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Research paper

Novel and widely spread citrus rootstocks behavior in response to salt stress

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ABSTRACT

Irrigation water salinity is a major limiting factor in arid and semi-arid environments. The use of rootstock is one of the appropriate agronomic techniques that attempt to support and limit the negative effect of salinity. The objective of this study was to evaluate the behavior of eight one-year-old rootstocks grown in pots under salt stress conditions (30 and 60 mM NaCl). Some genotypes, i.e. C35 citrange, Bitters, Carpenter and Furr citrandarins were recently released or spread and poor information on their behavior in saline conditions is available, while Carrizo citrange, Swingle Citrumelo, *Citrus volkameriana* and *Citrus macrophylla* are spread since long ago in the Mediterranean basin. The results clearly demonstrated that the most salt sensitive genotypes were Carrizo and C35 citrange, that reduced morphological and gas exchanges performances. Furr, Bitters and Carpenter citrandarins revealed good physiological and hormonal behavior. An intense antioxidant enzymatic activity was noted in C35 and Carrizo citranges, while Furr showed a decrease in malondialdehyde and antioxidant enzymatic activities at 60 mM NaCl. Its tolerance to saline water was also confirmed by transcriptomic analyses.

1. Introduction

The scarcity of water resources and its salinity in many cultivated areas are the main environmental constraints affecting agriculture, especially in semi-arid and arid regions (Ferlito et al., 2014; Stagno et al., 2015; Othman et al., 2023). These aspects, combined with climate change, extend dry and hot periods in different world regions, limiting crop productivity (Garcia-Sanchez et al., 2002; Vanella et al., 2023). Plants adopt multiple strategies to tolerate salt stress: including regulation of various physiological mechanisms, osmotic adjustment, maintenance of photosynthesis rates, activation of antioxidant enzyme activities (Arbona et al., 2003; Arbona et al., 2008), and regulation of hormones and metabolites in roots and leaves (Vives-Peris et al., 2023). Among fruit tree crops, citrus species are considered highly sensitive to salt and their responses to stress are strictly dependent on the genotype (species, cultivar, and scion/rootstock combination) (López-Climent et al., 2008; Shahid et al., 2019).

Saline water has negative effects on citrus plants growth and development that are associated with physiological disorders, such as low osmotic potential, low transpiration rate and reduction of the CO_2 availability for photosynthesis (Othman et al., 2023). It is known that salinity causes several damages in citrus plant, such as tissue burning and leaf abscission; therefore, one of the responses to osmotic stress is the accumulation of abscisic acid in plant tissues which seems to be a mediator between stress perception and the synthesis of 1-aminocyclopropane-1-carboxilic acid which leads to ethylene production and leaf abscission (Gómez-Cadenas et al., 2002).

Among agronomic practices, the use of tolerant rootstocks is a strategy that enables the cultivation of citrus under saline water conditions. Thus, grafting of commercial cultivars on salt-tolerant rootstocks allows the containment of the effect of salt stress on the scion.

Several works report that Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.), used as citrus rootstock in Spain and in other citrus growing countries, is tolerant to drought stress and saline conditions (Garcia--Sanchez, et al., 2002; García-Sánchez et al., 2007). Other authors reported that *Citrus volkameriana* outperforms sour orange in terms of plant vigour when grown in saline environment (Othman et al., 2023). Carrizo citrange is one of the most spread citrus rootstocks in the Mediterranean basin, even if it is highly susceptible to drought and salt stress (Moya et al., 2002; Garcia-Sanchez et al., 2002; García-Sánchez et al., 2007; Carr, 2012). Shahid et al. (2019) reported a reduction in plant dry biomass in Kinnow mandarin grafted on different rootstocks

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caused by salt stress, but this decline was much higher in plants grafted onto Carrizo citrange and Sacaton citrumelo. Indeed, salt stress caused more severe oxidative damage in the above-mentioned rootstocks than in Rangpur lime and 'Rubidoux' trifoliate orange, described as salt-tolerant rootstocks. These authors reported that salt-tolerant rootstocks altered the O_2 scavenging potential, also enhancing superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activity in response to stress.

The recent advancement in the sequencing technologies enabled a precise study of the genetic mechanism underlying agronomical traits through the analysis of the DNA polymorfisms and/or the comparison of the transcriptome of individuals with different phenotypes. As for salt tolerance, a QTL analysis was performed on a segregating population obtained crossing the salt tolerant Cleopatra mandarin (*Citrus reshni*) and the male parent *Poncirus trifoliata* that is characterized by a high susceptibility to salinity. The analysis enabled the identification of a QTL on chromosome 6 (Asins et al., 2023). The analysis of the whole transcriptome can provide additional information on the genes up and down-regulated in response to salt stress condition as shown by Xie et al., (2017) to (2018)).

The aim of the present study was to evaluate the performance of eight rootstocks grown in pots under saline stress conditions. Some of these genotypes, i.e. C35 citrange, Bitters, Carpenter and Furr citrandarins, were recently released (Federici et al., 2009), and poor information on their behavior in saline conditions is available. These rootstocks were evaluated in comparison with others spread in the Mediterranean basin (Carrizo citrange, Swingle Citrumelo, *C. volkameriana* and *C. macrophylla*) under two levels of salinity stress (30 and 60 mM NaCl) through the analysis of the morphological, physiological, enzymatic, and hormonal response to salt stress. The salt concentration was chosen based on results reported by Arbona et al. (2003) and López-Climent et al. (2008). In addition, a RNAseq analysis was performed on Carrizo (salt susceptible) and Furr (salt tolerant) to better decipher the genetic regulation of such an important trait.

2. Materials and methods

2.1. Plant material and salt treatments

Seeds were extracted from mature fruits of eight citrus rootstocks including Carrizo citrange (*Citrus sinensis* cv. Washington navel x *Poncirus trifoliata*), *C. macrophylla* Wester, *C. volkameriana* Ten. & Pasq., Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata* [L.] Raf.), C35 [*C. sinensis* (L.) Osb. cv. 'Ruby' x *P. trifoliata* (L.) Raf.], and the citrandarins Bitters (C22), Carpenter (C54), Furr (C57) (hybrids of *C. sunki* x *P. trifoliata*. Seeds were sown into pre-moistened substrate composed by peat, coconut fiber, sand, and perlite (50:25:20:5). After transplant and during growth plants were irrigated and fertilized using a Hoagland solution as modified by Forner-Giner et al. (2011) until the beginning of the experiment.

Ten one-year-old plants in 9 L pots per treatment were selected homogeneous in size for each rootstock. Salt stress was imposed by dissolving different amounts of pure NaCl in irrigation water, which has a basal NaCl concentration of 9 mM, up to two concentrations: 30 mM and 60 mM. Water (280 mL) was supplied for all treatments three times a week to compensate for evapotranspiration; water volume was previously calculated by means of gravimetric water loss (Dichio et al., 2006). Air temperature and relative humidity (RH) were recorded hourly in the greenhouse by a datalogger (Elitech RC-51 H USB, London, UK) during the experiment from 185 to 262 day of the year (DOY).

2.2. Morphological analyses and total chlorophyll content

Immediately after the end of the experiment, all the ten plants per rootstock and treatment were used for morphological analyses. Leaf area (m^2) and root length (m) were measured after harvesting. For the analysis of leaf, shoot and root biomass, harvested plants were washed with distilled water and dried with filter paper. The dry weight (g, DW) of the roots and the shoots was obtained by putting samples in an oven at 70 °C until they reached a constant weight. Shoot/root ratio was determined as the ratio of shoot and leaf to root dry weights. The specific leaf area ($cm^2\ g^{-1}$, SLA) was determined as the ratio of leaf area to leaf dry weight. The specific root length ($m\ g^{-1}$, SRL) was determined as the ratio of fibrous root length to its respective dry weight.

The chlorophyll content (Clh_tot) of the leaves was assessed following the procedure described by Inskeep and Bloom (1985). Analyses were performed on two leaves per plant for each rootstock and for each treatment (n=20). Leaf chlorophyll index was measured on fully developed leaves (n=30) by a SPAD chlorophyll meter (Minolta Co., Osaka, Japan).

2.3. Physiological monitoring of the plants

Physiological parameters were measured fortnightly from 185 to 262 DOY. Leaf transpiration (E, mmol $H_2O\ m^{-2}s^{-1}$), photosynthesis (A, µmol $CO_2\ m^{-2}s^{-1}$) and stomatal conductance (g, µmol $CO_2\ m^{-2}s^{-1}$) were measured with a portable infrared gas analyzer (LCi, ADC Bioscientific Ltd., Hoddesdon, UK). Measurements were made in clear days, between 8:00 h and 10:00 h (solar time) on fully developed leaves (3 replicates per treatment for each rootstock).

Chlorophyll fluorescence (Fv/Fm) was measured using a portable modulated pulse fluorometer (Handy Pea, Hansatech Instruments Ltd Narborough, United Kingdom) on the same leaves after dark acclimation for at least 30 min to inhibit all light dependent reactions by completely oxidizing PSII electron acceptor molecules. The Fv/Fm ratio, used to express the chlorophyll fluorescence, was calculated according to Schreiber et al. (1986).

2.4. Malondialdehyde determination and antioxidant enzyme activities

At the end of the trial, 30 leaves per rootstock and treatment were harvested for biochemical analyses. Lipid peroxidation was estimated by measuring the concentrations of malondialdehyde (MDA) as reported by Heath and Packer (1968). Leaves were crushed in liquid nitrogen to obtain a fine powder. Afterwards, powdered plant tissues (0.5 g) were homogenized in 5 mL of potassium phosphate buffer 50 mM (pH 7.8), 1 mM EDTA, 1 mM DTT, 1 % PVP w/v, and 1 mM PMSF. Samples were filtered through 3 layers of gauze, and centrifuged at 15,000 rpm for 30 min at 4 °C.

The resulting supernatant was recovered, and the total proteins were precipitated with solid $(NH_4)_2SO_4$ at 55 % of saturation. Enzymatic activities were performed by using the total protein extract from leaves. Enzymatic aliquots were centrifuged at 13,000 rpm for 30 min at 4 °C, the supernatant was discarded, and the pellet was dissolved in 4 mL 50 mM sodium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 % (w/v) PVP-40 (w/v) and 1 mM PMSF (Donahue et al., 1997). Ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to Ushimaru et al. (1997) by assessing the decrease in absorbance at 290 nm, defining one unit (U) of APX equal to 1 mmol mL^{-1} ascorbate oxidized min^{-1} at 20 °C. Catalase (CAT, EC 1.11.1.6) was determined as described by Aebi (1984), by measuring spectrophotometrically (240 nm) the decomposition of H_2O_2. To avoid a rapid decrease of the initial velocity of the reaction, the assay was conducted using low concentrations of H_2O_2 (<0.05 M). The amount of enzyme able to decompose 1 mmol of H_2O_2 per minute represents one enzyme unit (U). Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) according to Masia (1998). One unit of activity (U) was defined as the amount of enzyme that would inhibit 50 % of NBT photoreduction at 560 nm. The total protein content was routinely determined by the Bradford (1976) method, using BSA as a standard curve.

The malondialdehyde (MDA) content was determined was calculated

by Heath and Packer (1968) modified.

$$MDA\ equivalents\ nmol\ mL^{-1} = (A532 - A600/155000) \bullet 106$$

532 nm is the absorbance peak of the TBA (Thiobarbituric Acid) -MDA complex, 600 nm is the correction factor for non-specific turbidity, 155000 the molar extinction coefficient of MDA.

2.5. Hormone analyses

Hormones analyzed were abscisic acid (ABA), phaseic acid (PA), salicylic acid (SA), indoleacetic acid (IAA), jasmonic acid (JA), JAIsoleucine (JA-Ile). Hormone extraction was carried out with 15 mg of freeze-dried leaves as described in Balfagòn et al. (2022). The resulting solution was filtered through 0.22 μm polytetrafluoroethylene membrane syringe filters (Albet S.A., Barcelona, Spain), diluted 1:4, and directly injected into an ultraperformance LC system (Acquity SDS; Waters Corp., Milford, MA, USA) connected online to a TQS triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) through an orthogonal Z-spray electrospray ion source. Chromatographic separations were carried out on a reversed-phase C18 column (Luna Omega Polar C18, 50 × 2.1 mm, 1.8-μm particle size; Phenomenex, Torrance, CA, USA) using an acetonitrile: water (both supplemented with formic acid at a concentration of 0.1 %) gradient at a flow rate of 300 μl min^{-1}. Hormones were detected following their specific precursor-to-product ion transition and quantitated using an external calibration performed with injection of standard solutions of known amount.

2.6. RNA extraction

RNA was extracted from fresh leaves sampled from three biological replicates at two stress conditions (control and 60 mM NaCl) and for the two genotypes Carrizo and Furr. Total RNA was extracted using the RNeasy® Plant Mini Kit of QIAGEN following the manufacture instructions.

The RNA concentration and quality were assessed using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). RNA integrity was verified by 1.3 % agarose gel electrophoresis.

2.7. RNA sequencing and identification of differentially expressed genes

Library preparation for Illumina sequencing was performed using standard Illumina protocols and Illumina paired-ends adapters to generate reads of 2 ×150 nucleotides. Sequencing has been performed at a depth of 20 M reads with Novaseq 6000 S4 on three biological replicates for each accession and thesis. RNA-seq reads obtained from the two rootstocks were aligned to the reference genome of *Citrus sinensis* (cv Valencia, GCF_000317415.1_Csi_valencia_1.0_genomic.fna) using the STAR (ultrafast universal RNA-seq aligner) software (Dobin and Gingeras, 2015). Reads count was performed using the FeatureCounts algorithm embedded in the R package Rsubread (Liao et al., 2019). Finally, the analysis of the differential expressed genes was carried out using the R package EdgeR (Empirical Analysis of Digital Gene Expression Data in R; Robinson et al., 2010).

To detect only those genes showing significant changes among each sample in the 2 theses (i.e. Carrizo control VS Carrizo stress and Furr control VS Furr stress) and showing differences in the salt response between the two samples (Carrizo stress VS Furr stress), a mixed model was followed taking into consideration both the variability across samples (Carrizo VS Furr) and across theses (control VS salt stress). The threshold for the identification of differentially expressed genes (DEGs) was set to 0.05 after correction for multiple testing. The raw data can be retrieved from NCBI under the following code PRJNA1119965.

2.8. Statistical analysis

Statistical analyses were performed using STATISTICA 6.0 (Statsoft Inc., Tulsa, OK) and used to test the significance of each variable ($P \leq 0.05$). A basic descriptive statistical analysis was followed by an analysis of variance test for mean comparisons. The method used to discriminate among the means (Multiple Range Test) was Fisher's Least Significant Difference (LSD) procedure at a 95.0 % confidence level. Principal component analysis (PCA) was performed using R software computing the "prcomp" function of the package 'tidyverse' (Wickham et al., 2019). The results were represented using the package 'ggplot2' (Wickham et al., 2016).

3. Results

3.1. Morphological measurements and leaf chlorophyll content determination in citrus rootstocks

Salt stress influenced plant growth and caused an evident defoliation of the rootstocks studied. At the end of the trial period, all rootstocks showed a decrease in leaf area in response to salt stress, except Bitters and Furr that did not show statistical differences compared to the control in response to 30 mM NaCl (Table 1). A severe foliar drop was observed already at 30 mM NaCl in Carrizo and C35 citranges and in *C. macrophylla* and *C. volkameriana.* All rootstocks subjected to 60 mM NaCl showed a significant decrease in leaf area (Table 1) and, in the last week of the trial, all plants of Carrizo citrange died.

Salt stress significantly reduced root length of C35 citrange at both concentrations by 28.1 % and 32.2 %, respectively, respect to control values. We also observed a decrease in root length at 30 mM NaCl for *C. macrophylla* (16.2 %), and at 60 mM NaCl for Carrizo citrange (31.7 %).

Generally, salt stress significantly reduced root and shoot dry weight with respect to the control values (Table 1). Moreover, reduction in specific leaf area in response to salt stress was observed for all the studied rootstocks with respect to control. In response to 60 mM NaCl, specific leaf area was always above 80 % of the control values, highest in Furr (97.7 %), followed by Carpenter (96.5 %) and Carrizo (95.2 %), the lowest reduction was observed in Volkameriana with 80.9 % and Citrumelo with 83 % respect to control values. When subjected to 30 mM NaCl, Carrizo citrange showed the highest reduction (91.4 %), followed by C35 (85.3 %), and Furr (77.9 %), whereas the lowest reduction was observed in Bitters (59.8 %), followed by Carpenter and *C. volkameriana* (60.8 % and 60.9 %, respectively).

A reduction of the specific root length (SRL) with increasing salt concentration was observed except for Furr, and Volkameriana that increased SRL values at both 30 and 60 mM NaCl (Table 1). C35, Citrumelo and Carpenter showed a significant reduction in SRL at the two NaCl or at least one of the concentrations, but, interestingly, Carrizo did not show any significant difference with the controls at any of the NaCl concentrations. Regarding shoot to root ratios, no statistical difference was observed in response to salt stress in any of the studied rootstocks (Table 1).

All citrus rootstocks showed similar performance of the SPAD index during the trial (Figure S1). We observed that SPAD values decreased compared to control when salinity concentration increased, especially after 213 DOY in Bitters. Carrizo citrange exhibited a high sensitivity to saline treatment not only with the lower SPAD values recorded, but also responding with a progressive phylloptosis. Total chlorophyll in leaves of *C. macrophylla* increased at 60 mM (Fig. 1), while decreased in *C. volkameriana* and Carrizo although the latter showed no statistical difference with control. At 30 mM NaCl we observed the highest chlorophyll reduction in Carrizo citrange (38 %), while at 60 mM NaCl the highest reduction was observed in *C. volkameriana* (35 %).

Table 1
Values of morphological parameters measured on 8 citrus rootstocks subjected for 78 days to different saline irrigation treatments.

	Leaf area (m^2)			Root length (cm)			Specific leaf area ($cm^2\ g^{-1}$)			Specific root length ($cm\ g^{-1}$)			Shoot to Root Ratio ($g\ g^{-1}$)		
	Control	30 mM NaCl	60 mM NaCl	Control	30 mM NaCl	60 mM NaCl	Control	30 mM NaCl	60 mM NaCl	Control	30 mM NaCl	60 mM NaCl	Control	30 mM NaCl	60 mM NaCl
Carrizo	3.18 a	0.36 b	0.04 b	40.00 a	38.00 a	27.33 b	451.8 a	38.7 b	21.6 b	1.50 a	1.60 a	1.51 a	1.8 a	1.8 a	1.7 a
C35	4.44 a	0.55 b	0.03 b	40.33 a	29.00 b	27.33 b	533.8 a	78.4 b	27.3 b	1.59 a	1.02 b	1.17 b	1.7 a	1.4 a	1.2 a
Bitters	1.07 a	1.05 a	0.10 a	45.67 a	45.00 a	40.33 a	299.4 a	120.3 b	20.0 b	2.44 a	2.16 a	2.37 a	2.2 a	2.1 a	1.7 a
Carpenter	1.82 a	1.30 b	0.07 b	45.33 a	41.00 b	40.00 b	445.2 a	174.3 b	15.5 b	3.27 a	2.92 b	2.02 b	2.1 a	1.9 a	1.3 a
Furr	2.47 a	1.62 a	0.14 b	39.00 a	39.00 a	32.00 b	578.0 a	127.5 b	13.1 b	1.78 b	2.68 a	2.38 a	2.0 a	2.0 a	2.0 a
Citrumelo	3.29 a	0.65 b	0.25 b	40.33 a	40.67 a	32.67 b	397.4 a	92.2 b	67.7 b	1.60 a	1.70 a	1.38 b	1.5 a	1.5 a	0.9 a
Macrophylla	1.70 a	0.45 b	0.09 b	33.00 a	27.67 a	26.00 a	126.6 a	35.7 b	14.1 b	1.48 a	1.38 a	2.04 a	1.6 a	1.5 a	1.6 a
Volkameriana	3.90 a	0.60 b	0.17 b	34.33 a	32.00 b	27.33 b	221.1 a	86.4 ab	42.1 b	1.19 b	1.55 ab	1.60 a	3.0 a	2.0 a	2.0 a

Values followed by the same lowercase letter, within the same row, are not significantly different according to Fisher's LSD procedure at a 95 % confidence level.

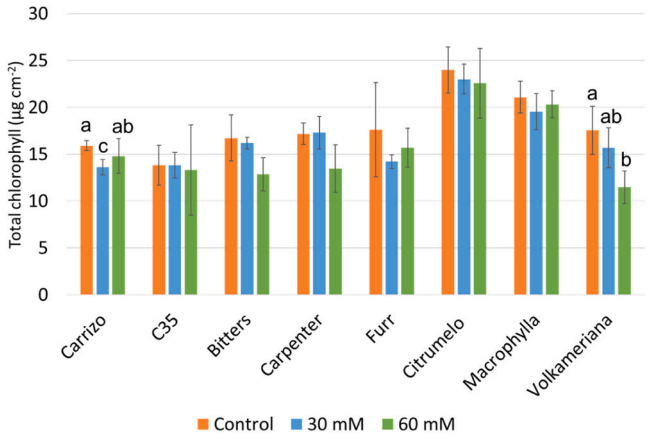

Fig. 1. Leaf chlorophyll content (µg cm^{-2}) measured at 262 DOY in 8 rootstocks irrigated with saline water. Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level.

3.2. Physiological monitoring of the plants

Net photosynthetic rate (A), transpiration (E) and stomatal conductance (g_s) decreased in response to increasing salt treatments in all rootstocks (Fig. 2 and S2) from 185 to 262 DOY. Regarding the net photosynthesis, at the end of the trial the highest decrease of A was observed in C35 (50.0 %) and in Carrizo citranges (40.7 %) at 30 mM NaCl, while Furr, Bitters and Carpenter citrandarins exhibited the lowest reduction compared with the control (10.9, 23.8 and 23.9 % respectively).

At the end of the experiment, both treatments induced a significant reduction of transpiration rates and stomatal conductance in almost all rootstocks (Figure S2). Furr plants watered at 30 and 60 mM NaCl recorded slight differences with respect to the control of E and g_s monitored during the investigation (Figure S2).

The Fv/Fm ratio decreased in C35 citrange compared to the control when grown in saline environment at the end of the surveys (from 233 to 262 DOY) (Fig. 3).

3.3. Malondialdehyde determination and antioxidant enzyme activities

The salt-susceptible rootstocks, i.e. C35 and Carrizo citranges, showed the highest values of MDA at 60 mM NaCl (2.3 and 3.3 mg g^{-1}, respectively) (Fig. 4). Also Bitters and *C. macrophylla* exhibited at 60 mM NaCl a significant increase with respect to the control, even if differences were slighter than values reported for the previous mentioned rootstocks. Contrarily, MDA values decreased in Carpenter and Furr rootstocks at increasing salinity levels.

Values of SOD activity significantly increased only in Carrizo citrange and Furr at the highest salt concentration, on the contrary SOD activity decreased in Bitters, Carpenter, and *C. macrophylla* (Fig. 4). Only Carrizo and C35 citranges increased CAT activity as stress increased, while Bitters, Carpenter, Furr, and *C. macrophylla* showed a progressive reduction of CAT activity in the highest salinity environment. The activity of APX decreased significantly in Carrizo citrange at 30 and 60 mM NaCl, on the contrary in C35 the highest APX value was recorded at 60 mM NaCl (Fig. 4).

3.4. Hormone analyses

At the end of the experiment, salt stress caused great accumulation of ABA in Carrizo citrange at 30 mM NaCl and in C35 citrange at 60 mM NaCl compared with control (Fig. 5), although the latter did not have a statistical difference with respect to the control. Bitters, Carpenter and Furr citrandarins showed a similar trend to the control, while a progressive increase in Citrumelo, *C. macrophylla* and *C. volkameriana* was recorded due to the increase in salt.

Bitters, Carpenter and Furr citrandarins exhibited a significant increase in PA at 60 mM NaCl.

Carrizo and C35 significantly increased JA at 30 mM, while a progressive decrease at increasing salinity levels was recorded in Carpenter, Citrumelo and *C. macrophylla*. Regarding JA-lle hormone a similar trend in respect of the control was observed in all rootstocks.

At 30 mM NaCl C35 showed the highest accumulation of SA, followed by Bitters, while at 60 mM NaCl the highest value of SA was observed in C35, Carrizo, Bitters and Carpenter, although no statistical difference was found for SA.

The inactive form of SA (as glucosides of SA, SAG) showed an increment in C35, Bitters, Carpenter and Furr at increasing NaCl levels.

A progressive increase of IAA was recorded in Carrizo citrange at increasing saline conditions, while Bitters and Furr showed an increment only at 60 mM NaCl.

3.5. Principal component analysis and Pearson's correlation test

A principal component analysis (PCA) on the physiological, chemical and hormones parameters was performed to assess the differential response of the eight rootstocks in analysis (Fig. 6, Supplementary Table 1). The first two principal components (PCs) accounted for the 57.9 % of total variance (PC1 = 31.1 %; PC2 = 26.8 %). The main variables contributing to PC1 were JA-lle- (0.360), followed by gs (0.267) and A (0.248). CAT (0.263), Clh_tot (0.236) and SPAD (0.230) were instead highly correlated with the variation in the PC2 values. Specifically, when analyzing the rootstocks, it was observed that Furr, Bitters and Carpenter citrandarins were greatly affected by physiological measurements and PA hormone. On the contrary, these parameters exhibited a negative correlation with C35 citrange. Swingle Citrumelo, *C. macrophylla* and *C. volkameriana* demonstrated a positive correlation with JA-lle, total chlorophyll content and SPAD, while Carrizo and C35

Fig. 2. Net photosynthesis values (A, µmol CO_2 m^{-2} s^{-1}) determined in 8 rootstocks irrigated with saline water from 185 to 262 day of the year (DOY). Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level.

citrange were greatly affected by malondialdehyde, antioxidant enzyme activities (APX, CAT and SOD), SA and ABA.

Correlation coefficients of physiological, chemical and hormones parameters were shown in Fig. 7. ABA value was positively correlated with APX activity and MDA levels, while JA presented a high positive correlation with E and A values and a negative correlation with CAT. (Fig. 7). JA-lle showed a negative correlation with ABA, APX and MDA values. SA was negatively associated with gs and total chlorophyll content; on the contrary, its inactive form, SAG, was positively correlated with PA, E, and SOD. IAA showed a positive correlation with SOD, while it was negatively associated with ABA. SOD, APX and MDA were highly correlated among them.

The accumulation of CAT was negative correlated with physiological parameters, especially A and E, because as CAT increases, gas exchange decreases. Indeed, we observed a negative correlation between MDA, A and E. Furthermore, the antioxidant enzyme activities and malondialdehyde were correlated positively with ABA and SA.

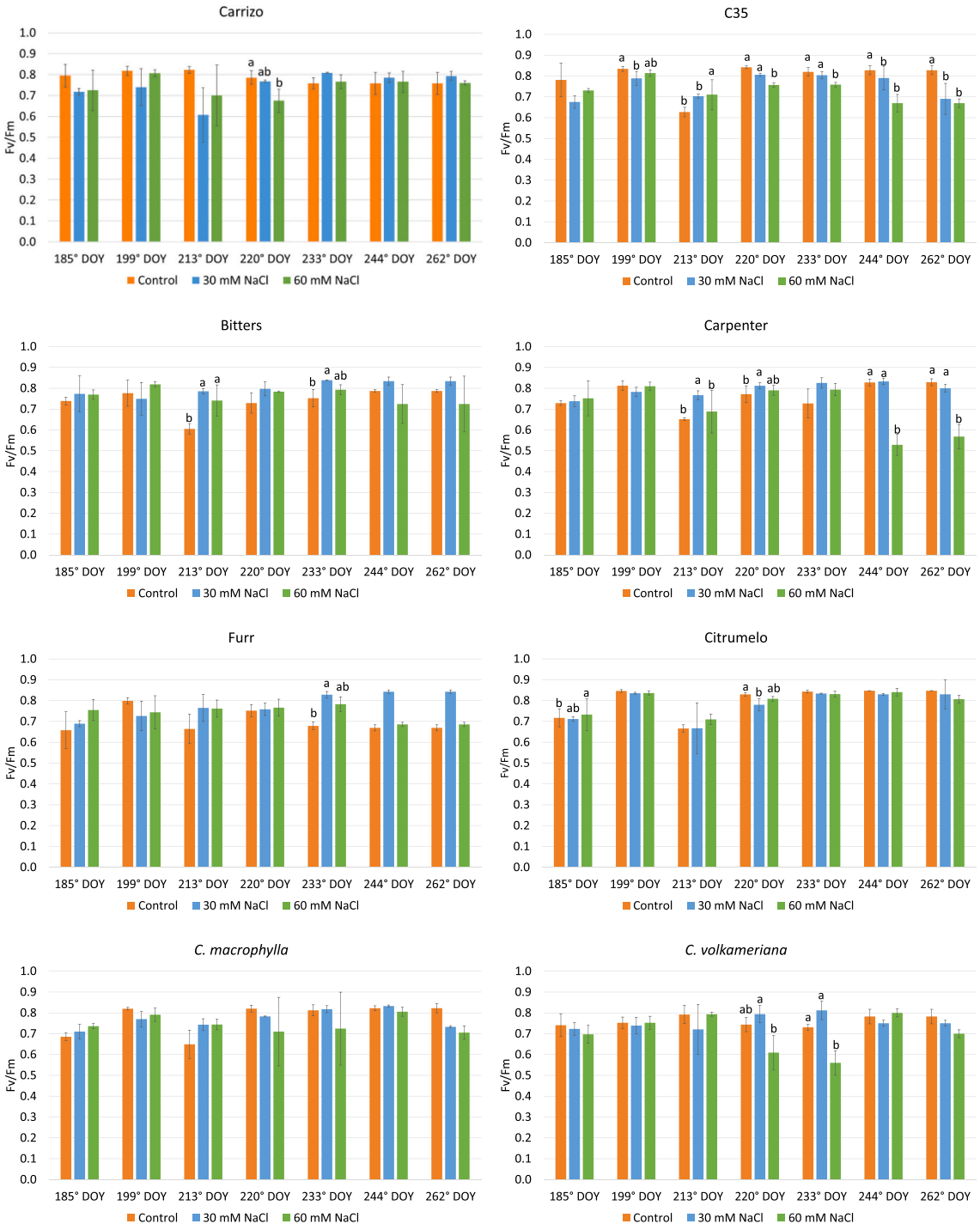

Fig. 3. Chlorophyll fluorescence (Fv/Fm) determined in 8 rootstocks irrigated with saline water from 185 to 262 day of the year (DOY). Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level.

3.6. RNAseq analysis

The RNAseq analysis was conducted on the two most divergent genotypes Carrizo (salt susceptible) and Furr (salt tolerant). This approach, aimed at understanding the molecular mechanisms involved in salt tolerance, was based on a differentially expressed genes analysis. For each biological replicate, an average of 18 Mb data was generated and raw reads were aligned against the reference genome of sweet orange (CV Valencia). The count of the reads for each gene was performed and the resulting log transformed data was employed for a PCA analysis to evaluate the variability within and between the thesis in exam (Fig. 8A). The first two principal components (PCs) explained the 56.4 % of the total phenotypic variability (PC1 = 33.6 %, PC2 = 22.8 %). The thesis showing the highest within-group variability was Furr under salt stress (furr_salt) while the others showed a lower variability between the three biological replicates. PC1 was mostly related with the response to salt stress with the controls of both rootstocks characterized by negative PC1 values while Furr, and especially Carrizo, under salt stress were characterized by higher PC1 value. Carrizo showed the highest variability among the control (PC1 < 50) and the stressed samples (PC1 > 125)

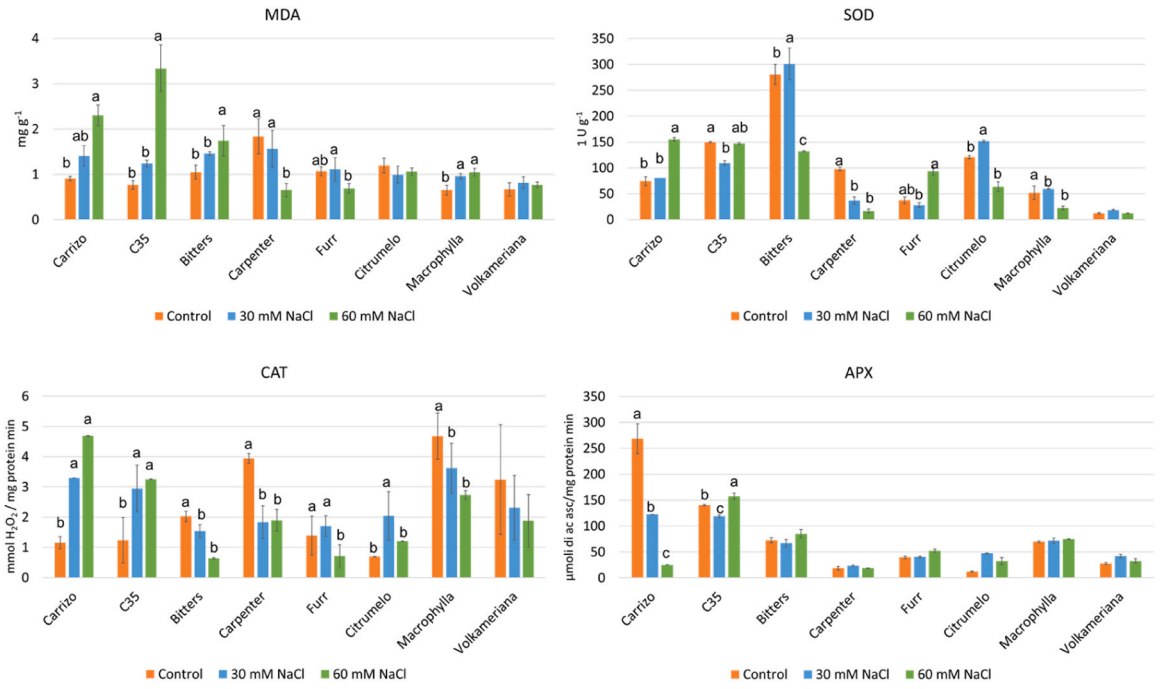

Fig. 4. Malondialdehyde content (MDA, mg g^{-1}), catalase (mmol H_2O_2/mg protein min), superoxide dismutase (SOD, 1 U g^{-1}), ascorbate peroxidase (APX, µmol of ascorbic acid/mg of protein min) measured at the end of the trial in the leaves of 8 rootstocks irrigated with saline water. Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level.

highlighting the higher susceptibility to the stress compared to Furr. The analysis of the differential expressed genes led to the detection of 851 significant DEGs (Fig. 8B). A functional annotation was carried out leading to the detection of a subset composing of 42 genes being functionally associated with the response to abiotic stress either in Citrus or in other species (Supplementary Table 2). A gene ontology analysis revealed the occurrence of three genes related to both response to stress (GO:0006950) and defense response (GO:0006952); in particular the genes NP_001275834.1 and XP_015386208.2 (expressing a chitinase and a NB-ARC domain respectively) were overexpressed in Carrizo, while the gene XP_006483958.2 belonging to the 'GDSL' lipolytic enzyme family were overexpressed in Furr. Compared to Furr, enriched GO terms in Carrizo involved the response to abiotic stimulus (GO:0009628; XP_015387379.1 gene) and to inorganic substance (GO:0010035; NP_001275834.1 gene; Supplementary Table 2).

4. Discussion

Citrus species are salt-sensitive and salinity affects plant growth and production (Vives-Peris et al., 2023); the effects of saline irrigation on citrus has been mostly investigated during plant growth and development (Vives-Peris et al., 2023), evaluating the physiological (Aparicio-Durán et al., 2021), biochemical (Arbona et al., 2003; Gómez-Cadenas et al., 2003) or molecular response (Alvarez-Gerding et al., 2015).

Recent studies (Colmenero-Flores et al., 2020 and references therein; Aparicio-Durán et al., 2021; Othman et al., 2023) reported that salt stress reduced citrus rootstock development, such as height, stem diameter, leaf area and root growth, although the symptomatic or asymptomatic responses of rootstocks depended on the saline concentration in the water. Previous studies demonstrated that the responses to salinity in terms of symptoms or mortality was related to the duration of the stress, the rootstock genotype and the age of the plant (Alvarez-Gerding et al., 2015; Aparicio-Durán et al., 2021). In fact, in relation to the damage caused by saline irrigation, the highest susceptibility was detected on plants grafted onto Carrizo citrange (Shahid et al., 2019; Vives-Peris et al., 2023). Similarly, we observed that salinity reduced plant growth, and a severe foliar drop occurred in Carrizo and C35 citranges and in *C. volkameriana* already at 30 mM NaCl (Table 1); contrarily, previous studies reported *C. volkameriana* being a moderately salt-tolerant rootstock (Othman et al., 2023).

Carrizo citrange is known to be a salt-sensitive rootstock (Shahid et al., 2019; Vives-Peris et al., 2023) and nearly all plants of Carrizo citrange treated with 60 mM NaCl had died at the end of the trial. In our results C35 citrange also exhibited a high sensitivity to salt stress, accordingly with Lopez-Climent et al. (2008).

SPAD values were mostly used to detect nutrient deficiencies (such as chlorosis and necrosis) or other physiological problems related to chlorophyll and nitrogen deficiency in the leaves (García-Sánchez et al., 2002). In this context, a progressive decrease in SPAD values was recorded in all rootstock genotypes when saline concentration increased (Figure S1) accordingly with previous work on mandarin cultivars grafted onto different rootstocks (García-Sánchez et al., 2002).

In general, citrus rootstocks irrigated with 60 mM NaCl, showed a marked reduction in leaf chlorophyll concentration, as previously stated (García-Sánchez et al., 2002), however, Carrizo citrange treated with 60 mM NaCl slightly increased both SPAD values and total chlorophyll content compared to the control, confirming previous studies (Aparicio-Durán et al., 2021).

The reduction in plant growth is closely related to the decline of physiological processes; previous researches described that the decrease in growth and gas exchanges in Carrizo were likely caused from ion toxicity of salt accumulated in leaves, as salinity reduced both leaf water potential and CO_2 assimilation (Shahid et al., 2019). A reduction in net photosynthesis in C35 and Carrizo citrange plants at 30 mM NaCl was observed, while a good response was detected in Furr, Bitters and Carpenter citrandarins that showed the lowest reduction compared to the control (Fig. 2).

Citrus macrophylla is often used in combination with lemon cultivars and it is considered salt tolerant, even if when soils have a salinity level above its tolerance threshold it may not exclude chloride absorption (Alvarez-Gerding et al., 2015). At the end of the experiment, plants of

Fig. 5. Hormones measured at the end of the trial in leaves of 8 rootstocks irrigated with saline water. ABA: Abscisic acid (ng g^{-1} dry weight), JA: Jasmonic acid (ng g^{-1} d. w.), SA: Salicylic acid (ng g^{-1} d. w.), SAG: Salicylic acid glucoside (ng g^{-1} d. w.), IAA: Indole acetic acid (ng g^{-1} d. w.), Ja-Ile: Jasmonoyl-Isoleucine (ng g^{-1} d. w.) and PA: Phaseic acid (ng g^{-1} d. w.). Values without letters have no significant differences according to Fisher's LSD procedure at 95 % confidence level.

C. macrophylla highlighted a similar response to the control at both stress levels, in particular for stomatal conductance and transpiration rates (Figure S2).

The study of chlorophyll fluorescence (Fv/Fm ratio) is widely used as a nondestructive diagnostic tool in photosynthesis research in order to investigate the adaptive mechanisms activated by biotic and abiotic stresses in plants (Bleda et al., 2011). A reduction in Fv/Fm ratio in C35 and Carpenter at 60 mM NaCl was noted (Fig. 3), confirming that salinity treatment inhibited chlorophyll fluorescence in salt-sensitive rootstocks (Bleda et al., 2011). Contrarily, Furr citrandarin showed an increase in chlorophyll fluorescence at both levels of stress.

Among the several ROS produced by stress, H_2O_2 formed in peroxisomes and chloroplasts might diffuse to the cytosol, where reacted with transition metal ions (Fe^{2+}) during the Fenton reaction, releasing hydroxyl radical (OH·) that is considered as the main cell-damaging product responsible for lipid peroxidation of membrane (Arbona et al., 2008). MDA is one of the final peroxidation products of polyunsaturated fatty acids in the cells, and its overproduction is linked to an increase in

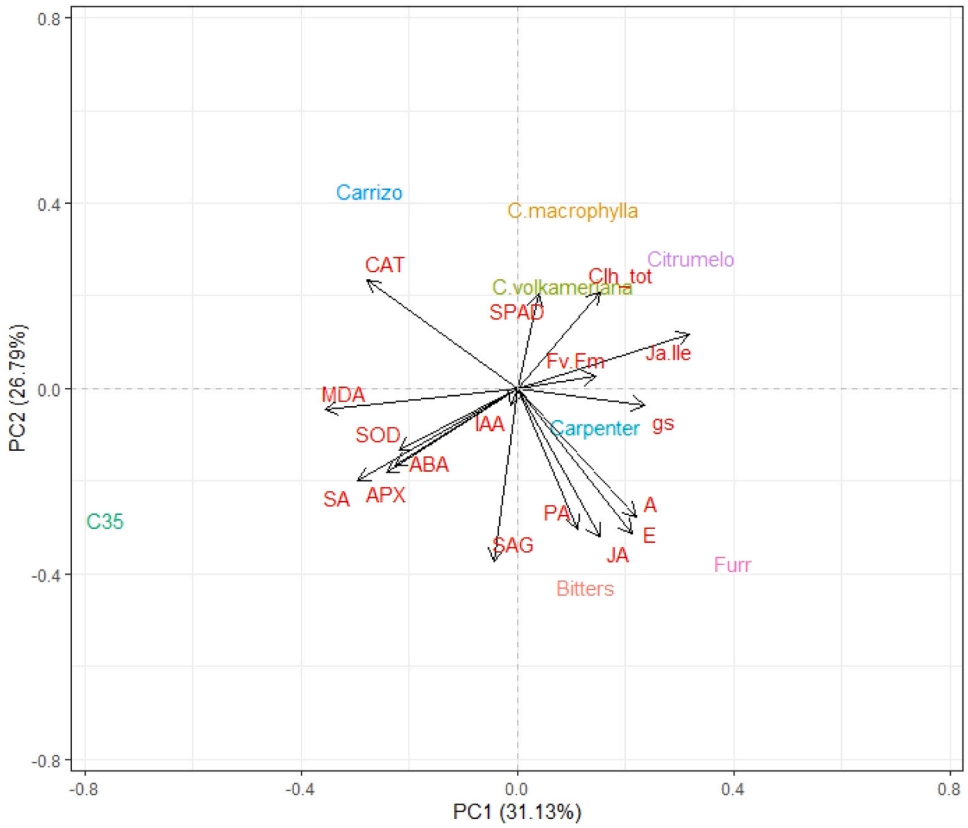

Fig. 6. Principal component analysis (PCA) showing the distribution of the eight rootstocks irrigated with saline water. The parameters measured were absicissic acid (ABA), jasmonic acid (JA), salicylic acid (SA), salicylic acid glucoside (SAG), indole acetic acid (IAA), jasmonoyl-Isoleucine (Ja-Ile), Phaseic acid (PA), physiological net photosynthesis (A), transpiration rate (E), stomatal conductance (gs), chlorophyll fluorescence (Fv/Fm), Chlorophyll index (SPAD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), malondialdehyde content (MDA), total chlorophyll (Clh_tot).

free radicals, hence MDA level is commonly known as a marker of oxidative stress in cells. Therefore, we observed that MDA showed a negative correlation with net photosynthesis, transpiration rate and stomatal conductance (Fig. 7). It was previously stated that salt-sensitive genotypes showed higher MDA content than tolerant genotypes under stress conditions (Arbona et al., 2003; Arbona et al., 2008). Indeed, the highest values of MDA were recorded in C35 citrange and Carrizo citrange under 60 mM NaCl stress, accordingly with above authors already reported the sensitivity of C35 citrange, an emerging rootstock in Italian citrus industry (Modica et al., 2022), to saline conditions. Salt treatments caused oxidative stress and interfered with the antioxidant activity of enzymes generated during plant metabolism. In fact, abiotic stress promotes an imbalance of metabolism and causes the over-accumulation of ROS limiting CO_2 availability due to stomatal closure. ROS-scavenging enzymes are used to prevent damage due to ROS accumulation, especially the enzymes belonging the ascorbate-glutathione pathway (Zandalinas et al., 2017). It is known that in the ascorbate-glutathione pathway two key enzymes were identified assuming to deal with the detoxification of ROS in plants. The first is superoxide dismutase (SOD) which converts superoxide to molecular oxygen and hydrogen peroxide. This latter toxic product is eliminated by catalase (CAT), converting the H_2O_2 into water and molecular oxygen, and by ascorbate peroxidase (APX), at expenses of oxidizing ascorbate to monodehydroascorbate. These enzymes responded to oxidative stress and were positively correlated to stress tolerance (Arbona et al., 2008; Zandalinas et al., 2017). Contrarily, the highest increase with respect to the control of antioxidant enzyme activities was recorded in Carrizo and C35 citranges, recording the highest amount of MDA accumulation. Nevertheless, according to our results showing that MDA and enzyme activities do not have always the same trend, other authors evidenced that in other species (*Oryza sativa*) a salt-sensitive genotype does not change the level of H_2O_2, while MDA content increased (Garcia-Caparros et al., 2021). Although, we observed a positive correlation between MDA and antioxidant enzyme activities (Fig. 7), linking oxidative damage to the induction of antioxidant defenses (Arbona et al., 2008).

The reduction of SOD and CAT activities in Bitters, Carpenter and *C. macrophylla* in response to salt stress suggests that the antioxidant activity is a genotype-related trait. These findings are in accordance with Almansa et al. (2002), who showed that lemon plants (cv. Verna) grafted on *C. macrophylla* or *C. reticulata* reduced SOD activity in response to salinity (28 mM NaCl) whereas they increased SOD activity when grafted on *C. aurantium*. Therefore, antioxidant activity related traits can also be transferred from the rootstock to the grafted variety. We also observed a high negative correlation of CAT with A and E, probably related to the induction of CAT and the salinity-induced reduction of A and E.

The progressive accumulation of salt ions in plant tissues leaded to an increase in antioxidant enzymes and ABA content decreasing stomatal conductance (Gómez-Cadenas et al., 2003), as ABA regulates stomatal closure to prevent water loss caused by osmotic stress for the elevated saline concentration in water (Gómez-Cadenas et al., 2003). ABA plays a pivot role to modulate stomatal opening and transpiration, greatly influencing citrus salt tolerance (Gómez-Cadenas et al., 2003). However, there was no correlation between stomatal closure and ABA, while gs was negatively correlated with SA, CAT and MDA. Indeed, significant accumulation of PA was recorded in Bitters, Carpenter and Furr at 60 mM NaCl. In addition, plant responses to salinity stress was explained by the positive correlation of ABA levels with APX activity and MDA content, as reported in Fig. 7, which could be a direct link

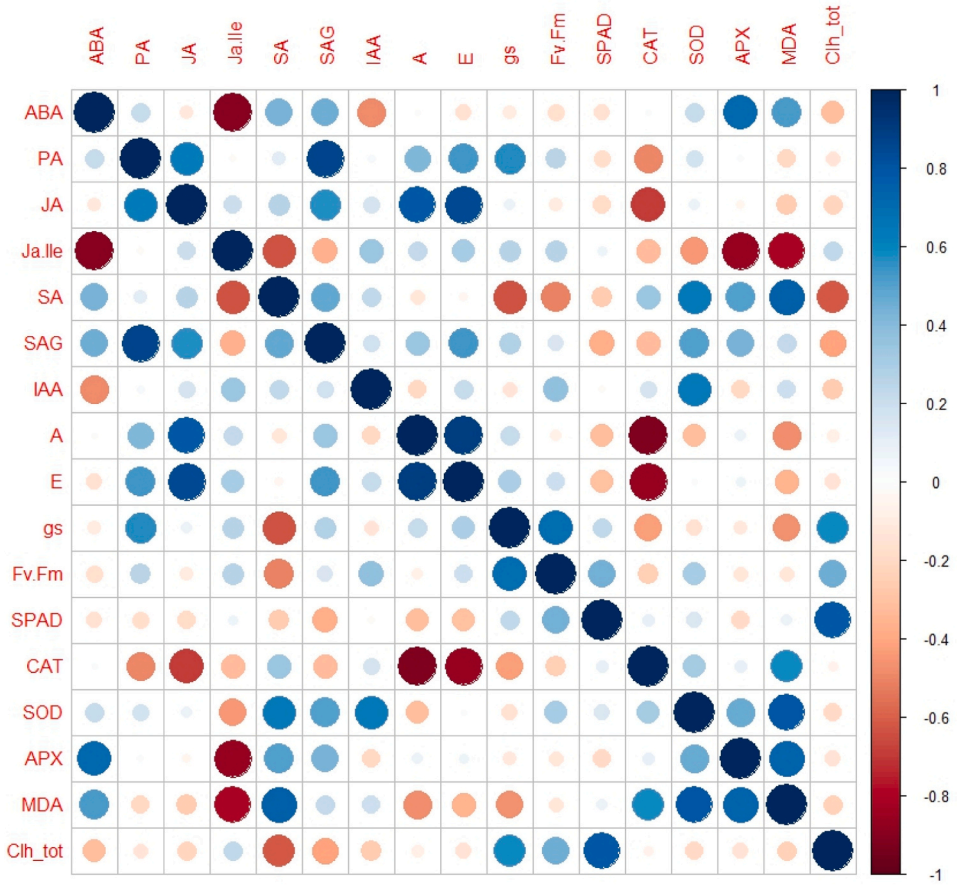

Fig. 7. Pearson's correlation coefficients among the parameters measured in the eight rootstocks irrigated with saline water. The parameters measured were absicissic acid (ABA), jasmonic acid (JA), salicylic acid (SA), salicylic acid glucoside (SAG), indole acetic acid (IAA), jasmonoyl-Isoleucine (Ja-lle), Phaseic acid (PA), physiological net photosynthesis (A), transpiration rate (E), stomatal conductance (gs), chlorophyll fluorescence (Fv/Fm), Chlorophyll index (SPAD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), malondialdehyde content (MDA), total chlorophyll (Clh_tot).

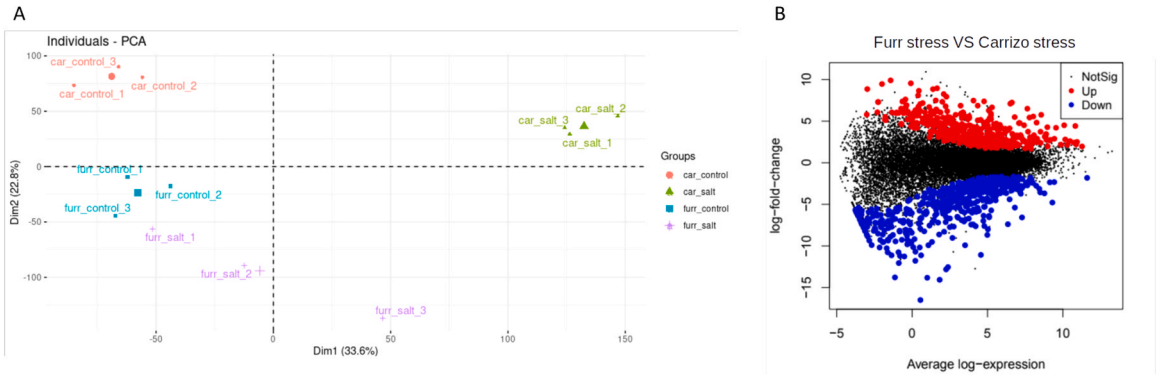

Fig. 8. A: Principal Component Analysis (PCA) of the log transformed expression data of 'Carrizo' and 'Furr' under salt stress (car_salt and furr-salt) and the respective controls (car_control and furr_control). B: Scatterplot highlighting the genes being significantly upregulated or downregulated (red and blue dots respectively) in Furr compared to Carrizo. In the x axis the differences in the number of reads between the thesis was reported while the y axis represented the fold change. The significant threshold was 0.05 after correction for multiple testing (FDR). Black dots represent genes whose expression did not pass the significant threshold.

ABA-ROS, causing oxidative damage, the production of H_2O_2 and stimulating antioxidant enzymatic activity (Arbona et al., 2003). As a whole, ABA content was similar in all treatments in leaves of Bitters, Carpenter and Furr citrandarins, while saline water caused great accumulation of ABA in Carrizo and C35 citranges (Fig. 5).

Jasmonic acid induces the transcription of several genes involved in defensive plant metabolism, including salt stress (Mahmoud et al., 2021). In particular jasmonates, e.g. JA and jasmonate iso-leucine conjugate (Ja-Ile), are oxygenated derivatives of fatty acids widely acknowledged as plant-signaling molecules. Jasmonates have been traditionally involved in plant-arthropod interactions, particularly those that cause a mechanical damage to plant tissues (de Ollas et al., 2013; Agut et al., 2016; Balfagòn et al., 2022) and also have been associated with resistance to abiotic stress; indeed, they are involved in the

activation of the antioxidant system, in the accumulation of amino acids and soluble sugars in plant tissues and in the regulation of stomatal opening and closing (Balfagòn et al., 2022). A different accumulation of JA between the rootstocks tested was clearly demonstrated in our results. More precisely, no significant difference was observed in Furr and *C. volkameriana* at 30 and 60 mM NaCl compared to the control. Previous investigations indicated that JA content did not change in salt-tolerant plants (Ali and Baek, 2020) and this could confirm the thesis that Furr rootstock has a good tolerance to salt stress. A significant increase in JA content was observed in Carrizo and C35 citranges at 30 and 60 mM NaCl compared to the control, and it corresponded to an increase in MDA content confirming their sensitivity to salt stress (Arbona et al., 2003; Arbona et al., 2008). Furthermore, we noticed that jasmonates had a negative correlation with the antioxidant enzyme activities and MDA (Fig. 7); probably hormonal signaling profiles were involved in the induction of other mechanisms, different than the enzymatic antioxidant mechanism, such as the production of secondary metabolites. These results confirmed that plants alter their metabolism in different ways, causing diverse secondary metabolite profiles, as reported by previous researcher (Balfagón et al., 2022).

The abiotic stress affected JA hormone which undergoes epimerization to form Ja-Ile, accumulating in the cytoplasm of the stressed leaves for defensive responses (Ali and Baek, 2020). A similar trend was observed in all rootstocks at 30 and 60 mM NaCl compared to the control in the experiment carried out. Moreover, it has been shown that SA plays an important role in abiotic stress inducing thermo-tolerance in citrus plants. SA accumulation was associated to a decrement in antioxidant activity and protection of the photosynthetic machinery avoiding electron leakage (Mahmoud et al., 2021). Although, it emerged that SA was positively correlated with antioxidant enzyme activities and MDA, while it was negatively correlated with gs, FvFm and chlorophyll content (Fig. 7). In the present study, SA levels increased in response to salt stress in all genotypes, although a decrement was observed in Furr at 30 and 60 mM NaCl compared to the control, although no statistical differences were detected. In any case, we observed an increase of the inactive form of SA (SAG) in C35 citrange, Bitters, Carpenter and Furr. Previous studies indicated that citrus rootstocks showed an increase in abiotic stress conditions of PA and DPA concentration, the main ABA degradation products, and a significant PA accumulation under abiotic stress in Carrizo citrange was observed (Mahmoud et al., 2021); in our results an increasing trend of PA as the amount of salt increased was observed in C35 and Carrizo citranges, even if the latter until 30 mM NaCl and no statistical difference was recorded. A significant accumulation of PA was observed in Bitters, Carpenter and Furr.

The result of PCA (Fig. 6) showed that Bitters, Carpenter and mostly Furr citrandarins grouped together demonstrating their positive behavior in response to salt stress, while Carrizo and C35 citranges, that showed overall a high sensitivity to saline conditions, positioned in the opposite side. The heatmap (Fig. 7) showed the consistence of many parameters among the studied ones, revealing that hormone analisys and antioxidant enzyme activities are helpful tools in searching salt tolerance.

The analysis of the transcriptomic profiles of Carrizo and Furr highlighted a differential genetic regulation on several genes that were already associated with the response to abiotic stress either in Citrus or in other species. In particular, the gene XP_006471729.1 belongs to the EF-hand type calcium binding domain that is involved in the salt overly sensitive (SOS) signaling pathway playing an important role in triggering the salinity tolerance response in plants (Ren et al., 2007; Zhang et al., 2016).

The gene NP_001275792.1 (overexpressed in Furr compared to Carrizo, fold change = 4.21, supplementary table 2) belongs to the NAC transcription factor gene family, and previous studies highlighted its involvement in the response against multiple abiotic stresses (especially salt and cold stress) in both leaves and roots of *Citrus reshni* (de Oliveira et al., 2011). Considering that the gene expression was induced by exogenous ABA treatments, it has been shown that NACs can regulate abiotic stress responses via both ABA-dependent and ABA-independent pathways (de Oliveira et al., 2011).

A second gene overexpressed in Furr compared to Carrizo encoded a glutathione S-transferase (GST, XP_015386930.1, fold change = 4.44, supplementary table 2). Ji et al. (2010) isolated the GsGST gene from wild soybean (*G. soja*) and observed an increase in drought and salt tolerance in transgenic tobacco overexpressing the gene. The role of GST in salt stress was also investigated in citrus in which played an important role in the cell protection against ROS (Mahmoud et al., 2021).

5. Conclusions

Salinity of irrigation water is one of the main environmental problems affecting citriculture. The high sensitivity in citrus causes a defoliation and a remarkable decrement in leaf area. Carrizo citrange had a severe foliar drop while C35 citrange showed the highest reduction of root length. Our findings reveal that salt stress showed a clear impairment in photosynthesis and decrement of transpiration, stomatal conductance, and chlorophyll fluorescence, while an increase in the concentration of MDA and CAT, especially in most sensitive rootstocks. The results of physiological, enzymatic and hormonal analyses could be useful to assume that the studied citrandarins, i.e. Bitters, Carpenter and especially Furr, could be considered as salt-tolerant rootstocks.

CRediT authorship contribution statement

Alberto Continella: Writing – review & editing, Project administration, Methodology, Funding acquisition. **Giulia Modica:** Writing – original draft, Investigation, Formal analysis. **Di Guardo Mario:** Writing – original draft, Methodology, Funding acquisition, Formal analysis. **Ivana Puglisi:** Writing – review & editing, Supervision, Methodology. **Andrea Baglieri:** Writing – review & editing, Supervision, Methodology. **Fortuna Sefora:** Investigation. **Fabio Arcidiacono:** Investigation. **Daria Costantino:** Investigation. **Stefano La Malfa:** Writing – review & editing, Supervision, Funding acquisition. **Alessandra Gentile:** Writing – review & editing, Supervision, Funding acquisition. **Vicente Arbona:** Writing – review & editing, Supervision, Methodology.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2024.105835.

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