

Detoxification of inorganic contaminants in transgenic *Arabidopsis thaliana*

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Abstract

In detoxification, the plant takes up the contaminant of concern from soil, water, or a mixture of both, converts it to a volatile form, and releases it to the atmosphere, usually through the leaf stomata. This technique is only suitable for contaminants that do not pose a significant air pollution hazard. Contaminants taken up by the roots pass through the roots to the leaves and are volatilized through stomata where gas exchange occurs. This experimental work examines the ability of transgenic *Arabidopsis thaliana* with P4502E1 gene from rabbit to detoxify for many soil contaminants. The P450 2E1 enzyme controls the rate-limiting step in the metabolism of multiple environmental pollutants. The percentage of transformation increased at the less diluted cultures of *Agrobacterium tumefaciens*. The ability of transgenic plants were tested for their potential in remediation of metals such as Sodium (Na), Magnesium (Mg), Potassium (K), Calcium (Ca), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Zn), and Nickel (Ni). In the present work, transgenic plants were more efficient achieving 31, 46, 42, 48, 33, 51, 35, 29 and 39% contribution of each to the total value of detoxification contaminants. [DOI: [10.22401/JNUS.20.1.14](https://doi.org/10.22401/JNUS.20.1.14)]

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Introduction

Geological and anthropogenic activities are two sources of heavy metal contamination [1]. Volcanic activities, industrial effluents, fuel production, mining, smelting processes, military operations, utilization of agricultural chemicals, and manufacturing products release enormous amount of heavy metals into the soil, water and air. Severe contamination of heavy metals in soils may cause a variety of environmental problems, including groundwater contamination and toxicity to plants, animals, and humans [2].

Toxic metals interfere with biochemical and homeostatic processes in the cell through the production of free radicals. The biological consequence of each metal depends on the target accumulation organ, the particular chemical pathway that disrupts the chemical form of the metal, and its oxidation state [3]. Kidney dysfunction is a common effect of exposure to Arsenic (As), Cadmium (Cd), Lead (Pb), Mercury (Hg) and Uranium (U) [4; 5] while chromosomal damage and cancer have been shown in humans exposed to Cd and Pb [6]. Neurological, hematological,

immune effects have also been shown in humans exposed to lead [7].

The potential of *A. thaliana* as a model organism for genetics was first recognized in 1943 by F. Laibach [8]. Although *A. thaliana* has no major agronomic significance, it offers several advantages for researchers in many areas of plant biology especially in molecular genetics [9; 10; 11]. Recognized as a tool for the discovery of genes in the role of transformation and phytoremediation, *A. thaliana* has been used for phytoformation studies. One such study examined the TNT metabolic pathways within *Tobacco* [12]. Heavy metal uptake using *A. thaliana* genes has been more extensively studied such as for arsenic [13] and the elements Zinc (Zn), Cobalt (Co), Copper (Cu), Lead (Pb) and Manganese (Mn) [14]. The use of this model plant in molecular studies has several advantages. By expressing organomercurial lyase (*merB*) within the same plant, the full pathway from methyl mercury to the least toxic metallic mercury was accomplished. *Arabidopsis* plants transformed with this gene were tolerant to concentrations of methyl mercury 50 times higher than the

concentrations to which wild-type plants were tolerant and 10 times higher than those to which plants transformed with *merB* alone were tolerant [15]. Other strategies are to overexpress ATP sulfurylase [16], glutathione synthetase [17], or γ -ECS [18] in Indian mustard (*Brassica juncea*). In a reported field trial on transgenic plants for enhanced remediation, three transgenic lines showed increased accumulation of selenium in leaves. Transgenic Indian mustard plants overexpressing the adenosine triphosphate sulfurylase (*APS*) gene accumulated 4.3-fold more selenium in the leaves than the wild-type plants. Transgenics overexpressing the γ -ECS gene or the *GS* gene accumulated 2.8- fold and 2.3-fold more selenium in their leaves than wild type respectively. The plants accumulated cadmium, lead, copper, zinc, nickel, and boron [19]. Due to the importance of enhancing transgenic plants for phytoremediation purposes, the aim of this study was to exploit *A. thaliana* that have been transformation [10] by the gene *cytochrome P450 2E1* from rabbit to enhance their phytovolatilization capabilities for detoxification of heavy metal pollutants in soil.

Materials and Methods

Plasmid Constructions

A 1.7-kb *EcoRI* fragment containing the P450 2E1 cDNA [20] kindly provided by Frank Gonzales (National Institutes of Health, Bethesda, MD) was subcloned between the Mac promote [21] and the mas terminator [22] in the vector pKH200, which was kindly provided by Luca Comai (University of Washington). The 3.8-kb *BglIII* fragment containing Mac-CYP2E1-mas 39 was subcloned into the *Bam* HI site of the binary vector pCGN1578, which was provided by Luca Comai. The resulting plasmid, pSLD 50-6, was introduced into *A. tumefaciens* by electroporation [23].

Bacterial strains

Agrobacterium tumefaciens strain C58C1 was used to transform *A. thaliana* plants. Strains were maintained on LB medium supplemented with the appropriate antibiotics, kanamycin sulfate (50 mg/l) and carbinicilline (100 mg/l) at 28°C.

Agrobacterium infection and co-cultivation

The proximal end of the cotyledon was gently pricked for ten times to make wounds using sterile needle (Dispovan India Ltd., 0.63 X 25 mm). Then the cotyledon explants were immersed in the bacterial culture for 10 min. After that the explants were removed, blotted dry using sterile Whatman no.1 filter paper and inoculated (one explants/culture tube) on MS medium containing IAA (1.0 mg/l) and Kn (1.5 mg/l). The co-cultivation was performed for 0, 1, 2, 3, 4 and 5 days under a 16h photoperiod with a light intensity of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ and kept at $25 \pm 2^\circ\text{C}$.

Arabidopsis transformation procedure

Fifteen-day-old seedlings were transplanted in plastic disposable pots (10x10x10cm) at a density of ~8-12 plants per pot and grown at 23°C in a controlled growth chamber under short days (10h photoperiod) to ensure rosette formation. Light was supplied by an equal mixture of Osram White L100/23 and Warm White L100/30 (2400mm) fluorescent tubes with a rating of 8600 lumen. After four weeks plants were removed to a glass house (16 h photoperiods) to induce flowering. Primary bolts were clipped to encourage secondary inflorescence growth and plants were used for infiltration 7-10 days later when approximately 30% of the flower buds were open. *A. tumefaciens* strain C58C1(p) bearing the binary vector was grown in 500ml LB supplemented with the appropriate antibiotics at 28°C for 48h in an orbital shaker (200-250 rpm). Cells were harvested by centrifugation at 4000g for 10min and pelleted cells were resuspended in 5% sucrose solution supplemented with Silwet-L77 (0.02%). Each plant pot was inverted into the *A. tumefaciens* suspension in a plastic container that was big enough to allow the pot to be gently moved in half-circles for 30 seconds. Excess medium was blotted from the dipped plants, which were then covered in a contained propagator for a few days and left to set seeds.

DNA isolation and amplification of *bar* and *CYP 2E1* gene

Isolation of Genomic DNA: Genomic DNA was isolated from young leaves of control and transformed plants using the method described

by Al-Daoude [8]. PCR confirmation: For PCR analysis, DNA samples from putative transformants were amplified by *bar* specific primers. The *bar* gene fragment (0.46 kb) was amplified by using the forward primer-5'-ATC GTC AAC TAC ATC GAG AC-3' and reverse primer 5'-CCA GCT GCC AGA AAC CCA CGT C-3'. All PCR reactions were performed using a Peltier effect thermal cycler (MJ Research Co., USA). Samples containing 50 ng genomic DNA were first heated at 94°C for 5 min followed by 30 cycles at 94°C for 30s, 55 C and 72°C for 30 s followed by 7 min final extension at 72°C. Fifty ng of plasmid DNA was used as positive control. The PCR reactions contained 10 pM of each primer, 10 mM dNTPs mix, 15 mM MgCl₂, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% (v/v) Triton X- 100, 2 U of Taq DNA polymerase and 50 ng of template DNA in 1X reaction buffer. The amplified DNA was analysed using 1.5% agarose gel electrophoresis. Transformed tissues that were able to regenerate and grow in medium supplemented with kanamycin and carbinicilline were used for genomic extraction and amplification by PCR with specific CYP 2E1 primers (THE 35S promoter with the sequence 5' CATCGGGAATCTTCTCCAGTTGG 3', and the reverse with the sequence 5' TGAAGGGTGTGCAGCCGACAA 3') using the standard protocols (Sambrook and Russell 2001) to determine the presence of the transgene in the plant genome. PCR was performed using a Mastercycler Gradient (BioRad, USA) machine. All the amplifications were carried out in 25 µl reaction volume containing template DNA (300 ng genomic DNA or 50 ng plasmid DNA), 1 X PCR buffer, dNTP mix, 1 mM MgCl₂, 20 pmole primer each, and 1.5 U *taq* DNA polymerase.

Transgenic experiments in the presence of metals

Samples of contaminated soil were collected from industrial contaminated sites. *A. thaliana* transgenic plants were cultured separately in pots containing soil: peat (1:1, V:V) with heavy metal concentrations with three replicates. Wild type plants of each plant species were treated with the same treatment

as the transgenic ones. All treatments were placed under greenhouse conditions at 25°C for 16/8 hrs. light/ dark photoperiod using day light inflorescents at a light intensity of 3000 lux for 12 days. One gram of tissues from each replicate was dried in an oven for 24 h at 105°C (weighed and re-weighed until a constant weight was reached). The samples were then allowed to cool at room temperature before the final weight was taken. The contents of the mineral elements for K, Ca, Na, Mg, Mn, Zn, Cu, Ni and Fe were determined using the standard method (aqua regain digestion method) and analyzed by Atomic absorption spectroscopy (AAS), as described by Page *et al.*[24]. Data analysis by Excel programmer and pie charts three-dimensional displays pie charts fragmented contribution of each to the total value of the total while emphasizing individual values.

Results and Discussion

Floral dipping method was used to transform *A. thaliana* with no need for plant tissue culture or regeneration techniques Fig.(1). The technique required culture medium, hormones, pH adjustment and *A. tumefaciens* mediated transformation which are applicable at a range of cell densities. Plants of *A. thaliana* were infected with the same *A. tumefaciens* strain using the floral dip method. Plants were dipped in a solution of *A. tumefaciens*, 5% sucrose and 0.02% of the surfactant, Silwet L-77. Plants were inoculated when numerous immature floral buds and few siliques were present. Plants were left to set up seeds and transgenic seeds were selected on Kanamycin containing medium.

Floral dip method is simple and can be conducted by submerging the above-ground parts of the plant into an *A. tumefaciens* solution for a few seconds. In nature, wounded sites secrete compounds (hexoses at low pH) that induce *vir* genes. In tissue culture transformation methods, the phenolic compound acetosyringone is sometimes added to induce expression of relevant *vir* genes. Bechtold *et al.* [25] and Al-Daoude [8] reported that *A. tumefaciens* C58Cl and pTiC56 genotypes are effective in *A. thaliana* transformation. Steven *et al.* [26] reported that

an increase in Silwet L-77 from 0.005% to 0.1% gave higher transformation rate, but high level surfactant (Silwet L-77) can cause plant tissue necrosis under various conditions.

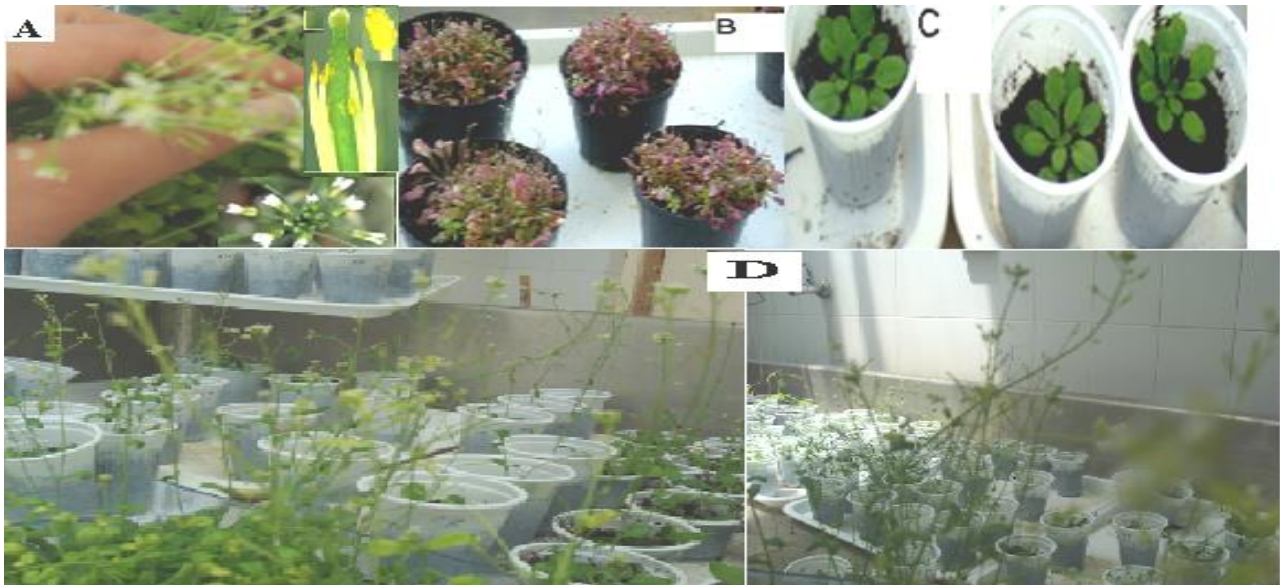


Fig.(1): *A. thaliana* growth stages using floral dipping method:

- A. Primary bolts clipped, secondary bolts about 2-10 cm (few open flowers)
- B. *A. thaliana* clusters were harvested after floral dipping with *A. tumefaciens*.
- C. *A. thaliana* germinated on soil containing 50 mg/l Kanamycin.
- D. *A. thaliana* F1 progeny was harvested and seeds were germinated under Kanamycin selection.

The CYP2E1-P1 and CYP2E1-P2 primers amplified a 410 bp band from both *CYP2E1* transgenic plants and pSLD50-6 plasmids, while no PCR products were detected using these two primers for plants and pKH200 plasmids.



Fig.(2): Agarose (2%) gel electrophoresis of PCR amplicons using *CYP2E1* specific primer (70 v, 60 minutes). Lane 1: DNA Ladder. DNA samples were extracted and tested for the presence of *CYP2E1* gene (a band of ~410 bp was seen). Control plants (2-7 wells) are transgenic plantlets (8-13 wells).

A. thaliana transgenic plants grown on soil containing heavy metals were healthy 12 days after the start of experiment and gained higher fresh reached 61% and dry weight reached

63% than wild type plants which recorded 37, 39% respectively Fig.(3).

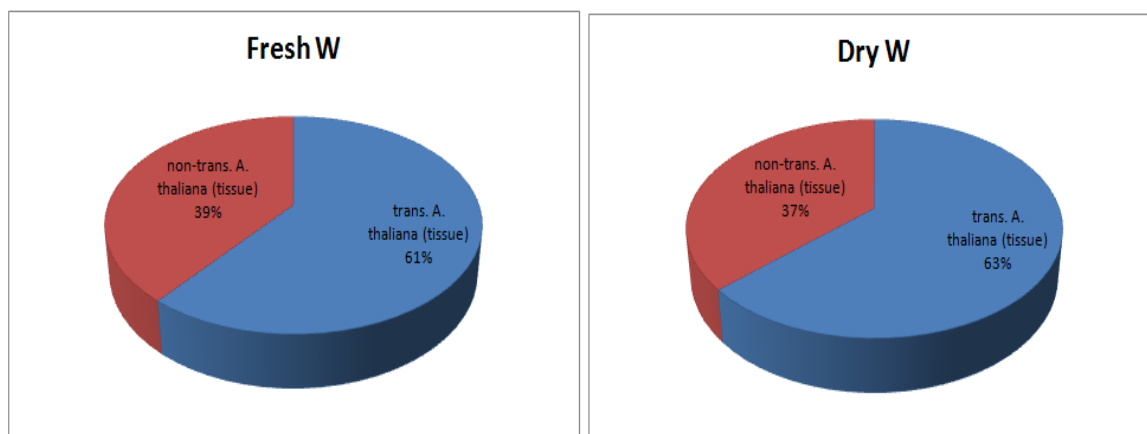


Fig.(3): Fresh and dry weights in transgenic and wild type plants of *Arabidopsis thaliana*.

Macronutrients

Arabidopsis transgenic lines showed increased accumulation of Na reached 31% compared with wild type plants reached 29%. However, decreases of Na concentration were 20% in all soils planted with *Arabidopsis* transgenic plant and wild type plant respectively Fig.(4). Results showed that increased accumulation up to 42% compared with wild type plants Fig.(4). While decreases in Ca concentration were 12% in soil planted with *Arabidopsis* transgenic plants compared with 5% in the same soils cultured in wild type plant. In contrast, an increase (46%) of Mg concentration in transgenic plants in soils planted with transgenic plant compared with wild type plants (41%). While the decrease in Mg concentration reached 12 and 1% in the same soil planted with transgenic and wild type plants respectively. Results showed increased accumulation of K in the transgenic *Arabidopsis* lines respectively 48% compared with wild type plants (15%). Decreases in K concentrations were 14% and 23% in soil planted with *Arabidopsis* transgenic plants and wild type plants respectively. The increase in macro elements accumulation could be explained that Ca, K, Na, and Mg are essential plant nutrients for plant growth and acquired from the soil solution by the root system and translocated to the shoots via the xylem. Roots must balance the delivery of calcium to the xylem with the need for individual root cells to use (M^{+2}) cytoplasmic for intracellular signaling. The current hypothesis assumes, that Ca^{+2} travels apoplastically across the root to the Casparian band which it then circumvents via the cytoplasm of the endodermal cell, although

Ca^{+2} channels and Ca^{+2} -ATPases are present and could catalyse Ca^{+2} influx and enfflux across the plasma membrane of endodermal cells, their transport, or interactions, between Ca^{+2} , Br^{+2} , Sr^{+2} or any cation for transport to the shoot [27;28; 29; 30;31].

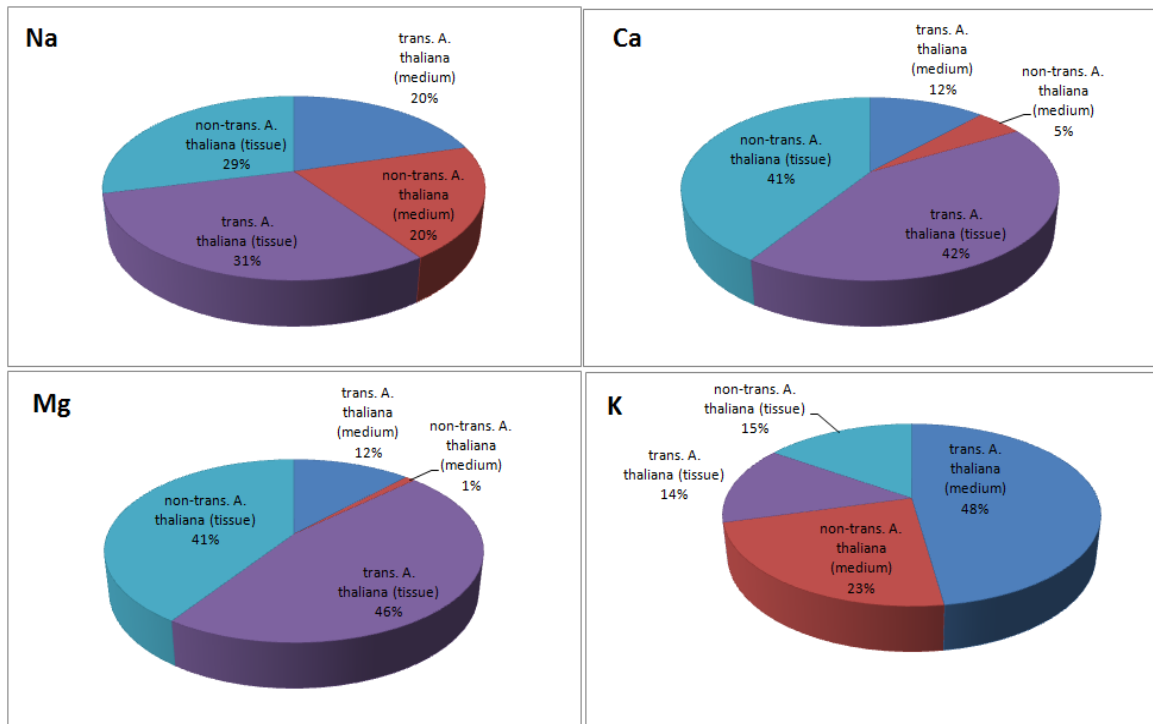


Fig.(4): Macronutrient concentration of Sodium, Calcium, Magnesium and Potassium in *Arabidopsis* transgenic lines planted on contaminant soils.

Heavy metals

Arabidopsis transgenic lines showed increased Cooper accumulations up to 35% compared with wild type plants (55%). On the other hand, decreases in Cu concentration was 7% and 3% in soil planted with transgenic *Arabidopsis* and wild type plants respectively Fig.(5). Results showed increased accumulation in Mn in transgenic plant (33%) compared with wild type plants (34%). Fig.(5) shows a decrease (22%) in Mn concentration in all soils contaminated with heavy metals and planted with transgenic *Arabidopsis* compared with wild type plants reached 11%.

Results show increased accumulation in Fe in transgenic *Arabidopsis* (51%) compared with wild type plants (41%) (Fig.5). In contrast, a decrease (6 and 2%) of Fe concentration in soils contaminated with heavy metals and planted with transgenic and wild type plants. Results also showed increased accumulations in Zn in transgenic *Arabidopsis* lines with increased accumulation percentage reached 29% compared with wild type plants which reached 27% Fig.(5). However, decreases in Fe concentrations were 33% and 11% in soil planted with *Arabidopsis* transgenic and wild type respectively. *Arabidopsis* transgenic lines showed again

increased accumulation up to 39% of Ni compared with wild type plants (25%) Fig.(5). A reduction in Ni concentration (18%) in all soils planted with transgenic *Arabidopsis* and wild type.

Toxic effects of metals were assessed inhibition of the primary root length increment as compared to the roots grown by in water. Metal localization in the root apex tissues, the metals were found in both the meristem and in the root cap. Also previous studies have shown that cytochrome P450 2E1 (CYP2E1)-dependent transcriptional up-regulation of glutamate-cysteine ligase (GCL). To identify sequences mediating constitutive and induced expression of the catalytic subunit of GCL (GCLC) [32; 33; 31;34].

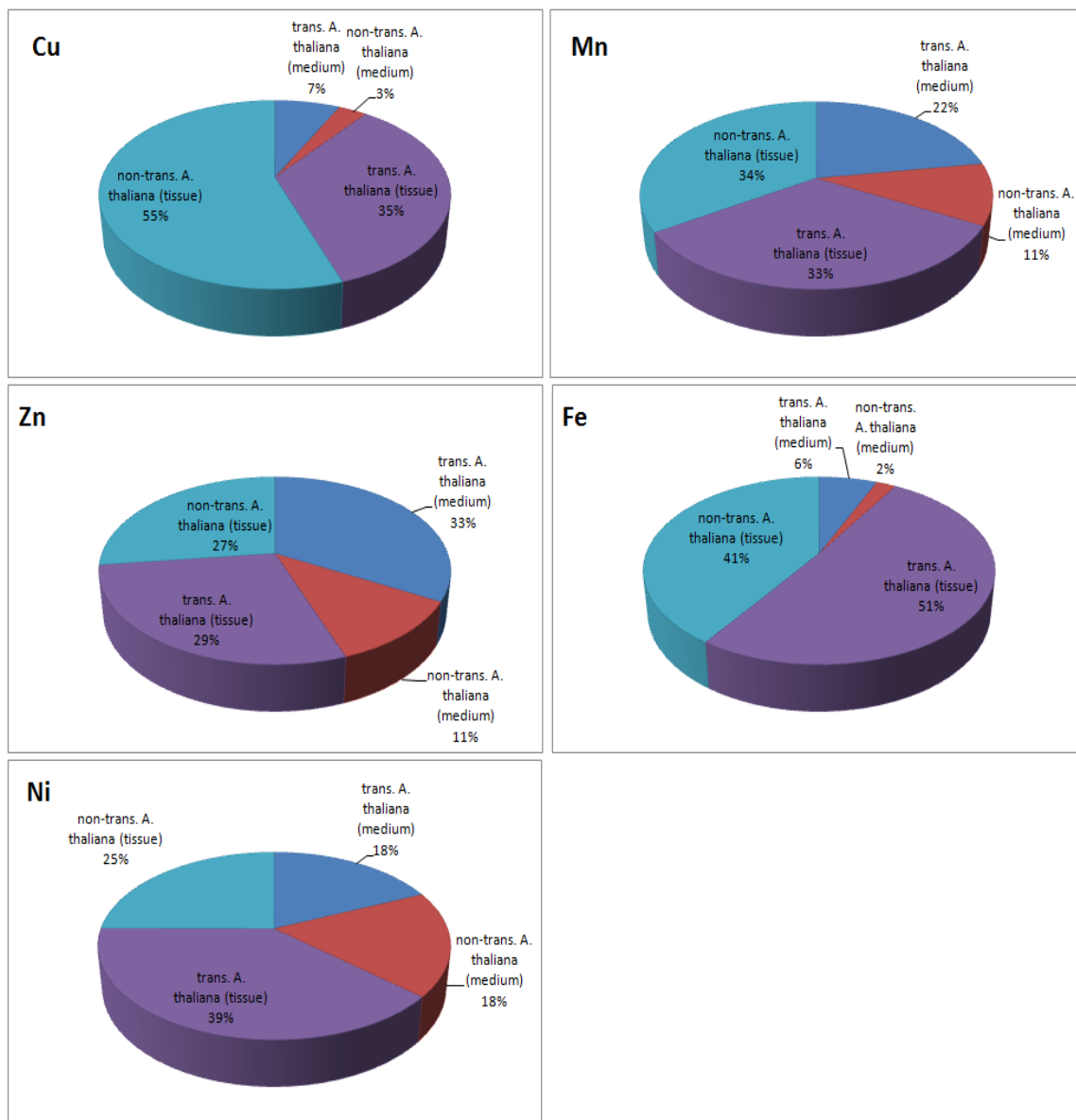


Fig.(5): Micronutrient concentration of Copper, Magnesia, Zinc, Iron and Nikel in Arabidopsis transgenic lines planted on contaminant soils.

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