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Effects of mycorrhizal and *Trichoderma* treatment on enhancing maize tolerance to salinity and drought stress, through metabolic and enzymatic evaluation

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Abstract

Background Nowadays, climate change has intensified environmental stresses, including salinity and drought stress. Salinity and drought significantly impair crop growth and yield by affecting physiological and biochemical processes. One of the ways to enhance environmental stress tolerance in plants is to improve their symbiotic relationships with soil microorganisms. This study investigates the impact of arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* (accession number: PV544806) inoculation on maize to trace the activated pathways under stress conditions. Maize plants were exposed to different stress conditions: salinity (S1D0), drought (S0D1), and a combination of both salinity and drought (S1D1). They received treatments with arbuscular mycorrhizal fungi (AMF) (M1T0), *Trichoderma* (M0T1), and a combination of both (M1T1).

Results Inoculation of maize plants with AMF and *T. harzianum* markedly enhanced root dry weight, root volume, and total biomass under stress conditions. Additionally, the simultaneous inoculation of AMF and *T. harzianum* under combined salinity and drought conditions significantly affected traits such as dry weight of aerial parts, total biomass, and root colonization percentage compared to the non-inoculated control. Physiologically, the results also indicated that the inoculation significantly increased the activity of antioxidant enzymes SOD and APX. Results from GC-MS analysis and metabolic pathway analysis showed that the combined inoculation of AMF and *Trichoderma* in maize plants stimulated the production of specific secondary metabolites such as oxaloacetate, Δ1-piperidine-6-carboxylate, and cadaverine under stress conditions.

Conclusions Based on this study's findings, the use of AMF and *T. harzianum* can enhance maize growth and performance under salinity and drought stress by stimulating the production of secondary metabolites.

Keywords Secondary metabolites, Microbial inoculation, Stress condition, GC-MS analysis, Antioxidant enzymes

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Introduction

Climate change intensifies environmental stresses such as salinity and drought by altering temperature and precipitation patterns. In arid and semi-arid regions, due to low rainfall, high temperatures, and elevated evaporation and transpiration rates, the impacts of heat, salinity, and drought stress occur concurrently, making it difficult to distinguish their individual adverse effects [1]. The incorporated effects of these stresses significantly impair various morphological, physiological, and molecular processes in plants [1], leading to irreversible damage and threatening food security [2]. An increased buildup of salts during salinity stress causes ion toxicity and lowers osmotic potential, which leads to physiological drought in plants. Additionally, osmotic stress causes stomatal closure, which reduces water uptake by the roots [3]. These deficiencies subsequently disrupt processes related to mRNA synthesis, protein synthesis, amino acid biosynthesis, and other essential cellular activities [4]. Drought stress, due to the limitation of available water for the plant, leads to disruptions in germination [5], reduced plant growth, increased plant temperature [6], stomatal closure, and consequently disturbances in carbon metabolism [7]. Under drought stress, the production of Reactive Oxygen Species (ROS) can exceed the capacity of ROS scavenging systems, resulting in cellular damage such as lipid peroxidation, protein denaturation, DNA damage, and reduced photosynthesis [8]. Various strategies are employed in plants to mitigate the adverse effects of these stresses. Increasing the extent of interaction and strengthening the symbiosis between plants and microorganisms is one of the methods to enhance plant tolerance and reduce the negative impacts of stress [9]. In nature, plants form symbiotic relationships with numerous beneficial microorganisms, especially bacteria and fungi, which play a crucial role in plant health, growth enhancement, and increased performance and productivity [10]. These endophytic, epiphytic, and rhizospheric microorganisms enhance plant tolerance to abiotic stresses by providing additional water and nutrients, producing various metabolites and growth-promoting compounds, and improving enzyme performance [11].

Arbuscular mycorrhizal fungi (AMF) and *Trichoderma* are rhizospheric microorganisms of significant abundance and importance. AMF forms symbiotic relationships with over 80% of plant species [12]. *Trichoderma* is also found in almost all natural ecosystems [13], and many species of it grow in the rhizosphere and on the roots of various non-pathogenic monocotyledonous and dicotyledonous plants [14]. In recent years, numerous studies have been conducted on the potential role of AMF fungi and *Trichoderma* species in enhancing plant stress tolerance. The results show that plants treated with AMF fungi and *Trichoderma* exhibit better morphological, physiological, and biochemical outcomes than control plants [15]. Through various mechanisms, AMF and *Trichoderma* fungi enhance plant growth and health under stress conditions. These include improving water and nutrient uptake [16], secreting secondary metabolites [17], influencing plant hormones [18], accumulating osmolytes [19], and affecting oxidative enzyme activities [20]. However, detailed information on how AMF and *Trichoderma* influence plants and their interaction when both fungi are applied simultaneously under salinity, drought, and particularly combined salinity and drought stress, concerning various physiological, biochemical aspects, and secondary metabolite synthesis, is still lacking.

Maize is a key agricultural crop with multiple applications and has lower tolerance to salinity and drought stresses compared to wheat and barley [21]. This plant has a relatively high symbiosis with rhizospheric microorganisms, particularly AMF. Therefore, this study aims to investigate the synergistic effects of *Trichoderma* and AMF on maize under salinity and drought stress by evaluating secondary metabolites and antioxidant enzymes and exploring their relationship with plant morphological parameters.

Materials and methods

Sampling and microorganism selection

Initially, 60 samples were collected from the roots and rhizosphere soil of wild plants in four districts of Kerman Province, including Shahdad (30° 25′ 1.54″ E, 57° 42′ 22.09″ N), Bam (29° 6′ 4.25″ E, 58° 20′ 39.37″ N), Rafsanjan (30° 24′ 48.04″ E, 55° 59′ 19.85″ N), and Zarand (30° 48′ 0″ E, 56° 34′ 12″ N), which were under salinity and drought stress. The collected roots from different areas were stained using the Koske & Gemma method to measure AMF colonization [22]. The level of root infection was assessed by counting the number of colonized root segments under a microscope, and the total percentage of colonization was calculated. Colonization was investigated by examining the roots under a light microscope, and the extent of AMF colonization was determined based on the presence of fungal structures such as hyphae, vesicles, and arbuscules. The colonization percentage was calculated as follows: the number of positive samples (those with observed fungal structures) was divided by the total number of samples and then multiplied by 100. The soil collected from around the plant roots was also transferred to the laboratory and air-dried, and then AMF spores were extracted and counted in all samples using the Jansa method [23]. The soil's electrical conductivity (EC) was determined using the water extracted from a saturated soil-water paste method and measured with a Jenway 4020 laboratory conductivity meter (Cole-Parmer, Staffordshire, UK). The pH of soil

samples was measured directly on the saturated soil-water paste using the Jenway 3020 pH meter. In this stage, 30 samples with a colonization percentage of 85% or higher and a higher number of spores (more than 50 spores in 100 gr soil) and EC higher than 10 ds/m were selected. These samples were then cultivated in three replicate pots (17 cm height, 18 cm diameter, filled with 1 kg of soil each) for 90 days on sorghum plants under greenhouse conditions at the Shahid Bahonar University of Kerman. Sorghum is a compatible host plant for all known genera of mycorrhizae [24]. Finally, five samples with root colonization rates above 85% and a minimum of 50 spores per 100 g of soil were selected as the primary mycorrhizal inoculum. These samples were mixed and used as an inoculum in the greenhouse cultivation.

A *Trichoderma* isolate was also separated from the final inoculum for use as a *Trichoderma* inoculum. Assuming that the microorganisms (mycorrhizae and *Trichoderma*) in these samples had become compatible and contributed to plant growth. To obtain a pure *Trichoderma* sample, a spore was transferred to a Potato Dextrose Agar culture medium and maintained in an incubator. After 7 days, conidia and conidiophores of *Trichoderma* were observed under a microscope. The spores were transferred to a liquid PDA medium and incubated at 30 °C, shaking at 180 rpm for 5 days to prepare the *Trichoderma* inoculum. The culture medium was centrifuged (5000 g, 20 min), and the concentration was adjusted to 10^6 spores/mL using sterile distilled water.

DNA isolation, amplification, and sequencing of *Trichoderma*

Pure cultures of isolates were subcultured on PDA and incubated at 25 °C for 8 to 16 days. Fungal mycelium (45 to 50 mg) was ground into powder using liquid nitrogen, and total DNA was extracted with an AccuPrep Genomic DNA Extraction Kit. The extracted DNA was analyzed on a 0.1% agarose gel and stored at − 20 °C. The amplification of the internal transcribed spacers (ITS1 and ITS2), along with the 5.8 S ribosomal RNA gene, was conducted to verify identity. The ITS region and TEF-1a gene were amplified utilizing the primer pairs ITS1/ITS4 [25]. Polymerase chain reaction (PCR) was conducted as described by Hashemi and Mohammadi [26] by Techne TC-312 Thermal Cycler (Techne, Cambridge, U.K.). The PCR products were visualized on 1% agarose gels (UltraPure Agarose; Invitrogen, Carlsbad, CA). A 100-bp ladder (GeneRuler DNA Ladder Mix, Fermentas, Vilnius, Lithuania) was used as a molecular weight marker to estimate the size of PCR products. PCR products were purified and sequenced in both directions by Bioneer Corporation (Daejeon, South Korea). Fungal species were initially identified using the MegaBLAST function of the National Center for Biotechnology Information's GenBank nucleotide database (https://www.ncbi.nlm.nih.gov).

Final experiment on maize

The experiment was conducted in a factorial design within a completely randomized design framework, with four inoculation treatments, four stress treatments, and four replications. The pots were divided into four groups based on the microorganisms used: inoculation with mycorrhizae (M1T0), inoculation with *Trichoderma* (M0T1), inoculation with a mixture of mycorrhizae and *Trichoderma* (M1T1), and a control treatment without inoculation (M0T0). Each group included 4 levels of stress: salinity at 8 dS/m (S1D0), drought equivalent to 35% FC (S0D1), combined salinity and drought stress (S1D1), and a control without stress with salinity at 1 dS/m and irrigation equivalent to 80% FC (S0D0). To the pots containing mycorrhizae, 100 g of AMF inoculum was added as a layer at a depth of 5 cm below the sterilized soil surface to each inoculated pot. 100 g of autoclaved (121 ∘C, 20 min) inoculum were used for the control pots. Maize seeds were initially sterilized with 70% ethanol for 30 min and 10% hypochlorite for 5 min, then rinsed with distilled water. The seeds were then germinated, and seven germinated seeds were planted in each pot containing 4 kg of soil (Table 1); after 2 weeks, five uniform plants were kept, and the rest were removed. *Trichoderma*, salinity, and drought stress treatments were applied two weeks after planting. For *Trichoderma* treatments, 70 mL of conidial suspension (10^6 CFU/mL) was applied to each pot by creating holes near the roots using sterilized rods by 75% ethanol. Salinity stress was applied using a saline solution containing NaCl (3.61 g/l), CaCl2 (1.02 g/l), and MgCl2 (0.49 g/l) to achieve a salinity of 8 dS/m and SAR values of 13. The achievement of the assigned soil salinity levels was verified using additional unplanted pots (data not shown). The molar ratio of the different ions in the saline solution was 70.5:20:9.5 (Na: Ca: Mg). Drought stress was applied by daily weighing each pot and watering until reaching the desired moisture level (FC 35% for stress treatment and FC 80% for non-drought stress treatments). The experiment included a total of 64 pots, which were maintained under greenhouse conditions (day length from 16 h at the start of the experiment to 11 h at the end), with temperatures

Table 1 Physical and chemical properties of soil

EC ds/m	pH	CaCo3 %	OC %	P_{ava} mg/kg	CEC Cmol(+)/kg	FC %	Sand %	Silt %	Clay %
1.3	7.56	20	0.48	7.9	12.4	25	67.1	16.3	16.6

EC: Electrical conductivity

ranging from 20 to 35 °C, and harvested after 75 days. First, each pot's aerial parts and roots were collected and washed, and excess water was removed. Fresh leaf samples were first collected and stored in Falcon tubes at -80 °C after being flash-frozen with liquid nitrogen to analyze antioxidant enzymes and secondary metabolites. The aerial parts and roots were then dried in an oven at 65 °C for 72 h, and their dry weights were measured using a precision balance of 0.001 g. Root volume was measured using a graduated cylinder. Additionally, in each pot, the number of spores and the percentage of mycorrhizal colonization of the roots were assessed using the method described above.

Protein extraction and Preparation of enzyme extracts

For protein extraction, 0.5 gram of fresh plant tissue was homogenized in a mortar with three milliliters of 50 mM phosphate buffer at pH 7.2, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1% polyvinylpyrrolidone (PVP). The homogenate was then centrifuged. The supernatant was used for enzyme activity assays and total protein quantification of the samples [27].

Determination of total protein content

Total protein content was measured using the Bradford method [28]. In test tubes, 25 µl of protein extract and 750 µl of Bradford reagent were added and quickly vortexed. Immediately, the solutions were read with a spectrophotometer at a wavelength of 595 nanometers, and the protein concentration was calculated in milligrams per gram of fresh weight.

Measurement of antioxidant enzymes

Superoxide dismutase (SOD) activity was assessed by inhibiting the photochemical reduction of Nitro Blue Tetrazolium (NBT) in the presence of a reaction mixture composed of 50 mL phosphate buffer, 13 mM methionine, 75 µmol/LNBT, 2 µmol/L riboflavin, 0.1 mM EDTA, and 0.1 mL of the enzymatic extraction. The reaction was initiated by adding 2 µmol L-1 riboflavin and exposing the mixture to 15 W fluorescent lamps for 15 min. The amount of the enzyme that led to a 50% inhibition of NBT photoreduction was defined as one unit of SOD [29]. Ascorbate peroxidase (APX) activity was measured by monitoring the specific absorbance of L-ascorbic acid at 290 nm. The assay mixture consisted of 1 mL containing 100 mM K-P buffer, 0.5 mM L-ascorbic acid, and 2% (v/v) crude enzymatic extraction. The amount of oxidized L-ascorbic acid per minute was determined as APX activity using the extinction coefficient (2.8 Mm/cm) [30].

Measurement of metabolites

Maize leaves were immediately frozen in liquid nitrogen for subsequent analysis. Approximately 50 mg of each frozen sample was placed into a 2-mL centrifuge tube, followed by adding 1 mL of 100% methanol (pre-cooled to – 20 °C). The samples were vortexed and ground using a 70-Hz grinding mill system for 5 min. The homogenates were then sonicated for 30 min at 70 °C. Afterward, the tubes were centrifuged at 14,000 g for 10 min at 4 °C. A 0.4 mL aliquot of the supernatant was transferred to a 2-mL screw-cap tube, to which 10 µL of an internal standard (0.02 mg·mL – 1 3,4-dichlorophenylalanine in methanol), 200 µL of chloroform (pre-cooled to – 20 °C), and 400 µL of demineralized water (Milli-Q) were added. The mixture was vortexed thoroughly and centrifuged for 15 min at 2,200 rcf at 4 °C. Subsequently, 200 µL of the aqueous and chloroform layers were transferred into a glass vial for vacuum drying at room temperature.

Additionally, blank samples were prepared using the extraction solution, as well as pooled samples created by combining aliquots from each biological replicate across the three maize leaf sample groups. These blank samples were included in every analytical run alongside the true samples. The dried samples were reconstituted and derivatized in a two-step process involving oximation and silylation before GC-MS analysis. First, 30 µL of a methoximation solution, consisting of methoxylamine hydrochloride dissolved in pyridine (20 mg·mL – 1), was added to the vial, vortexed for 30 s, and allowed to react for 90 min at 37 °C. This was followed by trimethylsilylation with 30 µL of N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), with the reaction kept at 70 °C for 60 min. Finally, the derivatized samples were cooled to room temperature before injection [31]. 1 µL of the polar phase was injected into the copper GC device. Gas chromatography analysis connected to the mass spectrometer (GC-MS) of corn plant extract was performed with a model QP-5050 made in Japan. The type of column was DB-5ms, the length of the column was 50 m, and the thermal programming of the column was from 60 to 250 degrees Celsius. Known compounds were confirmed by evaluation in the PubChem database.

Calculations and statistics

Statistical analyses were performed using two-way analysis of variance (ANOVA) after confirming the assumption of homoscedasticity. Duncan's multiple range test was applied as a post hoc test. All statistical procedures were carried out using SAS software (version 9.1 for Windows), and graphs were generated with SigmaPlot (version 14).

The phylogenetic tree was constructed using MEGA software (version 7), applying the Neighbor-Joining

method with the Tamura-Nei model. Bootstrap analysis with 1000 replicates was conducted to assess the robustness of the tree.

Results

Molecular characterization and phylogenetic analyses

Sequencing results indicated that the strain used in this study is part of the *Trichoderma* group of fungi (Fig. 1) and is closely related to *Trichoderma harzianum* (MN944479).

Morphological parameters

The main and interaction effects of stress and inoculation treatments on the morphological characteristics of maize, including the dry weight of aerial parts and roots, total biomass (sum of roots and aerial parts), root volume, and root colonization percentage, were significant at a p-value < 0.001(Table 2). Salinity stress significantly reduced the root dry weight, total biomass, and root volume (Table 3). Drought stress also decreased the dry weight of aerial parts. The combination of salinity and drought stress had a more severe detrimental effect on the aforementioned traits. Generally, stress treatment increased the percentage of colonization. Inoculation of the plant with microorganisms significantly enhanced all measured traits compared to the non-inoculated control. Although the simultaneous inoculation of AMF and *Trichoderma* had a significant increase compared to the control treatment, it only resulted in greater growth of aerial parts compared to other treatments. The combined

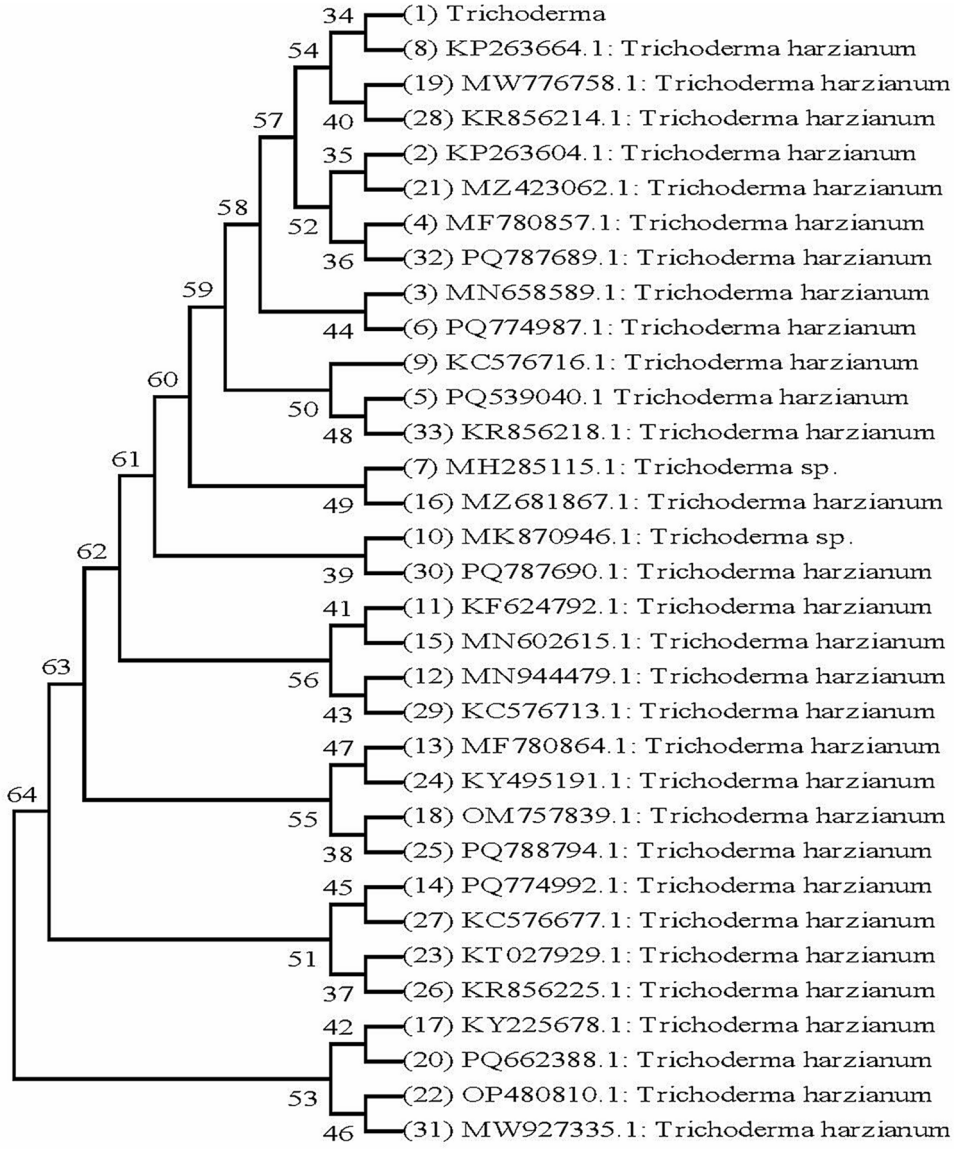

Fig. 1 Neighbor-joining phylogenetic tree based on ITS gene sequences showing the relationship between isolate T1 and various *Trichoderma* species. Isolate T1 clusters closely with *Trichoderma harzianum*

Table 2 Results of general linear model (GLM) analysis showing meansquare accompanied by p-value ranges for the effects of stress and inoculation treatments on the morphological and biochemical characteristics of maize

	DF	Root colonization	Shoot dry weight	Root dry weight	Root volume	Total biomass	APX activity	SOD activity
Microbial treatment	3	1881.30***	271.51***	368.51***	809.58***	1229.62***	7089.70***	3653.37***
Stress	3	10.93*	97.26***	175.51***	186.75***	522.70***	3184.65***	866.26***
Microbial treatment *Stress	9	21.37***	38.98***	21.93***	82.22***	48.05***	214.72***	180.02***
Error	48	2.85	0.93	0.43	1.39	1.48	39.75	14.29
CV		1.86	5.51	5.91	4.97	4.26	10.66	5.42

ns, $p \geq 0.05$. * $0.01 \leq p < 0.05$. ** $0.001 \leq p < 0.01$. *** $p < 0.001$. DF, degrees of free

Table 3 Results of the mean comparison of morphological characteristics of maize under stress and inoculation treatments

	Stress	Microbial inoculums			
		M0T0	M1T0	M0T1	M1T1
Root colonization (%)	S0D0	73 h	100a	89.5e	96bc
	S1D0	77.75f	100a	94.25 cd	94.25 cd
	S0D1	74.25gh	98.5a	92.75d	98.25ab
	S1D1	76.5 fg	99.25a	88.5e	100a
Dry shoot (gr/pot)	S0D0	12.25 g	23b	19.25d	23b
	S1D0	12.25 g	21c	23b	19.25d
	S0D1	10.75 h	21c	25.5a	14.25f
	S1D1	10.75 h	14.25f	14.25f	16.5e
Dry root (gr/pot)	S0D0	5.5 g	19.5a	17.5b	13.5c
	S1D0	4.5 h	16.75b	13.5c	11.5d
	S0D1	4.25 h	17.5b	11.5d	16.75b
	S1D1	3.25i	9.25e	6.5f	6.5f
Root vol (cm^3)	S0D0	22de	30.25b	24c	31b
	S1D0	15.75f	40.75a	21.25e	23.5 cd
	S0D1	11.75 g	30.25b	23.5 cd	30.25b
	S1D1	8.25 h	23.5 cd	22de	22de
Total biomass (gr/pot)	S0D0	17.75 g	42.5a	36.75c	36.5c
	S1D0	16.75 g	37.75bc	36.5c	30.75d
	S0D1	15 h	38.5b	37bc	31d
	S1D1	14 h	23.5e	20.75f	23e

M0T0 = Control M0T1 = *Trichoderma* M1T0 = AMF M1T1 = Trich + AMF

Means with the same letter are not significantly different at the 5% significance level

inoculation of *Trichoderma* with AMF was more effective than *Trichoderma* alone in terms of root volume and percentage of colonization. Generally, the AMF treatment had the greatest effect on vegetative traits.

Measurement of SOD and APX enzyme activity

The results from analyzing SOD and APX antioxidant enzyme activities in samples exposed to different treatments under stress conditions are shown in Fig. 2A and B. The data suggests that SOD enzyme activity increases in M0T0 samples when subjected to stress, and were no notable variations in SOD activity levels among stress types S0D1 and S1D1. Using different inoculation (M0T1, M1T0, M1T1) increased the SOD activity in maize plants under various situations (S0D0, S0D1, S1D0, S1D1). The results showed that samples treated with *Trichoderma* had the greatest SOD activity, and the enzyme activity increase did not vary significantly between different stress types (S1D0, S1D1). Although the M1T0 treatment increased SOD activity relative to the M0T0 sample, it was less effective than the M0T1 sample and exhibited no significant variations in enzyme activity under various stress conditions like S0D1, S1D0, and S1D1. The presence of both *Trichoderma* and mycorrhizae (M1T1) can potentially increase the activity of the SOD enzyme, indicating that the enzyme activity in samples treated with M1T1 under S1D1 stress was comparable to those treated with M1T0 under stress.

Analysis of APX activity in samples exposed to microorganisms (Fig. 2B) revealed increased enzyme activity in all samples compared to M0T0. No notable significant effect was seen in APX activity in the M0T0 under variable conditions of S0D1, S1D0, and S1D1. The samples showed the highest levels of APX activity when exposed to M0T1, M1T0, and M1T1 under S1D1 stress, with no noticeable variations between these treatments.

Investigation of metabolic compounds

Heat map analysis was employed to assess the concentrations of detected metabolites under varying conditions.

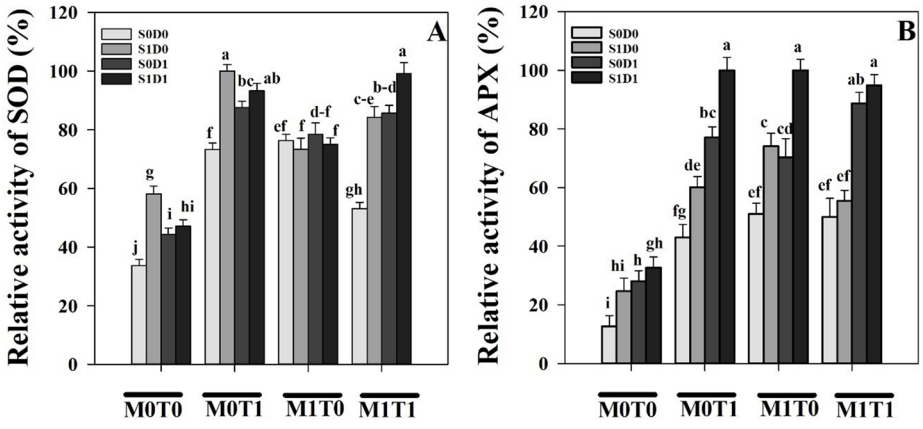

Fig. 2 Results of SOD (**A**) and APX (**B**) enzyme activity. This figure shows the activity of enzymes in samples treated with different treatments (M0T0, M1T0, M1T1) under various stress conditions (S0D0, S0D1, S1D0, S1D1)

As shown in Fig. 3, samples lacking microorganisms (M0T0) were classified according to their metabolite production levels when exposed to different stress conditions, S0D0, S0D1, S1D0, and S1D1. After experiencing various stresses, the samples exposed to M1T1 and M1T0 were categorized together. The Pathway Analysis module helped identify metabolic pathways associated with these compounds. An analysis of the proportion of identified compounds revealed three important metabolic pathways. The citric acid cycle, lysine breakdown, and tropane, piperidine, and pyridine alkaloids synthesis were linked with oxaloacetate, Δ1-piperidine-6-carboxylate, and cadaverine metabolites (Fig. 3).

As shown in Fig. 4A, the logarithm of Δ1-piperidine-6-carboxylate concentration decreased with stress in untreated corn plants. These values show an increase in plants treated with M1T0 and M1T1. Therefore, the lysine degradation pathway may be more active in the sample treated with microorganisms under stress, especially S1D1. The study found that the concentration of oxaloacetate decreased in stressed untreated corn plants but increased in stressed corn plants treated with M1T0 and M1T1 (Fig. 4B). Due to the presence of Cadaverine among the known compounds, the metabolic pathway of pyridine alkaloid biosynthesis was suggested. As shown in Fig. 4C, the logarithm of Cadaverine concentration in the untreated sample increases under stress induction. The highest concentration of this compound was seen in the treated sample M1T1 without stress and under stress S1D0.

Discussion

Salinity and drought are interrelated abiotic stresses that negatively impact crops' growth, yield, and quality by influencing physiological, morphological, and molecular factors [32]. Strengthening symbiotic relationships with microorganisms is a protective mechanism employed by plants. Mycorrhizae form beneficial symbiotic relationships in challenging environments, with AMF hyphae enhancing water and nutrient absorption and improving water use efficiency. The *Trichoderma* genus includes fungi that colonize substrates and interact with plants, leading to significant changes in plant metabolism, hormone levels, soluble sugars, phenolic compounds, and amino acids [33]. During this study, corn plants were exposed to AMF fungi and *Trichoderma*, which were obtained from regions experiencing salinity and drought conditions. Our findings indicated that stress levels on the plant without microorganisms (M0T0) caused a significant reduction in root dry weight, root volume, and total plant dry weight. The M0T0 samples displayed the most intense adverse morphological impacts when exposed to salinity and drought stresses (S1D1) simultaneously. Our findings indicated that introducing microbial inoculations under stress conditions notably boosted root size, root dry weight, and shoot biomass compared to the control group (M0T0). The plants displayed the greatest average root volume and dry weight when subjected to M1T0. This result is consistent with the findings of a previous study, which showed that root dry weight was higher in mycorrhizal-inoculated plants at all stress levels compared to non-inoculated plants [34]. Furthermore, our results indicate that the effect of M0T1 on shoot dry weight was greater than that of M1T0. Kaya et al. 2009 displayed that improved plant growth was likely due to the increased solubility of insoluble nutrients via *Trichoderma* species and increased accumulation of metabolites that protect photosynthetic pigments [35].

Another aspect examined in the current research involved evaluating how antioxidant enzymes in plants perform when faced with stress. Previous studies have shown that the activity of antioxidant enzymes, such as SOD, APX, and CAT, increases in reaction to various environmental pressures like soil salinity, drought,

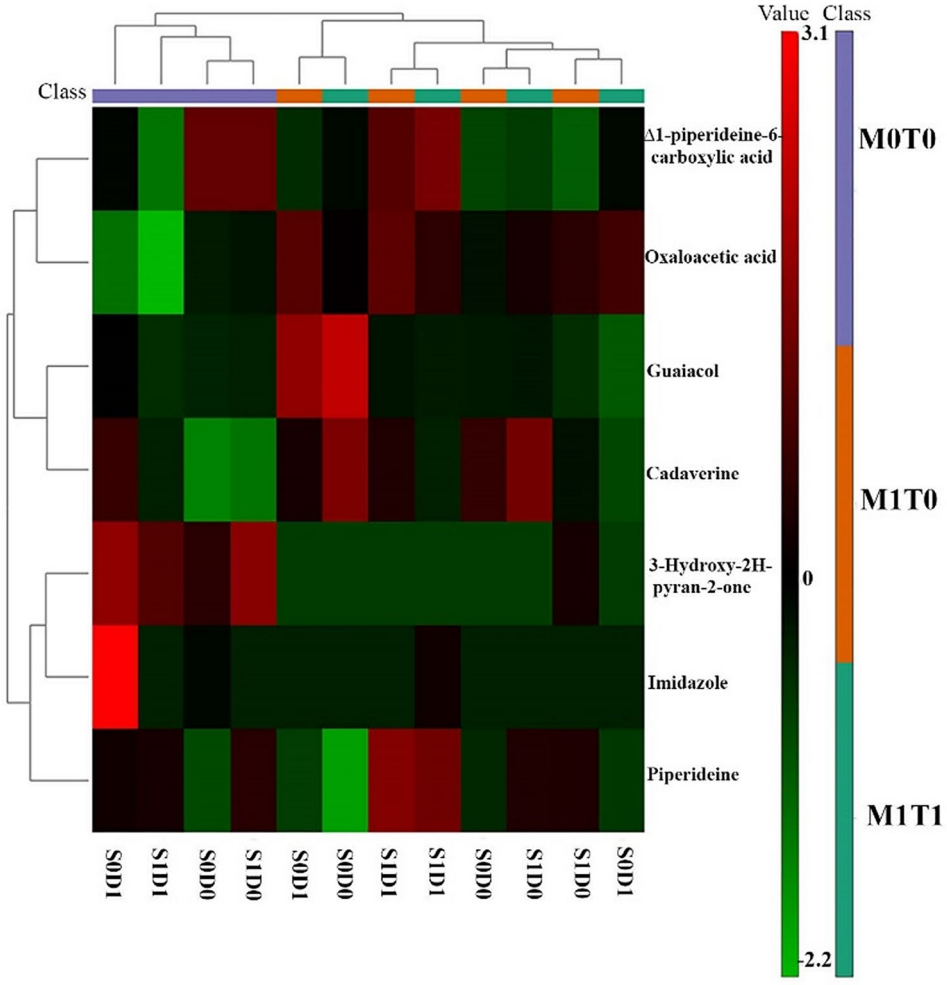

Fig. 3 Heat map of known metabolites. This figure shows the comparative concentration levels of various metabolites under different inoculation and stress conditions (M0T0, M1T0, M1T1) and stress conditions (S0D0, S0D1, S1D0, S1D1)

extreme temperatures, and heavy metals [36–39]. Salt stress triggers the production of ROS in plant cells through osmotic and ionic effects, with salt-sensitive plants showing increased levels of hydrogen peroxide (H2O2) in their leaves [40]. Superoxide dismutases (SODs), which consist of Cu–Zn-SOD, Fe-SOD, and Mn-SOD isoforms, are found in the cytoplasm, mitochondria, and chloroplasts. The stress from salinity prompts the generation of superoxide radicals (O_2^-) mainly from the plasma membrane and, to a lesser extent, from the electron transport chains in chloroplasts and mitochondria [41–42]. Salinity stress triggers the closure of stomata, limiting the absorption of CO_2 and resulting in electron transfer to O_2, which ultimately produces O_2^- and depletes $NADP^+$ [43]. SODs convert highly toxic O_2^- to less harmful hydrogen peroxide (H_2O_2) [44]. In a study involving corn subjected to saline conditions, it was noted that the increase of the SOD in the leaves corresponded with an elevation of other enzymes like CAT, GPX, and APX when compared to the control condition (without stress) [45]. Moreover, sorghum seedlings experiencing salinity stress exhibited heightened gene expression for all isoforms of SOD, underscoring the critical role of SODs in ROS regulation [46]. ROS produced under salinity conditions, such as H_2O_2, play a role in the lipid peroxidation of cellular membranes. The cooperative activity of peroxidative enzymes like CAT, GPX, and APX diminishes H_2O_2 concentrations in plants, thereby preserving a balanced condition. APX acts as a more efficient scavenger of H_2O_2 than CAT due to its strong affinity for H_2O_2. Therefore, APX regulates H_2O_2 levels within plant cells [47]. Consequently, APX activity increases in leaves that encounter salinity stress and remains elevated compared to those under control conditions (without stress) [45]. Our results indicated that plants (M0T0) exposed to different types of stress (S1D0, S0D1, and S1D1) exhibited increased activity levels of the enzymes SOD and APX when compared to the control group (S0D0). Various microbial inoculants, like *Trichoderma* and mycorrhiza, employ unique methods to

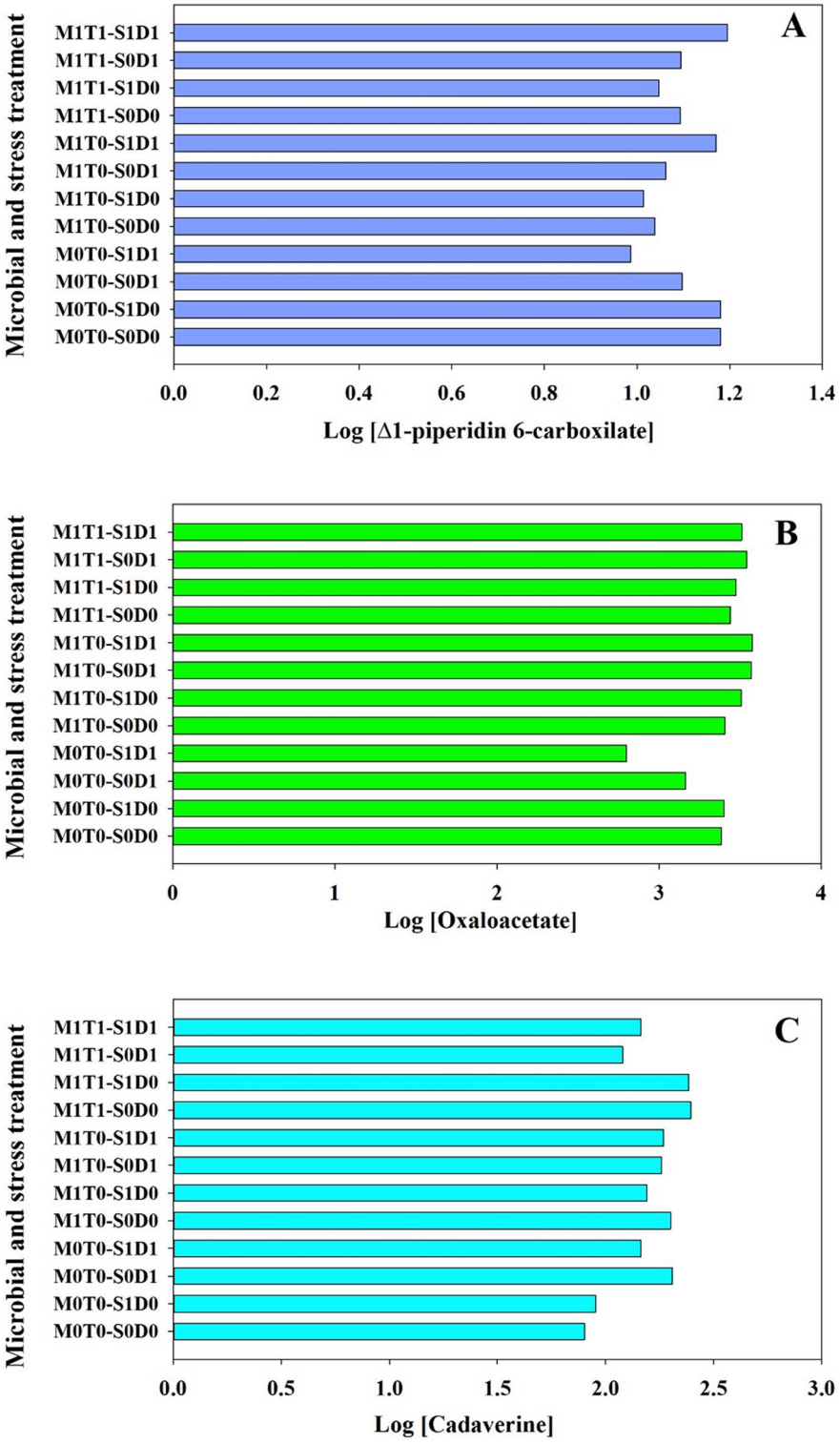

Fig. 4 logarithm diagram of the concentration of known compounds in corn plants treated with Mycorrhiza (M1T0) and combination Mycorrhiza and *Trichoderma*(M1T1) under normal (S0D0), salinity (S1D0), drought (S0D1), and salinity/drought (S1D1) conditions

enhance plant resistance to stress. Sahu et al. have studied microbes that may enhance plant metabolism when faced with salinity stress [48, 49]. They described one mechanism involving resistance to stress as the buildup of compatible solutes like proline. Research highlights the beneficial role of *Trichoderma* and mycorrhiz in promoting plant growth, particularly under saline conditions [25, 43, 50, 51]. Our investigation found that APX activity increased in the M0T1, M1T0, and M1T1 samples under stresses (S1D0, S0D1, S1D1), showing significant differences from the M0T0. The highest APX performance was observed in the M1T0 and M0T1 samples under stress condition S1D1. Alguacil et al. 2003 demonstrated that plants show elevated levels of antioxidant activity when they are colonized by mycorrhizal fungi, unlike those without mycorrhizal associations [52]. However, the type of antioxidant enzyme varies according to the plant species and fungal strains involved. This variation may stem from differences in the cofactors necessary for specific enzymes. Metalloproteins such as CAT, APX, and SOD require specific metal elements to function correctly. It seems that the microorganisms present in the M1T0 and M0T1 samples have successfully aided enzyme function under stress conditions S1D1 by enhancing APX activity and supplying essential cofactors. When both microorganisms were applied together (M1T1), the activity of the APX enzyme increased in both S0D1 and S1D1 conditions. Previous research has shown that different microbial inoculants employ various strategies to mitigate stress, highlighting the complementary functions of these inoculants in protecting host plants [49, 53]. In a holobiome, the host plant interacts actively with various microorganisms. These interactions can trigger responses in the plant, leading to variations in the activity levels of antioxidant enzymes influenced by the specific microbiomes present [53]. A comparable pattern was observed in our study; all antioxidant enzymes were stimulated to different extents depending on the treatments. The M0T1 sample showed the greatest SOD activity when exposed to S1D0 stress, and it had no notable difference in SOD activity with M0T1 and M1T1 samples under S1D1 stress. This activity was markedly higher than the sample treated solely with mycorrhiza (M1T0). Recently, researchers in this area have focused on combining *Trichoderma spp*. with other beneficial soil microorganisms to boost plant growth [54]. For example, pairing *Trichoderma asperellum* with AM fungi has increased plant biomass production even under challenging conditions [55]. Nadeem et al. (2014) have extensively examined the positive effects of the beneficial cooperative interactions of soil microorganisms on plant development and stress resilience in challenging environments [56]. These microorganisms are believed to act as important bio-ameliorators of stress by regulating nutritional and ionic equilibrium [57] and enhancing overall tolerance to stress [58]. Previous studies indicate that *Trichoderma* species enhance plant growth under optimal conditions, with even more pronounced positive effects observed in suboptimal conditions [14]. Fungi, particularly *Trichoderma spp*., form mutualistic relationships with host plants, enhancing gene expression and promoting growth under drought conditions through metabolites similar to phytohormones. Their colonization impacts physiological processes, such as photosynthesis, and regulates stress-related transcriptomics. Recent studies indicate that root proteomics can boost antioxidant enzyme function in *Trichoderma*-treated plants [14, 59]. According to Mastouri et al. 2010 [60] and Bailey et al. 2006 [61], the interactions between species of *Trichoderma* and plant seedlings influence the expression of genes associated with antioxidant enzymes and transcription factors, thereby improving resilience to water stress.

A study on tomatoes found that using AMF and *Trichoderma* significantly affected secondary metabolic pathways, leading to higher levels of phenolic compounds and changes in plant hormone levels like auxins and cytokinins [62]. AMF promotes phosphorus absorption in plants by colonizing the roots, leading to an increase in the levels of necessary precursors for the synthesis of secondary metabolites like NADPH, ATP, acetyl-CoA, pyruvate, glyceraldehyde-3-phosphate, erythrose-4-phosphate, and phosphoenolpyruvate [63].

Furthermore, Feng and coauthors 2002 [64] argued that plants exposed to AMF treatment exhibit enhanced resistance to stress due to higher amounts of soluble sugars. Soluble sugars act as energy and material sources, collaborating with signaling molecules like phytohormones to regulate plant growth and development [65]. Numerous studies show that *Trichoderma* and arbuscular mycorrhizal fungi (AMF) help plants cope with abiotic stresses like salt stress. However, the specific mechanisms by which these fungi enhance plant resilience are complex and poorly understood. Further research is needed to explore the relationship between these two types of fungi.

Based on GC-MS analysis, our results found that the pathways for lysine degradation, pyridine alkaloid synthesis, and the Krebs cycle were more active in maize plants when exposed to M1T0 and M1T1 than M0T0. These metabolic pathways and the relationship between them are shown in Fig. 5. Our results revealed a notable rise in the concentration of oxaloacetate in the M1T0 samples subjected to stress. This increase suggests a significant biochemical response of treated plants under stress. This finding aligns with Hu et al. (2020) who demonstrated a notable increase in organic acids within the Krebs cycle of corn seedlings treated with mycorrhizae under drought stress compared to untreated plants [66]. According to previous research, oxaloacetate is used as a

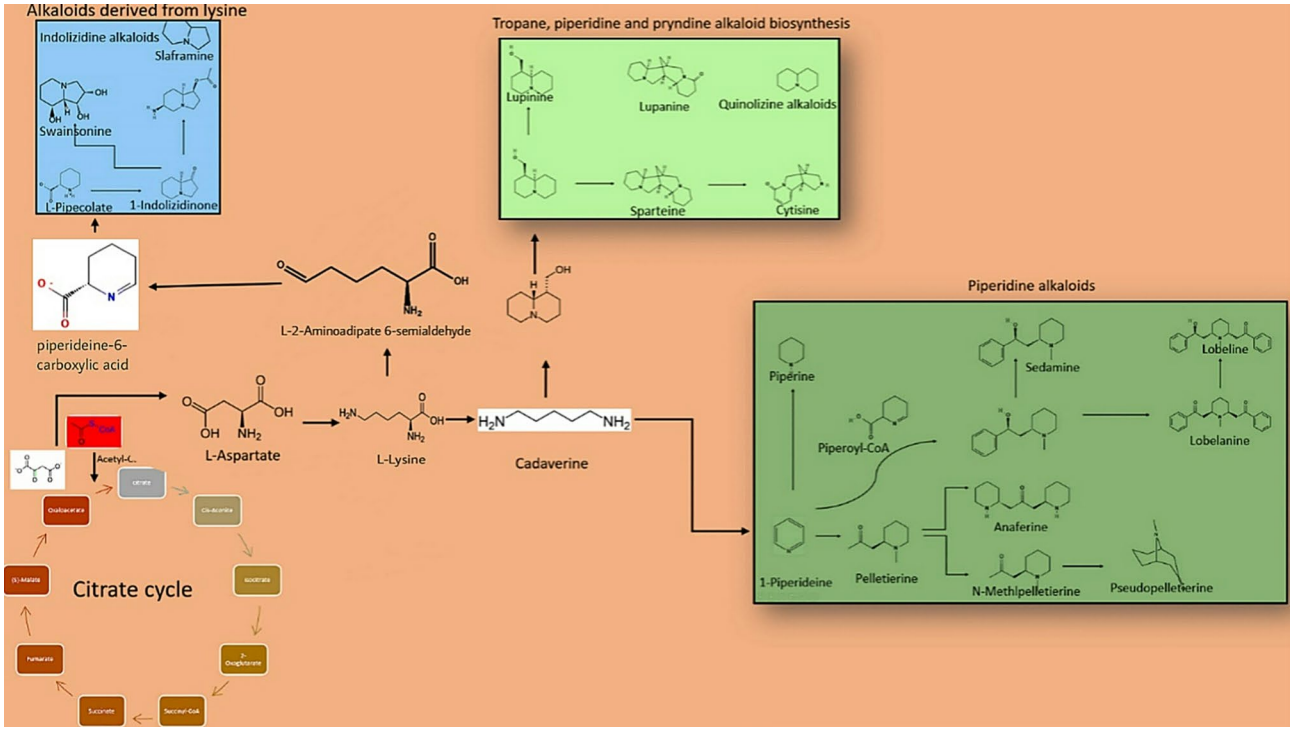

Fig. 5 Schematic diagram of the biosynthesis of alkaloids derived from lysine, such as piperidine, pyridine, and indolizidine, along with their relationship to the Krebs cycle, is presented using KEGG Pathway Database (https://www.genome.jp/entry/map01064) and reference [71]

precursor for citrate production in the Krebs cycle within mitochondria [67, 68]. The citrate produced can be an alternative carbon source for synthesizing fatty acids [69]. In a recent study by Zhang and colleagues (2019), the symbiotic function of AMF was verified in regulating gene expression of important metabolic enzymes like citrate synthase and citrate lyase [70]. However, more research is needed to confirm the influence of M1T0 and M0T1 treatment on plant citrate production.

Previous research has examined the metabolism of lysine in plants under stress. Currently, there are three recognized pathways for the breakdown of lysine in plants: the NHP, cadaverine, and SACPATH pathways. In the NHP pathway, lysine is converted to NHP through three reaction steps; the NHP pathway is triggered by pathogen invasion to enhance plant immunity [72]. Lysine is converted into the alkaloid cadaverine in the cadaverine pathway [73]. Cadaverine accumulates in plants in response to environmental stresses such as heat, drought, and salt, suggesting a potential role in stress mitigation [74]. It can support seed germination and seedling growth under stress. Plants can absorb cadaverine from their environment (through the microbiome's function), impacting their growth and stress responses. The molecular mechanisms of cadaverine's action remain unclear. However, its treatment has been linked to increased levels of putrescine and spermine while decreasing spermidine levels, indicating an interaction with putrescine-related pathways [74]. In the SACPATH pathway, which is common to plants, animals, and bacteria, lysine is converted to glutamate, then to proline by the action of the enzymes Δ1-pyrroline-5-carboxylate synthase (P5CS) and Δ1-pyrroline-5-carboxylate reductase (P5CR). At the same time, α-aminoadipate semialdehyde was cycled and formed Δ1-piperideine-6-carboxylate can be used to produce pipecolic acid [71]. Pipecolic acid, the downstream product of Δ^1-piperidine-6-carboxylate, is a critical signaling molecule in plants' systemic acquired resistance (SAR).

Numerous research efforts have been conducted to comprehend pipecolic acid biosynthesis, transport, and function in plants facing biotic stress. It has recently been found that pipecolic acid plays a role in plants' stress response when exposed to abiotic stress [75]. Under S1D1 stress conditions, the levels of Δ1-piperidine-6-carboxylate were increased, while the levels of cadaverine decreased in the M1T1 samples. On the flip side, the M1T1 samples showed high levels of cadaverine and low levels of Δ1-piperidine-6-carboxylate under S1D0 stress. The SACPATH and cadaverine pathways seem to be inactive at the same time in plants that are under stress. The saccharopine pathway (SACPATH), a crucial lysine catabolic route in plants, has been linked to the modulation of stress responses through the production of key intermediates, such as saccharopine and α-aminoadipic acid [71]. Although direct evidence linking SACPATH with

beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) and *Trichoderma spp.* remains limited, it is well established that these symbionts activate extensive defense signaling networks, including salicylic acid, jasmonic acid, and ethylene-mediated pathways [76]. Given that SACPATH contributes to redox regulation and energy balance during stress responses, it is plausible that its activation could complement the defense-related metabolic adjustments induced by AMF and *Trichoderma*. Combining metabolites from SACPATH with immune responses from microbes can improve a plant's metabolic adaptability, enhance the management of reactive oxygen species (ROS), and strengthen systemic resistance. Thus, SACPATH may serve as a significant yet underappreciated element within the intricate plant resilience mechanisms strengthened by symbiotic microorganisms.

Conclusion

The combined stress of salinity and drought had significantly more severe negative effects on morphological and enzymatic parameters compared to salinity or drought alone. The inoculation with AMF and *Trichoderma* led to significant improvements in root volume, dry weight of roots, and biomass production compared to non-inoculated controls in stress conditions. The microbial treatment showed the highest increases in these growth parameters. The study revealed that plants under stress conditions exhibited improved stress tolerance with increased activities of antioxidant enzymes such as SOD and APX. The highest levels of these enzymes were observed in plants treated with mycorrhizal treatment (M1T0) and combined AMF and *Trichoderma* (M1T1), suggesting that these microorganisms enhance the plant's ability to combat oxidative stress. We found that lysine catabolism pathways, specifically SACPATH and cadaverine pathways, were activated under stress conditions. The results indicate that these pathways contribute to stress mitigation by modulating the levels of osmolytes like proline and piperine. The results highlight the role of AMF and *Trichoderma* in modifying key metabolic pathways and stress response mechanisms. The observed increase in cellular concentrations of proline and piperine suggests that these microorganisms play a crucial role in enhancing the adaptive metabolism of maize plants.

Author contributions

F. E. conducted the experiments and wrote the manuscript. M.S. supervised the study and contributed to the manuscript revision. (**A**) L. performed the statistical analysis and reviewed the methodology. N. (**B**) contributed to the data interpretation and manuscript writing. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read and approved the final manuscript for publication.

Competing interests

The authors declare no competing interests.

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