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Research article

Morphological characteristics and transcriptome analysis of salt-tolerant *Trichoderma* sp. responding to salt-stress conditions

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Abstract

Importance of the work: *Trichoderma* spp., well known for their plant-growth-promoting characteristics and antagonistic activity against plant pathogens, present a viable solution for enhancing agricultural resilience to salinity. However, the molecular mechanisms for adaptation to salt stress in *Trichoderma* species are obscure.

Objectives: To monitor the morphological characteristics and antagonistic capacity of *Trichoderma* spp. against the fungal pathogen *Rhizoctonia solani* in hyper-saline conditions, as well as to investigate its transcriptomic analysis.

Materials and Methods: Three strains of *Trichoderma* (M4.1, V6.1 and CG4.2.3) were evaluated for their adaptability and their resistance to *R. solani* in hyper-saline conditions. Subsequently, transcriptomic analysis was applied to compare the control medium and *Trichoderma asperellum* V6.1, which had the highest adaptation under saline stress among the tested strains in hyper-saline conditions (3% NaCl).

Results: The *Trichoderma asperellum* V6.1 strain not only had considerable salt tolerance but also maintained its antagonistic potential against the plant pathogenic fungus *Rhizoctonia solani* under saline stress. The transcriptomic analysis of the *T. asperellum* V6.1 strain revealed 542 differentially expressed genes involved in molecular function (121 genes), cellular component (336 genes) and biological process (46 genes).

Main finding: The potential was highlighted of *Trichoderma* spp. as a microbial biocontrol agent in saline-affected agricultural landscapes. In addition, important insights were gained into the molecular mechanisms underlying the salt tolerance of *Trichoderma asperellum* V6.1.

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Introduction

Saline intrusion, a result of rising sea levels and other factors, poses major threats to agriculture, affecting soil health, crop growth and overall agricultural productivity (Lim et al., 2020; Marroquin et al., 2023). Regions with arid climates or coastal farmlands with insufficient rainfall are particularly vulnerable to inadequate crop water supply, which can lead to mineral salt leaching out of the root zone (Sahbeni et al., 2023). As saline water intrudes into freshwater systems, it leads to soil salinization, which affects the structure and fertility of the soil, impairs plant growth and reduces crop yield (M. Cheng et al., 2021). Currently, several approaches have been applied to minimize the effects of saline intrusion on agriculture. Together with salt-tolerant plants, salt-tolerant microorganisms play an essential role in promoting plant growth and performing better crop yield and quality in salinized areas (Egamberdieva et al., 2019).

Plant growth-promoting fungi (PGPFs) are a group of fungi that are non-pathogenic and inhabit the rhizosphere, while, as components of the soil-plant system, helping to maintain soil fertility, enhance plant growth, strengthen plant adaptability to environmental changes and induce plant defence systems against pathogen infections (Adedayo & Babalola, 2023). PGPFs include a diverse range of fungi from various classes and habitats, with some of the most commonly reported genera being *Aspergillus*, *Fusarium*, *Penicillium*, *Piriformospora* and *Trichoderma* (Adedayo and Babalola, 2023). These fungi are desirable due to the abundance of bioactive substances that help them overcome competition with other soil factors and survive under variable environmental conditions (Radhakrishnan et al., 2014). Among the various genera of fungi, *Trichoderma* stands out due to its remarkable ability to survive in challenging environmental conditions, such as salinized soil, and its ability to decrease the concentration of harmful ions in the soil (Boamah et al., 2021; Liu et al., 2023). Members of the genus *Trichoderma* are frequently described as PGPFs with their ability for defence against the plant-pathogens, phosphate and potassium solubilization and phytohormone production (Bononi et al., 2020; Illescas et al., 2021; Liu et al., 2023). The utilization of PGPFs, particularly salt-tolerant *Trichoderma* spp., is considered an effective strategy to enhance plant resilience. For example, the presence of *Trichoderma* help plant root development, antioxidant enzyme activity and photosynthesis under saline stress (L. Cheng et al., 2023). Treatment by *Trichoderma* was reported to have substantially improved plant height, root length and chlorophyll in cowpea under 200 mM NaCl (Liu et al., 2023). The effectiveness of *Trichoderma* spp. in promoting plant growth at high saline concentrations has been reported in tomato (Metwally and Soliman, 2023), cucumber (Zhang et al., 2022) and wheat (S. Zhang et al., 2019)

Fungi have had to develop underlying mechanisms to respond to the high-salt environment to survive and proliferate in high-salt conditions. However, the molecular mechanisms for adaptation to salt stress of *Trichoderma* species are unclear. Therefore, the aim of this study was to monitor the morphological characteristics and antagonistic capacity of *Trichoderma* spp. against the fungal pathogen *Rhizoctonia solani* in hyper-saline conditions and to carry out transcriptomic analysis in salinity culture conditions. By comparing the fungal transcriptome in standard growth conditions to that in salinity growth conditions, it was expected to observe the putative genes possibly associated with the salt-tolerant mechanism and the regulatory mechanisms related to salt-tolerance in *Trichoderma*.

Materials and Methods

Strains, media and growth conditions

All three *Trichoderma* strains (M4.1, V6.1 and GC4.2.3) and the *R. solani* CG5.1.1 strain belonged to the HCM Biotech Collection of Microorganisms (HBCM) in Biotechnology Center of Ho Chi Minh City, Vietnam. These fungal strains were isolated from mangrove land and showed ability to adapt to salt-stress growth conditions. Phylogenetic analysis indicated that the M4.1 and V6.1 strains were *T. asperellum*, while the GC4.2.3 strain was *T. caribbaeum*. All strains were grown on potato dextrose agar (PDA) medium, referred to as the standard medium. For morphological characterization under salt stress conditions, strains were grown on PDA plates containing 3%, 5%, 7% or 9% NaCl.

Fungal development assays

Spores of the three *Trichoderma* strains were collected from cultures aged 7 d on PDA plates, suspended in 0.1% Tween 80 in H_2O and adjusted to an approximate concentration of 1×10^7 spores/mL. Then, 1 μL of spore suspension (approximately 1×10^4 spores) was spotted on media plates with incremental NaCl concentrations (0–9% NaCl) and incubated at 25°C. The vegetative development of the strains was recorded based on colony diameter every 24 hup to 96 h. After 5 d of incubation, a 1 cm^2 agar plug was harvested from the center of each colony, excised and suspended in 1 mL 0.1% Tween 80 H_2O. Conidia were counted using a hemocytometer to assess conidia production. The inhibition rate percentage (IR) of NaCl on fungal growth was calculated based on Equation 1 (Liu et al., 2023):

$$IR = (D1 - D2) / D1 \times 100 \quad (1)$$

where D1 is colony diameter of the fungal strain on the standard medium and D2 is the colony diameter of the fungal strain on the NaCl-added medium.

The experiment was performed in triplicate.

Antagonistic activity against R. solani

All four fungal strains (the three *Trichoderma* strains and *R. solani*) were cultured on PDA plates at 25°C for 5 d. Then, a 5 mm plug of each strain was co-inoculated symmetrically on each side of the 90 mm Petri plate containing the PDA medium with either 0% or 3% NaCl at a distance of 4 cm apart. Individual inoculation of *R. solani* on the PDA plates with 0% and 3% NaCl was used as the control. The growth inhibition percentage (GI) was calculated based on Equation 2 (Kim et al., 2023):

$$GI = (G1 - G2) / G1 \times 100 \quad (2)$$

where G1 is the colony diameter of *R. solani* on the control plate and G2 is the colony diameter of *R. solani* on the co-cultured plate.

The experiment was performed in triplicate.

Statistical analysis

Data were analyzed using PRISM version 10 (GraphPad Software, San Diego, CA; www.graphpad.com). One-way ANOVA followed by Tukey's multiple comparison test were performed, with significance set at $p < 0.05$.

Total RNA extraction and sequencing

The fungal strain was inoculated on plates containing PDA and PDA with 3% NaCl. The fungal mycelia were harvested from culture aged 3 d and macerated with liquid nitrogen for RNA extraction. Total RNA was extracted using a Plant/Fungi Total RNA Kit (Norgen Biotek Corp.; Canada) according to the manufacturer's instructions. The extracted RNA was treated using a TURBO DNA-*free*™ Kit (Invitrogen; USA) to eliminate any contaminating residual DNA. The quantity and quality of the extracted RNA were verified using a nanodrop spectrophotometer based on ratios of absorbance at 260 nm/280 nm and 260 nm/230 nm, with gel electrophoresis. The RNA integrity samples were sent to Novogene AIT Genomics Singapore Pte. Ltd. for cDNA library construction and RNA sequencing. Raw reads from two biological replicates *of T. asperellum* grown on PDA and PDA with 3% NaCl are available on NCBI under accession numbers SRR29159167, SRR29159166, SRR29159165 and SRR29159164.

Differential gene expression and gene ontology enrichment analysis

The obtained sequencing data were analyzed using the Galaxy.org platform for gene expression profiling, quality control and trimming. Differential expression genes (DEGs) were identified based on the DESeq2 algorithm using a $\log_2$ transformation of the fold change ($\log_2(FC)$), according to Love et al. (2014). The statistical thresholds for significance were established with a p value criterion of less than 0.05 and a $|\log_2(FC)|$ exceeding zero, indicating substantial differential expression between sample groups. Subsequent to the identification of DEGs, gene ontology (GO) enrichment analysis was carried out utilizing top GO (Batut et al., 2021). Then, the gene list was analyzed to determine the frequency of each GO term, and a hypergeometric distribution was used to calculate the p values. *T. asperellum* CBS 433.97 (assembly Trias v.1.0, accession number GCF_003025105.1) was used as a reference genome.

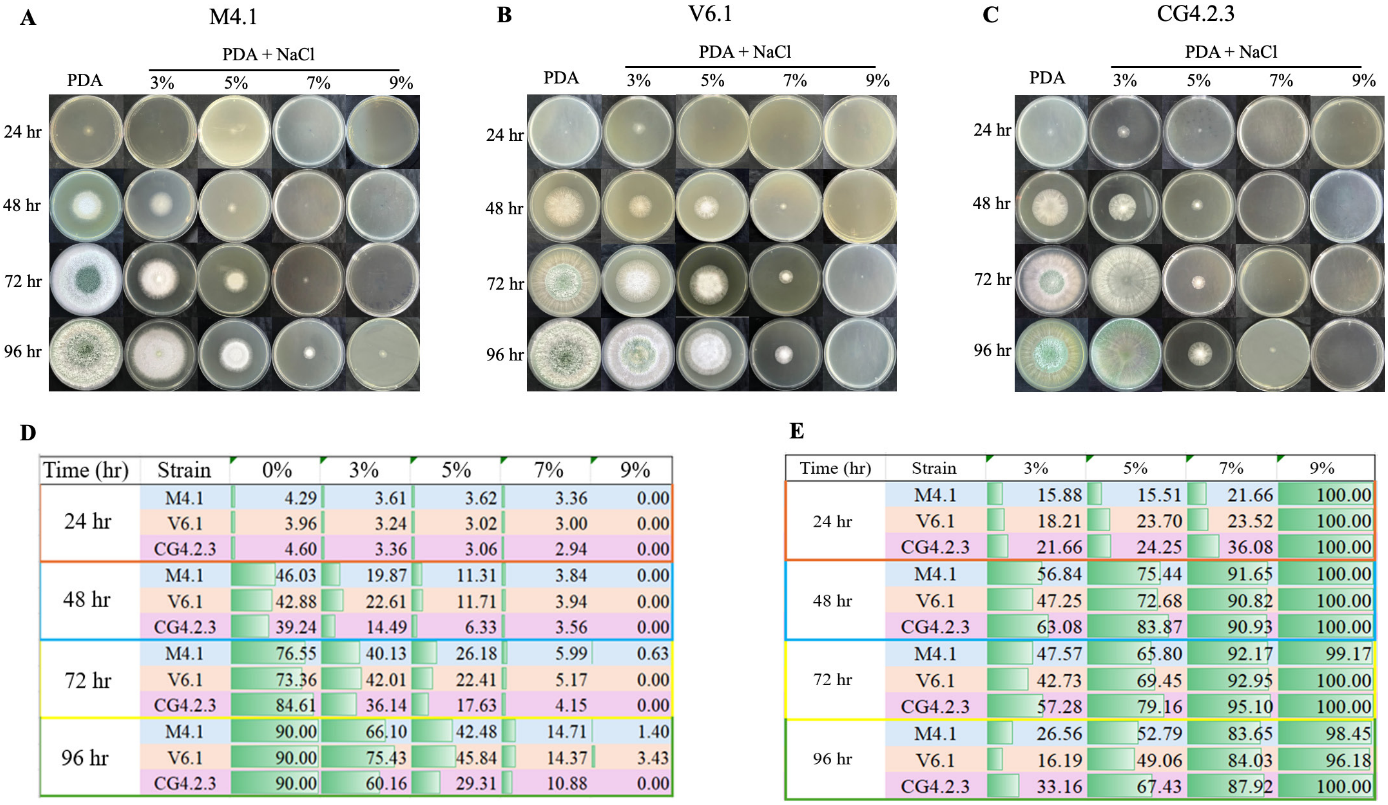
Results

Trichoderma V6.1 enhanced adaptation to salt stress conditions more than two other Trichoderma strains

The three fungal strains were grown in media with various concentrations of NaCl to assess the influence of salt stress conditions on fungal growth. Based on the results (Fig. 1), all three isolates demonstrated salt tolerance and were able to grow on salt-added media. There was a negative correlation between fungal adaptive development and NaCl concentration.

The adaptation to salt stress conditions differed among the three fungal strains. In the absence of NaCl stress, the mycelia of the *Trichoderma* strains proliferated extensively, covering the entire Petri dish within 3–4 d, creating a dense and wadded growth pattern. Conversely, increasing NaCl concentrations introduced a pronounced delay in the germination time of the *Trichoderma*. Salt stress conditions greatly impeded the hyphal growth rate and the aerial hyphae. Initially, at 24 hr, all strains exhibited minimal growth; however, at 96 hr, there was a substantially increase in growth diameter. At lower concentrations of NaCl, specifically 3% and 5%, mycelial growth was detectable after 24 hr. In the presence of 7% NaCl, the growth of CG4.2.3 was completely inhibited, while there was some colony formation in the M4.1 and V6.1 strains. By increasing the concentration of NaCl to 9%, no germination occurred in any of the three strains (Fig. 1A– 1D). Notably, V6.1 was more tolerant to salt stress than M4.1 and CG4.2.3. While the growth of V6.1 was reduced to approximately 16% in the presence of 3% NaCl, the reduction rates of M4.1 and CG4.2.3 were approximately 26% and 33%, respectively, which were more than 1.5 times and 2 times higher than that for V6.1 (Fig. 1E). Similarly, the inhibition of growth in V6.1 in the medium with 5% NaCl was lower than that of M4.1 and CG4.2.3. Overall, the presence of NaCl had less influence on the growth of V6.1 than on M4.1 and CG4.2.3.

Conidia formation was seen only on media containing up to 3% NaCl, with conidia production by M4.1 and V6.1 greatly reduced at the 3% NaCl concentration (Fig. 2A). Among the three strains, CG4.2.3 produced the lowest conidia formation at either 0% or 3% NaCl (Fig. 2A). Inspection of the three strains under a light microscope revealed a reduction in both vegetative growth and conidiation under NaCl-induced osmotic stress (Fig. 2B). Without NaCl, all strains produced robust mycelial structures and conidia. In contrast, at 3% NaCl, their spore production significantly diminished and was particularly evident at 3 d post-exposure (Fig. 1). In addition, the mycelia underwent structural changes, transitioning to a more sparse and fragmented morphology under saline stress. These findings suggested that while all strains exhibited some degree of salt tolerance, their resistance levels varied, with V6.1 having notably higher tolerance to salt-stress conditions than M4.1 and CG4.2.3.



D

Time (hr)	Strain	0%	3%	5%	7%	9%
24 hr	M4.1	4.29	3.61	3.62	3.36	0.00
	V6.1	3.96	3.24	3.02	3.00	0.00
	CG4.2.3	4.60	3.36	3.06	2.94	0.00
48 hr	M4.1	46.03	19.87	11.31	3.84	0.00
	V6.1	42.88	22.61	11.71	3.94	0.00
	CG4.2.3	39.24	14.49	6.33	3.56	0.00
72 hr	M4.1	76.55	40.13	26.18	5.99	0.63
	V6.1	73.36	42.01	22.41	5.17	0.00
	CG4.2.3	84.61	36.14	17.63	4.15	0.00
96 hr	M4.1	90.00	66.10	42.48	14.71	1.40
	V6.1	90.00	75.43	45.84	14.37	3.43
	CG4.2.3	90.00	60.16	29.31	10.88	0.00

E

Time (hr)	Strain	3%	5%	7%	9%
24 hr	M4.1	15.88	15.51	21.66	100.00
	V6.1	18.21	23.70	23.52	100.00
	CG4.2.3	21.66	24.25	36.08	100.00
48 hr	M4.1	56.84	75.44	91.65	100.00
	V6.1	47.25	72.68	90.82	100.00
	CG4.2.3	63.08	83.87	90.93	100.00
72 hr	M4.1	47.57	65.80	92.17	99.17
	V6.1	42.73	69.45	92.95	100.00
	CG4.2.3	57.28	79.16	95.10	100.00
96 hr	M4.1	26.56	52.79	83.65	98.45
	V6.1	16.19	49.06	84.03	96.18
	CG4.2.3	33.16	67.43	87.92	100.00

Fig. 1 Growth of three *Trichoderma* strains at 96 hr under NaCl concentrations (0%, 3%, 5%, 7%, 9%), with colony formation of: (A) M4.1; (B) V6.1; (C) CG4.2.3; (D) radial growth rate (in millimeters) of each *Trichoderma* strain under salt stress conditions; (E) Inhibition rate percentage of fungal growth under different salt stress conditions, where all values represent mean values of three replicates

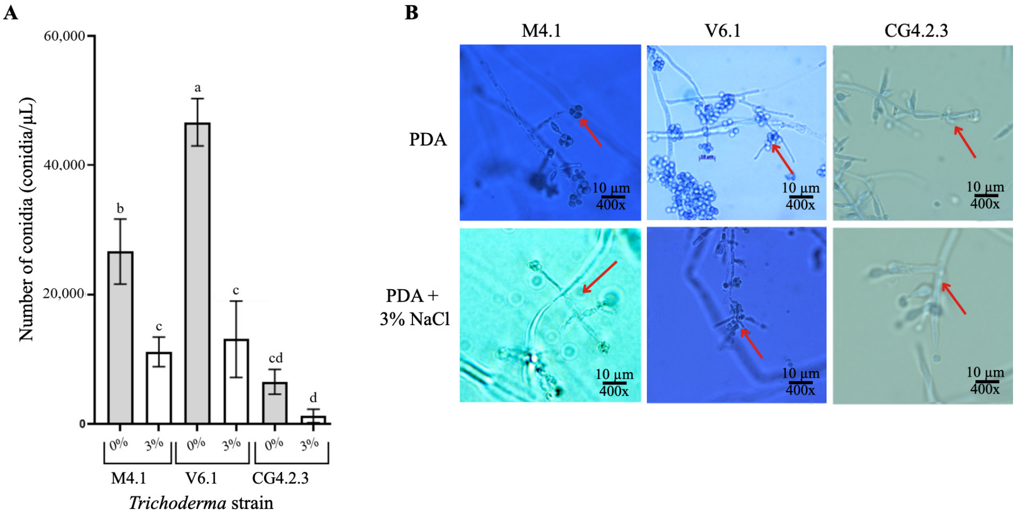


Fig. 2 Conidia production of three *Trichoderma* strains in potato dextrose agar (PDA) and PDA + 3% NaCl media: (A) conidia production, where data presented as mean ± SD from three independent experiments, different lowercase letters above columns indicate significant differences (Tukey's test, $p < 0.05$); (B) microscopic appearance of conidia formation (400× magnification), where red arrows indicate location of conidia formation and scale bars = 10 µm

Effect of salt-stress conditions on Trichoderma strains against R. solani

The pathogenic fungus *R. solani* causes critical damage to the growth and productivity of a wide range of economically important crops (Senapati et al., 2022). The current study analyzed the antagonistic activities against *R. solani* of the three *Trichoderma* strains to investigate whether this activity was affected by salinized conditions. Dual inoculations of each *Trichoderma* strain with the *R. solani* strain were conducted (Fig. 3). All three *Trichoderma* strains inhibited the growth of *R. solani* in both standard and salinized conditions. M4.1 and V6.1 suppressed approximately 90% of the growth of *R. solani* in the absence of NaCl (Figs. 3A, B). By increasing the NaCl concentration to 3%, the ability of these two strains to suppress the growth of *R. solani* slightly decreased to 80%, indicating robust antifungal activity even under salt stress (Fig. 3B). Furthermore, the growth morphology of *R. solani* at 3% NaCl was not distinguishable from that in the medium without NaCl, suggesting that the developmental decline of *R. solani* at 3% was due to the impact of the *Trichoderma* strains. On the other hand, even though CG4.2.3 inhibited the growth of the *R. solani* strain, its inhibition rates were lower than those of M4.1 and V6.1, which were about 80% (standard condition) and 50% (3% NaCl condition), respectively. These results indicated that M4.1 and V6.1 were less influenced than CG4.2.3 by hyper-salt levels regarding antagonistic activity against *R. solani*.

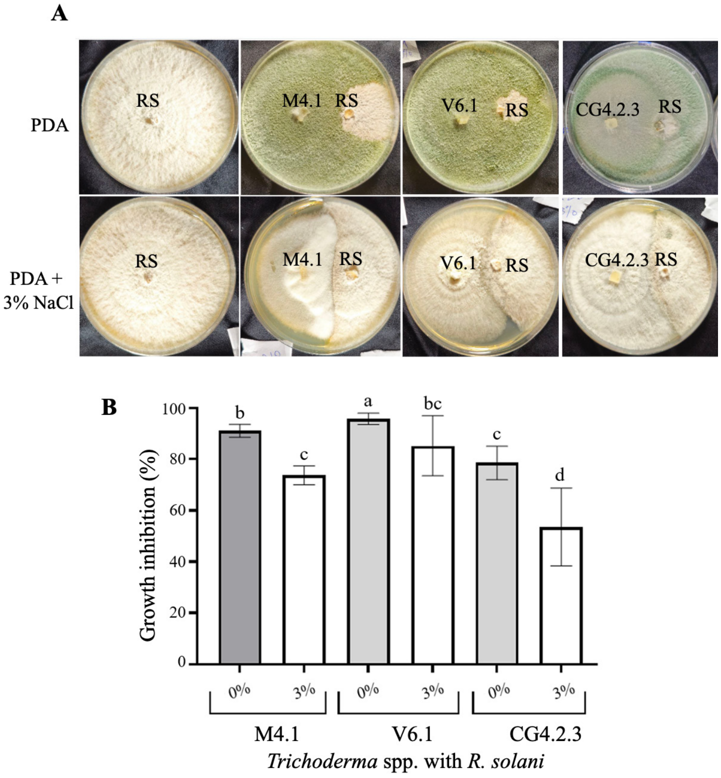


Fig. 3 Antagonistic effect of three *Trichoderma* strains against *Rhizoctonia solani*: (A) *in vitro* antagonistic activity of M4.1, V6.1 and CG4.2.3 strains against *R. solani* (RS) during 96 hr; (B) inhibition rate percentage of each *Trichoderma* spp. on growth of *R. solani*, where data presented as mean ± SD values from three independent experiments, different lowercase letters above columns indicate significant differences (Tukey's test, $p < 0.05$)

Differential expression of genes involved in fungal salt-stress tolerance

The developmental processes and antagonistic activity of the three *Trichoderma* strains were affected by the presence of NaCl. Based on the results, among the three *Trichoderma* strains, CG4.2.3 was more sensitive to salt-stress conditions than M4.1 and V6.1. In addition, V6.1 adapted better to the hyper-salt conditions than M4.1, where the vegetative development, conidiation and antagonistic activity of V6.1 in the NaCl-added media were higher than for M4.1. Based on these results, transcriptome analysis of V6.1 in the presence of 0% and 3% NaCl was analyzed to provide an overall view of the genetic adaptation of *Trichoderma* sp. in a salt-stress environment.

The RNA sequencing data for the V6.1 strain encompassed raw reads from two biological replicates, cultivated under both standard and 3% NaCl stress conditions. For the first replicate, sequencing generated 12,468,005 reads (equating to approximately 8.7 giga base pairs (Gbp) of data under standard conditions) and 11,500,731 reads (approximately 7.8 Gbp under 3% salt stress). The second replicate produced 11,956,509 reads (about 8.2 Gbp) and 10,283,129 reads (approximately 8.7 Gbp) under standard and salt stress conditions, respectively. Nucleotide composition was well balanced, with the GC contents consistently maintained between 50% and 53% across all samples and conditions, indicating high-quality sequencing data suitable for subsequent transcriptomic analysis. A rigorous analytical pipeline was implemented for the RNA-sequencing data of *Trichoderma* spp. strain V6.1 to determine differentially expressed genes in response to salt stress. The obtained data sequences were aligned with *T. asperellum* CBS 433.97. The resulting alignment had a pair ratio exceeding 90%, indicating an exceptional quality of read mappings to the reference, which is a prerequisite for accurate downstream analysis.

The DEGs were identified to learn the putative underlying mechanisms to adapt to salt-stress conditions in *Trichoderma* sp. The DEGs were analyzed using a volcano plot and heatmap, showing overall the most differentially distributed genes and the abundance of the two transcripts (Figs. 4A and 4B). In summary, there were no significant difference in expression level between the replicates in both the standard and salt-stress conditions.

In the GO analysis, a total of 29,935 genes were categorized, with 542 genes showing differential expression. These genes are classified into three primary functional categories: molecular function (MF, molecular activities of gene products), cellular component (CC, where gene products are active) and biological process (BP, pathways and larger processes made up of the activities of multiple gene products), as well as a No answer category (NA, genes that have not been classified into specific categories) (Table S2). Specifically, the number of genes in the MF ontology was 5,689, with 121 differentially expressed genes. In the BP ontology, there were 3,090 genes, with 46 differentially expressed. The CC ontology contained 18,247 genes, with 336 differentially expressed. Additionally, there were 2,909 genes in the NA ontology, with 39 differentially expressed genes.

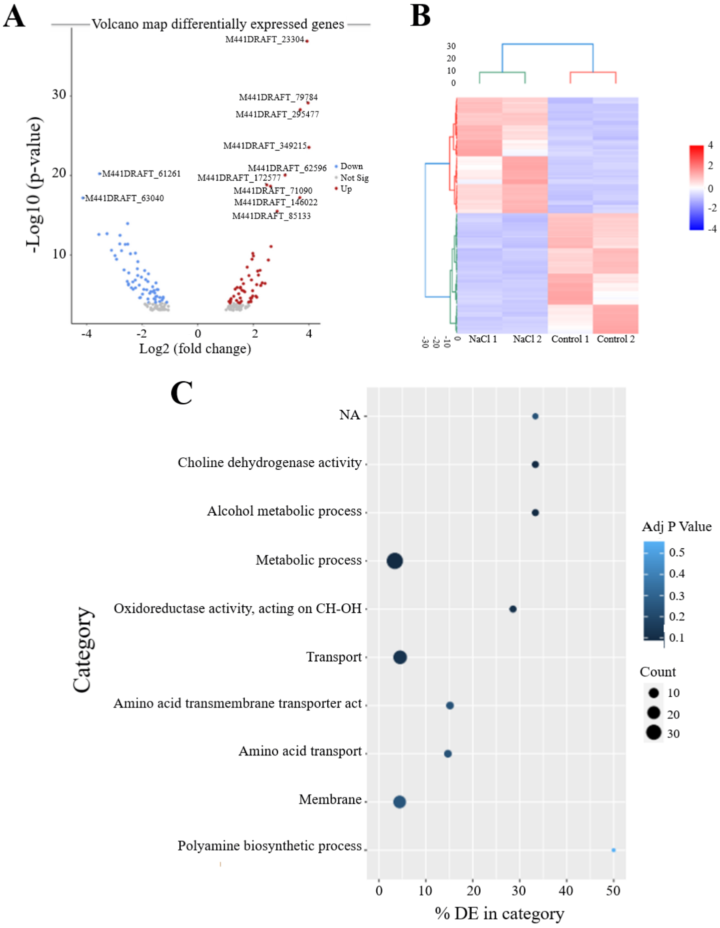


Fig. 4 Transcriptome analysis of *Trichoderma* V6.1 under salt-stress conditions: (A) volcano plot showing differential gene expression levels, where red dots = up-regulated genes, blue dots = down-regulated genes, gray dots = genes with no significant differences; (B) Heatmap of top 50 genes up-regulated and 50 genes down-regulated; (C) gene ontology term enrichment analysis (based on Wallenius method) of differential expression (DE) genes from *T. asperellum* V6.1 strain under control conditions and in presence of 3% NaCl

When running GO analysis for *Trichoderma*, it is expected to find over-represented GO categories related to critical processes for its adaptation to high salinity conditions. Ranking these functions based on over-representation analysis enables the determination of which predefined gene sets are more common (over-represented) in a subset of 'interesting' genes than would be expected by chance (Huang et al., 2009). The *p* value calculated in this analysis helps to determine whether a particular GO category (or term) is significantly overrepresented among the differentially expressed genes compared to what would be expected by chance. These terms indicate processes that are significantly more common among the DEGs than expected by chance, suggesting their potential importance in the stress response of *Trichoderma*. For example, in metabolic processes (GO:0008152), with 36 genes out of a total of 1,058 genes, the over-representation of metabolic processes indicates that *Trichoderma* might be extensively altering its metabolism to cope with high salinity. This could involve the up-regulation of genes involved in pathways that help the fungus manage osmotic stress, produce compatible solutes or detoxify harmful compounds generated due to salt stress. Catalytic activities (GO:0003824) had 28 genes out of 1019 genes, where an increase in genes associated with catalytic activities suggests that various enzymes are being up-regulated. These enzymes might be crucial for biochemical reactions that help *Trichoderma* adapt to the stressful conditions. Transport processes (GO:0006810) had 23 genes out of 508 genes, where the over-representation of transport processes indicates that the fungus is likely to enhance the transport of ions, solutes and other molecules across cellular membranes. This could help in maintaining ionic balance, removing excess salts or importing essential nutrients needed under salt stress conditions. Oxidoreductase activity (GO:0016491) had 21 genes out of 620 genes, where genes related to oxidoreductase activity being over-represented suggests that *Trichoderma* might be experiencing oxidative stress due to high salinity. Oxidoreductases are enzymes that participate in oxidation-reduction reactions, which can help in mitigating oxidative damage by detoxifying reactive oxygen species (ROS) generated under stress (Das et al., 2024).

Transcription factors (TFs) play pivotal roles in the response to environmental stimuli, including salt stress (Wang et al., 2021; Liu et al., 2023). The current study identified 473 genes associated with TFs, out of which 453 were related to DNA-binding transcription factor activity (GO:0003700). The high over-represented *p* value (0.85) indicated that DNA-binding transcription factor activity was not significantly overrepresented among the DEGs. This suggested that the presence of DEGs in this category was likely due to chance. Notably, among these 453 genes, 7 were differentially expressed, indicating this category was present (Table 1). Although the over-represented *p* values for DNA-binding transcription factor activity (0.85) were high, suggesting no significant overrepresentation, the fact that these genes were differentially expressed might still indicate a trend worth exploring. This presence may suggest that TFs could have major involvement in the regulatory mechanisms activated under salt stress conditions. Given the observed trend, even if not statistically significant, it is worth considering that DNA-binding transcription factor activity might play a crucial role in mediating *Trichoderma*'s response to salt stress.

Table 1 List of seven differential expression genes related to 'transcription factors' category

GENE ID	Log2 FC	Function
M441DRAFT_23514	-2.51	Enables DNA-binding transcription factor activity, RNA polymerase II-specific, enables zinc ion binding
M441DRAFT_348506	-3.41	Enables DNA-binding transcription factor activity, RNA polymerase II-specific, Enables zinc ion binding
M441DRAFT_24989	-1.165	Enables DNA-binding transcription factor activity, Enables DNA-binding transcription factor activity, RNA polymerase II-specific, Enables RNA polymerase II cis-regulatory region, sequence-specific DNA binding
M441DRAFT_89070	-1.95	Uncharacterized protein
M441DRAFT_31164	-2.66	Enables DNA-binding transcription factor activity
M441DRAFT_62192	-1.49	Enables zinc ion binding, Enables transcription cis-regulatory region binding, Enables DNA-binding transcription factor activity, RNA polymerase II-specific
M441DRAFT_203472	-3.54	Enables polygalacturonase activity

Further experimental validation and detailed analysis could help confirm whether specific TFs are indeed critical for the organism's adaptation to high salinity. Given the observed trend of overrepresentation of DNA-binding transcription factor activity under salt stress conditions, it is likely that specific TFs are crucial for mediating the plant's response to salt stress.

In the face of osmotic stress induced by high salinity, the integral component of membrane category (GO:0016021) had a significantly low over-represented *p* value (0.05, indicating that this category is overrepresented among the differentially expressed genes. This suggested that the genes involved in the integral component of membranes were likely important in the biological response under the studied condition. The 17 DEGs out of a total of 541 genes in this category suggested a notable involvement of membrane components in the condition being analyzed (Table 2). Membrane proteins play a critical role in plant responses to salt stress by mediating ion transport, osmoregulation and stress signaling (Rehman et al., 2022; Banik and Dutta, 2023).

Discussion

The *Trichoderma* genus is well-known for its widespread applications in agriculture, industry and medicine due to its advantages in plant growth promotion, biological control of plant diseases, biofertilizer production and commercial enzyme production (Fischer et al., 2021; Tyśkiewicz et al., 2022). Several studies have investigated the influence of saline stress on *Trichoderma's* biological characteristics and its antagonistic activity. For example, *T. longibrachiatum* strain isolated from saline-alkaline soil grew at 8% salt, but sporulation decreased substantially, with no obvious alteration in fungal morphology on 2% salt compared to that on untreated salt (Liu et al., 2023). Additionally, under 200 mM NaCl, *T. longibrachiatum* penetrated and inhibited about 68% of the growth of the plant pathogen *Fusarium oxysporum* (Liu et al., 2023). Similarly, a *T. atroviride* strain isolated from a sea mud sample grew on media with 9% NaCl, in which the colony diameter reached about 4 cm after 3 d of inoculation (Zhang, C. et al., 2022).

Table 2 List of 17 differential expression genes related to 'integral component of membrane' category

GENE ID	Log2FC	Function
M441DRAFT_23304	4.39	Enables carbohydrate:proton symporter activity
M441DRAFT_55122	-4.62	Enables transmembrane transporter activity
M441DRAFT_132651	1.49	Enables ATP binding, Enables ATP hydrolysis activity, Enables P-type divalent copper transporter activity, Enables copper ion binding
M441DRAFT_55602	-3.52	Enables phosphate transmembrane transporter activity
M441DRAFT_26261	2.75	Enables carbohydrate:proton symporter activity
M441DRAFT_139533	2.30	Enables transmembrane transporter activity, Enables myo-inositol:proton symporter activity
M441DRAFT_193082	1.61	Enables transmembrane transporter activity
M441DRAFT_170192	2.10	Enables transmembrane transporter activity
M441DRAFT_59384	-1.39	Enables ABC-type transporter activity, Enables ATP binding, Enables ATP hydrolysis activity
M441DRAFT_144956	2.11	Enables transmembrane transporter activity
M441DRAFT_147765	-2.74	Enables transmembrane transporter activity
M441DRAFT_30101	3.29	Enables carbohydrate:proton symporter activity
M441DRAFT_200931	2.71	Uncharacterized protein
M441DRAFT_201001	-2.66	Enables carbohydrate:proton symporter activity
M441DRAFT_50752	-2.18	Enables amino acid transmembrane transporter activity
M441DRAFT_154241	-6.39	Enables transmembrane transporter activity
M441DRAFT_154588	3.17	Enables ABC-type transporter activity, Enables ATP binding, Enables ATP hydrolysis activity

Under 100 mM salt stress, the vegetative development of *F. oxysporum* was suppressed significantly, up to 50% and 70%, by the volatile and non-volatile metabolites produced by *T. atroviride*, respectively (Zhang, C. et al., 2022). The presence of *T. citrinoviride* reduced leaf disease symptoms in strawberries caused by *R. solani* by 63%, even in salt-stress conditions (Sekmen et al., 2021). Two other salt-tolerant *Trichoderma* strains, *T. koningii and T. harzianum*, isolated from the rhizosphere of cucumber plants, inhibited the fungal pathogen *Sclerotinia sclerotiorum* and produced highly active cell wall lytic enzymes, such as chitinase and cellulose, under saline stress (Abdelrhim et al., 2024). In the current study, the variability in the salt tolerance ability within *Trichoderma* spp. revealed that the isolates had varied levels of resilience to NaCl concentrations in the three fungal strains, M4.1, V6.1 and CG4.2.3, with V6.1 showing remarkable adaptability. There was a correlation between increased NaCl concentration and inhibited growth dynamics such as delayed mycelia emergence, reduced colony size and thinner mycelia. Among the three strains, V6.1 not only withstood up to 9% NaCl but also had a high yield of sporulation at 3% NaCl, suggesting adaptive mechanisms counteracting salt stress. Furthermore, V6.1 had significant antagonism, achieving around a 90% inhibition rate against *R. solani* even under conditions of salt stress, highlighting its potential in agricultural applications where soil salinization and pathogenic threats coexist.

There were seven DEGs related to the category ‘transcription factors’. Notably, among these seven genes in terms of transcription factors, three genes are related to zinc-ion binding (Table 1), with two genes related to zinc-ion binding present in the list of 30 down-regulated genes (Table 4). Zinc is a fundamental element that is required for all domains of life. For example, in the nematode-trapping fungus *Drechslerella dactyloides*, mutation of the zinc finger transcription factor Crz1 increased the growth of *D. dactyloides* in the presence of NaCl and KCl (Zhao et al., 2022). In *Aspergillus nidulans*, the *aslA* gene, encoding a zinc finger transcription factor, was related to K^+ stress tolerance, while *aslA* deletion reduced the negative effects of K^+ stress on fungal growth and asexual development (Park et al., 2015). To date, there has been no published evidence on the role of zinc metabolism in fungal salt tolerance. However, GO analyses with the term ‘zinc-ion binding’ in *A. fumigatus*, *Candida albicans*, *C. neoformans*, *C. gattii* and *Sacharomyces cerevisiae* indicated that about 5% of proteomes were involved in zinc finger transcription factors and zinc-binding proteins. Additionally, 25% of these proteins were related to biological processes (Staats et al., 2013). In addition, zinc metabolism was associated with virulence against pathogenic fungi such as *A. fumigatus* and *C. albicans*. In plants, the essential role of zinc has been widely reported in responses to salinity stress. For example, zinc application mitigated the inhibitory effect of salt stress treatment through various cellular process, such as physiological and photochemical processes, in the antioxidant system (Al-Zahrani et al., 2021; Faizan et al., 2021; Noreen et al., 2021). As there is a close connection between zinc and biological processes in various species, there may be a link between zinc metabolism and fungal salt tolerance. However, more study is necessary to confirm this.

The M441DRAFT_84899 (GO:0008152, GO:0016491, GO:0006118, GO:0018658) and M441DRAFT_146866 (GO:0016491, GO:0050660, GO:0003824) gene is involved in enabling flavin adenine dinucleotide (FAD) binding and is important in various cellular processes requiring FAD as a cofactor (Rehman et al., 2022), as shown in Table 3. Based on the current results, the M441DRAFT_146866 gene was highly activated in the group affected by salt stress, while the M441DRAFT_84899 gene was also up-regulated under normal conditions. Based on the NCBI gene references and the GO analysis undertaken, M441DRAFT_146866 also enables monooxygenase activity, which is involved in secondary metabolite biosynthetic processes, oxidoreductase activity, electron transport and salicylate 1-monooxygenase activity. Additionally, M441DRAFT_146866 facilitates oxidoreductase activity. The up-regulation of M441DRAFT_146866 under salt stress conditions suggests its involvement in the plant’s adaptive response to high salinity. This gene’s activation may help in managing oxidative stress by facilitating oxidoreductase activity, which reduces ROS generated under salt stress (Ma et al., 2022; Banik and Dutta, 2023). Similarly, the M441DRAFT_84899 gene being up-regulated under normal conditions indicates its role in maintaining regular metabolic functions that are crucial for the plant’s growth and development. Its involvement in FAD binding ensures the proper functioning of various cellular processes under non-stress conditions (Ma et al., 2022). Studies have shown that FAD-binding proteins are involved in the plant’s response to environmental challenges. For example, in *Mycobacterium smegmatis*, FAD-sequestering proteins contribute to resistance against hypoxic and oxidative stress (Harold et al., 2019; Zhao et al., 2021) demonstrated that GmGPDH12, a FAD-binding protein, from soybean, played a crucial role in enhancing stress resistance by affecting the redox state and respiration (Zhao et al., 2021). Similarly, in the plant *Arabidopsis thaliana*, the FAD gene was reported to regulate the response to various abiotic stresses, including salt stress (Yuan et al., 2012). Transcriptome analysis in *Arabidopsis* and rice also indicated that the FAD_binding_4 gene has an influence on the drought and salt responsive pathways (Liu et al., 2021). These studies suggested that the regulation of FAD genes was related to plant tolerance to abiotic stresses, indicating a major role for FAD-binding proteins in responding to environmental challenges such as high salinity. The activation of the M441DRAFT_146866 gene under salt stress conditions underscores the importance of FAD-binding proteins in coping with environmental stressors, offering valuable insights into the adaptive strategies of organisms like *T. asperellum*. In contrast, the M441DRAFT_84899 gene was inactivated under these stress conditions, further suggesting its specific role under normal growth conditions.

Table 3 List of top-30 up-regulated genes and their functions

GENE ID	Log2FC	Function
M441DRAFT_146022	4.996045833	Enables transketolase activity
M441DRAFT_349215	4.98254586	Enables oxidoreductase activity
M441DRAFT_158208	4.921940984	Uncharacterized protein
M441DRAFT_70990	4.681014332	Uncharacterized protein
M441DRAFT_79784	4.64800092	Enables glycerol channel activity, Enables water channel activity
M441DRAFT_23304	4.399809217	Enables carbohydrate:proton symporter activity
M441DRAFT_109378	4.387771098	Uncharacterized protein
M441DRAFT_295477	4.214035071	Involved in glycerophospholipid biosynthetic process
M441DRAFT_58374	3.826298005	Uncharacterized protein
M441DRAFT_62596	3.613519079	Enables aldose reductase (NADPH) activity
M441DRAFT_447703	3.470523991	Uncharacterized protein
M441DRAFT_85133	3.340627737	Uncharacterized protein
M441DRAFT_30101	3.295810529	Enables carbohydrate:proton symporter activity
M441DRAFT_146093	3.262209293	Uncharacterized protein
M441DRAFT_151795	3.253064038	Enables ATP binding, Enables ATP hydrolysis activity, Enables P-type sodium:potassium-exchanging transporter activity
M441DRAFT_144988	3.234988351	Enables acyltransferase activity, Transferring groups other than amino-acyl groups
M441DRAFT_40903	3.222644525	Enables phosphatase activity
M441DRAFT_154588	3.175496568	Enables ATP hydrolysis activity, Enables ATP binding, Enables ABC-type transporter activity
M441DRAFT_29674	3.057442877	Uncharacterized protein
M441DRAFT_151250	2.995736436	Uncharacterized protein
M441DRAFT_59379	2.97387181	Uncharacterized protein
M441DRAFT_71090	2.88685634	Enables isomerase activity
M441DRAFT_47636	2.847290916	Enables serine-type endopeptidase activity, Enables tripeptidyl-peptidase activity
M441DRAFT_39359	2.837377658	Uncharacterized protein
M441DRAFT_145521	2.781375297	Enables potassium ion transmembrane transporter activity
M441DRAFT_91392	2.76154916	Uncharacterized protein
M441DRAFT_73728	2.760889526	Enables succinyl-coa:3-oxo-acid coa-transferase activity
M441DRAFT_26261	2.758136651	Enables carbohydrate:proton symporter activity
M441DRAFT_154065	2.724778176	Enables antiporter activity
M441DRAFT_200931	2.716387097	Uncharacterized protein

Table 4 List of top-30 down-regulated genes and their functions

GENE ID	Log2FC	Function
M441DRAFT_63040	-8.053982663	Uncharacterized protein
M441DRAFT_154241	-6.396267796	Enables transmembrane transporter activity
M441DRAFT_334617	-5.775915316	Enables heme binding, Enables monooxygenase activity Enables oxidoreductase activity, Acting on paired donors, With incorporation or reduction of molecular oxygen
M441DRAFT_180160	-5.741100485	Uncharacterized protein
M441DRAFT_55018	-4.838412699	Uncharacterized protein
M441DRAFT_84899	-4.796817532	Enables FAD binding, Enables monooxygenase activity
M441DRAFT_201221	-4.669715355	Enables agmatinase activity, Enables metal ion binding
M441DRAFT_55122	-4.628496853	Enables transmembrane transporter activity
M441DRAFT_63030	-4.456052922	Uncharacterized protein
M441DRAFT_41593	-4.453161699	Uncharacterized protein
M441DRAFT_63031	-4.417091438	Putative secondary metabolism biosynthetic enzyme
M441DRAFT_40049	-4.378545122	Putative secondary metabolism biosynthetic enzyme
M441DRAFT_61261	-4.315594612	Putative NRPS-like protein biosynthetic cluster
M441DRAFT_138309	-4.101437674	Uncharacterized protein
M441DRAFT_150506	-4.000575213	Uncharacterized protein
M441DRAFT_84900	-3.885357145	Involved_in secondary metabolite biosynthetic process
M441DRAFT_151574	-3.859140385	Enables chitin binding
M441DRAFT_196089	-3.75142696	Enables nucleotide binding, Enables oxidoreductase activity
M441DRAFT_141873	-3.621810743	Enables serine-type endopeptidase activity, Enables tripeptidyl-peptidase activity
M441DRAFT_203472	-3.545897113	Enables polygalacturonase activity
M441DRAFT_55602	-3.520848143	Enables phosphate transmembrane transporter activity
M441DRAFT_180171	-3.515646615	Uncharacterized protein
M441DRAFT_348506	-3.417876922	Enables DNA-binding transcription factor activity, RNA polymerase II-specific Enables zinc ion binding
M441DRAFT_145495	-3.407913665	Enables metalloendopeptidase activity, Enables zinc ion binding
M441DRAFT_69237	-3.305485155	Uncharacterized protein
M441DRAFT_84740	-3.287943564	Enables dioxygenase activity
M441DRAFT_30545	-3.235890301	Uncharacterized protein
M441DRAFT_445380	-3.153910149	Hypothetical protein
M441DRAFT_62191	-2.991528716	Enables transmembrane transporter activity
M441DRAFT_29935	-2.96918807	Uncharacterized protein

The M441DRAFT_146022 gene (GO:0003824, GO:0008152, GO:0004802), which was highly expressed under hyper-saline conditions in the current study, is involved in transketolase activity in the pentose-phosphate shunt and is located in the cytosol (Table 3). Transketolase is a multi-functional enzyme that is responsible for two cellular pathways: the pentose phosphate pathway and glycolytic pathway. For example, in the yeast-like fungus *Moniliella megachiliensis*, transketolase was related to both hyper-saline and osmotic stress adaptation (Iwata et al., 2017). In the presence of 1.2 M NaCl, the expression level of the transketolase-encoded gene increased rapidly in the first stage of cultivation up to 11 times and subsequently decreased gradually, suggesting the requirement of transketolase in response to salt stress. In the pathogenic bacterium *Staphylococcus aureus*, transketolase played an essential role in regulation for stress response during the cellular infection process of the bacterium (Tan et al., 2019). Other studies in human cells reported evidence on the critical role of transketolase in resisting oxidative and light stress (Xu et al., 2016; Chen et al., 2022) . Although there has been only limited publication regarding transketolase activity and stress resistance, the results from those studies and from the current study demonstrated an essential function of transketolase in adaptation to environmental stress and salt-stress in *T. asperellum*.

Notably, the expression level of the M441DRAFT_79784 (GO:0005215, GO:0006810, GO:0016020) gene, which functions in aquaporins (water channels) and glycerol channels activity, was greatly up-regulated under saline stress. Aquaporins mainly function in controlling water transport across cell membranes and are widely distributed in various species as cellular responses to stress conditions, especially in hyper-saline conditions. In the arbuscular mycorrhizal fungus *Glomus intraradices*, the increase in expression level of the aquaporin gene *Aqp1* was detected by treatment with 60 mM NaCl (Aroca et al., 2009). Genes encoding plasma membrane aquaporins (PIPs) were highly expressed when tomato seedlings were subjected to 150 mM NaCl (Jia et al., 2020; Li et. al., 2021) reported PIPs as a positive regulator for salt tolerance in sugarcane. Overexpression of a PIP from sugarcane in *Arabidopsis thaliana* enhanced the ability to resist salinity stress in the transgenic plants (Tang et al., 2021). Similarly, by introducing and inducing expression of a fungal aquaporins protein in soybean, the transgenic soybeans showed substantial salt tolerance in 250 mM NaCl (Li et al., 2021). Notably, the arbuscular mycorrhizal fungus *Paraglomus occultum* improved the growth of tomato seedlings in 150 mM NaCl, with the expression of tomato PIPs highly up-regulated by the presence of NaCl (Liang et al., 2022). The current study also identified that transcriptome analysis of V6.1 subjected to 3% NaCl had a high level of expression of the gene related to aquaporins action, revealing the involvement of aquaporins in salinity tolerance in *T. asperellum*.

The current study carried out analysis based on a specific function such as carbohydrate:proton symporter activity, which is crucial for the regulation of cellular homeostasis, particularly in the context of salt stress. Through transcriptomic analysis and subsequent GO enrichment, a total of 68 genes were annotated with carbohydrate:proton symporter activity; however, only four differentially expressed genes were identified in this category. Among them, three were up-regulated under salt stress conditions: M441DRAFT_23304 (GO:0005351, GO:0008643, GO:0016020, GO:0005215, GO:0006810, GO:0016021, GO:0008733), M441DRAFT_30101 (GO:0005351, GO:0008643, GO:0016020, GO:0005215, GO:0006810, GO:0016021, GO:0008733) and M441DRAFT_26261 (GO:0005351, GO:0008643, GO:0016020, GO:0005215, GO:0006810, GO:0016021, GO:0008733). One gene, M441DRAFT_201001 (GO:0005215, GO:0006810, GO:0016021, GO:0008733), was up-regulated under normal conditions. The up-regulation of these genes suggested a strategic cellular response aimed at augmenting the uptake of carbohydrates, possibly to accumulate compatible solutes that contribute to osmotic balance within the fungal cells, thereby enhancing tolerance to saline environments.

The current study provided insightful transcriptional data on the differential responses of *T. asperellum* V6.1 to salt stress. The data revealed several potential genes that may play critical roles in fungal hyper-saline response. Future studies should be carried out focusing on the function of individual genes to expand understanding of *Trichoderma* adaptation to salt stress. The *T. asperellum* V6.1 strain displayed the ability to inhibit the fungal pathogen *R. solani* in salt-added media, suggesting it may be a potential PGPF. More research should be considered on the application of the V6.1 strain as a PGPF under salt stress conditions, as well as transcriptome analysis of the V6.1 strain against *R. solani* under such conditions.

Conclusions

This study revealed, for the first time, transcriptome analysis of the *T. asperellum* under hyper-saline conditions. The analysis identified DEGs involved in molecular function (121 genes), cellular component (336 genes) and biological process (46 genes). It highlighted several biological processes contributing to adaptation of *T. asperellum* to high salinity, such as cellular membrane transportation, metabolic process and catalytic activity. The current findings should encourage further investigation regarding which genes in these biological processes are involved in the response of *T. asperellum* to salt stress.

Conflicts of interest

The authors declare no conflicts of interest.

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