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Bioaccumulation of copper and its effects on growth and biochemical parameters in bitter gourd (*Momordica charantia*) plants

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ABSTRACT: Heavy metal pollution has increased broadly over the globe due to industrial, anthropogenic activities and modern industrialization. Disturbing the environment which leads to causing various health hazards to our humankind. The present study is aimed to evaluate the bioaccumulation of copper and its effects on growth and biochemical parameters in Bitter Gourd (*Momordica charantia* L.) plants. Group 1 plants in polyethylene bag with soil served as control not received copper treatment, groups 2, 3 and 4 received copper treatment of 100, 200 and 400 mg throughout the experimental period. Our results revealed significant reduction in growth parameters such as germination percentage, root length, shoot length, fresh weight, dry weight and vigour index, biochemical parameters such as carbohydrate and protein contents and enzymic antioxidants such as catalase and super oxide dismutase, under copper treatment at various concentrations showed heavy metal toxicity in Bitter gourd plants. Therefore, it is essential to meticulously regulate the copper content in the soil to prevent any negative impacts on the growth and development of plants.

KEYWORDS: Bitter gourd, Copper, Germination, Heavy metals, Soil Contamination.

INTRODUCTION

Heavy metals and metalloids are playing a vital role in plant development by taking part in many metabolic processes and acting as micronutrients [1]. But when the heavy metals are exceeding its threshold concentrations, causing toxic effects in plants. Heavy metals can exert toxic effects that depends on the concentration and plant species. At high concentrations in soil, heavy metals having many adverse effects on plants [2], such as decreased seed germination, reduced root and shoot length, dry weight, membrane alteration, chlorophyll destruction, oxidative damage, nutritional disorders [3], mutations and reproductive disorders [4].

Copper (Cu) is considered as one of the most important micronutrients for the growth of plants. It plays a vital role in numerous physiological and enzymatic functions such as participation in electron flow, catalyze redox reactions in chloroplasts and mitochondria [5]. Contamination of soil due to copper is mainly caused by human activities such as mining, industrial, agriculture and so on. Copper is associated with metabolism of lipids and iron as well as protein transport, signal-regulating transcription and oxidative phosphorylation [6]. However, copper concentrations at elevated levels becomes toxic as it interferes with respiratory and photosynthetic processes, synthesis of proteins, plant organelles development [7], causing chlorosis, stunted root growth and damage in the function of plasma membrane permeability, overall resulting in the leakage of ions [8]. In addition, copper has also been reported to be toxic to plants by reactive oxygen species (ROS) generation leads to oxidative stress [9], thereby resulting in damage to DNA, RNA, lipids, photosystem pigment proteins, enzymes, changes in the composition of the thylakoid membrane, reduced mineral and chlorophyll contents and impaired meristem development. The effects of copper may vary depending on the plant species, duration of exposure and growing conditions [10].

In roots, the toxicity of copper occurs and then spreads to other plant parts, affecting various physiological processes. In the soil, copper at high concentrations causes impairment in the growth of roots resulting in the reduced water and nutrients uptake. In general, stunted growth of roots is associated with the ruptures of exodermis and epidermis. Thereby, copper toxicity causes destruction of the root cuticle, darkened color, reduced root hairs proliferation and growth inhibition. Some other studies have also reported that copper in excess concentrations can reduce the growth of

plant roots [11,12]. High concentrations of copper reflect on a reduction in root biomass, as well as in shoot biomass [13].

Bitter gourd (*Momordica charantia* L.), also known as bitter melon, bitter apple, or balsam pear, is a tropical vine classified under the order Cucurbitales, family Cucurbitaceae, and genus Momordica. The plant is widely cultivated for both its medicinal and nutritional value in regions such as India, China, and Southeast Asia [14]. Bitter gourd is recognized for its diverse pharmacological and bioactive properties, including antioxidant, anti-inflammatory [15], antidiabetic [16], anticancer [17], anticholesterolemic [18], antidementia [19], antibacterial and antifungal [20] activities.

At the cellular level, plants employ various biochemical mechanisms, including both enzymatic and non-enzymatic antioxidants, to safeguard their cells against oxidative stress [21]. By analyzing copper toxicity, its influence on plant vitality, and possible remedial approaches, this study aspires to provide significant insights that will aid in formulating effective strategies to alleviate copper toxicity and promote sustainable agricultural practices. Therefore, this present study is aimed to investigate the bioaccumulation of copper and its effects on growth and biochemical parameters in bitter gourd (*Momordica charantia*) plants.

MATERIALS AND METHODS

The experimental protocol formulated to meet the research objectives was executed following established methodologies and standardized procedures. Bitter gourd seeds obtained from agricultural shop, Puducherry. Copper sulphate was used to induce copper toxicity.

Polyethylene bag experiment

Polyethylene bag culture experiments were conducted to investigate the impact of copper toxicity on *Momordica charantia* (bitter gourd) plants. The growth medium in the polyethylene bags consisted of artificially contaminated soil, with copper (Cu) as the metal pollutant. To initiate the experiment, 2 cm deep holes were made in the soil using a sterile wooden dowel, and pre-sterilized seeds were sown in each bag. Each seed was subsequently covered with a thin layer of soil to optimize germination conditions. Soil moisture content was meticulously maintained by adjusting it to the soil's field capacity using dechlorinated tap water to ensure consistent hydration throughout the experiment.

Experimental design

Following the pretreatment phase, the bitter gourd plants were categorized into four distinct treatment groups. Group 1 bag with soil served as control, not received any copper treatment. In contrast, Bags of groups 2, 3 and 4 were subjected to copper treatments of 100 mg, 200 mg, and 400 mg, respectively, over the experimental period. The plants were cultivated under conditions of relative humidity, average temperature and natural photoperiod.

Germination parameters

Germination percentage was calculated by determining the daily germination rate, which was obtained by dividing the number of germinated seeds on each observation day by the total number of seeds sown, then multiplying by 100. The cumulative germination percentage was subsequently derived by summing the daily germination percentages over the entire experimental period.

Germination rate = Number of Seeds germination/Total Number of seeds

Germination %= Germination rate × 100

Root Length (in cm)

The root length, defined as the distance from the soil surface to the tip of the primary root, was measured using a calibrated linear measurement scale (centimeter ruler) with precision to the nearest millimeter.

Shoot Length (in cm)

The shoot length, defined as the vertical distance from the soil surface to the apical meristem of the shoot, was measured using a calibrated linear scale (centimeter ruler) with precision to the nearest millimeter.

Fresh Weight (in gm)

The fresh weight of the entire plant is measured using an electronic analytical balance.

Dry Weight (in gm)

The dry weight of the entire plant is determined by desiccating the plant tissue to remove all water content, and subsequently measuring the remaining biomass using an electronic analytical balance.

Vigour Index

The vigor index was assessed based on germination parameters, with data recorded on the germination percentage. The vigor index was calculated using the mean values of both root length and shoot length, following the methodology outlined by Baki and Anderson [22].

Vigour Index = (Mean Shoot length + Mean root length) × Germination %

Biochemical estimations

Carbohydrate Estimation

The carbohydrate content was estimated by the method of Hedge and Hofreiter [23]. For sample preparation, 1 g of freshly harvested leaf tissue was homogenized with 50 ml of potassium hydroxide (KOH) solution. The mixture was then

subjected to centrifugation for 15 minutes to separate the cellular debris. The supernatant was carefully made upto 100 ml to obtain the final sample solution. A volume ranging from 0.2 to 1 ml of the working standard solutions was accurately pipetted into separate test tubes, while 0.5 ml of the sample was placed into another test tube. Subsequently, the volume in each test tube was adjusted to 1 ml with distilled water. Following this, 4 ml of Anthrone reagent was added to each test tube. The contents of the test tubes were thoroughly mixed and then incubated in a boiling water bath for 20 minutes. After heating, the test tubes were allowed to cool to room temperature. The absorbance of the resulting green colored solution, indicative of the presence of carbohydrates, was measured spectrophotometrically at a wavelength of 640 nm.

Protein Estimation

The protein content was estimated by Lowry's method [24]. For sample preparation, 1 g of freshly collected leaf tissue was finely ground in 10 ml of trichloroacetic acid (TCA) to extract the soluble metabolites. The homogenate was then centrifuged for 15 minutes, and the supernatant was carefully discarded. The resulting pellet was resuspended in 5 ml of sodium hydroxide (NaOH) and subjected to further centrifugation. After centrifugation, the supernatant was collected and the volume was adjusted to 100 ml to prepare the final sample solution. A range of volumes from 0.2 ml to 1 ml of the working standard solution is pipetted into a series of test tubes. Additionally, 0.2 ml of the sample extract is added in separate test tube. Each of the test tubes is then adjusted to a final volume of 1 ml using distilled water, with 0.5 ml of distilled water serving as the blank control. The contents are thoroughly mixed and allowed to stand for 10 minutes to ensure proper equilibration. Subsequently, 0.5 ml of Folin-Ciocalteu reagent is added to all the test tubes and the solutions are mixed thoroughly. The test tubes are then incubated at ambient temperature in the dark for 30 minutes, allowing the development of a blue color. The absorbance of the resulting solution is measured spectrophotometrically at 660 nm.

Enzyme assays and analysis

Estimation of Catalase

The leaf tissue was homogenized in 100 mM phosphate buffer (pH 7.0) to extract the enzymes. The catalase (CAT, EC 1.11.1.6) activity was measured spectrophotometrically using the assay method described by Sinha [25]. To 0.9 ml of phosphate buffer, 0.4 ml of hydrogen peroxide solution, and 0.1 ml of the sample extract were added in separate test tubes. After predetermined time intervals of 30 and 60 seconds, 2 ml of dichromate reagent mixture was introduced to each tube to initiate the reaction. The tubes were then subjected to thermal treatment by placing them in a boiling water bath for 10 minutes, facilitating the development of a colored complex. The absorbance of the resulting solution was spectrophotometrically measured at 620 nm at both 0 and 60 seconds to monitor the color intensity. A series of standard solutions containing 2-10 micromoles were prepared and treated identically to the test samples. A blank control, containing only the reagent mixture, was also prepared to account for any background interference.

Estimation of Superoxide dismutase

The leaf tissue was homogenized in 100 mM sodium pyrophosphate buffer (pH 8.3) to extract the enzymes. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed using the method described by Kakkar et al. [26]. The assay solution consists of 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulfate (PMS), 0.3 ml of nitroblue tetrazolium (NBT), 1.0 ml of appropriately diluted enzyme preparation, and water, adjusting the final volume to 3 ml. The reaction is initiated by the addition of 0.2 ml of NADH. The mixture is then incubated at 30°C for 90 seconds. To halt the enzymatic reaction, 1 ml of glacial acetic acid is added. The reaction mixture is subsequently subjected to phase separation by shaking with 4ml of n-butanol. Then, allow the mixture to stand for 10 minutes and then centrifuged. The chromogenic intensity in the butanol layer is quantified by spectrophotometry at 560nm, using the butanol as a blank. A control sample, devoid of enzyme, is included to account for background interference.

Statistical analysis

Results were expressed as means±standard deviation of six plants per group. Data were analyzed by oneway analysis of variance and any significant differences among treatment groups were evaluated using Duncan's multiple range test. Results were considered statistically significant when $P<0.05$. All statistical analyses were performed using SPSS version 15.0 software package (SPSS, Tokyo, Japan).

RESULTS

Effect of Copper in Bitter Gourd plants on germination percentage, root length and shoot length

Table 1 represents the effect of copper on germination percentage, root length and shoot length in different experimental groups of bitter gourd plants. These observations were recorded on 30th day after sowing. Upon

increasing the copper concentration for about 100 mg, 200 mg and 400 mg, results revealed that gemination percentage, root length and shoot length were significantly decreased when compared to control plants.

Table 1. Effect of Copper on germination percentage (%), root length (in cm) and shoot length (in cm) in different experimental groups of Bitter Gourd plants.

Groups	Germination percentage (%)	Root length (cm)	Shoot length (cm)
Control (C)	85	8.54±0.70	15.34±1.10
Test (T1)	60	6.13±0.53	12.46±0.92
Test (T2)	45	5.55±0.42	11.75±0.76
Test (T3)	30	5.06±0.32	9.98±0.67

Values are expressed as mean±SD. Groups not sharing a common superscript letter differ significantly at p<0.05. Duncan's multiple range test (DMRT).

Effect of copper in bitter gourd plants on fresh weight, dry weight and Vigour index

Table 2 shows the effect of copper on fresh weight, dry weight and vigour index on different groups of bitter gourd plants. These observations are also recorded on 30th day after sowing. Fresh weight, dry weight and vigour index were significantly decreased under copper toxicity in bitter gourd plants as compared to control plants.

Table 2. Effect of copper on fresh weight, dry weight and vigour index in different experimental groups of bitter gourd plants.

Groups	Fresh weight (g)	Dry weight (g)	Vigour Index
Control (C)	7.65±0.64	2.04±0.17	2029.8±87.41
Test (T1)	6.3±0.54	1.26±0.11	1115.4±59.54
Test (T2)	5.5±0.43	1.13±0.09	778.5±41.39
Test (T3)	4.2±0.29	1.02±0.07	451.2±23.54

Values are expressed as mean±SD. Groups not sharing a common superscript letter differ significantly at p<0.05. Duncan's multiple range test (DMRT).

Effect of copper on carbohydrate and protein contents

Figure 1 illustrates the effect of copper on total carbohydrate levels, while figure 2 depicts the effect of copper on protein contents in different experimental groups of bitter gourd plants. These observations were recorded on 30th day after sowing the seeds. Results showed that Carbohydrates and Protein contents were significantly reduced in copper treated groups when compared to normal control plants.

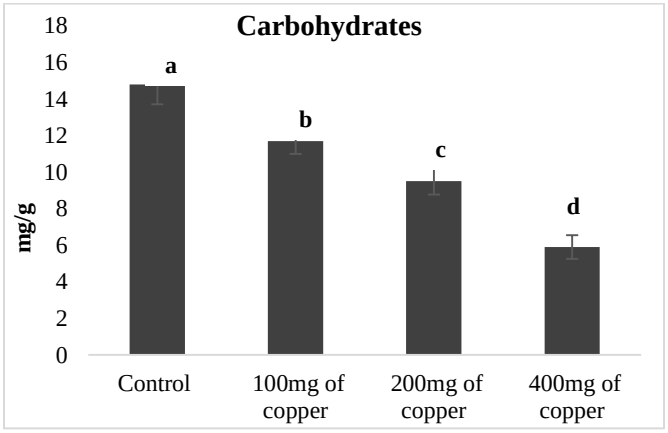

Figure 1. Effect of copper stress on carbohydrate contents in different experimental groups of bitter gourd plants. Values are expressed as mean±SD. Groups not sharing a common superscript letter differ significantly at p<0.05. Duncan's multiple range test (DMRT).

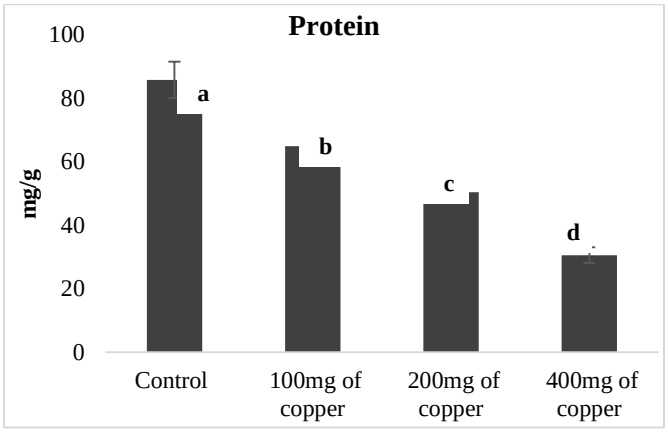

Figure 2. Effect of copper stress on protein contents in different experimental groups of bitter gourd plants. Values are expressed as mean±SD. Groups not sharing a common superscript letter differ significantly at p<0.05. Duncan's multiple range test (DMRT).

Effect of copper on enzymic antioxidants

Figure 3 illustrates the effect of copper on catalase activity, while figure 4 shows the effect of copper on superoxide dismutase levels in the different experimental groups of bitter gourd plants. Results suggested that decreased levels of these enzymic antioxidants such as catalase and superoxide dismutase were observed due to copper toxicity in bitter gourd plants.

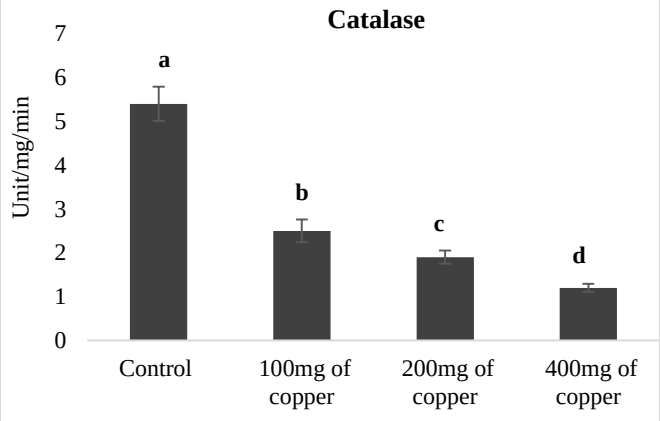

Figure 3. Effect of copper on Catalase levels in different experimental groups of bitter gourd plants. Values are expressed as mean±SD. Groups not sharing a common superscript letter differ significantly at $p<0.05$. Duncan's multiple range test (DMRT).

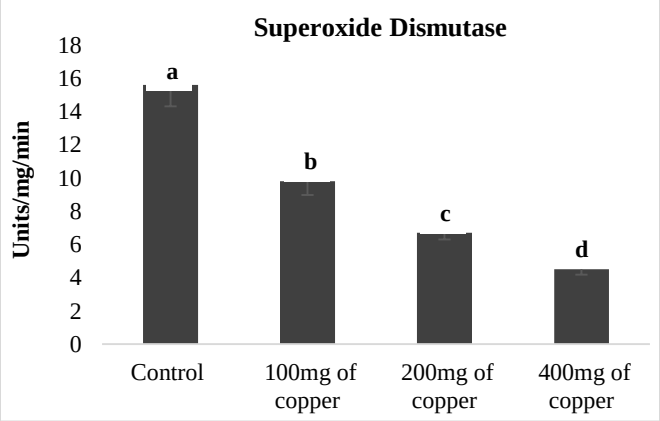

Figure 4. Effect of copper on Superoxide dismutase levels in different experimental groups of bitter gourd plants. Values are expressed as mean±SD. Groups not sharing a common superscript letter differ significantly at $p<0.05$. Duncan's multiple range test (DMRT).

DISCUSSION

The present study was carried out to elucidate the bioaccumulation of copper and its effects on growth and biochemical parameters in *Momordica charantia* (Bitter Gourd) plants.

Germination percentage, Root length and Shoot length

High concentrations of metals and chemicals affect plant germination, growth and production, which are mainly related to the biochemical, physiological and genetic components of the plant system. Seed germination and early seedling growth are most important stages in plant development, highly vulnerable to heavy metal toxicity [27], which disrupts physiological processes, cellular metabolism and enzymic functions. It is clear from the results, copper treatment decreases water content and its uptake in seed germination which leads to reduced germination rate when compared to control bitter gourd plants. Our current study correlate with those of the previous reports by Pena et al. [28], Hira et al. [29] revealed that copper stress leads to reduced germination rate.

Group 1 have high root length and shoot length when compared with group 2,3 and 4 plants. The control group exhibited the longest root and shoot length, whereas all copper tested plant groups showed reduced values under varying concentrations of copper toxicity which correlates with the previous findings by Marques et al. [30]. The percentage of germination, as well as the root and shoot length, has been adversely affected by copper and its values highly retrograde in the presence of high concentration than low concentration of control [31]. The observed growth reduction in the selected bitter gourd plants may be attributed to a loss of cellular turgor, which could lead to either inhibition of cell elongation or decrease in mitotic activity [32]. Moreover, the decrease in root length, resulting from copper accumulation within the roots, impairs the rate of mitosis in the meristematic regions, peculiarly by obstructing the stage of metaphase in meristematic cells. It is proposed that the morphological alterations observed in the roots and shoots, resulting from copper exposure, are associated with nutritional deficiencies in higher plants subjected to toxic concentrations of copper over both medium and long-term periods. Furthermore, it is well-established that elevated concentrations of heavy metals result in a significant reduction of growth and plant biomass [33], as heavy metal toxicity impairs physiological processes such as photosynthesis, nutrient uptake and cellular integrity, which also aligns with the findings of the present study.

Fresh weight, dry weight and vigour index

Metal stress directly impairs plant physiological processes, leading to a significant reduction in fresh weight, root length, shoot length, and overall biomass accumulation [34]. The seedling vigor index has been proposed as a reliable phytotoxicity indicator, reflecting the impact of heavy metal concentrations on plant health. This index is extensively utilized to assess the phytotoxic effects of heavy metals on seedling growth [35]. There was a direct relation between concentration of heavy metals and decline in vigour index, specifically as the level of heavy metals concentration increases, the vigor index correspondingly decreases [36]. It

was already reported that copper treatment produced toxic effects in *L Culinaris* plants which is evidenced by reduced dry weight and vigour index as compared to normal control plants [37]. This correlates with our study, suggested that copper treatment at various concentrations leads to decreased fresh weight, dry weight, and vigour index significantly in bitter gourd plants as compared to normal control plants. This notable decline in these parameters may be attributed to the metal toxicity associated with copper. This reduced vigor index underscores the detrimental effects of heavy metals exposure, indicating impaired seedling development and diminished resilience [38].

Carbohydrates and protein contents

Copper, being an important micronutrient, facilitates the growth of bitter gourd at lower concentrations; however, copper at elevated levels hinder growth by disrupting normal cellular metabolism. Carbohydrates perform a wide range of ecological functions in plants, playing a significant role in maintaining structural integrity and providing energy for various physiological processes. They are also essential for plant interactions with beneficial microbes, such as in symbiotic relationships, and for the plant's defense mechanisms against pathogenic microbes, contributing to both pathogen resistance and immune response [39]. Through photosynthesis, green plants synthesize carbohydrates from carbon dioxide and water, providing energy sources like glucose, starch, etc. Additionally, carbohydrates are integral components of other biomolecules which includes DNA, RNA, glycolipids, glycoproteins and ATP. The data shown in figure 1 indicates a significant reduction in carbohydrate levels in plants subjected to copper stress compared to normal control plants. This reduction is attributed to the copper absorbed by bitter gourd plant, and it is likely correlates with inhibited photosynthesis or an increased respiration rate. This disruption of photosynthesis leads to reduced agricultural productivity, especially in soils contaminated with copper. This corresponds to previous literature evidence by Duan et al. [40] who reported copper when present in lower concentrations functions as a nutrient in plants however concentration of copper at elevated levels resulted in toxic effects and negatively created impact on sugar contents in *Glycine max* plants.

The storage proteins mobilization is one of the most vital post-germinative process in the growth and development of seedlings. During the germination phase, various proteases facilitate the degradation of storage proteins and also transforming insoluble storage proteins into soluble peptides and amino acids. Then these products are transported to the embryonic axis, where they contribute to growth and provide energy through carbon skeleton oxidation after deamination. Proteins play a crucial role within cells and are especially vulnerable to damage when exposed to environmental stress conditions. Consequently, any alterations in these compounds can serve as significant oxidative stress indicator in plants. Our findings demonstrated varying changes in protein contents following different copper treatments, with a significant reduction in protein levels compared to the untreated control bitter gourd plants. This observation correlates with the study by Vijay et al. [41], who reported that increasing heavy metal concentrations lead to a significant decrease in protein levels in brinjal plants. Copper causes the decline in protein contents and the corresponding rise in the activity of hydrolytic enzymes such as protease due to heavy metal stress and this observation strongly suggests the catabolic activities. Furthermore, heavy metals also have the capacity to alter the protein composition of the cell membrane [42].

Catalase and Superoxide dismutase

Plants exhibit varying responses to different metal stresses, with exposure to elevated metal concentrations resulting in detrimental effects [43]. A primary consequence of metal stress is the accumulation of ROS within plant cells, leading to cellular damage induced by free radicals [44]. In plants, catalase plays a vital role in cellular defense mechanisms against oxidative stress by converting hydrogen peroxide into water and oxygen [45]. CAT is the most efficient enzyme as an antioxidative enzyme which lowers, hydrogen peroxide or superoxide to accumulate at toxic levels in plant growth [46]. Through a reducing cycle like fenton or Haber-weise reactions, copper causes oxidative stress. Moreover, copper can indirectly damage cells by interfering with defense systems, disrupting the electron transport chain and promoting lipid peroxidation [47]. High concentrations of copper cause toxicity in bitter gourd plant and therefore cause oxidative stress reflected in our present study which was evidenced by decreased catalase activity with different concentrations of copper, that was consistent with the findings of previous study by Khatun et al. [48]. Heavy metals, as contaminants, interact with the enzyme-substrate complex, leading to enzyme denaturation or interference with protein active sites, which results in reduced enzyme activity [49].

Cu at higher concentrations causes oxidative stress due to overproduction of ROS and reactive nitrogen species (RNS), which can be cytotoxic and damage important cell compounds [50]. To avoid oxidative damage, the antioxidant enzymes and specific metabolites found in plants might play a vital role in facilitating adaptation and ensuring plant

survival in stressful environments [51]. Superoxide dismutase (SOD) is a crucial antioxidant enzyme, serving as the primary and essential defense against ROS production. SOD catalyzes the dismutation of superoxide anions, which accumulate under stress conditions, converting them into hydrogen peroxide and molecular oxygen [52]. It was observed that as the copper concentration increased, the activity of SOD gets decreased in a dose-dependent manner. These results are in agreement with the findings of Zhao et al. [53], who reported that elevated heavy metal concentrations can reduce SOD activity. A decrease in SOD activity subjected to copper at higher concentration levels in bitter gourd plants as compared to control plants may be attributed to the inhibition of enzyme activity by more hydrogen peroxide (H_2O_2) contents present in various cellular compartments [54]. This impairs the plant's ability to adapt to environmental stressors, further compromising its growth and survival in contaminated conditions. Over time, copper contamination in agricultural soils can adversely affect overall biodiversity, destabilizing plant communities and leading to enduring ecological imbalances.

CONCLUSION

Based on the experimental results, copper at higher concentrations causes significant damage to bitter gourd plants as evidenced by reduction in the growth parameters such as germination percentage, root length, shoot length, fresh weight, dry weight and vigour index when compared to control plants. Further the data reveals that carbohydrates and protein contents in bitter gourd plants also gets reduced under copper treatment. Correspondingly, antioxidant enzymes such as catalase and super oxide dismutase levels gets decreased due to copper toxicity. Given the global occurrence of copper bioaccumulation in plants, it is essential to evaluate the biological activity of those plants prior to their application for medicinal purposes.

DECLARATION

Authorship contributions

Concept: V.M., Design: V.M., Data Collection or Processing: V.M., A.R., A.R., Analysis or Interpretation: V.M., Literature Search: V.M., A.R., A.R., Writing: V.M., A.R., A.R.

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Competing interests

The authors declared that there is no conflict of interest.

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