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Effects of foliar application of copper nanoparticles on the morphological and physio-biochemical traits of marigold (*Tagetes patula* L.) under salinity stress

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ABSTRACT

This study aimed to assess how foliar application of copper nanoparticles (Cu-NPs) influences the growth, morpho-physiological and biochemical traits of marigold plants subjected to salt stress. The study was conducted in Poldakhtar, Iran, using a greenhouse pot-based factorial experiment arranged in a completely randomized design with three replicates. Two factors were tested: foliar application of copper nanoparticles (Cu-NPs) at concentrations of 0, 100, 200, and 400 mg L^{-1}, and salinity stress induced by sodium chloride at levels of 0, 30, 60, and 90 mM. The study revealed that increasing salinity levels caused significant reductions in key growth parameters including plant height, stem diameter, leaf number, chlorophyll and carotenoid content, relative water content, root length and volume, shoot and root biomass, as well as flower size and longevity, demonstrating the pronounced negative impact of salinity stress. Concurrently, salinity stress induced physiological responses including elevated electrolyte leakage, malondialdehyde and proline levels, along with enhanced activities of antioxidant enzymes (catalase, peroxidase, ascorbate peroxidase). Foliar application of copper nanoparticles (Cu-NPs) at 100-200 mg/L effectively mitigated these stress effects by enhancing antioxidant enzyme activities, increasing proline accumulation, improving water balance, and enhancing photosynthetic pigments. The 200 mg/L Cu-NP treatment showed optimal efficacy, improving salinity tolerance through these mechanisms and demonstrating strong potential for enhancing marigold productivity in saline soils.

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1. Introduction

Agriculture faces increasing salinity conditions due to climate change and human effects (Mohit Rabari, 2023; Khaleghi, 2024). Salinity is among the most intense abiotic stresses, causing significant changes in plant structure and functional processes including metabolic activities, and gene regulation, which collectively hinder plant development and reduce agricultural productivity (Etesami et al., 2021; Guzma and Marques, 2023; Sharafi, 2024). Soil and water salinity often results in ion imbalance, osmotic pressure disruptions, and mineral limitations (Yang and Guo, 2018; Sharafi, 2024) that collectively can trigger oxidative stress in plants by enhancing the production of reactive oxygen species (Etesami et al., 2021). To counteract these effects, plants activate a complex detoxification system via amplifying the enzymatic and non-enzymatic antioxidants (Guzma and Marques, 2023), which coordinate cellular processes including water uptake and ion regulation for a better photosynthesis and performance (Yang and Guo, 2018).

In arid regions such as Iran, ornamental plants are often watered with high-quality water sources, a practice that conflicts with the nation's limited water availability. Beside water shortage, the climate change and associated global warming worsened the soil salinity status and plant production particularly in regions with arid and semi-arid climates (Hatamian et al., 2019). In sustainability point of view, the cultivation of tolerant crops and the use of alternative water resources may be suitable solutions (Souri and Hatamian, 2019; Khaleghi, 2024).

The marigold (*Tagetes patula* L.), a member of the Asteraceae family, is an ornamental plant that typically grows to a height of 20 to 30 cm. Beyond its ornamental value, marigold is an important source of natural pigments such as lutein, widely utilized in the food, cosmetic, and pharmaceutical industries. Additionally, various species possess bioactive compounds with insecticidal and medicinal properties, contributing to their significance in integrated pest management and herbal medicine (Faizi et al., 2008). The plant has high adaptability to different soil and climatic condition with an increasing demand for its cultivation in urban areas. Marigold is used as a seasonal and annual plant in green areas to decorate buildings facades, terraces, and balconies, and sometimes as cut flowers (Umar et al., 2017).

Over the past few years, the integration of nanotechnology into agriculture has gained significant importance, as it offers promising and rapid solutions for achieving sustainable farming (Ebrahimi et al., 2025). Nanoparticles possess unique physical and chemical properties that cause behaving differently from their conventionally sized counterparts (Safaei Far et al, 2024; Jafari et al, 2022; Zulfiqar and Ashraf, 2021;). Copper is an essential element that functions as a cofactor in enzymes that are important for plant growth and development, including superoxide dismutase, cytochrome oxidase, plastocyanin, and polyphenol oxidase (Marschner, 2012; Kazemi et al., 2024). However, in arid regons especially with saline and alkaline soils, copper deficiency is evident that can lead to nutritional disorders in plants and their products (Thounaojam et al., 2012).

Numerous studies have highlighted the positive impact of nanoparticles on plant growth under salinity stress (Hernández-Hernández et al., 2018; Noman et al., 2021; Tabatabaee et al., 2021; Jafari et al., 2023; Soufi et al., 2024); however, many aspects of their action are not clear. Further investigation is required to elucidate the precise mechanisms by which nanoparticles affect plants, their organs and tissues, and various biochemical processes, particularly under major agricultural stresses such as salinity or water deficit. Such knowledge might improve crop growth and production in salt-affected soils (Etesami et al., 2021) or in the use of brackish water for crop or ornamental plants production (Hatamian et al., 2019). In this context, the present study aims to evaluate the effectiveness of foliar application of copper nanoparticles in alleviating the adverse effects of salt stress on the morphophysiological and biochemical traits of marigold.

2. Materials and Methods

2.1 Experimental layout

The study was carried out in an agricultural greenhouse situated on a private farm in Poldakhtar city, Iran (47°42'N, 33°9'E). During the spring and summer months, the greenhouse environment was maintained at daytime temperatures of 20–28 °C and nighttime temperatures of 15–20 °C, with a relative humidity of 60–70% and a photosynthetic photon flux density (PPFD) of 400–500 µmol m^{-2} s^{-1}.

2.2 Treatments Application

The experiment was designed as a factorial study using a completely randomized layout, with three replications for each treatment. The experiment consisted of two main factors: the first being foliar application of copper nanoparticles (Cu-NPs) at four different doses (0, 100, 200, and 400 mg L^{-1}), and the second factor involving salinity induction through sodium chloride treatments at four levels (0, 30, 60, and 90 mM). Plants in the control group (Cu-NPs0-Salinity 0) received only distilled water without any Cu-NPs or sodium chloride. The Cu-NPs, characterized as colloidal particles with an average size ranging from 1 to 5 nanometers, were sourced from a commercial vendor (Pishgaman Nanomaterials Iran Co., Mashhad, Iran).

Marigold plants (F_1 seeds, cultivar 'Durango red') were sown in 1.5-liter pots containing a growing mixture of equal parts of field soil, well-decomposed manure, and sand. Foliar treatments with Cu-NPs began at the four-leaf stage, and repeated three times, each at two-week intervals and until flowering phase. Sodium chloride was introduced one week following the initial Cu-NPs foliar spray, and persisted for three months until the plants bloomed. For normal growth of marigold plants during the current study, the complete fertilizers were applied two times through irrigation. Afterward, various physiological and biochemical traits were evaluated.

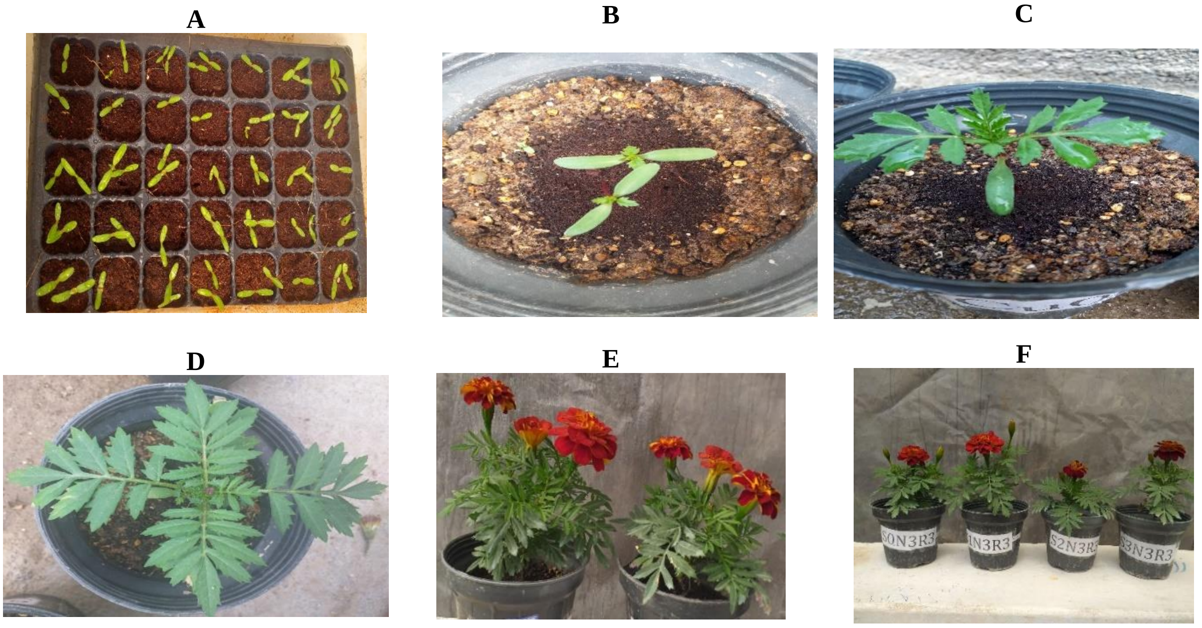


Figure 1 a. A marigold seed tray with cocopeat medium, b. Transplanting of seedlings into pots at the two true leaf stage, c. Marigold plants at the six true leaf stage, e. The stage of the fully opened flower, f. The experimental layout of treatments with copper nanoparticle foliar spray under salt stress.

2.3 Measurements:

2.3.1 Morphology and Biomass

The assessment of morphological traits and biomass distribution included measurements such as plant height (recorded in centimeters using a ruler), stem and peduncle diameters, diameter of the central flower (measured in millimeters with a caliper), number of leaves and nodes, and root length (also measured with a ruler in centimeters). Additionally, root volume was estimated, and the fresh and dry masses of both aerial parts (comprising stems, leaves, and flowers) and root system were determined using a precise digital scale.

2.3.2 Leaf Electrolyte Leakage

Cell membrane stability was assessed indirectly through electrolyte leakage (EL), applying a modified procedure based on Shakarami et al. (2024). Three circular leaf segments (2 cm in diameter) were excised from a fully developed leaf of each plant. These segments were gently washed three times (each for 3 minutes) with sterile distilled water to remove any surface impurities. After rinsing, the discs were transferred into test tubes containing 10 mL of double-distilled water. The samples were then maintained on a shaker at ambient room temperature (approximately 25°C) for 24 hours. After incubation, the first measurement of electrical conductivity (E1), indicating ion leakage into the water from extracellular spaces, was recorded using a digital conductivity meter (Crison 522, Crison Instruments, S.A., Barcelona, Spain).

To determine the total ionic content, the samples were exposed to high temperature by autoclaving at 120°C for 20 minutes. Once cooled back to room temperature, the second conductivity reading (E2) was taken. The extent of electrolyte leakage was computed based on the ratio of E1 to E2, representing the degree of membrane injury.

2.4 Plant Water Status

To assess leaf relative water content (RWC), samples were collected approximately two hours into the photoperiod (between 08:00 and 08:30 a.m.). The fresh weight (FW) of the detached leaves was immediately measured using a high-precision analytical balance (accuracy ±0.0001 g; Mettler AE 200, Giessen, Germany). Following this, the leaves were placed in 9-cm Petri dishes filled with double-distilled water, sealed, and kept in the dark for 24 hours to allow full rehydration. After this period, the saturated weight (SW) was recorded. To obtain the dry weight (DW), the samples were oven-dried at 80°C for 72 hours. The RWC was determined using the equation adapted from Ritchie and Nguyen (1990):

$$\mathbf{RWC}(\%) = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{SW} - \mathrm{DW})} \times 100$$

2.5 Estimation of Chlorophyll and Carotenoid Pigments

To assess chlorophyll and carotenoid levels, 0.1 g of freshly harvested leaf material was finely ground and extracted in 10 mL of pure acetone (100%) under dark conditions to minimize pigment breakdown. The mixture was then centrifuged at 14,000×g for 20 minutes. The resulting clear supernatant was subjected to absorbance measurements using a UV-1800 spectrophotometer (Mapada, Shanghai, China). Optical densities were recorded at wavelengths of 663, 646, and 470 nm to quantify chlorophyll a, chlorophyll b, and carotenoids, respectively, based on the formulas proposed by Lichtenthaler and Wellburn (1983). The chlorophyll stability index (CSI) was derived by comparing the chlorophyll content of stressed samples with that of the control group, following the method described by Vinaya and Parthiban (1995).

2.6 Determination of Malondialdehyde (MDA) Levels

Malondialdehyde (MDA), a common marker for oxidative degradation of lipids, was quantified using a modified TBARS (thiobarbituric acid reactive substances) method. Fresh leaf segments (2 cm^2) were ground and extracted in 5 mL of 20% (w/v) trichloroacetic acid (TCA) mixed with 0.5% (w/v) thiobarbituric acid (TBA). The homogenate was centrifuged at 6000×g for 15 minutes. The resulting supernatant was then incubated at 100°C for 25 minutes to facilitate formation of the MDA–TBA adduct, which has a characteristic pink color. After cooling to room temperature (approximately 25°C), the solution underwent a second centrifugation step (6000×g, 5 minutes) to eliminate any insoluble particles. MDA levels were estimated using a UV-visible spectrophotometer (Model UV-1800, Mapada Instruments, Shanghai, China), based on absorbance at 532 nm. To improve accuracy, the absorbance values at 450 nm and 600 nm were subtracted. The concentration was then determined using an extinction coefficient of 156 $mmol^{-1}$ L cm^{-1}, as described by Wang et al. (2009).

2.7 Quantification of Leaf Proline Content

Proline concentration in leaf samples was analyzed using the protocol established by Bates et al. (1973), with minor adjustment. Approximately 0.5 g of fresh leaf material was ground in 10 mL of 3% sulfosalicylic acid solution. The homogenate was then subjected to centrifugation at 14,000 rpm for 10 minutes at 4°C. A 2 mL aliquot of the resulting extract was mixed with 2 mL of ninhydrin solution and 2 mL of glacial acetic acid. This mixture was incubated in a water bath at 100°C for one hour to allow color development. After heating, the tubes were rapidly cooled on ice, followed by the addition of 4 mL toluene to extract the chromophore. After vigorous shaking for around 20 seconds, the upper toluene layer was isolated, and its absorbance was read at 520 nm using a UV-1800 spectrophotometer (Mapada, China). Proline concentration was calculated in µmol per gram of fresh weight using a standard curve.

2.8 Analysis of Potassium, Sodium, and Copper in Leaf Tissue

Fresh leaf samples were rinsed and washed thoroughly with deionized water, oven-dried at 80°C for 72 hours, and then finely ground into powder. A portion (1 g) of the powdered tissue was subjected to dry ashing by heating at 515°C for 6 hours. The resulting ash was reconstituted in 5 mL of 6.0 N hydrochloric acid and brought to a final volume of 50 mL using double-distilled water. Potassium and sodium contents were quantified using a flame photometer (Model PFP7, Jenway, Keison, UK), and their concentrations were determined with reference to standard curves, expressed as a percentage of the dry weight, following the approach described by Havre (1961). For copper measurement, a portion of the same acid extract was analyzed using an atomic absorption spectrophotometer (Model 240 FS, Agilent Technologies, Santa Clara, CA, USA), according to the method outlined by Oliveira et al. (2010).

2.9 Leaf Enzyme Activity Measurements

To assess catalase (CAT) and peroxidase (POD) activities, 0.3 g of fresh leaf material was ground in liquid nitrogen using mortar and pestle. The resulting powder was suspended in 1.5 mL of potassium phosphate buffer (pH 7.0), supplemented with 1 mM EDTA and 2% polyvinylpyrrolidone. The mixture was centrifuged at 14,000×g for 20 minutes at 4°C. CAT activity in the obtained supernatant was evaluated by monitoring the decline in absorbance at 240 nm over a 2-minute period, with measurements taken every 10 seconds. The assay mixture included phosphate buffer and hydrogen peroxide. The extinction coefficient of 39.4 M^{-1} cm^{-1} (Chance and Maehly, 1955) was used for calculating CAT activity.

POD activity was determined by observing the increase in absorbance at 470 nm, using a similar time course and interval as above. The reaction system contained phosphate buffer, guaiacol, and hydrogen peroxide. An extinction coefficient of 26.6 mM^{-1} cm^{-1} was used for quantification (MacAdam et al., 1992).

To analyze ascorbate peroxidase (APX) activity, 0.3 g of leaf tissue was homogenized in 1.5 mL of extraction buffer composed of 50 mM Na_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM EDTA, 5% polyvinylpyrrolidone, and 1 mM ascorbic acid. The homogenate was centrifuged under the same conditions as for CAT and POD. APX activity was assessed by recording the reduction in absorbance at 290 nm over 2 minutes, measured every 10 seconds, in a reaction containing ascorbate and hydrogen peroxide (Nakano and Asada, 1981).

All enzyme activities, CAT, POD, and APX, were expressed as micromoles of hydrogen peroxide decomposed per minute per gram of fresh tissue (μmol H_2O_2 min^{-1} g^{-1} FW).

2.10 Statistical Analysis and Experimental Design

All statistical analyses were carried out using SAS software, specifically version 9.4. Before proceeding with the analysis, the data were first examined for normal distribution using the Shapiro–Wilk test, ensuring that the assumption of normality was met. In addition, the data were checked for homogeneity of variances using Levene’s test, which assesses whether the variances across groups are consistent. To compare the means of different treatments, the Fisher’s least significant difference (LSD) test was employed, with a significance level set at 5%, meaning that any differences with a probability less than 5% were considered statistically significant. The graphical representations of the data were generated using Microsoft Excel, which was utilized to visually depict the results. Finally, the determination of statistical significance among the average values of the treatments was based on the outcomes of the LSD test.

3. Results

3.1 Growth Parameter

Exposure to elevated sodium chloride levels, particularly at 90 mM, caused a marked decline in several vegetative growth traits including plant height, number of nodes, stem diameter, as well as fresh and dry biomass of both stems and leaves, showing reductions of 45.51%, 59.30%, 44.10%, 50.55%, and 57.12%, respectively, when compared to untreated control plants (Table 1). However, the application of copper nanoparticles (Cu-NPs) at 200 mg L^{-1} notably alleviated these negative effects, resulting in respective increases of 23.56%, 35.93%, 16.18%, 27.48%, and 14.69% in the same parameters under saline conditions.

The Cu-NPs, regardless of the concentration used, positively influenced plant growth in salt-treated plants (Table 1). Although salinity led to a significant reduction in leaf number and its biomass, foliar spraying with Cu-NPs, particularly at the highest concentration, substantially improved leaf numbers and biomass.

Morphological and physiological characteristics such as peduncle diameter, central flower diameter, and fresh and dry weights of the central flower were also negatively impacted by increasing salinity levels. At 90 mM NaCl, reductions of 51.24%, 31.59%, 49.59%, and 43.35% were recorded in these traits, respectively, relative to the non-stressed control plants (Table 1). Conversely, treatment with 200 mg L^{-1} Cu-NPs enhanced peduncle diameter and the fresh and dry biomass of the central flower by 21.87%, 22.30%, and 21.25%, respectively. Lower concentrations of Cu-NPs (100 and 200 mg L^{-1}) also contributed to increased flower diameter under both saline and non-saline conditions (Table 1).

Table 1. Impact of salt stress and foliar-applied copper nanoparticles on selected morphological characteristics of marigold. Statistically significant changes between treatments are shown by different lowercase letters based on two-way ANOVA at $p < 0.05$.

NaCl (mM)	Cu-NPs (mgL^{-1})	Plant height (cm)	No. of Nodes	No. of Leaves	Stem diameter (mm)	Leaf dry weight	Stem fresh weight	Stem dry weight	Leaf fresh weight
			Per plant			(g)			
Control	0	18.57^{cd}	22.0^{bc}	81.3^{bc}	6.90^{cd}	1.420^{b}	4.253^{bc}	0.660^{bcd}	9.213^{bcd}
	100	21.13^{a}	22.0^{bc}	83.0^{ab}	8.33^{ab}	1.427^{b}	4.340^{abc}	0.693^{abc}	9.603^{abc}
	200	20.93^{ab}	24.0^{a}	85.7^{a}	9.13^{a}	1.603^{a}	5.043^{a}	0.757^{a}	10.310^{a}
	400	19.70^{bc}	23.7^{ab}	84.7^{ab}	7.90^{bc}	1.327^{bc}	4.477^{ab}	0.667^{bcd}	9.807^{ab}
30	0	16.67^{e}	18.7^{ef}	71.7^{e}	5.33^{fgh}	1.087^{de}	3.767^{bc}	0.600^{de}	8.130^{e}
	100	17.50^{de}	19.7^{de}	74.3^{de}	5.70^{e-h}	1.217^{cd}	3.790^{bc}	0.633^{cde}	8.793^{cde}
	200	19.40^{bc}	21.3^{cd}	83.0^{ab}	6.83^{cde}	1.440^{ab}	4.387^{abc}	0.723^{ab}	9.300^{bcd}
	400	17.37^{de}	20.7^{cd}	77.7^{cd}	6.17^{def}	1.280^{bc}	4.073^{bc}	0.657^{bcd}	8.570^{de}
60	0	12.63^{f}	16.3^{gh}	50.0^{h}	4.10^{jk}	0.847^{fg}	2.553^{df}	0.400^{g}	6.157^{fg}
	100	14.20^{f}	17.3^{fg}	54.3^{g}	4.87^{g-j}	0.970^{ef}	2.943^{d}	0.483^{f}	8.300^{fg}
	200	17.17^{de}	20.0^{de}	60.7^{f}	5.80^{d-g}	1.060^{de}	3.707^{c}	0.560^{ef}	8.300^{e}
	400	13.57^{f}	15.7^{gh}	51.7^{gh}	4.57^{h-k}	0.787^{g}	2.163^{ef}	0.343^{gh}	5.730^{gh}
90	0	9.83^{gh}	12.3^{i}	45.7^{i}	2.87^{l}	0.610^{hi}	2.057^{ef}	0.283^{hi}	4.970^{hi}
	100	10.87^{g}	12.7^{i}	49.0^{hi}	3.43^{kl}	0.730^{gh}	2.270^{def}	0.313^{h}	6.010^{fg}
	200	13.83^{f}	15.0^{h}	52.0^{gh}	4.33^{ijk}	0.837^{fg}	2.967^{d}	0.493^{f}	6.887^{f}
	400	9.23^{h}	11.3^{i}	45.0^{i}	2.50^{l}	0.470^{i}	1.663^{f}	0.223^{i}	4.167^{i}

Table 1. (continued)

NaCl (mM)	Cu-NPs (mgL^{-1})	Peduncle diameter (mm)	Flower diameter (mm)	Flower fresh weight (g)	Flower dry weight (g)	longevity (day)	Root Length (cm)	Root Volume (cm^3)	Root fresh weight (g)	Root dry weight (g)
		Central flower								
Control	0	11.55^{bc}	55.55^{a-d}	1.970^{abc}	0.323^{abc}	11.3^{ab}	25.33^{ab}	12.83^{abc}	11.730^{a}	0.630^{cd}
	100	12.24^{ab}	56.63^{abc}	2.097^{ab}	0.357^{a}	11.3^{ab}	24.80^{ab}	12.83^{abc}	12.517^{a}	0.710^{a}
	200	13.69^{a}	58.54^{a}	2.300^{a}	0.350^{ab}	11.7^{a}	27.17^{a}	14.33^{a}	12.323^{a}	0.700^{ab}
	400	12.26^{ab}	59.03^{a}	2.030^{abc}	0.353^{a}	11.7^{a}	25.33^{ab}	13.00^{abc}	11.927^{a}	0.683^{abc}
30	0	9.65^{cd}	50.49^{ef}	1.623^{de}	0.303^{bc}	9.0^{b-e}	21.17^{de}	10.33^{de}	9.883^{ab}	0.550^{ef}
	100	10.45^{bcd}	52.09^{de}	1.847^{bcd}	0.313^{abc}	10.3^{a-d}	23.50^{bcd}	11.67^{bcd}	11.133^{bc}	0.563^{ef}
	200	11.39^{bc}	57.70^{ab}	1.993^{abc}	0.347^{ab}	11.0^{abc}	26.17^{ab}	13.17^{ab}	12.387^{a}	0.647^{bcd}
	400	10.33^{bcd}	52.90^{cde}	1.713^{cde}	0.337^{ab}	8.7^{cde}	23.83^{a-d}	12.00^{bcd}	11.623^{a}	0.607^{de}
60	0	6.59^{efg}	44.31^{ghi}	1.230^{fg}	0.217^{efg}	8.0^{def}	16.67^{fg}	7.67^{fg}	6.427^{de}	0.363^{g}
	100	7.34^{ef}	47.45^{fg}	1.277^{fg}	0.230^{ef}	8.3^{de}	20.50^{de}	8.50^{f}	7.653^{d}	0.410^{g}
	200	8.40^{de}	54.24^{b-e}	1.397^{ef}	0.287^{cd}	9.0^{b-e}	23.67^{bcd}	11.33^{cd}	9.370^{d}	0.520^{f}
	400	5.82^{fg}	42.70^{hi}	1.133^{fgh}	0.213^{e-h}	6.7^{efg}	18.17^{ef}	7.67^{fg}	6.943^{c}	0.270^{h}
90	0	$5.50f^{g}$	38.00^{jk}	0.967^{gh}	0.173^{fg}	5.3^{g}	14.33^{g}	6.17^{gh}	4.483^{fg}	0.197^{i}
	100	6.41^{efg}	41.22^{ij}	1.010^{gh}	0.197^{fgh}	5.7^{fg}	17.93^{ef}	6.67^{gh}	5.177^{ef}	0.280^{h}
	200	7.07^{efg}	46.01^{gh}	1.393^{ef}	0.247^{de}	7.3^{efg}	21.83^{cd}	9.17^{ef}	6.813^{d}	0.370^{g}
	400	5.26^{g}	36.45^{k}	0.863^{h}	0.167^{h}	5.0^{g}	13.83^{g}	5.33^{h}	3.727^{g}	0.150^{i}

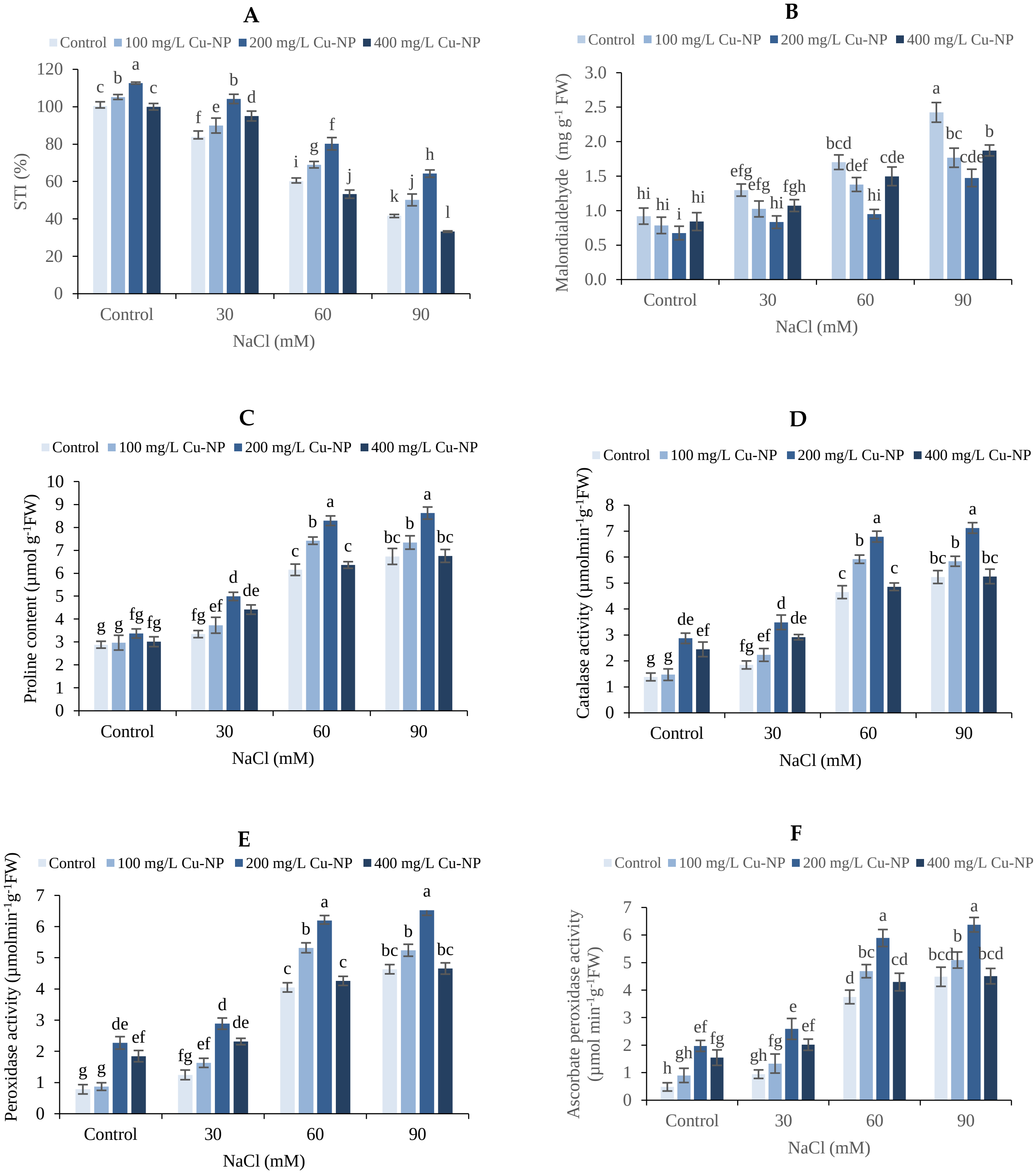


Figure 1. Effects of salinity and copper nanoparticle (Cu-NPs) spray treatments on marigold physiological responses: salinity tolerance index (STI, A), malondialdehyde content (MDA, B), proline levels (C), catalase enzyme activity (D), peroxidase enzyme activity (E), and ascorbate peroxidase activity (F). Results are shown as mean values ± standard error (SE). Treatment means were evaluated using the LSD test at a significance level of $p < 0.05$. Columns marked with the same letter are not significantly different.

In contrast, flower longevity was adversely affected by rising salinity levels, and foliar application of Cu-NPs did not produce a noticeable improvement in this parameter (Table 1).

Root growth characteristics including root length, root volume, and fresh and dry weights were significantly diminished (33.82%, 48.45%, 58.33%, and 68.73% reduction, respectively) under 90 mM salinity compared to the control plants (Table 1). Foliar application of Cu-NPs countered these effects, improving all root growth parameters, with the most pronounced improvements observed at 200 mg L^{-1}. Moreover, Cu-NP treatment enhanced root dry matter and the stress tolerance index (STI) under both salt-affected and non-salt-affected conditions (Figure 1A).

3.2 Relative Leaf Water Content, Electrolyte Leakage, and Malondialdehyde Levels

Analysis of variance indicated that both salinity levels and foliar treatments with Cu-NPs had significant impacts ($P < 0.01$) on leaf relative water content (RWC), electrolyte leakage (EL), and malondialdehyde (MDA) accumulation. However, a significant interaction between salinity and Cu-NPs was observed only for EL ($P < 0.01$). As salt concentration increased, a decline in RWC was noted, while EL and MDA content increased correspondingly. The most severe effects were seen at 90 mM NaCl, where RWC dropped to 62%, and EL and MDA reached peak values of 69% and 166.6 mg g^{-1} fresh weight, respectively (Table 2 and Figure 1B).

Foliar spraying with Cu-NPs mitigated EL under both saline and non-saline conditions (Table 2). At the optimal dose, the 200 mg L^{-1} Cu-NPs treatment led to the highest RWC (77%), the lowest EL (17%), and an MDA concentration of 0.983 mg g^{-1} fresh weight (Table 2 and Figure 1B).

3.3 Leaf Pigments, Chlorophyll Stability Index, and Leaf Proline Content

The analysis results of variance indicated a statistically significant interaction ($P<0.01$) between salinity levels and the use of copper nanoparticles (Cu-NPs) regarding both the chlorophyll stability index and leaf proline accumulation. Both salinity stress and foliar spraying with Cu-NPs had a marked influence ($P<0.01$) on the levels of chlorophyll (a, b and total), carotenoids, proline content, and the chlorophyll stability index of leaves.

Exposure to 90 mM salinity led to noticeable reductions in chlorophyll (a, b and total), and carotenoids by 56.37%, 49.01%, 53.83%, and 56.40%, respectively, when compared with the untreated control group (see Table 2). Conversely, foliar spraying with Cu-NPs, especially at concentration of 200 mg L^{-1}, resulted in significant increases in chlorophyll a (37.39%), chlorophyll b (35%), and total chlorophyll (36.53%) levels compared to salinity-treated plants without Cu-NPs application (Table 2).

Salinity stress significantly lowered the chlorophyll stability index and proline levels in marigold leaves; however, Cu-NPs application improved the leaves chlorophyll stability index and proline content irrespective of salinity or non-salinity conditions. The most pronounced enhancements, 117% in chlorophyll stability and 119% in proline concentration, were found in plants treated with 200 mg L^{-1} Cu-NPs under non-saline conditions, compared to the untreated control (see Table 2 and Figure 1C).

3.4 Antioxidant Enzymes and Mineral Contents

The variance analysis revealed that the combined effect of salinity and copper nanoparticles (Cu-NPs) had a high significant impact ($P<0.01$) on leaf ascorbate peroxidase activity and sodium content. Independently, the salinity and the foliar application of Cu-NPs also exerted a statistically significant influence ($P<0.01$) on the enzymatic activities of catalase, peroxidase, and ascorbate peroxidase, along with the concentrations of potassium, sodium, copper, and the potassium-to-sodium ratio.

An upward trend was observed in the activities of catalase, peroxidase, and ascorbate peroxidase in response to increasing salinity and Cu-NPs application. The highest enzyme activities, 415% for catalase, 733% for peroxidase, and 74% for ascorbate peroxidase relative to the control, were recorded under 90 mM salinity and 200 mg L^{-1} Cu-NP application (see Figure 1). Furthermore, the use of Cu-NPs under various salinity conditions consistently promoted the activity levels of these antioxidant enzymes (Figure 1).

Table 2. Effects of salinity conditions and copper nanoparticle foliar treatments on electrolyte leakage (%), relative water content (RWC), chlorophyll stability index (CSI), chlorophyll a, chlorophyll b, total chlorophyll (a+b), and carotenoid concentrations in marigold plants. Statistically significant changes between treatments are shown by different lowercase letters based on two-way ANOVA at $p < 0.05$.

NaCl (mM)	Cu-NPs (mgL^{-1})	RWC (%)	Electrolyte leakage (%)	CSI (%)	Chlorophyll a	Chlorophyll b	Chlorophyll ab	Carotenoid
					mg g^{-1} FW			
Control	**0**	89^{ab}	23^{jk}	100^{c}	13.441^{abc}	6.746^{a-d}	20.187^{abc}	3.579^{ab}
	100	88^{ab}	20^{jkl}	110^{b}	14.824^{ab}	7.451^{abc}	22.275^{a}	3.707^{ab}
	200	90^{a}	17^{l}	117^{a}	15.540^{a}	8.071^{ab}	23.611^{a}	4.175^{a}
	400	85^{ab}	19^{kl}	100^{c}	12.736^{bcd}	7.428^{abc}	20.164^{abc}	3.605^{ab}
30	**0**	78^{de}	32^{g}	82^{f}	10.499^{def}	5.957^{a-e}	16.456^{c-f}	2.806^{bcd}
	100	81^{cd}	27^{hi}	97^{c}	12.592^{bcd}	7.079^{abc}	19.671^{a-d}	2.911^{bcd}
	200	83^{c}	24^{ij}	110^{b}	13.792^{abc}	8.451^{a}	22.243^{ab}	3.309^{abc}
	400	76^{e}	29^{gh}	75^{f}	9.635^{ef}	5.557^{b-e}	15.192^{ef}	2.934^{bcd}
60	**0**	68^{f}	65^{b}	67^{g}	8.425^{fg}	5.024^{cde}	13.448^{fg}	1.916^{def}
	100	66^{f}	44^{e}	79^{ef}	9.895^{ef}	6.071^{a-d}	15.966^{def}	2.545^{b-e}
	200	70^{f}	40^{f}	91^{d}	11.793^{cde}	6.513^{a-d}	18.307^{b-e}	2.802^{bcd}
	400	61^{gh}	52^{d}	47^{i}	6.232^{gh}	3.378^{ef}	9.610^{gh}	1.893^{def}
90	**0**	61^{gh}	69^{a}	40^{j}	4.875^{hi}	3.295^{ef}	$8.170h^{i}$	1.494^{ef}
	100	62^{g}	58^{c}	56^{h}	6.987^{gh}	4.229^{def}	11.216^{gh}	$1.843^{de}f$
	200	67^{f}	51^{d}	76^{f}	10.040^{ef}	5.346^{cde}	15.385^{ef}	2.211^{c-f}
	400	57^{h}	65^{ab}	25^{k}	2.766^{i}	2.272^{f}	5.038^{i}	1.020^{f}

The highest sodium concentration (0.16 % of leaf DW and equal to 226% increase compared to the control), was observed at highest salinity level of 90 mM sodium chloride. The foliar application of Cu-NPs at 200 and 400 mg L^{-1} reduced leaf sodium concentrations under different NaCl salinity levels (Figure 2A).

The results also showed that leaf potassium concentration and the potassium-to-sodium ratio were decreased under salinity conditions (Figure 2C, D). However, copper nanoparticle (Cu-NPs) spray increased both potassium concentration and the potassium-to-sodium ratio in treated plants. At 200 mg L^{-1}, the highest leaf potassium concentration (3.014%) and potassium-to-sodium ratio (42%) were recorded (Figure 2). Nevertheless, foliar application of Cu-NPs increased leaf copper concentration, with the highest value (about 35 ppm equal to 421% increase compared to the control) observed at 400 mg L^{-1} Cu-NPs foliar spray (Figure 2).

The Pearson's correlation shows that there was an overall high correlation between leaf Na concentrations with EL and MDA in leaves, while vegetative parameters such as leaf number, leaf biomass, RWC and root growth parameters showed negative correlations mainly with leaf Na, EL and MDA.

Flower longevity, which is a major quality factor for those ornamental crops like marigold, showed negative correlations with Na levels and consequently with leaf MDA, EL, peroxidase, catalase, ascorbate peroxidase and proline, while it had positive correlation with leaf chlorophyll, K concentrations and biomass production. On the other hand, leaf RWC, as an important and basic physiological leaf traits especially under saline conditions, showed positive correlations with leaf biomass, leaf number, leaf chlorophylls and carotenoids, and leaf K, flower diameter, flower longevity, root length, volume and root biomass (Figure 3).

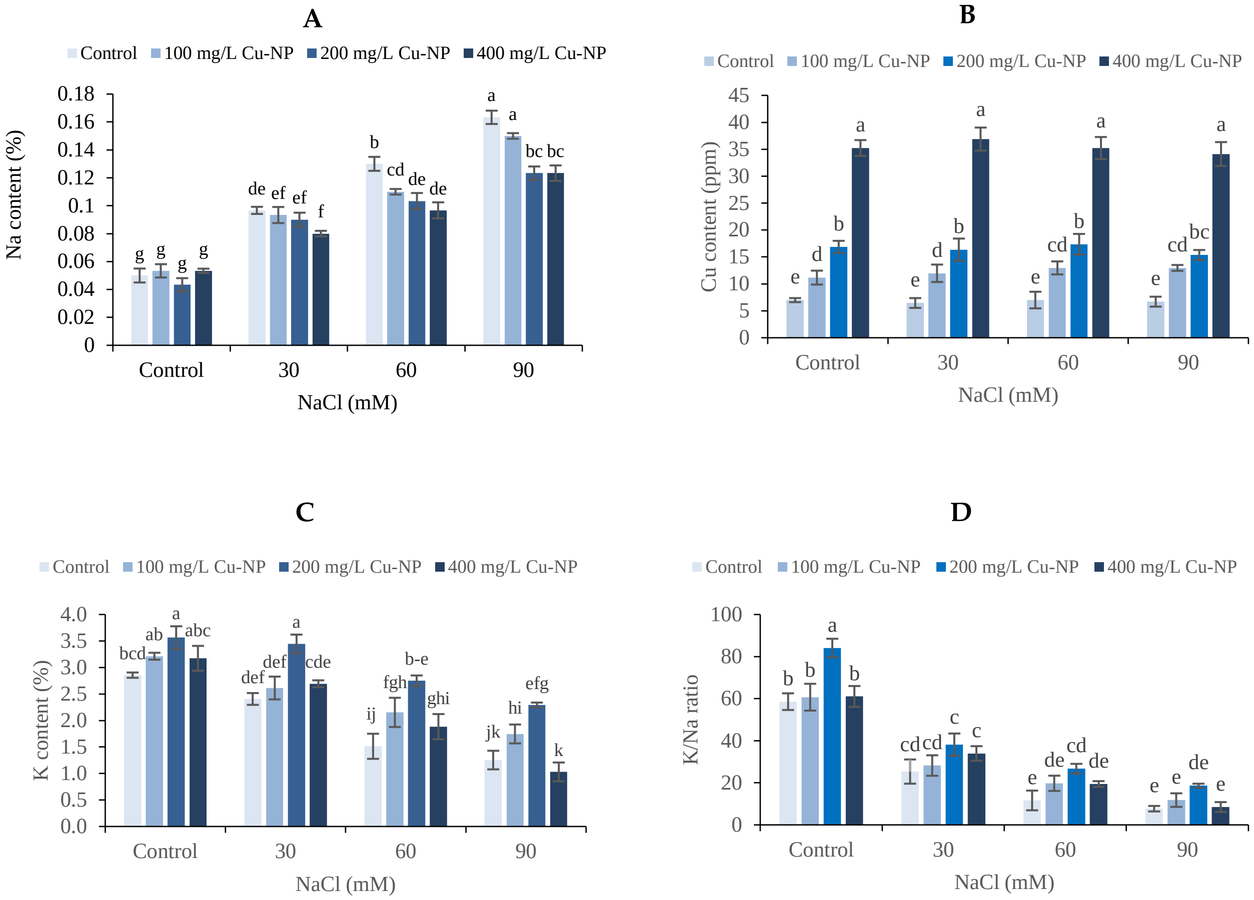
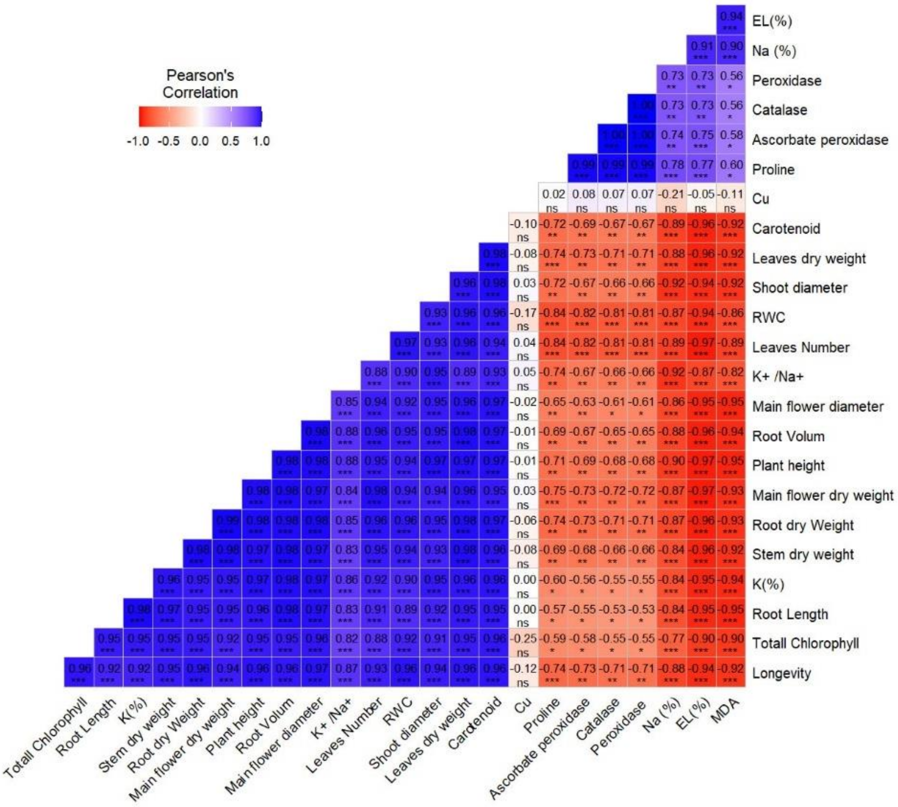


Figure 2. Effects of saline conditions and the foliar application of copper nanoparticles (Cu-NPs) on sodium (A), copper (B), potassium (C) concentrations, and the potassium-to-sodium ratio (D) in marigold plants. Values are expressed as mean ± standard error (SE). Statistical comparisons were conducted using the LSD test at the 0.05 significance level. Columns sharing the same letter denote no statistically significant differences among treatments.



ns p >= 0.05; * p < 0.05; ** p < 0.01; and *** p < 0.001

Figure 3. A Pearson's correlation heatmap was plotted for the studied morphological, phenological, and biochemical traits. In this diagram, groups of traits with positive and negative correlations are indicated by blue and red colors, respectively.

4. Discussion

4.1 Plant Growth Characteristics

This study found that exposure to salt stress adversely influenced various growth traits, including shoot length, stem thickness, leaf count, and both fresh and dry biomass of the marigold aerial parts. Conversely, foliar spraying with copper nanoparticles (Cu-NPs) contributed positively to these growth metrics. Previous research on ornamental species has indicated that irrigating with saline water, which elevates the electrical conductivity of the medium, can hinder growth due to osmotic stress and the build-up of harmful ions within plant tissues. For example, Oliveira et al. (2017) reported notable reductions in shoot length in species such as *Catharanthus roseus*, *Allamanda cathartica*, *Ixora coccinea*, and *Duranta erecta* under such saline conditions. Poor cell turgor, increased allocation of synthesized osmolytes to counteract salinity stress, shortened plant growth periods, and stress avoidance mechanisms may all hinder normal cell development and turgidity, thereby reducing plant growth (Raza et al., 2022).

The positive influence of copper oxide nanoparticles on marigold growth indicators may be linked to the gradual release of copper ions within plant tissues and the essential role of copper in various metabolic activities. These include its involvement in photosynthesis, nitrogen metabolism, amino acid synthesis, and the production of auxins, all of which contribute to cell elongation and expansion (Chrysargyris et al., 2018; Etesami et al., 2021).

It is noteworthy that in this study, foliar application at a concentration of 400 mg L^{-1} resulted in a reduction in most of the traits evaluated under salinity levels of 30, 60, and 90 mM, indicating the toxic effects of copper at this concentration. However, foliar application at

200 mg L^{-1} had a positive effect in alleviating the adverse effects of salinity stress. This may be due to the occurrence of copper toxicity at higher foliar application concentrations.

4.2 Flowering Characteristics

In present study, increasing salinity significantly reduced marigold flowering characteristics and its longevity; and the use of foliar spray of Cu-NPs has no significant effect on flower longivity. In ornamental as well as many agricultural crops, the flowering and development of reproductive structures are disrupted under unfavorable stress conditions (Souri and Hatamian, 2019; Guzman and Marques, 2023). Salinity stress has been reported to markedly impair floral development, reducing the size, quantity, and longevity of flowers in various ornamental species, including African marigold and floss flower (Zapryanova and Atanassova, 2009; Guzman and Marques, 2023). Similar reductions in plant height and flowering have been also observed in species such as *C. roseus*, *A. cathartica*, *I. coccinea*, and *D. erecta* (Oliveira et al., 2017). These effects may, in part, be driven by alterations in the activity of MADS-box transcription factors, key regulators of floral organ development, that are sensitive to salinity conditions (Kaashyap et al., 2022; Guzman and Marques, 2023). Research also indicates that copper plays an important role in reproductive success by boosting pollen viability, enhancing germination, and supporting seed development (Kaashyap et al., 2022). Additionally, the use of Cu-NPs may promote plant resilience to salinity by stimulating the synthesis of protective metabolites, such as amino and organic acids, as well as other osmolytes (Mohit Rabari et al., 2023).

4.3 Root Characteristics

Marigold root growth was severely influenced by salinity levels and application of Cu-NPs have improved root growth under both saline and non-saline conditions. Root development in plants like African marigold (*Tagetes erecta*) and *Catharanthus roseus* has been shown to be adversely affected by saline conditions (Oliveira et al., 2017; Chrysargyris et al., 2018). High salt concentrations in the soil can significantly lower the osmotic potential, making it difficult for plants to take up water, even when soil moisture is adequate. This inhibition of root growth under saline stress is likely due to a combination of factors, including restricted translocation of photosynthates from shoots, osmotic imbalance, and ion-induced toxicity (Mohit Rabari et al., 2023; Soufi et al., 2023). These stresses hinder cell enlargement as a result of poor water uptake by root cells and greater water retention within the soil matrix (Della Maggiora et al., 2023). The Cu-NPs treatment increased plant root characteristics that might be due to restriction of Na and Cl ion effects and better cell membrane and wall integrity (Souri and Hatamian, 2019). Studies on wheat seedlings have shown that the morphology and proliferation of root hairs are improved by Cu-NPs (Anderson et al., 2017).

4.4 Electrolyte Leakage, Malondialdehyde Concentration, and RWC contents

In this study, salinity stress led to elevated levels of leaf electrolyte leakage (EL) and malondialdehyde (MDA), accompanied by a marked decline in relative water content (RWC). Conversely, foliar treatment with copper nanoparticles (Cu-NPs) alleviated these harmful effects, resulting in decreased EL and MDA levels and improved leaf RWC. Under saline environments, plants often experience oxidative stress due to the overaccumulation of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Priyanka et al., 2019). These reactive molecules can compromise cell membrane integrity, trigger lipid peroxidation, reduce water content, and increase ion leakage (Chrysargyris et al., 2018). Application of Cu-NPs helped counteract these impacts by lowering the concentrations of hydrogen peroxide and MDA, thereby limiting oxidative damage to membranes and cell components (Noman et al., 2021; Tabatabaee et al., 2021). Additionally, nanoparticles are known to boost root water transport efficiency by upregulating aquaporin proteins, which may facilitate better water absorption and diminish oxidative injury and structural damage to cell membranes (Anderson et al., 2017; Priyanka et al., 2019; Etesami et al., 2021).

4.5 Photosynthetic Pigments and Proline

Salinity stress negatively influenced chlorophyll levels in leaves and limited photosynthetic efficiency, leading to reduced biomass production. At the same time, proline, an important osmo protectant and metabolic regulator, was found to accumulate in response to salt stress. Foliar application of copper nanoparticles (Cu-NPs) helped alleviate the detrimental impacts of salinity by improving chlorophyll content and enhancing photosynthetic activity in both saline and non-saline environments. Previous studies have highlighted the beneficial effects of Cu-NPs on promoting plant growth, preserving chlorophyll pigments under salinity, and stimulating proline accumulation in African marigold and other species (Chrysargyris et al., 2018; Priyanka et al., 2019; Etesami et al., 2021; Tabatabaee et al., 2021). A decline in chlorophyll content under salt stress is often attributed to disruptions in chlorophyll-protein complex stability, caused by excessive sodium and chloride ions, as well as structural changes in the chloroplasts, including thylakoid thinning, granal disorganization, and overall damage to chloroplast architecture (Della Maggiora et al., 2023). The ions deficiency (K, Mg, Fe, Zn, Mn and Cu) and enzymes instability may also has some roles in this regard (Souri and Hatamian, 2019). The reduction in chlorophyll and simultaneous increase in proline may be due to diversion of glutamate (a precursor for both chlorophyll and proline synthesis) toward proline production. The increase in proline content can enhance cell osmotic pressure and turgor, stabilize cell and membrane proteins, and ultimately protect membranes from oxidation and peroxidation (Chrysargyris et al., 2018; Souri and Hatamian, 2019). Copper, as a component of plastocyanin and numerous enzymes involved in electron transfer during mitochondrial and chloroplast oxidation-reduction reactions, plays a key role in pigment synthesis, plasma membrane permeability, and regulating amino acid (e.g., proline) and soluble sugar levels (Noman et al., 2021; Etesami et al., 2021; Zulfiqar and Ashraf, 2021).

4.6 Antioxidant Enzymes and Nutrients

The application of salinity and copper nanoparticles (Cu-NPs) led to a marked enhancement in the activity of antioxidant enzymes, specifically catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), in marigold plants. Previous studies have also documented similar enhancements in antioxidant enzyme function under salinity conditions and when copper nanoparticles were applied (Hernández-Hernández et al., 2018; Perez Labrada and Lopez-Vargas, 2019). Depending on their concentration, metal oxide nanoparticles such as copper can stimulate the generation of reactive oxygen species (ROS), which may play a dual role in plant systems. These ROS can serve as key signaling agents that trigger salt stress tolerance mechanisms by promoting both enzymatic and non-enzymatic antioxidant responses, potentially through the activation of the jasmonic acid signaling cascade (Hernandez-Hernandez et al., 2018; Zulfiqar & Ashraf, 2021).

Salinity stress leads to an excess of sodium ions around the root zone, which competes with potassium for uptake sites of the roots as well as within plant cells, ultimately reducing potassium absorption (Zhao et al., 2019; Pérez-Labrada et al., 2019; Noman et al., 2021, Soufi et al., 2023). The overall decline in plant growth under saline conditions is primarily attributed to ionic toxicity, especially sodium, which considerably limits K uptake through potassium channels or transporters (Souri and Hatamian, 2019, Soufi et al., 2024). These effects, in turn, impair nitrate uptake and hinder vegetative development (Zhao et al., 2019). Research on tomato plants experiencing salt stress has demonstrated that applying copper nanoparticles as a foliar spray enhanced plant growth and helped regulate the Na^+/K^+ balance, alleviating the adverse impacts of salinity (Perez-Labrada et al., 2019). Elevated sodium levels are also known to cause damage at the cellular level, including disruption of membrane integrity, organelle function, photosynthesis, respiration, and protein synthesis, as well as destabilization of protein structures (Noman et al., 2021).

Conclusion

Findings from this research revealed that exposure to sodium chloride-induced salinity adversely impacted marigold growth and quality. This was primarily due to osmotic stress, which led to reduction in main traits such as fresh and dry biomass, relative water content, and leaf photosynthetic characteristics, while it increased electrolyte leakage (61.7%), lipid peroxidation, and reactive oxygen species (ROS) accumulation. In contrast, spraying the plants with copper nanoparticles helped counteract many of these harmful effects. The treatment decreased membrane damage indicators like electrolyte leakage and lipid peroxidation while it enhanced physiological responses including higher levels of leaf chlorophyll, proline, and activity of antioxidant (CAT, POD, and APX). These changes collectively contributed to improved salinity stress resilience in marigold plants under foliar application of Cu-NPs. Overall, based on the results of this study, foliar application of Cu-NPs at a concentration of 200 mg L^{-1} is recommended as an optimal dose under salinity stress conditions, as it can positively influence the growth of Marigold plants and mitigate the adverse effects of salinity stress in both greenhouse and open-field cultivation.

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