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DETERMINATION OF ROLE OF GLUTATHIONE IN HEAVY METALS DETOXIFICATION IN *ARABIDOPSIS THALIANA*

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ABSTRACT

In this study, wild type *Arabidopsis* Col-0 and a glutathione deficient mutant *Cad2-1* were used to study the effect of low levels of glutathione (GSH) towards heavy metals stress. Seeds of the *Cad2-1* mutant and Col-0 were plated on normal half strength Hoagland media for seven days. Seven days after germination, the seedlings were subjected to low or high concentrations of sodium arsenate (100 and 200 µM), copper sulfate (50 and 100 µM) and nickel chloride (70 and 100 µM) for five days. After 5 days of growth in a growth chamber maintained at 22 ^{0}C, the root length of each seedling was measured. Both concentrations of sodium arsenate reduced the root length of *Cad2-1* significantly ($P<0.05$) as compared with Col-0. Neither concentrations of copper sulfate had significant adverse effect on the root length of *Cad2-1* as compared to Col-0. On the other hand, 70 µM concentration of nickel chloride did not cause significant reduction in the root length of *Cad2-1* as compared to Col-0. However, at 100 µM concentration, nickel chloride resulted in significant root length reduction of *Cad2-1* as compared to Col-0.

Key Words: *Arabidopsis thaliana, Col-0, Cad-2,* Glutathione, Heavy Metals

INTRODUCTION

Glutathione (GSH), a tri-peptide is most abundant low molecular weight thiol in all mitochondria-bearing eukaryotes including plants. In plants, GSH is involved in a plethora of cellular processes, including defense against ROS (Foyer and Noctor, 2005; Mullineaux and Rausch, 2005), sequestration of heavy metals (Cobbett and Goldsbrough, 2002; Freeman *et al*., 2004) and detoxification of xenobiotics (Dixon *et al.*, 1998). GSH also plays an important role in the regulation of developmental processes such as cell division (Vernoux*et al.*, 2000) and flowering (Ogawa *et al*., 2004**)**.Furthermore, GSH is a major transport and storage form of reduced sulfur. GSH is synthesized via two ATP dependent reactions, where γ-glutamyl cysteine synthetase (GSH1, E.C. 6.3.2.2) catalyzes the formation of a peptide bond between the carboxyl group of glutamate and the amino group of cysteine, to yield γ-glutamyl cysteine (γ-EC). This first step has been documented as a major control point under conditions of increasing demand for GSH (Noctor*et al*., 1998). In the second reaction, glutathione synthetase (GSH2, E.C. 6.3.2.3) ligates a glycine residue with γ-EC to form GSH. In *Arabidopsis thaliana* and *Brassica juncea*GSH1 is exclusively confined to the plastids, whereas GSH2 is found in both plastids and cytosol (Wachter*et al*., 2005). Glutathione exists in two form reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduction potential of glutathione depends on the intracellular GSH/GSSG ratio. Change in the redox ratio of glutathione mainly depends on the pH, total GSH

concentration, GSH biosynthesis and GSH catabolism (Mullineaux and Rausch, 2005). With the completion of Arabidopsis genome sequence, it has become clear that single genes encode GSH1 and GSH2 with predicted transit peptides for plastidic localization (The Arabidopsis Genome Initiative, 2000). GSH is a substrate for phytochelatin synthesis and crucial for detoxification of heavy metals such as cadmium and nickel (Freeman *et al.*, 2004).

Arsenic (As), a toxic metalloid found everywhere as in ground and surface waters has become worldwide concern due to geochemical weathering of rocks and microbial and human activities (Tripathi*et al*., 2007). It has no demonstrated biological function in plants. Its toxicity with many people has caused cancer, skin lesions and other symptoms (Dhankher, 2005; Mondal*et al.*, 2006). In the environment arsenic forms organic and inorganic complexes, but most importantly it is found in inorganic form as arsenate (AsV) and arsenite (AsIII). As (V) is taken up by phosphate transporters and rapidly reduced to As(III). As(III) can be used as the sole electron donor for an oxygenic photosynthesis in bacteria from hot spring biofilms (Kulp*et al*., 2008). The As also undergoes transformation within plant cells to other less phytotoxic As species (Meharg, 1994). In phytoplankton and macro algae, As is converted to arsenite, dimethylarsinic acid (DMA), and mono-methylarsinic acid (MMA). Such methylated forms of As are then metabolized to organophospholipids and arsenosugars (Phillips, 1990).

Nickel (Ni) is a transition metal and found in natural soils at trace concentrations except in ultramafic or serpentinic soils. However, Ni^{2+} concentration is increasing in certain areas by human activities such as mining works, emission of smelters, burning of coal and oil, sewage, phosphate fertilizers and pesticides (Gimeno-García*et al*., 1996). Ni^{2+} concentration in polluted soil may reach 20 to 30-fold (200–26,000 mg/kg) higher than the overall range (10–1000 mg/kg) found in natural soil (Izosimova, 2005). Excess of Ni^{2+} in soil causes various physiological alterations and diverse toxicity symptoms such as chlorosis and necrosis in different plant species (Zornoza*et al.*, 1999; Pandey and Sharma, 2002; Rahman*et al.*, 2005), including rice (Samantaray*et al.*, 1997).

Copper (Cu) is considered as a micronutrient for plants (Thomas *et al.*, 1998) and plays important role in CO_2 assimilation and ATP synthesis. Cu is also an essential component of various proteins like plastocyanin of photosynthetic system and cyto-chrome oxidase of respiratory electron transport chain (Demir-evska-kepova*et al*., 2004).However, the intracellular Cu level must be tightly regulated, as it is toxic for most plants when present in excess. Enhanced industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Cu is also added to soils from different human activities including mining and smelting of Cu-containing ores. Excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis *et al*., 2001). Exposure of plants to excess Cu generates oxidative stress and ROS (Stadtman and Oliver, 1991). Excess of Cu also effect the accumulation of other essential elements (Tsang *et al.*, 1996), inhibition of root elongation (Murphy and Taiz 1995), modification of protein and lipid composition of the root plasma membrane (Quartacci*et al.*, 2001), reduction of the thylakoid membrane structure of chloroplasts (Patsikka*et al.*, 2002) and alteration of cellular transport and content of several metabolites (Wintz and Vulpe, 2002).

MATERIALS AND METHODS

This research was conducted at Recombinant DNA Technology Laboratory, Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan.

Media preparation

Half strength Hoagland media were prepared for the germination of *Arabidopsisthaliana* plants. The composition of medium and concentration of each component are shown in table 1.

Stock	Component	Concentration
Macronutrients	$Ca(NO_3)_2$	2.5 mM
	KNO_3	2.5 mM
	$MgSO_4$ x $6H_2O$	0.5 mM
	KH_2PO_4	0.5 mM
Micronutrients		
	Fe-EDTA	40 µM
	H_3BO_3	2.25 µM
	$MnCl_2$ x 4 H_2O	2.25 µM
	$ZnSO_4$ x 7 H_2O	1.9 µM
	$CuSO_4$ x $5H_2O$	0.15 µM
	$(NH_4)_6Mo_7O_{24}$x $4H_2O$	0.05 µM
	Adjust PH 5.8 to 6.0 with HCl or NaOH	

Table 1: Composition and concentrations of ½ Hoagland medium

Plant material

Seeds of *Arabidopsis thaliana*, wild type Columbia (Col-0) and *cad2-1* mutantwere kindly provided by Dr. Muhammad Sayyar Khan.

Seed sterilization

For surface sterilizationappropriate amount of seeds of each line were placed in eppindorf tubes and remaining step were performed in sterile laminar flow hood. Seeds were soaked for 2 minutes in 700 µl sterile distilled water and were surface sterilized with 700 µl 70% ethanol for 40 seconds. Then seeds were treated with 15%bleach and 1 drop of tween-20 for 8 minutes. Finally the seeds were washed four times with sterile distilled water and dried on sterile filter paper. Seeds of each line were placed on solid half strength Hoagland medium for germination. Plates were wrapped with Para film.

Seed vernalization

Plates were transferred to refregerator and stored at 4 ^{0}C for vernalization treatment for 2 days

Seed transfered to growth chamber

After 2 days vernalization period at 4 ^{0}C, plates were shifted to growth chamber maintained at 22 ^{0}C. The plates were placed in vertical position for 7 days. In this period of time seeds germinated into seedlings.

Stress media preparation

Half strength Hoagland media containing sodium arsenate in 100 µM and 200 µM , Nickel chloride in 70 µM and 100 µM and copper sulfate in 50 µM and 100 µM concentrations were prepared .

Transferring of Seedling to stress media

To study the effect of sodium arsenate, nickel chloride and copper sulfate, 7 days old seedling of Col-0 and *Cad2-1* were transferred to stress media containing the above mentioned heavy metals for 5 days.

Parameter studied

In this study the phenotype and root length of the seedling grown on stress media and normal media were studied.

Statistical analysis

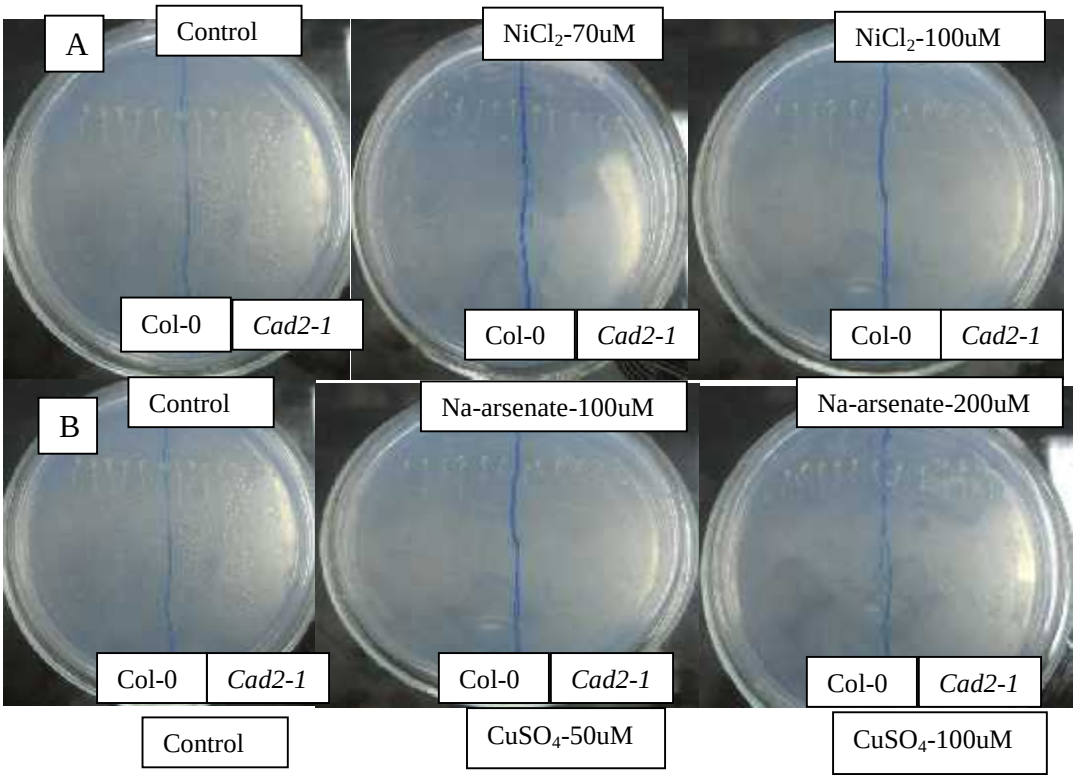
General t-test was performed for data analysis and graphs were prepared.

RESULTS AND DISCUSSIONS

This research was conducted to study the effect of sodium arsenate, nickel chloride and copper sulfate on *Arabidopsis thaliana*. Seeds of wild type Col-0 and *Cad2-1* mutant lines of *Arabidopsis* were first surface sterilized, plated on half strength Hoagland media and kept for two days at 4 ^{0}C for vernalization treatment. Then the Petri-plates were transferred to growth chamber maintained at 22 ^{0}C for seven days for germination. To study the effect of sodium arsenate, nickel chloride and copper sulfate, 7 days old seedling of Col-0 and *Cad2-1* were transferred to stress media containing different heavy metals concentrations for 5 days.

Effect on phenotype

To study the effect of sodium arsenate, copper sulfate and nickel chloride on phenotype of seedlings, 7 days old seedlings of wild type Col-0 and *Cad2-1* mutant were transferred to half strength Hoagland media containing sodium arsenate at 100 µM and 200 µM concentration, copper sulfate at 50 µM and 100 µM concentration and nickel chloride at 70 µM and 100 µM concentration for 5 days. After 5 days exposure of the seedlings to these concentrations indicated that *Cad2-1* mutant is more sensitive to nickel chloride and sodium arsenate compared to their wild type Col-0 control as shown by the paler color of their leaves and ultimately bleached seedlings (Figure 1 A and B). The *Cad2-1*mutant, however was not more sensitive than Col-0 at tested concentrations of copper sulfate (Figure 1 C).



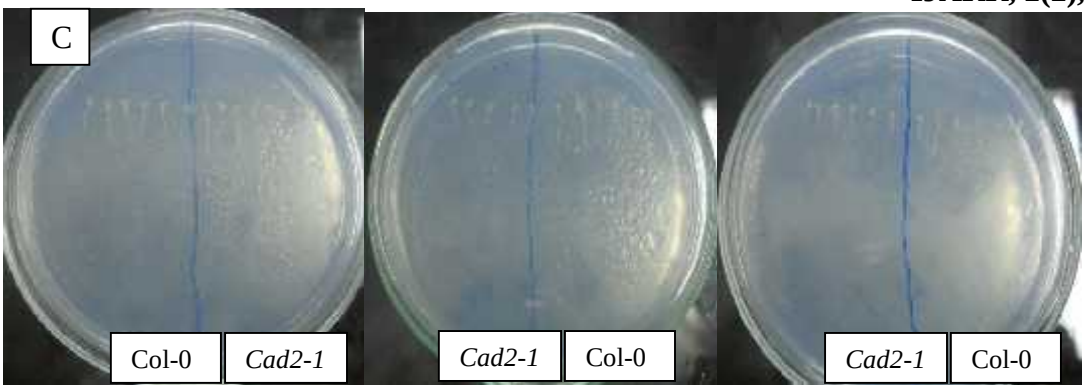


Figure 1: Effect of heavy metals on phenotype of *Arabidopsis thaliana.*
1A) Impact of nickel chloride on phenotype of wild type Col-0 and mutant *Cad*2-1, after 5 days exposure to 0 uM, 70uM and 100 uM concentrations of nickel chloride.
1B) Impact of sodium arsenate on phenotype of wild type Col-0 and mutant *Cad*2-1, after 5 days exposure to 0 uM, 100uM and 200 uM concentrations of sodium arsenate.
1C)Impact of copper sulfate on phenotype of wild type Col-0 and mutant *Cad*2-1, after 5 days exposure to 0 uM, 50uM and 100 uM concentrations of copper sulfate.

Effect of sodium arsenate on root length

To know the effect of sodium arsenate on root length of seedlings, 7 days old seedlings of wild type Col-0 and *Cad2-1* mutant were transferred to half strength Hoagland media plates containing 0 µM, 100 µM and 200 µM of sodium arsenate for 5 days. After 5 days of growth, on normal media (0 µM sodium arsenate), both wild type Col-0 and *Cad2-1* mutant of *Arabidopsis thaliana* gave similar root length (0.95 cm each). While, on media amended with 100 µM concentration of sodium arsenate, *Cad2-1* mutant produced 0.75 cm short root which was highly significantly ($P<0.01$) shorter than the root length of wild type Col-0(0.93cm). However, on media amended with double concentrations (200 µM) of sodium arsenate, *Cad2-1* mutant produced 0.70 cm root which was significantly ($P<0.05$) shorter than the root length of wild type Col-0 (0.86 cm) as shown in figure 2. These findings indicate that the wild type (Col-0) is more tolerant to the stress of sodium arsenate as compared to *Cad2-1* mutant. The low level of GSH in the *Cad2-1* mutant due to mutation has made it more sensitive to sodium arsenate toxicity even at low concentration.

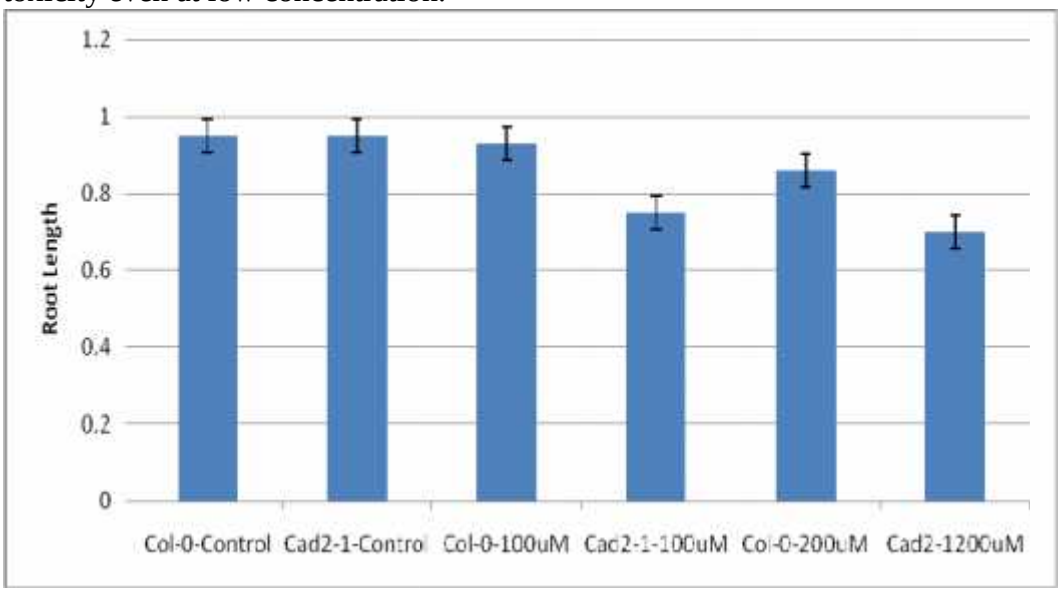


Figure 2: Effect of sodium arsenate concentration on the root length of wild type Col-0 and mutant *Cad2-1* ,after 5 days exposure of seedlings to 0 µM, 100 µM and 200 µM concentrations of sodium arsenate. P value for 100 µM concentration =0.006 and P value for 200 µM concentration = 0.021.

Effect of copper sulfate on root length

To study the effect of copper sulfate on root length of seedlings, 7 days old seedlings of wild type Col-0 and *Cad2-1* mutant were transferred to half strength Hoagland media plates containing 0 µM, 50 µM and 100 µM of copper sulfate for 5 days. On normal medium (0 µM copper sulfate), both wild type Col-0 and *Cad2-1* mutant registered similar root length (0.95 cm each) .However, the root length of Col-0 and *Cad2-1*(0.91cm and 0.83cm respectively) did not differ significantly (P>0.05) when treated with 50 µM concentration of copper sulfate. A non-significant difference was also recorded in the root lengths of the Col-0 and *Cad2-1* when a double concentration (100 µM) of copper sulfate was used (Figure 3). These findings show that, the mechanism of copper detoxification seems to be not directly related to GSH levels as indicated by non-significant difference between the root length of Col-0 and *Cad2-1*.

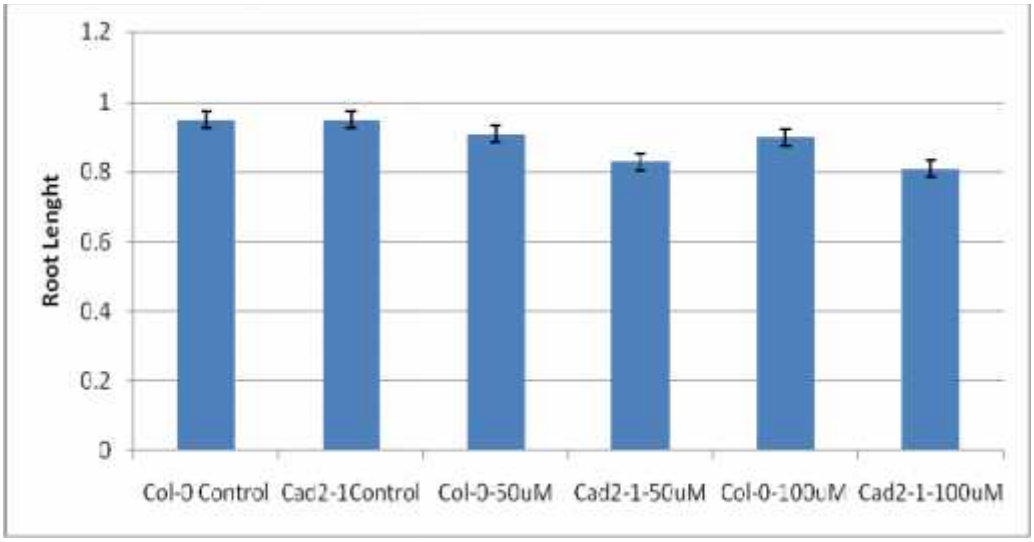


Figure 3: Effect of copper sulfate concentration on the root length of wild type Col-0 and mutant *Cad2-1,* after 5 days exposure of seedlings to 0 µM, 50 µM and 100 µM concentrations of copper sulfate. P value for 50 µM concentration =0.091 and P value for 100 µM concentration = 0.097.

Effect of nickel chloride on root length

To observe the effect of nickel chloride on the root length of seedlings, 7 days old seedlings of wild type Col-0 and *Cad2-1* mutant were transferred to half strength Hoagland media plates containing 0 µM, 70 µM and 100 µM of nickel chloride for 5 days. After 5 days of growth on normal medium (0 µM nickel chloride), both wild type Col-0 and *Cad2-1* mutant produced similar root length (0.95 cm each). However, treatment with 70 µM nickel chloride did not cause significant difference (P>0.05) in the root length of Col-0 and *Cad2-1*. However, on media amended with 100 µM concentration of nickel chloride *Cad2-1* mutant produced significantly (P<0.05) shorter (0.83 cm) roots compared to wild type Col-0 (0.93 cm) as depicted in figure 4. These findings suggest a role for GSH in the detoxification of nickel.

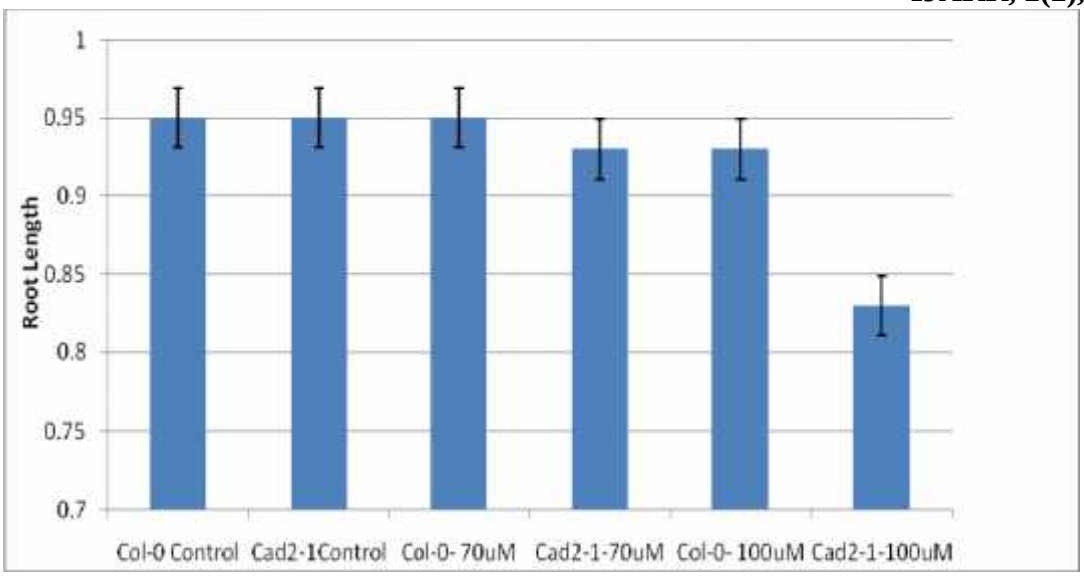


Figure 3: Effect of nickel chloride concentration on the root length of wild type Col-0 and mutant *Cad2-1*, after 5 days exposure of seedlings to 0 µM, 70 µM and 100 µM concentrations of nickel chloride. P value for 70 µM concentration =0.42 and P value for 100 µM concentration = 0.04.

CONCLUSIONS AND RECOMMENDATIONS

1) Both the low and high concentration of sodium arsenate can drastically reduce the root length of *Cad2-1* mutant of *Arabidopsis thaliana*.
2) Copper sulfate does not significantly affect the root length in *Cad2-1* mutant compared to wild type Col-0.
3) Nickel chloride is tolerated at low but not at high concentration by *Cad2-1* mutant.
4) Tolerence to arsenic and nickel seems to be mediated by the production of glutathione in *A. thaliana.*
5) Detailed studies are needed to elaborate the results of this research.

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