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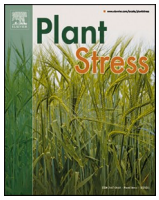

Water and nutrient availability modulate the salinity stress response in *Olea europaea* cv. Arbequina

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ABSTRACT

Salinity stress represents a key factor for global agriculture. Plants can respond to salinity stress by adapting their physiology in different ways with the aim of limiting reductions in growth and development. Importantly, moisture retention capacity, permeability and nutrient availability of substrates represent critical variables for plants as they may further influence the effect of osmotic stress. Here, a multidisciplinary approach was applied to evaluate the role of two different substrates, peat and perlite, on 2-year-old potted cuttings of *Olea europaea* (cultivar Arbequina) under different salinity stress conditions (0, 100 and 200 mM NaCl). Biometric and physiological data indicate that plants potted in perlite (AP) generally present lower growth and photosynthetic rates when compared with peat (AS) in combination with salinity stress. Ion measurements indicate a rise in Na^+ accumulation with increasing stress severity, which alters the ion ratio in both substrates. In addition, differences occurred in polyphenol contents, with a general increase in quinic acid and rutin contents in AS and AP samples, respectively. Metabolomic and biometric data were also coupled with metabarcoding analysis, which indicates that the moderate salinity treatment (100 mM NaCl, T100) reshaped the endophytic community of plants grown on both substrates. Taken together, the data suggest that the strategy used by a glycophytic species such as the olive tree to cope with salinity stress seems to be highly related to availability of water and nutrients. The lack of both may be simulated by perlite, enhancing the effect of salinity stress response in woody plants. Lastly, applying the beneficial endophytic bacterial taxa identified here could represent a step forward in increasing plant defence and nutrient uptake and reducing inputs for modern and more sustainable agriculture.

Introduction

Salinity stress is emerging as a pervasive challenge in modern agriculture, imperiling productivity and sustainability of food production around the world. Approximately 1.125 billion hectares of agricultural land and >52 % (4.03 billion) of the population are affected by salinity (Raza et al., 2023). In the Mediterranean area, soil salinity represents a problem for crop production in the Caspian Basin, Ukraine, the Carpathian Basin, the Iberian Peninsula (Tóth et al., 2008), and southern Europe (Daliakopoulos et al., 2016; Vittori Antisari et al., 2020). Saline irrigation is a practice employed with greater frequency in olive tree-growing regions throughout various countries of the

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Mediterranean, where water shortage is one of the major barriers to sustainable agriculture. Countries like Israel, Spain and Tunisia have also reported using saline irrigation, especially in arid and semi-arid areas with few freshwater resources. For example, in Israel, saline water is applied in new olive orchards in the arid part of Negev, allowing for the growing of olive trees on poor soils not meant for agriculture and demonstrating the moderate salinity tolerance of the olive tree (Kapulnik et al., 2010; Levin et al., 2007). Likewise, in Spain, olive production is favoured in the coastal zones where saline waters are normally employed, mainly due to the low water needs of the trees and their long drought tolerance (Loreto et al., 2003). In Tunisia, where water resources are extremely limited, olive trees are often irrigated with brackish water, especially in coastal and arid regions (Boussadia et al., 2023). Research suggests that saline water may enhance growth and yield within a defined salinity range, with horticultural studies showing that irrigating with water with a conductivity of 4.2 dS m^{-1} may improve the horticultural attributes of the olive cultivar 'Barnea' (Weissbein et al., 2008). The scarcity of water for irrigation is becoming even more acute due to climate change and leading to an increase in the saline lands with a lack of water for irrigation. Therefore, the amount of soil used for olive orchards characterized by low-quality water for irrigation is increasing (Hassan et al., 2020), which is also a consequence of the current climate change (Brito et al., 2019).

Plants can develop strategies to counteract the effect of salinity stress that can be characterized into two main groups: salt-sensitive glycophytes (Himabindu et al., 2016) and salt-tolerant halophytes (Munns and Tester, 2008). Halophytes differ from glycophytes partly in their adaptation through complex mechanisms to avoid salt damage (Tester and Davenport, 2003), including the development of strategies to integrate salinity perception and stress signalling with endogenous developmental cues (Vita et al., 2021). These adaptations can be summarized as strategies by which plants counteract salinity stress: tolerance to osmotic stress, which results in increased leaf area; Na^+ exclusion from roots and leaf blades; and tissue tolerance to accumulated Na^+ or, in some species, Cl^- (Munns and Tester, 2008) and confers the ability to complete their life cycle under saline conditions (Bazihizina et al., 2022), like consistent amounts of salt (>100 mM NaCl) in the rhizosphere (Flowers and Colmer, 2008, 2015). Based on this set of physiological mechanisms, woody plants respond in a similar physiological manner to non-woody plants when facing salinity stress (Llanes et al., 2021). However, an extended juvenile development phase and secondary growth in woody plants increases the transport capacity due to greater stem thickness (Liesche et al., 2017). The extended length of time and cost needed to obtain fruit yields means that woody crop tolerances have been mainly determined only for the vegetative growth phase (Maas and Grattan, 2015). Though most fruit trees and nut crops are considered salt sensitive, the salt tolerance definition in woody crops is complicated because of additional detrimental effects caused by specific ion toxicities. In contrast, olive and a few other species are generally thought to be salt-tolerant species (Gucci and Tattini, 2010; Maas and Grattan, 2015).

Olive is a long-living, evergreen, historically significant sclerophyll plant of the Mediterranean basin which dominated the corresponding rural economy and landscape for about six million years (Besnard et al., 2018; Diez et al., 2015). Olive trees growing in saline soils show reduced growth, shortened internodes, small leaves with thickened mesophyll and cell wall, and reduced blooming, pollen germinability, and lower number of fruits (Gucci et al., 1997). The main symptoms of salt stress in plants are chlorosis and necrosis of leaves, desiccation of flowers and new shoots, and leaf abscission after a long period of stress (Rugini et al., 2016). Accordingly, the premature drop of leaves may be the last defense mechanism against high salt concentrations, simultaneously reducing accumulations of toxic ions by dropping old leaves and the transpiration rate of the whole plant. Leaf drop occurs following the accumulation of toxic ions like Na and Cl, advancing from the bottom to the top (Loupassaki et al., 2002). Several studies have shown that the ability of olive trees to respond to high salt concentrations is closely linked with effective mechanisms of ion exclusion and retention by the root system (Chartzoulakis et al., 2002; Tattini et al., 2008). Considering the ion imbalance due to salinity stress, in olive it has been demonstrated that calcium plays a key role as a signalling ion (Sodini et al., 2022), and Na mobilization results in an ion imbalance (Gucci et al., 1997; Sodini et al., 2023). An ion imbalance leads to the activation of specific genes, including antiporters like *SOS1* (Sodini et al., 2023), *NHX* exchanger (Rossi et al., 2016) or genes involved in the phenylpropanoid pathway, including phenylalanine ammonia-lyase (PAL) (Rossi et al., 2016). However, plant responses to salinity stress are also linked to the substrate. In the work of Tavakkoli et al. (2010), authors determined that the severity of stress in barley (*Hordeum vulgare* L.) was different in soil and hydroponic systems since the soil mitigates the effect of stress according to the cation exchange capacity of soil colloid surfaces. This exchange capacity provides time for the plant to counteract stress. Since the capacity of the olive tree to mitigate the effect of stress is strongly associated with root capacity for sodium exclusion, inert substrates like perlite and vermiculite may be used experimentally, to test the effect of cation exchange capacity on tolerance to salinity stress (Lambardi et al., 2023) without any buffering effect due to soil properties or the presence of organic amendments (Ondrasek et al., 2022). Perlite as a substrate ensures good porosity, air and gas exchanges for plant roots, as well as water and nutrient holding capacity (Mahjoor et al., 2016). The present study aims to analyze the role of the substrate in mitigating the negative effects of salinity stress in olive *cv*. Arbequina by altering water and nutrient availability. This Spanish cultivar is considered a highly adaptable cultivar to different types of soil and environments (Ruiz et al., 2011) due of its low vigour (Farinelli and Tombesi, 2015), thus rendering it a good candidate for super high-density olive crops system (SHD) with a medium tolerance to salinity stress (Mousavi et al., 2019; Weissbein et al., 2008). Therefore, olive plants grown on two different substrates, peat and perlite, were tested and compared under salinity stress conditions with an integrated approach using biometric and physiological measurements combined with biochemical and metabolomics analyses. Additionally, the endophytic bacterial community was assessed to verify the impact of both substrate and stress on the plant-associated microbiota and to evaluate the role of the plant-microbiota interaction in the substrate-mediated response to salinity stress.

Materials and methods

Experimental setup

Two-year-old, self-rooted cuttings of the Arbequina **AS1®** (henceforth called Arbequina) olive cultivar was used in the present experiment, purchased from Agromillora (Sant Sadurní d'Anoia, Barcelona, Spain). Forty-five plants were used (n = 15 *per* substrate and salt treatment combination) to test each of the two substrates, peat (AS) and perlite (AP), for a total of ninety plants. The plants were grown in a greenhouse at the University of Florence, Italy (latitude 43°48′58.6″ N, longitude 11°11′58.1″ E) from June to September 2019 under semi-controlled conditions. Experimental conditions included natural, non-supplemented lighting (average 500 μmol m^{-2} s^{-1} PAR), a mean air temperature of 28 °C (maximum 34.5 °C, minimum 24.9 °C), a mean air humidity of 46 % (maximum 60.5 %, minimum 34.4 %), and an average photoperiod of 15:9 (L:D). The plants were watered using a circulating bench sub-irrigation system, which filled the benches to half the height of the pots (4 cm) in 5 min, with outflow occurring after 15 min. Following a one-month acclimation period after transplanting, different NaCl concentrations were applied: 0 mM (control), 100 mM, and 200 mM NaCl, using a half-strength Hoagland solution for nutrient supply (Hoagland and Arnon, 1950) in two different substrates, perlite (AP) and peat plus walnut fiber substrate (AS). These substrates are usually used in combination in olive tree nurseries. Perlite is a common substrate

employed for olive tree propagation in nurseries, characterized by high oxygenation and permeability. It is a naturally occurring, inorganic material that is lightweight and used for aeration and drainage. In contrast, peat is known for its moisture retention, providing a nutrient source and creating an ideal environment for root growth. The organic matter content of peat is crucial for enhancing nutrient exchange, which is essential for maintaining plant health (Fields et al., 2014). Unlike other materials, peat enhances the organic content of the substrate, thereby improving nutrient exchange. Moreover, peat serves as a nutrient source, although the nutrient content can vary significantly depending on the type of peat. Blond peat, often used in horticulture, is characterized by its relatively low nutrient content due to its less decomposed state than darker, more decomposed peat types. These two substrates were used singly here in order to determine the effects of salt stress without the interference of the complex mechanisms present with most common cultivated soils. By using an inert substrate such as perlite, the effects of organic matter on ion exchange capacity are reduced, with the limited ability of this substrate to regulate the availability of nutrients for uptake and assimilation by plants. To avoid osmotic shock, NaCl concentrations were increased gradually by adding 50 mM NaCl every two days until the final concentrations were reached (Ben Abdallah et al., 2021; Palm et al., 2024). The electrical conductivity (EC) and pH value of the solution reservoir were checked weekly with a portable conductivity meter and adjusted with HNO_3. The set values for EC/pH were 1.54 mS cm^{-1}/6.8 for 0 mM NaCl (control), 10.44 mS cm^{-1}/6.8 for 100 mM NaCl, and 19.07 mS cm^{-1}/6.8 for 200 mM NaCl.

Biometrics and physiological measurements

Ten plants of each treatment were dissected into roots, stems, and leaves, and their fresh weights (FW) were recorded immediately at the end of the experiment. The roots underwent a thorough wash to eliminate any residual substrate, and the tissues dried at 70 °C until constant weight to determine the dry weight (DW),

Gas exchange measurements were conducted using a portable LI-COR 6400XT instrument from LI-COR Inc. (Lincoln, NE, USA) following the same settings described by Palm et al. (2024). All the replicates collected during the analysis were grouped in a single dataset to determine the overall effects of salt on each cultivar.

Total phenolic compounds (TPC), total flavonoids (TFL) and malondialdehyde (MDA) were analyzed following a sequential extraction protocol and colorimetric measurements according to López-Hidalgo et al. (2021).

Ferric reducing antioxidant power

The ferric-reducing antioxidant power (FRAP) was determined according to Benzie et al. (1996) in triplicate ($n = 3$), and the values were expressed as Trolox equivalent antioxidant capacity (TE, μM Trolox equivalents per mg g^{-1} DW of dry plant).

Analysis of Na^+ and K^+content in the biomass

Na^+, K^+, and Ca^{2+} contents of the roots, stems, leaves were determined using an extraction method previously outlined in Guidi Nissim et al. (2021). Digested samples were diluted to a final volume of 50 mL using Milli-Q water and then analysed using a Flame Photometer Digiflame2000 DV 704 (Lab Services SAS, Rome, Italy), according to Palm et al. 2024.

LC-DAD-ESI-TOF-MS analysis of leaf metabolite extracts

Twenty leaves from the midsection of the primary axis were collected and pulverised in liquid nitrogen using a pestle and mortar. Subsequently, 0.5 g of the ground leaves were weighed and placed in a 15 mL bag (BIOREBA, Reinach, Switzerland) and proceeded as reported by Vergine et al. (2022). This process was replicated three times for each leaf sample collected after eight weeks from the beginning of the experiment.

Leaf extracts were analysed through LC-DAD-ESI-TOF-MS to characterize and quantify metabolites using an Agilent 1200 HPLC DAD ESI/MS-TOF system (Agilent Technologies, Palo Alto, CA, USA) equipped with a standard autosampler and an analytical column Agilent Zorbax extended C18 (5 × 2.1 cm, 1.8 μm), as previously reported by Palm et al. 2024. The HPLC system was coupled to an Agilent diode-array detector. Agilent 6320 TOF mass spectrometer was used, equipped with a dual ESI interface (Agilent Technologies). Compound detection was performed within a mass range of 50–1700 *m/z*. The accurate mass data of the molecular ions were processed through the software Mass Hunter (Agilent Technologies). The limit of quantification (LOQ) was determined as the signal-to-noise ratio of 10.1, and the limit of detection (LOD) was established as a 3:1 ratio.

Chlorophyll and proline analysis

Spectrophotometric analyses were carried out on 10 mg of fresh olive leaves collected from six plants ($n = 6$) for each treatment. The leaves were taken from the midpoint of the central leaf axis, frozen in liquid nitrogen, and then finely ground to a powder. Subsequently, 1 mL of cold methanol was added to the ground leaves, followed by shaking for 30 min and centrifugation at 10,000 x *g* for 10 min. The resulting supernatant was utilized for absorbance readings at 665, 652, and 470 nm to determine the levels of Chlorophyll a (Chl*a*), b (Chl*b*), and carotenoids, respectively. Absorbance readings were performed using a TECAN spectrophotometer with a 96 black multi-plate reader. Pigment quantification was conducted based on the equations provided by Wellburn (1994).

Proline concentrations were determined from 200 mg of fresh leaves collected ($n = 5$) and frozen with liquid nitrogen. 1.5 mL of 3 % w/v sulfosalicylic acid was added, and the samples vortexed and centrifuged at 14,000 x g for 15 min. The resulting supernatant was collected in glass tubes. The reaction mixture, consisting of 1.25 g ninhydrin, 30 mL glacial acetic acid, and 20 mL of 6 M phosphoric acid, was added to the extracted supernatant. Samples were incubated at 100 °C for 1 h, until their color changed to red-violet, depending on proline concentration; the supernatants were then cooled on ice to arrest the reaction. The samples were then extracted with 1 mL of toluene, vortexed to facilitate the separation of organic and water phases, and analysed using a Biorad SmartSpec Plus spectrophotometer (BioRad, Petaluma, CA, USA), with absorbance readings taken at 520 nm. Toluene was used as a blank. The calibration curve was established using a standard compound at various concentrations, and the proline concentration relative to fresh weight was calculated as outlined in Bates et al. (1973).

DNA extraction and sequencing – metabarcoding analysis

Extraction of DNA and subsequent metabarcoding analysis was undertaken on leaves collected at both the initial (T0) and concluding stages of the experiment (control T1 and salt-treated T100, T200), according to Vita et al. (2022). Roughly 1 gram of leaves was placed in BIOREBA extraction bags (Switzerland), to which 4 ml of Tris–HCl--based extraction buffer (0.2 M Tris–HCl pH 9, 0.4 M LiCl, and 25 mM EDTA) was added. Samples were homogenised using a semi-automatic homogeniser, following the procedure detailed by Vergine et al. (2019). Subsequently, DNA was extracted using a phenol-chloroform-isoamyl alcohol solution (25:24:1 ratio) following the protocol outlined by Edwards et al. (1991) with slight modifications.

The quantified DNA served as a template for PCR amplification using CS1–341F and CS2–806R primers targeting the V3-V4 variable regions of the 16S rDNA gene (Caporaso et al., 2011; Klindworth et al., 2013). A mixture of PNA (peptide nucleotide acid) blocker oligos (PNA Bio Inc., USA) was incorporated to enhance sequencing accuracy and prevent the

amplification of chloroplast and mitochondria sequences (Sillo et al., 2022; Vergine et al., 2024). Paired-end reads were obtained for each sample under each condition, resulting in 48 libraries: 12 for T0 (control) and 36 for T1 (control, T100, and T200). Sequencing was conducted on an Illumina MiSeq platform (v3 chemistry) at the Génome Québec Innovation Center, McGill University (Montréal, Canada).

QIIME2-pipeline

Initial analysis of paired-end sequences encompassing the V3–V4 regions of bacterial 16S rRNA was conducted using Quantitative Insights Into Microbial Ecology 2 (QIIME2) v. 2021.4 as previously described (Vita et al., 2022). Removal of adapter sequences was achieved through cutadapt (Leinonen et al., 2011), and sequences were truncated at 280 bases (forward library) and 200 bases (reverse library) from the start. Dada2 tool (Callahan et al., 2016), which is integrated into QIIME2, was applied for sequences denoising. The clustering process employed VSEARCH cluster-features-de-novo (–p-perc-identity 0.99) (Rognes et al., 2016) and sequence classification was assessed using a pre-trained naïve Bayes classifier (silva-138–99-nb-classifier.qza) with the sklearn feature classifier method (Pedregosa et al., 2011). To refine the dataset and remove contaminants, chloroplast and mitochondria sequences were filtered out from the resulting Operational Taxonomic Units (OTU) table using the "filter-features" parameter in QIIME2.

The output from QIIME2, including the amplicon sequence variance (ASV) table, taxonomy, metadata file, and tree, was converted into a . biom file and used in Microbiome Analyst (Chong et al., 2020) for the visualisation and statistical univariate and multivariate analysis. Data filtering criteria were assessed for eliminating low-quality and non-informative features; minimum count of features was set at "10″ (representing 10 % prevalence in samples), and 10 % of features with low variance in samples were removed based on the Interquartile Range (IQR). Total Sum Scaling method was utilised for data scaling. Statistical assessment of differences in taxa abundance among samples at the feature level utilised Shannon, Simpson, and Chao1 indexes for α-diversity and PermANOVA (100 permutations) for β-diversity, with an adjusted p-value cutoff (FDR) set at 0.05.

METAGENassist (Arndt et al., 2012) was then utilised to predict functions (metabolism) from metagenomic data. Before analysis, the data were filtered based on Interquartile Range (IQR) and normalised (mean-centered and divided by the standard deviation of each variable). Functions were associated with identified taxa, and differences were determined by ANOVA, with Tukey HSD as a *post-hoc* test. The 16S libraries are accessible at https://doi.org/10.6084/m9.figshare.25048388.

Statistical analysis

The data from biometric, photosynthetic, ion, pigments and polyphenolic analyses were assessed for normal distribution through a Shapiro Wilk test, and then analyzed using two-way ANOVA approach followed by Tukey's honestly significant difference (Tukey's-HSD) *post hoc* test ($p \leq 0.05$) within each substrate (peat, perlite).

Results

The present study was performed on one olive cultivar plant, Arbequina **AS1**®, grown on two substrates, peat (AS) and perlite (AP), which were exposed to control and two salt stress conditions (0 mM, 100 mM and 200 mM NaCl, respectively) according to the experimental scheme summarized in Fig. 1.

Morphological and physiological parameters

Biometric data

Variations in salinity treatments had no significant impact on the overall fresh weight of plants cultivated in two different substrates (Fig. 2). Nevertheless, plants grown in peat (AS) demonstrated a higher total fresh weight compared with those cultivated in perlite (Fig. S1–2). It was observed that using peat as the substrate reduced the inhibition of shoot growth due to increasing salinity. In AP, a general decrease in the fresh weight of leaves was observed for both treatments, compared to the corresponding control (Fig. 2C); in contrast, a significant reduction in AS was observed only with the highest treatment. Additionally, in AS, the leaf fresh weight was higher, although not statistically significant, than in AP across the diverse treatments, except for 200 mM of NaCl (Fig. 2C). Diverse salt irrigation had no effect on the development of stems and roots in either substrate (Fig. 2E and G). However, AS exhibited a greater stem fresh weight compared to AP in all treatments (Fig. 2E), while, except for the treatment with 100 mM of NaCl, no significant difference in root fresh weight was observed between AS and AP plants (Fig. 2G).

According to the two-way ANOVA results, salt treatments had a negative impact on total dry weights in AS and AP plants, particularly with 200 mM NaCl (Fig. 2B). As with the fresh weights of shoot tissues, the total dry weight and leaf dry weight was higher in AS plants across all three treatments. However, in AS plants, total and leaf dry weights decreased significantly in both 100 mM and 200 mM salt treatments relative to control. No significant changes were observed in AP plants in response to increasing salt concentrations (Fig. 2B and D). Although AS plants in control and 100 mM NaCl conditions had higher dry weight than AP plants, no differences between the two cultivars were observed

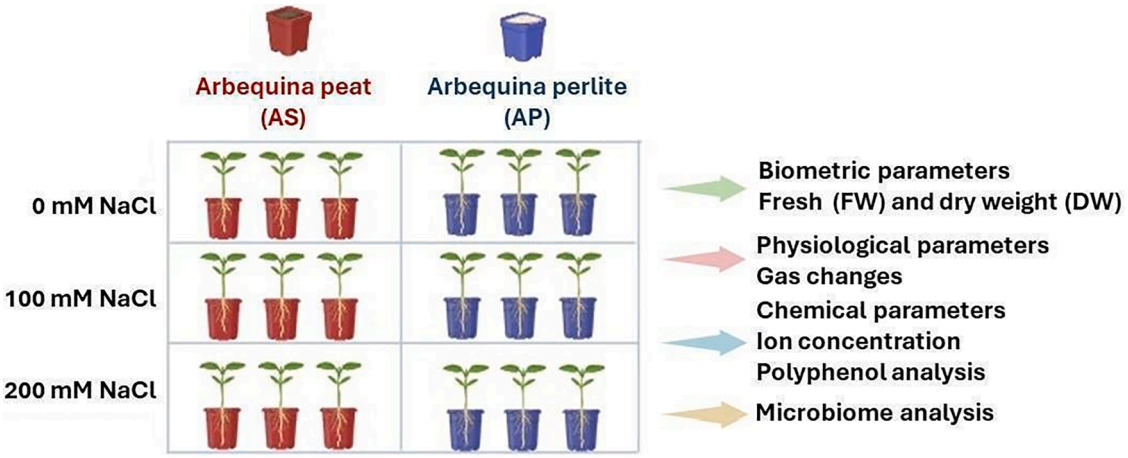

Fig. 1. Experimental setup scheme.

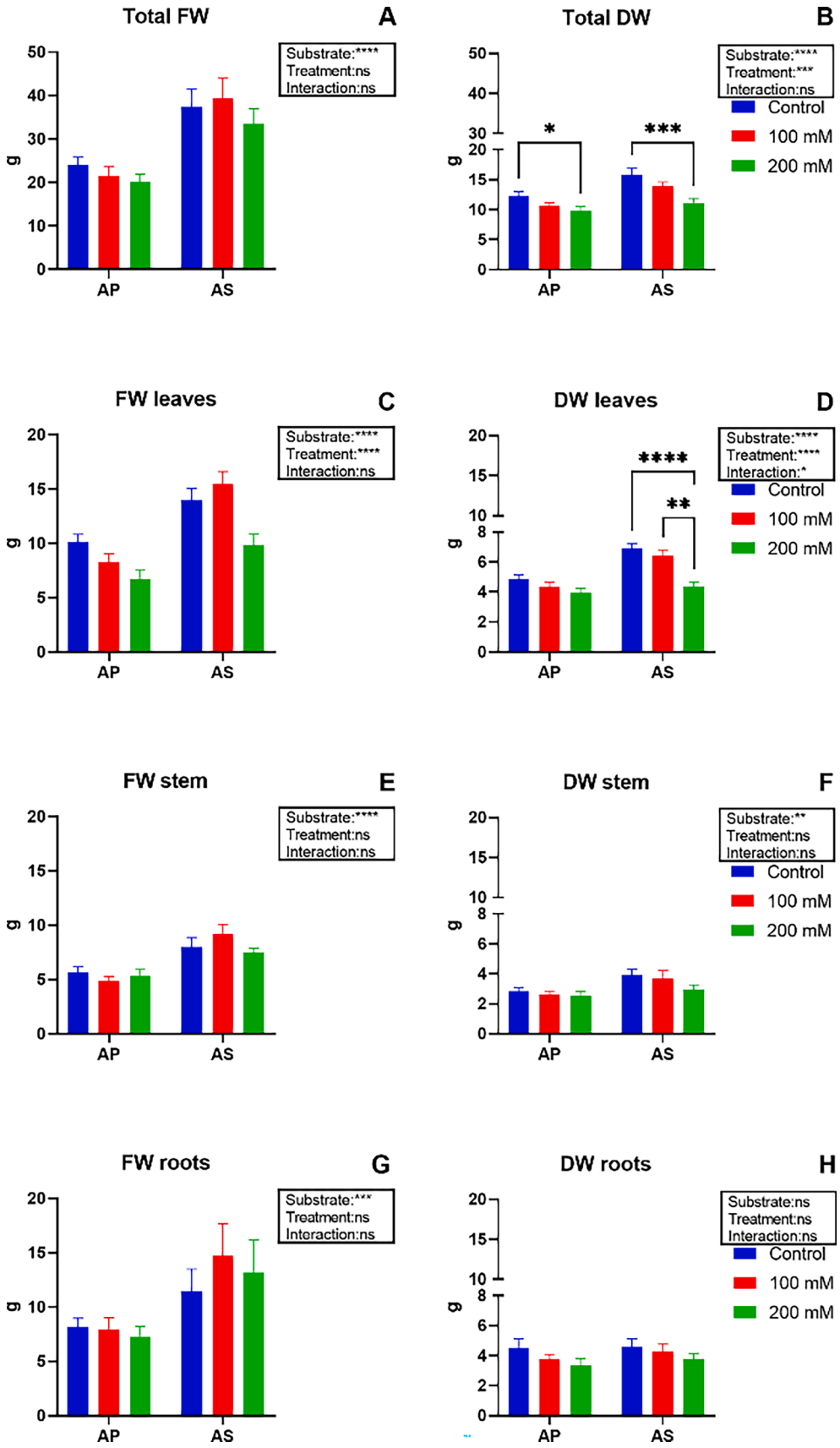

Fig. 2. Fresh (FW) and dry (DW) weight data of different tissues (leaf, root, stem) from Arbequina AS1® samples subjected to different salt stress condition. Samples were grown under different substrate; perlite (AS) and peat (AP). Tukey-HSD was performed as two-way ANOVA *post hoc* test according to the cultivar variable. **** < 0.0001 *** < 0.001 ** < 0.01 * < 0.05. Data were reported as mean ± *S*.E.M. ($n = 10$).

with 200 mM NaCl (Fig. 2B and D). Biochemical data reported in Fig. S3 showed that salinity stress led to an increase in the total polyphenols (TPC, Fig. S3A), flavonoid (TFL, Fig. S3B) and malondialdehyde (MDA, Fig. S3C) contents in both cultivars, with the highest values reported with 200 mM of NaCl in AS. The same trend was also reported for ferric-reducing antioxidant power (FRAP) data, with a general increase due to salinity stress in both cultivars (Fig. S3D).

Plants grown under control conditions significantly promoted their growth and leaf number in both substrates over the eight weeks of experimentation (Fig. 3A and Fig. S4). Notably, Arbequina plants exhibited a more substantial development in height and length of new shoots when grown in peat (AS) than in perlite (AP) (Fig. 3B). Furthermore, the number of leaves was higher in AS than AP, except with 200 mM NaCl, where no differences occurred between plants grown on the two substrates. Compared to the control, salt treatments adversely affected the height and length of new shoots, as well as the number of leaves in both substrates (Fig. 3). Though a significant decline in the number of leaves was observed only with 200 mM NaCl in AS, both salt stress conditions had similar inhibitory effects in AP (Fig. 3A).

Physiological data

The measure of the relative water content (RWC) of leaves showed that 200 mM NaCl induced a drastic reduction only in old leaves of AP plants (Fig. 4). On the other hand, the same salt stress did not change the RWC in the young leaves of AP and in both types of leaves of AS (Fig. 4).

Gas exchange parameters were collected every two weeks using the portable LI-COR 6400XT instrument (LI-COR Inc., Lincoln, NE, USA) for a period of eight weeks. The time course data are shown in the supplementary material (see Fig. S5). As reported in Table 2, salt treatments negatively affected gas exchange parameters of plants in perlite and peat (Table 1). Overall, higher values for gas exchange and photosystem efficiency parameters were supported by the use of peat as the growth substrate. However, plants grown in peat also demonstrated greater sensitivity to increasing salt concentrations, with greater reductions in all photosynthetic parameters than those of plants grown in perlite. After 8 weeks, the net carbon assimilation rate (A_n) and stomatal conductance (g_s) were significantly reduced compared with control samples in each substrate at 100 mM and 200 mM concentrations. In particular, the A_n reduction was dose-dependent in AS, with a 22.9 %

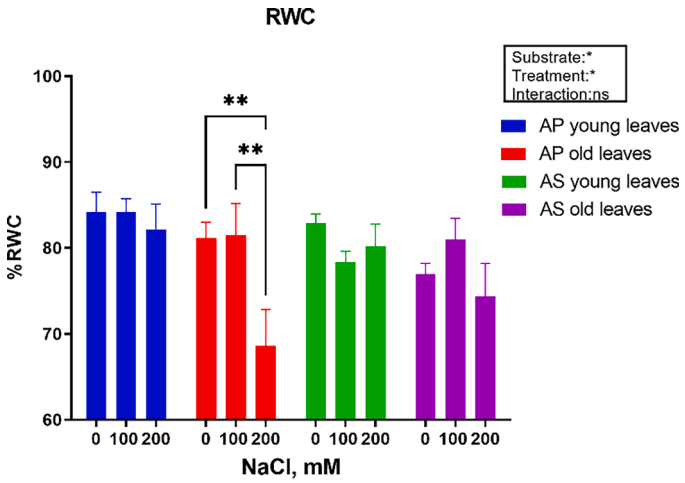

Fig. 4. Relative water content (RWC) of Arbequina AS1® plants grown on different substrates and subjected to different saltwater irrigation for 8 weeks. All values as presented as medium value + SEM (n = 5). Statistical significance of the difference between control and salt-stressed leaves was assessed by Tukey test (p-value <0.05) and indicated with different letter. **** < 0.0001 *** < 0.001 ** < 0.01 * < 0.05.

and a 38.4 % reduction compared with controls with 100 and 200 mM, respectively. In general, salt induced a significant decline in both treatments that is not dependent on the NaCl concentration in AP (Table 1). The same trend was observed for stomatal conductance (g_s), in which the salt effect was dose dependent in AS, with 33 % (100 mM treatment) and 59 % (200 mM treatment) reductions in g_s compared with control in AS. In addition, AS exhibited the highest photosynthetic rates compared with AP in all treatments.

The saline water irrigation did not significantly affect the intercellular CO_2 concentration (Ci) of plants in either substrate; however, AP plants had higher Ci values than AS plants. In contrast, the electron transport rate (ETR) was affected by the salt treatments. While AS exhibited a significant reduction in both salt treatments, AP revealed a significant decrease only at 200 mM NaCl. Even in this case, AS plants always had greater ETR values than AP plants. Maximum quantum yield

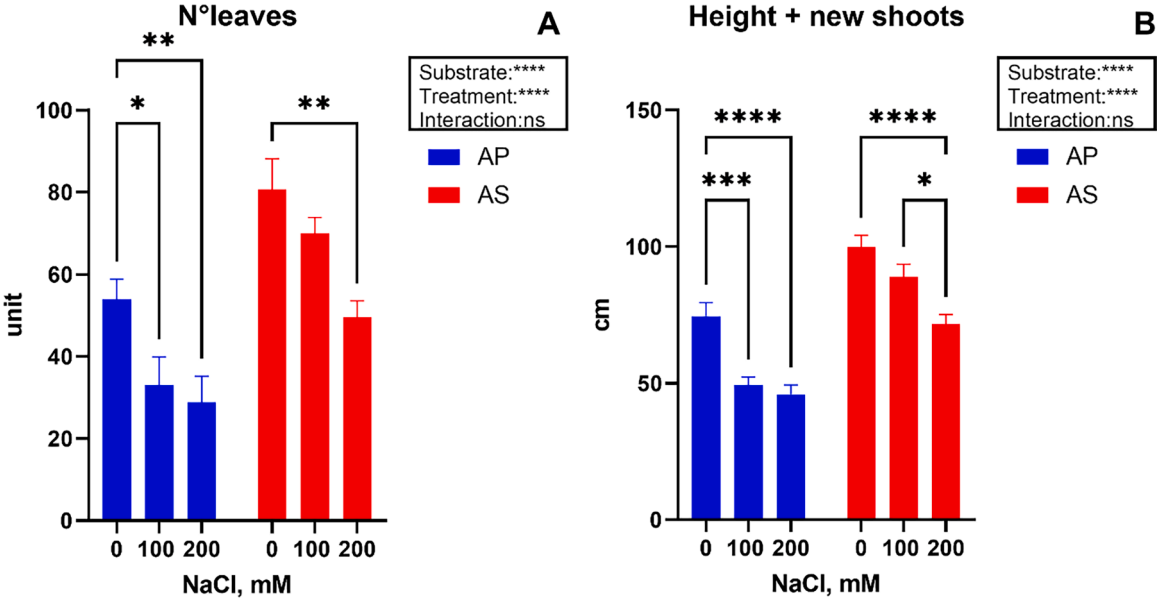

Fig. 3. Biometric data related to number of number leaves and related heights *per* plant of AS-1 cultivar grown on different substrate and subjected to different saltwater irrigation for 8 weeks. All values as presented as medium value + SEM (n = 10). Statistical significance of the difference between control and salt-stressed leaves was assessed by Tukey test (p-value <0.05) and indicated with different letter. **** < 0.0001 *** < 0.001 ** < 0.01 * < 0.05.

Table 1

Physiological parameters measured in the current experiment. Values are the averages of the values collected across the eight-week experiment once a week ($n = 6$). Values reported are the grand mean of all collected data (mean ± *S*.E.M, $n = 48$) for each treatment, relative to carbon assimilation rate (An), stomatal conductance (*gs*), and intercellular CO_2 concentration (Ci). Significance levels (*p*-values) of treatment, cultivar, and cultivar x treatment effect are shown. Different capital letters on the same column indicate significant differences due to treatments, according to Holm-Sidak test ($p \leq 0.05$). The differences (with the threshold of 5 %) between treatments (F, NF) and among cultivars are expressed by the symbols of comparison (>=<).

Treatments	Cultivar	An µmol CO_2 m^{-2} s^{-1} *Mean + S.E.M*		gs mmol H_2O m^{-2} s^{-1} *Mean + S.E.M*		Ci µmol CO_2 m^{-2} s^{-1} *Mean + S.E.M*		Fv/Fm		Fv'/Fm'		ETR	
0	AP	5.34 ± (0.18)		0.042 ± (0.004)		217.32 ± (11.27)		0.82 ± (0.003)		0.45 ± (0.09)		61.07 ± (3.25)	
	AS	10.04 ± (0.65)		0.093 ± (0.007)		183.51 ± (5.44)		0.82 ± (0.004)		0.50 ± (0.04)		117.91 ± (5.85)	
	Mean	7.69	**A**	0.07	**A**	200.42	**A**	0.82	**A**	0.48	**A**	89.49	**A**
100	AP	3.98 ± (0.22)		0.039 ± (0.004)		227.85 ± (8.77)		0.82 ± (0.006)		0.42 ± (0.07)		51.15 ± (3.46)	
	AS	7.74 ± (0.45)		0.062 ± (0.006)		165.54 ± (12.74)		0.81 ± (0.001)		0.44 ± (0.06)		91.12 ± (7.67)	
	Mean	5.86	**B**	0.05	**B**	196.70	**A**	0.82	**A**	0.43	**B**	71.14	**B**
200	AP	2.96 ± (0.27)		0.03 ± (0.004)		231.97 ± (9.53)		0.81 ± (0.006)		0.37 ± (0.09)		46.88 ± (2.59)	
	AS	6.18 ± (0.57)		0.038 ± (0.006)		133.37 ± (24.15)		0.82 ± (0.001)		0.42 ± (0.02)		64.71 ± (3.99)	
	Mean	4.57	**C**	0.034	**C**	172.67	**A**	0.82	**A**	0.4	**C**	55.79	**C**
2-way ANOVA	Substrate	**<0,0001**		**<0,0001**		**<0,0001**		0.1764		**<0,0001**		**<0,0001**	
(p-value)	Treatment	**<0,0001**		**<0,0001**		**0.5316**		0.9191		**<0,0001**		**<0,0001**	
	Treatment x substrate	0.1277		**<0,0001**		0.1204		0.1458		0.2605		**0.0004**	
Sidak's	Cultivar	AP (0 > 100=200)								AP (0 > 100>200)		AP (0 > 200)	
grouping		AS (0 > 100>200)		AS (0 > 100>200)						AS (0 > 100=200)		AS (0 > 100>200)	
	Treatment	0 (AS>AP)		0 (AS>AP)						0 (AS>AP)		0 (AS>AP)	
		100 (AS>AP)		100 (AS>AP)		100 (AS<AP)						100 (AS>AP)	
		200 (AS>AP)				200 (AS<AP)				200 (AS>AP)		200 (AS>AP)	

(Fv/Fm) remained stable between control, 100 mM, and 200 mM for both substrates.

On the other hand, salt treatments drastically reduced the efficiency of photosystem II (Fv'/Fm') of light-adapted leaves in both treatments, with a significant reduction between 100 and 200 mM NaCl in both AP and AS plants. Reductions in response to salinity were dose-dependent for plants grown in both substrates only for Fv'/Fm'. The aforementioned biometric data thus confirm the drop in physiological parameters occurring in stress conditions of AP compared to AS. By highlighting DW data (Fig. 2), the response differences between substrates are well correlated to carbon assimilation rate (An) and stomatal conductance (gs) data (Table 1), which is consistent during the eight weeks of the experiment (Fig. S4). Conversely, the intercellular CO_2 concentration (Ci) showed an opposite response trend, with higher values reported in AP samples that seem not to be much impacted by stress. This distinctness may be ascribed to different stomatal regulation, with a general reduction of transpiration and carbon dioxide uptake in AP plants due to perlite substrate.

Pigment and proline contents

Pigment analyses in Fig. 5 show that while salt treatments did not affect the Chl *a* concentration, the use of different substrates does lead to significant changes, with an increase in AS plants across all treatments relative with AP plants. On the other hand, salt treatments negatively influenced Chl *b* concentrations in both substrates (Fig. 5A, B). In particular, there was a significant decrease in Chl *b* in both treatments of AS plant compared with the control, while in AP, there was a significant decrease with only 200 mM NaCl. Salt treatments significantly affected leaf carotenoid concentrations only in 200 mM NaCl of AS compared with control (Fig. 5C). Proline content was quantified to highlight the response of a compatible osmolyte to salt stress (Fig. 5). Results indicated that only AP plants displayed a clear response pattern, with a significant increase of proline content in T200 compared with control. By contrast, the other substrate did not show any consistent change in response to stress.

Identification and quantification of polyphenolic compounds

Salt-induced changes in polyphenol contents were quantified in leaves by HPLC ESI/MS-TOF and data analyses were carried out using the Mass Hunter software (Agilent Technologies). The list of the eight polyphenolic compounds identified with retention time, experimental and calculated *m/z*, and molecular formula is reported in Table 2.

As illustrated in Fig. 6, irrigation with different saline water resulted in alterations in the concentrations of several polyphenolic compounds in Arbequina. Increasing NaCl negatively affects leaf quinic acid, with a significant drop after eight weeks of treatment with 100 and 200 mM NaCl in both substrates (Fig. 6A). However, statistical differences were observed between the substrates, with AP plants exhibiting a lower content of quinic acid compared with AS plants (Fig. 6A). Leaf rutin concentrations were influenced by the salt concentration (Fig. 6B). On the other hand, as reported by two-way ANOVA, AP plants exhibit a greater amount of rutin concentration compared with AS, regardless of the NaCl concentration. While verbascoside concentrations in AP plants decreased with 100 mM NaCl and increased with 200 mM NaCl, the concentration in AS was stable at 100 mM and increased at 200 mM (Fig. 6C). Furthermore, different salt treatments did not influence leaf quercetin glucoside concentrations (Fig. 6D). As observed in Fig. 6E, the irrigation with different saltwater decreases the oleuropein concentrations in AP, while increasing significantly in AS at 200 mM. In AP plants,

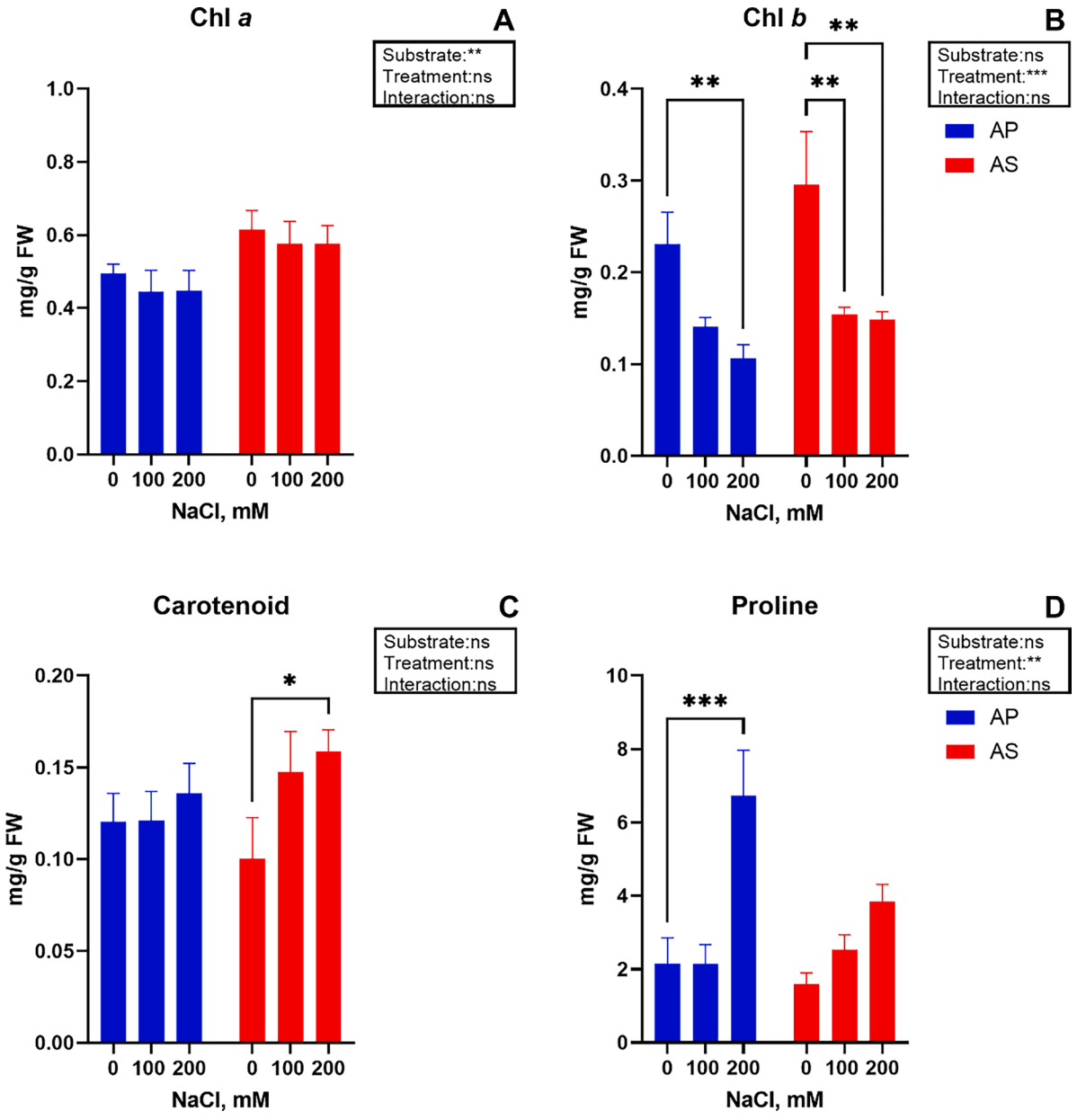

Fig. 5. Pigments (Chl *a*, *b* and carotenoids) and proline data of Arbequina AS1® plants. Data are the mean values ± *S*.E.M consisting of six ($n = 6$) and five ($n = 5$) independent replicates for pigments and proline analyses, respectively, for each experimental condition. 2-way ANOVA analyses coupled with Dunnet's Post-hoc tests (*p*-value <0.05, reference sample, T1 Control) were performed. **** < 0.0001 *** < 0.001 ** < 0.01 * < 0.05.

hydroxytyrosol glucoside content changed after salinity treatment, with a significant drop after treatment with 100 mM NaCl and an increase with 200 mM NaCl; by contrast, no significant salt-induced changes were observed in hydroxytyrosol glucoside content of AS plants (Fig. 6F). Luteolin glucoside levels drastically changed after different salt irrigation in plants grown on both substrates (Fig. 6G). In particular, compared to the control conditions, there was a significant decrease in leaf luteolin glucoside concentrations with 200 mM NaCl in AP plants, and after both salt treatments in AS plants. However, under the control condition, the level of luteolin glucoside in AP plants was higher than in AS plants (Fig. 6G). Lastly, leaf luteolin levels were affected by saline treatments with significant differences between AP and AS with 100 mM NaCl treatment (Fig. 6H).

By comparing physiological and metabolomic data, we may observe that the reduction in photosynthetic parameters like gas exchange seems to correlate to quinic acid, where the stress-related reduction is statistically significant in both substrates. This data fits with Chl *b* content, where a drop is still related to stress severity. Conversely, verbascoside and oleuropein showed a different trend, mainly in AS, with a salinity-induced increase. These differences in substrate-dependent responses are even more pronounced when comparing proline and carotenoid data, where AS and AP showed peculiar stress response patterns in terms of luteolin and hydroxytyrosol glucoside contents, in which the different substrates affect their production.

Results of ion measurements

Fig. 7 shows that irrigation with saline water caused a significant accumulation and translocation of sodium into the tissues of plants grown in perlite and peat. Independently from saline treatment, the highest sodium concentration was found inside the roots, then stems, and lastly, leaves (Fig. 7A–C). Compared with the control, after 200 mM NaCl, the Na concentration in roots was 10-fold and 11-fold higher in AP and AS, respectively. AP exhibited a significant sodium accumulation

Table 2
List of polyphenolic compounds from olive leaves identified and quantified in leaf samples by HPLC DAD ESI/MS-TOF using specific chemical standards, as reported in Palm et al. 2024.

Compound	(M-H)$^-$	*m/z* Exp[a]	*m/z* Clc[b]	Δm (ppm)[c]	RT (min)[d]	Score[e]	Reference
Quinic acid	$C_7H_{11}O_6$	191.0567	191.0609	−2.96	0.469	93.23	(Taamalli et al., 2013; Talhaoui et al., 2014)
Hydroxytyrosol glucoside	$C_{14}H_{19}O_8$	315.1098	315.1085	−3.94	1.025	84.88	(Taamalli et al., 2013; Talhaoui et al., 2014)
Rutin	$C_{27}H_{29}O_{16}$	609.1467	609.1461	−1.00	6.069	79.33	(Fu et al., 2010; Talhaoui et al., 2015)
Quercetin glucoside	$C_{21}H_{19}O_{12}$	463.0898	463.0882	−3.42	6.290	76.49	(Fu et al., 2010; Quirantes-Piné et al., 2013; Talhaoui et al., 2015, 2014)
Luteolin 7-O glucoside	$C_{21}H_{19}O_{11}$	447.0932	447.0933	0.23	6.432	88.75	(Fu et al., 2010; Taamalli et al., 2012)
Verbascoside	$C_{29}H_{35}O_{15}$	623.1985	623.1981	−0.64	6.993	77.09	(Lozano-Sánchez et al., 2010; Talhaoui et al., 2015)
Oleuropein	$C_{25}H_{31}O_{13}$	539.1791	539.1770	−3.92	8.829	58.69	(Lozano-Sánchez et al., 2010; Taamalli et al., 2013; Talhaoui et al., 2014)
Luteolin	$C_{15}H_9O_6$	285.0419	285.0455	−4.87	11.939	97.08	(Lozano-Sánchez et al., 2010; Taamalli et al., 2013)

[a] *m/z* experimental.
[b] *m/z* calculated.
[c] difference between the observed mass and the theoretical mass of the compound (ppm).
[d] retention time.
[e] isotopic abundance distribution match: a measure of the probability that the distribution of isotope abundance ratios calculated for the formula matches the measured data
*Compound positively identified with authentic chemical standards.

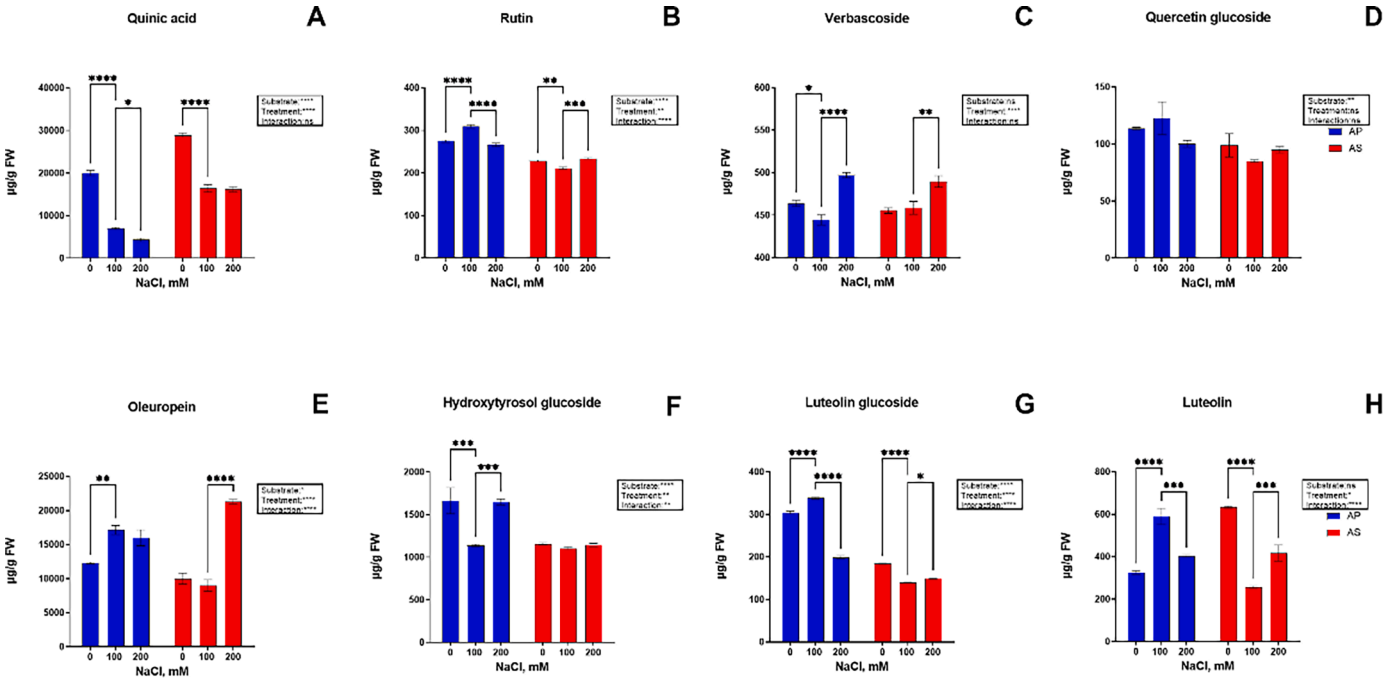

Fig. 6. HPLC-DAD ESI-MS results of the main polyphenolics identified in Arbequina AS1® olive leaves using reference standard compounds. *(A-H)* Two-way ANOVA coupled with Dunnet's test (*p*-value <0.05, reference sample, T1 Control) was performed. Values are mean ± *S*.E.M. ($n = 3$). **** < 0.0001 *** < 0.001 ** < 0.01 * < 0.05.

compared with AS in all salt treatments and tissues examined. AP leaves and stems showed a considerable sodium accumulation compared with AS in both treatments (Fig. 7A and B). On the other hand, there was no significant difference in the root sodium concentration between substrates after eight weeks of treatment (Fig. 7C).

Potassium concentrations were significantly reduced with the application of salt (Fig. 7). In the leaves (Fig. 7D), potassium concentrations significantly declined after salt was applied particularly in AP plants, where potassium concentrations were consistently lower than AS plants. Moreover, although salt irrigation reduced potassium concentrations in stems, there were no significant changes among treatments in either substrate (Fig. 7E). Root potassium levels significantly declined after salt treatment (Fig. 7F). Similar to the stems, potassium concentrations in the roots were significantly reduced between 100 mM and 200 mM NaCl compared with control plants (Fig 7F).

Additionally, the salt treatments drastically reduced the potassium-sodium ratio in leaves, stems, and roots, which declined in all tissues for both substrates compared to control values (Fig. 7G–I). Despite the observed changes in tissue ion concentrations among substrates, there was no significant difference in K/Na ratio between AS and AP plants after salt treatments (Fig. 7G–I).

Endophytic community profile

A careful analysis of the endophytic community was accomplished on leaf samples both at the initial (T0) and concluding (T1) (control, 100

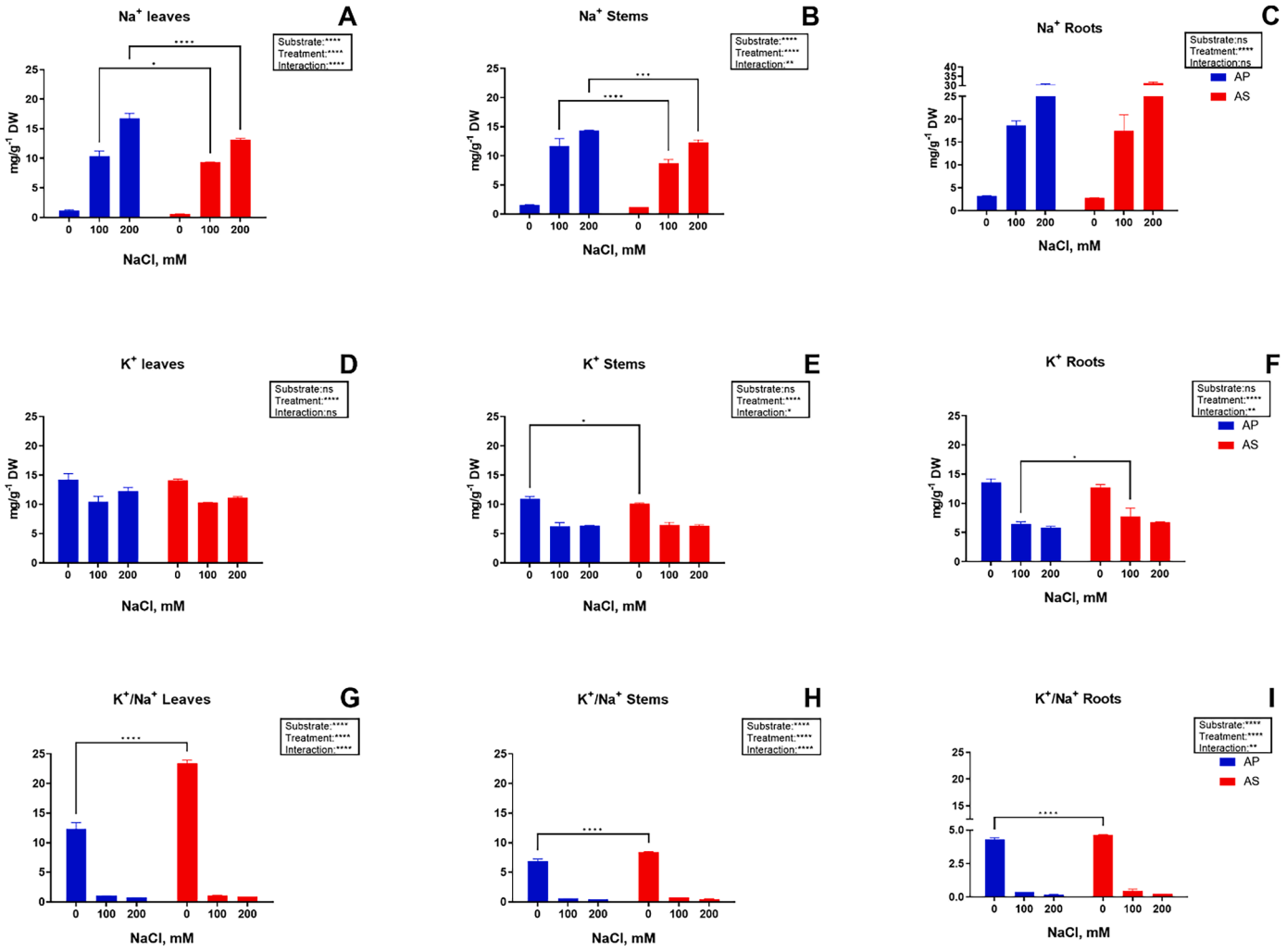

Fig. 7. Na^+ and K^+concentration, and K^+/Na^+ ratio values measured in different plant tissues (leaf, stem, root) of Arbequina AS1® samples under different experimental conditions (substrate, saline irrigation). (A,D,G) Leaf data. (B,E,H) Stem data. (C,F,I) Root data. Statistical analyses were performed according to two-way ANOVA coupled with Tukey post-hoc test (p-value <0.05). Values are mean ± S.E.M. ($n = 6$). **** < 0.0001 *** < 0.001 ** < 0.01 * < 0.05.

mM NaCl and 200 mM NaCl) stages of the experiment. Fig. 8 provides a representation of the identified ASV depending on the substrate and the saline treatment used, whereas data on libraries rarefaction is reported in Fig. S6. The α-diversity analysis on different substrates and after treatments, done at genus levels using three different indexes (Chao1, Simpson and Shannon), showed that there was no significant difference (Fig. 9).

Fig. 10A demonstrates the 2-D ordination plot, with statistical significance determined through Permutational ANOVA [PERMANOVA]. Beta-diversity was evaluated using PCoA based on Bray-Curtis distances and weighted UniFrac (Fig. 10A), capturing 95.2 % of the total variance (63.2 % PC1, 32 % PC2). These analyses discern changes in the dataset's presence/absence or abundance of thousands of taxa. A comprehensive examination, involving the comparison of each sample to every other sample that resulted in a distance matrix, identified that bacterial communities in samples exposed to 100 mM NaCl for both substrates exhibited significant differences from others (PERMANOVA F-value: 4.4084; R-squared: 0.39805; p-value: 0.002, Fig. 10A). Notably, the T100 samples clustered distinctly from others when considering the treatment factor, affirming the existence of unique features among the different conditions. Phylogenetic reconstruction of detected ASV showed that T100 samples include related genus. The genus *Ezakiella* was detected almost exclusively in T100 of both substrates (AP and AS), as well as *Cutibacterium* and *Bradyrhizobium* (Fig. 10B). This result was confirmed by heatmap, and Top 25 taxa analysis shown in Fig. 11 and also by univariate analysis (Fig. 12) in which the sample related to AP T100 demonstrated that the genus of greatest abundance were *Staphylococcus* (log2FC 16.295, p-value 9.92E-05, FDR 0.0011903) and *Ezakiella* (log2FC 16.295, p-value 0.0058568, FDR 0.026851Results from ASV's identification is also depicted in Fig. S7 as a heat tree, with an overall representation (Fig. S7A) and head-to-head comparison based on different substrates (Fig. S7B), where no significant features have been reported.

The outcomes of METAGENassist analyses illustrate that the metabolism of endophytic bacteria inside the leaves is ascribed to different metabolism types (Fig.S8 and S9). The main metabolism types in both substrates were ammonia oxidizer, sulfate reducer, nitrite reducer, and dehalogenation. However, the abundance of these metabolism types changed when comparing the two substrates (Fig. S10). For example, the dehalogenation metabolism of AS bacteria (67.7 %) was lower, compared to that of AP (69.3 %). Moreover, the nitrite reducer metabolism was higher in AP bacteria (69.3 %) than in AS (67.8 %). Xylan degrader bacteria were greater in AP (0.9 %) than AS (0.2 %). On the other hand, the salt treatments seem to slightly change the bacteria's metabolism inside the leaves. In 100 mM NaCl, there was a decrease in ammonia oxidizer (96 %) compared with T0 and the rest of T1 (Fig. S9), as well as in the substrate comparison (Fig. S10) by comparing peat (≈98 %) and perlite (97 %).

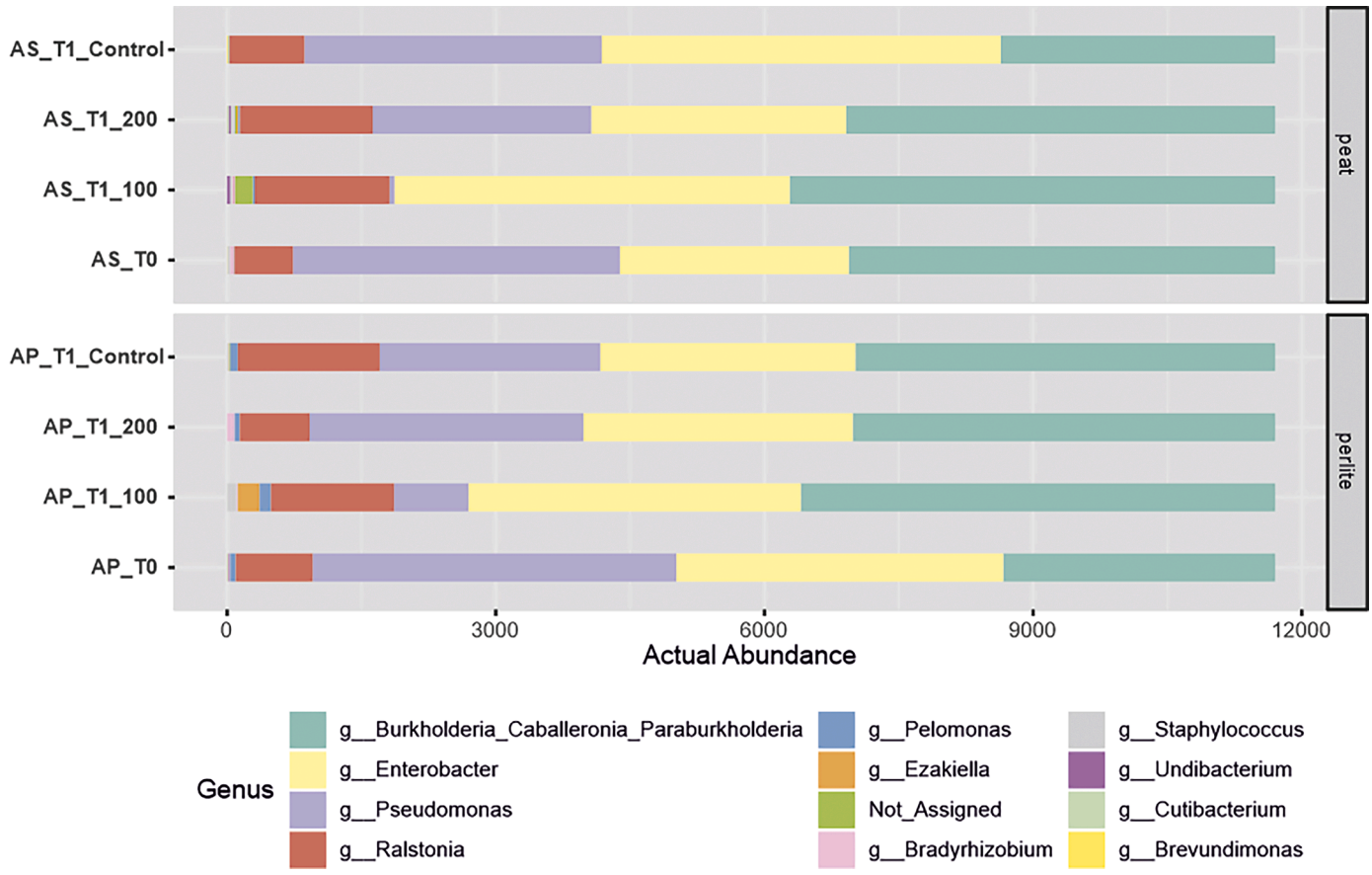

Fig. 8. Barplot of the identified ASV at the genus level. Samples were grouped for substrate and treatment. Represented ASVs were filtered (threshold = 0.5 %) to display only significant taxa.

Discussion

Olive tree responses to salinity have been studied in the last 30 years, encompassing a wide range of physiological, biochemical, morphological and molecular responses (El Yamani and Cordovilla 2024). In the pioneering work by Tattini et al. (1992), authors illustrated that forty-day-old self-rooted genotypes 'Frantoio' and 'Leccino' had shown different responses to salinity stress after 60 days of salinity stress at different concentrations (0, 12.5, 25, 50, and 100 mM). However, both cultivars exhibit a significant reduction in net photosynthetic rate, energy lost for salt exclusion mechanism, decrement of nutrient uptake, and finally, a reduction of plant growth. In particular, the 'Leccino' cultivar showed more significant potassium, calcium and magnesium reductions in its tissues with respect to 'Frantoio'. The K/Na ratio was also consistently higher in 'Frantoio' than in 'Leccino'. A subsequent study by Tattini et al. (1997) demonstrated that, despite similar Na uptake rates, 'Frantoio' and 'Leccino' diverged in terms of Na translocation to the shoots and Na exclusion by the roots. However, apical leaves of 'Frantoio' showed significantly higher K/Na ratio respect basal leaves, while this was not observed in 'Leccino'; for this reason, it was suggested that K/Na discrimination in favour of younger leaves is indeed correlated with salt tolerance in *Olea europaea* Munns (Tattini et al., 1997).

The role of calcium in salinity stress responses in olive was studied by Melgar et al. (2006), which demonstrated that calcium increases sodium exclusion in olive plants. In fact, the 'Picual' olive cultivar irrigated with saline water (75 mM NaCl + 40 mM $CaCl_2$) reduced sodium concentration in the leaf and did not show chlorosis symptoms with respect to plants that were not supplied with $CaCl_2$. Consequently, Melgar et al. (2006) assumed that calcium deficiency could facilitate sodium toxicity, and that Ca was directly involved in Na^+ exclusion and retention mechanism from the roots to the leaves. An extensive study of the effect of salinity stress on four olive tree cultivars, Arbequina included, was achieved in the work by Regni et al. (2019), where several physiological parameters were monitored. Differences in pigment contents, antioxidant enzymes activities (Glutathione reductase, GSH, and catalase, CAT) and gas exchange parameters were identified as direct consequences of the stress. More recently, Sodini and coworkers (2022) identified a specific molecular mechanism underlying the salt stress response in olive trees, which includes the overexpression of genes like *SOS1, ATPase11* and *ATPase8* in Frantoio plants treated with 120 mM of NaCl for 30 days.

The experimental set up used in these studies played a pivotal role in shaping the plants' reactions to such stress, which are influenced by several factors like plant age, environmental conditions, treatment duration, irrigation system, salt source, and substrate type, significantly impacting the results (Mousavi et al., 2019, 2022; Palm et al., 2024). Our investigation represents a pioneering effort to explore the influence of different substrates, specifically inert perlite (AP) and peat plus walnut fiber substrate (AS), on modulating salt stress in the Arbequina olive cultivar.

We discerned notable improvements in growth and leaf count in peat compared to perlite. This enhancement is likely tied to peat's superior water and mineral nutrient retention capacity, ensuring their availability to the plant as needed. Furthermore, the decreased growth rate in AP may be attributed to varying hydraulic conductivity compared with AS. Perlite exhibited substantially lower water conductivity than peat-based substrate at high matric potentials and exacerbated as potentials declined. This discrepancy implies that perlite's conductivity could constrain water uptake during periods of high transpiration rates

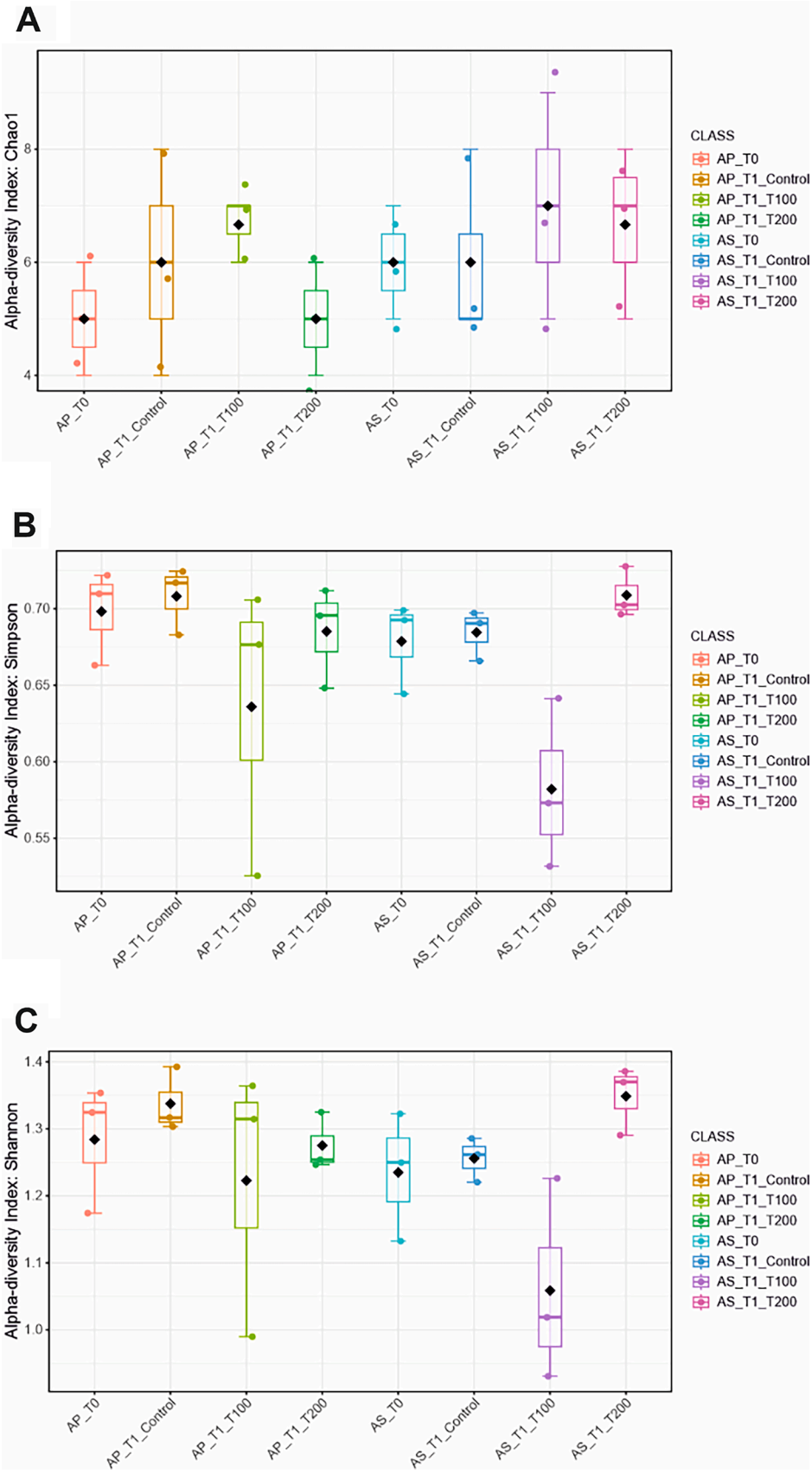

Fig. 9. Alpha diversity metrics of the analysed data. Data were computed based on three different indexes, (A) Chao1, (B) Shannon, and (C) Simpson. Samples were classified based on treatment and substrate variables.

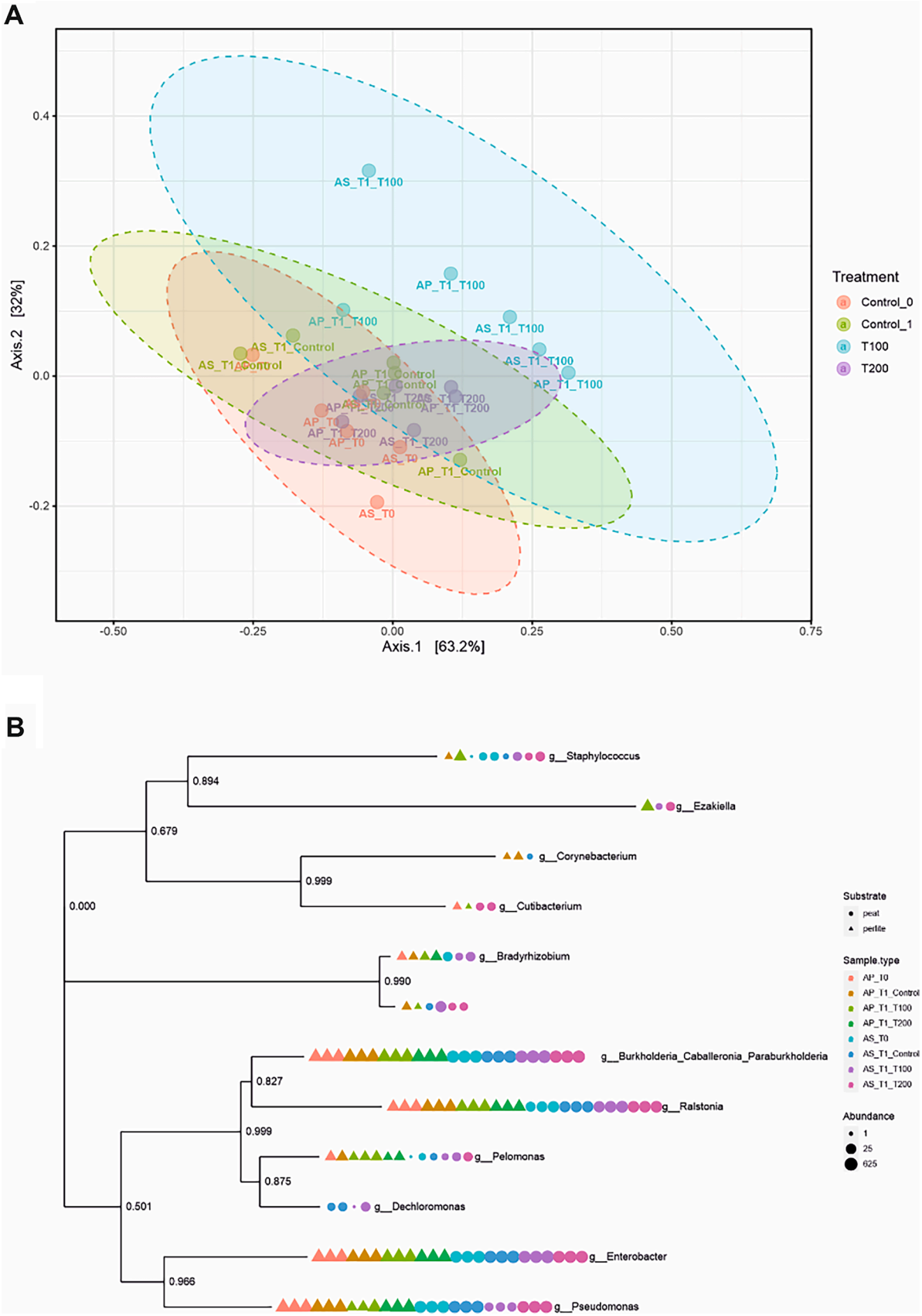

Fig. 10. Beta diversity analysis (PCoA) of samples based on Bray Curtis distances. The explained variances are shown in brackets. The statistical significance of clustering pattern was evaluated using Permutational ANOVA (PERMANOVA) analysis. Statistical significance is found out using [PERMANOVA] F-value: 4.4084; R-squared: 0.39805; *p*-value: 0.002.

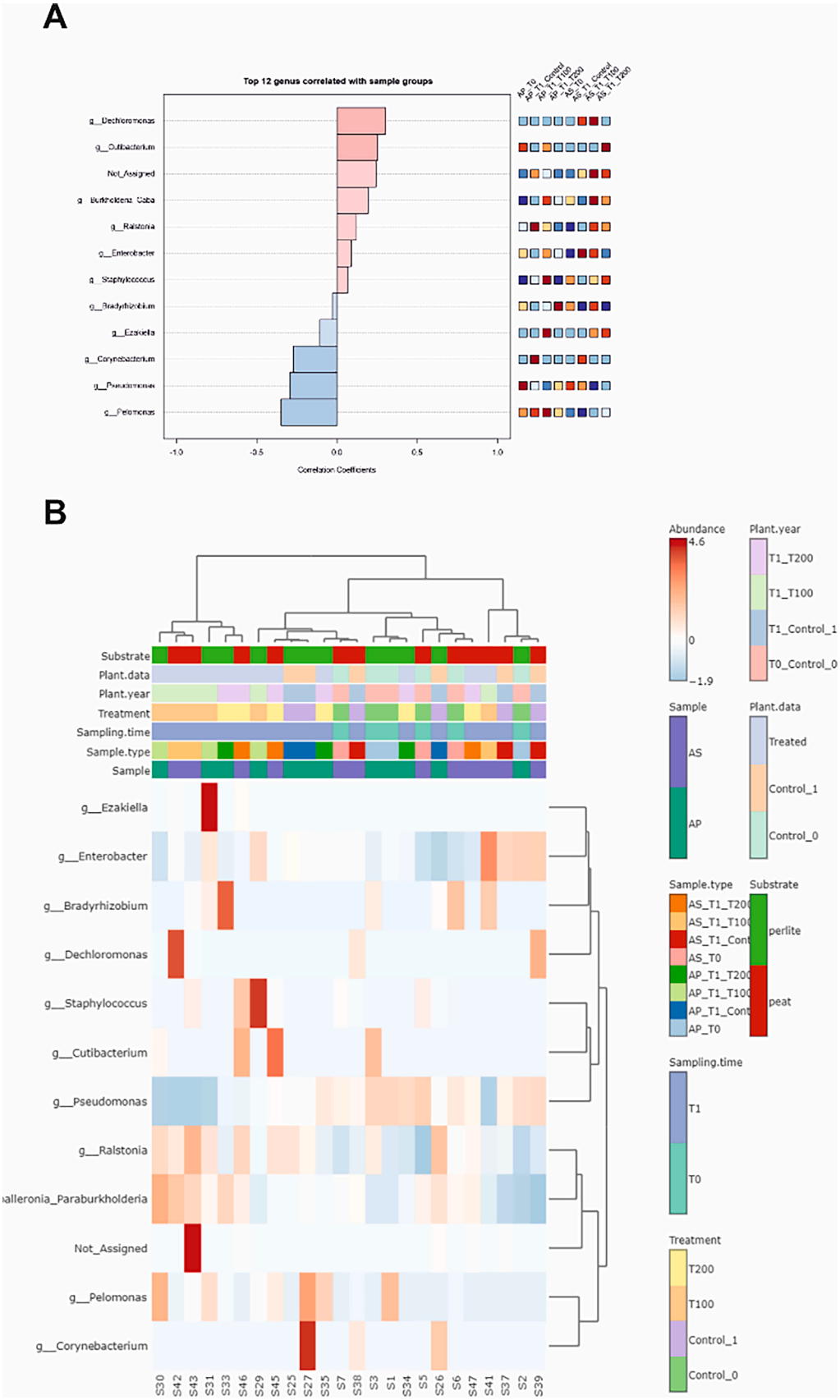

Fig. 11. Result from MicrobiomeAnalysst analysis. (A) Important features (at genus level) selected by correlation analysis with light purple indicates positive correlation and blue indicates negative correlations. **(B)** Clustering result shown as heatmap (distance measure using Euclidean and clustering algorithm using Ward distance at genus level).

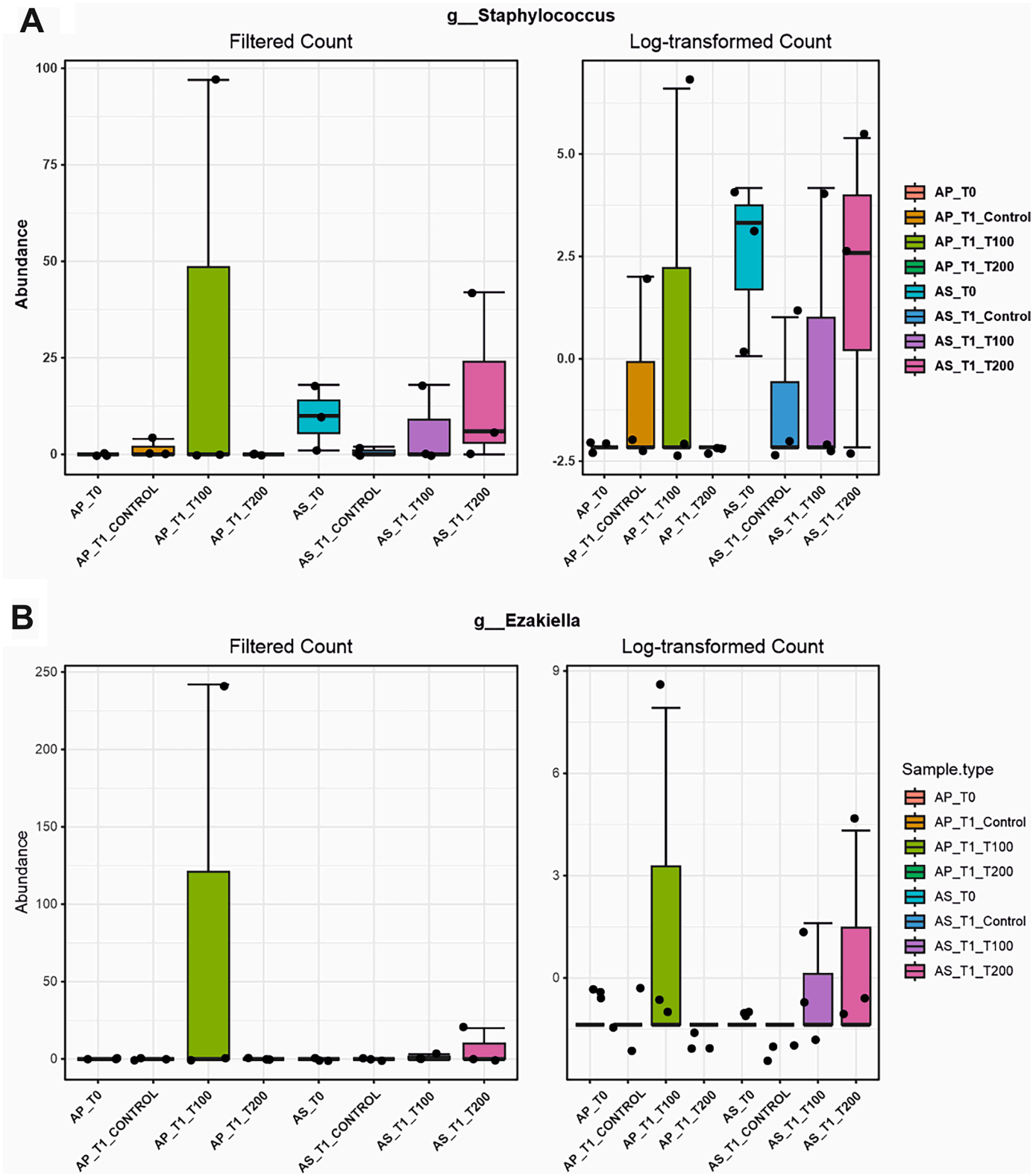

Fig. 12. The outcome from Microbiome Analyst distinctive features among treatments. Boxplots represent the abundance (filtered and log-transformed count) of the taxa Staphylococcus (A) and Ezakiella (B) in the eight experimental conditions.

(Jackson, 1974). Remarkably, salt stress curtailed the growth of plants in both substrates after the 2nd week, except in AS plants subjected to 100 mM NaCl irrigation, where olive trees exhibited a slight growth increase without significant leaf drop. This finding supported the hypothesis that peat efficiently alleviates salt effects compared to perlite. Nevertheless, plants can only endure until the stress source is removed, emphasizing the perilous consequences of whether salt effects surpass defense mechanisms of plants against stress, according to what has been previously reported (Munns and Tester, 2002; Tattini et al., 2008).

Moreover, prolonged exposure to salt stress inevitably diminished

photosynthetic activity and stomatal conductance, particularly evident in AP. This decline may be linked to limited water availability and the elevated sodium concentration inside leaves due to the absence of soil structure capable of retaining sodium and chlorine ions (Khondoker et al., 2023). Differences in AP responses can be due to substrate composition since peat and perlite are characterized by different water retention indexes (Markoska et al., 2018), with peat having a higher water-holding capacity due to smaller particle composition. It has also been demonstrated that a more pronounced water retention in soil significantly affects soil enzyme activity by increasing the diffusion of enzymes (Holz et al., 2019), which is beneficial for plant nutrient acquisition. Nevertheless, the prolonged duration of stress gradually diminished photosystem efficiency in both substrates, concurrently compromising the concentration of chlorophyll *b* within the leaves. Consequently, Arbequina endeavored to safeguard photosynthetic activity efficiency by fortifying the protection of photosystems through reactive oxygen species (ROS) metabolism and excess energy dissipation in response to high salinity irrigation. This was achieved through an elevation in the leaf concentration of carotenoids and some antioxidant polyphenols; the former phenomenon was more prominent in AS plants, while the latter occurred to a greater extent in AP plants. The natural shielding effect of peat-based substrate on the roots against the deleterious impact of toxic ions, such as sodium and chloride, coupled with the ready availability of water and nutrients, substantially contributed to preserving the photosynthetic system's efficiency. Water availability directly impacts the uptake of essential nutrients, thus representing a critical resource for maintaining ion homeostasis under saline conditions (Tadić et al., 2021). In this view, different substrates can create varying water and nutrient availability conditions, affecting the plant's ability to cope with salt stress. The choice of substrate is then a factor that can lead to variations in the salt stress response among different olive cultivars. For instance, the rooting potential of olive cultivars varies significantly across substrate types, which can lead to differences in water uptake and nutrient absorption, ultimately affecting the plants' resilience to salt stress (Villa et al., 2017). For example, substrates amended with biochar have been shown to enhance nutrient uptake while simultaneously reducing sodium accumulation, thereby improving the overall salt tolerance of plants (Çakmakcı et al., 2022; Jabborova, 2023). Further studies have also shown that olive trees grown in substrates with differing water retention capacities exhibit distinct physiological responses to salt stress, including variations in chlorophyll content and osmolyte accumulation (Regni et al., 2019; Bashir et al., 2021). Therefore, it is crucial to consider the substrate used when interpreting results from salt stress trials, as the substrate used can significantly influence the outcome and the physiological responses observed in olive cultivars. This knowledge can be useful also for the preparation of growth media in commercial olive cultivation, where optimizing substrate conditions can enhance plant resilience to salinity and improve overall yield (Aragüés et al., 2010).

According to Sodini et al. (2023) Fv/Fm data do not appear significantly affected by NaCl treatments. Fv/Fm$\geq$ 0.8 is considered as being indicative of a healthy photosystem II, and the higher values of electron transport rate (ETR) and Fv'/Fm' in AS compared with AP affirmed the prior assumptions.

Furthermore, the sodium concentration in AP exceeded that in AS. Consistent with Tattini et al. (1992), the olive tree's capacity to alleviate salt stress is intricately connected to the diverse abilities of roots to exclude sodium. In this instance, this mechanism was significantly reinforced by the buffering effect of the peat. Additionally, our observations revealed that peat enhanced potassium efflux compared with perlite. Increased potassium efflux appears to be employed as a strategic measure to curtail sodium penetration in both aerial parts and roots of the plants, aligning with the findings of Lutts et al. (1996), Pandolfi et al. (2017) and Tabatabaei (2006).

As a defence mechanism against oxidative stress triggered by reactive oxygen species (ROS) activity, olive trees are adept at generating various molecules, including the polyphenols MDA and proline. The role of MDA as a marker for identifying damages to photosynthetic machinery has been rereported in work by Sofo and coworkers (2004) in olive trees under drought stress conditions or more recently in the manuscript by Lima-Cabello et al. (2023), where Arbequina showed an increase in MDA content under stress, which is in agreement with our results for both substrates. Proline, identified as a secondary metabolite in numerous plants, compensates for osmotic imbalances within cells. Its multifaceted role encompasses deactivating singlet oxygen through physical quenching (Matysik et al., 2002). As a protein-compatible hydrotrope, proline mitigates cytoplasmic acidosis and helps to maintain favorable NADP+/NADPH ratios. This contributes to sustaining electron transport between photosynthetic centers, thereby minimizing damage to the photosynthetic apparatus (Hare and Cress, 1997; Sharma and Verslues, 2010). In recent years, the role of proline in olive tree responses to salinity has been investigated (Boulaem et al., 2019), where the stress increased proline production in cultivar Sigoise, thus suggesting a role in the osmotic regulation of cytoplasmic pH. Ayaz et al. (2021) investigated the role of proline in three Turkish olive cultivars and confirmed its role in mitigating the salinity stress effect.

A general increase in proline content was reported in AP and AS, although, in the case of AP, significant proline production was observed only after exposure to high salt concentrations. This observation suggests that AP experienced a pronounced osmotic imbalance, likely attributed to the elevated sodium concentration within the leaves. Consequently, it can be inferred that proline production may be genotype-specific and, furthermore, may occur only when the osmotic potential within the leaves surpasses a certain threshold. Conversely, our findings are not in agreement with those reported in the manuscript by Regni et al. (2019), where four olive tree cultivars (Arbequina, Koroneiki, Royal de Cazorla and Fadak 86) were studied in salinity stress conditions over a lengthy time period. The authors described that the proline content decreased in response to stress and was not affected by stress duration (180, 210 and 240 days), thus indicating that it does act as compatible osmolite in counteracting the stress effect. This discrepancy may be explained by the applied NaCl doses, stress duration, and the different methods used for proline determination (colourimetric or HPLC-based).

Total flavonoid content rises in AS and AP with a similar trend, despite it appearing to be slightly more consistent in AS samples with respect to AP, mostly in the 200 mM NaCl treatment. This increase may be correlated to the upregulation of genes involved in the phenylpropanoid pathway, as reported in the manuscript by Rossi et al. (2016). Also, our results are in agreement with those reported in the paper by Demir and coworkers (2020), where the increase in flavonoid contents reported in three Turkish cultivars under salinity stress was not followed by a comparable increase in total polyphenolic content.

The enhanced production of polyphenols within the leaves is notably pronounced in the perlite substrates, likely attributable to the unique characteristics of this substrate, as previously described. Additionally, we observed an increase in oleuropein, a compound pivotal in mitigating oxidative damage in olive leaves, particularly in AS subjected to 200 mM of NaCl. Oleuropein also functions as a reserve substitute for sugars, balancing osmotic imbalances within root cells (Petridis et al., 2012). Mechri and colleagues proposed that the augmented production of hydroxytyrosol is strongly linked to limited water availability of plants (Mechri et al., 2020). This phenomenon was not observed here for AP and AS plants experiencing salt stress. Conversely, a significant reduction in hydroxytyrosol levels was observed in AP plants exposed to 100 mM NaCl but not to 200 mM NaCl. The reason behind this discrepancy remains unknown, with possibilities including an inverse relationship between hydroxytyrosol and luteolin levels or salt concentration values being too high to determine augmented metabolite production.

In the present study, leaf quercetin levels did not change significantly in plants grown on either substrate under salt stress, which is in

agreement with previous observations (Rossi et al., 2016). Quercetin positively affects the K^+/Na^+ ratio and can act as an activator of the SOS1 Na^+/H^+ exchanger, favouring sodium efflux from the leaf mesophyll of AP-grown plant (Ismail et al., 2015; Parvin et al., 2019). Conversely, luteolin plays a role in alleviating the harmful effects of salt stress by enhancing water uptake and stimulating α-amylase for increased soluble sugars in the leaves. This leads to a reduction in the expression of SOD and CAT enzymes, subsequently decreasing the levels of free radicals responsible for oxidative stress (El-Shafey and AbdElgawad, 2012). The heightened production of both polyphenols under 100 mM NaCl suggests Arbequina attempts to restrict the damage and adapt to hostile conditions, limiting the scavenging effects of ROS. However, under 200 mM NaCl, it appears that the plants may prioritize conserving energy, directing their limited resources towards preserving photosystem activity and producing specific antioxidants like oleuropein.

In the present study, we also investigated the potential interaction between the endophytic bacteria community in plants, the ground substrate (perlite or peat) and the saltwater treatments. Additionally, we noticed that water availability and cation exchange capacity did not significantly influence the bacterial composition inside the leaves, as the bacterial profiles did not differ between AS and AP grown plants. On the contrary, the bacterial community was altered by variations of salt concentration. In particular, we observed a significant decrease of *Pseudomonas* genus and a significant increase of the *Burkholderia* genus in both AP and AS plants challenged with 100 mM NaCl but not 200 mM NaCl (Fig. 8). Earlier studies have investigated the possible mutualistic role of these various bacterial groups in plants subjected to saline stress. The potential role of PGPM (plant growth-promoting microorganisms) in mitigating the effect of salinity stress in crops and woody plants has already been investigated (Cardoni et al., 2023; Kumar et al., 2020). However, it is worth noting that this particular study attempted to use two olive-associated *Pseudomonas* sp. rhizobacteria strains synthesizing 1-aminocyclopropane-1-carboxylic acid deaminase (ACD). This enzyme degrades 1-aminocyclopropane 1-carboxylic Acid (ACC), thus reducing stress-induced ethylene in host plants and preventing increased salinity tolerance in young olive trees of Picual cultivar (Montes-Osuna et al., 2021). Jha et al. (2011) confirmed that *P. pseudoalcaligenes* could induce the accumulation of glycine betaine and reduce proline production in the early growth rate of *Oryza sativa* subjected to saline irrigation.

Conversely, the role of species belonging to *Burkholderia* genus in colonizing host plants is well documented (Pal et al., 2022), like *Burkholderia phytofirmans* PsJN which has been demonstrated to promote growth and yield in a halophytic species like quinoa (Yang et al., 2020), by triggering gene expression responses to salinity stress in *Arabidopsis thaliana* (Pinedo et al., 2015). Akhtar and coworkers (2015) studied the effects of two endophytic bacteria, *B. phytofirmans* PsJN and *Enterobacter* sp. FD17, in mitigating salinity stress according to ACC-deaminase and exopolysaccharide production. Bacterial inoculation using biochar as a carrier can lead to a drop in Na^+ uptake in maize plants under elevated salinity conditions by maintaining nutrient balance or by improving iron nutrition with a drop of oxidative stress reduction in quinoa plants grown in saline soils (Naveed et al., 2020).

Nevertheless, the physiological reason behind the observed reduction of the taxa mentioned above with 100 mM NaCl but not 200 mM NaCl is unclear and, here, not easily explained. As previously done, we can simply hypothesize that an increase of osmotic potential and a stomatal closure might drastically reduce the xylem efflux inside the leaves, thus affecting the composition of the corresponding bacterial community (Vita et al., 2022) by affecting the role of potential PGPMs, like *Burkholderia* sp., which are also involved in the accumulation of osmolytes and antioxidants (Vaishnav et al., 2019) until the host osmotic imbalance becomes unchallenging. However, the capacity of olive trees to maintain a sufficient water content can lead to preserving bacterial niche inside the leaf`s petioles despite the high sodium concentration inside the leaves. Moreover, this salt concentration-dependent effect could be in principle related to the corresponding high production of specific polyphenols, such as rutin, oleuropein, verbascoside and luteolin, which could inhibit the multiplication of specific bacteria belonging to the *Pseudomonas* genus, as it was widely demonstrated under in vitro conditions (Borjan et al., 2020; Pereira et al., 2007).

Conclusion

The present study offers a comprehensive description of olive trees grown under conditions varying in water availability and cation exchange capacity and experiencing diverse salinity stress conditions. A multidisciplinary approach using physiological data coupled with biochemical analyses, metabolomics, and metagenomics allowed us to describe the different adaptation strategies used to counteract the effects of osmotic stress. Differences in biometric parameters occurred mainly in plants grown under limited water availability due to reduced retention of the media in stress and non-stress conditions, with marked differences in fresh weight rather than dry weight of analyzed plants. The same trend was also depicted for pigments, ions and metabolomic profiles, where the general trend indicates that greater cation exchange capacity and higher organic content buffer the osmotic imbalance due to salinity as a consequence of the reduced efficiency of the photosynthetic machinery according to gas exchange data. Metabarcoding results indicated that the stress condition was more significant in determining an alteration of the microbiota composition than the properties of the growth media, with intermediate challenging conditions (100 mM NaCl) being more suitable and more rapid for plant adaptation to saline stress in both substrate conditions. Lastly, the overall data indicate that the used substrate can significantly impact the response to osmotic stress. This may require a thorough review of the information from previous studies. Variation in physiological and metabolomic data may be the result of both substrate and the level of salinity stress imposed, depending on the adaptation of the root system to different growth conditions. Our data indicate that the properties of the substrate used often represent under-estimated key factors in plant response to salinity in that they may be involved in mitigating the effects of stress. Also, using PGPMs inoculated along with substrate could represent a promising option for strengthening plant defences and increasing nutrient uptake from soil.

CRediT authorship contribution statement

Marzia Vergine: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Emily Rose Palm:** Writing – review & editing, Validation, Investigation, Formal analysis. **Anna Maria Salzano:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Carmine Negro:** Investigation. **Werther Guidi Nissim:** Writing – review & editing, Formal analysis, Conceptualization. **Leonardo Sabbatini:** Formal analysis, Conceptualization. **Raffaella Balestrini:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology. **Maria Concetta de Pinto:** Writing – review & editing, Visualization, Validation. **Nunzio Dipierro:** Writing – review & editing, Validation. **Gholamreza Gohari:** Validation, Supervision, Resources, Investigation. **Vasileios Fotopoulos:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition. **Stefano Mancuso:** Project administration, Funding acquisition. **Andrea Luvisi:** Writing – review & editing, Supervision, Resources. **Luigi De Bellis:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Andrea Scaloni:** Supervision, Resources, Project administration, Funding acquisition. **Federico Vita:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Investigation, Formal analysis, Data curation, 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.2024.100648.

Data availability

The data that supports the findings of this study are available in the Supporting Information.

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