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OPTIMIZING SAFFLOWER GROWTH UNDER COPPER STRESS VIA APPLICATION OF SALICYLIC ACID, CHITOSAN, AND NANOPARTICLE

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Abstract

Excessive copper (Cu) levels in plants cause toxicity, stunted growth, root damage, and deficiencies in essential minerals. It also generates oxidative stress, disrupts microbial activity, and alters soil structure, making it harder for roots to grow and absorb nutrients. However, using salicylic acid (SA), chitosan (Chi), and nanoparticles (NP) can be effective, environment-friendly amendments to mitigate copper stress in plants. Nanoparticles significantly enhance plant growth by improving nutrient availability, boosting photosynthesis, and increasing stress tolerance. Salicylic acid acts as a plant hormone, improving seed germination, root development, and photosynthesis. Chitosan improves seed germination, root growth, and shoot expansion by enhancing cell multiplication and nutrient uptake. That's why the current study aims to explore the potential of salicylic acid, chitosan, and nanoparticles as an amendment on three safflower varieties (Saff-64, Saff-50, and Saff-62) cultivated under Cu stress. Treatments include control, 400Cu, 0.01SA, Chi, 400Cu+0.01SA, 400Cu+Chi, 0.01NP, 0.1NP, 400Cu+0.01NP, and 400Cu+0.01NP in three replicates following a completely randomized design (CRD). Results showed that 0.01NP caused the highest increase in shoot length (~14%), shoot fresh weight (~51%), shoot dry weight (~55%), root fresh weight (~45%), and root dry weight (~10%) of variety Saff-62 compared to the control over the other varieties. The increase in chlorophyll a (~1%), chlorophyll b (~18%), and total chlorophyll (~2%) of variety Saff-62 above the other varieties under Cu stress also validated the effect of 0.0NP. It is concluded that variety Saff-62 is a resistant variety in contrast to the other varieties, and 0.01NP is an effective amendment for alleviating Cu stress in safflowers. Under Cu stress, farmers are suggested to maximize their crop growth by using the Saff-62 variety and applying 0.01NP as an amendment.

Key words: Salicylic acid; Chitosan; Nanoparticles; Chlorophyll content; Copper stress

Introduction

Copper is an essential micronutrient required for plant growth, playing a vital role in enzyme activation, photosynthesis, and protein synthesis (Shabbir *et al.*, 2020, Mir *et al.*, 2021). However, excessive copper in the soil can be highly toxic, negatively impacting both plants and soil health. The major sources of copper accumulation include industrial activities like mining and smelting, excessive use of copper-based pesticides and fertilizers, and the application of sewage sludge in agriculture (Simate & Ndlovu, 2014, Cacciuttolo & Atencio, 2022). When copper levels exceed the required amount, plants show signs of toxicity, such as chlorosis (yellowing of leaves), stunted growth, and root damage (Cruz *et al.*, 2022). High copper concentrations interfere with nutrient uptake, leading to deficiencies of essential minerals like iron, zinc, and manganese (Ahmed *et al.*, 2010, Bowszys *et al.*, 2015). Moreover, excessive copper generates oxidative stress in plants, damaging cell membranes and reducing overall growth and productivity. Elevated copper levels in soil affect microbial activity, disrupting essential processes such as the decomposition of organic matter and nutrient cycling (Fagnano *et al.*, 2020). Additionally, copper alters soil structure, reducing water infiltration and increasing compaction, making it harder for plant roots to grow and absorb nutrients (Rodrigues *et al.*, 2013).

Salicylic acid plays a crucial role in enhancing plant growth by regulating physiological and biochemical processes (Kaya *et al.*, 2023). It acts as a plant hormone, improving seed germination, root development, and photosynthesis. Salicylic acid helps plants tolerate environmental stresses such as drought, salinity, and heavy metal toxicity by boosting antioxidant activity and reducing oxidative damage (Li *et al.*, 2022). It also enhances nutrient uptake, strengthens the plant's immune system, and promotes flowering and fruit production (Yang *et al.*, 2023). By activating defense mechanisms, salicylic acid supports growth and increases resistance against pests and diseases, making it an essential compound for healthy plant development (Kaya *et al.*, 2023).

Nanoparticles significantly enhance plant growth by improving nutrient availability, boosting photosynthesis, and increasing stress tolerance (El-Saadony *et al.*, 2022). Due to their small size, nanoparticles can easily penetrate plant cells, facilitating efficient nutrient absorption and utilization. Certain nanoparticles, such as zinc, iron, and silica, help in root and shoot development, chlorophyll production, and overall plant metabolism (Rajput *et al.*, 2021). They also enhance water uptake and protect plants from environmental stresses like drought and salinity. Additionally, nanoparticles can act as carriers for fertilizers and pesticides, reducing chemical usage and minimizing environmental pollution (Hazarika *et al.*, 2022). Their application in agriculture offers a promising approach to improving crop yield and sustainability.

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The natural biopolymer Chi originates from chitin because it helps enhance plant growth and tolerance against stress factors (Shahrajabian *et al.*, 2021). The compound drives seed germination, root growth, and shoot expansion by enhancing cell multiplication and nutrient uptake (Saad Ullah *et al.*, 2023). Plants receive strengthened immune defense when they take chitosan because it promotes activation of defense-related enzymes, thus enabling better protection against fungal, bacterial, and viral infections. Applying chitosan results in increased water retention capacity of soil and allows plants to resist heavy metal uptake and produce more chlorophyll, which supports better photosynthetic activity (Sadak *et al.*, 2022). Chitosan is important in boosting crop yields and protecting plants' well-being through hormone regulation and antioxidant promotion (Sun *et al.*, 2023).

The oilseed crop safflower (*Carthamus tinctorius*) produces an outstanding oil product that includes abundant unsaturated fatty acids and serves multiple industries from kitchen to pharmaceutical use (Kurt *et al.*, 2025). Safflower exists as an essential medicinal plant along with its ability to grow successfully in dry and semi-arid regions (Emongor & Emongor, 2023). The presence of excessive copper in soil causes severe problems for safflower development as it disrupts nutrition absorption processes and leads to essential mineral element deficiencies such as iron and zinc (Korkmaz *et al.*, 2024). When copper reaches high levels, it generates oxidative stress that leads to chlorosis and damage to roots, as well as stunted growth of the plants. High copper levels inside soil hinder microbial processes, which subsequently decrease soil nutrient supplies and diminish plant productivity (Korkmaz *et al.*, 2024).

The study aims to evaluate the impact of copper stress on safflower growth, assess the effectiveness of salicylic acid, nanoparticles, and Chi, elucidate underlying mechanisms for enhanced tolerance, determine their influence on metal accumulation in safflower tissues, and explore their potential to enhance the quality and safety of safflower-derived products in the context of heavy metal contamination. By filling this knowledge gap and offering a sustainable way to mitigate the negative effects of copper stress on safflower production, our work advances the larger objective of crop and ecosystem preservation.

Materials and Methods

Experimental site: The experiment was conducted at the research area of the Botany department at Islamia University, Bahawalpur, to examine the effect of salicylic acid, nanoparticles, and chitosan on the growth, antioxidant activity, and nutrient concentration of different cultivars of safflower grown under copper stress. Random soil sampling was done from the research area to characterize soil physicochemical properties. The pre-experimental soil characteristics are provided in Table 1.

Soil spiking: Loam soil was used as control (T0), while Copper was spiked in the soil for three different copper treatments (50mg/kg, 200 mg/kg and 400 mg/kg), and hence two way (4×10) full factorial arrangement based on a randomized complete block design with three replicates for each treatment was applied. Sowing was done in November 2020.

Table 1. Pre-experimental soil and irrigation characteristics.

Soil	Values
pH	8.46
EC*e* (dS/m)	3.33
SOM (%)	0.42
Available phosphorus (µg/g)	5.78
Extractable potassium (µg/g)	111
Total nitrogen (%)	0.0025
Extractable sodium (µg/g)	145
Texture	Clay loam
Irrigation	**Values**
pH	7.85
Sodium (mg/L)	1044
Ca+Mg (meq./L)	3.18
Bicarbonates (meq./L)	5.25
Carbonates (meq./L)	0.00
EC (µS/cm)	463
Chloride (meq./L)	0.01

Treatment plan: The treatment includes a control, 400Cu (400mg/kg), 0.01SA (Salicylic acid), Chi (Chitosan), 400Cu+0.01SA, 400Cu+Chi, 0.01NP (Nanoparticles), 0.1NP, 400Cu+0.01NP, 400Cu+0.01NP. All these treatments were applied in three replicates following a completely randomized design (CRD). Salicylic acid, chitosan, and nanoparticles were added through foliar application.

Pot preparation and seed sowing procedure: The pots used in the experiment were uniformly filled with an equal amount of homogenized spiked soil. Seeds of three safflower genotypes (Saff-64, Saff-50, and Saff-62) were obtained from an affiliated seed supplier from the Punjab government in Multan, Punjab. Before sowing, these seeds underwent a thorough decontamination process that involved immersion in 70% ethanol, followed by brief exposure to chlorox (10%) for 2 to 3 minutes. Subsequently, the seeds were meticulously washed with distilled autoclaved water to ensure cleanliness and eliminate external influences.

Synthesis of chitosan NPs: The ionic gelation process was used to synthesize VSA-CS nanoparticles (NPs). Prepared a 0.4% w/v chitosan solution by dissolving chitosan in a 0.5% acetic acid solution, followed by a 100 mL dilution with ultrapure water, all under constant stirring at 100 rpm. The solution was passed through a 125 mm Whatman filter paper to eliminate impurities present. The production of a 0.2% TPP solution in ultrapure water required an independent preparation followed by filtration steps. The addition of TPP solution to the chitosan mix containing 0.1% salicylic acid took place at a constant stirring rate of 100 rpm. A purification process by centrifugation at 10,000 ×g for 10 minutes at 4°C yielded a clear nanoparticle suspension, which received thorough cleaning with ultrapure water. The developed nanoparticles went through freeze-drying before being evaluated for characterization purposes.

Characterization of SA-loaded chitosan nanostructures: We employed multiple advanced techniques in our analysis to thoroughly examine the basic characteristics and behavioral aspects of chitosan nanostructures loaded with SA. SEM provided detailed observation of both surface features and particle size details about the structures under investigation. The crystalline structure of these nanostructures was analyzed through X-ray Diffraction (XRD). In contrast, Fourier Transform Infrared Spectroscopy (FTIR) identified functional groups to prove the presence of salicylic acid together with chitosan and to describe their molecular binding patterns. The particle size distribution and zeta potential were measured through Dynamic Light Scattering (DLS) because these parameters determine the stability of colloids. The thermal stability assessment of nanoformulations, including DSC with TGA techniques, revealed their testing results. BET analysis enabled us to study the surface area and porosity properties of the nanostructures because these characteristics heavily influence their surface properties.

Harvesting and data collection: The data was collected 21 days after the plants' seeding. The weights of fresh shoots and roots were measured using a digital balance after harvesting. The samples were oven-dried for 72 hours at 65°C to calculate the dry weights of the shoots and roots.

Stress tolerance indices: The following formula was used to measure the promptness index (PI) and gemination stress tolerance index (GSTI) (Zafar *et al.*, 2015).

$$PI = nd1\ (1.00) + nd2\ (0.75) + nd3\ (0.50) + nd4\ (0.25)$$

where nd1, nd2, nd3 and nd4 are the number of seeds germinated on 1st, 2nd, 3rd and 4th day, respectively. A germination stress tolerance index (GSTI) was calculated in terms of percentage as follows:

$$GSTI = \frac{\text{PI of stressed seeds}}{\text{PI of control seeds}} \times 100$$

Stress tolerance indices for different growth parameters (shoot length, shoot and root fresh/dry weight) were calculated by using the following formula Wilkins (1957).

$$\text{Stress tolerance index (STI)} = \frac{\text{Value of stressed plants}}{\text{Value of control plants}} \times 100$$

Chlorophyll and carotenoid content: The study measured the amounts of chlorophyll in freshly collected leaves using Arnon's method (Arnon, 1949). The extraction process was carried out using an 80% acetone solution, and the amounts of carotenoids (Kirk & Allen, 1965) and chlorophyll (Arnon, 1949) were measured at 480, 663, and 645nm wavelengths.

Relative water contents (RWC): A standardized method described [27] was used to measure relative water content by using the following formula:

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} \times 100$$

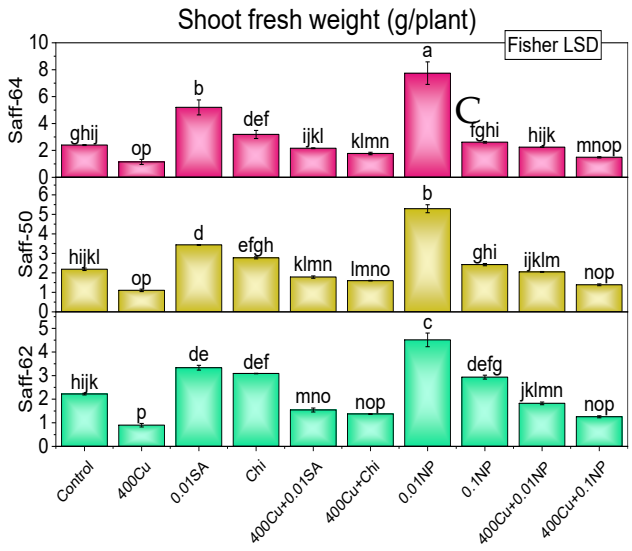
FW is the fresh weight, DW are the dry and TW is the turgid weight of the sample.

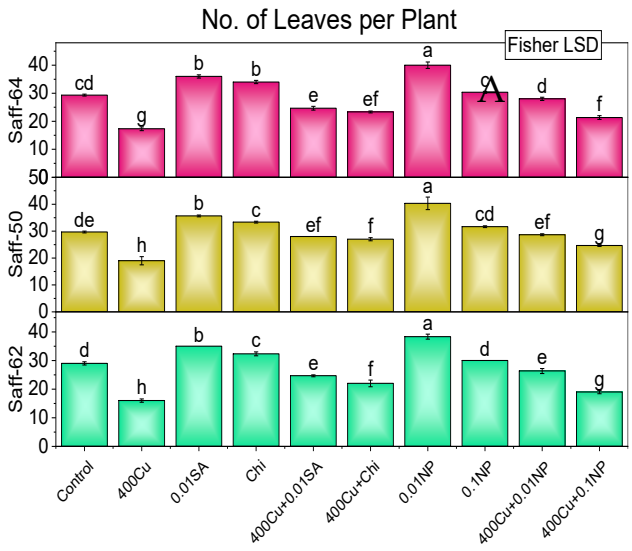
Total soluble proteins: The Biuret method (Racusen & Johnstone, 1961) measured total soluble protein levels. To determine the total protein content, a protein standard curve was made using bovine serum albumin, and the optical density was measured at a frequency of 545 nm using a UV spectrophotometer.

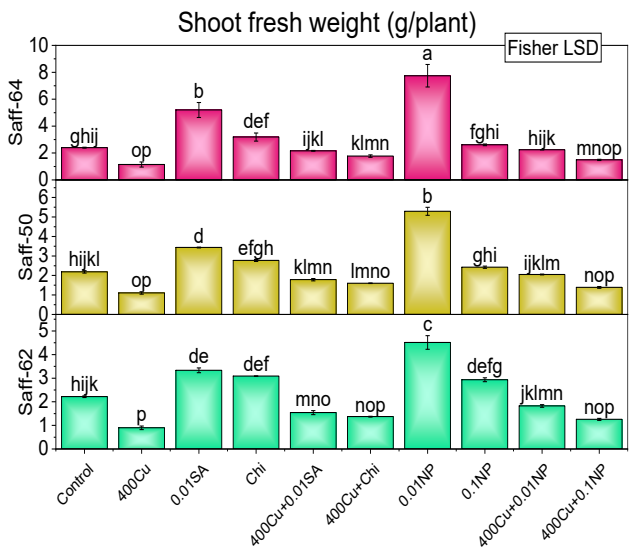
Statistical analysis: The complete dataset was presented as the mean of three replicates. To assess the statistical significance of diverse treatments, an Analysis of Variance (ANOVA) was performed using OriginPro 2021 (OriginLab Corporation, 2021). Treatments were considered significantly different if the comparisons yielded P values ≤ 0.05.

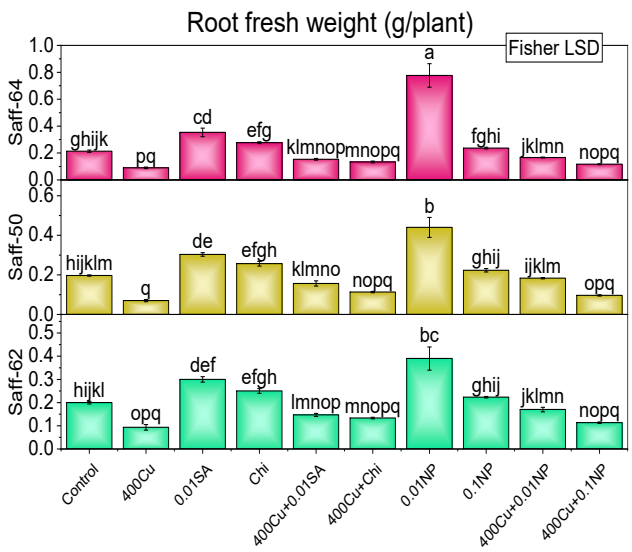
Results

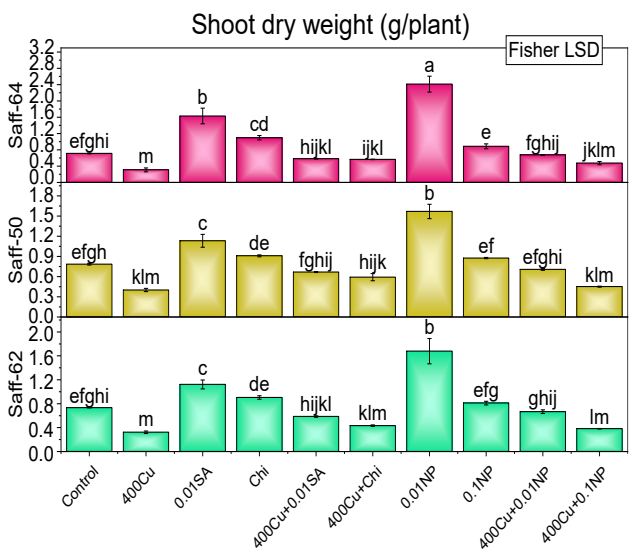
Shoot length, Shoot fresh and dry weight: Adding 400Cu treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in shoot length (~60%, ~37, and ~62%), shoot fresh weight (~52%, ~50%, and ~60%), and shoot dry weight (~57%, ~49%, and ~56%) over control. Applying 0.01SA treatment in varieties Saff-64, Saff-50, and Saff-62 showed an increase in shoot length (~22%, ~15%, and ~11%), shoot fresh weight (~117%, ~58%, and ~50%), and shoot dry weight (~129%, ~44%, and ~23%) more than the control. A significant increase in shoot length (~15%, ~9%, and ~7%), shoot fresh weight (~33%, ~45%, and ~39%), and shoot dry weight (~38%, ~25%, and ~31%) was recorded by applying Chi treatment in Saff-64, Saff-50, and Saff-62 varieties above the control. Treatment 400Cu+0.01SA caused a decrease in shoot length (~17%, ~8%, and ~15%), shoot fresh weight (~19%, ~34%, and ~29%), and shoot dry weight of varieties Saff-64, Saff-50, and Saff-62 compared to the control. Applying 400Cu+Chi treatment resulted decrease in shoot length (~28%, ~14%, and ~25%), shoot fresh weight (~26%, ~45%, and ~55%), and shoot dry weight (~34%, ~25%, and ~41%) in comparison to the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.01NP caused an increase in shoot length (~33%, ~22%, and ~20%), shoot fresh weight (~222%, ~143%, and ~103%), and shoot dry weight (~238%, ~100%, and ~65%) than the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.1NP showed an increase in shoot length (~10%, ~5%, and ~5%), shoot fresh weight (~15%, ~12%, and ~33%), and shoot dry weight (~128%, ~34%, and ~87%) of Saff-64, Saff-50, and Saff-62 varieties compared to the control. Treatment 400Cu+0.01NP caused a decrease in shoot length (~11%, ~3%, and ~14%), shoot fresh weight (~35%, ~50%, and ~51%), and shoot dry weight (~30%, ~45, and ~55%) of Saff-64, Saff-50, and Saff-62 varieties than the control. Adding 400Cu+0.1NP treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in shoot length (~39%, ~3%, and ~5%), shoot fresh weight (~38%, ~37%, and ~55%), and shoot dry weight (~34%, ~43%, and ~78%) over the control (Fig. 1A, B, and C).











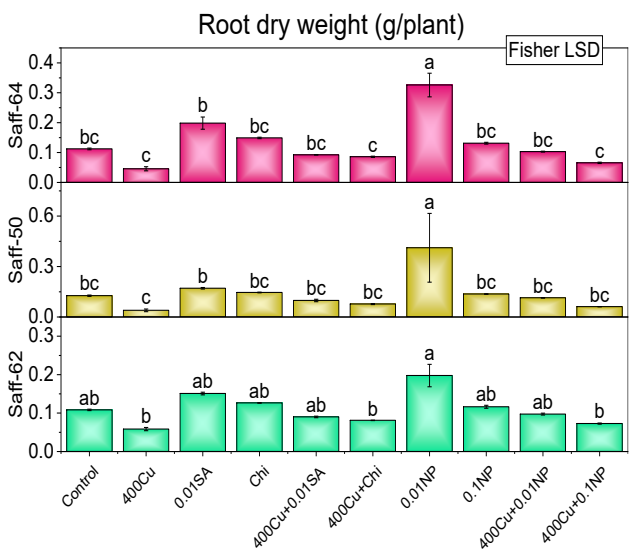
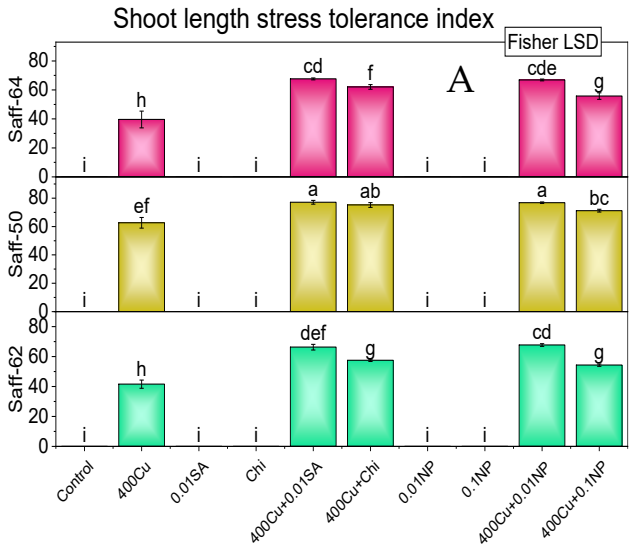
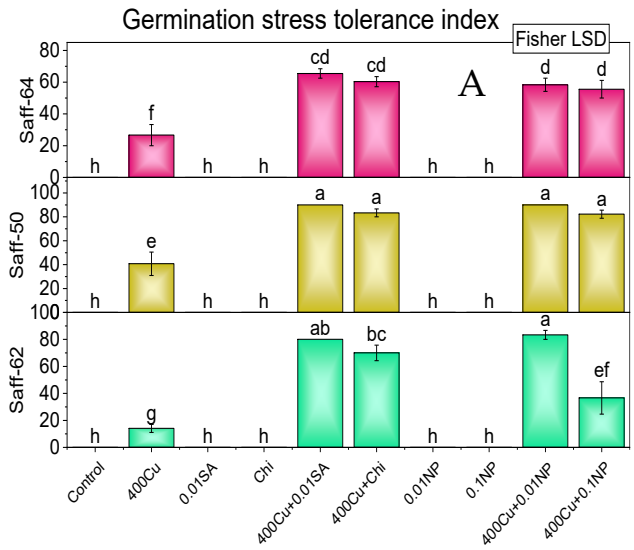
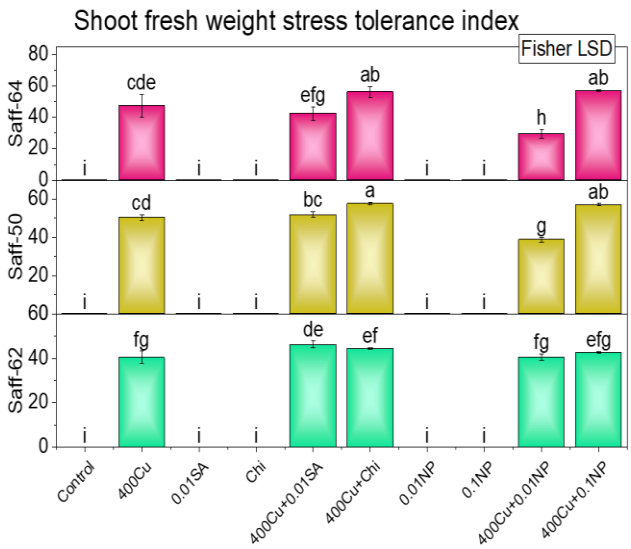


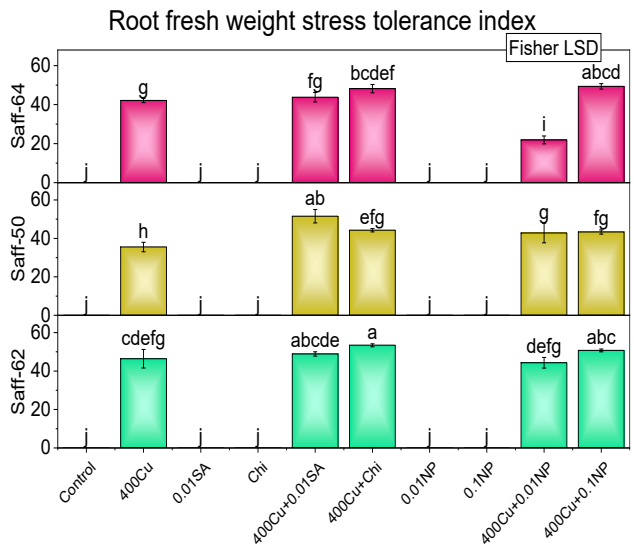
Fig. 1. The effect of different treatments on shoot length (A), shoot fresh (B), and dry weight (C) of different types of safflowers (Saff-64, Saff-50, Saff-62) cultivated with and without Cu (Copper) stress. The Fisher LSD test was used to measure the significant differences at ($p<0.05$); distinct letters on the bars represent the mean of three replicates ± SE. SA=Salicylic acid, NP=Nanoparticles, Chi= Chitosan

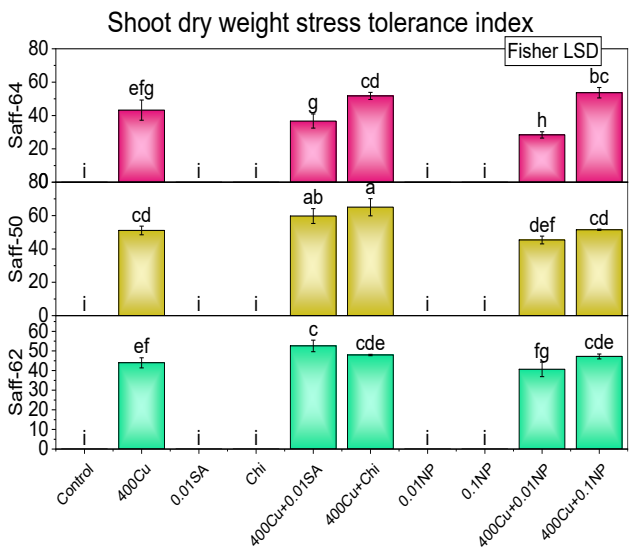
Fig. 2. The effect of different treatments on number of leaves/plant (A), root fresh (B), and dry weight (C) of different types of safflowers (Saff-64, Saff-50, Saff-62) cultivated with and without Cu (Copper) stress. The Fisher LSD test was used to measure the significant differences at ($p<0.05$); distinct letters on the bars represent the mean of three replicates ± SE. SA=Salicylic acid, NP=Nanoparticles, Chi= Chitosan











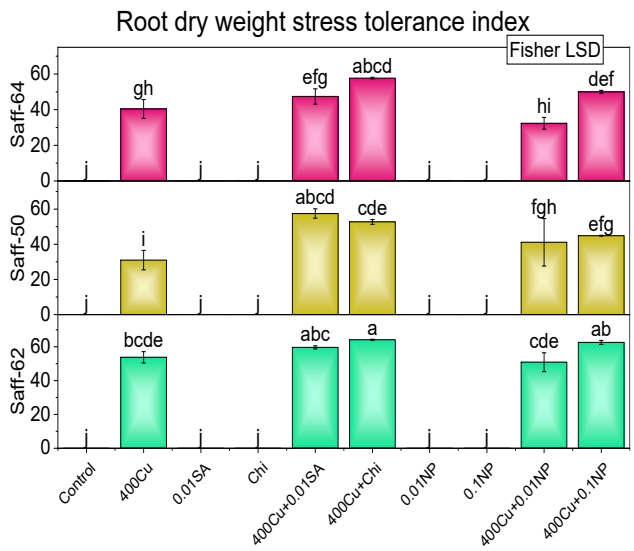


Fig. 3. The effect of different treatments on shoot length stress tolerance index (A), shoot fresh weight stress tolerance index (B), shoot dry weight stress tolerance index (C) of different types of safflowers (Saff-64, Saff-50, Saff-62) cultivated with and without Cu (Copper) stress. The Fisher LSD test was used to measure the significant differences at ($p<0.05$); distinct letters on the bars represent the mean of three replicates ± SE. SA=Salicylic acid, NP=Nanoparticles, Chi= Chitosan

Fig. 4. The effect of different treatments on germination stress tolerance index (A), root fresh weight stress tolerance index (B), and root dry weight stress tolerance index (C) of different types of safflowers (Saff-64, Saff-50, Saff-62) cultivated with and without Cu (Copper) stress. The Fisher LSD test was used to measure the significant differences at ($p<0.05$); distinct letters on the bars represent the mean of three replicates ± SE. SA=Salicylic acid, NP=Nanoparticles, Chi= Chitosan

Number of leaves/plant, Root fresh and dry weight: Applying 400Cu treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in number of leaves/plants (~50%, ~36%, and ~45%), root fresh weight (~58%, ~65%, and ~53%), and root dry weight (~59%, ~69%, and ~46%) over control. Applying 0.01SA treatment in varieties Saff-64, Saff-50, and Saff-62 showed an increase in number of leaves/plants (~22%, ~20%, and ~21%), root fresh weight (~66%, ~54%, and ~50%), and root dry weight (~77%, ~35%, and ~39%) more than the control. A significant increase in number of leaves/plants (~15%, ~12%, and ~12%), root fresh weight (~30%, ~31%, and ~25%), and root dry weight (~33%, ~15%, and ~17%) was recorded by applying Chi treatment in Saff-64, Saff-50, and Saff-62 varieties above the control. Treatment 400Cu+0.01SA caused a decrease in number of leaves/plants (~16%, ~19%, and ~15%), root fresh weight (~28%, ~30%, and ~27%), and root dry weight (~18%, ~22% and ~16.9%) of varieties Saff-64, Saff-50, and Saff-62 compared to the control. Applying 400Cu+Chi treatment resulted in an decrease in number of leaves/plants (~20%, ~9%, and ~24%), root fresh weight (~38%, ~42%, and ~33%), and root dry weight (~23%, ~39%, and ~25%) in comparison to the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.01NP caused an increase in number of leaves/plants (~12%, ~24%, and ~26%), root fresh weight (~264%, ~123%, and ~95%), and root dry weight (~190%, ~225%, and ~83%) than the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.1NP showed an increase in number of leaves/plants (~3%, ~7%, and ~39%), root fresh weight (~86%, ~39%, and ~91%), and root dry weight (~17%, ~8%, and ~7%) of Saff-64, Saff-50, and Saff-62 varieties compared to the control. Treatment 400Cu+0.01NP caused a decrease in number of leaves/plants (~16%, ~19%, and ~35%), root fresh weight (~44%, ~53%, and ~45%), and root dry weight (~8%, ~10% and ~10%) of Saff-64, Saff-50, and Saff-62 varieties than the control. Adding 400Cu+0.1NP treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in number of leaves/plants (~20%, ~17%, and ~9% %), root fresh weight (~45%, ~51%, and ~43%), and root dry weight (~42%, ~52%, and ~33%) over the control (Fig. 2A, B, and C).

Shoot length stress tolerance index, Shoot fresh weight stress tolerance index, Shoot dry weight stress tolerance index: Adding 400Cu treatment in varieties Saff-64, Saff-50, and Saff-62 demonstrate an increase in shoot length stress tolerance index (~40%, ~63%, and ~42), shoot fresh weight stress tolerance index (~48%, ~51%, and ~40%), shoot dry weight stress tolerance index (~43%, ~51%, and ~44%) over control. Applying 0.01SA, Chi. 0.01NP, and 0.1NP treatments showed no significant results for shoot length stress tolerance index, shoot fresh weight stress tolerance index, shoot dry weight stress tolerance index of Saff-64, Saff-50, and Saff-62 varieties. Treatment 400Cu+0.01SA caused an increase in shoot length stress tolerance index (~68%, ~77% and ~67%), shoot fresh weight stress tolerance index (~43%, ~52%, and ~46%), shoot dry weight stress tolerance index (~42%, ~52%, and ~46%) of varieties Saff-64, Saff-50, and Saff-62 compared to the control. Applying 400Cu+Chi treatment demonstrates an increase in shoot length stress tolerance index (~62%, ~75%, and ~58%), shoot fresh weight stress tolerance index (~56%, ~58%, and ~44%), shoot dry weight stress tolerance index (~56%, ~58%, and ~44%) in comparison to the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 400Cu+0.01NP caused a decrease in shoot length stress tolerance index (~30%, ~77%, and ~68%), shoot fresh weight stress tolerance index (~30%, ~39%, and ~41%), shoot dry weight stress tolerance index (~30%, ~39%, and ~41%) of Saff-64, Saff-50, and Saff-62 varieties than the control. Adding 400Cu+0.1NP treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in shoot length stress tolerance index (~57%, ~71%, and ~55), shoot fresh weight stress tolerance index (~57%, ~57%, and ~43%), shoot dry weight stress tolerance index (~57%, ~57%, and ~43%) over the control (Fig. 3A, B, and C).

Germination stress tolerance index, Root fresh weight stress tolerance index, and Root dry weight stress tolerance index: Applying 400Cu treatment in varieties Saff-64, Saff-50, and Saff-62 demonstrate an increase in germination stress tolerance index (~27%, ~41%, and ~14%), root fresh weight stress tolerance index (~42%, ~56%, and ~46%), and root dry weight stress tolerance index (~43%, ~50%, and ~46%) over control. Applying 0.01SA, Chi. 0.01NP, and 0.1NP treatments showed no significant results for shoot length stress tolerance index, shoot fresh weight stress tolerance index, shoot dry weight stress tolerance index of Saff-64, Saff-50, and Saff-62 varieties. Treatment 400Cu+0.01SA caused an increase in germination stress tolerance index (~66%, ~90% and ~80%), root fresh weight stress tolerance index (~44%, ~52%, and ~49%), and root dry weight stress tolerance index (~44%, ~52%, and ~49%) of varieties Saff-64, Saff-50, and Saff-62 compared to the control. Applying 400Cu+Chi treatment demonstrates an increase in germination stress tolerance index (~60%, ~83%, and ~70%), root fresh weight stress tolerance index (~48%, ~58%, and ~53%), and root dry weight stress tolerance index (~48.2, ~58%, and ~53%) in comparison to the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 400Cu+0.01NP caused a decrease in germination stress tolerance index (~70%, ~80% and ~89%), root fresh weight stress tolerance index (~30%, ~39%, and ~44%), and root dry weight stress tolerance index (~30%, ~39%, and ~44%) of Saff-64, Saff-50, and Saff-62 varieties than the control. Adding 400Cu+0.1NP treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in germination stress tolerance index (~66%, ~87%, and ~83%), root fresh weight stress tolerance index (~58%, ~58%, and ~51%), and root dry weight stress tolerance index (~57%, ~57%, and ~51%) over the control (Fig. 4A, B, and C).

Chlorophyll contents and carotenoids: Adding 400Cu treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in chlorophyll a (~27%, ~32%, and ~18%), chlorophyll b (~29%, ~60%, and ~36%), total chlorophyll (~28%, ~40%, and ~25%), and carotenoids (~39%, ~54%, and ~40%) than the control. Applying 0.01SA treatment in varieties Saff-64, Saff-50, and Saff-62 showed an increase in chlorophyll a (~15%, ~19%,

and ~13%), chlorophyll b (~28%, ~19%, and ~25%), total chlorophyll (~21%, ~21%, and ~9%), and carotenoids (~38%, ~36%, and ~20%) than the control. A significant increase in chlorophyll a (~11%, ~14%, and ~13%), chlorophyll b (~20%, ~7%, and ~17%), total chlorophyll (~15%, ~12%, and ~15%), and carotenoids (~21%, ~29%, and ~17%) were recorded by applying Chi treatment in Saff-64, Saff-50, and Saff-62 varieties above the control. Treatment 400Cu+0.01SA caused a decrease in chlorophyll a (~4%, ~6%, and 8%), chlorophyll b (~4%, ~25%, and ~10%), total chlorophyll (~3%, ~13%, and ~8%), and carotenoids (~8%, ~9%, and ~16%) of varieties Saff-64, Saff-50, and Saff-62 compared to the control. Applying 400Cu+Chi treatment resulted decrease in chlorophyll a (~4%, ~14%, and 28%), chlorophyll b (~8%, ~29% and ~9.6%), total chlorophyll (~5%, ~21%, and ~7%), and carotenoids (~25%, ~26%, and ~20%) in comparison to the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.01NP caused an increase in chlorophyll a (~23%, ~35%, and ~23%), chlorophyll b (~48%, ~34%, and ~34%), total chlorophyll (~34%, ~37%, and ~7%), and carotenoids (~56%, ~62%, and ~37%) than the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.1NP showed an increase in chlorophyll a (~1%, ~9%, and ~4%), chlorophyll b (~8%, ~7%, and ~8%), total chlorophyll (~4%, ~8%, and ~28%), and carotenoids (~3%, ~14%, and ~8%) of Saff-64, Saff-50, and Saff-62 varieties compared to the control. Treatment 400Cu+0.01NP caused a decrease in chlorophyll a (~2%, ~1%, and ~1%), chlorophyll b (~9%, ~17%, and ~18%), total chlorophyll (~11%, ~7%, and ~2%), and carotenoids (~5%, ~14%, and ~13%) of Saff-64, Saff-50, and Saff-62 varieties than the control. Adding 400Cu+0.1NP treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in chlorophyll a (~9%, ~23%, and ~10%), chlorophyll b (~16%, ~42%, and ~26%), total chlorophyll (~2%, ~28%, and ~16%), and carotenoids (~26%, ~42%, and ~37%) over the control (Fig. 5A, B, C, and D).

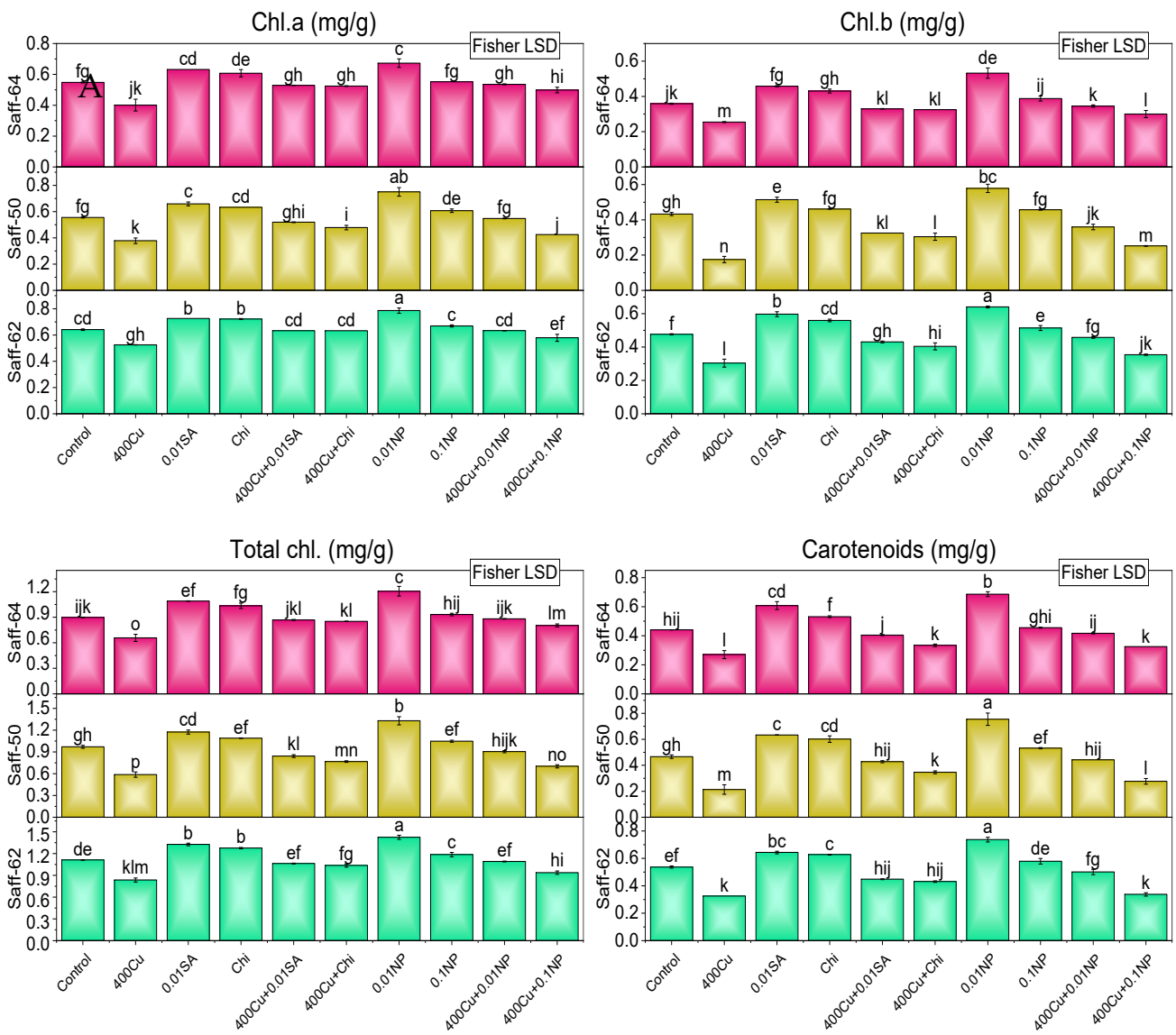


Fig. 5. The effect of different treatments on chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), and carotenoids (D) of different types of safflowers (Saff-64, Saff-50, Saff-62) cultivated with and without Cu (Copper) stress. The Fisher LSD test was used to measure the significant differences at ($p<0.05$); distinct letters on the bars represent the mean of three replicates ± SE. SA=Salicylic acid, NP=Nanoparticles, Chi= Chitosan

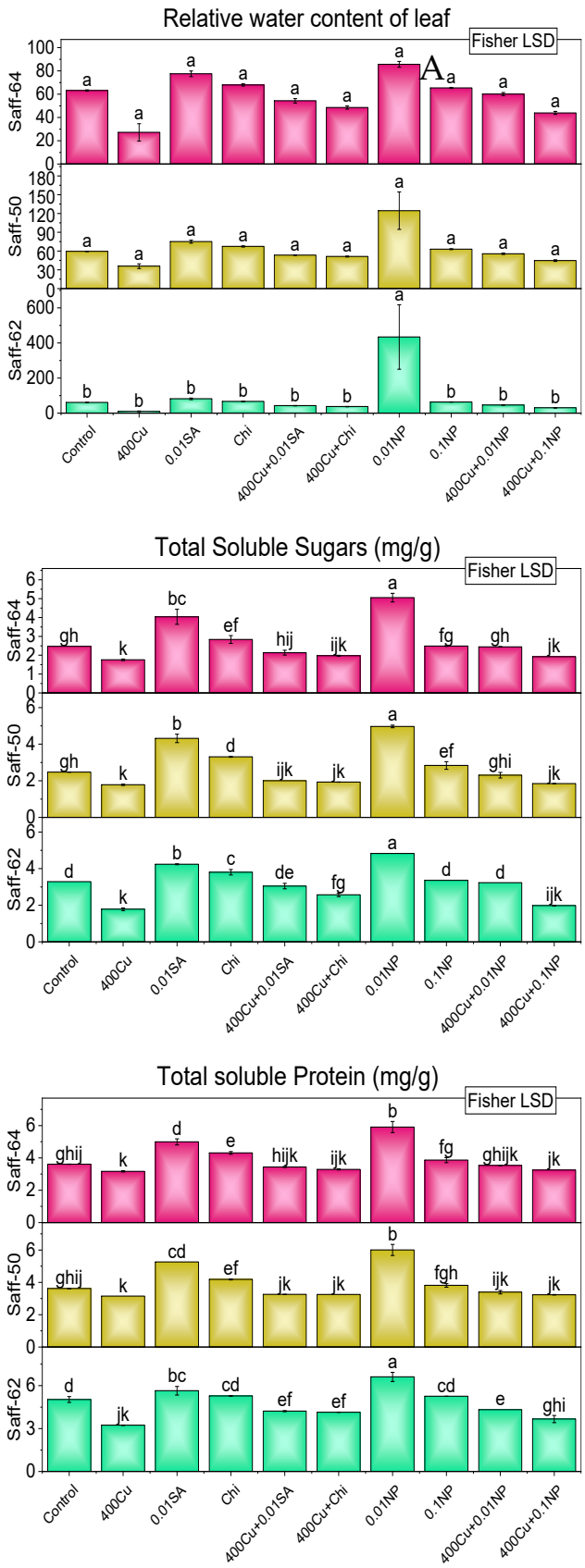


Fig. 6. The effect of different treatments on the relative water content of leaf (A), total soluble sugars (B), and total soluble protein (C) of different types of safflowers (Saff-64, Saff-50, Saff-62) cultivated with and without Cu (Copper) stress. The Fisher LSD test was used to measure the significant differences at ($p<0.05$); distinct letters on the bars represent the mean of three replicates ± SE. SA=Salicylic acid, NP=Nanoparticles, Chi= Chitosan

Relative water content of leaf, Total soluble sugars, and Total soluble protein: Varieties Saff-64, Saff-50, and Saff-62 caused a decrease in relative water content of leaf (~57%, ~40%, and ~83%), total soluble sugars (~29%, ~28%, and ~36%), and total soluble protein (~12%, ~13%, and ~36%) with 400Cu treatment than the control. Applying 0.01SA treatment in varieties Saff-64, Saff-50, and Saff-62 showed an increase in relative water content of leaf (~23%, ~27%, and ~34%), total soluble sugars (~63%, ~75%, and ~12%), and total soluble protein (~38%, ~45%, and ~12%) than the control. A significant increase in relative water content of leaf (~8%, ~12%, and ~8%), total soluble sugars (~15%, ~34%, and ~5%), and total soluble protein (~19%, ~16%, and ~5%) were recorded by applying Chi treatment in Saff-64, Saff-50, and Saff-62 varieties above the control. Treatment 400Cu+0.01SA caused a decrease in relative water content of leaf (~23%, ~14%, and ~30%), total soluble sugars (~14%, ~19%, and ~16%), and total soluble protein (~5%, ~10%, and ~16%) of varieties Saff-64, Saff-50, and Saff-62 compared to the control. Applying 400Cu+Chi treatment resulted decrease in relative water content of leaf (~6%, ~10%, and ~39%), total soluble sugars (~20%, 22%, and ~18%), and total soluble protein (~9%, ~10%, and ~18%) in comparison to the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.01NP caused an increase in relative water content of leaf (~36%, ~109%, and ~614%), total soluble sugars (~105%, ~101%, and ~31%), and total soluble protein (~64%, ~66%, and ~31%) than the control of Saff-64, Saff-50, and Saff-62 varieties. Treatment 0.1NP showed an increase in relative water content of leaf (~4%, ~6%, and ~5%), total soluble sugars (~1%, ~15%, and ~5%), and total soluble protein (~7%, ~5%, and ~5%) of Saff-64, Saff-50, and Saff-62 varieties compared to the control. Treatment 400Cu+0.01NP caused a decrease in relative water content of leaf (~5%, ~7%, and ~24%), total soluble sugars (~1%, ~7%, and ~14%), and total soluble protein (~2%, ~6%, and ~14%) of Saff-64, Saff-50, and Saff-62 varieties than the control. Adding 400Cu+0.1NP treatment in varieties Saff-64, Saff-50, and Saff-62 caused a decrease in relative water content of leaf (~21%, ~25%, and ~50%), total soluble sugars (~22%, ~26%, and ~27%), and total soluble protein (~10%,~11%, and ~27%) over the control (Fig. 6A, B, and C).

Discussion

Chitosan: Chitosan plays a pivotal role in improving plant growth and tolerance under heavy metal stress conditions, including copper toxicity (Krupa-Małkiewicz & Ochmian, 2024). In the present study, the application of chitosan significantly enhanced shoot length, shoot fresh weight, and shoot dry weight by promoting cell elongation and division, which are often suppressed under Cu-induced oxidative stress. Chitosan facilitated an improved number of leaves per plant, possibly by modulating hormonal signaling pathways, particularly auxin and gibberellins, which are crucial for leaf expansion and shoot development. Root growth was also positively influenced, as evident by increased root fresh weight and root dry weight. This enhancement can be attributed to chitosan's ability to improve water uptake and ion homeostasis, preventing copper-induced root damage

(Rafique *et al.*, 2024). The increase in stress tolerance suggests that chitosan mitigates Cu-induced oxidative damage by activating antioxidant defense mechanisms such as the upregulation of peroxidase and catalase enzymes. The application of chitosan elevated chlorophyll content and carotenoid levels in plants because it functions to stabilize thylakoid membranes, thus preventing chlorophyll degradation from metal stress (Panahirad *et al.*, 2023). Chitosan maintained leaf water content, which supported cell turgor pressure and cellular physiological activities. Total soluble sugars and total soluble proteins accumulated under chitosan treatment provided osmoprotection by ensuring an adequate energy supply together with structural stability to cellular components, which is essential for plant survival during Cu toxicity.

Nanoparticles: Nanoparticles (NPs) have emerged as promising agents in mitigating metal toxicity and improving plant physiological responses (Ulhassan *et al.*, 2022). In safflower plants subjected to copper stress, the application of nanoparticles significantly improved shoot length, shoot fresh weight, and shoot dry weight, likely due to enhanced nutrient uptake and improved water use efficiency (Singh *et al.*, 2024). The increase in the number of leaves per plant under NP treatment suggests that nanoparticles regulate phytohormone balance, particularly by modulating cytokinin and gibberellin pathways, which are vital for leaf expansion and chlorophyll biosynthesis. Furthermore, the improvement in root fresh weight and root dry weight indicates that nanoparticles facilitated root growth by reducing metal toxicity and enhancing nutrient transport efficiency. Nanoparticles act as ROS scavengers, reducing oxidative stress and promoting cellular homeostasis (Sanati *et al.*, 2022). Moreover, the chlorophyll content and carotenoids were markedly increased, indicating that nanoparticles protect the photosynthetic machinery from Cu-induced oxidative damage. The positive effect of nanoparticles on the relative water content of leaves highlights their role in stomatal regulation and osmotic balance, ensuring water conservation under stress conditions (Khan *et al.*, 2021). The elevated levels of total soluble sugars and total soluble proteins further demonstrate that nanoparticles enhance metabolic stability and provide an adaptive advantage in Cu-stressed safflower plants (Katarina *et al.*, 2021).

Salicylic acid: Salicylic acid (SA) serves essential functions in plant growth regulation, together with stress protection mechanisms. SA applications under copper toxicity led to increased shoot length and fresh weight, and dry weight through enhanced auxin signaling, which promoted cellular growth by stimulating cell division. SA probably stimulated the expression of photosynthetic genes, which produced better leaf expansion results in increased leaf numbers (Ghassemi-Golezani & Farhadi, 2021). SA improved both root fresh and dry weight, indicating that this compound modifies root structures while helping plants avoid copper toxicity. The stress tolerance index reduction indicates that SA intensifies antioxidant enzyme functioning, which stops Cu-induced oxidative damage to cells while protecting overall cellular structure. Under Cu stress, SA increases chlorophyll content and carotenoids because it supports photosystem stability and chlorophyll biosynthesis, thus maintaining effective photosynthesis (Naz *et al.*, 2022). The application of SA enhanced leaf relative water content through its probable mechanism of regulating stomatal transpiration and enhancing leaf water balance (Parveen *et al.*, 2021).

Conclusion

In conclusion, applying 0.01NP treatment can potentially improve safflower growth under Cu stress. Adding 0.01NP treatment showed the greatest improvement in photosynthetic efficiency and stress tolerance in the Saff-62 variety under Cu stress compared to the other. A significant improvement in the growth attributes and chlorophyll contents also validated the effect of 0.01NP under Cu stress. More investigations at the field level are suggested to explore these varietal differences and identify cultivars that are particularly suited for 0.01NP application under specific environmental challenges.

References

Ahmed, H.K, H.A. Fawy and E.S. Abdel-Hadi. 2010. Study of sewage sludge use in agriculture and its effect on plant and soil. *Agric. Biol. J. North Amer.*, 1(5): 1044-1049.

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. *Plant Physiol.*, 24: 1.

Bowszys, T., J. Wierzbowska, P. Sternik and M. Busse. 2015. Effect of the application of sewage sludge compost on the content and leaching of zinc and copper from soils under agricultural use. *J. Ecol. Eng.*, 16: 1-7.

Cacciuttolo, C. and E. Atencio. 2022. Past, present, and future of copper mine tailings governance in chile (1905–2022): A review in one of the leading mining countries in the world. *Int. J. Environ. Res. Public Health*, 19: 13060.

Cruz, F.J.R., S.S. da Cruz Ferreira, R.L. Conceição, E.U. Lima, C.F. de Oliveira Neto, J.R. Galvão, S. da Cunha Lopes and I.D.J.M. Viegas. 2022. Copper toxicity in plants: Nutritional, physiological, and biochemical aspects. In: *Advances in Plant Defense Mechanisms*. IntechOpen.

El-Saadony, M.T., A.M. Saad, S.M. Soliman, H.M. Salem, E.-S.M. Desoky, A.O. Babalghith, A.M. El-Tahan, O.M. Ibrahim, A.A.M. Ebrahim, A. El-Mageed and others. 2022. Role of nanoparticles in enhancing crop tolerance to abiotic stress: A comprehensive review. *Front. Plant Sci.*, 13: 946717.

Emongor, V.E. and R.A. Emongor. 2023. Safflower (*Carthamus tinctorius* L.). In: *Neglected and Underutilized Crops*. Elsevier, 683-731.

Fagnano, M., D. Agrelli, A. Pascale, P. Adamo, N. Fiorentino, C. Rocco, O. Pepe and V. Ventorino. 2020. Copper accumulation in agricultural soils: Risks for the food chain and soil microbial populations. *Sci. Total Environ.*, 734: 139434.

Ghassemi-Golezani, K. and N. Farhadi. 2021. The efficacy of salicylic acid levels on photosynthetic activity, growth, and essential oil content and composition of pennyroyal plants under salt stress. *J. Plant Growth Regul.*, 1-13.

Hazarika, A., M. Yadav, D.K. Yadav and H.S. Yadav. 2022. An overview of the role of nanoparticles in sustainable agriculture. *Biocatal. Agric. Biotechnol.*, 43: 102399.

Katarina, K., E. Masarovičová and J. Jampilek. 2021. Risks and benefits of metal-based nanoparticles for vascular plants. *In*: *Handbook of Plant and Crop Physiology*. CRC Press, 922-963.

Kaya, C., F. Ugurlar, M. Ashraf and P. Ahmad. 2023. Salicylic acid interacts with other plant growth regulators and signal molecules in response to stressful environments in plants. *Plant Physiol. Biochem.*,.

Khan, M.T., S. Ahmed, A.A. Shah, A. Noor Shah, M. Tanveer, M.A. El-Sheikh and M.H. Siddiqui. 2021. Influence of zinc oxide nanoparticles to regulate the antioxidants enzymes, some osmolytes and agronomic attributes in *Coriandrum sativum* L. grown under water stress. *Agronomy*, 11: 2004.

Kirk, J.T.O. and R.L. Allen. 1965. Dependence of chloroplast pigment synthesis on protein synthesis: Effect of actidione. *Biochem. Biophys. Res. Commun.*, 21: 523-530.

Korkmaz, K., R. Kiliç, M. Akgün and S.M. Kara. 2024. Effects of combining phosphorus (P) and zinc (Zn) fertilization on P-Zn distribution and yield in safflower. *J. Plant Nutr.*, 47: 1585-1595.

Krupa-Małkiewicz, M. and I. Ochmian. 2024. Sustainable approaches to alleviate heavy metal stress in tomatoes: exploring the role of chitosan and nanosilver. *Agronomy*, 14: 2477.

Kurt, C., M.T. Altaf, W. Liaqat, M.A. Nadeem, A.N. Çil and F.S. Baloch. 2025. Oil content and fatty acid composition of safflower (*Carthamus tinctorius* L.) Germplasm. *Foods*, 14: 264.

Li, A., X. Sun and L. Liu. 2022. Action of salicylic acid on plant growth. *Front. Plant Sci.*, 13: 878076.

Mir, A.R., J. Pichtel and S. Hayat. 2021. Copper: uptake, toxicity and tolerance in plants and management of Cu-contaminated soil. *BioMetals*, 34: 737-759.

Naz, S., A. Bilal, B. Saddiq, S. Ejaz, S. Ali, S.T. Ain Haider, H. Sardar, B. Nasir, I. Ahmad, R.K. Tiwari and others. 2022. Foliar application of salicylic acid improved growth, yield, quality and photosynthesis of pea (*Pisum sativum* L.) by improving antioxidant defense mechanism under saline conditions. *Sustainability*, 14: 14180.

OriginLab Corporation. 2021. OriginPro. OriginLab, Northampton, MA, USA.

Panahirad, S., G. Gohari, G. Mahdavinia, H. Jafari, M. Kulak, V. Fotopoulos, R. Alcázar and M. Dadpour. 2023. Foliar application of chitosan-putrescine nanoparticles (CTS-Put NPs) alleviates cadmium toxicity in grapevine (*Vitis vinifera* L.) cv. Sultana: modulation of antioxidant and photosynthetic status. *BMC Plant Biol.*, 23: 411.

Parveen, A., M. Arslan Ashraf, I. Hussain, S. Perveen, R. Rasheed, Q. Mahmood, S. Hussain, A. Ditta, A. Hashem, A.-B.F. Al-Arjani and others. 2021. Promotion of growth and physiological characteristics in water-stressed *Triticum aestivum* in relation to foliar-application of salicylic acid. *Water*, 13: 1316.

Racusen, D. and D.B. Johnstone. 1961. Estimation of protein in cellular material. *Nature*, 191: 492-493.

Rafique, M., Z. Noreen, S. Usman, A.A. Shah, H. Taj, V. Raja and M.A. El-Sheikh. 2024. Mitigation of adverse effect of cadmium toxicity in lettuce (*Lactuca sativa* L.) through foliar application of chitosan and spermidine.

Rajput, V.D., T. Minkina, M. Feizi, A. Kumari, M. Khan, S. Mandzhieva, S. Sushkova, H. El-Ramady, K.K. Verma, A. Singh and others. 2021. Effects of silicon and silicon-based nanoparticles on rhizosphere microbiome, plant stress and growth. *Biology (Basel).*, 10: 791.

Rodrigues, D.F., D.P. Jaisi and M. Elimelech. 2013. Toxicity of functionalized single-walled carbon nanotubes on soil microbial communities: Implications for nutrient cycling in soil. *Environ. Sci. Technol.*, 47: 625-633.

Saad Ullah, M., A. Mahmood, M.M. Javaid, M. Naqve, S. Bibi, Z.U. Abidin, I. ul Haq and S.R. Khan. 2023. Chitosan for plant growth and stress tolerance. In: *Climate-Resilient Agriculture, Vol 2: Agro-Biotechnological Advancement for Crop Production*. Springer, 259-280.

Sadak, M.S., M.M. Tawfik and G.S. Bakhoum. 2022. Role of chitosan and chitosan-based nanoparticles on drought tolerance in plants: probabilities and prospects. In: *Role of Chitosan and Chitosan-Based Nanomaterials in Plant Sciences*. *Elsevier*, 475-501.

Sanati, M., A.R. Afshari, P. Kesharwani, V.N. Sukhorukov and A. Sahebkar. 2022. Recent trends in the application of nanoparticles in cancer therapy: The involvement of oxidative stress. *J. Control. Release*, 348: 287-304.

Shabbir, Z., A. Sardar, A. Shabbir, G. Abbas, S. Shamshad, S. Khalid, Natasha, G. Murtaza, C. Dumat and M. Shahid. 2020. Copper uptake, essentiality, toxicity, detoxification and risk assessment in soil-plant environment. *Chemosphere*, 259: 127436.

Shahrajabian, M., C. Chaski, N. Polyzos, N. Tzortzakis and S.A. Petropoulos. 2021. Sustainable agriculture systems in vegetable production using chitin and chitosan as plant biostimulants. *Biomolecules*, 11: 819.

Simate, G.S. and S. Ndlovu. 2014. Acid mine drainage: Challenges and opportunities. *J. Environ. Chem. Eng.*, 2: 1785-1803.

Singh, A., A. Sharma, O. Singh, V.D. Rajput, H.S. Movsesyan, T. Minkina, A. Alexiou, M. Papadakis, R.K. Singh, S. Singh and others. 2024. In-depth exploration of nanoparticles for enhanced nutrient use efficiency and abiotic stresses management: present insights and future horizons. *Plant Stress*, 100576.

Sun, W., M.H. Shahrajabian, S.A. Petropoulos and N. Shahrajabian. 2023. Developing sustainable agriculture systems in medicinal and aromatic plant production by using chitosan and chitin-based biostimulants. *Plants*, 12: 2469.

Ulhassan, Z., I. Khan, M. Hussain, A.R. Khan, Y. Hamid, S. Hussain, S.I. Allakhverdiev and W. Zhou. 2022. Efficacy of metallic nanoparticles in attenuating the accumulation and toxicity of chromium in plants: Current knowledge and future perspectives. *Environ. Pollut.*, 120390.

Yang, H., R. Fang, L. Luo, W. Yang, Q. Huang, C. Yang, W. Hui, W. Gong and J. Wang. 2023. Uncovering the mechanisms of salicylic acid-mediated abiotic stress tolerance in horticultural crops. *Front. Plant Sci.*, 14: 1226041.

Zafar, S., M.Y. Ashraf, M. Niaz, A. Kausar and J. Hussain. 2015. Evaluation of wheat genotypes for salinity tolerance using physiological indices as screening tool. *Pak. J. Bot*, 47: 397-405.