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BIOGENICALLY SYNTHESIZED ZNO-NPS CONFERRED DROUGHT TOLERANCE IN MUNG BEAN (*VIGNA RADIATA* L. WILCZEK) BY MODULATING GROWTH, PHYSIO-BIOCHEMICAL ATTRIBUTES, AND ANTIOXIDANT STATUS

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Abstract

Nanoparticles have showcased prime importance in reducing the negative effects of drought stress in plants. The study aimed at biogenic synthesis of zinc oxide nanoparticles (ZnO-NPs) using Aspergillus flavus and to test ZnO-NPs against drought tolerance in two mung bean varieties (AZRI-2021, NM-54). After isolation and characterization of fungus, cell free filtrate and zinc acetate was used to synthesize ZnO-NPs followed by their characterization. After one week of seed germination, water stress was maintained at 60% of field capacity for fourteen days in a pot experiment. Foliar application of ZnO-NPs at varying levels (0, 5, 10, and 15 mg/L) was performed on the test plants. Data regarding growth, enzymatic activities, photosynthetic pigments, and biochemical components were recorded after two weeks of ZnO-NPs application. Drought stress significantly abridged growth attributes and photosynthetic pigments of all subjects. A substantial increase in shoot fresh weight (64.36%) and chlorophyll content (73.55%) of var. AZRI-2021 was observed at 10 mg/L ZnO-NPs treatment. A maximum increase in root fresh weight (51.5%), root dry weight (65.35%), and shoot length (39.92%) of var. NM-54 was recorded under the same level of application. Both vars. under study exhibited a significant difference regarding total free amino acids (TFAA), total soluble protein (TSP), total soluble sugars (TSS), and proline contents under drought stress. The plants treated with 5 mg/L ZnO-NPs demonstrated a maximum increase in TFAA (37.42 and 30.72%), while 10 mg/L treatment exhibited a maximum rise in TSP (46.26 and 46.39%), proline (47.48 and 33.04%), and TSS (40.10 and 40.20%) contents in var. AZRI-2021 and var. NM-54, respectively, under drought. The treatment of ZnO-NPs at 10 mg/L was found to be most effective in both mung bean vars. and restored the negative impacts of drought stress. In future, molecular research will reveal more about the induction of drought stress tolerance by biogenically synthesized ZnONPs.

Key words: Lipid peroxidation; Antioxidative mechanism; Drought stress; Growth; Osmoprotectant; Mung bean; ZnO-NPs

Introduction

Sustainable farming methods are becoming more critical than ever before due to the challenges of climate change and depletion of global freshwater resources (Seleiman *et al.,* 2021). Among all the environmental stresses, drought is the major factor in decreasing crop yield worldwide. Like all other crops, drought is deleterious for mung bean (*Vigna radiata* L. Wilczek), a crop with high economic importance and prime nutrient value. Water deficient environment not only limits its yield but also deprives the poor communities from a source of nutritious food. Global food security threats are expected to prevail more commonly in arid regions with water deceit conditions, and previously have caused major famines (Okorie *et al.,* 2019).

Many strategies are being employed including improved irrigation system, fertilization management, and application of nanoparticles (NPs) to manage the stress impacts. Among all these, the NPs application has earned researcher's confidence due to its un-paralleled efficiency in mitigating abiotic stresses (Juhel *et al.,* 2021). Nanotechnology has opened new avenues to improve performance under fluctuating environmental conditions (Aghdam *et al.,* 2021; Dimkpa *et al.,* 2019). The unique chemical and physical features of ZnO-NPs, as well as their ability to release Zn ions, make them perfect for shifting the metabolism to address environmental challenges (Prasad *et al.,* 2022; Kah *et al.,* 2018). Recent investigations demonstrate that ZnO-NPs have vital nutrient capacity and can improve growth and productivity under harsh conditions (Burman *et al.,* 2013). These have improved plant health not only by regulating enzymes like polymerases but also increase cell division, chlorophyll biosynthesis and cell stability (Abbasifar *et al.,* 2020; Vaghar *et al.,* 2020). Moreover, they are involved in carbonic anhydrase metabolism, which catalyzes CO_2 and HCO_3 in C4 photosynthetic pathway (Faizan *et al.,* 2021). Chemical and biogenic synthesis pathways are two predominant techniques being employed for synthesis of ZnO-NPs. The chemical synthesis raises numerous ecological and biocompatibility problems, whereas biogenic synthesis produces nanoparticles by extracting plants, bacteria, or other living systems (Akl *et al.,* 2020). The biogenic synthesis not only improves safety profile of nanoparticles but also is a sustainable agricultural practice. Biologically synthesized approaches may disclose dynamic details about their efficiency and utility in agriculture, especially when employed to boost crop resilience to drought stress, such as mung bean (Sheiha *et al.,* 2020). Fungi can produce larger quantity of metabolites than other microorganisms that particularly makes them more ideal for nanoparticle production (Sheiha *et al.,* 2020). It has now been observed

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that a large number of fungi, including *Cladosporium cladosporioides*, *Trichoderma asperellum*, *Aspergillus* spp., and *Fusarium* spp. can produce nanoparticles (Juhel *et al.,* 2021). Among all of them, filamentous fungi have drawn attention as their ZnO-NPs are highly stable and possess strong morphological properties that can lead for a broad range of applications.

A multivariant study covering different areas of environment science, plant physiology, and nano technology may reveal the effectiveness of chemically and biogenically synthesized NPs. Drought stress effects different biochemical and physiological attributes of plants like antioxidant defense system, stomatal conductance, and photosynthesis (Siddiqi & Husen, 2016; Kambe *et al.,* 2021). It is important to understand that how differentially produced ZnO-NPs interact with these processes. The varying effectiveness of chemically and biogenically synthesized NPs is not yet established, and present research concentrates on the benefits of employing ZnO-NPs in general, missing sufficient details about how their synthesis technique effects their performance (Juhel *et al.,* 2021; Klofac *et al.,* 2023).

ZnO-NPs are the most widely produced NPs in the world, second only to carbon nanotubes, titanium dioxide, silver, and gold (Farooq *et al.,* 2022). Due to their wide range of application, these have demonstrated a beneficial effect on plant growth and physiology (Burman *et al.,* 2013). Zinc (Zn) is an essential micronutrient that supports multiple functions in plant development (Nasrallah *et al.,* 2022), including chlorophyll biosynthesis, maintenance of cell membrane structure, cell division as well as also regulate the enzymes activity like DNA and RNA polymerase (Abbasifar *et al.,* 2020; Vaghar *et al.,* 2020). Moreover, ZnO-NPs improve the efficiency of plants antioxidant defense machinery in scavenging free radicals during stress by either modifying microRNA expression or modulating various in plant physiological, metabolic, and morphological processes (Siddiqi & Husen, 2016; Kambe *et al.,* 2021).

Mung bean (*Vigna radiata* L. Wilczek) is a short-term legume that is rich in proteins, carbohydrates, amino acids, minerals, and vitamins (Schafleitner *et al.,* 2015; Ganesan & Xu, 2018). The production of mung bean crop is about 3 million tons annually, making up about 5% of the world's pulse production (Sehrawat *et al.,* 2019). Almost 90% of this production globally comes from East, South and Southeast Asia (Nair *et al.,* 2013). Being summer-time leguminous crop, it is very susceptible to both abiotic and biotic stresses. The mung bean response towards drought stress is very complicated involving different biochemical and physiological modifications. Drought stress causes oxidative stress and to improve drought tolerance, oxidative damage must be reduced (Kathirvelan *et al.,* 2025). Different studies have revealed that applications of ZnO-NPs enhance antioxidant enzyme production thus decreasing oxidative damage in faranjmoshk and wheat (Karimian & Samiei, 2023; Pandya *et al.,* 2023). In recent study cobalt oxide (CoO) and nickel oxide (NiO) nanoparticles were used to check their biological and magnetic impacts on mung bean seedlings (Joshi *et al*., 2025). Exploring how biogenically generated ZnO-NPs influence the adaptation mechanism of mung bean to resist drought stress is an important research issue that will generate new knowledge and future directions for yield improvement (Guzel *et al.,* 2025). The main objective of present study was to evaluate biogenically synthesized ZnO-NPs with particular emphasis on their possible use to enhance drought tolerance in mung beans, hence, closing the gap between nanotechnology and agronomy. The aims of the research work were to figure out the specific modulations while evaluating their real-world implications for agricultural sustainability under drought stress.

Materials and Methods

Isolation and identification of strain: Soil samples were collected from rhizosphere of soybean plants grown in research area of university between longitude 73°E and latitude 31.15°N. These samples were milled and suspended in sterilized saline solution (100 mL) followed by serial dilutions from 10^{-1} to 10^{-6}. After that 100 µL of each dilution were spread on 2% potato dextrose agar (PDA) plates supplemented with streptomycin (10 mg/L). The petri plates were then incubated for one week at 28 ± 2°C. Pure cultures of fungal colonies were obtained through repeated sub-culturing on fresh PDA media. The isolated fungus was then identified on basis of microscopic and molecular characteristics. For microscopic identification, iodine-glycerol solution was used to stain fungal spores and observed under × 40 and × 100 magnifications.

For molecular identification, DNA extraction of fungal mycelium was performed using phenol-chloroform method as described by (Zare *et al.,* 2013). The isolated DNA was used to amplify ITS region through PCR using ITS 1 (5' - TCCGTAGGTGAACCTGCGG- 3') and ITS 4 (5' - TCCTCCGCTTATTGATATGC- 3') primer sets as given by (Rafiq *et al.,* 2022). Afterward, the resultant PCR product was got custom sequenced and homology identification was done using Basic Local Alignment Search (BLAST) tool.

Synthesis and characterization zinc oxide nanoparticles (ZnO-NPs): Fungal biomass was cultured in malt extract (100 mL) broth medium in an Erlenmeyer flask (250 mL) and placed in an orbital shaker at 150 rpm and 28 ± 2°C. After 5 days, biomass was harvested by Whatman filter paper No. 1 and then washed thoroughly with sterile distilled water to eliminate medium contamination. About 15 g of the fresh biomass was taken in an Erlenmeyer flask containing 100 mL of double distilled water. The flask was then placed in rotary shaker for 2 days at 28 ± 2°C, 150 rpm and again filtered fungal biomass to produce cell-free filtrate that was further used for biosynthesis of ZnO-NPs (zinc oxide nanoparticles). 100 mL of cell-free filtrate was mixed with same amount of zinc acetate solution (2 mM final concentration) and kept in shaker at 150 rpm, pH 10, 28 ± 2°C for 24 h. Finally, the reaction mixture was centrifuged for 10 min at 3000 rpm, dried at 150°C in hot air oven, resulting in ZnO-NPs formation (Fig. 1C). The characterization of ZnO-NPs was done through Fourier Transform Infrared spectra (FTIR) ranges from 400-4000 cm^{-1} at 4 cm^{-1} resolution, Scanning Electron Microscopy (SEM) and X-ray diffraction (XRD) to determine functional group, shape, and size of ZnO-NPs.

Plant material and experimental design: The experiment was conducted in the botanic garden of Govt. College University Faisalabad, located between longitude 73°E and latitude 31.15°N to study the changes in growth, biochemical and physiological attributes of mung bean under water deficit conditions. Mung bean seeds of AZRI-2021 and NM-54 were obtained from pulses division of AARI (Ayub Agricultural Research Institute), Faisalabad, Pakistan.

Ten seeds of each mung bean variety were sown in plastic pots; each filled with 10 kg of soil arranged in a completely randomized design (CRD) with four replicates. Water stress was applied after one week of seed germination and maintained for next two weeks. The stress group was maintained at 60% field capacity. Foliar application of ZnO-NPs (0, 5, 10, 15 mg/L) was done at seedling stage after one week of stress imposition. After one week of treatment, samples were collected to determine growth attributes, biochemical parameters, and enzymatic and non-enzymatic antioxidants at vegetative stage.

Growth parameters: Shoot and root lengths were determined by using measuring scale while root, shoot fresh and dry weights were recorded using an electrical weighing balance. For dry weight calculations, the shoots and roots were placed in an oven at 65°C for 72 h as previously reported (Singh *et al.,* 2021).

Estimation of enzymatic antioxidants: 1.0 g of fresh leaf sample was homogenized with 0.5 M phosphate buffer (pH 7.2) in a pre-cooled mortar and pestle, then filtered through muslin cloth and centrifuged at 12,000 rpm, 4°C for 10 min. The resultant supernatant was used to estimate enzymatic activities of antioxidants. The SOD and POD activity was calculated by Zhang (1992) method.

The CAT activity was calculated by Aebi (1984) method. The reaction mixture volume was set to 3 mL by adding enzyme extract (100 µL), 2 mM of EDTA-Na_2, 100 µL of 300 mM H_2O_2 and 2.8 mL of potassium phosphate buffer (50 mM, pH 7.0). The CAT activity was measured by the reduction in absorbance at 240 nm resulting from H_2O_2 depletion through spectrophotometer (Hitachi U-2001, Japan).

APX activity was calculated by Nakano & Asada, (1981) method. The reaction mixture volume was set to 3 mL by adding enzyme extract (100 µL), 100 µL of 7.5 mM ascorbate, 2.7 mL of potassium phosphate buffer (25 mM, pH 7.0), 100 µL of 300 mM H_2O_2 and 2 mM of EDTA. The absorbance was recorded through spectrophotometer (Hitachi U-2001, Japan) at 290 nm.

The GR (glutathione reductase) activity was calculated by (Barata *et al.,* 2000) method. The reaction mixture volume was set to 2.9 mL by adding enzyme extract (100 µL), 100 mM of potassium phosphate buffer (pH 7.0), 0.1 mM of NADPH, 1 mM of oxidized glutathione (GSSG) and 1 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The absorbance was recorded for 2 min through spectrophotometer (Hitachi U-2001, Japan) at 412 nm.

The Guaiacol Peroxidase (GPX) activity was calculated by (Rao *et al.,* 1996) method. The reaction mixture volume consisted of enzyme extract (100 µL), 1.5 mL of potassium phosphate buffer (100 mM, pH 7.0), 1 mM of EDTA, 200 µL of H2O2 (10 mM) and 200 µL of 10 mM guaiacol. The absorbance was recorded for 2 min through spectrophotometer (Hi-tachi U-2001, Japan) at 470 nm.

Photosynthetic pigments: To calculate photosynthetic pigments i.e. chlorophyll *a*, *b*, chlorophyll *a/b*, total chlorophyll and carotenoids, a leaf sample of 0.5 g was homogenized in 80% acetone (4 mL) and then resulting solution was placed in dark for 2 h. The solution optical density (OD) was recorded through spectrophotometer at 645 nm, 663 nm, and 470 nm for chlorophyll *a*, *b* and carotenoids, respectively. The calculation of photosynthetic pigments was done as given by (Arnon 1949).

Total soluble sugars, protein, and free amino acids: Anthrone reagent was used to calculate the sugar content (Dubois *et al*., 1956). The total soluble sugar (TSS) content was quantified using an analytical grade glucose standard curve. After homogenizing the fresh leaf material using liquid nitrogen, 10 mL of cooled 50 mM potassium phosphate buffer (PPB) with pH 7.5 was added. The homogenate was then centrifuged at 12,000 ×g for 5 min. The supernatant was collected and used to measure the total soluble protein (TSP) as described by Bradford (1976). Total free amino acids (TFAA) were determined according to the method García *et al*., (2004).

Proline: For proline content, leaf sample (0.5 g) was crushed and homogenized using 10 mL of 3% sulfosalicylic acid and then centrifuged the resultant mixture at 4°C, 10,000 rpm for 10 min. The assay mixture was made by adding supernatant (2 mL) with glacial acetic acid (2 mL) and acid ninhydrin (2 mL), kept the solution at 100°C for 60 min in water bath. After that the reaction mixture was cooled and then mixed with 5 mL toluene. The solution was vortexed for 30 s and absorbance was recorded at 520 nm using spectrophotometer and calculations were made as described by (Bates *et al.,* 1973).

Phenolic contents: To measure the phenol contents, the reaction mixture contained 20 µL leaf extract, folin-ciocalteu reagent (90 µL) and 20% of $Na2CO_3$ (90 µL) and then absorbance of solution was recorded at wavelength of 630 nm as described by (Iqbal *et al.,* 2020).

Flavonoid contents: To measure the flavonoid contents, the reaction mixture contained 200 µL of methanol extracts with 5% NaNO2 (60 µL) and 10% of AlCl3 (60 µL). Then finally 400 mL of 1 M of NaOH were added to reaction solution and absorbance through spectrophotometer was recorded at wavelength of 510 nm as reported by (Al-Farsi *et al.,* 2008).

Statistical analysis

The experimental data were statistically analyzed by three-way ANOVA (analysis of variance). LSD (least significant difference) test at 0.05% probability ($p \leq 0.05$) level was performed using Minitab v. 21.4.1. Graphical and

pictorial illustrations were done using Origin 2021 software version 9.8 while Pearson's correlation analyses were performed by using R software version 4.4.1 to describe the correlation between plant morphological, enzymatic antioxidant and biochemical attributes treated with ZnO-NPs foliar application under drought stress.

Results

Identification of strain: The isolated strain was identified as member of genus Aspergillus based on its unique colony characteristics i.e. white peripheral hyphae and dense spores deposit at the colony's center along with micromorphology shows hyphae bearing conidia with conidiophores (Fig. 1A). Furthermore, molecular identification revealed that the ITS sequence has 100% homology with *Aspergillus flavus* (accession number MT529182.1) in the GenBank database. A phylogenetic tree was constructed by MEGA 11 using the ITS sequence further confirmed (Fig. 1B) this identification.

Characterization of ZnO-NPs: The color change from pale yellow to yellowish-white solution was considered as an indicator of ZnO-NPs formation in present work (Fig. 2A). The characterization of ZnO NPs was executed using XRD (X-ray diffraction), SEM (scanning electron microscopy) and FTIR (fourier transform infrared spectroscopy). The analysis of ZnO nanoparticles produced by biological method using X-ray diffraction (XRD) is essential for confirming their phase composition and crystalline structure. The XRD pattern depicted in the figure 1 demonstrates distinct peaks at various 2θ angles, which correspond to specific planes in the crystal structure of ZnO nanoparticles. The data were collected over a 2θ range from 20° to 80°, with a scanning rate of 0.02° per second using Cu Kα radiation (λ = 0.1540 nm). The identified peaks at 31.08°, 34.30°, 37.41°, 46.35°, 56.90°, 62.20°, 66.78°, 72.48°, and 77.64° correspond to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) reflections, respectively. Additionally, the JCPDS (Joint Committee on Powder Diffraction Standards) card number for the wurtzite phase of ZnO is JCPDS No. 36-1451. The average size of ZnO NPs was estimated via Debye–Scherer equation (1) ($D=K\omega\beta\cos\theta$), where ω represents the X-ray wavelength (1.540560 Å), β is the full width at half maxima of the diffraction peak in radians, θ is the Braggs' angle in degrees, and K, the shape factor, is 20 nm.

The SEM image of biologically synthesized ZnO nanoparticles were seen to have a spherical or nearly spherical shape, which is a common morphology observed in biologically synthesized ZnO nanoparticles. The FTIR spectra of ZnO nanoparticles displayed multiple absorption peaks at 3433.29, 2943.27, 2133.27, 1672.28, 1265.30, 719.44, 592.14, and 474.48 cm^{-1}. The absorption peak near 2943.27 cm^{-1} is associated with C-H stretching in alkane (C-H) groups, while the broad band at 3433.29 cm^{-1} reflects the presence of hydroxyl (OH) groups. An absorption peak at 719.44 cm^{-1} corresponds to the stretching vibration of alkene (C=C) groups, and the peak at 2133.27 cm^{-1} indicates the presence of aromatic (C=C) group. Amide's carbonyl (C=O) group stretching vibrations are observed at 1672.28 cm^{-1}. The formation of ZnO nanoparticles is confirmed by absorption peaks at 592.14 and 474.48 cm^{-1}. Additionally, the band at 1265.30 cm^{-1} suggests C-O stretching. Thus, FTIR spectrum suggested that ZnO-NPs synthesized using fungus are capped by various phytochemicals, which serve as both stabilizing and reducing agents for nanoparticles (Fig. 2B).

Growth attributes: The results demonstrated a significant ($p\leq0.001$) decline in shoot fresh and dry weight of two mung bean vars. AZRI-2021 and NM-54 under drought stress. Both varieties showed significant differences ($p\leq0.001$) regarding shoot fresh and dry weight under drought, while a maximum decline was noted in shoot fresh weight (51.96%) and dry weight (40.34%) in sensitive var. NM-54. Foliar application of biologically synthesized ZnO-NPs highlighted a significant ($p\leq0.001$) improvement in shoot fresh and dry weight of mung bean varieties over untreated ones under drought stress. However, application of ZnO-NPs (10 mg/L) exhibited a maximal betterment in shoot fresh weight (64.36 and 49.58%) and dry weight (34.95 and 50.35%) in var. AZRI- 2021 and NM- var. 54, respectively under drought. Shoot length, root length and root fresh and dry weight were also reduced notably ($p\leq0.001$) in mung bean varieties during stress conditions. There was a significant ($p\leq0.001$) difference between the two varieties concerning these attributes, while a greater abridge was found in root fresh weight (39.87%), root dry weight (40.88%), shoot length (46.03%), and root length (34.71%) in sensitive var. NM-54 on drought exposure (Fig. 3). Mung bean varieties exhibited a remarkable ($p\leq0.001$) increase in root fresh and dry weight and shoot and root length in both varieties relative to untreated under drought conditions. Mung bean varieties under 10 mg/L treatment displayed maximum increase in root fresh weight (25.22 and 51.5%), root dry weight (37.63 and 65.35%), shoot length (38.16 and 39.92%), and root length (37.24 and 36.99%) in var. AZRI-2021 and NM-54, respectively under water stress.

Photosynthetic pigments: Drought stress displayed a highly significant ($p\leq0.001$) decline in chlorophyll *a,* chlorophyll *b*, total chlorophyll, chlorophyll ab^{-1}, and carotenoids, in both mung bean varieties under stress. There was a significant ($p\leq0.001$) difference between the two varieties concerning photosynthetic pigment levels under drought. A higher drop in chl *a* (55.87%), chl *b* (48.16%), and total chl (53.48%), chlorophyll ab^{-1} (15.32%), and carotenoids (39.94) was seen in drought-stressed sensitive var. NM-54. Foliar application with different levels of ZnO-NPs (5, 10 and 15 mg/L) illustrated a considerable ($p\leq0.001$) improvement in both varieties over untreated under stress conditions. Mung bean varieties displayed a maximum rise in chlorophyll *a* (93.55 and 75.89%), chlorophyll *b* (54.41 and 40.12%) and total chlorophyll (80.85 and 63.22%) under 10 mg/L in var. AZRI- 2021 and var. NM- 54, respectively on exposure to drought. However, both mung bean varieties showed the same enhancement to chlorophyll ab^{-1} (26.30 and 26.30%) and carotenoids (66.53 and 66.53%) with 10 mg/L treatment under drought (Fig. 4).

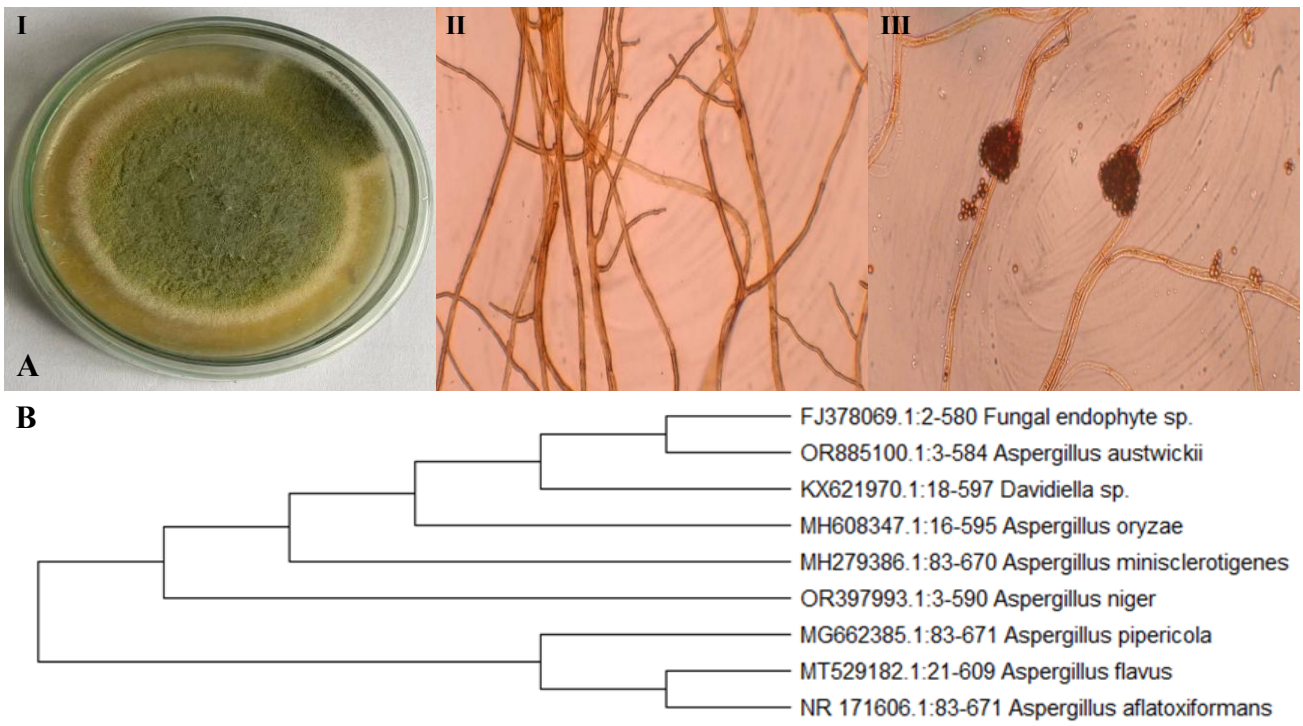

Fig. 1. Culture of Aspergillus strain on PDA media isolated from rhizosphere of soybean plants (A). (I) Macroscopic view (7 days, 28°C); (II) & (III) Microscopic image (x 100 magnification) of septate hyphae and globose conidia with conidiophore which are slightly roughened. (B) Phylogenetic tree (Neighbor-joining method) formed by using internal transcribed spacer (ITS) sequence.

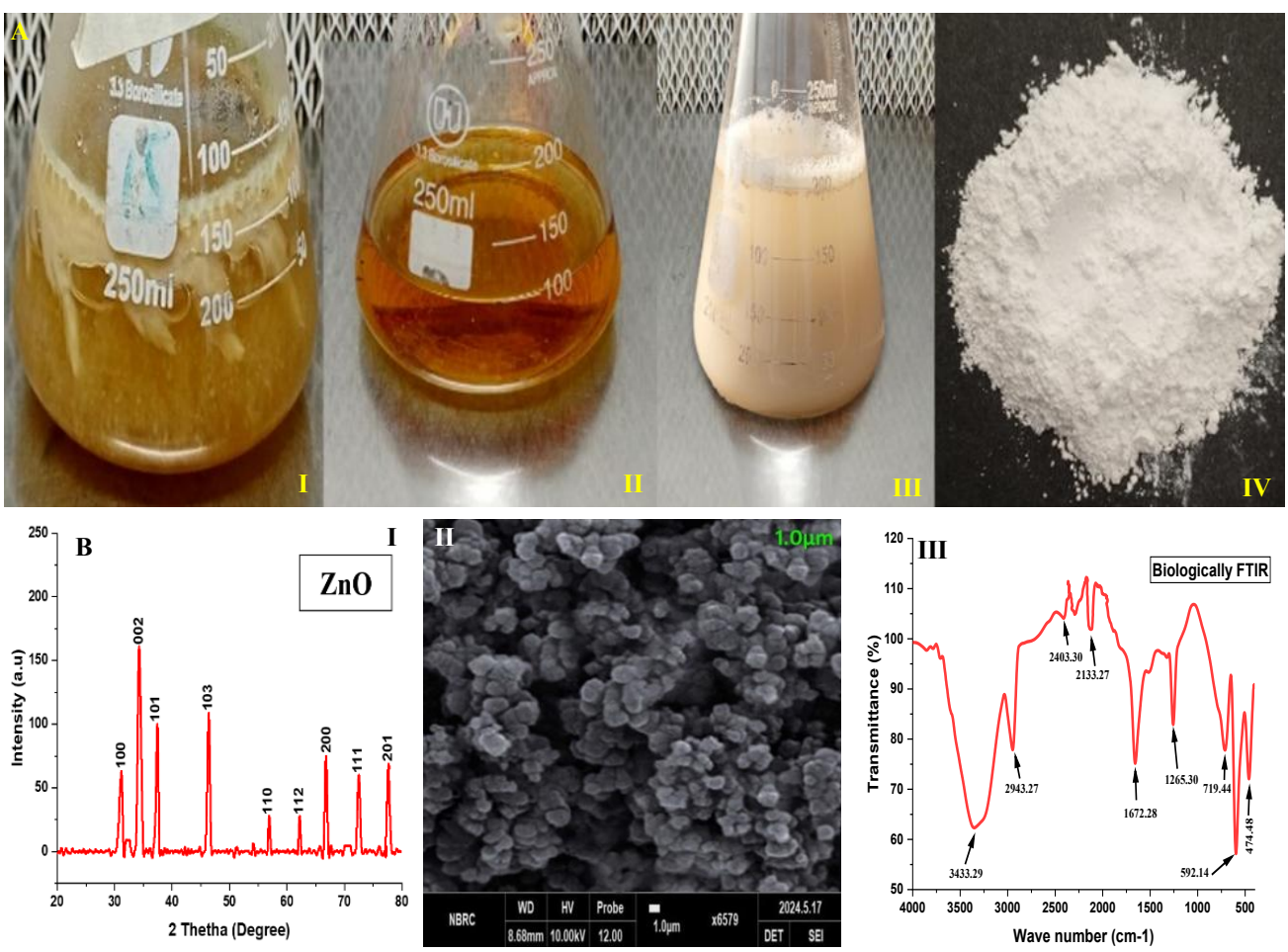

Fig. 2. (A) *Aspergillus flavus* culture in malt extract broth medium (I), Cell-free filtrate (II), Cell-free filtrate + zinc acetate dihydrate (III &IV), ZnO-NPs. (B) Zinc oxide nanoparticles (ZnO-NPs) characterization through (I) XRD (X-ray diffraction) analysis (II) SEM (Scanning electron microscopy) at 10.00 KV and (III) FTIR (Fourier Transform Infrared) spectrum of ZnO-NPs.

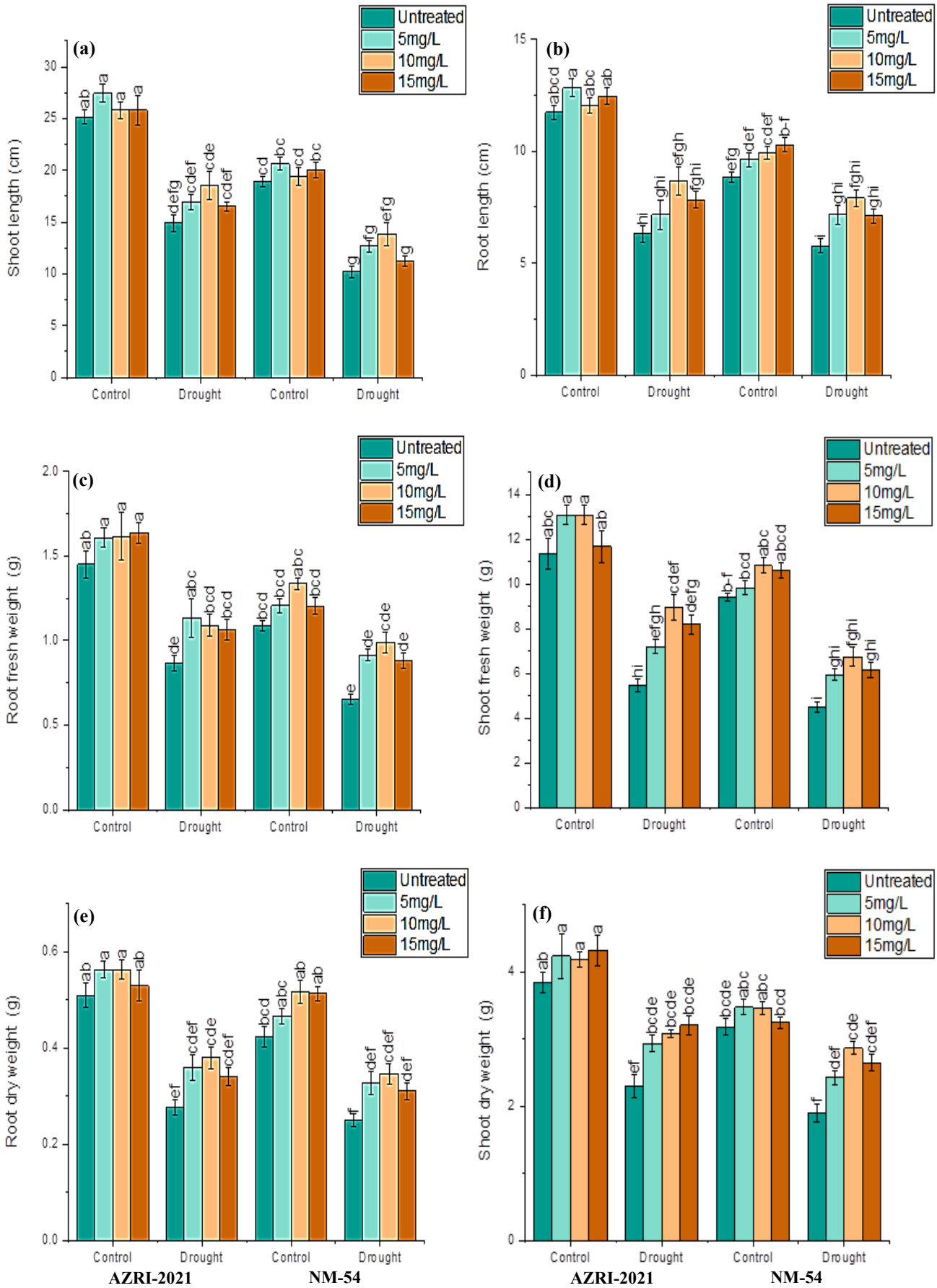

Fig. 3. Effect of ZnO-NPs foliar application on (a) shoot length (SL), (b) root length (RL), (c) root fresh weight (RFW) (d) shoot fresh weight (SFW) (e) root dry weight (RDW) and (f) shoot dry weight (SDW) of two Mung bean varieties, AZRI-2021 and NM-54 grown in pot condition under drought stress. Data presented mean values ±SE (standard error) of four replicates; mean values with distinct letters designate statistically significant differences among ZnO-NPs treatments at a significance level of 0.05.

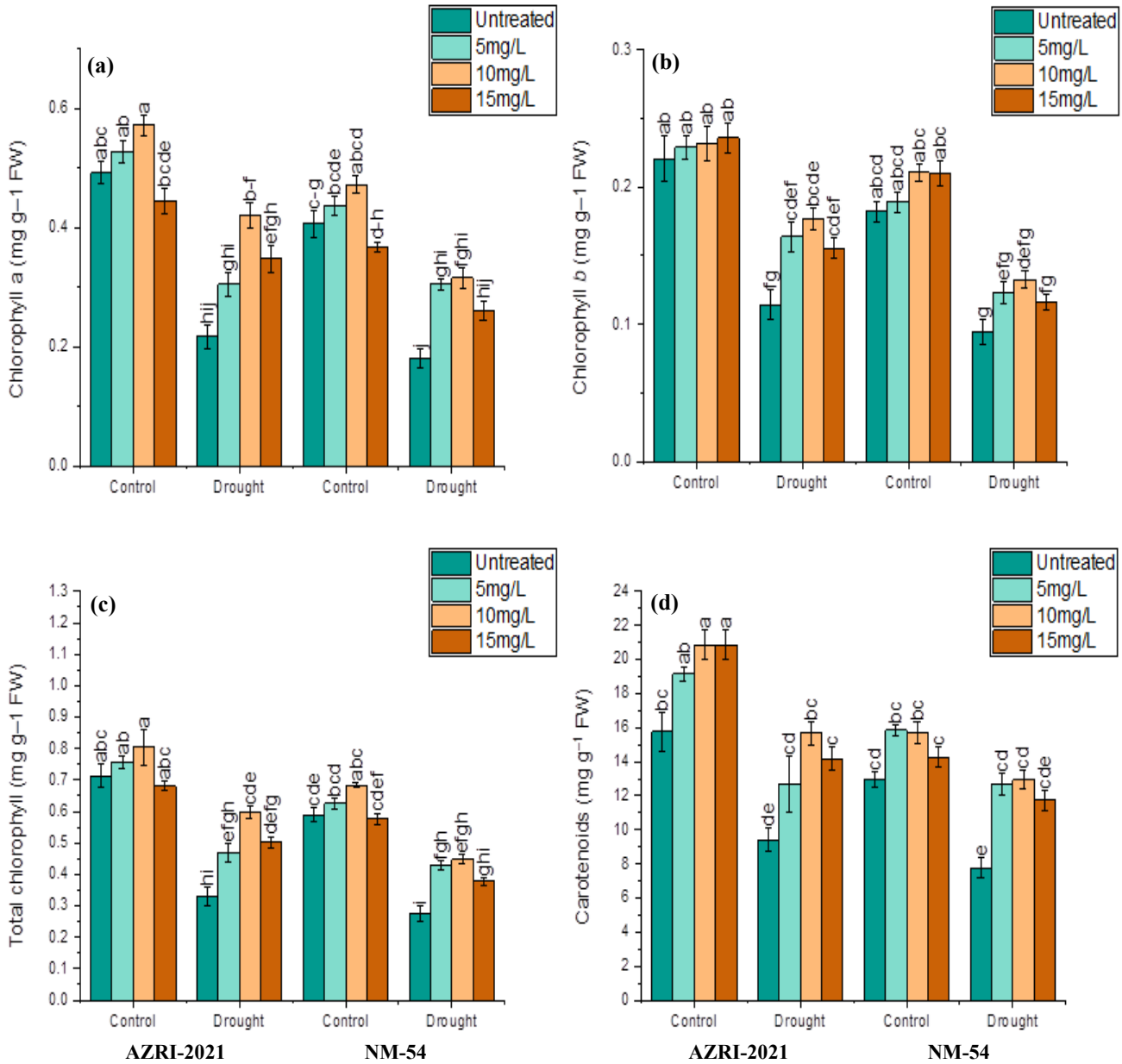

Fig. 4. Effect of ZnO-NPs foliar application on (a) Chlorophyll *a*, (b) Chlorophyll *b*, (c) Total chlorophyll, and (d) Carotenoids of two Mung bean varieties, AZRI-2021 and NM-54 grown in pot condition under drought stress. Data presented mean values ±SE (standard error) of four replicates; mean values with distinct letters designate statistically significant differences among ZnO-NPs treatments at a significance level of 0.05.

Osmoprotectants: Drought exhibited a significant ($p \leq 0.001$) rise in TFAA, TSP, TSS and proline levels in both mung bean varieties compared to their respective control. Both varieties displayed an enormous ($p \leq 0.001$) difference concerning TFAA, TSP, TSS and proline content under stress, whereas a greater rise was recorded in TFAA (33.25%), and proline (19.5%) in sensitive var. NM-54; TSP (21.08%) and TSS (15.71%) in a tolerant var. AZRI-2021 exposed to drought. In this context, plants treated with 5 mg/L manifested a maximum accumulation of TFAA (37.42 and 30.72%), 10 mg/L of ZnO NPs showed maximum rise for TSP (46.26 and 46.39%), proline (47.48 and 33.04%) and TSS (40.10 and 40.20%) in var. AZRI-2021 and var. NM-54, respectively grown under drought. Phenolics and flavonoids content were tremendously ($p \leq 0.001$) increased in both mung bean varieties under drought stress. A significant ($p \leq 0.001$) difference was evident in both varieties concerning these non-enzymatic antioxidant compounds in response to drought. A greater upsurge was documented in flavonoids (25.37%) in a sensitive var. NM-54 and phenolics (23.36 and 23.36%) level in both varieties showed equal enhancement when plants were subjected to drought stress. The administration of ZnO-NPs further evidently ($p \leq 0.001$) enhanced these variables in both mung bean varieties over untreated plants under drought (Fig. 4). Mung bean varieties supplemented with 10 mg/L foliar application presented a maximum improvement in phenolics (28.05 and 40.09%) and flavonoids (52.53 and 51.73%) in var. AZRI-2021 and var. NM-54, respectively grown under water deficit conditions (Fig. 5).

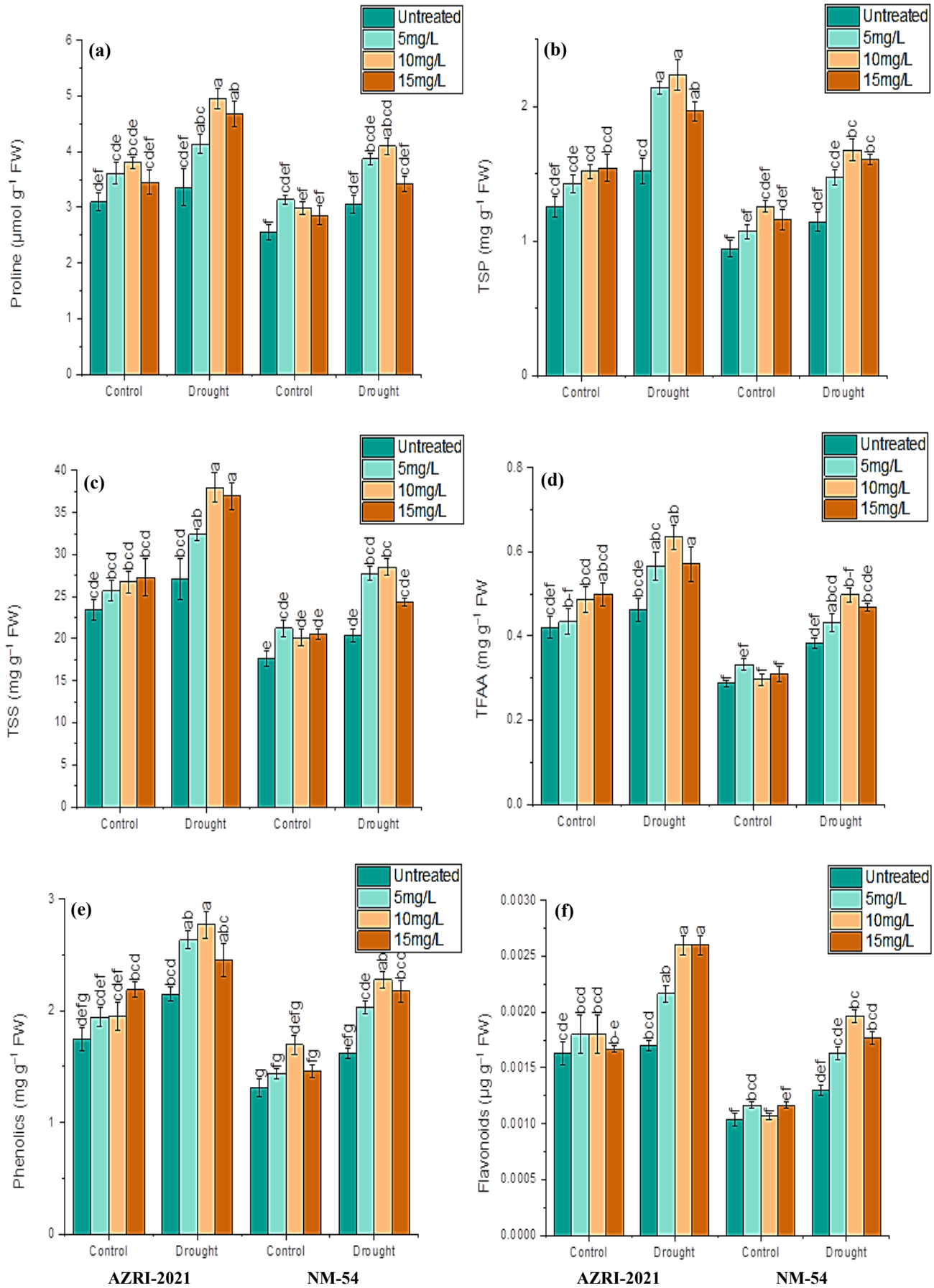

Fig. 5. Effect of ZnO-NPs foliar application on (a) proline, (b) TSP; total soluble proteins, (c) TSS; total soluble sugars, (d) TFAA; total free amino acids, (e) phenolics, and (f) flavonoids of Mung bean varieties, AZRI-2021 and NM-54 grown in pots under drought stress. Data presented mean values ±SE (standard error) of four replicates; mean values with distinct letters designate statistically significant differences among ZnO-NPs treatments at a significance level of 0.05.

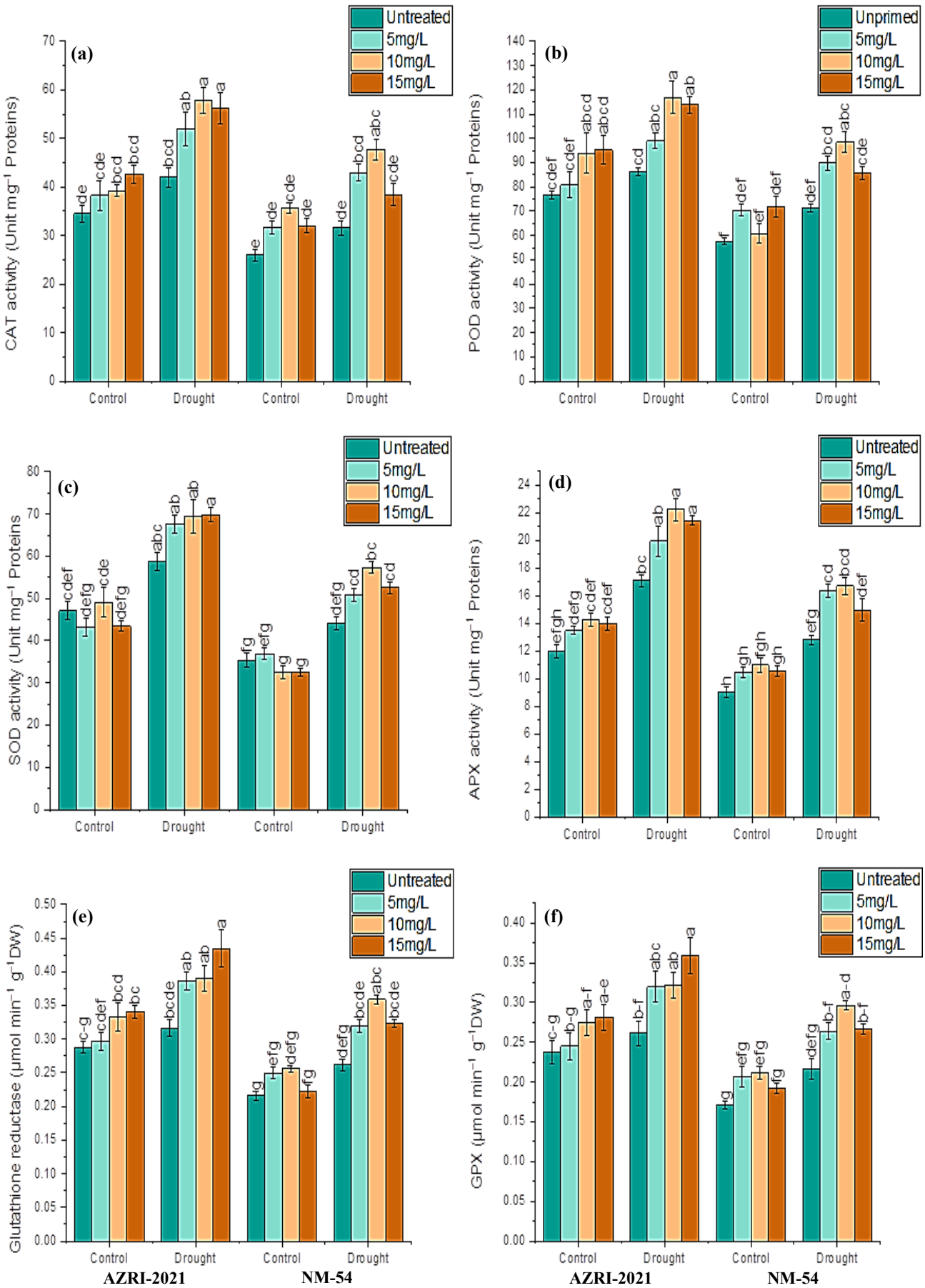

Fig. 6. Effect of ZnO-NPs foliar application on (a) CAT, (b) POD, (c) SOD, (d) APX, (e) GR, and (f) GPX of two Mung bean varieties, AZRI-2021 and NM-54 grown in pots under drought stress. Data presented mean values ±SE (standard error) of four replicates; mean values with distinct letters designate statistically significant differences among ZnO-NPs treatments at a significance level of 0.05.

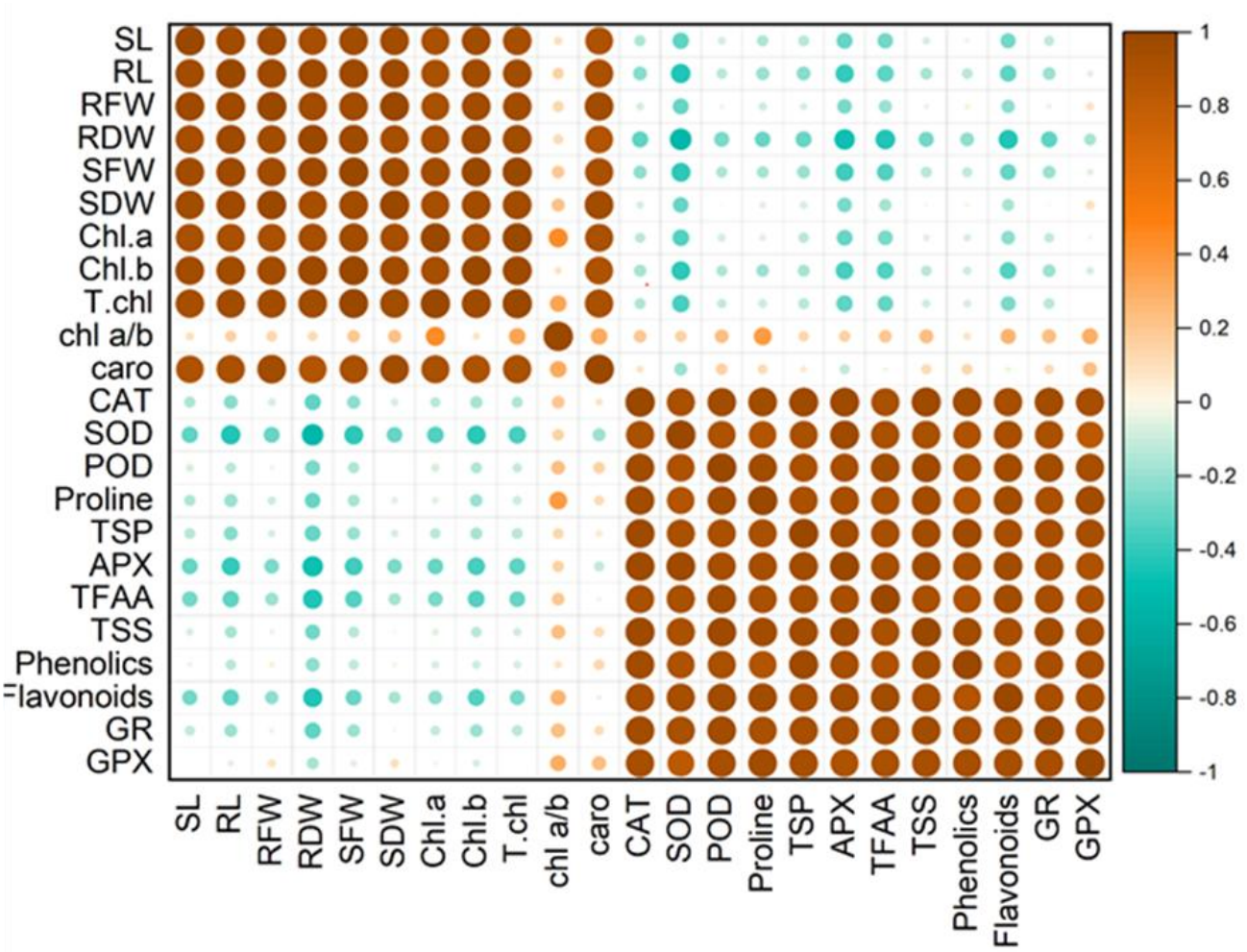

Fig. 7. Pearson's correlation analysis (r values) among the different studied parameters of mung bean varieties (AZRI-2021 and NM-54) grown under drought stress and foliar applied ZnO nanoparticles. Correlations are presented in orange (positive) and green (negative) while the circle size and color intensity are directly proportional to correlation coefficient. SL = root length; RL = shoot length; RFW = root fresh weight; RDW = root dry weight; SDW = shoot dry weight; Chl *a* = chlorophyll *a*; Chl *b* = Chlorophyll *b*; T. Chl = Total Chlorophyll; Chl *a/b* = Chlrophyll *a/b* ratio; Caro= carotenoids; CAT = catalase; SOD = Superoxide dismutase; POD = peroxidase; Proline; TSP = total soluble proteins; APX = ascorbate peroxidase; TFAA = total free amino acids; TSS = total soluble sugars; GR = glutathione reductase; GPX = glutathione peroxidase.

Antioxidants: Drought displayed a significant ($p \leq 0.001$) surged in the antioxidant enzymes activities in mung bean varieties confronted under drought. Both varieties differed greatly concerning the activities of enzymes in response to water deficit conditions, whereas a greater increment was seen in the activities of SOD (24.8%), POD (23.7%), CAT (21.21%), and APX (42.51%) in a tolerant var. AZRI-2021 under drought stress. Plants of both varieties treated with different levels (5 mg/L, 10 mg/L and 15 mg/L) significantly ($p \leq 0.001$) improved antioxidant enzymes activities in mung bean plants relative to untreated plants under drought. However, maximum enhancement in the activities of SOD (18.52 and 30.72%), POD (35.71 and 38.35%), CAT (37.32 and 51.76%), and APX (30.01 and 30.54%) in var. AZRI-2021 and var. NM-54, respectively facing drought under the treatment of 10 mg/L of ZnO-NPs was recorded (Fig. 6).

The activities of GR (glutathione reductase) and glutathione peroxidase (GPx) were noticeably ($p \leq 0.001$) enhanced in both mung bean varieties under drought stress. There was a profound ($p \leq 0.001$) difference between the two varieties regarding GR and GPX activity responding to drought. However, a greater rise was observed in GR (21%) and GPX (26.2%) levels in var. NM- 54 under drought. Mung bean plants depicted a significant ($p \leq 0.001$) improvement in GR and GPX activities in both varieties over untreated plants under drought. From this perspective, 10 mg/L concentration of ZnO-NPs demonstrated a maximal betterment in GR (23.35 and 37.62%) and GPX (23.45 and 37.62%) activities in var. AZRI-2021 and var. NM-54, respectively exposed to drought.

The Pearson's correlation analysis illustrates the correlation between growth, biochemical and physiological traits of two mung bean varieties during drought stress and foliar applied ZnO nanoparticles. A positive correlation between growth parameters such as root length (RL), shoot length (SL), fresh and dry weights of roots and shoots was noted, indicating that these parameters responded similarly to the treatments received. A strong positive correlation was noted the growth attributes and photosynthetic pigments i.e. Chl *a*, Chl *b*, total Chlorophyll and carotenoids that indicates that enhanced photosynthetic activity was responsible for better growth. Biochemical stress response indicators including,

antioxidant enzymes (CAT, SOD, POD, APX, GR, GPX), osmolytes (proline, TSS, TFAA) and secondary metabolites (phenolics, flavonoids) also indicated a strong intercorrelations among themselves, forming a unique cluster of evidence of a concerted biochemical stress response (Fig. 7). Although the stress indicators exhibited a negative correlation with growth and photosynthetic attributes but still there was an interaction between the growth and stress tolerance mechanism of plant. Overall, ZnO NPs application enhanced plant growth and stress tolerance response under drought stress, but plant will priorities defense over the growth with prolonged and enhanced drought stress.

Discussion

Plants being sessile are most affected by environmental hazards and have developed many mechanisms to perceive signals and regulate responses under abiotic stresses (Dudziak *et al.,* 2019; Nawaz *et al.,* 2024) that are specific to type of stress and its intensity, plant species and even genotype specific. Most high yielding crop genotypes are not resilient to stress, especially drought, which results in yield reductions. The yield of crop plants mainly depends on the biomass production and the function of physio-biochemical mechanisms. Though different strategies, including the use of micronutrients through different methods, are being employed for the stress tolerance induction in crop plants but these are not found up to mark in one or other way. With many advantages, such as the sustainability, economic feasibility, and environmental friendliness of NPs make them the principal choice to be used for stress tolerance induction in crop plants. The biological pathway of their synthesis provides new avenues and a sustainable future in this area of research since they have the ability to reduce the negative impacts of abiotic stress in agricultural crops (Younes *et al.,* 2020; El-Saadony *et al.,* 2022) with better yield and nutritional quality as well as for better transport and mobilization of micro-nutrients to fulfil their deficiency. Present study, in view of sustainability, environmental issues and soil degradation issues, was conducted to induce the water stress tolerance in differentially drought sensitive mung bean genotypes by foliar application of biologically synthesized ZnO-NPs. Zinc is a vital micronutrient for healthy growth and development of plant; thus, it plays an important role in maintaining cell turgor, homeostasis, cell structures and anatomy of plants under stress conditions (Rizwan *et al.,* 2019). Furthermore, it also contributes in maintaining cell integrity, and detoxifying free radicals during drought stress (Dimpka *et al.,* 2019). However, its availability and translocation are problems in crop plants that results in significant yield losses worldwide. Though different sources of Zn are being applied through different modes to overcome this problem with minimum impacts of environment.

In our investigation, both mung bean types demonstrated a rise in shoot fresh weight and dry weight by 64.36% and 50.35%, respectively, when foliarly supplied with biologically synthesized ZnO-NPs. This improvement was also found in root fresh and dry weights and 10 mg/L remained most effective in improving these growth attributes in comparison with plants that remained untreated with ZnO-NPs application. The application of ZnO-NPs at 15 mg/L caused a significant reduction in growth attributes. This shows the treatment level specific response of mung bean genotypes to biologically synthesized ZnO-NPs. Previously conducted studies exhibited that ZnO-NPs foliar application enhanced all plant growth parameter in wheat (Du *et al.,* 2019) and maize (Sun *et al.,* 2021) under drought stress when compared with control ones. This increase describes the role of Zn, because earlier it was found that Zn concentration in plant organs increases when exposed to Zn application in the form of ZnO-NPs (Akmal *et al.,* 2023). It is involved in boosting auxin metabolism that also found beneficial in maintaining cell membrane integrity. The Zn concentration also influences activity of carbonic anhydrase, a Zn-dependent enzyme, that regulate CO_2 sensing pathway and plays role in improving the drought tolerance (Tewaei *et al.,* 2019). The improvements in drought tolerance regarding maintaining better fresh and dry biomasses of mung bean genotypes can be correlated well with these findings. Excessive concentration of Zn in plant damages plant growth attributes and even may lead to death. This is because of hyper active antioxidant system, deteriorated nitrogen metabolism and cytostructural changes and same has been reported in Beans, Brassica and wheat (Michael, & Krishnaswamy, 2011; Blasco *et al*., 2019; Paunov, 2018).

It is well narrated that drought stress results in enhanced ROS production, which causes oxidative stress (Wang *et al.,* 2019) and results in lipid peroxidation of cellular membranes including the chloroplast membranes containing the photosynthetic pigments. These structural changes in photosynthetic membranes are marked as increased MDA production (Dudziak *et al.,* 2019). The supply of ZnO-NPs against drought stressed plants lowered MDA and H_2O_2 by regulating cellular metabolism, which is a significant finding in the current work, as has been reported in chickpea (Burman *et al*., 2013). Results of this study revealed that all the photosynthetic pigments decreased under drought stress in both mungbean genotypes which seems the function of over production ROS that caused damages to chloroplast membranes and resulted in reduced photosynthetic pigments (Mafakheri *et al.,* 2010). These damaging impacts of drought stress on photosynthetic pigments reduced significantly under foliar application of ZnO-NPs in both mung bean varieties as compared to non-treated ones. Earlier it was reported by Leopald *et al.,* (2022) that 100-200 nm ZnO-NPs elevated the leaf chlorophyll contents in soybean plants under stress and the variation in effective dose seems plant species specific response to Zn dose. Previous studies reported that imposition of drought resulted in agitations of chloroplast and disturbs chlorophyll *a* to *b* ratio thus hindering photosynthesis (Mejiri *et al.,* 2016). In contrast, this the reduction in chloroplast might also be the strategy to avoid photooxidation. Because chlorophyll plays a key role in photosynthesis by harvesting light and Zn stimulated chlorophyll synthesis is the function of increased levels of cytokinin (Amer, 2024) that resulted in increased photosynthesis and better biomass production. However, studies also reveal that plant chlorophyll concentrations not affected even with ZnO nanoparticles which likely might be the specific to applied dose of ZnO-NPs and type of plant species (Singh *et al.,* 2016). The present study demonstrating well that biologically synthesized ZnO-NPs application not only led to an enhancement in chlorophyll contents but also

resulted in a significant increase in carotenoid levels, with an observed maximum rise of up to 66.53% in mung bean showing the improvement in antioxidative defense mechanism because carotenoids other than the accessory pigment, also play strong role as antioxidant.

It is widely established fact that osmoprotectants accumulation in plants is a response toward drought tolerance (Sadak *et al.,* 2020). Drought stress significantly elevated the levels of proline, total soluble sugars, total free amino acids and total soluble proteins and foliar-applied ZnO-NPs application at 10 mg/L significantly further enhanced the levels of proline, TSS, TFAA and TSP when compared with non-treated plants. It confers the upregulation of osmotic adjustment to regulate the cellular water relations with their role as osmotica. This increased accumulation of these metabolites correlating well with growth improvements in both mung bean varieties. This study satisfies the findings of El-Bassiouny *et al*., (2022) in which they explored well effects of TiO_2 and ZnO in mitigating the drought stress effects in wheat. Findings of Soliman *et al*., (2015) in a study stated that ZnO-NPs application enhanced the total soluble sugars and proline concentrations, that resulted in improved salt tolerance in maize and resulted in an increased TSS and proline indicating well that ZnO-NPs played significant role in mitigating stress effects in maize by maintaining the cell turgor through osmotic adjustment and stabilizing proteins.

Present study demonstrates well that drought stress significantly enhanced the levels of phenolic and flavonoids in both mung bean cultivars indicating the improved defense against oxidative stress because they play strong role as non-enzymatic antioxidants. The elevated levels of phenolic and flavonoid indicate their key role in plant oxidative stress tolerance ability, because these are ROS scavengers. These observations align with the study of Ghani *et al.,* (2022). Furthermore, biologically synthesized ZnO-NPs by foliar application further advanced the levels of flavonoid and phenolic in both mung bean varieties under drought stress that is correlating well with decreased levels of MDA and H_2O_2 and improved growth of both varieties. Plants protect the oxidative damages not only by non-enzymatic antioxidants but also by activating enzymatic antioxidant system (Sun *et al.,* 2020). In this regard, in present study the antioxidant enzymes activities were studied and results showed acceleration in SOD, CAT, POD and APX activity. The SOD is thought to act as primary line of defense against reactive oxygen species (ROS) production because it scavenges superoxide into oxygen and H_2O_2 being produced as first mark of ROS synthesis (Carvalho 2008). The SOD activity increased with application of ZnO-NPs which might be due to increased expression level of SOD synthesis genes (Pandya *et al.,* 2023). Furthermore, ZnO-NPs application induced antioxidant enzymes activity was maximum in plants applied with 10 mg/L ZnO-NPs foliar application. Our outcomes are similar to the prior study that documented the increased concentration of SOD and POD in tomato plants supplied with 25 mg/L of ZnO-NPs (El-Zohri *et al.,* 2021). APX eradicates H_2O_2 and glutathione reductase provides substrate for it by enzymatic activity. High level of APX activity may be related to enhanced APX activity under ZnO-NPs application as compared to control. Similarly, earlier reported that expression levels of Cu/Zn, SOD, APX and CAT increased in maize experiencing drought, treated with ZnO-NPs (Sun *et al.,* 2020).

The results of present study also revealing that GR and GPx activity also increased when plant experienced water deficit environment. While such rise in the activity of these enzymes tend to increase significantly in those plants under ZnO-NPs application. Our investigations are similar with Abd El Mageed *et al.,* (2023) who reported glutathione influenced antioxidant activity of common beans during drought stress because GR plays a crucial role in reducing oxidative stress in plants as it is a component of plant antioxidant system thus its activity increases under water deficit conditions. Moreover, GR also helps in the regeneration of reduced glutathione (GSH) from its oxidized form under stress (GSSD) (Averill-Bates, 2023). This improvement in antioxidative defence mechanism in mung bean plants by foliar-applied ZnO-NPs down regulated the process of oxidative damages through scavenging the overly produced ROS as resulted from lowered MDA levels. It resulted in better maintenance of cellular membranes as depicted well from higher levels of chlorophyll and better osmotic adjustment through reduced leaky membranes as a result better growth of mung bean plants under water deficit stress.

Conclusively, biologically synthesized zinc oxide nanoparticles (ZnO-NPs) application significantly alleviated the negative effects of drought stress in mung bean varieties differing in drought tolerance. Application of ZnO-NPs, particularly at the 10 mg/L concentration, led to substantial improvements in plant growth parameters, that significantly associated with enhanced photosynthetic pigment concentrations, osmolyte accumulation, and antioxidative defense mechanisms. ZnO-NPs application was found to alleviate oxidative stress through boosting both non-enzymatic and enzymatic antioxidant responses. Furthermore, ZnO-NPs significantly enhanced the levels of chlorophyll, TFAA, TSS, proline, and TSP, have significant role in maintaining cellular homeostasis and osmotic balance under drought stress. Importantly, the biogenic synthesis of ZnO-NPs via *Aspergillus flavus* not only provides a sustainable and eco-friendly nanotechnological approach but also enhances zinc bioavailability, bypassing soil-related limitations and reducing potential environmental risks associated with conventional fertilization. The observed genotype-specific responses to ZnO-NPs further underline the need for targeted nano-interventions based on varietal traits. In conclusion, biogenically synthesized ZnO-NPs offer a promising, cost-effective strategy to enhance drought tolerance in mung bean by modulating growth, physiology, and antioxidant capacity. These findings pave the way for future translational research into nanoparticle-based sustainable agriculture under abiotic stress conditions. Furthermore, genetic basis and the gene expression pattern are also not documented. In future, based on these findings, study could be conducted to dug out the associated genes expression levels and molecular pathways activated by the application of ZnO-NPs to tolerate drought stress in mungbean.

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