***In vitro* selection for salt tolerance, and characterization of fifteen citrus rootstock**

**Ait El Aouad Bouchra1,2, Aderdour Tarik1,2, Chetto Ouiam1, Talha Abdelhak1, Rachid Benkirane2 and Hamid Benyahia1**

1. Department of Plant Breeding and Germplasm Conservation, National Institute for Agricultural Research (INRA), Kenitra 14000,Morocco

2. Department of Biology, Faculty of Science, Ibn Tofail University, Kenitra 242, Morocco

**Abstract**

In Morocco, citrus is an important fruit crop, however salt stress frequently limits its development. In order to understand the physiological response of fifteen citrus rootstocks to salt stress, this study evaluated the in vitro performance of the rootstocks at different salinity concentrations over two months. To determine their salt sensitivity, several growth and physiological parameters were evaluated. These included proline content, sugar content and chloride content. The results apparently show that the most salt sensitive genotypes accumulated high concentrations of chloride Cl− and a decrease in growth rate. Conversely, salt-tolerant genotypes accumulated less chloride Cl− and maintained a fair growth rate decreased their growth. For that Carrizo citrange 28608 **(**F7) and Troyer citrange (Morocco) (F33) were the most sensitive species, suggesting that these rootstocks are less tolerant to salt stress, while Citrumelo Winter Haven B2 (F1), Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9) were the most tolerant species, indicating that they can withstand higher levels of salt stress while maintaining their growth. Salt-tolerant rootstocks accumulated proline and soluble sugars as part of their adaptive response to salinity. In contrast, salt-sensitive rootstocks did not show the same level of accumulation. Overall, this study provides valuable insights into the salt stress tolerance of different citrus rootstocks, which can be important for citrus cultivation in regions like Morocco where salt stress is a common issue. Identifying and utilizing salt-tolerant rootstocks can help improve citrus production in such challenging conditions.

**Key words:** Salt tolerance,*In vitro,* Citrus, Rootstocks, NaCl, Salt stress.

1. **Introduction**

Salinity is indeed a significant abiotic stress that can have a detrimental impact on crop productivity and quality, and it's a particularly pressing issue in arid and semi-arid regions like Morocco, where citrus production is important. Nearly 35 % of Morocco's irrigated land is thought to be damaged by salt (Benyahia *et al*., 2007). In arid and semi-arid regions where citrus is produced, salinity is currently rising alarmingly. In fact, a study on irrigation water in a Moroccan region that grows citrus fruits was conducted (Benyahia *et al*., 1998). The investigation found that the irrigation water in these Moroccan citrus-growing regions is relatively salty. Salinity in irrigation water is a critical factor as it can directly affect the salt content in the soil and, consequently, the growth and health of citrus crops. Additionally, the salinity levels in the irrigation water typically ranged from 1.5 to 2 grams per liter (g/L). This indicates a moderate to high salinity level, and such water can lead to soil salinization over time if not managed properly. (Benyahia *et al*., 1998, 2007).

Citrus fruit is indeed one of the most important horticultural crops produced worldwide, and it plays a significant role in the agriculture and economy of many countries. However, this important crop is widespread in arid and semi-arid areas, where increased salinity is one of the major constraints on crop productivity. Salinity stress, caused by elevated levels of salt in the soil or irrigation water, can adversely affect citrus production. It can lead to reduced fruit yield and quality, and in severe cases, it may even result in crop failure. The need to produce plants with increased salt tolerance has been greatly accentuated by increased crop research (Munns *et al*., 2002; Flowers., 2004). Salt stress is a major environmental factor that can severely limit agricultural production. It can negatively impact plant growth and crop yields. The studies and research such as those by Zhu (2006), Guo et al. (2018), and Min et al. (2018), contribute to our understanding of salt stress and the mechanisms plants employ to respond to it. This knowledge is essential for developing strategies to mitigate the impact of salt stress on agriculture and ensure food security in regions affected by salinity. Due to regulated environmental conditions and predefined culture media, the effects of salt stress on plants could be studied using plant tissue culture techniques (Thorpe and Harry., 1997). In vitro screening studies for salinity tolerance in citrus becomes imperative as it is one of the most important commercial fruit crops. In vitro culture has taken on increased importance in plant breeding programs for selection of salinity tolerant genotypes (Fathi and Prat., 1989). This approach is a quick and early test to assess and evaluate the behavior of plant species under saline stress Plant tissue culture techniques provide a controlled environment and predefined culture media for the examination of salt stress effects on plants, as indicated by Thorpe and Harry in 1997. In the case of citrus, a pivotal commercial fruit crop, there is a compelling need for in vitro screening studies to assess salinity tolerance. In plant breeding programs, the significance of in vitro culture has grown substantially for the selection of salt-tolerant genotypes, as highlighted by Fathi and Prat in 1989. This method serves as a swift and early evaluation of how plant species respond to saline stress (Pourrat and Dutuit., 1994). Sodium chloride (NaCl) represent the major component of salt in irrigation water and soil solutions (Turkan and Demiral., 2009). Extensive research has been carried out on the utilization of in vitro techniques for genotype screening, adaptation, and selection in the face of various abiotic stresses. These studies have demonstrated the practicality of using in vitro methods for assessing salt tolerance in numerous fruit species, which includes pomegranate cultivars, citrus species, rootstocks of sweet cherry, and pistachio (El-Agamy *et al*., 2010; Chelli-Chaabouni *et al*., 2010).

The salinity of the soil can significantly affect citrus cultivation. Irrigation with saline water can be detrimental to citrus growth, making it crucial to select genotypes with greater tolerance potential (Brito *et al*., 2014). Consequently, in order to expand citrus agriculture in regions of Morocco affected by salt, it becomes imperative to develop resistant genotypes. As a result, citrus research programs are currently giving high priority to the discovery of new sources of salt tolerance, as noted in studies by Ollitrault et al. in 2000, Benyahia et al. in 2007, and Benyahia et al. in 2011. Tolerance to salinity varies among species, genotypes and even with crop development stages (Ayers and Westcot., 1985). This tolerance can vary among different factors, including species, genotypes, and crop development stages. This means that not all plants or crops respond to salt stress in the same way. Salt stress can cause growth reduction due to induced drought, specific ion toxicity, ionic imbalance or a combination of these factors (Hussain *et al*., 2012; Syvertsen and Garciasanchez., 2014; Hussain *et al*., 2015). In other words, salt stress can harm plants through different mechanisms. The tolerance level of different citrus species can be determined by their ability to exclude potentially toxic ions, specifically sodium (Na+) and chloride (Cl-) ions (Khoshbakht *et al*., [2015](https://www.tandfonline.com/doi/full/10.1080/15538362.2019.1674762); Moya et *al*., [2003](https://www.tandfonline.com/doi/full/10.1080/15538362.2019.1674762)). This suggests that some citrus species have mechanisms to prevent the uptake of these harmful ions when they are exposed to salinity. To classify the tolerance of citrus rootstocks to salinity, many criteria have been used over several decades based on physiological and biochemical parameters that have provided rapid tests to diagnose the varietal salt-tolerance (Ait El Aouad *et al*., 2015; Sykes., 2011; Fernández-Ballester *et al*., 2003). These criteria include the analysis of various factors, such as growth, nutrition, and mineral uptake, with a focus on the accumulation of sodium (Na+) and chloride (Cl-) ions, as well as the accumulation of proline and sugars. These parameters help in determining the level of salt tolerance in citrus rootstocks. The study mentioned in this work is centered around the use of in vitro culture techniques to evaluate the degree of salt sensitivity in fifteen different citrus rootstocks. This suggests that the researchers are using controlled laboratory conditions to assess how these rootstocks respond to salt stress, which can provide valuable insights into their salt tolerance.

1. **Materials and methods**

This study was carried out in El Menzeh Experimental Station of the National Institute for Agricultural Research in Kenitra (Morocco).

**2.1 Plant Materials:** Fifteen rootstocks were used throughout this study. Seeds were extracted from mature healthy fruits of rootstocks (Table 1), which are grown in the orchard of the experimental fields of the institute.

**2.2 Culture Conditions:** Seeds for each rootstock were subjected to continuous flow of water for about 90, in order to remove viscous materials covering the seeds were sterilized by using 50 % sodium hypochlorite (NaOCl) solution for 30 min. and rinsed three times (10 min for each rinse) in sterile distilled water. By using a scalpel (blade no. 11) and forceps to removed outer and inner off seeds on sterile filter paper. The cotyledons (seeds after removing the seed coat) were cultured.

**2.3 Callus establishment:** Callus was induced from embryos (one embryo is taken from each seed) on Murashige and Skoog (1962) medium supplemented with 30 g.L-1 sucrose, enriched with 1 mg / l of 2,4-D auxin (2,4-dichlorophenoxy acetic acid) combined with 0,5 mg / L of cytokinin BAP (benzylaminopurine) to induce callogenesis.

**2.4 Callus induction:** Embryo excision is performed under sterile conditions. Seeds were surface sterilised with 70 % ethanol for 10 s, 0.2 % HgCl for 10 min, and rinsed five times in sterile distilled water then the excised embryos were inoculated in petri dishes containing cultures sealed with Lab film (Para film) were incubated under darkness at 25 ± 2°C and subcultured after four weeks. The embryos were cultured by four in Petri dishes containing 20 mL of nutrient medium at a rate of more than ten boxes per condition. The boxes were sealed with two rounds of parafilm and incubated four weeks in the dark in a ventilated oven set at 30 ° C. The callogenic explants obtained were then subcultured with all of their callus three times for four weeks on the same medium.

**2.5 Application of saline treatment:** Friable calluses with an initial mass of 0.5 g are transferred to the saline media. The saline medium that used for *in vitro* evaluation experiments consisted of Murashige and Skoog (MS) salts and vitamins at full strength plus different salt concentrations of sodium chloride (NaCl) at 0, 2 and 5 g/L. The medium solidification was achieved by 8 g/L Agar, the pH of different concentrations was adjusted to 5.7 using KOH and HCl before autoclaving. The media were autoclaved at 121°c for 20 min, then left to cool and stored at 25 °C ±2 for 2 days before being used. Twenty explants were used for each treatment and visual observations were taken every three days.

**2.6 Growth measurement:** The response of the calli to salt stress was studied in terms of relative growth rate (RGR). The experiments were performed with three replicates.

**RGR= (final fresh weight−initial fresh weight)/initial fresh weight.**

Determination of tissue water content: The fresh weight (FW) of the calli was determined immediately after removal from the medium and blotted with tissue paper to remove excess water. Dry weight (DW) was recorded after drying the calli at 60 °C in the hot air oven for

48 h.

The percent tissue water content of the calli was determined as follows:

**TWC (%) =[(FW−DW)/FW] ×100.**

**2.7 Biochemical Analysis:** After eight weeks of treatment, chloride was extracted from dry callus using hot water and determined by titration according to the method of Cotlove. (1965). Proline was determined according to the method of Monneveux and Nemmar. (1986). The absorbance was then measured at 528 nm and obtained values were expressed in μmol. g-1 DW using the following equation: Y = 0,1043 × X. Where X is the optical density. For sugar content, we used the method of Dubois *et al*. (1956). The optical density is measured at 585 nm. The obtained values were expressed in mg. g-1 DW using the equation of the standard curve: Y= 4,3918 × (X – 0,194). Where Y refer to total soluble sugar content and X refer to the absorbance.

**2.8 Statistical analyzes:** The “Statistical Analysis System” program (SAS, 1988) was used to perform all analyzes of the variance, the comparison of means between the control sample and the treated sample for each parameter analyzed. Means were compared using the Duncan multiple range test for p ≤ 0.05.

**Table 1.** List of the citrus rootstock genotypes used in the experiment

|  |  |  |
| --- | --- | --- |
| **Rootstocks** | **Code (INRA)** | **Origine** |
| **Citrumelo 57-98-502** | 1 | CRC Riverside |
| **Swingle citrumelo F9-22-55 (80-11)** | 2 | CRC Riverside |
| **Citrumelo 57-98-506** | 3 | CRC Riverside |
| **Swingle citrumelo 74-1** | 4 | CRC Riverside |
| **Citrumelo Winter Haven B2** | F1 | SRA INRA/Cirad Corse |
| **Carrizo citrange 28608** | F7 | SRA INRA/Cirad Corse |
| **Troyer citrange C35B6A11** | F8 | SRA INRA/Cirad Corse |
| **Troyer citrange B2 31655** | F9 | SRA INRA/Cirad Corse |
| **Citrumelo 4475 B2G3** | F11 | SRA INRA/Cirad Corse |
| **Citrumelo 4475 B B6A5** | F12 | SRA INRA/Cirad Corse |
| **Citrumelo 4475 A B6A4** | F13 | SRA INRA/Cirad Corse |
| **Sacaton citrumelo 30057** | F14 | SRA INRA/Cirad Corse |
| **Gou-Tou SRA 506** | F23 | SRA INRA/Cirad Corse |
| **Volkamer lemon B2 28613** | F25 | SRA INRA/Cirad Corse |
| **Troyer citrange (Morocco)** | F33 | INRA Morocco |

1. **Results**

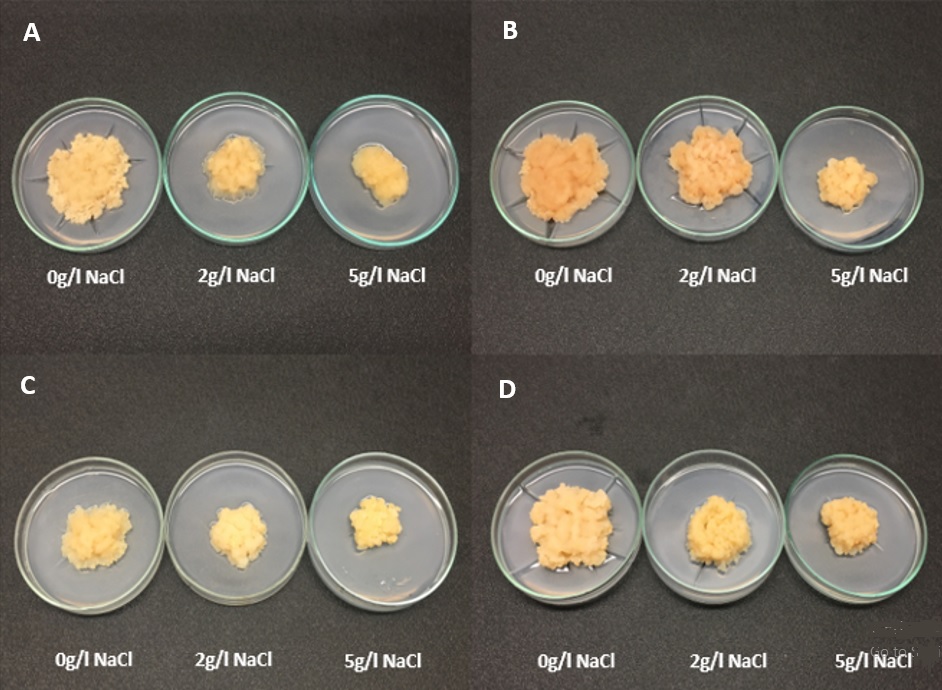
**3.1 Effect of NaCl on growth parameters:** Salt stress caused a significant reduction in all the growth parameters considered (Table 2 and 3). These parameters include fresh weight, dry weight, growth rate, and water content. The extent of reduction in these growth parameters was greater at higher NaCl concentrations, particularly at 2 and 5 g/L NaCl. This indicates a dose-dependent response to salt stress, where higher salt concentrations have a more pronounced negative impact on growth. Both fresh weight and dry weight of the citrus rootstocks decreased gradually with an increase in NaCl concentration (Table 2). The reduction was more pronounced at 5 g/L NaClin all the citrus rootstocks. The growth rate and water content were also affected by NaCl treatment, with a greater reduction as NaCl concentration was increased in all the rootstocks studied. Different citrus genotypes exhibited varying responses to salt stress. Maximum reduction in fresh and dry weights of callus was observed in genotype Carrizo citrange 28608 **(**F7), Troyer citrange B2 31655 (F9) and Troyer citrange (Morocco)(F33). While the genotypes Citrumelo 57-98-506 (3), Citrumelo Winter Haven B2 (F1) and Sacaton citrumelo 30057 (F14) were more tolerant and exhibited the minimum reduction in this regard (Table 2). In the presence of salt (NaCl) stress, a decreasing trend in RGR was observed as the NaCl concentration increased (Table 3). Different citrus genotypes displayed varying responses to salt stress. Compared to the control, Citrumelo Winter Haven B2 (F1) and Sacaton citrumelo 30057 (F14) callus showed an increase in growth at 2 g/L NaCl, In contrast, most other citrus genotypes experienced growth inhibition by nearly 50% at this concentration. Whereas at higher concentrations (5 g/L NaCl), the callus growth was significantly reduced, with nearly an 85% decrease observed for Carrizo citrange 28608 (F7), Troyer citrange B2 31655 (F9), and Troyer citrange (Morocco) (F33). This indicates that these genotypes were particularly sensitive to high salt concentrations. Interestingly, for Troyer citrange C35B6A11 (F8) and Troyer citrange B2 31655 (F9), there was only a small decrease in callus growth even at the highest NaCl concentration. These genotypes appeared to exhibit some degree of tolerance to salt stress. The percent tissue water content of salt-treated calli decreased significantly as the salt concentration increased compared to control calli(Table 3). This suggests that salt stress not only affects growth but also influences the water balance within the callus tissue.

**3.2 Effect of Salinity Stress on chloride Accumulation:** The application ofsalt treatment resulted in an increase in chloride concentration in the callus for all fifteen tested genotypes. This indicates that salt stress caused the accumulation of chloride ions in the plant tissue. (Table 4). Although salt treatment increased chloride concentration in all genotypes, there were differences in the maximum levels of chloride reached. Some genotypes accumulated higher levels of chloride than others. The genotypes Carrizo citrange 28608 **(**F7), Swingle citrumelo F9-22-55 (80-11) (2) and Swingle citrumelo 74-1 (4) accumulated the highest levels of chloride, especially at the higher salinity level 5 g/L NaCl followed by Citrumelo 57-98-502 (1), Citrumelo 4475 A B6A4 (F13) and Troyer citrange (Morocco)(F33). In contrast, some genotypes, including Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9), Citrumelo Winter Haven B2 (F1), and Volkamer lemon B2 28613 (F25), recorded the lowest chloride contents in their callus even under salt stress conditions. Under normal, non-stressed conditions (control), all the genotypes showed low levels of chloride content in their callus.

**3.3 Effect of Salinity Stress on Proline Accumulation:** Salinity stress led to an increase in proline levels in all tested citrus rootstocks (Table 4). This suggests that proline plays a role in osmotic adjustment in response to salt stress in citrus callus. The maximum proline accumulation was observed at the highest salinity level of 5 g/L NaCl, followed by 2 g/L NaCl. This indicates that proline accumulation is positively correlated with the severity of salt stress. Different citrus rootstocks showed varying degrees of proline accumulation in response to salt stress. Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9), Citrumelo 4475 B B6A5 (F12), and Citrumelo 4475 A B6A4 (F13) exhibited the highest proline accumulation, especially under the high 5 g/L NaCl concentration. Whereas Carrizo citrange 28608 (F7) showed the lowest proline accumulation, particularly at the 5 g/L NaCl concentration as shown in Table (4). The interaction between the rootstock and the salt treatment was found to be significant (p < 0.05), indicating that different rootstocks responded differently to salt stress in terms of proline accumulation. Citrumelo 57-98-506 (3) had the highest proline content under controlled conditions (no salt stress), indicating its ability to maintain higher proline levels even without stress. In contrast, Citrumelo Winter Haven B2 (F1) showed the lowest proline accumulation under controlled conditions. Thus, Citrumelo 4475 B2G3 (F11) was identified as the best performer due to its high proline accumulation, especially under salt stress conditions. On the other hand, Sacaton citrumelo 30057 (F14) was considered the poorest performer because it had the lowest proline accumulation under 2 g/L NaCl salinity level.

* 1. **Effect of Salinity Stress on soluble sugar Accumulation:** Salinity stress led to an increase

in the accumulation of total soluble sugars in all tested citrus rootstocks (Table 4). This suggests that the presence of salt stress prompts citrus plants to produce more soluble sugars. The maximum concentration of total soluble sugars was observed under the highest salinity treatment (5 g/L NaCl) compared to control conditions. This indicates that the severity of the salt stress positively correlates with the level of soluble sugar accumulation (Table 4). There were significant differences among the citrus rootstocks in terms of total soluble sugar accumulation. This suggests that different rootstocks respond differently to salt stress in relation to sugar production. The rootstock × treatment interaction was found to be significant (*p* < 0.05), indicating that different rootstocks had distinct responses to salt stress with regard to soluble sugar accumulation. Among the citrus rootstocks, Citrumelo Winter Haven B2 (F1), Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9), Sacaton citrumelo 30057 (F14), and Gou-Tou SRA 506 (F23) exhibited the highest accumulation of total soluble sugars under salt stress conditions. On the other hand, Citrumelo 57-98-502 (1), Citrumelo 57-98-506 (3), Swingle citrumelo 74-1 (4), and Troyer citrange (Morocco) (F33) had the lowest levels of total soluble sugars. Some rootstocks, like Swingle citrumelo F9-22-55 (80-11) (2), showed the lowest quantities of total soluble sugars under control conditions, while Citrumelo 57-98-506 (3) had the highest levels of soluble sugars without salt stress. Under the lower salt stress condition of 2 g/L NaCl, Gou-Tou SRA 506 (F23) exhibited the highest total soluble sugar content, while Citrumelo 4475 A B6A4 (F13) had the lowest content of soluble sugars.



**D**

**C**

**B**

**A**

**Figure 1: A: Carrizo citrange 28608 (F7); B: Troyer citrange (Morocco) (F33); C: Troyer citrange C35B6A11 (F8); D: Troyer citrange B2 31655 (F9**

**2g/l NaCl**

**Table 2: Effect of salt stress on fresh and dry weight**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Rootstock | Frech weight (mg) | | | Dry weight (mg) | | |
|  | **0 g/L NaCl** | **2 g/L NaCl** | **5 g/L NaCl** | **0 g/L NaCl** | **2 g/L NaCl** | **5 g/L NaCl** |
| 1 | 4216 a | 2187 c | 1521 e | 201 a | 129 b | 105 c |
| 2 | 3873 a | 2405 c | 2197 cde | 224 a | 135 b | 116 bc |
| 3 | 2713 a | 2200 c | 1784 de | 130 a | 168 b | 121 c |
| 4 | 4467 a | 3825 bc | 2466 bcde | 203 a | 162 b | 137 bc |
| F1 | 3272 a | 3175 bc | 2007 cde | 199 a | 178 ab | 164 bc |
| F7 | 8145 a | 5127 b | 2558 bcde | 391 a | 219 ab | 172 bc |
| F8 | 3776 a | 2614 bc | 1655 de | 163 a | 137 b | 138 bc |
| F9 | 9846 a | 8058.9 a | 6916 a | 385 a | 339 a | 289 a |
| F11 | 7111 a | 4486 bc | 3040 bcd | 360 a | 232 ab | 161 bc |
| F12 | 6123 a | 3624 bc | 3617 b | 309 a | 237 ab | 199 b |
| F13 | 6031 a | 3809 bc | 2268 bcde | 348 a | 220 ab | 201 b |
| F14 | 2493 a | 3199 bc | 1919 cde | 172 a | 125 b | 116 c |
| F23 | 6145 a | 4227 bc | 3307 bc | 311 a | 219 ab | 172 bc |
| F25 | 4873 a | 2905 bc | 2246 bcde | 224 a | 179 ab | 135 bc |
| F33 | 8846 a | 5059 b | 2216 bcde | 445 a | 222 ab | 160 bc |

*\*Means followed by the same letter in the same rows do not differ significantly at P≤0,05 (0ne-way-ANOVA, separated by Duncun test).*

**Table 3: Effect of salt stress on relative growth rate and water content**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Rootstock | Growth rate | | | Water content | | |
|  | **0 g/L NaCl** | **2 g/L NaCl** | **5 g/L NaCl** | **0 g/L NaCl** | **2 g/L NaCl** | **5 g/L NaCl** |
| 1 | 7.42 i | 3.37 o | 2.04 o | 95.22 g | 94.14 l | 93.09 j |
| 2 | 6.75 j | 3.81 m | 3.39 j | 94.22 m | 95.17 e | 93.85 g |
| 3 | 4.42 m | 3.39 n | 2.57 m | 95.24 f | 92.36 o | 93.21 i |
| 4 | 7.93 h | 6.65 g | 3.93 f | 95.45 d | 95.76 b | 94.44 d |
| F1 | 5.54 l | 5.35 j | 3.01 k | 93.92 n | 94.42 j | 91.88 l |
| F7 | 15.29 b | 9.25 b | 4.11 e | 96.79 a | 95.73 c | 93.31 h |
| F8 | 6.55 k | 4.23 l | 2.31 n | 95.68 c | 94.76 i | 91.66 n |
| F9 | 16.69 a | 15.12 a | 12.83 a | 96.09 b | 94.80 h | 91.70 m |
| F11 | 13.22 c | 7.97 e | 5.08 d | 94.50 k | 94.83 f | 94.71 b |
| F12 | 11.24 e | 9.25 c | 9.23 b | 94.95 i | 93.46 n | 94.52 c |
| F13 | 11.06 f | 6.62 h | 3.54 g | 94.23 l | 94.25 k | 91.14 o |
| F14 | 3.99 n | 5.39 i | 2.84 l | 93.10 o | 96.09 a | 93.95 f |
| F23 | 11.29 d | 7.45 f | 5.61 c | 94.94 j | 94.82 g | 94.83 a |
| F25 | 8.75 g | 4.81 k | 3.49 h | 95.40 e | 93.82 m | 93.99 e |
| F33 | 16.59 a | 9.12 d | 3.43 i | 94.97 h | 95.63 d | 92.82 k |
| *\*Means followed by the same letter in the same rows do not differ significantly at P≤0,05 (0ne-way-ANOVA, separated by Duncun test).* | | | | | | |

**Table 4: Effect of salt stress on chloride, proline and soluble sugar content**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Rootstock | Chloride g/L | | | Proline | | | Soluble sugar | | |
|  | **0 g/L NaCl** | **2 g/L NaCl** | **0 g/L NaCl** | **0 g/L NaCl** | **0 g/L NaCl** | **5 g/L NaCl** | **0 g/L NaCl** | **2 g/L NaCl** | **5 g/L NaCl** |
| 1 | 0,001 j | 0,001 k | 0,006 c | 0,044 f | 0,079 gf | 0,169 e | 0,092 b | 0,080 c | 0,081 f |
| 2 | 0,002 e | 0,003 e | 0,007 b | 0,037 g | 0,037 k | 0,221 a | 0,047 l | 0,061 i | 0,046 l |
| 3 | 0,003 b | 0,004 c | 0,005g | 0,120 a | 0,139 c | 0,195 cd | 0,102 a | 0,082 b | 0,083 e |
| 4 | 0,003 d | 0,002 j | 0,007 b | 0,048 f | 0,205 a | 0,219 ab | 0,083 c | 0,063 h | 0,064 h |
| F1 | 0,001 ij | 0,002 j | 0,003 l | 0,110 b | 0,127 d | 0,166 e | 0,052 j | 0,063 h | 0,096 bc |
| F7 | 0,002 f | 0,005 gh | 0,007 k | 0,055 f | 0,082 b | 0,114 gh | 0,055 d | 0,078 g | 0,080 ij |
| F8 | 0,004hij | 0,004 h | 0,005 h | 0,069 g | 0,119 i | 0,185 ab | 0,051 e | 0,071 k | 0,088 hi |
| F9 | 0,003 ij | 0,003 i | 0,004 e | 0,047 c | 0,072 gf | 0,217 a | 0,060 e | 0,069 l | 0,095 j |
| F11 | 0,002 h | 0,002 f | 0,004 j | 0,047 h | 0,171 l | 0,104 gf | 0,074 g | 0,066 e | 0,061b |
| F12 | 0,001 k | 0,002 g | 0,005 f | 0,035 i | 0,068 k | 0,219 h | 0,072 c | 0,050 a | 0,062 a |
| F13 | 0,001 b | 0,002 c | 0,006 k | 0,090 i | 0,076 j | 0,224 f | 0,070 k | 0,048 ih | 0,060 g |
| F14 | 0,001 hi | 0,003 i | 0,004 d | 0,020 g | 0,019 h | 0,116 bc | 0,062 f | 0,072 j | 0,097 k |
| F23 | 0,001 g | 0,003 a | 0,006 a | 0,012 e | 0,034 f | 0,090 gf | 0,081 i | 0,087 d | 0,106 f |
| F25 | 0,003 a | 0,004 b | 0,004 g | 0,010 d | 0,058 e | 0,125 d | 0,050 j | 0,062 e | 0,073 d |
| F33 | 0,001 c | 0,002 d | 0,006 i | 0,034 f | 0,075 hi | 0,205 ab | 0,066 h | 0,053 f | 0,052 c |
| *\*Means followed by the same letter in the same rows do not differ significantly at P≤0,05 (0ne-way-ANOVA, separated by Duncun test).* | | | | | | |  |  |  |

1. **Discussion**

Soil salinity can have various detrimental effects on plants. Depending on the degree and duration of the stress, they often respond to salt stress using different mechanisms. Plant development is initially affected by soil salinity through osmotic stress firstly (hyperosmotic stress) (Munns *et al*., 2006), This is the initial impact of soil salinity on plants. When the soil has a high salt concentration, it creates an osmotic potential that makes it difficult for plants to take up water. This causes dehydration and reduced turgor pressure in plant cells. To cope with this, plants may accumulate organic solutes (osmoprotectants) such as proline and soluble sugars to maintain water uptake and turgor pressure. Then by ion toxicity (hyperionic stress) (Munns and Tester., 2008; Ali et al., 2017). As salinity persists, sodium (Na+) and chloride (Cl-) ions can accumulate in plant tissues, causing toxicity. High levels of sodium can replace essential potassium (K+) and calcium (Ca2+) ions, leading to disruptions in various cellular processes. To counter this, plants have developed ion exclusion mechanisms, such as selectively absorbing and excluding specific ions from the roots, and ion compartmentalization to minimize the damage caused by excess ions. Finally interfere with soil nutrient balance (Yan *et al*., 2006) affecting plant growth and physiological functions. High salt concentrations can disrupt the balance of essential nutrients in the soil, particularly by reducing the availability of important ions like calcium, magnesium, and potassium. This can lead to nutrient imbalances in plants, affecting their growth and physiological functions. To mitigate this, plants may enhance their nutrient uptake mechanisms, but in severe cases, nutrient deficiencies can still occur. In addition to these primary responses to salt stress, plants also trigger various secondary adaptive mechanisms to enhance their salt tolerance, such as the synthesis of antioxidants to combat oxidative stress caused by salinity, changes in the root system architecture to explore deeper soil layers, and the activation of stress-related genes.Plant responses to salt stress can vary depending on the species, the duration of exposure, and the severity of the stress. Plant breeders and researchers are actively working on developing salt-tolerant crop varieties and improving our understanding of these mechanisms to help mitigate the negative effects of soil salinity on agriculture.

Significant differences among the tested rootstocks in response to salinity were noted. Salinity through osmotic and ionic stresses exerts negative effect on growth, development and plant metabolic machinery (Munns and Tester., 2008). The differences in stress tolerance among different varieties can be difficult to evaluate in the field where plants are exposed to both biological and climatic variable conditions (Tal., 1993). Therefore, the *in vitro* culture methodology may be preferred for carrying out a quick comparison of citrus rootstock with regards to their salinity tolerance, under controlled conditions. In vitro cultures allow precise control over environmental conditions, including temperature, humidity, light, and nutrient availability. This control minimizes the influence of external factors and ensures that the observed effects are primarily due to salinity stress. However, it's important to note that while in vitro culture provides valuable insights into the relative salt tolerance of different plant varieties, it may not fully replicate the complexity of real-world field conditions. Therefore, findings from in vitro studies should be validated in field trials to ensure that the results are applicable to practical agricultural settings. Additionally, the genetic and physiological traits observed in vitro should be considered in the context of the target environment and management practices for the specific crop, as interactions with field conditions can affect salt tolerance.

Studies performed on other species, under saline conditions, have revealed that growth inhibition in plants may be attributed to nutrient uptake alteration (Chelli-Chaabouni *et al*., 2010; Sibole *et al*., 2003; Mohammadi *et al*., 2008). Nutrient Uptake Alteration: High salt levels in the soil can disrupt the normal uptake of essential nutrients by plant roots. Sodium ions, which are abundant in saline soils, can compete with other essential nutrients like potassium (K+), calcium (Ca2+), and magnesium (Mg2+) for uptake by plant roots. This competition can lead to imbalances in nutrient availability, affecting the overall growth and health of the plants. When essential nutrient uptake is compromised, it can lead to nutrient deficiencies and hinder various metabolic processes necessary for plant growth. This inhibition is may be due to the accumulation of toxic sodium ions (Na+) and chloride ions (Cl-) within plant cells (Sannazzaro *et al*., 2007), toxic Sodium and Chloride Accumulation: Excess sodium and chloride ions can be toxic to plant cells. When plants take up excessive amounts of these ions, they can accumulate in plant tissues and disrupt cellular processes. The accumulation of sodium and chloride ions can lead to osmotic stress, which causes water to move out of plant cells, leading to cell dehydration. Additionally, these ions can interfere with various biochemical reactions, ultimately impairing plant growth and development. The results of your study indicate that salt-treated callus cultures showed a significant decrease in both fresh and dry weight compared to the control group. The most significant reduction in fresh weight was observed at a high concentration of sodium chloride (NaCl), particularly at 5 g/L NaCl. This reduction in weight is probably due to osmotic effects and ion cytotoxicity, both of which are associated with high salt levels. The significant decrease in weight at the high NaCl concentration (5 g/L) suggests that this particular concentration had a more pronounced negative impact on the callus cultures. It's important to consider that different plant species and even different cell lines within the same species can vary in their tolerance to salt stress. To mitigate the negative effects of salt stress on callus cultures, strategies such as optimizing the culture medium composition, adjusting the salt concentration, or using osmoprotectants can be employed. These approaches aim to reduce the osmotic stress and ion cytotoxicity while maintaining the viability and growth of the cultured cells. The observations in our work are consistent with the well-established effects of salt stress on plant tissues and callus cultures. The observed decline in callus growth can likely be attributed to two main factors: the first is the cellular dehydration resulting from a lowered water potential, Callus growth, which is a mass of undifferentiated plant cells, requires a suitable water supply for its development. If the water potential in the growth medium is too low, it can lead to dehydration of the callus cells. Water potential is a measure of the energy status of water in a system, and when its low, water tends to move out of cells, potentially leading to cell damage or inhibition of growth. The second one is an imbalance in essential nutrients caused by the disruptive influence of saline ions. Saline ions, like sodium and chloride ions, can interfere with the uptake of essential nutrients by plant cells. When the concentration of these ions is too high, it can disrupt the balance of nutrient uptake, leading to a nutritional imbalance. This can negatively affect the growth and development of callus tissue, as essential nutrients are crucial for cell division, differentiation, and overall growth. This phenomenon has been previously documented by Farooq and Azam (2006).

The present study observed a reduction in both callus growth and water content in calli subjected to salt stress when compared to control calli (Table 3). This implies that salt stress had a negative impact on the growth and water status of the callus tissue. It's interesting to note that the observed reduction in callus growth under salt stress in our study is consistent with findings from other research. Watad *et al*. (1991) and Zhao *et al*. (2009) likely discuss similar effects in different plant species, indicating that salt stress can indeed have a detrimental impact on plant growth, including callus cultures and also that the negative effects of salt stress on plant growth and development are not limited to the specific plant species studied in our research but are a broader trend observed across different plants. The correlation between the reduction in tissue water content and increased cell membrane permeability under salt stress, as discussed in the work by Farooq and Azam (2006), provides a mechanistic explanation for the observed reduction in water content in salt-stressed plants. This membrane permeability increase likely allows the loss of water from plant cells, leading to cell dehydration and ultimately affecting plant growth negatively. These references and correlations with our research contribute to a more comprehensive understanding of the impact of salt stress on plants and highlight the importance of addressing this stress factor when working with various plant species, including in plant tissue culture and callus growth studies. Researchers can use this knowledge to develop strategies for mitigating salt stress and promoting healthier plant growth under adverse conditions. In our study, we observed a reduction in water content with increasing salt concentration, aligns with the concept that salt stress can indeed lead to cellular dehydration. This connection between salt concentration and reduced water content underscores the importance of managing salt stress when working with plant tissue culture to ensure optimal growth and development. Salt stress can have detrimental effects on plant cells, including dehydration, which can hinder growth and overall plant health. To address this issue, researchers and practitioners in plant tissue culture should consider various strategies to manage and mitigate salt stress, such as adjusting growth medium composition, using salt-tolerant plant lines, monitoring and watering practices and genetic engineering. These strategies can help ensure that plant tissue culture experiments yield healthy and robust callus cultures, even when dealing with the challenges posed by salt stress.

Previous studies on several species showed that the presence of NaCl in the growth medium caused a disturbance in membrane permeability which was expressed by an increase in solute leakage (Masood *et al*., 2006; Khayyat *et al*., 2009), The presence of NaCl in the growth medium has b een observed to disrupt the membrane permeability of plant cells. This disruption is typically indicated by an increase in solute leakage, which suggests that salt stress negatively affects the integrity of the cell membranes. This phenomenon is consistent with the idea that salt stress can lead to increased cell membrane permeability, as previously discussed. Thus, considering cell membrane stability of stressed tissues as an index of tolerance. The stability of cell membranes under salt stress is considered an important indicator of a plant's tolerance to such stress. This means that plants or plant tissues with more stable cell membranes are better equipped to withstand the adverse effects of salt stress. (Kaya *et al*., 2007; Lutts *et al*., 1996; Xue and Liu., 2007). In the context of our study, understanding how salt stress affects cell membrane stability and permeability is crucial for assessing the tolerance of callus cultures to such stress. It can also help researchers identify and select salt-tolerant plant lines or develop strategies to enhance tolerance, ultimately leading to improved plant growth and tissue culture outcomes under saline conditions.

Tolerance of citrus rootstocks to salinity has been related to the ability to restrict or, at least, reduce the uptake of chloride ions to the shoots which is an important factor contributing to their salt tolerance. Chloride ions are a component of salt, and their accumulation in plant tissues can have harmful effects, disrupting various physiological processes (Moya *et al*., 1999, 2002, 2003; Storey and Walker., 1999; Arbona *et al*., 2006; Garcia-Sanchez *et al*., 2006). is an important factor contributing to their salt tolerance. Chloride ions are a component of salt, and their accumulation in plant tissues can have harmful effects, disrupting various physiological processes. The results from our current research highlight the differences in chloride accumulation among different rootstock genotypes when subjected to salt stress. Specifically, the results indicate that the following rootstock genotypes, Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9), Citrumelo Winter Haven B2 (F1), and Volkamer lemon B2 28613 (F25), were less affected by chloride accumulation compared to other rootstocks (Table 4). This observation serves as a crucial physiological parameter that confirms the greater salt tolerance of these specific genotypes. It indicates that these genotypes have developed mechanisms to either restrict the uptake or enhance the exclusion of chloride ions when exposed to saline conditions. These rootstock genotypes may be considered more suitable for cultivation in salt-affected soils or environments, and they could be valuable for citrus production in regions where salinity is a concern. The ability to identify and select rootstock genotypes with enhanced salt tolerance is important for sustainable agriculture, as it can lead to improved crop yields and quality in salt-stressed conditions. It also demonstrates the practical significance of your research in contributing to the development of salt-tolerant citrus rootstocks.

Proline and soluble sugar are are known as osmoprotectants that play a significant role in osmotic adjustment, and thus the accumulation of proline and soluble sugars can enhance a plants tolerance to various abiotic stresses, including salinity (NaCl stress) allowing them to continue growing and developing even in adverse environments. This can lead to improved growth and overall performance of stressed plants. (Chen and Murata., 2002 ; Rontein *et al*., 2002 ; Nishizawa *et al*., 2008 ; Rosa *et al*., 2009 ; Hayat *et al*., 2012 ; Wang *et al*., 2013). To assess the relationship between changes in the contents of proline and soluble sugars and the salt (NaCl) stress tolerance of plantlets, were quantitatively assayed. This allows us to measure and analyze the levels of proline and soluble sugars in plant tissues under different salt stress conditions and correlate these levels with the plantlets ability to tolerate salt stress. Proline serves as an important molecule in osmoregulation, helping plants maintain water balance and osmotic potential in their cells. It also plays a crucial role in enhancing osmotolerance, allowing plants to cope with osmotic stress caused by factors like high salinity and drought (Rontein *et al*., 2002). The accumulation of proline is widely used as a selection criterion for assessing the salinity and drought tolerance of plants. In this context, measuring proline levels can provide valuable information about a plants ability to withstand and adapt to these challenging environmental conditions (Cui *et al*., 2010). These studies contribute to our understanding of the physiological and molecular processes that enable plants to thrive under adverse environmental conditions. In the present study, we have used the accumulation of proline as a biochemical marker to characterize the selected callus for its tolerance to salt stress (NaCl). The increase in proline levels in the calli exposed to NaCl serves as a valuable indicator of their response to salt stress. Notably, we observed that the increase in proline content was most pronounced in the calli selected at 2 g/L NaCl and at 5 g/L NaCl, as indicated in Table 4. This result suggests that the calli exposed to higher salt concentrations (2 g/L and 5 g/L NaCl) exhibited a more significant proline accumulation response to salt stress. Proline accumulation is a common plant response to osmotic stress, and the increase in its content indicates that the calli are actively trying to manage osmotic stress caused by salt exposure. This biochemical response is an essential component of salt tolerance in plants and is associated with their ability to maintain osmotic balance and protect cellular structures. The increase in proline levels in our study suggests that proline may play a significant role in salt tolerance and osmotic adjustment, protecting plants from salt-induced damages (Szabados and Savouré.,2010; Lehmann *et al*., 2010). The positive correlation between proline concentration and salt tolerance, as highlighted in the research of Hokmabadi et al. (2005), further supports the idea that proline accumulation is a critical factor in enhancing a plant's ability to tolerate salt stress. In our previous study (Ait El Aouad *et al*., 2015), it has been demonstrating that with increasing NaCl concentration, the proline content recorded in all rootstocks significantly increased, as many researchers have already reported (Amini and Ehsanpour., 2005; Mohamed *et al*., 2007; Chelli-Chaabouni *et al*., 2010; Torabi and Halim., 2010; Aghaleh and Niknam.,2009; Szabados and Savouré., 2010).

In this study for most of all the fifteen genotypes, proline content of calli increased in response to salt stress. Specifically, Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9), Citrumelo 4475 B B6A5 (F12) and Citrumelo 4475 A B6A4 (F13) showed the highest proline accumulation at the highest salt concentration 5 g/L of NaCl (Table 4). These results indicate that the increase in proline accumulation is a response to the elevated chloride levels in the callus cultures. This observation aligns with previous research of Khoshbakht et al. (2014), which reported a significant increase in proline content in the leaves of citrus rootstocks in response to increasing salt levels. Proline is considered an organic compound that can facilitate osmotic adjustment under salinity stress. It serves as a compatible solute to help maintain osmotic balance and protect cells from dehydration. Furthermore, proline can also serve as an energy sink, meaning it absorbs excess energy in the plant cells, helping to prevent damage from oxidative stress. Additionally, proline's role as an enzymatic regulator during stress conditions, as noted by Rontein et al. (2002), emphasizes its importance in the plant's response to challenging environmental conditions. our study results and those researches contribute proline's role in salt tolerance and its potential as a valuable indicator for assessing salt stress responses in plant callus cultures and citrus rootstocks.

Under stress conditions, such as salt stress, plants tend to accumulate total soluble sugars. This is a common response to various types of stress. (William *et al*., 2000; Murakeozy *et al*., 2003). There are considerable variations in the accumulation of soluble sugars in response to salt stress not only between different plant species (inter-specific) but also within the same species (intra-specific). This variation suggests that different plants or even different lines within a plant species may have varying responses to salt stress (Thanaa and Nawar.,1994). Lower osmotic potential of plant cells can be beneficial for plants when dealing with saline substrates. Osmotic potential refers to the pressure required to prevent the flow of water across a semipermeable membrane, and a lower osmotic potential means that plant cells can absorb water and saline substrates more effectively. Hence, the increased concentration of reducing and total sugars in response to salinity stress could be seen as a form of osmotic adjustment. In other words, plants may accumulate more sugars to lower the osmotic potential of their cells, allowing them to better manage the challenges posed by saline conditions. (Thanaa and Nawar.,1994). This information suggests that the accumulation of soluble sugars is one of the strategies employed by plants to adapt to salt stress, and the extent of this accumulation can vary among different plant species and even among different lines within a species. This adaptation helps plants maintain water balance and overall cellular health in saline environments.

Our study found that the concentration of total soluble sugars increased as salinity levels increased. This increase in total soluble sugars occurred irrespective of the specific citrus rootstock being studied. In other words, higher salinity levels generally led to higher levels of total soluble sugars in all the citrus rootstocks examined. Although total soluble sugars increased in response to salinity in all the rootstocks, some rootstocks exhibited a greater accumulation of these sugars compared to others. Specifically, Citrumelo Winter Haven B2 (F1), Troyer citrange C35B6A11 (F8), Troyer citrange B2 31655 (F9), Sacaton citrumelo 30057 (F14), and Gou-Tou SRA 506 (F23) showed higher levels of total soluble sugar accumulation under salinity stress (Table 4). The results of this study also indicated that amount of total soluble sugars was higher than other solutes, such as proline, glycinebetaine, and total free amino acids. We found that the total soluble sugar content was higher than the other solutes. This suggests that, among the solutes studied, total soluble sugars were more prominent in their response to salinity stress in these citrus rootstocks, many reports confirm these findings (Fougere *et al*., 1991; Hsu *et al*., 2003 the accumulation of sugars plays a significant role in osmotic adjustment under saline conditions. Osmotic adjustment is a plant's ability to regulate water and solute balance to adapt to challenging environmental conditions,such as salinity (Popp and Smirnoff., 1999; Atienza *et al*., 2004). The accumulation of sugars contributes more to this adjustment compared to other solutes, according to our study's findings. This results show that total soluble sugars in citrus rootstocks increase in response to salinity stress, with some rootstocks exhibiting a more significant increase than others. These sugars play a key role in osmotic adjustment under saline conditions, which helps the plants adapt to the stress. Additionally, the study found that total soluble sugars were more prominent in their response compared to other solutes like proline, glycinebetaine, and total free amino acids.

1. **Conclusion**

In conclusion, increasing levels of NaCl (salt) in the growth media had a detrimental effect on the growth of all tested rootstocks. In other words, as the salinity in the growth media increased, plant growth was reduced across the board for all rootstocks. Among the tested rootstocks, Citrumelo Winter Haven B2 (F1), Troyer citrange C35B6A11 (F8), and Troyer citrange B2 31655 (F9) were found to be more tolerant to the elevated NaCl levels compared to the other tested rootstocks. This means that these specific rootstocks exhibited better growth or were less affected by the high salinity conditions. However, it was observed that high concentrations of NaCl had a negative impact on all the parameters that were studied. This reinforces the idea that excessive salinity is detrimental to plant growth and various plant-related factors.

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