



Article

Effective Categorization of Tolerance to Salt Stress through Clustering *Prunus* Rootstocks According to Their Physiological Performances

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Abstract: The effects of climate change on traditional stone fruit producing areas, together with the generation of new varieties with lower chilling requirements that allow the cultivation of previously unexplored areas, are setting up a challenging scenario for the establishment of productive orchards that must be more efficient in their capacity to adapt to new edaphoclimatic conditions. In this context, the rootstock breeding programs are a key piece in the agronomic strategy to achieve this adaptation through the development of rootstocks compatible with the new varieties and capable of transferring their tolerance to stress. An effective categorization of phenotypes within the germplasm involved in a plant breeding program is of utmost importance. Through the measurement of physiological parameters in both roots and leaves, tolerance to saline stress (120 mM NaCl) was evaluated in seven *Prunus* rootstocks whose genetic background included representatives of the subgenera *Prunus*, *Cerasus*, and *Amygdalus*. To group the genotypes according to their physiological performance under salt stress, an agglomerative hierarchical clustering was applied. The genotypes were grouped into three clusters containing rootstocks very sensitive ('Mazzard F12/1'), moderately tolerant ('Maxma 60', 'Cab6P' and 'AGAF 0204-09'), and tolerant ('Mariana 2624', 'Garnem' and 'Colt') to salt stress. 'Mariana 2624', a plum-based rootstock, was identified as the most tolerant *Prunus* rootstock. The information reported is valuable both in the productive context, for the selection of the most appropriate rootstocks to establish an orchard, and in the context of plant breeding programs, when choosing parents with outstanding traits to obtain progenies tolerant to salt stress.

Keywords: *Prunus*; rootstock; salt stress; gas exchange; WUE; root respiration; agglomerative hierarchical clustering; germplasm characterization



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1. Introduction

Among the abiotic factors that affect the productivity of fruit orchards, the excess of salts at the rhizosphere is recognized as one of the more detrimental ones. The soil salinization is given majorly by natural factors but also by anthropic factors derived from some agricultural procedures, such as inappropriate practices of irrigation or overuse of fertilizers. These factors contribute to a continuous salinization process, which is a growing menace that currently is affecting around 20% of irrigated lands [1] and estimations foresee an increment close to 50% by the middle of this century [2]. Saline soils are defined as those with an electrical conductivity of the saturation extract (EC_e) equal to or exceeding 4 dS/m [3]. This value determines significant reductions in the yield of the main crops worldwide [4].

In glycophyte plants, which include most crops, the impact of high salinity on plant growth is well known. Such a negative impact occurs in both the acute and chronic

phases of salt stress. In first place, there is a reduction in the root water uptake ability as a consequence of the fall in the osmotic potential of the saline soil. The consequent osmotic stress triggers a reduction in cell expansion affecting root growth and leaf expansion alongside a drop in the stomatal conductance and photosynthesis rate [5]. In second place, the chronic accumulation of ions in plant tissues, such as sodium, imposes an ionic stress to the plant. At the leaf level, this ion toxicity could affect the enzyme activities, the production of photoassimilates, and, ultimately, the plant growth [6–8].

In general terms, the *Prunus* species are crops classified as sensitive to salinity within a range from moderate to highly salt sensitive [9], which implies the existence of a gradient of tolerance within the genetic background of this genus. Greater genetic diversity included in cultivated germplasm is pivotal for sustained crop improvement [10]. The characterization of this genetic diversity and its interaction with environmental factors are crucial for its adequate incorporation into plant breeding programs. The development of new stone fruit varieties with lower chilling requirements [11,12] has allowed to establish orchards in climatic zones with earlier harvesting seasons, such as semiarid regions [13] but with soils prone to saline conditions impairing the fruit production. In this context, the identification of germplasm with a higher ability to tolerate saline soils is particularly important to make viable the agriculture in such soil conditions and to perform studies aiming to reach a deeper insight into the mechanisms underlying their salt tolerance in woody fruit trees. Regarding the above mentioned, the development of rootstocks more tolerant to environmental challenges imposed by global climate change is key to adapting the modern fruit orchard production to adverse scenarios such as an excess or deficit of water, alkaline soils, and high salinity [14–17].

The root is the first organ to deal with the salt excess in soil, and given its functions of prospecting and transporting water, nutrients, and ions, it can play a key role in determining the sensitivity or tolerance to salt stress of the whole plant. In woody fruit trees with different levels of salt tolerance, a reduction in root growth under salt stress has been indistinctly evidenced [18–21]. Saline stress can imbalance the metabolism of reactive oxygen species (ROS) [22,23] and favor the accumulation of these molecules with deleterious effects at multiple levels such as DNA, lipids, proteins, photosynthetic pigments, cell membrane, among others [24–26]. In addition, the oxidative damage induced by excessive ROS accumulation has been involved as part of the decline of root growth in plants under saline stress [27,28]. Along with the detrimental effects on root growth, a reduction in root respiration has been reported as a high salinity effect [29]. Root respiration provides the energy necessary for both root growth and nutrient absorption [30], therefore, the inhibition of this process would imply a series of detrimental effects for physiological functions at the whole plant level [29]. At the aerial part of the plant, one of the consequences more widely reported of the salt stress is the negative impact on the photosynthetic rate (P_n), which in an early stage of the stress is given by stomatal limitations [31,32] and, latterly, by nonstomatal limitations [33].

Several studies covering different genotypes of the *Prunus* spp. have evidenced the impacts of the excess of salt on growth, biochemical and photosynthetic parameters, among others in order to characterize the ability of these woody species to adapt to salinity [21,34–40]. Although these studies have found different levels of tolerance to salt stress within the genus *Prunus*, it is important to consider that they usually include a small number of genotypes and that both the salinity levels and the methodologies for the imposition of saline stress differ between the investigations, which makes it difficult to estimate comparatively the ability to tolerate saline conditions and establish a well-defined gradient of salt tolerance among the *Prunus* species.

In this article, we characterize the responses of seven *Prunus* rootstock genotypes to salt stress imposed by watering with NaCl solutions ranging from 0 to 120 mM. These genotypes have different genetic backgrounds which aimed to perform a systematic study of the plant response to salt stress in the *Prunus* rootstocks germplasm in terms of root development, root respiration, oxidative damage, gas exchange, and water use efficiency.

The salt tolerance coefficients of these genotypes were used to classify them according to their relative tolerance to high salinity (120 mM NaCl). This is a study that performed a systematic assessment of salt tolerance on the genotypes of rootstocks with a widely diverse genetic background that included species from the three subgenera of the *Prunus* genus and their hybrids. Such an evaluation is crucial to guide both the establishment of orchards with suitable rootstocks and the efforts of breeding programs for improving the ability of the *Prunus* rootstocks to better adapt to saline soils in order to sustain fruit production.

2. Material and Methods

2.1. Plant Material and Salt Stress Treatment

One-year-old, clonally propagated, and virus-free plants of the *Prunus* rootstock of ‘Mariana 2624’ (*Prunus cerasifera* × *Prunus munsoniana* W. Wight & Hedrick), ‘Cab 6P’ (*Prunus cerasus* L.), ‘Colt’ (*Prunus avium* (L.) L. × *Prunus pseudocerasus* Lindl.), ‘Maxma60’ (*Prunus mahaleb* L. × *Prunus avium*), ‘Garnem’ (G × N15) (*Prunus dulcis* (Mill.) D.A. Webb × (*Prunus persica* (L.) Batsch × *Prunus davidiana* (Carrière) N.E.Br.)), ‘Agaf 0204-09’ (*Prunus persica* × (*Prunus dulcis* × *Prunus persica*)), and ‘Mazzard F12/1’ (*Prunus avium*) genotypes were acquired from a commercial nursery and immediately transplanted to 3 L plastic pots. A mixture of sand, vermiculite, and perlite in a 1:1:1 ratio (v/v/v) was used as a substrate. Plants were watered three times a week with 200 mL of tap water and fertilized every two weeks with 1 g/pot of commercial fertilizer containing N:P:K (25:10:10) (Ultrasol™, Soquimich, Chile). Plants were grown in the CEAF’s (Centro de Estudios Avanzados en Fruticultura) experimental field (latitude 34°19′21.02″ S; longitude 70°50′02.26″ W) under semi controlled conditions under a plastic net where the PAR on the top of the plants was 700 mol m⁻² s⁻¹, and the temperature fluctuated between 27 and 30 °C in the day and 15 and 18 °C at night. The photoperiod during the experiment averaged 16/8 h day/night.

For salt stress treatment, sets of three plants of each genotype of the *Prunus* rootstocks were submitted to three salinity levels by watering with 0, 60 and 120 mM NaCl solutions with 0.83, 5.50 and 6.43 dS/m, respectively. These electrical conductivity values were recorded by an ExStick EC500 conductivimeter (Extech Instruments, Waltham, MA, USA). Plants were placed in greenhouse within controlled temperature ranging from 22 to 26 °C and watered regularly each third day with 400 mL of each solution for four weeks.

2.2. Root Length Measurement

At the end of the leaf gas exchange measurements, the plants were harvested, and their roots were carefully cleaned with water and shaken gently to remove excess water. Immediately, the roots were deployed on a smooth surface to be extended in order to record the maximum root length of their root systems. The maximum root length was determined as the length of the longest roots in each plant of the different genotypes under saline and control conditions. Finally, root samples were obtained for respiration rate and malondialdehyde content measurements.

2.3. Root Respiration Rate

Three rootstocks of each genotype submitted to three salinity levels were taken for root respiration measurements at the end of the experiment. To estimate root respiration rate (RRR), two sets of 2 cm segments of 10 root tips were excised from whole root system of each biological replicate. The root tips were enclosed and transferred in the dark to a root respiration airtight cuvette containing 3 mL of an oxygen-saturated nutrient solution without Fe to avoid element precipitation. The root respiration rate was measured as the O₂ consumption from the root tips using a liquid-phase O₂ electrode Clark-type (Hansatech Co. Ltd., Norfolk, UK) connected to a constant temperature circulating water bath (Labtech, Singapore) at 25 °C. For each measurement point, the dissolved O₂ concentration into the cuvette was continuously monitored and recorded every 5 min for 20 min. The nutrient

solution into the root respiration cuvette was continuously homogenized with a magnetic stir bar at the bottom of the pots.

2.4. Malondialdehyde Determination

For the malondialdehyde (MDA) measurements, frozen roots (0.2 g) of each biological replicate were homogenized in 80% cold ethanol using a cold mortar and pestle. The homogenates were centrifuged, and two aliquots (technical replicates) of the supernatants were mixed with either 20% trichloroacetic acid or 20% trichloroacetic acid plus 0.5% thiobarbituric acid. Both mixtures were incubated at 90 °C for 1 h, cooled on ice and centrifuged at $3000\times g$ for 10 min. The absorbance of each supernatant was measured at 440, 534, and 600 nm, and the MDA concentration was calculated according to [41].

2.5. Leaf Gas Exchange Measurements

The leaf gas exchange was measured according to [42], using a CIRAS-2 portable IRGA photosynthesis system (PPSystem, Hitchin, UK) with a controlled environment CIRAS PLC cuvette (broad windows 2.5 cm^2) warmed at 25 °C. The incident PAR ($1000\text{ mmol m}^{-2}\text{ s}^{-1}$) was supplied by a LED light cuvette unit. The CO_2 concentration in the cuvette was adjusted to 400 ppm, and the relative humidity was set at 50%. The stomatal conductance (g_s), photosynthetic rate (A), transpiration rate (E), and internal CO_2 concentration (C_i) were measured from 10:00 to 11:30 am on clear days on two fully expanded leaves from three plants of each genotype in the three experimental conditions at 0, 10, 20, and 30 days. The intrinsic water use efficiency (WUE_i) was estimated from the ratio between the photosynthetic rate and stomatal conductance (A/g_s).

2.6. Statistical Analysis

A randomized block design was used in the experiment. The means of two technical replicates of three independent biological replicates were subjected to one-way ANOVA and LSD pairwise comparisons using Statistica 4.0 software (Statsoft Inc., Tulsa, OK, USA).

Agglomerative hierarchical clustering was generated using salt tolerance coefficients (STCs). The STCs were calculated for growth, physiological, and biochemical parameters from their mean values registered at 30 days of treatment. $STC = \bar{x}_{\text{salt}} / \bar{x}_{\text{control}}$, where $\bar{x}_{\text{salt}}$ is the mean value of the parameter under saline conditions, and $\bar{x}_{\text{control}}$ is the mean value of the parameter under saline conditions. Dissimilarities among genotypes were established by the Euclidean distance and aggregated by Ward's minimum variance method.

3. Results

3.1. Root Phenotype in *Prunus* spp. Rootstocks under Salt Stress

Different root phenotypes were observed among the seven *Prunus* rootstocks genotypes after 30 days of salt stress (Figure 1). As expected, the degree of root damage was related to the salinity level of the irrigation applied during this study.

At the end of the assay, among plants under the control condition (0 mM NaCl), two rootstock genotypes ('Agaf 0204-09' and 'Maxma60') presented the longest root systems with 27 cm approximately. The rest of the rootstocks exhibited similar root sizes ranging from 23.4 to 19.1 cm (Table 1).

In general terms, all genotypes of the *Prunus* rootstocks reduced the length of their root systems when exposed to 60 mM NaCl irrigation, but such reductions were more evident in plants treated with the 120 mM NaCl solution. In this saline concentration, the greatest reduction in the root length was recorded by the 'Mazzard F12/1' rootstock, decreasing to 55.67% with respect to the length measured in the control condition (Table 1). Interestingly, the two rootstock genotypes with the longest root systems registered in control conditions, exhibited opposite trends in salt stress. With NaCl 120 mM, 'Agaf 0204-09' was the rootstock with the least reduction of its root system size (20.35%). On the other hand, 'Maxma60' registered the second greatest reduction (48.44%) (Table 1).

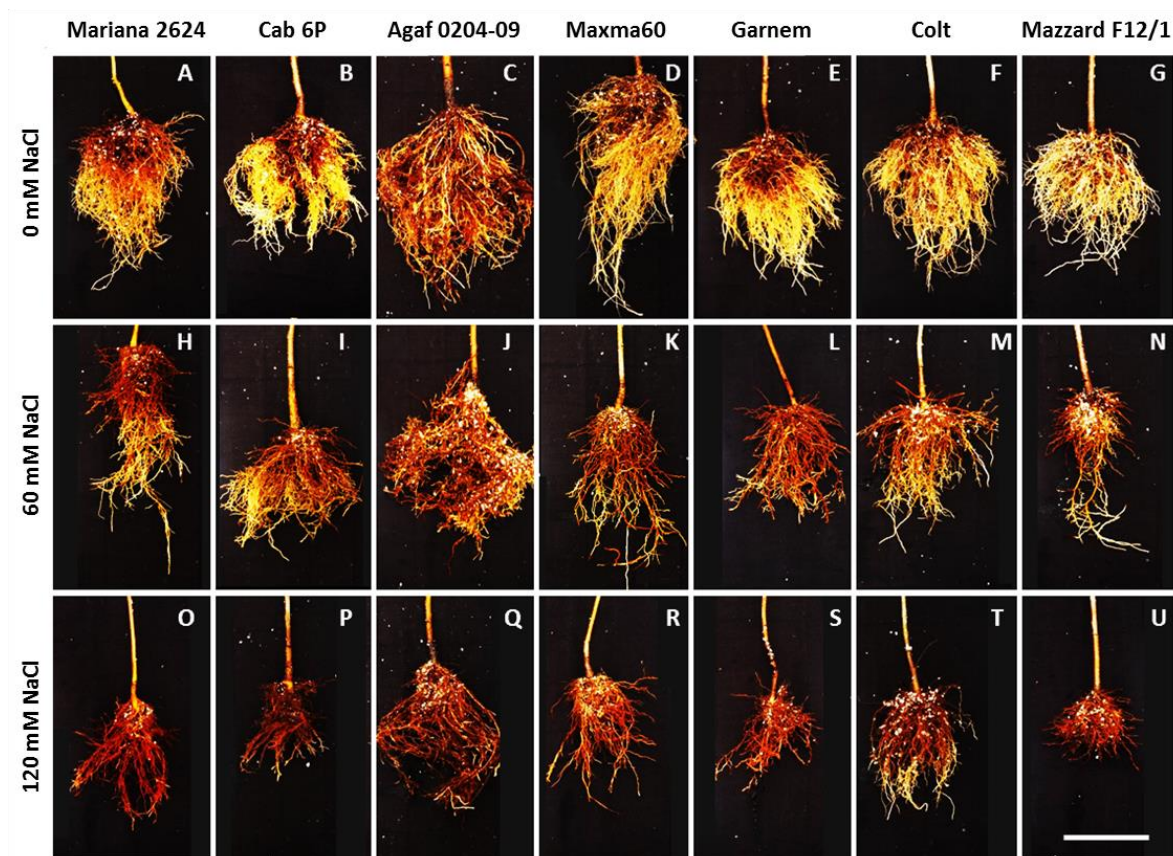


Figure 1. Representative phenotypes of the seven *Prunus* rootstock genotypes assayed after 30 days of saline treatment. Roots under control irrigation with distilled water: (A) ‘Mariana 2624’, (B) ‘Cab6P’, (C) ‘AGAF 0204-09’, (D) ‘Maxma 60’, (E) ‘Garnem’, (F) ‘Colt’, and (G) ‘Mazzard F12/1’. Roots under saline irrigation with 60 mM NaCl solution: (H) ‘Mariana 2624’, (I) ‘Cab6P’, (J) ‘AGAF 0204-09’, (K) ‘Maxma 60’, (L) ‘Garnem’, (M) ‘Colt’, and (N) ‘Mazzard F12/1’. Roots under saline irrigation with 120 mM NaCl solution: (O) ‘Mariana 2624’, (P) ‘Cab6P’, (Q) ‘AGAF 0204-09’, (R) ‘Maxma 60’, (S) ‘Garnem’, (T) ‘Colt’, and (U) ‘Mazzard F12/1’. Scale bar: 10 cm.

Table 1. Variations of the root system length of *Prunus* rootstocks under salt stress treatments.

	Maximum Root Length (cm)					
	NaCl Solution Concentration					
	0 mM		60 mM		120 mM	
Mariana 2624	23.4 a	±1.9	16.6 b	±3.7	13.9 b	±1.7
Cab 6P	20.2 a	±3.0	17.0 a	±1.1	15.0 b	±0.8
Agaf 0204-09	27.4 a	±1.8	23.0 b	±1.3	21.9 b	±1.3
Maxma60	27.8 a	±2.9	13.7 b	±2.8	14.4 b	±1.6
Garnem	19.6 a	±2.0	14.7 b	±1.0	12.4 b	±2.4
Colt	21.0 a	±1.6	17.0 b	±0.8	15.1 c	±0.4
Mazzard F12/1	19.1 a	±1.8	13.9 b	±1.8	8.5 c	±2.1

The values represent the mean and standard error of three biological replicates. The different letters indicate significant ($p < 0.05$) differences among treatments within a genotype after 30 days of salt stress treatments.

Because the saline treatment with 120 mM NaCl revealed the largest impacts on the lengths of the root systems and established more clearly the differences in the tolerance/sensitivity degree among all the rootstock genotypes of the *Prunus* spp. in comparison to the root lengths under control conditions, the present study was focused on this saline stress level.

3.2. Malondialdehyde (MDA) Content in Roots

Along with the reduction of the root system length, salt-induced damage was also evidenced as a reddish coloration likely associated with the oxidation of the root tissue, which was more marked in roots under 120 mM NaCl irrigation at 30 days. In order to quantify the extension of this symptom, the MDA content was used as a biochemical marker of oxidative damage. In control conditions, the roots exhibited different MDA basal levels among the rootstocks analyzed, ranging from $0.88 \text{ nmol g}^{-1} \text{ FW} \pm 0.37$ ('Colt') to $2.15 \text{ nmol g}^{-1} \text{ FW} \pm 0.28$ ('Mazzard F12/1') (Figure 2). Expectedly, under salt stress, all genotypes showed a significant increase in their root MDA contents compared to those levels detected for the control plants. However, this increase in the MDA contents evidenced different magnitudes among the roots of the *Prunus* genotypes assessed. Thus, four out seven *Prunus* rootstocks did not double the amount of MDA produced in response to saline stress when compared to the levels detected in the control condition. In this group, 'Mariana 2624' roots evidenced the lowest MDA increment (54.58%) followed by 'Garnem' (63.24%), 'Cab 6P' (72.25%), and 'Mazzard F12/1' (81.44%). Instead, the other three rootstocks at least doubled their MDA contents, reaching the highest value in 'Colt' (154.64%), followed by 'Maxma60' (138.16%) and 'Agaf 0204-09' (107.02%) (Figure 2). Interestingly, the 'Colt' roots showed the highest percentage of increase in MDA contents despite being the genotype with the lowest basal levels of this marker of oxidative damage.

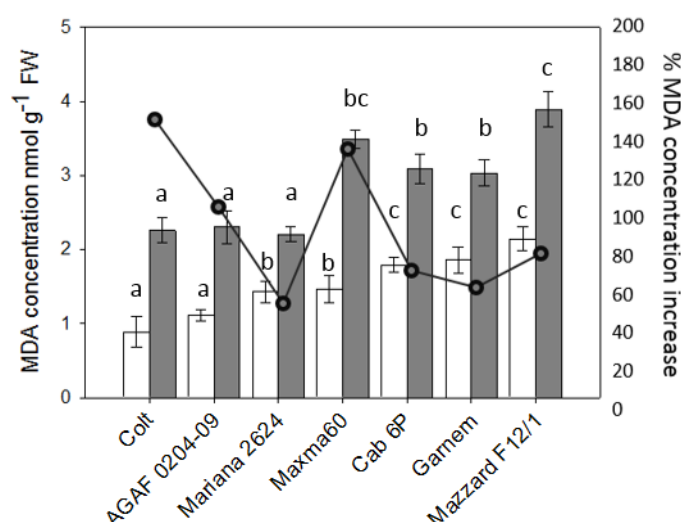


Figure 2. MDA concentrations in the roots of *Prunus* rootstocks under salt stress treatments. Levels under control condition (white bars) and 120 mM NaCl (gray bars) saline irrigation. The values and error bars represent the mean and standard errors of three biological replicates with two technical replicates. Different letters indicate significant ($p < 0.05$) differences among genotypes within the same treatment. Dots represent the percentage of increase in MDA levels between control and salt treatments for each rootstock genotype.

3.3. Root Respiration Rate

In control conditions, all the *Prunus* spp. rootstocks analyzed in this study showed similar RRRs, but when exposed to high salinity conditions (120 mM NaCl), this parameter was affected in a different extension depending on the genotype. Thus, the rootstocks evidencing the most marked drop in their RRRs were 'Mazzard F12/1' (78.62%) followed by 'Garnem' (78.26%, Figure 3). On the other hand, the 'Mariana 2624' roots exhibited the least decrease in RRR in saline conditions (52.97%, Figure 3) along with the lowest percentage of MDA concentration increase (Figure 2), however, this rootstock genotype suffered a noticeable reduction in its root length as a consequence of salt stress (Table 1).

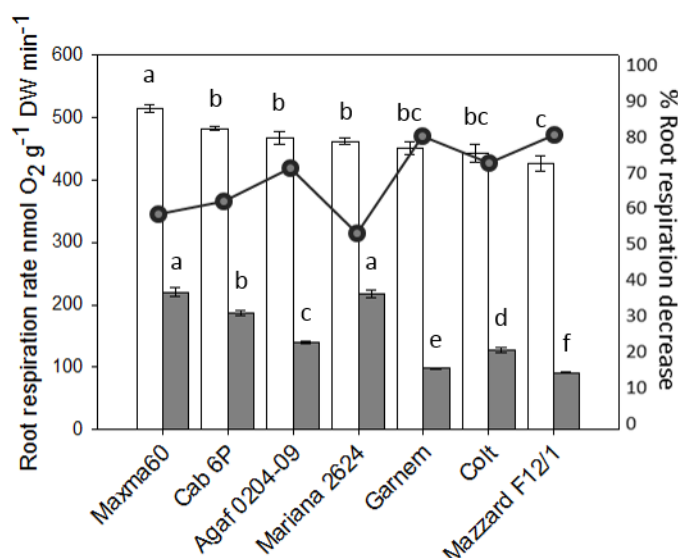


Figure 3. Root respiration rate of *Prunus* rootstocks under salt stress treatments. Levels under control condition (white bars) and 120 mM NaCl (gray bars) saline irrigation. The values and error bars represent the mean and standard errors of three biological replicates with two technical replicates. Different letters indicate significant ($p < 0.05$) differences among genotypes within the same treatment. Dots represent the percentage of decrease in root respiration levels between control and salt treatments for each rootstock genotype.

3.4. Gas Exchange Parameters

The plants under control conditions showed stable values of photosynthetic rate (A) within genotypes in the measurements performed during the experiment (Figure 4A–G). As a consequence of salt stress, the photosynthetic rate was negatively affected in all rootstock genotypes (Figure 4H–N). However, the ‘Mazzard F12/1’ rootstock exhibited the more rapid and dramatic drop since the assessment of the photosynthetic parameters evidenced an early A reduction of 68.73% (10 days of salinity), which was maintained until the end of the experiment (69.70% of A at 30 days) (Figure 4N). On the contrary, ‘Mariana 2624’ had the least fall in its values of photosynthetic rate in saline conditions showing reduced values in only 38.74% with respect to those detected in the control condition after 30 days of treatment (Figure 4H).

In a similar way as in the photosynthetic rate parameter, the stomatal conductance (g_s) had similar values for the same genotype under control conditions throughout the experiment (Figure 5A–G). After 30 days of 120 mM NaCl irrigation, the largest reduction in g_s values was evidenced in the ‘Maxma60’ genotype (88.30%, Figure 5K), followed by ‘Agaf 0204-09’ (85.79%, Figure 5J) and ‘Mazzard F12/1’ (81.79%, Figure 5N). Notably, the latter showed the strongest and most drastic stomatal closure, reaching its minimum values of g_s as early as at 10 days of saline condition and maintaining them until the end of the assay (Figure 5N). In addition, ‘Mazzard F12/1’ evidenced the highest internal CO₂ concentration (287.00 $\mu\text{L L}^{-1}$) among all the salt stressed rootstocks (Suppl. Table S1).

On the other hand, ‘Garnem’ (67.55%, Figure 5L), ‘Mariana 2624’ (73.02%, Figure 5H), and ‘Colt’ (73.21%, Figure 5M) were the rootstock genotypes with the least reduction in g_s . Interestingly, ‘Mariana 2624’ showed the highest value in this parameter (102.00 mmol H₂O m⁻² s⁻¹) in comparison to the rest of the analyzed genotypes (Figure 5H–N). Beside this, ‘Mariana 2624’ was the rootstock genotype that evidenced the smaller drop in transpiration rate values (E , 51.95%) derived from saline irrigation at the end of the experiment (Suppl. Table S2).

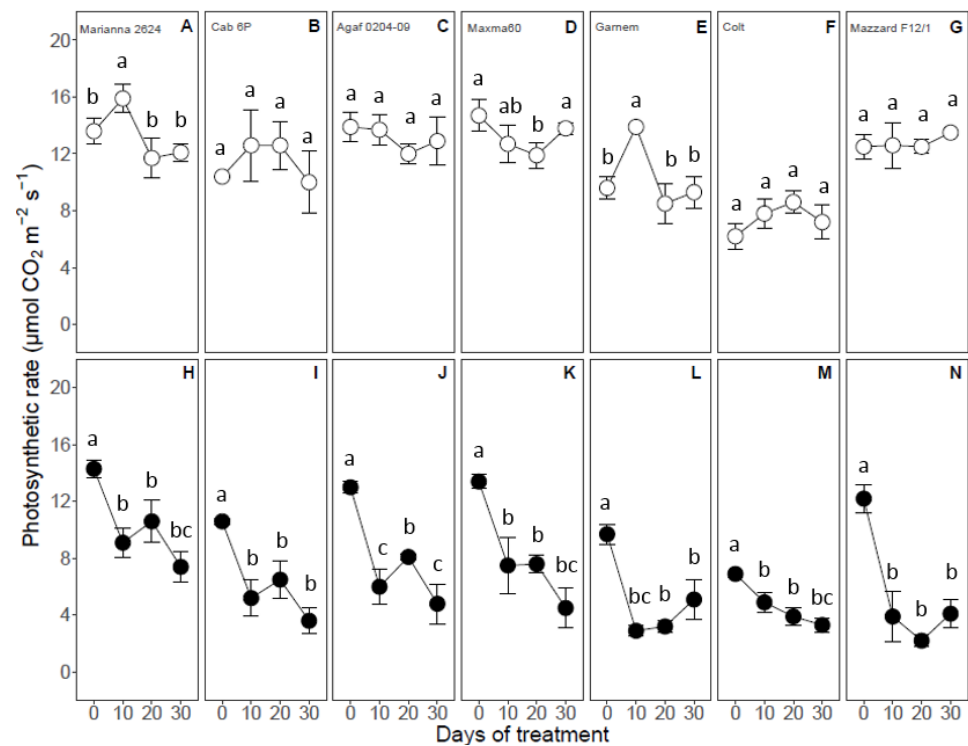


Figure 4. Photosynthetic rate (A) of *Prunus* rootstocks under control (A–G) and 120 mM NaCl salt stress (H–N) treatments. The values and error bars represent the mean and standard errors of four biological replicates with three technical replicates. Different letters indicate significant ($p < 0.05$) differences within a genotype during 30 days of treatments.

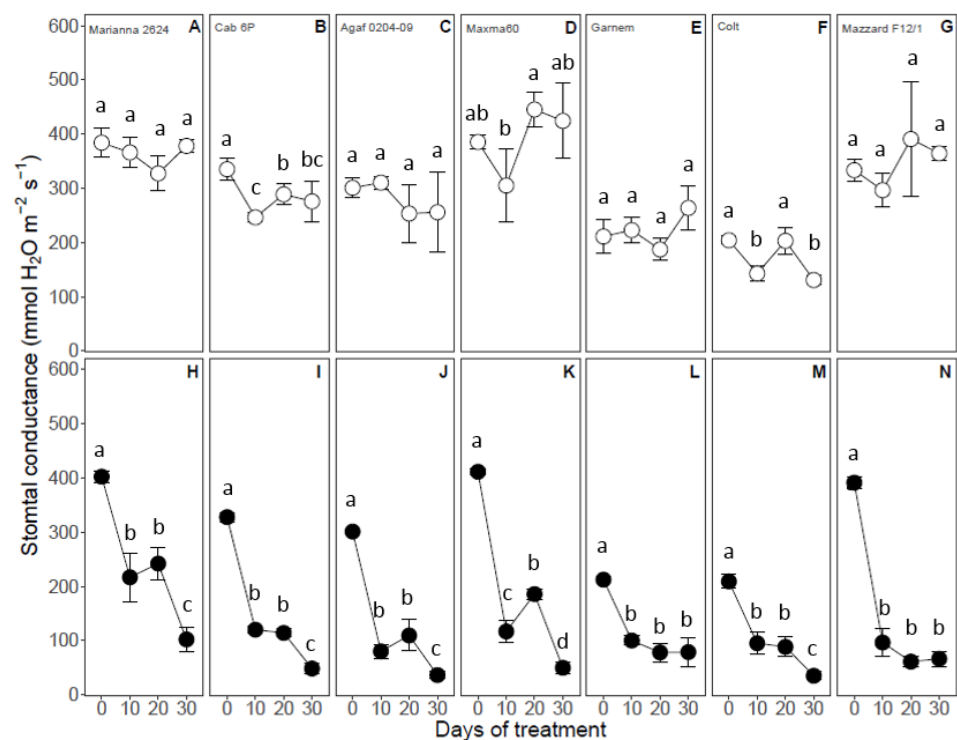


Figure 5. Stomatal conductance (g_s) of *Prunus* rootstocks under control (A–G) and 120 mM NaCl salt stress (H–N) treatments. The values and error bars represent the mean and standard error of four biological replicates with three technical replicates. Different letters indicate significant ($p < 0.05$) differences within a genotype during 30 days of treatments.

The intrinsic water use efficiency (WUE_i) was also calculated in order to further characterize the photosynthetic response of the *Prunus* spp. rootstocks to salt stress. Thus, the 'Maxma 60' and 'Mariana 2624' rootstocks reached the highest increments in WUE_i values after 30 days of saline irrigation with 146.13% and 146.06% in comparison to the control values, respectively. The lower increment in WUE_i values was recorded for 'Mazzard F12/1' with barely 63.63% (Figure 6).

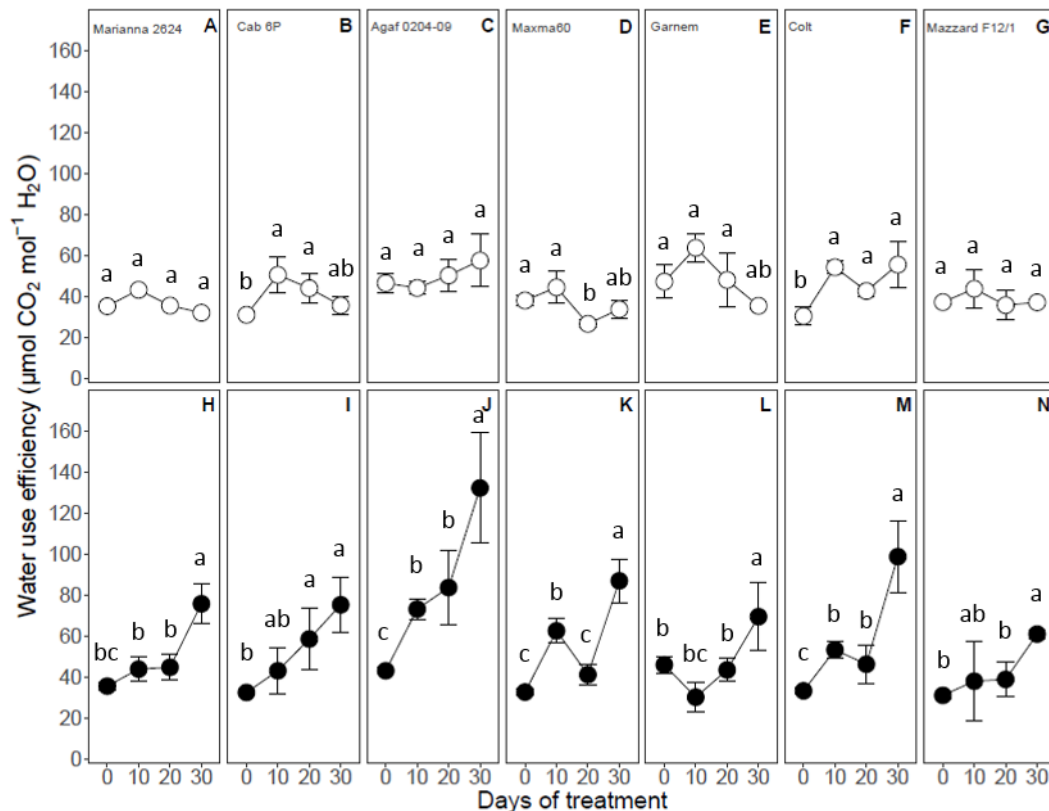


Figure 6. Intrinsic water use efficiency (WUE_i) of *Prunus* rootstocks under control (A–G) and 120 mM NaCl salt stress (H–N) treatments. The values and error bars represent the mean and standard errors of four biological replicates with three technical replicates. Different letters indicate significant ($p < 0.05$) differences within a genotype during 30 days of treatments.

3.5. Salt Tolerance Coefficients and Cluster Analysis

For each rootstock genotype, values from three parameters associated to root (maximum root length, root MDA, and root respiration rate) and six associated to leaf (leaf MDA, A , g_s , C_i , E , and WUE_i) were used to determine a ratio among their values recorded for plants under control and salt stressed conditions at the end of experiment after 30 days of treatments. These ratios denominated salt tolerance coefficients (STCs) were used as input for agglomerative hierarchical clustering. According to this analysis, the different *Prunus* spp. rootstocks were grouped into three major clusters: (I) 'Mazzard F12/1', (II) 'Maxma 60', 'Cab6P', and 'AGAF 0204-09'; and (III) 'Mariana 2624', 'Garnem', and 'Colt' (Figure 7).

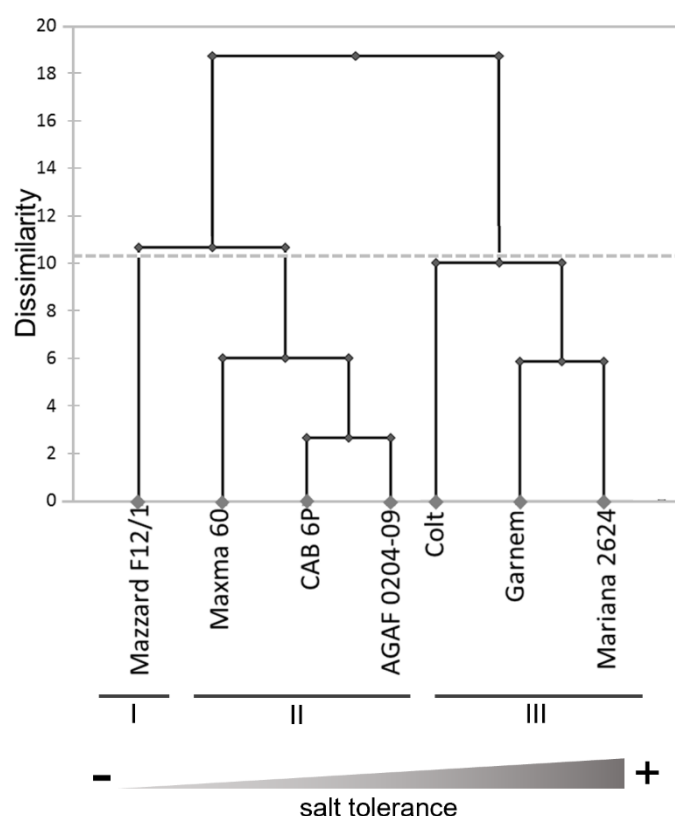


Figure 7. Dendrogram of the agglomerative hierarchical clustering of *Prunus* spp. rootstocks based on salt tolerance coefficients (STC). Dashed line indicates the dissimilarity threshold defining the number of rootstocks clusters within the analysis.

4. Discussion

Salt stress may cause a dramatic reduction in the productivity of different crops species present in arid and semiarid regions [43], but this problem is not restricted to those zones since it also affects some species, e.g., stone fruit trees grown in Mediterranean areas. In general terms, species of the *Prunus* genus are considered salt sensitive plants [9], however, it is feasible to detect some gradient of tolerance in commercial genotypes and wild relatives belonging to this genus [40]. Thus, in order to cope in adverse soil conditions that limit the development and production of these fruit trees, the use of rootstocks tolerant to biotic and/or abiotic factors turns out to be one of the best agronomic strategies to favor the establishment of orchards in such conditions [44,45].

In this study, we assessed the tolerance to long-term salt stress among seven *Prunus* genotypes used as rootstocks for cherries, plums, apricots, peaches, and almonds. The roots are the first plant organ in direct contact with excess salt and, despite that, they are considered more tolerant to salt stress compared to leaves; excess salt also triggers negative effects on the growth of the root system, such as reductions in biomass, elongation, and lateral development [46]. The root growth trait decreases in different magnitudes depending on the level of stress imposed and the intrinsic sensitivity of the genotypes at intra and interspecies levels. Usually, this trait can be expressed in terms of biomass or root length. Fruit trees with a degree of tolerance to salinity show a decrease in root biomass (dry weight) at NaCl concentrations as high as 200 and 300 mM as in the case of pomegranate (*Punica granatum*; L.) [47]. In *Vitis vinifera* accessions, the root relative growth rate (RGRr) fell between 36% and 40% under 150 mM NaCl [48]. In species very sensitive to salinity, e.g., avocado (*Persea americana* Mill.), the biomass (fresh weight) and root length decreases have been reported at low NaCl concentrations such as 9 mM [49] and 15 mM [18], respectively. Likewise, in olive (*Olea europaea* L.), a moderately salt tolerant plant, a more extensive reduction in the root biomass has been associated with more salt sensitive cultivars [19],

and even the specific root length (SRL) fell from an electrical conductivity of 6 dS m^{-1} [50]. Moreover, in pistachio (*Pistacia vera* L.), a relatively salt tolerant fruit tree [51], a significative decrease of the root length at 150 mM NaCl has been reported [52]. Within fruit trees of the *Prunus* genus, the hybrid rootstock ‘GF667’ (*P. amygdalus* Batsch $\times$ *P. persica* (L.)) evidenced a reduction in root growth parameters at NaCl concentrations ranging between 75 mM [37] and 120 mM [53]. The same was reported for the sour cherry rootstock ‘CAB 6P’ (*Prunus cerasus* L.) at 60 mM NaCl [21]. Moreover, an in vitro screening for the early detection of salt tolerance in *Prunus* spp. evidenced a reduction in the root length of rootstocks of the *Cerasus* (‘CAB 6P’ and ‘Masto de Montaña’) and *Amygdalus* (‘GF-667’) subgenera but not in those of the *Prunus* subgenus (‘Adesoto 101’ and ‘Mariana 2624’) [20]. In our work, we detected reductions of the root length in both saline levels but as expected, more markedly under 120 mM NaCl (Figure 1 and Table 1). Here, the hybrid ‘Agaf 0204-09’ (*Amygdalus* subgenus) was the rootstock with the least reduction in root length. On the other hand, ‘Mazzard F12/1’ turned out to be the genotype with the largest reduction in root length, which belongs to the subgenus *Cerasus*, this being the most affected in this trait as reported in the in vitro screening of [20].

As with other abiotic stresses, salt excess increases the production of reactive oxygen species (ROS), which triggers oxidative damage with detrimental impacts for plant tissue integrity [54]. The malondialdehyde (MDA), a lipoperoxidation product of disintegrated cell membranes, has been assessed as a biochemical marker of oxidative damage in roots by salt stress [55]. In maize (*Zea mays* L.), salinity-induced high MDA levels and oxidative stress affected more markedly the root tissues than the leaves [23]. However, an increase in MDA levels in the *Prunus* roots is not strictly coincident with a reduction in root length recorded in the rootstocks analyzed. For example, ‘Agaf 0204-09’, despite being the genotype with the lowest reduction in root length, is among the rootstocks that at least doubled their MDA levels in roots in response to saline stress (107.02% of increase relative to the control condition). On the other hand, ‘Mazzard F12/1’ showed a lower relative increase in MDA content (81.44%) but the greatest reduction in root length among genotypes. On the other hand, ‘Maxma 60’ showed a consistently negative impact on both root length and MDA levels detected in response to 120 mM NaCl.

Root respiration is sensitive to high levels of salinity in soils [56]. In the present study, all genotypes reduced root respiration under 120 mM NaCl, but a genotype-dependent variability was evidenced (Figure 3). According to [56], the respiratory rate responses under salinity conditions in plant tissues are complex, with 37% of studies reporting increases, 34% reporting decreases, and 29% reporting no consistent change in respiratory rate. In barley (*Hordeum vulgare* L.) cultivars with contrasting adaptive response to salinity, similar levels of root respiration were reported in the control condition. However, those genotypes showed an opposite behavior in their root respiration under salt stress (10 mM NaCl or KCl). The salt sensitive cultivar doubled its values of this parameter, but the salt tolerant one barely increased its root respiration to 50% and, additionally, showed a faster recovery after removal of the salt excess [57]. Conversely, in oak seedlings under high salinity (250 mM NaCl), root respiration was strongly inhibited, which was related to the arrest of root growth [58]. On the other hand, the halophyte species may increase root respiration in saline environments [59]. Root respiration is the major sink for carbohydrates [60], and it is separated into two principal components, “maintenance respiration” involved in the conservation of already existing tissue and “growth respiration” of new tissue [56]. In general, maintenance root respiration is key to providing energy and coping with stressful conditions, such as salinity [43,56]. The biomass accumulation in normal growth conditions represents between 10–40% of the total photosynthesis, while the maintenance mechanism represents the major investment [56]. Under saline conditions, root respiration has been observed to be increased but related principally with maintenance processes more than growth [43]. The elevated percentage of root respiration has been observed as dedicated to maintenance in plants under salt stress, principally associated to a significant demand of ATP provision to maintain transport processes against concentration gradient [61].

Thus, the *Prunus* spp. rootstock genotypes showed a variability in the impact of salt stress imposition on the components of their root respiration. A higher percentage of root respiration decrease was observed in ‘Garnem’, ‘Mazzard F12/1’, ‘Colt’, and ‘Agaf 0204-09’ than in ‘Cab 6P’, ‘Maxma 60’, and ‘Mariana 2624’ (Figure 3). Salinity showed the most detrimental effect on this parameter in the ‘Mazzard F12/1’ rootstock, whose roots also evidenced the largest decrease in root length (Table 1) and, additionally, the highest MDA concentration (Figure 2). Conversely, as happened with the MDA levels, ‘Mariana 2624’ showed the best response since it was the genotype that suffered the least reduction in the respiration of its roots, which possibly allowed for sufficient input energy to maintain its tissues and processes at the root level that are decisive in adaptation to saline stress.

The gas exchange is not only affected at the root level in plants under high salinity concentrations [61–63]. According to [43], the stress onset occurs when the amount of energy acquired by plants is reduced as a consequence of decreased *A*. In *Prunus salicina*, an experiment of long-term salt stress showed a complete inhibition of *A* under 28mM salt (mix NaCl and CaCl₂) [64] and serious damage has been observed in the photosynthetic apparatus in the sweet almond [36], which may be an effect of fast stomatal closure as was observed in the grafted *Prunus* [53]. In our experiment, salinity stress (120 mM NaCl) reduced the *A* of all *Prunus* genotypes (Figure 4), ‘Mariana 2624’ being the rootstock with the least drop in this parameter and ‘Mazzard F12/1’ being the most affected. The reduction in photosynthesis was coupled with a drastic reduction in the *g_s* (Figure 5). This may be associated with a CO₂ limitation more than a biochemical one because a *g_s* reduction may restrict the gas exchange as was observed in cotton plants by [65] and reviewed by [56]. Whereas here at the end of the saline stress treatment ‘Maxma60’ showed the greatest reduction in *g_s*, the genotype that closed its stomata earlier was ‘Mazzard F12/1’. The fast reduction of *g_s* under salt stress has been reported in previous studies [53,63,66] and showed that it could be beneficial to improve the *WUE_i*. The *WUE_i* reflects the balance between production (kg of biomass produced or moles of CO₂ assimilated) and water costs (m³ of water used or moles of water transpired) by each plant and is commonly used to characterize the genetic effect and variability on a certain stress condition [67]. A comparative study including four wild *Prunus* species (*Prunus maritima* Marshall, *P. salicina* Lindl., *P. cerasifera* Ehrh., and *P. persica* (L.) Batsch) evidenced a consistent decrease in their gas exchange parameters in all genotypes after irrigation with a 100 mM NaCl solution although water use efficiency increased in the beach plum (*P. maritima* Marshall), a species that inhabits sandy coastal soils and is considered highly tolerant to salinity and drought [40]. Our results showed that *WUE_i* increased in all the *Prunus* rootstock genotypes at the end of the salt stress experiment (Figure 6) but with variability among them. Thus, ‘Mariana 2624’ showed the highest increase in *WUE_i* values in saline stress along with ‘Maxma 60’. On the contrary, ‘Mazzard F12/1’ showed the smallest increase in this parameter. The increasing *WUE_i* observed is principally explained by the reduction in the *g_s* (Figure 5) with respect to the *A* (Figure 4) as was observed in different studies [53,63,66]. A high *WUE_i* indicates a better performance of the photosynthetic apparatus despite the stomatal closure triggered by the stress condition. In addition, a high *WUE_i* may reduce the uptake of salt and alleviate the water deficiency induced by salinity [62] and thereby may be used as a good indicator of salt tolerance [63].

From the results obtained in this characterization of the adaptive response to salinity stress in rootstocks of the *Prunus* spp., both at the root and leaf levels, the ‘Mazzard F12/1’ rootstocks showed to be the genotype most negatively affected in most of the parameters evaluated. However, the best indices expected to be registered in a salinity tolerant genotype were not always indisputably associated with a single genotype. For instance, even though ‘Mariana 2624’ showed the best performances in several of the measurements, it also exhibited a significant reduction in root length under the 120 mM NaCl treatment. On the other hand, ‘Agaf 0204-09’ was the rootstock that showed the least reduction in root length, which could be clear evidence of a better tolerance of excess salt at the level of the root tissue present in this genotype, but it does not register the best

values in other parameters in both root and leaf levels. In addition, the greatest increase in the *WUEi* value was detected in ‘Maxma 60’. As mentioned above, this parameter has been proposed as a good physiological index to characterize tolerance to stresses, such as drought and salinity, therefore, it would be expected that this rootstock would be among the *Prunus* genotypes most tolerant to saline stress. However, it was the second genotype with the greatest reduction in root length and accumulation of MDA under the 120 mM NaCl treatment. However, it was the second genotype with the greatest reduction in root length and accumulation of MDA under the 120 mM NaCl treatment. Given this complex scenario, an agglomerative clustering analysis was applied in order to group together data points according to their similarities and thus be able to discriminate the degree of tolerance to salt stress among the rootstocks analyzed. This type of analysis has been successfully used to characterize genotypes or accessions based on traits of interest in plant breeding programs [68–70].

In our analysis, the *Prunus* spp. rootstocks were grouped into three major clusters which were named considering the performance of the plant’s response to salt stress evidenced by the genotypes of each group. In this way, the most contrasting phenotypes in their response to saline stress (120 mM NaCl) were easily identifiable as ‘Mazzard F12/1’ (*Prunus avium* (L.) L.) (cluster I) being a very salt sensitive rootstock and ‘Mariana 2624’ (cluster III) being a salt tolerant rootstock. Although it shares cluster III with ‘Garnem’ and ‘Colt’, it is important to mention that ‘Mariana 2624’, a plum-based rootstock, showed better values in its parameters under stress conditions than its groupmates. This finding is consistent with previous reports that have indicated plum trees, members of the *Prunus* subgenus, as the species with the highest tolerance to salinity within the genus *Prunus* [9,35,40]. However, it is important to note that ‘Garnem’ (*Prunus dulcis* (Mill.) D.A. Webb × (*Prunus persica* (L.) Batsch × *Prunus davidiana* (Carrière) N.E.Br.)) and ‘Colt’ (*Prunus avium* (L.) L. × *Prunus pseudocerasus* Lindl.), as members of cluster III, represent excellent alternatives of salt tolerant rootstocks for productive varieties of the subgenera *Amygdalus* and *Cerasus*, respectively. In a classification of moderately tolerance to salt stress, cluster II mainly grouped rootstocks belonging to the *Cerasus* subgenus, i.e., ‘Cab 6P’ (*Prunus cerasus* L.) and ‘Maxma60’ (*Prunus mahaleb* L. × *Prunus avium*). These genotypes differ from the very salt sensitive genotype ‘Mazzard F12/1’, a cherry-based rootstock, for having a different or hybridized genetic background instead of only *P. avium*.

5. Conclusions

In the present work, an effective categorization of the adaptive response to salt stress of *Prunus* rootstocks was achieved from physiological measurements developed both at the root and leaf levels. An important aspect of this study is the inclusion of rootstocks (species and hybrids) whose genetic background covered the three subgenera (*Prunus*, *Cerasus*, and *Amygdalus*) with species of productive importance of the genus *Prunus*. The application of a multivariate analysis of agglomerative hierarchical clustering identified three clusters where the rootstocks were grouped based on their similarities in the behavior of their values in the physiological parameters analyzed. A tolerance gradient was established where the most contrasting genotypes in their tolerance to salt stress (120 mM NaCl) were ‘Mazzard F12/1’ (*Prunus avium* (L.) L.; subgenus *Cerasus*) and ‘Mariana 2624’ (*Prunus cerasifera* × *Prunus munsoniana* W. Wight & Hedrick; subgenus *Prunus*), being the most salt sensitive and the most salt tolerant, respectively.

An effective categorization of the adaptive response of plants is of crucial interest for the selection of parents with characteristics of interest in the framework of plant breeding programs. Moreover, it is valuable information when recommending rootstock alternatives to establish orchards in soils with edaphic problems, e.g., salinity due to a possible expansion of stone fruit orchards to new cultivation areas (arid or semiarid) due to the development of new productive varieties with lower chilling requirements.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/horticulturae7120542/s1>. Table S1: Internal CO₂ concentration (Ci) of Prunus rootstocks under control and 120 mM NaCl salt stress treatments. Table S2: Transpiration (E) of Prunus rootstocks under control and 120 mM NaCl salt stress treatments.

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