Protective Role of Salicylic Acid and Sodium Nitroprusside Foliar Application Against Copper Stress in Okra (*Abelmoschus esculentus*)

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Abstract

Copper (Cu) stress is one of the *abiotic stressors* that can severely damage plant cells. At elevated concentrations, copper becomes a toxic element within plants, triggering the generation of oxidative molecules and disrupting enzymatic activities. Salicylic acid (SA) is a plant growth regulator, while sodium nitroprusside (SNP) is a nitric oxide-releasing compound. Both play critical function**s** in modulating plant metabolism, growth, development, and mechanisms.They also influence gene expression and signaling pathways, with effects that may be either beneficial or detrimental depending on the context. The application of these compounds at appropriate doses significantly contributes to alleviating abiotic stresses in various plant species, particularly by counteracting the toxic effects. In this study, the individual and simultaneous effects of SA (500 µM) and SNP (150 µM), alongside varying concentrations of copper sulfate (600 and 1200 µM), were evaluated on the physiological and morphological responses of the Clemson variety of okra (*Abelmoschus esculentus*) in a hydroponic culture system. The experiments were conducted using a completely randomized design with three replicates per treatment. The results revealed that high copper concentrations reduced growth parameters and essential nutrient levels, while increasing malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and cell death, protein content, proline, soluble carbohydrates, and activities of enzymes, along with copper content in the shoots and roots of okra plants compared to the control. In contrast, foliar application of SA and SNP improved the uptake of essential elements, increasing Mg (up to 41%), Fe (51%), and Zn (50%). It also enhanced antioxidant enzyme activities (67–92%) and significantly reduced copper concentration (58%), MDA content (15%), H_2O_2 levels (26%), and cell death (49%) in the shoots and roots of okra plants. Therefore, based on the results, the individual and combined application of SA and SNP significantly mitigated the adverse effects of copper sulfate stress, improving tolerance of okra plants. According to the findings of this study SA demonstrated a considerably greater effect compared to SNP in in enhancing the resistance of okra plants to copper-induced stress.

Introduction

Okra (*Abelmoschus esculentus*) is an annual crop belonging to the Malvaceae family (1). This species demonstrates high adaptability to a wide range of soil types and is classified as a warm-season vegetable. It thrives best in tropical and subtropical climates, although it is sensitive to cold nighttime temperatures and drought conditions. The natural distribution of okra has been documented in the Middle East and surrounding regions (2, 3). Heavy metal (HMs) contamination presents significant environmental and ecological challenges worldwide (4). The primary issue with HMs arises from their non-biodegradable nature, which, unlike organic contaminants, causes them to persist in the environment. This characteristic renders HMs among the most hazardous groups of environmental pollutants (5). The toxicity of metals to plants is typically linked to their availability in soils and their chemical form within the soil matrix (6). The widespread use of copper-based compounds, such as fertilizers, pesticides, and nematicides, has led to extensive copper contamination of agricultural soils (7, 8). While copper is a vital micronutrient for plants, its excess accumulation may disrupt photosynthetic and respiratory processes, enzymatic functions, and cellular membrane integrity, leading to membrane

damage, reduced metabolism, and, ultimately, cell death (9). The most prominent impact of copper toxicity is the induction of oxidative stress by stimulating the production of harmful reactive oxygen species (ROS), such as superoxide and hydrogen peroxide. Due to their high reactivity with cellular components, these free radicals cause damage to cells and DNA while inhibiting ATP production. The presence of toxic levels of HMs in the plant environment induces physiological changes that reduce growth potential and, in severe cases, lead to plant mortality. Sensitive plants suffer damage and perish under these conditions, while resistant plants continue to grow and reproduce (10, 11). The generation of ROS, induced by oxidative stress from high copper concentrations, can lead to lipid peroxidation of cell membranes. Additionally, plants undergo alterations in their metabolic pathways, affecting the synthesis of essential biomolecules such as proteins and lipids (9, 12). HMs-induced stress can interfere with the uptake of essential elements like calcium (Ca), phosphorus (P), magnesium (Mg), and other metals, disrupting the elemental balance in plants (13, 14). In response to HMs stress, plants activate antioxidant defense systems, including enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), to mitigate the excessive accumulation of free radicals (15). Other defense mechanisms plants employ under HMs stress include the accumulation of proline (16), increased carbohydrate storage (13), and enhanced protein synthesis (17). Proline helps protect plants against stress through mechanisms like membrane stabilization, osmotic regulation, and the preservation of enzyme structure and function (18). Studies by Rather et al. (2020) (19) demonstrate that the using plant growth regulators, hormones, and other signaling molecules is a key strategy for alleviating copper toxicity in plants. Salicylic acid (SA), a plant growth regulator, is crucial in modulating various stages of plant growth and development, including ion uptake, photosynthesis, germination, enzymatic activity, and nitrogen regulation (20, 21). As a naturally occurring phenolic compound, SA effectively mitigates the adverse effects of environmental abiotic stresses (22, 23). Numerous studies have shown that SA application alleviates the negative impacts of drought stress (24), cold stress (25), salinity stress (26, 27), and HMs stress (28, 29). Research by Wei et al. (2020)(30) has conclusively demonstrated that SA significantly reduces the toxic effects of HMs stress and, in interaction with nitric oxide, ameliorates the detrimental impacts of HMs toxicity on plants.

Nitric oxide (NO) is a vital signaling molecule involved in numerous biochemical and physiological processes in plants, which has led to its classification as a phytohormone. NO acts as a concentration-dependent regulator, participating in key processes such as hypocotyl growth, defense responses, stomatal movement, seed germination, programmed cell death, hypersensitive responses, photosynthesis, growth and development, and phytoalexin production under various stress conditions (31). Sodium nitroprusside (SNP), an NO donor, is a bioactive molecule that can be applied through irrigation, foliar spraying, or injection into the apoplast of the leaf (32). It has been reported that SNP effectively mitigates the adverse effects of abiotic stresses, including HMs stress. In recent studies, SNP has been used as a source of NO due to its unique ability to release nitric oxide in plant tissues over an extended period, as well as its cost-effectiveness (33). Although limited research has been conducted on the effects of foliar application of SNP and SA on enhancing okra plant tolerance to copper sulfate stress, this study investigates their individual and combined effects under copper toxicity. The study primarily focuses on the physiological and biochemical changes in okra plants, alongside their mineral element

content, under copper toxicity. The primary aim of this research is to evaluate the enhancement of plant tolerance by stimulating related mechanisms, such as antioxidant activity, while also reducing copper accumulation and limiting its translocation from roots to shoots through the use of SNP and SA.

Materials and Methods

The plant studied in this research was okra, with seeds of the Clemson Spineless cultivar obtained from the Dutch company Bakker Brothers. Initially, the seeds were disinfected in a 5% sodium hypochlorite solution for 10 minutes, then rinsed several times with tap water and once with distilled water. Four seeds were sown in plastic pots (20 × 30 cm^2) filled with perlite, with three independent replicates per treatment, arranged in a completely randomized design. Until the emergence of the first sprout, the pots were irrigated with distilled water and kept in darkness. Upon observing the initial signs of germination, the pots were transferred to a growth chamber with a minimum temperature of 25°C and a maximum of 30°C, 80% relative humidity, and a photoperiod of 14 hours of light and 10 hours of darkness. At the four-leaf stage, the plants were irrigated with Hoagland nutrient solution (34) at 1/4 and 1/2 strength (each concentration applied for one week), followed by full-strength Hoagland solution alternated with distilled water every other day. At the 6–8 leaf stage, the plants were subjected to foliar application of SNP at 150 µM and SA at 500 µM, each applied separately and also in combination, with treatments repeated twice at a one-week interval. Twenty-four hours after the foliar treatments, the plants were exposed to copper sulfate ($CuSO_4$) stress at concentrations of 600 and 1200 µM for 14 days. After 35 days (Fig. 1), both shoot and root samples were collected for analysis. Fresh samples were immediately separated and stored at -80°C. The fresh weight of shoots and roots was measured using a digital scale with a precision of 0.001 g. The dry weight of shoots and roots was determined by oven-drying the samples at 75°C for 24 hours. Plant height was measured using a ruler.

Determination of malondialdehyde content and hydrogen peroxide levels

To determine the MDA content, 0.2 g of fresh shoot or root tissue was thoroughly homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged (5 min, 10,000 g), and the supernatant was collected. An equal volume of TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added to the supernatant. The reaction mixture was incubated in a water bath at 95°C for 30 min and then rapidly cooled in an ice bath. After cooling, the samples were centrifuged again (10 min, 10,000 g), and the absorbance of the clear supernatant was recorded at 532 and 600 nm using a spectrophotometer. Finally, the MDA concentration was calculated and expressed as (µmol g^{-1} FW) (35). Additionally, H_2O_2 levels were measured by homogenizing 0.5 g of shoot and root tissues in 0.1% TCA. The resulting homogenate was mixed with potassium phosphate buffer and potassium iodide to form the reaction mixture. Absorbance was then recorded at 390 nm (36).

Determination of Cell Death

To assess cell death, three 1-cm segments from the root tips of copper sulfate-treated plants and control samples were immersed in a 0.025% Evans blue solution for 30 minutes to specifically stain non-viable cells. Excess dye was removed by thoroughly washing the samples with distilled water for 15 minutes. To extract the dye bound to dead cells, the samples were homogenized in 1 mL of 50% methanol and incubated in a water bath at 50°C for 15 minutes. After centrifugation at 14,000g, the percentage of cell death was determined by measuring the absorbance of the supernatant at a wavelength of 600 nm and comparing it to the untreated control sample (37).

Determination of Soluble Sugar, Proline and Total Soluble Protein Content

In this study, soluble sugar content was quantified in 0.5 g samples of root and shoot tissues using the phenol-sulfuric acid method (38). Proline content was determined according to a previously established protocol with slight modifications (39). For this, 0.2 g of leaf or root tissue was homogenized in 3 mL of 3% sulfosalicylic acid. The homogenate was then mixed with ninhydrin reagent and glacial acetic acid, followed by incubation at 100°C for 1 hour. The reaction mixture was rapidly cooled in an ice bath, after which 4 mL of toluene was added. The absorbance of the upper organic phase was measured at 520 nm using a spectrophotometer. Total soluble protein content was measured using the Bradford method (40), and the resulting protein extract was subsequently used for the determination of antioxidant enzymes activities.

Determination of Antioxidant Enzymes Activities

The activities of catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), and superoxide dismutase (SOD, EC 1.15.1.1) were determined following the method described by Chance and Maehly (41). CAT activity was assessed by measuring the decrease in absorbance at 240 nm due to the decomposition of H_2O_2, while POD activity was determined by monitoring the oxidation of guaiacol at 470 nm. SOD activity was evaluated based on its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) at 560 nm (42). Enzyme activities were expressed as units per milligram of protein per minute.

Determination of Elemental Content

The total elemental content was assessed using a modified Sagner et al. (1998) (43) method. Plant root and shoot samples were dried at 70°C for 72 hours, and 0.5 g of each was digested in 10 mL of 65% (w/v) ultrapure nitric acid (Merck). After digestion, the volume was adjusted to 50 mL with deionized water, and concentrations of Cu, Fe, Mg and Zn were measured via atomic absorption spectrophotometry (AA6200 Shimadzu). To assess the translocation of Cu from roots to shoots, the translocation factor (TF) was calculated based on the following Eq. (44).

$$TF = \frac{Cu\ concentration\ in\ the\ shoots}{Cu\ concentration\ in\ the\ roots}$$

The results of plant growth indices (fresh weight, dry weight, shoot length, and root length)

Copper stress significantly reduced the fresh and dry weights of shoots and roots, as well as their lengths, compared to the control, with the most pronounced effects at 1200 μM. Cu stress also led to a significant reduction in shoot and root lengths, highlighting its inhibitory effect on plant growth. At 1200 and 600 μM Cu, shoot fresh weight decreased by 2.41- and 3.77-fold, root fresh weight by 2.16- and 3.84-fold, shoot dry weight by 2.22- and 7.06-fold, and root dry weight by 2.46- and 6.55-fold, respectively. However, applying SA and SNP, alone or combined with Cu, significantly enhanced growth indices under stress. The application of SA and SNP alleviated Cu-induced growth inhibition and improved shoot and root development. The greatest improvements in shoot fresh and dry weights were observed in Cu1200 + SA (31.63%) and Cu1200 + SA + SNP (50.03%), respectively, while root fresh and dry weights peaked in Cu600 + SA + SNP (36.92%) and Cu1200 + SA (63.46%), respectively, compared to Cu-stressed plants without SA or SNP (Table 1).

Table 1
Interactive Effects of Copper Stress, SA, and SNP on Growth Parameters in Okra

Treatments	Root fresh weight/plant (g)	Root dry weight/plant (g)	Root length (cm)	Shoot length (cm)	Shoot fresh weight/plant (g)	Shoot dry weight/plant (g)
Control	8.32 ± 0.28b	0.83 ± 0.020a	28.2 3 ± 0.23a	40.16 ± 1.16 ab	13.75 ± 0.55c	2.93 ± 0.21 a
500 µM SA	9.29 ± 0.15a	0.81 ± 0.0 4a	26.43 ± 0.23b	40. 83 ± 1.0 ab	16.14 ± 0.49ab	2.83 ± 0.0 8a
150 µM SNP	8.25 ± 0.14b	0.73 ± 0.024a	25.25 ± 0.25c	39.66 ± 0.69b	15.19 ± 0.87 abc	3.18 ± 0.13a
SA + SNP	8.95 ± 0.36ab	0.93 ± 0.02 a	29.3 ± 0.3a	42.16 ± 0.92 a	16.75 ± 0.55 a	3.15 ± 0.25 a
600 µM $CuSO_4$	3.85 ± 0.35 cd	0.33 ± 0.02 d	20 ± 0.57e	25.63 ± 0.31 e	9.73 ± 0.87 de	1..32 ± 0.12c
600 µM $CuSO_4$ +SA	4.31 ± 0.29c	0.55 ± 0.023b	22.59 ± 0.30d	28.33 ± 0.61 d	14.23 ± 1.07bc	1.73 ± 0.05 bc
600µM $CuSO_4$ + SNP	4.58 ± 0.46 c	0.43 ± 0.023c	25.96 ± 0.39bc	29.76 ± 0.62 d	11.22 ± 0.58d	1.62 ± 0.19bc
600µM$CuSO_4$ + SA + SNP	4.66 ± 0.24 c	0.43 ± .0.028c	26.67 ± 0.33b	32.23 ± 0.52c	13.73 ± 0.38c	1.92 ± .0.03b
1200 µM $CuSO_4$	1.16 ± 0.20 e	0.12 ± .0.030e	14.96 ± 0.20g	16.80 ± 0.73h	3.64 ± 0.30 f	0.416 ± .0.04d
1200µM$CuSO_4$ + SA	3.40 ± 0.21d	0.26 ± 0.012d	18.5 ± .0.76 f	23.96 ± .0.54ef	8.18 ± .0.46e	0.649 ± 0.04d
1200µM$CuSO_4$ + SNP	3.03 ± 0.29d	0.18 ± 0.020e	18 ± 0.28 f	20.6 3 ± 0.63 g	4.69 ± 0.64 f	0.569 ± 0.05d
1200µM$CuSO_4$ + SA + SNP	4.33 ± 0.23c	0.34 ± 0.024d	22.5 ± 0.28d	23..13 ± 0.40f	5.32 ± 0.59 f	0.044 ± 0.12d

Three different letters indicate a significant difference at the 5% level based on Duncan's test.

Results of Malondialdehyde Content, Hydrogen Peroxide Levels, and Cell Death

This study revealed that Cu stress significantly increased H_2O_2 levels and MDA content in shoots and roots, peaking in the 1200 µM Cu treatment and lowest in the control. However, applying SA and SNP, alone or combined, significantly lowered these parameters in stressed plants. The greatest H_2O_2 reduction occurred in the Cu600 + SA treatment (26.93% in shoots), Cu600 + SA + SNP (18.79% in roots), while MDA decreased most in Cu600 + SA + SNP for shoots (15.42%) and Cu600 + SA for roots (14.99%) compared to Cu-only treatments. Under non-stress conditions, SA, SNP, or their combination showed no significant impact on H_2O_2 or MDA levels **(**Tables 2 and 3**).** Increasing Cu in the nutrient solution elevated root cell death, but SA and SNP, applied individually or in combination, mitigated this effect. The highest cell death was observed in the 1200 µM Cu treatment, while SA and SNP proved effective across various Cu concentrations. The combination of SA and SNP in the $CuSO_4$ (600 µM) + SA treatment resulted in the reduction (45%). Under non-stress conditions, these compounds had no effect **(**Tables 3**).**

Results of Soluble Sugar, Proline and Protein Content

The analysis of copper stress effects on okra showed a significant increase in soluble carbohydrate content in both shoot and root tissues compared to the control, with the highest increase observed in the 1200 µM copper treatment. The use of SA, SNP, and their combination under both copper stress levels resulted in a significant reduction in carbohydrate content. The greatest reduction was observed in the $CuSO_4$ (600 µM) + SA treatment, with decreases of 10.04% in the shoots and 9.70% in the roots compared to copper treatments alone. When SNP was co-applied with copper, carbohydrate content decreased further, with reductions of 4.77% and 5.94% in the shoots and 7.08% and 6.15% in the roots under Cu 600 + SNP and Cu 1200 + SNP treatments, respectively. Under non-stress conditions, SA and SNP treatments had no effect on soluble carbohydrate content in the shoots. However, in the roots, SNP treatment significantly increased carbohydrate content in the 600 µM treatment **(**Tables 2 and 3**).**

Our research findings demonstrated that copper stress led to an increase in proline content in both the shoots and roots of the plant. The highest proline accumulation was observed in the shoots of the 1200 µM Cu treatment, showing a sixfold increase compared to the control. The application of SA reduced proline content in okra plants under copper stress. However, SNP treatment, in combination with 600 and 1200 µM Cu stress, significantly increased proline content in the shoots by 81.96% and 85.51%, respectively, and in the roots by 37.53% and 47.31% compared to the control group. Under non-stress conditions, SNP application did not affect proline content **(**Tables 2 and 3**).**

The results of the (Cu) effect assessment revealed that protein content significantly increased in shoots by 34.82% and 59.95% at 600 and 1200 µM Cu treatments, respectively, and by 47.47% in roots at 1200 µM Cu, while no significant change was observed in roots at 600 µM Cu. The application of (SA) and (SNP), individually or in combination, further increased protein content in shoots and roots under Cu stress. The greatest increases were recorded in the Cu1200 + SA + SNP treatment for shoots (63.65%) and in the Cu1200 + SNP treatment for roots (57.63%). Under non-stress conditions, protein content in treated plants also rose compared to the control **(**Tables 2 and 3**).**

Table 2
Interactive Effects of Copper Stress, SA, and SNP on MDA Content, H_2O_2 Levels, and Proline, Sugar, and Protein Content in Shoot

Treatments	MDAShoot (µmol/g FW)	H_2O_2 Shoot (µmol/g FW)	Soluble Sugar Shoot (mg/g FW)	Proline Shoot (nmol/g FW)	Total Proteins Shoot (mg/g FW)
Control	0.445 ± 0.022e	0.95 ± 0.03e	32.05 ± 1.05f	0.903 ± 0.071d	7.22 ± 0.415f
500 µM SA	0.454 ± 0.019e	1.03 ± 0.04 e	32.40 ± 0.30f	1.31 ± 0.151d	9.39 ± 0.231e
150 µM SNP	0467 ± 0.020e	1.08 ± 0.07 e	34.80 ± 0.30e	1.053 ± 0.103d	9.12 ± 0389e
SA + SNP	0.454 ± 0.012e	0.99 ± 0.07 e	32.87 ± 0.416f	0.76 ± 0.069d	9.45 ± 0.410e
600 µM $CuSO_4$	0.662 ± 0.0402c	2.3 ± 0.013c	40.88 ± 0.458c	4.14 ± 0.081b	11.08 ± 0.289d
600 µM $CuSO_4$ +SA	0.595 ± 0.024 cd	1.07 ± 0.05d	36.91 ± 0.471d	3.07 ± 0.133c	13.22 ± 0.170 c
600µM $CuSO_4$ + SNP	0.616 ± 0.035 cd	1.92 ± 0.09d	38.45 ± 0.54d	5.01 ± 0.156b	12.68 ± 0.612c
600µM$CuSO_4$ + SA + SNP	0.573 1 ± 0.028d	1.84 ± 0.11 d	38.38 ± 0.428d	4.45 ± 0.223b	13.49 ± 1.075c
1200 µM $CuSO_4$	0.949 ± 0.0201a	3.12 ± 0.10a	49.82 ± 0.858a	5.75 ± 0.138a	18.47 ± 0.252 b
1200µM$CuSO_4$ + SA	0.848 ± 0.0287b	2.51 ± 0.17bc	45.08 ± 0.103b	4.49 ± 0.248b	18.13 ± 0.470 b
1200µM$CuSO_4$ + SNP	0.858 ± 0.204b	2.76 ± 0.11b	47.44 ± 0.585b	6.23 ± 0.107a	18.13 ± 0.967 b
1200µM$CuSO_4$ + SA + SNP	0.848 ± 0.0315b	2.62 ± 0.14 bc	46.59 ± 0.409b	6.05 ± 0.285a	19.87 ± 0.537a

Three different letters indicate a significant difference at the 5% level based on Duncan's test.

Table 3
Interactive Effects of Copper Stress, SA, and SNP on MDA, H_2O_2, Proline, Sugar, Protein Content, and Cell Death Percentage in Roots

Treatments	MDA Root (µmol/g FW)	H_2O_2 Root (µmol/g FW)	Cell death Root (% of control)	Soluble sugar Root (mg/g FW)	Proline Root (nmol/g FW)	Total Proteins Root (mg/g FW)
Control	0.209 ± 0.0012 e	0.087 ± 0.030d	91.35 ± 7.42g	23.94 ± 0.408 gh	4.51 ± 0.265f	2.97 ± 0.433f
500 µM SA	0.198 ± 0.0013e	0.072 ± 0.073d	109.50 ± 8.79g	22.96 ± 0.577g	4.84 ± 0.099 def	4.01 ± 0.217d
150 µM SNP	0.201 ± 0.009e	0.087 ± 0.004d	134.73 ± 3.93g	22.55 ± 0.360h	5.23 ± 0.315de	3.37 ± 0.301ef
SA + SNP	0.195 ± 0.0009e	0.085 ± 0.008d	96.56 ± 16.21 g	24.08 ± 0.451gh	4.53 ± 0.188ef	4.23 ± 0.304de
600 µM $CuSO_4$	0.2 95 ± 0.014c	0.138 ± 0.003b	398.52 ± 10.10c	28.21 ± 0.927cde	6.59 ± 0.223c	3.45 ± 0.422ef
600 µM $CuSO_4$ +SA	0.251 ± 0.0121d	0.121 ± 0.003c	212.23 ± 20.20f	25.38 ± 0.508fg	5.291 ± 0.118d	3.31 ± 0.238ef
600µM $CuSO_4$ + SNP	0.265 ± 0.0128 cd	0.123 ± 0.002c	319.40 ± 24.59de	26.48 ± 0.608ef	7.346 ± 0.192b	4.23 ± 0.158de
600µM$CuSO_4$ + SA + SNP	0.281 ± 0.009cd	0.112 ± 0.006c	274.26 ± 21.09ef	27.10 ± 0.459def	5.55 ± 0.248d	4.47 ± 0.307d
1200 µM $CuSO_4$	0.414 ± 0.0138a	0.163 ± 0.003a	736.07 ± 48.90a	32.76 ± 1.240a	8.212 ± 0.164a	5.66 ± 0.423c
1200µM$CuSO_4$ + SA	0.356 ± 0.008b	0.144 ± 0.002b	372.57 ± 30.71cd	28.64 ± 0.266bcd	7.48 ± 0.280b	6.68 ± 0.207b
1200µM$CuSO_4$ + SNP	0.379 ± 0005b	0.148 ± 0.005b	537.13 ± 23.28b	30.44 ± 0.526b	8.564 ± 0.262a	7.02 ± 0.408a
1200µM$CuSO_4$ + SA + SNP	0.378 ± 0.011 b	0.144 ± 0.004b	400.84 ± 21.09c	29.87 ± 0.440bc	7.984 ± 0.248ab	6.49 ± 0.239ab

Three different letters indicate a significant difference at the 5% level based on Duncan's test.

Results of Antioxidant Enzymes Activity

According to the results obtained from Table 4, SOD enzyme activity increased by 35.55% and 53.59% in the shoots and by 73.93% and 89.52% in the roots under 600 and 1200 µM Cu treatments, respectively,

compared to the control. Foliar spraying of SA and SNP, either individually or in combination, further enhanced SOD activity under Cu stress. The highest SOD activity was observed in the Cu1200 + SA + SNP treatment, with increases of 67.11% in shoots and 92.17% in roots compared to the control. With increasing Cu concentrations, POD enzyme activity rose by 59.18% and 84.17% in shoots and by 73.44% and 84.79% in roots. Application of SA and SNP, either separately or together, significantly enhanced POD activity in Cu-treated plants compared to the control. The greatest increases were recorded in the Cu1200 + SA + SNP treatment for shoots 88.40% and in the Cu1200 + SA treatment for roots 86.80%. In non-stress conditions, foliar application of SA and SNP had no significant effect on POD activity in shoots; however, in roots, the combined SA + SNP treatment resulted in an increased POD activity. The results for CAT enzyme activity indicated increases at both Cu levels, with rises of 29.68% and 57.94% in shoots and 19.54% and 27.61% in roots compared to the control. In plants subjected to foliar application of SA and SNP (either individually or in combination), CAT activity was higher compared to plants exposed to Cu stress alone. The highest increases in CAT activity were observed in the Cu1200 + SA + SNP treatment, with enhancements of 67.04% in shoots and 67.02% in roots relative to the control (Table 4).

Table 4
Interactive Effects of Copper Stress, SA, and SNP on Antioxidant Enzyme Activity in Shoots and Roots

Treatments	SOD Shoot unit mg^{-1} protein	CAT Shoot unit mg^{-1}protein	POD Shoot unit mg^{-1}protein	SOD Root unitmg[1]protein	CAT Root unit mg^{-1}protein	POD Root unit mg^{-1} protein
Control	12.63 ± 1.33e	18.01 ± 0.533i	0.789 ± 0.058g	5.46 ± 0.417g	11.71 ± 0.572h	1.47 ± 0.073f
500 µM SA	11.93 ± 0.481f	20.83 ± 0.440 h	0.997 ± 0.0827g	7.48 ± 0.736 g	12.50 ± 0.449gh	1.82 ± 0.231ef
150 µM SNP	10.83 ± 1.03f	20.98 ± 0.768 h	0.799 ± 0.085 fg	5.92 ± 0.499g	14.39 ± 0.811fg	1.59 ± 0.305f
SA + SNP	14.00 ± 0.576 e	21.44 ± 0.446h	1.03 ± 0.136f	5.66 ± 1.94g	15.14 ± 0.751f	3.06 ± 0.408e
600 µM $CuSO_4$	19.61 ± 1.92d	25.62 ± 0.881g	1.93 ± 0.211e	19.81 ± 1.66f	19.54 ± 0.443e	5.561 ± 0.149d
600 µM $CuSO_4$ + SA	22.78 ± 1.37d	28.35 ± 0.548f	2.98 ± 0.292d	29.75 ± 1.87e	22.98 ± 1.09d	6.708 ± 0.733c
600µM $CuSO_4$ + SNP	26.93 ± 0.885c	30.94 ± 0.580ef	2.88 ± 0.219d	28.29 ± 2.60e	24.51 ± 0.846d	6.65 ± 0.414c
600µM$CuSO_4$ + SA + SNP	29.32 ± 0.735bc	32.01 ± 1.01ef	3.28 ± 0.374d	37.39 ± 1.55d	24.17 ± 0.554d	7.60 ± 0.210c
1200 µM $CuSO_4$	27.23 ± 1.51c	42.84 ± 1.31d	5.18 ± 0.313c	51.17 ± 3.39c	27.61 ± 1.05c	9.71 ± 0.182b
1200µM$CuSO_4$ + SA	32.18 ± 2.11b	45.88 ± 0.843c	6.12 ± 0.351ab	59.29 ± .2.29b	32.96 ± .1.03b	11.197 ± 0.678a
1200µM$CuSO_4$ + SNP	36.21 ± 1.62a	51.15 ± 1.78b	6.02 ± 0.239b	60.58 ± 2.26b	33.78 ± 0.874ab	10.39 ± 0.232ab
1200µM$CuSO_4$ + SA + SNP	38.43 ± 1.71a	54.67 ± 1.20a	6.80 ± 0.187a	68.48 ± 2.20a	35.51 ± 0.572a	11.14 ± 0.400a

Three different letters indicate a significant difference at the 5% level based on Duncan's test.

Results of Elemental Levels (Cu, Zn, Mg, and Fe) and TF

The results showed that Cu stress reduced Zn, Mg, and Fe contents in the shoots and roots of okra plants at 600 and 1200 µM Cu levels. However, applying SA and SNP significantly elevated these elements in specific treatments. The largest increases were recorded for Zn in the Cu600 + SA + SNP treatment (50.40%) and Mg in the Cu600 + SA treatment (41.78%). Likewise, Fe content increased in the Cu600 + SA (38.37%) and Cu600 + SNP (51.38%) treatments. In contrast, Cu content in plants under 1200 µM Cu

stress rose substantially compared to the control, yet SA and SNP application significantly lowered Cu levels in shoots (58.46%) and roots (73.42%) in the Cu600 + SA + SNP treatment. Data of Table 5 indicated that the translocation factor (TF) decreased with higher Cu concentrations, due to Cu-induced stress and reduced plant ability to transfer Cu from roots to shoots. These results suggest that okra is not a hyperaccumulator under Cu stress, as its TF remained below 1 **(**Tables 5 and 6**).**

Table 5
Interactive Effects of Copper Stress, SA, and SNP on Elemental Content in Okra Shoots

Treatments	Mg Shoot (mg/g DW)	Fe Shoot (mg/g DW)	Cu Shoot (µg/g DW)	Zn Shoot (mg/g DW)	TF
Control	4.29 ± 0.065b	0.151 ± 0.005a	36 ± 1.616g	0.028 ± 0.0003a	0.393 ± 0.05a
500 µM SA	4.66 ± 0.140a	0.152 ± 0.006 a	35.73 ± 2.082g	0.029 ± 0.0007a	0.273 ± 0.023b
150 µM SNP	4.63 ± 0.110a	0.147 ± 0.003a	36.8 ± 1.890fg	0.028 ± 0.0007a	0.276 ± 0.027b
SA + SNP	4.74 ± 0.14a	0.153 ± 0.008a	38.13 ± 3.014gf	0.030 ± 0.0004a	0.273 ± 0.039b
600 µM $CuSO_4$	2.23 ± 0.109d	0.07 ± 0.005 d	130.6 ± 6.150d	0.018. ±0.0004de	0.206 ± 0.012 bc
600 µM $CuSO_4$ + SA	3.84 ± 0.072c	0.11 ± 0.002b	99.6 ± 11.60e	0.021 ± 0.0004bc	0.161 ± 0.02c
600µM $CuSO_4$ + SNP	3.56 ± 0.104c	0.12 ± 0.007 b	54.26 ± 2.755gf	0.020 ± 0.0003bcd	0.202 ± 0.005 bc
600µMCuSO4 + SA + SNP	3.62 ± 0.096c	0.12 ± 0.007b	65.33 ± 5.871f	0.023 ± 0.0008b	0.182 ± 0.023 bc
1200 µM CuSO4	1.69 ± 0.065e	0.052 ± 0.003 e	265.0 ± 8.340a	0.014 ± 0.0004f	0.206 ± 0.003bc
1200µMCuSO4 + SA	2.08 ± 0.080d	0.108 ± 0.006bc	182.5 ± 13.33c	0.019 ± .0.0002cde	0.156 ± 0.012c
1200µMCuSO4 + SNP	2.293 ± 0.153d	0.092 ± 0.008cd	224.1 ± 12.66b	0.016 ± 0.0003ef	0.220 ± 0.015 bc
1200µMCuSO4 + SA + SNP	2.382 ± 0.167d	0.096 ± 0.006c	203.2 ± 20.38bc	0.018 ± 0.0003cde	0.203 ± 0.017bc

Three different letters indicate a significant difference at the 5% level based on Duncan's test.

Table 6
Interactive Effects of Copper Stress, SA, and SNP on Elemental Content in Okra Roots

Treatments	Mg Root (mg/g DW)	Fe Root (mg/g DW)	Cu Root (µg/g DW)	Zn Root (mg/g DW)
Control	2.86 ± 0.035b	1.51 ± 0.059a	93.73 ± 7.62g	0.047 ± 0.0005a
500 µM SA	3.183 ± 0.084a	1.151 ± 0.063a	130.4 ± 6.34g	0.045 83 ± 0.005a
150 µM SNP	3.11 ± 0.109ab	1.47 ± 0.034a	135.7 ± 10.55g	0.047.66 ± 0.0018a
SA + SNP	3.21 ± 0.087a	1.57 ± 0.10a	142.5 ± 8.873g	0.048.16 ± 0.0012a
600 µM $CuSO_4$	2.10 ± 0.052de	0.864 ± 0.036c	628.8 ± 9.75d	0.024.63 ± 0.0016cd
600 µM $CuSO_4$ + SA	2.34 ± 0.116cd	1.10 ± 0.031b	595 ± 66.36 d	0.035 ± 0.001b
600µM $CuSO_4$ + SNP	2.59 ± 0.117c	1.07 ± 0.059b	269.8 ± 22.28f	0.029 ± 0.001cd
600µMCuSO4 + SA + SNP	2.38 ± 0.167cd	1.20 ± 0.074b	360 ± 16.16e	0.029 ± 0.003c
1200 µM CuSO4	1.55 ± 0.083f	0.438 ± 0.061e	1290.9 ± 40.31a	0.017 ± 0.0008e
1200µMCuSO4 + SA	1.87 ± 0.037e	0.695 ± 0.074cd	1151 ± 20.24b	0.026 ± .0.0005cd
1200µMCuSO4 + SNP	1.82 ± 0.061e	0.596 ± 0.073de	1025 ± 47.20c	0.027 ± 0.0004d
1200µMCuSO4 + SA + SNP	1.91 ± 0.058e	0.689 ± 0.070cd	878 ± 11.93c	0.026 ± 0.001cd

Three different letters indicate a significant difference at the 5% level based on Duncan's test

Discussion

Copper exposure substantially reduced growth, height, and both the fresh and dry biomass of shoots and roots in okra plants. These detrimental effects arise from the heightened sensitivity of roots to copper toxicity, as they are the first organs exposed to contaminants and consequently sustain the greatest damage. The presence of copper in the rhizosphere causes morphological alterations in the root apex, leading to shorter and thicker roots. Additionally, copper stress impairs root growth and the absorption of water and nutrients by decreasing the rate of mitotic division (45, 46). Comparable effects were observed in Indian jute (*Corchorus capsularis* L.) and *Aegilops tauschii* under copper stress (47, 48). Foliar application of SA enhanced plant growth and markedly improved the fresh and dry biomass of shoots and roots in okra, which is consistent with similar findings observed in bean and *Salvia officinalis* L. under

copper stress (49, 50). SA likely promotes vegetative growth by stimulating indole acetic acid (IAA) and gibberellin synthesis, while boosting stem and root development through improved nutrient uptake (51). Foliar SNP mitigated copper toxicity, enhancing okra growth and yield. SNP's role in alleviating HM stress and improving growth was also noted in perennial ryegrass and Chinese cabbage under cadmium toxicity (52, 53). Hajihashem et al. (2022) found that combined SA + SNP treatment enhanced plant growth by increasing metabolite synthesis and carbon fixation, while stimulating growth via cell wall acidification (54). In this study, simultaneous SNP + SA application increased okra growth, biomass, and height. In this study, copper toxicity markedly elevated the levels of H_2O_2, MDA, and cell death in both the shoots and roots of okra plants. Despite higher Cu accumulation in the roots, the shoot tissues exhibited greater MDA content, indicating their heightened sensitivity to Cu-induced oxidative damage. Similar increases in H_2O_2 and MDA under copper stress have been reported in *Boehmeria nivea* (55) and *Brassica juncea* (56), along with enhanced cell death in pea (*Pisum sativum*) roots (57). The application of 500 and 150 µM levels of SA and SNP significantly reduced cell death, MDA, and H_2O_2 levels in both the shoots and roots of okra plants exposed to copper toxicity. These findings highlight the effectiveness of these treatments in alleviating copper-induced oxidative stress. SA is believed to confer protection by inhibiting lipid peroxidation, maintaining membrane integrity, and enhancing the antioxidant defense system (58). Similar protective effects of SA have been reported in cotton under copper stress (59), in soybean roots under aluminum toxicity (60), and in Canola subjected to arsenic stress (61), where reductions in MDA, H_2O_2, and cell death were observed. SNP functions as a signaling molecule that regulates the expression of antioxidant defense genes, thereby enhancing the production of enzymes that neutralize ROS. This action contributes to reduced oxidative damage, improved membrane stability, and a decrease in cell death (62). These findings align with similar results observed in plants such as soybean (63) and rice (64) under HMs stress. Additionally, the simultaneous application of SA and SNP in safflower under zinc stress led to a significant reduction in H_2O_2 and MDA levels in both the roots and shoots of okra (65). Under copper stress conditions, okra plants exhibited an effective adaptive response by increasing proline and soluble sugar content in both roots and shoots, with a more prominent increase observed in the roots. Proline accumulation, as an osmotic and oxidative stress protectant (16), has been previously reported in *Boehmeria nivea* (55) and *Linum usitatissimum* (66). Additionally, an elevation in soluble carbohydrates, contributing to osmotic osmotic regulation and carbon storage, has been observed in *Spartina alterniflora* under copper stress (67, 68). The increase in soluble sugars may result from factors such as growth cessation, breakdown of polysaccharides like starch, non-photosynthetic sugar synthesis, and reduced exchange of photosynthetic products between plant parts. These results underscore the essential role of these compounds in plant adaptation to HMs stress(13). The evaluation of individual and joint treatments of SA and SNP in the roots and shoots of okra under copper stress, compared to the control, showed an increase in proline and soluble sugars. However, a noticeable decline in osmolyte levels under SA treatment was observed compared to the stress levels. This decrease in proline in the SA treatment is likely due to the fact that, in the presence of SA, there is less need for free proline as a stress protectant. A reduction in soluble sugars in plants such as *Oryza sativa* L. (Rice) (69) and *Helianthus annuus* L. (70) treated with SA under copper stress has been reported. Ahmad et al. (2016) (71) indicated that NO helps maintain cell turgor and water retention in plants by stimulating the accumulation of

osmotic regulators, including proline and soluble sugars, thereby enhancing plant resilience against environmental stressors. In our study, the separate and combined application of SNP to the roots and leaves of okra under copper stress resulted in increased proline and soluble carbohydrates levels relative to the control. Our findings are consistent with those reported for tomato (*Solanum lycopersicum*) (72). The increase in protein content in the shoot and root organs of okra plants under copper stress may be attributed to the compensatory synthesis of proteins that have been inactivated by binding to HMs, or to the enhanced production of stress-related proteins such as heat shock proteins (HSPs) and enzymes involved in antioxidative defense (73). This result has similarly been documented for wheat variety (*Triticum aestivum* L.) (17). In the present study, both separate and combined applications of SNP and SA led to an increase in protein content in the shoot and root organs of okra plants under copper stress. This increase seems to be related to the role of SA in strengthening the antioxidant system and membrane protection, in addition to the effect of NO released from SNP in reducing oxidative stress and maintaining cellular redox homeostasis. The results are consistent with findings reported in *Oryza sativa* L. (64). Antioxidants, both enzymatic and non-enzymatic, are integral components of the plant defense system, developed to eliminate excess ROS and preserve cellular redox homeostasis (74, 75). In the present study, copper stress led to an increased activities of CAT, POD, and SOD in both shoot and root tissues of okra, which is consistent with findings reported for *Hibiscus cannabinus* L. under similar conditions (76). Similarly, a study by Wang et al. (2024) revealed that copper toxicity triggered a significant increase in antioxidant enzyme activity in both the shoots and roots of *Aegilops tauschii* (48). Exposure of okra plants to SA and SNP, either individually or in combination, significantly enhanced the activity of antioxidant enzymes (CAT, POD, and SOD) in both shoot and root tissues under copper-induced stress. This enhancement was particularly pronounced in the combined Cu + SA treatment, highlighting the protective role of SA in reducing oxidative stress induced by HMs exposure. Previous studies have also demonstrated that SA improves the efficiency of the plant's antioxidant system by suppressing ROS generation and mitigating lipid oxidative stress (77), in addition to inducing antioxidant enzymes or regulation of their gene expression (78). A similar increase in antioxidant enzyme activity in response to SA treatment has also been reported in wheat (79). SNP as a NO donor enhances the plant's defense mechanisms and mitigates damage caused by various abiotic factors by inducing the expression of antioxidant-related genes (80). In the present study, SNP application in okra under copper stress led to an increase in antioxidant enzyme activity, a response that has also been observed in sorghum under copper stress (72). In our study, the simultaneous application of SA and SNP led to an increase in antioxidant enzyme activity in okra plants, a finding that aligns with the results reported by Mostofa et al. (2019) (81) in rice plants under cadmium stress. This current research demonstrated that copper accumulation in okra plants under copper stress was significantly greater in the roots compared to the shoots. This indicates a slow movement of copper upward from roots to shoots and suggests that okra is not a hyperaccumulator species. These findings are consistent with those of Ibrahim (2017) (82) who reported greater copper accumulation in the root system than in the stem tissues of *Gynura procumbens* (Lour). Furthermore, in okra, increasing copper concentrations in the growth medium led to a reduction in essential nutrients such as Mg, Zn and Fe likely due to competitive uptake mechanisms. These observations align with those of Es-sbihi et al. 2020 (50), who investigated *Salvia officinalis* under copper

stress. In this research, foliar application of SA, SNP and their combination SA + SNP significantly increased the content of Mg, Zn, and Fe while reducing Cu accumulation in the roots and shoot parts of okra plants under Cu stress. These results indicate the inhibitory effect of SA and SNP on the process of Cu uptake and transfer to different plant parts. Moreover, treatment with SA and SNP reduced Cu toxicity by decreasing the transfer of this element and enhancing antioxidant enzyme activity in the plant. Similar results have been confirmed in cotton (*Gossypium hirsutum*) and rice (59, 83). A research by Bai et al. 2015 (84) showed that SA treatment increased the levels of Mg, Zn, Mn, and Fe in *Lolium perenne* under cadmium stress. It has been reported that SNP reduced heavy metal accumulation and increased the levels of Fe, Mn, and Zn in soybean plants under mercury stress (63). This effect was likely due to an increase in internal NO content, which activates membrane transporters, leading to metal removal and increased absorption of essential mineral elements (85). In another research, it was confirmed that the combination of SA and SNP significantly increased the levels of essential elements such as K, Ca, Zn, Fe, and Mn in rose plants under alkaline stress (86).

Conclusion

This study demonstrated that foliar application of SA and SNP whether applied individually or in combination significantly enhanced the tolerance of okra plants to copper stress. These protective effects were associated with reduced copper accumulation in plant tissues, mitigation of copper-induced oxidative stress, and improved regulation of nutrient uptake. Treatment with SA and SNP led to a decrease in cellular damage markers (e.g., MDA and H_2O_2), preservation of membrane integrity, enhancement of the antioxidant defense system, and improved uptake of essential nutrients. Overall, the results of the comparative analysis indicated that SA was more effective than SNP in alleviating copper toxicity and enhancing okra's physiological tolerance.

Declarations

Approval for plant experiments

All experimental procedures involving plants were carried out in accordance with institutional, national, and international regulations and ethical standards.

Data availability

The datasets used and /or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

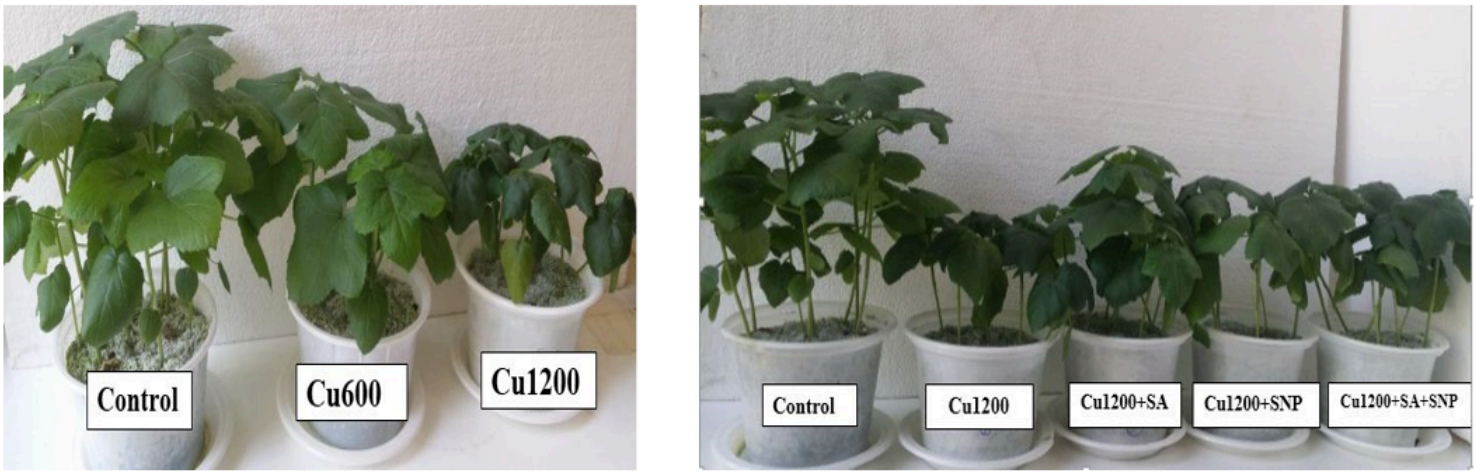

Figure 1

Okra plants under foliar application of salicylic acid and sodium nitroprusside in hydroponic conditions under copper stress after 35days.