RESEARCH | Egyptian Journal of Agricultural Research | AGRONOMY

Physiological, biochemical, and enzymatic implications of "salt and lead" tolerance in *Cicer arietinum* under hydroponic culture condition

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Received:07-04-2022; **Accepted**:06-08-2022; **Published:**17-08-2022 DOI: 10.21608/ejar.2022.131925.1226

ABSTRACT
Cicer arietinum is a legume harvested worldwide for its high protein content (20%), which makes it a great meat substitute. Chickpea generally accumulates lead in contaminated soil, particularly when contaminated with Pb(NO3)2. In hydroponic culture conditions, germinated seeds were grown in Hoagland's nutrient medium for 7 d at 23 ± 2°C and 2 h of light. A salt (NaCl) and a metal (Pb) treatment were then applied to the grown plants for 7-8 days while they were in Hoagland's nutrient medium. One test sample was grown in normal Hoagland's mixture of nutrients as control (C), another three were treated with 1 M (T1), 3 M (T2), and 5 M (T3) (Pb) metal concentrations and the remaining three were treated with 20 M, 40 M, and 60 M (salt concentrations), and the remaining three plants were treated with a combination of metal and salt concentrations of 1 + 40 M (T7), 3 + 40 M (T8), and 5 + 40 M (T9) respectively. The control plants showed the best output as compared to all the treatments. The biochemical aspects such as total soluble sugar, and total amino acids provided drastic conditions as treated with salt and metals. The proline, and phenol productions were indicating the presence of stress. The mineral contents and the enzymatic activities also contributed positively due to the toxicity contributions of metal and soil toxicity. Thus, it appears that the combination of metal and salt stress has a greater reduction in biomass on the growth of C. arietinum.
Keywords: *Cicer arietinum*, Hydroponics, Lead, Metal, Toxicity

INTRODUCTION
Agriculture covers 60.3 percent of India's total land area, making it the world's second-largest. Soil provides structural and mineral support to plants, as well as organic matter, water, and other elements. Today, soil pollution has become a major issue. Chemicals used in industrial operations, residential garbage, municipal waste, agrochemicals, and petroleum-derived products are the main sources of soil pollution. Heavy metals and metalloids, pesticides, polycyclic aromatic hydrocarbons, persistent organic pollutants, salts, and other contaminants contribute to a considerable quantity of contaminated agricultural land. Because plants can regrow and adapt, natural pollution has no effect on the ecosystem (Kim *et al.,* 2011). Heavy metal pollution in agricultural land is exacerbated by industrialization, mine tailings, dumping of high metal wastes, and industrialization. However, no instances of heavy metal pollution have been made. Even a little concentration of heavy metals has an impact on humans, plants, animals, and other biological systems (Nataraj *et al.,* 2009). Some heavy metals, such as As, Cd, Cr, Se, Ni, and Pb, have been found to be more frequent in the soil around industrial regions, mines, and cities around the world (Shrivastava *et al.,* 2018). Because these heavy metals are persistent and non-degradable, they build up in the body and become harmful chemicals. Metals cause physiological harm to plants by inhibiting germination, growth, and yield (Guala, 2010). Furthermore, it interferes with electron transport in the photosystem of plants by lowering membrane permeability (Qadir,2004). ; Lead is the most poisonous of all metalloids, and it can be found in both natural and man-made environments. The most prevalent forms of the element in the earth's crust are PbS, $PbSO_4$, and oxygen ($PbCO_3$), with concentrations ranging from 10 to 30 mg Kg^{-1}. Ionic lead, Pb (II), lead oxide, and lead hydroxide are most commonly found in solvent, groundwater, and surface water. Too much lead in plants affects seed germination and root elongation, which limits chlorophyll synthesis and diminishes enzyme activity, depending on the environment (Pourrut, 2011) and Another major issue with agricultural soil contamination is salinity, which reduces farming capacity. High salinity impacts 20% of all agricultural fields and 33% of irrigated lands worldwide, according to prior estimates (Shrivastava, 2014). Plants use salt as a means of nutrient absorption, but excessive quantities have a variety of negative effects, including reduced leaf development, chlorophyll, and photosynthesis (Oves *et al.*, 2016).

The chickpea or Garbanzo bean (*Cicer arietinum* L.) is the world's third most mass-produced crop, because of its high protein content (20%), which makes it an excellent meat substitute (Lev-Yadun *et al.,* 2000). Chickpeas are a good source of both linoleic acid (51.2%) and monounsaturated oleic acid (32.6%), which are vital PUFAs for nutrition. Chickpeas (44.4 percent LA; 20.9 percent OA) have more linoleic and oleic acids than lentils (45.6 percent LA; 23.2 percent OA) and beans (46.7 percent LA; 28.1 percent OA) (Wang and Daun, 2004). When soil is contaminated with $Pb(NO_3)_2$, chickpeas tend to acquire lead (Dasgupta *et al.*, 2011). In light of the aforementioned issues, the purpose of this study was to demonstrate the effect of lead and salt as stressors on chickpea seedlings in hydroponic culture.

MATERIAL AND METHODS

Seed collection, germination conditions, and salt and metal treatment:

Chickpea seeds (GG5) from Gujarat's Anand Agriculture University were used in the research. The plant tissue culture lab of Shri A. N. Patel PG Institute of Science and Research, Anand, was used for seed germination and further testing. The seeds were steeped in water for 1-2 days before being moved to petri plates for sprouting, where they were kept wet for another 3-4 days. Germinated seeds were grown in hydroponic culture conditions for 7 days at 23+ 2°C with 2 hours of light in Hoagland's nutrient mix. Using Hoagland's nutritional medium, the plants were subsequently treated with salt (NaCl) and metal (Pb) treatments for 7-8 days. We took ten test samples, one of which was grown in standard Hoagland's nutrient solution, three of which were treated with 1 M (T1), 3 M (T2), and 5 M (T3) metal (Pb) concentrations, another three of which were treated with 20 M (T4), 40 M (T5), and 60 M (T6) salt concentrations, and the remaining three plants were treated with a combination of metal and salt concentrations of 1 + 40 M (T7), 3 Plants were harvested after 7-8 days of treatment for further physiological, biochemical, and enzymatic studies.

Growth Parameters:

Plant height was measured after harvesting, and the fresh weight of every component of the plant was taken in three replicates per treatment, including leaves, stems, and roots. The samples are covered in aluminium foil and dried in a hot air oven at 70°C for two days. After that, the dry weight was recorded.

Leaf relative water content (RWC %):

Sample leaves were gathered from three distinct plants for the replicates, and the fresh weight was recorded. After immersing the leaves in deionized water for 7-8 hours, the turgid weight of the leaves was studied. The samples were wrapped in aluminium foil and dried for two days at 80°C in a hot air oven. The relative water content was determined, according to Parida and Jha (2013), based on the following formula:

RWC= {(FW-DW)/(TW-DW)} ×100

Where,

FW = Fresh weight; DW = Dry weight; TW = Turgid weight.

Determination of mineral ion contents:

Using Panda *et al.,* (2017) methodology, the content of mineral ions such as Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, B, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+}, was determined.

Biochemical analysis:

Ethanolic or acetonic extract:

Total soluble sugar, phenol, total amino acids, chlorophyll, and carotenoids were all measured biochemically using ethanol or acetone extract. Plant extract preparation required 80% acetone for estimates of chlorophyll and carotenoids. One gram of plant leaves was weighed and crushed using a mortar and pestle in 5 ml of 80% ethanol or 80% acetone per treatment to make plant extract. The mixture was spun for 10 min at 10,000 rpm in a centrifuge tube. For thorough extraction, the supernatant was centrifuged again with 5 ml ethanol. Keep the supernatant at 4°C for future use and discard the pellet.

Sulfosalicylic extract:

The sulphosalicylic extract was used to calculate proline levels. One gram of leaf sample and 10 ml of 3% sulphosalicylic acids were required for each replicate. Using a mortar and pestle, a fine mixture of sample and sulphosalicylic acid was created. Centrifugation at 10,000 rpm for 10 min yielded the supernatant. The sample was centrifuged again until the solution was colcolorlessnd the supernatant was kept at 4°C.

Estimation of total free amino acid:

Using the ninhydrin method, the total free amino acid was calculated (Yemm *et al.*, 1955). In a test tube, 1 ml of ninhydrin reagent was mixed with 0.5 ml of ethanolic extract. With distilled water, the volume was increased to 3 mL. The reaction mixture containing test tubes was heated for 15 min in a boiling water bath. After that, 1.5 mL of n-propanol (1:1 v/v) was added to the mixture. The test tube that contained all of the liquids except the plant extract was designated a blank. The hue of the reaction mixture changed as soon as it was finished. After cooling at room temperature, the color variations were measured using a spectrophotometer (Systronics, India) at 570 nm absorption. The standard was prepared by using glycine (100 µg/ml). The graph was plotted by using the absorbance value to get the total free amino acid content in µg/g of the plant sample.

Estimation of total soluble sugar:

Using an anthrone reagent, Richterich, (1969) proposed a method for determining total soluble sugar (prepared from 95 percent H2SO4). An aliquot of 0.5 ml plant extract (ethanol extract) was taken and dilute with distilled water to a volume of up to 2 ml. After adding 2 ml of anthrone reagent to the tubes, they were

incubated in a boiling water bath for 8 minutes. Using the same process as stated above, we generated a working standard solution of glucose (100 g/ml) and used 0.2, 0.4, 0.6, 0.8, and 1 ml for standard estimation. A spectrophotometer was used to measure the absorbance of the reaction mixture at 630 nm after it had been cooled to room temperature in this experiment. The graph was made by putting all of the data together. The estimated value of soluble sugar was converted into µg/g of plant sample.

Estimation of Proline:

Using a ninhydrin reagent, Chinard, (1952) proposed a method for estimating proline. The plant extract was produced with 3 percent sulfosalicylic acid and measured at 0.5 mL. The 2 ml container was then filled with glacial acetic acid. Following that, 2 mL of ninhydrin was added to the sample. After adding the ninhydrin, a 20-min incubation period in a boiling water bath aids in the completion of the reaction and formation of the color. After cooling the reaction tube completely to room temperature, the spectroscopic examination was performed at 520 nm. A blank sample was prepared by removing the plant sample from the reaction tube. L-proline (100 g/ml) was used in the standard analysis, which followed the same protocol as before. The graphical presentation helped to analyze the data obtained from this experiment and the proline concentration was defined by the µg/gm of the plant sample.

Estimation of phenol:

FCR (Folin Ciocalteu Reagent) can be used to estimate phenol levels (Ainsworth and Gillespie, 2007). The plant extract was evaporated and mixed with distilled water to make a volume of up to 5 mL. It was used as an aliquot to determine the amount of phenol in the sample. 0.5 mL aliquot and 2.5 mL distilled water were used to make the tubes. Each reaction tube received 0.2 mL of FCR reagent. 2 ml of 20 percent Na_2CO_3 solution was added to the reaction mixture after 3 min of room temperature incubation. The standard was made with catechol (100 g/ml) and followed the same technique as before. After 1 min of incubation in a boiling water bath, the color of the reaction tubes was changed. and those changes were measured by a spectrophotometer at 560 nm absorbance. A graph was made and the concentration was converted to µg/gm of the plant sample.

Chlorophyll estimation:

Chlorophyll estimation from the plant is easy to assay by using the procedure of Arnon *et al.*, (1974). A known amount of sample was taken and crushed with 80% acetone for extract preparation and after that, the value was noted down at 663 nm and 645 nm absorbance by using a spectrophotometer. The chlorophyll value was calculated by using the formula, which is given below:

Chlorophyll A = [12.7 × OD at 663 nm] – [2.69 × OD at 645 nm]

Chlorophyll B = [22.9 × OD at 645 nm] – [4.08 × OD at 663 nm]

Total chlorophyll = [20.2 × OD at 645 nm] + [8.02 × OD at 663 nm]

Carotenoids estimation:

Carotenoids were estimated using Butnariu's (2016) technique. After the plant extract had completely evaporated, 10 ml of ethanolic KOH was added. Before adding KOH, we also measured the amount of evaporated material. At room temperature, the tubes were incubated for 20 min. After that, the mixture was poured into the separating funnel. The addition of 5% NaCl helped to bring the process to a halt. After the addition of NaCl, a new phase developed in the funnel, which was used to collect the lower phase. As a dilution factor, 3 mL acetone was added. A spectrophotometer was used to measure the absorbance at 445 nm. The following equation was used to compute the number of carotenoids:

C=D ×V ×F×10/2500

Here, C = Total amount of carotenoids (mg), D = Absorbance at 445 nm, V = Volume of the evaporated sample, F = Dilution factor (amount of acetone).

Enzymatic estimation:

Catalase estimation was followed by Braber, (1980) and peroxidase estimation was done by Sumner and Gjessing, (1943).

Catalase enzyme extract and assay:

0.1M potassium phosphate buffer was used to make the enzyme extract at pH 7. One gram of leaf sample was ground in a prechilled mortar and pestle. The supernatant was deemed enzyme extract after centrifugation at 15,000 rpm for 30 min at 4°C. In the reaction tube, 1 ml of enzyme extract, 2 ml of 0.005 M H_2O_2, and 3 ml of 0.1 M phosphate buffer were combined. After 1 min of incubation at 20°C, the reaction was stopped by adding 0.7N H_2SO_4. The reaction mixture was titrated by adding 0.01N $KMnO_4$ until a faint purple tint developed. It gives a clear indication of the presence of H_2O_2. A blank sample was prepared by adding H_2SO_4 to the enzyme extract at zero time. The enzyme activity was expressed in units/min, whereas the specific activity was expressed in units/min/g of the sample weight.

Peroxidase enzyme extract and assay:

One gram of plant sample was homogenized in 0.1 M potassium phosphate buffer using a pre-chilled mortar and pestle (pH.6). The supernatant was collected after centrifuging for 20 min at 15,000 rpm to assess enzyme activity. 1 ml of 0.01 M o-dianisidine, 0.5 ml of 0.02 M H_2O_2, 1 ml of 0.1 M phosphate buffer, and 2.4 ml distilled water were combined in one test tube to achieve the reaction. In the blank, we didn't use H_2O_2, instead choosing pure water. Pouring 0.2 ml of the enzyme into the tubes and incubating them at 30°C for 5 min kicked off the reaction. The reaction was stopped by 1 ml of 2N H_2SO_4. At 430 nm, the absorbance was measured by a spectrophotometer. The enzyme activity is expressed as units/min/g of the sample.

Biostatistical analysis:

The trials employed three replicates, with the data provided as mean standard deviation (SD) in a random design. The distribution of the mean ± values is depicted by the standard error bars in the figures. To evaluate the results, one-way analysis of variance (ANOVA) was used, followed by Duncan multiple comparisons ($p< 0.05$) (Duncan, 1955) using SPSS software (SPSS for windows 20.0, SPSS Inc., USA). According to Pang *et al.*, (2021) principal component analysis (PCA) was also employed by MetaboAnalyst 5.0 software to detect lead and salt caused modifications in leaf, stem, and root.

RESULTS

Growth parameters:

The chickpea plant was affected by metal and salt stress in a variety of ways, including alterations in physical, biochemical, and enzymatic properties. One obvious outcome was a change in the height of chickpea plants, as shown in **Fig. 1A**. The control plant reached a height of 51.0 ± 3.21 cm, which was higher than all of the other salt and metal-treated plants. T9 (5 + 40 M) metal and salt combined stress caused the plant to grow to the shortest height of 21.33 ± 1.52 cm. We can see that when the metal and salt concentrations grew, the plant height declined. When comparing the height loss of metal and salt-stressed plants, the height loss of salt-stressed plants was higher than that of metal stressed plants, whereas the combined salt and metal affected plant height was lower than the individual salt and metal affected plants.

Changes in growth characteristics such as the fresh and dry weight of leaves, stems, and roots were also determined, and the weight variation is shown in **Fig. 1B-D and 2 A-C**. The fresh weight of the leaf sample from the control plant was 0.156 ± 0.01 g, while the dry weight was 0.052 ± 0.01 g. The fresh weight of T9 (5 + 40 M) metal and salt combination stressed plant leaves were 0.046 ± 0.01 g, and the dry weight was 0.0153 ± 0.01 g, which was significantly less than the other treated sample plants. Plants that are salt-stressed weigh more than plants that are metal stressed, and combined salt and metal impacted plants weigh less than individual salt and metal affected plants. The stem and root's fresh and dry weights reacted similarly. The control plant stem and root had fresh weights of 0.9106 ± 0.01 g and 0.298 ± 0.01 g, respectively, and dry weights of 0.4933 ± 0.01 g and 0.0757 ± 0.01 g, which were higher than all other plants. T9 (5 + 40 M) metal and salt-stressed plants had a stem and root fresh weights of 0.354 ± 0.01 g and 0.0947 ± 0.01 g, respectively, and stem and root dry weights of 0.0637 ± 0.01 g and 0.0157 ± 0.01 g. Salt stress harmed plant growth more than metal stress, and the combined effects of salt and metal stress were far more damaging to plant growth than other stresses

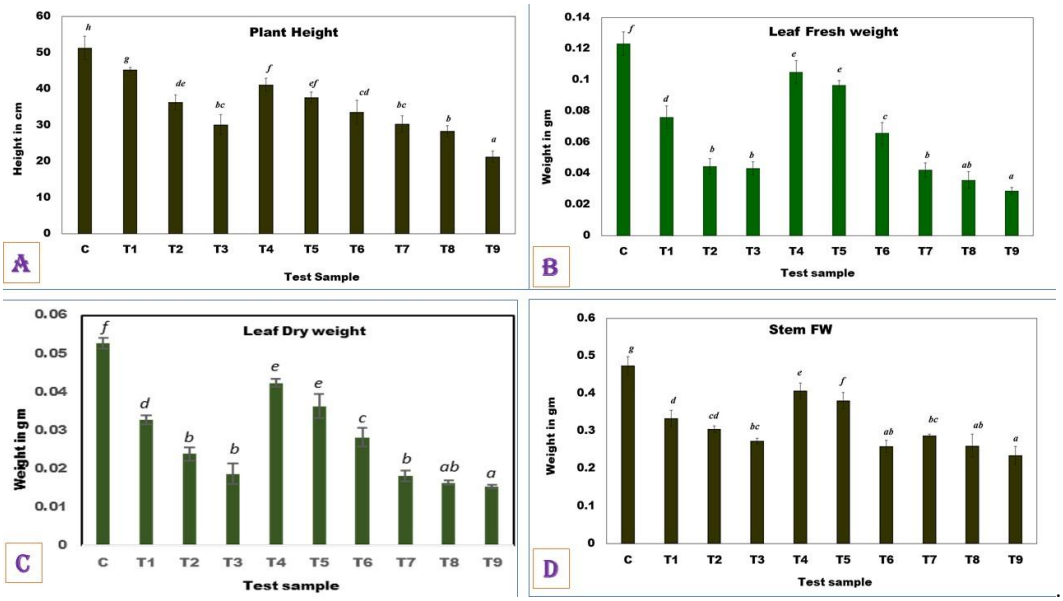

Fig. 1. The effect of various concentrations of metal, salt and combined metal and salt stress on the **(A)** height; **(B)** Leaf fresh weight; **(C)** Leaf Dry weight; and **(D)** Stem fresh weight of chickpea plant. The vertical bar indicates mean ± standard

deviations. The columns with different letters are significant at P< 0.05 based on one-way analysis of variance followed by Duncan's multiple range test (Duncan, 1955).

Relative water content (RWC) %:

The RWC is a salt tolerance indicator that considers turgor potential, water potential (WP), and osmotic adjustment in addition to turgor potential (OA). The RWC percent of all treated and non-treated plants is presented in **Fig. 2D**. The RWC % for the control plant was 92.98 ± 6.08 percent, while the RWC percentage for the T9 (5 + 40 M) metal and salt combination treatment plant was 35.69 ± 0.67 percent. As salt and metal concentrations increased, the RWC % dropped dramatically. When the data is examined as a whole, we may conclude that metal stress reduced RWC by a factor of two more than salt stress and that the combined effect was greater than either metal or salt stress individually.

Tolerance and/or adaptation to abiotic stress are achieved by two fundamental physiological events: preserving water status (measured as RWC % in this study) and sustaining cell metabolism and function (Bhushan *et al.*, 2007). As previously stated, salt stress causes an osmotic imbalance and destroys a cell's membrane, which could be the cause of a decrease in RWC % under salt and metal stress. Plant damage was minimal at lower levels of salt stress, and no severe symptoms were observed, which explains why the RWC % was so high when compared to other stresses.

Mineral ion contents:

Osmosis allows a plant to take water from the soil, whereas active transport collects mineral ions, altering the concentration gradient and mineral content of the soil. The fluctuation in Ca^{2+}, K^{+}, Mg^{2+}, Na^{+} as seen in Figures 3A-D, followed by B, Mn^{2+}, Fe^{3+}, Zn^{2+} in **Fig. 3E-H**, and Cu ion in **Fig. 4** if the soil has a considerable quantity of salt and metal contamination. The alterations in calcium ions as a result of salt and metal stress are depicted in **Fig. 3A**. The most Ca^{2+} ion was observed in T4 (20 M) salt-treated plants, at 1.666 mg/g dry weight, and it decreased significantly as the salt concentration increased. The salt-stressed T6 (60 M) plant contained the least amount of Ca^{2+} ion, 0.795 mg/g dry weight. All stressed plants, with the exception of T1, T2, and T4 showed lower Ca^{2+} ion levels than the control plant.

As metal stress increased, the ion concentration increased to specific values. The combined action of salt and metal stress wreaked havoc on the plant, having a higher impact than either stress alone. Calcium is required for metabolic control, cell division, and the activation of particular enzymes. The effects of potassium ions in different plants treated with varying salt and metal concentrations were shown in **Fig. 3B**. Plants treated with T2 (3 M) metal have the highest concentration of K^{+} ions, with 54.261 mg/g dry weight. The plant treated with T6 (60 M) salt had a lower level of 31.77 mg/g dry weight. Salt stressed plants and combined metal and salt-stressed plants have a reversible influence, which means that as the concentration rises, the concentration of K+ ions decreases under salt stress while increasing under combination metal and salt stress. As demonstrated in **Fig. 3C**, the magnesium ion concentration may fluctuate depending on the stress. The T4 (20 M) salt-stressed plant had a maximum Mg^{2+} concentration of 5.922 mg/g dry weight, which was greater than the control plant. Plants treated with T6 (60 M) salt exhibited a reduced level of 4.02 mg/g dry weight. As the level of stress grew, the concentration of Mg^{2+} ions decreased.

Fig. 3D shows the amount of sodium measured in several stressed plants. In the control plant, the lowest amount of salt in dry weight is 3.411 mg/g. The T6 (60 M) salt-stressed plant had the greatest sodium concentration of 73.045 mg/g dry weight. Plant stress has a significant impact on ion concentrations, which rises as the stress level rises. Between the control and stressed plants, there was a substantial difference. The concentration of boron in plant tissue is depicted in **Fig. 3E**. The control plant had a greater boron level of 0.0827 mg/g dry weight. Plants exposed to T6 (60 M) salt exhibited a decreased boron content of 0.0429 mg/g dry weight. In the case of metal stress, the ion concentration increased as the stress level grew, but the ion concentration decreased as the stress level increased in other forms of stress. The manganese concentrations in various plants were also shown in **Fig. 3F**. The maximum level of manganese observed in the T4 (20 M) salt-stressed plant was 0.0975 mg/g dry weight. The lowest concentration of manganese detected in the T6 (60 M) salt-stressed plant was 0.0453 mg/g dry weight. The number of ions rose as the metal and salt stress increased. The iron content of the control and treated plants were presented in **Fig. 3G**.

The T2 (3 M) metal stressed plant exhibited a higher dry weight iron content of 2.667 mg/g. The iron content of T6 (60 M) salt-stressed plants was lowered to 1.107 mg/g dry weight. When exposed to salt, the iron concentration drops, but when exposed to both metal and salt, it rises. However, in the case of metal tension, it can vary greatly. The zinc concentration of plants fluctuates as a result of salt and metal stress, as seen in **Fig. 3H**. The maximum concentration of zinc was found in the T3 (5 M) metal stressed plant, at 0.237 mg/g dry weight, while the lowest concentration was found in the T6 (60 M) salt-stressed plant, at 0.141 mg/g dry weight. The amount of zinc in the body increased with metal stress and reduced with salt stress as the pressures increased. **Fig. 3I** shows the amount of copper found in various plants. The highest amount of copper found in a T5 (40 M) salt-stressed plant was 0.0515 mg/g dry weight, while the lowest amount was 0.0253 mg/g dry weight in a T8 (3 + 40 M) metal and salt combination stressed plant.

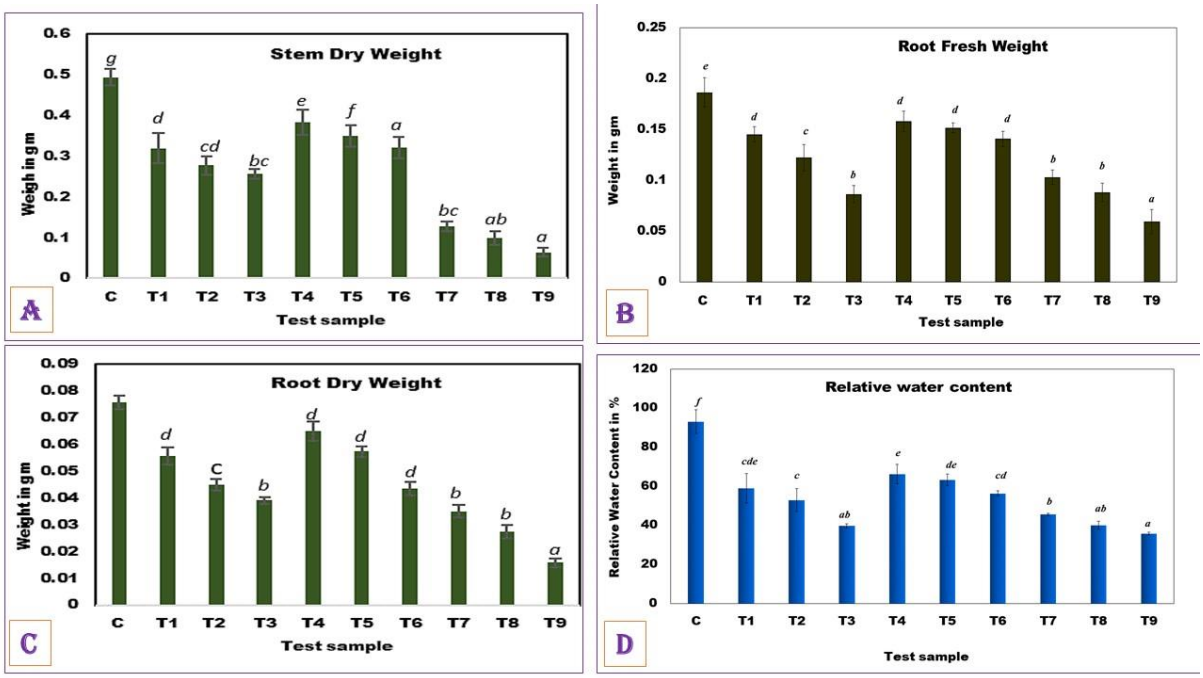

Fig.2. The effect of various concentrations of metal, salt and combined metal and salt stress on the **(A)** Stem dry weight; **(B)** Root fresh weight; **(C)** Root Dry weight; and **(D)** Relative water content of chickpea plant. The vertical bar indicates mean ± standard deviations. The columns with different letters are significant at P< 0.05 based on one-way analysis of variance followed by Duncan's multiple range test (Duncan, 1955).

Biochemical parameters:

Total free amino acids:

The variability in total free amino acid concentrations as a result of salt and metal stress levels is depicted in **Fig. 4A**. The metal stressed T3 (5 M) plant had a higher amino acid content of 145.1 µg/g than the other treated and control plants. The metal and salt combined stressed T9 (5 + 40 M) plant had the lowest concentration of 59.58 µg/g. Total free amino acid levels are raised by metal stress, however, the combined effect of salt and metal stress on total free amino acid levels was completely reversible with metal stress. The number of free amino acids changed with salt stress, although it remained lower than in the control plant.

Estimation of Total soluble sugars (TSS):

The concentration of soluble sugar changes substantially when salt and metal stress levels rise, as shown in **Fig. 4B**. The graph shows that when metal stress increases, so do the total soluble sugar level (TSS). TSS levels in T3 (5 M) metal stressed plants were greatest at 51.33 µg/g, which was higher than the control and all other plants. TSS levels in T5 (40 M) salt-stressed plants were lower, at 24.87 µg/g. The amount of TSS reduced as the combined impact of salt and metal increased. The amount of TSS changed as the salt stress increased.

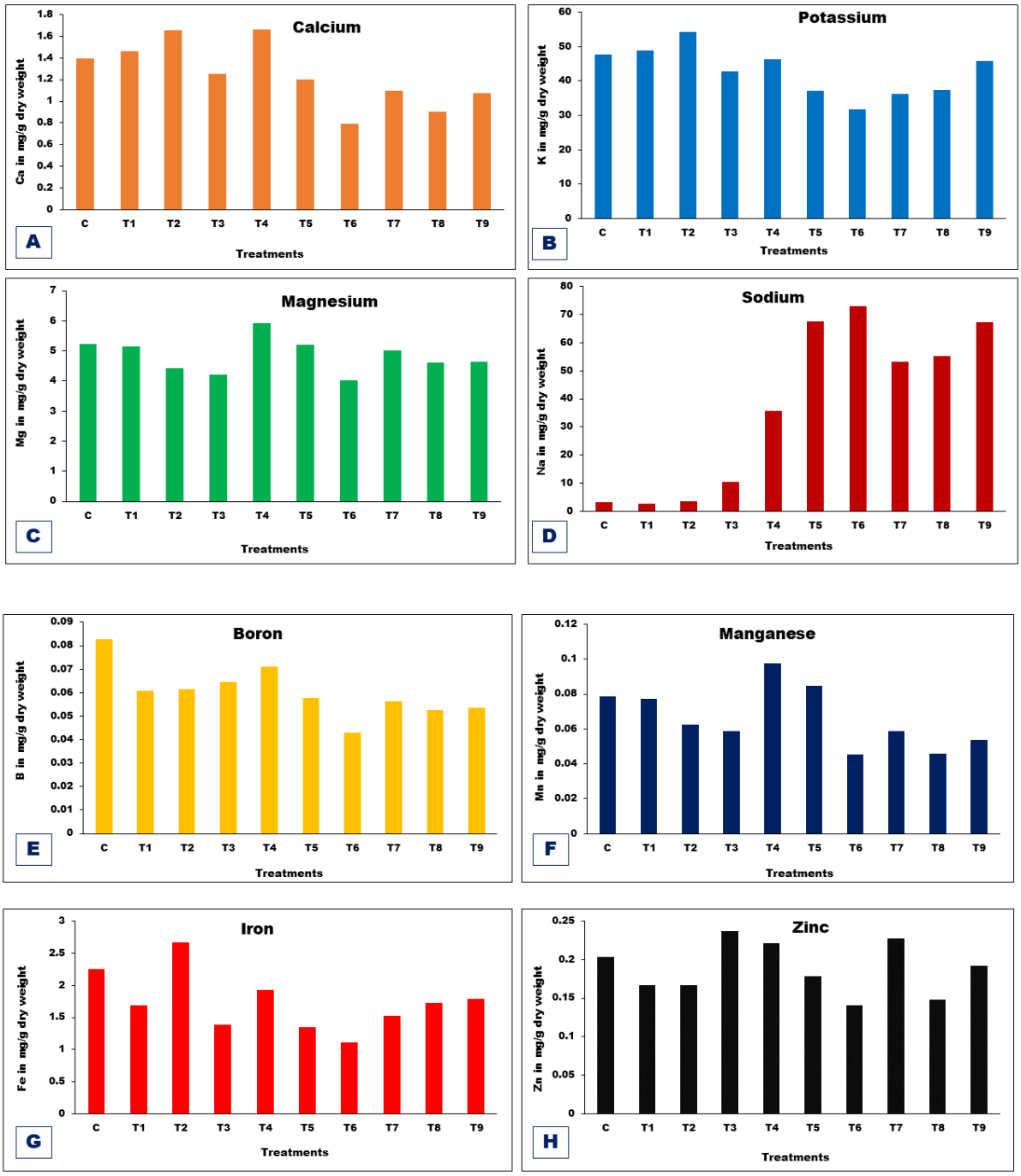

Fig. 3. The effect of various concentrations of metal, salt and combined metal and salt stress on the **(A)** Ca^{2+}, **(B)** K^{+}, **(C)** Mg^{2+}, **(D)** Na^{+}; **(E)** B, **(F)** Mn^{2+}, **(G)** Fe^{3+}, **(H)** Zn^{2+} content of chickpea plant.

Estimation of proline:

The current findings reveal that salt and metal stress have a significant impact on proline concentration in chickpea seeds. **Fig. 4C** depicts the amount of proline in the body. The proline concentration in the control plant was 17.57 µg/g. While the T6 (60 M) salt-treated plant has the highest proline content (87.69 µg/g), proline levels climb as metal and salt concentrations rise. Chickpea plants are more susceptible to salt stress than metal stress. While combined salt and metal stress have a variety of effects on the plant, as the combined concentration rises, the proline level decreases.

Estimation of phenol:

The total amount of phenol was calculated and displayed in **Fig. 4D**. The control plant had the least amount of phenol, 23.5 µg/g, whereas the T6 (60 M) salt-treated plant had the most, 50.87 µg/g, according to the figures. As the combined metal and salt stress increases, the amount of phenol drops, whereas the amount of phenol decreases as the combined metal and salt stress grows. Plants are much more sensitive to salt stress than they are to metal stress.

Photosynthetic pigments:

The total chlorophyll contents:

The effects of salt and metal stress on chlorophyll content are shown in **Fig. 5A-C**. As stress levels rise, the amount of chlorophyll in the plant decreases. Control plants have the highest levels of chlorophyll a, chlorophyll b, and total chlorophyll, with 0.16733 mg/g, 0.15373 mg/g, and 0.2739 mg/g, respectively. The T9 (5 + 40 M) metal and salt combined treated plant had the lowest amount of chlorophyll a (0.03575 mg/g), while the T6 (60 M) salt-treated plant had the lowest amount of chlorophyll b (0.04621 mg/g). Plants treated with T9 (5 + 40 M) metal and salt combined showed a reduced total chlorophyll content of 0.08486 mg/g.

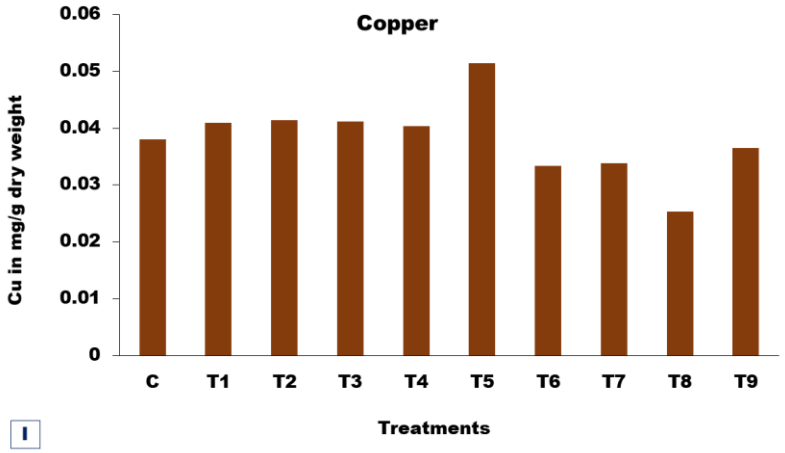

Fig. 3I The effect of various concentrations of metal, salt and combined metal and salt stress on the Cu ion content of chickpea plant.

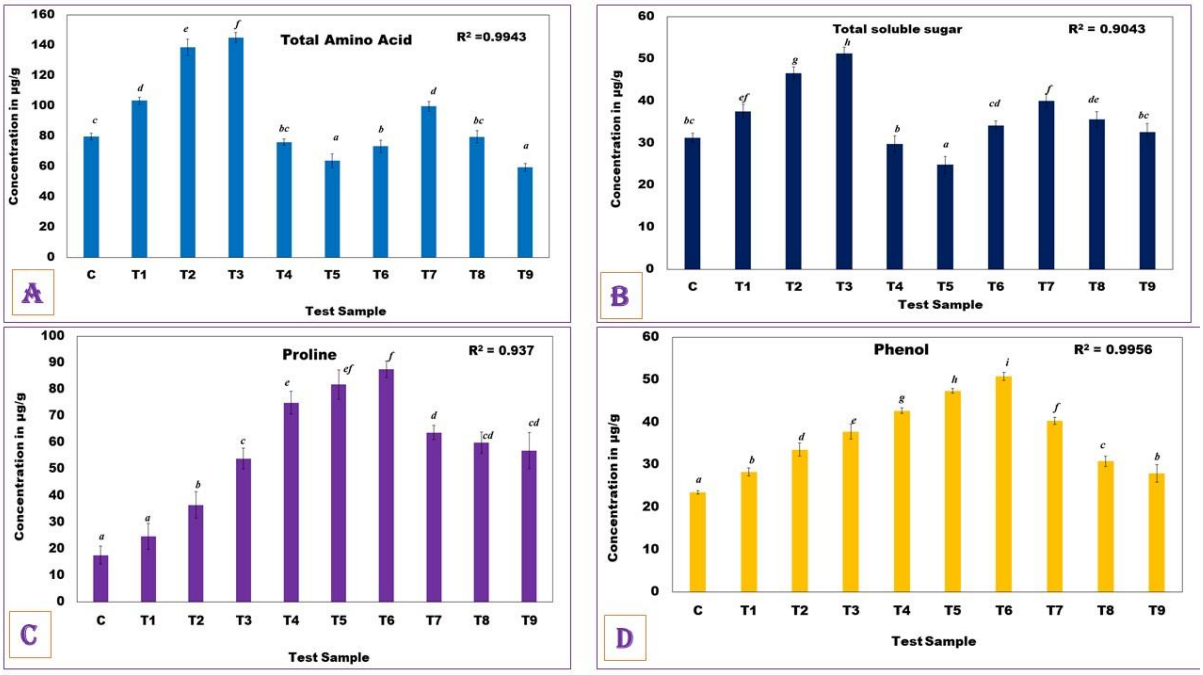

Fig. 4. The effect of various concentrations of metal, salt, and combined metal and salt stress on the **(A)** Total Amino acid; **(**B) Total Soluble sugar; **(**C) Proline; and **(**D) Phenol content of chickpea plant. The vertical bar indicates mean ± standard deviations. The columns with different letters are significant at P< 0.05 based on one-way analysis of variance followed by Duncan's multiple range test (Duncan, 1955).

Estimation of carotenoids:

When exposed to salt and metal stressors, the average carotenoid concentration was much lower than the control plant, as seen in **Fig. 5D.** With 0.04518 mg/g, the amount of carotenoid detected in control plants is the highest. Plants treated with T9 (5 + 40 M) metal and the salt combination had 0.0168 mg/g less carotenoid. The rate of carotenoid degradation rises as stress levels rise.

Enzyme activity:

Catalase activity:

The findings of all chickpeas treated with salt and metal stress exhibited significant variance in catalase activity, as shown in **Fig. 6A**. Under salt stress, the plant treated with T6 (60 M) salt had an enhanced enzyme activity of 3.86 units/min/g of catalase. The control plant, on the other hand, showed specific enzyme activity of 0.743 units/min/g. When salt and metal stress levels rise, specific enzyme activity rises dramatically. In comparison to plants that have been treated with metal, plants that have been treated with salt have the highest enzyme activity. While salt-treated plants have much lower enzyme activity than metal-stressed plants, salt-treated plants have significantly higher enzyme activity than metal-stressed plants.

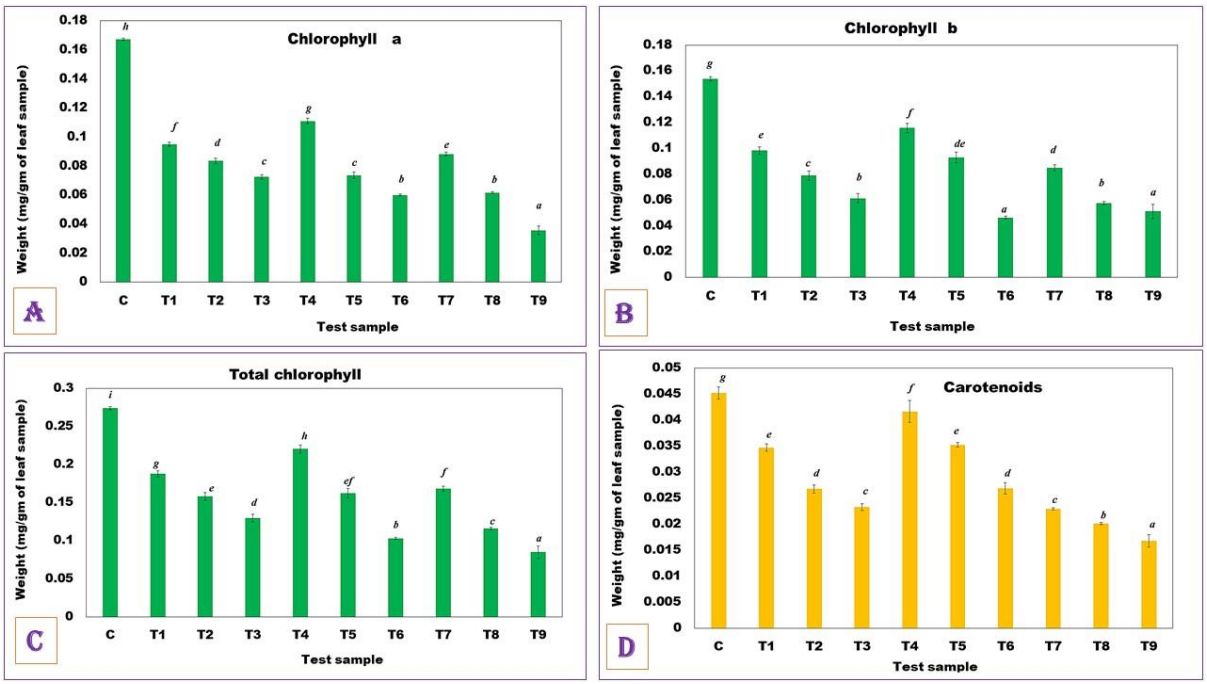

Fig. 5. The effect of various concentrations of metal, salt and combined metal and salt stress on the **(A)** Chlorophyll a; **(B)** Chlorophyll b; **(C)** Total Chlorophyll; and **(D)** Carotenoid content of chickpea plant. The vertical bar indicates mean ± standard deviations. The columns with different letters are significant at P< 0.05 based on one-way analysis of variance followed by Duncan's multiple range test (Duncan, 1955).

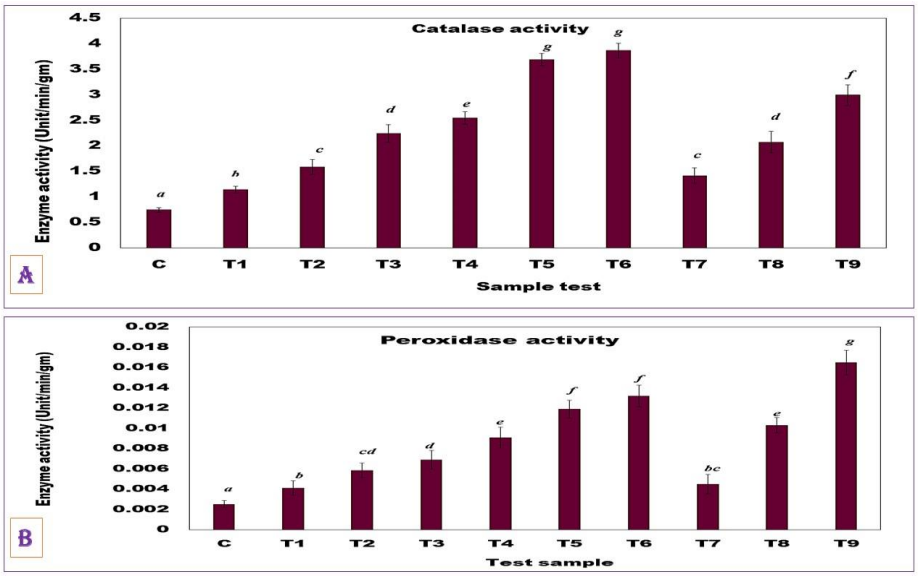

Fig. 6. The effect of various concentrations of metal, salt and combined metal and salt stress on the **(A)** Catalase activity; and **(B)** Peroxidase activity of chickpea plant. The vertical bar indicates mean ± standard deviations. The columns with different letters are significant at P< 0.05 based on one-way analysis of variance followed by Duncan's multiple range test (Duncan, 1955).

Peroxidase activity:

Peroxidase activity rose dramatically when chickpea plants were exposed to various amounts of salt and metal stress, as expected in **Fig. 6B**. Plants treated with T9 (5 + 40 M) metal and salt mix had greater peroxidase activity, with 0.0165 units/min/g. The control plant's enzyme activity was 0.00254 units/min/gm. According to the data in the figure, when stress levels rise, peroxidase activity rises as well.

Morphological analysis by Principal Component Analysis (PCA):

Fig. 7A-B depicts the examination of morphological parameters. Using data from PC1 and PC2, the 2D graph depicts the variability between the several plants under stress. The most critical morphological data information is contained in PC1. PC1 and PC2 account for 96.1 % and 3.9 % of the total variance, respectively, according to the PCA score plot. According to the PCA score plot, salt has little effect on the morphology of T4 (20 M) salt-stressed plants and T1 (1 M) metal stressed plants since they are identical to the control plant. According to the graph, T9 (5 + 40 M) – the highest among the metal and salt combination stressed plants and T3 (5 M) – the highest among the metal stressed plants have a substantial impact on the plant. PC3 has a variation of 0% in the 3D score plot, showing that when the original factors are "pulled out," only PC1 and PC2 are important.

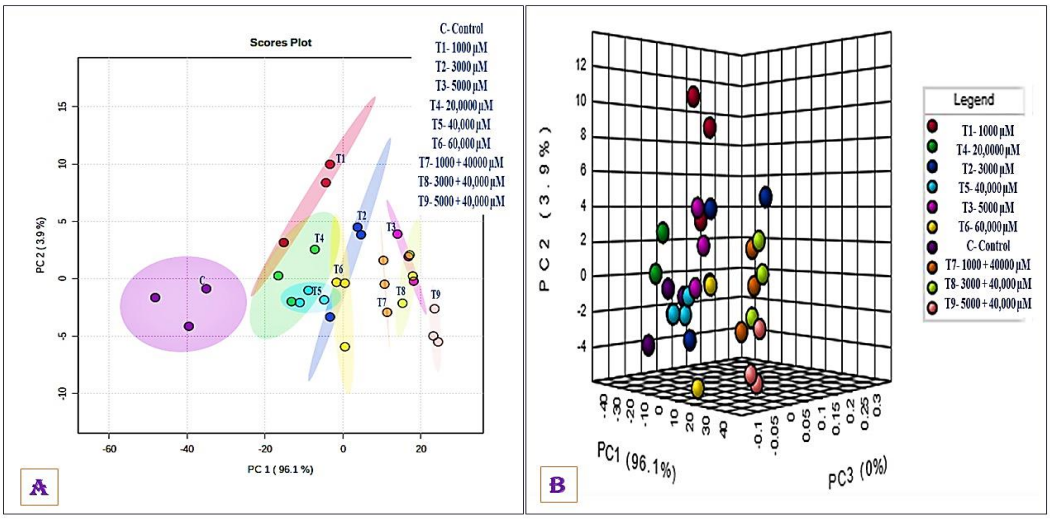

Fig. 7. PCA analysis **(A)** 2D and **(B)** 3D score plots for morphological analysis of chickpea under various concentrations of metal, salt, and combined metal and salt stress.

Biochemical analysis by PCA:

Fig. 8 A-B depicts the assessment of biochemical parameters. To explain the variation and similarities observed between control and treated plants, biochemical data such as amino acids, proline, phenol, and TSS estimation are displayed in the 2D and 3D score plots of PCA. The PCA reduces dimensionality by calculating relevant data. PC1, PC2, and PC3 have total variances of 69.8%, 28.5 percent, and 1.3 percent, respectively. T1 (1 M) plants with minimal metal stressors have significantly fewer effects on various biochemical parameters and are correlated with the control plant, as shown in the graph. High metal-stressed T3 (5 M) and high salt-stressed T6 (60 M) plants had a stronger impact on biochemical parameters than control plants.

Photosynthetic pigments analysis by PCA:

The PCA analysis of photosynthetic pigments is shown in **Fig. 9 A-B**. The amount of photosynthetic pigments in treated and control plants differs significantly. PC1, PC2, and PC3 have 98.1 percent, 1.2 percent, and 0.4 percent variance, respectively. This suggests that PC3 has a lower data impact. All of the treated plants have a higher amount of stress effect on photosynthetic pigments, as seen in the graph. All of the data show a lot of diversity in photosynthetic pigment when compared to the control plant. Except for the control and T9 (5 + 40 M) high combination metal and salt stress plant, all of the treated plants have similar photosynthetic content. This implies that photosynthetic pigments are affected by all conditions, with high combined metal and salt stress having a major influence.

DISCUSSION

Growth parameters:

Plants are typically sensitive to the accessibility of specific heavy metal ions, both high and low. Heavy metal, according to Reeves and Baker (2000), has a deleterious impact on the soil ecosystem, as well as plant fertility, growth, and development. Osmotic imbalance, nutritional insufficiency, an increase in reactive oxygen species (ROS), and enzymatic inhibition are all symptoms of salt and metal stress in plants. ROS damage cell membranes and other essential macromolecules like protein, nucleic acid, and lipid molecules (Hernández-Garca *et al.,* 2010; Oves *et al.,* 2016). That is why under salt and metal stress, chickpea plants slow down their growth, which results in a reduction in the plant's fresh and dry weight.

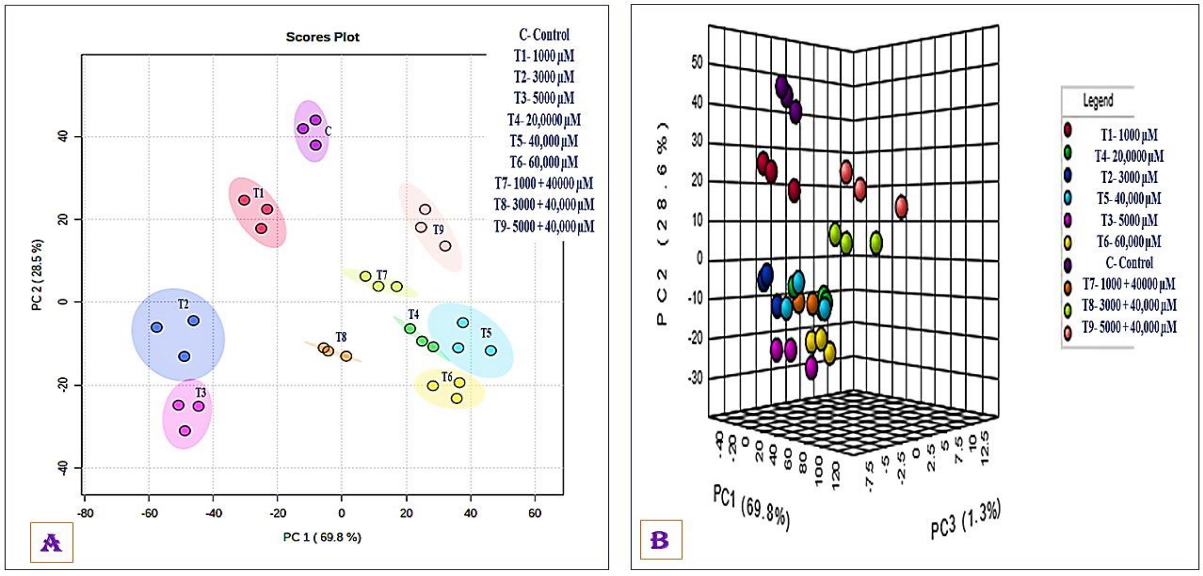

Fig. 8. PCA analysis **(A)** and 2D **(B)** 3D score plots for biochemical analysis of chickpea under various concentrations of metal, salt, and combined metal and salt stress.

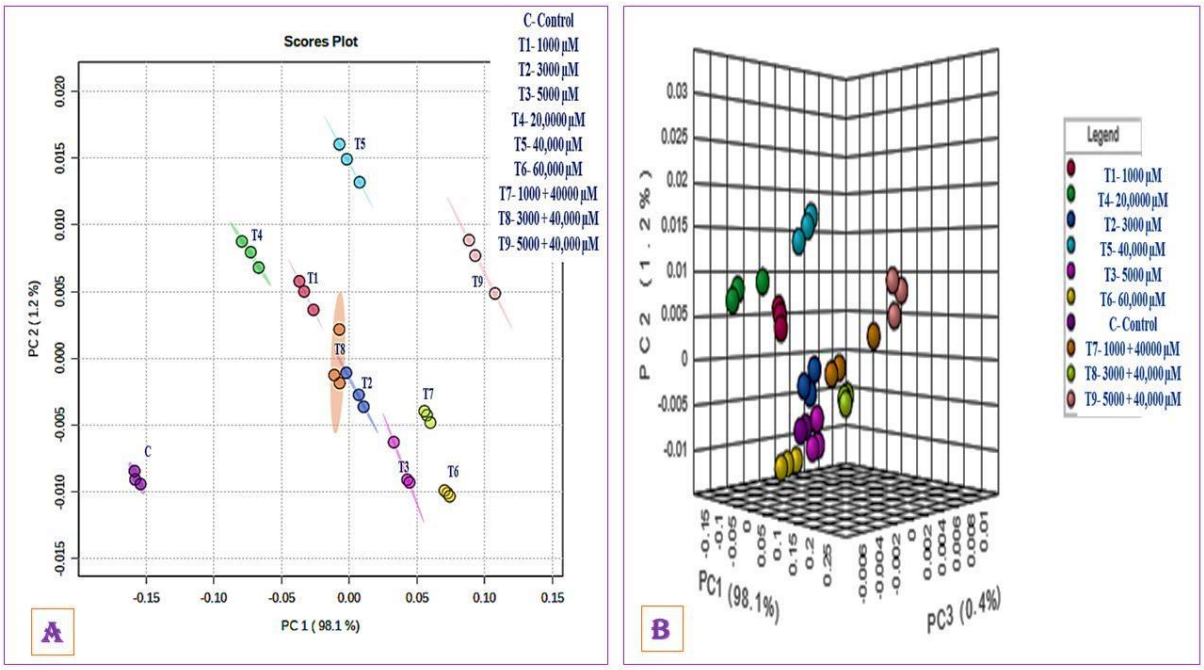

Fig. 9. PCA analysis **(A) and** 2D **(B)** 3D score plots for pigments analysis of chickpea under various concentrations of metal, salt, and combined metal and salt stress.

Relative water content (RWC) %:

Tolerance and/or adaptation to abiotic stress are achieved by two fundamental physiological events: preserving water status (measured as RWC % in this study) and sustaining cell metabolism and function (Bhushan et al., 2007). As previously stated, salt stress causes an osmotic imbalance and destroys a cell's membrane, which could be the cause of a decrease in RWC percent under salt and metal stress. Plant damage was minimal at lower levels of salt stress, and no severe symptoms were observed, which explains why the RWC % was so high when compared to other stresses.

Mineral ion contents:

Plants respond to salt stress by lowering their Ca^{2+} content, which is recognized as the main reaction by root cells. By reducing Ca^{2+} activity and boosting Na+ activity, salinity lowers Ca^{2+} transit instantly. While Shankar *et al.,* (2015) found a considerable number of Ca^{2+} ions in plants, they found the exact reverse in terms of Ca^{2+} content. Potassium promotes protein synthesis, stomata opening and shutting, enzyme activation, and cell turgidity. Salt stress causes a decrease in K^{+} ion, which is comparable to Mann *et al.,* (2018) findings. K^{+} ion levels rise when metal toxicity is present because K^{+} improves photosynthesis and the antioxidant defense mechanism. Magnesium aids in photosynthesis as well as DNA and RNA synthesis. It is a part of the chlorophyll ring structure and aids in ribosome structure maintenance. As a result, every stress that is applied to the chickpea plant lowers Mg^{2+} concentration because the stress damages photosynthetic pigments, nucleic acid, and protein content in the plant, lowering Mg^{2+} content. High levels of Na^{+} in the cytoplasm are detrimental to cell metabolism, including photosynthetic activities in plants. As a result, the cytoplasmic Na^{+} is separated. It is present in great amounts in plant vacuoles, where it serves as an osmoticum. Na^{+} is not an essential element in most plants, with the exception of a few halophytes. As previously stated, as salt stress increases, the activity of the Na^{+} ion increases while the Ca^{2+} ion's activity decreases. An increase in sodium ions occurs during salt stress as a result of the high concentration found in the root's surrounding media, which eventually promotes ion osmosis. Metals have a lower effect on sodium content than salt stress. Boron is involved in pollen germination, cell elongation and differentiation, and glucose transfer. Boron needs are generally higher throughout a plant's reproductive phase of life. In all of the treatments, the boron content was lower than that of the control plant. This suggests that salt and metal stress have an impact on the plant's life cycle and cell lengthening. Manganese is well recognized for its involvement in photosynthesis, where it splits water to release oxygen. While the photosynthesis process and photosynthetic pigments are reduced as the stress increases in the case of salt and metal stress, the amount of Mn^{2+} required for splitting water is also lowered. Iron is found in proteins like ferredoxin and cytochromes that engage in electron transport. It also aids in the production of chlorophyll by activating the catalase enzyme. Shankar *et al.,* (2015) showed that induced salt stress causes a decrease in iron content. We all know that when plants are stressed by salt or metal, their water intake drops, and iron transportation through the soil to the plant's tissue declines. The zone of the root

between cell elongation and maturity absorbs the greatest iron. Several enzymes, including carboxylase, are activated by zinc. It's also necessary for auxin biosynthesis. In a variety of plants, zinc aids in the decrease of heavy metal stress. Plants produce an antioxidant defense mechanism that protects them from oxidative damage, which minimizes heavy metal toxicity. It also improves the properties of plant development by lowering metal toxicity (Hassan *et al.,* 2017). As a result, as metal concentrations grow, so does the amount of zinc in plants. In the presence of salt, the zinc build-up was reduced (Aktaş *et al.,* 2007). The concentrations of all stressed and control plants were nearly identical. Copper is linked to specific enzymes engaged in redox reactions. It also plays a role in plant metabolism. Copper is both beneficial and harmful to plants. Impaired photosynthetic competency, low PSII quantum efficiency, and reduced cell elongation are all linked to copper poisoning. There is no visible difference between the control and treated plants since copper is both essential and harmful to plants.

Biochemical parameters:

Total free amino acids:

Amino acids have recently been discovered to serve a number of roles in plants, including serving as regulatory and signaling molecules, according to numerous studies. Amino acids that increased stomatal opening also boosted K^+ influx into guard cells in Rai and Sharma's (1991) investigation, whereas amino acids that limited stomatal opening decreased K^+ inflow into guard cells. As a result, amino acids could influence ion transport across membranes. Chickpeas' amino acid content has grown as a result of increasing metal stress. Toxic metals drive plants to synthesize amino acids with potentially beneficial properties. When plants are exposed to heavy metals, amino acids accumulate and function as signaling molecules and osmolytes, regulate ion transport, and aid in detoxification (Xu *et al.,* 2012). When chickpea plants are exposed to salt stress, amino acids like cysteine, arginine, and methionine, which make up about 55% of total free amino acids, decrease in concentration, while proline concentrations rise.

Estimation of Total soluble sugars:

Carbohydrates help in osmoprotection, osmotic balance, carbon preservation, membrane integrity, and radical scavenging under stressful situations by acting as metabolic signals (Parvaiz and Satyawati, 2008). To put it another way, as metal stress rises, metabolism shifts to a single form, and the number of sugars in the body rises with it. Several studies have found that mature leaves can release up to 80% of photosynthetically fixed carbon. The process of carbon reduction via photosynthesis, partitioning of starch synthesis in the chloroplast and triose-phosphates export from chloroplasts, and temporary storage of sucrose in the vacuole all influence sucrose export from source leaves. When one of these variables shifts, the amount of sucrose available for export shifts as well, resulting in tissue build-up. High salt and metal concentrations have an impact on photosynthesis and a variety of other functions. TSS rises as a result of salt and metal stress. Because a decline in photosynthesis may impair sugar production, the amount of TSS decreases when combination salt and metal concentrations are available.

Estimation of proline:

A considerable rise in proline accumulation by the cell is used as a stress signal, according to Hare and Cress (1997). Metal stress in chickpea seedlings is diagnosed via increased proline buildup. Increased expression of proline-related genes could be to blame (La *et al.,* 2019). Proline accumulates in the presence of metal stress, promoting the synthesis of phytochelatins, which chelate metals and reduce their harmful effects (de Knecht *et al.,* 1994). Proline regulates the production of several genes associated to antioxidant enzymes during salt stress, which is why a higher level of proline is detected during the stress (Kim and Nam, 2013).

Estimation of phenol:

Stress, regardless of its form, has been demonstrated to enhance the production and accumulation of phenolic acids and flavonoids (Manquián-Cerda *et al.,* 2018; Davies *et al.,* 2018). When there is a drought, the amount of phenol in the soil reduces. The combination of metal and salt stress lowers the plant's RWC and generates a water deficit, both of which have a direct impact on phenol concentration.

Photosynthetic pigments:

The total chlorophyll contents:

The lower the amount of chlorophyll in the plant, the faster it degrades. It's possible that an increase in harmful enzymes called chlorophyllase is to blame. The pigment system is reduced as the synthesis of the protein-pigment-lipid complex is diminished and the activity of the chlorophyllase enzyme is raised (Rahdari *et al.,* 2012). As a result, our findings show a strong association with Rahdari *et al.,* (2012), demonstrating that chlorophyll content is decreasing.

Estimation of carotenoids:

Carotenoid depletion is influenced by salt and metal stress. Due to an increase in reactive oxygen species, the considerable decrease in carotenoid concentration at high salinity causes oxidative damage and membrane

lipid peroxidation (ROS). Carotenoid levels appear to climb or fall in response to salt stress, depending on the plant species tested (Silva *et al.,* 2013; Parida *et al.,* 2004; Kholova *et al.*, 2009; Cha-um *et al.,* 2012).

Enzyme activity:

Catalase activity:

The CAT degrades the H_2O_2 produced by SOD and other activities (Foyer *et al.,* 1994). The activity of CAT rose considerably in stressed plants in this study, indicating that these plants are far more efficient at scavenging H_2O_2 than control plants and can give stronger protection against H_2O_2. As a result, under stressful conditions, the amount of H_2O_2 produced increases, as does catalase activity.

Peroxidase activity:

Because it consumes H_2O_2, peroxidase activity rises in parallel with stress levels. Peroxidase is another important antioxidative enzyme against stress. GPX appears to be capable of scavenging H_2O_2 generated by salt and metal stress, according to our findings. Stroinski's (1994) discovery that peroxidase induction is a general response of higher plants to hazardous metal absorption is consistent with our findings.

Principal Component Analysis (PCA):

Principal Component Analysis, or PCA, is a dimension-reduction technique for reducing the dimensionality of big data sets by reducing the number of variables in a large set to a smaller number of variables that yet contain the most information over a wide range. The data collected under metal and salt stress, such as morphological parameters, biochemical parameters, and pigments, were subjected to PCA analysis to reduce their dimensionality. PCA was used to simplify the difficult data. The correlation and variance in a data set are displayed using PCA.

CONCLUSION

In conclusion, the findings suggest that a high concentration of combined metal and salt stress has a greater influence on *C. arietinum* growth. This implies that the plant requires more energy for the synthesis and transportation of organic solutes from root to other parts of the plant, implying that the plant requires more energy for the synthesis and transportation of organic solutes from root to other parts of the plant. As a result, photosynthetic pigments in plants are reduced, and the concentration of Na+ ions in plant tissue increases. Because of the low transpiration rate in plants, which causes drought stress in plant tissue at varying salt concentrations, the relative water status of the plant drops as the biomass declines under salt stress. Cell turgidity is also influenced by K^+ ion concentration, therefore when salt stress rises, the amount of K^+ ion in the cell drops, lowering stomata opening and shutting. A low salt concentration has little effect on growth, but as the salt concentration rises, the effect on plant activities rises as well. Proline and phenols are recognized to be defensive mechanisms in plants, therefore when metal and salt stress increases, so does the level of proline and phenol. Metal stress has a lesser influence on the number of total amino acids and total soluble sugars in plant tissue than salt stress, and combined metal and salt stress have a smaller impact on the number of total amino acids and total soluble sugars in plant tissue. As we know, salt and metal stress toxicity causes ROS activity in plant tissue, and diverse enzyme activity increases in response to the stress to protect plants from ROS.

Finally, we can conclude that *C. arietinum* can grow in low salt and metal concentrations without harm, but it struggles to thrive in high salt, metal, and combination of metal and salt concentrations. T3 (5 M) and T6 (60 M) plants with severe metal and salt stress exhibited a greater influence on biochemical parameters than control plants. To avoid such extreme conditions, growers should employ high metal stresses and high slat stress-tolerant seeds.

Funding: Not applicable.

Conflict of Interest: The authors declare no conflict of interest

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