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Research Article

Effect of seed priming by 24-epibrassinolide in alleviation of cadmium and water stresses in basil (*Ocimum basilicum* L.)

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Abstract

Basil plants are highly sensitive to environmental stresses, which can negatively impact their growth and productivity. In order to explore whether 24-epiBL seed priming could alleviate the adverse effects of cadmium (Cd) and water stress on basil, an experiment was conducted in the greenhouse with a completely randomized design in three replications. The basil seeds were primed with two concentrations of 24-epiBL (0 and 10^{-2}µM). Plants were treated with three levels of watering regimes consisting of 100% field capacity (FC) as control (no stress), 50% of FC (moderate stress), and 25% of FC (severe stress). The Cd stress was applied at concentrations of 0, 10, 20, and 30 mM every two days. Results indicate that upon severe water and 30 mM of Cd stresses, chlorophyll a, b, and carotenoid declined by 33, 26, and 20% compared to the control condition. The hydrogen peroxide (H_2O_2), malondialdehyde (MDA), Cd, proline, and soluble sugar contents were raised at severe water and Cd stresses by 130%, 42%, 580%, 41%, and 33% in comparison to the non-stressed control plants. However, the pre-treated 24-epiBL plants, subjected to combined severe water and Cd stresses, displayed an increment in chlorophyll a (50%), b (26%), carotenoid (53%), H_2O_2 (160%), and proline (82%) contents with a decrement in MDA (16%), Cd (346%), and soluble sugar (25%) contents compared to non-primed basil plants under severe water and Cd stresses. These findings suggest that 24-epiBL seed priming can be a promising strategy for enhancing basil's resilience against environmental stresses, which ultimately contributes to improved crop productivity and sustainability.

Keywords: Field capacity, Heavy metal, Hormone presoaking, Water stress, Toxicity

Introduction

In recent years, environmental stresses such as heavy metal contamination and water scarcity have emerged as significant challenges affecting plant growth and productivity (Moreno-Marin *et al*., 2018). Cadmium (Cd), a heavy metal pollutant, is known to accumulate in the soil through industrial and agricultural practices, as well as atmospheric deposition. When present in excessive amounts, Cd can disrupt plant growth and development, impair nutrient uptake, and induce oxidative stress (Huang and Deng, 2020). Additionally, water stress, caused by inadequate water availability, further exacerbates plant vulnerability and reduces crop productivity. Both Cd toxicity and water stress are major concerns in worldwide agricultural systems, necessitating effective mitigation strategies (Khalid *et al*., 2023).

To mitigate the harmful impacts of these stressors, researchers have explored various strategies, including the application of plant growth regulators and priming techniques. Among these, brassinosteroids (BRs) are a group of plant hormones that play essential roles in various physiological processes, including seed germination, cell elongation, stress responses, and photosynthesis. 24-epiBL, a naturally occurring brassinosteroid hormone, has demonstrated promising effects in enhancing plant tolerance to abiotic stresses (Heidari *et al*., 2021). 24-epiBL application has been reported to improve seed germination, elevate root and shoot growth, regulate stomatal behavior, osmotic adjustment, and enhance antioxidant defense mechanisms in plants (Amarasinghe *et al*., 2022; Mu *et al*., 2022; Manghwar *et al*., 2022).

Seed priming, a pre-sowing treatment, involves the partial hydration of seeds to initiate metabolic processes without triggering germination. It improves seed vigor, accelerates germination, and improves seedling establishment and performance under adverse conditions. Priming seeds with 24-epiBL has been shown to be an effective approach to improve stress tolerance, as it activates various physiological and biochemical pathways that contribute to plant resilience (Galviz *et al*., 2021).

Among the various plant species, basil (*Ocimum*

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basilicum L.) holds remarkable importance due to its diverse applications in the culinary, medicinal, and aromatic industries. However, the adverse effects of Cd toxicity and water stress on basil plants have raised concerns regarding their survival and quality (Sakr and Nooh, 2013). The impact of stress on such a valuable crop has direct economic implications, making it an ideal candidate for studies aimed at improving stress tolerance. Understanding the effects of 24-epiBL seed priming on basil plants under Cd and water stress conditions will not only contribute to expanding our knowledge of plant stress physiology but also provide valuable information for developing sustainable agricultural practices.

The study aims to provide valuable insights into the effects of 24-epiBL seed priming on the physiological and biochemical characteristics of basil plants under water shortage and Cd heavy metal toxicity. This study could pave the way for the effective management of Cd-contaminated and water-prone environments, ensuring the successful cultivation of basil and potentially other crop species in similar agroecological settings (Feng and Gins, 2022; Barboza da Silva and Marcos-Filho, 2020).

Materials and methods

Basil seeds were obtained from the Urmia Agricultural Jihad Organization (Urmia, Iran). The seeds were immersed in 10% sodium hypochlorite solution for 5 minutes. After washing with deionized water, the seeds were primed in solutions with two concentrations of brassinosteroid (0 and 10^{-2} μM) at a temperature of 21°C; then, after 14 hours, they were taken out of the priming solutions and dried with a fan and cultivated in pots (20 cm diameter) in a completely randomized design with three replications. The pots were filled with soil composed of perlite and sand (volume 1:1) and placed in greenhouse conditions based on previous studies, under a temperature of 20 to 30°C (Gharebaghi *et al*., 2017), relative humidity of 60 to 80%, photoperiod (light/darkness 16/8 h), and light intensity (420 μmol $m^{-2}s^{-1}$) (Mehrian *et al*., 2023). Three levels of watering regimes, including 100% of FC (no stress), 50% of FC (moderate stress), and 25% of FC (severe stress), were applied using the FC method. The Fc refers to an agronomic measure in irrigation management that allows determination of water content held in soil without excessive leaching. For the determination of FC, pots were filled with water until reaching the saturation point and free drainage from the bottom. Afterward, the pots were weighed to determine the weight of the soil at field capacity. This value minus the dry weight of the soil was considered as 100% FC (Van Lier, 2017). In order to apply Cd toxicity stress, pots corresponding to Cd application were irrigated with specific levels of Cd chloride ($CdCl_2.5H_2O$) consisting of 0, 10, 20, and 30 mM every two days. Plants were harvested after 90 days and kept in a -30°C freezer for further biochemical analyses.

Photosynthetic pigments: Chlorophyll and carotenoid measurements were performed according to the method of Arnon *et al*. (1949). First, 0.05 g of the fresh leaves was ground with 80% acetone and filtered. The final volume of acetone used was 2 ml. Finally, the optical absorption of the obtained extract was read at wavelengths of 470, 645, and 663 nm using a spectrophotometer. The content of chlorophyll a and b, as well as the amount of carotenoid, were calculated using the following formulas in terms of mg per gram of fresh weight.

1. Chlorophyll a = [12.7 (D 662) – 2.6 (D 645)]
2. Chlorophyll b = [22.9 (D645) – 4.68 (D 662)]
3. Carotenoid = [1000(D470)-1.82 (chlorophyll a)-85.02 (chlorophyll b)]/198

(D: optical absorption content, V: volume of extract, W: sample weight)

H_2O_2 content: In order to measure the content of H_2O_2, 1 g of the fresh sample was extracted with 4 ml of absolute acetone and centrifuged at 6000 g for 8 min. Then, 1 mL of supernatant, 100 μL of 5% titanium sulfate, and 200 μL of ammonia solution were mixed. The resulting precipitate was dissolved in 5 ml of 2 M sulfuric acid and centrifuged again at 3500 g for 8 min. The absorbance of the obtained extract was read at 415 nm, and the hydrogen peroxide content was calculated in micromoles per gram of fresh weight (Xu *et al*., 2022).

Lipid peroxidation: Investigating the level of lipid peroxidation by measuring the content of MDA using thiobarbituric acid (TBA) was done according to the method of Meir *et al*. (1992) with some modifications (Meir *et al*., 1992). In order to prepare the required extract, 0.1 g of the fresh leaf tissue was ground well in 2 ml of 50 mM phosphate buffer solution with pH = 7 (this step should be done in the ice bath), and then the obtained suspension was centrifuged at 12000 rpm for 40 minutes. Then, 1 ml of a solution containing 0.5% thiobarbituric acid and 20% trichloroacetic acid was added to 1 ml of the supernatant extract, and the resulting solution was kept in a hot water bath at 95°C for half an hour. Immediately after cooling in ice for 5 minutes, centrifugation was performed at 10000 rpm for 10 minutes, and absorption of the colored solution obtained was read at 532 nm. The extinction coefficient of MDA was 1.56×10^5 $M^{-1}cm^{-1}$.

Cd concentration: The atomic absorption was used to measure the amount of Cd in plant samples. Extraction from dry plant powder was done by acid digestion using 65% nitric acid and hydrogen peroxide at 90°C (Hatamnia *et al*., 2014). The Cd concentration of the extracts was measured by an atomic absorption device (GBC Avanta, Australia) and calculated in comparison with standard Cd solutions.

Proline content: The method of Bates *et al*. (1973) was used to measure proline content. The amount of 0.05 grams of fresh leaf tissue and 2 ml of 3% sulfosalicylic acid were ground in a mortar until a completely homogeneous solution was formed. The

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obtained extract was filtered; this extract was mixed with ninhydrin and glacial acetic acid solution in the ratio of 1:1:1 and kept in a hot water bath (100°C) for one hour. After boiling for one hour at the mentioned temperature, the samples were placed in an ice bath. Then, 1 ml of toluene was added to each of the samples and vortexed by hand until they were mixed together. After placing the samples at room temperature for half an hour, the absorbance of the supernatant solution was read at a wavelength of 520 nm using a spectrophotometer. Proline content is calculated in micromoles per gram of fresh weight (Bates *et al*., 1973).

Soluble sugar content: To measure soluble sugars (Pquine and Lechasseur, 1979), 0.5 g of fresh leaf tissue was ground with 5 ml of 96% ethanol, homogenized with 10 ml of 70% ethanol, and centrifuged. Then, 0.1 ml of alcoholic extract was mixed with 3 ml of anthrone and placed in a bain-marie at 100°C for 10 minutes. The absorbance was read with a spectrophotometer at a wavelength of 625 nm and was obtained by following the equation:

4. Soluble sugars (μg/g FW) = [leaf (0.5 g)/extract (15 ml)] × [OD/1000]

(OD: optical density, FW: fresh weight)

Statistical analysis: The experiment was conducted as a factorial based on a completely randomized design. Data analysis was performed using a square statistical model with the general linear model procedure in SAS 9.2 software. Mean comparisons were conducted using Duncan's test at a significance level of $P \leq 0.05$. Graphs were plotted using the average data and standard error.

Results and discussion

Photosynthetic pigments : According to the obtained results, the maximum and minimum amounts of chlorophyll a content were obtained in the plants that received 100% FC + BR + Cd (20 mM) and 100% FC + BR + 30 mM of Cd (0.41 and 0.11 $mgg^{-1}FW$), respectively (Figure 1). 24-epiBL is effective in maintaining higher chlorophyll a content under moderate cadmium stress, but its protective effect is limited at higher cadmium concentrations. There is a clear threshold in cadmium concentration (between 20 mM and 30 mM) where the beneficial effects of 24-epiBL are significantly diminished, indicating severe stress that overwhelms the plant's defense mechanisms. While 24-epiBL can be utilized to improve plant resilience and photosynthetic efficiency in moderately contaminated environments, it is crucial to manage cadmium levels to prevent severe toxicity and ensure optimal plant health and productivity. Some studies have shown that brassinosteroids can influence chlorophyll content and photosynthetic efficiency in plants. For example, application of exogenous brassinosteroids has been found to increase chlorophyll content and improve photosynthetic performance in various plant species (Ma *et al*., 2018), but BR effectiveness is limited by the severity of cadmium stress.

Based on the acquired results, the plants that received 100% FC + BR + 30 mM of Cd yielded the highest chlorophyll b content (3.19 $mgg^{-1}FW$). Conversely, the lowest value of chlorophyll b (1.20 $mgg^{-1}FW$) was observed in the treatment of 50% FC-BR + 30 mM of Cd (Figure 2). 24-epiBL effectively maintains chlorophyll b content under high cadmium stress when no water stress is present, indicating its potential as a stress alleviator in cadmium-contaminated environments. This could be due to its role in enhancing antioxidant activity, stabilizing chloroplast structures, and promoting photosynthetic processes. The protective effect of 24-epiBL diminishes under combined water and cadmium stress, highlighting a limitation in its efficacy under multiple simultaneous stress conditions. For optimal use of 24-epiBL in agriculture, it is essential to manage water availability, particularly in cadmium-contaminated soils, to ensure the growth regulator's maximum effectiveness in maintaining chlorophyll content and overall plant health. Water stress can lead to a decrease in chlorophyll content. Water scarcity limits the availability of water molecules for photosynthesis and reduces the synthesis of chlorophyll, causing chlorophyll degradation (Sivakumar *et al*., 2017).

Cd stress can also lead to a decrease in chlorophyll content. Cd is a heavy metal that is toxic to plants and disrupts various physiological processes, including chlorophyll synthesis. Cd interferes with the enzymes involved in chlorophyll biosynthesis, leading to reduced chlorophyll production (Moustakas *et al*., 2019). The metal can also inhibit the activity of enzymes such as magnesium chelatase, which is essential for chlorophyll formation. Additionally, Cd-induced oxidative stress can accelerate chlorophyll degradation and breakdown, leading to a decrease in chlorophyll content (Paunov *et al*., 2018; Moustakas *et al*., 2019). Both water and Cd stress tend to lower chlorophyll content in plants. However, it's important to note that the severity of the stress, the duration of exposure, and the plant species can influence the magnitude of the impact on chlorophyll content. Monitoring chlorophyll content can serve as an indicator of plant stress and provide insights into the physiological state of plants under water and Cd stress conditions (Saadaoui *et al*., 2022).

It seems that the presence of 24-epiBL was able to partially cover the effect of increasing the Cd level in the absence of water stress. Upon Cd stress of 30 mM, it can be seen that the treatments with 24-epiBL showed higher chlorophyll a content than the absence of 24-epiBL at different levels of water stress, which is probably due to the success of 24-epiBL in reducing the stress effects. It has been shown that brassinolide boosts plant tolerance to water stress by regulating stomatal closure, improving water use efficiency, and enhancing antioxidant systems. While the direct effects of brassinolide treatment on chlorophyll content under water stress are not extensively studied, it can be

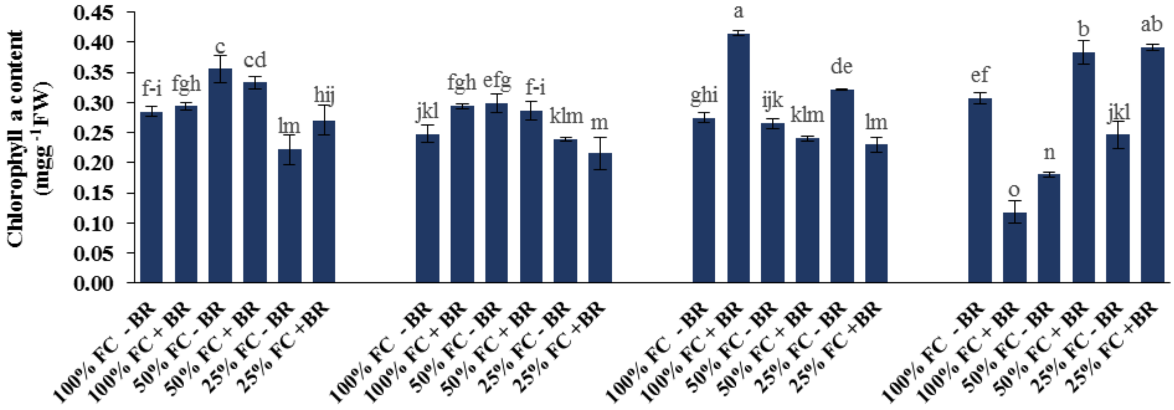

Figure 1. Effect of different concentrations of Cd and watering levels on chlorophyll a in the leaves of basil (*Ocimum basilicum* L.). The values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

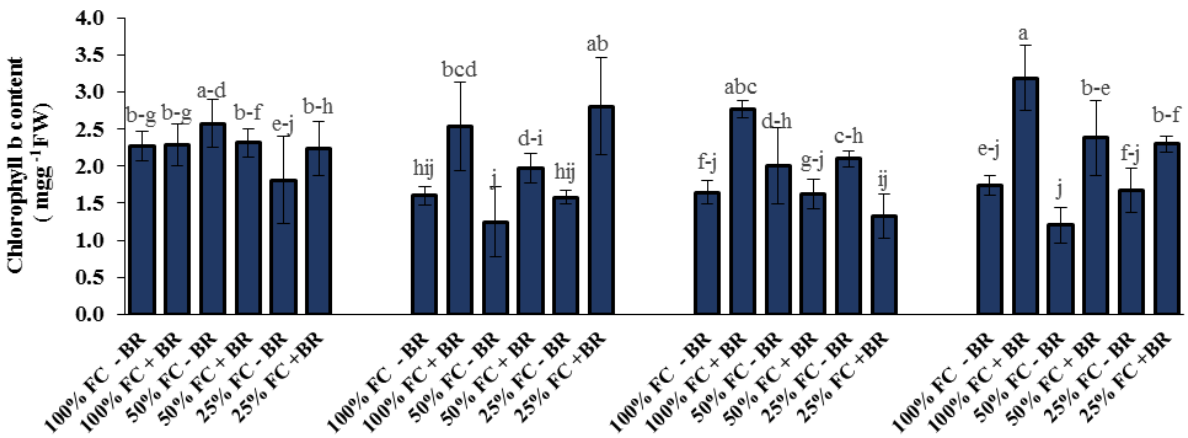

Figure 2. Effect of different concentrations of Cd and water stress levels on chlorophyll b in the leaves of basil (*Ocimum basilicum* L.). The values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

inferred that bassinolides may help maintain chlorophyll levels by improving overall plant health and reducing water-induced damage (Li *et al*., 2021).

Based on the obtained results, samples under treatments with 100% FC + BR + Cd (20 mM) yielded the highest carotenoid content (47.7 mg g^{-1} FW), which was recorded as a 1.37-fold rise in comparison to the control. Conversely, the lowest value of this parameter was observed in the treatment of 50% FC–BR + Cd (0 mM) (Figure 3). The study demonstrates that 24-epiBL pre-treatment, combined with adequate water supply, significantly enhances the content of chlorophyll a and carotenoids in basil under cadmium stress. This highlights the potential of BR as a valuable tool in agricultural practices aimed at improving plant resilience to environmental stresses. The pretreatment with BR could have primed the basil plants, making them more resilient to subsequent cadmium exposure. The presence of adequate water likely ensured that the metabolic processes required for BR-induced stress tolerance were not hindered. Adequate water availability supports the synthesis of chlorophyll and carotenoids by ensuring the efficient functioning of the photosynthetic machinery. BR may further enhance this by stabilizing these pigments and protecting the photosynthetic apparatus. 24-epiBL is highly effective in maintaining high carotenoid content under moderate cadmium stress and optimal hydration, highlighting its potential as a stress mitigator and suggesting its role in the alleviation of oxidative damage and possibly stimulating carotenoid biosynthesis pathways. Water stress significantly reduces carotenoid content, especially in the absence of 24-epiBL, indicating the need for effective stress management strategies. For plants exposed to cadmium contamination and potential water conditions, the application of 24-epiBL could be a beneficial strategy to improve stress tolerance and maintain high carotenoid levels, which are essential for

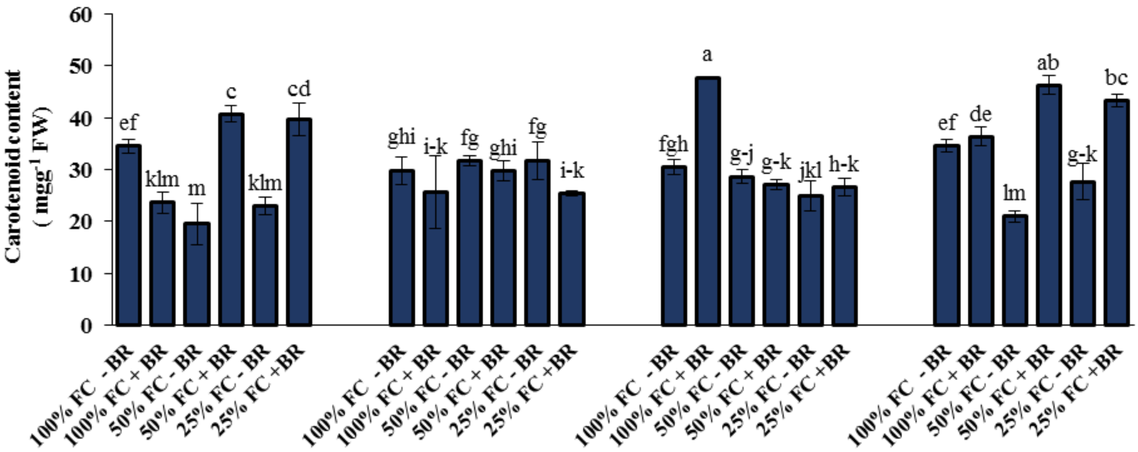

Figure 3. Effect of different concentrations of Cd and water stress levels on carotenoid in the leaves of basil (*Ocimum basilicum* L.). The values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

protecting photosynthetic efficiency and overall plant health. Both severe water stress and Cd exposure can induce oxidative stress in plants (Manghwar *et al*., 2022; Li *et al*., 2022). Carotenoids serve as antioxidants and play a crucial role in scavenging reactive oxygen species (ROS) generated during stress conditions. As a protective mechanism, plants may increase carotenoid synthesis to counteract the oxidative damage caused by water and Cd (El-Yazied *et al*., 2022). Moreover, carotenoids are essential pigments involved in photosynthesis. Under stress conditions such as severe water and Cd toxicity, the photosynthetic apparatus can be impaired. Carotenoids help to dissipate excess energy and protect the photosynthetic system from damage, thus maintaining photosynthetic efficiency (Faraloni, and Bonetti, 2021). Water stress and Cd exposure can disrupt the integrity of cell membranes. Carotenoids have been shown to stabilize and protect cell membranes from oxidative damage and lipid peroxidation. They preserve membrane structure and function, which is crucial for maintaining cellular integrity and overall plant health. Carotenoids also serve as signaling molecules in plants. They can regulate gene expression and signal stress-related pathways (Gori *et al*., 2021). Increased carotenoid levels may trigger specific biochemical and molecular responses that help plants to adapt and cope with water stress and Cd toxicity. These pigments can participate in the detoxification of heavy metals like Cd. They can form complexes with Cd ions, reducing their toxic effects on plant cells. This detoxification process may involve the synthesis of phytochelatins, which are small peptides derived from glutathione and can sequester heavy metals (Gori *et al*., 2021; Patane *et al*., 2021).

H_2O_2 content: The results indicated that the treatment with 25% FC + BR + Cd (30 mM) yielded the maximum concentration of H_2O_2 content (5.96 μmolg^{-1} FW), which was calculated as 6.20 times higher than the control and 2.55 times higher than 25% FC-BR+ Cd (30 mM) (2.33 μmolg^{-1} FW). In contrast, the lowest amount of this parameter (0.693 μmolg^{-1} FW) was detected in the level of 100% FC + BR + Cd (0 mM) (Figure 4). A combination of severe water stress and high Cd levels induces significant oxidative stress, leading to elevated ROS, including H_2O_2 levels. Low H_2O_2 content with 24-epiBL at Cd (0 mM) indicates that under optimal conditions (no water stress, no Cd), 24-epiBL helps maintain low levels of H_2O_2, suggesting effective regulation of oxidative stress and maintenance of redox homeostasis. The ability of 24-epiBL to keep H_2O_2 levels low under no stress conditions indicates its role in enhancing the plant's antioxidative defense mechanisms, which are crucial for protecting cellular structures from oxidative damage (Yu *et al*., 2020). Under severe stress conditions, 24-epiBL may provide some protective effects; it might also be involved in signaling pathways that increase H_2O_2 production. This could be a part of a complex stress response mechanism where H_2O_2 acts as a signaling molecule to trigger other defensive pathways. Cd can directly stimulate ROS production, while water stress can disrupt cellular processes and impair the balance between ROS generation and scavenging (Shahzad *et al.*, 2018). As a consequence, the accumulation of H_2O_2 occurs. A study indicates the occurrence of transcriptional and translational reprogramming in response to EBL, which acts as a signaling compound

In different metabolic and physiological processes to improve plant growth and development under salt stress (Tanveer *et al*., 2018). The antioxidant defense system in plants includes enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidases that scavenge ROS, including hydrogen peroxide. However, under severe water stress and high Cd concentration, the activity of these antioxidant enzymes may be compromised (Nadarajah, 2020; Adam *et al*., 2021). The reduced efficiency of the antioxidant defense system leads to the accumulation of H_2O_2 in plant tissues (Nazir *et al*., 2019). In a study by Mehrian *et al*. (2024), H_2O_2 content was reduced as a result of

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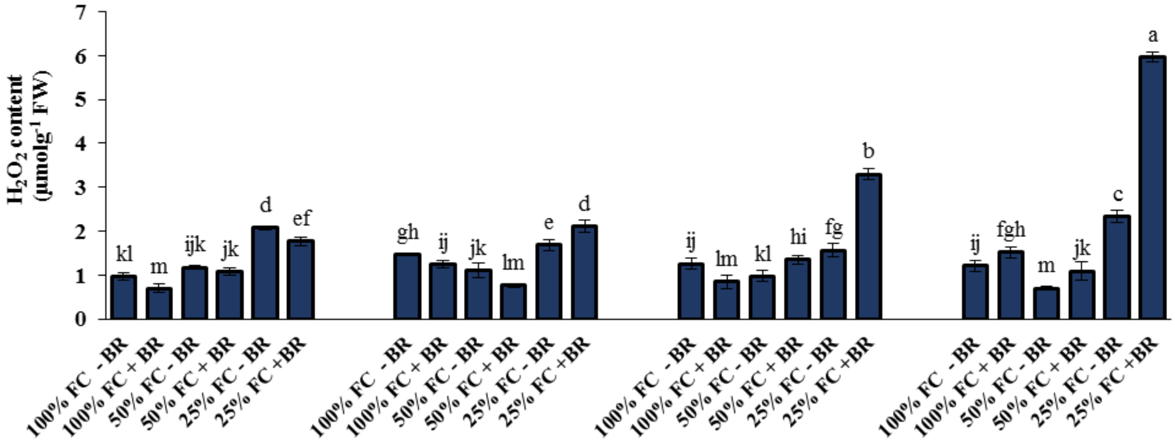

Figure 4. Effect of different concentrations of Cd and water stress levels on the H_2O_2 content of basil leaves. The values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

elevation in the activity of antioxidant enzymes. It seems in this study the combination of two stressors in their severely applied levels has resulted in elevated content of H_2O_2.

Lipid peroxidation: The analysis of MDA levels revealed that the highest content of this factor was found in the FC (25%) + BR + 0 mM of Cd (1.427 $\mu molg^{-1}$ FW), FC (25%) + BR + 10 mM of Cd (1.31 $\mu molg^{-1}$ FW), and FC (25%) - BR + 20 mM of Cd (1.29 $\mu molg^{-1}$ FW). These concentrations showed a 1.92, 1.77, and 1.74-fold increase compared to the control. The lowest concentration (0.645 $\mu molg^{-1}$ FW) was detected in samples treated with FC (100%) + BR + Cd (10 mM) (Figure 5). Severe water stress is a strong inducer of oxidative damage and lipid peroxidation, indicated by high MDA levels. Moderate cadmium levels in the absence of water stress, combined with 24-epiBL, lead to reduced oxidative damage.

Brassinolide has been reported to enrich the antioxidant defense system in plants. It can upregulate the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases, and glutathione reductase. By enhancing the activity of these enzymes, brassinolide helps to scavenge ROS and reduce oxidative stress. Lower oxidative stress levels can subsequently lead to a decrease in MDA formation. MDA is a product of lipid peroxidation induced by ROS, so reducing ROS levels can mitigate lipid peroxidation and lower MDA content (Manivannan *et al*., 2021). Cd and water stresses can disrupt the integrity of cell membranes, leading to increased membrane permeability. Brassinolide has been shown to augment the stability and integrity of membranes by reducing lipid peroxidation (Bolduc and Jumarie, 2004; Apostolova *et al*., 2006). In several studies, a decrease of MDA was observed due to the scavenging of ROS through the induction of antioxidant enzyme activity (Sharma *et al*., 2015; Li *et al*., 2022; Sharma *et al*., 2017). In this study, 24-epiBL plays a significant role in reducing oxidative stress and lipid peroxidation under no water and moderate Cd conditions, leading to lower MDA content. However, under severe water stress, its protective effects are limited, resulting in high MDA levels.

Brassinolide can also regulate the expression of stress-responsive genes involved in antioxidant defense and membrane stability. By activating the expression of these genes, brassinolide enhances the plant's ability to combat oxidative stress and maintain membrane integrity. This regulation of gene expression can contribute to the reduction of MDA accumulation under Cd and water stresses (Cai *et al*., 2022). Insights from this study can guide the development of crop management practices that incorporate 24-epiBL to improve plant health and yield, especially under moderate stress conditions.

Cd concentration: As the results depicted in Figure 6, data regarding the Cd levels showed that the 25% FC – BR + 20 mM of Cd samples, exhibited the highest concentration of this parameter (5.76 mg L^{-1}), which was 288 times greater than the control (0.02 mg L^{-1}). However, this amount decreased with the increase of Cd concentration up to 30 mM. Severe water stress likely leads to more uptake and accumulation of cadmium in plants. Water conditions can cause plants to have a more concentrated root zone solution due to reduced water availability, leading to higher absorption of available cadmium (Ma *et al*., 2024). The absence of 24-epiBL manifests that without this growth regulator, plants might be more susceptible to stress conditions, leading to higher Cd uptake. 24-epiBL might have a role in regulating or mitigating Cd uptake under stress conditions through enhancing plant stress tolerance by modulating stress response pathways. Under optimal growing conditions (no water, no Cd), the plants do not have external stressors forcing them to uptake and accumulate Cd. This result shows that in the absence of Cd in the environment and with sufficient water, plants naturally have low or negligible Cd content. Adequate water availability typically dilutes soil solutes, reducing

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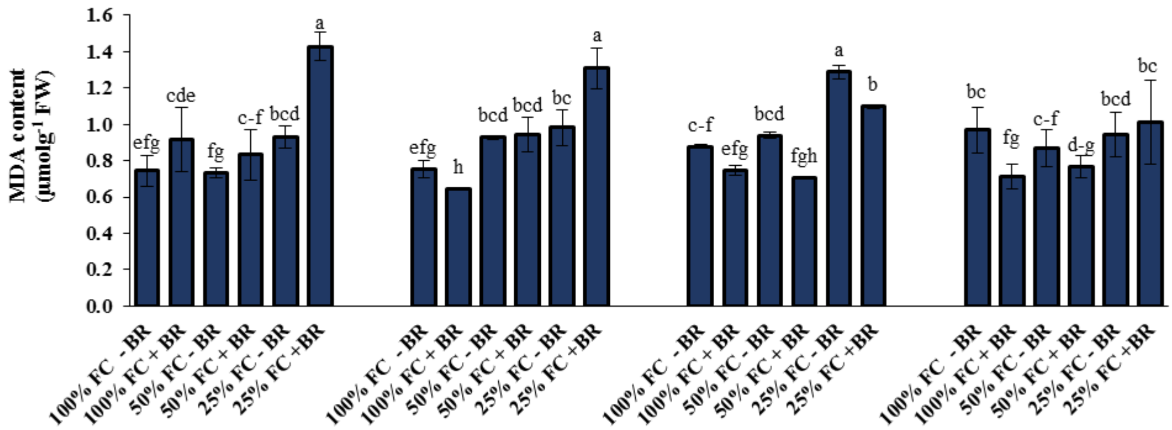

Figure 4. Effect of different concentrations of Cd and water stress levels on MDA content of basil leaves. The values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

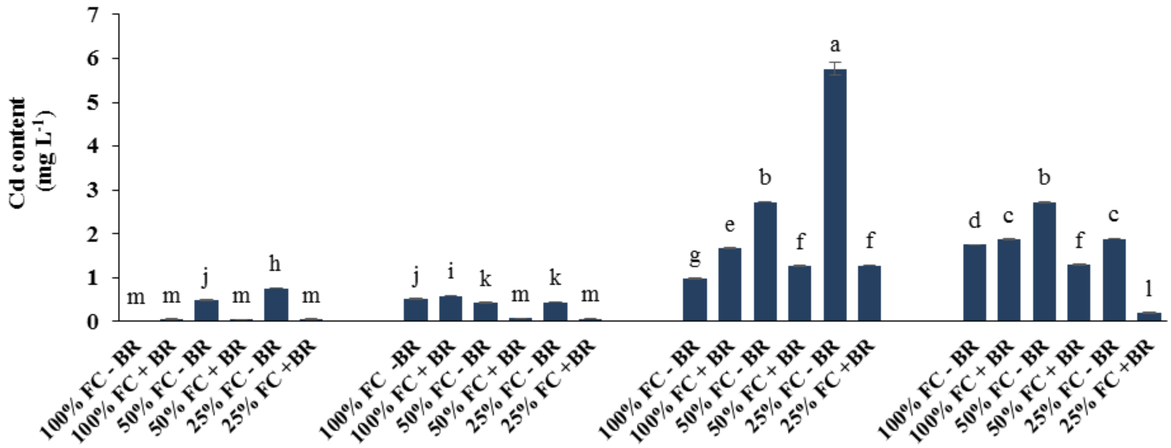

Figure 6. Effect of different concentrations of Cd and water stress levels on Cd concentration in basil leaves. Values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

the concentration of Cd in the soil solution and, consequently, its uptake by plants. It seems that under high Cd concentration (30 mM), the reduction of Cd accumulation in aerial organs can be related to the lower transfer of this metal to aerial organs and the high tendency of Cd ions to form a complex with elements on the cell wall (Zhang *et al*., 2013; Ghabriche *et al*., 2017). At the level of 20 mM of Cd, the samples pretreated with 24-epiBL showed a lower accumulation of Cd, which can be attributed to the increased fixation of Cd in the hemicellulose of the root cell wall, following the influence of 24-epiBL. Moreover, BRs reduce the expression of genes involved in Cd absorption and transport and upregulate Cd-sequestering genes (Sun *et al*., 2024). As Sun *et al.* (2024) demonstrated, 24-epiBL decreased Cd accumulation in rice by mediating the cell wall's fixation capacity to Cd, which might have relied on the buildup of the gibberellin (Sun *et al*., 2024). These findings highlight the importance of managing environmental stressors to control heavy metal uptake in plants. Strategies to mitigate water stress, such as irrigation management and the use of plant growth regulators like 24-epiBL, could be essential in reducing cadmium accumulation in crops. Incorporating 24-epiBL in agricultural practices might help in reducing cadmium uptake under stressful conditions, contributing to safer and healthier crop production.

Proline content: Primed plants with 25% FC + BR + Cd (30 mM) exhibited the maximum proline content (3.13 µmolg^{-1} FW) with a 2.54-fold rise compared to control and non-primed conditions (Figure 7). The combination of severe water (25% FC) and high Cd concentration (30 mM) imposes a significant stress on the plants, which likely triggers maximum proline accumulation as a defensive response. High levels of proline under severe water and high Cd stress indicate that proline is being heavily utilized by the plant as a protective measure against the combined stressors. This suggests that proline is a key component of the plant's defense strategy under such extreme conditions (Patanè *et al*., 2022; Dar *et al*., 2016; Ashraf and Foolad, 2007). Proline synthesis during water stress is primarily regulated by the enzyme pyrroline-5-carboxylate synthetase (P5CS). This enzyme catalyzes the conversion of glutamate to pyrroline-5-carboxylate

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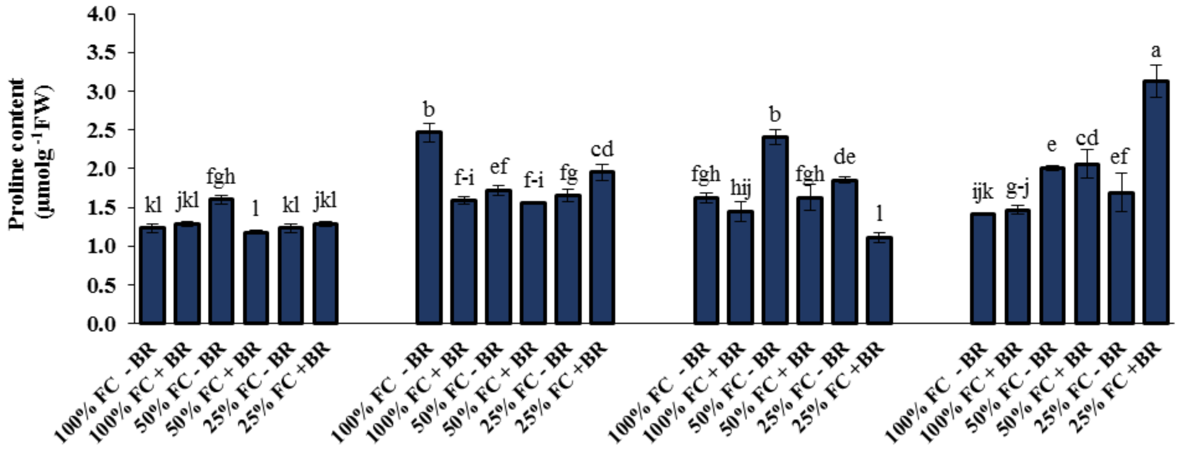

Figure 7. Effect of different concentrations of Cd and water stress levels on the proline content of basil leaves. Values represent the mean ± SE and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

(P5C), which is then rapidly reduced to proline (Hosseinifard *et al*., 2022). The lower proline levels at moderate cadmium stress conditions imply that the stress is less intense, and therefore, the plant does not need to synthesize as much proline. The consistent presence of 24-epiBL in both maximum and minimum proline content scenarios infers its role in modulating the plant's stress response. 24-epiBL likely helps in optimizing the proline synthesis according to the severity of the stress, ensuring efficient stress management.

Proline accumulation is considered an essential adaptive response to Cd toxicity (Dar *et al*., 2016). Cd stress induces oxidative stress in plants, leading to the production of ROS. Proline acts as an antioxidant and helps scavenge ROS, thereby reducing oxidative damage. Additionally, proline can also chelate Cd ions, reducing their toxicity and preventing their accumulation in vital plant tissues. Consequently, under Cd stress, 24-epiBL pretreatment plants tend to increase proline synthesis and accumulation in comparison to the plants without 24-epiBL pretreatments as a protective mechanism (Zdunek-Zastocka *et al*., 2021). Zafari *et al*. (2020) observed that the foliar application of 24-epiBL at the flowering stage improved the proline content of *Carthamus tinctorius* L. under water stress, which is in accordance with this study.

The obtained data indicates that pre-treatment with 24-epiBL significantly enhances the content of H_2O_2 and proline in basil under severe water and high Cd stress. This highlights the role of BR in modulating stress responses and augmenting plant resilience. These findings suggest that seed priming with BR could be a valuable tool in agricultural practices aimed at improving plant tolerance to combined abiotic stresses. The pre-treatment with 24-epiBL likely primed the basil plants to respond more robustly to the combined stresses. This hormonal priming effect would have enhanced the plant's ability to produce stress-related metabolites, such as H_2O_2 and proline.

Soluble sugar content: According to the obtained results, the maximum amount of soluble sugar was detected in the treatment of 25% FC + BR + 0 mM of Cd (30.79 µg/g FW), which was 2.09 times higher than the control level. However, plants experiencing 100% FC + BR + Cd (10 mM) appeared with the lowest content of this parameter (10.58 µg/g FW) (Figure 8). Soluble sugars are crucial for osmotic adjustment and stress signaling in plants (Saddhe *et al*., 2021). Under severe water stress, plants accumulate soluble sugars to maintain cell turgor and protect cellular structures. The increase in soluble sugar content during stress is part of a plant's adaptive response to maintain cell turgor, provide energy for survival, protect cellular components, and improve stress tolerance. Plants need to maintain a balance of water and solutes (substances dissolved in water) within their cells (Yetik and Candogan, 2023). 24-epiBL, likely boosts the plant's ability to produce and accumulate soluble sugars as part of its adaptive response to severe water stress (Kawaguchi *et al*., 2021). Osmotic potential refers to the concentration of solutes in the cell sap, which affects the movement of water into or out of the cells. Plants can effectively lower the osmotic potential in their cells by raising the concentration of soluble sugars (such as glucose, fructose, and sucrose). This increase in solute concentration creates a gradient that retains water within the cells, helping to maintain cell turgor pressure (Lambers and Oliveira, 2019). This turgor pressure is critical for plant structure and function, as it supports upright growth and allows the plant to withstand mechanical stresses. Soluble sugars serve as essential sources of energy for plants (Haeussinger and Sies, 2007).

During stressful conditions, when photosynthesis may be impaired or restricted, plants rely on stored sugars to meet their energy demands. The breakdown of these sugars through cellular respiration provides energy for vital metabolic functions, repair processes, and the synthesis of stress-related proteins and compounds

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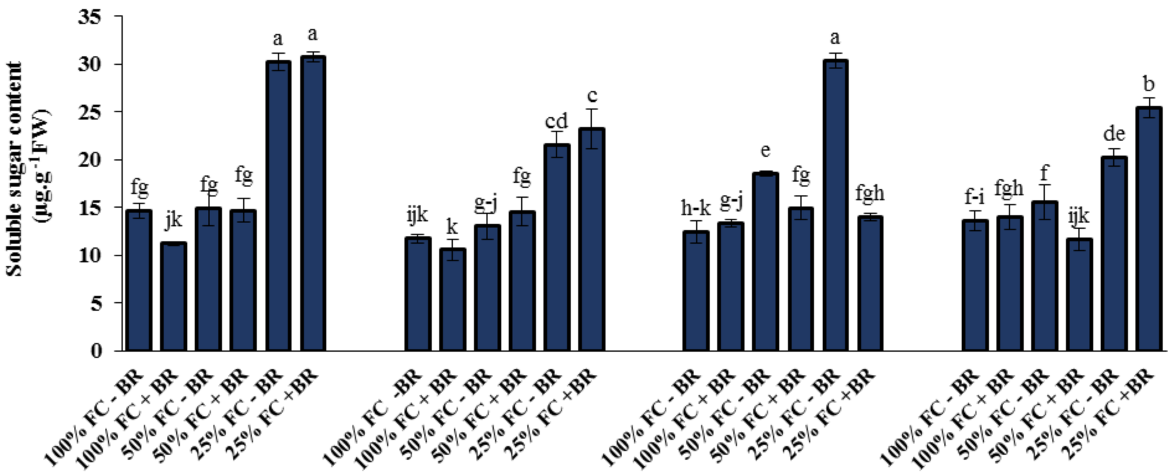

Figure. 8. Effect of different concentrations of Cd and water stress levels on the soluble sugar content of basil leaves. Values represent the mean ± SE, and different letters in the columns indicate the significance of the means based on Duncan's test (P<0.05).

(Afzal *et al*., 2021). Increased sugar content can also have protective effects on cells and cellular components. Soluble sugars act as osmoprotectants, helping to stabilize proteins, membranes, and other cellular structures under stress. They can scavenge ROS generated during stress and prevent oxidative damage to cells (Gil *et al*., 2013; Saddhe *et al*., 2021). The absence of Cd eliminates heavy metal stress, allowing the plant to focus its metabolic resources on managing water stress alone, which could explain the high accumulation of soluble sugars at 25% FC + BR + Cd (0 mM) treatment. The consistent presence of 24-epiBL in both maximum and minimum scenarios suggests its significant role in modulating stress responses. High soluble sugar levels under severe water stress is in accordance with the study of Niu *et al*. (2016), which implies that brassinolide (0.1 mg L^{-1}) foliar application rises the content of soluble sugars. 24-epiBL elevates the plant's ability to synthesize and accumulate soluble sugars under severe water stress. Under optimal water conditions, the presence of 24-epiBL might not significantly boost soluble sugar levels unless additional stress factors, like Cd, are present. Understanding the role of soluble sugars and 24-epiBL in stress tolerance can inform agricultural practices and might improve water resilience in crops. In the case of soluble sugar content and lipid peroxidation, provide information pointing out that 24-epiBL priming enhances the plant's ability to accumulate soluble sugars, which are crucial for stress mitigation. However, the increase in lipid peroxidation suggests that the oxidative stress induced by Cd is significant, even under optimal water conditions. 24-epiBL seems to play a dual role, enhancing stress tolerance mechanisms while also possibly increasing metabolic activity that leads to higher ROS production (Tanveer *et al*., 2018). The moderate level of Cd (10 mM) is enough to induce stress responses, leading to both protective (soluble sugars) and damage indicators (lipid peroxidation), and the presence of Cd under non-water conditions allows the plant to maintain its metabolic functions, contributing to the accumulation of soluble sugars. Understanding the balance between protective mechanisms and oxidative damage is crucial for optimizing stress management strategies in plants. These results highlight the potential of 24-epiBL in enhancing stress tolerance through the accumulation of protective compounds, but also the need to monitor and manage oxidative stress (Li *et al*., 2022).

Conclusion

This study highlights the potential of 24-epiBL seed priming as a beneficial technique to enhance the physiological and biochemical characteristics of basil (*Ocimum basilicum* L.) when exposed to Cd and water stress. The findings clearly demonstrate that 24-epiBL seed priming can alleviate the negative impacts of these stressors on basil plants. The improved plant growth observed in 24-epiBL-primed plants suggests that 24-epiBL promotes root and shoot development, enabling plants to better cope with stress conditions. Moreover, the reduction in lipid peroxidation levels in primed plants indicates improved membrane integrity and stability. 24-epiBL seed priming can contribute to the overall health and productivity of basil crops in stressful environments. This technique holds promise for sustainable agriculture, as it offers a means to mitigate the adverse effects of environmental stressors on basil production.

However, further investigations are needed to elucidate the precise mechanisms underlying the beneficial effects of 24-epiBL seed priming on basil plants. Additionally, exploring the long-term effects and potential interactions with other stressors would provide a more comprehensive understanding of the practical applications of 24-epiBL seed priming in crop production.

Overall, this study provides valuable insights into

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the potential of 24-epiBL seed priming as a sustainable and effective strategy for improving the tolerance of basil plants to Cd and water stress. Implementing this technique has the potential to contribute to the resilience and productivity of basil crops, ultimately benefiting farmers and ensuring a stable supply of this valuable herb in the face of challenging environmental conditions.

Statements and declarations

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Competing Interests

The authors declare no competing interests.

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