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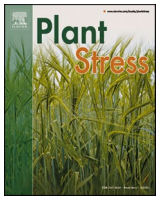

Amelioration of salt stress in broccoli seeds by an innovative biostimulant based on orange carrot cell cultures

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ABSTRACT

Adverse edaphoclimatic conditions resulting from climate change pose a serious threat to global food production, as they hinder plant development and reduce crop yields. Among these stress factors, soil salinization is one of the major constraints affecting seed germination, a critical stage in the crop life cycle. Seed priming has emerged as an effective strategy to enhance germination under stress conditions, particularly when employing higher plant-derived biostimulants (hPDBs), which are notable for their rich composition of bioactive compounds and their ability to modulate plant physiology. In this work, we produced an orange carrot cell culture-derived biostimulant (OCB) through elicitation techniques, resulting in a product enriched in phytosterols and phenolic compounds, and evaluated its effect on the germination of broccoli seedlings subjected to salt stress. OCB improved seed germination and vitality, avoiding H_2O_2 accumulation and reducing the oxidative stress-induced damage (-11 % MDA) and toxicity of salt stress. This allowed to prevent an aggressive response to stressful conditions which could worsen the germination process, thereby attenuating the disruption in the hormonal and proteomic profile induced under salt stress by downregulating the accumulation of stress-related hormones and antioxidant enzymes. Thus, OCB constitutes a novel plant biostimulant with significant potential to promote seed vigour and germination efficiency in broccoli under salinity stress.

1. Introduction

Extreme climate events, which are becoming increasingly frequent due to climate change, lead to reduced crop yields, decreasing food availability, and posing a risk to global food security (United Nations, 2019). These adverse phenomena can be diverse, such as heat and drought waves, as well as the salinization of the soil and freshwater used for irrigation (Balasubramaniam et al., 2023; Hassani et al., 2021).

The Region of Murcia, located in southeastern Spain, within the Mediterranean basin, is a world-leading producer of crops of agronomic interest. It is the first national producer of broccoli (*Brassica oleracea* var. *italica* Plenk), leading its exports and having a production of more than 200 tons annually (Ministerio de Agricultura, Pesca y Alimentación de España, 2023). Spain, in turn, is positioned as the 5th producer of broccoli and cauliflower in the world and the 1st in Europe (FAO, 2023). The consumption of this vegetable is increasing due to its health benefits, as it contains a wide variety of bioactive compounds, including glucosinolates, phenolic compounds, carotenoids, vitamins and minerals, as well as a high content of antioxidants, which contribute to its anti-inflammatory, anti-carcinogenic and cardioprotective properties (Borja-Martínez et al., 2025, 2024; Gudiño et al., 2024; Syed et al., 2023). However, extreme weather events are increasingly affecting the arable land in the Region of Murcia, particularly due to the ongoing process of desertification. Low rainfall and heat waves, combined with soil degradation due to the overuse of agrochemicals, contribute to an ever-increasing expansion of soil salinization, limiting crop productivity (Acosta et al., 2011; Daliakopoulos et al., 2016).

Soil salinization represents one of the most severe forms of land degradation, resulting from the excessive buildup of soluble salts including potassium and sulphate, but primarily sodium and chloride ions. Current projections indicate that by 2050, salinity could impact up to half of the arable land in arid and semi-arid regions (Hassani et al., 2021). The 90 % of crops of agronomic interest are classified as glycophytes, sensitive to salinity. The excess salinity severely affects plant

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growth, altering crop yield (Balasubramaniam et al., 2023). Specifically, germination is one of the most critical stages in a crop's life cycle, and it is strongly affected by salinity, as it takes place in the top layer of the soil, which is the most saline layer. Elevated salt levels in the water surrounding the seed decrease its osmotic potential, limiting water uptake, which in turn reduces germination rate and seedling growth. Moreover, ion toxicity resulting from the excessive buildup of sodium and chloride severely perturbs cellular homeostasis, leading to widespread disruption of key biochemical and metabolic functions, causing hormonal imbalance and an oxidative burst that can be damaging to cellular components, compromising the viability of the germination process (Balasubramaniam et al., 2023; Ibrahim, 2016; Ma et al., 2024; Uçarlı, 2020).

One of the most effective approaches to improve germination under adverse conditions is seed priming, a pre-sowing technique in which seeds are partially hydrated through controlled soaking, allowing metabolic activation without initiating germination or radicle protrusion. After this, they are dried-back, allowing them to be stored for later use. Seed priming gives seeds the ability to germinate faster than non-primed seeds, since they have undergone an activation of the pre-germinative metabolism that gives them greater efficiency for germination once they are rehydrated. Among the plant physiological processes activated by seed priming are the stimulation of primary metabolism, modulation of water and nutrient transport, regulation of hormonal balance, modification of seed ultrastructure, DNA repair, and enhancement of antioxidant metabolism. Several priming methodologies have been developed, differing mainly in the type of solution used for seed soaking, with treatments involving plant-derived biostimulants being particularly noteworthy due to their high content of bioactive compounds (Gupta et al., 2022; Ibrahim, 2016; Lutts et al., 2016; Uçarlı, 2020).

Plant biostimulants are defined as bioproducts capable of enhancing plant physiological processes such as nutrient use efficiency and tolerance to abiotic stress, independently of their nutrient content (*Regulation (EU) 2019/1009 of the European Parliament*, 2019). There are numerous bioproducts classified as plant biostimulants (du Jardin, 2015), however, those derived from higher plants (higher plant-derived biostimulants, hPDBs) are particularly noteworthy due to the wide variety of bioactive compounds they contain, which have the ability to stimulate multiple physiological processes in plants, promoting mineral nutrition, growth regulation, improved photosynthetic processes and stress tolerance, among others. Furthermore, they offer numerous advantages in terms of sustainability, and they do not constitute a risk to arable lands (Martínez-Lorente et al., 2024). Despite their advantages, commercially available plant-based biostimulants are relatively scarce. This is due to the difficulty in maintaining homogeneity in the production of these types of bioproducts, since they are made from plant raw materials, which are subject to variations in the edaphoclimatic conditions in which they are obtained. These variations can lead to significant changes in their composition and, consequently, affecting their effectiveness (Albaladejo-Marico et al., 2025), limiting the development of specific formulations and their ability to access the market.

The application of *in vitro* plant cell culture systems has emerged as a promising alternative to conventional plant-derived raw materials for the controlled and sustainable production of bioactive compounds, as they provide a stable and homogeneous system that is independent of environmental and geographical constraints. Additionally, the use of biotechnological tools, such as elicitor applications, can enhance the production of these bioactive compounds. Our research group has demonstrated this in previous studies, increasing the production of phenolic compounds in grapevine cell cultures (Belchí-Navarro et al., 2012) using the combination of methyl jasmonate (MJ) and cyclodextrins (CD), as well as phenylpropanoids and triterpenes in *Silybum marianum* (Corchete et al., 2022) and tomato (Sabater-Jara et al., 2022) cell cultures, respectively, using CD.

The results obtained in different carrot cell lines were of particular interest, as it was possible to increase the production of phytosterols (mainly campesterol, stigmasterol, β-sitosterol, and fucosterol), phenolic compounds (such as isoeugenol, eugenol, and vanillin, among others), and extracellular defence-related proteins in green (Miras-Moreno et al., 2016a), orange (Sabater-Jara et al., 2014; Sabater-Jara and Pedreño, 2013) and yellow carrot cell lines (Martí-Guillén et al., 2025a) using CD alone or combined with MJ. Importantly, these bioactive metabolites have been reported to contribute to enhance abiotic stress tolerance, particularly salt stress tolerance, by modulating antioxidant defence and phytohormone content (Kumar et al., 2023; Miras-Moreno et al., 2016b; Xu et al., 2024). The coordinated accumulation of these metabolites in carrot cell cultures, especially in response to CD and MJ elicitation, suggests that products rich in these compounds could serve as natural biostimulants capable of enhancing germination and early seedling performance under saline environments.

Therefore, the production of hPDBs from *in vitro* plant cell cultures and their application to seeds through seed priming represents a biotechnological tool for promoting stress tolerance during seed germination. Given the agronomic importance of broccoli in Region of Murcia, the aim of this study is to evaluate the biostimulant effect of a new bioproduct derived from orange carrot cell cultures (orange carrot biostimulant, OCB), enriched in bioactive compounds, on the acquisition of salt stress tolerance in broccoli seeds.

2. Materials and methods

2.1. Production and characterization of orange carrot biostimulant

2.1.1. Orange carrot biostimulant production

The orange carrot (*Daucus carota* L.) cell line used in this study was previously established in our laboratory. Suspension-cultured cells were maintained in culture medium optimized for biomass production, with periodic subculturing in liquid medium as described by Sabater-Jara and Pedreño (2013). To obtain the orange carrot biostimulant (OCB), cell cultures were elicited with 25 mM CD and 100 μM MJ for 7 days. After the elicitation period, the cell cultures were homogenized for 5 min using a blender and then, and a broad-spectrum preservative composed of 0.05 % (w/v) sodium benzoate and 0.1 % (w/v) potassium sorbate was subsequently added to ensure microbiological stability of the bioproduct during storage. Unelicited orange carrot cell cultures were grown in parallel and processed similarly to OCB, as a control (uOCB). The resulting bioproducts were used for seed treatment.

2.1.2. Physicochemical and biochemical characterization

The resulting bioproducts were characterized according to their physicochemical properties, including pH, °Brix, and electrical conductivity. The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for quantified spectrophotometrically the total protein content employing a BSA calibration curve. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method as described in Martí-Guillén et al. (2025b), and results were expressed as μg gallic acid equivalents (GAE) per mL.

For the determination of specific bioactive compounds, frozen orange carrot lysates were homogenized in 80 % (v/v) methanol using previously cooled porcelain mortar and pestle, and supernatants were recovered after centrifuge at 13,600 × *g* for 15 min. Supernatants were subjected to twice liquid-liquid extraction with (1:1, v/v) ethyl acetate. Then, recovered organic phases were evaporated to dryness using a rotary evaporator (Heidolph, Germany) under reduced pressure at 40 °C. The dried residues were reconstituted in 1 mL of methanol and stored at −20 °C. The analysis of bioactive compounds was performed as described in Sabater-Jara and Pedreño (2013) and Martí-Guillén et al. (2025a). Compound identification was carried out using ChemStation software by comparing the obtained mass spectra and retention times with those reported in the NIST11 spectral library and previously published reference data. The concentration of the main fatty acids and

phytosterols was determined as equivalents of linoleic acid and β-sitosterol, respectively, using standard curves. Eugenol concentration was determined using an appropriate standard. All standards were acquired from Merck Life Science S.L. (Madrid, Spain).

2.2. Plant material, seed priming and experimental design

For the entire experimental development, commercial broccoli seeds (*Brassica oleracea* var. *italica* Plenck cv. Naxos) kindly provided by Sakata Seed Iberica SLU (Valencia, Spain) were used.

2.2.1. Seed priming treatment

Seeds were subjected to priming treatments using different concentrations of uOCB and OCB (v/v): 10 % (uOCB10 and OCB10), 1 % (uOCB1 and OCB1) and 0.1 % (uOCB0.1 and OCB0.1) Seeds were soaked in each solution for 2 h under continuous orbital agitation at 200 rpm, in darkness, and at room temperature. Hydropriming (HP), consisting of seed hydration in distilled water, was used as a control treatment. Following priming, seeds were air-dried in a ventilated oven at room temperature until their initial moisture content was restored.

2.2.2. Evaluation of OCB biostimulant effect under salinity conditions

Primed seeds were placed in Petri dishes containing a sterile filter paper disk hydrated with 5 mL of either autoclaved distilled water (control) or a 100 mM NaCl solution to generate salt stress. Germination tests were carried out in darkness at 25 °C, using 25 seeds per dish with four independent replicates for each treatment. A batch of non-primed seeds (NP) was also included as an experimental control. Germination was recorded daily over a period of five days, considering a seed as germinated once radicle emergence reached approximately 1 mm. The germination parameters -Mean Germination Time (MGT), Germination Index (GI), and Vitality Index (VI)- were subsequently calculated following standard procedures as described Martí-Guillén et al. (2025b). Seedlings were collected after five days, their fresh weight (FW) measured, and the samples were immediately frozen in liquid nitrogen before being stored at −80 °C until further analysis.

2.3. Determination of oxidative stress markers

2.3.1. Hydrogen peroxide (H_2O_2) quantification

H_2O_2 content was determined using an iodometric colorimetric assay, following Junglee et al. (2014). Ground plant material was extracted in cold 0.1 % (w/v) trichloroacetic acid (TCA) at a 4:1 (w/v) ratio. The homogenate was centrifuged at 14,600 × *g* for 15 min, and the supernatant was used for analysis. For the reaction, defined volumes of extract, potassium phosphate buffer (50 mM, pH 7.0), KI (1 M), and 0.1 % TCA were combined (100, 50, 250, and 100 μL, respectively). Samples were incubated in the dark at room temperature for 1 h. Absorbance was read at 390 nm, and H_2O_2 concentration was calculated from a standard curve (0–500 μM).

2.3.2. Lipid peroxidation

Lipid peroxidation was estimated through malondialdehyde (MDA) formation using a thiobarbituric acid-reactive substances (TBARS) assay as described by Heath and Packer (1968), with slight modifications. An aliquot of the extract used for H_2O_2 quantification (150 μL) was mixed with 20 % (w/v) TCA containing 0.5 % (w/v) TBA (450 μL). The mixture was heated at 95 °C for 25 min and rapidly cooled. Absorbance was recorded at 532, 600, and 440 nm. MDA levels were calculated using the correction procedure of Hodges et al. (1999) and the coefficients described by Landi (2017).

2.3.3. Proline quantification

Proline concentration was measured using the modified ninhydrin-based colorimetric method described by Bates et al. (1973). Ground tissue was homogenized in 3 % (w/v) sulfosalicylic acid at a 2:1 ratio and centrifuged at 14,600 × *g* for 15 min. Equal volumes of the supernatant, acidic ninhydrin reagent, and glacial acetic acid were mixed and incubated at 100 °C for 1 h. After cooling on ice, the chromophore was extracted with 1 mL toluene, and absorbance was recorded at 520 nm. Proline concentration was determined from an L-proline standard curve (0–500 $\mu g \cdot mL^{-1}$).

2.4. Determination of hormone profile

Hormone profile determination was evaluated following the methodology described in Müller and Munné-Bosch (2011) with slight modifications. Briefly, around 100 mg of fresh grounded tissue were extracted with 400 μL of 20:79:1 (v/v/v) methanol:isopropanol:glacial acetic acid. Samples were vortexed for 10 min, sonicated for 30 min and centrifuged at 14,000 × *g* for 15 min. Supernatants were collected and stored. The process was repeated twice adding 200 μL of extractant each time and combining the supernatants obtained. Finally, volumes were adjusted up to 1 mL with extractant, filtered with 0.22 μm nylon filters, and stored at −80 °C until quantification. To avoid photo- or thermodegradation of phytohormones, all the procedure was performed in darkness and 4 °C. Hormone quantification was performed using an Agilent 1290 Infinity II HPLC coupled to Agilent 6550 Q-TOF Mass Spectrometer (HPLC/ESI-Q-TOF-MS/MS, Agilent Technologies, Santa Clara, USA), from Sección de Metabolómica y Proteómica of Área Científica y Técnica de Investigación (ACTI) of University of Murcia, using the method described in Müller and Munné-Bosch (2011).

The following hormones were determined: indole-3-acetic (IAA), indole-3-butyric (IBA), melatonin (MEL), gibberellic acid (GA_3), gibberellins A_4 (GA_4) and A_7 (GA_7), zeatin (Z), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), MJ and 1-aminocyclopropane-1-carboxylate (ACC); and were quantified using commercial standards purchased in Merck Life Science S.L. (Madrid, Spain) and Duchefa Biochemie B.V. (Haarlem, Netherlands).

2.5. Proteomic analysis

2.5.1. Protein extraction and sample preparation

Protein extraction was performed following Sánchez-López et al. (2016) method with slight modifications. For this, 200 mg of fresh and grounded plant material was extracted using 400 μL of a cooled extraction buffer, consisting of: 150 mM Tris–HCl (pH 8), 6 M urea, 2 % (w/v) SDS, 5 % (v/v) glycerol, 5 mM Tris(2-carboxyethyl) phosphine and 1X protease inhibitor cocktail (Cat. No. 11,873,580,001, Roche, Sigma-Aldrich, Spain). After 5 min at 95 °C, samples were centrifuged at 14,000 × *g*, for 10 min at 4 °C, and supernatants were recovered.

Then, protein was precipitated using methanol. Briefly, 200 μL of the protein extracts were mixed with 800 μL of cooled methanol and incubated for 30 min at −20 °C. Then, the mixture was centrifuged at 17,000 × *g* for 10 min at 10 °C and the supernatant was discarded. The precipitate was resuspended in 200 μL of 50 mM ammonium bicarbonate (pH 8) with 0.02 % 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate and sonicating. Samples were centrifuged at 17,000 × *g* for 10 min at 10 °C and supernatants were collected.

Total protein concentration was estimated using Pierce™ Coomassie Plus™ (Bradford) Protein Assay Kit (Cat. No. 23,236, Thermo Fisher Scientific, Waltham, MA, USA). Protein separation using one-dimensional 12 % denaturing SDS-PAGE was performed to verify the correct processing and state of the samples (Supplementary Fig. S1).

2.5.2. Protein digestion and identification

To develop protein identification, a label-free quantitative proteomics analysis was performed following the method described in Shevchenko et al. (1996). Briefly, 100 μg of protein extract was used for in-solution trypsin digestion and 0.01 % (v/v) ProteaseMAX™ Surfactant (Cat. No. V2071, Promega Corporation, Wisconsin, USA) was added to enhance the process. Protein samples were reduced by incubating

with 10 mM dithiothreitol at 56 °C for 20 min, alkylated by adding 25 mM iodoacetamide during 30 min at room temperature in the dark. Finally, samples were digested using Trypsin Gold MS Grade (Cat. No. V5280, Promega Corporation, Wisconsin, USA). The dried peptides generated were resuspended in 20 μL of buffer A [water/-acetonitrile/formic acid, 94.9:5:0.1 (v/v/v)] and separated using an Agilent 1290 Infinity II HPLC coupled to Agilent 6550 Q-TOF Mass Spectrometer (HPLC/ESI-Q-TOF-MS/MS, Agilent Technologies, Santa Clara, USA). Samples were injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 μm, 100 × 2.1 mm, Agilent Technologies, Santa Clara, USA), thermostated at 50 °C, at a flow rate of 0.4 ml min^{-1}. After the injection, the column was washed with buffer A for 3 min. During injection, peptide elution was performed using a linear gradient from 0 % to 40 % buffer B [water/acetonitrile/formic acid, 10:89.9:0.1 (v/v/v)] over 40 min, followed by a subsequent gradient from 40 % to 95 % buffer B over 8 min. The final composition of 95 % buffer B was maintained for an additional 3 min. Prior to each injection; the column was re-equilibrated to its initial conditions, consisting on 100 % buffer A, for 6 min. The mass spectrometer was operated in positive ionization mode.Quality controls (QC) consisted of 10 μg of bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. 23,209), which were subjected to the same processing as the samples.

All proteomic analysis were performed in the Servicio de Biología Molecular (SBM) of Área Científica y Técnica de Investigación (ACTI) of the University of Murcia.

2.6. Bioinformatic analysis

Protein identification was performed comparing the profile data acquired for both MS and MS/MS scans with the last version of broccoli proteome in Uniprot database (https://www.uniprot.org/uniprotkb?query=%28taxonomy_id%3A36774%29), using the Spectrum Mill MS Proteomics Workbench (Rev. B.06.00.20) software (Agilent Technologies, Santa Clara, USA). The Scored Peak Intensity (SPI) was used to filter high quality matches with database, and only protein identifications with SPI ≥ 60 % were selected for subsequent bioinformatic analysis. Three biological replicates were analyzed for each treatment and condition. Comprehensive data on protein and peptide identification and quantification are provided in Supplementary Tables S1–S13. QC were subsequently compared to mammal proteome Uniprot database and a coverage higher than 80 % was ensured.

The mass spectrometry-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2025) under the data set identifier PXD069532.

LFQ intensities of detected proteins were transformed to a log2 scale to stabilize variance and normalize data distribution. Filters were applied to exclude proteins detected in less than 70 % of replicates, and missing values were imputed using an MNAR approach (MiniProb, q = 0.01) to ensure the robustness of the analysis. To evaluate the overlap between replicates, heatmap graphs were generated (Supplementary Fig. S2–4). Protein coverage was assessed before and after filtering, guaranteeing data integrity for subsequent analyses. A principal component analysis (PCA) was performed to visualize the global variability and separation of the samples, as well as a partial least squares discriminant analysis (PLS-DA), identifying patterns that maximize the separation between conditions. Furthermore, a correlation matrix was calculated using Pearson's coefficient to explore the relationships between protein abundances and detect shared regulatory modules (Supplementary Fig. S5–7).

To perform the differential accumulation analysis, differentially abundant proteins (DAPs) were identified using an empirical Bayesian statistical model using *DEP2* package. Significance thresholds (adjust p-value < 0.05) and fold change (Ilog2 Fold ChangeI ≥ 1) were applied to define the relevant proteins in each comparison. Heatmaps (Supplementary Fig. S8–10) and volcano plots were used to represent the results.

Raw data processing was performed by División de Apoyo Estadístico (SAE) of Área Científica y Técnica de Investigación (ACTI) of the University of Murcia.

Gene ontology (GO) pathway enrichment was performed using the platform ShinyGO (v.0.85) (https://bioinformatics.sdstate.edu/go/), with Benjamini−Hochberg false discovery rate (FDR) significance threshold set at 0.05 (Ge et al., 2020).

2.7. Statistical analysis

All data analyses related to germination parameters, oxidative stress markers, and hormonal profiles were performed using the SPSS software package (version 28.0; SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) was conducted, followed by Duncan's multiple range test, and differences were considered statistically significant at $p < 0.05$. Principal Component Analysis (PCA) was carried out using the Minitab software package (version 21.0; Minitab Inc., State College, PA, USA). Hierarchical Cluster Analysis (HCA) was conducted using the ClustVis web tool (Metsalu and Vilo, 2015).

3. Results

3.1. Evaluation of the biostimulant effect of OCB on broccoli seeds germination

Before performing the seed priming treatments, both uOCB and OCB were characterized to assess the enrichment in bioactive compounds induced by the elicitation with 25 mM CD and 100 μM MJ (Table 1). OCB resulted in a bioproduct enriched in proteins (9.34 mg mL^{-1}) and

Table 1
Characterization of unelicited (uOCB) and elicited (OCB) orange carrot biostimulants. TPC: total phenolic content; GAE: gallic acid equivalents; LLA eq.: linoleic acid equivalents; n.d.: non-detected.

		Unelicited Orange Carrot Biostimulant (uOCB)	Orange Carrot Biostimulant (OCB)
pH		5.86	5.58
Conductivity (mS cm^{-1})		2.79	3.3
Soluble sugars (°Brix)		1	4.5
Total protein content (mg mL^{-1})		4.48	9.34
TPC (μg GAE mL^{-1})		65.96	125.30
Eugenol (μg mL^{-1})		n.d.	6.83
Fatty acids (μg LLA eq. mL^{-1})	Methyl palmitate	0.962	1.071
	Palmitate	1.299	3.817
	Ethyl palmitate	0.259	0.467
	Isopropyl palmitate	0.302	0.299
	Methyl inoleate	0.257	0.805
	Methyl linolenate	3.183	0.245
	Methyl stearate	0.366	0.487
	Ethyl linoleate	1.385	0.220
	Total fatty acid content	8.015	7.411
Phytosterols (μg β-sitosterol eq. mL^{-1})	Squalene	0.209	2.144
	Cholesterol	n.d.	0.741
	Campesterol	2.466	9.049
	(E)-14α-Methyl-5α-ergosta-8,23-dien-3β-ol	n.d.	1.363
	Stigmasterol	3.477	11.730
	25,26-Dihydroelasterol	n.d.	0.701
	γ-Ergostenol	n.d.	0.808
	β-Sitosterol	3.575	9.254
	Fucosterol	n.d.	4.152
	Stigmast-8(14)-en-3β-ol	n.d.	0.749
	Cycloartenol	n.d.	2.880
	Total phytosterol content	9.727	43.571

phenolic compounds (125.30 μg GAE mL^{-1}), as both total protein content and TPC were 2-fold higher compared to uOCB. Particularly, the elicitation process triggered the accumulation of eugenol, a phenylpropanoid characteristically found in carrots, reaching a concentration of 6.83 μg mL^{-1}. Furthermore, although total fatty acid content was not affected by the elicitation process, the results showed an increased total phytosterol content of 43.57 μg β-sitosterol eq. mL^{-1}, nearly 4.5-fold higher than in uOCB. Among the main phytosterols accumulated, the enrichment in campesterol, stigmasterol, β-sitosterol and fucosterol particularly stood out, as they correspond to the 78 % of total phytosterol content. Additional physicochemical characterization of OCB revealed a pH of 5.58, a soluble sugar content of 4.5 °Brix, and an electrical conductivity of 3.3 $mS{\cdot}cm^{-1}$.

Then, the biostimulant effect of OCB on broccoli seed germination under saline conditions (100 mM NaCl) was evaluated. For this purpose, broccoli seeds were primed with three different concentrations of the product: 10 %, 1 %, and 0.1 % (v/v). Furthermore, to demonstrate that the enrichment of bioactive compounds in the elicited product is responsible for its biostimulant properties, the seeds were also primed with uOCB at the same concentrations as those used with OCB.

Regarding the germination parameters evaluated, no significant differences were found in the MGT or GI of broccoli seeds germinated in the absence or presence of 100 mM NaCl, regardless of the priming treatment used (Fig. 1A-B). However, a clear reduction in seedling VI and FW was observed in non-primed (NP) seeds germinated under saline conditions, with decreases of −25 % and −22 %, respectively. In the absence of salinity, all priming treatments showed a positive effect on broccoli seedling development compared to the control (NP treatment), increasing VI and FW by up to +13 % and +10 %, respectively. No significant differences were observed between HP treatment and the biostimulant treatment (both uOCB and OCB) (Fig. 1C-D).

However, the bioproduct's effect under saline conditions is particularly noteworthy. With the application of 100 mM NaCl, the lowest dose of OCB (OCB0.1) achieved the greatest effect of all the priming treatments tested, reversing the negative impact of salinity. However, high OCB doses (OCB10 and OCB1) failed to achieve this effect. OCB0.1 increased seedling VI and FW up to 27 % compared to untreated seeds (NP seeds), restoring these parameters to the same levels as those observed in NP seeds germinated in the absence of salinity (Fig. 1C-D). This effect was only observed with the enriched bioproduct, since none of the uOCB doses showed any significant difference from HP treatment.

Therefore, based on the results obtained, 0.1 % (v/v) dose of OCB (OCB0.1) was selected for further analysis, as it successfully restored the vitality and weight of broccoli seedlings grown under saline conditions.

3.2. *Evaluation of the biostimulant effect of OCB for ameliorating salt stress*

To assess the effect of OCB alleviating salt stress, the analysis of different oxidative stress markers in the broccoli seedlings was carried out.

H_2O_2 is a molecule involved in cell signaling, but under oxidative stress conditions, its levels increase and can lead to damage to cellular components. As shown in Fig. 2A, germination under saline conditions increased H_2O_2 content compared to germination in the absence of salinity, both in non-primed (NP) seeds and in seeds subjected to hydropriming (HP). However, OCB treatment prevented H_2O_2 concentration increase under saline conditions, maintaining levels comparable to those observed in seeds germinated in the absence of salinity.

Lipid peroxidation, associated with cell membrane damage caused by reactive oxygen species (ROS), can be estimated through MDA content. The results showed an increase of approximately 40 % in MDA content with the application of 100 mM NaCl, in both NP and HP seeds. Conversely, seeds primed with OCB and germinated under salt stress conditions showed a lower MDA content (−11 %) than NP and HP seeds (Fig. 2B).

Proline is an osmoregulatory compound that may also be involved in oxidative stress response. As shown in Fig. 2C, proline concentration

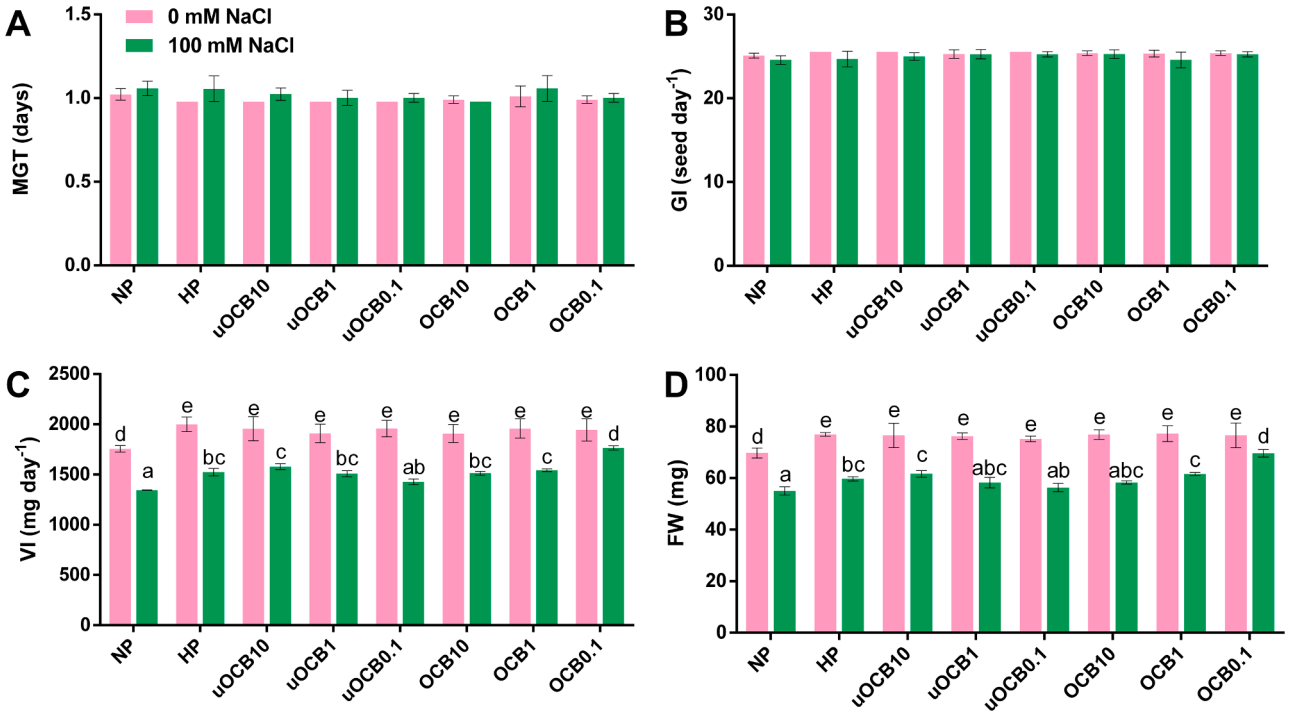


Fig. 1. Effects of the different seed-priming treatments on the germination performance of broccoli under non-saline (0 mM NaCl) and saline (100 mM NaCl) conditions. Mean Germination Time (MGT, A), Germination Index (GI, B), Vitality Index (VI, C), and Fresh Weight (FW, D) are shown. Data correspond to mean ± SD ($n = 4$). Distinct letters above bars denote significant differences among treatments ($p < 0.05$; Duncan's multiple range test). Abbreviations: NP, no priming; HP, hydropriming; uOCB, unelicited orange carrot biostimulant; OCB, orange carrot biostimulant.

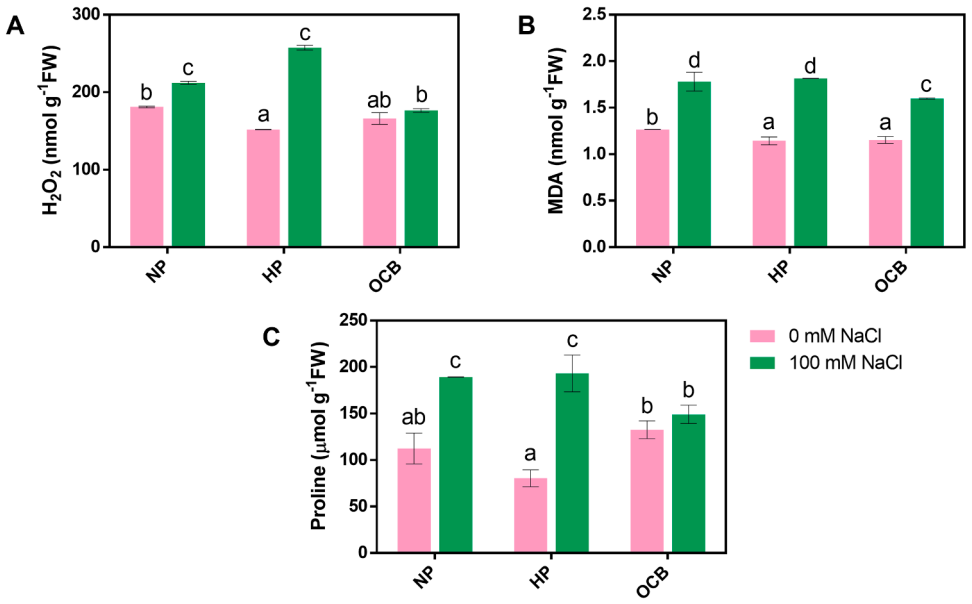


Fig. 2. Effect of OCB-based seed priming on oxidative stress indicators in broccoli seedlings germinated under control and saline (100 mM NaCl) conditions. Hydrogen peroxide (H_2O_2, A), malondialdehyde (MDA, B), and proline (C) contents are shown. Bars represent mean ± SD ($n = 3$). Different letters indicate statistically significant differences among treatments ($p < 0.05$; Duncan's test). Abbreviations: NP, no priming; HP, hydropriming; OCB, orange carrot biostimulant at 0.1 % (v/v).

increased by 68 % in NP seeds subjected to salt stress, compared to those germinated in the absence of salinity. Regarding primed seeds, those subjected to HP showed a similar response to non-primed seeds, while in OCB-primed seeds, proline levels were similar both in the absence and presence of salt, the latter being 22 % lower than those in NP seeds germinated in the presence of 100 mM NaCl.

3.3. Evaluation of the effect of OCB in the hormonal profile of broccoli seedlings subjected to salt stress

Given the crucial role that phytohormones play during seed germination and seedling development, as well as during stress response, a hormonal profile analysis of broccoli seedlings was conducted.

In general, a variation in the phytohormone balance was observed after the application of salt stress (Table 2). Regarding the main groups of altered hormones, a significant increase in the concentration of Z, GA_4, GA_7, IBA, and MEL was observed in the presence of salinity in non-primed seeds (NP S, Table 2). In contrast, OCB pre-treatment induced a different hormonal response in seeds grown under saline conditions (OCB S), which was not observed in seeds subjected to HP. Overall, a decrease in the concentration of several phytohormones, whose levels had been increased by salt stress in NP seeds, was observed, largely reversing the salinity effects previously described in untreated seeds (NP C). Thus, a lower concentration of the auxin IBA, as well as the gibberellins GA_3, GA_4, and GA_7, was observed. In addition, the decrease in the amount of stress-related phytohormones, such as MEL, ABA, MJ, and SA, is particularly noteworthy (Table 2).

To better understand the physiological, biochemical, and hormonal changes in broccoli seedlings after OCB treatment and salt stress exposure, an HCA and PCA were performed, integrating the obtained data. As shown in Fig. 3A, there is a clear separation between seeds germinated in the absence (C) and presence (S, 100 mM) of salt stress. Furthermore, among the seeds subjected to salt stress, differences were observed between those previously treated with OCB, which clustered together with the seeds germinated in the absence of salinity, and separating from NP and HP seeds. All these differences reflect the deregulation observed in the profile of the measured parameters, characterized by a decrease in growth parameters (FW, VI) and an increase in the concentration of oxidative stress markers and various phytohormones (mainly ABA, IBA, MEL, and gibberellins) in NP and HP seeds germinated under salinity conditions. However, in OCB-treated seeds, an attenuation of this deregulation under salt stress conditions was observed.

These findings are consistent with the results obtained from the PCA.

Table 2
Hormonal profile evaluation of broccoli seedlings grown in absence (C) or presence of 100 mM NaCl (S) and subjected to different seed priming treatments expressed as µg g^{-1}FW. Data represents mean ± SD ($n = 3$). Different letters in each row denote significant differences ($p < 0.05$) in germination parameters between salinity treatments according to Duncan's test. NP: no priming; HP: hydropriming; OCB: orange carrot biostimulant at 0.1 % (v/v); Z: Zeatin; GA_3: Gibberellic Acid; GA_4: Gibberellin A_4; GA_7: Gibberellin A_7; IAA: Indoleacetic Acid; IBA: Indole-3-butyric Acid; MEL: Melatonin; ABA: Abscisic Acid; ACC: 1-Amino-cyclopropane-1-carboxylic Acid; JA: Jasmonic Acid; MJ: Methyl-jasmonate; SA: Salicilyc Acid.

	NP C	NP S	HP C	HP S	OCB C	OCB S
Z	2.403 ± 0.167 a	2.789 ± 0.033 b	2.545 ± 0.104 ab	2.354 ± 0.231 a	2.724 ± 0.037 ab	2.596 ± 0.124 ab
GA_3	0.744 ± 0.094 c	0.741 ± 0.067 c	0.166 ± 0.079 a	0.719 ± 0.011 c	0.185 ± 0.009 a	0.568 ± 0.081 b
GA_4	2.938 ± 0.060 a	5.184 ± 0.138 c	3.427 ± 0.899 ab	5.128 ± 0.255 c	3.529 ± 0.491 ab	4.005 ± 0.280 b
GA_7	1.649 ± 0.108 d	2.177 ± 0.066 e	0.967 ± 0.007 a	1.476 ± 0.068 c	1.319 ± 0.011 b	1.260 ± 0.070 b
IAA	0.904 ± 0.144 a	0.994 ± 0.047 ab	1.621 ± 0.003 c	1.069 ± 0.229 ab	0.980 ± 0.018 ab	1.250 ± 0.236 b
IBA	83.806 ± 1.239 ab	124.380 ± 3.647 d	90.718 ± 8.073 bc	91.514 ± 6.140 bc	76.033 ± 4.785 a	98.377 ± 4.999 c
MEL	0.251 ± 0.024 ab	0.334 ± 0.004 d	0.222 ±0.013 a	0.320 ± 0.008 cd	0.228 ± 0.020 a	0.279 ± 0.023 bc
ABA	0.296 ± 0.025 b	0.288 ± 0.006 b	0.340 ± 0.030 b	0.659 ± 0.063 c	0.170 ± 0.018 a	0.199 ± 0.012 a
ACC	12.554 ± 0.020	12.724 ± 0.350	13.523 ± 1.149	11.411 ± 0.830	12.533 ± 0.725	10.777 ± 2.717
JA	1.671 ± 0.447	1.790 ± 0.018	1.404 ± 0.063	1.612 ± 0.255	1.897 ± 0.124	1.924 ± 0.161
MJ	7.034 ± 0.170 b	7.820 ± 1.150 b	7.723 ± 0.114 b	6.948 ± 0.399 b	7.001 ± 0.254 b	5.897 ± 0.083 a
SA	3.816 ± 0.608 b	3.884 ± 0.204 b	3.658 ± 0.058 b	3.525 ± 0.013 b	3.808 ± 0.402 b	2.898 ± 0.008 a

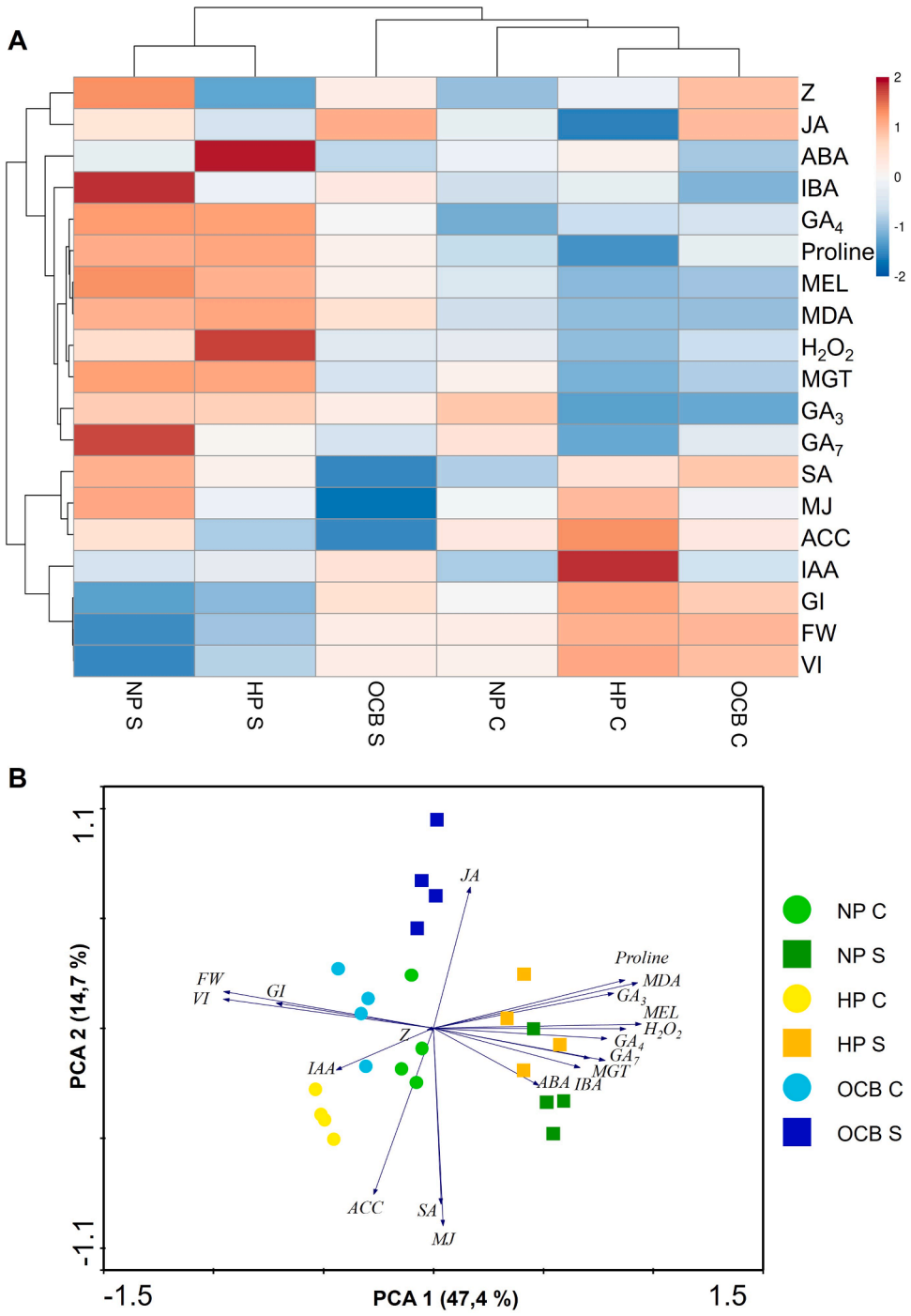


Fig. 3. Multivariate analysis of physiological, oxidative, and hormonal parameters in primed broccoli seedlings germinated under control (C) or saline (S, 100 mM NaCl) conditions. (A) Hierarchical Cluster Analysis (HCA) and heatmap visualization of phenotypic, oxidative, and hormonal datasets. The colour scale represents the normalized mean value of each trait. (B) Principal Component Analysis (PCA) based on the correlation matrix of all measured parameters. Seeds germinated under control and saline conditions are displayed as circles and squares, respectively. Vector length reflects the relative contribution of each variable to the principal components, while the angle between vectors denotes the degree of correlation (smaller angles indicating closer association). Abbreviations: PC, principal component; NP, no priming; HP, hydropriming; OCB, orange carrot biostimulant at 0.1 % (v/v); FW, Fresh Weight; VI, Vitality Index; Z, Zeatin; GA_3, Gibberellic Acid; GA_4, Gibberellin A_4; GA_7, Gibberellin A_7; IAA, Indoleacetic Acid; IBA, Indole-3-butyric Acid; MEL, Melatonin; ABA, Abscisic Acid; ACC, 1-Aminocyclopropane-1-carboxylic Acid; JA, Jasmonic Acid; MJ, Methyl Jasmonate; SA, Salicylic Acid.

As shown in Fig. 3B, principal component 1 (PC1) accounted for 47.4 % of the total variance and was primarily associated with fresh weight and vitality parameters (FW, VI) on the negative side of the X-axis, and with oxidative stress markers (H_2O_2, proline, and MDA) as well as several phytohormones (mainly ABA, MEL, and gibberellins) on the positive side of the X-axis. On the other hand, principal component 2 (PC2) explained 14.7 % of the variance and was mainly influenced by SA and MJ on the negative side of the Y-axis, and JA on the positive side. In agreement with the clustering observed in the HCA, sample projection along the principal components revealed a clear separation according to salinity treatment. Notably, among the seeds germinated under saline conditions, those treated with the biostimulant (OCB S) were clearly

distinguished from non-primed (NP S) and hydroprimed (HP S) seeds, and their distribution was closer to that of the non-stressed control seeds (NP C).

Therefore, NP seeds and OCB-treated seeds, germinated both in the presence and absence of salt stress, were selected for proteomic analysis in order to investigate in greater detail the changes in specific protein accumulation induced by the biostimulant.

3.4. Proteomic changes triggered by OCB in broccoli seedlings under saline conditions

To determine the changes in the accumulation of specific proteins induced by the biostimulant, a label-free quantitative proteomics analysis was performed. Approximately 150 proteins were identified for each one of the conditions analyzed. Samples from NP seedlings grown in the absence of salinity (NP C) were used for data normalization and to identify DAPs (Supplementary Tables S14–16).

Regarding non-primed seeds germinated under stress conditions (NP S), our results showed a total of 56 DAPs (35 upregulated and 21 downregulated) (Supplementary Fig. S8 and Supplementary Table S14). A GO enrichment analysis revealed that the main biological processes upregulated by salinity were related to removal of superoxide radicals (GO:0019,430), reactive oxygen species metabolic process (GO:0072,593) and detoxification (GO:0098,754), but also translation (GO:0006,412) and small molecule metabolic processes (GO:0044,281). However, no significant enrichment was found for downregulated DAPs (Fig. 4).

Then, we analyzed the effect of the biostimulant treatment under non stress conditions by determining the DAPs of OCB C (Supplementary Fig. S9 and Supplementary Table S15). We found a total of 30 DAPs (13 upregulated and 17 downregulated), mainly enriched in proteolysis and protein catabolism (GO:0045,732; GO:0045,862; GO:0061,136), miRNA processing (GO:0035,196), intracellular transport (GO:0051,169; GO:0006,913; GO:0046,907) and localization (GO:0051,179; GO:0051,234; GO:0051,649) biological processes among upregulated DAPs. However, in downregulated DAPs, the main GO terms regarding biological processes were related to intracellular lipid transport (GO:0015,918; GO:0032,366; GO:0032,365; GO:0015,850; GO:0006,869; GO:0010,876) and translation (GO:0006,412) (Fig. 5).

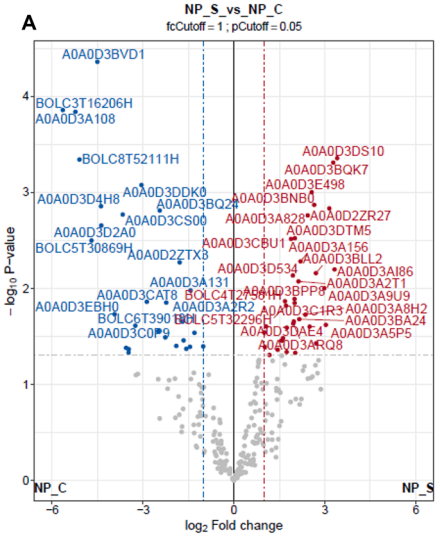
Ultimately, OCB treatment effect under salt stress conditions (OCB S) was assessed. Here, 34 DAPs (19 upregulated and 15 downregulated) were found in total (Supplementary Fig. S10 and Supplementary Table S16), a much lower number than in NP S seedlings. Among upregulated DAPs, it was found an enrichment in photosynthesis and photosynthesis regulation (GO:0015,979; GO:0042,548; GO:0,042,548; GO:0042,548), translation (GO:0006,412) and positive regulation of superoxide dismutase activity (GO:1901,671; GO:1901,668; GO:0051,353). The GO enrichment analysis of downregulated DAPs showed that the main biological processes were related to the regulation of protein metabolic processes (GO:0051,353), mRNA modification (GO:0016,556) and detection of stimulus and signaling processes (GO:0007,602, GO:0009,585; GO:0009,583; GO:0030,522; GO:0009,581; GO:0009,582) (Fig. 6).

Due to the enrichment in ROS metabolic processes-related DAPs in NP seeds under saline conditions, a specific analysis on the main enzymes detected and involved in this metabolism was performed. As Fig. 7 shows, NP seeds subjected to salinity conditions overaccumulated a wide range of enzymes involved in ROS detoxification, including catalase, glutathione transferase, peroxirredoxin and different isoforms of superoxide dismutase. However, in OCB-treated seeds, both germinated in absence or presence of 100 mM NaCl (OCB C and OCB S), the level of these enzymes was not significantly different from NP seeds germinated in control conditions (0 mM NaCl), indicating that OCB treatment is able to reverse the negative effect of salt stress on the activation of oxidative metabolism.

4. Discussion

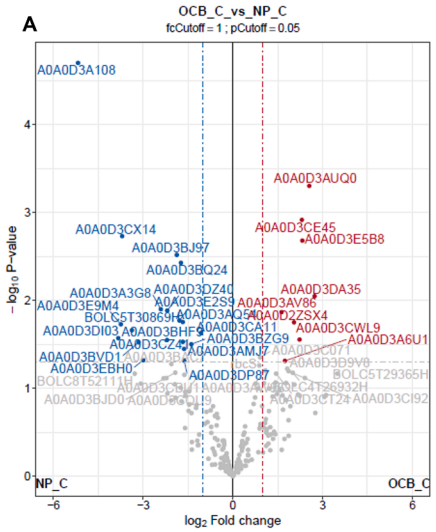
Soil salinization has become a widely extended problem with severe consequences for the development of crops of agronomic interest, decreasing their germination rate and preventing correct seedling development. Consequently, seed priming has emerged as a valuable approach that has gained substantial attention in recent years, as it enhances germination performance under adverse conditions and improves plant tolerance to abiotic stress (Ibrahim, 2016).

In this study, a biostimulant derived from elicited orange carrot cell cultures was used to enhance the germination performance of broccoli seeds under saline conditions. Carrot is a vegetable of special interest due to its antioxidant properties (Ahmad et al., 2019), as a result of the high content of bioactive compounds it possesses, mainly phenolic compounds, carotenoids, vitamin C and phytosterols (Ahmad et al.,



ID	Description	FDR enrichment
GO:0072593	Reactive oxygen species metabolic processes	6.0E-04
GO:0098869	Cellular oxidant detoxification	6.0E-04
GO:0097237	Cellular response to toxic substance	6.0E-04
GO:1990748	Cellular detoxification	6.0E-04
GO:0044281	Small molecule metabolic processes	6.0E-04
GO:0019430	Removal of superoxide radicals	1.3E-03
GO:0071450	Cellular response to oxygen radical	1.3E-03
GO:0071451	Cellular response to superoxide	1.3E-03
GO:0098754	Detoxification	1.3E-03
GO:0006412	Translation	1.3E-03

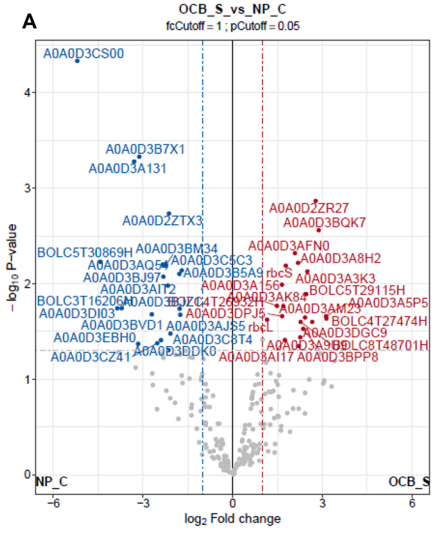
Fig. 4. Differentially abundant proteins (DAPs) in non-primed (NP) broccoli seedlings under salt stress (100 mM NaCl, S) compared to control conditions (0 mM NaCl, C). (A) Volcano plot representation of DAPs. The y-axis represents the significance level (expressed as $-\log_{10}$ p-value) and the x-axis represents the fold-change (expressed as $\log_2$). Each point represents individual protein, with grey points indicating proteins that showed no significant difference in abundance; blue points indicate downregulated DAPs; red points indicate upregulated DAPs. (B) GO terms of biological processes of upregulated DAPs.



ID	Description	FDR enrichment
GO:0051169	Nuclear transport	1.3E-02
GO:0006913	Nucleocytoplasmic transport	1.3E-02
GO:0035196	miRNA processing	3.6E-02
GO:0045732	Pos. reg. of protein catabolic processes	3.6E-02
GO:0045862	Pos. reg. of proteolysis	3.6E-02
GO:0061136	Reg. of proteasomal protein catabolic processes	3.6E-02
GO:0046907	Intracellular transport	3.6E-02
GO:0051649	Establishment of localization in cell	3.6E-02
GO:0051234	Establishment of localization	3.6E-02
GO:0051179	Localization	3.6E-02

ID	Description	FDR enrichment
GO:0006412	Translation	1.1E-04
GO:0006869	Lipid transport	2.4E-02
GO:0010876	Lipid localization	2.4E-02
GO:0015918	Sterol transport	2.9E-04
GO:0032366	Intracellular sterol transport	2.9E-04
GO:0032365	Intracellular lipid transport	3.8E-04
GO:0015850	Organic hydroxy compound transport	3.8E-04
GO:0007602	Phototransduction	3.9E-02
GO:0030522	Intracellular receptor signaling pathway	3.9E-02
GO:0016556	mRNA modification	3.9E-02

Fig. 5. Differentially abundant proteins (DAPs) from OCB-treated broccoli seedlings germinated in the absence of salt stress (0 mM NaCl, C) compared to non-primed (NP) seeds. (A) Volcano plot representation of DAPs. The y-axis represents the significance level (expressed as $-\log_{10}$ p-value) and the x-axis represents the fold-change (expressed as $\log_2$). Each point represents individual protein, with grey points indicating proteins that showed no significant difference in abundance; blue points indicate downregulated DAPs; red points indicate upregulated DAPs. (B) GO terms of biological processes of upregulated DAPs. (C) GO terms of biological processes of downregulated DAPs. OCB: orange carrot biostimulant at 0.1 % (v/v).



ID	Description	FDR enrichment
GO:0051353	Pos. reg. of oxidoreductase activity	1.0E-02
GO:1901668	Reg. of superoxide dismutase activity	1.0E-02
GO:1901671	Pos. reg. of superoxide dismutase activity	1.0E-02
GO:1905156	Neg. reg. of photosynthesis	1.0E-02
GO:0006412	Translation	1.0E-02
GO:0051341	Reg. of oxidoreductase activity	1.4E-02
GO:0042548	Photosystem ii stabilization	1.6E-02
GO:0015979	Photosynthesis	1.6E-02
GO:0042548	Reg. of photosynthesis light reaction	2.6E-02
GO:0043467	Reg. of generation of precursor metabolites and energy	2.6E-02

ID	Description	FDR enrichment
GO:0006556	S-adenosylmethionine biosynthetic processes	5.0E-02
GO:0046500	S-adenosylmethionine metabolic processes	5.0E-02
GO:0007602	Phototransduction	5.0E-02
GO:0009585	Red far-red light phototransduction	5.0E-02
GO:0009583	Detection of light stimulus	5.0E-02
GO:0030522	Intracellular receptor signaling pathway	5.0E-02
GO:0009581	Detection of external stimulus	5.0E-02
GO:0009582	Detection of abiotic stimulus	5.0E-02
GO:0016556	mRNA modification	5.0E-02
GO:0051246	Reg. of protein metabolic proc.	5.0E-02

Fig. 6. Differentially abundant proteins (DAPs) from OCB-treated broccoli seedlings germinated in presence (100 mM NaCl, S) of salt stress compared to non-primed (NP) seeds germinated in control (0 mM NaCl, C). (A) Volcano plot representation of DAPs. The y-axis represents the significance level (expressed as $-\log_{10}$ p-value) and the x-axis represents the fold-change (expressed as $\log_2$). Each point represents individual protein, with grey points indicating proteins that showed no significant difference in abundance; blue points indicate downregulated DAPs; red points indicate upregulated DAPs. (B) GO terms of biological processes of upregulated DAPs. (C) GO terms of biological processes of downregulated DAPs. OCB: orange carrot biostimulant at 0.1 % (v/v).

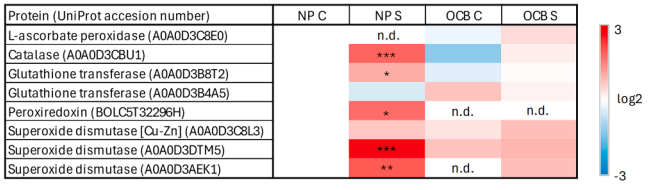


Fig. 7. Heatmap of relative accumulation of proteins involved in oxidative metabolism (expressed as $\log_2$) of broccoli seedlings germinated in absence (C, 0 mM NaCl) or presence of salt stress (S, 100 mM NaCl) after normalization with NP C. Asterisks denote significant differences from NP C (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). NP: no priming; OCB: orange carrot biostimulant at 0.1 % (v/v); n.d.: non-detected.

2019; Miras-Moreno et al., 2016a), providing this plant material with biostimulant potential. In fact, the effectiveness of carrot-derived biostimulants has recently been demonstrated in different plant species, achieving an improvement of the germination process (Martí-Guillén et al., 2025a; Teixeira et al., 2025). Thus, given the interest of this crop, orange carrot cell cultures, previously established in our research group, have been subjected to elicitation techniques (Sabater-Jara et al., 2014; Sabater-Jara and Pedreño, 2013), consequently obtaining a bioproduct, named OCB, enriched in phytosterols and phenolic compounds (Table 1). Furthermore, this biostimulant shows great potential for being produced uniform- and homogeneously over time, as it does not depend on the seasonal and geographical factors that influence the composition of traditional hPDBs.

To evaluate the potential of OCB as a seed-priming agent for

improving germination and reducing salt stress effects, several concentrations of the biostimulant were tested under both saline and non-saline conditions. In addition, the same doses of an unelicited product (uOCB) were applied. Our biostimulant, OCB, completely reversed the negative effects of salinity on germination parameters at a dose of 0.1 % (v/v), restoring the vitality and weight of developed seeds to the levels present in seeds germinated in the absence of salinity. This effect was exclusive to the bioproduct generated through elicitation techniques and was not achieved by HP treatment or any of the treatments with uOCB (Fig. 1). This demonstrates that the achieved effect is due to the enrichment in bioactive compounds triggered by the elicitation treatment, thus validating the effectiveness of this technology in generating a bioproduct with biostimulant potential. In fact, our group has already described the effectiveness of this technique in enriching cell cultures of yellow carrot (Martí-Guillén et al., 2025a) and grapevine (Martí-Guillén et al., 2025b) in bioactive compounds, providing them with biostimulant capacity. However, high OCB doses (10 % (v/v) and 1 % (v/v)) did not improve germination parameters. It has been widely described in literature that biostimulant effectiveness is commonly dose-dependent, based on their composition in bioactive compounds, as they can induce phytotoxicity when applied at high concentrations, inhibiting plant growth (Broutin et al., 2025; Han et al., 2024). Thus, the enrichment in bioactive compounds present in OCB could be restraining seedling growth when applied at high doses.

One of the reasons why salt stress compromises germination parameters is due to the damage to cellular components caused by the overaccumulation of reactive species, mainly ROS (Balasubramaniam et al., 2023; Martí-Guillén et al., 2022). Our results showed that seeds germinated under salinity conditions accumulated H_2O_2, also increasing MDA and proline content (Fig. 2). It has been widely described in the literature that H_2O_2, under basal conditions, is an essential molecule in signaling pathways for a large number of plant physiological processes (Mittler, 2017). However, under saline conditions, the accumulation of this molecule rises to phytotoxic levels, since it disrupts cell metabolism by damaging cellular components, such as lipid peroxidation, due to its high oxidative capacity (Martí-Guillén et al., 2022). Proline is an osmoregulatory metabolite that is accumulated under hyperosmotic conditions (Delauney and Verma, 1993), but it can also act as an oxidative stress marker, since it also accumulates under oxidative stress conditions, mediating ROS detoxification pathways (Anwar Hossain et al., 2014). Our results show that OCB managed to decrease H_2O_2 content under stress conditions to the same level as non-stressful conditions. Moreover, our bioproduct was also capable of decreasing both MDA and proline levels in seeds germinated under saline conditions (Fig. 2). It has been demonstrated that phenolic compounds can act as antioxidant molecules and ROS scavengers, playing a fundamental role in alleviating the negative impact of abiotic stress (Kumar et al., 2023). In fact, numerous studies have demonstrated the effectiveness of these types of compounds as biostimulants against salt stress. Specifically, recent studies have shown that the use of phenolic compound–rich extracts as seed-priming agents is capable of improving germination in numerous species of agronomic interest under salinity conditions, decreasing H_2O_2 and MDA levels by promoting antioxidant defences (García-Sánchez et al., 2012; Huang et al., 2021; Rady et al., 2013; Shalaby, 2024). Particularly, Xu et al. (2024) demonstrated that eugenol can alleviate salt stress in tobacco seedlings by reinforcing antioxidant defence and decreasing oxidative damage. Furthermore, phytosterols have also been classified as key molecules during germination (Suo et al., 2021) and stress response (Aboobucker and Suza, 2019), in addition to having a potential antioxidant effect (Shen et al., 2024). Specifically, it has been demonstrated that both fucosterol and β-sitosterol are capable of acting as biostimulants, decreasing ROS and MDA content in plants subjected to abiotic stress (Hanafy and Sadak, 2023; Yu et al., 2024). On the other hand, different biostimulants have been shown to exert a negative modulation of proline levels under salt stress conditions (Bulgari et al., 2019; Hmaeid et al., 2019; Kovács et al., 2024; Vangenechten et al., 2025). Therefore, the OCB would be exercising a more efficient management of salt stress due to its antioxidant-rich composition, which prevents the need for an increase in proline levels to counteract oxidative stress.

Furthermore, the effect of the OCB on the hormonal profile of germinated seeds under salinity conditions was also determined. Maintaining a balanced phytohormone profile is essential to ensure proper plant development and an effective stress response (Verma et al., 2016). In fact, numerous biostimulants have been described with the ability to modulate phytohormone content, helping to cope with abiotic stress, through the activation or deactivation of their synthetic pathways (Martínez-Lorente et al., 2024; Montesinos et al., 2024). Thus, OCB could be regulating hormonal profile through these mechanisms. Particularly, ABA levels are especially important during the germination process, as this hormone maintains seed dormancy, preventing seed germination (Miransari and Smith, 2014). Our results show that OCB triggers a decrease in ABA levels (Table 2). This decrease in ABA levels could be related to the greater promotion of seed development observed in treated seeds due to a lesser repression of the germination process. In fact, decreased ABA levels have been linked to a break of seed dormancy, leading to the start of germination (Sano and Marion-Poll, 2021).

Regarding auxin and gibberellin content, under salt stress conditions, an increase in IBA and gibberellin content was observed in untreated seeds. On the other hand, a decrease in the levels of these phytohormones was detected in OCB-treated seeds (Table 2). Although auxins *per se* are not essential for germination, it has been described that a repression of AUXIN RESPONSE FACTORS occurs during the process, which is essential for germination (Liu et al., 2007; Miransari and Smith, 2014). Specifically, Monroe-Augustus et al. (2003) described that the mutation of *ibr5* (indole-3-butyric acid-response 5) gene in Arabidopsis, which also belongs to Brassicaceae family, generated plants with an increased germination rate and a lower sensitivity to IBA. The lower concentration of IBA in treated seedlings, thus, would be related to a lower auxin-triggered response, promoting seed development. However, gibberellins have been widely described as germination promoters, acting contrary to ABA (Miransari and Smith, 2014). Nevertheless, different studies have shown that Arabidopsis mutants with high gibberellin content showed greater sensitivity to stress, while those with lower gibberellin levels showed greater stress tolerance (Achard et al., 2006; Colebrook et al., 2014). In fact, Magome et al. (2008) demonstrated that, in Arabidopsis, the response to high salinity concentrations depended on the expression of *GAox7*, a gibberellin deactivating gene. Therefore, the lower concentration of gibberellins found in OCB-treated seedlings could be related to their greater tolerance to salt stress.

With regards to MEL, SA and MJ, all these are stress-related phytohormones which content decreased under salinity conditions in OCB-primed seeds, like ABA (Table 2). All these hormones are involved in defence signaling pathways, regulating gene expression and antioxidant mechanisms (Hakeem et al., 2025; Martínez-Lorente et al., 2022; Song et al., 2023). However, the increase in their concentration may have a deleterious effect on the germination process, as high doses of these phytohormones can inhibit germination and plant growth (Bhavanam and Stout, 2021; Escobedo-Álvarez et al., 2024; Martínez-Lorente et al., 2022; Zalewski et al., 2020); so the better seedling growth and germination in OCB-treated seeds, even under salt stress, could be related to the reduced concentration of these phytohormones. This could be related to the lower ROS content present in OCB-treated seedlings (Fig. 2 and 3), which would prevent a pronounced defence response, compared to NP seeds.

Finally, a proteomic study of the seedlings was carried out. During germination, there is a significant remodeling of the proteome due to the degradation of storage proteins and the synthesis of necessary proteins for the activation of the germinative metabolism (Diaz-Vivancos et al., 2013). Along these lines, OCB-treated seeds germinated in the absence of salinity showed an accumulation of proteins related to proteolysis and protein catabolism (Fig. 5), which could be related to the greater vitality

present in these seedlings compared to NP. In contrast, this enrichment is not present in seeds germinated in the presence of salinity, both NP and OCB-treated, which would indicate a lower activation of proteolysis and protein mobilization processes due to the negative impact of salt stress. This lower mobilization of reserves in seeds germinated under salinity conditions coincides with that described by Sghayar et al. (2023), which demonstrated an inhibition of amylase and protease activities in seeds germinated under salt stress conditions. However, under these conditions, NP seeds showed an accumulation of oxidative metabolism-related enzymes that were not found in OCB-treated seeds (Fig. 7). In addition, OCB-treated seedlings also presented fewer DAPs than NP seeds under salt stress, indicating a lower proteome response, as well as a decrease in the accumulation of stress response-related proteins (Fig. 6). These results, together with the lower accumulation of stress markers described above, would indicate that OCB treatment prevents an exacerbated response to saline conditions. Furthermore, the modulation of the proteome observed in treated seeds correlates with the changes caused in the hormonal profile by the OCB treatment, given that the lower concentration of stress-related phytohormones would cause a lesser activation of defence pathways, resulting in a lower accumulation of antioxidant and stress-response proteins.

Therefore, our bioproduct is capable of dissipating salt stress-induced damage in broccoli seeds due to its composition rich in bioactive compounds with antioxidant capacity, improving the germination and physiological quality of the obtained seedlings and preventing an aggressive response to salinity that negatively affects the germination process (Fig. 8). Furthermore, the results obtained in this study on the effectiveness of OCB in broccoli seeds, as well as previous studies using other plant cell culture-derived biostimulants in tomato seeds (Martí-Guillén et al., 2025b, 2025a), constitute an effective alternative to produce biostimulants enriched in bioactive compounds, with the capacity to ameliorate abiotic stress in different crop species.

5. Conclusions

In this study, we developed a novel bioproduct (OCB) derived from orange carrot cell cultures subjected to elicitation treatments, resulting in a bioproduct enriched in phenolic compounds and phytosterols that confer enhanced antioxidant capacity. This bioproduct exhibited a strong biostimulant, fully counteracting the detrimental impact of salt stress on germination. Moreover, OCB treatment reduced oxidative damage and mitigated the alterations in hormonal and proteomic profiles induced by salinity in the resulting seedlings by downregulating the accumulation of stress-related hormones and antioxidant enzymes. Overall, these results demonstrate that OCB, as a biostimulant enriched in bioactive compounds, effectively promotes seed germination and early seedling vigour under saline conditions by alleviating salt-induced toxicity and preventing excessive stress responses that could impair plant development. Future research validating its effect in other crops of agronomic interest, as well as other phenological stages, will be conducted.

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CRediT authorship contribution statement

Sara Esperanza Martínez-Lorente: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José Manuel Martí-Guillén:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lorena Almagro:** Writing – review & editing, Funding acquisition. **María Ángeles Pedreño:** Writing – review & editing, Funding acquisition. **Ana Belén Sabater-Jara:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

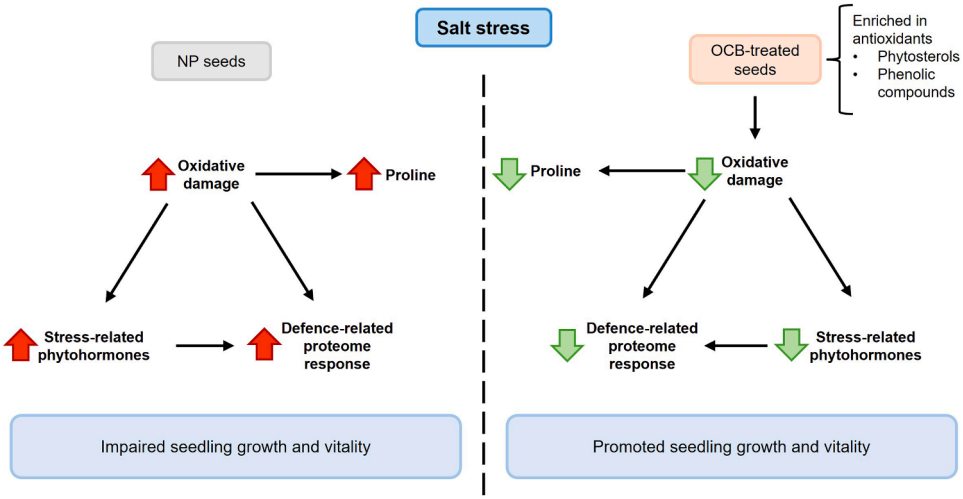

Fig. 8. Integrated model showing OCB effects on broccoli seedlings subjected to salt stress. Salinity induces oxidative damage in the seedlings, which in turn triggers the accumulation of proline and stress related phytohormones, promoting a shift in the proteome to accumulate defence-related proteins and impairing seedling growth and vitality. OCB, as a consequence of its enrichment in antioxidant compounds, is able to decrease oxidative damage, thus preventing the accumulation of stress-related phytohormones which downregulates defence-related proteome response. Thus, OCB allows a promotion of seedling growth and vitality. NP: no priming; OCB: orange carrot biostimulant at 0.1 % (v/v).

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2026.101278.

Data availability

Proteomics data are submitted to PRIDE (PXD069532) and the rest of the data will be available on request.

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