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BRASSINOLIDE ALLEVIATES THE DROUGHT STRESS OF *OXALIS CORNICULATA* BY ENHANCING THE GROWTH AND REDUCING THE OXIDATIVE STRESS

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Abstract

Drought stress poses a significant environmental challenge that can severely impact plant growth and productivity, primarily by inducing oxidative stress. In response to this challenge, brassinolide, a plant hormone, has gained increasing attention for its potential to mitigate the adverse effects of drought stress through the regulation of antioxidants. This study aimed to assess the impact of brassinolide on *Oxalis corniculata* under drought-stress conditions. There were 2 levels of brassinolide i.e., 0 and 0.1 µM brassinolide applied under drought stress and no drought stress. All treatments were applied in 4 replicates. Results showed that the brassinolide + drought stress group exhibited a marked increase in plant height (18.77%), stem diameter (21.78%), leaf area (24.65%), and biomass (22.76%) compared to those subjected to drought stress alone. Oxidative stress parameters, represented by MDA and H_2O_2 contents, exhibited a significant elevation in the drought stress group when compared to the control group. However, the administration of brassinolide significantly lowered MDA (29.41%) and H_2O_2 (28.88%) contents within the brassinolide + drought stress group, as opposed to the drought stress-only group. Moreover, antioxidant enzyme activities, including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), displayed a substantial increase in the brassinolide + drought stress group relative to the drought stress group, with percentage increases ranging from approximately 20.92% to 33.47%. In conclusion, brassinolide treatment significantly enhanced *Oxalis corniculata* tolerance to drought stress by promoting growth and reducing oxidative stress, emphasizing its potential as a valuable tool in sustainable agriculture.

Key words: Bassinolide; *Oxalis corniculata*; Oxidative stress; Drought stress.

Introduction

Drought stress poses a pressing global challenge to agricultural productivity, emphasizing the need for innovative strategies to enhance plant resilience in water-scarce environments (Bhutto *et al.*, 2023; Hussain & Shah, 2023; Jabborova *et al.*, 2023; Shahzadi *et al.*, 2023). One of the primary consequences of drought stress is reduced water uptake, as the soil's water deficit hinders the plant's ability to absorb water through its roots. To cope with limited water availability, plants respond by closing their stomata, small openings on the leaf surface, in an attempt to conserve water (Zhang & Kirkham, 1994; Latif *et al.*, 2016). However, this also restricts the plant's capacity to intake carbon dioxide for photosynthesis, leading to inhibited photosynthetic processes. As a result, the plant may exhibit visible signs of distress, such as wilting, leaf curling, or drooping. Furthermore, extended drought stress can inflict cellular damage due to the accumulation of reactive oxygen species (ROS), causing oxidative stress that harms cell components like membranes, proteins, and DNA (Liu *et al.*, 2019; Verma *et al.*, 2019). This cellular damage contributes to decreased plant growth, including reduced plant height, stem diameter, leaf area, and biomass production. Additionally, drought stress interferes with nutrient uptake, making it challenging for plants to access essential nutrients from the soil (Ashraf *et al.*, 2011, 2013).

Plant secondary metabolites and growth regulators contribute to drought tolerance, including auxin, jasmonic acid, abscisic acid, plant steroids, and ethylene (Joshi & Karan, 2013). Brassinosteroids (BRs) are regarded as plant hormones that govern plant growth and production, and they are among the substances utilized to treat plant stress (Asgher *et al.*, 2015; Vardhini, 2016). BRs, polyhydroxylated steroidal plant hormones, regulate plant growth and development. Many studies have demonstrated their central role in handling various physiologic processes, including cell division, male fertility, growth, aging, leaf creation, and vascular differentiation(Basit *et al.*, 2021). These chemicals' diverse biological actions provide novel opportunities for boosting agricultural yields by modulating plant metabolism and shielding plants from the variability of stressors (Basit *et al.*, 2021).

Furthermore, BRs are well-known regulators of translation and transcription pathways that increase seed production and boost total protein and enzyme levels (Mazorra *et al.*, 2002). BRs serve a regulatory function in plant development and contribute to forming defence systems to deal with numerous abiotic and biotic challenges (Ke *et al.*, 2022). Numerous BRs containing brassinolide as the significant constituent have been examined in the field and have significantly boosted crop yields. Tolerance to salt, drought, high/low temperatures, and heavy metals have all been enhanced with the exogenous application of BRs.

Even if there aren't many articles, BRs have been linked to identifying metabolic and gene pathways that confer drought tolerance in *O. corniculata* (Vardhini, 2016; Ahanger *et al.*, 2018; Santos *et al.*, 2018). Comparing the responses of drought-tolerant and -sensitive genotypes to exogenously applied BRs should reveal the BR-induced alterations, particularly in the susceptible genotypes, because the tolerant genotypes should be less affected by the drought. Unfortunately, existing evidence on the involvement of BRs in plant drought reactivity is not very definitive since it comes from the few research conducted with genotypes with recognized drought sensitivity (Sakauchi *et al.*, 2022). A similar pattern should be seen when BRs are administered exogenously to plants subjected to droughts of varying severity; BRs always have a higher effect on more severely stressed plants. Several studies have observed instances where the drought-tolerant genotype responded more strongly to BRs than the drought-sensitive genotype. Therefore, the situation is more nuanced than it initially

appears, and it likely varies between plant species and the mechanism associated with drought resistance/sensitivity in a given genotype (Tariq *et al.*, 2022).

Recent research has shown that some active chemicals isolated from herbal medications, herbal formulations, and herbal extracts impact in vitro and in vivo models of Parkinson's disease. *Oxalis corniculata* (*O. corniculata*) is a plant used to treat psychological and neurological disorders (Silalahi, 2022). It is a member of the family Oxalidaceae and is most frequently referred to as Indian sorrel (Puliiyarai in Tamil). In addition to being an effective antipsychotic, it stimulates the central nervous system and inhibits seizures. Traditional Indian medicine employs the usage of *O. corniculata* leaves in the treatment of epilepsy (Silalahi, 2022). However, coupled with weakened defenses, drought-stressed *Oxalis corniculata* become more susceptible to pests and diseases, compounding their challenges.

This study addresses the knowledge gap by focusing on *Oxalis corniculata*, a prevalent weed species, and its response to brassinolide, a plant hormone, under drought conditions. While brassinolide role in stress mitigation has been explored in various crops, its application in non-crop plants like *Oxalis corniculata* remains understudied. The novelty of this research lies in its context-specific examination of a weed species, shedding light on the potential use of brassinolide for weed management in water-limited ecosystems. The study aims to quantify morphological and physiological changes induced by brassinolide, assessing its effectiveness in ameliorating drought stress effects on *Oxalis corniculata*. We hypothesized that brassinolide treatment might enhance growth parameters and reduce oxidative stress markers, offering insights into its broader applicability in diverse plant species and ecosystems, ultimately contributing to sustainable weed management strategies in the face of increasing water scarcity.

Materials and Methods

Plant material and growth conditions: The *O. corniculata* seeds were obtained from a local seed supplier. Initially damaged seeds were screened out manually.

Preparation of growth medium and pots: Plastic pots (12 cm diameter) were filled with soil and vermicompost (3:1 v/v). The soil was sieved to remove any stones and debris, and vermicompost was obtained from a local source. In an autoclave, the soil mixture was sterilized at 120°C for 2 h.

Seed germination and plant growth conditions: The sterilized soil mixture was moistened with distilled water, and 10 *O. corniculata* were sown in each pot. The pots were located in a development chamber under controlled conditions of 25 ± 2°C temperature, 60% relative humidity, and a 16 h light/8 h dark photoperiod. After germination, the number of seedlings was reduced to five per pot to avoid competition. The plants were watered with distilled water as required to preserve soil moisture.

The drought stress treatment was applied by withholding water for 14 days, starting the third day after sowing. The soil moisture content was monitored daily using a soil moisture sensor (Delta-T Devices Ltd., Cambridge, UK) and maintained at 60% of field capacity in the control and brassinolide groups.

Experimental site and design: The experiment was conducted at the campus of Anhui Science and Technology University, Fengyang, Anhui Province China in 2022. The control group represented plants that received water without exposure to drought stress or brassinolide treatment. The drought stress group comprised plants subjected to a 14-day water deprivation regimen without brassinolide treatment. In contrast, the brassinolide group consisted of plants receiving distilled water and a weekly application of 0.1 µM brassinolide for 14 days, yet not exposed to drought stress. Lastly, the brassinolide + drought stress group encompassed plants that experienced both drought stress and the weekly application of 0.1 µM brassinolide for 14 days, allowing for a comprehensive assessment of the combined effects of brassinolide treatment and drought stress on plant responses.

Brassinolide application method and concentrations: Brassinolide (Sigma-Aldrich, Product Number: B1439, Batch Number: SLCR5476, Brand: SIGMA, CAS Number: 72962-43-7 St. Louis, MO, USA) was dissolved in distilled water to prepare a stock solution of 1 mM. The stock solution was diluted to a desired concentration of 0.1 µM to create the working solution. The brassinolide solution was applied with foliar spray once a week for 14 days, starting the third day after sowing.

Data collection: The study conducted a comprehensive assessment of growth parameters and oxidative stress parameters on day 14, following the initiation of the treatments. Plant height was determined as the length of the main stem from the soil surface to the tip of the apical bud, providing insight into vertical growth. Stem diameter, representing the maximum diameter of the stem at the plant's base, was also measured, indicating stem thickness and robustness. Leaf area was quantified by measuring the fifth leaf from the top of the plant using a leaf area meter (Li-Cor Inc., Lincoln, NE, USA), reflecting the plant's photosynthetic capacity and surface area for gas exchange. Additionally, biomass was assessed by harvesting the plants, recording fresh weights, and then measuring dry weights after 48 hours of drying at 70 degrees Celsius, offering insights into the overall plant mass and productivity.

Malondialdehyde (MDA) content: The formation of MDA indicates the presence of oxidative stress due to lipid peroxidation. Heath and Packer's method determined the amount of MDA present (Heath & Packer, 1968). To sum up, we homogenized 0.5 g of leaf tissue in 5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged it at 12,000 rpm for 10 minutes. After heating the supernatant at 95°C for 30 minutes, 2 mL of 0.5% thiobarbituric acid (TBA) was added. The resultant complex was tested for its pink absorbance at 532 nm, and the non-specific absorbance at 600 nm was subtracted. We determined the MDA concentration using an attenuation value of 155 mM-1 cm-1.

Hydrogen peroxide (H_2O_2) content: H_2O_2 is another indicator of oxidative stress. H_2O_2 content was measured (Velikova *et al.*, 2000). Briefly, 0.5 g of leaf tissue was homogenized in 5 mL of 0.1% TCA and centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with 1 mL of 1 M K.I. and 0.5 mL of 0.1 M phosphate buffer (pH 7.0). The absorbance of the resulting, yellow-coloured complex was calculated at 390 nm. The H_2O_2 content was considered using a standard curve of known H_2O_2 concentrations.

Antioxidant enzyme activities: Catalase, superoxide dismutase, and peroxidase activities were calculated according to the protocols of Aebi (1984), Beauchamp & Fridovich (1971), and (Chance & Maehly, 1955). The leaves were homogenized in 5 mL of ice-cold extraction solution (50 mM phosphate buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP)). Enzyme tests were performed on the supernatant obtained after centrifuging the homogenate at 12,000 rpm for 10 minutes. Hydrogen peroxide degradation at 240 nm was used as a proxy for CAT activity. The oxidation of guaiacol at 470 nm was used as a proxy for POD activity. Nitroblue tetrazolium (NBT) reduction inhibition at 560 nm was used as a proxy for superoxide dismutase (SOD) activity. Protein activity was measured in terms of units per gram of enzyme.

Statistical analysis

Standard statistical procedures were adopted for statistical analysis of collecting data (Steel *et al.*, 1997). To analyze the collected data, statistical methods were applied using Origin Pro software (OriginLab Corporation, 2021). Specifically, a two-way analysis of variance (ANOVA) was conducted, followed by Tukey's post-hoc test to compare group means across the different treatments.

Results

In the absence of drought stress, the application of brassinolide led to a significant increase in plant height, exhibiting a remarkable percentage increase of approximately 27.2% compared to the control. Conversely, under no drought stress, plants in the control group exhibited a mean height of 11.13 cm. However, in the presence of drought stress, both the control and brassinolide-treated groups experienced reduced plant height. Specifically, the control group exhibited a decrease of approximately 45.3% in plant height when subjected to drought stress. Conversely, the brassinolide-treated group displayed a smaller reduction in plant height under drought stress, with a decrease of approximately 38.8% compared to its counterpart in the control group (Fig. 1A).

In our investigation of stem diameter in response to drought stress and brassinolide treatment, we observed significant variations in stem diameter when compared to the control group under no drought stress conditions. In the absence of drought stress, the application of brassinolide resulted in a noteworthy percentage increase of approximately 40.3% in stem diameter compared to the control group. Specifically, the control group exhibited a mean stem diameter of 2.36 mm, while the brassinolide-treated group displayed a larger diameter, measuring approximately 3.31 mm. Conversely, under conditions of drought stress, both the control and brassinolide-treated groups exhibited reduced stem diameter compared to their respective counterparts under no drought stress conditions. In the control group, the stem diameter experienced a substantial decrease of approximately 48.9% when subjected to drought stress, with a mean stem diameter of 1.21 mm. On the other hand, the brassinolide-treated group demonstrated a less severe reduction in stem diameter under drought stress, experiencing a decrease of approximately 43.5% compared to its counterpart in the control group, resulting in a mean stem diameter of 1.88 mm (Fig. 2A).

In the absence of drought stress, the application of brassinolide resulted in a noteworthy percentage increase of approximately 28.20% in leaf area compared to the control group. Specifically, the control group had a mean leaf area of 56.07 cm², whereas the brassinolide-treated group exhibited a larger leaf area, measuring approximately 71.89 cm². Conversely, when subjected to drought stress, both the control and brassinolide-treated groups experienced a reduction in leaf area compared to their respective counterparts under no drought stress conditions. The control group displayed a substantial decrease of approximately 65.50% in leaf area when exposed to drought stress, resulting in a mean leaf area of 19.28 cm². In contrast, the brassinolide-treated group showed a less severe reduction in leaf area under drought stress, with a decrease of approximately 49.20% compared to its counterpart in the control group, resulting in a mean leaf area of 36.60 cm² (Fig. 3A).

The use of brassinolide resulted in a substantial percentage increase in biomass compared to the control group of about 44.51% when drought stress was not present. For example, the control group showed a mean biomass of 3.07 g, but the brassinolide-treated group showed a greater biomass, measuring about 4.43 g. In contrast, biomass decreased in the control and brassinolide-treated groups under drought stress compared to their respective counterparts under no drought stress circumstances. When the control group was under drought stress, their biomass significantly decreased by about 64.60%, with a mean biomass of 1.51 g. With a fall in biomass under drought stress of almost 46.70 percent compared to its counterpart in the control group, the brassinolide-treated group, however, showed a less severe response, with a mean biomass of 2.38 g.

Under no drought stress conditions, the control group exhibited a mean POD activity of 14.77 U/g protein, while the brassinolide-treated group showed a lower POD activity, measuring approximately 6.45 U/g protein. This represented a substantial decrease of approximately 56.33% in POD activity when brassinolide was applied compared to the control group. Conversely, when subjected to drought stress, both the control and brassinolide-treated groups exhibited increased POD activity compared to their respective counterparts under no drought stress conditions. The control group displayed a significant increase of approximately 77.42% in POD activity under drought stress, with a mean activity of 26.14 U/g protein. On the other hand, the brassinolide-treated group also experienced an increase in POD activity, albeit to a lesser extent, with a mean activity of 20.77 U/g protein. This represented an increase of approximately 220.64% compared to the brassinolide-treated group under no drought stress conditions (Fig. 2A).

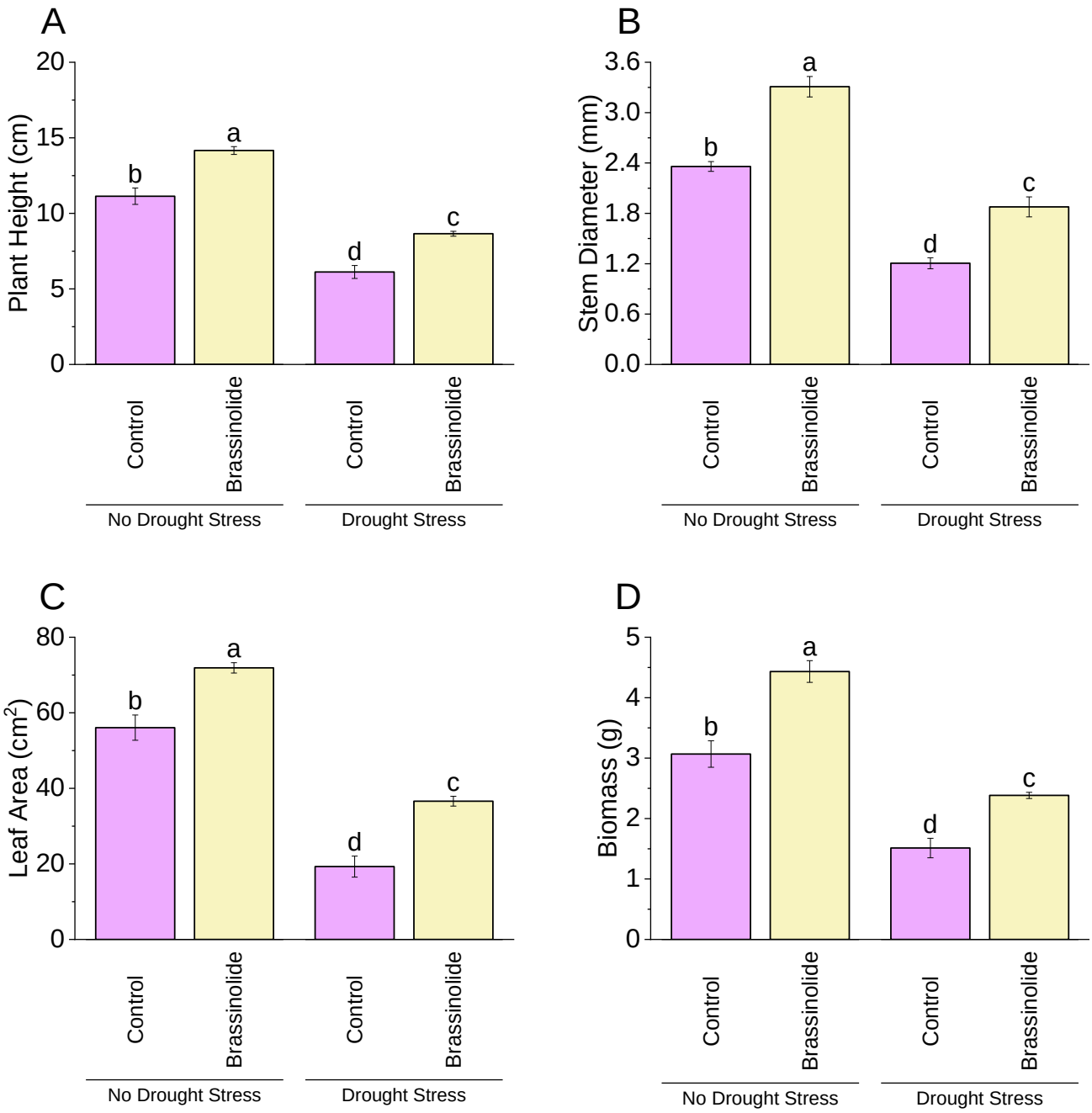

Fig. 1. Effect of brassinolide on plant height (A), stem diameter (B), leaf area (C), and biomass (D) of *O. corniculata* under no drought stress and drought stress. Bars are means of 4 replicates ± SE. Different letters on bars showed significant differences at $p \leq 0.05$ compared to the Tukey test.

In the case of no drought stress conditions, the control group exhibited a mean SOD activity of 21.05 U/g protein, while the brassinolide-treated group showed a lower SOD activity, measuring approximately 14.35 U/g protein. This represented a substantial decrease of approximately 31.95% in SOD activity when brassinolide was applied compared to the control group. The control group displayed a significant increase of approximately 48.32% in SOD activity under drought stress, with a mean activity of 31.21 U/g protein. On the other hand, the brassinolide-treated group also experienced an increase in SOD activity, with a mean activity of 25.88 U/g protein. This represented an increase of approximately 80.32% compared to the brassinolide-treated group under no drought stress conditions (Fig. 2B).

For no drought stress conditions, the control group exhibited a mean CAT activity of 5.86 U/g protein, while the brassinolide-treated group showed a slightly lower CAT activity, measuring approximately 4.83 U/g protein. This represented a decrease of approximately 17.68% in CAT activity when brassinolide was applied compared to the control group. The control group displayed a significant increase of approximately 50.58% in CAT activity under drought stress, with a mean activity of 8.83 U/g protein. On the other hand, the brassinolide-treated group also experienced an increase in CAT activity, with a mean activity of 7.83 U/g protein. This represented an increase of approximately 62.12% compared to the brassinolide-treated group under no drought stress conditions (Fig. 2C).

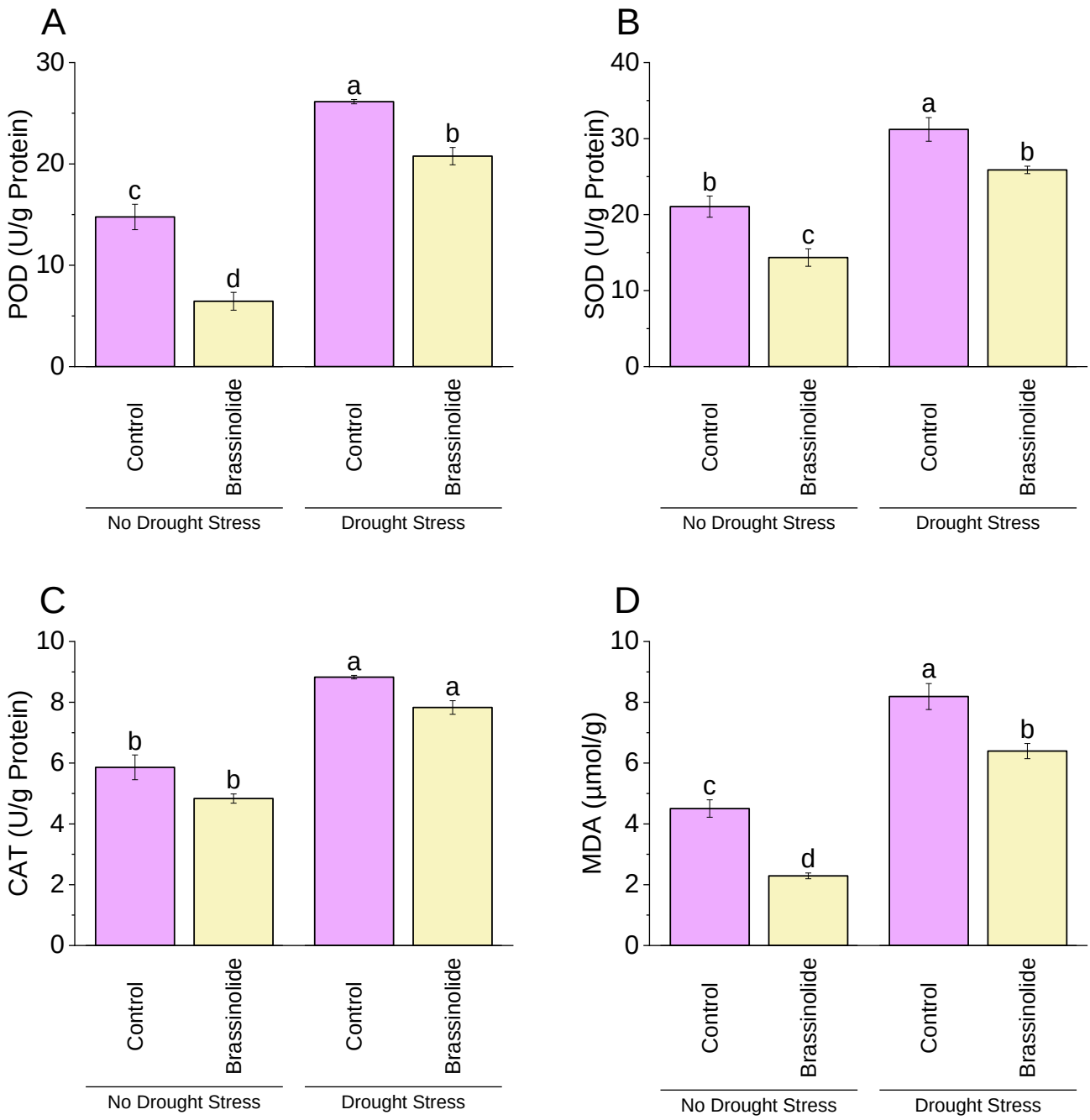

Fig. 2. Effect of brassinolide on POD (A), SOD (B), CAT (C), and MDA (D) of *O. corniculata* under no drought stress and drought stress. Bars are means of 4 replicates ± SE. Different letters on bars showed significant differences at $p \leq 0.05$ compared to the Tukey test.

The control group exhibited a mean MDA concentration of 4.50 µmol/g, while the brassinolide-treated group showed a lower MDA concentration, measuring approximately 2.29 µmol/g at no drought stress. This indicated a substantial decrease of approximately 49.07% in MDA concentration when brassinolide was applied compared to the control group.

Under drought stress, the control group showed a considerable rise in MDA concentration of around 81.97%, with a mean concentration of 8.19 mol/g. However, the MDA concentration increased in the brassinolide-treated group as well, with a mean concentration of 6.40 mol/g. This was an increase of about 179.68% in comparison to the group that received brassinolide treatment when there was no drought stress (Fig. 4D).

Under no drought stress conditions, the control group exhibited a mean Chlorophyll a content of 0.866 mg/g FW, while the brassinolide-treated group showed a slightly higher Chlorophyll a content, measuring approximately 0.931 mg/g FW. This represented an increase of approximately 7.51% in Chlorophyll a content when brassinolide was applied compared to the control group. The control group displayed a decrease of approximately 24.13% in Chlorophyll a content under drought stress, with a mean content of 0.657 mg/g FW. On the other hand, the brassinolide-treated group also experienced a decrease in Chlorophyll a content, with a mean content of 0.729 mg/g FW. This represented a decrease of approximately 21.69% compared to the brassinolide-treated group under no drought stress conditions (Fig. 3A).

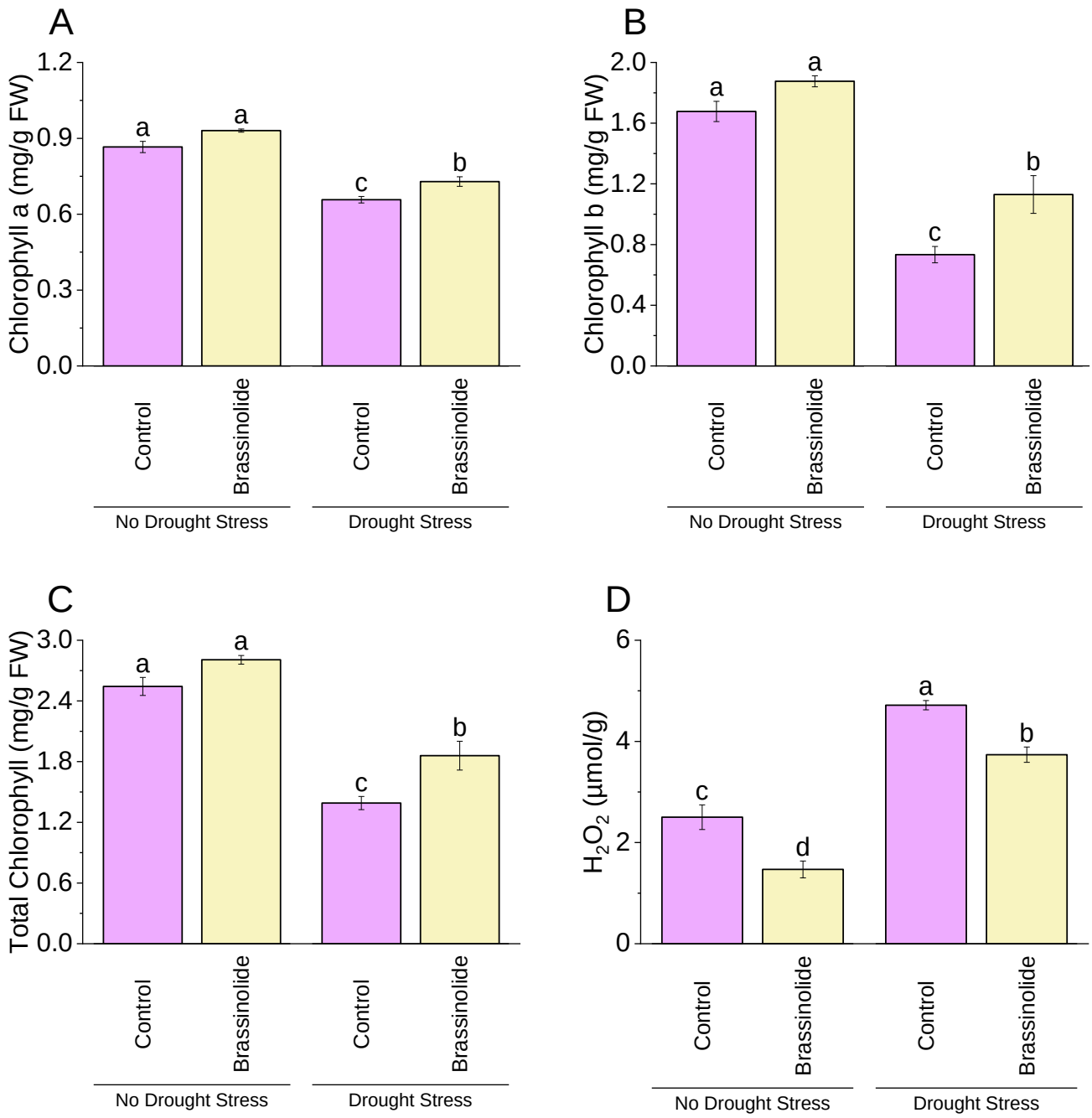

Fig. 3. Effect of brassinolide on chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), and H_2O_2 (D) of *O. corniculata* under no drought stress and drought stress. Bars are means of 4 replicates ± SE. Different letters on bars showed significant differences at $p \leq 0.05$ compared to the Tukey test.

In the absence of drought stress, the control group demonstrated an average Chlorophyll b content of 1.678 mg/g FW, whereas the brassinolide-treated group exhibited a slightly higher Chlorophyll b content, measuring approximately 1.876 mg/g FW. This corresponded to an approximately 11.77% elevation in Chlorophyll b content when brassinolide was administered in comparison to the control group. Specifically, the control group experienced a substantial decrease of about 56.23% in Chlorophyll b content under drought stress conditions, resulting in an average content of 0.734 mg/g FW. Conversely, the brassinolide-treated group also encountered a decline in Chlorophyll b content, recording an average content of 1.130 mg/g FW (Fig. 3B).

The control group exhibited a mean total chlorophyll content of 2.543 mg/g FW, while the brassinolide-treated group showed a slightly higher total chlorophyll content, measuring approximately 2.807 mg/g FW at no drought stress. This represented an increase of approximately 10.37% in total chlorophyll content when brassinolide was applied compared to the control group. The control group displayed a significant decrease of approximately 45.31% in total chlorophyll content under drought stress, with a mean content of 1.391 mg/g FW. On the other hand, the brassinolide-treated group also experienced a decrease in total chlorophyll content, with a mean content of 1.859 mg/g FW. This represented a decrease of approximately 33.46% compared to the brassinolide-treated group under no drought conditions (Fig. 3C).

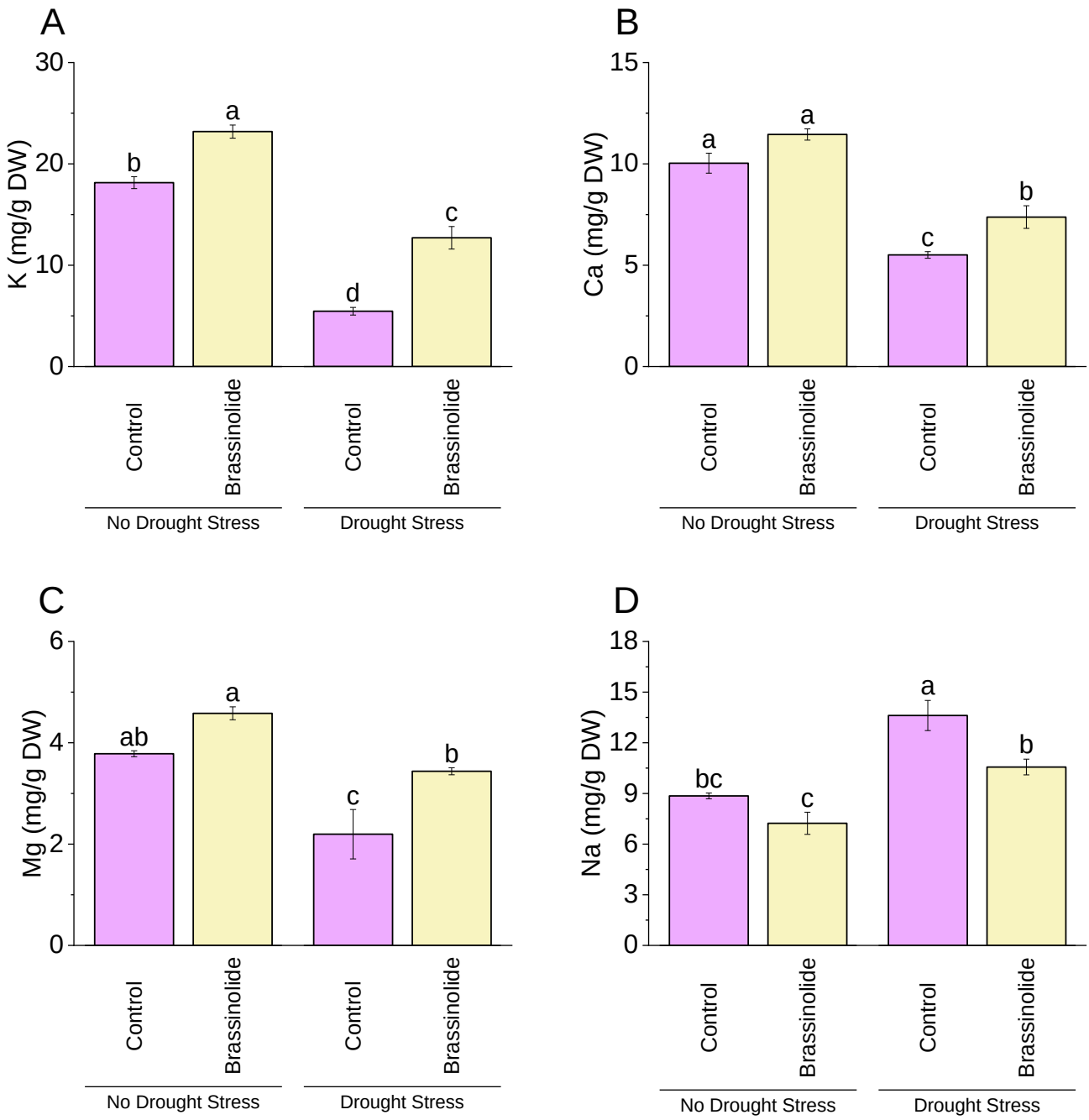

Fig. 4. Effect of brassinolide on K (A), Ca (B), Mg (C), and Na (D) of *O. corniculata* under no drought stress and drought stress. Bars are means of 4 replicates ± SE. Different letters on bars showed significant differences at $p \leq 0.05$ compared to the Tukey test.

In the case of no drought stress conditions, the control group exhibited a mean H_2O_2 concentration of 2.50 µmol/g, while the brassinolide-treated group showed a slightly lower H_2O_2 concentration, measuring approximately 1.47 µmol/g. This indicated a decrease of approximately 41.29% in H_2O_2 concentration when brassinolide was applied compared to the control group. The control group displayed a significant increase of approximately 88.90% in H_2O_2 concentration under drought stress, with a mean concentration of 4.72 µmol/g. On the other hand, the brassinolide-treated group also experienced an increase in H_2O_2 concentration, with a mean concentration of 3.74 µmol/g. This represented an increase of approximately 154.27% compared to the brassinolide-treated group under no drought stress conditions (Fig. 3D).

Under non-drought stress, the control group demonstrated a mean K concentration of 18.15 mg/g dry weight (DW), while the brassinolide-treated group exhibited a higher K concentration, measuring approximately 23.19 mg/g DW. This constituted an increase of approximately 27.83% in K concentration when brassinolide was administered compared to the control group. Specifically, the control group experienced a significant reduction of approximately 69.97% in K concentration under drought stress, resulting in a mean concentration of 5.46 mg/g DW. On the other hand, the brassinolide-treated group also encountered a decrease in K concentration, recording a mean concentration of 12.71 mg/g DW. This represented a decrease of approximately 45.21% compared to the brassinolide-treated group under non-drought stress conditions (Fig. 4A).

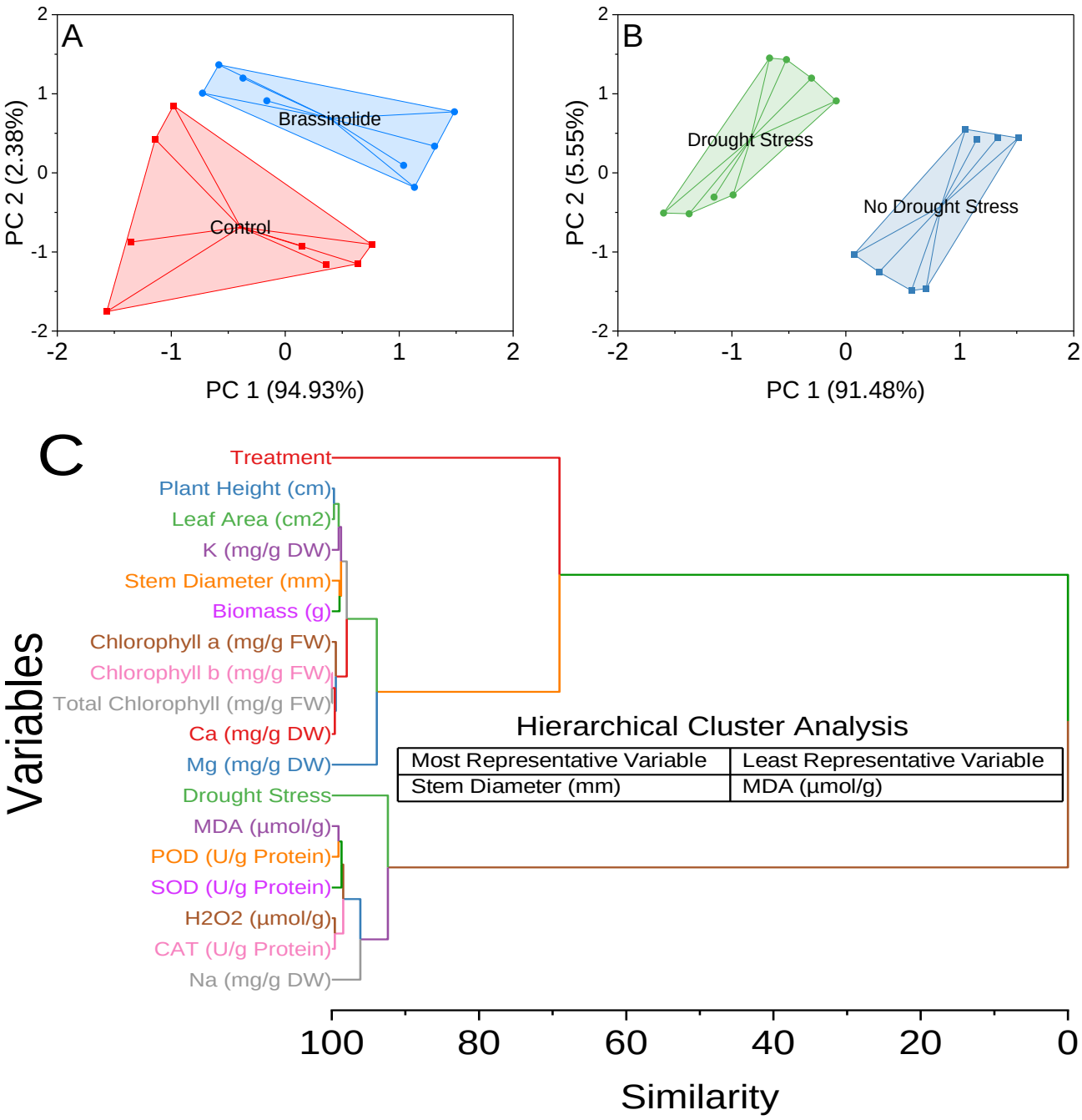

Fig. 5. Convex hull cluster plot for treatment (A), drought (B), and hierarchical cluster plot for studied attributes.

In conditions where drought stress was absent, the control group displayed an average Ca concentration of 10.04 mg/g dry weight (DW), while the brassinolide-treated group manifested a slightly higher Ca concentration, measuring at approximately 11.45 mg/g DW. This observation indicated a rise of approximately 13.97% in Ca concentration with the application of brassinolide when compared to the control group. The control group demonstrated a substantial decline of roughly 45.20% in Ca concentration under drought stress, yielding an average concentration of 5.51 mg/g DW. Conversely, the brassinolide-treated group also displayed a reduction in Ca concentration, with an average concentration of 7.37 mg/g DW. This equated to a decrease of approximately 35.79% in comparison to the brassinolide-treated group under non-drought stress conditions (Fig. 4B).

Under conditions devoid of drought-induced stress, the control group exhibited an average magnesium (Mg) concentration of 3.78 mg/g dry weight (DW), whereas the brassinolide-treated group presented a slightly elevated Mg concentration, measuring approximately 4.58 mg/g DW. This delineated an increment of roughly 21.05% in Mg concentration attributable to the application of brassinolide in comparison to the control group. The control group registered a substantial reduction of approximately 42.00% in Mg concentration under drought stress conditions, resulting in an average concentration of 2.20 mg/g DW. Conversely, the brassinolide-treated group also witnessed a decline in Mg concentration, with an average concentration of 3.44 mg/g DW. This signified a reduction of approximately 24.86% when compared to the brassinolide-treated group under non-drought stress conditions (Fig. 4C).

Under non-drought stress conditions, the control group displayed an average Na concentration of 8.85 mg/g dry weight (DW), while the brassinolide-treated group exhibited a slightly lower Na concentration, measuring approximately 7.24 mg/g DW. This indicated a decrease of approximately 18.16% in Na concentration when brassinolide was applied compared to the control group. The control group exhibited a significant increase of approximately 54.02% in Na concentration under drought stress, resulting in an average concentration of 13.62 mg/g DW. On the other hand, the brassinolide-treated group also experienced an increase in Na concentration, with an average concentration of 10.56 mg/g DW. This represented an increase of approximately 46.12% compared to the brassinolide-treated group under non-drought stress conditions (Fig. 4D).

A convex hull cluster plot was constructed to visualize the distribution of data points in a two-dimensional space defined by principal components PC 1 and PC 2. The percentages of variance explained by PC 1 (94.93%) and PC 2 (2.38%) were indicative of the significance of these components in capturing the data's variability. The plot displays individual data points as scores, with each point corresponding to a specific treatment group. The data points were labelled according to their treatment groups, with control and brassinolide representing the two treatment conditions. The convex hulls, which enclose data points of the same treatment group, highlight the clustering patterns within the dataset. Notably, the plot illustrates that data points within the control and brassinolide treatment groups tend to form distinct clusters, indicating separability between the two treatments based on the principal components PC 1 and PC 2 (Fig. 5A). Each data point corresponds to a specific condition, either no drought stress or drought stress. The convex hulls were used to enclose data points belonging to the same condition, illustrating the clustering patterns within the dataset. For the no drought stress condition, the data points form a distinct cluster within the convex hull, indicating the close grouping of these points in the principal component space. Similarly, data points representing the drought stress condition were enclosed within their convex hull, suggesting a separate clustering pattern (Fig. 5B). The hierarchical cluster plot presents a comprehensive view of the relationships and similarities among variables within the dataset. It highlights distinct clusters and associations between various parameters. Notably, Chlorophyll b (mg/g FW) and Total Chlorophyll (mg/g FW) emerge as closely related variables, sharing a remarkably low similarity score of 0.02465, suggesting their strong association. Similarly, Plant Height (cm) and Leaf Area (cm2), both growth-related parameters, cluster together with a similarity score of 0.29868. Ca (mg/g DW) exhibits moderate similarity with several variables, particularly Total Chlorophyll, emphasizing its role in plant health. Oxidative stress indicators H2O2 (µmol/g) and CAT (U/g Protein) form a tightly connected cluster, reflecting their shared attributes. Chlorophyll a (mg/g FW) associates moderately with Leaf Area, shedding light on their relatedness. In contrast, MDA (µmol/g) and POD (U/g Protein) show a strikingly high similarity score of 0.91912, placing them in a distinct cluster due to their close interdependence. Growth-related factors, such as K (mg/g DW), Stem Diameter (mm), and Biomass (g), share a profound degree of similarity, highlighting their interconnectedness. SOD (U/g Protein) forms its cluster, showcasing its significance in the dataset. Na (mg/g DW) exhibits a moderate association, primarily with Drought Stress, indicating a potential link. Mg (mg/g DW) shows a strong connection with Drought Stress and Treatment, underscoring its role in these contexts. The Treatment variable stands out as significantly different from all others, notably associated with Drought Stress and Mg (mg/g DW). Drought Stress forms a distinct cluster, with notable connections to Mg (mg/g DW) and Treatment (Fig. 5C).

Discussion

The application of brassinolide resulted in significant improvements across various growth parameters, including increased biomass, stem diameter, plant height, leaf area, water content, and chlorophyll content. These enhancements were accompanied by a reduction in oxidative stress indicators such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), as well as an increase in the activities of antioxidant enzymes (Anjum *et al.*, 2011; Zeng *et al.*, 2019; WU *et al.*, 2022). These combined results provide compelling evidence that brassinolide plays a pivotal role in enhancing the drought tolerance of *O. corniculata* by promoting its growth and ameliorating oxidative stress (Wang *et al.*, 2018).

Morphology and nutrient concentration are integral aspects of plant physiology that significantly impact a plant's growth, development, and overall well-being. Plant morphology encompasses the physical characteristics, such as size, shape, and arrangement of organs like leaves, stems, roots, and flowers (Anjum *et al.*, 2011; Wang *et al.*, 2018; Zeng *et al.*, 2019). These traits are closely linked to a plant's function and adaptation to its environment. For instance, leaf morphology influences a plant's ability to capture sunlight for photosynthesis, while root morphology determines its access to water and nutrients in the soil. A deep-rooted plant is better suited for surviving in drought-prone regions (Wang *et al.*, 2018; Maghsoudi *et al.*, 2019).

On the other hand, nutrient concentration refers to the levels of essential elements and compounds present in a plant's tissues, measured as the concentration of nutrients per unit of plant tissue. This parameter is vital for assessing a plant's nutritional status and health (Maghsoudi *et al.*, 2019). Adequate nutrient uptake and balanced nutrient concentrations are essential for various plant functions, including growth, reproduction, and defense against diseases and pests. For example, nitrogen concentration is crucial for photosynthesis and overall growth, while phosphorus is vital for root development and flowering, and potassium plays a role in enzyme activation and drought tolerance (Maghsoudi *et al.*, 2019).

The connection between morphology and nutrient concentration lies in how a plant's physical characteristics can influence its capacity to absorb and utilize nutrients efficiently. A well-developed root system, for instance, can enable a plant to access nutrients in deeper soil layers, enhancing its overall nutrient acquisition (Zeng *et al.*, 2019; WU *et al.*, 2022). Moreover, leaf morphology can

impact nutrient absorption through stomata and the efficiency of photosynthesis, which is closely tied to nutrient assimilation. The underlying mechanisms behind the effects of brassinolide on stress tolerance and plant growth have been extensively studied (Zhang *et al.*, 2007; Khan *et al.*, 2021). brassinolide exerts its influence by stimulating cell elongation, division, and differentiation, while also modulating gene expression associated with growth regulation. These findings align with previous research, where brassinolide treatments have demonstrated similar growth-promoting effects and stress tolerance improvements in various plant species facing different stress conditions. For instance, studies have reported that brassinolide treatment enhanced the growth and salt stress tolerance of maize seedlings (Choudhary *et al.*, 2019) and alleviated cadmium stress-induced growth inhibition and oxidative stress in tomato plants (Khan *et al.*, 2021).

Conclusions

In conclusion, this study provides compelling evidence of the capacity of brassinolide treatment to enhance the drought stress tolerance of O. Corniculata. The application of brassinolide not only significantly improved various growth parameters but also reduced oxidative stress markers, highlighting its potential as a valuable tool for crop management under drought-stress conditions. These findings underscore the dual functionality of brassinolide as both a plant growth regulator and a stress alleviator, holding promise for agriculture in the face of escalating drought challenges. Looking ahead, future research should prioritize the evaluation of brassinolide treatment in real-world field conditions to assess its practical efficacy and safety. Additionally, exploring the effects of brassinolide on a wider range of crop species is essential to fully grasp its applicability and potential benefits across diverse agricultural contexts. By continuing to investigate the role of brassinolide in plant stress responses and growth promotion, we can harness its potential to bolster crop resilience and yield in an era characterized by changing environmental conditions and increasing water scarcity.

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