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MICROBIAL ASSISTED PHYTOEXTRACTION OF METALS AND GROWTH OF SOYBEAN (*GLYCINE MAX* L. *MERRILL*) ON INDUSTRIAL WASTE WATER CONTAMINATED SOIL

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Abstract

Pots experiments were made to investigate the role of effective microorganisms (EM) in improving phytoextraction of metals (Cd^{+2} and Mn^{+2}) and growth of soybean plant in industrial waste water polluted soil. Waste water applications to soil were made in four different dilutions (i.e. 25%, 50%, 75% and 100%). Effective microorganisms were added into waste water prior to application. Effect of treatments on growth parameters was studied. The Cd^{+2} and Mn^{+2} concentrations in different parts of plant were measured by Atomic Absorption Spectrophotometer. Plant height significantly increased at all treatments except at 25% waste water treatment. Plant dry biomass and oil contents in seed significantly increased with all treatments compared to control but were higher at low concentration of waste water. Waste water treatments significantly increased the Cd and Mn accumulation in plant while inoculation of EM further enhanced the metals accumulation. The maximum accumulation of Cd and Mn found in plant treated with 100% waste water in combination with effective microorganisms. At harvest, the Cd^{+2} concentration decreased in leaves but increased in roots followed by stem > seeds, while, Mn^{+2} accumulation increased in leaves followed by roots > stem > seeds. Conclusively, EM enhanced the phytoextraction of Cd and Mn and also increased the oil contents in soybean on polluted soil. These findings suggest further investigation to find out a suitable concentration of industrial waste water in combination with EM for better growth of soybean and improving phytoextraction of metals.

Introduction

The untreated waste water is a growing concern throughout the world (Hernandez *et al*., 1991) and Pakistan is threatened by soil and water pollution by uncontrolled and untreated industrial effluents, house hold sewage and saline drainage water. Wastewater comprises industrial wastewater, domestic wastewater, storm water etc (Mahmood & Maqbool, 2006). Waste water contains trace elements, some heavy metals and dissolved salts and its use can impose negative impacts on ecosystems (Ghafoor *et al*., 1994). Around the world, different scientists are working to find ways to utilize the waste water and one of the ways is to use the waste water for irrigation purposes. In developing countries, approximately 80% of urban waste-water is used for irrigation (Mara & Cairncross 1989; Cooper, 1991). The industrial effluents are considered not only a rich source of organic matter but also contain heavy metals and the use of such effluents for irrigation may cause soil pollution. The restoration of heavy metals polluted soil is challenging because unlike organic compounds metal cannot be degraded, and removal of metals is usually require for soil clean up (Lasat, 2002). Various methods such as excavation, acid leaching, and electro-reclamation are used to restore metal contaminated soil. However, these methods are ineffective because of high cost, low efficiency, and destruction of soil structure and fertility (Jing *et al*., 2007). One of the new, effective, and promising processes is phytoremediation, which proposes the use of plants to extract, sequester and detoxify pollutants (Jing *et al*., 2007). Phytoremediation include phytoextraction, phytovolatilization, rhizofiltration and phytostabilization (Chaney *et al*., 1997). Scientists and engineers have started to generate cost-effective technologies that include the use of microorganisms and green plants in the cleaning process of effluents polluted areas (Boyajian & Carreira, 1997; Dushenkov *et al*., 1997). Previous studies have showed that some microbes enhance plant growth on polluted soil and subsequently increase heavy metals phytoextraction (Fazal & Bano, 2009).

The heavy metals uptake in plants varies with the mobility and concentration of metal in soil (Chen *et al*., 2010) and microbes enhances the metal uptake (Fazal & Bano, 2010). Present study was designed to see the effect of industrial waste water alone and in combination with effective microorganisms (EM) on physiology of soyabean. EM is a mixure of beneficial microorganisms including lactic acid bacteria, photosynthetic bacteria, yeast, fungi and actinomycetes, mixed culture of these microbes could enhance plant growth and yield (Higa & Parr, 1994). The waste water for this study was collected from industrial area of Gujranwala, from main drain (Nullah) along Lahore-Gujranwala road in Pakistan, where effluent from dyeing, plastic, ceramics, leather and paper industry etc in addition municipal waste water is added. Objective of the present study was to find out (1) the effect of industrial waste water at different concentrations alone and in combination with Effective microorganisms on growth of soybean plant. (2) the role of effective microorganisms (EM) in phytoextraction of cadmium (Cd^{+2}) and manganese (Mn^{+2}).

Materials and Methods

Plant materials: Seeds of Soybean [*Glycine max* (L.) cv. Ajmeri] were obtained from NARC (national agricultural research centre) Islamabad. Soil was prepared by mixing clay, sand and humus in the ratio of 2:1:1 respectively, Prior to sowing seeds in pots (18 x 24 cm) seeds were surface sterilized with 0.1% $HgCl_2$, followed by 5-6 washings with sterile distilled water. Five seeds per pot were used and after germination single healthy plant was selected per pot and other seedlings were removed. Three replicate pots were used for each treatment and control and no additional fertilizers were added throughout the experiments. Irrigation to each pot was made at weekly bases according to soil water holding capacity.

Preparation of EM extended solution and treatments: Effective microorganism inoculum was obtained from Dr. T. Higa (EM research Organisation (EMRO) Kishaba, Kitanakagusuku-Son Nakagami-Gun Okinawa, Japan). EM extended solution was prepared in phytohormone laboratory of Department of Plant Sciences, Quaid-i-Azam University Islamabad. For preparation, a mixture of 3ml EM and 5ml molasses in 92ml water were mixed in an air tight container. It was then left to ferment for 1 to 2 week. The dilution of industrial effluent for each treatment was made and applied to pots at the rate of water holding capacity of soil and the EM extended dilution (1:1000) was made with each concentration of effluent (waste water) and applied twice after germination on weakly bases. The following treatments were made during the experiment, T0 (Control (tap water), T1 (25% waste water), T2 (50% waste water), T3 (75% waste water), T4 (100% waste water), T5 (25% waste water + EM), T6 (50% waste water + EM), T7 (75% waste water + EM), T8 (100% waste water + EM).

Waste water analysis: The water samples were brought to the laboratory within 5-10 hours of collection and kept under refrigeration in 1.5L plastic bottles. A portion of this sample was preserved for heavy metal analysis by acid preservation method (Alexander, 1989). Sample of waste water was filtered through Whattman's filter paper No. 41 and was analyzed on atomic absorption spectrophotometer (Shimadzu AA-670) for heavy metals. Bicarbonate ions were extracted from rhizospheric soil by Reitemeier (1943) method. Soil suspension (1:1) was prepared by adding 10g soil into 10ml of distilled water and shaking for 15 minutes following filteration with whatman no.42 filter paper. The soil extract was taken in a conical flask, 1 drop of phenolphthalein indicator was added, but pink colour did not appear. The reading was taken as "Y". Then the soil extract was titrated against 0.01N sulphuric acid after adding 2 drops of 0.01% methyl orange indicator until the colour turned to orange. The reading was taken as "T". Measurement of bicarbonate ion of rhizospheric soil was done by using formula given below:

$$\text{HCO3 ions (Milliequivalents/litre)} = \frac{\text{(T-2Y) x normality of H2SO4 used x1000}}{\text{Volume of soil extract}}$$

Plant growth and oil contents in seeds: The plant height was measured with a cm ruler from the base of the stem to the top of the apical leaf. The roots and shoots were dried at 60°C for 48 h in an oven and dry weight was noted, similarly the dry weight of hundred air dried seeds was measured. The oil contents in seeds were estimated using the method of Robertson & Morrison (1979).

Plant sample analysis for heavy metals: The accumulation of metals in different plant tissues was determined by Perchloric-acid digestion method (Allen, 1974). From each sample, 0.25g were placed in a 50mL flask and 6.5mL of mixed acid solution (nitric acid + sulfuric acid + perchloric acid in the ratio of 5:1:0.5) were added and digested on hot plates until white fumes came out. The digested samples were transferred into 50mL volumetric flasks and the volume rose up to 50mL with distilled water. Thereafter, the samples were filtered and filtrates were analyzed for heavy metals in different parts of plant by atomic absorption spectrophotometer (AA-670 Shimadzu). Total accumulation of each metal in the entire plant was calculated as; Metal (mg g-1) x total dry biomass (g).

Statistical analysis: Data were subjected to Analysis of Variance (Steel & Torrie, 1980) and significance of mean values at 5% significance level was tested by Duncan's Multiple Range Test (Duncan, 1955) by programme MSTAT.

Results and Discussion

Plant height and biomass: Plant height increased significantly in all the treatments except T1 (25% waste water) and T5 (25% wastewater + EM) when compared to control (Table 1). Table 1 showed that when waste water was applied at low concentration, plant height increased. 25%, 50% and 75% application of waste water increased plant height, however in treatment of waste water without dilution decreased plant height. The EM enhanced plant height and 50% dilution of waste + EM (T6) showed maximum height followed by T7 (75% waste water + EM). Previous results support these findings (Abd-Alla *et al*., 1999; Breckle, 1991). At 100% waste water the decline in growth was observed which might be due to toxic effect of heavy metals (Cd^{+2} and Mn^{+2}) in waste water. It has been demonstrated that heavy metals generally reduce the plant growth (Dudka *et al*., 1996; Ouzounidou *et al*., 1997; Fazal *et al*., 2010). Present study showed that all treatments increased the biomass of plant significantly when compared with control (Table 1) but among the treatments the biomass decreased linearly with increasing concentration of waste water. Addition of waste water + EM showed comparatively higher biomass than waste water applied alone at each treatment. The EM is a composite of many beneficial plant growth promoting microbes, therefore, EM might have ameliorated the adverse effect of heavy metals taken up from waste water.

Effect on pods number, hundred seeds weight and oil contents: The number of pods increased significantly in all the treatments when compared to control plant applied with tap water only (T0). The treatments with 25% and 50% dilution of waste water increased the number of pods but gradual decline in pods was found in 75% and 100% waste water treatments (Table 1). The EM application further enhanced the number of pods in waste water treatments. The magnitude of EM induced of pods from waste water was maximum, when 50% dilution of waste water was applied. The weight of hundred seeds was increased upto 75% dilution and at 100% waste water, the hundred seeds weight (HSW) was decreased. Application of EM increased the HSW and the magnitude of EM stimulated HSW was maximum when dilution of waste water was applied at 50%. Table 1 showed that all the treatments increased the oil contents significantly compared to control. The oil contents was maximum, when 25% waste water was applied in combination with EM (T5). The oil contents was decreased with increasing concentration of waste water and minimum value was shown at 100% waste water (Table 1), This might be due to high metal concentration in concentrated waste water. Similar findings were reported by Ammar (1999) and also at higher concentration, yield might be reduced due to Cd^{+2} and Mn^{+2}(Malan & Farrant 1998).

Table 1. Effect of different treatments on plant growth and yield.

Treatments	Plant height (cm)	Dry biomass (g)	Number of pods	100 seeds weight (g)	Oil contents in seed (%)
Control (tap water) (T0)	98.60D ± 0.812	25.66C ± 0.081	23.33E ± 0.921	14.23D ± 0.083	19.07C ± 0.009
25% IWW (T1)	101.1D ± 0.633	40.38A ± 0.061	30.67CD ± 1.023	15.40BCD ± 0.067	21.72A ± 0.021
50% IWW (T2)	117.8B ± 0.391	40.35A ± 0.103	36.67AB ± 2.009	17.01AB ± 0.089	21.54AB ± 0.006
75% IWW (T3)	121.9AB ± 0.409	38.67AB ± 0.111	31.00CD ± 1.708	15.05CD ± 0.162	20.68B ± 0.032
100% IWW (T4)	113.2BC ± 1.092	34.57B ± 0.314	28.67D ± 1.991	15.26BCD ± 0.193	20.10B ± 0.015
25% IWW + EM (T5)	106.6CD ± 0.543	41.43A ± 0.234	35.67BC ± 2.099	16.14ABC ± 0.213	22.14A ± 0.021
50% IWW + EM (T6)	128..7A ± 0.725	40.43A ± 0.017	41.00A ± 0.989	17.25A ± 0.102	21.79A ± 0.008
75% IWW + EM (T7)	120.4AB ± 0.233	39.94A ± 0.098	35.67BC ± 1.923	16.10ABC ± 0.098	21.65A ± 0.017
100% IWW + EM (T8)	111.7BC ± 0.676	37.42AB ± 0.212	30.33D ± 2.111	15.18BCD ± 0.023	20.62B ± 0.003
LSD Value at 0.05	10.70	4.50	5.237	1.840	0.609

IWW= Industrial waste water, EM= Effective microorganisms, Different letters indicate significantly different mean values (DMRT, $p<0.05$, n = 3, ± SE)

Table 2. Characteristics of the industrial waste water and soil used for the experiments.

Waste water analysis			
Zn^{+2}	0.102 (mg / l)	Ca^{+2}	34.23 (mg / l)
Mg^{+2}	12.61 (mg / l).	Carbonates	Absent
Chromium	0.342 (mg / l)	Bicarbonates	6.5 meq/l
Nickel	0.24 (mg / l)	K^{+}	42.92 (mg / l)
Cadmium	0.384 (mg / l)	Na^{+}	34.91 app (mg / l)
Mn^{+2}	0.486 (mg / l)	pH	7.4
BOD	376 mg/l	COD	402 mg/l
Soil analysis before treatment with waste water			
Cadmium	1.1 mg kg-1	Mn^{+2}	87 mg kg-1

Analysis of industrial waste water prior to application: The waste water analysis showed the Cd^{+2} and Mn^{+2} concentrations (Table 2) above the maximum limit recommended by World Health Organization (Pandey, 1997). Apparently it is due to the presence of industries along the drain from where effluents had been collected for this study. Murtaza *et al*., (2003) also found the higher concentration of Cd^{+2} and Mn^{+2} from the effluent collected from Faisalabad in the vicinity of Karnailwala and Judgewala.

Accumulation and distribution of Cd in plant: The uptake and translocation of Cd increased linearly with the increase in the concentrations of waste water applied and EM application further enhanced Cd translocation. The maximum Cd accumulation was found in T8 (100% waste water + EM) after 60 days of sowing (Figs. 1a & b) and also similar increase in Cd accumulation was found at harvest (Figs. 1c & d). Previous reports indicate increased concentration of Cd in plant with increasing concentration of waste water applied (Ramachandran & Desouza, 1999; Paiva *et al*., 2001; Franz & Cornelia, 2011). T8 (EM with 100% waste water) showed maximum accumulation of Cd^{+2} in roots after 60 days of sowing (Fig. 1a) and at harvest (Fig. 1c) further accumulation occurred in its in roots and stem while translocation was decreased in leaves (Figs. 1a & c). This is perhaps due to high concentration of cadmium in 100% waste water added to soil and EM contains plant growth promoting microbes might enhance the process of phytoremediation (Whiting *et al*., 2001; Carlot *et al*., 2002). Total accumulation at both the stages (60 days after sowing (DAS) and at harvest) was higher due to EM application at all concentration of waste water. Measurement at 60 DAS both in root and leaves revealed that EM application at low concentration of waste water decreased the Cd accumulation as compared to control though the value was still higher than that of T0 (Control) but at higher concentration of waste water Cd accumulation was greater in root and leaves. Not much significant effect of EM was observed in stem. However measurement made at harvest showed a linear increase in Cd accumulation when waste water was used along with EM whereas in stem less Cd was translocated from root and in leaves, no significant difference in Cd accumulation was recorded in the treatment as compared to control. Achakzai *et al*., (2011) reported that wastewater used for irrigation around the peri-urban area of Quetta was polluted with Pb^{2+}& Cd^{2+} metals.

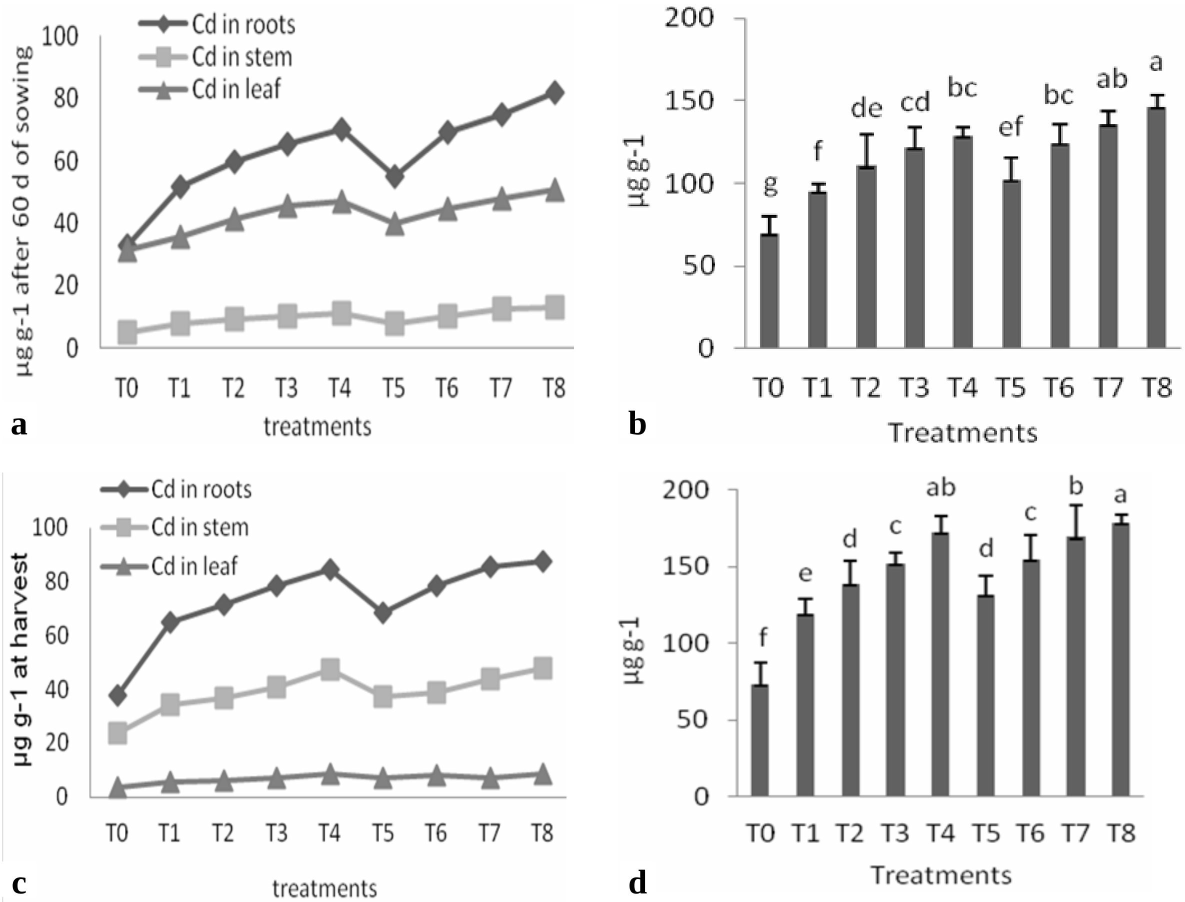

Fig. 1. (a-d) Effect of different treatments on Cd accumulation after 60 days of sowing (a= in different parts, b= total accumulation) and at harvest (c= in different parts, d= total accumulation). T0 is control (tap water only), T1 to T4 are 25%, 50%, 75%, 100% waste water respectively, T5 to T8 are waste water in combination with effective microorganisms. Bars indicate standard error and different letters indicate significantly different mean values (DMRT, $p<0.05$, n = 3).

Uptake and distribution of Mn in plant: Plants treated with different concentration of waste water alone and with EM showed linear increase in accumulation of Mn^{+2} in plants (Figs. 2a-d). The Mn^{+2} accumulations increased at harvest stage as compared to that of after 60 days of sowing. Measurement made 60d after sowing, revealed maximum accumulation of Mn^{+2} in roots except treatment T8 (EM + 100% waste water) in which case leaves and roots showed about similar magnitude of accumulation (Fig. 2a). Cardoso *et al*., (2003) reported similar findings that increase in external concentration of Mn^{+2} enhanced accumulation of Mn^{+2} in roots. At harvest, maximum accumulation of Mn^{+2} was found in leaves than other parts of plant (Fig. 2c). The observed increase of the Mn^{+2} in leaves at harvest might be attributed to the mobility of the Mn^{+2} from root to shoot, which might have occurred after a threshold level was attained in root due to addition of waste water and EM in soil. Mn^{+2} is a micro-nutrient and is involved in biochemical reactions e.g. photosynthesis (Salisburry & Ross 1985). Leaves are the most active metabolic site of plant and uptake of nutrients in greater concentration. The Mn^{+2} accumulation in different tissues also depend on the plant type, and organ e.g., Caines *et al*., (1985) and Albers *et al*., (1993) reported maximum accumulation of Mn^{+2} in shoots rather than root.

Concentrations of Cd and Mn in seeds: The Cd and Mn translocation into seed increased linearly with increase in the concentration of waste water in soil either alone or in combination with EM. The total accumulation of Cd was found higher than Mn (Fig. 3a) but both the metals showed a positive and significant correlation (R^2=0.893) in seeds (Fig. 3b. The Mn in seeds also bear positively and significantly positive correlation with Mn in leaf at harvest (Fig. 3c). Many researchers have reported Cd accumulation in seeds (Malan & Farrant, 1998; Yashida, 1986). This may possibly be due to the fact that nutrients get mobilized to the developing reproductive parts, possibly alongwith the assimilates.

Distribution of total Cd and Mn concentrations in different parts of plant: The total accumulation of Cd after 60 days of sowing was maximum in roots followed by leaf but at harvest the concentration of Cd declined in leaf, and the stem showed higher Cd than that of leaves (Fig. 4a). For Mn the pattern of distribution was different than Cd where the Mn concentration in stem after 60 days of sowing and at harvest was similar, but at 60 d the maximum Mn was in root whereas at harvest roots have less accumulation than leaves (Fig. 4b). The total accumulation of Cd and Mn in entire plant showed a positive and significant correlation (Fig. 4c).

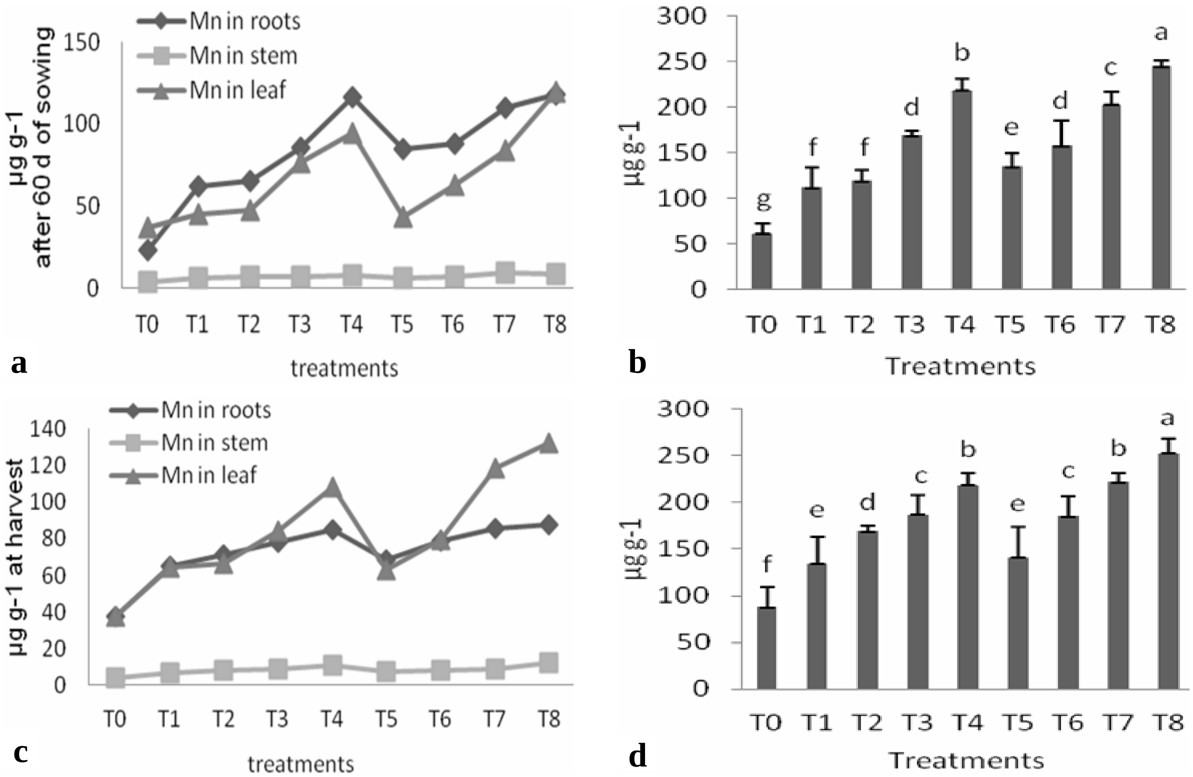

Fig. 2. (a-d) Effect of different treatments on Mn accumulation after 60 days of sowing (a= in different parts, b= total accumulation) and at harvest (c= in different parts, d= total accumulation. T0 is control (tap water only), T1 to T4 are 25%, 50%, 75%, 100% waste water respectively, T5 to T8 are waste water in combination with effective microorganisms. Different letters indicate significantly different mean values (DMRT, p<0.05) and bars indicate the standard error (n = 3).

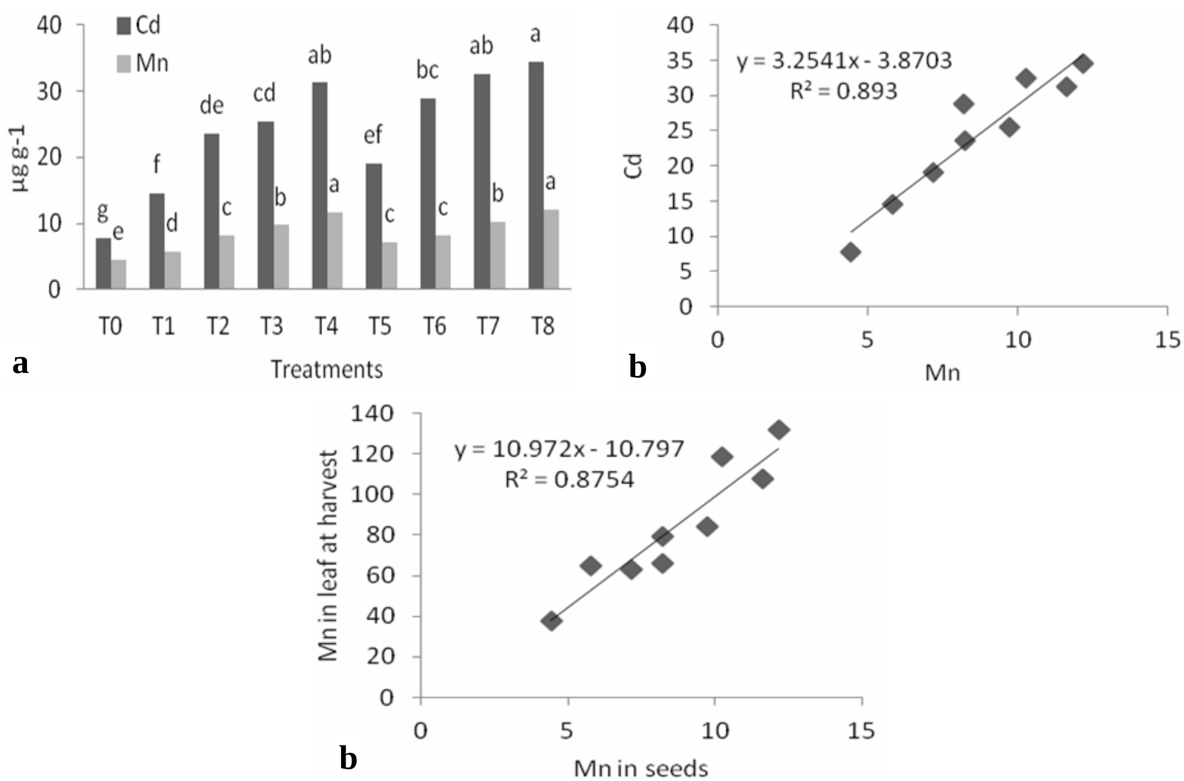

Fig. 3. Accumulation of Cd and Mn in seeds (a), Correlation between Cd and Mn in seeds (b) and Mn in leaf and seeds (c). Different letters indicate significantly different mean values (DMRT, p<0.05, n = 3).

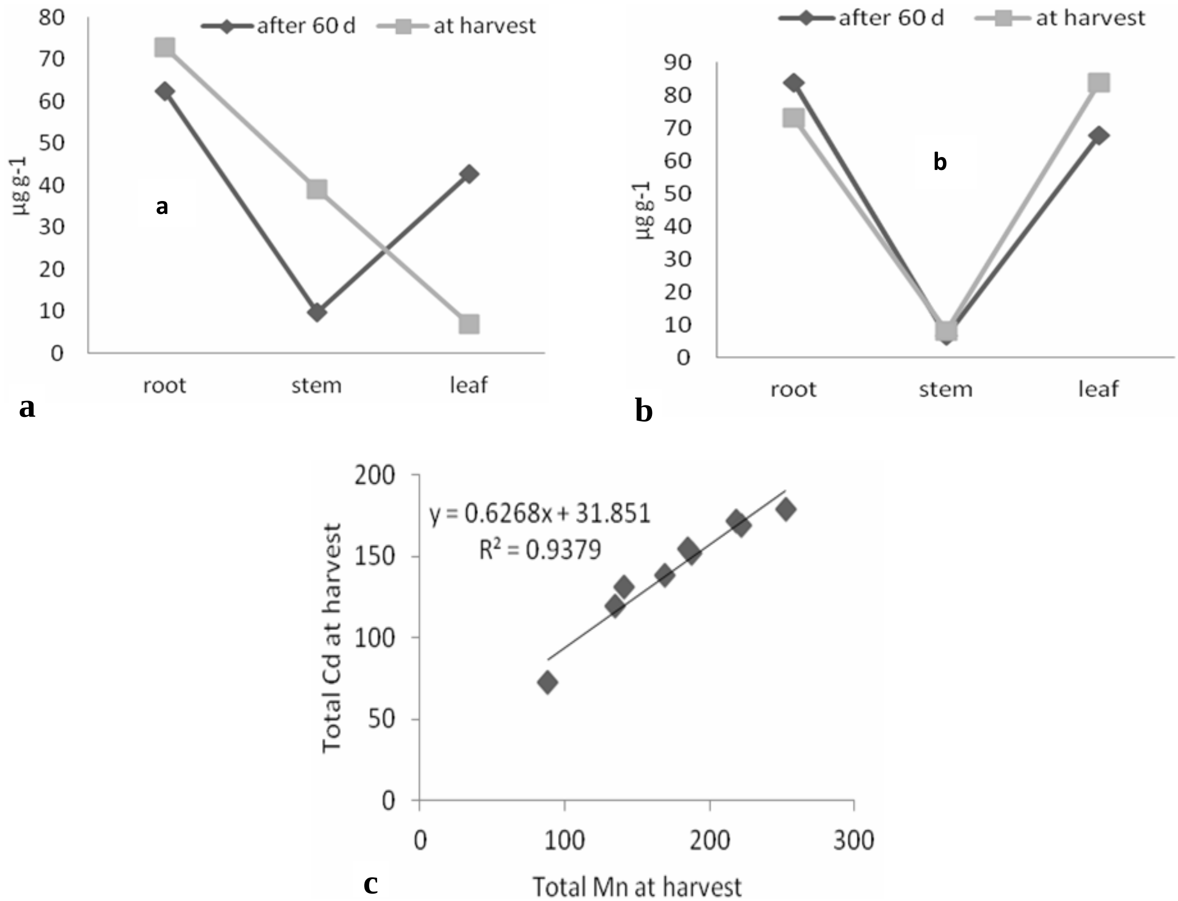

Fig. 4. Mean of all treatments and control values for Cd (a) and Mn (b) concentration in different parts of plant. Correlation between total Cd and Mn at harvest (c).

Conclusions

The application of waste water at 50% and 75% concentrations significantly increased the plant growth. The oil contents in seeds increased significantly with decreasing concentration of waste water and at 25% waste water the oil contents was maximum and EM application further augmented the accumulation of metals found in plant treated with 100% waste water, the inoculation of effective microorganisms (EM) further enhanced the phytoextraction of Cd and Mn when applied in combination with waste water. Therefore the present study suggests further research to study the role of EM in combination with waste water on plant growth and heavy metals phytoextraction from polluted area.

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