria remain at a microscale and are more dependent on others (Buée *et al.* 2009A, Xiao *et al.* 2017). Indeed, bacteria can disperse by cell division, motility ability (e.g. flagella), water or gas flow in the soil pores, mesofauna (e.g. earthworms) and macrofauna (e.g. larger animals) (Vos *et al.* 2013). Moreover, migration of bacteria through the fungal hyphae (fungal highways) has been reported (Simon *et al.* 2015).

In terms of composition, our study found that bacterial communities were dominated by Proteobacteria, Acidobacteria, Verrucomicrobia and Actinobacteria, while Basidiomycota and Ascomycota were the most predominant fungal phyla in the studied forest soil at a microscale. Previous studies of the oak rhizosphere have found similar bacterial composition (Uroz *et al.* 2010, Cobo-Diaz *et al.* 2017, Lasa *et al.* 2019) in acidic temperate forest but based on a larger soil composite. These results suggest dominant microbes' composition does not decrease linearly together with the scale (Vos *et al.* 2013). Within the ten predominant bacterial genera from these grains, six remain unidentified, hence, the ecology and the roles of these genera remain poorly documented. Particularly, the most abundant genus, DA101, has been reported as dominant and widespread in soil although its function is still unknown (Pershina *et al.* 2018). In contrast, the predominant fungal genera from bulk soil and rhizosphere are well documented in literature,

## *Taxonomy of microbial communities and cultivable bacterial species from grains of soil*

belonging to a wide diverse trophic modes and growth morphology. Among the most dominant fungi, we found saprotrophic yeast (*Cryptococcus*) (Sterkenburg *et al.* 2015), saprotroph (*Oidiodendron*) (Newsham 2011) and symbiotroph ectomycorrhiza (*Piloderma* and *Cortinarius*) (Rinaldi *et al.* 2008).

## **Conclusions and Perspectives**

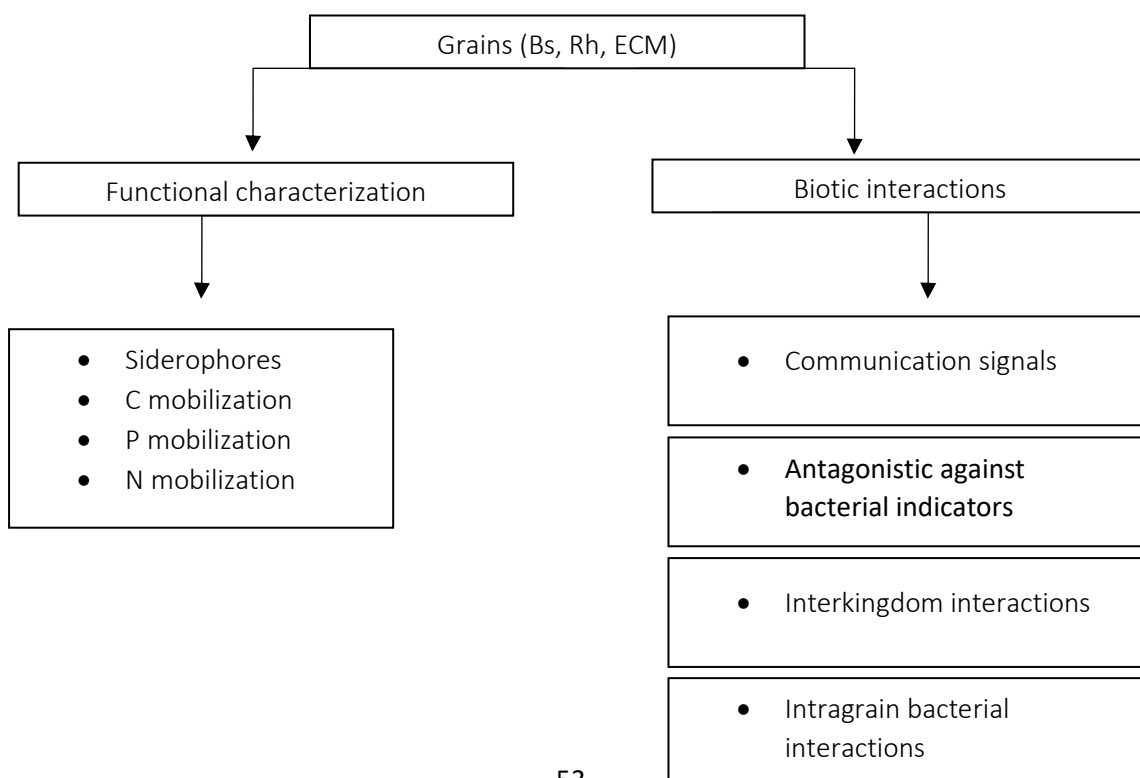
The first part of this work was dedicated to describe the microbial composition at the microscale level with one-grain soil samples. DNA sequencing analysis retrieved a high microbial diversity at this scale, where bacterial communities were more diverse than fungal communities. Grains were similar in dominant bacterial composition, but different in dominant fungal composition. In the sense of microbial interactions, actively transcribed RNA sequencing would have presented a more realistic picture of the active members of the soil community. For the moment, there are no metatranscriptomics studies from soil at a microscale likely linked to the challenges attributed to the RNA isolation, which yields small amounts of mRNA requiring an increased amount of starting material (Carvalhais *et al.* 2012). Low bacterial diversity of well-known cultured representatives was possible to cultivate compared to the initial bacterial community. A higher cultivable bacterial diversity naturally occurring in these microhabitats could have been retrieved through cultivable approaches, if mimicking soil media were improved and applied longer period of incubation time to allow slow growers. The next step would be to explore more ideal media to cultivate soil bacteria such as a microcosm where nutrients are varied but in low concentrations, with a complex physical structure. Moreover, a higher number of samples at a microscale would contribute to statistically represent better their origin.

# Chapter II

## Potential functional diversity and biotic interactions

## Context

In forest soil ecosystems, lignocellulose, organic matter and rocks constitute an important stock of organic and inorganic nutrients unavailable for plants and microbes. Certain bacterial enzymatic machineries are able to degrade and make these nutrients accessible. To determine if cultivable bacteria coming from a grain of soil encode these functions and to evaluate how functionally similar are the strains from this bacterial collection, we chose to characterize 11 degradation and solubilization processes. Moreover, this will provide insights of their functional abilities that may use for survival in forest soils. Besides, bacteria form complex network interactions belowground exchanging signal molecules and metabolites that modify the local environment altering soil microbial communities' structure and plant development. To obtain insights of the impact of these biotic interactions, we focused on bacteria that were functionally diverse. In order to reach this goal, we evaluated their ability to produce and disrupt one type of communication signals, to be active against specific gram positive and negative bacterial indicators and against intragrain bacterial members and their ability to modify plantlet development. This chapter will be divided in three sections: i) potential bacterial functions for nutrient acquisition ii) potential for biotic interactions: communication (quorum sensing and quenching molecules), antibacterial production against bacterial indicators, interkingdom interaction (plant hormone: auxin and phenotyping *Populus*) and iii) pairwise bacterial interactions within grains of bulk and rhizosphere soil. The latter results come from an article (submitted to Microbial Ecology – Springer) titled “**Insights in bacterial interactions isolated from forest soil microhabitats**”.



## Introduction

Microbial communities are key components of forest soil ecosystems since they perform critical functions such as nutrient cycling, organic matter decomposition and mineral weathering (Uroz *et al.* 2009B, 2013, Lladó *et al.* 2017). Yet, how these complex communities coexist and function in soil is still underexplored. Abiotic and biotic parameters (e.g. nutrient availability, plant diversity and pH) have been frequently reported to strongly influence soil bacterial communities' distribution, structure, composition and functions (Urbanová *et al.* 2015, Rousk *et al.* 2010). Thus, a heterogeneous distribution of nutrients will form multiple habitats likely distributing biotic components according to their functional capabilities.

In forest soil ecosystems, the largest proportion of organic carbon come from lignocellulose and soil organic matter (Martin 2014). Lignocellulose is mainly composed by cellulose, hemicellulose and lignin from dead plant biomass (Lopez-Mondejar *et al.* 2016). Soil organic matter derives from dead plants, litterfall, decaying wood, dead animals and microbes (Martin 2014). In addition, important stocks of inorganic nutrients are entrapped in soil minerals (Uroz *et al.* 2009B). Hence, forest soils constitute important nutrients such as carbon, nitrogen, phosphorous, iron, and other minerals cations. These nutrients in soil are in recalcitrant forms bound in organic and inorganic molecules, which makes them directly unavailable for plants. Certain bacteria and fungi can degrade them and make them accessible through specific metabolic machineries (Jacoby *et al.* 2017). The characterization of these enzymatic activities could provide insights of their ecological contributions. Reports based on culture-dependent approaches have shown a functional distribution of microbes in soil. For example, rhizosphere bacteria mobilize nutrients more effectively than bulk soil bacteria, suggesting that trees select for bacteria that can provide soluble nutrients (Nicolitch *et al.* 2016). Colin and collaborators also found differences in mineral weathering bacteria from rocks and the surrounding soil, indicating that only bacteria with the right enzymatic machinery will colonize mineral dense substrates (Colin *et al.* 2017). Knowing what different types of enzymatic machinery bacteria encode and how bacteria interact with others would explain their functional role in soil.

Additionally, bacteria have developed a wide array of strategies to survive in their ecological niche, including antibiotic production, nutrient scavenging, quorum sensing and signal disruption (Galet *et al.* 2015, Deveau *et al.* 2018, Uroz *et al.* 2009A, Tyc *et al.* 2014). Importantly, these different strategies are often specifically deployed in response to specific interactions (Traxler *et al.* 2013) and are more likely to occur between closely related species that share same resource requirements (Perez-Gutierrez *et al.* 2013, Russel *et al.* 2017). These metabolic dialogues, the constitutive and induced specialized metabolites produced during these interactions, stimulate strong community dynamics in soil among bacteria (Vetsigian *et al.* 2011), by modifying bacterial growth, motility, gene expression,



### *Potential functional diversity and biotic interactions*

sporulation and interfering in communication (Uroz *et al.* 2013, Tyc *et al.* 2017b, Helfrich *et al.* 2018). Moreover, these interactions occur in direct cell-to-cell contact (Tecon *et al.* 2018) or in a range of distance by diffusible compounds (Nunan 2017, Tecon *et al.* 2018), affecting directly at the scale of individual microbes or indirectly by modifying their environment (Deveau *et al.* 2018). Thus, knowing when, where and which soil bacterial interaction occurs is important to predict in situ traits of bacteria and their potential influence within soil microbiome (Müller *et al.* 2018).

While bacterial interactions have a key role in the functioning of natural ecosystems (Mesquita Braga *et al.* 2016), they have been mostly and described for model strains (e.g. *Streptomyces*: Vetsigian *et al.* 2011, *Bacillus*: Perez-Gutierrez *et al.* 2013), strains that were not isolated from same the habitat (Russel *et al.* 2017), and rarely tested for bacterial strains from the same habitat in an appropriate functional microscale. To our knowledge, such approaches were mainly addressed in larger soil scale (Davelos *et al.* 2004) or in the search for antibiotic production against pathogenic bacteria (Tyc *et al.* 2014), yet, bacterial interactions at the microscale and their impact on local community structure are still poorly understood.

We hypothesized that the isolation of strains at the microscale would allow capturing bacteria that share the same biotic and abiotic conditions and are likely interacting. Indeed, bacterial survival and bacterial interactions (i.e. competition and cooperation) are mainly linked to space and resources (i.e. obtained nutrients and public goods) (Hibbing *et al.* 2010). In this context the main aim was to determine whether cultivable bacteria with the same 16S sequences coming from a limited space (grain of soil) share the same functional abilities to obtain resources or not to further choose which strains to work for the biotic interactions. Hence, we investigated nutritional preferences, if bacteria are likely to communicate or to disrupt communication signals, if they produce antimicrobials and if the bacteria in the grain can also interact with its surroundings (e.g. plants). To evaluate bacterial functional diversity, we chose (i) to characterize cultivable bacterial isolates by the potential degradation and mobilization abilities of organic and inorganic soil compounds (ii) to evaluate the ability to produce quorum sensing molecules and to interfere in one type of quorum quenching molecule, (iii) to evaluate the possibility to modify *Populus* development and (iv) to assess antibacterial production (against indicator strains and against members that are likely to meet). Since bacterial interactions occurring inside the grain would provide insights of their community structure, we emphasized in i) analyzing the ability of bacteria, living in the same grain of soil, to interact, (ii) in determining the frequencies and intensities of the interactions between strains and (iii) in comparing potential colonization strategies. For this last section, we chose to work with bacteria coming from two different grains, bulk soil and rhizosphere, based on their potential metabolic functions. Then, we tested interactions through two complementary approaches: one in which two bacteria would

## *Potential functional diversity and biotic interactions*

colonize at the same time a habitat (co-inoculation) and one where a second bacteria would reach the habitat after a first one has already been established (delayed inoculation).

## **Materials & Methods**

### *Preparation of bacterial inocula for functional characterization and bacterial interactions assays*

Non-actinobacteria were grown from glycerol stock on 1/10 TSA plates at 25°C for 48 h. Then 2-3 bacterial colonies were picked, suspended in 10 mL of liquid TSB and grown at 25°C under agitation (200 rpm) for 48 h. The bacterial cultures were spin down and the pellets were washed 3 times with 1X phosphate buffered saline (PBS) and resuspended in 1X PBS to obtain an estimated suspension of  $OD_{600nm} = 0.6$ . For actinobacteria, the spore solution was diluted to reach an approximate of  $10^8$  cfu/mL and heat-shock treated (50°C, 10 min) to induce spore germination before further inoculation (Kieser *et al.* 2000).

### *Functional bioassays: agar tests and fluorogenic assays*

For all agar tests, 5  $\mu$ L drops of normalized bacterial or spore suspension were inoculated in triplicate on Petri dishes containing further described agar media. Agar plates were then incubated at 25°C for 7 days. At the end, diameters of halo were measured except if stated otherwise. The fluorescence assays were done as described (Pritsch *et al.* 2004; Courty *et al.* 2005) and adapted by (Uroz *et al.* 2013A) using 4-methylumbelliferone (MU) – linked to exo-enzyme substrates with a bacterial or spore suspensions. Briefly, substrates coupled with MU were incubated with bacterial suspension for 16 hours, suspensions were shaken under the dark and reactions were stopped using 1 M Tris solution pH 11. Results of agar tests and fluorogenic assays were normalized by dividing dataset by the maximum value per assay. All experiments were performed in triplicate.

### *Degradation of organic matter*

The potential ability of bacterial isolates to degrade organic matter on agar tests was determined by the capacity to hydrolyze cellulose and cleave proteolytic bonds. The hydrolysis of cellulose was tested as described (Ulrich *et al.* 2008) using carboxy-methyl-cellulose (CMC) agar, (5 g L<sup>-1</sup> carboxy-methyl-cellulose, 1 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 1 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 g L<sup>-1</sup> NaCl and 15 g L<sup>-1</sup> agar). To reveal the enzymatic activity, the agar plates were flooded with 3 mL of 1 mg/mL Congo red solution for 40 min, then poured off and washed with 1 M NaCl for 15 min. Transparent halos were measured as positive hydrolyzed cellulose. The cleavage of proteolytic bonds was tested using casein

### *Potential functional diversity and biotic interactions*

agar (10 g L<sup>-1</sup> milk, 5 g L<sup>-1</sup> tryptone, 2.5 g L<sup>-1</sup> yeast extract, 1 g L<sup>-1</sup> glucose and 15 g L<sup>-1</sup> agar). Clear halos were measured as indicators of protease activity.

To measure the production of exo-enzymes potentially involved in organic matter degradation, six MU-bound exo-enzyme substrates (Sigma Aldrich) were tested: MU-β-D-glucopyranoside to measure β-glucosidase (EC 3.2.1.3), MU-β-D-glucuronide hydrate for β-glucuronidase (EC 3.2.1.31), MU-β-D-xylopyranoside for xylosidase (EC 3.2.1.37), MU-β-D-cellobioside for cellobiohydrolase (EC 3.2.1.91) and MU-N-acetyl-β-D-glucosaminide for exochitinase (EC 3.2.1.14) (Uroz *et al.* 2013A).

#### *Siderophore production*

The potential ability to produce siderophores was assessed using chrome azurol S (CAS) agar by adapting the method of (Alexander and Zuberer 1991). Due to toxicity of the medium against certain bacterial isolates, bacterial strains were first grown on R2A agar (Difco) for 24 h (Loaces *et al.* 2011). CAS agar was then poured on top of the colonies and incubated at 25°C for 6 additional days. Diameter of pink/orange halos was measured as a reflection of the ability of bacteria to mobilize iron.

#### *Phosphorous mobilization*

Potential ability to solubilize inorganic phosphorus was assessed as described (Lepleux *et al.* 2013), using tri-calcium phosphate agar bioassay (TCP). Potential stepwise dephosphorylation of phytate carried out by phytases was measured using phytase agar bioassay method (Kerovuo *et al.* 1998). In both cases, clear halos were measured as indicator of the ability of bacteria to mobilize inorganic or organic phosphorous. MU-phosphate substrate was used to detect the production of acid phosphatases (Uroz *et al.* 2013A).

### *Biotic interactions*

#### *Communication signals: Quorum sensing and quenching*

To determine the ability of bacteria to detect quorum sensing (QS) molecules, we chose to work with one kind of molecules: *N*-acyl-homoserine lactones (AHLs) with *N*-acyl chain length from C<sub>4</sub>-C<sub>8</sub>, and detect them with the biosensor assay method using *Chromobacterium violaceum* CV026 as described (McClellan *et al.* 1997). QS signals (i.e. AHLs) were detected with the presence of violacein, a violet pigment that *C. violaceum* produces in presence of exogenous AHLs. To detect the interference of quorum sensing, i.e. the quorum quenching (QQ) we chose to work with one molecule: *N*-Hexanoyl-L-homoserine lactone (C<sub>6</sub>-HSL) and the same biosensor assay was performed. This molecule was detected by the presence of violacein and its degradation was revealed by a colorless zone.

## *Potential functional diversity and biotic interactions*

### *Antimicrobial production*

Antibiotic production against bacterial indicators *Escherichia coli*, *Micrococcus luteus* and *Bacillus subtilis* was determined by the overlay method (Maricic and Dawid 2014). First, bacterial isolates were grown on 1/10 TSA for 48 h at 25°C, then an overlay of Lysogeny broth (LB) soft agar (0.6%) supplemented with the bacterial indicator ( $OD_{600nm} = 0.004$ ) was poured on top and incubated 48 additional hours. Antibacterial activity was determined by the zone of inhibition surrounding the colony.

### *Pairwise intragrain bacterial interactions*

The ability of bacterial isolates to interact with each other was assessed using two complementary approaches: i) simultaneous co-inoculation and ii) delayed co-inoculation on modified Murashige & Skoog (mMS) agar plate (Murashige and Skoog 1962) in which sucrose was replaced by glucose to allow bacterial growth (per liter: 1 g glucose, 1 g MES, 100 mL of Murashige and Skoog basal salt micronutrient solution (M0529, Sigma-Aldrich), 50 mL of Murashige and Skoog basal salt macronutrient solution (M0654, Sigma-Aldrich), 1 mL of Gamborg vitamins (Sigma-Aldrich), 10 mL of 100X supplementary vitamins (20 mg.mL<sup>-1</sup> L-Glutamine, 0.1 mg.mL<sup>-1</sup> Ca-Pantothenate, 0.1 mg.mL<sup>-1</sup> L-cysteine hydrochloride, 0.1 mg.mL<sup>-1</sup> Biotin) and 10 g of agar; pH 5.8 adjusted with 3% HCl) (mMS agar).

First, 5 µL of bacterial or spore suspension ( $OD_{600nm} = 0.6$ ) were streaked on mMS agar along the center of a square petri dish and then 5 µL of bacterial or spore suspension ( $OD_{600nm} = 0.6$ ) were cross-streaked at around 5 mm away from the center to produce a 5 cm long streak either immediately after the deposition of the central streak (co-inoculation, Supplemental Figure 1A, Annexes Chapter II) or 48 h after the inoculation of the central streak (delayed-inoculation, Supplemental Figure 1B, Annexes Chapter II). Plates were incubated 5 days in total at 25°C and growth inhibition (length of the cross-streaks) or stimulation and morphological changes (i.e. color of colonies, aerial mycelium production and sporulation ability) were recorded. Combinations were done from bacteria originating from the same soil compartment only. Negative controls consisted of single species streak inoculation against 1X PBS. Self-interactions were also tested. For each method, three independent assays were analyzed. Cytoscape software was used to visualize bacterial interactions networks (Shannon *et al.* 2003).

### *Pairwise bacterial interactions through volatile compounds*

The ability of bacterial isolates to interact within each other using volatiles as signal molecules was assessed using split Petri dish. Five microliters of bacterial or spore suspension ( $OD_{600nm} = 0.6$ ) were

## *Potential functional diversity and biotic interactions*

streaked on mMS agar in each side in all the combinations with *Pseudomonas* Bs17. Plates were incubated 5 days in total at 25°C and growth inhibition or stimulation (length of streaks) and morphological changes (i.e. color of colonies, aerial mycelium production and sporulation ability) were recorded. Negative controls were 1X PBS. For each strain, three replicates were done.

### *Interkingdom interactions*

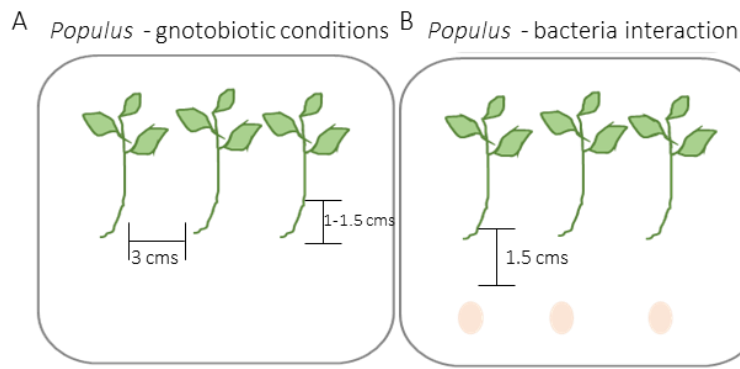
#### *Indolic compounds production*

Auxin production from tryptophan was tested as described (Frey-Klett *et al.* 2005). Bacterial isolates were grown on 1/10 TSA supplemented with 5mM tryptophan. After inoculation and drying, plates were covered by a nitrocellulose membrane and incubated at 25°C for 72 h. Then the membranes were removed and soaked on Salkowski reagent (2% 0.5M FeCl<sub>3</sub> in 35% perchloric acid) at room temperature for 1 h. Bacterial isolates producing auxin were identified by a formation of pink/red coloration in the membrane.

#### *Populus seedlings – bacteria interaction*

*Populus* seedlings (*Populus tremula* x *P. alba* 717-1B4) (*Populus*) were prepared as described (Felten *et al.* 2009). First, *Populus* cuttings obtained from 2-month-old *in vitro* seedlings were grown on mMS supplemented with Indole-3-butyric-acid (IBA)(mMSIBA) for two weeks at 25°C with 16h photoperiod. When *Populus* cuttings started rooting, the plantlets were transferred onto mMS agar and incubated for two more weeks at 25°C with 16h photoperiod. For the interaction assay, poplar roots were removed to keep only 1 root of around 1-1.5 cms long. Three of these plantlets were then transferred into new mMS agar square Petri dishes with 3 cms in between each other. A small agar cube was put on top of the plantlets to keep them standing on the plates when put vertically in the incubator. Then, five microliters of bacterial suspension (OD<sub>600</sub>: 0.6 or spore dilution) was inoculated 1.5 cms away from the tip of the root (Figure 15). *Populus*-bacteria interactions were done in 9 replicates per strain and incubated together for 21 days. The incubation period is limited based on the size of the Petri dish plate, the size of plantlets, the nutrient availability over time and the water content in the agar. Plates were scanned every 7 days, and images were analyzed using ImageJ to measure root system over time. Data analyzed consisted of the difference between T21 – T0.

## Potential functional diversity and biotic interactions



**Figure 15.** Schematic representation of *Populus* – bacteria interaction experiment. A. *Populus* in mMS agar in gnotobiotic conditions. B. *Populus* in interaction with bacteria.

### Statistical analyses

Statistical analyses were conducted with R software version 3.4.3 (R core Team 2016). Variables were verified for normality using Shapiro-Wilk's method (Royston 1982). If data was normally distributed, ANOVA test was used, if not normal then data was analyzed using non-parametric test Kruskal Wallis test by ranks and p-value was adjusted with Bonferroni test (Conover 1999). The threshold of significance was fixed for  $p < 0.05$ .

## Results

### 2.1 Potential bacterial functional abilities

In this study, we wonder whether the selected cultivable bacteria coming from a limited space (grain of soil) share the same functional abilities to obtain resources. Seventy-three bacterial isolates out of 77 were tested since four *Streptomyces* strains failed to grow from the glycerol stock. Twenty-two belonged to bulk soil grain, 25 to rhizosphere and 26 to mycorrhizosphere grains. Eleven functional traits were analyzed involving iron uptake (siderophores), phosphorus solubilization (acid phosphatase, inorganic P and phytases), nitrogen mobilization (proteases and N-acetylglucosaminidase/exochitinase), plant cell wall degradation and carbon mobilization ( $\beta$ -xylosidase,  $\beta$ -glucuronidase,  $\beta$ -glucosidase, cellulase).

Among all strains sharing same genus, variations of all 11 functions were recorded (Figure 16). For instance, only one *Bacillus* strain among 26 presented cellulase activity (ECM25), all *S. olivochromogenes* produced  $\beta$ -glucuronidase and cellobiohydrolase, except one (Bs1), 2 out 5 strains of *Bacillus drentensis* produced proteolytic exoenzymes while the three others did not in our experimental conditions (Figure 16). Moreover, strains sharing both 16S and functions showed different levels of activity. For example, proteolytic enzymes from *Bacillus* strains varied between each other (Figure 17). Overall, none of the potential activities measured was specific to a taxonomic group or to the origin (bulk soil, rhizosphere or mycorrhizosphere) (Figure 16).

## Potential functional diversity and biotic interactions

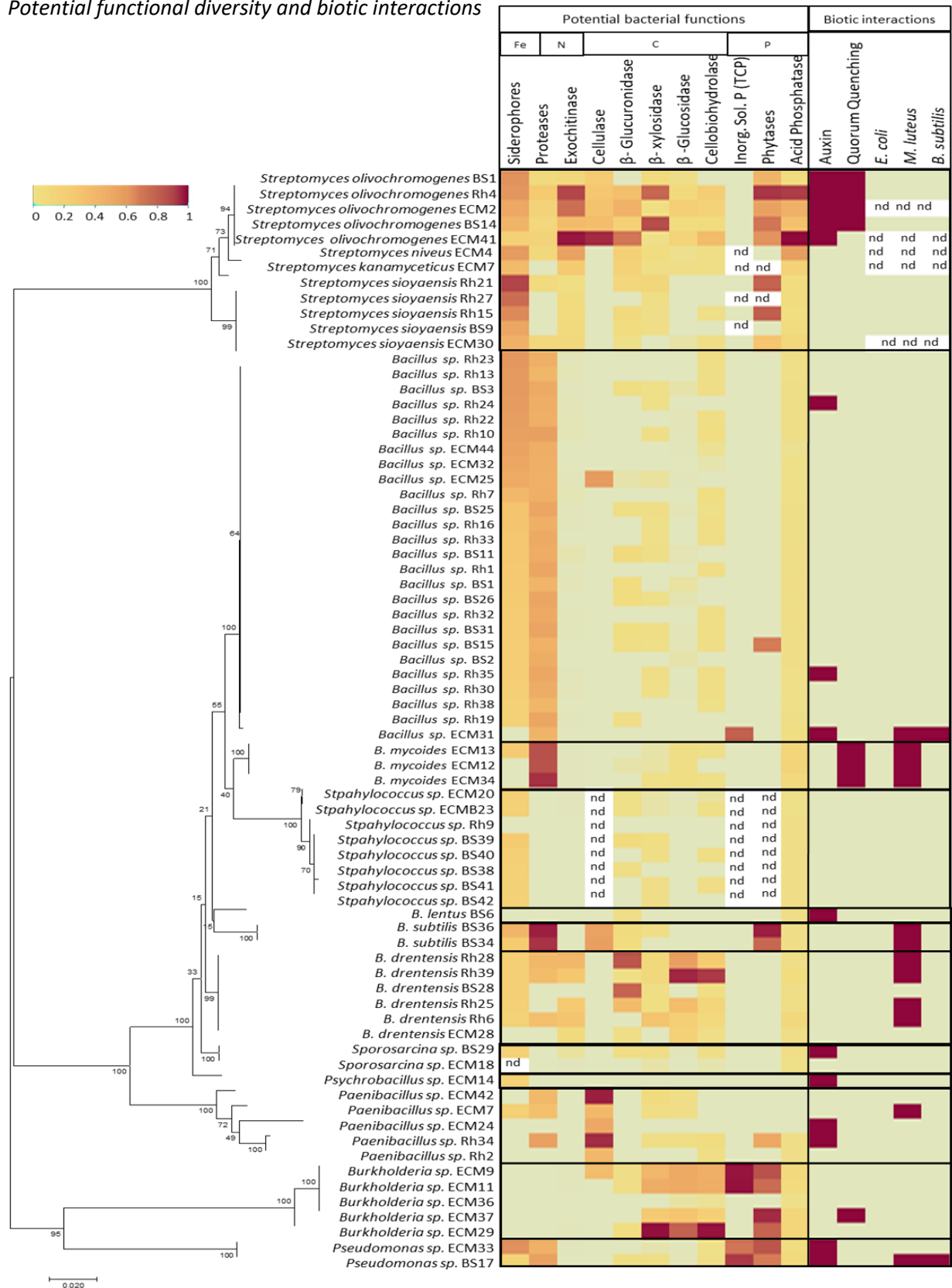
Since the potential abilities to degrade organic matter and to obtain nutrients are important for bacterial survival in soil, we analyzed the functional abilities bearing in mind that actual functions in soil may be different than under laboratory conditions. In detail, the number of active bacteria varied between grains and between assays (Table 1). For instance, no rhizospheric isolate from the collection contributed to the solubilization of inorganic phosphorus tested by TCP (Table 1). In the bulk soil grain, most of bacterial isolates produced siderophores (95.5%), followed by  $\beta$ -glucuronidase (72.7%) and proteolytic exoenzyme (59.1%) (Table 1, Figure 16). In the rhizosphere grain, the most abundant activity shared was siderophores produced by 88% of bacterial isolates followed by proteolytic exoenzymes (80%) and cellobiohydrolases (68%) (Table 5, Figure 16). In the mycorrhizosphere grain, 57% of bacteria produced acid phosphatases, and 53,8% produced siderophores and proteolytic exoenzymes Table 5, Figure 16). Even though most bacterial isolates presented all 11 functions tested, most strains had a low potential activity compared to the most active ones (Figure 16).

If we consider all the collection, the production of siderophores was the most commonly shared by 79% of bacterial isolates showing different levels of activity, while the least shared was phosphatases (TCP) (7%) (Figure 17). Among the extracellular enzymes involved in carbon mobilization, the most commonly produced was cellobiohydrolase (46%), for phosphorus solubilization the most produced enzyme was acid phosphatases (46%) and proteolytic exoenzymes were the most common for nitrogen mobilization with 65% of bacteria (Figure 17). Among the most active bacteria in bulk soil, *Streptomyces olivochromogenes* Bs14 and *Pseudomonas* Bs17 presented 10 and 8 functional enzymatic activities, respectively. In rhizosphere, *S. olivochromogenes* Rh4 and *Bacillus drentensis* Rh39 and Rh28 were active in 10 and 8 activities, respectively. In mycorrhizosphere, *S. olivochromogenes* ECM2 and ECM41 and *Burkholderia sp.* ECM9, were the most actives producing 10, 9 and 8 functions respectively. On the contrary, the least active bacterium in bulk soil was *Bacillus lentus* BS6, in rhizosphere, *Staphylococcus sp.* Rh9 and in mycorrhizosphere *Burkholderia sp.* ECM36 and *Sporosarcina sp.* ECM18 (Figure 16).

**Table 5.** Number of potential active isolates in each of the functional assays.

Origin	Total of isolates	Siderophores	Proteases	Exochitinase	Cellulase	$\beta$ -Glucuronidase	$\beta$ -Xylosidase	$\beta$ -Glucosidase	Cellobiohydrolase	Inorg. Sol. P (TCP)	Phytases	Acid Phosphatases	Total of present activities
BS	22	21	13	3	4	16	8	1	7	1	6	11	11
RH	25	22	20	8	3	6	6	6	17	0	4	8	10
ECM	26	14	14	6	7	7	9	13	9	4	8	15	11

Potential functional diversity and biotic interactions

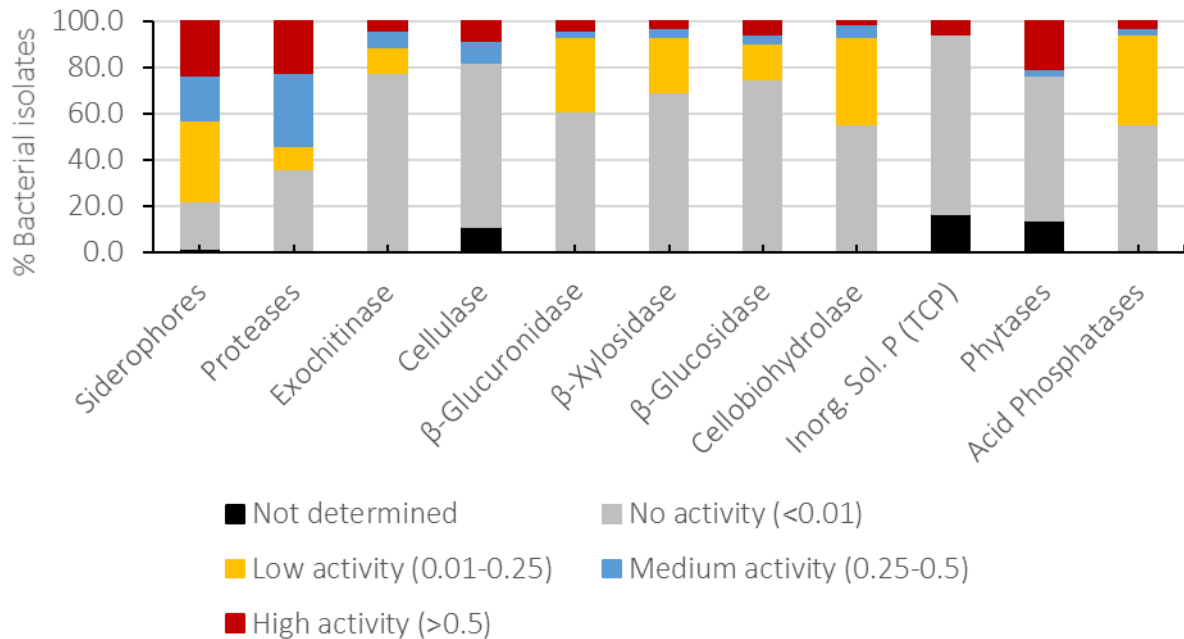


**Figure 16.** Functional potentials and phylogenetic tree of the strains isolated from grains of bulk soil (Bs), rhizosphere (Rh) and mycorrhizosphere (ECM) grains of soil. Taxonomical classification of all phenotypically looking different isolates (n=73) was built based on 16S rRNA sequences using Neighbor-Joining method. The numbers of each branch nodes are bootstrap values from 100 re-samplings. Horizontal branch lengths are proportional to evolutionary distance according to Kimura's 2 parameter distance. For strains with identical 16S rRNA sequences, ordering of strains was organized based on similarity of potential functional activities by hierarchical clustering. For each potential activity, data were normalized to the maximal intensity measured and



## Potential functional diversity and biotic interactions

intensity is represented as a gradient of color from no activity (green) to 100% activity (dark red). Nd – not determined.



**Figure 17.** Eleven potential functional activities of all the bacterial isolates from three grains of soil (n=73). Data was normalized by dividing the highest per activity. Red indicates activity higher than 0.5, Blue indicates medium activity (between 0.25-0.5), Yellow indicates low activity (0.01- 0.025), Grey indicates no activity (<0.01) and black indicates it could not be determined since bacterial isolates could not grow in the medium.

## 2.2 Biotic interactions

### 2.2.1 Communication signals

According to Bettenworth and collaborators, quorum sensing systems are almost ubiquitous in bacteria, having at least one QS system, and many even have several (Bettenworth *et al.* 2019). The best characterized QS system is driven by AHL molecules. To test if Gram negative bacterial isolates were able to produce AHLs molecules, we used *Chromobacterium violaceum* CV026 as a biosensor. This strain possesses a mutation allowing the production of violacein when it detects exogenous AHLs with *N*-acyl side chains from C<sub>4</sub> to C<sub>8</sub> in length. In these conditions, from the seven bacterial isolates tested (two *Pseudomonas* and five *Burkholderia*), none of them were able to produce AHL molecules, although they might be able to produce other signals (such as AHLs with longer acyl chain) that this biosensor cannot detect.

Competitors might get advantage if they interfere with the communication of other bacterial members, a mechanism called quorum quenching (QQ). Since *N*-Hexanoyl-L-homoserine lactone (C<sub>6</sub>-HSL), a kind of AHLs signal, is one of the most widely studied QS molecules in gram negative bacteria, we tested the QQ ability of bacterial isolates using this molecule and *C. violaceum* CV026 as a biosensor. If the isolate tested would interrupt the communication, then the biosensor would not be able to detect the molecule and there would not be production of violacein. In these conditions, 11%

## **Potential functional diversity and biotic interactions**

(8 out of 73 bacterial isolates) lost violet production: all *Bacillus mycoides* (3), one *Burkholderia* (ECM37), and all *Streptomyces olivochromogenes* (4) (Figure 16). Since this test combined the bacterium and the biosensor, these results might involve antimicrobial production and inhibition of growth of the biosensor making impossible to detect the C<sub>6</sub>-HSL. To discard this possibility, an ethyl acetate extraction of the C<sub>6</sub>-HSL after being incubated with the bacterial isolate was performed. From this test, C<sub>6</sub>-HSL was absent indicating a possible degradation of the molecule (data not shown), nevertheless the mechanism of degradation is still unknown. We hypothesize the action of an AHL acylase from the part of *Streptomyces* since genome data presents gene encoding a putative AHL Acylase and further studies are still ongoing (Chapter III).

### **2.2.2 Antibacterial production against bacterial indicators**

The potential ability to produce antibacterial compounds is of a great importance in the medical field (Tyc *et al.* 2014), however, it is strongly dependent on the growth conditions. As a screening trial, we evaluated if these bacteria can produce antimicrobials against specific Gram positive (*B. subtilis*, *M. luteus*) and Gram negative (*E. coli*). After co-incubation period, an inhibitory halo was measured. In our study, none of the bacterial isolates had an effect against *E. coli*, while few bacteria presented inhibitory activity against the other two bacterial indicators. Twelve bacterial isolates inhibited *M. luteus* and two inhibited *B. subtilis* (Figure 16). Inhibitors active only against only *M. luteus* were all *Bacillus mycoides* (3), all *Bacillus subtilis* (2), 4 out 6 *Bacillus drentensis* (Rh6, Rh25, Rh28, Rh39), *Paenibacillus* ECM7, *Pseudomonas* Bs17 and *Bacillus* ECM31. The latter two strains also inhibited *B. subtilis*, being active against two out of three bacterial indicators, a wider spectrum compared to the other bacteria in the collection. We then compared the intensities of these inhibitions to assess the effectiveness of the antimicrobial activities. All bacteria presented a weak inhibitory effect (0.5-1 cms halo), except *B. subtilis* strains which had a strong inhibitory effect (>3 cms) against *M. luteus* (data not shown). Although the inhibition of growth of the bacterial indicators was not specific to any taxonomic group or to the origin of grains in our experimental conditions, most of the inhibitors belonged to Firmicutes.

### **2.2.3 Interkingdom interactions**

#### **2.2.3.1 Indolic compounds production by bacteria**

While the ability to obtain nutrients is important for bacterial survival in soil, bacterial adaptations to stressful conditions lead to improved survival the environment. The ability to produce indolic compounds provides bacteria a selective advantage in soil and plant environments, and moreover confers protection against UV, salt and acidity (Duca *et al.* 2014). To test if bacteria were able to

### ***Potential functional diversity and biotic interactions***

produce indole related compounds involved in plant hormonal signaling, we performed the Salkowski test. From the 73 bacterial isolates tested, only 15 isolates were positive indole producers (Figure 16). The production of auxin was not specific to any taxonomic group or to the origin of grains in our experimental conditions. The producers were *S. olivochromogenes* (all), three *Bacillus* sp., *B. lentus*, one *Sporosarcina* sp., *Psychrobacillus*, 2 *Paenibacillus* and all *Pseudomonas* strains.

Expecting that different bacteria will reproduce different phenotypes on other bacteria and on Poplar development, we selected at least one bacterial strain per taxonomic group and functional profile. Based on the 11 functional activities tested and 16S rDNA sequences, a subset of 10 out of 22 bulk soil isolates, 15 out of 25 rhizosphere isolates and 10 out of 26 from mycorrhizosphere were chosen to perform interkingdom interactions with *Populus*. This subset was composed of 15 *Bacillus*, 6 *Streptomyces*, 4 *Paenibacillus*, 4 *Burkholderia*, 2 *Staphylococcus*, 2 *Pseudomonas* and 1 *Sporosarcina* strains.

#### ***2.2.3.2 Bacteria influences Populus' root architecture***

In order to evaluate bacterial influence on *Populus'* development, we first need to study poplar's growth on mMS in gnotobiotic conditions (Figure 15 A). Here we evaluated the generation of new roots, new lateral roots, lateral root density (lateral root per root), the length of the main root, the size of the root network (the sum of the length of all the roots), the length of the stem, and the size of poplar (root and stem systems combined). Modified Murashige and Skoog (mMS) agar contained enough nutrients to allow poplar root network to have a linear growth ( $R^2=0.99$ ) over 21 days of incubation. *Populus* phenotype was characteristic to be highly variable between replicates but generally presented deep root system where on average per plant the main root length was around  $5.7 \pm 2.7$  cms, there were few new roots ( $1.3 \pm 1.3$ ), few new lateral roots ( $1.4 \pm 2.1$ ), and the stem was around  $3.6 \pm 0.5$  cms. Moreover, *Populus* root network size was the double in Spring compared to beginning of Fall ( $p=0.0246$ ), and this was only attributed to four-time higher number of lateral roots ( $p=0.008$ ). However, no difference was found in length of main root nor number of new roots between the different experiments. Although these experiments were done only once, this could indicate seasons may interfere in the designing of root architecture and it is an important factor to be considered when analyzing plant interactions. It would be necessary to reproduce this experiment over years to demonstrate this hypothesis. In addition, no statistical differences in the overall growth of poplar were found between Fall experiments hence we could attribute the variability to the origin of the cutting, the age of the stock plants or the stress generated in the manipulation of plants.

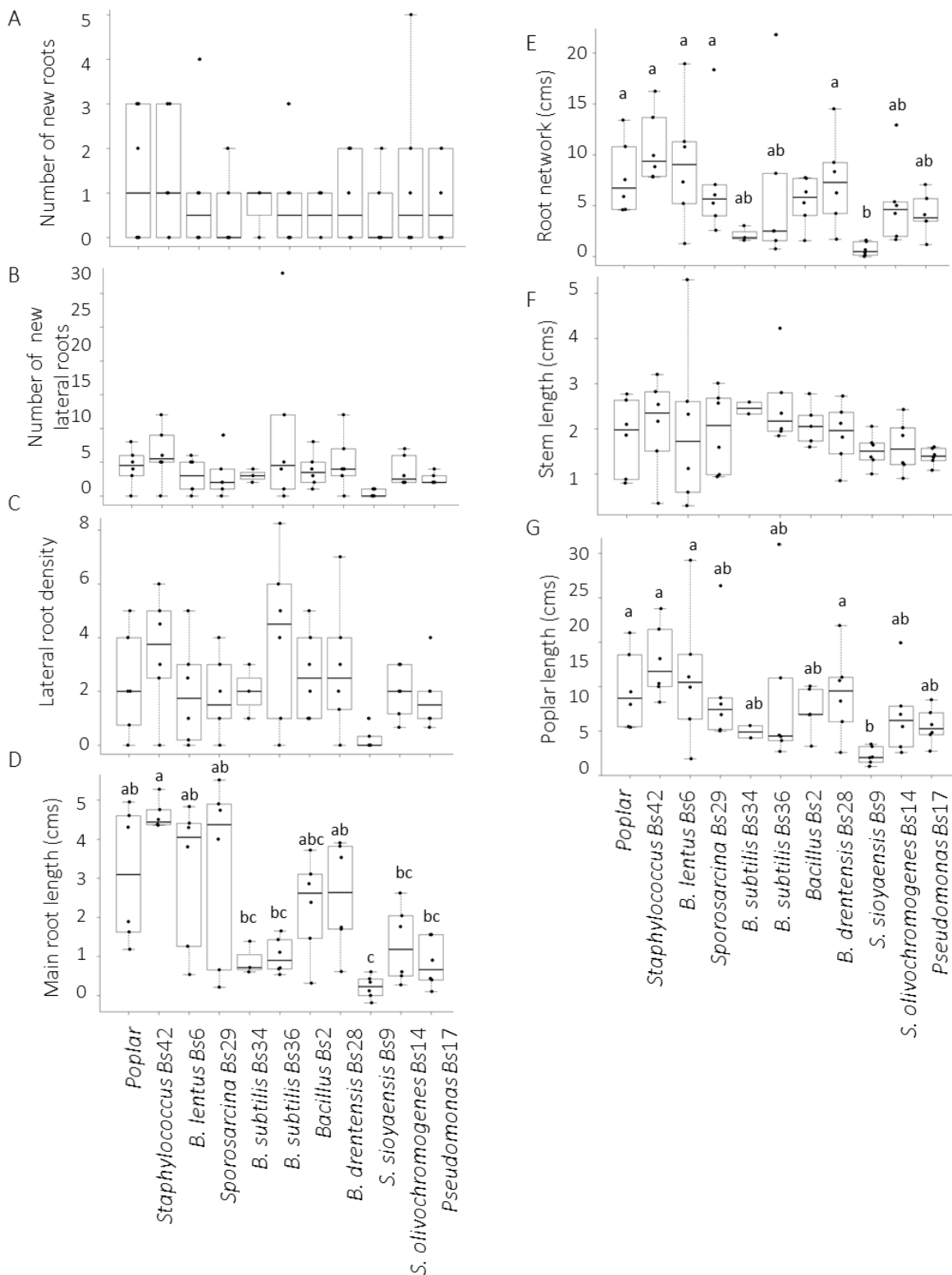
In order to assess the influence of bacterial isolates on *Populus'* development, 35 bacterial isolates belonging to bulk soil, rhizosphere and mycorrhizosphere grains were independently inoculated next

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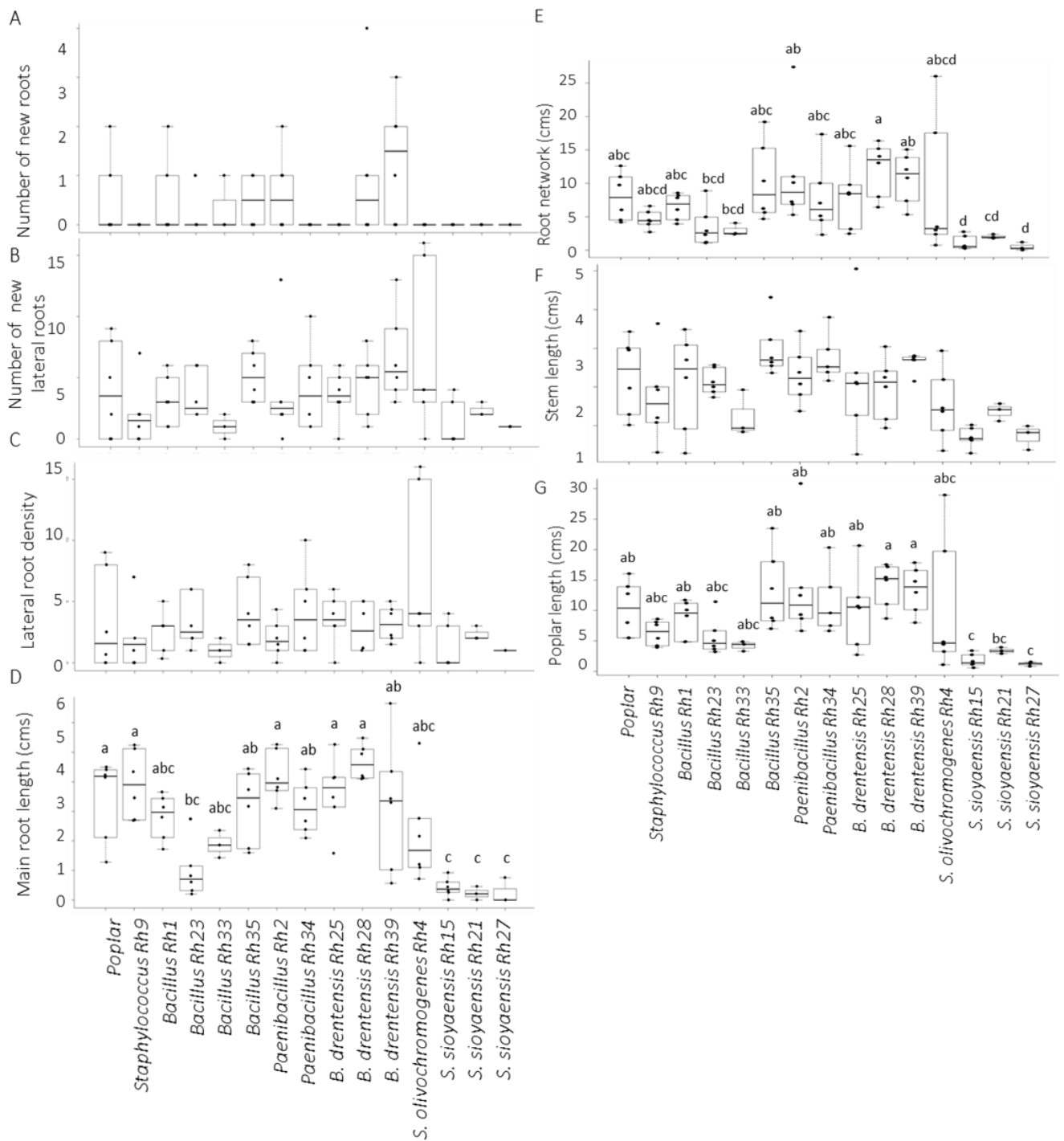
to *Populus* on mMS agar (Figure 15 B). Among the 35 strains analyzed, four did not grow on mMS medium when inoculated alone or with poplar (*Bacillus lentus* Bs6, *Sporosarcina* sp. Bs29, *Staphylococcus* sp. Bs42, and *Staphylococcus* sp. Rh9), and one rhizospheric strain (*Bacillus* Rh24) got contaminated in the process. Significant changes in poplar growth was difficult to measure since each plantlet replicate grew considerably differently (Figures 18, 19 and 20). Overall, none of the bacteria had a significant positive effect in the general growth of poplar, and few bacteria significantly reduced the root network in these experimental conditions (Figures 18, 19 and 20). Only five bacterial isolates had a significant negative effect reducing 5 times the size of poplar (Figures 19 D, E and G). All the strains involved in this inhibition of growth belonged to *Streptomyces sioyaensis* species (Bs9, Rh15, Rh21 and Rh27) and one *Pseudomonas* ECM33. These strains produced smaller poplars by affecting the root system reducing the size of the root network 10 times and producing necrosis (Figures 19 and 20). The only bacterium causing a significant positive effect aboveground was *Burkholderia* ECM9 (Figure 20F), although this increment did not affect the overall growth of poplar (Figure 20G). In addition, *Burkholderia* ECM37 was the only bacterium increasing the lateral root density significantly (Figure 20C), however, this change did not affect the overall size of poplar significantly (Figures 20E and G).

In general, we did not observe a significant improvement in growth (e.g. larger plants with bigger root networks), but we did observe an alteration of root architecture with high variabilities within the replicates. For example, *B. subtilis* Bs36 and *S. olivochromogenes* Rh4 produced smaller main roots with a high variability in lateral roots compared to untreated poplar (Figure 18D and Figure 19D). In addition, *Paenibacillus* ECM7 produced high variable poplars in terms of main root and new lateral roots (Figures 20C and 20D). Even though these changes were not significant, we observe trends of smaller poplars with an increased lateral root production and high variabilities within replicates.

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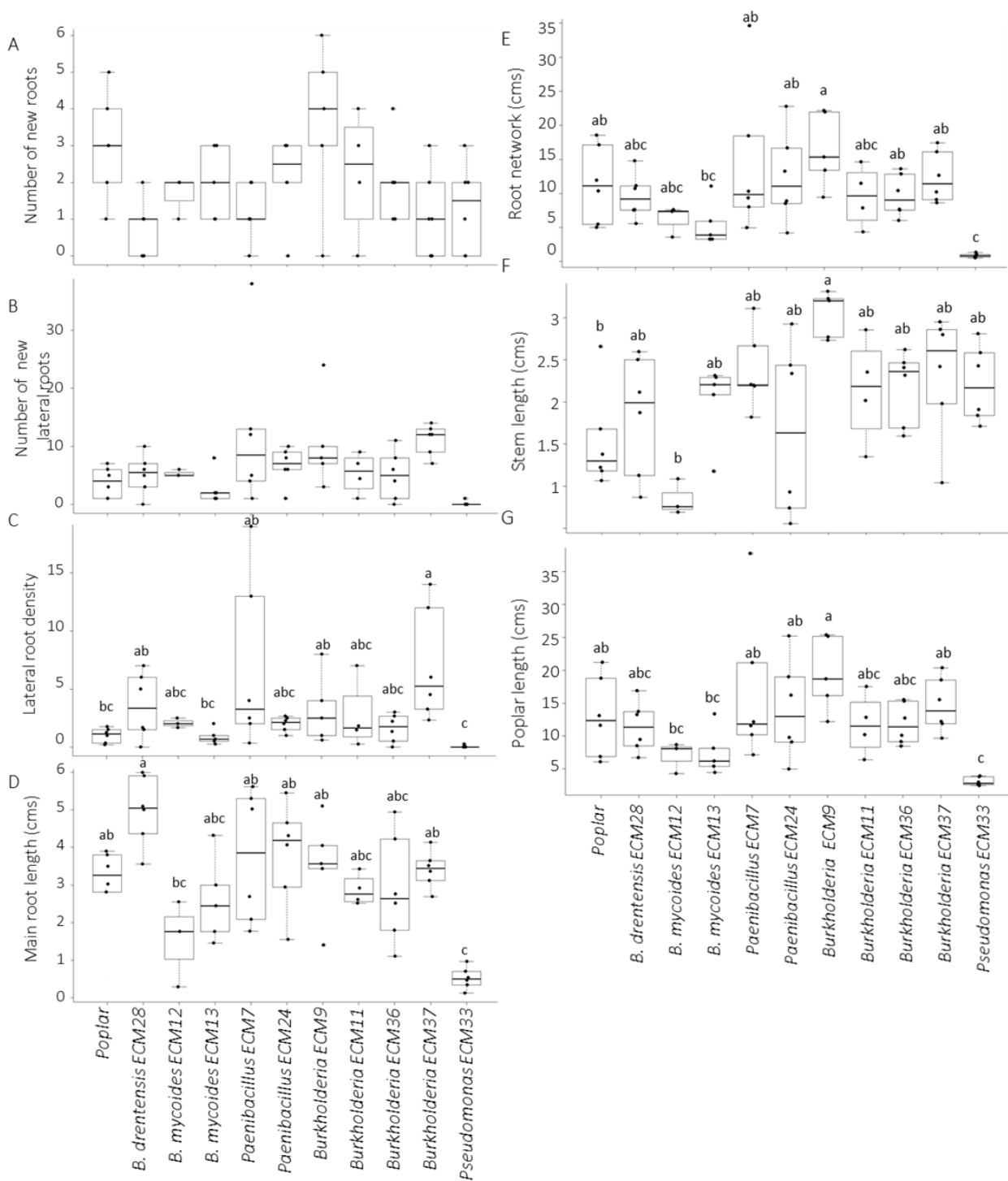


**Figure 18.** Poplar's growth interacting or not with bulk soil (Bs) bacteria (n=10) after 21 days of incubation. **A:** Number of new roots, **B:** Number of new lateral roots, **C:** Lateral root density, **D:** Length of the main root. **E:** Root network, **F:** Stem length and **G:** Poplar size. Different letters on top of the bars indicate significance based on Kruskal-Wallis adjusted with Bonferroni test. No letter indicates there were no significant changes.



**Figure 19.** Poplar’s growth interacting or not with rhizospheric (Rh) bacteria (n=15) after 21 days of incubation. **A:** Number of new roots, **B:** Number of new lateral roots, **C:** Lateral root density, **D:** Length of the main root. **E:** Root network, **F:** Stem length and **G:** Poplar size. Different letters on top of the bars indicate significance based on Kruskal-Wallis adjusted with Bonferroni test. No letter indicates there were no significant changes. **Note:** One bacterial strain (*Bacillus Rh24*) got contaminated over the 21 days of experiment, hence it is not included in this data.

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**Figure 20.** Poplar's growth interacting or not with mycorrhizosphere (ECM) bacteria (n=10) after 21 days of incubation. **A:** Number of new roots, **B:** Number of new lateral roots, **C:** Lateral root density, **D:** Length of the main root. **E:** Root network, **F:** Stem length and **G:** Poplar size. Different letters on top of the bars indicate significance based on Kruskal-Wallis adjusted with Bonferroni test. No letter indicates there were no significant changes.

## **2.2.4 Intragrain bacterial interactions**

### **2.2.4.1 Pairwise interactions between bacteria isolated from the same grain**

To evaluate potential interaction behaviors among bacteria living in the same ecological niche, pairwise combinations between strains from the same grain (sympatric) were tested on mMS. Here we only studied bulk and rhizospheric grains in order to reduce the amount of possible combinations and test priority effects. Since soils are dynamic environments, bacteria having the opportunity to colonize first will have priority to consume fresh nutrients. To assess the importance of priority effect on the output of the interactions, bipartite combinations were done in two different ways: using a simultaneous co-inoculation and with 48 hours delayed inoculation. After co-incubation period, potential interactions between strains isolated from the same grains of soil were recorded. Three phenotypes were observed: effect i) on the size of bacterial streak (i.e. effect on the bacterial growth), ii) on the ability to produce pigment (color of the colony) and iii) on morphological differentiation (aerial mycelium and sporulation) for the *Streptomyces* strains. In total, we performed 650 combinations, 325 combinations per approach, 100 for bulk soil (n=10) and 225 for rhizosphere (n =15), including self-interactions (Supplemental figure 1, Annexes Chapter II).

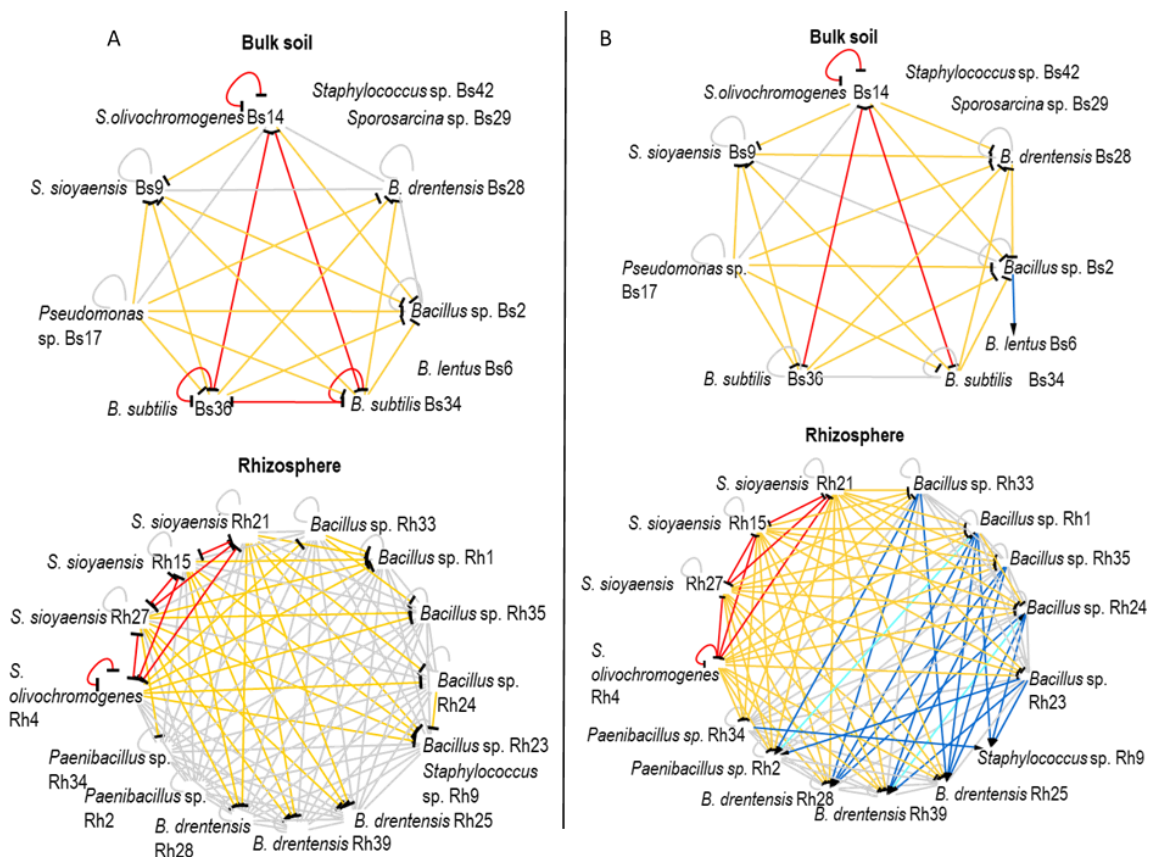
Among the 25 strains analyzed, 4 did not grow on mMS medium when inoculated alone (*Bacillus lentus* Bs6, *Sporosarcina* sp. Bs29, *Staphylococcus* sp. Bs42, and *Staphylococcus* sp. Rh9). Thus, only 245 out of the 325 possible interactions were recorded for the simultaneous co-inoculation experiment. No phenotypic change could be observed in 185 interactions (75.5% out of 245 possible interactions), while 60 (24.5%) led to a reduction of streak size (Figure 21A). From these inhibitory activities, 13 interactions (5.3 % out of 245) occurring either between strains from the same genus (*Streptomyces* against *Streptomyces*) or between certain *Streptomyces* and *Bacillus* strains led to reciprocal inhibition of growth (Figure 21A). The 47 other interactions (19.2 %) involved unidirectional inhibitions and were not taxa specific (Figure 21A). Interestingly, all *S. olivochromogenes* and *B. subtilis* strains showed a self-inhibition behavior (Figure 21A). Lastly, pigmentation of the colonies was not visibly affected by any of the interactions analyzed. No effect on sporulation was observed for the *Streptomyces* strains either.

In the 48 hours delay experiment, inhibitory and stimulatory interactions were observed for both soil grains. Additionally, we observed effects on aerial mycelium formation and sporulation and on pigmentation of the colonies in some cases. In details, 250 out of the 325 possible interactions were recorded. From these 250 observed interactions, 153 (61%) had no visible effect and 97 cases (39%) led to a change of streak size. From the latter, 75 were inhibitory and 22 were stimulating (Figure 21B). Seventeen (6.8% out 250) inhibitory interactions were reciprocal inhibitions and all involved strains from the *Streptomyces* genus. The remaining 58 (23.2% out of 250) inhibitory interactions



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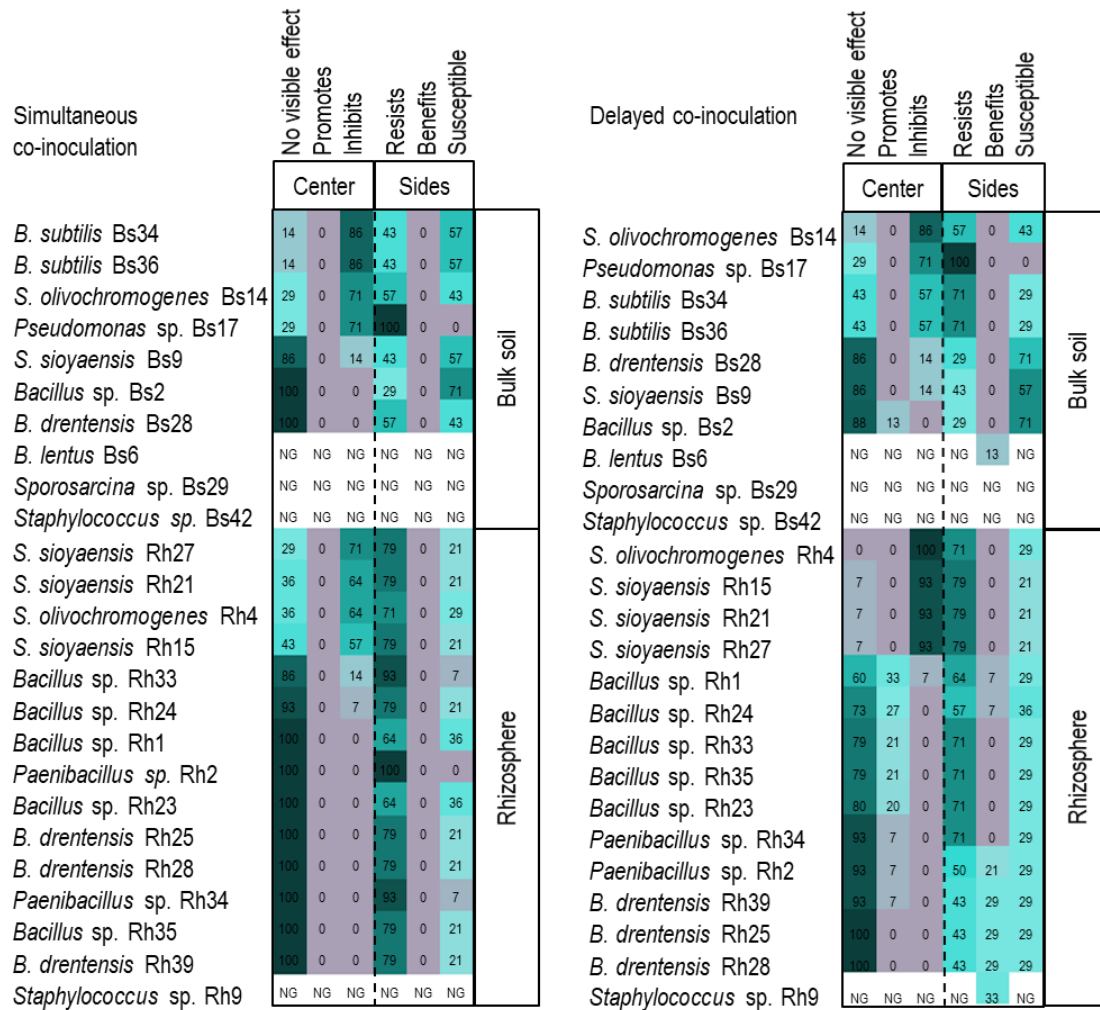
affected only one of the interacting strains. Specifically, *Bacillus* sp. strains no longer inhibited other *Bacillus* sp. strains, and mainly *Streptomyces* strains inhibited the other bacterial members. Interestingly, only *S. olivochromogenes*, showed a self-inhibition behavior regardless the origin. In the case of the positive interactions, only 4 (1.6 %) stimulated the growth of each other (*Bacillus* sp. Rh1 - *Paenibacillus* sp. Rh2 and *Bacillus* sp. Rh24 - *B. drentensis* Rh39), while 18 (7.2 %) increased the growth unidirectionally (mainly *Bacillus* sp. towards *B. drentensis*). Interestingly, *B. lentus* Bs6 was not able to grow alone on the medium but was able to form a colony when co-inoculated near a 48h old colony of *Bacillus* sp. Bs2 (Figure 21B). Similarly, the strain *Staphylococcus* sp. Rh9 was able to grow after being inoculated with delay with *Paenibacillus* sp. Rh34 or *Bacillus* sp. strains Rh1, Rh23, Rh24 and Rh35. However, none of the strains rescued the growth of *Staphylococcus* sp. Bs42 and of *Sporosarcina* sp. Bs29 in the tested conditions. Finally, a delay in the production of aerial mycelium and sporulation of *S. olivochromogenes* B14 was observed when inoculated 48h after *B. subtilis* Bs34 and Bs36 strains. Conversely, the two *B. subtilis* no longer produced red pigment when inoculated 48h after *S. olivochromogenes* Bs14.



**Figure 21.** Networks of pairwise interactions between bacterial strains isolated from bulk soil and rhizosphere bacteria. **A:** simultaneous co-inoculation, **B:** co-inoculated with a 48hrs delay. The direction of the interaction is marked by a bar for inhibition and an arrow for promotion of growth. Color of connecting lines indicate the type of interaction: red – mutual inhibition, yellow – unidirectional inhibition, turquoise – mutual promotion of growth, blue – unidirectional growth promotion, grey – no visible effect on growth. Loops figure potential self-interaction phenotypes.

2.2.4.2 Frequency and intensity of interactions: few aggressive strains but a large potential for interactions.

In a second step, we asked whether these interactions were due to a small number of highly active bacteria or whether most of the strains were engaged in interactions. Overall, all the strains interacted with at least one other bacterial strain in at least one of the two tested conditions. However, the numbers of strains with which bacteria interacted strongly varied from strain to strain and depended on the conditions of the interaction (Figure 22).



**Figure 22.** Degrees of activities and susceptibilities of bulk soil and rhizosphere bacterial strains when co-inoculated simultaneously or with a 48hrs delay. The abilities to promote/inhibit the development of other strains were determined for strains placed as a central streak (“Center” column) in the Petri dish while sensitivity to the activities of strains from the central steak was measured on strains streaked perpendicularly (“Side” column, Suppl. Fig 1). For each strain, the degree of activity (no visible effect / promotion /inhibition) and of susceptibility (no visible effect-resist / benefits / susceptible) was expressed as the percentage of strains impacted among the total number strains tested (n=10 and 15 for bulk soil and rhizosphere, respectively). Percentages are given as numbers and color-coded from 0 (grey) to 100% (dark blue) to facilitate visualization. NG indicates undetermined values because the strains did not grow on the medium. For each bacterial library (bulk soil and rhizosphere), strains are presented in the order of the most to the less aggressive strains.

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In detail, from a total of 21 interacting bacteria, 10 strains (47.6% out of 21) co-inoculated at the same time had no effect on other strains, three (14.3%) inhibited the growth of 1-2 bacterial strains, two (9.5%) inhibited between 3-5 bacterial strains and six (28.6%) inhibited more than 6 strains. Delayed inoculations led to two (9.5% out of 21) bacterial strains with no effect on other strains, three (14.3%) inhibited 1-2 strains, three (14.3%) inhibited 3-5 bacterial strains and five (23.8%) inhibited more than 6 bacterial strains. Additionally, in this later approach, four (19%) of the strains promoted the growth of one isolate while five (23.8%) promoted the growth of 3 to 5 bacterial isolates. All these promoting strains belonged to the *Bacillus* genus (Figure 21) and none of these strains showed inhibitory activity against other strains except *Bacillus* sp. Rh1 which inhibited the growth of *Bacillus* sp. Rh24. Correlations between “aggressiveness” of strains (defined in this study as strains that inhibit more than 4 strains) and their “susceptibility” to other strains (strains that get inhibited by more than 4 strains) could be drawn but the pattern was strongly dependent on the inoculation approach considered (Figure 22). In delayed assays, “aggressive” strains isolated from both soil compartments were more resistant to inhibition while “less aggressive” strains were more prone to be inhibited. Yet, no correlation between aggressiveness and susceptibility was found among strains when co-inoculated simultaneously whatever their origin.

Bacterial strains were categorized into 11 classes based on their behaviors (Table 6). These categories were built based on the total number of strains that each was able to promote, to benefit from, to inhibit, and was susceptible to. Connections between taxonomic affiliations of the strains and their activities could be drawn. For instance, the most active bacterial strains co-inoculated were *B. subtilis* Bs34 and Bs36, *Pseudomonas* sp. Bs17, *S. olivochromogenes* Bs14 and *S. sioyaensis* Rh27. These strains were able to inhibit the growth of at least 70% to 85.7% of the bacterial strains. While in delayed inoculation, inhibitory interactions were steered by *S. olivochromogenes* Rh4 (100% of strains inhibited), *S. sioyaensis* Rh15, Rh21 and Rh27 (>90% of strains inhibited) and *Pseudomonas* Bs17 (>70% of strains inhibited) (Figure 22, Table 7). Important variations occurred at the strain level: *S. sioyaensis* could be either poorly or highly aggressive depending on the strain with which it interacted, while *B. drentensis* could benefit or not from interaction depending on the identity of the other interacting strain.

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**Table 6.** Classification of interactive phenotypes as a function of levels of activities and susceptibilities of bacterial strains. HA, MA, LA: High, medium, low aggressiveness, respectively; HS, MS, LS: High, medium, low susceptibility, respectively. OP, P: Absence, presence of promoting effect. OB, B: does not, does benefit from another strain.

Type of interaction	Strains
HA – HS / OP – OB	<i>Streptomyces olivochromogenes</i> Rh4
HA – LS / OP – OB	<i>Pseudomonas</i> sp. Bs17
HA – MS / OP – OB	<i>Streptomyces sioyaensis</i> Rh15, Rh21, Rh27
	<i>Bacillus subtilis</i> Bs34, Bs36
	<i>Streptomyces olivochromogenes</i> Bs14
LA – HS / OP – OB	<i>Bacillus drentensis</i> Bs28
	<i>Streptomyces sioyaensis</i> Bs9
LA – HS / OP – B	<i>Bacillus drentensis</i> Rh25, Rh28
LA – HS / P – OB	<i>Bacillus</i> sp. Rh23, Rh35
	<i>Bacillus</i> sp. Bs2
LA – HS / P – B	<i>Bacillus drentensis</i> Rh39
	<i>Bacillus</i> sp. Rh24
LA – MS / P – OB	<i>Paenibacillus</i> sp. Rh34
LA – MS / P – B	<i>Paenibacillus</i> sp. Rh2
MA – HS / P – B	<i>Bacillus</i> sp. Rh1
MA – MS / P – OB	<i>Bacillus</i> sp. Rh33

We then compared the intensities of these inhibitions to assess the effectiveness of the antimicrobial activities. Most bacteria showed weak inhibition when co-inoculated, and when bacteria were inoculated with delay most showed intermediate and strong inhibitory interactions. From a total of 135 negative interactions for both approaches, 66 consisted of weak inhibition reducing the streak size at most by 20%, 52 had an intermediate inhibitory effect reducing by 20 to 40% of the streak size and only 17 consisted of strong inhibitory effect reducing the streak by more than 60%. Our data revealed that aggressive bacterial isolates (i.e. *Pseudomonas*, *B. subtilis* and *Streptomyces*) had also a strong inhibitory effect (reduction of > 60% of the streak size) towards 1 or 2 bacterial members (*Streptomyces* and *Bacillus*) (Figure 21, Table 7). In addition, *Pseudomonas* sp. Bs17 inhibitory effect against *S. sioyaensis* Bs9 was the strongest inhibitory interaction (Figure 21, Table 7), but this occurred in the co-inoculation approach.

Since *Pseudomonas* sp. Bs17 had the widest and strongest inhibitory effect towards bulk soil isolates, we wondered if this inhibition was due to volatile compounds. For this reason, we performed the same bacterial interactions assay, but in a split petri dish avoiding the possible contact of diffusible compounds through the agar. After 5 days of incubation, none of the bulk soil isolates was affected by *Pseudomonas* Bs17, excluding the effect of volatile signals.

### **2.2.4.3 pH alteration is involved in rescuing the growth of some bacterial strains**

Finally, we wondered if pH could be responsible for the rescue of growth provided by *Bacillus* sp. Bs2 to *B. lentus* Bs6 and by some Bacilli strains and by *Paenibacillus* sp. Rh34 to *Staphylococcus* sp. Rh9., Indeed, pH is an important driver for bacterial growth and survival in soil (Fierer *et al.* 2017). To test this hypothesis, we first cultivated on mMS pH 6.5, 7.0 and 7.5 bacteria that could not grow on mMS pH 5.8. *B. lentus* Bs6 and *Sporosarcina* sp. Bs29 were able to grow successfully at pH 6.5 and higher, while *Staphylococcus* Bs42 and Rh9 strains could not grow at any of these higher pH on this medium (Supplemental Table 1, Annexes Chapter II). Next, we questioned if the six helper bacteria were able to change environmental pH. *Bacillus* sp. Bs2 was able to increase the pH of the medium, suggesting that rescue of growth of *B. lentus* Bs6 could be due to pH conditions (Supplemental Table 1, Supplemental Figure 4, Annexes Chapter II). Yet, it is possible that pH increment was not sufficient to rescue *Sporosarcina* or that other complex metabolic processes were involved in this rescue. The rhizosphere strains that rescued the growth of *Staphylococcus* sp. Rh9 showed various behaviors: some reduced the pH (e.g. *Paenibacillus* sp. Rh34) while other slightly increased it (e.g. *Bacillus* sp. Rh23) (Supplemental Table 1, Annexes Chapter II). Altogether with the fact that pH increase did not permit to rescue the growth of *Staphylococcus* sp. Rh9 on mMS, it is very likely that pH alteration was not involved in the rescue of growth of *Staphylococcus* sp. Rh9 by *Bacillus* strains and *Paenibacillus* sp. Rh34. Lastly, we wondered if the ability to change environmental pH was a widespread phenomenon among our soil bacterial isolates. Out of the 38 strains that we isolated from bulk and rhizospheric grains of soil and that were able to grow on mMS, most bacteria (66%) tended to acidify the surrounding environment (<5.7) (Supplemental Table 1, Annexes Chapter II). These bacteria included *B. subtilis*, *B. drentensis*, some *Bacillus* sp., *Pseudomonas*, *Streptomyces* and *Paenibacillus* strains. Only 13% of the strains, all belonging to the *Bacillus* sp. genus increased the pH (>5.9) of their surrounding environment when grown in mMS, suggesting that this “alkalization” behavior is restricted to a subgroup of *Bacillus* sp. strains.

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**Table 7.** Levels of aggressiveness of the most active bacterial strains isolated from bulk and rhizosphere soils. Bacterial strains were classified according to their abilities to inhibit the development of other strains co-inoculated simultaneously or with a 48hrs delay. Bacterial strains isolated from bulk soil and rhizospheric are indicated by the Bs and Rh letters, respectively.

Inoculation	Origin	Bacterial strains	Total number of bacteria affected (%)	Weak inhibitory effect (<20%)	Intermediate inhibitory effect (20-40%)	Strong inhibitory effect (60-80%)	Very strong inhibitory effect (>80%)
Simultaneous	Bulk soil	<i>B. subtilis</i> B34	85.7	57.1	14.3	14.3	0
		<i>B. subtilis</i> B36	85.7	57.1	14.3	14.3	0
		<i>S. olivochromogenes</i> B14	71.4	42.9	28.6	0	0
		<i>Pseudomonas</i> sp. B17	71.4	14.3	28.6	14.3	14.3
		<i>S. sioyaensis</i> B9	14.3	14.3	0	0	0
	Rhizosphere	<i>S. sioyaensis</i> R27	71.4	64	7.1	0	0
		<i>S. sioyaensis</i> R21	64.3	42.9	21.4	0	0
		<i>S. olivochromogenes</i> R4	64.3	50	14.3	0	0
		<i>S. sioyaensis</i> R15	57.1	50	7.1	0	0
		<i>Bacillus</i> sp. R24	7.1	7.1	0	0	0
Delayed	Bulk soil	<i>S. olivochromogenes</i> B14	85.7	14.3	57.1	14.3	0
		<i>Pseudomonas</i> sp. B17	71.4	0	71.4	0	0
		<i>B. subtilis</i> B34	57.1	14.3	14.3	28.6	0
		<i>B. subtilis</i> B36	57.1	14.3	14.3	28.6	0
		<i>B. drentensis</i> B28	14.3	14.3	0	0	0
		<i>S. sioyaensis</i> B9	14.3	14.3	0	0	0
	Rhizosphere	<i>S. olivochromogenes</i> R4	100.0	42.9	50	7.1	0
		<i>S. sioyaensis</i> R15	92.9	14.3	71.4	7.1	0
		<i>S. sioyaensis</i> R21	92.9	50.0	28.6	14.3	0
		<i>S. sioyaensis</i> R27	92.9	14.3	50.0	28.6	0
		<i>Bacillus</i> sp. R1	6.7	6.7	0	0	0

## Discussion

A cultured dependent approach has been used to determine the functional diversity of soil bacterial cultivable species sharing the same ecological niche. Although this approach gave access to a limited portion of the total community, it provides essential information on their potential abilities. Therefore, functional abilities and biotic interactions captured in this work are representative of a small group of soil bacterial members with a particular lifestyle from single grains. Despite the low taxonomic diversity of the strains isolated (Chapter I), analyses of functional potentials revealed an important diversity of behaviors among the strains. This is in accordance with previous studies where bacteria have identical 16S rDNA-coding gene sequences but still showed important variations in their phenotypic traits (Vetsigian *et al.* 2011, Antony-Babu *et al.* 2017, Vicente *et al.* 2018). Additionally, a recent study based on genome comparison of a clonal population of *Streptomyces* in forest soils aggregates showed a high diversity in terms of accessory genome including biosynthetic gene clusters (Tidjani *et al.* 2019). A high flux of genetic information promotes variability of the phenotypic traits allowing niche adaption (Tidjani *et al.* 2019). If we assume gene transfer, mutations and homologous recombination by other members of the bacterial community at the microscale, we could hypothesize that soil grains are hot spots for genetic information exchange in order to adapt to ecological pressures. Such interactions are suggested to occur in soil aggregates, where space restriction brings bacteria into close proximity and specially gene transfer is promoted (Vos *et al.* 2013).

Moreover, we found a wide functional diversity where levels of activity were highly variable as well. Among all the activities tested, bacterial isolates from grains of soil had a high potential to uptake iron, produce proteolytic exoenzymes and solubilize phosphate through acid phosphatases. While the ability to produce acid phosphatases and proteases is commonly shared by soil bacteria in forest soils (Richardson *et al.* 2009; Uroz *et al.* 2013A), iron mobilization has been shown to be restricted to a subset of strains in soil (Colin *et al.* 2017; Nicolitch *et al.* 2016). This may reflect an iron deficiency at the microscale of our sample leading to the selection of strains able to take up iron through siderophores. Since iron is essential for all life forms, microorganisms have evolved efficient uptake mechanisms to obtain this element (Andrews *et al.* 2003). Thus, alternatives to siderophores may involve xenosiderophores, ferric citrate, heme, and iron permease, that are not captured by the CAS assay (Materials and Methods) (Cornelis and Andrews 2010). The least substrate used was inorganic tri-calcium-phosphate (TCP). Active strains able to solubilize TCP (*Burkholderia* and *Pseudomonas*) have been found in previous studies (Colin *et al.* 2017). Based on the high production of acid phosphatases, we could hypothesize that these strains are adapted to obtain phosphorus by mineralization of organic phosphate rather than solubilize it from inorganic metal complexes (Uroz *et*

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*al.* 2013A). Likewise, enzymatic contribution of the non-cultivable bacteria and other soil microorganisms must be considered for the general overview.

While these functional traits tested are relevant for bacterial nutrient acquisition, they also contribute as public goods to plant growth and development (e.g. P solubilization, production of hormones). In recent years, one of the most studied traits is the production of a plant hormone: auxin (Spaepen *et al.* 2007). Salkowski reagent detects a broad range of indole related compounds (e.g. Indole-3-Acetic-Acid (IAA), Indole-3-Pyruvic-Acid (IPyA) and Indole-3-acetamine (IAM)), that bacteria can produce through tryptophan (Trp) as a precursor (Glickmann and Dessaux 1995). Indole producers found in this study (*Pseudomonas*, *Paenibacillus*, *Psychrobacillus*, *Streptomyces*, *Sporosarcina*) are consistent with other reports (Malik and Sindhu 2011; Grady *et al.* 2016; Fonseca Garcia *et al.* 2016; Viaene *et al.* 2016). The low frequency of bacteria with the ability to synthesize indole compounds found in this study was unexpected since many bacteria have this ability thanks to the broad distribution of biosynthetic genes involved in these pathways, from phytopathogenic to plant growth promoting bacteria (Spaepen *et al.* 2007). Indeed, a study retrieved 7282 complete or draft genomes from soil and root environments, where 82.2% of analyzed bacterial genomes were potentially capable to synthesize Trp-dependent IAA and this biosynthetic pathway was widely distributed among different bacterial phyla (Zhang *et al.* 2019). The impact of indole compounds is dose-dependent ranging from positive to negative effects and the outcome is highly dependent on the amount and the sensitivity of the plant tissue (Spaepen *et al.* 2007). For example, low doses of IAA production by bacteria may act as a signal molecule increasing root biomass (Spaepen and Vanderleyden 2011), root elongation (Glick *et al.* 1998) and root hair development (Timm *et al.* 2016), while high doses may enlarge cells and induced tumors (e.g. *Agrobacterium*: Link and Eggers, 1941).

In this study, bacteria producing indole related compounds did not significantly improved nor deteriorate poplar development and changes in root architecture were irrespective of the presence of this trait. Several hypotheses can be envisioned: first since mMS agar did not contained tryptophan, we could expect bacteria lack the tryptophan biosynthetic pathway or bacteria that do have these metabolic systems could not produce it on mMS conditions. Although, there are bacteria that comprise a Trp-independent IAA biosynthetic pathway, these are still underexplored (Zhang *et al.* 2019). Second hypothesis is that the production of these indole related compounds is not constitutive in bacteria and need the presence of the plant to be induced and synthesized. Previous studies have found tryptophan in the root exudates (Kravchenko *et al.* 2004; Persello-Cartieaux *et al.* 2003), making it possible to induce bacterial auxin. However, we must consider concentration and stability of the molecule in an *in vitro* setting can range from beneficial to deleterious since it is a dose-dependent molecule (Persello-Cartieaux *et al.* 2003). It would be necessary to test for bacteria and



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Poplar - bacteria interaction if auxin can be detected in the medium and its concentration and likewise to evaluate the interaction on mMMS agar supplemented with tryptophan. A third hypothesis is that auxin effect is influenced by the interaction with other hormones (Fukaki and Tasaka 2009; Persello-Cartieaux *et al.* 2003). For instance, exogenous auxin treatment on *Arabidopsis* has been showed to stimulate lateral root formation (Felten *et al.* 2010). However, only IAA effect was not comparable to an ECM fungal treatment known to produce IAA, suggesting a synergistic effect between several hormonal signals (Frey *et al.* 2003, Felten *et al.* 2009). Indeed, Spivallo and collaborators could mimic *Arabidopsis* root morphology effect from black truffle by applying a mixture of ethylene and IAA (Spivallo *et al.* 2009). While most bacteria had no significant influence in poplar growth, we could wonder if bacteria isolated from *Quercus robur* can interact with *Populus*. Although *Quercus* and *Populus* are woody plants that live in temperate forest ecosystems (Müller *et al.* 2013), associations are influenced by complex factors and host specificity may be involved. Lastly, no visible phenotype does not necessarily indicate an absence of gene and protein expression response in plants (Cheng *et al.* 2010). It would be necessary to further study these associations by transcriptomics analysis.

Furthermore, poplar response to bacterial interactions might be underestimated due to limitations of the experimental procedure and inability to reproduce same phenotypes. Indeed, root formation is environmentally, physiologically, and hormonally regulated (Fukaki and Tasaka 2009) affecting the reproducibility and consistency of these experimental results. Although we did not take into account base and top cuttings, it has been described that cuttings coming from the base produced longer and heavier roots than cuttings from the top, increasing the deviation between replicates (Desrochers and Thomas, 2003). In addition, it has been reported regeneration of poplar varies seasonally due to carbohydrates and hormonal content imbalances (Frey *et al.* 2003), limiting the comparison between experiments. Moreover, age of the stock plant affects the next plantlet rooting ability, since older plants accumulate less energy (Zhao *et al.* 2014). Despite the difficulties to find bacterial behavior trends in poplar, among bacteria that had a positive effect in poplar's growth, *Burkholderia* is pertained in literature (Timm *et al.* 2016). Although very few plant diseases are caused by *Streptomyces* (Viaene *et al.* 2016), in this study we found *Streptomyces sioyaensis* strains causing necrotic effect in poplar roots which has not been reported for the moment, to the best of my knowledge.

In a next step, we wonder about the ability of bacteria to communicate through quorum sensing signals. Our ability to detect them depend on the type of molecule, the size, the composition and structure of the molecule and the biosensor used. It is now well known there are specific quorum sensing circuits for Gram negative (e.g. LuxR/I-type system with acyl homoserine lactone as signaling

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molecule (AHLs)), for Gram positive (e.g. peptides and butyrolactones) and for both (e.g. luxS/AI-2) (Uroz *et al.* 2009A). Although most bacterial isolates in this study are Gram positive, we decided to evaluate the presence/absence of AHL class since it is the most common and extensively studied molecule (Venturi 2005; Uroz *et al.* 2009A) and it has been previously detected in the mycorrhizosphere of forest soils (Uroz *et al.*, personal communication). Based on *C. violaceum* tests, none of the Gram negative isolates (*Pseudomonas* and *Burkholderia*) showed the production of AHLs molecules with *N*-acyl chain of C<sub>4</sub>-C<sub>8</sub> length. Since QS systems have been identified in these strains previously (Venturi 2005, Elasri *et al.* 2001; Uroz *et al.* 2009A; Eberl 2006), it is likely these isolates communicate through other molecules, a combination of molecules, or AHL molecules with longer acyl chains that inhibit violacein production (Uroz *et al.* 2009A). For instance, several studies have reported a broad heterogeneity of AHL QS systems in soil *Pseudomonas* (Venturi 2005; Elasri *et al.* 2001). Furthermore, a mass spectrometry analysis has revealed the production of C<sub>12</sub>-HSL by *Pseudomonas frederiksbergensis* from a tropical forest (Chong *et al.* 2012). Similarly, *Burkholderia* strains have been found to produce multiple species of AHL molecules (Eberl 2006; Yao *et al.* 2006). Additionally, a study reported that *Burkholderia* strains produced insufficient amounts of AHL molecules to be detected by *C. violaceum* (Yao *et al.* 2006), suggesting that isolates from this study may actually use AHLs molecules but could not be detected by this assay.

The ability of bacteria to prevent others to communicate serves competitors as an antimicrobial strategy. In this study, *Bacillus mycoides*, *Burkholderia* sp. and *Streptomyces olivochromogenes* strains could prevent the action of C<sub>6</sub>-HSL molecule. Several studies have already reported *Bacillus* strains to be able to degrade the lactone ring from AHLs (Dong *et al.* 2000, Dong *et al.* 2002) and also to oxidate AHLs (Chowdhary *et al.* 2007) eliminating their quorum sensing role. Another strategy to prevent quorum sensing, which has been reported previously by *Burkholderia* strains, is the production of bigger AHLs molecules (Eberl 2006). These AHLs analogues with longer acyl chain compete for binding to the LuxR-like receptor preventing QS to occur (Uroz *et al.* 2009A). While several studies have indicated the AHL-degrading activity in soil bacteria from Firmicutes, Proteobacteria and Actinobacteria (Dong *et al.* 2000, Dong *et al.* 2002, D'Angelo-Picard *et al.* 2005, Uroz *et al.* 2009A), the number of studies of such activity in *Streptomyces* is still very low (Park *et al.* 2005, Chankhamhaengdech et al. 2013). One study reported an AHL-acylase degrading activity from an endophytic *Streptomyces* sp. with a broad spectrum for C<sub>6</sub>-C<sub>12</sub>-HSL (Chankhamhaengdech et al. 2013). Another study identified an extracellular AHL-acylase from a soil *Streptomyces* sp. (Park *et al.* 2005). Interestingly, genome analysis (RAST automatic annotation) of *Streptomyces olivochromogenes* predicted the presence of a putative penicillin acylase that could be involved in the degradation of *N*-acyl-homoserine lactones (Park *et al.* 2005).

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An alternative for bacteria to compete is the production of antimicrobial compounds against other microorganisms. Because soil bacteria are well known to be good producers of growth inhibitory secondary metabolites for survival purposes (Hibbing *et al.* 2010; Tyc *et al.* 2014, 2017a), many studies focus on the discovery of novel antibacterial compounds for medical purposes (Tyc *et al.* 2014, 2017a). In our study, we found no inhibition against *E. coli*, and few inhibitory activities against *M. luteus* (15%) and *B. subtilis* (3%). Contrary to our study, Tyc and collaborators found soil bacterial communities that can inhibit *E. coli* (Tyc *et al.* 2014; 2017a), although this antimicrobial activity was enhanced when induced by co-culturing bacterial pairs involving beta-Proteobacteria, Actinobacteria and Bacilli (Tyc *et al.* 2014).

Such proposed induced inhibitory effect (Tyc *et al.* 2014) leads to question about the importance of bacterial composition during interactions and their effect in natural communities' structure. Previous reports have remarked antagonism as the major mode of interaction among soil bacteria (Hibbing *et al.* 2010; Foster and Bell 2012; Cornforth and Foster 2013; Pérez-Gutiérrez *et al.* 2013; Tyc *et al.* 2014). Thus, in an effort to understand how bacteria are influenced by the inoculation of a different strain from a common origin, we selected at least one bacterial strain per taxonomic group and functional profile. Ten bulk soil isolates and fifteen rhizospheric isolates were chosen to interact in pairwise combinations including self-interactions. Here, we found that between 60-75% of pair combinations induced no visible effect (Figures 21 and 22). This phenotype can be regarded as neutral (where there were no antagonistic compounds produced and therefore bacteria did not affect each other) or as resistant (where a bacterium is able to grow regardless the presence of antagonistic compounds). In our experimental approach, we cannot exclude the presence of an undetectable response (e.g. such as a metabolomic response (Traxler *et al.* 2013)). In this sense, Perez-Gutierrez and collaborators observed that 90% of *Bacillus* interactions were resistant to others (Perez-Gutierrez *et al.* 2013) and Davelos and collaborators found 67% of interactions between Streptomycetes were not leading to any inhibition (Davelos *et al.* 2004). While these preliminary results together with previous findings obtained by other groups, suggest that antagonistic and cooperative interactions may not dominate among microorganisms in soils (Davelos *et al.* 2004; Tyc *et al.* 2014). Nevertheless, bacterial interactions within the community in complex and heterogeneous conditions of soil may differ from experimental conditions.

In addition, in our study, the strength of inhibitions was weak or intermediate in majority, and only one case of very strong antagonism was observed (*Pseudomonas* sp. Bs17 against *S. siyoensis* Bs9). Low frequency of high intensity antagonism has been also reported previously in *Vibrio* interactions (Cordero *et al.* 2012). According to previous studies, this low frequency suggests that antibiotics functions are not necessarily bacterial weapons, but instead, that they would be public goods to

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control population dynamics in order to maintain the community structure (Celiker and Gore 2012; Cordero *et al.* 2012) and ecological stability (Coyte *et al.* 2015; Ghoul and Mitri 2016). Dantas and collaborators have even found a wide variety of bacteria able to subsist on antibiotics as the only carbon source (Dantas *et al.* 2008). Further investigations will be necessary to test whether those hypotheses apply to our system.

Although many mechanisms of mutualistic or cooperative interactions between bacteria have been described up to now (D'Onofrio *et al.* 2010; Ren *et al.* 2015, Herschend *et al.* 2018), the proportion of beneficial interactions among other types of interactions occurring at the microscale is poorly documented. In this study, 9% of interactions ended up in promotion of bacterial growth and those only occurred in delayed co-inoculation. By comparison, Vetsigian and collaborators reported a rate of 19% of promoting interactions among *Streptomyces* strains from grains of soils (Vetsigian *et al.* 2011). Altogether these data tend to support the hypothesis that cooperation behavior would be reduced in soil microhabitats compared to other interactions. In the present case, promotion behaviors occurred between strains of the same order (*Bacillus*, *Paenibacillus* and *Staphylococcus*) but not across Phyla. In accordance with other studies incriminating pH as an important regulator of bacterial interactions (Ratzke and Gore 2018), preliminary data suggest that the increase of pH could be responsible for some but not all of these beneficial interactions (Supplemental Table 1, Supplemental Figure 4, Annexes Chapter II). Changes in pH within the local environment has been described for oral (Burne and Marquis, 2000) and vaginal microbiomes (Ravel and Brotman, 2016) and in *in vitro* settings (Ratzke and Gore, 2008 Herschend *et al.* 2018). Thus, bacteria affecting each other by altering the pH of environment through their metabolism is not surprising. However, in this study the ability to increase the pH of the medium was restricted to a subgroup of *Bacillus* strains. Since changes of pH is via consumption and excretion of modified resources, secretion of metabolites, and other metabolic activities (Herschend *et al.* 2018), it may be influenced to culture conditions and medium composition (Ratzke and Gore, 2008). In addition, other mechanisms besides pH could also be in play for the observed behavior. Whether this type of interaction is relevant in natural communities needs to be further established.

As previously noticed in other studies, most of the interactions recorded were antagonistic (Davelos *et al.* 2004; Vetsigian *et al.* 2011; Foster and Bell 2012). Interestingly, highly antagonistic strains tended to be also highly resistant while susceptible strains were in general not antagonist. In this regard, *Pseudomonas* sp. Bs17 strain was the most efficient antagonist, as it inhibited the growth of all bulk soil members with a variety of intensities and did not suffer from any reciprocal inhibitory activity. This is not unexpected since the *Pseudomonas* genus regroups bacteria that are generally highly competitive for nutrients (Gross and Loper 2009; Galet *et al.* 2015; Deveau *et al.* 2016), have

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the ability to produce a wide range of antibacterial compounds (Galet *et al.* 2014; Hass *et al.* 2005; Schwechheimer and Kuehn 2015), prevent antibiotic production from other bacteria (Galet *et al.* 2014), while also being able to resist to antibiotics thanks to multidrug efflux pumps (Kim *et al.* 2015). *Streptomyces* are also famous for their ability to produce a wide range of antibiotics and this genus is therefore considered as highly bioactive and competitive to maintain a dynamic population (Vetsigian *et al.* 2011). In agreement, we observed that antagonism among *Streptomyces* strains was maintained over both inoculation approaches, suggesting the existence of a constitutive inhibitory activity. However, we noticed variations in the frequency and the intensity of the inhibitory activity among strains, suggesting potential differences in mechanisms of antagonisms (e.g. regulation pathways, stimuli for induction) (Davelos *et al.* 2004; Pérez-Gutiérrez *et al.* 2013; Traxler *et al.* 2013; Kinkel *et al.* 2014). Important variations in the activities of *Bacillus* strains were also visible. While *B. subtilis* strains strongly antagonized other *Bacillus* strains as well as *Streptomyces*, most *Bacillus* sp. strains were weakly aggressive and even rather stimulated the development of other strains (Figures 21 and 22). Such results corroborate with the hypothesis of Perez-Gutierrez and collaborators who proposed that interference between *Bacillus* is linked to genetic distance and resource use (Perez-Gutierrez *et al.* 2012).

### **Conclusions and Perspectives**

To summarize, at a microscale, all bacterial strains had at least one potential degradation activity, and among the active ones most of them had relative low levels of activity in the conditions tested. Moreover, no correlation was found between microbial functions and taxonomy or origin. As microbial functions appeared to be diverse at the microscale, it would be useful to have more replicates and obtain a more diverse cultivable bacterial library that could potentially explain more about soil functions at this scale. In addition, Gram negative bacteria from these grains were likely to communicate with molecules different from C<sub>4</sub>-C<sub>8</sub>-HSLs. Moreover, few bacteria from these grains could inhibit C<sub>6</sub>-HSL. A better understanding of how bacteria communicate would explain better bacterial dynamics, therefore, it would be useful to test the presence of other signal molecules with several biosensors, and their respective disruption mechanism.

Among sympatric interactions, few bacteria interacted, and most interactions were inhibitory. These inhibitions were mostly weak when co-inoculated, and intermediate when inoculated with delayed. Only cooperative interactions occurred in delayed inoculation and some were likely to be influenced by pH. In an effort to extrapolate these results into natural soil communities, it would be useful to localize and characterize living bacteria within the grain and have mimicking soil medium setup.

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In addition, our study indicated that few bacteria were able to produce auxin, and these strains were not involved in any significant alteration of root development. It would be necessary to test if adding exogenous tryptophan aids bacteria to produce auxin. Furthermore, phytohormones like gibberellin, abscisic acid, cytokinin and ethylene, are key players in the signaling networks involved in plant immunity and growth. Besides, their interaction also interferes in plant development (Fukaki and Tasaka, 2009). Hence, it would be useful to verify if bacteria can synthesize other phytohormones and evaluate their effect on poplar root development in single and in combination.

Although few bacteria could induce root growth and none had a significant effect in the whole growth of poplar, we observed an alteration of the root architecture. This small number of interactions may be related to the plant host origin. To validate this hypothesis, we could test bacterial interactions with *Quercus in vitro*. Although micropropagation of oak has been a challenge because of genetic heterogeneity in seed populations leading to highly different shoot culture and rooting systems, extensive studies have improved these experimental constraints (Hermann *et al.* 1998, Hermann and Buscot 2007). In addition, the microscale used may be appropriate to study cell-to-cell interactions, but it might not be appropriate for plant interactions by just sampling few grains. Yang and Crowley reported different bacterial communities associated with the rhizosphere from different root zones (primary root tip, non elongating secondary root tips, lateral root emergence, older roots) suggesting root exudate levels change from root location (Yang and Crowley, 2000). More evidence needs to be collected in this regard. Lastly, our study found one *Streptomyces species* able to significantly stop root growth and to induce necrosis in roots. More evidence needs to be collected regarding the molecular mechanisms of this antagonistic strain.

# **Annexes**

## **Chapter II**

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## **Insights in bacterial interactions isolated from forest soil microhabitats**

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### **Abstract**

Soil is a complex environment made of multiple microhabitats in which a wide variety of microorganisms co-exist and interact to form dynamic communities. While the abiotic factors that regulate the structure and the activities of these communities are now quite well documented, our knowledge of how bacteria interact together within these communities at a microscale is still deficient, partly because tracking microorganisms, their activities and interactions in situ at the scale of microhabitats is highly challenging in soils. To start filling this gap, we isolated bacterial strains from grains of soil assuming these bacteria would be likely interacting in their natural habitat. We identified strains at the taxonomic level, analyzed their functional potential abilities, as well as the outputs of their interactions in an in vitro set up. We compared the effects of simultaneous versus delayed co-inoculations, allowing or not one strain to modify first its environment. The majority of cultivable bacteria engaged in a few number of interactions with other bacteria isolated from the same microhabitat. A few bacteria were highly aggressive and most inhibitory activities were of low intensity. The proportion of beneficial interactions was also reduced and these beneficial interactions, including growth rescue, occurred only during delayed co-inoculations. Altogether, our data suggest that a few strains may regulate the activities of the full community.

**Keywords:** bacterial interactions, microhabitat, grain of soil, antagonism, beneficial interactions, forest soil



## **Introduction**

Microbial communities are key components of forest soil ecosystems since they perform critical functions such as nutrient cycling, organic matter decomposition and mineral weathering [75,45]. Microbial community structure in forest soils is strongly influenced by abiotic parameters (e.g. temperature, pH, precipitation) [86,46]. For example, soil pH is considered the main abiotic factor that drives soil bacterial communities' changes [43,32]. Biotic interactions are also important drivers of microbial community structure and composition. Indeed, microbes are involved in complex intra- and interkingdom interactions with bacteria, fungi, protists, vegetation, among others [17,19].

At a microscale, soil particles form microhabitats that provide unique environmental conditions and that are colonized by unique microbial communities [62]. In these microhabitats, inter-cell distances get shorten, allowing microbes to meet other microbial cells and interact physically or through the diffusion of active compounds [59]. A recent study showed that bacteria interact with around 11 ( $\pm 4$ ) other species for low bacterial density samples and 97 ( $\pm 24$ ) species in high bacterial density [53]. To do so, bacteria have developed a wide array of strategies, including antibiotic production, nutrient scavenging, quorum sensing and signal disruption [71,27,49,18]. Importantly, these different strategies are often specifically deployed in response to specific interactions [70] and are more likely to occur between closely related species that share same resource requirements [55,65]. These metabolic dialogues stimulate strong population dynamics among bacteria [79]. By modifying bacterial growth, motility, gene expression, sporulation and communication, the constitutive and induced specialized metabolites produced during these interactions can thus influence the composition and dynamics of microbial communities in soil [72,33]. Although there are several numbers of studies on microbial interactions [15,55,65,79], bacterial interactions at the microscale and their impact on local community structure are still poorly understood. For instance, the relative contributions of cooperation and competition in the interactions between strains is debated and strongly varies between studies. Knowing when, where and which soil bacterial interactions occur is important to predict in situ traits of bacteria and their potential influences within soil microbiome [50]. In addition, most studies performed so far on bacteria-bacteria interactions focused on model strains or strains that were not isolated from the same habitats [65,79], limiting our ability to infer whether the interactions identified truly occur in nature. Moreover, libraries of bacterial strains isolated from soil are generally made from large samples of soils to cover as best as possible the natural diversity of strains. As soils are made of multiple microhabitats that are not necessarily interconnected [62], this technique likely leads to the creation of libraries of strains that may never have encountered each other and are thus not good targets to analyze microbial interactions. Instead, we hypothesized that the isolation of strains from grains of soils would allow capturing bacteria that naturally interact together and permitting to evaluate their behaviors towards their relatives. Our aims were to (i) identify the bacterial taxonomic and functional diversity of individual grains of soil, (ii) to analyze the ability of bacteria, living in the same grain of soil, to interact, (iii) to determine the frequencies and intensities of the interactions between

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strains and (iv) to compare potential colonization strategies. We chose to work with bacteria coming from two different microhabitats, bulk soil and rhizosphere, in order to increase the amount of possible interactions. To do this, we first evaluated the potential metabolic functions important for the survival in forest soil conditions, and then, we tested interactions through two complementary approaches: one in which two bacteria would colonize at the same time a habitat (co-inoculation) and one where a second bacteria would reach the habitat after a first one has already been established (delayed inoculation). We found that most bacteria engaged in a small number of interactions and that few bacteria were highly aggressive.

### **Materials and methods**

#### *Site description and collection of grains of soil*

Soil samples were collected in a mixed forest with mainly oaks (*Quercus robur*) and beeches (*Fagus sylvatica*) in Champenoux, France in October 2016 (GPS coordinates Lambert93: dir N 6852020.9 dir E 945689.4 280). Litter was removed next to the roots of an oak; a square of 8 cms<sup>3</sup> of topsoil was extracted with a shovel and wrapped in sterile bag and then taken to the laboratory in an icebox. Samples were collected from two soil compartments: bulk soil (5 cms away from any root) and rhizosphere (soil adherent to the root). Grains of soil (approx. 5 mg) were collected under a binocular using a sterile clamp.

#### *Collection of bacterial strains from bulk soil and rhizosphere compartments*

Each grain of soil was resuspended in 0.9% NaCl and mixed with a vortex. Each solution was divided in two subsamples that were treated independently. First subsamples were used to select specifically for sporulating actinomycetes, since they are important active members of soil and rhizosphere communities [80] and only spores can be kept over long period of time in laboratory collections. To do so, a heat treatment (50°C for 1 hour) was performed as described [5] and then serial dilutions were inoculated on SIM agar (1 g L<sup>-1</sup> starch, 0.4 g L<sup>-1</sup> casein, 0.1 g L<sup>-1</sup> CaCO<sub>3</sub>, 0.25 g L<sup>-1</sup> KNO<sub>3</sub>, 0.1 g L<sup>-1</sup> MgSO<sub>4</sub>, 0.2 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 20 g L<sup>-1</sup> agar pH 7.0). Only sporulating *Streptomyces*-like strains were selected from SIM plates and spread on soy flour medium (SFM) (20 g L<sup>-1</sup> soy flour, 20 g L<sup>-1</sup> mannitol and 20 g L<sup>-1</sup> agar, pH 6.6) and grown for 7 days at 25°C [37] before collecting the lawn of spores using sterile water. The spore solution was vortexed, filtered using cotton, centrifuged (2400 g, 10 min, 4°C) resuspended in 20% glycerol, and stored at -80°C.

Second subsamples were used to isolate a broad range of strains using 1/10 strength tryptic soy agar (TSA) as a non-selective medium with low level of nutrients [75] (3 g L<sup>-1</sup> tryptic soy broth from Difco and 15 g L<sup>-1</sup> of agar pH 7.3). Plating on 1/10 TSA was made without pre-treatment. All agar plates were incubated at 25°C for 10 days. One colony per morphotype was picked on Petri dish plates to obtain the broadest diversity of strains. Initial plates were further incubated for 8 additional days to recover slow growers. Each isolate from 1/10 TSA plate was re-streaked on 1/10 TSA 3 times for purification purposes. All the bacterial strains were cryopreserved at -80 °C in 20% glycerol.

#### *Molecular identification of the tree root*

To identify the species to which belonged the root from which was isolated the rhizospheric grain of soil, DNA extraction from the root was performed using REExtract-N-Amp Plant PCR Kit using manufacturer

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recommendations (Sigma-Aldrich/Merck). The Internal Transcribed Spacer 2 (ITS) was amplified by PCR with the primers ITS3 (5'- GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [83,85]. Band with a size corresponding to the expected size of plant ITS2 (approx. 400 bp) was extracted from 1% agarose electrophoresis gel, purified with QIAquick PCR purification kit according to manufacturer recommendation (Qiagen) and sequenced using Sanger sequencing method (Eurofins, Germany). Taxonomy affiliation was determined by BLASTn on All plant EST database [3].

### *Molecular identification of bacterial isolates and phylogenetic analysis*

For the non-actinomycete bacteria, DNA extraction was performed using Wizard genomic DNA purification kit using manufacturer recommendations (Promega, France). The 16S rRNA gene sequence of these bacterial strains was amplified by PCR with the primers 16S\_fD1 (5'- AGAGTTTGATCCTGGCTCAG-3') and 16S\_rP2 (5'- AAGGAGGTGATCCAGCC-3') [82]. For *Streptomyces*-like isolates, 16S rRNA gene PCR amplification was done using 100% DMSO-cell suspension mix [77] and the StrepB forward (5'- ACAAGCCCTGAAACGGGGT-3') and StrepF reverse (5'- ACGTGTGCAGCCCAAGACA-3') primers as described [63]. The PCR products were then sequenced by Sanger sequencing method (Eurofins, Germany). Forward and reverse sequences were assembled using CLC Main Workbench (CLC bio) and taxonomical affiliations of bacterial isolates were determined using Ribosomal Database Project classifier [81]. MEGA7 software was used to aligned nucleotides using the MUSCLE algorithm and to build a phylogenetic tree based on neighbor-joining method, with Kimura's 2 parameter distance correction with 100 bootstrap correction [39,41]. All sequences are available on Genbank database under the accession numbers MK734019 to MK734040 (bulk soil strains) and MK801941-MK801965 (rhizosphere strains).

### *Composition of bacterial communities in grains of soil by high throughput sequencing*

Illumina Miseq sequencing of soil 16S rRNA was used to characterize the initial composition of the bacterial communities of each grain of soil. DNA was extracted using FastDNA Spin Kit for soil (MP Biomedicals). In order to obtain DNA from vegetative cells and spores, extraction protocol was modified to include three sequential bead-beating steps as described [84]. The V4 hypervariable region of the 16S SSU rRNA was amplified in triplicate using specific forward 515F (5'-GTGCCAGCMGCCGCGTAA-3') and reverse 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers as described [66,69]. PCR product size (expected size of around 291 bp) and concentration were verified using an agarose gel electrophoresis (1%) and then pooled. PCR products without addition of microbial DNA (negative control), mock communities of known bacterial compositions were added as quality controls. Samples of 50  $\mu$ L (30 ng DNA per  $\mu$ L) were sent for tagging and MiSeq Illumina Next Generation Sequencing (GeT PlaGe INRA sequencing platform, Toulouse, France). Resulting bacterial sequences were further processed with FROGS (Find Rapidly OTU with Galaxy Solution) [21] based on Galaxy metagenomic analysis platform [1] as described [66].

### *Preparation of bacterial inocula for functional characterization and bacterial interactions assays*

Non-actinobacteria were grown from glycerol stock on 1/10 TSA plates at 25°C for 48 h. Then 2-3 bacterial colonies were picked, suspended in 10 mL of tryptic soy broth (TSB) and grown at 25°C under agitation of 200 rpm for 48 h. The bacterial cultures were spin down and the pellets were washed with 1X phosphate buffered

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saline (PBS) 3 times and resuspended in 1X PBS to obtain an estimated suspension of  $A_{600\text{nm}} = 0.6$ . For actinobacteria, the spore solution was diluted to reach an approximate of  $10^8$  cfu/mL and heat-shock treated ( $50^\circ\text{C}$ , 10 min) to induce spore germination before further inoculation [37].

#### *Functional bioassays: agar tests and fluorogenic assays*

For all agar tests, 5  $\mu\text{L}$  drops of normalized bacterial or spore suspension were inoculated in triplicate on Petri dishes containing further described agar media. Agar plates were then incubated at  $25^\circ\text{C}$  for 7 days. At the end, diameters of halo were measured except if stated otherwise. The fluorescence assays were done as described [56, 11] and adapted [75] using 4-methylumbelliferone (MU) – linked to exo-enzyme substrates with a bacterial suspension of  $A_{600\text{nm}} = 0.6$  or for Actinobacteria, heat-shocked spore suspensions. Briefly, substrates coupled with MU were incubated with bacterial suspension for 16 hours, shaken under the dark and stopped using 1 M Tris solution pH 11. Results of agar tests and fluorogenic assays were normalized dividing by the maximum unit per assay. All experiments were performed in triplicate.

#### *Degradation of organic matter*

The potential ability of bacterial isolates to degrade organic matter on agar tests was determined by the capacity to hydrolyze cellulose and cleave proteolytic bonds. The hydrolysis of cellulose was tested as described [73] using carboxy-methyl-cellulose (CMC) agar, ( $5 \text{ g L}^{-1}$  carboxy-methyl-cellulose,  $1 \text{ g L}^{-1}$   $\text{K}_2\text{HPO}_4$ ,  $1 \text{ g L}^{-1}$   $(\text{NH}_4)_2\text{SO}_4$ ,  $0.5 \text{ g L}^{-1}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $0.5 \text{ g L}^{-1}$  NaCl and  $15 \text{ g L}^{-1}$  agar). To reveal the enzymatic activity, the agar plates were flooded with 3 mL of 1 mg/mL Congo red solution for 40 min, then poured off and washed with 1 M NaCl for 15 min. Transparent halos were measured as positive hydrolyzed cellulose. The cleavage of proteolytic bonds was tested using casein agar ( $10 \text{ g L}^{-1}$  milk,  $5 \text{ g L}^{-1}$  tryptone,  $2.5 \text{ g L}^{-1}$  yeast extract,  $1 \text{ g L}^{-1}$  glucose and  $15 \text{ g L}^{-1}$  agar). Clear halos were measured as indicators of the production of proteases.

To measure the production of exo-enzymes potentially involved in organic matter degradation, six MU-bound exo-enzyme substrates (Sigma Aldrich) were tested: MU- $\beta$ -D-glucopyranoside to measure  $\beta$ -glucosidase (EC 3.2.1.3), MU- $\beta$ -D-glucuronide hydrate for  $\beta$ -glucuronidase (EC 3.2.1.31), MU- $\beta$ -D-xylopyranoside for  $\beta$ -xylosidase (EC 3.2.1.37), MU- $\beta$ -D-cellobioside for cellobiohydrolase (EC 3.2.1.91) and MU-N-acetyl- $\beta$ -D-glucosaminide for exochitinase (EC 3.2.1.14) [75].

#### *Siderophore production*

The potential ability to produce siderophores was assessed using chrome azurol S (CAS) agar by adapting the method [2]. Due to toxicity of the medium against certain bacterial isolates, bacterial strains were first grown on R2A agar (Difco) for 24 h [47]. CAS agar was then poured on top of the colonies and incubated at  $25^\circ\text{C}$  for 6 additional days. Diameter of pink/orange halos were measured as a reflection of the ability of bacteria to mobilize iron.

#### *Phosphorous mobilization*

Potential ability to solubilize inorganic phosphorus was assessed as described [44], using tri-calcium phosphate agar bioassay (TCP). Potential stepwise dephosphorylation of phytate carried out by phytases was measured using phytase agar bioassay method [36]. In both cases, clear halos were measured as indicator of the ability of

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bacteria to mobilize inorganic or organic phosphorous. MU-phosphate substrate was used to detect the production of acid phosphatases [75].

### *Biotic interactions*

The potential ability to interact with some other organisms was determined by measuring two parameters: auxin production and quorum quenching. The auxin production from tryptophan was tested following the method described [25]. Bacterial isolates were grown on 1/10 TSA supplemented with 5mM tryptophan. After inoculation and drying, plates were covered by a nitrocellulose membrane and incubated at 25°C for 72 h. Then the membranes were removed and soaked on Salkowski reagent (2% 0.5M FeCl<sub>3</sub> in 35% perchloric acid) at room temperature for 1 h. Bacterial isolates producing auxin were identified by a formation of pink/red coloration in the membrane.

To detect the ability to quench quorum sensing (QS) molecules *N*-acylhomoserine lactones (AHLs) with *N*-acyl chain length from C<sub>4</sub>-C<sub>6</sub>, the biosensor assay method using *Chromobacterium violaceum* CV026 was performed as described previously [49]. QS signals were detected with the presence of violacein (a violet pigment) while inhibition of QS was detected by a colorless zone.

### *Pairwise bacterial interactions*

The ability of bacterial isolates to interact within each other was assessed using two complementary approaches: i) simultaneous co-inoculation and ii) delayed co-inoculation on modified Murashige & Skoog (mMS) agar plate [51] in which sucrose was replaced by glucose (per liter: 1 g glucose, 1 g MES, 100 mL of Murashige and Skoog basal salt micronutrient solution (M0529, Sigma-Aldrich), 50 mL of Murashige and Skoog basal salt macronutrient solution (M0654, Sigma-Aldrich), 1 mL of Gamborg vitamins (Sigma-Aldrich), 10 mL of 100X supplementary vitamins (20 mg mL<sup>-1</sup> L-glutamine, 0.1 mg mL<sup>-1</sup> Ca-pantothenate, 0.1 mg mL<sup>-1</sup> L-cysteine hydrochloride, 0.1 mg mL<sup>-1</sup> Biotin) and 10 g of agar; pH 5.8 adjusted with 3% HCl).

First, 5 µL of bacterial or spore suspension ( $A_{600nm} = 0.6$ ) were streaked on mMS agar along the center of a square petri dish and then 5 µL of bacterial or spore suspension ( $A_{600nm} = 0.6$ ) were cross-streaked at around 5 mm away from the center to produce a 5 cm long streak either immediately after the deposition of the central streak (co-inoculation, Supplemental Fig. 1A) or 48 h after the inoculation of the central streak (delayed-inoculation, Supplemental Fig. 1B). Plates were incubated 5 days in total at 25°C. Interactions (effect of the central streak on the side streaks) were determined by measuring the size of the bacterial streaks on the sides and morphological changes (i.e. color of colonies, aerial mycelium production and sporulation ability) were recorded. Combinations were done from bacteria originating from the same grain only. Negative controls consisted of single species streak inoculation against 1X PBS. Self-interactions were also tested. For each method, 3 independent assays were analyzed. Cytoscape software was used to visualize bacterial interactions networks [68].

### *Determination of pH*

To determine if bacterial growth depends on pH on mMS agar medium, bacteria that were not able to grow on regular mMS medium were grown on mMS agar with adjusted pH. mMS agar medium was prepared as mentioned before except pH was adjusted to 6.5, 7.0 or to 7.5 instead of 5.8 using 3% HCl. Five microliters of

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bacterial suspension ( $A_{600\text{nm}} = 0.6$ ) were streaked on pH adjusted mMS agar and incubated for 5 days at 25°C. To evaluate if bacteria were able to change environmental pH, bacteria were grown on mMS agar (pH 5.8) supplemented with 5  $\mu\text{M}$  of bromocresol purple, a pH indicator [64]. Changes of environmental pH were considered by the change of color. mMS bromocresol agar at pH 5.8 presented a dark red color, it turned yellow or violet if pH decreased or increased, respectively. Five microliters of bacterial suspension ( $A_{600\text{nm}} = 0.6$ ) were inoculated on bromocresol purple mMS agar and incubated for 5 days at 25°C. To determine if changes in environmental pH is widespread in the bacterial collection, five microliters of bacterial suspensions ( $A_{600\text{nm}} = 0.6$ ) were inoculated in liquid mMS (pH 5.8), incubated at 25°C under agitation of 200 rpm for 5 days and then pH was measured with a pH meter.

## **Results**

### *Taxonomic and functional characterization of bacteria from grains of soils*

To characterize the potential interactions between bacteria living in the same ecological niche and likely interacting in their natural environment, we isolated strains from two grains of soils: one from the rhizosphere of a lateral root that was considered to be metabolically active as it further harbored turgescient ectomycorrhizae (data not shown) from *Quercus robur* (identified by ITS sequencing, data not shown) and one from bulk soil taken 5 cm away from any root. A total of 137 and 154 colonies were isolated from a single grain of bulk and rhizospheric soil after 10 days of incubation, respectively. Initial Petri dish plates were further incubated for an additional 8 days in the hope of recovering slow growing members of the bacterial communities, but no more colonies could be isolated. Twenty-two and 25 strains showed phenotypic differences (i.e. color, shape, sporulation), respectively and were kept for further analyses. Molecular identification showed that these strains were affiliated to ten different species belonging to six genera and three phyla, according to taxonomic assignment of 16S rDNA sequences (Fig. 1). The collections were dominated by bacteria from the phyla Firmicutes (83%) and Actinobacteria (15%) and contained one strain of the Proteobacteria phylum (genus *Pseudomonas*). The largest diversity was found among Firmicutes as they regrouped strains belonging to *Sporosarcina* (3%), *Paenibacillus* (6%), *Staphylococcus* (16%) and *Bacillus* (75%). Actinomycete strains belonged to two *Streptomyces* species: *Streptomyces olivochromogenes* and *Streptomyces sioyaensis*. Strains of the genera *Streptomyces*, *Staphylococcus*, and *Bacillus* were isolated from the two microhabitats (bulk and rhizospheric soils). *Sporosarcina* and *Pseudomonas* were only found in the grain of bulk soil while *Paenibacillus* strains were only found in the grain from rhizosphere in our screening methodology (Fig. 1).

Illumina Miseq sequencing of 16S rRNA was performed in parallel to determine the potential initial composition of the communities of the two grains of soil. According to this approach based on DNA extracts, each grain harbored a highly diverse bacterial community with 1245 and 1178 species-level operational taxonomic units (OTUs) in bulk soil (B) and rhizosphere (R), respectively. The compositions of the communities were relatively similar between both grains of soil and were dominated by Proteobacteria (B: 30% & R: 29%), Verrucomicrobia (B: 15% & R: 25%), Acidobacteria (B: 14% & R: 20%) and Actinobacteria (B: 18% & R: 13%) (relative abundance >1%). Most abundant OTUs were affiliated to unknown genera from DA101 soil group family (Verrucomicrobia)

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and subgroup 2 of Acidobacteriaceae (Supplemental Fig. 2). Cultivated bacterial strains that were isolated from these grains were also found in this analysis but belonged to the rare taxa (Supplemental Fig. 2).

In a second step, we evaluated the potential functional activities of all the isolated strains to determine the variability between strains sharing the same microhabitat. We analyzed several traits important for the survival in soil and rhizosphere environments such as the ability to produce siderophores, to acquire nutrients from organic and inorganic matter or to interact with other organisms (with plants via auxin production or with other microorganisms via quorum quenching). The production of acid phosphatases, siderophores and proteolytic exoenzymes were the most commonly shared, but notable differences in the efficiency of these activities were measured (Fig. 1). For instance, the most efficient producers of proteolytic exoenzymes were *B. subtilis* strains, while *S. sioyaensis* were producing larger amount of siderophores (Fig. 1). None of the potential activities measured was specific to a taxonomic group or to the origin (bulk soil or rhizosphere). Instead, variations among all strains sharing an identical 16S rDNA sequence were recorded. For instance, 3 out 5 strains of *Bacillus drentensis* produced proteolytic exoenzymes while the two others did not in our experimental conditions (Fig. 1). Similarly, the ability of producing phytases was not shared by all strains of *S. sioyaensis* (Fig. 1). Based on the functional tests and 16S rDNA sequences, a subset of 25 bacterial strains ( $n = 10$  for bulk soil,  $n = 15$  for rhizosphere) was selected for further analyses to test pairwise interactions. This bacterial collection was composed of 13 strains of *Bacillus*, 6 strains of *Streptomyces*, 2 strains of *Staphylococcus* and *Paenibacillus* and one strain of *Sporosarcina* and *Pseudomonas* (in red Fig. 1).

### *Pairwise interactions between bacteria isolated from the same microhabitat*

To evaluate potential interaction behaviors among bacteria living in the same ecological niche, pairwise combinations between strains from the same collection subset (either bulk soil or rhizospheric soil) were tested on mMS, a synthetic medium that allows the growth of plants in vitro [22]. We tested interactions on this medium to explore the influence of plant nutrients in bacterial interactions in a next step. To assess the importance of priority effect on the output of the interactions, bipartite combinations were done in two different ways: using a simultaneous co-inoculation and with a 48 hours delayed inoculation (Supplemental Fig. 1). After co-incubation period, potential interactions between strains isolated from the same grains of soil were recorded. Three phenotypes were observed: effect i) on the size of bacterial streak (i.e. effect on the bacterial growth), ii) on the ability to produce pigment (color of the colony) and iii) on morphological differentiation (aerial mycelium and sporulation) for the *Streptomyces* strains. In total, we performed 650 combinations, 325 combinations per approach, 100 for bulk soil ( $n=10$ ) and 225 for rhizosphere ( $n =15$ ), including self-interactions.

Among the 25 strains analyzed, 4 did not grow on mMS medium when inoculated alone (*Bacillus lentus* B6, *Sporosarcina* sp. B29, *Staphylococcus* sp. B42, and *Staphylococcus* sp. R9). Thus, only 245 out of the 325 possible interactions were recorded for the simultaneous co-inoculation experiment. No phenotypic change could be observed in 185 interactions (75,5% out of 245 possible interactions), while 60 (24,5%) led to a reduction of streak size (Fig. 2, Supplemental Fig. 3). From these inhibitory activities, 13 interactions (5.3 % out of 245)

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occurring either between strains from the same genus (*Streptomyces* against *Streptomyces*) or between certain *Streptomyces* and *Bacillus* strains led to reciprocal inhibition of growth (Fig. 2). The 47 other interactions (19.2 %) involved unidirectional inhibitions and were not taxa specific (Fig. 2, Supplemental Fig.3). Interestingly, all *S. olivochromogenes* and *B. subtilis* strains showed a self-inhibition behavior (Fig. 2). Lastly, pigmentation of the colonies was not visibly affected by any of the interactions analyzed. No effect on sporulation was observed for the *Streptomyces* strains either.

In the 48 hours delay experiment, inhibitory and stimulatory interactions were observed for both soil grains. Additionally, we observed effects on aerial mycelium formation and sporulation and on pigmentation of the colonies in some cases. In details, 250 out of the 325 possible interactions were recorded. From these 250 observed interactions, 153 (61%) had no visible effect and 97 cases (39%) led to a change of streak size. From the latter, 75 were inhibitory and 22 were stimulating (Fig. 3., Supplemental Fig. 3). Seventeen (6.8% out 250) inhibitory interactions were reciprocal inhibitions and all involved strains from the *Streptomyces* genus. The remaining 58 (23.2% out of 250) inhibitory interactions affected only one of the interacting strains. Specifically, *Bacillus* sp. strains no longer inhibited other *Bacillus* sp. strains, and mainly *Streptomyces* strains inhibited the other bacterial members. Interestingly, only *S. olivochromogenes*, showed a self-inhibition behavior regardless the origin. In the case of the positive interactions, only 4 (1.6 %) stimulated the growth of each other (*Bacillus* sp. R1 - *Paenibacillus* sp. R2 and *Bacillus* sp. R24 - *B. drentensis* R39), while 18 (7.2 %) increased the growth unidirectionally (mainly *Bacillus* sp. towards *B. drentensis*). Interestingly, *B. lentus* B6 was not able to grow alone on the medium but was able to form a colony when co-inoculated near a 48h old colony of *Bacillus* sp. B2 (Fig. 3). Similarly, the strain *Staphylococcus* sp. R9 was able to grow after being inoculated with delay with *Paenibacillus* sp. R34 or *Bacillus* sp. strains R1, R23, R24 and R35. However, none of the strains rescued the growth of *Staphylococcus* sp. B42 and of *Sporosarcina* sp. B29 in the tested conditions. Finally, a delay in the production of aerial mycelium and sporulation of *S. olivochromogenes* B14 was observed when inoculated 48h after *B. subtilis* B34 and B36 strains. Conversely, the two *B. subtilis* no longer produced red pigment when inoculated 48h after *S. olivochromogenes* B14.

### *Frequency and intensity of interactions: few aggressive strains but a large potential for interactions.*

In a second step, we asked whether these interactions were due to a small number of highly active bacteria or whether most of the strains were engaged in interactions. Overall, all the strains interacted with at least one other bacterial strain in at least one of the two tested conditions. However, the numbers of strains with which bacteria interacted strongly varied from strain to strain and depended on the conditions of the interaction (Fig. 4). In detail, from a total of 21 interacting bacteria, 10 strains (47.6% out of 21) co-inoculated at the same time had no effect on other strains, three (14.3%) inhibited the growth of 1-2 bacterial strains, two (9.5%) inhibited between 3-5 bacterial strains and six (28.6%) inhibited more than 6 strains. Delayed inoculations led to two (9.5% out of 21) bacterial strains with no effect on other strains, three (14.3%) inhibited 1-2 strains, three (14.3%) inhibited 3-5 bacterial strains and five (23.8%) inhibited more than 6 bacterial strains. Additionally, in this later



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approach, four (19%) of the strains promoted the growth of one isolate while five (23.8%) promoted the growth of 3 to 5 bacterial isolates. All these promoting strains belonged to the *Bacillus* genus (Fig. 3) and none of these strains showed inhibitory activity against other strains except *Bacillus* sp. R1 which inhibited the growth of *Bacillus* sp. R24. Correlations between “aggressiveness” of strains (defined in this study as strains that inhibit more than 4 strains) and their “susceptibility” to other strains (strains that get inhibited by more than 4 strains) could be drawn but the pattern was strongly dependent on the inoculation approach considered (Fig. 4). In delayed assays, “aggressive” strains isolated from both soil compartments were more resistant to inhibition while “less aggressive” strains were more prone to be inhibited. Yet, no correlation between aggressiveness and susceptibility was found among strains when co-inoculated simultaneously whatever their origin.

Bacterial strains were categorized into 11 classes based on their behaviors (Table 1). These categories were built based on the total number of strains that each was able to promote, to benefit from, to inhibit, and was susceptible to. Connections between taxonomic affiliations of the strains and their activities could be drawn. For instance, the most active bacterial strains co-inoculated were *B. subtilis* B34 and B36, *Pseudomonas* sp. B17, *S. olivochromogenes* B14 and *S. sioyaensis* R27. These strains were able to inhibit the growth of at least 70% to 85.7% of the bacterial strains. While in delayed inoculation, inhibitory interactions were steered by *S. olivochromogenes* R4 (100% of strains inhibited), *S. sioyaensis* R15, R21 and R27 (>90% of strains inhibited) and *Pseudomonas* B17 (>70% of strains inhibited) (Fig. 4, Table 2). Important variations occurred at the strain level: *S. sioyaensis* could be either poorly or highly aggressive depending on the strain with which it interacted, while *B. drentensis* could benefit or not from interaction depending on the identity of the other interacting strain.

We then compared the intensities of these inhibitions to assess the effectiveness of the antimicrobial activities. Most bacteria showed weak inhibition when co-inoculated, and when bacteria were inoculated with delay most showed intermediate and strong inhibitory interactions. From a total of 135 negative interactions for both approaches, 66 consisted of weak inhibition reducing the streak size at most by 20%, 52 had an intermediate inhibitory effect reducing by 20 to 40% of the streak size and only 17 consisted of strong inhibitory effect reducing the streak by more than 60%. Our data revealed aggressive bacterial isolates (i.e. *Pseudomonas*, *B. subtilis* and *Streptomyces*) had also a strong inhibitory effect (reduction of > 60% of the streak size) towards 1 or 2 bacterial members (*Streptomyces* and *Bacillus*) (Fig. 2, Table 2). Also, *Pseudomonas* sp. B17 inhibitory effect against *S. sioyaensis* B9 was the strongest inhibitory interaction (Fig. 2, Table 2), but this occurred in the co-inoculation approach.

### *pH alteration is involved in rescuing the growth of some bacterial strains*

Finally, we wondered if pH could be responsible for the rescue of growth provided by *Bacillus* sp. B2 to *B. lentus* B6 and by some Bacilli strains and by *Paenibacillus* sp. R34 to *Staphylococcus* sp. R9., Indeed, pH is an important driver for bacterial growth and survival in soil [23]. To test this hypothesis, we first cultivated on mMS pH 6.5, 7.0 and 7.5 bacteria that could not grow on mMS pH 5.8. *B. lentus* B6 and *Sporosarcina* sp. B29 were

able to grow successfully at pH 6.5 and higher, while *Staphylococcus* B42 and R9 strains could not grow at any of these higher pH on this medium (Supplemental Table 1). Next, we questioned if the six helper bacteria were able to change environmental pH. *Bacillus* sp. B2 was able to increase the pH of the medium, suggesting that rescue of growth of *B. lentus* B6 could be due to pH conditions (Supplemental Table 1, Supplemental Fig. 4). Yet, it is possible that pH increment was not sufficient to rescue *Sporosarcina* or that other complex metabolic processes were involved in this rescue. The rhizosphere strains that rescued the growth of *Staphylococcus* sp. R9 showed various behaviors: some reduced the pH (e.g. *Paenibacillus* sp. R34) while other slightly increased it (e.g. *Bacillus* sp. R23) (Supplemental Table 1). Altogether with the fact that pH increase did not permit to rescue the growth of *Staphylococcus* sp. R9 on mMS, it is very likely that pH alteration was not involved in the rescue of growth of *Staphylococcus* sp. R9 by *Bacillus* strains and *Paenibacillus* sp. R34. Lastly, we wondered if the ability to change environmental pH was a widespread phenomenon among our soil bacterial isolates. Out of the 38 strains that we isolated from bulk and rhizospheric grains of soil and that were able to grow on mMS, most bacteria (66%) tended to acidify the surrounding environment (<5.7) (Supplemental Table 1). These bacteria included *B. subtilis*, *B. drentensis*, some *Bacillus* sp., *Pseudomonas*, *Streptomyces* and *Paenibacillus* strains. Only 13% of the strains, all belonging to the *Bacillus* sp. genus increased the pH (>5.9) of their surrounding environment when grown in mMS, suggesting that this “alkalization” behavior is restricted to a subgroup of *Bacillus* sp. strains.

## **Discussion**

Bacteria are metabolically versatile and possess a large toolbox to interact with other microbes [28,72]. Our ability to predict how microorganisms react in natural environments such as soils is very limited, as our knowledge of when, how and with whom microorganisms interact in situ is still poor. To get insights into how bacteria that colonized the same microhabitats could interact, we isolated cultivable bacteria from grains of soil and selected all strains that showed a divergent phenotype. Our cultivation method allowed the isolation of well-known cultured representatives from Actinobacteria, Firmicutes and Proteobacteria phyla, consistent with previous studies [16], but a low bacterial diversity was obtained compared to the original community from the grains of soil [16,32,78]. Indeed, DNA metabarcoding analysis of the microbiome of each grain of soil showed that the strains that were isolated were among the least abundant species from the community (Supplemental Fig. 2). The type of media used and short incubation time (10 days extended to 18 days) favored fast aerobic growers, spreading phenotype, with a wide pH tolerance range [35]. Same media have been used extensively in the past to isolate bacterial strains from soil and root habitats and allowed to isolate a much larger taxonomic diversity of strains [8,75]. Large composite samples were used in those studies while we focused here on single grains of soils, suggesting that the low diversity obtained is not a bias of the media used. Indeed, soil sample size influences the retrieved cultivable bacterial community structure [20] and the DNA analysis [54,57]. Moreover, most abundant OTUs according to metabarcoding analyses were affiliated to taxa that are not cultivable yet [35,78], limiting our chances to isolate them.

## Potential functional diversity and biotic interactions

Nevertheless, rare microorganisms can be very active and play important roles within microbial communities [74,76]. In addition, DNA metabarcoding analysis potentially provides a biased view of the real composition of the bacterial community as it captures active microorganisms as well as dead and spore dormant cells [42]. In this regard, reads corresponding to the genera isolated in this study were retrieved in metatranscriptomic analysis performed on nearby soils indicating that the microorganisms we isolated were likely to be active members of the community (Maillard, Auer, Buée *et al.* unpublished). Therefore, interactions captured in this work are representative of the activities of a small group of soil bacterial members with a particular lifestyle from single grains. Despite the low taxonomic diversity of the strains isolated, analysis of functional potentials revealed an important diversity of behaviors among the strains. This is in accordance with previous studies where bacteria have same genomic content but still show important variations in their phenotypic traits [4]. No correlation was found between potential functional activities and any taxonomic group (Fig. 1) as previously shown by Uroz and collaborators between bulk soil and mycorrhizal isolates [75]. Bacterial isolates from the two grains of soil had a high potential to uptake iron, produce proteolytic exoenzymes and solubilize phosphate through acid phosphatases. While the ability to produce acid phosphatases and proteases is commonly shared by soil bacteria in forest soils [61,75], iron mobilization has been shown to be restricted to a subset of strains [6,52]. This may reflect an iron deficiency at the micro-scale leading to the selection of strains able to take up iron through siderophores or xenosiderophores. The least substrate used was cellulose. Even though cellulose is highly abundant in forest topsoil, cellulolytic abilities are limited to certain bacterial taxa including Betaproteobacteria, Bacteroidetes and Acidobacteria which were not isolated in this study [48]. Based on different taxonomic and functional profiles, 10 and 15 strains from the two grains of soil were selected to test their ability to interact.

Among soil bacteria, antagonism is generally seen as the major mode of interaction [10,24,34,55,71]. Here, we found that between 60-75% of pair combinations induced no visible effect (Fig. 2, Fig. 3). Although, we cannot exclude the presence of undetectable responses in our experimental approach (e.g. such as a metabolomic response [70]), this result is in line with previous findings obtained by other groups, suggesting that antagonistic and cooperative interactions may not dominate among microorganisms in soils [15,71]. For instance, Perez-Gutierrez and collaborators observed that 90% of *Bacillus* interactions were resistant to others [55] and Davelos and collaborators found 67% of interactions between Streptomycetes were resistant to others [15]. In addition, in our study, the strength of inhibitions was weak or intermediate in majority, and only one case of very strong antagonism was observed (*Pseudomonas* sp. B17 against *S. sioyaensis* B9). Low frequency of high intensity antagonism has been also reported previously in *Vibrio* interactions [9]. According to previous studies, this low frequency suggests that antibiotics functions are not necessarily bacterial weapons, but instead, that they would be public goods to control population dynamics in order to maintain the community structure [7,9] and ecological stability [12,29]. Dantas and collaborators have even found a wide variety of bacteria able to subsist on antibiotics as the only carbon source [14]. Further investigations will be necessary to test whether those hypotheses apply to our system.

### *Potential functional diversity and biotic interactions*

Although many mechanisms of mutualistic or cooperative interactions between bacteria have been described up to now [13,60], the proportion of beneficial interactions among other types of interactions occurring at the microscale is poorly documented. In this study, 9% of interactions ended up in promotion of bacterial growth and those only occurred in delayed co-inoculation. By comparison, Vetsigian and collaborators reported a rate of 19% of promoting interactions among strains from grains of soils [79]. Altogether these data tend to support the hypothesis that cooperation behavior would be reduced in soil microhabitats compared to other interactions. In the present case, promotion behaviors occurred between strains of the same order (*Bacillus*, *Paenibacillus* and *Staphylococcus*) but not across Phyla. In accordance with other studies incriminating pH, an important regulator of bacterial interactions [58], preliminary data suggest that alteration of pH could be responsible for some but not all these beneficial interactions (Supplemental Table 1). However, this ability to increase the pH of the medium was restricted to a subgroup of *Bacillus* strains suggesting that it may not be a frequent phenomenon occurring in natural communities.

As previously noticed in other studies, most of the interactions recorded were antagonistic [15,24,79]. Interestingly, highly antagonistic strains tended to be also highly resistant while susceptible strains were in general not antagonist. In this regard, *Pseudomonas* sp. B17 strain was the most efficient antagonist, as it inhibited the growth of all bulk soil members with a variety of intensities and did not suffer from any reciprocal inhibitory activity. This is not unexpected since the *Pseudomonas* genus regroups bacteria that are generally highly competitive for nutrients [17,27,30], have the ability to produce a wide range of antibacterial compounds [26,31,67], prevent antibiotic production from other bacteria [26], while also being able to resist to antibiotics thanks to multidrug efflux pumps [38]. *Streptomyces* are also famous for their ability to produce a wide range of antibiotics and this genus is therefore considered as highly bioactive and competitive to maintain a dynamic population [79]. In agreement, we observed that antagonism among *Streptomyces* strains was maintained over both inoculation approaches, suggesting the existence of a constitutive inhibitory activity. However, we noticed variations in the frequency and the intensity of the inhibitory activity among strains, suggesting potential differences in mechanisms of antagonisms [15,40,55,70]. Important variations in the activities of *Bacillus* strains were also visible. While *B. subtilis* strains strongly antagonized other *Bacillus* strains as well as *Streptomyces*, most *Bacillus* sp. strains were weakly aggressive and even rather stimulated the development of other strains (Fig. 2, Fig. 3). Such results corroborate with the hypothesis of Perez-Gutierrez and collaborators who proposed that interference between *Bacillus* is linked to genetic distance and resource use [55].

In summary, we found in this study that at the scale of a grain of soil, the majority of cultivable bacteria engaged in a few number of interactions with other bacteria. A few bacteria were highly aggressive, but the proportion of beneficial interactions was also reduced. It remains to determine if these interactions really occur in soils and how they impact community dynamics. Elucidating the molecular mechanisms by which bacteria interact together may help predicting their activities in situ. Genome sequencing to find biosynthetic gene clusters with important

bioactive functions combined with metabolomics may help identify targets to survey how bacterial interactions affect natural complex microbial communities.

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## **Conflict of Interest**

The authors declare that they have no conflict of interest.

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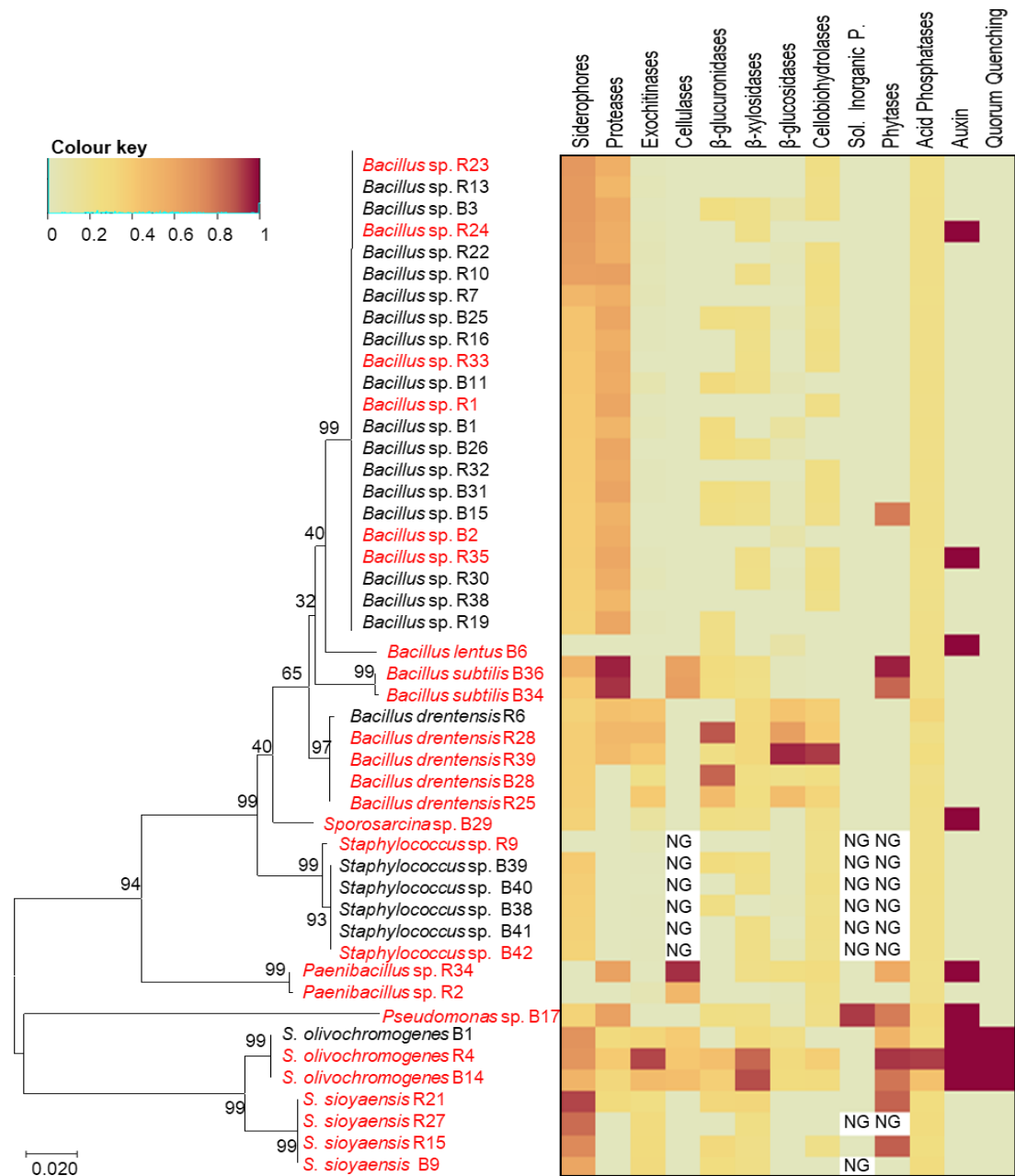
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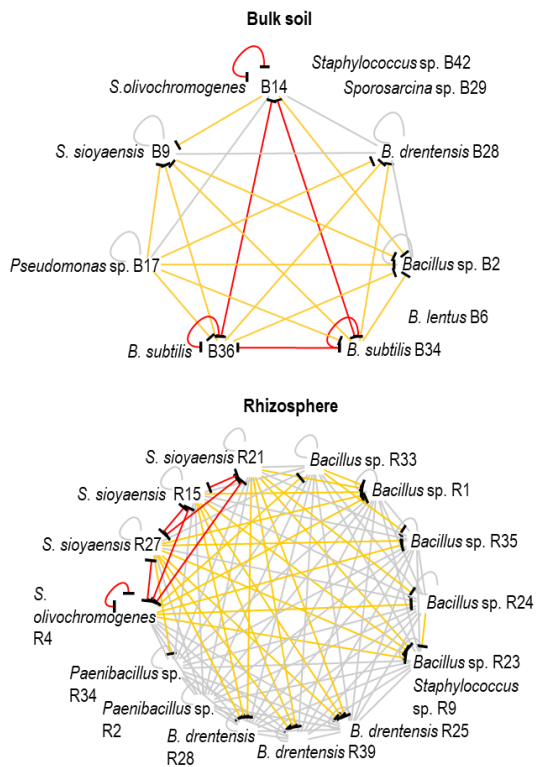
**Figures.**



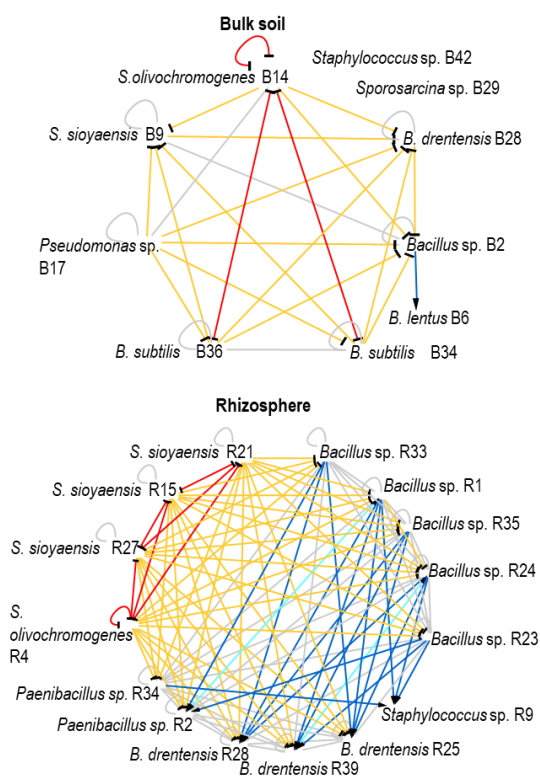


**Figure 1. Functional potentials and phylogenetic tree of the strains isolated from grains of bulk (B) and rhizospheric (R) soils.** Taxonomical classification of all phenotypically looking different isolates (n=47) was built based on 16S rRNA sequences using Neighbour-Joining method. The numbers of each branch nodes are bootstrap values from 100 re-samplings. Horizontal branch lengths are proportional to evolutionary distance according to Kimura's 2 parameter distance. For strains with identical 16S rRNA sequences, ordering of strains was organized based on similarity of potential functional activities by hierarchical clustering. For each potential activity, data were normalized to the maximal intensity measured and intensity is represented as a gradient of color from no activity (green) to 100% activity (dark red). NG – absence of growth on the respective media used. Strains in red letters correspond to strains that were further analyzed for binary interactions.

Potential functional diversity and biotic interactions

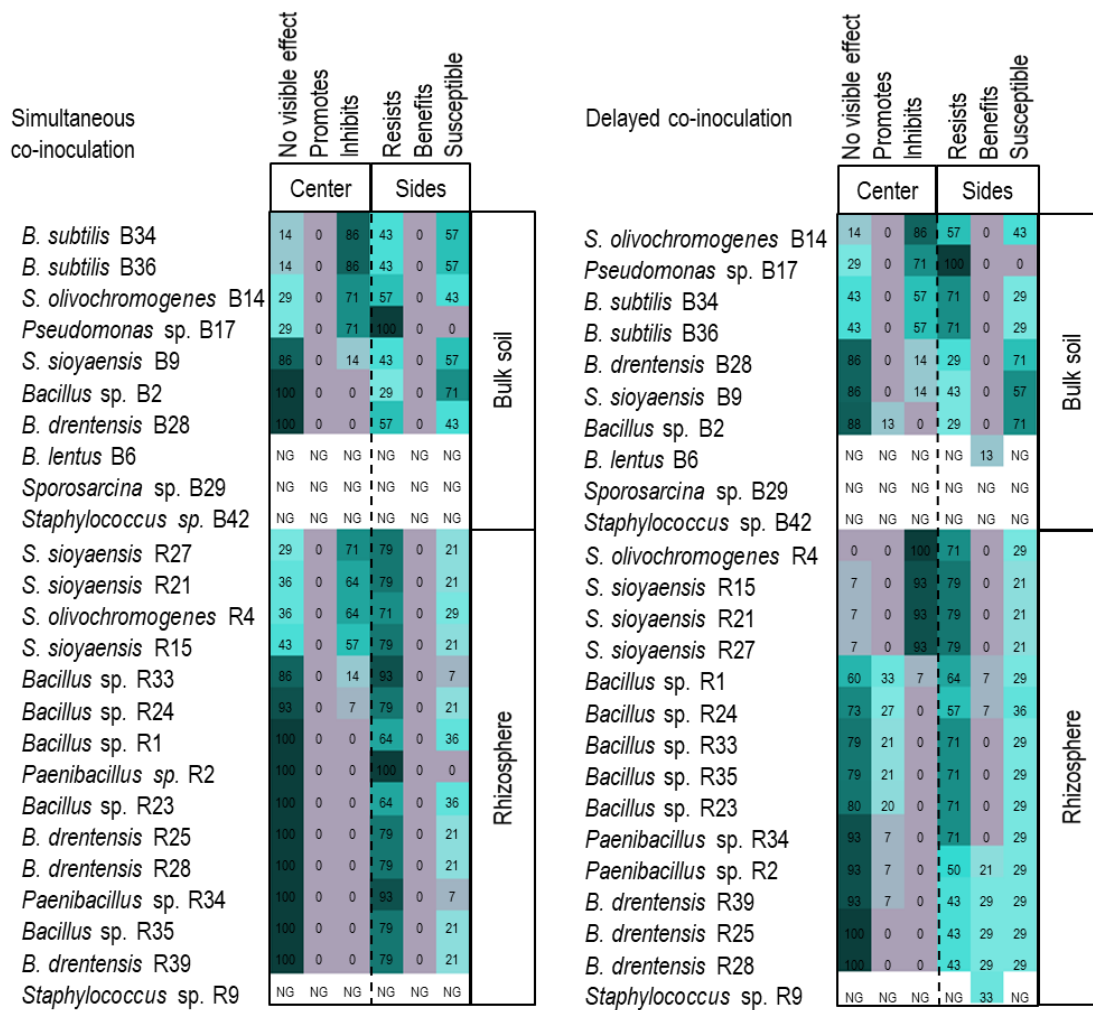


**Figure 2. Networks of pairwise interactions between bacterial strains isolated from bulk soil and rhizosphere bacteria co-inoculated simultaneously.** The direction of the interaction is marked by a bar for inhibition. Color of connecting lines indicate the type of interaction: red – mutual inhibition, yellow – unidirectional inhibition, grey – no visible effect on growth. Loops figure potential self-interaction phenotypes.

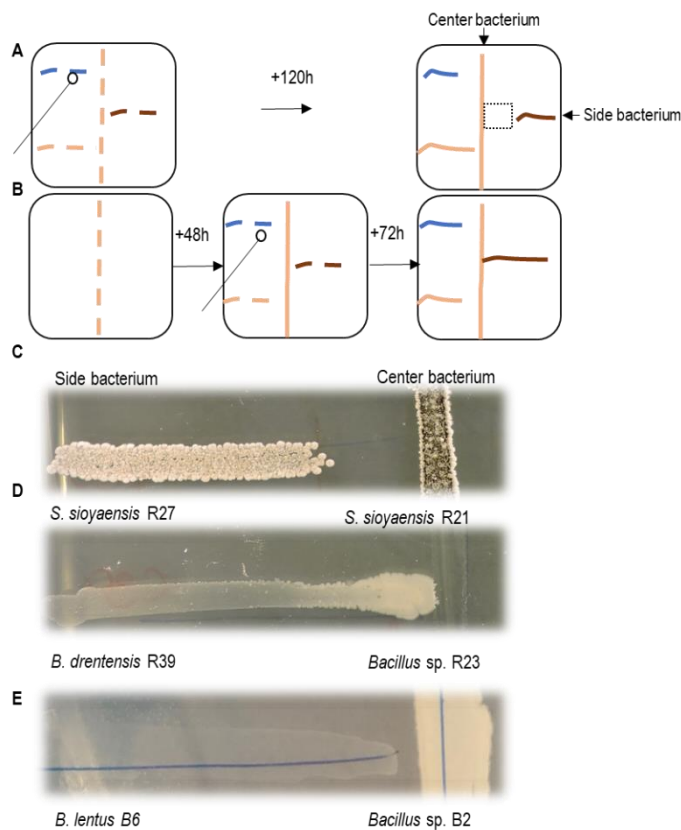


**Figure 3. Networks of pairwise interactions between bacterial strains isolated from bulk soil and rhizosphere bacteria co-inoculated with a 48hrs delay.** The direction of the interaction is marked by a bar for inhibition and an arrow for promotion of growth. Color of connecting lines indicate the type of interaction: red – mutual inhibition, yellow – unidirectional inhibition, turquoise – mutual promotion of growth, blue – unidirectional growth promotion, grey – no visible effect on growth. Loops figure potential self-interaction phenotypes.

Potential functional diversity and biotic interactions

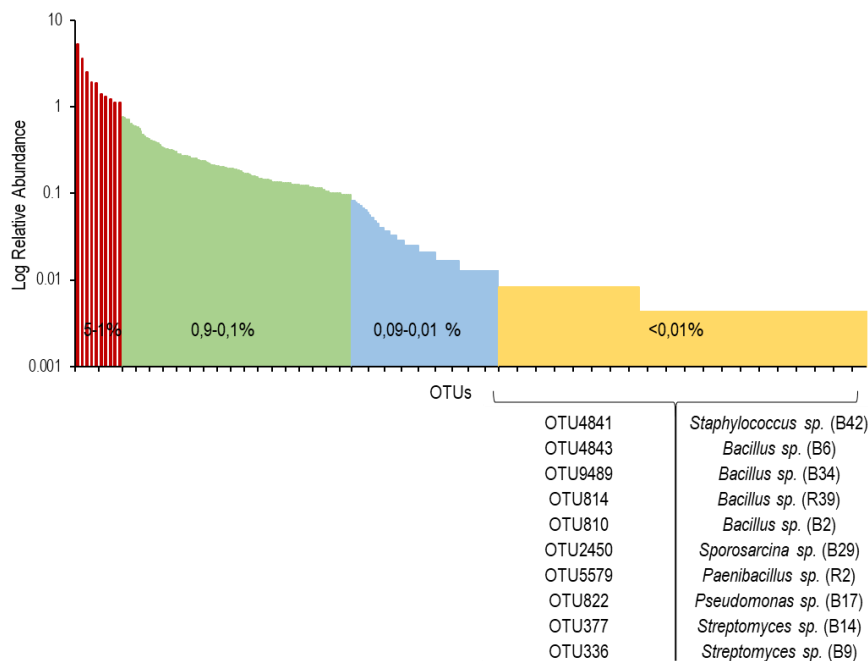


**Figure 4. Degrees of activities and susceptibilities of bulk soil and rhizosphere bacterial strains when co-inoculated simultaneously or with a 48hrs delay.** The abilities to promote/inhibit the development of other strains were determined for strains placed as a central streak (“Center” column) in the Petri dish while sensitivity to the activities of strains from the central steak was measured on strains streaked perpendicularly (“Side” column, Suppl. Fig 1). For each strain, the degree of activity (no visible effect / promotion / inhibition) and of susceptibility (no visible effect-resist / benefits / susceptible) was expressed as the percentage of strains impacted among the total number strains tested (n=10 and 15 for bulk soil and rhizosphere, respectively). Percentages are given as numbers and color-coded from 0 (grey) to 100% (dark blue) to facilitate visualization. NG indicates undetermined values because the strains did not grow on the medium. For each bacterial library (bulk soil and rhizosphere), strains are presented in the order of the most to the less aggressive strains.



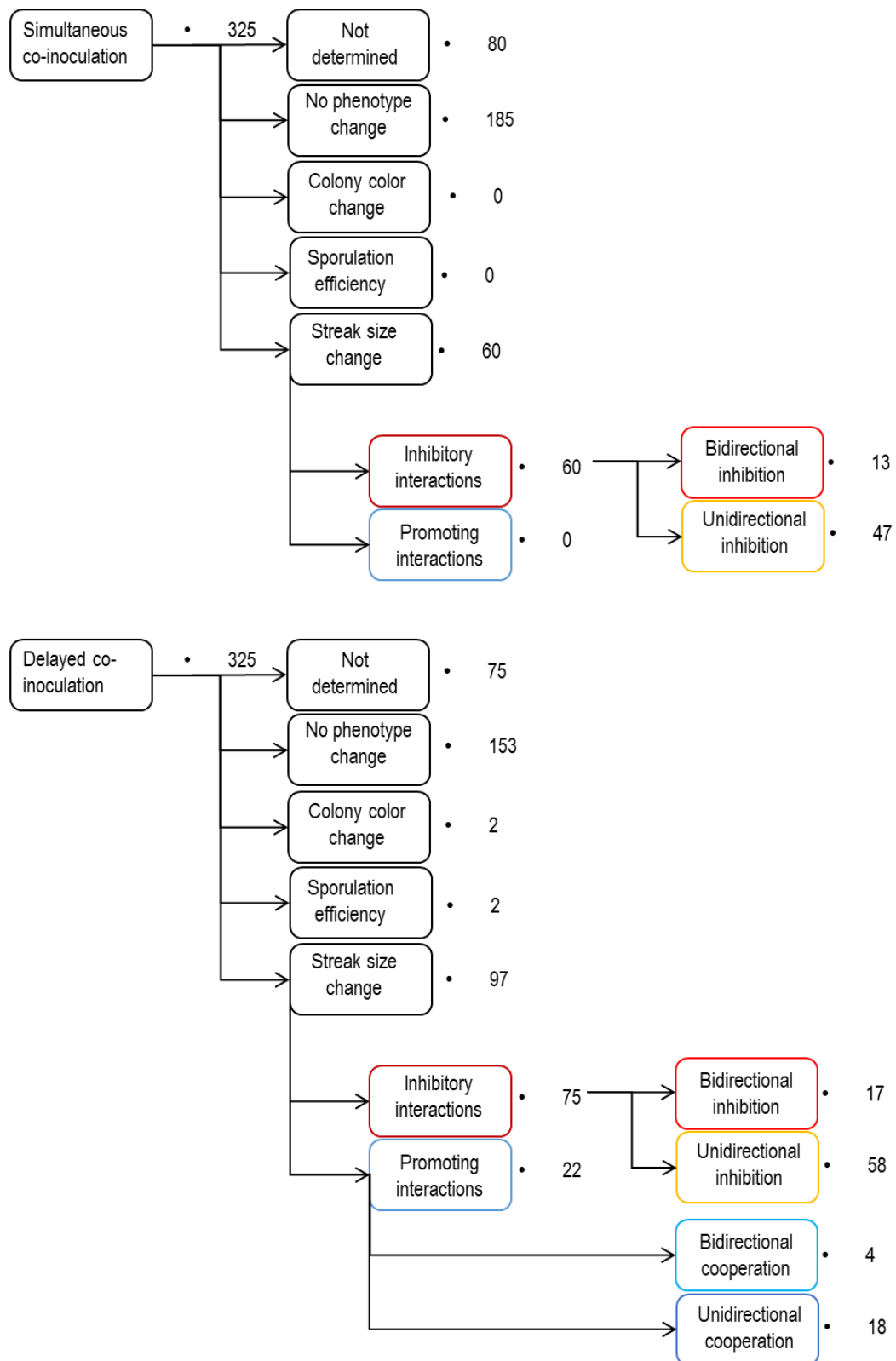
### Supplemental Figures.

**Supplementary Figure 1. Scheme of the set up used to analyse pairwise bacterial interactions and examples of the outputs obtained.**



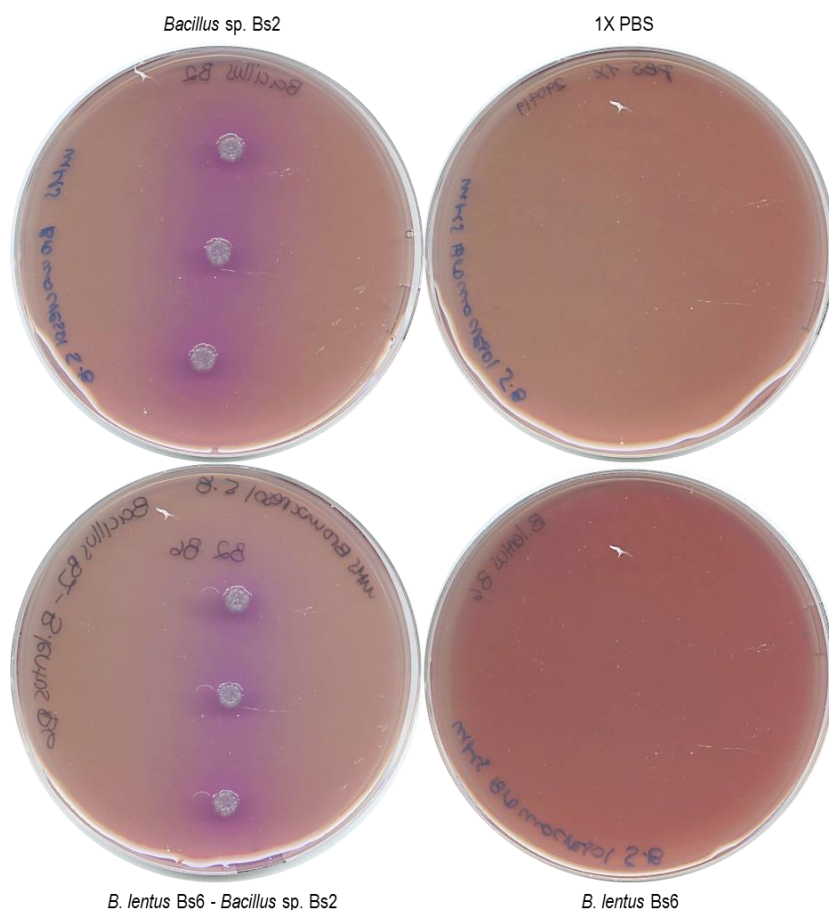
**Supplementary Figure 2.** Representativeness of isolated bacterial strains within the bacterial communities according to 16S rRNA metabarcoding analysis by Illumina Miseq sequencing. The bar plot represents the distribution of the relative abundance of OTUs from bulk soil. OTU sequences that are >95% identical to the 16SrRNA bacterial isolates are indicated.

Potential functional diversity and biotic interactions



**Supplementary Figure 3.** Flow chart indicating the number of interactions tested and occurring in both co-inoculation methods including all strains from both soil compartments. "Not determined" interactions were due to the inability to grow of some bacterial isolates on mMS agar medium.

## Potential functional diversity and biotic interactions



**Supplementary Figure 4.** Example of rescue of growth of *B. lentus* B6 from *Bacillus* sp. B2 on mMS – bromocresol agar. Purple halo indicates alkalinisation of the medium.

## Tables

**Table 1. Classification of interactive phenotypes as a function of levels of activities and susceptibilities of bacterial strains.** HA, MA, LA: High, medium, low aggressiveness, respectively; HS, MS, LS: High, medium, low susceptibility, respectively. OP, P: Absence, presence of promoting effect. OB, B: does not, does benefit from another strain.

Type of interaction	Strains
HA – HS / OP – OB	<i>Streptomyces olivochromogenes</i> R4
HA – LS / OP – OB	<i>Pseudomonas</i> sp. B17
HA – MS / OP – OB	<i>Streptomyces sioyaensis</i> R15, R21, R27
	<i>Bacillus subtilis</i> B34, B36
	<i>Streptomyces olivochromogenes</i> B14
LA – HS / OP – OB	<i>Bacillus drentensis</i> B28
	<i>Streptomyces sioyaensis</i> B9
LA – HS / OP – B	<i>Bacillus drentensis</i> R25, R28
LA – HS / P – OB	<i>Bacillus</i> sp. R23, R35
	<i>Bacillus</i> sp. B2
LA – HS / P – B	<i>Bacillus drentensis</i> R39
	<i>Bacillus</i> sp. R24
LA – MS / P – OB	<i>Paenibacillus</i> sp. R34
LA – MS / P – B	<i>Paenibacillus</i> sp. R2
MA – HS / P – B	<i>Bacillus</i> sp. R1
MA – MS / P – OB	<i>Bacillus</i> sp. R33

**Table 2. Levels of aggressiveness of the most active bacterial strains isolated from bulk and rhizosphere soils.**

Bacterial strains were classified according to their abilities to inhibit the development of other strains co-inoculated simultaneously or with a 48hrs delay. Bacterial strains isolated from bulk soil and rhizospheric are indicated by the B and R letters.

Inoculation	Origin	Bacterial strains	Total number of bacteria affected (%)	Weak inhibitory effect (<20%)	Intermediate inhibitory effect (20-40%)	Strong inhibitory effect (60-80%)	Very strong inhibitory effect (>80%)
Simultaneous	Bulk soil	<i>B. subtilis</i> B34	85.7	57.1	14.3	14.3	0
		<i>B. subtilis</i> B36	85.7	57.1	14.3	14.3	0
		<i>S. olivochromogenes</i> B14	71.4	42.9	28.6	0	0
		<i>Pseudomonas</i> sp. B17	71.4	14.3	28.6	14.3	14.3
		<i>S. sioyaensis</i> B9	14.3	14.3	0	0	0
	Rhizosphere	<i>S. sioyaensis</i> R27	71.4	64	7.1	0	0
		<i>S. sioyaensis</i> R21	64.3	42.9	21.4	0	0
		<i>S. olivochromogenes</i> R4	64.3	50	14.3	0	0
		<i>S. sioyaensis</i> R15	57.1	50	7.1	0	0
		<i>Bacillus</i> sp. R24	7.1	7.1	0	0	0
Delayed	Bulk soil	<i>S. olivochromogenes</i> B14	85.7	14.3	57.1	14.3	0
		<i>Pseudomonas</i> sp. B17	71.4	0	71.4	0	0
		<i>B. subtilis</i> B34	57.1	14.3	14.3	28.6	0
		<i>B. subtilis</i> B36	57.1	14.3	14.3	28.6	0
		<i>B. drentensis</i> B28	14.3	14.3	0	0	0
	<i>S. sioyaensis</i> B9	14.3	14.3	0	0	0	
	Rhizosphere	<i>S. olivochromogenes</i> R4	100.0	42.9	50	7.1	0
		<i>S. sioyaensis</i> R15	92.9	14.3	71.4	7.1	0
		<i>S. sioyaensis</i> R21	92.9	50.0	28.6	14.3	0
		<i>S. sioyaensis</i> R27	92.9	14.3	50.0	28.6	0
<i>Bacillus</i> sp. R1		6.7	6.7	0	0	0	

Supplemental Tables

**Supplemental Table 1. Environmental pH changes of bacterial strains isolated from bulk and rhizosphere soils.** Bacterial strains were grown on mMS liquid to evaluate their capabilities of changing environmental pH. Bacterial isolates isolated from bulk soil and rhizospheric are indicated by the B and R letters. Bold letters highlight strains that were able to rescue the growth of *B. lentus* B6 and *Staphylococcus* R9.

Soil compartment	Bacterial strain	Growth	pH
Bulk soil	<i>Bacillus</i> sp. B1	+	5.77
	<i>Bacillus</i> sp. B11	+	5.52
	<i>Bacillus</i> sp. B15	+	5.63
	<i>Pseudomonas</i> sp. B17	+	5.02
	<b><i>Bacillus</i> sp. B2</b>	+	5.93
	<i>Bacillus</i> sp. B25	+	5.69
	<i>Bacillus</i> sp. B26	+	5.31
	<i>B. drentensis</i> B28	+	4.89
	<i>Sporosarcina</i> sp. B29	-	5.80
	<i>Bacillus</i> sp. B3	+	6.01
	<i>Bacillus</i> sp. B31	+	5.66
	<i>B. subtilis</i> B34	+	5.65
	<i>B. subtilis</i> B36	+	5.59
	<i>Staphylococcus</i> sp. B38	-	5.80
	<i>Staphylococcus</i> sp. B39	-	5.80
	<i>Staphylococcus</i> sp. B40	-	5.80
	<i>Staphylococcus</i> sp. B41	-	5.80
	<i>Staphylococcus</i> sp. B42	-	5.80
	<i>B. lentus</i> B6	-	5.80
	<i>Streptomyces olivochromogenes</i> B1	+	5.70
<i>Streptomyces sioyaensis</i> B9	+	4.52	
<i>Streptomyces olivochromogenes</i> B14	+	5.33	
Rhizosphere	<b><i>Bacillus</i> sp. R1</b>	+	5.77
	<i>Bacillus</i> sp. R10	-	5.80
	<i>Bacillus</i> sp. R13	+	5.55
	<i>Bacillus</i> sp. R16	+	5.75
	<i>Bacillus</i> sp. R19	+	5.25
	<i>Paenibacillus</i> sp. R2	+	5.05
	<i>Bacillus</i> sp. R22	+	5.55
	<b><i>Bacillus</i> sp. R23</b>	+	6.05
	<b><i>Bacillus</i> sp. R24</b>	+	6.05
	<i>B. drentensis</i> R25	+	5.75
	<i>B. drentensis</i> R28	+	5.75
	<i>Bacillus</i> sp. R30	+	5.48
	<i>Bacillus</i> sp. R32	+	5.51
	<i>Bacillus</i> sp. R33	+	5.90



Potential functional diversity and biotic interactions

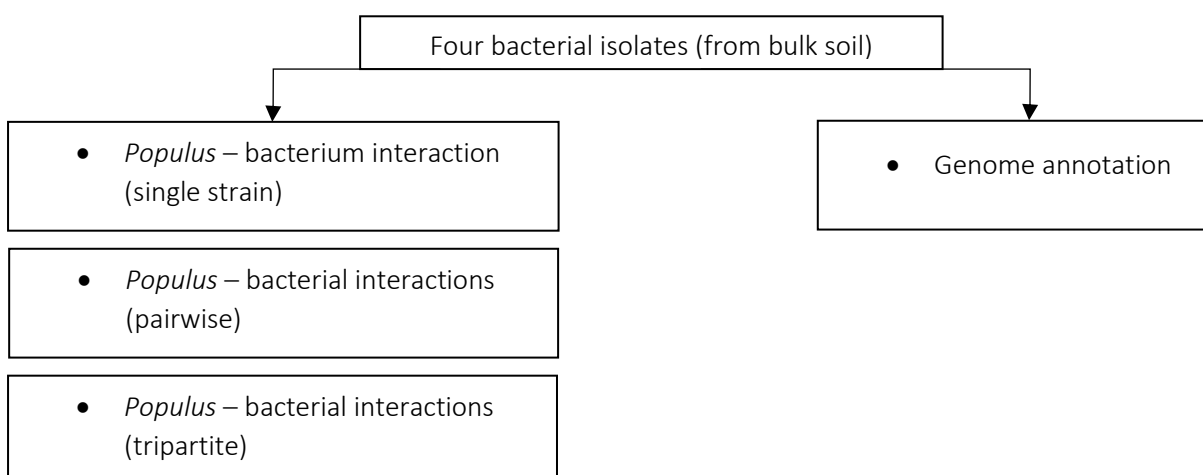
<i>Paenibacillus</i> sp. R34	+	4.85
<b><i>Bacillus</i> sp. R35</b>	+	5.40
<i>Bacillus</i> sp. R38	+	5.45
<i>B. drentensis</i> R39	+	5.75
<i>B. drentensis</i> R6	+	5.68
<i>Bacillus</i> sp. R7	+	5.88
<i>Staphylococcus</i> sp. R9	-	5.80
<i>Streptomyces olivochromogenes</i> R4	+	5.31
<i>Streptomyces sioyaensis</i> R15	+	5.65
<i>Streptomyces sioyaensis</i> R21	+	5.65
<i>Streptomyces sioyaensis</i> R27	+	5.65

# Chapter III

## Potential dialogues between bulk soil bacteria and *Populus*

## Context

Plants adapt to their environments and grow accordingly to optimize the acquisition of resources. Especially when stress is present (e.g. nutrient depletion, water limitation, competition), the root system modifies its architecture in order to explore and reach limited resources and, in parallel, exudates metabolites altering soil microbiome likely for the benefit of the tree. In return, plant associated bacteria can facilitate the fixation of atmospheric nitrogen, increase phosphorous uptake, produce phytohormones as growth promoter signals, inhibit pathogens and be involved in many other functions. Since soils are physically and chemically heterogeneous and comprise a mixture of habitats, there are plenty of possible bacterial interactions and biotic scenarios to unravel. The main goal in this chapter was to understand the effects at the molecular level of interactions between cultivable forest soil bacterial strains and with poplar trees. In this context, we performed interactions with ten, five and three bacterial strains isolated from a grain of bulk soil to study community effects on poplar development. Poplar root network were highly variable in the presence of the smallest consortium. In addition, poplar responses differed between single associations and consortium association. Thus, based on these preliminary experiments, we chose to work more in detail with four bacterial isolates coming from a grain of bulk soil. We analyzed poplar morphological and physiological changes in response to bacterial inoculation, individually and in combination. Effects of poplar on the size of bacterial colonies were also tracked. Finally, genome and metabolomics data were examined to provide insights of their behaviors. This chapter will be divided in two sections: i) plant – bacteria interactions ii) genome annotation and possible molecular dialogues.



## Introduction

Forest ecosystems are economically relevant for wood and biomass production (Achat *et al.* 2017), but most importantly, they support natural biodiversity, prevent soil erosion and are involved in mitigating the effects of climate change acting as CO<sub>2</sub> sinks (Lladó *et al.* 2017, Achat *et al.* 2017). Tree associated microbial communities are critical to sustain the health and growth of trees by enhancing nutrient assimilation and protecting against several abiotic and biotic constraints (Mercado-Blanco *et al.* 2018). These microbial associations confer such important functions, that in recent years, plants were no longer considered as standalone entities, but follow the holobiont concept i.e. as ecological units assembled with their microbiota (Vandenkoornhuyse *et al.* 2015, Cregger *et al.* 2018). DNA and RNA sequencing advances have enabled researchers to describe the composition of forest tree microbial communities and their structuring factors in order to better understand soil processes and its influence on trees (Lladó *et al.* 2017, Lasa *et al.* 2019).

Moreover, a wide variety of studies on cultured bacterial strains associated with plants have shown the effect of their metabolic feedbacks: for example, their ability to facilitate the fixation of atmospheric nitrogen to the host plant (Lira *et al.* 2015), to increase phosphorous uptake (Oteino *et al.* 2015), to produce phytohormones as growth promoter signals (Egamberdieva *et al.* 2017), and many others. Still, little is known about the effects of bacteria-bacteria interactions themselves on tree development. Here, we defined bacterial interactions as the production of specialized metabolites either soluble or volatile which can act as inhibitory, beneficial or neutral to other bacteria. Soluble and volatile compounds can act in direct physical contact or within a short distance diffusion (Tecon *et al.* 2018), while volatile organic compounds can also diffuse through air and gas pores in soil in relative 'longer'-distance interactions (Schmidt *et al.* 2019). For this reason, we wonder if bacteria inhabiting the same grain of soil, which are more likely to be interacting, are also affecting tree development. Several scenarios can be envisioned here assuming that bacteria interact and release these specialized metabolites: (i) neutral effect i.e. no observable effect on plant or surrounding microbes, (ii) a direct effect on plant growth (negatively or positively), (iii) these compounds affect other members of the soil microbiome that are essential for plant development, and therefore, there is an indirect effect on plant growth (negatively, positively), (iv) or more complex feedbacks driven by specific species-to-species interactions, where plant growth alters soil conditions that induce different specialized metabolites.

Because plants are immobile organisms, the degree of variability in plant architecture is high (Malamy 2005). Developmental plasticity allows plants to adapt to their environments and grow accordingly to optimize the acquisition of resources (White *et al.* 2013). Abiotic stresses (e.g. nutrient depletion,

water limitation) modulate the root system, changing the root length, number, angle and overall its architecture in order to reach limited resources (Koevoets *et al.* 2016). Hence, root patterns provide information about the variations in their surroundings. Additionally, as a response, plants exude metabolites modifying soil microbiome for their advantage (Canarini *et al.* 2019). Thus, we hypothesize that trees have an impact on bacterial interactions in return.

As soil microbiome can have complex and synergistic effects on plants (Timm *et al.* 2016), it is helpful to simplify the interaction system by selecting small numbers of bacterial isolates. Indeed, bacteria and plants in an *in vitro* interaction can provide essential information to disentangle the complexities of soil ecology. In this context, we studied poplar morphological changes in response to the inoculation of ten, five and three bacteria to see community effect on poplar development. Based on these preliminary experiments, we chose to work with consortia of three bacterial members as the major effect of plant was observed with this size of consortia. One consortium was composed by *Bacillus* sp. Bs2, *Pseudomonas* sp. Bs17 and *Streptomyces olivochromogenes* Bs14 (referred as *Streptomyces* Bs14), and in the second consortium *S. olivochromogenes* Bs14 was substituted by *Streptomyces sioyaensis* Bs9 (referred as *Streptomyces* Bs9) because of their contrasted behaviors. With these four bacterial isolates, we analyzed poplar morphological and physiological response to individual bacteria, pairwise combinations and tripartite consortia. We focused on important microbial mediated plant traits such as root size and architecture (Henning *et al.* 2016). Moreover, since carbon fixation by plants occurs through photosynthesis, we measured physiological traits such as chlorophyll content and the stress compound anthocyanin. In parallel, we also studied the effect that poplar had on bacterial colony size and on pairwise bacterial interactions. To have an idea of the nature of the metabolites involved in the interactions, we sequenced the four bacterial genomes and analyzed them.

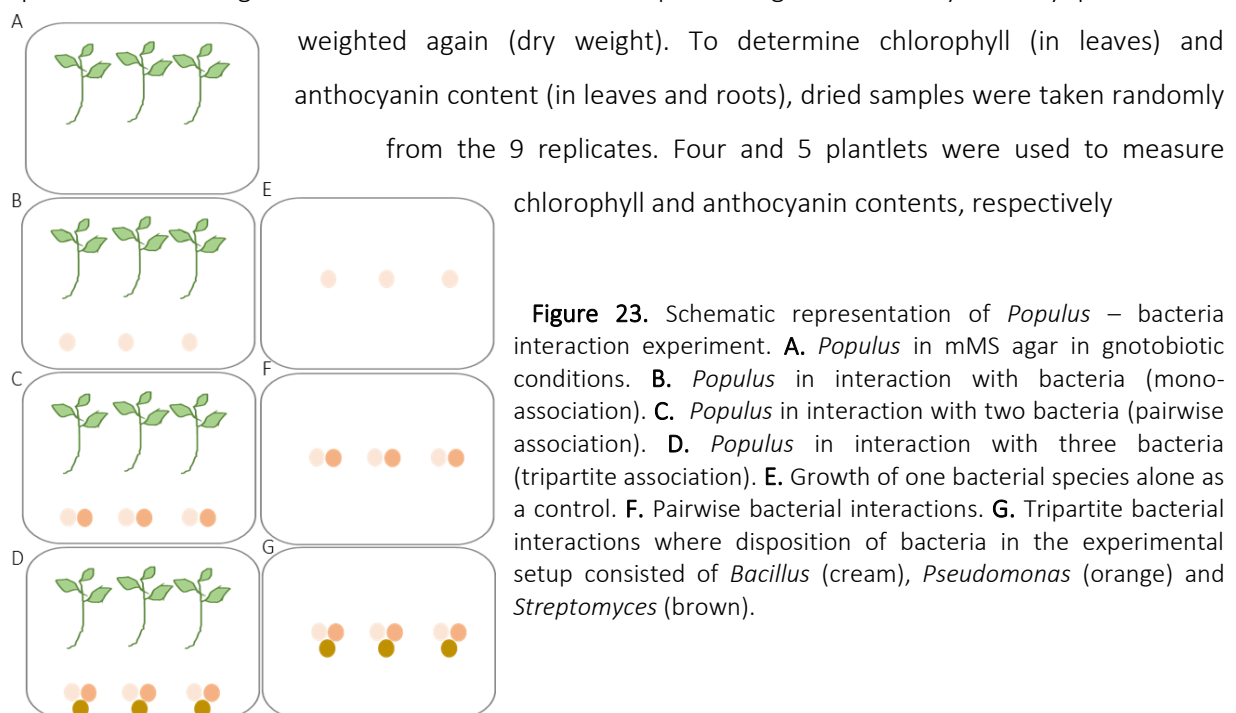
Overall, we found that the bacterial isolates could alter poplar root architecture. For instance, *Bacillus* Bs2 and *Streptomyces* Bs14 produced poplars with significantly more lateral roots, while *Pseudomonas* Bs17 produced poplars with significantly more lateral roots but smaller main root length and *Streptomyces* Bs9 produced smaller root networks and eventually necrosis. In pairwise combinations, regardless the combined isolate with *Streptomyces* Bs9, poplar root network remained significantly shorter, but no necrosis was evidenced. In tripartite association, the consortium was successful to reduce significantly *Streptomyces* Bs9 negative effects. Moreover, *Streptomyces* Bs14 colonies were significantly bigger when inoculated with poplar compared to inoculated alone. Additionally, bacterial interactions were different with and without poplar, suggesting poplar can interfere in the molecular dialogues between bacteria.

## Materials & Methods

### *Populus* seedlings – bacteria interaction

*Populus tremula* x *Populus alba*, clone INRA 717-1B4 (further named *Populus*) plantlets were prepared as described in Materials and Methods, Chapter II. *Bacillus* Bs2 and *Pseudomonas* Bs 17 were grown individually on 1/10 TSA from glycerol stocks and then transferred into TSB for 48h. The bacterial broth was washed three times and a bacterial suspension of OD<sub>600</sub>: 0.6 was prepared. *Streptomyces* Bs9 and *Streptomyces* Bs14 were diluted from spore glycerol stocks to prepare a suspension of 10<sup>8</sup> spores/ml, and heat-shock treated (50°C, 10 min) to induce spore germination before further inoculation (as described in Materials and Methods, Chapter II). Five microliters of bacterial/spore suspensions were inoculated 1.5 cms away from the tip of the root.

Inoculations were done individually, in all possible binary combinations and in tripartite associations (exchanging *Streptomyces* isolates) with and without *Populus* for comparison purposes (Figure 23). Plant-bacteria and bacterial interactions were done in 9 replicates per strain or stated otherwise. They were incubated together at 25°C with 16h photoperiods for 21 days. The incubation period is limited based on the size of the Petri dish plates, the size of plantlets, the nutrient availability over time and the water content in the agar. Plates were scanned every 7 days. Scan images were analyzed using ImageJ to measure the *Populus* root system, shoot and overall growth and the diameter of the bacterial colonies over time. After 21 days, all leaves and all the root system per plantlet were weighted fresh and then frozen in liquid nitrogen. Then they were lyophilized and



**Figure 23.** Schematic representation of *Populus* – bacteria interaction experiment. **A.** *Populus* in mMS agar in gnotobiotic conditions. **B.** *Populus* in interaction with bacteria (mono-association). **C.** *Populus* in interaction with two bacteria (pairwise association). **D.** *Populus* in interaction with three bacteria (tripartite association). **E.** Growth of one bacterial species alone as a control. **F.** Pairwise bacterial interactions. **G.** Tripartite bacterial interactions where disposition of bacteria in the experimental setup consisted of *Bacillus* (cream), *Pseudomonas* (orange) and *Streptomyces* (brown).

## *Potential dialogues between bulk soil bacteria and Populus*

### *Chlorophyll content from leaves*

Chlorophyll content was determined as previously described (Hall and Rao, 1999). Dried leaves were ground twice using metal beads and a tissue lyser for 30 secs. Dried leaf powder was resuspended in 500  $\mu$ L of 80% acetone and vortexed for 1 min. The mixture was centrifuged for 5 min at maximum speed. To measure chlorophyll content,  $A_{652\text{nm}}$  of the supernatant was measured using a spectrophotometer (Tecan Ultimate Pro).

### *Anthocyanin content from leaves and roots*

Anthocyanin content was determined as previously described (Ticconi *et al.* 2001). Dried leaves and dried roots were ground twice using metal beads and a tissue lyser for 30 secs. Then dried matter was resuspended in 500  $\mu$ L isopropanol/HCl 12N/H<sub>2</sub>O in proportion 18/1/81. Mixture was shaken and warmed in a water bath at 99°C for 3 minutes. The solution was centrifuged for 10 min at 10.000 g at 4°C. To measure anthocyanin content, difference of dual wavelength  $A_{650-535\text{nm}}$  was measured to the supernatant using a spectrophotometer (Tecan Ultimate Pro).

### *Volatile compounds from Streptomyces Bs9*

Since *Streptomyces* Bs9 had a strong antagonistic effect towards poplar, we assessed the ability to interact through volatiles as signal molecules using split Petri dishes. Bacterial spores were inoculated in one compartment and *Populus* in the other, and they were incubated as described previously for the *Populus* seedlings – bacteria interactions. Experiment was performed in triplicate.

### *Poplar recovery from Streptomyces Bs9*

Since *Streptomyces* Bs9 inhibited root growth and produced eventual necrosis, we assessed the ability of poplar to recover after transfer in fresh medium. Hence, we performed Bs9-poplar interaction as described previously. After 21 days of incubation together, we transferred poplar into a new fresh mMS agar and observed poplar development for 21 extra days.

### *Constitutive or induced effects of Streptomyces Bs9*

To assess if the antagonistic effect on poplar was constitutively produced by Bs9 or induced by poplar, we performed plant – bacteria interaction in tubes containing liquid mMS and glass beads. Five microliters of spore suspension ( $10^8$  spores/ml) was inoculated in 50 mL liquid mMS and growth was performed for 7 days under agitation (200 rpm) at 25°C. Bacterial suspension was diluted 1/10<sup>th</sup> in liquid mMS and used for inoculation. Supernatant of the Bs9 strain culture was obtained by filtering (0.22  $\mu$ m) the diluted suspension. Poplar in gnotobiotic conditions (negative control) was grown in 10

### *Potential dialogues between bulk soil bacteria and Populus*

mL liquid mMS. Poplar treated with the Bs9 strain was grown with 10 mL diluted Bs9 bacterial suspension, or with 10 mL of filtrated Bs9 culture supernatant. Tubes were incubated at 25°C with 16h photoperiod for 21 days and analyzed at the end. Experiments were performed in triplicate.

### *Statistical analyses*

Statistical analyses were conducted with R software version 3.4.3 (R core Team 2016). Data were verified for normality using Shapiro-Wilk's method (Royston 1982). If data was normally distributed, ANOVA test was used, if not normal then data was analyzed using non-parametric test Kruskal Wallis test by ranks and p-value was adjusted with Bonferroni test (Conover 1999). The threshold of significance was fixed for  $p < 0.05$ .

### *Genome sequencing*

Genomic DNAs of the *Streptomyces* strains were individually extracted as previously described (Pospiech and Neumann 1995). Genomic DNAs of *Bacillus* and *Pseudomonas* were extracted as described (Cheng *et al.* 2006). DNAs were sent for sequencing at a concentration of 10 ng/ $\mu$ L. Genomes of *Bacillus*, *Pseudomonas* and of the two *Streptomyces* were sequenced at I2BC (Paris, France) through Next Generation Sequencing platform with an Oxford Nanopore technology. Sequences were assembled using Canu 1.7.1 (Koren *et al.* 2017) and corrected using Nanopolish 0.11.1 (Loman *et al.* 2015). Genome annotations were obtained from Rapid Annotation using Subsystem Technology server (RAST) (Aziz *et al.* 2008, Brettin *et al.* 2015), biosynthetic gene clusters were predicted using antiSMASH 5.0 (Kai *et al.* 2019), bacteriocins and ribosomally synthesized and posttranslationally modified peptides (RiPPs) using BAGEL3 (van Heel *et al.* 2013). Genes were confirmed by searching in the NCBI non-redundant protein sequences database using Position-Specific Iterated BLAST.

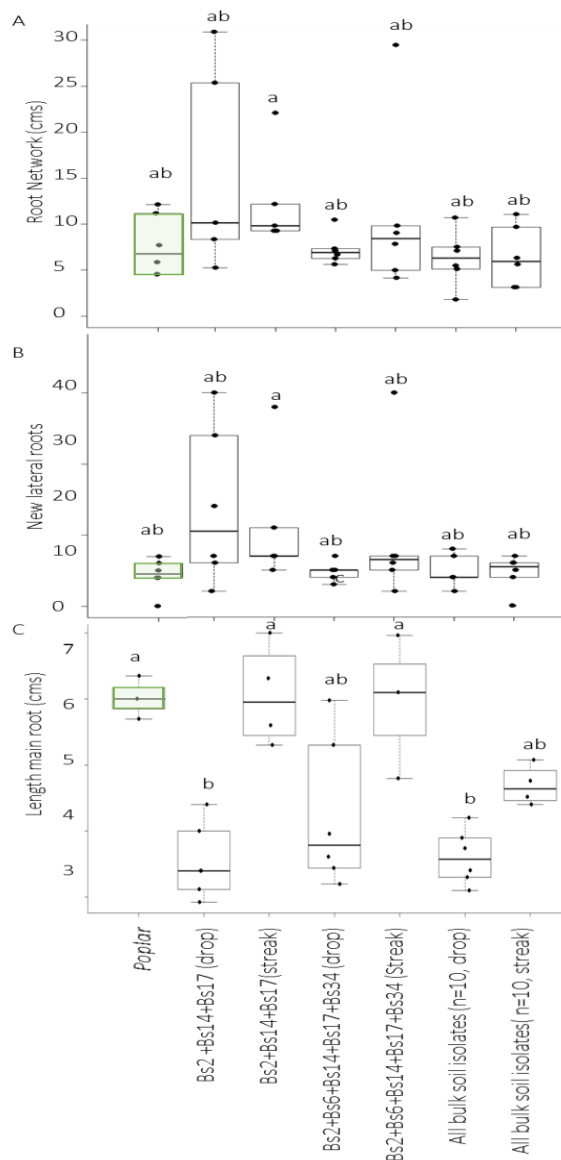


## Results

### 3.1 *Populus* – bacteria interactions

#### 3.1.1 First community trial

In order to simplify the plant-bacteria interaction system, we selected a defined bacterial community from one grain of soil. We inoculated all bulk soil isolates (n=10) and reduce the complexity of the community into intermediate (n= 5) and small (n=3) complexity levels, picking the isolates randomly from the previous batch. Here, bacteria were grown individually, mixed at the same OD<sub>600</sub> and then co-cultured either in the form of a drop or a streak. None of these synthetic communities had a significant effect on *Populus*'s root network development, but we noticed a high variability in the root network size and an alteration of the root architecture when incubated with the smallest community (Figure 24). Interestingly, we found that the length of the main roots was two times smaller when



bacteria were inoculated in drop compared to streaks. Colony forming unit counts (CFU) from the three mixtures showed that the bacterial isolates were inhibiting strongly each other (data not shown). Bacterial CFUs belonged mostly to *Pseudomonas* and few were *Streptomyces*.

**Figure 24.** Poplar physical traits measured (**A:** Root network, **B:** New lateral roots formation, **C:** Length of the main root in cms) together with bacterial mixtures (drop and streak) incubated on mMS for 21 days. Values represent the mean, dispersal and standard deviation of 6 replicates, and boxplots are organized in descendent order. As a negative control, poplar was incubated alone (indicated in green). Significance (letter on top of each boxplot) was calculated with Kruskal-Wallis and adjusted with Bonferroni test. Different letters mean they are significantly different ( $p < 0.05$ ).

**Note:** All bulk soil isolates consisted of *Bacillus* sp. Bs2, *Bacillus lentus* Bs6, *Streptomyces sioyaensis* Bs9, *Streptomyces olivochromogenes* Bs14, *Pseudomonas* sp. Bs17, *Bacillus drentensis* Bs28, *Sporosarcina* sp. Bs29, *Bacillus subtilis* Bs34, *Bacillus subtilis* Bs36, *Staphylococcus* sp. Bs42  
Five bulk soil isolates consisted of *Bacillus* sp. Bs2, *B. lentus* Bs6, *S. olivochromogenes* Bs14, *Pseudomonas* sp. Bs17, *B. subtilis* Bs34  
Three bulk soil isolates consisted of *Bacillus* sp. Bs2, *S. olivochromogenes* Bs14, *Pseudomonas* sp. Bs17

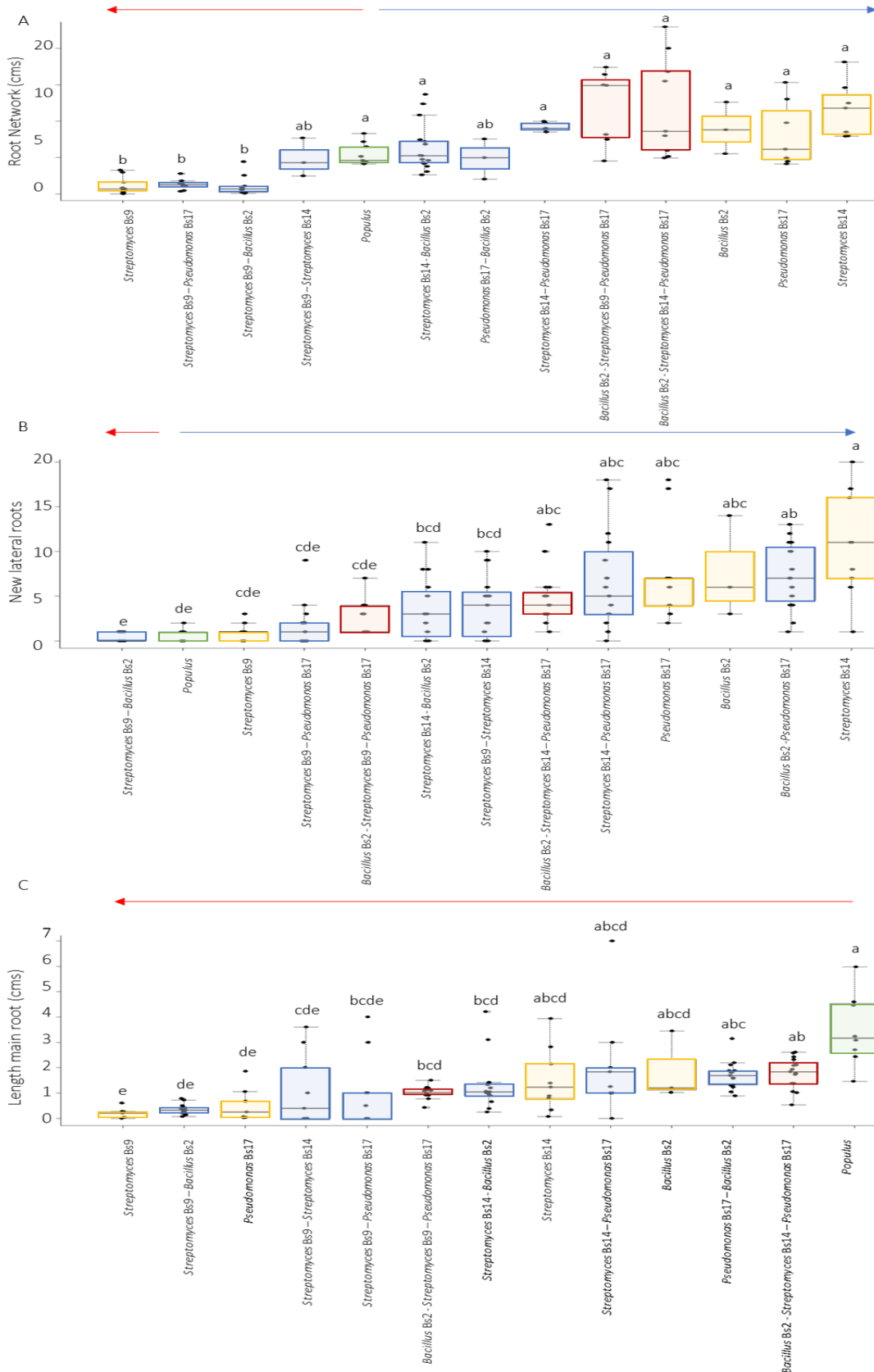
For this reason, we decided to repeat the interaction with the small community (n=3, *Bacillus* Bs2, *Streptomyces* Bs14 and *Pseudomonas* Bs17), but inoculating them separately next to each other in drops (Figure 23). Here, the spatial distribution, by colony, would allow bacteria to interact with each other by producing molecules that may impact poplar development and to still co-exist. In parallel, since there are few *Streptomyces* causing plant diseases in literature, we decided to include *Streptomyces* Bs9 to evaluate if bacterial interactions can attenuate its inhibitory effect on root growth (Chapter II). In order to understand how this consortium affects poplar, we deconstructed this community into their single bacterial strains (mono-associations) and the binary combination (pairwise interactions) and evaluated their ability to alter plant phenotype in all the possible combinations.

### **3.1.2 Second community trial**

#### **3.1.2.1 Mono-associations**

*Populus* response to the incubation with four different bacterial strains individually showed several root pattern alterations. *Streptomyces* Bs9 reduced significantly the root network size and induced the production of necrotic tissues (Figure 25A), while the other bacteria increased significantly new lateral root production (Figures 25A and 25B). In addition, *Pseudomonas* Bs17 and *Streptomyces* Bs9 decreased significantly the length of the main root (Figure 25C). Overall, each bacterial strain changed the dynamic of the root architecture of *Populus*. However, the root and shoot biomass were not significantly different between *Populus* treated and non-treated (Figures 26A and 27A). Additionally, none of the strains influenced chlorophyll or anthocyanin content in leaves of *Populus* after 21 days of incubation (Figures 26B, 26C and 27B). By contrast, anthocyanin content in the roots was significantly increased in *Populus* roots inoculated close to *Streptomyces* Bs9 and *Pseudomonas* Bs17 compared to gnotobiotic conditions (Figure 27B). Altogether, the data suggest that poplar reacts differently to different individual bacterium, but mostly showed alteration of the root architecture.

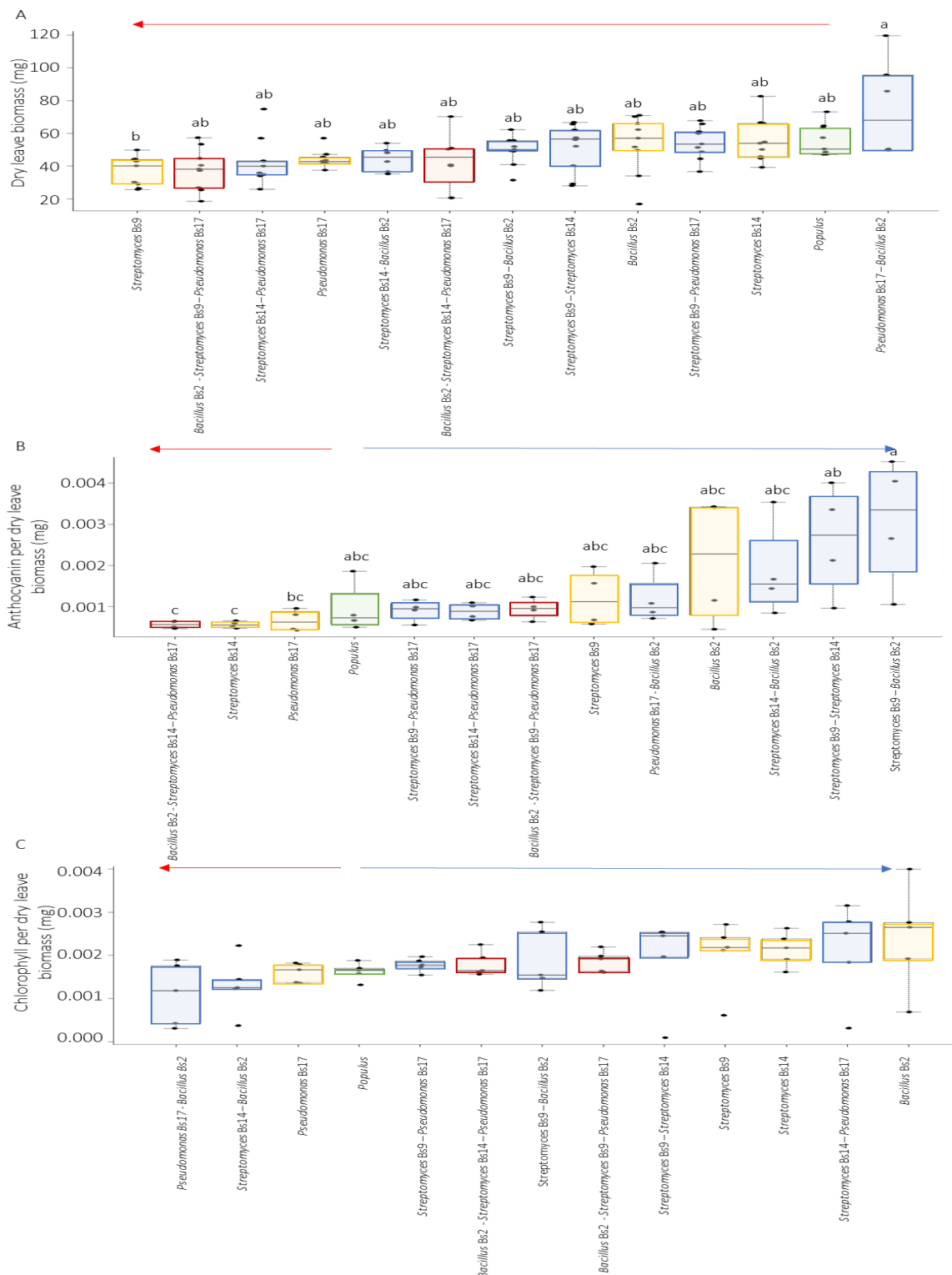
Potential dialogues between bulk soil bacteria and *Populus*



**Figure 25.** Poplar root physical traits measured (**A:** Root network, **B:** New lateral roots formation, **C:** Length of the main root in cms) together with a single bacteria (yellow), or with binary (blue) and tripartite bacterial associations (red) incubated on mMS for 21 days. As a negative control, poplar was incubated alone and it is denoted in green. Values represent the mean, dispersal and standard deviation of 9 replicates, and boxplots are organized in descendent order. Significance (letter on top of each boxplot) was calculated with Kruskal-

Potential dialogues between bulk soil bacteria and *Populus*

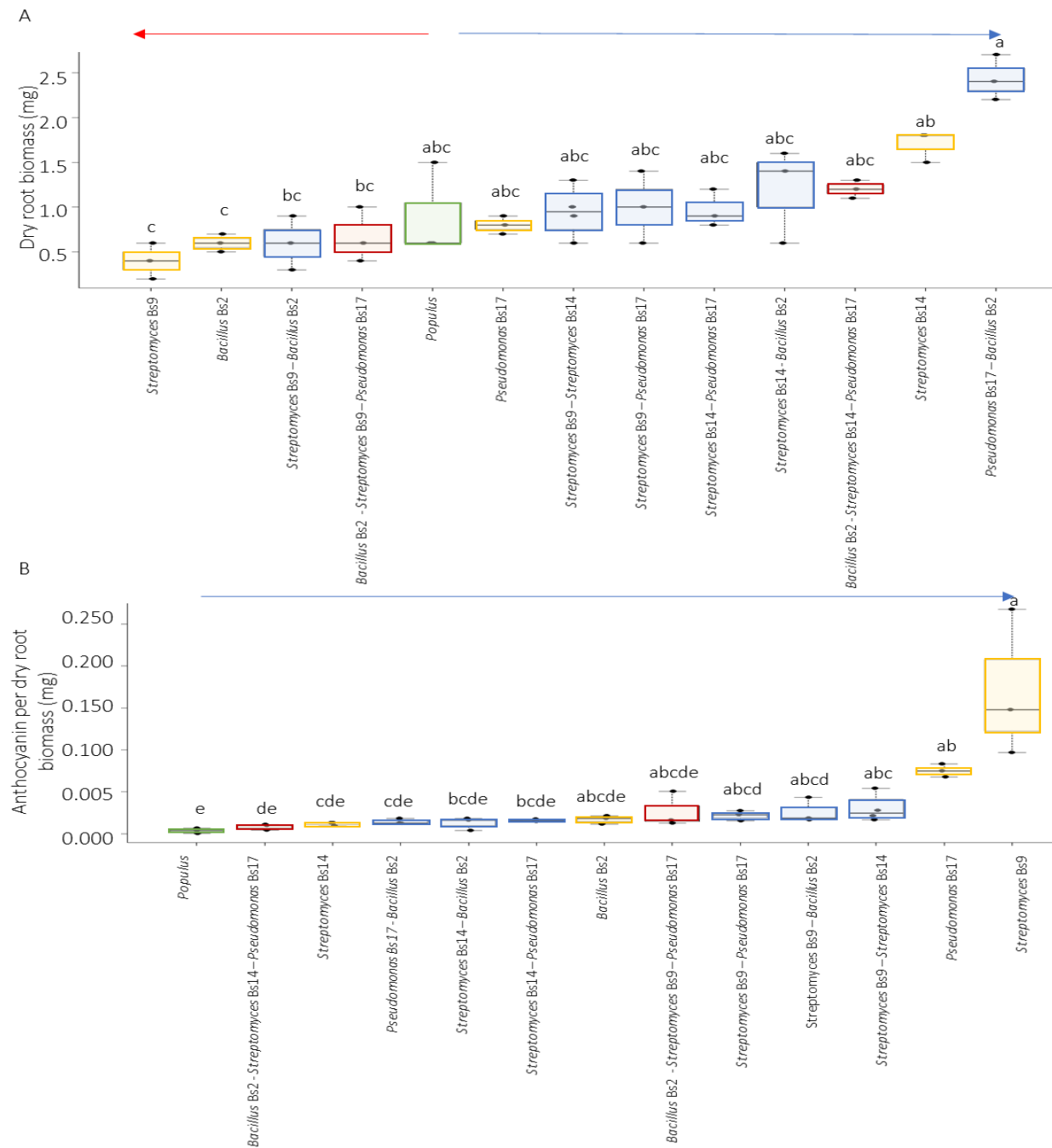
Wallis and adjusted with Bonferroni test. Different letters mean they are significantly different ( $p < 0.05$ ). Blue arrows indicate higher than negative control and red arrows indicate lower than negative control. **Note:** for poplar inoculated with the pair *Streptomyces* Bs9 - *Pseudomonas* Bs17, number of replicates was 8 instead of 9 due to *Pseudomonas* Bs17 inhibitory effect.



**Figure 26.** Poplar leaf physiological traits measured (**A**: Biomass of leaves in milligrams (9 replicates), **B**: Anthocyanin content in leaves per milligram of leaf (4 replicates), **C**: Chlorophyll content in leaves per milligram of leaf (5 replicates) together with a single bacteria (yellow), or with binary (blue) and tripartite bacterial associations (red) incubated on mMS for 21 days. As a negative control, poplar was incubated alone and it is denoted in green. Values represent the mean, dispersal and standard deviation of the replicates, and

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boxplots are organized in descendent order. Significance (letter on top of each boxplot) was calculated with Kruskal-Wallis and adjusted with Bonferroni test. Different letters mean they are significantly different ( $p < 0.05$ ) and no letter indicates there were no significant changes. Blue arrows indicate higher than negative control and red arrows indicate lower than negative control.



**Figure 27.** Poplar root physiological traits measured (**A**: Biomass of roots in milligrams, **B**: Anthocyanin content of roots per milligram of root) together with a single bacteria (yellow), or with binary (blue) and tripartite bacterial associations (red) incubated on mMS for 21 days. As a negative control, poplar was incubated alone and it is denotated in green. Values represent the mean, dispersal and standard deviation of 4 replicates, and boxplots are organized in descendent order. Significance (letter on top of each boxplot) was calculated with Kruskal-Wallis and adjusted with Bonferroni test. Different letters mean they are significantly different ( $p < 0.05$ ). Blue arrows indicate higher than negative control and red arrows indicate lower than negative control.

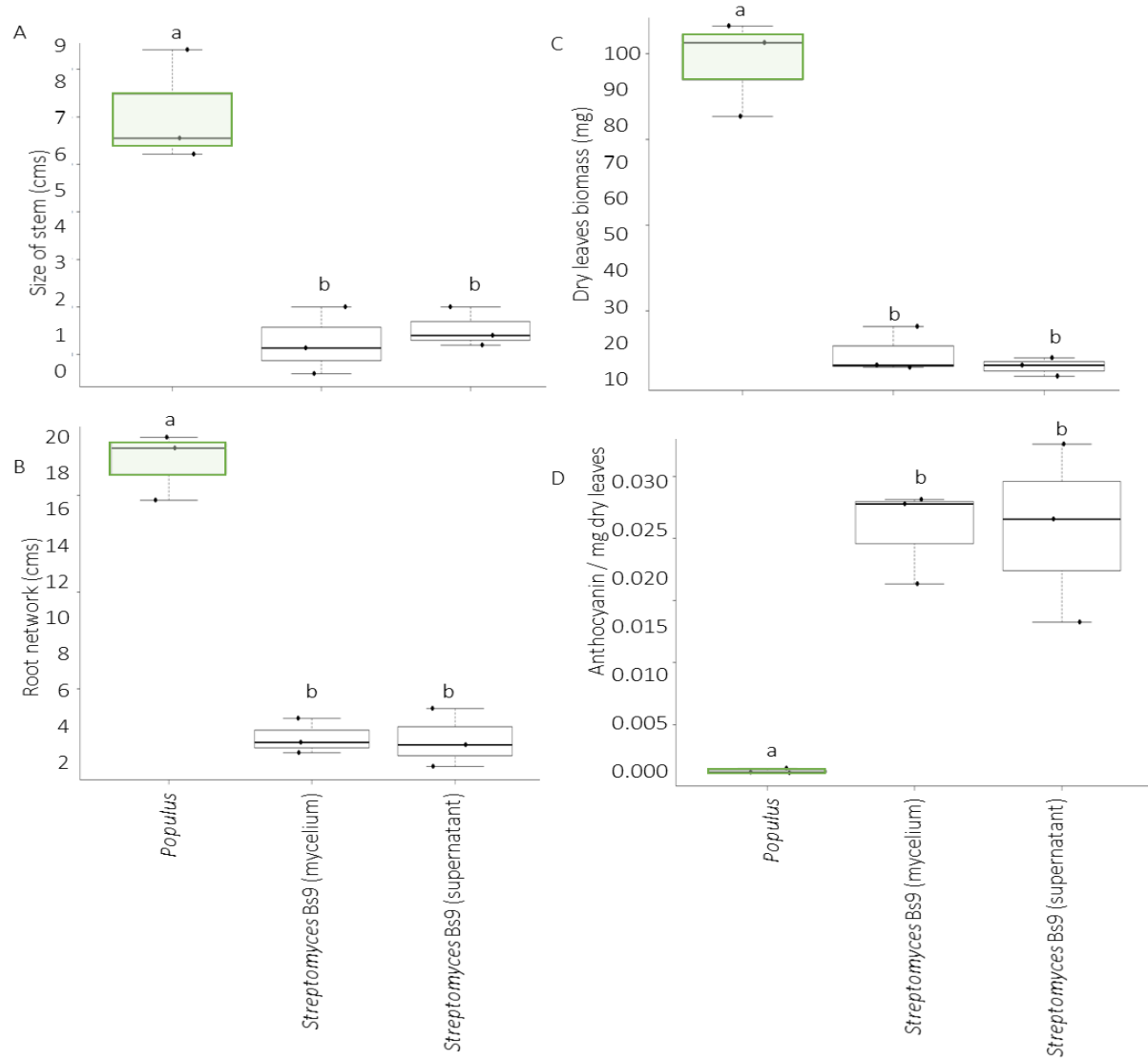
### ***Streptomyces Bs9 effect in details***

Since there are very few antagonistic actinomycetes in literature affecting plant development (Locci 1994) and the effect of *Streptomyces Bs9* on *Populus* was consistent and reproducible, we wanted to investigate deeper this effect.

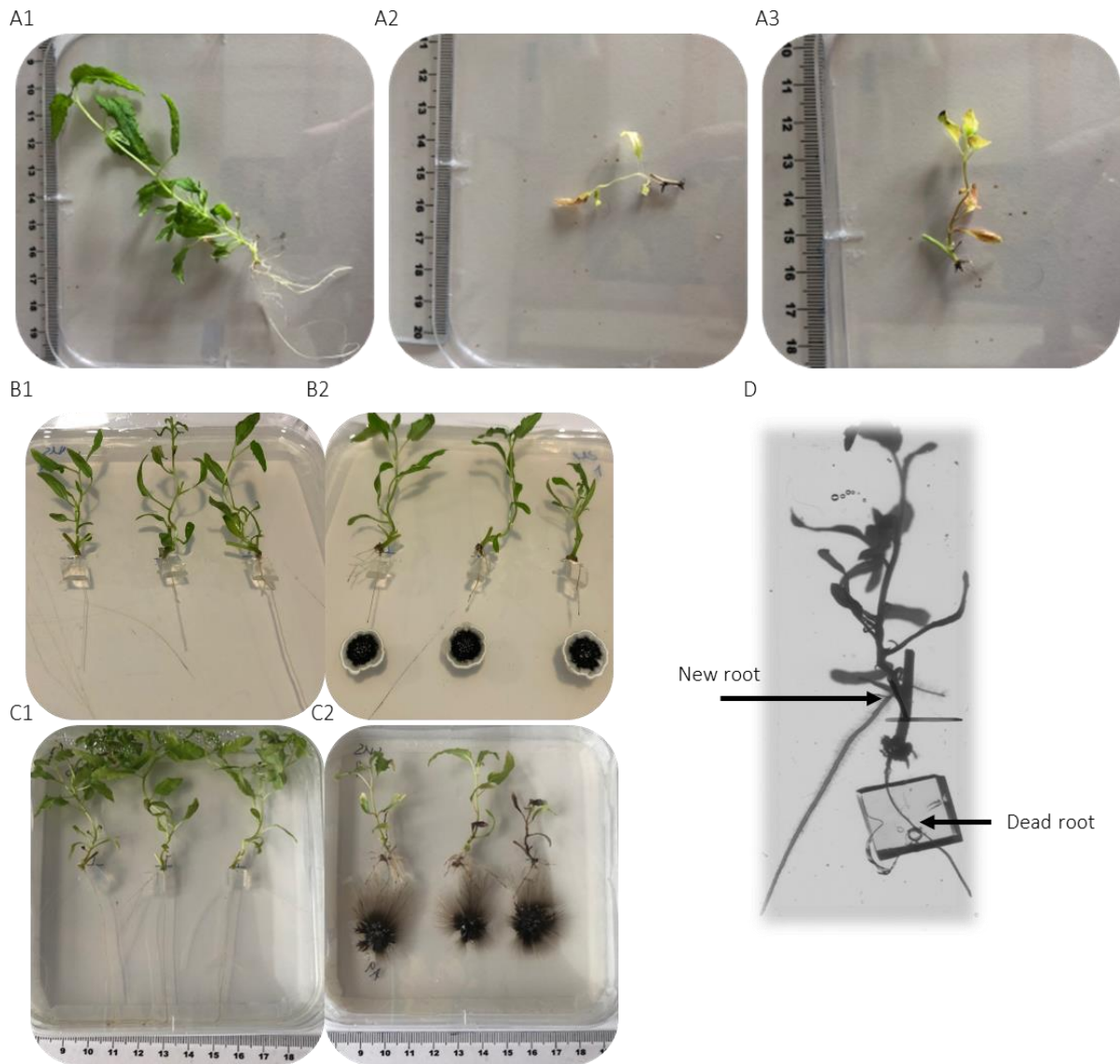
First, we tested its ability to inhibit poplar growth in separate compartments to evaluate the potential impact of volatile organic compounds in this phenotype. In this setup, *Streptomyces Bs9* did not affect the growth of poplar (data not shown) suggesting that the antagonistic effect does not occur through volatile compounds but through soluble molecules. In order to evaluate if the damaging compound was constitutively produced by *Streptomyces Bs9* or induced by the presence of the plant, *Populus* was grown with 7-day old *Streptomyces Bs9* mycelium, with a supernatant of 7d culture of Bs9 and with liquid mMS alone as a negative control. After 21 days of incubation, *Populus* treated with mycelium and supernatant were both significantly smaller in size and in biomass (roots and shoots) than *Populus* growing in fresh mMS (Figures 28A, 28B and 28C). Moreover, anthocyanin in leaves was significantly (16 times) higher in *Populus* inoculated with the bacterial culture and with the supernatant (Figure 28D). Root system was intensively damaged; thus, it was not possible to measure anthocyanin content in this case (Figure 29A). Altogether, the data suggest that the phytotoxic compound is constitutively produced by the bacterial strain in the tested conditions.

Next, we wondered how permanent the damage from *Streptomyces Bs9* towards *Populus* was. While treated *Populus* had a significant smaller root system from day 7 and necrotic roots after 21 days of incubation (Figure 29B), longer incubation period with *Streptomyces Bs9* (2 months) was lethal (Figure 29C). Interestingly, at this time point, *Streptomyces Bs9* had a different phenotype with hyphae pointing towards poplar roots, indicating a possible chemoattraction. In order to observe if the damage was permanent, we worked with *Populus* plants incubated with *Streptomyces Bs9* for 21 days since the damage was already located only in the roots. Then, these poplars were transferred into new fresh mMS agar for another 21 days to evidence recovery or not. In this test, roots did not recover, however, from the next healthy stem node, *Populus* could produce a total new root system (Figure 29D), suggesting the damage was localized to the apex of the existing roots.

Potential dialogues between bulk soil bacteria and *Populus*



**Figure 28.** Poplar traits measured (A: Size of stem, B: Root network, C: Dry leaves biomass, D: Anthocyanin content per dry leaves) together with *Streptomyces Bs9* mycelium or supernatant incubated in mMS for 21 days. As a negative control, poplar was incubated alone indicated in green. Values represent the mean, dispersal and standard deviation of 3 replicates. Significance (letter on top of each boxplot) was calculated with Kruskal-Wallis and adjusted with Bonferroni test. Different letters mean they are significantly different (p < 0.05).



**Figure 29.** Poplar interactions with *Streptomyces* Bs9 in different settings and incubation times. **A1.** Poplar grown in glass beads supplemented with mMS liquid for 21 days, **A2.** Poplar grown in glass beads supplemented with mMS liquid inoculated with *Streptomyces* Bs9 supernatant for 21 days, **A3.** Poplar grown in glass beads supplemented with mMS liquid inoculated with *Streptomyces* Bs9 bacterial suspension for 21 days, **B1.** Poplar grown on mMS agar for 21 days, **B2.** Poplar grown together with *Streptomyces* Bs9 after 21 days of incubation had the shortest root system and started to have necrosis, **C1.** Poplar grown on mMS agar for 2 months, **C2.** Poplar grown together with *Streptomyces* Bs9 after 2 months of incubation suffered necrosis in aboveground organs, **D.** Poplar grown together with *Streptomyces* Bs9 on mMS agar for 21 days of incubation transferred into fresh mMS agar can regenerate new roots from the top of the stem, while the original root system remained non-growing and in a necrotic state.

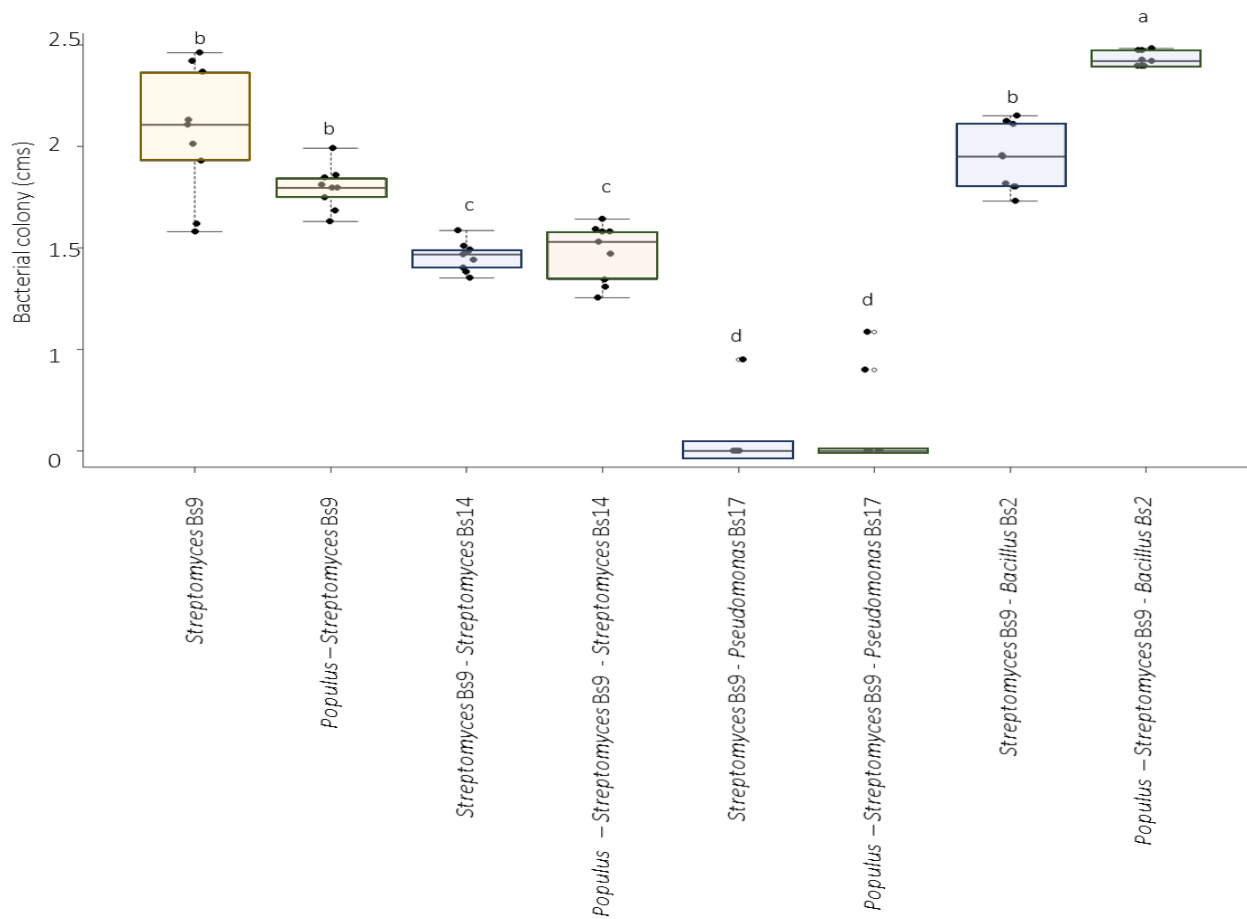


### **3.1.2.2 Pairwise associations**

All binary combinations with *Streptomyces* Bs9 decreased significantly the root network size compared to uninoculated control, indicating that the antagonistic effect of *Streptomyces* Bs9 remained active. However, roots were no longer suffering necrosis, suggesting a possible reduction of the negative effect (Figure 25A). Interestingly, *Streptomyces* Bs9 was strongly inhibited by *Pseudomonas* Bs17, having colonies four times smaller and in some cases Bs9 strain could not form visible colonies (Figure 30), nevertheless, the antagonistic effect remained. It is possible that the antagonistic compound from *Pseudomonas* Bs17 against Bs9 strain can have a detrimental effect on poplar roots .

Bacterial combinations that did not include Bs9 strain had no significantly effect on the root network size compared to the uninoculated control (Figure 25A). Moreover, *Pseudomonas* Bs17 - *Streptomyces* Bs14 and *Pseudomonas* Bs17 - *Bacillus* Bs2 combinations increased significantly the production of new lateral roots compared to uninoculated control (Figure 25B). All pairwise combinations with *Streptomyces* Bs9 and additionally the pair *Streptomyces* Bs14 – *Bacillus* Bs2 reduced significantly the length of main root compared to gnotobiotic conditions (Figure 25C).

Some pairwise interactions changed the behavior towards plants compared to the individual interaction. For instance, *Streptomyces* Bs14 in pair with either *Bacillus* Bs2 or *Streptomyces* Bs9 produced significantly fewer lateral roots compared to individual Bs14 strain – poplar interaction (Figure 25B). Likewise, *Bacillus* Bs2 produced fewer lateral roots together with *Streptomyces* Bs9 (Figure 25B). Poplar inoculated with only *Pseudomonas* Bs17 showed short main roots, but when accompanied with *Bacillus* Bs2 poplar main root was significantly longer (Figure 25C). No combination had an effect on chlorophyll content (Figure 26C), and only pair combinations with *Streptomyces* Bs9 significantly stimulated the production of anthocyanin in the roots (Figure 27B).



**Figure 30.** Bacterial colony size of *Streptomyces* Bs9 inoculated alone (yellow) and in binary combination (blue) with poplar (green outline) or without poplar incubated on mMS for 21 days. Values represent the mean, dispersal and standard deviation of 9 replicates. Significance (letter on top of each boxplot) was calculated with Kruskal-Wallis and adjusted with Bonferroni test. Different letters mean they are significantly different ( $p < 0.05$ ). Note: *Streptomyces* Bs9 number of replicates were 8 instead of 9 due to *Pseudomonas* Bs17 inhibitory effect.

### 3.1.2.3 Tripartite associations

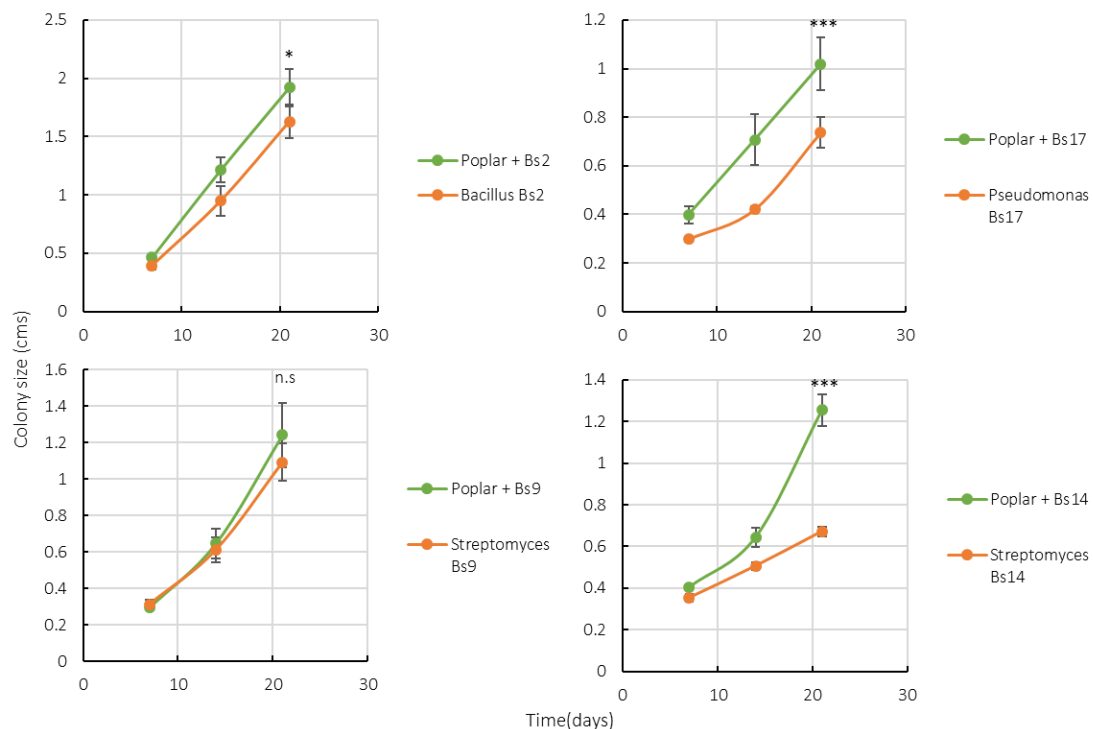
Both tripartite associations performed in this experimental design showed no significant changes in the size of the root network compared to poplar alone. Bs2Bs14Bs17 combination increased significantly lateral root formation, while Bs2Bs9Bs17 reduced the length of the main root, meaning that the different interactions between bacteria led to various modifications of the root architecture compared to poplar alone (Figure 25). While pairwise interactions did not reduce the Bs9 strain detrimental effect on poplar, interestingly, the consortium including *Streptomyces* Bs9 did significantly limit the damage. This consortium produced poplars with significantly bigger root network mainly due to a significantly increased of the length of the main root compared to Bs9 strain alone and Bs9 in binary combinations. Moreover, poplars incubated with this consortium did not show necrosis in roots. It is important to mention at this point that, even though *Pseudomonas* Bs17 had an inhibitory effect on *Streptomyces* Bs9, Bs9 strain was able to grow in all the replicates for the

tripartite association. Altogether, the data suggest that Bs9 consortium limited *Streptomyces* Bs9 antagonistic effect by interfering with the production of chemical compounds.

The consortium with Bs14 did not differ from the binary combinations, suggesting that bacterial interactions did not affect the overall development of poplar, in this case. Finally, there was no significant effect in the biomass of roots, shoots and neither the chlorophyll and anthocyanin content (Figures 26 and 27).

### 3.1.3 *Populus* influences bacterial colony size and interactions

Plants modify their local environment by releasing chemicals through the root system (Raynaud *et al.* 2007). For this reason, we evaluated the effect that poplar might have in bacterial colony size. In order to determine if *Populus* can influence bacterial behaviors, *Populus* was inoculated with one single bacterial strain each time and bacterial colony area was measured over time. Results showed that *Populus* had no antagonistic effect on any strain. Instead, bacterial colonies from *Bacillus* Bs2, *Streptomyces* Bs14 and *Pseudomonas* Bs17 were significantly bigger when inoculated with *Populus* after 21 days of incubation compared to when inoculated alone (Figure 31). On the contrary, the colony size of *Streptomyces* Bs9 did not significantly differ when grown alone or together with Poplar (Figure 31).



**Figure 31.** Poplar effect on the diameter of colonies of single bacterial isolates incubated on mMS agar over a period of 21 days. Values represent the mean of 9 replicates and error bars are standard deviations. Significance was calculated with student's t-test. (\*\*\*:<0.001, \*\*:0.001-0.01, \*:0.01-0.05, n.s:>0.05)

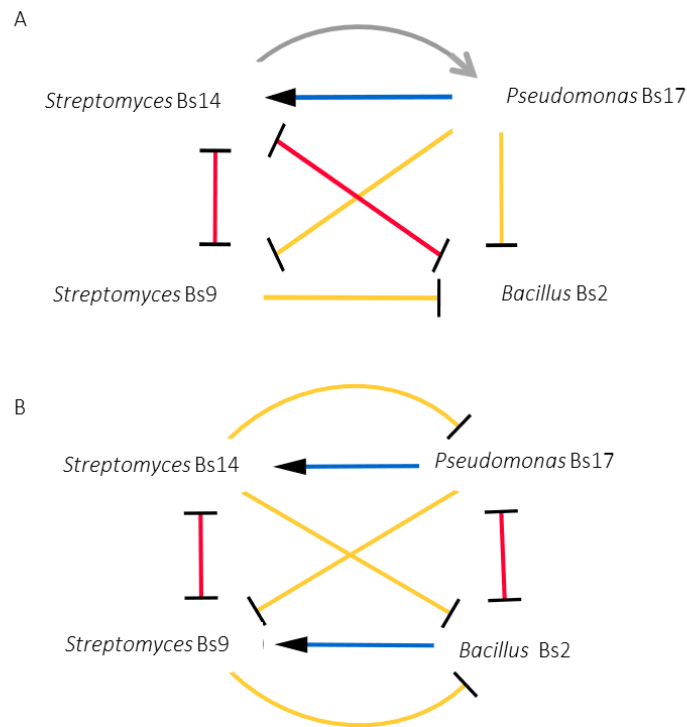
In a next step, we compared pairwise interactions on mMMS agar to explore the influence of poplar exudates (soluble and volatiles) on bacterial interactions (with and without poplar present in the set up) (Figure 23).

Bacterial pairwise interactions with *Streptomyces* Bs9 including or not *Populus* allowed us to check if interactions can influence Bs9 antagonistic behavior towards *Populus*. *Streptomyces* Bs14 and *Pseudomonas* Bs17 inhibited the growth of Bs9 significantly regardless the presence of *Populus*, and *Bacillus* Bs2 did not have any effect on Bs9 (Figures 30 and 32). Even though *Streptomyces* Bs14 and *Pseudomonas* Bs17 inhibited the growth of *Streptomyces* Bs9, root growth inhibition of *Populus* still remained (Figure 25). Interestingly, anthocyanin content tended to be reduced in poplar in binary combinations including Bs9 compared to *Streptomyces* Bs9 alone (Figure 27). However, the effect was not statistically significant (Figures 26 and 27). We could wonder if longer time of incubation would allow the recovery of poplar among these interactions. In addition, *Streptomyces* Bs9 colony was significantly bigger together with *Populus* and *Bacillus* Bs2 compared to any other combination (Figure 30), suggesting a possible advantage in this interaction.

Among pairwise bacterial interactions after 21 days of incubation without Poplar, *Pseudomonas* Bs17 was the strongest competitor reducing the size of *Bacillus* Bs2 and *Streptomyces* Bs9 colonies (Figure 32). Moreover, *Pseudomonas* Bs17 was not inhibited by any other bacterial strain and also promoted an increase of the colony size of *Streptomyces* Bs14 in the absence of poplar. This later also inhibited *Streptomyces* Bs9 and *Bacillus* Bs2, but it was reciprocally inhibited by these two strains. *Bacillus* Bs2 only inhibited *Streptomyces* Bs14, and was inhibited by all the other partners, being the most susceptible (Figure 32).

When bacterial interactions occurred in the presence of *Populus*, some interactions changed. It is interesting to notice how *Pseudomonas* Bs17 became more susceptible in the presence of poplar. For instance, *Streptomyces* Bs14 changed from neutral to inhibition against *Pseudomonas* Bs17 in this condition and *Bacillus* Bs2 went from neutral to inhibition against *Pseudomonas* Bs17. In addition, *Bacillus* Bs2 stopped inhibiting *Streptomyces* Bs14 and started to promote the growth of *Streptomyces* Bs9 (Figure 32).

### Potential dialogues between bulk soil bacteria and *Populus*



**Figure 32.** Scheme of the outputs of pairwise interactions between 4 bacterial strains isolated from bulk soil co-inoculated by pair simultaneously. **A:** mMMS agar after 21 days of incubation; **B:** mMMS agar after 21 days of incubation together with *Populus*. The direction of the interaction is marked by a bar for inhibition and an arrow for promotion of growth. Color of connecting lines indicate the type of interaction: red – mutual inhibition, yellow – unidirectional inhibition, grey – no visible effect on growth, blue – promotion of growth.

### 3.2 Draft genomes of bacterial isolates.

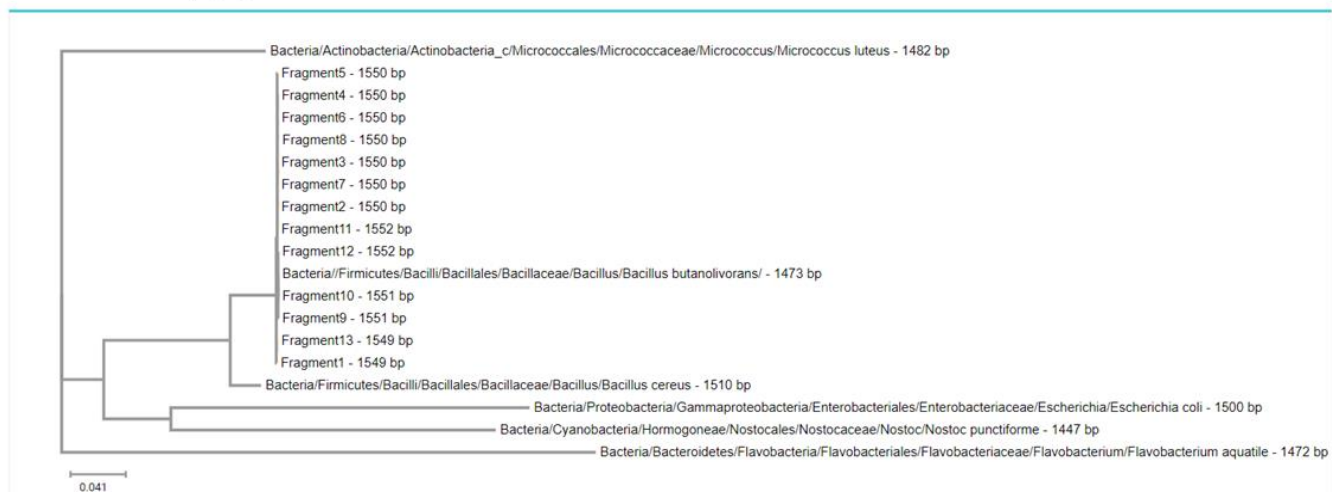
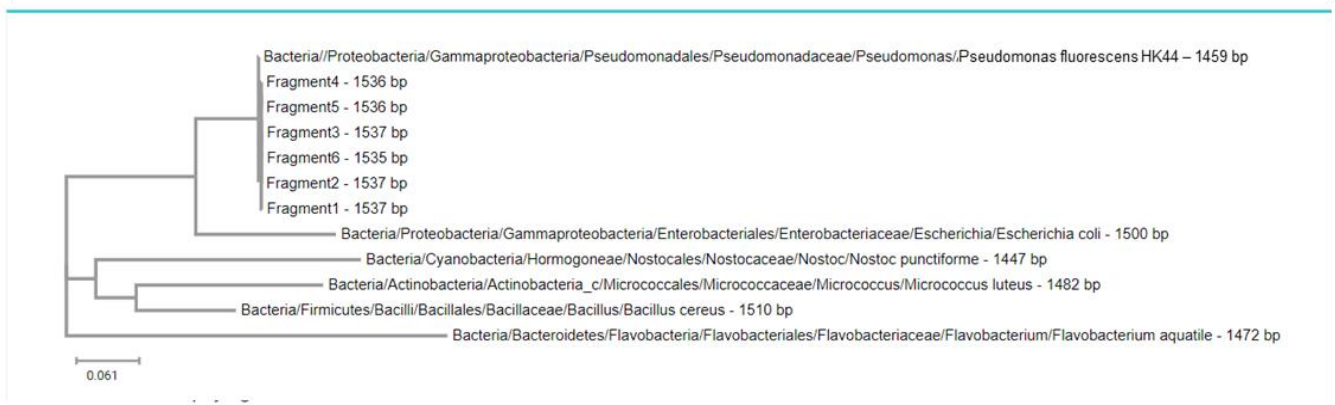
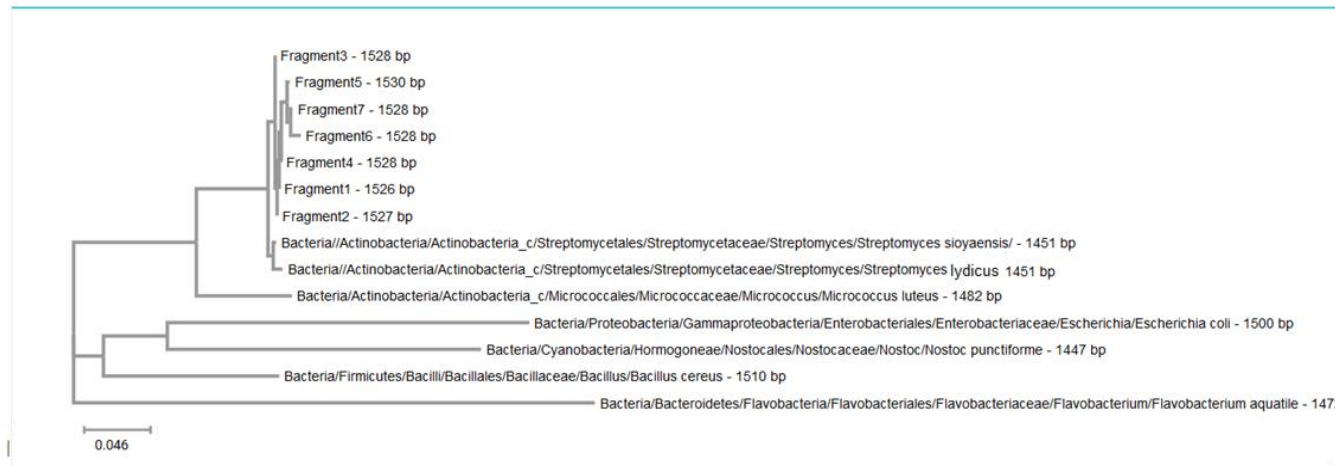
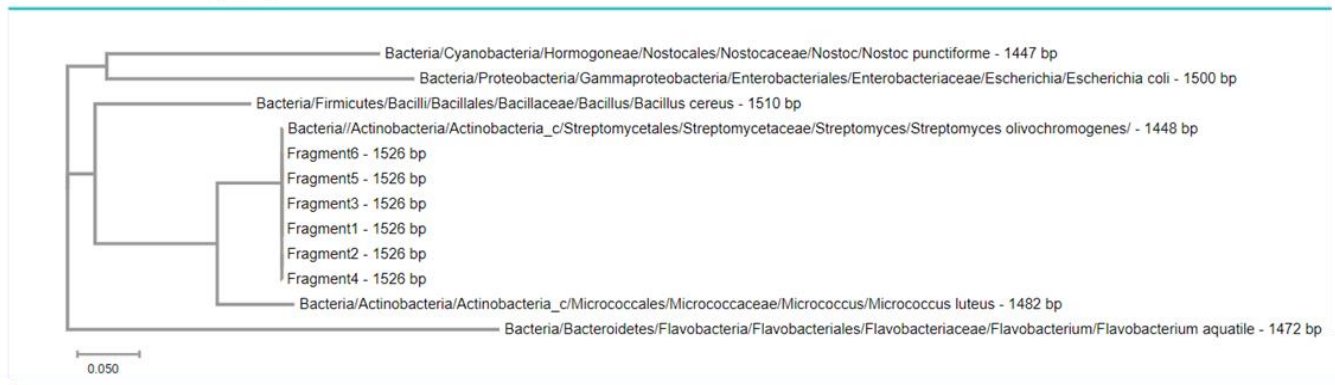
In order to obtain a preliminary idea of the molecules that could be involved in these interactions, the genomes of the four strains were sequenced. Both *Streptomyces* had a relatively large genome (Bs9: 8.45 Mb and Bs14: 11.2 Mb) compared to *Pseudomonas* (6.2 Mb) and *Bacillus* (5.1 Mb) (Table 8). Overall, genome size and G-C content of bacterial strains were consistent with literature from other related strains (Harrison and Studholme, 2014, Silby *et al.* 2011, Kunst *et al.* 1997). However, comparison with other related sequenced genomes and comparison of genome size with predicted number of open reading frames (ORFs) suggest that the predicted number of genes is likely overestimated for at least two genomes: *Bacillus* Bs2 and *Streptomyces* Bs9. Thus, further curation will be necessary to identify the origin of this problematic annotation: contamination, assembly problems, codon usage bias.

**Table 8.** Genome information of bacterial isolates analyzed in this work.

Strain	Contigs	G+C content (%)	Genome Size (Mb)	Predicted number of Open Reading Frames - ORFs (RAST)	Secondary Metabolite Biosynthetic gene clusters (antiSMASH)
<i>Bacillus</i> Bs2	2	38.2	5.1	7929	6
<i>Pseudomonas</i> Bs17	1	58.7	6.2	6947	8
<i>Streptomyces</i> Bs9	2	70	8.45	10533	24
<i>Streptomyces</i> Bs14	6	70.3	11.2	10432	33

Then whole genomes and full 16S sequences were used to identify closest described bacterial strains in the databases. All copies of 16S sequences were retrieved from the assembled genomes and compared by BLAST alignment to cured 16S rRNA and genome sequences of the EzBioCloud (Yoon *et al.* 2017). Based on this analysis, closest sequenced isolates were *Bacillus butanolivorans* for Bs2, *Streptomyces olivochromogenes* for Bs14 and *Pseudomonas fluorescens* HK44 for Bs17 (Figure 33). Species affiliation was not possible using this method for Bs9 as two species had the same 16S sequences: *S. siyoaensis* and *S. lydicus*. Since important differences can exist at the genome level between strains sharing the same 16S identity, we used whole genome comparison based on genome average nucleotide identity (ANI) to further affiliate the strains. Bacterial members from the same population are expected to share high sequence identity ranging from 94-100% genome ANI (Rodriguez-R and Konstantinidis, 2014). Thus, between genomic datasets, the average nucleotide identity can be used to affiliate bacteria at the species level. According to Rodriguez and Konstantinidis 2014, values below 75% should not be trusted (Rodriguez-R and Konstantinidis, 2014). Genomes from closest sequenced strains based on 16S rRNA analysis were used for paired ANI comparison. As Bs9 affiliation was not well resolved with 16S rRNA, both *S. siyoaensis* DSM 40032 and *S. lydicus* 103 genomes were compared to the one of Bs9. Best hit was obtained against *S. siyoaensis* DSM40032 based on the ANI calculator: identity between *Streptomyces* Bs9 and *S. siyoaensis* DSM 40032 was 86.8% and between *S. lydicus* 103 was 85.72%. However only 56% of the genome nucleotides aligned. Similarly, low alignment percentage between *Pseudomonas* Bs17 genome and *P. fluorescens* HK44 was obtained in ANI analysis (91.2 % of identity for 50.7% of coverage). Better results were obtained for *Bacillus* Bs2. Bs2 was affiliated to *Bacillus butanolivorans* DSM1892 with 97.22% identity (with 84% of the genome nucleotides aligned). Lastly, ANI for *Streptomyces* Bs14 and *Streptomyces olivochromogenes* DSM40451 was 94.43% with 74% of genome nucleotides aligned. However, since there are still regions of sequences that are not identified, further analyses must be performed.

Maximum likelihood phylogenetic tree



**Figure 33.** Maximum-likelihood phylogenetic tree of the 16S rRNA gene fragments extracted from the bacterial isolates genome A: *Streptomyces* Bs14, B: *Streptomyces* Bs9, C: *Pseudomonas* Bs17, D: *Bacillus* Bs2. Bar indicates changes per position. Ezibiocloud is a web-based service which detects contaminations from a whole genome

assembly using ContEst16S algorithm ([www.ezbiocloud.net/tools/contest16s](http://www.ezbiocloud.net/tools/contest16s)) aligning nucleotides with >50 % of bases (non-gaps) providing multiple sequence alignment (Lee *et al.* 2017). Based on these fragments, the website generates a maximum-likelihood phylogenetic tree using the RaxML software (Stamatakis 2006).

The search for specialized metabolite biosynthetic gene clusters was performed by a combined analysis using BAGEL and antiSMASH 5.0. In order to identify specialized metabolite biosynthetic gene clusters in bacteria, our criteria consisted on choosing regions that have all the genes involved in the synthesis, relying on the affiliations with already known functions in the database. Thus, the similarity percentage provided by antiSMASH given compared to the number of genes conserved with the reference cluster was confirmed by curating the genes on NCBI database.

In *Bacillus* Bs2, antiSMASH identified 6 putative regions encoding for specialized metabolites (Supplemental Table 1, Annexes Chapter III). Only one cluster contained all the expected genes (i.e. five) involved in the biosynthesis of a known compound (Table 9,). This region was predicted to be responsible for the biosynthesis of a lassopeptide similar to paeninodin (*pade*CAKB1B2D gene cluster) that has been described in *Paenibacillus dendritiformis* C454. Moreover, BAGEL algorithm predicted the presence of a gene encoding for the biosynthesis a bacteriocin (UviB). The amino acid predicted sequence was 94% identical (73% query cover) to a putative UviB-like bacteriocin from *Bacillus butanolivorans*.

**Table 9.** Analysis of paeninodin biosynthetic gene cluster from *Bacillus* Bs2 genome using *Paenibacillus dendritiformis* C454 as a reference genome.

Predicted metabolite	Encoded protein	Annotation in reference genome	AA Identity(%)	Query cover (%)
Paeninodin	PADEC	asparagine synthase	47	98
	PADEA	Precursor peptide	53	98
	PADEK	HPr kinase	51	95
	PADEB1	hypothetical protein	51	92
	PADEB2	hypothetical protein	65	94
	PADED	ABC transporter	57	98

In *Pseudomonas* Bs17, antiSMASH identified 8 putative regions (Supplemental Table 1, Annexes Chapter III). Two clusters showed high similarities to well described specialized metabolite biosynthetic gene clusters. The first one encoded a non-ribosomal peptide synthetase cluster (NRPS) that is possibly involved in the production a pseudomonine-like molecule, similar to the one produced by *Pseudomonas fluorescens* (Table 10). The second cluster was 71% similar to a mangotoxin biosynthetic gene cluster from *Pseudomonas syringae* *pv.* *Syringae*. Moreover, BAGEL identified two putative bacteriocins possibly colicins.



**Table 10.** Analysis of pseudomonine biosynthetic gene cluster from *Pseudomonas* Bs17 genome using *P. fluorescens* as a reference genome.

Predicted metabolite	Encoded protein / Protein id	Annotation in reference genome	AA Identity (%)	Query cover (%)
Pseudomonine	ABS50193.1	putative ferric siderophore ABC transporter periplasmic siderophore binding protein	85	76
	ABS50192.1	putative ferric siderophore ABC transporter ATP-binding protein	91	100
	ABS50191.1	putative ferric siderophore ABC transporter permease protein	85	99
	ABS50190.1	putative transport system permease protein	95	100
	ABS50189.1	putative ABC transporter permease/ATP-binding protein	88	100
	ABS50188.1	putative ABC transporter permease/ATP-binding protein	87	100
	ABS50187.1	putative RNA polymerase sigma-70 factor	81	98
	ABS50186.1	non-ribosomal peptide synthetase	81	100
	ABS50185.1	lysine 6-monooxygenase-like protein	89	77
	ABS50184.1	non-ribosomal peptide synthetase	96	100
	PMSC	isochorismate synthase	91	91
	PMSE	ATP-dependent activating enzyme	95	92
	PMSA	pyridoxal-dependent histidine decarboxylase	95	100
	PMSB	salicylic acid biosynthesis	95	100

In *Streptomyces* Bs9, antiSMASH identified 24 putative regions encoding the synthesis of specialized metabolites (Supplemental Table 1, Annexes Chapter III). Only one cluster contained all the genes (four) corresponding to biosynthetic cluster that have been previously identified in *Streptomyces anulatus* (Table 11). In addition, one region was 83% similar to spore pigment biosynthetic gene cluster from *Streptomyces avermitilis*. Moreover, BAGEL found one lanthipeptide.

**Table 11.** Analysis of ectoine biosynthetic gene cluster from *Streptomyces* Bs9 genome using *Streptomyces anulatus* as a reference genome.

Predicted metabolite	Encoded protein	Annotation in reference genome	AA Identity (%)	Query cover (%)
Ectoine	THPA	diaminobutyrate acetyltransferase [Streptomyces]	70	96
	THPB	diaminobutyrate--2-oxoglutarate transaminase [Streptomyces]	84	100
	THPC	L-ectoine synthase	91	94
	THPD	ectoine hydroxylase	67	69

Lastly, in *Streptomyces* Bs14, antiSMASH identified 33 putative regions (Supplemental Table 1, Annexes Chapter III). Nine clusters showed high similarities to well described metabolite biosynthetic

gene clusters. Four of these regions coded for biosynthetic clusters that are possibly involved in the production of desferrioxamin B, alkylresorcinol, 2-methylisoborneol and geosmin similar to the ones produced by *Streptomyces griseus* subsp. *griseus* NBRC 13350, one possibly involved in the production of albaflavenone similar to the one produced by *Streptomyces coelicolor* A3(2) and an ectoine biosynthetic gene cluster from *Streptomyces anulatus* (Table 12). Additionally, one region was 92% similar to hopene biosynthetic gene cluster from *Streptomyces coelicolor* A3(2), one region was 83% similar to spore pigment biosynthetic gene cluster produced by *Streptomyces avermitilis* and lastly one region was 80% similar to AmfS biosynthetic gene cluster from *Streptomyces griseus* subsp. *griseus* NBRC 13350. Additionally, BAGEL analysis showed one metalloendopeptidase (326-aa, 100% identity).

**Table 12.** Analysis of the biosynthetic gene clusters from *Streptomyces* Bs14 genome using *S. griseus*, *S. coelicolor* and *S. anulatus* as reference genomes.

Predicted metabolite	Encoded protein/ Protein id	Annotation in reference genome	AA Identity (%)	Query cover (%)
Alkylresorcinol	SRSC	monooxygenase	76	100
	SRSB	methyltransferase	71	98
	SRSA	type-III PKS	71	100
2-methylisoborneol	BAG18097.1	putative methyltransferase	57	99
	BAG18098.1	conserved hypothetical protein	53	97
Albaflavenone	SCO5222	putative lyase	84	100
	SCO5223	putative cytochrome P450	79	100
Geosmin	SCO6073	putative cyclase	78	92
Desferrioxamin B	BAG21580.1	putative siderophore-interacting protein	56	98
	BAG21579.1	putative pyridoxal-dependent decarboxylase	80	100
	ALCA	putative monooxygenase	81	100
	ALCB	putative acetyltransferase	64	92
	ALCC	putative siderophore biosynthetic enzyme	74	99
Ectoine	THPD	Ectoine hydroxylase	84	96
	THPC	L-ectoine synthase	95	97
	THPB	aminotransferase class-III	84	100
	THPA	diaminobutyrate acetyltransferase	71	100

## Discussion

Besides physical support, the main role of roots in trees involves the uptake and transport of water and minerals and this process is strongly related to the surface area covered in soil (Gou *et al.* 2010). The root system by branching out increases in root surface area, hence lateral roots constitute a major element of the root system (Torres-Martinez *et al.* 2019). Belowground, roots are in constant interaction with soil constituents, fauna and microorganism. Mycorrhizal associations are especially important for tree health since they help roots to uptake most of the nutrients (Marschner and Dell, 1994), and the overall soil microbiome has been reported to strongly influence plant development (Friesen *et al.* 2011; Vandenkoornhuysen *et al.* 2015). In addition, many bacterial strains (Proteobacteria, Firmicutes and Actinomycetes) have been reported to promote arbuscular and ectomycorrhizal symbioses (Frey-Klett *et al.* 2007). Many studies have shown bacterial influence on plant traits and physiology, however little is known about the influence of bacterial interactions. In this study, we explored how morphological traits (root and shoot systems) and physiology (chlorophyll and anthocyanin contents) of *Populus* responded to inoculation of four bacterial strains individually (*Bacillus*, *Pseudomonas*, two different *Streptomyces*) and in different combinations. Likewise, we investigated *Populus* influence in the bacterial interactions.

Simplified models with cultured bacteria inoculated onto gnotobiotic plants under controlled laboratory conditions can provide insights into the function of microorganisms and their interactions. Overall, none of the bacterial strains improved *Populus* growth significantly when incubated individually or in combinations. However, we found an alteration of the root architecture with an increased formation of lateral roots from *Bacillus* Bs2, *Pseudomonas* Bs17 and *Streptomyces* Bs14. Although there was an observed change of root phenotype, there was no visible physiological alterations in poplar. Similarly, an *in vitro* study exploring the response of root endophytes (*Pseudomonas* and *Burkholderia*) from *Populus trichocarpa* on plant growth related traits showed similar results, in which bacteria modified morphology but had no influence on biomass or photosynthetic traits after 21 days of incubation (Henning *et al.* 2016). These results suggest that poplar roots are responding to bacterial stimuli, either by consumption of primary metabolites or by production of secondary metabolites. More data needs to be collected to decipher the interaction.

The medium used in this study (mMS agar) contains nutrients that are not in complex forms and that are easily available for the plant and the bacteria. Thus, it is likely that in this *in vitro* setting, roots respond to bacterial consumption/modification of the medium and signal molecules. Nutrient concentration and water limitation have been reported to be involved in the production of lateral roots and the modification of carbon allocation to maximize their ability to gain limiting resources

Gou *et al.* 2010, Canarini *et al.* 2019). For example, Linkohr and collaborators reported that *Arabidopsis* root architecture is highly responsive to changes in phosphorous and nitrogen availability (Linkohr *et al.* 2002). Thus, we could expect that nutrient concentration, the intermediates released by resource consumption (e.g. organic acids) and the trade-offs between root exudation and bacterial resource-modifications and secretions may affect *Populus* root system (Herschend *et al.* 2018).

Although we observed different bacterial phenotypes in the pairwise bacterial interactions (antagonistic, neutral and beneficial (Figure 32)), all bacteria influenced root architecture through lateral root formation and length of the main root (Figure 25). Lateral root formation is regulated by developmental program and environmental signals, in which phytohormones and specially auxin play a major role (Gou *et al.* 2010). In our study, only *Pseudomonas* Bs17 and *Streptomyces* Bs14 produced auxin, suggesting a possible hormonal effect. Thus, auxin production in mMMS agar should be verified. Moreover, environmental cues can regulate phytohormone composition and concentration within the plant leading to a change in root architecture (Niu *et al.* 2013). For instance, at least for *Arabidopsis*, iron stimulated lateral root development by altering auxin distribution (Giehl *et al.* 2012). In this context, based on specialized metabolite biosynthetic gene cluster analyses using antiSMASH and functional analyses (Chapter II), all four bacterial isolates had a family of metabolites in common that uptake iron; the siderophores. Thus, it is tempting to think about iron as a potential driver of interactions. The similar case occurs with low phosphorus conditions (Niu *et al.* 2013). Likewise, all four isolates were able to utilize acid phosphatases. This suggests that different nutrient deficiencies may activate the auxin pathway to rewire the root system in order to acquire the nutrients needed.

In addition, we found *Streptomyces* Bs9 strain showed an antagonistic behavior towards poplar roots. Likewise, another *Streptomyces* strain with identical 16S rRNA sequence, but isolated from rhizosphere grain, exhibited also root inhibitory effect on *Arabidopsis thaliana* plantlets (data not shown, Canihac, 2018 personal communication). This suggests that the antagonistic behavior is not specific towards *Populus* and may act in diverse flowering plants. Moreover, our results indicated that this actinomycete produces constitutively an extracellular compound which acts as an inhibitor to the development of the root system and eventually produces necrosis. The accumulation of the antagonistic compound in the culture medium or its high diffusibility through the agar was high enough to be active over few centimeters (1-1.5 cms) preventing the growth of the root up to the bacterial colony. Despite that other bacterial strains could inhibit the growth of this *Streptomyces*, the root growth inhibition was not attenuated by bipartite interactions, but these roots did not presented necrosis. Moreover, root growth inhibition and necrosis were prevented by a tripartite consortium. Similar results have been evidenced previously in Finkel studies where complex bacterial synthetic

communities were responsible for root growth inhibition attenuation performed by one isolate (Finkel *et al.* 2019).

While few streptomycetes have been found to damage tree branches from Norway maples and decompose phloem from Douglas fir (Locci 1994), pathogenic species have not been reported in forest undergrounds. One of the best studied *Streptomyces* with phytopathogenicity activity is *Streptomyces scabies* damaging potato crops (Li *et al.* 2019). The mechanisms of plant pathogenicity in these *Streptomyces* strains involve phytotoxic specialized metabolites (phytotoxins) such as thaxtomins and concanamycins (Li *et al.* 2019). Although *S. scabies* mainly infect tubers, studies using model plants such as *A. thaliana* and *Nicotiana tabacum* have shown root stunting, necrosis and seedling death (Loria *et al.* 2006). Moreover, thaxtomin A has been shown to affect expression of genes involved in cell wall biogenesis, cell wall composition, and cause ectopic lignification in *A. thaliana* seedlings (Bischoff *et al.* 2009). Thus, it is possible that *Streptomyces* Bs9 possesses other phytotoxins that act against tree roots. In addition, in forests soil conditions, the interaction with other biomes, or microbial composition controlling the pathogenic population via bioactive compounds may reduce its phytopathogenic efficiency.

On the other side, *Bacillus* Bs2, *Pseudomonas* Bs17 and *Streptomyces* Bs14 colonies were significantly bigger together with poplar compared to when inoculated alone (Figure 31) and poplar had no effect on *Streptomyces* Bs9. Previous studies have reported plant roots exude primary metabolites (mainly sugars, amino acids and organic acids) and antimicrobials in order to select an appropriate microbiome (Raynaud *et al.* 2007, Hu *et al.* 2018, Canarini *et al.* 2019). Since there was no bacterial inhibition from poplar, we could hypothesize that bacteria were able to use root exudates for their benefit. Nevertheless, the molecular dialogue involved in this beneficial interaction requires further investigation. In addition, poplar modified some pairwise bacterial interactions (Figure 32). Based on mono-association interaction effect on colony size, several hypothesis could be envisioned: we could wonder if i) certain bacteria can benefit more from root exudates as additional nutrients and they provide an advantage to grow, or ii) root exudates change medium conditions that are not thus ideal for the steady growth (e.g. pH) and lastly iii) root exudates act as antibacterial against certain isolates, changing the dynamics of the interaction.

Still, the effect (direct or indirect) and the mechanism (molecules involved and signal transduction pathways) remain to be further explored. Several reviews have discussed the importance of interkingdom communications (Faure *et al.* 2009, Schirawski and Perlin 2018, Kai *et al.* 2016), however, more experimental validations are needed to decipher specific molecular dialogue pathways. Since soil microbiome is the result of complex, simultaneous, complementary and synergistic interactions, deciphering these pathways remains a challenge.

Overall, bacteria have been involved in the root design by metabolic signal interference (Finkel *et al.* 2019), creating hormonal imbalances through the production of their own phytohormones (Gou *et al.* 2010), altering the local environment through diffusible compounds (e.g. consumption of specific resources, secretion of metabolites) (Herschend *et al.* 2018). In addition, they may collaborate by making nutrients available (Linkohr *et al.* 2002, Browne *et al.* 2009) or compete for nutrients (Zhu *et al.* 2016). In return, poplar can respond to environmental stimuli via root growth modification and carbon allocation, exudation of primary metabolites (Canarini *et al.* 2019) and immunity and defense mechanisms (Hu *et al.* 2018), affecting their root microbiome.

## Conclusions and Perspectives

Our study provides important insights for phenotyping plant – bacteria interactions individually and in combinations in an *in vitro* setting. Overall, in these conditions, the four bacteria selected influenced poplar root architecture. More evidences need to be collected about the nature of these root modifications and the molecular dialogues responsible for them. A transcriptomic analysis of the root system and bacteria could be done to follow gene expression, and LC-MS and IMS techniques to evaluate the molecules diffused during the interactions.

Additionally, we have identified one *Streptomyces* strain with a strong antagonistic effect on *Populus*, and *Arabidopsis*, specifically reducing the growth of roots and eventually causing necrosis. More evidences need to be collected about the nature of this antagonistic compound and its biosynthetic molecular pathway and mode of action *in planta*. For this, LC-MS and IMS techniques could be used to determine the molecules produced during the interaction. Then specific fractions can be tested for direct plant interaction.

Furthermore, genome analyses predicted important functions that need experimental validation. Heterologous expression of specific gene clusters could elucidate some of their roles in intraspecies, interspecies and interkingdom interactions.

Another aspect to consider and that was not addressed in this study is the intraspecific root – root interaction of different individuals of poplar within the same enclosed petri dish (Faget *et al.* 2013). In certain cases where nutrients may be depleted, poplar individuals may compete increasing the variability in their phenotypes. To avoid this type of errors, experiments can be done separately, although this entitles a high cost of materials.

# **Annexes Chapter III**

**Supplemental Table. 1**  
AntiSMASH and Bagel  
predictions using bacterial  
genomes.

Bacterial strain	Region	Type of cluster	Most similar known cluster	Similarity
<i>Bacillus</i> Bs2	Region 1	NRPS- betalactone	Fengycin	46%
	Region 2	Other	Desferrioxamine B	40%
	Region 3	Terpene		
	Region 4	LAP		
	Region 5	Lassoptide	Paeninodin	100%
	Region 6	Terpene		
	Region 7	Bacteriocin	UviB	
<i>Pseudomonas</i> Bs17	Region 1	Other	siderophore	
	Region 2	NRPS-like	Mangotoxin	71%
	Region 3	T1pks	arylpolyene	40%
	Region 4	Bacteriocin		
	Region 5	Bacteriocin		
	Region 6	Butyrolactone		
	Region 7	NRPS	Pseudomonine	100%
	Region 8	NRPS-betalactone	Fengycin	13%
	Region 9	Bacteriocin	Colicin	
	Region 10	Bacteriocin	Colicin	
<i>Streptomyces</i> Bs9	Region 1	Terpene	-	-
	Region 2	T1pks	Aurafuron	42%
	Region 3	Terpene	Thiotetronate Tü 3010	14%
	Region 4	NRPS	siderophore	3%
	Region 5	NRPS,other	Skyllamycin	12%
	Region 6	NRPS-transatpks-butyrolactone	Griseoviridin / viridogrisein	5%
	Region 7	Bacteriocin	-	-
	Region 8	T1PKS, NRPS	Salinomycin	18%
	Region 9	terpene	Hopene	53%
	Region 10	NRPS, t2pks	Ishigamide	22%
	Region 11	T1pks,t3pks	Herboxidiene	3%
	Region 12	Terpene,bacteriocin	Pentalenolactone	15%
	Region 13	T1pks,butyrolactone	Coelimycin	12%
	Region 14	NRPS	Deimino- antipain	66%
	Region 15	Terpene	Isorenieratene	71%
	Region 16	LAP,thiopeptide	Cyclothiazomycin	38%
	Region 17	NRPS	Surugamide A / surugamide D	19%
	Region 18	T1pks/T3PKS	Herboxidiene	3%
	Region 19	NRPS,bacteriocin	Conglobatin	10%
	Region 20	T2PKS	Spore pigment	83%
	Region 21	NRPS	Crocagin A / crocagin B	10%
	Region 22	Other	Siderophore	27%
	Region 23	T1PKS	Tetronasin	23%
	Region 24	Other	Ectoine	100%
	Region 25	Lanthipeptide		
<i>Streptomyces</i> Bs14	Region 1	NRPS,T1PKS	Enduracidin	8%
	Region 2	Other	Desferrioxamine B	83%
	Region 3	Other	Ectoine	100%
	Region 4	NRPS-like	Octacosamicin	12%
	Region 5	T1pks,t3pks	Herboxidiene	8%
	Region 6	NRPS,T1PKS	Foxicins A–D	24%
	Region 7	NRPS-like	A54145	3%
	Region 8	Other	Melanin	42%
	Region 9	T3PKS	Alkylresorcinol	100%
	Region 10	Terpene	-	-
	Region 11	Fused	-	-
	Region 12	Terpene	Napyradiomycin	7%
	Region 13	NRPS-like	Stenothricin	13%
	Region 14	Terpene,lanthipeptide	2-methylisborneol	100%
	Region 15	NRPS	Diisonitrile antibiotic SF2768	66%
	Region 16	Terpene	Albaflavenone	100%
	Region 17	NRPS-like	Stenothricin	13%
	Region 18	T1PKS	Teicoplanin	4%
	Region 19	Other	siderophore	-
	Region 20	NRPS	Capreomycin	6%
	Region 21	terpene	Hopene	92%
	Region 22	T2PKS, NRPS-like	Spore pigment	83%
	Region 23	Lanthipeptide	AmfS	80%
	Region 24	Bacteriocin	Informatipeptin	42%
	Region 25	NRPS-like,t1PKS	Herboxidiene	4%
	Region 26	Terpene,t1pks	Galbonolides	10%
	Region 27	Bacteriocin	-	-
	Region 28	T2PKS,butyrolactone	Rabelomycin	31%
	Region 29	NRPS	siderophore	3%
	Region 30	NRPS	Skyllamycin	30%
	Region 31	Bacteriocin	-	-
	Region 32	Terpene	Geosmin	100%
	Region 33	NRPS-t1pks	Pristinamycin	10%
	Region 34	Metalloendopeptidase		



# **General Discussion and Perspectives**

## General Discussion and Perspectives

Despite recent advances in our knowledge of the composition of forest soil microbial communities thanks to the exponential use of high throughput DNA sequencing techniques, soil remains a black box. Indeed, we lack information on how microorganisms behave and interact *in situ*. My PhD project had for aim to get insights into the potential activities and the interactions of microorganisms in forest soil and thus to shed a little bit of light into this black box. This knowledge is important to further understand and predict how tree associated microorganisms respond to perturbations such as climate change. Moreover, the investigation of biotic interactions may lead to the discovery of new specialized metabolites since most secondary metabolites are mainly produced during interactions with other (micro)organisms. In this context, this project could contribute with the finding of new bioactive natural products for sustainable agriculture, tree plantations and medical solutions.

The goals of my work were to gain insights into the impact of molecular dialogues between bacteria on the structure of bacterial communities, on tree development and how plants respond to these metabolic exchanges. To do so, we i) isolated bacteria from grains of forest soil and described their microbial community, ii) characterized potential functional abilities of cultivable isolates, iii) investigated the effect bacteria has on other bacteria sharing the same microhabitat, iv) investigated the effect bacteria have on poplar and reciprocally the effect of poplar on bacteria and v) started to identify specialized metabolites involved in the molecular dialogues between bacteria and between bacteria and poplar through genome mining and metabolomics. Here I discuss and connect the major findings of this thesis in respect to the recent literature and suggest potential ecological perspectives.

### ***Can bacteria and trees communicate?***

Most literature on bacteria – plant interactions have focused on main crops and *Arabidopsis thaliana* as a model plant. Few studies have reported bacterial effects on development of forest trees; however, many have worked with endophytes, ignoring the soil microbiome effect (Henning *et al.* 2016, Timm *et al.* 2016). Moreover, those that have focused on forest soil microbiome have studied extensively one of the most important associations for tree development: the mycorrhiza (Felten *et al.* 2009, Plett *et al.* 2014). Additionally, the mycorrhiza helper bacteria have also been extensively studied (Frey-Klett *et al.* 2007, Frey-Klett *et al.* 2011). Thus, this thesis aims to provide insights to fill certain gaps by evaluating *in vitro* the effects of soil bacterial isolates coming from the same microhabitat on tree development, and likewise, tree effects on bacteria.

Here we found that bacteria, isolated from a grain of bulk soil, were able to modify the architecture of the root system of *Populus*, by inducing lateral root formation. Conversely, we also found that when

considering the phenotype of these bacteria, the colony sizes were bigger when inoculated individually next to *Populus*. Although colony size is a rough trait depending both from growth and the production of extracellular compounds (e.g. exopolysaccharide), it is noteworthy that this parameter was modulated during both bacteria-bacteria and bacteria-poplar interactions. Based on these findings, we evidenced that poplar and different bacteria influence each other.

The alteration of the root development occurred when bacteria were at close proximity to the roots, thus, we suspect an exchange of molecules stimulating the alteration of the root system. Unfortunately, so far, our results did not explain how the influence occurs (e.g via the secretion of metabolites or consumption of nutrients). Nevertheless, there is phenotypic response from both partners, thus suggesting a possible dialogue. Interestingly, even though these bacteria induced similar root phenotype, they belong to different bacterial species with distinct functional abilities (Chapter II). Either the molecular mechanism leading to secondary root development is shared by many bacteria or there are several ways to stimulate root development. Understanding the molecular mechanisms controlling root architecture is essential for improving nutrient uptake efficiency. Many of the signals regulating root architecture influence the auxin pathway. At least in *Arabidopsis thaliana*, different signals have been reported to control auxin pathways modifying root architecture (e.g. phosphorus (Niu *et al.* 2012), iron (Giehl *et al.* 2012), nitrate (Sun *et al.* 2017)). Thus, metabolic profiling showing presence as well concentration variation of molecules during interaction and dissecting the molecular basis by the creation of poplar mutants (e.g. auxin signaling) would help to decipher the molecular mechanisms in which bacteria can control root architecture. However, other mechanisms could be involved. For instance, it is known that roots respond to changes in pH with transcriptional alterations in the expression of a large number of auxin-responsive genes, suggesting that pH changes may mediate root architecture (Lager *et al.* 2010). Indeed, several of the strains isolated here acidified the medium while growing (e.g. *Streptomyces*, *Pseudomonas*) and other strains increased the pH of the medium (e.g. *Bacillus*). Although these bacteria could alter pH levels, the overall change was still under 7.0, thus the medium was still considered as acidic. One could wonder whether this phenomenon was involved in the interaction with poplar. Finally, determining whether bacterial influence on root development provides any benefit for trees and which bacteria are beneficial would be of interest to further design a synthetic community with the purpose of improving sustainable practices.

### ***Can trees participate in bacterial interactions?***

The effect of trees on bacterial communities have been determined through culture-independent approaches (Uroz. *et al.* 2010). It has been recognized that bulk soil bacterial communities differ from

those of the rhizosphere due to rhizodeposition (Buée *et al.* 2009A, Paterson *et al.* 2007, Bais *et al.* 2006). In addition, it is also known that bacterial communities are different depending on the species of the tree host (Dukunde *et al.* 2019). Based on these studies, we have an overall idea about the impact of trees on bacteria. However, little is known about the molecular influence between trees and bacteria and on bacterial interactions.

In this study, we showed that poplar presence changed the outputs of bacterial interactions. For instance, *Pseudomonas* Bs17 was originally considered to be the most aggressive competitor and the most resistant strain among our library, based on the extent and intensity of inhibitions towards most bacterial isolates (Chapters II and III). Interestingly, *Pseudomonas* Bs17 remained aggressive and became more susceptible in the presence of poplar, and *Streptomyces* Bs14 and *Bacillus* Bs2 were able to inhibit Bs17 strain in this condition. *Streptomyces* Bs14 was also considered as an aggressive competitor since it was found to inhibit many other bacterial isolates, but it was less resistant than Bs17 (Chapter II). When *Streptomyces* Bs14 was in interaction with other bacteria in the presence of poplar, the intensity of the antagonistic activities of *Streptomyces* Bs14 was increased. Moreover, we also observed a consistent mutual beneficial interaction between *Streptomyces* Bs14 and poplar. Our results showed that *Streptomyces* Bs14 induced the highest amount of new lateral roots and *Streptomyces* Bs14 colony size was influenced by the presence of poplar (Chapter III). *Bacillus* Bs2 was the least aggressive yet the most susceptible strain (Chapter II). Moreover, Bs2 strain started to show antagonistic effect with longer period of incubation (Chapter III), and together with poplar, Bs2 strain changed bacterial targets. For instance, Bs2 strain did not inhibit anymore *Streptomyces* Bs14 but became antagonistic towards *Pseudomonas* Bs17. In addition, the presence of poplar did not change *Bacillus* Bs2 susceptibility from the other grain inhabiting members. Finally, *Streptomyces* Bs9 was slightly aggressive against bacteria and very susceptible to other bacterial strains (Chapter II). With addition of incubation time, Bs9 strain became more aggressive but remained susceptible, and together with poplar its behavior did not change (Chapter III). Overall, each strain had a different interaction profile, suggesting interactions are species specific and dependent on determined conditions (e.g. period of interaction, presence or absence of poplar).

Based on poplar inoculated with individual strains, there was no visual evidence of antagonism from the roots towards bacteria, although we cannot discard the possibility of antimicrobial production with no effect on bacteria. Based on the bacterial colony size, roots produced molecules that may contribute preferentially to certain isolates. Indeed, not only bacteria can produce antimicrobials, but root exudates also comprise a molecular cocktail of organic acids, sugars and bioactive compounds that may regulate microbial community dynamics in the rhizosphere, select a 'healthy' microbiome and increase resistance to abiotic stresses (Haichar *et al.* 2008, Shi *et al.* 2011, Hu *et al.* 2018). For

instance, the addition of glucose to bacterial communities induced few changes in the composition of the community suggesting that a large proportion of soil microorganisms consume this sugar (Shi *et al.* 2011). However, organic acids such as citric acid and oxalic acid from roots have been reported to have a great impact on bacterial communities for two reasons. One consists on the proportion of microorganisms that are specialized in decomposing these carbon inputs. The second reason is pH. Organic acids can significantly change the soil pH, potentially contributing to shifts in bacterial communities (Shi *et al.* 2011). It is possible that bacterial communities are influenced by bacterial interactions, and additionally, the presence of trees alter bacterial communities by modifying the interactions. Understanding how bacterial interactions are influenced by trees is essential for the design of synthetic communities to improve nutrient uptake efficiency. Thus, we initiated metabolomics analyses to elucidate the molecules of dialogues involved during these interactions, however preliminary results were not sufficient and need to be further explored.

### ***Streptomyces*: more foes than friends?**

Here I showed for the first time that a *Streptomyces* strain had a constitutive antagonistic effect on poplar roots (Chapter III). The *Streptomyces* Bs9 strain, isolated from bulk soil of an oak forest, and affiliated to *S. sioyaensis* based on 16S rRNA gene sequence, did not allow poplar roots to grow and eventually produced necrosis on the root system. Moreover, this strain was also found to affect in the same way *Arabidopsis* plantlets. Interestingly, when this strain was inoculated next to any of the other bacteria studied, its detrimental effect towards poplar was still active but necrosis was possibly delayed. Indeed, *Pseudomonas* Bs17 and *Streptomyces* Bs14 could inhibit Bs9 strain with different intensities. However, *Bacillus* Bs2 could not inhibit Bs9 strain. Thus, lack of necrosis could be associated with an interference of the molecular mechanism or a delay/reduction of growth. More importantly, a tripartite association including Bs9 strain did not affect poplar roots in the same way, suggesting bacterial interactions interfered with Bs9 activities either by reducing directly its growth or by interfering with the production of antagonistic compounds. In order to elucidate the molecules produced constitutively and during interactions, we performed LC/MS and IMS techniques, however preliminary results did not reveal so far the identity of antagonistic compound(s) and further analyses need to be done.

Few *Streptomyces* have been reported to cause plant diseases. The most studied species infect economically important crops (Bignell *et al.* 2013). For instance, *Streptomyces scabies* has been found to cause 'scab disease' on potato, but also on carrots, beets, radish and parsnips (Dees and Wanner 2012). Potential virulence factors, phytotoxins, phytohormones and secreted proteins from *Streptomyces* are involved in the molecular mechanisms that damage these tubers (Li *et al.* 2019). For

instance, phytotoxins Thaxtomin A and B are responsible for the growth abnormalities and necrosis, and the gene cluster responsible for their biosynthesis has been found in a mobile pathogenicity island on *Streptomyces* genome (Kers *et al.* 2005). In addition, few streptomycetes have been found to damage trees. These have been found in branches from Norway maples and decomposing phloem from Douglas fir (Locci 1994). However, pathogenic *Streptomyces* species have not been reported in forest soils. Thus, further analyses need to be carried out to characterize the mechanisms by which Bs9 strain causes necrosis and growth inhibition and in which extent it affects forest tree roots.

Interestingly, *Streptomyces* has been extensively studied and used for the production of herbicides (Nakajima *et al.* 1990). A number of commercially used herbicides are produced by *Streptomyces* strains (Harir *et al.* 2018). Little is known about the natural targets of these herbicides and if they are naturally produced during interactions with plants or with other eukaryotes (e.g. fungi, insects...). Herbicides are usually designed to target herbaceous plants, however, it has been shown that they can have selective activity towards dicotyledonous plants (Shi *et al.* 2019), alga (Takiguchi 1979A) and fungi (Takiguchi 1979B). Indeed, targets of herbicides are not specific to herbaceous plants: herbicides impair processes such as cell wall synthesis, protein synthesis or lipid membranes, constituents that are shared with vascular plants (Bo *et al.* 2019). Interestingly, preliminary genome mining analysis suggests that Bs9 strain may be able to produce nigericin, an antibiotic that inhibits the growth of Gram-positive bacteria but that also has herbicide properties (Heisey and Putnam 1986). However, several genes seemed to be truncated and/or contained inappropriate stop codons. Whether this is due to mis-annotation needs to be verified and deeper *in silico* analyses are required to decipher what the cluster likely codes for. In the case that Bs9 strain truly produces this compound and whether it is involved in its phytotoxic activity need to be further explored. Thus, we can wonder whether herbicides produced by *Streptomyces* can have an effect on the root of trees and particularly on seedlings. Indeed, poplar plantlets used in this work were not yet completely lignified and one can wonder whether they are not thus more sensitive to herbicides compared to older trees. Altogether it is tempting to speculate that *Streptomyces* Bs9 produces a phytotoxic/herbicide metabolite which interferes with tree root development. Further analyses need to be done to characterize this molecule with antagonistic effect in forest tree roots.

### ***Mining genomes for specialized compounds***

Bacterial genomes contain an enormous resource of data about the species features. Unfortunately, often little can be predicted based on genome sequences when it comes to specialized metabolites. A first limitation comes from the completeness and accuracy of the databases: despite the efforts made to discover, describe and define the biosynthesis pathways of specialized metabolites across the

world, complete information is only available for a few thousands of compounds. Secondly, if automatic annotations of genomes allow for the detection of putative biosynthetic gene clusters, it is often not possible to predict what they code for based on the sequences in most cases. Nevertheless, such methods provide first hints on the potential abilities of the microorganisms. In our case, a few clusters could be identified with a relatively high confidence. For instance, based on antiSMASH, genome of *Bacillus* Bs2 contains a paeninodin biosynthetic gene cluster. Paeninodin is a lasso peptide mostly produced by proteobacteria and actinobacteria and recently was found also in firmicutes with a unique organization (Zhu *et al.* 2016). Lasso peptides can have a broad range of actions including antimicrobial activities and, for the moment, the role of paeninodin in our context remains elusive.

For *Pseudomonas* Bs17, antiSMASH analysis allowed to find a biosynthetic gene cluster potentially encoding for a siderophore named pseudomonine. Interestingly, it has been shown in other *Pseudomonas* strains that pseudomonine was produced in association with salicylic acid (SA) (Mercado-Blanco *et al.* 2001). SA is a potent siderophore that is used by bacteria to capture iron but it is also a phytohormone involved in defense against pathogens and that is responsible for disease suppression (Mercado-Blanco *et al.* 2001). Preliminary genome analysis suggests that Bs17 would have the full *pmsCEAB* cluster necessary for the production of SA and that it would be located close by the pseudomonine cluster. Intriguingly, Poplar is well known to constitutively accumulate high levels of SA (Tsai *et al.* 2006). Moreover, production of SA is altered in poplar tissue in the presence of different microorganisms, and it has been found that SA affects root architecture via auxin distribution (Pasternak *et al.* 2019) Thus, it would be interesting to test if Bs17 produces SA and if it is active against other strains, or whether the bacterial strains alter SA signaling in poplar.

Unfortunately, but interestingly, analysis of *Streptomyces* Bs9 genome did not reveal well known clusters for the biosynthesis of specialized metabolites except ectoine and a spore pigment. Ectoine enables the organism to survive under high osmotic pressure conditions and other environmental stresses like ultraviolet radiation (Prabhu *et al.* 2004). Thus, it is likely not involved in the interactions studied here. The second cluster shows high similarity both to a cluster involved in spore pigment production and in cluster involved in the biosynthesis of curamycin (Gaisser *et al.* 1997). Pigment production is expected since the Bs9 strain forms dark spores (Figure 29). However, we did not observe any change of pigment production in Bs9 strain during any interaction. Most promising finding is the discovery of a cluster of genes that could code for the biosynthesis of nigericin-like molecule as described above.

Lastly, the analysis of *Streptomyces* Bs14 genome revealed multiple genes cluster potentially responsible for the production of specialized metabolites. Nine out of the 33 predicted clusters could be attributed with good confidence and some could be involved in the interactions between bacteria

or with Poplar. For instance, albaflavenone is a potent antibiotic against *B. subtilis* (Gurtler and Pedersen, 1994). *Streptomyces* Bs14 was found to strongly inhibit the two *B. subtilis* strains isolated from bulk soil (Chapter II). Whether albaflavenone is responsible for this activity remains to be tested but it sounds to be a good candidate. Conversely, alkylresorcinol is involved in protection against  $\beta$ -lactam antibiotics (Funabashi *et al.* 2008). One could wonder if the production of this compound could explain the relative high resistance of this strain towards the activities of other bacteria (Chapter II). Indeed, only few bacteria were able to inhibit *S. olivochromogenes* strains in our studies. At least for the genomes sequenced, no  $\beta$ -lactam cluster was found. Similarly, hopene protects aerial mycelium against stresses (Siedenburg and Jendrossek, 2011). One can wonder whether they also protect Bs14 against other strains. Further analyses will be needed to demonstrate that the strain does produce these compounds and that they do protect it against the activities of other microorganisms. An intriguing compound is 2-methylisoborneol. The biological function of this compound is unknown, but it is studied since a while because of it is a smelly compound that cause troubles in potable water by giving an “earthy” taste (Giglio *et al.* 2010). We have overlooked the role of volatiles in the interactions during our studies, except for showing that they were not involved in some cases. It would be interesting to explore this aspect in the future.

Altogether, genome mining opens many paths to be further explored. However, many potential clusters could not be annotated (e.g. gaps in the genome sequence, quality of the sequences), suggesting that even more compounds could be involved in the interactions. Preliminary metabolomic experiments were initiated to identify the compounds possibly involved in the interactions. Despite a fantastic amount of bad luck and after four different attempts, a number of metabolites could be seen to be produced when the bacteria were grown alone or in interactions. However, variation in metabolic profiles between bacteria growing alone or in interactions were not clear. None have been identified yet, but optimization and additional experiments may allow to uncover the identity of the active compounds. Additionally, transcriptomic and/or mutagenesis experiments may allow to discover among the many potential targets which one are involved in the interactions and to further study them. The obtained results of this thesis set the *in vitro* settings basis for further understanding plant-bacteria molecular dialogues. The next step would be to discover the molecules involved and to unravel their ecological role in plant development. Deciphering these molecules could potentially be used in sustainable practices in the form of natural biocontrol.



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## Characterization of forest soil bacteria isolated at the microhabitat level and of their interactions with Poplar

Soil borne microbes are vital contributors to forest ecosystems through soil services such as organic matter decomposition, nutrient cycling and sustaining plant growth. Bacteria and fungi form complex networks of interactions belowground interconnected with roots and assisting plants with water, nutrients, and some can help overcome pathogenic damages. During these biotic interactions, there is an exchange of signal molecules and metabolites that modify the local environment and can affect the presence and growth of surrounding microbial neighbours. These alterations are key processes in the structuration of soil microbial communities and plant development. In soil, abiotic and biotic factors such as pH, organic matter and vegetation have been extensively studied to govern microbial communities' structure. However, some important biotic interactions are still poorly described. Especially molecules produced during bacterial interactions that affect microbial communities' structure and plant health and growth are still underexplored. To obtain insights of the impact of these interactions, we focused on bacteria that share a habitat with the same abiotic and biotic conditions and that are likely interacting. In order to reach this goal, we isolated bacteria from different grains of soil, described their functional abilities, evaluated the behaviours against each other and their impact on the growth of *Populus*. In this study, bacteria from a grain of soil had a high functional diversity where few bacteria were interacting. Most interactions were antagonistic and few were strong inhibitions. Moreover, most bacteria altered the root architecture of *Populus*, and interestingly one *Streptomyces* strain was able to cause necrosis in the root system.

Keywords: soil microbial functions, bacterial interactions, molecular dialogues, Poplar

## Caractérisation des bactéries du sol forestier isolées au niveau des microhabitats et de leurs interactions avec le peuplier.

Les micro-organismes du sol jouent un rôle majeur dans le fonctionnement des écosystèmes forestiers notamment par le biais de leurs actions dans les sols tels que la décomposition de la matière organique, le cycle des éléments nutritifs et la promotion de la croissance des plantes. Les bactéries et les champignons forment sous terre des réseaux d'interactions complexes ainsi qu'avec les racines des plantes qu'ils aident pour prélever de l'eau, des nutriments ou encore pour contrecarrer l'action de pathogènes. Au cours de ces interactions se produisent des échanges de molécules signal et de métabolites qui modifient l'environnement local et peuvent affecter la présence et/ou la croissance des autres microorganismes qui partagent le même habitat. Ces altérations sont des processus clés dans la structuration des communautés microbiennes du sol et ont un impact sur le développement des plantes. Dans le sol, les facteurs abiotiques et biotiques tels que le pH, la matière organique et la végétation ont été largement étudiés pour comprendre leurs rôles dans la structuration des communautés microbiennes. En revanche, le rôle joué par les interactions biotiques, et notamment entre microorganismes, reste méconnu. Peu de choses sont connues quant aux molécules produites au cours des interactions bactériennes et leur impact sur la structure des communautés microbiennes ainsi que sur la santé et la croissance des plantes. Pour mieux comprendre l'impact de ces interactions, nous nous sommes focalisés sur les bactéries qui partagent un même habitat et sont donc soumises aux mêmes conditions abiotiques et biotiques et dans lequel elles interagissent très probablement. Pour ce faire, nous avons isolé des bactéries de différents grains de sol, décrit leurs capacités fonctionnelles, évalué les comportements des unes par rapport aux autres et leur impact sur la croissance du peuplier. Nous avons montré que les bactéries d'un même grain de sol se caractérisaient par une grande diversité fonctionnelle et que peu de bactéries interagissaient les unes avec les autres. La plupart des interactions étaient inhibitrices et peu d'entre elles étaient de forte intensité. De plus, la plupart des bactéries induisaient une modification de l'architecture racinaire du peuplier. Enfin, fait intéressant, une des souches de *Streptomyces* isolée bloquait la croissance du système racinaire et y causait des nécroses.

Mot clés: fonctions microbiennes du sol, interactions bactériennes, dialogues moléculaires, peuplier