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# Impact of a biostimulant enriched in betalain degradation products on ROS signaling, proline accumulation, and phytohormone homeostasis

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ABSTRACT

This study investigates the potential of a biostimulant derived from *Selenicereus undatus* peel waste and enriched in betalain degradation products (BDP), to influence *Arabidopsis thaliana* seedling development. Notably, lower BDP concentrations enhanced seedling development, while higher dosages exhibited adverse effects. Assessment of mitochondrial activity in both seeds and purified organelles showed that the tested biostimulant did not affect mitochondrial activity or integrity, highlighting its independence from mitochondrial performance. Mechanistically, BDP-enriched biostimulant modulated ROS-signaling, diminishing $H_2O_2$ by regulating the enzymatic activity and gene expression of SOD, CAT, GPX, and GR. Particularly, analyzing their different isoform via qRT-PCR, the primary cellular compartment where detoxification occurred were identified. Furthermore, biostimulant was able to influence proline-accumulation, altering both the expression of metabolism (*PC5S*, *P5CR* and *OAT*) and catabolism (*PDH* and *P5CDH*) related genes. Finally, the BDP-enriched biostimulant altered phytohormone levels, mainly affecting ABA/ABA-glu, tZea/tZea-rib, and tZea/IAA. Concerning GAs, the increase in GA4 and GA7 suggested an involvement of GA13ox, a hypothesis encouraged by qRT-PCR analysis. In summary, this study underscores the potential of BDP-based biostimulant as sustainable promoters of plant growth, influencing critical regulatory pathways during germination. Further research is necessary to explore their extensive applications in agricultural practices.

## 1. Introduction

Seed germination and subsequent seedling establishment constitute critical developmental stages in the life cycle of higher plants. The favorable conditions for growth trigger the breaking of seed dormancy, thereby initiating germination. This intricate process involves a highly orchestrated sequence of biochemical events, each meticulously regulated by a complex interplay of internal and external signals [85]. Among the internal regulators, changes in mitochondrial activity represent a fundamental internal event during seed germination. As the dormant seed transitions to an active metabolic state, there is a surge in mitochondrial respiration, providing the necessary energy for the cellular processes. This heightened mitochondrial activity indicative of the shift in specific metabolic requirements of the commencement of germination, could compromise the cellular redox balance, negative affecting the early stages of plant development [20]. Indeed, the delicate equilibrium between reactive oxygen species (ROS) production and antioxidant defense mechanisms significantly influences the success of germination. Additionally, among the internal regulators involved in seed germination, phytohormones play a key role. Indeed active interactions among auxins, gibberellins, abscisic acid, and cytokinins serve as a masterful orchestration, guiding cellular responses and exerting influence over crucial processes like cell elongation, division, and differentiation [53].

While mitochondrial activity, redox balance, and phytohormones have been recognized as essential regulators of germination, a growing body of research suggests that the regulatory network extends beyond these factors. Recent studies show the active participation of secondary metabolites, potential enhancers of various plant physiological processes, including seed germination [51]. These compounds, thanks their biostimulant action, are actually included in commercial biostimulants predominantly derived from waste products from the agri-food industry, aligning with the principles of a circular economy [54].

Pitaya (*Selenicereus undatus*) fruits, known for their peculiar

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characteristics, remarkably share a similarity with sugar beets and prickly pear in their substantial production of betalains [40,48,83]. Nevertheless, in contrast to sugar beets, which produce minimal waste at the agro-industrial scale, pitaya and prickly pears, with their substantial portion of inedible spongy peel, not only have limited utility in the food industry but also generate larger quantities of organic material for disposal [36,68].

Betalains, known as water-soluble nitrogenous pigments, confer red-violet (betacyanins) and yellow (betaxanthins) hues to select fruits and vegetables. They arise as ammonium conjugates of betalamic acid with cyclo-DOPA and amino acids or amines [40,62] and are acclaimed for their chromatic vibrancy, exert profound influences on plant physiological processes. Functioning as robust antioxidants, they mitigate cellular oxidative stress induced by environmental factors such as intense luminosity and drought conditions [62]. Additionally, betalains safeguard the photosynthetic machinery by absorbing light energy and ensuring efficient energy production [34]. Beyond cellular functions, their colours can attract pollinators, aid in plant communication, and potentially serve as a defense mechanism against herbivores, enhancing plant survival and reproduction [46].

Over time, interest in reuse of waste enriched in betalains has grown, leading to various innovative applications, including for human and animal purposes [15,44,47]. However, one of the primary challenges associated with the circular economy perspective of betalains is their inherent instability. During storage times of waste materials, betalains undergo degradation, giving rise to several degradation products (BDPs), including betalamic acids, quinones, amino-acids, and decarboxylated betalains [31,30]. These compounds, while losing the characteristic color of betalains along with a portion of their antioxidant properties, have limited applications compared to the originating predecessors [31]. Current research endeavors focus on developing strategies to enhance the stability and preserve the chemical integrity of betalains [15]. Despite these limitations, BDPs show promise in alternative fields. Notably, certain BDPs are traditionally acknowledged for their roles in various plant physiological processes. For instance, recent investigations suggest that dopamine, cDOPA and L-DOPA (three of the different degradation products of late-stage BDPs) can function as signaling molecules. They influence crucial biochemical events that guide different phases of plant growth and development [58,67,70,71], although the precise mechanisms through which they exert their effects are still being explored.

To investigate the potential effects of a biostimulant formulation enriched in BDPs on key events during seed growth and development, we used pitaya fruit peels to extract betalains and increase BDP concentration through a thermal process. We then explored potential alterations in *Arabidopsis thaliana* seeds, focusing on well-established regulators such as mitochondrial activity, ROS levels, and phytohormones. Moreover, we aim to address the gap subsisting in the both the chemical profiling of biostimulant and the underlaid mechanism, performing an accurate chemical profile of the tested biostimulant using advanced chromatographic techniques. Our research seeks to explore the potential applications of BDPs in influencing crucial events during seed growth and development. Through this comprehensive investigation, we aspire to contribute to a deeper understanding of the biochemical and molecular pathways that drive the germination process, paving the way for sustainable agricultural practices and the repurposing of agri-food waste.

## 2. Materials and methods

### 2.1. Extraction and formulation of BDP-enriched biostimulant

Fruits of *S. undatus* (var. Costa Rican Sunset) were obtained from 10-years old plants grown in Vivai Torre (Milazzo, Sicily, Italy; 38°19' N, 15°24' E; 20 m a.s.l.). Flowers at anthesis were manually pollinated during the night, and fruit harvesting was done at the stage of maturity suitable for human consumption (32 days after pollination), and specifically when the fruits were characterized from changes in elasticity and fruit coloration [23,59]. After fruit collection (three fruits from five different plants), the peel was separated from the pulp. BDPs-enriched biostimulant was obtained after extracting the 100 g of peels in 1 L of water, using a ratio of 1:10 *(w/v)*. To facilitate the degradation of betalains into their corresponding BDPs, the extraction process was performed using a close hot reflux apparatus for 1 hours. Following an hour of heat treatment, the apparatus was unsealed, facilitating water evaporation and simultaneous concentration of the formulation until the total volume decreased 10-times (occurred in an additional hour) and the final volume was meticulously measured using a graduated cylinder. After cooling, the BDPs-containing solution was filtered using type-2 Whatman filter paper having a pore diameter of 8 μm (Merk Life Science S.r.l., Milan, Italy). The BDP-enriched biostimulant obtained following this procedure was aliquoted and stored at −80°C until further analyses were performed.

### 2.2. Chemical characterization of the BDP-enriched biostimulant

Before testing the biostimulant enriched in BDPs on seed germination, a comprehensive chemical profiling was performed using an High-Performance Liquid Chromatography (HPLC; 1200 Agilent Technologies, Santa Clara, USA) coupled with Diode Array Detector (DAD; 1200 Agilent Technologies, Santa Clara, USA) and Ion Trap Mass Spectrometer (MS/MS; Bruker Daltonics, Billerica, MA, USA). The ion source was an Electron Spray Ionization (ESI). A binary gradient composed of MilliQ $H_2O$ acidified with 0.1 % *(v/v)* formic acid (solvent A) and acetonitrile acidified with 0.1 % *(v/v)* formic acid (solvent B) was employed. During the chromatographic run, the compounds were separated on a reverse phase C18 Luna column (3.00 μm, 150 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, USA) maintained at 25 °C by an Agilent 1100 HPLC G1316A Column Compartment. The running flow was 0.2 mL $min^{-1}$ while the chromatographic gradient was set to 90 % *(v/v)* A and 10 % *(v/v)* B for the starting 5 min. Subsequently, the percentage of solvent B was increased to 55 % *(v/v)* of A for 25 min, followed by a final concentration of 70 % *(v/v)* B in 25 min. To ensure the cleaning of the column before the next injection, the concentration of solvent B was gradually increased and maintained at 3 % *(v/v)* for 5 min. The injection volume of each sample was 10 μL. The UV–VIS spectra were recorded between 220 and 800 nm. The tandem mass spectrometry analyses were performed in positive mode for the Betalain and its related BDPs [19]. The nitrogen flow rate was set to 5.0 mL $min^{-1}$ and maintained at 325 °C throughout the analysis. The capillary voltage was set to ±1.5 kV. Analysis were performed in triplicate. The identification of molecules was performed through inspection of UV/Vis spectra, MS/MS fragmentation, and comparison with previously reported literature data related to BDPs [30,69]. Similar to previous studies [82,84], the quantification of betalains and BDPs was conducted using standard from our previous betalain studies [25,49,57,78,79]. Conversely, commercially available molecules (L-DOPA and Dopamine from VWR International; cyclo-DOPA from Pharmaffiliates, UK) were utilized for quantifying compounds whenever feasible.

### 2.3. In vitro germination test

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) wild-type (WT) seeds were surface-sterilized using 70 % *(v/v)* ethanol (EtOH) for 15 min. Seeds were then washed thoroughly with 100 % *(v/v)* ethanol twice. Seeds were individually plated in sterile conditions on half-strength Murashige and Skoog (MS/2) medium (pH 5.8), supplemented with 0.1 % *(w/v)* sucrose and 0.8 % *(w/v)* plant agar. To evaluate the effects of BDP-enriched biostimulant on the germination of *A. thaliana* seeds, 200 seeds per condition (40 seeds per plate with a total of 5 plates per condition) were grown in solid MS/2 medium. In order to achieve the different experimental conditions, the MS/2 medium was

supplemented either with water (control) or with different concentrations of the formulation (0.02 – 20 mg $L^{-1}$). Following the preparation of the culture media, plates were filled with MS/2 medium supplemented with the BDPs-enriched extract, and the seeds were then placed into the plates. Consequently, plates were sealed with Micropore tape to facilitate gas exchange and prevent condensation. After vernalization at 4°C for 48 hours, plates were exposed to light for 16 hours before being kept in darkness for the remaining 8, as previously described [39]. Plates were then exposed to 120 μmol $m^{-2}$ $s^{-1}$ of light provided by a tunable LED lighting system source (PHYTOFY RL 150 W, Osram, Munich, Germany) at 22°C (±1.5°C). Temperature was controlled throughout the experiment using air conditioning. Root length (RL) was continuously monitored throughout the experimental trial using a high-resolution camera (EOS 1000D, Canon, Tokyo, Japan), and images were analyzed with the ImageJ software [77]. Fresh weight (FW) and final germination percentage (FGP) were instead obtained at the end of experimentation. FGP was determined by dividing the number of germinated seeds by the total number of seeds sown and multiplying by 100 [39]. On the final day, seedlings were harvested and immediately flash-frozen in liquid nitrogen. They were subsequently stored at −80°C until biochemical and molecular analyses could be conducted.

### 2.4. Mitochondrial activity

#### 2.4.1. On purified organelles

Intact and functional mitochondria were isolated following a previously reported protocol adapted to *A. thaliana* seeds [3]. Briefly, about 150 mg of viable *A. thaliana* seeds were weighed and homogenized with a mortar and pestle using pre-cooled Grinding Extraction Buffer (0.5 M sucrose, 1 mM EDTA, 70 mM $KH_2PO_4$, pH 7.5) supplemented with 0.8 % *(w/v)* bovine serum albumin (BSA) and 0.1 % *(w/v)* β-mercaptoethanol (β-ME) just before use. Once a homogeneous mixture was obtained, it was transferred to a clean Eppendorf tube and centrifuged at 1800 g for 7 min at 4°C, to pellet nuclei, chloroplasts, and cellular debris. The supernatant was then transferred to a new and clean Eppendorf tube and centrifuged at 20,000 g for 17 min. Again, the supernatant was discarded and the pellet was resuspended in appropriate amounts of grinding buffer supplemented with BSA and β-ME by pipetting with extreme caution. To eliminate the remaining nuclei, chloroplasts, and cellular debris, the suspension was centrifuged again at 1800 g for 7 min and the supernatant transferred to a new Eppendorf tube, discarding the pellet. The obtained supernatant was centrifuged twice: (i) once at 4000 g for 20 min to pellet residual chloroplasts, (ii) a second time at 20,000 g for 17 min to pellet mitochondria. The mitochondrial pellet was then resuspended with extreme caution by pipetting slowly up and down several times in 300 μL of Grinding Medium supplemented with BSA, and in the absence of β-ME. The suspension was then centrifuged at 2000 g for 5 min and the supernatant transferred to a new tube. To further purify the mitochondria, three serial centrifugations at 20,000 g for 17 min were performed, resuspending the pellet each time with fresh and clean Grinding Medium supplemented with BSA.

To assess mitochondrial activity following treatment with the BDP-enriched biostimulant, the previously purified mitochondria were seed in a 96-multiwell plate along with a 0.5 mg $mL^{-1}$ MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and a properly diluted solution of the biostimulant using a 1:1:1 (*v/v/v*) ratio. Following a 30-minute incubation at 30°C, 2 volumes of isopropanol were added at room temperature for 20 min [37]. The final absorbance was measured at 570 nm using a microplate reader (NB-12–0035, Neo Biotech, Nanterre, France).

#### 2.4.2. On A. thaliana seeds

Mitochondrial activity was also tested on healthy seeds of *A. thaliana* following the previously published protocol [63], with some modification. Briefly. approximately 20 mg of *A. thaliana* seeds were sterilized for 10 min using a mixture of 70 % *(v/v)* ethanol supplemented with 0.01 % *(v/v)* Triton-X. Subsequently, they were washed with sterile water and resuspended at a density of 10 g $L^{-1}$ in 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) with the addition of 0.1 % *(w/v)* plant agar (pH adjusted to 7.5). Fifty microliters of this suspension (equivalent to approximately 10–12 seeds) were dispensed into the wells of a 96-multiwell plate, and the seeds were allowed to stratify for 48 hours at 4°C. After stratification, 50 μL of a solution containing the biostimulant at various concentrations (1 – 1000 μg $mL^{-1}$) were added to the wells, and the plate was incubated at 24°C for 72 hours. Following incubation, the test solution was removed from each well, taking care to leave the seeds intact at the bottom. Subsequently, 100 μL of 5 mg $mL^{-1}$ MTT was added to each well and incubated for an additional 24 hours in the dark. Consequently, MTT solution was removed and the seeds were incubated for 24 hours at 30°C on an orbital shaker with 200 μL of a lysis buffer containing 10 % *(v/v)* tween-20, 40 % *(v/v)* dimethyl sulfoxide (DMSO), and 40 mM HCl in isopropanol. At the end of the incubation period, the content of each well was transferred to a new 96-well multiwell plate, and the absorbance was read at 570 nm using a plate reader (NB-12–0035, Neo Biotech, Nanterre, France).

### 2.5. Hydrogen peroxide ($H_2O_2$) quantification

The determination of $H_2O_2$ content was conducted utilizing a MAK311 Peroxide Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) [60]. 50 mg of seedlings grown on solid MS/2 medium containing varying concentrations of the BDP-enriched biostimulant (as detailed in Section 2.3) were pulverized using liquid nitrogen and then extracted in a 1:10 *(w/v)* ratio with mQ water. Following centrifugation at 15,000 g for 10 min, the resulting supernatant was employed for the assay. In a 96-well plate with a flat bottom, 200 μL of detection reagent was combined with 40 μL sample. After a 30-minute incubation period, the absorbance was measured at 600 nm using a microplate reader (NB-12–0035, Neo Biotech, Nanterre, France). The $H_2O_2$ content was subsequently determined from a standard curve generated through dilutions of a 3 % *(v/v)* $H_2O_2$ standard.

### 2.6. Proline quantification

The quantification of proline content was conducted in accordance with the method previously described [1], with minor modifications tailored to our experimental conditions. Specifically, 0.1 g of seedlings grown on solid MS/2 medium containing varying concentrations of the BDP-enriched biostimulant (as detailed in Section 2.3) were homogenized in 0.5 mL of 3 % *(w/v)* sulfosalicylic acid, and then centrifuged at 5000 g per 10 min at 4°C. The resultant supernatant was carefully collected, and a mixture of 2 mL of glacial acetic acid and 1 % *(w/v)* ninhydrin was added to initiate the reaction. The mixture was heated for 30 min at 80℃ in a water bath, after which it was promptly transferred to an ice bath to arrest further chemical reactions. Upon cooling, the mixture was additionally centrifuged at 10,000 rpm for 5 min to separate any residual. The absorbance of the resulting solution was then recorded at 520 nm using a spectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). The proline content was quantified and expressed as μmol per gram of fresh weight (μmol $g^{-1}$ FW) using an external calibration curve of pure proline (Sigma-Aldrich, Milan, Italy).

### 2.7. Activity of antioxidant enzymes

Germinated seeds subjected to various experimental conditions were pulverized and homogenized using a mortar and pestle. The resulting ground material was then extracted in a solution comprising 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 to perform enzymatic assays [60]. Total protein

concentration was determined according to the method outlined by Lowry et al. Kruger [42] and was subsequently used to normalize the enzymatic activities. The enzymatic activities of Superoxide Dismutase (SOD; ab65354), Catalase (CAT; ab83464), Glutathione Peroxidase (GPX; ab102530), and Peroxidase (POX; ab155895) were assessed using commercial kits, following the manufacturer's instructions (Abcam, Cambridge Biomedical Campus, UK) as previously described [2].

### 2.8. RNA preparation, cDNA Cloning and qRT-PCR Assays

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate and purify total RNA, while quality and quantity were assessed using a nanospectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). Starting from 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), following the manufacturer's instructions [60]. qRT-PCR experiments were conducted on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR green I and ROX as an internal loading standard. All runs were followed by a melting curve analysis from 55 to 95°C. After analysis of amplification plots by MX3000PTM software, Ct values of reference (*yls8, glox, pex4, ppr,* and *ubq*) and target genes for antioxidant balance (*CuZnSOD, MnSOD1, MnSOD2, MnSOD3, CAT1, CAT2, CAT3, GPX1, GPX2, GPX3, GPX4, GR1*, and *GR2*), proline metabolism (*P5CS1, P5CS2, P5CR, OAT, P5CDH, and PDH*) or gibberellin biosynthesis (*GA13ox*) were calculated. Expression analysis on target genes was performed by first assessing the stability of reference genes under the different experimental conditions (Figure S1). Consequently, *ppr* was used to calculate gene expression using the Pfaffl method [61]. Primers for qRT-PCR were designed using the Primer-BLAST software and are provided in Table S1 along with relative locus information.

### 2.9. Phytohormones quantification

Approximately 100 mg of seedlings grown on solid MS/2 medium containing varying concentrations of the BDP-enriched biostimulant (as detailed in Section 2.3) were pulverized using liquid nitrogen and then extracted with 1 mL of 1:1 *(v/v)* Methyl-t-Butyl Ether (MTBE)/Methanol (MetOH). After vortex mixing, samples were sonicated for 15 min at 4 °C, and 0.5 mL of 0.1 % *(v/v)* HCl acidified water was added. After 5 min, samples were centrifuged at 10,000 g for 10 min at 4 °C. The upper MTBE phase was transferred, dried under nitrogen flow, and resuspended in 100 µL 50 % *(v/v)* propanol. Analytical separation was achieved using HPLC-DAD-ESI-MS/MS by flushing a binary solvent system consisting of water acidified with 0.1 % *(v/v)* formic acid (solvent A) and methanol acidified to 0.1 % *(v/v)* with formic acid (solvent B) and using a Kinetecs C18 column (130 Å, 1.7 µm, 2.1 × 100 mm) (Phenomenex, Aschaffenburg, Germany). The gradient was: 0–8 min 95 % *(v/v)* A; 8–12 min A decreased to 75 % *(v/v)*; 12–16 min A decreased to 55 % *(v/v)*; 16–32 min A decreased to 25 % *(v/v)*; and after, reduced to 5 % *(v/v)* at 45 min. Solvent A was maintained at this concentration for 10 min. During the chromatographic run, the flow rate was set to 0.2 mL $min^{-1}$, while the injection volume was 10 µL. Before the next injection, chromatographic conditions were restored to initial ones and maintained for 8 min. Multiple Reaction Monitoring (MRM) analysis was employed to monitor the different phytohormones, using positive polarity for Indole Acetic Acid (IAA), *trans*-Zeatin (*t*ZEA), and *trans*-zeatin riboside (*t*ZEA-rib), and melatonin (5-HTP), or negative for Abscisic Acid (ABA), ABA-glucoside, and Gibberellic Acids. Standard solutions of all phytohormones (Sigma Aldrich, Milan, Italy) were prepared by diluting stock solutions of each hormone in 50 % *(v/v)* methanol to achieve a range of concentrations from 0.01 to 1000 ng $mL^{-1}$ [21]. Calibration curves were generated by plotting the peak areas of the hormones against their concentrations. Linear regression was applied to fit the curves, and the limits of detection (LOD) and limits of quantification (LOQ) were calculated for each standard [5].

### 2.10. Statistical analysis

Quantitative data from chemical, biochemical and molecular assay were presented as mean ± standard deviation (SD). Normality of residuals and homogeneity of variance were performed via visual inspection of histograms and Q-Q plots before to asses further statistical analysis. ANOVA was employed for the analysis of data, followed by Tukey's post-hoc test when appropriate. A $P \leq 0.05$ threshold was used to identify statistically significant differences among the different treatments. All statistical analyses were conducted using SPSS Statistics 29.

## 3. Results and discussion

### 3.1. Identification and quantification of BDPs and related compounds

Chemical characterization plays a key role in unraveling the potential behind the efficacy of bioactive compounds, offering invaluable insights into their possible action mechanisms. Formulation containing secondary metabolites are gaining prominence in modern agriculture for their purported ability to biostimulate plant physiological processes. Consequently, understanding their composition is crucial to correlate specific molecules with observed effects, paving the way for targeted improvements and optimization of these commercial products [24]. Despite the promising interest in biostimulant research, a noticeable gap exists in studies that delve into the profiling of bioactive molecules intricacies of these formulations. Many investigations focus primarily on the end effects—improved plant growth, yield, and stress resilience—without adequately exploring the secondary metabolites responsible for these outcomes. This lack of in-depth chemical characterization impedes our ability to unlock the full potential of biostimulants and hinders advancements in formulating highly effective and targeted products [24].

To address this knowledge gap, we undertook a target analysis by HPLC-DAD-ESI-MS/MS on the biostimulant obtained from pitaya peel and that we hypothesized to be enriched in BDPs. Consequently, we successfully monitored the ionization and fragmentation of 21 distinct molecules (Table 1) that have been previously reported to be present in extracts after thermal degradation of betalains [82,84,83]. Among these, four were identified as intact betalains (betanidin (**#5**), betanin (**#8**), and their respective (di)stereoisomers (**#6** and **#9**), together accounting for about 10 % of the monitored molecules. Additionally, four small molecules likely resulting from the complete degradation of the betalain scaffold were identified (dopamine (**#3**) and closely related compounds, including L-DOPA (**#2**), cyclo-DOPA (**#1**), and its glycosylated form (**#4**) (Table 1**,** Figure S2). These compounds, while not representing the predominant BDPs, together constitute approximately 19 % (*w/v*) of the detected compounds. These compounds naturally occur in pitaya peel extract from the fruit's primary metabolism, rather than solely from betalain degradation. Their hormonal role at trace-level suggest they contribute to BDP-enriched biostimulants primarily through betalain degradation [50,72].

Regarding the additional compounds detected and quantified in the BDP-enriched biostimulant, the predominant components were Neobetanin (**#17**) and its respective decarboxylated products (**#12** and **#15**), collectively constituting around 35 % (*w/V*) of the identified molecules. These compounds originate from the loss of the sugar moiety, initiating a betalamic ring saturation process that leads to the formation of **#17**. The subsequent decarboxylation contributes to the generation of **#12** and **#15**, respectively [43,76]. A representative illustration of the chemical structure is present in Figure S2**.** The occurrence of decarboxylated forms is a well-documented phenomenon in the literature, particularly subsequent to the heating of betalain-rich extracts [30,69]. For instance, Sławomir Wybraniec in 2005 not only identified

**Table 1**
Identification and quantification of betanin degradation products (BDPs) within the biostimulant. The table includes retention time (RT) in min, maximum absorption wavelength (λ) for each molecule in nm, molecular weight (MW), corresponding fragmentations (MS/MS), chemical formula, and CAS-ID. The data are presented as the mean ± standard deviation in μg per g of fresh pitaya agri-food waste.

| # | RT [min] | λ (nm) | MW | MS/MS | Compound(s) | Chemical Formula | CAS-ID | Amount (μg $g^{-1}$ of FW) |
|---|---|---|---|---|---|---|---|---|
| **1** | 4.2 | 260; 280 | 198 | 135 (100) | cyclo-DOPA | $C_{15}H_{19}NO_9$ | 18766–67–1 | 654.31 (25.45) |
| **2** | 4.9 | 260; 280 | 198 | 152 (100); 135 (20) | L-DOPA | $C_9H_{11}NO_4$ | 59–92–7 | 400.11 (14.26) |
| **3** | 9.4 | 260; 280 | 137 | 119 (100); 91 (54) | Dopamine | $C_8H_{11}NO_2$ | 51–61–6 | 44.26 (1.75) |
| **4** | 12.2 | 260; 280 | 358 | 196 (100); 150 (30) | cyclo-DOPA-glucoside | $C_{15}H_{19}NO_9$ | 71242–23–4 | 592.14 (23.34) |
| **5** | 13 | 564 | 389 | 177 (100) | Betanidin | $C_{18}H_{16}N_2O_8$ | 2181–76–2 | 222.18 (6.19) |
| **6** | 14.9 | 564 | 389 | 177 (100) | Isobetanidin | $C_{18}H_{16}N_2O_8$ | 4834–32–1 | 113.79 (5.37) |
| **7** | 17.5 | 260; 280 | 507 | 345 (100) | 17-Decarboxybetanin | $C_{23}H_{26}N_2O_{11}$ | 676320–08–4 | 138.55 (2.12) |
| **8** | 19.4 | 535 | 551 | 389 (100) | Betanin | $C_{24}H_{26}N_2O_{13}$ | 7659–95–2 | 501.16 (8.37) |
| **9** | 21.2 | 535 | 551 | 389 (100) | Isobetanin | $C_{24}H_{26}N_2O_{13}$ | 15121–53–6 | 160.61 (5.82) |
| **10** | 21.3 | 260; 280 | 507 | 345 (100) | 15-Decarboxybetanin | $C_{23}H_{26}N_2O_{11}$ | 676320–08–2 | 423.13 (9.22) |
| **11** | 23 | 410 | 212 | 166 (100); 167 (15) | Betalamic Acid | $C_9H_9NO_5$ | 18766–66–0 | 467.11 (5.48) |
| **12** | 23.1 | 260; 280 | 505 | 343 (100); 459 (40); 344 (31) | 17-Decarboxyneobetanin | $C_{23}H_{24}N_2O_9$ | | 1117.25 (21.46) |
| **13** | 23.8 | 410 | 212 | 166 (100); 167 (15) | Isobetalamic Acid | $C_9H_9NO_5$ | | 188.57 (3.68) |
| **14** | 24.2 | 260; 280 | 507 | 345 (100) | 2-Decarboxybetanin | $C_{23}H_{26}N_2O_{11}$ | 340720–01–6 | 662.52 (25.62) |
| **15** | 25.2 | 260; 280 | 505 | 344 (100); 459 (40); 344 (31) | 15-Decarboxyneobetanin | $C_{23}H_{24}N_2O_9$ | | 1173.83 (38.74) |
| **16** | 29.5 | 260; 280 | 505 | 345 (100); 459 (40); 344 (31) | 2-Decarboxyneobetanin | $C_{23}H_{24}N_2O_9$ | | 323.53 (5.39) |
| **17** | 29.9 | 260; 280 | 549 | 387 (100) | Neobetanin | $C_{24}H_{24}N_2O_{13}$ | 71199–29–6 | 1255.25 (18.81) |
| **18** | 35.9 | 260; 280 | 387 | 196 (100); 150 (30) | Neobetanidin | $C_{18}H_{14}N_2O_8$ | | 234.18 (3.91) |
| **19** | 41.4 | 260; 280 | 344 | 196 (100); 150 (30) | 17-Decarboxybetanidin | $C_{17}H_{16}N_2O_6$ | 15032–15–2 | 237.31 (8.86) |
| **20** | 42.9 | 260; 280 | 344 | 196 (100); 150 (30) | 15-Decarboxybetanidin | $C_{17}H_{16}N_2O_6$ | 1286196–08–4 | 558.13 (18.68) |
| **21** | 45.6 | 260; 280 | 344 | 196 (100); 150 (30) | 2-Decarboxybetanidin | $C_{17}H_{16}N_2O_6$ | 32101–25–0 | 484.82 (9.64) |

mono-decarboxylate related products but also characterized bi- and tri-decarboxylates, thereby proposing and elucidating the underlying mechanisms of their formation [82].

Due to the intricate phytochemical composition of plant-derived extracts, attaining a complete comprehension of their chemical profile is profoundly challenging. Here, we have started the foundational steps

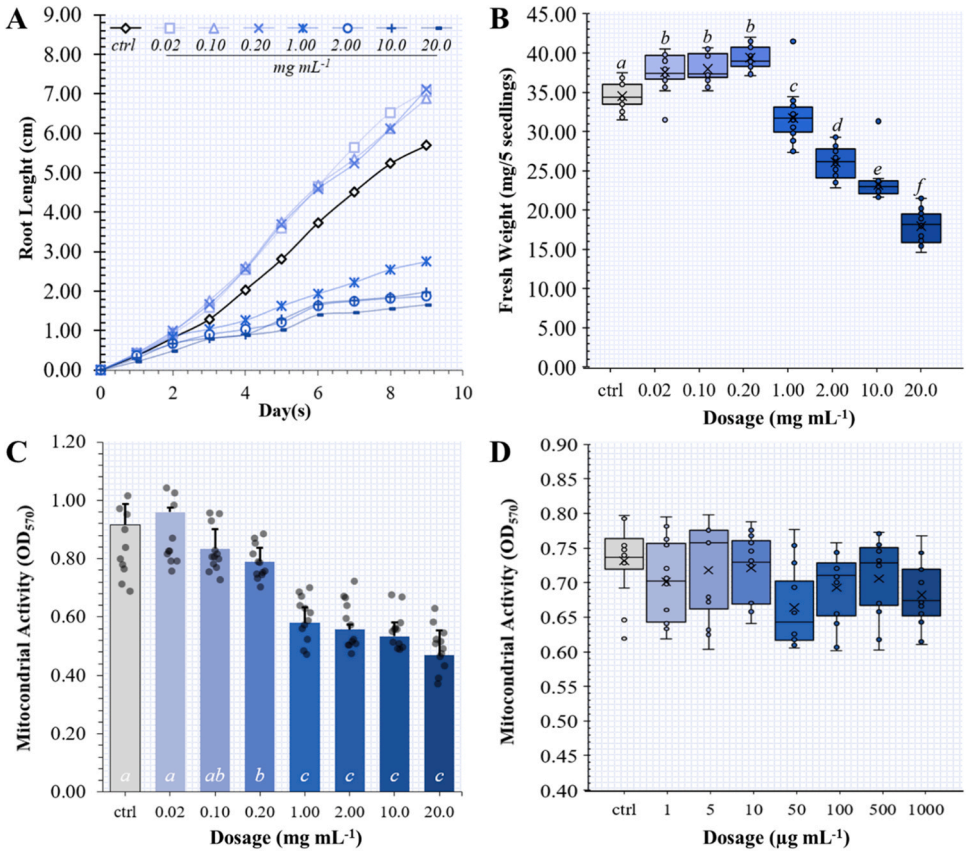


**Fig. 1.** Root development and mitochondrial activity of *Arabidopsis thaliana* seedlings in response to the biostimulant formulation enriched in BDPs. Panel A shows Root Length (cm), consistently measured throughout the experimental, while Panel B reports Fresh Biomass (mg per 5 seedlings) assessed at the trial's conclusion. Panels C and D depict mitochondrial activity assessed using the MTT assay, respectively, on seeds or the purified mitochondrial fraction. For Panel B and D, within each box, horizontal black lines indicate median values, while boxes extend from the 25th to the 75th percentile of the distribution of values for each group. The extended vertical lines indicate the standard deviations. For all the panels, statistical differences ($P < 0.05$) are indicated by various lowercase letters and determined using one-way ANOVA followed by the Tukey post hoc test.

towards characterizing the primary bioactive compounds prevalent in the biostimulant originating from pitaya peels. Nevertheless, it is plausible that the formulation harbors additional molecules beyond betalains and their derivatives, encompassing both primary and secondary metabolic pathways. However, it is important to note that many plants able to produce betalains, including *S. undatus*, experience a significant reduction in the phenylpropanoid pathway, which otherwise leads to the production of various polyphenolic compounds. This occurs for several reasons. Firstly, (i) tyrosine is a common substrate for both pathways, leading to direct competition for the substrate. Secondly, (ii) betalains perform some physiological functions similar to those of phenolic compounds, including anthocyanins and other flavonoids. This has led to transcriptional compensation mechanisms, where betalains not only inhibit many enzymes involved in polyphenol synthesis but also suppress elements that would otherwise initiate transcription [73,87]. Despite these consideration, further comprehensive investigations employing chemical profiling methodologies are imperative to elucidate the potential presence of bioactive compounds and other molecular entities.

### 3.2. Treatment with BDP-enriched biostimulant affects the development of *A. thaliana* seedling and mitochondrial activity

In order to investigate the impact of the biostimulant enriched in BDPs on seed germination, *A. thaliana* seeds were placed on MS/2 medium across a spectrum of concentrations ranging from 0.02 to 20 mg $mL^{-1}$. None of the tested dilutions affected the number of germinated seeds, germination speed, or FGP over the 9-day experimental period (data not shown). Unexpectedly, a distinctive pattern emerged in the development of the radicle over the experimental duration (Fig. 1**A**). Particularly interesting was the observation that the three lower concentrations (0.02 – 0.2 mg $mL^{-1}$) stimulated the main radicle's growth from third day, resulting in an average length 1.30-fold greater than the untreated control. This increase remained constant throughout the subsequent days, up to the conclusion of the experimental trial on the ninth day (Fig. 1**A**). On the contrary, although not directly influencing the FGP, the higher dosages (within the concentration range of 1 – 20 mg $mL^{-1}$) significantly constrained the development of the main root. This restriction became more pronounced, with an observed reduction of approximately 2-fold at the highest dosage (Fig. 1**A**). As expected, a strong Pearson correlation coefficient (PCC) was observed (PCC = 0.899) between root length and fresh weight (Fig. 1**B**), thus supporting an augmentation in root mass at lower concentrations and a pronounced inhibition with increasing concentration.

In order to understand if the biological effects could be related to potential cytotoxic effects of the BDP-enriched biostimulant, we performed an MTT assay on seeds (Fig. 1**C**). This method, originally introduced by [63] and later adapted by [80], serves as a valuable tool for assessing metabolic activity within the embryo, suggesting potential cytotoxic effects of molecules or extracts. In particular, MTT (yellow in color) functions as a metabolic marker whose reduction to purple formazan crystals can be quantified spectrophotometrically [63,80]. Remarkably, seeds treated with the BDP-enriched biostimulant in the lower concentration range exhibited mitochondrial activity comparable to the control (in the case of 0.02 mg $mL^{-1}$) or slightly lower (for 0.10 or 0.20 mg $mL^{-1}$). This data implies that the biostimulant enriched in BDPs is not cytotoxic at the tested concentrations. Conversely, when exploring the concentration range between 1.00 and 20.0 mg $mL^{-1}$, a pronounced inhibition of mitochondrial activity was observed (Fig. 1**C**). This effect is strongly correlated with both Root Length (PCC = 0.75) and Fresh Weight (PCC = 0.82), confirming the potential cytotoxic or germination-inhibiting action of the BDP-enriched biostimulant at high dosage. Furthermore, employing a Four Parameter Logistic Curve Analysis with dilution replicates, dose–response curves were generated, and the cytotoxic concentration ($CC_{50}$) was determined to be 17.25 mg $mL^{-1}$. Previous studies have demonstrated the efficacy of assessing the potential impact of biostimulants through this assay. For instance, Prado and colleagues formulated a biostimulant using *Aspergillus flavipes* (ATCC® 16814™), which effectively induces the production of indole-3-acetic acid (IAA) and has been confirmed to be safe, as it does not lead to mitochondrial inhibition in germinating seeds [16].

However, the results obtained from MTT assay on *A. thaliana* seeds could be reflective of the potential action of the molecules content in the biostimulant on mitochondrial performance. Consequently, we extended the MTT assay beyond *A. thaliana* seeds, including its application to a purified fraction of mitochondria, following the methodology outlined by [37]. As shown in Fig. 1**D**, the biostimulant enriched in BDPs within the examined concentration range do not exhibit an impact on the mitochondrial activity of the purified organelle fraction. This strongly suggests that the observed effects are primarily associated with cytotoxic actions or inhibitory pathways distinct from mitochondrial processes.

### 3.3. The biostimulant enriched in BDPs treatment influences ROS scavenging machinery

During the germination and development of seeds, the delicate equilibrium of ROS, specifically $H_2O_2$, plays a critical role in cellular signaling and metabolic processes. While $H_2O_2$ naturally arises as a byproduct of aerobic metabolism, it also acts regulating the activity of enzymes responsible for breaking down stored reserves and provides the expression of genes essential for the correct germination and development of seeds. However, an excessive production of $H_2O_2$ disrupts the cellular redox balance, potentially leading to fatal consequences [22,86]. Therefore, in order to explore whether potential shifts in the cellular redox balance may contribute to the observed biological effects, we quantified the $H_2O_2$ content of the seedlings after the treatment time (Fig. 2**A**). Under our experimental conditions, the untreated seedlings exhibited $H_2O_2$ content of 0.2240 ± 0.015 μmol $g^{-1}$ FW. This finding aligns with the recorded $H_2O_2$ levels previously reported in *A. thaliana* [64]. Upon exposure to the BDP-enriched biostimulant within the low concentration range (0.02 – 0.10 mg $mL^{-1}$), no marked variability was observed. Despite this, a slight but statistically non-significant decrease ($P > 0.05$) was detected when the seedlings were grown in MS/2 medium supplemented with 0.20 mg $mL^{-1}$ of the biostimulant. On the contrary, a 1.65-fold increase emerged when concentrations higher to 1.00 mg $mL^{-1}$ were tested. The augmentation in $H_2O_2$ production displayed a direct correlation with the concentration of the biostimulant, increasing proportionally as the BDP-enriched biostimulant rose. This increase peaked at levels 3.48-fold higher than those untreated seedlings, when measured at the highest concentration tested (Fig. 2**A**).

Under physiological conditions, cellular mechanisms regulate $H_2O_2$ levels through a sophisticated enzymatic system involving SOD, CAT, GPX, and GR. Consequently, in order to elucidate the physiological reasons for the observed changes, we measured their enzymatic activity. This analysis was conducted both in seedlings treated with biostimulant concentration that varied slightly (0.10 mg $mL^{-1}$) or strongly (10.0 mg $mL^{-1}$) the levels of $H_2O_2$ production, as well as control condition (Fig. 2**B**).

SOD is an enzyme with a fundamental role in cellular defense against oxidative stress. Its primary function is to catalyze the dismutation of superoxide radicals anion ($O_2^{\cdot-}$) into molecular oxygen ($O_2$) and $H_2O_2$. Consequently, CAT and GPX complete the scavenging by catalyzing the breakdown di $H_2O_2$ into $H_2O$ and $O_2$. In contrast to CAT, GPX relies on the coordinated activity of GR to regenerate glutathione (GSH) from its disulfide form (GSSH) [29]. Under our experimental conditions, SOD levels exhibited significant alterations following treatment with BDP-enriched biostimulant. Specifically, exposure to 0.10 mg $mL^{-1}$ resulted in a 1.28-fold rise, while higher concentrations led to modulation reaching up to a 2-fold increase compared to the control (Fig. 2**B**). The data on SOD activity implies a potential increase in $H_2O_2$, at both concentrations. Nevertheless, our prior experiment indicated that the

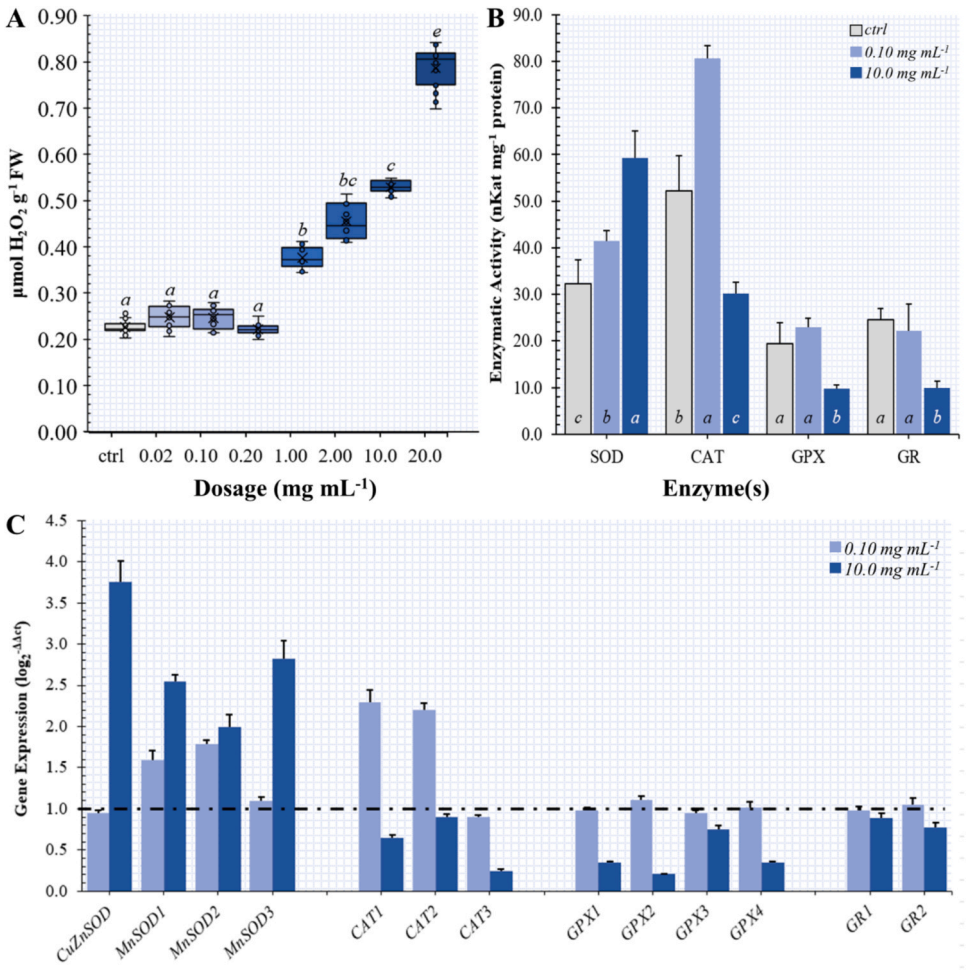


**Fig. 2.** Cellular redox balance in *Arabidopsis thaliana* seedlings in response to the biostimulant formulation enriched in BDPs. In Panel A, the total hydrogen peroxide ($H_2O_2$) content is expressed as µmol per $g^{-1}$ FW (fresh weight). For each box, horizontal black lines signify median values, with boxes extending from the 25th to the 75th percentile of the value distribution in each group. Extended vertical lines indicate standard deviations. Panel B showcases the activity of enzymes involved in $H_2O_2$ scavenging (SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase), reported as nKat $mg^{-1}$ protein. Panel C visually represents the expression of different isoforms of antioxidant enzymes, normalized on reference gene and reported as Fold Change values calculated using $\log_2^{-\Delta\Delta ct}$ on untreated seedlings. Values for all panels represent the mean ± SD of at least three different biological replicates. Statistical differences ($P < 0.05$) are indicated by various lowercase letters, determined using one-way ANOVA followed by the Tukey post hoc test.

$H_2O_2$ levels in the lower treatment, including 0.10 mg $mL^{-1}$, were comparable to those observed under control conditions (Fig. 2**A**). This effect could be attributed to the observed increase in CAT activity, observed when the seedlings were exposed to 0.10 mg $mL^{-1}$. On the other hand, due to the increase in GPx and GR activity in seedlings treated with 0.10 mg $mL^{-1}$ were non-significant ($P > 0.05$). The increased CAT activity seems the primary detoxification mechanism for $H_2O_2$ following treatment with the BDP-enriched biostimulant (Fig. 2**B**). Regarding the treatment with the higher dosage (10 mg $mL^{-1}$), the observed increased $H_2O_2$ levels in the seedlings appear to be a direct consequence of both the enhancement of SOD enzymatic activity (+1.88) and a lack of detoxification by CAT (-1.63) and GPX (-1.58), which were strongly inhibited along with GR (-2.08) activity (Fig. 2**B**). Our findings on the effect of the biostimulant enriched in BDPs on enzymatic activity align with observations in other studies evaluating biostimulants on plants. Similar to our results with the lower dose (0.10 mg $mL^{-1}$), some biostimulants, like humic substances, have been shown to induce a moderate rise in SOD activity, potentially leading to a slight increase in $H_2O_2$ production [4]. This uptick in $H_2O_2$ can be beneficial, triggering defense mechanisms and priming plants for stress tolerance [66].

The evaluation of enzymatic activity, as determined through biochemical assays, is indicative of changes in the activity levels of individual enzymes but not necessarily indicate the primary cellular compartment involved in scavenging reactions [45]. Because the diverse isoforms of antioxidant enzymes have a precise and distinct cellular localizations, the examination of their expression offers insights into the cellular compartment where the scavenging reaction occurs [17]. To address this, we assessed the gene expression of four distinct isoforms of SOD isoforms, three of CAT, four of GPX, and two of GR (Fig. 2**C**). This assessment was carried out on the same conditions used for the biochemical evaluation of the enzymatic activity. In this case, seedlings subjected to 0.10 mg $mL^{-1}$ showcased a significant rise in the transcription of *MnSOD1* (+1.5) and *MnSOD2* (+1.6), while *MnSOD3* and *CuZnSOD* exhibiting no significative changes compared to the control. At the same time, both *CAT1* and *CAT3* displayed increased expression levels, respectively recording +2.2 and +2.1 increment, whereas the transcription of the *GPX-GR* system remained constant (Fig. 2**C**). Unlike CuZnSOD, MnSOD can be localized in both mitochondria or cytosol, but has exclusive activity in mitochondria [33,41]. In our experimental conditions, the upregulation of *MnSOD1* and *MnSOD2* suggests a protective effect of BPDs against potential oxidative challenges originating from this organelle during respiratory processes. On the other hand, because *MnSOD3* is initially localized in the apoplast and only later

transported to the mitochondria, it is mainly upregulated in response to prolonged and excessive oxidative threats [33,41]. Concerning CAT isoforms, they display diverse cellular localization. In particular, while CAT1 and CAT3 are distributed across all other cellular compartments (including mitochondria), current evidence on CAT2 suggest its lack in this organelle [75]. The strong up-regulation of *CAT1* and *CAT3* (approximately 2.2-fold) along with the unaltered level di *CAT2* transcripts after the lower treatment with the biostimulant enriched in BDPs, strongly suggests that these enzymes are triggered in response to the upregulation of *SOD* gene transcription (Fig. 2**C**).

Conversely, exposure of seedlings to 10.0 mg $mL^{-1}$ of the biostimulant resulted in a substantial upregulation of all *SOD* isoforms, including *CuZnSOD* (+3.7). Among the four forms of SOD under examination, CuZnSOD has been suggested to localize to the outer mitochondrial membrane. As a result, its activity is primarily associated with cellular compartments other than the mitochondria, including the cytosol and cytoplasm [33,41]. Its upregulation, along with the Mn-related form of the other SOD, aligns with the heightened levels of $H_2O_2$ noted in this experimental condition (Fig. 2**A**), indicating a pronounced cellular redox imbalance also in the extra-mitochondrial environment. Furthermore, not only all isoforms of the CAT experience a significant reduction in transcription (-1.5), but also those of GPX-GR scavenging system (-3.3) (Fig. 2**C**).

The ability of biostimulants to modulate cellular redox balance is a topic of great interest and relevance in agronomic and biotechnological research. For example, several studies have demonstrated that the application of biostimulants can enhance the activity of plants' endogenous antioxidant enzymes, such as SOD, CAT, and GPX, thus contributing to maintaining an optimal redox balance within plant cells [11,14,18]. A concrete example of this phenomenon was reported by Hernández-Herrera and colleagues, where they evaluated the effects of a biostimulant based on marine algae extracts on oxidative stress resistance in tomato plants. The results showed a significant increase in the activity of antioxidant enzymes and a higher accumulation of non-enzymatic compounds, such as anthocyanins and vitamin C, in plants treated with the biostimulant. This suggests that the biostimulant effectively acted in modulating the cellular redox balance of plants, improving their ability to respond to environmental stress [32].

In summary, numerous studies have highlighted the importance of biostimulants in maintaining redox homeostasis during biotic and abiotic stress conditions [13,38,65]. For instance, the ability of biostimulants to modulate cellular redox balance represents an important mechanism through which these compounds can enhance plant physiological and adaptive responses to environmental stresses, thereby contributing to improving crop performance and the sustainability of agricultural systems.

### 3.4. The treatment with the biostimulant enriched in BDPs alters proline levels affecting the expression of proline metabolism-related genes

During the germination of seeds, the significance of proline takes center stage. This amino acid, primarily acknowledged for its multifaceted functions, plays a critical role in different biochemical pathways, including mitigating the impact of ROS generated during germination [26]. The dynamic regulation of proline levels is intricately tied not only to the adjustment of cellular osmotic balance but also to genes responsive to cellular redox status [26]. To evaluate if the observed biological effect due to treatment with the biostimulant enriched in BDPs could be attributed to altered proline levels following treatments, a quantification of the amino acid was performed Fig. 3**A**. Control seedlings demonstrated a proline content to that reported in other previously studies, and typical of non-stressed seedlings [26]. In our experimental conditions, the proline content measured in seedlings grown with lower concentrations (0.02 – 0.20 mg $mL^{-1}$) exhibited non-significant changes compared to the control. Despite this, also the treatment with 1.00 mg $mL^{-1}$ recorded a proline content similar to the control one (Fig. 3**A**). A distinctly different scenario unfolded when seedlings were exposed to higher BDP-enriched biostimulant concentrations. In these instances, a significant ($P < 0.05$) increase of 1.3-, 1.5-, and 2.10-fold was observed when the seedlings were exposed to 2.00, 10.0, and 20.0 mg $mL^{-1}$ of BPDs, respectively (Fig. 3**A**).

In order to understand the molecular mechanisms underpinning the observed alterations in proline content, we delved into the analysis of key genes involved its metabolism and catabolism (Fig. 3**B**). Proline synthesis takes place in the cytosol, starting from the amino acid glutamate. This process involves the reduction of glutamate to glutamate-semialdehyde (GSA), that spontaneously or by $\delta^1$-pyrroline-5-carboxylate synthetase (P5CS) leads to pyrroline-5-carboxylate (P5C). Then, P5C is reduced to proline by $\delta^1$-pyrroline-5-carboxylate reductase (P5CR). Additionally, the examination of the ornithine pathway adds complexity to this exploration. Indeed, as an alternative route, proline synthesis can begin within the mitochondria using ornithine as the precursor. Ornithine is first oxidated by ornithine-$\delta^1$-aminotransferase (OAT), to GSA. Then, GSA is converted to P5C. Similar to the cytosolic

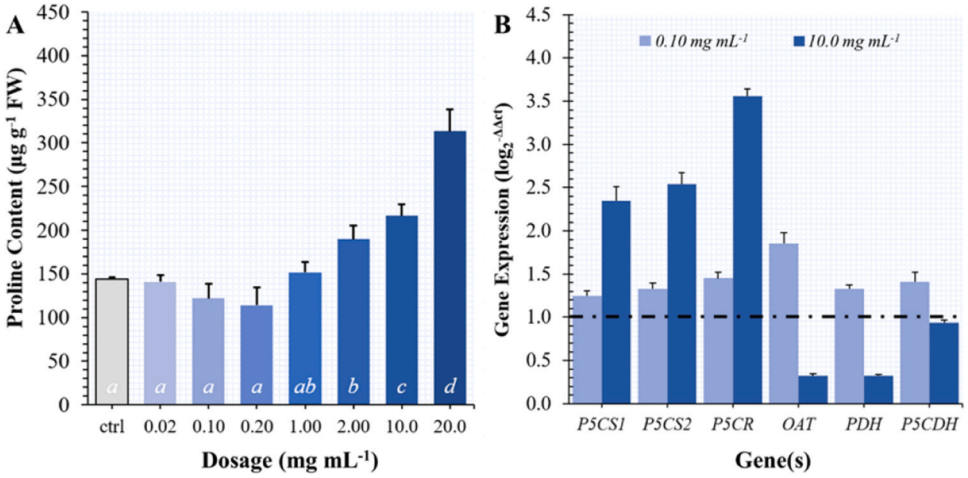


**Fig. 3.** Proline Content and Regulation in *Arabidopsis thaliana* Seedlings in response to the biostimulant formulation enriched in BDPs. In Panel A, the total proline content is quantified as µg per $g^{-1}$ FW (fresh weight). Panel B presents the expression of various genes involved in proline metabolism (*P5CS1, P5CS2, P5CR, OAT,* and *PDH*) and catabolism (*P5CDH*). Gene expression was normalized on reference gene and reported as Fold Change values calculated using $\log_2^{-\Delta\Delta ct}$ on untreated seedlings. Values for all panels represent the mean ± SD of at least three biological replicates, with statistical differences ($P < 0.05$) indicated by various lowercase letters, determined using one-way ANOVA followed by the Tukey post hoc test.

biosynthetic pathway, also during the ornithine pathway P5C is converted to proline by P5CR [6]. Proline levels are subject to regulation not only through *de novo* synthesis but also in response to shifts in its catabolism. Notably, proline catabolism unfolds in the mitochondria through the sequential actions of proline dehydrogenase (PDH) generating P5C from proline, and P5C dehydrogenase (P5CDH) converting P5C to glutamate [6]. Considering these physiological processes, we examined the expression patterns of enzymes associated with proline metabolism and catabolism in control seedlings, as well as those subjected to 0.10 mg $mL^{-1}$ or 10.0 mg $mL^{-1}$ treatments (Fig. 3**B**).

In our experimental conditions, we noted a subtle upregulation in the expression of genes associated with cytoplasmic proline metabolism in seedlings treated with 0.10 mg $mL^{-1}$ (approximately +1.3). However, a marked upregulation (approximately +2.5) was observed when seedlings underwent treatment with 10.0 mg $mL^{-1}$ of the biostimulant enriched in BDPs, as illustrated in Fig. 3**B**. Notably, the expression of *OAT* exhibited a significant and pronounced distinction between the two experimental conditions compared to the control group. Specifically, in the treatment involving the lowest dose of the BDP-enriched biostimulant, *OAT* displayed a robust upregulation (approximately +2.0), suggesting an augmented proline production at the mitochondrial level, likely as a response to increased $H_2O_2$ production. Conversely, despite a substantial and significant increase in proline content in conditions where seedlings were treated with 10.0 mg $mL^{-1}$, the gene expression of OAT was markedly reduced. This evidence implies that the heightened proline levels after exposure to the highest concentration primarily stem from cytoplasmic production. This inference is supported by the upregulation of both isoforms of *PCS* and *P5CR*, coupled with the reduced expression of *OAT*. Finally, the increase in proline under these experimental conditions is associated with the downregulation of gene transcription involved in its catabolism. Notably, significantly lower transcription levels of *PDH* were detected in conditions where seedlings were treated with 10.0 mg $mL^{-1}$, in contrast to both the control and the condition with the lowest treatment.

Our findings on proline accumulation following the treatment with the biostimulant enriched in BDPs aligns with previous studies highlighting its role in stress mitigation during seed germination. Similar to our control group, proline levels in seedlings treated with lower BDP-enriched biostimulant concentrations (0.02 – 0.20 mg $mL^{-1}$) were comparable to those reported in non-stressed seedlings [52]. This suggests that these lower doses did not induce significant stress responses. However, seedlings exposed to higher BDP-enriched biostimulant concentrations (2.00, 10.0, and 20.0 mg $mL^{-1}$) exhibited a significant increase in proline content. This is in line with observations where plants subjected to various environmental stresses accumulate proline [56,81], thus suggesting that these dosages may have triggered a stress response, prompting proline biosynthesis. To delve deeper, we analyzed the expression of genes involved in proline metabolism, and we observed that seedlings treated with the lower BDP-enriched biostimulant dose (0.10 mg $mL^{-1}$) displayed a subtle upregulation of genes associated with both metabolism and catabolism of proline in cytosol. This is consistent with reports where mild stress can stimulate proline production in the cytosol [81]. Interestingly, the higher dosage of BDP-enriched biostimulant (10.0 mg $mL^{-1}$) caused a more pronounced upregulation of genes involved in metabolism, particularly those encoding ornithine-δ1-aminotransferase (OAT) in the mitochondrial pathway, and a strong downregulation of those related to its catabolism, particularly proline dehydrogenase (PDH). This aligns with previous findings where stress conditions can suppress PDH activity, leading to proline accumulation [56]. This suggests that the substantial proline increase at this concentration might be due to enhanced production in both the cytoplasm and mitochondria.

### 3.5. The treatment with the BDP-enriched biostimulant orchestrate the synthesis of phytohormones crucial for seed germination

Throughout the intricate phases of germination and early seedling development, hormones emerge as pivotal orchestrators, finely tuning key processes that govern plant growth and development. To unravel the intricate relationship between these molecules and the observed effective biological effects after the treatment with the biostimulant enriched in BDPs, we performed HPLC-MS/MS analysis to quantify the content of the most critical phytohormones involved in seed germination and development (Fig. 4**A**, Table S2).

Under our experimental conditions, the content of tZea in seedlings grown without treatment was measured equal to 135.79 μmol $g^{-1}$ FW. The concentration of tZea tended to decrease with increasing dosage, reaching values three times lower under conditions where BDP-enriched biostimulant were at the maximum dosage (20.0 mg $mL^{-1}$). Simultaneously, a strong inverse correlation (PCC = 0.805) with its respective riboside form was also observed. tZea is one of the most important cytokines involved in seedling development, and it assumes a key role in cell division and differentiation, positively influencing cell proliferation and expansion. This contribution is essential for the establishment of root and shoot meristems during the seedling developmental phase. Notably, tZea has the capacity for reversible glycosylation, potentially enhancing its stability through the addition of sugar moieties, following in the loss of its biological activity [27]. A similar yet opposite trend was recorded for ABA and ABA-glu. In this case, ABA tended to increase with the rise in biostimulant concentration (raising from 24.77 to 68.69 μmol $g^{-1}$ FW in the control and the 20.0 mg $mL^{-1}$ dosage, respectively), while ABA-glu decreased following a strong negative correlation (PCC = 0.904). In this scenario, also ABA operates as a key regulator of seed dormancy, acting as a germination suppressor. High ABA levels during the initial seed development stages maintain dormancy, preventing premature germination. As seeds mature, ABA levels decrease, lifting dormancy restrictions and enabling the seed to progress into the germination phase. Similar to tZea, ABA is susceptible to reversible glycosylation processes. The addition of a glucose moiety can influence ABA's stability, transport, and activity, with glycosylation often linked to inactivation [35].

Since the 1970s, many researchers focused their attention to the intriguing interplay between betacyanins and auxins [74]. Auxins, well-established as crucial plant hormones governing growth and development, have been hypothesized to engage in an antagonistic relationship with betacyanins [88]. This theory is fueled by observations where tissues rich in betacyanins exhibit distinct growth patterns compared to those solely influenced by auxins. However, the intricate mechanisms underlying this potential interaction and its broader biological significance remain largely uncharted territory. Unlike tZea and ABA, IAA does not play a constant role in the germination and development of seedlings. Indeed, low concentrations of IAA generally stimulate germination, while higher concentrations can act as inhibitors. The effectiveness of IAA is contingent on its concentration and the developmental stage of the seed. Additionally, IAA emerges as a crucial regulator of root development, fostering the formation of lateral roots and contributing to the establishment of a robust root architecture [28]. In this context, we observed a consistent maintenance of IAA levels during treatments with the lowest doses (0.02 – 1.00 mg $mL^{-1}$) of BDP-enriched biostimulant, with a median content of approximately 55.65 μmol $g^{-1}$ FW, comparable to the control seedlings. However, a notable increase in IAA was observed when exploring concentrations within the range of 2.00–20.0 mg $mL^{-1}$ (Fig. 4**A**). Considering IAA's active role in shaping the proper root morphology of seedlings and its involvement in abiotic stress situations linked to a reduced efficiency in water and nutrient absorption, these heightened IAA levels may be attributed as a potential consequence for lack in the elongation of primary root (Fig. 1**A**).

Regarding GAs, they serve as the primary hormone that mitigates the

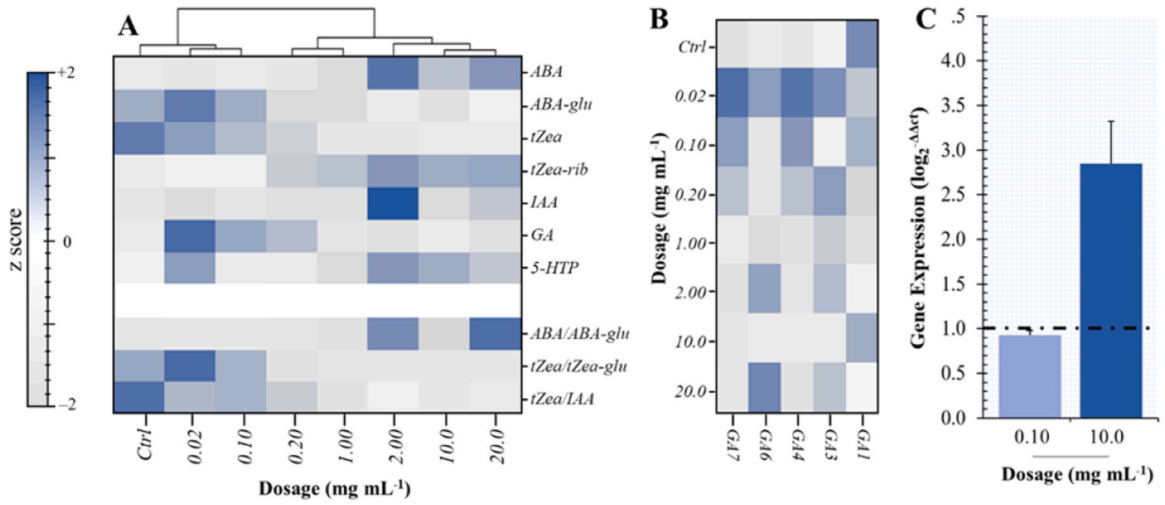


**Fig. 4.** Phytohormone Content and Variations in *Arabidopsis thaliana* Seedlings in response to the biostimulant formulation enriched in BDPs. In Panel A, the content of various phytohormones (ABA: abscisic acid; ABA-glu: ABA-O-glucoside; tZea: trans-zeatin; tZea-rib: trans-zeatin riboside; IAA: indoleacetic acid; GA: gibberellic acids; 5-HTP: melatonin) is presented, while Panel B displays the specific content of individually quantified GAs. The heatmaps illustrate quantitative variations calculated as z scores, ranging from gray (low content) to blue (high content). Quantitative data, represented as the mean ± SD of at least three biological replicates, are detailed in Table S2. Panel C outlines the expression of the gene involved in transforming GA12 into GA53, a key step in determining the production of specific GAs. Gene expression was normalized on reference gene and reported as Fold Change value calculated using $\log_2^{-\Delta\Delta ct}$ on untreated seedlings.

adverse effects of ABA on germination. Maintaining equilibrium between GAs and other hormones is critical for coordinating developmental processes in seedlings. As seed development progresses, GA levels rise, breaking seed dormancy and facilitating the germination process. Furthermore, GAs exert influence over the expression of genes involved in cell wall modifications and the hydrolysis of stored reserves, thereby facilitating radicle emergence and subsequent seedling growth [12]. Under our experimental conditions, the total GAs content was notably higher in the seedlings treated with the lowest dosages, implying a potential role of these hormones in the observed biological effects of the biostimulant enriched BDPs. More specifically, within the concentration range from 0.02 to 0.20 mg $mL^{-1}$, the total GAs content was approximately twice as much as in seedlings grown under control conditions. Notably, the levels of GAs recorded in treatments with the highest dosages were only slightly elevated compared to the control situation, and these differences did not reach statistical significance ($P < 0.05$) (Fig. 4**A**). However, there are numerous active GAs, with particular significance attributed to GA1, GA3, GA4, GA6, and GA7. Consequently, in order to quantify the punctual GAs we extended HPLC analysis investigating the different qualitative composition (Fig. 4**B**). The analysis revealed a different pattern of GAs between the different experimental conditions. In particular, seedlings treated with the lower amount of BAs (0.02 – 0.20 mg $mL^{-1}$) tends to selectively increase the production of GA7 and GA4 in comparison to both control and treatments with the higher dosage. Conversely, when the concretion raised up 1.00 mg $mL^{-1}$ a concomitant increment of GA1, GA3 and GA6 was observed (Fig. 4**B**). However, it's worth noting that GA4 and GA7 stand out due to their distinct biosynthetic pathways. Specifically, in the biosynthetic process, GA12 can be converted, through the coordinated action of GA20ox and GA3ox, into GA4 and GA7. Alternatively, GA12 can undergo transformation by GA13ox into GA55, which subsequently leads to the formation of GA1, GA3, and GA6 by GA20ox and GA3ox [12]. Consequently, in order to corroborate the HPLC findings, we conducted gene expression studies, assessing the expression of *GA13ox* (Fig. 4**C**). Nevertheless, our qRT-PCR data only partially align with the findings from HPLC-MS/MS analysis. While the chemical data propose a pronounced upregulation of *GA13ox* in seedlings treated with a higher dose and a downregulation in those treated with a lower dose, the obtained qRT-PCR analysis did not show significant differences between 0.10 mg $mL^{-1}$ and control. This discrepancy suggests that the transcription of *GA13ox* might only partially regulate the production of selective GAs, and other factors may be influencing the pathway.

Our analysis also included the quantification of melatonin, a molecule increasingly recognized for its role in plant physiology, particularly its potential interactions with common phytohormones and its influence on various developmental processes, including seed development [9,55,7,10,8]. Overall, the experimental results indicate that melatonin levels in *A. thaliana* seedlings responded differently to varying concentrations of the BDP-enriched biostimulant, with significant increases observed at higher concentrations compared to control and lower concentrations (Fig. 4**A**). Since melatonin serves as one of the most potent natural antioxidants in plants, our findings suggest that its increased synthesis could contribute to preventing oxidative stress induced by treatments with high doses [55]. However, further investigation is needed to fully elucidate its role and mechanisms under these conditions.

## 4. Conclusion

In conclusion, our thorough investigation into the influence of a biostimulant enriched in BDPs *on A. thaliana* seed germination has unveiled valuable insights into various facets of mitochondrial activity, ROS scavenging mechanisms, and molecular responses throughout this pivotal developmental stage. Through detailed chemical analysis using HPLC-DAD-ESI-MS/MS, we successfully identified and quantified a range of compounds, including intact betalains like betanidin and betanin, as well as their degradation products and other secondary metabolites. Furthermore, our biological assays with *A. thaliana* seedlings revealed concentration-dependent effects of the BDP-enriched biostimulant on root development and mitochondrial activity. Lower concentrations stimulated radicle growth without impacting germination rates, while higher concentrations exerted inhibitory effects on root growth. Our findings underscore its influence on ROS scavenging mechanisms, where we observed a dose-dependent increase in $H_2O_2$ levels with higher concentrations of the biostimulant. This was accompanied by upregulated enzymatic activities of SOD, CAT, GPX, and GR, indicative of an intensified cellular response to oxidative stress. Concurrently, the treatment altered proline levels, particularly notable at higher concentrations, suggesting a role in stress mitigation and adaptation. Furthermore, the biostimulant orchestrated complex hormonal responses in seedlings, influencing the levels of phytohormones

critical for germination and growth. Notably, it modulated cytokinins like tZea, essential for cell division and differentiation, and ABA, pivotal in seed dormancy regulation. The treatment also affected auxin (IAA) levels, potentially influencing root development, and demonstrated differential effects on GA biosynthesis, impacting processes like seedling growth and dormancy release. In conclusion, while our study demonstrates the potential of an biostimulant enriched in BDPs to influence key regulatory pathways during *Arabidopsis thaliana* germination and seedling development, it also highlights the necessity for future research. Specifically, further studies utilizing pure BDPs are crucial to establish more precise mechanistic insights and confirm the direct effects of these bioactive molecules on the observed physiological processes. These findings not only may contribute to our understanding of plant germination processes but also propose potential applications for enhancing seed germination efficiency by harnessing BDPs derived from agri-food waste.

## CRediT authorship contribution statement

Conceptualization, G.M., C.G.; data curation, G.M., C.M.B., N.G. and C.G.; formal analysis, G.M.; N.G., G.S. funding acquisition, G.M., C.M.B.; investigation, G.M. and C.G.; methodology, G.M. and C.G.; project administration, G.M. and C.G.; software, G.M. and N.G.; supervision, G. M., C.M.B. and C.G.; validation, G.M. and G.S.; visualization, G.M. and G.S.; writing—original draft, G.M., N.G., and C.G.; writing—review and editing, G.M., N.G., G.S, C.M.B. and C.G. All authors have read and agreed to the published version of the manuscript.

## 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.

## Data Availability

Data will be made available on request.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2024.100373.

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