



Unraveling the influence of salinity on physiological and biochemical parameters in citrus (*Citrus* spp.) rootstocks

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ABSTRACT

The present study was carried out during 2018–19 and 2019–20 at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana to examine the impact of salt stress on physiological parameters of 9 distinct rootstocks of citrus (*Citrus* spp.) The experimental design followed a completely randomized design (CRD) accompanied with 3 replications, enclosing 45 combinations with 9 citrus rootstocks (Pectinifera, Cleopatra mandarin, Rangpur lime, Alemow, Rough lemon, NRCC-4, Volkamer lemon, CRH-12 and NRCC-3) exposed to 5 NaCl salt stress levels, viz. control (0.07), 2.5, 4.0, 5.5 and 7.0 dS/m. Among the different rootstocks, Rangpur lime exhibited the highest leaf (12.65 mg/g DW) and root (12.42 mg/g DW) total soluble carbohydrates at the 7.0 dS/m salinity level. Additionally, Rangpur lime showcased minimal reduction in chlorophyll stability index (17.2%), leaf and root relative water content (18.7 and 18.9%, respectively), relative stress injury (32.0 and 33.0%, respectively) and leaf and root (Malondialdehyde) MDA content (8.46 and 8.12 μ moles/g DW, respectively) at the same salinity level. Overall, Rangpur lime, Volkamer lemon and CRH-12 demonstrated superior performance by exhibiting relatively higher buildup of total soluble carbohydrates and less drop in CSI, RWC, RSI and MDA content at 7.0 dS/m as compared to control. Conversely, Cleopatra mandarin, Rough lemon and NRCC-3 displayed a relative moderate response, while Pectinifera, Alemow, and NRCC-4 showcased substandard performance, exhibiting contrasting behaviour at 7.0 dS/m as compared to control, particularly concerning physiological parameters at the seedling stage.

Keywords: Chlorophyll, Citrus, MDA, Rootstock, RSI, RWC, Salinity

Citrus (*Citrus* spp.), an economically important horticultural crop, is widely cultivated and recognized for its numerous health benefits (Zou *et al.* 2016). However, it is highly susceptible to salt stress and convened as a glycophyte, particularly in tropical and subtropical regions facing increasing challenges of drought and salinity (Simpson *et al.* 2015, Sahin-Çevik *et al.* 2020). In India, citrus holds the third position among fruit crops, contributing significantly to fruit production (13.7% of entire production) after banana and mango. India boasts an extensive citrus cultivation area spanning 1,098 thousand hectares, which contributes to a remarkable production of 14,757 thousand metric tonnes (Anonymous 2023). The state of Haryana, with a substantial citrus cultivation area encompassing 24.40 thousand hectares and 570.88 thousand metric tonnes of production, is confronted with salinity issues affecting millions of hectares of cultivated land. Salinity

poses a significant environmental constraint, hampering plant efficiency in arid and semi-arid climates (Hussain *et al.* 2009). Excessive soil salt disrupts vital physiological processes, reduces water intake and disrupts nutrient balance (Sharma *et al.* 2013). In response to salinity, plants employ adaptive strategies involving morphological, physiological, and biochemical changes to mitigate the negative impacts on growth and yield (Abadi *et al.* 2010). Accumulation of total soluble sugars plays a crucial role in salt tolerance, as they help balance osmotic potential and maintain enzyme activities in the existence of toxic ions (Xue *et al.* 2009). Chlorophyll content serves as a biochemical indicator of salt tolerance, with salt-tolerant species either maintaining or exhibiting an increase in chlorophyll levels under salinity conditions (Ashraf and Harris 2013). The choice of a suitable rootstock is vital, as it acts as the first filter for salt ions entering the root system. Proper rootstock selection enhances antioxidant activities, increases osmo-protectant concentrations, and regulates Na⁺ and Cl⁻ levels in leaves, thereby improving salt tolerance in citrus plants (Stover *et al.* 2018, Shahid *et al.* 2019). Developing salt-tolerant citrus cultivars and rootstocks through breeding programmes remains challenging due to the intricate mechanisms

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involved (Ruiz *et al.* 2018). Therefore, evaluating potential rootstocks such as Volkamer lemon, NRCC-3, Pectinifera, CRH-12, Alemow and NRCC-4 for salinity tolerance in the western agroclimatic zone of Haryana is necessary. In light of this, the existing research aims to explore the impact of salinity on physiological parameters of citrus rootstock seedlings, providing valuable insights for the development of resilient citrus varieties in salt-affected regions.

MATERIALS AND METHODS

The experiment was conducted during 2018–19 and 2019–20 at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana which experiences a semi-arid climate characterized by hot and dry summers and extremely cold winters. Soil samples were collected from the sand dunes of Balasmand village in Hisar and underwent chemical and mechanical examination after sieving through a 2 mm sieve. The soil was found to be sandy in texture, low in organic carbon, with medium nitrogen and phosphorus availability. It exhibited an alkaline reaction and had a saturation capacity of 25%. For the experiment, plastic pots filled with 10 kg of sand were used, and the crops were supplied with Hoagland nutrient solution at consistent intermissions. Salinity was induced in the soil using artificial water with diverse ionic constituents (Table 1). The desired salinity levels were achieved by adding chloride and sulphate salts of Na^+ , Ca^{2+} and Mg^{2+} in appropriate quantities. The salts were dissolved in water and the solution was adjusted to a final volume of 75 litre for respective salinity level. A volume of 2.6 litre of the solution was added to each pot containing 10 kg of soil after thorough mixing and drying to maintain each salinity level. The study involved 9 diverse citrus rootstocks and 5 levels of NaCl dominated salinity. Seeds of Cleopatra mandarin, Pectinifera, Rangpur lime, Alemow, Rough lemon, NRCC-4, Volkamer lemon, CRH-12 and NRCC-3 were collected from single tree of each rootstock from CCS Haryana Agricultural University, Hisar and ICAR-Central Citrus Research Institute, Nagpur. The seeds were washed, air-dried under shade, and preserved with Bavistin. They were sown at a depth of 1 cm in pots with 3 replications, containing 10 seeds per replication in the month of November. The pots were covered with soil having salinity levels of 0.07 (control), 2.5, 4.0, and 7.0 dS/m and supplied with good quality irrigation water throughout the experiment. Physiological parameters of the one-year-old

seedlings were documented at the experiment end.

To estimate the chlorophyll stability index (CSI), the formula prescribed by Sairam *et al.* (1997) was used. Chlorophyll content was determined following the technique of Hiscox and Israelstam (1979) using dimethyl sulfoxide (DMSO):

$$\text{CSI (\%)} = \frac{\text{Total chlorophyll under stress}}{\text{Total chlorophyll under control}} \times 100$$

Relative water content (RWC) was estimated using the procedure suggested by Barrs and Weatherley (1962):

$$\text{RWC (\%)} = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Turgid weight} - \text{Dry weight})} \times 100$$

Relative stress injury (RSI) was calculated as the percentage proportion of ion leakage to the external aqueous medium relative to the total ion concentration of the stressed tissue (Sullivan and Ross 1979):

$$\text{RSI (\%)} = \frac{\text{EC1}}{\text{ECI}} \times 100$$

Total soluble carbohydrates (TSC) were measured using the process described by Dubois *et al.* (1951) with anthrone reagent and the data was expressed as mg/g DW. Lipid peroxidation amount was assessed by quantifying malondialdehyde (MDA) content in both leaf and root tissues. MDA content was determined using a modified version of the Heath and Packer (1968) method, which involves the thiobarbituric acid (TBA) reaction. The amount of MDA was estimated using its extinction coefficient of 155 m²/mol and expressed as $\mu\text{moles/g DW}$. The collected data were analyzed using a two-factorial completely randomized design (CRD) with three replications with a 5% critical difference (CD), and statistical analysis was performed using OP Stat software, provided by CCS Haryana Agricultural University, Hisar, Haryana (Sheoran *et al.* 1998).

RESULTS AND DISCUSSION

Total soluble carbohydrate (TSC): In the current study, the content of total soluble carbohydrates (TSC) in both leaves and roots exhibited a significant increase as the salinity levels were raised (Fig. 1). Rangpur lime demonstrated the highest TSC content in leaves and roots (12.65 and 12.42 mg/g DW, respectively). This was followed by leaves and roots of Volkamer lemon (12.13 and 11.89 mg/g DW), respectively. In contrast, Pectinifera exhibited the lowest TSC content (5.67 and 5.44 mg/g DW) in leaves and roots, respectively at a salinity level of 7 dS/m. TSC in leaves and roots of Rangpur lime was (45.9 and 49.2%), followed by Volkamer lemon (45.1 and 48.5%), respectively were least responsive to the salinity stress with accumulation of total soluble carbohydrates, as compared to other rootstocks. On the other hand, Pectinifera (37.2 and 43.6%) was found most responsive when subjected to highest salinity level compared to the control. Previous studies have also reported enhanced TSC content under salt

Table 1 Composition of different ions (me/l) for preparing chloride dominated saline water

EC _e level (dS/m)	Total dissolved salts (TDS)	Na ⁺	Ca ²⁺	Mg ²⁺	Cl ⁻	SO ₄ ²⁻
2.5	30.50	15.25	3.81	11.43	21.35	9.15
4.0	50.0	25.00	6.25	18.75	35.00	15.00
5.5	66.50	33.25	8.31	24.93	46.55	19.95
7.0	86.0	43.00	10.75	32.24	60.20	25.80

stress conditions. Pérez-Jimenez and Perez-Tornero (2020), observed increased TSC levels in *Citrus macrophylla* and its mutants at different salt stress levels (60 and 100 mM NaCl), while Murkute *et al.* (2010) found significantly higher total sugar increments in *C. karna* (43.04%) compared to *C. jambhiri* (41.40%) under the highest salinity level (100 mM NaCl) over the control, which leads to more salt tolerance.

Chlorophyll stability index (CSI%): The impact of increasing salinity stress on the chlorophyll stability index (CSI%) was evident across all the rootstocks analysed (Table 2). Rangpur lime exhibited the highest percentage of CSI (0.83%), which was statistically similar to Volkamer lemon (0.81%), CRH-12 (0.80%), Cleopatra mandarin (0.76%), and Rough lemon (0.75%). In contrast, Pectinifera displayed the lowest CSI (0.62%) at the highest salinity level of 7 dS/m. The present study observed a minimal reduction in CSI in the leaves of Rangpur lime (17.2%) and Volkamer lemon (18.8%), while Pectinifera experienced the maximum reduction (38.0%) with increased salt level from control to 7.0 dS/m. The decline in CSI under salinity stress can be attributed to the decreased rate of photosynthesis resulting from the reduced activity of oxygenase enzymes and photosystem II (Ma *et al.* 2020). These findings are in

Table 2 Effect of salinity on chlorophyll stability index (CSI %) in leaves of citrus rootstocks

Rootstock	Salinity level (dS/m)					
	0.07 (Control)	2.5	4.0	5.5	7.0	Mean
Rough lemon	1.00	0.97	0.94	0.87	0.75	0.91
Cleopatra mandarin	1.00	0.98	0.95	0.88	0.76	0.91
Pectinifera	1.00	0.94	0.90	0.76	0.62	0.84
Rangpur lime	1.00	0.99	0.97	0.91	0.83	0.94
Alemow	1.00	0.97	0.92	0.80	0.65	0.87
Volkamer lemon	1.00	0.98	0.96	0.90	0.81	0.93
NRCC-3	1.00	0.96	0.91	0.85	0.72	0.89
NRCC-4	1.00	0.95	0.92	0.84	0.70	0.88
CRH-12	1.00	0.97	0.95	0.89	0.80	0.92
Mean	1.00	0.97	0.94	0.86	0.74	
CD ($P=0.05$)	Rootstock = 0.04 Salinity = 0.03 Rootstock × Salinity = 0.08					

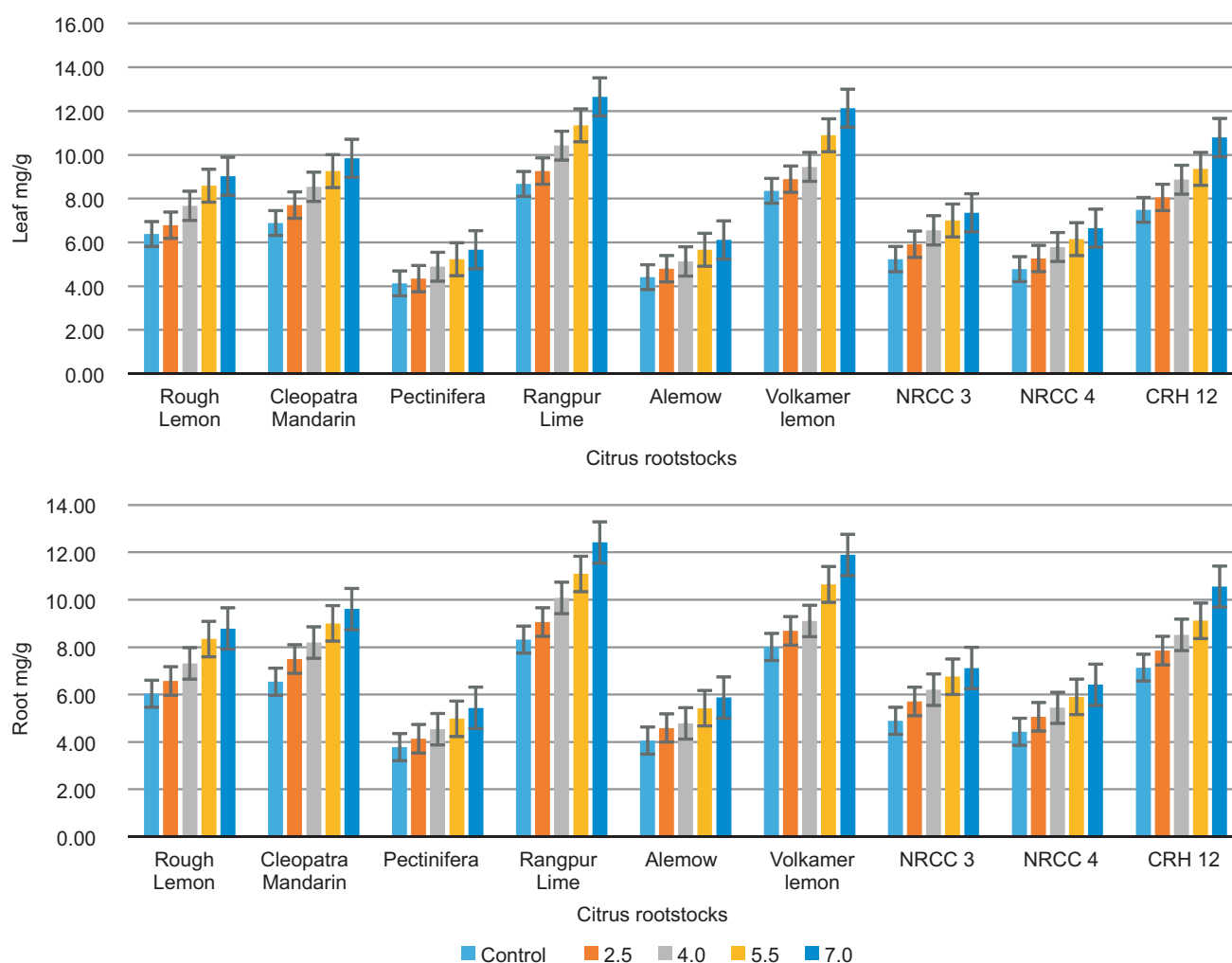


Fig. 1 Effect of salinity on total soluble carbohydrates (mg/g DW) in leaves and roots of citrus rootstocks.

line with those of Alam *et al.* (2020), who stated a maximum chlorophyll decrease in Calamansi (60%) and Pomelo (38%), while Cleopatra mandarin exhibited the minimum decrease (34%) when subjected to increasing saline irrigation from 0.75 to 16 dS/m levels.

Relative water content (RWC %): Significant reductions in the relative water content (RWC%) of both leaves and roots were observed across all the rootstocks analysed as salinity levels increased (Table 3). The maximum leaf and root relative water content (72.95 and 70.29%) were observed in Rangpur lime, respectively. This was statistically similar to Volkamer lemon (71.22 and 68.68%), CRH-12 (69.90 and 67.27%), and Cleopatra mandarin (68.25 and 65.57%), respectively. In contrast, Pectinifera displayed the lowest RWC values of 58.35% in leaves and 50.00% in roots at a salinity level of 7 dS/m. The decline in RWC in both leaves and roots was most pronounced in Pectinifera (25.2 and 25.5%, respectively), as salinity enhanced from

the control up to 7.0 dS/m. Conversely, Rangpur lime exhibited the least reduction in leaves (18.7%) and roots (18.9%) RWC. This decrease in RWC can be attributed to the accumulation of salt ions within the cells, disrupting the osmotic equilibrium and impeding water absorption and movement. Excessive salt concentration in the soil hinders plant metabolism and disturbs water relations, necessitating plants to expend more energy to extract water from saline solutions, resulting in reduced turgor pressure (Hassine and Lutts 2010). The discoveries of the existing research were consistent with the results reported by Khoshbakht *et al.* (2014), who observed the highest relative water content (RWC) in Sour orange (79%), while Trifoliate orange (54.5%) exhibited the lowest RWC of under 75 mM NaCl salt stress.

Relative stress injury (RSI %): Elevated salinity levels resulted in significantly greater relative stress injury (RSI) across all the rootstocks, reaching the highest values at

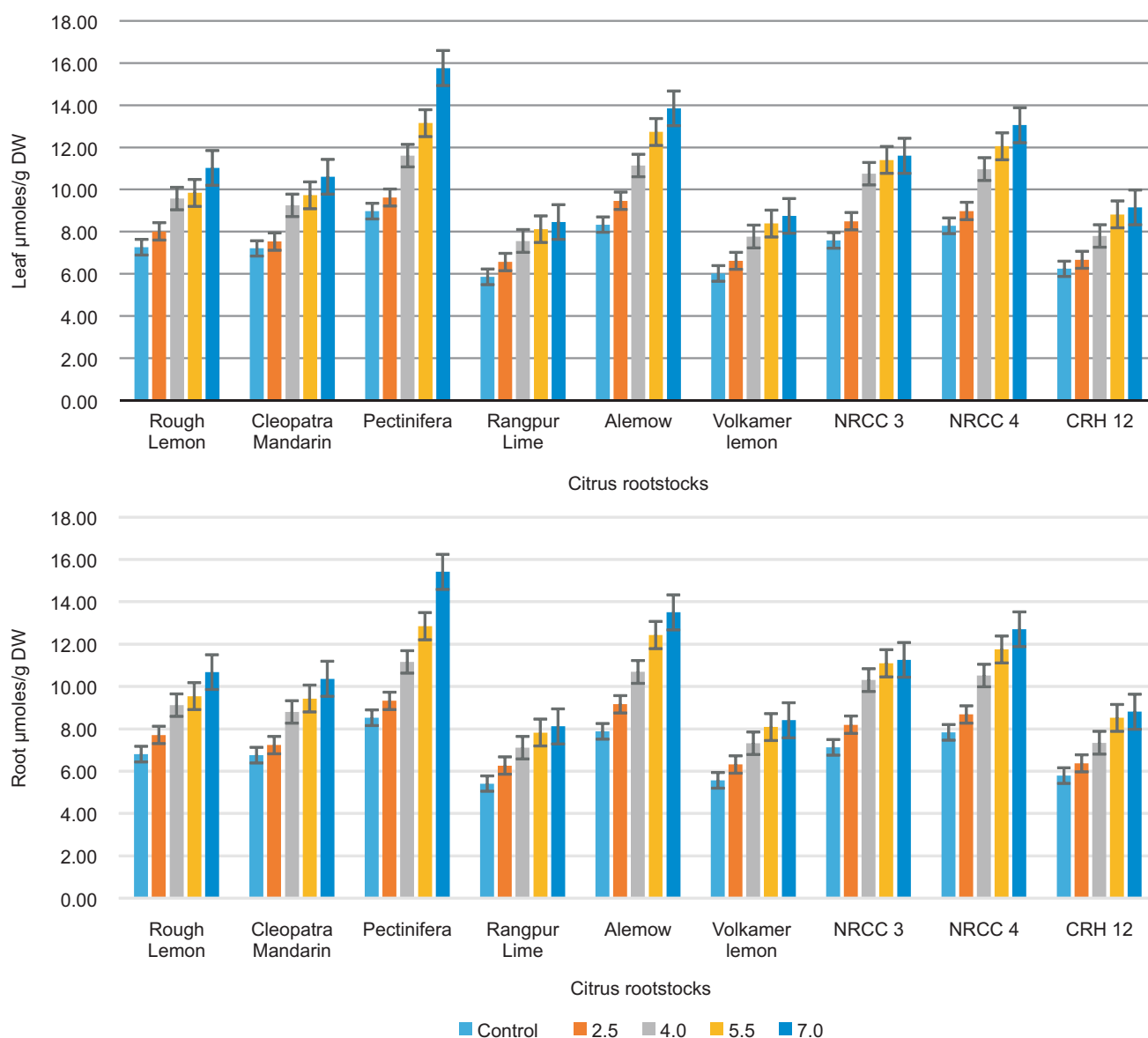


Fig. 2 Effect of salinity on (Malondialdehyde) MDA (µmoles/g DW) content in leaves and roots of citrus rootstocks.

Table 3 Effect of salinity on relative water content (RLC%) and relative stress injury (RSI%) of leaves and roots in citrus rootstocks

Rootstock	Salinity level (dS/m)																								
	Relative water content (RLC%)									Relative stress injury (RSI%)															
	Leaves					Roots				Leaves					Roots										
	Con-trol	2.5	4.0	5.5	7.0	Mean	Con-trol	2.5	4.0	5.5	7.0	Mean	Con-trol	2.5	4.0	5.5	7.0	Mean	Con-trol						
Rough lemon	82.78	80.93	78.58	74.80	68.25	76.60	80.78	78.97	76.66	72.99	63.30	74.54	26.69	26.94	28.42	32.55	36.58	30.24	24.24	24.41	24.87	28.96	33.48	27.19	
Cleopatra mandarin	85.21	83.43	81.54	75.69	58.35	78.82	83.11	81.37	79.53	73.82	65.57	76.68	26.50	27.13	27.82	32.18	36.05	29.94	24.15	24.60	25.18	28.41	33.11	27.09	
Pectinifera	77.98	76.78	74.47	67.34	72.95	70.98	76.18	75.00	72.75	65.78	56.00	69.14	29.34	30.45	31.33	36.67	42.78	34.11	26.87	27.90	28.95	32.18	39.47	31.07	
Rangpur lime	89.81	88.93	87.95	83.15	61.27	84.56	87.76	86.90	85.64	81.25	70.29	82.37	25.50	25.76	25.81	29.85	33.67	28.12	22.98	23.23	23.68	26.33	30.58	25.36	
Alemow	79.07	77.90	76.29	69.67	71.22	72.84	76.98	75.84	74.27	67.82	58.65	70.71	28.98	30.34	31.16	35.74	40.87	33.42	26.50	27.50	28.63	31.81	37.65	30.42	
Volkamer lemon	88.15	87.11	85.77	81.05	65.21	82.66	86.25	85.23	83.92	79.30	68.68	80.68	25.75	26.21	26.19	30.16	34.26	28.51	23.48	23.68	23.96	26.69	31.29	25.82	
NNRCC-3	82.21	80.69	78.74	72.24	63.05	75.82	80.21	78.73	76.82	70.49	62.62	73.78	27.23	27.80	28.78	32.64	37.68	30.82	24.80	25.27	25.84	29.25	34.58	27.95	
NNRCC-4	80.60	79.97	78.69	72.29	69.90	74.92	78.65	78.03	76.79	70.54	60.52	72.91	28.56	29.08	29.83	34.50	39.67	32.33	26.11	26.55	27.20	30.51	36.53	29.38	
CRH-12	86.56	85.82	84.74	80.12	68.25	81.43	84.54	83.82	82.76	78.25	67.27	79.33	26.10	26.52	26.73	30.91	35.27	29.11	23.61	23.99	24.40	27.28	32.15	26.29	
Mean	83.60	82.39	80.75	75.15	58.35		81.61	80.43	78.79	73.36	63.66		27.18	27.80	28.45	32.80	37.43		24.75	25.24	25.86	29.05	34.32		
CD	Rootstock = 2.18 Salinity = 1.96					Rootstock = 2.42 Salinity = 2.18					Rootstock = 0.99 Salinity = 0.65					Rootstock = 0.97 Salinity = 0.62									
(P=0.05)	Rootstock × Salinity = 5.79					Rootstock × Salinity = 6.12					Rootstock × Salinity = 2.33					Rootstock × Salinity = 2.26									

7 dS/m (Table 3). The minimum leaf and root relative stress injury (33.67 and 30.58%) was observed in Rangpur lime, respectively. This was comparable to Volkamer lemon (34.26 and 31.29%) and CRH-12 (35.27 and 32.15%), respectively, whereas RSI (%) was recorded highest (42.78 and 39.47%) in Pectinifera at 7 dS/m, respectively. The increase in RSI with escalating salt levels from control to 7.0 dS/m was most pronounced in leaves and roots of Pectinifera (45.8 and 46.9%). Conversely, Rangpur lime displayed the lowest increase in RSI (32.0 and 33.0%), followed by Volkamer lemon (33.0 and 33.2%) in leaves and roots, respectively. The accumulation of salt ions within the cell could potentially be responsible for disrupting osmotic equilibrium, leading to a reduction in water absorption and movement. The results of the present research align with results reported by Khoshbakht *et al.* (2014), who observed the lowest electrolyte leakage (35.9%) in Sour orange, while Trifoliate orange exhibited the highest electrolyte leakage (66.2%) under 75 mM NaCl salt stress.

Lipid peroxidation in terms of malondialdehyde (MDA) content: The results of present experiment in regarding MDA content in both leaves and roots (Fig. 2) revealed an increment with increasing salinity levels (control to 7.0 dS/m). The minimum leaf and root MDA content (8.46 and 8.12 $\mu\text{moles/g DW}$) was exhibited in Rangpur lime, following closely by Volkamer lemon with MDA content (8.75 and 8.41 $\mu\text{moles/g DW}$), while MDA content (15.76 and 15.42 $\mu\text{moles/g DW}$) was found highest in Pectinifera at 7 dS/m salinity level, respectively. Among rootstocks, leaf (75.6%) and root (80.9%) of Pectinifera, followed by leaf (66.2%) and root (71.3%) of Alemow was observed comparatively more responsive to salt concentrations over control in terms of MDA content, respectively as compared with the others, however, leaf MDA content in Rangpur lime (44.4%), Volkamer lemon (45.3%) and CRH-12 (46.7%) and root MDA content Rangpur lime (50%), Volkamer lemon (50.9%) and CRH-12 (52.1%) were relatively least responsive to increased salt concentration over control. The discoveries of the current investigation are in concurrence with those of Perez-Tornero *et al.* (2009) and Montoliu *et al.* (2009) who noted an increase in malondialdehyde (MDA) contents after saline treatment in explants of *C. macrophylla* and *in vitro* grown citrus, which leads to more salt tolerance.

After considering the entire study, it was observed that Rangpur lime, Volkamer lemon and CRH-12, displayed superior performance in terms of higher accumulation of total soluble carbohydrates and lesser reduction in chlorophyll stability index, relative water content, relative stress injury and lipid peroxidation in terms of malondialdehyde (MDA) content at 7 dS/m in compared to the control. On the other hand, Cleopatra mandarin, Rough lemon and NRCC-3, exhibited a moderate response to salinity stress. In contrast, Pectinifera, Alemow and NRCC-4 were considered substandard as they demonstrated contradictory behaviour at 7 dS/m in compared to the control in relation to the physiological parameters at the seedling stage. This study reveals numerous avenues for future research. These

efforts aim to enhance the resilience of citrus cultivation, enabling it to thrive under salinity stress and ensuring long-term productivity and profitability in the face of evolving environmental conditions.

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