Plant Stress 10 (2023) 100264

Contents lists available at ScienceDirect

Plant Stress

journal homepage: www.sciencedirect.com/journal/plant-stress

Early salinity response in root of salt sensitive *Olea europaea* L. cv Leccino

Mirko Sodini, Alessandra Francini *, Luca Sebastiani

Crop Science Research Center, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, I-56127 Pisa, Italy

ARTICLE INFO

Keywords:
Sodium antiporter
SOS
P-ATPase
Olea europaea

ABSTRACT

The present study evaluates the molecular mechanisms involved, under salt stress, in the roots of *Olea europaea* L. cultivar Leccino (salt sensitive). We hypothesized that during the first phase of salt application the inactivation of proton pumps H^+-*ATPases* and sodium antiporter Na^+/H^+ in roots determine the sodium translocation in the shoot. To verify this hypothesis, plants of olive tree (cv. Leccino) were treated with 60 mM NaCl salt and sampled after 24 h, 48 h, and seven days.

Data proved that Leccino's stem accumulate Na^+ after seven days of exposure. In root, the relative expressions of eight genes (five *P-ATPase*, one *V-ATPase sub E, SOS1,* and *NHX*) indicated that NaCl treatment led to a reduction in *P-ATPase 1* and *SOS1* expressions after 24 h, then *NHX* and *V-ATPase sub E* after 48 h and finally *P-ATPase 8* after 7 days. The reduction of the electrochemical proton gradient due to the under-expression of *P-ATPase* is consistent with the accumulation of sodium in the roots and inactivation of *SOS1* and the vacuolar Na^+/H^+ exchanger. These two genes have a clear biological role in the exclusion of sodium from the cytosol, mobilizing it outwards.

In conclusion, these results provide the evidences that H+-pumping expression was decreased by salinity treatment in the early phase of root salt response in sensible olive cultivar Leccino.

Introduction

Salt stress is a major problem for crop yield in the Mediterranean and semi-arid regions (Askari et al., 2006) that will also face an increase in soil salinity due to climate change. Plants exposed to salinity undergo to sodium mobilization and exclusion mechanisms (Munns and Tester, 2008; Shabala et al., 2013). These mechanisms induce the compartmentation of sodium at the cellular and intracellular levels avoiding toxic concentration in the cytoplasm. Moreover, sodium could be retained in the roots ensuring a limited translocation into the shoots (Munns and Tester, 2008).

The responses to salinity stress occurs in two phases: a) osmotic phase and b) ionic phase (Munns and Tester, 2008). Usually, the osmotic stress has an immediate effect on growth rates compared to ionic stress. In sensitive species the ionic effect dominant the osmotic one due to the lack of the ability to control Na^+ transport (Munns and Tester, 2008). Under salinity stress, the Na^+ accumulation in plant cells is determined by the ion-exchange activity of Na^+ efflux and influx membrane pumps. The most widely studied are plasma membrane Na^+/H^+ antiporter (SOS1), vacuolar Na^+/H^+ antiporter (NHX), P-type plasma membrane H^+-ATPase (P-ATPase), V-type vacuolar H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (PPase) (Apse and Blumwald, 2007; Barkla et al., 1995; Munns and Tester, 2008; Zhao et al., 2021). These pumps are responsible for the compartmentalization in the tonoplast, xylem Na^+ loading, and the exclusion of Na^+ in the extracellular environment (Pardo et al., 2006; Tester and Davenport, 2003; Zhu, 2002).

The olive tree is a relevant crop in the Mediterranean and semi-arid regions, and regarding salinity has an intermediate behavior between glycophyte and halophytes. The tolerance/sensitivity of olive trees to salinity is cultivar dependent (Tattini et al., 1992; Tattini, 1994; Chartzoulakis, 2005; Perica et al., 2008; Kchaou et al., 2010) and also depends on plant age and agronomic condition (Chartzoulakis, 2005). The saline susceptibility of the Leccino cultivar is known since the early 1990s (Tattini et al., 1992, 1995, 1997; Tattini, 1994; Gucci et al., 1997) indicates a limit threshold for Leccino growth at 50 mM of NaCl after 60 days of treatment. Over the years this cultivar has been used as a sensitive variety model (Moretti et al., 2018, 2019; Rossi et al., 2015, 2016; Pandolfi et al., 2017; Hill et al., 2013; Erel et al., 2019; Sodini et al., 2022). Under salt stress, Rossi et al. (2016) reported information about the expression of *NHX* gene in leaves of Leccino and Frantoio cultivars showing that only Leccino had a significant increase in *NHX* transcription under salt exposure. Bazakos et al. (2015) indicated an

* Corresponding author.
E-mail address: a.francini@santannapisa.it (A. Francini).

https://doi.org/10.1016/j.stress.2023.100264
Received 28 June 2023; Received in revised form 14 September 2023; Accepted 17 October 2023
Available online 18 October 2023

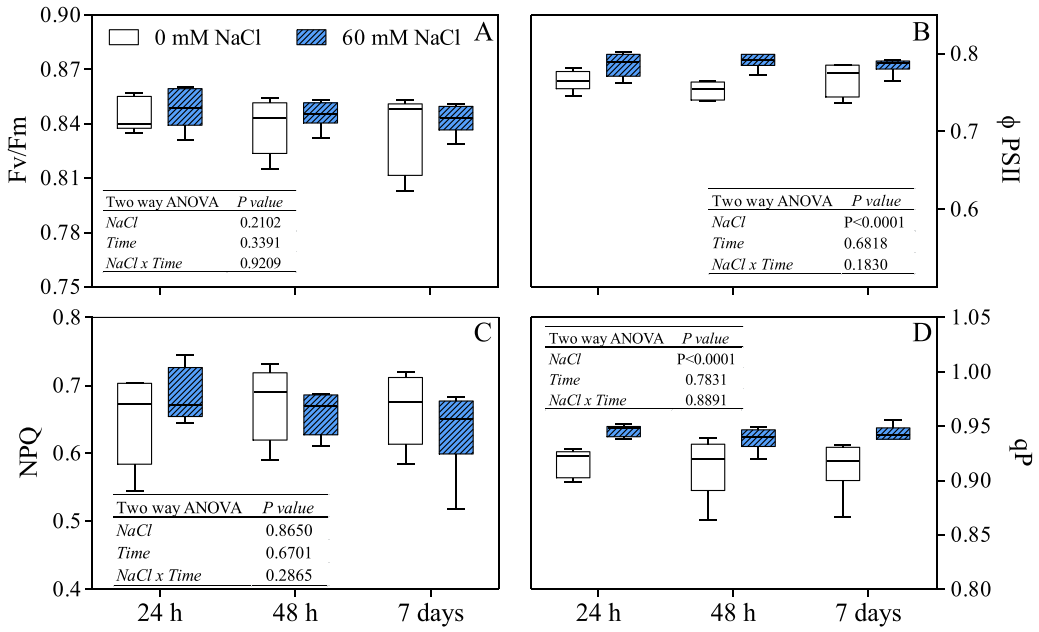

Fig. 1. Chlorophyll *a* fluorescence parameter ($n = 6$) of: A) maximum quantum efficiency of photosystem II (Fv/Fm); B) quantum yield of photosystem II (ΦPSII); C) non-photochemical quenching (NPQ); D) photochemical quenching (qP) and after 24 h, 48 h, and 7 days in leaves of olive tree cultivar Leccino treated with 0 and 60 mM of NaCl.

over-expression of *SOS1* gene in the roots of cv Kalamon under salt stress, but no significant difference in expression in the leaves underline the importance of tissue specificity in plant salt-stress tolerance/sensitivity. Mousavi et al. (2021) investigated four olive cultivars exposed to high salt stress conditions (200 mM of NaCl). Authors demonstrated a key role of *OeNHX7* gene in the activation of a salt tolerance responses.

The salt transport from the root to the shoot is considered the key point to discern tolerance/sensitive mechanism for numerous plant species (Balasubramaniam et al., 2023) including olives (Chartzoulakis, 2005). Leccino has less capacity to maintain the Na^+ in the roots compared to others (Rossi et al., 2015) and the early phase of salt stress could be the turning point between the accumulation and the mobilization. Roots are the first plant's organ exposed to salinity and they perceive and react early to this stress; for this reason our objective is to understand the contribution of some key genes involved in Na^+ root mobilization in the early phase of salt treatment which has remained unexplored in previous investigations in salt sensitive olive cultivar. We hypothesized that during the first phase of salt application in sensitive Leccino's cultivar, the inactivation of root's proton pumps *H^+-ATPases* and sodium antiporter *Na^+/H^+ SOS1* are responsible for the sodium translocation in the apical part of plants.

Material and methods

Plant material

One-year-old olive tree (*Olea europaea* L.) cultivar Leccino (salt-sensitive) from self-rooted cuttings were grown in pots of 15 cm of diameter (2 L volume) filled with expanded clay and perlite (v/v, 50/50). Plants were acclimated in Phytotron chamber (temperature 26–20 °C; relative humidity 55–75% day-night; photoperiod 16–8 h light-dark; light intensity 400–500 μmol $m^{-2}s^{-1}$). After three weeks of acclimation, plants ($n = 6$) were randomly assigned to treatments and irrigated every two days with OM (Rugini, 1984) at half strength (Control) and with OM at half strength plus 60 mM of NaCl. After 24 h, 48 h, and seven days from the start of treatments, plants were sampled and divided into roots, stem, and leaves. We further divided the leaves into lower leaves and upper leaves, measured from the middle point between the first fully expanded leaf in the apical and the basal. Samples of each organ were frozen in liquid nitrogen and stored at −80 °C to perform biochemical and molecular analysis but also dried in an oven (70 °C) until a constant weight was obtained and used to perform Na^+ concentration.

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured to evaluate the efficiency of photosystem II (PSII), in 30 min dark-adapted leaves, by pulse amplitude modulated (PAM) fluorometer (Hansatech FM2, Hansatech, Inc., UK). One completely expanded leaf per plant in the middle position of the stem was marked, and we monitored the chlorophyll *a* fluorescence at each sampling time. The minimum fluorescence yield in dark-adapted state (F_0) was detected under modulating beam; maximum fluorescence (F_m) was measured after application of a saturating light pulse [8000 μmol (photon) m^{-2} s^{-1}; 700 ms] and maximum quantum efficiency of photosystem II was determined as F_v/F_m, where F_v is the variable fluorescence, calculated as the difference between F_m and F_0. After the saturating light pulse, were measured: quantum yield of photosystem II [ΦPSII= (Fm'-Fs)/Fm', where Fm' is the maximal fluorescence in light-adapted state and Fs is the steady-state fluorescence yield], photochemical quenching [qP=(Fm'-Fs)/(Fm'-F0)], and non-photochemical quenching [NPQ=(Fm-Fm')/Fm'].

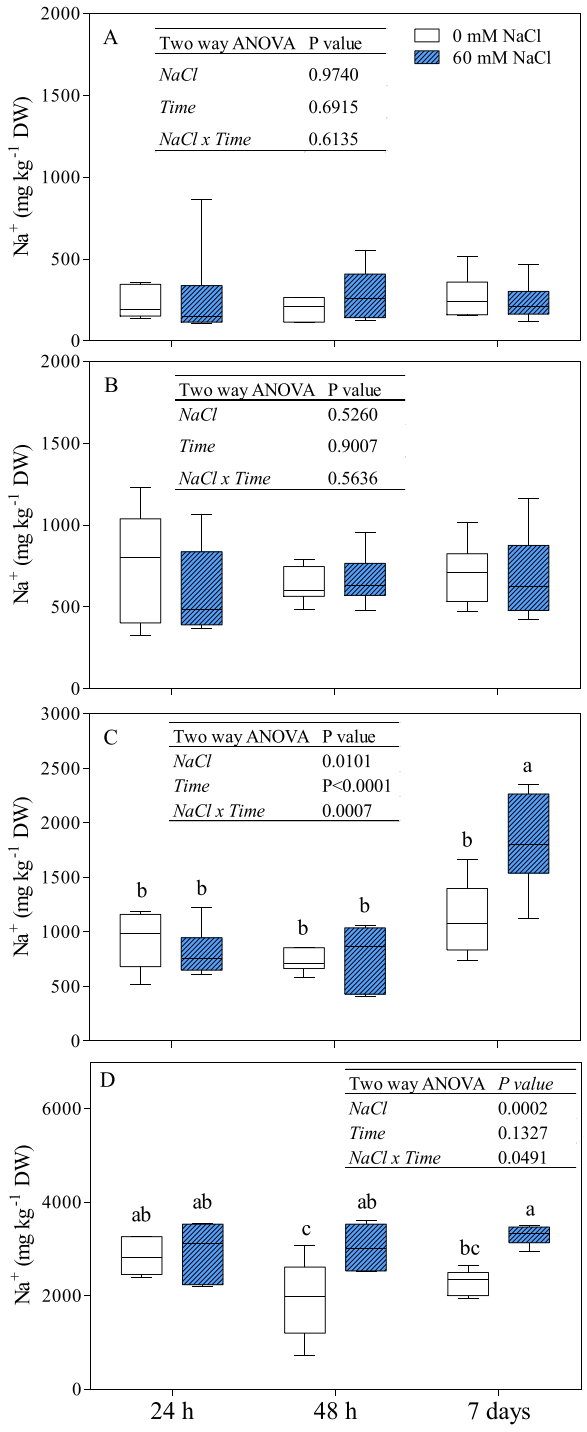

Fig. 2. Na^+ concentration (mg kg^{-1} of DW) after 24 h, 48 h, and 7 days in upper leaves (A), lower leaves (B), stem (C) and roots (D) olive tree cultivar Leccino treated with 0 and 60 mM of NaCl. Box plot showing variation in Na^+ concentration ($n = 6$). The horizontal line within the box represents the median of the data. Box limits on either side of the median represent 25% (lower) and 75% (upper) percentiles. Whiskers represent minimum (lower) and maximum (upper) values. Statistical significance was determined with Two-way ANOVA followed by Tukey's multiple comparison test. Different letter indicates statistically significant differences among treatments and time.

Table 1
Malondialdehyde – MDA - (nmol g^{-1} FW) and L-proline content (mg g^{-1} FW) in the roots of *O. europaea* cv Leccino treated with 0 and 60 mM of NaCl after 24 h, 48 h and 7 days of treatments. Data are means of 4 replicates (±SD). Statistical significance was determined with Two-way ANOVA followed by Tukey's Multiple Comparison test ($P \leq 0.05$).

		NaCl (mM)		ANOVA	*P value*
	Time	0	60		
MDA	24 h	5.75 ± 0.66	5.29 ± 0.67	*NaCl*	0.909
	48 h	6.58 ± 1.45	6.52 ± 0.76	*Time*	0.025
	7 days	5.27 ± 0.78	5.78 ± 0.42	*NaCl x Time*	0.474
L-proline	24 h	0.096 ± 0.014	0.077 ± 0.010	*NaCl*	0.103
	48 h	0.073 ± 0.017	0.073 ± 0.017	*Time*	0.003
	7 days	0.065 ± 0.014	0.057 ± 0.010	*NaCl x Time*	0.360

Table 2
Concentration (mg kg^{-1} DW) of Ca^{2+}, K^+ and Mg^{2+} in roots of *O. europaea* cv Leccino treated with 0 and 60 mM of NaCl after 24, 48 h, and 7 days of treatments. Data are means of 6 replicates (±SD). Statistical significance was determined with Two-way ANOVA followed by Tukey's Multiple Comparison test ($P \leq 0.05$).

		NaCl (mM)		ANOVA	*P value*
	Time	0	60		
Ca^{2+}	*24 h*	8647 ± 1798.6	9037 ± 2154.3	*NaCl*	0.022
	48 h	7347 ± 811.9	6273 ± 395.7	*Time*	0.012
	7 days	11,949 ± 4453.0	6886 ± 545.0	*NaCl x Time*	0.859
K^+	*24 h*	12,108 ± 2257.3	11,696 ± 3288.8	*NaCl*	0.139
	48 h	9974 ± 501.1	8612 ± 1095.7	*Time*	0.002
	7 days	11,512 ± 2735.6	8910 ± 876.2	*NaCl x Time*	0.129
Mg^{2+}	*24 h*	2572 ± 420.9	2211 ± 250.9	*NaCl*	0.232
	48 h	2738 ± 292.5	2043 ± 521.5	*Time*	0.233
	7 days	2467 ± 803.7	2324 ± 554.5	*NaCl x Time*	0.119

Element's determination

Na^+ concentration in roots, stems, lower and upper leaves, and Ca^{2+}, K^+, Mg^{2+} in roots, were determined on 0.2 g of dry material digested in 5 mL of 65% HNO_3 and subsequently, in 1 mL of 70% $HClO_4$. The resulting solution was filtered and opportunely diluted with Milli-Q H_2O and then analyzed. We used two analytical reference standards (WEPAL IPE, Wageningen University) with certified concentrations as controls: *Daucus carota* (L.) leaf (852±126.7 mg kg^{-1} of Na^+) and shoot (10,600 ±1010 mg kg^{-1} of Na^+). Na^+ quantification has been performed in an atomic absorption spectrometer (model 373; PerkinElmer, Norwalk, CT, USA) equipped with specific lamps.

Lipid peroxidation analyses

Lipid peroxidation was estimated by measuring the content in malondialdehyde (MDA) in the fresh roots (Hodges et al., 1999). This method takes into account the interference in the absorbance between the complex MDA and 2-thiobarbituric acid (TBA) but also interfering molecules like protein and sugar. Roots tissue (100 mg) was homogenized in ice with 2 mL of methanol 80% and then centrifuged for 10 min at 3000 g at 4 °C. The supernatant was divided into two 0.8 mL aliquots: one contained 0.8 mL of solution with 20% TCA and 0.65% of TBA, and the other 0.8 mL of 20% TCA solution. The tubes were vigorously mixed and incubated for 25 min at 95 °C. After the incubation, the tubes were cooled in ice and centrifuged for 10 min at 3000 g and 4 °C (Allegra 64R, Beckman). The supernatant was read at 440, 600, and 532 nm. The MDA

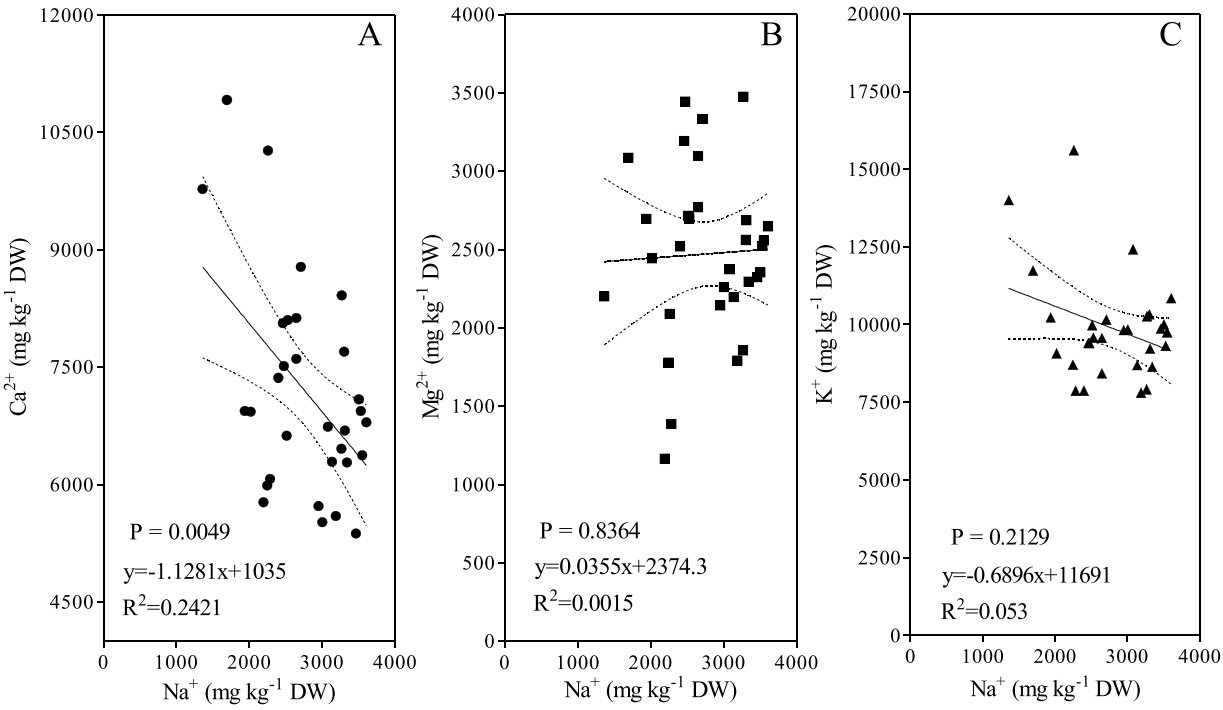

Fig. 3. Linear regression analysis between Na^+ concentration (mg kg^{-1} DW) and A) Ca^{2+}, B) Mg^{2+} (mg kg^{-1} DW) and, C) K^+ in roots of Leccino plants exposed to 0 and 60 mM NaCl.

Table 3
Comparisons of 3 linear models for the relative gene expression of 7 genes expressed in roots of Leccino. ANOVA table with the significance of each factor's model (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, n.s. not significant), the significance of the model (P-value), the accuracy of the model expressed in R^2, and the Baeysian Information Criterion (BIC)) as a tool for model selection. The factors tested in the model were: the treatment with NaCl "Treatment", the sampling time "Time", and the Na^+ concentration in the roots of olive tree "[Na^+]Roots". The selected models with the lowest BIC are highlighted in the gray box.

Model 1	*SOS1*	*V-ATPaseSub.E*	*ATPase 3*	*ATPase 8*	*ATPase 1*	*ATPase 11*	*NHX*
	Gene expression = [Na^+]Roots x Treatment						
[Na^+]Roots							
Treatment	**	*					
[Na^+]Roots x Treatment							
R^2	**0.23**						
P-value	**0.01**	**n.s.**	**n.s.**	n.s.	**n.s.**	**n.s.**	n.s.
BIC	**101.5**	**108.9**	**115.0**	80.3	**113.8**	**96.9**	114.3
Model 2	*Gene expression* = [Na^+]Roots x Treatment x Time						
[Na^+]Roots							
Treatment	**						
Time	**						
[Na^+]Roots x Treatment		*					
[Na^+]Roots xTime							*
Time x Treatment							*
R^2	0.35						
P-value	0.02	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BIC	113.7	126.2	132.8	96.3	137.1	114.1	119.9
Model 3	*Gene expression* = Time x Treatment						
Treatment	*	*		*		*	
Time	**						
Time x Treatment							**
R^2	0.29			**0.24**			**0.27**
P-value	0.008	n.s	n.s.	**0.02**	n.s.	n.s.	**0.009**
BIC	105.3	111.1	123.4	**77.4**	121.5	100.0	**108.0**

concentration was calculated according to the equation of Hodges et al. (1999):

1) [(Abs_{532}+TBA)) (Abs_{600}+TBA)) (Abs_{532}) TBA) Abs_{600}) TBA)] = A
2) [(Abs_{440}+TBA) Abs_{600}+TBA) 0.0571] = B
3) MDA equivalents (nmol mL^{-1}) = (A-B/157 000) 10^{-6}

Levels of MDA were expressed as nmol g^{-1} FW.

Proline content determination

Proline content was evaluated according to the method of Bates et al. (1973) with minor modification. Fresh roots (100 mg) were homogenized with 5 mL of 3% aqueous sulfosalicylic acid. The homogenated solution was filtered, and 1 mL was added to an equal volume of glacial acetic acid and ninhydrin. The mixture was incubated at 100 °C for 1 hour, and the reaction terminated in an ice bath before being extracted

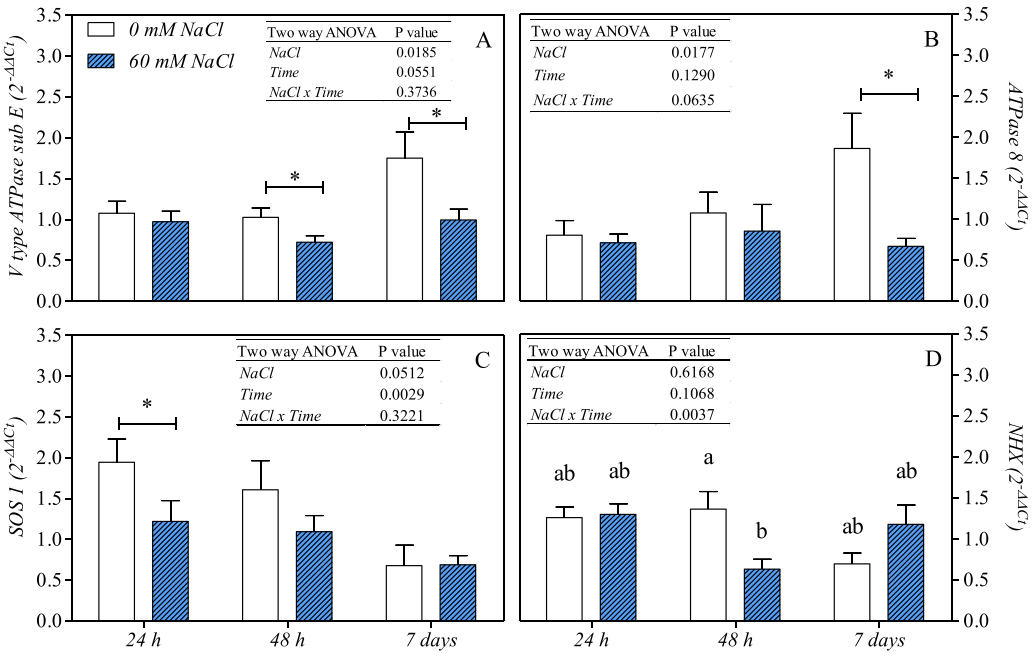

Fig. 4. Relative expression of *V type ATPase sub E* (A), *ATPase 8* (B) *SOS1* (C) and *NHX* (D) genes in *Olea europaea* cv Leccino's roots treated with 0 and 60 mM of NaCl. The genes expression was measured after 24, 48 h, and 7 days from the beginning of the treatments. *EF1-α* and *OUB2* were used as the reference gene for normalization. Statistical significance was determined with ANOVA followed by Tukey's Multiple Comparison test ($P \leq 0.05$). A *t-test* analyses was performed between control and 60 mM NaCl treated roots on *ATPase 8, SOS1* and *V type ATPase sub E* relative expression (*; $P<0.05$).

with 2 mL of toluene under mixed. The chromophore was removed from the toluene phase, and the absorbance read at 520 nm. The proline content was determinate from a standard curve in the range of 0.1–10 μmol.

RNA extraction and real-time PCR (qPCR)

Total RNA was isolated from olive tree's root using the Total RNA Purification kit (Sigma-Aldrich), according to the manufacturer's protocol. RNA concentration and purity were determined through a Nanodrop spectrophotometer. Genomic DNA contamination was eliminated by using On-Column DNase I Digestion Set (Sigma-Aldrich) and was further confirmed by gel electrophoresis. First-strand cDNA was synthesized employing First-strand cDNA Synthesis Kit (Sigma-Aldrich), according to the manufacturer's instructions. Specific primer pairs for V-ATPase (*V type ATPase sub E*), vacuolar Na^+/H^+ antiporter (*NHX*), plasma membrane Na^+/H^+ antiporter (*SOS1*), P-ATPase (*P-ATPase*), were designed based on the alignment of genes of genetically similar organisms, using Primer3web to design the primer and NCBI Primer-BLAST to verify the specificity of the amplicon (Supplementary Table S1). Real-time PCR was performed on a CFX Connect Real-Time PCR (Bio-Rad) using as indicator SYBR-Green PCR Master Mix (Bio-Rad). Melting curves with variations of 1 °C were performed to detect the primer specificity. All reactions were assayed in triplicate. The relative expression was normalized as $2^{-\Delta\Delta CT}$ using two reference genes: *EF1-α* and *OUB2*, chosen from three tested genes: *EF1-α, 60S RBP L18–3,* and *OUB2* (Ray and Johnson, 2014).

Amplicon sequencing

The fragments of selected genes to be sequenced were obtained from PCR reaction Mix (Sigma-Aldrich). The amplicons were sent to Eurofins Scientific (Italy) for sequencing using the Sanger method. Resulted were analyzed on NCBI Nucleotide-BLAST (Supplementary Table S2).

Statistical analysis

The experiment was set up in a completely randomized design with six biological replicates ($n = 6$) for each treatment. Statistical analysis was performed with R software (https://cran.r-project.org/). The elements concentration, proline concentration, MDA concentration, and chlorophyll a fluorescence, were analysed with two-way ANOVA with treatment and time as factors, followed by the Tukey test, for a $P \leq 0.05$. For the gene expression's analysis, we compared 3 different models containing treatment, time and roots sodium concentration as factors. We selected the model that minimized the value of Baeysian Information Criterion (BIC). Models were then validated, checking the distribution of the residuals.

The Hierarchical cluster analysis was based on the correlation matrix analyzed with Pearson's test. The data of the gene expressions and mineral element concentration were scaled with values between 3 and −2. A correlation matrix was then calculated with Pearson's coefficients. The correlation matrix was arranged with a Hierarchical cluster analysis, using Euclidean distance and Ward linkage methods. The number of branches into which to divide the cluster (k) was obtained utilizing the function "NbClust" (package "NbClust") which performs cluster assessment, and the optimal k values was 2. The heatmap of the correlation coefficients arranged by the hierarchical cluster was built with the package "pheatmap".

Results

The photosynthetic efficiency of Photosystem II was tested to understand the effect of salt treatments on the photosynthetic activity of cv

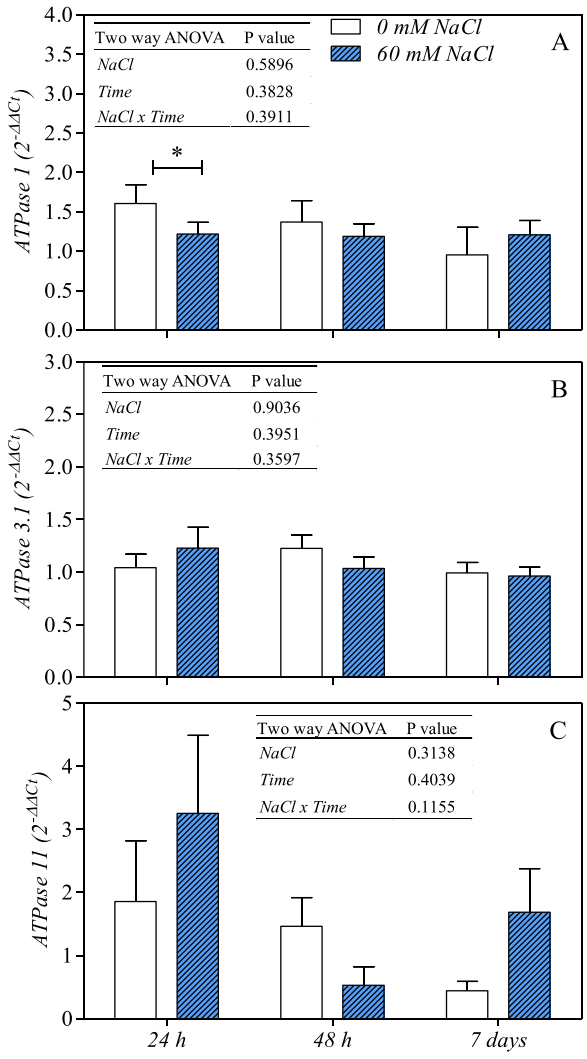

Fig. 5. Relative expression of *ATPase 1, ATPase 3.1 and ATPase 11* genes in *Olea europaea* cv Leccino's roots treated with 0 and 60 mM of NaCl. The genes expression was measured after 24, 48 h, and 7 days from the beginning of the treatments. *EF1-α* and *OUB2* were used as the reference gene for normalization. Statistical significance was determined with ANOVA followed by Tukey's Multiple Comparison test ($P \leq 0.05$).

Leccino. Chlorophyll *a* fluorescence measurement showed that during the experiment, Fv/Fm ratio was not significantly affected by 60 mM NaCl treatments. In fact, Fv/Fm was ≥ 0.8, that is considered the value of a healthy photosystem II (Fig. 1A). In leaves of treated plants, a significant salt effect ($P<0.0001$) on ΦPSII was observed and an increment of 3, 5 and 2% after 24 h, 48 h and 7 days was recorded in comparison to control leaves (Fig. 1B). Similar effect of Na^+ treatments ($P<0.0001$) was observed for the photochemical quenching (qP) and the increase was of 3% at each sampling time (Fig. 1D).

The Na^+ concentration in the upper leaves showed no significant difference among treatments during the time and the Na^+ average values were 229 mg kg^{-1} *vs* 257 mg kg^{-1} in control and treated plants respectively after 24 h, 195 mg kg^{-1} *vs* 282 mg kg^{-1} after 48 h, and 269 mg kg^{-1} *vs* 293 mg kg^{-1} DW after 7 days (Fig. 2A). Similarly, for the lower leaves, the values of Na^+ did not differ during the three-sampling time and the average values of control leaves were 759, 633, and 703 mg kg^{-1} DW after 24 h, 48 h and 7 days respectively, while in treated plants the values were 593, 667 and 684 mg kg^{-1} DW (Fig. 2B). Notably, the Na^+ concentration in the lower leaves was approximately three times higher than that observed in the upper leaves. Na^+ concentration in the stem was in the range of control plants after 24 and 48 h (Fig. 2A-C) and accumulate significantly after 7 days at 60 mM NaCl (Fig. 2C), reaching the concentration of 1832±404 mg kg^{-1} DW (+63% compared to control). Under 60 mM NaCl olive's roots accumulate Na^+after 48 h of exposure (+57% compared to control), and +43% after 7 days (Fig. 2D).

Focusing our attention on roots, measurement of lipid peroxidation of Leccino treated plants were carried out during the experiment (Table 1). The differences were not significant at each sampling time and data of MDA were in average of 5.7, 6.5 and 5.2 nmol g^{-1} FW in control roots and 5.2, 6.5, 5.7 in 60 mM NaCl treated roots during 24, 48 h and 7 days of exposure. Also, L-proline content in the roots ranged between 0.057 and 0.096 mg g^{-1} FW without difference among treatments (Table 1).

The concentration of K^+ and Mg^{2+} in the roots never varied between the tested groups (Table 2) and NaCl treatments and time were significant ($P = 0.022$; $P = 0.012$) only for Ca^{2+} concentration. Linear regression analysis between Na^+ and Ca^{2+} concentration in roots (Fig. 3A) was also significant ($P = 0.0049$).

To investigate the role of proton pumps and Na^+/H^+ exchangers under salt stress, we measured the relative gene expression using real-time quantitative PCR (qPCR), with specific primers for each *P-ATPase, V type ATPase sub E, SOS1*, and *NHX* gene. Couples of primers were designed using as target the available *O. europaea* genome and other tree species (*Populus trichocarpa, Populus alba, Vitis vinifera, Populus persica*). When aligned with functional genes from other species, the *O. europaea* genome potentially encodes the following predicted genes:

- A single predicted gene of a plasmalemma Na^+/H^+ antiporter: *sodium/hydrogen exchanger 7-like.*
- Two predicted genes for vacuolar Na^+/H^+ transporters: *sodium/hydrogen exchanger 1-like, sodium/hydrogen exchanger 2-like.*
- A single predicted gene for vacuolar subunit E ATPase: *V-type proton ATPase subunit E-like.*
- A gene family composed of many predicted genes for plasma membrane H^+-ATPase (*ATPase* genes family): *plasma membrane ATPase 1-like* to *plasma membrane ATPase 11-like.*

In the case of a multi-genic family such as *plasma membrane -ATPase*, we selected the genes that were observed to be expressed in the roots. The relative expression was analyzed comparing 3 different models. We selected the models that better explain the variability of the genes expressions containing the degree of freedom. The best models selected shows the treatment to be significant in the *ATPase 8,* the interaction "treatment x time" was significant in the models selected for the genes: *V type ATPase sub E* and *ATPase 8*, while the model selected for *SOS1* showed a significant effect of "roots [Na^+]" and the interaction "roots [Na^+] x treatment" (Table 3).

In general, a down regulation of relative expression of the genes tested was observed. When *V type ATPase sub E* was analyzed (Fig. 4A) the relative expression decrease significantly (−29%) in the roots of salt treated plants (*t-test* $P = 0.0266$) after 48 h of treatment, and after 7 days (−43%) of treatment (*t-test* $P = 0.0186$) (Fig. 4A). Also *ATPase 8* and *ATPase 1* relative expression decrease significantly (−64% and −24%) in the roots of plants treated with 60 mM compared to the control (*t-test* $P = 0.0155$ and *t-test* $P = 0.0268$ respectively), after 7 days of treatment

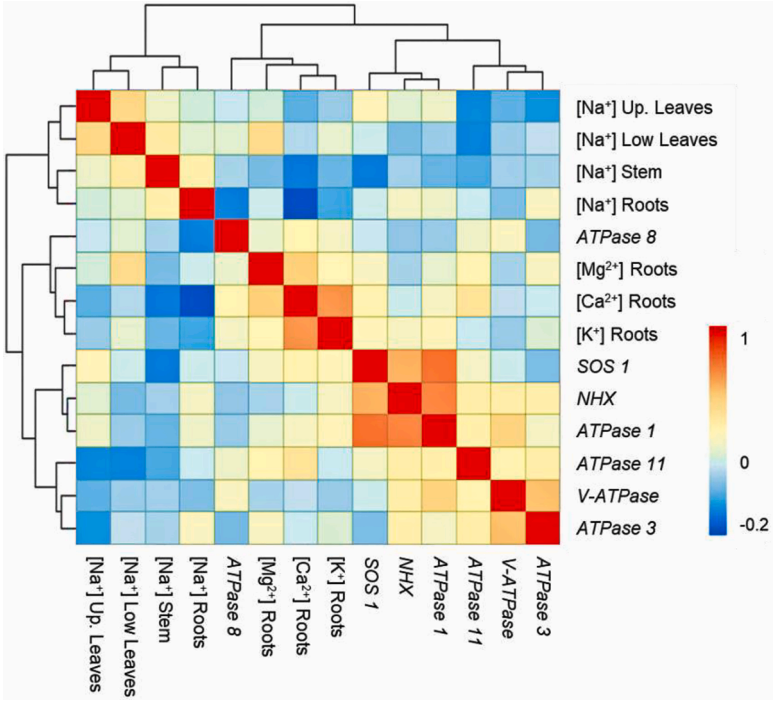

Fig. 6. Hierarchical cluster analysis based on the correlation matrix, of mineral elements content and gene's expression, based on Euclidian distance, and "Ward" method of cluster. The number of cluster $k = 2$ was chosen from the cluster validation process. Values of high correlation are shown in red while negative correlations are shown in blue.

(Fig. 4B) and after 24 h of treatment (Fig. 5A). Moreover, *SOS1* relative expression decrease after 24 h (−37%) between control and NaCl treatment (Fig. 4C). The only significant interaction "Treatment x Time" in plants treated with 60 mM NaCl was observed for *NHX* relative expression (Fig. 4D) with a significant decrease under 60 mM NaCl (−53% compared to control roots). The other relative genes expression of *ATPase 3.1 and ATPase 11* did not shows difference between control and NaCl treatment (Fig. 5).

The correlation between all the genes studied and the concentration of Na^+, Ca^{2+}, K^+ and Mg^{2+} in the roots were analyzed. The correlation matrix indicates a strong positive correlation between *SOS1, ATPase 1,* and *NHX* (Fig. 6). Furthermore, the hierarchical cluster analysis separates the correlation between genes expression and elements content (Fig. 6). The strong relationship between *SOS1, ATPase 1,* and *NHX* are shown in detail in the Fig. 7.

Discussion

Olea europaea Leccino cultivar is considered less tolerant to salt stress compared to other olive cultivars like Frantoio. Leccino easily mobilizes Na^+ in the higher part of the plant, such as stem and leaves. The mobilization of Na^+ into the shoot induces water imbalance (Gucci et al., 1997), reduction of leaves pigments, stomatal closure, and a considerable drop in photosynthesis performances (Wu, 2018). The mechanism that occurs in the early phase of the stress of a sensitive cultivar could be considered a key moment to understand how these plants drive the mobilization of Na^+ from the root to the shoot. Previous studies show the behavior of Leccino cultivar in consequence of long-term salt stress focusing on water balance and photosynthesis changes (Gucci et al., 1997; Rossi et al., 2016; Tattini et al., 1997). Tattini et al. (1994) report differences in salt accumulation between treatments at 60 and 120 mM NaCl in Leccino, showing a higher uptake rate of Na^+ in the treatment with 60 mM when compared to 120 mM NaCl.

Under our experimental condition, Leccino plants were at the beginning of the translocation stage: Na^+ accumulate in root and stem, but did not significantly increase in basal and apical leaves. As a result, we found no change in the ratio of variable to maximum fluorescence of Chl *a* after dark-adaptation (Fv/Fm). The value of Fv/Fm was around 0.8, which is the optimum for non-stressed plants (Murchie and Lawson, 2013). Considering the relative reduction state of first stable electron acceptor of PSII (QA), reflecting the fraction of open PSII reaction center, control mature leaves possessed 91% of reaction centers open and salt treated leaves 94% of reaction centers open. The slight increase under salt stress could be related to a more oxidized redox state of QA under salt stress.

Leccino accumulated Na^+ in the roots under 60 mM NaCl and the Na^+ concentration reached values similar to Rossi et al. (2015, 2016) after 40 days. On the contrary we found lower leaves Na^+ concentration than Rossi et al. (2015) confirming that the roots' uptake starts after a few hours while the translocation towards the shoot requires more than seven days.

Lower concentration of Ca^{2+}, K^+, Mg^{2+} induced by salt stress has been observed by many authors (Loupassaki et al., 2002; Tattini and Traversi 2008; Larbi et al., 2020). This experiment showed that as the Na^+accumulate in roots, the Ca^{2+} concentration decrease thus confirming our previous work data (Sodini et al., 2022). Despite Na^+ was accumulated after 24 h, proline contents remained unaltered, confirming that osmotic imbalance did not reach significant values during this short period of treatments.

The relative expression analysis showed a down-regulation of genes *P-ATPase 8, P-ATPase 1,* and *SOS1*. The enzymes codified by these genes are necessary to maintain the cell homeostasis (Pardo et al., 2006) and

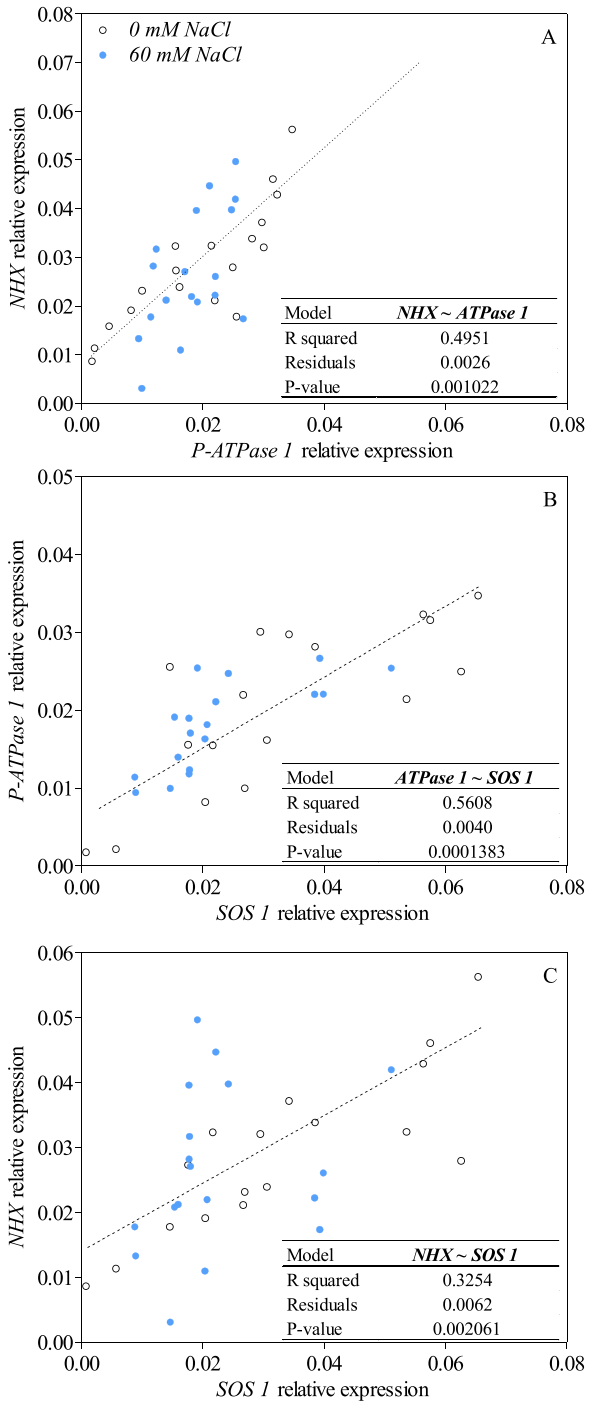

Fig. 7. Linear regression analysis between relative gene expression *NHX vs. ATPase 1, ATPase 1 vs. SOS1*, and *NHX vs. SOS1* in roots of Leccino plants exposed to 0 and 60 mM NaCl.

essential to preserve a proton gradient between inside-outside of the membranes. These down expressions confirms the already known physiological link between these trans-membrane pumps (Tester and Davenport, 2003).

The plasma membrane Na^+/H^+ antiporter SOS1 takes advantage of the proton gradient produced by the pumps ATPases to exclude Na^+ ions from the cytoplasm. Modulation of the H^+ATPase is widely reported in the literature (Janicka-Russak and Kabala 2015). As indicated by Balasubramaniam et al. (2023) P-type H^+ATPase has an housekeeping functions such as facilitating the turgor pressure and cell wall extension and for this could be the reason of their increased expression during root growth in control condition in our experimental data. Membrane *H^+ATPase* genes are often under-expressed, and proteins are inactivated or quantitatively reduced in response to salinity (Zhang et al., 2018). The salt treatment seems to block the activity of these pumps over time.

The *SOS1* gene in the roots is considered to have a dual role in the sodium translocation (Shabala et al., 2013; Munns and Tester 2008). *SOS1* is proposed to exclude Na^+ from the roots to the circulating solution (Wu, 2018). In the second role, *SOS1* charges sodium into the xylem, allowing delivery to higher organs (Shabala et al., 2013). This second mechanism can lead to an accumulation of sodium in the leaves (Tester and Davenport, 2003). Mutants of tomato plants (*Solanum lycopersicum*) under expressing *SOS1* accumulate more sodium in the leaves compare with wild type (Olìas et al., 2009). Moreover, the expression of *SOS1* in 4 ecotypes of Arabidopsis was negatively correlated with the plant Na^+accumulation, supporting the role of Na^+ efflux (Jha et al., 2010). The under expression of *SOS1* found in the roots of Leccino, supports the greater uptake of Na^+in the stem after 7 days of treatment. Our results on Leccino's *SOS1* seems to support the role of efflux sodium from the plant in the short time frame. After 24 h of salt treatment even a down expression of *ATPase 1* has been observed, furthermore, a positive correlation between *ATPase 1* and *SOS1*. Recently a down expression of *ATPase 1* has been recorded in leaves of Leccino treated with 120 mM of NaCl (Sodini et al., 2022), however no positive correlation between *ATPase 1* and *SOS1* has been reported, indicating how this mechanism is related to the roots (Fig. 8). Interestingly, as reported for seedlings of *M. crystallinum* that are not salt-tolerant such as Leccino, authors do not show transcriptional changes of the *V*−ATPase subunits under salt stress (Golldack and Dietz, 2001). These data indicate that the ability to respond to salinity stress could be a characteristic of salt tolerance in plants. Considering control and treated plants, the down expression of *V type ATPase sub E* and *NHX* found after 48 h in the salt treated plants is consistent with the mobilization mechanism discussed for *SOS1*. These genes are involved in the mechanisms of roots Na^+ storage, and their down expression is in line with the lack of sodium accumulation in the roots (Seidel, 2022). Consistently, a positive correlation between of *V type ATPase sub E* and *NHX* was found in leaves of Leccino, while these two genes were over expressed in both Leccino and Frantoio treated with 120 mM of NaCl (Sodini et al., 2022). In this previous study the over expression supported the Na^+compartmentalization in the leaves, while in the present work the down expression support the efflux of $Na^+$to the shoots. The two clusters of the correlation matrix well frame the global differences between the variables, with the membrane transporter genes negatively correlated with the Na^+accumulation and positively correlated each other. The under-regulation of membrane transporters gene in short-term stress on Leccino seem consistent with the sodium susceptibility of this cultivar.

In conclusion, the present work investigates for the first time the early response of a susceptible olive tree cultivar to salinity. It was shown for the first time that the Na^+ translocation occurred in the early phase of salt treatment, together with the Ca^{2+} depletion. Two couples of genes were found down expressed in the roots of *Olea europaea* cv. Leccino: *ATPase 1* and *SOS1* after 24 h and *V type ATPase sub E* and *NHX* after 48 h and the down expression of this two couple of genes can be associated to the efflux of $Na^+$to the shoots at the start of salt stress.

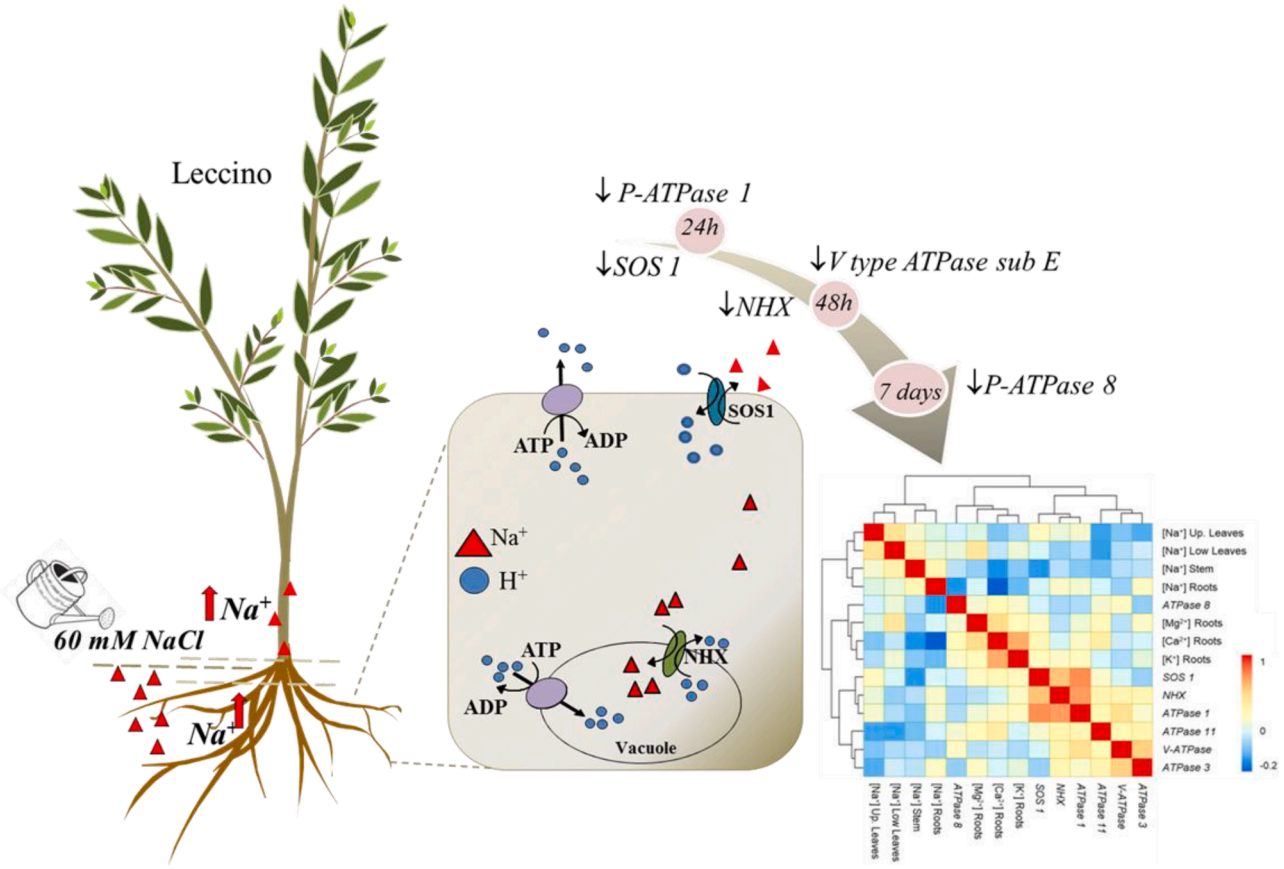

Fig. 8. Schematic summary of the results obtained in Leccino plants to avoid salt stress.

Author contribution statement

MS, AF and LS contributed to the planning of experiment;
MS, collected the biochemical, physiological and molecular data;
MS, AF and LS contributed to statistical elaboration;
MS, AF and LS contribute to the manuscript draft and approved the final version.

Consent for publication

All Authors have given approval for publication

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgements

This study was carried out within the Agritech National Research Center and received funding from the European Union NextGenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 14 – DD 1032 17/06/2022, CN00000022) and in part by "VIRMA: Velivolo Intelligente Robotico per il Monitoraggio Agro-Ambientale - finanziato dal MUR nell'ambito del Fondo per la promozione e lo sviluppo delle politiche del Programma nazionale per la ricerca_PNR, in coerenza con il Regolamento UE n 241/2021 e con il PNRR 2021–2026, misura: Iniziative di ricerca interdisciplinare che esplorino temi di rilievo trasversale per il PNR, senza restrizioni basate sull'aderenza a settori scientifici di riferimento o ad aree tematiche prioritarie" This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them Financial support was provided by PiAnta project granted (CUP J54I19000770002) by Regione Toscana, POR FESR 2014–2020 for part of the equipment used (Allegra 64R-Beckman) Financial support was provided by Agrobioscience PhD program at Scuola Superiore Sant'Anna of Pisa to Mirko Sodini.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2023.100264.

References

Apse, M.P., Blumwald, E., 2007. Na^+ transport in plants. FEBS Lett. 581 (12), 2247–2254. https://doi.org/10.1016/j.febslet.2007.04.014.
Askari, H., Edqvist, J., Hajheidari, M., Kafi, M., Salekdeh, G.H., 2006. Effects of salinity levels on proteome of Suaeda aegyptiaca leaves. Proteomics 6 (8), 2542–2554. https://doi.org/10.1002/pmic.200500328.
Balasubramaniam, T., Shen, G., Esmaeili, N., Zhang, H., 2023. Plants' Response Mechanisms to Salinity Stress. Plants 12, 2253. https://doi.org/10.3390/plants12122253.

Barkla, B.J., Zingarelli, L., Blumwald, E., Smith, J.A.C., 1995. Tonoplast Na^+/H^+ antiport activity and its energization by the vacuolar H^+-ATPase in the halophytic plant Mesembryanthemum crystallinum L. Plant Physiol. 109 (2), 549–556. https://doi.org/10.1104/pp.109.2.549.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39 (1), 205–207. https://doi.org/10.1007/BF00018060.

Bazakos, C., Manioudaki, M.E., Sarropoulou, E., Spano, T., Kalaitzis, P., 2015. 454 Pyrosequencing of Olive (Olea europaea L.) transcriptome in response to salinity. PLoS One 10 (11), e0143000.

Chartzoulakis, K.S., 2005. Salinity and olive: Growth, salt tolerance, photosynthesis and yield. Agric. Water Manage. 78 (1–2), 108–121. https://doi.org/10.1016/j.agwat.2005.04.025.

Erel, R., Eppel, A., Yermiyahu, U., Ben-Gal, A., Levy, G., Zipori, I., Schaumann, G.E., Mayer, O., Dag, A., 2019. Long-term irrigation with reclaimed wastewater: Implications on nutrient management, soil chemistry and olive (Olea europaea L.) performance. Agric. Water Manage. 213, 324–335. https://doi.org/10.1016/j.agwat.2018.10.033.

Golldack, D., Dietz, K.J., 2001. Salt-induced expression of the vacuolar H+-ATPase in the common ice plant is developmentally controlled and tissue specific. Plant Physiol. 125 (4), 1643–1654. https://doi.org/10.1104/pp.125.4.1643. Apr.

Gucci, R., Lombardini, L., Tattini, M., 1997. Analysis of leaf water relations in leaves of two olive (Olea europaea L.) cultivars differing in tolerance to salinity. Tree Physiol. 17, 13–21. https://doi.org/10.1093/treephys/17.1.13.

Hill, A., Rewald, B., Rachmilevitch, S., 2013. Belowground dynamics in two olive varieties as affected by saline irrigation. Sci. Hortic. 162, 313–319. https://doi.org/10.1016/j.scienta.2013.08.032.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207 (4), 604–611. https://doi.org/10.1007/s004250050524.

Janicka-Russak M., Kabała K. (2015). The Role of Plasma Membrane H+-ATPase in Salinity Stress of Plants. In: Lüttge, U., Beyschlag, W. (eds) Progress in Botany. Progress in Botany, 76. Springer, Cham. https://doi.org/10.1007/978-3-319-08807-5_3.

Jha, D., Shirley, N., Tester, M., Roy, S.J., 2010. Variation in salinity tolerance and shoot sodium accumulation in Arabidopsis ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport. Plant Cell Environ. 33 (5), 793–804. https://doi.org/10.1111/j.1365-3040.2009.02105.x.

Kchaou, H., Larbi, A., Gargouri, K., Chaieb, M., Morales, F., Msallem, M., 2010. Assessment of tolerance to NaCl salinity of five olive cultivars, based on growth characteristics and Na^+ and Cl^- exclusion mechanisms. Sci. Hortic. 124, 306–315. https://doi.org/10.1016/j.scienta.2010.01.007.

Larbi, A., Kchaou, H., Gaaliche, B., Gargouri, K., Boulal, H., Morales, F., 2020. Supplementary potassium and calcium improves salt tolerance in olive plants. Sci. Hortic., 260108912 https://doi.org/10.1016/j.scienta.2019.108912.

Loupassaki, M.H., Chartzoulakis, K.S., Digalaki, N.B., Androulakis, I.I., 2002. Effects of salt stress on concentration of nitrogen, phosphorus, calcium, magnesium and sodium in leaves, shoots and roots of six olive cultivars. J. Plant Nutr. 25, 2457–2482. https://doi.org/10.1081/PLN-120014707.

Moretti, S., Francini, A., Hernández, M.L., Martínez-Rivas, J.M., Sebastiani, L., 2019. Effect of saline irrigation on physiological traits, fatty acid composition and desaturase genes expression in olive fruit mesocarp. Plant Physiol. Biochem. 141, 423–430. https://doi.org/10.1016/j.plaphy.2019.06.015.

Moretti, S., Francini, A., Minnocci, A., Sebastiani, L., 2018. Does salinity modify anatomy and biochemistry of Olea europaea L. Fruit during Ripening? Sci. Hortic. 228, 33–40. https://doi.org/10.1016/j.scienta.2017.10.003.

Mousavi, S., Mariotti, R., Valeri, M.C., Regni, L., Lilli, E., Albertini, E., Proietti, P., Businelli, D., Baldoni, L., 2021. Characterization of Differentially Expressed Genes under Salt Stress in Olive. Int. J. Mol. Sci. 23 (1), 154. https://doi.org/10.3390/ijms23010154.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911.

Murchie, E.H., Lawson, T., 2013. Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. J. Exp. Bot. 64 (13), 3983–3998. https://doi.org/10.1093/jxb/ert208.

Olìas, R., Eljakaoui, Z., Li, J., De Morales, P.A., Marín-Manzano, M.C., Pardo, J.M., Belver, A., 2009. The plasma membrane Na^+/H^+ antiporter SOS1 is essential for salt tolerance in tomato and affects the partitioning of Na^+ between plant organs. Plant Cell Environ. 32 (7), 904–916. https://doi.org/10.1111/j.1365-3040.2009.01971.x.

Pandolfi, C., Bazihizina, N., Giordano, C., Mancuso, S., Azzarello, E., 2017. Salt acclimation process: A comparison between a sensitive and a tolerant Olea europaea cultivar. Tree Physiol. 37 (3), 380–388. https://doi.org/10.1093/treephys/tpw127.

Pardo, J.M., Cubero, B., Leidi, E.O., Quintero, F.J., 2006. Alkali cation exchangers: Roles in cellular homeostasis and stress tolerance. J. Exp. Bot. 57 (5), 1181–1199. https://doi.org/10.1093/jxb/erj114.

Perica, S., Goreta, S., Selak, G.V., 2008. Growth, biomass allocation and leaf ion concentration of seven olive (Olea europaea L.) cultivars under increased salinity. Sci. Hortic. 117, 123–129. https://doi.org/10.1016/j.scienta.2008.03.020.

Ray, D.L., Johnson, J.C., 2014. Validation of reference genes for gene expression analysis in olive (Olea europaea) mesocarp tissue by quantitative real-time RT-PCR. BMC Res. Notes 7, 304. https://doi.org/10.1186/1756-0500-7-304.

Rossi, L., Borghi, M., Francini, A., Lin, X., Xie, D.Y., Sebastiani, L., 2016. Salt stress induces differential regulation of the phenylpropanoid pathway in Olea europaea cultivars Frantoio (salt-tolerant) and Leccino (salt-sensitive). J. Plant Physiol. 204, 8–15. https://doi.org/10.1016/j.jplph.2016.07.014.

Rossi, L., Francini, A., Minnocci, A., Sebastiani, L., 2015. Salt stress modifies apoplastic barriers in olive (Olea europaea L.): A comparison between a salt-tolerant and a salt-sensitive cultivar. Sci. Hortic. 192, 38–46. https://doi.org/10.1016/j.scienta.2015.05.023.

Rugini, E., 1984. In vitro propagation of some olive (Olea europaea L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci. Hortic. 24, 123–134. https://doi.org/10.1016/0304-4238(84)90143-2.

Seidel, T., 2022. The Plant V-ATPase. Front. Plant Sci. 13, 931777 https://doi.org/10.3389/fpls.2022.931777.

Shabala, S., Hariadi, Y., Jacobsen, S.E., 2013. Genotypic difference in salinity tolerance in quinoa is determined by differential control of xylem Na^+ loading and stomatal density. J. Plant Physiol. 170 (10), 906–914. https://doi.org/10.1016/j.jplph.2013.01.014.

Sodini, M., Astolfi, S., Francini, A., Sebastiani, L., 2022. Multiple linear regression and linear mixed models identify novel traits of salinity tolerance in Olea europaea L. Tree Physiol. 5 (42), 1029–1042. https://doi.org/10.1093/treephys/tpab158.

Tattini, M., 1994. Ionic relations of aeroponically-grown olive genotypes, during salt stress. Plant Soil 161 (2), 251–256. https://doi.org/10.1007/BF00046396.

Tattini, M., Bertoni, P., Caselli, S., 1992. Genotipic responses of olive plants to sodium chloride. J. Plant Nutr. 15 (9), 1467–1485. https://doi.org/10.1080/01904169209364412.

Tattini, M., Gucci, R., Coradeschi, M.A., Ponzio, C., Everard, J.D., 1995. Growth, gas exchange and ion content in Olea europaea plants during salinity stress and subsequent relief. Physiol. Plant. 95 (2), 203–210. https://doi.org/10.1111/j.1399-3054.1995.tb00828.x.

Tattini, M., Lombardini, L., Gucci, R., 1997. The effect of NaCl stress and relief on gas exchange properties of two olive cultivars differing in tolerance to salinity. Plant Soil 197 (1), 87–93. https://doi.org/10.1023/A:1004241628878.

Tattini, M., Traversi, M.L., 2008. Responses to changes in Ca^{2+} supply in two Mediterranean evergreens, *Phillyrea latifolia* and *Pistacia lentiscus*, during salinity stress and subsequent relief. Ann. Bot. 102, 609–622. https://doi.org/10.1093/aob/mcn134.

Tester, M., Davenport, R., 2003. Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot. 91 (5), 503–527. https://doi.org/10.1093/aob/mcg058.

Wu, H., 2018. Plant salt tolerance and Na^+ sensing and transport. Crop J. 6 (3), 215–225.

Zhang, M., Chen, Q., Zhou, P., Zhang, Q., Fang, Y., 2018. NaCl-induced changes in vacuolar H^+-ATPase expression and vacuolar membrane lipid composition of two shrub willow clones differing in their response to salinity. Plant Growth Regul. 86 (3), 445–453. https://doi.org/10.1007/s10725-018-0442-0.

Zhao, S., Zhang, Q., Liu, M., Zhou, H., Ma, C., Wang, P., 2021. Regulation of Plant Responses to Salt Stress. Int. J. Mol. Sci. 22, 4609. https://doi.org/10.3390/ijms22094609.

Zhu, J.K., 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53 (1), 247–273. https://doi.org/10.1146/annurev.arplant.53.091401.143329.