



Functional Analysis of *Lolium perenne* L. in Response to Fungal Endophyte Inoculation and Salinity Stress

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Abstract

Lolium perenne L., is a widely cultivated cool-season grass, is an important turf and forage species worldwide, with moderate salt tolerance. However, soil salinity resulting from poor water management, inadequate drainage of fields and high temperatures significantly impair its yield. This study explores effects of two endophytic fungi on enhancing the growth of non-host *L. perenne* under salt stress. Two salt-tolerant, plant growth-promoting fungal endophytes, *Stereum gausapatum* (ENDO_1) and *Parasarocladium gamsii* (ENDO_2), were inoculated in *L. perenne* under salt stress conditions (0 mM, 150 mM, and 300 mM NaCl). The plant growth performance, physiological responses, and transcriptomic changes were analyzed in two ryegrass varieties, Indiana and Greensky, to assess the endophytes' effects on plant resilience and adaptation to salinity stress. The two fungi significantly increased growth and biomass with beneficial physiological effects in *L. perenne*: ENDO_1 reduced lipid peroxidation and increased total antioxidants, while ENDO_2 increased proline accumulation. Weighted gene correlation network analysis revealed that stress-responsive pathways were differentially regulated in plant organs (leaves and roots) and after fungal inoculation in both varieties. The representative processes included amino acid metabolism, cell wall macromolecule metabolism or biogenesis, organic acids and compatible solutes, plant stress-related, generation of precursor metabolites and energy, transport, and plant nutrition. Salinity-induced growth reductions were ameliorated by fungi leading to greater biomass and increased plant growth compared to non-inoculated plants. Salinity stress-related pathways namely lipid metabolism, carboxylic acid biosynthesis, alpha amino acid catabolism were regulated by endophytes, which can be potential biomarkers used for bioinoculant selection.

Keywords Salt stress · Grass · Plant growth-promoting · Transcriptome · Molecular function · Biological process

Introduction

Lolium perenne L. (common name: perennial ryegrass, family *Poaceae*), is an important cool-season turf and forage grass that is native to southern Europe, North Africa, the Middle East, and eastern Asia (Matzrafi et al. 2021). Among its commercial cultivars, perennial ryegrass has moderate salinity tolerance, very low drought tolerance and high cold tolerance (Tang et al. 2013a, b). The germination and growth of *L. perenne* are challenged by high salinity and poor water management for irrigation, thus drastically affecting yields (Tang et al. 2013a; Uddin and Juraimi 2013). Salt stress impairs various physiological and biochemical processes in plants by inducing osmotic stress, increasing ionic toxicity, and triggering a combination of osmotic stress-related effects, nutrient deficiency, water-deficit, leaf chlorosis, inhibition of metabolic activities and changes in gene expression (Parida and Das 2005; Hanin et al. 2016).

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Endophytic microbes colonizing living plants are often beneficial to their host plant, particularly under unfavorable biotic and abiotic conditions (Rho et al. 2018). *Lolium perenne* is naturally colonized by the clavicipitaceous asexual endophyte *Epichloë* spp. (formerly *Neotyphodium* spp.), which are transmitted to succeeding host generations via vertical transmission to seeds (Leuchtmann et al. 2014). Many studies have shown that *Epichloë*-infected grasses deter grazing herbivores, enhance defense against phytophagous insects and pathogens and produce bioprotective alkaloids (Schardl et al. 2013; Chen et al. 2020). However, it is reported that *Epichloë* spp. has no role in salinity tolerance in perennial ryegrass (Koch et al. 2017; Pereira et al. 2021; Kalske et al. 2022).

Thus, our study aimed to examine the effects of two halotolerant endophytic fungi on the growth of nonhost grass *L. perenne* under salt stress conditions. We hypothesize that inoculation with two halotolerant and plant growth-promoting endophytic fungal strains (*Stereum gausapatum* ISK3-11 and *Parasarocladium gamsii* CR1-9) would establish in a new host plant and may be beneficial in mitigating salinity stress. In our previous study, the two fungal strains isolated from *Salicornia europaea* L. were screened for their plant growth-promoting properties (Furtado et al. 2019b). *Stereum gausapatum* ISK3-11 showed higher protease, cellulase, and lipase enzyme activity than *P. gamsii* CR1-9, which displayed higher chitinase activity. Both fungal strains produced siderophores, indole acetic acid (IAA), and polyamines. Based on the above criteria, a pilot pot experiment was conducted with the two fungal strains in *L. perenne*, using the same two grass varieties as in the present study, under no salt stress conditions. The pilot pot experiment showed that the strains were compatible with the new host plant and promoted *L. perenne* growth (Furtado et al. 2019b). The present study uses the same two *L. perenne* varieties Indiana and Greensky, to examine how colonization by these nonhost endophytes impacts plant growth performance and physiological responses under salinity stress. To further elucidate the molecular basis of plant–endophyte interactions, we conducted a transcriptome analysis to identify gene expression changes and key pathways regulated by these fungal endophytes that may enhance plant salt tolerance. Identifying genes that are positively or negatively regulated by each endophyte offers insights into their functional roles and host-specific interactions, providing essential information for selecting effective bioinoculants. Furthermore, the results from this study could support the use of an endophytic route in mitigating crop stress by revealing not only the underlying molecular basis of plant–endophyte interactions but also how artificially introduced fungi colonize the new host *L. perenne*.

Materials and Methods

Fungal strain selection and inoculum preparation

The two fungal endophytes *Stereum gausapatum* ISK3-11 (**ENDO_1**) (NCBI acc. no. MK460970) and *Parasarocladium gamsii* CR1-9 (**ENDO_2**) (NCBI acc. no. MK460909), were previously isolated from the root of *S. europaea* growing at two salt-affected sites in Poland (Furtado et al. 2019b). For fungal inoculum preparation, mycelium disks (5 mm in diameter) were cut from fresh potato dextrose agar and placed in 100 mL of malt extract broth (BD Difco, USA). The flasks were incubated at 28 °C on an orbital shaker (rotating speed 150 rpm) for 7 days. After cultivation, mycelia were harvested by centrifugation at 5000 g at 20 °C for 15 min. Mycelia were subsequently washed twice with sterile distilled water and dispersed in 100 mL of sterile distilled water using sterile glass beads.

Plant Materials

Seeds of two *L. perenne* varieties were provided by DLF Seeds A/S, Denmark. The two grass plant varieties were the forage grass Indiana (**Variety 1**), and the turfgrass Greensky (**Variety 2**).

Experimental Design and Treatments

The pot experiment included a factorial arrangement of three salt concentrations (0 mM, 150 mM and 300 mM NaCl), two plant varieties (Variety 1 and Variety 2) and three inoculation treatments: control (no fungal inoculation), ENDO_1 and ENDO_2; 18 treatment combinations in total. Plants were sown in pots (6×6 cm) filled with sterile sand:vermiculite (1:1) mixture and placed in rectangular collection trays (Supplementary information B Fig. S1). After 1 week of germination, the seedlings were inoculated with 200 µl of freshly prepared fungal suspensions (ENDO_1 and ENDO_2) (see inoculum preparation above) and 200 µl of distilled water was added to the control plants. Salinity treatment was applied 9 days after seed germination and following fungal inoculation. The plants were irrigated with their corresponding salt (NaCl) solution, and the electrical conductivity (EC_e) of the substrate was analyzed until the desired salt concentration was attained. The collection trays placed under the pots ensured that the salt solution did not drain off. For each variant of the experiment, 54 plant replicates were prepared and analyzed (2 plant varieties×3 salt concentrations×3 variants of inoculation with endophytic fungi×54 replicates—a total of 972 plants). The plants were cultivated under aseptic conditions in a plant growth

chamber at a constant temperature of 24 °C under white light (450 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and a variable photoperiod of 12/12 h (day/night). Sterile Hoagland's solution was added to the pots throughout the experiment for 60 days (Hoagland and Arnon 1950).

Plant Growth and Physiological Parameters

After 60 days, the plants were harvested, washed in sterile distilled water to remove substrate particles and dried using sterile blotting paper. Three plant replicates per endophyte inoculation and salt concentrations were immediately quick-frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ for transcriptome analysis of leaves and roots. The remaining plant samples were measured for plant growth and physiological traits as follows:

- (a) *Plant length and biomass*: plants were assessed for the length and fresh weight of leaves and roots (six biological replicates per treatment). Furthermore, the samples were oven-dried at $60\text{ }^{\circ}\text{C}$ for 24 h, and their dry weights were measured. Relative leaf water content (RWC) was determined according to Barrs and Weatherley (1962) (six biological replicates per treatment).

$$\text{RWC (\%)} = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$$

- (b) *Chlorophyll estimation*: chlorophyll was estimated using 100 mg leaf sample, which was homogenized in liquid nitrogen. The chlorophyll was extracted and quantified according to Witham et al. (1971). The samples were extracted with 2 mL of 80% acetone. The extraction was repeated until the residue was colorless. The absorbance of the extract (whole extracted volume) was read at 645 and 663 nm against 80% acetone blank. Six biological replicates per treatment were analyzed. The amount of total chlorophyll in the sample was calculated using the formula:

$$\text{Total chlorophyll } (\mu\text{g}) = 20.2 \times (A_{645}) + 8.02 \times (A_{663}) \times (V / (1000 \times W))$$

where, V —final volume of the extract and W —weight of the leaves.

- (c) *Lipid peroxidation*: The extraction of lipid-soluble components from 100 mg leaf and root samples was performed using 90% MeOH and 0.1% butylated hydroxytoluene (BHT). The extraction of lipid soluble components from 100 mg of leaf and root tissue was performed using 90% MeOH and 0.1% butylated hydroxytoluene (BHT). The lipid peroxides in extracts were measured using PeroxiDetect kit following the manufacturer's protocol (Sigma–Aldrich Catalog Num-

ber PD1). The nmole of peroxide/mL was calculated from a standard curve with 200 μM tert-BuOOH as a standard. The absorbance of each sample at 560 nm was determined on a UV–visible spectrophotometer system. Three biological and three technical replicates for each sample per treatment were analyzed.

- (d) *Proline concentration*: proline was measured according to the procedure of Abrahám et al. (2010). 100 mg of frozen leaf and root samples were homogenized separately in liquid nitrogen and extracted with 3% sulfosalicylic acid (500 μL /100 mg fresh weight). The homogenate was centrifuged at 12,000 rpm for 10 min, and the supernatant was collected in a fresh tube. 100 μL of the extract was mixed with 100 μL of 3% sulfosalicylic acid, 200 μL glacial acetic acid, and 200 μL acidic ninhydrin. The mixture was incubated at $96\text{ }^{\circ}\text{C}$ for 60 min, terminated on the ice and extracted with 1 mL toluene. The absorbance of chromophore was measured at 520 nm using toluene as a reference. Three biological and three technical replicates for each sample per treatment were analyzed. Proline concentration in the sample was estimated by referring to a standard curve made from known concentrations of proline.
- (e) *Total antioxidant capacity*: the antioxidants in plant tis-

sue were quantified according to the Antioxidant assay SIGMA® kits protocols CS0790. 100 mg of leaf and root tissue was homogenized separately with liquid nitrogen and cold Assay Buffer (~ 100 mg of tissue/0.5 mL of buffer) and then centrifuged at 12,000g for 15 min at $4\text{ }^{\circ}\text{C}$. The supernatant was collected in 1.5 mL tubes and kept in working aliquots at $-80\text{ }^{\circ}\text{C}$. Some samples were diluted with 1X buffer to bring the antioxidant level within range. The assay was performed in multiwell plates and absorbance was measured using multiplate reader (Sunrise basic, Tecan 16039400

microplate reader). Three biological and three technical replicates for each sample per treatment were analyzed. The amount of antioxidant was calculated using the standard curve by plotting the average absorbance of each Trolox standard as a function of the final Trolox concentration (mM).

Statistical Analysis

The effect of two fungal inoculations, three salt concentrations and two plant varieties and their interactions on the

plant growth and physiological parameters (length and biomass of leaves and roots, RWC, chlorophyll, lipid peroxidation, proline concentration, and total antioxidants) were analyzed by the one-way and three-way ANOVA analysis using STATISTICA v.13.3.721 (StatSoft, Poland). The data were normally distributed and checked using Shapiro–Wilk test. The Newman–Keuls method was used as a post-hoc test to determine significant differences between fungal inoculations, three salt concentrations and the plant varieties used (defined as $p < 0.05$). For the bar plots, Student's T-test was used to determine significant differences between the fungal endophyte and non-inoculated control (defined as $p < 0.05$). The data are presented as means $\pm$ standard deviations from six biological replicates. The bar plots were generated in Microsoft Excel.

Endophyte Detection in *L. perenne* Using Microscopy and Real-Time Quantitative Polymerase Chain Reaction (RT–qPCR)

Three plant replicates from all treatments were surface sterilized as described by Hryniewicz et al. (2010). For endophyte staining, sterile root fragments were cut into 1 cm fragments, cleared in 5% KOH at 90 °C for 15 min, neutralized in 1% HCl at 20 °C overnight and stained with trypan blue (Phillips and Hayman 1970; Koske and Gemma 1989).

RT–qPCR was performed to check fungal colonization in the two *L. perenne* cultivars. Total RNA (1 µg) after DNAase I treatment (Chomczynski and Sacchi 2006) was reverse transcribed using cDNA synthesis kit (Transcriptor High Fidelity cDNA Synthesis Kit, Roche). qPCR was performed using LightCycler 480 FastStart SYBR Green I Master Mix and a LightCycler® 480 Instrument II (Roche). Each reaction mixture (10 µL) consisted of 5 µL of 2× SYBR Green I Master Mix (Roche), 3.5 µL of H₂O, 0.25 µL of each specific primer (10 pmol/µl) and 1 µL of template (10 ng/µl for leaves and roots). The cycling conditions included initial denaturation for 5 min at 95 °C, then 40 cycles of 10 s of denaturation at 95 °C, 20 s of annealing at the optimal temperature for the primer pair, 20 s of elongation at 72 °C, and finally a melting curve with a continuous temperature increase from 65 to 95 °C. Positive (pure DNA of specific fungal strains) and negative (molecular grade water) controls were analyzed in parallel with experimental samples. The primer pair used for amplification of target genes for *S. gausapatum* ISK3-11 (Internal transcribed spacer (ITS) region: 275–294, 386–367, Tm (°C): 60.04–60.11): Forward: 5'-CGCATCGATGAAGAACGCAG-3', and Reverse: 5'-GTGTGCCCTTCGGAATACCA-3'. In the case of *P. gamsii* CR1-9 (ITS region: 265–284, 391–372, Tm (°C): 60.11–60.67), Forward: 5'-TGAAGAACGCAGCGAAATGC-3', and Reverse: 5'-GGGTTGAAATGACGCTCGGA-3'. The translation elongation factor 1-alpha (tef1

α) gene for *P. gamsii* and Actin 1 for *S. gausapatum* were used as a reference (retrieved from the National Center for Biotechnology Information (NCBI: <http://www.ncbi.nlm.nih.gov/>) nucleotide database; see Supplementary information A Table S2). The analyses were performed in three biological and technical replicates. The average threshold cycle (Ct) from biological replicates was employed to calculate the gene expression fold change and are presented as the mean $2^{-\Delta\Delta C_T}$ value $\pm$ standard deviation (Pfaffl 2001). The data were analyzed using ANOVA using STATISTICA v.13.3.721 (StatSoft, Poland). The Newman–Keuls method was used as a post-hoc test to determine significant differences between fungal colonization in leaf and roots.

Transcriptome Sequencing

Total RNA was extracted from 100 mg of leaves and roots separately using TriPure reagent (EurX), followed by DNase treatment (DNase I, Thermo Scientific) (Chomczynski and Sacchi 2006). The quality of RNA was checked on NanoDrop spectrophotometer (Thermo Scientific) and stored at –80 °C until further processing. Two replicates were maintained for each treatment. The RNA samples were outsourced to Novogene for library preparation and sequencing using the Illumina HiSeq platform (<https://en.novogene.com/>). RNASeq datasets generated during the current study are available in the NCBI repository under BioProject no. PRJNA490634.

Gene Expression and Differential Expression Analysis

Adapter sequences, low-quality terminal nucleotides and unpaired reads were removed by Trimmomatic (v0.33, Bolger et al. 2014). Read pairs longer than 60 nt were kept for further processing. HISAT2 (Kim et al. 2015) was used to align trimmed reads to the *Lolium*_2.6.1 chromosome-scale reference genome with gene models of the version V2 annotation (Nagy et al. 2022) to produce spliced RNASeq alignments. The PCR duplicates were removed from the resulting bam files using the bammarkduplicates tool of the biobambam2 package (<https://gitlab.com/german.tischler/biobambam2>). Short-read alignments were processed by StringTie (v1.3.4b, Pertea et al. 2015) to obtain transcript counts and abundances. Gene-based read count information was extracted from the transcript abundance files by the prepDE.py script of the StringTie package. Differential gene expression analysis and hierarchical clustering of differentially expressed genes were conducted by EdgeR (Robinson et al. 2010) and DESeq2 (Love et al. 2014), applying a fold change threshold of 2 and a false discovery rate (FDR) of 0.01.

The networks of co-expressed genes in relation to quantitative gene expression data to phenotypic data were analyzed via the weighted gene coexpression network analysis (WGCNA) method using the WGCNA package in R (Langfelder and Horvath 2008). Construction of gene co-expression networks and identifying modules were carried out with the following WGCNA parameters: Soft threshold power: $\text{softPower} = 18$; Minimum number of genes in clusters: $\text{minModuleSize} = 75$. Heatmaps were generated using the 'labeledHeatmap' function of the WGCNA package. The moduleeigen (ME) genes were used to represent each module, which was calculated by the first principal component, capturing the maximal amount of variation of the module. The eigen genes were then used to estimate the correlations between module eigen genes and the traits of interest: salt concentrations (0, 150, and 300 mM), fungal inoculation (ENDO 1 and ENDO 2), length, fresh weight and dry weight of leaves and roots. BLASTP searches with transcript protein sequences as queries were performed on a local server. An *E*-value cutoff of $1e^{-3}$ was applied, and the top hit sequences were collected for further comparative analyses. For target reference sequences, a reduced-redundancy plant protein sequence database (all UniProtKB/TrEMBL Viridiplantae protein sequences clustered at a 75% similarity level, resulting in 4,098,635 sequences, plus 553,231 sequences from UniProt/SwissProt) was used.

The protein sequences of differentially regulated genes were further analyzed using the InterPro databases for their protein family relationships, signal peptides, transmembrane domains, and Gene Ontology (GO) terms were identified via local InterPro searches (InterProScan v5.28–67.0, Jones et al. 2014). Per gene GO-term information was collected from the InterProScan outputs using custom scripts. GO term enrichment analysis was carried out with the Python library GOATOOLS (Klopfenstein et al. 2018). Statistically overrepresented GO term categories were identified with multiple comparisons and a false discovery rate (FDR) of 0.05 using the Benjamini–Hochberg method (Benjamini and Hochberg 1995).

Validation of RNASeq-Based Expression Quantification via qRT–PCR

A total of 1 µg of total RNA (the same extract used for transcriptome analysis) was used in cDNA synthesis (Transcriptor High Fidelity cDNA Synthesis Kit, Roche). qRT–PCR was performed using FastStart SYBR Green I Master mix and a LightCycler® 480 (Roche LightCycler 480 Instrument II). The primer pairs for three selected transcript sequences were designed using Primer3 software (Untergasser et al. 2012) (Supplementary information A, Table S1). The beta-tubulin gene of *L. perenne* was used as a reference. All data are the means of three biological and technical replicates.

Relative expression differences (fold changes) were calculated according to Pfaffl (2001).

Results

Effect of different salt concentrations on *L. perenne* growth with or without fungal inoculation

In general, the inoculation with two nonhost fungal endophytes (*S. gausapatum* ISK3-11 (ENDO_1) and *P. gamsii* CR1-9 (ENDO_2)) in two *L. perenne* varieties significantly improved plant growth and biomass in response to increased salinity when compared to control (Fig. 1, Supplementary information B Fig. S2 and S3). A significant effect of inoculation in the two varieties was observed for root length, while for the same parameter, an opposite trend on the effect of salinity in the two varieties was observed (Table 1). The effect of inoculation on salinity was significant for plant growth and biomass parameters, except for root length and root dry weight (Table 1). Both fungal species performed better at 0 mM and 150 mM NaCl (as no significant difference between length and weight was observed between these two salt concentrations), while 300 mM NaCl displayed a significant decline in length and weight parameters (Table 1).

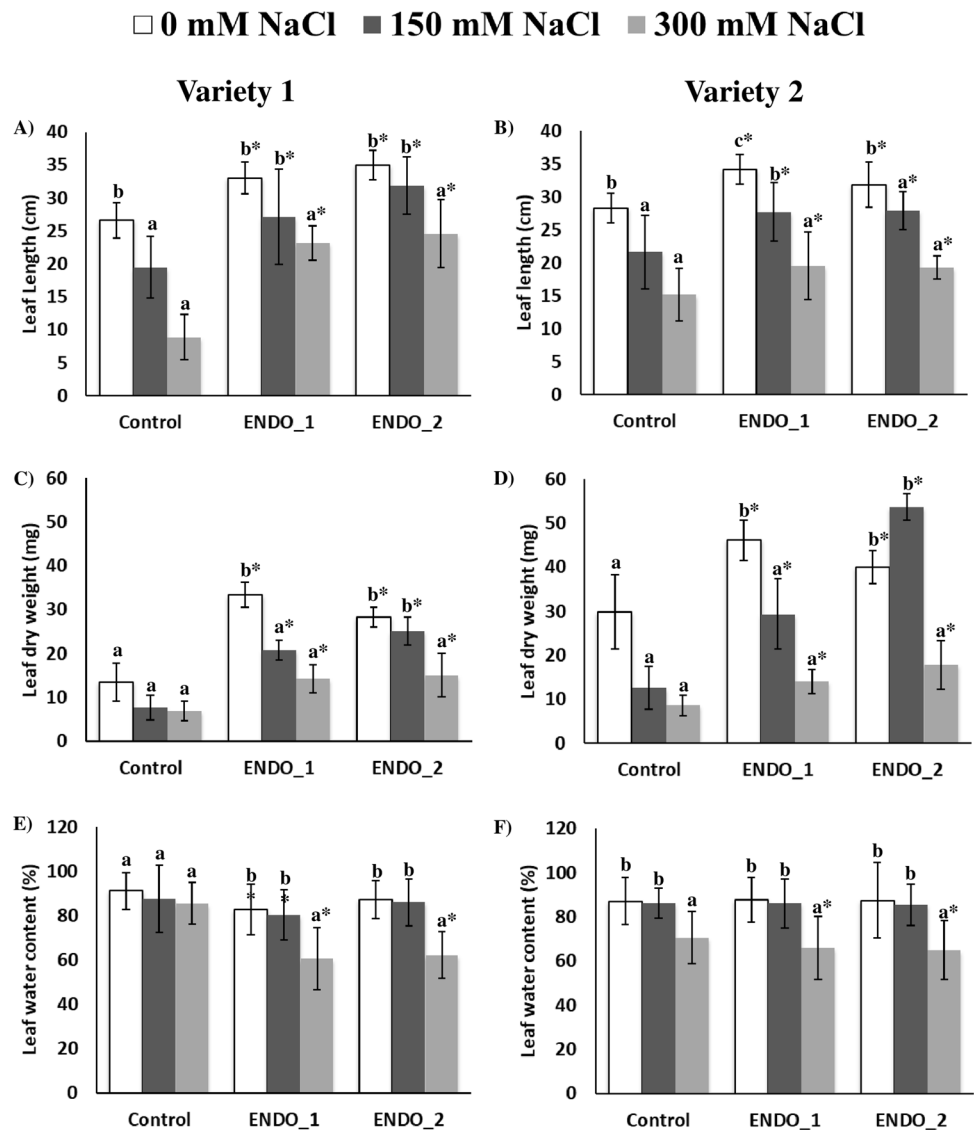
Fungal Endophyte Colonization in *L. perenne*

Trypan blue staining of Variety 1 and Variety 2 roots showed fungal colonization in endophyte-inoculated roots (Supplementary information B Fig. S4). The roots of the two inoculated grass varieties showed fungal hyphae colonizing intercellular spaces for ENDO_1, and in the case of ENDO_2, microsclerotia were observed inside the cortical root cell, some occupying the whole cortical cell volume.

To quantify fungal colonization in leaf and root, the RT-qPCR primers were designed to efficiently amplify the target sequences, respectively (primers listed in material and methods section). The average threshold cycle (Ct) from biological replicates was employed to calculate the gene expression fold change and is presented as the mean $2^{-\Delta\Delta\text{CT}}$ value $\pm$ standard deviation. The ITS region of ribosomal RNA from *S. gausapatum* ISK3-11 (ENDO_1) and *P. gamsii* CR1-9 (ENDO_2) was also included as the ITS region has often been used in the development of PCR primers for the detection of various fungi (He et al. 2020). The reference genes Actin 1 (for *S. gausapatum*) and *tef1α* (for *P. gamsii*) were used for normalization.

In case of both varieties, RT-qPCR amplification yielded no detectable amplification in non-inoculated control samples (Fig. 2). Overall, based on the relative expression ratio a higher fungal colonization was observed

Fig. 1 Effect of fungal endophyte inoculation on the leaf length (A, B), leaf dry weight (C, D) and relative water content (E, F) of two *L. perenne* varieties in response to fungal inoculation and salinity stress. Data represent the mean $\pm$ standard deviations, $n = 6$. a, b, c—Different letters indicate significant differences between three salt concentrations (0 mM, 150 mM and 300 mM NaCl) for each inoculation treatment. ($p < 0.05$, one-way ANOVA with Newman–Keuls post hoc comparisons). ‘*’—Indicate significant difference between *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasaroctadium gamsii*_CR1-9 (ENDO_2) and non-inoculated control ($p < 0.05$; Student’s *t*-test)



in roots than in leaves for all endophyte-inoculated plants in both varieties, except in 300 mM NaCl. A significant difference between the two grass varieties, Variety 1 and Variety 2 was observed in the leaf colonization by ENDO_1 at 0 mM (higher in Variety 2) and 150 mM (higher in Variety 1). Root colonization by ENDO_2 was significant at 300 mM (higher in Variety 2). The effect of increasing salinity led to a significant decrease in fungal colonization in the roots of the two varieties, specifically in 300 mM NaCl compared to 0 mM and 150 mM NaCl. The Ct values achieved in roots of Variety 1 (ENDO_1: 25–27.5 and ENDO_2: 25.2–27) and Variety 2 (ENDO_1: 29.9–33.3 and ENDO_2: 30–32.6) indicated more initial target DNA in the latter.

Physiological Responses to Fungal Inoculation and Salinity

No significant effect of fungal inoculation was observed on the relative leaf water content (RWC) in either treatment (Table 2, Fig. 1). However, for both the varieties significant differences were observed between the salinity treatments, specifically for ENDO_1 and ENDO_2 at 300 mM showing low RWC while values for 150 mM were similar to 0 mM. A gradual increase in salinity had negative effects on the leaf chlorophyll content; but a positive effect of inoculation was observed in both varieties (Table 2, Supplementary information (SI-B) Fig. S5). A significant difference in the parameters described below was observed in ENDO_1 and

Table 1 Effect of fungal endophyte inoculation on the growth and biomass of two *L. perenne* varieties under salt stress

	Length (cm)				Wet weight (mg)				Dry weight (mg)			
	Leaf		Root		Leaf		Root		Leaf		Root	
	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Variety	131.8**	7.2	54.6**	14.1	176,261**	28.2	2781.6**	7.4	2717.6**	26.2	50.7**	7.4
Inoculation	304.0**	16.5	105.4**	27.3	286,467**	45.9	3445.2**	9.2	2632.2**	25.3	52.4**	7.6
Salinity	944.4**	51.2	89.7**	23.3	103,228**	16.5	3673.9**	9.8	3162.4**	30.4	135.0**	19.6
Variety*inoculation	29.0	1.6	26.6**	6.9	21,589*	3.5	551.7	1.5	129.8	1.2	3.7	0.5
Variety*salinity	64.5*	3.5	1.0	0.3	11,902	1.9	1761.6**	4.7	381.0*	3.7	62.1**	9.0
Inoculation*salinity	52.6*	2.9	9.3	2.4	27,597**	4.4	1696.7**	4.5	563.1**	5.4	16.0	2.3
Variety*inoculation*salinity	8.9	0.5	4.5	1.2	8652	1.4	780.2	2.1	194.9	1.9	9.8	1.4
Error	18.4		3.9		6247		374.3		103.9		6.9	
Mean value												
(1) Variety												
Variety 1	27.41 b		5.77 a		128.09 a		18.15 a		18.32 a		3.48 a	
Variety 2	25.20 a		7.20 b		208.89 b		28.30 b		28.35 b		4.85 b	
(2) Inoculation												
Control	23.0 a		4.56 a		111.59 a		12.39 a		13.70 a		2.77 a	
ENDO_1	27.49 b		7.04 b		175.99 b		25.86 b		26.32 b		4.84 b	
ENDO_2	28.43 b		7.85 b		217.89 c		31.41 b		30.00 b		4.88 b	
(3) Salinity												
0 mM	31.52 c		7.85 c		256.94 c		30.84 b		31.84 b		6.27 c	
150 mM	26.13 b		6.85 b		169.98 b		27.07 b		24.88 b		3.76 b	
300 mM	21.28 a		4.76 a		78.551 a		11.76 a		13.29 a		2.46 a	

Different letters denote significant differences within each variety, inoculation and salinity analyzed by the Newman–Keuls test (three-way ANOVA analysis). **Values are significant at $p < 0.01$, *values are significant at $p < 0.05$. Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasaroeladium gamsii*_CR1-9 (ENDO_2)

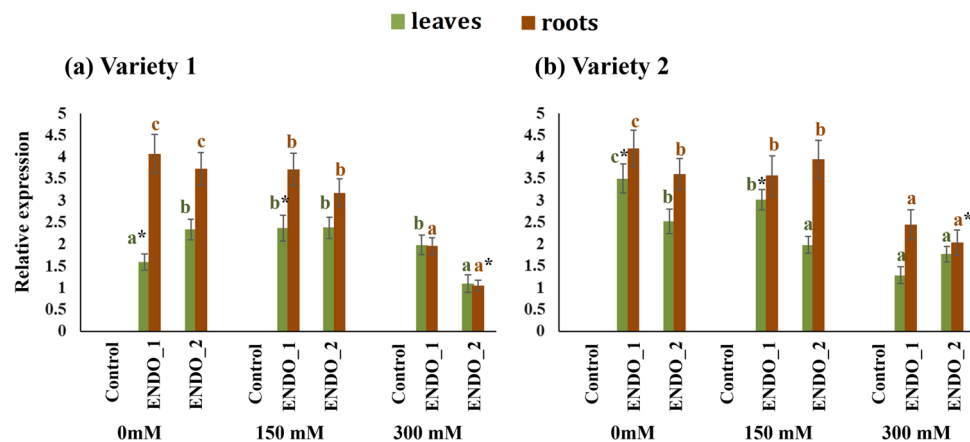


Fig. 2 qPCR detection of fungi in leaves and roots of Variety 1 (a) and Variety 2 (b). Relative expression levels with *tef1* α for *P. gamsii* and *actin 1* for *S. gausapatum* were calculated from the *Ct* values and the primer amplification efficiencies using the formula described by Pfaffl (2001). Error bars indicate the mean $\pm$ standard deviations = 3 replicates. Different letters (a, b, c) indicate significant differences ($p < 0.05$, one-way ANOVA with Newman–Keuls post hoc) for each

inoculation (ENDO_1 or ENDO_2) between the salt concentrations and ** denotes significant differences ($p < 0.05$) between the two varieties for the same salt concentration (ENDO_1 at 0 mM, or ENDO_1 at 150 mM or ENDO_2 at 300 mM). Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasaroeladium gamsii*_CR1-9 (ENDO_2)

Table 2 Effect of inoculation with two fungal endophytes on the physiological parameters of two *L. perenne* varieties under salt stress

	RWC (%)	Chlorophyll (µg/100 mg leaf)				Proline (µg/100 mg fresh weight)				Lipid peroxides (nmole g ⁻¹ fresh weight)				Total Antioxidant (mM/100 mg leaf)			
		Leaf		Root		Leaf		Root		Leaf		Root		Leaf		Root	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Variety	648.4	2.0	0.004**	333.1	5685.9	2.2	4825.9**	33.8	4012.6**	626.1	5877.2**	3019.6	6.8E+24	1.0	2.3**	66.5	
Inoculation	972.7	3.0	0.001**	84.6	10,597.5*	4.0	2503.4**	17.5	1972.4**	307.7	800.5**	411.3	6.8E+24	1.0	0.7**	21.5	
Salinity	493.6	1.5	0.0062**	502.1	34,800.5**	13.2	2845.9**	19.9	212.0**	33.1	85.8**	44.1	6.8E+24	1.0	0.8**	23.1	
Variety*inoculation	971.0	3.0	0.0002**	13.4	108.9	0.0	2982.5**	20.9	690.2**	107.7	929.1**	477.4	6.8E+24	1.0	1.4**	39.1	
Variety*salinity	135.8	0.4	0.0003**	27.8	1246.2	0.5	686.7**	4.8	87.2**	13.6	54.9**	28.2	6.8E+24	1.0	0.3**	9.0	
Inoculation*salinity	109.0	0.3	0.0004**	30.0	10,292.7**	3.9	194.2	1.4	23.4**	3.7	72.8**	37.4	6.8E+24	1.0	0.2**	7.1	
Variety*inoculation*salinity	94.0	0.3	0.0002**	15.9	249.7	0.1	855.4**	6.0	60.8**	9.5	18.6**	9.5	6.8E+24	1.0	0.7**	20.1	
Error	322.4		0.000012		2643.3		143.0		6.4		1.9		6.8E+24		0.03		
Mean value																	
(1) Variety																	
Variety 1	80.76 a		0.011 a		43.54 a		12.45 a		25.47 a		-1.88a		5.0E+11a		2.00 b		
Variety 2	85.66 a		0.024 b		29.03 a		25.82 b		37.66 b		12.88 b		2.35 a		1.71 a		
(2) Inoculation																	
Control	86.69 b		0.011 a		17.53 a		10.59 a		38.20 c		8.98 c		2.35 a		1.70 a		
ENDO_1	77.23 a		0.019 b		51.20 b		19.57 b		23.58 a		0.13 a		2.72 a		1.99 c		
ENDO_2	85.70 b		0.022 c		40.11 ab		27.25 c		32.91 b		7.38 b		7.5E+11a		1.87 b		
(3) Salinity																	
0 mM	87.23 a		0.032 c		7.73 a		13.07 a		28.76 a		7.28 b		7.5E+11a		1.89 b		
150 mM	82.45 a		0.014 b		31.71 a		14.99 a		32.98 b		4.68 a		2.55 a		1.98 c		
300 mM	79.94 a		0.007 a		69.41 b		29.34 b		32.95 b		4.53 a		2.25 a		1.69 a		

Different letters denote significant differences within each variety, inoculation and salinity analyzed by the Newman–Keuls test (three-way ANOVA analysis). RWC relative water content, **values are significant at $p < 0.01$, *values are significant at $p < 0.05$ Abbreviations: *Stereum gausapatium*_ISK3-11 (ENDO_1) and *Parasarcocladium gamsii*_CR1-9 (ENDO_2)

ENDO_2 compared to the controls. The effect of high salinity (300 mM) on proline concentration in leaves and roots was clear (Table 2, SI-B Fig. S6). The proline concentration in the leaves and roots of the two varieties after inoculation was notably higher with increasing salt concentration than in the control (0 mM). The root proline concentration was significantly different in plants with ENDO_2. The lipid peroxidation in Variety 1 and Variety 2 (both leaves and roots) significantly decreased with increasing salt concentration, specifically in ENDO_1 plants compared to the control and ENDO_2 plants (Table 2, SI-B Fig. S7). Conversely, a significant decrease in total antioxidants in roots with ENDO_2, while in leaves no significant difference was observed (Table 2, SI-B Fig. S8). In comparison to the control plants, ENDO_2-inoculated plants displayed higher proline concentration, while ENDO_1 displayed lower lipid peroxides and high antioxidants in both the leaves and roots.

RNASeq Data Processing and Quality Assessment

For each sample, in average 15 million RNASeq reads were obtained in 150 bp paired-end mode in two biological replicates. In the quality trimming step 3 to 5% of the read pairs were removed because of being shorter than 60 nt length threshold or completely losing one of the pairs after removing low-quality terminal sequences. The average GC content of the reads was 57% (minimum: 53%, maximum: 60%). Mapping of RNASeq reads to the *L. perenne* reference genome (Nagy et al. 2022) resulted in an overall alignment rate of 70.69% (minimum: 64.49%, maximum: 73.67%).

Expression Profiling of Differentially Expressed Genes in *L. perenne* Leaves and Roots Based on Fungal Inoculations and Different Salt Concentrations

As shown in Fig. 3, a total of 1485 (Variety 1) and 7471 (Variety 2) differentially expressed genes (DEGs) were identified after comparing inoculated treatments to their controls

(i.e., Control vs. ENDO_1 and Control vs. ENDO_2) (Supplementary information (SI-B) Fig. S9). The Venn diagram for Variety 1 shows ENDO_1 with higher number DEGs in leaves and roots compared to ENDO_2. Among these, 224 DEGs in leaves and 180 in roots were common to both endophytes. Similar observations were also made in Variety 2 with higher DEGs in ENDO_1 plants. Overall, ENDO_1 showed more DEGs (Variety 1—60% and Variety 2—72%) in both grass varieties than ENDO_2. To avoid comparing redundant treatment categories in two different genetic backgrounds, DEG analyses were carried out separately for the two varieties. Gene expression heatmaps (Fig. 4) and expression profile groups (subclusters in SI-B Fig. S10 a, b) derived from the heatmap showed most DEGs in the Variety 1 ENDO_1 plants were downregulated in response to salt treatments (SI B- Fig. S10a: 517 genes); in contrast, most DEGs of Variety 1 ENDO_2 were upregulated (SI B- Fig. S10c: 254). In Variety 1, the leaves (SI-B Fig. S10a–c) displayed no significant endophyte effect, while roots (SI-B Fig. S10e–j) showed a differential pattern of gene regulation between control and inoculated plants. A similar trend in the subclusters of Variety 2 leaves displayed gene clustering according to salt treatments, although an endophyte effect was observed (SI-B Fig. S10a–d). A clear differentiation between the effects of the two fungal inoculations was observed in Variety 2 roots. Under salt treatments, the ENDO_1 roots displayed more upregulated genes in subclusters e–g while ENDO_2 in subcluster h in comparison to the control. In summary, the gene expression was more significant between no salt (0 mM) and with salt (150 mM and 300 mM) and not between 150 and 300 mM. Leaves and roots displayed similar responses to 150 and 300 mM NaCl, with notable differences from 0 mM. Moreover, more significant differences between ENDO_1 and ENDO_2 were observed than the salinity treatments for both varieties. So, the transcripts were analyzed by comparing the non-inoculated control and the two endophytes- ENDO_1 and ENDO_2 for each grass variety in leaves and roots separately.

Fig. 3 Venn diagram comparing differentially expressed genes (DEGs) in *L. perenne* varieties (Variety 1 and Variety 2) and plant organs (green color denotes leaves and brown color denotes roots). Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasarocladium gamsii*_CR1-9 (ENDO_2)

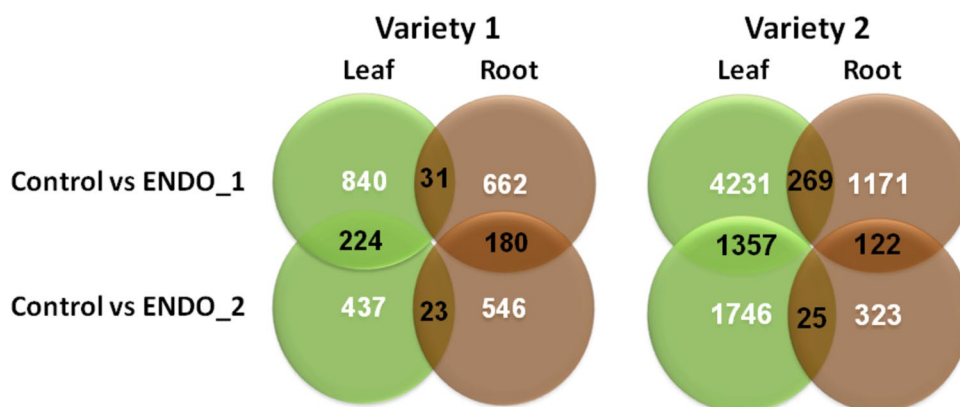




Fig. 4 Differential expression profiles of *L. perenne*: Variety 1 (A) and Variety 2 (B) in leaves and roots under different salt concentrations (0 mM, 150 mM, 300 mM NaCl). Expression values are presented as logarithmic base 2 of normalized counts per million (CPM) values across samples (control vs. ENDO_1 and control vs. ENDO_2 for leaves and roots). Highly expressed genes are colored green, whereas genes expressed at lower levels are colored red. The information on the total number of genes present in each subcluster (a–j) in the heatmap is given in Supplementary information B Figure S10. Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasarcocladium gamsii*_CR1-9 (ENDO_2), control (C), leaves (l), root (r), replicates (1, 2) (Color figure online)

Weighted Gene Co-expression Network Modules in *L. perenne* Leaves and Roots

Using the WGCNA approach, nine co-expression modules were identified in leaves and roots of Variety 1 and seven modules in Variety 2 (Fig. 5a–d). In the module trait relationships plots, correlations between quantitative gene expression profiles of identified modules and phenotype trait values are represented in the form of heatmaps. The colors and color intensities of the heatmap blocks show the direction (positive or negative correlation, red/blue) and value categories of the calculated correlation coefficients. The biological processes (BP) of the top enriched GO terms (p values < 0.0001) for each variety are illustrated in the results (Figs. 6, 7). A detailed list of enriched GO terms and their representative pathways of molecular functions and BP in leaves and roots of Variety 1 and Variety 2 are listed in Supplementary Information C.

Variety 1 Leaf Transcriptome

In Variety 1 leaves, four modules showed a clear negative connection with plant traits, in contrast five modules had a noticeable positive relationship (Fig. 5a). Two modules with a high number of enriched GO terms, the MEpurple module displayed a significantly strong positive correlation with the length and fresh and dry weight of leaves and roots; while the MEBrown module displayed a significantly strong negative correlation for the same. Between the two endophytes, ENDO_1 showed a positive correlation with a high number of enriched genes in modules MELightyellow (82 enriched GO terms) and MERed (66), which were also negatively correlated by ENDO_2. Conversely, ENDO_2 displayed a positive correlation for genes in module MEpurple (126 enriched GO terms).

In Fig. 6a, some of the important enriched GO terms in the biological processes (BP) in the MEpurple module represented processes related to carbohydrate metabolism, catalytic activity, cell wall macromolecule metabolism, cell wall organization or biogenesis, drug metabolism, heterocycle metabolism, lipid biosynthesis, lipid transport, nitrogen compound metabolism, and polysaccharide metabolism.

The MEBrown module was related to amino acid metabolism, cellular biosynthesis, drug metabolism, heterocycle metabolism, lipid biosynthesis, L-phenylalanine catabolism, macromolecule biosynthetic process, nucleobase-containing compound metabolic process, protein phosphorylation, and reactive oxygen species metabolism. The MELightyellow module corresponds to carboxylic acid biosynthesis, cellular response to stress, drug metabolism, generation of precursor metabolites and energy, photosynthesis, and RNA processing.

Variety 1 Root Transcriptome

In Fig. 5b, positive correlations between modules MEagenta, MEgrey, and MEblue of Variety 1 root at 0 mM and 150 mM NaCl for plant growth and biomass measurements were observed. In contrast, the modules MEpurple, MEpink, and MEblack were negatively correlated for the above-mentioned traits. A high number of enriched GO terms was observed in the MEblue module with a significantly strong positive correlation for the length and fresh and dry weight of leaves and roots, followed by the MEblack module displaying a significantly strong negative correlation for the same. In Fig. 6b, both endophytes showed a positive correlation for genes in the MEblue module (208 enriched GO terms) with high enriched GO terms related to processes in alpha-amino acid metabolic process, amide metabolic process, antibiotic metabolism, carbohydrate metabolism, cellular localization, cofactor metabolism, drug metabolism, erythrose 4-phosphate/phosphoenolpyruvate (E4P/PEP) family amino acid metabolism, heterocycle metabolism, macromolecule biosynthesis, nucleic acid metabolism, phosphorus metabolism, phosphorylation, polysaccharide metabolism, and reactive oxygen species metabolism. The MEblack and MEBrown modules were negatively correlated by both endophytes consisting of processes in DNA replication, macromolecule biosynthesis, lipid biosynthesis, generation of precursor metabolites and energy, and nucleic acid metabolism.

Overall, the results show that the role of two fungal endophytes in Variety 1 is unique to the leaves and/or the roots. In leaves, both endophytes positively regulated processes in cell recognition, phosphorylation, and transport, while a negative correlation for processes in amino acid metabolism, cellular biosynthesis, antibiotic and drug metabolism, heterocycle metabolism, L-phenylalanine catabolism, macromolecule biosynthesis, and reactive oxygen species metabolism was found. ENDO_1 and ENDO_2 also positively regulated processes that were only observed in the roots, such as alpha amino acid metabolism, amide metabolism, erythrose 4-phosphate (E4P) amino acid metabolism, glutamate metabolism, and phosphorous metabolism.

Fig. 5 Co-expression analysis (WGCNA) for Variety 1 (**a**, **b**) and Variety 2 (c and d) leaves and roots. A matrix with the module–trait relationships (MTRs) (correlation coefficients) and corresponding p values (in brackets) between modules on the y-axis and traits on the x-axis. The MTRs are colored based on their correlation: red indicates a strong positive correlation, while blue indicates a strong negative correlation. Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasaroeladum gamsii*_CR1-9 (ENDO_2), module eigen (ME) (Color figure online)

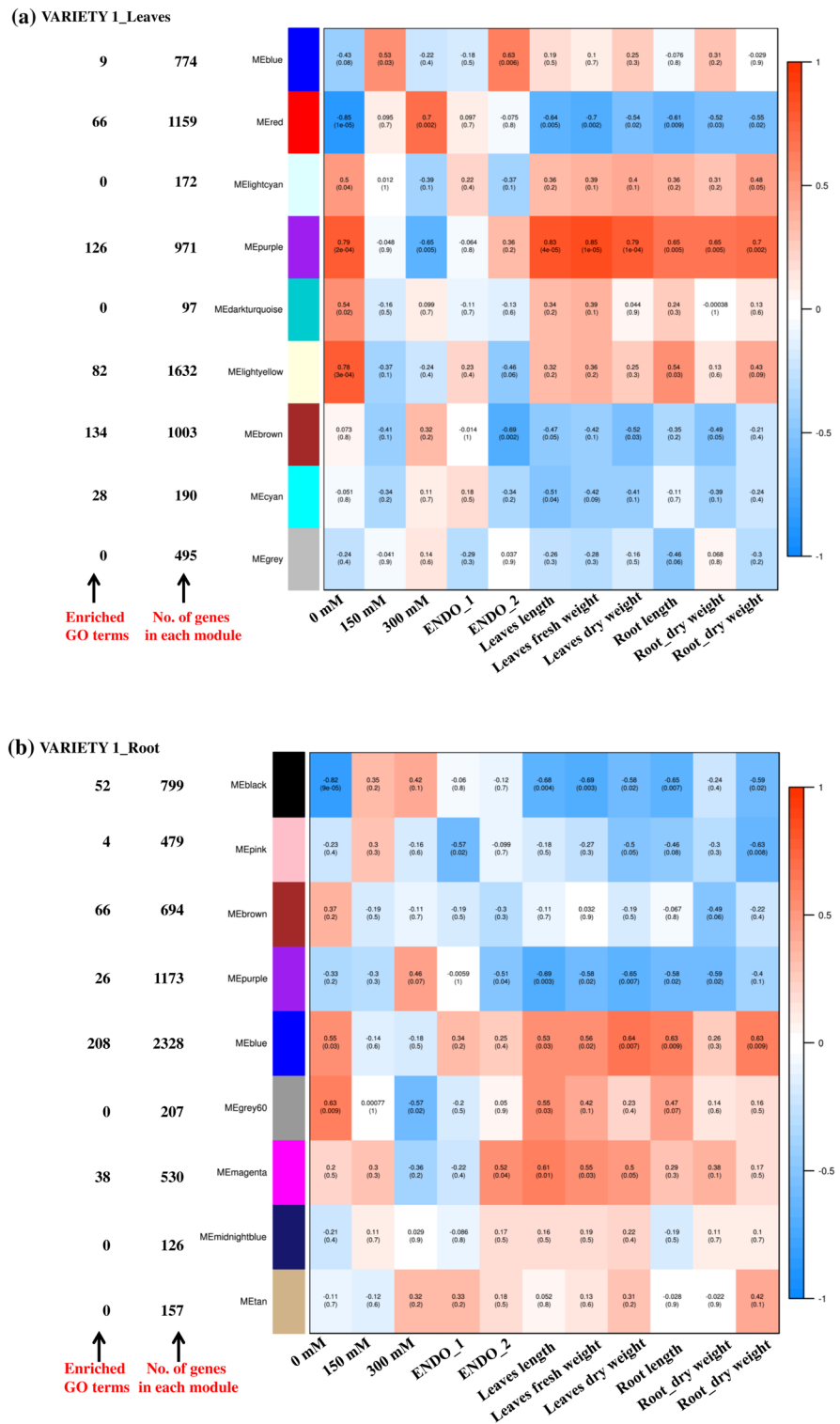
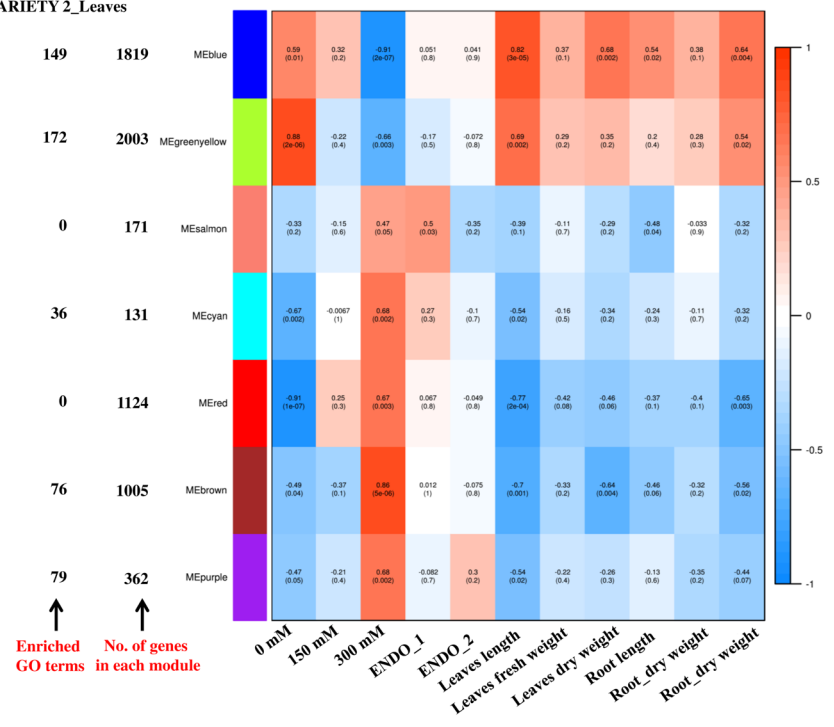
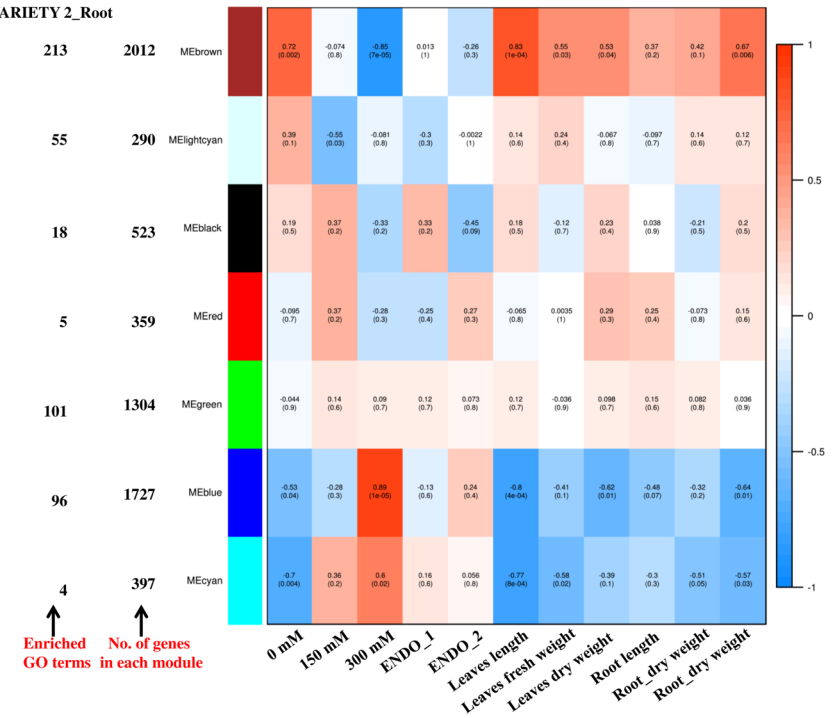


Fig. 5 (continued)

(c) VARIETY 2_Leaves



(d) VARIETY 2_Root





of enriched GO terms were found in modules MEblue (149 enriched GO terms) which was positively correlated by ENDO_1 and ENDO_2, and MEGreenyellow (172 enriched GO terms) which was negatively correlated by both endophytes (Fig. 5c). Positive correlations observed for ENDO_1

Fig. 6 Biological processes of the top enriched GO terms in Variety 1 (A) leaves and (B) roots related to salinity and fungal inoculation. The bar color of each functional description corresponds to the module color in WGCNA. Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasaroeladium gamsii*_CR1-9 (ENDO_2). Representative processes in Variety 1 (A) and (B) marked as (1) alpha-amino acid metabolism, (2) amide metabolism, (3) amino acid metabolism, (4) antibiotic metabolism, (5) carbohydrate metabolism, (6) carboxylic acid biosynthesis, (7) catabolism, (8) cell recognition, (9) cell wall organization or biogenesis, (10) cellular biosynthetic process, (11) cellular localization, (12) cellular response to stress, (13) cofactor metabolism, (14) DNA replication, (15) drug metabolism, (16) erythrose 4-phosphate amino acid metabolism, (17) generation of precursor metabolites and energy, (18) transport, (19) heterocycle metabolism, (20) lipid biosynthesis, (21) L-phenylalanine catabolism, (22) macromolecule biosynthesis, (23) nitrogen compound metabolism, (24) nucleic acid metabolic process, (25) polysaccharide metabolism, (26) phosphorylation, (27) photosynthesis, (28) reactive oxygen species metabolism, (29) reproductive process

(MEblue, MEbrown, MEcyan) and ENDO_2 (MEblue, MEpurple). As seen in Fig. 7a, the BP negatively correlated by both endophytes for the BLUE module included processes related to processes in carbohydrate metabolism, carboxylic acid biosynthesis, cell wall macromolecule metabolism, cell wall macromolecule metabolism, cellular metabolic process, cofactor metabolism, drug metabolism, heterocycle metabolism, lipid biosynthesis, macromolecule biosynthetic process, nucleic acid metabolic process, phosphorus metabolic process, phosphorylation, photosynthesis, polysaccharide metabolism, reactive oxygen species metabolism and vesicle-mediated transport. In contrast, the MEgreenyellow module was negatively correlated with ENDO_1 and ENDO_2 for processes in amino acid metabolic process, carbohydrate metabolism, cellular localization, chlorophyll biosynthesis, cofactor metabolism, drug metabolism, heterocycle metabolism, lipid biosynthesis, L-phenylalanine metabolism, nucleic acid metabolic process, peptide biosynthetic process, and protein phosphorylation.

Variety 2 Root Transcriptome

A strong negative correlation was observed for the modules MEcyan and MEblue for plant growth and biomass measurements, while the MEbrown module showed a strong positive correlation (Fig. 5d). The highest number of enriched GO terms were found in modules MEbrown (213 enriched GO terms) and MEgreen (101 enriched GO terms). Significant positive correlations were observed with ENDO_1 in the module MEbrown, while ENDO_2 in the module MEblue (55 enriched GO terms), and for both endophytes in modules MEgreen and MEcyan (4 enriched GO terms). The top enriched GO terms for the BP category, MEbrown module

included main processes in alpha-amino acid catabolism, amide metabolic process, ammonium ion metabolic process, antibiotic metabolism, carbohydrate metabolism, cell wall macromolecule metabolism, cellular localization, cofactor metabolism, drug metabolism, heterocycle metabolism, lipid biosynthesis, L-phenylalanine catabolism, nitrogen compound metabolism, nucleic acid metabolic process, peptide biosynthetic process, polyamine biosynthetic process, polysaccharide metabolism, reactive oxygen species metabolism, reproduction, response to oxidative stress, and secondary metabolic process (Fig. 7b). Module MEblue represented processes in amino acid metabolism, autophagy, cellular component organization, heterocycle metabolism, L-phenylalanine catabolism, macromolecule biosynthetic process, nitrogen compound metabolism, nucleic acid metabolic process, organic substance metabolism, phosphorus metabolic process, and protein phosphorylation.

Overall, similarly to Variety 1, the effect of ENDO_1 and ENDO_2 differed between the Variety 2 leaves and roots. In leaves, both endophytes positively regulated processes that were exclusive to the leaves, including carboxylic acid biosynthesis, drug metabolism, photosynthesis, reactive oxygen species metabolism, and RNA metabolism. Some processes were unique only to the roots, namely alpha amino acid catabolism, amide metabolic process, autophagy, generation of precursor metabolite and energy, nitrogen metabolism, peptide biosynthesis, polyamine biosynthesis, response to oxidative stress, and secondary metabolite process.

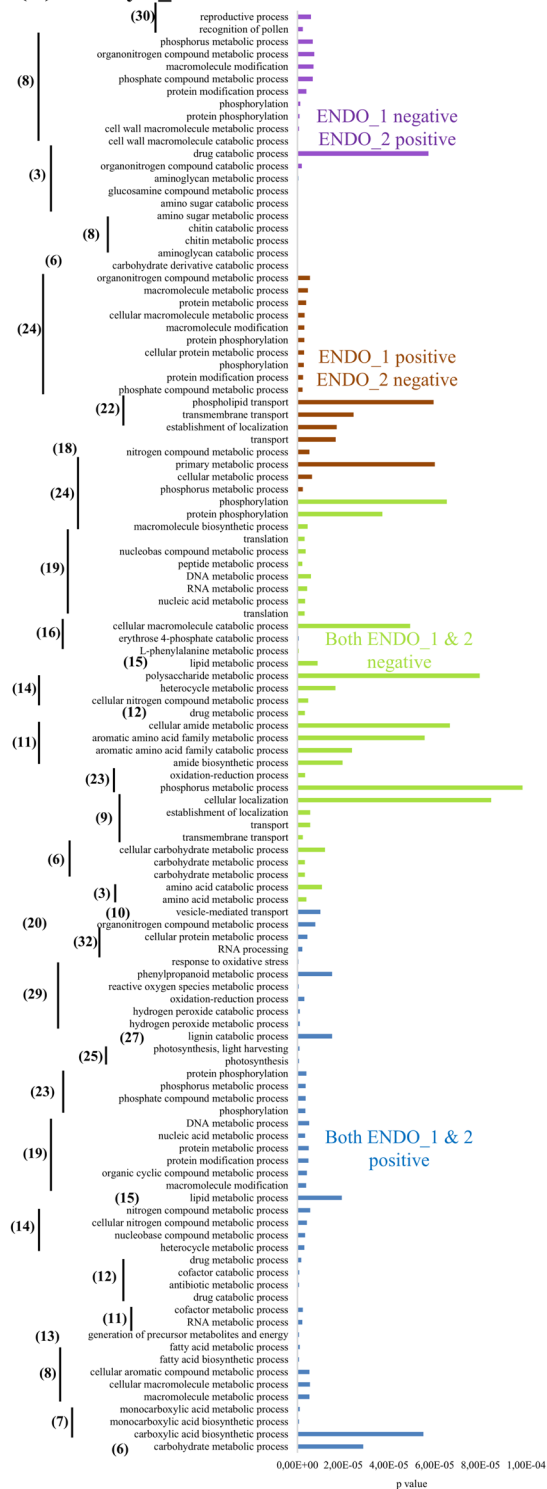
RNASeq Data Validation

The three genes for RT-qPCR were selected from DEGs that were expressed in all comparison groups. The results of RT-qPCR and RNA seq for the selected genes are shown in Supplementary Information B Fig. S11. We observed that the trend of gene expression from the RT-qPCR analysis was consistent with the RNASeq results.

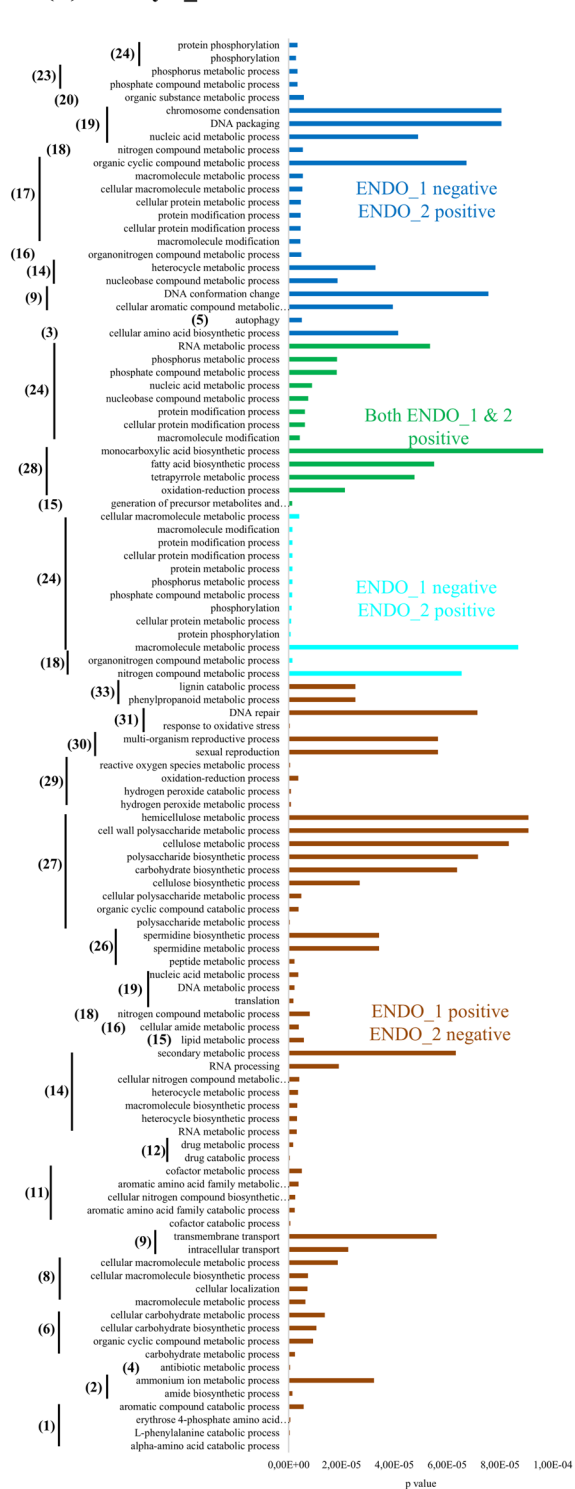
Discussion

Many physiological and biochemical processes can significantly impede plant growth under high salinity conditions. Thus, managing plant growth and reducing damage from salinity stress is an important issue in agricultural science. This study aimed to assess whether two selected fungal endophytes isolated from *S. europaea* could alleviate salt stress in the non-host *L. perenne*. The two halotolerant and plant growth-promoting endophytic fungal strains (*S. gausapatum* ISK3-11 (ENDO_1) and *P. gamsii* CR1-9 (ENDO_2))

(A) Variety 2 leaves



(B) Variety 2 roots



established in the *L. perenne* (non-host plant), and promoted growth in two varieties (Variety 1 and Variety 2) under salt stress. Furthermore, this work provides a comprehensive transcriptomic analysis, identifying key pathways in leaves and roots that are differentially regulated in response to

endophyte inoculation. Weighted gene co-expression network analysis (WGCNA) identified specific gene modules differing between ENDO_1 and ENDO_2, correlating gene modules with physiological and morphological features to pinpoint molecular responses underlying observed growth

Fig. 7 Biological processes of the top enriched GO terms in Variety 2 (A) leaves and (B) roots related to salinity and fungal inoculation. The bar color of each functional description corresponds to the module color in WGCNA. Abbreviations: *Stereum gausapatum*_ISK3-11 (ENDO_1) and *Parasaroeladium gamsii*_CR1-9 (ENDO_2). Representative processes in Variety 2 (A) and (B) are marked as (1) alpha-amino acid catabolism, (2) amide metabolic process, (3) amino acid metabolic process, (4) antibiotic metabolism, (5) autophagy, (6) carbohydrate metabolism, (7) carboxylic acid biosynthesis, (8) cell wall macromolecule metabolism, (9) cellular component organization, (10) vesicle-mediated transport, (11) cofactor metabolism, (12) drug metabolism, (13) generation of precursor metabolites and energy, (14) heterocycle metabolism, (15) lipid biosynthesis, (16) L-phenylalanine metabolism, (17) macromolecule biosynthetic process, (18) nitrogen compound metabolism, (19) nucleic acid metabolic process, (20) organic substance metabolism, (21) peptide biosynthetic process, (22) phospholipid transport, (23) phosphorus metabolism, (24) phosphorylation, (25) photosynthesis, (26) polyamine biosynthetic process, (27) polysaccharide metabolism, (28) porphyrin-containing compound biosynthesis, (29) reactive oxygen species metabolism, (30) reproductive process, (31) response to oxidative stress, (32) RNA processing, (33) secondary metabolic process, and (34) small molecule catabolic process

improvements. By leveraging the distinct gene expression profiles of each variety in response to fungal inoculation, we attributed differentially expressed genes (DEGs) to various biological processes and molecular functions. These insights into gene regulation and pathway activity deepen our understanding of plant-endophyte interactions under salt stress and highlight potential targets for bioinoculant selection to improve crop resilience in saline environments.

Fungal Endophyte Inoculations Improve Growth and Physiology in *L. perenne* Under Salinity Stress

Previous studies have demonstrated that salt-treated *L. perenne* shows reductions in growth, transpiration rates, chlorophyll content, and turf quality (Hu et al. 2012; Li et al. 2017). Our results align with these findings, indicating that salinity dramatically decreased *L. perenne* growth specifically in the non-inoculated plants (control). The two fungal endophytes inoculated in the two grass varieties significantly improved overall plant growth and biomass (wet and dry weights) notably in the no salt (0 mM NaCl) and moderate salt (150 mM NaCl) concentrations. Comparable studies by Toghueo et al. (2022) on the role of fungal endophytes in plant growth stimulation were made, who reported on the endophyte *Diaporthe* that promoted *L. perenne* growth under salinity stress.

Endophytic fungal colonization is known to mitigate salinity stress by promoting protective metabolites, antioxidant activity, and osmoprotectants. These functions protect against reactive oxygen species, lower salt-induced root respiration, and alter phytohormone profiles to enhance growth under stress (Porrás-Alfaro and Bayman 2011; Jogawat et al. 2013; Ghaffari et al. 2016; Li et al. 2017; Gupta et al.

2021). Reduced leaf water contents in plants are generally the first notable effect of salt and osmotic stresses (Win et al. 2016). In this study, high salinity (300 mM NaCl) in the substrate reduced plant water uptake, which was observed in the low relative water content (RWC) in the leaves of both *L. perenne* varieties. This may indicate that ENDO_1 and ENDO_2 may not directly play a role in influencing water uptake in leaves under salinity stress.

Major osmolytes such as proline can protect plants against oxidative damage and are indirectly responsible for enhancing chlorophyll content and stimulating antioxidant enzymes (to maintain cell membrane integrity) (Ghosh et al. 2022). The high chlorophyll contents detected in the leaves of the two inoculated *L. perenne* varieties under salt stress indicated that symbiotic interactions play a crucial role in regulating photosynthetic activity. Under increasing salt concentrations, the proline concentration in the leaves and roots of both varieties significantly increased after fungal inoculation. For both varieties at high salinity (300 mM NaCl), ENDO_2 showed higher proline, while only ENDO_1 displayed lower lipid peroxidation and strongly increased total antioxidants. Similar findings have been reported for the fungus *Aspergillus aculeatus* isolated from the rhizosphere of Bermuda grass that improved ryegrass photosynthetic efficiency, increased antioxidant enzyme activity, and attenuated lipid peroxidation induced by salinity stress (Li et al. 2017). These results suggest that both ENDO_1 and ENDO_2 negate the salinity stress effect by mediating the accumulation of osmolytes, consequently improving ryegrass water status, chlorophyll content, and antioxidants. Overall, the above findings support our hypothesis that the two halotolerant and metabolically active endophytic fungal strains (*S. gausapatum* ISK3-11 and *P. gamsii* CR1-9) can mitigate salinity stress in a nonhost plant species.

Fungal Inoculation Induces Specific Processes in *L. perenne* Leaves and Roots in Response to Salinity Stress

Weighted gene correlation network analysis (WGCNA) is an effective tool for data mining that enables trait-related genetic screening and classification of highly biologically significant coexpression clusters (Langfelder and Horvath 2008). In this study, WGCNA identified nine gene modules in the leaves and roots of Variety 1 and seven in Variety 2 that were closely correlated between transcriptional levels with morpho-physiological characteristics. Overall, key biological processes in *L. perenne* were differentially regulated in response to endophyte inoculation under salt stress in both plant varieties. However, some metabolic pathways were preferentially regulated in leaves or roots depending on the specific endophyte and variety, suggesting distinct mechanisms of salt tolerance.

Pathways Regulated by ENDO_1

1. Amino acid metabolism and nitrogen utilization: ENDO_1 positively regulated pathways to 2-oxoacid biosynthesis in the leaves of both Variety 1 and Variety 2. The activation of the 2-oxoacid biosynthetic pathway was reported in a halophytic turf grass *Sporobolus virginicus*, which enhances nitrogen metabolism under salt stress (Yamamoto et al. 2015). The 2-oxoacids are key compounds in metabolic networks, forming numerous reactions to amino acids, carbohydrates, and coenzymes (O'Leary et al. 2011). In glycolysis and the TCA cycle, 2-oxoacids such as pyruvate, oxaloacetate, and ketoglutarate are generated and used in amino acid biosynthesis to make asparagine and glutamate (O'Leary et al. 2011). Under stress conditions, glutamate is consumed for proline and polyamine production, as well as a compatible solute that contributes to the cell osmotic regulation (El-Shintinawy and El-Shourbagy 2001; Roberts 2005; Hakim et al. 2014). A study by Garg et al. (2014) highlighted the key role of amino acid biosynthesis in salt stress tolerance in wild halophyte rice *Porteresia coarctata*. Increased nitrogen assimilation by ENDO_1, particularly via amide metabolism, was observed in Variety 1 (MEblue) and Variety 2 (MEbrown) roots, supporting efficient nitrogen use and protein synthesis (González-Moro et al. 2021). Amides such as glutamine and asparagine are essential nitrogen carriers, critical for maintaining nitrogen homeostasis in saline conditions (González-Moro et al. 2021). In saline conditions, Na⁺ and Cl⁻ ions compete with ammonium ions for uptake by plant roots, which reduces the efficiency of ammonium absorption, thus affecting the nitrogen supply for the synthesis of amides. These disruptions lead to reduced nitrogen use efficiency, impacting plant growth, and productivity.
2. Cell wall adaptation- flexibility-driven strategy: ENDO_1 modulated cell wall macromolecule metabolism or biogenesis differently in the two plant varieties. In Variety 1 leaves (MEpurple), it negatively regulated processes related to cell wall macromolecule metabolism or biogenesis, which may indicate a strategy where cell wall flexibility is increased, potentially facilitating cellular expansion, water retention, or stress signaling rather than reinforcement. In contrast, Variety 2 leaves (MEblue), ENDO_1 positively regulated these processes, indicating a reinforcement strategy, where structural components such as cellulose and hemicellulose are accumulated to withstand salt-induced mechanical stress (Colin et al. 2023).
3. Organic acids and compatible solutes for ion homeostasis: Another important pathway detected in both Variety 1 (MElightyellow) and Variety 2 (MEblue) in leaves for

carboxylic acid biosynthesis was positively regulated by ENDO_1. Carboxylic acids, such as malate and citrate, function as osmolytes and play a role in ion homeostasis by regulating the intracellular sodium and other ions (Che-Othman et al. 2017; Shelke et al. 2019).

Pathways regulated by ENDO_2

1. Proline accumulation and osmotic adjustment: ENDO_2 significantly increased proline levels in roots under salinity stress, reinforcing its role as an osmoprotectant. The upregulation of erythrose 4-phosphate/phosphoenolpyruvate (E4P/PEP) family amino acid metabolism in the *L. perenne* roots suggests a metabolic shift towards stress adaptation through phenylalanine biosynthesis (Ashraf and Harris 2004). Phenylalanine (Phe) is crucial for plant reproduction, growth, development, and defense against stresses (Echevarria and Vidal 2003; Pascual et al. 2016; Feduraev et al. 2020). It is a substrate for many different secondary metabolites, including phenylpropanoids, flavonoids, anthocyanins, phytoalexins and cell wall lignin (Shadle et al. 2003). The enzyme phenylalanine ammonia-lyase catalyzes Phe to trans-cinnamic acid, induced by biotic and abiotic stresses (MacDonald and D'Cunha 2007). However, this process was negatively regulated in the *L. perenne* leaves.
2. Cell wall strengthening and structural reinforcement: Unlike ENDO_1, ENDO_2 upregulated cell wall biosynthesis in Variety 2 leaves, enhancing structural integrity under salt stress. This process may be associated with increased lignin and hemicellulose accumulation, suggesting an adaptive strategy for mechanical stress resistance (MacDonald and D'Cunha 2007).
3. Lipid metabolism for membrane stability: ENDO_2 strongly induced lipid metabolism pathways under salt stress in Variety 1 leaves (MEpurple module). Lipid metabolism is a crucial process for salt stress tolerance in plant cells and plays an important role in ion transport, signal transduction, and plant adaptation to salinity stress (Okazaki and Saito 2014; Mansour et al. 2015; Natera et al. 2016; Wang et al. 2022).

Overall, ENDO_1 predominantly modulated amino acid metabolism, nitrogen assimilation, cell wall adaptation and ion homeostasis, while ENDO_2 exerted a greater influence on proline accumulation and osmotic adjustment, cell wall strengthening and oxidative stress responses. Their effects were tissue-specific, with ENDO_1 being more involved in metabolic resource allocation and stress adaptation in roots, while ENDO_2 played a key role in enhancing structural integrity and antioxidant defenses, particularly in leaves.

Common Pathways Regulated by Both Endophytes

1. Oxidation-stress and reactive oxygen species (ROS) metabolism: Both the endophytes regulated oxidative stress pathways in a variety-specific manner. In Variety 2 leaves (MEblue), ROS detoxification mechanisms were positively regulated, likely mitigating oxidative damage (Biswas and Mano 2015). In contrast, in Variety 1 (MEbrown), the ROS metabolic process and hydrogen peroxide metabolic process were negatively regulated by both the endophytes. Although ROS are common enzymes that play dual roles in plants, acting as both harmful agents and crucial signaling molecules. Down-regulation of ROS metabolism in plants can lead to several consequences, like increased oxidative damage, altered growth and development, and impaired signaling pathways, thus impacting both their physiological and biochemical processes (Singh et al. 2019). The negative regulation of ROS by both endophytes indicates a reduced reliance on ROS scavenging pathways and potential trade-offs in stress adaptation.
2. Energy metabolism and carbohydrate accumulation: salinity can negatively affect the efficiency of photosystem II, thus potentially influencing photosynthetic efficiency and carbohydrate metabolism. The accumulation of carbohydrates enables plants to thrive under saline environments by increasing osmotic adjustment, improved energy production and grass yield, which was previously demonstrated in diverse perennial grasses (Tang et al. 2013b). In this study, carbohydrate and photosynthesis were positively regulated by ENDO_1 and ENDO_2 (MEred– Variety 1 and MEpurple– Variety 2), which was accompanied by higher grass yield. The energy metabolism of plants is sensitive to salinity stress (Zhang et al. 2011). In this study, the process of generation of precursor and energy metabolism was positively regulated (Variety 1– MELightyellow and Variety 2– MEblue) in leaves, which would provide *L. perenne* more energy to withstand salt damage. In roots, however, precursor metabolite generation was negatively regulated in both varieties. Thus suggesting tissue-specific energy partitioning strategies, which were reported in halophyte plants such as *Mesembryanthemum crystallinum* and *S. europaea* (Oh et al. 2015; Furtado et al. 2019a).
3. Organic acids and compatible solute metabolism: ENDO_1 and ENDO_2 negatively regulated alpha-amino acid catabolism in roots of Variety 1 (MEblue) and Variety 2 (MEbrown). This could be part of the plant's response to cope with the adverse effects of salinity and is an essential adaptive mechanism that helps plants manage osmotic stress and maintain cellular homeostasis (Batista-Silva et al. 2019). By accumulating

alpha-amino acids such as proline and glycine betaine, plants can withstand the osmotic pressure caused by salt, protect cellular structures, and minimize damage from water loss, which are crucial for maintaining cellular homeostasis under salt stress.

Endophyte-Mediated Salt Tolerance as a Dynamic, Plant Host-Specific Interaction

Despite both endophytic fungal strains promoting growth, their influence on *L. perenne* gene expression and physiological responses varied between Variety 1 and Variety 2. The differential regulation of key stress-response pathways suggests that endophytes modulate salt tolerance in a host-dependent manner, likely due to genotypic differences in stress perception, metabolism, and signaling.

1. Cell wall adaptation strategies: Variety 1 exhibited a flexibility-driven response, as ENDO_1 downregulated cell wall macromolecule metabolism, facilitating cell expansion and water retention under stress. Conversely, in Variety 2, both endophytes upregulated cell wall biosynthesis pathways, reinforcing structural integrity to mitigate salt-induced mechanical damage. The changes in cell wall composition during salt stress are critical for maintaining cell wall integrity, regulating water movement, and preventing ion toxicity. These differences in regulation suggest that host-specific interactions shape how endophytes modulate stress responses, either by prioritizing flexibility (Variety 1) or strengthening cell walls (Variety 2) to enhance stress tolerance (Van Zelm et al. 2020; Colin et al. 2023).
2. Reactive oxygen species (ROS) regulation and antioxidant activity: a similar divergence was observed in oxidative stress management; while both endophytes downregulated ROS metabolic pathways in Variety 1, suggesting a shift away from ROS detoxification in favor of osmolyte accumulation. Both endophytes upregulated oxidative stress response pathways in Variety 2, supporting an active ROS detoxification mechanism helping maintain redox homeostasis under salt stress. This highlights divergent oxidative stress coping mechanisms, with Variety 1 relying less on ROS scavenging and more on metabolic adjustments, while Variety 2 prioritizes antioxidant defenses.
3. Amino acid and osmoprotectant metabolism: both varieties showed upregulation of 2-oxoacid biosynthesis, supporting amino acid production crucial for osmotic regulation and stress adaptation. ENDO_2 enhanced proline accumulation in high salinity conditions (300 mM NaCl), while ENDO_1 significantly increased total antioxidant capacity, highlighting endophyte-specific roles in osmotic balance. Additionally, positive regula-

tion of amide metabolism in roots of Variety 1 suggests a stronger focus on nitrogen assimilation and transport, helping maintain cellular function under stress. While Variety 2 showed enhanced E4P/PEP metabolism and phenylpropanoid pathways, facilitating lignin biosynthesis and stress resilience.

4. Energy metabolism and photosynthesis: both endophytes improved photosynthetic efficiency in both varieties, correlating with increased chlorophyll content and carbohydrate accumulation. However, energy metabolism showed contrasting trends, with negative regulation of energy generation pathways in the roots of Variety 1, suggesting resource allocation shifts toward other protective mechanisms, whereas precursor metabolism was upregulated in the leaves of Variety 2, likely sustaining energy production under salt stress. These findings suggest a trade-off between energy conservation (Variety 1) and enhanced metabolic activity (Variety 2) in response to endophyte colonization.

The differential gene regulation observed between Variety 1 and Variety 2 underscores the idea that endophyte-mediated salt tolerance is highly genotype-dependent rather than a uniform response. Instead, endophytes fine-tune their influence based on the host plant's inherent stress response mechanisms. This highlights several critical insights:

- The effectiveness of fungal inoculation depends on genotypic factors that shape stress adaptation strategies.
- Endophytes employ multiple strategies to enhance salt tolerance, including structural modifications (reinforcing or loosening cell walls), metabolic shifts (accumulating osmolytes and organic acids), and enhanced antioxidant defenses—each of which is differentially regulated depending on the plant variety.
- Identifying host-specific responses to fungal inoculation can optimize biofertilizer applications, ensuring compatibility between plant varieties and microbial strains for maximum stress resilience.

Overall, the intricate coordination of gene expression and metabolic pathways between inoculated variants of *L. perenne* enables plants to adapt under salinity conditions. This study suggests that fungal endophytes not only improve plant survival under salinity stress but also modulate stress tolerance pathways in a variety-specific manner. Understanding these complex interactions provides a foundation for precision agriculture approaches, where endophyte selection can be tailored to specific crop varieties to enhance crop yield and mitigate the negative effects of salinity stress. While

increasing salt concentration in the substrate limited *L. perenne* growth, fungal inoculation significantly alleviated these detrimental effects. Our results suggest that the two selected fungal strains can improve plant health and alleviate some of the debilitating effects of salt stress via the accumulation of osmolytes, consequently improving ryegrass water status, increasing chlorophyll content, lowering lipid peroxidation, and increasing antioxidants. Moreover, the transcriptome analysis identified key genes involved in abiotic stress-related pathways that were differentially regulated by the two fungi in the leaves and roots, indicating potential biomarkers for bioinoculant selection. Finally, understanding the long-term impacts of fungal endophyte inoculation on plant growth, reproduction, and sustainability under saline conditions is crucial for developing resilient agricultural systems. Additionally, field trials or larger-scale studies would provide valuable insights into the real-world applicability of these findings, assessing the stability of endophyte-plant interactions across different environmental conditions. Beyond plant responses, further investigations elaborating on the transcriptome or proteome of the fungi alone could provide insight into potential fungal mechanisms, which could aid in developing practical strategies for using fungal endophytes in agriculture.

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Author Contributions BF participated in all analyses and wrote the first version of the manuscript. IN designed bioinformatics pipelines, performed computational analyses and participated in the preparation of the manuscript. JT designed protocols for biochemical estimations and analyzed the statistical outputs. NR selected plant varieties for the experiment and provided input on the manuscript. TA and KH conceived the project, designed and supervised lab experiments and analyses and participated in the manuscript preparation. All authors revised the manuscript and approved it for publication.

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Data Availability RNASeq datasets generated during the current study are available in the NCBI repository under BioProject no. PRJNA490634.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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