Dynamique de colonisation des racines du peuplier par les communautés de micro-organismes

I. Contexte général

La composition et la structure du microbiote racinaire d'une plante sont très dynamiques et peuvent être influencées par différents facteurs abiotiques tels que les propriétés du sol et le climat, ainsi que par des facteurs abiotiques tels que le génotype de l'hôte et l'exsudation racinaire (Berg & Smalla, 2009 ; Mendes et al., 2013).
Le microbiote racinaire se compose de communautés de micro-organismes originaires du sol environnant qui représente le réservoir principal de micro-organismes pour la formation du microbiote racinaire (Lareen et al., 2016). Ces micro-organismes colonisent alors différentes parties de la racine (rhizoplan et endosphère) en fonction de leur capacité de pénétration et de leur fonction (Gottel et al., 2011). La production d'exsudats racinaires riches en sucres, acides aminés, protéines et métabolites secondaires joue un rôle important dans le recrutement des membres du microbiote racinaire. La qualité et la quantité de ces exsudats racinaires sont fortement influencées par l'espèce, la physiologie mais également le stade de développement de la plante hôte (Badri & Vivanco, 2009). Les arbres sont des plantes pérennes et ligneuses à longue durée de vie avec un mode de gestion des éléments nutritifs différent par rapport aux plantes herbacées et annuelles comme *Arabidopsis thaliana* ou les plantes de grandes cultures (Grayston et al., 1996 ; Nehls et al., 2008). De plus, les arbres sont capables de s'associer à des champignons ectomycorrhiziens, très abondants dans les sols des forêts boréales et tempérées (Baldrian, 2017) et à divers champignons saprotrophes et endophytes dont le rôle reste encore à élucider ainsi qu'à diverses communautés bactériennes (Liao et al., 2019 ; Terhoven et al., 2019). La présence de champignons mycorhiziens affecte également la composition des communautés bactériennes (Garbaye et al., 1994 ; Frey-Klett et al., 2007).

L'établissement du microbiote est un processus dynamique dans lequel les communautés microbiennes issues essentiellement du sol colonisent progressivement le système racinaire de l'hôte. Des travaux ont été réalisés sur la compartimentation des communautés microbiennes du sol, de la rhizosphère et des racines du peuplier et d'autres arbres afin de comprendre les mécanismes de sélection et de mise en place des communautés de micro-organismes (Gottel et al., 2011 ; Shakya et al., 2013 ; Cregger et al., 2018 ; Uroz et al., 2010 ; Uroz et al., 2016). D'autres études se sont uniquement concentrées sur une seule espèce de bactérie ou de champignon et ont uniquement été fondées sur l'inoculation microbienne en laboratoire (Lilleskov et al., 2003 ; Noirot-Gros et al., 2018 ; Bueno de Mesquita et al., 2018; Mesanza et al., 2019). Or, l'étude de la dynamique de colonisation directement liée au développement racinaire des arbres dans le sol, principal réservoir de micro-organismes, est nécessaire pour aider à comprendre les interactions complexes existantes entre le microbiote et la plante hôte.

II. Objectifs

Dans ce contexte, l'objectif de cette étude est donc de mettre en évidence les différentes étapes de la colonisation des racines du peuplier par les micro-organismes du sol. Plus précisément, deux questions principales se posent :

- Quelle est la dynamique de colonisation des racines de peuplier par les communautés bactériennes et fongiques du sol ?
- Cette colonisation est-elle simultanée ou successive ?

III. Démarche expérimentale

Afin de répondre à notre questionnement, nous avons transplanté des vitroplants de peupliers cultivés en conditions axéniques dans du sol naturel de peupleraie. Huit prélèvements racinaires ont été effectués après 2, 4, 7, 15, 21, 30 et 50 jours de croissance en conditions contrôlées. Les échantillons de racines ainsi prélevés nous ont permis d'étudier la colonisation fongique et microbienne, d'une part, par séquençage MiSeq haut débit des amplicons 16S et ITS, et, d'autre part, par marquage puis visualisation des hyphes fongiques par microscopie confocale.

Les résultats de cette étude sont décrits sous la forme d'un article scientifique actuellement en préparation.
Les tableaux supplémentaires sont disponibles en Annexe (Annexe 1 de la page 1 à la page 8).

Colonization dynamic of *Populus tremula x alba* roots by soil microbial communities

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Abstract

A wide diversity of microorganisms belonging to various trophic guilds (i.e mutualists, endophytes, saprophytes) colonize roots of trees in natural conditions. The soil offers the main reservoir of microorganisms from which roots are colonized and select for specific microbial communities that contribute to the tree nutrition, development and protection against stresses. If it is well known that the formation of the root microbiome is a dynamic process, little is known on how different types of microorganisms colonize the root system and how the selection occurs. We hypothesized that the final composition of the microbiome is the product of several waves of colonization by different guilds of microorganisms To test this hypothesis, we planted sterile rooted cuttings of Grey Poplar obtained from plantlets propageted in axenic conditions in natural soil taken from a poplar stand. We analyzed the root microbiome at different time points between 2 and 50 days of culture by combining high throughput Illumina MiSeq sequencing of fungal rDNA ITS and bacterial 16S rRNA amplicons with Confocal Laser Scanning Microscope observations.

We observed that the colonization dynamic of *Populus* roots was different between bacterial and fungal communities. The rhizosphere effect was visible as early as two days after plantation for bacteria; roots were colonized very quickly and massively by members of the *Burkholderiaceae* family. By contrast, if fungi were also already colonizing the roots after 2 days, the initial communities were very close to the one of the soil and were dominated by saprotrophs and endophytes. Those were slowly replaced by both arbuscular and ectomycorrizal fungi . The replacement of the most abundant fungal guilds and bacterial community members observed over the time of our monitoring of the tree root colonization could suggest potential competition effect between microbial communities and/or a selection by the host tree.

Keywords : microbial communities, root colonization, metabarcoding, microscopy, *Populus*

Introduction

Plants have been recognized as metaorganisms possessing specific microbiomes. Plant microbiomes can be compartimentalised regarding the different plant organs (e.g. leaves, root microbiomes) and is a key determinant of plant health and productivity (Turner et al., 2013; Llado et al., 2017). Associated microorganisms, especially bacteria and fungi undergo different types of relationships with the host plant (Mendes et al., 2013; Hacquard & Schadt, 2015). The root-microbial interactions can be beneficial such as with mycorrhizal symbionts, by promoting plant nutrition and resistance against biotic and abiotic stresses, or detrimental such as with pathogens (Raaijmakers et al., 2009; Meena *et al.*, 2017; Naylor & Coleman-Derr, 2018).

The root microbiome is characterized by its composition and its structure. The composition corresponds to the identity of microbial members of the community while the structure is the product of the combination of the composition of the microbial community and the abundance of individual members. Composition and structure of root microbiomes are highly dynamic and are shaped by abiotic factors such as soil properties and climate, and by biotic factors such as the host genotype, root exudates and plant secondary metabolites (Berg & Smalla, 2009; Mendes et al., 2013).

The root microbiome is mainly recruited from the microbial communities present in the bulk soil which is considered as the main reservoir of microorganisms for plant root microbiome composition (Lareen et al., 2016). Roots provide different habitats for microorganisms that can be divided in three main compartements: the rhizosphere, the soil area directly under the influence of plant roots and the rhizoplane (i.e. the surface of the roots) are mainly colonized by subsets of microorganisms originating from the bulk soil, while the endosphere, i.e. the inner tissues of the roots, is colonized by both a subset of these rhizospheric communities and endophytic microorganisms coming from other plant tissues. The rhizosphere is enriched in compounds that are naturally produced by plant roots and attract microorganisms. Root exudates include sugars, amino acids, proteins and secondary metabolites and form a substantial source of nutrients for the microorganisms (Badri & Vivanco, 2009 ; Huang et al., 2014, Sasse et al. 2018). The quality and quantity of root exudates is strongly influenced by the species and the physiology of the host plant but also by plant developmental stages (Badri & Vivanco, 2009).

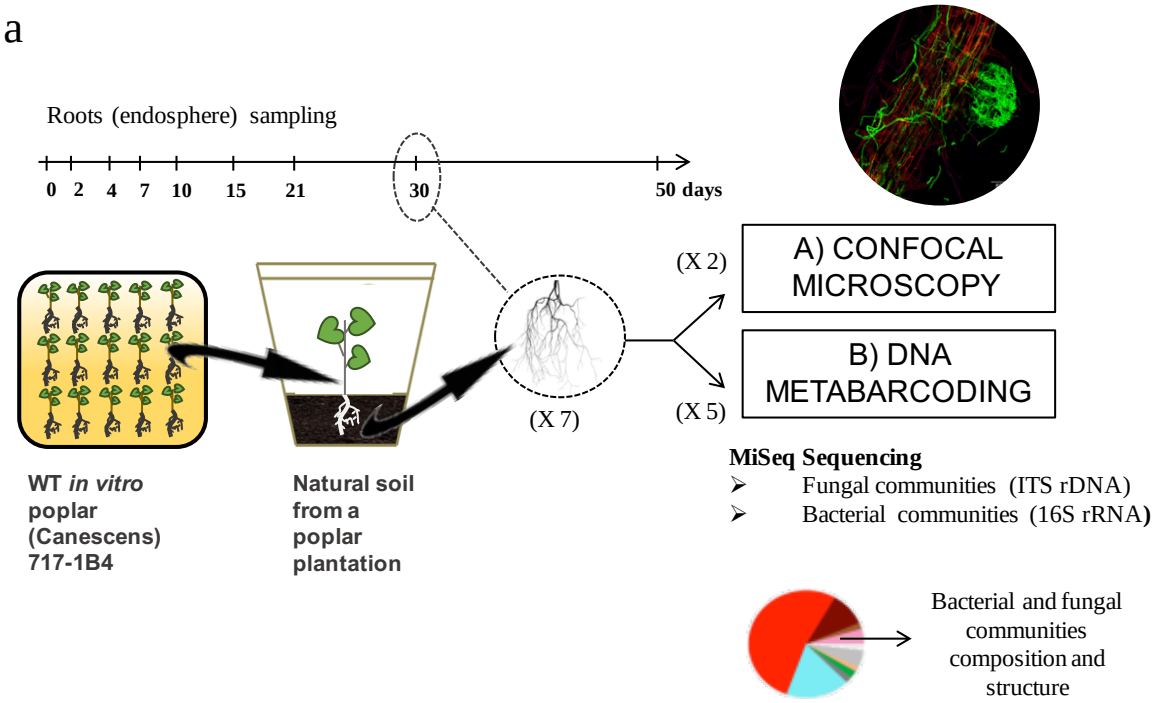
Trees are long-lived woody perennial plants with a different way of management of the nutrient allocation compared to herbaceous and annual plant species such *Arabidopsis thaliana* or crop plants (Grayston et al., 1996; Nehls et al., 2008). Tree root microbiome is also composed of a plethora of microorganisms with potential different functional capacity compared to herbaceous plants. Indeed, tree root are colonized by ectomycorrhizal (EcM) fungi in temperate and boreal forest ecosystem (Baldrian et al., 2017) but also by endophytes and saprotrophic fungi even though their role still remains elusive (Terhoven et al., 2019; Liao et al., 2019). The presence of mycorrhizal fungi also affect the composition of the bacterial part of the microbiome by offering a different habitat than nacked roots to bacteria named the mycorhizosphere (Frey-Klett et al., 2007, Marupakula et

al. 2017). Some rhizobacteria found in tree rhizosphere have been shown to improve mycorrhizal formation (mycorrhizal helper bacteria; Garbaye et al., 1994).

The establishment of the root microbiome is a dynamic process where specific microbial communities originating from the surrounding soil progressively colonise root systems under both the selection by the plant and the microorganisms communities. Previous works on root colonization have been carried out on trees to understand the mechanisms of the establishment of the tree root microbiome and of tree root selection (Lapeyrie et al., 1985; Chilvers et al., 1987; Lodge et al., 1989; Lilleskov et al., 2003; Noirot-Gros et al., 2018). For instance, aspen root colonization by the plant growth promoting bacteria *Pseudomonas* indicated that the spatial and temporal patterns of colonization of roots was different between the four strains of bacteria and was correlated with the ability of bacteria to form biofilm (Noirot-Gros et al., 2018). In pine roots, comparison of the dynamic of root colonization of two EcM fungi revealed different strategies. The ability of *Rhizopogon* to colonize roots rapidly from spores and its important early abundance constrasted with later root colonization and the slow increase in abundance of *Tomentella* (Lilleskov et al., 2003). The work of Lapeyrie et al. (1985) and Chilvers et al. (1987) on EcM and AM colonisation dynamic in eucalyptus roots showed a successionnal replacement of AM by EcM. Similarly, Lodge et al. (1989) showed negative associations among EcM and AM fungi leading to a depletion of AM and an increase in EcM in lateral roots in poplar. Nevertheless, these studies were focused on one or few bacterial or fungal species using microbial inoculation and did not look at the overall dynamic of the microbiome, including endophytes and saprophytes. Yet, pioneering studies on ectomycorrhizal and bacterial communities of the roots of Pines indicate that the full microbiome is likely subject to a complex dynamic during the colonization process (Marupakula et al. 2016). We hypothesized that fungal and bacterial communities originating from the natural soil colonized host roots with a successional turnover.

Investigating the succession of microbial communities colonizing the root system of young trees directly linked to the root development in natural soil, is needed to help the understanding of the complex interactions occurring between microbiota and host plant. To test this hypothesis, we assessed the dynamic of tree roots colonization by fungal and bacterial communities during the first 50 days of contact between naive tree roots and soil microbial communities. We used the grey poplar, *Populus tremula x alba* as a woody and perennial model organism. *Populus* root microbiome interactions and regulation have been studied in several genotypes (Shakya et al., 2013; Beckers et al., 2017; Cregger et al., 2018, Liao et al., 2019; Veach et al., 2019). Poplar trees have the specificity to host different types of mycorhizal fungi (ectomycorrhizal (EcM) and arbuscular mycorhizal (AM) fungi), fungal endophytes (Karlinski et al., 2010) and bacterial communities (Harquard & Schadt, 2015; Timm et al., 2018). Last but not least, poplar is an important species in the Northern hemisphere forestry with 80 million hectares of trees in the world (FAO, 2004). In France, poplar culture represent 23 % of the annual broad leaves trees yields and french industries should have difficulties in supply in 2023 (source CODIFAB).

a



b

	T0 Soil	T0 Roots		T2 Roots		T4 Roots		T7 Roots		T10 Roots		T15 Roots		T21 Roots		T30 Roots		T50 Roots	
Technical approach	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Number of samples collected	3	2	5	2	5	2	5	2	5	2	5	2	5	2	5	2	5	2	5
Completion of rarefaction (ITS)	3		0		5		4		5		4		4		5		5		5
Completion of rarefaction (16S)	3		0		4		5		5		5		5		5		5		4

Figure 15 - Experimental design (a) and number of samples used for each experimental approach in this study (b).

We characterized root colonization by fungal and bacterial communities of *Populus tremula x alba* cuttings cultivated in axenic conditions and transferred in natural soil taken from a *Populus* plantation using 16S and ITS rRNA gene-targeted Illumina MiSeq sequencing and Confocal Laser Scanning Microscopy (CLSM).

Material & Methods

Biological material and sample preparation

Populus tremula x alba (INRAE clone 717-1B4) vitroplants were cultivated on Musharige & Skood (MS) supplemented with IBA (2ml.L^{-1}) during one week before transfering them on MS for two weeks at 24 °C in growth chamber (photoperiodicity of 16h, light intensity of 150 umol.m^{-2}.s^{-1}) until root systems were developed as described in Felten et al (2009). Soil was collected from an 18-year-old poplar stand planted with *Populus trichocarpa* x *deltoides* and located in Champenoux, France (48° 51' 46'' N/2° 17' 15'' E). The first soil horizon (0-15 cm) was collected after pruning of brambles and adventitious plants and litter removal with a ratle. Then, soil was maintained at room temperature and homogenised through sifting at 2mm and fixed at 75% of humidity. Bulk soil was sampled in triplicate and stored at -20°C until DNA extraction.

Rooted vitroplants were selected to be homogeneous in terms of th size of the aerial part and the root system. Selected vitroplants were transplanted in natural soil in transparent plastic pots with a filtered cover allowing gas exchange and a dark area at the ground level to prevent algae development. Plants were cultivated in growth chamber (photoperiodicity of 16h, light intensity of 150 umol.m^{-2}.s^{-1}). Humidity in pots was maintained at 75% during all the experiment by regular wattering. Vitroplants were harvested after 0, 2, 4, 7, 10, 15, 21, 30 and 50 days of growth (Figure 15). At the beginning of the experiment (time-point T0) and at each time point, the root system (corresponding to the endosphere) of five plants were harvested, rinsed in sterile water, scanned to check root growth (WinRHIZO software v. 2009c, Regent Instrumentals, Québec, Canada), freezed in liquid nitrogen and stored at -20°C until DNA extraction. Two additional plants were harvested and roots fixed in a solution containing 3 volumes of 1X phosphate-buffered saline (PBS : 0,13 M NaCl, 7 mM Na2HPO4, 3 mM NaH_2PO_4, pH 7,2) for 1 volume of 3% para-formaldehyde (PFA) overnight at 4°C. At T30 and T50 time point, the root system was sufficiently developed to be splited in two equal part to performed these two technical approach on all plants.

Monitoring of vitroplant growth and EcM root colonization monitoring

Total area of root systems were measured for each vitroplant collected at the different time points on scan images using ImageJ (Schneider et al., 2012) before freezing in liquid nitrogen or PFA fixation. Mycorhization rate of each vitroplants was quantified as previously described (Labbé et al., 2011). Briefly, each root system was rinsed with tap water and analyzed under a dissecting microscope. For each root system, 100 short roots were randomly examined and assessed as mycorrhizal or non-mycorrhiza.

Confocal laser scanning microscopy

Staining of root systems and fungi were adapted from Vierheilig et al., (2005) protocol. In brief, fixed root systems were washed 3 times in one volume of 1X PBS and a last wash in 1 volume PBS / 1 volume of 90% ethanol before clearing them during 2h at 90°C in 20% KOH. After 3 washes in distilled water, samples were incubated overnight in 1X PBS containing 10 µg.ml-1 WGA-Alexa fluor 488, a specific marker of the chitine based fungi cell wall. Then, root systems were washed in 1X PBS and incubated for 15 min in 1X PBS containing 10 µg.ml-1 of propidium iodide, a marker of the root cell wall before 3 wash in 1X PBS. Samples were mounted between slide and cover slip with a drop of SlowFade solution (Life Technologies) containing the DNA staining DAPI. All root samples were observed with a ZEISS LSM 780 (ZEISS International) confocal laser scanning microscope (CLSM). WGA-AF488 was excited using 488 nm excitation wavelenght and detected at 500-540 nm whereas 561 nm excitation wavelenght and detection at 580-660 nm were used regarding propidium iodide. Maximum intensity projections were performed using the ZEN software with z-stack of 30 to 50 µm.

Optic Microscopy

Blue staining of fungal stuctures was adapted from Vierheilig et al., (2005) and Walker (2005). Cleared roots were incubated at 90°C in KOH 10% during 20 min. After few washes in distilled water, root systems were incubated for 10 min in 0,1 N HCL at room temperature. We removed HCL without washing and we incubated the root systems during 30 min at 90°C in acidified ink (5% Waterman ink, 20% lactic acid, 75% water). Finally, roots were washed in distilled water before being mounted between slide and cover slip with a drop of glycerol 20 % for observation under the OLYMPUS BX41 optic microscope.

DNA extraction, Illumina Miseq amplicon sequencing and quantification of microorganisms on roots

Approximatively 250 mg of bulk soil samples was used for each individual soil DNA extraction. Soil DNA was extracted using the DNeasyPowerSoil Kit following the protocol provided by the manufacturer (Quiagen, Venlo, the Netherlands). For root systems, fifty mg of root tissue were crushed in liquid nitrogen with mortar and pestle. DNA was extracted using the DNAeasy Powerplant Kit (Quiagen, Venlo, the Netherlands). All extractions were quantified on a Nanodrop 1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA).

A two-step PCR approach was performed in this study to barcode tag templates with frameshifting nucleotide primers. Forward and reverse primer mixtures were used to maximize phylogenetic coverage of bacteria and fungi. Primer mixtures for tagging bacterial amplicons were composed of 4 forwards and 2 reverses 515F and 806R primers screening the 16S rRNA V4 gene region in equal concentration (0,1µM; Mangeot-Peter et al., 2020). Primer mixtures for tagging fungal amplicons were composed of 6 forward and 1 reverse for ITS1 – ITS4 rRNA region at equal concentration (0,1 µM; Mangeot-Peter et al., 2020). To inhibit plant material amplification, a mixture of peptide nucleotide acid (PNA) blockers targeted plant mitochondrial and chloroplast 16S rRNA genes and plant 5.8S nuclear rRNA gene were added in PCR reaction mixes (Mangeot-Peter et al., 2020). Polymerase chain reaction (PCR) were performed for three replicates of eac sample (2µl isolated DNA at about 10 ng/µl) using 2.5x Phusion flash high fidelity master mix (ThermoScientific) with 1.5 µl of forward and reverse primer mix,

0.75 µl of PNA probe and 8.5 µl of 0.2µm filtered UV treated DNA free water (Carl Roth, France) in a total reaction volume of 30 µl per sample. Thermal cycler conditions for the primary PCRs for bacterial amplification in soil and root samples were 30 cycles of 98°C for 5s, 78°C for 10s, 52°C for 20s and 72°C for 15s. Primary PCR condition for fungal amplification in soil and root samples were 30 cycles of 98°C for 5s, 78°C for 10s, 55°C for 20s and 72°C for 15s. PCR products without addition of microbial DNA (negative control), mock communities of known fungal or bacterial compositions were added as quality controls. Samples of 50 µl (30 ng DNA per µl) were sent for tagging and MiSeq Illumina Next Generation Sequencing (GeT PlaGe INRAE sequencing platform, Toulouse, France).

Sequence processing

Bacterial sequences were further processed with FROGS (Find Rapidly OTU with Galaxy Solution; Escudié et al., 2018) based on the Galaxy analysis platform (Afgan et al., 2016). Sequences were demultiplexed, dereplicated, sequence quality was checked, oligonucleotides, linker, pads and barcodes were removed from sequences. Sequences were removed from data set, if they are non-barcoded, exhibited ambiguous bases or did not match expectations in amplicon size. Remaining sequences were clustered into operational taxonomic units (OTUs) based on the iterative Swarm algorithm, then chimeras and OTU containing only one, two, three or four one sequences were removed. Bacterial double affiliation was performed by blasting OTUs against SILVA database (Quast et al., 2012) and the ribosomal database project (RDP) classifier (Wang et al., 2007). OTUs with affiliation <100% at the phylum level (indicated by a RDP bootstrap value <1) and corresponding to chloroplasts or mitochondria were removed from the data set. OTUs at lower taxonomic ranks than the phylum level were considered as “unidentified” below when the RDP bootstrap value was < 0.70. OTUs with high abundances in negative controls were excluded from further analysis. Sequencing, and affiliation quality was also evaluated based on the results obtained for the bacterial mock community.

After demultiplexing and quality checking (QC quality score = 30, minimal size = 200 bp), bioinformatics analyses were performed using standard procedures as described in Pérez-Izquierdo et al. (2017).

For both fungal and bacterial data, per-sample rarefaction curves were produced to assess sampling completeness, using function *rarecurve()* in package Vegan v3.5-1 (Oksanen et al., 2015) in R (version 3.4.3 ; R Core Team, 2016). Reads assigned to AM fungi were extracted from the data set before the rarefaction due to the very small number of reads. Samples with insufficient number of sequences according to rarefaction curves were removed. Based on these, subsequent analyses of diversity and community structure were performed on datasets where samples had been rarefied with the Phyloseq (McMurdie and Holmes, 2013) package to achieve equal read numbers according to the minimum number of total reads in any sample (4,964 reads for fungi and 21,142 reads for bacteria). Microbial community composition and structure in bulk soil and roots data were further analysed by using Phyloseq package (McMurdie and Holmes, 2013).

FUNGuild (Nguyen et al., 2016) was used to classify each fungal OTU into an ecological guild. OTUs identified to a guild with a confidence ranking to “highly probable” or “probable” were conserved in our analysis, whereas

those ranking to “probable” or with multiple assignation were called “unclassified”.

Statistical analysis

Statistical analyses and data representations were performed using R software (R Core Team, 2016). Fungal and bacterial community structure was determined using permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity matrices and compositional differences were visualized using a non-metric dimensional scaling (NMDS) ordination. Analysis of similarities (PERMANOVA) was performed to test statistically whether there is a significant difference between fungal and bacterial communities detected in each sampling time. After checking normality of the data distribution with Shapiro-Wilk test, one-way ANOVA test followed by a Tukey post-hoc test were used to detect significant difference in the relative abundance of dominant fungal and bacterial phyla, orders and genera of the soil and across root systems collected at the different time points. This procedure was also used to compare relative abundance of fungal guild and mycorrhization rate between root samples.

Results

Microbial sequencing

MiSeq sequencing of ITS and 16S rDNA amplicons were performed on soil and roots DNA samples between T0 and T50. After quality filtering and chimera and singleton removal, a total of 450,000 fungal reads (10,714 ± 775 reads per sample) and 1,740,000 bacterial reads (40,510 ± 3,962 reads per sample) were kept for further analyses. After taxonomic assignment, elimination of contaminants and completion of rarefactions, 227 fungal OTUs (59 ± 5 OTUs per sample) and 8,572 bacterial OTUs (1,945 ± 121 OTUs per sample) were detected (Figure 16).

Plantlet development and composition of soil microbiome

In order to investigate the temporal colonisation dynamic of *Populus* roots by fungal and bacterial communities, axenic cuttings of poplar were propagated in vitro to obtain rooted plantlets. Two weeks-old plantlets with a single main root of about 2 cm was then planted in pots containing natural soil. Monitoring of the growth of the root system indicated a slow development of the roots during the first 15 days followed by an acceleration of the growth in the next weeks (Figure 17 a). First short roots and ectomycorrhizae (ECM) were observed at 10 and 15 days, respectively. The rate of ectomycorrhization regularly increased to reach 37 % at 50 days post plantation and nearly doubled between T15 and T50 (Figure 17 b).

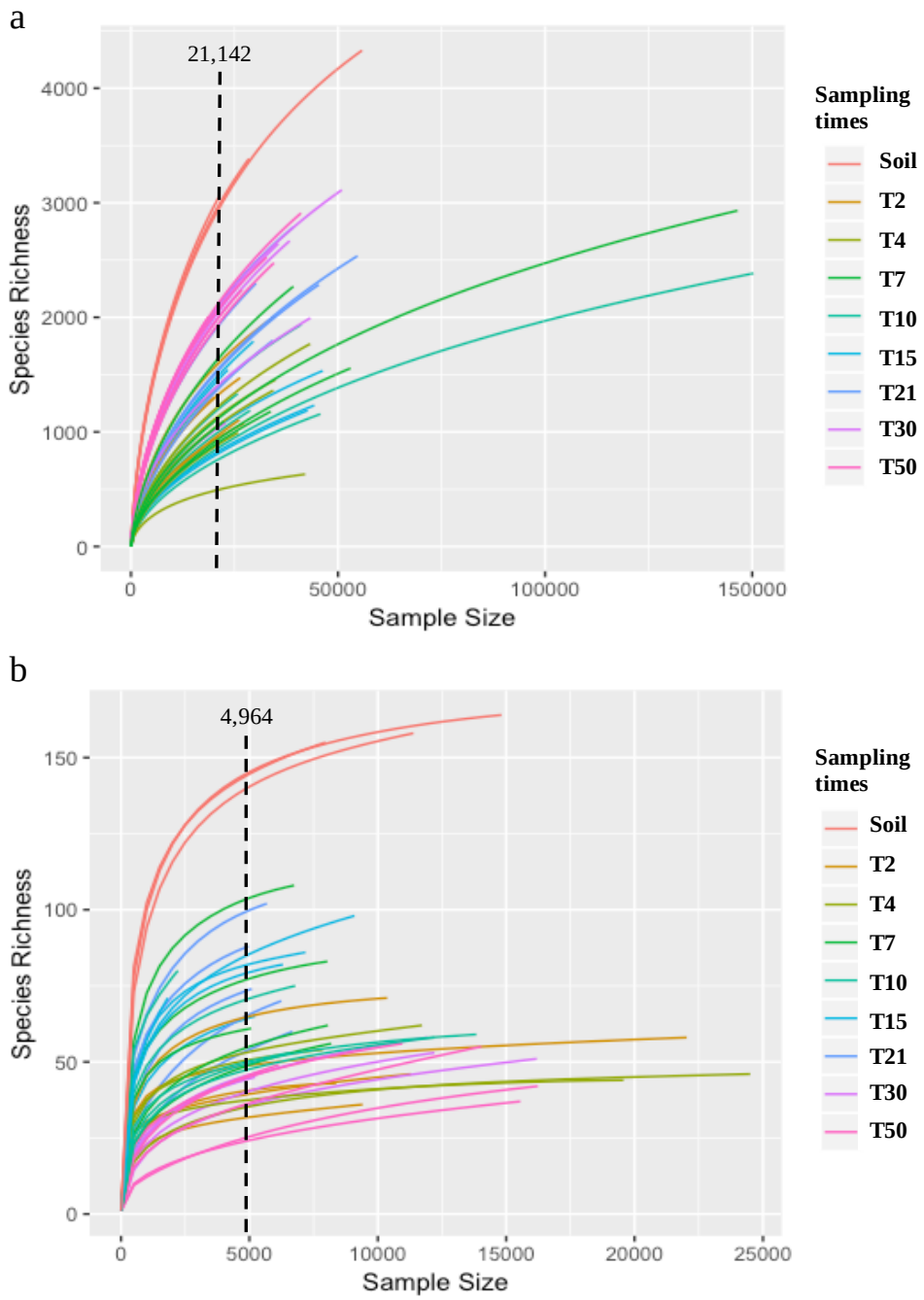


Figure 16 - Rarefaction curves of bacterial (a) and fungal (b) OTUs. Each curve represents one sample and sampling times are color-coded.

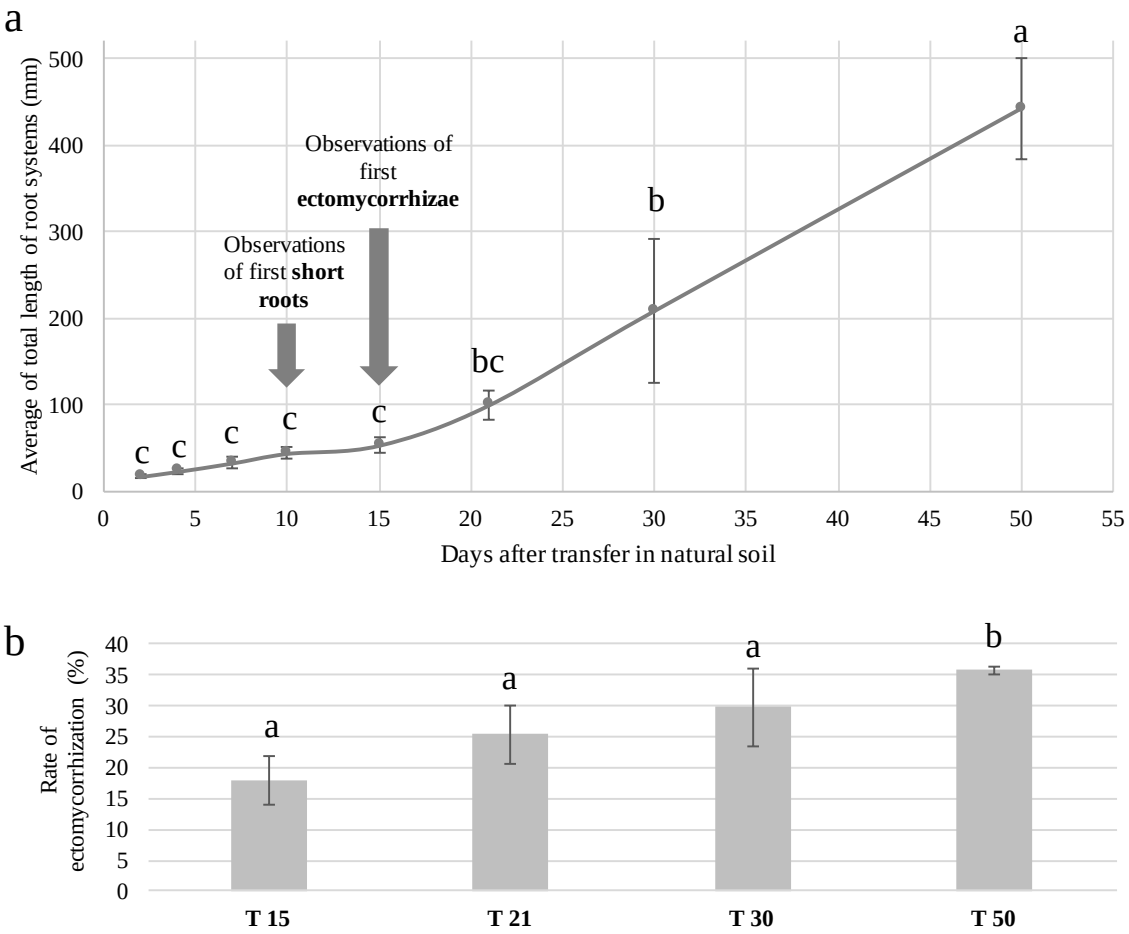


Figure 17 – Root development and ectomycorrhizae formation along time. Total length of the root system measured at each sampling time from T2 to T50 (a). Ectomycorrhization rate of Populus roots from T15 to T50 calculated as the number of fungal colonized lateral roots/ total number of lateral roots (b). Each given value is the average value of 7 replicates +/- SE. Different letters denote significant difference between each sampling time (One-way ANOVA, factor=sampling time, P<0.05).

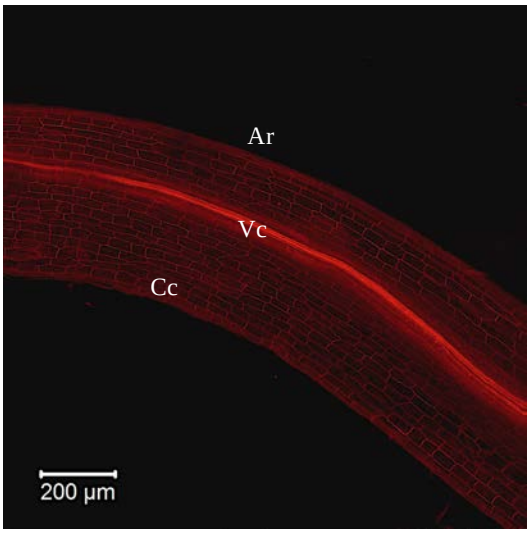


Figure 18 - Confocal microscopy image of Populus tremula x alba adventive root at the beginning of the experiment (T0). Plant cell walls were stained with propidium iodide and appear in red. Ar, Adventive Root; Vc, Vascular cylinder

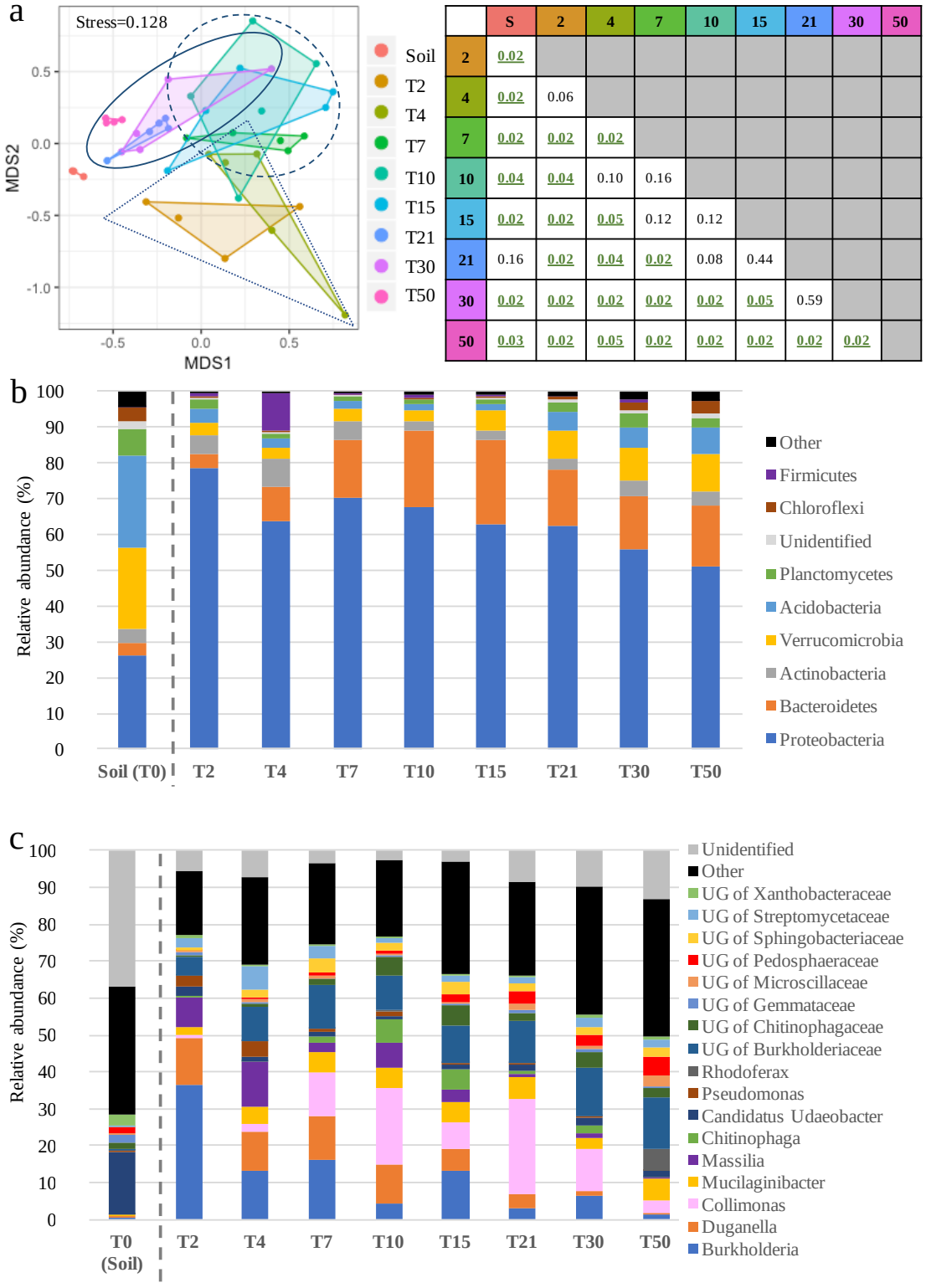
Fungal and bacterial colonization of the roots were tracked using two complementary methods: 16S and ITS rRNA gene-targeted Illumina MiSeq sequencing and confocal microscopy. We first validated the axenic status of the invitro plants: no amplification of ITS and 16S rDNA genes were obtained from samples of roots collected before vitroplants were transfered in natural soil. These results are in accordance with CLSM observations concerning fungal colonisation as no fungal structure could be visualised at T0 (Figure 18). By contrast, soil was heavily colonized by complex bacterial and fungal communities at T0, as expected from a previous study on soil taken from the same poplar plantation (Mangeot-Peter et al., 2020): a total of 4,028 bacterial and 165 fungal OTUs were detected in the soil. The soil inoculum was dominated by nine bacterial (> 1 % in relative abundance) and six fungal phyla. Proteobacteria (26.1 ± 0.4 %), Acidobacteria (25.9 ± 0.1 %) and Verrucomicrobia (22.6 ± 1.2 %) dominated the bacterial community while Basidiomycota (51.6 ± 2.9 %), Zygomycota (24.4 ± 1.3 %) and Ascomycota (20.1 ± 2.3 %) dominated the fungal soil community (Table S1, Table S2). Eight bacterial genera (>1 % in relative abundance) and 11 fungal genera were detected in bulk soil collected at T0. OTUs from *Candidatus Udaeobacter* largely dominated the soil bacterial community as they represented 17 % of the reads in average (Table S1). Members of a few genera also dominated the soil fungal community: *Sebacina* (EcM, 19.1 ± 0.8 %), *Umbelopsis* (saprophyte, 12.3 ± 0.8 %), *Mortierella* (saprophyte, 12.0 ± 1.1 %) and *Cryptococcus* (saprophyte, 8.3 ± 0.8 %) were the most abundant fungal genera detected in soil (Table S2). This initial soil fungal community was made of a mix of EcM fungi (34.9 ± 3.1 %), saprotrophic fungi (16.7 ± 1.8 %), fungal endophytes (13.0 ± 1.2 %) and AM fungi (0,3 %; 14 OTUs) (Table S3).

Structure and composition of bacterial communities associated to *Populus* roots along sampling time

Bacterial colonization of *Populus* roots was observed after two days of growth (T2). At the OTU level, the structure of bacterial communities significantly shifted between roots samples collected from T2 to T50 (except for root samples collected at T21) and bulk soil samples collected at T0 (PERMANOVA, $P<0.05$, Figure 19 a). In addition, significant shifts in the structure of bacterial communities were also observed between roots samples collected from T2 to T50 although close time points not statistically different (e.g T2-T4, T15-T21…, PERMANOVA test, $P<0.05$; Figure 19 a).

The number of bacterial OTUs detected in roots tended to increase after 21 days of growth in soil and almost doubled between T2 and T50 (Table 2). Similarly, diversity slowly increased over time as indicated by Shannon index values (Table 2).

A detailed analysis of bacterial community compositions across the different sampling times revealed an early selection of bacterial communities by the *Populus* roots. Community composition of the roots differed greatly from that of the soil as early as two days after planting. Proteobacteria, and particularly *Burkholderiaceae* (*Burkholderia, Duganella, Massilia*, *Cupriavidus*) massively colonized the roots, reaching 36 % of the reads at that time point (Figure 19 b, c).



	S	2	4	7	10	15	21	30	50
2	0.02								
4	0.02	0.06							
7	0.02	0.02	0.02						
10	0.04	0.04	0.10	0.16					
15	0.02	0.02	0.05	0.12	0.12				
21	0.16	0.02	0.04	0.02	0.08	0.44			
30	0.02	0.02	0.02	0.02	0.02	0.05	0.59		
50	0.03	0.02	0.05	0.02	0.02	0.02	0.02	0.02	

Figure 19 - Structure and composition of bacterial communities colonizing Populus roots across time. Non-metric multidimensional scaling (NMDS) ordinations of bacterial OTU across compartments (soil and roots) and sampling times (from T2 to T50). P-values of variances explanation based on permutational multivariate analysis using Euclidean dissimilarity matrix for bacterial communities. P-values < 0.05 appear in green) (a). Distribution of most abundant bacterial phyla (>2 % in relative abundance in at least one sampling time) detected in bulk soil and in Populus roots at each sampling (b). Distribution of most abundant bacterial genera (>1 % in relative abundance) detected in bulk soil and in Populus roots at each sampling time (c).

By contrast, Acidobacteria and Verrucomicrobia were strongly counter-selected compared to the bulk soil at T2. Then, the composition of the root bacterial communities clearly evolved over time from T2 to T50. While still dominant, the proportion of Proteobacteria significantly decreased from 78 % at T2 to reach 55 % at T30 (Figure 19 b, Table S1, ANOVA, $p<0.05$). Proteobacteria were slowly replaced by members of the Verrucomicrobia, Acidobacteria, Bacteroidetes and Chloroflexi. By contrast, proportion of Actinobacteria and Planctomycetes in the roots remained stable all along the time of the analysis. Different types of behaviours were detected at the genus level: some bacterial genera, mainly belonging to the *Burkholderiaceae* family strongly decreased in proportion along time. For instance, OTUs of the *Burkholderia-Caballeronia-Paraburkholderia* genus decreased from 36 % at T2 to 1.2 % at T50. By contrast, OTUs belonging to the *Chitinophaga* or *Sphingomonas* tended to pick between T10 and T15 and then decreased until T50. A third type of OTUs such as *Rhodoferax*, *Rhizobacter* or *Bradyrhizobium* reached their maximum relative abundance at T50. In addition, OTUs belonging to bacterial genera *Clostridium sensu stricto 9*, *Dyella*, and an unidentified genus of *Methylophilaceae* were not detected in the bulk soil but were found at different colonisation time of the experiment. *Clostridium sensu stricto 9* and *Dyella* relative abundance increased at T4 (Table S1) and fluctuated until the end of the expermiment wherease the unidentified genus of *Methylophilaceae* was the most abundant between T15 and T21 (Table S1). Finally, some genera strongly varied in abundance between root systems collected at the same time. For instance, *Collimonas* massively colonized some root systems between T7 and T30, reaching up to 25 % of the reads in some roots while being almost absent from other roots (Table S1).

Structure and composition of fungal communities associated to *Populus* roots

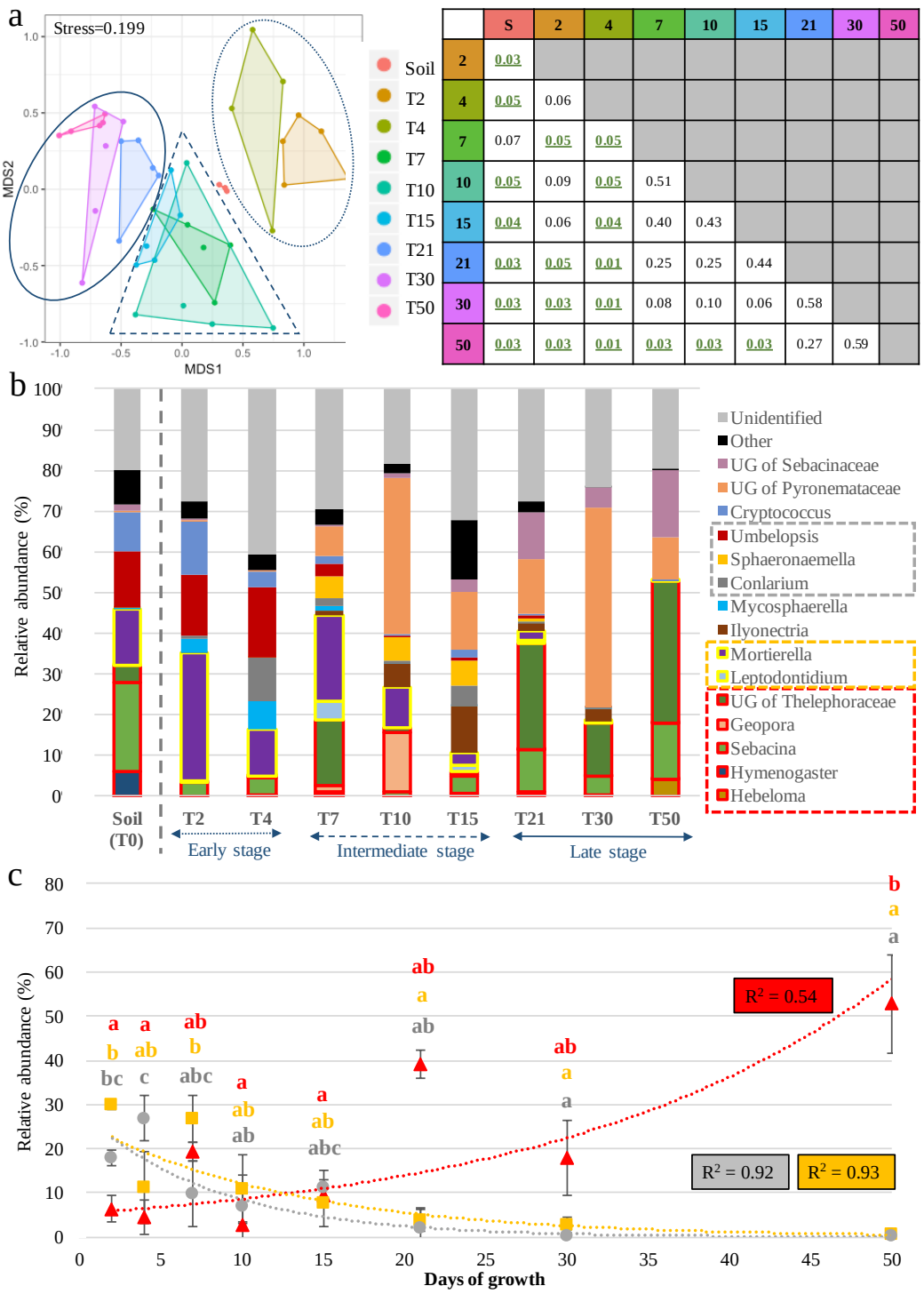
Like bacteria, we detected the presence of fungi on *Populus* roots after two days of growth in soil (T2) by Illumina Miseq sequencing of ITS amplicons. At the OTU level, the structure of fungal communities significantly shifted between roots samples collected from T2 to T50 (except for root samples collected at T7) compared to bulk soil samples collected at T0 (PERMANOVA, $P<0.05$, Figure 20 a). In addition, significant shifts in the structure of fungal communities were also observed between roots samples collected from T2 to T50 although close time points not statistically different (PERMANOVA test, $P<0.05$; Figure 20 a).

The number of fungal OTUs increased until 15 days of growth in natural soil then decreased until 50 days (Table 3). Diversity was stable between T2 and T10 and slowly tended to decrease over time between T10 and T50 as indicated by Shannon index values (Table 3).

A detailed analysis of fungal community composition across the different sampling times revealed a later selection of fungal communities colonizing *Populus* roots than for bacterial communities. As for bacteria, we observed a high variability in fungal colonization of the root system of *Populus*. This observation was made especially for the most abundant fungal genera in roots collected. However, we could distinguish three stages of root colonization: an early from T2 to T4, an intermediate from T7 to T15 and a late stage from T21 to T50 (Figure 20 b). The early fungal community was dominated by few saprotrophic fungi such as the yeast *Umbelopsis* and *Cryptococcus* and the filamentous saprotrophe/endophyte *Mortierella* that all together made

more than 50 % at T2 and 38 % at T4 of the reads (Table S3). Reads corresponding to EcM fungi were already detected at this stage but were in low abundance compared to the saprotrophs'ones and corresponded to 6 % of the reads at T2 and 4 % of the reads at T4 (for 9 genera detected). We detected the first Glomeromycota reads (*Claroideoglomus_sp*) after 4 days, even though their relative abundance remained very low (< 0.1 %, data not shown). The rhizosphere effect was also visible at this stage with a significant number of difference between soil and root composition. Indeed, we did not detect the fungal genera *Amylostereum*, *Daedaleopsis* and *Geopora* in the bulk soil, although these genera were found in the roots. A shift in the fungal community composition was observed at T7 with the significant increase of the relative abundance of some EcM fungi (e.g *Thelephoraceae* and *Hydnotrya*, Table S2) that all together made more than 19 % of the reads in T7 samples (Table S3). In addition, we observed that the relative abundance of saprotrophes and endophytes remained stable (37 % of the reads, Table S2) compared to the early stage of root colonization.

The relative abundance of AM fungi also increased from T7 (< 0.1 %) to T10 (around 0.5 %). *Archaeosporales_sp*, *Rhizophagus irregularis* and *Glomeromycota_sp* were present in a similar relative abundance at T7 while *R. irregularis* dominated at T10 (data not shown). At T10 and T15, the relative abundance of EcM fungi belonging to *Thelephoraceae* decreased while the relative abundance of EcM fungi of the *Sebacina, Hebeloma and Geopora* genera increased compared to T7 samples (Table S2). The total reads of EcM fungi reached 3 % at T10 and 10 % at T15 while the relative abundance of saprotrophes and endophytes reached 18 % at T10 and T15 (Table S3). *Paraglomus laccatum* was the only OTU belonging to the Glomeromycota detected in very low abundance in root samples (< 0.05 %) at T15 (data not shown). Finally, the relative abundance of Zygomycota decreased while the relative abundance of Basidiomycota increased in roots collected at T21, T30 and T50 (Table S2). The relative abundance of the fungal endophytes *Mortierella* and the saprotrophic fungi *Umbelopsis* significantly decreased at T21 and T30 and almost disappeared in roots collected at T50 while the relative abundance of the EcM fungi *Sebacina* and the EcM fungi belonged to *Thelephoraceae* significantly increased at T30 and T50 compared to the other sampling times (Figure 20 b, Table S2). In accordance with these observations, EcM fungi were significantly more abundant in roots collected at the late stage (52 % of the reads at T50, Table S3) while endophytes and saprotrophes were significantly more abundant during the early and the intermediate stages of colonization (Figure 20 c). From T21 to T30, we detected in very low abundance, two OTUs belonging to the Glomeromycota (*Paraglomus laccatum* and *Archaeosporales_sp*) before they vanished at T50 (data not shown).



	S	2	4	7	10	15	21	30	50
2	0.03								
4	0.05	0.06							
7	0.07	0.05	0.05						
10	0.05	0.09	0.05	0.51					
15	0.04	0.06	0.04	0.40	0.43				
21	0.03	0.05	0.01	0.25	0.25	0.44			
30	0.03	0.03	0.01	0.08	0.10	0.06	0.58		
50	0.03	0.03	0.01	0.03	0.03	0.03	0.27	0.59	

Figure 20 – Structure and composition of fungal communities colonizing Populus roots across time. Non-metric multidimensional scaling (NMDS) ordinations of fungal OTU across compartments (soil and roots) and sampling times (from T0 to T50). P-values of variances explanation based on permutational multivariate analysis using Euclidean dissimilarity matrix for fungal communities. P-values < 0.05 appear in green (a). Distribution of most abundant fungal genera (> 4 % in relative abundance in at least one sampling time) detected in bulk soil and in Populus roots at each sampling time grouped according to the stage of colonization: early, intermediate or late (b). Grey, yellow and red boxes highlight saprotrophic, endophytes and ectomycorrhizal fungi, respectively (b). Relative abundances of saprotrophic fungi (grey), fungal endophytes (yellow) and ectomycorrhizal fungi (red) detected in Populus roots at each sampling time (c). Each given value is the average of 4 to 5 replicates +/- SE. The dotted curves represent the exponential trend curves for each trophic. Letters denote significant difference between each sampling time (One-way ANOVA, factor=sampling time, P<0.05).

Table 2 – Diversity of the bacterial community detected in roots across time. Number of bacterial OTUs and Shannon indexes calculated for each root samples collected at the different sampling time from T2 to T50. Each given value is the average value of 4 or 5 replicates +/- SE. Different letters denote significant difference between each sampling time from T2 to T50 (One-way ANOVA, factor=sampling time, P<0.05).

Sampling time	Number of bacterial OTUs	Shannon index
T2	1019.3 ± 147.1 abc	3.5 ± 0.4 a
T4	828.4 ± 108.8 b	3.7 ± 0.2 a
T7	970.0 ± 120.0 abc	3.9 ± 0.2 ab
T10	906.2 ± 93.4 ab	3.5 ± 0.3 a
T15	963.2 ± 108.5 abc	4.3 ± 0.2 abc
T21	1477.8 ± 116.1 acd	4.4 ± 0.4 abc
T30	1507.4 ± 207.0 cd	4.9 ± 0.2 bc
T50	1738.5 ± 52.8 d	5.3 ± 0.1 c

Table 3 – Diversity of the fungal community detected in roots across time. Number of fungal OTUs and Shannon indexes calculated for each root samples collected at the different sampling time from T2 to T50. Each given value is the average value of 4 or 5 replicates +/- SE. Different letters denote significant difference between each sampling time from T2 to T50 (One-way ANOVA, factor=sampling time, P<0.05).

Sampling time	Number of bacterial OTUs	Shannon index
T2	43.0 ± 6.1 ab	2.1 ± 0.2 ab
T4	42.8 ± 4.3 abc	2.1 ± 0.2 ab
T7	65.8 ± 9.6 bc	2.4 ± 0.3 b
T10	53.0 ± 4.3 abc	2.1 ± 0.3 ab
T15	73.0 ± 4.9 c	2.6 ± 0.1 b
T21	68.0 ± 7.6 bc	2.3 ± 0.2 ab
T30	32.2 ± 2.1 a	1.4 ± 0.2 a
T50	32.4 ± 5.1 a	1.4 ± 0.3 a

Monitoring of fungal colonization in *Populus* roots by CLSM

MiSeq results brought global information about the structure and composition of microbial communities without knowledge about their spatial distribution. In order to deepen our understanding of the dynamic process of root colonisation, samples were also observed by CLSM.

The first fungal presence was detected by CLSM between 2 and 4 days of culture (Figure 21 a). We observed spores and extracellular hyphae colonising the surface of root system mainly from the apex (Figure 21 b). These colonisations were very heterogeneous from one sample to the other with some root apexes fully surrounded by fungal mycelia while other were only presenting few hyphae (Figure 21 c). The fungal extracellular hyphae were septed with a diameter under 1 µm and we observed a very low diversity of morphologies. During this time lap, we identified by light microscopy, as being melanised septed hyphae not stained by WGA-Alexa fluor 488, the early presence of dark septate endophytes (DSE) after 4 days of growth (Figure 22). Their hyphae were either extracellular or intercellular, but it was difficult to assess if they were located in the intracellular or apoplastic region. We detected an increased density of fungal morphologies by CLSM after 7 days of culture, in both the intercellular and intracellular root compartments. Fungal hyphae either developed between root cells, propagating in the apoplastic compartments particularly around epidermic regions or directly into root cells (Figure 23 a, b).

This apoplastic colonisation stayed heterogeneous along the root, and was dominantly present at the apex and in the root elongation zone. After 10 days, we observed an increase of the apoplastic and intracellular colonisation. Indeed, fungal hyphae were propagating from cell to cell by going through the root cell-walls and we were even able to see the pressure of the hyphae on the cell walls (Figure 23 b). Even though the global fungal diversity of morphologies remained poor at this stage of development, we noted the presence of septed and non-septed hyphae with diameters either inferior or superior to 1 µm and we still observed the presence of DSE. After 15 days of culture, we observed an important increase of fungal density and morphological diversity in the root systems. We identified within the same root region the occurrence of distinct fungal morphologies with the dominance of two major structures (Figure 23 c). We detected the first dominant morphology in the intracellular compartment propagating from cell to cell and displaying an « arbuscular mycorrhizal like » shape (Figure 23 d). Its hyphae diameter was inferior to 1 µm and developed going through the cell wall from the epidermic to the central cell forming a grid shaped network. The second dominant morphology was propagating in both the intracellular and intercellular compartments, with hyphae diameter closer to 5 µm and exhibiting « hand glove-like » structures (Figure 23 c). When in the intercellular compartment, this structure seemed to surround the root cell. The development of lateral roots after 15 days of culture was correlated with the establishment of the first distinct ectomycorrhizal structures (Figure 24). Most EcM root tips already exhibited a mantle and a Hartig net (Figure 24), however some EcM did not have a fully formed mantle and hyphal colonization originated either from the apical region or from the bottom of the main root system. The density of colonization and the occurrence of ectomycorrhizal structures were heterogeneous among the different root systems. Nevertheless, many fungal morphologies were present within the same region, both on lateral and main roots (Figure 24).

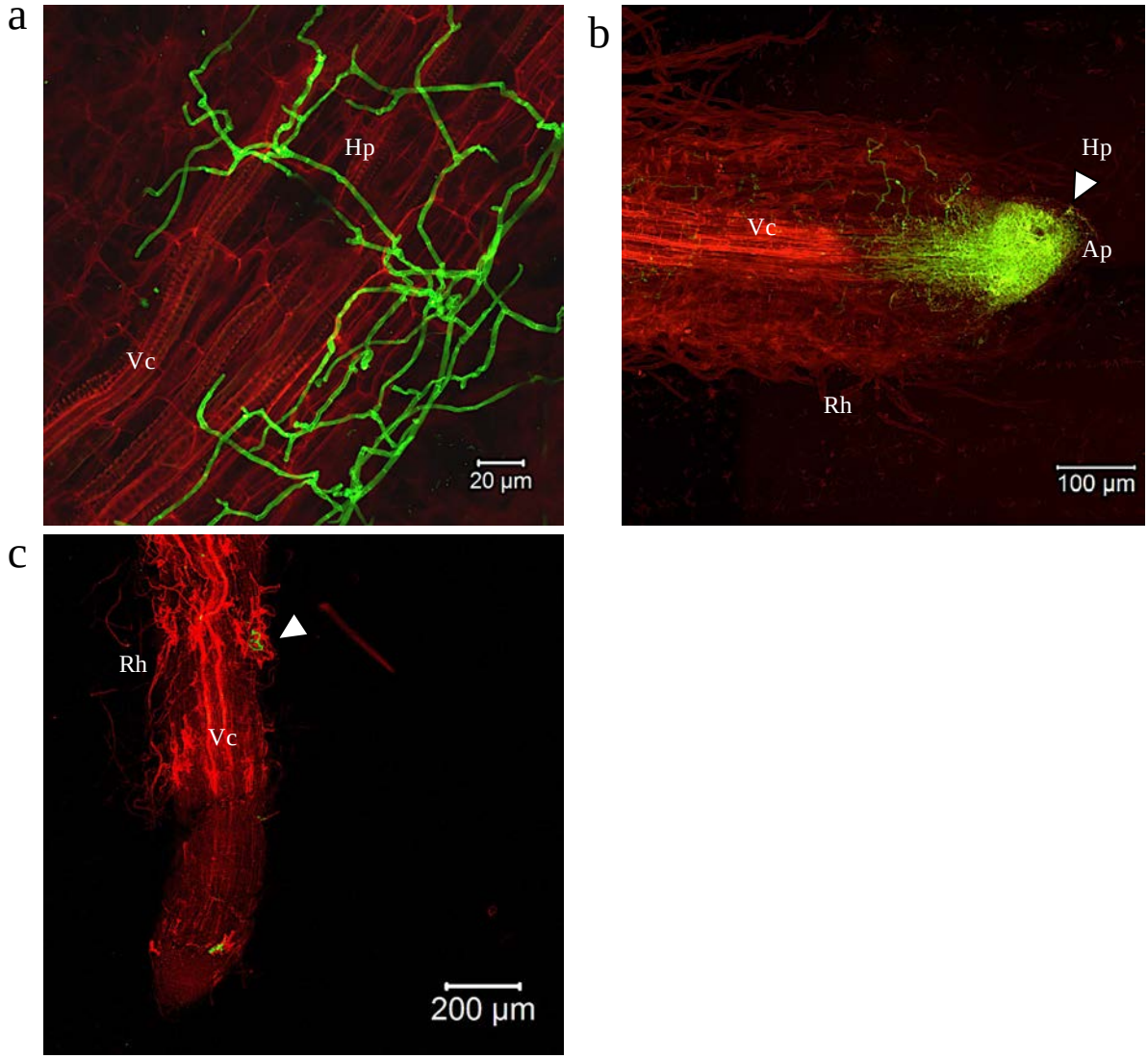


Figure 21 - Early stage of the fungal colonization dynamic. Confocal microscopy images of poplar roots colonized by fungi after 4 days of culture. (a) Extracellular hyphae surrounding a root after 4 days of culture. (b) Hyphae accumulation at the apex of the root after 4 days of culture. Hyphae on root hairs after 4 days of culture (c). Fungal structures appear in green through WGA-Alexa Fluor 488 staining while root cell-walls were stained with propidium iodide and appear in red. Ap, Apex; Vc, Vascular cylinder; Hp, Hyphae; Rh, Root hair.

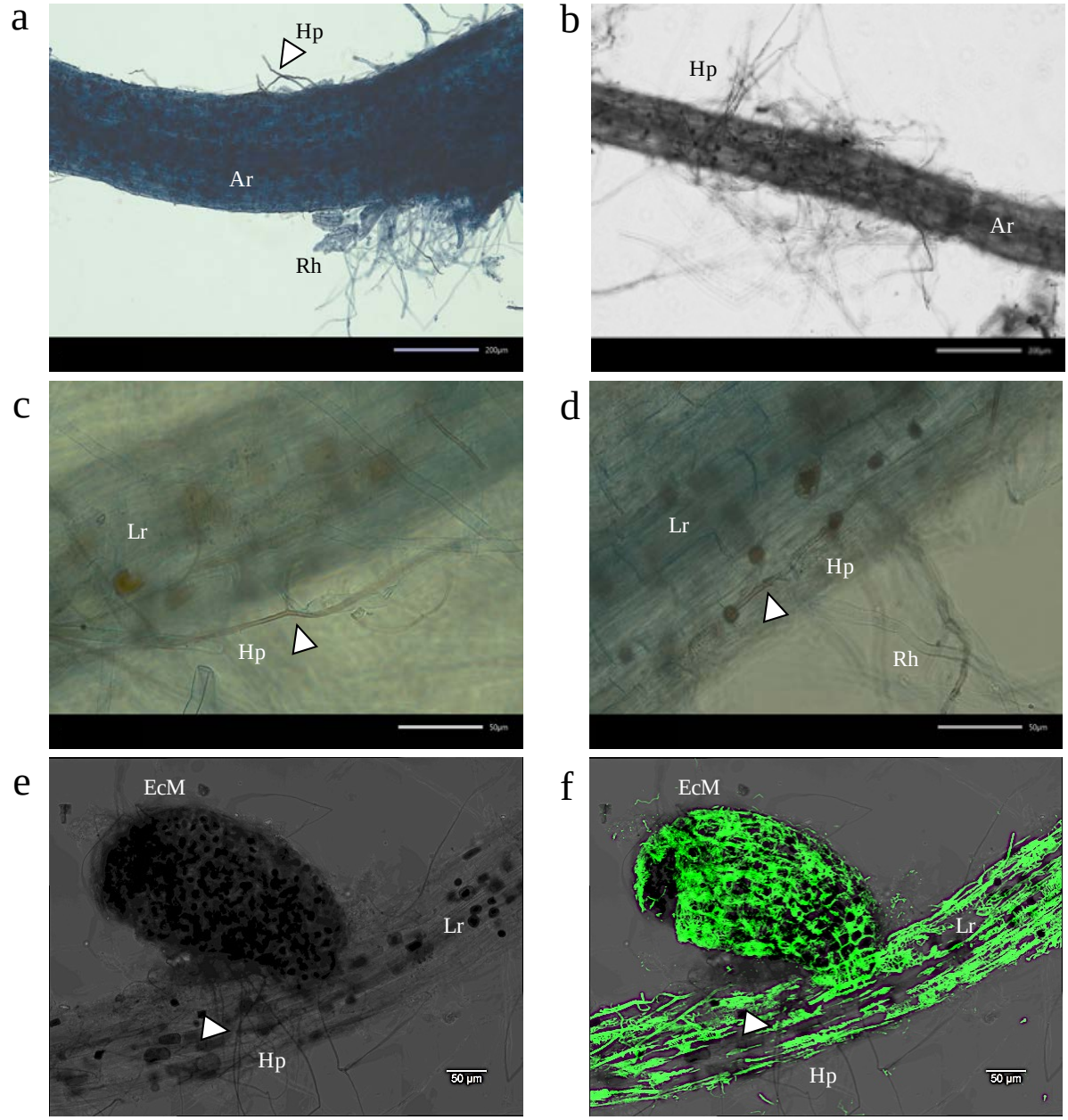


Figure 22 - Dark Septate Endophyte (DSE) colonizing poplar roots from 4 to 30 days of culture. Optic and confocal microscopy images of poplar roots colonized by DSE. (a) External colonization of roots by DSE after 4 days of culture. (b) Extracellular DSE hyphae surrounding an adventive root at 10 days of culture. (c) Extracellular DSE hyphae after 21 days of culture. Arrow indicates the DSE septa. (d) Intracellular DSE hyphae propagating in the apoplastic compartment after 21 days of culture. Arrow indicates the DSE septa. (e) Extracellular DSE hyphae surrounding an EcM forming on a lateral root after 30 days of culture. Arrow indicated the DSE hyphae. (f) Overlay of image (e) and the green track in order to visualize both melanized and non-melanized fungal structures. Non-melanized fungal structures appear in green through WGA-Alexa Fluor 488 staining. Ar, Adventive root; Hp, Hyphae; Lr; Lateral root; Rh, Root hair.

From 21 to 50 days of growth, we observed via CLSM a global increase of the fungal diversity and density within the same root region with some roots systems being colonised from the apex to the top of the root at 50 days, even though it remained heterogeneous within the different root systems. We still observed DSE, both inter or intracellular and we detected two new abundant fungal morphologies that were sometimes located within the same root region. The first structure was developing in the intracellular compartment in both adventive and lateral roots, displaying a globular shape with hyphae diameter inferior to 1 µm (Figure 25 a). The second morphology was only present in lateral and mycorrhized roots, with hyphae diameter superior to 1 µm and displaying a « maze like » structure (Figure 25 a). Its location between the inter/intra compartments, as well as its origin were difficult to determine, but it is noteworthy that it was often associated and seemed to develop within ectomycorrhizal structures (Figure 25 b). In addition, we observed fungal structures developing between the adventive and the lateral root forming a potential EcM (Figure 25 c). The apex of the lateral root was not colonized by any fungal structure suggesting that the EcM forming originated from pre-existing fungal structures on the adventive root. We also detected the presence of germinating spores with emerging hyphae colonizing the root cells (Figure 25 c). We observed an increase of EcM establishment (Figure 25 d) and we assessed by mycorrhizal counts under CLSM that 37 % of lateral roots were forming ectomycorrhizal structures at the end of the experiment, even if their presence was also variable depending on the root systems (Figure 17 b). We observed DSE until the end of the experiment at 50 days and we detected some of them in the same region where ectomycorrhization was taking place (Figure 22).

Regarding the lateral roots, we observed the successional replacement of the « arbuscular like » structures to the benefit of the « hand gloves like » structures and EcM. We did not observe this pattern in the main roots, where the « arbuscular like » morphologies continued to develop among the « hand gloves like » structures.

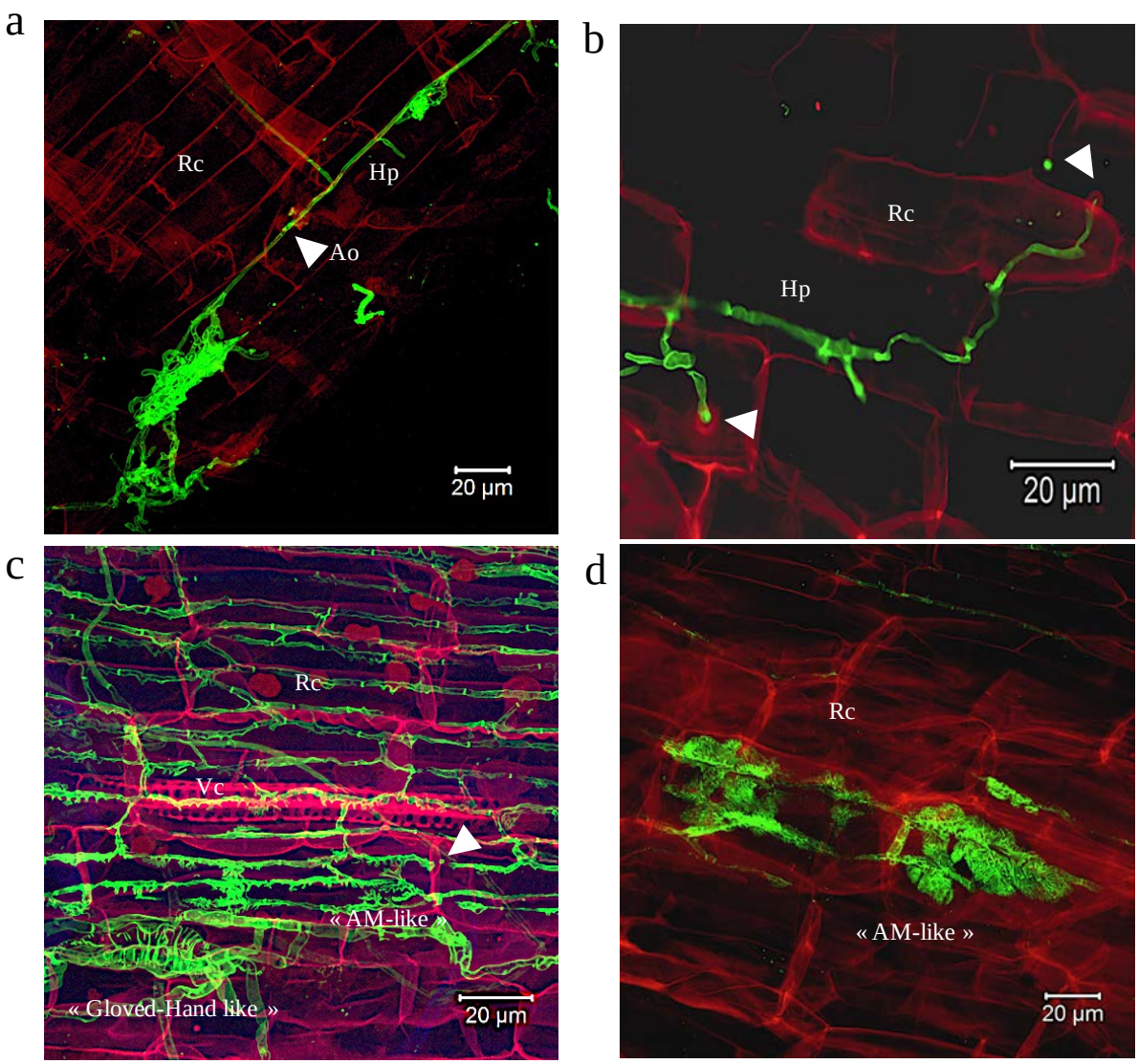


Figure 23 - Intermediate stage of the fungal colonization dynamic. Confocal microscopy images of poplar roots colonized by fungi after 7 to 15 days of culture. (a) Development of fungal hyphae in the apoplastic space of cortical cells of poplar after 7 days of culture. (b) Intracellular hyphal penetration in root cell after 7 days of culture. Arrows indicate the deformation of the root cell under the hyphal pressure. (c) Co-existing fungal morphologies (« arbuscular like » and « glove-Hand like »), within the same root region after 15 days of culture. Arrows indicate hyphal intracellular penetration. (d) « Arbuscular like » morphology observed in poplar root after 15 days of culture. Fungal structures appear in green through WGA-Alexa Fluor 488 staining while root membrane was stained with propidium iodide and appear in red. Ao, Apoplastic space; Hp, Hyphae; Rc, root cell; Vc, Vascular cylinder

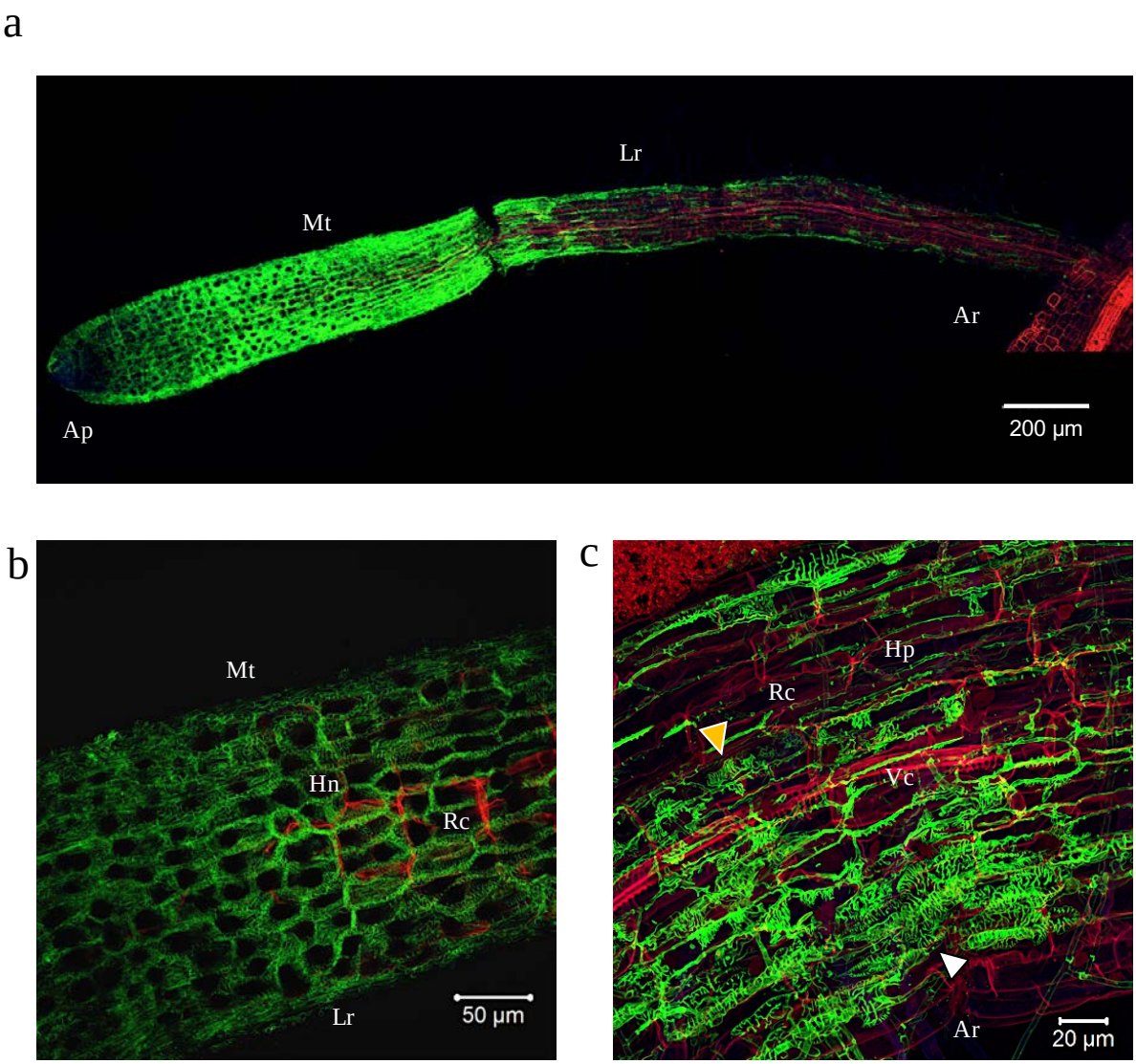


Figure 24 - Intermediate stage of the fungal colonisation dynamic. Confocal microscopy images of poplar roots colonised by fungi after 7 to 15 days of culture. (a) EcM formation on a lateral root after 15 days of culture. (b) Hartig net formation on EcM after 15 days of culture. (c) Co-existing and abundant fungal morphologies within the same root region after 15 days of growth. Orange arrow indicates the « arbuscular-like » and white arrow indicates the « hand glove-like » fungal structures. Fungal structures appear in green through WGA-Alexa Fluor 488 staining while root cell-walls were stained with propidium iodide and appear in red. Ar, Adventive root; Ap, Apex; Hn, Hartig net; Hp, Hyphae; Lr; Lateral root, Mt, Mantle; Rc, Root cell; Vc, Vascular cylinder

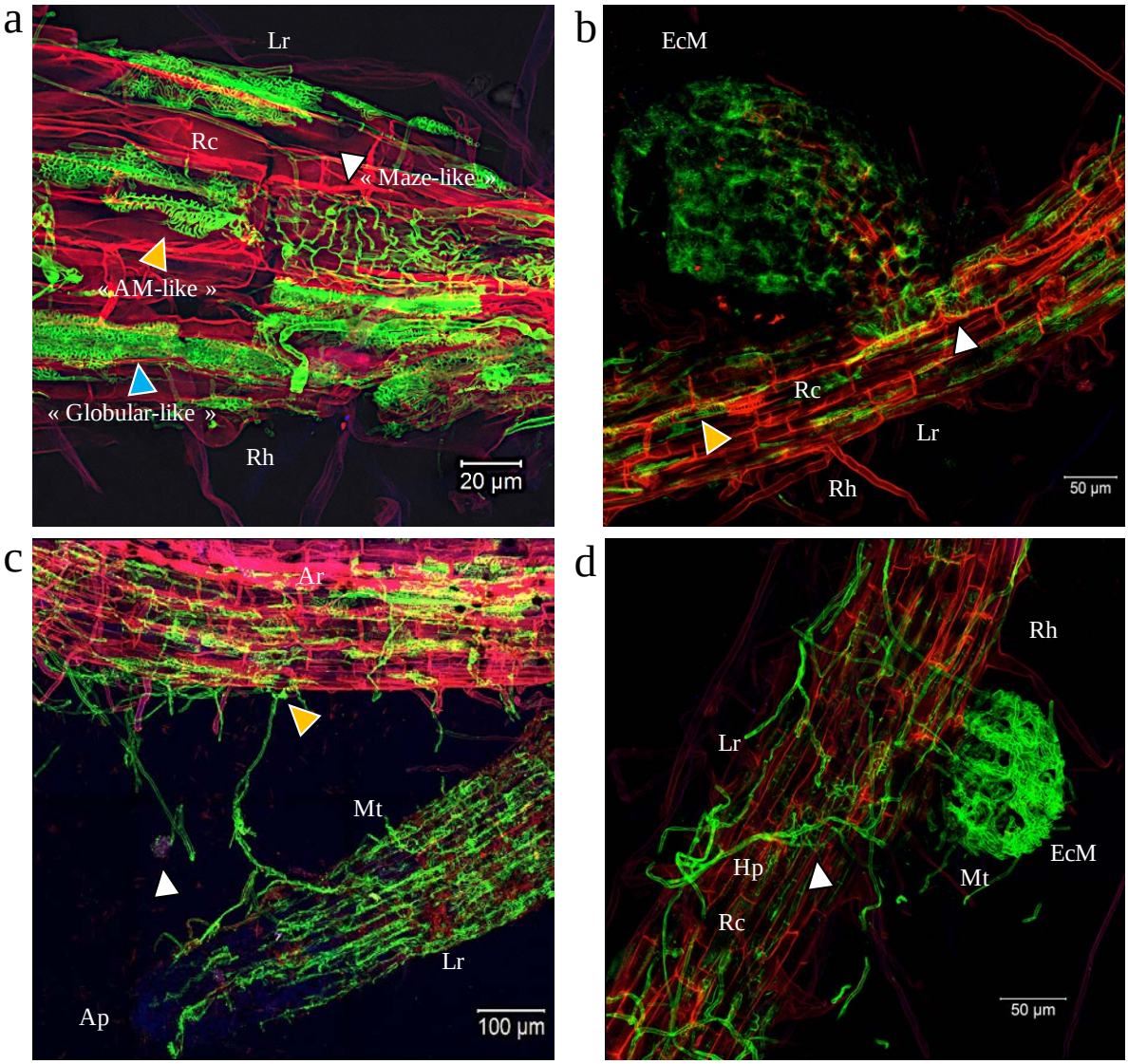


Figure 25 - Late stage fungal of the colonization dynamic. Confocal microscopy images of poplar roots colonized by fungi after 21 to 50 days of culture. (a) At least three different morphologies are co-existing within the same root region after 21 days of culture. White arrow indicates the « maze like » structure, the blue arrow shows the « globular like » and the orange arrow indicates « arbuscular like » structure. (b) Mycorrhizal formation with co-existing fungal morphologies after 30 days of culture. Orange arrow indicates the « arbuscular-like » structure and the white arrow the presence of the « maze like » fungal morphology, that seemed to be linked with the « glove-Hand like » morphology and the EcM forming structure. (c) Hyphal propagation at T30 between the adventive root to the lateral root forming a probable EcM. White arrow indicates a germinated spore and the orange arrow the « hand glove like » morphology. (d) EcM formation after 30 days of culture. Arrows indicate the « maze like » structure that seems to originate the EcM. Fungal structures appear in green through WGA-Alexa Fluor 488 staining while root cell-walls were stained with propidium iodide and appear in red. Ar, Adventive root; Ap, Apex; Hp, Hyphae; Lr, Lateral root; Mt, Mantle; Rc, Root cell; Rh, Root hair

Discussion

The establishment of the plant root microbiome is a dynamic process involving complex communities of microorganisms with distinct trophic mode and functions. These interactions, both between plant-microbes and microbes-microbes lead to microbial species turn over along with the tree development. If studies of the colonisation dynamic of roots by specific stains of bacteria or of single fungi have been performed in different plant species, especially to develop biocontrol strategies (Bueno de Mesquita et al., 2018; Noirot-Gros et al., 2018; Mesanza et al., 2019) , no study has been done so far to understand the colonisation dynamic of tree roots by complex microbial communities, so far. Here, we developed a microcosm to grow axenic Poplar in natural soil and to track the colonization of the root system by microorganisms. To do so, we used two complementary methods, CLSM and metabarcoding in order to investigate how the bacterial and fungal colonisation occurs in the root system of *Populus tremula x alba* grown in natural soil. The transfer of plantlets from axenic conditions to the microcosm did not induce visible stress to plants as they grew normally and developped short roots and ECM symbiosis at same rates and timing than in other systems (Vayssière et al. 2015). In addition, we observed a rapid but dynamic colonisation of the root system by both fungi and bacteria. We were able to track both arbuscular and ectomycorrhizal fungi, suggesting that our microscom allowed a normal development and colonization of the root system. To our knowledge, this is the first study investigating the primary steps of both bacterial and fungal colonization of axenic roots grown in a natural soil.

The dynamics of the rhizosphere effect was different between fungal and bacterial communities

Previous studies suggest that the root microbiome would be selected from the surrounding soil in a two steps process in which rhizodeposition would fuel an initial selection followed by a fine tuning of the communities of the rhizoplane and the endosphere by the plant (Bulgarelli et al. 2013). Our results are in accordance with this model but they suggest that this selection would occur through a different timing and a different process for bacterial and fungal communities. The structure and the composition of both fungal and bacterial communities were already clearly different from the one of the soil two days after planting poplar plantlets, suggesting that a very early selection is operating on both bacterial and fungal communities from the soil surrounding the roots. However, the degree of selection and the pattern of evolution differed between bacterial and fungal communities. A massive shift of the bacterial community composition already took place at day 2 while the fungal composition, although significantly different from the bulk soil, was still quite close to the one of the soil, at this stage. A gradual replacement of fungal species associated to a reduction of the richness and the diversity led to the domination of ECM species in the roots. By contrast, the bacterial richness and diversity increased over time and the change in the community composition was less pronounced as for fungi. Fungi and bacteria are already known to react differently to abiotic factors such as edaphic parameters and litter chemistry (Uroz et al. 2016). Our results would suggest that they also react differently to plant selection factors or that the plant would select the two communities through different processes. Such differencies may be explained by the existence of multiple signals

(e.g. flavonoids, nutrients, strigolactones) that would not be perceived by the same microorganisms (Bulgarelli et al. 2012, Martin et al. 2016).

Early fungal and bacterial communities are dominated by copiotrophs

Proteobacteria and particularly *Burkholderiaceae* dominated the early root microbial community. This is in accordance with previous studies on Poplar and other tree root microbiomes that showed a significant enrichment of OTUs from Proteobacteria and from *Burkholderia* in roots of trees (Gottel et al. 2011, Shakya et al., 2013, Uroz et al., 2010, Marupakula et al. 2016, Colin et al. 2017). Representative members of the *Burkholderiaceae* family have a high ability to develop on root exudates (Compant et al. 2008, Haicher et al. 2008). Similarly, dominant fungi at early time points were yeasts and filamentous saprotrophs/endophytes such as *Mortierella* that are likely able to quickly grow on root exudates. Members of the *Mortierella* genus are commonly detected in soils of forests and poplar plantation (Shakya et al., 2013; Bonito et al., 2014; Cregger et al., 2018). Although their ecological role is poorly understood, those fungi are charcterized by their rapid growth when encouterning rich media (Uehling et al. 2017). Yeast are commonly found in both natural and agricultural soil (Mestre et al. 2011, Yurkov et al., 2018). Some of the most frequently detected soil yeast belong to the genus *Cryptococcus* and have already been isolated from *Populus trichocarpa* roots (Gottel et al., 2011). Although their role remains largely unknown, plant growth promotion by soil yeast has been reported in different crops including maize (Sarabia et al., 2018), tomatoes, pepper and squash (Aziz Khan et al, 2012). This dominance of copiotrophs among both fungal and bacterial communities at early time points advocates for an important role of root exudates and particularly primary metabolites in the selection of the early root microbiome. In accordance with this hypothesis, fungal colonisation of the roots occurred mainly at the tip of roots (Figure 19), in the area where most of primary metabolites are exudated (Canarini et al. 2019). Preliminary analyses permitted to detect several sugars susceptible to attract microorganisms including mannitol, sucrose, glucose and arabinose, in root exudates of axenic *Populus tremula x alba* (data not shown). More unexpected is the very early detection reads corresponding to ECM fungi in the roots, long time before short roots start to develop. Similar early colonisation of roots by ECM fungi was found in *in vitro* experiments when inoculating eucalyptus roots with the ectomycorrhizal fungi *Pisolithus tinctorius* and *Paxillus involutus* (Horan et al., 1988) and between *Betula pendula* and *Paxillus involutus* (Brun et al., 1995). Indeed, both studies found evidence of hyphal attachment to the roots after two days of inoculation with an accumulation of hyphae at the root apex. These observations challenges the current model of the molecular dialog between ECM and roots leading to the establishment of the ECM symbiosis. In this model, the early molecular dialog between plant roots and ECM mycelium that lead to the formation of short roots and ECM colonization of the short roots rely on the production by both partner of diffusible compounds that act without physical contact between the root cells and the mycelium (Daguerre et al. 2016). Instead, our data would suggest that ECM fungi could colonize the main root before the formation of short roots and ectomycorrhizae. Further microscopy analyses will be needed to confirm this hypothesis.

The composition of bacterial communities colonizing *Populus* roots slowly evolved after seven days of root growth

We observed a significant shift in the structure and composition of the most dominant bacterial phyla and genera detetected in roots across time. Althought a strong selection of the bacterial communities from the soil occurred early on, the composition evolved slowly after seven days of root colonization. Marupakula et al. (2016) also observed that the bacterial colonization of single ECM root tips of *Pinus sylvestris* was highly dynamic and that bacterial community structure significantly differed depending on the sampling time. However, the time step chosen in this study was longer and main differences were visible between four and 24 weeks. This suggests that the bacterial communities may not have reached equilibrium by 50 days and that they could have evolved more if we had continued to sample longer. We observed the decrease of the relative abundance of members of the *Burkholderiaceae* family for the benefit of other well known tree root colonizers such as *Bradyrhizobium Rhizobacter* or *Sphingomonas* (Marupakula et al., 2016 ; Colin et al., 2017; Foulon et al., 2016; Bonito et al., 2019; Wang et al., 2019). Several phenomona could be involved in such evolution of the microbial communities: competition between microorganisms, slow growth of late comers, selection by the tree, cross-kingdom interactions with fungi… It is noteworthy the relatively high abundance of reads from *Collimonas* in some roots at intermediate time points (Figure 21). The genus *Collimonas* regroups a number of mycophagous bacteria (Leveau et al., 2010, Mela et al 2012). One could wonder if this increase is linked to specific interactions with fungi colonizing the roots. Further investigations will be needed to determine the mechanisms driving these dynamics.

The dominance of fungal endophytes and saprotrophs vs EcM fungi was reversed over time in *Populus* roots

The rhizosphere effect observed for fungal communities was not an isolated event and was followed by a time effect resulting in the successionnal replacement of fungal species within the root system over time. We distinguished three stages of root colonization: early from T2 to T4, intermediate from T7 to T15 and late from T21 to T50.

The relative abundance of endophytic and saprotrophic fungi tended to decrease over time, as mentionned in previous studies (Danielsen et al., 2012; Castano et al., 2019), in contrast to the relative abundance of EcM fungi which increased during the early and the intermediate stages of root colonisation (Figure 19). Both intra and intercellular colonisations by fungal hyphae were detected, with an increase of the density and diversity of fungal morphologies from 7 to 15 days. We observed the dominance and co-occurence of two major fungal morphologies “AM-like” and “Hand gloved-like” both in the adventive and lateral roots. Despite CLSM observations of AM morphologies within poplar root systems, their relative abundance was low in Miseq data, even if we detected their increase in roots collected after 10 days. However, these results must be confirmed by real-time PCR quantification of AM fungi (Voriskova et al., 2017). Indeed, our results as well as those of other studies showed that MiSeq high-throughput sequencing is not optimal for obtaining quantitative information on

AM fungi (Karlinski et al., 2010, Danielson et al., 2012, Bonito et al., 2014). We also detected by CLSM typical structures of dark septate endophyte in the roots system collected during the intermediate and late stage of colonization. These observations are in accordance with the detection of *Phialocephala*, *Cadophora* and *Leptodontidium* in poplar roots by amplicon sequencing. Berthelot et al, (2016) already isolated these DSE from poplar grown on a metal-polluted phytomanagement site and suggested their role in improving plant growth. As reported Jumpponen & Trappe (1998), we noted after CLSM observations that some DSE were often associated with EcM. Furthermore, we detected *Phialocephala* and *Leptodontidium* at the intermediate stage of colonisation whereas *Cadophora* was only present at the late stage according to amplicon sequencing. As recently described by Thoen et al. (2019), we also observed within a single ectomycorrhizal structure, a diverse range fungal morphologies and communities. These concurrent and successional occupation by distinct fungi also highlight the dynamics of the root colonising fungal communities.

From 15 to 21 days of *Populus* growth, CLSM observations and root DNA metabarcoding suggest a shift in the fungal composition, with the replacement of “AM-like” morphologies by the “Hand gloved-like” morphologies in lateral roots while “AM-like” morphologies remained present on adventive roots. This well known successional replacement of AM by EcM has been documented in different tree species such as eucalyptus (Lapeyrie et al., 1985; Chilvers et al., 1987; Castano et al., 2019) and poplar (Lodge et al., 1990). Nevertheless, these studies have been done on a long time scale, from 5 months to years, looking at the fungal colonisation dynamic of already grownup trees. According to the litterature, this is the first study attempting to characterise the fungal preliminary root colonisation from uncolonised root systems. The relative abundance of both saprotrophic and endophytic fungi decreased after 21 days, whereas the relative abundance of EcM fungi (e.g *Sebacina* and *Hebeloma*) tended to increase until the end of our root colonization monitoring. This correlates with the significant increase of the rate of ectomycorrhization until 50 days of root growth. The co-occurrence of distinct EcM fungi within the same root system has already been described in previous studies. For instance, Bahram et al. (2010) characterised the poplar EcM communities and recovered more than 122 EcM species within the same aspen root system. Furthermore, we observed the successional turnover of distinct EcM fungi in poplar root systems, going from *Sebacina* at the early stage of colonisation, replaced by Thelephoraceae and *Geopora* at an intermediate stage and dominated by Thelephoraceae, *Hebeloma*, and *Sebacina* in the late stage of colonisation. Interestingly, *Hebeloma* and *Telephora* have been discribed as “early-stage” EcM fungi colonisers in birch roots, with *Hebeloma* having the specificity to exclude colonisation by other mycorrhizal fungi (Fleming, 1985). Our results contrast with this priority concept for EcM colonisation as most changes in the EcM composition were due to fungal species present in soil that became more competitive over time (Kennedy et al., 2009). No changes in climatic or edaphic factors could explain these variations, thus plant-microbe related factors are responsible for the shift in EcM communities. Finally, we detected two new abundant fungal structures at the later time-point. The first one was intracellular in both adventive and lateral roots while the second one was only detected in mycorrhized roots. Further analysis using specific probes targeting a particular fungal species (such as Fluorescence In Situ Hybridization FISH) might help resolving the identification of the morphologies observed.

These observations infer the successional replacement of saprotrophic and endophytic fungi to the benefit of EcM fungi across the root development. It also points the important selection pressure undergoing between the plant root system and the soil microorganisms.

Conclusions

In conclusion, our data show that microbial communities of the natural soil successively colonized *Populus* roots punctuated by significant difference in the structure and composition of both fungal and bacterial communities. Bacteria were selected by the tree roots at a very early stage compared to fungi that were selected at a later stage. Our observations constitute a first phase of exploration of the establishment of tree-microbes' interactions as soon as roots appear and come into contact with the soil. Future studies on the interaction mechanisms of root microbiome could help us to understand how plant selection (and the mechanisms involved) and competition between microorganisms play a role in this dynamic of tree root colonization.

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IV. Conclusions

Nos données montrent que les communautés microbiennes issues du sol naturel de peupleraie ont colonisé les racines des jeunes plantules de peupliers de manière successive. Cette colonisation successive est ponctuée par des différences significatives en termes de structure et de composition des communautés bactériennes et fongiques entre les différents points de prélèvement. Les bactéries ont été sélectionnées par les racines à un stade très précoce par rapport aux champignons qui ont été sélectionnés à un stade plus tardif.

Nos résultats constituent une première phase d'exploration dans l'établissement des interactions entre les arbres et les micro-organismes dès l'apparition des racines et les premiers contacts avec le sol environnant. De futurs études sur la colonisation racinaire du peuplier et d'autres espèces d'arbres par les micro-organismes du sol pourraient aider les chercheurs à développer un inoculum microbien efficace pour améliorer la croissance et la santé des arbres et des plantes pérennes.