

Salt Stress Responses and Protein Expression Changes in Tomato (*Lycopersicon esculentum* Mill.)

I. I. Hamma¹, A. A. Aliero¹, H. M. Maishanu¹, and A.Y. Abbas²,

¹Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto.

²Department of Biochemistry, Usmanu Danfodiyo University, Sokoto.

ABSTRACT

Tomato is a model plant for studying the mechanisms of plant salt tolerance. Responses of Tomato (*Lycopersicon esculentum* Mill) to various concentrations of salt and changes in some protein expression levels were investigated. Seeds of three (3) varieties of tomato namely, Roma vf, Rio Grande and UC 82 B were germinated on a nursery bed and seedlings were transferred to polythene bags supplemented with 0, 20, 40 and 60 mM NaCl. After 24 days, growth parameters, floral number, free Proline content, Glycine betaine content, shoot dry weight were measured using standard methods and procedures. Leaf samples were collected for protein extraction and the alterations in expression levels induced in leaf proteins under salt stress treatments were studied by one-dimensional SDS-PAGE. Increasing concentration of NaCl decreased in all growth parameters (floral number, number of leaves, plant height and dry weight) evaluated. Free Proline and Glycine betaine content increased significantly ($P < 0.05$) in salt-treated plants compared with the control. The content ranges from 1.56-1.99 mM in Roma vf, 1.32-1.99 mM in UC 82 B, and 1.77-1.99 mM, 1.95-1.99mM in Rio Grande. SDS-PAGE showed up-regulation of at least nine proteins with a molecular weight of 46, 35, 25 and 12 kD in leaves of UC 82 B, 45, 25, 19 and 17 kD in leaves of Roma vf and 38 kD in leaves of Rio Grande. At least three proteins with a molecular weight of 29 kD in leaves of Roma vf and 45 and 53 kD in leaves of Rio Grande were down-regulated. From the results of this study, it was observed that Tomato Roma vf is more tolerant to salt stress followed by UC 82 B and the Rio Grande, and therefore could be selected as the salt-tolerant variety to ameliorate production loss.

Keywords: *Lycopersicon esculentum* - salt stress - proteome - SDS-PAGE

INTRODUCTION

Saline soils, is a major environmental issue, as it limits plant growth and development, causing loss of productivity, especially in crop species that are glycophyte (Hasegawa *et al.*, 2000; Qadir *et al.* 2008). Salt-affected soils are characterized by excessively high levels of water-soluble salts, including sodium chloride (NaCl), sodium sulfate (Na_2SO_4), calcium chloride (CaCl_2) and magnesium chloride (MgCl_2), among others (Tanji, 2002). NaCl has a small molecule size and when oxidized by water, produce sodium ions (Na^+) and chloride ions (Cl^-), which are easily absorbed by the root cells of higher plants and transferred to the whole plant using xylem uploading channels (Rodriguez- Navarro and Rubio, 2006). Salinity problem has been aggravated by the requirement of irrigation for crop production in arid and semiarid environments. Salt stress in plant cells is primarily caused by a combination of osmotic and ionic stress resulting from high Na^+ concentration in the soil (Hasegawa *et al.*, 2000). Metabolic acclimation via the accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Shabala and Cuin, 2006). Many plant species accumulate significant amounts of glycine betaine, proline, and polyols in response to high salinity (Di Martino *et al.*, 2003). Multiple functions for these compounds have been suggested. In addition to the conventional role of these compatible solutes in cell osmotic adjustment (Bray, 1993), they are also suggested to act as low molecular- weight chaperones, stabilizing the

photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS (Noiraud *et al.*, 2001). Tomato (*Lycopersicon esculentum* Mill.) belongs to the family *Solanaceae* or Nightshade and the Genus *Lycopersicon*. It is one of the most widely used vegetables in the world. It is an annual plant, which can reach a height of over two metres. Economically, tomato requires relatively cool weather for optimum yield (Rodriguez, 2007). Tomato is an important commercial crop in the world, has a nutritional value which makes it a widely accepted vegetable by consumers (Ijato *et al.*, 2010). Tomatoes contribute to a healthy, well-balanced diet.

This research aimed at investigating the salt stress responses of three (3) varieties of Tomato at a morphological, biochemical and molecular level and to identify a variety that is more tolerant, so that it could be selected as the salt-tolerant variety to ameliorate production loss.

MATERIALS AND METHODS

The study was conducted in the Biological Garden, Department of Biological Sciences and Central Laboratory, Usmanu Danfodiyo University, Sokoto-Nigeria. The seeds of tomato (*Lycopersicon esculentum*) namely Roma vf, Rio Grande and UC 82B were obtained from Sokoto Agricultural Development Programme (S.O.S.A.D.P), Sokoto State, Nigeria.

Plant Growth Condition

Seeds were surface-sterilized by soaking in 5% (v/v) sodium hypochlorite solution for 15 min followed by three washes with sterile distilled water. Viable seeds were first sown in a nursery bed for 3 weeks (21 days), after which uniformly germinated seedlings were transplanted into polythene bags containing the soil. Sodium chloride (NaCl) was weighed and dissolved in distilled water to make 20, 40 and 60 mM solutions of salt which were used to water the plants. The solutions were stored in airtight containers to prevent evaporation. The seedlings of Tomato were divided into four (4) treatments: the first represented the control where no NaCl was added to the water solution, the second, third and fourth treatments received 20, 40 and 60 mM NaCl respectively. The treatments were for 24 days. Each treatment was replicated three (3) times and each replicate consists of three (3) plants.

Evaluation of Floral and Vegetative parameters

After 24 days of salt treatment, the seedlings were harvested. Floral counts (number of flowers), number of leaves, shoot length (cm) and dry mass DM (g/plant) of plants were determined. For floral number, matured flowers borne by plants of the same treatment were counted. Shoot length was measured using meter rule at the horizontal length of the shoot. For dry mass determination, shoots and roots were left in desiccators at 80°C for 2 days and parameters were computed according to the formulas of Hunt (1981).

$$\text{Parameters/plant} = \frac{\text{Total number of parameter in treatment}}{\text{Number of plants in the treatment}}$$

Determination of Free Proline Accumulation

Extraction and determination of free proline were performed according to the methods of Bates *et al.*, (1973). Ground samples (1g) of plant material was extracted with 3% sulphosalicylic acid and filtered through Whatman No.1 filter paper and the extract (2 ml) was held for 1 hour in boiling water by adding 2 ml ninhydrin and 2 ml glacial acetic acid, after which cold toluene (4 ml) was added. Proline content was measured by a spectrophotometer at 520 nm and was calculated as $\mu\text{Mol g}^{-1}\text{DW}$ against standard proline.

Determination of Glycine Betaine Accumulation

Extraction and determination of betaine were carried out according to the method of Grieve and Maas (1984). Betaine was extracted by stirring finely ground-dried sample with demineralised water at 100°C for 1 hour. Betaine content was determined spectrophotometrically after reaction with potassium iodide (KI-I₂) at 365 nm.

Protein extraction for SDS-PAGE.

Approximately 1g of the fresh leaf was homogenized first in 5ml liquid nitrogen. Then, 2ml of extraction buffer (50mM Tris-HCl, 1mM PMSF, 2mM EDTA, 1mM 2-mercaptoethanol, pH 7.5) was added followed by stirring at 4°C for 5min. The homogenate was centrifuged for 25 min at 14000 rpm at 4°C. The supernatant was collected and mixed with 1ml of cold acetone. Then samples were kept at -80°C For 5min followed by centrifugation for 15 min at

14000 rpm at 4°C. The supernatant was then removed, and the pellets were air-dried at room temperature. Thereafter, the pellets were re-suspended in 1ml of extraction buffer and centrifuged for 5 min at 14000 rpm at 4°C (Amini *et al.*, 2007). Finally, the supernatant was collected for protein assay and the quality of protein was checked using ultraviolet absorption method and then SDS-PAGE.

SDS-PAGE

SDS-PAGE was carried out in 12.5% separating gel and 5% stacking gel according to the method of Hames and Rickwood (1990). Gels were run at the ambient temperature of (20°C) for 4 hours at a constant power of 80 mA until the tracking dye reached the end of the gel. After electrophoresis, the gel was stained with Coomassie blue according to the method of Reed *et al.* (1998). The relative molecular weight of each protein was determined using a standard protein marker.

Statistical Analysis

The result was expressed as a mean of three replicates and the data were subjected to one-way analysis of variance (ANOVA) test. Differences between means were determined by Tukey HSD All-Pairwise Comparisons test using Statistics 8.0 Statistical Software (Statistix 2007).

RESULTS AND DISCUSSION

The results for the salt stress responses of the three (3) different varieties of Tomato (*Lycopersicon esculentum* Mill.) namely Roma vf, Rio Grande and UC 82 B to different salt concentrations are summarized in Figures 1-5 and Table 1. In this work, reductions in plant height, number of leaves, number of flowers and dry weight were observed in the salt-stressed plants. NaCl treatment resulted in an overall decrease in growth measurements of all the cultivars also proline and glycine betaine accumulation were observed. In Tomato Roma vf, salinity stress significantly ($P < 0.05$) decreased the floral number. The control had the highest number of flowers averaging 15.3 per plant followed by 20 mM which averaged 5.3 flowers per plant while 40 mM and 60 mM treatments had 2.6 and 1.6 flowers/plant respectively (Fig. 1). However, comparisons of the means indicated that 40 mM and 60 mM treatments were not significantly different ($P > 0.05$). In Tomato UC 82 B, salinity stress also significantly ($P < 0.05$) decreased the floral number. The control had the highest number of flowers averaging 10.3 per plant followed by 20 mM which averaged 5.3 flowers per plant while 40 and 60mM treatments had averaging 1.3 flowers each per plant (Fig. 1). However, comparisons of the means indicated that 40 mM and 60mM treatments were not significantly different ($P < 0.05$). In Tomato Rio Grande salinity stress significantly ($P < 0.05$) decreased the floral number. The control had the highest number of flowers 5 per plant followed by 20 mM which averaged 2.3 flowers per plant while 40 and 60mM treatments had averaging 1.6 and 1 per plant respectively (Fig. 1). However, comparisons of the means indicated that 20mM, 40mM and 60mM treatments were not significantly different ($P > 0.05$). The number of flowers in all the salt-stressed cultivars of tomato (20-60 mM NaCl) was significantly lower than the control (0mM) plants. It was

shown that the number of flowers decreases with increasing concentration of salt. Gumi *et al.* (2013) reported the same results about the effect of salinity on flowering.

In Tomato Roma vf, the number of leaves per plant was markedly affected by salinity stress. The control (0mM) had the highest number of leaves (138.3/plant) followed by 20mM treatments with 75/plant. 40mM and 60mM treatment recorded 62.3 and 32 leaves per plant respectively (Fig. 2). Comparison of the means for the four different treatments revealed that there was a statistically significant difference between 0mM, 20mM and 60mM treatments but there were no statistically significant differences between 20mM and 40mM treatments. In Tomato UC 82 B, the number of leaves per plant was also markedly affected by salinity stress. The control (0mM) had the highest number of leaves (149.3/plant) followed by 20mM and 40mM treatments having 84.6 and 56.3 per plant respectively. 60mM treatment recorded 26 leaves per plant (Fig. 2). Comparison of the means for the four different treatments revealed that there was a statistically significant difference ($p < 0.05$) between the control and the three other treatments i.e 20mM, 40mM and 60mM. but there was no statistically significant differences ($p > 0.05$) between the three treatments i.e 20mM, 40mM and 60mM. In Tomato Rio Grande, the number of leaves per plant was markedly affected by salinity stress. The control (0mM) had the highest number of leaves (84.6/plant) followed by 20mM treatments with 56/plant. Treatment with 40mM and 60mM salt recorded 31.3 and 14 leaves per plant respectively (Fig. 2). Comparison of the means for the four different treatments revealed that there was statistically significant difference ($p < 0.05$) between 0mM and 20mM treatments but there was not significantly different ($p > 0.05$) between 40mM and 60mM treatments. These results corroborate the findings of Dolatabadian *et al.* (2011) while studying *G. max* observed that salinity stress significantly decreased leaf number, plant height. Elizamar *et al.* (2008) also observed the same in young umbu plants. This is because, in plants, where Na^+ and Cl^- build up in the transpiring leaves over a long period of time, the resulting high salt concentration may lead to leaf death. Leaf injury and death are attributed to the high salt load in the leaf that exceeds the capacity of salt compartmentation in the vacuoles, causing salt to build up in the cytoplasm to toxic levels (Munns, 2002, 2005; Munns *et al.*, 2006).

In Tomato Roma vf, with increasing concentration of salt, the height of the plant (cm) decreases. 60 mM treatment has the least plant height (30.3cm), while the control has the highest height (74.7cm). Treatment with 20 mM and 40 mM salt recorded (45cm) and (42.3cm) respectively (Fig. 3). The results between control (0mM) and 60mM differ significantly ($P < 0.05$) while there is no significant different ($p > 0.05$) between 20mM and 40mM. In Tomato UC 82 B, the height of the plant (cm) also decreased with increasing concentration of salt. The 60mM treatment has the least plant height (31.13cm), while the control had the highest height (73.46cm). The 20mM and 40mM recorded 40.13cm and 35.53cm respectively (Fig. 3). The results differ significantly ($P < 0.05$) between the control and the order treatments but there was no significant difference ($p > 0.05$) between treatment groups. In Tomato Rio Grande, the height of the plant (cm) also decreases with increasing

salt concentration. The control (0mM) has the highest length (48.2cm), while 60mM treatment has the least plant height (10.9cm). 20mM and 40mM recorded 34.1cm and 17.4cm respectively (Fig. 3). The results differ significantly ($P < 0.05$) between control and 40mM, 60mM treatments. This is consistent with the findings of Babu *et al.*, (2012) on Tomato cultivar pkm1; Shabani *et al.* (2012) on cherry Tomato; Hasanuzaman *et al.* (2009) in *Oryza sativa* and Elizamar *et al.* (2008) in young Umbu plants. In salt stress condition, reduced plant height may result from the toxicity of Na^+ and Cl^- . Disorder in translocation and distribution of minerals especially K^+ and Ca^{2+} can be another reason for growth reduction (Khalafalla *et al.*, 2010).

In Tomato Roma vf, the dry matter (g/plant) was reduced by increasing concentration of salt stress. The control (0mM) shows the highest dry matter (3.9 g/plant) followed by 20mM (1.9 g/plant) while 40mM and 60mM had 1.5 g/plant and 1.3 g/plant respectively (Fig 4). The result differs significantly ($P < 0.05$) between control (0mM) and the order treatments but there was no significant difference between treatments. In Tomato UC 82 B, the dry matter (g/plant) was also reduced by increasing concentration of salt stress. The control (0mM) shows the highest dry matter (2.8 g/plant) followed by 20mM (1.4 g/plant) while 40mM and 60mM had 1.3 g/plant and 1.0 g/plant respectively (Fig 4). The result differs significantly ($P < 0.05$) between control (0mM) and 60mM but there was no significant difference between. In tomato Rio Grande, the dry matter (g/plant) was also reduced by increasing concentration salt. 60mM treatment had the lowest dry matter (0.9 g/plant) while the control (0 mM) has the highest dry matter with (2.2 g/plant). 20mM and 40mM 1.5 g/plant and 1.4 g/plant. The result differs significantly ($P < 0.05$) between control (0mM) and 60mM but there was no significant difference between treatments. A similar result was reported by Gumi *et al.* (2013) and Amini *et al.* (2007) on tomato and Babu *et al.* (2012) also on tomato cultivar pkm1.

Effect of salt concentration on free proline ($\mu\text{Mol g}^{-1}$) and glycine betaine ($\mu\text{Mol g}^{-1}$) content of the three (3) varieties of tomato is presented in Table 1. The result revealed that there was significant difference ($p < 0.05$) in Roma vf proline content ($\mu\text{Mol}^{-1}\text{g}$) between control (0.52) and 20 mM (1.61) treatment and also Glycine betaine content ($\mu\text{Mol}^{-1}\text{g}$) shows significant difference ($p > 0.05$) between control (1.25) and 20 mM (1.56) concentrations. The results also show that there was a significant difference ($p < 0.05$) in Tomato UC 82 B proline content between control (0.58) and 20 mM (1.32) except for 40, and 60 mM shows no significant difference. However, in Glycine betaine content there was a significant difference ($p < 0.05$) between the control (1.26) and 60 mM (1.98). The results show that in Rio Grande proline content, there was a significant difference ($p < 0.05$) between the control (0mM) and other treatments but there was no significant difference ($p < 0.05$) among the treatments i.e (20, 40 and 60mM). There is also a significant difference ($p < 0.05$) in Glycine betaine content between the control (1.38) and 20 mM (1.87). The accumulation of osmolytes such as proline (Pro) is a well-known adaptive mechanism in plants against salt stress conditions. It has also been suggested that proline

accumulation can serve as a selection criterion for the tolerance of most species to stressed conditions (Ashraf and Foolad, 2007; Ahmad *et al.*, 2009). Since the first report on proline accumulation in wilting perennial ryegrass (Kemble and MacPherson 1954), a number of research works have been carried out concerning the role of proline as a compatible osmolyte and osmoprotectant and its roles in salt stress tolerance. Several studies have attributed an antioxidant feature to proline suggesting ROS scavenging activity and proline acting as a O₂ quencher (Matysik *et al.*, 2002). However, the content of free proline accumulation in all the cultivars and among the treated plants did not differ significantly but there is a significant difference between the treated and the control plant (0mM NaCl). This shows that there is higher proline accumulation in the stressed plants as compared to unstressed plant and therefore, free proline accumulated in response to salt stress. This finding is in line with the reports of Gumi *et al.* (2013); Ahmed *et al.* (2010); Deivanai *et al.* (2011); and Hogue *et al.* (2007). Glycine betaine (GB) is a small organic metabolite soluble in water and non-toxic at high concentrations which can potentially play a protective role against salt stress (Ashraf and Foolad, 2007; Chen and Murata, 2008). The major role of GB in plants exposed to salt is probably protecting cells by osmotic adjustment (Gadallah, 1999), protein stabilization (RuBisCo) (Makela

et al., 2000), photosynthetic apparatus protection (Allakhverdiev *et al.*, 2003; Cha-Um and Kirdmanee, 2010), and reduction of ROS (Ashraf and Foolad, 2007). These observed levels of glycine betaine (GB) content are in line with those reported by Cha-Um and Kirdmanee (2010) in *O. sativa* and Gumi *et al.* (2013) in tomato. Different concentration of NaCl on tomato plant revealed that salinity not only inhibits plant growth but also changes the protein levels. For SDS-PAGE, proteins were extracted from leaves of salt-treated and salt-untreated plants. Comparing the protein profiles between control plants and those treated with different salt concentrations using SDS-PAGE showed that NaCl treatment induced only a few changes in the expression pattern of proteins in all the cultivars. It was found that the intensity of the bands for 35, 25, and 12 kD proteins in leaves of UC 82 B (Fig. 5a), 45, 19 and 17 kD proteins in leaves of Roma vf (fig. 5b), and 38 kD proteins in leaves of Rio Grande (Fig. 5c) were increased in salt-treated plants. In contrast, the intensity of some other protein bands (e.g. 29, 45 and 53kD) in salt-treated leaves decreased dramatically (Fig. 5b and c). A similar result was reported by Amini *et al.* (2007) on the effects salinity on protein. This could be due to the inhibitory effects of salt stress on the protein (Riccardi *et al.*, 1998). Both control and treated leaves were harvested at the same time to minimize experimental error.

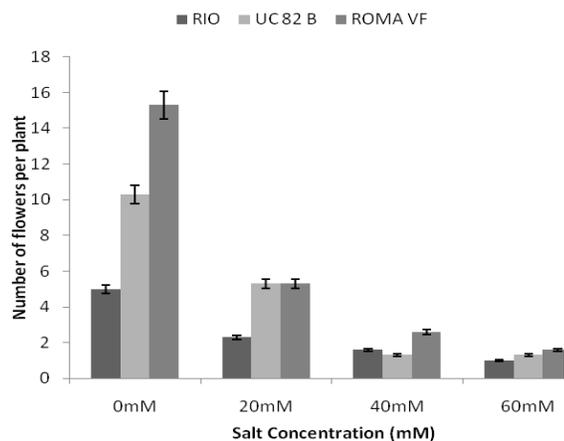


Fig. 1: Effect of Salt Concentration on the number of flowers of the three (3) varieties of tomato after 24 days of salt exposure ($P < 0.05$).

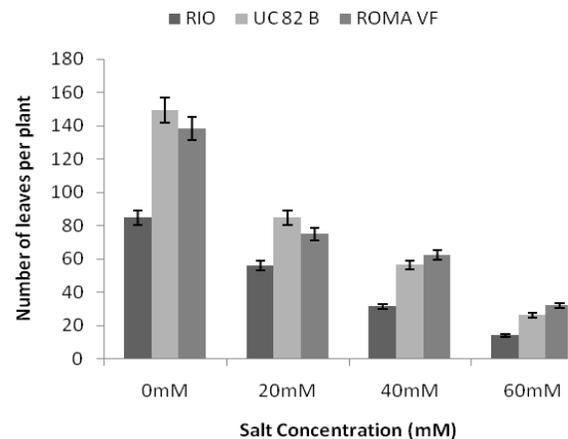


Fig. 2: Effect of Salt Concentration on the number of leaves of the three (3) varieties of tomato after 24 days of salt exposure ($P < 0.05$).

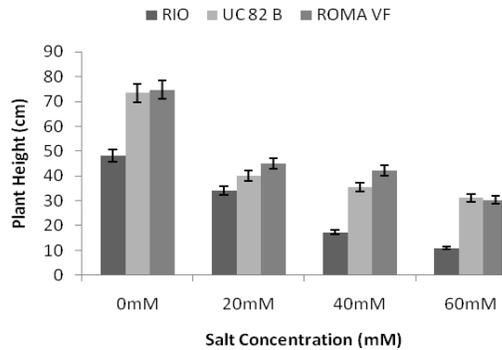


Fig. 3: Effect of Salt Concentration on plant height of the three (3) varieties of tomato after 24 days of salt exposure ($P < 0.05$).

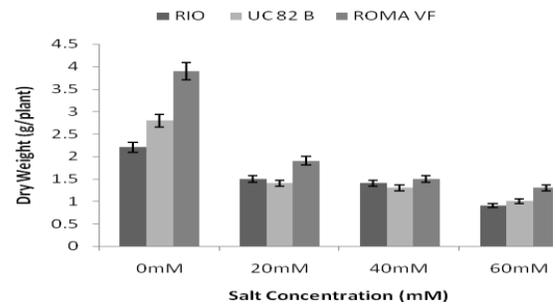


Fig. 4: Effect of Salt Concentration on dry matter accumulation (g/plant) of the three (3) varieties of tomato after 24 days of salt exposure ($P < 0.05$).

Table 1: Effect of salt concentration on free proline ($\mu\text{Mol g}^{-1}$) and glycine betaine ($\mu\text{Mol g}^{-1}$) content of the three (3) varieties of tomato

| Variety | Treatment (mM) | Proline ($\mu\text{Mol g}^{-1}$) | Glycine Betaine ($\mu\text{Mol g}^{-1}$) |
|------------|----------------|------------------------------------|--------------------------------------------|
| Roma vf | 0 | $0.52^c \pm 0.08$ | $1.25^c \pm 0.02$ |
| | 20 | $1.61^b \pm 0.13$ | $1.56^b \pm 0.05$ |
| | 40 | $1.92^{ab} \pm 0.01$ | $1.92^a \pm 0.03$ |
| | 60 | $1.99^a \pm 2.30\text{E-}03$ | $1.99^a \pm 8.81\text{E-}04$ |
| UC 82 B | 0 | $0.58^c \pm 0.02$ | $1.26^c \pm 0.09$ |
| | 20 | $1.32^b \pm 0.10$ | $1.47^{bc} \pm 0.08$ |
| | 40 | $1.95^a \pm 0.03$ | $1.79^{ab} \pm 0.10$ |
| | 60 | $1.99^a \pm 3.84\text{E-}03$ | $1.98^a \pm 7.00\text{E-}03$ |
| Rio Grande | 0 | $0.69^b \pm 0.07$ | $1.38^c \pm 0.03$ |
| | 20 | $1.77^a \pm 0.07$ | $1.87^b \pm 0.02$ |
| | 40 | $1.95^a \pm 0.03$ | $1.96^{ab} \pm 0.02$ |
| | 60 | $1.99^a \pm 1.66\text{E-}03$ | $1.99^a \pm 8.81\text{E-}04$ |

Values are means \pm standard error of three replications. ^{a,b,c} means in a column in each variety with different superscript are significantly different ($p < 0.05$).

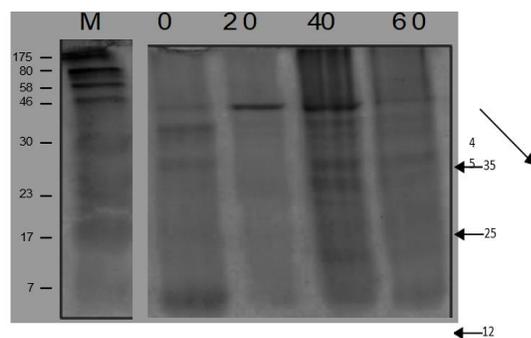


Fig. 5a: SDS-PAGE profile of leaf proteins from Tomato UC 82 B in 0, 20, 40, and 60 mM NaCl. M-protein marker, kD

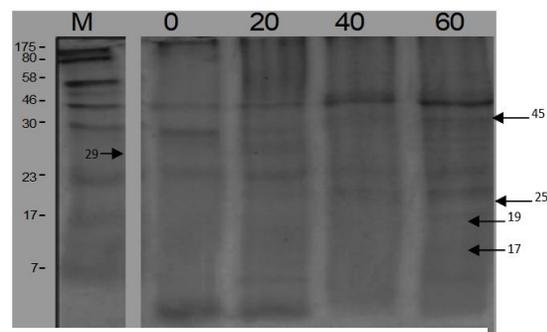


Fig. 5b: SDS-PAGE profile of leaf proteins from Tomato Roma Vf in 0, 20, 40, and 60 mM NaCl. M-protein marker, kD

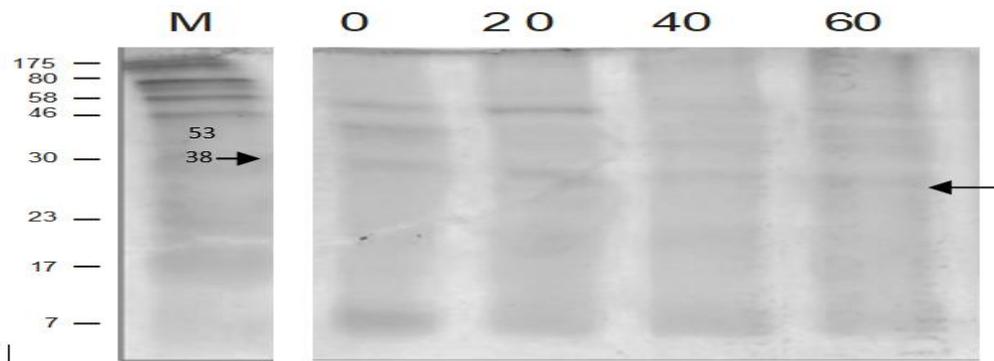


Fig. 5c. SDS-PAGE profile of leaf proteins from Tomato Rio Grande in 0, 20, 40, and 60 mM NaCl. M-protein marker, kD.

As a general conclusion in the current study, the results showed that NaCl decreased the growth of the three (3) cultivars of *L. esculentum*. From the results of this study, it was observed that Tomato Roma vf is more tolerant to salt stress with regards to the floral and vegetative parameters followed by UC 82B and then the Rio Grande. The results also showed that Tomato Rio grande accumulates more of

CONCLUSION

From this study, it can be concluded that among the three (3) cultivars studied, Roma vf is more tolerant to salt stress followed by UC 82B and then the Rio Grande.

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proline and glycine betaine content followed by Roma vf and then UC 82B while on the protein pattern changes, there is the expression of 4 proteins each in Tomato Roma vf and UC 82B on the stressed plants and only 1 protein in Tomato Rio Grande. The protein changes in all the cultivars of tomato leaves are attributed to the effect of salinity on the protein.

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