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Tolerance mechanisms and metabolomic profiling of *Kosteletzkya pentacarpos* in saline environments

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ABSTRACT

Kosteletzkya pentacarpos is a halophyte with significant potential for ecological restoration and phytoremediation in saline environments. This study investigated the growth, biochemical responses, metabolomic profiling and seed germination of plants under increasing sodium chloride (NaCl) concentrations from 0 to 0.3 M. Several vegetative growth parameters (plant height and root length, among others) along with some reproductive traits (flower and fruit number, seed production and germination rates), were determined. Treatment with high NaCl concentrations provoked a significant inhibition of growth. Germination tests revealed that seeds were affected by the highest salt concentrations tested, starting with 0.15 M NaCl and that seeds from plants exposed to 0.05 M NaCl exhibited higher germination rates than seeds germinated without salt. Significant alterations in ionic balance were detected, including increased sodium and chloride accumulation and potassium retention. The levels of osmolytes (proline and glycine betaine) and oxidative stress markers (malondialdehyde) increased under salt treatment conditions. A metabolomic profile of *K. pentacarpos* is presented for the first time, providing key insights into metabolites involved in salinity responses. The metabolomic profiling revealed significant changes in carbohydrates, amino acids, and other metabolites, suggesting metabolic reprogramming to mitigate salinity stress. This study emphasises *K. pentacarpos* adaptive mechanisms, including osmoprotectant accumulation, ionic regulation and metabolomic adjustments, to tolerate moderate salinity. Understanding these responses is essential for advancing the use of *K. pentacarpos* in saline agriculture and environmental management.

1. Introduction

Soil salinisation, an issue aggravated by population growth, agricultural intensification, and climate change, is a critical environmental problem affecting over 1.4×10^9 hectares worldwide, accounting for 10.7 % of the Earth's land surface (FAO, 2024). It remains one of the leading causes of soil degradation, capable of rendering land unsuitable for conventional agriculture and posing a significant threat to global food security (Shao et al., 2017). Salinity causes multiple stresses to plants, such as osmotic stress, ionic toxicity, oxidative damage, and protein degradation, for which a plant must develop various adaptive mechanisms (Atta et al., 2023). Vegetation restoration using halophytes and salt-tolerant plants offers a promising strategy to mitigate soil salinisation; these plants, which generally thrive well in saline environments, can be used not only to rehabilitate degraded soils but also to manage marginal lands sustainably (Vicente et al., 2024). Amongst these salt-tolerant species, *Kosteletzkya pentacarpos* (L.) Ledeb. also known by its synonym *Kosteletzkya virginica* (L.) C. Presl ex A.Gray is a

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facultative halophyte of the Malvaceae family, adapted to salinity levels up to 0.4 M NaCl (Blits and Gallagher; 1990a, 1990b). The genus *Kosteletzkya* C. Presl comprises 26 species, mainly distributed in Africa and the northern Neotropics, and to a lesser extent in the southeastern USA, Eurasia, and the Philippines (Blanchard, 2009).

The species *K. pentacarpos* is native to saline marshes along the Atlantic and Gulf coasts of the USA, serving as a model for research on salt tolerance. It has been introduced to other countries for various purposes, including the rehabilitation of saline soils and economic applications like oil production, animal feed, and industrial by-products (Ruan et al., 2008; Zhou et al., 2021). Outside its native area, it grows as a naturalised species in different European and Asian countries (Webb, 1968). In Europe, the species is protected under the Berne Convention and is included in the Habitats Directive 92/43/EEC (Council of the European Union, 1992) due to the small size of its populations (Pino et al., 2007).

Kosteletzkya pentacarpos is a salt tolerant species, valuable for saline agriculture and ecological restoration. This facultative halophyte can thrive in soils with sodium salt concentrations ranging from 0.05 M to 0.4 M NaCl (Zhang et al., 2014). It adapts to salinity through multiple physiological mechanisms, including effective sodium (Na^+) exclusion, potassium (K^+) retention, and osmotic adjustment via the accumulation of compatible solutes such as proline and glycine betaine (Blits and Gallagher, 1990c; Zhou et al., 2022). Additionally, it maintains stable photosynthesis under saline-alkaline conditions, further demonstrating its resilience to salt stress (Zhou et al., 2022). This salt tolerance, combined with its self-pollinating nature and ability to withstand heavy metals often found in contaminated coastal areas, makes *K. pentacarpos* a promising candidate for phytoremediation in metal-contaminated saline soils (Zhou et al., 2021).

The unique characteristics of *K. pentacarpos* make it an ideal model for studies in saline agriculture and coastal ecological engineering. Although considerable research has been carried out on this species, there is still insufficient information on the mechanisms behind its salt tolerance. Our research aimed to investigate its metabolomic, biochemical, and morphological responses to different salinity levels, and the maternal effects of such stress on seed germination and progeny performance. Addressing these issues is essential for advancing our understanding of the species' potential in ecological restoration and sustainable agriculture. In this context, the present study aims to fill these knowledge gaps by investigating the responses of *K. pentacarpos* to salinity treatments of 0, 0.05, 0.1, 0.2, and 0.3 M NaCl. The study evaluates the species' morphological, biochemical, and metabolomic parameters under these conditions, and assesses the germination performance of seeds produced by plants subjected to salt treatments (control and 0.05 M). Additionally, the study explores potential maternal effects on seedling performance. Our findings will contribute to a deeper understanding of the salt tolerance mechanisms and provide a basis for developing strategies for the conservation and sustainable production of this versatile species.

2. Materials and methods

2.1. Plant growth and greenhouse treatments

Adult *Kosteletzkya pentacarpos* plants were provided by the Centre for Forestry Research and Experimentation (CIEF) in Valencia, Spain, in March 2022. These plants had been grown from seeds sourced from the Germplasm Bank of the Wildlife Service and the Natura 2000 network of Generalitat Valenciana (reference 2213V3A4 - seeds collected from La Albufera Natural Park Valencia, Spain) and were cultivated in 17 × 17 cm pots filled with a substrate mixture of peat, perlite, and coconut fibre in a 4:1:1 ratio. During the acclimatisation period, the plants were irrigated with tap water twice a week until treatment initiation in May 2022, which coincided with the appearance of the first flower buds. The pots were arranged on five plastic trays (measuring 55 cm × 40 cm), with each tray assigned to one of the five treatments: a control group without salt (0 M) and four salinity levels (0.05 M, 0.1 M, 0.2 M, and 0.3 M NaCl), with five replicates each. From May 2nd to July 1st, 2022, the five plants contained in a tray were watered twice a week with 1.5 L of either tap water or the corresponding NaCl solution. From July 1st to October 12th, 2022, irrigation frequency increased to three times a week, using 2 L per tray and irrigation event—two waterings with the salt solution (or water for the controls) and one additional watering with tap water for all treatments. After 23 weeks, when the fruits and seeds had fully developed, the treatments were stopped, and the plants were harvested.

2.2. Substrate electrical conductivity

The electrical conductivity (EC) of the substrate contained in each pot ($n = 5$) was monitored every week using a WET-2 sensor (Delta-T Devices, Cambridge, UK). Additionally, EC values (1:5), expressed in mS cm^{-1}, were measured in the laboratory at the end of the treatment period using a Crison 522 conductivity meter (Crison Instruments SA, Barcelona, Spain).

2.3. Plant material sampling and storage

Flowers and fruits were counted every ten days during the treatment period. The aerial part and root system of each plant were sampled separately to determine its fresh weight. Part of the material was dried until it reached constant weight, and the water content percentage (WC %) was calculated.

The remaining fresh leaf material (0.05–0.15 g) was snap-frozen in liquid N_2 and stored at −80 °C in 2 mL Eppendorf tubes until used for biochemical and metabolomic analyses. The dry samples were used for ion content measurements.

2.4. Seed germination

During the sampling of plant material, the seeds of each plant were collected, counted and stored to be used in the germination experiments. The germination capacity of seeds produced by plants from the control (no salt) and 0.05 M NaCl treatments was tested in distilled water and 0.05 M, 0.1 M, 0.15 M and 0.2 M NaCl solutions.

The seeds were placed in standard Petri dishes (55 mm diameter) on a double layer of filter paper moistened with 1.5 mL of solution, 10 seeds per plate and five replicates. The plates were kept in an Equitec (EGCHS HR, Madrid, Spain) germination chamber, set to 16/8 h and 30/20 °C light/dark regimes. Once the radicle length was 1 mm or higher, the seeds were considered germinated and registered daily for 30 days. Germination capacity was expressed as the percentage of germination (*GP*), whereas the germination rate was represented by the mean germination time (*MGT*), which was calculated according to the formula by Ellis and Roberts (1981).

At the end of the germination period (30 days), the hypocotyl and radicle lengths of the germinated seeds were measured and analysed using Digimizer v.4.6.1 software (Med-Calc Software, Ostend, Belgium, 2005–2016). Several additional indexes were also calculated: SE, speed of emergence (Islam et al., 2009), GI, germination index, as indicator of the success and speed of germination (Kader, 2005), and SVI, seedling vigour index (Abdul-Baki and Anderson, 1973).

2.5. Biochemical analyses

2.5.1. Photosynthetic pigments

Photosynthetic pigments were extracted in 1 mL of ice-cold 80 % (v/v) acetone from approximately 0.1 g of ground fresh leaves and quantified according to the method and equations of Lichtenthaler and Wellburn (1983).

2.5.2. Ion contents determination

The concentration of monovalent ions Na^+, K^+, Cl^- and the bivalent Ca^{2+}, were measured separately in ground dry roots and leaves extracted in boiling Milli-Q water, following the protocol by Weimberg (1987).

2.5.3. Osmolytes

Proline (Pro) was quantified from fresh leaf material following the classical method by Bates et al. (1973). Glycine betaine (GB) concentration was determined as described by Grieve and Grattan (1983) with some modifications (Valadez-Bustos et al., 2016). Total soluble sugars (TSS) extracted in methanol were quantified according to the classical protocol by Dubois et al. (1956). The concentration of TSS was calculated using a standard curve that correlates glucose concentration and absorbance and was expressed as mg equivalent glucose g^{-1} DW

2.5.4. Oxidative stress marker and antioxidant compounds determination

Leaf contents of malondialdehyde (MDA), total phenolic compounds (TPC) and total flavonoids (TF), were determined in the same methanolic extracts prepared for TSS quantification.

MDA was determined according to Hodges et al. (1999), and concentrations were calculated following Taulavuori et al. (2002).

The total phenolic content (TPC) was determined by the reaction with the Folin-Ciocalteu reagent in the presence of sodium carbonate (Na_2CO_3), following the protocol by Blainski et al. (2013). The TPC values were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg eq. GA g^{-1} DW).

The total flavonoid (TF) content was determined according to the method by Zhishen et al. (1999). The flavonoid concentration was calculated using a standard curve based on catechin (C) and expressed as catechin equivalents (mg eq. C g^{-1} DW).

2.6. Metabolomic and pathway characterisation

For metabolic analysis, fresh leaves (0.1 g) were frozen and ground, followed by extraction, derivatisation, and GC–MS analyses according to the method of Lisec et al. (2006), with modifications from Misra et al. (2020). Briefly, enzymatic activity was halted using cold pure methanol, ribitol served as the internal standard, and chloroform as a solvent for extracting polar-phase compounds. After drying the samples in a vacuum concentrator, each was derivatised by adding methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). In addition, 10 μL of n-hydrocarbons mixture (alkane standard C10–C40, 50 mg mL^{-1} each, Sigma Aldrich, Switzerland) was included to monitor shifts in Retention Indices (RI).

A gas chromatograph (Agilent 7820A, GC) coupled with a single quadrupole mass spectrometer (Agilent 5975C, MS) and an RTX-5Sil MS capillary column (60 m × 0.25 mm × 0.25 μm) was used. The injector temperature was set at 200 °C, whereas the source temperature was maintained at 250 °C.

MS-DIAL software was used for deconvolution, calibration, baseline filtering, as well as peak extraction, alignment, identification and height integration (Tsugawa et al., 2015). Peak detection followed set thresholds for peak width, height, and spectral cut-offs, while retention time and mass tolerance were applied for accurate identification. Data annotation utilised publicly available libraries, including the Golm Metabolome Database (Kopka et al., 2005), MassBank (Horai et al., 2010) and MoNA (Mass Bank of North America). The analysis adhered to metabolomic initiative (MSI) guidelines, with compounds annotated at levels 2 and 3. Normalisation was done using ribitol, and quality control included alkane standards for RI calibration and blank samples for background correction.

2.7. Statistical analysis

The analysis of the germination, growth, and biochemical data was conducted using SPSS v. 18.0 software (SPSS Inc, Chicago, IL, USA) and Statgraphics Centurion XVII (Statgraphics Technologies, The Plains, VA, USA). Percentages of germination were arcsine transformed prior to the analysis of variance. A one-way ANOVA was carried out to check the effects of stress treatments on each trait analysed. When the ANOVA null hypothesis was rejected, the Tukey Honestly Significant Difference (HSD) post-hoc test was used to identify statistically significant differences in the mean values of the parameters between the treatments, with a significance level of $p < 0.05$.

To find the variables with the greatest influence on the species' response to salt stress, a Principal Component Analysis (PCA) was performed for all significant morphological and biochemical variables of the 25 plants analysed using pairwise Euclidean distances and visualized via SRplot (https://www.bioinformatics.com.cn), an online platform for bioinformatics data analysis and visualisation (Tang et al., 2023).

MetaboAnalyst 6.0 software was used for a log-transformation and Pareto-scaling of previously annotated metabolite data before statistical analysis. Unsupervised Principal Component Analysis (PCA) was conducted for group discrimination (score plot), and metabolites contribute identification, followed by supervised Partial Least Squares Discriminant Analysis (PLS-DA) and calculation of Variable Importance in Projection (VIP) scores to highlight key differentiating metabolites.

To detect metabolites that significantly differed across control and salinity treatments, univariate analysis was conducted using one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). The metabolites showing significant differences were visualised in a heatmap, with clustering performed using the Euclidean distance method and Ward's hierarchical algorithm for group classification. To further investigate the differences, Student's t-tests ($p \leq 0.05$) were applied to compare each salinity treatment (0.05, 0.1, 0.2, and 0.3 M NaCl) with the control. A pathway analysis of the significant metabolites was conducted using the MetPA module of MetaboAnalyst 6.0, which integrates pathway enrichment and topology analysis to evaluate the biological implications of the altered pathways (Araniti et al., 2017). A volcano plot was then created to emphasise the differential metabolites between the control and the highest salinity concentration (0.3 M NaCl), as well as a pattern detection analysis to identify which metabolites were positively and negatively correlated with the control and the treatment of the highest salinity. All raw and processed metabolomic data are available in the supplementary material (Table S1).

3. Results

3.1. Substrate analysis

Table 1 shows the electrical conductivity ($EC_{1:5}$) of the substrate after 23 weeks of treatments when plant material was sampled. The control group exhibited the lowest EC value (~5 mS cm^{-1}), significantly different from all treatment groups. As NaCl concentration increased, EC rose progressively, with significant differences observed between treatments. The highest EC was registered for the 0.3 M NaCl treatment (~31 mS cm^{-1}), indicating that high salinity substantially increases the ionic concentration in the medium.

3.2. Growth parameters

The NaCl treatments significantly affected the morphological traits of *K. pentacarpos* plants, including the number of leaves, fruits, and seeds

Table 1
Electrical conductivity upon completing the treatment period, EC values (1:5). Values shown are means per pot ± SE; $n = 5$. Different letters indicate significant differences between treatments ($p < 0.05$). Treatment (NaCl).

	0 M	0.05 M	0.1 M	0.2 M	0.3 M
$EC_{1:5}$ mS cm^{-1}	4.9 ± 0.5 a	16.2 ± 2.6 b	25.1 ± 3.7 bc	29.3 ± 2.8 bc	31.2 ± 2.3 c

Table 2
Effect of salt stress on growth parameters of *Kosteletzkya pentacarpos* after 23 weeks of salt treatments at the indicated NaCl concentrations. The values represent means ± SE ($n = 5$). For each plant part, different lowercase letters indicate significant differences between treatments according to the Tukey post-hoc test ($p < 0.05$).

Treatment NaCl	0 M	0.05 M	0.1 M	0.2 M	0.3 M
Root length (cm)	36.1 ± 4.6 a	46.2 ± 4.4 a	40.9 ± 3.2 a	48.8 ± 4.8 a	36.2 ± 4.3 a
Plant height (cm)	134 ± 10.1 c	117.8 ± 4.4 bc	89.4 ± 11.1 ab	75 ± 4.3 a	59.4 ± 2.8 a
Nr of leaves	146.4 ± 12.1 cd	169.2 ± 16.6 d	115 ± 11.8 bc	96.6 ± 5.4 ab	60.2 ± 2.8 a
Fresh weight roots (g)	117.6 ± 5.6 b	98.2 ± 5.0 b	92.6 ± 9.3 b	52.8 ± 5.6 a	28.4 ± 2.7 a
Fresh weight leaves (g)	54.0 ± 4.7 a	34.7 ± 9.4 ab	31.9 ± 9.1 ab	24.1 ± 4.7 a	12 ± 2.2 a
Fresh weight stem (g)	13.5 ± 6.4 a	8.3 ± 2.5 a	16 ± 7.4 a	13.5 ± 4 a	6.7 ± 1.5 a
Water content roots (%)	69.3 ± 0.8 a	68.8 ± 0.4 a	70.9 ± 0.3 ab	75.1 ± 1.6 b	70.9 ± 1 ab
Water content stem (%)	72.9 ± 1.4 a	73 ± 0.7 a	77.7 ± 1.1 ab	77.9 ± 3.1 ab	81.9 ± 0.8 a
Water content leaves (%)	79.7 ± 3.7 a	76.5 ± 1.7 a	81.2 ± 1 a	83.3 ± 0.6 a	79.2 ± 1.2 a
Nr of flowers	115.6 ± 11.1 c	98.6 ± 3.4 c	66 ± 4.7 b	33 ± 3.4 a	15.8 ± 3.1 a
Nr of fruits	18.6 ± 2.2 b	17.8 ± 2.2 b	6 ± 1.9 a	0.4 ± 0.4 a	0.4 ± 0.4 a
Nr of seeds	74.4 ± 8.9 b	71.2 ± 9.1 b	17 ± 4.8 a	1.6 ± 1.6 a	1.6 ± 1.6 a

(Table 2). Plants under control conditions exhibited the highest values for all three traits, and no significant reduction was observed at 0.05 M NaCl. A significant decline, however, was evident at 0.1 M NaCl, with the number of leaves, fruits and seeds decreasing. At 0.2 and 0.3 M NaCl, a drastic reduction occurred in all traits, with no significant differences between these two treatments. This indicates that low NaCl concentrations (up to 0.05 M) do not strongly affect plant morphology, whereas higher concentrations negatively impact vegetative and reproductive growth, particularly at 0.2 and 0.3 M NaCl.

The control group exhibited the tallest plants (~150 cm) and the longest roots (40 cm) without significant differences with the 0.05 M NaCl treatment (Table 2). Root length did not differ significantly from the control values at any of the salt concentrations tested. However, a significant decrease in plant height begins to be observed at 0.1 M NaCl, with the highest reductions detected in plants treated with 0.2 and 0.3 M NaCl. These results suggest that salt stress severely inhibits shoot development while having a negligible impact on root elongation.

Table 2 illustrates the fresh weights (FW) of roots, stems, and leaves under different salinity conditions. The control group exhibited the highest fresh weights for all plant organs, with roots contributing the most to total biomass (~120 g), followed by stems and leaves. Leaf FW did not show significant differences with the control values in any of the salt treatments. Similarly, at 0.05 and 0.1 M NaCl, root and stem FW remained relatively high, showing no significant reduction compared to the control plants grown in the absence of salt; however, a significant reduction was observed at higher NaCl concentrations (0.2 and 0.3 M). At 0.3 M NaCl, fresh weights for all plant parts were reduced to <30 g (Table 2).

Water content remained relatively constant across all treatments (Table 2), ranging between 70 % and 85 % for all organs and salt concentrations tested. Mean water content percentages were slightly higher in leaves and stems than in roots, but there were no significant reductions in water content with increasing salinity. These findings indicate that this species is highly resistant to salt-induced dehydration and that the observed reduction of root and stem fresh weight is due to growth inhibition, not differential water loss.

Table 3 shows the average number of flowers and fruits produced per plant over time, counted every 10 days, the newly produced ones, in the five plants grown per treatment. The influence of salinity on flowering and fruiting was notable, with increasing salinity progressively reducing flower and fruit production. Plants in the control group exhibited the highest flower counts, showing a consistent increase throughout the treatment period. Similarly, the plants treated with 0.05 M NaCl maintained relatively high flower production, following a pattern similar to the control group, suggesting that low salinity has little or no effect on the flowering potential of *K. pentacarpos*. At 0.1 M NaCl, the recorded number of flowers showed two peaks at 40 and 130 days after starting the treatment. Higher salinity levels (0.2 M and 0.3 M NaCl) led to a delayed start of flowering and a sharp reduction in flower production. This suggests that high salinity imposes physiological stress, significantly diminishing the plant's flowering capacity.

Regarding fruit production, plants in the control group also showed the highest value, as for the number of flowers, with a steady increase throughout the growing season. Similarly, plants exposed to 0.05 M NaCl produced a number of fruits similar to the control. At 0.1 M NaCl, fruit production was noticeably reduced compared to the control and 0.05 M treatments. Higher salinity levels (0.2 M and 0.3 M NaCl) severely inhibited fruit production.

Table 3
Average number of flowers and fruits per plant, counted at the indicated times after starting the treatments with increasing NaCl concentrations in five plants per treatment in the late spring and summer of 2022.

Treatment (NaCl)	0 M		0.05 M		0.1 M		0.2 M		0.3 M	
Day	Nr flowers	Nr fruits	Nr flowers	Nr fruits	Nr flowers	Nr fruits	Nr flowers	Nr fruits	Nr flowers	Nr fruits
1	0	0	0	0	0	0	0	0	0	0
10	1.6	0	3	0	0	0	0	0	0	0
20	6.2	0	3.8	0	3.2	0	0	0	0	0
30	6.6	0	7.6	0	3.6	0	0	0	0	0
40	7.4	0	6.4	0.4	7.4	0	0	0	0	0
50	6.8	0.4	6	0	4.2	0.4	1.2	0	0	0
60	7.2	0.6	4.8	0.2	4.8	0.2	2	0	0	0
70	7.6	0.8	5.8	1.4	1.8	0	3.8	0	0	0
80	8.2	1	5	1.4	1.4	0.8	3	0	0.2	0
90	7.8	1.4	7.8	1.8	3.8	0.2	4.2	0	0.6	0
100	11	1.4	6.6	2.2	5.2	0.4	2.2	0	1	0
110	11	1.8	11.4	2	6.6	1	3	0.4	4.6	0
120	11.4	2	9.8	2.6	7.8	1	2.2	0	2.8	0.2
130	10.4	1.8	8.2	3	8.8	1.4	3.8	0	3.6	0.2
140	9.2	2.4	8.2	2	4.8	0.4	3.8	0	2	0
150	3.2	2.6	4	0.8	2.6	0.2	3.6	0	0.8	0
160	0	2.4	0.2	0	0	0	0.2	0	0.2	0
Total	115.6	18.6	98.6	17.8	66	6	33	0.4	15.8	0.4

3.3. Seed germination

Parameters related to seed germination and seedling traits are summarised in Table 4. Seeds were produced by plants grown under control (0 M) and 0.05 M NaCl conditions. For seeds harvested from the control group, the germination percentage (GP) was highest in the presence of 0.1 M NaCl treatment (36.0 %) and the absence of salt (34.0 %) but decreased significantly at 0.15 M and 0.2 M NaCl. Mean germination time (MGT) progressively increased with salinity, indicating delayed germination. Similarly, the first germination day (FGD) and last germination day (LGD) were extended with increasing salinities, whereas the germination index (GI), seedling vigour index (SVI) and seedling size declined sharply, becoming negligible at 0.2 M since the seeds in this treatment did not germinate in the first two weeks.

When germinated under control conditions, seeds harvested from the 0.05 M NaCl-treated plants exhibited a better GP than seeds from plants grown without salt (82.0 % vs. 34 %), or in the presence of relatively low salt concentrations, 0.05 M or 0.1 M NaCl (Table 4), suggesting a possible adaptive response to moderate salinity. However, GP progressively declined at higher salinity, dropping to 10.0 % at 0.2 M NaCl. MGT, FGD, and LGD values were significantly higher in seeds exposed to higher salinity, reflecting slower germination rates. The speed of emergence (SE) significantly increased in the highest salt concentrations (0.15 and 0.2 M NaCl). When seeds from plants treated with 0.05 M NaCl were germinated under controlled conditions or low concentrations of 0.05 and 0.1 M NaCl, they had a higher germination percentage (GP) compared to seeds from plants grown without salt, as shown in Table 4, indicating a potential adaptive response to moderate salinity. However, the GP decreased considerably at the higher salt concentrations tested of 0.15 and 0.2 M NaCl, reaching only 10.0 % at 0.2 M NaCl. Additionally, the mean germination time (MGT), final germination distance (FGD), and length of germination delay (LGD) were notably higher in seeds exposed to greater salt concentrations, indicating slower germination. The speed of emergence (SE) was also significantly enhanced at the highest salt levels (0.15 and 0.2 M NaCl).

3.4. Biochemical parameters

The biochemical analysis underscores the substantial physiological adaptations that plants undergo in response to salinity stress. The performed biochemical analyses included the determination of leaf contents of photosynthetic pigments (chlorophylls and carotenoids), osmolytes (proline, glycine betaine, total soluble sugars), an oxidative stress biomarker (malondialdehyde), and antioxidant compounds (phenolic compounds and flavonoids), as well as ion (Na^+, Cl^-, K^+, Ca^{2+}) concentrations in roots and leaves.

3.4.1. Photosynthetic pigments

Leaf contents of chlorophyll *a* (Chl A), chlorophyll *b* (Chl B) and total carotenoids (Carot.) did not show clear patterns of variation with increasing salinity, although a general decreasing trend could be observed, more evident when comparing control plants with those subjected to the highest salt concentrations (Fig. 1). For example, the mean Chl A content in plants grown without salt was 1.1 mg g^{-1} DW and decreased to 0.4 mg g^{-1} DW at 0.3 M NaCl (Fig. 1A). Similarly, Chl B concentration under 0.3 M NaCl was around half of that measured in the control treatment (Fig. 1B) and in carotenoids only one-third (Fig. 1C). In any case, the differences between treatments were not statistically significant, indicating a moderate resistance of the photosynthetic machinery to salt stress.

3.4.2. Ions contents in roots and leaves

Sodium (Na^+) content in roots and leaves increased progressively and significantly with increasing salinity (Fig. 2A). For example, root Na^+ content in the presence of 0.3 M NaCl was ca. 7.5-fold higher than that measured in control plants; the difference was more pronounced in leaves, where a 32-fold increase was observed under the same conditions. Interestingly, under control conditions and at low salinity (0.05 M NaCl), Na^+ concentrations were higher in leaves than in roots, whereas the opposite trend was observed in plants treated with high (0.2 and 0.3 M) NaCl concentrations. These data suggest the activation of mechanisms blocking Na^+ transport to the plant aerial part at high salinities. Chloride (Cl^-) content variation followed a pattern similar to that of Na^+, with a significant, salt-dependent increase in roots and leaves and higher concentration in leaves than in roots under high salinity stress, although Cl^- levels were similar in both organs in the control plants and at low salinities (Fig. 2B).

Potassium (K^+) content increased slightly in roots and leaves in response to salt treatments, although accumulation patterns differed; the differences with the controls were statistically significant at all salt

Table 4
Germination parameters of seeds produced by the *Kosteletzkya pentacarpos* plants subjected to 0 (control) and 0.05 M NaCl treatments. Values shown are means per plate ± SE; $n = 5$. Different letters indicate significant differences between treatments within each seed source for each determined variable ($p < 0.05$). Abbreviations: GP, germination percentage; MGT, mean germination time; FGD, first germination day; LGD, last germination day; TSG, time spread of germination; SE, speed of emergence; GI, germination index; SVI, seedling vigour index.

Seed source	Treatment (NaCl)	GP (%)	MGT (days)	FGD (days)	LGD (days)	TSG (days)	SE	GI	SVI	Seedling size (mm)
0 M	0 M	34.0 ± 2.4 ab	4.3 ± 0.3 a	2.0 ± 0.0 a	6.8 ± 0.7 a	4.8 ± 0.7 a	35.0 ± 4.1 a	1.1 ± 0.1 c	10.1 ± 1.1 b	29.7 ± 1.1 d
	0.05 M	28.0 ± 3.7 ab	5.6 ± 1.2 a	3.2 ± 0.9 ab	7.8 ± 1.5 ab	4.6 ± 0.9 a	43.3 ± 4.1a	0.8 ± 0.2 bc	8.5 ± 1.1 b	30.5 ± 0.4 d
	0.1 M	36.0 ± 4.0 b	8.9 ± 0.7 b	5.0 ± 0.0 b	13.8 ± 1.7 bc	8.8 ± 1.7 a	34.0 ± 4.8 a	0.5 ± 0.0 ab	8.1 ± 1.0b	22.4 ± 0.4 c
	0.15 M	22.0 ± 3.7ab	10.7 ± 1.0 b	7.0 ± 0.0 c	14.4 ± 2.1 c	7.4 ± 2.1 a	53.3 ± 12.2a	0.2 ± 0.0 a	3.7 ± 0.8 a	16.4 ± 1,0 b
	0.2 M	20.0 ± 4.5 a	23.5 ± 0.4 c	21.8 ± 0.4 d	25.2 ± 1.1 d	3.4 ± 1.4 a	63.3 ± 15.3 a	0.1 ± 0.0 a	1.3 ± 0.4 a	6.3 ± 1.0 a
0.05 M	0 M	82.0 ± 9.2 d	4.5 ± 0.3 a	2.0 ± 0.0 a	8.2 ± 0.4 a	6.2 ± 0.4 a	22.2 ± 6.1 a	2.3 ± 0.3 c	28.8 ± 4.7 b	34.6 ± 2.3 c
	0.05 M	66.0 ± 2.4 cd	8.3 ± 0.9 a	2.8 ± 0.2 a	20.2 ± 2.2 b	17.4 ± 2.3 b	32.9 ± 4.9 a	1.3 ± 0.1 b	18.5 ± 2.3 b	27.7 ± 2.6 c
	0.1 M	44.0 ± 9.2 bc	15.5 ± 1.4 b	6.8 ± 1.2 a	25.6 ± 1.4 bc	18.8 ± 1.0 b	37.0 ± 6.2 a	0.4 ± 0.1 a	6.8 ± 1.6 a	15.6 ± 1.2 b
	0.15 M	18.0 ± 5.8 ab	18.2 ± 2.9 b	16.8 ± 2.4 b	18.6 ± 2.9 b	1.8 ± 1.4 a	75.0 ± 15.8 b	0.1 ± 0.0 a	2.4 ± 0.8 a	13.6 ± 0.5 b
	0.2 M	10.0 ± 0.0 a	28.4 ± 0.2 c	28.4 ± 0.2 c	28.4 ± 0.2 c	0.0 ± 0.0 a	100.0 ± 0.0 b	0.0 ± 0.0 a	0.3 ± 0.0 a	3.6 ± 0.5 a

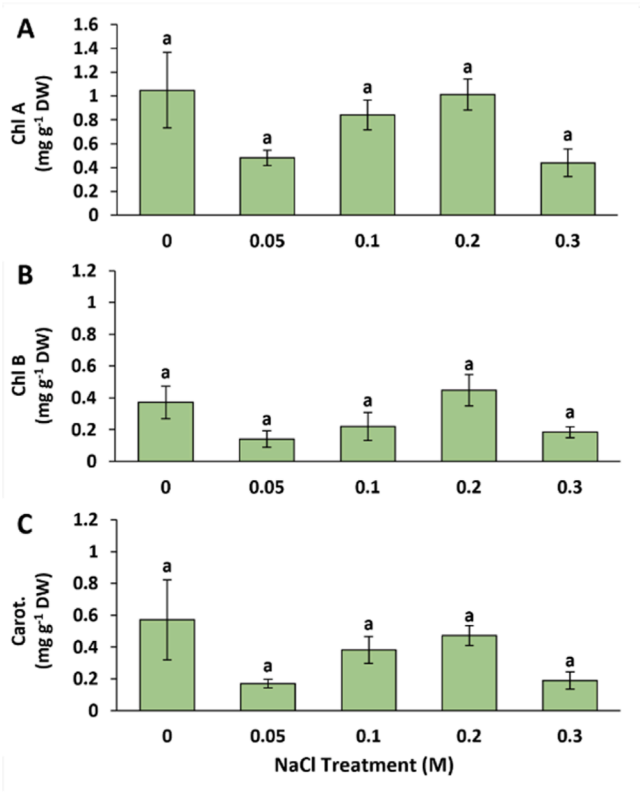

Fig. 1. Effect of NaCl stress treatments on the leaf contents of photosynthetic pigments after 23 weeks of treatment of *Kosteletzkya pentacarpos* plants with NaCl at the indicated concentrations. A) Chlorophyll *a* (Chl A), B) chlorophyll *b* (Chl B), and C) total carotenoids (Carot.). Values shown are means ± SE; $n = 5$. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$).

concentrations in the leaves, whereas in the roots only high salinity (0.2 and 0.3 M NaCl) caused significant differences. The measured K^+ concentrations were higher in leaves than in roots under all experimental conditions (Fig. 2C). Calcium (Ca^{2+}) contents in salt-treated plants showed different variation trends in roots and leaves, increasing and decreasing, respectively, with increasing salinity. The differences with the controls were better observed at high salinities but were, in all cases, relatively small and not always statistically significant. As for K^+, Ca^{2+} concentrations were higher in leaves than in roots under all salt concentrations tested (Fig. 2D).

3.4.3. Osmolytes

The accumulation of several osmolytes in response to the salt treatments was determined in leaf extracts of *K. pentacarpos* plants (Fig. 3). Proline (Pro) content augmented progressively and significantly with salinity, reaching a ca. 9-fold increase over the control value in plants treated with the highest salt concentration tested, 0.3 M NaCl (Fig. 3A). High glycine betaine (GB) levels were observed in the absence of salt and did not vary significantly in response to the salt treatments (Fig. 3B). Although high concentrations of total soluble sugars (TSS) were also measured for plants grown under control conditions, these were significantly higher in salt-treated plants, from 1.6- to 2.0-fold, with respect to the control plants (Fig. 3C). However, the observed differences between salt treatments were not statistically significant.

3.4.4. Malondialdehyde and antioxidant compounds

The concentration of malondialdehyde (MDA), a product of membrane lipid peroxidation and a reliable marker of oxidative damage, did not vary under low salinity conditions, up to 0.1 M NaCl, with values around 60 nmol g^{-1} DW, but rose significantly at higher salinity levels (0.2 and 0.3 M NaCl), reaching ca. 94 nmol g^{-1} DW (Fig. 4A).

Regarding antioxidant metabolites, total phenolic compounds (TPC) increased slightly with increasing salinity, although the differences with the control were statistically significant only at high salinity levels in the 0.2 and 0.3 M NaCl treatments (Fig. 4B). In contrast, total flavonoid (TF) contents showed no significant increase in response to the salt treatments (Fig. 4C).

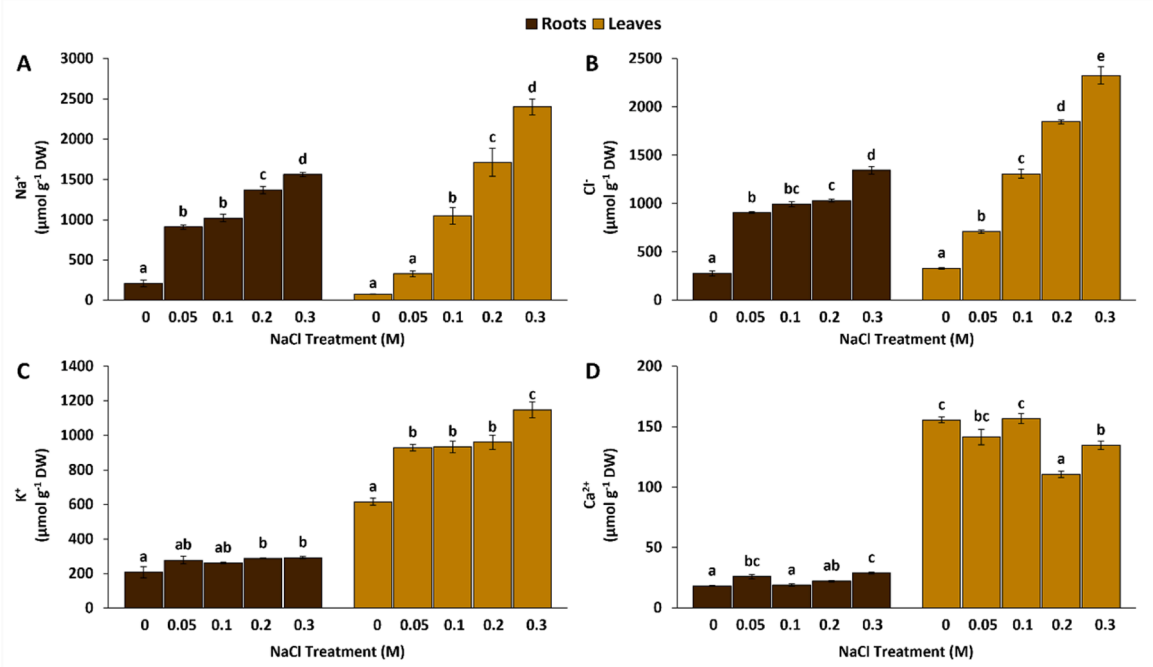

Fig. 2. Effect of NaCl stress treatments on ion concentrations in roots and leaves after 23 weeks of treatment of *Kosteletzkya pentacarpos* plants with NaCl at the indicated concentrations. A) sodium (Na^+), B) chloride (Cl^-), C) potassium (K^+), and D) calcium (Ca^{2+}). Values shown are means ± SE; $n = 5$. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$).

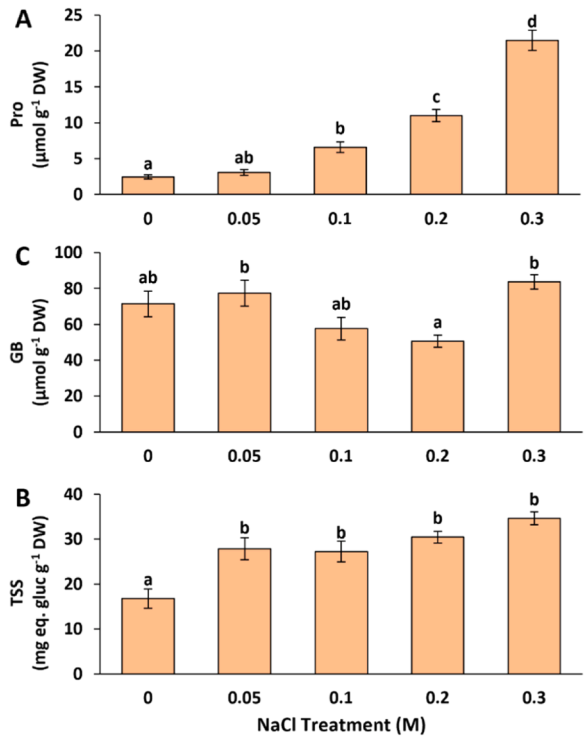

Fig. 3. Effect of NaCl stress treatments on the leaf contents of osmolytes after 23 weeks of treatment of *Kosteletzkya pentacarpos* plants with NaCl at the indicated concentrations. A) proline (Pro), B) glycine betaine (GB), and C) total soluble sugars (TSS). Values shown are means ± SE; $n = 5$. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$). Abbreviations: glucose (gluc).

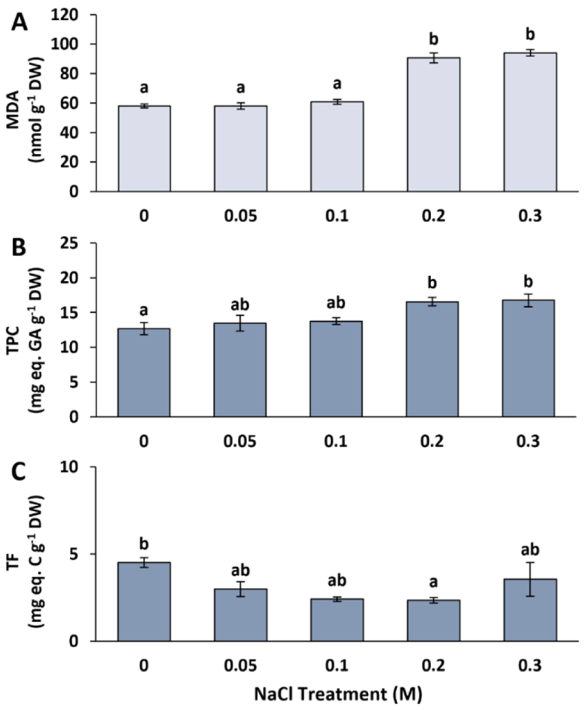

Fig. 4. Effect of NaCl stress treatments on the leaf contents of oxidative stress biomarkers and non-enzymatic antioxidant compounds after 23 weeks of treatment of *Kosteletzkya pentacarpos* plants with NaCl at the indicated concentrations. A) malondialdehyde (MDA), B) total phenolic compounds (TPC), and C) total flavonoids (TF). Values shown are means ± SE; $n = 5$. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$). Abbreviations: gallic acid (GA), catechin (C).

3.5. Principal component analysis

The PCA biplot (Fig. 5) illustrated the distribution of treatments (based on growth and biochemical parameters), with the first two principal components (PC1 and PC2) explaining 54 % and 9,9 % of the total variance, respectively. The clustering of treatments indicated distinct responses under varying salinity levels, as the treatments were clearly separated. Treatments with higher NaCl concentrations (0.3 M) were associated with elevated levels of Na^+ and Cl^- in roots and leaves, as well as increased electrical conductivity (EC) of the soil and increased malondialdehyde (MDA) and osmolytes (Pro and TSS) contents in plants. These traits correlate negatively along PC1, reflecting stress-induced biochemical responses. Glycine betaine (GB), being close to the origin of the PCA plot and negatively correlated to the PC2, indicated that it contributed moderately to the total variance and was not highly related to extreme salinity stress or optimal growth conditions.

In contrast, plants under control conditions (0 M NaCl) exhibited positive correlations with growth-related parameters, including root length, plant height, fresh weights (roots, stems, and leaves), and the number of leaves, flowers, and fruits. These parameters align positively along PC1, indicating optimal growth in the absence of salt stress. Moderate NaCl treatments (0.05 M and 0.1 M) were positioned between control and high-salt treatments, showing partial overlap.. The second principal component (PC2) captures additional variability, with fresh weight (FW) and water content (WC) in leaves showing moderate positive correlations with PC2. The tight clustering of data points within each treatment group indicates consistent responses amongst replicates regarding growth and biochemical parameters.

3.6. Metabolomic and pathway characterisation

The metabolomic analyses enable the annotation and quantification of 144 metabolites, along with the extraction of 346 unknown EI-MS features. The normalised data were subjected to univariate and multivariate analyses to evaluate the impact of the treatments on the metabolite and pathway profile.

3.6.1. Multivariate analysis of salt treatments

To reduce data complexity and visualise the differences between groups, Unsupervised Principal Component Analysis (PCA) was conducted. The first two components (PC1 and PC2) explained 40.5 % of the overall variability, with PC1 accounting for 28.9 % and PC2 for 11.6 % (Fig. 6A). The resulting score plot clearly separated the four treatments from the control group, with the highest salinity treatment (0.3 M) showing the highest deviation from the control, indicating the strong influence of salinity on the metabolic profile. The PCA loading analysis (Supplementary material Table S1) revealed that PC1, proline and aspartic acid emerged as key metabolites driving the separation in PC1, with both showing a higher accumulation across all salinity treatments (Fig. 6A). Conversely, compounds like quinic acid and gallic acid negatively correlated with PC1, reflecting their higher accumulation under control conditions. However, in the case of gallic acid, no significant differences were detected amongst treatments according to the ANOVA results. PC1 revealed a distinct pattern in the accumulation of specific metabolites as salinity increased, showing a strong positive correlation with these compounds, which play a key role in the plant's response to salinity stress. In contrast, PC2 was mainly influenced by galactitol and maltose, both negatively correlated and more abundant at intermediate salinity levels (0.1 and 0.2 M). This suggests that these metabolites contribute to salinity tolerance, with their effect being most prominent at moderate salt concentrations. Conversely, PC2 showed a positive correlation with metabolites such as gluconic acid (Fig. 6A), fumaric acid, ferulic acid, malic acid and citric acid, which accumulated

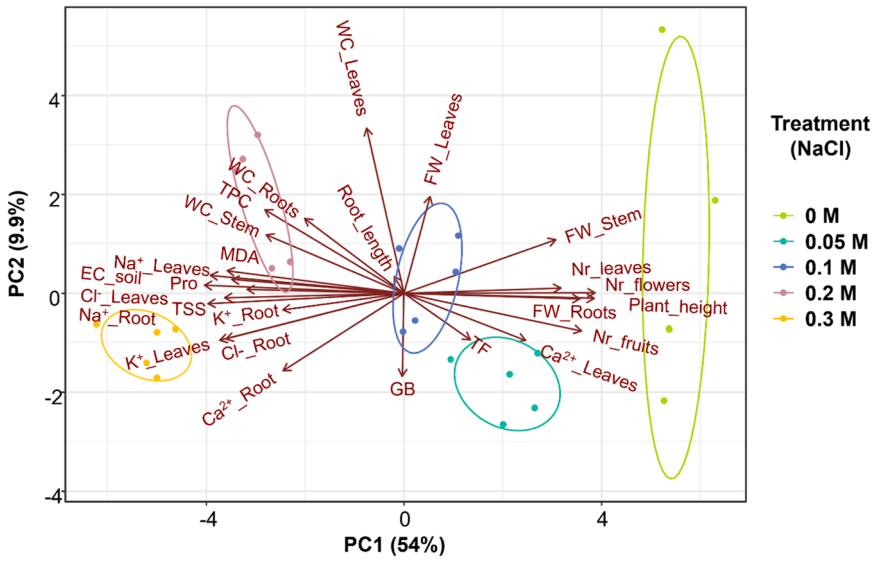

Fig. 5. Principal Component Analysis biplot of growth and biochemical data of *Kosteletzkya pentacarpos*. Abbreviations: fresh weight (FW), water content (WC), number (Nr), soil electro conductivity (EC_soil), proline (Pro), glycine betaine (GB), total soluble sugars (TSS), malondialdehyde (MDA), total phenolic compounds (TPC) and total flavonoids (TF).

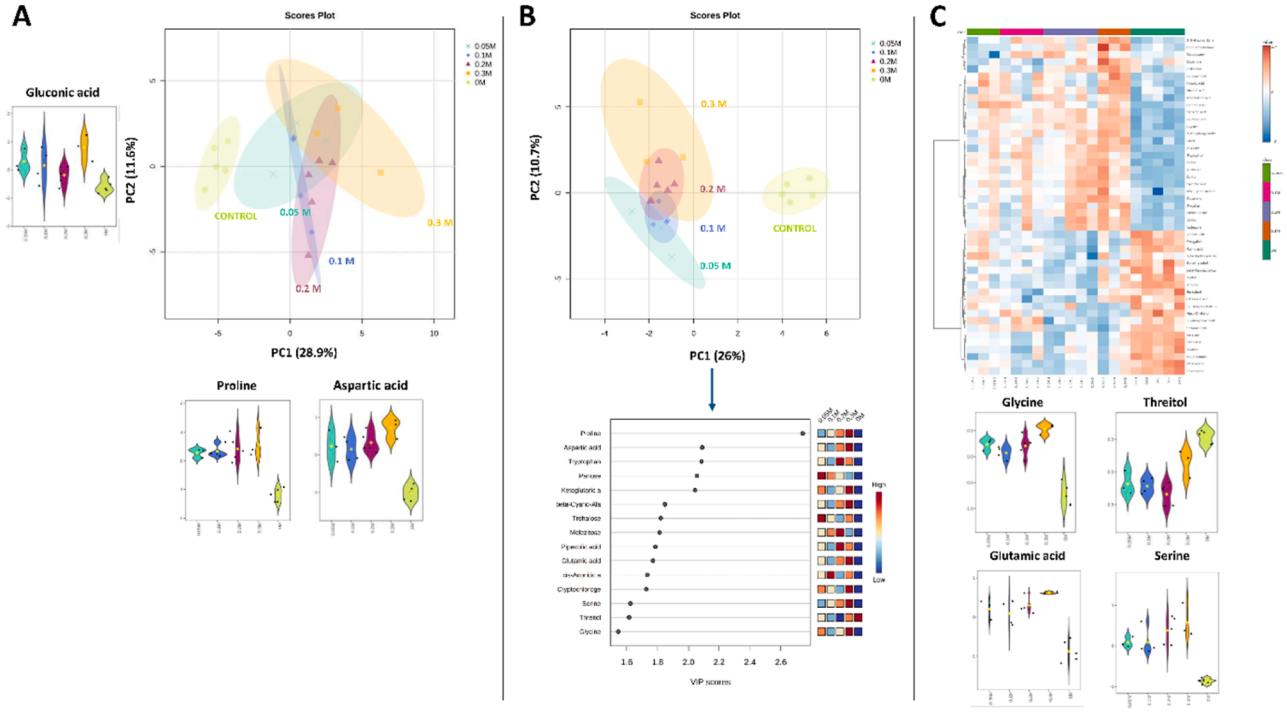

Fig. 6. Multivariate and univariate statistical analyses of the annotated metabolites found in K. pentacarpos leaves during NaCl treatments. A) PCA and box-plots of metabolites with the highest correlation values for PC1 (proline and aspartic acid) and PC2 (gluconic acid); B) multivariate PLS-DA and VIP of the metabolites(0 M, green colour; 0.05 M, turquoise colour; 0.1 M, blue colour; 0.2 M, violet colour; 0.3 M, orange colour); C) Overlay heatmap of the 47 statistically significant metabolites after one-way ANOVA (each row of the heatmap correspond to a metabolite, complete data in Supplementary material Table S1) in the control and the four treatments (each column corresponds to a replica) (LSD $p \leq 0.05$ and FDR ≤ 0.05). 0 M, dark green colour; 0.05 M, light green colour; 0.1 M, pink colour; 0.2 M purple colour; 0.3 M, orange colour.

in higher quantities under higher salinity (0.3 M NaCl).

The clustering observed in the PCA was further confirmed by the supervised PLS-DA (Fig. 6B), where the first two principal components (PC1 and PC2) accounted for 36.7 % of the total variance. PC1, explaining 26 % of the variation, effectively separated the control from the four salinity treatments. PC2, which explained 10.7 % of the variance, distinguished the treatments based on increasing salinity levels. Negative correlations of PC1 were observed with proline and aspartic acid, whereas quinic acid showed positive correlations. PC2 displayed negative correlations with gallic acid and sugars like panose, maltose, and turanose, while showing positive correlations with 3-amino-2-piperidine and methylnicotinate. The variable importance in projection (VIP) scores analysis highlighted the key role of proline in group separation, with a VIP score higher than 1.54, followed by aspartic acid, tryptophan

and panose (Fig. 6B).

One-way ANOVA identified that 47 out of 144 annotated metabolites were significantly affected by salt treatment (Supplementary material Table S1). These 47 metabolites were reported on a heatmap (Fig. 6C), providing an overview of their trends across the treatments and showing their relative accumulation patterns. Among the 47 altered metabolites, 24 showed a p-value lower than 0.001, indicating their high significance. The six metabolites with the highest F-values in the ANOVA analysis were glycine, threitol, glutamic acid, aspartic acid, proline, and serine (Figs. 6A and C). The concentrations of all these metabolites increased with rising salinity levels.

3.6.2. *Impact of salt treatments on metabolite profiles and pathways*

To compare the control with each salt treatment (0.05, 0.1, 0.2 and 0.3 M NaCl) a *t*-test analysis was performed (Fig. 7). The analysis of the 0.05 M NaCl pattern, compared with the control (Fig. 7A), revealed that 39 metabolites out of the 144 identified compounds were influenced by low salinity levels. A total of 25 metabolites showed increased accumulation at 0.05 M NaCl, with serine, proline, and tryptophan identified as the most abundant, while the other 14 compounds showed higher concentrations under control conditions (Supplementary material Table S1). According to the VIP scores (Supplementary material Table S1), lipid compounds, such as eicosane, docosane, and octadecane, were predominantly in the control group. In contrast, amino acids like serine and proline and other compounds such as citric acid and glycine accumulated more under the lowest salinity (0.05 M NaCl). These metabolites, exhibiting the highest VIP scores, were recognised as crucial biomarkers for distinguishing the control group from the low-salinity treatment.

A comparison between the control and 0.1 M NaCl salt treatment revealed significant differences in 33 out of the 144 metabolites detected (Fig. 5B). Amongst them, 15 metabolites, including proline, aspartic acid, and glycine, were more concentrated in plants under moderate salinity (0.1 M), while 18 metabolites, such as threitol, hexacosane, and docosane, were more abundant in the control. The VIP scores highlighted these compounds as key indicators, reflecting distinct metabolic profile changes between the control and 0.1 M of NaCl treatment.

In plants treated with 0.2 M NaCl, 53 altered metabolites were identified compared to control conditions, as shown in the heatmap in Fig. 7C. Of these, 25 metabolites, including aspartic acid, tryptophan, histidine, serine and glutamic acid, accumulated significantly more under the 0.2 M NaCl treatment. These metabolites also exhibited the highest VIP scores (Supplementary material Table S1), highlighting their importance in differentiating the two treatments. Conversely, 28 metabolites such as threitol and meso-erythritol (polyols or sugar alcohols) and pyrogallol, a phenol, were more abundant in the control group.

Finally, when comparing the control with 0.3 M NaCl treatment (Fig. 7D), 41 metabolites showed significant differences in accumulation. Thirty-two compounds, including 3-phosphoglycerate, aspartic acid, histidine and isoleucine, accumulated significantly more in salt-treated plants, with respect to control-grown plants, while 9 metabolites, including meso-erythritol and quinic acid as the most representative example, were more abundant under control conditions. VIP scores (Supplementary material Table S1) confirmed the importance of these metabolites in distinguishing between salt stress and control conditions.

A volcano plot (Fig. 8A) illustrates the differential metabolites between control and 0.3 M NaCl treatments, whereas pattern hunter (Fig. 8B) represents the correlation coefficients of metabolites, indicating that those with positive correlation coefficients (red bars) were more abundant in plants under high salinity treatment, and those with negative correlation coefficients (blue bars) were less abundant.

Although *t*-test analyses were performed across all salt treatments, no significant differences were found among the four salinity levels

Pathway analysis revealed a significant impact of salt treatments, particularly at the higher salinity levels (0.2 and 0.3 M NaCl). At the lowest level, 24 pathways were affected, with 9 showing an impact higher than 0.2. The metabolic routes with the highest scores, the most

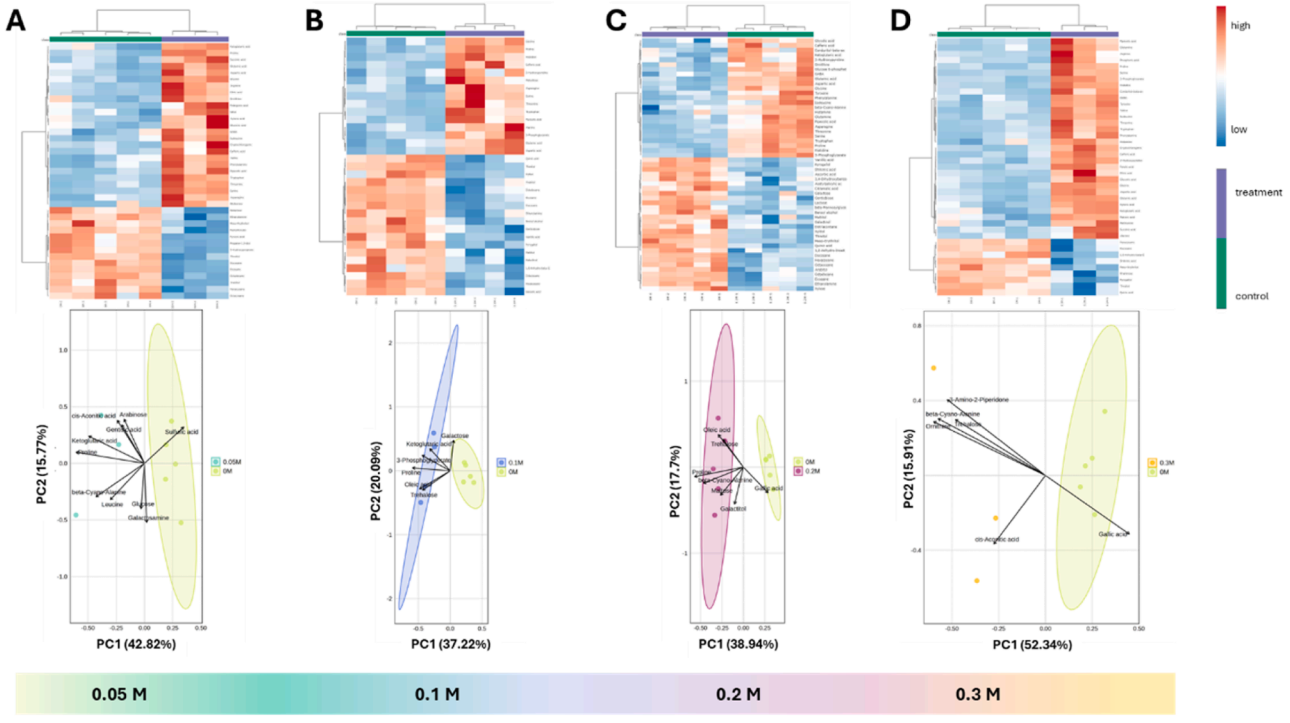

Fig. 7. At the top, overlay heatmaps of the significantly altered metabolites ($p \leq 0.05$) after the *t*-test between control and each salinity treatment, A: 0.05 M; B: 0.1 M; C: 0.2 M; D: 0.3 M (each row of the heatmap corresponds to a metabolite and each column correspond to a replica, complete data in Supplementary material Table S1). At the bottom, a biplot displaying the percentages of variance explained by the principal components of the PCA, along with the most important metabolites contributing to the separations.

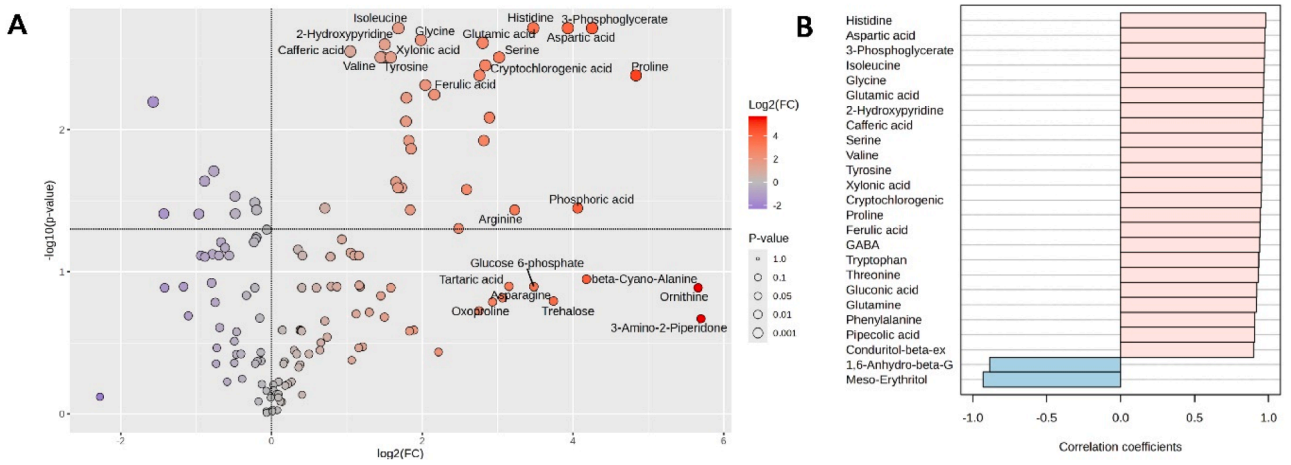

Fig. 8. A) Volcano plot showing differential metabolites with a p-value $\leq$ 0.05 and a Fold Change $>$ 1 between the control and the 0.3 M NaCl treatments. Upregulated (accumulated in 0.3 M NaCl) and downregulated (accumulated in Control)- metabolites are shown in red and blue, respectively. Grey dots represent non-significant metabolites. $n = 5$, control and $n = 3$, 0.3 M NaCl. B) Pattern hunter profile presenting the top 25 metabolites correlated with the treatments (Control and 0.3 M NaCl). Metabolites with positive correlation coefficients are pink, whereas metabolites with negative correlation coefficients are blue.

significantly altered, were alanine, aspartate, and glutamate metabolism, as well as glycine, serine, and threonine metabolism, with impacts greater than 0.05 (Table 5). At 0.1 M NaCl, 25 pathways were altered, with 13 demonstrating an impact score above 0.2. The most affected pathways included alanine, aspartate, and glutamate metabolism, and starch and sucrose metabolism (Table 5). The 0.2 M NaCl treatment influenced 29 pathways, 13 showing scores greater than 0.2. The most disrupted metabolic processes were isoquinoline alkaloid production, as well as alanine aspartate and glutamate metabolism. Finally, the highest salinity treatment (0.3 M NaCl) altered 30 pathways, with 11 exhibiting impact scores above 0.2. The two pathways with the highest score were the same as in the 0.2 M NaCl treatment, confirming a consistent trend. These findings suggest that increasing salinity progressively affects a broader range of metabolic pathways.

4. Discussion

4.1. Growth regulation and reproductive traits under salt stress

Plant growth inhibition is a general reaction to abiotic stress, which includes salinity. The presence of salts in the soil has a negative effect on plants, generating a decrease in the osmotic potential of the soil solution (osmotic stress), causing ion toxicity, and leading to nutritional imbalances (Atta et al., 2023). The growth of all glycophytes and many halophytes is optimal in the absence of salt but progressively declines as salinity levels increase. However, the growth of many dicotyledonous halophytes is stimulated by moderate salt concentrations, typically ranging from 0.05 to 0.25 M NaCl (Flowers et al., 1986). *K. pentacarpos* is a halophyte adapted to saline environments (Zhou et al., 2021). This species has been reported to exhibit optimal growth under moderate salinity of 0.085 M NaCl (Blits and Gallagher, 1990b) and to tolerate concentrations of up to 0.3 M NaCl (Islam et al., 1982). Our findings indicate no significant differences in growth parameters between the control plants and those treated with 0.05 M and 0.1 M NaCl and only higher salt concentrations induced significant reductions in growth parameters. Growth reduction under salinity stress is considered an adaptive response that allows the reallocation of cellular resources, such as energy and metabolic precursors, toward stress defence mechanisms (Zhu, 2001).

Salinity negatively impacts the reproductive development of plants by interfering with microsporogenesis, stamen filament elongation, ovule abortion, and the senescence of fertilised embryos (Shrivastava and Kumar 2015). However, in some species, salinity induces early flowering and increases flower production, though the quality of the flowers is often reduced (Al Hassan et al., 2014).

Under our experimental conditions, flowering and fruit set were significantly affected starting with the concentration of 0.1 M NaCl, and at 0.2 and 0.3 M NaCl, no fruits were produced at all. The significant reduction in flower and fruit production at elevated salinity levels can be attributed to salt stress-induced metabolic shifts, where plants allocate resources toward survival mechanisms rather than reproductive processes. This phenomenon may account for the observed delay and reduction in flowering and fruiting in the present study. Under salt stress, plants direct a substantial portion of their energy to maintaining

Table 5
Results from ingenuity pathway analysis with MetPa carried out on Kosteletzkya pentacarpos plants metabolites under control and four different salinity treatments. The table shows the five routes of each treatment with the highest impact (complete data in Supplementary material).

Pathways	Total Cmpd	Hits	0.05 M NaCl Raw *P*	0.1 M NaCl	0.2 M NaCl	0.3 M NaCl	Impact
Isoquinoline alkaloid biosynthesis	6	2	*	*	0.00428	0.00031	1
Alanine, aspartate and glutamate metabolism	22	10	2.11E-06	8.57E-07	1.02E-05	3.84E-06	0.85
Starch and sucrose metabolism	22	7	*	0.01287	3.30E-05	*	0.74
Galactose metabolism	27	12	*	0.00117	0.00051	*	0.70
Glycine, serine and threonine metabolism	33	8	2.08E-08	9.83E-09	4.00E-10	2.37E-09	0.55
Phenylalanine metabolism	12	1	1.42E-07	*	1.73E-12	2.07E-08	0.42
Cyanoamino acid metabolism	29	8	2.53E-08	2.16E-06	1.84E-09	1.69E-06	0.36
Glyoxylate and dicarboxylate metabolism	29	10	8.42E-06	6.87E-05	1.78E-05	4.82E-06	0.35

Total Cmpd: the total number of compounds in the pathway; Hits: the matched number from the annotated metabolites data; raw *p:* *p*-value calculated from the enrichment analysis; Impact: the pathway impact value calculated from pathway topology analysis. Only the pathways with an impact score higher than 0.1 are reported. The "*" indicates no significant differences because $p > 0.05$. The complete list of the significantly altered pathways is available in Supplementary Material.

ion homeostasis and protecting cellular structures from oxidative damage, thereby limiting resources available for growth and reproduction (Munns and Tester, 2008). Additionally, high salinity can impede nutrient uptake due to competition between ions in the soil, which may restrict the plant's access to essential nutrients required for growth (Läuchli and Grattan, 2007). The inhibition of flowering and fruiting may also be linked to the diminished availability of critical nutrients such as nitrogen, potassium, and phosphorus, which are vital for reproductive development (Jun et al., 2023). Furthermore, although photosynthetic pigment concentrations showed only a non-significant variation across all treatments, the physiological stress induced by high salinity could still compromise photosynthetic efficiency, ultimately reducing the availability of carbohydrates and energy necessary for reproductive processes, including flowering and fruit production.

Seed germination, which is particularly susceptible to soil salinity, represents a critical bottleneck in the plant's biological cycle. Not only glycophytes (Nouripour-Sisakht et al., 2022), but also most halophytes achieve maximum seed germination in non-saline environments, with substantial inhibition observed at salt concentrations significantly lower than those at which mature plants typically thrive (Flowers et al., 1986). However, extreme halophytes can germinate at salt concentrations comparable to or higher than seawater (Keiffer and Ungar, 1997), while others that grow in less saline environments cannot germinate at even low concentrations (Vicente et al., 2004). While salt tolerance during germination is crucial for seedling establishment, mature plants must develop additional adaptations, such as specialised ion transport mechanisms and enhanced antioxidant systems, to cope with longer-term salt stress. These complex mechanisms are not always reflected during early-stage germination but become essential as the plant grows and faces increasing salinity in the environment

Many halophytes found in temperate salt marshes begin to germinate when the soil salinity is alleviated by rainfall, usually in spring when the temperatures are higher (Gul et al., 2013). In the present study, only plants grown under control and 0.05 M NaCl conditions produced enough seeds for subsequent germination tests at different salt concentrations. These seeds exhibited the highest germination rates, seedling vigour indexes, seedling sizes and the fastest germination speed in the absence of salt or under low NaCl concentrations. However, a strong detrimental effect was observed starting at 0.15 M NaCl for seeds from both groups of maternal plants, salt-treated and non-treated. Therefore, in *K. pentacarpos*, maternal salinity did not enhance germination performance under high salinity conditions, as previously described for other halophytic species (El-Keblawy et al., 2016). These findings contradict the "seed memory hypothesis", which suggests that seeds from plants exposed to salt stress exhibit improved germination in saline environments, indicating an adaptive maternal effect documented in certain halophytes (Mohamed et al., 2020). Nevertheless, our experiments demonstrated that seeds produced by 0.05 M NaCl-treated plants show higher germination potential than those from non-treated mother plants under non-saline and low-salinity conditions. These results imply a potential transgenerational effect of salinity exposure, where seeds from stressed mother plants may be better primed for germination under low salinity but remain vulnerable to high salt stress levels. This indicates that while transgenerational priming can prepare seedlings for early growth stages, the long-term success of mature plants in saline conditions depends on the development of more complex, adaptive traits specific to adult plants.

4.2. Biochemical responses of Kosteletzkya pentacarpos to salinity

The biochemical analyses confirmed the relatively high salt tolerance of *K. pentacarpos* as photosynthetic pigments did not suffer a significant degradation under salt stress, and the concentration of MDA, a widely used marker of oxidative stress (Morales and Munné-Bosch, 2019), did not vary significantly up to the 0.2 M NaCl treatment, in agreement with previous reports (Wang et al., 2015a). *K. pentacarpos* employs osmotic adjustment mechanisms to maintain cellular turgor under salt stress conditions, allowing the plant to retain water and maintain growth despite high external salinity. The concentrations of proline and soluble sugars increased significantly in plants exposed to salt, contributing to the plant's defence against osmotic stress. Glycine betaine leaf contents did not vary significantly with salinity; however, they were relatively high even in the controls, pointing to the presence of constitutive mechanisms of osmotic adjustment based on changes in the intracellular localisation rather than the *de novo* synthesis of the osmolyte, as observed in other halophytes that use GB as a functional osmolyte, such as *Sarcocornia fruticosa*, or *Inula crithmoides* (Gil et al., 2014). Apart from their osmotic role, these compounds are essential for stress defence as they play a role in stabilising proteins and cellular structures, improve nutrient availability, and regulate redox balance (Ashraf and Foolad, 2007).

Salt stress induces the production of reactive oxygen species (ROS), which can damage cellular components if not properly managed. Halophytes possess enhanced antioxidant defence mechanisms that mitigate oxidative stress. In *K. pentacarpos,* a significant increase in the concentrations of MDA and TPC was detected only in plants subjected to 0.2 and 0.3 M NaCl treatments, indicating that lower salinity levels do not induce oxidative stress in this species. This aligns with previous findings where *K. pentacarpos* exhibited minimal lipid peroxidation under moderate salt stress (Wang et al., 2015b). The maintenance of cellular integrity, as indicated by stable MDA levels, underscores the species' ability to mitigate oxidative damage, a hallmark of effective salt tolerance mechanisms in halophytes. On the contrary, in glycophytes or salt-susceptible plants, even lower levels of salinity trigger a significant increase in MDA; for example, a concentration of 0.06 M NaCl induced a 3-fold increase in sesame (Bazrafshan and Ehsanzadeh, 2016). Maintaining cellular redox homeostasis under salt stress is essential for the plant's survival and growth in salt-affected environments.

4.3. Salt stress induced ion transport and compartmentalisation in Kosteletzkya pentacarpos

A critical strategy employed by *K. pentacarpos* to mitigate salt stress is the regulation of ion transport, particularly through the compartmentalisation of excess Na^+ and Cl^- ions into vacuoles. This effectively prevents the toxic accumulation of these ions in the cytoplasm, preserving cellular integrity and function. The regulation of ion homeostasis, combined with the preferential uptake and active transport of K^+, enables the plant to maintain both ionic balance and osmotic stability under saline conditions. This ion regulation mechanism is central to how halophytes cope with saline soils, as Na^+ and Cl^- toxicity, along with osmotic stress, are the two primary components of salt stress (Munns and Tester, 2008). Elevated Na^+ levels can interfere with the uptake and transport of K^+, an essential nutrient for growth and development. In many glycophytes, increased Na^+ concentration typically results in reduced K^+ levels, as Na^+ competes with K^+ for transport and uptake (Vaghar et al., 2024). However, in many halophytes, including *K. pentacarpos*, and in some glycophytic species, potassium levels are either maintained or even increase with increasing external salinity, suggesting the involvement of active potassium transport mechanisms to aerial tissues, which contributes to salt tolerance (Vicente et al., 2023). Potassium is the most abundant cation in plant cells, comprising about 10 % of the dry weight of plants (Szczerba et al., 2009). It plays a vital role in many physiological and biochemical processes, including enzyme activation, protein synthesis, and regulation of stomatal function (Johnson et al., 2022). The importance of K^+ is especially evident under salt stress, where its availability directly impacts plant productivity. Our findings reveal that K^+ concentrations in the leaves of *K. pentacarpos* were significantly higher than in the roots at all NaCl concentrations tested. Notably, K^+ levels increased substantially only at higher NaCl concentrations in roots, with leaves showing a marked increase even at the lowest NaCl treatment. These results are consistent with previous

reports in halophytes, where K^+ accumulation was observed in both roots (Al Hassan et al., 2016) and leaves (González-Orenga et al., 2021) under saline conditions. This suggests that *K. pentacarpos* employs active mechanisms to transport potassium to the aerial parts of the plant, where it plays a crucial role in osmotic adjustment and mitigating oxidative stress.

Interestingly, while Na^+ concentrations increased in the roots under salt stress, maintaining a high K^+/Na^+ ratio in the leaves indicates that the plant can effectively compartmentalise Na^+ in vacuoles while maintaining high K^+ levels in the photosynthetic tissues. This selective compartmentalisation is critical for maintaining cellular function under salinity stress. Previous studies have highlighted the strong affinity of *K. pentacarpos* for K^+, suggesting specialised ion transport systems that facilitate this process. Blits and Gallagher (1990b) first indicated the species' ability to selectively accumulate K^+, and subsequent research has shown that *K. pentacarpos* possesses specialised ion transport systems, including Na^+/H^+ antiporters, which actively expel Na^+ from the cytosol or sequester it in vacuoles (Blits et al., 1993).

Several genes related to salt tolerance have been identified in *K. pentacarpos*, underscoring the plant's ability to manage ionic stress. For example, the overexpression of the Na^+/H^+ vacuolar antiporter gene *KvNHX1* in transgenic tobacco plants has been shown to confer enhanced salt tolerance (Wang et al., 2018), as has the overexpression of *KvSOS1*, a plasma membrane Na+/H+ antiporter gene (Wang et al., 2014). These vacuolar Na^+/H+ antiporters (such as NHX proteins) play a pivotal role in Na^+ compartmentalization by transporting Na^+ into the vacuole, whereas plasma membrane-located Na^+/H^+ antiporters (SOS1) are responsible for excluding Na^+ from the cytosol to the apoplast. Furthermore, *KvCHX*, a gene from the cation/H^+ exchanger (CHX) family in *K. pentacarpos*, has been implicated in the selective accumulation and transport of K^+ from roots to leaves at both the cellular and whole-plant levels (Guo et al., 2023).

The selective uptake and transport of K^+, coupled with the maintenance of low Na^+/K^+ ratios under saline conditions and the compartmentalization of Na^+ and K^+ in the vacuoles of photosynthetic tissues, represent a key mechanism by which *K. pentacarpos* mitigates the detrimental effects of salt stress. This strategy of active potassium transport and ion homeostasis supports the plant's resilience to high salinity and underscores the crucial role of K^+ in osmotic regulation and stress tolerance (Blits et al., 1993).

4.4. Metabolic adjustments in Kosteletzkya pentacarpos to saline conditions

Metabolomics has become a crucial tool in understanding plant responses to environmental stress. Metabolic changes play a significant role in shaping plant development and adaptability, and gaining insights into the factors influencing metabolomic profiles is essential for advancing plant ecology. This includes enhancing our understanding of genetic adaptation, phenotypic plasticity, and resilience to abiotic stressors, such as salinity (Brunetti et al., 2013). Metabolic reprogramming in response to stress involves alterations in the concentrations of specific metabolites, including osmolytes that are critical for osmoregulation and stress tolerance (Sanchez et al., 2008).

The metabolic profiling of *K. pentacarpos* under increasing saline conditions provided valuable insights into adaptive metabolic responses. The analysis successfully discriminated between metabolic profiles at varying NaCl concentrations. Amongst the metabolites most strongly linked to salinity, proline and aspartic acid were particularly notable. Their concentrations increased progressively with higher salinity, making them key contributors to distinguishing control plants from those under saline stress. Proline, a well-established osmoprotectant, accumulated as expected, reinforcing its role in stress tolerance in halophytes, including *K. pentacarpos* (Wang et al., 2015a). Similarly, elevated levels of aspartic acid under saline conditions suggest its involvement in stress-related metabolic pathways, particularly within the alanine, aspartate, and glutamate metabolism pathways, which were significantly altered by salt treatment. These findings are consistent with previous studies that have highlighted the accumulation of proline and aspartic acid under salt stress, further supporting their critical roles in salt tolerance mechanisms (Parvaiz and Satyawati, 2008).

At intermediate salinity levels (0.1–0.2 M NaCl), *K. pentacarpos* exhibited increases in specific metabolites such as galactinol and maltose, which are associated with membrane stabilisation and osmoprotection. These results suggest that *K. pentacarpos* employs a metabolic strategy that balances stress management with continued growth under moderate salinity, a response also observed in other halophytes like *Aeluropus lagopoides* (Sobhanian et al., 2010). In response to severe salinity (0.3 M NaCl), the plant showed increased accumulation of metabolites involved in energy production and oxidative stress mitigation, including citric acid, fumaric acid, and gluconic acid. These findings align with previous studies that emphasise the role of these metabolites in maintaining cellular functions and reducing oxidative damage during salt stress (Gong et al., 2005).

Pathway enrichment analysis further revealed that salinity primarily disrupted amino acid metabolism, particularly pathways involving alanine, aspartate, glutamate, glycine, serine, and threonine. These pathways are critical for osmotic balance, redox homeostasis, and energy production, all of which contribute to enhancing salt tolerance (Sanchez et al., 2008). Similar disruptions in amino acid metabolism have been documented in other halophytes, including *A. lagopoides* and *T. halophila* (Sobhanian et al., 2010; Widodo et al., 2009). The significant alteration in amino acid metabolism under salt stress supports the hypothesis that halophytes reallocate resources toward essential protective pathways, a strategy commonly observed in such species.

Interestingly, the metabolite accumulation patterns observed in *K. pentacarpos* revealed both similarities and distinct differences when compared to other halophytes. While the general response, including the accumulation of proline and alterations in amino acid metabolism, appears conserved, distinct shifts in carbohydrate metabolism suggest species-specific adaptations (Sobhanian et al., 2010). These differences may be attributed to genetic variation, habitat-specific selective pressures, or evolutionary adaptations to saline environments (Brunetti et al., 2013).

In summary, this study highlights the metabolic flexibility of *K. pentacarpos* under salt stress, underscoring its potential applications in ecological restoration and sustainable agriculture in saline regions. The identified metabolic biomarkers, particularly proline and key carbohydrates, could serve as valuable indicators for breeding programmes aimed at developing salt-tolerant cultivars (Zhou et al., 2021). These findings align with previous research on halophytes and provide further evidence of the specific metabolic adaptations that contribute to the salt tolerance of *K. pentacarpos*.

5. Conclusion

Our data suggest that *K. pentacarpos* employs a multifaceted strategy to cope with salinity, including ion regulation, osmotic adjustment and antioxidant defence. The accumulation of osmolytes, such as proline and glycine betaine, coupled with increased antioxidant activity (evidenced by higher phenolic compound levels), demonstrates the plant's capacity to mitigate osmotic and oxidative stress. Additionally, this study represents the first metabolomic analysis of *K. pentacarpos*, revealing important metabolic pathways associated with salinity tolerance. The varying accumulation of sugars and amino acids emphasises the role of metabolic flexibility and resource reallocation in stress tolerance. As climate change worsens salinity stress in coastal ecosystems, knowing the salt tolerance mechanisms of halophytes like *K. pentacarpos* could help develop salt-tolerant crops and restore degraded coastal habitats. This study highlights the potential of *K. pentacarpos* in saline agriculture and phytoremediation, providing a foundation for sustainable agricultural practices. Future research should explore the genetic regulation of

these metabolic pathways to enhance salinity resilience and expand applications in saline environments.

CRediT authorship contribution statement

Diana-Maria Mircea: Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation. **Sara González-Orenga:** Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation. **Adela Sánchez-Moreiras:** Validation, Supervision, Resources, Funding acquisition. **Carla Díaz-Tielas:** Writing – review & editing, Software, Investigation. **P.Pablo Ferrer-Gallego:** Writing – review & editing, Resources, Methodology. **Ricardo Mir:** Writing – review & editing, Validation. **Jaime Prohens:** Writing – review & editing, Validation, Funding acquisition. **Oscar Vicente:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Monica Boscaiu:** Writing – original draft, Supervision, Project administration, Formal analysis, Conceptualization.

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.stress.2025.100856.

Data availability

All primary data has been deposited in Mendeley repository (htpps://data.mendeley.com) and will be available the February 2026

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