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Effects of Different Copper Concentration on Growth and Nodulation of Green Gram (*Vigna radiata* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

An investigation was conducted to study the influence of copper (Cu) on the growth of shoot, root, and nodulation of green gram (*Vigna radiata* L.). A field pot experiment was designed in a Completely Randomized Design with six treatments and four replicates at Palachcholai, Faculty of Agriculture, Eastern University, Sri Lanka from September to November 2023. Six levels of copper concentration (control, 50, 100, 150, 200, 250 mg copper/kg soil) was applied to the soil in the form of copper sulphate ($CuSO_4.5H_2O$) in which the green gram plants were grown. Exposure to copper at concentrations ranging from 100-250 mg copper/kg soil reduced total chlorophyll content,

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shortened root length, decreased plant biomass, and produced fewer leaves, and a lower number of nodules. Conversely, exposure to copper at 50 mg copper/kg soil increased the fresh shoot biomass, soil microbial respiration and soil pH. Additionally, the positive effects of copper were observed on tap root length, leaf area, and root volume. The copper at 50 mg copper/kg soil concentration enhanced the shoot biomass, the number of nodules and soil microbial respiration by 25% ,14%, and 22% respectively compared to the control while the copper concentrations ranging from 100 mg copper/kg soil to 250 mg copper/kg soil significantly inhibited the overall growth and nodule formation of green gram. The results clearly indicate that Cu is an important micro-nutrient, only it should be added in trace amount to induce plant growth and other physiological processes. However, copper at higher concentrations can act as a heavy metal and cause phyto-toxicity on plants.

Keywords: Soil microbial respiration; phyto-toxicity; heavy metal; Rhizobia spp; effective nodules.

1. INTRODUCTION

Green gram is one of the most significant grain legumes in traditional farming practices in Sri Lanka. It has become increasingly significant in Sri Lankan diet, serving as one of the most essential and economical sources of protein [1]. As a legume, green gram can fix atmospheric nitrogen (30–50 kg/ha) in collaboration with soil bacteria *Rhizobium* spp. Biological nitrogen fixation, occurring in the root nodules of legume plants through the activity of bacteria, is a pivotal process benefiting both the biological nitrogen cycle and global agriculture. This process involves the conversion of atmospheric nitrogen into ammonia. Rhizobia establishes symbiotic structures called root nodules on their host's roots, where they proliferate by acquiring nutrients from the host plant. In return, they contribute nitrogen resources through the fixation of nitrogen gas for the benefit of their host plants [2].

Copper (Cu) is a crucial micronutrient for plants. As a redox-active transition metal, it is essential for various physiological and biochemical processes in plants. Cu is known to be an essential micronutrient for all organisms when present in optimal amounts. Copper is essential for plants, playing a vital role in photosynthesis, the electron transport chain, mitochondrial respiration, cell wall metabolism, hormone signaling, and lignin production. Other crucial tasks carried out by Cu in plants include carbon dioxide absorption and ATP creation [3]. In plants, an excessive accumulation of copper may compromise membrane stability, diminish photosynthesis, and alter enzyme function, thereby impeding overall growth and causing other adverse effects [4]. The use of Cu-based agrochemical products, including fertilizers, pesticides, insecticides, herbicides, fungicides, miticides, and nematicides, is contaminating soil with high concentrations of copper [3,5]. Examples of anthropogenic sources of copper include heavy metal mining, metal processing, sewage sludge distribution, traffic emissions, pesticide use, and chemical fertilizer use [6].

Therefore, this study was initiated to evaluate whether the copper applied as its sulfate salts to soil have any toxic effect on nodules that affect the plant growth, or it enhance the plant growth and nodule formation on green gram crop. Hence, this experiment was carried to evaluate different concentrations of copper on plant growth and nodulation of green gram and to find out the concentration at which it acts as a micronutrient and as a heavy metal.

2. MATERIALS AND METHODS

The experiment was conducted at the crop farm, Eastern University, Sri Lanka, Vantharumoolai from September to November 2023. It is located at the latitude of 7º 48’36.64” N and longitude of 81º 35’30.76” E, which comes under the Agro-Ecological Zone of the Low Country Dry Zone (DL_2). The annual mean temperature of this area varies from 26 ºC to 37 ºC and the mean yearly rainfall ranges from 1,400 mm to 1,680 mm. The experimental area’s major soil type is sandy.

The experiment was carried out using Complete Randomized Design (CRD) with six treatments having four replicates. The experiment consisted of six treatments and four replications. The treatments were as follows:

Treatments	Description
T1	0 mg of copper (control)
T2	50 mg of copper per 1 Kg of soil
T3	100 mg of copper per 1 Kg of soil
T4	150 mg of copper per 1 Kg of soil
T5	200 mg of copper per 1 Kg of soil
T6	250 mg of copper per 1 Kg of soil

Green gram (var. Harsha) seeds were collected from the seed unit at the Farm of Eastern University of Sri Lanka. Copper was applied at rates of 0, 50, 100, 150, 200, and 250 mg/kg soil in the form of copper sulfate ($CuSO4·5H2O$), and the soil was watered to 50% of its water holding capacity. These pots with soil were allowed for 10 days before basal fertilizer application.

Two days after basal fertilizer application five uniform sized green gram seeds were directly sown in 2-3 cm depth in each pot and covered with soil. Irrigation was done twice daily in the morning and evening until the seeds germinated after that watering was limited to once a day. Two days before seed sowing, basal fertilizer application with urea, Triple Super Phosphate and Muriate of Potash was done in each pot and top dressing was done at the onset of flowering. All the plants were harvested at the onset of flowering stage. The flowering stage usually comes 40 days after sowing (DAS).

Plant height (cm), Chlorophyll content in leaves (SPAD 502, United States of America), Leaf area (cm^2) (LI-3100, United States of America), Shoot and root dry weight (g), Number of nodules, microbial and respiration in soil were measured.

Collected data were statistically analyzed using the statistical software Minitab 17, and the mean comparison within treatments was performed by Tukey's at a 5 % significant level.

3. RESULTS AND DISCUSSION

3.1 Plant Height (cm)

The plant height values of green gram corresponding to different copper concentrations were significantly ($p<0.05$) affected compared to the control (Table 1). Plant height was measured at weekly intervals up to 40 days. Plants treated with a low copper concentration (50 mg Cu/kg soil) exhibited the highest mean plant height (34.5 cm), followed by the control group (30.25 cm) at 5 weeks after sowing (WAS). Throughout the experiment, the recorded plant height at higher copper concentration ranges from 100-250 mg copper/kg soil consistently remained lower than the control plants. This trend persisted from the initial stages of the experiment until the harvesting stage (from 1st WAS to 5th WAS). Plant height was greatest at the low copper concentration of 50 mg Cu/kg soil and decreased as copper concentration increased.

Similar results were found by Kumar et al. [1], Syuhada et al. [2], and Manivasagaperumal et al. [3], where they reported that low copper level induces plant height compared to higher Cu level. According to Yruela [4], who reported that the increased plant height in 50 mg copper/kg soil than the control treatment might be due to Copper acts as a structural element in regulatory proteins and is involved in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism, and hormone signaling.

Tittonell et al. [5] stated that Plant height serves as a parameter to assess crop performance. Reductions in plant growth and development due to copper toxicity may result from reduced water absorption, nutritional deficiencies, and decreased metabolic activities. Elevated concentrations of copper (Cu) were observed to have adverse effects on both plant growth and biomass. It was noted that plants faced challenges in surviving when exposed to high Cu concentrations across various plant species [6,7,8].

3.2 Chlorophyll Content

The highest reduction in chlorophyll content, 22% lower than the control, was observed at 250 mg copper/kg soil Cu. However, the study also noted non-significant differences in total chlorophyll content. The maximum chlorophyll content (52.43) was observed at 50 mg copper/kg soil Cu, while the minimum (37.4) was observed at 250 mg copper/kg soil Cu.

Table 1. The effect of different copper concentration on plant height at different weeks

Treatment	1st WAS	3rd WAS	5th WAS
control	7.02 ± 0.025 ^{b}	18.62 ± 0.125 ^{b}	30.25 ± 0.250 ^{b}
50 mg copper/kg soil	8.07 ± 0.149 ^{a}	21.37 ± 0.239 ^{a}	34.50 ± 0.289 ^{a}
100 mg copper/kg soil	6.12 ± 0.075 ^{c}	17.27 ± 0.160 ^{c}	27.25 ± 0.144 ^{c}
150 mg copper/kg soil	5.2 ± 0.144 ^{d}	15.05 ± 0.166 ^{d}	26.30 ± 0.108 ^{c}
200 mg copper/kg soil	4.7 ± 0.144 ^{d}	14.87 ± 0.175 ^{d}	21.67 ± 0.284 ^{d}
250 mg copper/kg soil	3.62 ± 0.375 ^{e}	12.9 ± 0.10 ^{e}	20.25 ± 0.629 ^{d}

Each value represents mean ± standard error of four replicates

Table 2. The effect of different copper concentration on chlorophyll content of green gram at 40 DAS

Treatments	Chlorophyll content
Control	48.13 ± 0.395 [b]
50 mg copper/kg soil	52.43 ±0.0437 [a]
100 mg copper/kg soil	44.69 ± 0.287 [c]
150 mg copper/kg soil	40.15 ± 0.270 [d]
200 mg copper/kg soil	39.73 ± 0.312 [e]
250 mg copper/kg soil	37.40 ± 0.216 [e]

Each value represents mean ± standard error of four replicates

Table 3. The effect of different copper concentration on leaf area at 40 DAS

Treatments	Leaf area (cm^2)
Control	714.14 ± 6.56 [b]
50 mg copper/kg soil	771.1 ± 12.6 [a]
100 mg copper/kg soil	648.38 ± 2.70 [c]
150 mg copper/kg soil	566.8 ± 10.1 [d]
200 mg copper/kg soil	434.72 ± 8.67 [e]
250 mg copper/kg soil	431.48 ± 6.38 [e]

Each value represents mean ± standard error of four replicates

Chlorophyll content decreased significantly in leaves of elevated Cu-treated plants compared to that of leaves of Cu-untreated plants. Similar results were observed by Jahan et al. [9] in Arabidopsis plants at high copper concentration. These results suggest that Cu application might increase Chlorophyll accumulation in leaves and show positive function on light related reaction in plants. Syuhada et al. [10] stated that Cu involves in the structure of photosynthetic proteins and enzymes in plants.

According to Mir et al. [11], who stated that the increased chlorophyll content in Cu-treated plants might be due to Cu-induced enzyme might mediate photoreduction of O_2 by PS1. Padua [12] demonstrated that Cu effects the splitting of photosystem II (PSII) that necessary electrons are provided for photosynthesis. Excess copper, however, is detrimental to plants. It inhibits chlorophyll biosynthesis, reduces pigment composition in the chloroplast, affects photosynthetic gas exchange, inhibits photosystem II, and ultimately decreases photosynthesis in stressed plants [13].

3.3 Leaf Area (cm^2)

The impact of various copper concentrations on leaf area is presented in this study. The leaf area showed significant variation ($P < 0.05$) with different copper concentrations. Plants treated with 50 mg copper/kg soil exhibited the highest leaf area value (771.1 cm2), whereas those treated with 250 mg copper/kg soil showed the minimum leaf area value (431.48 cm2). However, the leaf area at 200 mg copper/kg soil and 250 mg copper/kg soil copper concentrations were statistically similar ($P > 0.05$) (Table 3).

Leaf area increased at lower doses and decreased at higher doses of Cu was observed in this present study. Similarly, Zeng et al. [14] reported that leaf area, leaf broadness and length were significantly reduced in maize plants exposed to greatest Cu levels in comparison with control plants. Reduction of leaf area due to copper was also observed by Zeng et al. [14], and Manivasagaperumal et al. [3]. Manivasagaperumal et al. [3] stated that reduced leaf area may be due to reduction in cell division, toxic effect of heavy metal on photosynthesis, respiration, and protein synthesis.

3.4 Shoot Dry Weight (g)

The influence of different copper concentrations on the dry weight of shoots at 40 days after sowing (DAS) is depicted in Fig. 1. As illustrated in the figure, various copper concentrations had a significant ($P < 0.05$) effect on the shoot dry weight of *Vigna radiata* (L.).

Among the treatments, plants treated with 50 mg copper/kg soil copper exhibited the Utmost shoot dry weight (17.94 g) compared to control plants (13.2 g). Conversely, among the treatments, plants treated with 250 mg copper/kg soil significantly displayed the lowest shoot dry weight (7.6 g). The findings indicated a reduction in shoot dry weight with increasing copper concentration. The shoot dry matter yield showed a slight decrease, consistent with findings reported by Kumar et al. [15] in wheat plants. Kong et al. [16] observed a reduction in the dry weight of sunflower, *Vigna radiata* (L.) Wilczek, and black oats due to copper.

The observed stimulatory responses in plant growth at low copper doses may be attributed to the involvement of copper in various physiological activities. These results align with the findings of Gopal [17] and Manivasagaperumal et al. [3], who also stated that copper is essential for plant growth. However, Muccifora [18] found that high doses of copper are toxic to plants, potentially damaging protein synthesis and enzyme activity, altering membrane permeability, and inhibiting photosynthesis.

3.5 Root Dry Weight (g)

The influence of various copper concentrations on the root dry weight of roots were significantly ($P < 0.05$) impacted by the application of copper. The application of increasing levels of copper to the soil had an impact on both fresh and dry root weights after 40 days of sowing (DAS). The lowest fresh root weight (2.71 g) was noted at 250 mg copper/kg soil, while the greatest root weight, 6.52 g, was documented at 50 mg copper/kg soil followed by control 5.42 g.

The reduction in fresh and dry weight of roots may be due to excess copper disrupting the cell membrane of the root cuticle and inhibiting root hair production, leading to root deformation. Long-term exposure of *Arabidopsis thaliana* to copper stress resulted in inhibited root and stem development by altering cell division, elongation, and expansion [11]. Additionally, auxin homeostasis was disturbed in both roots and stems, which could be a significant cause of growth inhibition [19]. This finding is consistent with Marques et al. [20], who observed detrimental effects on root growth and morphology in *Hymenaea courbaril* plants exposed to high levels of copper.

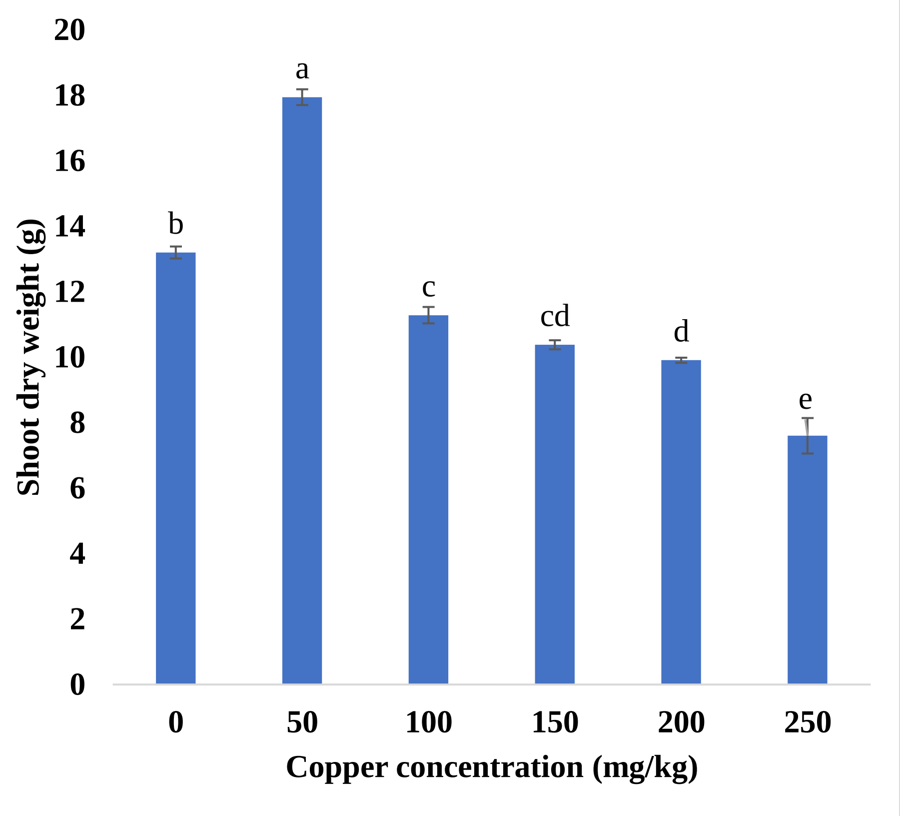

Fig. 1. The effect of different copper concentration on shoot dry weight at 40 DAS
Each value represents mean ± standard error of four replicates.

Table 4. The effect of different copper concentration on fresh weight and dry weight of roots at 40 DAS

Treatments	Root fresh weight (g)	Root dry weight (g)
Control	5.42 ± 0.0744 ^{b}	1.43 ± 0.0246 ^{b}
50 mg copper/kg soil	6.52 ± 0.176 ^{a}	1.61 ± 0.00913 ^{a}
100 mg copper/kg soil	4.72 ± 0.100 ^{c}	1.31 ± 0.0108 ^{c}
150 mg copper/kg soil	3.67 ± 0.120 ^{d}	1.25 ± 0.00479 ^{c}
200 mg copper/kg soil	3.45 ± 0.0806 ^{d}	1.14 ± 0.00854 ^{d}
250 mg copper/kg soil	2.71 ± 0.0435 ^{e}	0.31 ± 0.0178 ^{e}

Each value represents mean ± standard error of four replicates

Table 5. The effect of different copper concentration on number of nodules at 40 DAS

Treatments	Number of nodules
Control	94.50 ± 0.957 ^{b}
50 mg copper/kg soil	107.75 ± 1.11 ^{a}
100 mg copper/kg soil	87.00 ± 0.577 ^{c}
150 mg copper/kg soil	84.25 ± 0.479 ^{c}
200 mg copper/kg soil	65.50 ± 0.645 ^{d}
250 mg copper/kg soil	63.75 ± 1.11 ^{d}

Each value represents mean ± standard error of four replicates

3.6 Number of Nodules Per Plant

An evaluation of the number of nodules was conducted at the end of the experiment. It was found to have a significant ($P < 0.05$) effect of different copper concentration on the number of nodules of green gram after 40 DAS. As shown in Table 5 significant ($P < 0.05$) reduction in the number of nodules was observed in the plants grown in the presence of 100, 150, 200 and 250 mg copper/kg soil copper concentration. In fact, the maximum number of nodules (107.75) was produced by 50 mg copper/kg soil Cu when compared to non-treated plants (94.5). The lowest number of nodules was observed in the treatments treated with 200 mg copper/kg soil and 250 mg copper/kg soil copper concentrations, counting 65.5 and 63.75, respectively.

The above data confirmed that 50 mg copper/kg soil Cu^{2+} treatment was the maximum Cu concentration at which plants produce a greater number of nodules. A similar reduction in nodule number under copper treatment was reported for *Vigna unguiculata* [21]. This result is consistent with that of Tortosa et al. [22], who found that the formation of nodules was inhibited by adding high levels of copper to soil.

A decrease in the number of nodules in green gram plants, due to high level of copper, would be attributed to the reduction in the root development system as well as the direct toxicity of copper on soil microbes. Previous studies have indicated that most metal ions are toxic to soil microorganisms, even in small quantities [22].

3.7 Average Nodule Weight (g)

The impact of various copper concentrations on nodule weight was assessed at the end of the experiment. Significant ($P < 0.05$) variations in average nodule weight were observed among different copper concentrations (Table 6). Average nodule fresh weight showed significant increases in the presence 50 mg copper/kg soil (345 mg) Cu2+, in comparison to the untreated plants (control) (252 mg). No significant ($P > 0.05$) differences were observed in nodule weight between the plants treated with 150 mg copper/kg soil and 200 mg copper/kg soil of copper. The lowest nodule weight was recorded at 250 mg copper/kg soil (70 mg).

This data shows that the copper at low concentration improving the nodule formation and its weight. This finding is consistent with Kong et al. [16], who reported that the excess copper adversely affected the fresh nodule weight. These outcomes could be attributed to the capacity of toxic metals to diminish or impede the anticipated beneficial effects of rhizobia symbiosis on plant growth, potentially hampering both nodulation and nitrogen fixation processes [23].

Table 6. The effect of different copper concentration on nodule weight at 40 DAS

Treatments	Average nodule weight (mg)
Control	252 ±4.79 ^{b}
50 mg copper/kg soil	345.0 ± 11.9 ^{a}
100 mg copper/kg soil	200.0 ± 14.7 ^{c}
150 mg copper/kg soil	177.5 ± 10.3 ^{c}
200 mg copper/kg soil	125.0 ± 6.45 ^{d}
250 mg copper/kg soil	70.0 ± 4.08 ^{e}

Each value represents mean ± standard error of four replicates

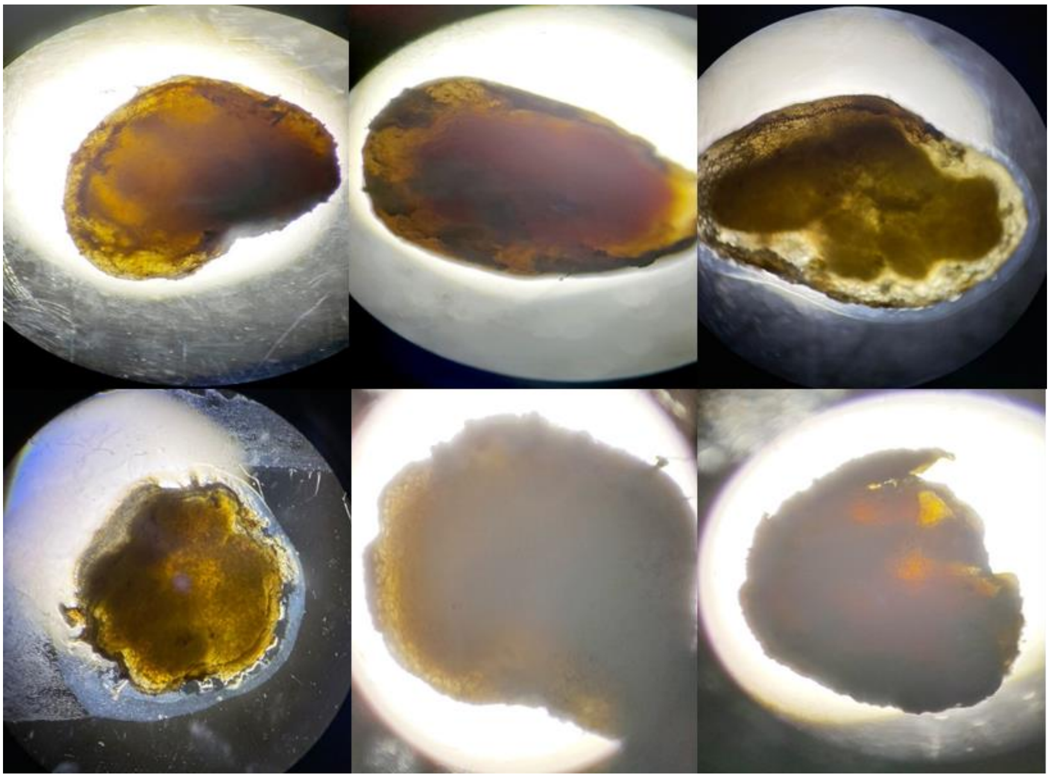

Plate 1. Cross section of nodules of green gram at each copper concentrations

A- Nodule at control, B- Nodule at 50 mg copper/kg soil, C- Nodule at 100 mg copper/kg soil, D- Nodule at 150 mg copper/kg soil, E- 200 mg copper/kg soil, F-250 mg copper/kg soil

Table 7. The effect of different copper concentration on tap root length at 40 DAS

Treatments	Tap root length (cm)
Control	45.50 ± 0.645 ^{b}
50 mg copper/kg soil	49.75 ± 1.25 ^{a}
100 mg copper/kg soil	39.75 ± 0.479 ^{c}
150 mg copper/kg soil	35.87 ± 0.515 ^{d}
200 mg copper/kg soil	34.37 ± 0.554 ^{d}
250 mg copper/kg soil	29.12 ± 0.591 ^{e}

Each value represents mean ± standard error of four replicates

3.8 Effective Nodules and In-effective Nodules

Effective nodules were assessed by cutting the nodules and evaluating reddish color. It was found that copper had an influence on the effectiveness of the nodules of green gram. Plate 1 shows the cross section of nodules which had a diameter above 2 mm, obtained from the plants treated with each copper concentration. The nodules obtained from the control (untreated) and 50 mg copper/kg soil copper exhibited effectiveness when compared to other treatments. The reddish-pink color was the indicator to sort out the effective nodules from other nodules.

The pink-red color of nodules could be observed, indicating the establishment of effective symbioses [24]. These effective nodules might be obtained due to the presence of leghemoglobin [16]. The -red color, because of the presence of leghemoglobin, was considered as an index of potential N fixation [25]. These results are supported by the statement of Fernandes and Henriques [26], who reported that Cu stress seems to be a negative factor for the legume-rhizobia symbiosis and the symbiotic nitrogen fixation (SNF) by reducing nitrogenase activity and the nodule viability could be the reason for the reduction in the number of effective nodules with increasing copper concentration.

3.9 Tap Root Length (cm)

The influence of varying copper concentrations on the tap root length of green gram is detailed in Table 7. The tap root length was significantly ($P < 0.05$) influenced by the application of copper. The utmost root length was recorded at 50 mg copper/kg soil (49.75 cm) followed by control (45.5 cm) while the lowest root length was recorded at 250 mg copper/kg soil (29.12 cm) copper concentration.

Further, the root length exhibited a decline in the range of 100 mg copper/kg soil to 250 mg copper/kg soil copper concentrations. Specifically, the treatment with 50 mg copper/kg soil of copper resulted in a 9% enhancement in root growth. Batool et al. [27] stated that a decrease in root growth, accompanied by reduced cell division due to heavy metal stress, leads to alterations in the root structure by enhancing cell wall thickness. This finding was also reported by Yuan et al. [28], where it was found that Cu stress led to inhibition in root and stem development by altering cell division, elongation, and cell expansion in *Arabidopsis thaliana.*

3.10 Soil Microbial Respiration

The table represents the effect of copper on soil microbial respiration. It was found that the soil microbial respiration was significantly ($P < 0.05$) affected by different soil copper concentration. The released Co_2 was highest at 50 mg copper/kg soil copper concentration and the respiration value was 9.8733 g/day. Therefore, the microbial respiration at low level of copper was higher when compared to the untreated soil (control). The minimum Co_2 was released at 250 mg copper/kg soil (3.8 g/day). However, there was no significant ($P > 0.05$) difference between the soil treated with 200 mg copper/kg soil and 250 mg copper/kg soil.

Aoyama and Nagumo [29] reported that amount of released Co_2 is proportional to the microbial respiration. Thus, higher microbial respiration was recorded at 50 mg copper/kg soil copper concentration. These results align with Vogeler et al. [30], who stated that heavy metals induce stress on the microbial biomass. This decreasing soil microbial respiration might be due to the size of the microbial populations having progressively decreased with increasing Cu contamination in the soil. According to Aoyama and Nagumo [31], who reported that high concentrations of heavy metals decrease the microbial biomass and functional diversity of soil micro-organisms, and thus the biological activity of soils [32].

Table 8. The effect of different copper concentration on soil microbial respiration

Treatments	Microbial respiration {Released CO_2 (g/day)}
Control/untreated	$8.3 \times 10^{-4} \pm 0.000025^{b}$
50 mg copper/kg soil	$9.8 \times 10^{-4} \pm 0.000009^{a}$
100 mg copper/kg soil	$8.1 \times 10^{-4} \pm 0.000030^{b}$
150 mg copper/kg soil	$6.9 \times 10^{-4} \pm 0.000009^{c}$
200 mg copper/kg soil	$4.6 \times 10^{-4} \pm 0.000038^{d}$
250 mg copper/kg soil	$3.8 \times 10^{-4} \pm 0.000015^{d}$

Each value represents mean ± standard error of four replicates

4. CONCLUSION

The research has shown that that 50 mg copper/kg soil copper concentration significantly increased the soil microbial respiration by 22% compared to the control. The copper concentration at 100mg copper/kg soil significantly reduced the tap root length and number of nodules by 12% and 7% respectively compared to the control. This experiment confirmed that copper concentration ranging from 100-250 mg copper/kg soil has a deleterious effect on overall growth performance including the nodule formation of green grams. As a result, fertilizer recommendations might incorporate copper to stimulate plant growth, fostering self-sufficiency and prevent micronutrient deficiencies in green gram production.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

I hereby declare that no AI have been used during writing the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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