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Effect of abscisic acid and epibrassinolide on physiological and hormonal responses of tomato plants subjected to water stress

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Abstract: In this study, the effect of abscisic acid (ABA; 150 μmol) or epibrassinolide (EBL, 3 μmol) in mitigating the adverse drought conditions was evaluated in tomato plants (*Solanum lycopersicum* L. cv. Vilma). Potted plants were subjected to two 6-day periods separated by a one-time rehydration. Results showed that water deficit increased the content of superoxide radical ($O_2^{\bullet-}$), malondialdehyde (MDA), proline, ABA and its metabolites. On the other hand, the studied cytokinins showed a rather opposite trend. ABA application maintained and later reduced the $O_2^{\bullet-}$ content. At the same time, the MDA level was lower but later increased, while the proline content was reduced compared to untreated plants. This indicates that ABA helps the plants cope with the initial stress phase. In addition, ABA-activated signalling pathways showed increased levels of ABA, auxins, salicylic acid or jasmonic acid. EBL even more increased $O_2^{\bullet-}$ and proline content. At the same time, EBL increased the content of auxins, jasmonic acid and later ABA. In contrast, a decrease in salicylic acid and cytokinins was monitored. These findings indicate that ABA contributed to improved stress responses through early phytohormone-mediated signalling and reduction of stress markers, whereas EBL appeared less effective under our experimental conditions.

Keywords: drought stress; oxidative stress; proline; tolerance; reactive oxygen species

Water imbalance represents the most important factor affecting plant growth and development. Drought triggers a wide range of responses, from alterations in gene expression and cellular metabolism to changes in growth, photosynthesis, or nutrient uptake (Seleiman et al. 2021). A common negative side effect is secondary oxidative stress caused by unbalanced production and reactive oxygen species (ROS) degradation. Excess ROS damages proteins and nucleic acids or impairs cell homeostasis mainly through the peroxidation of membrane lipids (Choudhury et al. 2017, Mukarram et al. 2021). During lipid peroxidation, several products are accumulated, among them, malondialdehyde (MDA) can be quickly and easily estimated and thus be considered a possible indicator of the degree of plasma membrane damage (Zhang et al. 2021).

Maintaining optimal water potential is essential for plant survival. One of the effective mechanisms is osmotic adjustment, an increase in the concentration of osmotically active solutes (e.g. amino acids or soluble sugars) with a decrease in osmotic potential, which maintains turgor in metabolically active cells (Mukarram et al. 2021, Ozturk et al. 2021). Proline plays a versatile role in plant metabolism, as it is a non-enzymatic antioxidant and stabiliser of sub-

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cellular structures, membranes, and proteins. High cellular content under stress conditions indicates its role as a key player in stress tolerance (Ozturk et al. 2021).

Phytohormones play an important role in the modulation of morpho-physiological, biochemical and molecular responses necessary for plant survival. Positive roles of several hormones in promoting plant acclimatisation, including abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), have been intensively investigated. Exogenously applied, they may act as chemical messengers improving plant tolerance to various stresses (Ullah et al. 2018). ABA, the major drought hormone, regulates stomata closure or modulation of root system morphology. Many studies showed enhanced endogenous ABA levels as well as increased activity of ABA-associated enzymes or transcription factors at osmotic stresses (Pál et al. 2018, Asghar et al. 2019). Besides the role of brassinosteroids (BR) in regulating plant growth and development, they also stimulate numerous responses to stress stimuli (Nawaz et al. 2017). Exogenously applied BR reduced ROS and MDA contents *via* enhanced scavenging activity of antioxidants, maintained membrane integrity or increased activity of photosynthetic enzymes (Chen et al. 2017).

Tomato (*Solanum lycopersicum* L.) is one of the most important economic crops worldwide. Abiotic stress, such as drought, extreme temperatures or salinity, is the main cause of huge loss of its production (Krishna et al. 2019). To improve production efficiency, it is necessary to know the physiological responses to drought, the subsequent recovery mechanism, and the possible modulation mechanism of exogenously applied phytohormones. Within the background information, the study aimed to evaluate whether exogenously applied ABA and BR have the potential to ameliorate the negative effect of drought conditions on tomato plants, which should be manifested by increased activity of specific phytohormones involved in signalling pathways and reduced accumulation of proline, ROS and MDA.

MATERIAL AND METHODS

Plant cultivation and experimental design. Tomato seeds (*Solanum lycopersicum* L., cv. Vilma; Nohel Garden a.s., Dobříš, Czech Republic) were sown in propagators filled with a commercially available growing substrate (AGRO, Říkov, Czech Republic), consisting of a peat-humus mixture (pH 5.5–6.5) with contents of plant-available phosphorus (P) 91.4 mg/kg, potassium (K) 203 mg/kg, magnesium (Mg) 202 mg/kg, and calcium (Ca) 1 840 mg/kg soil, as determined by Mehlich 3 extraction. Cv. Vilma is a richly fruiting compact bush tomato type producing cherry tomatoes. This cultivar is small in stature, space-saving, and low-maintenance, and it is directly designed to be grown in containers, which makes it possible to regulate irrigation carefully. Five pcs of nine-week-old plants were sown into a pot (99 × 17 × 14 cm) and cultivated in a growth chamber (Fitotron®, Weiss Technik, Reiskirchen-Lindenstruth, Germany) with 16/8 h photoperiod, temperature 25/20 °C and air humidity of 60%. Plants were watered 3 times per week with 500 mL of tap water. After one month, plants with an average height of 20 cm were divided into four groups: C – control; D – drought; ABA + D – ABA treatment and drought; EBL + D – EBL treatment and drought. Leaves were carefully hand-sprayed with 150 μmol aq. solution of ABA (Sigma-Aldrich, Louis, USA) or 3 μmol aq. solution of 24-epibrassinolide (Sigma-Aldrich, Darmstadt, Germany) in 0.1%Tween (Sigma-Aldrich, Darmstadt, Germany) until the solution began dropping off the leaves. The ABA and EBL concentrations were selected based on previous reports demonstrating their effectiveness in tomato under drought stress (Jangid and Dwivedi 2017, Yan et al. 2022). In addition, concentrations in the 100–200 μmol ABA range and 1–5 μmol EBL are commonly used to elicit reproducible physiological responses without causing phytotoxic effects. C and D groups were sprayed with the equivalent amount of distilled water. On the same day, watering of the plants was stopped. Fully developed leaves were sampled every 48 h (DAD – days after dehydration). On the 6th day (144 h), the substrate water-holding capacity was up to 25% and plants were one time rehydrated with 500 mL of tap water and subjected to the second drought period (DAR – days after rehydration), again sampling every 48 h (next 6 days).

Determination of the biochemical parameters. The content of superoxide radical ($O_2^{\bullet-}$) was determined in 50 mmol potassium phosphate buffer (pH 7.0) by measuring nitrite formation from hydroxylamine spectrophotometrically (530 nm) (Dučaiová et al. 2016). The level of membrane lipid peroxidation was determined as the amount of MDA using the thiobarbituric acid reactive substance assay at 532 nm (Dučaiová et al. 2016). The proline content was determined according to Bates et al. (1973). Sulfosalicylic acid extracts of fresh leaves were mixed with acidi-

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fied ninhydrin reagent and boiled in a water bath for 60 min. After rapid cooling, toluene was added and left overnight. The absorbance of toluene fractions was read at 520 nm. All used chemicals were purchased from Sigma Aldrich (Darmstadt, Germany).

Hormone analyses. Phytohormones were analysed according to Prerostova et al. (2020) with slight modifications. Frozen plant samples were homogenised with 1 mol/L formic acid, internal isotope-labelled standards and zircon balls (about 0.05 g) in a FastPrep-24 5G homogeniser (MP Biomedicals, Santa Anna, USA) for 40 s at 6 m/s. Isotope-labelled standards were added at 10 pmol per sample: $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Tewksbury, USA); $^2H_4$-SA (Sigma-Aldrich, St. Louis, USA); $^2H_3$-PA, $^2H_3$-DPA (NRC-PBI, Saskatoon, Canada); $^2H_6$-ABA, $^2H_5$-JA, $^2H_5$-tZ, $^{15}N_4$-cZ, $^2H_3$-DZ, $^2H_6$-iP (Olchemim, Olomouc, Czech Republic). Homogenates were centrifuged at 4 °C and 17 000 g for 10 min. A supernatant was applied to the well plate. SPE Oasis HLB 96 was prewashed with 100% acetonitrile, followed by H_2O and 1 mol HCOOH. The pellet was reextracted with 1 mol HCOOH, rehomogenised in FastPrep-24, centrifuged, and the supernatant was applied to the same SPE-well. Wells were washed with H_2O, and samples were eluted twice with 50% acetonitrile into a collection plate. The analysis was performed using an LC/MS system consisting of a UHPLC 1290 Infinity II (Agilent, Santa Clara, USA) coupled to a 6495 Triple Quadrupole Mass Spectrometer (Agilent, Santa Clara, USA), operating in MRM mode, with quantification by the isotope dilution method. Data acquisition and processing were performed with Mass Hunter software B.08 (Agilent, Santa Clara, USA).

Statistical analysis. Each treatment comprised five biological replicates, obtained by pooling one leaf from five individual plants grown under identical conditions. Five technical measurements were performed for each biological replicate using a standardised protocol and calibrated instruments. Replicates were randomly assigned to avoid bias. Before analysis, data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Treatment effects were evaluated using one-way ANOVA followed by Tukey's post hoc test at $P < 0.05$ (GraphPad Prism 9 Software, San Diego, USA).

RESULTS

Initially, all plants were uniform; the leaves were dark green and completely turgescent. After six days of dehydration (6 DAD), C plants were green, and some older lower leaves turned yellow. The D variant had severely wilted leaves; some turned pale green or yellow, and some abscised. In hormone-treated plants, leaves began to wilt and turned light green (EBL + D) or yellow (ABA + D). One-time rehydration did not significantly affect D plants (2 DAR), which showed severe dehydration damage at the end of the experiment (6 DAR). Most of the leaves were light yellow, turning to necrosis, and the leaves were wilted and curled. This negative manifestation was slightly slowed down by hormone application after rehydration (Figure 1).

With the prolonged water deficit, the levels of the $O_2^{\bullet-}$ increased in all stressed variants, mainly in EBL + D (Figure 2A). Surprisingly, on 6 DAD, a significant drop was observed. One-time rehydration did not reduce the levels; on the contrary, the values continued to rise to the maximum at 6 DAR. Overall, the lowest $O_2^{\bullet-}$ values were in control plants, and the highest were in EBL-treated plants. The addition of ABA showed an ameliorative effect only after rehydration, when the decrease relative to the D variant was observed, significant only at 4 DAR.

Overall, during dehydration, the lowest MDA levels (Figure 2B) were observed in ABA-treated plants, and the level gradually decreased with the sampling time. The level gradually rose during the second drought period, even above the D variant.

The proline level (Figure 2C) rose continuously with the prolonged drought period. The highest increase was recorded in plants treated with EBL. ABA treatment significantly reduced proline accumulation compared to variants D and EBL + D, especially after prolonged drought exposure (6 DAD, 6 DAR). Rehydration resulted in a drop in proline levels, mainly in D and EBL + D variants (2 DAR), but the second dry period resulted in a sharp increase, up 7-fold in the EBL + D variant (6 DAR).

In general, the ameliorative effect of ABA was more effective than EBL treatments.

Prolonged drought decreased the level of *trans*-zeatin (*t*Z), more significantly in ABA + D at 6 DAD. After rehydration (2 DAR), a sharp increase followed, after which the level gradually decreased again to a level similar to 6 DAD (Figure 3A). The exception was for EBL + D, where *t*Z changed minimally after rehydration. *Cis*-zeatin (*c*Z) values increased during prolonged drought (Figure 3B). After rehydration, a decrease occurred, most significantly in D (up to 2-fold), followed by a gradual increase until the end of the measurement.

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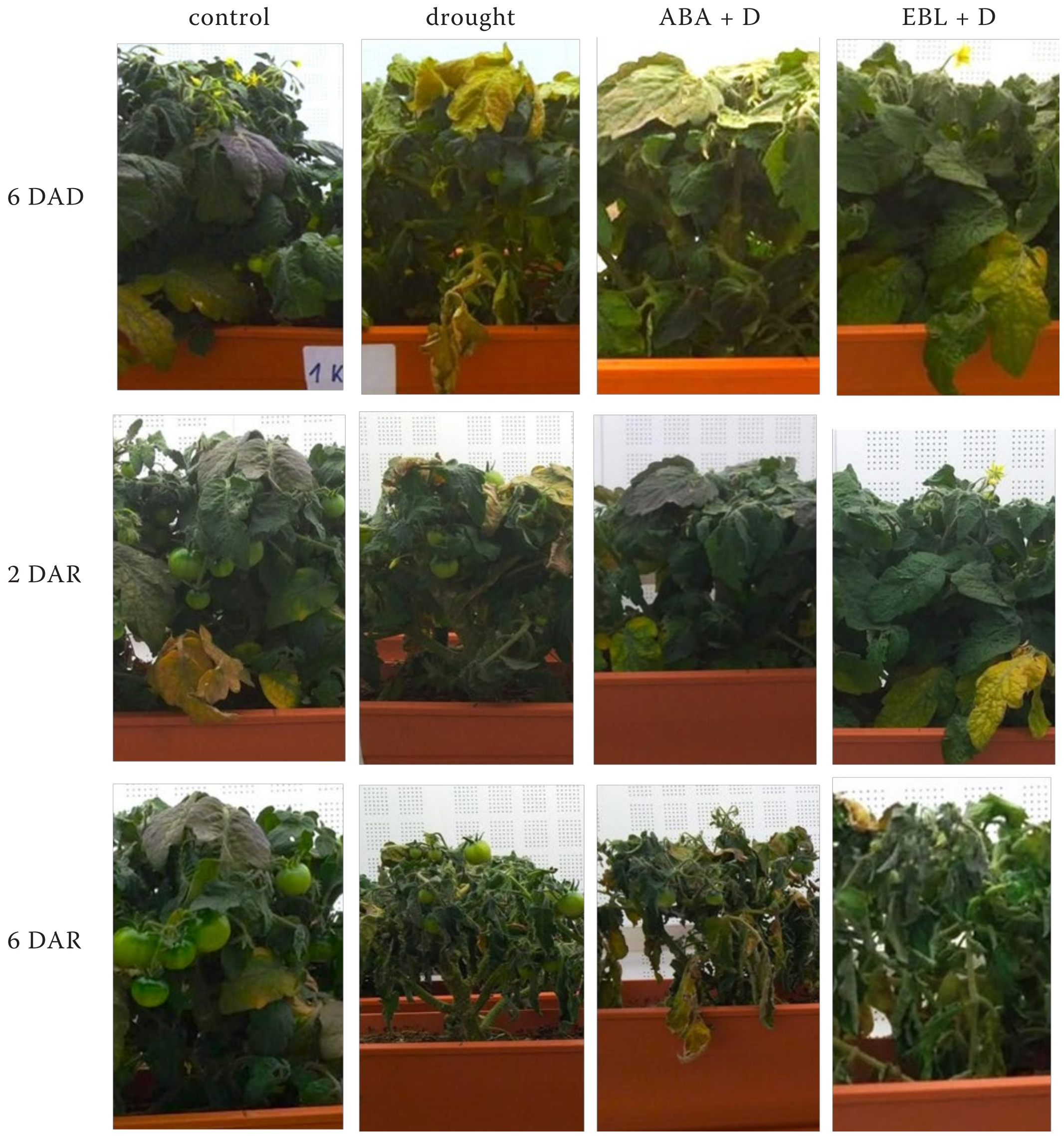

Figure 1. Effect of drought (D) and hormonal treatment on plant vitality status; ABA – abscisic acid; ELB – epibrassinolide; DAD – days after dehydration; DAR – days after rehydration

During dehydration, EBL increased the level of *c*Z in the first phase, while ABA only in the second phase. Significant changes in the content of isopentenyladenine (iP) appeared only at the end of the experiment (4 DAR and 6 DAR), especially in D and ABA + D (Figure 3C). In the presence of EBL, the values were lower than D. During the first phase of drought, the dihydrozeatin (DZ) level was relatively low. However, it increased sharply at the end of the second drought period (Figure 3D). However, when ABA and EBL were applied, the values remained low compared to D.

Individual cytokinin derivatives responded differently to dehydration and rehydration (Figure 3). The most pronounced changes were observed for *t*Z and DZ, while *c*Z and iP showed a gradual decrease and subsequent increase. ABA and EBL mostly modified these responses to attenuate extreme values.

The IAA content (Figure 4A) initially increased in the D variant and decreased later. On the contrary, the ABA + D and EBL + D variants exhibited relatively high IAA content, decreasing at later stress stages (6 DAR and 4 DAR, respectively).

Drought application reduced SA levels (Figure 4B), except for 6 DAD. On the contrary, the values rose after ABA addition, namely at 6 DAD, 2 DAR and 6 DAR.

The JA level (Figure 4C) varied throughout the experiment. In the first drought period, a relatively

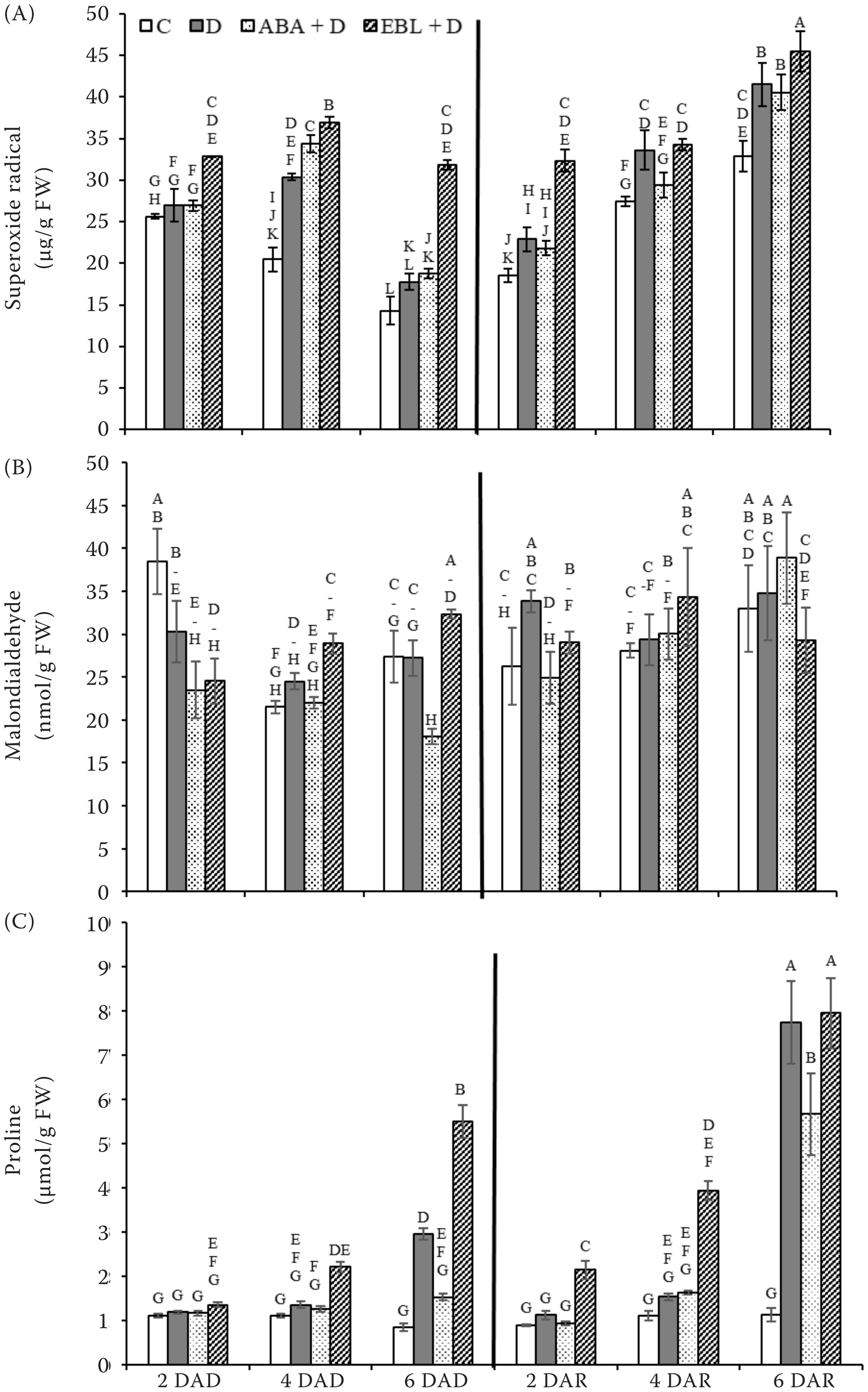

Figure 2. Effect of drought and hormonal treatment on (A) superoxide radical; (B) malondialdehyde, and (C) proline content in tomato plants. Data are means ± standard deviations ($n = 5$). Values within column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$). C – control; D – drought; ABA + D – abscisic acid treatment and drought; EBL + D – epibrassinolide treatment and drought; DAD – days after dehydration; DAR – days after rehydration; FW – fresh weight

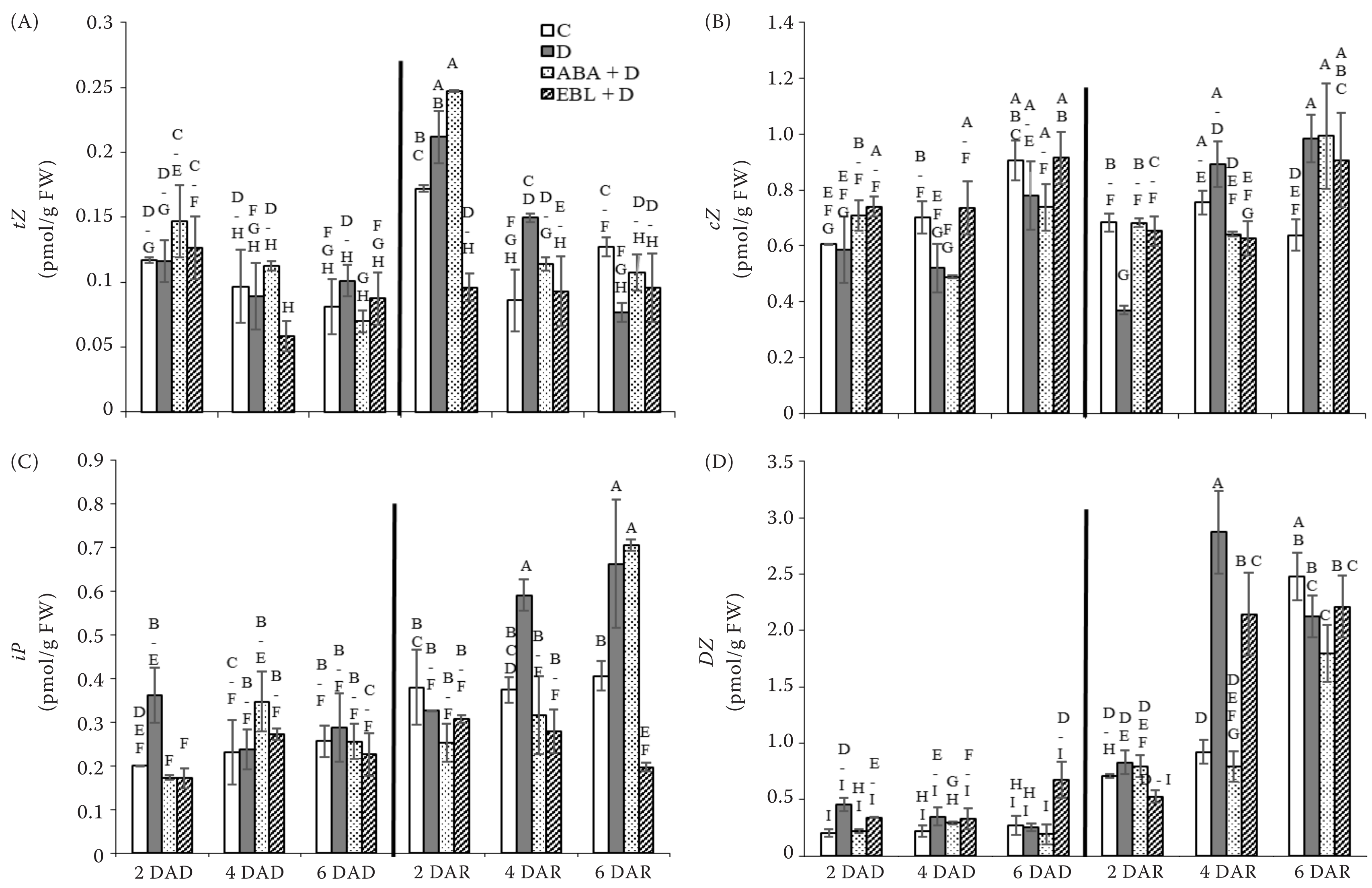

Figure 3. Effect of drought and hormonal treatment on (A) trans-zeatin (*tZ*); (B) cis-zeatin (*cZ*); (C) isopentenyladenine (*iP*), and (D) dihydrozetin (*DZ*) content. Data are means ± standard deviations (*n* = 5). Values within column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$). C – control; D – drought; ABA + D – abscisic acid treatment and drought; EBL + D – epibrassinolide treatment and drought; DAD – days after dehydration; DAR – days after rehydration; FW – fresh weight

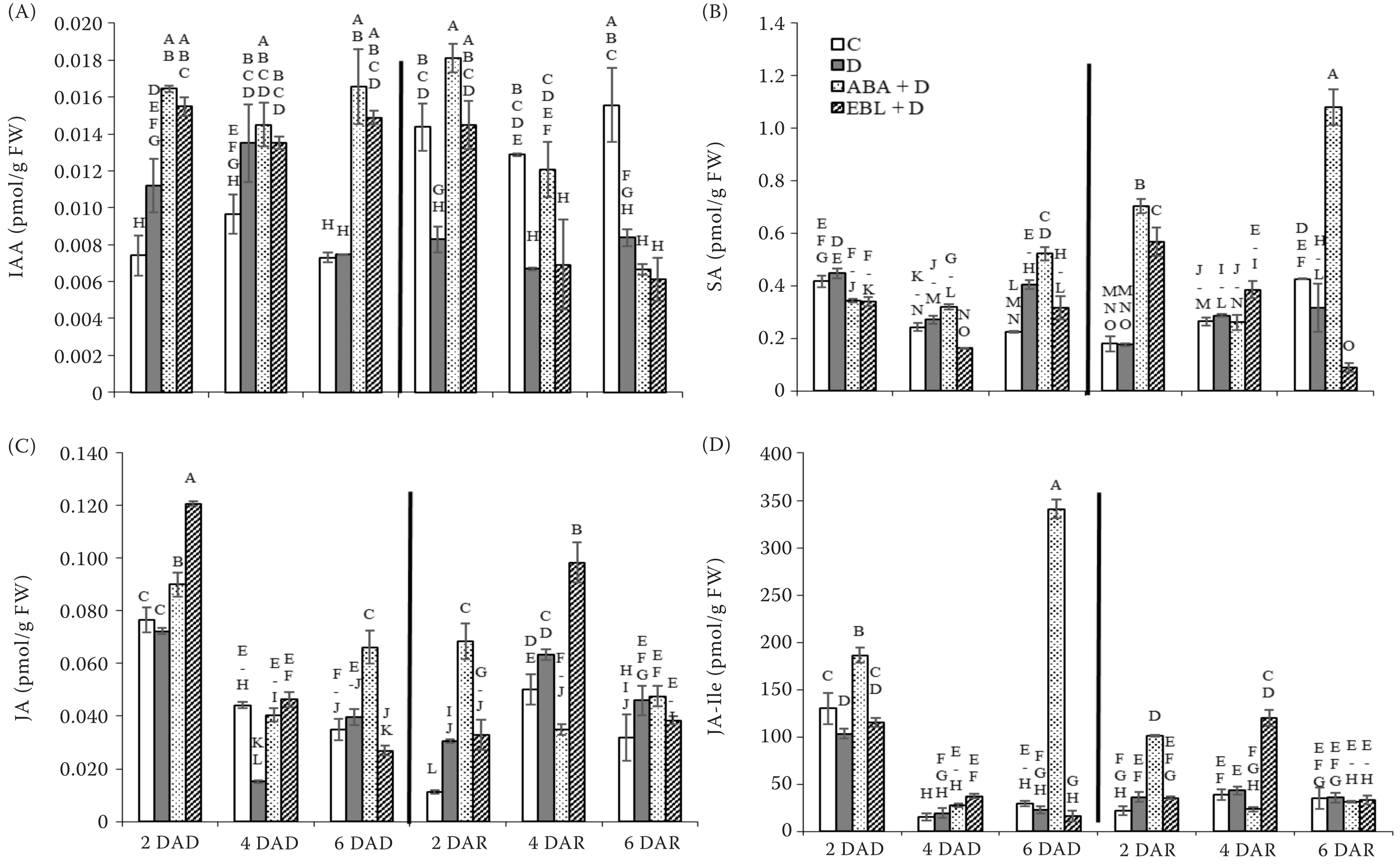

Figure 4. Effect of drought and hormonal treatment on the content of (A) indole-3-acetic acid (IAA); (B) salicylic acid (SA); (C) jasmonic acid (JA), and (D) JA-isoleucine (JA-Ile). Data are means ± standard deviations (n = 5). Values within column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$). C – control; D – drought; ABA + D – abscisic acid treatment and drought; EBL + D – epibrassinolide treatment and drought; DAD – days after dehydration; DAR – days after rehydration; FW – fresh weight

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gradual decrease was observed. The second drought period resulted in a slight increase, but it remained lower compared to 2 DAD. ABA and EBL mostly enhanced the JA level compared to D. The Bioactive metabolite JA-Ile decreased with time (Figure 4D). A significant increment was in ABA + D in 6 DAD and EBL + D in 4 DAR.

Hormonal responses differed significantly depending on the experimental variant and stress phase (Figure 4). While IAA and SA showed striking changes mainly at later times, JA and JA-Ile followed similar, gradual trends. Phytohormone treatment generally increased their levels compared to drought alone, with the effect of ABA being more pronounced than that of EBL.

High level of ABA (Figure 5A) was observed in the ABA + D variant (up to 4-fold), which slightly decreased after rehydration. Prolonged drought led to a simultaneous increase in ABA content in variants D and EBL + D. Rehydration reduced ABA level, which remained low in D. On the contrary, in EPI + D, the levels slowly increased with a prolonged drought period. From ABA metabolites, the contents of dihydrophaseic acid (DPA), phaseic acid (PA) and neophaseic acid (NeoPA) (Figure 5B–D) exhibited a transient increment during prolonged drought in the D variant. In the first dehydration phase, ABA and EBL maintained lower levels of PA. In contrast, in the second phase, they reached higher values than at D. Hormone application significantly reduced NeoPA and DPA values in all time points.

Overall, drought increased ABA content, with exogenous ABA further enhancing this effect, while both hormonal applications suppressed the accumulation of ABA metabolites.

DISCUSSION

Drought is one of the major abiotic stresses limiting plant growth, development, and production. Negative manifestations, such as increased production of ROS, MDA, osmolytes or alteration in hormonal profile, can be ameliorated *via* exogenously applied phytohormones, e.g. ABA, JA or EBL (Mukarram et al. 2021).

During dehydration, ROS act as signalling molecules and harmful substances, with $O_2^{\bullet -}$ being the first to generate ROS (Choudhury et al. 2017). Drought increased $O_2^{\bullet -}$ levels, as observed in previous studies (Li et al. 2018, da Silva Lobato et al. 2021, Galviz et al. 2021). The temporary reduction before rehydration (6 DAD) suggests the activation of antioxidant systems such as superoxide dismutase or antioxidants such as ascorbate and carotenoids (Zhou et al. 2019). Soares et al. (2016) reported that exogenous EBL reduces antioxidant activity, potentially explaining the higher levels of $O_2^{\bullet -}$ in our EBL-treated plants.

Excessive ROS damages membrane lipids and increases MDA content (Zhang et al. 2021). In our study, membrane damage was limited, probably due to the rapid activation of antioxidants or biosynthesis of membrane-stabilising molecules (proline, carbohydrates) (Andersen et al. 2011). This would explain MDA's simultaneous lower accumulation trend and sharp proline increase (Figure 2). BR application reduces ROS and MDA, with the strength of the effect increasing with higher hormone concentrations (Gill et al. 2017, Naveen et al. 2021). The effectiveness of our chosen EBL concentration may not have been sufficient, but it may also depend on the type of BR; for example, 28-epihomobrassinolide and 28-homobrassinolide were more effective than EBL in maize under water stress (Sun et al. 2022). ABA also reduced ROS and MDA in other studies (Wang et al. 2011, Ramasamy et al. 2022), but here the reduction was visible only for $O_2^{\bullet -}$ in the second drought period. Controversially, it increased its accumulation in the first period. Combined treatment with ABA and BR has been reported to reduce MDA more than either treatment alone (Jangid and Dwivedi 2017, Chen et al. 2018). Proline is an osmoprotectant, ROS scavenger, and membrane stabiliser (Ozturk et al. 2021). Its accumulation correlated with stress severity, peaking at the end of the dry period (6 DAD), in line with Ramasamy et al. (2022) and Galviz et al. (2021). Cultivar sensitivity influenced proline levels, with drought-adapted cultivars showing higher accumulation (Montesinos-Pereira et al. 2014, Moles et al. 2018), and re-irrigation reduced proline levels (Nguyen et al. 2020). ABA reduced proline in late drought (6 DAD and 6 DAR), consistent with Ramasamy et al. (2022), while combined ABA and EBL treatments or EBL alone increased proline, potentially *via* enhanced biosynthesis or gene expression (Chen et al. 2018, Pál et al. 2018, Kaur and Asthir 2020, Naservafaei et al. 2021, Naveen et al. 2021, Sun et al. 2022). Transcriptomic studies indicate that ABA alone may not stimulate drought-induced proline accumulation (Savouré et al. 1997, Sharma and Verslues 2010), suggesting that proline biosynthesis under drought involves both ABA-dependent and ABA-independent pathways. According to our results, ABA alleviated drought effects in the advanced

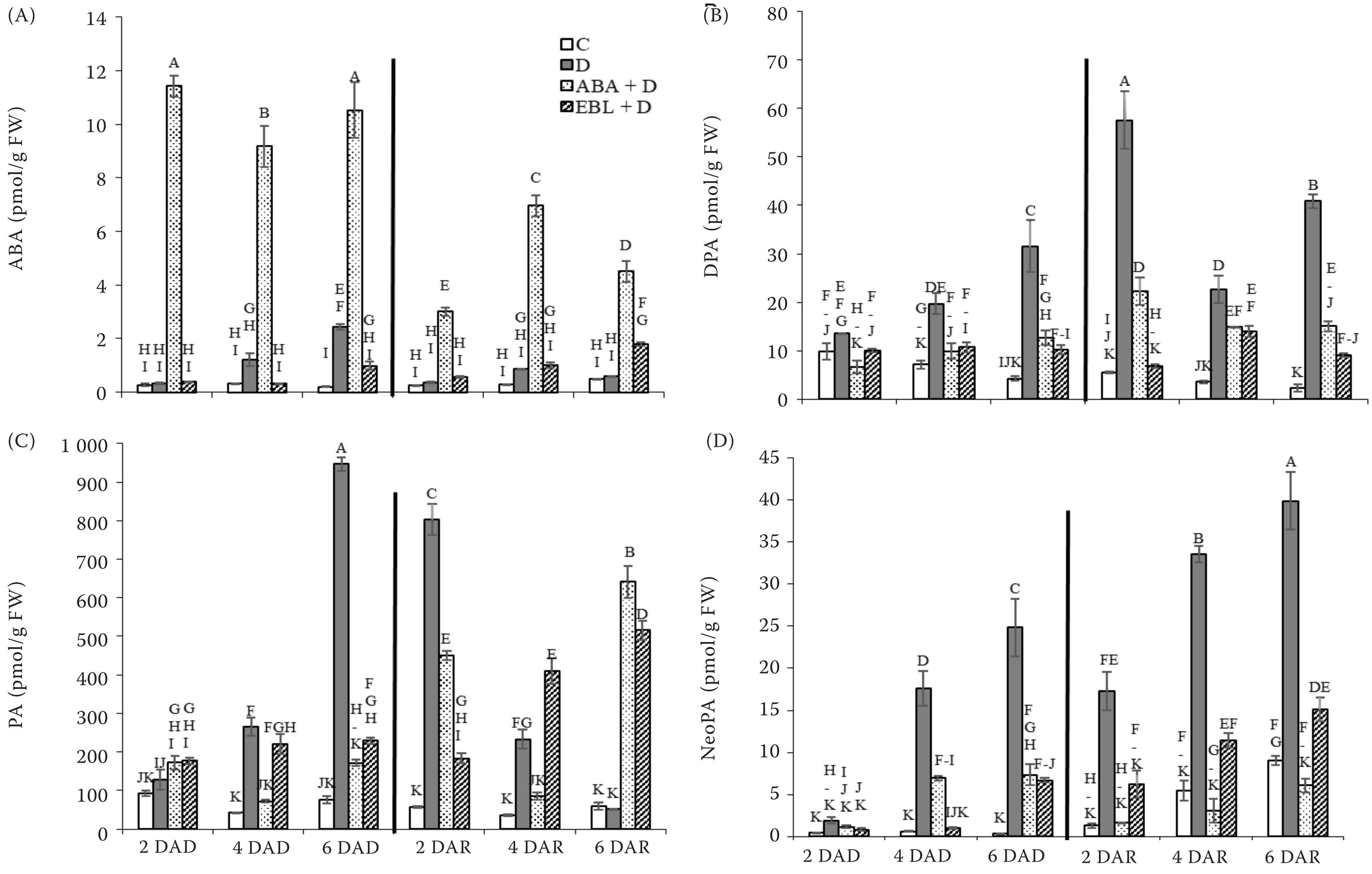

Figure 5. Effect of drought and hormonal treatment on the content of (A) abscisic acid (ABA); (B) dihydrophaseic acis (DPA); (C) phaseic acid (PA) and (D) neophaseic acid (NeoPA). Data are means ± standard deviations ($n = 5$). Values within column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$). C – control; D – drought; ABA + D – abscisic acid treatment and drought; EBL + D – epibrassinolide treatment and drought; DAD – days after dehydration; DAR – days after rehydration; FW – fresh weight

phase, while EBL promoted proline accumulation that may contribute to stress aggravation. Nevertheless, these conclusions are based on biochemical markers.

ABA accumulation is one of the first responses to drought, triggered by reduced water content and leaf turgor (Li et al. 2018, Moles et al. 2018). Prolonged drought induces ABA accumulation *via* up-regulation of NCED genes encoding key biosynthetic enzymes (Petrović et al. 2021). Elevated levels of ABA metabolites (PA, DPA, neoPA) were consistent with Tamang et al. (2021). Moreover, rehydration sharply reduced ABA content, as Hernandez-Espinoza and Barrios-Masias (2020) noted. Exogenous ABA application caused a strong increase in ABA levels (Yin et al. 2004, Zhang et al. 2009), while EBL enhanced ABA only at the final dehydration stage (6 DAR).

JA and its isoleucine conjugate (JA-Ile) are key stress-response signals. After an initial decrease, their concentrations increased during successive sampling periods, consistent with Wang et al. (2020) and Zhang and Huang (2013), though opposite trends were reported by De Ollas et al. (2015). Crosstalk between JA and ABA has been described, with ABA promoting JA accumulation, while JA presence does not affect ABA levels (Muñoz-Espinoza et al. 2015, Avramova 2017). Interestingly, JA/ABA-related genes were not induced during repeated dehydration (Liu et al. 2016), yet in our study, higher JA levels appeared in the second drought phase. Reported stimulation of JA synthesis in EBL presence (Zeng et al. 2022) was visible here only at 2 DAD and 4 DAR. Tomato cultivars differing in drought tolerance also vary in basal JA levels and accumulation rate (Zhang and Huang 2013, Wang et al. 2020), with tolerant cultivars showing faster accumulation. In our results, delayed JA accumulation and unchanged MDA content suggest that the tested cultivar may exhibit higher dehydration tolerance.

Increased SA accumulation is typical for stress response and tolerance mechanisms. Our data show only a transient accumulation of SA, which is in accordance with Soba et al. (2019). In contrast, De Diego et al. (2015) or Mirua and Tada (2014) presented a strong SA accumulation correlated with increased stomatal closure.

Auxin plays an important role in regulating plant development. Similarly, as in Du et al. (2013) or Tamang et al. (2021), enhanced IAA values continuously decrease at the beginning of the drought period. ABA treatment increases IAA content, which may promote primary root growth in deeper soil layers. According to Khan et al. (2020), EBL increased IAA content in early response to drought (up to 2 DAR).

Biochemical and transcriptomic studies show that CKs can positively and negatively regulate drought adaptation. After prolonged drought (6 DAD), the most active CK, *t*Z, decreased, likely reflecting growth inhibition and accelerated senescence, as De Diego et al. (2015) reported. In contrast, other bioactive CKs (*c*Z, iP, DZ) increased, consistent with Zhang et al. (2017), Soba et al. (2019), and Tamang et al. (2021). The antagonistic interaction of ABA and CK has been linked to reduced CK biosynthesis and improved drought tolerance (Cortleven et al. 2019). In our study, ABA or EBL did not markedly affect CK levels, differing from Khan et al. (2020), who observed CK enhancement after EBL application.

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