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Effect of *Glomus mossae* on accumulation efficiency, hazard index and antioxidant defense mechanisms in tomato under metal(loid) Stress

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ABSTRACT

In the present study, the phytoremedation potential along with growth, physiological and biochemical response of tomato (*Solanum lycopersicum*) was assessed under heavy metal(loid) (HM) and arbuscular mycorrhizal fungus (AMF) amendment. Effect of AMF on uptake and accumulation of metal(loid)s was assessed and accumulation characteristics were expressed in terms of bioabsorption coefficient (BAC), bioconcentration factor (BCF), translocation factor (TLF) and transfer factor (TF). Results showed that AMF-inoculated plants showed not only a better growth, chlorophyll content, strengthened non-enzymatic and enzymatic defense mechanism, but also accumulated higher concentration of metal(loid)s. The correlation between biochemical and physiological parameters was significant at 0.01 level. A significant difference ($p \leq 0.001$) in antioxidant enzyme activity was found on increasing metal(loid) dose and application of AMF. The accumulation of Cd and Pb in edible part exceeded the chronic reference dose stated by USEPA. The target hazard quotient (THQ) was >1 for Cd and Pb, whereas <1 for As. The study shows that tomato has good potential as Cd and Pb phytoremediator, hence must not be consumed when grown on Cd or Pb contaminated sites.

KEYWORDS

mycorrhiza; target hazard quotient; phytoremediation

1. Introduction

Heavy metal and metalloid (HM) contamination of soil is a threat to the ecosystem and has detrimental effects on human health since they enter the food chain through agricultural products. HM find their route into soil through a range of natural and anthropogenic activities. While natural calamities like volcanic eruptions, forest fires and weathering of rocks are out of human control, anthropogenic sources include mining processes, extensive industrialization and agricultural activities along with reckless sludge dumping. Even if the contamination level of HM in soil is low, they are known to be readily absorbed by roots of vegetable plants and accumulated in edible parts at high level (Yang *et al.* 2009). Consumption of such vegetables leads to Bioaccumulation and can pose a threat to consumers (Shi and Cai 2009). The threat resulting from cultivation of food crops on such HM polluted sites and health risks associated with consumption of such vegetables can be quantified using Target Hazard Quotient (THQ) (USEPA 2000). Whereas the same potential of such plants can be used for an ecologically pleasing and economically feasible technology called Phytoremediation. Phytoremediation, also called green remediation, botano-remediation, agroremediation, or vegetative remediation uses vegetation and associated microbiota, soil amendments and agronomic techniques to remove, contain, or render the heavy metal(loid)s harmless in the soil by stabilizing them (Vysloužilová *et al.* 2003; Helmisaari *et al.* 2007) and hence quite appealing.

Fungi of the phylum Glomeromycota can associate symbiotically with more than two-thirds of known plant species, including important crops to form arbuscular mycorrhizal symbiosis (Wang and Qiu 2006). This mutualistic association is probably the most ecologically and agriculturally important symbiosis in terrestrial ecosystems. They form an interface between the soil and the plant root and play an important role in soil fertility, plant nutrition, and terrestrial plant community composition (Bedini *et al.* 2010). The extraradical mycelium of the symbiont acts as an extension of the root system and increases the uptake of key nutrients, particularly phosphorus (Bucher 2007). Not only they are known to play an important role in phosphorous cycle, colonization by arbuscular mycorrhizal fungus (AMF) can also increase photosynthesis up to 20%, contributing to the global carbon cycle.

Hypha binding in the soil is an important sink for HM due to their large surface area. AMF plays a major role in the sequestration of HM which is supposed to be partly mediated by a glycoprotein (glomalin) released by them in soil (Gonzalez-Chavez *et al.* 2002, 2004; Cornejo *et al.* 2008). Although colonization by AMF is restricted to the root system, its effects are often detectable, even macroscopically, in the aboveground portion of plants (Smith and

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Read 2008). Furthermore, leaves are responsible for carbon uptake and transpiration, and they can be the site of accumulation of some HM (Zhao *et al.* 2000; Todeschini *et al.* 2011). Therefore, in order to better understand the mechanisms of tolerance, detoxification and stress response, the study of leaves of plants grown under HM stress is extremely relevant, both for basic knowledge and for application in phytoremediation approaches, especially for phytoextraction (Anjum *et al.* 2012).

Previous studies have shown that excessive accumulation of HM in plant tissues can decrease plant height, biomass and chlorophyll biosynthesis (Singh *et al.* 2010). Inside the cell, HM affects physiological reactions such as photosynthesis, respiration, etc. (Pourrut *et al.* 2011). A rather pronounced effect of HM toxicity in plants is increased production of reactive oxygen species (ROS). ROS production results from the interaction of HM with electron transport activities, particularly in the chloroplast and mitochondrial membranes (Shahid *et al.* 2014). Metal(loid)s such as Pb, Cd and As have the ability to induce the formation of ROS (Körpe and Aras 2011).

To overcome HM toxicity, plant cells are equipped with enzymatic mechanisms to eliminate or reduce their damaging effects. The anti-oxidant enzymes system, mainly including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11), has the ability to scavenge ROS and, thereby, prevent oxidative damage. AMF inoculation can improve plant performance under HM stress due to a number of mechanisms involving antioxidant enzymes, lipid peroxidation and soluble amino acid profile changes caused by the intimate relationship between AMF and host plant (Punamiya *et al.* 2010; Achakzai *et al.* 2012).

It is well known that AMF inoculation is beneficial for plants as AMF aids in plant establishment at HM contaminated or nutrient-poor sites, but the effect of AMF on HM uptake is still not clear. Based on the partition/promotion of HM uptake it can be of great utility to find out whether AMF-plant symbiosis needs to be applied on a contaminated crop site for sustainable agriculture or for remediation purpose of the same.

The aim of the present investigation is to study the effect of AMF fungus (1) in improving the overall performance of tomato plants grown under HM stress, (2) in alleviating HM stress and (3) on the accumulation of HM in edible parts and dietary toxicity that results due to HM accumulation.

2. Materials and methods

2.1. Experimental design and soil preparation

The study was conducted in Plant repository of Department of Botany, St. John's College, Agra, India using pot assays. Agra is situated at 164 MSL in North Central India (27°1939' N latitude and 78°0025' E longitude), around 200 km away from national capital New Delhi. The minimum temperature during the course of experiment ranged between 3.2–22.3° C and the maximum temperature ranged between 16.1–34.6° C. The humidity and light intensity ranged between 42–58% and 3.12–6.32 kWh/m^2 /day respectively. Soil used in this study was collected from the College botanical garden (0–20 cm depth), sieved with <5 mm mesh and thoroughly mixed to produce a homogenous soil composite. The soil was sandy-loam with an average pH value and electrical conductivity (EC) of 7.14 ± 0.04 and 0.51 ± 0.08 dS/m, respectively. Organic matter content of the soil (1.82%) was moderate while the available phosphate and available nitrogen contents of the soil were 21.5 and 118.9 kg/ha, respectively. Soil was kept in plastic bags, autoclaved thrice at 121° C, 15 lbs pressure for 30 mins duration of each cycle. Thereafter, it was air-dried before being artificially spiked with HM salts of $CdCl_2$, $PbNO_3$ and As_3S_2 as source of Cd, Pb and As respectively. Each 5 kg capacity pot had a few holes at the bottom for aeration, and the base was layered with pebbles before being filled with homogenous soil and respective HM salt mixture. The treatments were as follows: control, control + AMF, Cd (25 mg and 50 mg Cd kg^{-1} soil), Cd + AMF (25 mg Cd kg^{-1} soil + AMF), Pb (50 mg and 100 mg Pb kg^{-1} soil), Pb + AMF (50 mg Pb kg^{-1} soil + AMF), As (50 mg and 100 mg As kg^{-1} soil), As + AMF (50 mg As kg^{-1} soil + AMF), Multi metal(loid) combination (Mm: 25 mg Cd + 50 mg Pb+ 50 mg As kg^{-1} soil) and Mm + AMF. The experiment was carried out in a random block design. The pots were covered and kept for 15 days for stabilization. Simultaneously, certified seeds of *Solanum lycopersicum* L. (tomato, NBH-2424 var) were sown in trays containing control soil for about 15 days for germination. The plantlets were then transplanted into plastic pots containing metal (loid) spiked soil. Each pot was evenly watered with 500 ml tap water (pH-7.1, TDS- 480 mg/L) daily. A plastic tray was placed beneath each pot and any leachate collected was transferred back to the pot for possible loss of HM. Three replicates were maintained for each treatment. Inoculation of AMF was done at the rate of 50 g/pot (80 spores) while planting as per the treatment and spores of *Glomus mossae* were used as the inoculum. No fertilizers were added in the soil in order to investigate the effect of AMF solely on the growth of plants.

Parameters such as plant height, biomass, chlorophyll and proline content were recorded 30, 60 and 90 days after treatment (DAT). Antioxidative enzyme activities and metal(loid) content were analyzed 90 DAT. For the biochemical analysis, fresh leaves from replicates were plucked and stored in liquid nitrogen and for HM analysis whole plants were harvested, air dried and stored in zip locks.

2.2. HM content analysis and accumulation characteristics

Soil and HM treated plant tissues (root, shoot and fruit) were collected 90 DAT and dried in hot air oven at 65 °C for 24 hours to remove moisture content. The oven dried tissues were ground into fine powder and sieved through a fine mesh. Soil and dried plant samples (0.5 g) were digested at 80 °C for determination of metal(loid)s content, with 10 mL HNO_3 till the solution became transparent. The resulting solution was passed through Whatman No. 1 filter paper and the filtrate was analyzed for each metal(loid) by flame atomic absorption spectrophotometer (AAnalyst100, Perkin Elmer, USA) using an air-acetylene flame. Certified references (Virginia tobacco

leaves CTA-VTL-2, Polish Certified Reference Material and NIST 2709–San Joaquin Soil) were also used to check the accuracy of the results. The recovery rates for the elements analyzed are 93% for Cd, 71% for Pb and 92% for As.

The ability of tomato plants for HM absorption in soil was determined using bioabsorption coefficient (BAC) which is defined as metal content in shoot/metal content in soil and bioconcentration factor (BCF) which is defined as metal content in root/metal content in soil. The subsequent translocation of HM upwards was determined using translocation factor (TLF), which was used to track the extent of mobility and sequestration once the metal(loid) enters the plant system. Translocation factor was calculated as per Marchiol *et al.* (2004) which is defined as metal content in shoot/metal content in root. Plants exhibiting BAC and TLF values greater than 1 are considered to be promising phytoextractors (Fitz and Wenzel 2002), whereas plants possessing high BCF and low TLF values are considered to be potential phytostabilizers (Mendez and Maier 2008).

The plant and vegetable transfer factor (TF) was calculated as follows:

$$\text{Transfer Factor } [\text{TF}] = \frac{\text{HM concentration in plant/vegetable}}{\text{HM concentration in soil}}$$

TF_{plant} and TF_{veg} were used to analyze and assess the extent of contaminant accumulation in vegetative parts and subsequent storage in edible parts to give an idea that what fraction of metal(loid) present in soil is accumulated in the plant tissues.

2.3. Risk assessment by target hazard quotient (THQ) method

The potential health risks of HM consumption through vegetables were estimated based on the target hazard quotient method, which was stated by the United States Environmental Protection Agency (Storelli 2008; Wang *et al.* 2005). The THQ is given by the following equation:

$$THQ = \frac{EF * ED * FIR * C}{RFD * WAB * TA} * 10^{-3}$$

Where, EF is the exposure frequency 350 days/year (USEPA 2011); ED is the exposure duration (68.35 years, equivalent to the average lifetime of the Indian population as per World Bank Statistics 2015); FIR is the food ingestion rate (vegetable consumption values for adults and children are 350 and 220 g person^{-1} day^{-1}, respectively) (Song *et al.* 2015); C is the metal concentration in the edible parts of vegetables (mg kg^{-1}); RFD is the oral reference dose (Pb, Cd and As values were 0.0035, 0.001 and 0.050 mg kg^{-1} day^{-1}, respectively) (USEPA 2003); WAB is the average body weight (60 kg for adults and 25 kg for children); and TA is the average exposure time for non-carcinogens (ED = 365 days year^{-1}). If the THQ value is greater than 1, the exposure is likely to cause obvious adverse effects.

2.4. Physiological and biochemical analysis

2.4.1. Stress tolerance index

The Stress Tolerance Indices (TI_S) were determined for plant height and vegetative growth (biomass); referred to as TI_{PH} (Plant Height Tolerance Index) and TI_{VG} (Vegetative Growth Tolerance Index) respectively, using the equations (Wilkins 1978, Rawson *et al.* 1988):

$$TI_{PH} = \frac{Height\ of\ \ Treated\ plant}{Height\ of\ \ Control\ Plant} \times 100$$

$$TI_{VG} = \frac{Biomass\ of\ \ Treated\ plant}{Biomass\ of\ \ Control\ Plant} \times 100$$

TI_S helped to assess and compare the ability of AM inoculated and non-inoculated Tomato plants to grow in the presence of given concentrations of HM.

2.4.2. Pigment content

Leaf samples were ground with 10 mL of 80% (v/v) ice-cold acetone and the extract was collected by centrifugation at 4000 rpm for 5 min at 4°C. Chlorophyll a (chl a) and b (chl b) contents were determined using a UV-visible Spectrophotometer at wavelengths of 663 and 645 nm according to Lichtenthaler (1987) and expressed as mg g^{-1} FW.

2.4.3. Proline content

Free proline content was determined according to the method of Bates *et al.* (1973). Fresh leaf material (500 mg) was homogenized in 10 mL of 3% sulphosalicylic acid followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant (2 ml) was reacted with an equal volume of acetic acid and acid ninhydrin reagent, incubated in a water bath at 65 °C for 1 hour and cooled on an ice bath to terminate the reaction. Proline was extracted by adding 4 ml of toluene and vortexing it for 10–15 sec. The chromatophore containing toluene layer was separated and its absorbance was measured at 520 nm by a UV-Vis spectrophotometer using pure toluene as a blank.

The concentration of proline was estimated using a standard curve obtained after plotting the absorbance of pre-prepared L-proline concentration stocks and was expressed in μmol g^{-1}.

2.5. Enzyme activities

Preparation of Enzyme Extract – Fresh plant material (1 g) was homogenized in 10 mL of ice-cold extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA for determination of SOD and CAT; and 0.5 mM EDTA and 1 mM ascorbic acid for determination of APX) in chilled mortar and pestle. The extract was filtered and centrifuged, and the supernatant was used as a crude enzyme to determine the antioxidant enzyme activities.

2.5.1. CAT assay

The activity of CAT was assayed spectrophotometrically by measuring the decomposition of H_2O_2 in 1 minute at 240 nm as per Aebi's procedure (1984). The reaction mixture (3 ml) contained Potassium phosphate buffer (50 mM, pH 7.0), H_2O_2 (12.5 mM), enzyme extract (50 μL) and water to make up the

volume. The reaction was started by adding H_2O_2 and decrease in absorbance at 240 nm was recorded for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed.

The enzyme activity was expressed as Units (μmol of H_2O_2 destroyed per minute) per gram of fresh weight (Extinction Coefficient = 36.5 $mM^{-1}cm^{-1}$).

2.5.2. SOD assay

The activity of SOD was estimated by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to Dhindsa *et al.* (1981) method. The reaction mixture (3 mL) contained phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), methionine (13.33 mM), NBT (75 μM), Riboflavin (2 μM), sodium carbonate (50 mM) and enzyme extract (100 μL). The reaction mixture was kept 30 cm below the light source for 15 min. All reactants without enzyme extract which served as light blank were kept along with samples. The reaction mixture with 100 μL of enzyme extract incubated in the dark served as dark blank. The NBT reduction was estimated by monitoring the change in absorbance at 560 nm. The amount of enzyme extract that produced 50% inhibition of NBT reduction was considered as 1 U SOD. The enzyme activity was expressed as U SOD activity g−1 fresh weight.

2.5.3. APX assay

APX activity was measured as per Nakano and Asada (1981) method. The reaction mixture (3 mL) contained potassium phosphate buffer (50 mM, pH 7.0), ascorbic acid (0.5 mM), EDTA (0.1 mM), H_2O_2 (0.1 mM), enzyme extract (100 μL) and water (700 μL).

Ascorbate oxidation was monitored by the decrease in absorbance at 290 nm for 30 s in an UV-visible spectrophotometer. One enzyme unit determines the amount of enzyme necessary to decompose 1 μmol ascorbate per min and expressed as Units per gram fresh weight.

2.6. Statistical analysis

Correlation among plant metal(loid) uptake, physiochemical response and enzymatic activities was calculated by Pearson Product moment method using the software Sigma Plot 11 at a significance level of $p < 0.05$ and $p < 0.01$. One-way ANOVA with Student Newman Keuls test was carried out for comparisons between treatment and control group to analyze significant differences in treatments ($p \leq 0.001$).

3. Results and discussion

3.1. Metal(loid) uptake and accumulation characteristics

The metal(loid) content in plant tissues varied greatly due to different treatments given (Table 1). Uptake was found in the order Cd>Pb>As. Further, the highest accumulation of metal (loid) was seen in shoots followed by roots and fruit. The uptake and accumulation of metal(loid)s in plants increased on increasing the metal(loid) dose ($p \leq 0.001$) establishing a direct relationship between metal availability and accumulation.

The general trend of metal accumulation in plants is root> shoot, especially for Pb which is relatively immobile (Porrut *et al.* 2011). But all plants do not follow the same pattern. Further, the uptake and accumulation is affected by rhizospheric microbes and chelators. Phytoextractors are those plants that have the capability of translocating HM to harvestable parts. Tomato plants grown in dumpsites have shown metal accumulation in order leaf>stem>root>fruit (Adefemi and Awokunmi 2013). Crops from the family Solanaceae have been rendered unsafe for consumption when grown on polluted soil due to their phytoextraction ability. Significant amount of Pb has been shown to be accumulated in tomato, pepper and eggplants (Shilev and Babrikov 2005). In Pb contaminated soils planted with *G. max* L., plants mopped up substantial concentrations of Pb in the aboveground biomass compared to concentrations in the roots (TF>1) (Aransiola *et al.* 2013).

In most of the plant species, majority of the absorbed Pb is accumulated in the roots and only a small fraction is translocated to shoots. After entering the root, Pb mainly moves by apoplast and follows water stream until it reaches endodermis. Thereafter, it must follow symplastic transport as Pb is blocked in the endodermis by casparian strip. High concentrations of lead are known to destroy the casparian strip barrier (Porrut *et al.* 2011). Hyperaccumulator plants such as *Brassica pekinensis* and *Pelargonium* are capable of translocating higher concentration of Pb to shoots (Xiong *et al.* 2006, Arshad *et al.* 2008). These plants exude compounds from their roots that can solubilise metals in soil (Arshad *et al.* 2008) resulting in increased uptake and translocation by the involvement of metal cation transporters. Translocation of HM from root to shoots requires movement through the xylem (Verbruggen *et al.* 2009) driven by transpiration (Liao *et al.* 2006), whereby Pb can form complexes with amino or organic acids (Maestri *et al.* 2010) or may be translocated in inorganic form. After gaining entry into the vascular bundles, Pb can be transported via the apoplastic pathway and transported to leaves (Krzesłowska *et al.* 2010). Tomato plants in this study translocated a high amount of Cd and Pb in their shoots suggesting their applicability for phytoextraction.

AMF inoculation significantly increased the HM uptake by the plants in all treatments, but this difference was statistically not significant in edible part for Pb and Cd when given individually. Whereas, in case of As when given individually, significant increase was seen even at the fruit level. This suggests relative low mobility of Pb and Cd in comparison to As. Also it is possible that multi-metal when present together may form complexes and affect each other's mobility. Though AMF lead to a retarded translocation of metal(loid)s from root to shoot but the transfer factor from soil to plant and fruit was still higher than non-inoculated plants. The increase in metal accumulation in parts of plant under study, but decrease in translocation from root to shoot suggests the metal chelation ability of Glomalin in AMF colonized roots. Several mechanisms have been proposed to explain metal tolerance capability of AMF and its contribution in annulling the impacts of HMs stress on plants. Mechanisms at physiological level state the dilution effect of AMF on plants growing in HM stress i.e., by increasing the plants' biomass the strength and toxic effects of accumulated HM gets reduced. At molecular level it can be stated that

Table 1. Metal(loid) uptake and accumulation characteristics in *S. lycopersicum*.

Treatment	Root (mg kg^{-1})	Shoot (mg kg^{-1})	Fruit (mg kg^{-1})	BCF	BAC	TLF	TF_{plant}	TF_{veg}	Soil after harvest
Control	ND	ND	ND	—	—	—	—	—	ND
Cd $_{25}$	17.8 ± 0.76 ^{a}	39.93 ± 0.45 ^{a}	11.57 ± 0.51 ^{a}	0.71	2.06	2.89	2.77	0.46	22.28 ± 0.51a
Cd $_{50}$	22.79 ± 0.53 ^{b}	45 ± 0.51 ^{b}	11.68 ± 0.63 ^{a}	0.46	1.13	2.49	1.59	0.23	46.31 ± 0.78b
Cd (Cd_{25}+Pb_{50}+As_{50})	16.06 ± 0.45 ^{c}	35.79 ± 0.39 ^{c}	8.6 ± 0.49 ^{b}	0.64	1.78	2.76	2.42	0.34	23.39 ± 0.42c
Cd $_{25}$ +AMF	19.23 ± 0.81 ^{a}	42.35 ± 0.19 ^{d}	11.72 ± 0.34 ^{a}	0.77	2.16	2.81	2.93	0.47	21.85 ± 0.53a
Cd (Cd_{25}+Pb_{50}+As_{50} +AMF)	18.75 ± 0.63 ^{a}	40.22 ± 0.23 ^{a}	9.8 ± 0.31 ^{c}	0.38	2.00	2.67	2.75	0.39	22.91 ± 0.21ac
Pb $_{50}$	7.79 ± 0.34 ^{e}	18.14 ± 0.27 ^{e}	4.92 ± 0.23 ^{e}	0.16	0.46	2.96	0.62	0.10	46.81 ± 0.45e
Pb $_{100}$	9.59 ± 0.47 ^{f}	19.33 ± 0.36 ^{f}	5.63 ± 0.45 ^{e}	0.10	0.25	2.60	0.35	0.06	97.23 ± 0.32f
Pb (Cd_{25}+Pb_{50}+As_{50})	4.19 ± 0.24 ^{g}	17.52 ± 0.31 ^{g}	4.92 ± 0.39 ^{e}	0.08	0.45	5.36	0.53	0.10	48.21 ± 0.54g
Pb $_{50}$ +AMF	9.72 ± 0.39 ^{f}	19.43 ± 0.22 ^{f}	5.46 ± 0.19 ^{e}	0.19	0.50	2.56	0.69	0.11	45.32 ± 0.19h
Pb (Cd_{25}+Pb_{50}+As_{50}+AMF)	7.4 ± 0.42 ^{e}	19.21 ± 0.17 ^{f}	5.63 ± 0.29 ^{e}	0.15	0.50	3.36	0.64	0.11	47.19 ± 0.61eg
As $_{50}$	0.86 ± 0.02 ^{h}	0.80 ± 0.13 ^{h}	0.37 ± 0.08 ^{f}	0.02	0.02	1.36	0.04	0.01	48.9 ± 0.24h
As $_{100}$	0.87 ± 0.11 ^{h}	2.06 ± 0.19 ^{i}	0.78 ± 0.17 ^{g}	0.01	0.03	3.27	0.04	0.01	98.6 ± 0.25i
As (Cd_{25}+Pb_{50}+As_{50})	0.23 ± 0.04 ^{i}	0.56 ± 0.15 ^{h}	0.21 ± 0.09 ^{f}	0.01	0.02	3.35	0.02	0.01	49.5 ± 0.12j
As $_{50}$ + AMF	1.32 ± 0.09 ^{j}	2.34 ± 0.14 ^{j}	1.22 ± 0.21 ^{h}	0.03	0.07	2.69	0.09	0.02	48.4 ± 0.32h
As (Cd_{25}+Pb_{50}+As_{50}+AMF)	0.56 ± 0.07 ^{k}	0.74 ± 0.07 ^{h}	0.33 ± 0.13 ^{f}	0.01	0.02	1.91	0.03	0.01	49.1 ± 0.19k

Each value is a mean ± SD of triplicates; values followed by the same superscript letter (a-k) in each column are not significantly different from each other (Student Newman Keuls test, $p \leq 0.001$). ND: Not detected.

AMF may assist by encoding proteins involved in HM stress tolerance since mycorrhizal plants often show increased activity of enzymatic and non enzymatic antioxidants (Upadhyaya *et al.* 2010).

The BAC, BCF, and TF values (Table 1) help to identify the suitability of plants for phytoextraction and phytostabilization by explaining the accumulation characteristics and translocation behaviors of metals in plants. Since plants exhibiting BAC and TLF values >1 are considered promising phytoextractors, tomato plant can be stated as an efficient phytoextractor for Cd, whose efficiency can further be improved by synchronizing with AMF.

AMF inoculation led to an increase in TF_{plant} and $TF_{vegetable}$. This rules out the possibility that AMF can be applied to crop fields for sustainable agriculture. In contrast, this combination can be applied for on-site remediation of Cd and Pb contaminated sites.

3.2. Risk assessment

Based on the metal(loid) content in edible part of the plant and its consumption, THQ was calculated (Table 2). The THQ > 1 for Cd and Pb treatments, suggesting the high frequency of risks associated with cultivation of tomatoes on contaminated sites for consumption. Over time, Pb can get accumulated in blood and affect cardiovascular, renal and neurological system in consumers, whereas Cd can cause chronic toxicity to lungs, kidneys, bones and liver and impair immunity. THQ < 1 for As which suggests minimal risk associated with cultivation of tomatoes on As contaminated sites with range upto 100 mg kg^{-1}. The soil concentration of Pb and Cd should be monitored as both of them are common contaminants that enter soil through various industrial and agricultural effluents. Such soil should never be used for agricultural cultivation of tomatoes due to the resulting toxicity and risk of bioaccumulation. However, this ability of tomato plants can be harnessed to remediate soil and can be further enhanced by inoculation of AMF.

Table 2. Target Hazard Quotient.

Metal(loid)	Range mg kg^{-1}	THQ
Cd	8.6–11.72	7.21–9.83 (adults); 10.88–14.83 (children)
Pb	4.92–5.63	1.17–1.34 (adults); 1.77–2.03 (children)
As	0.21–1.22	0.05–0.34 (adults); 0.08–0.51 (children)

THQ>1 (likely to cause serious effects); THQ<1 (no risks associated)

3.3. Physiological and biochemical analysis

3.3.a. Plant height and biomass

The growth retarding effect of HM and the growth promoting behavior of AMF is well known. Higher the stress tolerance index, higher is the ability of plants to survive stress. Out of the 3 metal(loid) treatments given, exposure to Mm resulted in highest plant height reduction followed by As at 100 mg $kg^{-1,}$ whereas As at 50 mg kg^{-1} concentration proved to be the least toxic (Table 3). An interesting paradox related to As toxicity is its growth stimulating behavior at low concentration (Garg and Singla 2011). The sudden shift in toxicity on increasing the dose of As from 50 to 100 mg kg^{-1} can be attributed to this. AMF colonization resulted to a significant increase in plant height in both control and HM treated plants ($p \leq 0.001$). In control, the increase in plant height was up to 3% whereas in HM treated plants up to 10.7% for Cd treated, 8.6% for Pb treated, 5.5% for As treated and 9.1% for Mm treated plants respectively when compared to their non-AMF inoculated counterparts.

Similarly, a reduction in biomass was seen for HM treated plants (Table 3). The biomass decreased up to 23.44% for Cd, 22.14% for Pb, 24.74% for As and 25.26% for Mm combination respectively. The greatest shock was observed 30 DAT and thereafter the effect waned off. Mm stress proved to be most toxic, whereas As at 50 mg kg^{-1} concentration was least toxic in terms of biomass. AMF inoculation resulted in a significantly greater biomass of plants. Control plants showed 3.6% increase whereas Cd, Pb, As and Mm treated plants showed up to 4.6, 5.4, 1.2 and 5.4% increase in comparison to their non-inoculated counterparts.

Cadmium toxicity in plants results into stunted growth due to low water potential, disturbed nutrient uptake and oxidative stress. Lead can also affect microtubule

Table 3. Stress Tolerance Index of Tomato plants.

Treatments	TI_{PH}			TI_{VG}		
	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT
Control	100^{a}	100^{a}	100^{a}	100^{a} (3.84a)	100^{a} (7.32a)	100^{a} (10.12a)
Control+AMF	101.12^{a}	111.16^{b}	102.74^{b}	101.82^{b} (3.91b)	103.28^{b} (7.56b)	103.56^{b} (10.48b)
Cd_{25}	72.76^{b}	96.20^{c}	82.62^{cd}	83.85^{c} (3.22c)	95.90^{cd} (7.02cd)	92.69^{c} (9.38c)
Cd_{50}	69.03^{bc}	91.45^{d}	80.64^{e}	76.56^{de} (2.94de)	93.58^{e} (6.85e)	91.11^{d} (9.22d)
Cd_{25} +AMF	80.60^{d}	99.52^{a}	88.72^{fg}	87.76^{f} (3.37f)	97.54^{f} (7.14f)	95.75^{e} (9.69e)
Pb_{50}	71.64^{b}	90.74^{de}	83.54^{c}	81.77^{g} (3.14g)	93.31^{e} (6.83e)	93.77^{fg} (9.49fg)
Pb_{100}	69.78^{bc}	89.07^{e}	81.71^{d}	77.86^{dh} (2.99dh)	90.71^{g} (6.64g)	92.39^{c} (9.35c)
Pb_{50} +AMF	75.37^{b}	98.57_{a}	89.63^{f}	86.20^{f} (3.31f)	96.72^{cfh} (7.08cfh)	96.64^{h} (9.78h)
As_{50}	80.60^{d}	92.40^{d}	86.59^{h}	90.63^{i} (3.48i)	95.22^{d} (6.97d)	94.47^{f} (9.56f)
As_{100}	68.66^{bc}	85.99^{f}	82.77^{cd}	75.26^{e} (2.89e)	90.30^{g} (6.61g)	93.18^{cg} (9.43cg)
As_{50} +AMF	85.07^{d}	96.20^{c}	87.80^{g}	90.10^{i} (3.46i)	96.04^{dh} (7.03dh)	95.55^{e} (9.67e)
Pb_{50} +Cd_{25} +As_{50}	65.30^{c}	85.27^{f}	76.37^{h}	74.74^{e} (2.87e)	87.84^{i} (6.43i)	88.83^{h} (8.99h)
Pb_{50} +Cd_{25} +As_{50}+AMF	69.03^{bc}	92.16^{d}	83.38^{c}	78.65^{h} (3.02)	92.62^{e} (6.78e)	91.21^{d} (9.23d)

DAT: days after treatment Values followed by the same superscript letter (a-k) in each column are not significantly different from each other (Student Newman Keuls test, $p \leq 0.001$). Values in parenthesis indicate mean value of biomass in g.

organization in meristematic cells (Eun *et al.* 2000). Arsenic can severely inhibit plant growth by slowing or arresting expansion and biomass accumulation (Garg and Singla 2011). It is noteworthy that though As was least accumulated in comparison to Pb and Cd, but its effects on physiological and biochemical parameters were comparable to other metals under study. Cd was found to be more toxic than Pb in our study, which is in accordance with previous studies (John *et al.* 2009; Chibuike and Obiora 2014).

3.3.b. Chlorophyll and proline content

Chlorophyll a and b are essential for photosynthesis and very sensitive to environmental stress such as HM (Ekmekçi *et al.* 2008). Exposure to HM lead a statistically significant decrease in chlorophyll content of leaves as shown in Figure 1a.

Photosynthesis in higher plants is more sensitive to HM treatments, affecting biosynthesis of chlorophyll and accessory pigments (Gill *et al.* 2012). A negative relationship was observed between HM concentration and pigment levels in all

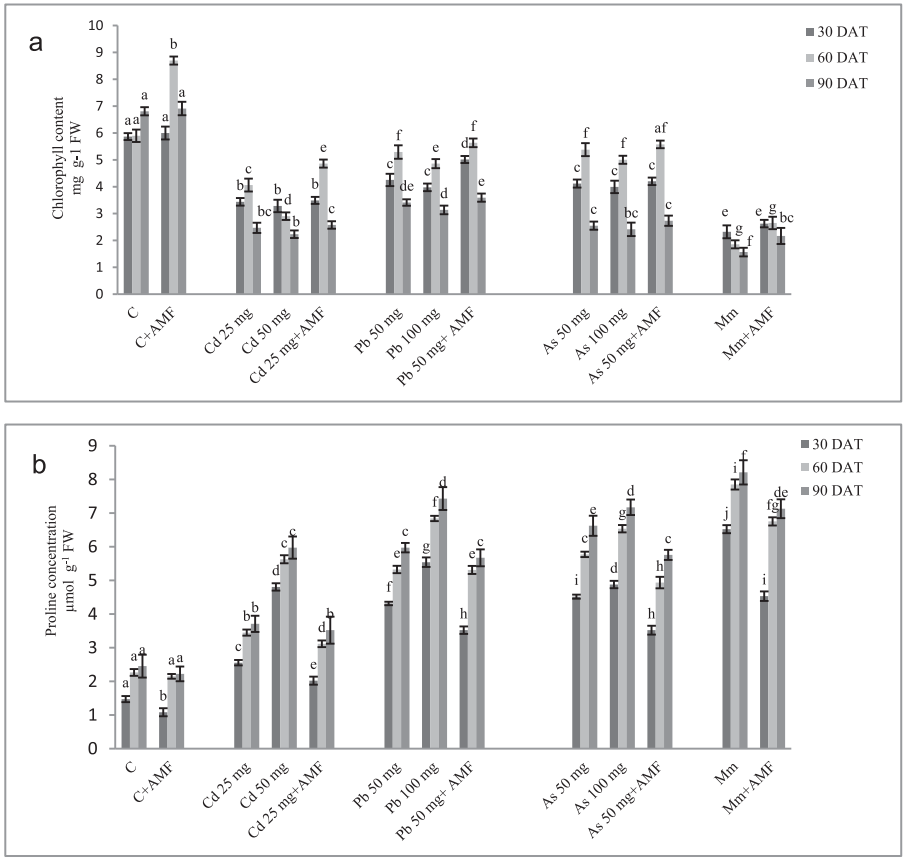

Figure 1. Plant biochemical characteristics (a)Total Chlorophyll content, (b) Proline concentration in Tomato leaves. Each value is a mean ± SD of triplicates; values followed by the same superscript letter (a-i) in each column are not significantly different from each other (Student Newman Keuls test, $p \leq 0.001$).

the plant. The data showed that total chlorophyll content decreased in order Mm (up to 76.9%) > Cd (up to 67.22%) > As (up to 64.54%) > Pb (up to 53.93%).

AMF inoculated plants also showed decrease in chlorophyll upon exposure to HM stress, but this reduction was lesser than their non-AMF inoculated counterparts. Control plants showed an increase up to 47.5% chlorophyll upon AMF inoculation. Similarly, plants showed up to 19.6, 17.8, 7.1 and 42.9% increase in chlorophyll under Cd, Pb, As and Mm stress, respectively when compared to their non-AMF inoculated counterparts.

Unlike chlorophyll, proline content increased in response to HM stress and decreased upon inoculation with AMF (Figure 1b). Plants showed up to 226, 276, 231 and 343% increase under Cd, Pb, As and Mm stress respectively. On comparison to their non-AMF inoculated counterparts, plants with AMF inoculation showed up to 20.9, 18.3, 22 and 30.5% reduction in proline content. Control plants showed upto 26.4% reduction in proline level when inoculated with AMF. It has been suggested that proline is an important amino acid that prevents oxidation of cell from inside by antioxidative properties, aids in osmoregulation and acts as a metal chelator. It also participates in reconstruction of chlorophyll (Carpena *et al.* 2003). It regulates cytosolic acidity and protects enzymes from denaturation (Gajewska and Skłodowska 2008). Proline is now even considered as a potent antioxidant and inhibitor of Programmed Cell Death (PCD), apart from being an osmolyte (Chen and Dickman 2005). It can be assumed that inoculation with AMF alleviated HM stress by its dilution effect i.e., by increasing the biomass and water content and hence the content of proline declined.

3.4. Enzyme activities

CAT, SOD and APX concentration was found to be upregulated both upon HM exposure and AMF inoculation ($p \leq 0.001$) (Figure 2). Enhanced level of antioxidative enzymes can be attributed to the over production of ROS or over expression of genes coding for antioxidant enzymes. The activity of these three enzymes was found to be increased in *Najas indica* (Singh *et al.* 2010) upon exposure to Pb, in *Brassica juncea* (Mobin and Khan 2007) upon exposure to Cd and in *Zea mays* and *Vicia faba* (Duquesnoy *et al.* 2010) upon exposure to As.

CAT is involved in the main defense mechanism against accumulation and toxicity of ROS. CAT enzymes eliminate H_2O_2 by breaking it down to water and oxygen. Plants showed up to 7.14, 32.14, 14.28 and 25% increase in the activity of CAT under Cd, Pb, As and Mm stress, respectively. Up to 7.14% increase was observed in the activity of CAT upon inoculation with AMF. Similarly, up to 21.42, 10.71, 32.14 and 50% increase was seen in plants under Cd, Pb, As and Mm stress upon AMF inoculation (Figure 2a).

SOD is considered to be the first line of defense against ROS and acts on superoxide free radicals produced in different cellular compartments (Alscher and Erturk 2002).

There was an increase up to 42.32, 23.29, 35.74 and 34.41% in the activity of SOD upon exposure to Cd, Pb, As and Mm, respectively as compared to control and up to 0.13, 32.9, 19.3, 34.45 and 35.03% increase in SOD concentration in control and Cd, Pb, As and Mm exposed plants with AMF inoculation, respectively (Figure 2b).

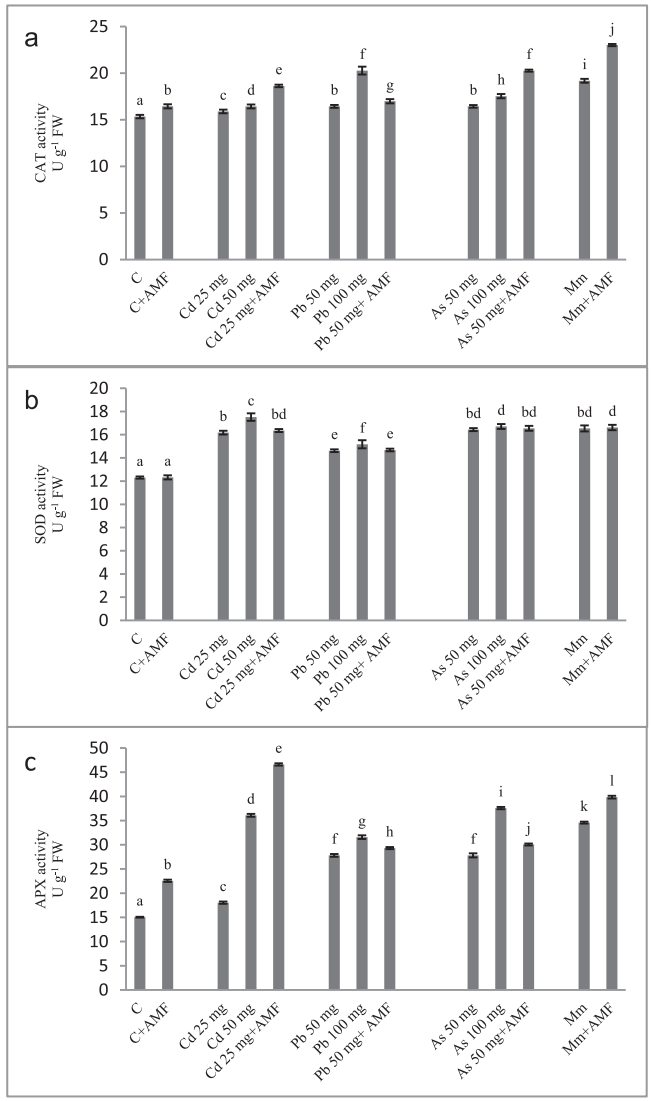

Figure 2. The activities of ROS antioxidant enzymes in Tomato plants. (a) CAT activity (b) SOD activity (c) APX activity. Each value is a mean ± SD of triplicates; values followed by the same superscript letter (a-l) in each column are not significantly different from each other (Student Newman Keuls test, $p \leq 0.001$).

Ascorbate enzymes are localized mainly in chloroplasts, but are also present in cytoplasm and other cellular organelles, where they play an important role combating oxidative stress (Malar *et al.* 2014). The increase in APX concentration was much higher than the rest two enzymes. Plants showed up to 140, 110, 150 and 130% increase in APX activity upon exposure to Cd, Pb, As and Mm stress respectively. AMF inoculation led to 50, 210, 95, 100 and 165% increase in APX activity in control and Cd, Pb, As and Mm exposed plants, respectively (Figure 2c). APX is believed to play the most essential role in scavenging ROS and protecting cells from oxidative stress. APX has a higher affinity for H_2O_2 (μM range) than CAT (mM range) and is thought to have a crucial role in ROS management (Gill and Tuteja 2010). This could be the reason for higher up regulation of APX than CAT and SOD in the present study.

Table 4. The Correlation coefficients among metal(loid) uptake, enzymatic activities, and plant physiochemical characteristics.

	Plant height	Biomass	Chlorophyll	Proline	CAT	SOD	APX
Uptake	−0.6*	−0.672*	−0.595*	0.294	0.204	0.536	0.502
Plant height		0.98**	0.924**	−0.82**	−0.196	−0.828**	−0.491
Biomass			0.905**	−0.81**	−0.244	−0.822**	−0.486
Chlorophyll				−0.749**	−0.267	−0.952**	−0.64*
Proline					0.534	0.646*	0.498
CAT						0.238	0.646*
SOD							0.659*

*Correlation is significant at the 0.05 level (two-tailed).
**Correlation is significant at the 0.01 level (two-tailed).

The present study clearly shows that exposure to HM triggers some of the key antioxidant enzymes in tomato plants. Antioxidant enzymes play a vital role in resisting oxidative damage; play a role in adaptive strategy of the plant and survival under stress (Zhang *et al.* 2007). In all the cases, AMF-inoculated plants showed significantly higher CAT and APX activity than their non-inoculated counterparts. This could be attributed to the contribution of mycorrhizal hyphae in aiding the transport of micronutrients such as Zn and Cu which act as co-factors for antioxidant enzymes (Subramanian et al. 2011). Mycorrhiza may provide protection against HM-induced oxidative stress and aid in ROS detoxification (Azcón et al. 2009).

3.5. Statistical analysis

Correlation was highly significant between plant height, biomass, chlorophyll, proline and SOD. Negative relationship was seen between HM uptake and parameters such as plant height, biomass and chlorophyll content (Table 4). Whereas, a positive relationship was seen between HM uptake and parameters such as proline content and antioxidative enzymes. Significant difference ($p<0.001$) in means was observed upon increasing HM dose and AMF inoculation in most of the treatments as shown in Figure 1 and 2.

4. Conclusion

The major response of tomato plants to HM stress was decreased plant growth and induction of oxidative stress. Proline, CAT, SOD and APX seem to play an indispensable role in combating HM stress. The results clearly showed that AMF inoculation resulted in a better growth, chlorophyll synthesis and a stronger osmoregulative and antioxidative defense mechanism.

Inoculation with AMF led to an increased phytoextraction potential of plants and hence the filtration hypothesis of AMF can be ruled out for this experiment. THQ of tomatoes was found to be >1 for Pb and Cd. This highlights the risks associated with cultivation of tomatoes for consumption on contaminated sites. The content of As in the fruit was found to be under safe limits as depicted by the THQ, hence does not pose a threat upon lifelong exposure.

The survival efficiency of *S. lycopersicum* in HM contaminated soil establishes it a potential candidate for soil remediation. Since AMF inoculation lead to a better defense response of the plants along with higher HM uptake, we conclude that AMF – tomato synchronization can be an economically feasible and potentially applicable process for on-site remediation of Cd and Pb contaminated sites.

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