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RESEARCH ARTICLE

Impact of mycorrhizal inoculation on the fungal root and rhizosphere communities, growth and salinity tolerance of *Alnus glutionosa* Gaertn. seedlings

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Abstract

Background and Aims *Alnus glutinosa* L. Geartn. is a valuable forest tree species capable of thriving in saline soils, making it suitable for afforestation in moist, saline areas which are expanding under the influence of climate change. Its high salinity tolerance may stem from its ability to form dual mycorrhizal symbioses with arbuscular (AM) and ectomycorrhizal (EM) fungi in a single root system. Since seedlings in forest nurseries can be inoculated with mycorrhizal fungi prior to planting, this study aimed to determine which inoculation provides the best protection against salinity.

Methods In an 11-month pot experiment, six-month-old A. glutinosa seedlings were inoculated with either AM fungi alone or a combination of AM + EM fungi under short-term (ST) and long-term (LT) salinity conditions.

Results Both treatments increased plant growth under ST salinity, whereas AM was more effective under LT salinity. Mycorrhization contributes to maintaining the water content, proline accumulation, and lipid peroxidation in leaves. Both inocula reduced the sodium bioconcentration in leaves and roots, with AM + EM being more effective under ST salinity. Inoculation significantly influenced fungal beta diversity in roots and soil, particularly under ST salinity, whereas fungal alpha diversity remained unchanged or increased under LT conditions. It also increased the abundance of specific fungal genera in the endosphere and rhizosphere.

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Conclusion These findings highlight the potential of mycorrhizal inoculation to enhance A. glutinosa resilience in saline environments and support sustainable ecosystem restoration amid climate change.

Keywords Microbiome · Soil salinity · Ectomycorrhizal fungi · Arbuscular fungi · Mycorrhizal inoculum · Amplicon analysis

Abbreviations

AM	Arbuscular
AM + EM	Arbuscular-ectomycorrhizal
ASV	Amplicon Sequence Variant
BCF	Bioconcentration factor
DW	Dry weight
EC	Electrical conductivity
FW	Fresh weight
ITS	Internal Transcribed Spacer
LT	Long-term salinity
MDA	Malonyl dialdehyde
NS1	Control LT, nonsaline soil 1
NS2	Control ST, nonsaline soil 2
NS2 10×	Experimental ST saline soil with salinity 10 times higher than that of NS2 variant
NS2 20×	Experimental ST saline soil with salinity 20 times higher than that of NS2 variant
S1	Experimental LT saline soil 1
S2	Experimental LT saline soil 2
SSU	Small subunit ribosomal
ST	Short-term salinity
TBARS	Thiobarbituric acid-reactive substances
Ti	Translocation index

Introduction

Soil salinity is one of the major and widespread challenges spreading to currently unaffected regions in the near future. This problem can adversely affect soil microbial activity and community structure, thereby altering the physiological, biochemical, and genetic properties of plants as a consequence of changes in gene expression and epigenetic modifications (Yan et al. 2015). Therefore, soil salinity mitigation is a key issue for agriculture and forestry worldwide. While the effects of salinity on crops in agriculture have already been relatively well studied (Alizadeh et al. 2021; Parihar et al. 2020; Qiu et al. 2020), knowledge of the effects of salinity on forest trees is still very poor. Moreover, mycorrhizal fungi, which can form mutualistic symbioses with most forest trees (Anthony et al. 2022), are the most important microorganisms that protect host plants from abiotic environmental stresses (Branco et al. 2015). However, only a few studies have demonstrated the positive impact of mycorrhizal inoculation on improving tree growth and enhancing tolerance in the condition of salt stress (Hoda et al. 2011; Thiem et al. 2020; Wang et al. 2022).

The role of mycorrhizal fungi in alleviating salt stress involves multiple mechanisms, including improved nutrient uptake, osmotic regulation, and modulation of biochemical responses (Evelin et al. 2009). Among the latter, the accumulation of proline is widely recognized as a key indicator of plant tolerance to osmotic and dehydration stress (Verbruggen and Hermans 2008). Likewise, malonyl dialdehyde (MDA), a product of membrane lipid peroxidation, serves as a marker of oxidative damage under abiotic stress conditions, with its reduction indicating enhanced cellular protection (Labidi et al. 2011). Measuring proline and MDA levels can thus provide insight into the physiological state of plants under salt stress and the potential mitigating effects of mycorrhizal symbiosis. Mycorrhizal fungi can help in maintaining the salt stress also by the reduction of toxic salt ions from the soil (Boorboori and Lackóová 2024). In understanding such an effect can help the analysis of bioconcentration and translocation factors that have been used so far to describe the plant responses to water-salt conditions (Ding et al. 2021).

Alnus glutinosa Gaertn., known as black alder, is one of the few forest tree species that tolerates saline soil environments, however soil salinity limits their growth by reducing growth in diameter and, to a lesser degree, the tree height (Deptuła et al. 2020). Studies conducted to date on this tree species indicate that the tolerance of the plant to salt stress may be closely related to the presence of a unique type of dual symbiosis which the tree forms with two types of mycorrhizal fungi: arbuscular (AM) and ectomycorrhizal (EM) (Thiem et al. 2018) in its roots. In addition to mycorrhizal associations, *A. glutinosa* is also capable of forming a third type of symbiotic relationship, with nitrogen-fixing actinobacteria of the genus *Frankia*. This actinorhizal symbiosis enhances nitrogen availability and has also been shown to contribute to the plant's resilience in saline and nutrient-poor

soils (Ngom et al. 2016) *A. glutionsa* inoculated with *Frankia* spp. together with the arbuscular fungus *Glomus intraradices* shows improved growth under saline stress conditions (Oliveira et al. 2005). However, in the present study, we focused exclusively on mycorrhizal fungi, due to their greater practical potential for use in seedling inoculation technologies in forest nurseries. Currently, a few AM fungi (Glomeraceae and Claroideoglomeraceae families) (Thiem et al. 2023) and approximately 20-30 EM species have been identified (for example, species of the tomentella-thelephora, hebeloma-alnicola, russula-lactarius, and paxillus lineages (Tedersoo et al. 2009) as the roots symbionts of *A. glutinosa* in different regions of Europe. Therefore, *A. glutinosa* is an excellent plant model for research on the role and occurrence of both types of mycorrhizal symbiosis simultaneously at saline sites, as well as for investigating the entire fungal root microbiome (Thiem et al. 2018). Although EM symbiosis seems to be the dominant in mature trees (Tedersoo et al. 2009), it is suggested that arbuscular fungi are preferred in the early stages of alder growth. In nature, plants often associate with multiple symbionts concurrently, yet the effects of multifactorial symbioses are not well understood (Larimer et al. 2010). It is not known whether dual mycorrhizae are better for growth under saline conditions than just one of them is. The following question arises: Will tree species that form both types of mycorrhizae, with the double protection of symbiotic partners, be more resistant to soil salinity than those associated with a single symbiont?

Studies on the effects of symbiotic microorganisms on plants are often conducted in sterilized soil in both agriculture and forestry research (Masrahi et al. 2020; Wang et al. 2022). It allows for the determination of the impact of an added inoculum but does not allow determination of whether the use of the inoculum under natural conditions will be as effective as in sterile conditions. Salomon et al. (2022) reported that many out of 25 commercial AM inoculations were not as effective under greenhouse as in sterile laboratory conditions. It seems that only those inoculations that revealed changes in the mycorrhizal community after inoculation were accompanied by increased plant biomass production (Salomon et al. 2022). For this reason, analysis of the fungal community after plant inoculation with mycorrhizal fungi is important and should be considered when planning pot experiments involving bioinoculation (Hart et al. 2017; Martignoni et al. 2020). Another important aspect in studies of the effects of mycorrhizal inoculation on tree seedling growth should concern the dual mycorrhizal status of plants. Recently, many shrub and tree species have been found to be associated with both AM and EM fungi, either simultaneously within the same root system or at different life stages or in different environments (Heklau et al. 2021). This phenomenon seems to be common among woody plants. An extensive search of the literature performed by Teste et al. (2020) revealed that 84% of the plant genera with confirmed dual-mycorrhizal status were woody taxa. Moreover, studies on both mycorrhizal communities at the same time are very limited (*e.g.*, Boeraeve et al. 2019).

Planning the study and experiments, we posed the following questions: (i) how will inoculation of forest trees with endo- and/or ectomycorrhizal fungi affect the autochthonous fungal microbiome in saline soils with a stable and well-formed halophilic microbiome because of long-term (LT) salinity exposition, (ii) how does inoculation with mycorrhizal fungi affect the fungal microbiome not adapted to salinity after introduced soil salinization lasting short period of time (ST). In order to answer for these questions, we performed two experiments that refer to both time of soil salinity exposition on the soil microbiome (ST and LT).

We aimed to (i) determine the effects of two mycorrhizal inocula (AM and AM +EM) on the growth and salinity adaptation of *A. glutinosa* seedlings growing in long-term (LT) and short-term (ST) saline soils; (ii) analyse the bioconcentration factors and translocation indices of sodium ions; and (iii) describe how the application of mycorrhizal inoculants changes the alpha diversity, taxa abundance and community structure of root endophytic and rhizospheric fungi. We hypothesized that the both types of mycorrhizal inocula would enhance ecophysiological growth parameters, improve water content, as well as increase proline accumulation and reduce MDA in leaves. Furthermore, we expected that one type of inoculum (either single or dual) would be more effective under short-term salinity conditions, while the other would perform better under long-term salinity conditions. We assumed that the application of both mycorrhizal inocula would significantly reduce the sodium bioconcentration factors for leaves and roots,

and translocation indices. In relation to performed amplicon analysis, we hypothesized that introduced mycorrhizal fungi would be more frequently observed in the roots and/or rhizosphere of inoculated plants, but their presence would not cause changes in the overall alpha diversity of rhizospheric and endophytic fungi but change the relative abundance of some fungal genera.

Materials and methods

Experimental design

The pot study was divided into two experiments. First, we considered effects of mycorrhizal fungi on *A. glutinosa* seedlings growing in long-term (LT) saline soils, in which the fungal microbiome can adapt to environmental conditions through long-term exposure to salinity. In the first experiment, three growth substrates were used: nonsaline soil, originating from the Dobrzejewice Forest District in Toruń (53°02′68.1″ N, 18°71′45.2″ E; control for LT saline soils) – NS1; experimental LT saline soil 1 from Szubin Forest District in Słonawy (53°01′26.6″ N, 17°37′47.3″ E) – S1, and experimental LT saline soil 2 from the edge of the salt meadow in Inowrocław (52° 45′22.932″ N, 18 13′26.004″ E) – S2. Soil samples for growth substrate were selected by the following criteria: (i) the soil originating from the neighborhood of black alder roots, (ii) the soils should differ in salinity levels, and (iii) they should come from sites with similar climatic and geological conditions. The salinity of soils in the Słonawy area follows from the impact of saline springs in contact with Zechstein (Permian) salt deposits (Thiem et al. 2018), whereas the soil salinity at the Inowrocław site was caused by improper storage of industrial waste from the soda factory, which was founded in 1879 and is still in operation. Wastewater infiltration into the soil leads to land degradation, such as alkalinization and salinization (Szymańska et al. 2013).

The second experiment analysed the effects of mycorrhizal fungi on alder seedlings growing in natural forest conditions when the soil salinity level could increase suddenly and influence the fungal microbiome violently, preventing the adaptation of microbes to abiotic factor. The second experiment of study allowed us to observe the effects of short-term (ST) salinity on alder seedlings and their fungal microbiomes. In this case, the control soil (NS2-nonsaline soil 2) was obtained from Szubin Forestry in Pszczółczyn (53°00′22.9″ N, 17°54′57.2″ E). Soil NS2 was sampled under black alder trees. In this part of the experiment, we assumed that the soil parameters should be similar for all experimental variable except the level of salinity, where we would like to receive soil that is approximately ten times more saline (NS2 10×) and twenty times more saline (NS2 20×) than the control soil (NS2).

The whole pot study was performed over eleven months under semiopen conditions in an experimental garden with a plastic cover to prevent the rinsing of salt from the pots during rainfall (Fig. 1). In October 2017, six month-old seedling of *A. glutionosa* were planted in pots filled with four types of substrate (5 L volume). The trees were 18 ±2 cm high. The fresh biomass of the organs was as follows: leaves (1.34 ±0.24 g), shoots (1.02 ±0.08 g), and roots (1.28 ±0.25 g), and the dry biomass was as follows: leaves (0.35 ±0.06 g), shoots (0.50 ±0.07 g), and roots (0.46 ±0.10 g). The seedlings were obtained from a forest nursery located in Dobrzejewice Forest District (Toruń, Poland). In April 2018, when the trees had adapted to the experimental growth conditions and new leaves appeared, the NS2 soil was salinized. Salinization of the experimental variants (ST: NS2 10×, NS2 20×) was performed by watering the plants with modified Hoagland's medium (Hoagland and Arnon 1950 without KH_2PO_4 solution), which was freshly prepared in our laboratory, supplemented with sodium chloride (NaCl) at concentrations of 100 mM and 200 mM. The plants were watered from the bottom, which allowed for the salts to gradually accumulate in the substrate and saturate it to the desired concentration. Other variants (LT: NS1, S1, S2; ST: NS2) were watered with Hoagland's medium without salt. Salinization was performed over three weeks: (1) first, the soil was watered, and the soil salinity was measured; (2) a break was made to observe the effect of salinization; and (3) a second watering was applied, and the soil salinity was measured. The level of salinity was measured one day after watering via a conductivity meter (CPC-505, Elmetron) according to the procedure of van Reeuwijk et al. (2006). In the fourth week from the start of salinization, when the final assumed salinity levels were confirmed in the NS2 10×, and NS2 20 ×variants, the plants from

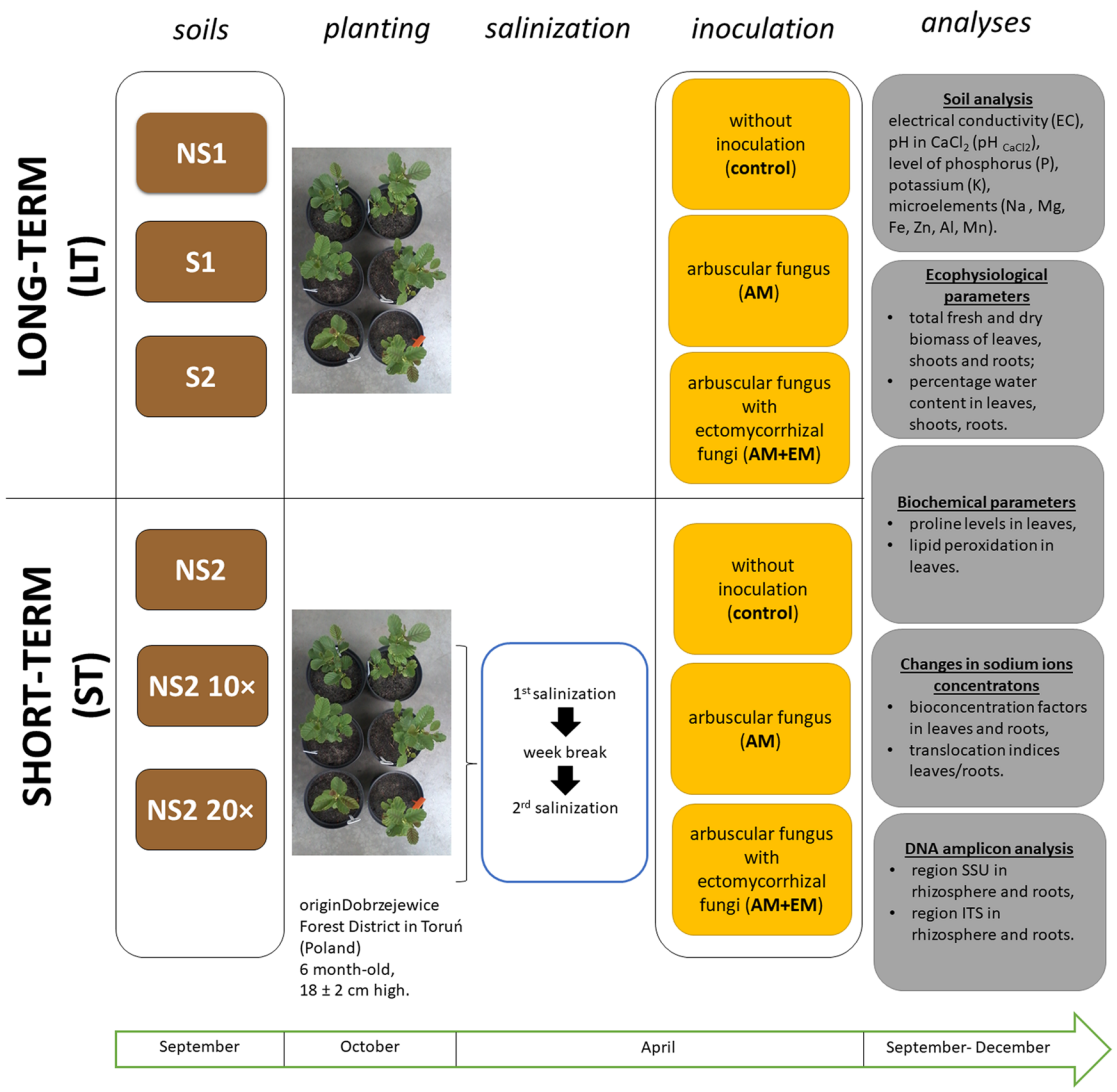


Fig. 1 Workflow of experimental variants and analyses for two types of salinity (long-term – LT, short-term – ST) with the use of three variants of inoculation (control, AM—arbuscular, AM + EM—arbuscular-ectomycorrhizal

two experiments were inoculated with two types of inocula: AM with *Rhizophagus irregularis* (INOQ GmbH, Germany) or arbuscular-ectomycorrhizal (AM + EM) with *R. irregularis*, *Amanita muscaria*, *Boletus edulis*, *Hebeloma crustuliniforme*, *Laccaria laccata*, *Paxillus involutus*, *Thelephora terrestis*, and *Imleria badia* (INOQ GmbH, Germany), and 20 ml of inoculum was added to each pot, which was mixed gently with the substrate. Over the next four months, the plants were watered as needed by adding tap water to the trays on which the pots were placed to prevent salt leaching. After this time, the experiments were finished. The plants were carefully removed from the pots, and the rhizosphere soil from all variants was collected from the root surfaces and placed in 1.5 volume tubes via a sterile scalpel for metagenomic analysis and measurement of the Na^+ content in the substrate. The roots were then washed with distilled water, dried on sterile paper, and used for further analysis. Ecophysiological parameters were then measured, including total fresh weight (FW) of leaves, shoots and roots, as well as the length of shoots and roots was measured using a ruler. Subsequently, approximately 2 g of root material-including

main roots, lateral roots, and fine roots-was cut with a sterile scalpel, transferred into sterile 50 mL tubes, rinsed five times with sterile water, and lyophilized for 24 h. Additionally, the leaves were flash-frozen in liquid nitrogen for analysis of proline and lipid peroxidation levels. The soil samples were air dried for analysis of their physicochemical parameters. Plant material from three plants per variant was dried for 24 h at 60 °C and weighed to measure dry weight (DW) and percentage water content in leaves, shoots, and roots. Then, the Na^+ concentration in leaves and roots was measured, and the percentage water content was calculated according to the following formula: (FW-DW)/FW) $\times 100$. The statistical significance of each growth parameter between inoculated and noninoculated variants was tested via one-way ANOVA and Newman-Keuls test for post hoc comparisons ($p \leq 0.05$, R.4.3.1).

Soil analysis

Air-dried soil was used to determine the soil parameters for each variant in triplicate. The electrical conductivity (EC) was measured conductometrically, and the soil pH was measured in 0.01 M $CaCl_2$ (pH_{CaCl2}; w/v = 1:2.5) via the potentiometric method. The concentrations of phosphorus (P), potassium (K), sodium (Na), magnesium (Mg), iron (Fe), zinc (Zn), aluminium (Al) and manganese (Mn) were measured via inductively coupled plasma-optical emission spectroscopy (ICP-OES; Perkin Elmer, USA). The statistical significance of differences in the soil physicochemical parameters was tested by one-way ANOVA and Tukey's HSD tests as post hoc comparisons ($p \leq 0.05$) (R software, version 4.3.1).

Biochemical parameters

Biochemical measurements of proline levels in leaves were performed in triplicate from six plants per variant according to procedure described by Abrahám et al. (2010) using 0.5 g of plant material. The obtained values were recalculated as µg of proline per gram of fresh weight of leaves according to a standard curve (0–20 µg/ml). Lipid peroxidation was determined in triplicate from three plants by measuring the production of thiobarbituric acid-reactive substances (TBARS) and expressed in equivalents of malonyl dialdehyde (MDA) according to DeLong et al. (2002) in which 0.25 g leaf sample was used. The absorbances at 532, 600, and 450 nm (*A*532, *A*600, and *A*450, respectively), were determined spectrophotometrically. The MDA concentration was calculated according to the formula: $6.45 \times (A532 - A600) - 0.56 \times A450$. The MDA content (µmol g^{-1}) was calculated as $C \times V/(1.000 \times W)$, where V denotes the sample extraction liquid (ml) and W denotes the sample fresh weight (g) (Krupa-Małkiewicz and Calomme 2021). The statistical significance of differences between inoculated and noninoculated variants for both biochemical parameters (proline, MDA) was tested by one-way ANOVA and Newman-Keuls test for post hoc comparisons ($p \leq 0.05$) (R software, version 4.3.1).

Sodium concentrations in the soil and plants

One hundred milligrams of dried (24 h, 60 °C) soil and plant organs (leaves and roots) from each variant in triplicate (from three plants) was mineralized in a 10 ml mixture of nitric and hydrochloric acids (v/v 1:3) via the mineralizer NovaWAVE SA (SCP Science, Canada), and demineralized water was added to obtain a 15 ml final volume ($1.5 \times$dilution). The Na^+ concentration was measured via a standard curve (0 – 100 mg/l NaCl) via a BWB-XP flame photometer (BWB Technologies, UK). The results are presented as indicators of phytodesalinization efficiency for (i) the bioconcentration factor ($BCF = [Na]_{Plants}/[Na]_{Soil}$) and (ii) the translocation index ($Ti = [Na]_{Leaves}/[Na]_{Roots}$). The statistical significance of differences between inoculated and noninoculated variants for both factors (BCF, Ti) was tested by Kruskal-Wallis tests and Newman-Keuls tests as post hoc comparisons ($p \leq 0.05$) (R software, version 4.3.1).

Amplicon and bioinformatic analyses

One hundred milligrams of lyophilized soil or 50 mg of lyophilized roots were placed in reinforced tubes containing 100 mg of sterile quartz sand or sterile metal beads, respectively. Next, total DNA was isolated using Power Soil DNA isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's protocol. Isolation was performed in triplicate (from three plants). The DNA concentration was measured spectrophotometrically (Nanodrop 2000), and diluted to 1 ng/µl. Next, two libraries were constructed: one

for the ITS (internal transcribed spacer) region and the second for the SSU (small subunit ribosomal) region. ITS amplicon libraries were generated under the conditions described by Thiem et al. (2018), whereas the SSU region was amplified using 0.5 U Phusion polymerase (Thermo Scientific), 0.1 mM dNTPs, buffer with 1.5 mM $MgCl_2$, and 0.25 pM each primer (uNS31 and uAML2). The cycling conditions were as follows: 98 °C for 30 s; 40 cycles of – 98 °C for 10 s, 58 °C for 30 s, and 72 oC for 60 s; and then 5 min at 72 °C. All PCR products were checked on 1.5% agarose gels in TBE and then purified using Ampure (Beckmann Coulter, USA) according to the manufacturer's protocol. Next, the concentration of the PCR products were measured by Nanodrop, diluted to 4 ng/µl and subjected to a second round of PCR via Taq PCR Master Mix (EurX, Poland) according to the manufacturer's protocol. The reaction conditions were as follows: 95 °C for 5 min; 14 cycles of 95 °C for 30 s, 54 °C for 15 s, 72 °C for 30 s; and, ultimately, 72 °C for 1 min. The products were checked again on a 1.5% agarose gel and pooled in equimolar amounts. The quality of the pooled libraries was assessed on a Bioanalyzer chip (Agilent, USA). The final pool was diluted to 4 nM, denatured, mixed with 5% of the PhiX control library and sequenced with the use of a 2 ×300 cycles kit v.3 on a MiSeq machine (Illumina). Sequencing was performed using HPLC-purified versions of forwards and reverse first-round primers (R1 and R2) as well as the reverse-complement reverse primer (I7 index read).

The raw reads were processed and analysed as described earlier by Thiem et al. (2023) using dada2 v.1.32.0, but the analyses were streamlined in the mgmisc1 package (Gołębiewski 2025, unpublished) in R v.4.4.0. R. Script can be found in Online Resource 1. The sequencing statistics for the ITS and SSU regions are described in detail in Online Resource 2.

Results

Differences in soil parameters

The level of salinity in the control soil for the long-term experiment (NS1), expressed as EC, did not exceed 41.69 (µS cm^{-1}) and the soil had a lower pH than the saline soils (S1, S2). Compared with the S1 soil, the NS1 soil had significantly higher phosphorus (P), iron (Fe), manganese (Mn) and zinc (Zn) contents. Compared to the NS1 and NS2 soils, the S1 soil was characterized by a significantly higher concentration of sodium (Na), whereas the S2 soil contained significantly higher levels of P and most analysed elements(Ca, Fe, Mg, Mn, Zn, K) compared to the NS1 and S1 soils (Table 1a). In the short-term salinized treatments, the EC and the level of Na increased significantly (NS2 < NS2 10× < NS2 20×), as expected (Table 1b).

The effect of mycorrhizal inocula on alder growth and water content under short- and long-term salinity

Analysis of black alder ecophysiological parameters revealed that plants inoculated with mycorrhizal fungi (AM and AM + EM) had significantly higher shoot and roots length (Online Resource 2, Fig. 1), total fresh biomass (Fig. 2a, b, c) and dry biomass (Fig. 2d, e, f) of leaves, shoots and roots in most treatments. The effects of mycorrhizal fungi (AM and AM + EM) varied according to the type of salinization and treatment. In the LT salinity (S1, S2), AM inoculum significantly increased the fresh and dry biomass of the plants, whereas the AM + EM inoculum improved these parameters, mainly in the S2 variant. The most notable differences between noninoculated control and inoculated with mycorrhizal fungi variants (AM and AM + EM) were observed in the control soil (without salinity) - NS1 for both fresh and dry biomass of organs. (Fig. 2).

Inoculation with fungi significantly changed the percentage of water content in leaves and shoots (Fig. 2g, h). The AM fungal inoculum significantly decreased the water content in the shoots of NS1 (LT) and the NS 20× variant (ST). The opposite was true in LT saline soils, in which the water content significantly increased in the leaves (S1) and shoots (S2), which may indicate that the AM inoculum works better. The AM + EM inoculum significantly increased the water content in the leaves of the NS1 (LT) variant and in the shoots of the NS1 (LT) and NS2 (ST) variants and significantly decreased the water in shoots of the NS2 10× and NS2 20× (LT) variants.

Table 1 Results of one-way ANOVA and Tukey's HSD tests as post hoc comparisons ($p \leq 0.05$) for a) long-term (LT) and b) short-term (ST) soil parameters. Variants with the same letters are not significantly different

a)

salinity	variant	pH_{CaCl2}	EC (µS/cm)	Al (mg/g)	Ca (mg/g)	Fe (mg/g)	Mg (mg/g)	Mn (mg/g)	P (mg/g)	Zn (µg/g)	K (µg/g)	Na (µg/g)
LT	NS1	6.19 ± 0.04 [a]	34.67 ± 7.02 [a]	3.57 ± 0.49 [ab]	3,79 ± 1.12 [a]	4.23 ± 0.63 [b]	0.66 ± 0.09 [a]	0.15 ± 0.02 [b]	0.32 ± 0.01 [b]	27.00 ± 0.86 [b]	517.57 ± 91.38 [a]	175.21 ± 21.64 [a]
	S1	6.55 ± 0.19 [ab]	153.33 ± 30.53 [ab]	2.27 ± 0.10 [a]	3,08 ± 0.14 [a]	2.13 ± 0.13 [a]	0.54 ± 0.01 [a]	0.05 ± 0.01 [a]	0.22 ± 0.02 [a]	13.54 ± 0.62 [a]	580.60 ± 13.98 [a]	1247.23 ± 23.15 [c]
	S2	6.82 ± 0.22 [b]	183.00 ± 90.37 [b]	4.42 ± 0.37 [b]	65,84 ± 2.32 [b]	8.06 ± 0.09 [c]	2.64 ± 0.06 [b]	0.30 ± 0.01 [c]	0.43 ± 0.03 [c]	33.65 ± 0.45 [c]	1078.41 ± 113.38 [b]	433.22 ± 42.10 [b]

b)

salinity	variant	pH_{CaCl2}	EC (µS/cm)	Al (mg/g)	Ca (mg/g)	Fe (mg/g)	Mg (mg/g)	Mn (mg/g)	P (mg/g)	Zn (µg/g)	K (µg/g)	Na (µg/g)
ST	NS2	6.08 ± 0.04 [a]	80.00 ± 11.00 [a]	2.67 ± 0.41 [a]	9,04 ± 2.00 [a]	7.64 ± 1.68 [a]	0.55 ± 0.05 [a]	0.18 ± 0.04 [a]	0.54 ± 0.09 [a]	10.45 ± 0.44 [a]	255.38 ± 54.30 [a]	179.72 ± 22.05 [a]
	NS2 10 ×	5.93 ± 0.07 [a]	723.33 ± 14.74 [b]	2.69 ± 0.40 [a]	8,80 ± 1.33 [a]	7.40 ± 1.01 [a]	0.55 ± 0.05 [a]	0.17 ± 0.02 [a]	0.54 ± 0.09 [a]	10.91 ± 1.45 [a]	309.09 ± 64.72 [a]	975.74 ± 256.20 [b]
	NS2 20 ×	5.92 ± 0.12 [a]	1345.33 ± 28.45 [c]	2.30 ± 0.30 [a]	8,33 ± 1.15 [a]	7.15 ± 1.08 [a]	0.52 ± 0.02 [a]	0.16 ± 0.02 [a]	0.50 ± 0.07 [a]	10.36 ± 0.83 [a]	286.77 ± 36.53 [a]	1929.38 ± 237.48 [c]

NS1 control LT nonsaline soil 1, *S1* experimental LT saline soil 1, *S2* experimental LT saline soil 2, *NS2* control ST, nonsaline soil 2, *NS2 10*× experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant, *NS2 20*× experimental ST saline soil with a salinity 20 times higher than that of the NS2 variant, pH_{CaCl2} pH in calcium chloride, *EC* electrical conductivity, *Al* aluminium, *Ca* calcium, *Fe* ferric, *Mg* magnesium, *Mn* manganese, *P* phosphorus, *Zn* zinc, *K* potassium, *Na* sodium

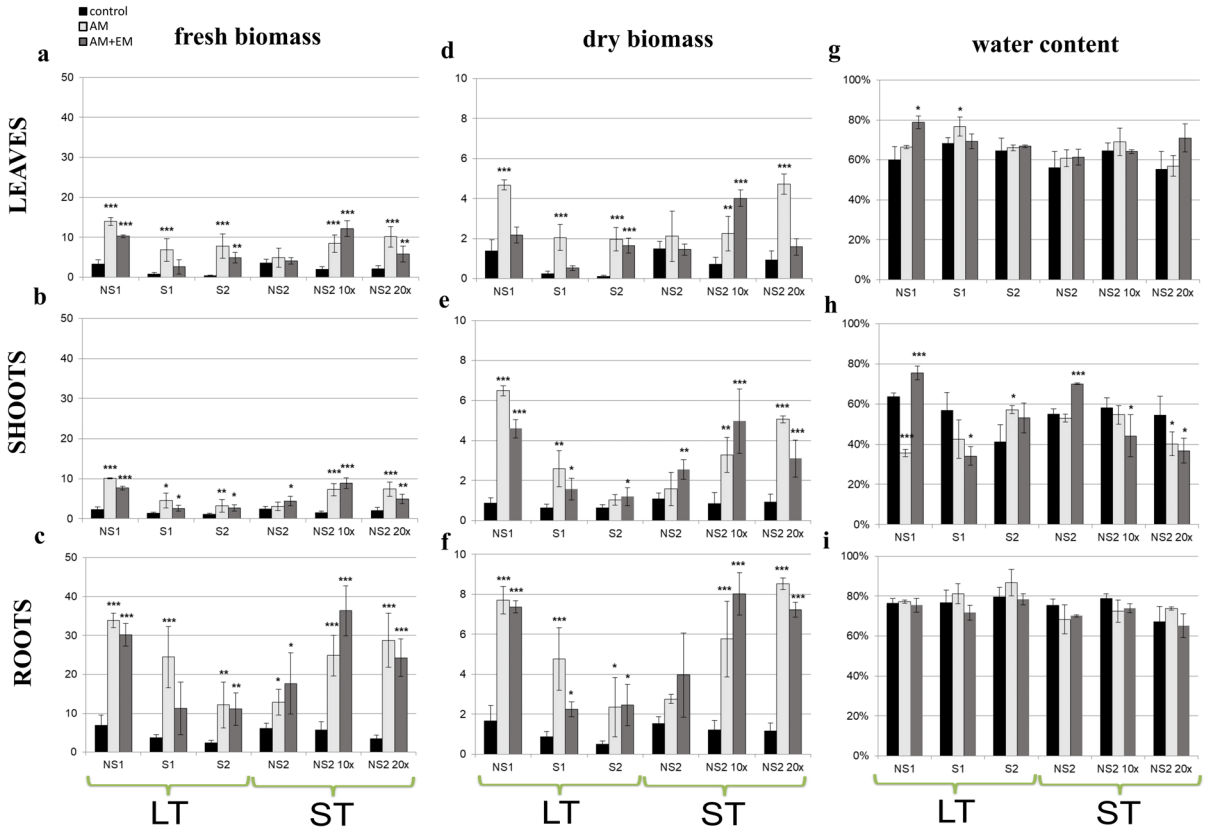


Fig. 2 Effects of mycorrhizal inoculation (control, AM – arbuscular, AM +EM – arbuscular-ectomycorrhizal) on (**a**) fresh biomass of leaves, (**b**) shoots, (**c**) roots (in grams); and (**d**) dry biomass of leaves, (**e**) shoots, (**f**) roots (in grams); and (**g**) percentage water content in leaves, (**h**) shoots, (**i**) roots of *A. glutinosa* seedlings for two types of salinity: long-term (LT) and short-term (ST). Stars denote significant differences between inoculated variants (AM or AM +EM) and uninoculated control variants on the basis of ANOVA and Newman–Keuls tests. Abbreviations: NS1 – control LT, nonsaline soil 1; S1 – experimental LT saline soil 1; S2 - experimental LT saline soil 2; NS2 – control ST, nonsaline soil 2; NS2 10× - experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant; NS2 20× - experimental ST saline soil with a salinity 20 times higher than that of the NS2 variant; * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$

The effect of mycorrhizal inocula on biochemical parameters under short- and long-term salinity

Inoculation increased proline levels regardless of the type of soil or salinization. The greatest increase in proline levels compared with those in the uninoculated control soils was observed in the LT saline soils in both inoculation variants (S1 and S2). Plants from the ST salinized soil variants (NS2 10×and NS2 20×), despite the higher soil salinity, presented almost four to five lower levels of proline in leaves than those from the S1 and S2 variants, and this occurred only when the plants were inoculated with two types of mycorrhizal fungi (AM +EM). Interestingly, we also found an increase in the concentration of proline in the leaves of trees from the soil samples without salinity (NS1 and NS2). In addition, a greater effect of combined inoculation (AM +EM) was observed in NS2 soil (control and saline variants of this soil, NS2 10 ×and NS2 20×) than in nonsaline soil (NS1) and naturally saline soils (S1 and S2) (Fig. 3a).

Compared with the control treatment, the application of AM did not significantly affect the levels of malonyl dialdehyde (MDA) in the leaves of all LT saline plants or in the ST salinity variant NS2 10×and its decreased the MDA level in the saltiest variant ST salinity (NS2 20×). Moreover, the application of the AM +EM inoculum increased the MDA level in all the variants except for the most saline experimental variant (NS 20×) (Fig. 3b).

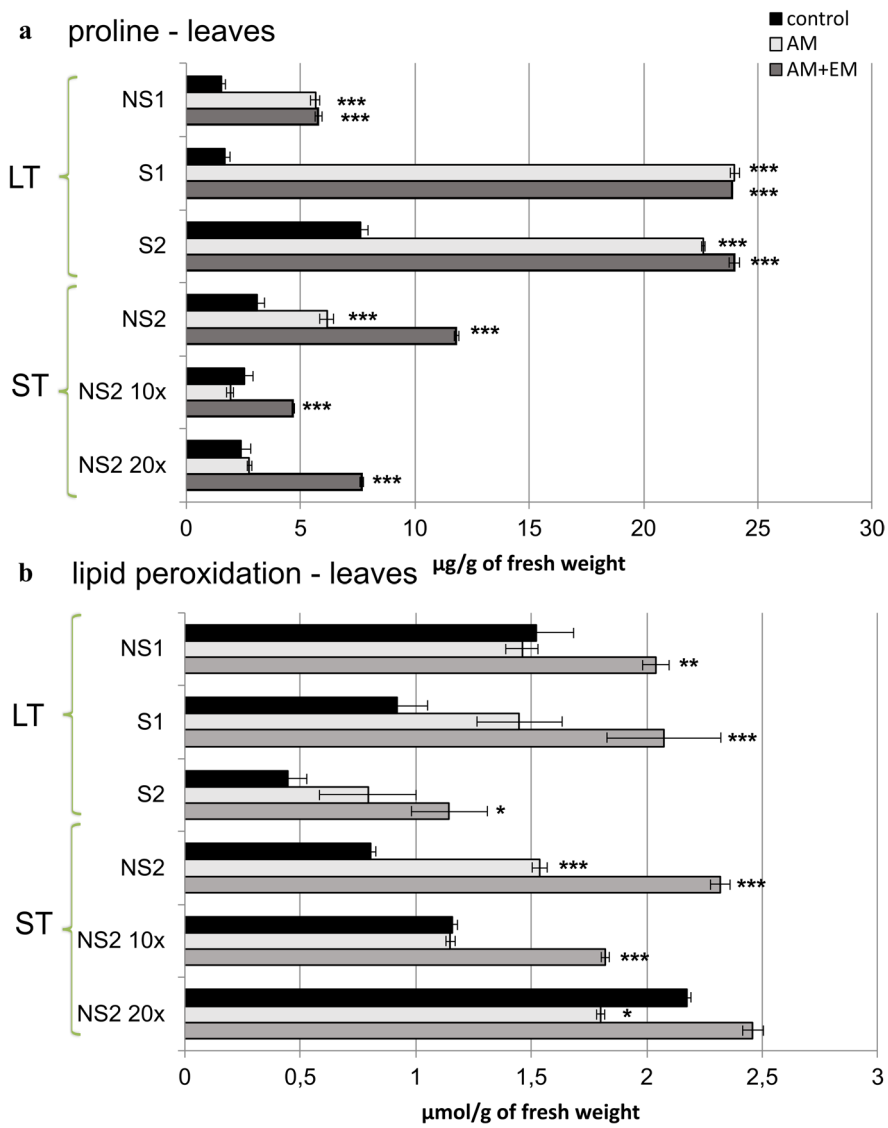


Fig. 3 Effects of mycorrhizal inoculation (control, AM – arbuscular, AM + EM – arbuscular-ectomycorrhizal) on (**a**) the level of proline (µg/g of fresh weight) and (**b**) lipid peroxidation (µmol/g of fresh weight) in the leaves of *A. glutinosa* seedlings for two types of salinity: long-term (LT) and short-term (ST). Stars denote significance difference between inoculated variants (AM – arbuscular, AM + EM – arbuscular–ectomycorrhizal) and uninoculated control variants on the basis of one-way ANOVA and Newman–Keuls tests. Abbreviations: NS1 – control LT, nonsaline soil 1; S1 – experimental LT saline soil 1; S2 - experimental LT saline soil 2; NS2 – control ST, nonsaline soil 2; NS2 10× - experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant; NS2 20× - experimental ST saline soil with a salinity 20 times higher than that of the NS2 variant; * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$

In the LT salinity variant, a differential effect of AM or AM + EM inoculation on the Na^+ bioconcentration and transfer from the soils to plants was observed, depending on the type of saline soil (S1 or S2). AM inoculation significantly reduced the BCF for leaves and roots and Ti leaves/roots in the S1 soil variant, whereas the application of the AM + EM inoculum significantly reduced the BCF for leaves and roots in the S2 soil variant (Table 2). In the ST salinity variant, significant reductions in BCF parameters for leaves and roots were observed after AM + EM combined inoculation for the NS2 20× and NS2 10× soil variants, respectively. AM inoculation significantly reduced only Ti leaves/roots in the NS2 10× soil variant (Table 2).

Effects of mycorrhizae on the fungal microbiome under short- and long-term salinity

More than 90 percent of the microorganisms found in the roots of the initial alder could not be classified. Fungi of the genera *Linnemannia*, *Solicoccozyma* and *Thelephora* were also found in the roots. A similar microbiome was observed in the rhizosphere in the NS1 soil and additionally contained *Acephala* spp. and *Fusarium* spp. The NS2 control soil was the most

Table 2 Bioconcentration factors (BCFs) for leaves and roots and translocation indices (Ti) for all experimental variants for two types of salinity: long-term (LT) and short-term (ST). Arrows denote a significant increase (↑) or decrease (↓) between inoculated variants (AM – arbuscular, AM + EM – arbuscular-ectomycorrhizal) and uninoculated control variants according to the Kruskal–Wallis tests ($p \leq 0.05$)

		BCF for leaves			BCF for roots			Ti leaves/roots		
salinity	variant	control	AM	AM + EM	control	AM	AM + EM	control	AM	AM + EM
LT	NS1	×	×	×	×	×	132.08 (32.59)	0.08 (0.01)	×	**0.40**↑ (0.02)
	S1	3.15 (0.35)	**0.23**↓ (0.06)	0.82 (0.33)	7.89 (0.50)	**3.60**↓ (0.32)	5.68 (0.15)	0.40 (0.02)	**0.06**↓ (0.01)	0.14 (0.05)
	S2	354 (1.68)	0.61 (0.33)	**0.24**↓ (0.08)	21.12 (10.64)	8.43 (0.98)	**2.90**↓ (0.60)	0.17 (0.02)	0.07 (0.04)	0.09 (0.04)
ST	NS2	×	×	0.07 (0.04)	×	×	0.67 (0.00)	0.08 (0.02)	0.54 (0.08)	0.10 (0.06)
	NS 10×	0.36 (0.07)	0.26 (0.15)	×	7.47 (2.47)	33.50 (9.01)	**4.58**↓ (0.23)	0.05 (0.01)	**0.01**↓ (0.00)	×
	NS2 20×	1.01 (0.10)	×	**0.07**↓ (0.05)	4.12 (0.31)	12.05 (3.56)	8.75 (4.40)	0.25 (0.01)	×	**0.01**↓ (0.00)

Values of error bars are given in parentheses; NS1 – control LT, nonsaline soil 1; S1 – experimental LT saline soil 1; S2—experimental LT saline soil 2; NS2 – control ST, nonsaline soil 2; NS2 10× - experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant; NS2 20× - experimental ST saline soil with a salinity 20 times higher that of the NS2 variant, (↑) – significant increase, or (↓) significant decrease between inoculated variants and the uninoculated control, × - no sodium ions in the analysed samples

rich in the mycorrhizal fungi belonging to *Tomentella* spp. and *Acephala* spp. While the least dominant fungal genera were found in the S1 soil, the S2 soil was the most diverse. In the S2 soil, among the dominant fungi *Oliveonia* spp. *Chaetosperum* spp., *Pleotrichocladium* spp. were found (Online Resource 2, Fig. 2).

Fungal community structure in roots and soils under both LT (permutational test of a dbRDA model (anova.cca), roots: $F_{2.25} = 2.18$, $p = 0.001$; soil: $F_{2.26} = 2.95$, $p = 0.002$) and ST salinity (permutational test of dbRDA model (anova.cca), roots: $F_{2.25} = 2.52$, $p = 0.001$; soil: $F_{2.26} = 6.78$, $p = 0.001$) differ significantly between the control, non-inolculated and inoculated variants (AM, AM + EM) (Online Resource 2, Fig. 3). Inoculation explained changes in the fungal community structure more in the rhizosphere than in the roots and more under ST salinity than under LT salinity both in the roots (LT: 9.26% < ST: 11.20%) and the soil (LT: 13.96% < ST: 32.03%).

The ITS sequencing data were divided into tables containing long-term (LT) and short-term salinity (ST) variants and then into two sources of isolation (roots and soil). The Shapiro–Wilk test of alpha diversity data showed they were not normally distributed, and Levene's test indicated deviation from homoscedasticity; therefore the Kruskal–Wallis test was used. Inoculation had no significant impact on alpha diversity indices (observed number of ASVs, Shannon's E and Shannon's H) in the majority of the analysed variants. Moreover, inoculation (AM or AM + EM) decreased fungal alpha diversity in roots in long-term saline soils S1 and S2 (Fig. 4d, g), whereas mixed inoculation (AM + EM) increased fungal alpha diversity in rhizosphere of the NS1, S1 and S2 variants (Fig. 5a-i). In contrast to the results for the LT saline treatments, the AM + EM inoculum increased the Shannon's E and H' indices for root samples of the ST control (NS2) (Fig. 6b-c), and both types of inocula significantly increased the number of observed ASVs for root samples in the case of the NS 10× variant (Fig. 6d-f). In the soil samples, regardless of the ST saline variants, inoculation increased all the diversity indices; expect for nonsaline soil NS2 (Fig. 7b-c).

The SSU amplicon analysis revealed the presence of five ASVs (Amplicon Sequence Variants) representing Glomeromycota fungi. Among them, two were identified as *Rhizophagus irregularis* (ASV2, ASV49), and three were identified as *Rhizophagus intraradices* (ASV1, ASV36, ASV47). The presence

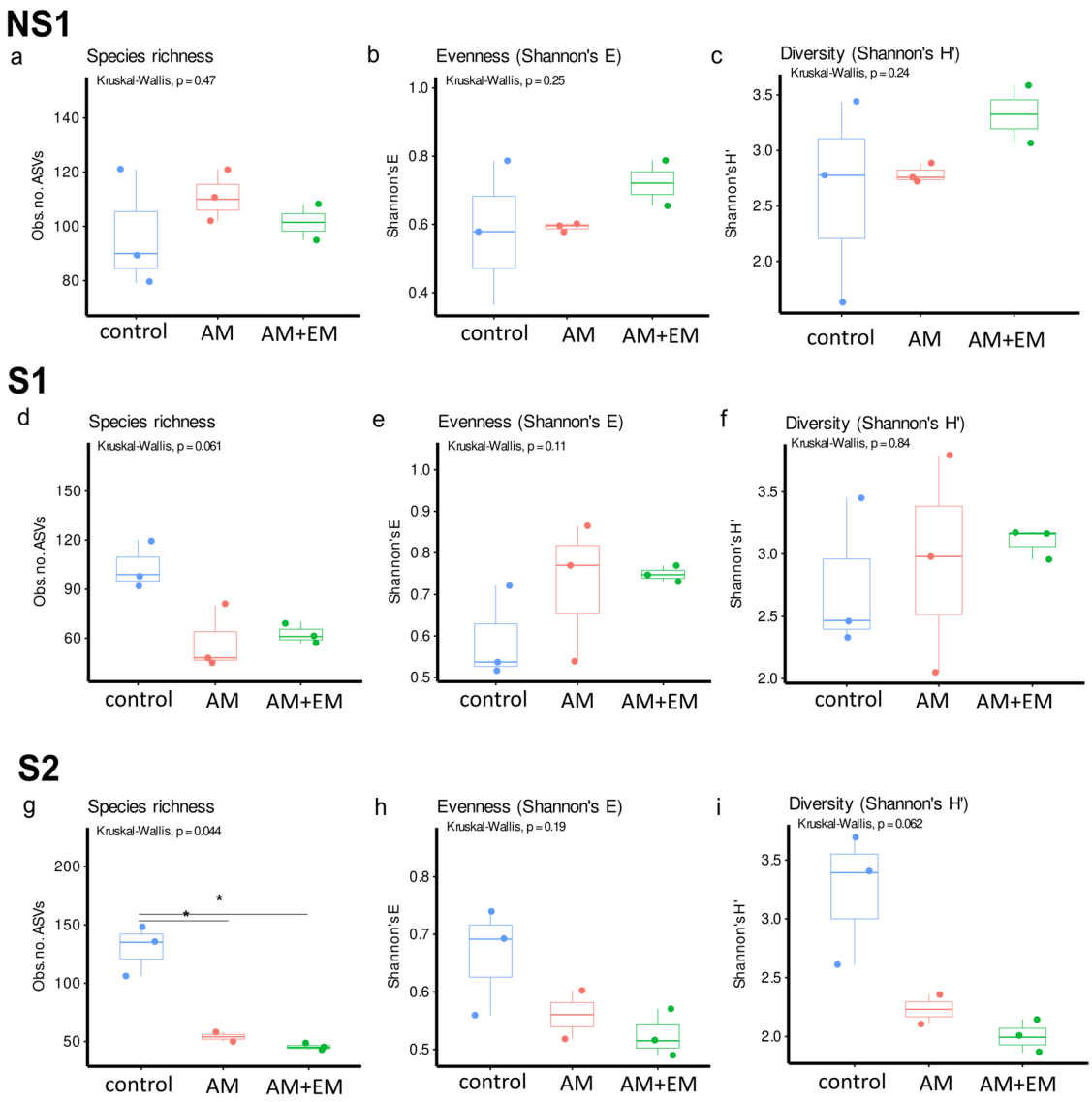


Fig. 4 Fungal alpha diversity in roots for long-term salinity variants presented as the number of ASVs (**a**, **d**, **g**), Shannon's E (**b**, **e**, **h**) and Shannon's H indices (**c**, **f**, **i**) with the results of Kruskal–Wallis tests between the inoculation variants (control – noninoculated, AM – arbuscular, AM + EM – arbuscular–ectomycorrhizal). Abbreviations: NS1 – control LT, nonsaline soil 1; S1 – experimental LT saline soil 1; S2 - experimental LT saline soil 2; * – $p \leq 0.05$

of *R. irregularis* DNA was confirmed in the NS1, S1 and NS2 soils in the pots of the control plants. The addition of AM inoculum caused the presence of *R. irregularis* in the roots of plants growing in ST saline variants (NS2, NS2 10×, NS2 20×) but not in LT saline variants (NS1, S1, S2). *R. irregularis* was confirmed in all the variants inoculated with the AM + EM inoculum in addition to the NS2 variant (Online Resource 2, Table 1).

Among the fungi that formed mix inoculum according to ITS sequence analysis, *Thelephora* spp. was the main genus found in the rhizosphere and alder roots. Few sequences of *Paxillus*, *Hebeloma* and *Amanita* spp. were recorded in the roots, whereas other fungi were not identified in analysed samples. The charts illustrating relative abundance are presented in the "rare" group because the sequences represented less than 2% of all identified fungal genera.

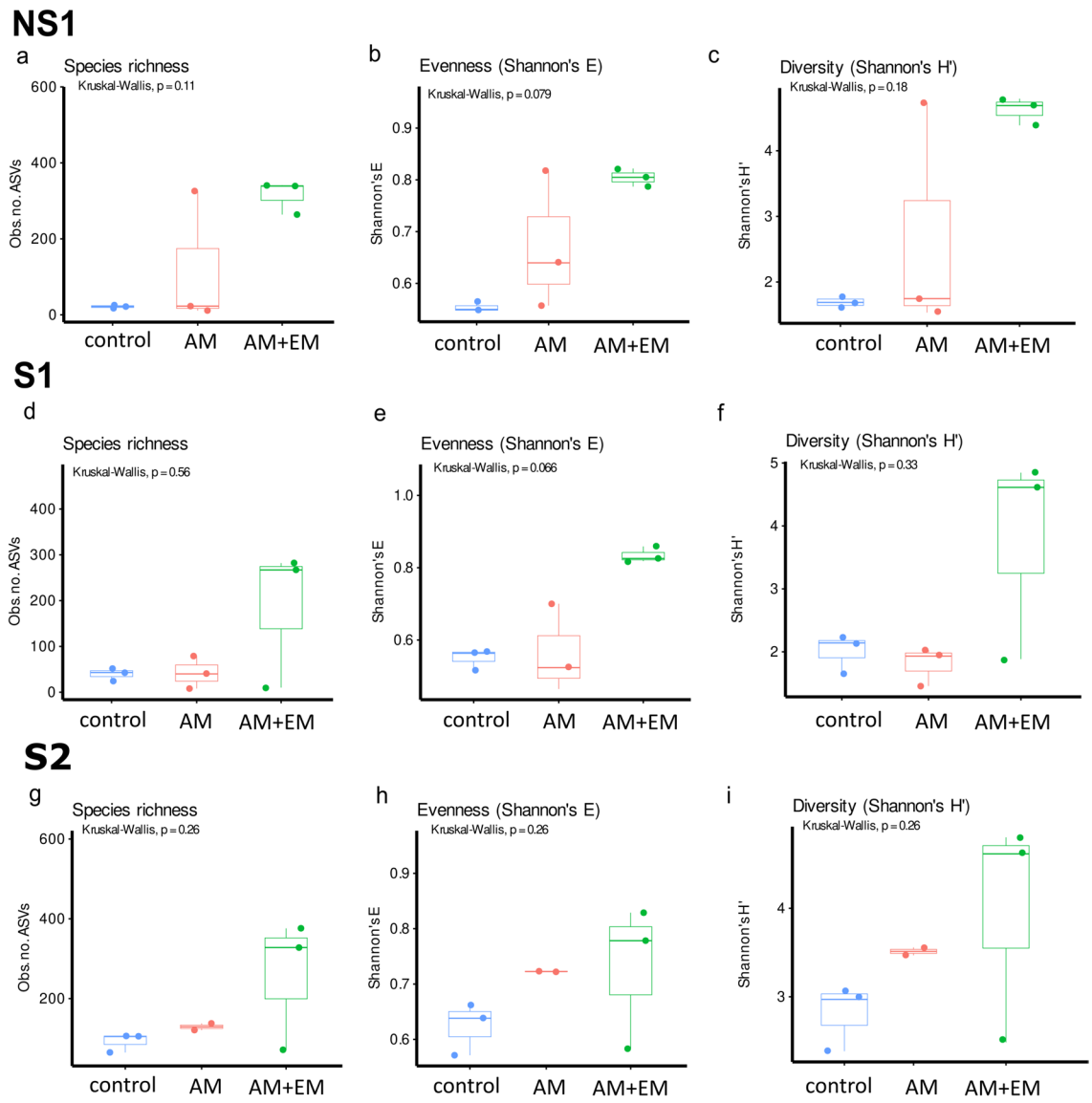


Fig. 5 Fungal alpha diversity in soil for long-term salinity variants presented as the number of ASVs (**a**, **d**, **g**), Shannon's E (**b**, **e**, **h**) and Shannon's H indices (**c**, **f**, **i**) with the results of Kruskal–Wallis tests between the inoculation variants (control – noninoculated, AM – arbuscular, AM + EM – arbuscular–ectomycorrhizal). Abbreviations: NS1 – control LT, nonsaline soil 1; S1 – experimental LT saline soil 1; S2 - experimental LT saline soil 2; * – $p \leq 0.05$

The relative abundance of *Thelephora* spp. in the roots was significantly lower in the AM inoculated treatment than in the control treatment in case of LT – NS1, S2 and ST – NS 20× (Figs. 8a and 9a). A similar effect was observed in the rhizosphere for the AM + EM inoculum but only in the case of the LT variant S1 (Fig. 8b). The opposite was observed in the soil of NS1 and S2 (LT), where the mixed inoculum significantly increased the relative abundance of *Thelephora* spp. (Fig. 8b). In all the ST variants, two types of inocula (AM or AM + EM) caused an increased incidence of *Thelephora* spp. in the rhizosphere compared with the control except for the AM inoculum in the NS2 10 × treatment (Fig. 9b).

The most significant differences in the relative abundance of the most numerous fungal genera

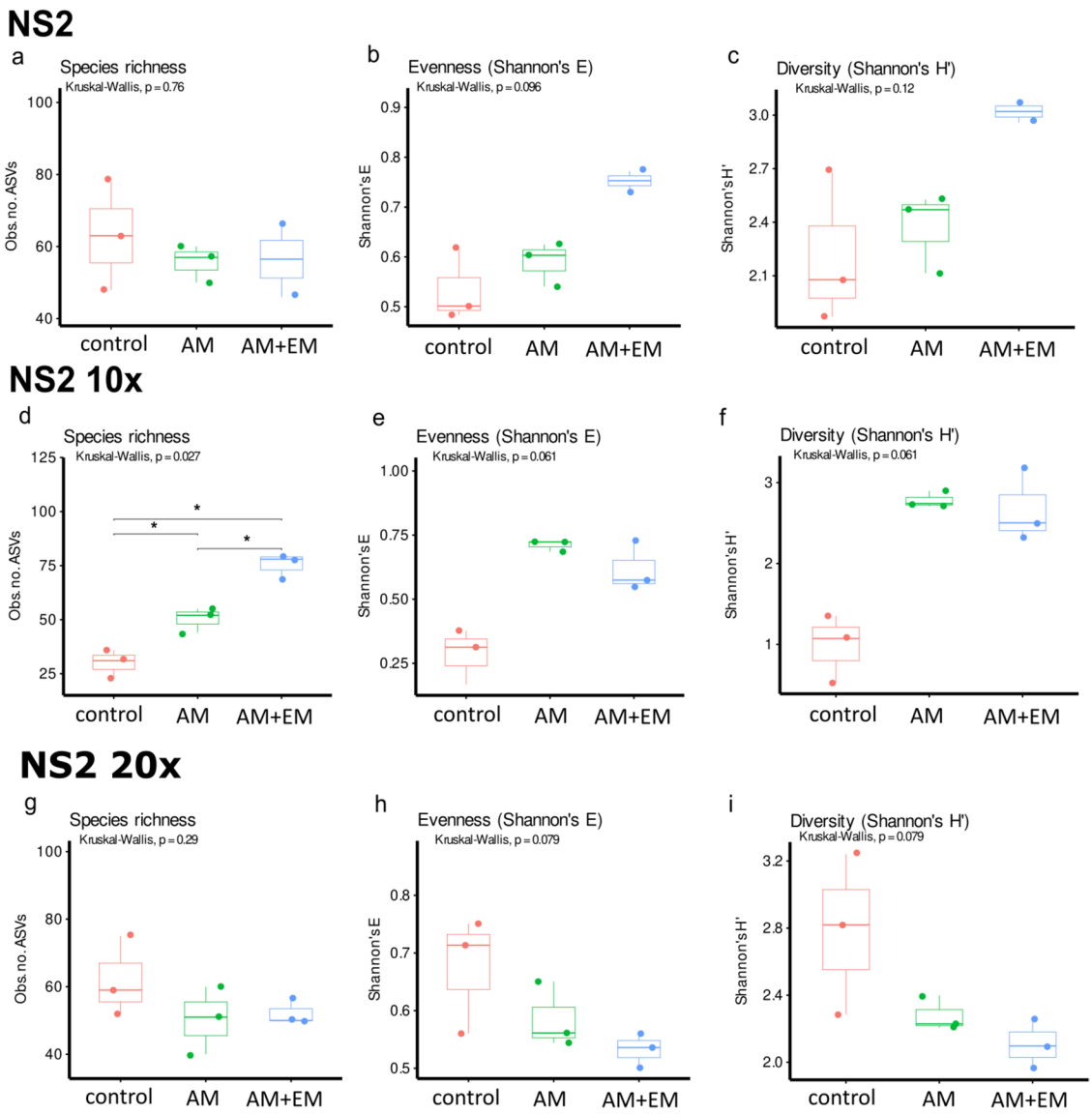


Fig. 6 Fungal alpha diversity in roots for short-term salinity variants presented as the number of ASVs (**a**, **d**, **g**), Shannon's E (**b**, **e**, **h**) and Shannon's H indices (**c**, **f**, **i**) with the results of Kruskal–Wallis tests between the inoculation variants (control – noninoculated, AM – arbuscular, AM + EM – arbuscular–ectomycorrhizal). Abbreviations: NS2 – control ST, nonsaline soil 2; NS2 10× - experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant; NS2 20× - experimental ST saline soil with a salinity 20 times higher than that of the NS2 variant; * – $p \leq 0.05$

were recorded in the variant inoculated with AM + EM in roots growing in NS1, S1 or S2 soils (LT). In this case, the increase or decrease in relative abundance affected 14 fungal taxa. In the remaining experimental groups, a comparable effect of both inocula on the relative abundance of the main genera of fungi was noted (4–9 taxa) (Fig. 8a).

In the LT salinity variants the use of both inoculates (AM or AM + EM) resulted in an increase in the occurrence of fungi belonging to the *Fusarium* and *Exidiopsis* genera (S1), and the use of AM inoculum increased the presence of *Tomentella* spp. in roots (S2). A significantly lower abundance of six fungal genera was observed in roots inoculated

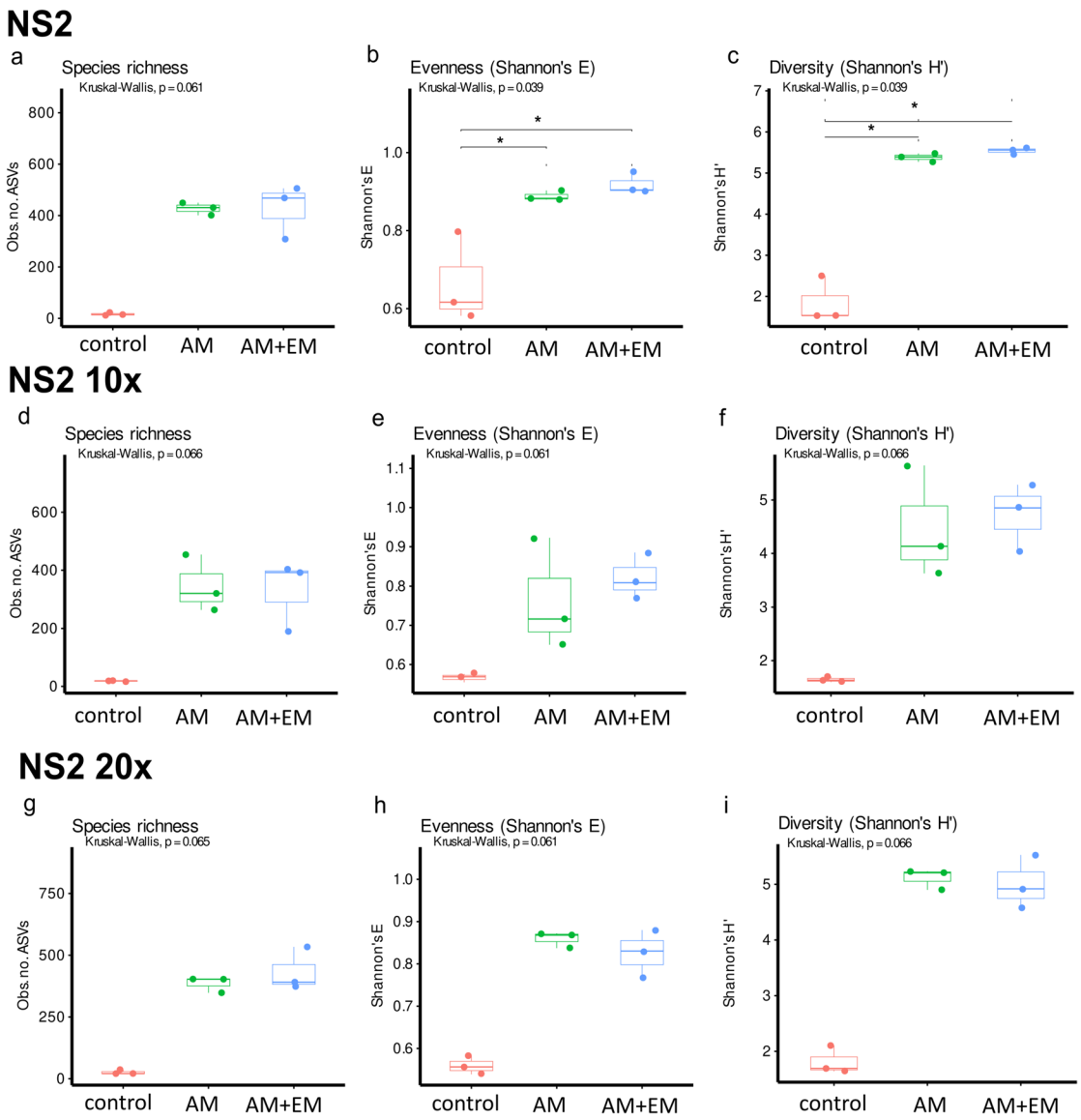


Fig. 7 Fungal alpha diversity in soil for short-term salinity variants presented as the number of ASVs (**a**, **d**, **g**), Shannon's E (**b**, **e**, **h**) and Shannon's H indices (**c**, **f**, **i**) with the results of Kruskal–Wallis tests between the inoculation variants (control – noninoculated, AM – arbuscular, AM + EM – arbuscular–ectomycorrhizal). Abbreviations: NS2 – control ST, nonsaline soil 2; NS2 10× - experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant; NS2 20× - experimental ST saline soil with a salinity 20 times higher than that of the NS2 variant; * – $p \leq 0.05$

with the double inoculum (AM + EM) and from the S1 variant, which may be related to the higher Na^+ concentration in this soil variant than in S2. Among them were *Exidiopsis*, *Paraphoma*, *Dactylonectria*, *Ilyonectria*, *Tetracladium*, and *Phialocephala* spp. (LT-S1). Moreover, in the S2 variant, with a lower concentration of Na and higher K concentration, the abundances of two genera, *Paraphoma* and *Tetracladium* spp. (LT-S2, AM + EM), were lower than those in the noninoculated variant (Fig. 8a). In the short-term salinity treatments, both inocula significantly increased the abundance of *Alternaria* spp. in the roots, but not in the same treatments: the AM inoculum in the NS2 and NS2 20× and the AM + EM inoculum in the NS2 10×. Additionally, compared with the control, the application of the mixed

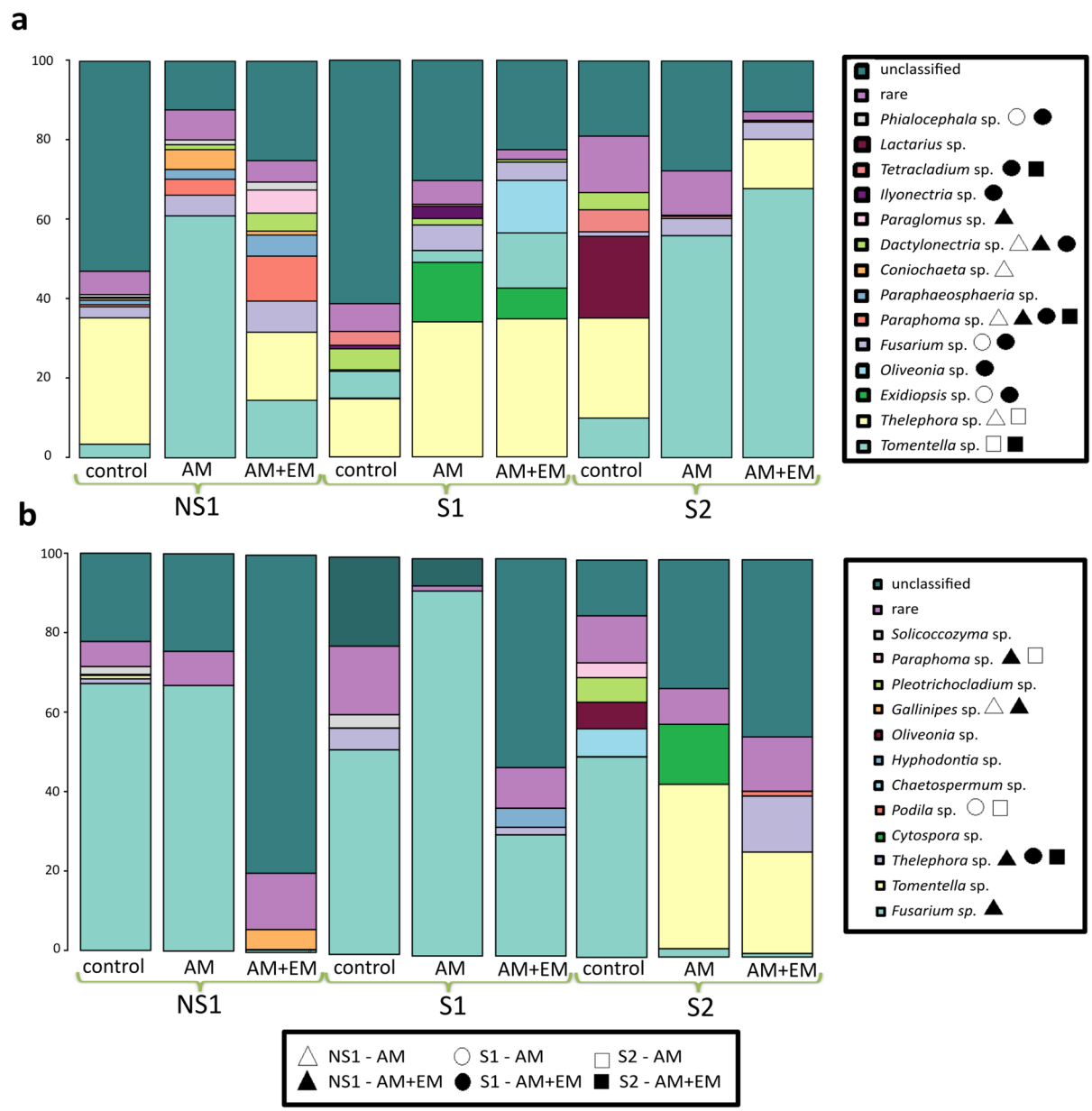


Fig. 8 Effects of mycorrhizal inoculation (control, AM – arbuscular, AM +EM – arbuscular-ectomycorrhizal) on fungal taxonomy structure at the genus level (**a**) in roots and (**b**) in the rhizosphere of *A. glutinosa* for long-term salinity treatment. "Rare" indicates taxa with less than 2% relative abundance. The shapes represents the results of DESeq2 tests, which were used to determine the significant differences in the relative abundance of fungal genera between inoculated variants (AM or AM +EM) and noninoculated variants (control) ($p_{adj} \leq 0.05$). Abbreviations: NS1 – control LT, nonsaline soil 1; S1 – experimental LT saline soil 1; S2 - experimental LT saline soil 2

inoculum increased the abundance of three other genera, namely, *Saccharomyces*, *Dactylonectria*, and *Leptodophora* spp., in the NS2 10× treatment (Fig. 9a).

In the LT saline soil, the use of AM inoculum caused a significant decrease in the relative abundance of *Podila* spp. (S1, S2) and increased the occurrence of *Paraphoma* spp. (S2). Other

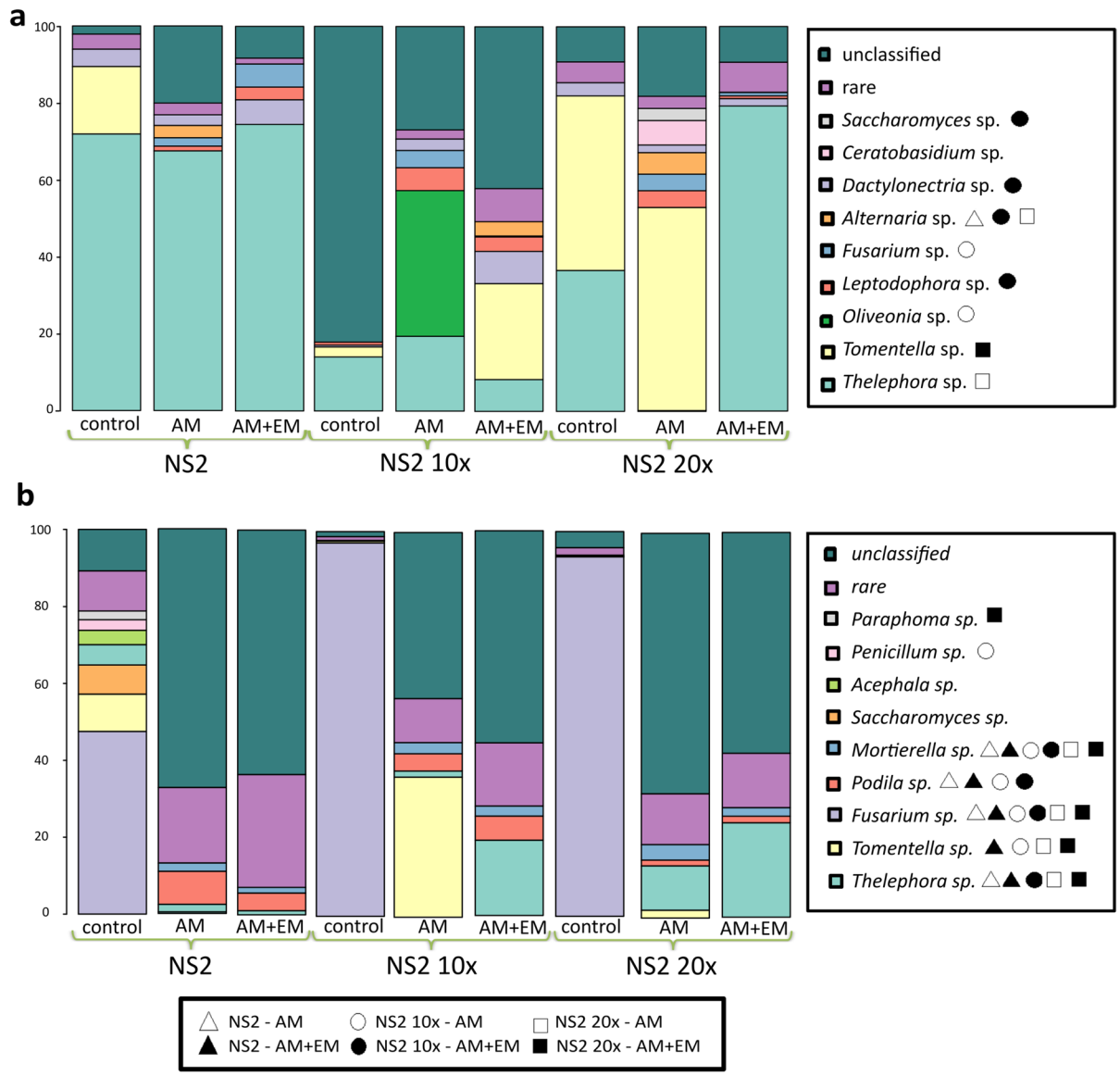


Fig. 9 Effects of mycorrhizal inoculation (control, AM – arbuscular, AM + EM – arbuscular-ectomycorrhizal) on fungal taxonomy structure at the genus level (**a**) in roots and (**b**) in the rhizosphere of *A. glutinosa* for short-term salinity treatments. "Rare" indicates taxa with less than 2% relative abundance. The shapes represents the results of DESeq2 tests, which were used to determine checking the significant differences in the relative abundance of fungal genera between inoculated variants (AM or AM + EM) and noninoculated variants (control) ($p_{adj} \leq 0.05$). Abbreviations: NS2 – control ST, nonsaline soil 2; NS2 10 × - experimental ST saline soil with a salinity 10 times higher than that of the NS2 variant; NS2 20 × - experimental ST saline soil with a salinity 20 times higher than that of the in NS2 variant

significant changes in relative abundance where detected at the nonsaline site 1 (NS1), where the mixed inoculum significantly increased the presence of *Paraphoma* spp. and decreased the occurrence of *Fusarium* spp. However, both inocula significantly increased the presence of *Gallinipes* spp. (Fig. 8b). Independent of the inoculation variants, the relative abundance of *Mortierella* spp. significantly increased, whereas that of *Fusarium* spp. significantly decreased in the NS2 soils. Both inocula caused a significant increase in the presence of *Podila* spp. in the NS 10× and at the same time,

the mixed inoculum increased occurrence of *Paraphoma* spp. in the NS2 20× (Fig. 9b).

Discussion

The duration of exposure to salinity changes the effectiveness of mycorrhizal inoculum in improving alder growth

In relation to the first hypothesis, the present study confirmed that the growth of black alder seedlings can be improved by both AM and AM + EM inocula. It can be the consequence of dual mycorrhizal status of *Alnus* that inoculated with both AM fungi and EM fungi may benefit from a synergistic effect under stressful conditions. Our observation can be supported by a review by Teste et al. (2020) suggesting that *Acacia*, *Eucalyptus*, *Fraxinus* and *Pinus* trees typically respond positively to single and dual inoculation with both AM and EM fungi. The mentioned synergistic effect have been also confirmed for *Alnus* trees inoculated simultaneously with *Frankia* bacteria and one type of mycorrhizal fungi (AM or EM), but not for dual mycorrhiza. In example, Chatarpaul et al. (1989) found that *Alnus incana* produced more biomass when inoculated with a combination of one AM fungus, one EM fungus, and *Frankia*, rather than *Frankia* alone.

Although we initially assumed that each inoculum would perform better under different types of salinity, both inocula improved plant growth under ST and LT salinity, and the effect depended on the soil treatment. One possible explanation for this is that salinity may reduce the effectiveness of mycorrhizal inocula with increasing salt exposure time. For example, a meta-analysis of experimental data by Dastogeer et al. (2020) revealed that AM fungi tend to be more effective under longer-term salt stress (more than 4 weeks), while their effects are often not apparent under short-term stress (less than 2 weeks), which is consistent with our findings regarding the dual inoculum. On the other hand, the influence of sodium toxicity on EM fungi appears to be an important aspect to consider when selecting inocula, as many EM species are described as salt-sensitive. We suggest that the salt sensitivity of some EM fungi forming the dual inoculum may be the reason for the lack of growth improvement observed in the LT-S1 variant, where salinity resulted from a relatively high sodium concentration close to 1270 µg/g. This result could be caused by the inhibition of ectomycorrhizal hyphal growth, which in turn diminished their ability to mobilize nutrients for the plant partner (Branco et al. 2022; Tang et al. 2009). Additionally, we suggest that the observed effect may be due to the competition for nutrients between AM and EM fungi, which ultimately reduced the individual performance of the mixed inoculum in the context of higher concentrations of toxic sodium ions and associated adverse environmental conditions.

Mycorrhizal inocula can regulate the water content in alder leaves under salinity

Our pot experiment revealed that plants growing in LT saline soil and inoculated with AM fungus presented a relatively high water content in their leaves (S1) or shoots (S2). The higher water content of AM-inoculated plants could be explained by an improved hydraulic conductivity attributed to mycorrhizae induced altered root morphology, as well as their ability to explore soil well beyond depletion due to external hyphae, which can help the host plant absorb more water from the soil (Navarro et al. 2024). The higher water content may help explain the higher plant biomass found in the mentioned variants, compared to the corresponding noninoculated-AM variant. To date, similar to our observation the positive effects of AM fungal inoculation on the relative water content or water use efficiency have been described by other researchers, who have tested the effects of AM fungal inoculation on different tree plants under drought (*e.g.*, Aganchich et al. 2022; Birhane et al. 2012) and saline stress (*e.g.*, Scambato et al. 2010; Navarro et al. 2024). In both experiments conducted, AM + EM inoculation also increased water content in leaves and shoots, but such an effect was noted only in the non-saline variants. However, to the best of our knowledge there is no literature confirming positive effect of dual mycorrhizal inoculation on water content in tree plants. The opposite phenomenon to that described for AM + EM inoculation in non-saline treatments was observed under long-term (S1) and short-term salinity (NS2 10×, NS2 20×) and was associated with a significant decrease in the shoot water content. This effect may be due to the incorporation of protective mechanism that limits long-distance transport from

roots to shoots which may be observed in glycophytes under salt stress conditions. This mechanism involves limiting salt water uptake by regulating the osmotic potential, which involves the production and accumulation of soluble compounds (Horie et al. 2012).

Accumulation of proline in *Alnus* leaves as a response to mycorrhizal inoculation

In leaves of plants inoculated with AM (ST, LT) or AM + EM (ST), the higher level of proline compared to that of noninoculated controls was corresponded with the positive effect of plant inoculation on plant growth. A similar effect in the case of AM-inoculated plants was noted in the study by Dastogeer et al. (2020), where the accumulation of proline was observed when the plants inoculated with AM fungi experienced moderate salinity stress. Similarly, Zarei and Paymaneh (2014) reported that *Glomous intraradices*-colonized and *G. etunicatum*-colonized seedlings presented significantly higher proline contents than noninoculated control at salinities of 4, 6 and 8 dS m^{-1}. Under saline conditions, increased levels of proline in leaves of *Alnus* seedlings inoculated with ectomycorrhizal fungus *P. involutus* compared to noninoculated ones was observed also in our earlier studies (Thiem et al. 2020), which is in the line with present founding about AM + EM inoculum which contained *P. involutus*. The fact that the double inoculum increased the amount of proline only in the ST variants may be related to the reduced water content of the shoots recorded in these treatments (NS2 10×, NS2 20×). As we mentioned earlier, glycophytes can recompense for the reduced amount of water in the plant by accumulating soluble compounds in the cells.

Despite higher salt levels in the ST variants, a greater effect of proline accumulation was observed under LT than under ST treatments. It is possible that the longer exposure time of LT plants (since the beginning of the experiment) compared to ST plants (since spring, when the salt was introduced) made possible to achieve the greater levels of proline in the leaves. Our observation is in the line with El-Danasoury et al. (2010) which described that the accumulation of free proline in the spearmint tissue (*Mentha spicata*) continued to increase over time at 200 mM NaCl concentration. The authors explained this by the inhibition proline dehydrogenase activity.

In our experiments, mycorrhization caused an increase in proline levels also under control conditions, and during exposure to short salinity the effect was greater for ST control treatment (NS2) than for saline variants. In our previous studies, we also observed significantly higher levels of proline in the leaves of alder seedlings inoculated with ectomycorrhizal fungi (*A. muscaria* and *P. inovlutus*) compared to noninoculated plants (Thiem et al. 2020). Overproduction of proline may be a response of the host plant to interaction with mycorrhizal fungi, which can regulate water relations in the plant, *e.g.* by inducing different degrees of osmotic regulation.

AM inoculum limits or reduces lipid peroxidation in *Alnus* leaves under salinity

Other observations regarding the mechanisms of improving the growth of alder seedlings under saline conditions can be drawn by analysing the results of the MDA levels. In this case, AM inoculation seems to have better efficiency regardless of the type of salinity because it generally did not cause a significant increase in the MDA level or even decreased the MDA content in the saltiest variant of ST compared to noninoculated controls. Likewise, Dastogeer et al. (2020) found that AMF-inoculated plants presented significantly lower MDA levels than noninoculated plants did as salinity stress increased. A similar to our studies, the decreasing trend in lipid peroxidation was reported by Duc et al. (2023) under drought stress in tomato plants inoculated with different AM fungi (*R. irregularis*, *Funneliformis verruculosum*, *Septoglomus deserticola*, and *S. constrictum*), and by Labidi et al. (2011), who described the significant involvement of AMF in the protection of chicory plants against carbonate stress. The explanation for the significantly higher MDA concentration in AM + EM-inoculated plants may be the less effective metabolism of antioxidant enzymes than in the case of plants inoculated with the AM fungus alone. Simultaneously, the interaction between mycorrhizal fungi and plants can increase reactive oxygen species (ROS) production under certain stress conditions, which may lead to increased lipid peroxidation. A similar observation was described by Alvarez et al. (2009) for *Nothofagus dombeyi* (Mirb.) Oerst. plants inoculated with the EM fungus *Descolea antartica* (Sing.), in which the MDA concentration was significantly

higher under drought stress conditions than under optimal supplementation with water. However, it is not understood why, in our experiment, such an effect of the AM + EM inoculum was also observed under control conditions as well as in the case of AM inoculum in control NS2 soil.

The effectiveness of inocula in decreasing BCFs and Ti depends on type of salinity

In our study, the beneficial effect of mycorrhizal fungi on alder growth under salt stress conditions may be related to the participation of these symbionts in sodium ion transport and/or in preventing or inhibiting ion transport from the soil, since in our experiments BCFs and Ti calculated for sodium ions were significantly lower in mycorrhizal plants than for noninoculated plants under both salinity conditions. Specifically, AM inoculum significantly decreased the BCF and translocation indices mainly in the saline LT variants, whereas AM + EM did the same in the ST saline variants.

Similar to our experiment, also Estrada et al. (2013) reported lower sodium ion levels in AM-inoculated maize plants (*Claroideoglomus etunicatum*) compared to uninoculated plants under salinity equal to 100 mM NaCl. In that case, the applied inoculate reduced the uptake of Na^+ by shoot tissue, which is the same as our results on reduced sodium bioconcentration factors in alder roots and leaves. Also studies of Han et al. (2024) described that AM inoculation resulted in an average Na^+ decrease by 18% in the roots of poplar trees, which is in the line with our results about the reduced bioconcetration factors for roots in AM-inoculated plants under LT salinity conditions. The significant reduction of sodium translocation between roots and leaves observed in inoculated-AM plants under LT salinity can be explained by the involvement of the AM symbiosis in accelerating Na^+ shoot-to-root translocation. Wang et al. (2021) described similar phenomenon induced by arbuscular fungus *Funneliformis mosseae* in maize plants, in which AM symbiont mediating changes in the Na^+/K^+ distribution between shoots and roots. AM inoculation caused an increase in the Na^+/K^+ ratio in roots and a decrease in shoots, which resulted in maintaining the structural and functional integrity of chloroplasts in mesophyll cells.

Although there is a lack of studies on the effect of double inoculate on sodium ion concentrations in plants under salt stress conditions, it seems that also EM fungi can help to reduce the Na^+ concentration in inoculated plants, in which increased activity of the membrane antiporter Na^+/H^+ was detected by Li et al. (2012). In this study, EM inoculation reduced the influx of sodium ions into the poplar roots both after short- (24 h) and long-term (7 days) exposure to salinity, which is consistent with our observation about the reduction of BCF for leaves and roots after AM + EM inoculation under short-term salinity conditions.

Fungal communities response to introduce mycorrhizae under saline conditions

Although our study demonstrated positive effects of both types of mycorrhizal inocula (AM and AM + EM) on the growth and salt stress tolerance of *A. glutinosa*, among the taxa of fungi forming inoculates, fungi belonging to the genus *Thelephora* dominated in the roots and soil whereas AM fungus was not found in both root and rhizosphere under LT salinity conditions. For this reason, our hypothesis that mycorrhizal fungi are more common in the roots and/or rhizosphere of inoculated plants should be rejected. One reason may be the timing of the introduction of mycorrhizal inoculants and the completion date of the pot experiment. It is possible that the mycorrhizal inoculate has served its purpose and that only those fungi that pair well with the alder host have become a permanent part of the fungal community. However, depending on the soil and plant context the extent to which the introduced mycorrhizal fungi are established remains unknown (Lutz et al. 2023; Salomon et al. 2022). For the other hand, arbuscular fungi can affect the structure and diversity of microbial communities by affecting the type and amount of plant root exudates and in this way stimulating other soil microbes (Hu et al. 2024). For this reason, despite the fact that AM fungus itself did not penetrate from the substrate into the studied rhizosphere and alder root, it can be speculated that AM inoculum could activate other biological factors with positive effects on plant growth, such as plant growth-promoting bacteria in the LT soils. However, confirmation of this assumption

would require further microbiological qualitative testing of LT soils.

Moreover, in our studies permutation test of dbRDA models clearly revealed that inoculation significantly influenced the fungal community structure in the rhizosphere and roots of black alder. It explained the greater fraction of variance in the short-term salinity treatment than in the long-term, especially in the case of rhizosphere. These results may be explained by a lower adaptation of the microbiome under ST salinity than that of the LT microbiome, which was previously exposed to salinity. Our observations regarding changes in the fungal alpha diversity of inoculated plants showed that, in line with our assumption, the diversity of fungi in the roots and rhizosphere did not change significantly in most of the analysed variants. A similar effect was observed in studies by Yang et al. (2024), in which *R. intraradices* had no significant effects on the richness or abundance of the bacterial community in both their rhizosphere soil or the root endosphere. However, a more detailed analysis of our results revealed that in the short-term experiment (NS2, NS 10×), mycorrhizal inoculation significantly increased the diversity of fungi in the roots and in the rhizosphere. Since significant improvements in root growth and water content in organs were also observed in these variants, we suggest that in this case the increase in diversity may have contributed to the improved growth of alder plants. In the case of long-term salinity (LT), mycorrhization increases the diversity of fungi in the rhizosphere while reducing it in the roots, *e.g.*, in variant S2, where the positive effect on alder growth involved only the leaves. Similarly, the use of *R. intraradices* to inoculate soybean plants reduced the values of the Chao1 and Shannon indices in the root endosphere compared with those in the rhizosphere soil (Yang et al. 2024). Increased diversity may implicate that a larger pool of fungi with diverse array of plant growth-promoting traits is available fort the host plant, and chances of finding an organism, which fits requirements imposed by the environment are higher.

In addition to the observed changes in alpha diversity, we observed significant changes in the abundance of different fungal genera after plant mycorrhization. Under short-term salinity, the use of mycorrhizal inocula generally did not change the amount of *Thelephora* spp. in the roots but increased it in the rhizosphere, which may have a beneficial effect on alder growth under stress conditions because improved growth and water content were noted in these plants. The previously described increase in the overall alpha fungal diversity as well as the increase in the relative abundance of *Thelephora* spp. in the *Alnus* rhizosphere may be the reason for the better alder growth under ST salinity conditions. The hypothesis that *Thelephora* spp. may play a significant role in improving seedling growth also under long-term salinity conditions may be evidenced by the reduced relative abundance of *Thelephora* spp. in the LT variants where at the same time no significant improvement in seedling growth was noted. However, this lack of growth improvement may also be related to the generally unfavorable disturbance of the fungal community structure in this experimental variant, as a decrease in the abundance of five other fungal genera was noted.

The lack of stimulation of tree growth by mycorrhizal fungi introduced in the form of inoculum may also be related to the overall unfavorable disruption of the fungal community structure in this soil variant, as a decrease in the abundance of five other fungal genera was recorded in this variant. Such an adverse decrease in abundance was also noted in other experimental variants exposed to long-term salinity, where mycorrhization did not improve plant growth. This concerned, for example, fungi of the genera *Paraphoma* spp. and *Tetracladium* spp. (roots, LT – S2, AM + EM) or *Podila* spp. (soil, LT – S2, AM). The opposite trend was observed in the case of plants exposed to short-term salinity, where many increases in the abundance of various fungal genera were noted in the endosphere, *i.e*. *Alternaria* spp., *Saccharomyces* spp., *Dactylonectria* spp., *Leptodophora* spp. and in the rhizosphere *Podila* spp. and *Paraphoma* spp.

Conclusions and outlooks

The most beneficial effect was observed with inoculation using the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, particularly under conditions of sudden soil salinization. The selection of fungal inoculants should be guided by both the physicochemical and microbiological characteristics

of the soil. The mechanisms by which inoculants enhance plant growth vary depending on the salinity exposure history of the fungal microbiome. The protective mechanism likely involves a reduction in transpiration, which limits sodium uptake along with saline water. Simultaneously, osmotic adjustment is enhanced through the increased production of osmoprotectants as a response to mycorrhizal inoculation. Even small changes in the abundance of specific fungal genera can lead to substantial physiological changes in the host plant. The less favorable outcomes observed with dual inoculation may be attributed to competition between arbuscular (AM) and ectomycorrhizal (EM) fungi, EM-induced alterations in the plant microbiome and physiology, and a subsequent reduction in plant responsiveness to AM fungi.

Further research is needed to elucidate the complex mechanisms underlying plant–fungus interactions, particularly at the molecular level. In this context, transcriptomic approaches hold great promise. Moreover, the hypothesis that arbuscular mycorrhizal fungi confer protection by reducing transpiration requires direct testing through precise physiological measurements.

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Author contributions D.T. and K.H. conceived and conceptualized the work. K.H. supervised experiment and manuscript preparation. D.T. obtained alder seedlings, established the pot experiment, collected plant material and performed all laboratory analyses. M.G performed bioinformatic analysis. D.T. and M.G. performed the statistical analyses. J.T. supervised the measurement of biochemical parameters and soil salinization. T.K. supervised the measurement of sodium ions in the plant material. C.B. performed the soil analysis. D.T., K.H., M.G., C.B. and J.T. critically revised the article.

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Data availability ITS and SSU sequences were deposited in NCBI's SRA under BioProject accession number PRJNA1120626.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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