Chromium, Nickel and Zinc Tolerance in *Leucaena leucocephalla* (K8)

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Abstract

In vitro shoot bud regeneration via callogenesis was achieved from hypocotyl explants of *Leucaena leucocephalla* (K8) collected from both contaminated (chromium, nickel and zinc) and uncontaminated sites. The proliferated calli raised from hypocotyl were tested *in vitro* for their relative tolerance to chromium, nickel and zinc. The calluses derived from contaminated sources were tolerant to metal and showed better growth on MS basal medium supplemented with 1.0 mg l^{-1} kinetin, 4.0 mg l^{-1} NAA and either 0.015 mM chromium, 0.13 mM nickel or 0.053 mM zinc than those derived from uncontaminated seeds. The specificity of metal tolerance noted in the parent materials was observed in the calluses. The calli derived *L. leucocephalla* growing on contaminated soil exhibited higher catalase and peroxidase activities than those from the uncontaminated soil. Biochemical studies provided evidences that plant material from the contaminated sources were physiologically distinct from the uncontaminated ones. This study indicated that seeds of *Leucaena leucocephalla* collected from contaminated sites were tolerant to chromium, nickel and zinc and may have the advantage of being used in sustainable revegetation programmes on metalliferous minewastes.

Key words: Chromium, heavy metal stress, metal tolerance, nickel, zinc, tree.

Abbreviations: BA, benzylaminopurine; Kn, kinetin; MS, MURASHIGE and SKOOG's (1962).

Introduction

Mining as well as processing of mineral ores produce vast tracts of derelict land which are both visually unattractive and possible sources of environmental pollution. In many parts of the world, these problems are accentuated because the derelict lands are often adjacent to agricultural fields or areas of great aesthetic and amenity value. Smelting of mineral often leads to the production of acid, metal contaminated soils which are toxic for plant growth (ARCHAMBAULT and WINTERHALDER, 1995). Heavy metal pollution can cause severe phytotoxic action, and may act as a powerful force for the evolution of tolerant populations (BAKER and WALKER, 1990). The phenomenon of heavy metal tolerance in plants has attracted the interests of plant ecologists, plant physiologists and evolutionary biologists. Tolerance by plant populations to metals e.g. cadmium, copper, lead and zinc has been well documented (BAKER, 1987). Metal tolerance is under genetic control, usually being polygenically determined (GARTSIDE and MCNEILLY, 1974). In recent years, considerable research has been focused by using cellular techniques for assessing metal tolerance in plants (MEREDITH, 1978a and b; WU and ANTONOVICS, 1978; MACNAIR and CHRISTIE, 1983). Cell cultures are useful for obtaining stress-tolerant cell lines in a relatively short time (VAN SINT JAN et al., 1997). Thus, metal toxicity appears to be one area where emerging methods in somatic cell genetics may complement conventional crop improvement programmes by providing additional means of screening and/or selecting for improved levels of tolerance (PETOLINO and COLLINS, 1985).

Most of the monocots or dicots, have been reported to possess populations tolerant to specific metals (BAKER and WALKER, 1990). Multiple tolerance to two or more metals (COX and HUTCHINSON, 1981) and co-tolerance or cross-tolerance (VERKLEIJ and PRAST, 1989) have been reported. Major reviews on various aspects relating to heavy metal tolerance in plants are availables including reviews on evolutionary aspects (ANTONOVICS et al., 1971; BAKER and WALKER, 1990; VEKEMANS and LEFEBVRE, 1997), methodology (WILKINS, 1978) and mechanisms of tolerance (VERKLEIJ and SCHAT, 1990). However, there are few reports on development of metal tolerant calli through *in vitro* (CLAIRMONT et al., 1986; VAN SINT JAN et al., 1997; TAYLOR, 1989, 1995; ROUT et al., 1998). Recently, it has been pointed out that metal tolerant plants can provide a simple and economical solution to many of the problems encountered in metalliferous minelands. The present investigation was designed to assess the chromium, nickel and zinc tolerance to plants through callus cultures of *Leucaena leucocephalla* (K8), a fuel wood tree, derived from hypocotyl explants from contaminated and uncontaminated sites.

Materials and Methods

Description of plant

Leucaena leucocephalla (K8) is a fast growing leguminous tree species native to Mexico and introduced to India and provides nutritious forage, firewood, timber and enriches the soil through nitrogen fixation. It is one of the major sources for paper pulp, construction material and is also used for reforestation program in the tropics. It is reported to grow on poor soils and chromite overburdens and hence, might be used as an effective cover to prevent leaching of heavy metals from the minewaste dumps to the neighbouring environments. Propagation through seed is unreliable due to poor gemination/and death of young seedlings under natural conditions (Anonymous, 1989).

Plant material

Seeds of *Leucaena leucocephalla* (K8) were collected from plants growing on metalliferous mine (Sukinda, Orissa, India) overburdens (contaminated site) and the Regional Plant Resource Centre (uncontaminated site), Bhubaneswar, India. Seeds from both sources were surface sterilized in 0.1% $HgCl_2$ solution for 20 min., rinsed 3 to 4 times with sterile distilled water and were aseptically germinated on semi-solid MS (MURASHIGE and SKOOG, 1962) basal medium under 16 h photoperiod in cool, white fluorescent light (55 µE m^{-2} s^{-1}) at 25 °C ± 2 °C.

Determination of heavy metals in hypocotyl

Seeds collected from trees growing on contaminated and uncontaminated site were germinated *in vitro* on basal MS

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salts with 3% sucrose. The hypocotyls derived from the seedlings (both contaminated and uncontaminated sites) were digested at 150°C (1 g fresh weight of sample digested in 2 ml nitric acid followed by 1 ml perchloric acid). The heavy metal content (Cr, Ni and Zn) in the hypocotyl were determined by Inductively Coupled Plasma Spectrometry (ICP Model-8410, Labtam, Australia) at 205.6 nm, 352.5 nm and 213.9 nm for chromium, nickel and zinc respectively.

Callus culture

For callus induction, cultures of *Leucaena leucocephalla* (K8) were initiated from hypocotyl explants derived from 15-day-old seedling grown *in vitro* on MS basal medium containing 3% (w/v) sucrose, 0.8% agar (w/v), 1.0 mg l^{-1} kinetin (Kn) and 4.0 mg l^{-1} NAA (Rout et al., 1995), at pH 5.8. The cultures were incubated under a 16-h photoperiod (55 µE $m^{-2}s^{-1}$, cool, white fluorescent light) at 25°C ± 2°C. Determination of callus growth was determined on the basis of initial and final weight. Calluses were subcultured on fresh media with same composition at 4-week intervals. Stock solutions of $K_2Cr_2O_7$, $NiSO_4$. H_2O and $ZnSO_4$. $7H_2O$ were filter sterilised (0.45 µm millipore membrane). The concentrations of test metals used (i.e. LD_{50}, sub-lethal dosage; one concentration below LD_{50}; another concentration above LD_{50}) were determined in separate experiments in non-tolerant seed-based nutrient culture (Unpublished).

Determination of tolerance of callus cultures

Approximately 100 mg of callus, initiated from contaminated and uncontaminated sources, were separated and placed into weighed vials containing 15 ml of similar fresh culture medium (mentioned above) along with various concentrations of either chromium (0, 0.075, 0.15 and 0.3 mM), nickel (0, 0.13, 0.25 and 0.5 mM) or zinc (0, 0.053, 0.10 and 0.31 mM). Morphological observations and callus growth measurements were determined at 4-week intervals. Pre-weighed culture vials containing 15 ml of culture medium were inoculated with similar quantities of callus, and the inoculated vials were re-weighed to obtain the initial fresh weight of the callus inoculum. The final weight minus initial weight of calluses in different treatments was expressed as percentage of callus growth against control. The cultures were incubated at 25°C ± 2°C under cool, white fluorescent lamps (55 $\mu Em^{-2}s^{-1}$) for 8 weeks. The above experiment had 20 cultures per treatment and was repeated four times.

Determination of dry weight of callus

Callus samples of known fresh weight (100 mg) from each treatment were dried to constant weight at 70°C in an oven. The water content was expressed as mg water per mg dry weight of callus.

Metal content of callus

The amount of metal accumulated by callus during the culture period (4 weeks) was determined. The calluses were removed from culture vials, washed with sterile deionised water and digested at 150°C (1 g fresh weight of sample digested in 2 ml nitric acid followed by 1 ml perchloric acid). All glassware and apparatus were washed with 0.1 N HNO_3 before use. The residues were dissolved in deionised water and concentration of metal was measured by Inductively Coupled Plasma Spectrometry (ICP Model-8410, Labtam, Australia) at 205.6 nm, 352.5 nm and 213.9 nm for chromium, nickel and zinc respectively. Cr, Ni and Zinc Standard Reference Material (SRM) was prepared in our laboratory from E.MERCK, chromium (Product No. 17511 – $K_2Cr_2O_7$,1000 mg l^{-1}), nickel (Product No.17567-$NiSO_4.6H_2O$, 1000 mg l^{-1}) and BDH, zinc (Product No.30621-$ZnSO_4.7H_2O$, 1000 mg l^{-1}) standard solution. Distilled water was used as the base solution. The detection limits were 0.0061 µg ml^{-1} for Cr, 0.045 µg ml^{-1} for Ni and 0.0018 µg ml^{-1} for Zn.

Differentiation of shoot buds from callus

The 4-week old tolerant and non-tolerant callus (100 mg ± 50 mg) grown in the light were transferred to differentiation medium containing half-strength MS basal salts supplemented with various concentrations and combinations of 6-benzylaminopurine, kinetin and 1-naphthaleneacetic acid or indole-3-acetic acid along with different concentrations of chromium (0.075, 0.15 and 0.3 mM) or nickel (0.13, 0.25 and 0.5 mM) or zinc (0.053, 0.10 and 0.31 mM). The pH was adjusted at 5.8. Cultures were grown in 25 mm x 150 mm glass culture tubes (Borosil, India) and incubated at 25°C ± 2°C in a growth room under cool, white fluorescent lamps (55 $\mu Em^{-2}s^{-1}$) for 8 weeks. The experiments had 20 cultures per treatment and were repeated four times.

Biochemical analysis

Chlorophyll and protein determination

Callus samples (100 mg ± 20 mg) fresh weight from each treatment were collected at 4-week intervals for chlorophyll determination. The callus was homogenized with 80% acetone in the dark. The amount of chlorophyll was estimated according to Vernon (1960). Pigment content was expressed as $mg.g^{-1}$ fresh weight of sample. For the study of protein content, fresh callus samples (100 mg ± 20 mg) were analysed by conventional microkjeldahl method for the estimation of total nitrogen. Soluble nitrogen was determined by the above method after precipitating the protein in the extract of the fresh material with trichloroacetic acid (TCA) (Anonymous, 1970).

Enzyme extraction and assay

Peroxidase

Fresh callus samples (100 mg) from each treatment were collected at 2 week intervals and homogenized with mortar and pestle in cold 0.1 M phosphate buffer (pH 6.1) containing 30 mg of insoluble PVP and 15 mg sodium ascorbate. The homogenate was filtered through four layers of miracloth and centrifuged at 12000 g for 10 min at 4°C. The supernatant was used for the peroxidase assay. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H_2O_2 and 0.4 ml of crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbance (OD) at 420 nm was measured using a UV-Spectrophotometer (Jasco, UVIDEC-650, Japan). The levels of enzyme activity were expressed as µmol H_2O_2 destroyed.mg $protein^{-1}$ min^{-1}.

Catalase

Fresh callus samples (100 mg) from each treatment were collected at 4-week intervals and homogenised in 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 1,000 g for 10 min at 4°C. One ml of the supernatant was added to a reaction mixture containing 1 ml of 0.1 M H_2O_2 and 3 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction was stopped by adding 10 ml of 2% H_2SO_4 after 1 min incubation at 20°C. The acidified reaction mixture with or without supernatant was titrated against 0.01 M $KMnO_4$ to determine the quantity of H_2O_2 utilised by the enzyme. The catalase activity was expressed in enzyme units as µmol H_2O_2 destroyed.mg $protein^{-1}$ min^{-1}.

Statistical analysis

The data pertaining to mean percentage of explant with calli/treatment, chlorophyll accumulation, total protein, cata-

lase and peroxidase activity and metal content in the callus derived from tolerant and non-tolerant population were statistically analysed by the ANOVA (SOKAL and ROHLF, 1973). Between the treatment, the average figures followed by same letter within a column are not significantly different at the $p< 0.05$ level (Post-Hoc Multiple Comparison test).

Results and Discussion

Determination heavy metals in hypocotyl

Analysis of heavy metals (Cr, Ni and Zn) content in hypocotyl were found in the seedling derived from contaminated source. The result showed that Cr, Ni and Zn were found to be 0.57 ± 0.08 mg.kg^{-1}, 0.65 ± 0.06 mg.kg^{-1} and 0.71 ± 0.05 mg.kg^{-1} respectively in hypocotyl derived from contaminated sources; the hypocotyl derived from the un-contaminated sources contained only Zn (0.35 ± 0.10 mg.kg^{-1}). The evidence suggests that there was difference in heavy metal accumulation in seedlings derived from contaminated and uncontaminated sources.

Initiation of callus

Callus growth from hypocotyl explants derived from seeds from contaminated and uncontaminated sites varied on MS medium supplemented with 1.0 mg l^{-1} kinetin, 4.0 mg l^{-1} NAA and different concentrations of chromium, nickel and zinc *(Table 1)*. Callus derived from plants at uncontaminated sites and cultured in presence of elevated levels of chromium (0.3 mM), nickel (0.5 mM) and zinc (0.31 mM) developed a dark brown coloration as compared to the creamy white calluses when the same metals were supplemented at low concentrations. Callus derived from the non-tolerant (uncontaminated sites) sources showed a marked reduction (35.1%) in callus initiation in the treatments supplemented with 0.50 mM of Ni, while 44% and 34.5% reduction was noted with 0.075 mM chromium and 0.31 mM Zn respectively. Nickel at low concentration (0.13 mM), induced better callus initiation in comparison with the control, as reported earlier (BROWN et al., 1987). In the presence of low concentrations of chromium, nickel and zinc, enhanced callus initiation was recorded in the calluses derived from the 'contaminated sources'. Maximum initiation was found at 0.015 mM Cr, 0.13 mM Ni and 0.053 mM Zn. The results from this study demonstrated that the calli derived from contaminated sources showed tolerance to chromium, nickel and zinc in comparison with the ununcontaminated sources. WU and ANTIONOVICS (1978) found callus derived from Cu- and Zn-tolerant clones of *Agrostis stolonifera* to be more tolerant to the same metals added to the media than callus derived from sensitive clones of the same species, taking the growth inhibition into consideration.

Dry weight of callus

Under the influence of Cr, Ni, Zn, the proportion was different in callus derived from tolerant and non-tolerant sources. Dry weight of calluses enhanced with increasing in the metal concentration in the medium. Calluses from the tolerant clones showed a greater dry weight than those derived from the non-tolerant plants *(Figs. 1A to C)* which could be due to high accumulation of metals (FOY et al., 1978; BURKE et al., 1990; MORAL et al., 1994; SAMANTARAY et al., 1997). Similar observation was made by MARSH and PETERSON (1990) who reported that shoot dry weight increased with the increase of manganese concentration in the test solution which was largely due to high manganese in the shoot.

Pigment synthesis

An interesting observation was that the chlorophyll content in callus derived from uncontaminated sources declined on medium containing high concentrations of chromium or nickel or zinc as compared to the control *(Table 2)*. The callus derived from contaminated sources showed significantly higher chlorophyll content (Chlorophyll-a and b) which varied from 6.44 mg.g^{-1} to 6.73 mg.g^{-1} in Cr, 5.23 mg.g^{-1} to 8.02 mg.g^{-1} in Ni and 5.16 mg.g^{-1} to 6.85 mg.g^{-1} in Zn containing medium. Similar observations were made earlier on chlorophyll synthesis due to managanese toxicity in tobacco callus (PETOLINO and COLLINS, 1985; CLAIRMONT et al., 1986). Chlorophyll content in the callus derived from the tolerant (contaminated sites) sources showed a reduction (32.3%) in callus initiation in the treatment supplemented with 0.31 mM Zn, while 31.4% and 11.7% reduction was noted with 0.50 mM Ni and 0.30 mM Cr respectively. The

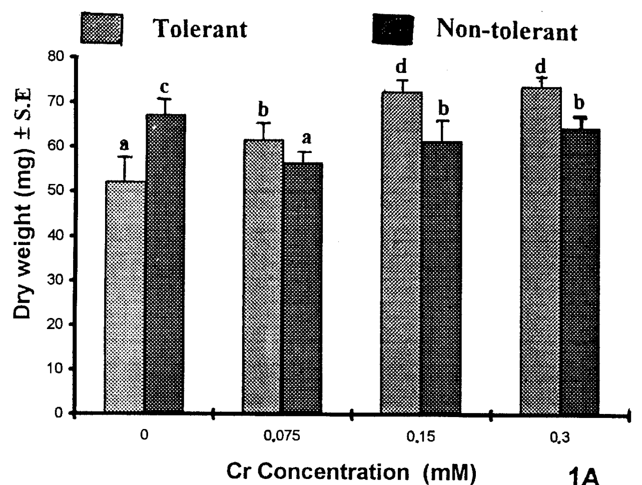
Table 1. – Effect of different concentration of heavy metals (Cr, Ni and Zn) on callus production in hypocotyl explants of *Leucaena leucocephalla* (K8) after 4 weeks of culture. Parenthesis indicates the percentage of reduction (–) / increase (+) relative to control.

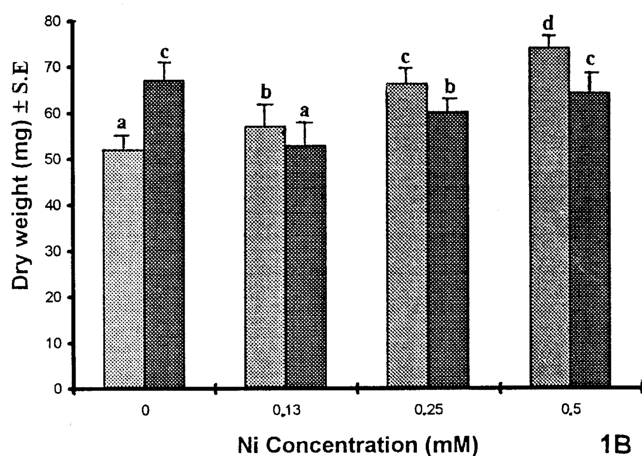
Metal Concentration (mM)			% of explant forming callus (Mean ± SE)*	
			Source of explant	
Cr	Ni	Zn	Uncontaminated	Contaminated
0	0	0	67.5 ± 0.8f	56.6 ± 0.6b
0.075	0	0	52.8 ± 0.3d (-21.77)	60.7 ± 0.8d (+7.24)
0.15	0	0	48.7 ± 0.7c (-27.85)	56.2 ± 0.3b (- 0.71)
0.3	0	0	37.8 ± 0.4a (-44.00)	51.3 ± 0.4a (-9.36)
0	0.13	0	68.6 ± 0.8f (+1.63)	69.2 ± 0.6f (+22.26)
0	0.25	0	57.2 ± 0.5e (-15.26)	63.2 ± 0.2e (+11.66)
0	0.50	0	43.8 ± 0.3b (-35.11)	56.4 ± 0.8b (-0.35)
0	0	0.053	57.6 ± 0.4e (-14.67)	58.3 ± 0.6c (+3.00)
0	0	0.10	51.8 ± 0.6d (-23.26)	56.5 ± 0.7b (-0.18)
0	0	0.31	44.2 ± 0.5b (-34.52)	50.4 ± 0.5a (-10.95)

*) 20 replicates/treatment; repeated four times.

a to f Means having the same letter within a column were not significantly different by Post-Hoc Multiple Comparison Test. $P < 0.05$.

chlorophyll-a and b content in the callus derived from tolerant (contaminated site) sources increased 5.25% of chlorophyll-a and 3.56% of chlorophyll-b in the presence of 0.13 mM nickel. Protein content increased in the callus derived from tolerant (contaminated site) sources whereas callus derived from uncontaminated sources showed decline in the protein content *(Table 3)*.





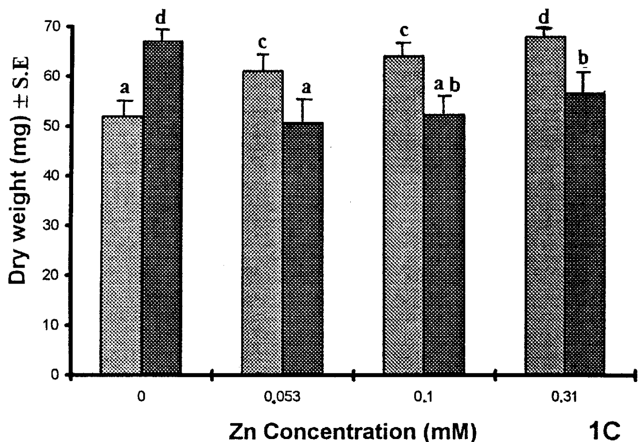


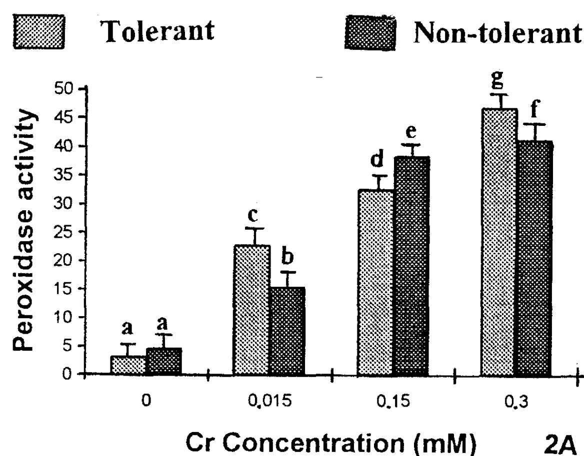
Fig. 1A to 1C. – Effect of chromium, nickel and zinc on dry weight of tolerant and non-tolerant callus of *Leucaena leucocephalla* (K8) after 4 weeks of culture. Mean of the four experiments; 20 cultures/treatment. Mean having the same letter were not significantly different at $p < 0.05$.

Enzyme activity

Acceleration in the activities of specific enzymes is to play an important role in plant metabolism under conditions of metal stress (VAN ASSCHE and CLIJSTERS, 1990) and therefore may have a subtle role in metal tolerance. During a period of 4-weeks in culture, the activities of both catalase and peroxidase were significantly higher in callus derived from tolerant plants in comparison with the uncontaminated ones *(Figs. 2A, B to 4A, B)*. Greater activity of catalase and peroxidase indicated that the tolerant plants were under oxidative stress, a feature often associated with metal tolerance (VAN ASSCHE and CLIJSTERS, 1990). NASHIKKAR and CHAKRABARTI (1994) reported that both catalase and peroxidase activity were generally high in crops grown on heavy metal polluted soil.

Accumulation of metal in callus

Accumulation of metal in the callus after 4-week of growth in the presence of the metals increased significantly with the increase in metal concentrations in the medium *(Table 4)*. The Cr concentration in callus grown in medium containing 0.30 mM Cr typically reached to 4.26 $mg.Kg^{-1}$ and 2.34 $mg.Kg^{-1}$ respectively in case of tolerant and non-tolerant one. However, 2.66 $mg.Kg^{-1}$ and 0.92 $mg.Kg^{-1}$ chromium were found in case of tolerant and non-tolerant callus grown to 0.075 mM Cr. Tolerant calluses showed high accumulation of chromium, nickel and zinc as compared to non-tolerant ones at all the concentrations tested *(Table 4)*. Heavy metal content in the callus deriv-



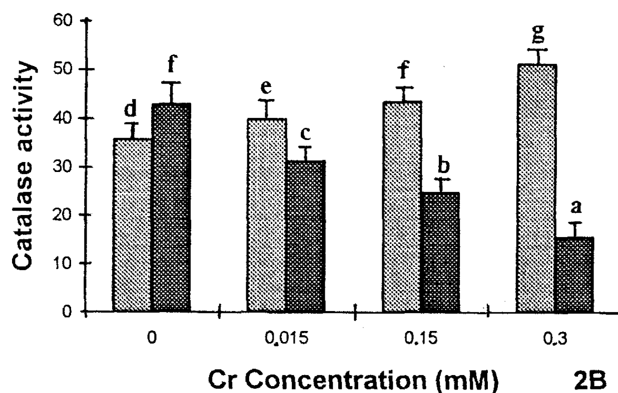


Fig. 2A to B. – Catalase and Peroxidase activity of tolerant and non-tolerant callus of *Leucaena leucocephalla* (K8) grown on different concentration of chromium after 4 weeks of culture. Mean of the four experiments; 20 cultures/treatment. Mean having the same letter were not significantly different at $p < 0.05$.

Table 2. – Chlorophyll content (mg.g^{-1} fresh weight) of callus derived from contaminated and uncontaminated sources of *Leucaena leucocephalla* (K8) cultured on MS medium supplemented with 1.0 mg.l^{-1} kinetin, 4.0 mg.l^{-1} NAA and different concentrations of chromium, nickel and zinc after 4 weeks of culture.
Parenthesis indicates the percentage of reduction (–) / increase (+) relative to control.

MS + Different concentration of metals (mM)	Source of callus	Chlorophyll content (mg.g^{-1} fresh weight) (Mean ± SE.*) Chl-a	Chl-b	Total Chl(a+b)
	Contaminated (Tolerant)			
Control		7.62 ± 0.3f	8.14 ± 0.5f	15.76 ± 0.8f
Cr 0.075		6.44 ± 0.6c(-15.5)	6.92 ± 0.4d (-15.0)	13.36 ± 1.0c (-15.2)
Cr 0.15		6.86 ± 0.3d (-9.47)	7.01 ± 0.3d (-13.9)	13.87 ± 0.5e (-12.0)
Cr 0.30		6.73 ± 0.2d (-11.7)	6.54 ± 0.6c (-19.7)	13.27 ± 0.8c (-15.8)
Ni 0.13		8.02 ± 0.8g (+5.25)	8.43 ± 0.4g(+3.56)	16.45 ± 1.2g (+4.38)
Ni 0.25		7.44 ± 0.2e (-2.36)	7.92 ± 0.2e (-2.71)	15.36 ± 0.4f (-2.54)
Ni 0.50		5.23 ± 0.2a (-31.4)	5.54 ± 0.5a (-32.0)	10.77 ± 0.7a (-31.7)
Zn 0.053		6.85 ± 0.5d (-10.2)	6.73 ± 0.3c (-17.4)	13.58 ± 0.8d (-13.9)
Zn 0.10		5.38 ± 0.5b (-29.4)	5.87 ± 0.6b (-27.9)	11.25 ±1.1b (-28.6)
Zn 0.31		5.16 ± 0.3a (-32.3)	5.64 ± 0.3a (-30.7)	10.80 ± 0.6a (-31.5)
	Uncontaminated (non-tolerant)			
Control		7.74 ± 0.4h	8.05 ± 0.3f	15.79 ± 0.7g
Cr 0.075		6.42 ± 0.3f (-17.0)	6.27 ± 0.6e (-22.1)	12.69 ± 0.9e (-19.7)
Cr 0.15		6.32 ± 0.5f (-18.4)	6.16 ± 0.2e (-23.5)	12.48 ± 0.7e (-21.0)
Cr 0.30		2.81 ± 0.6a (-63.7)	2.63 ± 0.3b (-67.4)	5.44 ± 0.9a (-65.6)
Ni 0.13		7.12 ± 0.8g (-8.01)	6.16 ± 0.4e (-23.5)	13.28 ± 1.2f (-15.9)
Ni 0.25		6.04 ± 0.8e (-21.9)	5.42 ± 0.6d (-32.7)	11.46 ± 1.4d (-27.4)
Ni 0.50		2.92 ± 0.3a (-62.3)	2.56 ± 0.3b (-68.2)	5.48 ± 0.6a (-65.3)
Zn 0.053		5.65 ± 0.4d (-27.0)	5.31 ± 0.6d (-34.0)	10.96 ± 1.0c (-30.6)
Zn 0.10		4.36 ± 0.6c (-43.7)	3.27 ± 0.5c (-59.4)	7.63 ± 1.1b (-51.7)
Zn 0.31		3.07 ± 0.5b (-60.4)	2.16 ± 0.2a (-73.2)	5.23 ± 0.7a (-66.9)

*) 20 replicates/treatment; repeated four times.

a to h Means having the same letter within a column were not significantly different by Post-Hoc Multiple Comparison Test. $P < 0.05$.

Table 3. – Total protein content (µg.g^{-1} fresh weight basis) of callus derived from contaminated and uncontaminated sources of *Leucaena leucocephalla* (K8) grown on MS medium containing 1.0 mg.l^{-1} kinetin, 4.0 mg.l^{-1} NAA in absence or presence of metal after 4 weeks of culture.
Parenthesis indicates the percentage of reduction (–) / increase (+) relative to control.

MS + Concentration of metal (mM)	Source of callus: Contaminated	Un-contaminated
	(Total protein content) (Mean ± SE *)	
Control	3.01 ± 0.7b	4.11 ± 0.2f
Cr 0.075	3.33 ± 0.6c (+10.6)	3.14 ± 0.4e(-23.6)
Cr 0.15	3.12 ± 0.3b (+3.66)	2.22 ± 0.5c (-46.0)
Cr 0.30	3.55 ± 0.2d (+17.9)	2.08 ± 0.6b (-49.4)
Ni 0.13	2.56 ± 0.5a (-14.9)	2.53 ± 0.7d (-38.5)
Ni 0.25	3.51 ± 0.4d (+16.6)	2.40 ± 0.2d (-41.6)
Ni 0.50	3.47 ± 0.5c (+15.3)	1.81 ± 0.3a (-55.9)
Zn 0.053	3.85 ± 0.8e (+27.9)	2.22 ± 0.7c(-45.9)
Zn 0.10	3.14 ± 0.5b (+4.31)	2.02 ± 0.4b (-50.8)
Zn 0.31	3.32 ± 0.4c (+10.3)	1.82 ± 0.6a (-55.7)

*) 20 replicates/treatment; repeated four times.

a to f Means having the same letter within a column were not significantly different by Post-Hoc Multiple Comparison Test. $P < 0.05$.

ed from the tolerant (contaminated sites) sources showed an increased (610.0%) in callus growth after 4 weeks in the treatment supplemented with 0.30 mM Cr, while 364.7% and 336.8% increase was noted with 0.50 mM nickel and 0.31 mM Zn respectively. Similar observations were made by QURESHI et al. (1981) in *in vitro* studies for copper and zinc tolerance. BAKER (1987) suggested two basic strategies of tolerance; metal exclusion, where metal uptake and transport is restricted, and

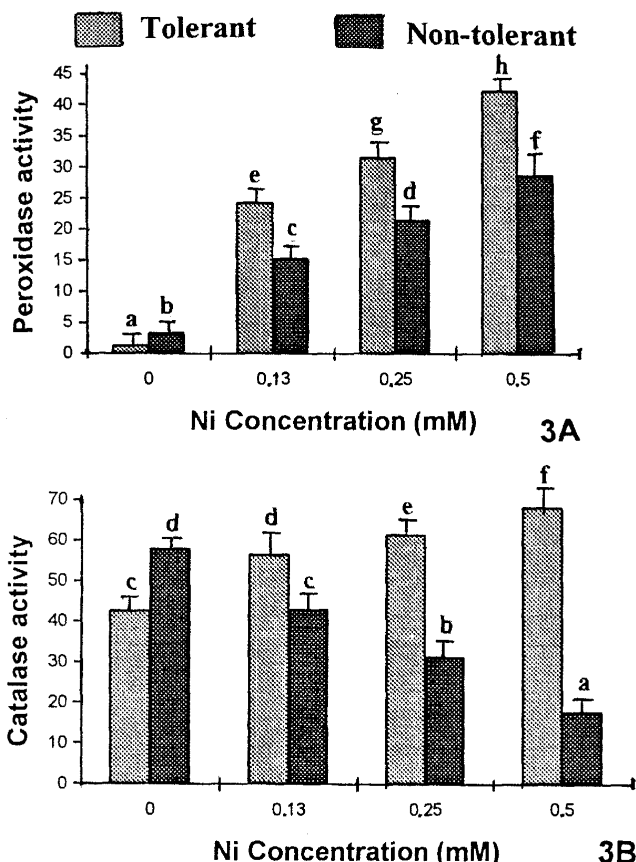


Fig. 3A to B. – Catalase and Peroxidase activity of tolerant and non-tolerant callus of *Leucaena leucocephalla* (K8) grown on different concentration of nickel after 4 weeks of culture. Mean of the four experiments; 20 cultures/treatment. Mean having the same letter were not significantly different at $p < 0.05$.

metal accumulation where there is no such restriction and metals are accumulated in a detoxified form. Detoxification may result from cell wall binding, active pumping of ions into vacuoles, complexing by organic acids and possibly by specific metal-binding proteins, alteration of metal compartmentation patterns, cellular metabolism and membrane structure (BAKER and WALKER, 1990; VERKLEIJ and SCHAT, 1990).

Differentiation of shoots and rooting

Small globular protuberances developed on the entire surface of the callus derived from contaminated and uncontaminated sources and gave rise to shoot buds. A maximum of 12 to 18 shoot buds developed within 4 weeks of culture on regeneration medium containing 1/2 MS + 2.5 $mg.l^{-1}$ kinetin + 1.0 $mg.l^{-1}$ BA + 0.5 $mg.l^{-1}$ NAA + 0.15 mM Cr or 0.25 mM Ni or 0.10 mM Zn + 3% sucrose. On the other hand, calluses derived from uncontaminated sources did not show regeneration ability on medium containing similar composition. Callus derived from uncontaminated sources became brown on medium containing 0.15 mM Cr to 0.30 mM Cr, 0.013 mM Ni to 0.5 mM Ni or 0.053 mM Zn to 0.31 mM Zn. The elongated shoots were rooted on medium containing ½ strength MS supplemented with 0.25 mg/l IBA + 2% sucrose within 10 to 12 days of culture.

Our data suggest that the callus derived from contaminated sources showed tolerance to Cr, Ni and Zn in comparison with the uncontaminated sources. Pigment accumulation and enzyme activity (Peroxidase and Catalase) were significantly higher in callus derived from tolerant plants (contaminated sources). The calli derived from tolerant plant accumulate more metal than non-tolerant plants (uncontaminated sources). The present findings can be explored to other plant species growing at the contaminated site unless tested specifically. The studies may help in the screening for metal tolerant cell lines in different species. The growth responses of calli to elevated levels of

Table 4. – Chromium, Nickel and Zinc content ($mg.kg^{-1}$) of tolerant and non-tolerant calluses of *Leucaena leucocephalla* (K8) after 4 weeks growth in the presence of each metal.
Parenthesis indicates the percentage of reduction (–) / increase (+) relative to control.

Concentration of metal in medium (mM)	Metal content ($mg.kg^{-1}$)*	
	Source of callus	
	Tolerant	Non-tolerant
Chromium		
0	0.60 ± 0.06 a	0
0.075	2.66 ± 0.9b c (+343.3)	0.92 ± 0.7 a
0.15	3.41 ± 0.8c d (+468.3)	2.15 ± 0.8 d
0.30	4.26 ± 1.1d e (+610.0)	2.34 ± 1.0cd
Nickel		
0	0.68 ± 0.12a	0
0.13	1.13 ± 0.9 b (+66.2)	0.96 ± 0.7 b
0.25	2.51 ± 1.1c (+269.1)	1.32 ± 0.5 c
0.50	3.16 ± 1.2d (+364.7)	1.46 ± 0.9 c
Zinc		
0	0.76 ± 0.10a	0.42 ± 0.06 a
Zn 0.053	1.48 ± 0.9b (+97.7)	1.12 ± 1.0 b (+166.6)
Zn 0.10	2.59 ± 1.0c (+240.8)	1.56 ± 0.6 c 9+271.4)
Zn 0.31	3.32 ± 0.8d (+336.8)	2.01 ± 0.4 d (+378.6)

*) 10 replicates/ treatment; repeated three times.

a to d Means having the same letter within a column were not significantly different by Post-Hoc Multiple Comparison Test. $P < 0.05$.

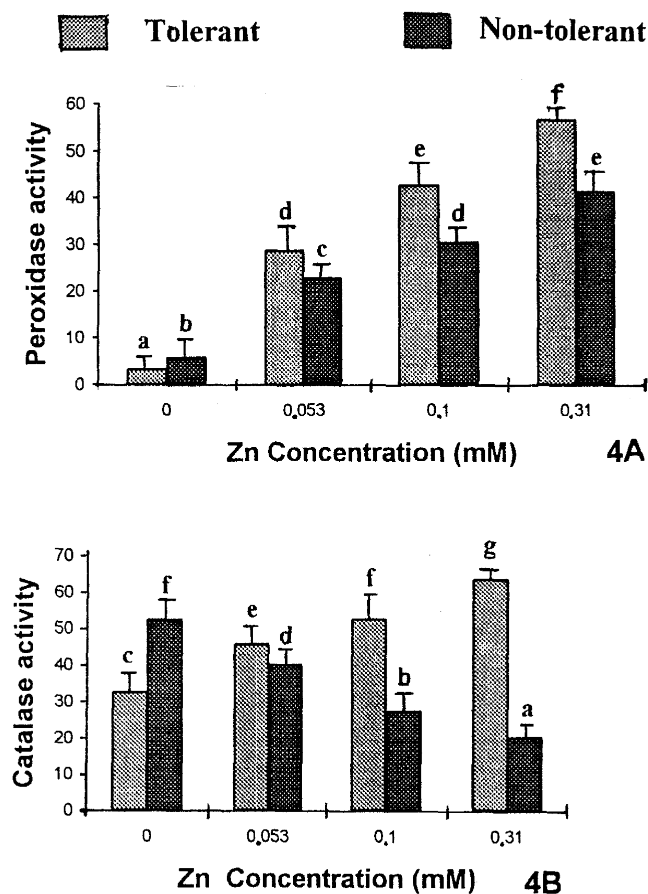


Fig. 4A to B. – Catalase and Peroxidase activity of tolerant and non-tolerant callus of *Leucaena leucocephalla* (K8) grown on different concentration of zinc after 4 weeks of culture. Mean of the four experiments; 20 cultures/treatment. Mean having the same letter were not significantly different at $p < 0.05$.

heavy metals may help in understanding the physiological and biochemical mechanism of metal tolerance in plants.

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