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Multivariate data analysis for bioremediation of contaminated soil through Interactions between heavy metals, microbes and plants

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Abstract: Plant-assisted bioremediation (phytoremediation) is a promising technique for in-situ remediation of contaminated soils. Enhancement of phytoremediation processes requires a sound understanding of the complex interactions in the rhizosphere. This work presents a Pot experiment was conducted under green house conditions to test the effect of fungal inoculation on remediating heavy metal (HM) contaminated soil treated with sewage effluent for several years. Canola crop was used as accumulator plants. Results demonstrated that the dry matter yield of tested crops were significantly higher in soil irrigated for 50 years with sewage effluent than that in 20 years sewage effluent irrigated soil. Metal uptake and accumulation in different plant parts (shoot and root) was enhanced after inoculation with *Aspergillus parasiticus* (F1) and *Fusarium oxysporum* (F2). The reate of HM accumulation as higher in in soil treated irrigated sewage effluent for 50 years than that in 20 years sewage irrigated soil.

Keywords: *Bioremediation, Contaminated Soil, Heavy Metals, Canola plant, Multivariate Statistical Analysis*

INTRODUCTION

Soil contamination represents one of the most important environmental problems all over the world (Evans and Furlong 2003; Harrison 2001). Anthropogenic sources including industrial activities, metalliferous mining, smelting and waste disposal have increased soil contamination (Gianfreda and Rao 2004). Among the various contaminants which are released to the soil are the heavy metals (HM) such as Ag, As, Au, Cd, Co, Cr, Cu, Hg, Ni, Pb, Pd, Pt, Rd, Sn, Th, U and Zn which are found in high concentrations and cause harmful effects on human health and the environment.

In recent years, bioremediation techniques were developed and improved to remove hazardous contaminants from polluted soils (Romantschuk *et al.* 2000). These techniques provide the possibility to destroy or render the adverse effects of various contaminants using natural biological activity (Sriprang 2007). Additionally, they use relatively low-cost technology, which generally have a high public acceptance and can often be carried out on site (Chen *et al.* 2005; De *et al.* 2008). One type of bioremediation that is widely known is phytoremediation; where certain species of higher plants can accumulate considerable concentrations of HM in their tissues without showing toxicity (Bennett *et al.* 2003). Such plants can be used successfully to clean up heavy metal polluted soils if their biomass and metal content are large enough to complete remediation within a reasonable period (Ebbs and Kochian 1998). In fact, such process represents an integrated multidisciplinary approach to remediate contaminated soils, which combines the disciplines of plant physiology, soil chemistry, and soil microbiology (Cunningham and Ow 1996). Mechanisms by which microorganisms act on heavy metals include biosorption, bioleaching, biomineralization, intracellular accumulation and enzyme-catalyzed transformation (redox reactions) (Lloyd 2002) which finally leads to the decrease of contaminants concentration.

Due to multivariate nature of environmental data, together with their complex interrelation, multivariate statistics could be used to analyze or decipher any structure within the data. It can give hidden information about quality of environmental samples or about similarity between different environmental samples (Voncina 2009). (Juahir *et al.* 2008) used multivariate statistical analysis to determine number of sampling sites which appear significantly different from each other and hence reduce cost and sampling time.

The aim of the present study was to evaluate the role of *Aspergillus parasiticus (F1)* and *Fusarium oxysporum (F2)* fungi in remediating heavy metal contaminated soils cultivated with canola as tested plant and at the same time acts as hyper HM accumulator. Additionally, to determine the levels of Pb, Cu, Zn, Cr, Cd and Fe to identify the possible sources of these metals using multivariate statistical analysis, for Best Management Practice (BMP) in order to reduce the risk of exposure and possible health effects.

MATERIAL AND METHODS

Study area

Al Gabal Al Asfar, (30.2 (30° 11' 60 N31° 22' 0 E), is a spot feature (railroad station) located in the area / state of Al Qalyubiyah in Egypt. The location is situated 380 kilometers (km) north (20°) of the approximate center of Egypt and 20 km north east (34°) of the capital Cairo. A 10 square km area around Al Gabal Al Asfar has an approximate population of 62434 and an average elevation of 15 meters above the sea level.

Experimental design

Pot experiment was carried out under greenhouse conditions at Soil and Water Research Department, Cairo, Egypt. The experiment tried to explore and evaluate the synergetic role of fungal inoculation and canola on remediation of heavy metals contaminated soils.

Soil

Two types of soil samples were collected from AL Gabal Al Asfar. The first is sample (20 Y) was irrigated for 20 years with sewage effluents. The second sample (50 Y) was irrigated for 50 years with sewage effluents. Plants were irrigated with 200 mL per pot twice a week until harvesting after 90 days.

Isolation and identification of microbial inoculants

Fungal Isolates: *Aspergillus parasiticus strain I9-(F1)*. Identification was carried out as mentioned in Raper and Fennell (1965) *Fusarium oxysporum isolate I1 (F2)* was identified according to the method of Booch (1977); Cazpek-Dox`s medium (Czapek 1902-1903; Dox 1910) was used for culture and maintenance of both fungal isolates.

Seeds inoculation

The selected fungal isolates *(F1*and *F2)* were allowed to grow on plates containing Czapek Dox-Agar medium (CDAM) for 7 days at 30°C. A spore suspension of each fungus was prepared by shaking agar discs grown with the fungus in 100 mL of liquid Cyapek Dox media on a rotary shaker (150 rpm) for 30 min. Spore suspensions were then added to seeds at two time intervals (20 and 40 days before germination) at a concentration of 2×10^8 spores /seed. The seeds were then sawn in plastic pots (30 cm diameter).

Fertilization

Recommended doses of NPK required for the crop growth were applied during preparation of soil. Nitrogen, Phosphorous and Potassium fertilizers were applied as shown in Table (1).

Table 1. fertilization doses used during the experiment

Fertilizer	Formula	Active ingredient	Dose per domum (kg)
ammonium sulphate	**$(NH_4)_2SO_4$**	**20.6% N**	**25 kg**
calcium Super phosphate	**$Ca(HPO_4)$**	**15.5% P_2O_5**	**37.5 kg**
potassium sulphate	**K_2SO_4**	**48% K_2O**	**10 kg**

Analytical Methods

Chemical characteristics (pH, electrical conductivity and organic matter) of sewage soils were analyzed according to (Jackson 1958). Mechanical analysis and total potential toxic elements were conducted according to (Piper 1950) and (Cottonie *et al.* 1982), respectively. Potential Toxic Elements (PTEs) were also fractionated to water soluble, exchangeable, carbonate-bound, Fe-Mn oxides-bound, organic-bound and residual fraction as mentioned in (Zaghloul 2002). The concentrations of HM were determined using atomic absorption as described in (Cottenie *et al.* 1982).

Data analysis

Multivariate statistical data analysis and calculations were carried out using 'Unscrambler' v. 9.2 (Camo, ASA, Oslo, Norway), a statistical software package for multivariate data analysis. Principal Component Analysis (PCA) was used for the experiment data. PCA is one of the multivariate techniques, and it is used to identify patterns in a data set derived from recording several characteristics at a time to eliminate redundancy in univariate analyses The PCA is explained by Principal Components (PCs), which are composite variables, since they are linear functions of the original variables, estimated to contain the main structured information in the data. PCs are also called latent variables, and a PC is the same as a score vector (Esbensen *et al.* 2001).
For investigating the significant difference of heavy metals at different treatments, XLStat program (Addinsoft Software) with one-sample student's t-test / right-tailed test was used.

RESULTS AND DISCUSSION

Initial characteristics of untreated soil

Chemical and physical characteristics

Sewage soil samples collected from Al-Gabal Al-Asfar farm were initially analyzed to study the effect of fungi on sewage soil treatment especially on their key chemical and physical properties after sewage farming for extended periods (20 and 50 years). Results presented in Table 2 show remarkable chronological fluctuations in the estimated soil chemical and physical characteristics. However, the soil properties particularly texture were the main factor directly prejudiced other estimated chemical and physical characteristics of sewage soils. In the present study, the soil mechanical analysis (texture) showed a slight difference between the two soil samples.

No effect of irrigation period was recorded. The pH measured (7.5 and 7.3) was slightly but not significantly different in 20 Y and 50 Y soils, respectively. Several studies revealed that irrigation period have influenced the soil chemical and physical characteristics. (Saber 1986) found that the pH values decreased from 7.9 to 5 after 60 years of sewage irrigation at the same farming area. Moreover, the total soluble salts increased from 1751 to 3256 ppm (Saber 1986) and after 70 years it reached 4550 ppm (Abdel-Mottaleb *et al.* 1993). In addition to that, sewage farming increased the organic carbon content in soil from 0.51 to 0.86% (Masto *et al*. 2009) and from 0.19 t0 0.56% (Abdel-Shafy *et al.* 2003). Additionally, irrigation with sewage effluent was found to increase the organic matter content in the soil to 3.5% after 8 years (Aboulroos *et al*. 1991) and to 13.0% after 60 years (Saber 1986). Our results showed that soil texture was changed due to sewage farming for extended periods.

Heavy metals

The collected sewage soil samples from Al Gabal Al Asfar were also analyzed for their total and available heavy metals (HM) contents (Tables 3). Clearly, the total and available concentrations of HM in the studied soil samples were influenced by the duration of sewage farming (20 and 50 years), types of HM and land use. According to our results, data representing the different HM concentrations in the sewage soil samples might be classified into four groups.

The first group contained high concentration of Fe (10252 and 10485 ppm) and available HM of 26.48 and 63.67 ppm in the two soils types, respectively. The second group contained medium concentrations of Zn and Mn. The HM concentrations were 2562, 5100 (for Zn) and 2753, 7167 (for Mn) and the available HM concentration were 27.31, 64.16 (Zn) and 53.92, 73.3 ppm (Mn), respectively. The third group contained low concentrations of Cu and Co. with total HM concentration of 482, 520 (Cu) and 419, 477 (Co) and available HM concentrations were 9.46, 11.73 (Cu) and 6.97, 8.33 ppm (Co), respectively. In the fourth group very low concentrations of Cd in sewage loamy sandy soil 136 and 206 ppm in the sewage soils, and the available HM concentration was 2.96 and 5.61ppm were recorded.
The results obtained were in agreement with Abo-el-Abbas (2001) who proved higher accumulation of PTEs such as Fe, Zn, Mn, Cu, and Pb in the upper soil after 85 years of sewage farming at Al Gabal Al Asfer. It was also found that the longer the period of irrigation with sewage effluent resulted in

higher level of PTEs accumulation in soil (Abdel-Shafy *et al.* 2003; Abdel-Sabour 1994). Moreover, Kamel and Husein (2007) found that soil irrigated for 75 years with sewage effluent showed an increment in the total content of PTEs. Abdel-Mottaleb *et al.* (1993) indicated that DTPA-extractable contents of Fe, Mn, Zn, Cu, Pb, Ni and Cd increased during a 70 years period of sewage effluent irrigation.

Canola dry matter yield (CDMY)

Plants and microorganism can adapt to high concentrations of toxic pollutant. Phyto- / rhizoremediation processes that remove pollutants (phytoextraction, -degradation, -volatilisation) contribute to alleviation of toxicity by decreasing the pollutant concentration in the rhizosphere. Fungal inoculation *(F1, F2* and combination*)* resulted in increased shoot and root dry matter of canola plant grown on soil irrigated with sewage effluent for 20 and 50 years (Figure.1). In this work, CDMY were significantly higher in soil irrigated for 50 years than that irrigated for 20 years with sewage effluent. The increased biomass of canola plants (Figure. 1) under in soil irrigated with sewage effluent for 50 years might be attributed to higher soil fertility after inoculation with the fungi. (Wang *et al.* 2005) found increased biomass production of *Elsholtzia splendens* inoculated with arbuscular mycorrhizal. Beneficial effects of arbuscular mycorrhizae and a Penicillium fungi on biomass production of *Elsholtzia splendens* was confirmed under field conditions (Wang *et al.* 2007).

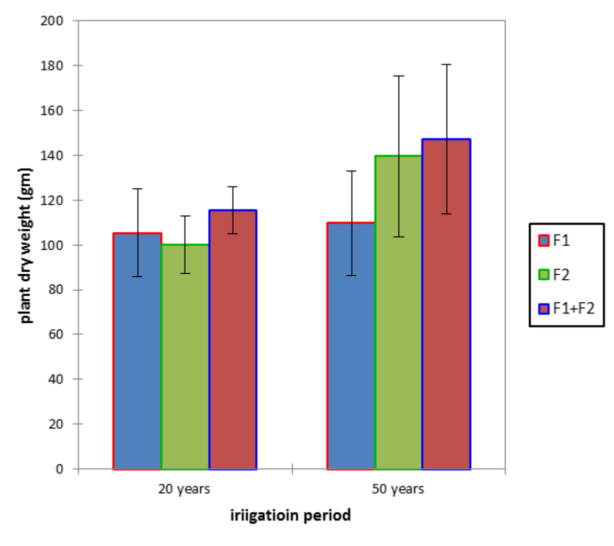

Figure 1. Canola plant dry matter yield after harvest were grown in soil for 20 and 50 years irrigated with sewage effluent and inoculants with fungi.

Heavy metal uptake by canola

Soil samples from Al Gabal Al Asfer sewage farms grown with canola plant and treated with fungal inoculation were analyzed for total HM contents (Table 4). Total concentrations of HM in the studied sewage soil samples were influenced with the period of sewage irrigation (Tables 3. and 4). The plant and fungi played a significant role in absorption of considerable amounts of HM and reduced HM contents in the soil.

Table 2. Some Chemical and physical Characteristics of Al Gabal Al Asfer farm soils * (Oven dry basis).

Soil No.	Irrigation Period	Texture	EC (ds/m)	pH	OM %	OC %	F.C %	Sand %	Silt %	Clay %
1.	20	Loamy sand	0.3	7.5	0.8	0.47	8.5	89	8.3	2.7
2.	50	Loamy sand	0.4	7.3	1.9	1.3	9.2	84	11	5

Table 3. Total and Available Heavy Metals contents (µg g-1) before cultivation (ppm oven dry basis).

Soil No.	Period of irrigation (years)	Heavy Metals	Fe	Zn	Mn	Cu	Co	Cd
1.	20	Total	10252	2562	2753	482	419	136
		Available	26.48	27.31	53.92	9.46	6.97	2.96
2.	50	Total	10485	5100	7167	520	477	206
		Available	63.67	64.16	73.30	11.73	8.33	5.61

Table 4. Total contents of Heavy metals (µg g^{-1}) remained in soil after harvest of canola plants (ppm oven dry basis).

Soil No.	Sewage irrigation period (years)	Treatment	Fe	Zn	Mn	Cu	Co	Cd
1.	20	Un inoculant	193.7	7.2	4.3	6.8	13.3	n.d
		F1	151.4	7.0	4.0	6.3	14.8	n.d
		F2	100.1	7.0	4.4	6.3	10.5	n.d
		F1+F2	81.1	6.1	4.5	6.2	10.4	n.d
		Sum	**526.3**	**27.3**	**17.2**	**25.6**	**49**	
2.	50	Un inoculant	178.3	8.7	6.7	27.8	10.3	n.d
		F1	141.9	8.7	7.2	28.7	13.8	n.d
		F2	168.8	8.8	6.2	28.5	14.8	n.d
		F1+F2	209.3	8.2	7.0	16.4	14.4	n.d
		Sum	**698.3**	**34.4**	**27.1**	**101.4**	**53.3**	
		Sf	Sf	Sf	Sf	Sf	Sf	Nsf

F1: Fusarium oxyspourum *F2: Aspirugellus paraciticus*

Sf: significant, Nsf: not-significant

The scores plot of PCA is shown in Figure 2. Full cross validation were carried out on the data. Two PCs explain 77% of variation of the data. The Figure shows a clear classification between experimental sewage soil irrigated for 20 and 50 years and remediated by fungi.

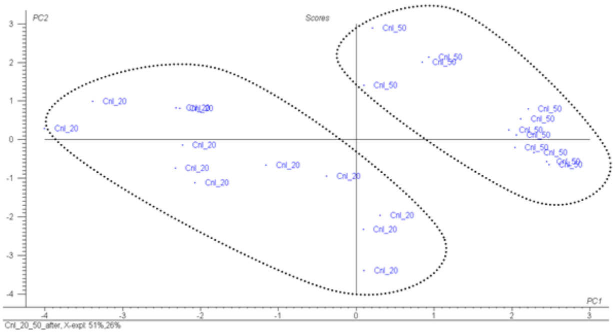

Figure. 2. Score plot of principal component analysis (PCA).

The use of plants for environmental restoration is an emerging cleanup technology. Some plants phytostabilise heavy metals in the rhizophere through root exudates immobilization (Blaylock and Huang 2000).,Other species incorporate them into root tissues (Khan 2001) and Other plants transfer metals to their above ground tissues allowing potential soil decontamination by harvesting the above groundparts. Phytoextraction process reduces soil metal concentrations by cultivating plants with a high capacity for metal accumulation in shoots (Barceló and Poschenrieder 2003). The plants must extract large concentrations of HM into their roots, translocate the heavy metals to above ground shoots or leaves and produce large quantity of plant biomass that can be easily harvested.

The result presented in this work (Table 3) indicated significant role of fungi and canola on remediating and cleaning up the contaminated soil. Canola, effluent applications and fungal inoculation treatments have significant effects on iron uptake by canola plants. These effects were significantly varied according to interaction between treated soils and inoculation treatments (Table 4). The Fe concentration in 20 and 50 Y soil samples (10252 and 10485 ppm) decreased to 526.3 and 698.3 ppm after treatment (Tables 3 and 4).

Fungal inoculation on Zn values Zn concentrations to 27.3and 34.3 in both soil samples. Zn is an essential trace element to all high plants and animals. it is required for enzymes activities (Mengel and Kirkby 1982) and plays an essential role in DNA transcription. Zn toxicity often leads to leaf chlorosis (Cobbett and Goldsbrough 2002).

Mn contents in soil before treatment were 2753, 7167 in the two soil respectively. Canola shoots and roots were affected by fungal inoculation according to the duration of sewage effluent irrigation. Higher reduction in Mn uptake was recorded in the two soils (17.2 and 27.1, respectively). Mn, under the given conditions, has the ability to move upward from the root to the shoot system.

Cu values in soil before treatment were 482, 520 and after treatment and uptake by plant were significantly reduced mainly after fungal inoculation to 25.6, 101.4. No increase in Cu values after 20 and 50 years of irrigation was recorded. Cu is essential

micronutrient for plants, but it can be toxic at higher concentrations. It contributes to several physiological processes in plants including photosynthesis, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, seed production including also disease resistance (Kabata-Pendias and Pendias 2001). The higher concentration of Cu may account for the suppressed root growth, leaf chlorosis observed among plants (Kuzovkina *et al.* 2004).
Co values before treatments were 419 477 and decreased to 49 and 53.3 in the treated soil respectively.

The HM Cd was not detected in the sewage loamy sandy soil and its concentration was very low in the experimental sewage soil samples. Cd is not an essential element for plant metabolism and is considered phytotoxic causing rapid death.

It can be conclude from our work that the use of combination of bioremediators (canola plants and fungi) can be applied for cleaning HM from polluted soils. Metal stabilization may be in many cases good enough to eliminate serious environmental risks.

Rhizospheric microorganisms may interact symbiotically with roots to enhance potential metal uptake. Some microorganisms may secret organic compounds which increase bioavailability of nutrients and facilitate root absorption of essential metals, such as Fe and Mn as well as nonessential metals, such as Cd (Crowley *et al.* 1991; Barber and Lee, 1974; Salt *et al.* 1995). It was demonstrated by Sayer and Gadd 2001) that citric and oxalic acid and the *A. niger* culture filtrates can bind Co^{2+} and Zn^{2+}.

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