sustainability

MDPI

Article

Insight into Membrane Stability and Physiological Responses of Selected Salt-Tolerant and Salt-Sensitive Cell Lines of Troyer Citrange (*Citrus sinensis* [L.] x *Citrus trifoliata* [L.] Raf.) under Salt Stress

Houda ElYacoubi [1], Fatine Mouhssine [1], Hamada Imtara [2,*], Imane Ouallal [1], Sara Ech-cheddadi [1], Ayolié Koutoua [1], Mohamed Lagzouli [1], Badriyah S. Alotaibi [3], Omkulthom Al kamaly [3], Mohammad Khalid Parvez [4] and Atmane Rochdi [1,*]

[1] Natural Resources & Sustainable Development Laboratory, "AgroPhysiology, Biotechnology & Environment" Research Unit, Faculty of Science, Ibn Tofail University, Kenitra 14000, Morocco
[2] Faculty of Arts and Sciences, Arab American University Palestine, P.O. Box 240, Jenin 44862, Palestine
[3] Department of Pharmaceutical Sciences, College of Pharmacy, Princess Nourah Bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia
[4] Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
* Correspondence: hamada.imtara@aaup.edu (H.I.); atmane.rochdi@uit.ac.ma or atmanerochdi@gmail.com (A.R.)

Citation: ElYacoubi, H.; Mouhssine, F.; Imtara, H.; Ouallal, I.; Ech-cheddadi, S.; Koutoua, A.; Lagzouli, M.; Alotaibi, B.S.; Al kamaly, O.; Parvez, M.K.; et al. Insight into Membrane Stability and Physiological Responses of Selected Salt-Tolerant and Salt-Sensitive Cell Lines of Troyer Citrange (*Citrus sinensis* [L.] x *Citrus trifoliata* [L.] Raf.) under Salt Stress. *Sustainability* **2022**, *14*, 9583. https://doi.org/10.3390/su14159583

Academic Editors: Muhammad Naveed and Adnan Mustafa

Received: 24 May 2022
Accepted: 28 July 2022
Published: 4 August 2022

Abstract: The aim of this study was to evaluate the membrane integrity and some physiological responses of rootstock citrus calli under exposure to different concentrations of NaCl. Selected salt-tolerant cell lines were compared with salt-sensitive calli of Troyer's citrange (*Citrus sinensis* [L.] x *Citrus trifoliata* [L.] Raf.) (TC) with respect to growth, water content, Na^+, K^+ and Cl^- ion content as well as cell membrane stability under exposure to different NaCl concentrations. The results show that the stressed sensitive lines have a consistently high ion efflux. The values recorded for these sensitive calli are 3 to 6 times higher than those of the tolerant calli. Thus, only selected halotolerant calli were able to maintain the integrity of their membranes under salt stress conditions. In the sensitive calli, NaCl always induces a slowing down of growth even from 4 g $L^{-1,}$ and the reduction in the relative growth rate is higher than 50% and reaches more than 90% for the three culture durations at 8 g L^{-1} NaCl. For the salt-tolerant selected lines, the relative growth rate seems to be slightly slowed down until the second month of culture but becomes equal to that of the control at the third month, whether at 4 or 8 g L^{-1} NaCl. At the end of the third month, the relative growth rate of the selected calli is 100% at 8 g L^{-1} NaCl. The water content is twice as high in the selected tolerant calli as in the sensitive ones after three months of salt treatment at 8 g L^{-1} NaCl. After long-term culture, the halotolerant calli absorbed similar or even higher amounts of Na^+ and Cl^- than the salt-sensitive lines. However, by the 3rd month, the recorded accumulation rate dropped in the unselected but continued to increase in the tolerant calli (4-fold higher at 12 g L^{-1} NaCl than the control). Furthermore, exposure of both types of calli (salt-sensitive and salt-tolerant) to equal concentrations of NaCl resulted in greater loss of K^+ by the NaCl-sensitive lines. However, for tolerant lines, K^+ uptake is not affected at 4 g L^{-1} NaCl and the decrease in tissue content is less than 25% at 8 g L^{-1} NaCl. From this observation, it can be concluded that growth and the ability to retain high levels of internal K^+ are correlated.

Keywords: citrus rootstock; in vitro culture; electrolyte leakage; ion accumulation; physiological parameters; salt tolerance; tolerant cell lines

1. Introduction

Salinity is a major stress that causes huge economic losses to agricultural productivity in arid and semiarid areas around the world. Affected plants suffer from impaired osmotic adjustment, nutrient uptake and transport and ion toxicity [1]. Salt stress influences

crop growth, survival and yield [2]. In the presence of stress, plants may exhibit different physiological and metabolic responses [3,4]. However, the degree to which plant species are affected by stress is related to the metabolic changes developed as part of their physiological and biochemical responses. Osmotic adjustment is one of the main mechanisms leading to salt stress tolerance [5]. Therefore, the development of some selection criteria for tolerant species is possible by evaluating these different responses in plants [6,7].

To produce salt-tolerant genotypes, new techniques need to be developed, which requires appropriate breeding methods. Tissue culture can help to produce new cultivars against environmental stressors [8]. It has been shown that variant cell lines with a desired trait can be selected from a somatic cell population. As a result, many researchers have been able to obtain important crop cells that exhibit useful agricultural traits, such as salt tolerance [9–12], water stress [13], herbicide tolerance [14] or cold tolerance [15]. Cell lines provided the initial material and are a practical tool to elucidate tolerance mechanisms at the cellular level. In fact, to quantify the stress tolerance of various crops, in vitro protocols have been adopted using different concentrations of the stressor as selection tools.

Citrus is a major fruit crop of great economic importance and is widely distributed throughout the world. However, citrus cultivation is seriously threatened by various biotic and abiotic stresses such as salinity. In relation to abiotic stress and especially salinity, some regions will suffer in the coming years from salt ion accumulation in the soil due to salt water intrusion [16]. Furthermore, in arid and semiarid regions, which are the areas usually prone to salinity, citrus productivity levels are even lower, which can be avoided by using scion/rootstock combinations tolerant to this type of stress, as well as by proper crop management [17,18]. This combination influences several characteristics of the scion variety, such as fruit quality and quantity, vigor and plant size, tolerance to abiotic factors and resistance/tolerance to biotic factors [17].

Troyer Citrange is resistant to the Tristeza virus, and therefore is one of the most used rootstocks in citrus cultivation for the replacement of the sour orange tree, which is unfortunately susceptible to this viral disease. However, despite its resistance to several diseases, it is sensitive to salinity stress. It is therefore essential to improve this rootstock for a better response to variations in agro-ecological conditions and a better tolerance to abiotic constraints, including salinity and water-deficit stress.

Thus, this study aimed to understand some physiological mechanisms by which selected TC cell lines can tolerate different levels of NaCl.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

Troyer's citrange (*Citrus sinensis* [L.] x *Citrus trifoliata* [L.] Raf.) embryos were cultivated in vitro and NaCl-tolerant or NaCl-sensitive cells were obtained from the callus as previously described [19]. Briefly, embryos were collected from several Troyer Citrange seeds and were cultivated on Murashige and Tucker (1969) medium supplemented with 1 mg L^{-1} 2,4-D and 5 mg L^{-1} BAP. Tolerant cells were screened from calli, following application of salt-stress selection pressure at 8 g L^{-1} NaCl. Cultures were incubated in the dark at 30 °C and subcultivated every three weeks on fresh medium.

Homogeneous 200 mg fresh weight (FW) fragments of calli (tolerant or sensitive) were grown for three durations (1, 2 and 3 months) in the presence (4, 8 and 12 g L^{-1} NaCl) and absence (0 g L^{-1} NaCl) of salt to quantify the effects of salinity stress on cell growth, water content, mineral nutrition (Cl^-, Na^+ and K^+) and cell membrane stability.

2.2. Membrane Stability

For the membrane stability assay, selected salt-tolerant cell lines (S) subcultured on 8 g L^{-1} NaCl, as well as unselected (NS) calli which were maintained in the absence of salt stress (0 g L^{-1} NaCl) 'untreated control', in addition to sensitive calli that were transferred under salt stress (NSS) for 1 month onto the medium containing 8 g L^{-1} NaCl, are used.

Samples of 0.1 g FW calli (S, NS, and NSS) were weighed and washed briefly three times in test tubes containing deionized water to remove any electrolytes adhering to surface tissues or collected in the apoplast [20,21]. Each sample was placed in a final volume of 10 mL of deionized water and allowed to stir for 24 h at 25 °C.

The electrical conductivity (ECt) of the samples was measured using an EC meter and the tubes were then heated to 120 °C for 15 min. The samples were cooled to 25 °C and the final electrical conductivity (EC0) was measured. Twelve repetitions of each type of calli were used. The electrolyte leakage (EL) was calculated according to the following equation [20]:

$$EL\ (\%) = (ECt/EC0) \times 100$$

For solute leakage, the same protocol as before was used to obtain the initial solutions from calli samples (12 repetitions for each type of callus). However, the evaluation is performed according to Redman et al. [22] by measuring the optical density (ODt) of UV-absorbing substances. The tubes containing the samples were then placed in a freezer (at −30 °C) for 4 h; the optical density (OD0) was then measured again after temperature equilibration (at 25 °C). The solute leakage (SL) was evaluated by the following equation [22]:

$$SL\ (\%) = (ODt/OD0) \times 100$$

2.3. Cell Growth and Water Content

Fragments of 0.2 g FW of both callus types were plated on fresh media supplemented with salt (0, 4, 8 and 12 g L^{-1} NaCl), and growth was measured after 1, 2 and 3 months of culture. At the end of each period, a total of 96 calli (i.e., 4 calli replicates of each combination: callus type × salt treatment × culture duration) were used for biomass determination.

The dry weight (DW) was determined after drying at 85 °C to obtain a constant dry weight of the calli [23,24]. The growth rate (GR) and water content (TE) were then calculated based on the biomass results.

$$\text{Growth Rate (GR)} = (\text{final weight} - \text{initial weight})/\text{initial weight}$$

$$\text{Relative Growth Rate (RGR)} = 100 \times \text{GR(stress)/GR (control)} \quad (1)$$

2.4. Ion Determination

A total of 0.25 g DW callus sample was calcined in a muffle furnace at 500 °C for 4 h. The resulting ash was taken up with 5 mL of an acid mixture (HNO_3 + H_2SO_4 10%). The solution obtained was then filtered into a volumetric flask and made up to 50 mL with demineralized water. The concentrations of sodium and potassium were determined by flame photometry at 590 nm for Na^+ and 680 nm for K^+ [25]. In total, 96 samples, corresponding to 4 callus replicates of each combination (callus type × salt treatment × cultivation time) were used for chloride determination.

A further 96 callus samples of 0.25 g DW were used for the determination of chloride ions after dry mineralization in the presence of calcium oxide. The chlorides were extracted with hot water and then measured according to the technique described by Cotlove [26] by silver nitrate titration, using yellowish potassium chromate as a color indicator which turns into reddish silver chromate as soon as all Cl^- ions have been precipitated.

2.5. Statistical Analysis

The experimental device is a random model. Quantitative data were tested by analysis of variance and means were compared by Tukey's studentized range test for $p < 0.05$. All data were expressed as mean ± standard error.

3. Results

3.1. Action of NaCl on Membrane Stability

Our results show that the unselected calli cultured in the presence of salinity (NSS) showed high solutes (OD measured) and electrolytes (EC measured) efflux. The recorded values for these sensitive calli are 3–6 times higher than for the selected tolerant calli. In contrast, the salt-tolerant selected calli cultured on 8 g L^{-1} NaCl (S) showed membrane stability equivalent to that of unselected calli that were maintained in the absence of NaCl 'untreated control' (NS) (Figures 1 and 2) for the two methods (OD and EC) tested. The salt-selected calli maintain the integrity of their membranes (low permeability) in the presence of NaCl.

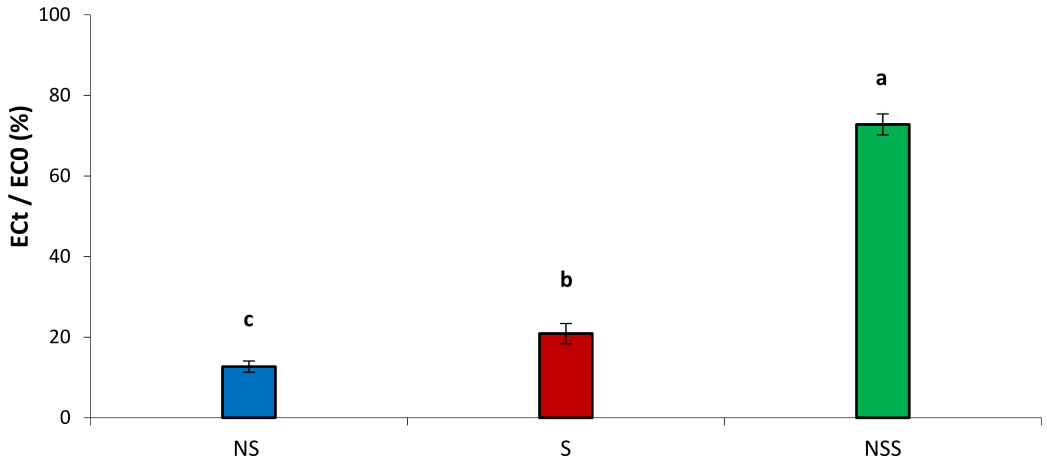

Figure 1. Leakage of electrolytes in selected calli cultured in the presence of 8 g L^{-1} NaCl (S) and unselected calli maintained in the absence (NS) or stressed by NaCl (NSS). The letters (a, b, c) represent the classes generated by the comparison of the means. Means linked by different letters are significantly different.

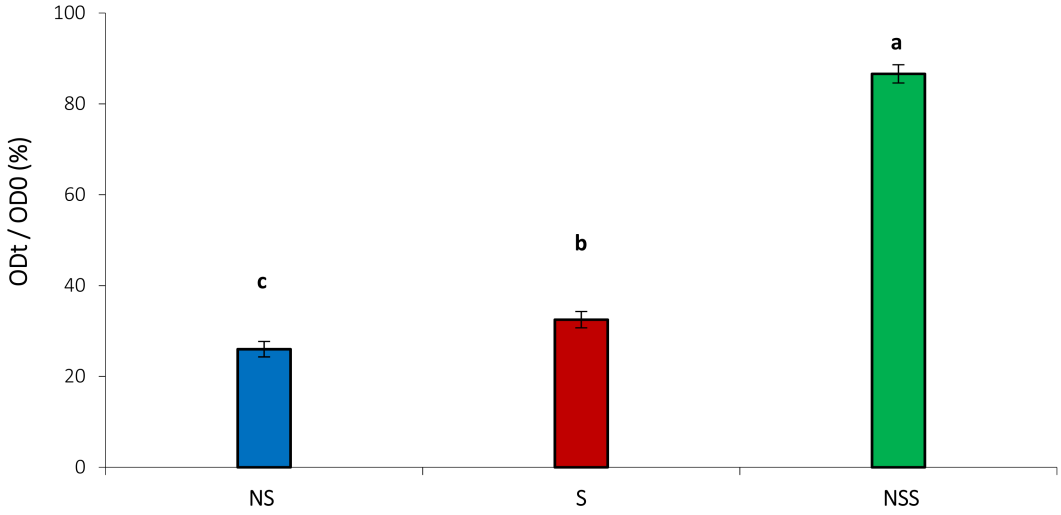

Figure 2. Leakage of solutes in selected calli grown in the presence of 8 g L^{-1} NaCl (S) and unselected calli maintained in the absence (NS) or stressed by NaCl (NSS). The letters (a, b, c) represent the classes generated by the comparison of the means. Means linked by different letters are significantly different.

The results illustrated in the two precedent graphs show the same appearance, suggesting that the preliminary washing of the samples in deionized water removed every electrolyte adhering to the surface or accumulated in the apoplast.

Statistical analysis showed a highly significant effect of callus type on membrane stability, for both criteria (electrical conductivity ECt/EC0 and optical density ODt/OD0) tested (Tables 1 and 2).

Table 1. Results of the analysis of variance testing the membrane stability estimated by electrical conductivity and optical density.

Variable	Source of Variation	Degree of Freedom	Sum of Squares	F. Value	Pr > F
ECt/EC0	type	2	2.54801906	214.92	<0.001 ***
	Error	33	0.19561525		
	Total	35	2.74363431		
ODt/OD0	type	2	2.65338039	335.88	<0.001 ***
	Error	33	0.13034517		
	Total	35	2.78372556		

***: significant effect at 0.1%.

Table 2. Means of membrane stability estimated by electrical conductivity (EC) and optical density (OD) for selected calli grown in the presence of 8 g L^{-1} NaCl (S) and unselected calli maintained in the absence 'untreated control' (NS) or in the presence 'stressed' of NaCl (NSS).

	Membrane Stability Measured by	
	Electrical Conductivity (ECt/EC0 × 100)	**Optical Density (ODt/OD0 × 100)**
NS	0.1267 ± 0.0139 c	0.2604 ± 0.0168 c
NSS	0.7278 ± 0.0258 a	0.8660 ± 0.0196 a
S	0.2094 ± 0.0249 b	0.3253± 0.0179 b

Values are means ± standard errors of 12 replicates. Significant dissimilarities between columns based on Tukey's studentized range test; the values assigned by different letters differ at the 5% level.

3.2. Cell Growth

3.2.1. Fresh Weight

The growth of the NaCl-selected and unselected cell lines is shown in Figure 3. Without NaCl, there is no difference between these two types of calli, even after three months of cultivation, and an increase in fresh weight of 10 times the initial fresh weight is recorded. However, at all NaCl concentrations tested and the three durations of experimentation, all salt-selected cell lines grew much better than the unselected. Even at 8 g L^{-1} NaCl, the fresh weight of the selected calli becomes equal to that of the control at month 3.

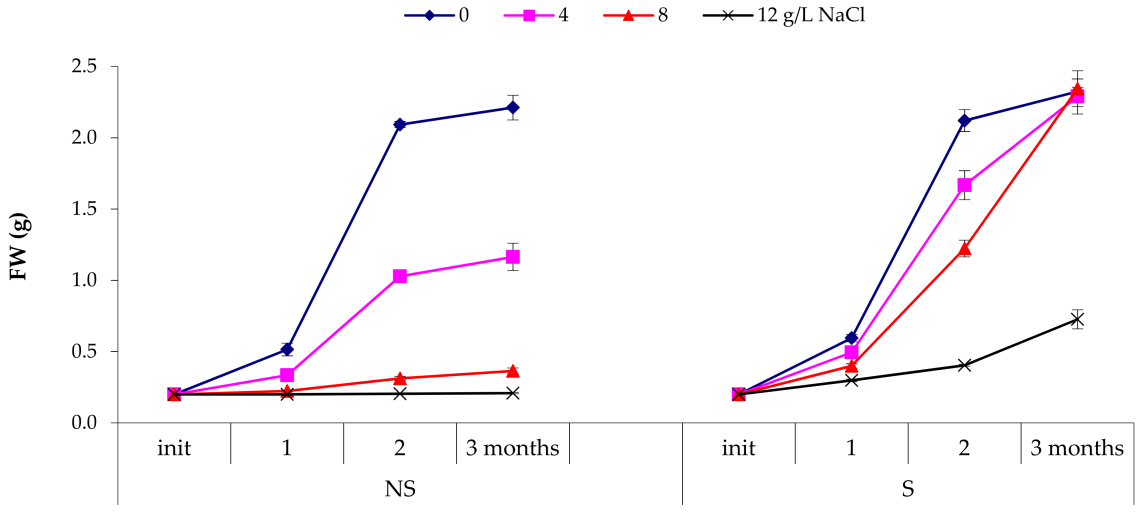

Figure 3. Variation of fresh weight (FW) of selected (S) and non-selected (NS) calli as a function of NaCl concentration and the culture duration.

In unselected cell lines, NaCl always induced a slowdown in growth, even at 4 g L^{-1} NaCl (the Relative Growth Rate was dramatically reduced by over 50%), and at 8 g L^{-1} NaCl, the growth was almost completely inhibited from the first months (the reduction was over 90% for the three durations of culture). However, in the selected cell lines, the RGR seemed to slow down up to 2 months of culture but reached the control level by the 3rd month, at 4 and 8 g L^{-1} NaCl. Unfortunately, with 12 g L^{-1} NaCl, the growth of the selected calli remained very low (at least 75% reduction) (Figure 4).

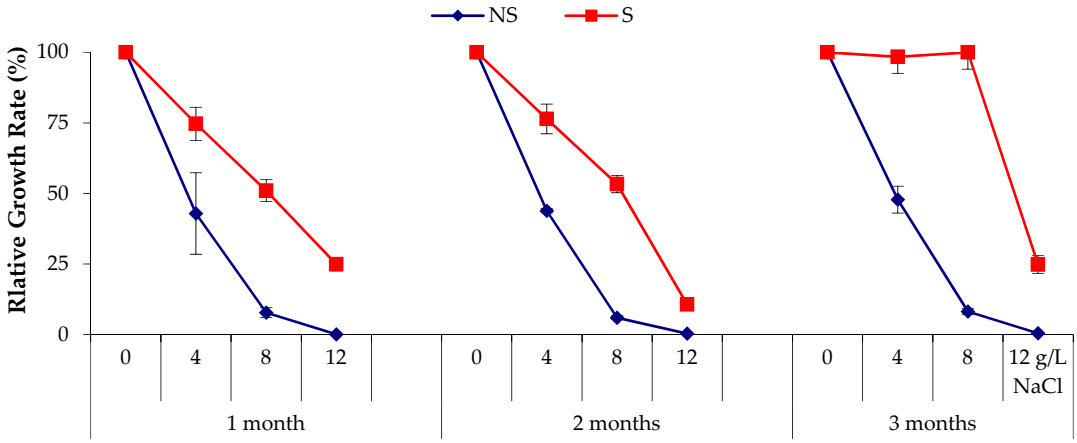

Figure 4. Variation in the relative growth rate of the fresh mass of selected and unselected calli depending on NaCl concentration and culture time.

The variance analysis performed for FW and its RGR revealed a very highly significant difference for the three variable-tested callus type factors, salt concentration and duration of treatment (Table 1). The Tukey's studentized range test distinguished three groups according to the effect of duration (3 > 2 > 1 month), four groups for the salt concentration (effect of 0 > effect of 4 > effect of 8 > effect of 12 g L^{-1} NaCl) and two groups for callus type (S > NS).

3.2.2. Dry Weight

Without NaCl, the dry weight of growth was the same for both types of calli. However, in the presence of salinity, selected cell lines had a DW greater than the unselected calli for three durations of culture (Figure 5).

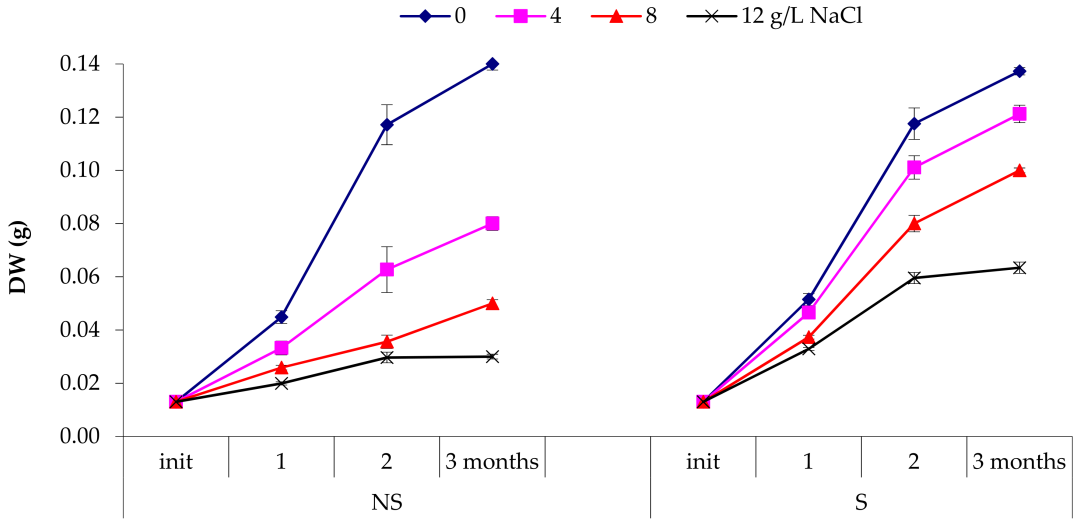

Figure 5. Variation in dry weight (DW) of selected (S) and unselected (NS) calli depending on NaCl concentration and culture time.

In unselected calli, salinity affected the DW at low concentrations of 4 g L^{-1} NaCl; the reduction in the RGR (DW) was 36%, 49% and 56%, respectively, for the 1st, 2nd and 3rd month. The decrease in growth was more pronounced with the salt concentration and duration of stress. At 8 g L^{-1} NaCl, the reduction reached 60%, 70% and 78%, respectively, at the 1st, 2nd and 3rd months. At 12 g L^{-1} NaCl, growth in dry weight was even lower for 3 months of experimentation. However, in selected cell lines, growth remained stable during the two months, but at 12 g L^{-1} NaCl, the DW was slowed, especially in the third month of culture. Thus, the reduced growth at 8 g L^{-1} NaCl was maintained at about 36% for the three durations of culture. However, at 12 g L^{-1} NaCl, the reduction is 48%, 54% and 63%, respectively, for three months of culture (Figure 6).

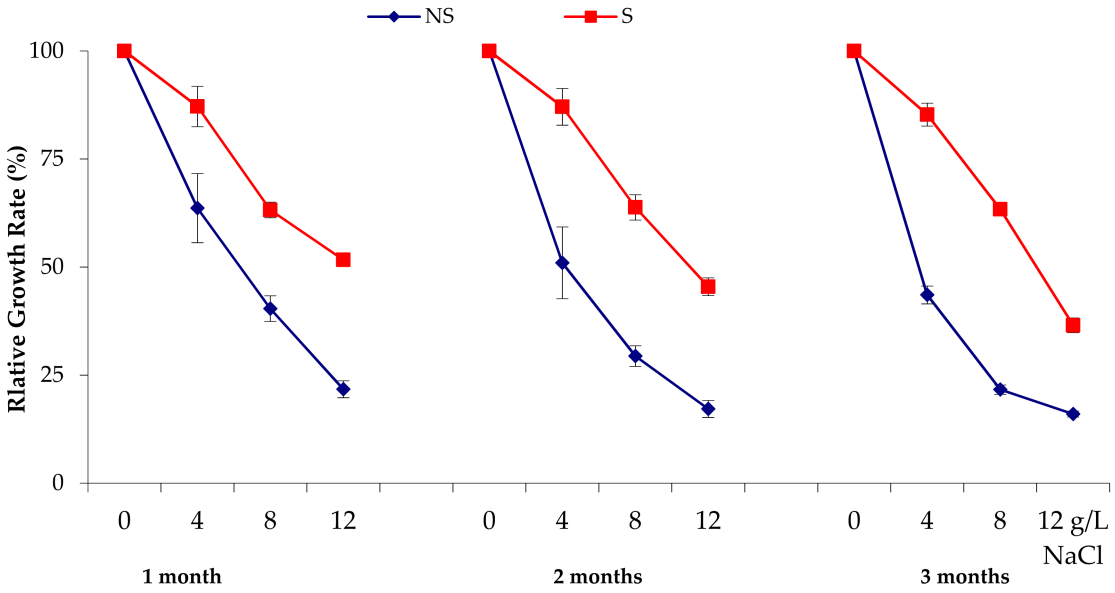

Figure 6. Variation in the relative growth rate of the dry weight of selected and unselected calli depending on NaCl concentration and culture time.

Variance analysis performed for DW and RGR showed a very highly significant difference for callus type factor, salt concentration and duration of treatment and their interactions (Table 3). The Tukey's studentized range test distinguished three groups about the effect of duration (3 > 2 > 1 month), four groups for the salt concentration (effect of 0 > effect of 4 > effect of 8 > effect of 12 g L^{-1} NaCl) and two groups for callus type (S > NS) (Table 4).

Table 3. Analysis of variance for the mass of fresh (FW) and dry (DW) weight of the calli and RGR of the selected and unselected calli under different salt concentrations and culture time.

Variable	Source of Variation	Degree of Freedom	Sum of Squares	F. Value	Pr > F
FW	duration	2	57.98119907	730.21	<0.001 ***
	Salinity	3	65.39775522	549.07	<0.001 ***
	type	1	18.12366167	456.49	<0.001 ***
	Error	264	10.48132183		
	Total	287	195.26001770		
DW	duration	2	0.15399009	578.82	<0.001 ***
	Salinity	3	0.15097049	378.31	<0.001 ***
	type	1	0.03257415	244.88	< 0.001 ***
	Error	264	0.03511769		
	Total	287	0.42376192		

Table 3. *Cont.*

Variable	Source of Variation	Degree of Freedom	Sum of Squares	F. Value	Pr > F
RGR (FW)	duration	2	1449.557520	730.23	<0.001 ***
	Salinity	3	1635.010314	549.10	<0.001 ***
	type	1	453.193144	456.60	<0.001 ***
	Error	264	262.029255		
	Total	287	4881.660805		
RGR (DW)	duration	2	911.200562	578.81	<0.001 ***
	Salinity	3	893.319721	378.30	<0.001 ***
	type	1	192.745341	244.87	< 0.001 ***
	Error	264	207.803853		
	Total	287	2507.487092		

***: significant effect at 0.1%.

Table 4. Growth and growth rate (GR) of fresh (FW) and dry (DW) selected (S) and unselected (NS) calli (treatment time and salt concentration confused).

	Growth (g)		RGR	
	FW	**DW**	**FW**	**DW**
NS	0.739 ± 0.059 b	0.057 ± 0.003 b	2.695 ± 0.299 b	3.389 ± 0.248 b
S	1.241 ± 0.071 a	0.078 ± 0.003 a	5.204 ± 0.354 a	5.025 ± 0.225 a

Values are means ± standard errors of 12 replicates. Significant dissimilarities between columns based on Tukey's studentized range test; the values assigned by different letters differ at the 5% level.

3.3. Water Content

In the absence of NaCl, both types of calli had the same water content throughout the test. In the first month of salinity, this amount does not differ between selected and nonselected calli. Water status was impacted, especially among unselected calli in the second and third month. In selected calli, water content was not affected to almost 12 g L^{-1} NaCl. In unselected calli, the water content was reduced from 4 g L^{-1} NaCl and was very low at 8 and 12 g L^{-1} NaCl. (Figure 7). There was a slowdown in the amount of water after two months of experimentation; the recorded value was 1.14 compared with the control which was 2, but in the third month the recorded values were 2.24, similar to the control.

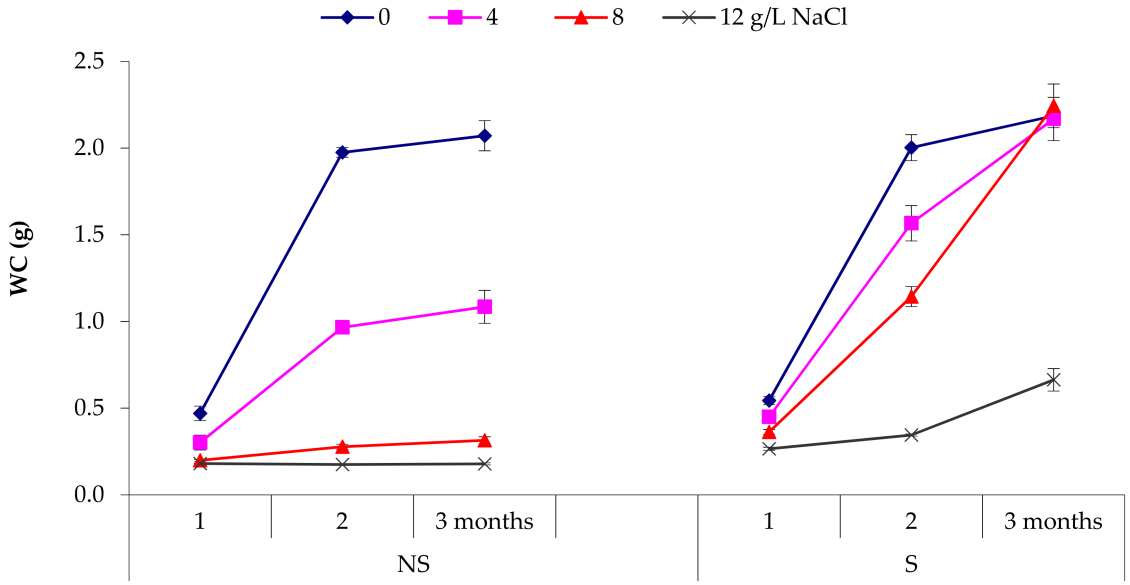

Figure 7. Variation in the water content (WC) of selected and unselected calli depending on NaCl concentration and culture time.

The effect of salinity, duration and type of calli was very significantly high at 0.001%. (Table 5). The Tukey's studentized range test showed two distinguished groups for callus type (S > NS) with the values 1.162 ± 0.068 for selected calli and 0.682 ± 0.057 for unselected calli at the 5% level.

Table 5. Analysis of variance for water content of selected and unselected calli depending on NaCl concentration and culture time.

Variable	Source of Variation	Degree of Freedom	Sum of Squares	F. Value	Pr > F
WC	duration	2	52.15924101	660.28	<0.001 ***
	Salinity	3	59.33360887	500.73	<0.001 ***
	type	1	16.61953422	420.77	<0.001 ***
	Error	264	10.42750111		
	Total	287	179.40942773		

***: significant effect at 0.1%.

3.4. Ion Accumulation

3.4.1. Sodium

The salinity of the external medium caused a remarkable accumulation of Na^+ in both types of calli. However, the levels recorded are higher in tolerant than in unselected calli (Figure 8).

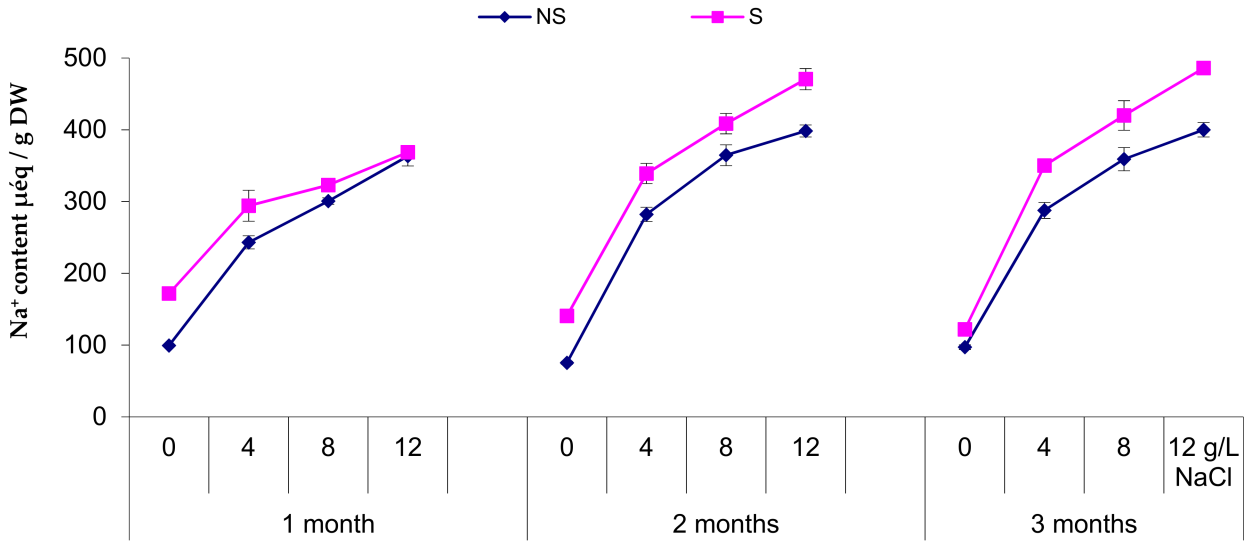

Figure 8. Variation of Na^+ content of selected and unselected calli as a function of NaCl concentration and culture duration.

Compared with the control, the accumulation of sodium in the unselected calli was much higher, especially during the first and second month (Figure 8). During the first month at 4 g L^{-1} NaCl, the accumulation rate was 2.4 and 1.8 times higher than in the controls, for unselected and tolerant calli, respectively. The accumulation of Na+ peaked for unselected calli during the 2nd month: it was almost 5 times higher (for this type of callus in media with 8 and 12 g L^{-1} NaCl) than the controls. While for the selected calli, the maximum accumulation rate, reached at the 2nd month, was almost 3 times higher (for this type of callus in media with 8 and 12 g L^{-1} NaCl) than the controls. However, at the 3rd month, the rate of accumulation declined in unselected but continued to increase in salt-tolerant selected calli.

The variance analysis on the Na^+ content showed a high significant effect of salinity level, duration and type of calli on sodium uptake (Table 6) and distinguished two groups based on type of calli (Table 7).

Table 6. Analysis of variance for sodium, chloride and potassium content of calli.

Variable	Source of Variation	Degree of Freedom	Sum of Squares	F. Value	Pr > F
Na^+	duration	2	35,379.30085769	35.24	<0.001 ***
	Salinity	3	1,188,351.16916883	789.04	<0.001 ***
	type	1	63,934.26181350	127.35	<0.001 ***
	Error	72	36,145.94993951		
	Total	95	1,374,860.89483050		
Cl^-	duration	2	45,158.68722765	30.44	<0.001 ***
	Salinity	3	743,102.91858333	333.98	<0.001 ***
	Type	1	8738.37292538	11.78	<0.001 ***
	Error	72	53,399.67134550		
	Total	95	862,502.24677133		
K^+	duration	2	48,613.093915	25.22	<0.001 ***
	Salinity	3	217,357.330958	75.17	<0.001 ***
	Type	1	79,926.041667	82.93	<0.001 ***
	Error	72	69,392.524700		
	Total	95	462,240.370183		

***: significant effect at 0.1%.

Table 7. Sodium, chloride and potassium ion contents in selected (S) and unselected (NS) calli (treatment duration and salt concentration combined).

	Na^+ (µeq/g DW)	Cl^- (µeq/g DW)	K^+ (µeq/g DW)
NS	271.516 ± 16.764 b	212.204 ± 13.618 b	242.130 ± 8.654 b
S	323.129 ± 17.323 a	231.285 ± 13.892 a	299.839 ± 9.725 a

Values are means ± standard errors of 12 replicates. Significant dissimilarities between columns based on Tukey's studentized range test; the values assigned by different letters differ at the 5% level.

3.4.2. Chloride

Curves of the Cl^- levels (Figure 9) followed an upward appearance with increasing the NaCl concentration in the culture medium. The content of the calli also increased with culture time. In addition, selected calli registered more chloride than the unselected calli. The variance analysis on the Cl-content (Tables 6 and 7) showed, like Na^+, a highly significant effect on the type of callus factor, the salt concentration and duration of treatment and two distinguished groups based on type of callus.

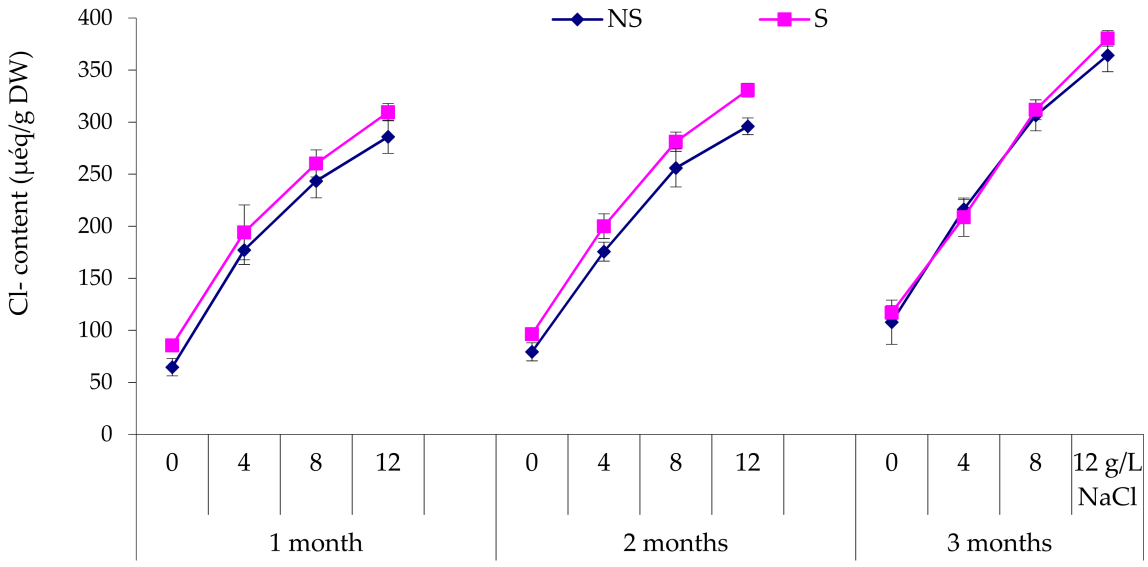

Figure 9. Variation of Cl^- content in selected and unselected calli as a function of NaCl concentration and culture time.

3.4.3. Potassium

As shown in Figure 10 and Table 6, in the absence of NaCl, the K^+ content in the selected calli's value passed from 335 µeq/g DW in the first month and increased until 391µeq/g DW in the second month, then decreased during the last month to register 326; the same allure was observed in the unselected calli. In addition, the salinity of the external medium caused a decrease in these levels in both types of calli. However, the values are relatively higher in tolerant calli. During the second month, a significant decrease in K^+ content was recorded in NS, it is 253 µeq/g DW at 4 g L^{-1} NaCl and 210 µeq/g DW at 8 g L^{-1} NaCl, while for S the values recorded it is 382 µeq/g DW and 335 µeq/g DW; for the same concentration, a remarkable decrease was noted at 12 g L^{-1} NaCl. During the third month, the K^+ values recorded are less low in both types of calli but remain higher in S callus than in NS callus.

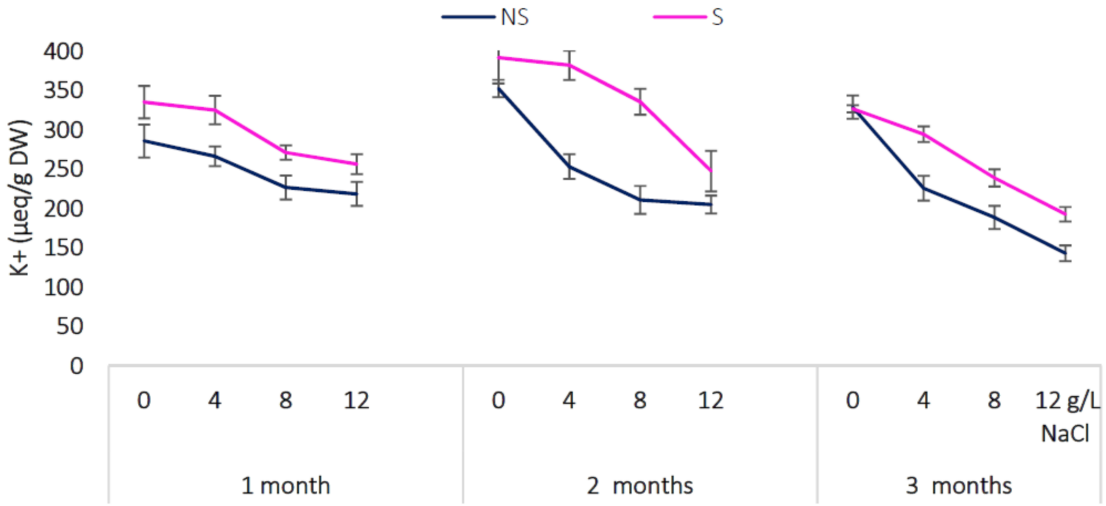

Figure 10. Variation of K^+ content in selected and unselected calli as a function of NaCl concentration and culture duration.

The analysis of variance (Tables 5 and 6) for the content of K^+ showed a highly significant effect of callus type, salt concentration and duration of treatment. Three groups are distinguished for duration (2 months > 1 months > 3 months), four groups for the concentration (effect of 0 > effect of 4 > effect of 8 > effect of 12 g L^{-1} NaCl) and two groups for the callus type (selected lines > NS).

4. Discussion

Salinity stress in the plant edaphic environment is a major constraint to agriculture as it negatively affects crop growth, development and yield. As a matter of fact, several studies have shown that the increase in electrical conductivity and thus salinity in the growing medium causes several problems (water deficiency by osmotic effect, nutrient deficiency, as well as sodium and chloride ions toxicity), which negatively affect the physiological state and productivity of plants [27,28]. Some plants have developed various morpho-physiological and biochemical mechanisms to survive in environments with high salt concentrations. The main mechanisms include ion homeostasis and compartmentalization [29], synthesis of antioxidant compounds and activation of antioxidant enzymes [30]. The maintenance of ion homeostasis through ion uptake and compartmentalization is an essential process for growth during salt stress [31].

Therefore, measurements of electrolyte leakage are a good way to assess damage to membranes in stressed plants. Indeed, membranes are the most sensitive to stress-induced deterioration, and their level of alteration is a good indicator of salt sensitivity/tolerance [32]. Similarly, Stevens et al. reported that maintaining the integrity of cell membranes under salt stress is an effective strategy for improving salinity resistance [33].

In our experiment, salinity caused an increase in electrolyte/solute leakage in the sensitive calli, but no difference was recorded between the tolerant cell lines and the controls regarding this same parameter.

Several researchers have proposed the membrane stability test as a means of measuring tolerance to water stress [34]. In addition, the restoration of cell wall stability and plasma membrane integrity increases K+/Na+ selectivity, increases Na+ exclusion and thus improves plant salt tolerance [35].

Consequently, salt induces a significant efflux of cellular electrolytes such as K^+ [36]. The magnitude of the damage depends on the salt concentration and the type of salt [22].

Furthermore, the response of the calli to the salt treatment was directly observed in their weights. Indeed, in the presence of NaCl, the fresh and dry weights evaluated in the selected lines were better than in the unselected calli. After long-term cultivation, the tolerant calli showed optimal growth on the selective medium containing the different salt concentrations. Our results are in agreement with those obtained by Hannachi et al. [24] in their in vitro work on eggplant. According to Shanthi et al., NaCl decreased callus growth, indicating that the inability of callus tissues and cells to adapt to salinity for sufficient periods may be caused by osmotic or ionic shock [37]. Others reported that altering membrane stability, inhibition of enzyme activity and slowing protein synthesis are the main causes of reduced growth in response to salinity [38].

Water content analysis indicated that the selected lines show considerable osmotic adjustment even at the 3rd month of culture in the presence of salinity less than or equal to 8 g L^{-1} NaCl. Furthermore, Binzel et al. showed that the osmotic potential and turgor of the cells vary along with the growth cycle in function of adaptation levels, and the maximum turgor occurred at approximately the beginning of the exponential phase [39]. In addition, these researchers suggested that adaptation to NaCl tends to reduce cell expansion and fresh weight and that changes in the turgidity of the cells during the growth cycle are higher in the adapted cells than in nonadapted cells to salinity. However, other researchers believed that this reduction in growth was due to the fact that cells need some time to adapt to the osmotic pressure of the culture medium before their growth rate reaches that of the cells in the control medium [40,41]. Atabaki et al. mentioned that plants under salt stress slow down their growth rate, which has been witnessed in a number of in vitro systems of halophytes and nonhalophytes [42].

Tissue water content is a good indicator of stress [24]. Plants can maintain high turgidity and cell expansion through an efficient plasmolytic process [43]. However, plant cell growth can be inhibited by salt stress as a result of decreased water potential, increased ion toxicity, inhibition of cell expansion and division or an ion imbalance. Indeed, the reduction in growth due to salt stress is a consequence of both osmotic and ionic stress effects on critical biochemical processes [35,44].

According to Binzel et al., cell tolerance to salinity is influenced by the stages: the highest degree of tolerance as expressed during the exponential phase [39]. However, other researchers felt that this reduction in growth was due to the fact that the cells needed time to adapt to the high osmotic pressure of the culture medium before their growth rate reached that of the cells in the control medium [40,41].

Understanding the mechanisms of salinity tolerance is crucial for the development of salt-stress-tolerant crops. Two mechanisms operate in the survival of plants under salinity stress: plants can either accumulate ions in response to high salt concentrations in their environment, or they can protect themselves by excluding salts and accumulating compatible solutes that do not interfere with normal biochemical reactions [45,46].

In addition, glycophytic plants do not support salinity and are therefore severely affected both at the cellular level and at the level of the whole plant [47]. Under salt stress, they appeared to be excluding Na^+ and Cl^- in response to moderate salt levels and could use the synthesis of organic compounds for osmotic adjustment [43]. The in vitro isolation of salt-tolerant cells from glycophytes has facilitated the study of responses to salinity stress.

In our work, the two types of calli cultured in the presence of salinity showed increasing levels of Na^+ ions and Cl^-. The accumulation rates also increased with the NaCl concentration in the culture medium. Furthermore, these calli absorbed larger amounts of sodium than chloride; this allowed us to assume that the Cl^- element is toxic for the cells of the rootstock studied. Other researchers have noted that in rice, for example, the tolerant lines accumulated more sodium and sensitive lines accumulated more Cl^- [20]. For Mahmoud et al., the sensitivity of citrus plants to salt is mainly related to their sensitivity to chloride ions, which causes oxidative stress [16].

As emphasized by Li et al., for most plant species, in the saline environment, sodium triggers stress, but for some plants, chloride is more toxic [48]. However, the mechanisms related to Cl^- tolerance are less well-known than for Na^+ [35,49]. Thus, it is essential to consider toxicity of both ions by studying their effect individually and combined as NaCl as stated in the study by Shelke et al. [50].

Our tolerant cell lines showed higher concentrations of Na^+ and Cl^- than the unselected calli. Similarly, other studies have shown that in sour orange [51], tobacco [52] and potato [53], tolerant calli have higher Na^+ and Cl^- levels than sensitive cells. Beloualy and Bouharmont noted a high accumulation of salt ions in calli of trifoliate orange, in contrast to Citrange troyer, which is relatively more sensitive to salinity stress [54].

Mechanisms for reducing cytoplasmic sodium content include impeding Na^+ uptake, increasing its efflux and compartmentalizing it within the vacuole. However, the levels of Na^+ and Cl^- accumulated by cells adapted to high levels of NaCl are considered to be inhibitory concentrations of metabolic function in the cytoplasm. It should be noted that the sequestration of salt ions in the vacuole not only reduces their cytosolic toxicity but also provides the opportunity to use them as a cheap osmoticum that participates in the water retention necessary for turgor and thus cell expansion under high salinity [55]. Therefore, it appeared that the salinity tolerance at the cellular level involved strict control of the level of cytoplasmic ions coupled with the compartmentalization of excess salt ions required for osmotic adjustment in the vacuole.

High Na+ concentration inhibits the uptake of K^+, which is a necessary element for growth and development. Thus, adaptation to salt stress appears to be correlated with the ability to control Na+ entry by selectively removing sodium and promoting potassium uptake to maintain a high K^+/Na^+ ratio in the intracellular content [56,57]. Under salt stress, K^+ helps maintain ion homeostasis and control the osmotic balance [58]. In our results, we found a decrease in K^+ content, parallel to the increase in the salt treatment concentration. This decrease was significant, especially in the unselected calli that also showed consistently lower contents than in the selected calli. Furthermore, in the selected cell lines, the reduction relative to the controls was only significant in the case of the treatment with the highest salt concentration (12 g L^{-1} NaCl). This shows that the selected cells favor net potassium uptake in a sodium-loaded medium. These results are consistent with the literature data regarding the sensitivity of citrus cell cultures subjected to salt stress [59].

According to Ben-Hayyim and Kochba, K^+ would be largely accumulated in the cytoplasm of citrus cells: in salinity condition, the volume of the vacuole increases, and thus the volume of the cytoplasm decreases, with a decrease in accumulated K^+ but not in the internal K+ concentration [60].

Leigh and Wyn Jones showed that K^+ concentrations between 100 and 200 mM in the cytoplasm are optimal for the functioning of metabolic processes and the potassium salts in the vacuole contribute in part to the generation of turgor [61]. The K^+ ion concentration in the vacuole can be replaced by other cations/Na^+ and Mg^{+2}. When the concentration of K^+ in the vacuole decreases below a critical level, the concentration in the cytoplasm is reduced, resulting in metabolic disturbance and decreased growth. The critical K^+ level in the plant (often expressed as a percentage of dry matter) varies among varieties in a range of 0.5 to 3.5% (0.130 to 0.900 mM/g DM).

Concerning the accumulation of sodium as a function of the duration of culture in the presence of salinity, we noted that the absorption is faster at the beginning (at the 1st month) in the unselected calli, which reach their maximum accumulation capacity relatively earlier (at the 2nd month) before showing lower quantities of accumulated sodium at the 3rd month. Thus, it appears that the tolerant calli better express their ionic selectivity (slow Na^+ uptake) and especially their good capacity for compartmentalization (larger accumulated quantity over time), and thus show a halophytic behavior. However, the unselected calli do not express selectivity, and Na^+ uptake would not be accompanied by efficient compartmentalization, which is indicated by the disruption of their growth and the decrease in the quantity recorded at the 3rd month of salinity. In fact, this result would indicate a high leakage of electrolytes (Na^+ and K^+) (decrease in the accumulation rate in the unselected ones) concomitantly with a decrease in their membrane stability (results corroborated later) due to the disruption and intoxication of the cells' metabolism.

Regulation of ion transport is one of the mechanisms involved in salt stress tolerance in plants. Another feature of tolerance is that Na+ can be potentially sequestered in vacuoles by sodium/proton antiporters (Na+/H+ antiporters, NHXs), which belong to the cation/proton antiporter (CPA1) family of transporters [62,63]. Among these chemical mediators, researchers proposed the existence of an ATPase stimulated by both Na^+ and K^+ which would be located at the level of the plasmalemma and the tonoplast. This ATPase allows the accumulation/sequestration of Na+ in the vacuole or its release into the external environment, which would maintain a higher cytoplasmic K+ concentration [64]. Other researchers suggest the existence of two distinct Na^+/H^+ and K^+/H^+ antiporters in the plasmalemma and tonoplast [65].

In addition, our study suggests that maintenance of membrane integrity, selective uptake and compartmentalization are among the factors related to the acquisition of salt tolerance. These results are in agreement with those obtained by other researchers [66–68]. We also believe that the loss of membrane integrity under salt stress in unselected lines promotes the loss of important nutrients for growth, such as K^+, while allowing the mineral ions Cl^- and Na^+ predominantly present in the saline environment to 'flood' the cytoplasm, thus causing damage to cell metabolism.

5. Conclusions

Salt stress is a major factor of hyperosmotic and ionic stress, leading to deterioration of the general health of citrus plants and reduction in their yield. Establishing salt-tolerant lines is an important way for overcoming soil salinity in the context of global climate change and for expanding our knowledge of the morpho-physiological and biochemical mechanisms involved in adaptation to salt stress. In our research, we have demonstrated the possibility of selecting at the cellular level the ones that can withstand high doses of NaCl, and we have shown by comparing the two types of cells the effects caused by salt stress and the possible mechanisms of tolerance. Indeed, our tolerant calli kept a normal growth and water content with a good maintenance of the membrane integrity and ionic balance.

Author Contributions: Conceptualization, H.E., A.K. and. A.R.; methodology, H.E., F.M., S.E.-c. and I.O.; validation, H.E. and A.R.; formal analysis, H.E., M.L., H.I., M.K.P. and B.S.A.; resources, O.A.k. and A.R.; data curation, H.I., B.S.A., O.A.k. and M.K.P.; writing—original draft preparation, H.E. and F.M.; writing—review and editing, H.E., H.I. and A.R.; supervisor, A.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2022R73), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available upon request.

Acknowledgments: The authors extend their appreciation to Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2022R73), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia for funding this work and to Researchers Supporting Project (no. RSP-2021/379), King Saud University, Riyadh. The authors also extend their appreciation to the Faculty of Sciences of the Ibn Tofail University (Kenitra, Morocco) for support and assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Al-Khateeb, S.A.; Al-Khateeb, A.A.; Sattar, M.N.; Mohmand, A.S. Induced in Vitro Adaptation for Salt Tolerance in Date Palm (*Phoenix Dactylifera* L.) Cultivar Khalas. *Biol. Res.* **2020**, *53*, 37. [CrossRef]
2. Shi, X.; Gupta, S.; Rashotte, A.M. Characterization of Two Tomato AP2/ERF Genes, SlCRF1 and SlCRF2 in Hormone and Stress Responses. *Plant Cell Rep.* **2014**, *33*, 35–45. [CrossRef]
3. Agır, S.U.; Kutbay, H.G.; Surmen, B.; Elmas, E. The Effects of Erosion and Accretion on Plant Communities in Coastal Dunes in North of Turkey. *Rend. Fis. Acc. Lincei* **2017**, *28*, 203–224. [CrossRef]
4. Ghanbari, M.; Modarres-Sanavy, S.A.M.; Mokhtassi-Bidgoli, A. Is Time Important in Response of Morpho-Physiological Parameters in Withania Coagulans L. Landraces to Water Deficit Stress? *Ind. Crops Prod.* **2019**, *128*, 18–28. [CrossRef]
5. Bündig, C.; Vu, T.H.; Meise, P.; Seddig, S.; Schum, A.; Winkelmann, T. Variability in Osmotic Stress Tolerance of Starch Potato Genotypes (*Solanum Tuberosum* L.) as Revealed by an In Vitro Screening: Role of Proline, Osmotic Adjustment and Drought Response in Pot Trials. *J. Agron. Crop Sci.* **2017**, *203*, 206–218. [CrossRef]
6. Kahlaoui, B.; Hachicha, M.; Misle, E.; Fidalgo, F.; Teixeira, J. Physiological and Biochemical Responses to the Exogenous Application of Proline of Tomato Plants Irrigated with Saline Water. *J. Saudi Soc. Agric. Sci.* **2018**, *17*, 17–23. [CrossRef]
7. Le Gall, H.; Philippe, F.; Domon, J.-M.; Gillet, F.; Pelloux, J.; Rayon, C. Cell Wall Metabolism in Response to Abiotic Stress. *Plants* **2015**, *4*, 112. [CrossRef]
8. Bezirganoglu, İ. Response of Five Triticale Genotypes to Salt Stress in in Vitro Culture. *Turk. J. Agric. For.* **2017**, *41*, 372–380. [CrossRef]
9. Hasegawa, P.M. Sodium (Na+) Homeostasis and Salt Tolerance of Plants. *Environ. Exp. Bot.* **2013**, *92*, 19–31. [CrossRef]
10. Hasegawa, P.M.; Bressan, R.A.; Handa, A.K. Growth Characteristics of NaCl-Selected and Nonselected Cells of *Nicotiana Tabacum* L. *Plant Cell Physiol.* **1980**, *21*, 1347–1355. [CrossRef]
11. Croughan, T.P.; Stavarek, S.J.; Rains, D.W. Selection of a NaCl Tolerant Line of Cultured Alfalfa Cells1. *Crop Sci.* **1978**, *18*, 959–963. [CrossRef]
12. Dix, P.J.; Street, H.E. Sodium Chloride-Resistant Cultured Cell Lines from Nicotiana Sylvestris and Capsicum Annuum. *Plant Sci. Lett.* **1975**, *5*, 231–237. [CrossRef]
13. Bressan, R.A.; Hasegawa, P.M.; Handa, A.K. Resistance of Cultured Higher Plant Cells to Polyethylene Glycol-Induced Water Stress. *Plant Sci. Lett.* **1981**, *21*, 23–30. [CrossRef]
14. Chaleff, R.S.; Parsons, M.F. Direct Selection in Vitro for Herbicide-Resistant Mutants of Nicotiana Tabacum. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 5104–5107. [CrossRef]
15. Dix, P.J.; Street, H.E. Selection of Plant Cell Lines with Enhanced Chilling Resistance. *Ann. Bot.* **1976**, *40*, 903–910. [CrossRef]
16. Mahmoud, L.M.; Dutt, M.; Vincent, C.I.; Grosser, J.W. Salinity-Induced Physiological Responses of Three Putative Salt Tolerant Citrus Rootstocks. *Horticulturae* **2020**, *6*, 90. [CrossRef]
17. Santana LG, L.; Souza ED, S.; Passos, O.S.; Gesteira AD, S.; Ledo CA, D.S.; Soares Filho WD, S. Vigor and Mortality of Citrus Progenies with Potential Use as Rootstocks. *Sci. Agric.* **2018**, *75*, 339–345. [CrossRef]
18. Brito ME, B.; Fernandes, P.D.; Gheyi, H.R.; Soares LA, D.A.; Soares Filho WD, S.; Suassuna, J.F. Screening of Citrus Scion-Rootstock Combinations for Tolerance to Water Salinity during Seedling Formation. *Acta Sci. Agron.* **2020**, *43*. [CrossRef]
19. EI Yacoubi, H.; Rochdi, A.; Ayolie, K.; Rachidai, A. Sélection et Évaluation de Lignées de Cals Stables et Tolérantes Vis-à-Vis Du Stress Salin Chez Le Citrange 'Troyer' [*Citrus Sinensis* (L.) × *Poncirus Trifoliata* (L.) Raf.]. *Fruits* **2004**, *59*, 325–337. [CrossRef]
20. Bajji, M.; Kine, J.-M.; Lutts, S. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regul.* **2002**, *36*, 61–70. [CrossRef]
21. Heyser, J.W.; Nabors, M.W. Osmotic Adjustment of Cultured Tobacco Cells (*Nicotiana Tabacum* Var. *Samsum*) Grown on Sodium Chloride. *Plant Physiol.* **1981**, *67*, 720–727. [CrossRef] [PubMed]
22. Redmann, R.E.; Haraldson, J.; Gusta, L.V. Leakage of UV-Absorbing Substances as a Measure of Salt Injury in Leaf Tissue of Woody Species. *Physiol. Plant.* **1986**, *67*, 87–91. [CrossRef]
23. Muchate, N.S.; Rajurkar, N.S.; Suprasanna, P.; Nikam, T.D. NaCl induced salt adaptive changes and enhanced accumulation of 20-hydroxyecdysone in the in vitro shoot cultures of *Spinacia oleracea* (L.). *Sci. Rep.* **2019**, *9*, 12522. [CrossRef] [PubMed]
24. Hannachi, S.; Werbrouck, S.; Bahrini, I.; Abdelgadir, A.; Affan Siddiqui, H. Agronomical, Physiological and Biochemical Characterization of In Vitro Selected Eggplant Somaclonal Variants under NaCl Stress. *Plants* **2021**, *10*, 2544. [CrossRef]
25. Novozamsky, I.; Houba VJ, G.; Van Der Lee, J.J.; Van Eck, R.; Mignorance, M.D. A convenient wet digestion procedure for multi-element analysis of plant materials. *Commun. Soil Sci. Plant Anal.* **1993**, *24*, 2595–2605. [CrossRef]

26. Cotlove, E. Determination of Clin biologicalmaterial. In *Methods of Biochemical Analysis*; Glick, D., Ed.; Interscience Publishing: New York, NY, USA, 1965; pp. 277–392.
27. Munns, R.; James, R.A.; Lauchli, A. Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot.* **2006**, *57*, 1025–1043. [CrossRef]
28. Al-Ashkar, I.; Alderfasi, A.; El-Hendawy, S.; Al-Suhaibani, N.; El-Kafafi, S.; Seleiman, M.F. Detecting Salt Tolerance in Doubled Haploid Wheat Lines. *Agronomy* **2019**, *9*, 211. [CrossRef]
29. Flowers, T.J. Improving Crop Salt Tolerance. *J. Exp. Bot.* **2004**, *55*, 307–319. [CrossRef]
30. Kumar, S.P.; Kumari, B.R. Impact of Ethyl Methane Sulphonate Mutagenesis in *Artemisia vulgaris* L. under NaCl Stress. *BioTech* **2021**, *10*, 18. [CrossRef]
31. Niu, X.; Bressan, R.A.; Hasegawa, P.M.; Pardo, J.M. Ion Homeostasis in NaCl Stress Environments. *Plant Physiol.* **1995**, *109*, 735–742. [CrossRef]
32. Yasar, F.; Ellialtioglu, S.; Yildiz, K. Effect of salt stress on antioxidant defence systems, lipid peroxidation, and chlorophyll content in green bean. Russ. *J. Plant Physiol.* **2008**, *55*, 782–786.
33. Stevens, J.; Senaratna, T.; Sivasithamparam, K. Salicylic Acid Induces Salinity Tolerance in Tomato (*Lycopersicon Esculentum* Cv. *Roma*): Associated Changes in Gas Exchange, Water Relations and Membrane Stabilisation. *Plant Growth Regul.* **2006**, *49*, 77–83. [CrossRef]
34. Premachandra, G.S.; Saneoka, H.; Kanaya, M.; Ogata, S. Cell Membrane Stability and Leaf Surface Wax Content as Affected by Increasing Water Deficits in Maize. *J. Exp. Bot.* **1991**, *42*, 167–171. [CrossRef]
35. Tavakkoli, E.; Rengasamy, P.; McDonald, G.K. High Concentrations of Na+ and Cl– Ions in Soil Solution Have Simultaneous Detrimental Effects on Growth of Faba Bean under Salinity Stress. *J. Exp. Bot.* **2010**, *61*, 4449–4459. [CrossRef]
36. Hansson, G.; Kuiper, P.J.C.; Kylin, A. Effect of Preparation Method on the Induction of (Sodium + Potassium)–Activated Adenosine Triphosphalase from Sugar Beet Root and Its Lipid Composition. *Physiol. Plant.* **1973**, *28*, 430–435. [CrossRef]
37. Shanthi, P.; Jebaraj, S.; Geetha, S. In Vitro Screening for Salt Tolerance in Rice (*Oryza Sativa*). *Electron. J. Plant Breed.* **2010**, *1*, 1208–1212.
38. Tester, M.; Davenport, R. Na+ Tolerance and Na+ Transport in Higher Plants. *Ann. Bot.* **2003**, *91*, 503–527. [CrossRef]
39. Binzel, M.L.; Hasegawa, P.M.; Handa, A.K.; Bressan, R.A. Adaptation of Tobacco Cells to NaCl 1. *Plant Physiol.* **1985**, *79*, 118–125. [CrossRef]
40. Rus, A.M.; Rios, S.; Olmos, E.; Santa-Cruz, A.; Bolarin, M.C. Long-Term Culture Modifies the Salt Responses of Callus Lines of Salt-Tolerant and Salt-Sensitive Tomato Species. *J. Plant Physiol.* **2000**, *157*, 413–420. [CrossRef]
41. Muralitharan, M.S.; Van Steveninck, R.F.M.; Chandler, S. Effects of NaCl and Na2SO4 on Growth and Solute Composition of Highbush Blueberry (*Vaccinium Corymbosum*). *Aust. J. Plant Physiol.* **1992**, *19*, 155–164. [CrossRef]
42. Atabaki, N.; Nulit, R.; Kalhori, N.; Lasumin, N.; Sahebi, M.; Abiri, R. In Vitro Selection and Development of Malaysian Salt-Tolerant Rice (*Oryza Sativa* L. Cv. MR263) Under Salinity. *Acta Sci. Agric.* **2018**, *2*, 8–17.
43. Yeo, A.R. Salinity Resistance: Physiologies and Prices. *Physiol. Plant.* **1983**, *58*, 214–222. [CrossRef]
44. Munns, R.; Tester, M. Mechanisms of Salinity Tolerance. *Annu. Rev. Plant Biol.* **2008**, *59*, 651–681. [CrossRef]
45. Ben-Hayyim, G.; Kochba, J. Growth Characteristics and Stability of Tolerance of Citrus Callus Cells Subjected to NaCl Stress. *Plant Sci.* **1982**, *27*, 87–94. [CrossRef]
46. Yadav, P.V.; Kumari, M.; Ahmed, Z. Seed Priming Mediated Germination Improvement and Tolerance to Subsequent Exposure to Cold and Salt Stress in Capsicum. *Res. J. Seed Sci.* **2011**, *4*, 125–136. [CrossRef]
47. Shahid, M.A.; Sarkhosh, A.; Khan, N.; Balal, R.M.; Ali, S.; Rossi, L.; Gómez, C.; Mattson, N.; Nasim, W.; Garcia-Sanchez, F. Insights into the Physiological and Biochemical Impacts of Salt Stress on Plant Growth and Development. *Agronomy* **2020**, *10*, 938. [CrossRef]
48. Li, B.; Tester, M.; Gilliham, M. Chloride on the Move. *Trends Plant Sci.* **2017**, *22*, 236–248. [CrossRef]
49. Britto, D.T.; Ruth, T.J.; Lapi, S.; Kronzucker, H.J. Cellular and Whole-Plant Chloride Dynamics in Barley: Insights into Chloride-Nitrogen Interactions and Salinity Responses. *Planta* **2004**, *218*, 615–622. [CrossRef]
50. Shelke, D.B.; Nikalje, G.C.; Chambhare, M.R.; Zaware, B.N.; Penna, S.; Nikam, T.D. Na+ and Cl- Induce Differential Physiological, Biochemical Responses and Metabolite Modulations in Vitro in Contrasting Salt-Tolerant Soybean Genotypes. *3 Biotech* **2019**, *9*, 91. [CrossRef]
51. Ben-Hayyim, G.; Spiegel-Roy, P.; Neumann, H. Relation between Ion Accumulation of Salt-Sensitive and Isolated Stable Salt-Tolerant Cell Lines of Citrus Aurantium. *Plant Physiol.* **1985**, *78*, 144–148. [CrossRef]
52. Binzel, M.L.; Hasegawa, P.M.; Rhodes, D.; Handa, S.; Handa, A.K.; Bressan, R.A. Solute Accumulation in Tobacco Cells Adapted to NaCl 1. *Plant Physiol.* **1987**, *84*, 1408–1415. [CrossRef] [PubMed]
53. Sabbah, S.; Tal, M. Development of Callus and Suspension Cultures of Potato Resistant to NaCl and Mannitol and Their Response to Stress. *Plant Cell Tissue Organ. Cult.* **1990**, *21*, 119–128. [CrossRef]
54. Beloualy, N.; Bouharmont, J. NaCl-Tolerant Plants of Poncirus Trifoliata Regenerated from Tolerant Cell Lines. *Theoret. Appl. Genet.* **1992**, *83*, 509–514. [CrossRef] [PubMed]
55. Mohamed Magdy, F.M. Role of Vacuolar Membrane Transport Systems in Plant Salinity Tolerance. *J. Plant Growth Regul.* **2022**, 732. [CrossRef]

56. Wang, N.; Qi, H.; Qiao, W.; Shi, J.; Xu, Q.; Zhou, H.; Yan, G.; Huang, Q. Cotton (*Gossypium hirsutum* L.) genotypes with contrasting K+/ Na+ ion homeostasis: Implications for salinity tolerance. *Acta Physiol. Plant* **2017**, *39*, 77. [CrossRef]
57. Ketehouli, T.; Carther, K.F.I.; Muhammad Noman, M.; Wang, F.W.; Li, X.W.; Li, H.Y. Adaptation of Plants to Salt Stress: Characterization of Na+ and K+ Transporters and Role of CBL Gene Family in Regulating Salt Stress Response. *Agronomy* **2019**, *9*, 687. [CrossRef]
58. Hasanuzzaman, M.; Bhuyan, M.B.; Nahar, K.; Hossain, M.S.; Mahmud, J.A.; Hossen, M.S.; Masud, A.A.C.; Moumita; Fujita, M. Potassium: A Vital Regulator of Plant Responses and Tolerance to Abiotic Stresses. *Agronomy* **2018**, *8*, 31. [CrossRef]
59. Rochdi, A.; ElYacoubi, H.; Rachidai, A. Comportement Vis-à-Vis de La Salinité de Cals de Porte-Greffes d'agrumes Citrus Aurantium, Citrange Troyer et Poncirus Trifoliata: Évaluation de Critères Certifiant La Réponse des Agrumes Au Stress Salin. *Agronomie* **2003**, *23*, 643–649. [CrossRef]
60. Ben-Hayyim, G.; Kochba, J. Aspects of Salt Tolerance in a NaCl-Selected Stable Cell Line of Citrus Sinensis. *Plant Physiol.* **1983**, *72*, 685–690. [CrossRef]
61. Leigh, R.A.; Jones, R.G.W. A Hypothesis Relating Critical Potassium Concentrations for Growth to the Distribution and Functions of This Ion in the Plant Cell. *New Phytol.* **1984**, *97*, 1–13. [CrossRef]
62. Jia, Q.; Zheng, C.; Sun, S.; Amjad, H.; Liang, K.; Lin, W. The role of plant cation/proton antiporter gene family in salt tolerance. *Biol. Plant* **2018**, *62*, 617–629. [CrossRef]
63. Al-Harrasi, I.; Jana, G.A.; Patankar, H.V.; Al-Yahyai, R.; Rajappa, S.; Kumar, P.P.; Yaish, M.W. A novel tonoplast Na+/H+ antiporter gene from date palm (PdNHX6) confers enhanced salt tolerance response in Arabidopsis. *Plant Cell Rep.* **2020**, *39*, 1079–1093. [CrossRef]
64. Assaha, D.V.M.; Ueda, A.; Saneoka, H.; Al-Yahyai, R.; Yaish, M.W. The Role of Na+ and K+ Transporters in Salt Stress Adaptation in Glycophytes. *Front. Physiol.* **2017**, *8*, 509. [CrossRef]
65. Silva, P.; Hernâni Gerós, H. Regulation by salt of vacuolar H+-ATPase and H+-pyrophosphatase activities and Na+/H+ exchange. *Plant Signal. Behav.* **2009**, *4*, 718–726. [CrossRef]
66. Peng, Z.; He, S.; Sun, J.; Pan, Z.; Gong, W.; Lu, Y.; Du, X. Na(+) compartmentalization related to salinity stress tolerance in upland cotton (*Gossypium hirsutum*) seedlings. *Sci. Rep.* **2016**, *6*, 34548. [CrossRef]
67. Mansour, M.M.F. Plasma membrane permeability as an indicator of salt tolerance in plants. *Biol. Plant.* **2013**, *57*, 1–10. [CrossRef]
68. Liu, J.; Zhang, W.; Long, S.; Zhao, C. Maintenance of Cell Wall Integrity under High Salinity. *Int. J. Mol. Sci.* **2021**, *22*, 3260. [CrossRef]