International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 3, Issue 3, 2012, pp 688-697
http://www.bipublication.com

THE EFFECT OF HEAVY METALS ZN AND NI ON GROWTH OF *IN VITRO* HAIRY ROOT CULTURES OF INDIAN MUSTARD *Brassica juncea* L.

A. Mohamed Ismail* and P. Agastian Theodor

*Research and Development Centre, Bharathiar University, Coimbatore,Tamilnadu- 641046.
Dept.of Plant Biology and Biotechnology,Loyola College (Autonomous), Chennai, Tamilnadu.
*Corresponding Author-E.mail I.D: biotechismail@gmail.com

[Received-29/06/2012, Accepted-28/07/2012]

ABSTRACT:

Roots are the plant parts which facilitate the primary contact between a plant and pollutant and culturing of genetically transformed hairy roots of hyperaccumulator plant sp.*Brassica juncea* L. grown *in vitro* was established to study the potential of *in vitro* hairy root cultures for the uptake and accumulation of heavy metals (Ni and Zn) for their growth and biomass from an aquatic environment. At elevated concentrations of Ni and Zn metals, hairy roots showed an exponential growth and accumulation. In lower concentrations, Zn might have no effect on growth of root biomass and also found to be it caused no dramatic decrease in root growth even they are accumulated. At higher concentrations of Zn, *B. Juncea* hairy roots was not only found to be tolerent to Zn and also had capacity to increase their root biomass and no growth retardation was seen. All concentrations of Ni showed a drastic change in root biomass growth irrespective of the duration of incubation periods. Ni in higher concentrations caused an exponential increase in root biomass growth and Ni showed no toxicity symptoms in hairy roots even at higher concentrations for longer time upto 8 weeks of our study period and Ni was found to be an essential micronutrient for the growth of hairy root biomass. From this study, it was cleared and concluded that hairy roots of *in vitro* grown hyperaccumulator plant culture system could be the useful and effective model (as it needs metals for their root biomass growth) to study the metal uptake and accumulation from an aquatic environments.
KEYWORDS: Hairy roots; *in vitro* culture ; Nickel (Ni); Zinc (Zn); *Brassica juncea*; hyperaccumulator.

INTRODUCTION

Metals are natural components in soil with a number of heavy metals being required by plants as micronutrients.However, pollution of biosphere by toxic metals dramatically since the beginning of the industrial revolution. As a result of human activities such as mining and smelting of metals, electroplating, gas exhaust, energy and fuel

production,fertilizer, sewage and pesticide application, municipal waste generation, etc.(1), metal pollution has become one of the most severe environmental problems today. Excessive accumuation of heavy metals is toxic to most plants.Heavy metals ions, when present at an elevant level in the environment,are excessively absorbed by roots and translocated to shoot ,leading to impaired metabolism and reduced growth (2) (3). According to their chemical properties and biological function ,heavy metal form a heterogeneous group ; toxicity varies by metals and concentrations. Many of them (Hg,Cd, Ni,Pb,Cu,Zn ,Cr,Co) are highly toxic both in elemental and soluble salt forms. Their presence in the atmosphere , soil and water ,even in traces can cause serious problems to organisms . Heavy metals bioaccumulation in the food chain especially can be highly dangerous to human health . The most common route of human exposure to heavy metals is through ingestion from both food and water sources (4).

Heavy metals , such as cadmium , copper , lead , chromium ,zinc , and nickel are important environmental pollutants , particularly in areas with high anthropogenic pressure (5).

Environmental pollution is a global problem that affects both the developing and developed countries(6). To a large extent , both human and natural processses contribute to environmental pollution and contaminants are commonly classified as either organic or inorganic . In general , inorganic contaminants originate from either natural processes of soil weathering or human activities including agriculture and mining (7). Subsequently ,both natural and human activities may promote the release of heavy metals e.g. manganese ,lead , copper , zinc , molybdenum , mercury , and nickel into soils and water posing a health threat to livestock and human populations. (8).

Agrobacterium rhizogenes (formerly *Phytomonas rhizogenes*) was first identified more than 70 yr ago (9)(10) (11). as the causative agent of the plant disease known as hairy-root syndrome or root-mat disease.Transformed plant roots, also called hairy roots, are adventitious roots that are caused by infection with the soil pathogen *Agrobacterium rhizogenes*, a gram- negative bacterium that belongs to the Rhizobiaceace family and the development of these roots is the result of natural genetic engineering in which a specific region of bacterial DNA contained in the Ri (Root inducing) plasmid is transferred from the bacterial cell to the plant cell and this fragment of DNA (T-DNA) is integrated into the plant genome and expressed.(12) (13).

Hairy roots extensively used in phytoremediation studies due to their ability to grow rapidly in microbe-free conditions, providing a greater surface area of contact between contaminat and tissue, they are genetically and metabolically more stable in comparison to wild type (14) (15).They are amenable to genetic transformation, making gene transfer and characterization possible in a system that may pose minimum health or environmental concerns. They also have ability to produce large quantities of exudates which are composed of enzymes and some metal chelating compounds that may detoxify or sequester harmful inorganic contaminants (14) (16) (17). Hairy roots have been applied to investigate heavy metal uptake and detoxification by rare plant species capable of growing in high-metal environments and accumulating elevated levels of specific metal ions. These species are known as “hyperaccumulators”that store heavy metal in their tissues at a concentration at least 100 times greater than those found in non accumulator plants.(18).

Hairy roots of several hyperaccumulators have been applied in metal uptake studies in liquid culture systems and these include *Alyssum bertolonii* and *Thlaspi caerulescens*,which were tested for hyperaccumulation of nickel and cadmium,respectively.(19).

Metal hyper accumulator plants have been found in wide range of families of vascular plants (20) (21). About 400 hyper accumulators have been identified and many hyper accumulators belong to the *Brassicacea* family. Indian mustard(*Brassica juncea*)belong to *brassicas* family have been tested for their ability to tolerate and accumulate heavy metals and it showed a strong ability to accumulate and translocate Cu,Cr VI,Cd,Ni,Pb and Zn to the shoots.

Indian mustard (*Brassica juncea*. Czern) is one of the most promising terrestrial plants for metal removal in water and the roots of Indian mustard are effective in removal of Cu, Cr VI, Cd, Ni, Pb and Ni (22)(23)(24)(25).

The *Brassicaceae* is very important group when heavy metal accumulation is concerned,with several species being able to hyperaccumulate more than one metal (26). *Brassica juncea* belongs to the family *Brassicaceae* and is a very important oil crop . Mustard oil is one of the major edible oils in India .Mustard oil has also got medicinal importance . Residual part of seeds is used as cattle feed and in fertilizer. India mustard (*Brassica juncea* L) is a fast growing plant which produces a high biomass even in heavy metal polluted soils . Thus this plant might be a potential candidate for phytofiltration and / or rhizofiltration of heavy metal contaminated wastewaters.

Brassica juncea (Indian mustard) – a high-biomass plant that can accumulate, Ni , Zn (22)(23)(24). The sources of Ni contamination are Volcanic eruptions, land fill, forest fire, bubble bursting and gas exchange in ocean, weathering of soils and geological materials Production of stainless steel,alloys,storage batteries,spark plugs,magnets and machinery. (27).

The sources of Zn contamination are Electroplating industry, smelting and refining,mining, biosolids Brass and bronze alloys production,galvanized metal production,pesticides and ink. (28)(29). Ni is an essential element that can be toxic and possibly carcinogenic in high concentrations(30). Ni toxity in humans usually results from repeated occupational exposure resulting in dermatitis ,asthma or headaches (31)(32).Ni ,Cu and Zn are three essential micronutrients for plant nutrition.

Ni is an essential component of the enzyme urease,but when Ni concentrations in vegetative tissues of plants exceed 50 mg/kg dry weight,plants may suffer from toxicity symptoms. Zn is essential microelement , but is toxic to animals and plants at high concentrations (33)(34).

MATERIALS AND METHODS

All the chemicals and reagents in this experiment were of Analytical grade and were obtained from Hi media laboratories Pvt ltd., Mumbai, India. Zinc and Nickel were used in the form of $ZnSo_4$ and $NiCl_2$.

Initiation or generation of In vitro cultures of Indian mustard

Indian mustard (*Brassica juncea* L) seeds were used as a source for initiating *in vitro cultures* for generating explant to form hairy roots by *A. rhizogenes* infection. The seeds were sterilized with 70% ethanol for 40 s followed by 0.1 % mercuric chloride for 5 min. The sterilized seeds were subsequently washed five times with sterile double distilled water and aseptically inoculated for germination on MS basal medium (35).supplemented and solidified with 3% sucrose and 0.8% agar respectively. Two to three weeks old sterile seedlings were used for infection by *A. rhizogenes*.

Culture of A.*rhizogenes*

Two wild type strains of *A. rhizogenes*, MTCC 2364 and MTCC 532 were obtained from Microbial Type Culture Collection Centre, (IMTECH) Chandigarh, India and were cultured on solid YMB medium. The bacterial suspension or 48-hrs old culture of A. *rhizogenes* was transferred to sterile centrifuge tubes and centrifuged at 4000- 5000 rpm for 5-10 min and

the resultant cell biomass was suspended in liquid MS medium supplemented with 3% sucrose and this culture of A. *rhizogenes* at exponential growth phase was used for transformation studies.

Infection with *A.rhizogenes*

A manual wound was made on the stems of *Invitro* grown seedlings of indian mustard with a fine needle attached to a syringe containing culture of *A. rhizogenes* and inoculated 1-2 drops onto the wounded portion of the tissue.the infected plants were placed in the same medium for co-cultivation for another 2-3 weeks without disturbing the media even without adding antibiotic to avoid contamination.

Induction or generation of Hairy roots

Hairy roots induced at the site of infection were individually isolated and cultured on phytohormone free MS medium supplemented with 500mg L cefotaxime to inhibit the growth of agrobacteria. Hairy roots were subcultured for another two passages on fresh MS medium with reduced level of cefotaxime to 250 mg L. After two passages, the clonal hairy roots were were transferred to MS liquid medium devoid of cefotaxime and maitained or kept on an orbital shaker operated at 50-70 rpm. Two to three weeks old hairy root cultures each one initiated from a single clone was used for uptake and accumulation of Ni and Zn from liquid medium.

Confirmation of transformation by PCR

The total genomic DNA was isolated from hairy root cultures of *Brassica juncea* L. for transformation confirmation. For amplification of the ORF-13 coding sequence of TL DNAwith a 498 bp domain, the primers used were(+)5' CAG CTT CTA AAT GTG GAG GCC and (-)5' CCT TGC CGA TTG CCA GTA TGG C. For amplification of *mas* 1' sequence of TR DNA with a 970 bp domain, the primers used were (+) 5' CGG TCT AAA TGA AAC CGG CAA ACG and (-)5' GGC AGA TGT CTA TCG CTC GCA CTC C.

For amplification of *vir* B10 coding sequence with a 644 bp domain, the primers used were (+)5' CAA TCC CGA TCA AGT CGT GCT C and (-) 5' AGA CGC CAA CCT CGT GAA ACC G.

DNA amplification was performed on an eppendorf thermal cycler in 25µL reaction mixture containing 25 ng template, 2.5 µL 10 x Taq DNA polymerase buffer (3-tris(hydroxymethyl) methylamine propane sulfonic acid pH 8.8, 15 mM $MgCl_2$, 500 mm KCl, 0.1 % gelatin).100 µM each of dNTPs, 0.2 µM primer and 0.5 U Taq polymerase (Bangalore Genei Pvt. Ltd). Following an initial denaturation step at 94^0C for 2 min, the amplification programme was 30 cycles of 30s at 94^oC, 30 s at 56^oC and 1 min at 72^oC. Amplification products were separated by electrophoresis on 1.5 % agarose gel in 1x TBE buffer, stained with ethidium bromide and visualized under UV trans-illuminator.

GROWTH STUDIES:

Metals uptake and accumulation in short-term and long term experiments:

Two sets of experiments were carried out in triplicate independently for lower concentrations with short period and higher concentrations with long term periods.

500 mg – 1 g of hairy root tissues was inoculated in 50 mL MS medium in 250 mL conical flasks and kept on a orbital shaker at 50-75 rpm. The rate of growth was measured from 1h upto 48 hr. At different periods the medium was drained off and the tissue was blotted briefly and its fresh weight was determined. The samples were then dried in an oven at 70^oC for a minimum period of 2 days and the final dry weight was determined. The initial dry weight was noted. The rate at which the tissue grew up was determined as grams per gram tissue per day.

Short-term experiments were performed over 48 h using hairy roots exposed to metal ions in MS medium. Ni in the form $NiCl_2.6H_20$ Zn in the

form of $ZnSo_4.4H_2O$ were used with *Brassica juncea* L roots at a concentration of 25 ppm,50 ppm,100 ppm, 250 ppm and 500 ppm.

Long-term experiments were performed over periods of 1-8 weeks. Flasks containing 50 mL MS medium with initial Ni and Zn concentrations of 500 ppm ,1000 ppm, 2000 ppm , 3000 ppm, 4000 ppm and 5000 ppm. The initial pH of two (Zn and Ni) metal solutions was 5.8 before autoclaving and no considerable change in pH was observed by the addition of $NiCl_2.6H_20$ solution, and $ZnSo_4.4H_2O$ solution,irrespective of different concentrations of Nickel chloride and Zinc sulfate respectively. For all short term and long term experiments, 1 g fresh weight biomass of hairy roots of *Brassica juncea* L.was added to 50 mL medium in 250-mL conical flasks.

After each sampling, the roots were filtered through Whatman No. 1 filter paper and dried at 70°C for measurement of dry weight. The sample was dried until the weight remained constant for a minimum of two consecutive weighing. The liquid medium was passed through a 0.45 ~tm filter and stored at -20°C for analysis of residual metal ion concentration. The dry biomass was digested in concentrated HNO_3 for 2 h at 140°C under pressure in a fume hood. After digestion the acid was completly removed by evaporation. Ni and Zn concentrations in hairy roots and medium were determined using an atomic absorption spectrophotometer (Varian, Australia). Uptake of Zn and Ni by hairy roots, their accumulation and hairy root biomass growth were measured periodically. The reduction of metal concentrations in the medium was attributed to its uptake by hairy roots.

RESULTS AND DISCUSSIONS

Induction of hairy roots by *A. rhizogenes*

Both wild type *A.rhizogenes* strains MTCC 2364 and MTCC 532 induced profuse root system from the wounded portions of *in vitro* grown seedlings or stem parts within 1- 4 weeks of incubation. The hairy roots were cultured on solid MS medium supplemented with cefotaxime antibiotic and roots grew plagiotropically with extensive branching while non transformed (control) roots failed to grow on MS medium devoid of phytohormones. A single fast growing clone of roots was selected for metal uptake.

PCR amplification

PCR amplification of hairy root DNA with primers specific for ORF 13 and mas 1' sequences indicated the expected fragment sizes of 498 and 970 bp, respectively. The control plant did not showed amplification with primers which indicated the integration of TL and TR DNA regions of *A. rhizogenes* with the genome of *B. Juncea* L .hairy roots. PCR analysis using primers specific to vir. B10, a non transformed region of Ri plasmid showed amplification only in the positive control (plasmid DNA) and not in DNA isolated from the hairy roots.

Uptake and accumulation of Zn and root biomass growth By hairy roots of *Brassica juncea* L.

In the short- term lower concentrations of Zn metal uptake studies of *B.juncea* hairy roots, 5 different concentrations of $ZnSo_4.4H_2O$ (25 ppm- 500 ppm) were tested. Uptake and accumulation of Zinc by *Brassica juncea* hairy roots from the initial periods of incubation to 2 days (48 hours) of incubation, upto 250 ppm showed no change in root biomass growth meanwhile the lower concentrations of Zn 25, 50, 100 and 250 ppm were uptaken from the liquid spent medium and accumulated by hairy roots and these lowest level of Zn was not utilized by hairy roots for their growth of root biomass and 500 ppm of Zn after 24 hours of incubation had a marked effect on root biomass growth of *B. Juncea* hairy roots.

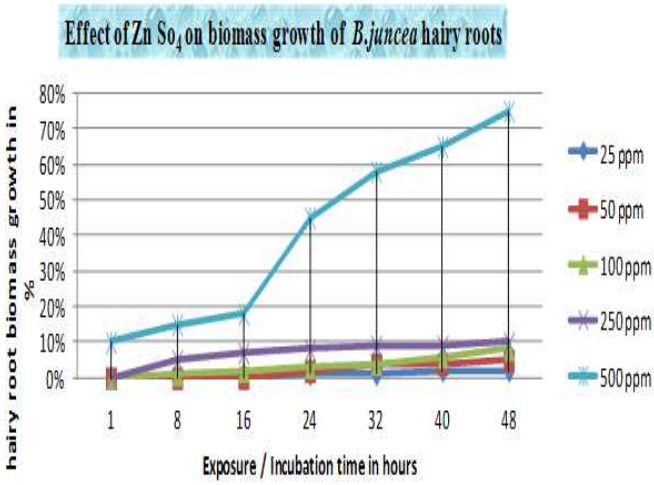

Figure 1 shows the efficiency of *Brassica juncea* hairy roots to uptake and accumulate $ZnSo_4$ metal for their biomass growth from 1 hour to 48 hours of incubation period. (Short time with low concentrations).

This result indicated that low levels of Zn had no influence on root biomass growth upto 2 days of incubation eventhough they were uptaken and accumulated by hairy roots meanwhile 500 ppm of Zn caused an gradual increase in hairy root biomass (150-200 mg / g dry weight) within a day of incubation. At the end of 48 hours exposure, 80% of root biomass growth was attained.From this analysis it was observed and cleared that Zn in higher concentration might be the nutrient for the growth induction of hairy roots of *Brassica juncea* and no growth inhibitory effect was seen at this level and in lower concentrations of Zn might have no effect on growth of root biomass and also found to be it caused no dramatic decrease in root growth even they are accumulated and hairy roots of *Brassica juncea* had the potential to uptake and accumulate Zn which was in higher concentrations used for their root biomass growth also.

In the long- term higher concentrations of Zn metal uptake studies of *B.juncea* hairy roots, 6 different concentrations of $ZnSo_4.4H_2O$ (500 ppm,1000 ppm, 2000 ppm, 3000 ppm, 4000 ppm and 5000 ppm) were tested. Uptake and accumulation of Nickel by *Brassica juncea* hairy roots from the initial periods of incubation –1 week to 8 weeks of incubation ,500 ppm to 2000 ppm of Zn had the same influence on root biomass growth within 2 weeks but at the end of fourth week, there was no significant changes in the growth of root biomass at the same concentrations. Root biomass was highly increased after 4 weeks of incubation period with the Zn concentrations of 3000 ppm to 5000 ppm. At higher concentrations of Zn, *B. Juncea* hairy roots was not only found to be tolerent to Zn and also had capacity to increase their root biomass (400- 500 mg / g dry weight) without losing their viability and no growth retardation was seen. This studies showed that there was a direct linear uptake relationship of Zn metal with *B. Juncea* hairy roots growth and transformed root system (hairy roots) of *B. Juncea* is a potential plant model system to study the removal of Zn from the metal contaminated water solutions.

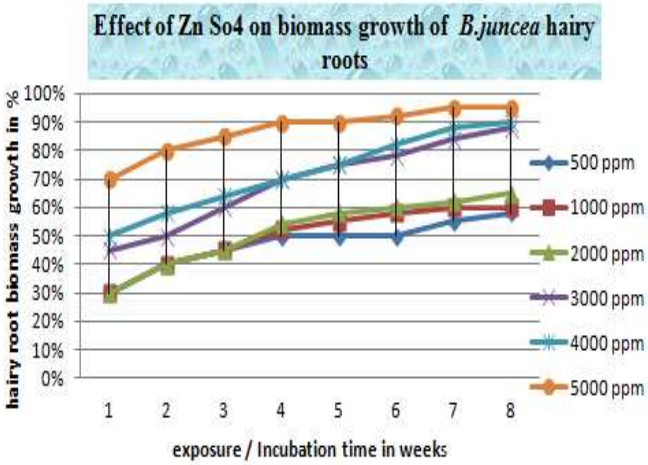

Figure 2 shows the efficiency of *Brassica juncea* hairy roots to uptake and accumulate $ZnSo_4$ metal for their biomass growth from 1 week to 8 weeks of incubation period. (long time with high concentrations).

Uptake and accumulation of Ni and root biomass growth By hairy roots of *Brassica juncea* L.

In the short- term lower concentrations of Ni metal uptake studies of *B.juncea* hairy roots, 5 different concentrations of $NiCl_2.6H_2O$ (25 ppm- 500 ppm) were tested. Uptake and accumulation of Nickel by *B.juncea* hairy roots from 1 hr to 48 hrs of incubation period ,all concentrations of Ni showed

a change in root biomass growth irrespective of the duration of incubation periods meanwhile the lower concentrations of Zn 25, 50, 100 ,250 and 500 ppm were uptaken from the liquid spent medium and accumulated by hairy roots and these lowest concentrations of Ni was utilized by hairy roots for their growth of root biomass unlike in the case of Zn. There was an gradual increase in root biomass with the increase in exposure time.

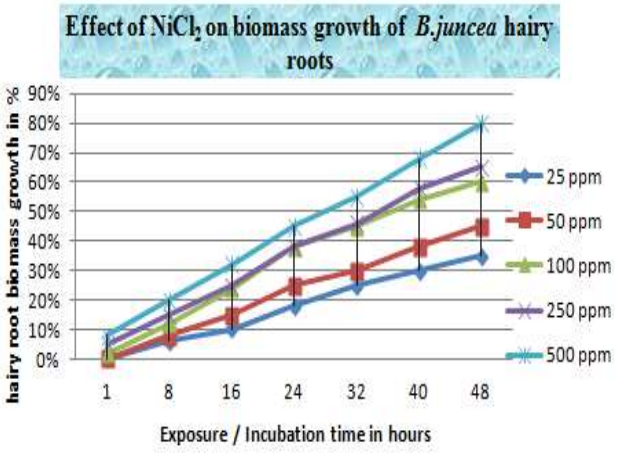

Figure 3 shows the efficiency of *Brassica juncea* hairy roots to uptake and accumulate $NiCl_2$ metal for their biomass growth from 1 hour to 48 hours of incubation period. (short time with low concentrations).

Ni in higher concentrations caused an exponential increase or saturation curve in root biomass growth as Ni level increases in medium with the increase in duration of incubation.Ni showed no toxicity symptoms or growth inhibitory effects in hairy roots even at higher concentrations for longer time upto 8 weeks of our study period and Ni was found to be an essential micronutrient for the growth of hairy root biomass.

The result showed that an increase in growth of hairy root biomass was directly proportional to the reduction of concentrations of Ni in MS liquid medium.

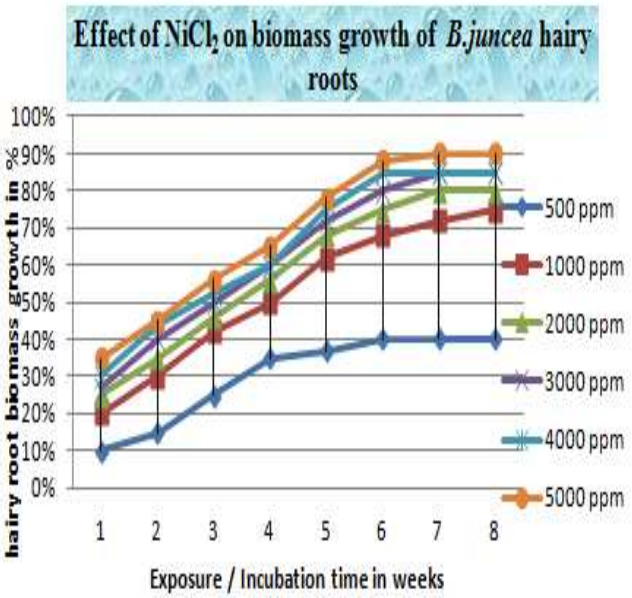

Figure 4 shows the efficiency of *Brassica juncea* hairy roots to uptake and accumulate $NiCl_2$ metal for their biomass growth from 1 week to 8 weeks of incubation period. (long time with high concentrations).

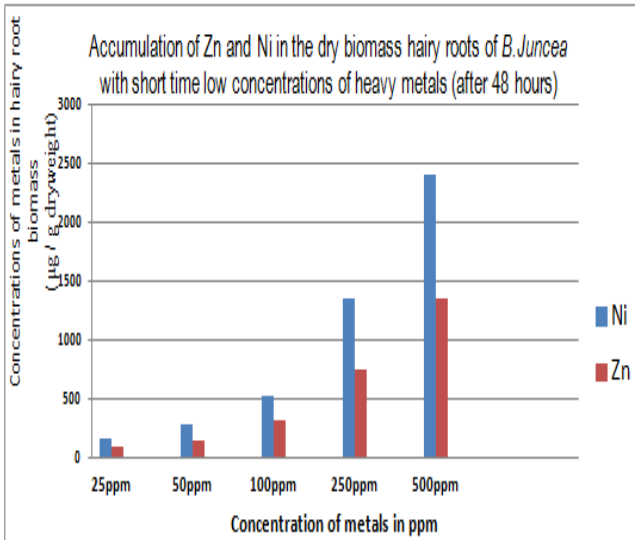

Figure 5 shows the metals accumulation potential of hairy roots in dry weight basis after 48 hours of incubation periods.

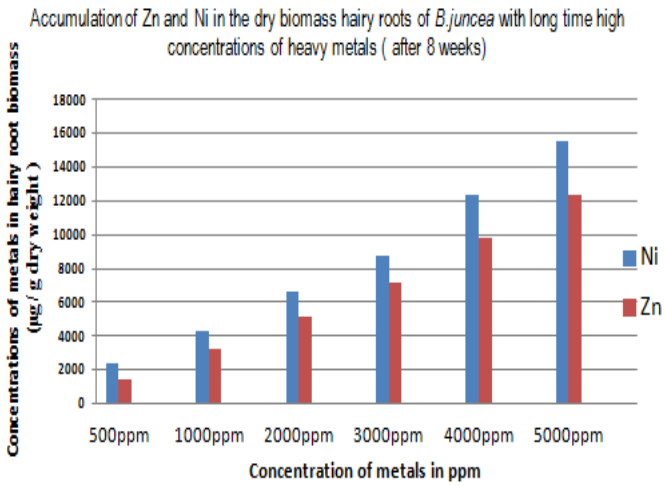

Figure 6 shows the metals accumulation potential of Brassica juncea hairy roots in dry weight basis after 8 weeks of incubation periods

CONCLUSION

It is essential to have plants with highly branched root system with large surface area for efficient uptake of toxic metals and hairy roots induced in hyperaccumulators were shown to have high efficiency for rhizofiltration of radionuclides (36). and heavy metals (19)(37). *In vitro* plant cultures are commonly used as tools for conducting basic laboratory studies of phytoremediation. *In vitro* cultures of roots(the main plant organ involved in uptake of metals) are of particular importance for studying the interaction of contaminants(heavy metals)with this plant organ and the drawback of root cultures is their low growth rate but the *Invitro* cultures of transformed roots (*hairy roots*) have the properties of fast growth, autotrophy in phytohormone and genotype and phenotype stability. Extensive root proliferation by *Agrobacterium rhizogenes*, generally considered an undesirable charecteristic, may find good utility for phytoremediation as roots for their larger penetrating ability to retrieve the heavy metal from aqueous solution.

In vitro cultures of roots may have the potential to study the interaction of metals with this plant organ and this type of isolated organ culture permits the charecterization of the uptake and accumulation capability of the roots while avoiding the interference of translocation to other plant tissues and the drawback of root cultures is their low growth rate but *in vitro* culture of transformed roots might be the suitable alternate to carry out this experiments and thus *in vitro* Culturing of hyperaccumulator hairy roots have proven to be suitable and successful experimental model system to study the uptake and accumulation potential for the metals from solution. Roots play a primary role in phytoremediation and phytomining, as they are the plant organs in direct contact with soil pollutants and heavy metals. Accordingly, there is a particular need to understand the biochemical and physiological functioning of roots in contaminated environments. Hairy root cultures are a convenient experimental system for such studies(19). As an experimental system, hairy root culture shares many of the advantages of hydroponics over soil- based cultivation, including better control over the conditions and nutrient concentrations experienced by the roots. There are additional benefits associated with hairy roots, however, such as the ability to use propagated tissues originating from the same plant throughout the experimental program, thus overcoming problems with variability between individual specimens (38). The influence of microbial contamination on metal uptake characteristics can also be eliminated using axenic root cultures, while the absence of aerial organs such as leaves and shoots allows identification of root-based mechanisms for metal uptake without interference from translocation effects.

Even the *Brassica juncea* L. Showed as a hyperaccumulator status for Zn and Ni accumulation in previous studies, its hairy roots may have more capacity / potential to uptake and accumulate such metals in higher level than that of in native form and this is may be due to the activation and synthesis of metal stressed gene

products such as nicotianamine, γ-glutamyl cysteine, citrate, phytochelatin, metallothionins,glutathione, thiols and forming various complexes with the metals at higher level for the efficient sequestration, translocation, uptake and detoxification of metals by transformed roots (hairy roots) than in non transformed roots.

AKNOWLEDGEMENT

I am grateful to Dr.Paul Agastian Theodor who had been Research advisor to carry out this work as a part of my Ph.D thesis and also thankful to the Management, Principal of Loyola college (A), Chennai, Tamilnadu for permitting me to do the research work at Plant biology and Biotechnology laboratory and also to all the technical co-operators for rendering assistance in Atomic absorption spectrophotometer to complete the research work successful.

REFERENCES

1. Kabata-Pendias., A., Pendias, H. (1989). Trace Elements in the Soil and Plants. CRC Press, Boca Raton, FL.
2. Foy, C.D., Chaney, R.L., White, M.C. (1978). The physiology of metal toxicity in plants.*Annu. Rev. Plant.Physiol.***29**: 511-566.
3. Bingham, F.T., Pereyea, F.J., Jarrell, W.M. (1986). Metal toxicity to agricultural crops. *Metal Ions Biol. Syst.*, **20**: 119-156.
4. Pickering, K.T, Owen, L.A. (1997). Water Resources and Pollution. In: *An Introduction to Global Environmental Issues.* 2nd (eds). London, New York. pp.187-207.
5. United States Environmental Protection Agency (USEPA) (1997). Cleaning Up the Nation's Waste Sites: Markets and Technology Trends. EPA/542/R-96/005. Office of Solid Waste and Emergency Response, Washington, DC
6. Suresh, B., Ravishankar, G.A. (2004). phytoremidiation-a novel and promising approach for environmental clean-up.*Crit Rev Biotechnol* **24**:97-124
7. Pilon-Smits, E.A. (2005). Phytoremediation. *Ann. Rev. Plant Biology*., **56**: 15-39.
8. Nedelkoska, T.V. and Doran, P.M. (2000a). Characteristics of heavy metal uptake by plant species with potential for phytoremediation and phytomining.*Miner.Eng*. **13**:549-561.
9. Riker, A.J., Banfield, W.M., Wright, W.H.,Keight, G.W., and Sasen, H.E.(1930). *J. Agric.Res*., **41**.507
10. Hildebrand, E.M. (1934). Life history of the hairy root organism in relation to its pathogenesis on nursery apple trees. *Journel of Agricultural research* **48**:857-885.
11. White,L.O., (1972). The taxanomy of the crown-gall organisms agrobacterium tumifaciens and its relationships to Rhizobia and other Agrobacteria. *Journel of general microbiology*, **72**:565-574.
12. Tepfer, M. (1984). Transformation of several species of higher plants by agrobacterium rhizogenes, sexual transmission of the transformed genotype and phenotype. *Cell* **37**: 959-967.
13. Zambryski, P. (1992) chronicles from the Agrobacterium –plant cell Dna transfer story. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43**:465-490.
14. Gujarathi, N.P., Haney, B.J., Park, H.J., Wickramasinghe, S.R., and Linden, J.C. (2005). Hairy roots of *Helianthus annuus*: A model system to study phytoremediation of tetracycline and oxytetracycline. *Biotechnol Prog.* **21**: 775-780
15. Georgiev, M.I., Pavlov, A.I., Bley, T. (2007). Hairy root type plant in vitro systems as sources of bioactive substances. *Appl. Microbiol Biotechnol* **74**: 1175-1185
16. Bais, H.P, Weir, T.L, Perry, L.G, Gilroy, S, Vivanco, J.M (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* **57**: 233-266
17. Doty, S.L (2008). Enhancing phytoremediation through the use of transgenics and endophytes. *New Phytol* **179**: 318-333.
18. Brooks, R.R. (1998).Plants that hyperaccumulate heavy metals. CAB Intl., Wallingford.
19. Nedelkoska, T.V., Doran, P.M. (2000). Hyperaccumulation of cadmium by hairy roots of *Thlaspi caerulescens*. *Biotechnol Bioeng*;**67**(5):607– 15

20. Reeves, R.D and A.J.M.Baker, (2000). Metal-Accumulating Plants. In: Phytoremediation of Toxic Metals-using Plants to clean up the Environment, Raskin, I.and B.D. Ensley (Eds.). Wiley, New york, pp: 193-229.
21. Prasad, M.N.V., Freitas, H.M.D.(2003). Metal hyperaccumulation in plants—Biodiversity prospecting for phytoremediation technology.*Electron. J. Biotechnol*., **93**(1):285-321.
22. Nanda-Kumar, P.B.A., Dushenkov, V., Motto, H. and Raskin, I. (1995). Phytoextraction: the use of plants to remove heavy metals from soils. *Environmental Science and Technology*, **29:** 1232-1238.
23. Raskin, I., P.B.A.N. Kumar, S. Dushenkov- and D.E. Salt: (1994). Bioconcentration of heavy metals by plants. *Curr. Opin. Biotechnol*., **5**: 285-290.
24. Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, B.D., and Chet, I.(1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology*, **13**:468–474.
25. Dushenkov, V., NandaKumar, P.B.A., Motto, H. and Raskin, I.(1995). Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. *Environmental Science and Technology*, , **29**(5):1239-1245.
26. Prasad,M.N.V. and H.Freitas,(2003). Metal hyperaccumulation in plants: Biodiversity prosoecting for phytoremediation technology, *Electron. J. Biotechnol*. **6**: 275-321.
27. Knox, A. S., Seaman, J., Adriano, D. C., and Perzynski, G. (2000). Chemophytostabilization of metals in contaminated soil. *Environ. Sci. Poll. Con.Series* **22**: 811–836.
28. Liu, H. Probst, A., and. Liao, B. (2005). Metal contamination of soils and crops affected by the Chenzhou lead/zinc mine spill (Hunan, China). *Science of the Total Environment*, **339**: 153- 166.
29. Liu, X.M., Wu, Q.T., Banks, M.K. (2005). Effect of simultaneous establishment of *Sedum alfridii* and *Zea mays* on heavy metal accumulation in plants. *Int. J. Phytoremediation*,**7**(1):43-53.
30. ATSDR.(2003).Top 20 Hazardous Substances. ATSDR/EPA Priority List for 2003. Agency for Toxic Substances and Disease Registry. US Department of Health and Human Services.
31. Davies, J. (1986). Occupational asthma caused by nickel salts. *J Soc Occ Med* **36**:29-31
32. Akeeson. B., Skerfing. S. (1985). Exposure in welding high nickel alloy. *Int Arch Occup Environ Health*, **56**:111-117.
33. Cobbett, C. and P. Goldsbrough: (2002). Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Ann. Rev. Plant Physiol. Plant Mol. Biol*., **53,** 159-182.
34. Gupta, U.C., Gupta, S.C. (1998). Trace element toxicity relationships to crop production and livestock and human health: Implications for management. *Commun Soil Sci Plant Anal* **29**:1491-1522.
35. Murashige, T., Skoog, F. (1962).A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, **15**: 473-497.
36. Eapen, S.,Suseelan, K., Tivarekar, S., Kotwal, S., Mitra, R.(2003). Potential for rhizofiltration of uranium using hairy root cultures of *Brassica juncea* and *Chenopodium amaranticolor*. *Environ Res*;**91**:127–33.
37. Eapen, S.,Suchita, K and Suseelan, K.(2007).Rhizofiltrationof Cadmium and lead by Hairy root cultures of *Brassica juncea* L. And the influence of the metals on antioxidative enzymes. *Plant cell biotechnology and Molecular biology* **8**(1&2): 73-78.
38. Pollard, A.J. and Baker, A.J.M. (1996). Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens.New Phytologist*, **132**: 113-118.