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Physiological and Biochemical Evaluation of Salt Stress Tolerance in a Citrus Tetraploid Somatic Hybrid

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Abstract: Somatic hybridization has emerged as a valuable tool for developing novel genetic combinations in citrus breeding programs, including the creation of salt-tolerant rootstocks. In this study, the performance of a tetraploid somatic hybrid, obtained by fusing protoplasts derived from salt-tolerant Cleopatra mandarin (*Citrus reshni* hort. ex Tanaka) and salt-sensitive Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf), was assessed under in vitro salt stress. Hybrid plants were characterized by leaf morphology, and ploidy level by flow cytometry and molecular markers. In vitro shoots were generated from the micropropagation of mature stem pieces of the somatic hybrid and its parents, and these were challenged by exposure to NaCl (0, 50, or 100 mM) supplemented to the media for three weeks to induce salt stress. The leaves of the somatic hybrid display intermediate morphology compared to the parental Cleopatra mandarin and Carrizo citrange rootstocks. All molecular markers successfully amplified DNA from the three cultivars; however, only 11 of 14 unequivocally confirmed somatic hybridity. The physiological and biochemical parameters, including chlorophyll content, lipid peroxidation, total phenolic compounds, antioxidants activity and proline content, were measured in the leaves. The somatic hybrid exhibited superior salt stress tolerance compared to the parent varieties, as evidenced by the reduced cellular membrane damage indicated by the lower levels of malondialdehyde and electrolyte leakage, particularly under 100 mM NaCl treatment. The somatic tetraploid hybrid also displayed higher total phenolic content than either parent, while Cleopatra mandarin exhibited the highest proline levels under 50 mm NaCl. These results demonstrate the enhanced salinity stress tolerance of the somatic hybrid compared to its parent lines, highlighting its potential as a valuable candidate for developing salt-tolerant citrus rootstocks.

Keywords: citrus rootstocks; flow cytometry; salt stress tolerance; somatic hybridization; tetraploids

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1. Introduction

Citrus, a prominent fruit crop belonging to the Rutaceae family, includes a variety of well-known fruits such as oranges, lemons, limes, grapefruits, and tangerines [1]. The citrus genus encompasses several tropical and subtropical species that are highly sensitive to environmental stressors, thereby restricting their distribution to specific latitudes [2]. The detrimental effects of these stresses are further compounded by climate change and global warming, which are predicted to result in extreme weather events such as heavy rainfall, droughts, rising temperatures, sea-level rise, and more frequent cold and heatwaves. These conditions pose a threat to citriculture sustainability in various regions and impair citrus growth, reduce fruit production, and cause significant economic losses [3–7].

Salinity, among these stressors, leads to increased osmotic pressure and reduced water availability in the root zone [8]. Moreover, elevated ion levels associated with salinity can

lead to toxicity and nutrient imbalance in plants. The excess of ions also disrupts the electron transport chain and impacts the functionality of mitochondria and chloroplasts [9,10]. As a consequence, the cell experiences excitation or incomplete reduction in molecular oxygen, leading to an excessive production of reactive oxygen species (ROS) [11,12].

Various agricultural approaches are employed to mitigate the negative impact of environmental stresses on crop production. These strategies encompass the implementation of optimal fertilization and irrigation methods [13–15], the utilization of conventional breeding techniques to enhance plant performance [7], and the application of genetic transformation methods to create novel genotypes with specific salt tolerance attributes [16]. The development of suitable rootstock plays a critical role in citrus production systems [17]. The citrus breeding program at the University of Florida has successfully generated numerous rootstocks, including both diploid and tetraploid varieties [18].

Somatic hybridization plays a crucial role in the breeding and enhancement of citrus cultivars [19–22]. Through the protoplast-mediated fusion process, citrus autotetraploid and allotetraploid parents can be generated by combining selected diploid varieties with great success [23,24]. The tetraploid citrus progenies can serve directly as improved rootstock cultivars [25,26], and they can also be utilized in the development of seedless triploid cultivars [24]. This technique enables the generation of extensive genetic diversity in offspring, making it a powerful tool for creating horticulturally desirable cultivars that may possess many of the necessary tolerance traits [24,27].

Cleopatra mandarin had significant commercial value as a rootstock in Florida due to its commendable tolerance to tristeza, exocortis, xyloporosis, salinity, cold, calcareous soils, and a low incidence of citrus blight [28]. However, limitations arise when using Cleopatra mandarin as a rootstock, including its susceptibility to nematodes and Phytophthora, as well as the reduced productivity of young trees grafted onto this rootstock [28]. Previous studies have identified Cleopatra mandarin as a rootstock with salt tolerance capabilities [29]. Previous studies have observed changes in metabolite profiles, including the accumulation of photoprotective antioxidant secondary metabolites, in Cleopatra mandarin under stress conditions [7]. This metabolic response was interpreted as an activation of energy metabolism and stress-mitigating pathways in Cleopatra mandarin, whereas Carrizo citrange exhibited the enzymatic means to cope with oxidative stress, thereby preventing the excessive accumulation of antioxidant metabolites [30].

It was hypothesized that a tetraploid somatic hybrid obtained by fusing Cleopatra mandarin with Carrizo citrange protoplasts can inherit the tolerance traits of each parent, respectively. This somatic hybrid may better tolerate salinity and oxidative stresses synergistically. This hypothesis was tested by assessing the physiological and biochemical performance of this somatic hybrid to salt stress in comparison to its parent plants under controlled laboratory conditions.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

A somatic hybrid was previously produced by fusing Cleopatra mandarin protoplasts obtained from embryogenic cell suspension cultures with Carrizo citrange protoplasts obtained from leaf mesophyll tissues [22] according to the protocol outlined by Grosser and Gmitter [31,32]. Certified mature cuttings from the somatic hybrid and its parents, free of known plant pathogens, were obtained from trees maintained by the Florida Department of Agriculture and Consumer Services (DPI) for subsequent analyses.

2.2. Flow Cytometry and Leaf Morphology Analysis

Ploidy analysis was performed using a tabletop CyFlow® Cube 6 flow cytometer (Sysmex America, Inc., Lincolnshire, IL, USA). A small leaf piece (approximately 0.4 cm^2) was chopped with a sharp blade in nuclei extraction buffer. This mixture was strained through a 45 µm nylon mesh screen and stained with fluorescent dye (DAPI) as per the instructions provided in a CyStain UV Precise P Automate kit. The position of the 2 N

peak was determined from nuclear DNA obtained from a known diploid standard on the machine's histogram. Diploid and tetraploid leaves were collected from mature trees. The leaf area was measured using ImageJ software to scanned photos at uniform A4 paper Twenty leaves randomly selected for image capturing per cultivar were analyzed.

2.3. Somatic Fusion Confirmation Using Simple Sequence Repeat (SSR) Marker Analysis

DNA was extracted from 100 mg of fresh leaves using the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The concentration of the extracted DNA was determined using a Nanodrop spectrophotometer and adjusted to a normalized concentration of 25 ng/µL. For the study, 14 SSR primer sets synthesized by Operon Technologies were utilized. PCR amplifications were performed using the T100™ Thermal Cycler by Bio-Rad Laboratories, Hercules, CA, USA and fragment separation was carried out using the ABI PRISM 3130 xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). The forward SSR primers in Table 1 were modified with a fluorescently labeled universal M13 primer (5′–GTTGTAAAACGACGGCCAGT–3′). Analysis of SSR markers was performed using the SoftGenetics GeneMarker 3.0.1 software (SoftGenetics LLC., State College, PA, USA).

Table 1. List of the primer sequences used for the SSR characterization of regenerated somatic hybrid and the parents.

Primer	Forward and Reverse Primer Sequences (5′ to 3′)
CX6F04	AGTGAACTGTCCATTGGATTTTCG GTGTTGAATCCCGACCTTCTACC
CX6F29	TTCACCACAAACGAAGACTCAGAC CTGTAATCCACTCGGTAATCCGAC
CX5F57	CCTCGCCAATGACCTTTGTATTTA CAATACGTTTGGGTTCTAGTTCCG
CX0010	AACCGAAGATGGAGGGAACT ACATTCATGGCCACATCTCA
CX0035	CCATTAACGAGAAAACCAAACA CAAAAAGGGGTTGCAAAGAA
CX2021	AAGGTCATGTCTTTAGCACTTTGA CAAGTTGCCAATTCAGGAGG
CX6F02	AACAGTGTAGCATCGCACTTTCAC GATACAAGGGACTTGCCCATCTC
CX6F16	GTCTTCACCCTCTCCATCTTCATC GGGACTATGGCAACAATAACTCCA
CX6F07	CTGTTACCGTTGAGGAAACCAAAG CTCTTCAGCTGGTTTCTCTTCCTG
CX6F13	AAACCCAAGTCATAAACGTCAGGA ATCTTCAATGCTTTTGGAGCAAAC
CX6F17	GATACAAATTAGCATTTGATTGAATGGA ATCGGGACTCGCATTAGGGT
CX6F21	CTACAAGTTCCCCAGTTATCCCG ACTTGACCCGCTCTAGGAGTGAC
CX6F18	GTCTTCAACGAAGTTGCAGGCT TACTATTTCGAGAGAGCAGCAGCA
CX2007	AAATCGGCTAGTTGCAAACG CCTTGACATTGTCGATGGTG

2.4. In Vitro Propagation and NaCl Treatments

Mature stem pieces were collected from the mother plants and cultured in vitro according to Mahmoud et al. [33]. The adventitious shoots were cultured in Murashige and Skoog (MS) medium [34] supplemented with 1 mg·L^{-1} BAP. The regenerated shoots were subcultured twice in the same medium to produce adequate numbers of shoots before salt screening experiments (Figure S1). The shoots were subsequently subcultured in MS medium supplemented with 0, 50, 100, and 150 mM NaCl to induce salt stress. The cultures

were incubated at 27 °C ± 1 °C and a 16 h photoperiod using Philips T8 Lamps with ALTO II Technology (2150–2040 Lumens) as a source of light for 4 weeks. Each treatment consisted of ten replicates. All the chemicals used for tissue culture media were obtained from Phyto Technology Laboratories, Shawnee Mission, KS, USA.

2.5. Physiological and Biochemical Variables

The in vitro cultivated shoots were harvested from each genotype, frozen in liquid nitrogen, and subsequently finely ground. Three biological replicates were sampled from each plant. The ground leaves were kept at −20 °C for biochemical assays. A total of 100 mg fresh weight was homogenized in 1 mL of absolute methanol, centrifuged at 10,000 rpm for 15 min at 4 °C, and further diluted 10× with fresh methanol. The mixture was analyzed for chlorophyll *a* and chlorophyll *b* by reading the absorbance at different wavelengths (665 nm for chlorophyll *a* and 653 nm for chlorophyll *b*) using a visible spectrophotometer (Thermo Scientific™ GENESYS™ 30 spectrophotometer). Quantification of chlorophyll *a*, chlorophyll *b*, carotenoids, and total chlorophyll content was conducted following the methodologies outlined by Lichtenthaler and Wellburn [35].

Malondialdehyde (MDA), the final product of the lipid peroxidation process [36], was measured following the methodology outlined by Heath and Packer [37]. Briefly, frozen leaf samples (100 mg) were suspended in 0.5 mL of 0.1% (*w*/*v*) trichloroacetic acid (TCA) and subsequently subjected to centrifugation at 12,000 rpm, 4 °C for 10 min. The resulting supernatant (0.5 mL) was combined with 1.5 mL of 2-thiobarbituric acid (TBA) in a 20% TCA solution, followed by an incubation at 95 °C for 25 min. The reaction was stopped by placing the mixture on ice for 25 min, and the absorbance of the supernatant was monitored at wavelengths of 532 nm and 600 nm.

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free-radical scavenging activity of leaf samples was measured using the method described by Blois [38]. A fresh solution of DPPH in methanol was prepared at a concentration of 1 mM. Equal volumes of the DPPH solution and leaf extracts were mixed and left to incubate in the absence of light for 30 min. Subsequently, the absorbance was measured at 517 nm using a spectrophotometer, with methanol used as the blank solution. As a control, a solution of DPPH in methanol was used in place of the leaf extract. This experimental process was repeated three times for validation. The inhibition of DPPH was quantified following this equation:

$$\text{DPPH inhibition \%} = (\text{A control} - \text{A sample})/\text{A control} \times 100$$

The phenolic compound content (TPC) in the leaf samples was estimated using the Folin–Ciocalteu method of Singleton and Rossi [39] with a few modifications. TPC extract was centrifuged at 12,000 rpm, 4 °C for 15 min. Next, 100 µL of Folin reagent (1:10) was mixed with leaf extract, vortexed, and incubated for 5 min at room temperature. Then, the reaction was induced by adding 300 mL of 20% sodium carbonate (Na_2CO_3) to the extract, and the tubes were incubated in the dark for 1 h. The absorbance of the reaction mixture was estimated at 765 nm. A standard curve was created using standard solutions of gallic acid (0–600 ppm).

Proline was extracted according to the method described by Bates et al. [40] in aqueous sulfo-salicylic (3% *w*/*v*) acid. The reaction mixture (2 mL supernatant, 2 mL of glacial acetic acid and ninhydrin reagent) was incubated for 1 h at 100 °C in a water bath, followed by incubation in an ice bath to stop the reaction. The reaction mixture was vigorously mixed with 4 mL of toluene in glass tubes. After warming at 25 °C, the color change was monitored at 515 nm using a UV/Vis spectrophotometer for proline content determination. All chemicals used for physiological and biochemical parameters were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.6. Statistical Analysis

The physiological and biochemical traits were investigated using a factorial-based complete randomized design with three salt levels (0, 50 and 100 mM NaCl) and three root-

stocks (Cleopatra mandarin, Carrizo citrange and somatic hybrid of Cleopatra + Carrizo) in ten replicates. Data were analyzed with analysis of variance using JMP Pro 16 software, with post hoc Tukey–Kramer HSD test to compare the means of the different treatments. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Leaf Morphology and Ploidy Confirmation

The leaves of the somatic hybrid display intermediate morphology compared to the parental Cleopatra mandarin and Carrizo citrange rootstocks. The somatic hybrid has larger leaves than the middle leaf of Carrizo citrange, whereas it is similar in size to Cleopatra mandarin (Figure 1A). Unlike the consistently trifoliate leaf morphology of Carrizo citrange, only a few leaves on the somatic hybrid shoots were trifoliate, suggesting only the partial dominance of this trait in the tetraploid background. When comparing the three, there were no significant mean differences in the leaf areas (Figure 1B). The ploidy levels of all regenerated plants were confirmed using flow cytometer analysis, based on the analysis of nuclear fluorescence intensities, as depicted in the representative histogram (Figure 1C–E).

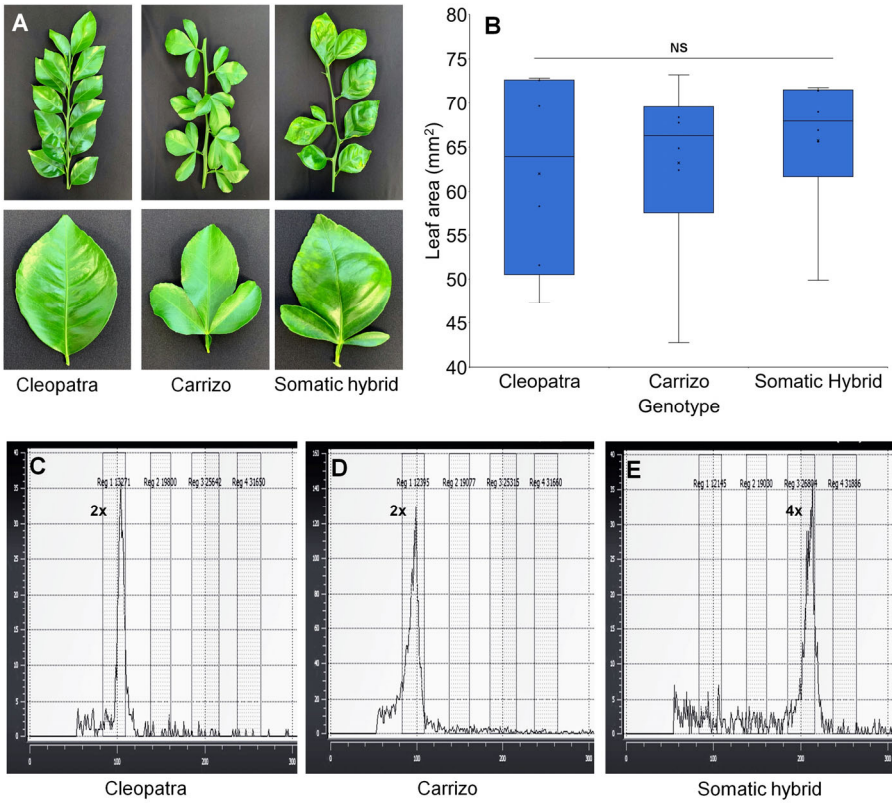

Figure 1. (**A**) Leaf morphology of somatic hybrids and regenerated plants. The upper image displays a shoot, and the lower image displays one leaf of each type. (**B**) Leaf area. The leaves were collected from mature trees growing in a certified greenhouse. The leaf area of Carrizo citrange included the area of the trifoliate leaves. The error bar indicates SE (n = 10). Ploidy analysis using flow cytometry. Peaks derived from Cleopatra mandarin (**C**), Carrizo citrange (**D**) and tetraploid somatic fusion hybrid (**E**). NS—Not significant.

3.2. Molecular Characterization of Donor Parents and the Somatic Hybrid Using SSR Markers

SSR markers were used to characterize the somatic hybrids and donor parents at 14 loci. All primer pairs successfully amplified DNA from the three cultivars; however, only 11 of 14 unequivocally confirmed somatic hybridity (Table 2, Figures S2 and S3). Specifically, one allele from Cleopatra mandarin was missing in the somatic hybrid at CX6F21 and

CX6F18 (assuming that the 166 and 167 fragments are identical), but no Cleopatra mandarin alleles were found at CX2007.

Table 2. Molecular analysis of regenerated plants through SSR primers. Numbers are allele-specific amplification fragment sizes.

Genotype/EST-SSR Marker		**CX6F04 ***				**CX6F29**		
Carrizo citrange	157	162			149	156		
Cleopatra mandarin	162	169			156	156		
Somatic hybrid	157	162	162	169	149	156	156	156
		CX5F57				**CX0010**		
Carrizo citrange	156	166			222	229		
Cleopatra mandarin	156	156			219	219		
Somatic hybrid	156	156	156	166	219	219	222	229
		CX0035				**CX2021**		
Carrizo citrange	172	186			150	157		
Cleopatra mandarin	172	172			150	150		
Somatic hybrid	172	172	172	186	150	150	150	157
		CX6F02				**CX6F16**		
Carrizo citrange	168	175			170	175		
Cleopatra mandarin	168	168			164	164		
Somatic hybrid	168	168	168	175	164	164	170	175
		CX6F07				**CX6F13**		
Carrizo citrange	104	110			172	178		
Cleopatra mandarin	104	104			178	178		
Somatic hybrid	104	104	104	110	172	178	178	178
		CX6F17				**CX6F21**		
Carrizo citrange	133	133			155	155		
Cleopatra mandarin	139	158			149	155		
Somatic hybrid	133	133	139	158	155	155	155	155
		CX6F18				**CX2007**		
Carrizo citrange	161	161			172	177		
Cleopatra mandarin	155	166			174	174.6		
Somatic hybrid	161		167		172	177		

* EST-SSR markers are written in bold.

3.3. Physiological and Biochemical Variables

Our results clearly indicate variations in growth among the different genotypes when subjected to salt stress conditions, as illustrated in Table 3 and Figure 2. In general, the in vitro application of NaCl caused an increase in MDA content. Carrizo citrange leaves accumulated 0.99 and 1.19 $nmol^{-1}$ MDA eq. g FW at 50 and 100 mM NaCl, respectively. The somatic hybrid exhibited comparable levels (0.68 and 0.93 $nmol^{-1}$ MDA eq. g FW) to or better levels than those of the standard salt-tolerant Cleopatra mandarin rootstock (0.81 and 1.03 $nmol^{-1}$ MDA eq. g FW) at 50 and 100 mM NaCl treatments, respectively (Figure 2).

Table 3. Significance analysis of the physiological traits using a two-way ANOVA assay.

Variables	Genotype	NaCl Treatments	Interaction
MDA content *	0.0077	0.0222	0.923
Chlorophyll *a*	<0.0001	<0.0001	0.001
Chlorophyll *b*	<0.0001	0.0008	0.0063
Carotenoids	<0.0001	0.0067	NS
Total Chlorophyll	<0.0001	<0.0001	0.0015
DPPH inhibition	NS	0.034	NS
Total phenolic compounds	<0.0001	0.0018	<0.0001
Proline content	0.0013	0.0013	NS

* All the parameters were measured in the shoots grown in vitro. NS—Not significant.

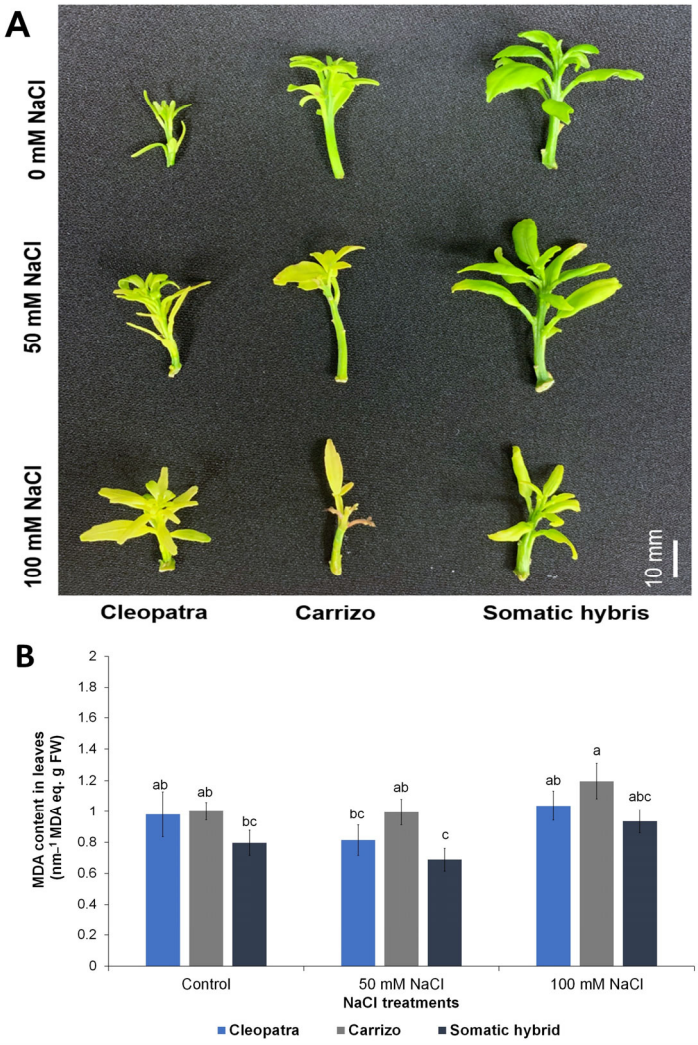

Figure 2. Effect of different concentrations of sodium chloride (NaCl) on shoot growth of Cleopatra mandarin and Carrizo citrange and tetraploid somatic fusion hybrid (**A**), MDA content (**B**). Means compared using Tukey–Kramer HSD test. Means followed by the same letter were not significantly different at ($p < 0.05$). The error bar indicates SE ($n = 10$).

A significant difference in foliar chlorophyll content ($p < 0.0001$) was observed when the effect of different rootstocks was compared, as indicated in Table 3. The somatic hybrid recorded the highest foliar chlorophyll *a* content under control and NaCl conditions, with values of 13.63, 9.35 and 4.95 mg^{-1} g FW, following 0, 50 and 100 mM NaCl treatments, respectively (Figure 3A). There was a slight reduction in the carotenoid response when the two levels (50, 100 mM) of NaCl were compared in all the rootstocks. There was no

significant difference of foliar chlorophyll content between Cleopatra mandarin and Carrizo citrange shoots under all the tested conditions. Under 100 mM NaCl, there was an obvious decrease in chlorophyll *b* in the somatic hybrid, and we recorded similar levels as Cleopatra mandarin or Carrizo citrange shoots under 100 mM NaCl (Figure 3C). The somatic hybrid displayed the highest foliar chlorophyll content, with values of 6.59 mg^{-1} g FW following 100 mM NaCl treatments, whereas there was no significant difference of foliar chlorophyll content between Cleopatra mandarin and Carrizo citrange shoots (3.44 and 2.61 mg^{-1} g FW) (Figure 3D).

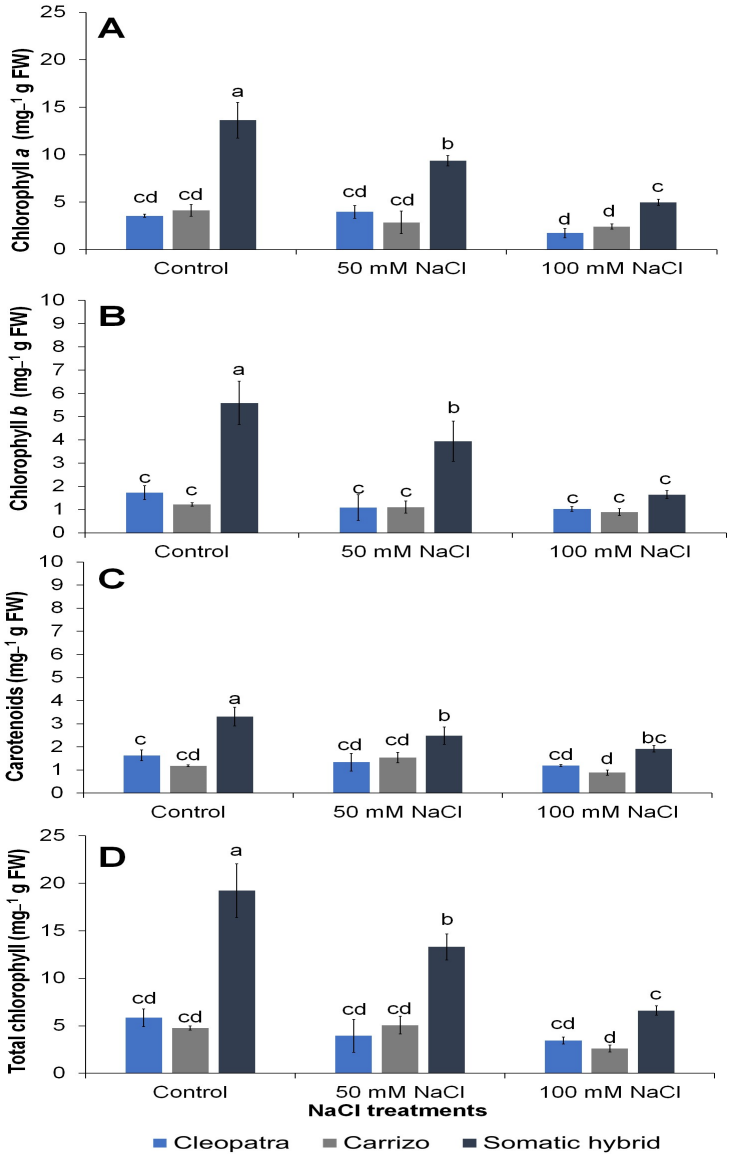

Figure 3. Effect of different concentrations of sodium chloride (NaCl) on the content of chlorophyll *a* (**A**), chlorophyll *b* (**B**), carotenoids (**C**), and total chlorophyll (**D**) of Cleopatra mandarin and Carrizo citrange and a tetraploid somatic fusion hybrid. Means compared using Tukey–Kramer HSD test. Means followed by the same letter were not significantly different at ($p < 0.05$). The error bar indicates SE ($n = 10$).

3.4. DPPH Radical Scavenging Activity, Total Phenolic Compounds, and Proline Content

The DPPH free-radical scavenging activity was slightly different among the rootstocks. The somatic hybrid recorded the highest DPPH content (59.35%) under 100 mM NaCl (Figure 4A). The foliar TPC content was significantly different ($p < 0.0001$) when the effect

of the different rootstocks was compared (Figure 4B). The somatic hybrid exhibited the highest TPC values (180.28 mg gallic acid g^{-1} FW), whereas Cleopatra mandarin recorded 115.44 mg gallic acid g^{-1} FW and Carrizo citrange recorded 124.66 mg gallic acid g^{-1} FW. There were no significant differences in proline content when the rootstocks were compared under 100 mM NaCl (Figure 4C); however, Carrizo citrange exhibited a significant increase in proline content (3.36 µmol g^{-1} FW) under 50 mM NaCl.

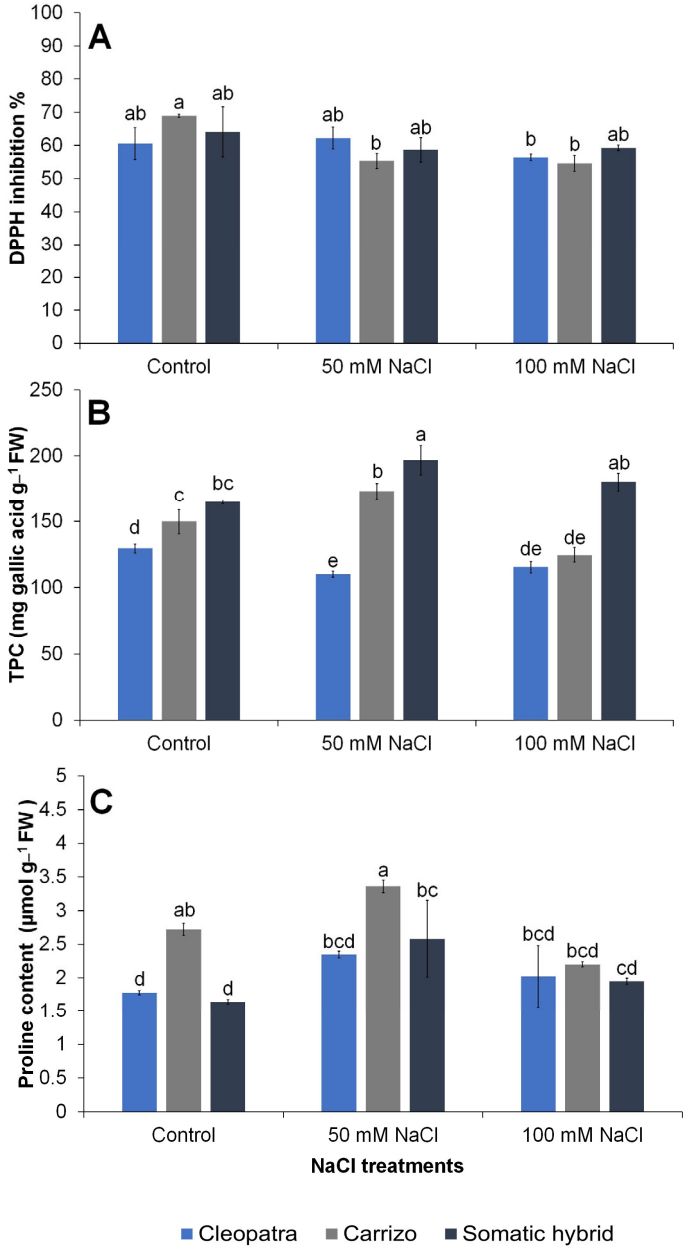

Figure 4. Effect of different concentrations of sodium chloride (NaCl) on DPPH inhibition% (**A**), total phenolic compounds content (**B**), and proline content (**C**) of Cleopatra mandarin and Carrizo citrange and a tetraploid somatic fusion hybrid. Means compared using Tukey–Kramer HSD test. Means followed by the same letter were not significantly different at ($p < 0.05$). The error bar indicates SE ($n = 10$).

3.5. Correlation Analysis

The chlorophyll *a* and *b* and total chlorophyll contents were positively correlated with total phenolic compounds content and DPPH inhibition%. The MDA content was significant and positively correlated with proline content (Table 4).

Table 4. Pearson's correlation matrix among the studied parameters of Citrus genotypes under NaCl stress.

Variables *	Chl *a*	Chl *b*	Caro	T Chl	DPPH	TPC	Proline	MDA
Chl *a*	1							
Chl *b*	0.9512	1						
Caro	0.9153	0.7867	1					
T Chl	0.9953	0.9767	0.8845	1				
DPPH	0.2445	0.1653	0.3311	0.2221	1			
TPC	0.5295	0.4503	0.4859	0.5102	−0.0104	1		
Proline	−0.2261	−0.3848	−0.0665	−0.2785	0.1009	0.184	1	
MDA	−0.4446	−0.3946	−0.4399	−0.4336	−0.4312	−0.2828	0.0005	1

* Numbers represent average values per rootstock and treatment. Chl *a*—chlorophyll *a* content; Chl *b*—Chlorophyll *b* content; Caro-Carotenoids; T Chl—total chlorophyll content; MDA—malondialdehyde; TPC—total phenolic compounds.

4. Discussion

Salt stress significantly affects plant metabolism, disrupting the photosynthetic machinery and inducing osmotic stress [41]. It triggers the excessive production of free oxygen radicals, which have the potential to disrupt the cell membrane and induce lipid peroxidation within the membrane [7,17]. Several studies have indicated that tetraploid citrus plants exhibit greater resistance to salt stress compared to their corresponding diploid relatives [42,43]. Consequently, there is an increasing appreciation of the adaptive advantages provided by tetraploid plants [44,45]. In the present study, we investigated the potential of a tetraploid somatic hybrid to alleviate salt stress in comparison with its diploid parents. We confirmed the ploidy of the somatic hybrid through flow cytometry and SSR markers. Despite specific primers not showing amplification, some other primers provide sufficient evidence to conclusively support allotetraploidy in the regenerated hybrid. This discrepancy could be attributed to somaclonal variation or mutation induction in the Cleopatra mandarin cell line suspension used during protoplast fusion, as well as genetic variation between the Cleopatra mandarin cell line and the plant source used in the SSR analysis.

The somatic tetraploid hybrid has been observed to exhibit higher chlorophyll content compared to the corresponding diploid parents. Tetraploid plants can often exhibit a darker coloration compared to their diploid counterparts [46]. This darkening in color can be attributed to a range of factors stemming from changes in gene expression, alterations in pigment production, and modifications in cell structure resulting from the increased chromosome count. The phenomenon can arise due to the accumulation of pigments such as chlorophyll, anthocyanins, and carotenoids, which play crucial roles in plant coloration. Moreover, the larger cell sizes and modified cell shapes found in tetraploid plants can impact how light is absorbed and reflected, potentially influencing color perception. The genetic changes induced by polyploidy can affect genes related to pigment biosynthesis, cell wall composition, and other color-associated processes.

The amount of chlorophyll present in a plant is intricately linked to its photosynthetic rate, and the ratio of chlorophyll *a* to chlorophyll *b* serves as an indicator of the plant's proficiency in utilizing light [47,48]. Consequently, the enhanced photosynthetic performance observed in somatic tetraploid leaves in comparison to their diploid parents can be elucidated by their possession of a greater photosynthetic surface area, owing to larger leaf dimensions, and elevated levels of photosynthetic pigments like chlorophyll and carotenoids. These findings were observed when the autotetraploid of apple 'Hanfu' leaves was compared with their diploid counterparts [49].

Tetraploid plants employ complex physiological and biochemical mechanisms to cope with salt stress, including photosynthetic rate, the regulation of protein, lipid, and carbohydrate metabolism, metal ion binding and transportation, and cell wall synthesis [50]. It also influences phenology, antioxidant response, and morphology [51]. Recent studies have highlighted the significance of ROS detoxification through the induction of antioxidant pathways in controlling salt stress. In our current study, we observed an elevation in the

DPPH free-radical scavenging capacity, suggesting their ability to mitigate the adverse effects of ROS compared to other rootstocks. Furthermore, we recorded decreased malondialdehyde (MDA) content and cellular damage in the somatic hybrid compared with the diploid parents. Phenolic compounds are important antioxidants that play essential roles as antimicrobial agents in response to abiotic stress [7,36,46]. An increase in TPC content was observed in the somatic hybrid, which is regulated by the polymerization of phenols. This process can reduce the levels of free phenols in plant tissues. Proline, acting as an osmolyte, plays a role in alleviating oxidative stress in plants subjected to salt stress [52]. Compared to control treatments, a decrease in proline content was recorded with NaCl supplementation at 100 mM, while an increase was observed in response to 50 mM NaCl, with the highest concentration recorded in Cleopatra mandarin. The somatic hybrid exhibited an increase in total phenolic content, regulated by phenol polymerization, which can reduce free phenol levels in plant tissues. Proline, functioning as an osmolyte, assists in mitigating oxidative stress in plants subjected to salt stress. Proline levels decreased with 100 mM NaCl supplementation compared to control treatments, while an increase was observed in response to 50 mM NaCl, with Cleopatra mandarin showing the highest concentration.

Previous studies have also indicated the advantages of tetraploid plants over their diploid counterparts. Carrizo citrange tetraploid seedlings showed superior salt tolerance, attributable to a combination of factors including reduced chloride uptake, modified root morphology, enhanced root histology, sustained photosynthetic capacity, and efficient water management [53]. Similarly, a tetraploid rootstock (4x Citrumelo 4475) exhibited enhanced tolerance to nutrient deficiency, as indicated by improved photosynthetic parameters, reduced organelle degradation, and a more efficient antioxidant system [54]. A transcriptomic investigation into the salt stress tolerance in tetraploid *Paulownia fortunei* (Seem.) Hemsl., compared to its diploid counterpart, provided valuable insights into the underlying molecular mechanisms and led to the identification of several differentially expressed genes associated with photosynthesis, plant growth, development, and osmolyte regulation of the tetraploid trees under saline conditions [55]. Similarly, autotetraploid *Ziziphus jujuba* Mill. had enhanced salt tolerance when compared to the diploid form [56].

5. Conclusions

The current study examined the response of a tetraploid somatic fusion plant obtained from the protoplast fusion of Cleopatra mandarin and Carrizo citrange and compared with its parental plants, to salt stress. The tetraploid hybrid exhibited reduced sensitivity to NaCl stress compared to diploid plants. Physiological and biochemical changes, such as increased chlorophyll content, decreased MDA, and total phenolic compounds, were observed in the tetraploid hybrid, contributing to its enhanced salt stress tolerance. Our findings highlight the potential of tetraploid hybrids in developing more resilient citrus varieties that can be a new source of salinity tolerance for salt-affected lands.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9111215/s1, Figure S1: In vitro propagation of Cleopatra mandarin, Carrizo citrange and the tetraploid somatic hybrid in Murashige and Skoog (MS) medium supplemented with 1 mg·L^{-1} BAP; Figure S2: A chromatogram of EST-SSR markers generated from ABI trace files by GeneMarker®software (SoftGenetics); Figure S3: A chromatogram of EST-SSR markers generated from ABI trace files by GeneMarker®software (SoftGenetics).

Author Contributions: L.M.M. Conceptualization, data curation, formal analysis, investigation, methodology, writing original draft, review and editing. P.H. and F.G.G.J. Simple Sequence Repeat (SSR) Marker Analysis. N.K., F.G.G.J., J.W.G. and M.D. resources, supervision, review and editing. All authors have read and agreed to the published version of the manuscript.

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