

## Fungal Endophytes of *Alnus incana* ssp. *rugosa* and *Alnus alnobetula* ssp. *crispa* and Their Potential to Tolerate Heavy Metals and to Promote Plant Growth

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### ABSTRACT

Soil contamination by metals is of particular interest, given that their retention times within the profile can be indefinite. Thus, phytostabilization can be viewed as a means of limiting metal toxicity in soils. Due to their ability to grow on contaminated soils, alders have repeatedly been used as key species in phytostabilization efforts. Alder ability to grow on contaminated sites stems, in part, from its association with microbial endophytes. This work emphasizes the fungal endophytes populations associated with *Alnus incana* ssp. *rugosa* and *Alnus alnobetula* ssp. *crispa* (previously *A. viridis* ssp. *crispa*) under a phytostabilization angle. Fungal endophytes were isolated from alder trees that were growing on or near disturbed environments; their tolerances to Cu, Ni, Zn, and As, and acidic pH (4.3, 3, and 2) were subsequently assessed. *Cryptosporiopsis* spp. and *Rhizoscyphus* spp. were identified as fungal endophytes of *Alnus* for the first time. When used as inoculants for alder, some isolates promoted plant growth, while others apparently presented antagonistic relationships with the host plant. This study reports the first step in finding the right fungal endophytic partners for two species of alder used in phytostabilization of metal-contaminated mining sites.

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## 1. Introduction

In a review, we made the case that alders could be key species in rehabilitating habitats that have been destroyed or which have been adversely affected by human activities, based upon their ability to grow on contaminated sites and sites containing low levels of nutrients [1]. The presence of alders on these sites could indeed stimulate the biodegradation of organic contaminants while stabilizing inorganic contaminants within soils. In addition, reduced migration of inorganic contaminants through phytostabilization is seen as a promising practice since inorganic contaminants are not degraded and their retention times in soil are difficult to establish [2,3]. Although they are not considered to be hyper-accumulators, alders have a demonstrated ability to limit metal migration in soils [4,5].

Alders are perennial trees and shrubs of the genus *Alnus* in the Family Betulaceae that grow primarily in north temperate ecosystems [1]. Alders are early successional species [6] that can grow in harsh environments [7]. They are less affected by nitrogen (N) and phosphorus (P) deficiencies in the soil than are other species [5], for several reasons.

As actinorhizal species, alders are able to access atmospheric nitrogen through the activity of their bacterial partners, i.e., *Frankia* spp. [8,9], which contributes to increasing soil nitrogen levels [10]. Alders are also important to improving the availability of phosphorus [11]. Alder tolerance of low soluble-P levels in the soil is due to the plant's ability to release active phosphatases in root exudates that break down organic-P compounds, together with its ability to form symbioses with a wide variety of fungi that facilitate P uptake [1,5,12]. Alders can form symbioses with ectomycorrhizal fungi (ECM) as well. Unlike several ectomycorrhizal plants, alders form such symbioses with a limited number of fungal species [13–17]. These fungi are believed to have specialized in the acquisition of P [12].

Xu et al. [18] also reported that *Alnus nepalensis* D. Don (Utis or Nepalese alder) formed associations with several dark septate endophytes (DSE), i.e., a miscellaneous, likely paraphyletic group of ascomycetous melanized, septate fungi that colonize roots [19,20]. The nature of the interaction between DSE and their host plants has been the subject of several studies, where effects on host plants have ranged

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 Supplemental data for this article can be accessed [here](#).

from negative [21–25] to positive [19,26–28]. DSE-host combinations, together with environmental and growth conditions, are considered as keys to understanding the variable effects of such interactions [25,27,29]. Yet, the potential ability of DSE to breakdown complex organic molecules, thereby increasing essential nutrient levels such as P in the rhizosphere [20,28,30,31], together with the ability of certain strains to produce auxin (IAA) [32] could promote plant growth even on metal-contaminated soils [32]. Thus, DSEs are potential of great interest in the development of rehabilitation strategies.

Evaluating the tolerance of alders and their symbionts to heavy metal stress and acidic conditions is necessary to develop new phytotechnologies, given that host plants and their microsymbionts must both survive for their symbiosis to be exploited. In this case, we aimed to alleviate the effects of metal-contaminated environments on alders, such as slow plant growth, using microbial partners [33]. Previous studies have addressed the tolerance of alders and the actinorhizal symbiosis. These studies demonstrated that alders and their actinorhizal symbionts are tolerant to heavy metals and, thus, good candidates for phytostabilization approaches [5,34]. However, the study of other microorganisms that form associations with alders, such as DSE and ECM is also warranted.

The study of the fungal symbionts of alder is of particular interest with respect to phytostabilization, as several studies have shown that ECMs can alleviate metal toxicity in plants [35–37]. Even if mechanisms underlying the relationship between DSE and host plants are not well understood, studies have definitely shown their association with plant roots on metal-contaminated sites [38–40]. Furthermore, they may be of interest, given that shifts in fungal community structure from ECM toward DSE have been reported in extreme environments [41] and colonization patterns of roots do not seem to have been affected by metal toxicity [42]. Furthermore, it has been reported that some plants that are colonized by DSE show increased growth and reduced metal uptake in metal-contaminated soils [43,44].

The aim of this study was to select metal-tolerant fungal endophytes that can form associations with alders and to find positive associations between these plants and fungi. This is the first step in developing optimal partnerships between alders and fungi to be used in phytostabilization strategies on metal-contaminated soils. Root endophytes were isolated from *Alnus incana* ssp. *rugosa* (Du Roi) R.T. Clausen and *Alnus alnobetula* (Ehrh.) K. Koch ssp. *crispa* (Aiton) Raus, which were located on various contaminated and disturbed environments. Inoculations of host plants with the isolated fungi were performed in order to validate their endophytic status. The tolerance of alder fungal endophytes to copper, nickel, zinc, and arsenic, together with acidic pH, was also investigated. Last, growth trials were performed to characterize the effects of the various endophytes on their hosts.

## 2. Materials and methods

### 2.1. Root sampling

Roots were collected from two alder species, *A. incana* ssp. *rugosa* (speckled alder, hereafter referred to as AR) and *A. alnobetula* ssp. *crispa* (green alder, hereafter referred to as AC), which were growing in soils affected by human activities in various regions of Quebec, Canada. The collected plants were growing in sites that had been perturbed by mining activities (tailing ponds and waste rock piles), and those within the vicinity of engineered structures (dike, bicycle path, unpaved road, highway, and cemetery). Table 1 summarizes the list of the sampled sites, which were labeled A to K. Plants from sites B, D, E, and F were growing in soils that had been contaminated with metals. The leachate from these sites exceeded regulatory levels for discharges into the environment, which were set by Quebec Ministry of Sustainable Development, Environment and the Fight against Climate Change [45].

Roots were sampled with the surrounding substrate to a depth of 5–15 cm beneath the ground surface, but large stones were carefully removed.

**Table 1.** Harvest sites used for the collection of roots from *Alnus incana* ssp. *rugosa* (AR) and *Alnus alnobetula* ssp. *crispa* (AC).

Site designation	Site description	Sub-region	Alder subspecies
A	Mining site – waste rock pile	Eastern Townships	AR, AC
B	Closed mining site – tailing pond	Northern Quebec	AR
C	Closed mining site – dike	Northern Quebec	AR
D	Closed mining site – tailing pond	Abitibi	AR
E	Mining site – waste rock pile	Abitibi	AR, AC
F	Mining site – tailing pond	Abitibi	AR
G	Mining site – waste rock pile	Côte-Nord	AR
H	Highway	Eastern Townships	AR
I	Cemetery – land fill	Eastern Townships	AR
J	Unpaved road	Eastern Townships	AR
K	Bicycle path	Eastern Townships	AR

Roots were stored at 4 °C for 1–2 weeks prior to fungi isolation.

## 2.2. Endophytic fungi isolation

Roots were carefully washed with tap water to remove soil and other debris. To guarantee that only alder roots were used for the isolation of fungi, roots that did not connect to a nodule were discarded. Roots were observed using a MSZ-1B Nikon binocular scope at 10× magnification to ensure that they were not damaged while being harvested. Selected roots were then sectioned into 2–3 mm lengths and surface sterilized. A total of 539 root segments were processed.

Four disinfection procedures were used to sterilize the surface of the root segments. This was done to lessen the chance that the choice of disinfection method would exclude certain types of endophytic fungi relative to others, introducing bias. We aimed to increase the observable fungal biodiversity. Half of the segments were surface sterilized as previously described by Molina and Palmer [46], with 0.01% Tween-80 solution used as detergent and 30% aqueous hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as the disinfection agent. Disinfection times of 10 and 30 s were used respectively for half of the segments in this group (a quarter of total segments). The other half of the segments were surface sterilized as previously described by Yamada et al. [47], with 0.01% Tween-80 solution and 0.005% Tween-80 solution used as detergents, and 1% aqueous calcium hypochlorite as the disinfection agent. Disinfection times of 15 and 60 s were used, respectively, for half of the segments in this group (a quarter of total segments). The efficacy of the disinfection methods on the number of isolates recovered was assessed using a Pearson correlation.

Root segments were placed individually on modified Melin-Norkrans medium plates (MMN, pH 5.7) and incubated at room temperature (about 22 °C) [48]. Plates were observed daily for the presence of fungal colonies. When a fungal colony reached a radius of about 3 cm, approximately 50 mg of mycelium were removed for fungal identification (cf. DNA extraction, below). Fungi were cultivated in the dark at room temperature on MMN medium. For isolation, streptomycin (50 µg/l) and ampicillin (50 µg/l) were added to the culture plates to prevent bacterial contamination.

After initial identification (see below), a 1-cm radius mycelial plug was collected from the edge of selected colonies and placed onto fresh antibiotic-free medium. This process was repeated 3–4 times before secondary identification. For conservation

purposes, colonies were transferred to fresh medium every 3 months.

The MMN was adjusted to lower pH values using 1 N HCl to test growth of the isolates under acidic conditions. Cultures were also maintained on this medium for long-term preservation.

## 2.3. Fungal identification

Phylogenetic analysis was based upon sequencing of the ITS1-5.8S-ITS2 region (ITS). Two strains isolated in this study, strains ACCG6 and ACCG15, were excluded from the analysis, as they were isolated from alders that were produced in a greenhouse and planted on waste rock piles as part of a rehabilitation effort, making it impossible to ascertain whether the fungi originated from the surrounding environment or from the greenhouse.

Total DNA from each colony was extracted using REDEExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St-Louis, MO) according to manufacturer instructions. PCR was initially carried in a volume of 25 µl containing 12.5 µl of REDEExtract-N-Amp™ PCR ReadyMix™, 1.25 µl of 10 µM primers ITS\_1F and ITS\_4 [49] and 10 µl of biomolecular-grade water. The amplifications were performed on an S1000 Thermal Cycler (Bio-Rad, Hercules, CA) under the following conditions: an initial pre-denaturing step at 95 °C for 2 min, 29 cycles at 95 °C for 40 s, 46 °C for 30 s, 72 °C for 60 s, and a final extension step at 72 °C for 7 min.

PCR amplification products were sequenced at the Plateforme de séquençage et de génotypage des génomes (Centre de recherche du CHU, Quebec City, Canada) using an ABI 3730 × 1 DNA analyser (Applied Biosystems, Foster City, CA). As a first step in screening, only sequencing with the forward primer was performed. Sequencing using both forward and reverse primers was performed later on samples appearing to be taxonomically associated with either mycorrhizal fungi or DSE. ITS sequences were compared to sequences from the GenBank database using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST). Neighbor-joining analysis [50] was performed using the MEGA version 7 software package (<https://www.megasoftware.net>) [51] with Kimura 2 parameter model [52]. Bootstrap analysis was performed using 1000 resamplings. *Spiromyces aspiralis* (Zoopagomycota) was used as an outgroup.

## 2.4. Validation of endophytic status

To validate whether or not isolates are actual endophytes, each isolate was used to inoculate either AR

or AC according to the plant species from which it was isolated.

Seeds of AR (National Tree Seed Centre of Canada [NTSC], lot 8421707.0) and AC (NTSC, lot 8421722) were sterilized and germinated, as previously described by Bélanger et al. [53]. Plant growth chamber conditions were as follows. A regime of 16 h light at 22 °C and 8 h dark at 18 °C; illumination of 500–600  $\mu\text{mol photons/m}^2/\text{s}$ . After a 6-week growth period, plants were transferred to pots containing a 1:1 sterilized sand:perlite mixture. Pots were placed inside clear sterilized containers with two openings to allow air flow. Openings were covered with a 0.20  $\mu\text{m}$  membrane (Millipore, Billerica, MA) to prevent contamination. An aliquot of 30 ml of fungal inoculum was mixed into the rooting medium prior to plant transfer. The plants were grown for an additional 16 weeks in a growth chamber. The plants were watered weekly with 5 ml of Hoagland's solution  $0.1 \times$  [54]. During the first 6 weeks following transfer, 5 g/l of dextrose were added to the watering solution.

After this growth period, roots inoculated with known symbionts that formed ECM hyphal mantles (HC5247, PI8235, AD6193, ACCG15 [identified as *Sebacina* sp.] and ACCG6 [identified as *Tomentella* sp.]) were harvested, carefully washed, and observed with a Nikon SMZ800 binocular scope at  $10\times$  magnification. All other roots were carefully washed and cleared by boiling them in a 10% w/v aqueous KOH for 20 min. Root segments were then observed at  $200\times$  and  $400\times$  with an Axio Imager M1 light microscope (Carl Zeiss Canada Ltd., Toronto, Canada). When no fungal hyphae were observed within the roots, they were stained using the ink and vinegar method [55] and observed again to detect the presence of endophytic fungal hyphae.

When endophytic status was confirmed for a given strain, isolation procedures were performed on the infected roots, as previously described. Thirty days following sterilization, a 5-mm mycelium plug was cut from the edges of the actively growing colonies and transferred to fresh MMN medium. Thirty days following this transfer, the phenotype of the reisolated strain was compared to that of the inoculated strain in order to validate Koch's postulates.

The numbers of fungal strains that were isolated in this study are listed in Table 2. In addition to these strains, three ECM strains were included in this study that had been obtained from the University of Alberta Mold Herbarium and Culture Collection (UAMH): *Paxillus involutus* 8235 (PI8235), *Hebeloma crustuliniforme* 5247 (HC5247) and *Alpova diplophloeus* 6193 (AD6193). These three strains belong to species that have been

**Table 2.** Origins of potential mycorrhizal fungi (MF) or dark-septate endophytes (DSE) per host plant per site. A total of 539 root segments were analyzed from the 11 sites.

Site <sup>a</sup>	Host plant	Number of root segments analyzed	Number of isolates	Number of potential MF or DSE <sup>b</sup>
A	AR <sup>c</sup>	90	73	2
	AC <sup>d</sup>	84	53	3
B	AR	29	17	4
C	AR	54	49	13
D	AR	22	20	4
	AR	12	5	0
F	AC	27	26	0
	AR	34	29	6
G	AR	33	21	4
H	AR	7	1	0
I	AR	10	7	3
J	AR	15	0	0
K	AR	122	64	11

<sup>a</sup>Site descriptions are in Table 1.

<sup>b</sup>Identification of potential MF or DSE was determined by comparing the ITS1-5.8S-ITS2 region that was sequenced to previously sequenced fungi that are available at GenBank (<https://www.ncbi.nlm.nih.gov/genbank>).

<sup>c</sup>*Alnus incana* ssp. *rugosa*.

<sup>d</sup>*Alnus alnobetula* ssp. *crispa*.

previously reported as symbionts of either AC or AR, and which were used as positive controls [13,14].

Fungal inocula that were used in plant colonization assays were prepared in a vermiculite carrier [43] as follows. A full fungal colony (about 5-cm radius) that was grown on an MMN plate was cut roughly in 0.5-cm pieces and added to vermiculite (500 ml) that had been moistened with 250 ml of MMN liquid medium before being incubated two weeks in the dark at room temperature.

## 2.5. Metal/metalloid and pH tolerance

Metal tolerance of the fungal isolates was also tested on MMN plates. The growth medium was amended with metals and prepared by adding 100 ml of metal solution ( $10\times$ ) to 900 ml of MMN ( $1.1\times$ ) that was adjusted to pH 4.3 to insure metal availability [56]. Metal solutions, which were filtered using a 0.45  $\mu\text{m}$  membrane (Millipore, Billerica, MA), were added after autoclaving to avoid precipitation. The final concentrations were  $\text{Cu}^{2+}$  as  $\text{CuSO}_4$  at 60 and 150 mg/l,  $\text{Ni}^{2+}$  as  $\text{Ni}(\text{CH}_3\text{COO})_2$  at 30 and 50 mg/l,  $\text{Zn}^{2+}$  as  $\text{ZnSO}_4$  at 125 and 225 mg/l, and  $\text{As}^{3+}$  as  $\text{Na}_2\text{HAsO}_4$  at 50 and 150 mg/l.

Selected isolates, together with the three ECM control strains (PI8235, HC5247, and AD6193) were tested for their tolerance to elements that were found in the contaminated sites, i.e., copper (Cu), zinc (Zn), nickel (Ni), and arsenic (As), as previously described by Blaudez et al. [56]. Briefly, 5-mm diameter plugs were collected from the edges of actively growing colonies and transferred to a fresh medium that had been amended with one of the four elements being tested. Tolerance of the strains

to each metal (refer to “culture conditions” above for tested concentrations) was estimated by determining the time required (in days) by the colonies to reach a 1-cm radius. Each experiment was carried out in 12 replicates. Controls, one per strain, were performed on MMN medium free of metals. As soils contaminated by heavy metals are often of low pH, a similar experiment was also performed to compare pH tolerance between isolates, with pH 5.7 being used as a control.

Non-parametric statistical methods were used to compare tolerance to metals and acidity both within and between isolates. For each test condition, the time (days) that was required by each individual replicate of the same isolate ( $n = 12$ ) to reach the 1-cm radius were ordered from highest to lowest. The same procedure was done for the negative control of each strain. Growth times for each rank of the tested conditions were then divided by the corresponding rank of the negative control. Statistical analyses performed on these ratios revealed that our data could not be normally distributed, whether our growth times were normally distributed or not. Therefore, conditions were reported using cumulative normal plots of the ratios that were summarized as boxplots, i.e., the median or 50th-percentile of the cumulative distribution, and the 25th and 75th percentiles delimiting the “box” (data summaries are shown in Figure 1 and Supplementary Figures 1–6).

The median ratios (considered as rank values) of the 12 replicates per strain were ranked for the 23 fungal strains, followed by Kruskal–Wallis test by ranks and subsequent multiple pairwise comparisons of median ratios (ranks). The responses ranged from ratios that were very low to zero, to values that were equal to or greater than 1.0, indicating that the performance of the isolate on metal-amended Petri dishes, or in acidified medium, was better than growth on the comparable control.

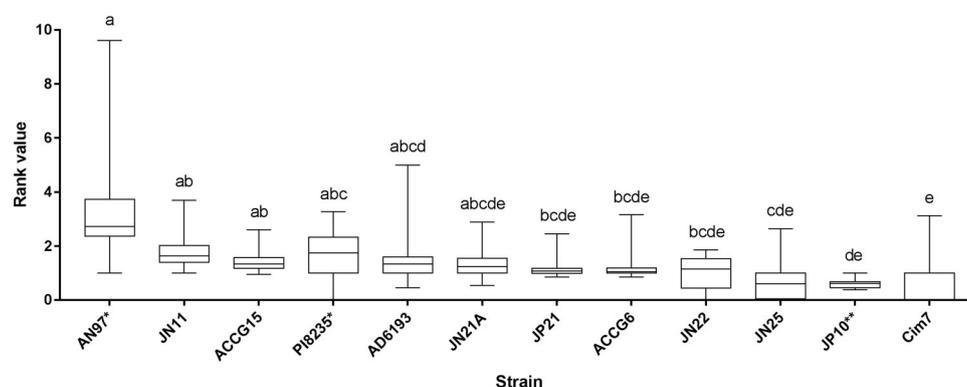
Rank-response plots were created by ordering each isolate from highest to lowest. From these rankings, the performance of each isolate could be globally compared across experiments.

A heatmap was created to visualize the sequencing data versus the responses of the isolates to heavy metal amendments and adjustments to growth medium acidity. In this two-dimensional depiction of responses, the isolates form rows and the relationships between their ITS sequences are visualized as a dendrogram. The dendrogram was derived using a complete linkage-clustering algorithm, employing the Manhattan metric as a measure of distance.

In this study, the columns represented the responses of the isolates to  $\text{Cu}^{2+}$  150 mg/l,  $\text{Ni}^{2+}$  50 mg/l,  $\text{Zn}^{2+}$  225 mg/l,  $\text{As}^{3+}$  150 mg/l, and pH 2.

## 2.6. Plant growth promotion

The plant growth promotion potential of 12 strains was evaluated using the same protocol as for the validation of endophytic status. The fungal strains were selected for their ability to colonize roots in the “validation of endophytic status” experiments. One member of each group of strains showing highly similar ITS regions and originating from a single site was chosen for the growth promotion experiment. Strains JN10, JN14, AN2, AN75, AN77, and AN115 were therefore excluded. The growth medium used was “Connaisseur Potting Soil for indoor plants” (Fafard; sphagnum peat moss, black soil, perlite, coconut husk fiber, sand, dolomitic limestone, and natural fertilizer). The nitrogen content of the growth medium was 0.35%, available phosphorus (as  $\text{P}_2\text{O}_5$ ) was 0.20% and soluble potassium (as  $\text{K}_2\text{O}$ ) was 0.15%. This growth medium contained 25% organic matter and a maximum (gravimetric) moisture content of 55%. The experiment was conducted with 16 replicates and the



**Figure 1.** Growth promotion of *Alnus incana* ssp. *rugosa* and *A. alnobetula* ssp. *crispa* by 12 fungal strains (two ECM strains for culture collection, PI8235, and AD6193, and ten strains isolated from metal-contaminated mining sites) illustrated by rank (median ratio) and interquartile range per strain. All strains showing higher growth rates which are statistically different from the control ( $p < .05$ ) are marked with an asterisk (\*). Strains showing lower growth rates which are statistically different from the control ( $p < .05$ ) are marked with\*\*.

growth period was 10 weeks. After the growth period, plants were harvested and the roots were carefully washed. Stem and root lengths were measured. Shoots and roots were dried for 72 h at 70 °C, and weighted. Statistical analysis was performed as described above.

### 3. Results

#### 3.1. Isolation, identification of fungal strains

In order to isolate fungal endophyte strains of potential interest for phytostabilization strategies using alders, 539 surface-sterilized alder root segments were placed on Petri dishes (one segment per Petri dish). Of these, 365 produced fungal colonies. Pearson correlation analysis between the number of isolates that were identified as mycorrhizal fungi or DSE per sterilization method *versus* the number of root segments that were treated per sterilization method yielded a result of  $r=0.91$  (Supplementary Table 1), suggesting that no disinfection procedure was particularly more efficient in the isolation of mycorrhizal or DSE fungi. A second correlation analysis between the total number of isolates per sterilization method *versus* the number of root fragments treated per sterilization method yielded similar results ( $r=0.94$ ). Furthermore, there were no significant shifts in the taxa obtained when using a sterilization method *versus* another.

Seventy-three Petri dishes were discarded as two or more fungal colonies overlapped (Supplementary Table 1). In addition, the PCR products of 83 fungal isolates were of insufficient quality and were discarded. Consequently, the ITS1-5.8S rDNA-ITS2 region of 209 fungal isolates (forward strand only)

was amplified and sequenced. The sequences were compared to available fungal sequences in GenBank, and 41 sequences showed similarity ( $\geq 95\%$ ) with those of known mycorrhizal fungi or DSE. Isolates that did not show sequence similarities with known fungi were set aside, and not studied further. Table 2 presents the provenance (sites) where the potential mycorrhizal fungi or DSE were isolated.

Refined identification of the 41 isolates selected for further study was accomplished by sequencing both strands of the ITS region (using forward and reverse primers). This led to the identification of 20 distinct strains corresponding to mycorrhizal fungi or DSE. Sixteen of these fungal isolates showed 100% sequence similarity with at least one other isolate. If isolates from the same sampling site shared 100% sequence similarity, we retained only one isolate from each group for further analyses. Nine isolates were rejected since the sequencing of both strands revealed that they were initially wrongly classified as mycorrhizal fungi or DSE. Therefore, a total of 35 isolates fell into this 20-strains classification. Table 3 lists the selected strains, together with their GenBank accession numbers.

Four distinct groups emerged from the phylogenetic analysis of the 35 selected strains (Figure 2). All were subgroups of the order *Helotiales* (*Ascomycota*). Group 1 contained closely related strains of the *Cryptosporiopsis* genus, a known DSE genus [57]. Groups 2 and 3 contained members of the genera *Phialocephala* and *Cadophora*, respectively; both genera are known to be DSE [58]. Finally, Group 4 was composed of various members of the *Rhizoscyphus ericae* aggregate, which contains species known to form ericoid mycorrhizae with ericoid hosts [59].

**Table 3.** Strains identified as potential mycorrhizal fungi or dark-septate endophytes and their nearest GenBank neighbors.

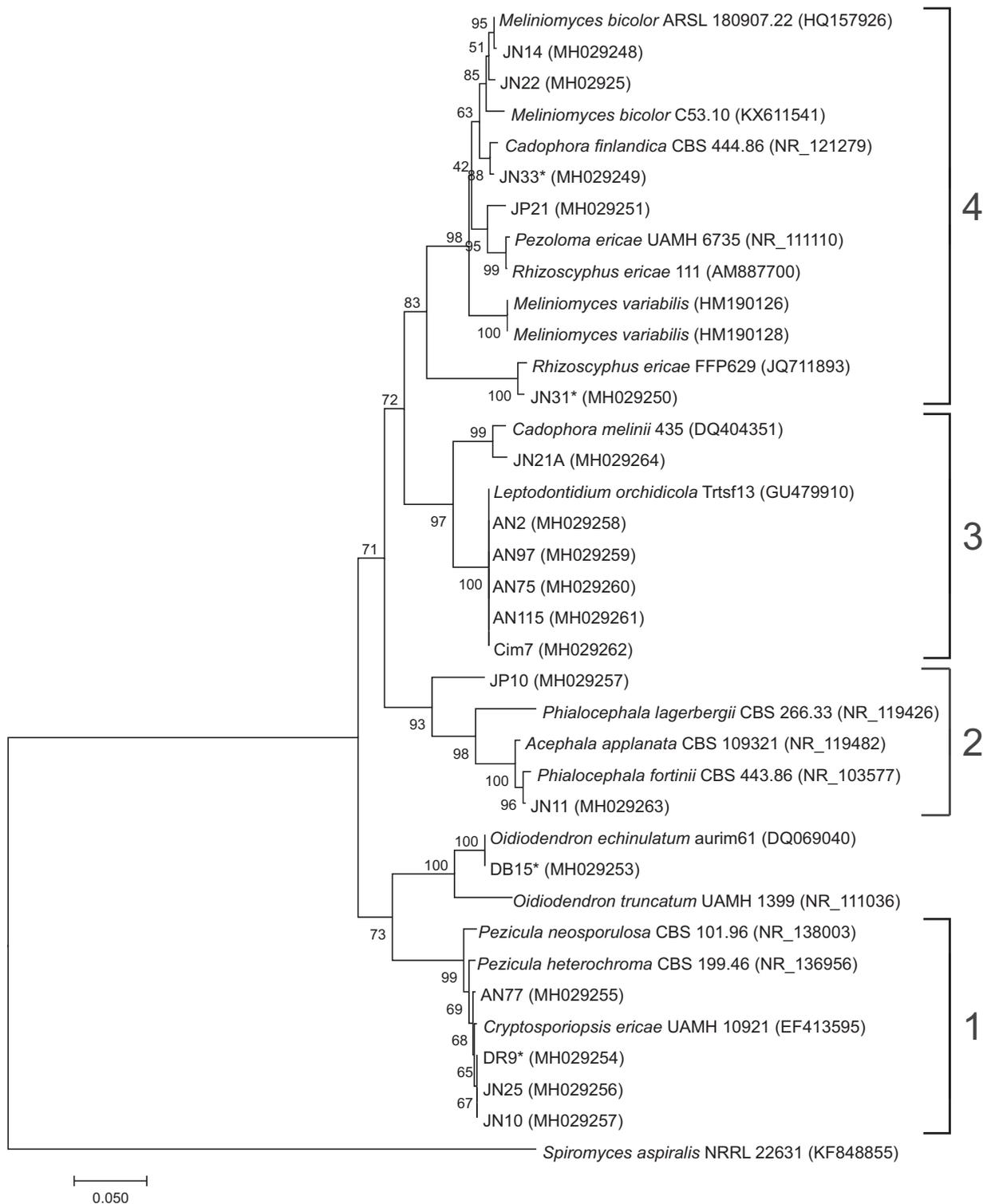
Strain	GenBank accession #	Number of isolates	Host	Site <sup>a</sup>	Nearest GenBank neighbor (accession #)	Identity (%)
ACCG6	MH029247	2	AC <sup>b</sup>	A	<i>Tomentella</i> cf. <i>ellisii</i> (KC782507)	96.2
ACCG15	MH016787	1	AC	A	<i>Sebacina</i> sp. (HQ154358)	98.6
JP10	MH029265	1	AR <sup>c</sup>	B	<i>Phialocephala bamuru</i> (MG195534)	98.5
JP21	MH029251	1	AR	B	<i>Rhizoscyphus ericae</i> (AM887700)	95.9
JN10	MH029257	2	AR	C	<i>Cryptosporiopsis</i> sp. (KT268764)	99.8
JN11	MH029263	4	AR	C	<i>Phialocephala fortinii</i> (LC131022)	99.5
JN14	MH029248	1	AR	C	<i>Meliniomyces bicolor</i> (HQ157926)	99.8
JN21A	MH029264	1	AR	C	<i>Phialocephala lagerbergii</i> (AB190400)	99.1
JN22	MH029252	1	AR	C	<i>Meliniomyces bicolor</i> (HM190124)	99.5
JN25	MH029256	1	AR	C	<i>Pezizula melanigena</i> (KY977594)	100.0
JN31 <sup>d</sup>	MH029250	1	AR	C	<i>Rhizoscyphus ericae</i> (JQ711893)	98.9
JN33 <sup>d</sup>	MH029249	1	AR	C	<i>Cadophora finlandica</i> (NR_121279)	99.6
DB15 <sup>d</sup>	MH029253	1	AR	D	<i>Oidiodendron echinulatum</i> (DQ069040)	100.0
DR9 <sup>d</sup>	MH029254	6	AR	F	<i>Pezizula melanigena</i> (KY977594)	100.0
Cim7	MH029262	3	AR	I	<i>Cadophora</i> sp. (MG076773)	100.0
AN2	MH029258	2	AR	K	<i>Cadophora</i> sp. (MG076773)	99.8
AN75	MH029260	1	AR	K	<i>Cadophora</i> sp. (MG076773)	99.7
AN77	MH029255	1	AR	K	<i>Cryptosporiopsis</i> sp. (KT268764)	99.8
AN97	MH029259	3	AR	K	<i>Cadophora</i> sp. (MG076773)	100.0
AN115	MH029261	1	AR	K	<i>Cadophora</i> sp. (MG076773)	99.7

<sup>a</sup>Site descriptions are in Table 1.

<sup>b</sup>*Alnus alnobetula* ssp. *crispa*.

<sup>c</sup>*Alnus incana* ssp. *rugosa*.

<sup>d</sup>These strains did not form endophytic structures in inoculation trials.



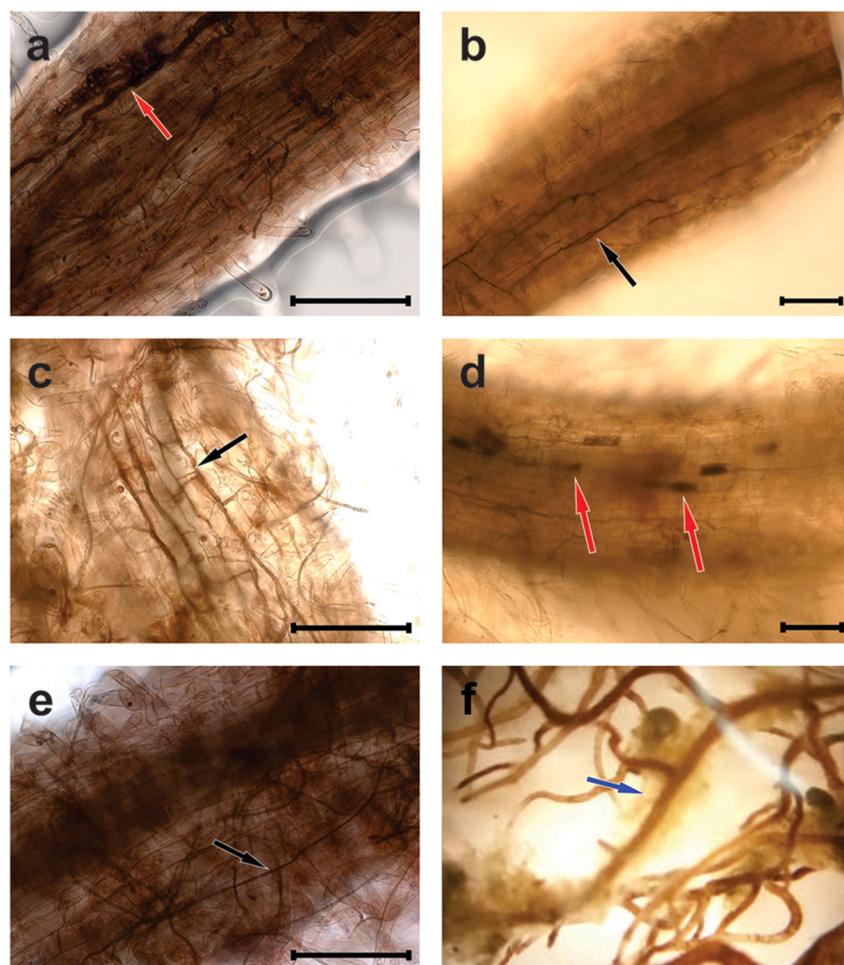
**Figure 2.** Neighbor-joining analysis showing the position of strains based upon ITS1-5.8S rDNA-ITS2 sequences, with *Spiromyces spiralis* as the outgroup. Strains not forming endophyte structures in the inoculation trial are marked with an asterisk (\*).

### 3.2. Endophytic status

As most of the strains identified were not known endophytes for AR and AC, root colonization assays were performed. This was meant to determine whether these fungi were simply rhizospheric, free-living fungi, or potentially interesting candidates for alder inoculation prior to their planting on disturbed sites.

We observed that all selected strains formed endophytic structures in alders, with the exception of strains JN31, JN33, DB15, and DR9. Similarly, we observed that one of the three ECM control strains obtained from UAMH did not form hyphal mantles on roots; strain *H. crustuliniforme*.

As was observed for control strains *P. involutus* 8235 and *A. diplophloeus* 6193, strains ACCG6 and



**Figure 3.** Root colonization by various endophytes as observed by microscope and binocular. Scale bars represent 100  $\mu\text{m}$ . Magnification was 100 $\times$  or 200 $\times$  (panels a–e), or 10 $\times$  (panel f). Black arrows indicate fungal hyphae, red arrows indicate microsclerotia and the blue arrow indicates a hyphal mantle surrounding a root. (a) strain JN25; (b) strain JN11; (c) strain JN14; (d) strain AN115; (e) strain JN22, and (f) strain ACCG15.

ACCG15 formed typical ECM hyphal mantles on roots. These structures, however, were few in number, their occurrence on roots varying between zero and two per plant.

Strain JN25 from Group 1 and all strains from Group 2 exhibited inter- and intra-cellular hyphal colonization and formed microsclerotia. All other strains showed a hyphal colonization pattern that was similar to Group 2. No particular structure, whether ericoid mycorrhiza or microsclerotium, were observed. Typical observations of root colonization are shown in Figure 3.

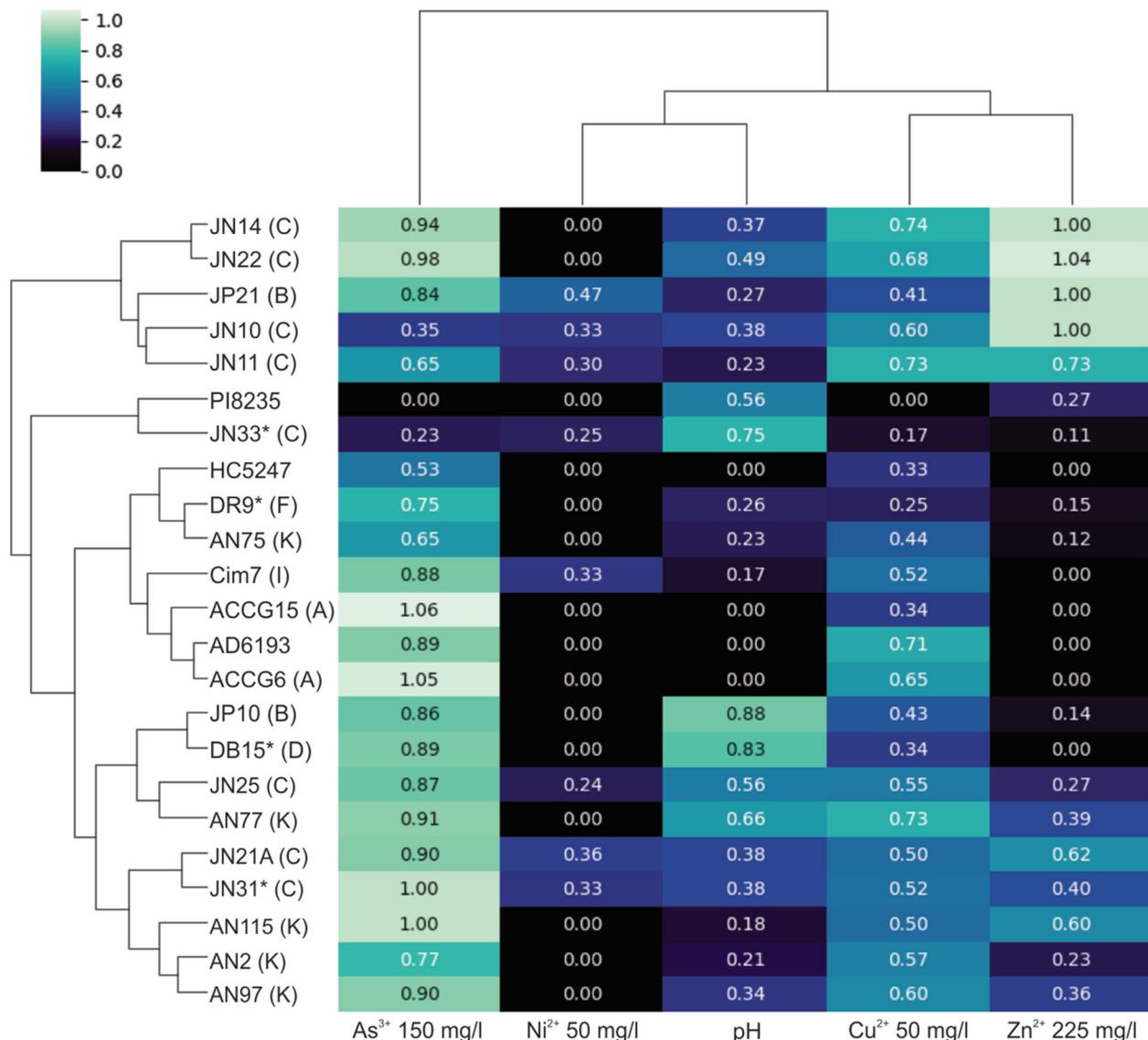
### 3.3. Copper, nickel, zinc, arsenic, and acidic pH tolerance

Since metal phytostabilization techniques depend on the tolerance of plants and microorganisms to contaminated soils, we conducted tolerance assays to four metals (As, Cu, Zn, and Ni) and various acidic conditions.

To ensure that the method used to evaluate metal tolerance and pH tolerance yielded consistent

results, a one-way ANOVA was performed on growth data obtained by three identical conditions, one from each of the three experiments performed (i.e., the two controls in the metal tolerance tests and the pH 4.3 conditions in the pH tolerance test). Twenty-three separate ANOVA were performed, one for each of the isolate. Although  $p$  values exhibited a broad range among strains, no strain showed results that were statistically significant from one test to another ( $p \geq .10$ ) (data not shown).

Detailed ranking results of strain tolerances can be found in Figure 4 and in the supplemental material. For all metals tested, fungal growth rates relative to the metal-free controls diminished as metal concentrations increased. Yet, inhibition of growth also varied relative to the metal that was being tested.  $\text{Ni}^{2+}$  strongly inhibited growth. Indeed, isolate growth relative to the control at 30 mg/l of  $\text{Ni}^{2+}$  was systematically lower than 75% (data not shown) and most strains (65%) showed no growth at 50 mg/l (see Supplementary Figure 2).  $\text{As}^{3+}$ , in contrast, had very few adverse effects on relative growth. At 50 mg/l  $\text{As}^{3+}$  (Supplementary



**Figure 4.** Heatmap of the rank per strain in media amended with metals (pH 4.3) or at pH 2 without metal, shown as a representation of the sequencing data versus the responses of the isolates to the growth stress applied. The relationships between the isolates, according to ITS sequencing, are pictured as a dendrogram. Site of origin of isolates is indicated into parentheses (see Table 1 for site details). Strains not forming endophytic structures in the inoculation trial are marked with an asterisk (\*). The heatmap was drawn using complete-linkage clustering and the Manhattan metric to calculate distances. Detailed figures can be found in supplemental material.

Figure 4), median growth rates of the isolates (as the percentage ratio of the amendment to the control) were higher than 1 (or 100%) for strains AN2 (151%), ACCG15 (147%), ACCG6 (144%), JN25 (141%), AN115 (143%), AN97 (126%), JN21A (115%), JN14 (111%), AN77 (110%), JN22 (108%), DB15 (107%), and Cim7 (107%). At 150 mg/l As<sup>3+</sup>, ACCG6, and ACCG15 also showed significant growth increases relative to their controls of 105% and 104%, respectively (Supplementary Figure 5). Even though As<sup>3+</sup> showed growth stimulation at 50 mg/l, it still followed the general trend observed for other metals, growth rates falling at 150 mg/l.

No particular patterns were observed for the lowest concentration for each metal that had been tested, in regards to isolate taxon or site of origin

(data not shown). This was also the case in tests that included Cu<sup>2+</sup> and As<sup>3+</sup> at 150 mg/l. However, growth trials with media spiked with 50 mg/l Ni<sup>2+</sup> or 225 mg/l Zn<sup>2+</sup>, revealed greater tolerance in strains from the Order *Helotiales*. Site of origin also appeared important in regards to Ni<sup>2+</sup> and Zn<sup>2+</sup> tolerance, given that all strains showing growth on 50 mg/l Ni<sup>2+</sup> (*cf.* Cim7), and all strains that were not inhibited on 225 mg/l Zn<sup>2+</sup> had been isolated from adjacent sites B and C. The presence of metals in the substrate of origin did not, however, affect tolerance ranking under any condition.

Growth of most fungi was similar to the control at pH values of 4.3 and 3. At pH 3, two strains exhibited relative growth lower than 75%, i.e., ACCG6 (60%) and HC5247 (58%), while two were

completely inhibited (AD6193 and ACCG15; data not shown). At pH 2, all fungi that were capable of growth belonged to the *Ascomycota*, except for PI8235. Rankings at pH 3 also showed the same pattern, with members of the *Basidiomycota* being at the lower end of the scale (except for PI8235), but still showing some growth.

Clustering, as shown in the heatmap in Figure 4, did not reveal particular patterns. Some closely related strains that originated from the same site clustered together, such as JN14 and JN22 or AN115, AN2, and AN97, but this pattern does not extend to similar taxa originating from different sites. While strains JN14, JN22, JP21, JN10, and JN11 originated from two contiguous sites and clustered together, other strains from these sites clustered with other non-related strains. Given that no other site-of-origin clustering occurs, this pattern cannot be generalized and could be attributable to chance events. Clustering of conditions in the heatmap did not yield significantly related results, with detailed examination of strain responses to each condition showing highly variable results.

### 3.4. Growth promotion

Stem to root dry weight ratios were typically of 10:1 with no statistically significant variations between conditions (data not shown). Total length and total dry weight were correlated ( $r^2 = 0.62$ ), but only dry weight data yielded statistically significant variations.

Figure 1 shows detailed ranking results for growth stimulation. Only strains AN97 and PI8235 yielded statistically significant increases in their host plant dry weights relative to their control ( $p = .0079$  and  $p = .0239$ , respectively). Strain JP10 was the only strain showing statistically significant growth inhibition ( $p = .0045$ ). Although surviving plants inoculated with strains JN25 and Cim7 did not show statistically significant variations in host plants dry weights, mortality was higher with 6 and 13 dead plants, respectively. This high mortality rates explain their low ranking in Figure 1. There was also no significant variation between plants inoculated with known ECM fungi and DSE (data not shown). This absence of significant results is mostly due to very high variations between replicates, as standard deviation was typically over 50% of mean values (data not shown).

## 4. Discussion

The main objective of the study was to find metal-tolerant fungal endophytes that could be used in further research using AR and AC for phytostabilization of metals. To do so, fungal strains naturally

in association with alders were isolated from various locations including mining sites with typically harsh soil conditions (contamination with heavy metals and low soil pH). Among the isolated fungal strains, potential microbial partners (mycorrhizal and DSE fungi) were selected and tested for heavy metals and low pH tolerance, as well as for their potential beneficial effects on alder growth.

Our results suggest that the use of different disinfection methods to improve the biodiversity of fungal isolates is not necessary. The lack of significant differences in results for various disinfection methods might be explained by the protection that was offered to the endophytes by the roots themselves.

Successful isolation of previously unknown AR and AC fungal endophytes was achieved from alder root samples from different sites with different substrate properties. None of the isolates was identified as a previously reported ECM endosymbiont of *Alnus* sp. [15], as the origins of strains ACCG6 and ACCG15 could not be ascertained. Presence of DSE fungi on poor and contaminated soils had been previously reported [29,40,43,44], and all collection sites had been disturbed by human activities. As such, the presence of DSE could be expected. The lack of ECMs, however, was rather surprising, given their ubiquity in roots that are growing in contaminated or disturbed soils has been widely reported, although not specifically for *Alnus* sp. [41,56,60]. One could argue that the sterilization method used was too stringent for ECM, allowing DSE to survive. This, however, does not seem likely. First, four distinct sterilization methods were tested and none showed significant variations as per the type of fungi isolated. Second, these methods have proven their usefulness to isolate ECM rather than DSE [46,47]. Finally, isolation assays conducted in order to validate Koch's postulates included four ECM strains (ACCG6, ACCG15, PI8235, and AD6193) and none of them were destroyed in the root surface-sterilization procedures employed. We hypothesize that low levels of active mycorrhizal propagules in mine tailings, combined with the ubiquitous presence of DSE in the environment, could explain their appearance on mining sites, while ECM fungi are still absent [30,61].

Soil properties have also repeatedly been identified as key drivers of the endophytic fungal communities of the genus *Alnus* [15,62,63]. Therefore, it should not be surprising that a shift could occur, away from previously described community structures that are mostly composed of ECM, with a large proportion belonging to *Tomentella*-*Thelephora* or *Hebeloma*-*Alnicola* lineages [15,62-64] in highly stressful environments, especially since *Alnus* root-associated fungi communities have shown some sensitivity to neighborhood

effects [65]. However, further studies regarding the development of alder fungal communities on disturbed sites would be required to fully understand these results.

Although not the primary focus of these assays, this study has yielded several results furthering our understanding of AC and AR's fungal endophytes. Three strains were included to this study as positive controls for inoculation trials. UAMH strains *A. diplophloeus* 6193 and *P. involutus* 8235 confirmed their capacity to form ECM with both *A. incana* [13,14] and *A. alnobetula* [14]. UAMH *H. crustulini-forme* 5247 did not form ECM structures on root tips, contrary to its symbiotic status that was previously reported by Godbout and Fortin [14]. As argued by Tedersoo et al. [15], some associations that are formed in aseptic trials using aggressively growing pioneer fungi could be described as "semicompatible," exhibiting fragmented mantles and minimal development of the Hartig net [66]. The low degree of colonization in the trial conducted by Godbout and Fortin [14] and the absence of other such results, to our knowledge, could explain the absence of ECM formation in our trial.

All other isolates were members of the Order *Helotiales* (*Ascomycota*). Putative *Ascomycota* generalist ECM fungi were identified by Tedersoo et al. [15] as symbionts of *Alnus* spp. Yet, the distribution of our isolates more closely reflected the DSE community associated with the roots of *A. nepalensis* that was described by Xu et al. [18], who found them in mine tailings. To our knowledge, we are reporting members of the genera *Cryptosporiopsis* and *Rhizocyphus* as root endophytes of *Alnus* for the first time.

DSEs are known to colonize roots of a wide variety of plants, with some of these associations being non-mycorrhizal [19,58], especially in stressful environments and polluted soils [29,40,43,44]. The penetration of the root system of *Asparagus officinalis* L. by *Phialocephala fortinii* C. J. K. Wang and H. E. Wilcox, which was described by Yu et al. [67], showed similarities to a weak pathogen or an opportunistic fungus. These peculiarities could suggest that the colonization of *Alnus* roots by DSE is not subject to developed signaling pathways, as is the case with many other root symbionts [68–70] and, therefore, the process is mostly under the control of the fungus. The DSE-*Alnus* relationship could thus fall outside the scope of the close reciprocal specificity that is shown by the *Alnus* spp.-ECM community.

Group 4 contained strains that were related to genera known to form ericoid mycorrhizae with ericoid hosts, such as *Rhizocyphus* and *Meliniomyces*, but no such structures were observed [59,71], as colonization patterns were similar to those observed

with several DSE strains. Mycorrhizal fungi are known to form different types of structures and to follow different colonization patterns, depending upon the plant host and environmental conditions [58]. Thus, a non-ericoid colonization pattern in a non-ericoid host was not unexpected. However, additional observations and studies are required to fully understand how these fungi interact with *Alnus* spp. and whether the colonization patterns that have been observed are typical of this interaction.

The identification of metal tolerant strains for use in phytostabilization strategies was conducted through metal tolerance trials. Our results showed that no individual strain was globally more metal-resistant than the others (i.e., the most resistant to all metals tested). High intra- and inter-specific variation in the tolerance of ECM and DSE fungi was however observed, as could be expected from literature [32,56,72–74]. Furthermore, metal tolerance of individual strains was mostly metal-specific [41,56]. The results of our metal tolerance trials, therefore, confirm previously reported findings.

As argued by Colpaert et al. [41], true metal tolerance should involve mechanisms that transport metal ions from the cytoplasm to the apoplast, as other defense mechanisms eventually could be overloaded. The presence of specific metal transporters, such as  $P_{1B}$ -ATPases [75–77], could actually provide an explanation for high tolerance levels to specific metal ions.

A case could, however, be made that some fungal taxa generally have a higher constitutive tolerance to metals. Certain tolerance mechanisms, such as sorption to components of the cell wall (e.g., melanins), are not metal-specific and some fungi can chelate a large array of metal ions [78,79]. Strains belonging to the Order *Helotiales* (*Ascomycota*) showed higher tolerance to  $Zn^{2+}$  and  $Ni^{2+}$  than their *Basidiomycota* counterparts. Whereas fungi did not show particular growth patterns in terms of  $Cu^{2+}$  and  $As^{3+}$  tolerance, *Ascomycota* still showed growth. These findings are supported by field observations, where DSE appear to dominate various metal-polluted environments [18,40,43,80] or exhibit an increasing presence with increasing metal concentrations [39]. Higher tolerance to acidic conditions for members of the *Helotiales* is consistent with previous findings, as members of this order are dominant in highly acidic substrates, such as Mor humus [58].

Interestingly, our study revealed that the presence of 50 mg/l arsenic could stimulate the growth of a large number of our strains, even though arsenic performs no known biological function [81]. There are regular reports in the literature suggesting growth-stimulation effects at lower metal concentrations in several fungi [82–85]. Whether this is due

to secondary effects of certain resistance mechanisms, such as methylation or activation of NADPH oxidase activity as seen in immortal human cell lines [86], further research would be needed to adequately address this question.

Growth assays in this study were performed to provide a baseline so results from further assays could be put into perspective, rather than to select endophytes for phytostabilization purposes. As environmental conditions significantly alter the relationship between endophytes and their host [27], results obtained in rich non-contaminated media cannot be extended to what could be observed in poor or contaminated soils. Even if very high intraspecific variation generally prevented the observation of statistically significant results, the identification of strains having negative effects on their host will be useful for further research. As growth inhibition is to be expected in poor or contaminated soils, identification of endophytes with negative impacts on growth in rich non-contaminated medium will allow better interpretation of results.

This study was intended as a first step in the selection of suitable strains for the inoculation of alders in a phytostabilization context. Sampling and isolation methods yielded an array of endophytic fungi of potential use on contaminated sites. No specific strain was observed as systematically forming part of the highest-ranking groups. However, results indicate that DSE could be of particular interest. First, they were found in both disturbed and contaminated soils were no ECM seemed present. Second, they showed higher resistance to a larger array of metals than their ECM counterparts. Finally, our limited growth assays showed that certain DSE endophytes had neutral to positive impacts on growth. However, as the impact of DSE colonization on host plants varies greatly, further growth assays in poor and contaminated media are the next step to select proper strains for rehabilitation purposes.

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