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Alleviation of copper phytotoxicity by acetylsalicylic acid and nitric oxide application in mung bean involves the up-regulation of antioxidants, osmolytes and glyoxalase system

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ABSTRACT

The study assessed the individual and interactive role of acetylsalicylic acid (ASA) and nitric oxide (NO) against excess copper (Cu) concentrations. Excess Cu significantly reduced growth and photosynthesis, although the application of ASA and NO significantly mitigated the reduction with much visible effect in their combination. The oxidative effects of Cu have been evident as increased reactive oxygen species (ROS) production, resulting in increased malondialdehyde (MDA) and reduced membrane stability index (MSI). Both ASA and NO reduced oxidative damage obvious as reduced MDA and lipoxygenase (LOX) down-regulation. Up-regulation of enzymatic and non-enzymatic antioxidants due to ASA and NO application contributed to the quick elimination of excess ROS. Oxidative effects of excess Cu were mitigated by osmolyte accumulation, up-regulation of the γ-Glutamyl kinase (γ -GK), glyoxalase, and down-regulation of proline oxidase (PROX). Reduced Cu accumulation in our study showed the crosstalk effect of ASA and NO resulting in the protection of mung bean growth against the damaging effects of Cu.

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Copper toxicity; oxidative damage; glyoxalase; osmolytes; phytohormones

Introduction

It is established that plant growth depends on the optimum availability of essential macro and microelements. Their optimal presence actively regulates various physiological and biochemical attributes (Elkelish et al. 2019a, 2019b; Ahanger et al. 2019). Copper (Cu) commonly exists as a trace element in rocks, soils, and sediments. Cu concentrations in soil range from 100 mg / kg to 200 mg / kg (Tiller 1983). Since it is essential for plants, animals as well as humans, Cu is usually supplied in small doses and its deficiency turns out to be a growth-restricting factor for several crops (Arnon and Stout 1939). Contrarily to the deficiency scenario, several anthropogenic activities, such as waste disposal, mining, smelting, overuse of Cu-rich agrochemicals, and soil amendment, result in phytotoxicity and toxic effects of Cu to the other soil biota (Naidu and Bolan 2008; Ballabio et al. 2018). The main source of nutrients for plants is soil. It is therefore evident that the availability of Cu at higher concentrations in the soil medium may lead to significant toxic effects on the growth, development, and productivity of crop plants. The harmful effects of toxic levels have been mainly attributed to their potential to change redox homeostasis, alter the functioning of the enzyme, damage proteins, lipids, and nucleic acids through excessive ROS production and interruption of tolerance mechanisms (Rehman et al. 2019).

Excessive accumulation of ROS has harmful effects on plant growth and development resulting in significant loss of yield (Elkelish et al. 2020; Soliman et al.2020; Khan et al.2017; Hasan et al.2020a). Key ROSs accumulated include: hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (OH^-), nitric oxide (NO), etc. Its beneficial role in processes such as stress signalling and tolerance has been reported when their concentrations are maintained at certain threshold levels (Saleem et al. 2020a). Contrary, they become key detrimental molecules when their concentrations increase and plants eliminate excess ROS by activating multiple defense mechanisms that work together to protect cellular structures and functions (Ahanger et al. 2017; Elkelish et al. 2019a; Soliman et al. 2019). Key ROS elimination pathways include the antioxidant and osmolyte accumulation system (Soliman et al. 2020). The functioning of enzymatic as well as non-enzymatic antioxidants and the accumulation of osmolytes, including proline, glycine betaine, sugars, etc., determine the tolerance potential of plants against stress factors by protecting the functional and structural stability of major cellular components (Ahanger et al. 2017; Soliman et al. 2019; Rasheed et al. 2020). The glyoxalase system is another intriguing pathway involved in removing toxic methylglyoxal (MG) for efficient cellular functioning (Mudalkar et al. 2017; Alharbi et al. 2021; Hasan et al. 2020b). Up-regulation of the antioxidant and glyoxalase systems, increased accumulation of osmolytes

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have been correlated with the efficient functioning of plants under various stress conditions (Nahar et al. 2016; Elkelish et al. 2019a, 2019b; Soliman et al. 2020; Rasheed et al. 2020).

Salicylic acid (SA) and nitric oxide (NO) have been recognized for their role in plant development, enzyme functioning, signalling, and stress tolerance (Khan et al. 2015a; Wu et al. 2011). These beneficial molecules have been reported to interact with other key molecules to elicit and strengthen stress reactions in plants (Ahmad et al. 2018a; Nahar et al. 2016). However, the interactive effect of ASA and NO for increased Cu tolerance has not been reported. Therefore, in the present study, the influence of exogenous ASA and NO (both individually and in combination) on key tolerance mechanisms such as antioxidants, glyoxalase system, and osmolyte accumulation in mung bean has been studied.

Mungbean (*Vigna radiata* L. wilczek) is an important legume crop in the Fabaceae family, commonly known as green gram or moong. Seeds are consumed in various forms throughout the world and are considered a rich source of protein (Ihsan et al. 2013). Mungbean starch extracted from the ground Mungbean is used to make cellophane noodles. Excess Cu can be a challenge for its optimum growth and productivity in polluted soils (Hasan et al. 2019a). Excessive Cu accumulation in food components can also prove toxic to consumers. In this backdrop present study envisages that excess Cu-induced growth and photosynthetic damages could be mitigated by exogenous ASA and/or NO application by up-regulating the tolerance mechanisms.

Material and methods

Experimental design and treatment

Vigorous mung bean (*Vigna radiata* L. wilczek) seeds were obtained from Agriculture Research Centre, Giza, Egypt. Seeds were surface-sterilized using $HgCl_2$ (0.001%, w/v) for five minutes, followed by repeated washing with distilled water. Sterilized seeds were sown in pots filled with sterilized sand. 200 mL of Hoagland nutrient solution was supplied to all pots at the time of sowing. The detailed concentration of the Hoagland nutrient solution is given elsewhere (Elkelish et al. 2019b) with 0.5 µM $CuSO_4$ as indigenous Cu concentration. After germination, the seedlings were regularly monitored for ten days and were fed with nutrient solution (100 mL) on alternate days. After that, pots were separated into two groups where one group was irrigated with normal nutrient solution containing 0.5 µM $CuSO_4$, and another group was supplied with 100 µM $CuSO_4$. Application of 50 µM nitric oxide (as sodium nitroprusside) and 100 µM salicylic acid (in the form of acetylsalicylic acid; ASA) was also started along with Cu treatment. NO was supplemented via nutrient solution while ASA was applied onto the foliage using 0.1% (v/v) teepol as a surfactant. The detailed experimental treatments included: (a) control (nutrient solution with 0.5 µM $CuSO_4$), (b) 100 µM $CuSO_4$, (c) ASA, (d) NO, (e) ASA + NO, (f) Cu + ASA, (g) Cu + NO, and (h) Cu + ASA + NO. Thirty-day old seedlings were uprooted and various parameters such as oxidative stress markers, osmolytes, secondary metabolites, antioxidants, and glyoxalase were analyzed.

Pots were kept in the greenhouse and the day/night temperature was 22/16^{0}C, with 60–70% relative humidity (RH), and the photoperiod was 16/8-h during the experimental period. Pots were arranged in a complete randomized block design with six replicates for each treatment.

Shoot length and dry weight

Shoot length was measured using the scale. Dry weight was taken after drying the plants in the oven at 70 °C for 48 hrs.

Determination of photosynthetic pigments, photosynthesis, and photochemical efficiency

The content of total chlorophylls and carotenoids were estimated by macerating fresh 0.1g leaf tissue in 80% (v/v) acetone by using pestle and mortar. The homogenate was centrifuged at 3000g for 20mins. The optical density of the supernatant was read at 480, 645, and 663 nm, respectively (Arnon 1949).

Net photosynthetic rate (P_n) data was taken by using a portable infra-red gas analyzer (CID-340, Photosynthesis System, Bioscience, USA). For measurement of maximal photochemical efficiency (Fv/Fm) Modulated Chlorophyll Fluorometer (PAM 2500; Walz, Germany) was used, and leaves were dark-adapted for 25 min.

Determination of RWC, total soluble sugars, and glycine betaine

Relative water content (RWC) was determined by punching leaf discs from fresh leaves. After recording their fresh weight, turgid weight was taken after floating the same discs in petri dishes containing distilled water and discs were oven-dried at 80 °C for 24 hrs for the dry weight (Smart and Bihgham 1974). RWC was calculated using the following formula:

$$\text{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

The total soluble sugar content was determined using the method of Shields and Burnett (1960). Briefly, 500 mg dry powdered plant samples were extracted in 80% (v/v) ethanol. After centrifugation at 5000g for 20 min, the supernatant was reacted with anthrone reagent and optical density was taken at 585 nm. For estimation of glycine betaine (GB), dry powdered tissue was extracted in distilled water and the extract was mixed with 2N H_2SO_4 followed by the addition of cold KI-I_2 reagent. The mixture was centrifuged at 10,000g for 15 min, and the supernatant was aspirated. The periodide crystals were dissolved in 1, 2-dichloroethane and the optical density (OD) was taken at 365 nm (Grieve and Grattan 1983).

Estimation of proline and assay of γ-glutamyl kinase, and proline oxidase

The content of proline was determined by extracting 500 mg dry powdered tissue in 3% (w/v) sulphosalicylic acid. After centrifuge, the extract for 20 min at 3000g, 2 mL supernatant was reacted with 2 mL glacial acetic acid and 2 mL ninhydrin reagent in a water bath at 100 °C for 1 h. After that, tubes were kept on an ice bath and proline was separated using

toluene. Optical density was recorded at 520 nm (Bates et al. 1973).

For assaying the activities of the γ-Glutamyl kinase (GK, EC 2.7.2.11) and proline oxidase (PROX, EC 1.4.3.1), fresh 500 mg leaf tissue was homogenized in Tris buffer (pH 7.5) using cold pestle and mortar. Homogenate was centrifuged for half an hour at 30,000g, and the activity of γ-GK (Hayzer and Leisinger 1980) and PROX (Huang and Cavelieri 1979) was determined in pellet following the respective methods. The reaction mixture for γ-GK contained Tris buffer (50 mM, pH 7.0), L-glutamate (50 mM), $MgCl_2$ (20 mM), ATP (10 mM), hydroxamate–HCl (100 mM), and the reaction was initiated by adding the enzyme. After terminating the reaction by adding stop buffer ($FeCl_3$ and TCA), the absorbance was taken at 535 nm, and μg of γ-glutamyl hydroxamate formed was considered as the activity of γ-GK and expressed as U mg^{-1} protein min^{-1}. Assay mixture for PROX contained Tris buffer (50 mM, pH 8.5), 1 mL $MgCl_2$, 100 μL of NADP, KCN, phenazine methanosulphate, 2, 6 – dichlorophenol indophenol (DCPIP), and proline. Change in absorbance was monitored for 3 min at 600 nm and activity was considered as mmol DCPIP reduced and expressed as U mg^{-1} protein min^{-1}.

Measurement of membrane stability index and lipid peroxidation

The membrane stability index (MSI) was measured by using the Sairam et al. (1997) method and the following formula was used for calculation:

$$MSI = [1 - (C_1/C_2)] \times 100$$

Lipid peroxidation was measured as the content of malonaldehyde (MDA) formation according to Heath and Packer (1968). Fresh 100 mg was extracted in 1% (w/v) trichloro acetic acid (TCA) using pestle and mortar, followed by centrifugation at 10,000g. After that, 1.0 mL supernatant was mixed with 0.5% (w/v) thiobarbituric acid and heated at 95° C for one hour. After cooling the samples on an ice bath and subsequently centrifuging for 5 min at 5000g, the optical density of supernatant was read at 532 and 600 nm.

Activity of lipoxygenase

The activity of LOX (EC 1.13.11.12) was determined according to Doderer et al. (1992). An increase in absorbance was recorded at 234 nm using linoleic acid as a substrate. The extinction coefficient of 25 mM^{-1} cm^{-1} was used for the calculation and activity was expressed as mg^{-1} protein equivalent to 1 μmol of substrate oxidized min^{-1}.

Estimation of hydrogen peroxide and superoxide

For the determination of hydrogen peroxide method of Velikova et al. (2000) was used. 100 mg tissue was extracted in 0.1% TCA. Homogenate was centrifuged for 15 min at 12,000g and 0.5 mL supernatant was mixed with 0.5 mL of potassium phosphate buffer (pH 7.0) followed by the addition of 1 mL potassium iodide. Absorbance was taken at 390 nm. The content of superoxide was estimated by extracting the fresh tissue in 65 mM potassium phosphate buffer (pH 7.8). After centrifuging the extract at 5000g, the supernatant was mixed with 10 mM hydroxylamine hydrochloride and allowed to stand for 20 min. Thereafter, sulphanilamide, and naphthylamine were added, followed by incubation for 20 min at 25 °C and optical density was taken at 530 nm. For calculation standard graph of $NaNO_2$ was used (Yang et al. 2011).

Activity of glyoxalase I and II, and content of methylglyoxal (MG)

For determination of glyoxalase I (EC: 4.4.1.5) and glyoxalase II (EC: 3.1.2.6) activity fresh 500 mg tissue was homogenized in cold 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM KCl, β-mercaptoethanol (5 mM), ascorbate (1 mM) and glycerol (10%, v/v). Homogenate was centrifuged at 11,500g for 15 min, and the supernatant was used as an enzyme. For the determination of Gly I method described by Hasanuzzaman et al. (2011) was used and the assay mixture contained 100 mM GSH, 0.1 M phosphate buffer, 16 mM $MgSO_4$, 35 mM methylglyoxal, and enzyme extract. Change in absorbance was recorded at 240 nm for 2 min and extinction coefficient of 3.37 mM^{-1} cm^{-1} was used for calculation and activity was expressed as μmol min^{-1} mg^{-1} protein. For the determination of Gly II activity method of Principato et al. (1987) was followed, and the formation of reduced glutathione (GSH) was recorded at 412 nm for 1 min. Reaction mixture (1 mL) containing 100 mM Tris–HCl buffer (pH 7.2), DTNB (5, 5'-dithiobis-2-nitrobenzoic acid, 0.2mM) and S-D-lactoylglutathione (1 mM). For calculation extinction co-efficient of 13.6 mM^{-1} cm^{-1} was used.

For estimation of methylglyoxal (MG), leaf tissue was homogenized in 5% (v/v) perchloric acid and centrifuged for 10 min at 11,000g. Subsequently, the supernatant was decolored by the addition of charcoal (10 mg/mL) and was neutralised with a saturated solution of potassium carbonate. Thereafter, MG content was estimated by adding 400 μL of sodium dihydrogen phosphate and 40 μL of N-acetyl-L-cysteine in the final volume of 1 mL. After 10 min, the formation of N-α-acetyl-S-(1-hydroxy-2- oxo-prop-1-yl) was recorded at 288 nm (Wild et al. 2012).

Assay of antioxidant enzymes

Antioxidant enzymes were extracted by homogenizing 1 gm fresh leaf tissue in chilled 50 mM phosphate buffer (pH 7.0) supplemented with 1% (w/v) polyvinyl pyrrolidine and 1 mM EDTA (Ethylenediaminetetraacetic acid) using pre-chilled pestle and mortar. After centrifuging the homogenate at 15,000g for 20 min at 4 °C, the supernatant was used as an enzyme source. Content of protein was estimated in the supernatant as per the method of Lowry et al. (1951) and bovine serum albumin was used as standard.

Bayer and Fridovich (1987) methods were used to determine the activity of superoxide dismutase (SOD, EC 1.15.1.1) and nitroblue tetrazolium (NBT) photochemical reductions were recorded at 560 nm in a 1.5 mL assay mixture containing sodium phosphate buffer (50 mM, pH 7.5), 100 μL EDTA, 13 mM L-methionine, 75 μM NBT, 60 μM riboflavin and 100 μL enzyme extract. After 15 min of incubation, the light was switched off. The activity was expressed as EU mg^{-1} protein. For the catalase assay (CAT, EC1.11.1.6) activity method of Aebi (1984) was followed and change in absorbance was monitored at 240 nm for 2 min. For the

calculation, an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ was used. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was tested by monitoring absorption change at 290 nm for 3 min in 1 mL reaction mixture containing potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.5 mM hydrogen peroxide, and 0.1 mL enzyme extract. The calculation of the extinction coefficient of 2.8 mM^{-1} cm^{-1} was used (Nakano and Asada 1981). The activity of glutathione reductase (GR; EC 1.6.4.2) was tested according to Foyer and Halliwell (1976) in an assay mixture containing sodium phosphate buffer (pH 7.8), 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione (GSSG) and 0.1 mL enzyme extract. Change in optical density was observed at 340 nm for 2 min and an extinction coefficient of 6.2 mM^{-1} cm^{-1} was used for calculation (Foyer and Halliwell 1976).

Estimation of ascorbate and reduced glutathione

Ascorbate (AsA) content was estimated using the method described by Mukherjee and Choudhuri (1983). Fresh plant material was homogenized in 6% TCA using pestle and mortar. After centrifugation, the extract at 5000g for 10 min, 2% dinitrophenylhydrazine, and 10% thiourea were added to the supernatant and the mixture was boiled for 15 min in a water bath. After cooling the samples, 5 mL of cooled 80% H_2SO_4 was added, and the absorbance was taken at 530 nm. The standard curve of ascorbate was used for calculations.

Reduced glutathione (GSH) content was estimated by Ellman (1959). Fresh tissue was homogenized in the phosphate buffer (pH 8.0) and the extract was centrifuged at 3000g for 15 min. Fresh tissue was homogenized in the phosphate buffer (pH 8.0) and the extract was centrifuged for 15 min at 3000 g.

Determination of phenols

For extraction of total phenols 500 mg dry powdered sample was homogenized in methanol, and the extract was subjected to centrifugation at 10000g for 10 min. 0.5 mL of supernatant was made to 2 mL using distilled water and reacted with 1 mL Folin–Ciocalteu reagent. Afterward, sodium carbonate was added and optical density was taken at 760 nm (Singleton and Rossi 1965; Hasan et al. 2018). The standard curve of gallic acid was used for calculation.

Estimation of copper

The dry plant sample was digested in acid (HNO_3: $HClO_4$, 4:1) until a colourless solution was obtained. After cooling, the solution was filtered and the concentration of Cu was determined using atomic absorption spectrophotometry (Perkin-Elmer3100).

Statistical analysis

Data are mean (±SE) of four replicates and least significant difference (LSD) at p <0.05 was calculated using Duncan's Multiple Range Test.

Results

Growth parameters

Table 1 reveals the effect of ASA and NO treatment on several growth parameters including plant length and shoot dry weight alone and in combination under normal and Cu stress conditions. Relative to control, plant height and dry weight decreased significantly due to excess Cu by 39.46% and 35.97% respectively. Application of ASA and NO either individually or in combination improved growth parameters, such positive influence was also evident under Cu toxicity. Application of ASA and NO significantly improved the reduction with a maximum improvement of 81.36 and 66.49% in Cu + ASA + NO treated seedlings (Table 1).

Lipid peroxidation, electrolyte leakage content, lipoxygenase activity, Cu content, and ROS production

Membrane stability index (MS) and ROS production like hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) in mungbean seedlings treated with ASA and NO under Cu toxicity are presented as shown in Table 1. Results show a significant decrease in hydrogen peroxide and superoxide generation under both normal and Cu stress conditions. Higher H_2O_2 and O_2^- accumulation resulted in increased lipid peroxidation (MDA) and reduced MSI. Relative to control, a significant increase in accumulation of H_2O_2 (146.34%) and O_2^- (77.79%) was observed due to Cu toxicity resulting in a 99.28% increase in MDA production and a 34.30% decrease in MSI. Treatment of ASA and NO mitigated the oxidative damage significantly by maximal reduction of 43.48% (H_2O_2), 43.94% (O_2^-), 36.72% (MDA) and 20.71% (MSI) in Cu + ASA + NO treated plants over the Cu stressed ones (Table 1).

Higher concentrations of Cu dramatically improved the activity of LOX while ASA and NO treatments individually or in combination reduced its activity. Under normal conditions, a decline of 52.37% in LOX was observed due to ASA + NO treatment, while as an increase of 104.92% was observed due to Cu treatment. The application of ASA + NO to Cu-treated seedlings resulted in a decline of 49.92% in LOX over the Cu treated plants (Table 1).

The content of Cu increased in the shoot (459.4%) and root (498.62%) over the control accompanied by a decline in Cu + ASA + NO treated seedlings exhibiting 42.49% and 34.79% decline in shoot and root over Cu stressed plants (Table 2).

Enzymatic and non-enzymatic antioxidant

The performance of antioxidant enzymes such as SOD, CAT, APX, and GR activity in mungbean leaves was evaluated under Cu stress and with or without ASA and NO is shown in Table 3. Significant enhancement in the activities of SOD (18.70%), CAT (15.46%), APX (25.55%), and GR (9.52%) was observed due to Cu stress. Application of ASA and NO increased the activities under normal conditions and caused further enhancement under Cu stress. Relative to control, a maximal increase of the activities of SOD (57.55%), CAT (93.90%), APX (72.22%), and GR (61.95%) was observed in seedlings treated with Cu + ASA + NO over the control (Table 3).

Table 1. Effect of excess copper (100 µM Cu) with and without exogenous application of acetylsalicylic acid and nitric oxide on shoot length (cm), shoot dry weight (gm/ plant), hydrogen peroxide (µmol /g FW), superoxide (µmol /g FW), lipid peroxidation (µmol /g FW), membrane stability index (percent) and lipoxygenase activity (µmol min^{-1} mg^{-1} protein) in mungbean (*Vigna radiata*). Values are mean (±SE) of four replicates and different letters represent significant difference at $P<0.05$.

Treatments	Shot length	Shoot dry weight	Hydrogen peroxide	Superoxide	Lipid peroxidation	MSI	Lipoxygenase activity
Control	28.63 ± 2.41^{d}	3.03 ± 0.41^{d}	10.12 ± 1.07^{d}	8.96 ± 0.94^{c}	30.66 ± 2.5^{d}	83.40 ± 4.1^{c}	16.86 ± 1.6^{d}
Cu	17.33 ± 1.69^{f}	1.94 ± 0.14^{f}	24.93 ± 2.83^{a}	15.93 ± 0.80^{a}	61.10 ± 3.1^{a}	56.10 ± 4.3^{f}	34.55 ± 2.8^{a}
ASA	33.86 ± 1.65^{b}	3.90 ± 0.26^{b}	7.80 ± 0.20^{e}	6.23 ± 0.65^{d}	23.66 ± 2.5^{e}	88.67 ± 3.3^{b}	11.83 ± 0.83^{e}
NO	33.13 ± 2.34^{b}	3.80 ± 0.20^{b}	8.22 ± 0.93^{e}	6.43 ± 0.58^{d}	24.33 ± 1.5^{e}	87.70 ± 4.7^{b}	12.10 ± 0.87^{e}
ASA + NO	37.26 ± 1.68^{a}	4.23 ± 0.45^{a}	6.16 ± 0.60^{f}	5.56 ± 0.45^{e}	19.33 ± 2.5^{f}	91.94 ± 1.0^{a}	8.03 ± 0.80^{f}
Cu + ASA	24.26 ± 1.34^{e}	2.86 ± 0.25^{e}	17.99 ± 1.26^{b}	11.76 ± 0.81^{b}	51.66 ± 3.0^{b}	64.78 ± 4.7^{e}	24.63 ± 1.9^{b}
Cu + NO	23.46 ± 1.43^{e}	2.73 ± 0.30^{e}	18.66 ± 1.56^{b}	12.40 ± 0.60^{b}	53.66 ± 4.9^{b}	62.00 ± 3.0^{e}	26.10 ± 1.8^{b}
Cu + ASA + NO	31.43 ± 1.85^{c}	3.23 ± 0.23^{c}	13.09 ± 0.88^{c}	8.93 ± 0.96^{c}	38.66 ± 3.7^{c}	76.76 ± 3.17^{d}	17.30 ± 1.3^{c}

The effect of Cu stress and the applied ASA and NO on the level of ascorbic acid and GSH is shown in Table 3. Comparable to control, Cu treatment resulted in decreased (16.11%) ascorbic acid and increased (6.67%) GSH content. Content of ascorbic acid and GSH were increased by 40.77% and 22.66% in ASA + NO treated seedlings over control under normal conditions. Relative to Cu stressed seedlings, the combined application of ASA and NO ameliorated the decline in ascorbic acid significantly by 25.81% and further enhanced the GSH content by 28.01% (Table 3). ASA and NO applications significantly improved ascorbic acid by 25.81% and further increase GSH content by 28.01% (Table 3).

Photosynthetic pigments

Mungbean plants exposed to Cu stress exhibited declined synthesis of total chlorophyll and carotenoids, and net photosynthesis (*Pn*) and maximum photochemical efficiency (F_V / F_m) dropped. The application of ASA and NO, however, significantly mitigated the decline. Relative to control, a decline in total chlorophyll, carotenoids, *Pn*, and *Fv/Fm* was 36.38%, 30.37%, 34.49%, and 28.24% respectively over the control. Application of ASA and NO resulted in a significant enhancement in the above parameters and amelioration of the decline. Maximal amelioration of 88.81% for total chlorophyll, 58.81% for carotenoids, 58.77% for *Pn*, and 58.11% for *Fv/Fm* was observed in Cu + ASA + NO treated plants over the Cu stressed ones (Figure 1).

Osmolytes, glutamyl kinase, proline oxidase, and RWC

Results showing the influence of Cu and application of ASA and NO on the accumulation of osmolytes including, proline, glycine betaine, and total soluble sugars, and proline metabolizing enzymes are shown in Figure 2. Relative to control, application of ASA and NO individually as well as combinedly increased the accumulation of osmolytes under normal growth conditions. Relative to control, proline and glycine betaine increased by 85.40% and 69.32% while the total soluble sugars were reduced by 36.15% due to Cu stress. Application of ASA and NO to Cu-stressed seedlings further increased their accumulation attaining maximal enhancement of 136.76% for proline and 118.58% for glycine betaine in Cu + ASA + NO treated seedlings over control counterparts (Figure 2(A,B)).

The activity of γ-GK and PROX increased and decreased respectively due to ASA and NO either individually or combinedly. Cu stressed plants exhibited an increase of 21.36% in γ-GK and a decrease of 16.87% in PROX activity over control. A maximal increase of 47.49% in γ-GK and a decline of 34.47% in PROX activity was observed in Cu + ASA + NO treated seedlings (Figure 2(D,E)).

Relative to control Cu stressed seedlings exhibited significant a decline in RWC (Figure 2(F)). A marked increase in RWC was recorded in plants treated with ASA + NO combinations. RWC maximally declined by 30.36% due to excess Cu treatment and amelioration of 20.52% in Cu + ASA, 20.40% in Cu + NO, and 33.59% in Cu + ASA + NO over the Cu treated seedlings were observed (Figure 2(F)).

Table 2. Effect of exogenous application of acetylsalicylic acid and nitric oxide on shoot and root Cu (mg/g DW) in mungbean (*Vigna radiata*). Values are mean (±SE) of four replicates and different letters represent significant difference at $P<0.05$.

Treatments	Shoot Cu	Root Cu
Control	0.631 ± 0.003^{e}	0.725 ± 0.006^{e}
Cu	3.53 ± 0.161^{a}	4.34 ± 0.14^{a}
ASA	0.667 ± 0.015^{d}	0.790 ± 0.024^{d}
NO	0.674 ± 0.014^{d}	0.778 ± 0.012^{d}
ASA + NO	0.611 ± 0.016^{e}	0.793 ± 0.015^{d}
Cu + ASA	2.61 ± 0.314^{b}	3.18 ± 0.151^{b}
Cu + NO	2.73 ± 0.203^{b}	3.03 ± 0.145^{b}
Cu + ASA + NO	2.03 ± 0.325^{c}	2.83 ± 0.183^{c}

Phenol content

Excess Cu treatment resulted in a significant decline (42.69%) in total phenol content over the control seedlings, however, the application of ASA and NO enhanced the phenol content attaining a maximal increase of 43.25% due to ASA + NO application. Relative to Cu stressed plants decline in phenol content was ameliorated by 73.52% in Cu + ASA + NO treated seedlings (Figure 3).

Glyoxalase I and glyoxalase II

The activity of Gly I (51.85%) and Gly II (52.18%) increased significantly due to Cu stress. under normal conditions and application of ASA and NO resulted in an enhancement in the activities of Gly I and Gly II, and this was maintained when applied to Cu stressed seedlings. Maximal increase of 129.62% and 122.64% in the activities of Gly I and Gly II was observed in Cu + ASA + NO treated seedlings (Figure 3 (A,B)). Content of MG decreased maximally by 44.64% in ASA + NO treated seedlings over the control. However, increased maximally by 97.32% due to Cu stress and was ameliorated maximally by 50.67% in Cu + ASA + NO treated seedlings (Figure 3(C)).

Table 3. Effect of excess copper (100 µM Cu) with and without exogenous application of acetylsalicylic acid and nitric oxide on the activity of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase and the content of ascorbate (nmol g^{-1} FW) and reduced glutathione (nmol g^{-1} FW) in mungbean (*Vigna radiata*). Values are mean (±SE) of four replicates and different letters represent significant difference at $P<0.05$.

Treatments	SOD	CAT	APX	GR	Ascorbic acid	GSH
Control	5.56 ± 0.602^{f}	29.36 ± 3.49^{f}	0.90 ± 0.10^{f}	0.4261 ± 0.047^{f}	252.6 ± 11.7^{e}	274.0 ± 8.5^{f}
Cu	6.60 ± 0.458^{e}	33.90 ± 3.97^{e}	1.13 ± 0.11^{e}	0.4667 ± 0.040^{e}	211.9 ± 6.9^{f}	292.3 ± 7.7^{e}
ASA	7.13 ± 0.321^{d}	39.33 ± 4.10^{d}	1.31 ± 0.08^{d}	0.5333 ± 0.035^{d}	300.1 ± 11.2^{b}	306.6 ± 11.5^{d}
NO	7.06 ± 0.251^{d}	39.76 ± 4.36^{d}	1.35 ± 0.172^{d}	0.5463 ± 0.022^{d}	292.6 ± 6.8^{b}	311.1 ± 7.9^{d}
ASA + NO	8.00 ± 0.360^{b}	50.43 ± 3.63^{b}	1.41 ± 0.204^{b}	0.6510 ± 0.020^{b}	355.6 ± 11.5^{a}	336.1 ± 11.2^{c}
Cu + ASA	7.96 ± 0.305^{c}	46.03 ± 3.27^{c}	1.26 ± 0.100^{c}	0.6230 ± 0.009^{c}	234.6 ± 9.4^{d}	350.6 ± 11.1^{b}
Cu + NO	7.66 ± 0.208^{c}	44.13 ± 2.74^{c}	1.23 ± 0.138^{c}	0.6200 ± 0.036^{c}	229.3 ± 5.6^{d}	342.4 ± 10.1^{b}
Cu + ASA + NO	8.76 ± 0.416^{a}	56.93 ± 3.70^{a}	1.55 ± 0.045^{a}	0.6901 ± 0.020^{a}	266.6 ± 11.0^{c}	374.2 ± 11.3^{a}

Discussion

With excessive anthropogenic disturbances, the accumulation of toxic concentrations of metal ions is becoming one of the serious threats to the sustainable development and productivity of maximum crop plants. Accumulation of Cu is one of the consequences of human activities, which has alarmed plant scientists to build up management strategies to improve crop yields under such circumstances. In this study, we have tried to understand both the efficacy of the individual and the combined use of ASA and NO to improve the harmful effects of excess Cu on mung bean through physiological and biochemical alterations. Cu phytotoxicity significantly reduced the growth and dry biomass production of mungbean. Reduced growth due to excess Cu has been reported earlier by Lamb et al. (2012) and Saleem et al. (2020a). Elleuch et al. (2013) have demonstrated a significant decline in shoot and radical length in fenugreek seedlings subjected to Cu stress. Excess Cu interrupts the cell cycle progression by inhibiting the transition point between G1 – S, thereby affecting the proliferation and morphology of meristematic cells (Doncheva 1998; Qin et al. 2015). In our present research, exogenous ASA and NO may have protected cell cycle functioning and cell proliferation and tissue growth either (a) by reducing the intake and accumulation of excess Cu or (b) by maintaining the content of tissue water. Both of these attributes are positively influenced by the exogenous application of ASA and NO. Earlier regulatory roles of NO (Novikova et al. 2017) and SA (Meguro and

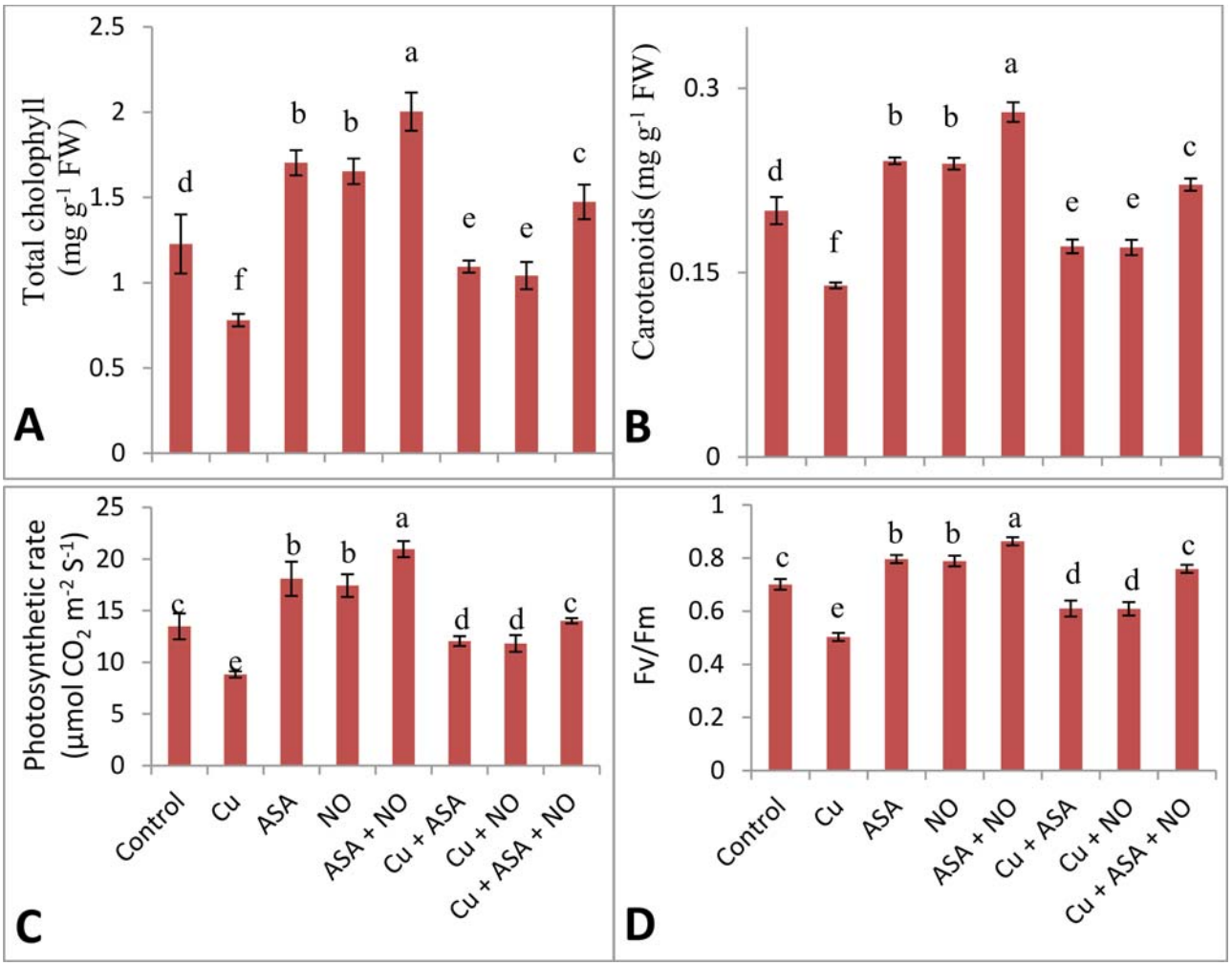

Figure 1. Effect of excess copper (100 µM Cu) with and without exogenous application of acetylsalicylic acid and nitric oxide on (A) total chlorophyll and (B) carotenoids (mg/ g FW), and (C) photosynthetic rate (µmol CO_2 m^{-2} S^{-1}), and (D) maximal photochemical efficiency (Fv/Fm) in mung bean (*Vigna radiata*). Values are mean (±SE) of four replicates and different letters on bars denote significant difference at $P<0.05$.

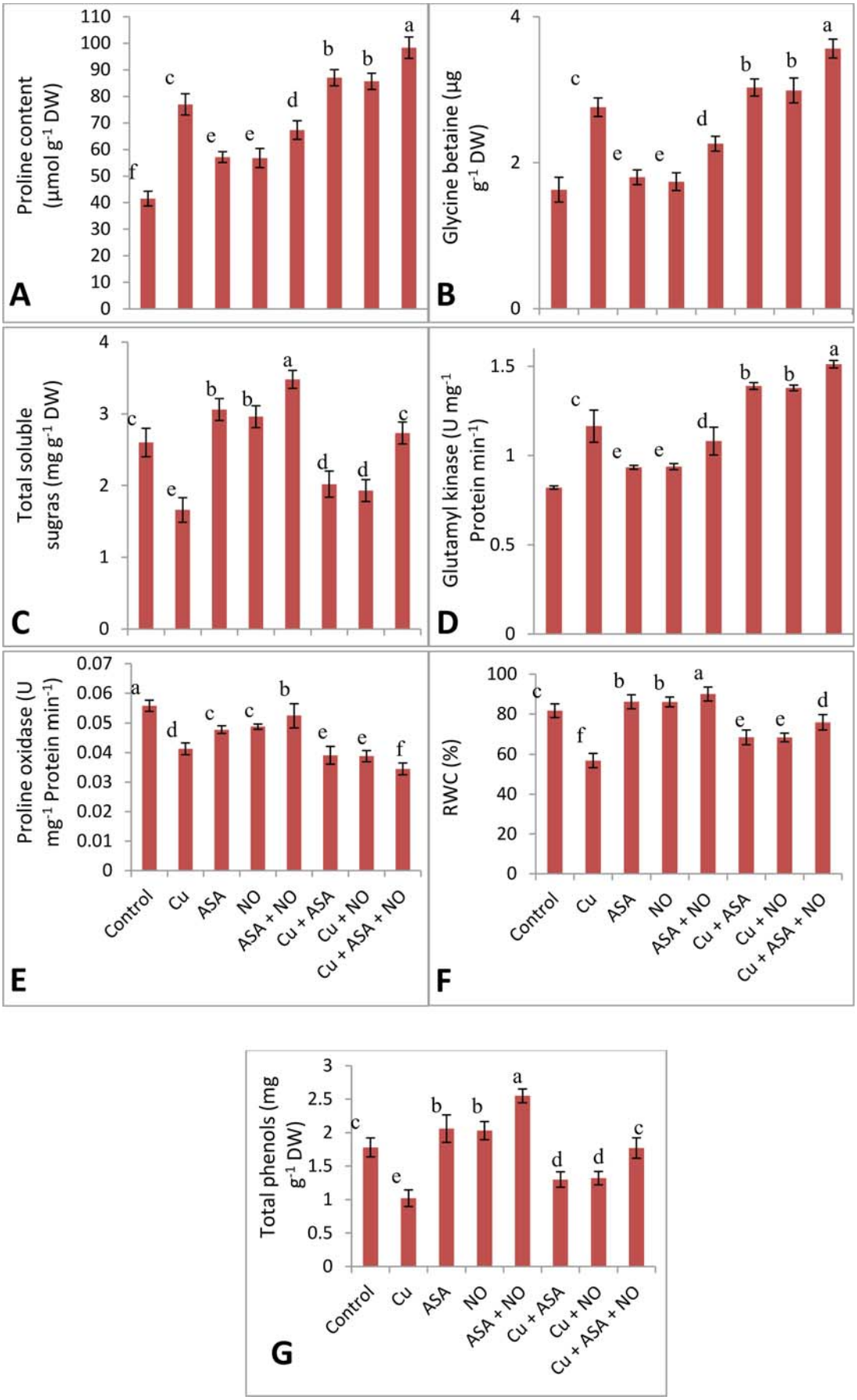

Figure 2. Effect of excess copper (100 µM Cu) with and without exogenous application of acetylsalicylic acid and nitric oxide on (A) proline content (µmol g^{-1} DW), (B) glycine betaine (µg / g DW), (C) sugars (mg / g DW), (D) glutamyl kinase (U mg^{-1} protein min^{-1}), (E) proline oxidase (U mg^{-1} protein min^{-1}), (F) Relative water content, RWC (%), and (G) total phenols (mg /g DW) content in mungbean (*Vigna radiata*). Values are mean (±SE) of four replicates and different letters on bars denote significant difference at $P<0.05$.

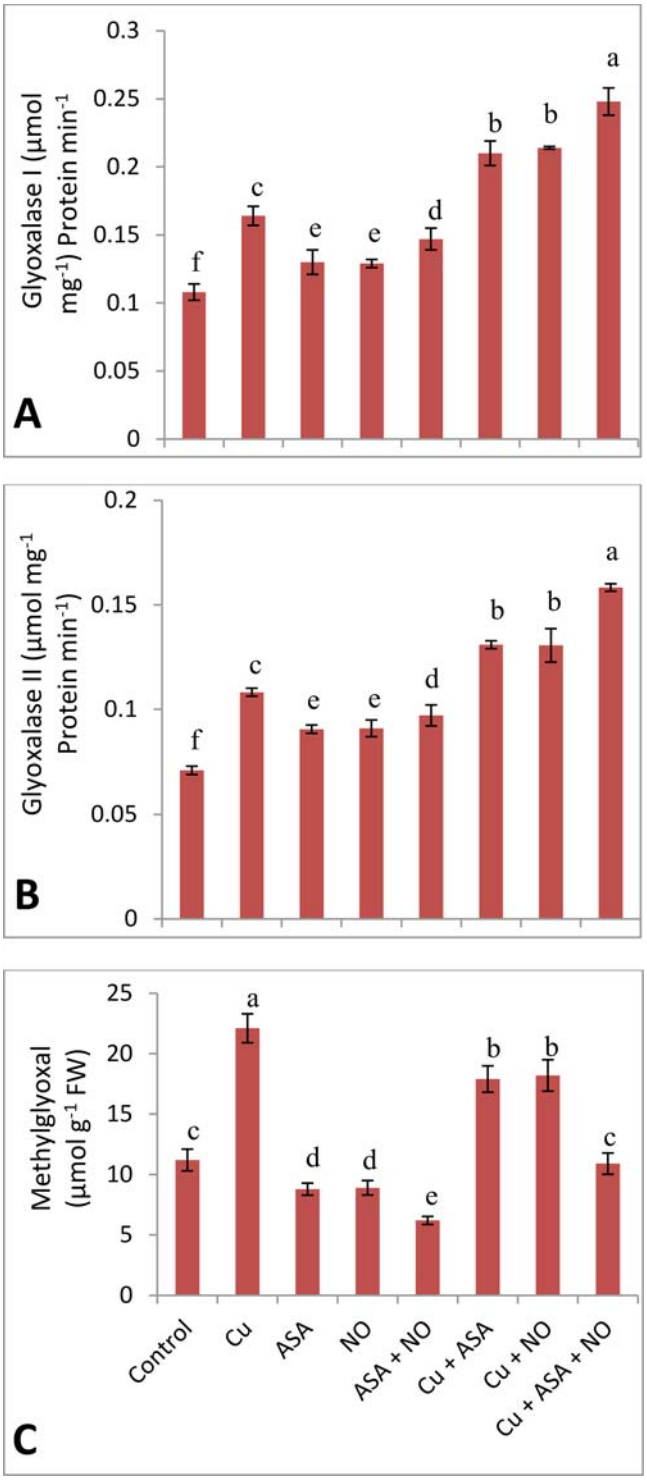

Figure 3. Effect of excess copper (100 µM Cu) with and without exogenous application of acetylsalicylic acid and nitric oxide on activity of (A) glyoxalase I, (B) glyoxalase II (µmol/mg protein /min), and (C) methylglyoxal (µmol /g FW) content in mungbean (*Vigna radiata*). Values are mean (±SE) of four replicates and different letters on bars denote significant difference at $P<0.05$.

Sato 2014) have been investigated and it has been shown that they can antagonize the inhibiting effects of different molecules on growth. In addition to the possible regulatory effect on cell division, exogenous ASA and NO significantly restricted the entry of excess Cu into tissue resulting in a significant increase in tolerance. Excess Cu significantly reduces the diameter of the stem, the thickness of the epidermis, the sclerenchyma, and the vascular bundles (Agami 2016). The decreased growth of several crops has been reported in excess of Cu aggregation (Saleem et al. 2020b; Elleuch et al. 2013; Lwalaba et al. 2020). Alleviation of toxic effects of Cu due to the individual application of NO (Mostofa et al. 2015) and SA (Mostofa and Fujita 2013) has been reported, although the effects of the combined application of ASA and NO have not yet been worked out. Further studies on the identification of the essential proteins involved in membrane-level Cu uptake as influenced by ASA and NO may be worthwhile.

Reduction in growth due to Cu toxicity was observed to be correlated with reduced pigment (chlorophyll and carotenoid) synthesis, reflecting a significant decrease in photosynthesis and PSII functioning. Previously reduced pigment synthesis due to Cu stress has been reported in fenugreek (Elleuch et al. 2013), rice (Mostofa and Fujita 2013). Like our findings, pigment formulation and photosynthetic activity due to Cu toxicity have been demonstrated by Saleem et al. (2020a) in *Linum usitatissimum* L. Both NO and ASA have been reported to have the potential to enhance chlorophyll production and photosynthetic efficiency levels under stressful conditions (Fatma et al. 2016; Ahmad et al. 2018a, 2018b). Alleviation of the decrease in chlorophylls, carotenoids, and photosynthesis due to the combined use of ASA and NO under iron deficiency (Kong et al. 2014), salinity (Ahanger et al. 2020), copper in *Corchorus capsularis* L and cadmium (Mostofa et al. 2019) stress has been reported, but reports on their interactive effect under Cu stress are not available. Under extreme situations, chlorophyll degradation is significantly increased (Dalal and Tripathy 2012) and the greater generation of toxic ROS impart damaging effects to the structural integrity of key photosynthetic sites within chloroplasts. Leaf photocatalytic functioning in ASA and NO treated seedlings under normal or Cu-stressed conditions could be ascribed to their considerable influence on stomatal and non-stomatal attributes. The oxidative effects of excess Cu were reflected as increased ROS build-up, lipid peroxidation, and LOX activity leading to loss of membrane integrity. However, the application of ASA and NO significantly enhanced the functioning of the membrane by lowering the regulation of LOX concomitantly with reduced ROS generation. Increased lipid peroxidation and LOX activity due to Cu toxicity concomitantly with higher accumulation of toxic ROS, including H_2O_2 and O_2, has been reported in Mostofa and Fujita (2013) rice. Increased LOX and lipid peroxidation activity lead to significant membrane damage through altered lipid content (Soliman et al. 2018;Nahar et al. 2016; El-Esawi et al. 2020). Exogenous application of SA (Mostofa and Fujita 2013) and NO (Nahar et al. 2016) has been reported to reduce Cu and Cd stress caused lipid damage by LOX and lipid peroxidation owing to enhanced MSI. Lowered levels of ROS in seedlings treated with ASA and NO may be attributed to greater antioxidant functioning, osmolyte accumulation, and restricted Cu uptake in them. Earlier exogenous applications of NO (Fatma et al. 2016) and SA (Nazar et al. 2011) have been demonstrated to ameliorate the photosynthetic decline by reducing the accumulation of ROS. Reduced ROS accumulation following combined application of ASA and NO resulted in enhanced membrane stability and photosynthetic functioning in mungbean under Cu toxicity.

One of the key reasons for reduced oxidative damage to ASA and NO supplemented seedlings can be attributed to increased antioxidant functioning. Both enzymatic and non-enzymatic antioxidant components were significantly enhanced due to the exogenous application of ASA (Soliman

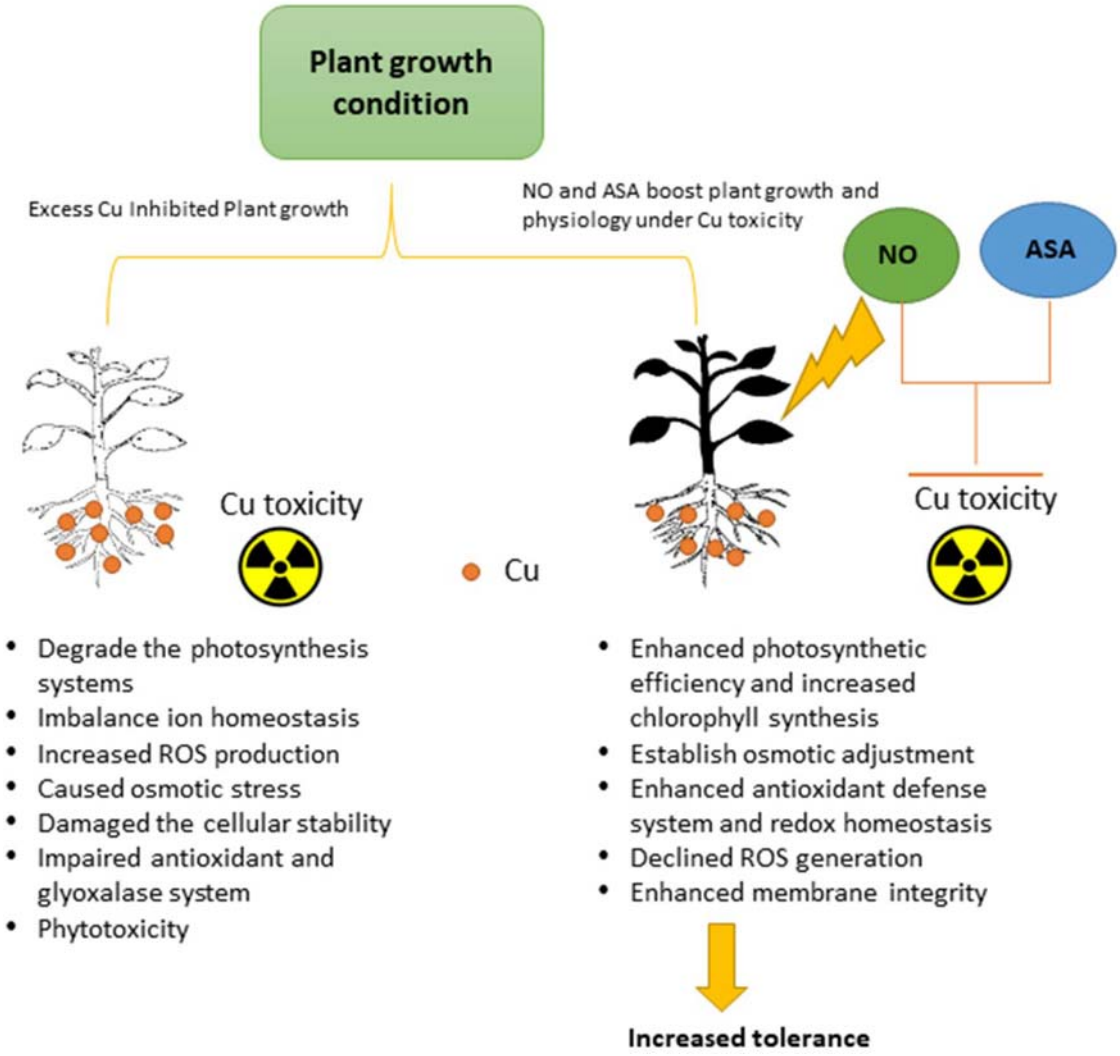

Figure 4. A proposed model is showing how SA and NO mitigates Cu toxicity in mung bean seedlings.

et al. 2018) and NO (Soliman et al. 2019). The antioxidant system promotes the neutralization of ROS to improve the protection of cellular functioning (Ahanger et al. 2017; Lwalaba et al. 2020; Jahan et al. 2021). SOD eliminates superoxide radicals from chloroplasts and mitochondria, thereby preventing damage to the electron transport system and structural components (Elkelish et al. 2019b; Soliman et al. 2019). Cu toxicity resulted in an up-regulation of the SOD isozymes in barley (Lwalaba et al. 2020). In confirmation with the results of this study increased antioxidant function due to NO (Ahmad et al. 2018b) and SA (Nazar et al. 2011) has been reported earlier. Excess H_2O_2 is nullified either by CAT or the AsA-GSH cycle. The upregulation of the cellular antioxidant under Cu stress due to exogenous SA has been demonstrated to prevent oxidative injury (Mostofa and Fujita 2013). In *Linum usitatissimum*, Saleem et al. (2020a) also showed higher SOD and POD activity due to Cu treatment.

Increased functioning of the AsA-GSH cycle due to SA and NO application may have contributed to enhanced growth protection in mung bean against Cu toxicity in two main ways: (a) sustaining redox homeostasis by facilitating more generation of GSH and AsA; and (b) maintaining the pace of electron transfer by securing improved NADP / NADPH leading to lower generation of O_2^- (Saleem et al. 2020a, 2020c). Clearly, such positive effects of SA and NO may have contributed to the rapid elimination of ROS, thereby preventing structural and functional deformities in major cellular organelles such as chloroplast (Fatma et al. 2016; Elkelish et al. 2020; Soliman et al. 2020). AsA and GSH protect the functioning of the enzymes, contribute to the cellular homeostasis and the scavenging of ROS (Noctor and Foyer 2016). In addition, they play a key role in stress signalling (Foyer and Noctor 2003).

Increased accumulation of compatible osmolytes further strengthened the alleviation of oxidative impacts of Cu toxicity due to ASA and NO. Compatible osmolytes contribute to the regulation of plant growth by maintaining tissue water content and protecting the proper function of the enzyme (Elkelish et al. 2019a), providing protection to major macromolecules and helping in stress signalling (Hasan et al. 2019b; Hasan et al. 2020b). Phytohormones play a crucial role in regulating the metabolism of osmolytes by integrating beneficial responses to the stress factor (Sharma et al. 2019; Hasan et al. 2021a; Hasan et al. 2021b) and it was evident in this study that exogenous application not only increased the content of osmolytes but also finely regulated their metabolism. For example, proline synthesizing enzyme (γ-GK) up-regulated while catabolizing (PROX) one was down-regulated to result in greater accumulation of proline. Similar to our results, higher accumulation of proline through the differential regulation of proline metabolizing

enzymes has been reported in cadmium (Khan et al. 2015b) and salinity (Elkelish et al. 2019a; Soliman et al. 2020).

Exogenous application of ASA and NO reduced the toxic accumulation of MG by up-regulating the activity of glyoxalase enzymes (Figure 4). Earlier upregulation of the glyoxalase pathway enzymes (Gly I and Gly II) has been reported in salinity-exposed plants (Hasanuzzaman et al. 2011), Cu (Mostofa and Fujita 2013), cadmium (Nahar et al. 2016), and nickel (Soliman et al. 2020). Reports on the interactive effect of ASA and NO on the accumulation and functioning of the glyoxalase system are not available. Up-regulation of glyoxalase due to SA (Mostofa and Fujita 2013) and NO (Soliman et al. 2020) results in reduced accumulation of MG preventing growth inhibition under stressed conditions. MG is a toxic product of lipid, amino acid, and glycolytic pathways that are detoxified by either aldo-keto reductase or glyoxalase . GSH plays a key role in improving the functioning of the glyoxalase pathway. Overall, ASA and NO could be improved plant growth and physiology and under copper toxicity (Cu) (Figure 4). Further studies are required to identify the actual mechanisms involved in the regulation of glyoxalase under Cu toxicity due to combined SA and NO application.

Conclusion

It can be concluded that excess Cu availability reduces the growth and photosynthetic functioning of mung bean by inducing oxidative damage. However, SA and NO application regulated the tolerance mechanisms leading to alleviation of the oxidative damage. Reduced lipid peroxidation and LOX activity in SA and NO treated seedlings reflect enhanced membrane stability, which was evident as a reduced accumulation of ROS. Upregulation of the antioxidant system, the glyoxalase pathway, and the accumulation of osmolytes depicts the beneficial role of SA and NO crosstalk in growth and photosynthetic protection under Cu toxicity.

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References

Aebi H. 1984. Catalase in vitro. Methods Enzymol. 105:121–126.

Agami RA. 2016. Pre-soaking in indole-3-acetic acid or spermidine enhances copper tolerance in wheat seedlings. South Afri J Bot. 104:167–174.

Ahanger MA, Aziz U, Alsahli AA, Alyemeni MN, Ahmad P. 2020. Influence of exogenous salicylic acid and nitric oxide on growth, photosynthesis, and ascorbate-glutathione cycle in salt stressed *Vigna angularis*. Biomolecules. 10:42, 1–16. doi:10.3390/biom10010042.

Ahanger MA, Qin C, Begum N, Maodong Q, Dong XX, El-Esawi M, El-Sheikh MA, Alatar AA, Zhang L. 2019. Nitrogen availability prevents oxidative effects of salinity on wheat growth and photosynthesis by up-regulating the antioxidants and osmolytes metabolism, and secondary metabolite accumulation. BMC Plant Biol. 19:479, 1–12. doi:10.1186/s12870-019-2085-3.

Ahanger MA, Tomar NS, Tittal M, Argal S, Agarwal RM. 2017. Plant growth under water/ salt stress: ROS production; antioxidants and significance of added potassium under such conditions. Physiol. Mol. Bio. Plants. 23:31–744.

Ahmad P, Ahanger MA, Alyemeni MN, Wijaya L, Alam P, Ashraf M. 2018b. Mitigation of sodium chloride toxicity in *solanum lycopersicum* L. by supplementation of jasmonic acid and nitric oxide. J Plant Inter. 13:64–72.

Ahmad P, Ahanger MA, Alyemeni MN, Wijaya L, Alam P. 2018a. Exogenous application of nitric oxide modulates osmolyte metabolism, antioxidants, enzymes of ascorbate-glutathione cycle and promotes growth under cadmium stress in tomato. Protoplasma. 255:79–93.

Alharbi BM, Elhakem AH, Alnusairi GSH, Soliman MH, Hakeem KR, Hasan MM, Abdelhamid MT. 2021. Exogenous application of melatonin alleviates salt stress in soybean seedlings by improving their growth, mineral uptake, antioxidant and photosynthetic capacities. Plant Soil Environ. 67:208–220. doi:10.17221/659/2020-PSE.

Arnon DI. 1949. Copper enzymes in isolated chloroplast polyphenol oxidase in beta vulgaris. Plant Physiol. 24:1–15.

Arnon DI, Stout PR. 1939. The essentiality of certain elements in minute quantity for plants with special reference to copper. Plant Physiol. 14:371–375.

Ballabio C, Panagos P, Lugato E, Huang JH, Orgiazzi A, Jones A, Fernández-Ugalde O, Borrelli P, Montanarella L. 2018. Copper distribution in european top soils: an assessment based on LUCAS soil survey. Sci. Total Environ. 636:282–298.

Bates LS, Waldre RP, Teare ID. 1973. Rapid determination of free proline for water stress studies. Plant Sci. 39:205–207.

Bayer WF, Fridovich JL. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem. 161:559–566.

Dalal VK, Tripathy BC. 2012. Modulation of chlorophyll biosynthesis by water stress in rice seedlings during chloroplast biogenesis. Plant Cell Env. 35:1685–1703.

Doderer A, Kokkelink I, van der Veen S, Valk B, Schram A, Douma A. 1992. Purification and characterization of two lipoxygenase isoenzymes from germinating barley. Biochim Biophys Acta. 112:97–104.

Doncheva S. 1998. Copper-induced alterations in structure and proliferation of maize root meristem cells. J Plant Physiol. 153:482–487.

El-Esawi MA, Elkelish A, Soliman M, Elansary HO, Zaid A, Wani SH. 2020. *Serratia marcescens* BM1 enhances cadmium stress tolerance and phytoremediation potential of soybean through modulation of osmolytes, leaf gas exchange, antioxidant machinery, and stress-responsive genes expression. Antioxidants. 9:43, 1–17. doi:10.3390/antiox9010043.

Elkelish AA, Alhaithloul HAS, Qaric SH, Soliman MH, Hasanuzzaman M. 2020. Pre-treatment with *trichoderma harzianum* alleviates waterlogging-induced growth alterations in tomato seedlings by modulating physiological, biochemical, and molecular mechanisms. Environ Exp Bot. 171:103946, 1–11.

Elkelish AA, Alnusaire TS, Soliman MH, Gowayed S, Senousy HH, Fahad S. 2019b. Calcium availability regulates antioxidant system, physio-biochemical activities and alleviates salinity stress mediated oxidative damage in soybean seedlings. J Applied Bot Food Quality. 92:258–266.

Elkelish AA, Soliman MH, Alhaithloul HA, El-Esawi MA. 2019a. Selenium protects wheat seedlings against salt stress-mediated oxidative damage by up-regulating antioxidants and osmolytes metabolism. Plant Physiol Biochem. 137:144–153.

Elleuch A, Chaabene Z, Grubb DC, Drira N, Mejdoub H, Khemakhem H. 2013. Morphological and biochemical behavior of fenureek (*trigonella foenum-graecum*) under copper stress. Ecotoxicol Environ Safety. 98:46–53.

Ellman GL. 1959. Tissue sulfhydryl groups. Arch Biochem Biophys. 82:70–77.

Fatma M, Masood A, Per TS, Khan NA. 2016. Nitric oxide alleviates salt stress inhibited photosynthetic performance by interacting with sulfur assimilation in mustard. Front Plant Sci. 7:521, 1–16. doi:10.3389/fpls.2016.00521.

Foyer CH, Halliwell B. 1976. The presence of glutathione and glutathione reductase in chloroplast: a proposed role in ascorbic acid metabolism. Planta. 133:21–25.

Foyer CH, Noctor G. 2003. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant. 119:355–364.

Grieve CM, Grattan SR. 1983. Rapid assay for determination of water-soluble quaternary ammonium compounds. Plant Soil. 70:303–307.

Hasan MM, Alharby HF, Hajar AS, Hakeem KR, Alzahrani T. 2018. Effects of magnetized water on phenolic compounds, lipid peroxidation and antioxidant activity of *Moringa* species under drought stress. J Animal Plant Sci. 28:803–810.

Hasan MM, Alharby HF, Hajar AS, Hakeem KR, Alzahrani Y. 2019b. The effect of magnetized water on the growth and physiological conditions of *Moringa* species under drought stress. Pol J Environ Stud. 539(28):1145–1155.

Hasan MM, Alharby HF, Uddin MN, Ali MA, Anwar Y, Fang XW, Hakeem KR, Alzahrani Y, Hajar AS. 2020a. Magnetized water confers drought stress tolerance in *Moringa* biotype via modulation of growth, gas exchange, lipid peroxidation and antioxidant activity. Pol J Environ Stud. 29:1–12.

Hasan MM, Ali MA, Soliman MH, Alqarawi AA, Abd_Allah EF, Fang XW. 2020b. Insights into 28-homobrassinolide (HBR)- mediated redox homeostasis, AsA–GSH cycle, and methylglyoxal detoxification in soybean under drought-induced oxidative stress. J Plant Inter. 15:371–385.

Hasan MM, Gong L, Nie Z, Li F, Ahammed GJ, Fang XW. 2021b. ABA-induced stomatal movements in vascular plants during dehydration versus rehydration. Environ Exp Bot. 186:104436, 1–8. doi:10.1016/j.envexpbot.2021.104436.

Hasan MM, Skalicky M, Jahan MS, Hossain MN, Anwar Z, Nie ZF, Alabdallah NM, Brestic M, Hejnak V, Fang XW. 2021a. Spermine: Its emerging role in regulating drought stress responses in plants. Cells. 10:261, 1–15.

Hasan MM, Uddin M, Ara-Sharmeen I, Alharby FH, Alzahrani Y, Hakeem KR, Zhang L. 2019a. Assisting phytoremediation of heavy metals using chemical amendments. Plants. 8:295, 1–14.

Hasanuzzaman M, Hossain MA, Fujita M. 2011. Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. Plant Biotechnol Rep. 5:353–365.

Hayzer DJ, Leisinger TH. 1980. The gene enzyme relationships of proline biosynthesis in *escherichia coli*. J. Gen. Microbiol. 118:287–293.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 125:189–198.

Huang AHC, Cavelieri A. 1979. Proline oxidase and water stress induced proline accumulation in spinach leaves. Plant Physiol. 6:531–535.

Ihsan MZ, Shahzad N, Kanwal S, Naeem M, Khaliq A, El-Nakhlawy FS. 2013. Potassium as foliar supplementation mitigates moisture induced stresses in mung bean (*Vigna radiata* L.) as revealed by growth, photosynthesis, Gas exchange capacity and Zn analysis of shoot. Int J Agron Plant Prod. 4:3828–3835.

Jahan MS, Shu S, Wang Y, Hasan MM, El-Yazied AA, Alabdallah NM, Hajjar D, Altaf MA, Sun J, Guo S. 2021. Melatonin pretreatment confers heat tolerance and repression of heat-induced senescence in tomato through the modulation of ABA and GA-mediated pathways. Front Plant Sci. 12:650955, 1–14. doi:10.3389/fpls.2021.650955.

Khan A, Anwar Y, Hasan MM, Iqbal A, Ali M, Alharby HF, Hakeem KR, Hasanuzzaman M. 2017. Attenuation of drought stress in brassica seedlings with exogenous application of Ca^{2+} and H_2O_2. Plants. 6:20, 1–13.

Khan MIR, Fatma M, Per TS, Anjum NA, Khan NA. 2015a. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. Front Plant Sci. 6:462, 1–17. doi:10.3389/fpls.2015.00462.

Khan MIR, Nazir F, Asgher M, Per TS, Khan NA. 2015b. Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat. J Plant Physiol. 173:9–18.

Kong J, Dong Y, Xu L, Liu S, Bai X. 2014. Effects of foliar application of salicylic acid and nitric oxide in alleviating iron deficiency induced chlorosis of *arachis hypogaea L*. Bot Studies. 55:1–12. doi:10.1186/1999-3110-55-9.

Lamb DT, Naidu R, Ming H, Megharaj M. 2012. Copper phytotoxicity in native and agronomical plant species. Ecotoxic Environ Safety. 85:23–29.

Lowry OH, Rosebraugh NS, Farrand AL, Randall RJ. 1951. Protein measurement with folin phenol reagent. J Bio Chem. 193:263–275.

Lwalaba JLW, Louis LT, Zvobgo G, Richmond MEA, Fu L, Naz S, Mwamba M, Mundende RPM, Zhang G. 2020. Physiological and molecular mechanisms of cobalt and copper interaction in causing phyto-toxicity to two barley genotypes differing in Co tolerance. Ecotoxic Environ Safety. 187:109866, 1–10.

Meguro A, Sato Y. 2014. Salicylic acid antagonizes abscisic acid inhibition of shoot growth and cell cycle progression in rice. Sci Rep. 4:4555, 1–11. doi:10.1038/srep04555.

Mostofa MG, Fujita M. 2013. Salicylic acid alleviates copper toxicity in rice (Oryza sativa L.) seedlings by Up-regulating antioxidative and glyoxalase systems. Ecotoxic. 22:959–973.

Mostofa GM, Rahman MM, Ansary MMU, Fujita M, Tran LSP. 2019. Interactive effects of salicylic acid and nitric oxide in enhancing rice tolerance to cadmium stress. Int J Mol Sci. 20:5798, 1–16.

Mostofa MG, Seraj ZI, Fujita M. 2015. Interactive effects of nitric oxide and glutathione in mitigating copper toxicity of rice (*Oryza sativa* L.) seedlings. Plant Sig Beh. 10:e991570. doi:10.4161/15592324.2014.991570.

Mudalkar S, Sreeharsha RS, Reddy AR. 2017. Involvement of glyoxalases and glutathione reductase in conferring abiotic stress tolerance to jatropha curcas L. Environ Exp Bot. 134:141–150.

Mukherjee SP, Choudhuri MA. 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Physiol Plant. 58:166–170.

Nahar K, Hasanuzzaman M, Alam MM, Rahman A, Suzuki T, Fujita M. 2016. Polyamine and nitric oxide crosstalk: antagonistic effects on cadmium toxicity in mungbean plants through up-regulating the metal detoxification, antioxidant defense and methylglyoxal detoxification systems. Ecotoxic Environ Safety. 126:245–255.

Naidu R, Bolan NS. 2008. Contaminant chemistry in soils: key concepts and bioavailability. In: Naidu R, editor. Chemical bioavailability in terrestrial environment. Amsterdam: Elsevier; p. 9–38.

Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach-chloroplasts. Plant Cell Physiol. 22:867–880.

Nazar R, Iqbal N, Syeed S, Khan NA. 2011. Salicylic acid alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant metabolism differentially in two mungbean cultivars. J Plant Physiol. 168:807–815.

Noctor G, Foyer CH. 2016. Intracellular redox compartmentation and ROS-related communication in regulation and signaling. Plant Physiol. 171:1581–1592.

Novikova GV, Mur LAJ, Nosov AV, Fomenkov AA, Mironov KS, Mamaeva AS, Shilov ES, Rakitin VY, Hall MA. 2017. Nitric oxide Has a concentration-dependent effect on the cell cycle acting *via* EIN2 in *arabidopsis thaliana* cultured cells. Front Physiol. 8:142, 1–11. doi:10.3389/fphys.2017.00142.

Principato GB, Rosi G, Talesa V, Govannini E, Uolila L. 1987. Purification and characterization of two forms of glyoxalase II from rat liver and brain of wistar rats. Biochem Biophys Acta. 911:349–355.

Qin R, Wang C, Chen D, Björn LO, Li S. 2015. Copper-induced root growth inhibition of *allium cepa* Var. agrogarum L. involves disturbances in cell division and DNA damage. Environ Toxicol Chem. 34:1045–1055.

Rasheed R, Yasmeen H, Hussain I, Iqbal M, Ashraf MA, Parveen A. 2020. Exogenously applied 5-aminolevulinic acid modulates growth, secondary metabolism and oxidative defense in sunflower under water deficit stress. Physiol Mol Biol Plants. 26:489–499. doi:10.1007/s12298-019-00756-3.

Rehman M, Liu L, Wang Q, Saleem MH, Bashir S, Ullah S, Peng D. 2019. Copper environmental toxicology, recent advances, and future outlook: a review. Environ. Sci Pollut Res. 26:18003–18016.

Sairam RK, Deshmukh PS, Shukla DS. 1997. Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. J Agron Crop Sci. 178:171–178.

Saleem MH, Ali S, Irshad S, Hussaan M, Rizwan M, Rana MS, Hashem A, Abd_Allah EF, Ahmad P. 2020b. Copper uptake and accumulation, ultra-structural alteration, and bast fibre yield and Quality of fibrous jute (*Corchorus capsularis* L.) plants grown under Two Di_erent soils of China. Plants. 9:404, 1–12. doi:10.3390/plants9030404.

Saleem MH, Ali S, Rehman M, Rana MS, Rizwan M, Kamran M, Imran M, Riaz M, Soliman MH, Elkelish A, Liu L. 2020c. Influence of phosphorus on copper phytoextraction via modulating cellular organelles in two jute (Corchorus capsularis L.) varieties grown in a copper mining soil of hubei province, China. Chemosphere. 248:126032, 1–12.

Saleem MH, Kamran M, Zhou Y, Parveen A, Rehman M, Ahmar S, Malik Z, Mustafa A, Anjum RMA, Wang B, Liu L. 2020a. Appraising growth, oxidative stress and copper phytoextraction potential of flax (*Linum usitatissimum* L.) grown in soil differentially spiked with copper. J Env Manag. 257:109994, 1–11.

Sharma A, Ahahzad B, Kumar V, Kohli SK, Sidhu GPS, Bali AS, Handa N, Kapoor D, Bhardwaj R, Zheng B. 2019. Phytohormones regulate accumulation of osmolytes under abiotic stress. Biomolecules. 9:285, 1–36. doi:10.3390/biom9070285.

Shields R, Burnett W. 1960. Determination of protein-bound carbohydrate in serum by a modified anthrone method. Anal Chem. 32:885–886.

Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphor-molybdic-phosphotungstic acid reagents. Amer J Enol Viti. 16:144–153.

Smart RE, Bihgham GE. 1974. Rapid estimates of relative water content. Plant Physiol. 53:258–260.

Soliman MH, Alayafi AAM, El Kelish AA, Abu-Elsaoud AM. 2018. Acetylsalicylic acid enhance tolerance of phaseolus vulgaris L. to chilling stress, improving photosynthesis, antioxidants and expression of cold stress responsive genes. Botanical Studies. 59:6, 1–17. doi:10.1186/s40529-018-0222-1

Soliman M, Alhaithloul HA, Hakeem KR, Alharbi BM, El-Esawi M, Elkelish A. 2019. Exogenous nitric oxide mitigates nickel-induced oxidative damage in eggplant by up-regulating antioxidants, osmolyte metabolism, and glyoxalase systems. Plants. 8:562, 1–17. doi:10.3390/plants8120562.

Soliman M, Elkelish A, Souad T, Alhaithloul H, Farooq M. 2020. Brassinosteroid seed priming with nitrogen supplementation improves salt tolerance in soybean. Physiol Mol Biol Plants. 26:501–511. doi:10.1007/s12298-020-00765-7.

Tiller KG. 1983. Micronutrients. In: Tiller KG, editor. Soils: An Australian viewpoint, chap. 25. CSIRO, melbourne. London: Academic Press; p. 365–387.

Velikova V, Yordanov I, Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain treated bean plants, protective role of exogenous polyamines. Plant Sci. 151:59–66.

Wild R, Ooi L, Srikanth V, Münch G. 2012. A quick, convenient and economical method for the reliable determination of methylglyoxal in milimolar concentrations: the N-acetyl- L-cysteine assay. Anal Bioanal Chem. 403:2577–2581.

Wu X, Zhu W, Zhang H, Ding H, Zhang HJ. 2011. Exogenous nitric oxide protects against salt-induced oxidative stress in the leaves from two genotypes of tomato (*lycopersicon esculentum* mill.). Acta Physiol Plant. 33:1199–1209.

Yang H, Wu F, Cheng J. 2011. Reduced chilling injury in cucumber by nitric oxide and the antioxidant response. Food Chem. 127:1237–1242.