ANATOMICAL ALTERATIONS OF *IN VITRO* GROWN PAULOWNIA (*PAULOWNIA TOMENTOSA* L) UNDER SALT STRESS

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Paulownia (*Paulownia tomentosa*) is an economically important tree, which is used in the manufacture of paper and furniture industry. Moreover, it considered a medicinal plant used for treating a diversity of diseases. Buds were cultured on Murashige and Skoog (MS) solid medium supplemented with benzyl amino purine (BAP) and kinetin (KIN). The resulting plantlets were cultured on MS medium containing different sodium chloride (NaCl) concentrations (50, 100 and 150 mM). The survival percentage and mean shoot length of plantlets were calculated after eight weeks. The results revealed that the survival percentage and mean shoot length were decreasing with increasing NaCl concentration. In order to provide a reference for investigating the mechanism of salinity resistance, variations of anatomy and chloroplast ultrastructure in the leaves of unstressed and stressed plantlets (50 and 150 mM NaCl) after 16 days from culture were examined under photonic and electron microscopy. The results showed that salinity treatments induced variations in leaf mesophyll, changes in vascular bundle, deformed trichomes, and appearance of starch granules in chloroplasts. The diversity in phenotypic and ultrastructure of leaves considered as genetic markers of paulownia plantlets against salinity.

Keywords: anatomy, *in vitro*, paulownia, salinity, ultrastructure

INTRODUCTION

Paulownia tomentosa L. is a member of the family Paulowniaceae. It is used as herbal medicine. Also, it considers a source of biologically active secondary metabolites (Fan et al., 2015; Cao et al., 2017 and Perry et al., 2021). Tissue culture technique offers valuable data to explain plant reply to salt stress. Micropropagation provides better control than *in vivo* growth conditions which illustrated the responses of shoot and root in the presence of stress (Shibli et al., 1992 and Sobieh et al., 2019). The micropropagation

technique of *Paulownia* species is a rapid resource of producing timbered biomass and planting store for afforestation, also it is an effective method to maintain the genetic gain (Park and Bonga, 1992). The success of micropropagation for several woody plants could be affected by altered factors such as plant growth regulators which are the most vital ones (Taha et al., 2008). Plants are exposed to several abiotic stresses, for instance salinity, drought, chilling, freezing and radiation, which could interrupt growth improvement and development, furthermore in severe cases may cause plant death (Krasensky and Jonak, 2012 and Kamran et al., 2020).

Salinity is a chief abiotic stress which considered one of the most harmful agents for the plant life cycle also imposing both osmotic and ionic stresses on plants (Jampeetong and Brix, 2009 and Alam et al., 2016). Additionally, salinity caused accumulation of reactive oxygen species (ROS), which producing an oxidative stress. Oxidative stress can cause DNA destruction, lipid peroxidation, and deactivate enzymes (Gill and Tuteja, 2010 and Youssef et al., 2020). Which affects plants at different levels including physiology, morphology, biochemistry and molecular pathways (Shavrukov, 2013 and Sobieh et al., 2019).

Cellular responses to stress contain alterations in cell cycle and cell division, adjustments in the membrane system and cellular organelles, as well as modifications in gene expression profiles (Sairam and Tyagi, 2004; De Oliveira et al., 2013 and Al-Safadi and Nakar, 2016). Additionally, adaptations of anatomical configurations are an acclimatization mechanism for the plant species (Grigore and Toma, 2007). The ultrastructure changes of chloroplasts were studied, which are a great significance organelle for plants. It executes photosynthesis by using carbon dioxide and water to produce different chemical components that are changed by the plant into sugars and other biomolecules (Chen et al., 2018 and Otegui, 2018). Moreover, it is involved in sensing and signaling stress to other cell compartments which lead to adaptations of growth and development of the plant (Chan et al., 2016 and Dogra et al., 2018). So, chloroplasts are the most sensitive organelles to abiotic stress and the modifications in ultrastructure of chloroplasts are considered as a biomarker to salinity (Zechmann, 2019). The target of this investigation is studying the modifications in cellular anatomy and the ultrastructure of leaves in *in vitro* propagated paulownia plantlets under salt stress, which would be suitable as stress marker.

MATERIALS AND METHODS

1. Plant Material

Paulownia tomentosa was obtained from the Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Terminal and lateral buds Buds were surface sterilized by washing with soap for 2 min then washing under tap water, subsequently dipping in 30% Clorox (containing 5% sodium hypochlorite) for 15 min, followed by three rinses in sterile distilled water.

2. *In vitro* Culture

The buds were cultured on 3.3 g l^{-1} Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.2 mg l^{-1} benzyl amino purine (BAP), 0.1 mg l^{-1} kinetin (KIN), 3% sucrose and 6 g l^{-1} agar. The pH of the culture medium was adjusted to 5.8 before autoclaving and the buds were incubated in a growth chamber at 25 °C ± 2 under 16 h photoperiod. The bud survival percentage (%) and mean shoot length (cm) and rooting were observed.

3. Salinity Treatments

Paulownia buds were cultured on 3.3 g l^{-1} MS medium supplemented with 0.2 mg l^{-1} BAP, 0.1 mg l^{-1} KIN, 3% sucrose, 6 g l^{-1} agar and containing different sodium chloride (NaCl) concentrations (50, 100 and 150 mM). The pH of the culture medium was adjusted to 5.8 before autoclaving and the buds were incubated in a growth chamber at 25 °C ± 2 under photoperiod 16 h. The bud survival percentage (%) and shoot length (cm) were recorded after 8 weeks of culture. Also, the abiltiy of shoots to form roots was observed. The medium without NaCl was served as control.

4. Microscopic Examination of Leaves

Specimens were prepared as determined by John and Lonnie (1998). One mm^2 of fresh leaves were fixed in 1% potassium permanganate for 5 min at room temperature, and then washed three times in distilled water for 15 min for each. Subsequently, specimens were dehydrated in graded ethanol (30% to 90%), then in absolute ethanol followed by passage through a graded propylene oxide ethanol series finally, maintained in pure propylene oxide. Dehydrated specimens were embedded in an epoxy resin composed of 20 ml dodecenyl succinic anhydride (DDSA) (hardener), 16 ml nadic methyl anhydride (NMA) (softener) and 8 ml 2, 4, 6-dimethylamin-ethylphenol (DMP) (accelerator). Finally, samples were polymerized in oven at 60°C for 48 h. Sections (1 µm) were cut and examination with Leica Microscope, Electron Microscope Unit, Center for Mycology and The Regional Biotechnology, Al Azhar University.

5. Scanning Electron Microscopy (SEM)

The fresh leaves samples were gold sputtered for 12 min by using the ion sputtering device model JEOL (JFC 1100 E). The sample surface was investigated by using scanning electron microscope JEOL-5400 in National Centre for Radiation Research and technology.

6. Transmission Electron Microscopy (TEM)

Fresh leaf specimens were prepared for TEM analysis according to John and Lonnie (1998). Sections (1 µm) were cut with Leica Ultra-microtome, mounted on copper grids and stained with 0.5% uranyl acetate and lead citrate for 15 min (for each) according to Reynolds (1963) in Electron Microscope Unit, Center for Mycology and the Regional Biotehnology, Al

Azhar University. Observations were examined by using JEOL TEM 100 CX, transmission electron microscope at80kV in National Centre for Radiation Research and Technology.

7. Statistical Analysis

The data were statistically analyzed using ANOVA analysis to determine the level of significant differences between means as compared to the control at $P \leq 0.05$ level of significance. The statistical software Costat (http://www.cohort.com/costat.html) was used for all analyses.

RESULTS AND DISCUSSION

1. *In Vitro* Propagation of Paulownia Under Salt Stress

Tissue culture is the ideal technique to simplify propagation and study salinity stress effects on paulownia. Paulownia *in vitro* grown shoots were cultured on solid MS medium supplemented with BAP and KIN and different salinity concentrations (50, 100 and 150 mM NaCl) to study morphological characters of shoots and the modifications in anatomical features and ultrastructure of young leaves. Morphological characters were negatively affected by increasing salinity levels in stressed shoots as compared with the unstressed. Salinity caused significantly decrease in the percentage of survival of micropropagated buds to 56.21, 24.32, 16.20 by a percentage of decrease of 40.45, 72.34 and 80.46% at 50, 100 and 150 mM NaCl, respectively, compared to the control. Moreover, the mean shoot length was significantly decreased with increasing salinity levels reaching 4, 2.45 and 0.61 cm, compared to the control. The results showed variances between unstressed shoots and all treatments of salinity, root growth more strongly than shoot growth. Increasing salinity concentrations leads to decrease in root formation. Additionally, the roots did not form at the concentration of 150 mM NaCl. So, 150 mM NaCl considered the lethal concentration. The concentration of 50 mM NaCl was the optimal concentration for paulownia plantlets survival under salinity and the sublethal concentration was 100 mM NaCl as shown in table (1) and (Fig. 1)

Table (1). Effect of different NaCl concentrations on bud survival and mean of shoot length in paulownia *in vitro* grown shoots.

NaCl concentrations (mM)	Bud survival percentage %	Mean shoot length (cm)
Control	96.66^{a}	$5.70^{a} \pm 0.23$
50	56.21^{b}	$4.00^{b} \pm 0.27$
100	24.32^{c}	$2.45^{c} \pm 0.16$
150	16.20^{d}	$0.61^{d} \pm 0.19$

Values (mean±SD) followed by different letters are significantly different at $P \leq 0.05$ level

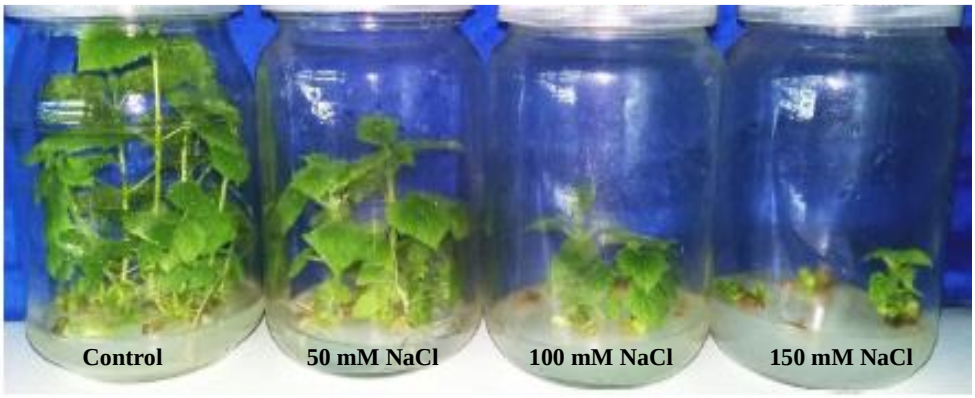

Fig. (1). Salinity concentrations impact on *in vitro* grown paulownia.

2. Effect of Salt Stress on the Anatomy of Leaves

Transverse sections of paulownia leaves illustrated the variations of morphological and anatomical features of the unstressed and stressed plantlets subjected to two salinity concentrations (the lowest concentration of 50 mM NaCl and the highest concentration of 150 mM NaCl). The leaf of unstressed plantlets was a symmetric and had a heterogeneous structure (Fig. 2 a, b, c and d). Furthermore, the semi sections of leaf under the salinity concentration of 50 mM NaCl illustrated that the epidermis was made up of a single layer of elongated cells that were closely packed. Cuticle was being thicker than control. Stomata were closed and surrounded by a pair of smaller guard cells than control. Fig. (2e, f, g and h) show that the mesophyll thicknesses increased due to the elongated palisade parenchyma cells and spongy parenchyma cells were more elongated with irregular shape. Both palisade parenchyma and spongy parenchyma contained less chloroplast compared with control. The intercellular spaces were small compared with control, which led to reduction of the size of mesophyll cells. Vascular bundles were surrounded by irregular parenchymatous cells and xylem in vascular bundles was more than control. Moreover, the semi section under concentration of 150 mM NaCl showed that the epidermis was made up of a single layer of elongated cells compared with control. Cuticle layer was being thicker than under 50 mM NaCl concentration and control. Stomata were surrounded by a pair of smaller guard cells than control. The palisade parenchyma cells were more vertically elongated cylindrical cells in one layer. Spongy cells were irregularly shaped. The intercellular spaces were smaller than under 50 mM NaCl concentration and control. Vascular bundles were surrounded by irregular parenchymatous cells more than under 50 mM NaCl and xylem in the vascular bundles was less than under 50 mM NaCl concentration and control. The phloem cells were more compared to 50 mM NaCl concentration and control as illustrated in fig. (2i, j, k and l).

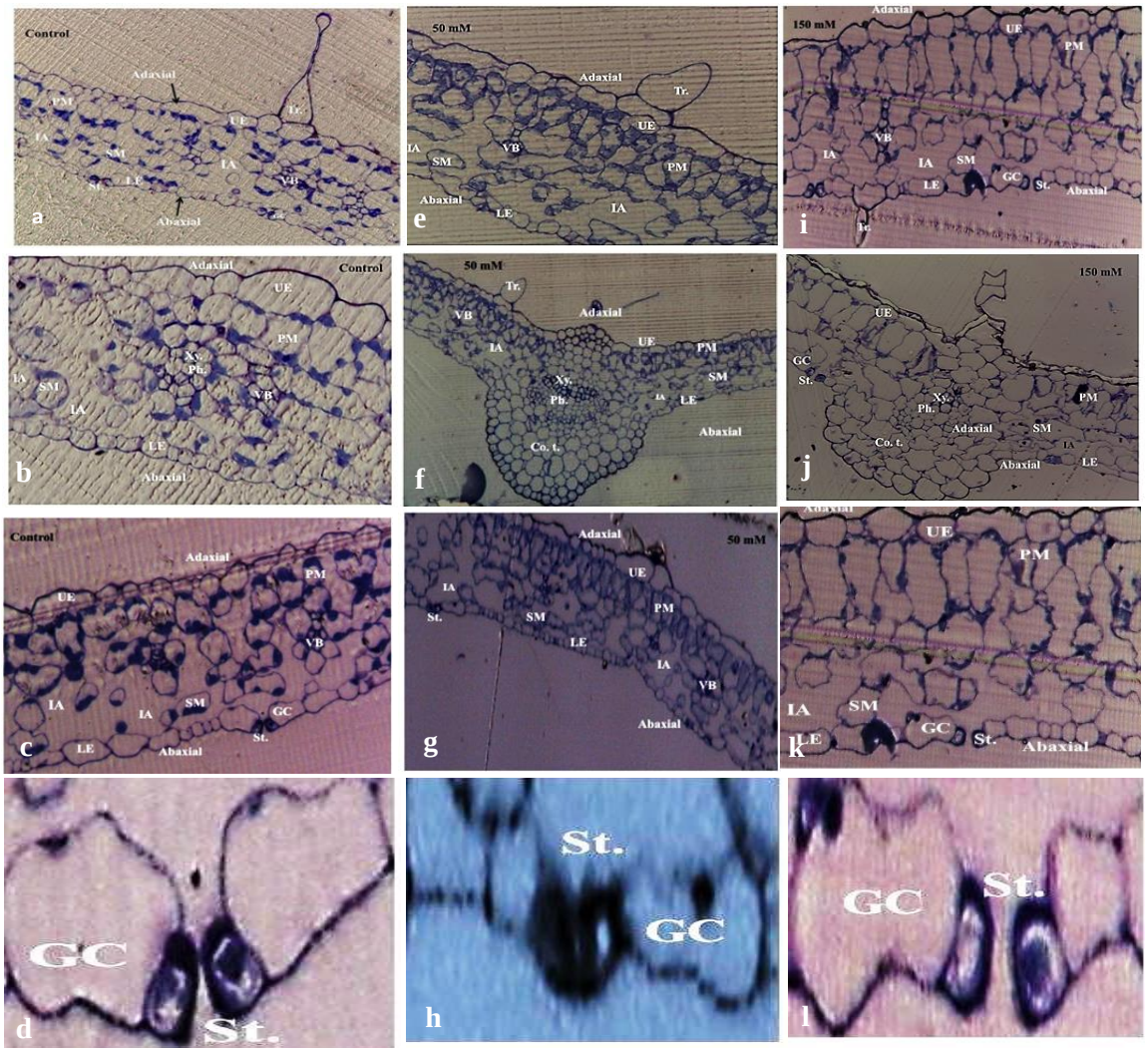

Fig. (2). Cross sections of paulownia unstressed leaf (a, b, c and d), and stressed leaves with salinity treatments of 50 mM NaCl (e, f, g and h) and 150 mM NaCl (i, j, k and l) . [IA: Intercellular airspace, Co.t: collenchyma tissue, GC: guard cell, LE: lower epidermis, Ph: phloem, PM: palisade mesophyll, SM: spongy mesophyll, ST: stomata, TR: trichome, UE: upper epidermis, VB: vascular bundle, X: xylem].

3. Scanning Electron Microscopy of Leaves Under Salt Stress

Trichomes and their traits can be an important source of information for plant identification and as a taxonomic tool (Antipin and Choob, 2019). The structure of trichomes of paulownia plantlets is a key for thoughtful how these plantlets adjusted to the stresses, they are known as general adaptive responses of plants to survive under biotic and abiotic stresses. Trichomes of unstressed plantlets were hair- or scale-like extensions of the epidermis. As shown in the SEM sections (Fig. 3). The structure of trichomes of paulownia leaves was capitate, straight, un-branched glandular with a one celled head. The unicellular base was arisen from epidermal cells. Trichomes had long stalk consisted of three long cells. The capitate glandular trichomes commonly had rounded to pear shaped heads (Fig. 3). On the other hand, salinity affected trichomes structure, salt concentrations of 50 mM NaCl and 150 mM NaCl induced a decrease in length of stalk cells compared to control and deformed the basal cell. Moreover, the cell of stalk no. 3 became thin and long in 150 mM NaCl of salt stress compared to control and concentration of 50 mM NaCl (Fig. 3).

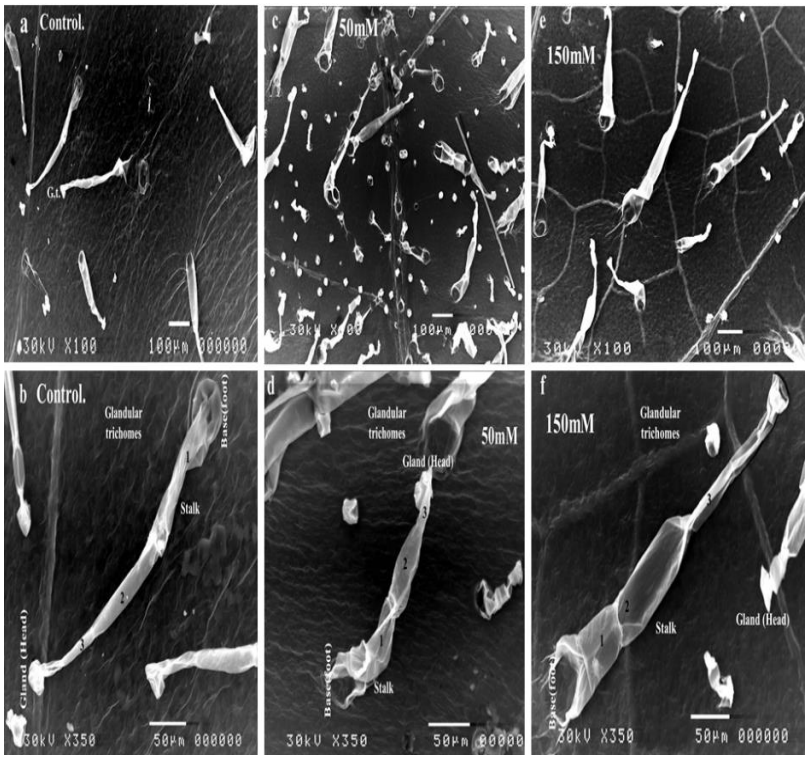

Fig. (3). Scan electron micrographs of glandular three-celled trichomes of paulownia leaf surface of (a & b) un-stressed plantlets, (c & d) deformed trichomes under 50 mM NaCl and (e & f) deformed trichomes under 150 mM NaCl.

4. Transmission Electron Microscopy of Leaves Under Salt Stress

The ultrastructure of leaves of unstressed and stressed paulownia plantlets showed the change in structure of some internal organs in the cells. Electron micrograph of unstressed leaves illustrated the majority of mesophyll cells and chloroplasts. There was a large intercellular space between mesophyll cells (Fig. 4). Under salinity, plasmolysis in some cells was attended by a reduction in mesophyll intercellular spaces. Plastids implement many vital utilities in plant metabolism including photosynthesis, synthesis of metabolites, and stress signaling. So, under salt concentration of 50 mM NaCl, disturbance of chloroplasts in mesophyll cells was occurred. At the highest salinity concentration of 150 mM NaCl, a number of cells seemed to be linked together without spaces compared with control as seen in fig. (5g). At both concentrations, lower number of chloroplasts and few small starch granules in chloroplasts were appeared as seen in fig. (5).

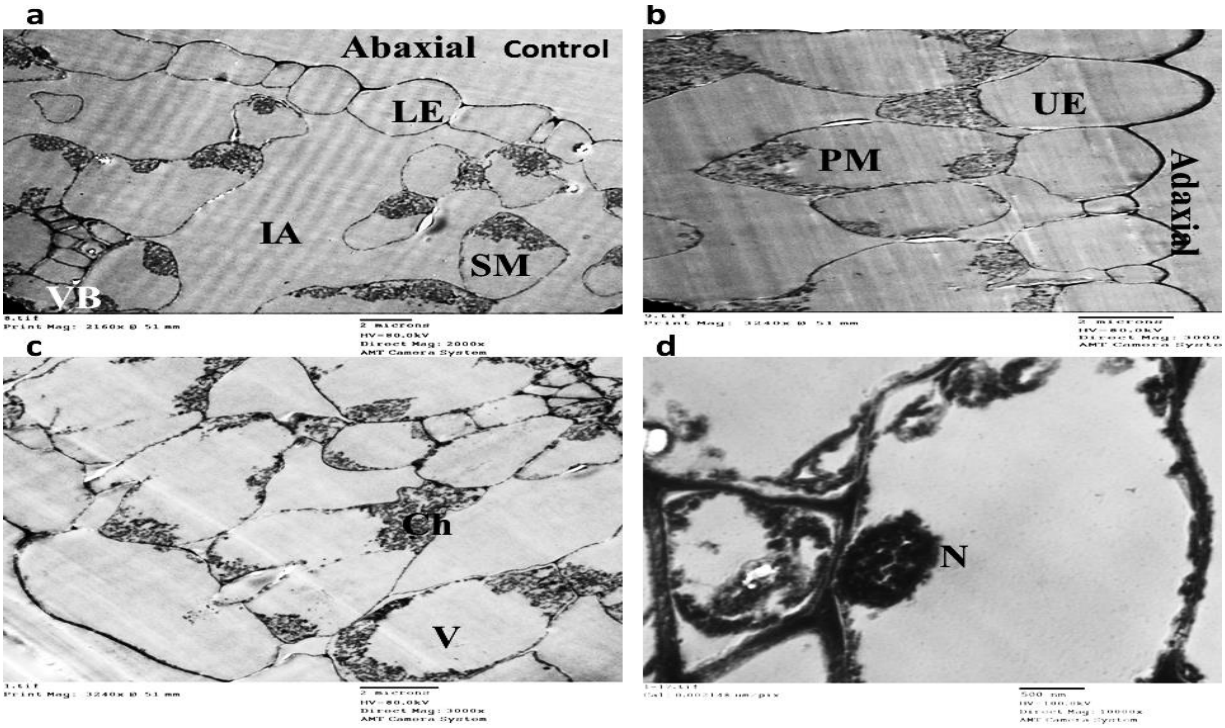

Fig. (4). Ultrastructural aspect of the leaf cells of control paulownia plantlets. [ch: chloroplast, GC: guard cell, IA: intercellular airspace, LE: lower epidermis, N: nucleus, PM: palisade mesophyll, St. g.: starch granule, SM: spongy mesophyll, ST: stomata, TR: trichome, UE: upper epidermis, V: vacuole, VB: vascular bundle].

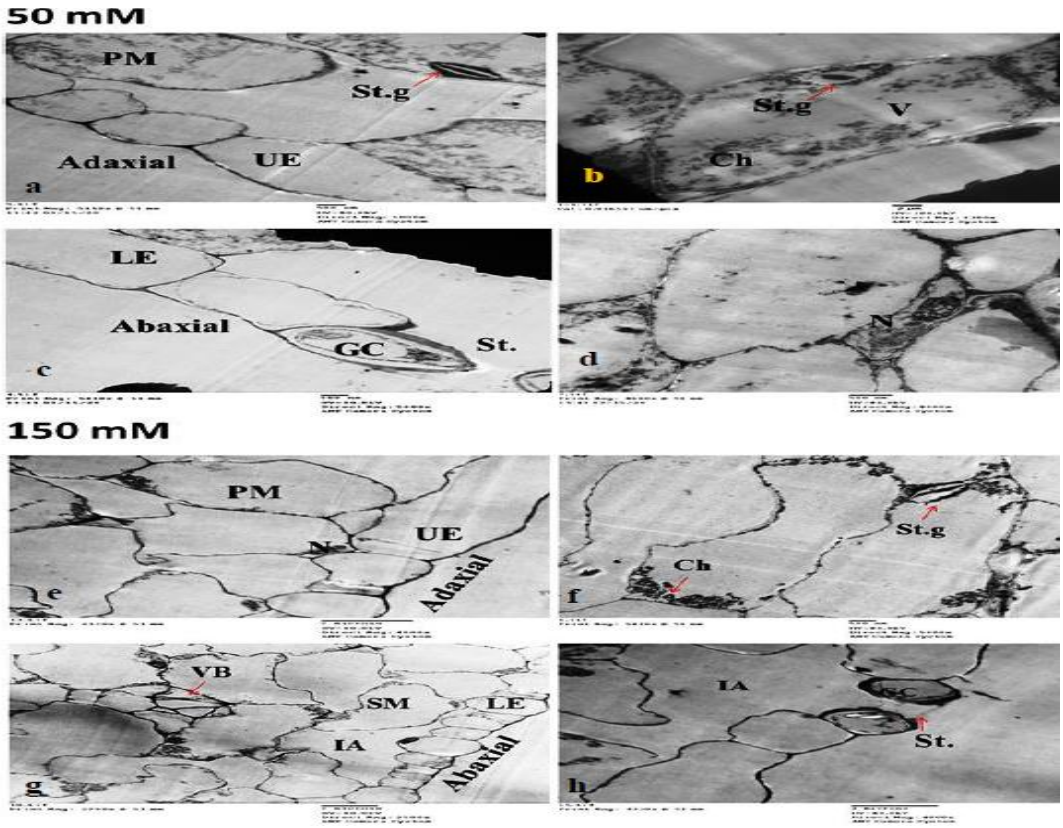

Fig. (5). Ultrastructural aspect of the leaf cells of stressed paulownia plantlets grown *in vitro* under 50 mM NaCl and 150 mM NaCl. [ch: chloroplast, GC: guard cell, IA: intercellular airspace, LE: lower epidermis, N: nucleus, PM: palisade mesophyll, St. g: starch granule, SM: spongy mesophyll, ST: stomata, TR: trichome, UE: upper epidermis, V: vacuole, VB: vascular bundle].

Tissue culture technique provides a powerful tool to study essential processes in plants. One of the most chief advantages of tissue culture is the production of insect and virus free plants Shukla et al. (2017). Abiotic stress, especially salinity stress is considered as the most serious growth preventive factor (Vinocur and Altman, 2005). The addition of NaCl to the culture media of paulownia caused depression in the osmotic potential of the media causing adverse effect as shown also in other plants growth as documented by Pour et al. (2009) and Aazami et al. (2010). In this study, by increasing NaCl levels,the morphological characters as bud survival, mean shoot length and root formation in paulownia plantlets were decreased. Plant growth and development is unfavorably affected by salinity. Jbir-Koubaa et al. (2015)

stated that initial signs of salt toxicity are observed in the root system, where the reduction in root growth was severely reduced due to salinity stress. Where Na^+ is a major ion causes the lessening of plant growth because of its ion toxicity. Furthermore, may vary plant's physiology and eventually causing plant death Soltabayeva et al. (2021). Moreover, salt stress causes complex changes in interactions of genes as reduction in photosynthesis efficiency, chlorophyll, total protein, biomass, stomata closure and increasing the oxidative stress (Gupta and Huang, 2014). Salinity tolerance is a polygenic trait, which has a range of variation by genetic and environmental factors, hence acts the phenotypic responses of plants under salinity stress (Al-Ashkar et al., 2019). Salt stress causes complex changes in plants through interactions of genes. The modified genetic regulation perturbs metabolic balance. Plants usually develop salt resistance mechanism and unique structures to continue under high saline-stress conditions (Roy et al., 2014 and Shabala et al., 2014). Therefore, to simplify the identification of saline tolerance mechanisms, the structural variations, ion distribution and physiological alterations in crop plants induced by salinity should be understanded (Roy et al., 2014).

The changes produced in leaf anatomy and ultrastructure of leaf cells is a suitable way to study saline tolerance mechanisms. Moreover, the leaf is the best adjustable organ in its response to environmental conditions (Marchi et al., 2008). Leaf structures reflect the effects of water stress more noticeably than those of stems and/or roots (Ennajeh et al., 2010). In this study, the mesophyll thickness increased due to greater spongy layers and increase in palisade cells length, these results agree with David and Parks (1979) on cotton and Atriplex and Hussein et al. (2012) on Jatropha. On the other hand, palisade cells and spongy layers stayed constant or had slight effect at the lower salinity concentration compared with the un-salinized plantlets (Hussein et al., 2012). The high accumulation of Na^+ and Cl^- ions by increasing salinity levels led to water loss of cell, the intercellular spaces of cell decreased with the increase of external salt concentration and plasmolysis occurred, which is in the same line with Navarro et al. (2007) and Gao et al. (2015) . Stomata play an essential role in the regulation the water and carbon cycle between the plants and the atmosphere (Weng et al., 2011). Salt stress negatively affects relative water content, leaf stomata/conductance, closure of stomata and transpiration (Fidalgo et al., 2004 and Özyiğit and Akinci, 2009). Stomata is another minor structure on plant leaves that orients plant to adjust to diverse stress through the opening and closing of the stomatal pore (Schlüter et al., 2003). Stomata replied speedily to the environmental variations by decreasing their dimensions and areas (Mehri et al., 2009). Also, Imtiyaz et al. (2012) stated that alterations of stomata aperture can be used for survival of mutagenic progeny in *in vitro* and *in vivo* systems.

Glandular trichomes are epidermal appendages with greatly flexible morphology and responsible for the creation of secretion with economic, medicinal and ecologic values (Argyropoulou et al., 2010 and Tozin et al.,

2015). Trichomes originate from the surface epidermal cell tissue of the plants. Trichomes are the first line of defense for plants and can play a key role between plants and biotic or abiotic stresses. The special structures and physiological functions of trichome are reflectance, energy balance, ultraviolet protection, abiotic resistance, gas exchange, insect resistance and disease resistance. They are essential for the growth and development and they have many important biological functions. Moreover, trichomes offer a model structure for studying the regulation of plant cell diversity. These results agree with the results of Yamane et al. (2003 and 2008), they found that ultrastructure of leaf cells was damaged by salinity. Yamane et al. (2003 and 2008) stated that variations observed in plastids are consistent with circumscribed organelle differentiation in the attendance of salt. The structural integrity of chloroplast is a foundation of photosynthesis process for plants. Therefore, salt stress reduces photochemical effectiveness and electron transport due to changed structure of thylakoids (Parida et al., 2003). Under unfavorable growth environments, the formation of grana is prevented by inhibition of protein synthesis, as well as the formation of plastid ribosomes and chlorophyll accumulation (Abdelkader et al., 2007). Moreover, chloroplasts are few in number and less developed in vascular tissues to decrease oxidative damage (Hameed et al., 2021).

In this investigation, the accumulation of starch granules in the chloroplast appeared in salinity concentrations of 50 mM NaCl and 150 mM NaCl, which agrees with Rahman et al. (2000). Also, Gao et al. (2015) reported in potatoes that salinity stress leads to a decrease in the chloroplast number, intercellular spaces, and rupturing of the cell wall. Moreover, the high external NaCl gradually decreases the size mesophyll cell, disorganization of chloroplast and starch takes place. The accumulation of starch granules in the chloroplast recognized to the destruction of the sucrose-phosphate synthase in the cytosol, leading the triose phosphate pathway towards starch synthesis or, to the damage of enzymes involved in starch degradation through variations in the ionic composition in the chloroplast. Additionally, these numerous responses involved in the synthesis of starch and sucrose that are regulated by orthophosphate concentration (Preiss, 1984 and Rahman et al., 2000).

At the ultrastructural level, the chloroplast structure presumably alterations to affect photosynthesis, resulting in increased starch in leaves, suppression of nitrate reductase activity and reduced growth (Ghosh et al., 2001). The acclimation of plant cells grown under salinity requires not only the accumulation of osmotically active solutes of low molecular mass, but also the accumulation of starch (Hasegawa et al., 2000). Starch accumulation under salt conditions was also observed in NaCl acclimated citrus cell line (Ferreira and Lima-Costa, 2008) and it is tempting to speculate that starch synthesis plays a role in moderating the osmotic condition. To conclude, Salinity had a negative impact on growth rate and ultrastructure of paulownia plantlets.

These ultrastructure changes are well suited as a stress marker for plants to salinity as an abiotic stress.

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التغيرات التشريحية في نبات الباولونيا النامي معمليًا تحت تأثير الملوحة

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الباولونيا هي شجرة اقتصادية مهمة تستخدم في صناعة الورق والأثاث. علاوة على ذلك، فهي تعتبر من النباتات الطبية المستخدمة في علاج مجموعة متنوعة من الأمراض. تم زراعة البراعم على بيئة موراشيج وسكوج مضاف إليها بنزيل أدنين وكينيتين. تمت زراعة الشتلات الناتجة على بيئة موراشيج وسكوج تحتوي على تركيزات مختلفة من كلوريد الصوديوم (٥٠، ١٠٠ و١٥٠ مللي مولار). أوضحت النتائج أن نسبة حيوية البراعم ومتوسط طول النبيتات تتناقص مع زيادة تركيز كلوريد الصوديوم وذلك بعد حساب نسبة حيوية البراعم ومتوسط طول النبيتات بعد ثمانية أسابيع من الزراعة وذلك للتأكد من آلية مقاومة النباتات للملوحة. ثم فحصت الاختلافات في تشريح البنية الخلوية للورقة والتركيب الدقيق للبلاستيدات الخضراء في أوراق النباتات غير المعاملة والمعاملة بتركيزات الملوحة (٥٠ و١٥٠ مللي مولار من كلوريد الصوديوم) بعد ١٦ يومًا من الزراعة تحت المجهر الضوئي والإلكتروني. أظهرت النتائج أن تركيزات الملوحة تسببت في ظهور تغيرات في النسيج الوسطي للأوراق، تغيرات في الحزم الوعائية، تشوه الشعيرات، وظهور حبيبات النشا في البلاستيدات الخضراء. يعتبر التنوع في النمط الظاهري والتركيب الدقيق للأوراق بمثابة علامات وراثية لنباتات الباولونيا لتحمل الملوحة.