

Original Research Article

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Potential of *Trichoderma* spp. on Growth Promotion and Mitigating Cadmium Uptake in Rice Plant under the Metal Stress Ecosystem

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ABSTRACT

This study was designed to investigate the efficacy of the biocontrol isolates on growth promotion and ability in lowering the metal uptake by rice plant (variety MTU 7029) with two *Trichoderma* isolates namely MT-4 and UBT-18 both having tolerance towards cadmium. Substantial variations had been found in different treatments. Significant increase in chlorophyll was observed in *Trichoderma* treated plants at 25ppm stress. Total protein estimation in *Trichoderma* treated and non-treated rice plants under cadmium stress showed that protein content decreased significantly with increasing metal concentration in *Trichoderma* non treated plants. *Trichoderma* isolates helped in construction of significantly more protein with cadmium gradient. Peroxidase activity showed increasing trend up to 25ppm followed by gradual decline. The enzyme activity in *Trichoderma* treated plants was always lower compared to non-treated plants. In *Trichoderma* non-amended treatment, cadmium concentration in plant increased with increased in level of cadmium contamination which was equivalent with decreasing biomass of the plants. The cadmium uptake by rice plants increased with increasing cadmium contamination ranging from 6.66 to 6.99 $\mu\text{g g}^{-1}$ plant biomass. The plant treated with *Trichoderma* on the other hand gained higher biomass which might be correlated with lower cadmium concentration in plants. It was observed that MT-4 treated plants contained lower cadmium coupled with higher biomass as compared to UBT-18.

Keywords

Rice,
Trichoderma,
Growth promotion,
Cadmium stress.

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Introduction

Metal containing pollutants are increasingly being released into the soil from industrial waste water as well as from wastes derived from chemical fertilizers and pesticides used in agriculture (Lopez and Vazquez, 2003; Ting and Choong, 2009). Major problem with metals is their tendency to persist indefinitely in the food chain (Gupta *et al.*, 2000; Aleem *et al.*, 2003). Their accumulation reduces soil fertility, soil microbial activity, plant growth

and quality of agricultural products. Cadmium is one of the most ubiquitous and potentially hazardous contaminants in the biosphere. Phosphatic fertilizers are widely regarded as being the most ubiquitous source of cadmium contamination of agricultural soils (Alloway, 1995). Cadmium is readily absorbed by plant roots within a few hours of its supply to roots media and from there is easily transported to other parts of the plants (Ghoshroy and

Nadakavukaren, 1990; Rauser and Meuwly, 1995). In most of the cadmium stressed ecosystem, the heavy metal easily penetrates the roots through cortical tissues and reaches the xylem through apoplastic and /or symplastic pathway (Salt *et al.*, 1995). Normally cadmium ions are retained in the roots and only small amount are transported to the shoot as also indicated by Cataldo *et al.*, (1983). The inhibitory effect of cadmium ions on root elongation is mediated through altered cell growth. Cadmium in cells gets associated with cell walls and middle lamella and increases the cross linking between the cell wall components resulting in inhibition of the cell growth (Poscherieder *et al.*, 1989). The reduction of biomass by cadmium toxicity was attributed to the direct consequence of inhibition of chlorophyll synthesis and photosynthesis (Padmaja *et al.*, 1990). Moreover, cadmium also alters the water relations in plants, causing physiological drought (Barcelo and Poscherieder, 1990) and metabolic dysfunctions such as reactive oxygen species (Asada, 1999). These and some other altered processes like pollen fertility, proline content, nitrate reductase activity lead to the decrease in the length and fresh and dry mass of the plants subject to cadmium stress (Parveen *et al.*, 2011).

Trichoderma species are imperfect filamentous fungi, with teleomorphs belonging to the hypocreales order of the ascomycete division. *Trichoderma* spp. have great role in ecology as they take part in decomposition of plant residues as well as biodegradation of man-made chemicals and bioaccumulation of high amount of different metals from waste water and soil (Ezzi and Lynch, 2005; Anand *et al.*, 2006). Evidence suggested that *Trichoderma* spp. exhibited considerable tolerance against metals and accumulate high amount of the metals from polluted habitats (Lopez and Vazquez, 2003;

Anand *et al.*, 2006). Therefore, metal tolerant *Trichoderma* species may become dominant organisms in some polluted and play an important role in environment friendly metal-removal technology (Ting and Choong, 2009). Also metal ions in soil may influence growth, sporulation and enzymatic activities of *Trichoderma* spp. (Jaworska and Dluzniewska, 2007) which can cause changes in the quantities of extra cellular enzymes produced and metabolites (Kredics *et al.*, 2001a, b) as well as overall biocontrol activities against plant pathogenic fungi and plant growth stimulating factors. However, the micro habitat behaviour of *Trichoderma* spp. upon exposure to each metal-containing compound may differ depending on type of metal and ability to detoxify it by *Trichoderma* isolates. In this background, the present study was undertaken to investigate the effect of *Trichoderma* on physical and biochemical attributes of rice plant under cadmium stress ecosystem and also to correlate the ability of the biocontrol agent for accumulation of the toxicant aimed towards reducing the chance of cadmium uptake by the plant.

Materials and Methods

Two cadmium tolerant *Trichoderma* isolates namely MT-4 and UBT-18 were taken to study their effect on growth promoting ability with concomitant decrease of cadmium uptake in rice plants under cadmium stress ecosystem. The rice (var. MTU 7029) seedlings were raised in perforated aluminium tray containing sterilized soil-farm yard manure mix (3:1). The recommended fertilizer dose (N: P: K 10:10:10) was applied before sowing. Irrigation was provided frequently. The seedling was ready for transplanting at 25 days of sowing. Soil and FYM mix containing different concentrations of cadmium (0, 5, 10, 25 and 50ppm) was prepared separately in earthen pots with the

metal standard solution made in sterilized double distilled water. Soil and FYM mix without any metal amendment was served as control. Fertilizer dose was applied in the form of balanced fertilizer @ 2g/kg of potting mix. The mixes were kept in pots for one month to stabilize the toxicity with intermittent application of the solution of respective concentration. Recommended fertilizer dose was applied before sowing and *Trichoderma* isolates were applied at the population level of 10⁷ cfu/g of soil. The *Trichoderma* non-amended metal containing soil served as control. The rice seedlings were transplanted and frequent irrigation was provided with tap water. Chlorophyll, protein and poly phenol oxidase content in leaf was estimated at 30 days after transplanting, whereas, cadmium concentration in plant was measured at 75 days of crop age.

Chlorophyll estimation

Portable Chlorophyll Meter SPAD-502 (Minolta Corporation, NJ, and USA) was used for spectral measurement of total chlorophyll in rice at six randomly chosen leaves per individual rice plant, taking two leaves from the top, middle and bottom, respectively, and the average value for each sample site was calculated.

Protein estimation

Fresh leaves weighing 0.2 g was crushed in a previously chilled mortar with pestle in 0.8 ml of sodium phosphate buffer (pH 7.1). The grind tissue was centrifuged at 4°C for 20 minutes at 10,000 rpm and the supernatant was used as crude protein. Total protein was estimated following Lowry's method (1951).

Peroxidase estimation

The peroxidase (PO) activity was determined by the method described by Sadasivam and Manickam (1996). The enzyme extract was

prepared by taking 0.2 g of fresh healthy tissue in 0.8 ml of 0.1M phosphate buffer (pH 7.1) by grinding with a pre cooled mortar and pestle at 0 degree c and the homogenate was centrifuged at 20,000 rpm at 4°C for 20 minutes. The supernatant was used as enzyme source.

In a cuvette 3ml buffer (0.05 M) Pyrogallol solution, 0.1ml enzyme extract and 0.5ml hydrogen peroxide were taken by using micropipette and were mixed well. Therefore the cuvette was placed in the Spectrophotometer and the absorbance was measured at 490 nm in a spectrophotometer against a reagent blank without enzyme extract at an interval of 30 seconds. Enzyme activity was expressed as change in absorbance min⁻¹ g⁻¹ tissue.

Analysis of cadmium in rice plant

To determine the cadmium concentration in rice at the time of flowering, the entire plants along with roots in each pot were carefully removed, washed free of adhering soil particles with water and air-dried. For dry biomass measurement the roots and shoots dried in a forced-air oven for 2 days at 50°C, followed by 3 days at 80°C and overnight at 105°C. Biomass of the roots and shoots was measured by using Sartorius LA8200S digital weight balance on the basis of wet weight and dry weight. Dried material was ground to homogenous powder using mill grinder. The sample was then digested by using triacid digestion method using a mixture of HNO₃:H₂SO₄:HClO₄ in the ratio of 9:4:1 following the method of Tandon (2005). 0.5 g ground plant material is placed in 100 ml clean beaker and to that 10 ml of triacid mixture was added and the content of the flask is mixed by swirling and keep it for overnight. The flask was placed on low heat hot plate at 60°C in a digestion chamber. Then, the flask is heated at higher temperature until the production of red NO₂ fumes ceases.

The content was further evaporated until the volume was reduced to about 2 to 3 ml but not to dryness. The completion of digestion is confirmed when the liquid becomes colourless.

After cooling the flask, it was transferred quantitatively to 50 ml capacity volumetric flask, diluted to 50 ml with distilled water and kept overnight. Next day it was filtered through Whatman no. 44 filter paper. The filtrate was stored properly and was analyzed for the estimation of cadmium using AA Flame Spectrophotometer (Model: AAnalyst 200, S/N 200S6040301 Autosampler Model). Each sample was analyzed two or three times at a wave length of 229 nm. Concentrations were expressed in terms of mg/kg.

Quantification of cadmium accumulation in *Trichoderma*

This study was carried out with one test isolate of *Trichoderma* sp. (UBT-18) to establish a correlation between the biomass production and cadmium accumulation. The test isolate was grown in metal amended PDA medium of different concentrations (0, 25, 50, 100 and 150 ppm) at $27 \pm 1^\circ\text{C}$ for 7 days. The biomass was harvested through cheese cloth and oven dried at 40°C . After complete drying the biomass was weighted. The cadmium accumulation by the *Trichoderma* isolate was quantified by taking 0.5g of dried biomass and following the triacid digestion method as described above.

Results and Discussion

The results directed towards the hypothetical inference of having significant role of *Trichoderma* in annulling the adverse effect of cadmium in rice plant. Attempt had been made to evaluate the growth promotion and to quantify the metal uptake in rice plants grown under cadmium stress at panicle emergence stage with two *Trichoderma* isolates namely MT-4 and UBT-18 both of having tolerance

towards cadmium. Substantial variations had been found in different treatments. The variation in chlorophyll content in MT-4 and UBT-18 treated plants were measured under elevated cadmium stress and the results obtained have been presented in figure 1. It was observed that cadmium stress had significant effect on reduction of chlorophyll content in leaf. The use of tolerant *Trichoderma* spp. helped in enhancing the chlorophyll irrespective of a particular cadmium concentration. The isolates varied in response to increase in the chlorophyll content under cadmium stressed condition. UBT-18 was found to induce significantly more chlorophyll at 50ppm cadmium stress. Significant decrease in protein content was observed with increasing concentration of cadmium irrespective of tolerant *Trichoderma* spp (Fig. 2). However, application of *Trichoderma* isolates aided in increasing amount of protein construction irrespective upon exposure to different level of metal stress. Among the isolates, UBT-18 was more potent in enhancing the protein level in plants at 50ppm cadmium stress (1.46 mg g^{-1} fresh wt) as compared to MT-4 (1.32 mg g^{-1} fresh wt).

Peroxidase activity in rice plants under the interaction of cadmium and *Trichoderma* isolates revealed that the enzyme activity in *Trichoderma* non-treated plants increased with increasing cadmium level up to 25ppm followed by modest decrease at 50ppm. In *Trichoderma* treated plants peroxidase activity was always comparatively low irrespective of isolates concerned. However, at initial level of cadmium contamination, peroxidase activity showed increasing trend in *Trichoderma* treated plants. With further increase in cadmium level significantly low peroxidase activity was recorded in *Trichoderma* treated plants except UBT-18 where gradual increase in peroxidase activity was found (Fig. 3). The result presented in table 1 revealed that in *Trichoderma* non-

amended treatment, cadmium concentration in plant increased with increase in level of cadmium which was equivalent with decreasing biomass of the plants. Simultaneously, the cadmium uptake by rice plants increased with increasing cadmium contamination ranging from 6.66 to 6.99 $\mu\text{g plant}^{-1}$. The plants treated with *Trichoderma* on the other hand gained higher biomass in comparison to the plant without *Trichoderma* which might be correlated with lower cadmium concentration in plants treated with *Trichoderma*. Comparing the ability of two isolates, it was observed that plants treated with MT-4 contained lower cadmium coupled with higher biomass than plants treated with UBT-18. The results depicted in figure 4 and 5 conferred that negative relation exists among plant cadmium concentration and its biomass.

In this present investigation, cadmium toxicity in rice plants were measured under dual interaction of *Trichoderma* and heavy metals and compared with *Trichoderma* non treated plants grown under only heavy metal stress. Cadmium damages the photosynthetic apparatus (Sidlecka and Baszynsky, 1993), lowers chlorophyll content (Larsson *et al.*, 1998), and inhibits the stomatal opening (Barcelo and Poschenrieder, 1990). With increase in metal contamination in *Trichoderma* non-treated treatment there was a significant reduction in chlorophyll content. Heavy metal stress in soils results in subtle changes of leaf chlorophyll concentration in rice, which are related to crop growth and crop yield (Liu *et al.*, 2010). High concentrations of heavy metals can degrade the activities of photosynthetic enzymes and block the photosynthetic electron transport chain, resulting in reduction of chlorophyll content (Thapar *et al.*, 2008). Decrease in chlorophyll content may be due to reduce synthesis of chlorophyll as a result of inhibition of enzyme activity such as δ -aminolevulinic acid dehydratase (Padmaja *et*

al., 1990) and protochlorophyllide reductase (Van Assche and Clijsters, 1990), replacement of Mg with heavy metals in chlorophyll structure, decrease in source of essential metals that involved in chlorophyll synthesis such as Fe^{2+} and Zn^{2+} (Kupper *et al.*, 1998), destruction of chloroplast membrane by lipid peroxidation due to increase in peroxidase activity and lack of antioxidants such caretenoids (Prasad and Strzalka, 1999), decrease in density, size and synthesis of chlorophyll and inhibition in the activity of some enzymes of Calvin cycle (Benavides *et al.*, 2005).

One possible mechanism by which excess heavy metals may damage plant tissues is the stimulation of free radical production, by imposing oxidative stress (Foyer *et al.*, 1997). Significant increase in chlorophyll was observed in *Trichoderma* treated plants in comparison to non-treated plants. MT-4 and UBT-18 were found to enhance chlorophyll production at 25ppm nickel and cadmium stress, respectively. Role of *Trichoderma* in inducing chlorophyll content in plant has been reported by several workers. *Trichoderma* treatments significantly increased the growth of maize plants as compared to the control. *Trichoderma*-treated plants were able to enhance nutrient uptake, resulting in increasing root and shoot growth, and improving plant vigour to grow more rapidly with enhanced plant greenness, which resulted in higher photosynthetic rates (Harman, 2006). Such increased carbohydrate production under stress condition facilitated to high biomass production of the plant. Plant genes respond to pathogens and elicitors. For this reason, plant defense mechanisms do not necessarily require stimulation by the living organism. The addition of *Trichoderma* metabolites may act as elicitors of plant resistance, or the expression in transgenic plants of genes whose products act as elicitors.

Table.1 Effect of *Trichoderma* isolates on cadmium uptake in rice plants under cadmium stressed condition

Isolate	Cadmium concentration (ppm)	Dry wt. of plants (g) <i>Trichoderma</i> (-)	Dry wt. of plants (g) <i>Trichoderma</i> (+)	Cadmium concentration in plants (µg/g) <i>Trichoderma</i> (-)	Cadmium concentration in plants (µg/g) <i>Trichoderma</i> (+)	Cadmium uptake (µg/plant) in <i>Trichoderma</i> (-)	Cadmium uptake (µg/plant) in <i>Trichoderma</i> (+)
UBT-18	5 ppm	5.82	6.31	1.14	0.90	6.63	5.68
	10 ppm	5.74	6.20	1.16	1.00	6.66	6.20
	25 ppm	5.52	5.95	1.24	0.90	6.81	5.36
	50 ppm	5.14	5.65	1.36	1.20	6.99	6.78
MT-4	5 ppm	5.82	6.68	1.14	0.50	6.63	3.34
	10 ppm	5.74	6.57	1.16	0.50	6.66	3.29
	25 ppm	5.52	6.37	1.24	0.70	6.81	4.46
	50 ppm	5.14	5.97	1.36	1.50	6.99	8.95

Fig.1 Total chlorophyll in rice leaves under influence of *Trichoderma* spp. and cadmium stress

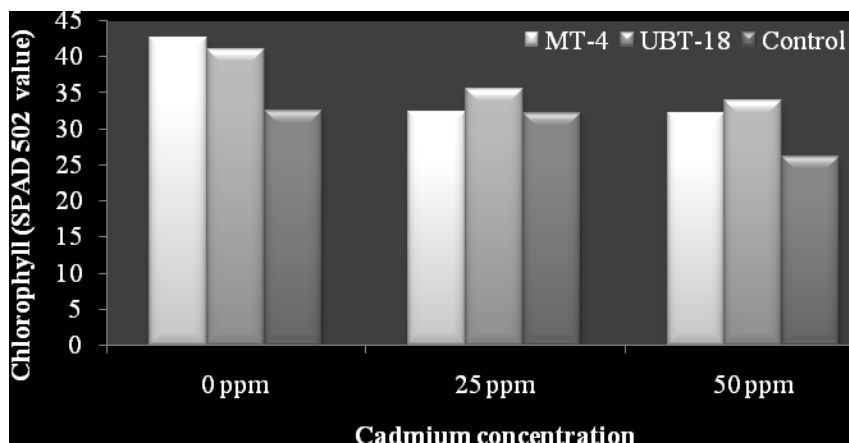


Fig.2 Variation in total protein under influence of *Trichoderma* spp. and cadmium stress

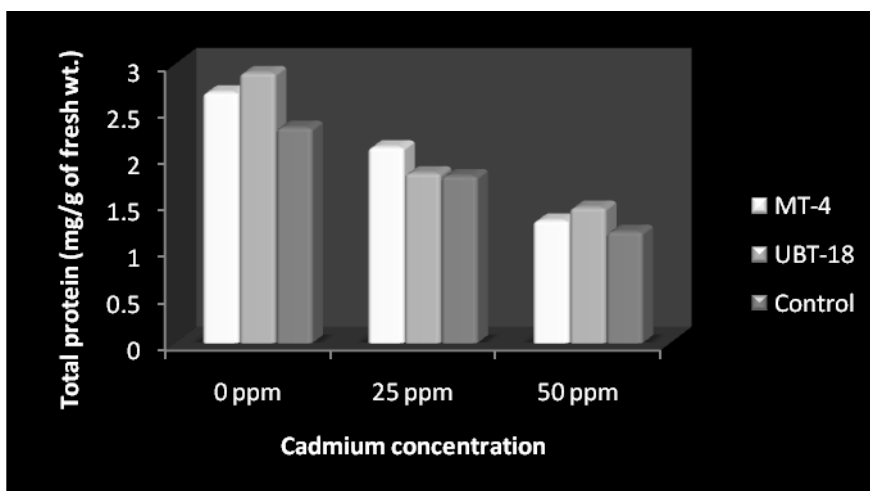


Fig.3 Peroxidase activity in rice leaves under influence of *Trichoderma* spp. and cadmium stress

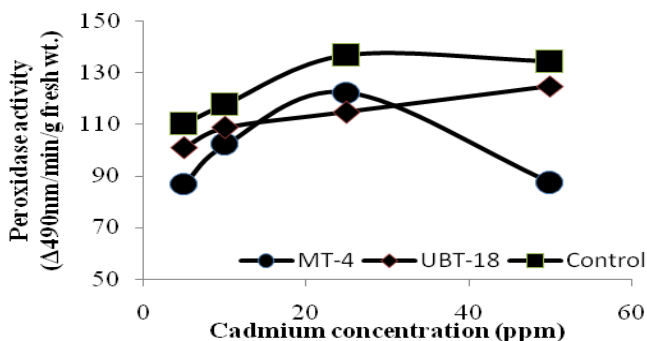


Fig. 4. Relation between plant biomass and cadmium uptake in presence of MT-4 under cadmium stress condition

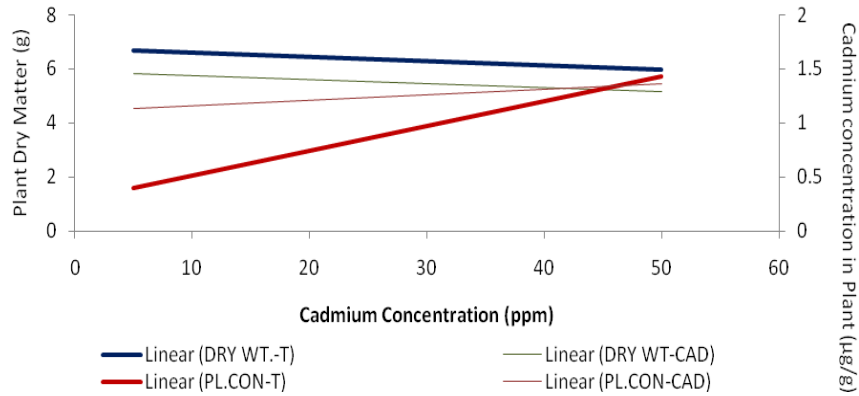
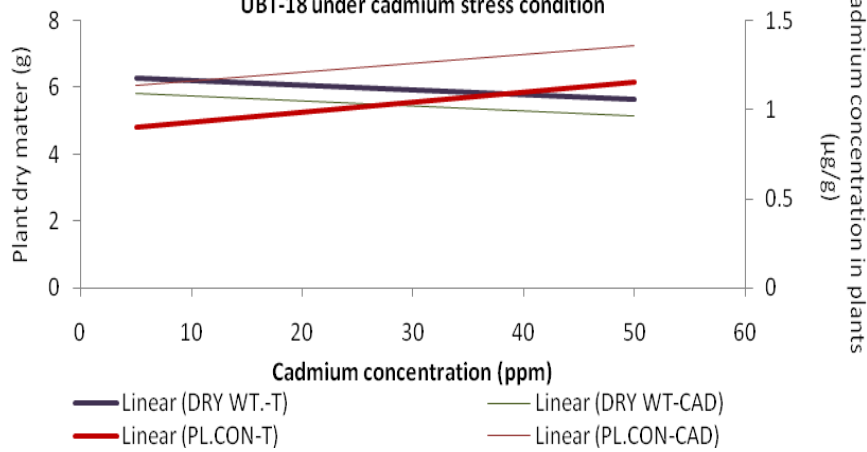
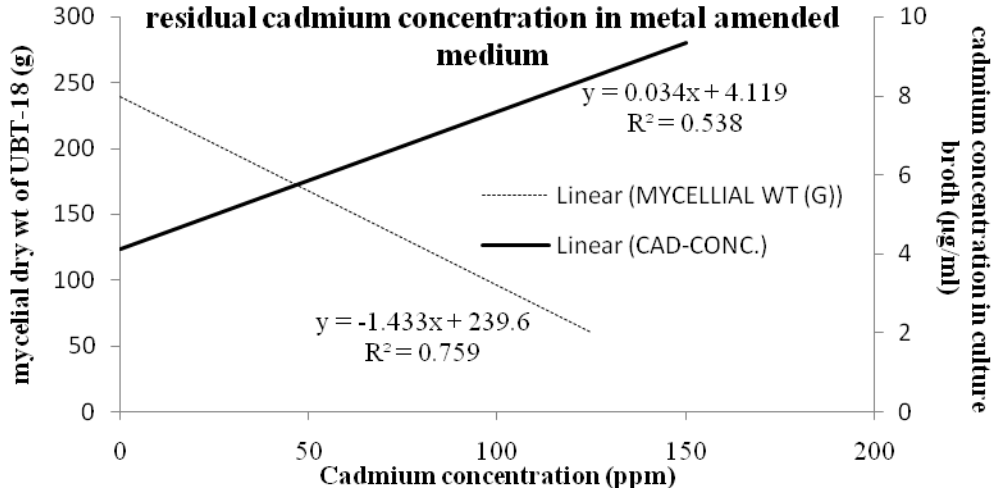


Fig. 5. Relation between plant biomass and cadmium uptake in presence of UBT-18 under cadmium stress condition



DRY WT.-T= dry weight of *Trichoderma* treated plants; DRY WT.-CAD= dry weight of *Trichoderma* non-treated plants only with different concentration of cadmium; PL.CON-T= cadmium concentration in *Trichoderma* treated plants; PL.CON-CAD= cadmium concentration of *Trichoderma* non-treated plants only with different concentration of cadmium.

Fig.6. Relation between UBT 18 biomass production and residual cadmium concentration in metal amended medium



It may also results in the synthesis of phytoalexins, PR proteins and other compounds, with consequence in an increase in resistance against several plant pathogens, including fungi and bacteria (Dana *et al.*, 2001), as well as resistance to hostile abiotic conditions (Harman *et al.*, 2004).

During the study, it was also observed that with increase in metal contamination in *Trichoderma* non-treated treatment there was a significant reduction in protein content in rice leaves. Lipids and proteins are important constituents of the cell that easily damage in environmental stress condition (Prasad, 1996). Hence any change in these compounds can be considered as an important indicator of oxidative stress in plants. Variable changes in soluble protein content in different metal treatments which reflected different level of antioxidant defence (Rastgoo and Alemzadeh, 2011). It is thought that decrease in total soluble protein content under heavy metal stress may be due to protease activity (Palma *et al.*, 2002), various structural and functional modifications by the denaturation and fragmentation of proteins (John *et al.*, 2009), DNA-protein cross links (Atesi *et al.*, 2004), interaction with thiol residues of proteins and replacement with heavy metals in metalloproteins (Pal *et al.*, 2006). In *Trichoderma* treated plants significant increase in protein content was noted since *Trichoderma* has great role in induction of protein content in plants as a general phenomenon of plant growth promotion. Proteins content of shoots and roots of maize plants treated with *T. harzianum* T22 were increased (Alkadious and Abbas, 2012) attributed to ability of *Trichoderma* spp. to increase uptake of nitrates and other ions (Harman, 2000). *Trichoderma* spp. increase biological nitrogen fixation in soil and nitrogen uptake by plant (Dordas and Sioulas, 2008). *T. harzianum* could produce nitrogen oxide (NO) which is that coding for enzyme

involved in L-arginine which is important precursor for protein biosynthesis (Gong *et al.*, 2007). *T. harzianum* inoculums in soybean grown gave higher percentage of crude protein (Egberongbe *et al.*, 2010). In addition, numerous proteins induced in response to *Trichoderma* were involved in stress and defense responses (Michal and Harman, 2008). The increase in total soluble protein content under heavy metal stress may be related to induction in the synthesis of stress proteins such as enzymes involved in Krebs cycle, glutathione and phytochelatin biosynthesis and some heat shock proteins (Verma and Dubey, 2003; Mishra *et al.*, 2006).

A common feature of environmental stress is their ability for production of toxic oxygen derivatives (Arora *et al.*, 2002). Reactive oxygen species (ROS) are continuously produced at low level during normal metabolic processes. But in biological systems, increasing the synthesis of ROS is one of the initial responses to different stress factors (Singh and Sinha, 2005). ROS induce damage to the biomolecules through peroxidation of membrane lipids, alteration of protein functions, DNA mutation, and damage to chlorophyll and disruption of some of the metabolic pathways (Semane *et al.*, 2010). Therefore, the tolerance of plants to stress conditions depends on their ability to make balance between the production of toxic oxygen derivatives and capacity of antioxidative defense systems. which include antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) and low-molecular weight quenchers (cycteine, ascorbic acid, thiols, proline (Singh and Sinha, 2005), atocopherol, glutation, carotenoids, phenolic and nitrogen compounds (Michalak, 2006). In the present

investigation, peroxidase activity was also found to be increased in *Trichoderma* non treated plants with increasing concentration of cadmium up to 25ppm followed by gradual decline. Peroxidase activity in *Trichoderma* treated plants was always lower compared to non-treated plants and MT-4. There are considerable evidences that activation of peoxidase activity plays a major function in biological control of pathogens and plant health management by *Trichoderma* spp. (Cherif *et al.*, 2007). Diversity in peroxidase activity under heavy metal stress depends on number of biotic and abiotic factors (Tamas *et al.*, 2008). Rastgoo and Alemzadeh (2011) observed that peroxidase activity increased with low level of metals like cobalt, lead, silver at 50µm concentration but enzyme activity decreased under severe stress due to blocking of essential functional groups, replacement of essential metals with heavy metals, changes in structure or the integrity of proteins and the interruption of signal transduction pathways of antioxidant enzymes because of poisonous active oxygen species derivatives (Schutzendube and Polle, 2002). The enhanced redox state of *Trichoderma* colonized plants could be explained by their higher activity of ascorbate and glutathione-recycling enzymes, higher activity of superoxide dismutase, catalase, and ascorbate peroxidase, in both root and shoot throughout crop growth. Similar enzymes were induced in uncolonized plants in response to stress but to a lower extent when compared with colonized plants. This orchestrated enhancement in activity of reactive oxygen species (ROS)-scavenging pathways in colonized plants in response to stress supports the hypothesis that enhanced resistance of colonized plants to stress is at least partly due to higher capacity to scavenge ROS and recycle oxidized ascorbate and glutathione, a mechanism that is expected to enhance tolerance to abiotic and biotic stresses (Mastouri *et al.*, 2010).

It was observed that the plant biomass was decreased with increasing concentration of heavy metal. These results are in accord with those observed in other agricultural crops inoculated with specific strains of *Trichoderma* spp. by Harman *et al.*, (2004). According to Hoyos-Carvajal *et al.*, (2009b) the increment in biomass related to production of plant growth hormones or analogues is another mechanism by which strains of *Trichoderma* spp. can enhance plant growth. Various species of fungi have been reported to produce auxins, which are key hormones effecting plant growth and development that can be produced by fungi in symbiotic interactions with plants (Gravel *et al.*, 2007). Cadmium as non-essential element was detected in *Trichoderma* treated and non-treated rice plants. Correlation between cadmium contamination in soil, plant biomass and cadmium uptake in rice plants suggested that plant biomass is negatively correlated with metal contamination with simultaneous increase in cadmium uptake by rice plants. *Trichoderma* aided in induction of defence response in plants which helped in production of more biomass and thereby reducing the cadmium uptake in plants. Negative correlation between higher biomass production by *Trichoderma* isolate (UBT-18) and lower residual cadmium concentration in metal amended growth medium (Fig. 6) supported the above findings in the way that cadmium was removed by the *Trichoderma* isolate and henceforth it remained available in lower quantity for uptake by the rice plant.

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