Effects of Combined Stresses of Salinity (NaCl) and Heavy Metal (NiCl₂) Levels on Seed Germination and Growth of Citrus colocynthis L.

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Abstract

Salinity and heavy metal stresses are a great hindrance to the germination and development of plants. Nickel (Ni) is an indispensable micronutrient for plants at low concentrations. However, the proficiency depends upon the plant types. Citrus colocynthis is a medicinal cucurbit weed. The contemporary target of the study is to appraise the influence of combined stresses by varying concentrations of sodium chloride and nickel chloride on germination, morphology, biochemical ions, and physiology of C. colocynthis. Germination percentage, root length, shoot length, fresh weight, dry weight, number of leaves, Na⁺, K⁺, Ca²⁺, Cl⁻, Ni²⁺, chlorophyll a and b, proline, SOD and CAT of C. colocynthis were measured with nine combined levels (100 mM NaCl + 50 uM NiCl₂), (200 mM NaCl + 50 uM NiCl₂), (400 mM NaCl + 50 uM NiCl₂), (100 mM NaCl + 100 uM NiCl₂), (200 mM NaCl +100 uM NiCl₂), (400 mM NaCl +100 uM NiCl₂), (100 mM NaCl +200 uM NiCl₂), (200 mM NaCl + 200 uM NiCl₂) and (400 mM NaCl +200 uM NiCl₂). This experiment was conducted in a completely randomized design with four replications. Germination percentage, root length, shoot length, fresh weight, dry weight, and the number of leaves, K⁺, Ca²⁺, chlorophyll a, and b decreased by increasing stress levels while Na⁺, Cl⁻, Ni²⁺, Proline, SOD, and CAT increased with increasing stress levels. This species has a positive morpho-physiological response under low(100mM NaCl, 50 uM NiCl₂) and moderate (200mM NaCl, 100 uM NiCl₂) combinations of salt and heavy metals while tolerance was observed under high-stress levels (400mM NaCl, 200 uM NiCl₂). It is concluded that this species can be recommended for phytoremediation against combined salt and heavy metal stresses.

Keywords: Cucurbit, Physiology, Biochemical ions, proline, SOD, CAT.

1. Introduction

Salinity and heavy metal are the most influential factors among environmental stresses limiting plant germination. The potential of water absorption in the root zone of plants and availability of water is reduced due to salinity and heavy metals in the soil. Avoidable ions impose toxic properties on plant physiological as well as biochemical procedures, which may lead to trepidation in nutrient ion uptakes by root epidermis and root hairs reducing seed germination. The sensitivity of the plant to salinity and heavy metal stresses varies at the different stages of growth (Huang et al., 2016). Salinity may induce other secondary stresses such as oxidative stress, through which active Radicles accumulation may lead to the oxidation of cell’s proteins & lipids which result in cell death (Molassiotis et al., 2006). Environmental stresses have been studied in numerous investigations (Asgari et al., 2002). Hanci and Cebci (2015) revealed that onion (Allium cepa L.) exhibited different responses to salinity. Germination indices of Triticum aestivum L. towards salinity stress revealed significant effects (Sofo et al., 2022). Increased salinity significantly decreased germination indices of Agropyron desertorum (Saeedi et al., 2013). Bhardwaj et al., (2017) recommended heavy metals as one of the leading cradles of ecological pollution causing numerous complications connected to industrial as well as agricultural activities. The amplified extent of heavy metals in soil and consequently, in plants produce toxic effects in animals and humans. The elements with a specific gravity above 5 while atomic weight from 63-200 are classified under heavy metals (Oldham, 2011). A low quantity of
Heavy metals is essential for plants generally non-toxic and their amount exceeding the required limit imposes toxicity. Examples of heavy metals may be cobalt, copper, iron, magnesium, molybdenum, nickel, and zinc (Co, Cu, Fe, Mg, Mo, Ni & Zn) beneficial in low quantity and reduce germination (Rizvi et al., 2020; Sengupta et al., 2014). Heavy metals diminished soil fertility, microbial activity, and plant yield (Sengupta et al., 2014). Heavy metals dissolved in water (from industrial sources and sewage) used for irrigation affect the plant reducing seed germination, growth as well as yield (Oguntade et al., 2015). Nickel, as a component of enzyme urease widespread plant types. An enzyme urease has a dynamic role during the germination of seeds (Mondal and Bose, 2019). In the absence of nickel, the urease enzyme is absent, so urea may be accumulated in the plants having toxic effects and forming necrotic lesions on the leaf tip. If a plant faces nickel deficiency then the urease enzyme will be deficient causing urea toxicity (Polacco et al., 2013). Nickel, with atomic number 28 and atomic weight of 58.71, is the 28th element in the periodic table. Nickel is a necessary micronutrient of plants for normal growth while its high absorption imposes adverse effects. Low concentration levels of nickel were positively affected while high concentrations of nickel chloride above 0.5mg/L showed adverse effects in various wheat (Triticum aestivum L.) cultivars (Shweti et al., 2018). Nickel is a catalyst in enzymes helping nitrogen fixation in leguminous plants and is also involved in the breakdown of urea (a nitrogenous compound). Ni may be deliberated micronutrient for cereals for example barley crop failed to complete its life cycle without nickel (Mondal and Bose, 2019). Some bacteria and fungi, friendly for plant growth also require nickel (Wang et al., 2013). Maintenance of proper cellular redox state, and physiological and growth responses of the cell also require the presence of nickel (Mir et al., 2018). It has been reported that higher concentrations of nickel may stop mitosis, inhibit the transport of carbohydrates and inhibit starch accumulation in the photosynthetic zones (Du et al., 2020). Nickel toxicity reduces the yield as it reduces the transport of food and nutrients from leaves toward flowers (Sachan and Lal, 2017). Medicinal plants are important for the preparation of many drugs as they comprise active ingredients for many drugs. Production of the drug ingredients by the plant is predominantly directed by the genotype of the plant while environmental factors may have a significant influence on the magnitude and value of their constituents (Witzler et al., 2018). Citrullus colocynthis L. is an important medicinal cucurbit weed having 53% oil and 28% proteins in its seeds (Kheiry, et al., 2017). The oil obtained from seeds of C. colocynthis L. is identical to Brassica oil and is being used for pharmaceutical diligence (OM and Emelike, 2018) due to its anti-microbial as well as anti-cancer potentials (Kapoor et al., 2020). C. colocynthis L. is also being used to treat urinary tract infections in indigenous medication by the people of the Mediterranean zone of the world (Gacem et al., 2019). The primary reason behind the harmful impact of salinity and heavy metals on the plants' seedlings is osmotic stress which inhibits the uptake of water and ion toxication (Lutts and Lefèvre, 2015). Responses of different plants toward different saline and heavy metal stress conditions are different (Gul et al., 2016). The capacity of the plants to tolerate and rest saline and heavy metal stresses are different which may assess through germination percentage and other growth parameters under salinity and heavy metal stress conditions (Saxena et al., 2016). It has been considered that salinity and heavy metals in the soil are critical issues for germination and growth of plants. Phytoremediation is eco-friendly technique for the rehabilitation of contaminated soils. It is need of time to explore plant species which can not only tolerate combined stresses of salt and heavy metals but also are easy to cultivate and have great medicinal importance. A cucurbit weed Citrullus colocynthis L. have great medicinal importance and is easily cultivated. So, it was selected in this study to evaluate its morphological, biochemical and physiological responses against salinity and heavy metal stresses.
2. Materials and Methods

Current study intended to evaluate the combined effects of sodium chloride and nickel chloride on seed germination and morpho-physiology of Tumma (Citrus colocynthis L.). For the present study, the seeds of desert weed C. colocynthis L. were obtained from the thal desert of district Layyah Punjab, Pakistan. The experiment was conducted in the Research laboratory and experimental area of the Botany department, The Islamia University of Bahawalpur, Pakistan. For germination percentage, a Laboratory experiment was conducted in Petri plates while for growth parameters pot experiment was conducted in the experimental area. Twenty selected healthy seeds with approximately uniform size were sterilized with 0.01% mercuric chloride and washed in disinfectant/distilled water (Sarkar et al., 2017) soaked in 1000 ppm dilute sulphuric acid solution for 15 hours to break dormancy (Ali et al., 2011). Seeds placed into separate Petri plates lined with the what-man filter paper no-1 accordingly; control plants T0=Distilled water, T1=100mMNaCl +50µM NiCl2 T2=200mMNaCl +50 µM NiCl2, T3=400mMNaCl +50µM NiCl2, T4=100mMNaCl +100µM NiCl2, T5=200mM NaCl +100µM NiCl2, T6=400mMNaCl +100µM NiCl2, T7=100mMNaCl +200µM NiCl2, T8=200mMNaCl +200 µM NiCl2 and T9=400mMNaCl +200µM NiCl2 (Bybordi et al., 2010; Pessarakli et al., 2001; Sriwanthana et al., 1994; Schuler and Relyea, 2018. ) The Petri plates were provided with distilled water and Hoagland’s nutrient solution prepared according to Hoagland and Arnon (1950) on daily basis to compensate the evaporation until recording of germination data. For morphological and physiological responses pot experiment was conducted in an experimental area with the same treatments on weekly basis. The experiment was designed completely randomized with four replicates of each treatment. Seeds were considered as germinated at attaining a minimum 1mm length by coleoptile and radicle. Germinated seeds were counted after ten days of soaking. Germination percentage (GP) was calculated by the following equation:

\[ \text{Germination percentage} = \frac{\text{number of germinated seeds}}{\text{total seeds}} \times 100 \]

Morphological characteristics like Shoot length and Root Length were measured with the help of a scale, fresh weight and dry weight were measured with electronic balance, and the number of leaves was counted after four weeks with four treatments. Meanwhile, shoot specimens were also collected for physiological parameters of the plants like Na+, K+, chlorophyll a and b, proline, SOD, and CAT. Sodium and Potassium cations were determined with a flame photometer (Jenway, PFP-7). A graded series of standards (ranging from 5 to 25 mg L⁻¹) of Na⁺, K⁺ and Ca²⁺ were prepared and standard curves were drawn. The values of Na⁺, Ca²⁺ and K⁺ from the flame photometer were compared with standard curves and total quantities were computed. Cl⁻ content is determined with a chloride meter (Jenway, PCLM 3). Ni²⁺ (aq) ion reacts with concentrated Cl⁻ ion in the presence of Ethanol medium to give tetrachloronickel (II) ion (NiCl₄)²⁻, a blue color solution is obtained. Nickel ion reacts with chloride ion in the presence of acid to give tetrachloronickel (ii) ion (NiCl₄)²⁻ gives a yellow-brownish color (Zhong et al., 2006; Salazar et al., 2011) The chlorophyll a, b and carotenoids were determined according to the method of Arnon (1949). Proline was estimated according to the method of Bates et al. (1973). The proline concentration in the sample was determined from a standard curve using analytic grade proline (SRL, Mumbai) and calculated on a fresh weight basis. Catalase (CAT) actions were measured by using the method of Chance and Maehly (1955). The activity of Superoxide dismutase (SOD) was analyzed by the method used by Giannopolitis and Ries (1977) by measuring the capacity of the enzyme to inhibit the photochemical reduction of nitrobluetetrazolium (NBT).
Statistical analysis: The trial was repeated twice with four replicates. Data analysis for (ANOVA) and (LSD) at 0.05 probability was done by using software STATISTIX 8.1. For the graphical presentation of the data, MS-Excel was used.

3. Results

Germination percentage

*Citrullus colocynthis* L. showed a significant increase in germination percentage as compared to control at combined lower salt and lower Heavy metal (100 mM NaCl+ 50 uM NiCl$_2$). A slight increase in germination percentage at a combined lower salt level with moderate-heavy metal (100 mM NaCl+ 100 uM NiCl$_2$) and moderate salt with lower heavy metal(200 mM NaCl+ 50 uM NiCl$_2$). A slight decrease in germination percentage was observed under combined moderate salt and moderate-heavy metal (200 mM NaCl+100 uM NiCl$_2$), lower salt with high heavy metal (100 mM NaCl+ 200 uM NiCl$_2$), and lower heavy metal with high salt (400 mM NaCl+50 uM NiCl$_2$). A significant decrease in germination percentage was recorded in higher levels of salt with moderate-heavy metals(400 mM NaCl+ 100 uM NiCl$_2$), moderate salt with high heavy metal (200 mM NaCl+ 200 uM NiCl$_2$), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl$_2$)(Graph-1).

![Graph-1](image-url)

*Graph-1: Germination percentage of Citrullus colocynthis L. under various levels of combined salt and heavy metal stresses*

Root Length

*Citrullus colocynthis* L. showed a significant increase in root length as compared to control at combined lower salt and lower Heavy metal (100 mM NaCl+ 50 uM NiCl$_2$). A slight increase in root length at a combined lower salt level with moderate-heavy metal (100 mM NaCl+ 100 uM NiCl$_2$) and moderate salt with lower heavy metal(200 mM NaCl+ 50 uM NiCl$_2$). A slight decrease in root length was observed under combined moderate salt and moderate-heavy metal (200 mM NaCl+100 uM NiCl$_2$), lower salt with high heavy metal (100 mM NaCl+ 200 uM NiCl$_2$), and lower heavy metal with high salt (400 mM NaCl+50 uM NiCl$_2$). A significant decrease in root length was recorded in higher levels of salt with moderate-heavy metals(400 mM NaCl+ 100 uM NiCl$_2$), moderate salt with high heavy metal (200 mM NaCl+ 200 uM NiCl$_2$), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl$_2$)(Graph-2).
Citrullus colocynthis L. showed a significant increase in Shoot Length as compared to control at combined lower salt and lower Heavy metal (100 mM NaCl+ 50 uM NiCl₂). A slight increase in Shoot Length was observed under combined moderate salt and moderate-heavy metal (200 mM NaCl+100 uM NiCl₂), lower salt with high heavy metal (100 mM NaCl+ 200 uM NiCl₂), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl₂)(Graph-3).

Citrullus colocynthis L. showed a significant increase in fresh weight as compared to control at combined lower salt and lower Heavy metal (100 mM NaCl+ 50 uM NiCl₂). A slight increase in fresh weight was observed under combined moderate salt and moderate-heavy metal (200 mM NaCl+100 uM NiCl₂), lower salt with high heavy metal (100 mM NaCl+ 200 uM NiCl₂), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl₂)(Graph-3).
NaCl+50 uM NiCl₂). A significant decrease in fresh weight was recorded in higher levels of salt with moderate-heavy metals (400 mM NaCl+ 100 uM NiCl₂), moderate salt with high heavy metal (200 mM NaCl+ 200 uM NiCl₂), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl₂) (Graph 4).

**Graph-4:** Response of Fresh weight of *Citrullus colocynth* L. under various levels of combined salt and heavy metal stresses

**Dry weight**

*Citrullus colocynth* L. showed a significant increase in dry weight as compared to control at combined lower salt and lower Heavy metal (100 mM NaCl+ 50 uM NiCl₂). A slight increase in dry weight at a combined lower salt level with moderate-heavy metal (100 mM NaCl+ 100 uM NiCl₂) and moderate salt with lower heavy metal (200 mM NaCl+ 50 uM NiCl₂). A slight decrease in dry weight was observed under combined moderate salt and moderate-heavy metal (200 mM NaCl+100 uM NiCl₂), lower salt with high heavy metal (100 mM NaCl+ 200 uM NiCl₂), and lower heavy metal with high salt (400 mM NaCl+50 uM NiCl₂). A significant decrease in dry weight was recorded in higher levels of salt with moderate-heavy metals (400 mM NaCl+ 100 uM NiCl₂), moderate salt with high heavy metal (200 mM NaCl+ 200 uM NiCl₂), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl₂) (Graph 5).

**Graph-5:** Response of Dry weight of *Citrullus colocynth* L. under various levels of combined salt and heavy metal stresses

**Number of leaves**

*Citrullus colocynth* L. showed a significant increase in the number of leaves as compared to control at combined lower salt and lower heavy metal (100 mM NaCl+ 50 uM NiCl₂). A slight increase in the number of leaves at a combined lower salt level with moderate-heavy metal (100 mM NaCl+ 100 uM NiCl₂) and moderate salt with lower heavy metal (200 mM NaCl+ 50 uM NiCl₂). A slight decrease in the...
number of leaves was observed under combined moderate salt and moderate-heavy metal (200 mM NaCl+100 uM NiCl₂), lower salt with high heavy metal (100 mM NaCl + 200 uM NiCl₂), and lower heavy metal with high salt (400 mM NaCl+50 uM NiCl₂). A significant decrease in the number of leaves was recorded in higher levels of salt with moderate-heavy metals (400 mM NaCl+ 100 uM NiCl₂), moderate salt with high heavy metal (200 mM NaCl+ 200 uM NiCl₂), and high salt with high heavy metal (400 mM NaCl+ 200 uM NiCl₂)(Graph-6).

**Graph-6: Response of Root length of Citrullus colocynthis L. under various levels of combined salt and heavy metal stresses**

**Potassium**

In comparison with control, *Citrullus colocynthis* L. revealed a low concentration of Potassium in plants grown under High salt + Low heavy metal (400 mM NaCl + 50 uM NiCl₂), High salt+Moderate heavy metal (400 mM NaCl + 100 uM NiCl₂) & High salt+High heavy metal (400 mM NaCl + 200 uM NiCl₂). A moderate concentration of Potassium was estimated in Moderate salt + Low heavy metal (200 mM NaCl + 50 uM NiCl₂), Moderate salt + Moderate heavy metal (200 mM NaCl + 100 uM NiCl₂) and Moderate salt + High heavy metal (200 mM NaCl + 200 uM NiCl₂). High amount of Potassium was observed in Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂) Low salt +Moderate heavy metal (100 mM NaCl + 100 uM NiCl₂) and Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂)(Graph-7)

**Graph-7: Potassium ions of Citrullus colocynthis L. under various levels of combined salt and heavy metal stresses**

**Sodium**

*Citrullus colocynthis* L. in comparison to control, revealed a significant increase in Sodium at High salt +Low heavy metal (400 mM NaCl + 50 uM NiCl₂), High salt +Moderate heavy metal (400 mM NaCl+ 100 uM NiCl₂) & High salt + high heavy metal (400 mM NaCl + 200 uM NiCl₂). A slight increase in
sodium content was observed in Moderate salt + Low heavy metal (200 mM NaCl + 50 uM NiCl₂), Moderate salt + Moderate heavy metal (200 mM NaCl + 100 uM NiCl₂) and Moderate salt + High heavy metal (200 mM NaCl + 200 uM NiCl₂). Low amount of sodium was observed in Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂), Low salt + Moderate heavy metal (100 mM NaCl + 100 uM NiCl₂) and Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂) (Graph-8).

![Graph-8: Sodium ions of Citrullus colocynth L. under various levels of combined salt and heavy metal stresses](image)

**Calcium**

*Citrullus colocynthis* L. in comparison with control, revealed a least amount of Calcium at Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂), Moderate salt + High heavy metal (200 mM NaCl + 200 uM NiCl₂) and High salt + High heavy metal (400 mM NaCl + 200 uM NiCl₂). A slight increase was observed in Low salt + moderate heavy metal (100 mM NaCl + 100 uM NiCl₂), Moderate salt + Moderate heavy metal (200 mM NaCl + 100 uM NiCl₂) and in High salt + Moderate heavy metal (400 mM NaCl + 100 uM NiCl₂). Moderate amount of Calcium was observed in Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂), moderate salt + Low heavy metal (200 mM NaCl + 50 uM NiCl₂) and High salt + Low heavy metal (400 mM NaCl + 50 uM NiCl₂) (Graph-9).

![Graph-9: Calcium ions of Citrullus colocynth L. under various levels of combined salt and heavy metal stresses](image)

**Chloride contents**

*Citrullus colocynthis* L. revealed a significant increase in Chloride contents at all levels of treatments as compared to control. (Graph-10)
Nickel

*Citrullus colocynthis* L. as compared to control, revealed a significant amount of Nickel at Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂), Moderate salt + High heavy metal (200 mM NaCl + 200 uM NiCl₂) and High salt + High heavy metal (400 mM NaCl + 200 uM NiCl₂). A slight increase in Low salt + moderate heavy metal (100 mM NaCl + 100 uM NiCl₂), Moderate salt + Moderate heavy metal (200 mM NaCl + 100 uM NiCl₂) and High salt + Moderate heavy metal (400 mM NaCl + 100 uM NiCl₂). Low amount of Nickel was observed in Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂), Moderate salt + Low heavy metal (200 mM NaCl + 50 uM NiCl₂) and High salt + Low heavy metal (400 mM NaCl + 50 uM NiCl₂) (Graph-11).

Chlorophyll a

In comparison with control, *Citrullus colocynthis* L. amount of chlorophyll a, was significantly decreased by increasing salinity and heavy metal stress levels. *Citrullus colocynthis* L. has variable results in variable stresses. Chlorophyll a level significantly increased in Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂). A slight decrease in chlorophyll a was recorded in Moderate salt + Low heavy metal (200 mM NaCl + 50 uM NiCl₂) Low salt + moderate heavy metal (100 mM NaCl + 100 uM NiCl₂) and Moderate...
salt + moderate heavy metal (200 mM NaCl + 100 uM NiCl₂). A considerable decline in chlorophyll a was observed at High salt + Low heavy metal (400 mM NaCl + 50 uM NiCl₂), High salt + Moderate heavy metal (400 mM NaCl + 100 uM NiCl₂), Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂), Moderate salt + High heavy metal (200 mM NaCl + 200 uM NiCl₂) and High salt + High heavy metal (400 mM NaCl + 200 uM NiCl₂) (Graph-12).

Graph-12; Chlorophyll a of Citrullus colocynthis L. under various levels of combined salt and heavy metal stresses

Chlorophyll b

In Citrullus colocynthis, results for chlorophyll b were slightly different from chlorophyll a in some cases while similar in most cases. In comparison with control, Chlorophyll b level significantly increased in Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂). A slight decrease in chlorophyll b was recorded in Moderate salt + low heavy metal (200 mM NaCl + 50 uM NiCl₂), Low salt + moderate heavy metal (100 mM NaCl + 100 uM NiCl₂) and Moderate salt + moderate heavy metal (200 mM NaCl + 100 uM NiCl₂). A noteworthy decline in chlorophyll b at High salt + Low heavy metal (400 mM NaCl + 50 uM NiCl₂), High salt + Moderate heavy metal (400 mM NaCl + 100 uM NiCl₂), Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂), Moderate salt+ High heavy metal (200 mM NaCl + 200 uM NiCl₂) and High salt + High heavy metal (400 mM NaCl + 200 uM NiCl₂)(Graph-13).

Graph-13; Chlorophyll b of Citrullus colocynthis L. under various levels of combined salt and heavy metal stresses

Proline

In Citrullus colocynthis L. Proline amplified using the increasing salinity level as well as Heavy metal. Maximum amount of Proline was recorded in High salt + High heavy metal (400 mM NaCl + 200 uM NiCl₂), Moderate salt + High heavy metal (200 mM NaCl + 200 uM NiCl₂), Low salt + High heavy metal (100 mM NaCl + 200 uM NiCl₂) High salt + Moderate heavy metal (400 mM NaCl + 200 uM
NiCl$_2$) and High salt ± Low heavy metal (400 mM NaCl + 50 uM NiCl$_2$) While minimum Proline was found in the plants grown under Low salt ± Low heavy metal (100 mM NaCl + 50 uM NiCl$_2$) While plants grown in Low salt ± Moderate heavy metal (100 mM NaCl + 100 uM NiCl$_2$) and Moderate salt ± Moderate heavy metal (200 mM NaCl + 100 uM NiCl$_2$) showed moderate concentrations of proline. (Graph-14)

**Graph-14:** Proline of *Citrullus colocynth* L. under various levels of combined salt and heavy metal stresses

**Catalase enzymes (CAT)**

In *Citrullus colocynth* L. as compared to control, maximum amount of Catalase (CAT) was recorded High salt ± High heavy metal (400 mM NaCl + 200 uM NiCl$_2$), Moderate salt ± High heavy metal (200 mM NaCl + 200 uM NiCl$_2$), Low salt ± High heavy metal (100 mM NaCl + 200 uM NiCl$_2$). High salt ± Moderate heavy metal and High salt ± Low heavy metal (400 mM NaCl + 50 uM NiCl$_2$). While minimum CAT was found in the plants grown under Low salt ± Low heavy metal (100 mM NaCl + 50 uM NiCl$_2$) While plants grown in Low salt ± Moderate heavy metal (100 mM NaCl + 100 uM NiCl$_2$) and Moderate salt ± Moderate heavy metal (200 mM NaCl + 100 uM NiCl$_2$) showed moderate concentrations of CAT. (Graph-15)

**Graph-15:** Catalase (CAT) of *Citrullus colocynth* L. under various levels of combined salt and heavy metal stresses

**Superoxide dismutase enzymes (SOD)**

In *Citrullus colocynth* L., It has been observed that antioxidant enzyme activities increased with the increasing level of salinity and Heavy metal. Maximum amount of Superoxidase dismutase (SOD) was recorded in High salt ± High heavy metal (400 mM NaCl + 200 uM NiCl$_2$), Moderate salt ± High heavy metal (200 mM NaCl + 200 uM NiCl$_2$), Low salt ± High heavy metal (100 mM NaCl + 200 uM NiCl$_2$), High salt ± Moderate heavy metal (400 mM NaCl + 100 uM NiCl$_2$) and High salt ± Low heavy metal
(400 mM NaCl + 50 uM NiCl₂). While minimum SOD was found in the plants grown under Low salt + Low heavy metal (100 mM NaCl + 50 uM NiCl₂) while Low salt + Moderate heavy metal (100 mM NaCl + 100 uM NiCl₂) and Moderate salt + Moderate heavy metal (200 mM NaCl + 100 uM NiCl₂) showed moderate concentrations of SOD (Graph-16).

Graph-16: Superoxide dismutase (SOD) of Citrullus colocynth L. under various levels of combined salt and heavy metal stresses

Table 1- Analysis of variance for effect of combined Salt +Heavy metal (NaCl + NiCl₂) Stresses on germination percentage & morphological characteristics (Root length, Shoot length, Fresh weight, dry weight, and Number of leaves) of Citrullus colocynth L.

| Sources of variation | Degree of freedom | Mean sum of squares |
|----------------------|-------------------|---------------------|
|                      | Germination percentage | Root length | Shoot length | Fresh weight | Dry weight | Number of leaves |
| Treatment            | 9                 | 58.3148            | 36.5519        | 872.759      | 4.64181    | 0.87411          | 183.407          |
| Error                | 20                | 0.8667             | 1.0000         | 33.367       | 0.06067    | 0.15900          | 0.867            |
| Grand Mean           |                   | 12.167             | 6.3667         | 32.833       | 2.2367     | 1.2900           | 16.000           |
| CV                   |                   | 7.65               | 15.71          | 17.59        | 11.01      | 30.91             | 5.82             |
| F                    |                   | 67.3               | 36.6           | 26.2         | 76.5       | 5.50              | 212              |

Table 2- Analysis of variance for effect of combined Salt +Heavy metal (NaCl + NiCl₂) Stresses on Biochemical ion contents in plant samples of Citrullus colocynth L.

| Sources of variation | Degree of freedom | Mean sum of squares |
|----------------------|-------------------|---------------------|
|                      | K⁺ | Na⁺ | Ca²⁺ | Cl⁻ | Ni⁺ |
| Treatment            | 9  |     |      |     |     |
| Error                | 20 |     |      |     |     |
| Grand Mean           |    |     |      |     |     |
| CV                   |    |     |      |     |     |
| F                    |    |     |      |     |     |
Table 3- Analysis of variance for effect of combined Salt +Heavy metal (NaCl + NiCl₂) Stresses on Physiological characteristics (chlorophyll a &b, proline, Catalase (CAT) and Superoxide dismutase (SOD) of Citrullus colocynthis L.

| Sources of variation | Degree of freedom | Chl. a | Chl. b | Proline | CAT | SOD |
|----------------------|-------------------|--------|--------|---------|-----|-----|
| Treatment            | 15                | 0.00140| 0.39133| 11.3422 | 48.1023 | 9.69399 |
| Error                | 32                | 0.00001| 0.00958| 0.0433  | 0.3300 | 0.31312 |
| Grand Mean           |                   | 54.37  | 1.0417 | 5.1458  | 10.240 | 4.2979 |
| CV                   |                   | 2.25   | 9.40   | 4.05    | 5.61  | 13.02 |
| F                    |                   | 775    | 40.8   | 262     | 146   | 31.0 |

4. Discussion

The outcomes of our present work revealed that C. colocynthis L. showed different responses against different salinity and heavy metal treatments. The influence of combined salinity and heavy metal stress was significant on germination percentage and other growth parameters of the plant. Combined higher salt and higher heavy metal have a significant negative impact on germination of seeds, root length, shoot length, fresh weight, and dry weight of the plant. It was determined that the co-effects of heavy metal and salinity were more severe in comparison to single stress (Gul et al., 2016). Correspondingly, previous works also suggest that high-stress levels of salinity and heavy metal might generate a punitive situation for seed germination (Sharma et al., 2020). It may be due to a drop in interior osmotic potential due to salt and water stress already been declared (Ahmad et al., 2022)), which had been recognized to variation between organic and mineral ions balance present in the cell sap and absorption of ions (Aqeel et al., 2021). The disturbance of nutrients absorption and uptake also has been described as the loss of germination percentage and growth parameters (Zahra et al., 2021). Diminution of germination percentage under salinity stress already observed (Onen et al., 2018), also may be the same under heavy metal stress and loss of water (AbdElgawad et al., 2020). The obstructive water content of seeds due to salinity and heavy metal stress leads to a decline in the activity of enzymes especially hydrolytic enzymes which have a key role to hydrolyze cotyledonous reserve food required for supplying energy by respiration during the early stages of seed growth. Limitation of water uptake, transfer of seed stocks, and direct influence on gene expression and organic structure is related to salinity and heavy metal stresses (Schillaci et al., 2019). An important reason behind the reduced seed germination may be the inhibited nutrient delivery from seed storage tissues to the developing embryo (Joshi, 2018). The minerals and heavy metals accumulation in the plant cells may lead to negative effects like ion toxicity induced mainly by salinity and drought stress (Kumar, 2020). Detaining absorbable water by seeds due to salinity and heavy metal stress can lead to reduced germination (Sukmarani et al., 2021). Decreased seed germination under salinity and heavy metal stress may be illuminated by turgor pressure limitation or dry material storage (Khosropour et al., 2022). High levels of salinity suppress seedling growth, imposing a negative impact of NaCl on cellular membranes, and destructing cytoplasmic membranes due to ion toxicity (Shahid et al., 2020; Shehzad et al., 2019). The main factor which limits the establishment of the plant under salinity and heavy metals is impaired germination (Ali et al., 2021). The adverse impact of salinity on germination and seedling parameters of different plants has been testified previously (Li et al., 2020). The debility in germination under salinity and heavy metals has been
endorsed as a collective outcome of the toxicity of salts and osmotic pressure (Kumar et al., 2022). In our experiment, combined stresses showed more adverse effects which may be due to the addition of increased chlorine ions from sodium chloride as well as nickel chloride resulting in a rise in osmotic stress inhibiting seed germination (Shahzad et al., 2019). Altered water relations due to high salt stress significantly delay germination (Zhu et al., 2019) because extra salts are accumulated in intercellular spaces (Oi et al., 2020). Sometimes embryo may be damaged due to higher levels of Na+/Cl- ions or exosmosis of water (Hakimi et al., 2022). Fresh weight, dry weight, and the number of leaves were significantly decreased under higher salt and heavy metal combinations (400 mM NaCl + 200 uM NiCl2) similarly as described by Dolatabadian et al. (2011) that total plant weight, plant height, and leaf number was decreased due to salinity stress. Alfaraas et al. (2016) reported that Paddy plants treated with heavy metals resulted in decreased roots and shoot length as well as several leaves. Increased levels of salts and heavy metals cause a reduction in water uptake by the roots which ultimately reduces the growth of roots and shoot (Gul et al., 2016). Ain et al. (2016) studied Plant growth of wheat under Ni and salinity stress. It was concluded that increasing the level of Ni along with salinity stress decreased plant growth. The highest reductions in the number of leaves and number of flowers were recorded in salinity compared to the control reported by Alam et al. (2016). Pant et al. (2014) reported that heavy metals had a considerable negative effect on the majority of the plant parameters of S. robusta; a high decrease in morphological parameters was noticed in the number of leaves, shoot length, and root length. It seems that the reduced root and shoot length, and fresh weight were because of decreased water intake, which as a result could cause a decrease in the amount of water in plant tissue (Anjum et al., 2011). In the present study germination percentage and growth factors showed positive response under lower concentrations of salt and heavy metal as the lower Ni concentration imposes positive effects on early seedling growth but higher concentrations have been documented to be enormously toxic and impose negative effects on seedling growth (Shafiq et al., 2008). Similarly, a low concentration of nickel promotes seed germination in rice (Das et al. 1978) and many crops (Welch, 1981). Germination parameters were significantly promoted in 10 and 20 mg L-1 Ni. Thereafter, a decreasing trend was observed and all these parameters were increasingly reduced at 50 and 60 mg L-1 of nickel. Of the cultivars, Hysun-33 showed a better germination rate, index as well as it took fewer days to achieve 50% germination. However, Ricinus communis was poor in all germination parameters appraised in this study. All other cultivars showed an intermediate response to exogenous Chromium application (Ali et al., 2022). In our biochemical studies, sodium ions concentration in Citrullus colocynthis L. was increased by increasing salinity and heavy metal while potassium level decreased by increased salinity and heavy metal stress levels. Sodium accumulates in plants during salt stress conditions resulting in ionic imbalance, specific ion effects, nutrient deficiency symptoms, and disturbed metabolism by the toxic effects of accumulated ions (Acharia et al., 2022) while potassium content decreased under salt stress. Salt stress-induced increase in sodium and depletion of potassium contents have been reported earlier in cucumbers (Tiwari et al., 2010). Calcium level of C.colocynthis L. was decreased in increased salt and heavy metal conditions in our experiment. This may occur because high salt stress imposes osmotic/ionic stress on plants as well as influences the uptake and transport of essential nutrients such as K and Ca (Yang and Guo, 2018). In our studies chlorophyll, a and b content was increased under lower concentrations of salt and heavy metals. An increase of chlorophyll content under salinity stress was reported by Pinheiro et al in 2008 on Ricinus Communis and also by Jamil et al in 2007 on Beta vulgaris L. The reason for increasing chlorophyll contents under salinity and heavy metal stresses as a result of the increased number of chloroplast in leaves during stress for maintaining plant
photosynthesis is evidence of resistance in proportion to environmental stress. non-decreasing chlorophyll content in stress conditions expressing plant resistance against light damage of chloroplast (Parvaneh et al., 2012). However, Chlorophyll content was decreased by increasing levels of salt and metals. The previous reports on cucumber (Yildrim et al. 2008) and tomato (Agong et al. 2003) support our results. In our studies, Citrullus colocynthis L. grown under higher levels of salinity and heavy metals showed more levels of proline as compared to those grown under lower levels of stress. It has also been recognized that plants adopt mechanisms to cope with stressful situations in which proline production is of great importance (Rajasheker et al., 2019). Parvaneh et al. 2012 also reported increased proline level in Purslane with increasing salinity imposed. It has been observed that proline works as a reducer component of osmotic pressure in high salinity in research on portulaca (Parvaneh et al., 2012). Increasing proline content with increasing salinity also has been reported in cotton (Desingh and Kanagaraj, 2007), Paulownia imperialis (Ayala et al., 2010), and wheat (Khan et al., 2009). Proline works as a consistent osmolyte to conserve macromolecules and remove active oxygen in stress (Awais et al., 2022). Regarding germination and growth attributes under salinity and heavy metals of C.colocynthis L. suggests dominance and greater capacity to manage stress. Previously it is also known that Salt-tolerant plant ecotypes accumulate Na+ at a lower rate than the salt-sensitive plant ecotypes as seen in Hordeum spp. (Hmidi et al., 2019). Still, early germination is strongly correlated and coordinated with growth parameters like root and shoot length, fresh and dry biomass production subjected to salinity, and heavy metals stresses. The most essential phase for the future life of the plant is its germination. The subsequent development of the plant depends upon germination and early seedling attributes. The resistance of the plant to heavy metals and salinity at the seedling stage produces tolerant juvenile plants. The current discoveries are similar to the verdict of Akhtar et al., 2021. In the present study high level of NiCl₂ (200uM) reduced the germination percentage of C.colocynthis L. same as described earlier that which a high level of Ni stress reduced seed germination of many plant types (AbdElgawad et al., 2020). The reason behind this justifies that high-stress levels of nickel have straight possessions on the events related to enzymes like amylases, urease, proteases, and ribonucleases, thus affecting the breakdown and utilization of food backup in sprouting seed (Tlhajoane, 2018). High-stress levels of Nickel chloride in our experiment reduced other attributes of seedlings of C.colocynthis L. like seedlings’ radicle projection, plumule protuberance; fresh and dry biomass similarly to the high levels of nickel inhibited the growth of soybean in an experiment (Aqeel et al., 2021). In present studies, low concentrations of Nickel (50uM) with lower salt (100mM NaCl) positively promoted germination percentage and other morphological, biochemical, and physiological parameters of C.colocynthis L. like root length, shoot length, fresh and dry biomass, several leaves, potassium ions, calcium ions, chlorophyll a and chlorophyll b similarly as low levels of nickel promoted germination in wheat (Sofo et al., 2022).

4. Conclusion
The outcomes of our experiment indicated that C. colocynthis L. performed better and tolerate the examined stress under lower combined stress conditions, better germination at combined lower salt and lower heavy metal (100mM NaCl + 50uM NiCl₂) was observed, tolerable at lower salt and moderate metal (100mM NaCl +100uM NiCl₂) acceptable at moderate salt and lower metal (200mM NaCl + 50uM NiCl₂), a slight decrease in moderate salt and moderate metal (200mM NaCl +100uM NiCl₂). Consequently, morphological, biochemical, and physiological parameters of C.colocynthis L. showed better performance under lower levels of stress as compared to control. Increased production of SOD and CAT indicates its adaptability trend to stress conditions. As C.colocynthis L. is a medicinal weed and has adaptability and strategic response against salinity and heavy metals it can be
recommended for further physiological studies of its allelopathic effects before its recommendation as a soil conserving plant. As this plant can tolerate and withstand salinity and heavy metal conditions, it may be recommended for restoration of rangelands indulged in salinity and heavy metal conditions after further advanced biochemical studies of its medicinal value under salinity and heavy metal stress. This research will open a gateway for future researchers to study its genome and explore it as a plant of multidimensional values.

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