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BIOREMEDIATION OF POTENTIAL TOXIC ELEMENTS IN VARIED SOILECOSYSTEMS (GREENHOUSE SCALE)

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Abstract

Bioremediation technologies are environmentally friendly and cost-effective approaches for potential toxic elements (PTEs) in polluted soil ecosystems. Results indicated that Kafr-el-Sheikh soil had a risky concentrations of the studied PTEs according to permissible limits according to FAO and WHO and the index of Geoaccumulation (I*geo*). In a pot experiment, four major bioremediation strategies, i.e., phytoremediation, natural attenuation, bio augmentation and bio augmentation with bio stimulation were tested. The experiment was performed using soil ecosystem exposed to pollution with PTEs for long periods, collected from Kafr-El-Sheikh Governorate. Tested bacterial strains used in bioremediation showed close proximity with *Pseudomonas aeruginosa KY549647* and *Enterobacter cloacae JX 885522* according to 16S rRNA gene sequence analysis and the yeast isolate showed close proximity with *Saccharomyces cerevisiae KF747750* according to universal fungal ITS gene analysis. Results indicated that tested microorganisms showed signs of growth promotion that might be proved on all tested treatments accompanied by decreasing soil pH. The highest reduction was recorded under the application of rock phosphate (RP) plus *Enterobacter cloacae* (PH 7.4). Radish was used as a test plant. Enhancement in radish growth, represented by increase in the total fresh biomass, which was observed under application of soluble organic matter amended with mixed culture consortium (T_2) (131g). Results also showed that some treatments enhanced phytoextraction of tested potential toxic elements leading to a reduction in their total concentration in the soil under the permissible limits set by FAO and WHO, as observed in mixed culture consortium treatment (T_1) and in soluble organic matter amended with mixed culture consortium (T_2). Other protocols led to a decrease PTEs uptake by radish plant as in *Saccharomyces cerevisiae KF747750* a (T_5) and *Pseudomonas aeruginosa KY549647* plus a mixture of kaolinite and bentonite (T_3) and these treatments are efficient in rapid decontamination of heavily contaminated soils for safe food production. Results confirmed that used microorganisms were promissing tools for heavy metals bioremediation.

Key words : Potential toxic elements, Bioremediation, Phytoremediation, bio augmentation with bio stimulation.

Introduction

Soil contamination with potential toxic elements (PTEs) exerts a high worldwide concern. Unlike organic contaminants, PTEs do not easily undergo microbial or chemical degradation; their concentration in soils persists for a long time. Potential toxic elements invade the food chain and cause negative consequences on human health and natural ecosystems (Popescu, *et al.,* 2009). Such situation drew the attention of scientists to innovate proper technologies for heavy metals bioremediation. Bioremediation is management biotechnology that makes use of a variety of microorganisms and hyper-accumulator plants solely or in associations for removing and/or neutralizing pollutants from soil ecosystems (Akcil *et al.,* 2015). In contrast to "traditional" soil remediation technologies, biological methods are environmentally friendly and particularly attractive because of their low cost and relatively simple maintenance (Mirsal, 2008). *Pseudomonas aeruginosa,* Yeast (*S. cerevisiae)* and phosphate dissolving bacteria are commonly used

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microorganisms for the decontamination of PTEs and for plant growth-promotion. They have been reported extensively in enhancing the plant-growth and soothe plants against various environmental stresses including metal stress (Mirabal *et al.*, 2008 and Li and Ramakrishna, 2011).

Natural attenuation, bio augmentation and phytoremediation are examples of successful biological remediation strategies that approved for the remediation of soils affected by different types of pollutants as the PTEs. Natural attenuation consists of the use of natural processes (e.g. biodegradation by indigenous microbial communities, dispersion, sorption, volatilization, (bio) chemical stabilization to contain and/or reduce the concentration of pollutants at contaminated sites (EPA, 1999, Declercq *et al.*, 2012). Bio augmentation is enhancing of bio degradative capacities of contaminated sites by the introducing of single strains or consortia of microorganisms in desirable conditions (Lebeau, 2011). Natural attenuation, bio augmentation and phytoremediation strategies can be used not only as remediation technologies in themselves but also in combinations. For example, bio augmentation can be combined with phytoremediation to strengthen clean-up processes (Glick, 2003).

On the other hand, soluble organic matter and clay minerals are cost-effective and eco-friendly chemical amendments from/with natural resources, which were used effectively in the bioremediation of several environmental pollutants (Saber et *al*., 2012b). Bolan *et al.*, (2003b) stated that application of organic and/or inorganic remediation amendments such as clay minerals, phosphates, lime, organic matter, iron and manganese oxides and coal fly ashes, etc., fixed up potential toxic elements in soil ecosystems, motivate their immobilization and reduce their availability to plants.

The present paper aims to study the remediation capacity of PTEs by pure and mixed bacterial cultures, for bioremediation process applications, by achieving a successful remediation process efficient in decontaminating potential toxic elements in soil ecosystems to the level ensuing safe food production.

Materials and Methods

Soil samples

Both uncultivated (B1) and cultivated (B2) surface soil samples (0-30 cm) collected from Kafr-El-Sheikh Governorate were irrigated with mixed sewage, agricultural and industrial effluents from Branch 62 - main canal pronged from Kitchener drain. The selected soil samples were mixed in large containers and air-dried at room temperature, then crushed and sieved through 2mm sieve to remove rocks and un-decomposed organic materials.

Plants

Radish (*Raphanus sativus*) were surface disinfected by immersion in 2% (v/v) hydrogen peroxide for 8 min (Qu *et al.*, 2011), in order to avoid the addition of non-indigenous microorganisms to the system. Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiment.

Microorganisms

Indigenous micro-organisms were locally isolated from Kafr-el-Sheikh soil according to (Atlas, 2005), and identified as *Pseudomonas aeruginosa* KY549647 according to Spilker *et al.,* (2004) *and Enterobacter cloacae* JX 885522 *according* to 16S rRNA gene sequence analysis (Lagacé *et al.,* 2004 and the yeast isolates showed close proximity with *Saccharomyces cerevisiae* KF747750 according to universal fungal ITS gene analysis (Luo and Mitchell, 2002). All microorganisms used in this study were grown in Bioflo & Celligen fermentor / bioreactor, each in its specific growth medium, to reach 10^6 cfu.

Chemical amendments

Soluble organic matter: was obtained from Agricultural Department, National Research Centre and it was extracted from solid compost composed of agricultural wastes including tomato leaves, rice straw and guava leaves.

Clay minerals used in remediation

Bentonite and kaolinite clay minerals were purchased from El-Nasr Company for minerals.

Soil Chemical Characterization

Soil chemical characters were determined according to Page *et al*., (1982) as follows:

1. Soil reaction (pH) was measured using glass electrode in a 1: 2.5 soil water suspension.
2. The electrical conductivity (EC) in dS m^{-1} at 25°C was determined in 1:5 soil water extract.
3. Total PTEs in soil and plant samples were determined as given in Page *et al.,* (1982) using Atomic Absorption Spectrophotometry (AAS) with a Perkin-Elmer Model-2380 instrument according to Saber *et al.,* (2016c).

Assessment of PTEs pollution in the studied soil

The index of Geoaccumulation (I*geo*) was used to asses soil contamination with potential toxic elements (Awadh, 2013) using the following equation (Muller1979)

$$I\,geo = \log2\,(C_m / 1.5 \times Bm)$$

Where C_m is the measured concentration of potential toxic element in soil and B_m is the geochemical background concentration of the PTEs (crustal average), soil is a part of the layer of the Earth's crust and its chemical composition is related to that of the crust (Rahman *et al.,* 2012)

Lu *et al.,* (2009) defined the constant 1.5 in equation as a constant introduced to minimize the effect of possible variations in the background values which may be attributed to lithologic variations in the sediments. Here the focus is between the concentration obtained and the concentration of elements in the Earth's crust.

Greenhouse experiment

A greenhouse experiment in a completely randomized design with three replicates was carried out at National Research Centre (NRC). Disinfected Radish seeds were cultivated in plastic pots filled with 5 kg of the contaminated soil from Kafr-el-Sheikh. The experimental design included mainly four experimental conditions: (a) natural attenuation (NA, intrinsic clean up ability of the soil) included Un-cultivated soil control (UC), (b) phytoremediation as cultivated soil control (CC); (PR, soil vegetated with Radish seeds were also applied to all the treatments), c) bio augmentation-assisted phytoremediation (BA+PR, soil vegetated with Radish and inoculated with *Saccharomyces cerevisiae* only (T_5), and Soil supplemented with a mixture of all tested microorganisms (*Pseudomonas aeruginosa, Enterobacter cloacae and Saccharomyces cerevisiae* (T_1) (d) bio augmentation-assisted bio stimulation BA+BS and soil vegetated with Radish seeds (PR), as included either pots supplemented with rock phosphate at the rate of 1.25 ton fed^{-1} and inoculated with 1 ml/pot of a 10^6 cfu/ml suspension of phosphate dissolving bacteria "*Enterobacter cloacae*" (T_7); or Soil supplemented with a combined mixture of bentonite and kaolinite each at the rate of 1.25 ton fed^{-1} and inoculated with a 10^6cfu/ml suspension of *Pseudomonas aeruginosa* at the rate of 10 l/ fed. (T_3), Or soil supplemented with a combined mixture of bentonite and kaolinite each at the rate of 1.25 ton fed^{-1} (10^6cfu/ml) plus a suspension of all tested microorganisms (T_4) ;or finally soil supplemented with soluble organic matter at a rate of a 20 l/fed (10^6cfu/ml) plus a suspension of all tested microorganisms (T_2).

Bio augmentation was performed every 15 days, i.e., during the experimental time, with the aim to maintain an elevated number of microorganisms throughout the experiment, as described by Huguenot *et al.,* (2015). Non-bio augmented pots received the same amount of sterile distilled water. Each condition was performed in triplicates. Plants were harvested after 45 days of growth in the contaminated soil (the different treatments were grown in parallel) and every time bioaugmentation was performed three days before. Plants were removed from pots, and roots and shoots were separated Soil samples were collected initially and after 45 day, their chemical and biological characteristics and heavy metals contents were estimated. Their vegetative characters were estimated at the maturity stage. Plant samples were thereafter dried at 70° C, grained to fin powder and analyzed for their heavy metals content.

Statistical analysis

Each individual treatment was taken in three replicates; Standard deviation (SD) among the three replicates was calculated using Microsoft Excel (2010).

Results and Discussion

Physicochemical characteristics of the studied soil ecosystem

Results in table 1 revealed that Kafr-el-Sheikh soil ecosystem had clay texture. Soil pH value was 8.08. Also the soil salinity level in the investigated soil samples was 2.013 ms/cm at Kafr El-sheikh soil samples. Kafr-el-Sheikh soil ecosystem contained risky concentrations of Ni, Cd and Cr reaching (65, 72), (13, 14) and (215, 280) ppm) in cultivated (B1) and uncultivated (B2) soil ecosystems, respectively, these values exceeding the permissible limits set by FAO and WHO as showed in (Table 2).

Assessment of Geo-accumulation (I*geo*) index and contamination level of tested soil samples

Table 1 also represents PTEs status in the tested soils. According to used index data, cadmium showed extremely pollution status compared to other measured PTEs in both samples tested, while Cr pollutant values put in moderate to strong pollution status.

The values of Cu showed moderate pollution status in B1, increased to moderate to strong pollution status in B2. Nickel I*geo* values were in moderately status in both sites. The Zn values, however, showed unpolluted to moderately status in all tested sites.

Results in table 1 represent the effect of pollution source of HMs in selected sites on the status of pollutants. The irrigation water of this farm is mixture of Nile water mixed with sewage and industrial effluents, the application of this mixture for a long period led to increase some of HMs concentration in used soil samples selected in this work.

Table 1: I_{geo} values and levels of pollution in different soil ecosystems.

Site	Zn		Cu		Ni		Cd		Cr	
	I_{geo} value	Pollution level	I_{geo} value	Pollution level	I_{geo} value	Pollution level	I_{geo} value	Pollution level	I_{geo} value	Pollution level
B1	0.74	Un-polluted to moderate	1.64	Moderate	1.2	Moderate	4.4	Extreme	2.03	Moderate to strong
B2	0.89	Un-polluted to moderate	2.04	Moderate to strong	1.36	Moderate	4.55	Extreme	2.36	Moderate to strong

B1: Kafr-Elsheikh cultivated soil B2: Kafr-Elsheikh un-cultivated soil.

Kafr-el-Sheikh samples had the higher I*geo* values for Cu , N i , Cd and Cr (1.64 , 2.04) , (1.2 ,1.36) , (4.4 , 4.55) and (2.03,2.36) for both cultivated and uncultivated samples, respectively, which indicated that the higher contamination level of tested potential toxic elements was found in Kafr-el-Sheikh samples.

Table 2: Effect of tested treatments on the total concentration of tested potential toxic elements and total fresh biomass of radish in greenhouse.

Type of treatment	pH	Total fresh biomass(g)	Total potential toxic elements concentration (ppm)				
			Zn	Cu	Ni	Cr	Zd
UC	8.08	-	133±5.69	73±5	69± 5.07	215±6	13.5±1.5
CC	7.75	60.3±5.2	99±6.56	66±.4.04	64±4.16	164±1	4.8 ±1
T_1	7.6	124.6±4.3	78 ± 2	41±1.4	36 ± 4	96±3.6	1.96± 0.3
T_2	7.6	131±6.1	83±3	46±3.6	62±4.16	88±4.3	2.2±0.18
T_3	7.7	104±3.3	104±2.52	55.5±2.1	60±3.05	187±1.5	6.18±0.3
T_4	7.7	97±2	86.33±3	54.4±2.3	58±4.7	163±3.6	3.5 ±0.4
T_5	7.6	76.3±4	125±3.79	54±3.46	64±3.2	2.8±3	8.6 ±0.35
T_6	7.5	91±7.2	91±2.08	47.3±3.5	46.6±2.0	116±7	3.4±0.15
T_7	7.4	80±0.9	87 ±2.52	49.3±2.5	61.2±1.4	122±3	5 ± 0.21
Permissible limits of tested potential toxic elements*			300	100	50	100	3

PL*: Permissible limits of potential toxic elements in soil according to FAO and WHO according to Chiroma *et al.*, 2014.

UC: Un-cultivated control sample, CC: Cultivated control, T_1: Mixed culture consortium, T_2: Soluble organic matter and mixed culture consortium, T_3: Mixture of kaolinite and bentonite and *Pseudomonas*, T_4: Mixture of kaolinite and bentonite and mixed culture consortium, T_5: *Saccharomyces cerevisiae*, T_6: *Pseudomonas aeruginosa*, T_7: *Enterobacter cloacae* +rock phosphate

Changes in radish growth and soil pH associated with the tested amendments applied in contaminated soils

All tested treatments enhanced radish growth and decreased the soil pH clearly which is one of critical factors affecting potential toxic elements availability in soil ecosystem and their uptake by plant compared to control. It is worthy to state that mixed treatments led to higher increase in radish fresh biomass than the single ones. The maximum increase was recorded by application of soluble organic matter amended mixed culture consortium (T_2) and mixed culture consortium protocols (T_1) (117, 106%) compared to control. The maximum depression in soil pH was recorded under the application of *Enterobacter cloacae* amended with rock phosphate (T_7) and *Pseudomonas aeruginosa* (T_6) treatments from 8.01 to 7.4 and 7.5., respectively.

Total concentration of PTEs as affected by treatments applied

Results in table 1 showed that, radish exhibited high reduction capacity for total concentration of Zn, Cd and Cr (99, 164, 4.8 ppm) compared to control soil (133, 215, 13.5 ppm), respectively. All tested treatments, displayed a marked reduction in total concentration of all tested PTEs compared to control uncultivated soil (UC), but mixed treatments were more effective than single ones. *Pseudomonas aeruginosa* (T_6) treatment was the most effective single pure culture isolate in reduction of total concentration of tested PTEs.

Application of mixed culture consortium treatment resulted in the highest reduction in total concentration of Ni, Cd and Cr (36, 96, 1.96 ppm) and decreased their total concentration below the permissible limits given by FAO and WHO. Furthermore, Addition of mixture of kaolinite and bentonite and soluble organic matter to mixed culture consortium (T_2 & T_4) led to a marked fixation of

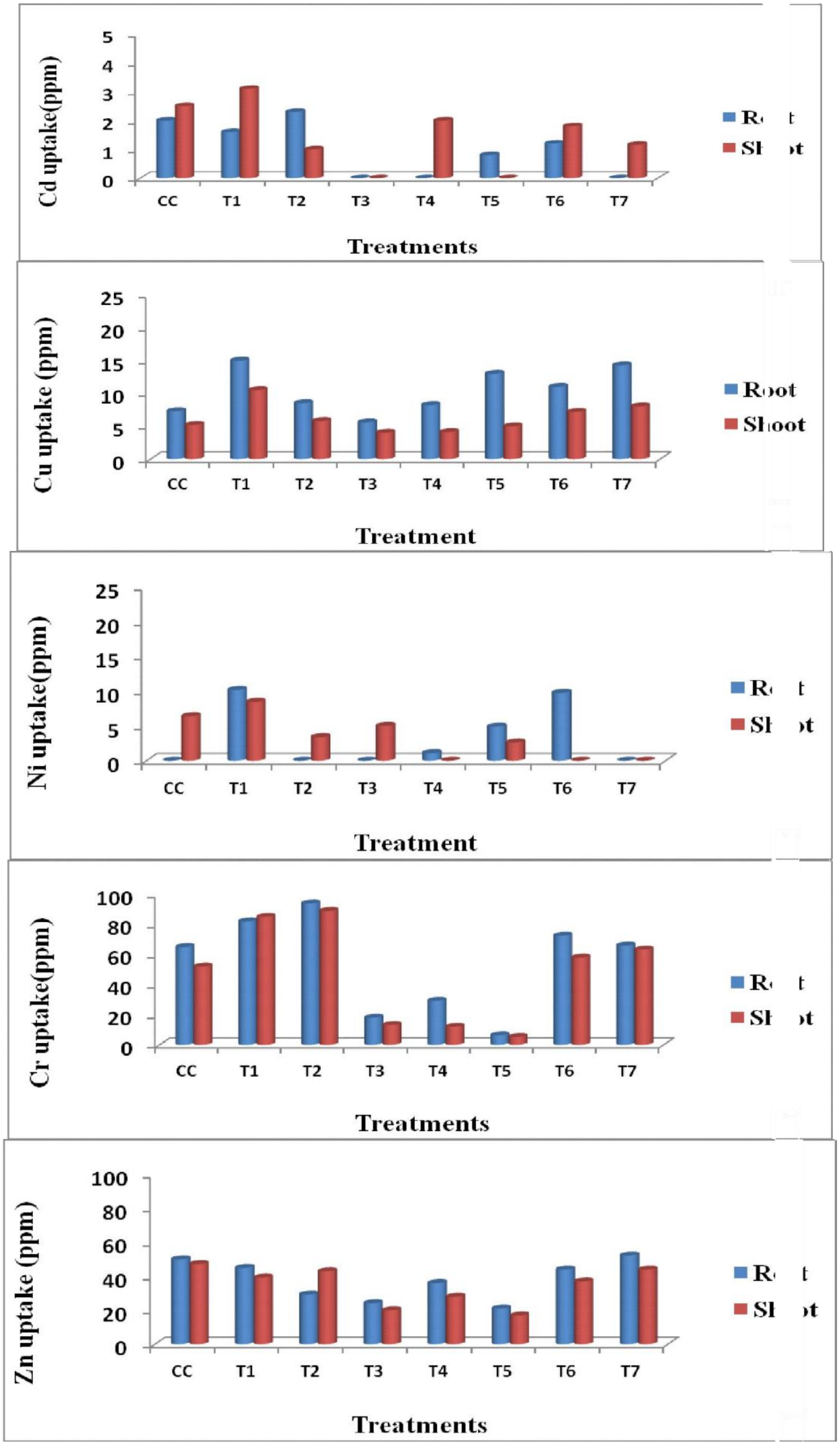

Fig. 1: Effect of different treatments applied on PTEs uptake by Radish.

tested potential toxic elements except for chromium, In case of soluble organic matter amended with mixed culture consortium (T_2) which led to the highest reduction in total concentration of chromium (88ppm) in soil and lowered Cr concentration below permissible limits set by FAO and WHO (100pm).

Effect of bioremediation material applied on PTEs accumulation in Radish plant

Results in Fig. (1) showed that radish was found to have good phytoremediation potential for tested PTEs, especially for Zn, Cd and Cr, (50,47ppm), (2,2.5 ppm), (65, 52ppm) in root and shoot, respectively. Tested treatments exhibited different scenarios for tested PTEs uptake by radish. Some tested treatments enhanced uptake of tested PTEs and their accumulation in radish, such as mixed culture consortium amended with soluble organic matter (T_2) and mixed culture consortium (T_1), *Pseudomonas aeruginosa* (T_6) and *Enterobacter cloacae* amended with rock phosphate (T_7), which exceeded the permissible limits for tested potential toxic elements in vegetables.

In this work, the applied treatments such as a mixture of kaolinite and bentonite plus *Pseudomonas aeruginosa* (T_3) lowered Zn, Cd and Ni to (20, 24ppm), (0.00, 0.00 ppm), (0.00, 5 ppm) in root and shoot respectively compared to control plant, which were below the safe limits (50, 0.2, 10) according to Kabata-Pendias and Pendias (2001). Application of *Saccharomyces cerevisiae* (T_5) lowered concentration of Zn and Ni to safe values in plant and achieved the highest reduction for chromium uptake (6.4, 5.3).

Discussion

Radish (*Raphanus sativus* L.) is well known as a hyper-accumulator plant always associated with different remediation technologies Mathe-Gaspar and Anton (2002). Results indicated that radish was a significant hyper-accumulator plant for all tested heavy elements, it had a significant role in the uptake of the majority of tested PTEs especially for Cd, Cr and Zn as previously reported by Cheng and Huang, (2006) who found high ability of radish to uptake and accumulate Cd in root and shoot.

Pseudomonas aeruginosa, *Saccharomyces cerevisiae* and *Enterobacter cloacae* used in this study are common used microorganisms for bioremediation of potential toxic elements and well known as plant growth promoting (PGPR) bacteria. Jha *et al.*, (2017) stated that *B. megaterium* and *P. aeruginosa* showed a positive growth response of maize in Zn, Pb contaminated soil and accumulated a significant amount of Zn and Pb (587, 227 mg kg^{-1}) in the plant.

Higher performance of *Pseudomonas aeruginosa* toward tested potential toxic elements might be related to multi-physiological, biochemical and metabolic mechanisms (Chien *et al.*, 2013) and contains at least four zinc or cadmium or Lead efflux transporters and two Cadmium, zinc and cobalt (CZC) chemiosmosis transporters Leedjarv *et al.*, (1996).

Enterobacter cloacae amended with Rock phosphate treatment proved that it is a promising agent in heavy metal remediation. It showed a noticeable reduction in all tested total heavy metal concentrations compared to control un-cultivated soil ecosystem mainly due to the depression in pH that led to their solubilzation with different affinities. Different solubilities reflected positively on their uptake in radish and this is in agreement with (Saber *et al.*, 2012) who confirmed that phosphate dissolving bacteria enhanced contaminants uptake minimizing the potential toxic elements in different studied soil ecosystems. Schalk *et al.*, (2011) mentioned that phosphate solubilizing bacteria chelated several potential toxic elements such as As, Cd, Ni and Zn with variable affinities.

Saccharomyces cerevisiae treatment caused a clear reduction in uptake of all tested potential toxic elements except for Cu which enhanced its accumulation in radish. *Saccharomyces cerevisiae* led also to appreciable improvement in total fresh biomass of radish compared to control which reflected that it is a promising growth promoting strain causing a little depression in pH compared to control that confirmed its ability of acid production.The effect of *Saccharomyces cerevisiae* in the remediation of potential toxic elements could be explained as the role of yeasts in plant growth promotion, since a diverse range of soil yeasts use similar mechanisms as soil bacteria (Mirabal *et al.*, 2008), including pathogen inhibition; phyto-hormone production; phosphate solubilization; siderophore production; N and S oxidation and stimulation of mycorrhizal-root colonization (Sansone *et al.*, 2005 and Nassar *et al.*, 2005). Yeast exhibits different mechanisms for bioremediation of potential toxic elements such as immobilization (Wang and Chen, 2009), since it has functional groups such as hydroxyl, amide, amino-sulphydryl and phosphate group (Gadd, 2010).

Application of mixed culture consortium (MCC) achieved the maximum reduction in total concentration of all tested potential toxic elements as well as the highest uptake and accumulation in radish hence it is promising in the phyto-extraction of potential toxic elements in soil ecosystem. Saber *et al.*, (2016b) stated that soil inoculation with *Thiobacillus thiooxidans* and Arbuscular mycorrhizae significantly enhanced the ability of *Brassica napus and Brassica juncea Czern* to uptake the studied potential toxic elements compared to single isolates. Kang *et al.*, (2016), confirmed the synergistic effect of bacterial mixtures on the bioremediation of a mixture of Pb, Cd and Cu from soil ecosystems using four strains. *i.e.*, *Viridibacillus arenosi* B-21, *Sporosarcina soli* B-22, *Enterobacter cloacae* KJ-46 and *E. cloacae* KJ-47. They found that the bacterial mixtures had greater resistance and efficiency in the remediation of potential toxic elements compared to single strain culture with remediation efficiencies of 98.3% for Pb, 85.4% for Cd and 5.6% for Cu recorded.

Addition of Soluble organic matter and mixed culture consortium, T_2, and mixture of kaolinite with bentonite and mixed culture consortium, T_4, led to a marked fixation of tested potential toxic elements except for chromium and this is in agreement with the Cr complexion with organic compounds which was involved in facilitating Cr availability to plants (Srivastava *et al.*, 1999). Marchiol *et al.*, (2004) determined the heavy metal phytoextraction efficiency of canola and radish and the phytoextraction coefficient for each metal calculated. Data indicated that both species were moderately tolerant to potential toxic elements and that radish was more so than canola, but these species showed relatively low phytoremediation potential of multi-contaminated soils.

Conclusions

1. Chemical and biological characteristics of the studied soil ecosystems pointed to that the long term irrigation with low quality water had risky effect on both biological and chemical properties of Kafr-el-Sheikh soil ecosystem. Kafr-el-Sheikh soil ecosystem had high concentrations of Cd, Cr, Ni exceeding the permissible limits given by FAO and WHO and higher geoaccumlation index,which reflect a potential risk for cultivation in this soil and urgent bioremediation is needed.

2. Results obtained from the pot experiment pointed to that the remediation of the tested heavy metals were achieved by different scenarios. Some tested treatments enhanced the phyto-extraction of tested heavy metals leading to reduction of their total concentration in the soil ecosystem to the permissible limits given by FAO and WHO. The most effective treatment was (MCC) treatment that diminished the total concentrations of Ni, Cd and Cr to the permissible limits given by FAO and WHO and attained the highest reduction in Zn and Cu concentrations compared to other tested remediation treatments. Application of soluble organic matter plus mixed culture consortium treatment also exhibited various actions towards the different tested heavy metals, it caused a moderate fixation of tested elements compared to mixed culture consortium treatment and it was more efficient in Ni fixation, however it decreased Cd and Cr concentrations to the permissible limits given by FAO and WHO and diminished the total concentration of Zn and Cu clearly. On the other hand further tested treatments decreased both availability and uptake of the tested heavy metals by plant and some of them reached to the permissible limits given by FAO and WHO and led to a safe vegetables production. *Saccharomyces cerevisiae* and mixture of kaolinite and bentonite plus *Pseudomonas aeruginosa* treatments led to fixing the tested heavy metals, leading to a safe food production. This treatment could be applied to treat heavily contaminated soil ecosystem triggering rapid their decontamination and a safe food production

3. Regarding to soil pH, all tested remediative amendments decreased soil pH, the highest reduction was measured under the application of *Pseudomonas aeruginosa* and *Enterobacter cloacae* plus RP (7.5 and 7.4) compared to control soil ecosystem (8.08).

4. All tested remediative amendments led to a growth promotion, the maximum growth promotion was observed by application of soluble organic matter plus MCC, and it increased total vegetative biomass by 117 %. The study concluded that *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* and *Enterobacter cloacae* could be used as effective inoculant for bioremediation of multi-heavy metal contaminated soil ecosystem, as well as for the reclamation of heavy metal contaminated soil ecosystem.

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