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The Effect of Cobalamin (Vitamin B_{12}) Application on Some Growth, Biochemical Parameters and *Pv-MATE14* Gene Expression Level in Bush Bean (*Phaseolus vulgaris* L.) Plants Exposed to Heavy Metal ($CuSO_4$) Stress

Ağır Metal ($CuSO_4$) Stresine Bırakılan Çalı Fasulyesi (*Phaseolus vulgaris* L.) Bitkisinde Kobalamin (Vitamin B_{12}) Uygulamasının Bazı Büyüme, Biyokimyasal Parametreler ve *Pv-MATE14* Gen Ekspresyon Seviyesi Üzerine Etkisi

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THE EFFECT OF COBALAMIN (VITAMIN B_{12}) APPLICATION ON SOME GROWTH, BIOCHEMICAL PARAMETERS AND *PV-MATE14* GENE EXPRESSION LEVEL IN BUSH BEAN (*Phaseolus vulgaris* L.) PLANTS EXPOSED TO HEAVY METAL ($CUSO_4$) STRESS

ABSTRACT

Copper (Cu) toxicity poses a significant threat to plant health, particularly as Cu levels in agricultural soils rise due to the use of Cu-based agrochemicals. The role of Vitamin B_{12} in mitigating this heavy metal stress in common bean has not been fully elucidated. Therefore, this study investigated the effects of copper(II) sulfate ($CuSO_4$), Vitamin B_{12}, and their combination treatments on growth parameters, key biochemical indices, and *Pv-MATE14* gene expression in bush bean (*Phaseolus vulgaris* L.) seedlings. $CuSO_4$ was applied at 50 and 125 ppm, and Vitamin B_{12} at 50 ppm. Solutions were applied to the soil in 60 mL aliquots every two days for a total of 14 days. At the end of the treatment period, we measured plant height, root and stem lengths, fresh weights, relative water content (RWC), proline, MDA contents, and *Pv-MATE14* expression. Results showed that $CuSO_4$ treatments significantly decreased all growth parameters and RWC, while drastically increasing proline and MDA content. $CuSO_4$ also significantly upregulated *Pv-MATE14* expression, severely inhibiting growth and development. Conversely, Vitamin B_{12} application increased growth parameters and RWC, decreased proline and MDA content, and significantly downregulated *Pv-MATE14* expression. These findings demonstrate that Vitamin B_{12} effectively mitigates the adverse effects of $CuSO_4$ stress by promoting growth, alleviating cellular damage, and normalizing the expression of the *Pv-MATE14* metal transporter gene in common bean.

Keywords: $CuSO_4$, *Phaseolus vulgaris* L., Vitamin B_{12}, MDA, Prolin, *Pv-MATE14.*

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AĞIR METAL ($CUSO_4$) STRESİNE BIRAKILAN ÇALI FASULYESİ (*Phaseolus vulgaris* L.) BİTKİSİNDE KOBALAMİN (VİTAMİN B_{12}) UYGULAMASININ BAZI BÜYÜME, BİYOKİMYASAL PARAMETRELER VE *PV-MATE14* GEN EKSPRESYON SEVİYESİ ÜZERİNE ETKİSİ

ÖZ

Bakır (Cu) toksisitesi, özellikle Cu bazlı zirai kimyasalların kullanımıyla toprak Cu seviyeleri arttıkça, bitki sağlığı için önemli bir tehdit oluşturmaktadır. Vitamin B_{12}'nin fasulye bitkilerinde Cu ağır metal stresini hafifletmedeki rolü tam olarak

aydınlatılmamıştır. Bu nedenle, bu çalışmada, çalı fasulye (*Phaseolus vulgaris* L.) tohumları materyal olarak kullanılmış ve bakır (II) sülfat ($CuSO_4$), Vitamin B_{12} ve kombinasyon uygulamalarının bazı büyüme parametreleri, biyokimyasal analizler ve *Pv-MATE14* gen ifadesi üzerindeki etkileri araştırılmıştır. Uygulanan konsantrasyonlar $CuSO_4$ (50 ve 125 ppm), Vitamin B_{12} (50 ppm) ve bunların kombinasyonları olup, konsantrasyonlar 14 gün boyunca ikişer gün aralıklarla 60 mL çözelti halinde bitkilerin toprağına uygulanmıştır. Bu sürenin sonunda, bitkiler hasat edilmiş ve uygulamaların bitki boyu, kök ve gövde uzunlukları (cm), kök, gövde ve yaprak yaş ağırlıkları (g), nispi su içeriği (%), prolin ve MDA içerikleri ve *Pv-MATE14* gen ifadesi düzeyi üzerindeki etkileri incelenmiştir. Çalışmanın sonuçlarına göre, $CuSO_4$ uygulamaları, tüm büyüme parametrelerini ve RWC'yi önemli ölçüde azaltmış, prolin ve MDA içeriğini şiddetli bir şekilde artırmış ve *Pv-MATE14* gen ifadesini belirgin olarak yukarı regüle ederek bitki büyüme ve gelişimini olumsuz etkilemiştir. Öte yandan, Vitamin B_{12} uygulaması, büyüme parametrelerini ve RWC'yi artırmış, prolin ve MDA içeriğini azaltmış ve *Pv-MATE14* ifadesini önemli ölçüde aşağı regüle ederek bitki büyüme ve gelişiminde olumlu bir etki göstermiştir. Bu bulgular, Vitamin B_{12}'nin $CuSO_4$ stresinin olumsuz etkilerini etkin bir şekilde hafiflettiğini ve fasulyede büyümeyi desteklerken hücresel hasarı azalttığını ve *Pv-MATE14* metal taşıyıcı geninin ifadesini normalleştirdiğini göstermektedir.

Anahtar Kelimeler: $CuSO_4$, *Phaseolus vulgaris* L., Vitamin B_{12}, MDA, Prolin, *Pv-MATE14*.

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1. INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the most important renewable legume crops worldwide, providing essential proteins, dietary fibers, B-complex vitamins, and rich micronutrients crucial for the nutrition of a large portion of the global population (Sangronis et al., 2006; Wu et al., 2020). As a member of the legume family, common bean is a significant source of calories and protein, particularly in developing countries in Latin America and Africa (Graham, 1978). Beyond its nutritional importance, common bean holds great economic significance as it provides income for millions of smallholder farmers (Colmenero-Flores et al., 1997). Furthermore, it is vital in agriculture due to its ability to form root nodules through symbiotic relationships with nitrogen-fixing bacteria (Broughton et al., 2003). Unfortunately, common bean production faces various significant threats from abiotic stresses (Schneider et al., 1997).

Heavy metals are among the most significant abiotic stresses, accumulating in the environment through human activities or natural processes, thereby posing a high risk to both human health and agriculture (Castro et al., 2011). Some

heavy metals, such as Cu, Zn, Mn, Fe, and Mo, are micronutrients required in small amounts for healthy plant growth. These metals act as cofactors for various enzymes and participate in essential physiological processes. However, high concentrations of both macro and micro heavy metals cause toxicity in plants, leading to growth inhibition (Gill et al., 2022). Since heavy metals are non-biodegradable, they accumulate excessively in the environment, causing contamination in the food chain. Therefore, understanding and mitigating the effects of heavy metals on plants is crucial for promoting sustainable agriculture (Liu et al., 2020).

Copper (Cu) is one of the heavy metals naturally found in trace amounts in soil (Kaźmierczak et al., 2024). Cu is an essential micronutrient for plant growth and development, participating in many physiological processes, and serves as a crucial cofactor for numerous enzymes involved in respiration, photosynthesis, and electron transport processes (Mendes et al., 2024). Excess Cu leads to various disorders in plants, such as inhibited growth, reduced biomass, and ultimately death. At high concentrations, Cu is highly toxic to all living organisms. Conversely, Cu deficiency in higher plants manifests through the activation of morphological changes in leaves and roots (Rengel et al., 2022). As a redox metal, Cu produces reactive oxygen species (ROS) via the Fenton reaction, which can lead to oxidative stress, causing peroxidation and damage to cell membranes, ultimately resulting in cell death (Kováčik et al., 2008). Therefore, plants have evolved a complex antioxidant system, genetically controlled and composed of enzymatic and non-enzymatic antioxidants, to regulate intracellular Cu levels and combat oxidative stress. They produce various compounds such as phenolic acids, flavonoids, polyamines, and proline as antioxidants (Badiaa et al., 2020).

Vitamin B_{12} is a relatively large molecule with a molecular weight of 1355.38 g mol^{-1}. The active form of Vitamin B_{12} is cobalamin or cyanocobalamin. In its natural habitat, agricultural soil is a rich source of Vitamin B_{12}, where 70% of certain bacteria and archaea can synthesize Vitamin B_{12}, which is then absorbed by plant roots. Consequently, soil is considered one of the richest natural sources of Vitamin B_{12}. Despite the general belief that plants cannot synthesize Vitamin B_{12}, there are reports indicating that some plants contain detectable amounts of Vitamin B_{12}. For instance, reports suggest that pea (*Pisum sativum* L.) can synthesize it (Lilly and Leonian, 1939; Lochhead and Thexton, 1951; Smith, 1960; Kononova, 2013).

The application of cyanocobalamin as a biostimulant promotes plant yield and, indirectly, plant metabolism by increasing rhizobia activity and improving plant growth, including leaf area, shoot, and root development (Mozafar, 1994; Soppelsa et al., 2019; Rehim et al., 2021). This vitamin also plays a role in inducing or suppressing the expression of specific genes and can potentially serve as an indirect stimulant of proline biosynthesis, which is linked to the pentose phosphate pathway activated during stress (Pitzschke et al., 2015). Furthermore, B-group vitamins

have been shown to function as powerful antioxidants, equivalent to Vitamins C and E, and are particularly active in quenching singlet oxygen (Burguieres et al., 2007; Hanson et al., 2016).

The MATE gene family is widely distributed in bacteria, archaea, animals, and plants (Wang et al., 2016). Most members of the MATE family possess 450 to 550 amino acid residues, while a few members can have up to 700. The sequence of MATE proteins generally contains 9–12 transmembrane helices, which enables their shuttling function across cell membranes and participation in physiological activities both inside and outside the cell (Moriyama et al., 2008). In plants, MATE proteins are subcellular proteins localized in plasma membranes, vacuoles, and thylakoids. These proteins have been proven to play a crucial role in the transport of inorganic anions, secondary metabolites, and heavy metals, in the regulation of iron homeostasis, in the detoxification of exogenous substances, in the regulation of growth and senescence, and in the response to disease and abiotic stress in many plants (Tiwari et al., 2014; Liu et al., 2016; Santos et al., 2017; Huang et al., 2021).

Relatively few studies have investigated the effects of heavy metals and their potential mitigation in common bean (*Phaseolus vulgaris* L.) plants. In this context, the aim of this study was to develop tolerance mechanisms in common bean plants exposed to different concentrations (50 and 125 ppm) of $CuSO_4$ toxicity. This was achieved by examining the effects of Vitamin B_{12} application (at a concentration of 50 ppm) on several growth parameters, biochemical analyses, and the expression level of the *Pv-MATE14* gene.

2. MATERIAL AND METHODS

2.1. Plant Growth Conditions and Treatments

This research was conducted in 2024 at the Kars Kafkas University Life Sciences and Technologies Application and Research Center. Common bean (*Phaseolus vulgaris* L.) seeds, sourced from local farmers, were used as plant material. Prior to sowing, the seeds were surface sterilized for 5 minutes in 5% sodium hypochlorite and 30 seconds in 96% ethanol, followed by thorough rinsing with distilled water. The seeds were sown to a depth of 2–3 cm in multiple trays (viols) filled with a mixture of peat, perlite, and soil (1:1:1) (Yiamthaisong et al., 2024). After sowing, the trays were transferred to a climate-controlled cabinet programmed for a 24/20 ∘C (day/night) temperature, a 16/8-hour (light/dark) photoperiod, and 55% humidity. Seedlings were irrigated exclusively with distilled water every other day until the application phase commenced. The application phase for $CuSO_4$ and Vitamin B_{12} began four weeks after sowing. Concentrations were determined based on literature review and preliminary experiments, establishing a total of 6 treatment groups

including a control (Table 1). The treatments included: $CuSO_4$ (50 and 125 ppm), Vitamin B_{12} (50 ppm), and the combination treatments ($CuSO_4$ 50 ppm+Vitamin B_{12} 50 ppm) and ($CuSO_4$ 125 ppm+Vitamin B_{12} 50 ppm). Concentration solutions were prepared solely with distilled water and applied to the soil as 60 mL aliquots every two days. The control group received only distilled water. The application period was terminated on the 14th day, based on morphological observations of the plants. Samples designated for biochemical analyses were immediately stored at -80 ∘C.

Table 1. Concentration applications

Applications	$CuSO_4$	+	Vitamin B_{12}
1.	Control		Control
2.	50 ppm		-
3.	125 ppm		-
4.	-		50 ppm
5.	50 ppm	+	50 ppm
6.	125 ppm	+	50 ppm

2.2. Some Growth Parameters

At the end of the 14th day, the common bean plants were harvested. Root, stem, and total plant lengths were measured using a precision ruler. Subsequently, the fresh weights of the roots, stems, and leaves were determined using a precision balance.

2.3. Determination of Proline Content

Proline content was determined using the method cited by Bates et al. (1973). Both root and leaf samples (0.2 g each) were weighed, ground to a fine powder in liquid nitrogen using a mortar, and then homogenized with the addition of 3 mL of 3% sulfosalicylic acid. The homogenate was centrifuged at 18.000 rpm for 15 minutes, after which 2 mL of the supernatant was transferred to a new test tube. To this, 2 mL of glacial acetic acid and 2 mL of acid ninhydrin solution were added. After incubation in a water bath at 100 ∘C for 1 hour, the mixture was cooled immediately on ice. Finally, 4 mL of toluene was added, mixed, and the colored layer was transferred to a glass cuvette. Absorbance was measured at 520 nm using a spectrophotometer in triplicate, and proline content was expressed as $\mu g\ g^{-1}$ fresh weight (FW).

2.4. Determination of Lipid Peroxidation Level

MDA content was determined according to the method of Heath and Packer (1968). Both root and leaf samples (0.2 g each) were weighed, ground to a fine powder in liquid nitrogen using a mortar, and then homogenized with the addition of 10 mL of 0.1% trichloroacetic acid (TCA). The resulting homogenate was centrifuged at 15.000 rpm for 5 minutes. Subsequently, a 1 mL aliquot of the supernatant was transferred, and 4 mL of 0.5% thiobarbituric acid (TBA) dissolved in 20% TCA was added. This mixture was then incubated in a 95 ∘C water bath for 30 minutes and rapidly cooled in an ice bath. After a final centrifugation at 10.000 rpm for 10 minutes, the supernatant was collected. Absorbance was measured in triplicate at 532 nm and 600 nm using a spectrophotometer, and MDA content was quantified as nmol g^{-1} fresh weight (FW).

2.5. Determination of Relative Water Content (RWC)

Relative Water Content (RWC) was determined using the method established method. Four random leaf samples were collected from common bean plants, and their fresh weights (FW) were immediately measured. The samples were then immersed in distilled water at room temperature for 2 hours until fully saturated, and their turgor weights (TW) were subsequently recorded. Finally, the samples were dried in an oven at 72 ∘C for 48 hours, and their dry weights (DW) were recorded (Barrs and Weatherley, 1962).

Relative Water Content (%) = [(Fresh Weight – Dry Weight) / (Turgid Weight – Dry Weight)] × 100

2.6. RNA Isolation, cDNA Synthesis, and qRT-PCR Analysis

Total RNA was extracted from 0.2 g common bean leaf samples using an RNA Mini Kit (PureLink™/Cat. No. 12183018A), following the manufacturer's instructions. The quality and quantity of the isolated RNA were determined using a nanodrop (Thermo Scientific Multiscan) device, specifically evaluating the 260/280 ratio.

For cDNA synthesis, the isolated RNA samples were used according to the protocol of the RevertAid First Strand cDNA Synthesis Kit (Thermoscientific/Cat. No. K1622). The 20 µL reaction mixture was composed as follows: 5×Buffer (4 µL), RNase inhibitor (1 µL), dNTP (2 µL), RevertAid (RT) (1 µL), random primer (1 µL), and RNase-free water and RNA sample (variable amounts to reach final volume). The reaction conditions were: incubation at 65 ∘C for 5 minutes, followed by 5 minutes at 25 ∘C and 60 minutes at 42 ∘C. After the reaction completed, 50 µL of dH_2O was added to the cDNA samples prior to the qRT-PCR stage.

qRT-PCR was performed using the Hot Firepol Evagreen Real-Time qPCR Supermix 5x (Solis Biodyne/Cat. No. 08-36-00001), following the kit protocol. The 10 µL reaction mixture contained: Solis Biodyne Evagreen Supermix (2 µL), Forward primer (0.2 µL), Universal primer (0.2 µL), cDNA sample (2 µL), and Nuclease Free Water (5.6 µL).

The thermal cycling conditions were:

1. Pre-denaturation: 95 ∘C for 12 minutes (1 cycle)
2. Denaturation: 95 ∘C for 15 seconds (40 cycles)
3. Annealing: 58 ∘C for 20–30 seconds (40 cycles)
4. Extension: 72 ∘C for 20–30 seconds (40 cycles)

The Common bean *ACTIN-11* gene was used as the reference gene for signal normalization (Du et al., 2022). The primers utilized for qRT-PCR are listed in Table 2 (Zhao et al., 2024). All reactions were conducted using triplicate biological repeats for each sample. Changes in gene expression were calculated from the resulting data using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2. Sequence information of primers used

ACTIN-11	
F-5'	TGCATACGTTGGTGATGAGG-3'
R-5'	AGCCTTGGGGTTAAGAGGAG-3'
Pv-MATE14	
F-5'	TGTTCCTGGTATCTGGTCTG-3'
R-5'	AAGCGATGCCTCTTTATTC-3'

2.7. Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software. The study was conducted with 3 biological replicates, and the data were presented as the mean±standard deviation (SD) of these replicates. Data were evaluated using One-way ANOVA followed by Duncan's Multiple Range Test, with a significance level set at $p<0.05$ (Field, 2024).

RESULTS AND DISCUSSION

Effect of $CuSO_4$, Vitamin B_{12}, and combined applications on plant height, root, and stem length in *Phaseolus vulgaris* L. seedlings

Our research findings indicate that plant height across all groups ranged from 23.49 to 37.39 cm (Table 3). Both concentrations of $CuSO_4$ heavy metal application significantly reduced plant height compared to the control, with this reduction becoming more pronounced as the concentration increased. Conversely, Vitamin B_{12} application significantly increased plant height compared to the control. The effects of both $CuSO_4$ and Vitamin B_{12} applications on common bean plant height were found to be statistically significant. The lowest plant height was observed in the 125 ppm $CuSO_4$ treatment, showing a 37% reduction compared to the control. In contrast, the highest increase in plant height, at 12%, was noted with the 50 ppm Vitamin B_{12} application ($p<0.05$). Combination treatments of both $CuSO_4$ concentrations with Vitamin B_{12} showed reduced plant height compared to the control. However, when compared to the respective $CuSO_4$ alone treatments, these combinations notably increased plant height (Table 3). Similar studies on the effects of heavy metal application on plant height corroborate our findings. A study on tomato (*Lycopersicon esculentum* L.) seedlings reported that Cu application (0,1000, and 2000 mg kg^{-1}) reduced plant height by 39% and 50% respectively, with the reduction increasing at higher concentrations (Sonmez et al., 2006). Another study on barley (*Hordeum vulgare* L.) indicated that 40–80 µM $CuSO_4$ promoted plant growth, while 400–1600 µM $CuSO_4$ applications significantly reduced plant height (Özcan, 2003). In contrast, exogenous application of Vitamin B_{12} (0, 50, 100 mg L^{-1}) to cabbage (*Brassica oleracea* var. *capitata*) was reported to increase plant height (Al-Ameri et al., 2022).

Root lengths across all treatment groups ranged from 6.43 to 15.03 cm (Table 3). Both concentrations of $CuSO_4$ heavy metal application significantly reduced plant root length compared to the control, with the reduction becoming more pronounced at higher concentrations. Conversely, Vitamin B_{12} application significantly increased root length compared to the control. The effects of both $CuSO_4$ and Vitamin B_{12} applications on common bean root length were statistically significant ($p<0.05$). The lowest root length was observed in the 125 ppm $CuSO_4$ treatment, showing a 46% reduction compared to the control group. The highest increase in root length, similar to the findings for plant height, was observed with the 50 ppm Vitamin B_{12} application, showing a 27% increase. Combination treatments of both $CuSO_4$ concentrations with Vitamin B_{12} resulted in reduced root lengths compared to the control. However, when compared to the respective $CuSO_4$ alone treatments, these combinations notably increased root length (Table 3). It is well-known that Cu heavy metal negatively affects plant growth and development. Since Cu translocation from roots to shoots is often limited due to its difficult passage via passive transport, high concentrations tend to accumulate in the roots (Trakal et al., 2015). Literature studies demonstrating that Cu applications lead to reductions in root length align with our findings. For instance, a study on flax (*Linum usitatissimum* L.) seeds reported a decrease in root length with increasing concentrations of app-

lied Cu heavy metal (0.01, 0.05, 0.1, 0.5, and 1 mM) (Soudek et al., 2010). Another study on eggplant (*Solanum melongena* L.) seeds reported that Cu heavy metal applied at different concentrations (30, 60, 120, and 240 mg L^{-1}) caused a reduction in plant root length, with this reduction being more pronounced at increasing concentrations (Körpe and Aras, 2011). In contrast, the application of B-group vitamins (250 mg L^{-1}) to maize (*Zea mays* L.) varieties (cv. Pearl and cv. Akbar) was reported to increase root length in both varieties (Atif et al., 2024). A study with common bean plants similarly found that 11 and 22 µM concentrations of Vitamin B_{12} increased root lengths compared to the control (Keshavarz et al., 2015).

When evaluating stem lengths, values across all groups ranged from 6.99 to 14.10 cm (Table 3). The lowest stem length was observed at the highest concentration (125 ppm) of $CuSO_4$ heavy metal application, showing a 31% reduction compared to the control. Conversely, the highest stem length was seen with the 50 ppm Vitamin B_{12} application, indicating a 39% increase. Both $CuSO_4$ and Vitamin B_{12} applications were found to be statistically significant compared to the control ($p<0.05$). When considering the combinations of Vitamin B_{12} with both concentrations of $CuSO_4$, it was observed that Vitamin B_{12} mitigated the reduction in stem lengths caused by the heavy metal, compared to the $CuSO_4$ alone treatments. Similar studies in the literature concerning stem lengths support these findings. One study reported a significant decrease in stem growth in common bean (*Phaseolus vulgaris* L.) seedlings exposed to Cu stress at concentrations of 1.5, 2, and 2.5 mM (Zengin and Munzuroğlu, 2005). It was also reported that Cu concentrations (50, 100, 150, 200, 250 mg kg^{-1}) applied to mung bean (*Vigna radiata* (L.) Wilczek) negatively affected cell division and reduced stem length (Manivasagaperumal et al., 2011). Another study investigating Cu heavy metal on Red Maple (*Acer rubrum* L.) also reported an adverse effect on stem length (de Silva et al., 2012). In contrast, a study on pumpkin (*Cucurbita moschata* Duch) found no change in stem lengths at 50 mM Cu application compared to the control, but an increase in stem lengths at 100 mM Cu concentration compared to the control (Sevgi, 2023). Regarding the effect of B-group vitamins on plant stem length, a study on marigold (*Calendula officinalis* L.) reported that thiamine (Vitamin B_1) concentrations (50 and 100 ppm) increased plant stem length (Soltani et al., 2014).

Based on the findings regarding plant height, root, and stem lengths in *Phaseolus vulgaris* L. plants, increasing concentrations of $CuSO_4$ heavy metal significantly reduced all measured parameters. Conversely, Vitamin B_{12} application significantly increased plant height, root, and stem lengths. These opposing effects were all found to be statistically significant ($p<0.05$). These results collectively suggest that Vitamin B_{12} may promote the synthesis of protective compounds that mitigate $CuSO_4$-induced toxicity and enhance plant tolerance under heavy metal stress.

Table 3. Effects of $CuSO_4$, Vitamin B_{12} and combination treatments on plant height, root and stem length of *Phaseolus vulgaris* L. seedlings

Applications	Plant Height (cm)	Root Length (cm)	Stem Length (cm)
Control	37.39±0.23^{b}	11.85±0.43^{b}	10.16±0.42^{b}
50 ppm $CuSO_4$	29.53±0.03^{d}	8.76±0.14^{c}	8.33±0.66^{bc}
125 ppm $CuSO_4$	23.49±0.84^{e}	6.43±0.17^{d}	6.99±0.55^{c}
50 ppm Vitamin B_{12}	41.98±1.06^{a}	15.03±0.33^{a}	14.10±0.05^{a}
50 ppm $CuSO_4$+ 50 ppm Vitamin B_{12}	33.56±0.72^{c}	9.74±0.52^{bc}	9.82±0.91^{b}
125 ppm $CuSO_4$+ 50 ppm Vitamin B_{12}	27.13±0.46^{d}	8.38±0.67^{cd}	8.83±0.21^{bc}

* $CuSO_4$: Copper (II) sulfate
*Values are presented as mean ± standard deviation of three parallel measurements (n=3)
*Differences between group means sharing different letters in the same column are statistically significant ($p<0.05$)

3.2. Effect of $CuSO_4$, Vitamin B_{12}, and Combined Applications on Root, Stem, and Leaf Fresh Weights of *Phaseolus vulgaris* L. Seedlings

Upon evaluating the root fresh weights of common bean plants, values across all groups ranged from 0.17 to 0.96 g (Table 4). We observed a decrease in root fresh weight as the concentration of $CuSO_4$ application increased, while Vitamin B_{12} application led to a corresponding increase. The lowest root fresh weight, representing a 78% reduction compared to the control, occurred at the highest $CuSO_4$ concentration (125 ppm). Conversely, the highest root fresh weight, showing a 26% increase compared to the control, was observed with the 50 ppm Vitamin B_{12} application ($p<0.05$). Combination treatments of both $CuSO_4$ concentrations with Vitamin B_{12} reduced root fresh weights when compared to the control. However, these combinations increased root fresh weights when compared to the respective $CuSO_4$ alone treatments (Table 4). Growth parameters are closely linked to weight gain in plants, and metal stress generally reduces plant weight, a phenomenon widely reported by many researchers (Abdelgawad et al., 2020). Studies on Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) reported that $CuSO_4$ applications (10, 25, and 50 µM) reduced the root fresh weight of plants compared to the control (Feigl et al., 2013). Similarly, a study on pumpkin (*Cucurbita moschata* Duch.) indicated that high concentrations (50 and *100* mM) of applied Cu heavy metal decreased root fresh weight, while a low concentration increased it (Sevgi, 2023). It has also been reported that boron (B) stress applied to wheat (*Triticum aestivum* L.) plants reduced fresh weight, but the application of B-group vitamins (thiamine) at 150 µM increased the fresh weight of the plants

(El-Shazoly et al., 2019). Likewise, a study on radish (*Raphanus sativus* L.) reported that Vitamin B_{12} concentrations (0.5, 1.0, and 1.5 mg L^{-1}) increased the fresh weight of the plant (Rehim et al., 2021).

An evaluation of stem fresh weights in common bean plants revealed that values across all groups ranged from 0.52 to 1.54 g (Table 4). The effects of both $CuSO_4$ and Vitamin B_{12} applications on common bean stem fresh weight were found to be statistically significant ($p<0.05$). Stem fresh weight decreased as the concentration of $CuSO_4$ application increased, while, conversely, Vitamin B_{12} application led to an increase. The lowest stem fresh weight, representing a 65% reduction compared to the control, occurred at the highest $CuSO_4$ concentration (125 ppm). The highest stem fresh weight, showing *a 3%* increase compared to the control, was observed with the 50 ppm Vitamin B_{12} application. Combination treatments of both $CuSO_4$ concentrations with Vitamin B_{12} reduced stem fresh weights when compared to the control. However, these combinations increased stem fresh weights when compared to the respective $CuSO_4$ alone treatments (Table 4). Literature studies concerning stem fresh weights and Cu heavy metal stress are generally consistent with our findings. A study on common bean (*Phaseolus vulgaris*) reported that low concentrations of $CuSO_4$ and $CdCl_2$ heavy metal stresses (10, 25, and 50 mg L^{-1}) increased stem fresh weight, but the weight subsequently decreased as the concentration was further raised compared to the control group (Stingu et al., 2009). Similarly, a study on pumpkin (*Cucurbita moschata* Duch.) indicated that high concentrations (50 and 100 mM) of applied Cu heavy metal decreased stem fresh weight, while a low concentration resulted in an increase (Sevgi, 2023). For cherry plum (*Prunus cerasifera*) plantlets, it was reported that concentrations up to 50 µM $CuSO_4$ (10, 50, and 100 µM) allowed the plantlets to tolerate heavy metal stress, but fresh weights decreased, and the growth rate slowed at the 100 µM $CuSO_4$ concentration (Lombardini and Sebastiani, 2005). Regarding the effect of Vitamin B_{12} on stem fresh weight, studies show that B-group vitamins increase stem fresh weight; for example, exogenous application of Vitamin B_{12} (50, 100, and 150 ppm) to potato (*Solanum tuberosum* L.) plants was reported to increase stem fresh weight compared to the control (Youssif et al., 2017).

When evaluating the leaf fresh weights of common bean plants, values across all groups ranged from 2.46 to 6.98 g (Table 4). Results for leaf fresh weight mirrored the trends observed for root and stem fresh weights. The effects of both $CuSO_4$ and Vitamin B_{12} applications on leaf fresh weight were found to be statistically significant ($p<0.05$). Compared to the control group, both concentrations of $CuSO_4$ application caused a decrease in leaf fresh weight, and this reduction intensified with increasing concentration. Conversely, Vitamin B_{12} application led to an increase in leaf fresh weight compared to the control group. The lowest leaf fresh weight, representing a 61% reduction, occurred with the 125 ppm $CuSO_4$ application. In contrast, the highest leaf fresh weight, showing an 11% increase compared to the

control, was observed with the 50 ppm Vitamin B_{12} application. Combination treatments of both $CuSO_4$ concentrations with Vitamin B_{12} reduced leaf fresh weights compared to the control, yet they increased leaf fresh weights when compared to the respective $CuSO_4$ alone treatments (Table 4). Studies report that heavy metal stress reduces leaf fresh weight, and high doses of Cu can lead to various physiological impairments, including disruptions in chlorophyll biosynthesis, photosynthesis, respiration, and stomatal closure (Alaoui-Sossé, 2004). A study on common bean (*Phaseolus vulgaris*) reported that low concentrations of $CuSO_4$ and $CdCl_2$ heavy metal stresses (10, 25, and 50 mg L^{-1}) increased leaf fresh weight, but as the concentration increased, leaf fresh weight decreased compared to the control group (Stingu et al., 2009). Another study reported that 50 µM $CuSO_4$ heavy metal stress applied to pepper (*Capsicum annuum* L.) plants affected and reduced leaf fresh weight, among other parts of the plant (Díaz et al., 2001). In contrast, concentrations of thiamine (Vitamin B_1) (50 and 100 ppm*)* applied to marigold (*Calendula officinalis* L.) plants were reported to increase fresh weight, with the highest increase occurring at the higher concentration (Soltani et al., 2014). Similarly, it was reported that 100 mg L^{-1} *Vitamin* B_{12} applied to maize (*Zea mays* L.) seedlings increased fresh weight compared to the control, suggesting that Vitamin B_{12} may play a protective role in plants against stress (Chi et al., 2021).

Based on the findings regarding the root, stem, and leaf fresh weights of *Phaseolus vulgaris* L. plants, increasing concentrations of $CuSO_4$ heavy metal significantly reduced the fresh weights of all three organs. Conversely, Vitamin B_{12} application significantly increased the fresh weights of these organs. Both the detrimental effects of $CuSO_4$ and the beneficial effects of Vitamin B_{12} were found to be statistically significant ($p<0.05$).

Table 4. Effects of $CuSO_4$, Vitamin B_{12} and combination treatments on root, stem and leaf wet weights of *Phaseolus vulgaris* L. seedlings

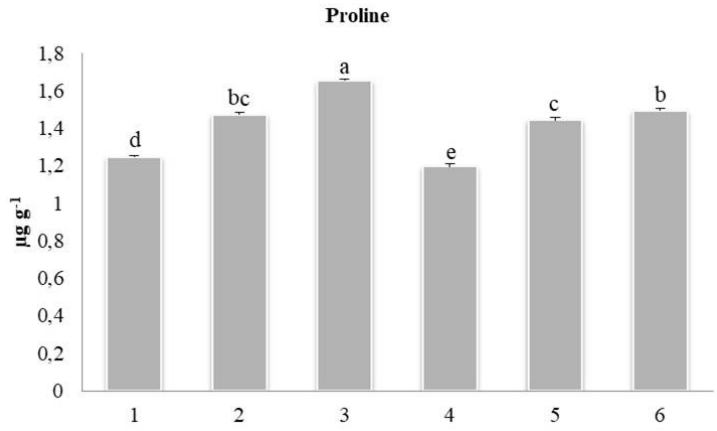
Applications	Root Fresh Weight (g)	Stem Fresh Weight (g)	Leaf Fresh Weight (g)
Control	0.76 ± 0.07^{b}	1.49 ± 0.00^{ab}	6.29 ± 0.34^{b}
50 ppm $CuSO_4$	0.42 ± 0.06^{c}	0.81 ± 0.00^{c}	3.86 ± 0.03^{c}
125 ppm $CuSO_4$	0.17 ± 0.04^{d}	0.52 ± 0.06^{d}	2.46 ± 0.23^{d}
50 ppm Vitamin B_{12}	0.96 ± 0.01^{a}	1.54 ± 0.02^{a}	6.98 ± 0.01^{a}
50 ppm $CuSO_4$+ 50 ppm Vitamin B_{12}	0.47 ± 0.05^{c}	0.85 ± 0.00^{c}	4.47 ± 0.15^{c}
125 ppm $CuSO_4$+ 50 ppm Vitamin B_{12}	0.25 ± 0.02^{d}	1.44 ± 0.04^{b}	2.76 ± 0.21^{d}

* $CuSO_4$: Copper (II) sulfate
*Values are presented as mean ± standard deviation of three parallel measurements (n=3)
*Differences between group means sharing different letters in the same column are statistically significant ($p<0.05$)

3.3. Determination of Proline Content

Proline, an amino acid, accumulates in plants to prevent the osmotic imbalance that results from damage to the cell membrane caused by ROS. Proline accumulation is thus considered a key indicator of cellular damage or stress. Along with total soluble proteins, proline is one of the most significantly altered amino acids in plants under stress conditions (Sharma et al., 2004; Valliyodan and Nguyen, 2006; Heidari and Moaveni, 2009). As shown in Figure 1, proline content ranged from 1.19 to 1.65 μg g^{-1} FW. Both concentrations of $CuSO_4$ application resulted in a significant increase in proline content compared to the control. The highest increase, at 33%, was observed with the 125 ppm $CuSO_4$ application. Conversely, the 50 ppm Vitamin B_{12} application led to a 4% decrease in proline content compared to the control. When comparing the combination treatments of Vitamin B_{12} with both $CuSO_4$ concentrations, these combinations consistently showed lower proline content than the respective $CuSO_4$ alone treatments, indicating a mitigating effect. Proline content was statistically significant across all applied treatments compared to the control ($p<0.05$). Literature studies on proline content support our findings. A study on rice (*Oryza sativa* cv.) seedlings reported that 0.5 mM Cu stress increased proline content in rice shoots (Chen et al., 2004). Similarly, it was reported that Cu heavy metal (0, 200, 300, 400, and 500 ppm) applied to tomato (*Lycopersicon esculentum* Mill.) plants increased proline content compared to the control, with this increase rising gradually with increasing Cu concentration (Badiaa et al., 2020). Likewise, a study on common bean (*Phaseolus vulgaris* L.) reported that Cu stress (0.1, 0.2, and 0.3 mM) applied for ten days increased proline content by 12.2%, 21.3%, and 30.9% respectively (Zengin and Munzuroğlu, 2005). Regarding the effect of B vitamins on proline in plants, a study on two pea (*Pisum sativum* L.) varieties reported that B-group vitamins increased osmolite activities (proline, soluble sugars) (Kausar et al., 2023). Similarly, it was reported that thiamine (0.3 mM) applied to wheat (*Triticum aestivum* L.) plants under salt stress severely increased proline content compared to the control (Al-Hakimi et al., 2001).

Our findings regarding proline content in *Phaseolus vulgaris* L. plants indicate that increasing $CuSO_4$ concentrations significantly elevated proline levels. This increase is primarily interpreted as the plant's attempt to counteract $CuSO_4$-induced osmotic imbalance and oxidative stress. Conversely, Vitamin B_{12} application led to a decrease in proline content, strongly suggesting its role in mitigating the adverse physiological and osmotic effects induced by $CuSO_4$ in the plant.



*1: Control, 2: 50 ppm $CuSO_4$, 3: 125 ppm $CuSO_4$, 4: 50 ppm Vitamin B_{12}, 5: 50 ppm $CuSO_4$ + 50 ppm Vitamin B_{12}, 6: 125 ppm $CuSO_4$ + 50 ppm Vitamin B_{12}

*Differences between group means sharing different letters in the same column are statistically significant ($p<0.05$)

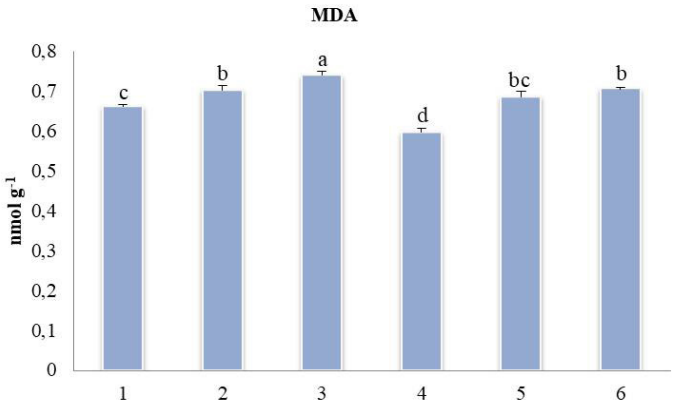
Figure 1. Proline content in bush bean (*Phaseolus vulgaris* L.) plants

3.4. Determination of Lipid Peroxidation Level

Stress factors (both biotic and abiotic) cause lipid peroxidation (Prasad et al., 1999). Oxidative stress, which is often triggered by the production of reactive oxygen species (ROS), is a primary effect induced by copper heavy metal. Oxidative stress can damage the structure of lipids, carbohydrates, proteins, and DNA, potentially leading to irreversible harm as the duration of stress increases. Malondialdehyde (MDA) is formed as an end product of the peroxidation of fatty acids containing three or more double bonds; therefore, MDA content serves as a widely used marker to determine the level of lipid peroxidation (Onat et al., 2002). As seen in Figure 2, MDA content ranged from 0.59 to 0.73 nmol g^{-1} FW. Both concentrations of $CuSO_4$ application (50 and 125 ppm) significantly increased MDA content compared to the control, with this increase becoming more pronounced at the higher concentration. The highest increase in MDA content, at 11%, was observed with the 125 ppm $CuSO_4$ application. Conversely, the Vitamin B_{12} application resulted in a 4% decrease in MDA content compared to the control. When comparing the combination treatments of Vitamin B_{12} with both $CuSO_4$ concentrations, these combinations exhibited lower MDA content than the respective $CuSO_4$ alone treatments, indicating a clear protective effect. MDA content was found to be statistically significant across all applied treatments compared to the control ($p<0.05$). Literature studies on MDA content support our findings. A study on tomato (*So-*

lanum lycopersicum L.) seeds reported that 0.1 mM Cu heavy metal application increased MDA content compared to the control (Zhang et al., 2022). Another study on fenugreek (*Trigonella foenum-graecum*) with different Cu concentrations (0.005, 0.1, 1, 10, and 20 mM) reported that high concentrations increased MDA content (Elleuch et al., 2013). Similarly, it was reported that Cu concentrations (0, 10, 50, and 100 µM) applied to rice (*Oryza sativa* L.) plants for five days increased lipid peroxidation, and this increase was concentration-dependent (Thounaojam et al., 2012). In addition, a study on alfalfa (*Medicago sativa* L.) under lead stress demonstrated that lead (II) acetate application at 100 and 1000 ppm significantly increased MDA content (Akçay, 2025). In contrast, exogenous application of Vitamin B to maize plants was reported to reduce MDA content under As stress (Atif et al., 2024). Similarly, it was reported that 10 and 20 µM concentrations of Vitamin B_{12} applied to chickpea (*Cicer arietinum* L.) plants under salt stress significantly reduced MDA content compared to the stress group (Manjunatha, 2018).

Our findings regarding MDA content in *Phaseolus vulgaris* L. plants indicate that increasing $CuSO_4$ concentrations significantly elevated MDA levels, consistent with enhanced lipid peroxidation and subsequent cellular damage within the plant. Conversely, Vitamin B_{12} application resulted in a decrease in MDA content, strongly suggesting that it mitigates the oxidative damaging effects induced by $CuSO_4$ in the plant.



*1: Control, 2: 50 ppm $CuSO_4$, 3: 125 ppm $CuSO_4$, 4: 50 ppm Vitamin B_{12}, 5: 50 ppm $CuSO_4$ + 50 ppm Vitamin B_{12}, 6: 125 ppm $CuSO_4$ + 50 ppm Vitamin B_{12}

*Differences between group means sharing different letters in the same column are statistically significant ($p<0.05$)

Figure 2. MDA content in bush bean (*Phaseolus vulgaris* L.) plants

3.5. Determination of Relative Water Content (RWC)

Relative water content (RWC) is a fundamental parameter of plant water physiology, widely used to assess the water status of plants significantly affected by stress conditions (Kardile et al., 2018). The RWC of common bean (*Phaseolus vulgaris* L.) plants is presented in Table 5. Considering all applied concentrations, RWC values ranged from 30.74% to 56.22%. Both heavy metal $CuSO_4$ and Vitamin B_{12} applications had a statistically significant effect on RWC compared to the control ($p<0.05$). The greatest reduction in RWC, at 41%, was observed with the highest concentration of $CuSO_4$ heavy metal stress. Conversely, the highest RWC, showing a 9% increase, occurred with the Vitamin B_{12} application. Metal stress reduces main root growth and capillary root density, which hinders water uptake from the soil and leads to water deficit in plants (Rucińska-Sobkowiak, 2016). Our findings are supported by heavy metal studies that determined RWC. For instance, Cu and Zn heavy metals applied to ice plants (*Mesembryanthemum crystallinum*) severely suppressed root exudation (Kholodova et al., 2011). Similarly, Cu heavy metal stress (50, 100, and 200 µM) applied to barley (*Hordeum vulgare* L.) plants reduced the plants RWC (Sığmaz, 2022). In contrast, thiamine (150 µM) applied against boron (B) stress increased RWC in wheat (*Triticum aestivum* L.) plants (El-Shazoly et al., 2019).

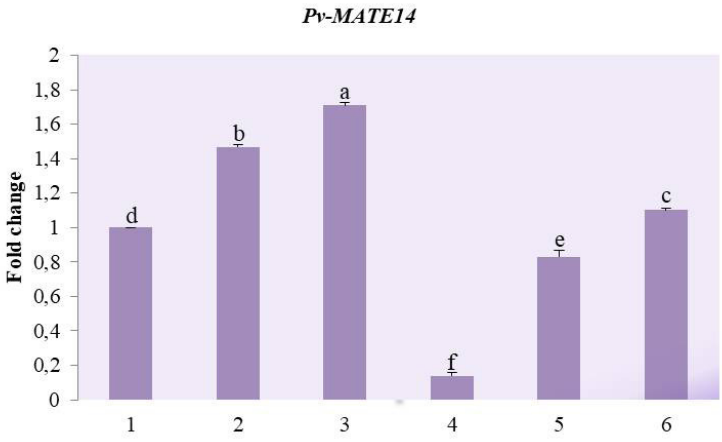
Table 5. Effect of $CuSO_4$, Vitamin B_{12} and combination treatments on relative water content (RWC) of *Phaseolus vulgaris* L. seedlings

Applications	RWC (%)
Control	51.70 ± 0.88^{b}
50 ppm $CuSO_4$	37.35 ± 0.01^{c}
125 ppm $CuSO_4$	30.74 ± 0.74^{d}
50 ppm Vitamin B_{12}	56.22 ± 0.83^{a}
50 ppm $CuSO_4$+ 50 ppm Vitamin B_{12}	38 ± 0.53^{c}
125 ppm $CuSO_4$+ 50 ppm Vitamin B_{12}	31.98 ± 0.43^{d}

* $CuSO_4$: Copper (II) sulfate
*Values are presented as mean ± standard deviation of three parallel measurements (n=3)
*Differences between group means sharing different letters in the same column are statistically significant ($p<0.05$)

3.6. qRT-PCR Analysis

Applications of $CuSO_4$, Vitamin B_{12}, and their combinations resulted in varying *Pv-MATE14* gene expression levels in common bean (*Phaseolus vulgaris* L.) (Figure 3). Compared to the control group, *Pv-MATE14* expression was significantly upregulated with both 50 and 125 ppm $CuSO_4$ applications. Conversely, the 50 ppm Vitamin B_{12} application downregulated *Pv-MATE14* expression compared to the control. The 50 ppm $CuSO_4$ application upregulated gene expression by 47%, corresponding to a 1.47-fold change. The 125 ppm $CuSO_4$ application resulted in the highest upregulation, with a 71% increase and a 1.71-fold change ($p<0.05$). The 50 ppm Vitamin B_{12} application led to the lowest downregulation, with an 86% decrease and a 0.13-fold change ($p<0.05$). In combination treatments, the 50 ppm $CuSO_4$+50 ppm Vitamin B_{12} application downregulated expression with a 0.83-fold change, suggesting Vitamin B_{12} completely mitigated the stress response. However, the 125 ppm $CuSO_4$+50 ppm Vitamin B_{12} application still resulted in slight upregulation, showing a 1.10-fold change ($p<0.05$) (Figure 3). Various abiotic factors, especially heavy metal pollution as a major environmental concern, restrict plant growth (Mustafa and Komatsu, 2016). Unfortunately, plants absorb heavy metals along with essential elements from the soil, leading them to develop different strategies to cope with the harmful accumulation of heavy metals. For instance, the families of heavy metal ATPases (HMA), multidrug and toxic compound extrusion (MATE), yellow stripe-like (YSL), and metal tolerance proteins (MTP) are constitutively overexpressed transmembrane transport proteins that facilitate the uptake of heavy metals, transport them to leaves, and ultimately sequester them in vacuoles or cell walls (Rascio and Navari-Izzo, 2011). MATE transporters are widely distributed in plants (Omote et al., 2006). Studies on MATE gene expression levels related to heavy metal stress support our findings. For example, in rice (*Oryza sativa*) plants, exogenous application of 200 µM arsenic (As) heavy metal to different tissues resulted in the upregulation of *OsMATE2* in both leaves and seeds, while it was downregulated in roots (Das et al., 2018). In *Arabidopsis*, the upregulation of *OsMATE* was emphasized to ameliorate diversity in vegetative tissues and be important in the plants growth and development process (Tiwari et al., 2014). Another study reported that among all MATE genes, seven were upregulated in rice (*Oryza sativa*) plants exposed to 13.3 µM arsenic heavy metal (Norton et al., 2008). Additionally, a study with *Arabidopsis* showed that overexpression of the cotton DTX/MATE gene could regulate the amount of reactive oxygen species, thereby minimizing the effects of various oxidative stresses (Lu et al., 2019). Finally, research on common bean (*Phaseolus vulgaris* Linn.) investigating *Pv-MATE* expression levels under NaCl, $CuCl_2$, $HgCl_2$, $CdCl_2$, and PEG6000 applications revealed that *Pv-MATE14* expression was upregulated under all applied abiotic stresses (Zhao et al., 2024).



*1: Control, 2: 50 ppm $CuSO_4$, 3: 125 ppm $CuSO_4$, 4: 50 ppm Vitamin B_{12}, 5: 50 ppm $CuSO_4$ + 50 ppm Vitamin B_{12}, 6: 125 ppm $CuSO_4$ + 50 ppm Vitamin B_{12}

*Differences between group means sharing different letters in the same column are statistically significant ($p<0.05$).

Figure 3. Variation in *Pv-MATE14* gene expression level in bush bean (*Phaseolus vulgaris* L.) plants

4. CONCLUSION

This study investigated the effect of Vitamin B_{12} application on specific growth parameters, biochemical properties, and *Pv-MATE14* gene expression in common bean (*Phaseolus vulgaris* L.) plants subjected to $CuSO_4$ induced heavy metal stress. Our findings reveal that both concentrations of $CuSO_4$ (50 and 125 ppm) negatively impacted the common bean plants overall physiology. This was evidenced by significant reductions in growth parameters (plant height, root and stem lengths, and root, stem, and leaf fresh weights) and detrimental alterations in biochemical parameters (increased proline and MDA content) and *Pv-MATE14* gene expression (upregulation), collectively resulting in the slowing of plant growth and development. Conversely, Vitamin B_{12} application exhibited a positive and mitigating influence across all measured parameters. It promoted growth (increased lengths and fresh weights), alleviated stress indicators (decreased proline and MDA content), and normalized the $CuSO_4$ induced changes in *Pv-MATE14* gene expression, both compared to the control and in combination with $CuSO_4$ treatments. This strongly suggests that Vitamin B_{12} effectively alleviates the physiological and molecular damage caused by $CuSO_4$ heavy metal, thereby promoting plant growth and development under stress conditions. To more definitively elucidate the protective effects and mechanisms of Vitamin B_{12}, future studies incorporating different

common bean varieties, a wider range of stress conditions, and various Vitamin B_{12} concentrations are crucial. Such research would significantly contribute to the existing literature on phytoremediation and aid in finding sustainable solutions to this environmental challenge.

Conflict of Interest

The author declare that there is no conflict of interest.

Ethics

This study does not require ethics committee approval.

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