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Salinity Effect on Plant Physiological and Nutritional Parameters of New Huanglongbing Disease-Tolerant Citrus Rootstocks

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Abstract: Salinity is a major agriculture problem for citrus in the Mediterranean basin, which is a major global producer region. Citrus crops are also threatened by emerging diseases such as Huanglongbing (HLB). The use of different rootstocks increases the variability of citrus plant material in orchards, thus preventing extensive damage caused by abiotic and/or biotic diseases. In this work, we have evaluated the salinity response of five citrus rootstocks (US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange) some of which have known tolerance to HLB, under Mediterranean conditions. Four treatments with different salt concentrations (0, 25, 50 and 75 mM of NaCl) were applied by watering the plants three times per week for eleven weeks. Chlorophyll index (SPAD), growth and plant symptom parameters were recorded on a biweekly basis. At the end of the trial, roots, stem and leaves biomass and plant mineral content were obtained. The increasing concentration of NaCl resulted in visible leave damage symptoms for all citrus rootstocks assayed, hindering plant growth in all citrus rootstocks assayed, except for X639. The highest concentration of toxic ions in leaves was detected in Carrizo citrange and US897 for Cl^-, while the lowest concentration of Na^+ was obtained in X639. These results provide growers with information about the sensitivity to salinity of different citrus rootstocks.

Keywords: chloride; HLB; NaCl; plant growth; salt-stress tolerance; SPAD

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1. Introduction

Citrus (*Rutaceae* family) is one of the most economically important fruit crop production in the Mediterranean basin, yielding more than 26 million tons and accounting for 13% of the worldwide *Citrus* production [1]. Due to climate change, the Mediterranean basin is seeing the rise of soil desertification [2–6], and most recently the emerging risk of diseases which have never been reported on citrus crops in this region, such as Huanglongbing disease (HLB) [7,8].

Salinity is a major negative abiotic factor in agriculture, and around 6% of the total land on the Earth and 20% of its arable area is under salinity conditions [9,10]. The Mediterranean basin is a semiarid region [11], where the low levels of water resources require usage of poor quality water from rivers, aquifers and/or treatment plants with high salt concentrations (chloride and/or sodium) [12,13]. Hence, these water resources often contain high electrical conductivity levels of over 3 dSm^{-1}, which further increase soil salinity and produce negative effects on citrus crops [14,15]. Crops respond differently to salinity, and *Citrus* is a sensitive crop [16]. In citrus, salt stress can cause reduction of plant growth, fruit quality and production [16–19]. Salinity symptoms are due to the accumulation of chloride (Cl^-) and sodium (Na^+) ions in leaves [20]. This accumulation causes osmotic disorders,

where long-term exposure can modify plant water relations [10]. Moreover, the increase of salt concentration in plant tissue leads to stomatal closure, decreases conductance, reduces leaf area, and produces chlorotic and necrotic patches on leaves [21–23]. As a result, the chlorophyll content and photosynthesis decrease [21–23]. Furthermore, the main negative effects related to transpiration, physiological and molecular aspects are involved with Cl^- [24–26]. Tolerant *Citrus* rootstocks can avoid salinity disorders, due to the fact that they are capable of excluding Cl^- and Na^+, showing different responses compared to salt-susceptible rootstocks [27,28]. In addition, salt stress produces *Citrus* plant disorders such as macronutrient deficiency [29].

In addition, HLB, caused by three bacterial species, *Candidatus* Liberibacter asiaticus (Jagoueix, Bové & Garnier) (CLas), *Candidatus* Liberibacter americanus (Teixeira, Saillard, Eveillard, Danet, da Costa, Ayres & Bové) (CLam), and *Candidatus* Liberibacter africanus (Jagoueix, Bové & Garnier) (CLaf) [30–34], is one of the most devastating citrus diseases in the world. Trees affected by this pathogen become unproductive and, as time elapses, they may eventually die [30,35]. *Candidatus* Liberibacter spp. are proteobacteria Gram-negative, obligate parasite, phloem-restricted and unculturable under laboratory in vitro conditions [32–36]. HLB affects production and fruit quality, and can occasionally cause tree death [30]. The most commonly used strategies for controlling the bacteria are the application of chemical antibiotics and a three-pronged system (TPS) [30], based on chemical and/or biological control of the psyllid vector complementary with planting healthy nursery trees and removing HLB-infected trees. However these solutions show moderate efficacy, [37] and the requisite chemical compounds are not currently approved for use in agriculture by the European Union authorities [38]. Thus, citrus plant breeding has become a major strategy to combat this disease [39]. This pathogen is widely distributed throughout all citrus producer regions in the world, except in Australia and the Mediterranean basin [7,8]. These bacterial species may spread by grafting from infected citruses to healthy citruses; and/or naturally by psyllids vectors [30]. *Diaphorina citri* (Kuwayama) has been described as the insect vector of CLas and CLam, whereas *Trioza erytreae* (Del Guercio) as the insect vector of CLaf [30]. However, *T. erytreae* has been recently reported as carrying CLas in Ethiopia [40]. Furthermore, this insect vector has recently appeared in mainland Mediterranean basin countries, such as Portugal and Spain [41–43].

Against this background, the accurate selection of citrus rootstocks is necessary to improve new trends and requirements of citrus crops [44,45]. Hence, new rootstocks have a huge importance due to their influence on multiple factors, such as tree vigor, production, quality and tolerance to abiotic and biotic agents [46]. Citrus rootstocks diversification reduces the risk of suffering enormous losses due to abiotic or biotic stresses [46,47]. Currently the Carrizo citrange [*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.] is the most common citrus rootstock used in Spain [48], although this rootstock is described as having an intermediate level of sensitivity to HLB [49] and it is also sensitive to salinity [28]. Nowadays, different citrus breeding programs continue to develop improved new citrus rootstocks for different factors [46]. Consequently, new trifoliate citrus hybrids such as US897 (*C. reticulata* 'Blanco' x *P. trifoliata*), US942 (*C. sunki* Hort. ex Tan. 'Sunki mandarin' x *P. trifoliata*) and X639 (*C. reshni* Hort. ex Tan. 'Cleopatra mandarin' x *P. trifoliata*) have been reported, with better tolerance response to HLB than Carrizo citrange [50–52] and with positive response against salinity stress [22,49,53]. To our knowledge, these citrus rootstocks have never been proposed as a viable option in Mediterranean basin citrus orchards.

Therefore, this work aims at studying the development of new citrus rootstocks, with known tolerance to HLB disease, under one of the most limiting abiotic factors in Mediterranean basin—salinity—in order to extend the variability of rootstocks for citrus growers and provide useful knowledge for the development of sustainable HLB control strategies.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

A total of 160 plants belonging to five different citrus rootstocks were used in this work. Two referenced comparative rootstocks including Carrizo citrange and Forner Alcaide No. 5 [FA No. 5; *C. reshni* 'Cleopatra mandarin' x *P. trifoliata* (L.) Raf.], and three citrus rootstocks with described HLB tolerance [49–52] as US942, US897, X639. Six-month-old citrus rootstocks from in vitro culture were obtained and provided by Agromillora Group nursery (Subirats, Barcelona, Spain).

The experiment was carried out in 2018 summer season under greenhouse conditions (30 °C average temperature and 56% average relative humidity) located in "Las Torres" Center of the Andalusian Institute for Agricultural and Fisheries Research and Training (IFAPA), in Alcalá del Río, Seville, Spain (Figure 1) (37°30′43.3″ N; 5°57′47.4″ W). Rootstocks were planted in 1.6 L pots with silica sand; this substrate allows leaching the solute and roots aeration. Plants were maintained under an acclimation period for two weeks where the rootstocks were irrigated three times a week with Hoagland and Arnon solution [54], with brief modification to citrus (3 mM of KNO_3, 3 mM $Ca(NO_3)$, 1 mM $MgSO_4$ $7H_2O$, 1.2 mM H_3PO_3 85%, 20 µM Fe-EDDAH, 54.4 µM $MnSO_4$ H_2O, 7.64 µM $ZnSO_4$ $7H_2O$, 0.5 µM, $CuSO_4$ $5H_2O$, 46.25 µM H_3BO_3, 0.55 µM MoO_3). The nutritive reagents were weighted with a digital scale and dissolved in a tank in the laboratory, and finally this primary solution was diluted in the greenhouse before of the irrigation process.

Figure 1. Satellite image of the greenhouse location.

2.2. Treatments and Experimental Design

A total of four salinity treatments (0, 25, 50 and 75 mM of NaCl) were established for all citrus rootstocks. To this end, a factorial experimental design was set on with four repetitions (block) and an elemental plot of eight plants (n = 8). The first application was carried out at the beginning of the experiment (W1) with eight plants per rootstock and treatment and after an acclimation period of two weeks. Each plant was irrigated with 500 mL of Hoagland and Arnon solution three times per week for ten weeks. Each treatment was prepared in a specific tank using nutritive solution amended with each salt concentration as described previously.

2.3. Variables Recorded

2.3.1. Evaluation of Plant Symptoms

The above-ground leaves symptoms were evaluated for each rootstock/plant and treatment to estimate the effect of the different salinity treatments, using a symptom scale of 0–4: plants without symptoms = 0, plants with 25% leaves affected by chlorosis = 1, 50% leaves affected by chlorosis = 2, over 50% leaves affected by chlorosis = 3, and fully

desiccated and dead plants = 4. This evaluation process was repeated every two weeks, the values were recorded for each plant for 11 weeks and starting from the first salinity application until two months later. These values were used to calculate the standardized area under the disease progress curve (SAUDPC, [55]), which increases in the same proportion as the symptom scale.

2.3.2. Plant Growth

Plant growth was determined in six plants per rootstock and treatment based on tree height. This parameter was measured with a measuring tape from the beginning of the assay (W1), with a frequency of every two weeks (W3, W5, W7, W9 and W11).

2.3.3. Chlorophyll Index (SPAD)

The leaf chlorophyll index was measured for a total of six plants by a SPAD chlorophyll meter (Minolta Co., Osaka, Japan). Two expanded leaves per plant were measured in four different weeks (W1, W5, W9 and W11).

2.3.4. Biomass

At the end of the experiment (W11), all plants (eight plants per treatment and citrus rootstock) were harvested and separated into three sections: roots, stem, and leaves. The fresh plant samples were then rinsed in distillated water, dried with filter paper, and weighted using a digital scale (COBOS precision, CB-3000C, L'Hospitalet de Llobregat, Barcelona, Spain). The different plant samples were introduced in a labeled paper envelope and dried in an oven at 60 °C for 48 h, and then weighted again to record the dry weight. The percentage of biomass reduction (PBR, %) was calculated for dry weight (DW) and fresh weight (FW) in each section plant for each sample, according to Vincent's equation [56]:

$$\text{PBR } (\%) \; = \; 100 \times \frac{(\text{CW} - \text{TW})}{\text{CW}}$$

where: CW = weight (g) of control treatment plants (0 mM of NaCl) averaged eight replicates from dry weight (DW) and/or fresh weight (FW) per each section plant. TW = weight (g) of treated plants at each concentration of salt tested (25, 50 and 75 mM of NaCl) from dry weight (DW) and/or fresh weight (FW) per each sample replicate and section plant.

2.3.5. Mineral Contents in Leaves and Roots

Analysis of Cl^-, Na^+ and macronutrients (N, P, K, Ca, Mg and S) in three leaves and for each citrus rootstock and treatment were carried out by a certified agriculture laboratory (Laboratorio Agrama S.L, La Rinconada, Seville, Spain) for plant analysis. The methodology analyses technique used for macro- and microelements was inductively coupled plasma optical emission spectroscopy (ICP-OES), for nitrogen a potentiometric technique was used and chloride was determined by Dumas's method.

2.4. Data Analysis

All data obtained were subjected to variance analysis (ANOVA) using the STATISTICA 10 software (StatSoft, Palo Alto, CA USA). Means separation were obtained using Fisher's test ($p < 0.05$). Normality and homogeneity assumptions were tested before ANOVA, using the Kolmogorov-Smirnov and Cochran's test, respectively. For non-observance of the normality and/or homogeneity assumptions, a non-parametric Kruskal-Wallis test was adopted. Two-way ANOVA and one-way ANOVA analysis were performed for SAUDPC data and the other results obtained respectively.

3. Results

3.1. Plant Symptoms

Plant symptoms for all five rootstocks assayed showed different SAUDPC value responses among salt treatments and rootstocks. Treatment application at 0 mM of salt

did not produce any stress symptoms. However, with a higher concentration of salt treatment, the values of SAUDPC saw a directly proportional increase as the salt treatment increased. No significant differences were detected at 25 mM among the citrus rootstocks assayed, in which US897 and FA No. 5 showed similar response than the results of control treatment (0 mM of NaCl). When concentration increased at 50 mM, significant differences in SAUDPC appeared among the rootstocks assayed, thus Carrizo citrange showed the lowest salinity rate even with a similar response as in the previous dosage described. On the contrary, FA No. 5 was the highest damaged rootstock at a 50 mM concentration, followed by US897, US942 and X639, with the latter showing a similar response as Carrizo citrange. Moreover, under the highest salt concentration (75 mM) X639 was the strongest rootstock against salinity, whereas US897 showed the highest symptoms values followed by FA No. 5, Carrizo citrange and US942 (Table 1). Similarly, mortality response started at 50 mM, with the same value for US897, X639 and FA No. 5. This parameter increased at 75 mM, in which US897 showed the highest percentage of plant death, followed by US942, FA No. 5 and Carrizo citrange; X639 plants did not die at the highest concentration treatment throughout experiment evaluation (Table 1).

Table 1. Mean standardized area under the disease progress curve (SAUDPC) ± standard error (SE) and Percentage of plant mortality (%) due to the effect of salinity treatments (0, 25, 50 and 75 mM NaCl) on leaves in five citrus rootstocks (US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange) over eleven weeks of treatments.

		Salinity Treatments (mM)			
	Rootstock	**0**	**25**	**50**	**75**
SAUDPC ± SE	**US942**	0 i	0.47 ± 0.07 h	0.69 ± 0.10 efg	1.01 ± 0.13 bcd
	US897	0 i	0.24 ± 0.04 hi	0.79 ± 0.06 def	1.31 ± 0.14 a
	X639	0 i	0.26 ± 0.06 h	0.66 ± 0.14 fg	0.74 ± 0.07 efg
	FA No. 5	0 i	0.23 ± 0.06 hi	0.92 ± 0.10 cde	1.25 ± 0.19 ab
	Carrizo citrange	0 i	0.25 ± 0.06 h	0.58 ± 0.10 gh	1.07 ± 0.18 abc
Mortality (%)	**US942**	0	0	0	25
	US897	0	0	12.50	37.50
	X639	0	0	12.50	0
	FA No. 5	0	0	12.50	12.50
	Carrizo citrange	0	0	0	12.50

Values with different letters mean significant differences among rootstocks and treatments assayed by Fisher's test ($p < 0.05$).

3.2. Plant Growth

A different response in growth among the citrus rootstocks and salt concentrations was shown compared with the treatment control (0 mM of NaCl) during all the experiment periods. Generally, all citrus rootstocks reduced in height when salt treatment concentration increased, compared with the control treatment. US942 and growth of Carrizo citrange began to reduce significantly from the third week evaluated between the highest concentration of salt tested salinity concentration (75 mM of NaCl) and the control treatment. Whereas FA No. 5 and US897 showed significant growth reduction in height between control and the highest concentration tested from the seventh and ninth week, respectively. Otherwise, X639 did not show significant differences in growth among all the treatments assayed, although growth was slightly reduced with the two highest treatments assayed (50 mM and 75 mM of NaCl) at the end of the experiment evaluation. At the end of experiment, US942 and X639 reduced their growth in height by less than 30% at the highest salt concentration compared with the control treatment. On the contrary, FA No. 5, Carrizo citrange and US897 showed a reduction of 35%, 38% and 47% at a concentration of 75 mM, compared with the control treatment in the eleventh week, respectively (Figure 2).

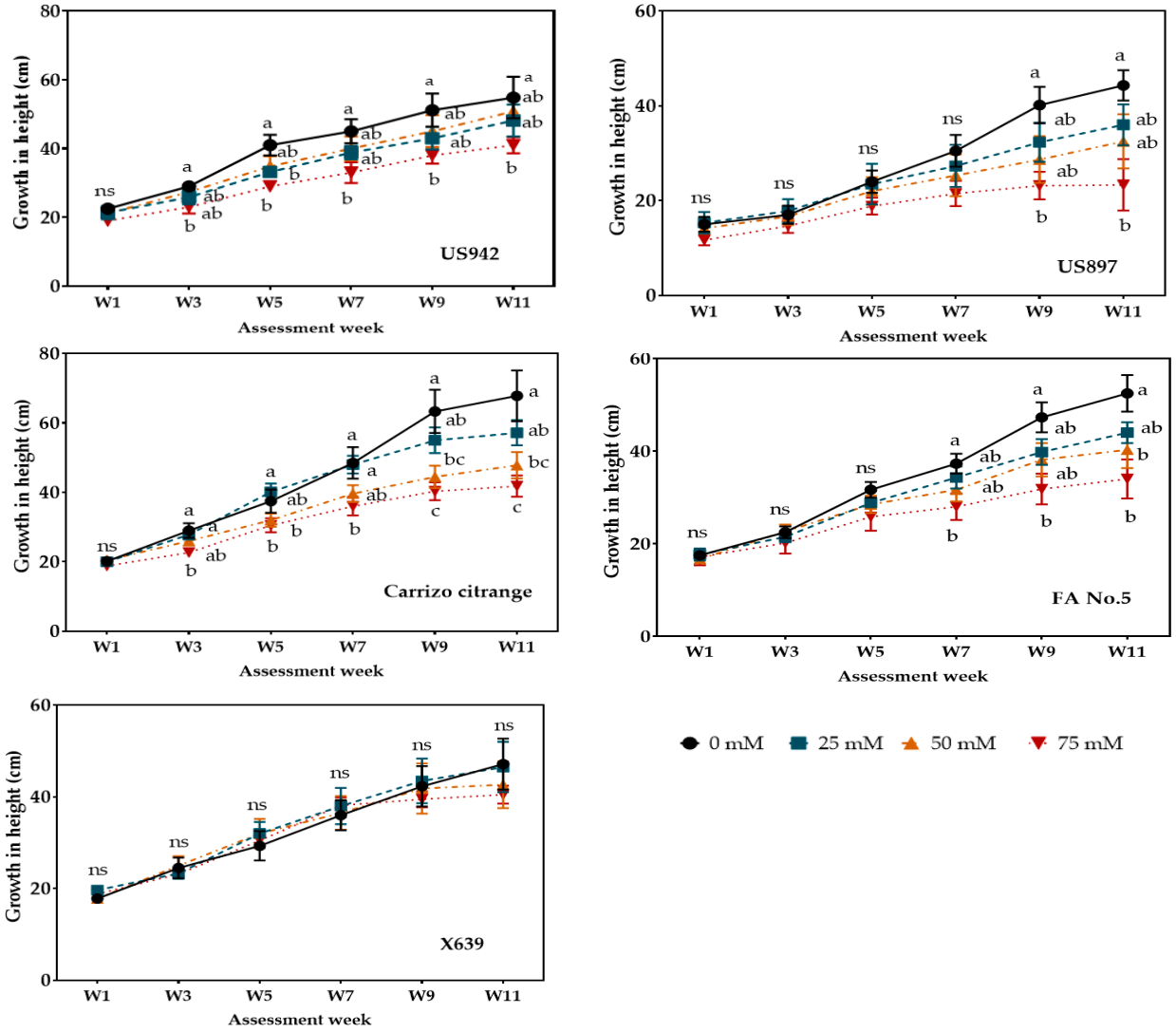

Figure 2. Time course assessment for the mean plant growth in height (cm) under the effect of four salinity treatments (0; 25; 50 and 75 mM of NaCl) on five citrus rootstocks plants (US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange) during eleven weeks of evaluation. Values in points with different letters mean significant differences among treatments for each citrus rootstock and evaluation time by Fisher's test ($p < 0.05$). FA No. 5: Forner Alcaide No. 5; ns: not significant differences.

3.3. Chlorophyll Index (SPAD)

All citrus rootstocks showed similar SPAD responses without significant differences among the treatments at the beginning of the experiment compared with the control treatment (0 mM of NaCl). However, SPAD values decreased when exposure time and salinity concentration increased. No rootstock significantly reduced this index in the first week evaluated. Only US942 and US897 reduced significantly the SPAD response with the highest salt concentration (75 mM) compared with the control treatment in the fifth week. In the nineth week evaluated, US942, US897 and X639 plants reduced the SPAD index at the highest concentration tested significantly, while the two others rootstocks assayed reduced this parameter slightly without significant differences at the highest dosage tested, compared with the salt concentration of 0 mM. Finally, only US897 plants reduced the SPAD values significantly, with a reduction rate of 47% at the highest concentration assayed compared with the control treatment at the eleventh week, the other rootstocks tested reduced slightly this parameter without significant differences at the same concentration and time compared with the control treatment, showing a reduction rate of 28%, 27%, 25% and 18% for FA No. 5, Carrizo citrange, US942 and X639, respectively (Figure 3).

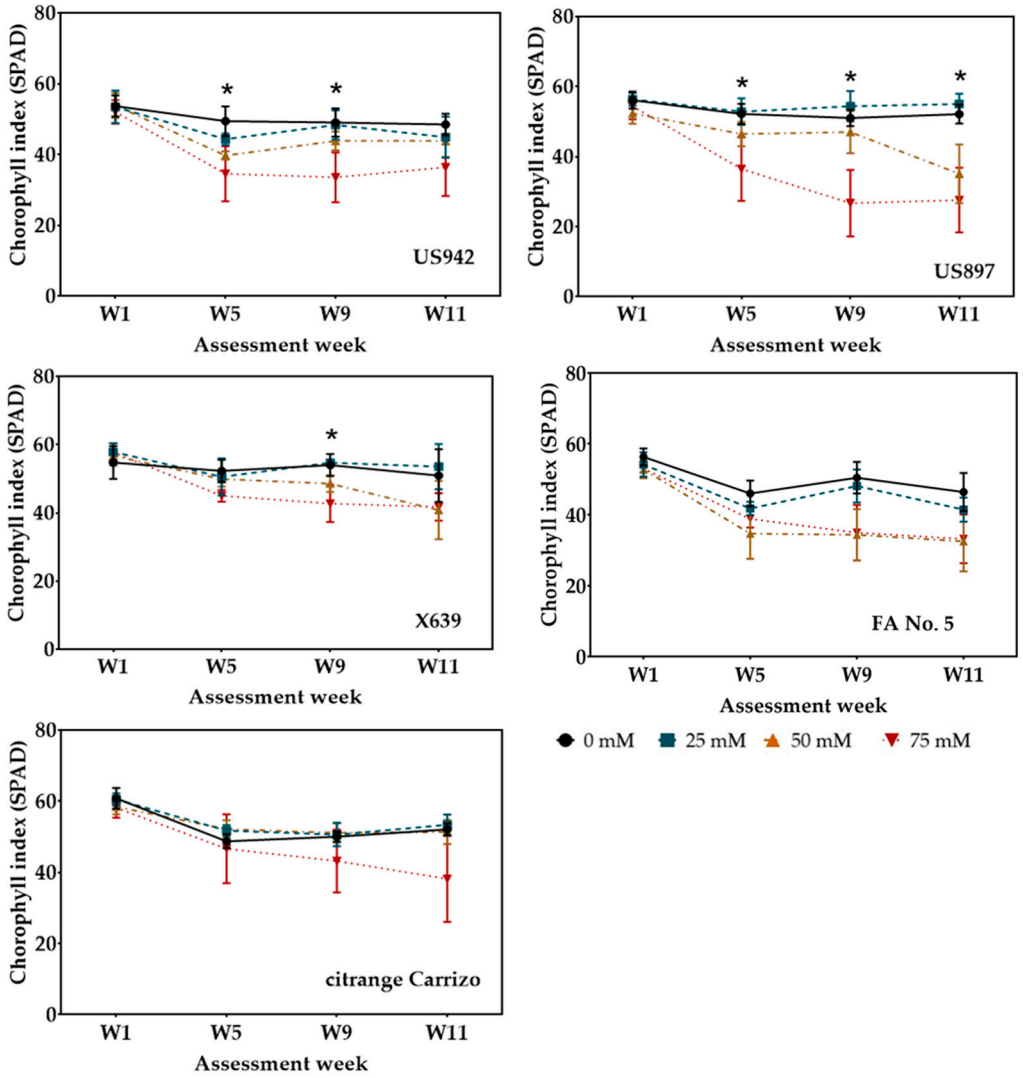

Figure 3. Time course for the mean of chlorophyll index (SPAD) values ± standard error (SE) under the effect of different salinity treatments (0, 25, 50 and 75 mM NaCl) on five citrus rootstocks plants (US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange) during eleven weeks of evaluation. (*) indicate significant differences among treatments for each citrus rootstock and evaluation time by Fisher's test ($p < 0.05$). FA No. 5: Forner Alcaide No. 5.

3.4. Biomass

At the end of the experiment, all evaluated rootstocks reduced fresh weight (FW) and dry weight (DW) for all plant sections evaluated with different level of significance compared with the control treatment (0 mM of NaCl).

All rootstocks tested showed significant differences among them for fresh weight of roots (FWR) and dry weight of roots (DWR) at a salt concentration of 25 mM. In this concentration, Carrizo citrange showed the highest significant PBR in FWR, with 46.8% followed by US942, US897, FA No. 5 and X639 with 28.06%, 27.12%, 16.96% and 15.04% respectively. However, percentage of reduction in DWR, X639 (41.23%) and Carrizo citrange (33.92%) displayed the highest reduction, followed by US942 (25.98%) and Fa No. 5 (29.39%), while US897 showed the least reduction (7.95%) (Table 2).

Table 2. Mean Percentage of biomass reduction (%, PBR) ± standard error (SE) on leaf (L), stem (S) and root (R) in dry (DW) and fresh weight (FW) after eleven weeks of four salinity treatment (0, 25, 50 and 75 mM of NaCl) in five citrus rootstocks, US942, US897, X639, Forner-Alcaide No. 5 and Carrizo citrange.

NaCl	Rootstock	FWR ± SE	FWS ± SE	FWL ± SE	DWR ± SE	DWS ± SE	DWL ± SE
25 mM	US942	28.06 ± 11.05 ab	41.09 ± 9.91 ns	24.35 ± 9.86 ns	25.98 ± 10.13 ab	41.85 ± 7.94 ns	20.67 ± 8.12 ns
	US897	27.12 ± 9.47 ab	34.94 ± 11.23 ns	22.14 ± 11.67 ns	7.95 ± 7.95 b	28.00 ± 13.14 ns	6.25 ± 6.25 ns
	X639	15.04 ± 6.26 b	23.21 ± 7.36 ns	18.31 ± 8.07 ns	41.23 ± 5.38 a	28.18 ± 7.59 ns	12.50 ± 6.55 ns
	FA No. 5	16.96 ± 5.61 b	23.57 ± 7.76 ns	25.00 ± 8.33 ns	29.39 ± 11.97 ab	31.84 ± 10.28 ns	25.38 ± 12.15 ns
	Carrizo citrange	46.84 ± 4.96 a	40.85 ± 7.78 ns	8.09 ± 6.57 ns	33.92 ± 5.64 a	20.41 ± 9.33 ns	11.31 ± 5.90 ns
50 mM	US942	48.67 ± 4.04 ns	56.06 ± 6.99 ns	31.76 ± 7.19 ab	41.67 ± 5.76 b	59.51 ± 5.91 ns	37.50 ± 0.07 b
	US897	62.57 ± 12.26 ns	52.24 ± 12.23 ns	51.43 ± 12.56 a	49.24 ± 12.63 ab	54.00 ± 12.69 ns	45.31 ± 0.13 b
	X639	50.48 ± 10.71 ns	45.13 ± 11.56 ns	39.79 ± 13.6 ab	67.86 ± 7.88 a	57.45 ± 9.42 ns	38.10 ± 0.15 b
	FA No. 5	53.97 ± 7.30 ns	51.43 ± 8.33 ns	61.90 ± 11.76 a	73.65 ± 8.75 a	68.42 ± 4.43 ns	78.46 ± 0.08 a
	Carrizo citrange	69.04 ± 3.51 ns	64.15 ± 4.05 ns	14.46 ± 6.00 b	64.34 ± 6.91 ab	57.19 ± 6.38 ns	32.9 4± 0.11 b
75 mM	US942	64.36 ± 7.48 ab	77.10 ± 4.90 a	68.24 ± 9.39 a	55.88 ± 10.99 ns	78.26 ± 5.90 ns	66.35 ± 09.39 a
	US897	67.91 ± 10.64 ab	76.60 ± 4.45 a	84.29 ± 4.55 a	57.58 ± 8.26 ns	67.00 ± 7.47 ns	71.88 ± 10.76 a
	X639	56.51 ± 4.83 ab	56.49 ± 4.43 b	39.61 ± 5.32 b	70.78 ± 6.01 ns	63.04 ± 4.26 ns	36.31 ± 6.36 b
	FA No. 5	53.09 ± 10.35 b	55.43 ± 9.63 b	64.29 ± 10.86 a	62.84 ± 5.91 ns	63.68 ± 6.98 ns	68.46 ± 7.30 a
	Carrizo citrange	77.17 ± 3.38 a	74.04 ±2.41 a	27.94 ± 8.16 b	71.33 ± 2.35 ns	69.42 ± 3.52 ns	50.79 ± 8.20 ab

Values with different letters means significant differences among rootstocks for each treatment by Fisher's test ($p < 0.05$). FA No. 5: Forner Alcaide No. 5; ns: not significant differences.

At a 50 mM of NaCl treatment, roots only reported a significant reduction among rootstocks in DWR but not in FWR. FA No. 5, and X639 showed the highest reduction (73.65% and 67.86%, respectively), followed by Carrizo citrange (64.34%), US897 (49.24%) and US942 (41.67%). At this concentration, stem did not report a reduction of biomass among rootstocks significantly. In contrast, leaves displayed significant reductions in FWL and dry weight in leaves (DWL). In FWL highest reduction occurs in US897 (51.43%) and FA No. 5 (61.9%), intermediate reduction appeared in US942 (37.76%) and X639 (39.79%), with a low reduction in Carrizo citrange (14.46%), whereas DWL showed 78.46% of reduction in FA No. 5 and the same reduction in the rest of citrus rootstocks (Table 2).

75 mM treatment generated significant difference in roots, stem and leaves fresh weight and only in leaves by dry weight. In FWR, the highest reductions occurred in Carrizo citrange (77.17%), whereas the lowest was in FA No. 5 (53.09%), with the reduction in US942 (64.36%), US897 (67.91%) and X639 (56.51%) being intermediate. However, in fresh weight of stem (FWS) a reduction higher than 70% of biomass was obtained in Carrizo citrange, US942 and US897, while in X639 and FA No. 5 it occurred below 60%. X639 and Carrizo citrange displayed less than 40% reduction in FWL whereas the remaining citrus rootstocks reported more than 60% reduction. Finally, significant differences among rootstocks were not found in DW of roots and stem. However, significant differences were achieved in DWL, where X639 reduced its biomass by less than 40%, but the remaining had an influence on salinity which led to a reduction higher than 50% (Table 2).

3.5. Concentration of Salt Ions (Chloride and Sodium) in Leaves and Roots

Overall, chloride concentration significantly increased, while salt concentration of the treatments increased in both plant tissues analyzed (roots and leaves) at the end of the plant experiments. Thus, Carrizo citrange displayed the highest significant concentration of Cl^- in leaves at 75 mM, compared with the other salt treatment concentrations, whereas US897 and US942 showed the highest leaf Cl^- concentration at 50 of salt; significant differences only compared with the lowest salt concentration (0 mM) for each rootstock. X639 and FA No. 5 displayed the highest levels of Cl^- in leaves at 75 mM of salt, with significant differences compared with the control treatment for each (Figure 4A).

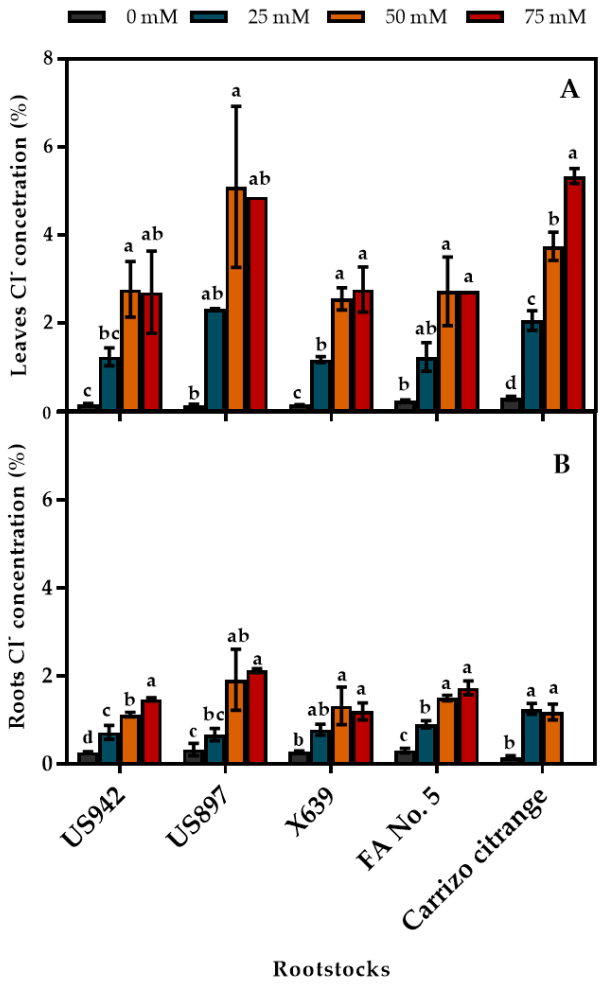

Figure 4. Mean Cl^- concentration (%) ± standard error (SE) after eleven weeks for all five rootstocks (US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange) under four concentrations of salt treatments (0, 25, 50 and 75 mM of NaCl). (**A**) Mean Cl^- concentration in leaves. (**B**) Mean Cl^- concentration in roots. Values in columns with different letters mean statistical differences among treatments for each rootstock by Fisher's test ($p < 0.05$). FA No. 5: Forner Alcaide No. 5; ns: not significant differences.

US942 showed the highest concentration of Cl^- in roots at 75 mM of salt, with significant differences compared with the remaining salt treatments. US897 and FA No. 5 reported a significant highest concentration of Cl^- in roots at 75 mM of salt compared with the control treatment for each rootstock. In addition, X639 showed the highest Cl^- in roots at a salt concentration of 50 mM, with significant differences compared with the control treatment. Finally, Carrizo citrange showed the significant highest concentration of Cl^- with a salt treatment of 25 mM, compared with the control treatment (Figure 4B).

Similarly, the sodium concentration increased significantly, while the salt treatments concentration increased for all plant tissues analyzed (roots and leaves) at the end of the plant experiments. Hence, Carrizo citrange and US942 showed the highest level of Na^+ in leaves and roots at 75 mM of salt, which was significantly different where compared with the other treatments for each rootstock. Additionally, US897, X639 and FA No. 5 recorded the significant highest concentration of Na^+ in leaves and roots, with the salt treatment of 75 mM compared with the control treatment for each rootstock (Figure 5).

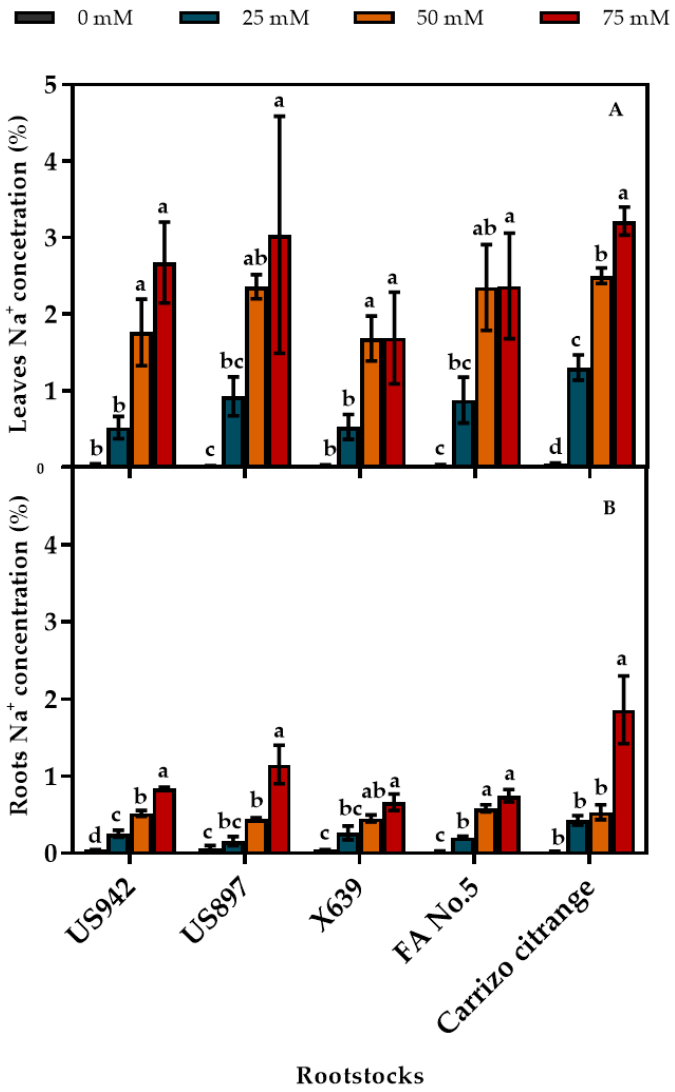

Figure 5. Mean Na^+ concentration (%) ± standard error (SE) after eleven weeks for the five rootstocks (US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange) under four concentrations of salt treatments applied (0, 25, 50 and 75 mM of NaCl). (**A**) Mean Na^+ concentration in leaves. (**B**) Mean Cl^- concentration in roots. Values in columns with different letters mean statistical differences among treatments for each rootstock by Fisher's test ($p < 0.05$). FA No. 5: Forner Alcaide No. 5; ns: not significant.

3.6. Macronutrient Concentration in Leaves

All evaluated *Citrus* rootstocks showed different response of macronutrients concentration in leaves between salinity treatments (Table 3). Nitrogen concentration did not differ significantly among salinity treatments within each rootstock, with the highest nitrogen concentration being found under the treatments of 25 and 50 mM of NaCl in all rootstocks, except for X639, which showed the highest nitrogen concentration in the control treatment (0 mM of NaCl). The phosphorus content increased in leaves with the treatment of higher salt concentration (75 mM of NaCl) compared to the control treatment (0 mM of NaCl), showing significant differences in all rootstocks except for US897. All citrus rootstock increased the leaf potassium concentration in intermediate salinity treatments (25 and 50 mM of NaCl), showing statistical differences among treatments within each rootstock, except for US942. The concentration of calcium in leaves was significantly higher in the control treatment compared with the other treatments applied for each rootstock tested, except for Carrizo citrange. When the treatment concentration of salt increased, the calcium level in leaves diminished. The highest leaf magnesium concentration was reported in

control (0 mM of NaCl) in all evaluated rootstock. Thus, the leaf magnesium concentration significantly decreased with the salt concentration for all rootstocks except for FA No. 5. Regarding the sulfur content, FA No. 5 and C. citrange were the only rootstocks that showed significant differences among the salt treatments applied, with a trend to increase concentration of this nutrient in leaves when the salt concentration increased (Table 3).

Table 3. Effect of salinity treatments (0, 25, 50 and 75 mM of NaCl) on leaf macronutrients (nitrogen, phosphorous, potassium, calcium, magnesium, and sulfur) expressed as the mean concentration (%) ± standard error (SE) for five rootstocks tested: US942, US897, X639, Forner Alcaide No. 5 and Carrizo citrange after eleven weeks.

		Macronutrients Concentration (%)					
Rootstock	**NaCl (mM)**	**N ± SE**	**P ± SE**	**K ± SE**	**Ca ± SE**	**Mg ± SE**	**S ± SE**
US942	0	3.74 ± 0.10 ns	0.15 ± 0.02 b	2.14 ±0.05 ns	2.11 ± 0.11 a	0.18 ± 0.00 a	0.26 ± 0.02 ns
	25	4.06 ± 0.10 ns	0.19 ± 0.00 ab	2.32 ±0.13 ns	1.51 ± 0.07 b	0.14 ± 0.02 b	0.23 ± 0.02 ns
	50	3.37 ± 0.43 ns	0.23 ± 0.02 a	2.21 ± 0.06 ns	1.53 ± 0.08 b	0.14 ± 0.01 b	0.31 ± 0.04 ns
	75	3.71 ± 0.23 ns	0.21 ± 0.01 a	2.19 ± 0.07 ns	1.47 ± 0.17 b	0.16 ± 0.02 ab	0.31 ± 0.02 ns
US897	0	3.28 ± 0.19 ns	0.18 ± 0.01 ns	1.90 ± 0.08 ab	2.23 ± 0.10 a	0.17 ± 0.00 a	0.25 ± 0.01 ns
	25	3.54 ± 0.16 ns	0.22 ± 0.00 ns	2.04 ± 0.06 a	1.75 ± 0.09 b	0.17 ± 0.02 a	0.24 ± 0.01 ns
	50	3.55 ± 0.01 ns	0.22 ± 0.00 ns	2.03 ± 0.03 a	1.41 ± 0.03 c	0.15 ± 0.01 ab	0.25 ± 0.00 ns
	75	3.46 ± 0.08 ns	0.21 ± 0.08 ns	1.45 ± 0.46 b	0.87 ± 0.09 d	0.11 ± 0.02 b	0.21 ± 0.06 ns
X639	0	3.55 ± 0.07 ns	0.13 ± 0.02 b	1.93 ± 0.07 b	2.09 ± 0.02 a	0.22 ± 0.03 a	0.23 ± 0.02 ns
	25	3.46 ± 0.33 ns	0.20 ± 0.01 a	2.33 ± 0.12 ab	1.73 ± 0.08 b	0.15 ± 0.01 b	0.24 ± 0.02 ns
	50	3.32 ± 0.18 ns	0.19 ± 0.01 a	2.72 ± 0.14 a	1.69 ± 0.05 b	0.18 ± 0.01 ab	0.26 ± 0.00 ns
	75	3.29 ± 0.04 ns	0.21 ± 0.01 a	2.18 ± 0.15 b	1.40 ± 0.09 c	0.16 ± 0.02 b	0.25 ± 0.01 ns
FA No. 5	0	3.05 ± 0.09 ns	0.17 ± 0.01 b	1.57 ± 0.03 b	1.97 ± 0.08 a	0.20 ± 0.01 ns	0.20 ± 0.02 b
	25	3.39 ± 0.08 ns	0.21 ± 0.01 a	2.08 ± 0.04 a	1.75 ± 0.07 ab	0.16 ± 0.01 ns	0.23 ± 0.00 ab
	50	3.17 ± 0.39 ns	0.20 ± 0.02 ab	1.82 ± 0.38 ab	1.38 ± 0.12 b	0.11 ± 0.02 ns	0.24 ± 0.03 ab
	75	3.09 ± 0.17 ns	0.22 ± 0.01 a	1.82 ± 0.14 ab	1.40 ± 0.27 b	0.15 ± 0.04 ns	0.25 ± 0.01 a
Carrizo citrange	0	3.98 ± 0.23 ns	0.19 ± 0.01 c	1.56 ± 0.06 c	2.49 ± 0.17 ns	0.21 ± 0.01 a	0.21 ± 0.01 b
	25	4.02 ± 0.23 ns	0.24 ± 0.01 b	1.80 ± 0.04 b	2.12 ± 0.08 ns	0.14 ± 0.01 b	0.29 ± 0.01 a
	50	4.02 ± 0.07 ns	0.24 ± 0.01 b	2.35 ± 0.07 a	2.36 ± 0.08 ns	0.16 ± 0.02 b	0.31 ± 0.02 a
	75	3.39 ± 0.08 ns	0.28 ± 0.01 a	1.77 ± 0.12 bc	NA	0.17 ± 0.01 ab	0.27 ± 0.02 a

Values with different letters mean significant differences among treatments for each rootstock by Fisher's test ($p < 0.05$). ns = not significant differences. N: nitrogen; P: phosphorous; K: potassium; Ca: calcium; Mg: magnesium; and S: sulfur. NA: Not available data. FA No. 5: Forner Alcaide No. 5; ns: not significant differences.

4. Discussion

Salinity stress is one of the most common plant-limiting factors in the Mediterranean region, and the crop surface affected by this issue increases every year [57]. Furthermore, this factor can influence plant physiological development [58]. Different studies have reported that *Citrus* crops can be symptomatic or asymptomatic in response to salinity problems, depending on the salt level [59]. In this study, we have successfully evaluated the influence of four salt concentrations by several plant parameters under Mediterranean conditions in five citrus rootstocks, some of which possess described tolerance to HLB disease [49,52,60]. These rootstocks showed different symptom and mortality responses to salinity stress, which could be related to genotype [61]. As in previous studies, X639 showed an adequate response of plant symptoms against salinity under the different assayed salt treatments. In addition, US942 and Carrizo citrange only showed high symptoms levels at the highest salt treatment [22,62]. However, FA No. 5 and US897 have been described as salinity tolerant citrus rootstocks in similar studies, [63,64] but these two rootstocks showed a high symptoms index upon moderate and severe salt treatments in our study.

As reported in prior research, salinity stress induces plant growth reduction of citrus rootstocks [13,22,58]. However, in our study X639 showed a similar plant growth response in height among the assayed salt treatments. According to Syvertsen and Bandaranayake [53], our results showed a better growth response under salinity for X639 than the other tested citrus rootstocks. On the other hand, US942 and US897 only reduced their growth at the highest treatment.

Moreover, plant growth reduction is related to photosynthesis reduction and changes in plant-water relations [16,65,66]. Some plant symptoms, such as chlorosis and necrosis, are associated with chlorophyll concentration in leaves, with the SPAD index being an indirect measure of this concentration [67]. SPAD index decreased in salt presence depending on the citrus rootstocks [14]. This response can occur due to chlorophyll synthesis process interruptions [68]. SPAD values between 50 to 60 denote non-stressed citrus plants [67]; all experiment plants were in optimal conditions at the beginning of the experiment. In contrast, at the end all citrus, rootstocks displayed a loss of SPAD units under severe treatment. US897 was the only citrus rootstock affected by salt stress on the last days of the experiment, with treatments reaching SPAD values below 30, which suggests a loss of chlorophyll in leaves. SPAD values are related with nutrients deficiencies [24,69].

Reduction of fresh and dry biomass is triggered by salinity treatment, which is also influenced by rootstock. The first organ in a plant affected by osmotic stress as a result of the presence of salt are roots. Carrizo citrange showed a high sensitivity to salt stress, showing a reduction of biomass in roots with all salt treatments, whereas US942, US897, X639 and FA No. 5 has less PBR. Additionally, Carrizo citrange and Forner-Alcaide No. 5 display this reduction at the high and mild salt treatments. Regarding leaves, PBR was similar in all citrus rootstocks at mild treatment, whereas it was similar at 50 mM in FA No. 5 in dry and fresh weight. In contrast, under the highest concentration of salts, US897 decreased above 70% its fresh and dry biomass when compared with control, which indicates a major susceptibility to salts in growth terms. Furthermore, salt stress leads to a reduction both in citrus growth and in biomass development over time. Our percentage of biomass reduction values ranged from 8 to 85%, higher than described in previous research [70,71]. These differences among works may be traced to different growth conditions and climatology.

All citrus rootstocks showed differences in nutrient concentration in the control treatment; this variety could be explained because of different absorption capacities and roots systems [72]. Nutritional imbalance under salinity exposure appears to be due to affections on membrane selectivity [12] and/or competitive antagonist interactions [73,74]. Salinity tolerance in citrus is due to the ability of exclude or avoid to uptake or transport the salt ions from the roots to the shoots [75]. Several studies state that a high Cl^- concentration is related to sensitivity to salt stress, and the lower presence in leaves of this ion indicates an exclusion mechanism, which is associated with a tolerant phenotype [63,66]. Low salt concentration entails a low accumulation, although the differences on ion accumulation found in our study depend on citrus rootstock. On the other hand, our results showed that all citrus rootstocks treated with salts (25 mM, 50 mM, 75 mM of NaCl) exceed the limit Cl^- concentration (more than 0.7% according to Morgan and Obreza [76]). The accumulation of Cl^- in the leaves increased from salt concentration of 25 to 75 mM in US942, which is described as a suitable rootstock for a mild salinity level, but not moderate or severe levels [22]. Carrizo citrange and US897 displayed the highest Cl^- concentration in leaves at 75 and 50 mM of salt, respectively. This Cl^- accumulation is related to less tolerance to salt environments, which renders these citrus rootstocks options less suitable for areas with soils and/or water with salt excess [75].

Our results showed a proportional accumulation of Na^+ in leaves when salt treatments increased. In addition, this accumulation increased in roots, which can be related to a partial accumulation of this ion in roots in order to diminish the accumulation in leaves where Na^+ caused more damage. This exclusion is described in *P. trifoliata* citrus rootstocks, which is the parental for all rootstocks studied in this work [77]. However, there are clear differences in accumulation of Na^+ in leaves and roots.

In keeping with Syvertsen et al. [53], leaf nitrogen contents were not impacted by salt treatments in our study. There is a competitive relationship between Na^+ and potassium; hence as the concentration Na^+ increases in leaves, potassium accumulation reduces in plant tissues [78,79]. In our study, this effect occurs in US897, which saw its potassium accumulation in leaves drop when the level of salt concentrations increased [12,75]. However, X639 increased potassium accumulation in leaves under high salinity treatments,

which is associated with a mechanism to reduce osmotic stress caused by Cl^- accumulation within cell leaves [14,78]. The concentration of calcium and magnesium was reduced in all rootstocks with salinity treatments higher than 0 mM [78,80]. This response was aggravated in US897 for calcium accumulation, while magnesium accumulation reported a similar decrease in all citrus rootstocks studied, except for FA No. 5, with no variations in the magnesium percentage in leaves [81]. The low absorption of nutrients, such as potassium, calcium and magnesium, may be associated with the high concentration of Na^+ in roots [82]. As opposed to other research, leaf phosphorus content decreases as salinity concentration increases in all assayed citrus rootstocks, except for FA No. 5, the leaf phosphorus content of which was not impacted by salinity [81,83]. The results obtained report that X639 showed a better physiological and nutritional behavior under salt stress that the remaining citrus rootstocks studied; similar results were reported by previous studies [53,64,84] where X639 appears as sound citrus rootstocks for salinity environment.

5. Conclusions

Few studies have tested new citrus HLB tolerant rootstock against salinity problems under Mediterranean conditions. The proper choice of citrus rootstocks is a determinant factor against salinity, which can largely solve the salinity issues of citrus crops in this area. In addition, citrus selection should be done in light of other limiting factors and threats in this region, increasing the availability of citrus rootstocks against abiotic and biotic stress. Thus, X639 demonstrated an optimal response against high salinity concentration of the Mediterranean Basin conditions. Likewise, US942 and FA No. 5 were reported as optimal citrus rootstock for mild salinity concentration conditions. Conversely, US897 and Carrizo citrange are not shown as the best option for Mediterranean salinity conditions. Our findings provide interesting information for citrus growers about new citrus rootstocks for Mediterranean basin conditions in order to increase the diversity of citrus rootstocks and address current and future abiotic and biotic stress problems.

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