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Full Length Research Paper

Synergistic effect of *Glomus fasciculatum* and *Trichoderma pseudokoningii* on *Heliathus annuus* to decontaminate tannery sludge from toxic metals

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The effect of two soil amendments of tannery sludge (10% and 20%) on growth and metal uptake of *Helianthus annuus* L. was studied under three treatments of rhizosphere and mycorrhizal fungi. *Trichoderma pseudokoningii* Rifai was used as rhizosphere fungal inoculum (F) and *Glomus fasciculatum* (Thax.) Gerd. & Trappe as the mycorrhizal inoculum (M). The third treatment comprised of combined inoculation (F+M). The control (C) treatment was without any inoculum of the fungi.

The plants given both the fungus and mycorrhizal (F+M) treatment showed the maximum growth among all treatments. Plants given only fungus (F) and only mycorrhizal (M) treatment also showed significantly better growth as compared with control (C) treatment. Among the two sludge amendments, the statistical analyses of the results showed increase in all growth parameters in lower (10%) sludge amendment ratio.

The accumulation of potentially toxic metals (Cd, Cr, Na and Zn) in different parts of *H. annuus* grown on tannery sludge amended soil increased with increasing concentration of sludge in the soil. The plants treated with both fungus and mycorrhizal (F+M) treatment showed the maximum uptake of metals and thus the synergistic effect of these fungi can be exploited in decontamination of metals from tannery sludge.

Key words: Phytoextraction, tannery sludge, heavy metals, resistant rhizosphere fungi, Arbuscular mycorrhizal fungi.

INTRODUCTION

In the developing countries heavy metal pollution has become serious due to mining, mineral, smelting and tannery industry (Wang et al. 2007). The leather industry is recognized as a serious environmental threat all over the world because it is associated with the generation of huge amounts of heavy metal contaminated solid wastes and disposal of these wastes is a serious problem (Haroun et al. 2009). The discharge of untreated effluents and sludge from treatment plants of tanneries into nearby dumping areas is a growing problem in Pakistan (Khan, 2001).

The Kasur, an area of major tanning concentration in Pakistan, has a treatment plant for treating the tannery effluents named the Kasur Tannery Waste Management Agency (KTWMA) located at Depalpur Road. The sludge obtained from the treatment plant is being dumped into permanent sludge lagoons, thereafter to be shifted to a nearby landfill site after drying. This method of disposal is considered a low cost solution but it merely shifts the contamination problem elsewhere. The sludge is considered a source of potentially toxic elements and its disposal is problematic due to the presence of several heavy metals (Gupta and Sinha, 2007). The production of sludge is increasing day by day as a result of wastewater treatment.

Environmental restoration of polluted soils by conventional technologies demands large economic resources (Nascimento and Xing, 2006; Ahluwalia and Goyal, 2007), while, affordable approaches to decontaminate soils are increasingly gaining interest. Phytotechnologies involving use of plants for pollutant removal has

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gained importance during the last two decades (Dhir, 2010). Phytoextraction - the use of plants to remove toxic metals from soils by storing them in above-ground biomass - has been developed as an economical and environmentally attractive technology to decontaminate heavy metal polluted soils (Terry and Bañuelos, 2000). Although it is cheaper than the conventional methods, it is in fact a highly technical strategy, requiring expert project designers with field experience, choosing the proper species and cultivars for particular metals and regions (Alkorta et al. 2004).

Plants such as the Indian mustard, corn and sunflower show high tolerance to heavy metals and therefore have been used in phytoremediation (Tang et al. 2003; Pilon-Smits, 2005). Sunflower is reported to be a deep rooted and fast growing industrial oil crop (Prasad, 2004) with a high biomass (Zhuang et al. 2005). Ximenez-Embun et al. (2001) have also shown that sunflower is effective in removing Pb, Cr, Zn, Cd and Ni. The plant material may be used for non-food purposes or may be burned followed by recycling of the metals or as disposal in a landfill (Bennett et al. 2003; Angel and Linacre, 2005).

Accumulation of heavy metals in plants depends on many factors, including the transport of metals from the soil to the plant. Among these factors, bacteria and fungi, have received special attention. Fungal strains like *Aspergillus niger* and *Trichoderma pseudokoningii* directly link soil and roots and can be of great importance in heavy metal availability and toxicity to plants (Kapoor et al. 1999; Mungasavalli et al. 2007)**.** Considerable attention has been focused on the potential role of fungal inoculum in the protection of plants against heavy metal toxicity. According to Khan (2001), the protection provided to roots and enhanced capability for greater uptake of minerals results in greater biomass production, a prerequisite for successful remediation. Spores of *Glomus mosseae* and *G. intraradices* significantly increased the growth and uptake of N, P, Zn, and Pb in a greenhouse trial using *Vetiveria zizanioides* (Wong et al. 2007).

A wide range of fungi from all major taxonomic groups are reported in metal-polluted habitats and are capable of surviving and growing in the presence of toxic concentrations of heavy metals (Gadd, 1993). Korda et al. (2004) reported that reintroduction of indigenous microorganisms isolated from the contaminated sites after culturing seems to be a highly effective bioremediation method.

This study was designed to elucidate the role of an autochthonous fungal isolate along in association with an AM fungus to enhance the metal uptake in *Helianthus annuus,* a well known high biomass producing plant from metal contaminated soil.

MATERIALS AND METHODS

Tannery sludge was collected from the “Permanent Sludge Lagoon” constructed by KTWMA (Kasur Tannery Waste Management Agency) in Kasur, Pakistan. The sludge was transported to the Department of Botany, University of the Punjab, Lahore and was air dried in metallic troughs under sunlight. Then it was crushed and sieved through a sieve of mesh size $1mm^2$. Three types of tannery sludge amended soils (10%, 20% and 50%) were prepared by mixing crushed and sieved sludge in appropriate ratios (w/w) with soil collected from the Botanical Garden, University of the Punjab, Lahore. Crushed and sieved sludge (100%, without soil) was also used in the preliminary experiment.

For analysis of amended soil, the soil saturation extract was prepared by the method given by Rhoades (1982) and was used to determine the soil characteristics like pH, conductivity and sodium chloride percentage of the soil samples by using an auto ranging portable water proof microprocessor EC/ TDS/ NaCl/ ^{o}C meter (Model HI 9835). Carbonates, bicarbonates and chlorides were determined using the titremetric methods given by Saeed (1980). The organic matter content of the soil was measured using ASTM (1987) D2974-87 procedure. The determination of Na was done on a flame photometer (PFP7&PFP7/C, England) while the determination of Cr, Cd, and Zn was done on an atomic absorption spectrophotometer (AA-1275/VARIAN, Australia). Soil samples were digested by the method given by Mench et al. (1994).

Certified seeds of *Helianthus annuus* were collected from the Pulse Centre, NIAB, Faisalabad, Pakistan. Five hundred grams of each soil type was filled in plastic pots and five replicates of each were taken. Three seeds were sown each pot and the germination was observed daily in different concentrations of tannery sludge amended soils (10%, 20%, 50%, and 100%). Seeds were unable to tolerate 50% and 100% sludge amendments. Thus, two sludge amendments were selected (10% and 20%). *Glomus fasciculatum* maintained as a pot culture using sterilized sand: soil mix (1:1 by volume) as the substrate and onion plants used as the host was used in the experiments. After 75 days of growth, roots of onion plant were severed and the pot ball consisting of the root system plus substrate was finely chopped and air dried to constitute the mycorrhizal inoculum. Twenty- five g of this inoculum containing approximately 150 spores/g was used for inoculation of each pot.

Pure culture of *Trichoderma pseudokoningii,* isolated from tannery sludge, was grown in 500 ml conical flasks containing potato dextrose broth for 8 days. The cultures were then filtered through Whatman no. 1 filter paper and the mycelial mat was macerated using a Waring blendor for 1 min and mixed with 250 ml of 0.1 M $MgSO_4.7H_2O$ solution. Ten ml of this inoculum containing 5 X 10^4 c.f.u. /ml was used for inoculating each pot. The experiment was set up in a wire house having a glass roof in a Completely Randomized Design. The pots were watered on alternate days. Thinning and weeding was done after 08-10 days and one healthy plant was kept in each pot. The seeds were sown on 02 January 2007 and plants were harvested on 13 March 2007 (After 52 days).

Morphological parameters like root length, seedling length, total number of leaves, total number of roots and dry weight of plants were observed. Harvested plants were oven dried to a constant weight and crushed in a grinding machine. The roots and shoots were digested separately according to the method given by Greenberg et al. (1992) for metal estimations.

Statistical analyses were applied on the results of morphological and elemental assays. The software Analyse-it Free, available on web was used to determine the values of two way ANOVA for different fungal and soil treatments.

RESULTS

Among the five different combinations of soil and tannery sludge, pure sludge was the most toxic. Toxicity showed

Table 1. Some physico-chemical properties and amount of different metals in soil and different concentrations of tannery sludge (TS).

Parameter	Soil	1:10 (10%) (TS)	1:5 (20%) (TS)	1:1 (50%) (TS)	TS
pH	7.1	7.9	8.1	8.5	8.8
ECe (dS/cm)	0.02	0.31	0.95	1.65	2.90
NaCl (%)	2.9	47.2	130.9	244.7	450.5
Bicarbonates (mg/L)	103.7	160.9	210	290.5	365
Carbonates (mg/L)	-	-	-	-	-
Chlorides (mg/L)	62.1	355	990	1150	2,118
Ca (mg/kg)	1350	1,500	1,720	2,500	3,800
Cd (mg/kg)	55	780	875	1,720	5,590
Cr (mg/kg)	110	9,150	11,210	15,500	19,230
K (mg/kg)	1,560	1,250	1,725	2,250	3,190
Na (mg/kg)	995	4,580	4,645	8,350	9,500
Zn (mg/kg)	200	950	1,500	2,150	3,230
Organic matter (%)	2.11	2.29	2.81	3.10	3.72

TS, Tannery sludge; 10%, 10% tannery sludge mixed with soil; 20%, 20% tannery sludge mixed with soil; 50%, 50% tannery sludge mixed with soil.

a decreasing trend with decreasing ratio in the soil amendments (Table 1). The NaCl percentage, chlorides and bicarbonates were much greater than normal soil. The conductivity and pH value were also significantly high, the value being 2.9 dS/cm for pure sludge. Among the metals, a significantly greater amount was observed in pure sludge and all sludge amended ratios. Chromium was observed to be the highest followed by Na and Cd. Organic matter was found to be the maximum in tannery sludge (3.72%) and the minimum value was observed for soil (2.11%), while intermediate values were found for 10, 20 and 50% sludge amended soil (Table 1).

The highest growth of plants was observed in normal soil which showed retardation with increasing amendment of sludge (1:5 and 1:10) (Table 2), while the comparison between treatments (Control, F, M, and F+M) showed the highest growth in F+M when *Glomus fasciculatum* and *Trichoderma pseudokoningii* applied together as inoculum. Increase in all growth parameters was observed in lower sludge amendment ratio (1:10). Number of leaves and roots were greatest in soil treatment. However, among the treatments, the maximum increase was found in F+M treatment over their respective controls, M and F. The plants grown in (F + M) also showed flowering as compared to fungal treatment (F), mycorrhizal treatment (M) and Control treatment (without any fungal inoculum).

The uptake of heavy metals Cd, Cr and Zn conformed to their concentration of tannery sludge ratio in soil (Table 3). Statistical analysis showed that there was a greater uptake of metals by plants at higher sludge concentration in roots compared to shoots. The maximum uptake in roots was observed for Zn (990 mg/kg) and the maximum uptake for shoots was observed for Na (590 mg/kg), both in the 20% sludge amended ratio. The best root to shoot translocation was observed for Na, followed by Zn, Cr and Cd.

Among microbial treatments, the metal uptake was higher in F+M treatment as compared to their controls, M and F for all concentrations of tannery sludge amended soils (Table 3). The metal accumulation after 52 days of growth of the plants was found to be in the order of Zn>Cd>Na>Cr in roots, and Na>Zn>Cr>Cd in shoots. The accumulation of toxic metals (Cd, Cr, Na and Zn) in the plants was found to increase with increasing sludge ratio being the maximum in 20% tannery sludge amended soil.

DISCUSSION

Heavy metal toxicity and tolerance in plants is a subject that has been broadly reviewed (Mishra and Dubey, 2006; Panda, 2008, Pal and Rai 2009). Heavy metals are also included in the main category of environmental pollutants as they can remain in the environment for long periods; their accumulation is potentially hazardous to humans, animals and plants (Upadhyaya et al. 2010). Plant associated microorganisms have been studied for their ability to degrade a number of contaminants (Suresh and Ravishankar, 2004; Macek et al. 2004). These microorganisms increased the efficiency of plants to decontaminate the toxic environment. The occurrence of Arbuscular Mycorrhizal (AM) fungi in metal hyperaccumulating plants have been reported by Regvar et al. (2003) and Turnau and Masjasz Przybylowics (2003), so these fungi can be used as practicable biotechnological tools for enhancing phytoremediation of heavy metal

contaminated soils (Gaur and Adholeya, 2004). The majority of bioaugmentation studies of historically contaminated sites have been conducted with allochthonous fungi (Pointing, 2001 and Šašek, 2003). However, the use of autochthonous species for the cleanup of a contaminated site has been shown to be a successful approach (Atagana, 2004; Garon et al. 2004). Such findings have led to investigation of any synergistic effect of indigenous (*Trichoderma pseudokoningii)* and AM (*Glomus fasciculatum*) fungi on *Helianthus annuus* to clean up multi metal contaminated tannery sludge.

Although the tannery sludge has a reasonable concentration of organic matter but analysis of tannery sludge amended soils showed a high degree of pollution with metals especially Cd, Cr, Na and Zn. Increasing toxicity was observed with increasing amount of sludge. *Helianthus annuus* was found to be effective due to its fast growth rate and large amount of biomass as already advocated by Schmidt (2003) Pilon-Smits (2005) and Jadia and Fulekar (2008, 2009). It could tolerate the lower sludge concentrations well (10% and 20 %), but failed to even germinate at higher concentration (50% and 100%). These findings are in accordance with Fabig (1982) who stated that high amounts of heavy metals in soil can decrease plant growth but others (Arriagada et al. 2007) have shown that AM fungi protect plants against toxic actions of heavy metals.

In the present study, an autochthonous fungus (*Trichoderma pseudokoningii*), obtained from the sludge itself, was used as inoculum alone and in combination with the mycorrhizal fungus *Glomus fasciculatum* and both have shown promising results in aiding the metal uptake in plants. These finding are in accordance with Davies et al. (2002) who observed that mycorrhizal fungi increase chromium uptake by sunflower plants. Furthermore, a high uptake of metals was observed in roots as also reported by Salt et al. (1995) and Flathman and Lanza (1998) who believe that plants for phytoextraction should accumulate metals only in the roots.

A significantly high amount of metals was observed in (F+M) in higher concentration of sludge amended soils followed by (F) treatment in all experiments but in some cases (M) treatment showed better uptake of metals than (F). The minimum uptake of metals was observed in control. The concentration of metals in the plants was found to increase with increasing concentration of sludge in soil. These findings conform to the findings of Gupta and Sinha (2006), showing that the accumulation of heavy metals (Cr, Ni and Cd) in the plants was found to increase with increasing tannery sludge concentration. The two groups of fungi have shown synergistic effects on the growth of *H. annuus.* The morphological results indicated the maximum growth of seedling and greater dry weight in (F+M) treatment as compared to (F) or (M) treatment alone. Minimum growth was observed in the control treatment without any of these fungi. These findings are in line with Bareen and Nazir (2010) while working on *Tagetes patula* a member of the same family, Asteraceae.

According to Gryndler (2000) microbial populations in the rhizosphere are known either to interfere with or to benefit the establishment of mycorrhizal symbioses. The symbiotic relationship between mycorrhizal and autochthonous fungi (*T. pseudokoningii*) was confirmed by the maximum growth in treatment with dual inoculum. The rhizosphere fungi existing in heavy metal contaminated soils have important implications for phytoremediation. Since heavy metal uptake and tolerance depends on both plant and soil factors, including soil microbes, interactions between plant root and their symbionts such as AM fungi can play an important role in successful survival and growth of plants in contaminated soils.

There have been many reports that AM fungi contribute to phytoextraction (Davies et al. 2002; Agely et al. 2005; Wang et al. 2005). Wei et al. (2003) have already claimed that rhizosphere is a special ecological remediation unit to treat contaminated soils containing a great number of microorganisms such as fungi and rhizobacteria living with plant roots.

Plants used for phytoextraction should develop a large amount of biomass quickly and be easy to cultivate and harvest, preferably multiple times per year (Tong et al. 2004). In the present study, from the morphological data of the plants grown in different tannery sludge amended soils, it was observed that the plants treated with both fungal and mycorrhizal inoculum showed significant growth even under stressed conditions. The flowering was observed only for (F+M) treatment. The plants treated with rhizosphere fungus (F) only also showed good results but no flowering was observed. The plants treated with mycorrhizal infection only (M) showed good results but less significant as compared to F only. The plants grown in the control treatment did not show good morphological results although they were growing under normal conditions.

Conclusion

In view of growth parameters and metal accumulation in sunflower plants, it was observed that lower amendments (10%) of tannery sludge were more suitable for the removal of most of the metals present. Thus sludge remediation can be achieved by mixing it in normal soil at excavation sites and planting well known hyperaccumulators of metals. The tolerance exhibited by the sludge-grown plants of *Helianthus annuus* in the present study may be attributed to the enhanced level of the metal uptake and growth with the aid of fungal and mycorrhizal inocula under metal stressed conditions. After sufficient plant growth and metal accumulation, the plants can be harvested, resulting in the permanent removal of metals from the site. The harvested plants can be incinerated and the ash can either be buried in a landfill or used to recycle the metals.

Table 2. Various morphological parameters observed in 52 days old Plants of *Helianthus annuus* growing in different concentrations of tannery sludge amended soils.

Treatment	Parameter														
	No. of leaves			No. of roots			Root length (cm)			Seedling length(cm)			Dry weight (g)		
	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5
Control	6±0.31	5± 0.11	4± 0.27	10± 0.11	9± 0.45	7± 0.09	16.1±0.45	11± 0.11	9.2± 0.18	29.3± 0.31	20.2± 0.09	18.2± 0.16	5.6± 0.12	6.2± 0.32	4.3± 0.44
M	7± 0.44	6± 0.32	5± 0.19	9± 0.37	8± 0.21	6± 0.06	18.1±0.31	11.9± 0.41	8.4± 0.16	34.7± 0.21	23.4± 0.04	18.0± 0.16	7.8± 0.16	7.5± 0.11	3.4± 0.30
F	9± 0.11	7± 0.21	6± 0.30	11± 0.16	10± 0.15	8± 0.12	24.2±0.09	12.2± 0.32	7.6± 0.31	43.2± 0.43	27.4± 0.37	18.9± 0.14	6.3± 0.16	6.5± 0.21	5.2± 0.05
M±F	6± 0.08	10± 0.16	9± 0.01	13± 0.36	11± 0.19	10± 0.11	27.3±0.07	13.9± 0.37	11.2± 0.16	51.4± 0.51	32.1± 0.21	24.9± 0.13	10.2± 0.31	9.5± 0.41	7.1± 0.37
L.S.D (P=0.05)															
For Conc.	3.12			0.56			5.25			7.24			0.24		
For treatments	2.70			0.48			4.54			6.27			0.20		

L.S.D = Least significant difference; control, without any treatment; M, mycorrhizal treatment; F, fungal treatment; F+M, fungal + mycorrhizal treatment; 1:10, tannery sludge amended; soil (10% tannery sludge mixed with soil); 1:5, tannery sludge amended soil (20% tannery sludge mixed with soil).

Table 3. Metal uptake in roots and shoots of 52 days old plants of *Helianthus annuus* growing in different concentrations of tannery sludge amended soils.

Treatment	Amount of Cd (mg/kg)						Amount of Cr (mg/kg)						Amount of Na (mg/kg)						Amount of Zn (mg/kg)					
	Roots			Shoots			Roots			Shoots			Roots			Shoots			Roots			Shoots		
	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5	Soil	1:10	1:5
Control	45	250	600	30	125	350	5	500	650	2	230	310	310	410	550	210	310	450	145	450	710	95	210	350
M	65	500	690	45	200	390	7	540	710	3	260	350	370	490	580	250	340	490	210	480	840	110	190	440
F	80	610	750	40	210	395	8	590	690	5	290	390	410	550	595	270	390	510	250	525	910	125	225	485
M+F	125	680	850	75	300	450	12	625	810	6	410	460	610	710	840	340	410	590	310	690	990	150	310	555
L.S.D (P=0.05)																								
For Conc.	161.9			53.61			74.54			82.7			43.36			26.8			74.38			74.25		
For Treatments	140.2			46.42			64.55			71.69			37.57			23.24			64.41			64.30		

L.S.D = Least significant difference; control, without any treatment; M, mycorrhizal treatment; F, fungal treatment; F+M, fungal + mycorrhizal treatment; 1:10, tannery sludge amended; soil (10% tannery sludge mixed with soil); 1:5, tannery sludge amended soil (20% tannery sludge mixed with soil).

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