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Original Article

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Phytotoxic effects of Cobalt, Copper and Cadmium on Seed Germination and Seedling Vigour in Mungbean [*Vigna radiata* (L) Wilczek]

Nand Lal

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Abstract

Investigations were carried to study the influence of 0 (control), 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mM of cadmium (Cd++), cobalt (Co++), and copper (Cu++) ions in an aqueous solution (as chloride salts) on germination, root elongation, hypocotyl growth, mobilization efficiency, tolerance index and vigour index of mungbean (*Vigna radiata* (L.) Wilczek) variety K-851 seeds and seedlings. The extent of inhibition of germination was found to depend on, metal concentration, metal type and incubation period. In comparison to control, there was a complete inhibition of root and hypocotyl growth in germinating mung bean seeds at Co and Cu concentration ≥ 6.0 mM. The order of these metals on inhibition of germination, root elongation and hypocotyl growth was Cu > Cd > Co. The phytotoxic effects of tested metal ions were also evident in case of mobilization efficiency, tolerance index and vigour index. All the tested metal concentration could be efficiently tolerated by *V. radiata* seedlings. Variation in these physiological parameters has role in combating the metal chlorides mediated stress, and could be used for measuring the phytotoxicity and degree of tolerance of the test system.

Keywords: Cadmium; Cobalt; Copper; Germination; Mungbean; Phytotoxic; Seedling Vigour; Vigna radiata.

Introduction

Metals are continuously released into the biosphere by volcanoes, natural weathering of rocks, industrial activities, the combustion of fossil fuels and the release of sewage. Though some metals, e.g., Mn, Cu, Zn, Mo, and Ni are essential or beneficial micronutrients for micro-organisms, plants and animals, at high concentrations all metals have toxic effects and pose environmental threat[1] *(Nedelkoska and Doran, 2000)*. This threat is first experienced by plants, the primary producers, mostly through the contamination of soil and water. Like other stresses, plant species differ markedly in their sensitivity to metals. The highest risk for human health occurs when plants develop tolerance against metals and metals are incorporated into the food chain.

During processing of minerals and waste products, plenty of metals in form of finally

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powdered stuff are disposed off in the surrounding areas and enter into biogeochemical cycle. Most of metals occur as insoluble organic compound or are bound to organic matter, clays and hydrous oxides of other divalent and trivalent metals in soil. Because of this, only few heavy metals are in available form and show toxicity. The frequent metals are Zn, Cu, Ni and their phytotoxic effects are evident in definite magnitude in different plant systems[2] *(Lal, 2010)*. Cereals are relatively tolerant and in other plants responses are highly genotype specific.

The most characteristic symptoms of metal toxicity appear as stunting and chlorosis. The chlorosis result due to imbalance in Fe nutrition due to interaction of toxic metals with Fe and is affected by climatic and soil conditions, phosphate status of the soil and plants and Fe status of the soil because all these factors regulate the activity of competing metal ions. The stunting observed is generally due to specific toxicity of the metal to the crop which induces antagonism/synergism for the efforts of essential ions or due to inhibition of root penetration. In the second case, the effect is specific to the root tip cells which ultimately restricts the root length and supply of essential ions via diffusion. The seedling stage is generally more sensitive to metal toxicity than other stages where in the toxic symptoms are modified by metal interaction and external factors. Metal ions also enter into cell cytoplasm where they interfere with several essential life processes. Such processes are not clearly known as they require study of ion effects on isolated organelles. However, cumulatively metals disturb the organic acid pool, amino acid pool, cation exchange across membrane and pH of cellular compartments[2] (*Lal, 2010)*. Certain organelles like dictyosome show deposition of metals at preferential level than other organelles and thus seem to be an important metal excretion processes[3] *(Malone et al., 1974)*.

Seed coat is the main barriers to metals and prevent contamination of embryos until the seed coat is torn apart by the germinating embryonic root. The effects of metals on germination of seeds depend on interspecies differences in seed structure, particularly seed coats, because seed coats have a wide range of anatomic forms that exist in no other plant organ or tissue[4] (*Wierzbicka and Obidziniska, 1998)*. Leguminous plants are very sensitive test system for heavy metal toxicity. There are reports on toxic effects of Cu, Cd, Co and other heavy metals in several legumes including lentil, Soybean[5] (*Bazzaz et al., 1974)*, gram[6] *(Lal and Mishra, 2004)*, pea[7] *(Hernandez et al., 1996, 1997)*, Rajamas[9] *(Van Assche and Clijsters, 1990)*, cowpea[10] *(Lal and Mishra, 2006)* etc.

Considering this, the present investigations were carried with mungbean (*Vigna radiata* (L.) Wilczek) variety K-851 to study the phytotoxic effects of Co, Cu and Cd with special references to seed germination, root elongation, hypocotyl growth, mobilization efficiency, tolerance index and vigour index therein. Seed germination stage constitutes the first and foremost important stage of plant life and lays the foundation for future crop health and yield. So the present investigation at germination would be of pivotal importance for future studies on heavy metal effects on plants in general and mungbean in particular.

Materials and Methods

Mung (*Vigna radiata* (L.) Wilczek) variety seeds, procured from authorized shop of Natural Seed Corporation Limited, New Delhi, were used as experimental material. The chloride salt formulations of cadmium (Cd^{++}), cobalt (Co^{++}), and copper (Cu^{++}) metals used as heavy metal source were from Merck Chemicals and comprised of $CdCl_2.½H_2O$, $CuCl_2.2H_2O$ and $CoCl_2.6H_2O$.

Seeds were sown in a series of 7 glass petridishes each containing 20 ml distilled water (dH_2O) or metal chloride solution of different concentrations [0.0 (control), 0.5, 1.0, 2.0, 4.0, 6.0 & 8.0 mM, respectively] and each treatment was replicated to five. The seeds were imbibed by immersing them into 20 ml metal solutions in plant growth cabinet in dark at 25°C for 4 hours, the swelling period. Swollen seeds were then sown in petridishes lined with double layer of filter paper wetted with 20 ml metal chloride solutions. The petridishes were kept at 25°C in the dark in growth cabinets for 96 hours, for determining the germination rate for every 24 hours. A 1-mm radicle emergence from seeds was considered seed germination. The root, shoot and hypocotyl lengths, root and shoot fresh weights, and dry weight of seedling and cotyledons, however, were measured only after 96 hours of incubation. At 96 hours, observations were recorded for germination and seedlings growth. Different parameters (root length, shoot length, hypocotyl length, root fresh weight, shoot fresh weight, dry weight of seedling and cotyledons, Mobilization Efficiency, Tolerance Index and Vigour Index) were recorded at specified stages. The time taken for initiation of germination and % germination data were recorded for each metal with each treatment after every 24 hours.

Five randomly selected seedlings were harvested separately from each treatment at 96 hours' stage, respectively, washed with dH_2O and dried on a blotting sheet. The root and shoot portions were separated and observations were recorded on Root length (cm), Shoot length (cm), Hypocotyl length (cm), Root fresh weight (gm) and Shoot fresh weight (gm). For evaluating the dry weight of cotyledons and seedling, the cotyledons and seedling portions were separated and these portions were placed on aluminium foils. All the aluminum foils containing portions of cotyledons and seedling for each treatment were placed in the hot air oven at 72°C temperature for 48 hours in order to achieve constant dry weight.

Mobilization efficiency[11] *(Mohan et al., 1996)* in germinating seedlings for each treatment was calculated by following formula:

$$\text{Mobilization Efficiency} = \frac{\text{Dry weight of seedling}}{\text{Dry weight of cotyledon}} \times 100$$

Tolerance index[12] *(Mishra and Choudhury, 1998)* of seedlings obtained from each treatment was calculated by following formula:

$$\text{Tolerance Index (TI)} = \frac{\text{Mean length of longest root in a treatment}}{\text{Mean length of largest root in a control}} \times 100$$

Vigour index[13] *(Abdul-Baki and Anderson, 1973)* of resulting seedlings from each treatment was calculated by the following formula:

$$\text{Vigour Index (VI)} = \%\ \text{germination} \times \text{average hypocotyl length.}$$

Data are based on five replications for each parameter, randomly selected from 50 seeds in each treatment. The data were subjected to appropriate statistical analysis and all values are expressed as mean±SD.

Results and Discussion

The results for the effect of selected metals (Co, Cu, Cd) on germination of mungbean seeds are summarized in table 1. The extent of inhibition of germination was found to depend on metal concentration, metal type and incubation period. When the concentration of metals exceeded certain levels, (e.g., at concentrations between 6.0-8.0 mM for both Co and Cu, 0.5-8.0 mM for Cd), an abnormal germination was resulted (e.g. testa torn by radicle, but no development of root at further incubation periods).

In control, at 24 hours' incubation period, 62% germination was found which increased at 48 hours to 98% and at 72 hours and 96 hours to 100% (maximum). The germination percentage (%) gradually increased from 24 hours to 96 hours in control and same pattern was followed by other treatments as well.

In case of $CoCl_2$, germination rate of mungbean at 24 hours' incubation period was maximum (80%) at 1.0 mM. As $CoCl_2$ concentration increased further, there was decrease in germination. At 2.0 mM, it decreased from 80% to 72% and the trend continued till 4.0 mM and reached to minimum i.e. 42%.

In general, the effective concentrations of metals required for inhibition of seed germination to a certain degree increased with increasing incubation period. The effect of metals investigated for germination inhibition was in the following sequence: Cu > Cd > Co. A study by *Munzuroglu and Geckil*[14] *(2002)*, using six metals (Hg, Cd, Co, Cu, Pb and Zn) showed, effect of metals for germination inhibition was in the following sequence: Hg > Cd > Cu > Pb > Co > Zn in wheat grains (monocot) and Hg > Cu > Cd > Pb > Zn > Co in cucumber seeds (dicot). These findings with mungbean largely confirm to above observations with cucumber seeds.

At 6.0 mM concentration, germination again increased up to 66% and further decreased at 8.0

Table 1: Percent germination of *V. radiata* cv. K-851 in the presence of varying concentrations of Cobalt, Copper and Cadmium.

Metal Chloride (mM)		% germination			
		24 hours	48 hours	72 hours	96 hours
0.0 (control)		62	98	100	100
CoCl2	0.5	76	96	98	98
	1.0	80	100	100	100
	2.0	72	96	98	98
	4.0	42	98	100	100
	6.0	66	92	98	100
	8.0	56	92	94	100

table cont...

CuCl2	0.5	68	98	98	98
	1.0	64	94	98	100
	2.0	70	96	100	100
	4.0	66	88	94	100
	6.0	12	22	22	48
	8.0	02	02	06	26
CdCl2	0.5	64	98	98	98
	1.0	46	74	96	98
	2.0	42	42	46	98
	4.0	30	40	40	74
	6.0	28	44	48	62
	8.0	32	44	48	60

mM (56%). At 48 hours, maximum germination was found at 1.0 mM (100%) and minimum (92%) at 6.0 mM and 8.0 mM (Table 1). At 72 hours, maximum germination was found with control, 1.0 and 4.0 mM $CuCl_2$ (100%) followed by 0.5, 2.0 and 6.0 mM (98%) and minimum at 8.0 mM (94%). At 96 hours, germination at 0.0, 1.0, 4.0, 6.0, 8.0 mM reached 100% except at 0.5 mM and 2.0 mM (98%). It is known that Co is an essential element only for some plants, and it affords both beneficial effects and toxicity to plants[15] (Liu *et al.*, 2000). That is why this metal caused a lesser inhibition of germination in mungbean seed.

In case of $CuCl_2$, at 24 hours there was alternate increase and decrease in germination % till 4.0 mM. Germination was maximum (70%) at 2.0 mM, at minimum (2%) at 8.0 mM. Germination at 48 hours was maximum at control and 0.5 mM (98%). Germination at 72 hours was maximum at 2.0 mM (100%). Germination at 96 hours was maximum and equal at 1.0, 2.0, 4.0 mM (100%). Minimum germination was found at 8.0 mM for all the incubation periods. Except for mungbean seeds at 24 hours' incubation with 0.5-4.0 mM, all concentrations of this metal reduced the germination substantially. Copper has been rated as one of the most unfavourable metal for plant growth by other workers as well[16] (Quarity et al., 1997). In case of $CdCl_2$, at 24 hours, maximum germination was found at 0.5 mM (64%). Upon further increase in $CdCl_2$ concentration, germination decreased gradually till 6.0 mM and reached to minimum 28% (Table 1). At 8.0 mM concentration germination rate increased slightly (by 4%) in comparison to 6.0 mM. The germination at 48 hours was maximum at control and 0.5 mM (98%) and minimum at 4.0 mM (40%). It decreased from 0.5 mM to 4.0 mM and then slightly increased at 6.0 mM (44%). At 72 hours, maximum germination was 100% (at control). As the concentration increased, the germination decreased till 4.0 mM and increased further at 6.0 and 8.0 mM up to 48%. At 96 hours, germination was maximum at control in comparison to other $CdCl_2$ concentrations. The 0.5, 1.0 & 2.0 mM concentrations showed 98% germination. Upon further increase in $CdCl_2$ concentration, germination reduced reaching minimum at 8.0 mM (60%).

In general, $CoCl_2$ treatment did not inhibit the germination response. In case of $CuCl_2$, germination response remained unaffected till 4.0 mM after which there was a drastic reduction. In case of $CdCl_2$ there was a general inhibition/ reduction in germination response of mungbean seeds. Overall it appears from the results that Cu was most inhibitory metal for germination at higher concentration (6.0 and 8.0 mM). Cd was less inhibitory for germination (magnitude wise) than Cu but at all the levels. Co metal caused least/ no inhibition of germination in mung bean and showed promotory effect on seed germination at lower incubation periods. The order of these metals on inhibition of germination was Cu > Cd > Co.

Effect of different $CoCl_2$, $CuCl_2$ and $CdCl_2$ concentrations on length of root, shoot and hypocotyls and biomass (fresh weight) of root and shoot portion of V. radiata seedlings are shown in table 2. In control the root and shoot length were 1.92±0.13 cm and 2.92±0.17 cm, respectively. At 0.5 mM CoCl2, the root length increased to 2.10±0.17 cm but there was no change in FW (Table 2). At 1.0 mM concentration, root length and biomass increased to 2.14±0.76 cm and 0.01±0.00 gm. Upon further increase in $CoCl_2$, the root length and fresh weight decreased to 0.633±0.047 cm and 0.002±0.001 gm, respectively (Table 2). At 6.0 mM and 8.0 mM $CoCl_2$, no measurable roots and hypocotyls were available. Shoot length was promoted at 0.5 mM and 1.0 mM $CoCl_2$ but further levels of $CoCl_2$ caused a significant reduction in shoot length.

Root length, root FW, shoot length, shoot FW and hypocotyl length were maximum at 1.0 mM $Cocl_2$ concentration. Shoot length and biomass were maximum at 1.0 mM and minimum at 8.0 mM. Hypocotyl length was maximum (1.58±0.23 cm), at 1.0 mM and it was reached to 0.66±0.29 cm (minimum) at 4.0 mM. It appears that root is more sensitive than shoot to Co metal.

Table 2: Growth response of root, shoot and hypocotyl of germinating seeds of *V. radiata* cv. K-851 in the presence of varying concentrations of Cobalt, Copper and Cadmium.

Metal Chloride(mM)		Root		Shoot		Hypocotyl length (cm)
		Length (cm)	FW (gm)	Length (cm)	FW (gm)	
CoCl2	0.0	1.92 ± 0.13	0.008 ± 0.002	2.92 ± 0.17	0.150 ± 0.013	1.00 ± 0.24
	0.5	2.10 ± 0.17	0.008 ± 0.003	3.18 ± 0.37	0.141 ± 0.022	1.08 ± 0.38
	1.0	2.14 ± 0.76	0.011 ± 0.003	3.72 ± 0.77	0.172 ± 0.020	1.58 ± 0.23
	2.0	1.52 ± 0.18	0.006 ± 0.003	2.72 ± 0.34	0.160 ± 0.029	1.20 ± 0.19
	4.0	0.63 ± 0.05	0.002 ± 0.001	1.22 ± 0.22	0.131 ± 0.028	0.66 ± 0.29
	6.0	*	*	0.76 ± 0.19	0.132 ± 0.022	*
	8.0	*	*	0.54 ± 0.05	0.122 ± 0.023	*
CuCl2	0.5	1.56 ± 0.23	0.012 ± 0.004	3.20 ± 0.65	0.154 ± 0.026	1.54 ± 0.65
	1.0	1.92 ± 0.29	0.011 ± 0.002	3.76 ± 1.07	0.154 ± 0.010	1.84 ± 0.95
	2.0	1.72 ± 0.23	0.008 ± 0.004	2.86 ± 0.41	0.146 ± 0.010	1.14 ± 0.48
	4.0	0.40 ± 0.10	0.0021	0.90 ± 0.43	0.126 ± 0.019	1.00 ± 0.20
	6.0	*	*	0.32 ± 0.07	0.093 ± 0.025	*
	8.0	*	*	0.26 ± 0.05	0.092 ± 0.018	*
CdCl2	0.5	*	*	0.48 ± 0.07	0.146 ± 0.017	*
	1.0	*	*	0.36 ± 0.05	0.121 ± 0.012	*
	2.0	*	*	0.32 ± 0.07	0.125 ± 0.009	*
	4.0	*	*	0.28 ± 0.07	0.102 ± 0.016	*
	6.0	*	*	0.36 ± 0.05	0.133 ± 0.036	*
	8.0	*	*	0.36± 0.08	0.129 ± 0.013	*

* No measurable root and hypocotyl available.

1 S.d. not shown due to lack of replicated data.

In case of 0.5 mM $CuCl_2$, root length was decreased to 1.56±0.23 cm but there was increase in FW. At 1.0 mM concentration, root length increased but FW slightly decreased from 0.012±0.004 gm to 0.011±0.002 gm (Table 2). Upon further increase in $CuCl_2$, the root length, root FW, shoot length, shoot FW and hypocotyl length showed a decreasing trend. Shoot length and hypocotyl length were maximum at 1.0 mM $CuCl_2$. Root length was maximum at control and at 1.0 mM $CuCl_2$. Root fresh weight was maximum at 0.5 mM but shoot fresh weight maximum and equal at 0.5 mM and 1.0 mM. At 6.0 and 8.0 mM, no measurable root and hypocotyl lengths were available but shoot length was evident reaching to minimum at 8.0 mM (0.26±0.05) and same was true for shoot biomass (Table 2).

In case of $CdCl_2$ ranging 0.5-8.0 mM, no measurable roots and hypocotyls were available, however, shoot growth was clearly evident. Between 0.5-4.0 mM $CdCl_2$, shoot length showed decreasing trend, but shoot length increased further to 0.36±0.08 at 8.0 mM. The maximum shoot length and shoot FW were at control and these reached to minimum at 4.0 mM (Table 2). Comparative metal toxicity studies in wheat revealed that Cd had toxic effect on plants at concentrations as low as 50 mgL-1, whereas lead did not show the same effect at 500 mgL^{-1} concentrations[17] *(Oncel et al., 2000).*

In comparison to control, there was a complete inhibition of root and hypocotyl growth in germinating mungbean seeds at Co and Cu concentration ≥ 6.0 mM. The 0.5 and 1.0 mM concentration of Co had promotory effect on root, shoot and hypocotyl growth (Table 2). In case of Cu, maximum root length was available at control and 1.0 mM. Shoot length and hypocotyl length were maximum at 1.0 mM. At 0.5 mM maximum root fresh weight was found but shoot fresh weight was maximum at 0.5 and 1.0 mM. However, other

concentrations of Cu showed inhibitory effect on growth of seedlings/seedling parts. In case of Cd, no root length & hypocotyl length was available because of complete inhibition of root and hypocotyl growth, however, shoot growth was well marked which was maximum at control and decreased upon increase in $CdCl_2$ concentration. The order of these metals on inhibition of root elongation, shoot elongation and hypocotyl growth was Cu > Cd > Co.

It appeared that above certain concentrations of these metals, the germination of seeds was not normal in the sense that roots and hypocotyls were not properly developed, although they appeared to process a distinct radicle. This might be an indicator of sensitivity of plant's early development stage to these metals. The order of these metals on inhibition of root elongation and hypocotyl growth was Cu > Cd > Co for mungbean. The order of these metals on inhibition of root elongation and hypocotyl growth, reported in the present study show resemblance with results obtained earlier[14] by *Munzuroglu and Geckil (2002)*. Root which is the site of entry of metal ions was much affected in comparison to the shoot which receives metal ions quite late due to metal translocation.

Table 3 summarizes the data on variation in mobilization efficiency (ME), tolerance index (TI) and vigour index (VI) of seedlings of *V. radiata* obtained in the presence of varying concentrations of Cobalt, copper and Cadmium respectively. At control, ME was 120.79±2.83. In case of Co, ME was maximum at 0.5 mM i.e. 121.326±7.41 marginally higher than control. ME showed a decreasing trend due to increase in $CoCl_2$ concentration. In case of Cu, ME was maximum at 1.0 mM but at all other levels it was less than control, and decreased to 101.92± 0.76 at 8.0 mM. In case of Cd, maximum ME was evident at control and minimum ME was recorded at 2.0 mM. Highest level of tested metals showed ME value in close proximity. More growth of seedlings was recorded with Co which shows correspondence with mobilization efficiency. Effect of metals investigated on ME in *V. Radiata* was in the following sequence: Co > Cd > Cu.

At control, TI was 100. In case of Co, TI was maximum at 1.0 mM (111.46). At concentrations between 0.0 to 1.0 mM, TI was increased indicating that the metal was beneficial to root growth rather than being inhibitory/toxic. However, further increase in $CoCl_2$ decreased TI to 32.97. TI could not be determined due to lack of measurable root length

Table 3: Variation in Mobilization efficiency, Tolerance index and Vigour index of seedlings of *V. radiata* cv. K-851 in the presence of varying concentrations of Cobalt, Copper and Cadmium.

(mM)		Mobilization Efficiency (ME)	Tolerance Index (TI)	Vigour Index (VI) (at 96 hours)
0.0 (control)		120.79 ± 2.83	100.00	100.00
CoCl2	0.5	121.33 ± 7.41	109.38	105.84
	1.0	113.56 ± 3.51	111.46	158.00
	2.0	110.75 ± 5.58	79.17	117.60
	4.0	104.98 ± 3.10	32.97	66.000
	6.0	102.96 ± 0.94	*	*
	8.0	102.12 ± 0.89	*	*
CuCl2	0.5	120.73 ± 5.21	81.25	150.92
	1.0	124.12 ± 8.56	100.00	184.00
	2.0	117.74 ± 6.45	89.58	114.60
	4.0	110.93 ± 5.67	20.83	100.00
	6.0	101.60 ± 1.00	*	*
	8.0	101.92 ± 0.76	*	*
CdCl2	0.5	102.44 ± 1.54	*	*
	1.0	102.05 ± 0.96	*	*
	2.0	101.43 ± 1.04	*	*
	4.0	101.96 ± 1.55	*	*
	6.0	102.28 ± 0.81	*	*
	8.0	102.04 ± 0.81	*	*

*Value of TI could not be determined due to lack of measurable root length and value of VI could not be determined because no differentiated/ well marked hypocotyl present.

at 6.0 and 8.0 mM. In case of Cu, TI was maximum at control and 1.0 mM i.e. 100. TI decreased at 0.5 mM to 81.25. Upon increase in $CuCl_2$ beyond, 1.0 mM, TI decreased to 20.83. At 6.0 and 8.0 mM, value of TI could not be determined due to lack of measurable root length.

In comparison to control, Co treated seeds/ seedlings were more tolerant to metal toxicity as evident from TI data. Cd treated seeds were more susceptible to metal toxicity as evident from failure of radicle emergence and root growth. The order of these metals for TI was Co > Cu > Cd.

At control, VI was 100. In case of Co, VI was maximum at 1.0 mM (158.00) and reached to minimum at 4.0 mM. The 0-2.0 mM range of Co metal appears to have a positive effect on growth and health of seedlings (Table 3). At 6.0 and 8.0 mM, value of VI could not be determined as no differentiated/well marked hypocotyls were present.

In case of Cu, VI was maximum at 1.0 mM (184.00) and minimum at 4.0 mM (100.00, equal to control). Like Co, this metal also caused a complete inhibition of VI due to absence of differentiated/ well marked hypocotyl length at 6.0 and 8.0 mM. In case of Cd, value of VI could not be determined at either of $CdCl_2$ concentration as no differentiated/ well marked hypocotyl present. In comparison to control, Co and Cu treated seeds showed same pattern for VI (Table 3). The order of these metals for VI was Cu > Co > Cd.

The selection range of metal chloride concentrations is based on metal concentrations commonly used by other workers, and encountered in environment to their several fold higher concentrations. The type of application (incubation of seeds on filter paper soaked with metal) is a common method for testing metal toxicity effects in seed germination. This method reduces the effect of counter anions associated with other metals/ metal cations normally present in soil. In soil, the effect of a metal is determined synergistically or antagonistically due to interaction with other metal cations and their associated anions. In the present study, the effect of counter anion (Cl^-) associated with these metals has not been further considered as all the metals were used as chloride salt. The effect of metals on development and reproduction of plants can first be quantified by determining the germination characteristics of seeds. The metal treatment used here is simple (no need for highly skilled personnel, plant seeds are simply allowed to germinate in metal and control solutions), rapid (requires a few days), and easy to perform (does not need physical methods using expensive apparatus). All three metals used in this study caused a decrease/ delay in germination of mungbean seeds, though to different extents. In general, the germination inhibition increased with increasing concentrations of metals and each metal at certain concentration level lowered germinability. These three metals also caused complete inhibition of hypocotyls and roots in germinated seeds at certain concentrations. Inhibition of root elongation is considered to be the first evident effect of metal toxicity in plants. Cell division at the root tip and cell elongation in the extension zone are two different mechanisms in root growth, both of which are affected by the presence of metals *(Arduini et al., 1994)* and cause inhibition of root elongation.[18]

Conclusions

The study concludes that 96 hours' exposure to different metal chlorides shows differential phytotoxic effects on different physiological parameters. The shoot length and biomass are little affected due to metal chlorides treatments in comparison to root length and biomass.The phytotoxic effects of metal chlorides are also evident in case of mobilization efficiency, tolerance index and vigour index. All the tested metal chlorides concentration could be efficiently tolerated by *V. radiata* seedlings. Variation in these physiological parameters has an important role in combating the metal chlorides mediated stress, and could be used for measuring the phytotoxicity and degree of tolerance of the test system.

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Antioxidant Activity of Bark and Leaf Extracts of *Anthocephalus cadamba* (Roxb.) Mique using FRAP Assay

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Abstract

Phytochemicals derived from various herbs hold a promise in treating the oxidative stress in various diseases. The aim of this study was to measure the antioxidant activity of various extracts prepared from the bark and leaves of *Anthocephalus cadamba* (Roxb.) Mique (Rubiaceae) plant. The Ferric reducing ability of plasma (FRAP, also Ferric ion reducing antioxidant power) assay was used to assess the oxidative potential of various extracts. The results show that methanol extracts of the bark and the leaves give the higher TAC FRAP values. The results obtained have the potential of bark and leaf extracts of *A. cadamba* to be exploited pharmacologically in future.

Keywords: Invitro; Antioxidant Activity; Anthocephalus Cadamba; Bark and Leaves.

Introduction

The process of oxidative metabolism is crucial for the survival of most cells. However, the indispensability of oxygen as the element that gives life has a flip side to it. The fame of oxygen (O_2) stems from its capacity to generate free radicals, which, in the majority of instances, are associated with a multitude of negative outcomes. The examples of such reactive oxygen species are highly reactive radicals having surplus of electrons such as hydroxyl electron ($OH^{\cdot}$), the superoxide radical (O_2), the nitric oxide radical ($NO^{\cdot}$) and lipid peroxyl radical ($LOO^{\cdot}$)[1] (Bagchi and Puri, 1998).

Cells are proving to be adversely affected by the reactive oxygen species which may be generated as unwanted products of oxidative metabolism in cells. Cells have evolved a variety of mechanisms to protect themselves from the oxidative stress they may be subjected to either during the course of disease or as a part of their routine metabolism. Some of the most important defences of a cell against the oxidative damage resolve around the antioxidant activities of vitamins such as ascorbic acid, tocopherol, coenzyme Q or glutathione etc. Other vital players in the antioxidant defence of our body are the enzymes superoxide dismutase (SOD), catalase and peroxidase etc.

Plants, being a rich source of antioxidant substances, have been frequently used to repair oxidative damage occurring during the course of various diseases[2] (Singh *et al.*, 2000). Plants are a rich source of antioxidants because they possess

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numerous phytochemicals which may have the ability to remove or neutralize harmful radicals. Plant metabolites are considered to be as superior to modern medicines because their consumption pre-empts a variety of side effects which are associated with synthetic medicines. This is the reason why natural products are in demand these days, and this demand is growing day by day (Gupta, 1994).

Anthocephalus cadamba is one such herb that has been extensively used in traditional medicine to treat various conditions.

Description of the Plant

The diverse culture of our country is a rich source of traditional medicines, many of which are of plant origin. *Anthocephalus cadamba* (Roxb.) Mique, also known as Kadam or Kadamba, is a tropical tree species that is native to South Asia and Southeast Asia, including Indonesia. In India, it is found on the slopes of evergreen forests up to 500 meters. It is commonly distributed in the sub-himalayan tract from Nepal eastwards on the lower hills of Darjeeling terai in West Bengal; in Chhota Nagpur (Bihar), Orissa, and Andhra Pradesh; in damp places along large streams in the Andamans; in Karnataka and Kerala on the west coast, and at low level in wet places of the western ghats. The bark is gray, smooth in young trees, but rough and longitudinally fissures in old trees. Leaves simple, opposite, elliptic–oblong; flowers in solitary globose head, orange and yellow; fruits are pseudocarps[4,5] (Anonymous, 1985; Naithani and Sahni, 1997).

In folk medicine *Anthocephalus cadamba* is used in the treatment of fever, uterine complaints, blood diseases [6] (Majumdar, 2002), skin diseases[7] (Bhandary *et al.*, 1998), eye inflammation and diarrhoea[8] (Pal and Jai, 2000). anaemia, leprosy, dysentery and stomatitis, menorrhagia and in improvement of semen quality[9] (Salkar *et al.*,1992).

Chemical constituents

The major constituents of bark have reported triterpenes, triterpenoids, glycosides, saponins, indole alkaloids, cadamabine, 3 alpha (symbol) dihydrocadambine, isocadambine and isodihydrocadambine[10,11] (Sahua *et al.*, 2000; Rastogi *et al.*, 1993). Quercetin-3-rhamnoglucoside, kaempferol and chromogenic acid are isolated from the leaves[12] (Kapil *et al.*, 1995).

Materials and Methods

Collection of Plant Sample

Fresh bark and leaves of *Anthocephalus cadamba* were collected from the medicinal plants garden, C.S.J.M. University, Kanpur. Collected samples were immediately broken into small fragments in the field to hasten drying and reduce bulks. Packed samples were further chopped to reduce size for drying and then processed for drying under shade. Before further size reduction of the samples by grinding, the crude chopped samples (100-200 gm) were preserved in plastic bags with proper label and kept as voucher samples. They were completely dried and then mounted on a sheet with proper labelling. To convert dried samples into coarse powder, grinding of samples was done with the help of a milling machine. After grinding, the samples were kept in a cool and dry place for further use.

Chemicals

The chemicals used in the experiment were TPTZ (2,4,6–tripyridyl–s–triazine), Ferrous Sulphate and Ferric Chloride for antioxidant estimation were purchased from Sigma Solvents. Hexane, Methanol, Chloroform, Ethyl Acetate used for extraction was purchased from Merck Chemicals Ltd. All chemicals used were of analytical grades.

Method of Extraction

Extraction was done by Soxhlet apparatus using four different solvents in increasing order of their polarity: Hexane, Chloroform, Ethyl Acetate and Methanol. Finally, the aqueous extract was prepared and immediately 5.0 ml methanol was added to avoid fungal growth in aqueous extracts. These extracts were properly weighed, labelled and kept in refrigerator at low temperature for further use in estimating in vitro antioxidant activity.

Method for in vitro Antioxidant Assay

For in vitro antioxidant assays the most widely used assay – Ferric reducing ability of plasma (FRAP) as μmol ferrous ion equivalents was performed for measuring the total antioxidant activity[13] (Benzie and Strain, 1966).

Reagents for FRAP Assay

a. Acetate buffer 300mM, pH 3.6: Weigh 3.1 g Sodium acetate trihydrate and added 16 ml of glacial acetic acid and made the volume to 1.0 L with distilled water.

b. TPTZ (2,4,6–tripyridyl–s–triazine): (MW 312.34), 10 mM in 40 mM HCl (MW 36.46). 0.031 g of TPTZ was added to 10 ml of 40 mM HCl and dissolved at 50 °C.

c. $FeCl_3.6H_2O$: (MW 270.30), 20 mM. 0.054 ***g of*** $FeCl_3$ was dissolved in 10 ml of distilled water.

The usable FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1 just before the test. The standard was $FeSO_4.7H_2O$:0.1 – 1.0 mM in methanol.

FRAP Assay Procedure

FRAP solution (3.6 mL) was added to distilled water (0.4 mL), incubated at 37 °C for 5.0 minutes and this solution was mixed with a certain concentration of the plant extract (80 mL) and incubated at 37 °C for 10 minutes. The absorbance of the reaction mixture was measured at 593 nm. For the construction of the calibration curve, five concentrations of $FeSO_4.7H_2O$ (0.1, 0.2, 0.4, 0.6, 0.8 and 10 mM) were used, and the absorbance values were measured for the sample solutions. Table 1 shows the amount of plant extract, distilled water and FRAP reagents required for the assay.

Table 1: The amount of plant extract, distilled water and FRAP reagents taken for the assay

Reagents	Samples	Standards	Blank
Plant extract	20 µL	20 µL ($FeSO_4.7H_2O$)	x
Deionized water	30 µL	30 µL	30 µL
FRAP reagents	3000 µL	3000 µL	3000 µL

Results and Discussion

The results for total antioxidant capacity (TAC) of *Anthocephalus cadamba* bark and leaves by the FRAP method are presented in Table 2. The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability equivalent to that of 1 mM of $FeSO_4$.

The sensitivity of the method is determined by the strong absorbance of FRAP. After observing the results of FRAP assay between different extracts, the highest TACFRAP value is obtained for methanol extract of leaves of *Anthocephalus cadamba* followed by Methanol extract of bark of *Anthocephalus cadamba* > Chloroform extract of leaves of *Anthocephalus cadamba* > Water extract of leaves of *Anthocephalus cadamba* > Hexane extract of leaves of *Anthocephalus cadamba* > Water extract of bark of *Anthocephalus cadamba* > Ethyl acetate extract of leaves of *Anthocephalus cadamba* > Hexane extract of bark of *Anthocephalus cadamba* > Ethyl acetate extract of bark of *Anthocephalus cadamba* > Chloroform extract of bark of *Anthocephalus cadamba* recorded lowest TACFRAP value.

Despite the fact that *Anthocephalus cadamba* and several other herbs have extensively been used in medicinal preparations of Ayurveda, the biochemical basis of the beneficial effects of such herbs remains unexplored in most of the cases. These necessities the need to identify phytochemicals present in various parts of a medicinal plant and the effects they may have on cells. Such knowledge may be of critical use in developing herbal drugs for ailments.

Table 2: FRAP activity of different extracts of bark and leaves of *Anthocephalus cadamba*.

Sample	0 sec	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	Blank
KBM	0.509	0.538	0.557	0.567	0.576	0.584	0.589	0.595	0.601	0.180
KBH	0.383	0.387	0.391	0.393	0.394	0.397	0.398	0.399	0.401	0.176
KBC	0.384	0.383	0.383	0.384	0.383	0.384	0.387	0.387	0.388	0.176
KBEA	0.395	0.398	0.399	0.402	0.402	0.403	0.406	0.406	0.406	0.178
KBW	0.332	0.334	0.336	0.340	0.344	0.346	0.349	0.352	0.353	0.180
KLM	0.606	0.662	0.691	0.716	0.733	0.748	0.762	0.773	0.784	0.173
KLH	0.353	0.360	0.363	0.367	0.371	0.372	0.373	0.375	0.377	0.174
KLC	0.388	0.406	0.418	0.420	0.423	0.425	0.426	0.428	0.430	0.174
KLEA	0.370	0.376	0.380	0.383	0.384	0.386	0.387	0.389	0.390	0.181
KLW	0.367	0.372	0.375	0.379	0.381	0.384	0.387	0.391	0.393	0.179

KBM - KadambaBark Methanolic extract; KBH - Kadamba Bark Hexane extract; KBC - Kadamba Bark Chloroform extract; KBEA - Kadamba Bark Ethyl Acetate extract; KBW - Kadamba Bark Water extract; KLM - Kadamba Leaf Methanolic extract; KLH - Kadamba Leaf Hexane extract; KLEA - Kadamba Leaf Ethyl Acetate extract; KLW - KadambaLeaf Water extract.

The past few decades have been a burst of knowledge regarding the processes of ROS generations and disposal. Some plant metabolites (e.g. Phenolic compounds and flavonoids) have been shown to be excellent scavengers of ROS produced in vitro such as superoxide radical (O_2), H_2O_2, hydroxyl radical ($OH^{\cdot}$) and singlet oxygen[14] (Rice – Evans *et al*., 1996).

The FRAP assay, which tests the ability of herbal extracts to reduce ferric ions (Fe^{3+}), has been used to measure the antioxidant potential of various plant extracts. The test relies on changer in absorbance at 593 nm owing to the formation of blue coloured Fe^{2+}/ 2,4,6–tripyridyl–S–triazine from the colourless ferric ions (Fe^{3+}). The FRAP values are early obtained by measuring absorbance and are reproducible. Besides, the reducing power of extracts linearly increases with the concentration of phytochemicals that are implicated in antioxidant activity.

There are many possible ways in which the plant extracts can prevent oxidative damage. These include chelation of metal ions involved in catalysis of oxidation reactions, decomposition of peroxides, scavenging of free radicals and interruption of chain initiation reactions triggered by free radicals.

The in vitro assays performed in this study establish the efficacy of various *A. cadamba* bark and leaf extracts in managing oxidative stress of cells and could serve as free radical inhibitors or scavengers, which may act as primary antioxidants. The fact that methanol extracts of bark and leaves have high antioxidant activity is of great pharmacological interest. Further studies in this line can help in tapping the therapeutic potential of this herb.

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Counselling Considerations for Chromosomal Mosaicism after Embryo Biopsy PGT-A: A Case Report

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Abstract

Pre-implantation genetic testing for aneuploidy PGT-A is a genetic test performed on Day 5 or Day 6 embryos obtained through Invitro fertilization (IVF). Embryo Biopsy is a procedure where the part of Trophoectoderm of the embryo is taken and sent for PGT-A analysis. PGT-A is indicated in couple who has been through previous miscarriage, maternal age >35 years and previous child anomalies. Mosaicism is a condition in which the presence of two or more abnormal cell lines or a normal and an abnormal cell line has observed. Embryonic mosaicism might play a significant role in pregnancy loss after IVF, cytogenetic and array based analysis of miscarriages following spontaneously conceived pregnancies commonly reveal chromosomal mosaicism. The degree of Embryonic Mosaicism negatively affects the implantation rate.

Keywords: PGT-A; Genetic Counselling; Embryo Biopsy; Aneuploidy.

INTRODUCTION

Pre-implantation genetic testing for aneuploidy PGT-A is a genetic test performed on Day 5 or Day 6 embryos obtained through In vitro fertilization (IVF). Embryo Biopsy is a procedure where the part of the trophoectoderm of the embryo is taken and sent for PGT-A analysis. PGT-A is indicated in a couple who has been through a previous miscarriage, maternal age >35 years, and previous child anomalies.[1,2]

Chromosomal Mosaicism is a condition of an embryo that contains a normal cell line and an abnormal cell line. Chromosomal Mosaicism is a condition more often due to the non-disjunction of chromosomes post-zygomatically which leads to a monosomic line and a trisomic line. Chromosomal Mosaicism of each chromosome has been reported with a wide variety of morphological defects. However, some studies showed that chromosomal mosaicism may not affect the further development as there might be auto correction of the embryo genetics.[3,4]

CASE PRESENTATION

Based on the case study, we have observed a

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couple in which a female patient of age 36 and a male patient of age 43 years have participated in an IVF cycle. The patient has previously undergone 2 IVF cycles which have failed and after approaching us for 3rd cycle, they were interested to undergo PGT–A Analysis. As per the Antagonist protocol, the ovarian stimulation was performed and the ovum pick up (OPU) was conducted at the 34th hour after triggering with HCG. A total of 10 follicles were obtained and subjected to Intra Cytoplasmic Sperm Injection (ICSI) where the male patient's sperm count was observed as 55 million/mL and progressive motility as 40%. On day 5, five full blastocysts were formed and the trophoectoderm biopsy was performed for all the samples and sent to the laboratory for Comparative Genomic Hybridization (CGH).

PGT-A Analysis Report of Five Embryos (ECR/1301/Inst/TG/2019)

ID	QC	Results (CGH)
E1	PASS	Segmental loss of 5mb and 8mb has been observed in chromosome 11 & 18 Multiple mosaic gain and loss (more than 30mb) has been observed.
E2	PASS	Segmental loss of 2-10mb has been observed in chromosome 17 & 20
E3	PASS	Multiple aneuploidy has been observed
E4	PASS	Mosaic loss of 109mb has been observed in chromosome 1
E5	PASS	3mb loss of chromosome 9 & 19

Based on the following laboratory report, both patients were counseled about the aneuploidy, segmental loss, and Mosaicism of Embryos. After 2 months, the frozen embryos such as E1, E2, and E5 were thawed and were transferred to the female patient. The pregnancy test was conducted after 14 days of embryo transfer and it showed a positive result. The ultrasound scanning was performed after 6 weeks and it was observed by imaging to have a single fetal sac and after 9 months the female patient delivered a healthy baby.

Counselling Considerations for PGT-A

As per the protocol before going for the PGT-A, these guidelines were followed accordingly such as:

Pre-test genetic counselling: The patient before going for the PGT–A Analysis has to be informed about the risks, benefits and limitations while undergoing with this technology.[3]

Post-test genetic counselling: In this method, the euploid embryos has to be the first option for transferring as well as the degree of mosaicism, segmental loss or gain of embryos has to be analyzed. The embryo which is having the least aneuploidy embryo has to be selected for this transfer. Based on the laboratory results and conditions, the embryos whose genetic analysis is correlating with any known syndrome has to be reported or informed to the patient as well as with concern cautiously it has to be advised to the patient not to go for the transfer.[3,4,5]

Pre-natal Testing: Before going for the pre-natal testing, the couple has to be provided with a counselling to explain about the constraints and advantages of prenatal screening. Chorionic villi sampling (CVS) is of placental origin which offers the initial prenatal diagnosis for aneuploidy. Amniocentesis represents more of fetal tissues embryonic ectoderm and amnion Amniocentesis is more accurate, however normal amniocentesis does not interpret low level of mosaicism.[3,4]

DISCUSSION

As per the observations and growth of embryos, E1, E2, and E5 have been chosen for embryo transfer. It has been observed from the genetic analysis that Embryo E1 has a Segmental loss of 5 mb and 8 mb which is observed in chromosome 11 & 18 and multiple mosaic gain and loss of more than 30 mb has been observed. In this same manner, embryo E2 has a Segmental loss of 2-10 mb in chromosomes 17 & 20, and embryo E5 has a 3 mb loss of chromosomes in 9 & 19. Due to the multiple aneuploidy nature, the embryo E3 has not been chosen and also E4 has been not chosen in the same cycle transfer due to the presence of mosaic loss of 109 mb in chromosome.[1]

An abnormal cell line. The percentage of mosaicism may or may not correlate with the mosaicism of the embryo as the test is done by trophoectoderm biopsy which forms the placenta of the embryo. Mosaicism is a condition more often due to the non-disjunction of chromosomes post-zygomatically which leads to a monosomic line and a trisomic line. Mosaicism of each chromosome has been reported with a wide variety of morphological

defects. Patients have to be counseled for the most common syndrome with mosaic embryos which includes down syndrome, syndromes including x and y, 13 and 18 trisomy, Syndromes including Intra Uterine Growth Retardation, and Uni Parental Disomy. However, some of the Mosaic aneuploid Embryos might show Auto correction on further division and might become a normal embryo.[7,8]

CONCLUSION

PGT-A is a pre-implantation genetic screening for aneuploidy and it is a procedure that is recommended in Advanced Maternal age, Previous Miscarriages, and Previous child anomalies. Mosaicism is a common phenomenon that is observed in most Embryo biopsy cases. The patient should be counseled properly before going to PGT-A and informed regarding the risk of having an abnormal baby after Embryo transfer. In our case, a Healthy baby was born, even though, the transferred embryos has Mosaicism and segmental loss/gain.[3]

PGT-A Embryos which are reported with chromosomal mosaicism have to be dealt with carefully. Patients should be informed regarding the risks of chromosomal mosaicism. Patients have to be counseled for the most common syndrome with mosaic embryos which includes Down syndrome, syndromes including x and y, 13 and 18 trisomy, Syndromes including Intra Uterine Growth Retardation and Uni Parental Disomy, and counsel them not to go for embryo transfer in such cases. Patients after PGT-A have to go under Amniocentesis for further evaluation of the fetus and if any abnormality is reported has to for medical termination of pregnancy (MTP).

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