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Effect of fungal biosorbed and nonbiosorbed copper and zinc metal solutions on growth and metal uptake of leguminous plants

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Abstract
Effect of Zn, Cu and Cu + Zn at 10, 25, 50, 100, 200 and 500ppm concentrations of fungal untreated and treated metal solutions on seed germination and seedling vigor of *Cicer areietinum* (Chick pea), *Macrotyloma uniflorum* (Horse gram), *Vigna radiata* (Green gram) and *Vigna unguiculata* (Cowpea) were evaluated. Heavy metal solutions were prepared in increasing concentration up to the concentration critical to the soil. Increased metal concentrations reduced the seed germination and growth of test plants. Low metal concentrations of 10, 25, 50 ppm, stimulated the shoot, root and seed germination in test plants. Untreated and treated effluent was not acutely toxic to the seed germination and plant growth. In *Aspergillus niger* and *Aspergillus flavus* biosorbed metal ions, reduced metal toxicity with increased seedling vigor was observed. Efficiency of metal biosorption by fungal biomass and metal ions tolerance and accumulation ability in test plants were analyzed by Atomic Absorption Spectrophotometer (AAS).

Keywords: Heavy metals; Biosorption; Leguminous plants; *Aspergillus niger*; *Aspergillus flavus*; Pretreatment

INTRODUCTION

Heavy metals biosorption by microbial biomass has been suggested as a potential alternative to the physicochemical technology for detoxification and recovery of toxic and valuable metals from waste waters (1). Non-viable microbial biomass exhibits a higher affinity for metal ions compared with viable biomass due to the absence of competing protons. It avoids the metal toxicity for microbial growth and inhibition of metal accumulation by nutrient or excreted metabolites (2). The metal loading capacity of dead cells depends on the fungus under consideration, pretreatment methods and type of metal ions. Dead cells offers advantages of easy desorption of biosorbed metal ions and reusability of biomass (3). Biosorption is relatively rapid, reversible and prepared from the naturally abundant waste biomass (4). The fungal biomass provide better adsorbent surface for metal ions due to relatively small particles, low density, low mechanical strength and rigidity (5). Cu and Zn are important micronutrients at low concentration but in high concentrations these metals become toxic to plants (6, 7). Copper, Zinc, Chromium, Lead, Cadmium taken up by plants in higher concentrations, they not only inhibit metabolic process and reduce crop production, also become incorporated in to the food chain and potentially cause human liver and brain disorders (8). Green gram is a well known pulse crop in India. It is a short duration and drought resistant crop with stand adverse environmental conditions. Green gram is a digestible, high in protein (22-24%), vitamin A, B, niacin and minerals necessary for human body. It is good substitute of animal protein and form a balanced diet when taken with cereals (9). Horse gram is cultivated in several parts of south India as dry land legume crop. It is relatively cheaper than cereals and reduced feed cost in poultry feeds (10).Horse gram is rich in protein (20%) which can be further processed to obtain starch, it has variety of applications in food industry (11). Cowpea is an important legume food crops in Asia compatible as an intercrop. It is rich in vitamins, essential minerals and 100g contain 44 calories of energy. It fix atmospheric nitrogen and grow well in poor soils containing >85% sand, <0.2% organic matter and low phosphorous. Chickpea is source for protein and very high dietary fibers for insulin sensitivity persons. India is the world leader in chickpea production. The objective of this research work was to test the fungal treated and untreated metal toxicity on seed germination and seedling vigor of chick pea, horse gram, green gram and cow pea plants. The choice of metals was made with regard to their industrial use and potential pollution impact on the environment. Phytoremediation involves diverse use of plants for *in situ* treatment of metal contaminated soils, sediments, water and air (12). Metals resistance and uptake by selected plants was statistically evaluated for its application in phytoremediation.

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MATERIALS AND METHODS

Microorganisms and culture media

Aspergillus niger and *Aspergillus flavus* were isolated from paper mill effluent (13). *A.niger* was cultured in liquid Minimal Salt Medium (14) and *A.flavus* in Czapek-Dox growth medium (15) to produced biomass for metal biosorption study.

Preparation of heavy metal solutions

The stock metal solutions of 1000mg/l Cu and Zn were prepared by dissolved A.R. grade salts of $CuSO_4.5H_2O$ and

$ZnSO_4.6H_2O$ in double distilled water. Working metal solutions of 10, 25, 50, 100, 200 and 500ppm were prepared from stock solutions.

Preparation of microorganisms for biosorption study

Fungal biomass was harvested from the growth medium by filtering through Whatman No.1 filter paper and washed thoroughly in distilled water till it was free from the media components. Collected biomass was initially pretreated by autoclaving for 20 min. at 121^{0}C (124kPa). This biomass was boiled in 0.5N NaOH solution for 15 min. and washed with generous amounts of deionized water till the pH of the washed solution was near the neutral range (7.0-7.2). Finally the biomass was dried at 60^{0}C for overnight and used for metal biosorption study (16).

Elution of biosorbed heavy metals from fungal biomass

Pretreated biomass was treated with metal solutions in rotary shaker at 125 rpm for 15 min. and biosorbed metal ions were eluted from fungal biomass using 0.05N nitric acid solution. The biomass was used for five cycles of metal sorption and elution (17).

To check fungal biosorbed and non biosorbed heavy metals on seed germination and plant growth

Cicer areietinum (Chick pea), *Macrotyloma uniflorum* (Horse gram), *Vigna radiata* (Green gram) and *Vigna unguiculata* (Cow pea) were used as test plants. Seeds of the test plants were purchased from Agro seed trader; Shimoga Dist. Obtained seeds were immersed in 3% V/V formaldehyde/deionized water for five minutes to eliminate the exogenous microbial contamination. After that, the seeds were washed with deionized water and used for seed germination study. Selected healthy seeds were scattered on Petri plates (9 cm diameter) and blotting paper (paper towel method) containing two sheets of Whatman No.1 filter. Subsequently, 15ml of fungal treated and untreated heavy metal solutions as control were added on to the seeds to expose the germinating seeds for heavy metals (6). Metal treated seeds were placed in a controlled temperature of 24±1^{0}C (day) and 18±2^{0}C (night) and regularly treated with metal solutions. Seed germination and growth was measured every 24 h. until no further growth noted. Shoot and root length of plants was measured at the end of the germination trial. (15).

Physicochemical analysis of effluent and its effect on plant growth

Untreated and treated paper mill effluent was collected from Bhadravati city before discharge into the Bhadra River. After acidification of effluent with concentrated HNO_3 (1.5ml/L), physicochemical and heavy metal (Cu^{2+}, Zn^{2+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Pb^{2+}) analysis were carried out according to standard methods of American Public Health Association (18). Seed germination and seeding vigor was checked in presence untreated and treated effluent.

To detect accumulated metals in fungal biomass and plants

Eluted metal solutions were acid digested using concentrated HNO_3 and HCl (5:5) and concentration of metals in the solution was determined by AAS (19). Each plant was divided into stem, leaves and root region and digested separately to ascertain the metal content in the individual section. Samples were dried overnight at 65^{0}C in an oven and 1g of milled plant tissues was soaked in 20 mL pure nitric acid for six hours. The mixture was then boiled to 50% of the original volume and 4mL of perchloric acid (70 vol %) was added and the mixture was refluxed for 90 min. Finally solution was diluted with distilled water to 20 ml and analyzed metal content by Atomic Absorption Spectrophotometer (AAS) (20). The student t-test was applied to statistically compare the difference between fungal treated and untreated metals on plant growth ($P < 0.05$).

RESULTS AND DISCUSSION

Physicochemical and heavy metals content of effluent sample

The physicochemical and heavy metal characterization of untreated and treated paper mill effluent was recorded (Table 1) and results were compared with the standard BIS and WHO values.

Table 1. Physicochemical parameters of untreated and treated paper mill effluent

Sl. No.	Parameter	Observed values		Tolerance limit (IS 10500)	Tolerance limit (WHO 2006)
		Untreated effluent	Treated effluent		
1	pH	3.5±0.03	7.8±0.08	5.5-9	No guideline
2	Temperature	32±0.30	32.4±0.29	40	No guideline
3	Color	Dark yellow	Light yellow	--	--
4	Odor	Pinching	Pinching	--	--
5	BOD (3days)	602 ±2.0	60 ±1.51	30	No guideline
6	OD	0	4.3 ±0.28		No guideline
7	COD	1488 ±2.1	202 ±2.26	250	No guideline
8	Hardness	687 ±1.8	359 ±2.76	300	No guideline
9	Total acidity	157± 1.9	67 ±1.24	--	--
10	Total alkalinity	164 ±1.2	63± 0.96	--	--
11	Free CO_2	81 ±0.9	53 ±1	--	--
12	TDS	765 ±1.3	803± 2.1	500	No guideline
13	Conductivity	1528± 2.4	1598± 3.1	--	--
14	Chlorine	101 ±0.7	75 ±1.29	--	--
15	Calcium	95 ±0.8	79 ±0.86	--	--
16	Magnesium	110 ±0.8	45 ±1.23	--	--
17	Chloride	1768± 1.8	686± 2.64	--	--
18	Sulfate	446 ±1.3	192 ±2.64	--	--
19	Copper	8.01± 0.08	7.72± 0.11	3.0	2.0
20	Chromium	63.15 ±0.23	59.02± 0.43	0.1	0.05
21	Nickel	3.76 ±0.14	3.49± 0.13	3.0	0.07
22	Zinc	3.64 ±0.13	2.90 ±0.15	5.0	2.0
22	Lead	1.92 ±0.10	1.54 ±0.06	0.1	0.01
23	Cadmium	1.71 ±0.01	1.44± 0.01	2.0	0.03

Parameter unit -mgl^{-1}, except pH, temperature and conductivity,
± -Standard Error, --= Not applicable
WHO (2006)-World Health Organization
BIS (1998)-Bureau of Indian Standard for effluent discharge to inland surface water

The untreated effluent had dark yellow colored, pinching odor, acidic nature with high COD, BOD, TDS and total hardness. BOD, total hardness, TDS and heavy metals content was higher than the permissible limit in treated industrial effluent. The presence of lignin and its derivatives impart color to the effluent. High organic matter and chemical content of the paper mill effluent favored seed germination. Use of lime and chemical treatment reduce effluent content to the safer level which in turn increases the heavy metal concentrations in effluent.

Effect of heavy metals on seed germination

The percentage seed germination in tested species was reduced at 100 and above metal concentrations (Fig.1). Seed germination favored at low metal concentrations was observed.

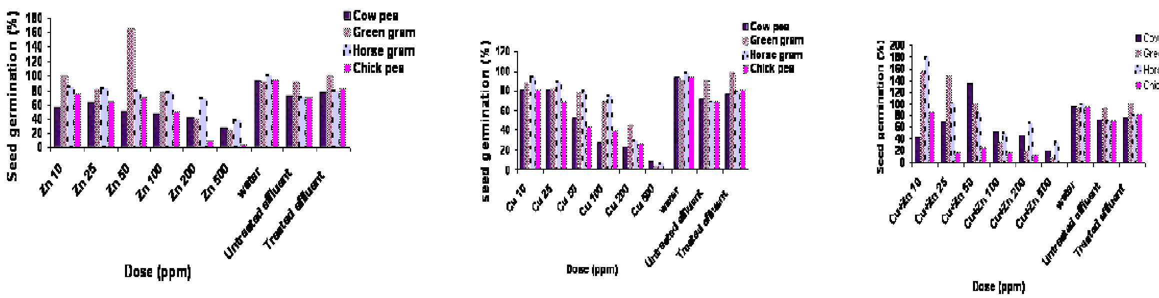

Fig 1.Effect of Cu, Zn and Cu+Zn metals on seed germination

Effect of heavy metals on shoot and root growth of plants

Effect of Cu, Zn and Cu +Zn on shoot growth was observed (Fig.2). Cu increased the shoot growth of cowpea compared to control at 125- 68%, horse gram at 97 -55%, chickpea at 268-140% The dose of Zn increased the cowpea germination at 146-138%, green gram at 97- 82%, horse gram at 79-82% and chickpea at 25-118%. Combination of Cu and Zn increased cowpea germination to 53-66%, green gram to 103-97%, Horse gram to 155-87% and Chickpea 268-50%. 100,200,500ppm concentrations of all treated metal ions were significantly reduced the shoot growth.

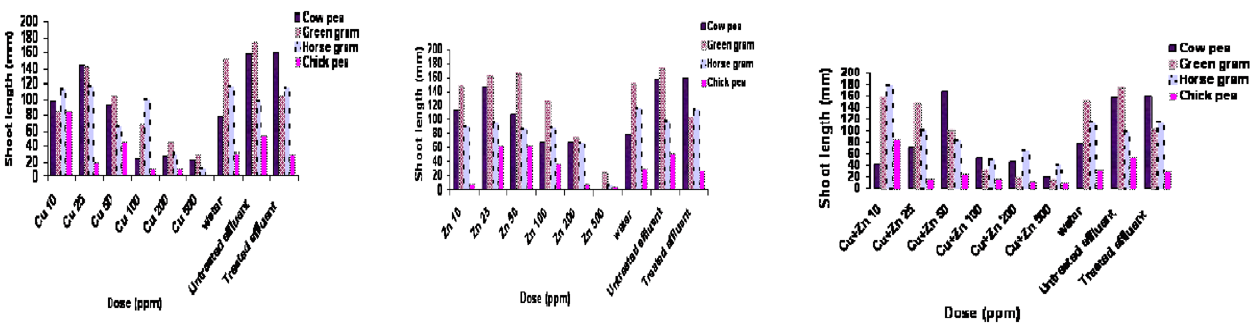

Fig 2. Effect of Cu, Zn and Cu+Zn metals on plants shoot growth

Metal toxicity symptoms like apical yellowing, chlorosis, shortened internodes and smaller leaves were observed due to readily take up and mobility of metals in the plants (21). The effect of plants root growth vs. different metal concentrations was reported (Fig.3). Root germination in cowpea was more at 50ppm of Cu+Zn. 100ppm of Cu+Zn significantly reduced the root growth in test plants.

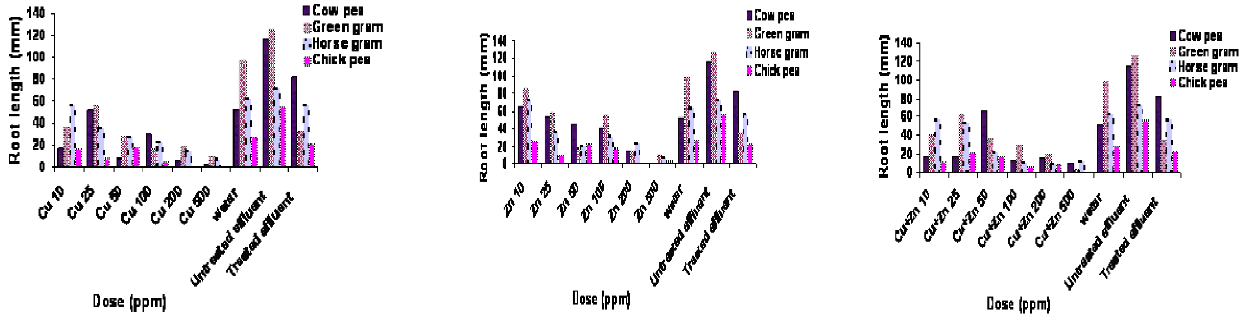

Fig 3. Effect of Cu, Zn and Cu+Zn metals on plants root growth

Cu increased the root germination of cowpea to 19-15% followed by green gram to 38%, horse gram to 91%, and chickpea to 62% and decreased root growth observed at 100,250 and 500 ppm concentrations. Zn increased the cowpea germination to 123-15% followed by green gram to 87%, horse gram to 116-22%, and chickpea to 92-81%. Combination of Cu and Zn increased the cowpea germination to 34-125%, green gram to 40%, horse gram to 90-35% and Chickpea to 40%-81%. Root and shoot growth of test plants were stimulated at 10, 25,50ppm metal concentrations. Cowpea was able to germinate efficiently in all treated metals at 25ppm. Increased green gram growth observed at 10-100ppm of Zn compared to Cu and Cu+Zn at same concentrations. Growth of horse gram favored by 25-50ppm Cu compared to same Zn concentration. 10ppm Cu promoted the root growth in all tested plants. Decreased root growth of chickpea observed in combination of Cu and Zn treated metals. Obtained results indicated that the plants were able to grown in moderated amount of individually metals. Higher concentration of Cu causes deficiency of other essential nutrients like Zn by competitive exclusion at uptake sites. This reduces enzyme production and suppresses membrane functioning in plants (8). The greater reduction in root growth than in stem growth and seed germination was due to, heavy metals were more readily absorbed into the cells through thin root epidermis than in to the seed and stem. Roots, the primary plant organs that sense, contact, and accumulate heavy metals from the substrate are thought to be reliable indicator of metal tolerance in plants (6).

Effect of fungal biosorbed metal solutions on plant growth and uptake of metals

Eluted metal ions from *A.niger* and *A.flavus* biomass showed decreased metal concentration (Table 2). Metal elution in fungi is possibly due to ion-exchange reactions. Autoclaved fungal biomass showed maximum metal removal by cell wall rupture and allows free access of metal ions to binding sites (15).

Table 2.Biosorption efficiency of 10-500 mg l^{-1} Cu (II) and Zn (II) metal ions from aqueous solution by pretreated *A.niger* and *A.flavus* biomass

Cu $(mg^{-1}l)$	Biosorption by *A.niger*	Biosorption by *A.flavus*	Zn $(mg^{-1}l)$	Biosorption by *A.niger*	Biosorption by *A.flavus*
10	8.2±1.6	7.8±1.4	10	7.3±2.1	6.9±3.9
25	19.2±2.4	16.2±2.3	25	17.2±2.4	15.2±2.3
50	29.4±2.9*	23.9±3.1*	50	25.3±2.9*	21±2.1
100	59.4±5.2*	49.4±4.8	100	54±3.5*	42±2.9
200	58.3±4.0	48.7±4.2	200	47.7±3.9	41.7±4.2
500	57.7±3.9	47.3±3.9	500	46.9±3.7	47.5±3.9

All values are the mean of three replicates ±SD. LSD* ($P<0.05$)

100ppm of fungal eluted metals was used in seed germination. Increased seed germination percentage in fungal treated metal solution was observed compared to control (Fig.4). Killing and alkali pretreatment increases the fungal heavy metals bisorption by destroy autolytic enzymes which cause fungal putrefaction. Lipid and proteins that mask binding sites remove by pretreatment and releases certain biopolymers towards cell wall that have a high affinity towards heavy metal ions (3).

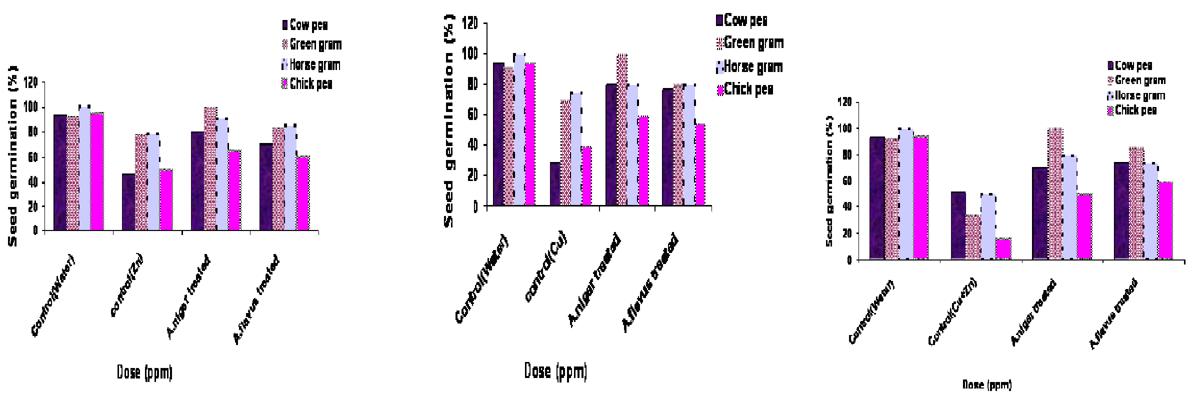

Fig 4. Effect of *Aspergillus niger* and *Aspergillus flavus* biosorbed metal solutions on seed germination

Observed percentage seed germination in 100ppm Cu, Zn and Cu+Zn metals treated with *A.niger* was 52%, 34%, 18% in cowpea, 30%, 22%, 66% in horsegram, 5%, 12%, 66% in greem gram and 20%, 15%, 34% in chickpea. In *A.flavus* treated metal solutions, 48%, 24%, 18% in cowpea, 10%, 6%, 66% in green gram, 5%, 7%, 30% by horse gram and 15%, 10%, 34% in chickpea. Combination of Cu and Zn metals favored less seed germination compared to individual Cu and Zn metals. Increased seedling vigor and biomass weight (Fig.5) was observed in plants treated with *Aspergillus niger* and *Aspergillus flavus* biosorbed metal solutions.

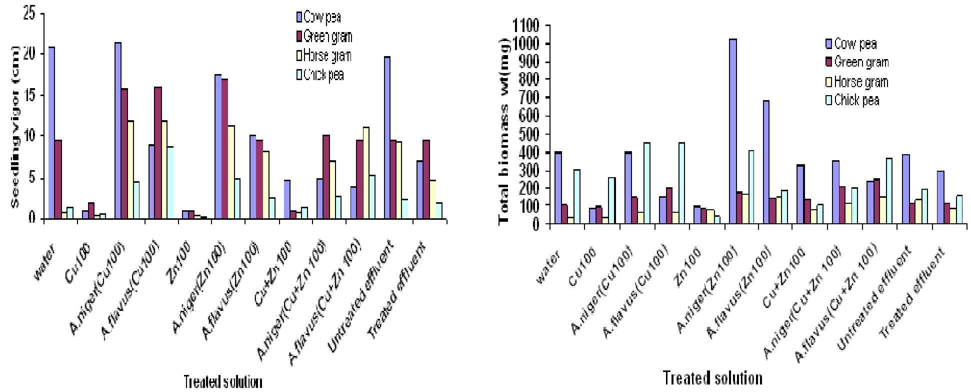

Fig 5. Effect of *Aspergillus niger* and *Aspergillus flavus* biosorbed Cu and Zn metal solutions on seedling vigor and total biomass weight of plants

Dry weight of root in metal treated plants was reduced more than the shoot weight. This is supported by the earlier findings that the influence of higher Zn, Cu, Pb and Cd in wheat resulted decreased shoot growth but the most evident symptoms were on the roots (22). Reduction in root growth is probably occurs due to heavy metals are more readily absorbed into cells through the thin root epidermis than into the stem and seeds. Thus roots are thought to a reliable indicator of metal tolerance in plants (23). Low Cu transfer to the aboveground plant tissues may be due to storage mechanism of Cu in the root tissues or by the low mobility of Cu in plants due to binding to the xylem (24). Physiological strategies were employed by the plants in specific developmental stage to cope with external toxic ions (25). The reduced metal accumulation was observed in the root and stem parts of the plant treated with *A.niger* (Table 3) and *A.flavus* (Table 4) biosorbed metal solutions.

Table 3. Accumulation of Cu in different plant parts after the addition of untreated (Control) and *Aspergillus niger* treated metal solutions

Added Cu (mg⁻¹l)	Plant parts	Control plant	*Aspergillus niger* treated metal solutions — Metal ions accumulated by test plants (mg plant⁻¹) *C.areietinum*	*M.uniflorum*	*V.radiata*	*V.unguiculata*
10	Root	6.9±1.49	1.1±0.47	1.4±0.67	0.9.±0.08	0.8±0.05
	Arial	3.1±1.57	0.7±0.06	0.3±0.07	0.6±0.09	1.1±0.87
25	Root	13±2.37	2.9±0.92	2.4±0.81	1.9±0.81	3.1±1.09
	Arial	11±2.04	1.6±0.71	1.5±0.52	2.1±0.49	1.6±0.89
50	Root	26±2.32	11±1.71	10±1.36	09±1.40	08±1.47
	Arial	19±2.09	08±1.42	07±1.79	08±1.18	10±2.31
100	Root	49±5.20*	22±2.78*	20±3.16	19±1.79	22±2.36*
	Arial	29±3.29	16±2.61	18±2.21	16±1.28	15±1.91
200	Root	68±5.29	49±4.09	59±4.58*	43±4.43*	39±3.21
	Arial	59±4.17	41±4.23	43±4.49	37±3.75	29±2.71
500	Root	71±3.94	52±5.13	57*±5.17	41±4.12	38±3.47
	Arial	69±4.01	43±2.14	39±4.42	35±3.71	26±2.98

All values are the mean of three replicates ±SD. LSD* (P<0.05)

Table 4. Accumulation of Zn in different plant parts after the addition of untreated (Control) and *Aspergillus flavus* treated metal solutions

Added Zn (mg⁻¹l)	Plant parts	Control plant	*Aspergillus flavus* treated metal solutions — Metal ions accumulated by test plants (mg plant⁻¹) *C.areietinum*	*M.uniflorum*	*V.radiata*	*V.unguiculata*
10	Root	5.1±1.74	1.9±0.87	1.1±0.47	0.9±0.32	1.2±0.21
	Arial	3.0±1.63	1.1±0.46	0.9±0.04	0.7±0.09	0.8±0.06
25	Root	15±2.13	3.9±1.32	4.1±1.17	3.7±1.28	3.1±1.39
	Arial	11±2.49	2.4±1.21	3.7±1.32	2.4±1.29	2.0±1.19
50	Root	23±2.12	19±2.71	14±1.36	13±2.04	10±1.9
	Arial	18±2.39	15±1.72	11±1.19	09±1.18	12±1.15
100	Root	29±3.27	27±3.18*	23±2.06	19±2.09*	20±2.03*
	Arial	18±2.29	19±2.01	15±1.21	17±2.18	13±2.06
200	Root	56±4.21*	49±3.29	52±4.58*	39±2.43	42±3.21
	Arial	37±3.27	37±3.53	44±4.09	27±3.15	35±3.11
500	Root	59±4.4	40±3.78	43±3.93	37±3.05	39±3.24
	Arial	41±3.21	33.±2.77	40±3.79	26±2.98	27±3.08

All values are the mean of three replicates ±SD. LSD* (P<0.05)

Aspergillus sp. were the most prevalent fungal strain in the waste water and able to tolerate and detoxify toxic metal ions in the effluent. Earlier findings showed that *Aspergillus niger* removed 91% copper and 70% zinc by solubilized insoluble compound from waste water (26, 27). The metal concentration in the roots was significantly higher than in the shoots. Metal exclusion and metal accumulation are two strategies adopted by the plants to tolerate heavy metals. Exclusion strategies avoid excessive metals uptake and restrict its transport to the roots. Transport of heavy metals form root to shoot depends on compartmentation in vacuoles or complexation by organic ligands such as organic acids, amino acids, metallothioneins proteins and peptides (28). Results showed that autoclaved *A.flavus* and *A.niger* biomass may be used as an inexpensive and effective biosorbent for the removal of toxic metal ions form aqueous solutions. Metal sorption depends on factors like pH, concentration of biomass, concentration of the metal ions, temperature and other ions in test solution (29). The mobility of metals through the plant can be related to the root/shoot ratio of accumulated metal ions. Hyper accumulator species are characterized by high amount of metals in the shoots, but biomass production is usually not as high as control, which concentrate moderate amounts of metal in their tissues. Zn concentration in shoot and root ration was higher than Cu, either supplied alone or in combination of both metal ions (30). Results showed that both susceptibility and response to metals differed among crop species and various metals (30) and related to both adaptation to the environment and genetic traits of the crops (31).

CONCLUSION

Seed is a stage in the plant life cycle that is well protected against various stresses including metals. Seeds still germinated in the presence of high metal concentrations, but the subsequent seedling growth after breakage of seed coat was severely inhibited by low metal concentrations. Plants can be used as metal accumulator and indicator for metal toxicity. Fungal biomasses are potential heavy metals biosorbent results in reduced metal toxicity and able to enhance the plants growth.

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