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

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**Abstract**

*In vitro* shoot bud regeneration via calllogenesis 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 *Leucaena 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 available including reviews on evolutionary aspects (Antonovics et al., 1971; Baker and Walker, 1990; Veekmans and Leferve, 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 chrome 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 germination 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), Bhubeswar, India. Seeds from both sources were surface sterilized in 0.1% HgCl₂ 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
salts with 3% sucrose. The hypocotyls derived from the seedlings (both contaminated and unconsontaminated 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 hypocotyls 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 *Lecuena 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⁻²s⁻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. Pre-weighed culture vials containing 15 ml of similar fresh culture medium (mentioned above) along with various concentrations of either chromium (0.075, 0.15 and 0.3 mM), nickel (0.0, 0.13, 0.25 and 0.5 mM) or zinc (0.0, 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 was expressed as percentage of callus growth against control. The cultures were incubated at 25°C ± 2°C under cool, white fluorescent lamps (55 µEm⁻²s⁻¹) for 8 weeks. The above experiments had 20 cultures per treatment and were repeated four times.

**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 chromium (0.0, 0.075, 0.15 and 0.3 mM), nickel (0.0, 0.13, 0.25 and 0.5 mM) or zinc (0.0, 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 was expressed as percentage of callus growth against control. The cultures were incubated at 25°C ± 2°C under cool, white fluorescent lamps (55 µEm⁻²s⁻¹) for 8 weeks. The above experiments had 20 cultures per treatment and were 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 cultures 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 HNO₃ 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 Zn Standard Reference Material (SRM) was prepared in our laboratory from E.MERCK, chromium (Product No. 17511 – K₂Cr₂O₇,1000 mg l⁻¹), nickel (Product No.17567-NiSO₄.7H₂O, 1000 mg l⁻¹) and zinc (Product No.30621-ZnSO₄.7H₂O, 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 µEm⁻²s⁻¹) for 8 weeks. The experiments had 20 cultures per treatment and were repeated four times.

**Biochemical analysis**

**Chlorophyll and protein determination**

**Catalase**

Fresh callus samples (100 mg ± 20 mg) fresh weight from each treatment were collected at 4-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 H₂O₂ 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, UVSCED-650, Japan). The levels of enzyme activity were expressed as µmol H₂O₂ destroyed mg protein⁻¹ min⁻¹.

**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 both the proteins. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H₂O₂ 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, UVSCED-650, Japan). The levels of enzyme activity were expressed as µmol H₂O₂ 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 uncontaminated 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. IA 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>
<thead>
<tr>
<th>Metal Concentration (mM)</th>
<th>% of explant forming callus (Mean ± SE)*</th>
</tr>
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<tbody>
<tr>
<td>Cr</td>
<td>Ni</td>
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<td>---</td>
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</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.075</td>
<td>0</td>
</tr>
<tr>
<td>0.15</td>
<td>0</td>
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<tr>
<td>0.3</td>
<td>0</td>
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<tr>
<td>0</td>
<td>0.13</td>
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<tr>
<td>0</td>
<td>0.25</td>
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<tr>
<td>0</td>
<td>0.50</td>
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</tbody>
</table>

*) 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).

**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 3A, 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). Naskikar 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-
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

Table 2. – Chlorophyll content (mg.g⁻¹ fresh weight) of callus derived from contaminated and uncontaminated sources of Leucaena leucocephala (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.

<table>
<thead>
<tr>
<th>MS + Different concentration of metals (mM)</th>
<th>Source of callus</th>
<th>Chlorophyll content (mg.g⁻¹ fresh weight)</th>
<th>(Mean ± SE*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chl-a</td>
<td>Chl-b</td>
<td>Total Chl(a+b)</td>
</tr>
<tr>
<td>Contaminated (Tolerant)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.62 ± 0.3f</td>
<td>8.14 ± 0.5f</td>
<td>15.76 ± 0.8f</td>
</tr>
<tr>
<td>Cr 0.075</td>
<td>6.44 ± 0.6c(-15.5)</td>
<td>6.92 ± 0.4d(-15.0)</td>
<td>13.36 ± 1.0c(-15.2)</td>
</tr>
<tr>
<td>Cr 0.15</td>
<td>6.86 ± 0.3d(-19.7)</td>
<td>7.01 ± 0.3d(-13.9)</td>
<td>13.87 ± 0.5e(-12.0)</td>
</tr>
<tr>
<td>Cr 0.30</td>
<td>6.73 ± 0.2d(-11.7)</td>
<td>6.54 ± 0.6c(-19.7)</td>
<td>13.27 ± 0.8c(-15.8)</td>
</tr>
<tr>
<td>Ni 0.13</td>
<td>8.02 ± 0.8g(+5.25)</td>
<td>8.43 ± 0.4g(+3.56)</td>
<td>16.45 ± 1.2g(+4.38)</td>
</tr>
<tr>
<td>Ni 0.25</td>
<td>7.44 ± 0.2e(-2.36)</td>
<td>7.92 ± 0.2e(-2.71)</td>
<td>15.36 ± 0.4f(-2.54)</td>
</tr>
<tr>
<td>Ni 0.50</td>
<td>5.23 ± 0.2a(-31.4)</td>
<td>5.54 ± 0.5a(-32.0)</td>
<td>10.77 ± 0.7a(-31.7)</td>
</tr>
<tr>
<td>Zn 0.053</td>
<td>6.85 ± 0.5d(-10.2)</td>
<td>6.73 ± 0.3c(-17.4)</td>
<td>12.58 ± 0.8d(-13.9)</td>
</tr>
<tr>
<td>Zn 0.10</td>
<td>5.38 ± 0.5b(-29.4)</td>
<td>5.87 ± 0.6b(-27.9)</td>
<td>11.25 ± 1.1b(-28.6)</td>
</tr>
<tr>
<td>Zn 0.31</td>
<td>5.16 ± 0.5a(-32.3)</td>
<td>5.64 ± 0.3a(-30.7)</td>
<td>10.80 ± 0.6a(-31.5)</td>
</tr>
</tbody>
</table>

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.

Table 3. – Total protein content (µg.g⁻¹ fresh weight basis) of callus derived from contaminated and uncontaminated sources of Leucaena leucocephala (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.

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

*) 20 replicates/treatment; repeated four times.

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
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 1/2 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

\[\text{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.}\

<table>
<thead>
<tr>
<th>Concentration of metal in medium (mM)</th>
<th>Metal content (mg.kg(^{-1}))(^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of callus</td>
<td>Tolerant</td>
</tr>
<tr>
<td>Chromium</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.60 ± 0.06 a</td>
</tr>
<tr>
<td>0.075</td>
<td>2.66 ± 0.9b c (+343.3)</td>
</tr>
<tr>
<td>0.15</td>
<td>3.41 ± 0.8c d (+468.3)</td>
</tr>
<tr>
<td>0.30</td>
<td>4.26 ± 1.1d e (+610.0)</td>
</tr>
<tr>
<td>Nickel</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.68 ± 0.12a</td>
</tr>
<tr>
<td>0.13</td>
<td>1.13 ± 0.9 b (+66.2)</td>
</tr>
<tr>
<td>0.25</td>
<td>2.51 ± 1.1c (+269.1)</td>
</tr>
<tr>
<td>0.50</td>
<td>3.16 ± 1.2d (+364.7)</td>
</tr>
<tr>
<td>Zinc</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.76 ± 0.10a</td>
</tr>
<tr>
<td>Zn 0.053</td>
<td>1.48 ± 0.9b (+97.7)</td>
</tr>
<tr>
<td>Zn 0.10</td>
<td>2.59 ± 1.0c (+240.8)</td>
</tr>
<tr>
<td>Zn 0.31</td>
<td>3.32 ± 0.8d (+336.8)</td>
</tr>
</tbody>
</table>

\(^{a}\) 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. \(P < 0.05\).
significant at p < 0.05.

ments; 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.**

**Acknowledgement**

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**References**