The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
21(1): 75-83, 2026 www.thebioscan.com

Drought stress alleviation in rice by endophytic *Trichoderma* sp. via modulation of physiological and antioxidant responses

Abhishek R. R., Kumaraswamy H. M.*
Laboratory of Experimental Medicine, Department of Biotechnology, Kuvempu University, Shankaraghatta-577 451, Karnataka, India

***Corresponding Author:** Dr. Kumaraswamy H M, E-mail: drhmklab@gmail.com

DOI: 10.63001/tbs.2026.v21.i01.pp75-83

Keywords
Trichoderma sp., Rice IR-64, Drought stress, Endophytic fungi.

Received on:
07-11-2025

Accepted on:
19-12-2025

Published on:
10-01-2026

ABSTRACT

Drought is a major constraint to rice production, and beneficial endophytic fungi offer a promising strategy to enhance plant resilience under water-limited conditions. In this study, a drought-tolerant fungal endophytic *Trichoderma* sp. was evaluated for its ability to improve the physiological and biochemical performance of rice (*Oryza sativa* L. cv. IR-64) under greenhouse-imposed drought stress. Plants were grown under four treatment combinations with and without endophyte inoculation and drought. Endophyte-treated plants showed improved growth under optimal conditions and exhibited strong protection against drought-induced declines in chlorophyll content, SPAD index, and leaf water status. Enhanced soluble sugar accumulation and elevated antioxidant enzyme activities further indicated improved osmotic balance and strengthened defense responses in inoculated plants exposed to drought. Overall, the *Trichoderma* sp. strain substantially mitigated drought-induced damage by enhancing water retention, maintaining photosynthetic capacity, and activating antioxidant and osmoprotective mechanisms. These findings highlight the potential of endophytic fungi as effective biological tools for improving drought tolerance in rice.

Introduction

Globally, about one-third of cropland experiences drought stress[7]. Rice (*Oryza sativa* L.) is one of the major food crops consumed by half of the world's population[5]. A study reported that around 1,900 to 5,000 L of water are required to produce 1 kg of rice grain, and that about 10% of irrigated rice areas are expected to experience water shortages in 2025[8]. The intensity and severity of drought depends on several interacting factors, including rainfall frequency, evaporation rates, and soil moisture content, making the predictions difficult. Drought stress creates an ion imbalance and osmotic stress, often leading to oxidative stress, causing the excessive production of reactive oxygen species (ROS), which can severely damage cellular components and impair plant growth[14].

According to the Intergovernmental Panel on Climate Change (IPCC), drought events are expected to increase and be severe by the end of this century as it is a direct consequence of global climate change[13]. Physiologically, drought leads to reduced cell expansion, loss of membrane stability, and premature leaf senescence, all of which negatively affect plant growth and productivity[19]. It

also impairs photosynthesis by promoting stomatal closure to reduce water loss, which in turn damages cell membranes and disrupts the activity of key enzymes involved in ATP generation[9]. Additionally, drought alters chlorophyll content, increases ethylene production, and triggers structural adjustments in the photosynthetic apparatus, ultimately suppressing photosynthetic efficiency and energy production[10].

Considering the complexity and increasing frequency of abiotic stress combinations under climate change, there is an urgent need to develop innovative and sustainable strategies to enhance crop resilience. In recent years, the role of beneficial microorganisms, particularly fungal endophytes, has been increasingly studied for improving plant resilience under stress. Endophytic fungi colonize inter or intracellular spaces of plant cells without causing visible symptoms[4]. They protect plants by increasing the water and nutrient uptake in arid or water-limited environments[12]. *Trichoderma* sp., the fungal endophyte isolated from plants in the Himalayan cold deserts was found to impart drought stress tolerance to plants. Hence exploring the functional potential towards drought stress represents a promising avenue for developing sustainable, biologically based solutions. This study elucidates the physiological and biochemical functions of *Trichoderma* sp. induced drought tolerance in the IR-64 variety of rice. Understanding how this fungal endophyte mitigates the detrimental effects of water deficit conditions not only advances our knowledge of plant-microbe interactions but also offers potential strategies in developing drought-resilient crops in a changing climate.

Materials and methods

Plant material and endophyte inoculation

The drought-sensitive rice cultivar *Oryza sativa* L. cv. IR-64 was used for this study. The endophytic fungus, *Trichoderma* sp., previously isolated from the cold regions of the Himalayas and characterized for drought tolerance, served as the fungal inoculant. Seeds were surface-sterilized and pre-germinated for 48 hours. *Trichoderma* sp. was cultured on PDA medium for ten days, and the mycelia were gently washed with sterile deionized water to obtain a suspension standardized to 2×10^6 spores/mycelial fragments mL^{-1}. Pre-germinated seedlings were incubated in the fungal suspension for three hours, while control seedlings were treated with sterile deionized water for the same duration[16].

Greenhouse establishment, stress imposition, and trait assessments

Endophyte-inoculated (E+) and uninoculated (E−) seedlings (2 days old) were transplanted into plastic pots (12 × 12 cm) and maintained under greenhouse. Plants were irrigated regularly for the first 30 days to ensure uniform establishment. Drought stress (D+) was imposed by withholding irrigation until soil moisture declined and drought symptoms appeared, while non-stressed plants (D−) continued to receive regular watering. This resulted in four treatment combinations: E−D− (control), E+D− (endophyte only), E−D+ (drought), and E+D+ (endophyte + drought), arranged in a completely randomized design with ten replications. Plant growth parameters, physiological and biochemical assessments were done after the completion of drought stress imposition.

Estimation of RWC, SPAD and total chlorophyll content

Relative Water Content (RWC), SPAD chlorophyll index, and total chlorophyll concentration were measured to assess the physiological responses of plants under different treatments. Fully expanded leaves from each plant were sampled for RWC estimation. Fresh weight (FW) was

recorded immediately after excision, after which the leaves were floated on distilled water for 4 h under low light to obtain turgid weight (TW). Samples were then oven-dried at 70 °C for 48 h to determine dry weight (DW), and RWC was calculated using the formula[20]: RWC (%) = ((FW − DW) / (TW − DW)) × 100. Leaf chlorophyll index was quantified non-destructively using a SPAD meter, with three readings taken per leaf and averaged for each plant. Total chlorophyll content was estimated spectrophotometrically by extracting 0.1 g of fresh leaf tissue in 80% acetone, followed by centrifugation at 10,000 rpm for 10 min. Absorbance of the supernatant was measured at 645 and 663 nm[11] and total chlorophyll (mg g^{-1} FW) was calculated using Arnon's equation ($20.2 \times A_{645} + 8.02 \times A_{663}$). All measurements were carried out under dim light conditions to prevent pigment degradation [2].

Estimation of soluble sugar content

Soluble sugar content was quantified using the anthrone reagent method. Fresh leaf tissue (0.1 g) was homogenized in 5 mL of 80% (v/v) ethanol and incubated in a water bath at 80 °C for 30 minutes. The samples were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected for analysis. An aliquot of 1 mL of the extract was mixed with 4 mL of freshly prepared anthrone reagent (0.2% anthrone in concentrated sulfuric acid) and heated in a boiling water bath for 10 minutes. After rapid cooling on ice, absorbance was measured at 620 nm using a UV–Vis spectrophotometer. Total soluble sugar concentration was determined using a D-glucose standard curve and expressed as µg g^{-1} fresh weight[21].

Antioxidant enzyme assays (SOD, CAT, POD)

Antioxidant enzyme activities were quantified from fresh leaf tissue following standard spectrophotometric protocols. For enzyme extraction, 0.5 g of fresh leaf tissue was homogenized in 5 mL of ice-cold extraction buffer (50 mM phosphate buffer, pH 7.0, containing 1% polyvinylpyrrolidone). The homogenate was centrifuged at 12,000 rpm for 15 minutes at 4 °C, and the supernatant was used as the enzyme source. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of nitro blue tetrazolium (NBT) photoreduction at 560 nm, with one unit of SOD defined as the amount of enzyme required to inhibit 50% NBT reduction. Catalase (CAT) activity was quantified by monitoring the decomposition of hydrogen peroxide at 240 nm, and activity was expressed as the change in absorbance per minute per gram fresh weight. Peroxidase (POD) activity was assayed by measuring the oxidation of guaiacol at 470 nm, using an extinction coefficient of 26.6 mM^{-1} cm^{-1}, and expressed as units per gram fresh weight. All assays were performed at room temperature, and enzyme activities were calculated on a fresh weight basis [1].

Statistical analysis

All data were analyzed using a completely randomized design (CRD) with ten replicates per treatment. One-way analysis of variance (ANOVA) was performed to evaluate the effects of treatments on all measured growth, physiological, biochemical, and enzymatic parameters. Tukey's HSD test ($p < 0.05$) was employed for post-hoc mean separation.

Results

Effects of *Trichoderma* sp. on plant growth under drought stress

Endophytic fungal (*Trichoderma* sp.) treatment significantly enhanced plant growth parameters under both optimal and drought-stressed conditions (Fig. 1). Under well-watered control conditions, endophyte inoculated plants (E+D-) showed substantial improvements across all growth traits: shoot length increased by 20%, root length increased by 20% and biomass accumulation was markedly enhanced with 40% higher shoot dry weight and 78% higher root dry weight. Photosynthetic capacity, as reflected by leaf area (29% increase) and leaf number (20% increase), also demonstrated a significant improvement with endophytic colonization (Table 1).

Drought stress severely impaired plant development, reducing shoot length, root length, shoot dry weight, root dry weight, leaf area, and leaf number by 42–60% compared to controls. However, endophytic inoculation substantially ameliorated drought-induced growth inhibition, with E+D+ plants showing 39–50% recovery in shoot and root length, and improvements in shoot and root dry biomass relative to drought-only plants. Leaf area and leaf number in E+D+ plants recovered to 61% and 54% of drought-alone values, respectively. These results indicate that endophytic colonization promotes growth under favorable conditions and activates stress-amelioration mechanisms that restore plant development under drought conditions, with particularly pronounced effects on root architecture and biomass accumulation (Table 1).

Fig. 1. Effect of *Trichoderma* sp. on rice phenotype under greenhouse conditions

Table 1. Effect of *Trichoderma* sp. on the growth parameters of rice under drought stress

Parameter	E−D−	E+D−	E−D+	E+D+
Shoot length (cm)	30.67 ± 0.34^{b}	36.81 ± 0.36^{a}	17.67 ± 0.39^{d}	24.54 ± 0.54^{c}
Root length (cm)	15.67 ± 0.34^{b}	18.81 ± 0.34^{a}	7.67 ± 0.37^{d}	11.54 ± 0.52^{c}
Shoot dry weight (g)	0.472 ± 0.011^{b}	0.661 ± 0.011^{a}	0.189 ± 0.012^{d}	0.385 ± 0.017^{c}
Root dry weight (g)	0.287 ± 0.014^{b}	0.510 ± 0.011^{a}	0.119 ± 0.013^{d}	0.248 ± 0.020^{c}
Root volume (ml)	3.672 ± 0.325^{b}	3.813 ± 0.340^{a}	1.166 ± 0.367^{d}	2.535 ± 0.515^{c}
Leaf area (cm^2)	18.90 ± 0.43^{b}	24.42 ± 0.45^{a}	9.56 ± 0.49^{d}	15.38 ± 0.69^{c}
Leaf number	7.7 ± 0.26^{b}	9.2 ± 0.25^{a}	3.7 ± 0.21^{d}	5.7 ± 0.37^{c}

Growth parameters of rice seedlings grown under four treatments: E−D− (control), E+D− (*Trichoderma* sp.), E−D+ (drought), and E+D+ (*Trichoderma* sp.+ drought). Values are mean ± SE (n = 10). Different letters indicate significant differences (Tukey's HSD, p < 0.05).

Relative water content, SPAD Index, and total chlorophyll

Relative Water Content (RWC), SPAD chlorophyll index, and total chlorophyll concentration showed clear treatment-dependent differences. Under well-watered conditions, both E−D− and E+D− plants maintained high RWC (83.35 ± 0.26% and 85.63 ± 0.25%, respectively). Drought stress (E−D+) sharply reduced RWC to 53.34 ± 0.21%, indicating a 36% decline relative to E−D− plants, while inoculation under drought (E+D+) significantly improved leaf hydration to 69.07 ± 0.35%, a 29% increase compared to E−D+ (Fig. 2).

SPAD values followed a similar pattern. E+D− plants exhibited the highest leaf greenness (36.56 ± 0.36), which was 9% higher than E−D− (33.50 ± 0.35). Drought stress lowered SPAD values to 17.84 ± 0.41 (a 47% reduction relative to E−D−), whereas E+D+ plants showed a significant recovery, corresponding to a 56% improvement over drought-only plants (Fig. 2).

Total chlorophyll content also declined under drought but improved with endophyte inoculation. E+D− plants recorded the highest chlorophyll concentration (3.48 ± 0.09 mg g^{-1} FW), representing a 30% increase compared to E−D− (2.68 ± 0.09 mg g^{-1} FW). Drought stress reduced chlorophyll to 1.11 ± 0.10 mg g^{-1} FW (a 59% decrease), while E+D+ plants showed a substantial recovery to 2.18 ± 0.14 mg g^{-1} FW, relative to E−D+. Overall, these results indicate that *Trichoderma* sp. enhances water retention, pigment stability, and photosynthetic capacity, particularly under drought conditions (Fig. 2).

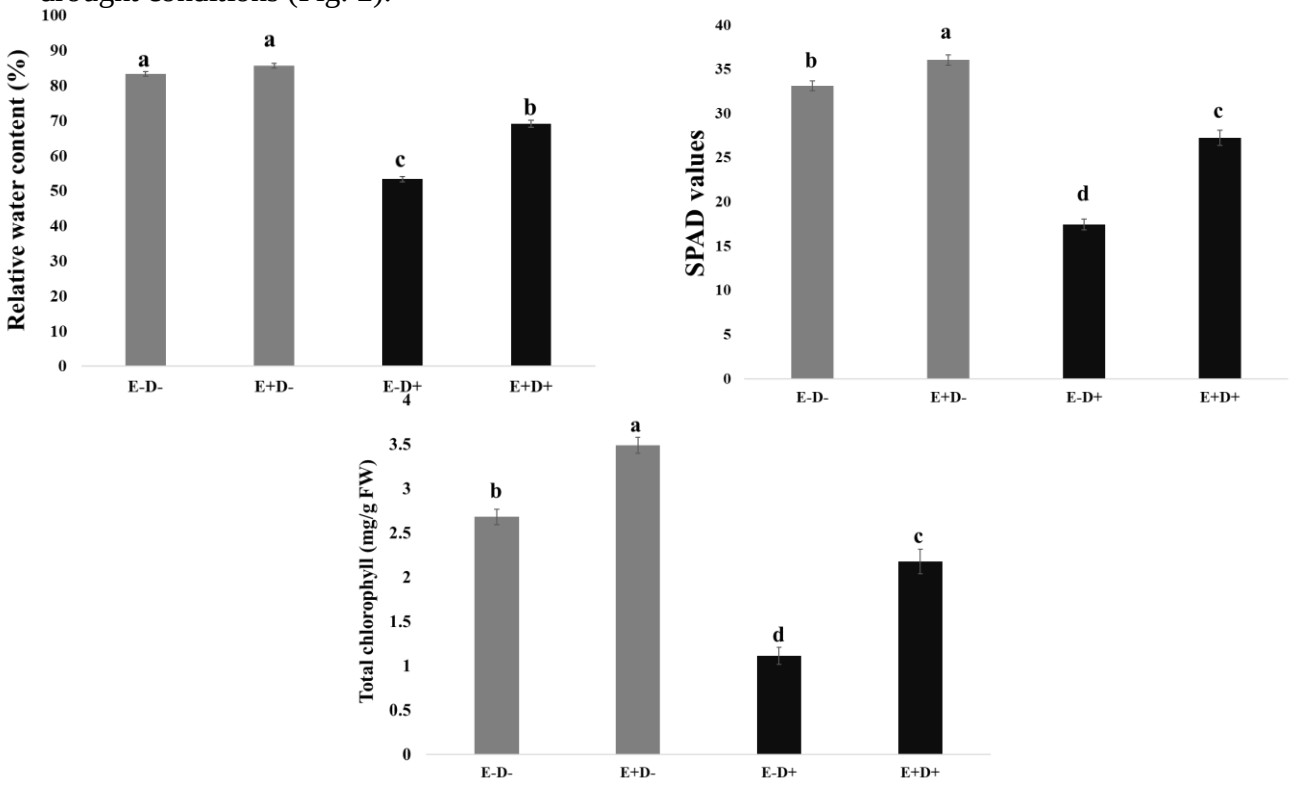

Fig 2. Relative Water Content (RWC), SPAD chlorophyll index, and total chlorophyll content of rice seedlings grown under four treatments: E−D− (control), E+D− (*Trichoderma* sp.), E−D+ (drought), and E+D+ (*Trichoderma* sp. + drought). Values are mean ± SE (n = 10). Different letters indicate significant differences (Tukey's HSD, p < 0.05).

Soluble sugar accumulation under drought stress

Soluble sugar levels differed markedly across treatments. Under well-watered conditions, endophyte inoculation (E+D−) resulted in a modest increase in sugar content (6.15 ± 0.30 µg g^{-1} FW) compared to the uninoculated control E−D− (4.58 ± 0.20 µg g^{-1} FW), corresponding to a 34% increase. Drought stress (E−D+) caused a rise in sugar accumulation (11.73 ± 0.29 µg g^{-1} FW), relative to E−D− plants, reflecting osmotic adjustment under water deficit. Endophyte inoculation under drought (E+D+) further enhanced soluble sugar content to the highest level (16.83 ± 0.37 µg g^{-1} FW), representing a 44% increase compared to drought-stressed plants (E−D+). Tukey's HSD placed treatments into distinct groups (E+D+ > E−D+ > E+D− > E−D−), indicating that *Trichoderma* sp. strongly enhances osmolyte accumulation, particularly under drought conditions (Fig. 3).

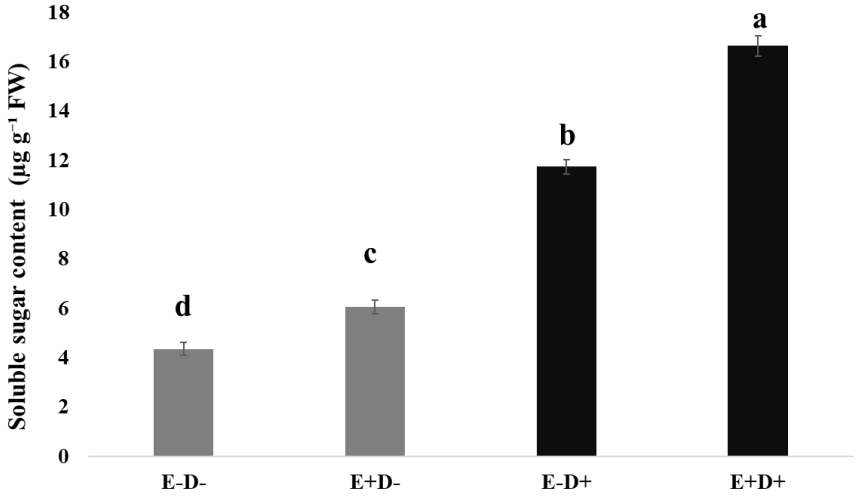

Fig 3. Soluble sugar content (mg g^{-1} FW) in rice plants under four treatments: E−D− (control), E+D− (*Trichoderma* sp.), E−D+ (drought), and E+D+ (*Trichoderma* sp. + drought). Values are mean ± SE (n = 10). Different letters indicate significant differences (Tukey's HSD, p < 0.05).

Antioxidant enzyme responses to endophyte inoculation and drought

Antioxidant enzyme activities increased strongly under drought stress and were further enhanced by endophyte inoculation. Superoxide dismutase (SOD) activity increased from 144.60 ± 2.01 U g^{-1} FW in E−D− plants to 166.30 ± 2.18 U g^{-1} FW under drought (E−D+), while E+D+ plants exhibited the highest activity (182.97 ± 2.75 U g^{-1} FW), representing a 25% increase relative to drought-only plants. A similar trend was observed for catalase (CAT), where activity rose from 102.85 ± 2.75 U g^{-1} FW in E−D− to 181.79 ± 2.80 U g^{-1} FW in E−D+, and further to 217.47 ± 3.37 U g^{-1} FW in E+D+, indicating a 20% enhancement due to endophyte inoculation under stress. Peroxidase (POD) activity also showed a progressive rise, increasing from 1.46 ± 0.08 U g^{-1} FW in E−D− to 3.01 ± 0.10 U g^{-1} FW in E−D+, and reaching the highest levels in E+D+ plants (3.86

± 0.12 U g^{-1} FW), corresponding to a 28% increase relative to drought-stressed plants. Across all three enzymes, statistical comparisons clearly separated treatments, reflecting a consistent pattern in which *Trichoderma* sp. significantly boosts antioxidant capacity, particularly under drought stress (Fig. 4).

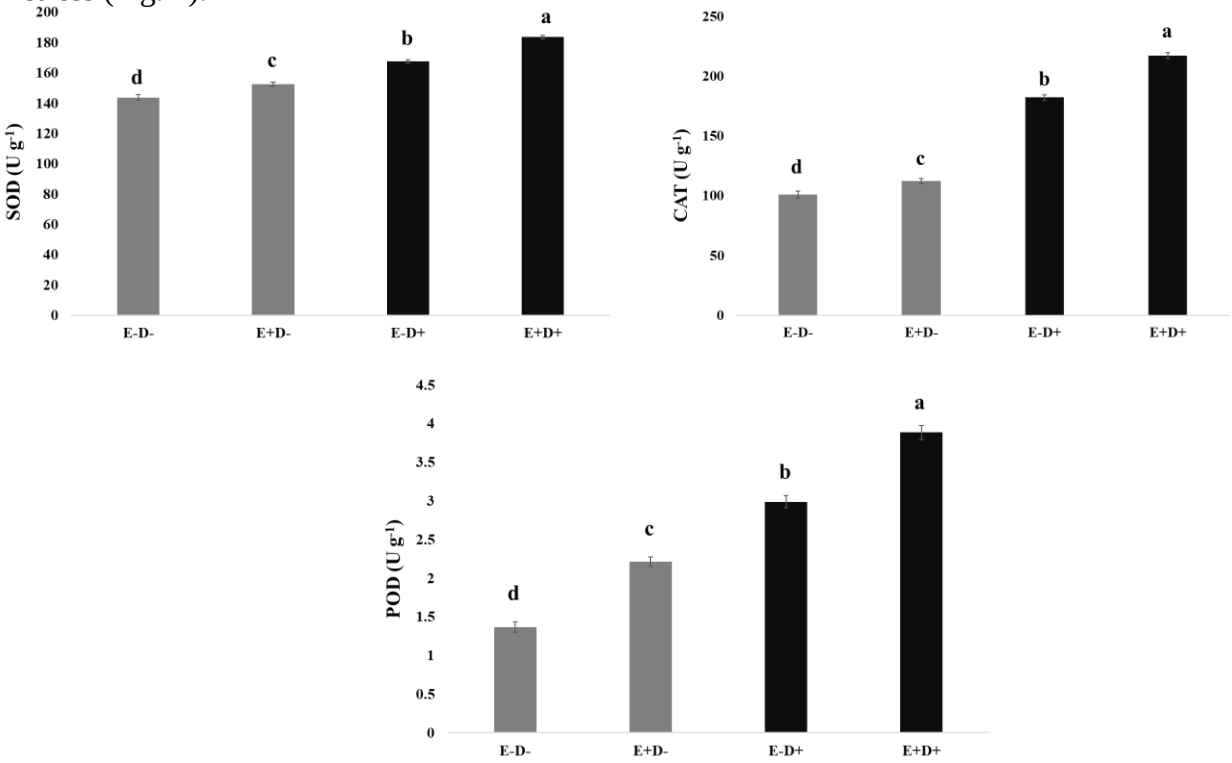

Fig 4. Antioxidant enzyme activities (SOD, CAT, POD) in rice plants four treatments: E−D− (control), E+D− (*Trichoderma* sp.), E−D+ (drought), and E+D+ (*Trichoderma* sp. + drought). Values are mean ± SE (n = 10). Different letters indicate significant differences (Tukey's HSD, p < 0.05).

Discussion

The present investigation establishes that endophytic *Trichoderma* sp. from the Himalayan region significantly enhances drought tolerance in rice (*Oryza sativa* L. cv. IR64) by regulating diverse physiological and antioxidant defence pathways crucial for maintaining plant vigour under stress[3]. Inoculation not only preserved shoot and root growth during drought but also restored chlorophyll content, leaf greenness, and relative water content compared to non-inoculated plants, indicating improved photosynthetic performance and water status[15,17].

These findings are consistent with recent studies demonstrating that local *Trichoderma* isolates provide protection against water deficit by improving growth parameters and recovery under 25–75% water availability, with significant increases in grain yield ranging from 15–24% under well-watered conditions[15,18]. Interestingly, *Trichoderma* inoculation led to pronounced accumulation of soluble sugars under water deficit—an osmoprotective response facilitating cellular hydration and supporting metabolic stability[14].

Moreover, endophyte-treated plants exhibited robust activation of antioxidant enzymes (SOD, CAT, POD), resulting in detoxification of reactive oxygen species and reduced oxidative damage, consistent with mechanisms reported for other beneficial plant–microbe systems[17,6]. These antioxidant enzyme enhancements are of particular interest given that drought stress elevates ROS generation, posing substantial threats to cellular structures and metabolic processes[14].

The enhanced upregulation of these biochemical and molecular processes suggests that *Trichoderma* sp. promotes stress adaptation response enabling rice plants to overcome drought-induced growth inhibition through simultaneous modulation of photosynthetic genes, secondary metabolite pathways, and osmotic homeostasis mechanisms[3]. These results show that fungal endophytes, especially *Trichoderma* sp., help plants cope with drought in several ways—by increasing osmolytes, improving antioxidant activity, supporting better root growth for water uptake, and changing the expression of genes involved in stress response[18]. Integrating such microbial inoculants into crop management not only augments physiological resilience but also holds promise for sustainable agriculture under exacerbating climate stress, particularly as recent investigations utilizing multi-omics approaches continue to elucidate the sophisticated molecular networks underlying endophyte-mediated drought tolerance[15,6].

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