



Biochemical and biomolecular response of Arabidopsis seedlings to osmotic and salt stress: Mitigation by biostimulant formulation enriched in betalain degradation products

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ABSTRACT

Abiotic stress, such as salt and osmotic stress, negatively impacts plant growth and productivity, making it crucial to explore sustainable solutions that can improve plant resilience. In this study, we investigated the effects of a biostimulant formulation containing betalain degradation products (BDPs) on the growth and stress resistance of *Arabidopsis thaliana* seedlings subjected to salt stress (SS) and osmotic stress (OS) conditions. Specifically, we fortified the medium culture of stressed seedlings with 0.02, 0.10, and 0.20 mg/mL of BDPs-enriched biostimulant, revealing significant improvements in growth and biomass under both SS and OS conditions, with the most notable effects in OS condition. In this study, we applied these findings to stress conditions, demonstrating that treatment with the formulation containing BDP-related compounds significantly enhanced seedling growth and biomass in both salt stress (SS) and osmotic stress (OS) environments, with the most significant effects observed in OS conditions. Specifically, seedlings treated with 0.20 mg/mL of BDP-enriched biostimulant exhibited growth increases of up to 150 % under OS conditions and a 116 % increase under SS conditions, restoring growth metrics comparable to control conditions. We analyzed the impact of the formulation on the dynamics of reactive oxygen species (ROS) and the activities of antioxidant enzyme, revealing that BDP-containing formulation effectively balanced ROS levels by modulating superoxide dismutase (SOD), catalase (CAT) and the glutathione peroxidase-glutathione reductase (GPX-GR) system in both stress conditions. Furthermore, BDP-containing formulation affected proline metabolism by reducing its accumulation under stress conditions and enhancing proline catabolism, as demonstrated by the upregulation of the genes for proline dehydrogenase (PDH), pyrroline-5-carboxylate dehydrogenase (P5CDH), and ornithine aminotransferase (OAT). These findings suggest that the biostimulant enriched in BDP-related compounds enhanced the resilience of *Arabidopsis* seedlings to abiotic stress by regulating both oxidative stress responses and proline metabolism, highlighting their potential applications for improving crop tolerance to challenging environmental conditions.

1. Introduction

The climate change experienced by our planet in recent decades have resulted in serious environmental consequences, creating an urgent need to explore how organisms respond to these stresses. This investigation is important not only for enhancing our basic understanding but also for developing strategies to alleviate their impacts [4,54]. Among these challenges, osmotic and saline stresses represent the most critical

environmental pressures faced by plants, as they pose a substantial threat to global plant production [7,48]. Osmotic stress (OS) specifically arises when plants encounter water deficit conditions caused by insufficient soil moisture or high solute concentration in the soil. This leads to decreased water uptake, desiccation and changes in physiological cellular processes, including photosynthesis, nutrient uptake, and hormonal regulation [10,54]. On the other hand, salt stress (SS) occurs when soil salinity levels exceed the tolerance threshold of plants,

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resulting in ionic imbalances within cells. This disruption of ion homeostasis affects critical physiological processes, including enzyme activity, protein synthesis, and membrane integrity, ultimately hindering plant growth and development [24,45]. Although OS and SS are driven by distinct biochemical and molecular mechanisms, they often activate similar response pathways in plants. For example, both stresses prompt morphological and anatomical adjustments as adaptive strategies to minimize water loss and regulate ion uptake [12,31,43]. These adaptations may involve modifications in root architecture to explore deeper soil layers for water and to prevent salt accumulation in the rhizosphere [31,43], as well as stomatal closure and leaf senescence to conserve water and decrease transpiration loss under stress conditions [12]. Biochemically, both stresses activate similar stress-response pathways, which include the synthesis of osmoprotectants like proline, glycine betaine, and sugars. These compounds play a crucial role in maintaining the cell turgor and stabilizing proteins and membranes under stress conditions [6,9]. Additionally, plants employ antioxidant defense mechanisms to scavenge reactive oxygen species (ROS) that may arise during stress, thereby mitigating potential oxidative damages [30]. Although traditional agricultural practices have historically provided solutions to reduce the impact of environmental stresses on crop production, they often lead to significant environmental consequences. Conventional approaches, such as irrigation and fertilization, are often suggested to alleviate OS and SS in crops; however, their extensive use leads to salt accumulation in the soil through evaporation, particularly in arid and semi-arid regions [34]. Furthermore, the indiscriminate use of chemical fertilizers not only leads to soil salinization but also disturbs soil microbial communities, ultimately compromising long-term soil fertility [3]. As a result, traditional agricultural practices feed a cycle of degradation, where short-term increases in crop productivity are compensated for by long-term environmental degradation and reduced agricultural resilience [39,50]. Additionally, in order to increase production while reducing processing costs, traditional farming methods often depend on monocultural cropping systems. However, these systems are more vulnerable to OS and SS due to their lack of genetic diversity, which is essential for resisting and adapting to changing environmental conditions [22,37]. To address these challenges, biostimulants have emerged as promising solutions for enhancing plant resilience to environmental stresses while promoting sustainable agricultural practices. Biostimulants are defined as substances or microorganisms applied to plants, seeds or the rhizosphere, aimed at improving nutrient uptake, stress tolerance and overall crop quality [40]. Unlike fertilizers that directly provide essential nutrients, biostimulants are recognized for their ability to influence multiple physiological processes in plants, thereby enhancing stress tolerance and overall productivity [16]. Furthermore, these formulations are usually derived from natural sources, including plant extracts, microbial metabolites, or organic compounds, which makes them environmentally friendly and consistent with organic farming practices [44]. In our recent study, we developed a novel biostimulant formulation derived from agri-food waste that was enriched in betalain degradation products (BDPs). While the formulation retained some naturally occurring phytochemical compounds from the original plant matrix, the majority consisted of thermally generated BDPs [15]. Expanding on our previous research conducted under optimal growth conditions, we showed a comprehensive investigation to evaluate various concentrations of this innovative formulation, identifying both effective and cytotoxic doses. Our assessment included morphometric parameters, mitochondrial performance, and the metabolism and catabolism of reactive oxygen species (ROS), osmoprotectants, and hormones. Consequently, we identified potential mechanisms that contribute to the phytostimulatory effect on germinating seedlings, as well as those that lead to cytotoxic effects [15].

Despite significant advances in the understanding of plant responses to stresses, effective strategies to increase crop resilience to abiotic stresses without the use of chemicals that may be hazardous to the environment need continued investigation. Previous research has

demonstrated the beneficial effects of biostimulants, while our preliminary investigation has shown that formulations enriched in BDP-related compounds can improve plant growth under non-stress conditions. However, the potential of BDP-enriched formulations to improve plant performance under abiotic stress conditions has not been fully explored. This study aims to fill this knowledge gap by analyzing how BDPs-enriched biostimulants can influence key physiological and molecular mechanisms, such as reactive oxygen species (ROS) management and proline metabolism, under salt (SS) and osmotic stress (OS) conditions, providing new insights into the mechanisms underlying how BDPs-enriched biostimulant can induced stress tolerance. The results of this study could pave the way for the development of new biostimulant-based agricultural strategies to improve crop productivity under increasingly harsh environmental conditions.

2. Materials and methods

2.1. *In vitro* germination test

For the *in vitro* germination test, about 2000 seeds of the *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) wild type (WT) were sterilized through surface treatment with 70 % (v/v) ethanol for 15 min, followed by two thorough washes with pure ethanol. After sterilization, the individual seeds were placed under sterile conditions onto square plates filled with 70 mL of Murashige and Skoog (MS) medium at pH 5.8, differently supplemented according to the specific experimental conditions outlined in Table 1. Specifically, all plates contained MS medium with 0.1 % (w/v) sucrose and 0.8 % (w/v) plant agar as a base. To simulate the different stress conditions, one-third of these plates ($n = 20$) was supplemented with 100 mM NaCl to induce salt stress, while another third ($n = 20$) with 150 mM mannitol to mimic osmotic stress. Moreover, to evaluate the effect of BDP-containing biostimulant on stress responses, some plates were further supplemented with the formulation at three different concentrations: 0.2, 0.1 or 0.02 mg mL⁻¹. Control plates, containing MS medium with only sucrose and agarose, were used to grow *Arabidopsis* seedlings under standard conditions. Table 1 provides a detailed summary of these experimental conditions. Each plate contained 30 *Arabidopsis thaliana* seeds, and 5 plates were used for each condition, for a total of 150 seeds per condition. The total number of seeds used for the experiment was equal to 1800. After seedling, the plates were sealed with micropore tape to facilitate gas exchange while preventing condensation. After vernalization at 4 °C for 48 h, the plates were subjected to a 16-h light period followed by 8 h of darkness, as previously described [15], using a tunable LED lighting system source (PHYTOFY RL 150 W, Osram, Munich, Germany) that 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of light at a controlled temperature of 22 °C ($\pm 1.5^\circ\text{C}$). Morphological parameters were monitored throughout the experiment time (12 days), and the Final Germination Percentage (FGP) was evaluated at the end. On the final day (day 12), the seedlings were collected in liquid nitrogen and stored at -80 °C until subsequent biochemical and molecular analyses were performed.

2.2. Hydrogen peroxide (H₂O₂) quantification

The H₂O₂ content was quantified using a MAK311 Peroxide Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). For each experimental condition, 50 mg of germinated seedlings were sampled and ground into fine powder using liquid nitrogen. The powder was extracted in a 1:10 (w/v) ratio with milli-Q water and centrifugated at 15,000 g for 5 min. H₂O₂ content was determined incubating the supernatant with the detection reagent and monitoring the reaction at 600 nm for 30 min, as previously reported [36].

2.3. Proline quantification

The total proline content was quantified using a previously

Table 1

Experimental conditions used to evaluate the effects of the BDP-containing formulation on *Arabidopsis thaliana* seedlings under control, salt stress (SS), and osmotic stress (OS) conditions. Stress conditions were simulated using either 100 mM NaCl (SS) or 150 mM mannitol (OS), with or without the addition of the BDP-containing formulation at varying concentrations. Each row represents a specific experimental setup, with details on the concentration of NaCl, mannitol, and the BDP-containing formulation (reported as dry weight). The table also includes the final concentration of the formulation, the number of plates (n°), the volume per plate (mL), the number of seeds per plate (n°), and the condition label used to identify each treatment. All plates contained MS medium with 0.1 % (w/v) sucrose and 0.8 % (w/v) plant agar as a base.

NaCl (mM)	Mannitol (mM)	BDP-containing formulation (mg d.wt)	Final concentration	Plate (n°)	Volume Plate (mL)	Seeds (n°)	Condition Labelling
-	-	-	0	5	70	30	ctrl
-	-	14	0.20 mg/mL	5	70	30	0.20 mg/mL
-	-	7.0	0.10 mg/mL	5	70	30	0.10 mg/mL
-	-	1.4	0.02 mg/mL	5	70	30	0.02 mg/mL
100	-	-	0	5	70	30	SS
100	-	14	0.20 mg/mL	5	70	30	SS + 0.20 mg/mL
100	-	7.0	0.10 mg/mL	5	70	30	SS + 0.10 mg/mL
100	-	1.4	0.02 mg/mL	5	70	30	SS + 0.02 mg/mL
-	150	-	0	5	70	30	OS
-	150	14	0.20 mg/mL	5	70	30	OS + 0.20 mg/mL
-	150	7.0	0.10 mg/mL	5	70	30	OS + 0.10 mg/mL
-	150	1.4	0.02 mg/mL	5	70	30	OS + 0.02 mg/mL

established method [1], with minor modifications [2]. Specifically, 0.1 g of germinated seeds from each treatment were homogenized in 0.5 mL of 3 % (w/v) sulfosalicylic acid and centrifuged at 5000 g for 10 min at 4°C. The resulting supernatant was incubated in a solution containing 1 % (w/v) ninhydrin in 2 mL of glacial acetic acid and heated for 30 min at 80°C. After cooling, the absorbance was monitored at 520 nm. Proline content was quantified and expressed as micromoles per gram of fresh weight ($\mu\text{mol g}^{-1}$ FW) using an external calibration curve prepared with pure proline (Sigma-Aldrich, Milan, Italy).

2.4. Activity of antioxidant enzymes

Seeds that had germinated under different experimental conditions were ground and homogenized using a mortar and pestle. The resulting material was then extracted in a solution containing 62.5 mM Tris-HCl (pH 7), 10 % (v/v) glycerol, 2 % (w/v) Sodium Dodecyl Sulfate (SDS), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Following centrifugation at 5000 g for 10 min at 4°C, the supernatant was aliquoted for enzymatic assays [28]. The total protein concentration was determined using the method described by Lowry et al. Fiorillo et al., [13] and was subsequently used to standardize the enzymatic activities. The activities of Superoxide Dismutase (SOD; ab65354), Catalase (CAT; ab83464), Glutathione Peroxidase (GPX; ab102530), and Glutathione Reductase (POX; ab155895) were evaluated using previously described protocols [28].

2.5. RNA preparation, cDNA cloning and qRT-PCR assays

Total RNA was isolated and purified using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), with its quality and quantity assessed via a nanospectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). Starting with 500 ng of total RNA, cDNA was synthesized for quantitative real-time polymerase chain reaction (qRT-PCR) analyses using random primers and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's protocol [29]. The qRT-PCR assays were performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) utilizing SYBR green I and ROX as internal loading standard. Each run was followed by a melting curve analysis ranging from 55 to 95°C. mplification plots were analyzed using MX3000PTM software to determine the Ct values for reference genes (*yls8*, *glox*, *pex4*, *ppr*, and *ubq*) as well as target genes associated with antioxidant balance (*CuZnSOD*, *MnSOD1*, *MnSOD2*, *MnSOD3*, *CAT1*, *CAT2*, *CAT3*, *GPX1*, *GPX2*, *GPX3*, *GPX4*, *GR1*, and *GR2*) and proline metabolism (*P5CS1*, *P5CS2*, *P5CR*, *OAT*, *P5CDH*, and *PDH*). The expression analysis of target genes included an assessment of the stability of reference genes under

various experimental conditions (Pfaffl, 2001). Primers for the qRT-PCR were designed using the Primer-BLAST software and are detailed in our previous work [15].

2.6. Statistical analysis

Quantitative results obtained from chemical, biochemical, and molecular assays were presented as mean $\pm$ standard deviation (SD). Data analysis was performed using ANOVA, followed by Tukey's post-hoc test where applicable. Statistical significance was established with a threshold of $P \leq 0.05$ to identify differences among the various treatments. All statistical analyses were performed using SPSS Statistics 28.

3. Results and discussion

In a previous study, we assessed the phytostimulatory effects of BDP-containing formulation on *A. thaliana* seedling germination, identifying both cytotoxic concentrations (>1.00 mg/mL) and concentrations that positively influenced growth [15]. We found that within the optimal range of 0.02–0.20 mg/mL, the formulation effectively promoted root system development and increased fresh biomass [15]. However, this prior research was limited to evaluating seedlings under standard growth conditions, without considering the impact of abiotic stressors. In the current study, we investigated whether the novel biostimulant could enhance seedling resilience under salt (SS) or osmotic (OS) stress, experimentally induced by 100 mM NaCl or 150 mM Mannitol, respectively. Along with these stress conditions, we also included normal growth scenarios, where seedlings were neither stressed nor treated with BDP-enriched formulation (Fig. 1). Consistent with previous findings, the formulation led to a + 125 % increase in seedling development compared to untreated controls, with the most significant effect observed at 0.20 mg/mL [15]. This effect was apparent early on, with statistical differences ($P < 0.05$) emerging from day 5 (Fig. 1, Panel A). As observed before, enhanced seedling growth also resulted in increased biomass accumulation by the end of the experiment (Fig. 1, Panel B), with a final biomass increase of + 133 % in seedlings treated with BPD-enriched biostimulant. The lowest increase was observed at 0.02 mg/mL (+126 %), and the highest at 0.20 mg/mL (+144 %) (Fig. 1, Panel B).

3.1. Treatments with BDPs-containing biostimulant enhanced *Arabidopsis* seedling growth and biomass under salt and osmotic stress conditions

In relation to stress conditions, OS or SS significantly affected root development in seedlings (Fig. 1, Panels C and D). Seedlings simply grown under OS or SS conditions displayed inhibited development

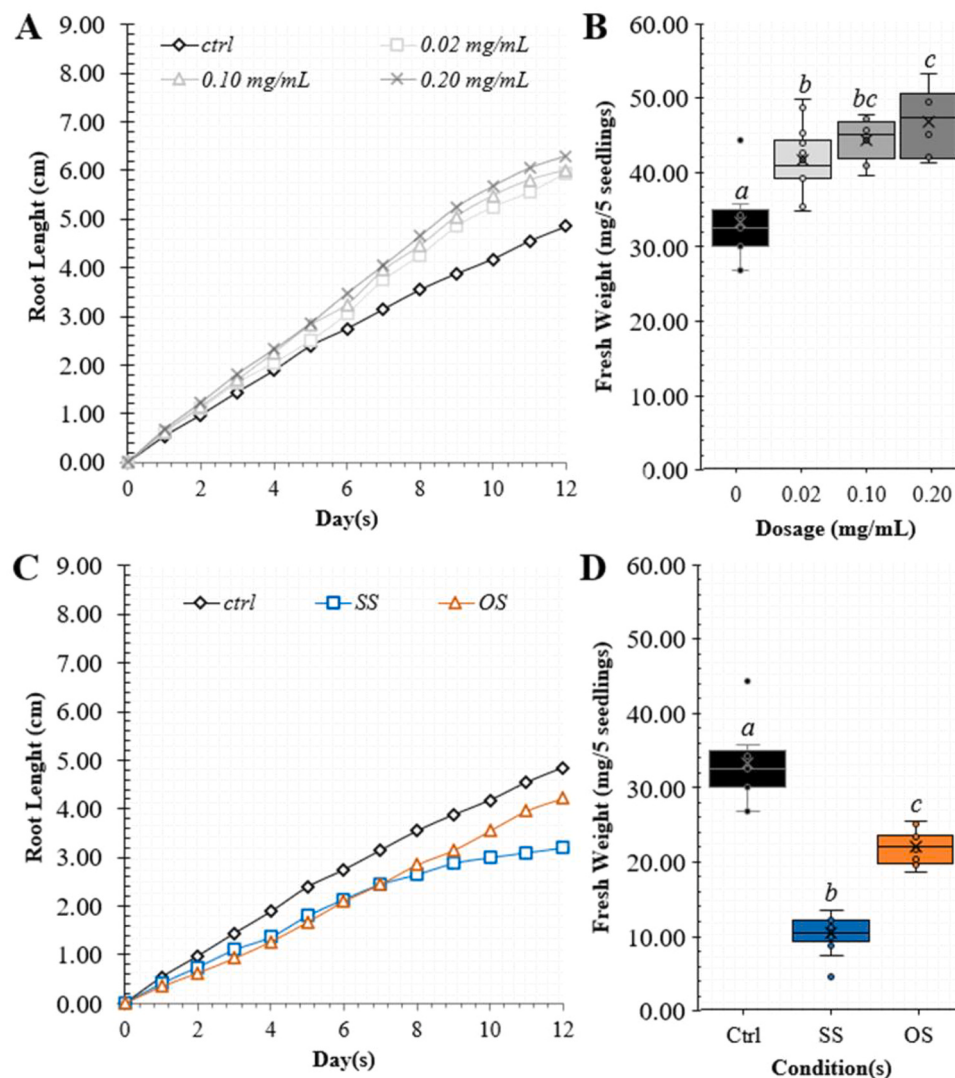


Fig. 1. Effects of BDP-Enriched Biostimulant and Stress Conditions on Root Length and Biomass in *Arabidopsis thaliana* Seedlings. Panel A illustrates Root Length (cm) measured consistently throughout the experiment in response to different concentrations (grey lines, 0.02 – 0.20 mg/mL) of the BDP-enriched biostimulant, compared to seedlings grown under standard conditions without the inclusion of BDP-enriched biostimulant (black line). Panel B presents Fresh Biomass (mg per 5 seedlings) assessed at the end of the trial under the same experimental conditions and in comparison with seedlings grown under standard conditions without the inclusion of BDP-enriched biostimulant (black box). Panel C shows the effects of Salt Stress (blue line) and Osmotic Stress (orange line) induced experimentally by 100 mM NaCl and 150 mM mannitol, respectively. Panel D displays Fresh Biomass (mg per 5 seedlings) measured at the trial's conclusion for Salt Stress (blue box) and Osmotic Stress (orange box) compared to untreated controls (black box). In Panel B and D, for each box, horizontal black lines signify median values, with boxes extending from the 25th to the 75th percentile of the value distribution in each group. Extended vertical lines indicate standard deviations. Lowercase letters on the boxes indicate statistical differences among the experimental conditions, as determined by one-way ANOVA followed by Tukey's test. Raw data are included in [Table S1](#) and [Table S2](#).

compared to control. In particular, under SS conditions, the seedlings exhibited 30 % growth reduction throughout most of the experimental period, with the inhibition becoming progressively more severe over time, peaking at around 40 % by the 10th day (blue trend line in [Fig. 1](#), Panel C). A similar trend was also observed under OS. Initially, these seedlings exhibited a more pronounced growth inhibition of 36 % compared to both the control and SS groups; however, by the 8th day, they displayed slight improvement, with the growth inhibition percentage decreasing to 25 % (orange trend line in [Fig. 1](#), Panel C). This improvement resulted in 131 % increase in root development over time compared to SS, as well as greater biomass. In particular, under SS a 70 % reduction in biomass was observed in comparison to the control, while those exposed to OS showed a lesser reduction of 30 % ([Fig. 1](#), Panel D). The variations in seedling development between SS and OS can be explained by the distinct physiological responses of plants to these stresses. Both SS and OS lead to a reduction in soil water potential and

hinder the plants' ability to absorb water, which results in a rapid decline in the rate of cell expansion in developing tissues. This slowdown impedes the flow of assimilates to the plant's meristematic and growing tissues, ultimately affecting root development [[19,26,33](#)]. However, over time, salt can exert an additional inhibitory effect on growth. When excessive amounts of Na^+ or Cl^- penetrate the seedlings, they may reach toxic levels, causing further damage. These effects can be quantitatively assessed over time, as demonstrated in a previous laboratory study that indicated a biphasic impact on plant growth under constant salt level [[14,25,26](#)]. Initially, the slowing of growth is attributed to external salt effects, where neither Na^+ nor Cl^- accumulates in growing tissues at concentrations that inhibit growth. This occurs because meristematic tissues primarily receive most of their nutrients through the phloem, which effectively excludes salt. Furthermore, rapidly elongating cells can sequester salt from the xylem into their expanding vacuoles. However, with prolonged exposure to salts, a

second phase emerges, characterized by growth inhibition due to the toxic effects of internalized salt. The amount of accumulated salt exceeds the cells' ability to compartmentalize it within vacuoles [25,33]. Consequently, in order to evaluate whether incorporating the formulation containing BDP-related compounds could alleviate the negative effects of SS or OS conditions, we monitored the development of seedlings throughout the same experimental period (Fig. 2). Seedlings subjected to SS and supplemented with the formulation enriched in BDP-related compounds exhibited growth improvements only at concentrations of 0.10 mg/mL and 0.20 mg/mL. Specifically, these two conditions showed growth increases of 125 % and 150 %, respectively (Fig. 2, Panel A). In line with this trend, the biomass measured on the 14th day showed a significant statistical improvement ($P < 0.05$) for seedlings treated with 0.20 mg/mL under SS, with their total fresh biomass nearly double that of untreated seedlings subjected to the same type of stress. In contrast, seedlings treated with the lowest

concentration did not demonstrate any significant effects on growth performance. Instead, an intermediate response was observed for seedlings treated with 0.10 mg/mL (Fig. 2, Panel B). Notably, among the three tested concentrations, only the treatment with 0.20 mg/mL of the extract containing BDP-related compounds enabled the seedlings to attain a development and final fresh weight that were fully comparable to those of the control.

Concerning OS, the addition of the formulation containing BDP-related compounds to the growth media did enhance growth performance; however, this effect was more moderate compared to what was observed in SS seedlings and did not appear to be dose-dependent (Fig. 2, Panel C). Specifically, all tested concentrations resulted in an approximate 110 % increase in seedling development, with the highest concentration reaching a peak of 116 % (Fig. 2, Panel C). As expected, this enhanced growth performance led to a statistically significant ($P < 0.05$) 1.5-fold increase in final fresh biomass by the 14th day of

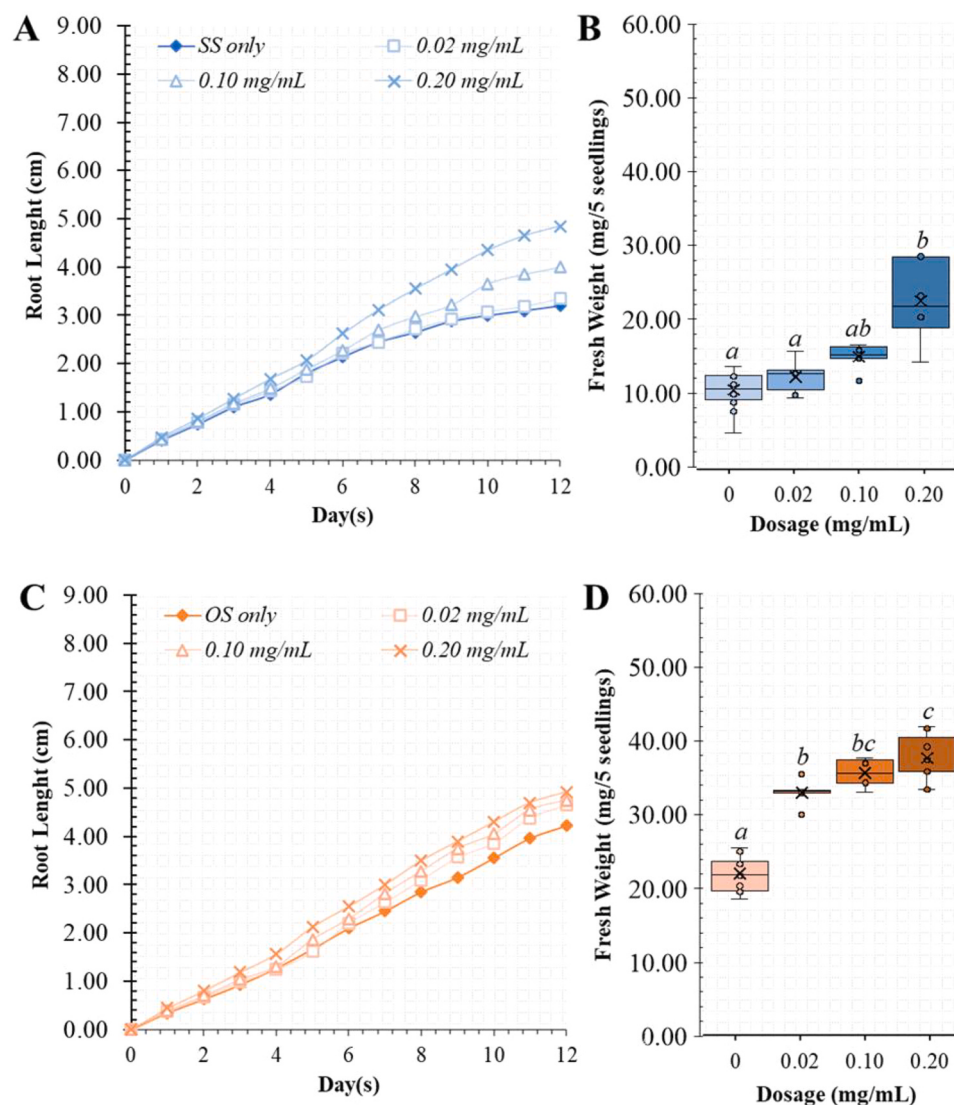


Fig. 2. Root development and fresh biomass of *Arabidopsis thaliana* seedlings grown under salt (SS) and osmotic (OS) stress condition. Panel A illustrates Root Length (cm) measured consistently throughout the experiment of seedlings grown under Salt Stress (SS only) and in response to different concentrations (light blue lines, 0.02 – 0.20 mg/mL) of the BDP-enriched biostimulant, compared to untreated controls (blue line). Panel B presents Fresh Biomass (mg per 5 seedlings) assessed at the end of the trial under the same experimental conditions. Panel C illustrates Root Length (cm) measured consistently throughout the experiment of seedlings grown under Osmotic Stress (OS only) and in response to different concentrations (light orange lines, 0.02 – 0.20 mg/mL) of the BDP-enriched biostimulant, compared to untreated controls (orange line). Panel D presents Fresh Biomass (mg per 5 seedlings) assessed at the end of the trial under the same experimental conditions. In Panel B and D, for each box, horizontal black lines signify median values, with boxes extending from the 25th to the 75th percentile of the value distribution in each group. Extended vertical lines indicate standard deviations. Lowercase letters on the boxes indicate statistical differences among the experimental conditions, as determined by one-way ANOVA followed by Tukey's test. Raw data are included in Table S1 and Table S2.

growth (Fig. 2, Panel D). Interestingly, unlike the treatment under SS, seedlings grown under this condition and treated with the same concentrations of the formulation not only showed similar growth improvement but also restored their growth and biomass levels to those of seedlings grown under unstressed conditions without treatment with the biostimulant (Fig. 1).

3.2. Inclusion of the formulation containing BDPs balanced ROS dynamics in *Arabidopsis* seedlings under abiotic stress

In *Arabidopsis* seedlings subjected to abiotic stress, significant disruption in cellular homeostasis often occurs, leading to an increased production of reactive oxygen species (ROS). These ROS, which include superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$), serve as a crucial signaling molecules that coordinate a cascade of molecular events in response to stress [20]. Beyond their role in metabolic alterations, ROS function as secondary messengers that

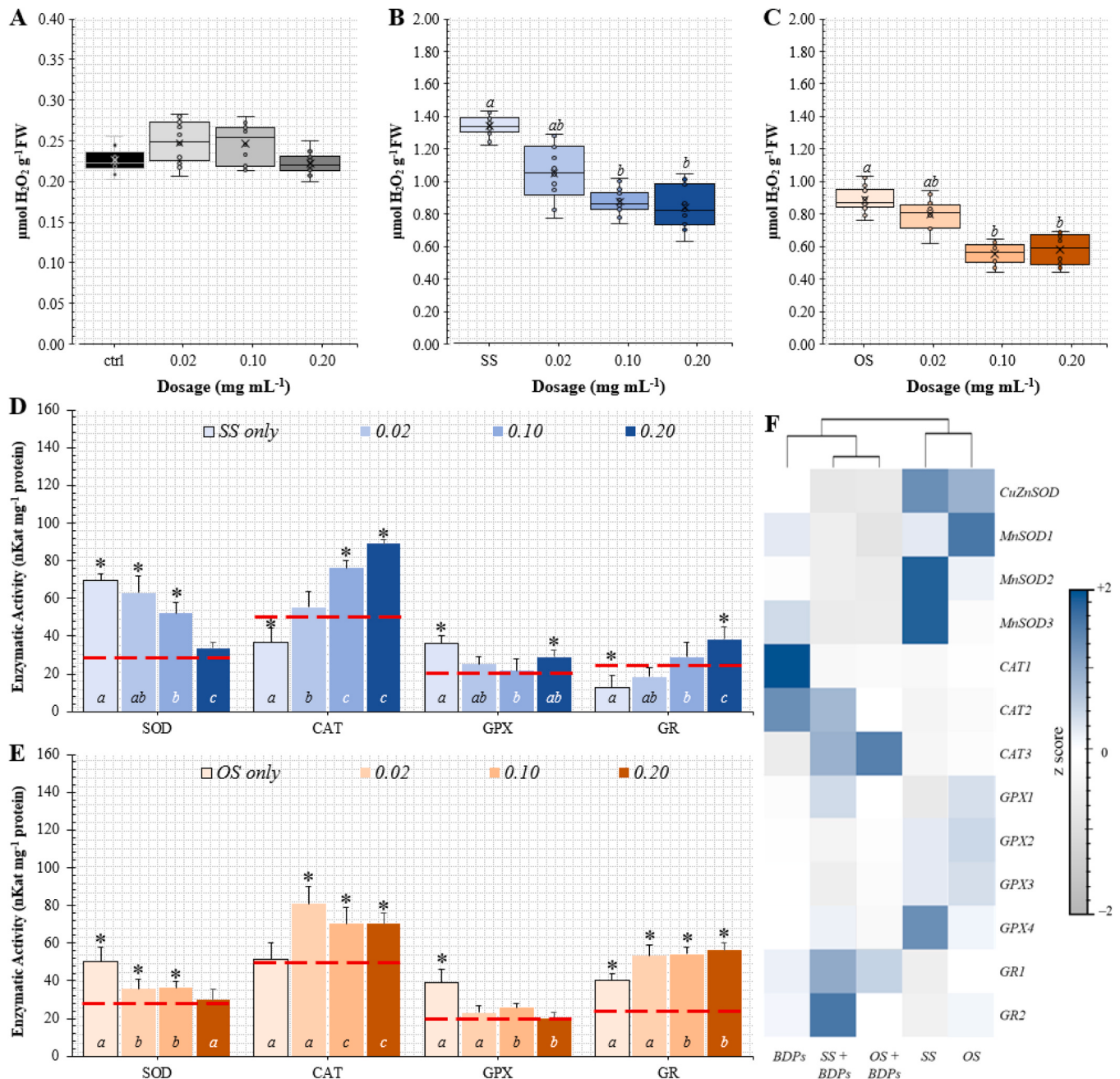


Fig. 3. Cellular redox balance in *Arabidopsis thaliana* seedlings grown under standard conditions or stress conditions and in response to BDPs. Total hydrogen peroxide (H_2O_2) content in *Arabidopsis* seedlings subjected to standard conditions (Panel A), salt stress (Panel B), or osmotic stress (Panel C) with varying concentrations of biostimulant enriched in betanin degradation products (BDPs) (0.02 – 0.20 mg/mL) compared to untreated controls. In the box plots, horizontal black lines represent median values, while boxes indicate the interquartile range (25th to 75th percentile). Extended vertical lines reflect standard deviations. Panels D and E illustrate the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) under salt stress (Panel D) or osmotic stress (Panel E) measured in nanokatal (nKat) per mg of protein. Panel F displays the expression levels of different isoforms of antioxidant enzymes, normalized to a reference gene and reported as Fold Change values calculated using the $\log_2\Delta\Delta Ct$ method in comparison to untreated seedlings.

activate various stress response pathways essential for plant adaptation and survival in challenging environmental conditions [41]. The interplay between ROS production and scavenging mechanisms significantly affects the cellular redox state, thereby influencing the outcomes of stress responses. While moderate levels of ROS are essential for triggering stress acclimation mechanisms, excessive accumulation can lead to oxidative damage to biomolecules, impairing cellular functions and ultimately threatening plant viability [47]. In our previous study, we demonstrated that the addition of a BDP-containing formulation to the seedling growth medium influences the balance of ROS through specific mechanisms. Notably, we found that doses exceeding 1.00 mg/mL resulted in an excessively oxidized cellular environment by upregulating genes related to H_2O_2 production while simultaneously downregulating detoxification systems. In contrast, sub-cytotoxic doses (less than 1.00 mg/mL) led to the upregulation of the same H_2O_2 production genes, but this was accompanied by a concurrent increase in the activity of catalase (CAT) and the glutathione scavenging system (GPX-GR). This coordinated response effectively maintained stable levels of cellular oxidation, thereby preventing excessive oxidative stress [15]. These findings were further validated in the current study (Fig. 3, Panel A). Building on these findings, we aim to investigate the effect of SS or OS on cellular antioxidant machinery and to determine whether the incorporation of non-cytotoxic doses of the formulation containing BDP-related compounds can protect seedlings during their development. Consistent with existing literature, our results showed that seeds exposed to both SS and OS exhibited increased H_2O_2 levels, with a more pronounced effect under SS conditions, where the H_2O_2 level increased 6-fold compared to a four-fold increase under OS (Fig. 3, panels B and C). The application of BDP-containing biostimulant positively influenced the redox balance in both stress scenarios. Under OS, the BDP-containing biostimulant effectively reduced ROS levels across all tested concentrations, demonstrating consistent efficacy. In contrast, under SS conditions, a dose-response effect was observed, with the highest concentration of the formulation proving to be the most effective in reducing ROS levels. Conversely, the lowest concentration did not show any statistically significant improvement compared to untreated SS conditions (Fig. 3, Panel B and C).

The production and scavenging of ROS are typically the results of various enzymatic activities. Among these enzymes, SOD plays a critical role by directly converting H_2O_2 through dismutation, thereby neutralizing radical threats from other oxidized molecules. The resulting H_2O_2 can then be scavenged by CAT, which independently reduces H_2O_2 into water and O_2 , or by the combined GPX-GR system. In this case, GPX functions similarly to CAT, using GSH as electron acceptor, while GR is responsible for regenerating glutathione into its reduced form. This combined system operates effectively only when both enzymes are fully functional and sufficiently reduced glutathione, allowing for a more efficient detoxification of the H_2O_2 [17]. In our experimental conditions, the observed increase in H_2O_2 in both SS and OS is directly linked to enhanced SOD activity, as demonstrated by biochemical assays (Fig. 3, Panels D and E). The primary difference between SS and OS lies in the types of SODs that are activated in response to each stress and their respective subcellular localization. In SS, there is a notable increase in the production of *CuZnSOD* and mitochondrial SODs (*MnSOD2* and *MnSOD3*). *CuZnSOD*, which is localized on the outer mitochondrial membrane, primarily targets oxidative stress in the cytoplasm [8,21,42]. In contrast, *MnSOD2* and *MnSOD3*, which are initially found in the cytosol and apoplast, are transported to the mitochondria and activated only under condition of prolonged and excessive oxidative stress [42]. Our findings suggest that seedlings subjected to SS experience sustained oxidative damage, with the mitochondria serving as the primary site of ROS production (Fig. 3, Panel F). Conversely, OS primarily induces upregulation of *CuZnSOD* and *MnSOD1*, while other SODs show only minimal regulation (Fig. 3, Panel F). This suggests that the oxidative challenge associated with OS is less severe than that of SS, and that the oxidative stress is mainly confined to extramitochondrial compartments.

Another factor influencing the changes in H_2O_2 levels during the seedling development under SS or OS is the activity of the CAT and GPX-GR system. Under SS conditions, although GPX activity increased, there was a corresponding decrease in GR function, which reduced the overall effectiveness of the scavenging system (Fig. 3, Panel D). Additionally, CAT activity was significantly reduced (Fig. 3, Panel D), further contributing to the increased level of ROS (Fig. 3, Panel B). In contrast, under OS conditions, CAT activity was maintained at levels similar to those of the control, while the GPX-GR system was upregulated. This combination of factors led to a more favorable cellular redox balance in OS compared to SS, resulting in lower levels of H_2O_2 (Fig. 3, Panel B and C) and improved seedling development (Fig. 2). Consistent with the enzyme activity data, CAT isoforms were slightly downregulated at the transcriptional level in SS, whereas they remained unchanged in OS (Fig. 3, Panel F). Additionally, the transcriptional levels of GPX varied significantly depending on the stress type. Specifically, *GPX1*, *GPX2* and *GPX3* were most upregulated in seedlings subjected to OS, while *GPX4* showed strong upregulation in seedlings exposed to SS (Fig. 3, Panel F). These GPX isoforms have different subcellular localizations and functions: *GPX1* is mainly cytoplasmic, *GPX2* is mitochondrial, and *GPX3* is peroxisomal. Consequently, these isoforms are specialized to optimize the efficiency of specific physiological processes, including photosynthesis, mitochondrial respiration, or photorespiration [27,35]. In contrast, *GPX4* is localized on the plasma membrane and sometimes in the nucleolus, with its transcription primarily induced by prolonged stress conditions that lead to lipid peroxidation [23,27]. Therefore, the qRT-PCR data indicate that the modulation of GPX in SS and OS serves different functions. In SS, GPX is upregulated to mitigate redox threats that could cause significant damage on cellular structures, while in OS, it plays a role in restoring the redox balance resulting from enhanced physiological processes (Fig. 2, Panel F). Finally, transcriptional data indicated that both GR isoforms, *GR1* and *GR2*, were downregulated in SS, while OS led to a slight upregulation of *GR2* (Fig. 2, Panel F). In seedlings subjected to SS or OS and treated with a BDP-containing formulation, a significant decrease in SOD activity was observed (Fig. 1, Panels D and E). This reduction in SOD activity was associated with a downregulation of all four analyzed SOD isoforms, as demonstrated by qRT-PCR analysis (Fig. 1, Panel F). While SS exhibited a concentration-dependent effect, the reduction in SOD activity was evident in seedlings treated with the biostimulant under both SS and OS conditions. Furthermore, the treatment enhanced the efficiency of CAT and the GPX-GR system. Specifically, CAT activity was enhanced to be up to four times more effective in seedlings treated with 0.20 mg/mL of the formulation under SS, and about twice as effective under OS. Although no significant differences in GPX activity were observed, the treatment facilitated H_2O_2 detoxification by increasing transcriptional levels and GR activity, thereby effectively restoring glutathione necessary for redox reactions (Fig. 3, Panel F).

3.3. The treatment with BDP-containing formulation affected proline dynamics and regulatory machinery in *Arabidopsis* seedlings grown under salt or osmotic stress conditions

Proline is a multifunctional amino acid that plays a pivotal role in plant responses to stress, especially under SS or OS. Its accumulation is a key adaptive strategy that helps plants mitigate the harmful effects of environmental stressors. As an osmoprotectant, proline functions as a compatible solute, facilitating osmotic adjustment to maintain cellular water balance and turgor pressure [5,49]. Beyond its osmoprotective functions, proline also participates in diverse regulatory functions within stress signaling pathways. It interacts with various stress-responsive genes and transcription factors, thereby affecting gene expression patterns related to stress adaptation and tolerance [18,52,56]. In our previous study, we demonstrated that the addition of a BDP-containing formulation to the culture medium significantly improved the growth of *Arabidopsis* seedlings, particularly at

concentrations below 1.00 mg/mL [15]. While the overall proline levels in these seedlings were comparable to those in the control group, a significant increase in proline production was observed specifically in the mitochondria, as evidenced by the upregulation of the *OAT* gene (Fig. 4, Panel D). This mitochondria-specific increase in proline synthesis is particularly beneficial, as it suggests that proline is being produced to support improved seedling development and to mitigate ROS at the mitochondrial level, rather than serving as osmolyte regulator [32, 52]. Furthermore, this increase in mitochondrial proline synthesis was accompanied by enhanced catabolism at the cytoplasmic level, involving *PDH1* and *P5CDH*, which facilitated a balanced turnover of osmolytes within the cell. In our current study, we further confirmed these observations. Specifically, at the tested concentrations (0.02 – 0.20 mg/mL), the biostimulant did not alter the total proline levels (Fig. 4, Panel A); however, it did result in increased *OAT* expression, along with a statistically significant upregulation of *PDH1* and *P5CDH* (Fig. 4, Panel D). Building on these results, we aim to investigate the effects of SS and OS on proline metabolism and catabolism, specifically examining the role of the biostimulant enriched in BDP-related

compounds under these stress conditions. Our data suggest that seedlings actively regulate proline biosynthesis pathways in response to SS and OS, resulting in a threefold increase in proline content (Fig. 4, Panel B), which subsequently accumulates in the cytoplasm and vacuoles. The increased proline levels are primarily driven by a significant upregulation of genes involved in its cytoplasmic metabolism, particularly *P5CS1* and *P5CS2*. These genes encode enzymes that catalyze the rate-limiting step in proline biosynthesis, converting glutamate to glutamate-5-semialdehyde, which is subsequently converted to proline by *P5CR*. Since *P5CR*, *P5CS1*, and *P5CS2* are primarily localized in the cytoplasm, they play a crucial role in proline synthesis in response to osmotic stress [52,53]. Furthermore, the upregulation of these metabolism-related genes, along with the downregulation of genes related to proline catabolism-related genes (*P5CDH* and *PDH*), is essential for regulating proline synthesis in the cytoplasm. This regulation allows the plant to accumulate proline as an osmolyte within this cellular compartment (Fig. 4, Panel D). Additionally, proline functions as a molecular chaperone, helping to stabilize proteins and membranes under stress conditions. Its well-documented ability to mitigate ROS also

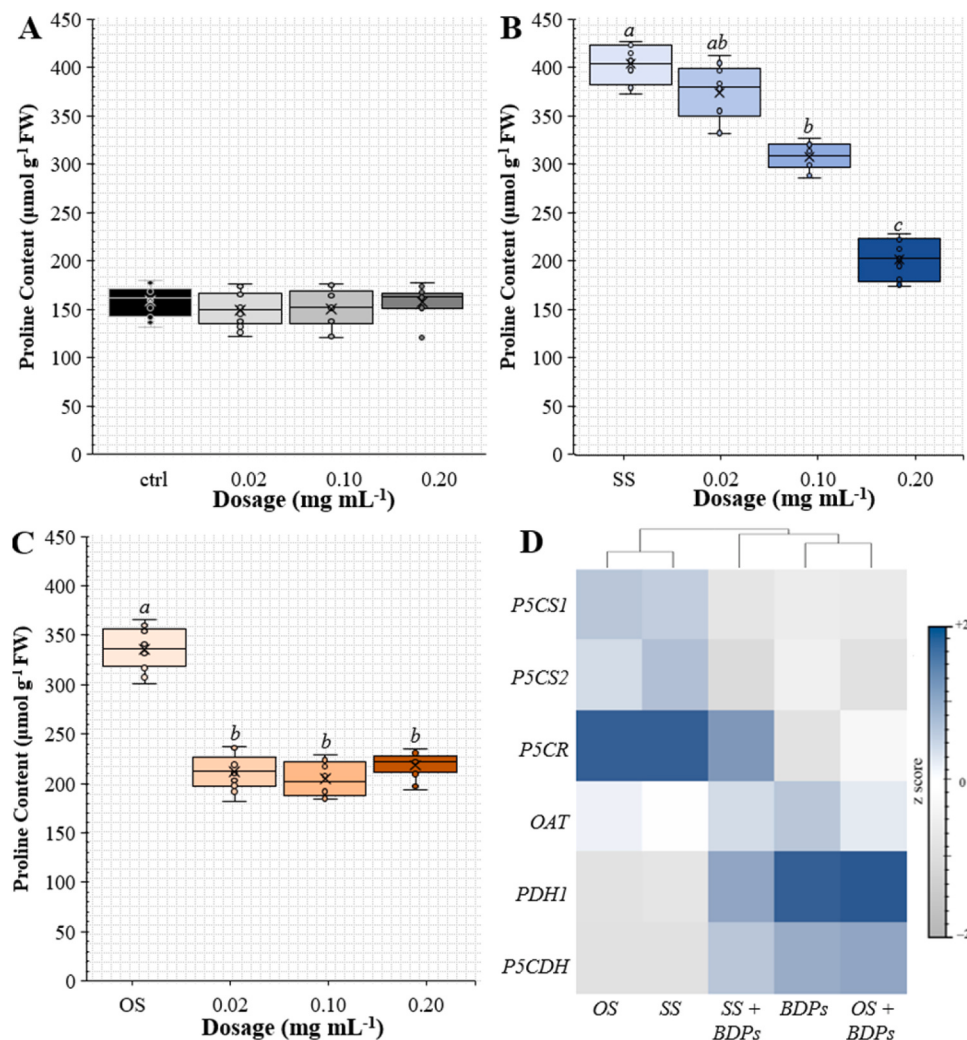


Fig. 4. Proline Content and Regulation in *Arabidopsis thaliana* Seedlings grown under stress conditions and in Response to BDPs. Panel A shows the total proline content in *Arabidopsis* seedlings grown under standard conditions and after the treatment with biostimulant enriched in betalain degradation products (BDPs) (0.02 – 0.20 mg/mL). Panel B and C presents the total proline content of *Arabidopsis* seedlings grown under the same experimental condition but subjected to salt (Panel B) or osmotic (Panel C) stress. For each box, horizontal black lines signify median values, with boxes extending from the 25th to the 75th percentile of the value distribution in each group. Extended vertical lines indicate standard deviations. Lowercase letters on the boxes indicate statistical differences among the experimental conditions, as determined by one-way ANOVA followed by Tukey's test. Panel D reports the expression of various genes involved in proline metabolism (*P5CS1*, *P5CS2*, *P5CR*, and *OAT*) and catabolism (*PDH* and *P5CDH*). Gene expression was normalized on the reference gene and reported as Fold Change values calculated using $\log_2^{\Delta\Delta Ct}$ on untreated seedlings.

plays a significant role in reducing oxidative damage, thereby enhancing cellular resilience to salt-induced oxidative stress [18,46]. However, the upregulation of the proline synthesis pathway in the cytoplasm did not coincide with the regulation of the *OAT* gene, which is generally associated with proline production at the mitochondrial level (Fig. 4, Panel D). When proline is synthesized through this pathway, it primarily functions as an antioxidant molecule, improving the efficiency of the electron transport chain and promoting overall development. The absence of *OAT* regulation suggests that the increased proline levels in seedlings are not meant to support normal physiological functions in mitochondria; instead, they serve as an osmolyte or antioxidant molecule within the cytoplasm [11,38].

The application of the biostimulant enriched in BDP-related compounds to seedlings grown under SS or OS resulted in increased proline levels. Notably, under SS conditions, proline content decreased as the concentration of the formulation in the growth medium increased, with the most significant effects observed at 0.20 mg/mL, where proline levels were approximately half of those observed in seeds grown under SS without the treatment (Fig. 4, Panel B). A similar pattern was also observed in experiments conducted under OS conditions (Fig. 4, Panel C). In this case, all tested concentrations effectively reduced proline levels, returning them to values comparable to those of the untreated and unstressed control ($154.58 \pm 23.14 \mu\text{g/g FW}$). Interestingly, in seedlings subjected to OS or SS without treatment with the BDP-enriched formulation, the increased proline content was associated with enhanced transcription of *P5CS1*, *P5CS2*, and *P5CR*. However, when the formulation was added to the growth medium, the transcription of these enzymes was slightly downregulated compared to the control conditions. At the same time, a significant upregulation of *OAT* was observed, along with increased transcripts expression of the two main enzymes involved in proline catabolism, *PDH* and *P5CDH* (Fig. 4, Panel D). *PDH* and *P5CDH* are key enzymes in the proline degradation pathway. *PDH* catalyzes the first step in proline catabolism by converting proline into pyrroline-5-carboxylate (P5C), which is subsequently oxidized to glutamate by *P5CDH* [51,55]. This process not only regulates proline levels but also enriches the cellular pool of glutamate, a critical amino acid involved in various metabolic processes [38,52]. These findings suggest that the incorporation of the biostimulant enriched in BDP-related compounds modulates proline metabolism by reducing its accumulation under stress conditions through enhanced catabolism. The upregulation of *OAT*, along with *PDH* and *P5CDH*, indicates a transition towards increased proline turnover, which may help the plant in balancing osmotic and oxidative stress by preventing excessive proline accumulation. This regulated balance likely plays a crucial role in maintaining cellular homeostasis and improving stress adaptation.

4. Conclusion

In conclusion, this study highlights the significant role of BDP-enriched formulation in enhancing the resilience of *Arabidopsis* seedling under salt and osmotic stress conditions. The application of this biostimulant not only promoted growth and biomass accumulation under normal conditions but also supported seedling development and redox balance during abiotic stress events. Our findings demonstrate that the biostimulant enriched in BDP-related compounds effectively modulated ROS dynamics, promoting antioxidant mechanisms, crucial for seedling survival in challenging environments. The use of the formulation resulted in a clear dose-dependent response in seedlings subjected to SS, with higher concentrations leading to significant reductions in ROS levels and enhanced seedling growth compared to untreated controls. Notably, the most pronounced effects were observed at a concentration of 0.20 mg mL^{-1} , under both OS and SS conditions. However, lower concentrations also exhibited a positive impact on seedling growth and resilience, suggesting their potential applicability. This concentration (0.20 mg mL^{-1}) may be particularly useful and

experimentally viable; nonetheless, adjustments will be necessary when evaluating other model plants beyond *Arabidopsis*, as well as food crops intended for agricultural use and human consumption. Additionally, BDP-containing biostimulant influenced proline metabolism, reinforcing its role as a protective osmolyte while preventing excessive accumulation that could disrupt cellular homeostasis. Overall, this study highlights the potential of BDP-containing biostimulants as an effective strategy for improving plant resilience against abiotic stresses, particularly in the context of global climate change and the need for sustainable agricultural practices. Specifically, we proposed that BDP-related compounds act as secondary messengers in the modulation of stress responses. However, we cannot entirely rule out the possibility that other minor components in the formulation may contribute to the observed effects, potentially through synergistic interactions. To address this, future research aim to isolate BDP-related compounds from the whole extract to determine their specific roles and determine their contributions relative to other components. These efforts will help clarify the molecular mechanisms underlying the observed biostimulator effects and provide important insights for future applications.

CRediT authorship contribution statement

Conceptualization: GM, NG, CMB; Methodology: GM, GS, NG; Development or of methodology: GM, CG; Software: GM, MM; Validation: GM; Formal analysis: GM; Investigation: NG, GM, CMB, GC, GS; Resources: GC, GM; Data Curation: GM, NG, GS; Writing - Original Draft: GM, GS, NG; Writing - Review & Editing: GM, CG, CMB, MM; Visualization: GM, MM; Supervision: GM, CMB, CG; Project administration: GM; Funding acquisition: GM

Declaration of Competing Interest

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2025.100438.

Data availability

Data will be made available on request.

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