Environment and Ecology 43 (1) : 16—23, January—March 2025
Article DOI: https://doi.org/10.60151/envec/GUSZ8343
ISSN 0970-0420

In-vitro Evaluation on the Germination Potential and Seedling Growth of *Vigna radiata* (L.) under Heavy Metal Stress

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Received 18 October 2024, Accepted 6 December 2024, Published on 14 January 2025

ABSTRACT

Copper (Cu) and zinc (Zn), both are essentially required to maintain cellular functions in all living beings but whenever their concentrations cross a certain threshold, they become toxic and behave as 'Heavy metals'. Their excess levels in soil diminish agricultural productivity, prompting humanity to investigate new plant species with enhanced tolerance levels for cultivation on metal-contaminated soil, with the aim of reclamation and mitigation. In present study, *in-vitro* trials were conducted to analyze the tolerance level of mungbean seedlings against Cu and Zn in March 2023. Metal concentrations were selected on the basis of target limits (50 ppm for Zn, 36 ppm for Cu) in soil as approved by the World Health Organization (1996), which were further enhanced two fold to induce stress conditions as 50, 100 and 200 ppm for zinc and 36, 72 and 144 ppm for copper. Surface sterilized viable seeds were allowed to germinate in selected concentrations with a control set up in triplets. Final observations were recorded on the 15th day of germination. Experimental findings revealed that Zn exerted a greater impact on early seedling development than on seed germination. Greatest inhibition in germination was observed in copper-treated seed at highest concentration (144 ppm). Seedling length and biomass (fresh and dry) were stimulated at lowest concentrations (50 ppm) of zinc, but for copper, positive impacts were observed up to 72 ppm. A concentration-dependent progressive decrease was observed in biomass (fresh/dry) in Zn treatment. As a result of the investigation, the toxicity order for test metals can be concluded as Zn > Cu. Findings suggest long-term cultivation of test variety in natural field setting to explore its potential against heavy metal stress.

Keywords Germination, Heavy metal tolerance, Seedling development, *Vigna radiata*.

INTRODUCTION

Heavy metals, among the most persistent environmental contaminants, get released into the atmosphere as a result of both natural and anthropogenic activities (Weldeslassie *et al.* 2018). The world's most pressing concern right now is heavy metal toxicity in plants, humans, and food. Some heavy metals are vital nutrients (Cu, Mg, Zn and Ni) to perform different metabolic reactions, however, their elevated concentrations can induce

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toxicity symptoms in plants (Mitra *et al.* 2022). Heavy metals are known to negatively impact plant life cycles, but some plant species are capable enough to establish tolerance against elevated levels of heavy metals via controlling the supply of micronutrients and metabolically inactivating non-essential elements (Yan *et al.* 2020).

Copper (Cu) as a heavy metal selected in this study is one of the micronutrients known to play an important role in CO_2 assimilation and ATP synthesis. The functions of copper include secondary cell wall formation, lignin synthesis, stress tolerance and photosynthesis (Mir *et al.* 2021). Despite being necessary, it becomes poisonous to plants when found in excessive quantities due to its high density and toxicity at elevated levels, hindering plant growth and harming vital cellular functions such as photosynthetic electron transport (Kumar *et al.* 2021). Specifically, excess copper can cause chlorosis, inhibition of root growth and damage to plasma membrane permeability leading to ion leakage (Bouazizi *et al.* 2010, Katare *et al.* 2015). Zinc (Zn), as another heavy metal, serves as an essential micronutrient for the growth and development of living beings (plants, animals and humans) and a significant component of various proteins (cofactors or coenzymes) (Hussain *et al.* 2022) but inadequate amounts of zinc in plants can impair physiological and developmental processes (Song *et al.* 2019, Agarwal *et al.* 2022). Earlier reports are also available on inhibited germination and seedling growth of legume cover crops (*Coronilla varia, Lotus corniculatus* and *Trifolium arvense*) under metal toxicity (Zn, Pb, Ni, Cu and Cd) (Bae *et al.* 2016). Agricultural lands are reported as becoming less fertile due to heavy metal poisoning, which is restricting plant output globally (Rashid *et al*. 2023). Therefore, it is essentially required to manage and monitor the level of heavy metals in the environment to prevent their harmful consequences. Under these circumstances, it is crucial to choose the right plant species by taking into account accelerated quantities of heavy metals in the soil and how susceptible the crop becomes to soil contamination. Globally, these days more attention is being paid to the idea that plants could be able to tolerate the accelerated limit of pollution and contamination. This offers us the foundation to conduct a study on the impact of heavy metals on a particular legume crop and to determine whether it is possible to cultivate that crop on heavy metal contaminated soils.

One of the significant legume crops, mung bean, also known as green gram or *Vigna radiata*, is valued for both its nutritional worth and its ability to be grown sustainably. Many biotic (weeds, pests and microorganisms) and abiotic (temperature, humidity, heavy metals, light and nutrient cycles) factors have an impact on the production of legumes via direct or indirect interference with the environment (Gharde *et al.* 2018). Due to small water requirement and high dietary value, expansion of this plant (*Vigna radiata*) cultivation seems to be important. So, the task of assessing *in vitro* impact of heavy metals (Cu, Zn) on seed germination along with seedling growth parameters has been undertaken in order to examine how a particular variety of *Vigna radiata* can thrive at various normal and stressful concentrations of copper and zinc.

This study was designed to evaluate the tolerance level of *Vigna radiata* var 'Pusa Vishal' against heavy metals (Cu and Zn), considering the unavailability of sufficient information regarding *in vitro* studies of test variety growing under selected metal concentrations. Present study will assess the toxicity of Cu and Zn on seed germination, seedling growth and development of *Vigna radiata*. The selected parameters will help in determining the critical and toxic levels of test metals (Cu, Zn) for the test species. This investigation can also be useful for scanning the resistant or susceptible nature of test seeds.

MATERIALS AND METHODS

Plant materials and treatment details

Experiments for *in-vitro* analysis were carried out with three replicates in March 2023 at the Botany Department of CCS University (29º18' N and 78º47' E) in Meerut, UP, India. Seeds of *Vigna radiata* were procured from Indian Agricultural Research Institute (IARI), New Delhi, India. Metal stress was

Table 1. Target limits of different heavy metals in agricultural soil (WHO 1996).

Heavy metal	Concentration
Cu	36 ppm
Zn	50 ppm
Ni	35 ppm
Cd	0.8 ppm

created by applying solutions of heavy metal salts: $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$, respectively. Metal concentrations were adopted on the basis of target limits (desirable maximum levels) as 50 ppm for zinc (Zn) and 36 ppm for copper (Cu) as directed by World Health Organization (WHO 1996) (Table 1). These concentrations were enhanced two folds (twice) to induce stress conditions and three varying concentrations of each metal were prepared. Thus, the final concentrations were 36 ppm, 72 ppm, and 144 ppm for copper which were denoted as T1, T2 and T3. Similarly, for zinc, 50 ppm, 100 ppm, and 200 ppm were the final concentrations and labeled as T4, T5 and T6, respectively. Metal solutions were prepared using double-distilled water with a control (without metal). Seeds were disinfected by sodium hypochlorite (0.1%) for fifteen minutes, followed by repeated washing to remove any trace particles and drying on blotting paper. Ten healthy seeds were placed in filter paper-contained sterilized Petri dishes. Three replications per treatment were used. Five ml of double-distilled water was used as control. The filter papers were kept moistened with the respective metal concentrations for two weeks. The control and treatments were analyzed as follows:

Control (C): 10 seeds: 5 ml double-distilled water
Treatment 1 (T1): 10 seeds: 5 ml of 36 ppm of copper solution
Treatment 2 (T2): 10 seeds: 5 ml of 72 ppm of copper solution
Treatment 3 (T3): 10 seeds: 5 ml of 144 ppm of copper solution
Treatment 4 (T4): 10 seeds: 5 ml of 50 ppm of zinc solution
Treatment 5 (T5): 10 seeds: 5 ml of 100 ppm of zinc solution
Treatment 6 (T6): 10 seeds: 5 ml of 200 ppm of zinc solution

Seed germination assay

Seed germination is a character of the prime significance and consists of a complex phenomenon of many physico-biochemical changes leading to the activation of embryo (Farooq *et al.* 2022). Seed germination parameters were calculated to evaluate its performance under the applied treatments, as followed by Baruah *et al.* (2019):

Germination % = (Germinated seeds)/(Total number of seeds) × 100

Germination index (GI)

$$= 2\times(7\times n1)+(6\times n2)+(5\times n3)+(4\times n4)+(3\times n5)\ (2\times n6)+(1\times n7)$$

Where,

n1, n2….n7 are number of germinated seeds on first, second and subsequent days.

Seed vigour index

Seed vigour determines the overall development and activity of the seed during seedling emergence. It was observed on length and dry weight basis as SV-I and SV-II as described by Abdul-Baki and Anderson (1973) :

SVI = Seedling length (cm) × Germination percentage
SVII=Seedling dry weight (mg)×Germination percentage

Seedling measurements

Randomly selected ten seedlings were used for recording their length and weight. After seed germination, 8 hrs of artificial light was given to the seedlings as suggested by Niinemets and Keenan (2012) and on 15th day of germination, their respective radical, hypocotyls and cotyledon lengths were recorded by measuring scale. After fresh weight observations, similar seedlings were put in hot air oven at 72°C for 48 hrs for dry weight. Fresh and dry weights were expressed in milligrams.

Tolerance index

It was calculated on dry weight basis as described by

Amin *et al.* (2014) :

$$\text{Tolerance index} = \frac{\text{Value in treatment} \times 100}{\text{Value in control}}$$

Data analysis

The statistical analysis and graph preparation was computed using Microsoft Excel (2017). One-way Anova compared the data between more than two groups at one variable at a time point. Each mean was calculated from three different values $p < 0.05$ was considered a significant difference. The standard deviation and standard error were calculated against the obtained values.

RESULTS AND DISCUSSION

The present study evaluated the effect of different concentrations of Cu and Zn as heavy metals on seed germination and early seedling growth of *Vigna radiata* var Pusa Vishal, aiming to establish their phytoremediation potential. The results of present study are summarized in Figs. 1–4, revealing that zinc (Zn) had more detrimental impacts on the growth activity of test species than that of copper (Cu).

Germination attributes

Heavy metal exposure exhibited highly significant (one-way ANOVA, $p < 0.0001$) impact on the test species during germination stage. Among the heavy metals evaluated, copper at 144 ppm exhibited greatest suppression (22%) on *Vigna radiata* seed germination as maximum germination decline. However, 36 and 72 ppm concentrations stimulated germination percentage by 15 and 24%. In case of zinc, germination percentage decreased gradually with increasing zinc concentration (Table 2). Zinc at 50 ppm showed maximum germination percentage (96.67%) as compared to other treatments, but 200 ppm showed slight reduction (6%) (Fig. 1). The germination percentage decrease in the presence of high concentrations of heavy metals could be due to the accelerated decomposition of reserve materials in the endosperm or changes in the permeability properties of the cell membrane (Sobrero and Ronco 2004, Baruah *et al.* 2019). Reduction in seed germination properties is also often seen as a result of metal toxicity on certain enzymatic (protease, amylase) functions that suppress nutrient supply to growing seed (Singh *et al.* 2007). Wang *et al.* (2010) also reported the similar deleterious effects of heavy metals (Pb, Cu, Zn) on *Triticum aestivum* germination. Reduced germination in seedlings of *Brassica pekinensis* due to copper toxicity (0.5 Mm) was also confirmed by Xiong and Wang (2005).

Germination index (GI) is directly related to the germination performance of seed. On addition of different concentrations of heavy metal solutions, both kinds of responses, i.e., positive or negative, were observed on derived germination index. Copper gradually enhanced germination index up to 72 ppm, with the highest value among all treatments. In case of zinc treatment, a concentration-dependent gradual decrease was observed in germination index values, where 50 ppm and 100 ppm gave positive values, but 200 ppm led to negative results as compared to the control (Fig. 1). Increase in germination index is attributed to the triggering effect of copper and zinc as micronutrients on the germination of seeds, enhancing α-amylase activity as one of the important enzymes involved in the germination process (Sarma *et al.*

Table 2. Effect of different heavy metals (Cu, Zn) concentrations on seed germination attributes (GP%: Germination percentage, GI: Germination index, SV: Seed vigour index) of *Vigna radiata*. (± = SD).

	Germination attributes			
Treatments	GP%	GI	SV-I	SV-II
Control	76.67 ± 4.71	35.34 ± 0.98	615.03 ± 35.54	1448.00 ± 139.54
T1	88.33 ± 2.36	43.01 ± 1.45	879.17 ± 29.20	1908.33 ± 75.90
T2	95.00 ± 2.36	45.65 ± 0.46	1069.78 ± 40.38	2223.67 ± 108.89
T3	60.00 ± 4.71	32.82 ± 4.46	372.32 ± 2.44	1038.33 ± 90.04
T4	96.67 ± 3.54	42.82 ± 2.61	1153.63 ± 68.41	2308.00 ± 16.97
T5	81.67 ± 2.36	36.67 ± 3.31	620.68 ± 35.58	1772.67 ± 85.80
T6	71.67 ± 7.07	30.34 ± 1.39	365.50 ± 38.89	1195.33 ± 87.68

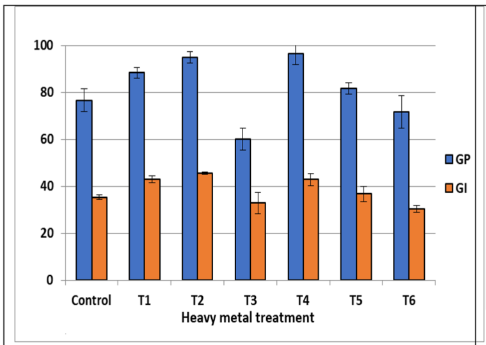

Fig 1

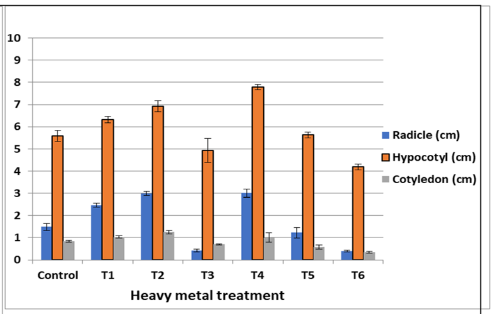

Fig 2

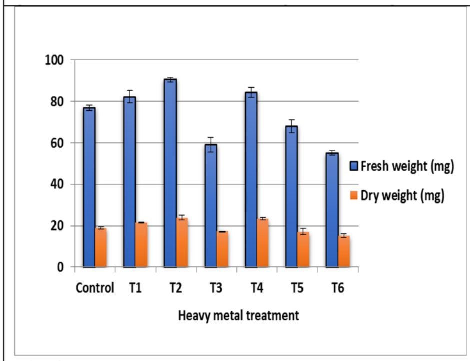

Fig 3

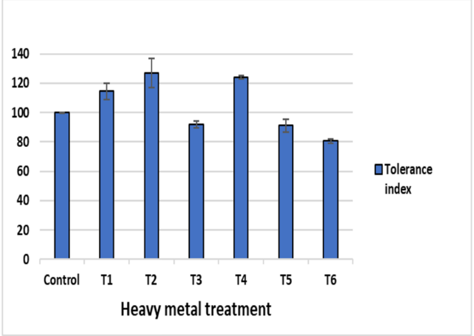

Fig 4

Fig. 1. Effect of different concentrations of Cu and Zn on germination percentage (GP) and germination index (GI) of *Vigna* seedlings. **Fig. 2.** Seedlings length in the form of radicle, hypocotyl & cotyledon under different concentrations of copper and zinc. **Fig. 3.** Effect of different concentrations of Cu and Zn on seedling biomass (mg). **Fig. 4.** Effect of different concentrations of Cu and Zn on tolerance index of *Vigna* seedlings.

2014). The detailed results of germination attributes are given in Table 2. Enhancing and decreasing effects of lower and higher doses of cobalt on wheat germination have been previously reported by Sarma *et al.* (2014). Our results are also in accordance with the findings of Parveen and Muthukumaran (2023) on *Vigna mungo* germination growth under excessive doses of heavy metal copper.

Seed vigour index

The seed vigour index values were found to be decreased at highest concentration of copper and zinc in comparison to control. Zinc-treated seeds showed the highest values for both SV-I and SV-II at 50 ppm in T4. However, the lowest values for the same (SV-I, SV-II) were recorded in T6 (200 ppm/Zn) and T3 (144 ppm/Cu) (Table 2). The recorded increased vigour index in test species indicated that exposure of Cu and Zn has a stimulatory effect on meristematic cell growth, resulting in plumule and radical elongation at concentrations up to 72 ppm and 100 ppm as compared to control. Concentrations higher than these values caused hindrance in radicle and plumule growth leading to reduced vigour index. Increased vigour index is a sign of meristematic tissue growth during cell division, however, reduction in vigour index often results due to nutrient limitation or unavailability to growing seedlings due to enzyme (hydrolytic) inhibition (Ashraf *et al.* 2011).

Seedling length

Radical length was significantly affected ($p < 0.0001$) with positive and negative responses by both metals as compared to the control, where an increase of

64% and 98% was observed under Cu treatment in T1 and T2 and 99% with 50 ppm of Zn (T4). The increasing zinc concentration had a pronounced effect on radical reduction, as 19% in T5 and 74% (maximum decrease) in T6 (Fig. 2). A similar kind of pattern was also recorded in hypocotyl length with a maximum enhancement (39%) and maximum reduction (25%) in zinc-treated seedlings at 50 and 200 ppm. In case of Cu, an increase of 13% and 23% was observed in T1 and T2 but T3 reduced hypocotyl length by 12% (Fig. 2). Cotyledon length was positively affected in Cu at 36 ppm and 72 ppm, reaching values significantly higher than those of control with an increase of 18% and 42%. On the other hand, in zinc-treated seedlings, cotyledon length increased (12%) only with 50 ppm (T4), but 100 & 200 ppm (T5 & T6) gave significant reduction by 36% and 61% as compared to control (Fig. 2). Copper application positively affected radical, hypocotyl, and cotyledon growth of test seedlings evaluated at 36 and 72 ppm, however, a strong growth reduction in all parts of seedlings was observed at elevated concentration (144 ppm). On the other hand, zinc enhanced seedlings' length only at lowest concentration (50 ppm), whereas all remaining concentrations caused a significant reduction in seedlings' length. The detailed results are given in Fig. 2. Observations demonstrated a correlation between highest concentrations of metals (144 ppm/Cu and 200 ppm/Zn) and delayed seedling elongation. Our results are in accordance with the finding of Kamberi *et al.* (2023), where they observed reduced growth at high concentration of zinc in pea plant. Excess copper has also been reported to inhibit young seedling growth and root elongation by damaging root epidermal cells and root cell membranes (Adrees *et al.* 2015, Gautam *et al.* 2016). High levels of copper have been shown to impede the radical development as it binds to root surface, obstructing the translocation of water and other ion absorption (Shabbir *et al.* 2020). Our studies are supported by the findings of Dey *et al.* (2014). Decreased length is attributed to the fact that despite their status as essential micronutrients, both Cu and Zn in excess have a detrimental effect on enzyme activities associated with plant metabolism and mineral nutrition (Farid *et al.* 2021, Kamberi *et al.* 2023).

Seedling weight (fresh and dry weight)

Vigna seedlings' biomass was highly significant ($p < 0.0001$) with respect to heavy metal treatment. Maximum percent reduction for both fresh and dry biomass was observed in Zn at 200 ppm in T6. However, the maximum percent increase for fresh/dry weight was observed in Cu treatment at 72 ppm. Copper had a more stimulatory effect on seedling biomass than zinc. The findings revealed that plants treated with copper up to 72 ppm and Zn with 50 ppm showed a significant increase in biomass production but above these concentrations, biomass accumulation is severely hampered (Fig. 3). Findings are also corroborated by Verma *et al.* (2011) and Xiong *et al.* (2006). Same results were obtained by Parveen and Muthukumaran (2023) with reduced biomass and growth in blackgram under copper stress. Similarly, zinc is another important vital trace nutrient for plant development, but several investigations have also confirmed its excessive concentrations as hazardous and phytotoxic (White and Pongrac 2017, De Oliveira and Tibbett 2018). Tolerance index (TI) reflects the resistance of plants against the effects of phytotoxic compounds. The TI values of whole seedlings in natural conditions, i.e., without any treatment, were used as 100%. Maximum toxicity impacts were observed in highest concentrations of copper and zinc in T3 and T6, approving these concentrations (144 ppm for zinc, 200 pm for zinc) toxic for dry matter accumulation in *Vigna* seedlings (Fig. 4).

CONCLUSION

Screening for germination as well as early seedling development is a rapid and feasible approach to gain insights about heavy metal stress to plant species. The current study sheds light on how *Vigna radiata* seedlings respond differentially to heavy metal types (Cu and Zn) and concentrations during the early growth stage when exposed for a short time period. The test variety had no toxic effect on seedling growth under copper up to 72 ppm and zinc at 50 ppm, but as the concentration increased above these levels, all growth-associated parameters significantly impacted adversely. Highest concentrations of both metals (144 ppm/Cu, 200 ppm/Zn) lead to inhibitory effects on germination potential and seedling growth, including biomass (fresh/dry) and length (shoot/root). Early seedling growth was found to be more susceptible to zinc exposure than germination. Copper was more effective for overall seedling development including germination and growth than zinc. The findings of present investigation concluded *Vigna radiata* var Pusa Vishal as a relatively more tolerant leguminous crop against heavy metal, copper than that of zinc. Moreover, growing seedlings exhibited greater sensitivity towards zinc as compared to copper. Given the preceding findings, the authors reported copper at 144 ppm and zinc at 200 ppm are toxic enough to pose lethal effects on young seedlings, however, long-term field research with specific metal concentrations is required to explore the *Vigna* plant's potential against copper and zinc stress and to uncover the surprising results.

ACKNOWLEDGMENT

The authors extend their gratitude to the HOD and all faculty members of the Botany Department, CCS University Campus Meerut, for providing all of the resources needed for conducting this experiment.

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