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Cette thèse intitulée

Manipulation du microbiome rhizosphérique et son application en phytoremédiation

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Résumé

Le microbiome de la rhizosphère fait généralement référence aux communautés bactériennes, archées et fongiques ainsi qu'à leur matériel génétique entourant étroitement les systèmes racinaires des plantes. Le métagénome de ce microbiome a été appelé le deuxième génome de la plante puisqu'elle est capable de profiter de plusieurs fonctions dont elle manque. La communauté microbienne de la rhizosphère inclue entre autres des microorganismes ayant développé des interactions intimes et spécifiques de longue durée avec les racines des plantes. Il s'agit d'une communauté dynamique de microorganismes, à partir de laquelle une partie d'espèces a développé des interactions intimes et spécifiques de longue durée avec les racines des plantes. Les progrès récents dans l'étude des interactions plantes-microbes ont démontré leur impact considérable sur la croissance, la nutrition et la santé des plantes. Le microbiote de la rhizosphère est complexe avec une structure spatio-temporelle dynamique qui s'adapte rapidement en fonction des stress biotiques et abiotiques. Considérant l'importance du microbiome de la rhizosphère pour la santé des plantes, des informations précises sur leurs microbes associés sont d'une importance capitale pour déchiffrer les mécanismes d'adaptation des plantes aux stress médiés par le microbiome et comprendre comment les plantes recrutent des taxons microbiens clés pour mieux faire face aux conditions stressantes. Pour ce faire, nous avons mené trois études afin de faire la lumière sur les facteurs qui jouent un rôle dans le recrutement et la structure du microbiome de la rhizosphère de plantes dans les milieux stressés.

Dans un premier lieu, nous avons testé si des inoculations répétées avec des protéobactéries influençaient la productivité des plantes et les communautés microbiennes associées à la rhizosphère de quatre espèces végétales poussant dans des sédiments contaminés par des hydrocarbures pétroliers. Une expérience de mésocosme a été réalisée en conception de blocs randomisés avec deux facteurs : 1) la présence ou l'absence de quatre espèces végétales collectées dans un bassin de sédimentation d'une ancienne usine

pétrochimique, et 2) l'inoculation ou non avec un consortium bactérien composé de dix isolats de Protéobactéries. Les plantes ont été cultivées en serre pendant quatre mois. Le séquençage d'amplicon MiSeq, ciblant le gène de l'ARNr 16S bactérien l'ITS fongique, a été utilisé pour évaluer les structures de la communauté microbienne des sédiments provenant de mesocosmes plantés ou non plantés. Nos résultats ont montré qu'alors que l'inoculation provoquait un changement significatif dans les communautés microbiennes, la présence de la plante et de son identité spécifique avait une influence plus forte sur la structure du microbiome dans les sédiments contaminés par les hydrocarbures pétroliers.

Ensuite, en utilisant le même dispositif expérimental, nous avons utilisé le séquençage d'amplicon MiSeq ciblant le gène de l'ARNr 18S pour évaluer les structures communautaires AMF dans les racines et la rhizosphère de plantes poussant dans des substrats contaminés et non contaminés. Nous avons également étudié la contribution de l'identité spécifique des plantes et du biotope (racines des plantes et sol rhizosphérique) dans la formation des assemblages AMF associés. Nos résultats ont montré que si l'inoculation provoquait un changement significatif dans les communautés AMF, la contamination du substrat avait une influence beaucoup plus forte sur leur structure, suivie par le biotope et l'identité végétale dans une moindre mesure. De plus, l'inoculation augmentait considérablement la production de biomasse végétale et était associée à une diminution de la dissipation des hydrocarbures pétroliers dans le sol contaminé. Le résultat de cette étude fournit des connaissances sur les facteurs influençant la diversité et la structure communautaire de l'AMF associée aux plantes en milieux stressés à la suite d'inoculations répétées d'un consortium bactérien.

Finalement, nous avons testé l'effet d'une inoculation d'arbres avec des champignons mycorhiziens spécifiques sur leur survie et croissance, ainsi que l'extraction de métaux traces. Pour ce faire, une expérience sur le terrain a été menée dans laquelle nous

avons cultivé le clone de *Salix miyabeana* "SX67" sur le site d'une décharge industrielle déclassée, et inoculé les arbustes avec le champignon arbusculaire mycorhizien *Rhizophagus irregularis*, le champignon ectomycorhizien *Sphaerosporella brunnea*, ou un mélange des deux. Après deux saisons de croissance, les saules inoculés avec le champignon *S. brunnea* ont produit une biomasse significativement plus élevée. Le Ba, le Cd et le Zn se sont avérés être accumulés dans les parties aériennes des plantes, où le Cd présentait les valeurs de facteur de bioconcentration les plus élevées dans tous les traitements. De plus, les parcelles où les saules ont reçu l'inoculation de *S. brunnea* ont montré une diminution significative des concentrations de Cu, Pb et Sn dans le sol. L'inoculation avec *R. irregularis* ainsi que la double inoculation n'ont pas influencé de manière significative la production de biomasse et les niveaux d'éléments traces du sol.

Le résultat de cette étude apporte des connaissances sur la diversité et l'écophysiologie des microbes de la rhizosphère associés aux plantes de croissance spontanée à la suite d'inoculations répétées. De plus ils montrent le potentiel de l'utilisation de champignons mycorhiziens afin d'améliorer la santé et croissance des plantes dans des milieux pollués et toxiques. Ils soulignent aussi l'importance de la sélection des plantes afin de faciliter leur gestion efficace et accélérer les processus de remise en état des terres.

Mots-clés: Interactions Plant-microbes, écologie microbienne, microbiome rhizosphérique, bioaugmentation, hydrocarbures pétroliers contamination; séquençage d'amplicons;

Abstract

The rhizosphere microbiome generally refers to the bacterial, archaea, and fungal communities and their genetic material that closely surrounds the root systems of plants. The metagenome of this microbiome has been called the second genome of the plant because it is able to take advantage of several functions that it lacks. It is a vibrant community of microorganisms, from which part of the species has developed long-lasting, specific and intimate interactions with plant roots. Recent advances in the study of plant-microbe interactions have demonstrated their considerable impact on plant growth, nutrition and health. The rhizosphere microbiota is complex with a dynamic spatio-temporal structure which adapts rapidly to biotic and abiotic stresses. Considering the importance of the rhizosphere microbiome to plant health, accurate information about their associated microbes is of utmost importance in deciphering the mechanisms of plant adaptation to microbiome-mediated stress, and understanding how plants recruit key microbial taxa to better cope with stressful conditions. To do this, we conducted three studies to shed light on the factors that play a role in the recruitment and structure of the microbiome of the rhizosphere of plants in stressed environments.

First, we tested whether repeated inoculations with *Proteobacteria* influenced the productivity of plants and the microbial communities associated with the rhizosphere of four plant species growing in sediments contaminated with petroleum hydrocarbons. A mesocosm experiment was carried out in design of randomized blocks with two factors: 1) the presence or absence of four plant species collected in a sedimentation basin of a former petrochemical plant, and 2) inoculation or not with a bacterial consortium made up of ten isolates of *Proteobacteria*. The plants were grown in the greenhouse for four months. MiSeq amplicon sequencing, targeting the bacterial 16S rRNA gene and the fungal ITS, was used to assess the microbial community structures of sediments from planted and

unplanted microcosms. Our results showed that while inoculation caused a significant change in microbial communities, the presence of the plant and its specific identity had a stronger influence on the structure of the microbiome in sediments contaminated with petroleum hydrocarbons.

Next, using the same experimental setup, we used MiSeq amplicon sequencing targeting the 18S rRNA gene to assess AMF community structures in the roots and rhizosphere of plants growing in contaminated and uncontaminated substrates. We also studied the contribution of the specific identity of plants and the biotope (plant roots and rhizospheric soil) in the formation of associated AMF assemblages. Our results showed that while inoculation caused a significant change in AMF communities, substrate contamination had a much stronger influence on their structure, followed by biotope and plant identity to a lesser extent. In addition, inoculation dramatically increased plant biomass production and was associated with decreased dissipation of petroleum hydrocarbons in contaminated soil. The result of this study provides knowledge on the factors influencing the diversity and community structure of AMF associated with plants in stressed environments following repeated inoculations of a bacterial consortium.

Finally, we tested the effect of inoculating trees with specific mycorrhizal fungi on their survival and growth, as well as the extraction of trace metals. To do this, a field experiment was carried out in which we cultivated the *Salix miyabeana "SX67"* clone on the site of a decommissioned industrial landfill and inoculated the shrubs with the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, the ectomycorrhizal *fungus Sphaerosporella brunnea*, or a mixture of both. After two growing seasons, willows inoculated with the fungus *S. brunnea* produced a significantly higher biomass. Ba, Cd and Zn were found to accumulate in the aerial parts of plants, where Cd had the highest bioconcentration factor values in all treatments. In addition, the plots where the willows

were inoculated with *S. brunnea* showed a significant decrease in the concentrations of Cu, Pb and Sn in the soil. The inoculation with *R. irregularis* as well as the double inoculation did not significantly influence the biomass production and the soil trace elements levels

The result of this study provides insight into the diversity and ecophysiology of rhizosphere microbes associated with spontaneously growing plants following repeated inoculations. In addition, they show the potential of using mycorrhizal fungi to improve plant health and growth in polluted and toxic environments. They also stress the importance of plant selection to facilitate their efficient management, in order to speed up land reclamation processes.

Keywords: Plant-microbes interactions, microbial ecology, rhizosphere microbiome, bioaugmentation, petroleum hydrocarbon contamination; amplicon sequencing;

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ANOVA	analyse de la variance
ADN	acide désoxyribonucléique
AMF	arbuscular mycorrhizal fungi
ARN	acide ribonucléique
ARNr	ARN ribosomal
BLASTN	basic local alignment search tool nucleotide
bp	base pairs
Cd	Cadmium
cm	centimètre
CMA	champignons mycorhiziens à arbuscule
Cu	cuivre
DNA	desoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dNTP	deoxynucleotide triphosphate
HAP	hydrocarbures aromatiques polycycliques
HP	hydrocarbures pétroliers
ITS	Internal transcribed spacer
kg	kilogramme
KOH	hydroxyde de potassium
L	litre
MDDELCC	ministère du Développement durable, de l'Environnement et de la Lutte contre les changements climatiques
mg	milligramme
$MgCl_2$	chlorure de magnésium
MHB	mycorrhiza helper bacteria

min	minute
mL	millilitre
mM	millimolaire
MT	Métaux traces
NCBI	National center for biotechnology information
NSERC	Natural Science and Engineering Research Council of Canada
OTU	operational taxonomic unit
PAH	polycyclic aromatic hydrocarbons
Pb	plomb
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PERMANOVA	Permutational Multivariate Analysis of Variance
PHC	petroleum hydrocarbons
PSB	phosphate-solubilizing bacteria
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
TM	Trace metal
sp.	species (espèce)

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1- Chapitre 1 | Introduction Générale

Les microorganismes habitent presque tous les habitats de notre planète, incluant les plus extrêmes tel que les glaciers, les cheminées hydrothermales sulfureuses, ainsi que les roches sous-terraines où règnent des températures et pressions élevées (Rothschild and Mancinelli, 2001). Ils sont présumés d'avoir été les premiers organismes à coloniser les habitats terrestres il y a quelque 3,48 milliards d'années (Djokic et al., 2017; Homann et al., 2018). D'ailleurs, les traces de la plus ancienne symbiose entre les plante et microbes remontent à plus de 400 millions d'années (Selosse et al., 2015), et impliquent des champignons dis mycorhiziens arbusculaires. Ce type de symbiose est présumé d'avoir joué un rôle majeur dans la conquête végétale des milieux terrestres, ainsi que dans l'évolution des plantes (Selosse et al., 1998). La dépendance nutritionnelle des plantes sur leur symbiontes racinaires a été postulée et décrite aussi tôt que 1885 par B. Frank (Frank and Trappe, 2005). Depuis, les microbiologistes ont porté une grande attention sur les microorganismes vivant à proximité des plantes, mais ce n'est que lors du développement des technologies de séquençage de l'ADN que la grande abondance et diversité de ces microbes a été vraiment révélée (Hannula et al., 2012). En effet, les racines des plantes agissent comme de véritables pôles d'attraction pour les microorganismes du sol, notamment à travers leurs exsudats tels que les acides aminés, les acides organiques, et les hydrates de carbone (Kowalchuk et al., 2002; Berg et al., 2005; Berendsen et al., 2012b; Mendes et al., 2013; Peiffer et al., 2013; Bucci, 2015). Ceci implique que la zone rhizosphérique est un milieu très sélectif puisque chaque profil d'exsudats va attirer et favoriser un assemblage de microbes différents (Kumar and Dubey, 2020). L'ensemble de ces communautés microbiennes est appelé le microbiome de la rhizosphère de la plante. Il peut être considéré comme un ensemble de gènes et de fonctions qui peuvent être bénéfiques, neutres, ou nocifs pour la plante (Berendsen et al., 2012a). En conséquence, il

est également appelé deuxième génome de la plante et est considéré comme une communauté dynamique de micro-organismes, qui ont développé des interactions intimes et spécifiques de longue durée avec les racines des plantes. Les progrès récents dans l'étude des interactions plantes-microbes ont démontré leurs effets profonds sur la croissance, la nutrition et la santé des plantes (Barea et al., 2002b; Mendes et al., 2011; Hrynkiewicz and Baum, 2012; Bakker et al., 2013). Une grande partie de nos connaissances actuelles sur les interactions et les processus dans le microbiome de la rhizosphère est issue d'études sur les systèmes agricoles et horticoles. Cependant, notre compréhension de ces interactions lorsque les plantes poussent dans des conditions stressantes en est encore à ses débuts. De telles études nous aideront à comprendre les stratégies des plantes pour recruter des taxons microbiens clés, leur permettant de mieux s'adapter aux conditions de stress, et pourraient également aider à concevoir de nouvelles stratégies de réhabilitation des écosystèmes. Par exemple, la remise en état des terres par phytoremédiation est une pratique courante qui repose sur l'utilisation de plantes introduites et de leurs micro-organismes associés pour stabiliser ou réduire les polluants dans les sols (par exemple *Salix spp.*) (Bell et al., 2013). Bien que cette pratique se soit avérée être efficace pour décontaminer les sols légèrement contaminés, elle pourrait être compromise en raison d'une mauvaise croissance des plantes à des niveaux de pollution plus élevés (Pulford and Watson, 2003), causée au moins en partie par des variations dans leur capacité de s'associer aux microbes résidents (Bell et al., 2013). Ces associations jouent donc un rôle non négligeable pour l'établissement et la survie des plantes, d'où l'intérêt de pouvoir gérer ces microbes rhizosphériques afin qu'ils bénéficient à la plante. Les pratiques courantes en phytoremédiation sont axées sur l'introduction de plantes qui présentent une croissance rapide, produisant une biomasse élevée ainsi qu'une tolérance au stress des contaminants et un système racinaire extensif (Pilon-Smits, 2005a). Ces plantes sont souvent des arbustes comme des saules (*Salix*) et des peupliers (*Populus*) arbustifs par exemple.. Des études antérieures sur les patrons de

végétalisation naturelle dans des environnements fortement stressés (Desjardins et al., 2014) (par exemple les sols pollués par le pétrole) ont montré une grande diversité de végétation spontanée, suggérant que de nombreuses plantes poussant localement sont adaptées pour survivre dans des conditions de stress. Si elles s'avèrent efficaces pour la phytoremédiation, elles pourraient remplacer l'introduction d'arbustes lorsque la situation le permet. Des informations précises sur leurs microbes associés sont donc d'une importance capitale pour déchiffrer les mécanismes d'adaptation des plantes au stress, afin fournir des services écologiques adaptés à la spécificité du site.

1.1 Le microbiome rhizosphérique et ses composantes

La notion de 'superorganisme' fut proposée il y a un peu plus d'un siècle par William Morton Wheeler pour décrire une colonie de fourmis étant une association entre plusieurs individus de la même espèce pour accomplir une fonction spécifique, agissant comme un seul organisme (Wheeler, 1911; 1923). Avec le temps certains ont étendu la notion de superorganisme pour désigner une symbiose entre deux espèces différentes qui coopèrent comme un seul organisme (Gordon et al., 2013). Il y avait donc une nécessité de pouvoir plus clairement catégoriser et distinguer ces interactions complexes; En effet, plusieurs types de relations peuvent survenir pendant le cycle de vie d'un organisme allant d'une association permanente jusqu'à une relation transitoire de courte durée (Zilber-Rosenberg and Rosenberg, 2008). En 1993, Lynn Margulis proposa que toute association physique entre des individus d'espèces différentes, pendant des portions importantes de leur cycle vital constitue une symbiose où tous les individus participants sont des *biontes*, et que l'assemblage qui en résulte est le *Holobionte* (Margulis, 1993) et ce terme fut adopté par les biologistes depuis. Comme déjà mentionné, la rhizosphère est la zone étroite du sol qui est influencée par les exsudats des racines des plantes, et abrite une gamme impressionnante de micro-organismes qui se développent grâce à cette proximité (Berendsen et al., 2012b). La plante et son microbiome rhizosphérique peuvent être considérés comme un méta-

organisme, où la plante dépend des microbes pour certaines fonctions et en retour, dépose une partie de son carbone grâce à la photosynthèse dans l'interface racine-sol, ce qui servira de nutrition pour le microbiote (Mendes et al., 2011). La grande majorité des micro-organismes habitant la rhizosphère sont des champignons et des bactéries. La zone rhizosphérique a un effet direct sur leur abondance, qui est jusqu'à 20 fois plus élevée que dans le sol en vrac adjacent (Hrynkiewicz and Baum, 2012). Par conséquence, on y retrouve une compétition intense pour les ressources nutritives, mais également, les différents organismes développement des stratégies de survie distinctes donnant lieu à de nombreuses interactions antagonistes, mais aussi synergiques entre eux et avec la plante (Hrynkiewicz and Baum, 2012). Les plantes jouent donc un rôle majeur dans la détermination de la structure des communautés microbiennes du sol. elles peuvent recruter et influencer l'abondance des microorganismes du sol par exsudation racinaire de composés carbonés, d'acides organiques, d'hormones végétales, ainsi que d'autres molécules bioactives (Kowalchuk et al., 2002; Berg et al., 2005; Berendsen et al., 2012b; Mendes et al., 2013; Peiffer et al., 2013; Bucci, 2015). Les plantes influencent les propriétés chimiques du sol et par conséquence les profils microbiens par l'intermédiaire de composés racinaires spécifiques, ce qui va ensuite affecter la croissance et la productivité des plantes (Bezemer et al., 2006). Ce phénomène est largement décrit dans la littérature comme étant *Plant-Soil Feedback* (Bever, 1994). Les exsudats des racines sont déposés de manière différentielle dans la rhizosphère selon l'identité de l'espèce végétale et le stade de développement de la plante dans un environnement donné (Hertenberger et al., 2002; Chaparro et al., 2013), ce qui conduirait à des structures de communautés microbiennes rhizosphériques distinctes. L'attraction de microbes dans leur rhizosphère peut profiter aux plantes de multiples façons, comme par exemple en améliorant leur nutrition minérale, puisque les microbes sont responsables de l'acquisition de 5 à 80% d'azote et jusqu'à 75% de phosphore nécessaire à la croissance et au développement des plantes (Heijden et al., 2008).

1.1.1 Bactéries

Parmi les microbes du sol, les bactéries sont sans aucun doute les organismes les plus nombreux (Fierer and Jackson, 2006). Elles sont d'une importance capitale pour le bon fonctionnement des écosystèmes du sol car elles présentent un vaste éventail de capacités fonctionnelles: par leurs activités métaboliques et enzymatiques, elles jouent un rôle central dans le cycle biogéochimique des nutriments, la croissance des plantes et la productivité. La rhizosphère abrite des membres de tous les embranchements bactériens dont plusieurs peuvent exercer des effets favorables à la santé des plantes. Ces bactéries sont généralement en contact avec la surface des racines et augmentent la croissance des plantes en améliorant leur nutrition minérale et leur production de phytohormones, ainsi qu'en supprimant les maladies (Tarkka et al., 2008). Par exemple, certaines peuvent stimuler la croissance des plantes en mobilisant des nutriments inorganiques, par fixation de l'azote, par solubilisation des phosphates via des acides organiques exsudés et des enzymes, ainsi que par la production d'auxines, de cytokinines et de substances volatiles (Barea et al., 2002b). Celles qui peuvent lutter contre les agents pathogènes des plantes peuvent le faire par antagonisme direct ou bien en induisant une résistance accrue des plantes. Les bactéries rhizosphériques peuvent également interagir avec les champignons par le biais d'interactions simples comme l'attachement à la surface des hyphes, et des interactions plus complexes telles que la symbiose obligatoire, faisant de cette synergie un facteur important dans l'écologie de la rhizosphère (Perotto and Bonfante, 1997). Certaines bactéries sont capables d'encourager et d'augmenter les associations mycorhiziennes entre les plantes et les champignons en améliorant les capacités d'association des deux partenaires : Quelques mécanismes pour l'effet auxiliaire leurs effets auxiliaires ont été proposés, comme par exemple, la production de vitamines, d'acides aminés, de phytohormones et d'enzymes hydrolytiques de la paroi cellulaire. Certains de ces effets pourraient influencer directement la germination et le taux de croissance des structures fongiques, tandis que d'autres pourraient agir sur le

développement des racines et leur susceptibilité à la colonisation fongique (Garbaye, 1994). Finalement, des bactéries associées avec des spores de certains champignons mycorhiziens fournissent la capacité de contrôler la croissance de certains phytopathogènes in vitro (Bharadwaj et al., 2008).

1.1.2 Les champignons

Les champignons habitent tous les écosystèmes terrestres et jouent un rôle majeur dans le cycle des nutriments (Hoffland et al., 2004). Certains sont capables de décomposer des molécules organiques complexes, notamment la cellulose, l'hémicellulose et la lignine, minéraliser la matière organique et ainsi contribuer à la nutrition des plantes (de Boer et al., 2005). Dans la rhizosphère des plantes, les champignons saprotrophes aident à mobiliser les nutriments et à les rendre facilement disponibles pour être absorbés par les racines grâce à la production d'acides organiques et d'autres enzymes (Dutton and Evans, 1996). L'hyphosphère agit également comme un pôle d'attraction pour une communauté bactérienne diversifiée, qui utilise le réseau mycélien dense comme vecteur de dispersion et source d'exsudats organiques nutritifs (El Amrani et al., 2015). Certains champignons agissent également comme des agents de biocontrôle et réduisent l'incidence de certaines maladies (Elad et al., 1982; Nordbring-Hertz et al., 2006).

1.1.3 Les champignons mycorhiziens arbusculaires

Les champignons mycorhiziens arbusculaires (CMA) sont des microorganismes du sol omniprésents qui forment des associations mutualistes avec les racines de 72% des espèces végétales terrestres, et jouent un rôle important dans leur fonctionnement biologique (Brundrett, 2017). Cette association symbiotique est obligatoire pour le partenaire fongique, dont le mycélium pénètre dans les cellules corticales des racines et forme des structures caractéristiques, les arbuscules, qui agissent comme une interface pour

les échanges plantes-champignons (Parniske, 2008). Cette association permet au champignon d'avoir un accès direct au carbone produit par la plante; en retour, celle-ci profite de la capacité du mycélium à s'étendre au-delà de la zone racinaire permettant l'absorption une grande quantité d'eau et de minéraux (Solaimanand and Saito, 1997). Les CMA secrètent également des enzymes qui mobilisent l'azote et le phosphore, augmentant ainsi l'absorption de ces nutriments essentiels par leur hôte. De plus, la résistance des plantes aux maladies, leur taux de photosynthèse ainsi que leur succès d'établissement dans des environnements stressants, sont également accrus (Harrier and Watson, 2004; Parniske, 2008; Zamioudis and Pieterse, 2012). Dans le cadre de cette thèse, ces champignons seront scrutés, étant donné que la réalisation de toute application de phytoremédiation dépend de la réussite de l'établissement et de la croissance des plantes, afin de produire une grande biomasse de racineaire et aérienne. Les CMA et les microbes associés deviennent des acteurs de base dans ce domaine car ils peuvent atténuer le stress induit par les polluants, tels que les métaux traces et les hydrocarbures pétroliers (Kuiper et al., 2004; Zhang et al., 2006; Al-Amri, 2013). Dans l'assainissement des contaminants inorganiques comme les métaux traces, les CMA peuvent faciliter la solubilisation et biodisponibilité des métaux traces, par l'exsudation d'enzymes et d'acides organiques, augmentant ainsi l'efficacité d'extraction (Kumar et al., 1995; Garbisu and Alkorta, 2001; Pulford and Watson, 2003). Il faut cependant noter que les AMF peuvent aussi diminuer l'absorption de certains éléments par la plante, agissant comme un mécanisme de protection. Comme il n'y a aucune information sur la capacité de des CMA à dégrader les polluants organiques en raison de leur manque d'enzymes nécessaires pour les décomposer (d'où leur biotrophisme supposé obligatoire pour assurer la nutrition et la reproduction), leur activité au niveau de la rhizosphère est susceptible d'augmenter l'exsudation des racines des plantes et d'influencer des taxons microbiens. Ces derniers pourraient alors poursuivre la transformation de

produits organiques et inorganiques (Barea et al., 2002a; Jeffries et al., 2003; Li et al., 2006).

1.1.4 Champignons ectomycorhiziens

La symbiose ectomycorhizienne est une association entre les plantes ligneuses et les membres des phyla fongiques Basidiomycota et Ascomycota. Ces champignons forment un réseau d'hyphes ramifiées entre les cellules corticales racinaires (appelé réseau de Hartig) qui permet d'augmenter l'interface d'échange avec la plante, améliorant ainsi le transport de métabolites (Smith and Read, 2010). Le mycélium recouvre aussi la surface externe des racines sur lesquels il forme un manteau (ou manchon) d'hyphes qui se propagent vers le sol environnant, et multiplient la surface d'absorption de l'eau et des éléments nutritifs de la plante. Ces fins filaments peuvent aussi pénétrer dans les moindres recoins des particules du sol pour mobiliser et absorber les minéraux qui sans le mycélium, ne seraient pas accessibles pour les plantes (Landeweert et al., 2001). Les ectomycorhizes peuvent aussi assister leur plantes hôtes à résister aux pathogènes racinaires, notamment en empêchant ces derniers de pénétrer dans les racines protégées par le manchon mycélien, ou bien en les inhibant au moyen de la production des certain composés (Dutton and Evans, 1996; van der Heijden et al., 2015). En plus, certains exsudats fongiques vont attirer vers la rhizosphère d'autres microorganismes en quête de sources de carbone (Liu et al., 2018). La symbiose ectomycorhizienne améliore aussi la résistance des plantes contre les facteurs abiotiques, tel que la sécheresse, la salinisation, et les éléments traces (van der Heijden et al., 2015), jouant ainsi un rôle important dans l'établissement et la survie des plantes dans des milieux stressés. Dans les sols contaminés par des éléments traces, les champignons ectomycorhiziens peuvent réduire leur toxicité en les rendant moins biodisponibles pour la plante, et ce à travers plusieurs mécanismes : entre autres par précipitation extracellulaire de ces éléments où ils deviennent insolubles, par réduction de leur absorption et par

séquestration dans des compartiments spécialisés (Hartley et al., 1997; Kabata-Pendias, 2000; Bellion et al., 2006). Ces traits physiologiques font des champignons ectomycorhiziens de potentiels candidats pour améliorer l'efficacité des plantes dans la remédiation de sols contenant des contaminants xénobiotiques.

1.2 Manipulation du microbiome

Dans le contexte de cette thèse, le terme 'manipuler' signifie toutes les interventions humaines permettant de modifier certains paramètres physico-chimiques ou biologiques qui induiront des changements de la composition et/ou la structure des communautés microbiennes. Afin de pouvoir manipuler le microbiome de la rhizosphère, il faut d'abord connaitre et comprendre les différents facteurs qui influencent sa composition et sa diversité. Plusieurs paramètres abiotiques, comme l'humidité, le pH l'azote ou le phosphore disponibles influencent les communautés microbiennes du microbiome rhizosphérique (van der Voort et al., 2016; Taktek et al., 2017). Les interactions avec les microbes avoisinants, ainsi qu'avec la plante jouent aussi un rôle déterminant dans la sélection des microbes de la rhizosphère. Considérant la complexité de ces interactions, plusieurs approches ont été proposées et développées pour manipuler le microbiome rhizosphérique. En général, trois stratégies sont adoptées, soit l'approche à travers le microbiome, l'approche à travers la plante hôte et l'approche à travers le métaorganisme que sont la plante hôte et ses microbes associés (Kumar and Dubey, 2020). L`approche à travers le microbiome est basée sur une intervention au niveau des microbes de la rhizosphère, principalement en inoculant cette dernière avec des souches uniques ou des consortia de microbes (Quiza et al., 2015b). Les candidats microbiens (bactériens et fongiques) sont sélectionnés pour leur capacité à accomplir une fonction spécifique pour améliorer la santé de la plante; Par exemple, certaines bactéries induisent les systèmes de résistance systémique, renforçant ainsi l'immunité de la plante (Bakker et al., 2013), alors que d'autres agissent sur la nutrition des plantes, en solubilisant le phosphore et le

potassium (Khan et al., 2009), ou bien en fixant l'azote atmosphérique (Bhattacharjee et al., 2008; Mia and Shamsuddin, 2010), qui deviennent ainsi disponibles à la plante. Des bactéries recombinantes peuvent aussi être introduites afin de transférer horizontalement un gène d'intérêt qui induira des fonctions bénéfiques tel que la résilience, la résistance aux maladies et autres (Lynch et al., 2004; Ryan et al., 2009). Les chances de réussite de ces inoculation de la rhizosphère peuvent être augmentées en introduisant préalablement une perturbation des microorganismes résident, à travers des moyens mécaniques tel que labourage du sol, ou l'introduction de fongicides et/ou antibiotiques qui vont cibler des microbes spécifiques (Brussaard et al., 2007; Ryan et al., 2009; Bakker et al., 2012). La deuxième approche pour manipuler le microbiome rhizosphérique à travers des interventions sur la plante hôte comporte plusieurs stratégies possibles; Cela peut se faire à travers un programme de croisement et sélection artificiel des plantes pour la production de cultivars avec des propriétés utiles : par exemple, une exsudation augmentée ou optimisée afin de possiblement recruter des microbes désirables dans la rhizosphère (Hartmann et al., 2009; Dubey et al., 2019), ou bien des cultivars qui présentent de plus grande efficacité à établir des associations bénéfiques avec un inoculum qui sera appliqué (Champignons mycorhiziens et autres endophytes) (Solaiman et al., 2000). Une autre option est la modification génétique des plantes, visant encore une exsudation spécifique qui augmentera le potentiel à induire les microbes à produire des sidéropores, des antibiotiques et des antifongiques par exemple (Bakker et al., 2012; Imam et al., 2016). Enfin, l'approche à travers le métaorganisme plante/microbes consisterait à développer et sélectionner à la fois l'hôte et les microorganismes afin qu'ils soient complémentaires (Quiza et al., 2015b; Thijs et al., 2016). Par exemple, l'utilisation de la rotation de cultures avec remplacement de jachère par une culture de légumineuses, qui vont augmenter la concentration d'azote dans le sol grâce aux microorganismes auxquels ils sont associés (Gan et al., 2015). Un autre exemple serait la sélection artificielle de cultivars de plantes exsudant un composé

spécifique, puis introduire un microorganisme modifié pour pouvoir métaboliser le composé (Dessaux et al., 1998; Savka et al., 2002).

1.3 Stress abiotique sélectionné pour la thèse

Comme mentionné plus haut, nous visons comprendre la réaction du microbiome de la rhizosphère de plantes poussant dans des milieux stressés. Les expériences se dérouleront dans des sédiments hautement contaminés aux hydrocarbures pétroliers, ainsi qu'un ancien dépotoir industriel contenant des concentrations de métaux traces élevées.

1.3.1 Les hydrocarbures pétroliers

Dans les écosystèmes terrestres, la pollution par les hydrocarbures pétroliers (PHC) a eu un impact négatif prononcé sur la productivité des cultures. Ces composés toxiques affectent la germination et la croissance de nombreuses plantes, diminuent également la fertilité du sol, et empêchent la croissance des cultures (Tang et al., 2011). La contamination des terres par le pétrole brut affecte certains paramètres du sol tels que la teneur en matières minérales et organiques, la capacité d'échange cationique, les propriétés redox et le pH. Comme il crée une condition anaérobie dans le sol, couplée à la saturation de l'eau et aux métabolites acides, le résultat peut être une forte accumulation de certains ions, qui sont toxiques pour la croissance des plantes (Onwurah et al., 2007).

1.3.2 Pollution aux métaux traces (MT)

Les activités industrielles et minières peuvent conduire à l'accumulation de fortes concentrations de métaux et d'oligo-éléments dans les sols. Comme les MT ne sont pas dégradables, ils s'accumulent dans les écosystèmes et posent un grave risque pour l'environnement et la santé humaine (Giller et al., 1998; 1999; Garbisu and Alkorta, 2001). Cela fait de l'assainissement des terres contaminées par les MT un enjeu important, afin de limiter la propagation et de diminuer les concentrations de ces éléments potentiellement

toxiques. Les méthodes conventionnelles de traitement des sols impliquent des traitements perturbateurs in situ et ex situ tels que l'excavation et la mise en décharge, le lavage du sol, la stabilisation chimique, l'incinération du sol et le lessivage acide entre autres. Ces pratiques présentent des limitations majeures en termes de coût, de main-d'œuvre et d'empreinte écologique, car elles nécessitent machineries lourdes et coûteuses ainsi que des procédures techniques complexes. Ils induisent également des altérations irréversibles des propriétés physico-chimiques du sol et des communautés microbiennes (Tchounwou et al., 2012; Yao et al., 2012; Suzuki et al., 2014). Par conséquent, des techniques plus durables et plus respectueuses de l'environnement sont recherchées pour l'assainissement des sols contaminés par des métaux traces. Une de ces méthodes est la phytoremédiation, qui consiste à utiliser des plantes et la microflore du sol associée pour dégrader, extraire, ou stabiliser les polluants. Elle est moins coûteuse car elle ne nécessite pas beaucoup de main-d'œuvre et contribuera à terme à la végétalisation naturelle des sites pollués, bien qu'elle soit beaucoup plus lente que les méthodes mécaniques et chimiques.

1.4 Raisonnement et objectifs de la thèse

À travers les travaux présentés dans cette thèse, je teste premièrement l'effet d'une perturbation biotique du microbiome rhizosphérique de plantes poussant dans des sédiments hautement contaminés avec des hydrocarbures pétroliers. Quatre espèces de plantes sont utilisées afin de comparer l'effet de la perturbation sur leur croissance, repérer des différences entre leurs microbiomes. La perturbation biotique sélectionnée est un ensemencement de mésocosmes contenant les sédiments et les plantes, avec un consortium de bactéries isolées des mêmes sédiments. Le raisonnement est que l'introduction d'une quantité massive de cellules bactériennes causera une perturbation dans la structure du microbiome déjà établi. Ce changement de structure pourrait aussi se traduire par des effets sur la croissance des plantes et l'état des sédiments, notamment la dissipation des

hydrocarbures pétroliers. Les bactéries ensemencées ne sont pas criblées pour une fonction spécifique, puisque le but est de rompre l'équilibre dans le microbiome par leur ajout massif et non pas à travers une fonction sélectionnée puisque cela dépend de la survie et persistance des inocula qui souvent sont exclus par compétition. Les microorganismes que nous avons analysés sont les bactéries et les champignons, avec une attention particulière sur les CMA vu leur rôle important dans la santé des plantes. De plus, l'analyse de CMA inclue aussi des échantillons provenant d'une réplication exacte de l'expérience mais dans un sol naturel non contaminé, afin de comparer les changements suite à la perturbation dans les deux milieux différents. Un objectif secondaire de cette expérience est d'explorer la possibilité de l'utilisation de plantes locales de croissance spontanée dans des milieux pollués, afin d'accélérer la décomposition d'hydrocarbures; La pratique courante étant l'introduction de plantes à croissance rapide, produisant une grande biomasse et un système racinaire extensif. Finalement, une expérience sur le terrain est conduite où des saules arbustifs sont plantés dans un ancien dépotoir industriel. La couche organique recouvrant le dépotoir contient des concentrations toxiques de plusieurs éléments traces. Les arbres sont inoculés avec un champignon ectomycorhizien, un CMA ou bien une combinaison des deux, et l'effet de ces inoculations sur la croissance des arbres et les niveaux d'éléments traces dans le sol et la partie aérienne des saules est mesuré.

2 Chapitre 2 | Modification de la structure du microbiome rhizosphérique de plantes poussant dans des sédiments hautement contaminés avec hydrocarbures pétroliers, par l'intermédiaire d'inoculations bactériennes du substrat

Compte tenu de l'importance du rôle que joue le microbiome de la rhizosphère dans le fonctionnement et la santé des plantes, il est primordial de connaitre les facteurs majeurs qui déterminent sa composition et structure. Ces connaissances nous permettront de moduler la performance et productivité des plantes à travers la manipulation de leur microbiome rhizosphérique sans l'utilisation d'intrants tels que les engrais, ou des composés tels que les hormones végétales synthétiques. Comme la majorité des connaissances actuelles sur le microbiome rhizosphérique des plantes proviennent de contextes naturels ou agricoles, nous avons choisi de travailler avec des plantes soumises à un stress abiotique. Dans ce chapitre, nous avons testé la possibilité d'induire un changement dans la structure du microbiome de la rhizosphère de plantes, poussant dans un substrat contaminé aux hydrocarbures pétroliers. Ceci a été réalisé par l'intermédiaire d'inoculations répétées avec un consortium de bactéries isolées du même substrat contaminé.

Plant identity shaped rhizospheric microbial communities more strongly than bacterial inoculation in petroleum hydrocarbon-polluted sediments

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Abstract

Manipulating the plant-root microbiota has the potential to reduce plant stress and promote their growth and production in harsh conditions. Community composition and activity of plant-roots microbiota can be either beneficial or deleterious to plant health. Shifting this equilibrium could then strongly affect plant productivity in anthropized areas. In this study, we tested whether repeated inoculation with *Proteobacteria* influenced plant productivity and the microbial communities associated with the rhizosphere of four plant species growing in sediments contaminated with petroleum hydrocarbons. A mesocosm experiment was performed in randomized block design with two factors: 1) presence or absence of four plants species collected from a sedimentation basin of a former petrochemical plant, and 2) inoculation or not with a bacterial consortium composed of ten isolates of *Proteobacteria*. Plants were grown in a greenhouse over four months. MiSeq amplicon sequencing, targeting the bacterial 16S rRNA gene and the fungal ITS, was used to assess microbial community structures of sediments from planted or unplanted microcosms. Our results showed that while inoculation caused a significant shift in microbial communities, presence of plant and their species identity had a stronger influence on the structure of the microbiome in petroleum hydrocarbons contaminated sediments. The outcome of this study provides knowledge on the diversity and behavior of rhizosphere microbes associated with indigenous plants following repeated inoculation, underlining the importance of plant selection in order to facilitate their efficient management, in order to accelerate processes of land reclamation.

Keywords: Plant-microbes interactions, microbial ecology, rhizosphere microbiome, bioaugmentation, petroleum hydrocarbon contamination; amplicon sequencing;

2.1 Introduction

The rhizosphere microbiome usually refers to bacterial, archaeal and fungal communities as well as their genetic material closely surrounding plant-root systems (Mendes et al., 2013). The metagenome of this microbiome has been referred to as the second genome of the plant (Berendsen et al., 2012b). It is a dynamic community of microorganisms, from which a part of the species have developed long-lasting intimate and specific interactions with plants roots (Vandenkoornhuyse et al., 2015). Recent advances in plant-microbe interactions demonstrated their profound effects on the growth, nutrition, and health of plants (Barea et al., 2002b; Mendes et al., 2011; Hrynkiewicz and Baum, 2012; Bakker et al., 2013). The rhizosphere microbiota is complex with a dynamic spatiotemporal structure that adapts rapidly depending on biotic and abiotic stresses (Marschner et al., 2002; Mendes et al., 2013).

Recent advances in next-generation sequencing and bioinformatics allow the unraveling of taxonomic composition and functions of complex communities in a wide range of habitats and environmental conditions (Hiraoka et al., 2016). Much of our current knowledge regarding interactions and processes in the rhizosphere microbiome has emerged from studies in natural and agricultural environments (Quiza et al., 2015a; Busby et al., 2017). On the other hand, the knowledge of rhizospheric microbial communities associated with plants growing under stressful conditions in highly anthropized areas is still in its early stages. Although, in this decade, there has been an increase in research attention to these habitats (Bell et al., 2013; Bell et al., 2014; Yergeau et al., 2014; Yergeau et al., 2015; Bell et al., 2016; Gonzalez et al., 2018; Yergeau et al., 2018), more studies are needed to understand how plants recruit key microbial taxa to better cope with stressful conditions, and to help design new green strategies for ecosystem restoration. For instance, land reclamation based on phytoremediation is a technique that relies on the use of plants (e.g. *Salix* spp. or hyperaccumulator plants) and their associated microorganisms to

stabilize or reduce pollutants in soils (Bell et al., 2013). Although, this practice has been shown to be effective to rehabilitate mildly contaminated soils, phytoremediation efficiency might be compromised at higher levels of pollution due to poor growth of introduced plants (Pulford and Watson, 2003), caused at least in part by variations in their strength of association with resident microbes (Bell et al., 2013). An ecologically friendly and low carbon footprint method for the remediation of petroleum hydrocarbons (PHCs) is phytoremediation using a monoculture of fast growing, contaminant-tolerant high-biomass plants, with very developed root system (i.e. willows or poplars) (Pilon-Smits, 2005b; Hassan et al., 2014) that can recruit and maintain an increased biological activity, which in turn will accelerate the biodegradation of the pollutants. Local plant species have been shown to develop more cooperative interactions with indigenous microbes than foreign-introduced plant species (Johnson, 2010), making the use of naturally tolerant local plants attractive for the phytoremediation of petroleum compounds. A previous survey of natural revegetation patterns in sediments highly polluted with petroleum by-products reported a high diversity of spontaneously-arising plants (Desjardins et al., 2014), suggesting that many species are adapted to survive under stressful conditions. Precise information regarding their associated microbes is of paramount importance to decipher microbiome-mediated mechanisms of plant adaptation to stress, that should provide ecological services adapted to site specificity. Inoculation, which is the inoculation with indigenous or allochthonous microorganisms to enhance desired biological functions, has been used in many applications, such as wastewater treatment, food production or bioremediation of polluted soil (Vogel, 1996; Limbergen et al., 1998; Singer et al., 2005). The inoculation of a given environment can affect the established indigenous microbiota by triggering and/or accelerating community shift dynamics (Morgante et al., 2010; Zhang et al., 2014).

The aim of this study was to test whether repeated inoculation with bacterial consortium composed of ten isolates of *p,* influenced the rhizosphere microbial communities associated with the roots of four plant species growing in sediments contaminated with petroleum hydrocarbons, as well as their productivity. To address this, we performed an experiment in a greenhouse during a four-month growth period, using mesocosms filled with petroleum hydrocarbon-polluted sediments collected from a decantation basin of a former petrochemical plant. The microcosms were planted or not with a mixture of four plant species and were inoculated or not with a bacterial consortium of ten strains belonging to six genera of *Proteobacteria* that were isolated from the polluted sediments. The four plant species (*Persicaria lapathifolia*, *Lythrum salicaria*, *Lycopus europaeus* and *Panicum capillare*) were selected among the spontaneously growing vegetation thriving in the highly polluted sedimentation basin. We hypothesized that inoculation of the rhizosphere of plants already able to tolerate pollutant stress with *Proteobacteria* retreived from the polluted sediments will result in an increased biodegradation of the PHCs and cause a shift in the structure of the rhizosphere microbiome toward the bioaugmented taxa.

2.2 Material and Methods

2.2.1 Contaminated sediments

Sediments contaminated with petroleum-hydrocarbons were collected in October 2013 from a by-product sedimentation basin of a petrochemical plant located at Varennes, on the south shore of the St-Lawrence River near Montreal, Canada (45°41’56” N, 73°25’43” W). Basic chemical characteristics of the sediments have been previously described (Desjardins et al., 2014). Contaminated sediments were collected from the 0-10 cm layer of the decantation basin and brought back to the laboratory where they were

thoroughly homogenized and transferred into 60×29×12 cm trays to a final volume of 18 L per tray, and used as the growth substrate for plants. One kilogram of the homogenized contaminated sediments was kept at -80 °C and subsequently used for bacterial isolation, as described below. At the end of the experiment and after plant harvesting, the substrate was homogenized in the trays and a composite sample made up of three subsamples was taken per each tray and conserved at -20°C until used for PHC levels measures. Initial hydrocarbons concentrations were 3055 ± 188 mg/kg for C10-C50 and 35.4 ± 2.6 mg/kg for PAHs. From here on, the *contaminated sediments* will be referred to as the *substrate.*

2.2.2 Seeds harvesting and germination

Seedpods from four plant species, *Persicaria lapathifolia*, *Lythrum salicaria*, *Lycopus europaeus* and *Panicum capillare*, naturally growing within the same contaminated basin used to collect the sediments, were harvested in October 2013. These species were chosen based on the availability of seedpods, as well as on their germination success. The seeds were stratified in sterilized damp sand at 4° C for 8 weeks, after which they were germinated in a 1:1 (v:v) sterile sand/calcined montmorillonite clay (Turface®; Profile Products LLC, Buffalo Grove, IL, US) mix incubated at room temperature (~22° C). Germinated seeds were hand-selected and planted in 50 ml multi-cell compartments filled with an all-purpose commercial potting soil mix (Scotts Canada ltd., Mississauga, On, Canada) autoclaved twice (121° C for 45 minutes). After three weeks of growth, seedlings were carefully transferred to the trays containing the substrate following the design described below.

2.2.3 Isolation and identification of bacteria from the substrate, and preparation of the consortium

A 10% strength tryptic soy agar medium (3g/L) (Sigma-Aldrich, Oakville, ON, Canada) was prepared and autoclaved for 30 min at 121 ºC. The medium was supplemented with 100 mg/L cycloheximide before solidification to inhibit fungal growth and it was poured in Petri dishes. An inoculum was prepared by serially diluting down to 10^{-7} a thoroughly vortexed stock suspension composed of 1 g of the substrate in 9 ml of sterile demineralized water. Aliquots of 100 µl from dilutions of 10^{-6} and 10^{-7} were spread on the culture medium and incubated at 27º C for one week while being checked daily for bacterial growth. All growing colonies were subcultured on the same medium in order to obtain a pure culture. The bacterial isolates were then stored at 4ºC on the same solid growth medium for three days until use. Bacterial isolates were identified by sequencing the 16S rRNA gene, which was PCR-amplified using primers 27f and 1492r (Klindworth et al., 2013). A DNA sample was picked from each colony using a 1-µl sterile inoculation loop and directly added to the PCR master mix, that was made up of 1× PCR buffer, 0.5 mg BSA, 2mM $MgCL_2$, 0.2 µM of each primer, 0.2 mM of deoxynucleotide triphosphate (dNTPs), and one unit of the Qiagen *Taq* DNA Polymerase (Qiagen, Canada) in a total volume of 20µl per reaction. Thermal cycling conditions were as follow: initial denaturation at 94°C for 3 min; 30 cycles at 95°C for 30 sec., 55°C for 30 sec., and 72°C for 1 min, and a final elongation step at 72°C for 10 min. PCR reactions were performed on an Eppendorf Mastercycler ProS thermocycler (Eppendorf, Mississauga, ON). Sanger DNA sequencing was achieved using a commercial service provided by Genome Quebec Innovation Centre at McGill University (Montreal, QC).

To prepare the consortium, ten bacterial isolates from six genera belonging to the phylum *Proteobacteria* were selected (Supplementary Table S1). *Proteobacteria* were chosen because they have been found as a dominant and active bacterial group within the same

sediments in previous studies (Bell et al., 2014; Pagé et al., 2015; Stefani et al., 2015a). Bacterial isolates were selected based on their growth vigour and stability after subculturing. Isolates were individually cultured in 2 L flasks containing each 1 L of 10% strength tryptic soy broth (3 g/ L) (Sigma-Aldrich, Oakville, ON, Canada) at 27° C for 72 hours with agitation, after which the liquid cultures were centrifuged at 5000 g for 10 min at 4° C. The resulting bacterial cell pellets were re-suspended in 1 L of a sterile isotonic 0.154 M NaCl solution. Cell counts for each isolate were performed using a Neubauer improved haemocytometer (Sigma-Aldrich, Oakville, ON, Canada), and the inoculum was made up by suspending all the selected isolates in equal amounts in a final volume of 2.4 L at a final concentration of 2.4 x 10^9 CFU/ml.

2.2.4 Experimental design of the mesocosm experiment

The experiment was setup in a randomized block design in four blocks, with two factors: microcosms were planted or not, and inoculated or not, resulting in four treatment-combinations. The treatments were the following: not planted and non-inoculated (P-B-), planted and non-inoculated (P+B-), not planted and inoculated (P-B+), and planted and inoculated (P+B+). Each planted tray contained three rows, separated by 7.5 cm from each other. Within each row, the four plant species were randomly distributed in four planting positions, each being a cluster of four individual seedlings of the same plant species (Supplementary Fig. S1). These clusters were placed at 12.5 cm intervals on each row. Planted and non-planted mesocosms were watered as needed, several times weekly throughout the experiment. No fertilization was applied. Inoculation with the bacterial consortium was performed twice in both P-B+ and P+B+ treatments, two weeks and four weeks after the seedlings were transplanted in the substrate. At both inoculations, each tray received 300 ml of the bacterial consortium, which gave a final concentration of 4 x 10^7 CFU/ml of dry soil, or 300 ml of sterile water as a control.

2.2.5 Data collection and harvest

The experiment was harvested after 16 weeks of growth. At harvest, each plant cluster was carefully removed from the substrate to avoid root damage and aerial parts were separated from the roots. The substrate was gently shaken-off from the roots and the rhizospheric substrate still attached was brushed into plastic bags, flash frozen in liquid nitrogen, and kept at -80° C until DNA extraction. Soil from each plant cluster was conserved individually and represented one sample. In non-planted mesocosms, two composite soil samples were collected, each made out of three soil subsamples taken following the scheme presented in (Supplementary Fig. S2) and preserved similarly. Aerial plant parts were oven-dried for 72 hrs at 60° C before being weighted.

2.2.6 DNA extraction, PCR amplification and Illumina MiSeq sequencing

Total genomic DNA was extracted from 96 samples of rhizospheric substrate from the planted mesocosms (4 blocks × 2 planted treatment (P+B- and P+B+) × 12 plant clusters per tray that formed 1 sample each, and from 16 bulk substrate samples from the unplanted mesocosms (4 blocks × 2 unplanted treatments (P-B- and P-B+) × 2 composite samples per tray), using the Nucleospin® Soil Kit (MACHEREY-NAGEL Inc. Bethlehem, PA, USA), following the manufacturer's instructions. The extracted DNA was then diluted ten folds in sterile PCR grade ultrapure water, to reduce the risk of PCR inhibition by PHCs and humic substances. A two-step PCR procedure was performed to generate amplicons of bacterial 16S rRNA and fungal ITS genes suitable for MiSeq sequencing. In the first PCR rounds, primers Bakt_341F and Bakt_805R targeting the V3-V4 region of bacterial 16S rRNA genes (Klindworth et al., 2013), and primers ITS1F and 58A2R targeting the fungal ITS-1 region (Martin and Rygiewicz, 2005) were used. To each primer was added the Illumina overhang forward and reverse adapters to which unique sample-specific indexes have been attached in the second PCR round. Amplicons were then purified using the

NucleoMag® NGS Clean-up and Size Select kit (Macherey-Nagel, Canada). In the second PCR round, the Nextera XT V2 Illumina MiSeq specific index kit was used to attach individual indexes to amplicons from each sample, using a limited cycle run as recommended by the manufacturer. The tagged sequences were then purified and normalized using the SequalPrep™ Normalization Plate Kit, after which they were pooled at equimolar concentration and sequenced on an Illumina MiSeq sequencer using the 600 cycle MiSeq Reagent Kit v.3 in 2×300 bp configuration (Illumina Inc., San Diego, CA, USA). The sequences for all primers can be found in supplementary Table S2, and the specific PCR conditions in supplementary information file section II.

2.2.7 Sequence processing and statistical analysis

The assembling of reads and primers trimming were done in Mothur (v.1.34.4), while the rest of the initial processing was performed in QIIME (v.1.9), following the Brazilian Microbiome Project 16S and ITS profiling pipeline (Pylro et al., 2013; Pylro et al., 2014). Additional details of the main steps of this pipeline can be found in the supporting information file Sections I.A and I.B. The Miseq sequences were deposited in the NCBI Sequence Read Archive and can be found under project number PRJNA507467. The effect of inoculation with the bacterial consortium on plant shoot biomass, and the effects of inoculation and of the presence of plants on the concentration of C10-C50 and polycyclic aromatic hydrocarbons (PAHs) at the end of the experiment, as well as on the alpha diversity indices from the samples (Chao1 estimator (Chao, 1984), Shannon's diversity (Magurran, 2013), and Pielou's evenness (Pielou, 1966)), were analysed using ANOVA or Student's T test in JMP statistical software (SAS Institute Inc.). The effects of inoculation with the bacterial consortium and of the presence of plants on microbial

communities in the rhizosphere were analysed in R (v3.2.0, The R Foundation for Statistical computing). PERMANOVA was used to test the effect of treatments on the beta diversity of microbial communities in the substrate, followed by a Permutational multivariate analyses of dispersion (PERMDISP) of the Bray-Curtis matrices of these communities to assess whether any significant difference detected was effectively a shift in the communities or just due to random dispersion of the samples. Redundancy analysis (RDA) was performed using the 'rda' function from the 'vegan' v2.5 package in R, and constrained OTU composition and relative abundance by plant presence, plant species and bioaugmentation. In order to assess which microbial taxa's relative abundance is significantly modified by the treatments, we used the Kruskal-Wallis non-parametric analysis followed by a false discovery rate (FDR) adjustment of the P-value using the Benjamini-Hochberg procedure. The new P-value threshold (q value) is 0.05 for both fungi and bacteria. Information regarding the settings used in JMP and the R code are available in supplementary section III

2.3 Results

2.3.1 Microbial diversity in the substrate

Sequencing of 16S rRNA gene amplicons generated a total of 1913451 reads, of which 291000 sequences with an average length of 399 ± 69 bp were retained after quality filtering and subsampling to 3000 sequences per sample. Fifteen samples did not meet the 3000 sequences cutoff and were ignored for the further analysis. Reads per sample initially ranged from 1309 to 39984 reads, and grouped after subsampling into 7192 operational taxonomic units (OTUs) at the 97% similarity threshold. Good's coverage indices ranged between 81.8% and 84.6% for all treatments after subsampling, indicating that most bacterial diversity in each sample was captured (Supplementary Fig. S3).

Sequencing of ITS1 fragment yielded 1407783 sequences with an average length of 324 ± 28 bp, of which 424000 sequences were retained following quality filtering and subsampling to 4000 sequences per sample. Six samples did not meet the 4000-sequence threshold and were excluded from the analysis. Read counts ranged between 945 and 31335 per sample and grouped after subsampling into 889 OTUs at a cut-off threshold of 97%. Goods coverage index values ranged between 98.8 % and 99.2% across all treatments after subsampling, showing that nearly all the fungal diversity in each sample was captured (Supplementary Fig. S4).

Bacterial Chao1 species richness in the substrate was significantly influenced by the presence of plants (P+B-, P+B+) (P>| t| = 0.0123), compared with the unplanted treatments (P-B-, P-B+), while inoculation did not affect Chao1 species richness (Supplementary Fig. S5). Plants also significantly increased the Shannon diversity index (P>| t| <.0001, Supplementary Fig. S6) and evenness (P>| t| p<0.0001, Supplementary Fig. S7) of bacterial communities.

Overall, *Alphaproteobacteria* were the most abundant group across all treatments with an average of 22% of the bacterial community, followed by *Planctomycetes* at 13% and *Acidobacteria* with 11.4%. Then, *Gammaproteobacteria* formed 9.4% of the community, *Actinobacteria* 7.7%, *Betaproteobacteria* 6.4%, *Chloroflexi* 4.6%, *Verrumicrobia* 3.9%, *Deltaproteobacteria* 3.6% (Fig 1A). *Deltaproteobacteria* (FRD-adj. P=0.001), *Planctomyces* (FRD-adj. P=0.002), and *Bacteroidetes* (FRD-adj. P=0.0002) had significantly higher relative abundances in both planted treatments than in the unplanted mesocosms. *Alphaproteobacteria* (FRD-adj. P=0.0006) were significantly more abundant following bioaugmentation only in the unplanted treatments. On the other hand, *Acidobacteria* (FRD-adj. P=0.0002), and *Gammaproteobacteria* (FRD-adj. P=0.0002) had

significantly different relative abundances in both planted treatments than in the unplanted mesocosms.

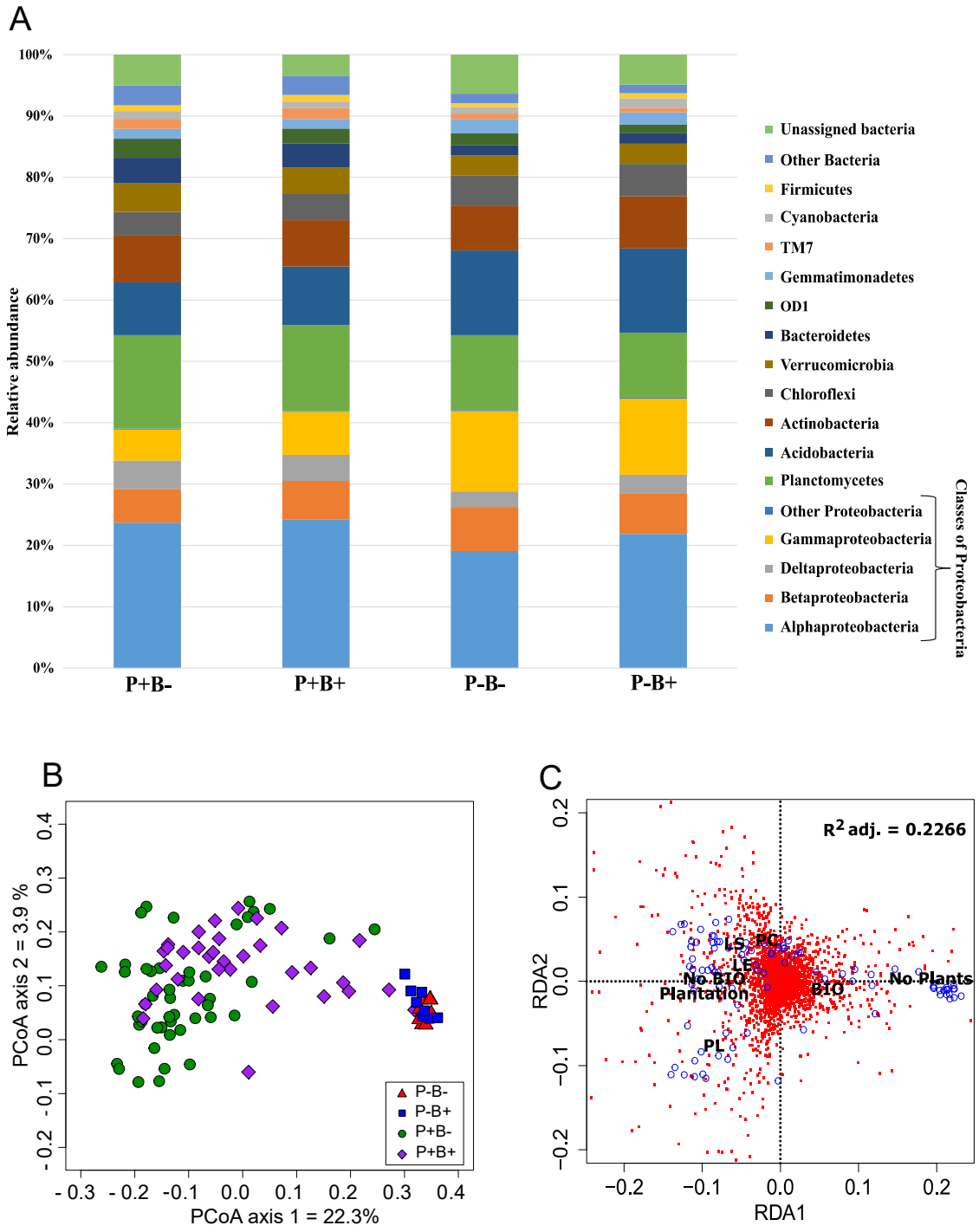

Figure 1. (A) Effect of the plantation and inoculation on the relative abundance of bacterial phyla or classes. (B) Principal coordinate analysis based on the Bray-Curtis dissimilarity of bacterial communities showing the effect of plantation and inoculation. (C) Redundancy analysis (RDA) showing the relation between the abundance of bacterial

OTUs and the factors Inoculation, Plantation, and Plant species identity. The adjusted R^2 value indicates the amount of variance in bacterial community composition accounted for by the constraining factors (Inoculation, Plantation, and Plant species identity). The location of labels represents factor centroids. Red squares indicate bacterial OTUs. Blue open circles represent individual samples. (Plt=Plantation; Bio=Inoculation; LE= *Lycopus europaeus*; LS=*Lythrum salicaria*; PC=*Panicum capillare*; PL=*Persicaria lapathifolia*).

For fungi, Chao1 richness estimator was significantly lower in planted than unplanted mesocosms (P> |t| <.0001), indicating that plant rhizosphere contained a lower number of fungal taxa than unplanted substrate (Supplementary Fig. S8). On the other hand, Pielou's evenness was significantly increased in the presence of plants (P> |t| = 0.003) (Supplementary Fig. S9).The Shannon diversity index did not change significantly in any of the treatments (Supplementary Fig. S10).

Ascomycota dominated the substrate in all treatments and formed 79.6% to 84.5 % of the fungal community (Fig. 2A). *Basidiomycota* ranged between 1.5% and 6.7%, and they showed significantly higher relative abundances in the planted mesocosms, regardless of the bioaugmentation (FRD-adj. P=0.04). *Sordariomycetes* were also significantly more abundant in the bioaugmented mesocosms, but only in the presence of plants (P+B+) (FRD-adj. P=0.03) and formed between 28% and 38% of the community. Unknown fungi formed between 14.8% and 8% of the community and the rest of the fungal diversity not mentioned here ranged from 0.4% to 1.5%.

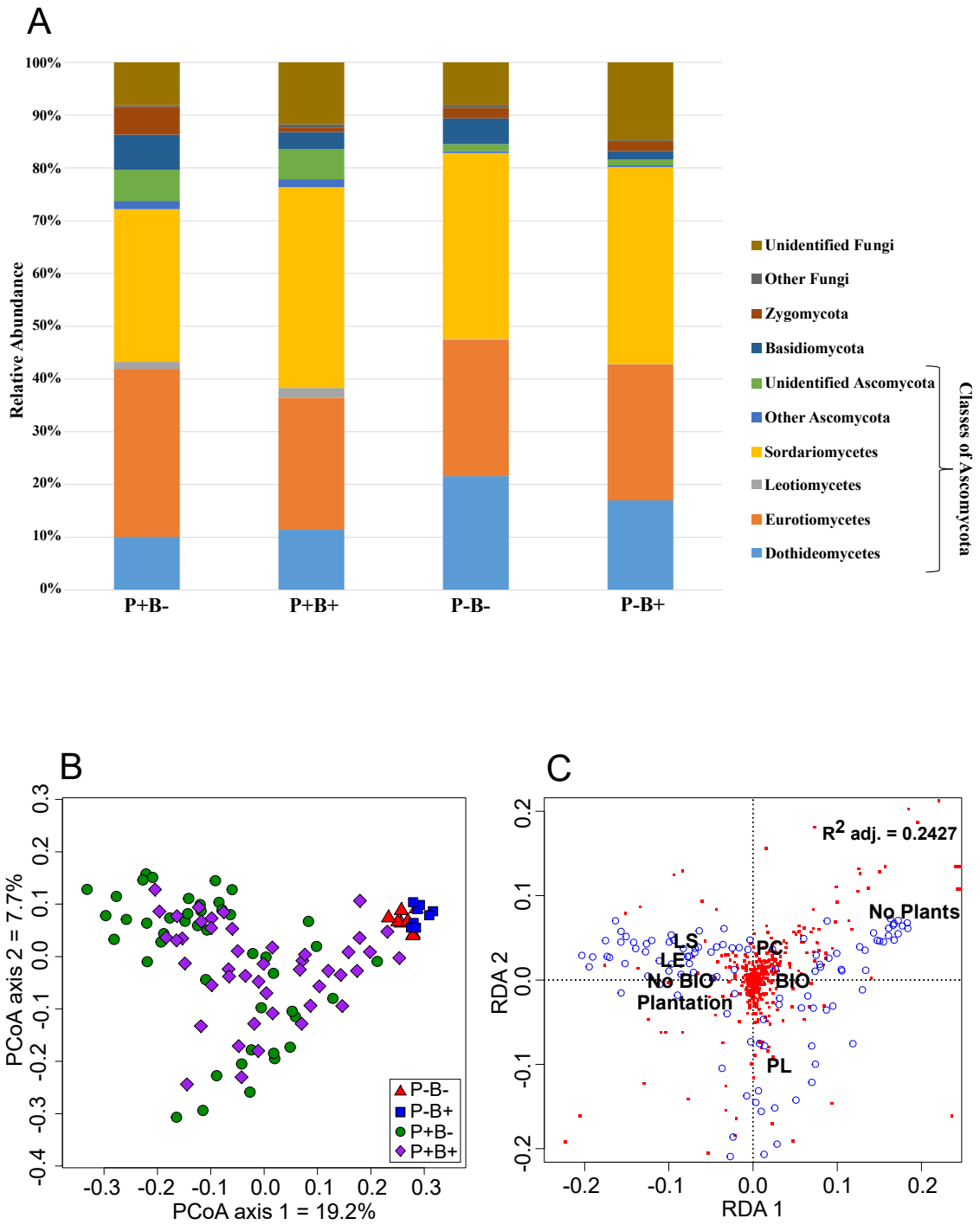

Figure 2. (A) Effect of the plantation and inoculation on the relative abundance of fungal phyla or classes. (B) Principal coordinate analysis based on the Bray-Curtis dissimilarity of fungal communities showing the effect of plantation and inoculation. (C) Redundancy analysis (RDA) showing the relation between the abundance of fungal OTUs and the factors Inoculation, Plantation, and Plant species identity. The adjusted R^2 value indicates the amount of variance in fungal community composition accounted for by the constraining factors (Inoculation, Plantation, and Plant species identity). The location of labels represents factor centroids (Plt=Plantation; Bio=Inoculation; LE= *Lycopus europaeus*; LS=*Lythrum salicaria*; PC=*Panicum capillare*; PL=*Persicaria lapathifolia*). Red squares indicate bacterial OTUs. Blue open circles represent individual samples.

2.3.2 Effect of inoculation and plantation on microbial community structures

A clear influence of the presence of plants in the bacterial communities' assemblages was revealed by principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity, with samples from unplanted and planted microcosms mostly clustering on opposite sides of the biplot (Fig. 1B). PERMANOVA analysis based on the Bray-Curtis dissimilarity matrix of bacterial communities showed that inoculation, presence of plants, and plant species identity all significantly influenced the rhizospheric bacterial community structure (Table 1). The presence of plants explained 14.1% of the observed variation in community structure, followed by plant species identity (Supplementary Fig. S11) and inoculation with 5.5 % and 3.3 % respectively, as accounted by the R^2 values (Table 1). Permutational multivariate analyses of dispersion (PERMDISP) were conducted to test for differences in bacterial community dispersion between treatments, since a significant PERMANOVA result may indicate either a difference in centroids or an unequal dispersion between treatments. Results showed that the dispersion in bacterial communities was not significantly affected among planted treatments (Supplementary Fig. S12), supporting that the difference detected by PERMANOVA was due to a shift in community composition. RDA analysis of the bacterial communities confirmed the stronger influence of plantation on the abundance of bacterial OTUs, and also showed that plant species identity had a differential influence on it. (Fig. 1C).

Table 1. PERMANOVA analysis of the effects of the plantation, plant species identity and inoculation on bacterial community structure in the substrate based on the Bray-Curtis dissimilarity indices.

Factor	*Df*	*SumsOfSqs*	*MeanSqs*	*F.Model*	R^2	*Pr(>F)*
Inoculation	1	0.5015	0.50154	3.9262	0.03276	0.001*
Plantation	1	2.1622	2.16222	16.9264	0.14122	0.001*
Plant Species	3	0.8382	0.27939	2.1871	0.05474	0.002*
Inoculation:Plantation	1	0.1747	0.17475	1.368	0.01141	0.094
Inoculation:Plant Species	3	0.5203	0.17344	1.3578	0.03398	0.051
Residuals	87	11.1136	0.12774		0.72588	
Total	96	15.3106			1	

Df: degree of freedom; **SumsOfSqs:** Sums of squares; **Meansqs:** Mean squares; **F.Model:** F-test value for model; **R^2:** R-squared; **Pr(>F):** p-value.

In the case of fungi, the PCoA of the Bray-Curtis dissimilarity indices showed that fungal communities in planted (P+B-, P+B+) distributed on the left side of the biplot while those from unplanted treatments (P-B-, P-B+) tightly clustered together on the right side of the biplot, regardless of the bioaugmentation treatment (Fig. 2B). PERMANOVA analysis showed that presence of plants, inoculation, and plant species identity all significantly influenced the fungal rhizospheric communities (Table 2), with both plant presence and plant identity (Supplementary Fig. S13) explaining 10.9% of the observed variation, followed by inoculation explaining 3.6% of the variation (Table 2). There was also a significant interaction between bioaugmentation and plant species identity, which was responsible of 3.5% of the variation in community structure (Table 2). PERMDIPSD analysis showed that the dispersion of the fungal rhizospheric communities was not significantly different between the unplanted and planted mesocosms (Supplementary Fig. S14), which confirms that the differences detected by the PERMANOVA were due to shifts in community structure. As with bacteria, RDA analysis confirmed that plantation had a strong relationship with the abundances of fungal OTUs, which also related differentially with plant species identity (Fig. 2C).

Table 2. PERMANOVA analysis of the effects of the plantation, plant species identity and inoculation on fungal community structure in the substrate, based on Bray-Curtis dissimilarity indices.

Factor	*Df*	*SumsOfSqs*	*MeanSqs*	*F.Model*	R^2	*Pr(>F)*
Inoculation	1	0.5675	0.56755	4.9734	0.03623	0.001*
Plantation	1	1.7199	1.71989	15.0.713	0.10978	0.001*
Plant Species	3	1.7212	0.57374	5.0276	0.10987	0.001*
Inoculation:Plantation	1	0.1512	0.15118	1.3248	0.00965	0.102
Inoculation:Plant Species	3	0.551	0.18366	1.6094	0.03517	0.005*
Residuals	96	10.9552	0.11412		0.6993	
Total	105	15.6661			1	

Df: degree of freedom; **SumsOfSqs:** Sums of squares; **Meansqs:** Mean squares; **F.Model:** F-test value for model; **R^2:** R-squared; **Pr(>F):** p-value.

2.3.3 Effect of treatments on plant shoot biomass and petroleum hydrocarbon concentrations

Inoculation significantly increased the average shoot dry weight across all plant species in comparison to the non-bioaugmented controls except for *Lythrum salicaria* (P=0.0001) (Fig. 3A). Inoculation also influenced the concentrations of aliphatic hydrocarbons (C10-C50 fraction) and aromatic polycyclic hydrocarbons (PAHs) in the substrate, at the end of experiment (Fig. 3B and C). Intriguingly, there was significantly less (P=0.0088) aliphatic petroleum hydrocarbons in the non-bioaugmented treatments compared to inoculation treatments (Fig. 3B). Values ranged from 1360 ± 147 mg/kg in the non-inoculated treatments to 2660 ± 686 mg/kg in the planted bioaugmented treatments. The same trend was observed for PAHs (P=0.0181) (Fig. 3C), with levels ranging from 6 ± 1.14 mg/kg in the non-bioaugmented treatments to 10.7 ± 1.11 mg/kg in the planted and bioaugmented substrate. Plantation did not have any significant effect on both final C10-C50 hydrocarbons and total PAH concentrations.

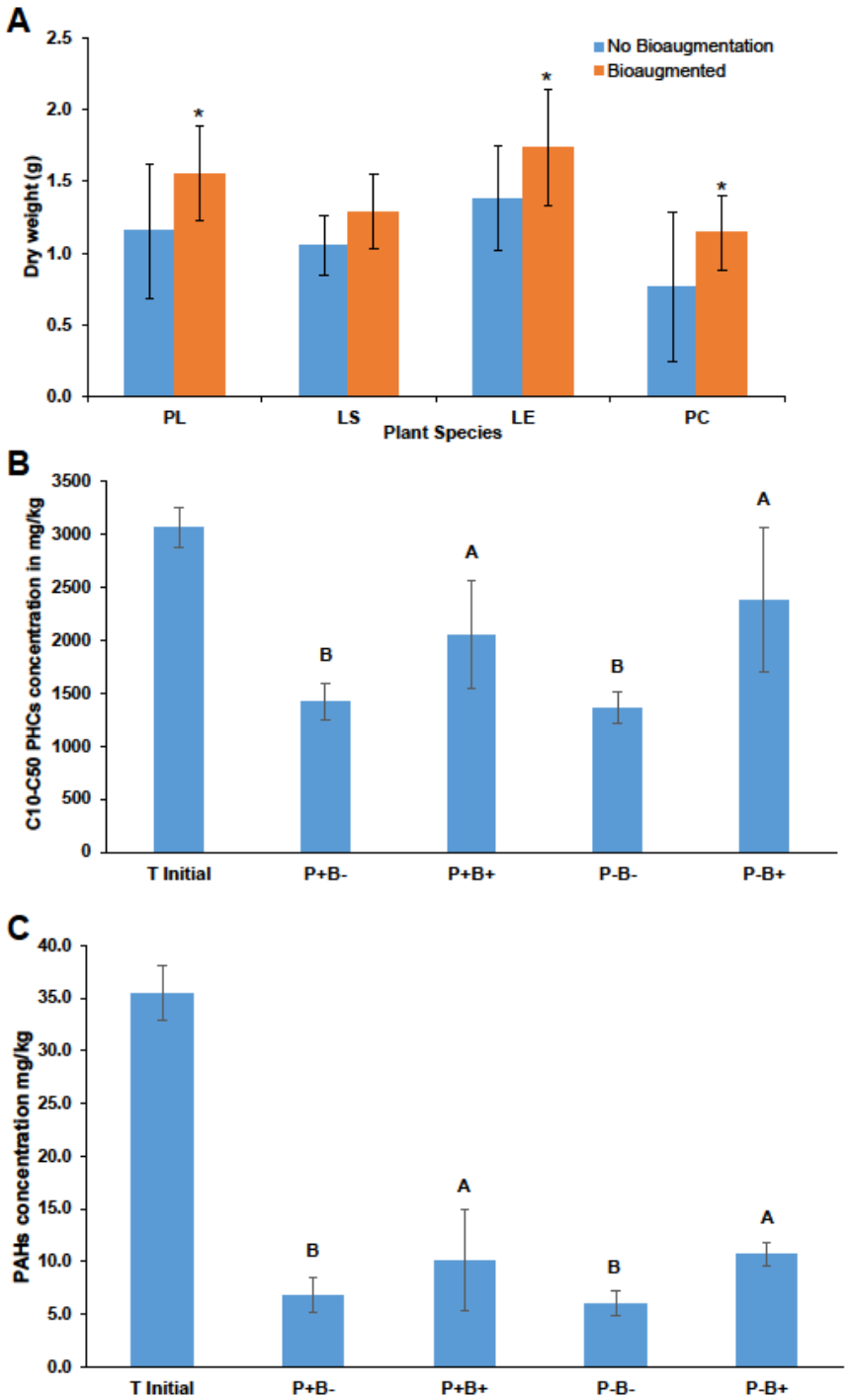

Figure 3. (A) Effect of inoculation on average plant shoot dry biomass for each species at the end of the experiment. Errors bars are standard deviation. PL: *Persicaria lapathifolia*; LS: *Lythrum salicaria*; LE: *Lycopus europaeus*; PC: *Panicum capillare*. N = 12 for each species. Average dry weights for each species were compared with or without inoculation using a Student's T test. Asterisks indicate that the dry weight was significantly different between treatments. (B) Average remaining concentration in the sediments of C10-C50 aliphatic hydrocarbons in each treatment (C) Average remaining polycyclic aromatic hydrocarbons (PAHs) at the end of the experiment. Errors bars represent standard deviation. N = 4 for each treatment. For (B) and (C), plantation and inoculation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different. We added the initial value for reference.

2.4 Discussion

In this study, we determined the effect of the inoculation with a consortium of *Proteobacteria* on the productivity and rhizosphere microbial communities of four spontaneous plant species growing together in sediments contaminated with petroleum hydrocarbons. We found that the presence of plants and plant species identity were the main drivers of bacterial and fungal communities in the substrate. Inoculation also significantly shift microbial community structure and increased the overall total plant shoot dry biomass, but reduced PHC degradation even in the unplanted bulk substrate. Therefore our hypothesis regarding the inoculation of the rhizosphere and increased biodegradation of the PHCs is rejected.

2.4.1 Plantation shaped the rhizosphere microbiome

Plants have been shown to be strong drivers of the abundance and structure of soil microbial communities. They can recruit and influence the abundance of soil microorganisms through root exudation of carbon compounds and other mechanisms (Kowalchuk et al., 2002; Berg et al., 2005; Berendsen et al., 2012b; Mendes et al., 2013; Peiffer et al., 2013; Bucci, 2015), making the rhizosphere a highly selective environment. In our study, bacterial and fungal communities' structures were significantly different between the rhizosphere and unplanted bulk substrate. Results showed that plants shaped significantly the bacterial and fungal communities under polluted conditions, thus altering the microbiome composition. Plants can attract rhizosphere colonizers in their direct vicinity by depositing a myriad of compounds and substances, however it is still unclear which mechanisms are used by plants to modify their exudation profile to recruit microbes. Studies using DNA Stable Isotope Probing approach (DNA-SIP) (Haichar et al., 2008), whole genome transcriptome approach (Mark et al., 2005), and whole genome microarray

(Shidore et al., 2012) have clearly demonstrated changes in bacterial transcriptional profiles under the influence of root exudates, which were also shown to influence soil fungal community composition and diversity (Broeckling et al., 2008), underlining the role of plants in driving rhizospheric bacteria and fungi (Hartmann et al., 2009).

Attracting microbes in their rhizosphere can benefit plants in multiple ways because microbes are responsible for the acquisition of 5-80% of nitrogen, and up to 75% of phosphorus that is necessary for plant growth and development (Heijden et al., 2008). Here, we found that among the 50 most abundant bacterial OTUs in the plant rhizospheres, nine were assigned to the order *Rhizobiales* versus none in the unplanted bulk substrate (Supplementary Table S2). Bacteria of this group are considered as plant growth-promoting rhizobacteria, that are able to enhance plant growth by increasing the nitrogen availability (Zahran, 2001). The second most abundant OTU in the plant rhizosphere was identified as *Kaistobacter* sp. The *Kaistobacter* genus was shown to be associated with the suppression of tobacco bacterial wilt caused by *Ralstonia solanacearum* (Liu et al., 2016). Plants can also benefit from associations with fungi. Strains belonging to the genera *Fusarium*, *Penicillium*, *Phoma*, and *Trichoderma* have been shown to induce systemic resistance in cucumbers, which lead to a greater resistance to multiple diseases (Koike et al., 2001; Benhamou et al., 2002). Moreover, some fungi were also reported to increase plant nutrients mobilization and uptake, as it is the case with symbiotic fungi such as the arbuscular mycorrhizal fungi, which can solubilize and translocate nutrients from the soil to the plant roots through their vast mycelial network (Parniske, 2008). Free living fungi are also able to increase nutrient availability. A strain of *Trichoderma harzianum* (Altomare et al., 1999), and other fungi such as *Trichosporon beigelii, Pichia norvegensis, Cryptococcus albidus* var *aerius*, as well as *Penicillium bilaii* (Kucey, 1987; Cunningham and Kuiack, 1992; Gizaw et al., 2017) have shown inorganic phosphate and other mineral solubilization capabilities. In our results, we observed that many of the 50 most abundant rhizospheric

fungal OTUs were related to *Phoma*, *Fusarium*, *Penicillium*, *Eupenicillium*, and *Trichoderma* species (Supplementary Table S3). These results suggest that plants naturally occurring in PHC contaminated sediments recruit potential growth and health promoting microorganisms. It should be noted however that these genera also contain known plant pathogens.

2.4.2 Plant species-specific effects on microbiome structure

Our results also showed that plants exerted species-specific effects on the structure of rhizospheric microbial assemblages (Supplementary Fig. S9 and Fig. S11), highlighting the differential selectivity of the plant rhizosphere. A phenomenon known as plant-soil feedback proposes that plants, through specific root-compounds, influence soil chemical properties and soil microbial profiles which subsequently influence plant growth and productivity (Bezemer et al., 2006). Exudates from plant-roots, which include amino acids, organic acids, and carbohydrates, are differentially deposited in the rhizosphere, depending on the plant species identity and plant development stage in a given environment (Hertenberger et al., 2002; Chaparro et al., 2013). This, in turn, would lead to distinct rhizospheric microbial community structures. A terminal-restriction fragment length polymorphism analysis of the rhizospheric microbial communities of seven coastal angiosperm congeners showed that plant species significantly correlated with the variation of the rhizosphere microbiome composition (Burns et al., 2015). In another recently published study, Schmid et al. (2018) grew offsprings of eight plant species that had been growing for 11 years in the field under monoculture and mixture planting. They performed 16S rRNA sequencing and found that the bacterial community structure in the rhizosphere of plants was determined by soil plantation history (monoculture vs. mixture) and plant

species identity (Schmid et al., 2018). In a context of phytoremediation of PHCs, the aim is to use the plant rhizosphere as stimulator of microbial activity, which will then biodegrade the petroleum compounds (Pilon-Smits, 2005b). The approach of using multiple plant species for the phytoremediation PHC compounds might not always prove to be the most effective, since different plant species might recruit different rhizospheric microbiomes, which might result in mitigated efficiency. Physico-chemical properties are the main drivers of the soil and rhizosphere microbiome (Fierer, 2017), but in a given environment, plant species can have an influence on the microbiome structure (Hartmann et al., 2009; Bell et al., 2014; Albert et al., 2015; Bulgarelli et al., 2015).

2.4.3 Inoculation induced a shift in the rhizosphere microbiome structure and increased plant growth

Inoculation is a common approach used for the remediation of contaminated environments which resulted in a variable and contradicting conclusions (Thompson et al., 2005). These inconsistencies were attributed (1) to the performance of microbial strains used in the inoculum, as successful inoculation being linked to the survival, persistence, and function of the selected organisms (Tyagi et al., 2011), (2) to the potential competition between the inoculum members and the resident community (Pala and Freire, 2002; Trindade et al., 2002; Mariano et al., 2009), but also to the aeration, nutrient content and soil type (Mrozik and Piotrowska-Seget, 2010).

Here, we observed that inoculation caused a shift in the rhizosphere microbiome structure (Fig. 5 and 6). However decrease of hydrocarbon pollutants in non-bioaugmented microcosms (P+B-) and P-B-) was significantly higher than in inoculation treatments P-B+ and P+B+ (Fig. 3A and 3B), suggesting that the biodegradation of hydrocarbons was less effective following the repeated amendments with the bacterial consortium. The consortium

candidates were selected based on previous studies on the site (Bell et al., 2014; Stefani et al., 2015b) which showed that *Proteobacteria* were the dominant group in these sediments. However, isolation usually recover from 2 to 5 % of the total species richness in any given site (Stefani et al., 2015a). Moreover, even when their capacity as hydrocarbon degraders is demonstrated, the persistence and activity of bioaugmented strains depends fundamentally on their ability to compete with indigenous microorganisms (Thompson et al., 2005), in spite of their concentration increase through the inoculation. Similar results had already been reported previously (Yu et al., 2005; Tongarun et al., 2008; Cunningham et al., 2009) and were attributed to biotic factors like predation and competition for limited carbon sources and space through different mechanism such as antibiotic production and resistance (Hibbing et al., 2010). In our study, several of the top 50 bacterial OTUs in the bioaugmented rhizosphere were related to taxa that have been identified as plant growth promoting such as *Kaistobacter* sp., *Devosia* sp., and other *Rhizobiales* spp. (Table S2). Similarly, plant beneficial fungi such as *Trichoderma* sp. were found among the most 50 abundant fungal OTUs in both the bioaugmented and non-bioaugmented treatments (Table S3). The significantly higher abundance of some of these beneficial groups such as *Trichoderma* and *Kaistobacter* sp. in the bioaugmented treatments might explain the positive effects on final plant shoot biomass in comparison with the non bioaugmented treatments (Fig. 2A and 2B). The positive effects might be related to different mechanisms, such as the production of phytohormones, protection from environmental toxicity and pathogens, as well as increased nutrient bioavailability (Compant et al., 2005; Berg and Hallmann, 2006; Zaidi et al., 2006).

2.5 Conclusion

Plants significantly influenced the structure of the microbial communities in the petroleum hydrocarbons-contaminated sediments, where taxa related to plant growth promoting microorganisms where among the most abundant OTUs. Additionally, plant species identity had a significant impact on the structure of rhizosphere microbiome, highlighting the importance of plant selection in phytoremediation strategies through the creation of new niches allowing introduced organisms to persist. On the other hand, while the inoculation influenced the structure of both fungal and bacterial communities in the rhizosphere, its effect was much weaker than the presence of plants and their identity which contradicts our hypothesis. One caveat is the use of a restricted set of organisms and the results cannot be generalized. There is a need for long-term *in situ* studies involving the use of autochthonous multiple-plant species compared to monoculture of species whose survival and remediation efficiency have been shown to decrease in highly contaminated environments (Pulford and Watson, 2003). However, any strategy regarding soil remediation should also consider both the degradation potential of the selected microorganisms and the interaction with host plants and the local microbiome. These studies will help to precisely select microorganisms which will improve plant health while accelerating the remediation process.

2.6 Acknowledgments

We thank Geoffrey Hall for the *in situ* identification of the plants during seed collection. We thank Alice Roy-Bolduc for help during seed collection, and David Denis for his help in the isolation and culturing of bacterial strains for the inoculation consortium.

Author contributions

DD and IP designed and performed the experiment; IP, FP, MSA and MH supervised the project; DD analyzed the data; MSA and MH provided material and analytic tools; DD, IP, FP, MSA and MH wrote the paper.

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Conflict of Interest Statement

The GenoRem project contains several industrial partners, but these partners have in no way influenced or modified this manuscript or the analysis of the results presented.

2.7 Supplementary information

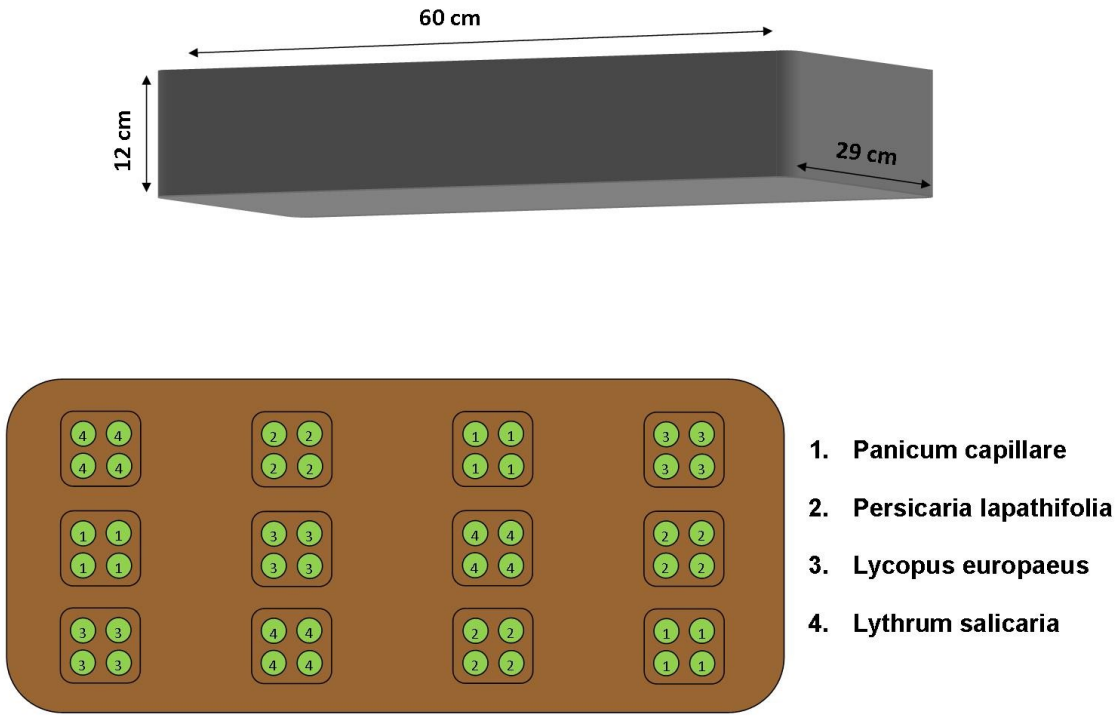


Figure S1. Experimental design and description of the plantation pattern in the mesocosms. Each tray contained twelve planting sites which consisted of a cluster of four individual plants of the same species for a total of four plant species with three planting sites each.

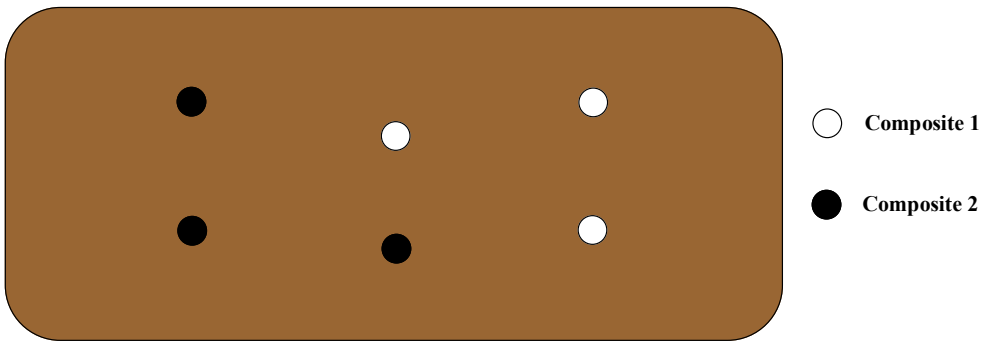


Figure S2. Sampling scheme for non-planted treatments

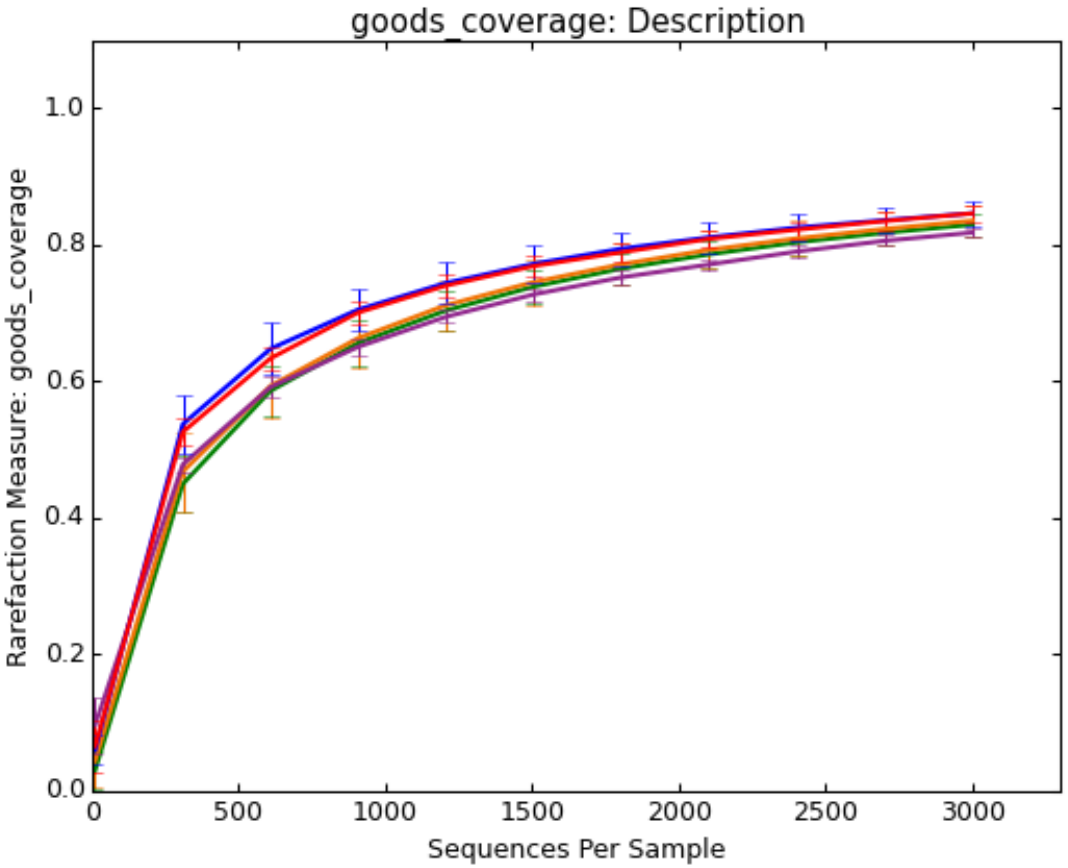


Figure S3. Good's coverage curve of the 16S sequences after subsampling to 3000 sequences per sample.

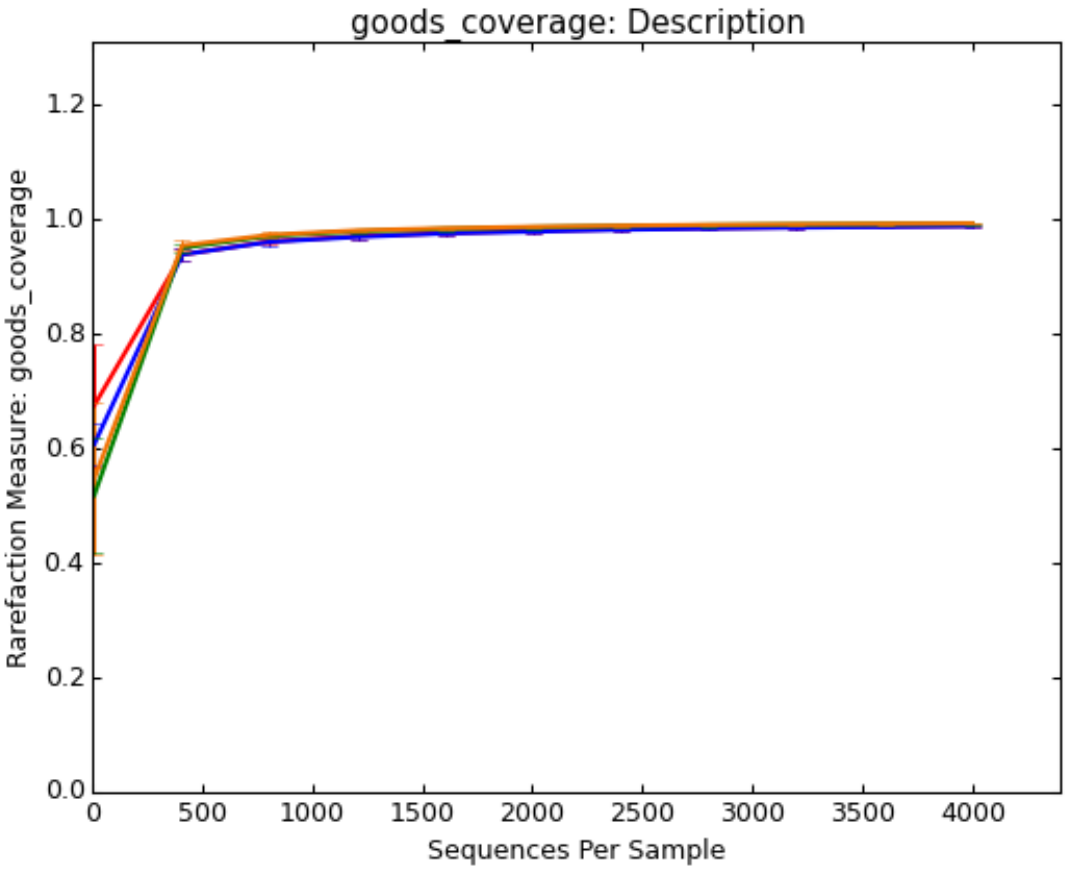


Figure S4. Goods coverage curve of the ITS sequences after subsampling to 4000 sequences per sample.

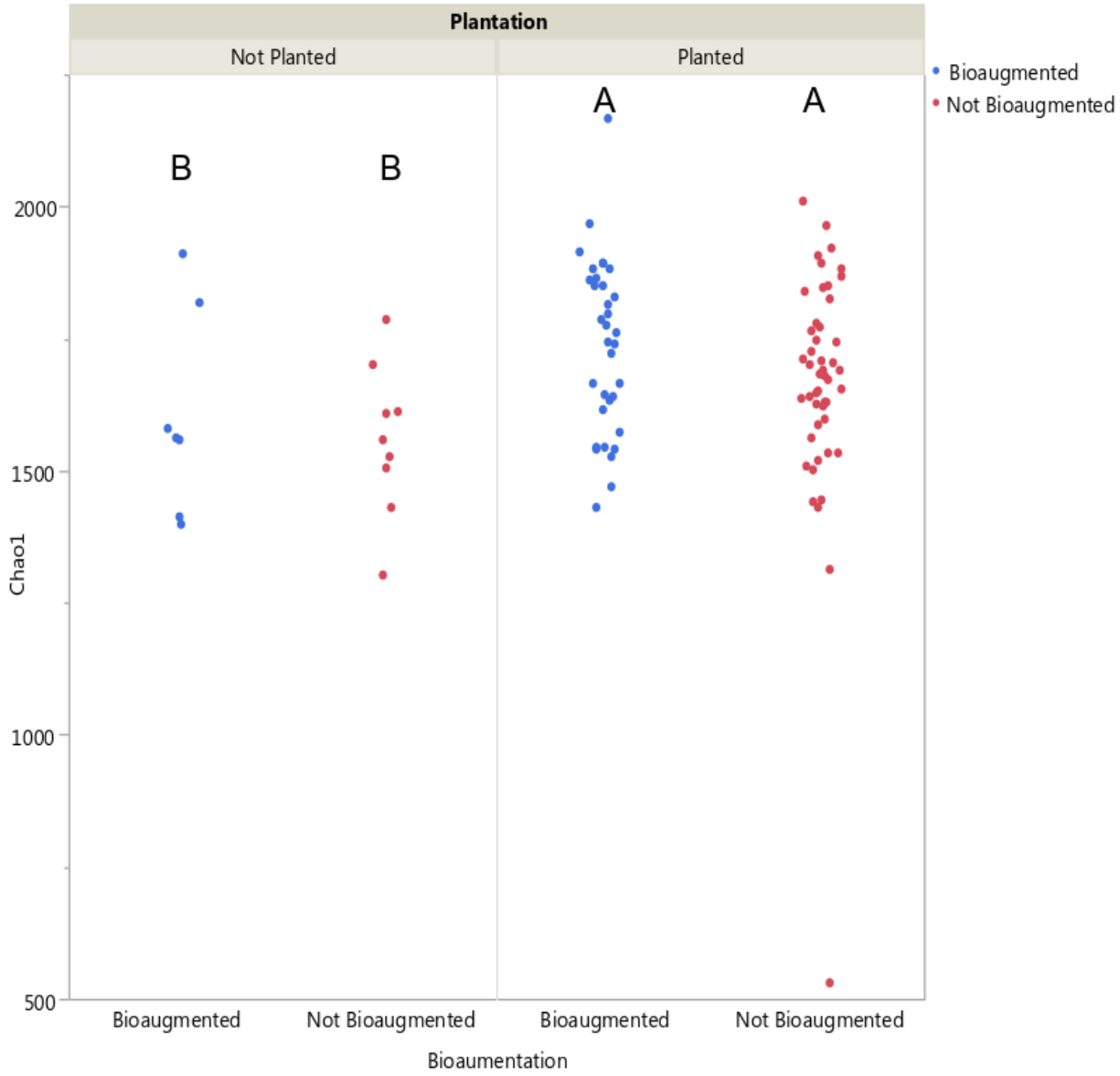


Figure S5. Chao1 estimate for bacterial communities in all samples across all treatments. Plantation and bioaugmentation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different.

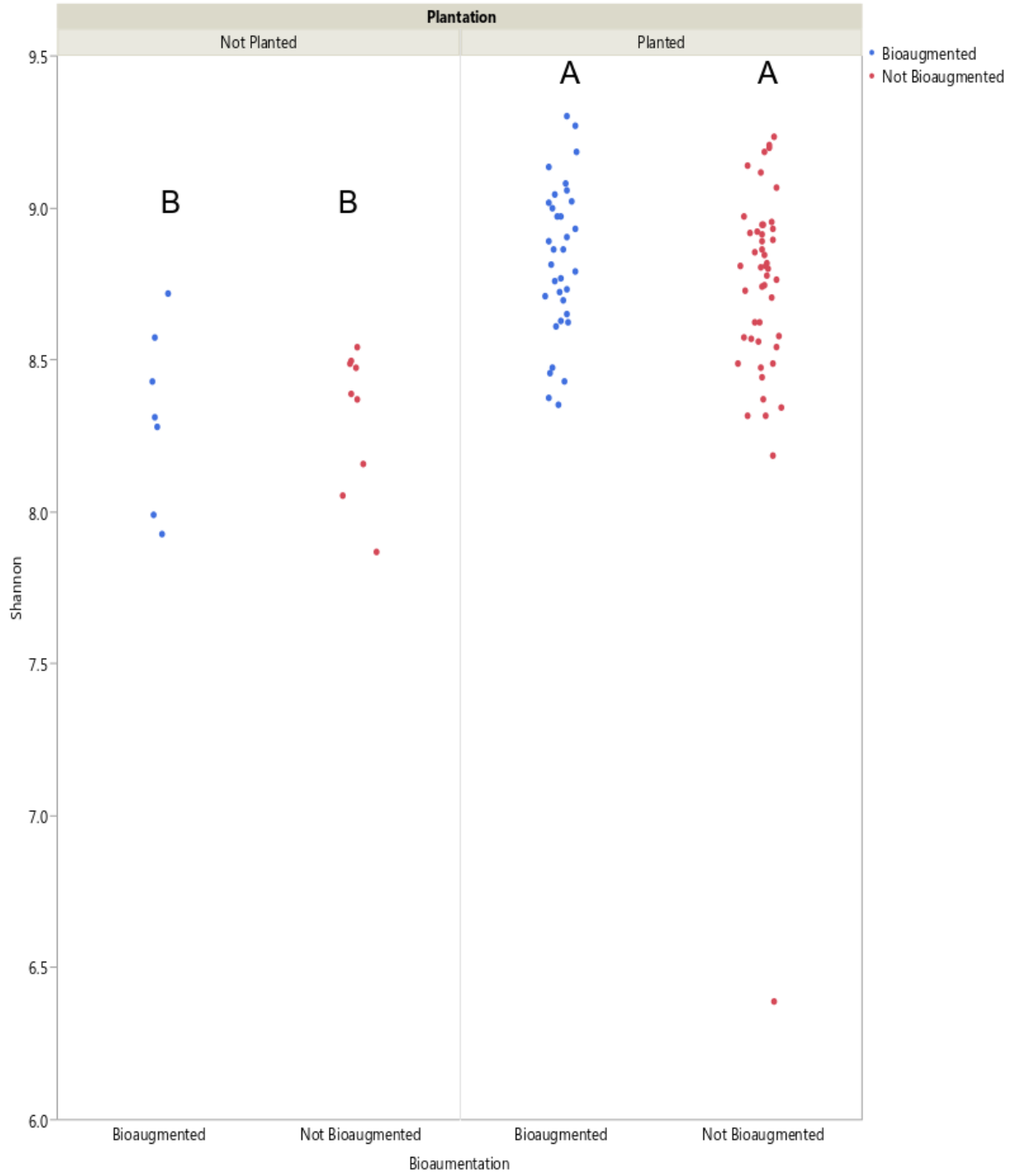


Figure S6. Shannon's diversity index for bacterial communities in all samples across all treatments. Plantation and bioaugmentation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different.

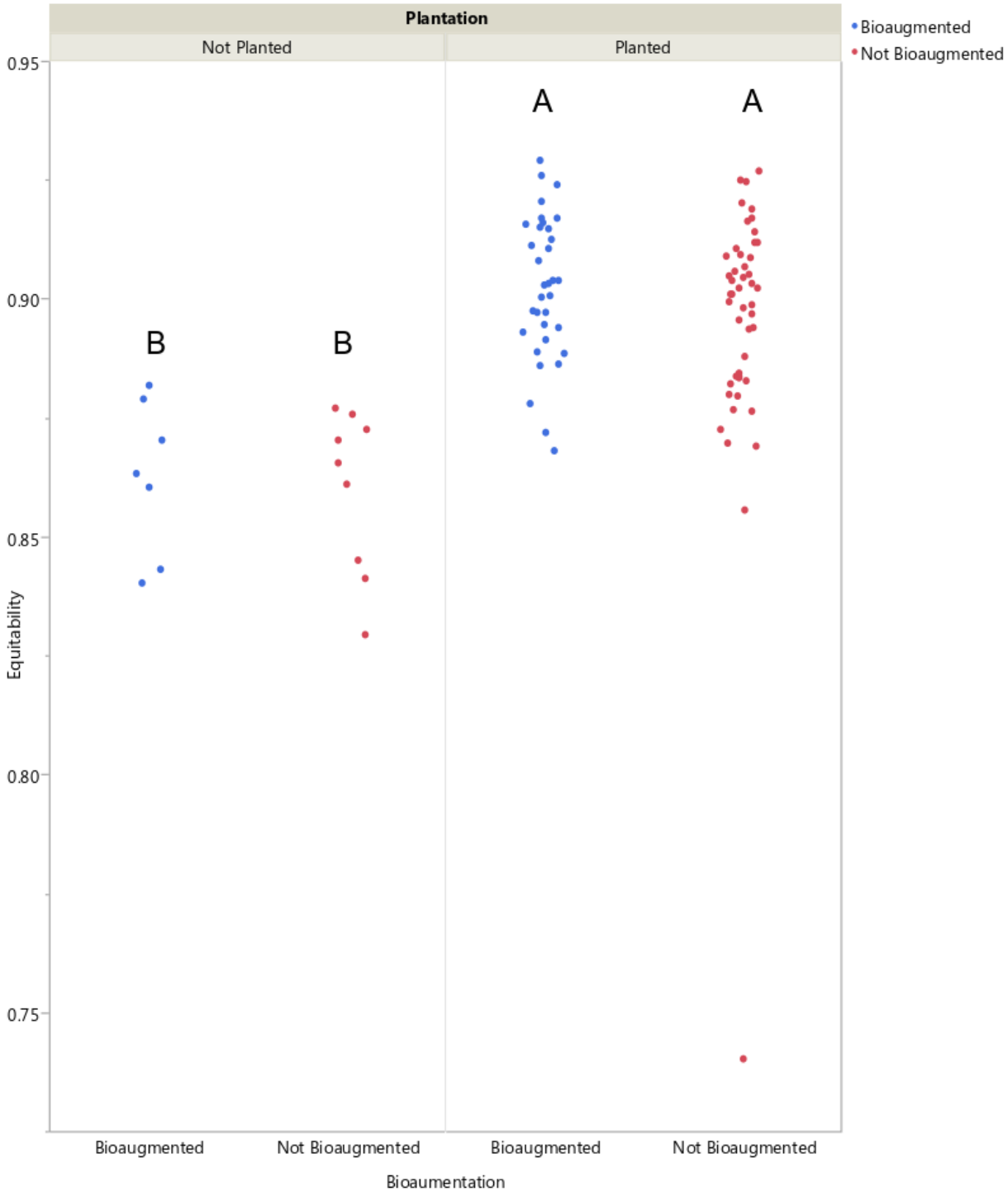


Figure S7. Pielou's evenness measure for the bacterial communities in all samples across all treatments. Plantation and bioaugmentation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different.

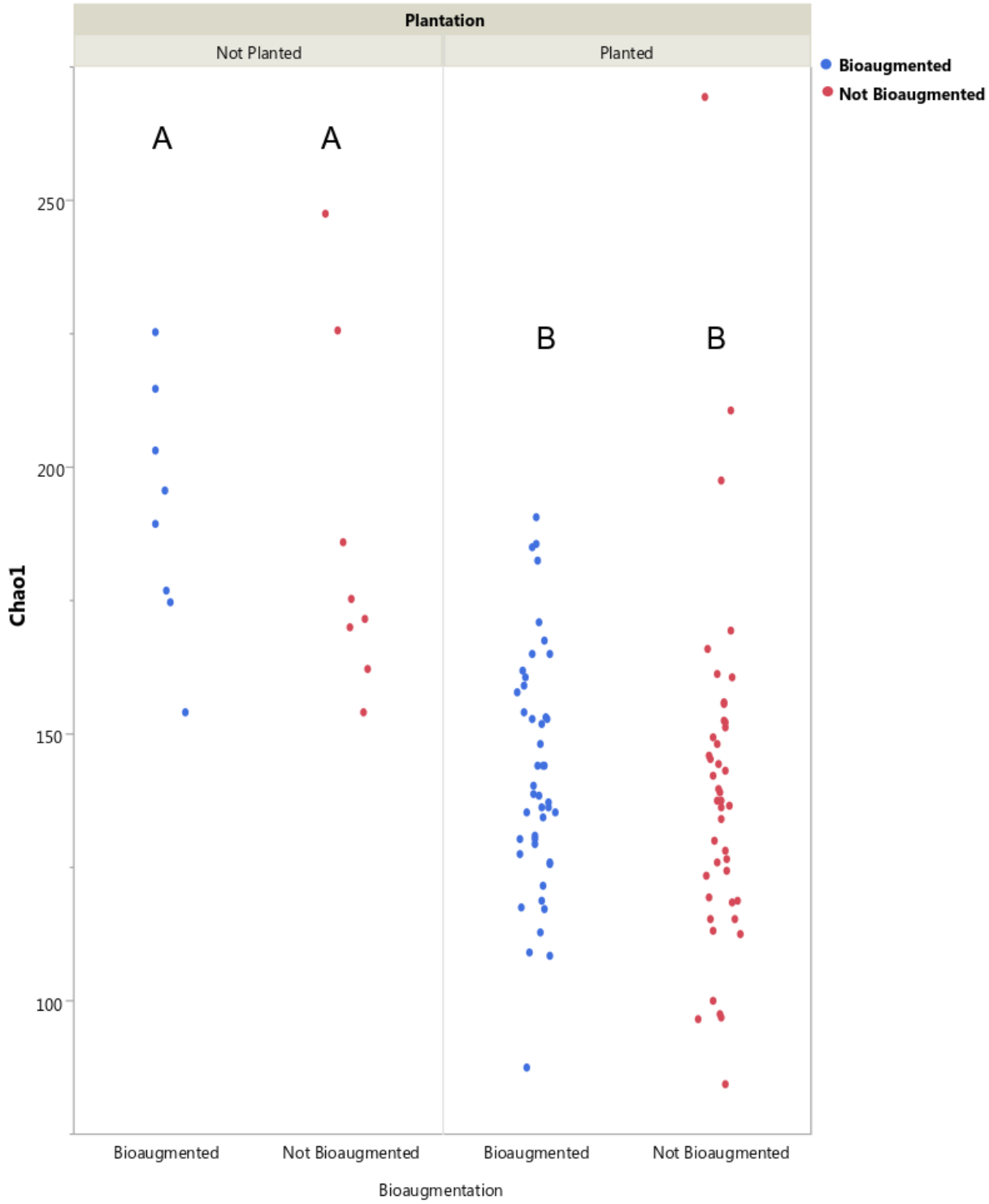


Figure S8. Chao1 richness estimate for fungal communities across the four treatments. Plantation and bioaugmentation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different.

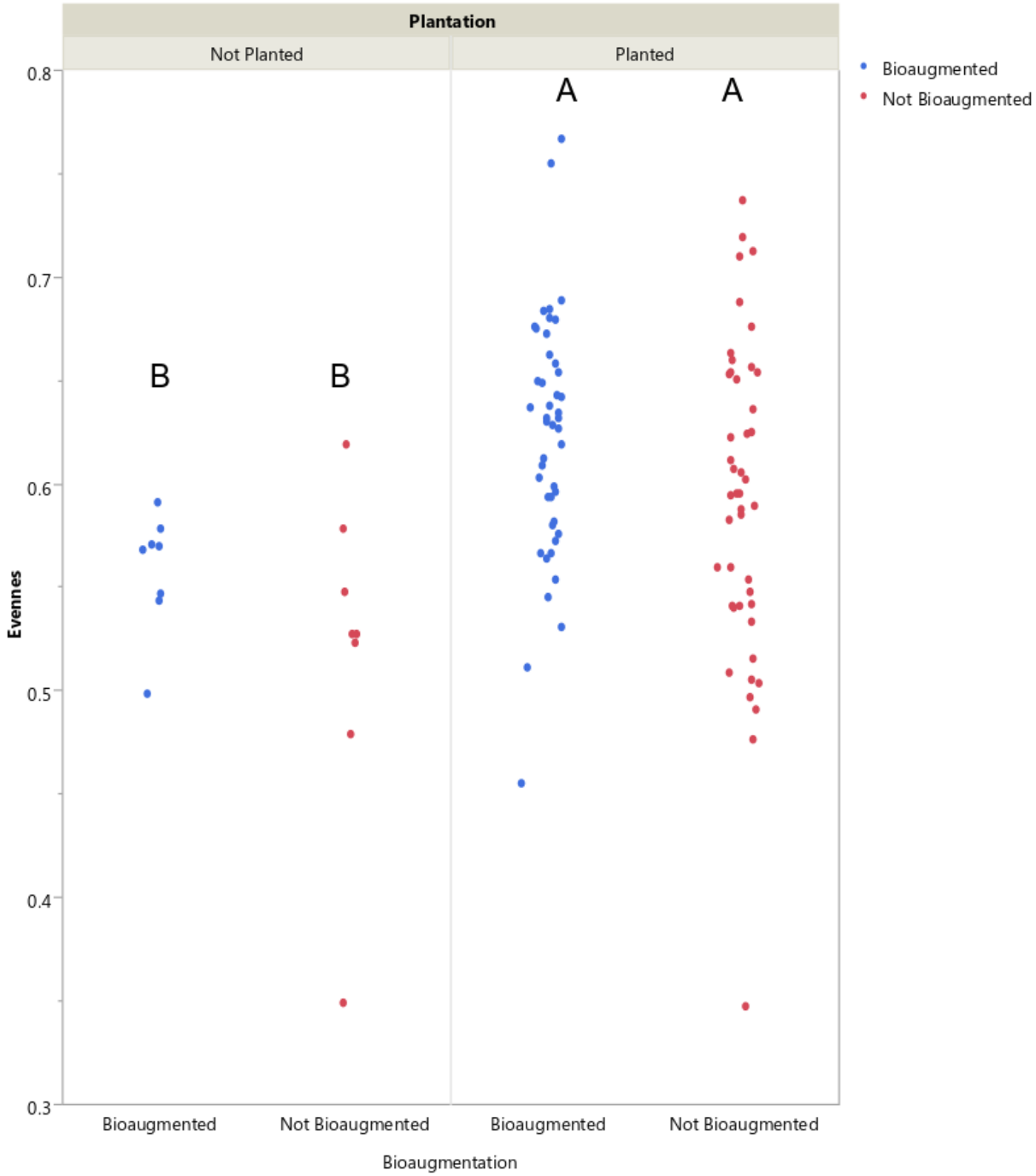


Figure S9. Pielou's evenness measure for the fungal communities in all samples across all treatments. Plantation and bioaugmentation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different.

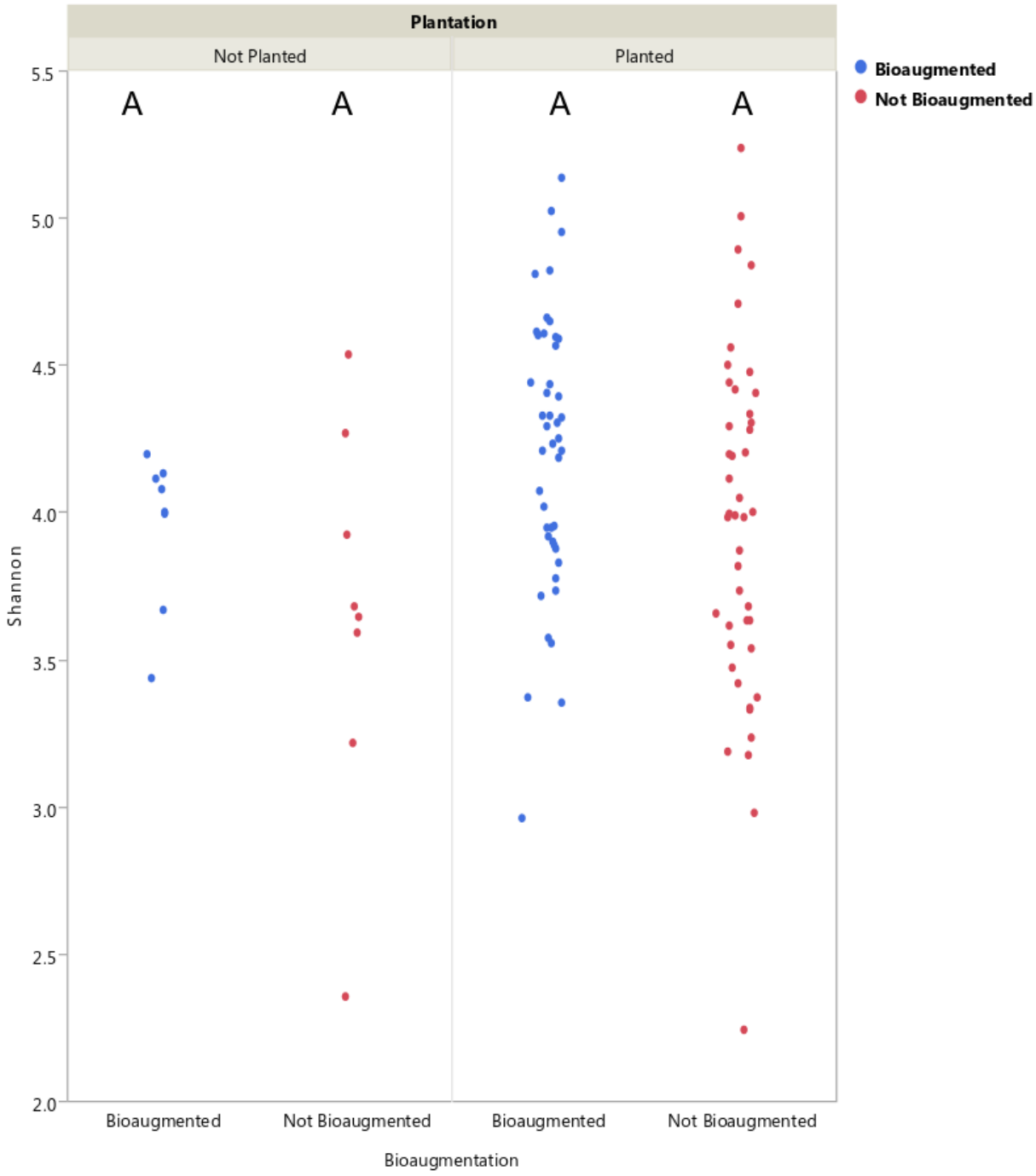


Figure S10. Shannon's diversity index for fungal communities in all samples across all treatments. Plantation and bioaugmentation effects were tested using a two-way full factorial ANOVA in JMP v.7. Treatments not sharing the same letter are significantly different.

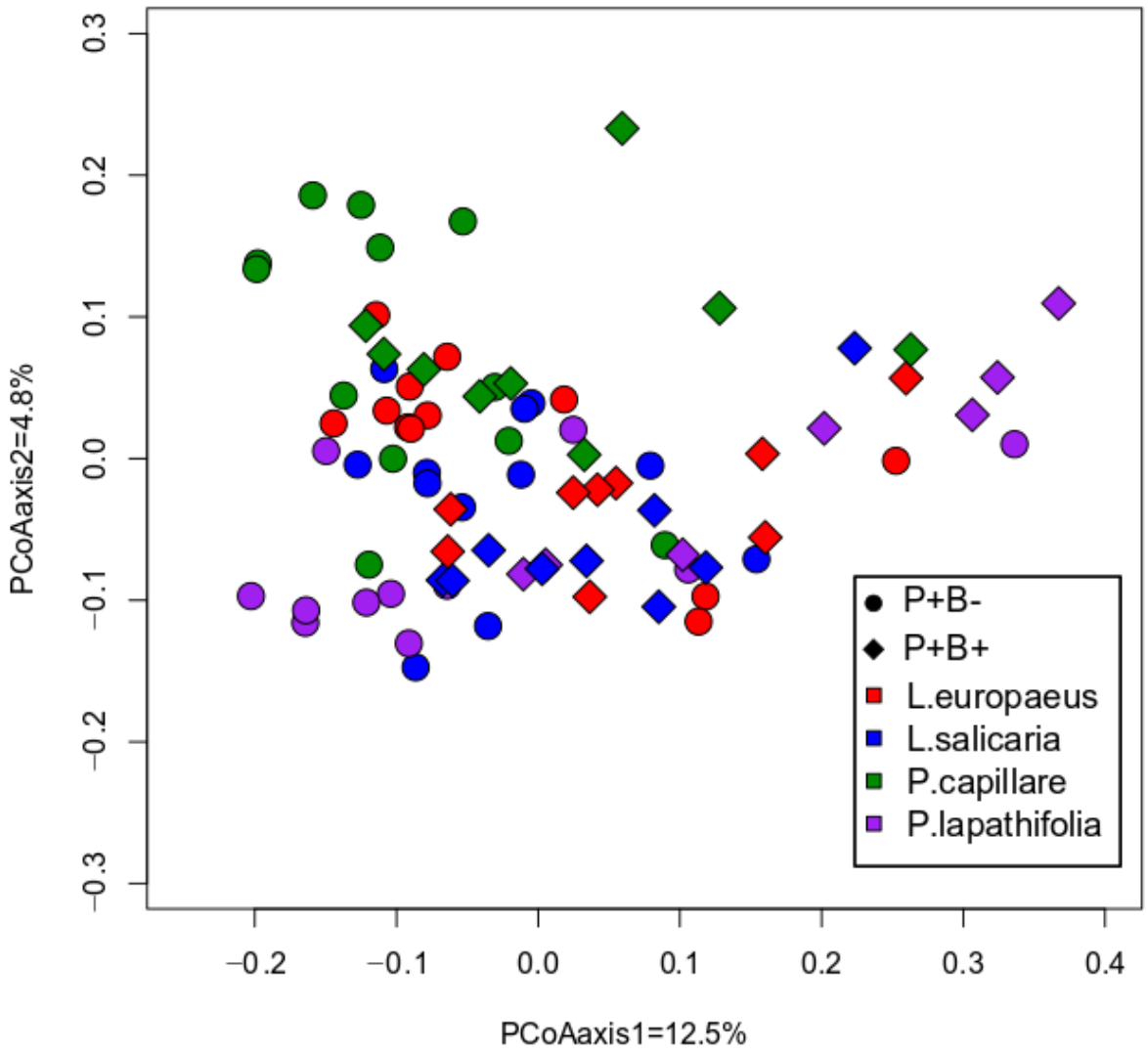


Figure S11. Principal coordinate analysis based on the Bray-Curtis dissimilarity of rhizospheric bacteria communities. **P+B-:** Planted and not bioaugmented, **P+B+:** Planted and bioaugmented.

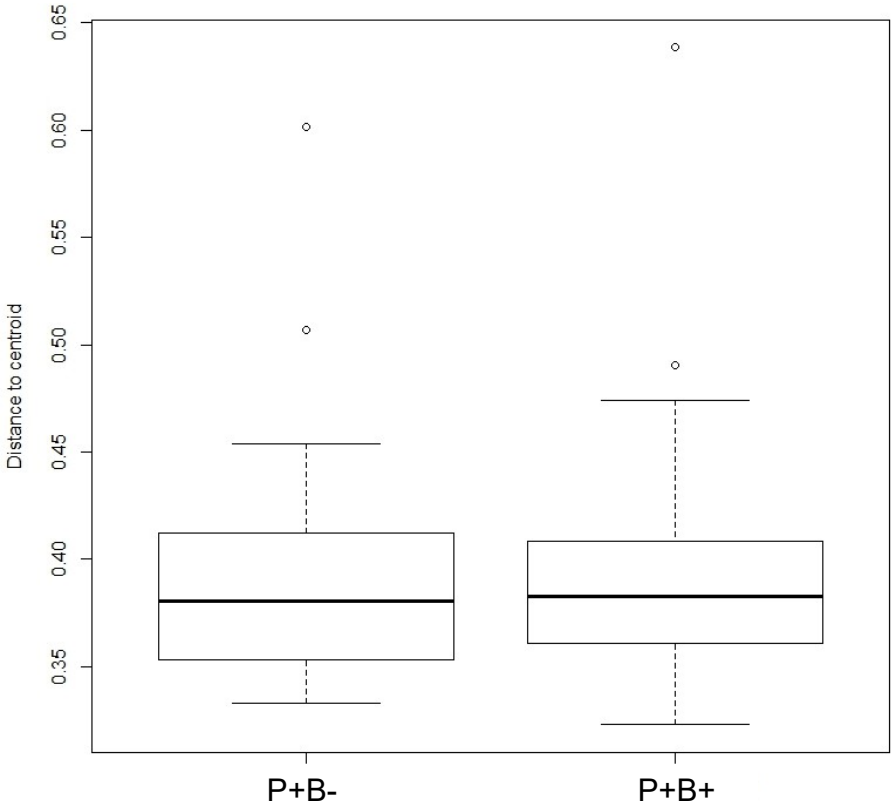


Figure S12. Boxplot of distance to centroid based on beta-dispersion analysis of bacterial community Bray–Curtis distance in both planted treatments.

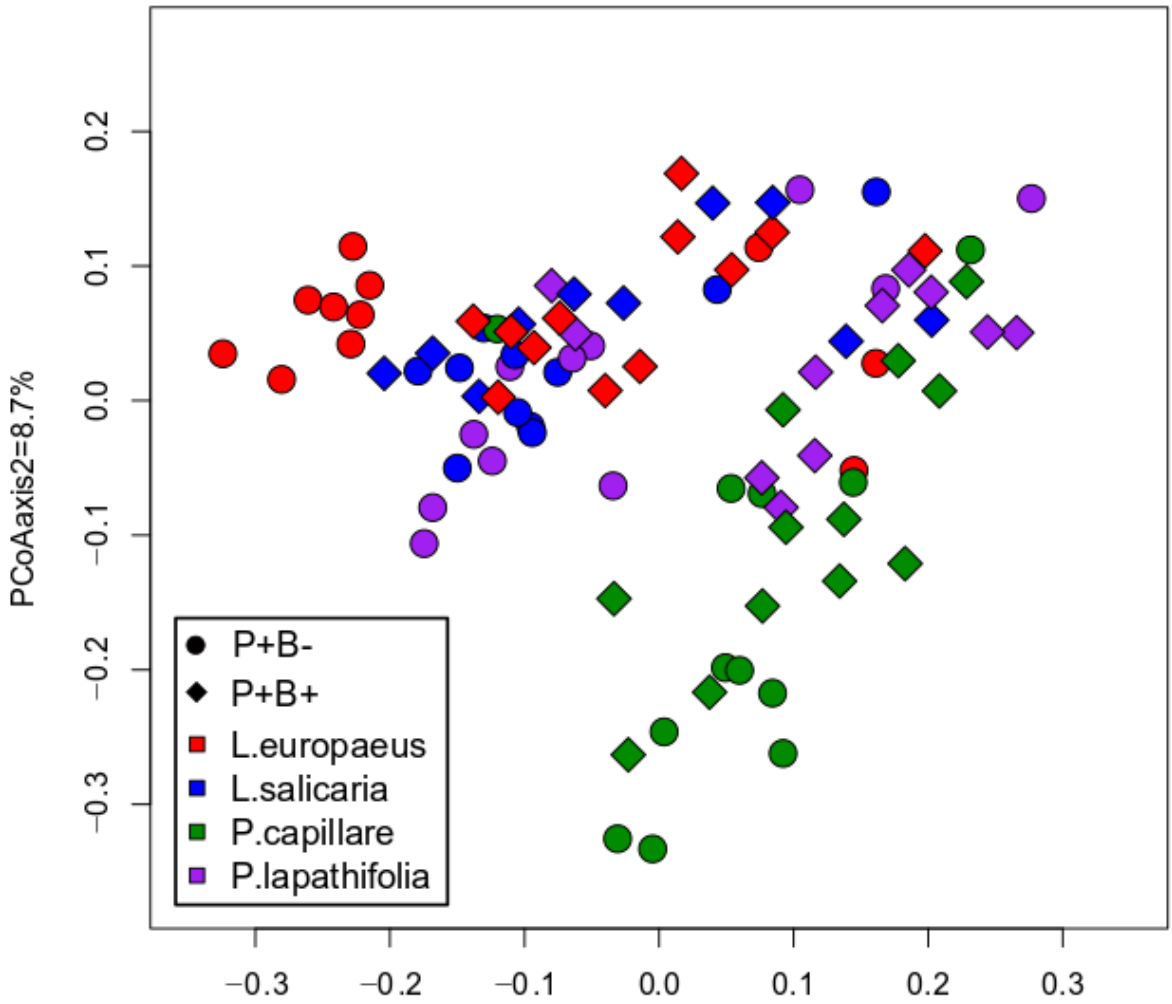


Figure S13. Principal coordinate analysis based on the Bray-Curtis dissimilarity of rhizospheric fungal communities. **P+B-:** Planted and not bioaugmented, **P+B+:** Planted and bioaugmented.

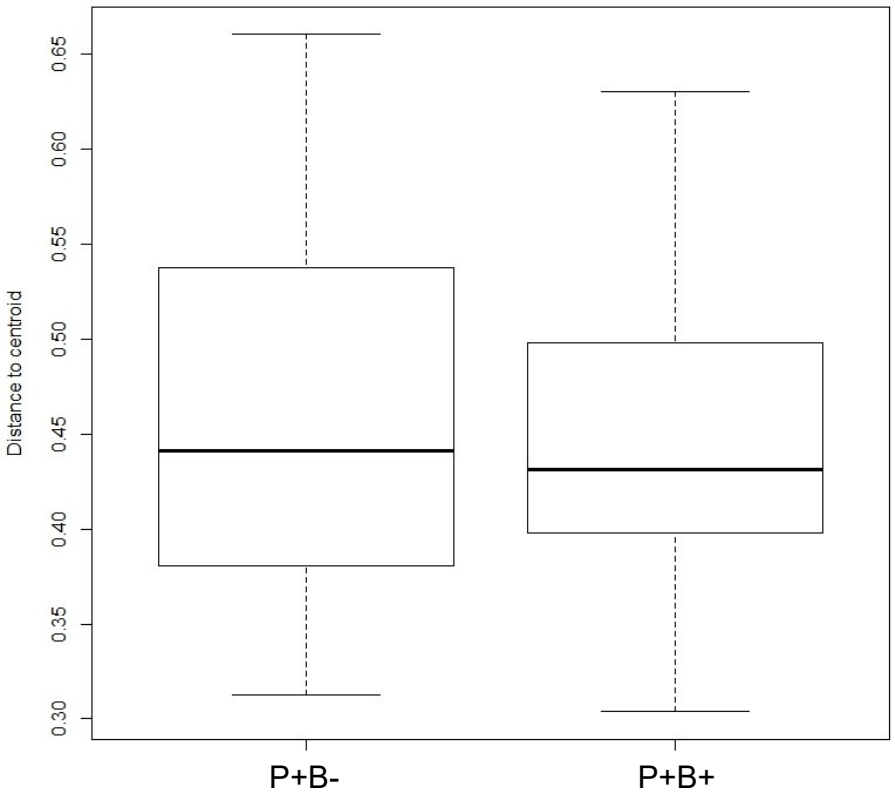


Figure S14. Boxplot of distance to centroid based on beta-dispersion analysis of fungal community Bray–Curtis distance in both planted treatments.

Section I

Details of the sequence processing pipeline

I.A: 16S rRNA sequences pipeline

In Mothur, the reads from each sample were assembled using the 'make.contigs' command. This generated a .fasta file containing the assembled reads. Primers were then removed with 'trim.seqs', after which he sequences were exported to QIIME. Special labels were then added for compatibility, with the 'add.qiime.labels.py' command. Using the Usearch7 sequence analysis tool QIIME implementation, the dataset was reduced to unique sequences using '–derep_fulllength', which we sorted by decreasing cluster size, and removed singletons with '-sortbysize'. The sequences were then clustered by OTU (Operational Taxonomic Unit) using a 97% identity threshold using the UPARSE method, and aligned using QIIME's implementation of the GreenGenes bacterial database 08/13 update. Further sequencing errors were removed with '–uchime_ref'. OTU's were assigned taxonomic identity using the UCLUST method and GreenGenes database with 'assign_taxonomy.py'. We then produced an OTU table at 97% similarity and sequences that classified as 'Mitochondria', 'Chloroplast', 'Archaea', 'Eukaryota', or 'unknown' were removed with 'filter_taxa_from_otu_table.py'. Finally, we subsampled the sequences, so each sample had the same amount (3000). Basic alpha diversity indices were generated using the "core_diversity_analyses.py" command. The OTU table was then exported to R for diversity and statistical analyses.

I.B: Fungal ITS sequences processing pipeline

In Mothur, the reads 1 and 2 from each sample were assembled using the 'make.contigs' command. This generated a .fasta file containing the assembled reads.

Primers were then removed with 'trim.seqs', after which the sequences were exported to QIIME. Special labels were then added for compatibility, with the 'add.qiime.labels.py' command. Using the Usearch7 sequence analysis tool QIIME implementation, the dataset was reduced to unique sequences using '–derep_fulllength', which we sorted by decreasing cluster size, and removed singletons with '-sortbysize'. ITS1 sequences were then selected with the ITSx Tool, and clustered by OTU (Operational Taxonomic Unit) using a 97% identity threshold with UPARSE method. Further sequencing errors were removed with '–uchime_ref'. OTU's were assigned taxonomic identity using BLAST method on QIIME and the UNITE reference database with 'assign_taxonomy.py'. We then produced an OTU table at 97% similarity and only sequences that classified as 'Fungi' were retained. Basic alpha diversity indices were generated using the "core_diversity_analyses.py" command. The OTU table was then exported to R for diversity and statistical analyses.

Section II

PCR conditions

16S amplification

First round reagents

Component	Final concentration
Buffer 10X (Provided with the Taq kit)	2 µl (1X)
Primer fwd.	0.2 µM
Primer rev.	0.2 µM
dNTP	200 µM each
MgCL2	1.5 mM (contained in the buffer 10X)
BSA	0.8 µl
DMSO	4% v/v
gDNA template	2 µl
Ultrapure H_2O	13.1
Qiagen Taq	0.5 unit

Total volume of one reaction	**20 µl**

First round cycling conditions

Step	Temperature	Time	Repeats
Initial denaturation	95 ºC	3 mins	1X
Denaturation	95 ºC	45 s	40X
Annealing	55 ºC	30 s	
Extension	72 ºC	45s	
Final extension	72 ºC	10 mins	1X

Second round reagents

Component	Volume
DNA (amplicons from first round)	5 µl
Nextera XT index primer 1 (N7xx)	2.5 µl
Nextera XT index primer 2 (S5xx)	2.5 µl
Kappa HiFi HotStart ReadyMix	12.5 µl
Ultrapure H_2O	2.5 µl
Total volume of one reaction	**25 µl**

Second round cycling conditions

Step	Cycling conditions	Time	Repeats
Initial denaturation	95 ºC	3 mins	1X
Denaturation	95 ºC	30 s	

Annealing	55 ºC	30 s	8X
Extension	72 ºC	30s	
Final extension	72 ºC	5 mins	1X

ITS amplification

First round reagents

Component	Final concentration
Buffer 10X (Provided with the Taq kit)	2 µl (1X)
Primer fwd.	0.2 µM
Primer rev.	0.2 µM
dNTP	200 µM each
MgCL2	1.5 mM (contained in the buffer 10X)
BSA	0.8 µl
DMSO	4% v/v
gDNA template	2 µl
Ultrapure H_2O	13.1
Qiagen Taq	0.5 unit
Total volume of one reaction	**20 µl**

First round cycling conditions

Step	Temperature	Time	Repeats
Initial denaturation	95 ºC	3 mins	1X
Denaturation	95 ºC	45 s	

Annealing	45 ºC	30 s	40X
Extension	72 ºC	45s	
Final extension	72 ºC	10 mins	1X

Second round reagents

Component	Volume
DNA (amplicons from first round)	5 µl
Nextera XT index primer 1 (N7xx)	2.5 µl
Nextera XT index primer 2 (S5xx)	2.5 µl
Kappa HiFi HotStart ReadyMix	12.5 µl
Ultrapure H_2O	2.5 µl
Total volume of one reaction	**25 µl**

Second round cycling conditions

Step	Cycling conditions	Time	Repeats
Initial denaturation	95 ºC	3 mins	1X
Denaturation	95 ºC	30 s	8X
Annealing	55 ºC	30 s	
Extension	72 ºC	30s	
Final extension	72 ºC	5 mins	1X

Section III

Setting used in JMP

When testing for significance of bioaugmentation and plantation, we first assessed the normality of the dataset using the "distribution" analysis. Data were transformed if needed. The ANOVA model was built using the "Fit model" option and looked like follows: "Bioaugmentation", "Plantation", "Bioaugmentation x Plantation". We also considered the block from which the samples came and marked it as a random factor. Once we established the significance of the factors, we compared the means using Tukey's HSD. For the effect of bioaugmentation on plant biomass, we used Student's T test to compare the two means for each plant species (with or without bioaugmentation).

R code for the vegan 2.5 package functions used for the analysis

Permanova

library (vegan)

#Import OTU table

ITS_perm = read.delim("ITS_FINAL4000_permanova.txt", header=TRUE, row.names=1)

#Import Metadata file

ITS_Meta = read.delim("ITS_Meta_permanova.txt", header=TRUE, row.names=1)

 # Hellinger transformation to deal with double zeros

ITS_perm_hel = decostand(ITS_perm, "hel")

 # Generate Bray-Curtis dissimilarity matrix

ITS_bray_perm = vegdist(ITS_perm_hel, "bray")

#Actual permanova

#Testing factors and their interaction

- adonis(ITS_bray_perm~Inoculation*Plant_Presence*Plant_Species, data = ITS_Meta, strata = ITS_Meta$Block, permutations = 999)

#Testing factors without interaction

- adonis(ITS_bray_perm~Inoculation+Plant_Presence+Plant_Species, data = ITS_Meta, strata = ITS_Meta$Block, permutations = 999)

#Generating PCoA data

Bac.pcoa<- cmdscale(bacteria_bray_perm, k=(nrow(bacteria_perm_hel)-1), eig=TRUE)

Bac.points <-Bac.pcoa$points[,1:2]

Bac.eig<-Bac.pcoa$eig

Bac.pcoa$eig[1:2]/sum(Bac.pcoa$eig)*100

Then use the ordiplot() command to draw and customize.

RDA Analysis

#Data

ITS_OTU<-read.delim("ITS_FINAL4000_permanova.txt", row.names=1, header=T, dec=".")

ITS_metadata2F<-read.delim("ITS_Metadata_RDA.txt",row.names=1, header=T, dec=".")

ITS_metadata2F<-ITS_metadata2F[,1:3]

str(ITS_metadata2F)

#Transform the data to get rid of the double-zero problem

ITS_OTU_hel2F<-decostand(ITS_OTU, "hel")

#RDA

ITS_rda_all2F<-rda(ITS_OTU_hel2F ~ ., data=ITS_metadata2F)

summary(ITS_rda_all2F)

#Calculate R2

(R2adjITS2F<-RsquareAdj(ITS_rda_all2F)$r.squared)

#Verify collinearity of factors (if two factors are collinear, must eliminate one of them)

vif.cca(ITS_rda_all2F)

#Test significance of Whole model

anova.cca(ITS_rda_all2F, step=999)

#Test which RDA axes are significant

anova.cca(ITS_rda_all2F, by="axis", step=999)

#Test which factor is significant

anova.cca(ITS_rda_all2F, by="term", step=999)

#Plot if needed

plot(ITS_rda_all2F, scaling=2, main="Fungi RDA - scaling 2", type="none", cex.axis=1.5, cex.lab=1.5, cex.sub=1.5, xlab=c("RDA1"), ylab=c("RDA2"), xlim=c(-0.6,0.6), ylim=c(-1.1,0.4))

Betadispertion

data("ITSbetadispPlanted1")

##Transform hellinger

ITSbetadispPlanted = decostand(ITSbetadispPlanted1, "hel")

```
## Bray-Curtis distances between samples
dis <- vegdist(ITSbetadispPlanted)

## Designate groups
groups <- factor(c(rep(1,45), rep(2,45)), labels = c("Planted","Planted_Inoculated"))
groups <- factor(c(rep(1,8), rep(2,8)), labels = c("Not_Planted","Not_Planted_Inoculated"))
## Calculate multivariate dispersions
mod <- betadisper(dis, groups)
mod
plot(mod)
## Perform test
anova(mod)
## Permutation test for F
permutest(mod, pairwise = TRUE)
## Tukey's Honest Significant Differences
(mod.HSD <- TukeyHSD(mod))
plot(mod.HSD)

## Plot the groups and distances to centroids on the
## first two PCoA axes
plot(mod)
## Draw a boxplot of the distances to centroid for each group
boxplot(mod)
```

Suplementary tables (Tables S3 and S4 are the OTU tables and too big to include in this document)

Table S1. Bacterial isolates selected for the bioaugmentation consortium

Isolate code	Closest match	GenBank accession	Identity
C10	Duganella nigrescens strain YIM H16	EF584756	99%
C17	Comamonadaceae bacterium KACHI12	LC094532	99%
C19	Pelomonas aquatica strain CCUG 52575	NR_042614	99%
C25	Massilia sp. DC2a-55	AB552860	99%
C26	Massilia sp. PO12	KC687079	99%
C30	Duganella sp. 13-D4	KU647207	99%
C45	Janthinobacterium sp. HP12G1	KM187404	99%
C53	Duganella sp. T53	KC464857	99%
C55	Janthinobacterium sp. HME1	HQ829835	99%
C65	Methylibium sp. YIM 61602	FJ615290	99%

Table S2. Primers sequences

Primer	Sequence	Target
27f	5'-AGAGTTTGATCMTGGCTCAG-3'	Bacterial 16S rRNA
1492r	5'-TACGGYTACCTTGTTACGACTT-3'	Bacterial 16S rRNA
Bakt_341F	5'-CCTACGGGNGGCWGCAG-3'	Bacterial 16S rRNA V3-V4 region
Bakt_805R	5'-GACTACHVGGGTATCTAATCC-3'	Bacterial 16S rRNA V3-V4 region
ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Fungal ITS1 region
58A2R	5'-CTGCGTTCTTCATCGAT-3'	Fungal ITS1 region
Illumina Fwd adaptor	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'	
Illumina Reverse adaptor	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'	

Table S5. Permanova analysis for fungal anf bacterial community: Here we perform the test without the interaction factors

ITS

adonis(formula = ITS_bray_perm ~ Inoculation + Plant_Presence + Plant_Species, data = ITS_Meta, permutations = 999, strata = ITS_Meta$Block)

Blocks: strata
Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Inoculation	1	0.5675	0.56755	4.8686	0.03623	0.001	***
Plant_Presence	1	1.7199	1.71989	14.7536	0.10978	0.001	***
Plant_Species	3	1.7212	0.57374	4.9217	0.10987	0.001	***
Residuals	100	11.6574	0.11657		0.74412		
Total	105	15.6661			1.00000		

16S

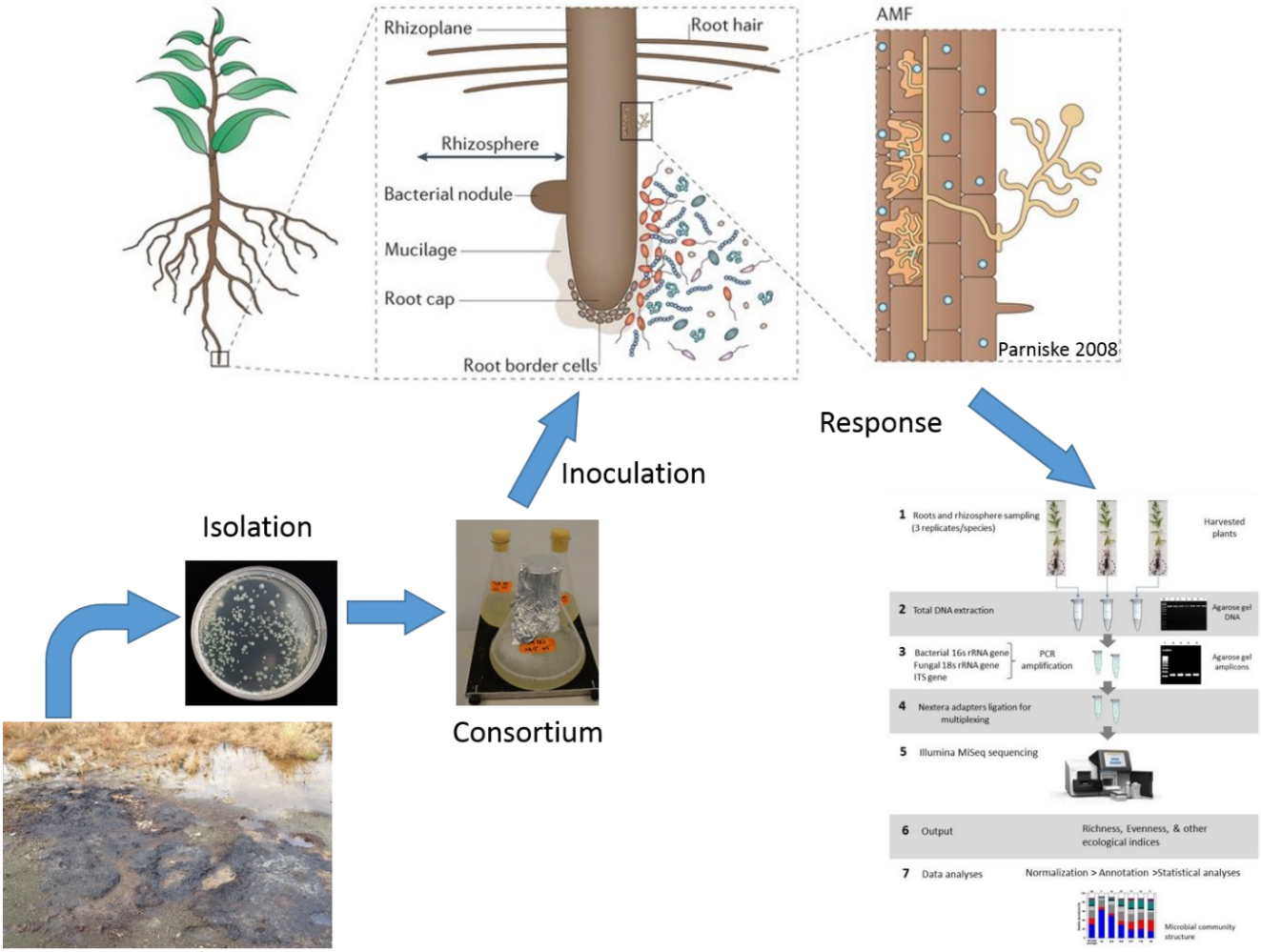
adonis(formula = bacteria_bray_perm ~ Inoculation + Plant_Presence + Plant_Species, data = bacteria_Meta, permutations = 999, strata = bacteria_Meta$Block)

Blocks: strata
Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Inoculation	1	0.5015	0.50154	3.865	0.03276	0.001	***
Plant_Presence	1	2.1622	2.16222	16.663	0.14122	0.001	***
Plant_Species	3	0.8382	0.27939	2.153	0.05474	0.001	***
Residuals	91	11.8087	0.12977		0.77127		
Total	96	15.3106			1.00000		

3 Chapitre 3 | Perturbation des communautés de champignons mycorhiziens arbusculaires (CMA) en association avec des plantes poussant dans des substrats contaminés et naturels



Dans le chapitre précédent nous avons constaté le rôle majeur que joue la présence des plantes dans la détermination de la structure du microbiome de la rhizosphère : elles agissent comme un véritable pôle d'attraction des microorganismes habitant le sol, majoritairement par le biais de leurs exsudats racinaires. En effet, une différence significative a été observée entre la structure des microbiomes en absence ou présence des plantes. Additionnellement, l'identité de l'espèce végétale, ainsi que la perturbation causée

par l'inoculation bactérienne influencent significativement la structure du microbiome, mais dans une moindre mesure.

À la suite de ces résultats, nous nous concentrons dans le présent chapitre sur un des éléments fongiques les plus importants de la rhizosphère, soit les champignons mycorhiziens arbusculaires. Nous visons ici de mieux comprendre comment influencer la structure de ces communautés dans la rhizosphère et dans les racines, et comparer leur comportement dans des milieux naturels et contaminés aux hydrocarbures. Pour ce faire, nous procédons à la perturbation de la rhizosphère à travers des inoculations bactériennes répétées de quatre espèces de plantes poussant dans des mésocosmes en serre. L'objectif principal est d'observer et de comparer le comportement des CMA dans un milieu naturel et contaminé au niveau de leur structure dans la rhizosphère et dans les racines, ainsi que l'effet de l'inoculation sur les taux de colonisation des racines et la croissance des plantes.

Arbuscular Mycorrhizal Fungal Assemblages Significantly Shifted upon Bacterial Inoculation in Non-Contaminated and Petroleum-Contaminated Environments

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Abstract

Arbuscular mycorrhizal fungi (AMF) have been shown to reduce plant stress and improve their health and growth, making them important components of the plant-root associated microbiome, especially in stressful conditions such as petroleum hydrocarbons (PHCs) contaminated environments. Purposely manipulating the root-associated AMF assemblages in order to improve plant health and modulate their interaction with the rhizosphere microbes could lead to increased agricultural crop yields and phytoremediation performance by the host plant and its root-associated microbiota. In this study, we tested whether repeated inoculations with a *Proteobacteria* consortium influenced plant productivity and the AMF assemblages associated with the root and rhizosphere of four plant species growing either in non-contaminated natural soil or in sediments contaminated with petroleum hydrocarbons. A mesocosm experiment was performed in a randomized complete block design in four blocks with two factors: (1) substrate contamination (contaminated or not contaminated), and (2) inoculation (or not) with a bacterial consortium composed of ten isolates of *Proteobacteria*. Plants were grown in a greenhouse over four months, after which the effect of treatments on plant biomass and petroleum hydrocarbon concentrations in the substrate were determined. MiSeq amplicon sequencing, targeting the 18S rRNA gene, was used to assess AMF community structures in the roots and rhizosphere of plants growing in both contaminated and non-contaminated substrates. We also investigated the contribution of plant identity and biotope (plant roots and rhizospheric soil) in shaping the associated AMF assemblages. Our results showed that while inoculation caused a significant shift in AMF communities, the substrate contamination had a much stronger influence on their structure, followed by the biotope and plant identity to a lesser extent. Moreover, inoculation significantly increased plant biomass production and was associated with a decreased petroleum hydrocarbons dissipation in the contaminated soil. The outcome of this study provides

knowledge on the factors influencing the diversity and community structure of AMF associated with indigenous plants following repeated inoculation of a bacterial consortium. It highlights the dominance of soil chemical properties, such as petroleum hydrocarbon presence, over biotic factors and inputs, such as plant species and microbial inoculations, in determining the plant-associated arbuscular mycorrhizal fungi communities.

Keywords: Arbuscular mycorrhizal fungi; plant–microbe interactions; microbial ecology; rhizosphere microbiome; bacterial inoculation; petroleum hydrocarbon contamination; amplicon sequencing; biodiversity; ribosomal RNA

3.1 Introduction

Plant-root associated microbes have been identified as major contributors to plant survival and health (van der Heijden et al., 2008), as they can increase growth through a myriad of functions like the secretion of compounds such as phytohormones, organic acids, and antibiotics, thus improving access to nutrition and resistance to pathogens (Ping and Boland, 2004; Berendsen et al., 2012a; Dangl et al., 2013; Jambon et al., 2018). Moreover, in polluted environments, microorganisms can alleviate soil toxicity by reducing contaminant levels and/or bioavailability (Gkorezis et al., 2016; Ojuederie and Babalola, 2017). Considering the benefits and services microbes provide, their proper management is seen as a way to improve plant health, agricultural yields, as well as the rate of microbe mediated bioremediation. The application of microbial inoculants to the soil in order to modify certain processes and functions is a practice that has grown in popularity over the past few years (Fox, 2015; Alori et al., 2017). While results obtained through function screening and cultivation in laboratory conditions are promising, the large-scale application in the field presents challenges in the establishment, persistence, and performance of microbes (Kaminsky et al., 2019). Influencing the composition and structure of the rhizosphere-associated microbial community by inoculating indigenous or allochthonous microorganisms is a promising research avenue. It has the potential to be applied at large scale because it does not necessarily rely on the persistence of the inoculants, so long as they induce lasting and desirable changes in microbiome. This could be accomplished through a modification in the soil environment, for example a pH change or nitrogen fixation (Kallala et al., 2018; Ratzke and Gore, 2018), or possibly from an interaction with the native rhizosphere microbes. This would lead to significant changes in the microbial community and plant growth (Yergeau et al., 2015; Dagher et al., 2019).

Arbuscular mycorrhizal fungi (AMF) are major actors in the rhizosphere. They are ubiquitous soil microorganisms that develop obligatory associations with the roots of most terrestrial plant species and play an important role in their biological functioning (Smith and Read, 2010). They provide many benefits to their host plants, notably expanding the plant root system through their extensive mycelial network, which improves plant access to water and mineral nutrition (Jakobsen et al., 2003; Abdel Latef and Chaoxing, 2011; Posta and Duc, 2019). Additionally, they can buffer abiotic stresses (i.e., salinity, drought, and trace metal toxicity) (Hajiboland et al., 2010; Wu et al., 2014b; Begum et al., 2019) and reduce the damages of root pathogens (St-Arnaud and Vujanovic, 2007; Song et al., 2015; Bruisson et al., 2016). Beyond their interaction with their hosts, AMF mycelia also contribute to soil stability and structure (Rillig and Mummey, 2006) through the secretion of glomalin (Rillig et al., 2002), a long-lasting glycoprotein known for increasing soil aggregation and water retention (Bedini et al., 2009). Previous experiments showed that plant rhizosphere inoculation with AMF improved the reduction of petroleum contaminants in comparison to non-inoculated plants (Joner et al., 2001; Joner and Leyval, 2003). As there is no information about the ability of AMF to degrade organic pollutants due to their lack of the required enzymes to break them down, the improved degradation of hydrocarbons is likely due to an AMF-mediated increase in plant roots exudation and/or stimulation of other microbial taxa. These taxa can then carry on the degradation of organic pollutants (Barea et al., 2002b; Jeffries et al., 2003; Li et al., 2006). Some studies have shown the specific attachment to AMF hyphae of certain types of bacteria isolated from AMF spores and agricultural soils (Scheublin et al., 2010; Lecomte et al., 2011), and the potential of these microbes to contribute to AMF P uptake (Taktek et al., 2017). Very few AMF taxa are commercially available for large-scale use (Emam, 2016). Moreover, the success of AMF inoculation in polluted sites relies on many parameters including the soil and pollutant characteristics and the ability of the inoculated strains to compete with

indigenous taxa. Therefore, in an effort to better understand how to influence plant-associated AMF communities, we investigated the contribution of plant species identity and repeated bacterial inoculation in shaping AMF assemblages in contaminated and uncontaminated substrates. We selected plants which were spontaneously growing in highly PHC-contaminated sediments, as this would increase their chances of survival. We measured plant growth and PHC dissipation at the end of the experiment. This inoculation-based approach could lead to a better understanding of the recruitment and management of the most widespread plant–fungus symbioses on earth in the service of phytotechnology.

3.2 Materials and Methods

Sediments contaminated with petroleum hydrocarbons were collected in October 2013 from a by-product sedimentation basin of a petrochemical plant located at Varennes, on the south shore of the St. Lawrence River near Montreal, Quebec, Canada (45°41′56” N, 73°25′43” W). This basin was artificially made to collect wastewater of the petrochemical plant. It was used for several decades. The petrochemical plant stopped its industrial activities in 2008, and the site was colonized by spontaneous vegetation. No information on the vegetation nor arbuscular mycorrhizal fungal diversity were available for the site before its exploitation. Basic chemical characteristics of the sediments have been previously described (Desjardins et al., 2014; Dagher et al., 2019). Contaminated sediments were collected from the 0–10 cm layer of the decantation basin and brought back to the laboratory where they were thoroughly homogenized and transferred into 60 × 29 × 12 cm trays to a final volume of 18 L per tray and used as the growth substrate for plants. Non-contaminated field soil was also collected from a land plot adjacent to the contaminated basins and transferred to similar trays. Chemical characteristics of both substrates are available in Table S1. Initial hydrocarbons concentrations were 3055 ± 188 mg/kg for C10–C50 and 35.4 ± 2.6 mg/kg for polycyclic aromatic hydrocarbons (PAHs) in the

contaminated treatments. Soils with these levels are considered to be significantly contaminated for agricultural, residential, and commercial use according to the Canadian soil quality guidelines.

3.2.1 Seeds Collection and Germination

We collected seeds from eight species of annual plants that spontaneously grew within the same contaminated basin in October 2013. Out of these eight species, we chose the following four plant species: *Persicaria lapathifolia* (L.) Delarbre, *Lythrum salicaria* L., *Lycopus europaeus* L., and *Panicum capillare* L., based on the availability of a sufficient number of seed pods on the day of collection, as well as on their germination success. The seeds were stratified in damp sand at 4 °C during 8 weeks, after which they were germinated in a 1:1 (*v*/*v*) sand/calcined montmorillonite clay (Turface, Buffalo Grove, IL, USA) mix incubated at room temperature. Germinated seeds were planted in 50 mL multi-cell compartments filled with double autoclaved at 121 °C all-purpose commercial potting soil mix (Scotts Premium potting soil, Rocky View County, AB, Canada). After three weeks of growth, seedlings were carefully transferred to the trays.

3.2.2 Media Preparation and Isolation of Bacteria from the Contaminated Sediments

One kilogram of contaminated sediments was mixed with one liter of sterile demineralized water and stirred for 24 h at room temperature. Subsequently, the supernatant was filtered through a 40 µm sieve and used to prepare a culture medium containing solely 8 g/L of agarose. After being autoclaved for 30 min at 121 °C and left to cool for 45 min, the medium was supplemented with 100 mg/L cycloheximide to inhibit any fungal growth. An inoculum was made up by serially diluting down to 10^{-7} a thoroughly vortexed stock suspension composed of 1 g of the same contaminated sediment in 9 mL of sterile demineralized water. From these serial dilutions, aliquots of 100 µL from

dilutions of 10^{-6} and 10^{-7} were spread on the culture plates, incubated at 27 °C for one week, and checked daily for bacterial growth. Each colony was subcultured on the same medium in order to obtain a pure culture. The bacterial isolates were then stored at 4 °C. The same procedure was repeated using an impoverished tryptic soy agar medium (3 g/L) to isolate more copiotrophic bacteria.

3.2.3 16S rDNA Amplification, Sequencing, and Identification of Bacteria

Bacterial isolates were individually picked with 1 µL sterile inoculation loops and directly added to a PCR master mix. 16S rRNA sequences were amplified using '27f' 5′AGAGTTTGATCMTGGCTCAG 3′ and '1492r' 5′ TACGGYTACCTTGTTACGACTT 3′ primers. The PCR master mix was made up of 1× PCR buffer, 0.5 mg BSA, 2mM $MgCL_2$, 0.2 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate, one unit of *Taq* DNA Polymerase (Qiagen, Toronto, ON, Canada), and 1 µL of bacterial cells as DNA template. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. PCR reactions were performed using an Eppendorf Mastercycler ProS (Eppendorf, Mississauga, ON, Canada). Sanger DNA sequencing was achieved using a commercial service provided at the Genome Quebec Innovation Centre (Montreal, QC, Canada). Briefly, a polymerase chain reaction (PCR) was first conducted using Big Dye 3.1 terminators and thermocyclers. The sequence was then determined by capillary electrophoresis, then analyzed using the ABI 3730xl Data Analyzer (Thermo Fisher Scientific, Mississauga, ON, Canada). Obtained sequences were identified using the NCBI nucleotide BLAST database. Identity was assigned based on the closest match with the highest coverage of our query sequences.

3.2.4 Selection of Isolates and Production of a Bacterial Consortium

A bacterial consortium that was used for the inoculation of plants was formed from a selection of bacterial isolates obtained from the contaminated sediments, as described above. To prepare this consortium, ten bacterial isolates belonging to the phylum *Proteobacteria* were selected (Table S2). Protobacteria were chosen because they have been identified as a dominant bacterial group within the same contaminated sediments in previous studies (Bell et al., 2014; Stefani et al., 2015a). Selected bacterial isolates were individually subcultured in impoverished Tryptic Soy Broth (TSB, 3 g/ L) at 27 °C for 72 h, after which the liquid cultures were centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was then discarded, and the resulting bacterial cell pellet was re-suspended in 1 L of a sterile isotonic 0.154 M NaCl solution. Cell counts for each isolate were then performed using a Neubauer improved hemocytometer (Sigma-Aldrich, Oakville, ON, Canada), and the inoculum was made up by suspending all the selected isolates in equal numbers in a final volume of 2.4 L at a final concentration of 2.4×10^9 CFU/mL.

3.2.5 Mesocosm Experiment Design and Inoculation

A randomized complete block design with two factors—substrate contamination and inoculation—with four replicates each was setup in four blocks (one replicate per block). The treatments were the following: non-contaminated field soil not inoculated (NB-), non-contaminated field soil inoculated with the bacterial consortium (NB+), contaminated sediments not inoculated (CB-), and contaminated sediments inoculated with the bacterial consortium (CB+). Each tray was planted with the plant species listed above in three rows, separated by 7.5 cm. Within each row, the four plant species were randomly distributed in four planting points, each composed of a cluster of four individual seedlings from the same plant species. These points were spaced at 12.5 cm intervals. Plants were watered several times weekly, as needed throughout the experiment. No fertilization was applied. Bacterial

inoculation was performed twice, two and four weeks after planting the trays. At both applications, each tray received 300 mL of the bacterial consortium, which gave a concentration of 4×10^7 CFU/g of dry soil. The planting design in each tray as well as pictures of the experimental setup are available in the Supplementary Material file, Figure S1.

3.2.6 Data Collection and Harvest

The plants were harvested after 16 weeks of growth. At harvest, each plant cluster was carefully removed from the substrate to avoid root damage, and aerial parts were separated from the roots. The substrate was gently shaken off from the roots, and the portion still attached (considered as the rhizospheric soil) was collected and used for DNA extraction. Subsequently, the root system was thoroughly washed with tap water to eliminate the remaining substrate particles. In total, there were 12 rhizospheric soil samples and 12 root samples from each tray (one sample per plant cluster, i.e., three samples for each plant species), hence 384 samples were taken for the whole experiment. All samples were stored at −80 °C until processing. Aerial plant parts were dried in the oven for 72 h at 60 °C and weighted. A plant sampling example can be found in Supplementary Figure S2. In addition, soil composite samples (3 subsamples) from each of the contaminated mesocosms were collected for petroleum hydrocarbon analysis. C10–C50 aliphatic hydrocarbons and polycyclic aromatic hydrocarbons levels analysis was performed using a commercial service (AGAT labs, Montreal, QC, Canada).

3.2.7 DNA Extraction, PCR Amplification and Illumina MiSeq Sequencing

Total genomic DNA was extracted from plant roots and rhizospheric soil samples using the Nucleospin® Soil Kit (Macherey Nagel, Bethlehem, PA, USA) following the manufacturer's instructions. The isolated DNA was then diluted tenfold in Milli-Q type 1

sterile water to reduce the risk of PCR inhibition by PHC contaminants and humic substances. A two-step nested PCR was performed in order to amplify part of the 18S rRNA gene of arbuscular mycorrhizal fungi using primers AML1 (5′-ATCAACTTTCGATGGTAGGATAGA-3′) and AML2 (5′-GAACCCAAACACTTTGGTTTCC-3′) according to Lee et al. (2008) for the first step, followed by an in-house set of internal primers: nu-SSU-0595-5' CGGTAATTCCAGCTCCAATAG / nu-SSU-0948-3' TTGATTAATGAAAACATCCTTGGC with overhang adapter sequences to produce a final amplicon size of ~400 bp (Renaut et al., 2020). Finally, Illumina Miseq-specific indexes were attached to the generated amplicons using the Nextera XT V2 kit using a limited-cycles PCR as recommended by the manufacturer. The indexed sequences were then purified and normalized using the SequalPrep™ Normalization Plate Kit (Thermo Fisher Scientific, St-Laurent, QC, Canada), after which they were pooled at equimolar concentration and sequenced on an Illumina MiSeq sequencer. The 384 samples were multiplexed in one flow cell for sequencing using the 600 cycle MiSeq Reagent Kit v.3 in 2×300 bp configuration (Illumina Inc., San Diego, CA, USA).

3.2.8 Evaluation of the AMF Colonization of Plants Roots

Composite root samples from each plant cluster were surface rinsed in tap water to remove any remaining soil debris and cut in fragments 1 cm long. They were then cleared in a 10% KOH solution, acidified using acetic acid, and stained with trypan blue, following a microwave-assisted protocol (Dalpé and Séguin, 2013). Root fragments were mounted onto microscope slides in lactoglycerol medium (300 mL lactic acid, 300 mL glycerol, 400 mL double-distilled water), and colonization percentages were evaluated using the magnified intersections method (McGonigle et al., 1990), with 150 intersects examined per plant cluster for the presence of mycorrhizal structures.

3.2.9 . Sequence Processing and details of the pipeline

Read assembly and primer trimming were done in Mothur (v.1.34.4). The rest of the processing was performed in QIIME (v.1.9), following the Brazilian Microbiome Project 18S profiling pipeline (Pylro et al., 2013; Pylro et al., 2014). In Mothur, the reads from each sample were assembled using the 'make.contigs' command. This generated a .fasta file containing the assembled reads. Primers were then removed with 'trim.seqs', after which the sequences were exported to QIIME. Special labels were then added for compatibility, with the 'add.qiime.labels.py' command. Using the Usearch7 sequence analysis tool via QIIME implementation, the dataset was reduced to unique sequences using '-derep_fulllength', which we sorted by decreasing cluster size and removed singletons with '-sortbysize'. The sequences were then clustered by operational taxonomic unit (OTU) using a 97% identity threshold using the UPARSE method, and further sequencing errors were removed with '– uchime_denovo'. Representative sequences of the OTUs were aligned with '-align.seqs' using the Silva eukaryote database (release 132), and filtered ('-filter.alignment' -e 0.10 and –g 0.80). We then produced an OTU table at 97% similarity, and sequences that did not classify as Glomeromycota were removed with 'filter_taxa_from_otu_table.py'. Finally, we subsampled the sequences, so each sample had the same number of sequences (150). Raw sequence data have been deposited in NCBI's Sequence Read Archive and can be found under BioProject number PRJNA591301. An operational taxonomic unit (OTU) table of the dataset used for the analyses can be found in Table S3.

3.2.10 Statistical Analyses

The effect of inoculation with the bacterial consortium on plant biomass and concentration of C10–C50 petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAH) at the end of the experiment were analyzed using ANOVA in JMP® 11.0.0 statistical

software (SAS Institute Inc.). The effect of inoculation and plant species identity on the alpha diversity indices of AMF communities (Chao1, Shannon, and Pielou's equitability) was also analyzed with ANOVA. The effects of the inoculation with the bacterial consortium and plant species identity on AMF community structure in the rhizosphere were analyzed using R (v3.2.0, The R Foundation for Statistical computing). Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity matrixes was used to reveal the effect of treatments and biotope (plant roots and rhizospheric soil) on AMF communities' structures, while Permanova was used to test the effect of treatments on the beta diversity of AMF communities in the substrate.

3.2.11 Phylogenetic Analysis

Sequences of the operational taxonomic units (OTUs) obtained from the bioinformatic pipeline were combined with all the AMF sequences from Kruger et al. (2012), along with the closest matches from the MaarjAM database (Öpik et al., 2010), and aligned using the MUSCLE v.3.8 (Edgar, 2004) plugin in Geneious v.6 (Kearse et al., 2012). We determined the DNA substitution model in jModelTest2 v.2.1.9 (Guindon and Gascuel, 2003; Darriba et al., 2012) using the Bayesian information criterion calculations. A phylogenetic analysis was then performed using the Mr.Bayes v.3.2.6 (Ronquist and Huelsenbeck, 2001) plugin in Geneious v.8 (Biomatters, Auckland, New Zealand). We adjusted the temperature parameter for heating chains in order to keep the swap acceptance rate between 10% and 70%. The number of trees saved was 6000, and the first 1000 trees were ignored before the computation of the consensus tree with the Bayesian posterior probabilities. The tree was rooted using outgroup sequences.

3.3 Results

3.3.1 Sequence Processing

Sequencing of 18S rRNA gene amplicons generated a total of 13,087,745 reads, of which 5,873,061 usable AMF sequences with an average length of 408 bp were retained after processing and quality filtering. All non-glomeromycotan sequences were excluded from the dataset. Reads number per sample ranged from 20 to 42,410. The dataset was subsampled to 150 sequences per sample for all analyses, in order to account for the disparity in reads numbers while minimizing the loss of samples. Sixteen samples out of 384 did not meet this cutoff criterion and were ignored for the analyses. Only OTUs with a cumulative abundance greater than 0.5% for the whole dataset were considered, yielding 23 OTUs at a 97% similarity threshold. Good's coverage indices ranged between 96% and 100% for all samples, indicating that most of the diversity was captured.

3.3.2 Diversity and Identity of AMF Taxa

All alpha diversity results can be found in Table 1. AMF diversity was significantly influenced by inoculation, soil contamination, plant species identity, and biotope, as shown by the Shannon diversity indices of AMF taxa (Table S4). There were also significant inoculation*contamination, plant species*biotope, and biotope*contamination interactions. Overall, AMF communities in the non-contaminated soil had significantly higher Shannon diversity indices than those in the contaminated soil. Inoculation was also significantly associated with higher scores, but this effect was only observed in the non-contaminated soil. Moreover, significantly different diversity scores between plant species were only observed in the roots and not in the rhizosphere: *P. lapathifolia*-associated AMF communities in the roots had a significantly lower Shannon diversity score than those associated with the other plant species (Table S4). Chao1 species richness estimator was significantly influenced by substrate contamination, biotope, and plant species (Table S5).

There was significant plant species*inoculation, plant species*biotope, and plant species*contamination interactions. Overall, Chao1 estimated species richness was significantly higher in the non-contaminated soil and in the roots in comparison to the contaminated sediments and rhizosphere respectively (Table S5). The estimated richness was differentially influenced by inoculation among plant species, biotope, and contamination as indicated by the significant interaction terms. There was no significant difference between plant species that received inoculation, whereas richness was significantly higher in *L. salicaria* than *P. capillare* and *P. lapathifolia* non-inoculated treatments. Additionally, estimated richness was similar between plant species in the non-contaminated soil, but significantly higher in *L. salicaria* than *P. capillare* and *P. lapathifolia* (Table S5). Pielou's equitability index reflects how evenly species (in this case OTU) are represented in each sample. It varies between 0 and 1, with 1 meaning that all OTUs in the samples are represented by the same number of sequences (Pielou, 1966). We found that AMF communities' evenness was significantly influenced by the substrate contamination, inoculation, and biotope (Table S6). Overall, inoculated AMF assemblages showed a significantly higher evenness than those from the non-inoculated communities. Additionally, there was significant plant species*biotope and biotope*contamination interactions. Evenness was not different among plant species in the rhizosphere, but there was significant difference in the roots, with *L. europaeus* showing higher scores than *P. lapathifolia*. Moreover, evenness was similar in the roots and rhizosphere of plants in the contaminated soil, but it was significantly higher in the roots of plants in the non-contaminated soil in comparison with the rhizosphere (Table S6). The 23 AMF OTUs belonged to 15 virtual taxa (VTX) based on the MaarjAM's database (Öpik et al., 2010). The taxonomic affiliation of each OTU was confirmed by the phylogenetic analysis (Supplementary Material, Figure S3). The OTUs belonged to Glomeraceae (16), Claroideoglomeraceae (4), Acaulosporaceae (1), Diversisporaceae (1), and

Paraglomeraceae (1) families. Overall, OTUs belonging to Glomeraceae and Claroideoglomeraceae accounted for most of the sequences, irrespective of inoculation, plant species, biotope (roots, rhizosphere), and contamination. They represented more than 96% of the sequences in the contaminated soil and more than 73% in the non-contaminated. In the roots of plants growing in non-contaminated soil, sequences belonging to *Glomus* sp. were the most abundant irrespective of inoculation. Relative abundances ranged from 27.8% (VTX00247) to 40.6% (VTX00143) in the contaminated mesocosms (Figure 1A). On the other hand, rhizospheric samples were dominated by *Paraglomus* sp. VTX00281 with values ranging between 39.4% and 41.6% (Figure 1A). In the non-contaminated sediments (Figure 1B), root samples were dominated by an OTU identified as VTX00114, which belong to *Rhizophagus irregularis* and ranged from 55.8% to 63%. The rhizosphere was dominated by *Claroideoglomus* VTX00193, representing 54.8% to 57.6% of the AMF community.

Table 1. Alpha diversity indices of AMF communities as calculated in the different treatments and biotopes. LE: *Lycopus europaeus*; LS: *Lythrum salicaria*; PL: *Persicaria lapathifolia*; PC: *Panicum capillare*. Std. Dev: Standard deviation.

				Shannon		Chao 1		Pielou's equitability	
Contamination	**Inoculation**	**Biotope**	**Plant Species**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**
Contaminated	**Inoculated**	**Rhizosphere**	**LE**	1.7	0.38	9.96	3.35	0.56	0.12
			LS	1.96	0.43	8.92	2.37	0.64	0.14
			PC	1.94	0.43	10.88	4.43	0.62	0.13
			PL	1.61	0.39	8.6	3.57	0.57	0.13
		Roots	**LE**	2.39	0.22	10.87	1.38	0.71	0.07
			LS	1.87	0.64	10.32	3.67	0.57	0.16
			PC	2.08	0.27	8.58	1.43	0.69	0.07
			PL	1.73	0.5	8.42	3.05	0.58	0.11
	Non-inoculated	**Rhizosphere**	**LE**	1.86	0.36	9.3	2.82	0.62	0.11
			LS	1.87	0.5	7.74	2.16	0.65	0.14
			PC	1.74	0.65	8.86	4.5	0.61	0.14
			PL	1.68	0.32	7.7	2.63	0.62	0.12
		Roots	**LE**	2.09	0.27	9.25	1.78	0.67	0.07
			LS	2.25	0.42	17.5	2.93	0.6	0.09
			PC	1.75	0.62	8.58	2.85	0.59	0.15
			PL	1.39	0.51	8.36	3.71	0.48	0.13
Non-contaminated	**Inoculated**	**Rhizosphere**	**LE**	2.51	0.28	13.52	4.42	0.71	0.07
			LS	2.49	0.25	12	2.5	0.71	0.04
			PC	2.6	0.34	12.68	2.08	0.73	0.07
			PL	2.79	0.31	15.3	5.96	0.76	0.06
		Roots	**LE**	2.92	0.67	14.94	2.87	0.76	0.15
			LS	3.05	0.22	14.43	1.65	0.8	0.06
			PC	2.82	0.34	14.24	3.34	0.77	0.08
			PL	2.79	0.52	13.49	1.95	0.75	0.12
	Non-inoculated	**Rhizosphere**	**LE**	2.33	0.37	14.87	4.16	0.65	0.09
			LS	2.43	0.33	12.27	1.69	0.68	0.08
			PC	2.28	0.44	10.69	2.55	0.68	0.09
			PL	2.46	0.57	14.24	3.1	0.67	0.12
		Roots	**LE**	2.7	0.93	14	5.29	0.73	0.23
			LS	2.82	0.46	14.18	2.82	0.77	0.11
			PC	2.59	0.47	12.95	2.43	0.73	0.11
			PL	2.47	0.31	13.06	1.85	0.69	0.07

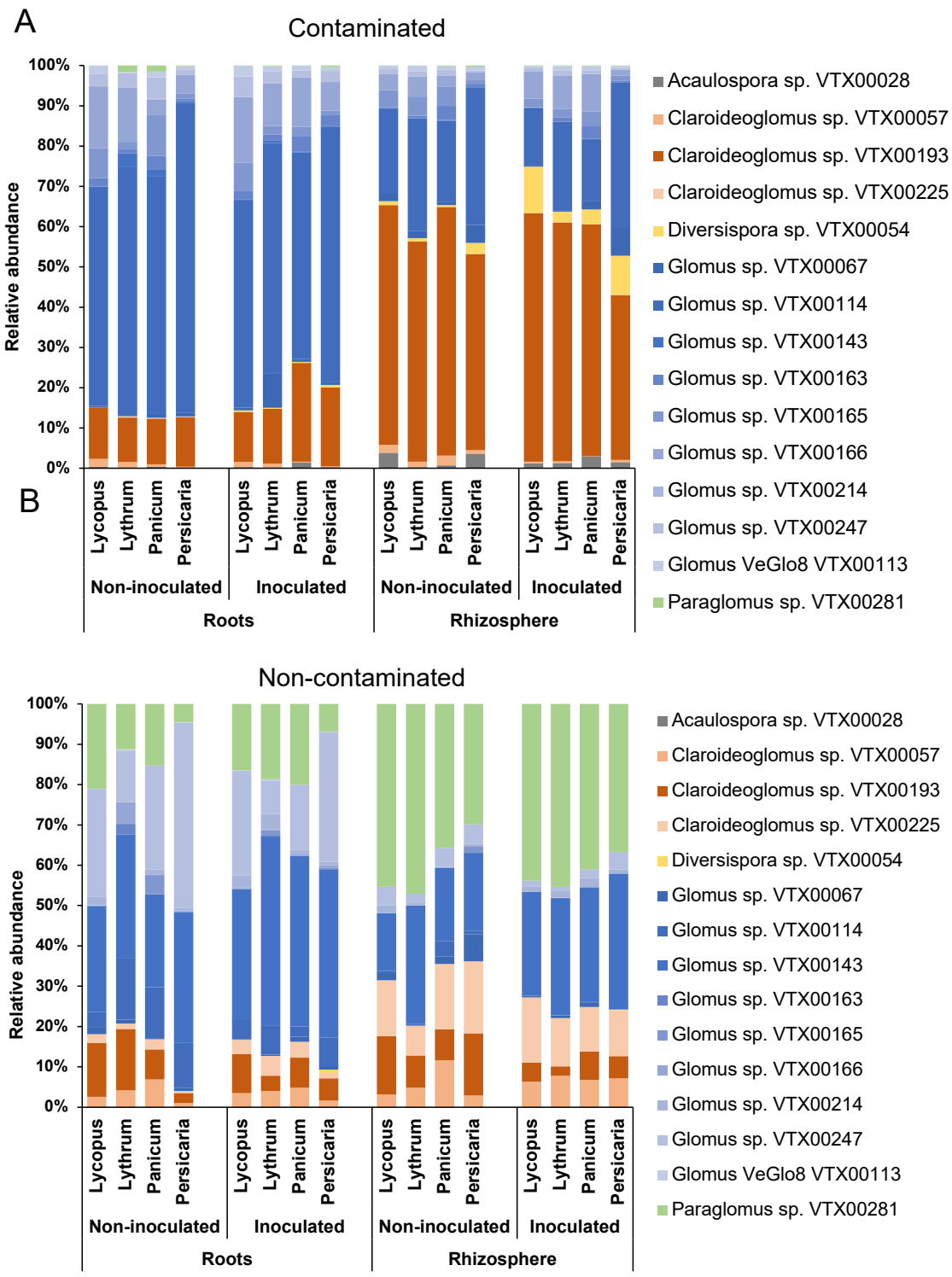

Figure 1. Relative abundance of AMF taxa detected in the roots and rhizospheres of plants from the contaminated substrate (A) and the non-contaminated substrate (B), inoculated or not-inoculated with the bacterial consortium. LE: *Lycopus europaeus*; LS: *Lythrum salicaria*; PC: *Panicum capillare*; PL: *Persicaria lapathifolia*.

3.3.3 AMF Community Structure

Substrate contamination influenced the AMF communities, as illustrated by the PCoA analysis of all samples showing that the samples from contaminated and non-contaminated soil mostly clustered closely together on opposite sides of the biplot (Figure 2A). Permanova analysis showed a significant effect of the substrate contamination level and biotope (Table S7). Noteworthy is the effect of contamination that explained 52.6% of the variation in the AMF communities ($p = 0.001$). There was also a significant contamination*biotope interaction. In each substrate contamination level, the plant biotope (roots vs. rhizosphere) significantly influenced the structure of AMF assemblages, as shown by the PCoA analysis, with a cleaner separation between communities in the non-contaminated soil (Figure 2B, C). Moreover, UPGMA (Unweighted pair group method with arithmetic mean) hierarchical clustering analysis with Bray-Curtis distance on the average relative abundance of each of the AMF OTUs in the experimental blocks also showed the influence of biotope on AMF assemblages, as roots and rhizosphere samples belonged to different clusters (Figure 3A,B). The biotope explained 3% of the variation in the AMF communities ($p = 0.001$) in the non-contaminated substrate, and 5.7% in the contaminated sediments ($p = 0.001$), as per the Permanova analysis (Table S7). In the non-contaminated substrate, AMF communities clustered together in the rhizosphere based on the inoculation, and to a lesser extent in the roots following UPGMA clustering analysis (Figure 3B). On the other hand, inoculation did not induce a clear clustering in the contaminated soil, especially in the roots (Figure 3A). PCoA analysis showed that the root and rhizosphere AMF communities from the non-contaminated substrate underwent a shift in structure following inoculation (Figure 2B), as confirmed by the Permanova with inoculation explaining 3.4% of the variation ($p = 0.003$) in the roots, while in the rhizosphere, inoculation influenced 7.2% of the community structure variation ($p = 0.001$). On the other hand, inoculation significantly influenced the AMF communities only in the roots from the contaminated substrate and accounted for 2% of the

variation (p = 0.028) and did not have a significant influence in the rhizosphere (Figure 2C). Since the interaction between substrate contamination level and plant biotope was significant, we also assessed the influence of plant species identity on the AMF community structure within each substrate contamination level and biotope using Permanova. In the non-contaminated substrate, plant species identity accounted for 9.5% (p = 0.001) of the variation in the roots and 6.7% in the rhizosphere (p = 0.003). Similarly, in the contaminated substrate, plant identity significantly explained 9.7% ($p \leq 0.001$) of the variance in the roots and 5.7% (p = 0.009) in the rhizosphere. More details on the Permanova results can be found in Table S7.

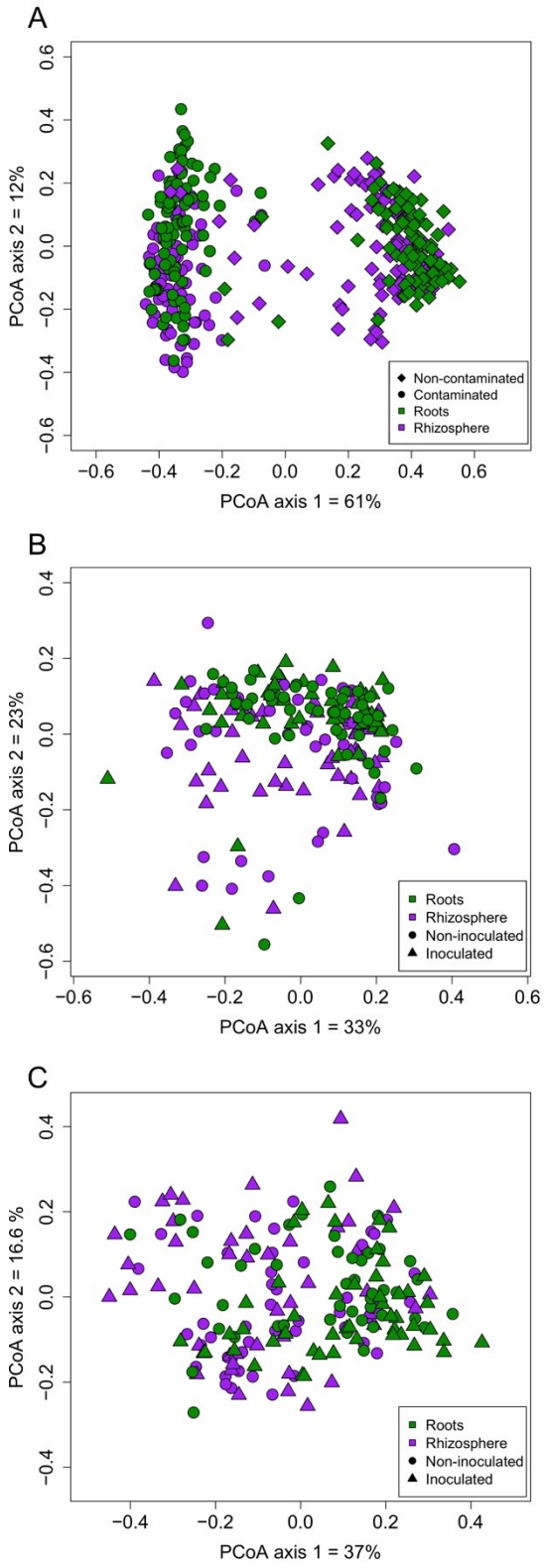

Figure 2. Principal coordinate analysis based on the Bray–Curtis dissimilarity of (**A**) roots and rhizosphere AMF communities together from both type of substrates (non-contaminated soil or contaminated sediments), and separately from (**B**) the non-contaminated soil and (**C**) the contaminated sediments.

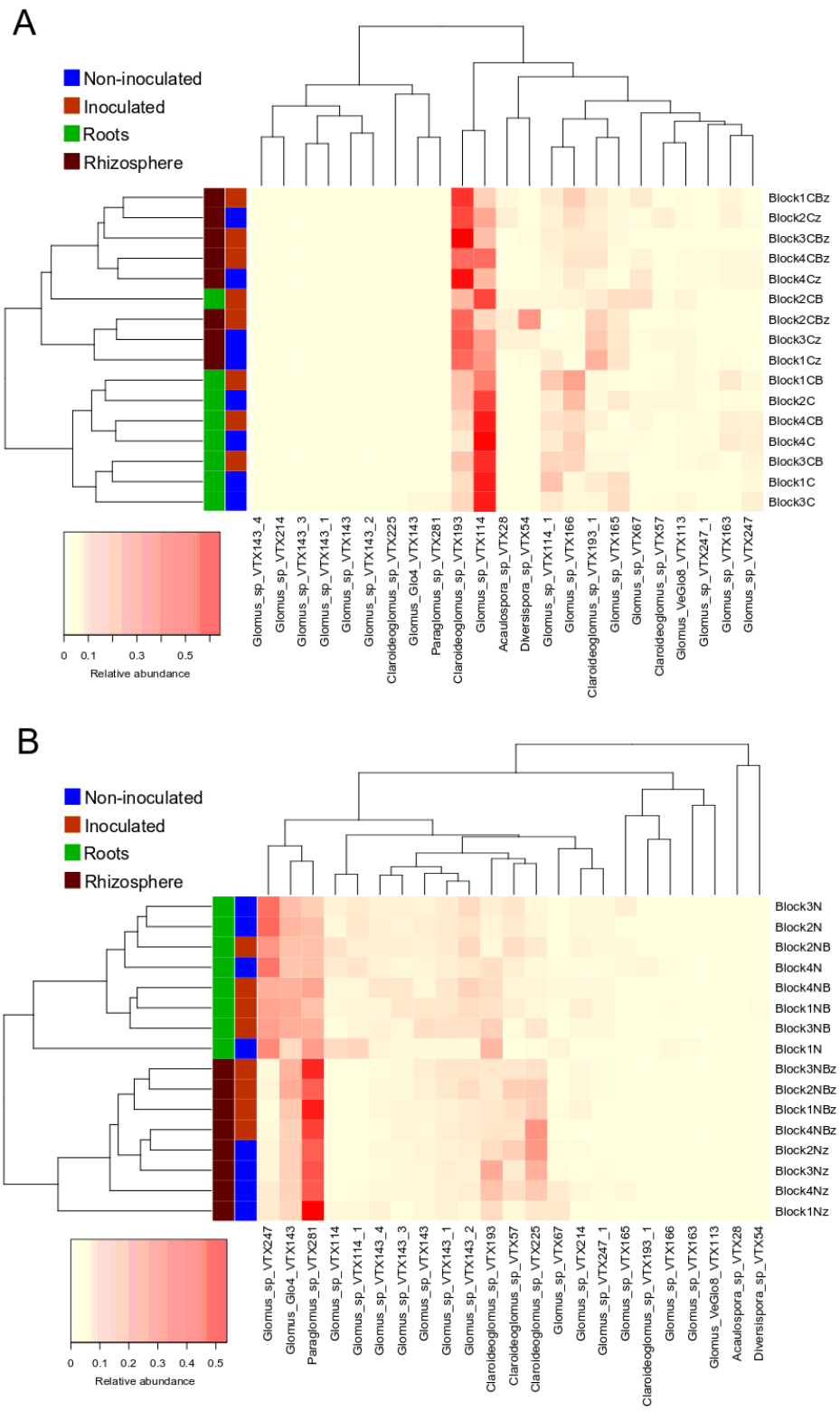

Figure 3. Heat map and UPGMA clustering based on the relative abundance of AMF OTUs in roots and rhizosphere in each block, from the different treatments in the contaminated soil (**A**) and the non-contaminated sediments (**B**). The colors of the last two squares on the left of rows represent the origin and treatment of each sample (biotope and inoculation). The dendrogram are based on the UPGMA.

3.3.4 Plant Dry Biomass

Substrate contamination significantly affected plant biomass since plants growing in the contaminated substrate produced significantly less dry biomass than those growing in the non-contaminated substrate for all plant species ($p < 0.0001$). Plant species identity also showed a significant effect ($p < 0001$), as well as inoculation ($p = 0.0043$) (Figure 4A, B). There was a significant contamination * plant species effect ($p < 0001$), where biomass production was significantly different between plant species in the non-contaminated soil, but not in the contaminated setting (Table S8).

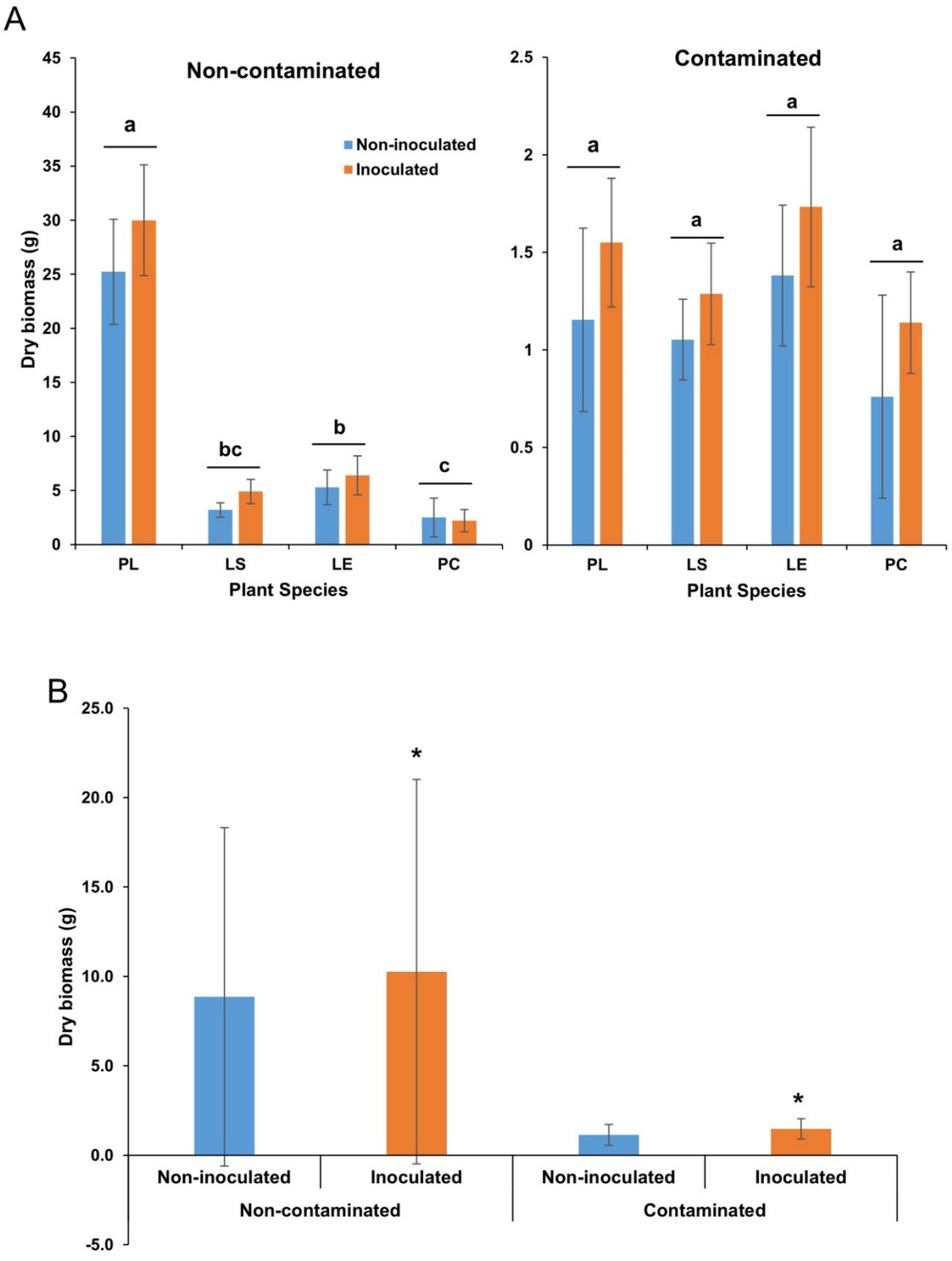

Figure 4. Effect of inoculation on average plant aerial dry biomass for each plant species (**A**) and overall (**B**), in the non-contaminated soil and the contaminated sediments. Errors bars are standard deviations. Within each contamination level, treatments not sharing the same letter are significantly different (**A**). Asterisks indicate a significant difference between the two treatments in each contamination level (**B**). PL: *Persicaria lapathifolia*; LS: *Lythrum salicaria*; LE: *Lycopus europaeus*; PC: *Panicum capillare*. $N = 12$ for each species.

3.3.5 AMF Root Colonization

The microscope examination of trypan blue-stained roots revealed the occurrence of AMF structures associated with the roots, such as hyphopodia, intraradical mycelium, arbuscules, and vesicles (Figure S4). Assessment of the percentage of root length colonized using the magnified intersect method showed that the colonization rate was significantly higher ($p < 0.001$) in the roots of plants that grew in the contaminated sediments than those in non-contaminated soil for all plant species (Table S9). Colonization ranged from 25% to 67% of root length in the contaminated sediments and from 12% to 23.5% in the non-contaminated soil (Figure 5A). Inoculation did not influence root colonization in the non-contaminated soil, but it significantly increased the root colonization in the contaminated sediments (Figure 5A).

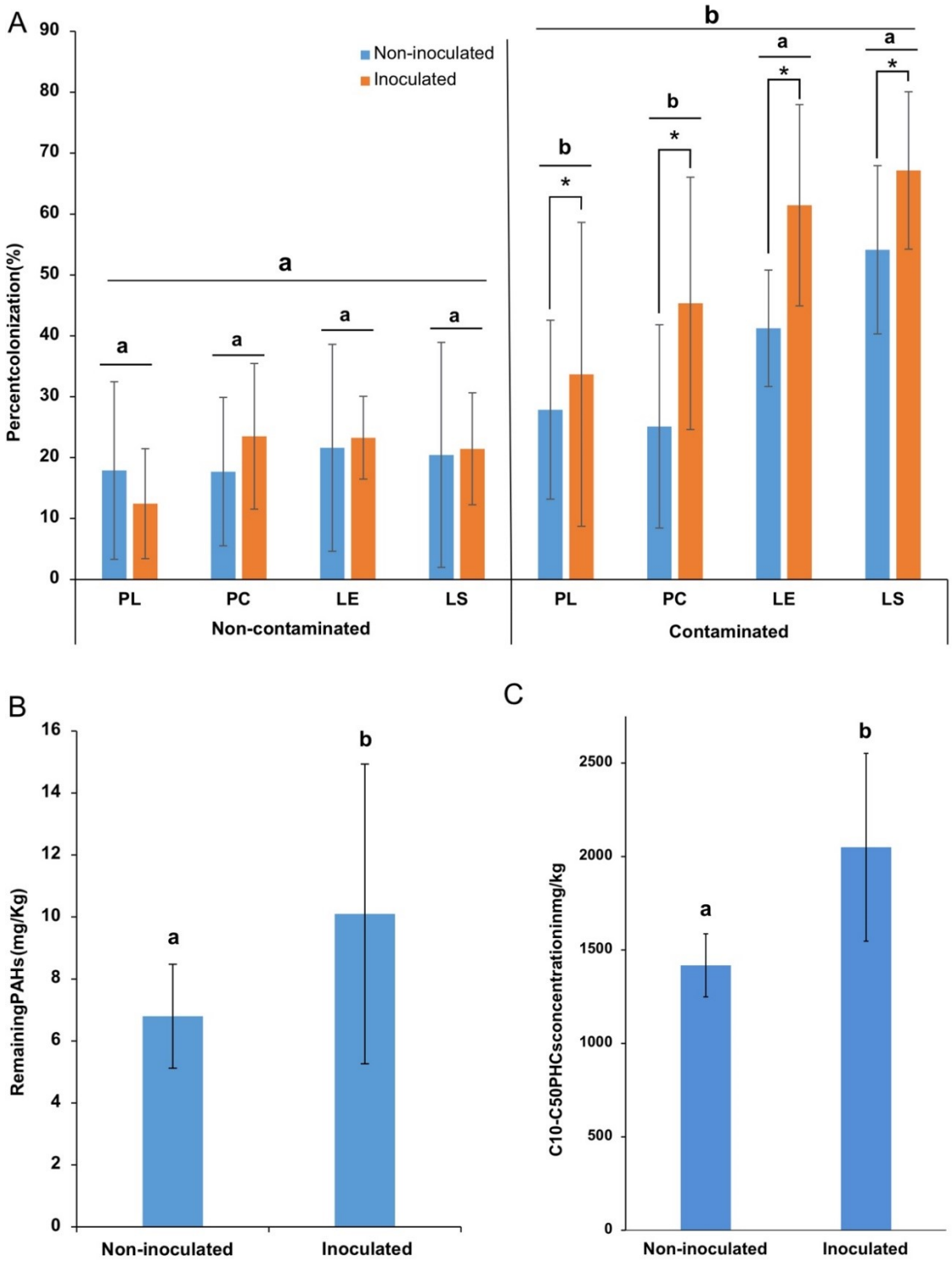

Figure 5. (**A**) Average AMF root colonization percentage for each plant species in both soil contamination levels**.** PL: *Persicaria lapathifolia*; LS: *Lythrum salicaria*; LE: *Lycopus europaeus*; PC: *Panicum capillare***.** Within each contamination level, plant species not sharing the same letter are significantly different. For each plant species, an asterisk denotes a significant difference between inoculation treatments. (**B**) Average remaining PAHs and (**C**) C10/C50 PHCs in the contaminated sediments at harvest. Errors bars are standard deviations.

3.3.6 Effect of Inoculation on PHC Concentrations

Bacterial inoculation also influenced the concentrations of PAHs (Figure 5B) and aliphatic hydrocarbons (C10–C50 fraction; Figure 5C) in the contaminated sediments at the end of the experiment. Intriguingly, there was significantly less PAHs (p = 0.0181) in the non-inoculated compared to the inoculated treatments (Figure 5B). Values ranged from 6 ± 1.14 mg/kg in the non-inoculated to 10.7 ± 1.11 mg/kg in the inoculated microcosms. The same trend was observed for aliphatic petroleum hydrocarbons (Figure 5C), with levels ranging from 1360 ± 147 mg/kg in the non-inoculated to 2050 ± 502 mg/kg in the inoculated microcosms.

3.4 Discussion

The present study shows that the physicochemical properties of the plant substrate that was contaminated or not with petroleum hydrocarbons strongly shaped the AMF communities associated with the plant roots and rhizosphere. Moreover, within a given substrate contamination level, plant identity, biotope, and inoculation with the bacterial consortium also had significant effects on the structure of AMF assemblages.

3.4.1 Contamination and Biotope Shape AMF Communities

In this experiment, AMF rhizospheric and root communities of plants growing in non-contaminated soil and PHC-contaminated sediments were inoculated using a bacterial consortium formed of multiple *Proteobacteria* isolates. Overall, neither plant species identity nor bacterial inoculation were significant determinants of AMF root and rhizosphere assemblages, obviously because the effect of soil contamination and the differences between biotopes were so large that it overwhelmed the more subtle effects of the other factors, which still were enlightened by interactions such as inoculation*biotope in the non-contaminated soil, inoculation*plant species in the rhizosphere, and contamination*inoculation in both roots and rhizosphere. However, looking at each

level of soil contamination, we found significant effect of biotope (rhizosphere vs. roots) and, to a lesser extent but also significant, plant species identity and bacterial inoculation effects. It is still unclear how to manipulate different environmental factors in order to influence the AMF communities to a predictable state, as multiple studies found different patterns. For example, Xu et al. (2017) sampled *Chenopodium ambrosioides* plants from five different sites and found, using high-throughput sequencing, that soil properties were the major factor explaining the variation in AMF communities, followed by soil habitat (rhizosphere vs. roots), while plants showed little effect (Xu et al., 2017). Contrarily, Krüger et al. (2017) found that it was the plant communities that shaped the AMF assemblages, rather than soil properties, during primary succession on mine spoils (Krüger et al., 2017). In another study, the authors analyzed rhizospheric soil from different plants sampled from five sites in the Tibetan alpine steppe; they found that plant species identity did not significantly explain the variation in the AMF assemblages, but rather it was the precipitation which was associated with an increased hyphal length density (Zhang et al., 2016). Finally, Dumbrell et al. (2009) sampled 28 plants species growing along a pH gradient and found that it was the pH, rather than plants, that structured AMF communities (Dumbrell et al., 2009). While of different conclusions, the aforementioned studies showed that the soil environment is generally at the center of the process of shaping the AMF communities; therefore, an approach where the soil parameters would be precisely manipulated might be a successful method to shape microbial assemblages. Such shaping of microbial communities has been observed in the human gut microbiome, where significant, reproducible, and long-lasting changes were induced by osmotic stress caused by the laxative polyethylene glycol, which disrupts the mucus barrier and causes an IgG response from the immune system against the resident highly abundant bacteria and a modification of the cytokinin levels (Tropini et al., 2018). This showed that a modification of the environmental parameters triggered a cascade of reactions that led to lasting and predictable changes. In the present experiment,

we tried to modify the community structure through a biotic disturbance in the form of a repeated inoculation of a bacterial consortium. We hypothesized that this inoculation could change the soil environment (at least temporarily) through the bacterial metabolites produced by the members of the consortium and their interaction with resident microorganism, as well as the decomposition of dead bacterial cells. As a result, the AMF community structure would be altered since AMF have been shown to interact with their surrounding microorganisms (Miransari, 2011; Taktek et al., 2015; Iffis et al., 2016; Iffis et al., 2017). In fact, the bacterial inoculation did cause significant shifts in the AMF community structure in both biotopes (roots vs. rhizosphere) growing in non-contaminated field soil, but in the contaminated sediments only the AMF community of roots exhibited significant shifts. The toxicity caused by the high concentrations of PHC might likely make the growing environment more selective for stress-tolerant strains of AMF, thus overriding the influence of the biotic disturbance. On the other hand, while insignificant in the rhizosphere, inoculation caused significant shifts in the AMF in roots, suggesting a possible plant-mediated restructuring of the assemblages. This further complicates the task of producing a reproducible and predictable method for shaping community structure, as it was shown that plant species identity counts in the selection of microbial associates.

3.4.2 Glomeraceae and Claroideoglomeraceae Dominate Most Samples

OTUs belonging to the Glomeraceae dominated the roots of all plant species in both contaminated and non-contaminated substrates, and with or without inoculation. OTUs related to *Glomus* sp. were the most abundant in the roots of plants in non-contaminated substrate, while in the contaminated substrate roots were dominated by *Rhizophagus* sp. VTX114 in. On the other hand, an OTU identified as *Paraglomus* sp. dominated the rhizosphere soil of plants in non-contaminated soil, while *Claroideoglomus* sp. was the most abundant in the rhizospheric soil in contaminated sediments. Previous field studies in the same geographical area from which the substrates used in this experiment were sourced have reported higher abundances of *Paraglomu*s in non-contaminated soil

and *Claroideoglomus* in contaminated sediments, as well as a dominance of OTUs related to *Rhizophagus* sp. in plant roots in comparison to rhizospheric soil (Hassan et al., 2014; de la Providencia et al., 2015; Iffis et al., 2016). We found that the structure of AMF in the roots of plants was different at each level of contamination; however, the communities were dominated by Glomeraceae strains in both cases, while the rhizosphere was dominated by non-Glomeraceae OTUs. AMF colonize roots using different strategies; for example, some species produce a larger mycelial network in the rhizosphere before penetrating the roots (e.g., *Gigaspora* spp.), while others readily colonize the roots without producing an extensive network of mycelium outside the roots (e.g., *Glomus* spp.)(Hart and Reader, 2005). This could be explained by the fact that root colonization strategies of AMF species vary with their taxonomic affiliation, and that members of the Glomeraceae family more readily colonize the roots than produce extraradical structures (Hart and Reader, 2002). Such species would be ideal candidates for inoculating plants in contaminated environments to assure adequate plant colonization.

3.4.3 Contamination Increases Root AM Colonization

We found that AMF root colonization percentages in the four plant species collected from the contaminated sediments were significantly higher than their counterpart grown in non-contaminated soil. de la Providencia et al. (2015) sampled during two successive years *P. capillare* plants from the same location from which the contaminated sediments for the present experiment originated. They found that in *P. capillare*, colonization percentages were 4.84% in 2011 and 12% in 2012 (de la Providencia et al., 2015), which were lower than the levels found in the same plant species in this experiment. Plants are subjected to the natural elements and different edaphic factors in situ, which might explain the observed difference. It should also be noted that contamination levels found at the site were much higher than those recorded in this greenhouse experiment, reaching up to 41,000 mg/kg of C10–C50 hydrocarbons. This difference likely was due to the natural degradation due to

exposure to the elements, microbial activity, and volatilization. Higher levels of PHCs might stimulate a stronger mycorrhizal colonization of the roots as observed here; nonetheless, extreme levels are likely to inhibit it. Cabello (1997) found that the AMF root colonization in plants sampled from two contaminated sites in Argentina and Germany was lower than in the same plant species sampled from non-contaminated sites (Cabello, 1997). On the other hand, colonization percentages of over 80% were recorded in plants growing in weathered crude oil in the Ecuador Amazon region (Garcés-Ruiz et al., 2017). Could the PHC-induced stress have driven the plants into engaging more symbioses and promoted the growth of the AMF, which in turn could help alleviate the toxic effects? During the colonization of the roots, AMF develops multiple structures such as arbuscules and vesicles, which play an important part in the interaction with the host plant and the nutrition of the fungus (Varela-Cervero et al., 2016). Defoliation such as grazing by herbivores induces plant stress, in part due to loss of nutrients present in the foliage (Tuomi et al., 1984). In a manual defoliation experiment, *Medicago truncatula* plants showed significantly lower vesicular colonization percentages than their non-defoliated counterparts (IJdo et al., 2010). Piippo et al. (2011) found that grazing simulation through defoliation of two varieties of the biennial grassland herb *Gentianella amarella* decreased arbuscular colonization in the early flowering type, but on the other hand increased it in late flowering type plants (Piippo et al., 2011). Saito et al. (2004) also observed a decrease of root colonization following the defoliation of grazing intolerant grass *Miscanthus sinensis* and at one sampling point for the grazing tolerant *Zoysia japonica*. Inversely, Ambrosino et al. (2018) noted little to no effect of defoliation on total root AMF colonization. These contradictory results suggest that plant/host responses to stress can vary from one plant species to the other, and that different stress types would induce different responses, but also point to the host plant identity as a potential key for influencing AMF root communities. The bacterial inoculation in this experiment was associated with significantly increased root colonization percentages, but only in the

contaminated substrate. AMF activity through its extra radical mycelium has been found to be influenced by soil microbiota assemblages (Svenningsen et al., 2018). The stress-inducing toxicity of PHC contaminants could explain why significant changes in root colonization were only observed in the contaminated substrate, as it might have led to more dynamic and responsive root/AMF interactions.

3.4.4 Inoculation Affects Plant Growth and PHC Attenuation

In this experiment, bacterial inoculation was associated with increased plant growth for some of the species; nevertheless, this did not translate to an increased PHC attenuation rate. On the contrary, it significantly decreased the degradation rate, resulting in greater concentrations in the inoculated compared to the non-inoculated trays at the end of the experiment.

One would assume that the health of plants is likely to be a key component of the success or failure of the process. Nonetheless, our results suggest that improved plant growth does not necessarily lead to a successful remediation of PHC contaminants. Wu et al. (2014a) used dual inoculation of ryegrass with a PAH-degrading bacteria and an AMF strain in order to attenuate PAHs levels, and they found that this approach was superior to single inoculation for the degradation of pyrene and phenanthrene. However, inoculation with the bacteria *Acinetobacter* sp. did not significantly increase ryegrass growth. Moreover, Bell et al. (2016) showed that specialized soil bacterial assemblages obtained in culture are less efficient at degrading crude oil than a diverse microbiome.

3.5 Conclusions

Our results highlight the important part that the soil properties can play in shaping the plant associated AMF communities. Moreover, in the presence of high stress due to the petroleum hydrocarbons, AMF root colonization rates were significantly higher than in the natural soil. This

suggests that AMF could play an important part in plant physiology when growing under stressful conditions. The bacterial inoculation without functional screening was correlated with plant biomass increase, and shifts in the AMF root communities. This shows that it could be a potential practice in order to alter AMF assemblages in polluted environments to assist plant health, without the necessity of the inoculum to persist in the rhizosphere. It remains to be seen whether the change in the AMF communities was due to a direct effect of the bacterial inoculation, or to the potential change in exudation of the plants that increased biomass production. Interestingly, inoculation and increased plant biomass were associated with a decrease in petroleum hydrocarbons dissipation, possibly due to the disruption of the local microflora following inoculation. Future research should be geared towards analyzing the functional genes and metabolites implicated in the changes in the rhizosphere and plant growth in order to have a better control over the management of rhizospheric AMF assemblages.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/8/4/602/s1

Table S1: Basic chemical characteristics of the substrates.

Table S2: Bacterial isolates selected for the inoculation of plants

Table S3: AMF taxa from the 18S sequences after subsampling (150 sequences). LE: *Lycopus europaeus*; LS: *Lythrum salicaria*; PL: *Persicaria lapathifolia*; PC: *Panicum capillare*. *Samples starting with A: Non-contaminated and non-inoculated; B: Contaminated and non-inoculated; C: Non-contaminated and inoculated; D: Contaminated and inoculated. The number after the first letter indicates the block from which the sample was taken. The number after the plant species designation indicates the plant species replicate in the treatment as there were 3 replicates of each plant species in each treatment (As shown in planting tray design in supplementary figure S1). Samples ending with a "z" come from the rhizosphere and those without the "z" represent root samples.*

Table S4: ANOVA analysis output from JMP for Shannon's index.

Table S5: ANOVA analysis output from JMP for the Chao1 diversity index.

Table S6: ANOVA analysis output from JMP for Pielou's equitability index.

Table S7: Permanova analysis of the AMF communities based on the Bray-Curtis dissimilarity matrix. **Table S8:** ANOVA analysis output from JMP for the plant dry biomass production.

Table S9: ANOVA analysis output from JMP for AMF root colonization percentages.

Figure S1: Planting scheme in each tray (A), experimental setup (B), and a comparison between the non-contaminated soil and contaminated sediments mesocosms (C).

Figure S2. Plant sampling example from a *Persicaria lapathifolia*.

Figure S3. Bayesian phylogenetic tree based on nuclear small subunit (SSU) rDNA consensus sequences showing the distribution of the 23 OTUs recorded in this experiment (red labels) among the Glomeromycota tree.Sequence data were analyzed with the SSU sequences (black labels) from Krüger et al. (2012) and the closest matches recovered from MaarjAM database. The numbers on the nodes represent the Bayesian posterior probabilities. The scale represents the branch length corresponding to expected substitutions per site.

Figure S4. Mycorrhizal structures observed in the plant roots under light microscopy

3.6 Acknowledgments

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Author contributions: D.J.D. and I.E.P. designed and performed the experiment; I.E.P., F.E.P., M.S.-A., and M.H. supervised the project; D.J.D. analyzed the data; M.S.-A. and M.H. provided material and analytic tools; D.J.D., I.E.P., F.E.P., M.S.-A., and M.H. wrote the paper.

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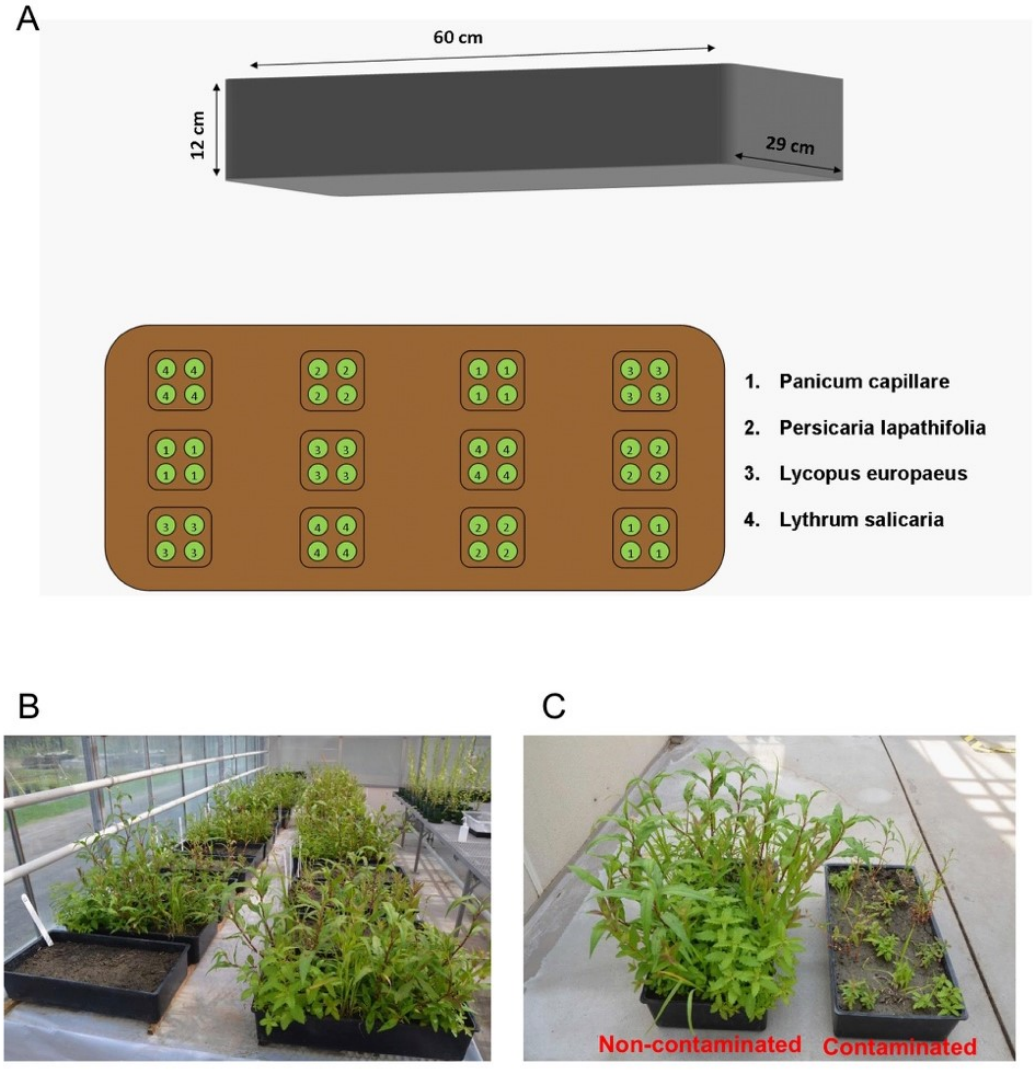
Conflicts of Interest: The authors declare no conflicts of interest.

3.7 Supplementary information

Supplementary tables can be found at: https://www.mdpi.com/2076-2607/8/4/602/s1



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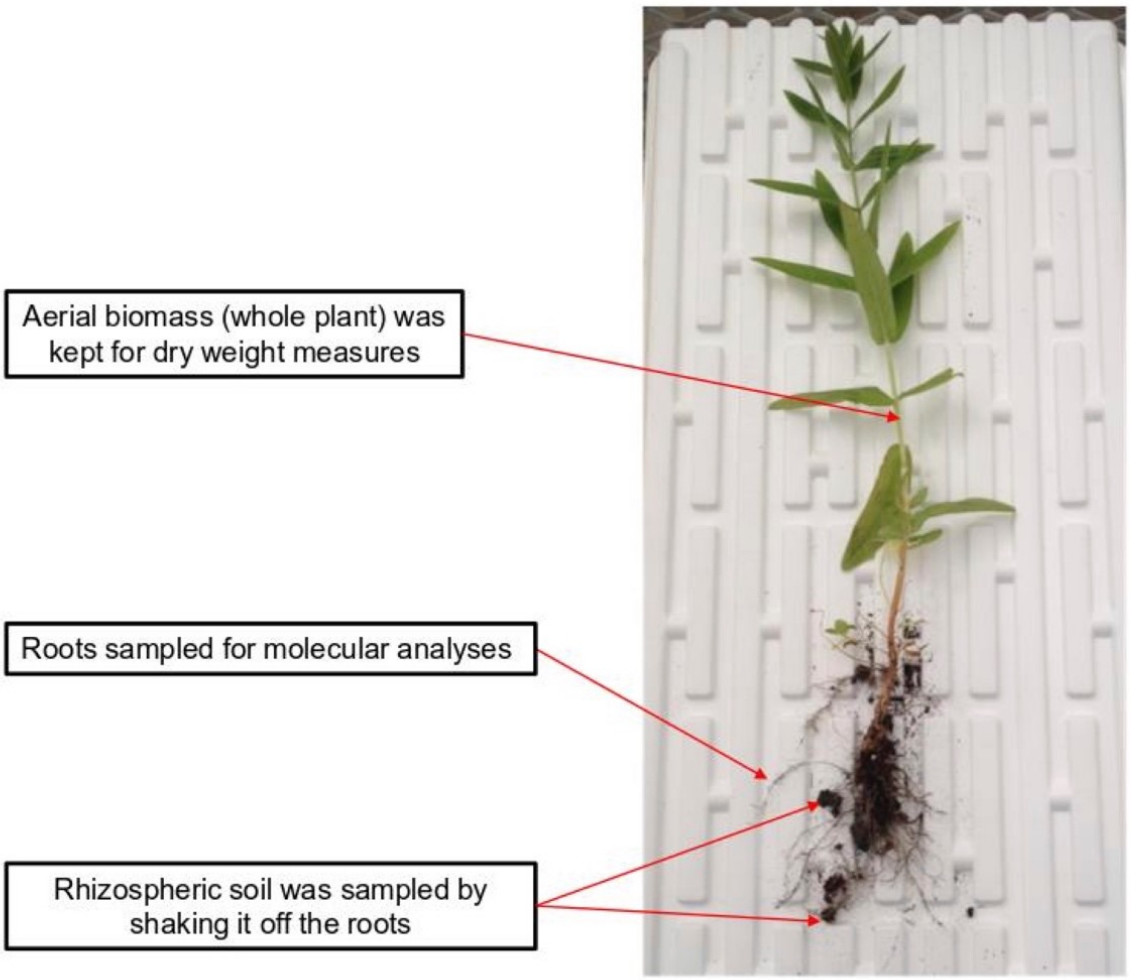
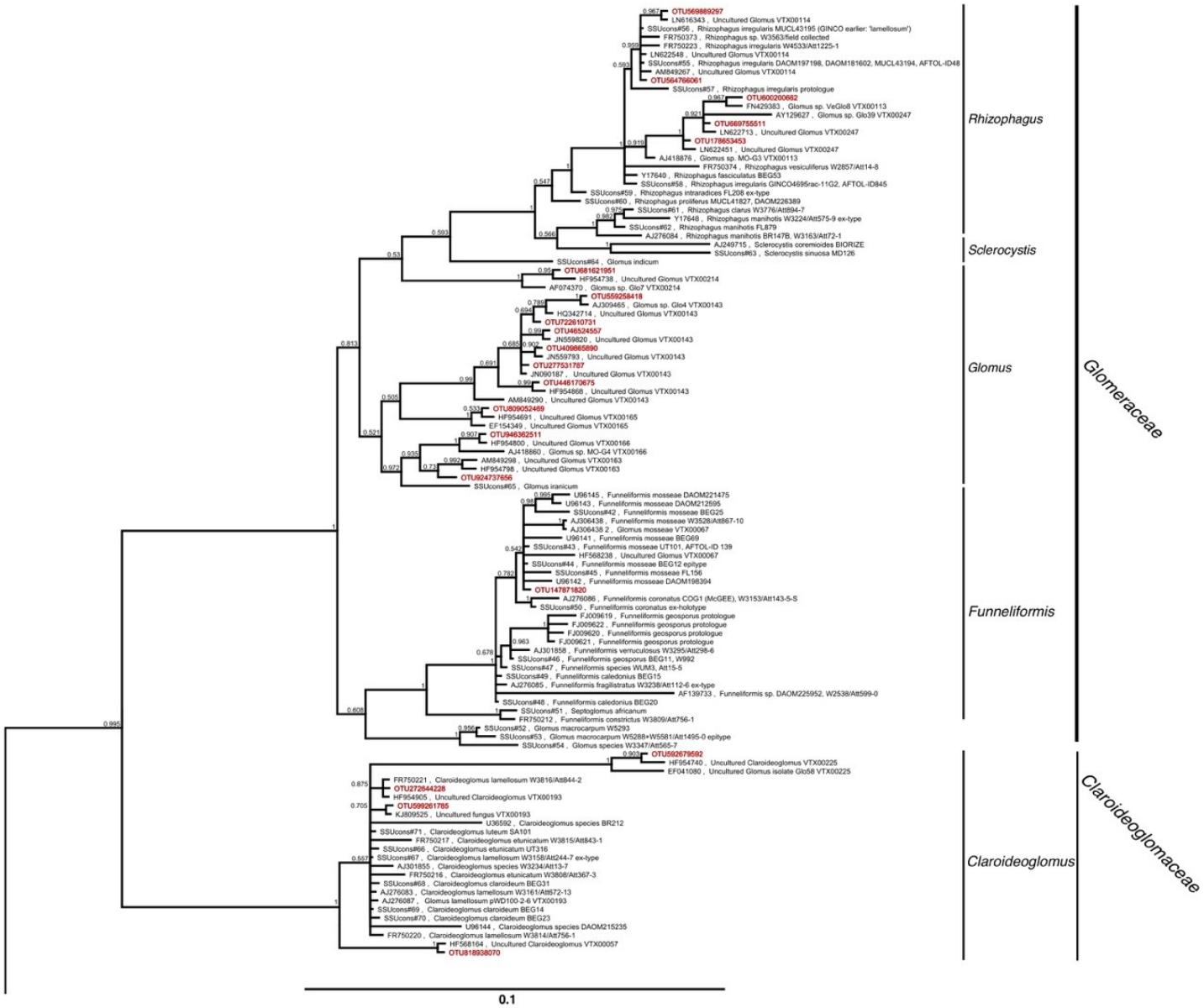


Figure S2. Plant sampling example from a *Persicaria lapathifolia*

Fig. S3


Rhizophagus
Sclerocystis
Glomus
Funneliformis
Claroideoglomus
Glomeraceae
Claroideoglomaceae
0.1

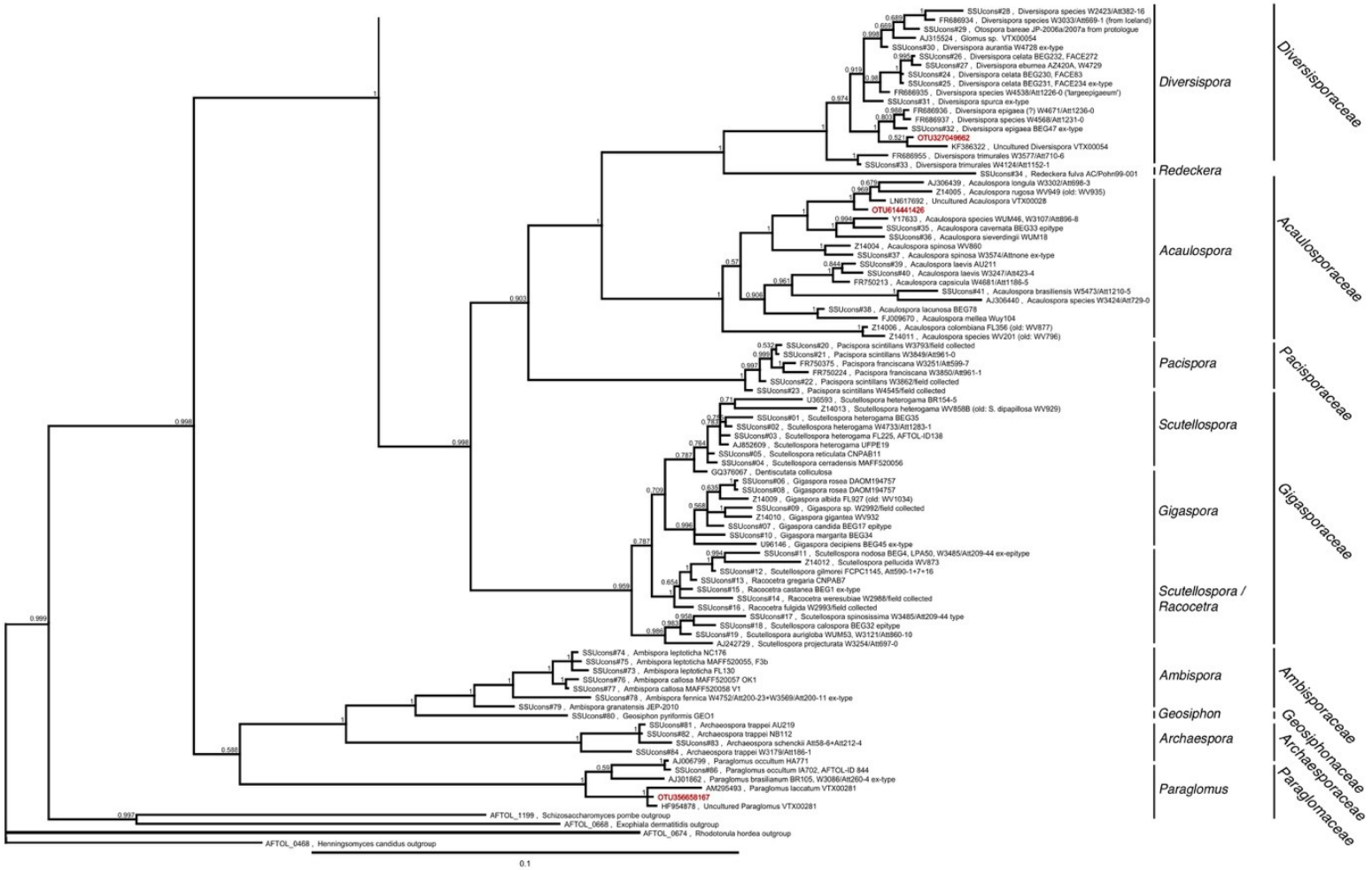


Figure S3. Bayesian phylogenetic tree based on nuclear small subunit (SSU) rDNA consensus sequences showing the distribution of the 23 OTUs recorded in this experiment (red labels) among the *Glomeromycota* tree.Sequence data were analyzed with the SSU sequences (black labels) from Krüger et al. (2012) and the closest matches recovered from MaarjAM database. The numbers on the nodes represent the Bayesian posterior probabilities. The scale represents the branch length corresponding to expected substitutions per site.

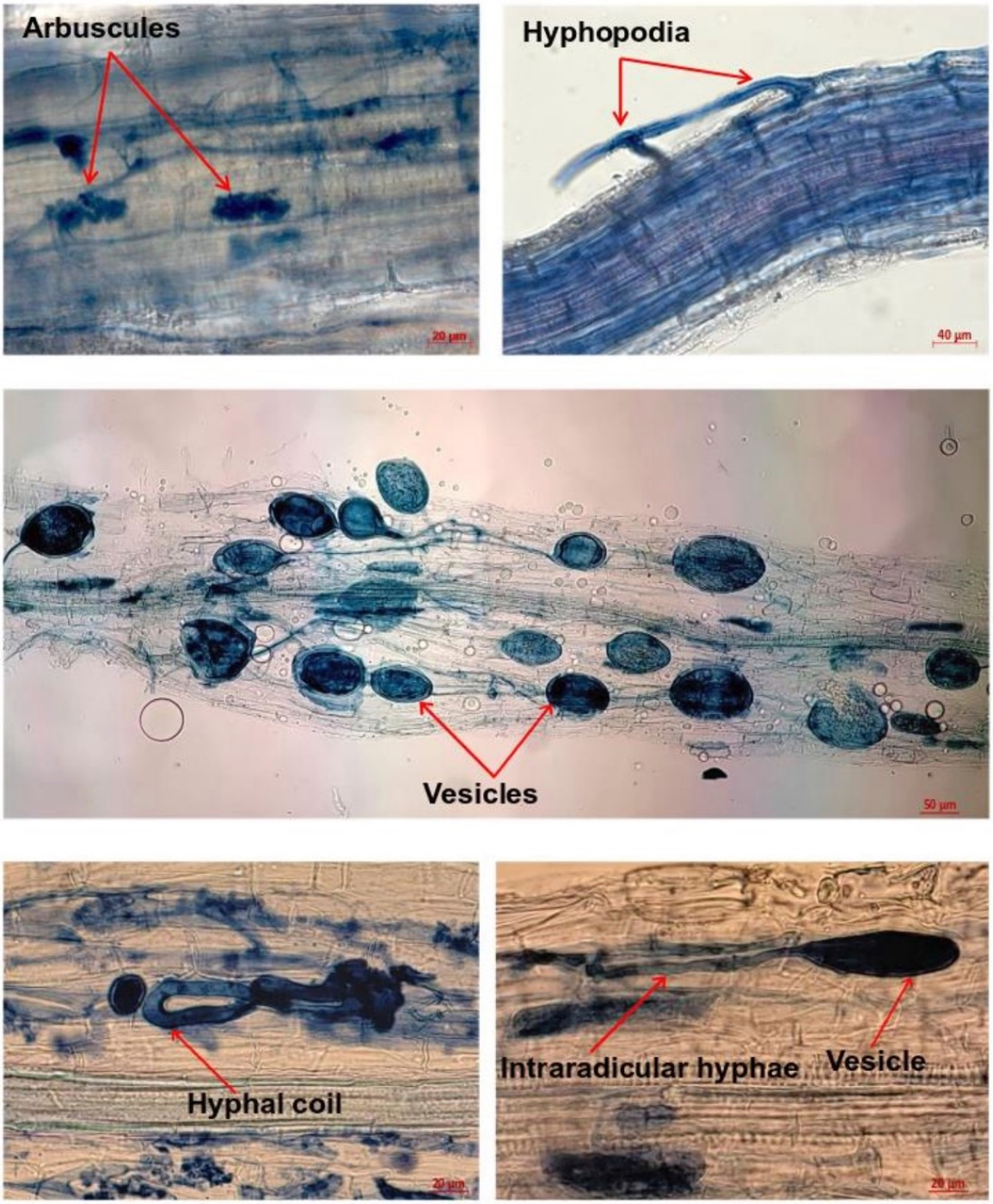


Figure S4. Mycorrhizal structures observed in the plant roots under light microscopy

Details of the sequence processing pipeline

In Mothur, the reads from each sample were assembled using the 'make.contigs' command. This generated a .fasta file containing the assembled reads. Primers were then removed with 'trim.seqs', after which he sequences were exported to QIIME. Special labels were then added for compatibility, with the 'add.qiime.labels.py' command. Using the Usearch7 sequence analysis tool QIIME implementation, the dataset was reduced to unique sequences using '-derep_fulllength', which we sorted by decreasing cluster size, and removed singletons with '-sortbysize'. The sequences were then clustered by Operational Taxonomic Unit (OTU) using a 97% identity threshold using the UPARSE method and further sequencing errors were removed with '– uchime_denovo'. Representative sequences of the OTUs were aligned with '-align.seqs' using the Silva eukaryote database (release 132), and filtered ('-filter.alignment' -e 0.10 and –g 0.80). We then produced an OTU table at 97% similarity and sequences that did not classify as *Glomeromycota* were removed with 'filter_taxa_from_otu_table.py'. Finally, we subsampled the sequences, so each sample had the same amount of sequences (150).

4 Chapitre 4 | Inoculation de clones de Saules avec des champignons mycorhiziens arbusculaires, ectomycorhiziens, et une combinaison des deux dans un ancien dépotoir industriel contaminé aux métaux traces

Dans le chapitre précédent, nous avons observé que la composition et structure des communautés de CMA associées aux plantes sont influencées par la présence de contaminants. De plus, les taux de colonisation racinaire sont plus élevés en sol contaminé, probablement dû au stress toxique que subissent les plantes. L'inoculation bactérienne a montré un effet perturbateur significatif des communautés de CMA, tout en causant une augmentation de la colonisation des racines en milieu contaminé. Cependant, la biomasse des plantes était significativement inférieure dans les traitements inoculés. Dans ce chapitre, nous inoculons des clones de saules dans un ancien dépotoir industriel avec des champignons symbiotiques capables de coloniser leurs racines, afin d'observer leur effet sur la survie, croissance, et extraction des métaux par les arbres.

Ectomycorrhizal fungal inoculation of *Sphaerosporella brunnea* significantly increased stem biomass of *Salix miyabeana* and decreased lead, tin, and zinc, soil concentrations during the phytoremediation of a trace elements-contaminated industrial landfill

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Abstract

Fast growing, high biomass willows (*Salix sp.*) have been extensively used for the phytoremediation of trace element-contaminated environments, as they have an extensive root system and they tolerate abiotic stressors such as drought and metal toxicity. Being dual mycorrhizal plants, they can engage single or simultaneous symbiotic associations with both arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (EM) fungi, which can improve overall plant health and growth. The aim of this study was to test the effect of these mycorrhizal fungi on the growth and trace element (TE) extraction potential of willows. A field experiment was carried out where we grew *Salix miyabeana* clone "SX67" on the site of a decommissioned industrial landfill and inoculated the shrubs with an AM fungus *Rhizophagus irregularis*, an EM fungus *Sphaerosporella brunnea*, or a mixture of both. After two growing seasons, the willows inoculated with the EM fungus *S. brunnea* produced significantly higher biomass. Ba, Cd and Zn were found to be phytoextrated to the aerial plant biomass, where Cd presented the highest bioconcentration factor values in all treatments. Additionally, the plots where the willows received the *S. brunnea* inoculation showed a significant decrease of Cu, Pb, and Sn soil concentrations. AM fungal inoculation and dual inoculation did not significantly influence biomass production and soil TE levels.

Keywords: Arbuscular mycorrhizal fungi; ectomycorrhizal fungi; trace elements; contamination; phytoremediation; willow

4.1 Introduction

With the surge of the human population and urban development in the past century, modern society has been generating an ever-increasing amount of industrial waste. Industrial residues and by-products are buried in the ground around urban centers, creating vast landfills, most often composed of non-organic waste such as glass, plastics, and trace elements (TE) such as lead. With extended weathering, TE are solubilized and leech into the environment, resulting in elevated levels in soil and water (Cunningham et al., 1995; Navarro et al., 2008). Because they are not degradable, they accumulate in the ecosystems and pose a serious environmental and human health risk (Giller et al., 1998; Garbisu and Alkorta, 2001). This makes the remediation of inorganic contaminated lands an important issue, in order to limit the spread and reduce the concentrations of these toxic elements.

Conventional methods for treating soils involve disruptive *in situ* and *ex situ* treatments such as excavation and landfill, soil washing, chemical stabilization, soil incineration, and acid leeching, among others (Yao et al., 2012). These practices present major limitations with respect to cost, labor, and ecological footprint, as they require heavy and expensive machinery as well as complex technical procedures (Ashraf et al., 2019). They also induce irreversible alterations to the soil's physicochemical properties and microbiology (Tchounwou et al., 2012). Therefore, more sustainable and ecologically friendly techniques are being sought after for the remediation of TE-contaminated soils.

One such method is phytoremediation, which is the use of plants and their associated soil microbiota to degrade, extract, and stabilize pollutants (Marchand et al., 2018; Ashraf et al., 2019; Robichaud et al., 2019a; Robichaud et al., 2019b). It is cost-efficient, requires much less labour, and will ultimately help the natural revegetation of polluted sites, although it is much slower than mechanical and chemical methods. Previous experiments have demonstrated the ability of plants to

assimilate and concentrate many TE such as zinc (Zn), copper (Cu), lead (Pb), cadmium (Cd), and nickel (Ni), reaching concentrations as high as 3000 mg/kg dry weight, which is a thousand-fold the normal values found in healthy plant tissue (Camelia et al., 2009; Sinhal et al., 2010; Laidlaw et al., 2012). Nevertheless, results vary greatly depending on the TE and plant species/cultivar (Laureysens et al., 2004; Laureysens et al., 2005; French et al., 2006; Tőzsér et al., 2017).

S*alix* is a genus comprising around 400 species of shrubs and deciduous trees that have a fast growth rate, produce high aerial biomass and deep roots, and are part of the pioneer vegetation that grows in disturbed and polluted environments (Argus, 1997; Pulford, 2003; Kuzovkina et al., 2007; Camelia et al., 2009; Hrynkiewicz and Baum, 2013). Being mycorrhizal shrubs and trees, they are able to form a symbiosis with fungi that colonize their roots (Van der Heijden, 2001). This association benefits the fungus with a direct access to the plant's carbohydrates; in return, the plant takes advantage of the mycelium's great capacity to extend beyond the root zone and absorb a greater amount of water and nutrients (Smith and Read, 2010). Moreover, arbuscular mycorrhizal (AM) fungi can improve plant health in stressful conditions such as drought (Wu et al., 2008) and trace element-polluted land (Glassman and Casper, 2012; Hassan et al., 2013).

Among mycorrhizal fungi, arbuscular (AM) and ectomycorrhizal (EM) fungi are the most commonly encountered. AM fungi are ubiquitous soil microorganisms that engage in an obligatory mutualistic association with the roots of most terrestrial plant species and play an important part in their biological functioning (Smith and Read, 2010). EM fungi, on the other hand, associate with only 10% of plant families and unlike AM fungi, their mycelium does not colonize the cortical cells, but intercalates between them. These mycorrhizal associations can play an important role in the process of phytoremediation (Khan, 2006). It has been hypothesized that since they increase the plants growth, resistance to abiotic stress and survival, they can improve the plants effectiveness in extracting TEs through a higher uptake and biomass production. In addition to these potential

benefits, the AM fungal's glycoprotein "glomalin", binds to a variety of metals and sequesters them, thus protecting the fungus and the plant from the toxic effects and immobilizing the contaminants (Rillig et al., 2002; Hrynkiewicz and Baum, 2013). Many studies have been conducted on the effects of AM fungal inoculation on phytoremediation with varying results that mostly depend on the plant/fungus combination. However, there is a paucity of information regarding the use of EM fungi in phytoremediation, and even less is known about the effect of a dual AM/EM fungal inoculation of capable trees, like *Salix* (Turnau et al., 2006; Mrnka et al., 2012).

In this work, we investigated the synergistic effects of the EM (*Sphaerosporella brunnea*) and AM (*Rhizophagus irregularis*) fungi in the enhancement of willow growth, soil TE extraction and uptake by plants in a contaminated industrial brownfield of the Montreal region (St-Hubert, QC, Canada). We aimed to find out whether the dual inoculation would improve or impede the willows aerial biomass production and TE accumulation in comparison to single or no inoculation

4.2 Material and method

4.2.1 The experimental site

The experiment took place is an industrial landfill that was decommissioned in the 1950's, located in the borough of St-Hubert, QC, Canada (45°30'05.5"N, 73°27'08.7"W). The average annual temperature is 6.2 ºC with 1010 mm of yearly precipitation. At the surface, the site is composed of filling material consisting largely of incineration residues, glass and metal debris. Underneath is a silty clay layer, followed by till and bedrock. Exploratory sampling of the upper layer (30cm) found to concentrations of barium (Ba), Cd, Cu, tin (Sn), Ni, Pb, and Zn which were above the set values by the local authorities for residential and industrial development; the "C" criterion of the *Ministère du développement durable, Environnement et lute contre les changements climatiques du Québec (MELCC)* (Table 1). Notably, Cu, Pb, and Zn were detected at very high concentrations, reaching 680 mg/kg, 2400 mg/kg, and 5400 mg/kg respectively (Table 1). The top layer of the landfill will be

referred to from here on as “soil”. At the beginning of the project the site was totally covered by *Phragmites autralis*, which we mowed before setting up the experiment. We also removed the shoot debris and covered the ground with a block geotextile membrane to control weed growth.

Table 1. Trace element (TE) concentrations and pH from the 2015 exploratory soil sampling of the Longueuil, Québec industrial landfill.

		Sampling Plot				
Metal	**Unit**	**1**	**2**	**3**	**4**	**5**
Silver (Ag)	mg/kg	<0.5	8.5	3.1	0.6	2.3
Arsenic (As)	mg/kg	8	17	27	13	17
Barium (Ba)	mg/kg	190	630	530	830	540
Cadmium (Cd)	mg/kg	1.6	37	12	4	7.4
Chrome (Cr)	mg/kg	65	74	88	71	93
Cobalt (Co)	mg/kg	18	13	21	18	16
Copper (Cu)	mg/kg	110	550	680	420	600
Tin (Sn)	mg/kg	56	310	730	88	440
Manganese (Mn)	mg/kg	530	670	1200	520	790
Molybdenum (Mo)	mg/kg	2	8	12	3	7
Nickel (Ni)	mg/kg	61	65	150	88	99
Lead (Pb)	mg/kg	150	1100	2400	430	1800
Zinc (Zn)	mg/kg	380	4400	5400	2000	2300
pH		7.18	7.09	7.40	7.12	7.09

4.2.2 Experimental design and biological material

Five experimental blocks were setup in the field site. Each block consisted of five plots measuring 5 m by 3 m for a total block size of 15 m by 3 m. Plots were chosen randomly to receive different treatments. One plot in each block was left unplanted as a total control (CN). The remaining four plots were each planted with willow (*Salix miyabeana* clone "SX67") and received one of the following treatments: **1)** AM fungal inoculation (AM), **2)** EM fungal inoculation (EM), **3)** AM + EM fungal inoculation (XX), **4)** no inoculation (SX). In June of 2015 we used 20 cm cuttings that were gently hammered into the ground using a rubber mallet to a depth of approximately 10 cm. When applicable, a pilot hole was prepared in which we added 15 mL of each inoculant before inserting the

cuttings. During the summer of the first year of growing season, willows were fertilized by hen manure ActiSol 5-3-2 (Notre-Dame-du-Bon-Conseil, QC, Canada), at the dose of 26 Kg per 100 m^2. This was repeated during July of the second year of growing season. Willow plantation was maintained in the two first years (2015 and 2016) where two weedings were done to control *Phragmites autralis*. The plantation was then left without any intervention nor fertilization until 2019, where a final sampling compaign was done at November 2019.

4.2.3 Rhizophagus irregularis

R. irregularis isolate DAOM-242422 (Varennes, QC, Canada) propagules were produced by cultivating AM fungal-infected transformed chicory roots on Minimal medium plates (Bécard and Fortin, 1988). After five weeks of growth, the solid medium was solubilized using Citrate buffer solution (Hijri and Sanders, 2004), and the obtained mycelium/spores/infected roots mix was blended in an Eberbach blender twice for 3 seconds. Finally, the propagule blend was counted using a stereomicroscope and diluted in an isotonic NaCl (0.9%) solution to a concentration of 400 spores and 800 infected roots fragments per 15 mL. Field inoculation was done by pouring 15 mL of the inoculum in each hole where the willows cuttings were to be planted.

4.2.4 Sphaerosporella brunnea

A pure culture of *S. brunnea* strain Sb_GMNB300 (NRRL 66913, Perugia, Italy) (Sánchez et al., 2014) was kindly provided by Dr. Sánchez and was cultured in malt extract broth under constant shaking for 10 days at 24° C. The fungal mycelial biomass was then washed with an isotonic sterile NaCl (0.9%) solution and blended in an Eberbach blender twice for two pulses of three seconds each. The propagules were then counted using a haemocytometer and diluted to 100000 propagules per 15 mL. Field inoculation was performed by adding 15ml of the propagule suspension in each hole where the willow clones were to be planted.

4.2.5 Sampling and plant measures

After two growing seasons (June 2015-October 2016), we randomly chose five plants in each of the plots for dry biomass production analysis. The selected willows were excavated from the ground and stored in individual bags for subsequent drying at 60°C for 48 hours. Due to the compacted nature of the field top layer it was not possible to consistently recover roots from the willows. For this reason, we only used the aerial biomass.

4.2.6 TE concentrations

The soil was sampled in October of 2016 and November of 2019 from all plots for analysis of TEs Two composite samples were prepared from each plot by combining three samples of soil for each. In 2019, five willows from each plot and were randomly chosen and all stems above the last node were collected. Each treatment plot was represented by a pool of the five sampled willows for a total of 25 tissue samples (1 composite of 5 willows per plot x 4 willow treatments x 5 experimental blocks). Willow shoots were then dried in the oven at 60°C for 72 hrs, after which it was ground then screened at 2 mm size. Chemical analysis was done using a commercial service (AGAT laboratories, St-Laurent, QC, Canada). A biological concentration factor (BCF) was also calculated for each of the tested elements. BCF is the ratio of the concentration of an element in an organism to the concentration of the element in the surrounding environment (Arnot and Gobas, 2006). In this case, it is the ratio of TE concentration in plant tissues to the TE concentration (HNO_3 extractable) in the ground.

4.2.7 Statistical analyses

Plant biomass, TE concentrations and BCF values were analyzed using ANOVA with Tukey's HSD post hoc comparisons with an alpha of 0.05 in JMP V.11 statistical software (SAS Institute Inc.

Cary, NC, USA). Distribution and normality were verified and transformations were performed when necessary. The block effect was tested using the Blocking function as a random factor.

4.3 Results and Discussion

After two growing seasons, all treatments showed high plant survival rates of *Salix myabeana* clone SX64, ranging from 94.4% ± 5.5 to 97.2% ± 3, with no significant statistical difference between treatments. Survival rate of *Salix* spp. clones in phytoremediation applications depends on many parameters such as pollution concentrations, climate, contamination depth, etc. For example, Guidi Nissim et al. (2012) tested two *Salix* clones "SX64" and "SX67" to remedy a deep, polluted plume contaminated by petroleum hydrocarbons and obtained a survival rate as low as 24.4% for *S. miyabeana* clone "SX67", which resulted in the failure of that phytoremediation assay (Guidi Nissim et al., 2012). The same clone "SX67" of *S. miyabeana* used in our study showed an excellent survival rate, in line with previous studies of phytoremediation trials in the field (Courchesne et al., 2017). Overall, we found that *S. miyabeana* clone "SX67" is a suitable candidate for revegetation projects of TE-contaminated landfills, as indicated by the high survival rates observed in all treatments. This is important given that the top layer of the landfill contains little to no soil and consists of mainly rubble and debris as indicated before.

Willows inoculated with the EM fungi *S. brunnea* produced significantly higher shoot biomass than all other treatments (*P=0.0191*) by the end of the second growing season, with an average of 72.6 g ± 8. *S. miyabeana* "SX67" shoot dry weight ranged between 14 g and 153 g, with the averages for the other treatments as follows: SX, 44.4g ± 18; XX, 53.2g ± 18.4; AM, 53.1g ± 7.6; EM, 72.6g ± 8 (Figure 1). Average dry shoot biomass varied considerably between blocks (*P=0.0002*), therefore we tested the interaction between the treatment and the block number using the “blocking” feature for ANOVA in JMP and the results were negative (no interaction), meaning the treatment effect was not

different between blocks. Recently, an extensive review on dual mycorrhizal plants by Teste et al. (2019) showed that the few existing studies on the subject indicate that the benefits/disadvantages of this tripartite symbiosis (including plant biomass production) vary depending on the contexts (Teste et al., 2019), including the plant and mycorrhizal species involved, as well as the life stage of the plant. On the other hand, we found that inoculation with the EM fungus *S. brunnea* significantly increased the dry stem biomass of the planted willow clones at the end of two growing seasons. EM fungi have been shown to increase plant biomass in metal-contaminated environments: Hrynkiewicz and Baum (2013) found that inoculation of *Salix dasyclados* clones with the EM fungus *Amanita muscaria* grown in a metal contaminated field increased the plant stem size and biomass (Hrynkiewicz and Baum, 2013). Similarly, Zong et al. (2015) saw increased biomass of pine and oak trees grown on copper mine tailings and inoculated with a consortium of EM fungi. Moreover, a pot experiment by Ma et al. (2014) showed that the inoculation of *Populus x canescens* clones with the ectomycorrhizal fungus *Paxillus involutus* increased growth and biomass production in comparison to non-inoculated plants. Our results seem to be aligned with these previous studies where EM fungi can increase plant biomass production in metal contaminated conditions.

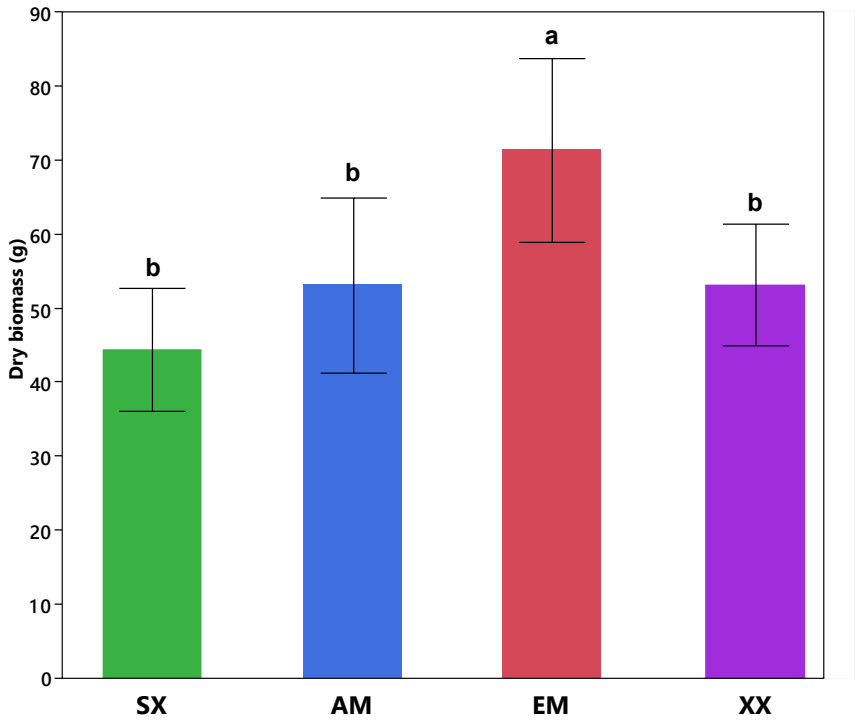

Figure 1. Mean dry aerial biomass production of *Salix miyabeana* "SX67" inoculated with *R. irregularis* (AM), *S. brunnea* (EM), both fungi (XX), or non-inoculated (SX), illustrates that only the EM treatment significantly increased biomass. Measures were taken from five plants in each plot, at the end of the second growing season (October 2016) after drying at 60 degrees C. Column values were obtained by calculating the average of five samples per treatment in each of the five blocks, then obtaining the mean value across all blocks for each treatment. Error bars represent standard error. Treatments not sharing a letter are significantly different.

Overall, *S. miyabeana* "SX67" inoculated with mycorrhizal fungi resulted in a decreasing trend in average soil concentrations for most metals between the 2016 and 2019 (Table 2). However, EM inoculation exhibited a significant decrease in the concentrations of Cu (P=0.003), Sn (P=0.045) and Pb (P=0.046) (Figure 2 and Table 3) in comparison with other treatments. There was no significant difference between the reduction of TEs in the other treatments (SX, AM, XX) and the non-planted control (CN). As for the TE content in the plant shoots, only Ba, Cd and Zn were detected in the tissue. Their average concentrations by treatment ranged between 26.2 mg/kg ± 13.3 to 31.8 mg ± 9.7 for Ba, from 8.7 mg/kg ± 3.3 to 10.7 mg/kg ± 4.1 for Cd, and from 380 mg/kg ± 92 to 474 mg/kg ± 81 for Zn (Figure 3). There was no significant difference between treatments in the mean shoot

concentrations and only the BCF of Cd was significantly higher among all treatments (*P<0.0001*) with values between 2.11 ± 1.1 to 2.25 ± 1.8. Zinc BCF values were between 0.31 ± 0.23 and 0.54 ± 0.36 and Ba had the lowest values that ranged from 0.10 ± 0.04 to 0.15 ± 0.09 (Figure 4). BCF values did not significantly differ between treatments for all metals.

Table 2. Mean TE concentrations and standard error of the soil per treatment as measured in 2016 and 2019 at the Longueuil, QC, and industrial landfill site.

Barium	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	262.7	38.33	337.5	47.94	339.8	52.62	464.8	64.19	362.9	39.5
2019	247.9	35.27	298.1	38.43	247.5	39.8	264.3 *	22.84	302.3	38.85
Cadmium	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	5.64	1.03	7.6	1.91	7.16	1.28	6.99	1.02	6.35	1.23
2019	5.4	1.08	7.17	1.71	7.55	1.58	4.83 *	0.72	4.89	0.75
Copper	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	375.4	74.65	377.8	88.09	948.8	295.88	1261.6	476.76	526.7	94.17
2019	601.4	265.34	324.2	68.66	581.6	105.64	351.4	65.09	390.3 *	61.04
Lead	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	587.2	128.68	781.5	208.51	1010.4	178.19	1334	258.36	1672.7	931.39
2019	557	123.23	713.7	194.74	792	175.93	594.3 *	156.94	622.7	124.49
Nickel	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	87.1	9.29	107.2	12.74	114.1	13.45	118.2	12.89	93.2	11.67
2019	88.6	13.04	68.5 *	6.99	95.9	16.47	79.5 *	9.98	62.6 *	4.42
Tin	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	225.5	60.46	337.6	111.24	559.6	117.66	542.8	101.18	497.5	155.08
2019	244.6	62.51	247	72.06	410.7	123.79	265.9 *	88.58	218.5	53.74
Zinc	**CN**		**SX**		**AM**		**EM**		**XX**	
Year	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**	**Mean**	**Std. Err.**
2016	1753.9	300.35	1697.7	324.05	1992.7	325.54	2063.6	249.48	2001.3	355.89
2019	2070.1	393.19	1346 *	280.25	1656.3	259.44	1363.6 *	236.71	1455.2	236.43

n = 5 for each year and treatment combination. For each treatment, asterisks (*) show that the mean TE concentration was significantly different from the previous measurement (2016 vs. 2019). CN = Non-planted control; SX = Willow non-inoculated; AM = Willow + AMF inoculation; EM = Willow + ECM inoculation; XX = Willow + AMF + ECM inoculation; Std. Err. = Standard error.

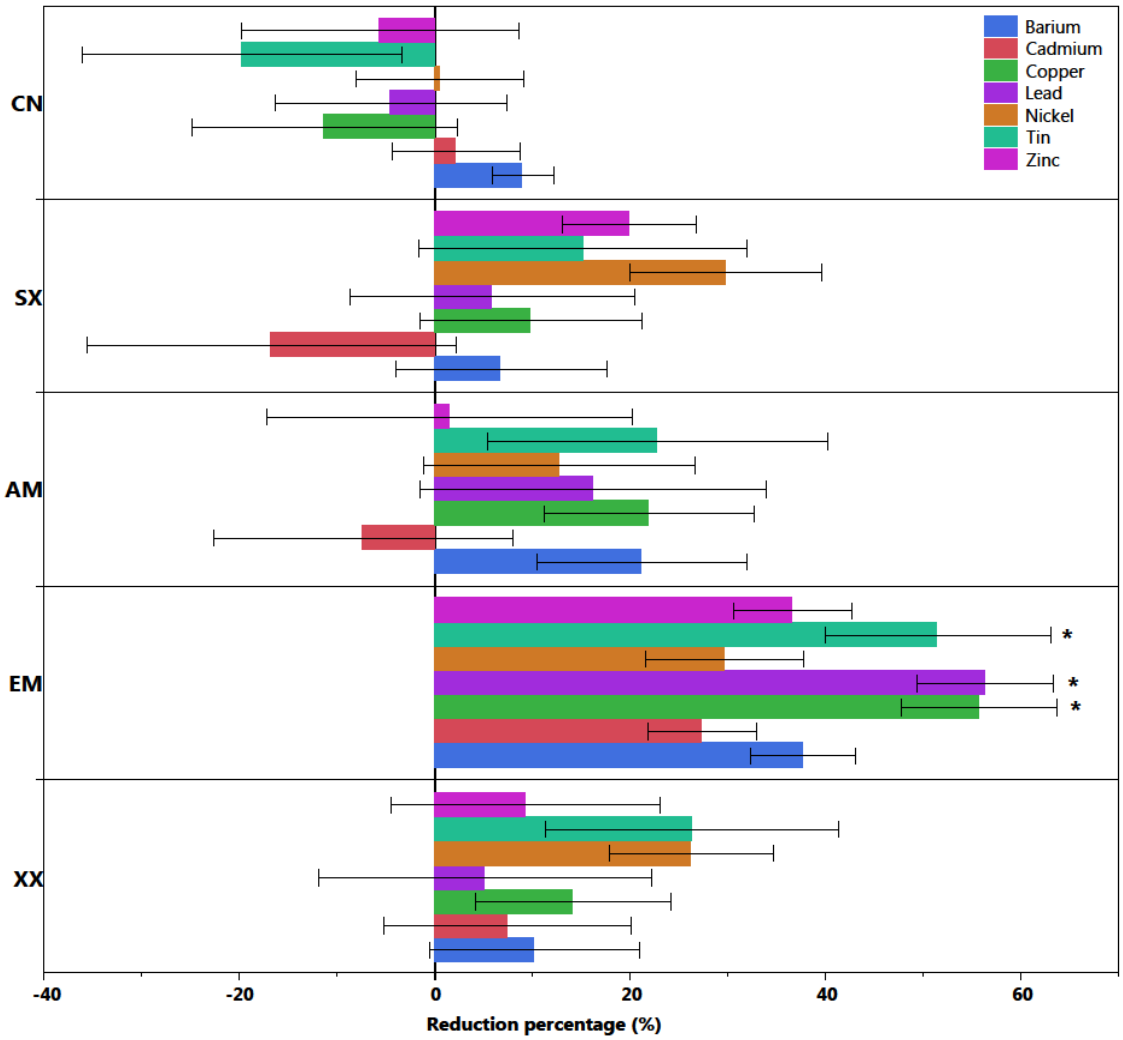

Figure 2. Average change in soil TE between 2016 and 2019 in five treatment plots of *Salix miyabeana* "SX67" (N = 5 for each treatment), shows that only the EM treatment had significant decreases (Sn, Pb, and Cu). Negative values indicate an increase in concentration. Error bars represent standard error. *SX = Willow non-inoculated; AM = Willow + AMF inoculation; EM = Willow + ECM inoculation; XX = Willow + AMF + ECM inoculation; CN = Non-planted control*. Asterisks (*) indicate that the percent reduction of TE concentration is significantly different from other treatments

Table 3. ANOVA analysis of the aerial dry biomass of *S. miyabeana* "SX67" and the TE percentage decrease/increase in the soil for each treatment.

Metal Decrease/Increase % and Plant Dry Biomass Tukey's HSD Comparisons					
	CN	**SX**	**AM**	**EM**	**XX**
Ba	N.S.	N.S.	N.S.	N.S.	N.S.
Cd	N.S.	N.S.	N.S.	N.S.	N.S.
Cu	B	B	AB	A	AB
Pb	B	AB	AB	A	AB
Ni	N.S.	N.S.	N.S.	N.S.	N.S.
Sn	B	AB	AB	A	AB
Zn	N.S.	N.S.	N.S.	N.S.	N.S.
BIOMASS		B	B	A	B

Within each row, treatments not sharing a letter are significantly ($p < 0.05$) different. N.S. = Not significant. CN = Non-planted control; SX = Willow non-inoculated; AM = Willow + AMF inoculation; EM = Willow + ECM inoculation; XX = Willow + AMF + ECM inoculation.

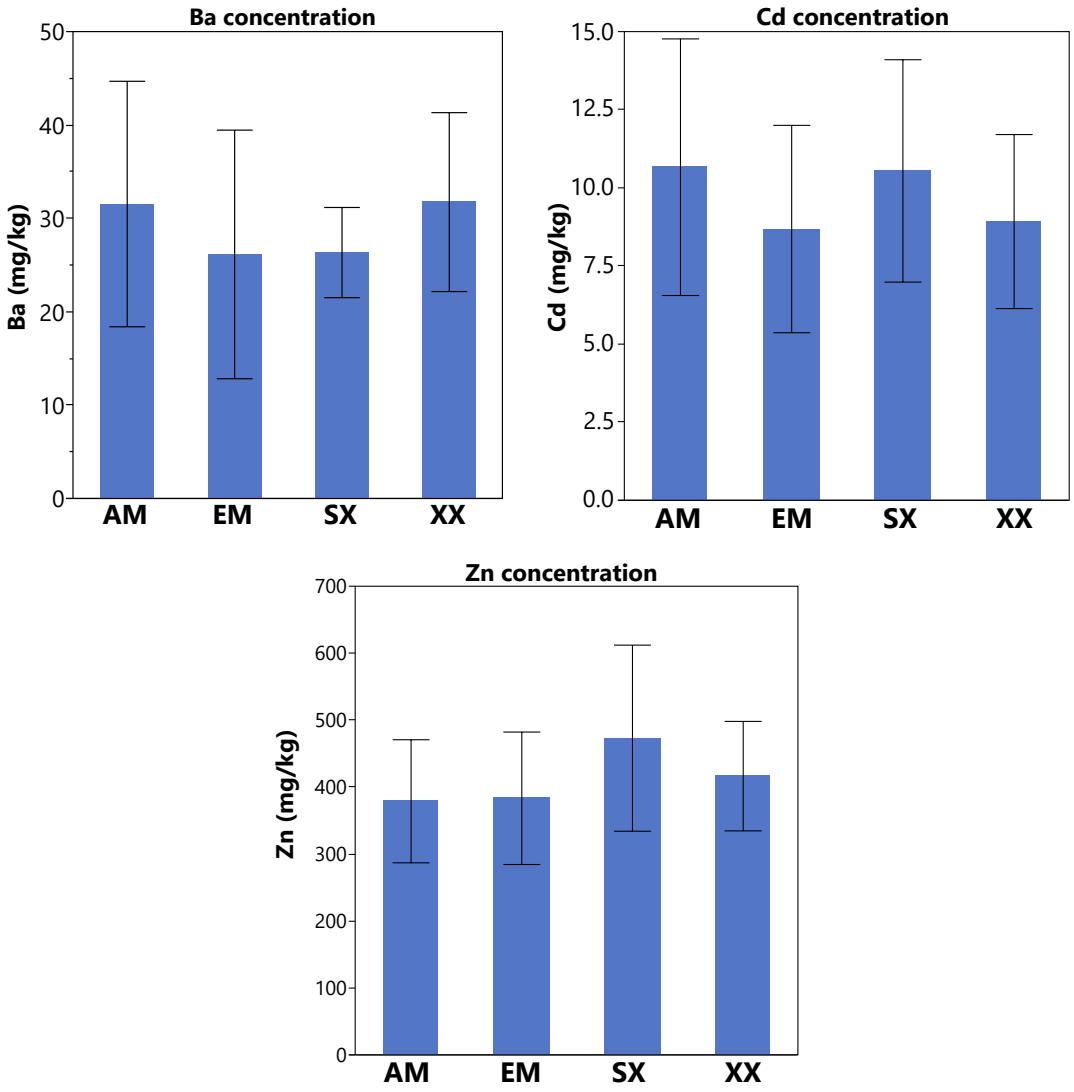

Figure 3. Ba, Cd, and Zn, were the only TE detected in the stem tissue of the *Salix miyabeana* "SX67" plots (N = 5 for each treatment), though there were no significant differences in the average concentrations between treatments. Error bars represent standard deviation. *SX = Willow non-inoculated; AM = Willow + AMF inoculation; EM = Willow + ECM inoculation; XX = Willow + AMF + ECM inoculation; CN = Non-planted control*.

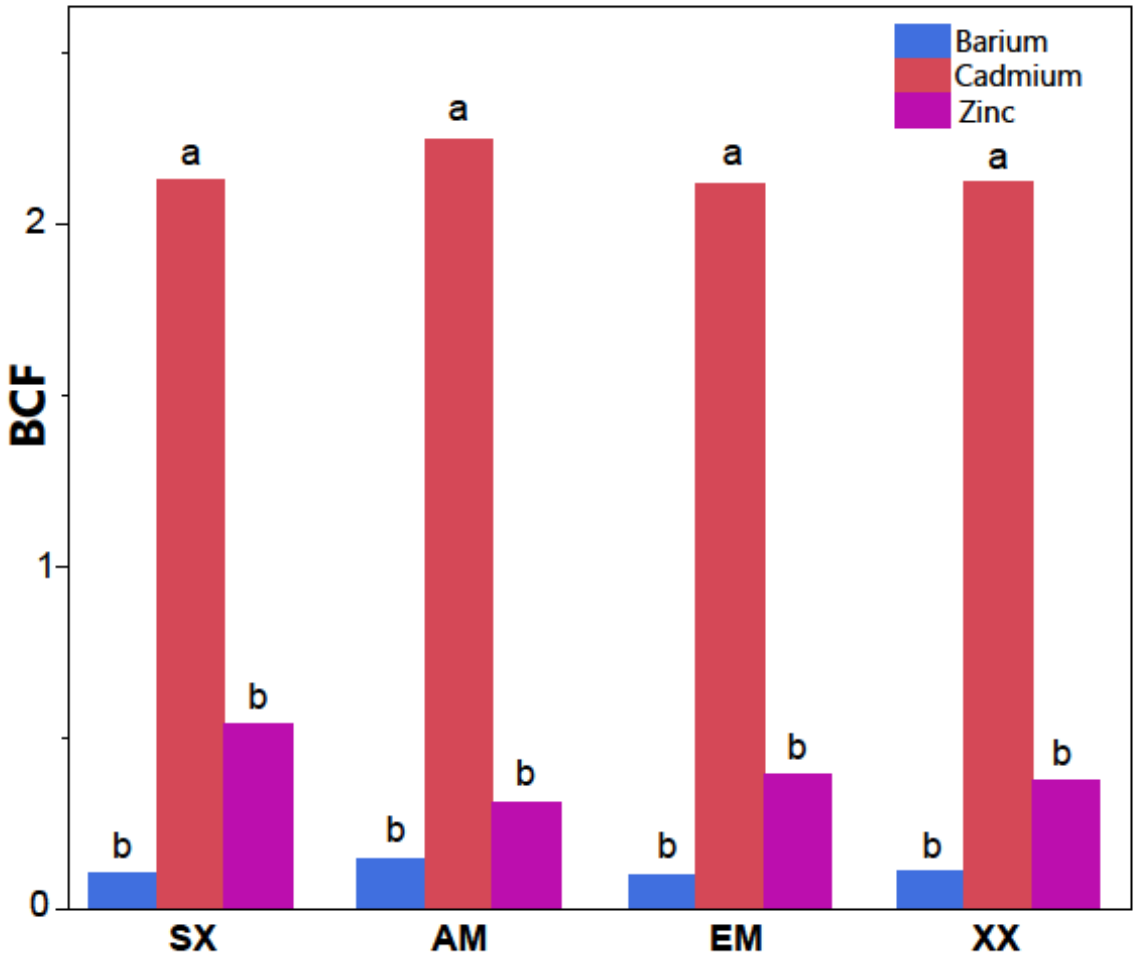

Figure 4. Cd had a significantly higher mean BCF value (*P<0.0001*) in each treatment of inoculated *Salix miyabeana* "SX67" (N = 5) than Ba, or Zn. BCF is calculated here as the ratio of TE concentration in stem tissues to the TE concentration (HNO_3 extractable) in the ground. Columns not sharing a letter are significantly different. *AM = AMF inoculation; EM = ECM inoculation; XX = AMF + ECM inoculation; SX = Non-inoculated S. miyabeana.*

Research has shown that willows are more efficient at accumulating Cd and Zn in their aerial parts relatively to other TEs that are preferentially accumulated in the roots such as Pb and Cu (Vandecasteele et al., 2005; Wang et al., 2014; Courchesne et al., 2017; Tőzsér et al., 2017; Desjardins et al., 2018). Moreover, edaphic factors, such as humic/fulvic acid content, and pH, increase or decrease the TEs bioavailability which influences uptake by plants (Violante et al., 2010; Alloway, 2013). Here, Ba concentrations were within the expected range for plants (4-50 mg/kg)

(Chaudhry et al., 1977), while Cd and Zn concentrations met and even surpassed the phytotoxic ranges: 5-30 mg/kg for Cd and 100-400 mg/kg for Zn (Kabata-Pendias, 2000). The dual inoculation did not confer any advantage in terms of shoot accumulation. However, previous studies have reported enhanced phytoextraction of TEs, induced by inoculation with either AM or EM fungi: In a pot experiment using *Populus canadensis* and *Salix viminalis* cultivars, Sell et al. (2005) inoculated the soil with three EM fungi *Hebeloma crustuliniforme, Paxillus involutus* and *Pisolithus tinctoriu*s and found that the association of *P. tinctorius* and *P. canadensis* significantly enhanced the extraction of Cd, with concentrations in the shoots reaching 2.73mg/kg (Sell et al., 2005). Another pot experiment using *populus x canescens* cultivars showed that inoculation of the soil with the EM fungus *Paxillus involutus* improved the uptake and tolerance of Cd (Ma et al., 2014). AM fungi have also been shown to increase extraction of metals, such as lead as shown by Yang et al. (2016) in a field experiment in Pb contaminated soil. They inoculated legume trees with the AM fungus *Rhizophagus intraradices* and found increased extraction of Pb relative to non-inoculated trees (Yang et al., 2016).

In the context of our experiment, inoculation strategies did not significantly increase TE extraction to the aerial parts by the willows. This could be in part attributed to the nature of the environment where the experiment was performed: the thin top layer of the decommissioned industrial landfill consisted of rubble and miscellaneous other materials, such as glass shards and incineration residues, making it a hostile environment that could affect the EM-plant symbioses. Among the TEs tested in this experiment, Cd showed interesting BCF values, reaching more than 3 in certain samples regardless of inoculation. Therefore, a high BCF for Cd and the increased biomass production of the EM inoculated willows suggests that the use of *S. miyabeana* "SX67" inoculated with *S. brunnea* is a useful approach for the phytoextraction of Cd. Moreover, although the BCF value of zinc showed an average under 1, the actual Zn concentration in the shoots was relatively

high (over 400 mg/kg), indicating that this willow cultivar can also be used for Zn contaminated environments.

In conclusion, we found that the *S. miyabeana* "SX67" clone is a potential candidate for the revegetation of industrial landfills containing high concentrations of TEs, as shown by the high survival rates of the clones. Moreover, they showed an interesting potential for the phytoextraction of Cd and Zn, and possibly for the immobilization of other TEs in the roots. Fungal inoculations did not have a significant impact on the extraction of TEs nevertheless, the EM treated plants showed a significant increase in biomass production, which can lead to an increase in the total amount of extracted TEs. Whether this effect was due to the direct influence of the EM inoculation on the willows through root colonization, or the result of a change in the local microbial assemblages in reaction to the inoculation, which in turn stimulated plant growth remains to be seen. As mentioned before, it was not possible to assess root colonization rates due to the nature of the terrain which prevented the recovery of enough fine roots. The persistence of laboratory cultivated microbial inocula in field conditions is one of the main concerns in such applications, as a host of factors modulate and influence soil and root-associated microbiota

Future studies should be directed at finding out whether inoculation of the "SX67" cultivar with mycorrhizal fungi in contaminated landfills can improve the stabilization of TEs in the roots. In addition, the fate of the indigenous soil microbes and inoculum organisms in the should be monitored through the evaluation of root mycorrhizal colonization, and molecular analyses of the rhizospheric microbiota using universal and specific markers, as they evolve in each treatment throughout multiple growing seasons. This will shed a light on the mycorrhizal succession dynamic and its relationship with plant growth and phytoremediation performance.

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Conflicts of Interest: The authors declare no conflict of interest.

5 Chapitre 5 | Conclusion

5.1 Rappel du contexte de l'étude

La manipulation du microbiote des racines des plantes a le potentiel de réduire le stress des plantes et de favoriser leur croissance et leur production dans des conditions difficiles. La composition et l'activité des microorganismes racinaires peuvent être bénéfiques ou néfastes pour la santé des plantes. La modification de cet équilibre pourrait alors affecter fortement la productivité des plantes dans les zones anthropisées. L'objectif principal des travaux effectués était de comprendre les facteurs qui peuvent déterminer et influencer la structure du microbiome de la rhizosphère de plantes poussant en milieux stressés, ainsi que tester l'effet d'une inoculation avec des champignons mycorhiziens sur la performance végétale en milieu contaminé. Pour ce faire, trois études ont été réalisées, soit deux expériences en serre dans des mésocosmes, et une troisième *in situ*.

5.2 Récapitulatif du Chapitre 2

L'objectif premier était de tester l'effet d'une inoculation des racines de plantes poussant dans un substrat contaminé aux hydrocarbures pétroliers, avec un consortium bactérien sur la structure du microbiome de la rhizosphère. Notre hypothèse stipulait que l'inoculation modifiera la structure du microbiome rhizosphérique, augmentera significativement la biomasse aérienne produite par les plantes, et mènera à une diminution significative des niveaux d'hydrocarbures. Les résultats ont

montré que plantes ont considérablement influencé la structure des communautés microbiennes dans les sédiments contaminés par les hydrocarbures pétroliers. Plusieurs séquences d'ADN représentant des membres de genres bactériens connus pour leur potentiel de favoriser la croissance des plantes étaient présents dans les sédiments. De plus, l'identité des espèces végétales a eu un impact significatif sur la structure du microbiome de la rhizosphère, soulignant l'importance de la sélection des plantes dans les stratégies de phytoremédiation pour potentiellement recruter des microbes spécifiques. En revanche, si l'inoculation a influencé la structure des communautés fongiques et bactériennes dans la rhizosphère, son effet était beaucoup plus faible que la présence de plantes et leur identité qui contredit notre hypothèse. De plus, nous avons observé que l'inoculation était significativement associée à une augmentation de la biomasse aérienne des plantes, mais ne mène pas nécessairement à une diminution plus élevée des concentrations d'hydrocarbures. Il existe un besoin d'études in situ à long terme impliquant l'utilisation d'espèces végétales multiples autochtones à la place de la monoculture d'espèces introduites dont l'efficacité de survie et de remédiation diminue dans les environnements fortement contaminés (Pulford et Watson, 2003). Cependant, toute stratégie d'assainissement des sols doit également prendre en compte à la fois le potentiel de dégradation des micro-organismes sélectionnés et l'interaction avec les plantes hôtes et le microbiome local.

5.3 Récapitulatif du Chapitre 3

Dans cette étude, nous avons testé si des inoculations répétées avec un consortium de Protéobactéries influencerait la productivité des plantes et les assemblages des champignons mycorhiziens arbusculaires (CMA) associés aux racines et à la rhizosphère de quatre espèces végétales, poussant soit dans un sol naturel non contaminé, ou dans des sédiments contaminés par des hydrocarbures pétroliers. Nos résultats ont montré que l'inoculation provoquait un changement

significatif dans les communautés CMA cependant, la contamination du substrat avait une influence beaucoup plus forte sur leur structure, suivie par le biotope et l'identité des plantes dans une moindre mesure. De plus, l'inoculation augmentait considérablement la production de biomasse végétale et était associée à une diminution de la dissipation des hydrocarbures pétroliers dans le sol contaminé. Le résultat de cette étude fournit des connaissances sur les facteurs influençant la diversité et la structure communautaire des CMA associée aux plantes indigènes à la suite de l'inoculation répétée avec consortium bactérien. Il met en évidence la prédominance des propriétés chimiques du sol, telles que la présence d'hydrocarbures pétroliers, sur les facteurs et intrants biotiques, tels que les espèces végétales et les inoculations microbiennes, pour déterminer les communautés des CMA associés aux plantes. Ceci mène à conclure que la première étape pour l'implantation de plantes et la gestion de leurs CMA associés serait d'analyser la nature du sol (pH, conductivité électrique, potentiel hydrique, composés organiques, pollution, etc...). Cette analyse mènera à un choix convenable de type de plantes adaptées ou tolérantes aux conditions données. Ensuite, l'identité de l'espèce de plante peut être déterminée puisqu'elle influence aussi la structure des CMA dans les racines. Des recherches visant le complexe réseau d'interactions entre les microbes du sol sont nécessaires, afin de comprendre leur rôle dans la détermination des communautés de CMA. Ces connaissances seront vitales pour l'élaboration d'une stratégie réussite pour manipuler ou gérer les CMA pour mieux bénéficier à leurs plantes associées.

5.4 Récapitulatif du Chapitre 4

Dans cette étude, nous avons testé l'effet d'inoculations fongiques avec des champignons éctomycorhiziens et endomycorhiziens (CMA), ainsi qu'un mélange des deux sur la survie, production de biomasse, ainsi que d'extraction et concentration des métaux d'un cultivar de saule. Les inoculations fongiques n'ont pas eu d'impact significatif sur l'extraction des métaux traces.

Néanmoins, les plantes traitées par le champignon ectomycorhize ont montré une augmentation significative de la production de biomasse, ce qui ultimement peut conduire à une augmentation de la quantité totale de métaux extraits. Reste à savoir si cet effet était dû à l'influence directe de l'inoculation EM sur les saules par la colonisation des racines, ou au résultat d'un changement dans les assemblages microbiens locaux en réaction à l'inoculation, qui à son tour aurait stimulé la croissance des plantes. De plus, nous avons aussi constaté que le clone de saule *S. miyabeana* "SX67" est un candidat potentiel pour la végétalisation de décharges industrielles contenant de fortes concentrations de métaux trace, comme le montrent les taux de survie élevés des clones et un potentiel intéressant pour la phytoextraction de Cd et Zn du sol. Ces résultats suggèrent que l'inoculation des racines de plantes avec des champignons symbiotiques spécifiques serait une méthode valable pour l'optimisation de la phytoremédiation. Il reste à vérifier s'il y a eu des changements au niveau du microbiome de la rhizosphère, puisqu'une colonisation des racines réussie par les champignons peut causer des modifications à sa structure, suite aux interactions des microorganismes introduits avec les communautés locales, causées par une compétition pour les ressources et l'exsudation de différents composés. La connaissance des microorganismes associés à la symbiose mycorhizienne, permettra de développer des inocula fongiques plus spécifiques et efficaces, puisque des bactéries sont connues pour s'associer à la mycosphère et pourraient avoir un effet bénéfique sur les processus visés.

5.5 Synthèse et perspectives

La majorité des études sur le microbiome rhizosphérique proviennent de contextes agricoles et naturels, mais l'intérêt sur son rôle et sa dynamique dans les milieux contaminés prend plus d'ampleur. Dans un premier temps, nous avons créé des mésocosmes contenant des sédiments contaminés et quatre espèces de plantes qui poussaient spontanément dans ces mêmes sédiments. Le point saillant de cette étude, c'est l'analyse de communautés bactériennes, fongiques, ainsi que les communautés de CMA des racines **et** de la rhizosphère provenant du même système, à travers le séquençage à haut débit. Cet examen fournit une vue complète dans un milieu contaminé sur les membres les plus importants de la rhizosphère et des indices sur le potentiel de modifier ces communautés à travers une perturbation biotique. De plus, nous avons aussi mis au point le même dispositif expérimental, mais dans un sol non contaminé, ce qui a permis de contraster les assemblages de CMA associées aux mêmes plantes, mais dans un contexte différent. La dominance du type de sol et de sa chimie dans la détermination des communautés microbiennes du sol et de la rhizosphère a été très évidente. Ceci concorde avec des conclusions d'études précédentes dans le même contexte, où les auteurs ont montré que la structure du microbiome rhizophérique dépendait de la nature du sol et de la contamination (Hassan et al., 2014; Iffis et al., 2017). Tardif et al. (2016) ont aussi constaté que le niveau de contamination déterminait les communautés microbiennes, avec un effet significatif du compartiment de la plante (racines, rhizosphère). Ceci est similaire à l'effet significatif du compartiment de la plante qui a été détecté dans cette étude concernant les CMA. Cet effet était présent dans les deux types de sol.

La manipulation, ou gestion du microbiome rhizosphérique des plantes se heurte à plusieurs obstacles qu'il faut contourner. De prime à bord, le sol contient une grande diversité de microorganismes tels que les virus, les protistes, les champignons, les bactéries, et les archées. L'état de la connaissance a l`heure actuelle ne représente qu'une infime partie de la diversité présente dans le sol, surtout concernant les aspects écologiques et physiologiques des interactions microbiennes et aussi avec les plantes (O'Callaghan, 2016, Kaminsky et al., 2019). Ces informations sont vitales pour pouvoir comprendre et mieux gérer les ressources du sol. Il faut aussi considérer la grande hétérogénéité du sol, qui peut présenter des profils chimiques et microbiens très dissimilaires, malgré qu'ils ne soient séparés que de quelques centimètres (Baer et al., 2020). De plus, le choix de l'identité de l'espèce de plante visée est difficile, considérant qu'il est compliqué pour le moment de prédire la structure de leur microbiome rhizosphérique, puisque cela va dépendre du milieu, tel que dicté par la chimie et la nature du sol, les microbes indigènes, ainsi que d'autres facteurs climatiques et édaphiques.

La bioaugmentation, ou inoculation avec un ou plusieurs microorganismes est une pratique répandue pour modifier le microbiome associé à la rhizosphère (Singer et al., 2005). Les microorganismes sont criblés et sélectionnés pour une fonction spécifique (nutrition, immunité, etc…) (Thompson et al., 2005). Mais la performance des inocula bactériens n'est pas souvent fiable. Pour la conception avec succès d'un inoculum microbien, il faut réussir la capture des microbes et leur isolation, leur production, leur établissement dans le milieu ciblé, et l'accomplissement avec succès de leurs fonctions. Chacune de ces étapes pose des défis à surmonter, et l'échec de l'une d'elles signifie un échec de tout le processus. Pourtant, la recherche se concentre plutôt sur les traits fonctionnels des microorganismes d'intérêt, alors que les études sur leur écologie et les mécanismes qui vont assurer leur survie et persistance sont moins nombreuses. La majorité des microorganismes du sol sont toujours résistants à la culture en laboratoire malgré les progrès des méthodes de culture, réduisant ainsi le puits d'éventuelles souches intéressantes (Stephani et al., 2015). L'isolation en laboratoire

capture en majorité des organismes qui dans leurs milieux naturels sont peu abondants et actifs (Shade et al., 2012). Les microbes isolés avec succès sont alors soumis à un criblage pour le processus de sélection, où il faut éviter que l'expression d'une fonction sélectionnée ne soit pas délétère pour la réussite des étapes suivantes, tel que l'établissement dans le sol. De plus, leur culture dans un milieu synthétique en conditions axéniques risque d'induire des modifications dans leur métabolisme qui pourrait alors devenir moins efficace dans le sol, où les microbes feront face à une compétition intense pour les ressources avec le microbiote local (Hottes et al., 2013). Ensuite, l'étape de production de l'inoculum impliquera plusieurs générations de cultures dans des fermenteurs, d'où l'importance de la stabilité génétique des candidats choisis, afin d'éviter la perte de fonctions. Une fois produit, l'inoculum est généralement inclus dans une matrice de matériel porteur, et peut inclure d'autres produits tels que des stimulateurs de croissance et/ou source de carbone pour favoriser l'implantation. La composition de la formulation joue donc un rôle important dans la survie et fonctionnement des membres de l'inoculum, sans lesquels les résultats escomptés ne seront pas obtenus. Dans le cadre de l'étude dans les mésocosmes, nous avons opté de ne pas cribler les candidats bactériens pour une fonction spécifique. Le but était de tester l'efficacité d'une altération dans la structure des microbes rhizosphériques, par le biais d'inoculations avec un consortium bactérien non criblé. Cette approche vise à réduire les obstacles décrits plus haut pour la production d'un inoculum efficace. Cette bioaugmentation a eu un effet positif significatif sur la production de biomasse des plantes, mais a ralenti considérablement la diminution des concentrations d'hydrocarbures, ce qui montre qu'une meilleure performance de la plante ne garantit pas une amélioration dans son potentiel de bioremédiation. Considérant l'extrême complexité des interactions dans le sol et la panoplie de composés impliqués, nous sommes encore loin de pouvoir prédire avec précision les effets d'une telle inoculation sans criblage. Par contre, des analyses des fonctions exprimées, ainsi que des protéines et métabolites produits et impliqués dans les changements de la

rhizosphère aideront à clarifier et identifier les procédés clés qui sous-tendent la dynamique microbienne du sol. Le suivi précis des membres des inocula une fois introduits doit être amélioré. En effet, dans les présents travaux, les membres du consortium ont été identifiés à travers une amplification et séquençage du gène de l'ARNr 16S au complet, en utilisant la technique Sanger. Par contre le séquençage des échantillons à la fin de l'expérience visait juste la région V3/V4 du gène de l'ARNr 16S, et a été réalisé sur la platforme Miseq d'Illumina qui est une technologie différente. Il n'est donc pas possible de déterminer si ce sont les microorganismes inoculés que nous avons détectés, ou bien d'autres souches ou espèces du même genre. Peut-être qu'avec les progrès très rapides dans la technologie de séquençage et la miniaturisation, nous pourront un jour avoir des puces implantées dans les plantes et le sol pour avoir des données en temps réel. La capacité de pouvoir suivre fréquemment la dynamique de la rhizosphère aidera à élucider plusieurs mécanismes, car à présent l'effort d'échantillonnage pour le faire devrait être trop intensif et empêche des suivis précis, surtout qu'on sait que la structure du microbiome de la rhizosphère varie aussi avec les stades de croissance des plantes.

En ce qui concerne l'inoculation de saules avec des champignons mycorhiziens, la double inoculation CMA-ECM n'a pas eu d'effet significatif sur la croissance des arbres et leur performance d'extraction des éléments traces par rapport aux autres traitements. Par contre, l'inoculation avec les ECM a été significativement associée à une plus grande production de biomasse aérienne, ce qui résultera en une quantité totale supérieure d'éléments extraits. Cet effet bénéfique a probablement été médié par un plus grand accès à la nutrition grâce au mycélium et/ou une protection de l'effet toxique du sol à travers le manchon de mycélium qui se forme autour des racines. Un exemple d'une formulation, qui pourrait améliorer les inocula dans des contextes similaires aux travaux effectués lors de ma thèse, serait un inoculum de champignons mycorhiziens accompagnés de certaines bactéries qui vont s'associer avec leur spores et mycélium, et qui présentent des fonctions

intéressantes. Les bactéries formant des biofilms sur le mycélium des CMA, et qui ont la capacité de solubiliser des minéraux en sont un bon exemple. L'inoculum peut être aussi accompagné de sources de carbone spécifiques pour stimuler la croissance de l'inoculum et la performance de la plante hôte. Un inoculum d'ECM est le plus souvent composé de mycélium qui n'est pas aussi résistant qu'une spore, ce qui réduit les chances de survie et d'établissement. Pour y remédier, il est possible d'englober le mycélium dans des billes de polymères, tel qu'un gel d'alginate de calcium (Lalaymia et al., 2014; Costa et al., 2019).

Les progrès réalisés dans l'étude des microorganismes du sol ont été catalysés en grande partie grâce aux nouvelles technologies de séquençage à haut débit, et les analyses qui en découlent tel que la génomique, la transcriptomique, la métabolomique, etc.Toutefois, bien que notre compréhension de l'écologie microbienne ait grandement évolué, l'extrême complexité de la microbiologie du sol limite encore notre capacité de gestion spécifique des microorganismes associés aux plantes peu efficace. Les progrès qui seront réalisés dans le future devront permettre de déchiffrer la communication et les interactions plante-microbes et microbes-microbes.

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7 Annexe

7.1 Chapitres 2 et 3 en images

Soil collection and treatment

- Approximately 350L of contaminated soil were collected from the top 15cm of Varennes site basin 1

- Also 350L of Non-contaminated soil were collected from the top 15cm of the Non-contaminated control parcel of the same site

- Both soils were emptied in 60x29x12 cm trays (16 for each type of soil) and resident seeds were left to sprout for removal

- Soil was then left to dry in the trays. It was then emptied on a tarp and broken down with a sledgehammer

- Finally, each soil type was homogenized and filled back in the trays

Plant material

- Seed pods from seven species of the basin 1 spontaneous vegetation were collected in October 2013
- Pods from only six species contained seeds
- Seeds were stratified in damp sand at 4°C for five weeks
- They were then sown on a 1:3 mixture of sand and turface and kept in humidity domes for germination at 21°C

- Seeds from four species germinated with very differential timing, ranging from one to three weeks
- Seedlings were then transferred to multicell units in sterile potting soil

- Seedlings from each species were also planted in the contaminated soil to assess their tolerance to high levels of hydrocarbons

- All seedlings survived but growth was slow
- Once all the plants were transferred to the multicells and their roots established, a pre-bioaugmentation was performed

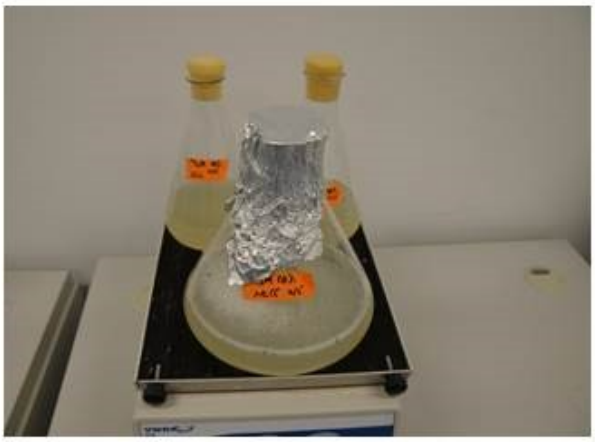
Experimental run

- 15 days after the pre-bioaugmentation, plants were transferred to the experimental tray, following the design previously described.

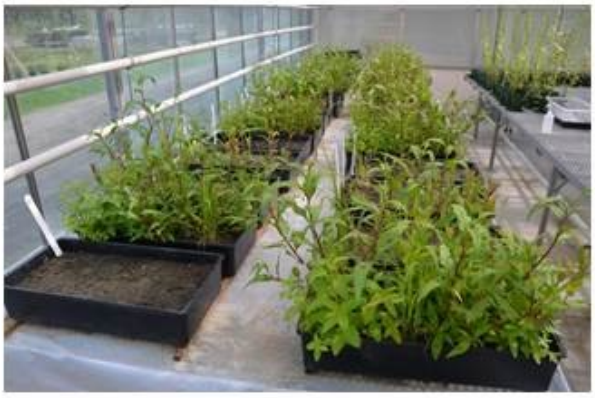
- They were left to acclimate for 2 weeks then the first bioaugmentation treatment was done after a first sampling round of the soil.

- 4 sampling rounds were done in total, including the initial round and final harvest
- David Denis and Mylène Durant helped with the intermediate sampling rounds

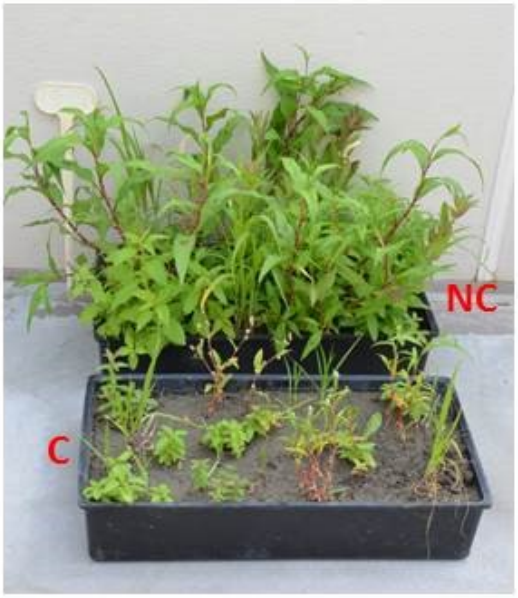
- Bacterial consortium growing in culture flasks



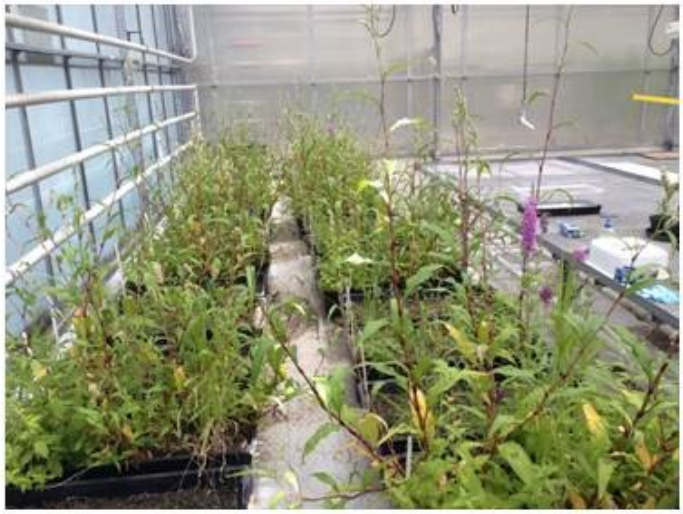
- Experimental setup at 1 month after first bioaugmentation treatment



- Contaminated **(C)** versus Non-contaminated **(NC)** trays



- Experimental setup 2 months after first bioaugmentation treatment



- Only bioaugmented non-contaminated trays presented the purple inflorescences on *Lythrum salicaria*

Final harvest

- Each plant cluster was excised from the trays using a big knife
- Roots, rhizosphere , and the aerial biomass were properly sampled