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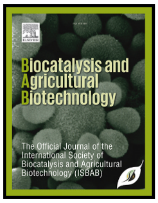

Reduction of heavy metals bioaccumulation in sorghum and its rhizosphere by heavy metals-tolerant bacterial consortium

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ABSTRACT

A consortium of three heavy metals tolerant-plant growth promoting bacteria (HMT-PGPB) (*Bacillus cereus* MG257494.1, *Alcaligenes faecalis* MG966440.1 and *Alcaligenes faecalis* MG257493.1) was tested for its beneficial effects on microbial activities in sorghum' rhizosphere (*Sorghum vulgare* L.). Moreover their ability to reduce heavy metals bioaccumulation in soil and plant during two successive experiments achieved in vitro and greenhouse. Firstly, the activities of HMT-PGPB consortium were estimated in vitro under mixture of four heavy metals (copper, cadmium, lead, zinc) at two concentrations (1000 and 1500 mgL^{-1}), to confirm its heavy metal tolerance and plant growth promoting ability, then applied as a biofertilizer for sorghum cultivated in soil contaminated with heavy metals. *In vitro* results, no antagonism among the tested stains was observed and the HMT-PGPB consortium produced all estimated compounds. Greenhouse results proved that the inoculation of sorghum with HMT-PGPB consortium enhanced the microbial activities like increasing dehydrogenase activity (DHA), reduction of heavy metal bioaccumulation in roots and plants and regulate heavy metals bioaccumulation factor (BAF) in rhizosphere and plant. Moreover, the inoculation of sorghum with HMT-PGPB consortium increased the microbial activities and reduced the accumulation of heavy metals in soil, besides decrease BAF values in rhizosphere and plant. The importance of this study, is that enable us to cultivate the contaminated soils with heavy metals and not neglect large areas of such soils with reducing the environmental pollution, through the inoculation of plants by bacterial consortia, that can remove the polluting elements and at the same time stimulate plant growth.

1. Introduction

Globally, vast areas suffering soil pollution which is mainly due to the industrial, agricultural, and urban wastes (Kacholi and Sahu, 2018). The application of agrochemicals like fertilizers and pesticides has contributed to a continuous accumulation of heavy metals in agricultural soils (Atafar et al., 2010), causing some of the geological and ecological changes (Batayneh, 2012). Although, the accumulation of heavy metals at high concentrations in soil causes negative effects on its ecosystem, heavy metals play an important role in modification of its biological properties (Smejkalova et al., 2003; Millàn et al., 2008; Goretti et al., 2018). To assess the extent of soil pollution, assessing its biological properties is a more sensitive and expressive method than its physical and chemical properties (Srivastava and Thakur, 2006; Garcia et al., 2011; Gall et al., 2015; Goretti et al., 2020).

The rhizosphere microbial communities that have a superior ability to live and achieve their vital activities under high concentrations of heavy metals play a vital role in heavy metal detoxification in contaminated soils (Mishra et al., 2017), among them bacteria has been documented as the most efficient category. Microbes developed its adaptive strategies to tolerate elevated metal levels by different transformation and immobilization processes (Voica et al., 2016). Microbes not only can remove heavy metals from contaminated sites but also convert toxic forms into harmless forms (Elmeihy et al. 2021). Microbes can use one or more of these mechanisms to eliminate nonessential metals and normalize essential metals concentrations in their cells (Vargas et al., 2008; Abou-Aly et al., 2019). Thus, understanding of mechanisms used by heavy metal tolerant microorganisms for remove pollutants may contribute to improving bioremediation processes (El-Meihy et al.,

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2019 a) and encourage us to applied as bio-remediators for polluted sites (Rajbanshi, 2008).

In this concern, the current study was divided into two parts, the first one was conducted in vitro to estimate the ability of HMT-PGPB consortium to produce plant growth promoters under two concentrations of heavy metals mixture. Then, the second part was achieved under green-house conditions to study the effect of HMT-PGPB consortium on microbial activities in sorghum' rhizosphere and the changes in heavy metals bioaccumulation in soil and plant.

The significance of the current study to examine the activities of three bacterial strains (*Alcaligenes faecalis* MG257493.1, *Bacillus cereus* MG257494.1 and *Alcaligenes faecalis* MG966440.1) under laboratory conditions makes them eligible to be used as heavy metals-tolerant plant growth promotors for sorghum (*Sorghum bicolor,* L.) cultivated in heavy metals polluted soils. Furthermore, these strains reduce the accumulation of heavy metals in soil that cause high yield with low environment pollution.

2. Materials and methods

2.1. *In vitro experiment (activities of three HMT-PGPB as a consortium)*

Bacterial strains under study was identified in our previous work (Hamed et al., 2019) and tested for their antagonism before applied as a consortium in the following experiments by cross-lining all strains on the same petri dish and noting the presence of an inhibition zone among them. Equal density (1.0 × 10^7 CFU mL^{-1}) of each bacterial culture was mixed and used as a consortium for the following testes. The plant growth promoting activities of HMT-PGPB consortium were estimated under a mixture of four heavy metals (copper, lead, zinc, cadmium) at two concentrations (1000 and 1500 mg L^{-1}) (Hamed et al., 2019) as follows:

a) Oxidative enzymes, catalase (CAT) (EC 1.11.1.6), polyphenol oxidase (PPO) (EC 1.10.3.1) and peroxidase (POD) (EC 1.11.1.9) were measured spectrophotometrically as described by Aebi (1984), Onsa et al. (2004) and Oktay et al. (1995), respectively.
b) Indole acetic (IAA) and gibberellic acids (GA3) production were estimated as reported by Gilickmann and Dessaux (1995) and Patel et al. (2015), respectively.
c) Proline (Pro), salicylic acid (SA), exopolysaccharides (EPS) and biosurfactants (BS) production were estimated as the method described by Bates et al. (1973), Lukkani and Reddy (2014), Emtiazi et al. (2004) and Suganya (2013), respectively.
d) Three types of siderophores *viz.* hydroxamate, catecholate and carboxylate were measured spectrophotometrically at 430, 495 and 280 nm, respectively as described by Carson et al. (1992).

2.2. *Green-house experiment*

2.2.1. *Experimental design and treatments*

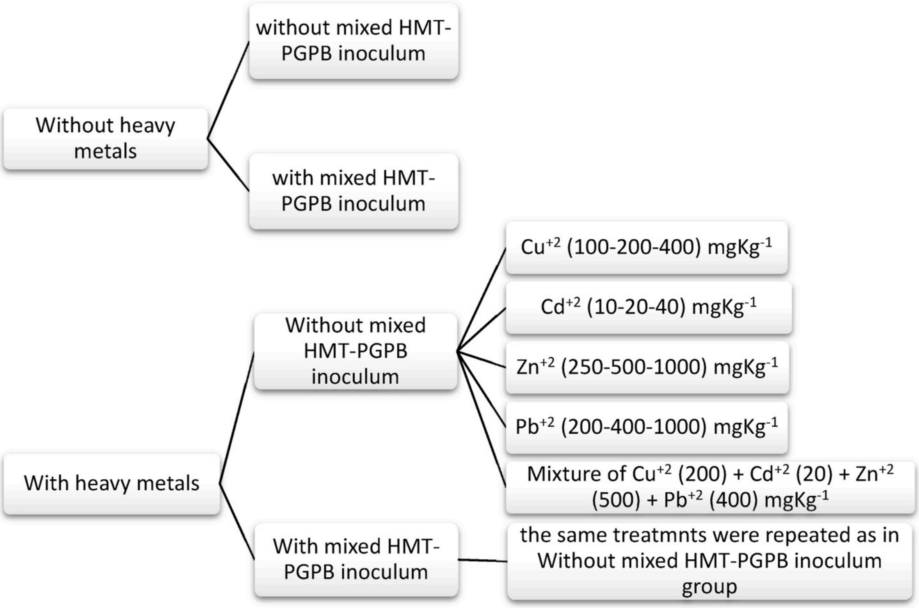
A green-house experiment was accomplished at Benha University. Faculty of Agriculture, Egypt using plastic pots (30 cm depth and 35 cm diameter) and filled with 10 kg pot-1 soil (clay loam, pH 8.2, organic matter 1.52%, N:P:K ratio 1:1.9:1.2). Pots were distributed in randomized complete block design (RCBD) with three replicates for each treatment as displayed in (Scheme 1).

2.2.2. *Preparation of heavy metal solutions for soil treatment*

Three concentrations of each heavy metal salt were prepared separately and added to the experimental soil for one week before cultivation (Setkit et al., 2014) as follows: 100, 200 and 400 mg L^{-1} of copper sulphate ($CuSO_4{\cdot}5H_2O$, M.W.: 249.68 g mol^{-1}), 10, 20 and 40 mg L^{-1} of cadmium chloride ($CdCl_{2,}$ M.W.: 183.32 g mol^{-1}), 200, 400 and 800 mg L^{-1} of lead acetate ($Pb(CH_3COO)_2{\cdot}3H_2O$, M.W.: 379.33 g mol^{-1}), 250, 500 and 1000 mg L^{-1} of zinc sulphate ($ZnSO_4{\cdot}7H_2O$, M.W.: 287.6 g mol^{-1}). And a mixture of (200 + 20 + 400 + 500 mg L^{-1}) of $CuSO_4{\cdot}5H_2O$, $CdCl_{2,}$ $Pb(CH_3COO)_2{\cdot}3H_2O$ and $ZnSO_4{\cdot}7H_2O$, respectively.

2.2.3. *Preparation of heavy metal-tolerant bacterial mixture inoculum*

Alcaligenes faecalis MG257493.1 and *A. faecalis* MG966440.1 at 9 × 10^8 CFU mL^{-1}, and *Bacillus cereus* MG257494.1 at 8 × 10^7 CFU mL^{-1} were prepared in Mueller-Hinton broth medium (Oxoid, UK) at 30 ± 2 °C for 48 h (El-Meihy et al., 2019 a; Abou-Aly et al., 2019). Equal volume of each cell suspension was mixed and used as a bacterial consortium.



Scheme 1. Experimental design and treatments.

2.2.4. Cultivation process

Firstly, 20 g of Arabic gum was added to 100 mL of bacterial consortium suspension and shaken till dissolving, then sorghum' seeds (*Sorghum vulgare* L.) Giza 1 (15 seed/pot) that purchased from Field Crops Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt were socked for 1 h before sowing. After a week of germination, ten vigorous seedlings were preserved for the extended experiment for 9-week. During the trial period, plants were irrigated and chemically fertilized according to the recommendations of the Ministry of Agriculture, Egypt (El-Meihy et al., 2019 b).

2.3. Determinations

2.3.1. Dehydrogenase activity (DHA)

Periodically changes in dehydrogenase activity (EC 1.1.1.) was assayed in sorghums' rhizosphere after 3, 6 and 9 weeks of cultivation according to Glathe' and Thalmann (1970) and calculated as μg TPF/g dry soil/day.

2.3.2. Heavy metals analysis

Heavy metals content was estimated after 3, 6 and 9 weeks in plant and soil as described by Reichman and Parker (2007) and Sahrawat et al. (2002) using atomic absorption, respectively.

2.3.3. Heavy metals bioaccumulation factor (BAF)

The bioaccumulation factor (BAF) of heavy metal was calculated using the following equations as reported by Cluis (2004):

$$\text{BAF}_{\text{Root}} = \text{C}_{\text{Root}} / \text{C}_{\text{Soil}}$$
$$\text{BAF}_{\text{Shoot}} = \text{C}_{\text{Shoot}} / \text{C}_{\text{Soil}}$$

where: C $_{Root}$: heavy-metal concentration in the sorghums' roots (mg Kg^{-1}), C sho$_{ot}$: heavy-metal concentration in the sorghums' shoots (mg Kg^{-1}), C $_{soil}$: heavy-metal concentration in sorghums' rhizosphere (mg Kg^{-1}).

Plants are classified as hyperaccumulators, accumulators, or excluders based on BAF values > 1, equal to 1, or <1 mg kg^{-1}, respectively.

2.4. Statistical analysis

Costate package program version 6.311 (cohort software, USA) was used for statistical analysis. Collected data were analyzed as recommended by Snedecor and Cochran (1980). Then, differences between means of treatments were analyzed using Duncan's multiple range tests (Duncan's, 1955).

3. Results and discussion

3.1. Laboratory experiment

3.1.1. Plant growth promoting activities by the mixture culture of HMT-PGPB strains

Regarding plant growth promotion activities, the HMT-PGPB consortium produced all estimated compounds (IAA, GA3, SA and Pro) under two concentrations of heavy metal mixture (Table 1), that reflecting the synergistic effect of bacterial strains being present as a consortium to mitigate the toxic effect of heavy metals besides no antagonism among them was recorded. Also, results indicated that most compounds were higher at 1000 mg L^{-1} of heavy metals than at 1500 mg L^{-1}, this might be due to that heavy metals act as inhibitors for bacterial growth and their biochemical processes (Aydinalpi and Marinova (2003)). Also, Dimkpa et al. (2012) observed that the presence of heavy metals in *Pseudomonas* sp. growth medium controlled IAA production, they found that zinc and copper increases and decreases IAA production, respectively. Furthermore, microorganisms have several mechanisms in which can resist lead like adsorption by exopolysaccharides and ion efflux to the cell surface (Jarosławiecka and Piotrowska-Seget 2014). Moreover, Karnwal and Bhardwaj (2014) demonstrated that BS have the potential for removal of metals from soil or sediment. These results encourage the application of HMT-PGPB strains as plant growth promotors and heavy metals removers at the same time.

Table 1

Plant growth promoting activities of the heavy metal-tolerant plant growth promoting bacterial strains **(HMT-PGPB) mixed culture**.

Parameters		Heavy metals mixture (mg L^{-1})	
		1000	1500
Oxidative enzymes as absorbance per min.	Catalase (CAT)	0.186	0.123
	Peroxidase (POD)	0.014	0.110
	Polyphenol oxidase (PPO)	0.880	0.156
Phytohormones (mg ml^{-1})	Indole acetic acid (IAA)	4.07	4.36
	Gibberellic acid (GA$_3$)	9.47	6.55
Salicylic acid (SA) (mg ml^{-1})		33.2	30.6
Proline (Pro) (μg ml^{-1})		162.8	146.1
Exopolysaccharides (ESP) dry weight (mg L^{-1})		51.0	213.0
Biosurfactants (BS) (mg L^{-1})		43.0	25.0
Siderophores as absorbance	Hydroxamate	0.475	0.412
	Catecholate	0.321	0.214
	Carboxylate	0.091	0.025

Concerning the enzymatic antioxidant activities by HMT-PGPB consortium, only peroxidase was higher under 1500 mg L^{-1} of heavy metals mixture than 1000 mg L^{-1} in contrast with catalase in which its highest value was recorded at 1000 mg mL^{-1} (Table 1). In this regard, Sharma and Dietz (2009) recorded a relationship between salicylic acid and plant tolerance to metals. Correspondingly, Popova et al. (2012) reported that proline production, lipid peroxidation, CO$_2$ fixation was affected by Cd$_2$ treatment. At the end of this part it can be concluded that heavy metal resistance and enhanced plant growth activity are two of the most important characteristics of bacteria that will be applied as a bioremediator. Therefore, isolating of bacteria having these characteristics from contaminated sites is useful and imperative to develop the effective bioremediation process.

3.2. Greenhouse experiment

3.2.1. Effect of different heavy metals concentrations on dehydrogenase activity

Although the inoculation of sorghum with HMT-PGPB consortium without heavy metals cause increase in DHA than uninoculated ones, the uninoculated plant's rhizosphere also exhibited DHA. This trend was true during all experimental periods and could be attributed to the respiration process of native microorganisms in soil which stimulated by root exudates. Otherwise, in control treatments (without heavy metals), the inoculation of sorghum with HMT-PGPB consortium cause increase in DHA than uninoculated ones. This trend was true during all experimental periods (Fig. 1).

DHA recorded highest values after 3 weeks of cultivation and then gradually decreased to reach its minimum activity after 9 weeks. This trend of results was logic and might be due to that root exudates increased during vegetative growth than other growth stages, the differences in multiplication rate of different soil microorganisms during flowering stage and the high root-colonizing ability of these strains as discussed by Chebotar et al. (2001) that the success of seed inoculation with beneficial microbes was closely related to their colonizing ability.

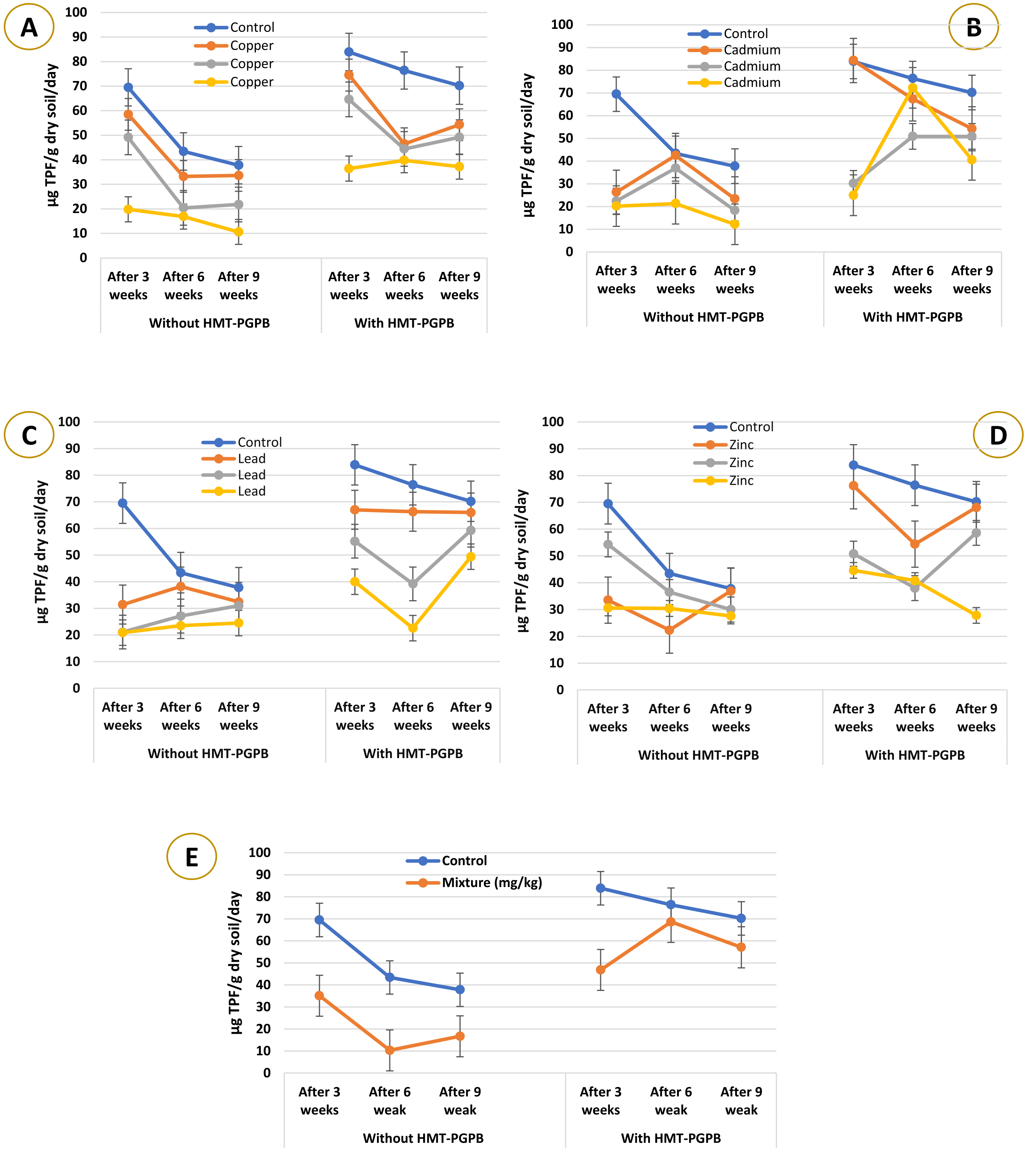


Fig. 1. Dehydrogenase activity (DHA) in sorghum rhizosphere under various treatments.

Heavy metals at high concentrations recorded toxic effects on microbial activities than low concentrations thus, dehydrogenase activity was inversely proportional to heavy metals concentration in both inoculated and non-inoculated plants grew in soil treated with heavy metals. The biological activities of soil microbial communities and their biodiversity were negatively affected by heavy metals in soil (Henry, 2000; Belikov et al., 2005).

Copper at 400 mg kg^{-1} soil was the toxic metal for non-inoculated plants and gave the lowest significant DHA values (Fig. 1 A), while cadmium at 40 mg kg^{-1} soil was the most significant toxic one for the inoculated treatments with HMT-PGPB consortium and gave the lowest significant DHA values after 3 weeks of cultivation (Fig. 1 B). This proved that cadmium is one of the most toxic metals that inhibits most of biological activities in soil and plant (Belimov et al., 2005). Concerning the beneficial effects of HMT-PGPB consortium in alleviation of heavy metals toxic effects, Rajkumar and Freitas (2008) demonstrated that soil microbes capable of resist toxicity by many mechanisms as transformation of toxic metals into harmless forms, immobilizing toxic metals on their outer membranes or binging intracellularly with polymers, and by precipitation or biomethylation. Excreting of organic acids by soil microbes play an important role in solubilization of unavailable forms of heavy metal-bearing minerals (Abou-Shanab et al., 2003) which enhanced their tolerance to heavy metals and increase its activity as bioremediator (Glick, 2003).

3.2.2. Accumulation of heavy metals in soil

Heavy metals concentration in soil was varied depending on different treatments, in this concern no heavy metals were recorded in control soil, this was logic and true because no heavy metals were recorded soil analysis before cultivation besides no heavy metals were added to it. Lead and zinc had accumulated at high concentrations in rhizosphere either in inoculated or uninoculated plants with HMT-PGPB consortium, this trend was observed in soil treated with them at different concentrations (Fig. 2). As reported by Darabi et al. (2016), the accumulation of cadmium in soil increased with the increasing of its concentration in irrigation water.

Heavy metals concentration was gradually decreased after 3 weeks of cultivation to reach their minimum values after 9 weeks (Fig. 2). This trend was observed in rhizosphere of either inoculated or uninoculated plants and may be related to soil structure and its microbiological properties or related to root exudates such as siderophores that increased with the progress of plant growth as discussed by Tak et al. (2013) that the availability of metal is closely related to physical, chemical and biological properties of soil in addition to microbial secondary metabolites. Thus, soil microflora can detoxify heavy metals either by direct or

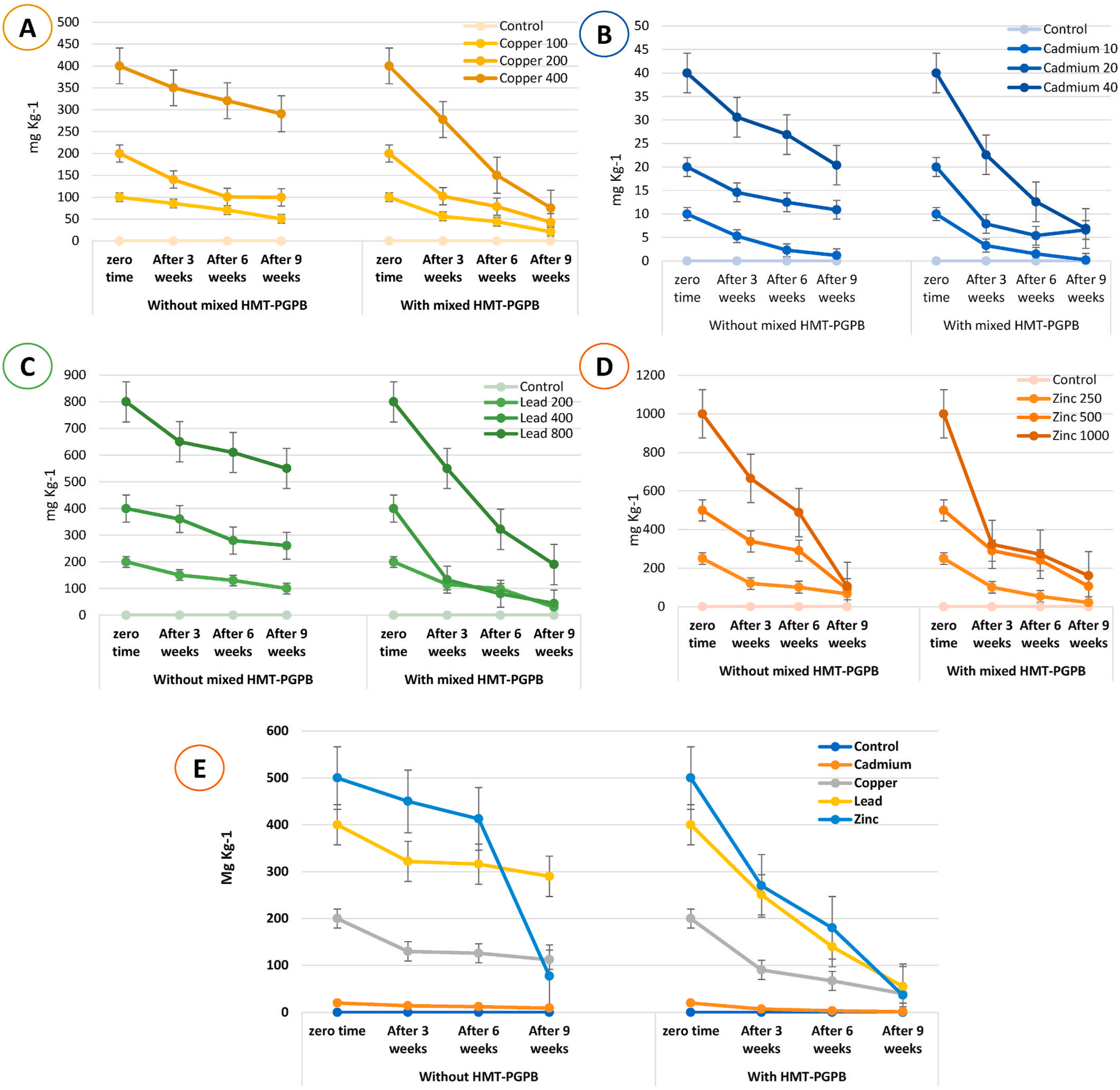


Fig. 2. Accumulation of heavy metals in sorghum rhizosphere under various treatments.

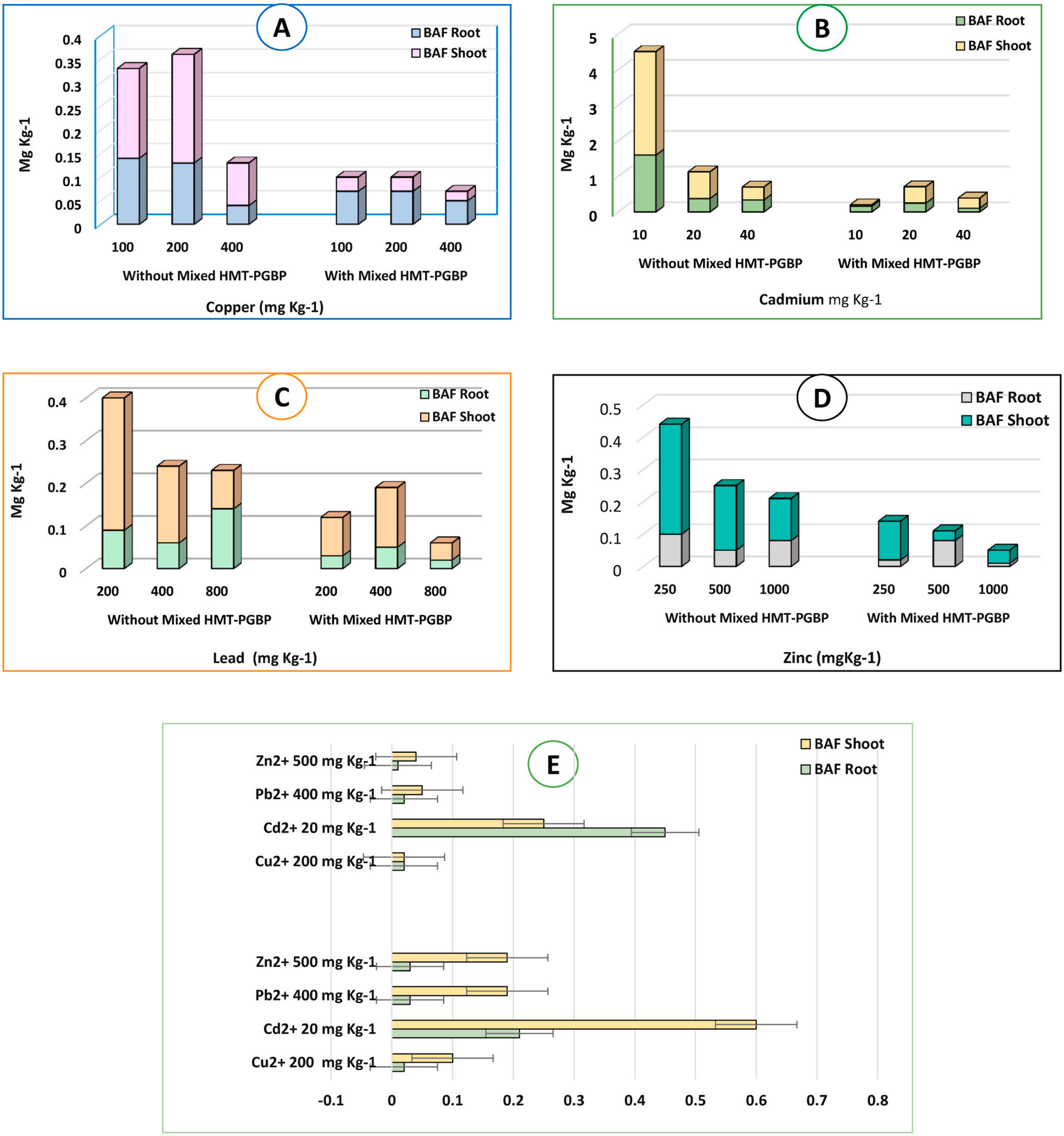


Fig. 3. BAF of heavy metals in sorghum rhizosphere and plant under various treatments.

indirect activities in soil like precipitation, acidification, chelation, immobilization besides effect on plant growth dynamics. Moreover, the application of heavy metals as a mixture cause significant decrease in their concentrations in soil than individually application of each heavy metal. This trend was true with inoculated and uninoculated plants, and because that the presence of heavy metals combined may be inhibits each other. Soil's ability to accumulate heavy metals depended on pH value and different compounds in soil like sulphates and this play an important role in soil pollution (Darabi et al., 2016).

The application of HMT-PGPB consortium as sorghum' inoculum cause decrease in concentrations of all estimated heavy metals, and might be due to the efficiency of heavy metal tolerant bacteria to alleviate toxic effects of heavy metal by maintaining soil structure, fertility, and in remediating soils. So, this copartner-ship between plants and soil microbes could be applied as an important way to enhance successful bioremediation of metal-contaminated soils (Tak et al., 2013). Additionally, the presence of siderophore-producing bacteria in polluted sites could directly or indirectly promote bioremediation process because siderophores protect bacterial bioremediators against the toxic effects of pollutants by reducing the formed reactive oxygen species (Adler et al., 2012).

3.2.3. Bioaccumulation factors (BAF) of different heavy metals as affected by inoculation with HMT-PGPB

BAF $_{Shoot}$ or $_{Root}$ expresses the relationship between heavy metals concentration in plant shoots or roots (mg Kg-1) and relationship with their concentrations in soil (mg Kg-1). BAF values of either shoots or rhizosphere of the uninoculated plants were higher than the inoculated ones (Fig. 3). This trend of results was true with all treatments. Except in uninoculated plants and cultivated in soil with cadmium at 10 mgKg^{-1} soil, BAF were lower than 1.0 and this means that sorghum was excluders as Cluis (2004) confirmed, he classified plants as hyperaccumulators, accumulators, or excluders based on BAF values $>$ 1 mg kg^{-1}, equal to 1, or $<$ 1 mg kg^{-1}, respectively.

Otherwise, BAF $_{Shoot\ or\ Root}$ values in uninoculated plants and cultivated in soil with cadmium at 10 mg kg^{-1} were more than 1.0 and this means that sorghum under this treatment was hyperaccumulator to cadmium. These results reflected the beneficial effects of HMT-PGPB consortium as a biofertilizer for sorghum in decreasing BAF values in shoots or in soil.

Regarding BAF $_{root}$, the highest values in uninoculated plants was recorded at lower concentrations of metal then gradually decreased with the increasing of its concentration except lead. Whereas in inoculated plants with HMT-PGPB, BAF $_{root}$ values were increased with the increasing of heavy metal concentration in soil except at highest concentration (Fig. 3 A, B, C, D). In plants cultivated in soil treated with heavy metals at mixture, cadmium was the highest accumulated metal in both uninoculated and inoculated plants. Also, no significant differences were observed between BAF $_{root}$ of copper in uninoculated and inoculated plants. Additionally, the inoculation of sorghum with HMT-PGPB decreased BAF $_{root}$ values (Fig. 3 E).

Concerning BAF $_{shoot}$, results showed that the inoculation of sorghum with HMT-PGPB caused sharp decrease in BAF values under various treatments. In plants cultivated in zinc-treated soil, the highest BAF shoot values were recorded at lower concentration of zinc and decreased with the increasing of its concentration (Fig. 3 D). Like BAF $_{root}$, BAF $_{shoot}$ had the same trend in inoculated plants at all metals and its mixture except in case of zinc (Fig. 3 A, B, C).

Similarly, heavy metals content was increased in various parts of sorghum plant with the increasing of heavy metals concentration either individually or as a mixture while, the application of HMT-PGPB as biofertilizer for sorghum help plants to neglect the toxic effects of heavy metals and enhance growth characteristics (El-Meihy et al. (2019 b)). Moreover, microorganisms in plant rhizosphere affected toxic metals mobility and availability to various parts of plant by acidification, releasing of chelators and redox changes (Abou-Shanab et al. 2003). Additionally, plants exudates can immobilize metals in rhizosphere by forming insoluble compounds or by help in absorption on root system (Kidd et al., 2009). Some plants can accumulate heavy metals in their tissues, so the contaminated sites become free of heavy metals (McGrath and Zhao, 2003).

4. Conclusion

Two experiments were designed in this paper to evaluate the activities of three heavy metals-tolerant plant growth promoting bacterial strains (HMT-PGPB) namely *Alcaligenes faecalis* MG257493.1, *Bacillus cereus* MG257494.1 and *Alcaligenes faecalis* MG966440.1 under laboratory conditions then, estimate their effects on sorghum rhizosphere (*Sorghum bicolor,* L.) in addition to their ability to enhance plant growth. Results of the in vitro experiment proved that the mixture of the three stains gave higher values of all estimated activities at two concentrations of Heavy metals mixture (1000 and 1500 mg l^{-1}). On the other hand, results of the green-house experiment indicated that the inoculated sorghum with HMT-PGPB cause increase in DHA than uninoculated ones during all experimental periods. Also, among all treatments, the application of Pb^{2+} and Zn^{2+} at different concentrations, sorghum rhizosphere can accumulate them in high concentrations either in inoculated or uninoculated plants. Comparison among the four heavy metals accumulation in sorghum rhizosphere, the following order: lead $>$ zinc $>$ cadmium $>$ copper. Moreover, heavy metals concentrations were gradually decreased from the first period (after 3 weeks) to reach their minimum values after 9 weeks. BAF values of either shoots or rhizosphere of the uninoculated plants were higher than the inoculated ones. This trend of results was true with all treatments.

Author's contributions

Hamed E. Abou-Aly[1], **Rasha M. El-Meihy**, and **Taha A. Tewfike** conceived and designed research. **Eman A. El-Alkshar** conducted experiments. **Rasha M. El-Meihy**, **Eman A. El-Alkshar**, and **Ahmed M. Youssef** contributed new preparation method and analytical tools. **Hamed E. Abou-Aly**[1], **Rasha M. El-Meihy**, **Taha A. Tewfike**, **Eman A. El-Alkshar**, and **Ahmed M. Youssef** analyzed data, wrote and edit the manuscript edit the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

No conflict of interest declared.

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