In vitro screening for salt stress tolerance of certain citrus rootstocks through the exposure of their roots

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ABSTRACT:

Salt stress is the most important abiotic stress limiting crop yield globally, and breeders are encouraged to test novel genotypes for resistance to this selection agent. However, getting a sufficient number of plants for the evaluation can be a lengthy and time-consuming procedure that might be shortened by applying *in vitro* techniques. An experiment was conducted under controlled saline circumstances using a tissue culture technique to investigate the influence of salt on several physic-biochemical parameters. Micro-shoots of Volkamariana lemon, sour orange, trifoliate orange, and Cleopatra mandarin rootstocks were re-cultured on MS media supplemented with NaCl from 25, up to 150 mM. Following the experiments, all of the tested rootstocks' morphological and biochemical characteristics, stomata behavior, and element accumulation were measured. As a result, adding NaCl from 25 to 150 mM to MS media caused a reduction in All morphogenetic parameters were measured and compared to the control, including shoot number, shoot length, leaf number, and survival percentage. Photosynthesis pigment, RWC, K, and Ca ions were decreased by increasing NaCl from 100 to 150 mM. On the contrary, proline, Cl, and Na were increased. Cleopatra mandarin and Trifoliate orange are more tolerant to salt stress than Volkamariana lemon and sour orange citrus.

Keywords: Citrus rootstocks; tissue culture; biotechnology; salinity; NaCl; EDX; Stomata.

INTRODUCTION

Salinity has developed into a significant hazard to agriculture, reducing agricultural output all around the world. According to estimates, the global consequences of soil salinization could result in up to 50% less land by 2050. (Wang et al., 2003). Salinity affects the production of proteins, carbohydrates, and lipids, which inhibits growth, causes wilting, or even results in death. (Aslam et al., 2017); (Hamouda et al., 2022). Some plants evolve defense mechanisms that either prevent salt from entering their cells or allow it to exist there. There are differences across species in how plants respond to the salt environment and how well their responses work (Galvan-Ampudia & Testerink, 2011). Crops are threatened by ion toxicity as well as high salinity, which makes it more difficult for roots to absorb water and causes stress similar to that caused by drought (osmotic impact). High concentrations of proline and glycine betaine, amino acids, inorganic ions, soluble sugars, and other compatible substances are found in halophyte plants; however, compatible solute concentrations that accumulate in glycophytes are not as high (Flowers & Colmer, 2015). As a result, screening salt-tolerant rootstocks gives an alternative source to improve the breeding program and to better understand the pathways involved in the salt tolerance mechanism. A genus of flowering plants in the *Rutaceae* family goes by the name "citrus." With 2n = 18, all citrus species are remarkably similar in size and karyotypic form. Furthermore, with 150-162 genera and 1500-2096 species, the *Rutaceae* family is a massive, mostly tropical and subtropical family.(Kubitzki, 2011); (Guerra et al., 1997). The primary breeding goals of the citrus industry are always to increase fruit quality and reduce biotic and abiotic stresses. (Cai et al., 2017). The highest growth rate, standing leaf area and survival rate of grafted citrus trees decreased with increasing tissue Na+ and Cl− concentrations(Simpson et al., 2014). Citrus is extremely sensitive to salinity, but we believe that rootstocks react to salt stress differently. Rootstock of citrus for decades, scientists have been studying salt tolerance. Volkamer Lemon (*Citrus volkameriana*) and Sour Orange (*Citrus aurantium* L.) are two multipurpose species that are typically grown as rootstock for sweet oranges(Srivastava & Singh, 2009). In a nutshell, the salinity tolerance range for the most popular rootstocks is as follows: *Citrus macrophylla* (*Citrus macrophylla* Wester) > Citrus reshni Hort. ex Tan. > Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) > *Citrus limonia* Osbeck > Rangpur lime (*Citrus limonia* Osbeck) > Sour orange (*Citrus aurantium* [L.]) > Sweet orange (*Citrus sinensis* [L] (Ferguson & Grattan, 2005). While *Poncirus trifoliata* and its hybrid seem to be sodium excluders, Rangpur lime and

Cleopatra mandarin appear to be chloride excluders(Zekri & Parsons, 1992). Citrus genotypes with the ability to confine chloride ions to the roots, like Cleopatra mandarin (CM) or Rangpur lime rootstocks, can be categorised as relatively tolerant, whereas others, like *Carrizo citrange* (CC) or citrumelo CPB4475 (Cit), have shown to be more sensitive to salinity(López-Climent et al., 2008). Tissue culture has emerged as a relevant tool for stress evaluation in recent decades since it allows for the management of huge populations in a small space while allowing for more stringent environmental control (Vives-Peris et al., 2017). Since plants of the same genotype responded to salinity similarly in both experiments, it was shown that tissue culture is a reliable method for determining whether a genotype is tolerant to salinity or not(Pérez-Jiménez & Pérez-Tornero, 2020). *In vitro* tests can now be used to test genotypes very early on while consuming very little time and space. In the current study, we looked at four citrus rootstocks to examine some physiological and biochemical factors, as well as the biochemical genetic diversity in the leaves, to better understand the mechanisms underlying citrus rootstocks' ability to withstand salinity.

MATERIALS AND METHODS

For three consecutive years—2019, 2020, and 2021—this study was conducted in the tissue culture lab of the horticulture department of the faculty of agriculture at Al-Azhar University in Nasr City, Cairo, Egypt.

Initiation and Stock:

Ripe fruits from four citrus rootstocks, including Cleopatra mandarin, trifoliate orange, Volkamariana lemon, and Sour orange, were sterilized by dipping them in tap water with soap for 30 minutes to remove dust. They were then dipped in 99.6% ethanol for 1/4 minute before being burned on the flame for 5 seconds under laminar flow conditions. Then, the fruits were placed on sterilized paper to extract the seeds from them under the laminar flow hood. Finally, seeds were removed from the bulbs of fruits, and seeds were prepared for culturing on MS media without plant growth regulators. During the preparation of the medium, we controlled the pH in the medium at 5.7 by adding a few drops of potassium hydroxide (KOH) or hydrochloric acid (HCL) at 0.1 N. 100-millilitre glass jars were filled with 25 milliliters of media each. Transparent polypropylene covers were used to protect the culture jars. The media were then sterilized by autoclaving the jars for 20 minutes at 121 °C under 1.5 Kg/cm2 pressure. The jars were refrigerated in a slant position in the culture cabinet until needed. They were transferred to the culture room and cooled until they could be used. The cultures were incubated for four weeks at 27 °C using cool white fluorescent lamps at an intensity of 3000 Lux for 16 h daily). Each treatment was repeated three times.

Proliferation of citrus rootstocks:

To create explants of all tested rootstocks, shoot tips or micro-cuttings of all tested rootstocks were cultured on MS media supplemented with BA at 1.0 and NAA at 0.01 mg/L. (Abdel Aziz et al., 2023). They were explants taken from seed germination above, and the cultures were incubated for four weeks at 27 °C using cool white fluorescent lamps at an intensity of 3000 Lux for 16 h daily). Each treatment was repeated three times.

Mass-production of citrus rootstocks:

Axillary shoots were moved from different citrus rootstocks to MS (Murashige and skoog,1962 basal medium) that was supplemented with 30 g/L sucrose and both BA (6-Benzylaminopurine) and KIN (kinetin) at 1.0 mg/L. (Alizadeh et al., 2010), and the cultures were incubated in a growth chamber at 27 °C using cool white fluorescent lamps with an intensity of 3000 Lux (luminous flux per unit are)for 16 hours daily for four weeks. Each treatment was repeated three times to achieve mass production of all tested rootstocks.

Stress treatments.

The goal of this study was to see how several citrus rootstocks, such as Volkamariana lemon, sour orange, trifoliate orange, and Cleopatra mandarin, responded in vitro to increasing salt stress in the culture media. Each treatment was performed three times with three plantlets in each duplicate, and uniformly developed explants from the multiplication medium were selected and transferred to MS media supplemented with sucrose, 30 g/l, BA at 1.0, NAA at 0.01, and with varying concentrations of (sodium chloride) (NaCl) (0.0, 25, 50, 75, 100, and 150 mM).

Data collection and morphological and biochemical characteristics

Determination of morphological characteristics

After 30 days, the growth of axillary shoots exposed to salt stress was assessed by counting the increase in previous morphological characteristics. The following equation by

Survival (%) = number of explants alive at end of period X100

Determination of photosynthetic pigments

According to Lichtenthaler (Lichtenthaler, 1987), the chlorophyll a, b, total chlorophyll, and carotenoid contents of the leaves were measured at the end of the experiment period (after 30 days). All previous photosynthetic pigments were measured after thirty days using the method of abrading about 0.2 g of fresh tissue of plant leaf in a mortar with 15 mL of acetone 80% and, after filtering, its absorption was measured by spectrophotometer UV-Vis model 715 Jenway at 470, 663, and 646 mm. For calibrating the device, we used acetone at 80%. The concentrations of pigments were calculated by applying the formula:

Chl. a (Chlorophyll a) = (12.25 A 663.2 - 2.79 A 646.8)

Chl. b (Chlorophyll b) = (21.21 A 646.8 – 5.1 A 663.2)

$$\text{Total carotenoids} = + \frac{(1000\text{A } 470 - 1.8 \text{ chl, a} - 85.02 \text{ chl. b Car})}{198 \text{ (µg per ml solution)}}$$

Where Chl. a Chl. b, T. Chl. (Total Chlorophyll) and car represent the concentrations of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids. The measurements of photosynthetic pigment content were based on fresh weight.

Determination Proline content:

To assess proline content, (Bates et al., 1973) used a quick colorimetric approach. Acid-ninhydrin was produced by heating 1.25 g of ninhydrin in 30 mT of glacial acetic acid and 20 mL of 6 M phosphoric acid until dissolved. When kept cold (at 4 °C), the reagent is stable for 24 hours (7 procedures). The resulting homogenate was then filtered through Whatman # 2 filter paper after being homogenized in 10 ml of 3 percent aqueous sulfosalicylic acid. 2) Two milliliters of the filtrate, two milliliters of acldninhdrin, and two milliliters of glacial acetic acid were combined in a test tube and heated to 100 °C for one hour before being stopped in an ice bath. 3) The reaction mixture was vigorously stirred with a test tube stirrer for 15-20 seconds before being extracted with 4 mL of toluene. A chromophore containing toluene was drawn out of the aqueous phase, warmed to room temperature, and its absorbance at 520 nm was measured using toluene as a control. 5) Using a standard curve, the proline concentration was calculated on a fresh weight basis as follows:

{(ag proline /ml X ml toluene) /115.5ixg /Izmole}/{(g sample)/5}- = moles proline/g of fresh weight material

Leaf relative water content:

Leaf relative water content (RWC) was calculated based on the (García-Mata & Lamattina, 2001) method. In each repetition, two leaves were chosen at random from the plants' middles. To begin, leaves were separated from stems and fresh masses (FM) were calculated. To determine the saturation mass (TM), they were immersed in distilled water in closed containers for 24 hours at 22° C in order to reach their maximum saturation mass, and then weighed. The leaves were then placed in an electrical oven for 48 hours at 80° C, and the dry mass of the leaves (DM) was obtained. All measurements were taken with 0.001g precision scales and entered into the following formula:

Relative water content (%)= FW-DW/TW-DWX100

Where (FW) denotes fresh weight, (DW) denotes dry weight, and (TW) denotes turgid weight.

Ions accumulation:

This inquiry was carried out to study the effect of MS media complemented with NaCl at different concentrations: 0.0, 25, 50, 75, 100, and 150 mM on ion accumulation in the leaves of all tested rootstocks. EDX (Energy-dispersive X-ray spectroscopy) was used to estimate the accumulation content of some elements such as Na, Cl, K, Ca, P, and some microelements in the leaves of some citrus rootstocks. The samples were examined under X-ray microanalysis (Module Oxford 6587 INCAx-sight) attached to JEOL JSM-5500 LV scanning electron microscopy at 20KV at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

Stomata behavior:

This experiment was carried out to study the effect of MS media complemented with NaCl at 0.0, 25, 50, 75, 100, 125, and 150 mM on stomata characteristics such as the number of stomata, inside length of stomata µm, inside

width of stomata μm, outside length of stomata μm and outside width of stomata μm of four tested grape rootstocks. The samples of the low surface of a leaf in some citrus rootstocks were coated by a gold sputter, coater (SPI-Module). For each imprint, the number of stomata/mm2 (average number of 3 fields) and stomata dimensions (10 stomata randomly selected per field) of four tested grape rootstocks were measured. At the Regional Centre of Mycology and Biotechnology in Cairo, Egypt, the samples were lastly examined using scanning electron microscopy (JEOL JSM-5500 LV) in high vacuum mode [13].

Statistical procedure.

Three replicates of each treatment were used for the analysis of variance (ANOVA), which was carried out using two methods of the ANOVA Co-stat software in a randomized design. According to (Stern, 1991), Duncan's test with a probability of 0.05 was used to show significant differences between treatments.

RESULTS

Evaluation of Growth characteristics.

The effects of NaCl treatments on morphological traits measured in the four tested citrus rootstocks are depicted in Fig. 1. It was clear that number of shoot, shoot length (cm), number of leaf, and survival percentage were decreased due to the increase in NaCl from 25 to 150 mM through direct treatment (shock treatment) compared with control. In contrast, increasing salt stress induced by NaCl from 25 up to 150 mM led to an increase in the mortality rate of all tested rootstocks. A significant difference between the tested rootstocks was noticed regarding the effect of NaCl at different concentrations through shock treatment on previous morphological characteristics, where trifoliate orange rootstocks recorded the highest value compared with other tested rootstocks.

Evaluation of photosynthesis pigment.

The effects of salt stress generated by NaCl by direct (shock treatment) on chlorophyll content as chlorophyll "a" chlorophyll "b," total chlorophyll, and carotenoids were shown in Fig. 2. A severe reduction in photosynthesis pigment as chlorophyll "a", chlorophyll "b", and total chlorophyll carotenoids was achieved when shoots of all tested citrus rootstocks were cultured on a high level of NaCl from 100 up to 150 mM in comparison with the control and lowest concentrations of NaCl. All of the citrus rootstocks evaluated showed substantial variations. The highest levels of chlorophyll and carotenoids were found in Trifoliate orange rootstock, followed by Cleopatra mandarin, Volkamer lemon, and sour orange citrus rootstocks.

Effect of NaCl on RWC.

Figure 3 shows the impact of salt stress generated by NaCl by direct (shock treatment) using various doses of sodium chloride from 25 up to 150 mM on relative water content (RWC) in leaves of certain citrus rootstocks. When micro-shoots of tested rootstocks were cultured on MS media with any level of NaCl, whether using different concentrations of sodium chloride by direct (shock treatment) at 25 up to 150 mM in comparison to control, RWC was reduced. When compared with other rootstocks, the highest reduction in RWC was obtained when shoots of Sour Orang and Volkamariana lemon were cultivated on a high level of NaCl at 150 Mm in comparison with other rootstocks. There were significant variations among all citrus rootstocks tested when they were cultured on MS media complemented at 125 plus 150 mM. The Cleopatra mandarin rootstock had the highest RWC values, followed by the Trifoliate orange rootstock compared to the other two citrus rootstocks.

Effect of NaCl on proline content.

The results in Fig. 4 show the effect of direct (shock treatment) salt stress caused by NaCl at different doses from 25 up to 150 mM on the proline content of four citrus rootstocks. When compared to the control, increasing salinity from 25 to 150 mM resulted in a significant increase in proline concentration in leaves for all the citrus rootstocks studied. There were substantial variations between all treatments of all citrus rootstocks evaluated, with MS medium enriched with 150 mM recording the highest accumulation proline concentration, followed by 125 mM, 100 mM, 75, 50, and both 25 and without sodium chloride. The accumulation of proline content differed significantly between all citrus rootstocks concerning salt stress, with the Cleopatra citrus rootstock recording the highest accumulation of proline content, followed in descending order by Trifoliate orange, Volkamer lemon, and Sour orange rootstocks.

Effect of NaCl on Ion content:

The establishment of ion homeostasis is required for plants to survive under salt-stress

circumstances. The data given in Figure 5 demonstrated the influence of NaCl-induced salt stress on the accumulation amounts of various elements such as Na,(Sodium) ,Mg (Magnesium), Cl (Chloride) , K(Potassium), Ca(Calcium), P(phosphors), Na/K ratio and some micro-elements in the leaves of all investigated citrus rootstocks. When citrus rootstock shoots were grown in NaCl-enriched MS medium at varying from 25 up to 150 mM, the accumulation of K, Ca, Mg, P, and certain micro-elements was significantly reduced in contrast to control. When the micro-plantlets of the four rootstocks under examination were grown in high concentrations of NaCl (125 and 150 mM), the least amounts of K, Ca, Mg, P, and some micro-elements were accumulated in comparison to control and the lowest value of sodium chloride. In contrast, the accumulation contents of Na, and Na/K ratio increased with increasing NaCl from 25 to 150 mM in comparison to the control. Compared to the control and alternative treatments, the largest element accumulation of Na, Cl, and Na/K ratio was obtained when the micro-plantlets were cultivated on an MS medium supplemented with high levels of sodium chloride (125 and 150 mM). In this regard, the Cleopatra mandarin citrus rootstock recorded the highest accumulation of K in the leaf petiole and the lowest accumulation values of Na and Cl by increasing the NaCl level in the culturing medium, followed by Trifoliate orange, Volkamer lemon, and Sour orange citrus rootstock.

All stomata characteristics, including the number of open stomata, inner length of stomata (m), inside width of stomata (m), outside length of cell guard (m), and outside width of cell guard (m), were negatively influenced by adding NaCl from 25 to 150 mM to the growth medium (shock treatment technique) were data presented in Figure. 6. All of the above-mentioned parameters of the four tested citrus rootstocks appeared to be strongly impacted by the citrus rootstocks, salt level, and their interaction, where all parameters of all tested citrus rootstocks were gradually lowered by raising the NaCl level in the growing medium. The magnitude of the decrease, however, differed from rootstock to rootstock. Re-cultivating the micro-plantlets of citrus rootstocks on MS medium complemented with 150 mM NaCl resulted in a decline in all parameters of all tested citrus rootstocks, with Cleopatra mandarin rootstock exhibiting the highest percentage of reduction in the number of open stomata, inside length of stomata (m), outside length of cell guard (m), and outside width of cell guard (m), followed in descending order by Trifoliate orange and those of Volkamariana lemon and Sour orang.

Principal Components Analysis (PCA)

PCA was clarified first two components by 10 variables where the first and second components were (92.7-5.6%), (91.2 - 7.2%), (89.9-9.1%) and (89.4-8.1%) Cleopatra mandarin, Sour orange, Trifoliate orange, and Volkamer lemon, respectively (Table 1) and (Figure. 7). Table 8 provides a summary of the individual values, variance %, cumulative percentage, and component loading. The effect of MS media supplemented with different concentrations of NaCl is mostly located right side of the plot and has a strong positive correlation with the first component while the (Cleopatra mandarin) is recorded on the upper right side of the plot.

DISCUSSION

Salt stress is a critical factor in plants that harms plant metabolism and development. As salt levels rose, there was a decrease in shoot length, leaf number, and shoot quantity (Fig. 1). The outcomes completely concur with those that many workers had previously reported (Ghaleb et al., 2010). *In vitro* studies on two citrus rootstocks (sour orange and Volkamer lemon) revealed that MS media supplemented with NaCl from 0.0 to 300 mM harmed plant growth in terms of leaf number, plant length, fresh weight, and dry weight. Increasing NaCl level from 150 up to 300 mM in the growth medium caused a severe reduction in the above growth parameters compared with control and other treatments. A similar study (رستميان et al., 2019) found that adding NaCl from 50 up to 200 mM caused a reduction in the shoot fresh weight of four tested citrus rootstocks compared with the control. Likewise, the negative effects of salinity on growth parameters can be related to ionic imbalance, changed availability and absorption of other ions, ion buildup in leaf cell vacuoles, a decrease in photosynthetic rate, and decreased carbon fixation (Prior et al., 1992). Otherwise, suppression of branch development has been seen as a whole-plant response to salt stress (Mulholland et al., 2003). Furthermore, these deleterious effects of salt stress might be attributable to a decrease in both cell division and cell growth (Sofo et al., 2015). While toxicity caused by an excessive amount of salt buildup in plant cells becomes

apparent later in the growth cycle, plant growth inhibition under salt stress is also primarily caused by the osmotic impact. (Munns, 2002). Plants grown in salty conditions are stunted due to a decrease in cell elongation and cell division, which are controlled by several auxins, the synthesis of which is slowed by salinity (Loreto et al., 2003); (Ndayiragije & Lutts, 2006). Plant growth is a key factor in assessing the salt tolerance of various citrus rootstocks. Under saline circumstances, the citrus rootstocks Cleopatra mandarin and Trifoliate orange maintained the highest growth parameters. The decrease in biomass increased with salt, owing to disruptions in physiological and metabolic activity under saline circumstances (Craine, 2005) ;(Munns et al., 2006), which may be related to a reduction in leaf area and the number of leaves (25). Despite being membrane-bound and dependent on membrane stability, chlorophyll typically survives unharmed in saltwater environments. (Shah, n.d.). Salinity has also been linked to a decrease in chlorophyll concentration e.g., (Gu et al., 2004). However, researchers summarized the findings by indicating that the decline in chlorophyll may be attributed to a difference in its production between plant species due to variance in particular enzymes under saltwater circumstances (Kreps et al., 2002); (Keutgen & Pawelzik, 2007). These criteria can be used to distinguish between salt-tolerant and salt-sensitive citrus rootstocks. In this regard, these results are in agreement with the findings of(رستميان et al., 2019) which reported that photosynthetic pigments such as chlorophyll a, chlorophyll b, and carotenoid have had a negative correlation with salinity concentration and concentration of sodium ions in the tissue of the leaves of four citrus rootstocks, namely Sour orange (*Citrus aurantium* L.), Poncirus (*Poncirus trifoliate* Raf.), Citromelo (Citrumelo), and Citrange (Citranges) by adding NaCl 50, 100 and 200 mM to MS media in comparison with control. It was clear that Citrange and Sour orange rootstock show better resistance to the damages, caused by salt stress than other citrus rootstocks. Change in chlorophyll content due to salinity is the most obvious biochemical response (Mustafa et al., 2021). The amount of chlorophyll decreases due to salinity stress because the glutamate which is the prefabricate matter of chlorophyll and proline is spent on the production of proline (Molazem et al., 2010). Furthermore, decreased chlorophyll concentration could be due to the inhibitory effect of ions accumulating in chloroplasts, chlorophyll breakdown caused by oxidative stress caused by salt, activation of chlorophyllase enzyme by salinity ions, and its negative effect on protophyzine. Increasing salinity decreases chlorophyll production through an increase in salt. Due to water constraint stress brought on by salinity, an increase in leaf temperature, and the formation of abscisic acid in the root and its transfer to the stomata, the stomata shut down as a result. (Garcia & Charbaji, 1993). Carotenoids have antioxidant characteristics and are vital in scavenging ROS as well as functioning as light-harvesting pigments (Pascale et al., 2001). Chlorophyll content reduction in abiotic stress plants may be due to altered pigment-protein complex lipid-protein ratios or elevated chlorophyllase activity [33]. Furthermore, the decrease in chlorophyll concentrations is most likely owing to the inhibitory impact of accumulated ions of various salts on the production of the various chlorophyll components (Sutee et al., 2008).

All rootstocks analysed showed a significant reduction in RWC at moderate and high salinities. Comparing the sour orange rootstock to the other tested rootstocks, this reduction was particularly apparent. RWC is a direct indicator of the water status of plants, and a decline in it demonstrates that salt contributed to a water deficit in plants. The results go in line with (رستميان et al., 2019) who found that MS media supplemented with NaCl at (0, 50, 100, and 200 mM) affected on RWC of four citrus rootstocks, namely Sour orange (*Citrus aurantium* L.), Poncirus (*Poncirus trifoliate* Raf.), Citromelo (Citrumelo), and Citrange (Citranges). It was clear that the RWC of all tested citrus rootstocks was decreased by increasing NaCl from 50 up to 200 mM compared with the control. In comparison to the control and other treatments, the lowest RWC was obtained when the micro-shoots of all tested cultivars were cultured on MS media complemented with NaCl at 200 mM. Also, (Hatami et al., n.d.). They stated that the increase of NaCl up to 150mM in the culturing medium caused the Seedless Red and Ghezel Uzum grape cultivars' leaves to possess the lowest RWC. Simple reductions in turgescence potential and pore closure are reversible when the relative water content is between 70% and 100%. Rewatering can restore the plant's chloroplast in the case where there is a relative water content of 30 to 70% (due to the optical block). When the relative water content is less than 30%, the chloroplast membrane suffers irreparable damage. (Damani et al., n.d.). An

increase in soluble salts, which slow the absorption of water and nutrients, creating osmotic effects and toxicity, caused a detrimental effect on plant water relations (Yang et al., 2009); (Jiang et al., 2014). The leaf area, dry weight of the leaf, amount of chlorophyll, and other growth rate indicators are primarily connected with relative water content Maintaining a high RWC in leaves is one way of coping with salt stress and keeping plant cells turgid (Walker et al., 2007). In addition, changes in the leaf water content are the key signal that causes changes in the plant's hormonal balance. Thus, despite the saltwater environment of the root, ABA content in the leaves does not grow when the leaf-water content does not fall significantly below the ideal, as in the saline-high humidity condition (Hatami et al., n.d.). So, It may be used as an enzyme protectant, a free radical scavenger, a cytosolic pH buffer stabilizer for subcellular structures, and a cell redox balancer (Kaur & Asthir, 2015).

When plants are under stress from salt, they can store osmoprotective solutes like proline and soluble carbohydrates in the form of a buildup (Azza et al., 2010). One of the most common responses of plants to changes in the external osmotic potential is accumulation of metabolites that act as compatible solutes [50]. Proline protects plants from salt stress in addition to acting as an appropriate osmolyte. Due to moderate and high salinity, the current study discovered that all rootstocks' leaves and roots had higher concentrations of free proline. This increase was especially noticeable for Cleopatra mandarin rootstocks. The results are in agreement with (El-Habashy, 2018) who clarified that the increase in sodium chloride levels in cultured medium (up to 5000 ppm) causes a significant buildup of proline in citrus rootstocks' leaves. More specifically, the Spanish sour orange is considered to be the citrus rootstock that is most tolerant to salinity stress due to its high survival rate and proline accumulation, while the *Brazilian*, *Russian*, Alemow, and Trifoliate oranges were found to have a moderate tolerance to salt. Also, (Gutierrez-Partida et al., 2021) found that MS media supplemented with NaCl at 0.0, 60, and 120 mM affected proline accumulation in leaves of five Persian pistachio genotypes including Akbari, Ahmad-Aghaee, Italyayi, Badami, and Ghazvini cultivars. In comparison to the control, all tested cultivars had significantly higher proline contents as salinity levels rose from 60 mM to 120 mM. Proline has been proposed to have a variety of functions related to salinity tolerance. One theory is that when a plant is freed from stress, it acts as a reservoir of energy that can be quickly used up. Another is that it performs the role of an osmolyte and lowers the cell's osmotic potential, preventing the cell from absorbing harmful ions. The latter is more likely in this situation because salt-tolerant plants typically do not significantly decrease their chlorophyll content and also produce more proline when stressed. This suggests that the proline increase is lessening the salt's harmful physiological effects. The glutamate ligase enzyme is activated in salt and drought stress to convert glutamine to proline. Osmoregulation by cellulose solute buildup, such as proline, has been postulated as a feasible strategy for combating salt stress situations (Tony H.H & Murata, 2002). Additionally, it has been proposed that proline functions as a molecular chaperone, stabilizing protein structure, and that proline accumulation can provide a mechanism to buffer cytosolic pH and control cell redox state. Proline may also play a part in limiting the damage caused by dehydration.

The Cl content of the leaves of all examined rootstocks rose considerably when exposed to salt. Also, with increasing salt concentration, the K+ level in the leaves of all tested rootstocks decreased significantly. It is widely assumed that higher K+ accumulation in plants leads to increased tolerance to rising Na+ (Mahmood-ur-Rahman et al., 2019). It is generally recognized that K+ can play a vital role in plant growth and development, as well as osmotic correction and cell turgor maintenance (Wenji et al., 2018). Additionally, it is the primary cation in plants that counteracts the negative charge of anions and is crucial for the activation of enzymes involved in the metabolism of proteins and carbohydrates as well as for controlling the movement of stomata. The results are in line with those that many researchers have previously reported, who found that raising the NaCl level in the growth medium caused an increase in Na and Cl accretion and a decrease in Ca concentrations in the plant tissue of two root-stocks (sour orange and Volkamer lemon). And also, (L et al., 2013) illustrated that re-culturing the micro-shoots of rough lemon *(Citrus jambhiri* Lush.) rootstock on MS media complemented with NaCl at 0.0, 17, 35, 51, 68, 86, 103, 120, 137, and 154 mM caused an increase in Cl and Na while the K+/Na+ ratio was decreased. When micro-

shoots of tested citrus rootstock were grown on MS media supplemented with a high level of NaCl at 154 mM, the highest values of Cl- and Na+, as well as the lowest K+/Na+ ratio, were obtained when compared to the control and other treatments.

CONCLUSIONS

In conclusion, after one month in culture, all tested rootstocks showed decreased plant growth (leaf number, plant length, and shoot number), as well as biochemical traits (chlorophyll content and RWC). At the highest salinity levels (150 mM), total death was seen. All rootstocks responded to an increase in NaCl concentration in the growth medium by accumulating more Na and Cl, while having less K and Ca in the plant tissue. Citrus rootstocks Volkamariana lemon and sour orange are less tolerant of salt stress than Cleopatra mandarin and Trifoliate orange.

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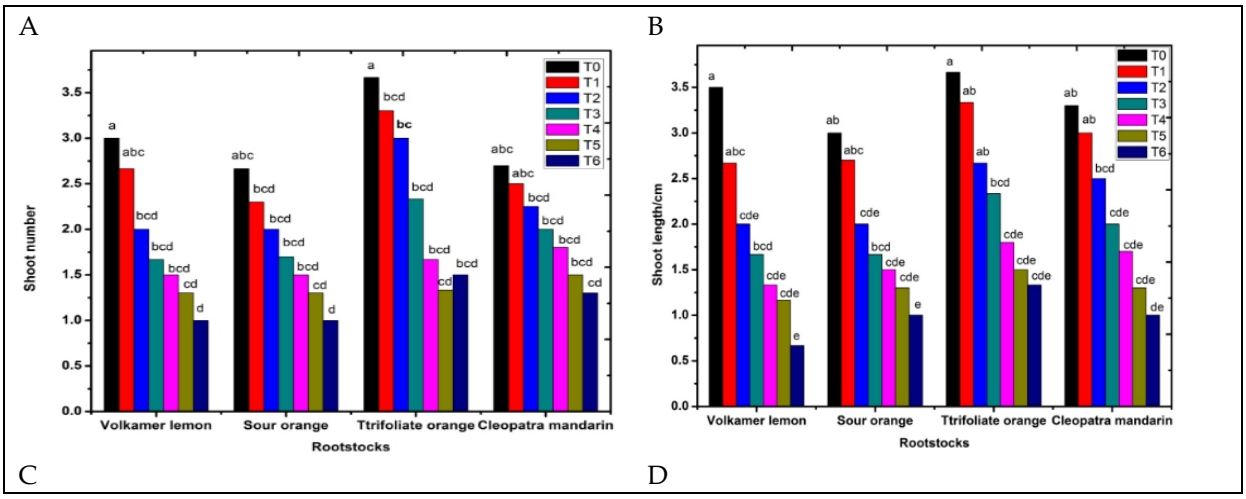
Table 1: Results of principal component analysis (PCA) Effect of MS media complemented with NaCl from 25 up to 150 mM on micro-shoots of Volkamer lemon, sour orange, trifoliate orange, and Cleopatra mandarin.

	Cleopatra mandarin			Sour orange		
Variable	PC1	PC2	PC3	PC1	PC2	PC3
Eigenvalue	9.2714	0.5646	0.075	9.1224	0.7171	0.0892
Variance	92.7	5.6	0.007	91. 2	7.2	0.009
Cumulative	92.7	98.4	0.991	91. 2	98.4	0.993
Components loadings						
Shoot number	0.328	0.046	-0.100	0.326	-0.195	0.112
Shoot length/cm	0.327	0.125	-0.017	0.318	-0.279	0.34
Leaves number	0.322	0.154	0.455	0.322	-0.063	-0.74
Survival %	0.253	-0.839	0.230	0.237	0.822	0.131
RWC %	0.324	0.080	-0.294	0.327	0.106	0.248
Chlorophyll a	0.320	0.225	0.416	0.328	-0.106	-0.17
Chlorophyll b	0.320	0.212	-0.266	0.327	-0.056	0.244
Total Chlorophyll	0.324	0.168	0.314	0.328	0.065	-0.351
Carotenoids	0.321	-0.005	-0.521	0.316	-0.335	0.175
Proline	-0.316	0.352	0.172	0.322	-0.25	-0.054

	Trifoliate orange			Volkamer lemon		
	PC1	PC2	PC3	PC1	PC2	PC3
Eigenvalue	8.9933	0.9101	0.0494	8.9435	0.8147	0.1682
Variance	89.9	9.1	0.005	89. 4	8. 1	0.017
Cumulative	89.9	99	0.995	89. 4	97. 6	0.993
Components loadings						
Shoot number	0.322	-0.264	0.18	0.329	-0.153	0.106
Shoot length/cm	0.328	-0.152	0.014	0.331	-0.142	-0.13
Leaves number	0.331	-0.114	0.168	0.326	0.002	0.491
Survival %	0.212	0.807	0.126	0.215	0.842	-0.16



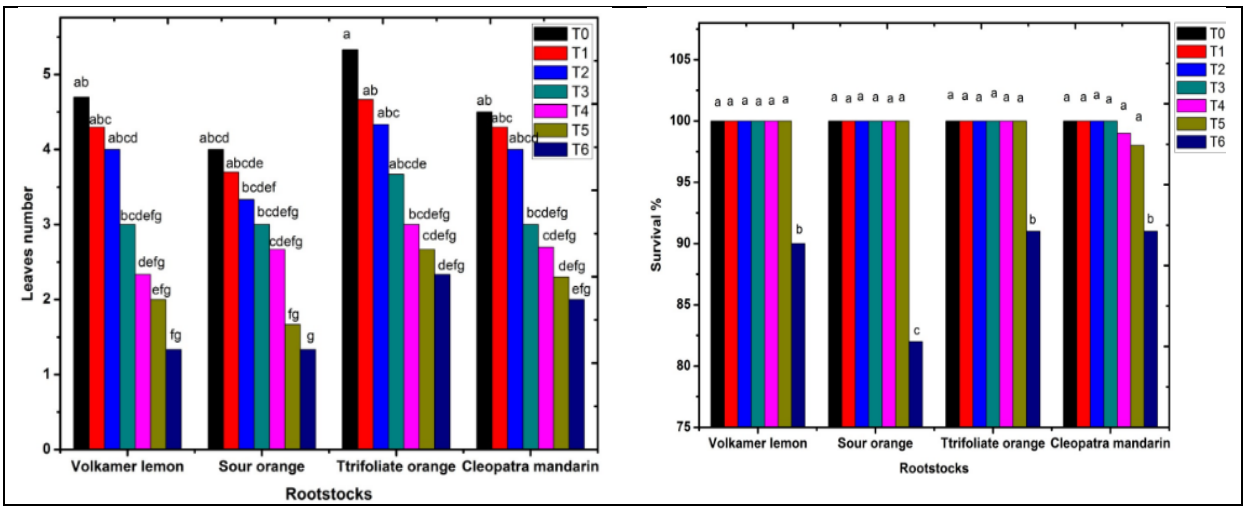


Figure 1: Effect of MS media supplemented with NaCl at different concentrations on morphological characteristics of some citrus rootstocks growing *in vitro*. T0: Control,T1 25mM(NaCl),T2: 50mM(NaCl),T3: 75mM(NaCl),T4: 100mM(NaCl),T5: 125mM(NaCl),T6: 150mM(NaCl). (A) Shoot number, (B) shoot length, (C) Leaves number,(D) survival%. Different letters above columns indicate significant differences among treatments at $P \leq 0.05$ ac-cording to Bartlett's test. Where (A) p-value of (R) rootstock 0.000; p-value of (T) treatments 0.000 and p-value of (Interaction between rootstocks and treatments) RxT = 0.152; (B) p-value of (R) = 0.000; (T) = 0.000 and RxT = 0.5988 ;(C) p-value of (R) = 0.000; (T) = 0.000 and RxT = 0.0.9139 and (D). p-value of (R) = 0.000; (T) = 0.000 and RxT = 0.0000

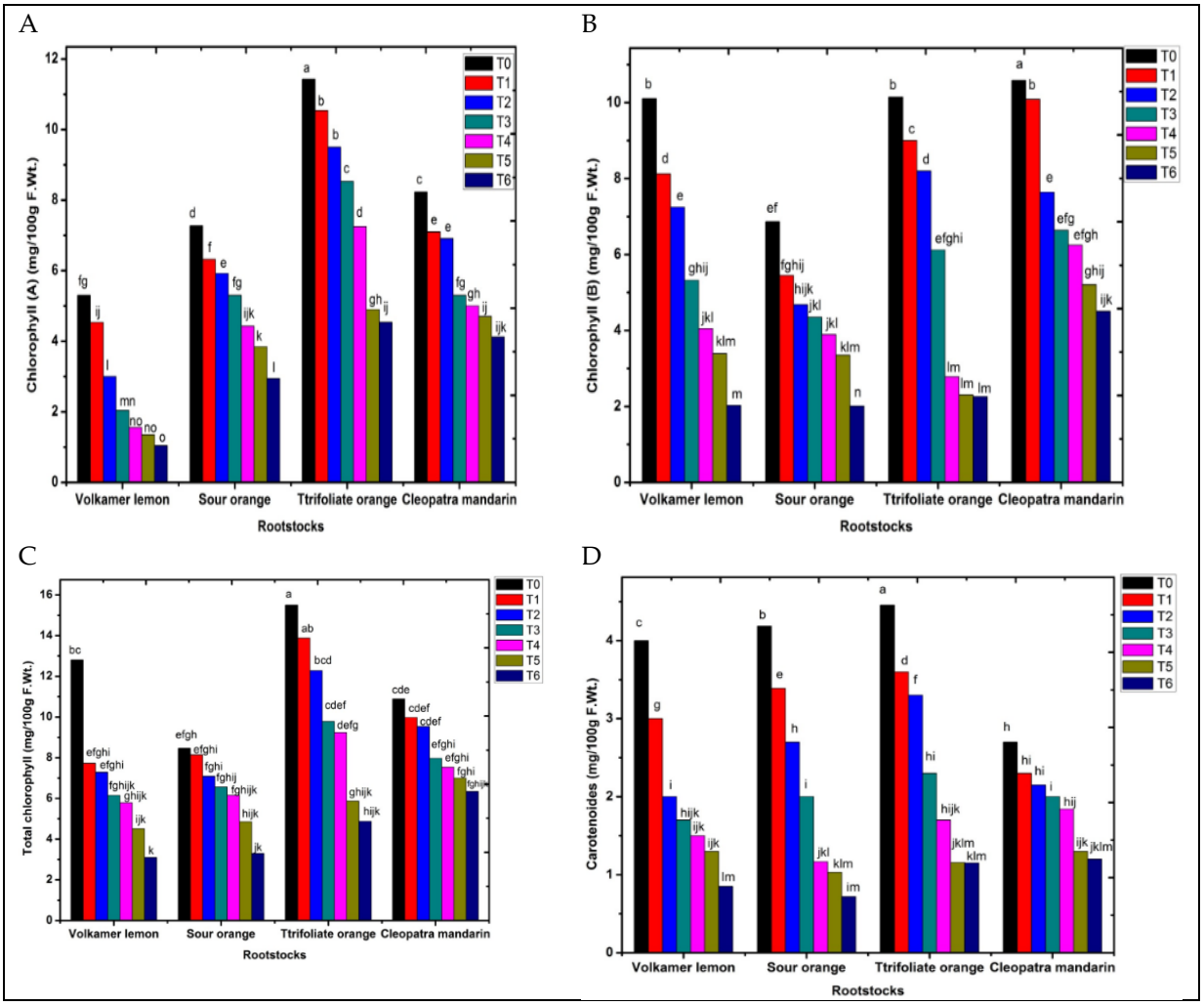


Figure 2: Effect of MS media supplemented with NaCl at different concentrations on leaf biochemical

characteristics of some citrus rootstocks grown *in vitro*. T0: Control,T1 25mM(NaCl),T2: 50mM(NaCl),T3: 75mM(NaCl),T4: 100mM(NaCl),T5: 125mM(NaCl),T6: 150mM(NaCl). (A) Chlorophyll (a) (mg/100g F.Wt.), (B) Chlorophyll (b) (mg/100g F.Wt.), (C) Total chlorophyll (mg/100g F.Wt.),(D) Carotenoides (mg/100g F.Wt.). Different letters above columns indicate significant differences among treatments at P ≤0.05 ac-cording to Bartlett's test Where (A) p-value of (R) rootstock 0.000; p-value of (T) treatments 0.000 and p-value of (Interaction between rootstocks and treatments) RxT = 0.000; (B) p-value of (R) = 0.000; (T) = 0.000 and RxT = 0. 000;(C) p-value of (R) = 0.000; (T) = 0.000 and RxT = 0.00009 and (D). p-value of (R) = 0.000; (T) = 0.000 and RxT = 0.0000

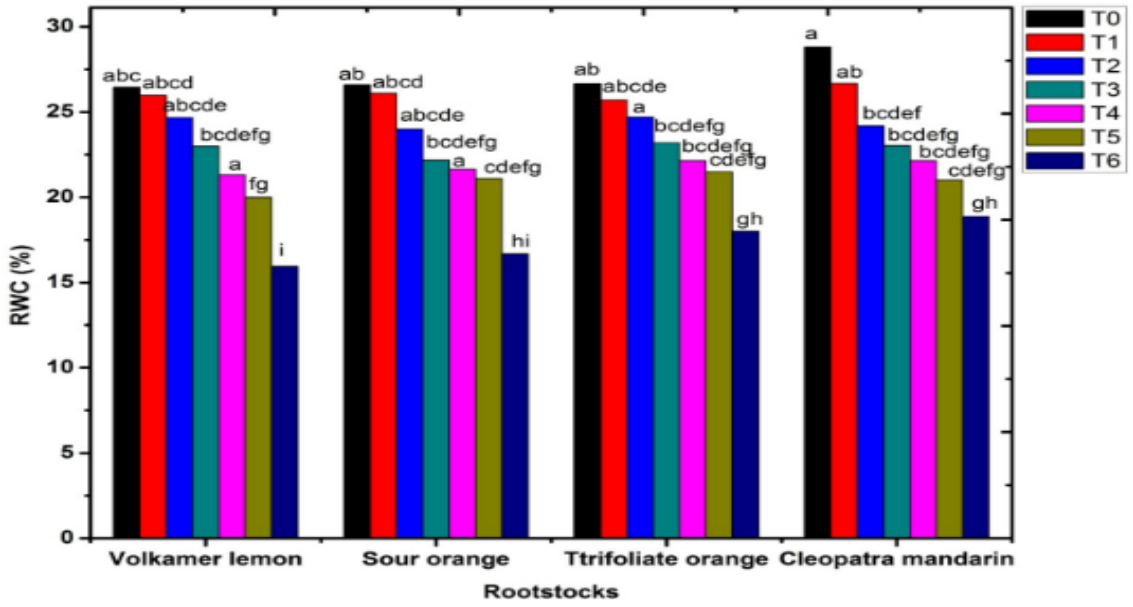


Figure 3: Effect of MS media supplemented with NaCl at different concentrations on leaf-relative water contents of some citrus rootstocks growing in vitro. T0: Control,T1 25mM(NaCl),T2: 50mM(NaCl),T3: 75mM(NaCl),T4: 100mM(NaCl),T5: 125mM(NaCl),T6: 150mM(NaCl). Different letters above columns indicate significant differences among treatments at P ≤0.05 ac-cording to Bartlett's test. Where p-value of (R) rootstock 0.000; p-value of (T) treatments 0.6596 and p-value of (Interaction between rootstocks and treatments) RxT = 0.2801

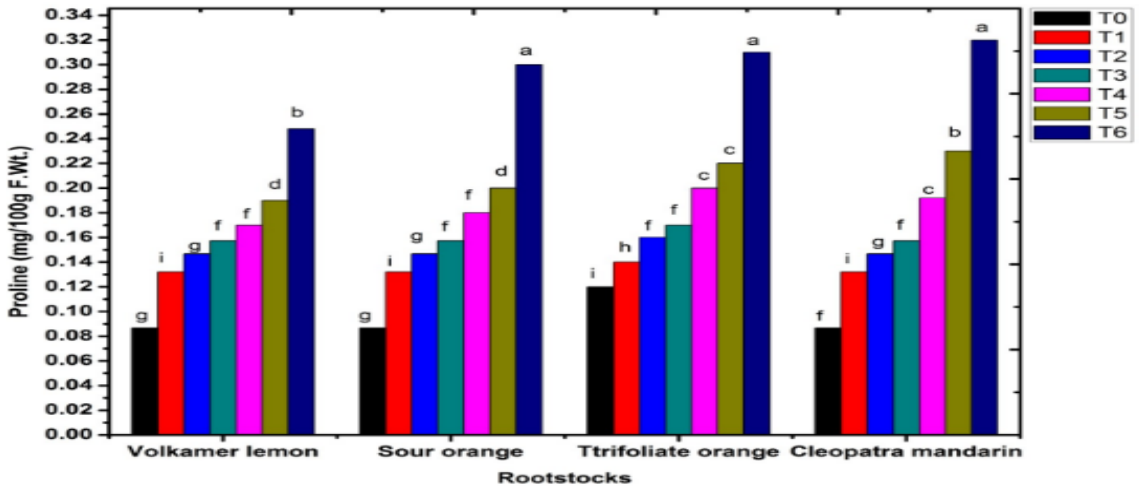
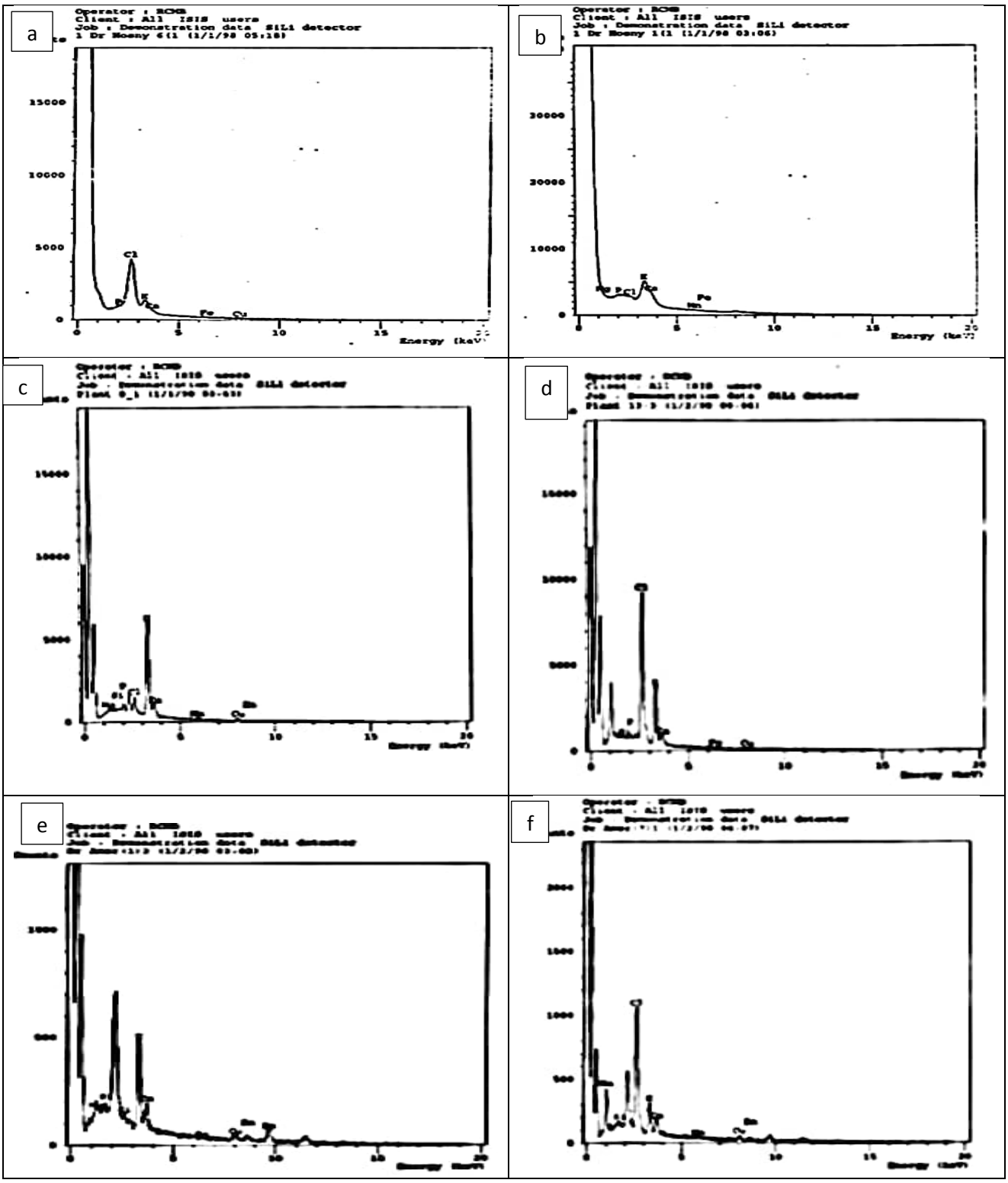


Figure 4: Effect of MS media supplemented with NaCl at different concentrations on leaf proline content of some citrus rootstocks growing in vitro. T0: Control,T1 25mM(NaCl),T2: 50mM(NaCl),T3: 75mM(NaCl),T4: 100mM(NaCl),T5: 125mM(NaCl),T6: 150mM(NaCl). Different letters above columns indicate significant differences among treatments at P ≤0.05 ac-cording to Bartlett's test. Where p-value of (R) rootstock 0.5381; p-value of (T) treatments 0.0103 and p-value of (Interaction between rootstocks and treatments) RxT = 0.9396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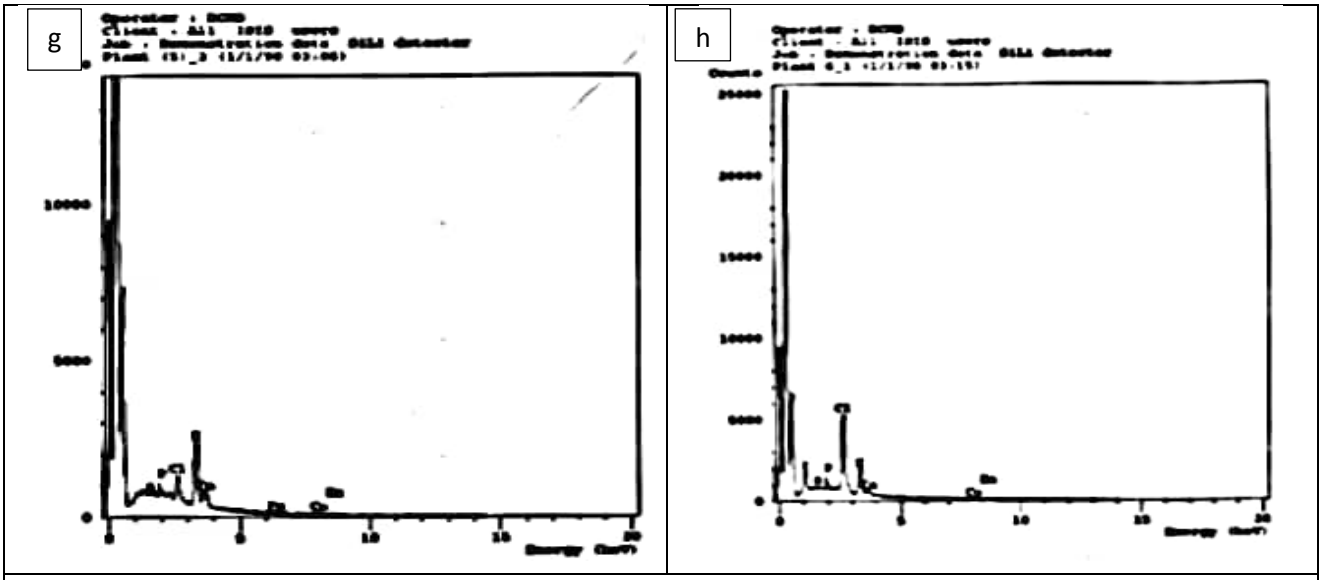

Figure 5: Effect of MS media supplemented with NaCl at different concentrations on leaf biochemical characteristics of some citrus rootstocks growing *in vitro*. a, b control and 150 mM NaCl respectively of volkamariana lemon rootstock; c, d (control and 150 mM NaCl respectively) of sour orange rootstock; e, f (control and 150 mM NaCl respectively) of trifoliate orange rootstocks and g, h (control and 150 mM NaCl respectively) of Cleopatra mandarin rootstocks.

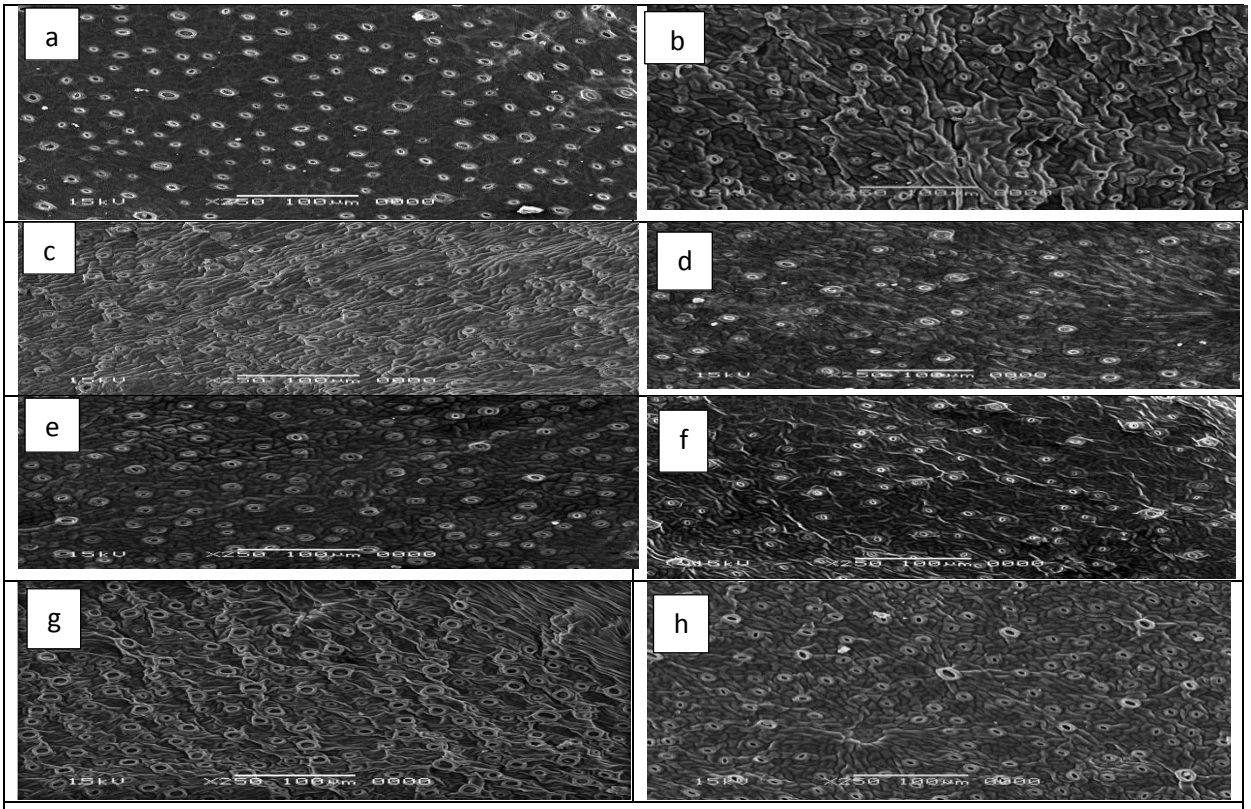

Figure 6: Effect of MS media supplemented with NaCl at different concentrations on leaf stomata behavior of some citrus rootstocks growing *in vitro*. a, b control and 150 mM NaCl respectively of volkamariana lemon rootstock; c, d (control and 150 mM NaCl respectively) of sour orange rootstock; e, f (control and 150 mM NaCl respectively) of trifoliate orange rootstocks and g, h (control and 150 mM NaCl respectively) of Cleopatra mandarin rootstocks.

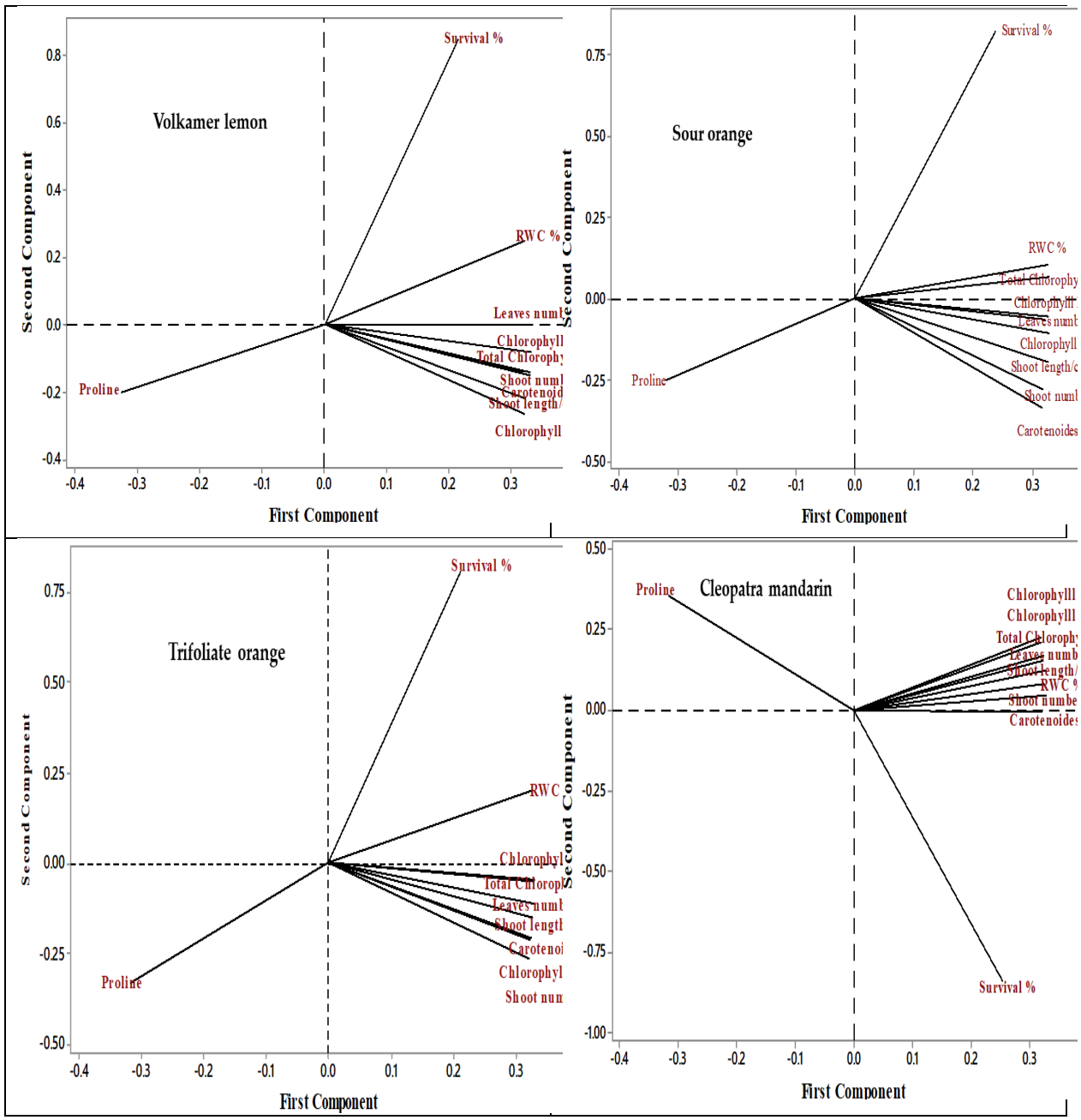

Figure 7: Principal Components Analysis (PCA) Effect of MS media supplemented with NaCl from 25 up to 150 mM on micro-shoots of Volkamer lemon, sour orange, trifoliate orange, and Cleopatra mandarin.

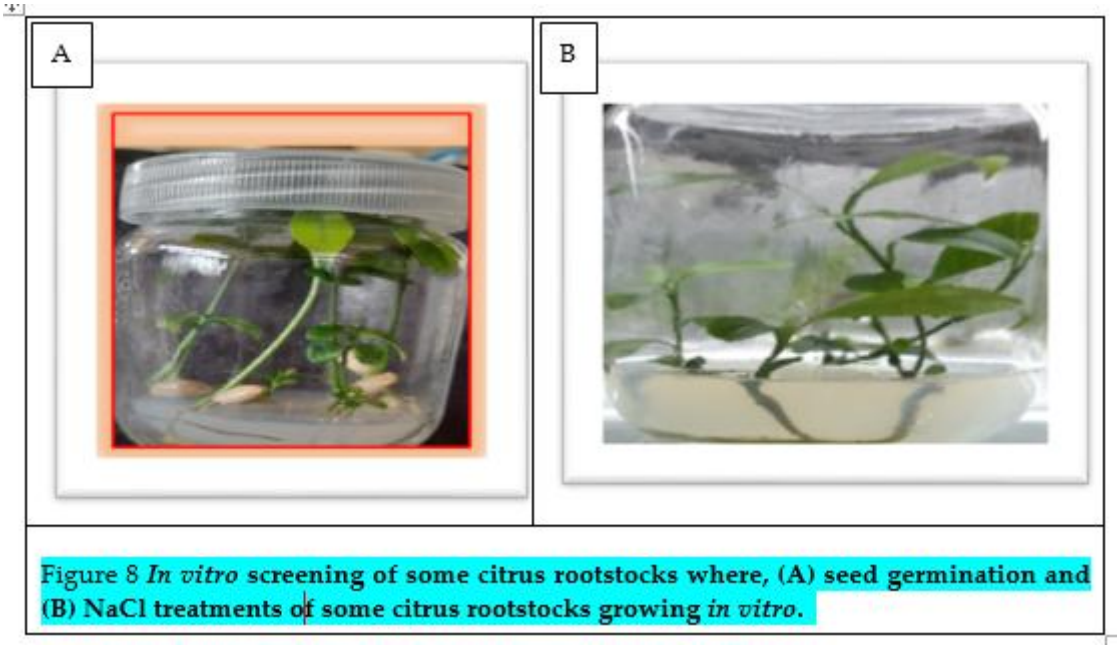

Figure 8 *In vitro* screening of some citrus rootstocks where, (A) seed germination and (B) NaCl treatments of some citrus rootstocks growing *in vitro*.

كشف مدى تحمل بعض أصول الموالح للاجهاد الملحي المنزرعة معمليا

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الملخص العربي:

يعد الإجهاد الملحي من أهم الاجهادات البيئية الذي تقلل من إنتاجية المحاصيل على مستوى العالم، حيث ذلك يشجع مربي النبات على انتخاب انماط جينية جديدة ذات قدرة عالية على تحمل الاجهاد الملحي. ومع ذلك، فإن الحصول على عدد كافٍ من النباتات المتحملة للاجهاد الملحي بالطرق التقليدية يحتاج لوقت طويل جدا ويمكن تقليل ذلك الوقت في انتخاب انماط جينية جديدة متحملة للملوحة وذلك عن طريق تقنية الزراعة المعملية في المختبر.اجريت هذه الدراسة تحت ظروف تقنية زراعة الانسجة لدراسة مدى تأثير الاجهاد الملحي على الصفات الطبيعية والبيوكيميائية لأصول الموالح محل الدراسة . تمت إعادة زراعة النبتات الصغيرة لبعض اصول الموالح مثل الفولكا ماريانا , النارنج, البرتقال ثلاثي الاوراق و اليوسفي كليوباترا على بيئة النمو (موراشيجي وسكوج) المزودة بكلوريد الصوديوم بتركيزات من 25 الى 150 مللي مولر . بعد اربع اسابيع من زراعة النبتات الصغيرة لاصول الموالح على البيئات السابقة تم قياس الصفات المورفولوجية والكيميائية الحيوية و محتور الاوراق النسبي للماء و محتواها ايضا من البرولين وسلوك الثغور وكذلك محتوى الاوراق من العناصر لأصول الدراسة ونتيجة لذلك، أدت إضافة كلوريد الصوديوم من 25 إلى 150 مللي مولر إلى بيئة النمو (موراشيجي وسكوج) إلى خفض في الصفات المورفولوجية (طول النمو – عدد النموات – عدد الاوراق – نسبة البقاء). كذلك نقص في معدل تركيز صبغات البناء الضوئي ، محتوى الماء النسبي في الاوراق ، انخفاض محتوى الاوراق من البوتاسيوم ، الكالسيوم ولكن في ذات الوقت نفسه زاد محتوى الاوراق من تركيز الصوديوم والكلور وارتفاع تركيز الحمض الاميني الحر (البرولين) في اوراق اصول الدراسة خاصة مع زيادة تركيز الاملاح في بيئة النمو من 100 الى 150 مللي مولر مقارنة بالكنترول والمعاملات الاخرى . في النهاية يعتبر اصل اليوسفي كليو بارتا والبرتقال ثلاثي الاوراق من افضل الاصول المتحملة للملوحة مقارنة بأصلي الفولكا ماريانا والنارنج.

الكلمات الاسترشادية : اصول الموالح, زراعة الانسجة, البيوتكنولوجى, الملوحة , كلوريد الصوديوم, الثغر.