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Synergistic effects of PGPR consortia and arbuscular mycorrhizal fungi on *Opuntia ficus-indica* tolerance against drought stress: physiological and biochemical responses

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ABSTRACT

Drought is a major constraint limiting the productivity of *Opuntia ficus-indica,* particularly in arid and semi-arid regions where sustainable strategies for enhancing stress tolerance are urgently needed. Plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) offer promising solutions to reinforce plant resilience. In this study, a greenhouse experiment was conducted to investigate the individual and combined effects of two PGP bacterial consortia (Cs1, Cs2), AMF, and their combinations (Cs1&AMF and Cs2&AMF) on growth, biochemical performance, and oxidative stress responses of *O. ficus-indica*, under two irrigation regimes, 95% field capacity (FC) and 20% FC. The overall results revealed that microbial inoculation enhanced multiple growth parameters, with combined treatments showing the greatest improvements in plant height, cladode area, and root and cladode biomass. Under drought, plants treated with Cs2, AMF, and combined inoculations maintained significantly better growth than stressed controls, indicating improved drought resilience. Inoculation with Cs2 and Cs1&AMF also promoted higher total chlorophyll content, supporting the maintenance of photosynthetic efficiency under water deficit, with increases of 88.34% for Cs2 and 41.11% for Cs1&AMF, respectively, compared with the control. Drought stress significantly increased proline and soluble sugar accumulation, whereas inoculated plants showed lower proline levels than the stressed control, with reductions of 19.23%, 20.44%, and 22.97% under AMF, Cs1&AMF, and Cs2&AMF treatments, respectively ($p < 0.05$), indicating partial alleviation of drought stress. Moreover, Cs2, AMF, and combined treatments effectively reduced malondialdehyde and hydrogen peroxide levels under stress, demonstrating reduced lipid peroxidation and reactive oxygen species accumulation, with reductions of 62.29% and 9.50% for Cs2, 58.33% and 12.11% for AMF, 35.05% and 18.99% for Cs1&AMF, and 54.90% and 18.62% for Cs2&AMF, respectively, compared with the stressed control. Antioxidant enzymes, catalase and ascorbate peroxidase, were generally induced by drought, but their activities did not differ significantly among treatments ($p \geq 0.05$). Overall, the results demonstrate that combined PGPR&AMF inoculation provides the most consistent benefits, improving growth, pigment stability, osmotic adjustment, and oxidative protection of *O. ficus-indica* under water deficit. These findings support the use of microbial combination of PGPR and AMF as an efficient biofertilization strategy for cactus cultivation in drought-prone environments.

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1. Introduction

Opuntia ficus-indica (L.) Mill., commonly known as the prickly pear cactus, is a cactus species of major agronomic, ecological, and economic importance. It is considered the most economically valuable cactus crop worldwide and the most widely distributed species within the *Cactaceae* family (Nobel et al., 2002). Owing to its remarkable capacity to adapt to harsh climatic conditions, this species is cultivated across numerous arid and semi-arid regions of the world, particularly in the Mediterranean basin, North Africa, and Latin America (Griffith, 2004). Beyond its agronomic value, *O. ficus-indica* plays a crucial ecological role in combating desertification and soil erosion by improving soil structure and stability through its extensive root system (Neffar et al., 2022; Rocha Filho et al., 2021; Rodrigues et al., 2023; Zouitane et al., 2025). Physiologically, *O. ficus-indica* exhibits a unique photosynthetic pathway known as crassulacean acid metabolism (CAM), which enables nocturnal CO_2 fixation and enhances water-use efficiency, contributing to its adaptation to arid environments (Al-Alam et al., 2023). This mechanism, together with the presence of aquifer parenchyma, confers an exceptional level of drought tolerance and water-use efficiency, positioning *O. ficus-indica* as a strategic crop for climate change adaptation in regions with limited water availability (Banga et al., 2022; Freitas Campos et al., 2021). Nevertheless, despite its intrinsic adaptation, prolonged or severe water deficits can significantly ($p < 0.05$) reduce its growth, biochemical composition, and productivity, resulting in osmotic imbalance, the accumulation of reactive oxygen species (ROS), and reduced biomass production (Freitas Campos et al., 2021; Guo et al., 2025; Lahbouki et al., 2022; Scalisi et al., 2016a).

To sustain the productivity of crops under water scarcity, eco-friendly biological strategies such as mycorrhizal and bacterial biofertilization have gained increasing attention (Khaliq et al., 2022; Mounaimi et al., 2024). Arbuscular mycorrhizal fungi (AMF) establish mutualistic associations with the roots of most terrestrial plants (Smith and Read, 2008). These symbiotic fungi expand the functional root surface by extending their hyphae into the soil beyond the rhizosphere, facilitating the uptake of water and essential nutrients (Porcel and Ruiz-Lozano, 2004; Silvestri et al., 2019). Additionally, AMF produce glomalin, a glycoprotein that enhances soil aggregate stability and water retention capacity (Bisht and Garg, 2022; Yang et al., 2017). From a physiological standpoint, AMF improve plant performance under drought by stimulating abscisic acid and osmolyte synthesis, regulating aquaporin expression, strengthening antioxidant defenses, and enhancing hydraulic conductivity (Begum et al., 2019; Meddich et al., 2022; Ouallal et al., 2022). Several studies have demonstrated that mycorrhizal inoculation mitigates the adverse effects of drought stress, as shown in cactus plants, where it increased soluble sugar levels, vegetative growth, and nutrient content (Lahbouki et al., 2023).

In parallel, plant growth-promoting rhizobacteria (PGPR) play a pivotal role in enhancing plant tolerance to both abiotic and biotic stresses. These beneficial bacteria employ multiple mechanisms, including phosphorus solubilization, biological nitrogen fixation, phytohormone production (e.g., indole-3-acetic acid, cytokinins, gibberellins), osmoprotectant synthesis, and activation of antioxidant enzyme systems (Bona et al., 2015, 2017; Schütz et al., 2018). They also stimulate root growth and protect against pathogens (Gianinazzi et al., 2010; Khoso et al., 2024; Lingua et al., 2013). Although their beneficial effects have been extensively documented in annual and herbaceous crops, few studies have focused on perennial woody species such as *O. ficus-indica* (Bashan et al., 2009; Ramírez-Moreno et al., 2017).

The simultaneous application of AMF and PGPR has emerged as a promising biotechnological strategy to enhance plant resilience under water deficit conditions (Zouitane et al., 2026a). PGPR can promote root proliferation and release metabolites that favor mycorrhizal establishment, while AMF enhance nutrient and water uptake and modulate host plant physiology (Bona et al., 2017). Several recent studies have reported additive or synergistic effects of such dual inoculations on plant growth, water-use efficiency, and oxidative stress regulation, although the extent of these benefits depends on the plant species, microbial strains, and experimental conditions (Kebede et al., 2024; Lahbouki et al., 2022; Lahbouki et al., 2023). However, research integrating both symbiotic systems (AMF and PGPR) in *O. ficus-indica* remains scarce, particularly under controlled drought conditions.

The mechanisms underlying PGPR-AMF co-inoculation and their synergistic effects on plant performance under water deficit are still poorly understood, despite their importance for optimizing sustainable biofertilization strategies in arid and semi-arid ecosystems. Therefore, the present greenhouse study was designed to evaluate the combined effects of two drought-tolerant PGP bacterial consortia and AMF on morphological growth, chlorophyll content, as well as biochemical and antioxidant responses of *O. ficus-indica* under both well-watered and water-deficit conditions. The overarching goal was to identify beneficial microbial interactions and synergistic mechanisms capable of enhancing productivity and drought tolerance in this emblematic cactus species cultivated in water-limited agroecosystems. Importantly, and to the best of our knowledge, this study is the first to investigate the use of a PGPR consortium isolated from the rhizosphere of *O. ficus-indica* alongside another consortium derived from a different rhizosphere, applied either alone or in combination with AMF. By integrating these microbial consortia with mycorrhizal symbiosis, this work provides novel insights into microbial compatibility, functional complementarity, and their combined impact on plant physiological and biochemical responses under drought stress, thereby advancing current knowledge beyond conventional inoculation approaches.

2. Material and methods

2.1. Plant material

Cladodes of *O. ficus-indica* were collected on February 19, 2023, from Aknoul station, in the Taza province (34°39′39″N; 3°52′08″W), a region characterized by a sub-humid climate. After harvesting, the cladodes were air-dried in the shade at ambient temperature for 15 days to ensure moisture reduction while preventing sun damage. All selected cladodes were collected from the same level of the plant to ensure uniformity in physiological age.

2.2. Microbial inocula

Two bacterial consortia and an AMF complex were evaluated for their potential to enhance the growth and development of *O. ficus-indica* under controlled conditions. The AMF complex, provided by the National School of Agriculture of Meknes (ENAM), was a consortium of three indigenous strains: *Rhizoglomus irregulare*, *Claroideoglomus etunicatum*, and *Funneliformis mosseae*. These strains were originally isolated from the date palm rhizosphere in the Tafilalt oasis (Morocco), as described by El Hilali et al. (2022). The inoculum was multiplied via trap culture using *Sorghum bicolor* as the host plant, reaching a final spore density of 1600 spores per 100 g of substrate. Regarding the bacterial treatments, the first bacterial consortium (Cs1), derived from the microbial collection of the Faculty of Sciences and Technologies of Fez (FST) at USMBA, consisted of four previously characterized strains: MFF3 (*Achromobacter insolitus*), MFE1 (*Pseudomonas putida* CP1V1-01), MFE3 (*Providencia rettgeri* RFFL-I), and MFE4 (*Alcaligenes* sp. BND-AN8). These strains were previously identified and described by Ferioun et al. (2024a, b). The second consortium (Cs2) was developed as part of the ProSmallAgriMed project and also comprised four bacterial isolates: ZN3 (*P. frederiksbergensis*), ZN4 (*P. koreensis*), ZN12 (*Bacillus halotolerans*), and ZSSp3 (*Priestia megaterium*) (Zouitane et al., 2026b). These isolates were obtained from the rhizosphere of *O. ficus-indica* collected from three distinct Moroccan sites: Tafrant, Fez, and Chichaoua (Zouitane et al., 2025).

All bacterial isolates were screened for multiple plant growth-

promoting (PGP) traits, including inorganic phosphate solubilization, nitrogen fixation, production of indole-3-acetic acid (IAA), siderophores, ammonia (NH_3), hydrogen cyanide (HCN), and exopolysaccharides (EPS). Additionally, their capacity to produce acid and alkaline phosphatases and hydrolytic enzymes (cellulase and pectinase) was evaluated. Their tolerance to water stress was assessed by simulating drought conditions, expressed in terms of osmotic potential (MPa) (Ferioun et al., 2024a, 2024b). The results of the PGP trait assays are summarized in Table 1.

In vitro antagonistic interactions among the isolates within each consortium were assessed. No inhibitory effects or antagonism were observed, confirming their compatibility.

Bacterial inoculants were prepared by culturing each isolate in Luria-Bertani (LB) broth at 30°C for 48 h under shaking conditions. The cultures were then centrifuged at 6000 × g for 20 min. The resulting pellets were washed twice with sterile physiological saline (0.85% NaCl), resuspended, and mixed in equal proportions for each bacterial strain, then diluted in sterile distilled water to obtain a final concentration of approximately 10^8 CFU/mL. For the AMF treatment, 10 g of the fungal inoculum (soil containing spores, mycelium, and colonized root fragments) was placed directly beneath each *O. ficus-indica* cladode at the time of planting to ensure immediate contact with the emerging roots. Regarding the PGPR, the inoculation was performed by soil irrigation near the base of each plant. A volume of 100 mL of the bacterial suspension was applied to each pot to ensure proper colonization of the rhizosphere.

2.3. Experimental design

Each experimental unit consisted of a plastic pot (35 cm in height and 30 cm in diameter) filled with 18 kg of a soil mixture composed of agricultural soil from the Fez region and sand in a 1:1 ratio. The physicochemical characteristics of the soil mixture were as follows: pH (H_2O) = 7.87, pH (KCl) = 8.16, electrical conductivity = 204.67 µS/cm, organic matter content = 4.93%, P = 74.18 µg/g, NH_4^+ = 5.43 µg/g, NO_3^- = 46.60 µg/g, SO_4^{2-} = 406.30 µg/g, Ca = 288.60 µg/g, Mg = 38.10 µg/g, Na = 598.03 µg/g, Fe = 41.82 µg/g, and Mn = 66.98 µg/g.

The experiment was conducted from March to September 2023 in a greenhouse at the Faculty of Sciences and Technologies, Sidi Mohamed Ben Abdellah University, Fez, Morocco, under natural light conditions, with an average photoperiod of approximately 16 h per day. The regional climate is Mediterranean, characterized by humid winters and dry summers. During the experimental period, the relative humidity inside the greenhouse ranged from approximately 50% to 70%. While *O. ficus-indica* is a perennial, slow-growing species, the experimental duration was sufficient to capture significant physiological, biochemical, and symbiotic responses to microbial inoculation under drought stress. These short-to-medium-term adaptations provide critical insights into early plant-microbe interactions, although long-term stable dynamics may justify extended investigation in future studies.

Two irrigation regimes were implemented: a well-watered regime corresponding to 95% of field capacity (FC), ensuring optimal water availability without excess saturation, thereby providing a reliable non-stress baseline for comparison and avoiding waterlogging conditions, and a drought-stressed regime corresponding to 20% of FC. This level was selected to impose a severe but non-lethal water deficit, based on previous studies indicating that severe drought stress typically occurs around 15% FC, while moderate stress occurs around 30% FC (Lahbouki et al., 2022). The target soil-moisture levels corresponding to 95% and 20% of field capacity were maintained using a gravimetric pot-weighing method. Briefly, each pot was weighed regularly, and the amount of water required to restore the soil to its predetermined target weight was calculated from the difference between the current pot weight and the target weight corresponding to the respective field-capacity level. Irrigation was therefore adjusted throughout the experiment to compensate for water losses resulting from evapotranspiration and plant growth. The field capacity of the soil mixture was determined beforehand by saturating the soil and recording the amount of water retained after 24 h of drainage. After an establishment period of 15 days, microbial inoculation was performed, and drought stress was subsequently imposed. This approach allowed plants to reach a minimum physiological stability before stress application, ensuring that the observed responses reflect true drought stress effects rather than differences in early establishment. The field capacity for each pot was previously determined by measuring the volume of water retained 24 h after saturation.

The experimental design followed a completely randomized design (CRD), comprising six microbial treatments and two irrigation regimes, with five independent biological replicates per treatment × irrigation combination. Each pot containing one plant was considered an independent experimental unit, resulting in a total of 60 experimental units. The microbial treatments were: (i) a non-inoculated control (Control); (ii) inoculation with Cs1 (MFF3, MFE1, MFE3, and MFE4) (100 mL per plant); (iii) inoculation with Cs2 (ZN3, ZN4, ZN12, and ZSSp3) (100 mL per plant); (iv) inoculation with an AMF complex (10 g per plant); (v) co-inoculation with Cs1 and AMF; and (vi) co-inoculation with Cs2 and AMF. The pots were randomly assigned to their respective treatment × irrigation combinations and randomly distributed within the greenhouse to minimize potential positional effects.

The harvest was carried out six months after planting, allowing sufficient time to detect measurable growth, physiological, biochemical, and early plant-microbe responses to water deficit and microbial inoculation under the experimental conditions used in this study (Lahbouki et al., 2023).

2.4. Plant growth measurements

At harvest, growth parameters were measured for both roots and newly developed cladodes. Root length, plant height, number of new cladodes, cladode fresh and dry (dried at 60°C for 12 days) weights, roots fresh and dry (dried at 60°C for 72 h) weights, and cladode area of plants (length, width, and thickness of each cladode were recorded using

Table 1
Plant growth-promoting traits and drought stress tolerance of bacterial isolates.

Isolates	PSI	N_2 fixation	IAA	Siderophores	NH_3^+	HCN	EPS	Cellulase	Pectinase	Alkaline Pase	Acid Pase	Drought tolerance (MPa)
ZN3	+	+	+	+	+	+	-	+	+	+	-	−0.15
ZN4	+	-	+	+	+	+	-	-	-	+	+	−0.05
ZN12	+	+	+	+	+	-	+	-	-	+	+	−0.15
ZSSp3	+	+	+	-	+	-	+	-	-	+	-	−0.15
MFF3	+	-	+	+	+	-	+	-	+	+	+	−0.49
MFE1	+	+	+	+	+	-	-	+	+	+	+	−0.49
MFE3	+	+	+	-	+	-	+	-	+	+	+	−0.73
MFE4	+	+	+	+	+	+	-	-	-	+	+	−0.73

PSI: phosphate solubilization index; N_2 fixation: nitrogen fixation; IAA: indole-3-acetic acid production; NH_3^+: ammonia production; HCN: hydrogen cyanide production; EPS: exopolysaccharides production; alkaline Pase: alkaline phosphatase activity; acid Pase, acid phosphatase activity. All tests were performed in triplicate. Qualitative traits are denoted by (+) for presence and (−) for absence, as consistently observed across all replicates.

a measuring ruler) were estimated using the formula for the area of an ellipse:

$$\text{Total cladode area per plant} = \sum_{i=1}^{n} \left(\frac{\text{Li}}{2}\right) * \left(\frac{\text{wi}}{2}\right) * \pi \ (\text{cm}^2)$$

(where π = 3.141592, Li and Wi represent the length and width of the i cladode, respectively, and n is the total number of cladodes per plant).

2.5. Chlorophyll content

Twenty milligrams of fresh plant tissue were placed in glass tubes containing 1.4 mL of dimethyl sulfoxide (DMSO) and incubated in a water bath at 65°C for 5 h. After incubation, the extract volume was adjusted to 2 mL with DMSO (Hiscox and Israelstam, n.d.). The absorbance of the extracts was measured at 645 nm and 663 nm using a spectrophotometer. DMSO without plant tissue was used as the blank. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents were calculated using the equations described by Arnon (1949):

Chlorophyll *a* (mg. g^{-1} FW) = ((12.7 * A663) – (2.69 * A645)) * (2 / (1000 * m))

Chlorophyll *b* (mg. g^{-1} FW) = ((22.9 * A654) – (4.68 * A663)) * (2 / (1000 * m))

Total chlorophyll (mg. g^{-1} FW) = ((20.2 * A645) + (8.02 * A663)) * (2 / (1000 * m))

The chlorophyll concentrations obtained from these equations were initially calculated on a fresh-weight basis (mg g^{-1} FW). For consistency with the dry-weight normalization adopted in this study, the values were subsequently converted to a dry-weight basis using the corresponding sample-specific FW/DW ratio. The final chlorophyll contents are expressed as mg g^{-1} DW.

2.6. Accumulation of osmolytes

a. Proline content

The proline concentration in plant tissues was quantified following the method described by (Bates et al. (1973). 0.5 g of fresh plant tissue was homogenized in 10 mL of 3% (w/v) aqueous sulfosalicylic acid. The homogenate was then filtered through Whatman No. 2 filter paper to obtain a clear extract. Two milliliters of the filtrate were transferred into a clean test tube and mixed with 2 mL of acid ninhydrin reagent and 2 mL of glacial acetic acid. The acid ninhydrin reagent was freshly prepared by dissolving 1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid with gentle heating and constant stirring until complete dissolution. The reaction mixture was incubated in a water bath at 100°C for 1 h, followed by immediate cooling in an ice bath to terminate the reaction. Subsequently, 4 mL of toluene was added to the reaction mixture. The solution was vigorously mixed for 15-20 s. The absorbance was measured at 520 nm using a UV-Visible spectrophotometer, with toluene as a blank. The results were expressed in μmol of proline per gram of fresh weight (FW) using the following formula:

Proline (μmol/g FW) = ((μg proline/mL×mL of toluene)/ 115.5 μg/μmol) ÷ (g of sample/5)

Proline concentration was determined using a standard curve and initially calculated relative to the fresh tissue mass used for extraction. The resulting values were normalized to the corresponding dry tissue mass using the sample-specific FW/DW ratio. The final proline content was expressed as mg g^{-1} DW.

b. Soluble sugars

To quantify soluble sugars in cactus tissues, approximately 0.075 g of fresh cladode tissue was homogenized in 2 mL of 80% (v/v) ethanol using a mortar and pestle. The homogenate was centrifuged at 10000 rpm for 15 min at 4°C. An aliquot of 1 mL of the supernatant was transferred to a test tube and mixed with 1 mL of 5% (w/v) phenol solution, followed by the rapid addition of 5 mL of concentrated sulfuric acid. The tubes were gently shaken and left at room temperature for 10 min to allow color development, then incubated in a water bath at 70°C for 30 min to facilitate the extraction of soluble sugars. The absorbance of the resulting-colored solution was measured at 485 nm using a spectrophotometer (Dubois et al., 1956). The concentration of soluble sugars was calculated by comparing the absorbance values to a standard curve generated with known concentrations of glucose. The soluble sugar concentration was initially determined from the glucose standard curve and calculated relative to the fresh tissue mass used for extraction. The resulting values were normalized to the corresponding dry tissue mass using the sample-specific FW/DW ratio. The final soluble sugar content was expressed as mg g^{-1} DW.

c. Total proteins

Approximately 0.2 g of fresh cladode tissue was homogenized in 2 mL of 100 mM sodium phosphate buffer (pH 7.5). The homogenate was centrifuged at 8000 rpm for 15 min at 4°C, and the resulting supernatant was collected for protein quantification (Ferioun et al., 2025). In a test tube, 100 μL of the protein extract was mixed with 100 μL of 2× diluted Folin-Ciocalteu reagent, followed by the addition of 1 mL of Solution A, which consists of a mixture of copper reagent, base reagent, and potassium-sodium tartrate in sodium carbonate (Na_2CO_3). The tubes were incubated at room temperature for 10 min. After this step, 100 μL of Solution B (containing sodium hydroxide (NaOH) and sodium dodecyl sulfate (SDS)) was added to each tube. The mixtures were gently shaken and left to incubate for 45 min at room temperature to allow for the development of the blue coloration. The absorbance of the resulting solutions was measured at 640 nm using a spectrophotometer. Protein concentration was determined by comparing the absorbance values to a standard curve prepared with bovine serum albumin (BSA) at known concentrations (0.2, 0.4, 0.6, 0.8, and 1 μg/mL) (Sarker et al., 2015). The protein concentration determined from the BSA standard curve was calculated relative to the fresh tissue mass used for extraction. The resulting values were normalized to the corresponding dry tissue mass using the sample-specific FW/DW ratio. The final protein content was expressed as mg g^{-1} DW.

2.7. Oxidative stress indicators

a. Malondialdehyde (MDA) content - lipid peroxidation

The MDA content was determined using the thiobarbituric acid (TBA) assay, as described by Heath and Packer (1968). 0.2 g of biomass was homogenized in 5 mL of a solution containing 0.5% (w/v) thiobarbituric acid prepared in 20% (w/v) trichloroacetic acid (TCA). The homogenate was transferred into centrifuge tubes, and the reaction mixture was incubated in a water bath at 95°C for 30 min. Following incubation, samples were cooled to room temperature and centrifuged at 10000g for 5 min at 25°C to remove insoluble debris. The absorbance of the supernatant was measured at 532 nm and 600 nm using a UV-Visible spectrophotometer. The concentration of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Mirbakhsh and Sohrabi sedeh, 2022). The MDA concentration was initially calculated relative to the fresh tissue mass used for extraction. The resulting values were subsequently normalized to the corresponding dry tissue mass using the sample-specific FW/DW ratio. The final MDA content is expressed as mg g^{-1} DW.

b. Hydrogen peroxide (H_2O_2) content

The hydrogen peroxide (H_2O_2) content in *O. ficus-indica* cladodes was quantified using the method described by Jana and Choudhuri (1981), with slight modifications. 1 g of fresh cladode tissue was homogenized in 15 mL of cold 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was then centrifuged at 6000g for 20 min. An aliquot of 0.5 mL of the supernatant was transferred to a test tube and mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The reaction mixture was incubated at room temperature for 10 min in the dark. The absorbance was recorded at 390 nm using a UV-Visible spectrophotometer. The concentration of H_2O_2 was determined by comparison with a standard calibration curve prepared from known concentrations of hydrogen peroxide and expressed as mg of H_2O_2 per gram of fresh weight (FW) of cladode tissue. The H_2O_2 concentration was initially calculated relative to the fresh tissue mass used for extraction. The resulting values were subsequently normalized to the corresponding dry tissue mass using the sample-specific FW/DW ratio. The final H_2O_2 content is expressed as mg g^{-1} DW.

2.8. Antioxidant Enzyme Activity

Around 500 mg of cladode samples was ground with 4 mL of 50 mM phosphate potassium buffer (pH 7) in an ice-cold mortar. The mixture was centrifuged at 20000 rpm for 30 min at 4 ∘C. The supernatant was considered as the enzyme extract (Ferioun et al., 2025).

a. Catalase activity (CAT)

Catalase (CAT) (EC:1.11.1.6) activity was determined by the method of Aebi (1984). The reaction was started by adding 50 μL of enzyme extract to the assay solution containing phosphate potassium buffer (50 mM, pH 7) and H_2O_2 (10 mM). The mixture was incubated for 5 min at room temperature, and the absorbance was measured at 240 nm. CAT activity was calculated using the molar extinction coefficient $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$. The activity was calculated from the decrease in absorbance at 240 nm and normalized to the dry tissue mass corresponding to the enzyme extract used in the assay. CAT activity is expressed as mM H_2O_2 min^{-1} g^{-1} DW.

b. Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) (EC: 1.11.1.11) activity was determined photometrically using the method described by Nakano and Asada (1980). Briefly, the reaction was started by adding 50 μL of enzymes solution to the assay solution containing phosphate potassium buffer (50 mM, pH 7), H_2O_2 (1 mM), ethylenediaminetetraacetic acid (EDTA) (0.2 mM), and ascorbic acid (0.5 mM). After 1 min at room temperature, the absorbance was measured at 290 nm. APX activity was calculated using the molar extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. The activity was calculated from the decrease in absorbance at 290 nm and normalized to the dry tissue mass corresponding to the enzyme extract used in the assay. APX activity is expressed as mM ascorbate oxidized min^{-1} g^{-1} DW.

2.9. Estimation of root mycorrhizal colonization

Root colonization by arbuscular mycorrhizal fungi was evaluated using the trypan blue staining method. Fine root fragments were thoroughly washed and cleared in 10% potassium hydroxide (KOH) at 90°C for 60 min, then rinsed with distilled water and acidified in 1% hydrochloric acid (HCl) for 5 min. The roots were subsequently stained in a trypan blue solution at 90°C for 20 min (Phillips and Hayman, 1970). After staining, excess dye was removed by transferring the roots to distilled water, followed by a brief destaining step to improve visualization. Stained root fragments were mounted on slides, and AMF colonization was quantified under a microscope. Mycorrhizal indices were quantified according to Trouvelot (1986). The frequency of mycorrhization (F %), reflecting the proportion of colonized root fragments, was calculated as:

$$F\ \% = [(N - n) / N] \times 100$$

where N is the total number of observed fragments and n the number of non-colonized fragments. The intensity of mycorrhization (M %), representing the degree of fungal development within the colonized roots, was determined using the formula:

$$M\ \% = [(95 \times n5) + (70 \times n4) + (30 \times n3) + (5 \times n2) + n1] / N$$

with n5, n4, n3, n2, and n1 corresponding to the numbers of fragments assigned to colonization classes 5 to 1, respectively. The arbuscular abundance (A %), indicating the proportion of root fragments containing arbuscules, was calculated as:

$$A\ \% = [(100 \times mA3) + (50 \times mA2) + (10 \times mA1)] / 100$$

where mA1, mA2, and mA3 represent the percentages of fragments presenting arbuscular structures of increasing abundance (classes A1, A2, and A3).

2.10. Data Analysis

To ensure consistency among samples differing in tissue water content, all biochemical parameters, including chlorophylls, soluble sugars, total proteins, proline, malondialdehyde (MDA), H_2O_2, total phenolics, flavonoids, and antioxidant enzyme activities, were expressed on a dry-weight (DW) basis. Although biochemical analyses were performed using fresh plant tissue, the final values were normalized to the corresponding dry tissue mass. For each sample, the fresh-to-dry weight ratio (FW/DW) was determined, and FW-based concentrations were converted to DW-based concentrations using the following equation:

$$C\ (DW) = C\ (FW) \times (FW/DW)$$

where C (DW) is the concentration expressed per unit of dry weight, C (FW) is the concentration expressed per unit of fresh weight, FW is the fresh mass of the sample, and DW is the corresponding dry mass of the same sample. The sample-specific FW/DW ratio was used for conversion.

All data collected from the different treatments and irrigation regimes were analyzed using two-way factorial analysis of variance (ANOVA), with irrigation regime and microbial treatment as fixed factors and their interaction included in the model. Statistical significance was assessed at $p < 0.05$. When significant differences were detected, mean comparisons were performed using Tukey's honestly significant difference (HSD) test at a significance level of $p < 0.05$. Differences with $p \geq 0.05$ were considered non-significant. All statistical analyses were performed using Minitab® 21.2 (64-bit). In addition, principal component analysis (PCA) was performed using XLSTAT® 2016.02.28451 software to evaluate patterns of variation and to reveal correlations among the measured morphological, physiological, biochemical, and mycorrhizal colonization parameters across different treatments. PCA enabled the identification of relationships between variables and grouping patterns among treatments based on their multidimensional data profiles.

3. Results and discussion

3.1. Analysis of variance

The mean squares obtained from the analysis of variance (ANOVA) were used to assess the contribution of water regime and inoculation factors, as well as their interaction, to trait variability (Fig. 1). The results indicated that the water regime factor was the main source of variation for most plant traits, accounting for more than 75% of the total

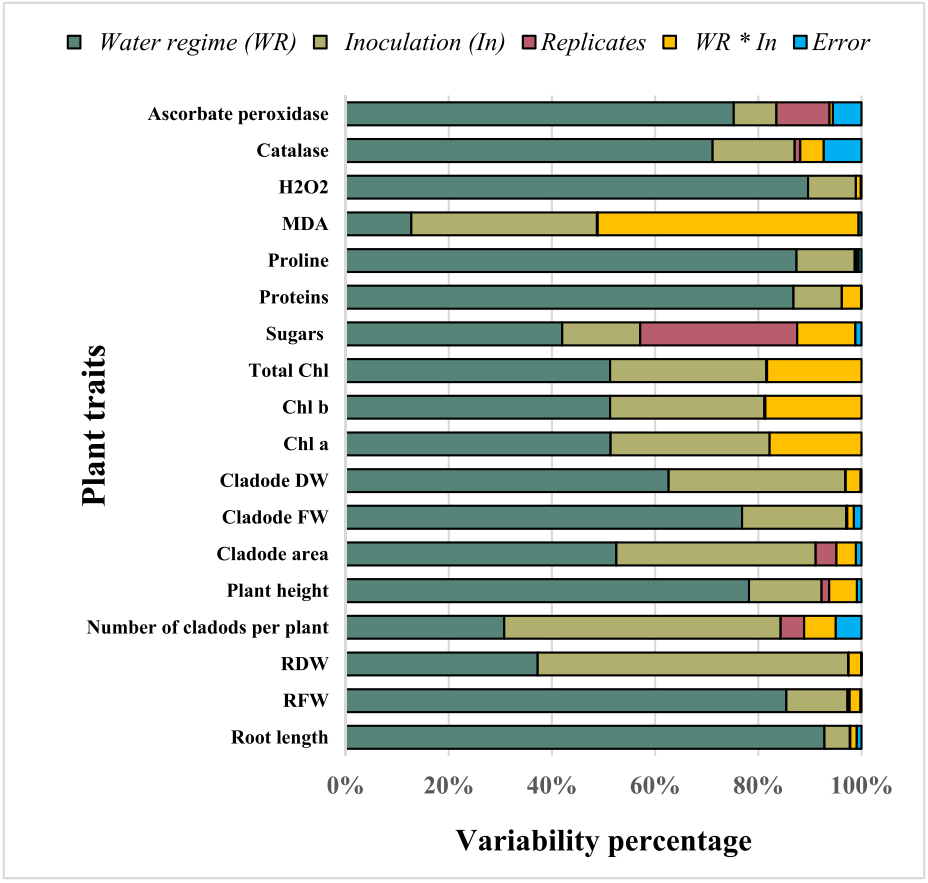


Fig. 1. Effect of treatment and inoculation factors and their combination on the variability of plant traits using the analysis of variance (ANOVA) multifactorial RFW: Root fresh weight; RDW: Root dry weight; CFW: Cladode fresh weight; CDW: Cladode dry weight; Nbr C: Number of cladodes; Chl a: Chlorophyll *a*; Chl b: Chlorophyll *b*; Chl total: Total chlorophyll; MDA: Malondialdehyde; APX: Ascorbate peroxidase; H_2O_2: Hydrogen peroxide; F%: Frequency of mycorrhization, M%: Intensity of mycorrhization; A%: Arbuscule frequency.

variability in root length, RFW, cladode FW, proteins, proline, H_2O_2, catalase, and ascorbate peroxidase. Moreover, the water regime factor explained more than 45% of the variability in cladode area and DW, as well as chlorophyll pigment content. In contrast, the contribution of the water regime factor was less than 35% of the total variability in RDW, the number of cladodes per plant, and MDA content. Consistent with our findings, several previous studies have identified the water regime factor as the primary source of variability for most plant traits (Ferioun et al., 2024a; Lahbouki et al., 2022). Our study also reflects the strong physiological and biochemical responsiveness of *O. ficus-indica* to changes in soil water availability (Najafinezhad et al., 2025). Under drought conditions, microbial inoculation maintained higher chlorophyll levels than the stressed control (chlorophyll *a*: +45.46%; chlorophyll *b*: +89.39%; total chlorophyll: +88.34%) and reduced proline accumulation (−19.23 to −22.97%), MDA levels (−35.05 to −62.29%), and H_2O_2 accumulation (approximately −18%), while partially maintaining protein content (+20.77 to +30.47%). CAT and APX activities increased under drought, although no significant differences among inoculation treatments were detected.

The inoculation factor was identified as the primary source of variability for RDW and the number of cladodes per plant, accounting for more than 50% of the total variability. In addition, this factor represented the second most important source of variation for MDA content, chlorophyll pigment content, and cladode area and DW, contributing approximately 35% of the total variability. Finally, the interaction between treatment and inoculation factors was the main source of variability for MDA content (about 50% of the total variability) and the third source of variability for chlorophyll pigment content (Fig. 1). Moreover, the pronounced effect of the inoculation factor on the variability of various plant traits, as reported in numerous studies, highlights the strong biostimulation potential of the tested microbial consortia. This effect is associated with improved cellular osmotic status, down-regulation of oxidative stress markers, upregulation of antioxidant enzyme activities, and enhanced photosynthetic efficiency through increased chlorophyll synthesis (Lahbouki et al., 2023; Shreshtha et al., 2025), ultimately leading to improved plant growth and biomass accumulation.

3.2. Plant growth responses to bioinoculation under drought stress

Growth parameters were analyzed to assess the influence of microbial inoculation and drought stress on the development of *O. ficus-indica*. Fig. 2 presents the effect of microbial inoculation on eight morphological parameters of *O. ficus-indica*: (A) plant height, (B) cladode number, (C) cladode area, (D) cladode fresh weight, (E) cladode dry weight, (F) root length, (G) root fresh weight, and (H) root dry weight.

Under well-irrigated conditions, combined inoculations actively stimulated vegetative growth. Cs1&AMF significantly ($p < 0.05$) increased plant height by 14.04% compared with the non-inoculated control, while Cs2&AMF and Cs1&AMF markedly enhanced cladode development. In particular, cladode number increased by 81.82% under Cs1&AMF and by 54.55% under Cs2&AMF treatments, reflecting enhanced vegetative propagation. Cladode area was also significantly ($p < 0.05$) enlarged under Cs2 (+28.11%), Cs1&AMF (+44.21%), and Cs2&AMF (+35.85%), indicating improved photosynthetic surface and growth capacity. These morphological improvements translated into

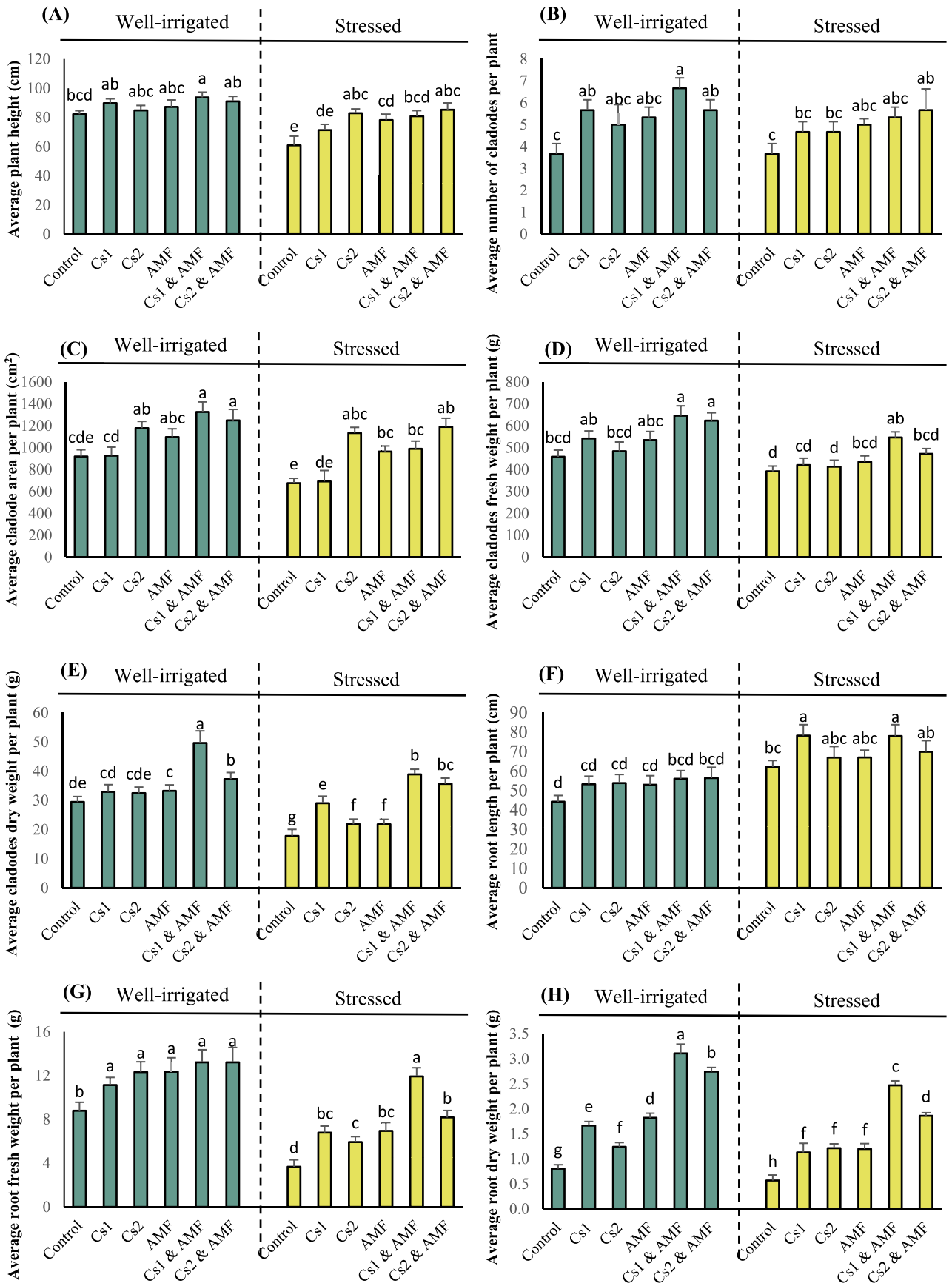


Fig. 2. Growth responses of *O. ficus-indica* to microbial inoculation under well-irrigated and drought stress conditions. Different letters indicate significant differences according to Tukey's test ($p < 0.05$). Cs: consortium; AMF: Arbuscular Mycorrhiza Fungi.

greater biomass accumulation, as cladode fresh weight increased by 40.87% and 36.00% under Cs1&AMF and Cs2&AMF, respectively, while cladode dry weight rose by 68.67% and 26.62% in the same treatments.

The present study demonstrates that microbial inoculation markedly improves the growth performance of *O. ficus-indica*, with the strongest effects recorded under the combined PGPR&AMF treatments (Cs1&AMF and Cs2&AMF). In both irrigation regimes, these combined inoculations enhanced multiple morphological traits (plant height, cladode area, cladode biomass, and root development) (Fig. 2) more effectively than single inoculations, confirming the synergistic interactions widely reported in other species such as *Olea europaea*, *Leymus chinensis*, *Zea mays*, and *Solanum lycopersicum* (Khan et al., 2024; Sbbar et al., 2025; Tahiri et al., 2022; Zhang et al., 2024). Such growth enhancement under optimal water availability likely reflects improved nutrient acquisition, particularly phosphorus and nitrogen. Phosphorus availability is known to stimulate photosynthesis, shoot growth, and dry matter accumulation, as demonstrated in soybean inoculated with *Bacillus velezensis* UFV 3918 (Luiz Santos et al., 2025). In parallel, nitrogen fixation and ammonia production by PGPR may have contributed to increased biomass production, consistent with observations in *Populus trichocarpa* clones inoculated with diazotrophic consortia (Knoth et al., 2014). Moreover, PGPR-derived phytohormones, especially indole-3-acetic acid (IAA), are known to stimulate root elongation and branching (Erturk et al., 2010; Sandhya et al., 2009), thereby improving water and nutrient uptake and supporting the observed enhancement of aboveground growth.

The positive effects of microbial inoculation were even more pronounced under drought stress, where growth inhibition was strongly mitigated by AMF-associated treatments. Under water deficit, Cs2, AMF, Cs1&AMF, and Cs2&AMF maintained significantly ($p < 0.05$) greater plant height than the stressed control, with increases ranging between approximately 30% and 40%. In contrast, the non-inoculated control and Cs1 treated plants showed marked height reductions under stress, highlighting the superior buffering capacity of AMF based treatments. Similarly, cladode area was substantially preserved under drought, increasing by 67.70% in Cs2, 42.69% in AMF, 46.38% in Cs1&AMF, and up to 75.96% in Cs2&AMF relative to the stressed control. This maintenance of the cladode surface under drought is particularly critical for CAM plants such as *O. ficus-indica*, in which cladodes are central to carbon assimilation, water storage, and overall productivity (Bartlett et al., 2014; Scalisi et al., 2016b; Wang et al., 1998).

Biomass-related traits provided the clearest evidence of synergistic effects under drought conditions. While drought markedly reduced cladode fresh and dry weights in the control, combined inoculations preserved biomass accumulation. Cs1&AMF maintained a 39.37% increase in cladode fresh weight under drought, while cladode dry weight increased dramatically by 118.62% in Cs1&AMF and by 100.35% in Cs2&AMF compared with the stressed control. Similar improvements in biomass maintenance under stress have been reported in cactus and AMF associations (Lahbouki et al., 2022; Lahbouki et al., 2023) and in drought or salt-stressed grasses subjected to microbial co-inoculation (Khan et al., 2024; Sbbar et al., 2025; Zhang et al., 2024).

Root traits further highlighted the functional complementarity between PGPR and AMF. Under well-irrigated conditions, root fresh weight increased in all inoculated treatments compared with the control, by 26.74% in Cs1, 40.13% in Cs2, 40.54% in AMF, 50.29% in Cs1&AMF, and 50.22% in Cs2&AMF, indicating a general stimulation of belowground growth. Under drought stress, combined treatments showed a remarkable capacity to preserve root biomass, with root fresh weight increases reaching 225.15% in Cs1&AMF and 122.92% in Cs2&AMF. Root dry weight showed a similar pattern, with Cs1&AMF and Cs2&AMF displaying significantly ($p < 0.05$) higher values under both irrigation regimes, reaching 335.53% and 228.18%, respectively, under drought. In addition, root length increased under stress in Cs1 (+25.84%), Cs1&AMF (+25.47%), and Cs2&AMF (+12.44%), indicating improved root elongation and soil exploration capacity.

These root responses are consistent with auxin-mediated root proliferation induced by PGPR and the expansion of the soil exploration zone driven by AMF hyphal networks (Chaudhary et al., 2025; Erturk et al., 2010). Enhanced root development likely improved water foraging efficiency and nutrient acquisition, thereby sustaining shoot growth and biomass accumulation under drought, as previously reported in maize and other drought adapted crops (Chandregowda et al., 2023; Danish et al., 2020). Additional mechanisms, such as the production of bacterial exopolysaccharides that improve soil aggregation and moisture retention (Sandhya et al., 2009), may also have contributed to the observed growth stability. Although hydrogen cyanide production was low, it may have indirectly enhanced plant performance by suppressing deleterious rhizosphere microorganisms (Lieberei et al., 1996).

Altogether, the consistent superiority of Cs1&AMF and Cs2&AMF across growth parameters and irrigation regimes demonstrates a strong synergistic interaction, in which PGPR may act as mycorrhiza-helper bacteria, enhancing AMF colonization and efficiency, while AMF improve rhizosphere conditions and nutrient dynamics that support bacterial activity. This synergy translates into enhanced plant vigor, sustained growth, and improved drought resilience. Such interactions, previously reported in soil restoration and stress mitigation contexts (Hnini et al., 2024), position combined PGPR&AMF inoculation as a promising and sustainable strategy for improving *O. ficus-indica* cultivation in arid and semi-arid environments.

3.3. Chlorophyll content

The effect of microbial inoculation on the photosynthetic pigments of *O. ficus-indica* is shown in Fig. 3, highlighting changes in chlorophyll *a* (A), chlorophyll *b* (B), and total chlorophyll (C) under well-irrigated and stressed conditions. Under well-irrigated conditions, the uninoculated control exhibited the lowest chlorophyll *a* content. Single bacterial inoculations improved this parameter moderately (25.30% and 53.36% for Cs1 and Cs2, respectively), while the treatment with AMF alone and the combinations produced the highest increases. In particular, the Cs2&AMF treatment reached the maximum value (383.60%), significantly outperforming all other treatments. Under water-stress conditions, chlorophyll *a* content decreased across all treatments compared with well-irrigated plants. Nevertheless, inoculated plants, especially those treated with Cs1&AMF, maintained significantly ($p < 0.05$) higher values than the stressed control, showing an increase of 45.46%, indicating that the inoculants contributed to the protection of chlorophyll pigments against drought-induced degradation (Fig. 3).

In well-watered plants, the Cs1&AMF treatment recorded the highest level of chlorophyll *b*, followed by Cs2&AMF, both of which were statistically distinct from the control (418.34% and 169.54%, respectively). Single bacterial treatments (Cs1 and Cs2) resulted in intermediate improvements. Under stress conditions, chlorophyll *b* contents dropped markedly, with the stressed control exhibiting the lowest values. However, plants inoculated with Cs2 alone and Cs1&AMF retained significantly ($p < 0.05$) higher chlorophyll *b* levels compared with the control (89.39% and 38.64%, respectively), demonstrating the protective role of microbial inoculations.

The pattern of total chlorophyll, which integrates chlorophyll *a* and *b*, followed a similar trend. In well-irrigated plants, Cs1&AMF produced the highest chlorophyll content, showing an increase of 405.86%, while Cs2&AMF also achieved a significant ($p < 0.05$) improvement, reaching a 170.70% increase compared with the control. Single inoculations displayed moderate increases relative to the control. Under drought stress, total chlorophyll content was strongly reduced, with the control recording the lowest value. Still, inoculated plants, particularly those treated with Cs2 and Cs1&AMF, maintained higher levels (88.34% and 41.11%, respectively), suggesting a capacity to mitigate chlorophyll loss under stress conditions.

The present findings show that microbial inoculation markedly

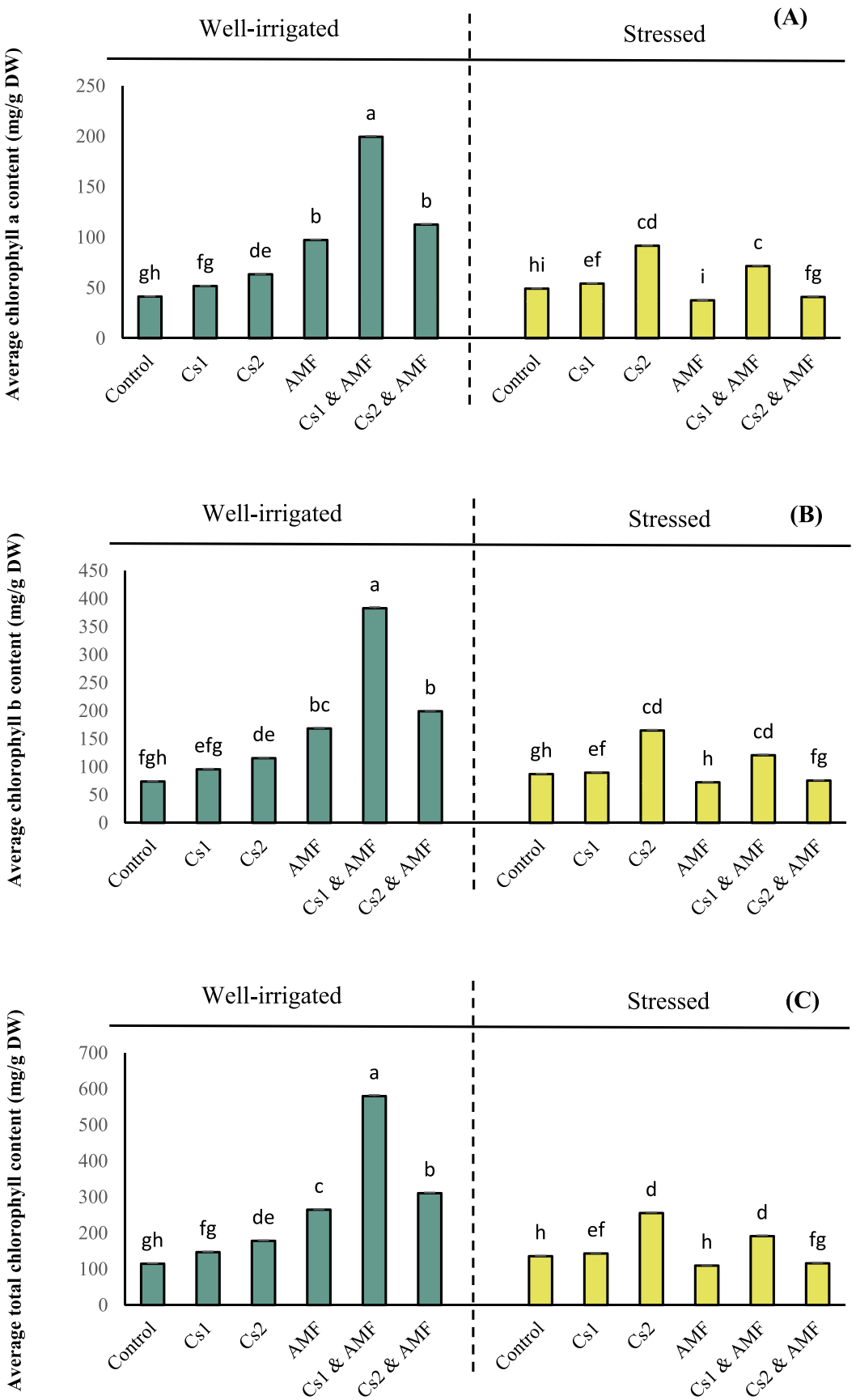


Fig. 3. Chlorophyll *a*, *b*, and total content of *O. ficus-indica* in response to microbial inoculation and non-inoculated control under normal and drought stress conditions. Different letters indicate significant differences according to Tukey's test ($p < 0.05$). Cs: consortium; AMF: Arbuscular Mycorrhiza Fungi.

improves chlorophyll preservation and accumulation in *O. ficus-indica* cladodes, with the strongest effects consistently observed in the combined treatments (Cs1&AMF and Cs2&AMF). Under well-irrigated conditions, both chlorophyll *a* and *b* significantly ($p < 0.05$) increased in co-inoculated plants, with Cs2&AMF recording the highest values. Such enhancement is commonly attributed to improved nutrient acquisition, particularly nitrogen, potassium, and phosphorus, which are essential components of chlorophyll biosynthesis (Boyd and Vickers, 1971). PGPR enhances nutrient availability in the soil by processes such as nitrogen fixation and phosphate solubilization (Glick, 2014), which are subsequently taken up and translocated by AMF hyphal networks, further improving plant access to both macronutrients (N, P) and micronutrients (Zn, Cu) (Chaudhary et al., 2025). These complementary mechanisms likely explain the superior pigment concentration observed in the combined treatments.

Under drought stress, chlorophyll *a*, chlorophyll *b*, and total chlorophyll decreased in all treatments, consistent with the well-established degradation of pigments due to oxidative stress, chloroplast damage,

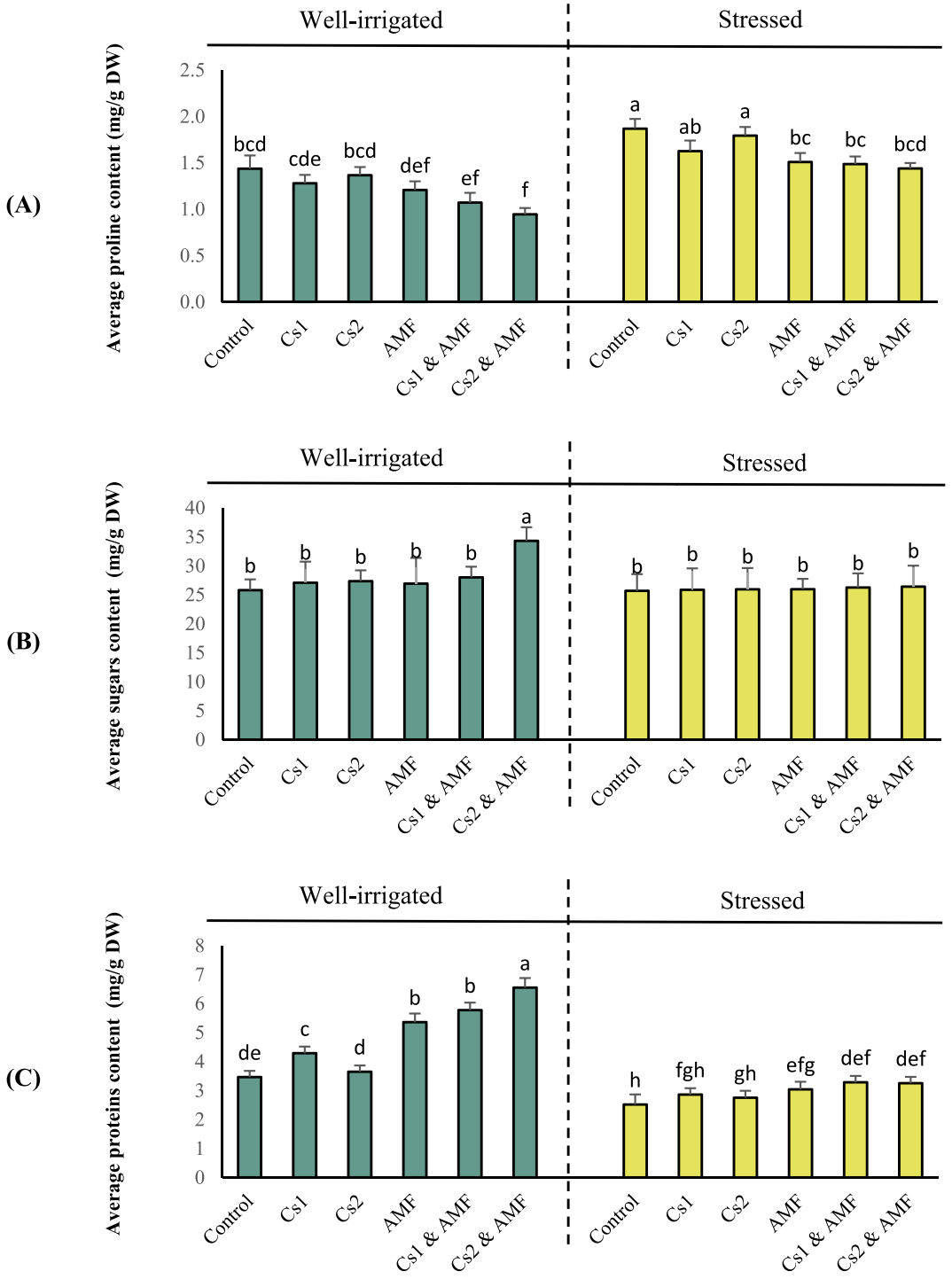


Fig. 4. Proline, soluble sugars, and total protein contents in *O. ficus-indica* cladodes in response to microbial inoculation and non-inoculated control under normal and drought stress conditions. Different letters indicate significant differences according to Tukey's test ($p < 0.05$). Cs: consortium; AMF: Arbuscular Mycorrhiza Fungi.

and reduced biosynthetic enzyme activities (Razi and Muneer, 2021). However, inoculated plants, especially those receiving Cs1&AMF and Cs2&AMF, retained significantly ($p < 0.05$) higher pigment levels than the stressed control. This indicates that both PGPR and AMF helped preserve the integrity of the photosynthetic machinery under water deficit. Our results align with previous observations in tobacco (*Nicotiana tabacum* L.) where co-inoculation with AMF and PGPR significantly ($p < 0.05$) increased chlorophyll contents under drought stress (Begum et al., 2022; Ferioun et al., 2025; Ferioun et al., 2024a), and with studies showing that AMF alone improves photosynthetic gas exchange in *Robinia pseudoacacia* (He et al., 2017).

Several physiological mechanisms may explain this protective effect. PGPR producing ACC-deaminase help regulate ethylene levels, and D-amino acids transported into plants can serve as nitrogen sources, modulating chloroplast division and ethylene production (Gamalero et al., 2023). Meanwhile, AMF enhance osmotic adjustment, stimulate antioxidant enzyme activities, and limit ROS accumulation, all contributing to reduced chlorophyll degradation during drought (He et al., 2017). Moreover, drought stress disrupts the photosynthetic apparatus by reducing key proteins such as Rubisco activase (RCA) and the Rubisco large-subunit binding protein and by decreasing chlorophyll *a*, chlorophyll *b*, pigment-protein complexes, and the structural integrity of chloroplasts and thylakoids, ultimately lowering carboxylation capacity and photosynthetic efficiency (Chieb and Gachomo, 2023). The superior performance of Cs2&AMF and Cs1&AMF under both irrigation regimes suggests strong synergistic interactions between the microbial partners (Sbbar et al., 2025). PGPR may act as mycorrhiza-helper organisms (Garbaye, 1994), enhancing AMF colonization and function, while AMF create a favorable rhizospheric environment that supports bacterial activity. Similar synergistic effects on photosynthetic pigments have been documented in tomato and maize (Danish et al., 2020; Tahiri et al., 2022). Overall, the ability of combined inoculations to maintain higher chlorophyll contents under drought highlights the potential of integrated PGPR & AMF biofertilization strategies to sustain the photosynthetic capacity of *O. ficus-indica* under arid and semi-arid conditions.

3.4. Osmolyte accumulation

The accumulation of osmolytes in *O. ficus-indica* under the different inoculation treatments is depicted in Fig. 4, including proline (A), soluble sugars (B), and total proteins (C), measured under both well-irrigated and drought-stressed conditions. Under well-irrigated conditions, proline accumulation remained low across treatments, with no major differences among inoculated plants and the control ($p \geq 0.05$). The highest values were observed in the control and Cs1 treatments, while the lowest level was recorded in the Cs2&AMF combination (reduced by 34.34% compared to the control). Under drought stress, however, proline content increased sharply in all treatments compared with well-irrigated plants. The stressed control showed the highest accumulation, reflecting a strong stress response. Inoculated plants also accumulated proline under stress but at lower levels, suggesting that microbial treatments partially alleviated the stress intensity, reducing the need for excessive proline accumulation. Compared with the stressed control, AMF, Cs1&AMF, and Cs2&AMF reduced proline accumulation by 19.23%, 20.44%, and 22.97%, respectively, confirming that combined microbial inoculation, especially PGPR&AMF associations, effectively alleviated the stress intensity, reducing the need for excessive proline accumulation.

In well-watered plants, soluble sugar content was moderate and did not vary significantly ($p \geq 0.05$) among most treatments, except for the Cs2&AMF combination, which displayed a higher accumulation of 32.95% compared to the control. Under drought stress, soluble sugar levels increased across all treatments. This indicates that sugar accumulation under stress is a general plant response to osmotic adjustment.

Protein content in well-irrigated plants (Fig. 4) showed clear improvements in inoculated treatments compared to the control. The highest levels were recorded in the Cs2&AMF combination, followed by Cs1&AMF and AMF alone, highlighting a positive effect of inoculation on protein metabolism under non-stress conditions, with increases of 89.21%, 66.83%, and 54.74%, respectively, compared with the uninoculated control. Under drought stress, however, protein levels decreased markedly in all treatments compared to well-irrigated plants. The stressed control had the lowest value, whereas inoculated plants maintained slightly higher protein contents, although differences among inoculated treatments were modest, with AMF, Cs1&AMF, and Cs2&AMF showing increases of 20.77%, 30.47%, and 29.25%, respectively, compared with the stressed control.

The modulation of osmolyte and protein metabolism in *O. ficus-indica* under microbial inoculation reveals clear drought-mitigation effects. Proline, a major osmoprotectant and reliable stress marker, increased sharply under drought across all treatments, with the highest accumulation recorded in the stressed control. In contrast, inoculated plants accumulated significantly ($p < 0.05$) less proline under stress, indicating lower perceived stress intensity. This pattern is consistent with findings showing that PGPR and AMF alleviate drought by improving plant water status and nutrient uptake, thereby reducing the plant's need to synthesize high proline concentrations (Gasemi et al., 2023; Zou et al., 2013). Mechanistically, AMF enhance water and phosphorus uptake through their extraradical hyphae (Chaudhary et al., 2025), while PGPR contribute through ACC-deaminase activity, EPS production, and nutrient mobilization, collectively lowering ethylene-mediated stress signaling and improving rhizosphere hydration (Batool et al., 2020; Bhagat et al., 2021; Danish et al., 2020).

Soluble sugars slightly decreased under drought across all treatments, highlighting the sensitivity of carbohydrate metabolism to water deficit in this CAM species, and demonstrating that soluble sugar dynamics are strongly influenced by water availability (De La Barrera and Nobel, 2004). However, inoculation did not create strong differences among stressed plants, suggesting that sugar accumulation is a conserved response that is less sensitive to microbial mediation than proline. Under non-stress conditions, the Cs2&AMF treatment induced higher sugar levels, in agreement with reports showing that AMF enhance soluble sugar accumulation in *O. ficus-indica* (Lahbouki et al., 2023) and increase glucose and fructose contents in several Citrus species by stimulating enzymes such as leaf acid invertase, sucrose synthase, and root neutral invertase (Wu et al., 2013).

Total proteins were significantly ($p < 0.05$) higher in inoculated plants under well-watered conditions, particularly in the Cs2&AMF combination, indicating enhanced nitrogen assimilation and biosynthetic activity supported by microbial nutrient mobilization. Under drought, protein levels decreased in all treatments, as commonly observed due to dehydration-induced protein degradation, but inoculated plants maintained comparatively higher levels. This aligns with studies showing that PGPR improve N nutrition through fixation and mineralization, while AMF enhance P and micronutrient uptake, ultimately protecting protein synthesis and limiting oxidative damage (Chaudhary et al., 2025; Glick, 2014; Gómez-Godínez et al., 2019). Similar increases in soluble proteins have been reported in *O. ficus-indica* and *Pistacia vera* inoculated with AMF, where microbial symbiosis mitigated RNA and protein degradation under stress (Abbaspour et al., 2012; Lahbouki et al., 2022).

Overall, the combined PGPR&AMF treatments appear to reduce reliance on extreme osmotic adjustment (lower proline), support moderate carbohydrate accumulation, and preserve protein pools, illustrating their synergistic role in enhancing physiological resilience of *O. ficus-indica* under water-limited conditions.

3.5. Oxidative stress indicators

Oxidative stress indicators in *O. ficus-indica* were assessed through the quantification of malondialdehyde and hydrogen peroxide, as

illustrated in Fig. 5 (A &B), under both normal and drought conditions with different inoculation treatments. Under well-irrigated conditions, MDA levels remained low, with no significant ($p \geq 0.05$) differences between the control and inoculated treatments except a slight reduction in the AMF treatment compared with the control. Under drought stress, however, MDA increased markedly in the control and Cs1 treated plants, reflecting enhanced lipid peroxidation and membrane damage. By contrast, inoculation with Cs2, AMF, and both combinations significantly ($p < 0.05$) reduced MDA accumulation, indicating their protective effect against oxidative stress. Specifically, drought-stressed plants inoculated with Cs2 showed a 62.29% reduction in MDA compared with the stressed control, while AMF, Cs1&AMF, and Cs2&AMF decreased MDA by 58.33%, 35.05%, and 54.90%, respectively. These reductions confirm that microbial inoculation, particularly treatments involving Cs2 and AMF, substantially mitigates drought-induced lipid peroxidation in *O. ficus-indica*.

Under well-irrigated conditions, the Cs2&AMF treatment reduced H_2O_2 content by 25.97% relative to the non-stressed control, demonstrating a strong basal antioxidant effect even in the absence of stress (Fig. 5). Under drought conditions, both PGPR&AMF combinations further decreased H_2O_2 accumulation by approximately 18% relative to the stressed control, confirming that these microbial associations efficiently limit ROS buildup under water deficit.

The marked increase in MDA and H_2O_2 in drought-stressed control plants clearly reflects enhanced lipid peroxidation and oxidative injury, since both markers are widely recognized indicators of membrane damage under abiotic stress conditions. In the present study, the strong accumulation of MDA and H_2O_2 in the untreated stressed plants indicates maximal damage to cellular structures, consistent with the concept that the build-up of these compounds reflects the extent of oxidative stress impacting membrane stability (Hossain et al., 2015; Sairam and Srivastava, 2002). Conversely, the significant ($p < 0.05$) reduction of both MDA and H_2O_2 in plants inoculated with Cs2, AMF, or their combinations demonstrates an improved capacity to modulate ROS production and maintain membrane integrity under drought conditions. This protective pattern aligns with earlier findings showing that co-inoculation with AMF and PGPR reduces lipid peroxidation in tobacco and *O. ficus-indica* by lowering MDA accumulation under stress (Begum et al., 2022; Ferioun et al., 2024a, 2025; Lahbouki et al., 2023). The ability of AMF and PGPR-treated plants to limit oxidative damage has also been reported in *Arizonica Cupressus*, where microbial inoculation led to markedly lower H_2O_2 and MDA concentrations under drought compared with non-inoculated plants (Aalipour et al., 2020). These parallels support the idea that the enhanced oxidative protection observed in the current experiment is a consistent and reproducible effect of microbial inoculation across plant species. In co-inoculated systems, synergistic effects between AMF and PGPR further strengthen membrane protection by improving plant physiological status and reducing the extent of oxidative burst associated with drought stress.

3.6. Antioxidant enzyme activities

The antioxidant defense system of *O. ficus-indica* was evaluated by measuring catalase and ascorbate peroxidase activities under different

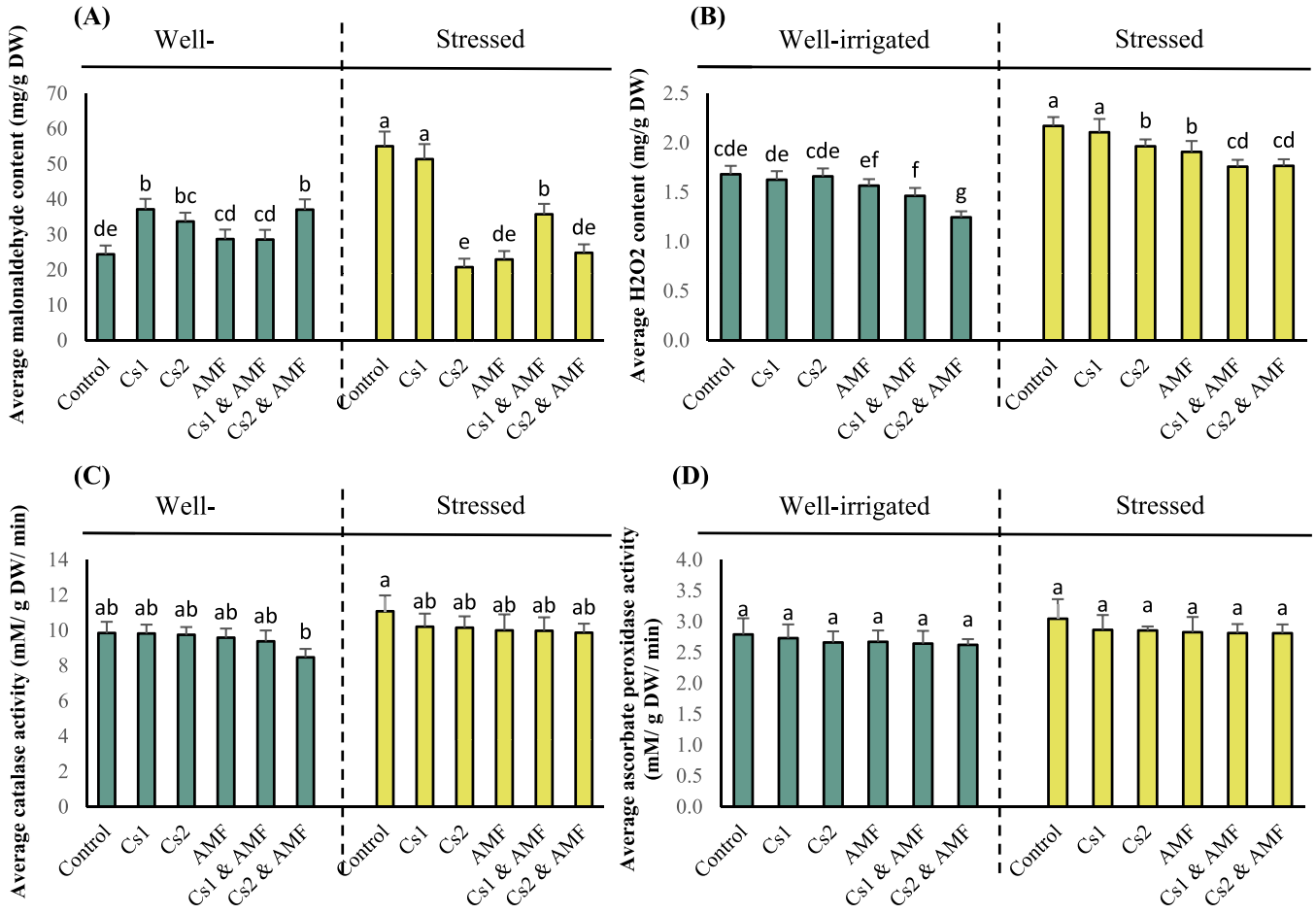


Fig. 5. Oxidative stress markers and antioxidant enzyme activities in *O. ficus-indica* in response to microbial inoculation and non-inoculated control under normal and drought stress conditions. Different letters indicate significant differences according to Tukey's test ($p < 0.05$). Cs: consortium; AMF: Arbuscular Mycorrhiza Fungi; DW: dry weight.

irrigation regimes and inoculation treatments, as shown in Fig. 5 (C & D). Catalase activity remained relatively stable under well-irrigated conditions, with no significant ($p \geq 0.05$) differences among treatments. Under drought stress, catalase activity increased slightly in all treatments, but differences among them were not statistically significant, suggesting that CAT activity was uniformly induced as a general protective response to oxidative stress.

APX activity showed a similar trend to catalase, with low baseline activity under well-irrigated conditions and a general increase under drought stress. However, no significant ($p \geq 0.05$) differences were observed among treatments in either condition, indicating that APX activity was broadly induced by stress but not strongly modulated by microbial inoculation.

The activities of catalase and ascorbate peroxidase in *O. ficus-indica* followed the typical antioxidant response pattern observed under drought stress. In our study, both enzymes displayed low baseline levels under well-irrigated conditions and were uniformly induced under drought, regardless of inoculation treatment. This generalized increase is consistent with the activation of antioxidant defenses known to occur when plants experience water deficit, during which ROS overproduction, particularly superoxide (O_2^-) and hydrogen peroxide (H_2O_2), triggers a metabolic shift toward protective enzymatic pathways (Armada et al., 2014a; Khanna et al., 2019; Ortiz et al., 2015). The observed increase of CAT and APX in all stressed plants reflects their central roles in detoxifying H_2O_2. Superoxide dismutase (SOD) first converts O_2^- into H_2O_2, which is subsequently reduced to water and oxygen through the concerted action of CAT, APX, POD, and other enzymes of the ascorbate-glutathione cycle (Chiappero et al., 2019; Mishra and Sharma, 2019; Rukmini et al., 2004). This induction represents a primary protective mechanism allowing plants to limit oxidative injury under drought (Fouad et al., 2014; Mirzaee et al., 2013).

Although microbial inoculation did not significantly ($p \geq 0.05$) differentiate CAT or APX activity in our experiment, the global increase across treatments remains consistent with numerous studies reporting enhanced antioxidant metabolism in plants exposed to water stress, including *O. ficus-indica* (Lahbouki et al., 2023), barely (Ferioun et al., 2025) and tobacco (Begum et al., 2022). Previous research has shown that AMF and PGPR frequently boost activities of CAT, APX, SOD, POD, and GR under drought, thereby reducing oxidative damage (Begum et al., 2022; Gururani et al., 2013; Ortiz et al., 2015). Co-inoculation with AMF&PGPR enhanced APX and SOD activities in drought-stressed *Arizonica Cupressus* (Aalipour et al., 2020), and similar increases in CAT, SOD, and APX were recently reported in drought-imposed wheat treated with PGPR (Chandra et al., 2019). Thus, while CAT and APX were not strongly modulated by specific inoculants in *O. ficus-indica*, their consistent induction under drought aligns with the widely documented antioxidant activation that enables plants to counteract ROS accumulation and maintain cellular integrity under water-limited conditions.

3.7. Root mycorrhizal colonization

The results of mycorrhizal colonization revealed a substantial enhancement in all treatments involving AMF inoculation, whether applied alone or in combination with PGPR (Table 2). While the non-inoculated control and PGPR-only treatments exhibited a baseline level of colonization due to the indigenous AMF community, the introduced consortium significantly ($p < 0.05$) outperformed the native population. Under well-irrigated conditions, the Cs2&AMF treatment increased the frequency (F%), intensity (M%), and arbuscule frequency (A%) by 127.97%, 589.47%, and 113.87%, respectively, compared with the non-inoculated control. Under water-stress conditions, although overall colonization levels declined in the control, they remained notably high in AMF-inoculated plants, indicating the resilience of the symbiosis. The PGPR&AMF combinations maintained some of the highest F%, M%, and A% values despite the stress. Specifically, AMF-based treatments consistently resulted in superior mycorrhizal colonization parameters. While A% variations between some treatments remained limited, the overall data indicates that mycorrhization was primarily driven by AMF inoculation. Notably, no significant ($p \geq 0.05$) differences were detected between AMF applied alone and AMF combined with PGPR, suggesting that bacterial inoculation neither enhanced nor inhibited the establishment of the fungal symbiont.

These results indicate that the AMF inoculum was the main factor responsible for the increase in root colonization in *O. ficus-indica*, whereas the associated PGPR were fully compatible with the mycorrhizal fungi used in this study. The absence of negative effects on mycorrhization confirms that the bacterial consortia did not interfere with AMF development or functioning. Such compatibility is particularly relevant for agroecological applications, where multi-microbial inoculants are expected to coexist without antagonistic interactions (Savastano and Bais, 2024a). Comparable observations have been reported in previous studies, where PGPR maintained or supported mycorrhizal functionality (Begum et al., 2022). For instance, in *Leymus chinensis* exposed to saline or alkaline stress, co-inoculation with PGPR and AMF significantly ($p < 0.05$) improved plant performance without necessarily increasing mycorrhizal colonization parameters, suggesting that PGPR benefits may operate through complementary mechanisms such as nutrient solubilization, phytohormone production, or stress alleviation rather than through direct stimulation of fungal colonization (Zhang et al., 2024). In contrast, other studies in maize indicate that PGPR&AMF co-inoculation promotes root colonization, enhances hydraulic conductance and mineral acquisition, reduces cellular damage, and increases drought tolerance (Khan et al., 2024; Tirry et al., 2023). Bacterial activity can enhance AMF colonization by interfering with plant-fungal signaling or modifying root architecture. For instance,

Table 2
Levels of AMF colonization (%) in the roots of *O. ficus-indica* plants grown under normal and water stress conditions. Different letters within the same column indicate significance at $p < 0.05$.

	Treatments	F%	M%	A%
Well-irrigated plants	**Control**	27.78 ± 1.92^{bc}	1.96 ± 0.93^{e}	21.41 ± 9.54^{a}
	Cs1	34.44 ± 5.09^{b}	4.23 ± 1.44^{cde}	54.30 ± 20.20^{a}
	Cs2	22.22 ± 10.18^{bc}	3.43 ± 2.70^{de}	25.60 ± 22.20^{a}
	AMF	53.33 ± 6.94^{a}	8.38 ± 1.23^{b}	28.32 ± 6.18^{a}
	Cs1&AMF	53.33 ± 1.92^{a}	7.12 ± 0.63^{bc}	45.16 ± 4.62^{a}
	Cs2&AMF	63.33 ± 3.33^{a}	13.69 ± 1.00^{a}	45.79 ± 3.90^{a}
Stressed plants	**Control**	20.00 ± 3.33^{c}	1.52 ± 0.11^{e}	38.80 ± 33.30^{a}
	Cs1	26.67 ± 5.77^{bc}	2.09 ± 0.85^{e}	34.65 ± 16.39^{a}
	Cs2	22.22 ± 1.92^{bc}	2.19 ± 0.63^{e}	36.40 ± 11.71^{a}
	AMF	54.44 ± 6.94^{a}	6.36 ± 1.08^{bcd}	56.38 ± 11.25^{a}
	Cs1&AMF	55.56 ± 1.92^{a}	6.97 ± 0.58^{bc}	43.18 ± 5.96^{a}
	Cs2&AMF	54.44 ± 1.92^{a}	9.62 ± 0.65^{b}	34.92 ± 12.05^{a}

F%, the frequency of root colonization; M%, the intensity of cortical colonization; A%, arbuscular frequency in roots, Cs: consortium, AMF: Arbuscular Mycorrhiza Fungi.

Bradyrhizobium japonicum promotes flavonoid production in soybeans via Nod factors, thereby stimulating mycorrhization (Xie et al., 1995). In our study, the findings highlight the pivotal role of AMF inoculation in promoting mycorrhization in *O. ficus-indica* and demonstrate that the selected PGPR consortia can be safely combined with AMF without compromising symbiotic establishment, an essential prerequisite for the development of integrated microbial inoculants. It is important to note that although some mycorrhizal colonization may occur in non-inoculated plants grown in non-sterilized soils, indigenous AMF communities are often limited in abundance and functional efficiency, and previous studies have shown that the introduction of selected microbial inoculants can enhance colonization, modify native community structure, and improve plant performance (Omirou et al., 2016), explaining the relatively low colonization observed in non-inoculated treatments in the present study.

Under water-deficit conditions, the maintenance of AM colonization in plants inoculated with AMF alone or in combination with PGPR highlights the resilience of the symbiosis. The extraradical mycelium of AMF, together with PGPR-mediated improvements in root morphology and plant physiology, likely facilitates access to water and nutrients under stress (Savastano and Bais, 2024b), thereby stabilizing root function and limiting dehydration-induced cellular damage. Several mechanisms may underlie this synergy. PGPR can enhance phosphate solubilization, synthesize phytohormones (auxins, cytokinins), and produce exopolysaccharides that modify the rhizosphere, collectively promoting root development (Chieb and Gachomo, 2023). In parallel, AMF hyphae expand the effective absorptive surface, improve root hydraulics, enhance water and nutrient uptake, and modulate the expression of aquaporins and mineral transporters, thereby reinforcing drought tolerance (Begum et al., 2019a; Meddich et al., 2022; Ouallal et al., 2022). However, increased AMF colonization does not always translate into proportional plant benefits. Recent reviews note that excessive colonization may impose additional carbon costs on the host, depending on environmental conditions and resource availability (Savastano and Bais, 2024b). In the present study, agronomic and physiological responses (growth, chlorophyll content, and reduced oxidative stress) indicate a clear net benefit, supporting the relevance of PGPR&AMF co-inoculation for enhancing the resilience of *O. ficus-indica* under drought stress.

3.8. Correlation among the growth, biochemical and physiological parameters: PCA analysis

The PCA biplot (Fig. 6) explains 73% of the total variability across the first two axes (F1: 63%, F2: 10%). A clear separation appears between stressed (S) and non-stressed (NS) conditions along F1. Stress-related variables, including proline, H_2O_2, MDA, and antioxidant enzymes (CAT, APX), are grouped in the negative F1 and positive F2 quadrant, aligning strongly with the Control stressed and to a lesser extent with Cs1 and Cs2 stressed, confirming elevated oxidative stress under water deficit in the absence of full microbial inoculation. Conversely, growth and performance variables (plant height, leaf area, fresh and dry biomass of cladodes and roots, sugars, proteins, chlorophyll pigments, and number of cladodes) are positioned on the positive side of F1 together with non-stressed treatments, showing that well-watered conditions favor morphological development. Importantly, AMF colonization parameters (F%, M%, A%) cluster closely with growth variables and with inoculated stressed plants (Cs1 & AMF, Cs2 & AMF, AMF), which occupy an intermediate position between the stressed and non-stressed groups. This pattern indicates that AMF and combined PGPR&AMF inoculation enhance growth even under drought and partially counteract oxidative stress. Overall, the PCA demonstrates that

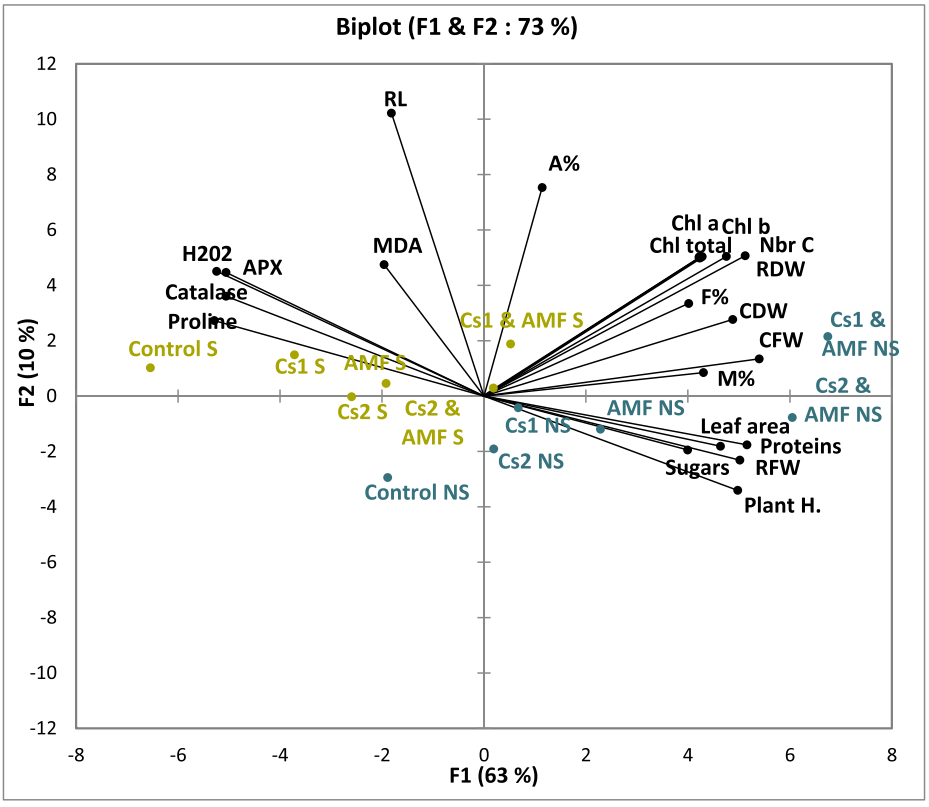


Fig. 6. Principal component analysis of *O. ficus-indica* growth and stress response under microbial inoculation and water-deficit conditions. S: stressed; NS: Non-stressed; RL: Root length; Plant H.: Plant height; RFW: Root fresh weight; RDW: Root dry weight; CFW: Cladode fresh weight; CDW: Cladode dry weight; Nbr C: Number of cladodes; Chl a: Chlorophyll *a*; Chl b: Chlorophyll *b*; Chl total: Total chlorophyll; MDA: Malondialdehyde; APX: Ascorbate peroxidase; H_2O_2: Hydrogen peroxide; F%: Frequency of mycorrhization, M%: Intensity of mycorrhization; A%: Arbuscule frequency.

water deficit strongly shapes the cactus response by increasing oxidative markers, while microbial inoculation, especially AMF and PGPR&AMF combinations, promotes physiological performance and contributes to maintaining higher mycorrhizal colonization even under stress, reflecting improved resource acquisition and partial stress alleviation.

The correlations revealed by the PCA highlight the antagonistic behavior between oxidative stress indicators and growth-related traits. The close association of proline, H_2O_2, MDA, CAT, and APX with stressed, non-inoculated treatments indicates that water deficit induces strong ROS accumulation, consistent with previous reports showing that drought enhances oxidative pressure and disrupts cellular stability (Armada et al., 2014b; Lahbouki et al., 2022; Ortiz et al., 2015; Porcel and Ruiz-Lozano, 2004). In contrast, positive loadings of proteins, sugars, chlorophyll pigments, and biomass parameters along F1 reflect improved physiological functioning in inoculated plants, supported by studies showing that PGPR and AMF increase protein stability, promote photosynthesis, and reduce oxidative degradation under stress (Begum et al., 2022; Gasemi et al., 2023; Sbbar et al., 2025; Tahiri et al., 2022). The correlation analyses indicate that the bioinoculants (PGPR and AMF) used in the present study stabilizes plant cellular physiology by reducing ROS accumulation and maintaining osmotic balance, thereby optimizing photosynthetic efficiency and promoting plant growth.

The strong correlation between AMF parameters (F%, M%, A%) and growth traits underscores the essential role of mycorrhizal colonization in enhancing nutrient and water uptake (Raklami et al., 2019; Smith and Read, 2008). Similar findings revealed that AMF improve P, N, Ca, Mg, Zn, and K acquisition, elements fundamental for osmotic adjustment, membrane integrity, and maintenance of photosynthesis under drought (Meddich et al., 2015; Ortiz et al., 2015; Tahiri et al., 2022). The intermediate positioning of the PGPR&AMF stressed treatments suggests synergistic mitigation, aligned with evidence that co-inoculation enhances ACC deaminase activity, EPS production, and phytohormone balance, resulting in reduced ROS accumulation and improved plant performance under water deficit (Ahemad and Kibret, 2014; Kang et al., 2014; Rolli et al., 2014). Altogether, the PCA highlights a coherent biological pattern: drought enhances oxidative disturbances, whereas microbial inoculation, particularly AMF and combined PGPR&AMF, supports resource uptake, maintains metabolic stability, and improves cactus resilience.

4. Conclusion

This study demonstrates that the integration of plant growth–promoting rhizobacteria and arbuscular mycorrhizal fungi constitutes an effective strategy to enhance the drought resilience of *O. ficus-indica*. Combined inoculations, Cs1&AMF and Cs2&AMF, consistently improved plant performance by promoting growth, maintaining photosynthetic pigments, enhancing protein accumulation, and reducing oxidative stress markers under severe water deficit. The reduction in malondialdehyde and hydrogen peroxide levels in inoculated plants highlights the role of microbial partners in limiting membrane damage and moderating ROS overproduction, while the stabilization of osmolytes suggests improved physiological homeostasis. Although antioxidant enzyme activities (CAT and APX) were broadly induced by drought across treatments, the lower oxidative damage in inoculated plants indicates that microbial symbioses helped alleviate stress before requiring strong enzymatic activation. Overall, these findings provide solid evidence that PGPR&AMF combination act synergistically to reinforce both growth and biochemical defense mechanisms in *O. ficus-indica*. From a practical perspective, the combined use of PGPR and AMF, particularly Cs1&AMF and Cs2&AMF, can be recommended as a sustainable bio-inoculation strategy to improve cactus productivity and resilience under water-limited conditions, especially in arid and semi-arid regions.

Future research should focus on validating these results under field conditions, assessing the long-term stability and performance of microbial consortia, and characterizing their functional traits and interactions with native soil microbiota. In addition, optimizing inoculation protocols and application strategies will be essential to facilitate their large-scale adoption in sustainable agricultural systems. Further long-term studies should also follow *O. ficus-indica* plants through the fruiting stage to determine whether the observed benefits of microbial inoculation translate into improved fruit yield and yield-related traits.

CRediT authorship contribution statement

Ilham Zouitane: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Mohamed Ferioun:** Writing – original draft, Visualization, Investigation, Conceptualization. **Khalid Derraz:** Supervision, Project administration. **Rachid Bouamri:** Project administration. **Saad Ibnsouda Koraichi:** Project administration. **Guido Lingua:** Visualization, Project administration. **Naïma El Ghachtouli:** Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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