Etude de l’expression hétérologue de l’effecteur fongique MiSSP7 sur la structuration et la composition du microbiote racinaire et sur le métabolome du Peuplier

I. Contexte général

Des mutations dans la voie de signalisation de l'AJ conduisent à des modifications de la composition du microbiote associé à la racine de la plante modèle herbacée, *Arabidopsis thaliana*, en modifiant la composition des exsudats racinaires (Carvailhais et al., 2015). L'activation de la voie de signalisation de l'AJ chez *A. thaliana* a également été liée à l'augmentation de l'abondance relative de certains membres de communautés bactériennes (Carvalhais et al., 2013). Ces études suggèrent que la voie de signalisation de l'AJ n'est pas seulement un élément clé du système de défense, mais qu'elle participe également à la formation du microbiote racinaire complet des plantes herbacées. Cependant, aucune étude explorant l'impact de la voie de signalisation de cette phytohormone sur le microbiote racinaire des arbres n'est, à l'heure actuelle, disponible dans la littérature scientifique. Il est connu que la modulation de la voie de signalisation de l'AJ serait nécessaire pour permettre l'établissement d'une symbiose ectomycorhizienne (EcM) entre *Laccaria bicolor* et les racines du peuplier (Plett et al. 2014). Le champignon ectomycorhizien sécrète une petite protéine de 7 kDa appelée MiSSP7 qui pénètre dans le noyau des cellules racinaires de l'hôte où elle interagit avec le répresseur PtJAZ6. Il a été démontré qu'en stabilisant le complexe PtJAZ6, MiSSP7 bloque le déclenchement de la voie de signalisation de l'AJ et, ainsi, limite les mécanismes de défense qui empêcheraient la colonisation des racines par *L. bicolor* (Martin et al. 2016). Néanmoins, nous ne savons pas si l'altération de la voie de signalisation de l'AJ module seulement l'établissement de la symbiose EcM ou si elle a un impact plus large sur le microbiote.

II. Objectifs

Dans ce contexte, les objectifs de ce chapitre sont, d'une part, de caractériser la composition et la structure taxonomique des communautés bactériennes et fongiques de la rhizosphère et de l'endosphère de peupliers exprimant de manière constitutive l'effecteur MiSSP7 en comparaison avec des peupliers sauvages (non génétiquement modifiés), et, d'autre part, d'évaluer si les potentielles modifications de la composition du microbiote racinaire sont également visibles au niveau du métabolome et du métatrancriptome racinaire des peupliers génétiquement modifiés.

Nous avons tenté de répondre à plusieurs questions :

- Est-ce que la répression de la voie de signalisation de l'AJ par MiSSP7 a un impact sur la physiologie du peuplier ?
- Est-ce que la répression de la voie de signalisation de l'AJ par MiSSP7 a un impact sur le métabolome racinaire du peuplier ?
- Est-ce que la répression de la voie de signalisation de l'AJ par MiSSP7 a un impact sur le métatranscriptome racinaire du peuplier ?

- Est-ce que les manipulations de la voie de signalisation de l'AJ par MiSSP7 influencent la composition et la structure de l'ensemble du microbiote racinaire du peuplier ou seulement la colonisation par *Laccaria bicolor* ?

III. Démarche expérimentale

Afin de répondre à ces questions, le sol de peupleraie précédemment décrit (Chapitre IV) a été utilisé comme substrat et inoculum naturel pour la culture de boutures de peupliers (*Populus tremula x alba*) exprimant de manière constitutive le gène fongique MiSSP7 (peupliers MiSSP7) et de boutures de peupliers non génétiquement modifiées (peupliers sauvages). Ces boutures ont été prélevées sur leur arbre mère en 2016 (Année 1) et en 2017 (Année 2), cultivées en hydroponie pour contrôler l'enracinement et ensuite, cultivées dans le sol naturel en serre durant 10 jours ou 6,5 semaines. La culture de ces boutures nous a permis de caractériser et comparer la structure et la composition taxonomique des communautés microbiennes (bactéries et champignons) associées aux racines ainsi que le métabolome racinaire des deux populations de peupliers (MiSSP7 et sauvages) cultivées l'année 1 et l'année 2.
Les communautés bactériennes et fongiques du sol et des racines ont été caractérisées par séquençage Illumina MiSeq haut débit des amplicons d'ADNr ITS et d'ARNr 16S tandis que le métabolome racinaire a été analysé par GC-MS. En plus de ces deux types d'analyses, nous avons comparé certains paramètres phénotypiques entre les deux populations de peupliers (MiSSP7 et sauvages) et réalisé une analyse des transcriptomes et du métatranscriptomes des racines de ces peupliers cultivés durant l'année 2.

L'ensemble des résultats de cette étude est présenté ci-après sous la forme d'un article scientifique. Les figures et tableaux supplémentaires sont disponibles en Annexe (Annexe 4 de la page 53 à la page 75).
Contrairement aux articles en préparation présentés dans les chapitres II et III, la publication de ce dernier chapitre n'est pas prévue à court terme.

Is Jasmonic acid signaling pathway a hub for controlling the *Populus tremula x alba* root microbiome colonization?

Introduction

The roots of terrestrial plants are an important habitat for microorganisms. Plants transfer up to 40 % of their photosynthetically-fixed carbon to their roots and 10 to 30 % of this carbon is allocates to the root microbiome (Dilkes et al., 2004; Kaiser et al., 2014). The root microbiome corresponds to the dynamic community of microorganisms associated with plant roots. It is mainly composed of bacteria and fungi recruited from the surrounding bulk soil, the area of soil outside the rhizosphere or vertically transmitted from generation to generation (i.e, the endophytes; Frank et al., 2017). Major interactions between micro-organisms and plant roots are known to be those between the microbial communities of the rhizosphere and the roots. The main players of the root microbiome are fungi and bacteria that can reach cell densities up to 10^8 cells, a number much greater than the number of plant cells (Mendes et al., 2013). These microorganisms play an important role for plant growth and health by increasing water and nutrient acquisition (Pii et al., 2015; Bowles et al., 2017) but also for plant resistance against biotic and abiotic stresses (Zelicourt et al., 2013). By contrast, some microbial members of the root microbiome are pathogenic and detrimental for plant growth and health (Raaijmakers et al., 2009). Additionally, thanks to its plasticity, its short-term dynamic and its large pool of genes, the tree root microbiome has a much higher potential capacity to adapt to environmental changes compared to the plants (Goh et al., 2013; Mendes et al., 2013) and thus offer potential interesting perspective of microbial-plant engineering.
Research on the interactions between tree roots and their microbiome has exponentially increased in the past 15 years. The development of next generation sequencing technologies and associated computational analysis tools allow more detailed investigation of factors which impact the composition and structure of root microbiome (Bulgarelli et al., 2012; Lundberg et al., 2012). Such analyses revealed the complexity of interactions between microorganisms and plant roots. This complexity, already perceptible in annual and crop plants, is exacerbated in trees and shrubs, where seasonal climatic variability, difference in life style compared to herbaceous plant in terms of nutrient allocation and root system development are known to differentially influenced microbial communities associated with roots.

Studies performed on tree species revealed that root microbiome of woody plant composition is influenced by both environmental factors such as soil properties and climate (Compant et al., 2010; Voriskova et al., 2014; Bonito et al., 2014; Mercado-Blanco et al., 2018) and host factors such as root exudates and root secondary metabolites (Tschaplinski et al., 2014; Hacquard & Schadt, 2015; Wagner et al., 2016; Gallart et al., 2018). In addition to these factors modulating and shaping root microbiome composition and functions, microbial communities may also have to deal with plant immunity and defence mechanisms. Indeed, plants have evolved a sophisticated immune system to detect and respond to potential microbial invaders (Jones & Dangl, 2006).

Cell-surface and intracellular localized pattern recognition receptors (PRR) can detect pathogens by recognizing microbe-associated molecular patters (MAMPs) which are molecules that are generally conserved through a wide range of microorganisms (Boller & Felix, 2009). Beneficial microorganisms possess MAMPs that are very similar to those of pathogens. During the initial contact with plant roots, beneficial microorganisms are also recognized by PRRs, activating plant immune system signalling pathways (Yu et al., 2019). Plant defence is partly mediated by phytohormones that trigger the activation of signalling pathways involved in defence mechanisms against pathogens. One of these defence phytohormone is jasmonic acid (JA). JA is involved in the control of cell development and cycling, of vegetative growth and in the mediation of plant defensive response against necrotrophic pathogens (McDowell & Dangl, 2000; Thomma et al., 2001). In addition, recent evidences suggest that these defence mechanisms could play important roles in helping plants to recruit microbiomes that enhance stress tolerance (Pineda et al., 2013). Furthermore, JA signalling influences the composition of the root-associated microbiome of the herbaceous plant model *Arabidopsis thaliana* by altering root exudate composition (Carvailhais et al., 2015). Activation of JA signalling pathway in *A. thaliana* was also linked to the increase of the relative abundance of bacterial communities closely related to taxa that are reported to supress phytopathogens colonisation (e.g., *Bacillus* population, *Paenibacillus amylolyticus* and *Lysinibacillus*-related population; Carvalhais et al., 2013). Altogether these studies suggest that JA signalling is not only a key element of the defence system but that it also participates to the shaping of the whole root microbiome of non-perennial plants.
Less is known regarding trees. However, the modulation of JA signalling would also be necessary to allow the establishment of ectomycorrhizal (EcM) symbiosis between *Laccaria bicolor* and poplar roots (Plett et al., 2014). The ectomycorrhizal fungus secretes a Mycorrhizal induced Small Secreted Protein of 7kDa (MiSSP7) that penetrates in the nucleus of *Populus* root cells where it interacts with PtJAZ6 and competes for the binding of jasmonate. By stabilizing PtJAZ6 complex, MiSSP7 is thought to block the triggering of JA signalling and thus to limit defence mechanisms that would preclude the colonization of *Populus* roots by *L. bicolor* (Martin et al., 2016). Whether the alteration of JA signalling modulates only the establishment of the EcM symbiosis or whether it has a broader impact on the microbiome is unknown.

In order to answer this question, we analysed the microbial communities of *Populus tremula x alba* lines constitutively expressing the fungal effector MiSSP7 and we compared it to the ones of wild type lines (WT). We hypothesized that, due to the alteration of the JA signalling pathway by MiSSP7 expression in *Populus*, the root microbiome composition and structure would vary compared to the control lines.
Rooted cuttings of WT and MiSPP7 lines were planted in pots containing natural soil taken from a poplar plantation and were grown in a greenhouse under controlled conditions. Bulk soil, rhizospheric and endospheric microbiomes were characterized using 16S and ITS rRNA gene-targeted amplicon Illumina MiSeq sequencing. Growth of each *Populus* line was monitored to observe the potential effect of MiSSP7 expression on tree development. Metabolomes of roots were also characterized by mass spectrometry (GC-MS) to determine the potential effects of MiSSP7 expression and the alteration of the microbiome on the physiology of the roots. Finally, a metatranscriptome analysis was performed on the roots of each *Populus* line to observe if MiSSP7

expression was responsible of potential differences in the root microbiome activity and in the activity of the root as well. Analyses of microbial community (i.e DNA metabarcoding) and root metabolome characterization was repeated over two consecutive years to control the reproducibility of the effects measured.

Material and Methods

Tree cutting origin and cultivation conditions

Different lines of *Populus tremula x alba* (clone INRA 717-1B4) were used in this study: wild type *P. tremula x alba* (WT) and four independent lines of *P. tremula x alba* constitutively expressing MiSSP7 (MiSSP7.1, MiSPP7.2, MiSSP7.3 and MiSSP7.6 lines, Plett et al., 2011). For each line, seven to 30 cuttings were taken from mother trees both years in February and were conserved at 4°C until being transferred in hydroponic solution to stimulate rooting and synchronize growth (Hoagland's nutrient solution; Hoagland & Arnon, 1950). Immersed parts of the cuttings were maintained in the dark and were aerated by air bubbling. Hydroponic cultivation was done in a class 2 GMO greenhouse at 20°C and with a 16h light period for a month. Rooted cuttings with similar root length were then transferred at the beginning of the summer of Year 1 and Year 2 (June 13th, 2016 and July 6th, 2017) to one litre pots containing natural soil taken from a poplar stand (see description below). Cuttings were further grown in the same glasshouse at 20°C and with a 16h light period for 6.5 weeks. Humidity in pots was maintained at approximately 75 % during all the experiments. At least 6 cuttings per line were planted independently each year (Table S1).

Seventy-two hours before the transplant of cuttings in pots in greenhouse, the top soil horizon used and characterized in our previous study (Mangeot-Peter et al., 2020) was collected. The soil was collected under the same tree in June 2016 and in July 2017 following the procedure described in Mangeot-Peter et al. (2020). Briefly, soil was stored at room temperature for 72 hours before being sieved at 2 mm and adjusted to 75 % of humidity. One litre of soil was transferred in each pot.

Monitoring of cutting growth

Phenotypic parameters of MiSSP7 and WT cuttings cultivated in Year 2 were monitored throughout the cultivation period, prior to sampling. Cutting diameters, length and fresh weight were measured before the establishment of the hydroponic culture. Number of roots and length of the main root were measured every week from the beginning of rhizogenesis to the end of the hydroponic culture. The number of leaves, the leaf surface and the stem length were monitored once a week from the transfer of cuttings in pots to the sampling time.

Sampling procedures

Samples were taken at three time points: at the transplanting time (T0), after 10 days (T1) and after 6,5 weeks of growth (T2) of growth in pots. At T0, samples of initial soils were collected in triplicates of 50 g, dried at 30 °C and stored at room temperature in an airtight container until physico-chemical analysis (see below). Samples of roots

collected after the hydroponic culture (T0) were freezed in liquid nitrogen and conserved at -20°C until DNA extraction.

At T1 and T2, five types of samples were collected on each *Populus* cuttings (i.e., WT and MiSSP7) in each pot. These samples correspond to the leaves, the bulk soil (BS, the soil in an area free of cuttings root in pot), the adherent soil of the roots (AS, the rhizosphere), the rhizoplane (Rh, the external surface of the roots) and the endosphere (E, the internal tissue of the roots).

BS, AS, Rh and E samples were collected according to the collection procedure detailed in Gottel et al., 2011 for microbiome analyses. Briefly, bulk soil samples were collected from each pot in an area free of roots and stored at -20°C until DNA extraction while five representative parts of the total root system were collected: (i) One part was cleaned with 10 mM NaCl solution, freezed in liquid nitrogen and stored at -80°C until being lyophilized then processed for metabolomic analysis. (ii) The second representative part of each root system was shaken to remove non-adherent soil, and washed in a centrifuge tube containing 35 mL of 10 mM NaCl solution to remove the root adherent soil (i.e, the rhizosphere). Then, tubes were centrifuged at 6,500 g for 15 min and the pellet was aliquoted in 2 mL tubes and stored at -20°C until DNA extraction. (iii) The surface of the third part of roots (i.e., the endosphere) were sterilized by using the sterilization steps described by Gottel et al., 2011: washed with sterile H_2O 3 times, 3% H_2O_2 for 30 s, 100% ethanol for 30 s, 6.15% NaOCl with 2 to 3 drops of Tween 20 per 100 mL for 3 min, 3% H_2O_2 for 30 s and finally 3 washed with sterile H_2O. Surface sterility was confirmed for all samples by touching a subsampled root from each collection onto LB plates and incubating overnight at 30°C. (iv) Another batch of roots were taken for qPCR assays and metatranscriptomic analyses. The roots were cleaned with sterile 10 mM NaCl solution and immediately dried freeze in liquid nitrogen and conserved at -80°C until RNA extractions. Finally, (v) the last sample of roots system collected in Year 2 at T2 was used to quantify the ectomycorhization rate according to the procedure detailed in Labbé et al., 2011. Briefly, each root system was rinsed with tap water and analysed under a dissecting microscope. For each root system, 100 short roots were randomly examined and assessed as mycorrhizal or non-mycorrhizal. In addition, ectomycorrhizae was harvested and conserved at -20°C until DNA extraction for identification.

All the leaves of each cuttings were sampled in each *Populus* cuttings, freezed in liquid nitrogen and stored at -80°C until RNA extraction. Detailed information about the number of each sample types collected in Year 1 and Year 2, at T1 and T2, and used for each experimental approach is available in Table 5. In total, thirteen tree cuttings (3 WT and 10 MiSSP7) were sampled at T1, 18 (4 WT and 14 MiSSP7) at T2 in Year 1, 14 (6 WT and 8 MiSSP7) at T1 in Year 2 and 22 (6 WT and 16 MiSSP7) at T2 in Year 2 (Table 5).

Table 5 - Number of samples used for each experimental approach performed in this study.

***Populus tremula x alba* cuttings used in Year 1 at T1**	**qpCR (Leaves samples)**	**16S amplification**	**ITS amplification**	**Root metabolome study (Roots samples)**	**Metatranscriptome study (Roots samples)**
WT	**0**	BS: 3 ; AS: 3 ; Rh: 3 ; E: 3	BS: 3 ; AS: 3 ; Rh: 3 ; E: 3	**3**	
MiSSP7 lines	0	BS: 10 ; AS: 10 ; Rh: 10 ; E: 8	BS: 10 ; AS: 10 ; Rh: 10 ; E: 9	6	
***Populus tremula x alba* cuttings used in Year 1 at T2**	**qpCR (Leaves samples)**	**16S amplification**	**ITS amplification**	**Root metabolome study (Roots samples)**	**Metatranscriptome study (Roots samples)**
WT	**4**	BS: 4 ; AS: 4 ; Rh: 4 ; E: 4	BS: 4 ; AS: 4 ; Rh: 4 ; E: 4	**4**	
MiSSP7_High exp	4	BS: 4 ; AS: 3 ; Rh: 4 ; E: 3	BS: 4 ; AS: 3 ; Rh: 4 ; E: 3	4	
MiSSP7_Low exp	8	BS: 8 ; AS: 7 ; Rh: 8 ; E: 8	BS: 8 ; AS: 8 ; Rh: 8 ; E: 8	7	
***Populus tremula x alba* cuttings used in Year 2 at T1**	**qpCR (Roots samples)**	**16S amplification**	**ITS amplification**	**Root metabolome study (Roots samples)**	**Metatranscriptome study (Roots samples)**
WT	**6**	BS: 6 ; AS: 6 ; Rh: 6 ; E: 6	BS: 6 ; AS: 6 ; Rh: 6 ; E: 6	**6**	
MiSSP7_High exp	3	BS: 3 ; AS: 3 ; Rh: 3 ; E: 3	BS: 3 ; AS: 3 ; Rh: 3 ; E: 3	3	
MiSSP7_Low exp	5	BS: 4 ; AS: 5 ; Rh: 3 ; E: 5	BS: 5 ; AS: 5 ; Rh: 5 ; E: 4	5	
***Populus tremula x alba* cuttings used in Year 2 at T2**	**qpCR (Roots samples)**	**16S amplification**	**ITS amplification**	**Root metabolome study (Roots samples)**	**Metatranscriptome study (Roots samples)**
WT	**6**	BS: 6 ; AS: 6 ; Rh: 6 ; E: 6	BS: 6 ; AS: 6 ; Rh: 6 ; E: 6	**6**	**6**
MiSSP7_High exp	9	BS: 8 ; AS: 9 ; Rh: 8 ; E: 6	BS: 8 ; AS: 8 ; Rh: 8 ; E: 8	7	9
MiSSP7_Low exp	5	BS: 4 ; AS: 4 ; Rh: 4 ; E: 3	BS: 5 ; AS: 5 ; Rh:5 ; E: 4	4	5

DNA extraction, amplification and Illumina MiSeq sequencing

To minimize DNA extraction bias, DNA was extracted in triplicate from the bulk soil. Approximately 250 mg of soil samples was used for each individual DNA extraction. DNA was extracted using the DNeasy PowerSoil Kit following the protocol provided by the manufacturer (Qiagen, Venlo, the Netherlands). Fifty mg of root tissues and isolated ectomycorrhizae were crushed in liquid nitrogen and pulverized tissue was extracted using the DNAeasy PowerPlant Kit (Qiagen, Venlo, the Netherlands). All extractions were quantified on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

A two-step PCR approach was chosen in this study to barcode tag templates with frameshifting nucleotide primers (Mangeot-Peter et al., 2020). 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 forward and 2 reverse 515F and 806R primers screening the 16S rRNA V4 gene region in equal concentration (0.1 μM). 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). To inhibit plant material amplification, a mixture of peptide nucleotide acid (PNA) blockers targeting 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). 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).

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 and sequences were filtered on the additional following criteria. Sequences were removed from data set, if non-barcoded, if sequences 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 singletons (OTUs containing only one sequence) 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 was 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 evaluated based on the results obtained for the bacterial mock community.

For fungal reads, after demultiplexing and quality checking (QC quality score = 28, minimal size = 180 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 calculated to assess sampling completeness, using function *rarecurve()* in package Vegan v3.5-1 (Oksanen et al., 2015) in R (version 3.4.3 ; R Core Team, 2016). Based on these, subsequent analyses of diversity and community structure were performed on datasets where samples had been rarefied with the Phyloseq (McMurdie & Holmes, 2013) package to achieve equal read numbers according to the minimum number of total reads in any sample (8,982 bacteria, 8,005 fungi). Microbial community composition and structure in bulk soil and roots data were further analysed by using Phyloseq package (McMurdie & Holmes, 2013).

FUNGuild (Nguyen et al., 2016) was used to classify each 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 assignations were called as "unclassified".

Molecular identification of EcM root tips

Ectomycorrhiza DNAs were amplified using the primer pair ITS1F and ITS4. PCR products (size and concentration) were controlled by gel electrophoresis, and successful amplifications were purified using the GeneMatrix PCR/DNA Clean-Up purification kit (EURx, Gdansk, Poland) as per the manufacturer's instructions. Purified products were then sequenced by the Sanger sequencing forward approach (Eurofins, Germany). Sequences were then blasted against UNIT database.

RNA extraction and analysis of the MiSSP7 expression in transformed lines

Approximately 80 mg of tissues (leaves collected in Year 1 and roots collected in Year 2) were used for total RNA extraction using the Plant RNAeasy kit (Qiagen) as per the manufacturer's instructions except the addition of 20 mg poplyethylene glycol 8000 per mL of RLC buffer. A on-column DNA digestion step was peformed with the DNase Removal kit (ThermoFisher Scientific, Carlsbad, CA, USA) to avoid DNA contamination. RNA were quantified by using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA quality was verified by Experion HighSens capillarity gels (Bio-Rad). Synthesis of cDNA from 400 ng of total RNA extracted from leaves and/or roots of each *Populus* cuttings was performed using the iScript kit (Bio-rad). Expression levels of *missp7* transcripts in transgenic poplars was compaired to the one of *missp7* in 2 weeks old ectomycorrhizae of *Laccaria bicolor* in *Populus tremula x alba* roots cultivated in axenic conditions. A Chromo4 Light Cycler Real-time PCR was used for real-time PCR analyses with two technical replicates per biological replicate using the SYBRGreen Supermix following the manufacturer's instructions (Bio-Rad; 5.5 µL of primers 10 µM [MiSSP7_Fwd: ATTGCTGCGTTAGCCATCTC and MiSSP7_Rev: GGAATTGGTCCTCTCTCAACC], 2 µL of cDNA at 400 ng, 7.5 µL of SYBRGreen Supermix). Thermal cycler conditions for qPCR were 40 cycles of 95°C for 15s and 60°C for 10s. Normalization in gene expression between the target gene MiSSP7 and the reference gene UBIQUITIN (Plett et al., 2011) were calculated. Relative expression of MiSSP7 in each cuttings and in the

control (i.e., 2 weeks old ectomycorrhizae of *Laccaria bicolor* in *Populus tremula x alba* roots) was normalized with the gene reference UBIQUITIN.

qPCR products were purified using the GeneMatrix PCR/DNA Clean-Up purification kit (EURx, Gdansk, Poland) as per the manufacturer's instructions. Purified products were then sequenced by the Sanger sequencing approach to check if all amplification products correspond to the sequence of MiSSP7.

All samples were then classified according to their degree of MiSSP7 expression : Not expressed, MiSSP7_Low expression and MiSSP7_Low expression.

Metabolome analysis

Root metabolome extraction and analyses were carried out as previously described by Tschaplinski et al. (2014). Briefly, between 20 and 100 mg fresh weight of roots and leaves samples were ground to a fine powder in liquid nitrogen, weighed and then transferred to an acid washed glass scintillation vial containing 2.5 mL 80% ethanol. Sorbitol was used before extraction as an internal standard to correct potential extraction efficiency difference and to normalized final concentration of metabolites in each sample. After overnight extraction and the solvent transferred into another vial, each sample pellets were re-extracted with another 2.5 ml of 80 % ethanol overnight and the supernatant combined with the prior extract. Using a nitrogen stream, 2 mL of this solution were dried and stored at -80°C until analysis. The samples were dissolved in 750 µL of aqueous 80% ethanol, from which 500 µL were dried in a nitrogen stream. Metabolites were trimethylsilyl derivatized and analysed using gas chromatography-mass spectrometry (GC-MS), as previously described (Tschaplinski et al., 2014). The peaks of known metabolites were extracted using a key mass-to-charge (m/z) ratio and scaled back up to the total ion current using predetermined scaling factors. The scaling factor for sorbitol was used for unidentified metabolites. Unidentified metabolites were denoted by their retention time as well as key m/z fragments. Peaks were quantified by area integration and were normalized to the quantity of the internal standard (sorbitol) recovered, accounting for the volume of sample processed, derivatization volume, and injection volume.

Meta-Trasncriptome analysis

Illumina sequencing was performed at the Joint Genome Institute (JGI; https://jgi.doe.gov). Stranded RNASeq libraries were created and quantified by qPCR using their standard protocol for low input RNA samples. Sequencing was performed using an Illumina NovaSeq. A read preprocessing was performed as following :raw fastq file reads were filtered and trimmed using the JGI QC pipeline (http://1ofdmq2n8tc36m6i46scovo2e.wpengine.netdna-cdn.com/wp-content/uploads/2013/11/Poster_Singan.pdf), raw reads were evaluated for artifact sequence by kmer matching (kmer=25), allowing 1 mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases). Filtered reads from libraries were aligned to the reference genome (*Populus trichocarpa* v3.0 assembly with v3.1 gene annotation) using HISAT2 version 2.1.0 (Kim et al., 2015). FeatureCounts (Liao et al., 2014) was used to generate the raw gene counts file

using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts. FPKM and TPM normalized gene counts are also provided. Raw gene counts were used to evaluate the level of correlation between biological replicates using Pearson's correlation and determine which replicates would be used in the DGE analysis.

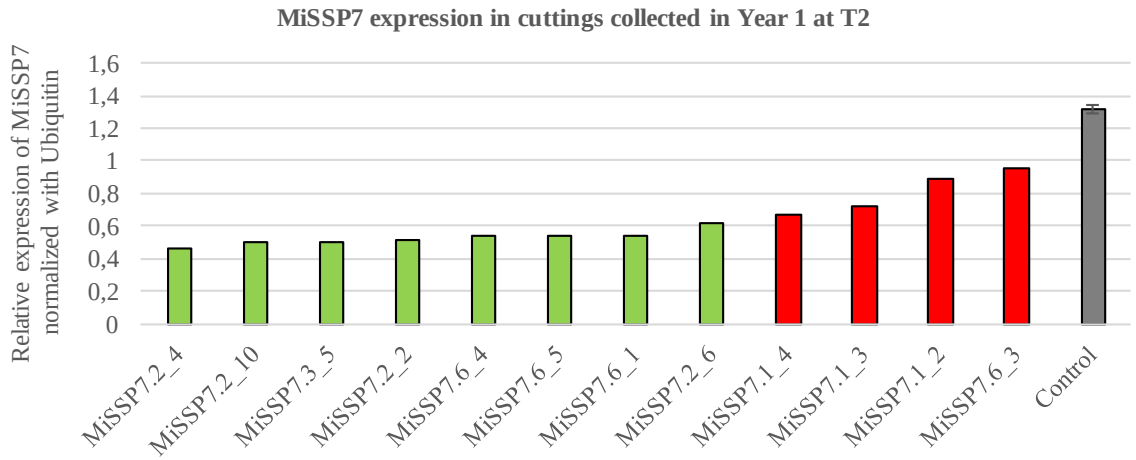
DESeq2 (version 1.18.1, Love et al., 2014) was subsequently used to determine which genes were differentially expressed between pairs of conditions. For the strandedness estimation, features assigned to the forward strand were also tabulated. Strandedness of each library was estimated by calculating the percentage of reverse-assigned fragments to the total assigned fragments (reverse plus forward hits).

Read pairs from all samples were used to perform a *de novo* co-assembly using Megahit version 1.1.3 with default parameters (Li et al., 2015). Contigs under 500 pb length were discarded. For each sample, reads were then mapped on the selected contigs using bowtie version 2.3.0 (Langmead et al., 2012), and counts were determined using SAMtools version 1.7 (Li et al., 2009). Contigs supported by less than 3 samples with at least 5 counts were then discarded. Remaining contigs were annotated using Diamond version 0.9.19 (Buchfink et al., 2015) with the parameters --more sensitive --max-target-seqs 1 --max-hsps 1 --evalue 0.00001 and JGI-Mycocosm fungal genomes (deposited before July 2018) as reference database. To check for fungal false positives, Diamond annotation was also used with NCBI-NR database (March 2018 version). Comparison between Mycocosm and NR was based on best bit-scores. Count tables and annotations were then analyzed using R version 3.4.3 and packages dplyR and ggplot2.

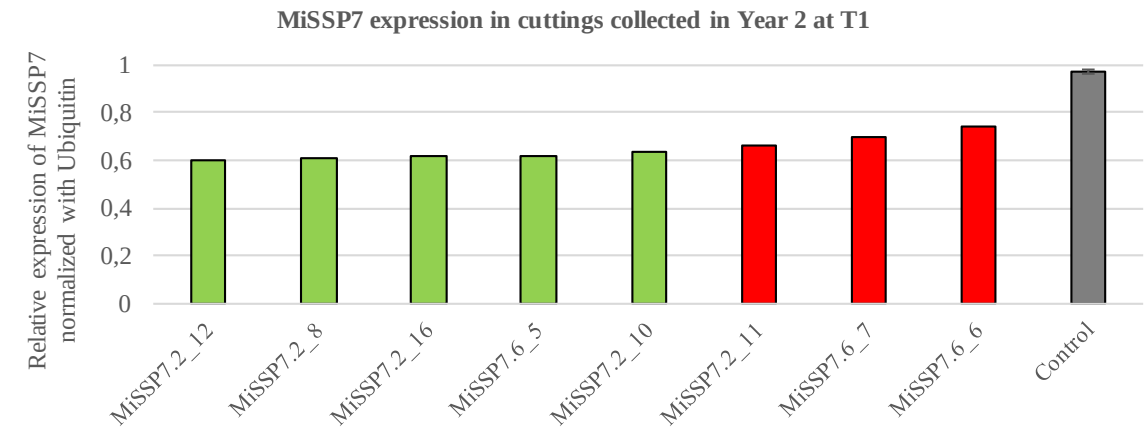
Statistical analysis

Statistical analyses and data representations were performed using R software (R Core Team, 2016). After checking normal distribution of each dataset with Shapiro-Wilk test, one-way ANOVA followed by a Tukey HSD multiple comparison test were used to determine if the relative abundance of dominant (>1%) bacterial and fungal phyla and genera differed in soil samples (BS and AS) and in roots samples (Rh and E) collected in Year 1 and Year 2 from *Populus* WT, MiSSP7_High expression and MiSSP7_Low expression cuttings. Microbial community structures were analysed using nonmetric multidimensional scaling analysis (NMDS) and permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity matrices. Wilcoxon test were used to determine difference in transcripts frequency of the most active fungal genera detected between *Populus*–expressing MiSSP7 (MiSSP7_Low expression or MiSSP7_High expression) and *Populus* WT roots collected in Year 2 at T2. The p-values of root metabolite differences were calculated using a One-way ANOVA and post-hoc HSD tests between the means of sets of MiSSP7 root samples (MiSSP7_Low expression or MiSSP7_High expression) and the means of sets of WT root samples collected in Year 1 and in Year 2 for each sampling time (T1 and T2), with the data expressed as fold changes relative to a WT samples.

a



b



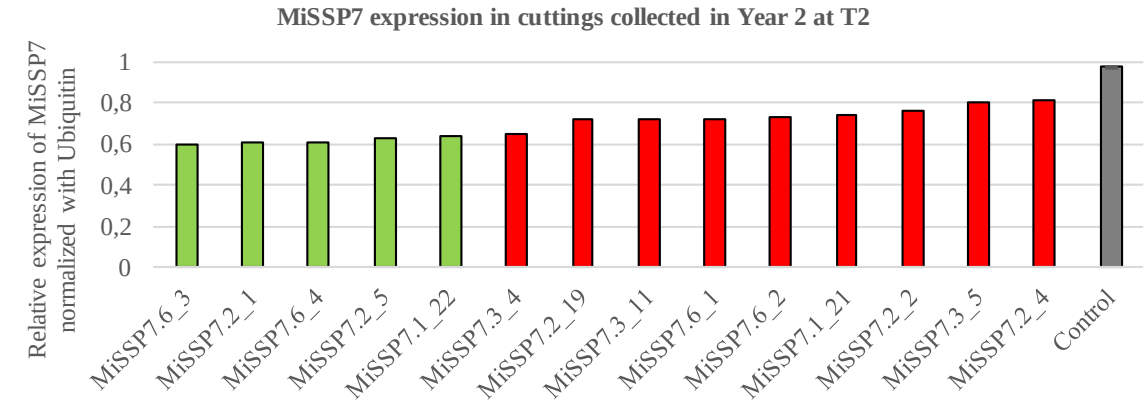


Figure 38 - The relative expression of MiSSP7 in each cutting used in this study. Relative expression of MiSSP7 normalized with Ubiquitin expression in the leaves of Populus-expressing MiSSP7 collected at T2 in Year 1 (a) and in the roots of Populus-expressing MiSSP7 collected at T1 and T2 in Year 2 (b). The green bars correspond to the cuttings with a low expression of MiSSP7 (between 0.40 and 0.60 in Year 1 and between 0.60 and 0.64 in Year 2) while the red bars correspond to the cuttings with a high expression of MiSSP7 (between 0.70 and 0.90 in Year 1 and between 0.65 and 0.80 in Year 2). The dark-grey bars correspond to the relative expression of MiSSP7 normalized with Ubiquitin in 2 weeks old ectomycorrhizae of Laccaria bicolor in Populus tremula x alba roots cultivated in axenic conditions (Control).

Results

The MiSSP7 expression in leaves and roots of *Populus* cuttings

qPCR analysis performed on the four MiSSP7 lines cultivated in natural soil revealed that several cuttings did not express the gene of interest anymore. In Year 1, only one cuttings of the MiSSP7.3 line collected at T2 did not express the gene of interest anymore. By contrast, 23% of the cuttings had lost the ectopic expression of MISSP7 on Year 2. Some lines were poorly impacted (e.g. MISSP7.1, MISSP7.6), while few cuttings kept expressing the gene in other lines (e.g. MISSP7.3, Table S1).

We observed that the expression of MiSSP7 in transgenic lines was lower compared to the one in the control (i.e, cDNA of 2 weeks-old *Laccaria*/*Populus* ectomycorrhizae) in Year 1 and in Year 2. (Figure 38). In addition, we observed an important variability in the expression level of MiSSP7 in each MiSSP7 cutting collected in Year 1 and Year 2, no matter the line considered (Figure 38). In Year 1, eight cuttings collected at T2 showed a low expression of MiSSP7 (between 0.40 and 0.60 after the normalization with the reference gene) and four cuttings showed a high expression of MiSSP7 (between 0.70 and 0.90 after the normalization with the reference gene). No qPCR analysis was performed on MiSSP7 cuttings collected at T1 in Year 1 because we could not successfully extract RNA from leaves and roots with these cuttings.

In Year 2, five cuttings collected at T1 showed a low expression of MiSSP7 (between 0.60 and 0.64 after the normalization with the reference gene) and three cuttings showed a high expression of MiSSP7 (between 0.66 and 0.75 after the normalization with the reference gene). At T2, five cuttings showed a low expression of MiSSP7 (between 0.60 and 0.64 after the normalization with the reference gene) and nine cuttings showed a high expression of MiSSP7 (between 0.65 and 0.80 after the normalization with the reference gene).

The impact of MiSSP7 ectopic expression on *Populus* growth and development

Phenotypic parameters were monitored before, during and after the hydroponic culture of MiSSP7 lines and WT to observe potential alterations of cuttings development and growth due to the constitutive expression of MiSSP7 in *Populus* (i.e. aerial parts and roots) in Year 2.

Significant differences were observed in phenotypic parameters monitored before the hydroponic culture of MiSSP7 and WT cuttings collected at T1. Indeed, the WT cuttings were longer and heavier than the MiSSP7 cuttings (ANOVA, $P<0.05$; WT > MiSSP7_Low > MiSSP7_High; Figure 39 a). In addition, we observed significant differences in the length of the main root between WT and MiSSP7 cuttings during the hydroponic culture for cutting collected at T1 (ANOVA, $P<0.05$; MiSSP7_Low > WT > MiSSP7_High; Figure 39 b). At T2, we also observed that WT cuttings were longer and heavier than the MiSSP7 cuttings (ANOVA, $P<0.05$; WT > MiSSP7_High > MiSSP7_Low; Figure 39 a). No significant differences were observed between WT and MiSSP7 cuttings collected at T1 and T2 for the phenotypic parameters monitored after the transfer in pots (i.e., number of leaves, leaf area, Figure 39 c) except for the length of the collected at T2 (ANOVA, $P<0.05$; WT < MiSSP_Low < MiSSP7_High, Figure 39 c).

*Table 6 - Permutational multivariate ANOVA results with Bray-Curtis distance matrices implemented to partition sources of variation in this study (compartments [BS, AS, Rh and E], sampling times [T1 and T2], treatment [MiSSP7 and WT cuttings] or expression level [WT, MiSSP7_Low exp and MiSSP7_High exp]) for fungal and bacterial communities at the OTU level. BS, AS, Rh and E samples of the WT and MiSSP7 cuttings collected in Year 1 were included in this analysis. Statistical significance (p-value) computed was based on sequential sums of square from 999 permutations (*** = P-value < 0.01, ** = P-value < 0.03, * = P-value < 0.05).*

Fungi_Year1			**PermANOVA**		
Compartment	**Time**	**Source of variation**	**F**	**R2**	**p-value**
BS, AS, Rh, E	T1 and T2	Compartment	12.45	0.220	***
		Time	14.16	0.080	***
		Treatment	3.19	0.018	***
BS	T1, T2 and T0	Time	9.37	0.215	***
		Treatment	2.04	0.094	*
BS	T1 and T2	Time	9.24	0.242	***
		Treatment	0.82	0.021	0.515
AS	T1 and T2	Time	11.38	0.280	***
		Treatment	1.26	0.030	0.227
Rh	T1 and T2	Time	5.04	0.145	***
		Treatment	2.34	0.067	*
E	T1 and T2	Time	2.44	0.084	**
		Treatment	1.69	0.058	0.060
BS	T1	Treatment	0.97	0.080	0.513
BS	T2	Expression	0.86	0.104	0.581
BS	T2	Treatment	0.78	0.048	0.520
AS	T1	Treatment	0.85	0.072	0.642
AS	T2	Expression	1.39	0.162	0.140
AS	T2	Treatment	1.36	0.079	0.197
Rh	T1	Treatment	1.96	0.151	*
Rh	T2	Expression	0.55	0.065	0.943
Rh	T2	Treatment	1.88	0.111	0.075
E	T1	Treatment	1.01	0.100	0.482
E	T2	Expression	1.01	0.121	0.432
E	T2	Treatment	1.59	0.096	0.095
Bacteria_Year1			**PermANOVA**		
Compartment	**Time**	**Source of variation**	**F**	**R2**	**p-value**
BS, AS, Rh, E	T1 and T2	Compartment	35.58	0.055	***
		Time	3.35	0.014	*
		Treatment	4.17	0.471	***
BS	T1, T2 and T0	Time	3.60	0.102	***
		Treatment	1.72	0.098	**
BS	T1 and T2	Time	3.59	0.118	***
		Treatment	0.84	0.027	0.684
AS	T1 and T2	Time	5.34	0.169	***
		Treatment	1.19	0.037	0.207
Rh	T1 and T2	Time	8.92	0.238	***
		Treatment	2.43	0.065	*
E	T1 and T2	Time	3.94	0.137	***
		Treatment	1.67	0.058	*
BS	T1	Treatment	0.72	0.061	0.836
BS	T2	Expression	1.23	0.081	0.135
BS	T2	Treatment	1.04	0.068	0.362
AS	T1	Treatment	0.85	0.072	0.662
AS	T2	Expression	1.21	0.082	0.170
AS	T2	Treatment	1.47	0.100	0.053
Rh	T1	Treatment	1.85	0.143	0.051
Rh	T2	Expression	2.45	0.146	*
Rh	T2	Treatment	1.27	0.076	0.197
E	T1	Treatment	1.87	0.172	*
E	T2	Expression	0.87	0.060	0.615
E	T2	Treatment	1.60	0.110	*

Concerning the ectomycorrhization rate calculated from MiSSP7 cuttings collected in Year 2 at T2, no significant differences were observed between *Populus* lines (WT vs. MiSSP7_Low vs. MiSSP7_High). However, the ectomycorrhization rate tended to be higher in MiSSP7_Low cuttings compared to WT and MiSSP7_High cuttings (ANOVA, P=0.07; Figure 40). Collected ectomycorrhizae mostly belonged to *Thelephora* sp. (Figure 40).

Microbial sequencing results

In order to characterize the structure and composition of soil- and root-associated microbiomes of *Populus* WT and *Populus*-expressing MiSSP7 lines, MiSeq sequencing of rDNA 16S and ITS rDNA were performed on DNA extracted from bulk soil, rhizosphere, rhizoplan and endosphere in two independent experiments performed over two years. After quality filtering, chimera and singleton removals, a total of 399,560 fungal reads (6,445 ± 16 reads per sample) and 1,777,000 (29,000 ± 814 reads per sample) for Year 1, and 363,130 fungal reads (5,044 ± 275 reads per sample) and 1,135,000 (15,500 ± 494 reads per sample) for Year 2 were kept for further analyses. After taxonomic assignment, elimination of contaminants and rarefactions of the sequences, 908 fungal OTUs (263 ± 13 OTUs per sample) and 1,938 bacterial OTUs (1087 ± 30 OTUs per sample) in Year 1, and 985 fungal OTUs (149 ± 8 OTUs per sample) and 2,030 bacterial OTUs (1,088 ± 24 OTUs per sample) in Year 2 were retained for further analyses.

Structure and composition of microbial communities of each compartment in *Populus* WT and *Populus*-expressing MiSSP7 line cultivated in Year 1

In Year 1, we observed a significant effect of the time, the treatment (WT vs MiSSP7) and the expression (WT, MiSSP7_Low exp and MiSSP7_High exp) on the structure of both fungal and bacterial communities associated to the soil (BS and AS) and roots (Rh and E) samples of the cuttings collected at T1 and T2 (Table 6). At T1, the structure of fungal communities associated to the rhizoplane of the cuttings was significantly impacted by the treatment at the OTU level. Concerning bacterial communities, the structure was significantly impacted by the treatment in the endosphere and tended also to be impacted by the treatment in the rhizoplane (P=0.051, PermANOVA, Table 6). At T2, the structure of fungal communities associated to the rhizoplane tended to be impacted by the treatment (P=0.075, PermANOVA, Table 6) while the structure of bacterial communities associated to the rhizoplane and the endosphere was significantly impacted by expression level of MiSSP7 and by the treatment (P<0.05, PermANOVA, Table 6).

Detailed analyses of the composition of fungal communities showed significant differences in the relative abundance of the most dominant fungal and bacterial genera colonizing the rhizosphere and the roots of WT and MiSSP7 cuttings at T1. For instance, the endophytes *Mortierella* and *Phialocephala* were significantly enriched in AS, Rh and E samples of the WT cuttings while *Cryptococcus* was significantly enriched in the Rh samples of the MiSSP7 cuttings (P<0.05, ANOVA, Figure S1, Table S2).

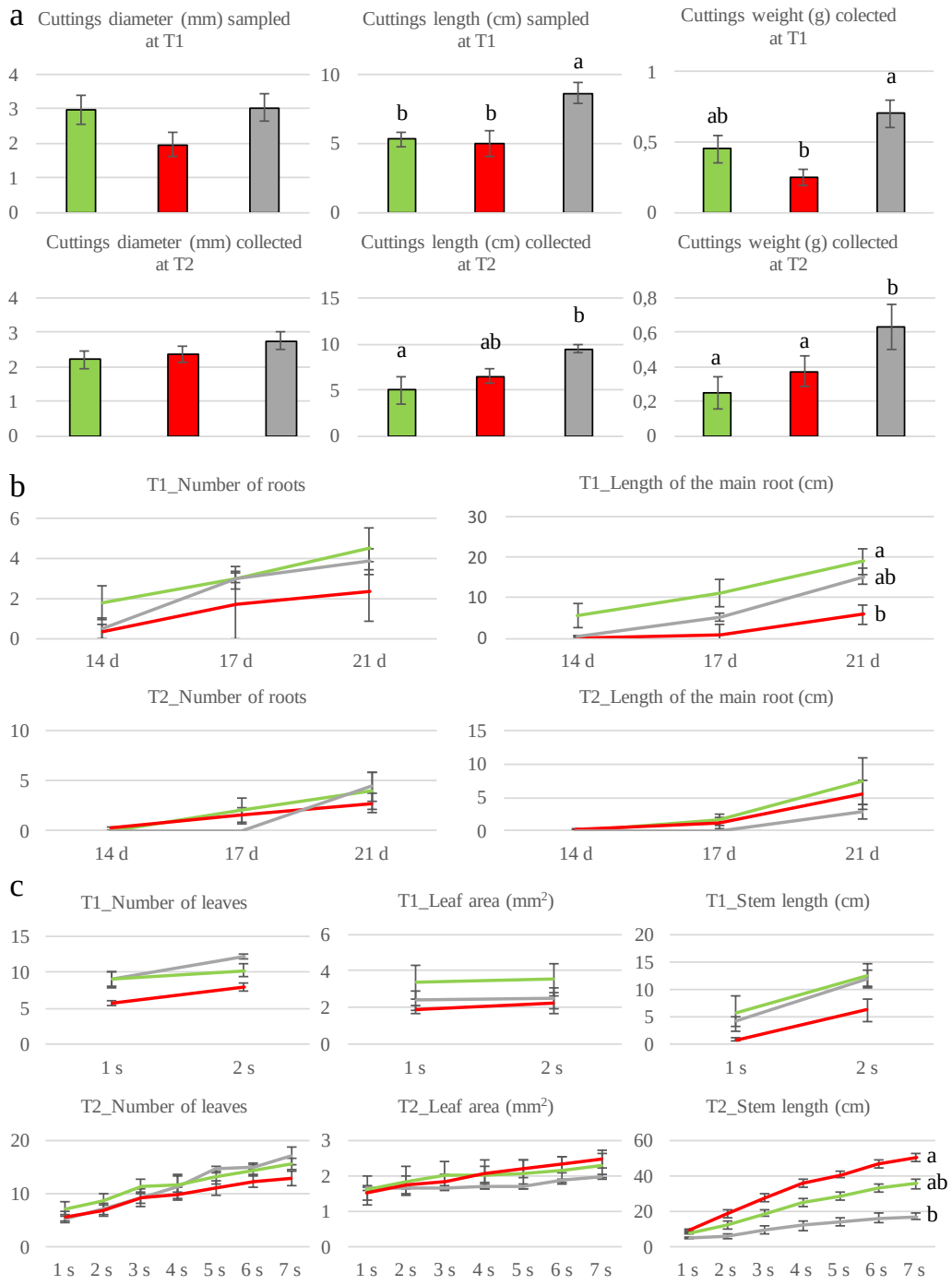


Figure 39 - The phenotypic parameters of Populus-expressing MiSSP7 and Populus WT monitored before, during and after the hydroponic culture in Year 2. Diameter, length and weight of Populus-expressing MiSSP7 and Populus WT cuttings collected at T1 and T2 measured before the culture in hydroponic system (a). Number of roots and length of the main root of Populus-expressing MiSSP7 and Populus WT collected at T1 and T2 monitored once a week during the culture in hydroponic system (b). Number of leaves, leaf area and stem length of Populus-expressing MiSSP7 and Populus WT collected at T1 and T2 monitored once a week during the culture of cuttings in pots (c). The green bars and lines correspond to the cuttings of Populus-expressing MiSSP7 with a low expression of MiSSP7 (average of 10 replicates ± SE) while the red bars and lines correspond to the cuttings of Populus-expressing MiSSP7 with a high expression of MiSSP7 (average of 12 replicates ± SE). The grey bars and lines correspond to the cuttings of Populus WT (average of 12 replicates ± SE). Letters denote significant differences in the phenotypic parameters between Populus-expressing MiSSP7 and Populus WT (P<0.05, ANOVA).

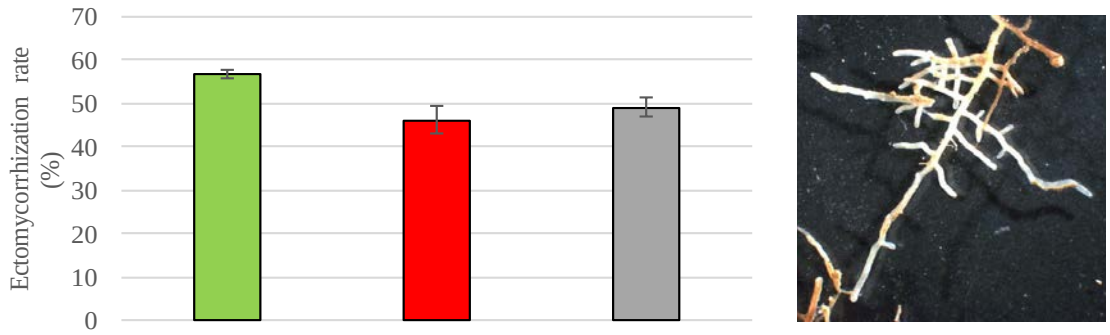


Figure 40 - Ectomycorrhization rate measured from the roots of Populus-expressing MiSSP7 and Populus WT collected at T2 in Year 2. The green bar corresponds to Populus-expressing MiSSP7 roots with a low expression of MiSSP7 (average of 5 replicates ± SE). The red bar corresponds to Populus-expressing MiSSP7 roots with a high expression of MiSSP7 (average of 9 replicates ± SE). The grey bar corresponds to Populus WT roots (average of 6 replicates ± SE). The picture corresponds to Thelephora ectomycorrhizae.

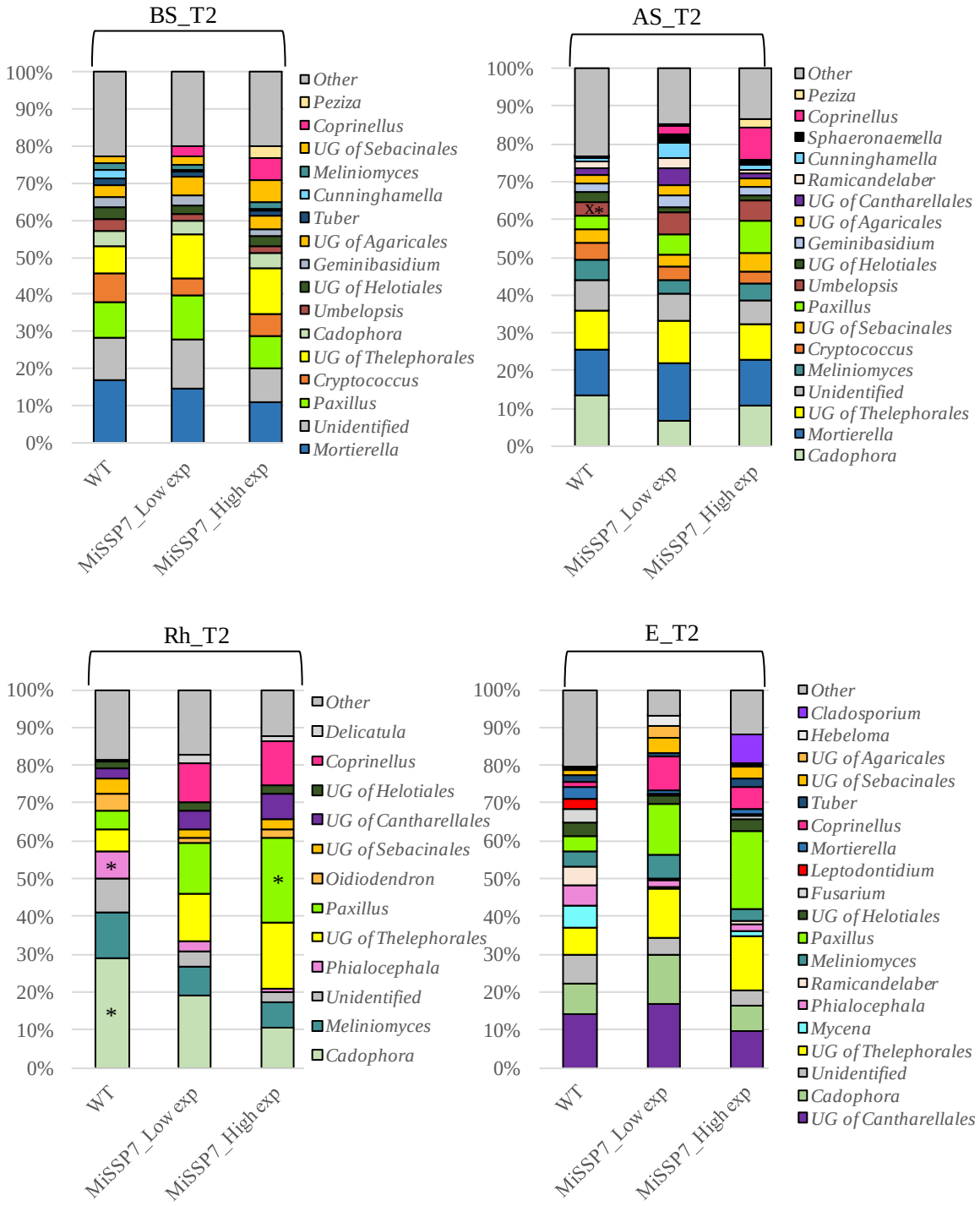


Figure 41 - The distribution of the most dominant fungal genera (>2 % in relative abundance) detected in BS, AS, Rh and E samples of Populus-expressing MiSSP7 and Populus WT cuttings collected at T2 in Year 1. The crosses (x) denote significant difference in the relative abundance of fungal genera detected between WT and MiSSP7_Low expression. The asterisks (*) denote significant difference in the relative abundance of fungal genera detected between WT and MiSSP7_High expression (P<0.05, ANOVA). Detailed information are available in Table S2.

Concerning bacterial communities, the relative abundance of Proteobacteria and Firmicutes was significantly enriched in the Rh and E of MiSSP7 cuttings while Saccharibacteria and Bacteroidetes were significantly more abundant in the endosphere of WT ($P<0.05$, ANOVA, Table S3). At the genus level, the relative abundance of *Massilia* (Proteobacteria) was significantly higher in the Rh and E samples of the MiSSP7 cuttings compared to the WT cuttings ($P<0.05$, ANOVA, Figure S2, Table S3).

At T2, we observed a significant enrichment of the saprotrophe *Umbelopsis* in the rhizosphere of WT cuttings compared to MiSSP7 cuttings. In the rhizoplane, the relative abundance of the endophytes *Cadophora* and *Phialocephala* was significantly higher in WT while the relative abundance of the EcM *Paxillus* was significantly higher in MiSSP7 cuttings with a high level of MiSSP7 expression (MiSSP7_ High exp, $P<0.05$, ANOVA, Figure 41, Table S2). These observations were confirmed by the relative distribution of fungal guilds in the rhizoplane of WT and MiSSP7 cuttings. Indeed, the relative abundance of fungal endophytes was significantly enriched in WT while the relative abundance of EcM fungi was significantly enriched in MiSSP7 cuttings (MiSSP7_Low exp and MiSSP7_High exp, $P<0.05$, ANOVA, Table S4). In addition, at the bacterial phylum level, we observed that the relative abundance of Acidobacteria, Planctomycetes and Proteobacteria (*Delproteobacteria* class) was significantly enriched in the Rh of MiSSP7_High exp compared to the WT ($P<0.05$, ANOVA, Table S3). At the genus level, the relative abundance of *Streptacidiphilus* was significantly enriched in the Rh of WT while the relative abundance of *Acidothermus* was significantly enriched in the Rh of MiSSP7_High exp cuttings ($P<0.05$, ANOVA, Figure 42, Table S3).

The root metabolome of *Populus* WT and *Populus*-expressing MiSSP7 lines cultivated in Year 1

We characterized by GC-MS the root metabolome of *Populus* WT and *Populus*-expressing MiSSP7 cuttings after 10 days and 6.5 weeks of growth in natural soil sampled on Year 1 to visualized if an alteration of the microbiome composition linked to MiSSP7 expression could induced alteration of the root metabolome. Some of the detected metabolites are known to be only produced by host tree while others can also be produced by bacterial and fungal members of root microbiome.

In roots of *Populus*-expressing MiSSP7 cuttings cultivating in Year 1, the main primary metabolites (>1 % of the total metabolites amount) were sucrose (22.4 %), palmitic acid (9.0 %), malic acid (7.6 %), glucose (6.4 %), succinic acid (1.5 %), phosphate (1.4 %) and galactose (1.1 %) while the main secondary metabolites (>1 % of the total metabolites amount) were α-salicyloylsalicin (12.1 %), tremulacin (6.8 %), salicin (6.7 %), tremuloidin (4.3 %), β-sitosterol (2.6 %) and catechin (1.4 %). All of these 15 metabolites were also found to be the main metabolites produced by *Populus* WT roots except for tremulacin and succinic acid which, respectively, corresponded to only 0.12 and 0.20 % of all root metabolites of *Populus* WT (Table S5).

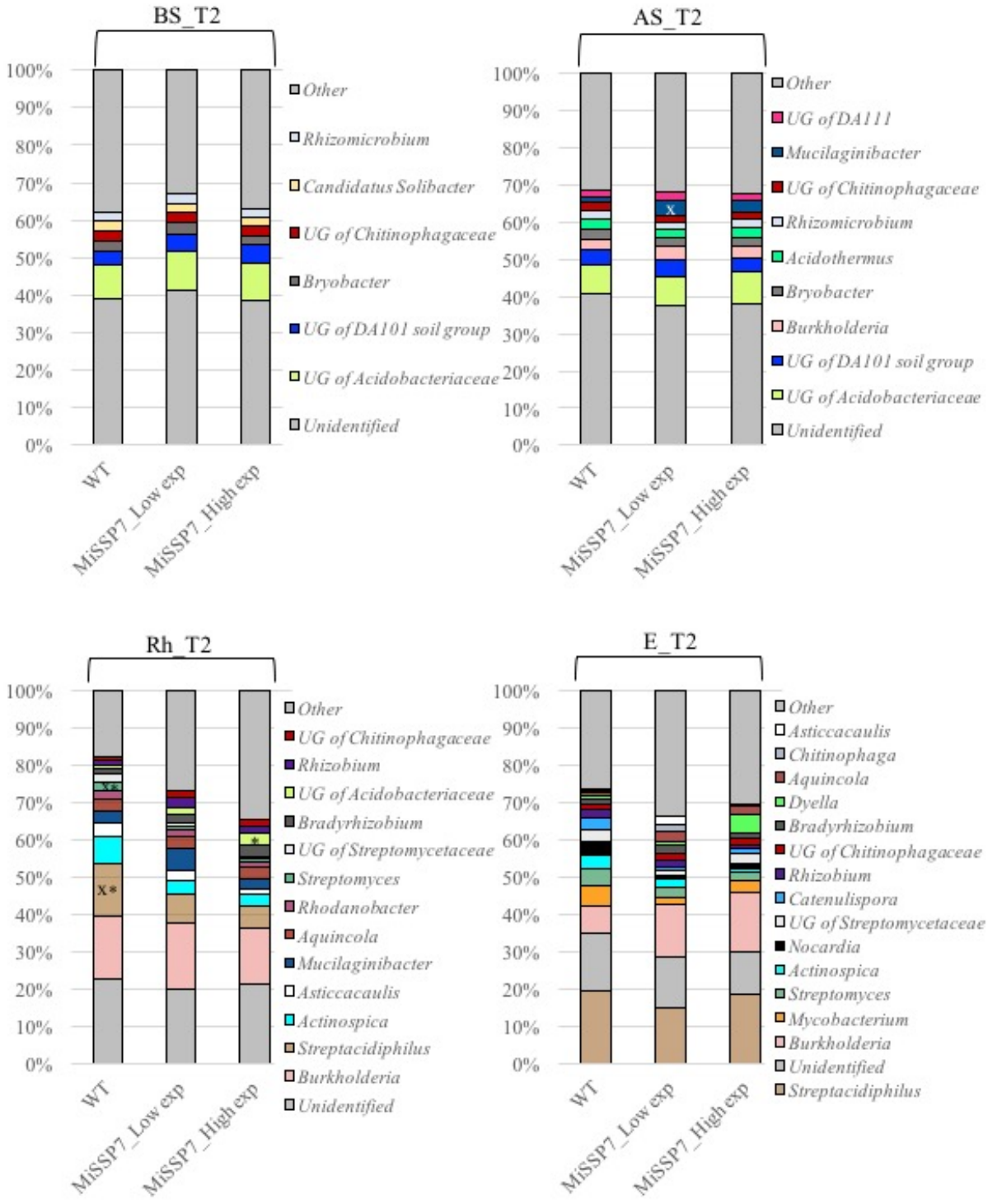


Figure 42 - The distribution of the most dominant bacterial genera (>2 % in relative abundance) detected in BS, AS, Rh and E samples of Populus-expressing MiSSP7 and Populus WT cuttings collected at T2 in Year 1. The crosses (x) denote significant difference in the relative abundance of bacterial genera detected between WT and MiSSP7_Low expression. The asterisks (*) denote significant difference in the relative abundance of bacterial genera detected between WT and MiSSP7_High expression (P<0.05, ANOVA). Detailed informations are available in Table S3.

Table 7 - List of metabolites detected in roots of Populus tremula x alba WT and Populus tremula x alba expressing MiSSP7 roots harvested in Year 1 after 10 days (T1) and 6.5 weeks (T2). Values indicate fold changes between WT and MiSSP7 line at each sampling time. The asterisks denote significant difference of concentration which has been measured between WT and MiSSP_High and between WT and MiSSP7_Low (= P<0.05, one way ANOVA). Metabolites highlighted in yellow and green are involved in primary, secondary metabolisms, respectively.*

Metabolite (RT-m/z)	Plant metabolite	Bacterial or fungal	WT/MiSSP7 (T1)	WT/MiSS7_Low (T2)	WT/MiSSP7_High (T2)
1,2,3-benzenetriol	X	X	ND	0.91	0.50
1,2,4-benzenetriol	X	X	ND	0.98	0.46
10.68 217 391 411	?		1.52	1.41	1.25
10.90 450 dehydro sugar	?		ND	1.40	2.29
11.22 450 dehydro sugar	?		0.77	0.97	0.58
11.29 393 303 257	?		ND	2.25	3.44
13.84 183 256 167	?		0.36	ND	ND
14.09 375 292 217	?		1.18	ND	ND
14.25 331 263 233 258 M+ glycoside	?		0.87	1.88	1.38
14.38 254 inositol conj	?		0.45	ND	ND
15.18 284 glycoside	?		0.82	0.93	0.35
15.24 284 glycoside	?		0.88	0.93	0.73
16.04 guaiacyl lignan	X		0.67	0.88	0.53
16.11 guaiacyl lignan	X		0.52	0.60 *	0.29
16.37 guaiacyl lignan	X		ND	0.64	0.56
17.65 418 179 193 91 glycoside	?		1.53	1.76	1.12
19.00 219 171 331	?		0.61	2.15	0.97
19.18 171 coumaroyl glycoside	?		0.63	1.51	0.79
19.69 171 caffeoyl glycoside	?		0.93	1.39	0.75
2-hydroxypentanedioic acid	X	X	0.89	0.61	0.14
2,5-dihydroxybenzoic acid-2-O-	X	X	ND	1.29	0.13
2,5-dihydroxybenzoic acid-5-O-	X		ND	1.17	0.18
4-hydroxybenzoic acid	X	X	0.69	0.73	0.29
5-oxo-proline	X	X	1.66	1.77	1.28
6-hydroxy-2-cyclohexenone alcohol	X		0.71	0.86	0.28
6-hydroxy-2-cyclohexenone-1-carboxylic acid	X	X	0.67	0.95	0.54
6.94 225 240 332 278	?		0.61	0.72	0.33
7.69 169 101 75 68	?		ND	1.27	0.79
8.34 256 167	?		ND	1.67	1.14
9.98 98 288 390	?		ND	0.00	0.0
a-linolenic acid	X	X	1.22	0.77	0.55
a-salicyloylsalicin	X		2.15	1.68	1.12
a-tocopherol	X	X	0.74	1.14	0.52
alanine	X	X	1.38	2.04	1.73
arabinose	X	X	1.15	ND	ND
arabitol	X	X	0.82	0.42	1.10
arbutin	X		0.63	0.59	0.77
B-sitosterol	X		0.73	0.93	0.51
caffeic acid	X		0.71	1.11	0.61
catechin	X		0.70	0.49 *	0.24
catechol	X		0.69	1.10	0.69
cis-aconitic acid	X	X	1.29	0.66	0.26
citric acid	X	X	1.19	0.73	0.21
digalactosylglycerol	X	X	0.56	1.09	1.38
erythronic acid	X		1.50	0.69 *	0.34
ethyl-phosphate	?		0.88	1.27	0.89
fructose	X	X	3.14	1.28	2.03 *
fumaric acid	X	X	1.03	0.53	0.22

Metabolite (RT-m/z)	Plant metabolite	Bacterial or fungal	WT/MiSSP7 (T1)	WT/MiSS7_Low (T2)	WT/MiSSP7_High (T2)
GABA	X	X	1.88	2.55	1.75
galactose	X	X	2.21	0.97	0.75
gallocatechin	X		0.62	0.72	0.32
glucose	X	X	2.29	0.98	1.17
glutamic acid	X	X	ND	3.26	1.44
glyceric acid	X	X	1.11	0.63 *	0.30
glycerol	X	X	0.62	0.64	0.44
glycerol-1/3-P	X	X	0.90	0.72	0.70
hydroquinone	X		0.68	0.72	0.11
lactic acid	X	X	0.79	0.62	0.52
linoleic acid	X	X	1.08	0.68	0.52
maleic acid	X	X	1.26	0.80	0.06
malic acid	X	X	1.56	0.74	0.15
mannitol		X	0.91	0.53	1.63
monogalactosylglycerol	X	X	1.08	0.91	0.86
myo-inositol	X		2.23	2.36	1.22
nonanoic acid	X		0.69	0.65	0.33
oxalomalic acid	X	X	1.21	0.74	0.52
palmitic acid	X	X	0.87	0.66	0.41
phluoroglucinol	X	X	0.62	0.67	0.09
phosphate	X	X	0.59	0.71	0.32
quinic acid	X	X	2.68	1.10	0.84
ribitol	X	X	ND	1.48	2.41
salicin	X		0.82	0.91	0.95
salicortin	X		1.52	1.00	0.51
salicyl alcohol	X		0.70	1.06	0.54 *
salicyl-salicylic acid-2-O-glucoside	X	X	2.43	1.16	0.59
salicylic acid	X	X	0.65	1.08	0.61
salicyltremuloidin	X		1.12	1.75	1.43
salireposide	X		1.25	1.58	1.09
shikimic acid	X	X	1.51	0.54	0.47 *
succinic acid	X	X	1.39	0.87	0.40
sucrose	X		1.59	0.67	0.60
threonic acid	X		3.50	0.42	0.24
threono-1,4-lactone	X	X	1.18	0.94	0.49
trehalose		X	2.08 *	0.24 *	0.42
tremulacin	X		0.89	1.52	1.09
tremuloidin	X		1.18	1.27	1.19
xylitol	X	X	0.84	1.00	0.85
xylono-1,4-lactone	X		1.26	0.82	0.58

At both T1 and T2, 33 of these detected metabolites were linked to primary metabolism while 36 were involved in primary metabolism while. In addition, 19 metabolites corresponded to unidentified compounds. In addition, 19 metabolites corresponded to unidentified compounds. At T1, the concentration of trehalose (primary metabolite, fungal disaccharide) was significantly higher in WT roots compared to MiSSP7 roots ($P<0.05$, Student-t-test, Table 7). In roots of cuttings collected at T2, the concentrations of the primary metabolites glyceric acid and of the secondary metabolite catechin were significantly higher in WT roots while the concentration of the primary metabolite trehalose were higher in the roots of MiSSP7_Low exp cuttings. The concentration of the secondary metabolite salicyl alcohol was significantly higher in the roots of MiSSP7_High exp cuttings while the concentration of fructose (primary metabolite) was significantly higher in the roots of WT cuttings ($P<0.05$, Student-t-test, Table 7).

Structure and composition of microbial communities of each compartment in *Populus* WT and *Populus*-expressing MiSSP7 line cultivated in Year 2

Experimental set-up used in Year 1 was conserved for a second experiment performed the following year (Year 2) to observe if the observations made in Year 1 were confirmed whatever the potential shifts in microbial community composition and structure of the natural soil.

Comparison of the structure of fungal and bacterial communities detected in soil (i.e., BS and AS samples) and in roots (i.e., Rh and E samples) of *Populus* WT and *Populus*-expressing MiSSP7 lines (i.e., MiSSP7_Low and MiSSP7_High) collected at T1 an T2 revealed significant differences between compartments and between sampling time at the OTU level (PermANOVA, $P<0.05$; Table 8). However, no significant shift in the microbial community structure was linked to the treatment effect (WT vs. MiSSP7_Low vs. MiSSP7_High; Table 8). These observations were confirmed in each compartment (i.e., BS, AS, Rh and E) and at each sampling time (i.e., T0, T1 and T2) (Table 8).

Detailed analyses of the fungal and bacterial community compositions in each compartment (i.e., BS, AS, Rh and E) revealed some significant differences between *Populus* WT and *Populus*-expressing MiSSP7 lines (i.e., MiSSP7_Low and MiSSP7_High) collected at T1 and T2 in Year 2 (ANOVA, $P<0.05$). Significant shifts in fungal community composition were only detected in the BS. At T2, Ascomycota were significantly enriched in the BS of *Populus* WT (74.4 ± 3.2 %) and MiSSP7_Low (75.3 ± 3.0 %) compared to MiSSP7_High (61.4 ± 2.9 %; Table S6) while Basidiomycota were significantly enriched in the BS of MiSSP7_High (18.1 ± 2.5 %) compared to WT (18.3 ± 1.9 %) and MiSSP7_Low (29.9 ± 2.2 %; Table S6). Concerning bacterial community, Proteobacteria (*Alphaproteobacteria* class) were significantly more abundant in the endosphere of MiSSP7_High (8.0 ± 1.2 %) compared to MiSSP7_Low (3.5 ± 0.6 %) and WT (3.7 ± 0.8 %) cuttings (Table S7). No significant change was observed between *Populus* WT and MiSSP7 lines collected at T1 at the fungal and bacterial phylum level.

At the genus level, the relative abundance of *Cryptococcus* was significantly enriched in the BS of WT compared to MiSSP7_Low cuttings collected at T1 (ANOVA, $P<0.05$; WT: 12.7 ± 1.0 % = MiSSP7_High: 10.5 ± 1.3 % > MiSSP7_Low: 9.0 ± 0.8 %, Figure S3, Table S6). In addition, we observed that the relative abundance of *Leptodontidium* was significantly enriched the Rh compartment of WT compared to MiSSP7_High line collected at

*Table 8 - Permutational multivariate ANOVA results with Bray-Curtis distance matrices implemented to partition sources of variation in this study (compartments [BS, AS, Rh and E], sampling times [T1 and T2] and expression [WT, MiSSP7_Low exp and MiSSP7_High exp]) for fungal and bacterial communities at the OTU level. BS, AS, Rh and E samples of the WT and MiSSP7 cuttings collected in Year 2 were included in this analysis. Statistical significance (p-value) computed were based on sequential sums of square from 999 permutations (*** = P-value < 0.01, ** = P-value < 0.03).*

Fungi_Year2			PermANOVA			Bacteria_Year2			PermANOVA		
Compartment	**Time**	**Source of variation**	**F**	**R2**	**p-value**	**Compartment**	**Time**	**Source of variation**	**F**	**R2**	**p-value**
BS, AS, Rh, E	T1 and T2	Compartment	45.13	0.486	***	BS, AS, Rh, E	T1 and T2	Compartment	31.56	0.397	***
		Time	7.15	0.025	***			Time	11.30	0.095	***
		Expression	1.44	0.087	0.110			Expression	1.08	0.009	0.326
		Treatment	1.10	0.003	0.318			Treatment	1.16	0.019	0.215
BS	T1, T2 and T0	Time	6.00	0.132	***	BS	T1, T2 and T0	Time	2.22	0.063	**
		Expression	1.18	0.087	0.0109			Expression	1.39	0.079	0.055
		Treatment	2.10	0.093	0.063			Treatment	1.45	0.087	0.065
Rh	T1, T2 and T0	Time	10.1	0.420	***	Rh	T1, T2 and T0	Time	15.43	0.488	***
		Expression	0.98	0.020	0.414			Expression	1.39	0.022	0.178
		Treatment	0.87	0.018	0.464			Treatment	1.25	0.036	0.209
BS	T1 and T2	Time	4.54	0.111	***	BS	T1 and T2	Time	2.19	0.069	***
BS	T1 and T2	Expression	1.44	0.142	*	BS	T1 and T2	Expression	1.42	0.045	0.053
BS	T1 and T2	Treatment	1.32	0.032	0.124	BS	T1 and T2	Treatment	1.37	0.135	0.067
AS	T1 and T2	Time	9.50	0.216	***	AS	T1 and T2	Time	3.53	0.108	***
AS	T1 and T2	Expression	1.05	0.047	0.342	AS	T1 and T2	Expression	1.00	0.066	0.405
AS	T1 and T2	Treatment	1.35	0.030	0.158	AS	T1 and T2	Treatment	0.91	0.030	0.618
Rh	T1 and T2	Time	8.40	0.197	***	Rh	T1 and T2	Time	6.65	0.193	***
Rh	T1 and T2	Expression	1.09	0.051	0.327	Rh	T1 and T2	Expression	0.79	0.023	0.635
Rh	T1 and T2	Treatment	0.94	0.022	0.411	Rh	T1 and T2	Treatment	0.66	0.023	0.845
E	T1 and T2	Time	3.87	0.111	**	E	T1 and T2	Time	4.00	0.137	**
E	T1 and T2	Expression	0.97	0.056	0.478	E	T1 and T2	Expression	1.16	0.040	0.260
E	T1 and T2	Treatment	1.00	0.028	0.428	E	T1 and T2	Treatment	0.94	0.073	0.521
BS	T1	Expression	1.01	0.094	0.082	BS	T1	Expression	1.07	0.089	0.125
BS	T1	Treatment	1.35	0.211	0.434	BS	T1	Treatment	1.00	0.167	0.421
BS	T2	Expression	1.08	0.157	0.313	BS	T2	Expression	1.35	0.077	0.086
BS	T2	Treatment	1.47	0.071	0.080	BS	T2	Treatment	1.12	0.194	0.166
AS	T1	Expression	0.91	0.125	0.558	AS	T1	Expression	0.98	0.082	0.562
AS	T1	Treatment	1.77	0.121	**	AS	T1	Treatment	0.90	0.153	0.940
AS	T2	Expression	1.33	0.129	0.182	AS	T2	Expression	0.98	0.057	0.462
AS	T2	Treatment	0.85	0.041	0.502	AS	T2	Treatment	1.01	0.118	0.373
Rh	T1	Expression	0.95	0.134	0.477	Rh	T1	Expression	0.79	0.078	0.732
Rh	T1	Treatment	1.29	0.091	0.181	Rh	T1	Treatment	0.85	0.085	0.645
Rh	T2	Expression	0.78	0.081	0.598	Rh	T2	Expression	0.81	0.048	0.716
Rh	T2	Treatment	0.75	0.038	0.676	Rh	T2	Treatment	0.72	0.087	0.959
E	T1	Expression	1.00	0.170	0.503	E	T1	Expression	0.77	0.066	0.673
E	T1	Treatment	0.73	0.062	0.843	E	T1	Treatment	0.92	0.156	0.572
E	T2	Expression	0.85	0.090	0.655	E	T2	Expression	1.25	0.094	0.202
E	T2	Treatment	1.29	0.067	0.215	E	T2	Treatment	1.08	0.164	0.363

T1 (ANOVA, P<0.05; WT: 7.2 ± 0.9 % > MiSSP7_High: 3.0 ± 1.1 % = MiSSP7_Low: 3.8 ± 1.0 % Figure S3, Table S6). *Cryptococcus* was also significantly enriched in the BS of MiSSP7_High line compared to WT and MiSSP7_Low line collected at T2 (ANOVA, P<0.05; MiSSP7_High: 27.4 ± 2.4 % > WT: 14.6 ± 1.8 % = MiSSP7_Low: 17.0 ± 1.8 %, Figure 43, Table S6). Significant changes were also observed in the distribution of fungal guilds at T1. Indeed, the fungal endophytes were significantly enriched in the R compartment of *Populus* WT compared to MiSSP7_High cuttings (ANOVA, P<0.05; WT: 8.0 ± 0.9 % > MiSSP7_Low: 4.6 ± 1.0 % = MiSSP7_High: 3.6 ± 0.8 %, Table S8). At T2, no significant change was observed in the distribution of fungal guild in the four-studied compartment between WT and MiSSP7 lines. Concerning bacterial community composition, no significant change was observed in the four studied compartments between WT and MiSSP7 lines collected at T1 (ANOVA, P<0.05; Figure S4, Table S8).

At T2, we observed that the relative abundance of *Candidatus Xiphinematobacter* and unknown genus of *Methylacidiphilaceae* was significantly higher in the rhizosphere of MiSSP7_High compared to WT cuttings (ANOVA, P<0.05; Figure 44, Table S7). In addition, the relative abundance of *Chthoniobacter* was significantly higher in the endosphere of MiSSP7_High cuttings compared to WT cuttings (ANOVA, P<0.05; Figure 44, Table S7).

The root metabolome of *Populus* WT and *Populus*-expressing MiSSP7 lines cultivated in Year 2

We detected the same main primary and secondary metabolites in the roots of *Populus*-expressing MiSSP7 cuttings sampled in Year 2 compared with those sampled in Year 1. However, the concentration of some of these metabolites was significantly different between Year 1 and Year 2. For instance, the concentration of sucrose and of four secondary metabolites (i.e., α-salicyloylsalicin, tremulacin, salicin and tremuloidin) was significantly higher in T1-samples collected in Year 2 while the concentration of glucose, fructose and galactose was significantly higher in T2-samples collected in Year 1 (Table S5).

At T1, the level of salicin, tremulacin and the unidentified compound 14.25 min (m/z 331 263 233 258 M+ glycoside) was significantly enriched in the roots of WT cuttings compared to MiSSP7_Low roots (ANOVA, P<0.05; Table S10). No significant difference was observed in the roots of WT and MiSSP7_High cuttings collected at T1 (Table S10). At T2, two primary metabolites (oxalomalic acid and threono-1,4-lactone) and three secondary metabolites (6-hydroxy-2-cyclohexenone-1-carboxylic acid, catechin and xylono-1,4-lactone) were significantly enriched in the roots of MiSSP7_Low compared to the roots of WT cuttings (ANOVA, P<0.05; Table S10). Comparison of the root metabolome of WT and MiSSP7_High revealed significant difference between the two lines of *Populus* collected at T2. Several secondary metabolites were more abundant in WT roots (e.g catechin, catechol, salicin and xylitol) while tremulacin was significantly more abundant in the roots of MiSSP7_High line with a fold change of 50 (ANOVA, P<0.05; Table S10). Concerning primary metabolites, we observed that the level of oxalomalic acid, cis-aconitic acid and sucrose was significantly enriched in WT roots compared to MiSSP7_High roots (ANOVA, P<0.05; Table S10).

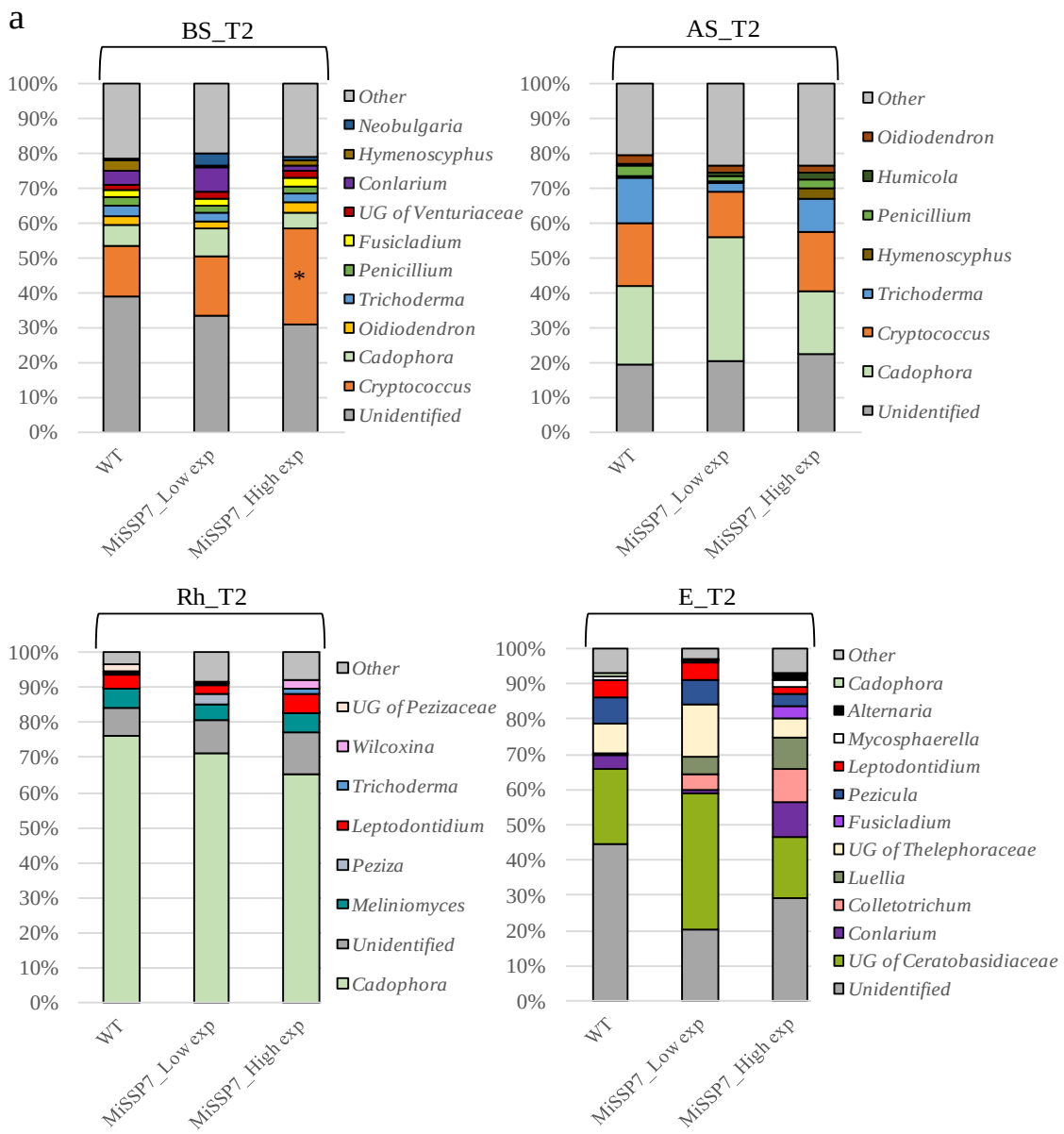


Figure 43 - The distribution of the most dominant fungal genera (>2 % in relative abundance) detected in BS, AS, Rh and E samples of Populus-expressing MiSSP7 and Populus WT cuttings collected at T2 in Year 2. The asterisks (*) denote significant difference in the relative abundance of fungal genera detected between WT and MiSSP7_High expression (P<0.05, ANOVA) (a). Detailed informations are available in Table S6.

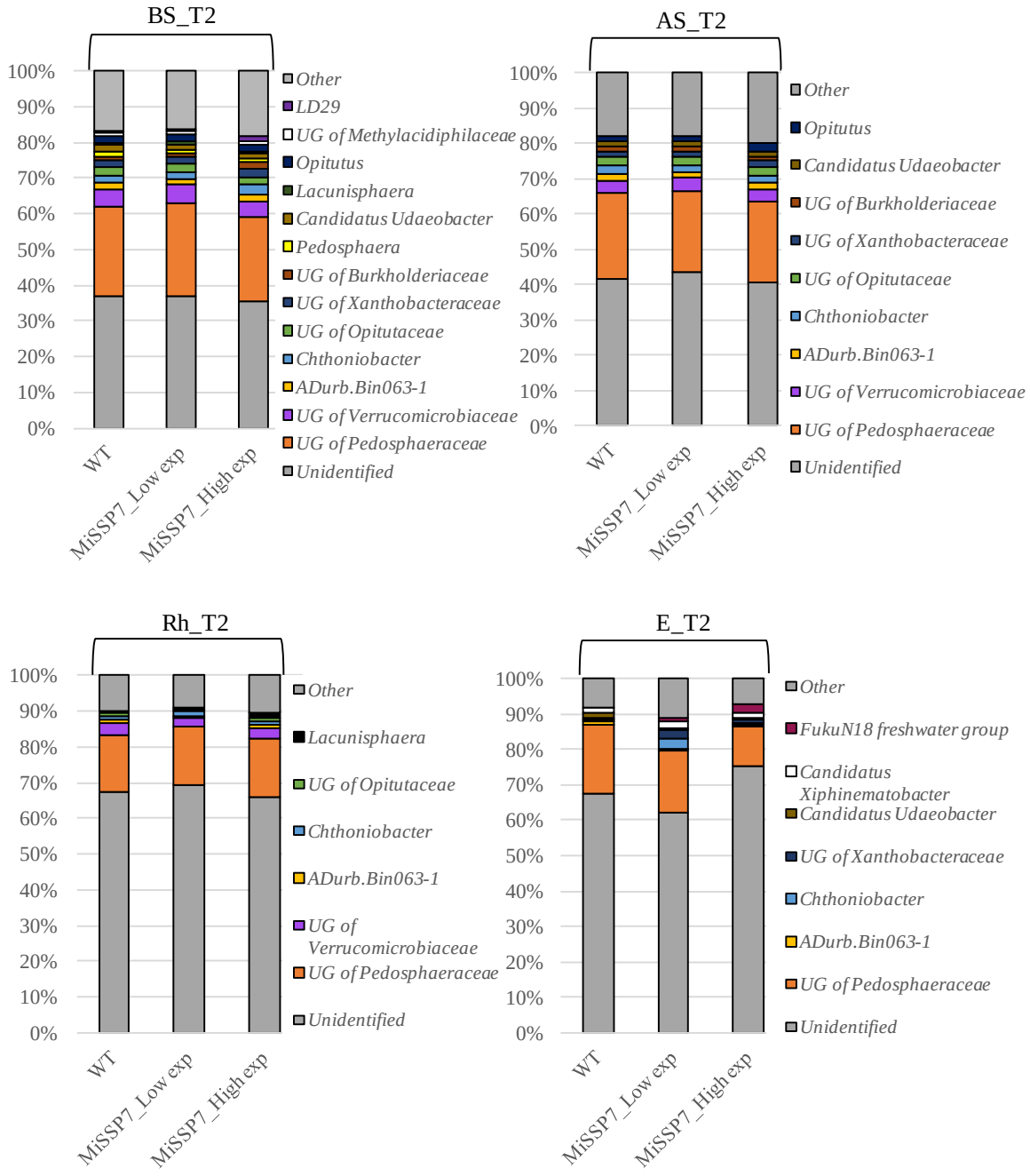


Figure 44 - The distribution of the most dominant bacterial genera (>2 % in relative abundance) detected in BS, AS, Rh and E samples of Populus-expressing MiSSP7 and Populus WT cuttings collected at T2 in Year 2. Detailed informations are available in Table S7.

The metatranscriptome of the *Populus* WT and *Populus*-expressing MiSSP7 roots collected in Year 2

A metatranscriptome analysis of the microbial communities colonizing the roots (i.e., both Rh and E compartments) of the WT and the *Populus*-expressing MiSSP7 lines (i.e., MiSSP7_Low exp and MiSSP7_High exp) was performed in order to highlight the active communities detected at T2.

We observed that the EcM fungi *Thelephora* were the most abundant fungal genus detected in the metatranscriptome of the roots of *Populus* cuttings (more than 50 % of the transcript detected in *Populus*-expressing MiSSP7 roots and 31 % of the transcript detected in the *Populus* WT roots, Figure 45 a, Table S11). No significant difference was observed in the proportion of *Thelephora*-related transcripts between the roots of *Populus*-expressing MiSSP7 and WT (Wilcoxon test, MiSSP7_Low exp vs WT, P=0.21; MiSSP7_High exp vs WT, P=0.23, Figure 45 b). In addition, we also did not detect any significant difference in the proportion of transcripts of the other most abundant fungal genera in the roots of *Populus*-expressing MiSSP7 and WT (Wilcoxon test, Figure 45 b). However, the abundance of *Tulasnalla*-related transcripts tended to be higher in the roots of MiSSP7_Low expression compared to WT (Wilcoxon test, P=0.06, Figure 45 b) while the abundance of *Meliniomyces*-related transcripts tended to be higher in the roots of WT compared to MiSSP7_High expression (Wilcoxon test, P=0.08, Figure 45 b). Although the dominance of the fungal endophytes *Phialocephala* and *Cadophora* and the yeast *Cryptococcus* in *Populus* roots, their transcripts were undetected.

Discussion

The composition of the root microbiome is influenced by the host physiology and metabolism (Berg & Smalla, 2009; Jacoby et al., 2017). Defence phytohormones (JA, SA and ET) are structurally diverse plant secondary metabolites involved in plant immune system but also in the regulation of plant growth and development. Previous studies examined the roles of defence hormones in shaping the root microbiome by using mutant lines defective in their biosynthesis or perception in combination with microbial culturing (Lebeis et al., 2015; Liu et al., 2017). Although these studies improved our knowledge concerning the effect of plant defence on root microbiome composition and structure, no study has been performed on woody perennial plant. Here, we asked if the ectopic expression of MiSSP7 in *Populus* impact the composition and the structure of the root microbiome. Based on the work of Plett et al., (2011), we hypothesized that alteration of JA signalling pathway by MiSSP7 influences the root-associated microbiome of *Populus* and if these potential modifications of the root microbiome are accompanied by alteration of root metabolism. For this, we took advantage of the ability of the action of MiSSP7, the most highly symbiosis-upregulated gene from the EcM fungi *Laccaria bicolor*, known for its interaction with the negative repressor JAZ6 maintaining repression of JA-induced genes (Plett et al., 2011; 2014).

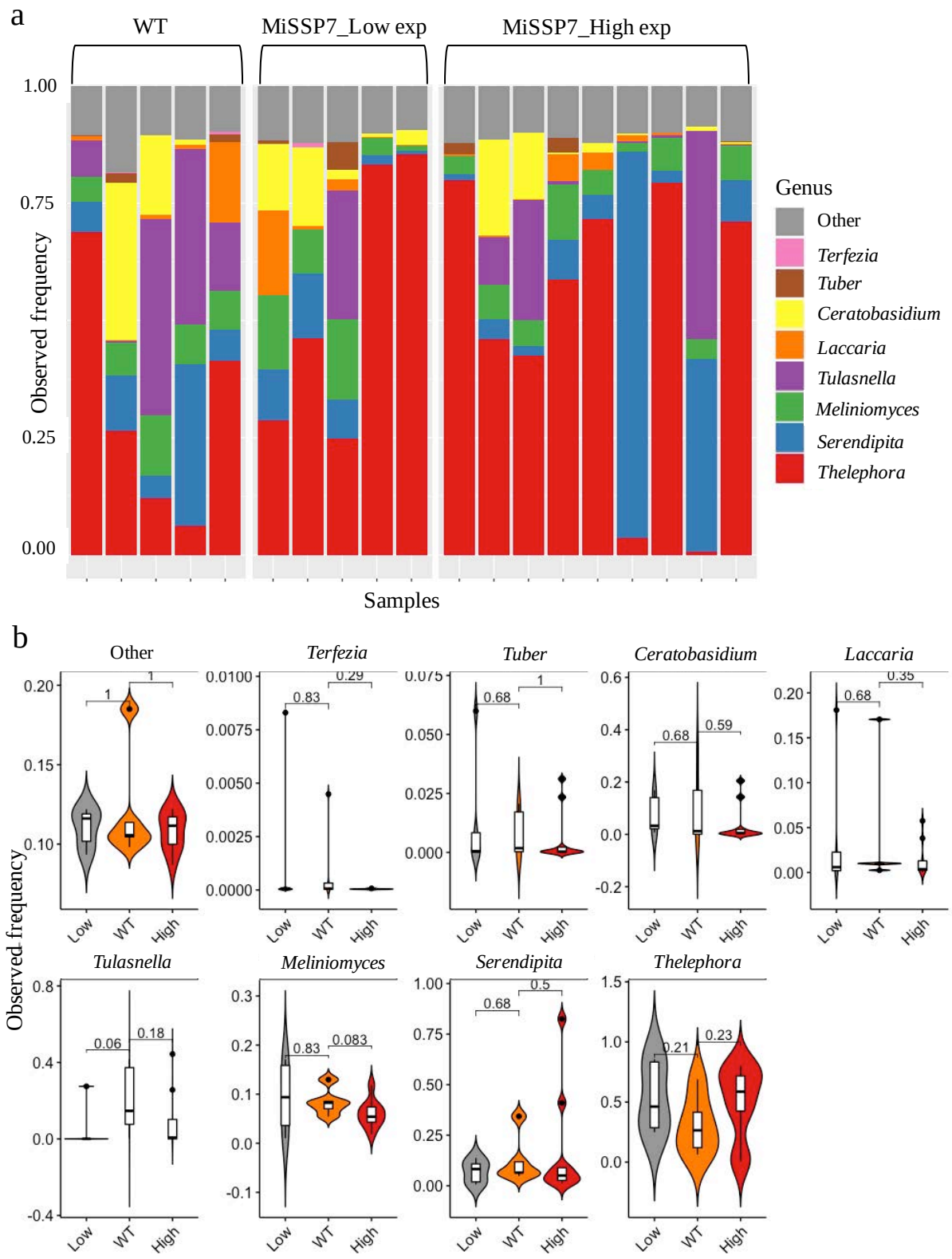


Figure 45 - Proportional distribution of the most active fungal genera detected in the roots of Populus-expressing MiSSP7 and Populus WT cuttings collected at T2 in Year 2. Each column corresponds to the observed frequency of the most active fungal genera based on metatranscriptomic rRNA data collected from the roots samples of WT, MiSSP7_Low expression and MiSSP7_High expression cuttings (a). Comparison of the observed frequency of the most active fungal genera detected in the roots of the different Populus lines (b). P-value are given for fungal genus and correspond to the comparison between WT (in orange) and MiSSP7_Low exp (in grey) cuttings and the comparison between WT and MiSSP7_High exp (in red) cuttings (Wilcoxon test).

We observed that MiSSP7 expression in *Populus* induced contrasted change in root microbiome composition according to the year of experiment (i.e., Year 1 and Year 2). Although we do not know the precise effect of MiSSP7 on the biosynthesis and the perception of JA in *Populus* roots, contrasted changes in the composition of bacterial and fungal communities associated with *Populus* roots detected between the two years of experiments was observed and was accompanied by contrasted changes in the root metabolome of *Populus* lines cultivated in Year 1 and Year 2. Indeed, the concentration of different metabolites was impacted by MiSSP7 expression in *Populus* roots between Year 1 and Year 2. Therefore, we could imagine that the root metabolome is more regulated by microorganisms than by MiSSP7 expression. Even if MiSSP7 expression in *Populus* significantly affected the root microbiome composition, microbial communities detected in the roots of *Populus*-expressing MiSSP7 were similar to those previously described in other *Populus* genotype (Gottel et al., 2011; Danielsen et al., 2012; Shakya et al., 2013; Bonito et al., 2014; Cregger et al., 2018). *Burkholderia* (Proteobacteria), *Mucilaginibacter* (Bacteroidetes) and *Streptacidiphilus* (Actinobacteria) dominated root bacterial communities while *Meliniomyces,* the fungal endophytes *Cadophora* and the EcM fungi *Paxillus* dominated the root fungal communities.

MiSSP7 expression in *Populus* was not responsible of modification of the host tree transcriptome

A transcriptome analysis of the roots of *Populus*-expressing MiSSP7 and *Populus* WT cuttings was performed with Year 2 samples in order to observed if the ectopic expression of MiSSP7 in Poplar could regulate the expression of other genes of interest (data not shown). Almost no significant differences were observed between the transcriptomes of the roots of *Populus*-expressing MiSSP7 and *Populus* WT cuttings. Indeed, only three and two genes were up-regulated (DHBP synthase RibB-like alpha [Potri.001G234900.1], kunitz trypsin inhibitor 1 [Potri.007G111500.1] and amino acid permease 2 [Potri.007G100100.1]) and down-regulated (cinnamate-4-hydroxylase [Potri.018G146100.1] and senescence-related gene 1 [Potri.001G355100.1]) with a maximum fold change of 1.2 between MiSSP7 and WT roots (data not shown). Similarly, no significant difference between the phenotype of *Populus*-expressing MiSSP7 and *Populus* WT was observed (Figure 39). Analysis of the transcriptome of poplar roots incubated with MiSSP7 protein induced the modulation of 200 transcripts mostly involved in the alteration of the root architecture (Plett et al., 2011). However, this study was performed on the roots of 2-weeks-old *Populus* cultivated in axenic conditions and incubated with MiSSP7 protein for 1 hour. In addition, transcriptome analysis was performed with only one replicates which could lead to misinterpretation. Therefore, we could easily imagine that after 6.5 weeks of growth, the ectomycorhization as well as the induction of other genes by MiSSP7 are already established in *Populus* roots.

MiSSP7 expression in Populus induces contrasted change in the composition and the structure of the root microbiome

We previously described that soil microbiome which is now considered as the main reservoir of microorganisms for tree roots (Lareen et al., 2016, Mangeot-Peter et al., 2020) was significantly altered in its composition and its

structure from year to year. These modifications were responsible for significant shifts of *Populus* root microbiome composition but also for changes in both primary and secondary metabolisms in the roots of the WT (Mangeot-Peter et al., 2020, Chapter 3). In the current study, we compared the composition and the structure of bacterial and fungal communities associated with the roots of genetically modified *Populus* line (*Populus*-expressing MiSSP7) and of non-genetically modified *Populus* (*Populus* WT) cultivated in the same soil across two following years. This natural soil was characterized in our previous work (Mangeot-Peter et al., 2020). Colonization of the roots by microorganisms is highly dynamic: bacterial communities as well as fungal communities assigned as saprotrophes and pathotrophes are able to colonize host roots in few days while the functional association of the roots with ectomycorrhizal fungi required several weeks (Smith & Reads, 2008; Marupakula et al., 2016, chapter 1 of this thesis). The sampling time (i.e, abiotic factor) affected OTU-level microbial soil and root community structure of the WT cuttings in Year 1 and Year 2 (Mangeot-Peter et al., 2020). In addition, the expression of MiSSP7 in *Populus* (i.e, biotic factor) significantly impacted fungal communities detected in soil and roots only in Year 1.

In our study, we expect that MiSSP7 expression was responsible of the alteration of the JA signalling pathway in *Populus*. Other studies were interested by the impact of SA manipulation on the bacterial communities colonizing *Arabidopsis* roots. Knowing that JA and SA signalling pathway are antagonistics (Koornbeef et al., 2008), the increase of SA could lead to the decrease of JA, mimicking the expected effect of MiSSP7 expression. Significant shifts in the relative abundance of the most dominant bacterial and fungal communities were observed but were contrasted according to the year of experiment (i.e., Year 1 and Year 2). In Year 1, the relative abundance of *Streptomyces* (Actinobacteria), *Acidovorax* and *Massilia* (Proteobacteria) was significantly enriched in the roots of *Populus*-expressing lines compared to the roots of *Populus* WT collected at T1 (Figure 42). These observations were in accordance with other studies which showed a significant increase of the relative abundance of Proteobacteria and a decrease of the relative abundance of Actinobacteria in roots of SA-deficient *Arabidopsis* plants (Lebeis et al., 2015). Exogenous SA application was responsible for the enrichment of *Streptomyces* in *Arabidopsis* roots (Lebeis et al., 2015). Although the antagonistically action of JA and SA (Koornneef et al., 2008), these two phytohormones seemed to have the same effect on bacterial members of the root microbiome. These results were in accordance with our study. However, the activation of JA signalling pathway had a more contrasted effect on *Streptomyces* populations in roots of wheat (*Triticum aestivum*) where some OTU increased and other decreased in relative abundance in response to JA. These observations performed on different plant species highlight that manipulation on JA signalling pathway could promote or prevent the root colonisation by bacteria. We could also hypothesize that the colonisation of the *Populus* roots by bacteria is mostly influence by fungal communities potentially impacted by MiSSP7 expression.

Concerning fungal communities, EcM fungi (e.g *Paxillus*) were more abundant in the roots of *Populus* expressing MiSSP7 line while fungal endophytes (e.g *Cadophora* and *Phialocephala*) were more abundant in the rhizoplane of *Populus* WT line in Year 1 at T2 (Figure 41). In addition, the same trend was observed in the endosphere suggested that MiSSP7 expression have the same effect on and in the roots. To our knowledge, it is the first work dealing with JA signalling pathway and fungal microbiome of tree roots. In Year 2, a significant enrichment of

fungal endophytes (e.g *Cadophora*) was observed in the roots of *Populus*-expressing MiSSP7 collected at T1 whereas no enrichment of EcM fungi was observed in *Populus*-expressing MiSSP7 (Table S8). However, the metatranscriptome analysis of *Populus* roots collected at T2 in Year 2 revealed that the EcM fungus *Thelephora* was the most active colonizers of the *Populus* roots (Figure 45). This result was in concordance with DNA metabarcoding approach which revealed that *Thelephoraceae* was the main EcM detected in *Populus* roots (10 % in relative abundance, Figure 43). It was also the fungus which formed the most identified ectomycorrhizae on the *Populus* roots (Figure 40). No significant difference was observed in the proportional distribution of *Thelephora* transcripts between WT and MiSSP7 roots suggesting that MiSSP7 expression had no effect on the colonization of this EcM fungus. Although JA signalling pathway seems to be the main defence pathway to protect tree the attack against necrotrophic pathogens and against *Laccaria bicolor* root colonisation (Plett et al., 2011; 2014), other signalling pathways or molecular mechanisms may also play this role. In addition, if this fungal effector of 7kDa involved in symbiosis establishment was specific of the EcM fungus *Laccaria bicolor* (Plett et al., 2014), other EcM fungi produced MiSSPs such as *Paxillus* (Kohler et al., 2015). Although the genome of *Thelephora* had just been sequenced from mycelium samples, no information is currently available regarding the ability of this EcM fungus to produce MiSSP. In vitro culture of *Populus* in the resence of this fungus may be carried out to allow the sequencing of ectomycorrhizae.

Taking together, these observations suggest that the alteration of JA signalling pathway induced by MiSSP7 expression in *Populus* was responsible of the modification of the balance between some EcM fungi and fungal endophytes only in Year 1. In addition, we could hypothesize that the EcM fungi *Paxillus* and *Thelephora* have different strategy to counterbalance defence mechanisms of the host to establishment symbiotic interactions which could be add to the initial effect of MiSSP7 in genetically-modified *Populus*.

Contasted change in the composition of the root microbiome induced by MiSSP7 expression was correlated with difference in the concentration of the main metabolites detected in *Populus* roots

We observed that MiSSP7 expression in *Populus* was correlated with changes in the distribution of most dominant fungal guilds in Year 1: EcM fungi colonizing root system was significantly enriched in *Populus*-expressing MiSSP7 line while fungal endophytes were significantly enriched in non-genetically modified *Populus*. These different types of fungi have different roles in terms of nutrition and protection against stresses (Van der Heijden et al., 2008; Baum et al., 2018). Analyses of the metatranscriptome revealed that the transports of amino acid, nucleotide, coenzyme and inorganic iron were significantly more active in EcM fungi while the transports of carbohydrate and lipid was significantly more active in endophytes. Secondary metabolites biosynthesis and transport tended to be increased in EcM fungi and endophytes compared to saprotrophes while defence mechanisms tended to be increased in EcM fungi compared to saprotrophes and endophytes (data not shown). In Year 2, the most dominant endophytes and EcM fungi detected in *Populus* roots were *Cadophora* and *Thelephoraceae* species. Bonito et al., (2016) showed that *Thelophoraceae* species interacted and compete for resources using a range of different strategy. These results highlight that specific members of the root

microbiome adopt different strategy to colonize tree roots which could also impact the assembly and the functioning of microbial communities (Bonito et al., 2019).

Significant differences were observed in the concentration of primary and secondary metabolites detected in *Populus*-expressing MiSSP7 roots collected in Year 1 and Year 2. This observation was concomitant with the significant shifts detected in root microbiome composition between Year 1 and Year 2. For instance, the concentration of fructose and glucose was significantly enriched in Year 1 compared to Year 2 while the concentration of sucrose remained stable between Year 1 and Year 2 in roots of *Populus*-expressing MiSSP7 harvested at T2 (Table S9). This is particularly interesting in the context of carbon fluxes occurring between host tree and EcM fungi; sucrose is excreted by plant root cells into the common apoplast of the host-fungus interface where it is hydrolysed into equimolar amount of glucose and fructose which could trigger fungal physiology in symbiosis (Nehls et al., 2004; 2008). By contrast, we observed that the concentration of tremulacin was significantly enriched in the roots of *Populus*-expressing MiSSP7 collected in Year 2 compared to those collected in Year 1 (Table S9). Tremulacin is a plant phenolic glucoside that may divert carbon flux through the lignin pathway and play a role in defence (Busov et al., 2006). According to these results, we could hypothesize that the root microbiome which significantly shifts in its composition from year to year, may be responsible of the differential accumulation of metabolites involved in nutrition or in defence mechanisms. In accordance with this hypothesis, the changes in the root microbiome between *Populus*-expressing MiSSP7 and *Populus* WT were concomitant with a modification of 15 % of the measured root metabolites, mostly observed after 6.5 weeks of growth in natural soil. The root metabolites significantly regulated between the two studied lines were different between Year 1 and Year 2: the root metabolites significantly regulated between the two *Populus* lines in Year 1 were involved in primary metabolism while the root metabolites significantly regulated between the two *Populus* line in Year 2 mostly belonged to secondary metabolism. In Year 1, the accumulation of a-linolenic acid and linoleic acid (i.e, the fatty acid substrates of JA biosynthesis; Vick & Zimmerman, 1984) in the roots of *Populus*-expressing MiSSP7 could be explained by the effect of MiSSP7 on the JA signalling and biosynthesis pathway (Plett et al., 2014). In addition, we observed the significant accumulation of SA in the roots of *Populus*-expressing MiSSP7 compared to *Populus* WT collected in Year 1 and the opposite in *Populus* lines collected in Year 2. The roots of *Populus* MiSSP7 cultivated in Year 1 being more colonized by EcM fungi compared to those cultivated in Year 2, these observations were in accordance with the work of Basso et al., (2019) who observed the accumulation of SA in ectomycorrhized roots of *Populus*. In Year 1, the significant accumulation of trehalose coupled with the significant decrease of fructose in the roots of *Populus*-expressing MiSSP7 was in accordance with the presence of EcM fungi (Lewis & Harley, 1965). In Year 2, the accumulation of mannitol, another sugar commonly produced by EcM fungi (Deveau et al., 2008), in the roots of *Populus*-WT could be correlated by the presence of EcM fungi (e.g, members of *Thelephoraceae*). In parallel, fungal endophytes are known to induce important changes in the expression of genes involved in TCA cycle in host cell of raygrass (Dupont et al., 2015). This information was concomitant with the increase of oxalomalic acid level in *Populus*-WT roots collected in Year 2, significantly more colonized by fungal endophytes compared to *Populus*-expressing MiSSP7 roots.

Taken together, these results show that modifications of the composition of the root microbiome observed between *Populus*-expressing MiSSP7 and *Populus* WT correlate with different change in the root metabolome. In Year 1, the significant increase of EcM fungi detected in *Populus*-expressing MiSSP7 roots was concomitant with the accumulation of metabolites, known to be highly regulated in the interactions between EcM fungi and tree host. In Year 2, the change in root metabolome potentially due to MiSSP7 expression was less clear as well as the change in the composition of root microbiome observed between *Populus*-expressing MiSSP7 and *Populus* WT.

Conclusions

The root microbiome of *Populus* cuttings with altered JA signalling pathway differed in the relative abundance of specific bacterial as well as specific fungal communities as compared with those of *Populus* WT. The repetition of our experimental approach over two consecutive years has allowed us to highlight the contrasting effect of JA on the root microbiome and host metabolism. Although the immune system of the tree is an important regulator of the composition and the structure of root microbiome, our study shows that the soil type and natural variations of its microbiome are the key factors involved in the formation of the tree root microbiome.

At this stage, it is difficult to conclude on the actual effect of the JA signalling pathway modulated by MiSSP7 expression on the root microbiome of *Populus*. Further work is needed to validate the observations made in Year 1 and understand those made in Year 2.

III. Conclusions

L'altération de la voie de signalisation de l'AJ via l'expression de MiSSP7 chez les peupliers génétiquement modifiés a engendré des changements au niveau de l'abondance relative des taxons fongiques dominants de la rhizosphère et de l'endosphère par rapport aux peupliers non génétiquement modifiés. Des résultats différents ont été obtenus à l'issue des deux répétitions de notre approche expérimentale sur deux années consécutives concernant l'effet de l'expression ectopique de MiSSP7 sur le microbiote et le métabolome de l'arbre hôte. Bien que le système immunitaire des plantes soit un régulateur important de la composition et de la structure du microbiote racinaire, notre étude montre que le type de sol et les variations naturelles de son microbiote sont les facteurs clés impliqués dans la mise en place et l'évolution du microbiote racinaire. Suite à l'obtention de ces résultats, il est difficile de conclure réellement sur l'effet de la voie de signalisation de l'AJ modulée par MiSSP7 sur la composition et la structure du microbiote racinaire du peuplier. D'autres travaux seraient nécessaires pour confirmer les observations et interprétations réalisées suite à l'expérimentation de l'Année 1 et comprendre celles réalisées suite à l'expérimentation de l'Année 2.

Chapitre VI

Conclusion générale, discussion et perpectives