

## Research Paper

## Physiological and metabolic profiling reveals distinct early salt resilience strategies among pre-commercial broccoli cultivars

Angel Almagro-Lopez<sup>a</sup>, Jose M. Mulet<sup>b</sup>, Rosa Porcel<sup>b</sup>, Marina Celdrán<sup>b</sup>, Micaela Carvajal<sup>a</sup>, Juan Nicolas-Espinosa<sup>a,\*</sup>

<sup>a</sup> Aquaporins Group, Centro de Edafología y Biología Aplicada del Segura, CEBAS-CSIC, Campus Universitario de Espinardo - 25, Murcia E-30100, Spain

<sup>b</sup> Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de Valencia-Consejo Superior de Investigaciones Científicas, Valencia 46022, Spain

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## ABSTRACT

Soil salinization represents a primary abiotic constraint to global agricultural productivity, necessitating the development of resilient cultivars. This study assesses the physiological and metabolic responses associated with salinity tolerance in four pre-commercial broccoli (*Brassica oleracea* var. *italica*) cultivars (BG1, BH1, BX1, and BQ1) during the vegetative stage under salt stress conditions. Among the genotypes, BQ1 demonstrated superior performance at the lowest salinity level assayed (80 mM NaCl), maintaining biomass accumulation and the highest Salt Tolerance Index (STI). This resilience is attributed to a constitutive physiological adaptation characterized by a low stable relative water content (RWC) and water potential, which prevents further salinity-induced dehydration. Furthermore, BQ1 exhibited effective ionic homeostasis by retaining phosphorus (P) and boron (B) in root tissues, alongside an early induction of ascorbate peroxidase (APX) and catalase (CAT) activities. Metabolic profiling revealed that BQ1 uniquely accumulates carbon-based osmolytes, specifically sucrose and maltose, which correlate with increased levels of Krebs cycle intermediates such as malate and aspartate. While certain unique adaptive responses in BQ1 reached a threshold at high salinity, its growth performance remained markedly superior to the other cultivars, suggesting a highly robust mechanism for sustaining metabolic flux and cellular homeostasis under extreme ionic and osmotic constraints.

## 1. Introduction

Abiotic stresses are currently a significant threat to global agricultural productivity that negatively impact plant growth, development, and yield. Recent assessments by the Food and Agriculture Organization (FAO) indicate that agricultural systems are facing unprecedented environmental constraints, with over one-third of global agricultural land suffering from degradation processes, including salinization and water scarcity, which are being exacerbated by climate change (FAO, 2021). Among these pervasive challenges, salinity stands out as one of the most critical factors limiting crop performance. It impairs essential physiological and biochemical processes, leading to diminished biomass accumulation, reduced reproductive success, and compromised seed quality, which in severe cases can result in plant mortality (Gull et al., 2019).

Salinity, defined by excessive accumulation of soluble salts in the soil, affects numerous regions worldwide (Gull et al., 2019). The

detrimental effects of salinity on plants are twofold: osmotic stress and ionic toxicity. Osmotic stress arises from the high solute concentration in the soil, which lowers the water potential and restricts water uptake by the roots. Simultaneously, ionic toxicity results from the excessive accumulation of specific ions, such as Na<sup>+</sup> and Cl<sup>-</sup>, within plant tissues, disrupting cellular homeostasis and metabolic functions (Safdar et al., 2019). To cope with these challenges, plants divert metabolic resources towards tolerance mechanisms, including osmotic adjustment, ion compartmentalization in tonoplast, and the activation of antioxidant systems, often at the expense of growth and development (D'Amelia et al., 2018). These responses are critical throughout the plant life cycle, from the highly sensitive stages of seed germination and seedling establishment to maturity (Almagro-Lopez et al., 2025; Ibrahim, 2016).

Plant responses to salinity are complex and depend on the specific tissue or organ affected, as well as the intensity and duration of the stress. A key determinant of plant survival under saline conditions is the ability to maintain water transport and turgor through osmotic

\* Corresponding author.

E-mail address: [jmmu-let@ibmcp.upv.es](mailto:jmmu-let@ibmcp.upv.es) (J. Nicolas-Espinosa).

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adjustment (Parida and Das, 2005). This is often achieved by accumulating compatible solutes, such as proline, in the cytoplasm (Ali et al., 2019; Verbruggen and Hermans, 2008). However, the photosynthetic machinery is also a primary target of salt stress. Salinity can lead to a reduction in chlorophyll content, impair the efficiency of photosystem II (PSII), and decrease stomatal conductance, which limits CO<sub>2</sub> assimilation and exacerbates the production of reactive oxygen species (ROS) (Zahra et al., 2022). To counteract the damaging effects of ROS on cellular components, plants have evolved a sophisticated antioxidant defence system, including enzymes such as catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (AP). Enhanced activity of these enzymes has been associated with increased salt tolerance in several plant species (Hernández et al., 2000). As stress severity increases, energy metabolism, when it is possible, undergoes progressive adjustments, reflecting an attempt to restore plant cellular homeostasis and sustain growth under adverse conditions (Cramer et al., 2011).

Broccoli (*Brassica oleracea* var. *italica*), a member of the Brassicaceae family, is a crop of significant economic and nutritional value. It is a rich source of essential minerals, vitamins, and dietary fiber (Nagraj et al., 2020). Furthermore, broccoli contains a wide array of bioactive compounds, including polyphenols, flavonoids, and glucosinolates, which have garnered scientific interest for their antioxidant, anti-inflammatory, and anti-carcinogenic properties (Li et al., 2022). Consequently, enhancing the resilience of broccoli to abiotic stressors like salinity is crucial for ensuring its sustainable production and preserving its health-promoting benefits. While broccoli is considered moderately sensitive to salinity, with reported growth inhibition at NaCl concentrations of 60–80 mM (Nicolas-Espinosa et al., 2024; Yepes-Molina et al., 2026), significant variations in tolerance exist among different cultivars. Understanding the physiological and molecular basis of these differences is essential for developing more salt-tolerant varieties.

Previous research identified several pre-commercial broccoli cultivars (BG1, BH1, BX1, and BQ1) with promising agronomic traits and superior performance during germination and early seedling development under saline conditions when compared to a known salt-tolerant species (Almagro-Lopez et al., 2025). The present study builds upon these findings by investigating the responses of these selected cultivars to a range of salt concentrations (80, 120, and 180 mM NaCl) during the vegetative growth stage in a hydroponic system. The primary objective is to determine whether the tolerance mechanisms observed during early development are maintained in mature plants or whether distinct physiological and molecular strategies are activated at later stages. To address this, we performed a comprehensive analysis of physiological parameters, mineral ion accumulation, photosynthetic efficiency, antioxidant enzymatic activity and the metabolomic profile of the selected cultivars. This research provided valuable insights into the developmental plasticity and adaptive strategies of broccoli in response to salinity.

## 2. Materials and methods

### 2.1. Plant growth conditions

Broccoli seeds (*Brassica oleracea* var. *italica*) from four cultivars (BG1, BH1, BX1, and BQ1) supplied by SAKATA were initially hydrated in distilled water with continuous aeration for 24 h. Subsequently, seeds were placed in trays containing vermiculite and allowed to germinate for 48 h in complete darkness at 28 °C. Following germination, seedlings were transferred to a controlled-environment growth chamber under a 16 h light/8 h dark photoperiod, with day/night temperatures of 24 °C/20 °C, relative humidity maintained at 60%, and grown for an additional 48 h.

Seedlings were then transplanted into a hydroponic culture system (8 liters) containing Hoagland nutrient solution under continuous aeration. Eight hydroponic tanks were prepared per cultivar (two tanks per

salinity treatment), with four plants per tank to minimize shading. Tanks were exposed to photosynthetically active radiation (PAR) at an intensity of 400 μmol m<sup>-2</sup> s<sup>-1</sup>.

After two weeks of growth under these conditions, salinity treatments were applied by supplementing the nutrient solution with NaCl at concentrations of 0 mM (control, ~2.0 dS m<sup>-1</sup>), 80 mM (~10.4 dS m<sup>-1</sup>), 120 mM (~14.3 dS m<sup>-1</sup>), and 180 mM (~19.9 dS m<sup>-1</sup>), which were maintained for 10 days. At the end of the treatment period, plants were harvested and separated into aerial and root tissues. Four plants per treatment were immediately frozen at -80 °C for further biochemical and molecular analyses, while the remaining plants were used for physiological measurements.

### 2.2. Physiological measures

After harvesting, fresh weight (FW) and root length were recorded for each plant. Samples were then dried in an oven at 60 °C to determine dry weight (DW) of both aerial and root tissues.

The salinity tolerance Index (STI) (Bakirov et al., 2022; Efe et al., 2024) was calculated using dry weight values according to the following formula:

$$STI = ((Y_p)(Y_s) / Y_p^2)$$

Where Y<sub>p</sub> represents the dry weight value of the genotype under unstressed conditions and Y<sub>s</sub> represents the dry weight of the plant under salinity stress, and Y<sub>p</sub><sup>-</sup> represents the dry weight mean value of the genotypes under unstressed conditions.

The osmotic potential (Ψ<sub>π</sub>) of three plants per treatment was measured using a freezing-point depression osmometer (Digital Osmometer, Roebling, Berlin) at 25 ± 1 °C (Navarro et al., 2003). The leaf water potential (Ψ<sub>w</sub>) was determined in the fourth fully expanded leaf from five plants per treatment from each cultivar (0, 80, 120, and 180 mM NaCl) using a Scholander pressure chamber (SKYE INSTRUMENTS LTD, United Kingdom) (Turner, 1988). Turgor potential (Ψ<sub>p</sub>) was calculated as the difference between Ψ<sub>w</sub> and Ψ<sub>π</sub> (Nonami and Schulze, 1989).

Relative water content (RWC) was determined from leaf discs collected at harvest minimize the need for entire plant sampling. Fresh weight (FW) was recorded immediately, followed by turgid weight (TW) after 24 h of immersion in deionized water, and dry weight (DW) after 48 h at 60 °C. RWC was calculated as:

$$RWC(\%) = ((\text{Fresh Weight} - \text{Dry Weight}) / (\text{Turgid Weight} - \text{Dry Weight})) \times 100$$

Chlorophyll fluorescence was assessed using a chlorophyll fluorimeter (OS-30p+ Chlorophyll Fluorimeter, OPTI-SCIENCES) following the OJIP protocol. Measurements were taken on four plants per treatment on leaves previously dark-adapted for 30 min. Evaluations were performed on days 2, 7, 9, and 10 after the initiation of salinity treatments.

### 2.3. Analysis of mineral nutrients

Macro- and micronutrient concentrations were measured using dried aerial and root tissues from three individual plants per treatment. The tissues were finely ground into a homogeneous powder before analysis. Nutrient quantification was performed using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) on a Thermo iCAP 6500 Duo instrument (Thermo Fisher Scientific, Waltham, MA, USA), following the methodology described by Nicolas-Espinosa et al. (2024). Macronutrient concentrations were expressed as mg 100 g<sup>-1</sup> DW, while micronutrient concentrations were expressed as mg g<sup>-1</sup> DW.

### 2.4. Preparation of extracts and enzyme assay

Antioxidant enzymes were extracted from 300 mg of frozen, ground

aerial and root tissues using 50 mM potassium phosphate buffer (pH 7.8) supplemented with 0.1 mM Na<sub>2</sub>-EDTA and 1% (w/v) insoluble polyvinylpyrrolidone (PVPP). For glutathione reductase (GR) assays, the extraction buffer was additionally supplemented with 10 mM  $\beta$ -mercaptoethanol, whereas for ascorbate peroxidase (APX) assays, 4 mM ascorbic acid was included, following the methodology described by Moran et al. (1994). Enzyme extracts were centrifuged, and the resulting supernatants were stored at  $-20^{\circ}\text{C}$  until analysis.

Catalase (CAT) activity was determined by monitoring hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) decomposition according to Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) and 10.6 mM H<sub>2</sub>O<sub>2</sub>, and the decrease in absorbance at 240 nm was recorded every 30 s for 3 min.

Ascorbate peroxidase (APX) activity was assayed by measuring the rate of ascorbate oxidation as described by Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM Na<sub>2</sub>-EDTA, and 0.5 mM ascorbic acid. The reaction was initiated by adding H<sub>2</sub>O<sub>2</sub>, and the decrease in absorbance at 290 nm was monitored every 30 s for 3 min.

Glutathione reductase (GR) activity was quantified following the protocols of Carlberg and Mannervik (1985). The assay mixture comprised 0.1 M HEPES buffer (pH 7.8), 1 mM Na<sub>2</sub>-EDTA, 3 mM MgCl<sub>2</sub>, and 0.5 mM oxidized glutathione (GSSG). The reaction was initiated by the addition of 0.2 mM NADPH, and the rate of NADPH oxidation was monitored spectrophotometrically at 340 nm every 30 s for 2 min.

Protein concentrations were determined using the Bradford method (Bradford, 1976). Enzyme activities (CAT, APX, GR) were expressed as  $\mu\text{mol}$  of substrate consumed per minute per mg of protein ( $\mu\text{mol substrate min}^{-1} \text{mg}^{-1} \text{protein}$ ). Three biological replicates per treatment and cultivar, each analyzed with three technical replicates, were included.

## 2.5. Lipid oxidative damage

Lipid peroxidation was quantified using the previously prepared enzymatic extract, following the method of Minotti and Aust (1987). The reaction mixture contained 20% (w/v) trichloroacetic acid (TCA), 0.5% (w/v) 2-thiobarbituric acid (TBA), 0.01% (w/v) butylated hydroxytoluene (BHT), and 0.25 N HCl. Equal volumes of extract and reaction mixture were combined and incubated at  $95^{\circ}\text{C}$  for 30 min. The reaction was terminated by rapid cooling on ice, followed by centrifugation to remove precipitates. The absorbance of the resulting supernatant was recorded at 535 nm using a spectrophotometer. A calibration curve was prepared using malondialdehyde (MDA) standards (0–10  $\mu\text{M}$ ), and lipid peroxidation levels were expressed as  $\mu\text{mol MDA min}^{-1} \text{mg}^{-1} \text{protein}$ .

## 2.6. Metabolomics analysis

Primary metabolites were determined by MNR. For the measurements, 50 mg of freeze-dried powder from each sample was analysed using proton nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR) using a 500 MHz Bruker BioSpin spectrometer (Rheinstetten, Germany), equipped with a 5 mm N<sub>2</sub> CryoProbe Prodigy BBO broadband cryogenic probe.

## 2.7. Statistical analysis

Statistical analyses and data presentation were conducted using Origin (Pro) (Version 2021 software package by OriginLab Corporation, Northampton, MA, USA). Significant differences among the values of all the parameters were determined via *post hoc* analyses. Data was analysed using one-way ANOVA. In all the analyses,  $p < 0.05$  was selected to represent statistically significant differences according to Tukey's test. The numbers of replicates are specified in the figure for each measure.

## 3. Results

### 3.1. Impact of salinity on plant growth and water status

Biomass values differed depending on the cultivar and the salinity conditions. All cultivars exhibited a reduction in fresh weight (FW) (Figure 1A) under salinity stress; however, no significant differences were detected between the control and the 80 mM NaCl treatment in BH1 and BQ1. Among the tested cultivars, BQ1 showed the highest FW under both control and 80 mM conditions with a value around 50% higher if compared with the ones observed in BG1 under the same conditions. A similar trend was observed for dry weight (DW) (Figure 1B), with all cultivars showing a decline as salinity increased.

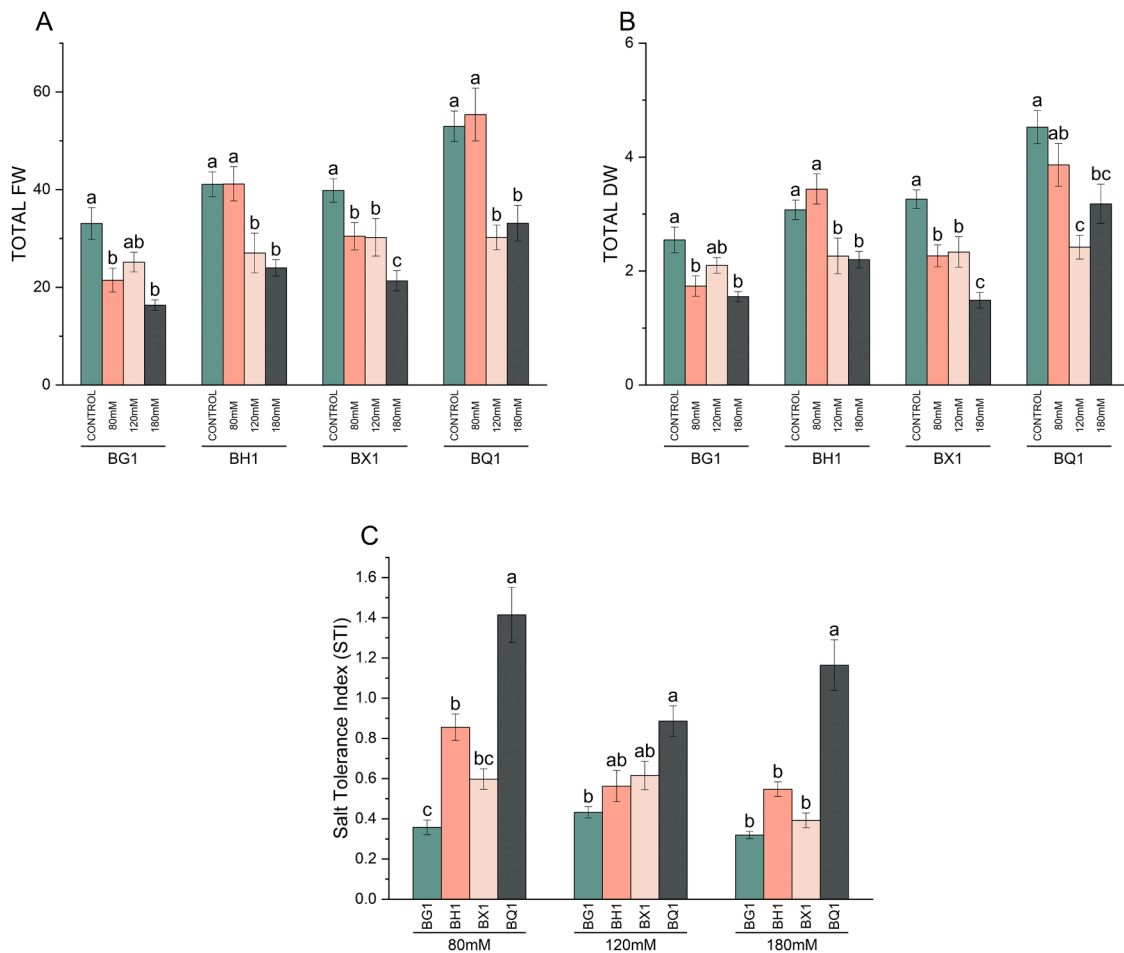
Regarding the Salt Tolerance Index (STI) (Figure 1C), under 80 mM NaCl, BG1 exhibited the lowest STI value. BH1 showed a higher STI at 80 mM compared with BG1, but not relative to BX1. BQ1 demonstrated the highest STI under this condition. At 120 mM NaCl, statistically significant differences were detected only between BG1 and BQ1. Under 180 mM NaCl, no significant differences were observed among BG1, BH1, and BX1. In contrast, BQ1 was the only cultivar that exhibited a significantly higher STI value at this salinity level.

If we observe the osmotic potential (Figure 2A), a progressive reduction was observed in BX1 and BQ1 under increasing salinity levels. In BG1, this decrease became evident only after exposure to 120 mM NaCl, whereas in BH1, a reduction was detected exclusively under the highest salinity treatment (180 mM). Regarding water potential (Figure 2B), cultivar BG1 exhibited a consistent decline with increasing salinity, while BH1 showed an increasing reduction only under 120 mM and 180 mM conditions. Notably, BX1 and BQ1 displayed the lowest water potential values under control conditions. In BX1, a marked decrease was observed at 180 mM, whereas in BQ1, the reduction occurred at 80 mM but remained stable at higher salinity levels. Turgor potential (Figure 2C) increased across all cultivars under saline conditions, indicating an active osmotic adjustment mechanism to maintain cell turgidity despite external osmotic stress. Regarding relative water content (RWC) (Figure 2D), BG1 showed a clear reduction under salinity, with the minimum value occurring at 120 mM. No significant changes were observed in BH1 across salinity treatments. BX1 exhibited a decrease in RWC only at 180 mM, whereas BQ1 showed a significant increase exclusively at 120 mM and no changes under 180 mM.

### 3.2. Ion partitioning and nutrient balance

In leaf tissue, a general decline in mineral concentrations was observed under saline conditions. Specifically, K, S, Ca and Mg levels decreased consistently in all cultivars upon salinity exposure (Figure 3A, D, F, G). Na<sup>+</sup> concentrations accumulated in all cultivars, reaching peak values at 180 mM, with the notable exception of BQ1, which showed no significant differences between salinity treatments (Figure 3B). A reduction in P content was detected exclusively in BG1 and BH1 (Figure 3C), while B concentrations declined in BH1 and BX1 (Figure 3E). Furthermore, Mo content was significantly reduced in BH1, BX1, and BQ1 under salinity stress, whereas BG1 remained unaffected (Figure 3H).

In root tissues, K, P, and Mo concentrations decreased under salinity across all cultivars (Figure 4A, C, H). The root Na<sup>+</sup> accumulation pattern mirrored that observed in leaf tissues, with BQ1 being the only cultivar to maintain stable Na<sup>+</sup> levels across all salinity treatments (Figure 4B). In BG1 and BH1, a progressive reduction in S content was observed with increasing salinity (Figure 4D), whereas levels remained stable in BX1 and BQ1. Similarly, B concentrations were unaffected by either genotype or treatment conditions (Figure 4E). All cultivars exhibited a decline in Ca concentration under saline conditions (Figure 4F). Notably, the high basal Ca levels observed in BG1 and BH1 under control conditions converged to values similar to those of BX1 and BQ1 under stress; a similar convergence pattern was noted for P levels in BG1 and



**Figure 1.** Physiological parameters in broccoli cultivars BG1, BH1, BX1 and BQ1 under control 80 mM, 120 mM and 180 mM NaCl conditions. (A) Fresh weight (n=4). (B) Dry weight (n=4). (C) Salt tolerance index (STI) (n=4). Different letters indicate significant differences according to one-way ANOVA followed by a post hoc Tukey's test ( $p < 0.05$ ) between treatments within the same species. Each bar represents the mean  $\pm$  SE.

BH1, and for Mo levels in BX1 and BQ1. Finally, an increase in Mg content was detected exclusively in BX1 and BH1 in response to salinity stress (Figure 4G).

### 3.3. Antioxidant defence and oxidative damage

The antioxidant enzymatic activity exhibited distinct patterns depending on the cultivar (Figure 5). Cultivar BG1 was the only genotype displaying no significant changes in enzymatic activity under salinity stress; however, activity levels remained moderate across all conditions and enzymes analysed. In contrast, cultivar BH1 exhibited high ascorbate peroxidase (APX) activity under control conditions, which decreased under salinity. Conversely, this cultivar showed no GR activity under control and 80 mM NaCl conditions, whereas activity markedly increased under 120 mM and 180 mM treatments. Catalase (CAT) activity remained stable across all salinity levels, except for a significant increase observed at 180 mM NaCl.

Cultivar BX1 exhibited low APX activity under control and 80 mM conditions, with a notable increase under 120 mM and 180 mM. Additionally, glutathione reductase (GR) activity remained moderate under most treatments, with a significant increase only under 180 mM. CAT activity showed no variation in this cultivar. For cultivar BQ1, APX and CAT activities increased only under 80 mM NaCl, while moderate levels were maintained under all other salinity conditions. GR activity remained unaffected by salinity in this cultivar (Figure 5A-C).

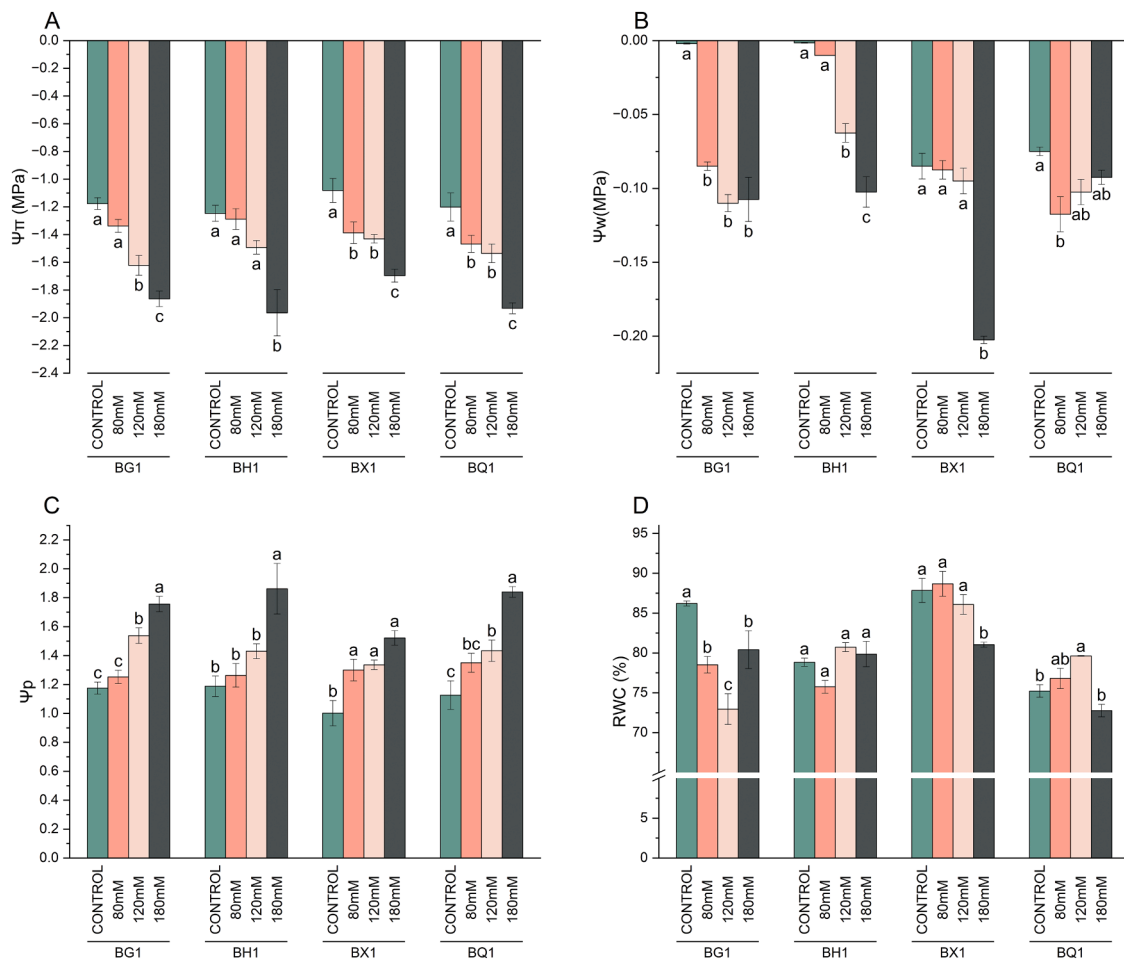
Regarding oxidative damage (Figure 5D), cultivar BG1 exhibited no significant changes under salinity stress. In BH1, oxidative damage

levels remained low under control conditions but progressively increased with salinity, reaching statistical significance at 180 mM NaCl. A similar trend was observed in BX1. BQ1 exhibited low oxidative damage levels under control conditions but showed a significant increase under 80 mM and 120 mM salinity, reaching the highest levels at 180 mM NaCl.

### 3.4. Metabolic profiling

The global PCA analysis (Figure 6) reveals a clear separation between control and 180 mM NaCl-treated samples, forming two distinct clusters (Figure 6A). The PCA biplot of the top 10 contributing metabolites indicates that salinity is strongly associated with changes in asparagine, proline, and serine concentrations (Figure 6B). When PCA was performed by cultivar, distinct clustering patterns were observed among the four cultivars, with BH1 exhibiting the smallest metabolic shift in response to increasing salinity levels (Figure 6C). PCA based on treatment further shows that samples from different cultivars exposed to 80 mM and 120 mM NaCl cluster together, whereas at 180 mM, BQ1 distinctly separates from the other cultivars (Figure 6D).

Among the 39 metabolites quantified in our metabolic profiling, those exhibiting statistically significant variations are depicted in Figure 7. A total of 15 metabolites displayed significant changes in response to salinity or between cultivars. Of these, eight were amino acids, where we include alanine, aspartate, glutamate, proline, serine, valine, threonine, and asparagine. We also include sugars such as glucose, maltose, fructose and sucrose. The remaining three metabolites



**Figure 2.** Potential values and water relations measured in BG1, BH1, BX1 and BQ1 broccoli cultivars under control, 80 mM, 120 mM and 180 mM NaCl conditions. (A) Osmotic potential ( $n=4$ ). (B) Water potential ( $n=4$ ). (C) Turgid potential ( $n=4$ ). (D) Relative water content ( $n=3$ ). Different letters indicate significant differences determined by one-way ANOVA followed by Tukey's post hoc test ( $p < 0.05$ ) between treatments within the same species. Each bar represents the mean  $\pm$  SE.

were myo-inositol, malate and glucosinolates. The concentrations of all eight amino acids increased with rising salinity levels across all cultivars, except in BX1, where alanine, aspartate and threonine levels remained unchanged (Figure 7A–H). All sugar concentrations were increased in BQ1 under the highest salinity (Figure 7K, M, N, O). Myo-inositol concentrations increased under saline conditions only in cultivars BH1 and BQ1 (Figure 7J), whereas malate accumulation under salinity was observed exclusively in BQ1 (Figure 7L).

#### 4. Discussion

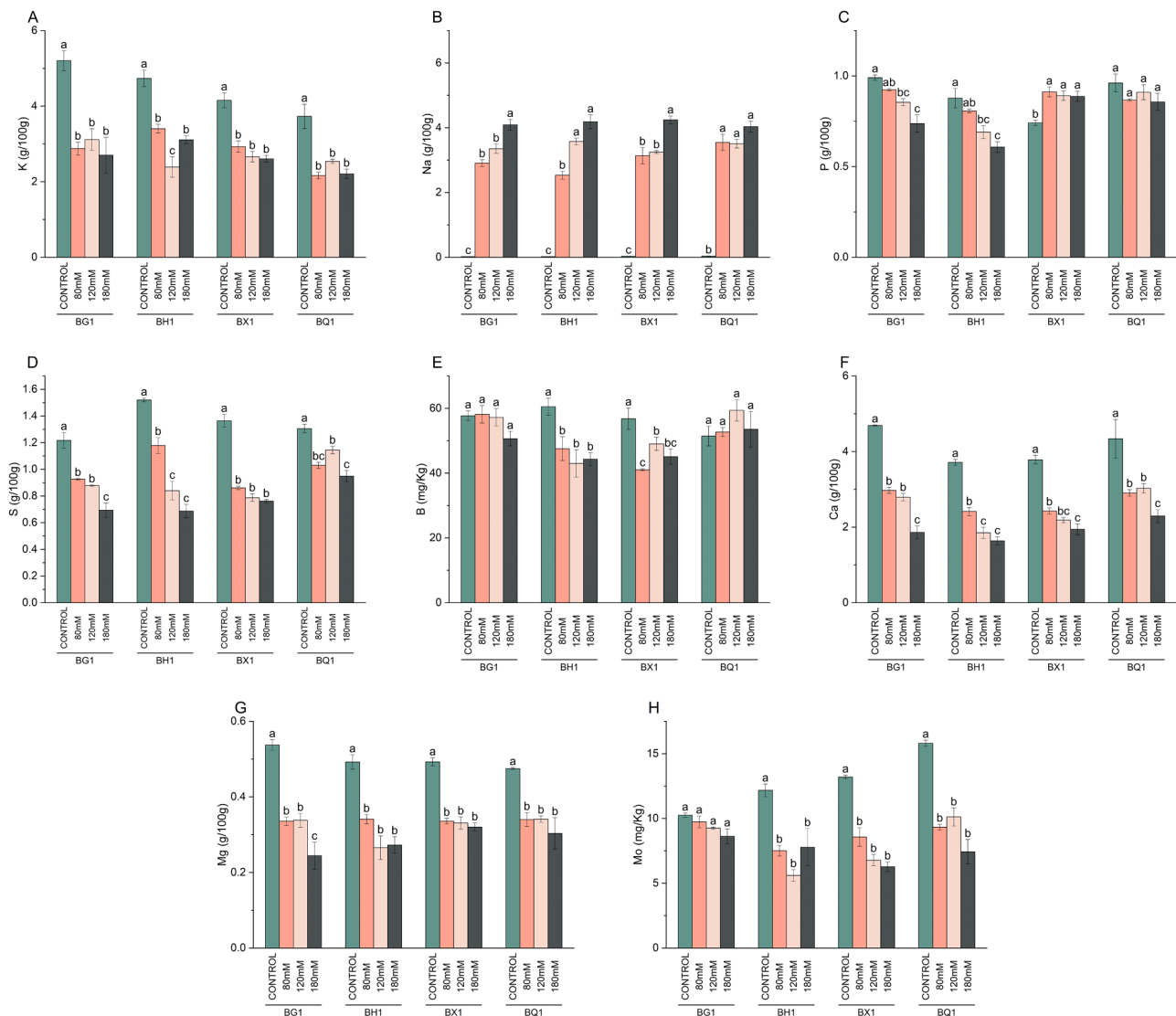
Abiotic stressors such as salinity significantly influence plant development by impairing fundamental processes, including seed germination, growth, and vegetative development. Consequently, there is an urgent need to identify and develop new cultivars with improved salinity tolerance to ensure successful early crop establishment and maintain global food security. In this context, we evaluated the early physiological resilience of four pre-commercial broccoli cultivars bred for enhanced salt tolerance, aiming to characterize their adaptive mechanisms during the vegetative stage and elucidate the underlying physiological and biochemical mechanisms. To achieve this, we assessed a range of parameters, including physiological traits, mineral nutrient composition, antioxidant enzyme activity, and metabolic profiles.

Previous studies in broccoli have reported comparable vegetative growth under control and salinity conditions (López-Berenguer et al., 2008; Nicolas-Espinosa et al., 2024). In agreement with these observations, BG1, BH1, and BX1 showed similar biomass accumulation under

80 mM NaCl in the present study (Figure 1A–B). In contrast, cultivar BQ1 displayed markedly higher biomass, reaching approximately 1.4–1.5-fold greater fresh weight, indicating enhanced growth performance.

Notably, this superior performance in BQ1 was maintained even under severe salinity stress (up to 180 mM NaCl), suggesting that growth is sustained despite strong ionic and osmotic constraints. In this context, BQ1 exhibited the highest Salt Tolerance Index (STI) values under the 80 mM and 180 mM salinity treatments (Figure 1C), consistent with its sustained fresh and dry biomass relative to the other cultivars. Under 120 mM, weight values are similar to those observed in BH1 and BX1 explaining why there are no differences in STI values. In contrast, BG1 showed a pronounced reduction in biomass under salinity, which was reflected in lower STI values, indicating greater sensitivity to salt stress. BH1 and BX1 displayed intermediate STI values with no significant differences between them, consistent with its ability to maintain biomass under 80 mM NaCl but not under higher NaCl concentrations, although physiological values in BH1 seem to be higher. The STI calculated from weight measurements has been previously demonstrated to be a reliable parameter for assessing salinity tolerance in different species (Bakirov et al., 2022; Efe et al., 2024).

With respect to osmotic potential, a progressive reduction was observed across all cultivars under saline conditions (Figure 2A), indicating the activation of osmotic adjustment mechanisms in response to increasing salinity, representing a common adaptive strategy to sustain water uptake and mitigate salinity-induced damage in this species (Muries et al., 2013). Consistently, turgor potential increased with rising



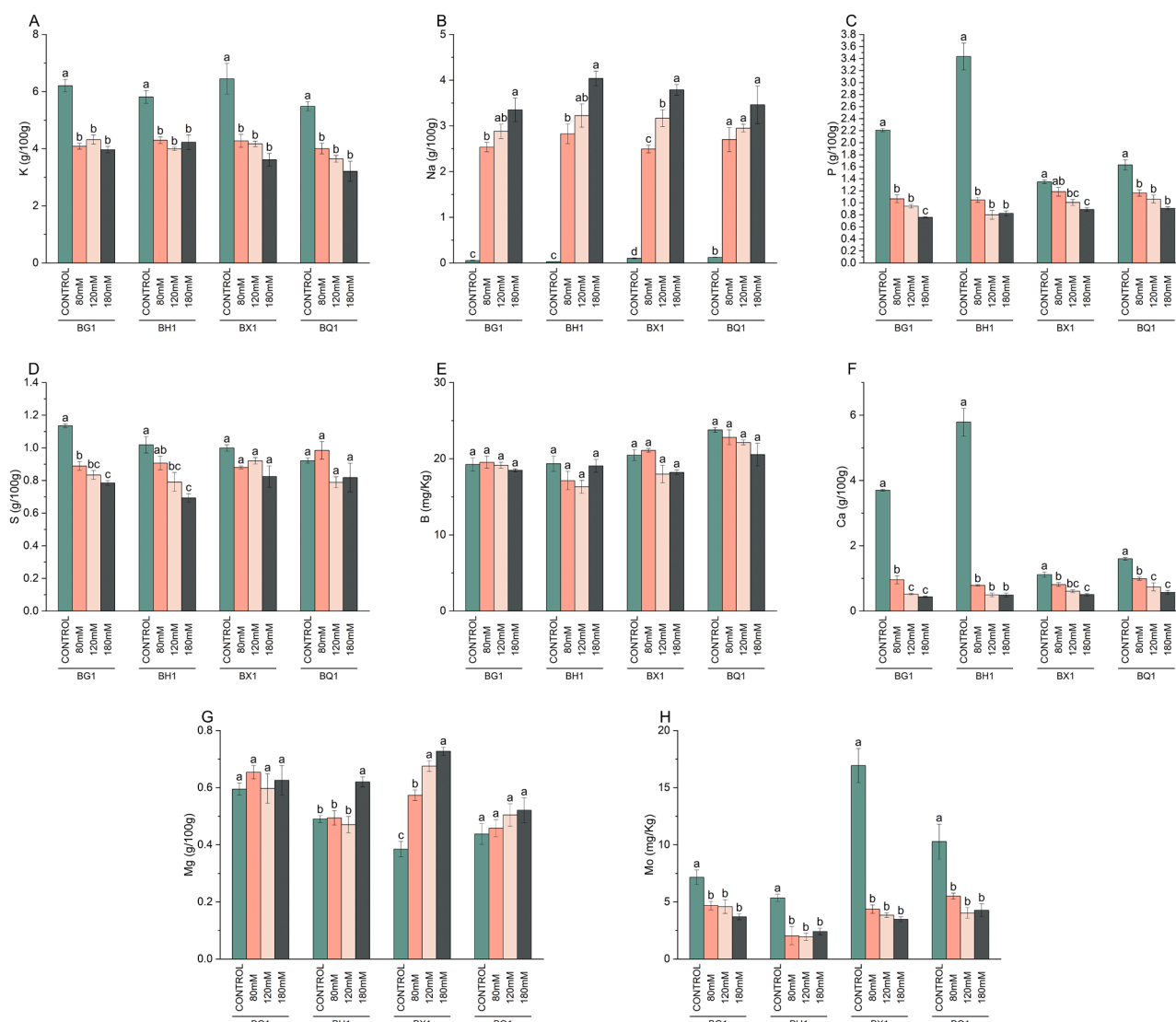
**Figure 3.** Nutrient concentration obtained from leaf tissue in BG1, BH1, BX1 and BQ1 broccoli cultivars under control, 80 mM, 120 mM and 180 mM NaCl conditions. Macronutrient concentration is expressed in (g/100g) and micronutrient concentration in (mg/Kg). Different letters indicate significant differences according to one-way ANOVA followed by a post hoc Tukey's test ( $p < 0.05$ ) between treatments within the same species. Each bar represents the mean  $\pm$  SE ( $n=3$ ).

NaCl concentrations in all cultivars, supporting the role of osmotic adjustment in maintaining cell turgidity under salt stress (Hand et al., 2017). Osmotic adjustment is a well-established adaptive response to salinity, mediated by the accumulation of compatible solutes that lower osmotic potential, thereby facilitating water uptake and preserving cellular hydration (Chen and Jiang, 2010).

In contrast, water potential responses revealed two distinct physiological profiles among the cultivars (Figure 2B). BG1 and BH1 showed a clear salinity-induced decline in water potential, with significant reductions already detected at the two lowest salinity concentrations (80 mM and 120 mM NaCl, respectively), indicating an early sensitivity to osmotic stress. Conversely, BQ1 exhibited a relatively stable water potential across all salinity treatments, suggesting a pre-adjusted water status that limits further decline even under severe stress. BX1 displayed an intermediate response, with water potential remaining stable under the 80 and 120 mM treatments and decreasing only under the highest NaCl concentration. This maintenance of water potential in BQ1, together with its sustained biomass accumulation, is consistent with the high STI values observed for this cultivar, reinforcing its classification as the most salt-tolerant genotype in the present study.

An interesting and contrasting feature of cultivar BQ1 is that, despite

exhibiting the highest STI, it consistently displayed the lowest relative water content (RWC) among the cultivars (Figure 2D). Importantly, this reduced RWC was already present under control conditions and did not further decline under increasing salinity when compared with the control conditions, indicating that BQ1 does not experience an additional salinity-induced water deficit. This constitutive low RWC and stable water potential suggest a conservative water-use strategy that functions as a pre-adaptive mechanism. By maintaining a reduced hydration state under control conditions, BQ1 minimizes the osmotic gradient between the plant tissues and the saline substrate, reducing the metabolic energy required for osmotic adjustment and preventing the catastrophic decline in water potential observed in sensitive genotypes (Munns and Tester, 2008), therefore allowing a more efficient water regulation to avoid changes under critical salinity concentrations. By contrast, cultivars such as BG1 showed a clear salinity-induced decline in RWC up to 120 mM, but in 180 mM a higher value is observed if compared to the 120 mM. This can suggest that under critical salinity exposure, the most sensitive cultivar activates water homeostasis mechanism to maintain water status in order to survive. BX1 showed a decline in RWC only under 180 mM, indicating a stress-driven disruption of water balance, while BH1 maintained RWC stability through apparent osmotic



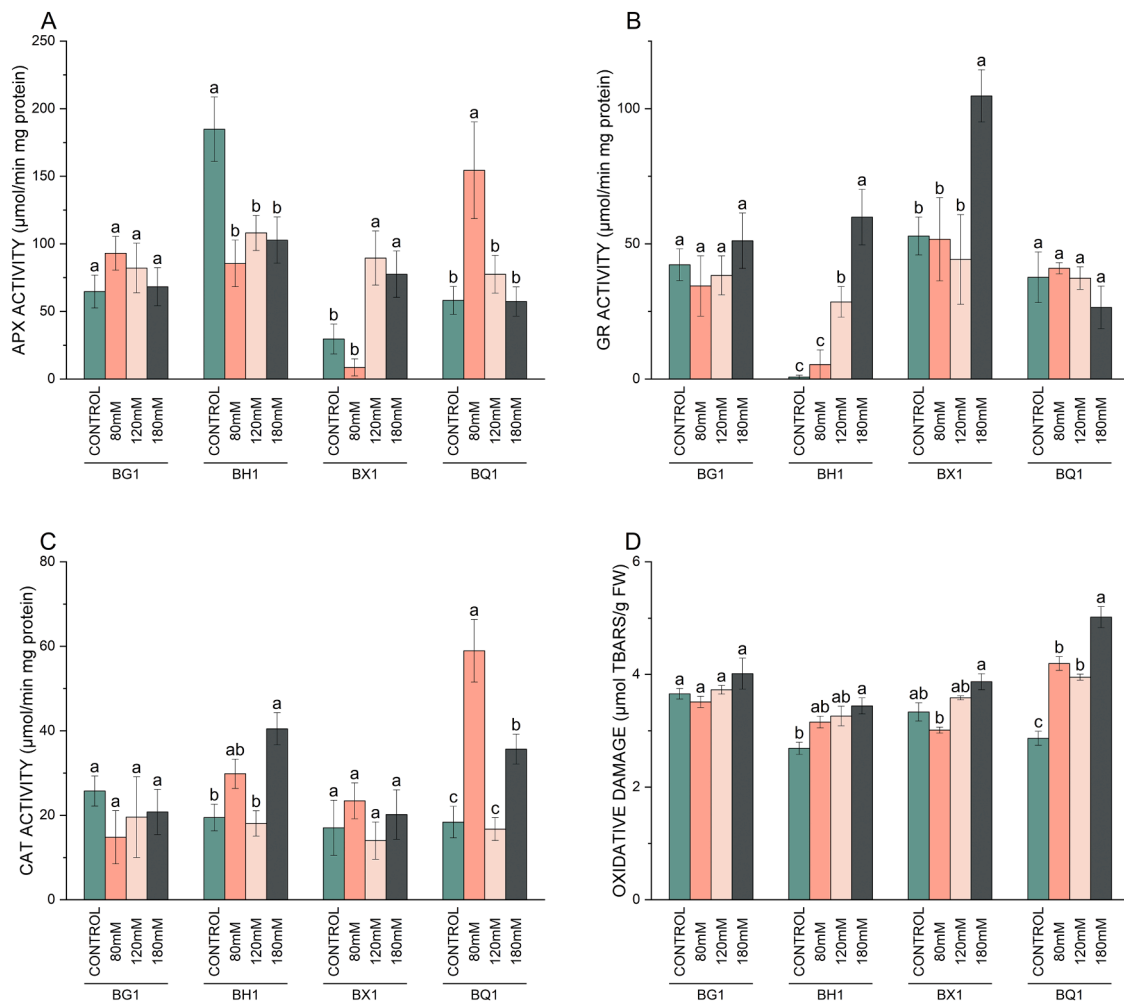
**Figure 4.** Nutrient concentration obtained from root tissue in BG1, BH1, BX1 and BQ1 broccoli cultivars under control, 80 mM, 120 mM and 180 mM NaCl conditions. Macronutrient concentration is expressed in (g/100g) and micronutrient concentration in (mg/Kg). Different letters indicate significant differences according to one-way ANOVA followed by a post hoc Tukey's test ( $p < 0.05$ ) between treatments within the same species. Each bar represents the mean  $\pm$  SE ( $n=3$ ).

regulation mechanisms. This maintenance of RWC appears to contribute to salinity tolerance in BH1, as reflected by relatively high STI values under 80 mM NaCl and can be described as a tolerance trait of this cultivar. Previous studies have reported that even relatively low salinity levels can reduce RWC in broccoli and related *Brassica* crops (Giuffrida et al., 2013). In this context, the ability of BH1 and BQ1 to maintain RWC under salinity suggests the activation of effective water-regulation mechanisms, likely associated with osmotic adjustment and cellular homeostasis.

Regarding photosynthetic performance, while all cultivars maintained stable  $F_v/F_0$  and  $F_v/F_m$  values under salinity, BQ1 uniquely exhibited a progressive decline in these parameters (Supplementary Figure 1). However, rather than indicating stress-induced photo-inhibition, this reduction reflects a structural reorganization. The stability of electron transport efficiency suggests a dilution effect caused by rapid leaf expansion in BQ1, where mesophyll growth outpaces the synthesis of new Photosystem II (PSII) reaction centers (Kalaji et al., 2016). The concurrent increase in  $ABS/RC$  and  $Dl_0/RC$  points to an effective photoprotective mechanism to manage excitation energy (Baker, 2008). Therefore, this decline in BQ1 highlights a structural constraint of accelerated growth rather than photodamage (Murchie and

Lawson, 2013), a strategy that effectively mitigates excessive ROS production (Foyer, 2018).

Analysis of mineral concentrations in leaves and roots revealed clear cultivar-dependent differences in ion homeostasis under salinity stress. While  $Na^+$  concentrations increased with rising salinity in all cultivars, BQ1 uniquely maintained stable  $Na^+$  levels in leaf tissues across treatments. This pattern suggests that  $Na^+$  accumulation in BQ1 is tightly regulated to prevent cytosolic toxicity. In *Brassica* species, this regulation relies heavily on the transport of  $Na^+$  to the aerial parts and its subsequent sequestration into vacuoles, a process primarily mediated by the tonoplasmic  $Na^+/H^+$  antiporter (NHX1) (Farooq et al., 2023). The superior tolerance of BQ1 may be mechanistically linked to a more efficient NHX1-mediated compartmentalization. Enhanced activity or basal expression of tonoplasmic ion transporters like NHX1 not only facilitates rapid vacuolar  $Na^+$  sequestration, thereby protecting critical cytosolic enzymes, but also utilizes vacuolar  $Na^+$  as a cheap osmoticum to maintain cell turgor (Munns and Tester, 2008). Although our total tissues ionic data indicate active transport in all genotypes, a heightened efficiency in this subcellular compartmentalization pathway in BQ1 would explain its ability to sustain growth and physiological function under severe ionic constraints. Nevertheless, future



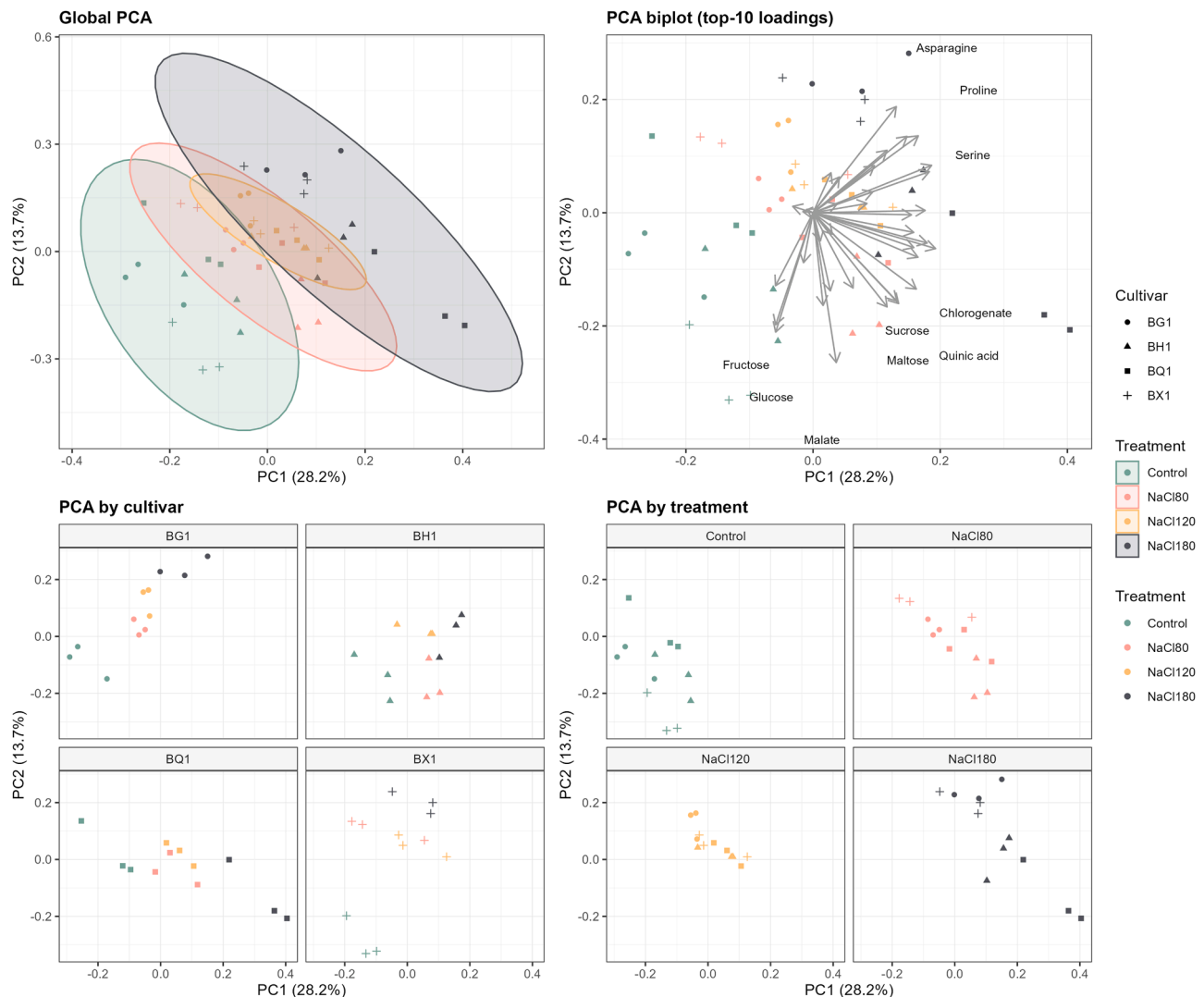
**Figure 5.** Enzymatic activity and lipid damage measure in BG1, BH1, BX1 and BQ1 broccoli cultivars under control, 80 mM, 120 mM and 180 mM conditions. (A) Ascorbate peroxidase activity. (B) Glutathione reductase activity. (C) Catalase activity. (D) Lipid oxidative damage. Different letters indicate significant differences according to one-way ANOVA followed by a *post hoc* Tukey's test ( $p < 0.05$ ) between treatments within the same species. Each bar represents the mean  $\pm$  SE ( $n=3$ ).

transcriptional and functional studies targeting *NHX1* and other ion compartmentalization genes will be required to fully elucidate the molecular basis underlying these cultivar-specific differences in salinity tolerance. Potassium content decreases and sodium increases upon rising salt tolerance. The pattern of accumulation and the K/Na ratio is similar in all cultivars. When we consider the other ions, BG1 and BH1 present higher P and Ca in root tissue under control conditions, and BX1 and BQ1 higher Mo. Although Ca and P are essential mineral nutrients, and can become limiting under different conditions, this does not seem to be the case here, because their higher accumulation does not correlate with higher salt tolerance (Munns and Tester, 2008).

In root tissue, a reduction in K, Ca, and Mo was observed in all cultivars under salinity stress. Similar decreases in K and Ca have been previously reported in broccoli (Del Carmen Rodríguez-Hernández et al., 2013), indicating that such mineral disruptions represent a general physiological response to salinity rather than a mechanism associated with salinity tolerance (Munns and Tester, 2008). Notably, BQ1 maintained P and B concentrations in leaf tissue under salinity, suggesting the presence of active mechanisms preserving P and B uptake, transport or retention under ionic stress. The maintenance of B was also reflected in root tissues. The root uptake and retention of B is particularly relevant given the role of B, together with  $\text{Ca}^{2+}$ , in cell wall structure and mechanical stability under salinity stress (Del Carmen Rodríguez-Hernández et al., 2013). Similarly, the maintenance of P levels in BQ1 under salinity indicates sustained phosphorus availability,

which may support energy metabolism, photosynthetic processes, and stress-associated metabolic adjustments, including the synthesis of compatible solutes such as soluble sugars, free amino acids, and proline (Khan et al., 2023). Regarding  $\text{Mg}^{2+}$ , the reduction observed in leaf tissue did not translate into decreased root  $\text{Mg}^{2+}$  concentrations; instead,  $\text{Mg}^{2+}$  levels in roots were maintained or even increased under salinity. This pattern suggests an active transport and remobilization of  $\text{Mg}^{2+}$  from roots to photosynthetically active tissues, likely contributing to the maintenance of chlorophyll structure and photosynthetic efficiency, in line with the central role of  $\text{Mg}^{2+}$  in these processes (Hauer-Jäckli and Tränkner, 2019).

Antioxidant enzyme profiling revealed distinct redox regulation strategies that align with the cultivars varying degrees of salinity tolerance. In the salt-sensitive BG1, the activities of APX, GR, and CAT remained largely unchanged across treatments, a lack of responsiveness typically associated with salt sensitivity in *Brassica* species (Joshi et al., 2010). Conversely, the intermediate cultivars (BH1 and BX1) exhibited a late, compensatory antioxidant response, significantly upregulating target enzymes only under severe stress (120–180 mM NaCl). This delayed activation coincided with a marked increase in oxidative damage, suggesting these defenses are triggered only when cellular homeostasis is compromised. In contrast, the highly tolerant BQ1 displayed an early induction of APX and CAT at 80 mM NaCl. Although this proactive response aligns with established protective mechanisms (Joshi et al., 2010; Tian et al., 2016), BQ1's overall low basal oxidative



**Figure 6.** Metabolomic PCA analysis in BG1, BH1, BX1 and BQ1 under control, 80 mM, 120 mM and 180 mM conditions. (A) Global PCA (mM). (B) PCA biplot including the top 10 metabolites. (C) PCA analysis sorting by cultivar. (D) PCA analysis sorting by treatment.

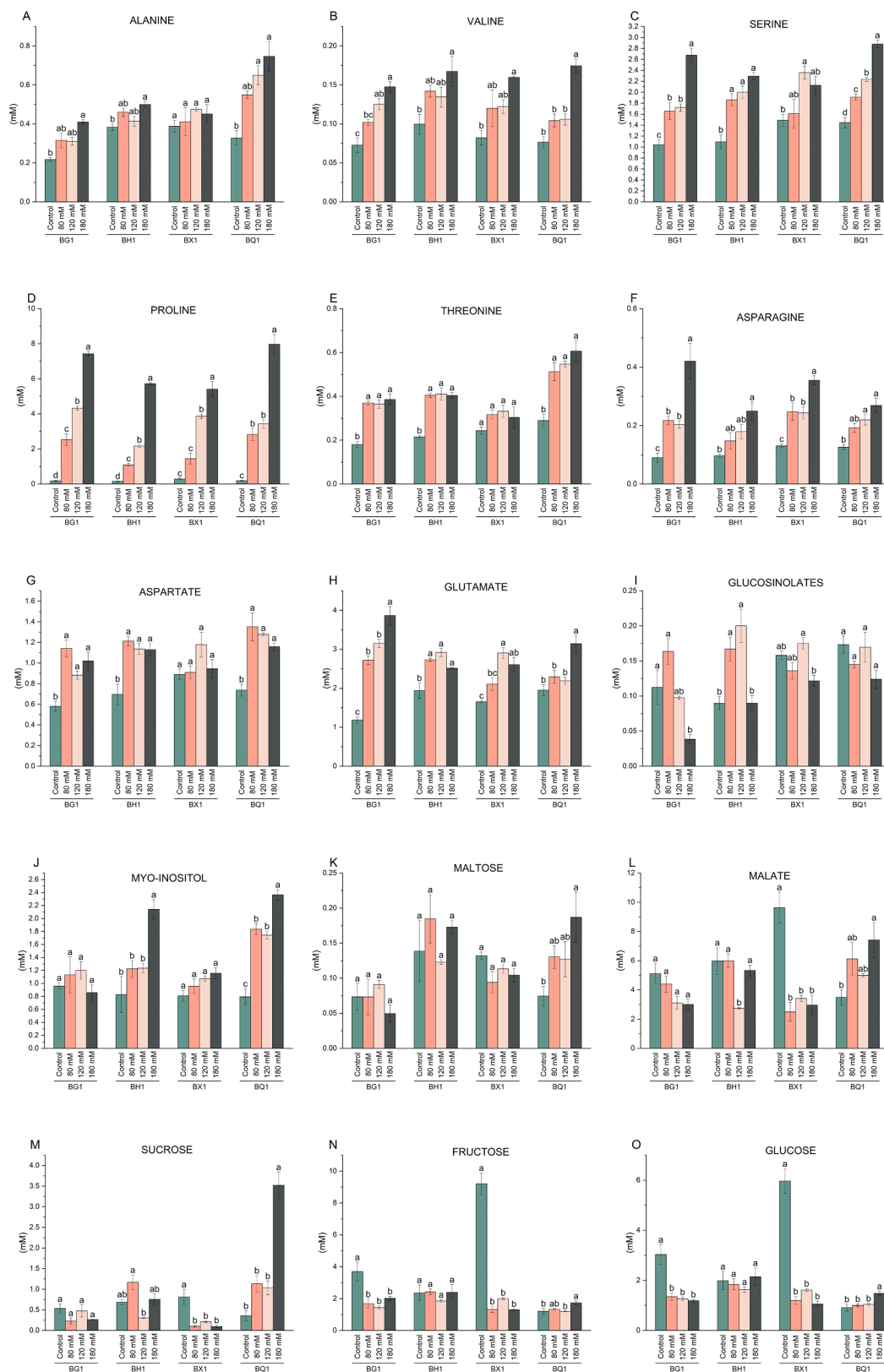
status and the subsequent attenuation of these enzymes at higher salinities indicate that classical ROS scavenging is not the primary determinant of its superior tolerance. Instead, the dissociation between BQ1's redox responses and its robust whole-plant performance suggests that its tolerance is predominantly driven by its constitutive water-use strategy and energetic metabolic reprogramming, which collectively minimize the initial perception of stress (Munns et al., 2020).

Given that antioxidant and ionic responses only partially explain the differential salinity tolerance, metabolic reprogramming emerged as a critical integrative component. The global metabolic shift was clearly evidenced by the Principal Component Analysis (PCA), which identified amino acid and sugar metabolism as the primary drivers of cultivar- and treatment-dependent clustering (Figure 6). Interestingly, the PCA demonstrated that metabolic divergence is dose-dependent; cultivars were distinctly separated under control, 80 mM, and 180 mM conditions, but lost clear differentiation at 120 mM NaCl. This convergence at 120 mM, which is consistent with the similar STI values observed at this concentration, suggests a transitional physiological bottleneck. At this threshold, the basal adaptive mechanisms of the tolerant cultivars are likely saturated, temporarily aligning their metabolic profiles with the sensitive genotypes before the extreme stress of 180 mM forces a final divergence, isolating the truly resilient profile of BQ1.

Among the metabolites driving this separation, proline stands out not

merely as a compatible solute, but as a multifunctional protectant whose accumulation is strongly linked to salinity tolerance in broccoli (Chevilly et al., 2021; Sardar et al., 2023). Beyond its primary role in osmotic adjustment, an in-depth analysis of proline's function reveals its critical role as a molecular chaperone stabilizing membrane proteins and as a reactive oxygen species (ROS) scavenger, buffering cellular redox potential (Verbruggen and Hermans, 2008). Its biosynthesis requires substantial metabolic coordination, as evidenced by the concurrent accumulation of its direct precursor, glutamate. Glutamate is also a key substrate for glutathione biosynthesis, thereby intimately linking proline-mediated osmotic adjustment with the broader antioxidative defense network (Ali et al., 2019). While this proline-glutamate axis, alongside the accumulation of general stress-responsive amino acids, provides essential basal survival across all cultivars, the exceptional resilience of BQ1 indicates that this nitrogen-based defense is further complemented by a unique carbon-based bioenergetic strategy.

Previous studies have indicated the central role of energetic metabolism in salinity tolerance, as stress adaptation requires a substantial diversion of metabolic resources towards protective mechanisms. Accumulation of sucrose and maltose has been widely associated with enhanced salinity tolerance. Comparative analyses of sucrose metabolism in salt-sensitive and salt-tolerant sweet sorghum genotypes have identified sucrose biosynthesis as a key contributor to salinity tolerance



**Figure 7.** Metabolites concentrations in BG1, BH1, BX1 and BQ1 under control, 80 mM, 120 mM and 180 mM conditions. (A) Alanine concentration (mM). (B) Valine concentration (mM). (C) Serine concentration (mM). (D) Proline concentration (mM). (E) Threonine concentration (mM). (F) Asparagine concentration (mM). (G) Aspartate concentration (mM). (H) Glutamate concentration (mM). (I) Glucosinolates concentration (mM). (J) Myo-inositol concentration (mM). (K) Maltose concentration (mM). (L) Malate concentration (mM). (M) Sucrose concentration (mM). (N) Fructose concentration (mM). (O) Glucose concentration (mM). Different letters indicate significant differences according to one-way ANOVA followed by a post hoc Tukey's test ( $p < 0.05$ ) between treatments within the same species. Each bar represents the mean  $\pm$  SE (n=3).

(Yang et al., 2020). Beyond its osmoprotective role, sucrose has been shown to stimulate antioxidant enzyme activity and promote anthocyanin accumulation, thereby alleviating oxidative damage caused by ROS (Qiu et al., 2014). Similarly, maltose accumulation has been reported to enhance tolerance to water stress in wheat (Ibrahim and Abdellatif, 2016). In the present study, sucrose and maltose levels increased under salinity exclusively in cultivar BQ1, supporting the hypothesis that the accumulation of carbon-based osmolytes represents a cultivar-specific tolerance mechanism.

Importantly, variations in these carbohydrates are known to directly influence cellular energy metabolism by increasing the availability of carbon and energy required to activate metabolically demanding tolerance mechanisms (Benito et al., 2023). If increased sugar availability effectively supports salinity tolerance, it would be expected to be accompanied by catabolic activity. Consistent with this expectation, BQ1 shows a net increase in malate, a key intermediate of the tricarboxylic acid (TCA) cycle, along with elevated levels of aspartate and glutamate. Both amino acids are involved in anaplerotic reactions that replenish TCA cycle intermediates, thereby sustaining respiratory flux.

This apparent reinforcement of energy metabolism may provide the resources required for the biosynthesis of compounds in salt stress responses, such as proline and serine, the latter serving as a precursor for sulfur-containing amino acids. A similar association between increased sugar availability, enhanced energetic metabolism, and salinity tolerance has been previously reported in broccoli (Chevilly et al., 2021) and *Arabidopsis thaliana*. Notably, these metabolites exhibited some of the highest loadings separating BQ1 samples under salinity in the PCA analysis (Figure 6), further highlighting their relevance as key metabolic markers of salt tolerance in this cultivar.

According to our results, it is clear that BH1 and BQ1 are the cultivars showing the highest salinity tolerance but the underlying physiological and metabolic bases of this tolerance differ between them. BH1 does not exhibit changes in osmotic potential at 80 or 120 mM NaCl, suggesting the absence of major osmotic adjustment response. This pattern is consistent with the observed stability in water potential at 80 mM, despite an increase in the accumulation of osmoregulatory metabolites such as proline. Relative water content (RWC) also remains unchanged. Furthermore, BH1 does not upregulate antioxidant enzyme activity at 80 mM; such activation is only detectable at 120 or 180 mM. Collectively, these findings suggest that BH1 presents limited salinity-induced stress at 80 mM, as neither oxidative stress markers nor indicators of water stress are altered. Glucosinolate concentrations increase under saline conditions in BH1, associated with a reduction in sulphur content. It has been demonstrated that glucosinolates have a direct effect on water transport and osmotic regulation (López-Berenguer et al., 2008; Martínez-Ballesta et al., 2014). This pattern suggests that BH1 may rely on alternative tolerance strategies, potentially including sodium compartmentalization and glucosinolate-associated pathways, to effectively mitigate NaCl-induced stress and ensure water transport.

Cultivar BQ1 exhibited the highest biomass accumulation and STI, identifying it as the genotype with the most robust early physiological resilience. Our initial hypothesis proposed a halophyte-like preventive strategy of BQ1; however, the lack of constitutive osmolyte or antioxidant accumulation argues against a truly preventive model. Instead, BQ1 relies on a highly efficient constitutive water-use strategy, coupled with metabolic reprogramming that delays the onset of stress. Importantly, while our study provides deep mechanistic insights into these early adaptive responses, we acknowledge that short-term physiological performance in young plants does not definitively predict final agronomic yield. Future long-term field trials evaluating the reproductive stage and harvest yield will be essential to confirm if these robust early physiological mechanisms translate into commercial agronomic tolerance.

## 5. Conclusion

Our results demonstrate that BG1 and BX1 are the most salt-sensitive cultivars, evidenced by pronounced biomass reductions and consistently lower STI values under saline conditions. In contrast, BH1 and BQ1 display greater early physiological resilience. However, the underlying bases of this tolerance differ markedly: while BH1 relies on delayed or alternative responses, BQ1 thrives through the coordinated activation of multiple adaptive mechanisms. Crucially, the superior performance of BQ1 is driven by a constitutive, conservative water-use strategy, maintaining a low but stable water status that remains unaltered by increasing salinity. Rather than relying solely on Na<sup>+</sup> exclusion, BQ1 exhibits highly effective ionic homeostasis, likely involving efficient vacuolar compartmentalization, alongside the preservation of essential nutrients like phosphorus and boron. This ionic stability supports an early induction of antioxidant defenses, ensuring optimal performance under 80 mM NaCl. At the metabolic level, BQ1 is distinguished by a unique reprogramming that integrates nitrogen-based osmolytes (proline, myo-inositol) with carbon-based protectants (sucrose, maltose). This specific accumulation of soluble sugars fuels central energy metabolism, providing the bioenergetic resources required to sustain growth under stress. Finally, while certain metabolic and physiological responses converge among cultivars under severe stress (120–180 mM NaCl), the robust energetic and osmotic buffer established by BQ1 allows it to maintain superior biomass accumulation even at the highest salinity levels. Ultimately, these findings highlight BQ1 as a highly resilient genotype and provide valuable physiological markers for early salt tolerance, though future field trials will be necessary to confirm how these early vegetative advantages translate to final agronomic yield.

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## Ethics approval and consent to participate

Nothing to declare.

## Consent for publication

Nothing to declare.

## CRediT authorship contribution statement

**Angel Almagro-Lopez:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jose M. Mulet:** Writing – review & editing, Validation, Supervision, Resources. **Rosa Porcel:** Writing – review & editing, Validation, Supervision. **Marina Celdrán:** Methodology. **Micaela Carvajal:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Juan Nicolas-Espinosa:** Writing – review & editing, Validation, Supervision, Formal analysis.

## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.scienta.2026.114792](https://doi.org/10.1016/j.scienta.2026.114792).

## Data availability

Data will be made available on request.

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