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The influence of environment on invasive *Carpobrotus* sp. populations across genetic clusters

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A B S T R A C T

The study aims to explore the natural variation in the metabolome of different populations of the invasive plant *Carpobrotus* from different genetic clusters and geographical origins to enhance our comprehension of its involvement in the adaptation process and phenotypic diversity. The metabolomic profile of shoots was analysed in four populations from two different genetic clusters (Cluster A: Cádiz and A Lanzada; Cluster B: La Marina and Samil) and two different biogeographical regions in Spain (Atlantic: Samil and A Lanzada; Mediterranean: Cádiz and La Marina), collected in the field and subsequently grown in the greenhouse. In addition, climatic, and physiological parameters were analysed. The Mediterranean populations (Cádiz and La Marina) showed lower initial weight and length measurements in morphological parameters than the Atlantic populations. On the contrary, only root parameters showed significant differences in growth parameters among populations. The analysis of ion levels revealed a consistent pattern of higher concentrations in shoots compared to roots, with significant differences among populations, particularly in sodium (Na^+) and chlorides (Cl^-) levels. Regarding metabolomic analysis, clear correlations between the metabolome, genetic and climatic conditions of *Carpobrotus* sp.pl populations are described. Pairwise comparisons using *t*-tests and Principal Component Analysis (PCA) indicated that the differences in metabolomic profile between the Samil and La Marina populations, which correspond to the same genetic cluster (cluster B), were smaller than in the rest of the comparisons indicating that populations from the same genetic cluster were more similar metabolically than those from the same climatic region. The study identified key metabolites representative of each cluster, with significant differences in amino acids, organic acids, and sugars contributing to the variation among populations. Pathway analysis highlighted the impact of climatic conditions on metabolic pathways, particularly in populations from Cluster A. In conclusion, the different populations were more similar according to the genetic cluster than to the climatic region of origin when studied at the metabolomic level. Consequently, the metabolites more representative of each cluster were also identified.

1. Introduction

Invasive alien species (IAS) are one of the main environmental problems, posing significant challenges in maintaining biodiversity and the sustainable management of natural resources (Wang et al., 2023). Furthermore, the possibility of invasion will predictably increase under climate change conditions. Some previous studies (Early et al., 2016; Tu et al., 2021) predicted that these environmental dynamics will likely favour certain invasive species. Consequently, climate change and biological invasions are two essential drivers compromising biodiversity and the provision of ecosystem services. In addition to threatening the stability of ecosystems, the total cost of IAS in Europe amounted to US $140.20 billion (or €116.61 billion) between 1960 and 2020 (Haubrock et al., 2021).

One of the most dangerous species worldwide is *Carpobrotus* sp.pl due to its invasive capacity (Battisti et al., 2021). *Carpobrotus* N.E.Br.

(family Aizoaceae) is a perennial, prostrate, succulent genus comprising between 12 and 25 species and lower rank taxa, most native to South Africa. It grows from multiple rooting axes and spreads radially at rates of up to 1 m per year, potentially forming clonal swarms with diameters of up to 20 m (Conser and Connor, 2009). It grows on coastal slopes and inland areas at low latitudes, influenced by a general dry-temperate climate with warm summers (Campoy et al., 2018). It was introduced to Europe, California, and Australia in the early 20th century as a measure to stabilise coastal dunes, as well as for ornamental purposes on all five continents (Albert et al., 1997).

Currently, species within the *Carpobrotus* genus are widely naturalised in many coastal habitats beyond their native distribution range (Campoy et al., 2018). Typically, it is observed in coastal habitats, such as sea cliffs or coastal dunes, that are highly threatened (Campoy et al., 2018). These ecosystems include extreme environmental conditions such as low soil moisture, high soil salinity, salt spray, intense solar irradiance and soil nutrient deficiencies (Maun, 2009). Therefore, only a limited number of highly specialised plants can prosper in such ecosystems, given the need for a high degree of adaptation. Consequently, hosts are rare and endemic communities of high conservation value. Therefore, with Carpobrotus as a primary example, invasive species pose a pronounced threat to these invaluable coastal ecosystems and their associated flora.

A previous study (Novoa et al., 2023) identified three genetically distinct *Carpobrotus* clusters, morphologically undifferentiated, present in native and non-native worldwide regions. Taxon-specific microsatellite markers were developed using a Next Generation Sequencing approach to analyse the population's genetic structure. Novoa et al. (2023) provided insights into genetic diversity metrics, including allelic richness, the number of alleles per microsatellite locus, and heterozygosity, and analysed population differentiation using F_ST, a measure of genetic differentiation among populations, and AMOVA (Analysis of Molecular Variance), to evaluate the distribution of genetic variation among populations or group. These findings illustrate the genetic variability and structure within *Carpobrotus* populations and emphasize the complex biogeographic patterns. The results of this study revealed that there are two clusters in the Iberian Peninsula, clusters A and B. Cluster A-related populations originate from the Western Cape Province (South Africa) and are found in Punta de Rons (Pontevedra), A Lanzada (Pontevedra), and Cádiz (Cádiz). On the other hand, Cluster B populations originate from Mdumbi (South Africa) and are found in La Marina (Alicante) and Samil (Pontevedra).

In the current climate change situation, it has become crucial to analyse and understand plant adaptation mechanisms in order to improve management practices and establish the most effective eradication mechanisms (Drenovsky et al., 2012). In this context, metabolomics may provide valuable insights. Metabolomics elucidates the phenotype by establishing correlations between the phenotype and alterations within the metabolome (Ritchie et al., 2015). Besides, metabolomic methods are important tools for corroborating the physiological responses of plant species to environmental changes and provide a chance to gain insights into mechanisms underlying the role of biodiversity–ecosystem relationships (Scherling et al., 2010). Individuals of the same species growing under different environmental conditions show significant differences in the production and accumulation of primary and specialised metabolites (Pavarini et al., 2012).

Primary metabolites, such as carbohydrates, lipids and amino acids, are key elements working as osmolytes and osmoprotectants for plants against diverse environmental stressors (Salam et al., 2023). Nevertheless, natural variation in the metabolome is mostly correlated with alterations in secondary or specialised metabolism, e.g. phenolics, alkaloids or terpenoids (Meijón et al., 2016). Plant specialised metabolites are frequently referred to as compounds with no direct role in maintaining fundamental life processes such as growth or development. Still, they are essential for the plant's interaction with its environment by protecting plants from biotic and abiotic factors (Kumar et al., 2023).

Typically, these metabolites exhibit responsiveness to environmental stresses such as soil salinity, drought, or extreme temperatures. Among these specialised metabolites with osmoregulation potential, osmolytes such as proline or glycine betaine, sugars and polyamines have a crucial role in the response of organisms to climate change, helping them to survive and adapt to adverse environmental conditions (Ghosh et al., 2021). Therefore, studying the metabolome and its changes between the four populations selected in this study can generate meaningful information and unveil the influence exerted by genetic factors and environmental conditions.

Our study aims to investigate the influence of genetics *versus* environment on two distinct *Carpobrotus* clusters in the Iberian Peninsula. By employing metabolomic techniques, we examine four populations from two genetic clusters (Cluster A and Cluster B) and two biogeographic regions (Mediterranean and Atlantic). This approach will allow us to assess the plasticity of these clusters in response to environmental conditions and identify specific metabolites that may be crucial for their adaptation and survival. Additionally, we analyse the climatic characteristics of the areas of origin, and morphological and physiological parameters in shoots and roots to gain a comprehensive understanding of how these factors contribute to the adaptation of *Carpobrotus* populations.

2. Materials and methods

2.1. Material collection and plant growth

Carpobrotus sp.pl N.E. Br ramets were collected randomly from the four different populations in Spain (Fig. 1), corresponding to two different genetic clusters (A and B) and different climatic environments (Table 1). Plant material was at the same stage of development without apparent damage. The populations selected were two related to cluster A, A Lanzada (Pontevedra) and Cádiz (Cádiz), and two corresponding to cluster B, La Marina (Alicante) and Samil (Pontevedra). The two different populations within each cluster came from two different biogeographic regions identified in Spain according to the classification of the European Environmental Agency (https://www.eea.europa.eu/data-and-maps/figures/biogeographical-regions-in-europe-2), e.g., Mediterranean (Cádiz and La Marina) and Atlantic (A Lanzada and Samil). From each location, similarly sized ramets (two or three whorls) were collected from individuals separated by a distance of at least 3 m to minimise inbreeding. All plants were measured for initial weight, length and number of whorls before transplanting. Seven ramets of each population were then planted (one per pot) in plastic pots (10 × 9 × 7 cm) with homogenized sand and grown in the greenhouse of the Facultade de Bioloxía, Universidade de Vigo (Vigo, Galicia, Spain), allowing to root for one month. The plants were then left to grow for 50 days with a natural photoperiod. During this time, plants were watered once a week with 100 mL of tap water per pot. After harvest, several parameters were measured, including final weight, length, water content of shoots and roots, and final number of whorls. Part of the fresh shoot material was immediately frozen in liquid nitrogen and stored at −80 °C until metabolome extraction. The remaining leaf material was weighed in a balance and dried in a stove at 65 °C, until a constant weight was reached. This dry material was used to determine water and ion content.

Climatic data (Table 1) were obtained from SIAR, the Agroclimatic Information System for Irrigation (https://servicio.mapa.gob.es/websiar/) of the Spanish Ministry of Agriculture, Fisheries and Food for Cádiz and La Marina populations, and from Meteogalicia website (https://www.meteogalicia.gal) for A Lanzada and Samil populations. Data on the mean temperatures, total rainfall, and mean relative humidity were collected from the nearest climatological stations, from Elche (Alicante) for La Marina, from Puerto de Santa Maria (Cádiz) for Cádiz, from A Lanzada (O Grove, Pontevedra) for A Lanzada, and from Vigo Port (Vigo, Pontevedra) for Samil. Data were collected for 2021, the previous year of the material recollection.

Fig. 1. Localisation in the Iberian Peninsula of the four *Carpobrotus* sp.pl populations used in this study.

De Martonne's annual index was calculated using the following formula:

$$DMIa = \frac{P}{T + 10}$$

where DMI_a is the De Martonne annual index, P is the annual sum of precipitation in mm, and T is the annual average temperature in °C according to the classification proposed by Dimkić et al. (2022).

2.2. Determination of water content

The shoot weight was measured in fresh and dry to calculate the water content using the formula:

$$WC\ (\%) = \left(\frac{FW - DW}{FW}\right) \times 100$$

where WC represents the water content in %; FW is the fresh weight in grams, and DW is the dry weight in grams.

2.3. Determination of ion content

Monovalent ions, including potassium (K^+), sodium (Na^+), and chloride (Cl^-), as well as the divalent cation calcium (Ca^{2+}), were individually assessed in plant water extracts from both roots and shoots. To extract samples, 100 mg of crushed dried material (seven replications for leaves and four for roots) was incubated in 2 mL of deionized water at 95 °C for 1 h using a water bath. Subsequently, the samples were cooled on ice, subjected to overnight mixing in a shaker, and centrifuged for 10 min at 13,300 g (Weimberg, 1987). K^+, Na^+, and Ca^{2+} concentrations were determined using a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), while Cl^- levels were measured using an MKII Chloride Analyser 926 (Sherwood, Inc., Cambridge, UK).

2.4. Soil analysis

Minor elements (sodium, calcium, phosphorus and potassium) were determined using an ICP-OES (Inductively Coupled Plasma - Optical Emission Spectrometry) instrument, specifically the iCAP-pro Thermo-Fisher model. Between 0.11 and 0.20 g soil samples were calcined in a muffle furnace at 500 °C (four replicates). Subsequently, 2 mL of nitric acid (HNO_3) was added, and the mixture was heated using a block heater. After this step, the solution was diluted to 40 mL with ultrapure water (mQ). The equipment was calibrated using standards and multi-patterns in a 2% HNO_3 aqueous matrix. The elemental analysis of nitrogen and carbon (%) in the samples was performed by weighing each sample in a tin capsule and analysing them using a Thermo NC2500 elemental analyser based on the Dumas combustion method. Sample calculation values were conducted against a calibration curve obtained using certified standards of AEDT (Analytical Environmental and Toxicological Data). Finally, nutrient determination of chlorides, nitrates and phosphates was conducted using an Ionic Chromatography system (940 Professional IC Vario model by METROHM). Homogenized samples were weighed into 50 mL Falcon tubes, followed by adding the extractant.

2.5. Extraction, identification, and quantification of metabolites

Metabolomic analyses were performed on the shoots of the different populations following the protocol proposed by Lisec et al. (2006) with some modifications. For that, 100 mg fresh material of each *Carpobrotus* sp.pl population were lyophilised for 48 h in a LyoAlfa (Telstar, Spain) and milled to a fine powder. Five replications per population were used, except for Cádiz, which had just four replicates. Extraction was performed by adding 1400 μL of methanol (at −20 °C) and vortexing for 10 s, followed by the addition of 60 μL of ribitol (0.2 mg/mL in ddH₂O) as an internal standard for the polar phase (Lisec et al., 2006). The samples

Table 1

Origin and climatic details of the populations. Climatic data were obtained from the Agroclimatic Information System for Irrigation (SIAR, 2023) for Cádiz and La Marina populations and Meteogalicia for A Lanzada and Samil populations for 2021.

Origin	Cluster	Latitude and longitude	Average annual temperature (°C)	Annual rainfall (mm)	Relative humidity (%)	Zonification (De Martonne index)	Climatic Region
A Lanzada	A	42°25'N 8°52'W	15	1132	82	Very Humid (45.6)	Atlantic
Samil	B	42°12'N 8°46'W	16	1106	77	Very Humid (42.9)	Atlantic
Cádiz	A[a]	36°34'N 6°13'W	18	492	78	Semiarid (17.6)	Mediterranean
La Marina	B	38°8'N 0°38'W	19	360	67	Semiarid (12.4)	Mediterranean

[a] Previous data suggest possible admission of clusters with a predominance of cluster A.

were then incubated in a thermomixer at 70 °C with shaking for 10 min at 950 rpm, followed by centrifugation at 11,000 g for 10 min. The supernatants were transferred to glass vials, and 750 μL of $CHCl_3$ (−20 °C) and 1500 μL of ddH_2O (4 °C) were sequentially added. The mixture was vortexed for 10 s and centrifuged for 15 min at 2200 g. The upper polar phase (150 μL) was collected, transferred to a 1.5 mL tube, and dried in a vacuum concentrator without heating. Next, 40 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) were added to the dried samples, which were then incubated for 2 h in a thermomixer at 37 °C with shaking at 950 rpm. Subsequently, 70 μL of MSTFA was added to the samples, which were shaken for another 30 min at 37 °C. The derivatized samples (110 μL) were transferred to glass vials suitable for GC/MS analysis.

For GC/MS analysis, the derivatized extracts were injected into a 5MS capillary column (30 m × 0.25 mm x 0.25 μm + 10 m of precolumn) using an Agilent gas chromatograph (8890 GC System) equipped with a single quadrupole mass spectrometer (5977C GC/MSD). The injector and source temperatures were set to 230 °C, the quadrupole at 150 °C and the transferline at 280 °C, respectively. One microliter of the sample was injected in splitless mode with a helium flow rate of 1 mL/min. The temperature program was as follows: isothermal at 70 °C for 5 min, followed by a ramp of 6 °C/min to 250 °C, held for 5 min. Mass spectra were recorded in electron impact (EI) mode at 70 eV, scanning in the range of 40–600 m/z with a scan time of 0.2 s. The mass spectrometric solvent delay was set to 9 min. Quality control samples, n-alkane standards (C8-C40 all even), and blank solvents were injected at scheduled intervals to monitor instrument performance and retention index shifts.

The obtained chromatograms were then analysed using the opensource software MS-DIAL 4 (Tsugawa et al., 2015), which was used for peak extraction, baseline filtering and calibration, peak alignment, deconvolution, peak identification, and peak height integration. An average peak width of 20 scans and a minimum peak height of 1000 amplitudes were used for peak detection, with a sigma window value of 0.5 and an EI spectra cut-off of 5000 amplitudes for deconvolution. For identification, the retention time tolerance was set to 0.2 min, the m/z tolerance to 0.5 Da, the EI similarity cut-off to 60%, and the identification score cut-off to 80%. In the alignment parameters, the retention time tolerance was 0.5 min, and the retention time factor was 0.5. Compound annotation was carried out using an in-house library that was built following the protocol proposed by Misra (2019). Metabolite annotation followed the Metabolomics Standards Initiative (MSI) guidelines for identification: Level 2 for spectral database matches with a match factor above 80%, and Level 3 for known compound groups based on specific ions and retention time regions. Peak intensities were used for the relative quantification of the annotated molecules.

2.6. Statistical analysis

A completely randomised design was employed in each experiment, with the number of replicates varying depending on the specific analysis (details provided below). After identifying and excluding outliers using Tukey's method in SPSS Statistics 25.0, the data underwent normality assessment via the Kolmogorov−Smirnov test and heteroscedasticity assessment using Levene's test. Statistically significant differences between populations were analysed by ANOVA, followed by Tukey's test as a post-hoc test for homoscedastic data. Kruskal-Wallis test was used for non-normally distributed data with Tamhane's T2 test as a post-hoc test ($p \leq 0.05$).

The metabolomic profile was analysed using the MetaboAnalyst 6.0 software. Data normalization was performed using the Lowessnormalization function available in the MS-DIAL software. Metabolite concentrations were assessed for integrity, and missing values were replaced by a small positive value (half of the minimum positive number detected in the data). Then, data were normalised by a reference sample (ribitol), Log_{10} transformed and Pareto-scaled. Unsupervised Principal

Component Analysis (PCA) was used to generate the score plot (for visualising group discrimination) and the loadings plot (for identifying metabolites contributing to group separation). Supervised Partial Least-Squares Discriminant Analysis (PLS-DA) was conducted to calculate the corresponding variable importance in the projection (VIP value). Data were then analysed through the univariate analysis one-way ANOVA with Tukey's test as post-hoc ($p \leq 0.05$) to highlight statistical differences among single metabolites and populations. All features significantly affected in the ANOVA test were presented as a heatmap and clustered using the Euclidean method for distance measurement and the Ward algorithm for group clusterisation. Additionally, a Student's *t*-test analysis ($p \leq 0.05$) was performed for each population (Samil, La Marina, A Lanzada and Cádiz) to identify potentially different metabolites between clusters (A and B) or between climatic zones (Atlantic and Mediterranean). Furthermore, a pathway analysis was conducted with MetPA, a web-based tool that combines results from pathway enrichment analysis and topology analysis, allowing the evaluation of the possible biological impacts on the perturbed pathways (Araniti et al., 2017). Finally, a volcano plot analysis was carried out to show the differential metabolites between clusters. All the metabolomic analyses were carried out using Metaboanalyst 6.0. The supplementary material reports all the raw and analysed metabolomic data (Table S1).

3. Results

3.1. Growth-related parameters

Populations from the Mediterranean biogeographic region (Cádiz and La Marina) exhibited notably significantly lower initial weight and length of the shoots than those from the Atlantic region (A Lanzada and Samil), especially in comparison with those from the Samil population (Table 2). Nevertheless, ramets from Cádiz showed a notably higher initial number of whorls compared to the other populations. In all initial parameters, plants from La Marina showed the lowest values.

After a fifty-day growth period, the plants from the different populations were harvested, and the final weight, length, shoot and root water content, and the final number of whorls were recorded for each population. Table 3 shows the values of the growth parameters for the four populations. For the plants for which initial data (shoot weight and length, root weight and length, and number of whorls) were available, the variation between the beginning and the end of the fifty-day growth is shown to give more representative information.

Carpobrotus plants from the four different locations showed similar growth parameters, with significant differences only in two root parameters: weight and water content. Specifically, A Lanzada showed the highest fresh root weight values, while Mediterranean populations generally showed lowers values in line with the parameters initially recorded. Relative to A Lanzada plants, La Marina and Cádiz plants (Mediterranean populations) exhibited 48% and 60% less root weight, respectively, while plants from Samil displayed a relatively modest 17% decrease in this parameter. Concerning root water content, Cádiz roots

showed less significant value, with a reduction over 10% compared to the other three populations.

3.2. Ion content

The analysis of ion levels revealed a consistent pattern of higher concentrations in shoots compared to roots (Fig. 2). Notably, significant differences were observed among populations, particularly in the concentration of sodium (Na^+) and chlorides (Cl^-) (Fig. 1a and b). Mean Na^+ concentrations in shoots were higher in La Marina than in the other populations, ranging from 1233.02 μmol g_{DW}^{-1} in La Marina to 814.05 μmol g_{DW}^{-1} in A Lanzada. Significant differences were exclusively found between these two populations. Cádiz and Samil plants showed intermediate values. Similarly, regarding Na^+ root concentration, intermediate levels were observed in Samil and Cádiz populations, falling between La Marina (339.73 μmol g_{DW}^{-1}) and A Lanzada (160.87 μmol g_{DW}^{-1}) (Fig. 2a). Chloride (Cl^-) concentrations in shoots did not vary among populations, whereas in roots, significantly increased Cl^- levels of 145.01 μmol g_{DW}^{-1} were found in La Marina with significant differences with A Lanzada and Samil chloride values (approximately 78 μmol g_{DW}^{-1}) (Fig. 2b). In comparison, the mean root concentration of Cádiz populations was 96.83 μmol g_{DW}^{-1}. Variations in ion accumulation among different populations were highlighted within these results, with La Marina emerging as a notable case in terms of sodium and chloride concentrations. Regarding potassium (K^+), concentrations did not differ among the four populations (Fig. 2c). Nevertheless, K^+ concentrations in shoots were reduced in Cádiz 1.5 times in comparison with the rest of the populations. In contrast, K^+ concentrations in roots were lower in A Lanzada (28.30 μmol g_{DW}^{-1}) compared to the other three populations (with a mean of 90 μmol g_{DW}^{-1}). In both cases, differences were not significant. Also, root and shoot calcium (Ca^{2+}) concentrations did not differ significantly (Fig. 2d).

3.3. Soil analysis

Considering the different biogeographic origins of the four populations, the soil was analysed to detect potential differences. In summary, the soil samples collected from the four populations showed several differences (Supplementary Tables S2 and S3). The most notable one was the elevated accumulation of minor elements, including sodium, potassium, phosphate, and other nutrients such as chlorides, nitrates and phosphates in the soils from Samil. Furthermore, calcium tended to accumulate more in soils from the Mediterranean than from the Atlantic region. Cádiz and La Marina showed calcium levels approximately 6 and 25 times higher than the populations from the Atlantic region. Regarding the elemental analysis presented in Supplementary Table S3, the nitrogen and carbon concentrations were notably higher in cluster B populations. Specifically, Samil displayed higher concentrations than La Marina. The higher accumulation in the analysed soil coming from the invaded area of Samil was detected for all the components, except for calcium.

3.4. Metabolomic profile

The MS-DIAL software identified 578 compounds, of which 389 were unknown, and 189 were putatively annotated. After the removal of falsely annotated metabolites, a total of 158 metabolites were considered reliable. Many similarities, but also some differences, were found among the four populations. Raw data were analysed through univariate and multivariate approaches to assess the impact of the populations on the metabolite pool.

3.4.1. Comparative analysis of the four populations

Unsupervised Principal Component Analysis (PCA) was used to reduce dimensionality and visualise groupings. Using the first (PC1) and second (PC2) components, a score plot was generated describing 81% of

Table 2
Weight (g), length (cm), and number of whorls (mean ± dev) in the ramets of the four populations of *Carpobrotus* sp.pl before transplanting.

Cluster	Region	Origin	Weight	Length	N° whorls
A	Atlantic	A Lanzada	9.28 ± 2.34 [b]	9.66 ± 1.30[c]	2.35 ± 0.53 [b]
B	Atlantic	Samil	11.60 ± 3.76[c]	11.19 ± 1.30 [d]	2.38 ± 0.62 [b]
A	Mediterranean	Cádiz	7.69 ± 2.38[a]	8.41 ± 0.88 [b]	3.19 ± 1.12[c]
B	Mediterranean	La Marina	8.11 ± 2.24[a]	7.64 ± 1.11[a]	2.10 ± 0.30[a]

Different letters indicate statistical differences between populations ($p \leq 0.05$). N = 112.

Table 3

Weight (g), length (cm), shoot and root water content (%), and number of whorls (mean ± dev) in the four populations of *Carpobrotus* sp.pl after the 50-day growth period.

Origin	Shoot weight variation	Root weight	Shoot length variation	Root length	Shoot WC	Root WC	Nº whorls variation
A Lanzada	4.6 ± 1.3	1.4 ± 0.2 [b]	1.4 ± 1.0	16.1 ± 2.7	92.4 ± 0.3	72.4 ± 2.2 [b]	2.1 ± 1.5
Samil	5.8 ± 1.4	1.2 ± 0.9 [ab]	1.4 ± 1.0	14.1 ± 3.1	92.0 ± 1.0	76.9 ± 3.7 [b]	2.8 ± 1.5
Cádiz	4.5 ± 1.3	0.6 ± 0.2 [a]	1.1 ± 0.5	16.7 ± 2.8	92.1 ± 1.0	63.7 ± 3.2 [a]	2.9 ± 1.5
La Marina	4.6 ± 1.9	0.7 ± 0.2 [a]	1.1 ± 0.4	18.4 ± 5.9	91.6 ± 0.9	75.3 ± 2.6 [b]	2.2 ± 1.2

Different letters indicate statistical differences between populations. ($p \leq 0.05$). N = 7.
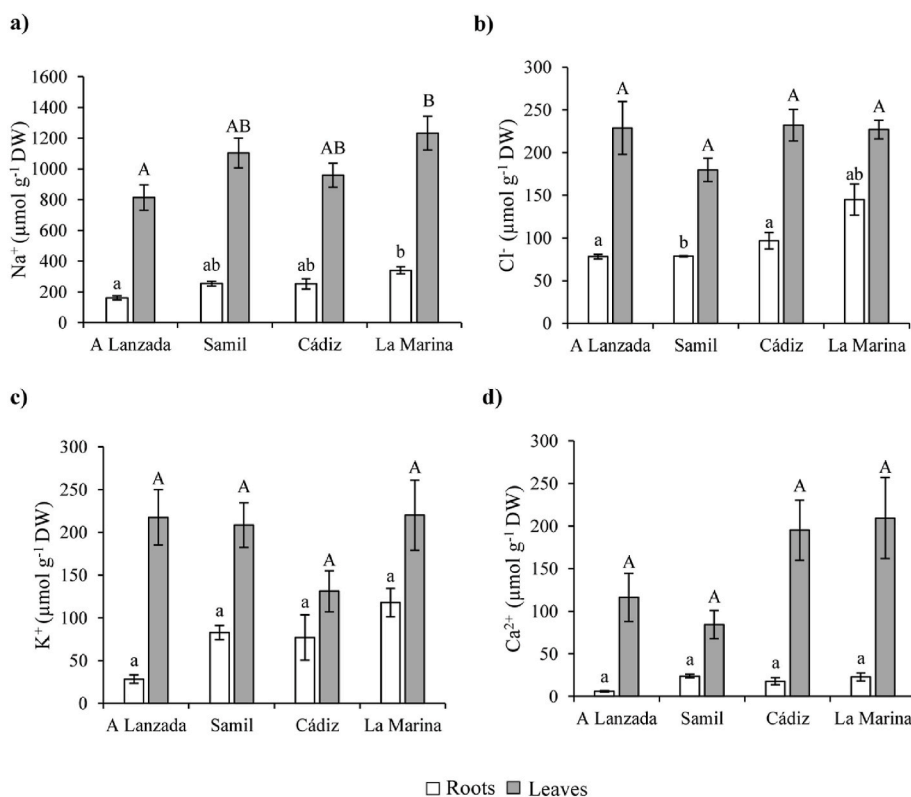

Fig. 2. a) sodium (Na^+), b) chloride (Cl^-), c) potassium (K^+) and d) calcium (Ca^{2+}) ion content in plants from the four populations of *Carpobrotus* sp.pl used in this study. Different letters indicate statistical differences between populations ($p \leq 0.05$). Capital letters indicate significant differences in the shoots, while lowercase letters indicate significant differences in the roots. N = 7 for shoots and N = 4 for root.

the total variability (Fig. 3a). PC1 accounted for most of the variance (73.9%), while PC2 accounted just for the 7.1% of the total variance. The PCA score plot showed a clear discrimination among the four populations. The PCA loading plot highlighted that PC1 was dominated by D-arabitol, D-galacturonic acid, hexanoic acid, 2-hydroxypyridine and nicotinic acid (Fig. 4a, first column), which accumulated significantly more in the plants from Cádiz ($p \leq 0.05$) followed by those from A Lanzada (both related to cluster A). According to PC1, Marina and Samil populations (cluster B) were more similar than those related to cluster A. However, Marina, Samil and A Lanzada were clearly separated from Cádiz (Fig. 3a, b and 3c). On the other hand, PC2 was defined by myo-inositol and derivates, salicylic acid, stearic acid, 3,4-dihydroxybenzoate, gluconic acid, glycerol-3-galactoside, phosphate, lactulose, and glucoheptulose, of which the last four compounds were significantly ($p \leq 0.05$) accumulated in plants from Samil, while gluconic acid accumulated more in Cádiz and Samil (Fig. 4a, second column). PC2 separated Samil from A Lanzada and Marina populations, whereas Cádiz samples showed an intermediate trend. The clustering observed during PCA analysis was subsequently validated by cluster analysis (Fig. 3c), which confirmed the formation of four main separated groups corresponding to the four populations. In this way, the analysis grouped Samil and La Marina populations (cluster B) in the same pool, and in a separate

unit from Cádiz and A Lanzada (cluster A). In fact, the supervised PLS-DA (Fig. 3b) confirmed the separation between the four populations, previously observed in the PCA. The division was accomplished by using the two initial principal components (PC1 as opposed to PC2), which accounted for a combined variation of 80.9%. Again, the first component clarified the maximum variation (73.7%), whereas the second component explained 7.2% of the total variation. Additionally, the variable importance in projection (VIP) scores analysis (built on the 15 metabolites with the highest VIP score, higher than 1.4), pointed out that D-arabitol, quinic acid and shikimic acid were the compounds mainly contributing to group discrimination (Fig. 3d). Still, some phenolic compounds, such as hydroquinone and 3-hydroxybenzoate, also contributed to the discrimination.

The univariate one-way analysis of variance (ANOVA) revealed that 130 out of 158 identified metabolites were significantly altered among the four populations. Those 130 metabolites were reported on a heatmap, which gave an overview of the trend of each metabolite among the four populations (Fig. 4b). Among the 130 altered metabolites, 111 showed a *p*-value lower than 0.001 (highly significant).

The ten compounds exhibiting the highest F-values in the ANOVA analyses included hexanoic acid, D-galacturonic acid, glucose-1-phosphate, 2-furoic acid, thymine, 3-hydroxybenzoate, nicotinic acid,
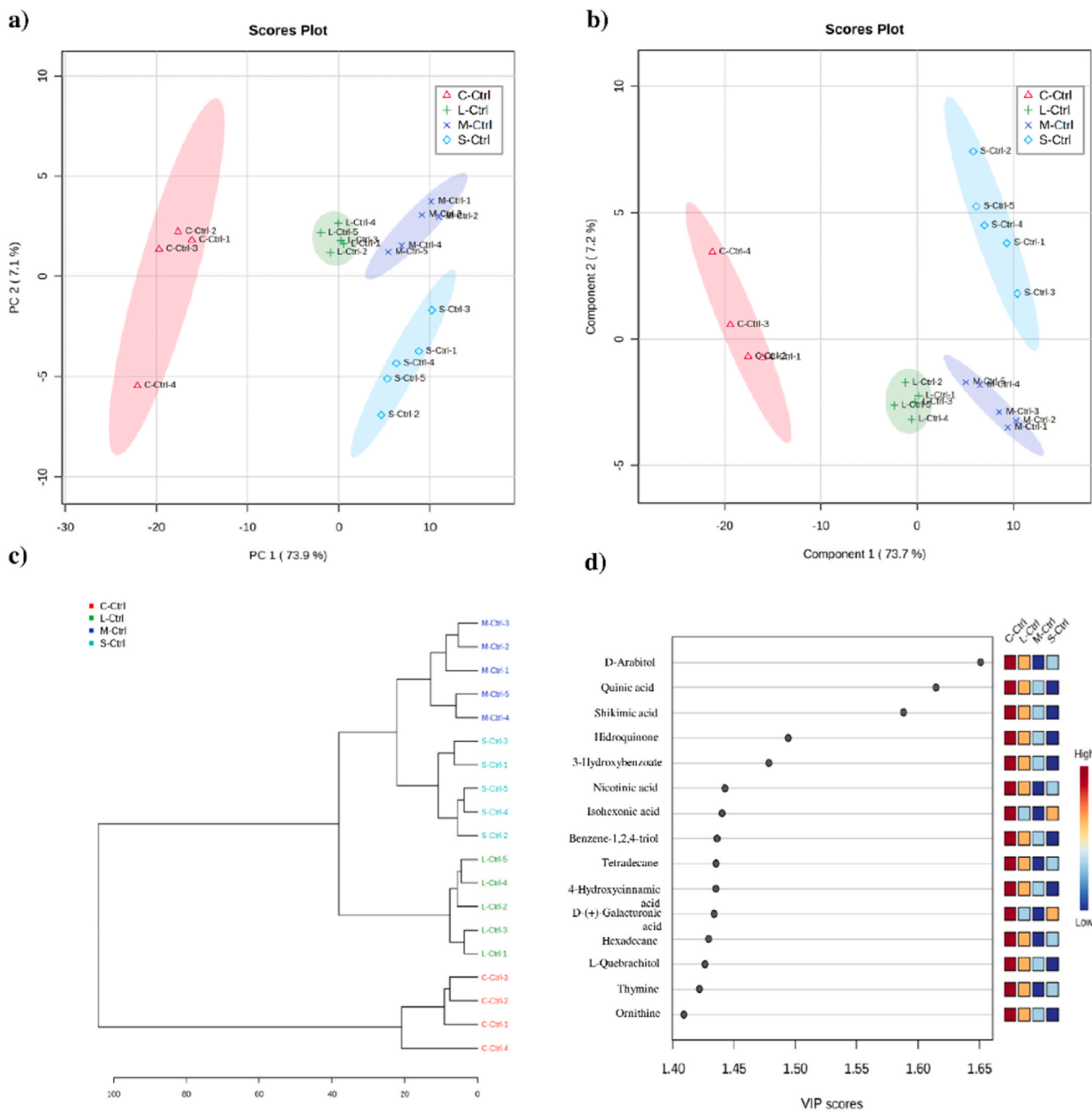
Fig. 3. a) Discrimination through principal component analysis (PCA) between the selected PCs (PC1 and PC2); b) multivariate partial least-squares discriminant analysis (PLS-DA) between the selected PCs (PC1 and PC2); c) Clustering results shown as a dendrogram (distance measure using euclidean, and clustering algorithm using ward); d) Variable importance of projection (VIP) features with a VIP score higher than 1.4 identified by PLS-DA analysis of the metabolites patterns for the four populations of *Carpobrotus* sp.pl (C-Ctrl = Cádiz, red colour; L-Ctrl = A Lanzada, green colour; S-Ctrl = Samil, light blue colour; M-Ctrl = La Marina, purple). N = 4 for Cádiz and N = 5 for A Lanzada, Samil and La Marina.

uracil, hydroquinone and benzene-1,2,4-triol. Moreover, 2-furoic acid, benzene-1,2,4-triol, glycerol, 6-deoxyglucitol, and 2-aminoethanol were the only metabolites that showed significant differences between the four populations, some of which corresponded to alcohols or sugar alcohols (Supplementary Table S1). In general, the highest accumulation of the identified metabolites was detected in the population of Cádiz, followed by A Lanzada (both related to cluster A), while Samil and La Marina showed lower values and almost no differences among the two

populations (both from cluster B). Although this was a clear and general pattern, there were some exceptions. A Lanzada showed a higher accumulation of citric acid than the other three populations, which showed similar concentrations of this compound. In addition, other compounds accumulated more in Samil' plants. The presence of organic and fatty acids such as oleic, picolinic, and succinic acids, among others, was particularly high. Finally, plants from La Marina did not accumulate any compound in higher concentrations compared to the rest of the
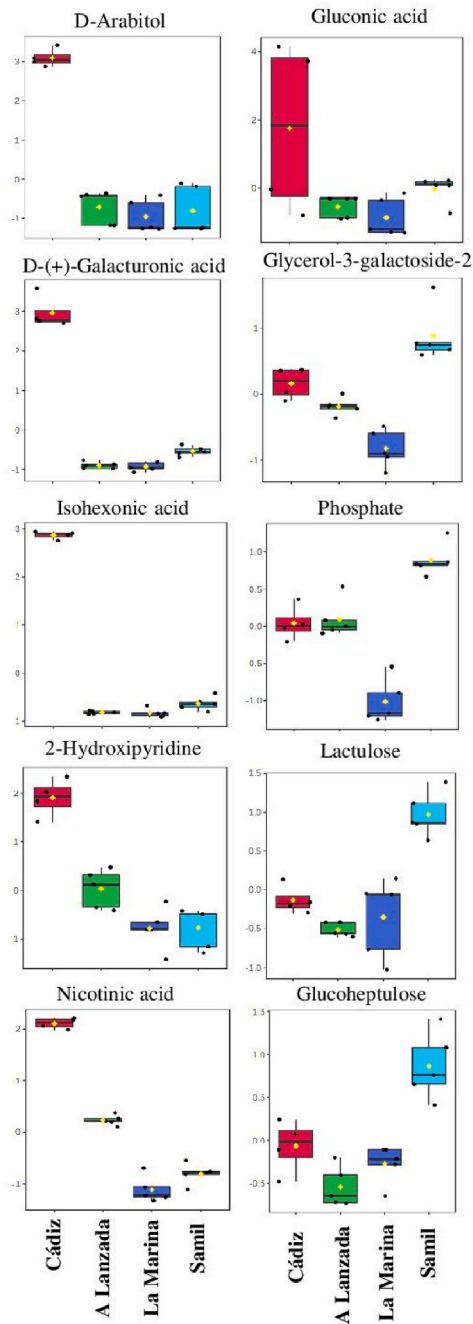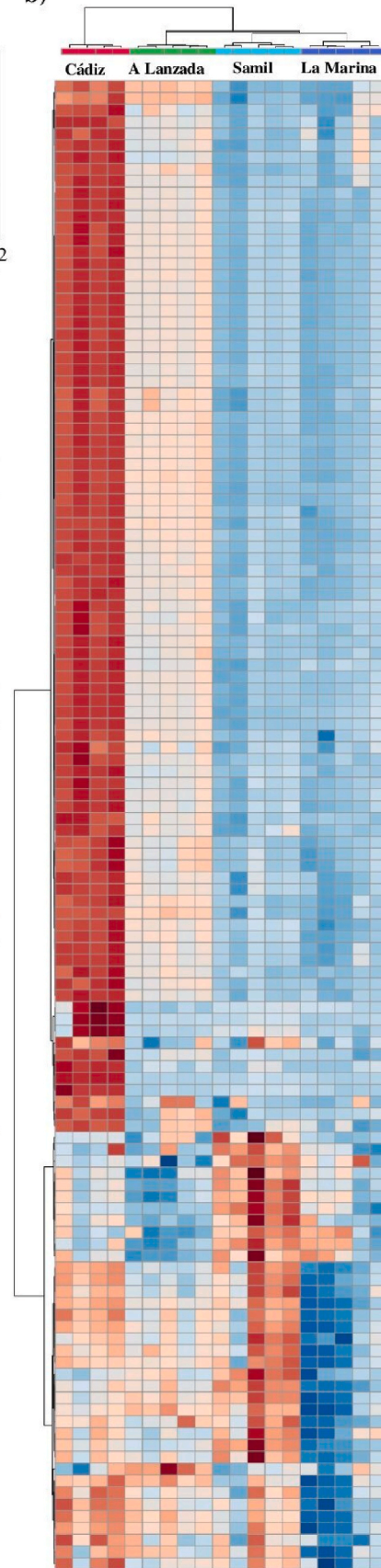
(caption on next page)

Fig. 4. a) Metabolites with the main loadings on PC1 (first column) and PC2 (second column) of the principal component analysis (PCA). Normalised metabolomic data were analysed through ANOVA using the Tukey test as post-hoc ($p \leq 0.05$). N = 5. b) Overlay heat map of the significantly altered 130 metabolites (each row of the heatmap corresponds to a metabolite, complete data in Supplementary Table S1) in the four populations (each column correspond to a replica) of *Carpobrotus* sp. pl resulted from the ANOVA test (LSD $p \leq 0.05$ and FDR ≤ 0.05). C-Ctrl = Cádiz, red colour; L-Ctrl = A Lanzada, green colour; S-Ctrl = Samil, light blue colour; M-Ctrl = La Marina, purple. N = 4 for Cádiz and N = 5 for A Lanzada, Samil and La Marina. c) Venn diagram showing the total number of significantly different metabolites detected by comparing the four populations in pairs.

populations.

The Venn diagram (Fig. 4c) shows the 158 metabolites detected. The intersections reflect the compounds that were significantly different according to the *t*-test carried out between the different populations based on belonging to the same genetic cluster or the same climatic region. (explained in detail in subsections *'Comparative analysis of the populations into each genetic cluster'* and *'Comparative analysis of the populations in each biogeographic region'*).

3.4.2. Comparative analysis of the populations into each genetic cluster

A *t*-test analysis was performed to compare populations within each genetic cluster. Individuals from A Lanzada and Cádiz are related to cluster A, and individuals from Samil and La Marina belong to cluster B.

The analysis of the cluster A-related populations revealed that 111 metabolites out of the 158 compounds detected were influenced by the climatic conditions in which *Carpobrotus* plants were living before the collection (Fig. 4c). In these two populations, all the altered metabolites accumulated more in Cádiz plants (Mediterranean region) than in those from A Lanzada (Atlantic region), except in the case of citric acid. The variable importance in projection (VIP) scores (Supplementary Table S1) showed that hexanoic acid, palmitic acid, threonine, 2-furoic acid, nicotinic acid, and fructose had the highest VIP scores and, as a consequence, were the compounds that most contributed to differentiate both populations. Differences between these two populations (cluster A) revealed a significant concentration of organic and fatty acids (hexenoic acid, palmitic acid, 2-furoic acid and nicotinic acid), and amino acids in Cádiz plants in comparison with the plants from A Lanzada (Fig. 5a).

On the other hand, the analysis between the populations of cluster B (La Marina and Samil) showed that only 38 metabolites out of the 158

metabolites detected differed among the two populations of this genetic cluster due to the different origin climatic conditions (Fig. 4c). For practically all compounds, the concentrations in both populations were much lower than those found in the populations related to cluster A (Cádiz and A Lanzada). All analysed compounds accumulated more in Samil than in La Marina plants. The VIP scores (Supplementary Table S1) revealed that phosphate, glycerol-3-galactoside, caffeic acid, salicylic acid, succinic acid, myo-inositol and sinapic acid, among others, had the highest values, pointing out the differences between plant metabolomic profiles in these populations. The results show the influence of fatty and organic acids on the differences in the metabolomic profile of cluster B populations. Specifically, the accumulation of these compounds was particularly higher in the case of plants collected from Samil (Fig. 5b).

3.4.3. Pathway impact of the biogeographic region on the different populations

A detailed analysis concerning the pathways altered by the biogeographic region (Mediterranean or Atlantic) was carried out using the Metaboanalyst module "MetPa". The pathway analysis revealed an important impact of the climatic region, especially in the populations related to the genetic cluster A. In particular, 33 pathways were affected by the climatic factor between populations of cluster A, and 12 showed an impact higher than 0.2. The routes with the higher scores were the starch and sucrose metabolism and glycine, serine and threonine biosynthesis pathways, with impacts higher than 0.5 (Fig. 6a and b). In starch and sucrose metabolic pathways, the compounds with higher significance were D-fructose, D-glucose-1-phosphate, D-glucose, sucrose, and maltose (Fig. 6a). Meanwhile, in glycine, serine and threonine
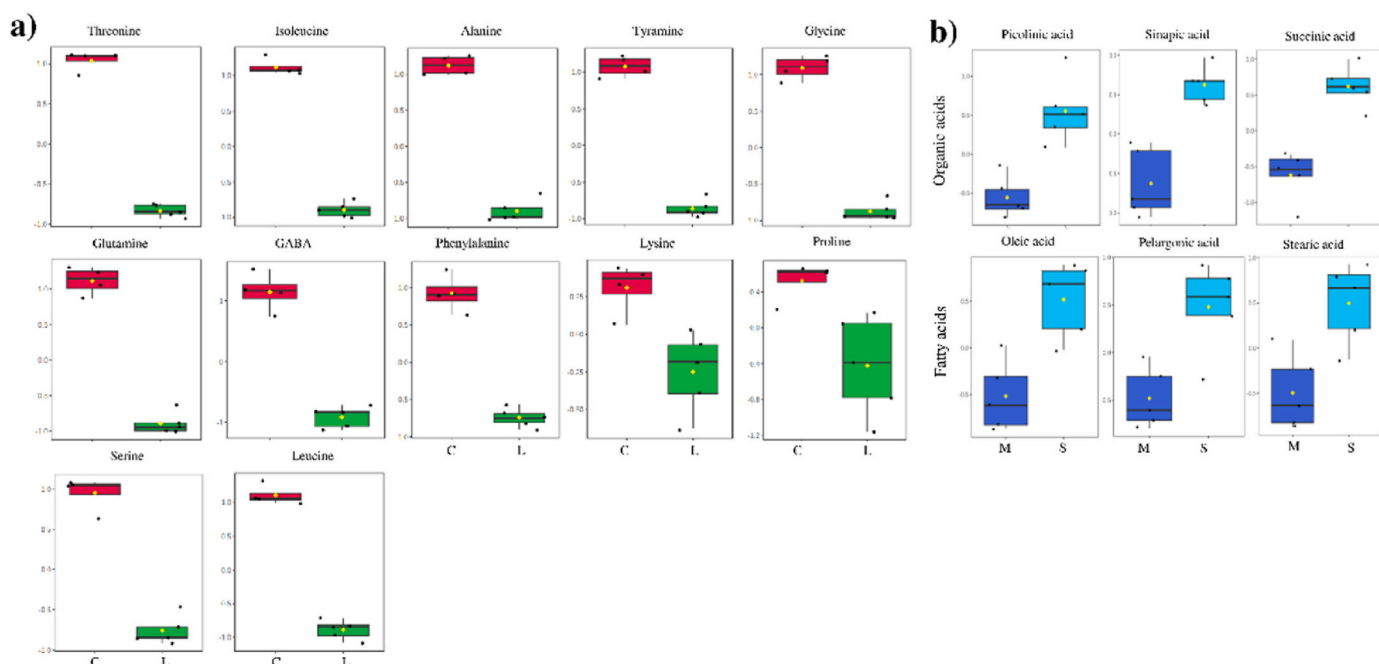

Fig. 5. a) Box plots reporting the amino acids with significant differences between *Carpobrotus* sp.pl plants from the cluster A-related populations: Cádiz (C, red colour) and A Lanzada (L, green colour). Normalised metabolomic data were analysed using Student's *t*-test ($p \leq 0.05$). N = 4 for Cádiz and N = 5 for A Lanzada. b) Box plots reporting the organic and fatty acids with significant differences between *Carpobrotus* sp.pl plants from the cluster B-related populations: La Marina (M, purple colour) and Samil (S, blue light colour). Normalised metabolomic data were analysed using Student's *t*-test ($p \leq 0.05$). N = 5.
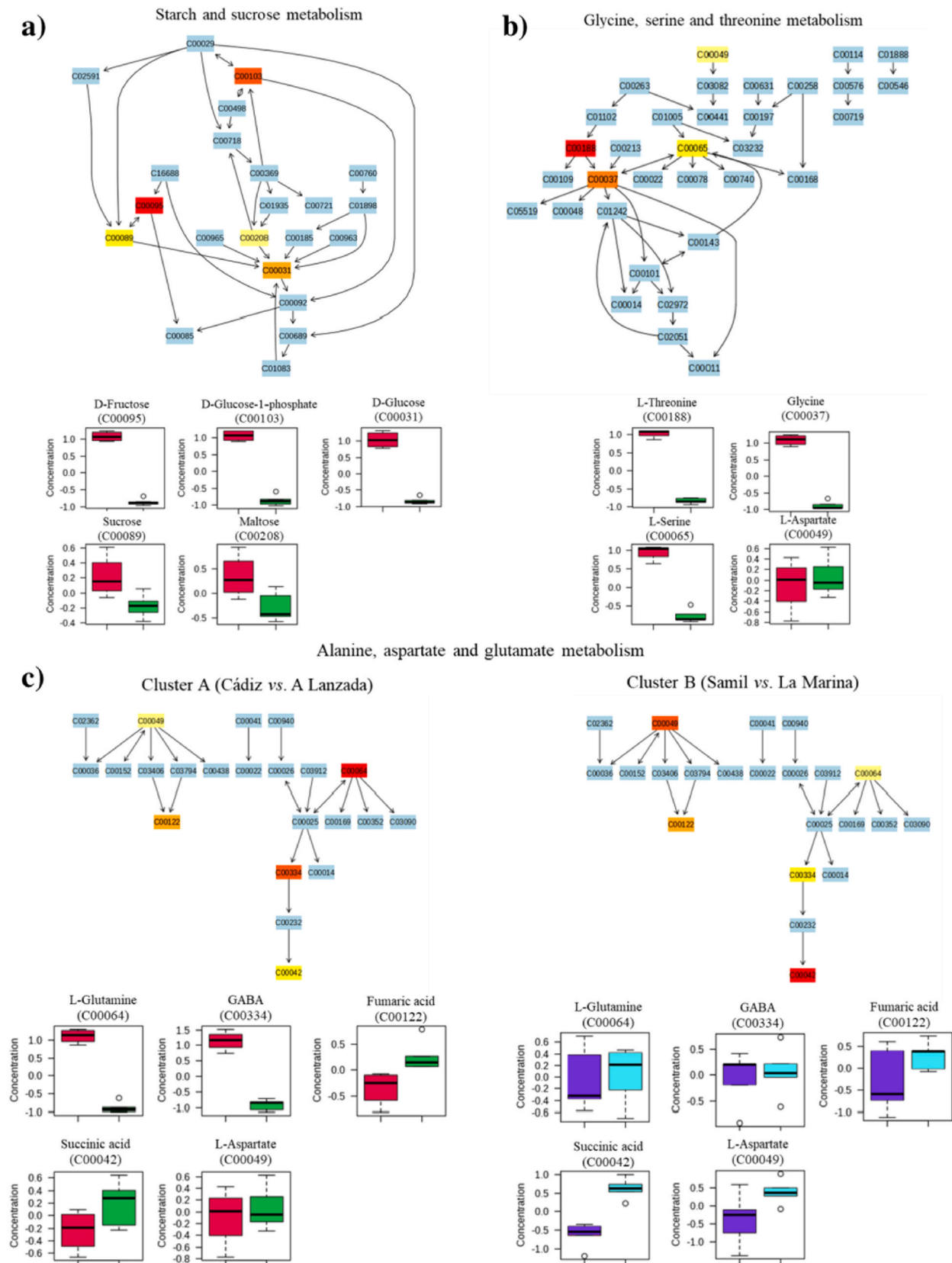
Fig. 6. Pathways with the higher impact scores (≥0.5) in the *Carpobrotus* sp.pl populations related to cluster A: Cádiz (red colour) and A Lanzada (green colour), and cluster B: La Marina (purple colour) and Samil (blue light colour). a) Starch and sucrose metabolism in cluster A; b) Glycine, serine and threonine metabolism in cluster B; c) Alanine, aspartate and glutamate metabolism in cluster A and cluster B. Regarding the compound colours within the pathway - light blue shows metabolites that are not in our plants but are used as background for enrichment analysis; other colours (varying from yellow to red) show metabolites found in our plants with different levels of significance.

pathways, the significant compounds were L-threonine, glycine, L-serine, and L-aspartate (Fig. 6b). These results aligned with the previous ones, emphasising the role of the difference in the content of amino acids in the populations related to cluster A (Fig. 6b). Nevertheless, the biogeographic region in cluster B affected 11 pathways, but only 7 had an impact score higher than 0.1. The route with the highest impact score was alanine, aspartate and glutamate metabolism (Fig. 6c), related to amino acid metabolism, with succinic acid (C00042) as the most significant metabolite. Table 4 includes the top 5 pathways according to the impact of cluster A and cluster B comparisons.

3.4.4. Comparative analysis of the populations in each biogeographic region

A comparative analysis was conducted on the populations collected from the two different biogeographic regions: Atlantic (A Lanzada and Samil), particularly in Pontevedra province, and Mediterranean (Cádiz and La Marina). The comparison involved paired *t*-test analyses to assess differences between each pair of populations.

The analysis of populations corresponding to the Atlantic region (A Lanzada and Samil), detected 114 altered metabolites (Fig. 4c). Among the affected compounds, 82 were found to be accumulated in significantly higher quantities in plants from A Lanzada, highlighting the presence of shikimic acid, 4-hydroxycinnamic acid, 6-hydroxynicotinic acid and quinic acid (Fig. 7a), which also exhibited the highest VIP scores (Supplementary material S1), indicating their significance in the differences between the Atlantic populations. A remarkable difference in amino acids content between populations was also evident, showing again A Lanzada plants the higher values of these compounds, especially of L-phenylalanine (Supplementary material S1). Conversely, just 37 metabolites, especially the sugars lactulose, maltose, glucoheptulose and sucrose, were significantly higher in Samil plants than in A Lanzada plants (Fig. 7b).

Relative to the Mediterranean region (Cádiz and La Marina), 117 metabolites showed significant differences between populations (Fig. 4c). In general, plants from Cádiz accumulated much more than La Marina, except for tagatose (Fig. 7c), which was accumulated more in plants from La Marina. Hexanoic acid, glucose-1-phosphate, meso-erythritol, 2′-deoxyadenosine and ribonic acid were the more significant metabolites in the differences between Mediterranean populations according to the *t*-test and the VIP scores (Fig. 7d and Supplementary Table S1). As expected, when comparing Cádiz, which corresponds to the population with the highest accumulation of metabolites, with La Marina, the population with the lowest accumulation, the differences were quite similar to the results found when the four populations were compared.

3.4.5. Pathway impact of the genetic cluster on the populations from each biogeographic region

A detailed analysis concerning the pathways altered by the genetic cluster (A or B) was carried out using the Metaboanalyst module "MetPa". Specifically, 35 pathways were affected between Atlantic populations due to the genetic cluster factor, with 13 pathways showing an impact higher than 0.2. The routes with the higher scores were starch and sucrose metabolism and glycine, serine and threonine metabolism, with impacts higher than 0.5. The most significant compound was D-glucose-1-phosphate (C00103), followed by maltose (C00208) (Supplementary material S1). Moreover, the genetic factor in the Mediterranean populations affected 33 pathways, and 12 had an impact score higher than 0.2. Starch and sucrose metabolism also showed the highest impact when comparing Mediterranean populations, but in this case, the more affected compounds were D-glucose-1-phosphate (C00103) and D-fructose. Table 5 includes the top 5 pathways according to their impact in the Atlantic and Mediterranean regions.

3.4.6. Comparative analysis of the genetic clusters (cluster A vs. cluster B)

A comparative analysis was conducted to identify the compounds more closely associated with a particular genetic cluster. The presence of specific compounds in the analysis could indicate that a population belonged to a particular cluster. The comparison involved paired *t*-test analyses to assess differences between the clusters.

The metabolic analysis of the clusters revealed a total of 98 altered metabolites. Among the affected compounds, 89 accumulated at significantly higher amounts in cluster A plants, with shikimic acid, quinic acid and cis-aconitic acid standing out (Fig. 7). The presence of the phenolic compound 4-hydroxycinnamic acid is also remarkable, as well as the presence of most essential amino acids. Conversely, just nine metabolites, highlighting the presence of sugars such as lactulose, maltose, alpha-lactose and glucoheptulose, were significantly higher in plants from cluster B (Fig. 8).

4. Discussion

Plant metabolism variation significantly influences plant development and adaptative response to the environment. Understanding the effect of genetic and environmental variables on metabolomic profiles is vital for metabolomics research applications. Its application includes the whole field of plant ecology, facilitating the comprehension of several processes, from community composition to genetic alterations in crops, as well as plant plasticity and adaptability (Brunetti et al., 2013). Nevertheless, relatively few studies of this nature have been conducted in non-model species and even fewer in invasive species.

In this study, our primary aim was to ascertain whether the different *Carpobrotus* sp.pl populations could be characterised by analysing their metabolomes and, if so, which compounds would contribute to this differentiation. The objective was to assess potential correlations between these metabolites and estimate the influence of both the genetic cluster and the climatic origin, as in Gomulkiewicz et al. (2010), where various adaptive mechanisms, including phenotypic plasticity and genetic adaptation, were analysed. Thus, our research aimed to discern variations in the metabolomic profiles among four different populations from two different genetic clusters and two different climatic conditions.

Table 4

Results from ingenuity pathway analysis with MetPa carried out on *Carpobrotus* sp.pl plants from four different populations corresponding to two genetic clusters (cluster A and cluster B). The table shows the 5 routes of each cluster with the highest impact (complete data in the *Supplementary Table S1*).

Pathways	Total Cmpd	Hits	Cluster A	Cluster B	Impact
			Raw *P*	Raw *P*	
Starch and sucrose metabolism	22	5	4.91E-07	//	0.60
Glycine, serine and threonine metabolism	33	4	1.63E-08	//	0.52
Isoquinoline alkaloid biosynthesis	6	2	7.02E-08	//	0.5
Alanine, aspartate and glutamate metabolism	22	5	2.48E-07	0.0146	0.45
Phenylalanine metabolism	12	1	3.59E-06	//	0.42
Galactose metabolism	27	9	1.12E-05	0.0103	0.42
Butanoate metabolism	17	2	6.38E-07	0.0006	0.14
Cutin, suberin and wax biosynthesis	18	1	2.36E-07	0.0030	0.13
Glycerolipid metabolism	21	2	3.16E-07	0.0092	0.12

Total Cmpd: the total number of compounds in the pathway; Hits: the matched number from the uploaded data; raw p: the original *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 were reported. The "//" indicates not significant differences because $P \geq 0.05$. The full list of the significantly altered pathways is available in Supplementary Table S1.
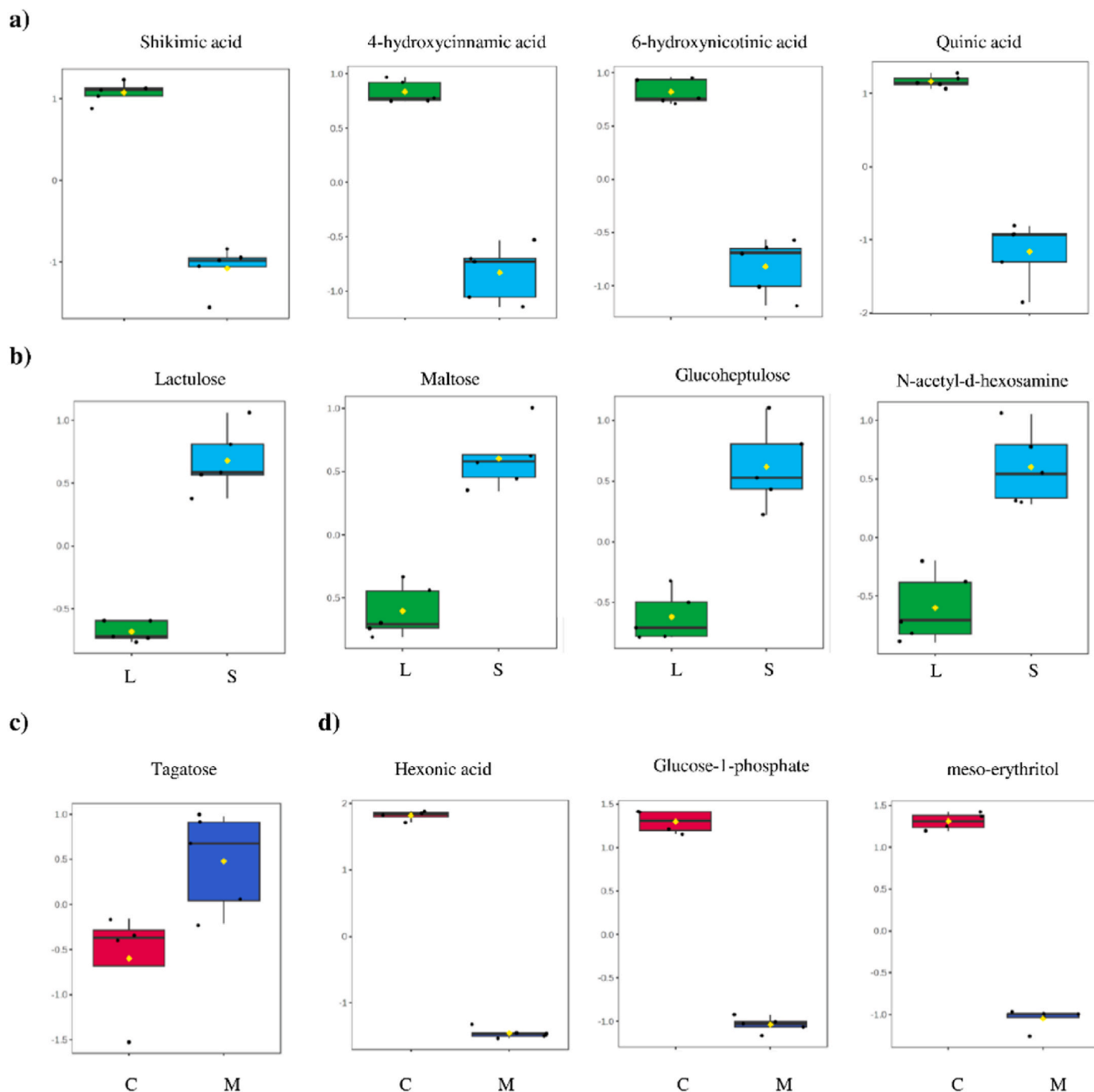
Fig. 7. a) Box plots reporting the compounds with the highest significance (*P*-value), accumulated in the populations of A Lanzada (a) and Samil (b). *Carpobrotus* sp. pl plants from A Lanzada (L) are represented with green colour and from Samil (S) with blue light colour. Normalised metabolomic data were analysed using Student's *t*-test (*p* ≤ 0.05). N = 5. b) Box plots reporting the only metabolite with higher accumulation in the population of La Marina compared to Cádiz (Tagatose) (a) and compounds, with the highest significance (*p*-value), accumulated in the populations of Cádiz (b). *Carpobrotus* sp.pl plants from Cádiz (C) are represented with red colour and from La Marina (M) with purple colour. Normalised metabolomic data were analysed through Student's *t*-test analysis (*p* ≤ 0.05). N = 5.

We focused on elucidating the relative influence of genetic and climatic factors on the observed differences among populations, discerning which variables have the greatest weight in shaping such distinctions.

Our study provides general evidence of a change in metabolomic, morphological traits and adaptation to environmental changes in populations of two clusters of *Carpobrotus* sp.pl. grown under different environmental conditions, including the Mediterranean and Atlantic biogeographic regions.

The shoots weight and length of the populations from the Mediterranean climate (populations from Cádiz and La Marina) were lower than

those from the Atlantic region, particularly when compared to the plants from Samil. The existence of water restrictions in the Mediterranean region may be the cause of these variations. Precipitation is vital for plant development, as increased precipitation improves water accessibility, ensuring the growth and elongation of plant roots, stems, and leaves during the seedling phase (Messier et al., 2010). In order to keep the balance between water supply and absorption, plants are likely to modify their morphological characteristics (Sun et al., 2022). These trait alterations in response to environmental conditions allow plants to adapt to a wider variety of environments, known as phenotypic

Table 5

Results from ingenuity pathway analysis with MetPa carried out on *Carpobrotus* sp.pl plants from four different populations corresponding to two biogeographic regions (Atlantic and Mediterranean). The table shows the four pathways of each cluster with the highest impact (complete data in Supplementary Table S1).

Pathways	Total Cmpd	Hits	Atlantic Region	Mediterranean Region	Impact
			Raw *P*	Raw *P*	
Starch and sucrose metabolism	22	5	1.20E-07	1.77E-06	0.60
Glycine, serine and threonine metabolism	33	4	4.59E-06	4.06E-05	0.52
Isoquinoline alkaloid biosynthesis	6	2	7.26E-05	8.56E-07	0.5
C5-Branched dibasic acid metabolism	6	1	0.00047	//	0.5
Alanine, aspartate and glutamate metabolism	22	5	1.38E-06	6.13E-07	0.45
Phenylalanine metabolism	12	1	2.56E-06	5.64E-06	0.42

Total Cmpd: the total number of compounds in the pathway; Hits: the matched number from the uploaded data; raw p: the original *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 were reported. The "//" indicates not significant differences because $p \geq 0.05$. The full list of the significantly altered pathways is available in Supplementary Table S1.

plasticity. While rainfall in the Atlantic region usually exceeds 1100 mm annually, the Mediterranean region did not reach more than 492 mm rainfall in Cádiz and 360 mm in La Marina in 2021, the year before the ramets were collected. Therefore, the La Marina region seems to be under more stressful conditions due to high temperatures and low precipitation, as evidenced by the lowest values of all the initial morphological parameters. Concerning growth parameters, populations did not differ much apart from root weight and water content. In particular, A Lanzada plants (Atlantic region) showed the highest values in root parameters. At the same time, Cádiz and La Marina (Mediterranean populations) revealed the lowest values in line with the parameters initially registered. It is already known (Xing et al., 2024) that variations in the precipitation pattern have a major effect on the growth of roots and their acquisition of resources. Under drought stress conditions, the root system can be as affected, or even more, than the aerial parts of the plant (Franco et al., 2011).

Regarding ion transport and accumulation, a continuous trend of higher concentrations in shoots was observed, which was inversely correlated with the root concentrations. The majority of dicotyledonous halophytes, especially tolerant succulent species, actively transport toxic ions – sodium and chlorides – from roots to shoots and store them in the leaf vacuoles to prevent harmful effects in the cytoplasm (Maathuis et al., 2014). The high levels of Na^+ and Cl^- in the four populations suggest the presence of active ion transport to the shoots under non-stressed salinity conditions, which could be related to salt tolerance, as previously found for other species such as *Aloe vera* L., or *Salicornia europaea* L. (Zheng et al., 2009). The storage parenchyma cells of *Carpobrotus rossi* (Haw.) Schwantes act as Na^+ sinks and have higher Na^+ sequestration with non-selective cation channels as the main pathway for Na^+ entry (Zeng et al., 2018), and is complemented by an intrinsically low slow vacuolar channel activity, which prevents the futile Na^+ cycle between the vacuole and the cytosol.

Concerning metabolomic analysis, a clear separation between populations was noted in the PCA and PLS-DA, also reflected in the heatmap built employing the 130 significantly altered compounds resulting from the ANOVA analysis. Plants from the Cádiz population generally manifested a notably higher accumulation of metabolites, followed by those from A Lanzada, both of these populations related to the genetic cluster A. Otherwise, plants from cluster B (Samil and La Marina) showed the lowest concentrations of compound accumulation without many differences when the four populations were compared.

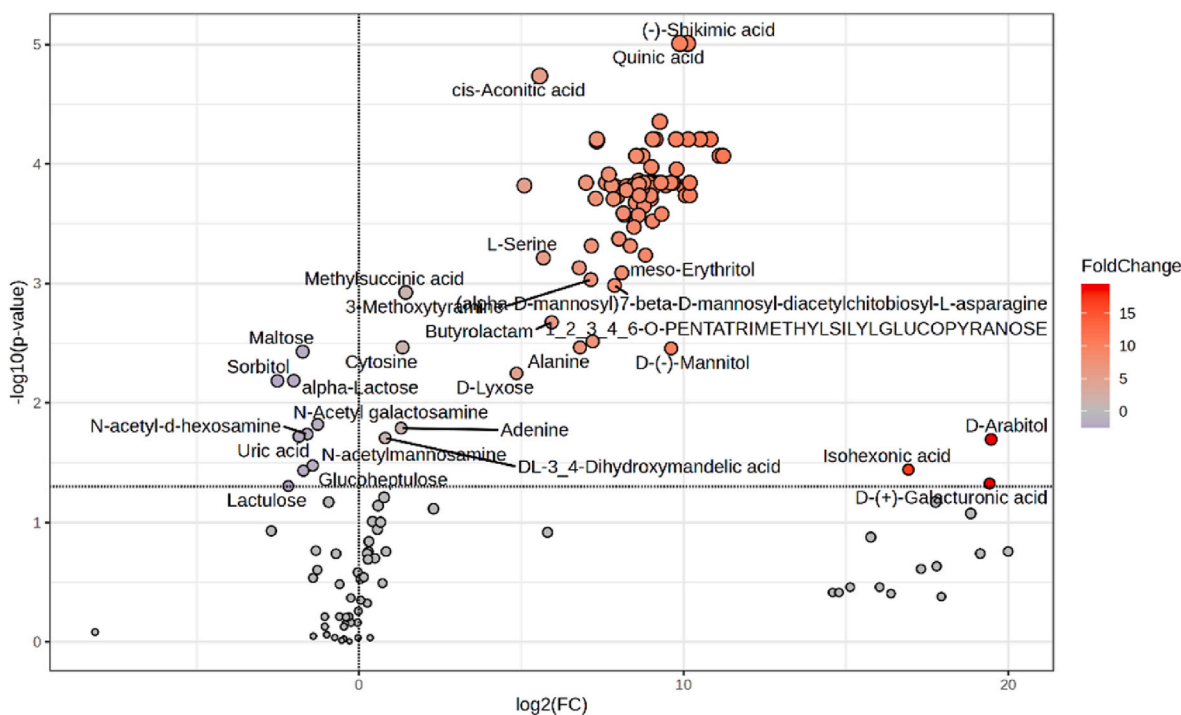The variation between the four populations was mainly based on the

Fig. 8. Volcano plot showing differential metabolites with a *P*-value of less than 0.05 and a Fold Change of greater than 1.5. between clusters. Upregulated-accumulated in cluster B- and downregulated-accumulated in cluster A-metabolites are in red and blue, respectively. Non-significant metabolites are represented by grey dots. N = 5.

accumulation of organic acids, fatty acids, phenolic compounds, sugars and sugar-alcohols (i.e., D-arabitol). Changes in the content of different organic acids have been related to drought tolerance due to stress conditions (Khan et al., 2020). The accumulation of phenolic compounds is related to the response of plants to abiotic and biotic stress due to their antioxidant properties (Kumar et al., 2020). Phenolic compounds, including sinapic acid, cacticin, hyperoside, 1,3-dicaffeoyl quinic acid, procyanidin, luteolin-7-O-beta-d-glucoside, rutin, epicatechin, isorhamnetin-3-O-rutinoside, chlorogenic acid, and myricetin were identified in *Carpobrotus edulis* (L.) N.E.Br. (Sabiu et al., 2021). There has been an increase in the biosynthesis of phenolic and polyphenolic compounds in plants to help them cope with a wide range of abiotic and biotic stresses. Related to this, Pereira et al. (2023) showed that despite the large number of polyphenols in *C. edulis*, they increased in spring and summer, the driest period in the Mediterranean climate. Carbohydrate accumulation is a strategic adaptation to maintain intracellular ion balance, avoiding excessive salt accumulation and protecting cellular structures from oxidative stress (Zaier et al., 2020). Concretely, elevated D-arabitol levels could reduce the cytoplasm's water potential without inhibiting the metabolism (Sadowsky et al., 2016). Carbohydrates have a seasonal distribution in plants, increasing in the invasive *C. edulis* during the dry season (Pereira et al., 2023). Thus, a higher carbohydrate concentration in spring and summer could be a strategy to cope with salt stress, which can increase during periods of higher temperatures and lower rainfall.

The comparative analysis by genetic cluster revealed different results depending on the analysed cluster, suggesting higher plasticity in cluster A than in cluster B populations. In fact, except for citric acid, all modified metabolites, a total of 111 compounds, in populations related to cluster A (Cádiz and A Lanzada) were more highly accumulated in the Mediterranean Cádiz plants than in the Atlantic A Lanzada plants. Citric acid is an intermediate of the mitochondrial tricarboxylic acid (TCA) cycle, which is commonly recognised to be responsible for the oxidation of respiratory substrate to drive ATP synthesis and contributes to biotic and abiotic stress responses (Zhang and Fernie, 2023). Plants from the Cádiz population showed a notable generalised increase in the content of amino acids with osmoprotective properties (i.e., threonine, L-isoleucine, alanine, and GABA, among others). In plants, amino acid metabolism plays an important role under abiotic stress conditions (Álvarez-Rodríguez et al., 2023). Nasir et al. (2010) confirmed an increase in free amino acid content in some halophytes under salinity stress in arid environment, as well as under drought conditions (Itam et al., 2020). High accumulation of amino acids, such as those found in this study, in particular branched-chain amino acids such as leucine and isoleucine, have already been determined to be associated with osmotic stress responses (Shim et al., 2023). In fact, in *Carpobrotus acinaciformis* L., protease activity gradually decreased with increasing NaCl concentrations, indicating protein depletion and a potential increase of free amino acids under stress conditions (Dikilitaş et al., 2019). These alterations in the levels of many amino acids were also reflected in the metabolic pathways analysis performed with these four *Carpobrotus* populations, where up to 5 pathways related to amino acid metabolism were altered (glycine, serine and threonine metabolism; alanine, aspartate and glutamate metabolism; phenylalanine metabolism; beta-alanine metabolism and arginine and proline metabolism). The differences between populations related to cluster A were also manifested by the high accumulation of some organic and fatty acids in plants from the Cádiz population, which was ten-fold higher than in A Lanzada plants for most of the compounds. Plant tolerance to salt and drought depends mainly on the inherent level of fatty acid unsaturation and/or the ability to maintain or adjust fatty acid unsaturation (Mikami and Murata, 2003). This aligns with the climatic conditions of the Cádiz populations. Nevertheless, starch and sucrose metabolism had a higher influence, with a special impact on D-fructose, D-glucose-1-phosphate and D-glucose as intermediary metabolites. They could interact with signalling molecules and other osmotic regulatory chemicals to modify

downstream reactions as occur in *Aquilegia vulgaris* under salt stress (Chen et al., 2023).

Otherwise, the comparative analysis between populations from cluster B revealed fewer differences due to climate conditions than in cluster A, as only 38 compounds were affected. Climate conditions factor in this genetic cluster had less influence than in the case of populations related to genetic cluster A, where the climate conditions caused differences in a higher number of metabolites related to stress and adaptation. The main difference between populations was found in the accumulation of phosphate, an inorganic anion. The absorption of phosphate by the plant could have been done in their natural habitat. It was confirmed by the analysis of the soil from the four populations, as soil from Samil contained ten times more phosphates than in the rest of the populations, with 88 mg kg^{-1} in Samil in front of the 8 mg kg^{-1} of average in the other three populations. The results also showed the influence of the significative accumulation of determinate metabolites in plants from Samil. This is the case of caffeic acid, which is actively involved in plant physiology and the primary stress tolerance mechanisms used by plants for the synthesis of lignin, responsible for cell wall thickening, making plants resistant to sodium and heavy metal stress, and helping in the absorption of high light energy in mesophyll cells during drought stress. (Riaz et al., 2019). Succinic acid, another accumulated compound in this study, is a metabolite that can promote plant growth (Yoshikawa et al., 1993) and also plays a crucial role in some altered pathways (alanine, aspartate and glutamate metabolism; butanoate metabolism and sulphur metabolism). The increment of succinic acid evidenced the alteration of alanine, aspartate and glutamate metabolism but also of L-aspartate, which serves as a precursor through different metabolic pathways for the biosynthesis of other amino acids, such as lysine (Jander and Joshi, 2009).

Moreover, the change in 114 compounds showed the differences between the Atlantic populations when the populations were compared from another point of view: the biogeographic/climatic zones. Plants from cluster A (A Lanzada) accumulated organic acids such as shikimic acid, a natural compound in various plants that plays a key role as an intermediary in the biosynthesis of aromatic amino acids, lignin and alkaloids (Bochkov et al., 2012). Phenolic compounds known for their antioxidant properties, like 4-hydroxycinnamic acid, also contributed to the population differentiation. Remarkable is also the accumulation of some amino acids in plants from A Lanzada, particularly L-phenylalanine, which has a crucial role in protein synthesis, is a precursor of other compounds and is involved in plant defence and photosynthetic activity (Kumari et al., 2023). Concerning the metabolites predominantly accumulated in Samil plants (cluster B) sugars notably stand out. Sugars (i.e., lactulose, maltose, and sucrose, among others) in plants have diverse functions such as sources of energy, precursors of various metabolomics pathways, osmoprotective compounds or ROS scavengers (Saddhe et al., 2021), and also play a crucial role in the most impacted pathway, starch and sucrose metabolisms.

Finally, 117 metabolites showed significant demographic differences concerning the Mediterranean region. Except for tagatose, which was accumulated more in plants from La Marina, plants from the Cádiz population generally accumulated more than those from La Marina. Tagatose is a rare sugar that has beneficial effects on plants, controlling a wide range of plant diseases (Mochizuki et al., 2020). There are no metabolites to highlight in reference to the accumulation in plants from Cádiz population because the results are similar to the general comparison.

According to the metabolic profile, the populations were more similar within the genetic cluster than the biogeographic region of origin. Consequently, a comparative analysis was carried out to determine the most representative metabolites of each genetic cluster. In a general overview, individuals belonging to Cluster A tend to accumulate a higher proportion of most of the metabolites identified, particularly those from Cádiz. At the same time, the concentration of most of the compounds in Cluster B populations is much lower, sometimes

unnoticeable. Specifically, organic acids such as shikimic acid and quinic acid, phenolic compounds like 4-hydroxycinnamic acid, hydroquinone, and 3-hydroxybenzoate, as well as essential amino acids, except lysine, are strongly associated with Cluster A. Conversely, the presence of sugars like alpha-lactose, maltose, and lactulose in the analysis could indicate the affiliation of the analysed population to Cluster B.

5. Conclusions

Plants have an intricate and complex system of metabolic pathways responsible for their survival. While specialised metabolism helps plants adapt once primary metabolism is stable, they typically increase specialised metabolites in response to environmental stress. In this study we find that most of the compounds of the cluster A increase in water-limiting climates, but we provide examples of specific specialised metabolites that either decrease or do not change. Conversely, cluster B maintains stable metabolite levels, with only a few compounds adjusting to environmental changes. This suggests differential metabolic pathway expression between clusters. Our results indicate, for the first time, that *Carpobrotus* cluster A show more plasticity against environmental conditions than cluster B. Plants from genetic cluster B, La Marina and Samil, despite their different origins, are more similar than plants from the same climatic regions or plants from other genetic clusters, as cluster A. These results point out that the influence of genetics in this case is higher than the environmental conditions. However, the results also highlight the strong plasticity of the invasive species *Carpobrotus* sp. to survive under different climatic conditions. Investigating metabolomic regulation in plants is crucial for understanding their adaptation and acclimation processes. Differences in metabolite profiles between plants of the same species but from different origins can shed light on how plants respond to different environmental conditions. This study can form the basis for upcoming metabolomic-based resistance testing to abiotic stress situations.

Data availability statement

The raw data have been deposited at Zenodo webpage https://zenodo.org/communities/(accessed on March 20, 2024), with the following DOI: https://doi.org/10.5281/zenodo.10845148 (accessed on March 20, 2024), as per the rules and regulation.

Author contributions

S.G-O, L.G, A.M.S-M. Conceptualization and Methodology. S.G-O, D.L-G, F.A. Formal analysis and Investigation. L.G, A.M.S-M Resources. S.G-O Data curation and Writing - original draft. S.G-O, F.A, L.G, A.M.S-M. Writing - review & editing. All the authors read and approved the manuscript.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109066.

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