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Partial overlap of fungal communities associated with nettle and poplar roots when co-occurring at a trace metal contaminated site

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HIGHLIGHTS

- *Urtica dioica* L. raises growing interest because it easily grows at metal contaminated sites and represents a source of high-quality fibres.
- We characterized the mycobiomes associated with nettle and poplar roots co-occuring at a TM-contaminated site.
- Nettle was dominated by endophytic and saprotrophic taxa while poplar was associated with ectomycorrhizal fungi.
- Pezizomycetes taxa were more represented in the highly TM-contaminated area whereas Agaricomycetes tended to be reduced.
- We detected a partial overlap of communities, suggesting some connexions between the poplar and the nettle root mycobiomes.

GRAPHICAL ABSTRACT

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ABSTRACT

Stinging nettle (*Urtica dioica* L.) raises growing interest in phytomanagement because it commonly grows under poplar Short Rotation Coppices (SRC) set up at trace-metal (TM) contaminated sites and provides high-quality herbaceous fibres. The mycobiome of this non-mycorhizal plant and its capacity to adapt to TM-contaminated environments remains unknown. This study aimed at characterizing the mycobiome associated with nettle and poplar roots co-occurring at a TM-contaminated site. Plant root barcoding using the fungi-specific ITS1F-ITS2 primers and Illumina MiSeq technology revealed that nettle and poplar had distinct root fungal communities. The nettle mycobiome was dominated by Pezizomycetes from known endophytic taxa and from the supposedly saprotrophic genus *Kotlabaea* (which was the most abundant). Several ectomycorrhizal fungi such

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as *Inocybe* (Agaricomycetes) and *Tuber* (Pezizomycetes) species were associated with the poplar roots. Most of the Pezizomycetes taxa were present in the highly TM-contaminated area whereas Agaricomycetes tended to be reduced. Despite being a known non-mycorrhizal plant, nettle was associated with a significant proportion of ectomycorrhizal OTU (9.7%), suggesting some connexions between the poplar and the nettle root mycobiomes. Finally, our study raised the interest in reconsidering the fungal networking beyond known mycorrhizal interactions.

1. Introduction

Phytomanagement is a remediation technique based on plant species to restore and revalorize polluted sites while producing biomass (Cundy et al., 2016; Evangelou et al., 2015). Fast-growing trees such as poplars (*Populus sp.*) allow the production of a high amount of valuable plant biomass for renewable energy (Kidd et al., 2015; Pandey et al., 2016). At the same time, trees limit the dispersal and risks of soil contaminants, while promoting the spontaneous biodiversity (Parraga-Aguado et al., 2014). Recent approaches on the optimisation of phytomanagement systems pointed out the importance of assemblages between crops and spontaneous species (Parraga-Aguado et al., 2013) to ensure a more efficient and gentle remediation (Boisson et al., 2016). The spontaneous vegetation biodiversity is generally restricted by the soil physico-chemical properties, limiting the panel of species able to develop and compete with the tolerant species (Macnair, 1987). Much less is known on how this plant biodiversity is shaping its root microbiota in this context.

The stinging nettle (*Urtica dioica* L.) colonizes a vast diversity of nitrophilic environments and particularly anthropogenic areas (Balabanova et al., 2015; Shams et al., 2010; Viktorova et al., 2016). It has thus been frequently observed as a companion species in a wide range of nitrogen-rich environments such as Salicaceous plantations at both natural (Cronk et al., 2016) and anthropized sites (Yung et al., 2019). Nettle has recently raised growing interest in the domain of phytomanagement because it (i) constitutes a new resource of high-quality herbaceous fibres for the manufacture of biomaterials (Bacci et al., 2009; Di Virgilio et al., 2015; Jeannin et al., 2019, 2020), (ii) promotes the biodiversity of local entomofauna (James et al., 2015; Perrin, 1975; Yung et al., 2019) and (iii) spontaneously grows in contaminated sites (Murtic et al., 2019; Paukszto and Mirosławski, 2019; Viktorova et al., 2016).

The success of phytomanagement depends on the capacity of plants to colonize harsh environments, which is partly related to their ability to interact with soil microorganisms, and especially to establish symbiotic relationships with some of them. Among these microorganisms, arbuscular mycorrhizal (AM) (Miransari, 2017), ectomycorrhizal (EM) (Gil-Martínez et al., 2018) fungi and Dark Septate Endophytes (DSE) (Deng and Cao, 2017) are major drivers of plant growth and response to abiotic stress and phytopathogens (Otero-Blanca et al., 2018; Selosse et al., 2004). The use of fungal inoculum has been described as a phytoremediation enhancer by improving uptake and accumulation of trace metals (TM) by plants in phytoextraction application or by limiting the translocation of TM from roots to shoots during phytostabilisation (Berthelot et al., 2016; Khan, 2005; Ma et al., 2019; Pepper et al., 2015). While the success of field inoculation is rather unpredictable (Gerhardt et al., 2017; Selosse et al., 1998, 1999) due to various abiotic (*e.g.*, sensitivity to TM) and biotic parameters (*e.g.*, competition with the local microbiome), the risks of failure may be mitigated by considering indigenous strains, which may have developed a tolerance for TM (Wubs et al., 2016). Characterization of *in-situ* plant-associated microbiome appears as an essential preliminary step for identifying rhizospheric microorganisms that may actively participate to phytomanagement processes.

In the temperate climate zone, poplars are among the few cultivated trees that form tripartite symbiotic associations with EM and AM fungi (Gehring et al., 2006; Teste et al., 2020). As symbiotic fungi play crucial roles in soil fertility, colonisation, plant nutrition, metal uptake, accumulation or restriction in plants (Smith and Read, 2010; van der Heijden et al., 2015), poplar microbiome have been well studied in phytomanagement contexts (Durand et al., 2017; Foulon et al., 2016b; Schmidt et al., 2018; Vitali et al., 2019; Zappelini et al., 2015). Recent studies highlighted the positive effects of EM and AM inoculation on poplar cultivars growing at a multi-contaminated site (Ciadamidaro et al., 2017; Phanthavongsa et al., 2017). However, mycobiome associated with nettle have been barely studied in natural or anthropogenic biota. A study combining *in-situ* and mesocosm approaches concluded that the nettle rhizosphere was devoid of any mycorrhizal structures and revealed that the AM fungus *Glomus mosseae* was not able to develop in the presence of nettle (Vierheilig et al., 1996). The authors speculated that a rhizome protein called *Urtica dioica* agglutinin (UDA) with anti-fungal properties (Broekaert et al., 1989) could be involved in AM avoidance. In addition, this inhibitory effect could even affect the mycobiome of neighbouring plants (Fontenla et al., 1999).

The present study aimed at characterizing, using Illumina Mi-seq sequencing, the mycobiome associated with nettle and poplar roots co-occurring in an agrosystem set up on a contaminated site, where two areas were clearly distinguished by their TM concentrations. We hypothesised that poplar and nettle would harbour distinct root-associated fungal communities: poplar mycobiome would be dominated by EM and AM fungi; at the opposite, nettle communities would be composed of endophytic fungi (*i.e.* fungi colonizing root tissues asymptomatically (Wilson, 1995) with a low fungal diversity as described above. We further hypothesised that the level of TM contamination would alter the root-associated mycobiomes by reducing root fungal diversity and by increasing the diversity and abundance of TM-tolerant fungi, as mentioned elsewhere (Giller et al., 2009; Zappelini et al., 2015).

2. Material and methods

2.1. Site description

The study site located at Fresnes-sur-Escaut (France, 50°25′47.9"N 3°35′07.8″E) is a nettle-poplar agrosystem on a dredged sediment disposal site, contaminated with TM, that operated between 1978 and 1989.

Approximately 200,000 m^3 of silt and sand from the dredging of l'Escaut canal were deposited, resulting in heterogeneous contamination with As, Cd, Cu, Ni, Pb and Zn within the surface horizons (0–50 cm). The experimental site is divided into two areas differing by the level of TM: the low trace-metal (LTM) and the high trace-metal (HTM) areas. The HTM area has mean soil concentrations of Zn (2231 mg/kg), Pb (425 mg/kg), Cu (108 mg/kg) and Cd (63 mg/kg), on average 20-, 9-, 4- and 100-fold higher than the LTM area, respectively (Phanthavongsa et al., 2017). A phytomanagement field trial was implemented in 2011 on this site (covering both areas) with plots of poplar Skado (*P. trichocarpa* × *P. maximowiczii*) or I214 (*P. deltoides x P. nigra*) as a short-rotation coppice (SRC; 2200 stems/ha). A detailed description of the experimental design is provided in Ciadamidaro et al. (2017) and Phanthavongsa et al. (2017). Five years after planting, the spontaneous herbaceous layer evolved in the same way as that of another similar

experiment set up in eastern France, with a dominance of the stinging nettle under poplars with a coverage rate depending on the poplar cultivar (Yung et al., 2019). In order to get rid of this variable and appreciate the contaminant effect on the microbiomes associated with nettle and poplar roots, we selected only one plot for each poplar cultivar (*i.e.* Skado or I214) for both the HTM and LTM areas, each of 504 m^2 (28 m x 18 m), *i.e.* 4 plots in all. These plots are of sufficient size to allow for replicate collection.

2.2. Sample collection and preparation

The sampling was conducted in November 2016, consisting of root samples from the upper 20-cm layer of soil from 9 random poplars and 9 adjacent nettles for each of the four studied plots. Comparisons between soil and poplar root mycobiomes were done and available in our previous studies (Durand et al., 2017; Foulon et al., 2016a, 2016b). A total of three pseudo-replicates of thin roots were sampled from each tree and mixed to obtain one composite per poplar individual, whereas the entire root system of nettles was collected. For each plant, the root samples were carefully sorted so as not to be mixed, the soil was carefully removed in three successive baths in sterilized distilled-water, and the smallest roots were selected and separated from larger roots by sampling them with a scalpel. The samples were freeze-dried and stored at −20 °C before molecular analysis. Thus, we considered both the endophytic and epiphytic fungi from the rhizoplane.

2.3. Molecular method

The environmental DNA from the roots was extracted with the PowerSoil DNA isolation Kit following the manufacturer's instructions (MoBio Laboratories, Inc., Carlsbad, CA, USA). The PCR was performed using the specific fungal primers ITS1F (5'-CTTGGTCATTTAGAGGAAG TAA-3′) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3′) (Gardes and Bruns, 1993; White et al., 1990). We selected these primers to allow for comparison with our previous dataset obtained on a similar plantation, at a different site (Durand et al., 2017). These primers has also been found as one of the most competent in uncovering the fungal diversity of plant root (Bainard et al., 2017; Huang et al., 2020). PCR was performed in a reaction volume of 35 μL using 0.5 μM forward and reverse primer each, 200 μM dNTPs, 1 μL of Phire hot start II DNA polymerase (1 U/μL), 26.25 μL of 5× Phire reaction buffer (Thermo Fisher Scientific, Inc. USA), 10 ng template DNA and sterilized water to reach a final volume of 35 μL. The PCR conditions were 98 °C for 30 s, followed by 25 cycles of 98 °C for 5 s, 57.2 °C for 5 s and 72 °C for 15 s, followed by a final extension step at 72 °C for 1 min. These primers targeted a short portion of the fungal ITS region, resulting in amplicons of small size (~300 bp), appropriated for Illumina sequencing. The DNA quality and quantity were assessed by agarose gel electrophoresis using the ImageLab software (Bio-Rad, USA) and with a Qubit™ dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) using the Qubit 4 fluorometer. The partial fungal ITS region (Wang et al., 2015) of 6 and 7 of nine poplar and nettle samples (*i.e.* with the concentration of environmental DNA required) were sequenced with the Illumina MiSeq platform (Microsynth AG, Switzerland).

2.4. Bioinformatic analysis

The sequence data were processed using Mothur (Schloss et al., 2009). First, all raw read pairs were joined at the overlapping region to create consensus sequences. Then several steps of filtering were performed, consisting in removing homopolymers, ambiguous sequences and sequences with an inappropriate length (shorter than 220 bp and greater than 400 bp). The 18S RNA region amplified by the ITS1F primer (Toju et al., 2012) was removed by *in-silico* PCR to target precisely the ITS1 region. After quality filtering, reads were then clustered into OTUs using the swarm tools (Mahé et al., 2015) with a difference value of 1 and fastidious option enable. The OTU clustering method has been repeatedly shown to be powerful for recovering the richness and composition of the fungal community (Bell et al., 2014; Durand et al., 2017; Foulon et al., 2016b; Huang et al., 2020; Nguyen et al., 2016). We removed singleton OTUs to avoid technical artefacts and overestimation of the number of species (Tedersoo et al., 2010). The taxonomic assignment of OTUs was performed using the UNITE (Kõljalg et al., 2013) database by a naïve Bayesian approach (Wang et al., 2007). The presence of plant sequences was verified using the BLAST tool of the GenBank database and removed. Each fungal OTU was further assigned to functional or morphological groups of fungi using FUNguild (Nguyen et al., 2016). For each assignment, three confidence rankings (*i.e.*, "possible": suspected, "probable": fairly certain, "highly probable": absolutely certain) referring to previously peer reviewed data were given. We only considered the functional and morphological assignation with at least a "probable" confidence ranking. Finally, the guilds related to a similar function were grouped to obtain a total of six main guilds, based on the literature.

2.5. Data analysis

Alpha diversity indices (OTU richness, Chao estimation, Shannon diversity index, inverse of the Simpson diversity index) were calculated using MOTHUR. Samples were rarefied at 33000 sequences, corresponding to the smallest number of sequences detected in a sample. No sample was excluded. To verify that sequencing depth allowed covering most of the fungal diversity of our root samples, Good's coverage was calculated as $C = [1 - (n / N)] \times 100$ (%), where "n" is the number of OTUs, and "N" is the number of sequences (Good, 1953).

Statistical analyses were performed with R software v.3.6.1 (R Development Core Team, 2019). All the considered variables were tested for their homoscedasticity (Bartlett or Levene tests) and normal distribution (Shapiro–Wilk test) and compared using analysis of variance (ANOVA) followed by a Tukey HSD test. Where necessary, data were "log" transformed to improve normality. Data that did not fit a normal distribution after transformation were analysed with non-parametrical tests (Kruskal-Wallis test by ranks). First we calculated and drew rarefaction curves for each experimental condition of nettle and poplar, using "rarecurve" function available in the R Vegan package. The shape of the curve is a graphic display of the relative estimated species accumulation rates. All diversity and richness indices and the mean number of OTUs were then compared between all experimental conditions and plants through ANOVA followed by a Tukey HSD test.

To summarize differences in fungal OTU composition between the experimental conditions for both the I214 and Skado plots, we used two-dimensional non-metric Multi-Dimensional Scaling (NMDS) plots based on Bray-Curtis dissimilarity matrix ($k = 2$) using the metaMDS function of the R Vegan package. Permutational multivariate analyses of variance (PERMANOVA) based on Bray-Curtis dissimilarity were applied to determine the potential effects of the plant species (nettle *vs* poplar) and the level of contamination (LTM *vs* HTM) on fungal communities for both I214 and Skado plots. To assess the influence of the studied factors on the fungal classes, genera, EM fungal taxa and guilds, the experimental conditions were compared using a Kruskal-Wallis test, followed by a post-hoc test with a Holm correction of the *p*-value.

NMDS and PERMANOVA analyses were further performed on the EM fungal guilds. For the whole fungal community and the six most abundant guilds taken individually, for nettle and poplar, we tested the correlation and the similarity between the OTU composition using Mantel test (ade4 package) and by calculating the Bray-Curtis similarity index (SpadeR package), respectively. The relative abundances of EM fungal OTUs were represented by a heatmap, from the pheatmap R package. Similarly, a hierarchical cluster analysis of the relative abundances of EM fungal OTUs from the various experimental conditions

was represented by a dendrogram expressing the linkage between species and between the experimental conditions.

3. Results

3.1. Diversity of mycobiome associated with nettle and poplar roots

Illumina MiSeq sequencing of ITS1 amplicons obtained from 26 root samples of poplar and 25 root samples of nettle generated a total of 3,481,223 non-chimeric fungal sequences. The initial total number of sequences obtained per sample ranged from 33,615 to 140,409. The sample with the lowest number of sequences was selected for subsampling, revealing a total number of 1833 non-singleton OTUs for 1,683,000 fungal reads, and constituting our final dataset.

Rarefaction curves calculated for each experimental condition of nettle and poplar tended to reach an asymptotic profile, suggesting that the sequencing depth was sufficient to cover the fungal diversity of our root samples. Additionally, the measured Good's coverage values were greater than 99% for each condition and in every sample, suggesting a representative sampling. Rarefaction curves for nettles clearly exhibited divergent trends in LTM *vs.* HTM areas. For a same number of reads on the asymptote, the HTM plots reached a number of OTUs reduced by approximately 30%, as compared to the LTM plots (Fig. S1). Concerning poplar, the curve for the I214-HTM plot was clearly lower than those corresponding to the three other experimental conditions.

The total number of fungal OTUs was comparable for nettle (1218) and poplar (1321). Among them, nettle and poplar shared 766 OTUs, representing 63% of the total number of OTUs for nettle and 58% for poplar. The mean number of fungal OTUs associated with the roots of nettle and poplar are detailed in Table 1 for each experimental condition. The highest mean number of OTUs was obtained for Skado roots from the HTM area, whereas the lowest was obtained for nettles within the same area, with a significant difference ($P < 0.05$). Only 7.1% and 7.5% of the total number of OTUs were shared between the four conditions for nettle and poplar, respectively. Additionally, the percentage of OTUs specific to each condition ranged between 8.5% and 19.9% for nettle and between 11.2% and 14.8% for poplar (Fig. 1). For nettle, this number of specific OTUs was 2-fold higher in the LTM area (35.4%) compared to the HTM area (17.3%), whereas it was comparable for poplar (23 *vs* 26%). Depending on the experimental condition, nettle shared between 47.3% and 63.0% of their OTUs with poplar, while poplar shared between 39.5% and 44.0% of their OTUs with nettle (Fig. 1).

While the obtained mean numbers of fungal OTUs were comparable between nettle and poplar, the estimated number of fungal OTUs (using the Chao model) showed a higher species richness in poplar roots, except for the I214-HTM condition (Table 1). The species richness of the HTM area was lower than the LTM area for nettle, while this tendency was only noticeable for I214 poplars, as suggested by the rarefaction curves (Fig. S1). For nettle, we collected an average of 65% and 54% of the estimated number of OTUs (Chao estimation) for the LTM and HTM areas, respectively, whereas we obtained 69% for HTM *versus* 48% for LTM for poplar. Overall, when considering the individual diversity estimators related to poplar or nettle, no apparent influence of the studied factors level of contamination was noticeable. However, NMDS analysis revealed a clear clustering of fungal communities according to the plant species (nettle *vs* poplar) and the level of contamination (LTM *vs* HTM) for the I214 and Skado plots (Fig. 2). Thus, while the fungal diversity did not seem to be significantly influenced by the experimental condition (Table 1), the fungal composition of nettle and poplar appeared contrasted and affected by the level of TM contamination and the plant species (Fig. 2). NMDS and PERMANOVA indicated substantial differences in the composition of fungal communities between nettle and poplar (Fig. 2), the plant species factor explaining 24% and 17% of the variance in the fungal community for I214 and Skado plots, respectively ($P < 0.001$; Table S1). The level of TM had a significant effect on the fungal communities, both for nettle grown under I214 (28%) and Skado (37%) and for poplar, although to a lesser extent (26% for I214, and 16% for I214 Skado) (all $P < 0.01$; Fig. 2, Table S1).

3.2. Taxonomic characterization of the mycobiome of nettle and poplar roots

The fungal communities associated with roots of plants growing at the sediment disposal site studied were dominated by the phylum *Ascomycota* in nettle roots (84.5% of total relative abundance) whereas *Basidiomycota* (43.2%) and *Ascomycota* (52.3%) were more equally represented in poplar roots (the *Ascomycota*/*Basidiomycota* ratio was significantly higher for nettle than for poplar; $P < 0.05$).

Among all samples, we detected 29 and 28 distinct fungal classes for nettle and poplar, respectively. Among them, the most abundant classes were *Pezizomycetes, Agaricomycetes, Leotiomycetes, Dothideomycetes, Sordariomycetes, Mortierellomycetes, Eurotiomycetes* and *Olpidiomycetes* (total relative abundance of each >0.5%; Fig. S2). For this study, *Dothideomycetes* and *Leotiomycetes*, as abundant classes, characterized the mycobiome of nettle roots. In contrast, poplar roots were characterized by *Agaricomycetes*, representing 20–66% of all sequences, depending on the experimental condition (Fig. S2). *Pezizomycetes* were also well represented and similarly abundant in both plants.

For poplars, 79% of the fungal sequences were successfully assigned to a genus (73% for I214 *vs* 85% for Skado), whereas only 54% were successfully assigned for nettle (Fig. 3). The genus *Kotlabaea* was the most abundant genus associated in nettle roots (26.5% of the total number of fungi associated with nettle), followed by three unknown genera belonging to Helotiales (17.5%), Didymosphaeriaceae (8.7%) and Phaeosphaeriaceae (5.8%). The genera *Tetracladium* (5.7%) and *Hymenoscyphus* (2.5%) were the most abundant nettle-associated *Leotiomycetes* and were also present in poplar roots with comparable relative abundances (Fig. 3). For poplar, the most abundant genera were *Inocybe* (17.1% of the total number of fungi associated with

Table 1
Results of the Illumina MiSeq sequencing of ITS1 amplicons, including richness and diversity indices of the mycobiomes associated to I214 and Skado poplar roots, or to nettle roots growing in the I214 or Skado plots, at low trace-metal (LTM) and the high trace-metal (HTM) areas. Mean values and standard deviations (mean ± SD) are provided for each experimental condition. The "total number of sequences" corresponds to datasets before subsampling at 33,000 sequences, while the other parameters are calculated after subsampling. Values designated with the same letters are not significantly different (Kruskal–Wallis test, P < 0.05).

	Nettle				Poplar			
	LTM		HTM		LTM		HTM	
	I214	Skado	I214	Skado	I214	Skado	I214	Skado
Number of samples	6	6	6	7	6	7	6	7
Mean total number of sequences	52,264	73,778	68,434	70,185	69,479	63,116	55,243	61,067
Mean OTUs observed	175^{ab} (± 56)	168^{ab} (± 69)	122^{b} (± 23)	109^{b} (± 52)	178^{ab} (± 19)	196^{a} (± 30)	156^{ab} (± 38)	204^{a} (± 43)
Chao estimation	248^{c} (± 56)	280^{bc} (± 61)	221^{c} (± 25)	210^{c} (± 64)	362^{ab} (± 31)	410^{a} (± 74)	232^{c} (± 33)	358^{ab} (± 70)
Shannon index	2.3^{ab} (± 0.8)	2.7^{a} (± 1.0)	2.2^{ab} (± 0.6)	1.4^{b} (± 1.0)	2.4^{ab} (± 0.5)	2.7^{a} (± 0.5)	2.9^{a} (± 0.7)	2.9^{a} (± 0.5)
Inverse of Simpson diversity index	6.0^{a} (± 5.4)	8.9^{a} (± 6.1)	5.8^{a} (± 3.4)	3.1^{a} (± 2.8)	5.7^{a} (± 2.3)	7.7^{a} (± 5.3)	10.1^{a} (± 5.7)	8.9^{a} (± 6.1)

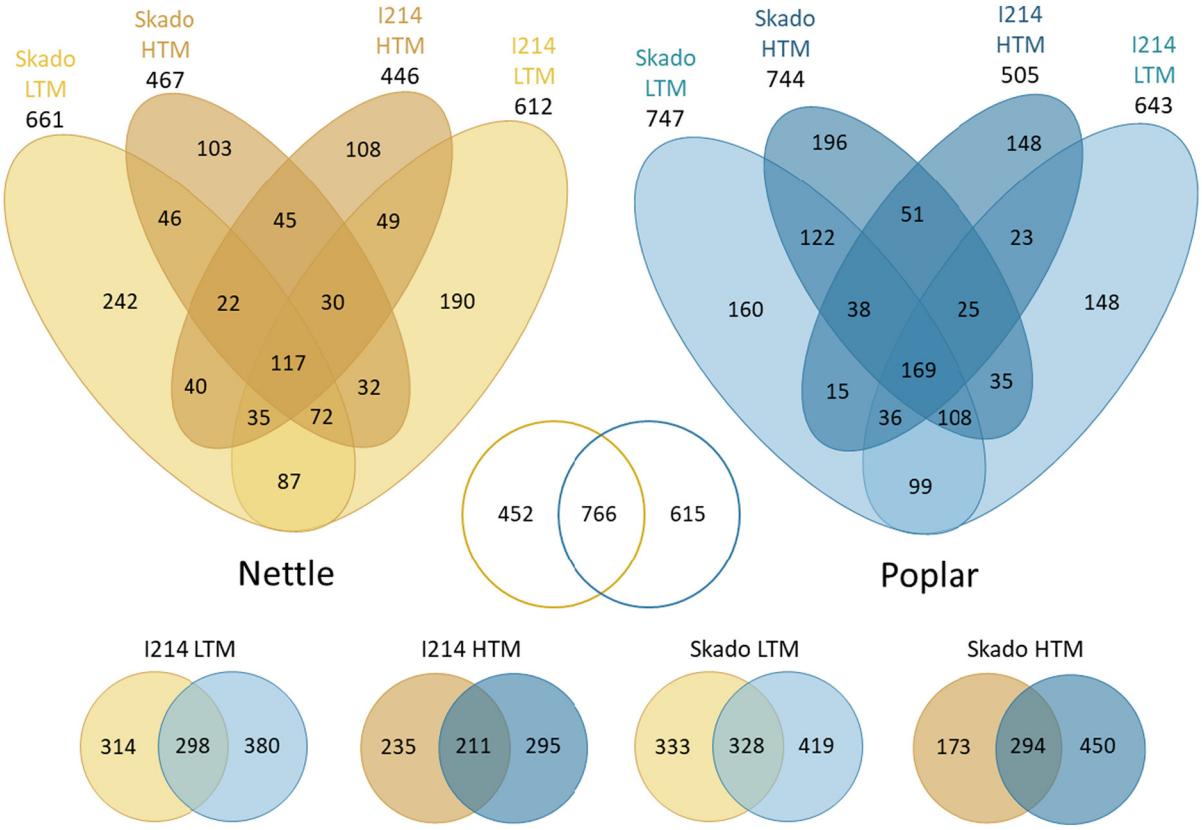

Fig. 1. Venn diagrams showing the overlap of the fungal communities according to the poplar cultivar plot (I214 and Skado) and the level of TM in soil (LTM and HTM), for nettle (left) and poplar (right). These diagrams present the number of total (above the ellipses), specific (within non-intersected ellipses) and shared OTUs (intersected ellipses). *The five couples of circles provide the total number of OTUs for nettle (left) and poplar (right) and the shared number (intersection) for the whole dataset (center) and for each experimental conditions (bottom).*

poplar), *Tuber* (10.8%), *Hebeloma* (7.3%), *Tomentella* (6.9%) and *Geopora* (7.1%), the latter being almost absent in nettle roots (< 0.02%) (Fig. 3). The genus *Olpidium* (*Olpidiomycetes*), accounting for 1.3% and 1.4% of the fungal sequences for nettle and poplar, respectively, was also well represented.

We detected significant differences in terms of fungal composition between the two poplar cultivars. *Tomentella, Geopora, Hymenogaster, Hebeloma* and *Clavulina* were more represented in roots of the Skado cultivar, while *Helvella* and *Kotlabaea* were mostly associated with I214 cultivar ($P < 0.05$), but each of these trends were only significant for one of the two considered areas (*i.e.*, LTM or HTM; $P < 0.05$; Fig. 3, Table S2). Differences were also noticed between mycobiomes of nettles grown in I214 plots (high density of nettle) and those grown in Skado plots (low density of nettle). *Inocybe*, *Genabea, Helvellosebacina* and *Hebeloma* were more abundant in roots of nettles grown in the Skado plot ($P < 0.05$), whereas *Olpidium* and *Calyptella* were more abundant in the I214 plot ($P < 0.05$). These results were significant for only one of the two considered areas (Fig. 3, Table S2). Pezizomycetes were significantly influenced by TM contamination: first, their relative abundance was significantly higher at the HTM area for nettles, both the I214 (10% for LTM *vs.* 32% for HTM; $P < 0.05$) and Skado (7% *vs.* 68%; $P < 0.05$) plots. Second, the same trend was observed on poplar but only statistically supported for the Skado cultivar, (6% *vs.* 41%; $P < 0.05$; Fig. S2). Among Pezizomycetes, the genus *Kotlabaea* significantly increased in the HTM area (for I214 plots: 0.1% *vs.* 31.7%; for Skado plots: 2.5% *vs.* 65.1%; $P < 0.05$; Fig. 3). Similarly, the genus *Tuber* was more represented in the poplar roots collected from the HTM area (for the I214 cultivar: 6.2% *vs.* 17.6%; for the Skado cultivar 4.6% *vs.* 15.2%; P), however, the high between-sample variability did not allow to statistically support this. *Agaricomycetes* associated with both poplar and nettle were influenced by TM as their abundance was significantly reduced in the HTM areas ($P < 0.05$), except for nettle in the I214 plots (Fig. S2). This reduction of *Agaricomycetes* in the HTM area was mainly due to the decrease of the *Inocybe* genus, the most abundant of all *Agaricomycetes* for nettle (34%) and poplar (40%) (Fig. 3). When considering nettle and poplar together, the relative abundance of *Inocybe* species decreased from LTM- to HTM area, namely from 31.9 to 1.5% for I214 plots and from 24.1 to 8.1% for Skado plots ($P < 0.05$ in each case; Fig. 3). A similar but less pronounced trend was also noticeable for the genus *Tomentella* within the I214 plots.

3.3. Functional characterization of the mycobiome of nettle and poplar roots

Among the 1218 and 1321 fungal OTUs recorded in nettle and poplar roots (Fig. 1), 70.4% and 83.1%, respectively, were successfully assigned to a functional guild with at least a "probable" confidence ranking using FUNguild (Nguyen et al., 2016). This functional assignment revealed a majority of saprotrophic fungi (from 18 to 69% depending on the condition), followed by plant pathogens (6–15%) and EM fungi (1–30%) in the nettle roots whereas EM fungi (26–75%), mainly represented by *Tomentella, Geopora, Hymenogaster, Hebeloma* and *Clavulina* genera, were the most abundant guild in poplar roots (Fig. 4). This guild was significantly more abundant in roots of Skado poplar, compared to I214 poplar (63% *vs* 26% for HTM area, $P < 0.05$; Fig. 4). EM fungi were abundant on nettle roots and, for the LTM area, even more on nettle grown with the Skado cultivar than on those grown with I214 (30% *vs* 2%; $P < 0.05$). Endophytes were poorly represented in the poplar roots in contrast to nettle (0.1% *vs.* 9.2% of all sequences; $P < 0.05$; Fig. 4). Twelve OTUs assigned to AM fungi were found in nettle and

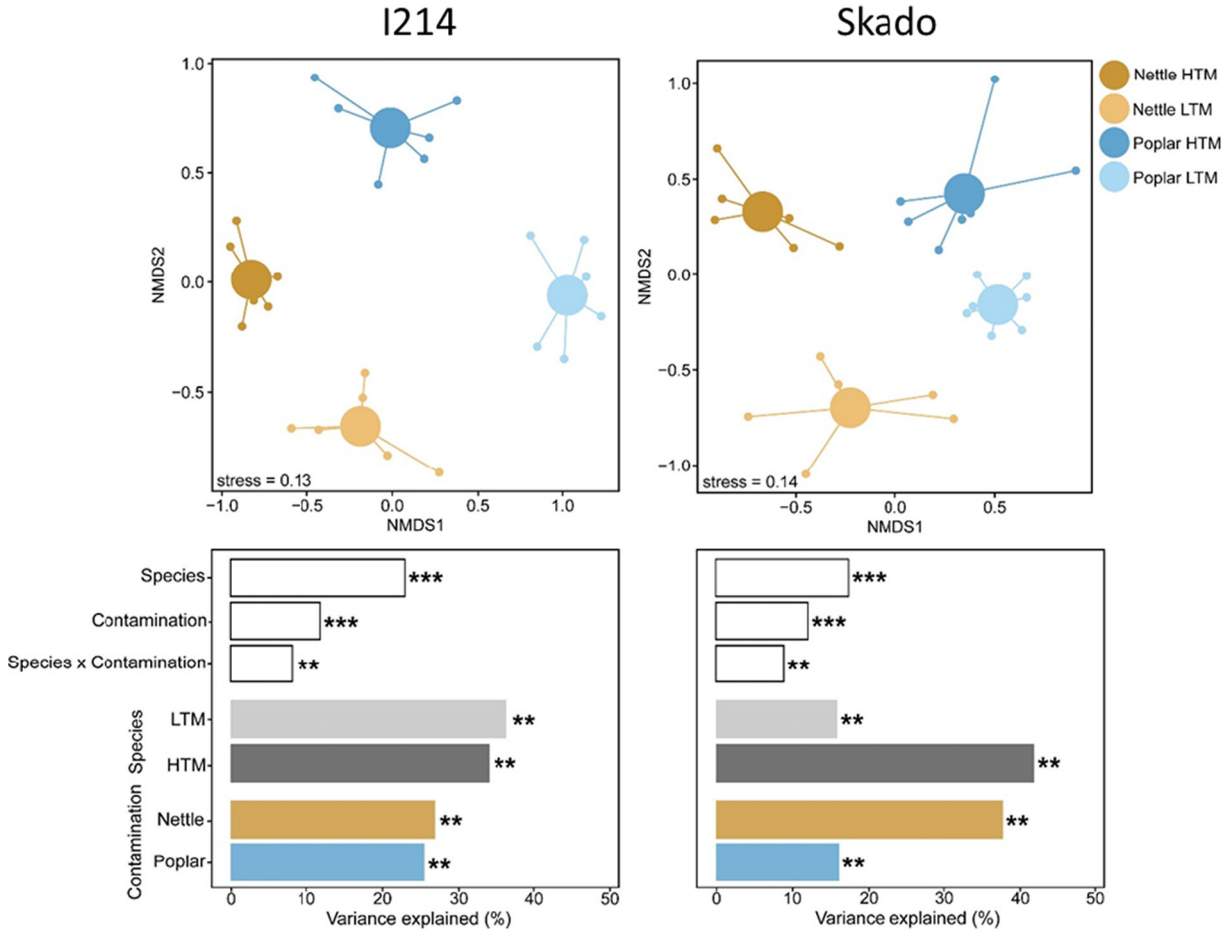

Fig. 2. Non-parametric multidimensional scaling (NMDS) plot of fungal communities associated with nettles and poplars grown at LTM and HTM areas, for I214 (left) and Skado (right) plots, using the Bray Curtis dissimilarity measure. Small and large circles represent the individual samples and the centroids of the different temperatures, respectively. Results from a PERMANOVA analysis for plant species ("species") and level of TM ("contamination") factors and their interaction, and conducted separately by species (contamination effect) and contamination (species effect) are provided under each NMDS, with the following legend: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

poplar roots, accounting for less than 0.05% of all sequences (not shown). Plant pathogenic fungi were equally represented in poplar and nettle roots, without any influence of the studied factors (Fig. 4).

Functional guild also revealed several differences between soil contamination levels. Indeed, the relative abundance of nettle saprotrophs was higher in the HTM area (statistically supported only for the Skado plots; $P < 0.05$) (Fig. 4). This result was driven by *Kotlabaea*, the most abundant genus within this guild (Fig. 3): an unidentified species from this genus, accounting for 66% of all the saprotrophs associated with nettle, was indeed significantly more present in the HTM areas ($P < 0.001$). EM fungi associated with poplar (particularly *Inocybe* and *Tuber*) and nettle (particularly *Inocybe*) tended to decrease in the HTM area, which was statistically supported for I214 plot in the case of poplar (75% *vs* 26%, $P < 0.05$) and for Skado plot in the case of nettle (30% *vs* 6%; $P < 0.05$; Fig. 4).

3.4. Overlap of poplar and nettle microbial communities

According to the Mantel test, nettle and poplar total fungal communities under I214 ($P < 0.05$) or Skado ($P < 0.01$) plots were significantly correlated (Fig. 5), with Bray-Curtis similarity index of 0.21 and 0.27, respectively. Mantel tests indicated that nettle and poplar root-associated animal pathogenic fungi, EM fungi and saprotrophs were significantly correlated (Mantel test, $P < 0.05$), with similarity indexes between 0.22 and 0.64, except for EM fungi under I214 plots (Fig. 5). Despite the low relative abundance of EM fungi on nettle under I214 plots (Fig. 4), nettle and poplar composition of EM fungi were strongly correlated ($P < 0.001$, Fig. 5) for I214, while it was not the case for Skado plots. Interestingly, the saprotrophic guild of the two plant species were only correlated at I214 plots, where the most abundant genera *Kotlabaea* was driving the trend. Consequently, these results suggested that nettle and poplar roots shared large part of their fungal communities.

Although there was a significant relationship between EM fungi associated with the two plants, the NMDS analysis showed that EM fungi of nettle and poplar clustered for each host, but not together, suggesting host differentiation (Fig. 6.A). EM fungi were significantly more represented (59.6% *vs* 9.7%; $P < 0.05$) and diverse (330 *vs* 146 OTUs; $P < 0.05$) in poplar than in nettle roots (Figs. 4 and 6.C). Moreover, the ratios between nettle and poplar EM richnesses were similar for each condition (ranging from 0.64 to 0.83). A Venn diagram showed a higher species richness of EM fungi associated with poplar and particularly with the Skado cultivar (Fig. 6.B), confirming poplar as the main host for EM fungi. As observed above for poplar, nettle EM fungi were more represented in terms of richness (115 *vs* 65 OTUs) and abundance (27.1 *vs* 1.7% of the total number of sequences) under the Skado plots as compared to the I214 plots. Among the 138 OTUs associated with nettle, 102 OTUs were shared between nettle and poplar (Fig. 6.B). Only 29 of the 115 EM OTUs (representing 1.6% of the total abundance of EM fungi) under the Skado plots and 8 of the 65 EM OTUs (11.7% of the total abundance) under the I214 plots were specific to nettle (Fig. 6.B).

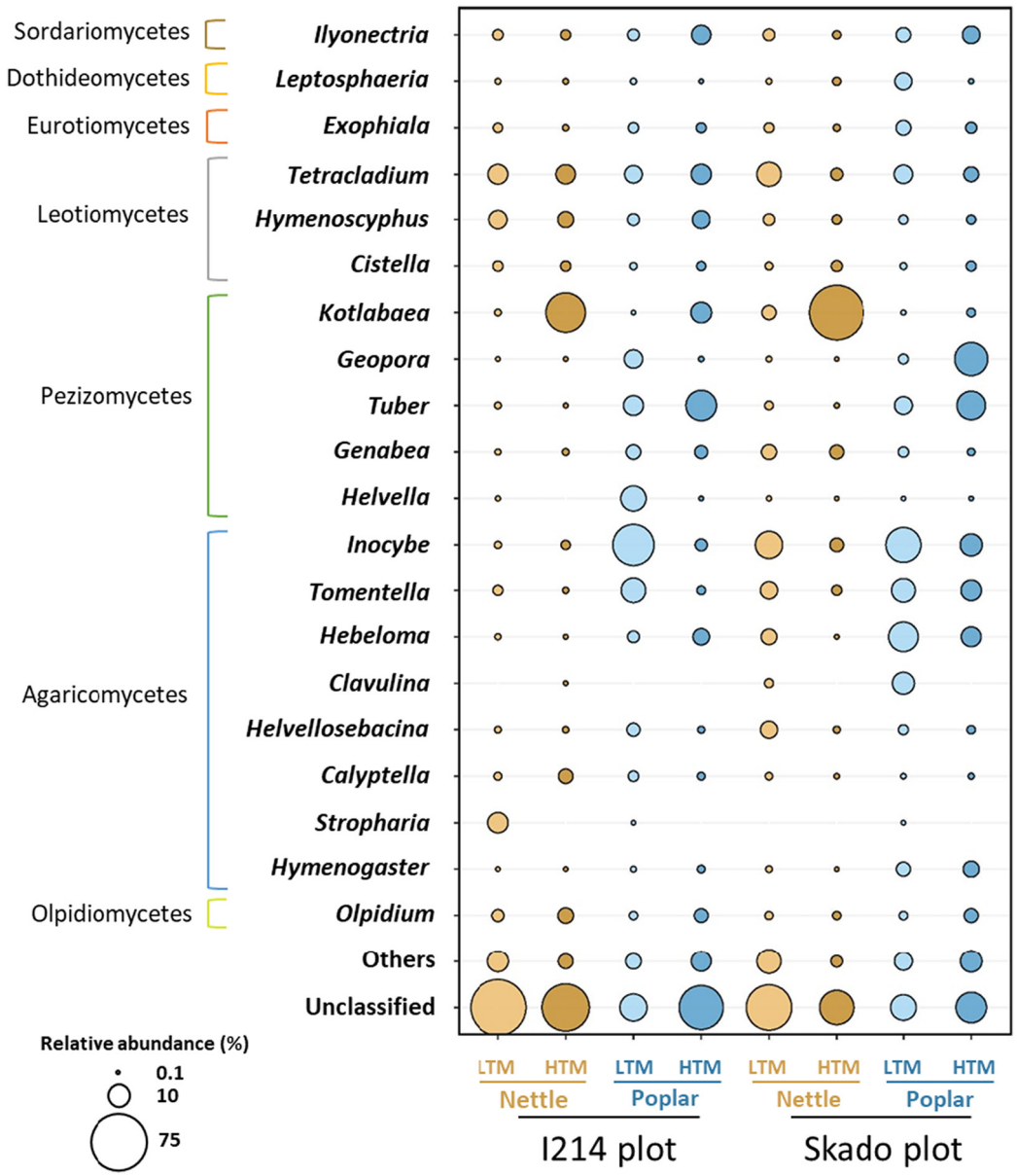

Fig. 3. Relative abundance of the 20 most represented fungal genera (based on the taxonomic assignment of OTUs), belonging to 7 classes, according to the level of TM in soil (LTM and HTM) for nettle (orange bubbles) and poplar (blue bubbles) grown in the I214 or Skado plots. The classes with a relative abundance <0.5% have been gathered in the "others" group. For each genus, the related results from Kruskal–Wallis pairwise or Tukey HSD tests are provided in the Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These results suggested that most of the EM fungi associated with nettle were related to these hosted by poplar.

According to a hierarchical cluster analysis of the relative abundances of EM fungal OTUs, LTM poplars were mostly colonized by the EM *Inocybe rimosa, Tomentella sp., Helvella elastica, Inocybe semifulva, Clavulina* sp. and *Hebeloma eburneum* (Fig. S3). The EM OTUs *Geopora cervina* and *Tuber rapaeodorum* were abundant in roots of poplar grown at the HTM area (Fig. S3). Most of these taxa were poorly represented in the nettle roots, except for *Inocybe rimosa*, which was the most abundant EM fungus in both plants (Fig. S3). Indeed, this species accounted for 4% of nettle sequences (95% from the Skado plots) and 12.3% of poplar sequences (52% from the Skado plots). Additionally, this species was more abundant in roots of nettle grown with the Skado cultivar ($P < 0.05$), and when associated with poplar, it was sensitive to the soil contamination ($P < 0.05$) (Fig. S3).

4. Discussion

4.1. EM fungi dominated the poplar mycobiome

In this study, the mycobiome associated with poplar roots were clearly dominated by several EM fungal species, such as the Agaricomycetes *Inocybe rimosa, Tomentella sp.*, and *Hebeloma spp.*, and the Pezizomycetes *Tuber rapeodorum* and *Geopora cervina*. We also detected the presence of AM fungi belonging to the *Rhizophagus* and *Glomus* genera, although with a rather low number of sequences (< 0.001%), partially due to the choice that was made to target the ITS1 region, which is not the most adapted region for the study of Glomeromycota (Beeck et al., 2014). The recorded EM fungal species are known fungal symbionts of poplar (Podila et al., 2009) that improve plant health by enhancing resistance to diverse stresses like drought,

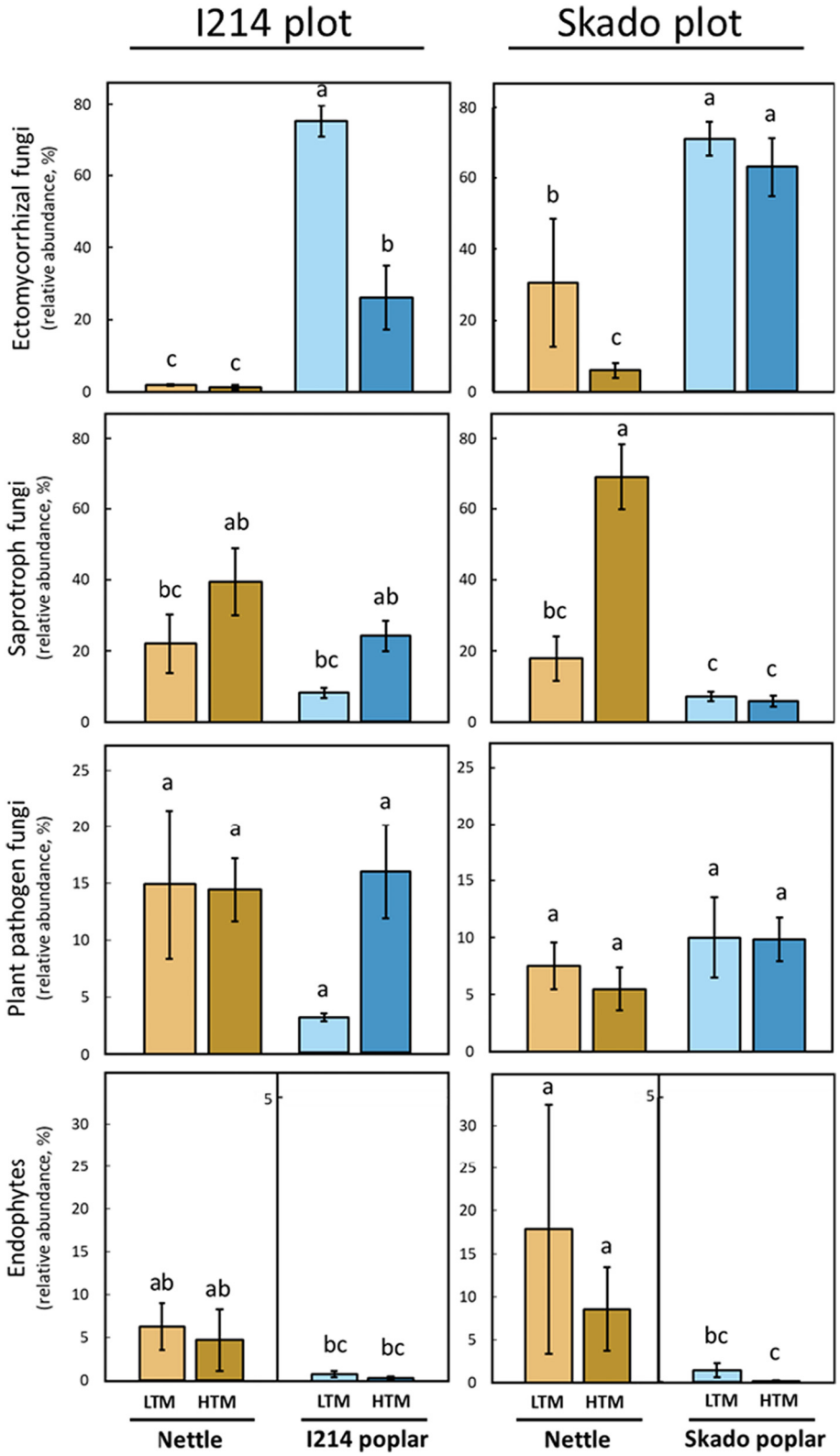

Fig. 4. Relative abundance of the four most abundant fungal guilds associated with nettles and poplars grown at the LTM or HTM area, for I214 (left) and Skado (right) plots. Data are the means ± SE ($n = 6$ or 7). For each class, bars designated with the same letter(s) are not significantly different (Kruskal–Wallis, $P < 0.05$).

salinity, heavy metals and pathogens, *etc.* (van der Heijden et al., 2015). Particularly, they facilitate the adaptation of plants to TE stress, promoting host growth and phytomanagement of TM-contaminated soils (Gil-Martínez et al., 2018).

In a previous study on a Hg-enriched sediment disposal site, the same experimental design was set up in 2011. Durand et al. (2017) showed that EM fungi, and particularly the *Tuber* and *Geopora* genera also dominated the roots of Skado poplar at this site. These taxa seemed

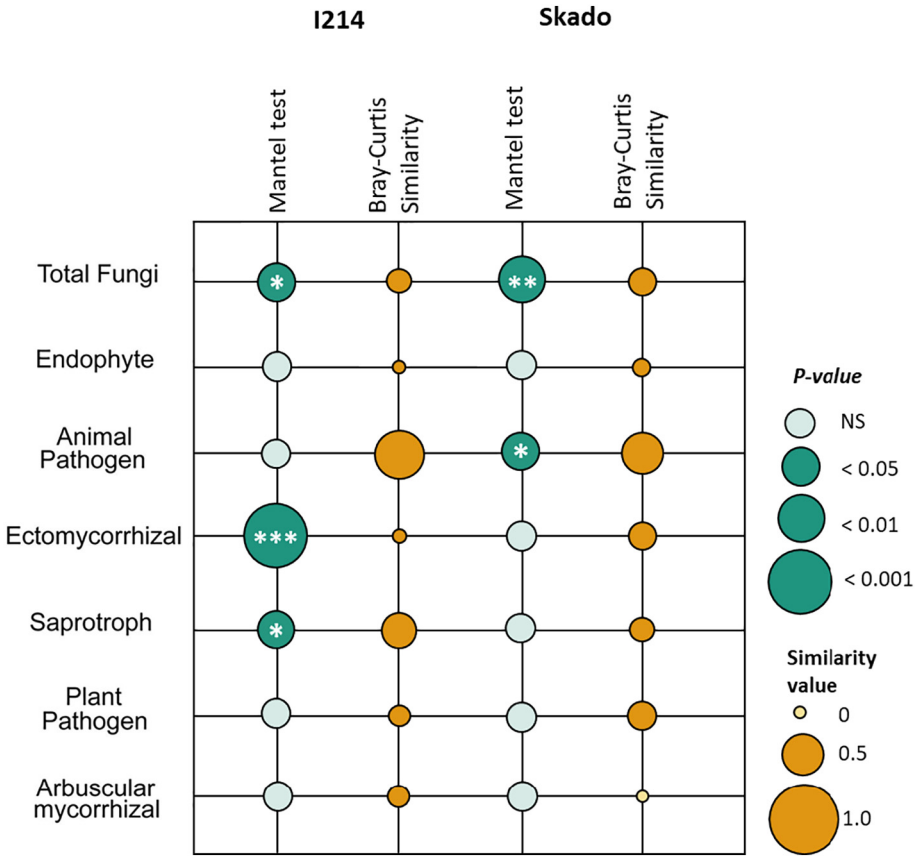

Fig. 5. Correlations (Mantel test) and similarity (Bray-Curtis) between the OTUs composition of nettle and poplar, for the whole fungal community ("Total Fungi") and the six most abundant guilds depending on the poplar cultivar plot. The size of the circles is proportional to the *P*-value for the Mantel test and to the index value for Bray-Curtis. P-value of the Mantel tests are represented with the following legend: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

to be largely abundant in TM contaminated sites (Bonito et al., 2014; Guevara et al., 2013; Lacercat-Didier et al., 2016). Moreover, these EM fungi were not only related to the physico-chemical properties of these sites, but rather to the juvenile tree stage that promotes some Pezizales (*Geopora spp., Tuber spp.*) or Agaricales (*Inocybe spp.*) (Foulon et al., 2016b; Hrynkiewicz et al., 2010; Lacercat-Didier et al., 2016). *Hebeloma* or *Inocybe* species, which were well represented at the studied site, are "early stage" fungi capable of colonizing roots of trees established in virgin or disturbed habitats (Smith and Read, 2010).

4.2. Saprotroph and endophytic fungi dominated the nettle root mycobiome

The overall fungal composition of nettle roots differed from that of poplar, despite their co-occurrence. Most of the dominant species associated with nettles were only assigned to the family or order level, reflecting a lack of information concerning the nettle mycobiome. Dominant species on nettle roots were mostly endophytic or pathogenic fungi, such as *Tetracladium sp.*, *Olpidium spp.*, or saprotroph belonging to the Helotiales and Pleosporales orders as previously observed for

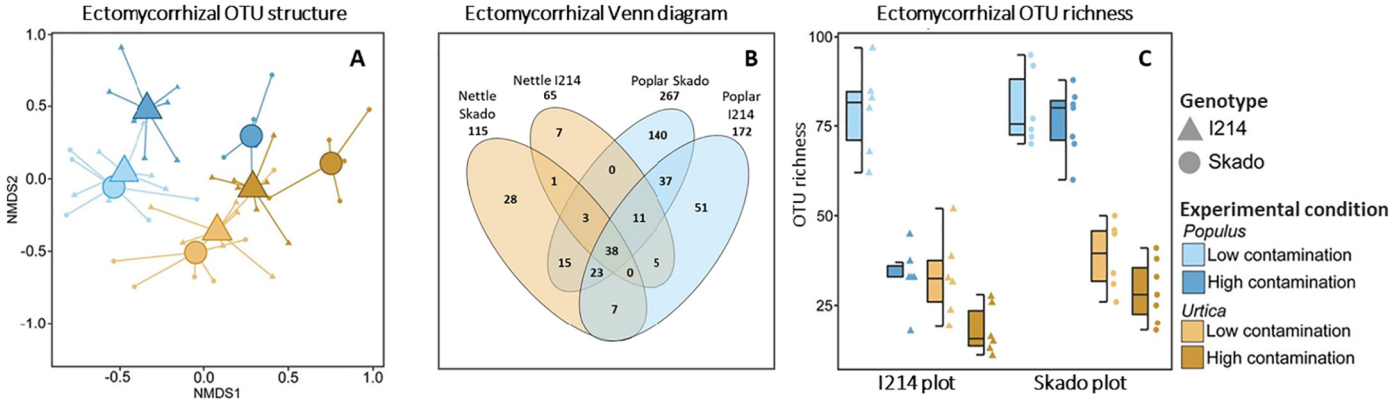

Fig. 6. (A) Non-metric multidimensional scaling (NMDS) analysis of the EM fungal guild structure associated to poplar or nettle roots, (B) a Venn diagram based on EM OTUs and (C) the EM OTU richness, depending on the plant species (poplar or nettle), the poplar cultivar plot and the level of contamination (LTM and HTM).

the non-mycorrhizal plant *Arabis alpina* (Almario et al., 2017). The most abundant OTU was assigned to the *Kotlabaea* genus, accounting for more than 26% of all sequences. This species belongs to the Pyronemataceae, one of the largest family of Pezizales traditionally considered saprotrophic, although the trophic strategies of most species remain undocumented (Perry et al., 2007). Indeed, an increasing number of them are being identified as EM (Tedersoo et al., 2010). We classified *Kotlabaea* spp. as saprotrophic based on (Tedersoo et al., 2014), but uncertainties remained. Overall, knowledge about taxonomy and functional role of these root-associated fungi of nettle needs to be enriched by fungal isolation and characterization experiments, which has not yet been performed to our knowledge.

4.3. The degree of contamination differently affected the mycobiomes of poplar and nettle

According to a previous pedogeochemical characterization of the soils from the two areas, Zn and Cd concentrations were the most contrasted parameters between the two areas (Phanthavongsa et al., 2017). Other soil parameters were rather comparable, suggesting that the level of TM is one of the main factor influencing the soil fungal communities.

A slight reduction of fungal diversity was noticed for plants grown at the most contaminated area (HTM), which is commonly observed (Bååth, 1989; Giller et al., 2009). Indeed, while certain fungal microorganisms can tolerate large concentrations of non-essential heavy metals (*e.g.* Al, As, Cd, Hg, Pb), plant mycobiome is generally affected (Gadd, 2010). Other studies showed that TM tended to reduce the amount of soil microbial biomass as well as the species richness, changing microbial structure in favor of tolerant taxa and modifying the soil functioning (Giller et al., 1998, 2009).

Some mycorrhizal fungi isolated from contaminated soils develop adaptations to tolerate metals (Colpaert et al., 2011; Gonçalves et al., 2009), which make them particularly suitable for phytomanagement applications. Indeed, plants inoculated with metal-tolerant EM fungi (Adriaensen et al., 2005; Redon et al., 2009) or DSE (Likar and Regvar, 2013) showed enhanced tolerance when compared to plants inoculated with non-tolerant strains. In our observations, Agaricomycetes, and particularly the *Inocybe* and *Tomentella* genera, when associated with nettle or poplar, were the most impacted by contamination. Although significantly reduced, these two EM fungi were still well represented at the heavily contaminated area on poplar roots. Additionally, the *Inocybe* genus has already been isolated (Repáč, 1996), and successfully tested as an inoculum strain on coniferous trees (Flores-Rentería et al., 2018), which supports its interest in bioremediation applications. Their usefulness and functional importance in nettle plantation deserves further analyses (see below).

In our study, members of the Pezizomycetes seemed to be the most tolerant fungi as the *Kotlabaea* genus associated with nettle and the *Tuber* genus, associated with poplars, were more represented in the area with the highest concentration of Cd, Zn, Cu and Pb. This increase of Perizomycetes related to soil contamination has already been detected in hydrocarbon-contaminated soils (Bell et al., 2014; Tardif et al., 2016). Indeed, an untested possibility is that hydrocarbons are also present at our site because canals dredged sediments generally contain various contaminants (Besser et al., 1996; King et al., 1987).

4.4. A significant fraction of EM fungi were shared by poplar and nettle roots

Despite the noticeable differences mentioned above in terms of fungal composition between nettle and poplar, our results suggested that their respective mycobiome could be influenced by the other when co-occurring. More specifically, fungal taxa associated with abundant plants seemed more likely to colonize neighbouring plants than if the later were alone. Indeed, taxa characteristics of the nettle mycobiome (*e.g. Helvella, Kotlabaea*) where more represented in the I214 plot where nettle covered up to 60% of the herbaceous layer, compared to the Skado plot where nettle and other plants were rare. Similarly, an influence of Skado poplar on the mycobiome of neighbouring nettles was possibly detected, since a significant part (9.7%) of EM OTUs, which were characteristic of poplars mycobiomes, contributed to the mycobiome of nettle roots, which might be surprising as this plant is a recognised non-mycorrhizal plant (Vierheilig et al., 1996). Nettle roots were carefully washed and sorted, making contaminations by the adjacent soil or poplar roots unlikely. Yet their presence on the rhizoplane or as true endophytes (*i.e.* with root penetration) deserves further investigations.

Indeed, the ability of some EM fungi to colonize AM (and more generally non-EM) plants has already been described for several clades based on molecular detection and direct observations, mainly for Sebacinaceae (Selosse et al., 2009; Weiß et al., 2011) and *Tuber* spp. (Gryndler et al., 2014; Schneider-Maunoury et al., 2018, 2020). In addition, some suspicion exists, based on molecular detection only, for Thelephoraceae and Inocybaceae (Schneider-Maunoury et al., 2018, 2020), Pyronemataceae (Hansen et al., 2013) and Helotiales (Wang et al., 2006). Such a dual, EM and endophytic interaction has been viewed as a persistence of the evolutionary past of EM fungi, if they evolved from endophytic species: in the so-called 'waiting room hypothesis', endophytism is considered as a niche from which the tighter and more elaborate mycorrhizal symbiosis can evolve (Schneider-Maunoury et al., 2018; Selosse et al., 2009; van der Heijden et al., 2015). It was recently commentated that many fungi have more complex niches (*e.g.*, dual niches) than what is commonly agreed, which could be explained by such evolutionary trajectories (Selosse et al., 2018; Thoen et al., 2020).

Recently, Taschen et al. (2020) demonstrated that *Tuber melanosporum* growth gains benefit from the non-EM plants it colonizes, while it has a negative effect on them. Additionally, the EM trees colonized by this fungus as ectomycorrhizal partners gain, at least in terms of nitrogen content, from the interaction between truffles and non-EM plants. The possibility of an overlooked interaction between EM- and AM- or non-mycorrhizal plants growing close to host trees is further supported by the fact that connection and the exchange of carbon between plant sharing the same belowground ectomycorrhizal network were demonstrated for trees (Klein et al., 2016; Selosse et al., 2006), even when one of the plant was herbaceous (Selosse et al., 2009). The functional implications for plants of an extensive sharing of non-mycorrhizal fungi remained unclear and highly dependant on the plant-fungus association. In case they are beneficial, and this happens even for non-mycorrhizal endophytic fungi such as dark septate endophytes in non-mycorrhizal plants (Liu et al., 2017; Yung et al., 2021), there may be mutual reinforcement of the two plant species by way of coordinated responses to stresses, nutritional sharing, *etc.* Yet, the benefit can be asymmetric, or even relevant for one plant species only. Moreover, an intriguing possibility is pathogen spillover (Mordecai, 2011), *i.e.* one plant promotes the spread of pathogens that harm competitors more strongly than itself. In this context, further investigations are now necessary to conclude about the exact nature and functionality of this poplar-nettle EM sharing and its consequence for this agroforestry system.

Inocybe rimosa, the most abundant EM fungal species found on nettle roots, accounting for approximately 4% of the total fungal sequences, is a major candidate for such studies. The presence of *I. rimosa* is reported in TM-polluted soils (Huang et al., 2012; Krpata et al., 2009) and, although its ability to colonize roots of non-EM plant has not been previously investigated, Inocybaceae were already detected in some AM plants (Schneider-Maunoury et al., 2018). Any facilitation mechanism through EM fungi could contribute to the dominance of nettles within Salicaceous SRC in natural (Cronk et al., 2016) and contaminated sites (Yung et al., 2019). However, further research about the mycobiome of nettle roots and particularly in the case of co-cropping at phytomanagement sites, especially to assess morphological (*e.g.* by

fluorescent *in-situ* hybridization; Schneider-Maunoury et al., 2020) and functional evidence of interactions with EM fungi, is now awaited. *I. rimosa* is present in the CBS Filamentous fungi and Yeast Collection as CBS 210.55. It will be used in further experiments, for poplar x nettle experiments to test its growth between the two plants and assess its positive or negative or no influence on nettle.

As a conclusion, we taxonomically and functionally characterized the fungal microbiomes of a spontaneous species (*i.e., Urtica dioica* L.) co-occurring with cultivated crops (*i.e., Populus* spp.) at a phytomanaged area contaminated with TM. Our results suggested that nettle and poplar had distinct root-associated fungal communities, albeit sharing many common ectomycorrhizal fungi and particularly the genus *Inocybe*, which is surprising given the non-mycorrhizal status of nettle. These results suggest to reconsider the ecological niches of fungi.

CRediT authorship contribution statement

Loïc Yung: data curation, formal analysis, investigation, roles/writing - original draft preparation; **Coralie Bertheau**: Methodology, funding acquisition, resources, supervision, writing - review & editing; **Flavien Tafforeau**: methodology, investigation software; **Cyril Zappelini**: investigation, methodology; **Benoit Valot**: software; **François Maillard**: formal analysis, writing - review & editing; **Marc-André Selosse**: visualization, conceptualization, writing - review & editing; **Chloé Viotti**: formal analysis, writing - review & editing; **Philippe Binet**: visualization, writing - review & editing; **Geneviève Chiapusio**: conceptualization, funding acquisition, supervision, writing - review & editing; **Michel Chalot**: conceptualization, funding acquisition, investigation, methodology, project administration, validation, writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146692.

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