ENHANCED BIO-REMOVAL OF CADMIUM FROM CONTAMINATED SOIL BY AUGMENTATION USING ELEPHANT GRASS RHIZOPHERIC BACTERIA

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The ability of bacteria associated with the rhizosphere of elephant grass to enhance removal of cadmium from contaminated soil was evaluated. Rhizosphere bacteria were isolated using the soil dilution plate method on nutrient agar, centrimide agar, yeast extract mannitol agar, and formulated media specific for *Acidobacterium* spp. and *Azospirrillum* spp. Isolated bacterial response to cadmium toxicity was tested at cadmium concentrations of 0.01, 0.1, 1.0 and 10 mg/L. Resistant isolates were further tested for their cadmium sorption capacity using Atomic Adsorption Spectrophotometer (AAS). Bacterial isolates with high tolerance and sorption capacity were subsequently inoculated as consortium and individually into duplicate soils samples planted with elephant grass containing 4000 g of soil in pots at concentration of 6.118 mg/Kg of cadmium. Same was done for uninoculated contaminated control. Rhizosphere soil in pots was then monitored for 60 days for residual cadmium levels. Results showed that cadmium resistance and sorption were highest in *Pseudomonas* sp., *Bacillus* sp. and *Azospirillum* sp., among rhizosphere isolates. Generally, cadmium levels decreased with time in soil. Cadmium removal was in the order: Bacterial consortium > *Bacillus* sp. > *Pseudomonas* sp. > *Azosprillum* sp. > Unaugmented (control) planted soils. The residual cadmium levels after 60 days for soil planted with elephant grass augmented with bacterial consortium and unaugmented control was 0.705 and 5.701 mg/Kg respectively. Bacteria associated with the rhizosphere of elephant grass thus enhanced the removal of cadmium from contaminated soil.

Key words: Cadmium, bioaugmentation, elephant grass, rhizosphere.

INTRODUCTION

Heavy metals are metals with specific gravity of more than 5 g/cm^3 (Ganesan, 2012). These include toxic metals such as Lead, Cadmium, Mercury, Chromium etc. The past decades have witnessed a steady increase in anthropogenic releases of heavy metals into the environment. These releases are traceable to increased use of agrochemicals, long term application of urban sewage sludge, indiscriminate industrial waste disposal, waste from car exhausts and incineration. Environmental contamination by heavy metals is of great concern due to their striking toxicity even at very minimal concentrations which may range between 1.0 to 10 mg/L (Ahemad, 2014).

The presence of heavy metals in the environment does not only disrupt important microbial processes and transformation in the biogeochemical cycles (Mahajeh and Kausha, 2018), their prolong exposure may cause them to accumulate in the food chain with consequential toxicity. In animals, heavy metals with particular reference to cadmium have been reported to cause renal and liver failures as well as 'itai itai' or bone disease (Baba et al., 2013). In soil, cadmium affects plant health. It has been implicated in the inhibition of certain cytoplasmic enzyme activities and damage of entire cell structure through oxidative stress (Mishra et al., 2017; Ojuedeire and Babalola, 2017).

In contrast to organic pollutants, heavy metals in the environment are not biodegradable. However, some plants such as *Oryzae sativa*, *Vetive grass*, *Leminar minor* and *Allium sativum* have been demonstrated to be excellent hyperaccumulators of metals, thus capable of removing them from the soil. Microorganisms can also independently remove heavy metals from the environment. Nevertheless, for efficient removal of heavy metals from the environment, plant-microbe

synergism is required. This strategy which is known as rhizoremediation (phytoremediation) involves the removal of pollutants from the soil through mutual interaction of plant roots and associated microorganisms. It is an efficient, low cost, environmental friendly, and a sustainable technique (Ubogu et al., 2019a). Rhizosphere microorganisms greatly enhance the process of phytoremediation by siderophore production, secretion of plant growth promoting substances, acidification and redox exchanges (Lee *et* al., 2008; Tchunwon et al., 2012; Oves and Zaidi, 2013; Kielak et al., 2016; Ojuedeire and Babalola, 2017).

As a result of possible toxicity in the use of food crops, the use of non-food plants in the removal of heavy metals from soil is a more acceptable option. *Pennisetum purpereum* (also known as elephant grass) is a non-food grass. The grass is widely naturalized in tropical and sub-tropical regions of the world. It is included in the global compendium of weeds where it is listed as agricultural and environmental weed as well as invasive species (Dubeux et al., 2014). Like most weeds it is well adapted to unfavourable conditions. It grows fast upon germination, establish populations rapidly, produce numerous seeds which are easily dispersed and have ability of recruiting none specific microbial species into its rhizosphere. This grass is particularly associated with diazotrophic bacteria. These bacteria colonize different niches in the plant. Endophytic bacteria reside inside while epiphytic bacteria colonize its external surface (Dubeux et al., 2014).

It is on the basis of the afore-mentioned qualities of the plant and the need to broaden the search for efficient and environmentally friendly removal of cadmium from contaminated soil that cadmium-tolerant bacteria in the rhizosphere of elephant grass were investigated in the present study.

MATERIALS AND METHODS

Sample collection

Soil sample and grass (elephant grass) used in this study were obtained from metallic/automobile waste deposition yard (Eku, Delta state, Nigeria). Composite, soil sample for physicochemical baseline study was collected within the depth of 5- 10 cm. It was introduced immediately into sterile black polyethylene bags and conveyed to the laboratory for analysis. Rhizhosphere soil from elephant grass was obtained by adopting the method of Ikediugwu and Ubogu (2012). Thirty plants of about 1 m height were uprooted and jolted gently to liberate soil weakly adhering to roots. Further vigorous agitation of plant roots was done in sterile polytene bag for the collection of rhizosphere soil. Soil samples were then transported to the laboratory and analysed within 30 minutes of collection. Prior to the collection of the rhizosphere samples, the whole grass was taken to the Department of Botany, Delta State University, Abraka, for identification.

Baseline physicochemical analysis of soil sample

The soil sample was analyzed for nitrogen (micro-Kjeldahl method as described by van Reeuwijk, 2002), pH (Hendershot et al., 2006), textural components (hydrometer technique as described by Aliyu and Oyeyiola, 2011). Cadmium was determined using atomic adsorption spectrophotometer (AAS).

Isolation and Identification of Rhizosphere bacteria

Bacteria associated with elephant grass, rhizosphere were isolated following ten-fold serial dilution of one gram of each rhizosphere soil sample. They were subsequently plated (0.1 ml of dilution factors ranging from 10^{-3} to 10^{-6}) into both nutrient agar and selective agar for isolation of *Acidobacterium* using the pour plate method. Nutrient agar plates were then incubated at ambient temperature for 24-48 h while plates for the isolation of *Acidobacterium* were incubated at 30°C for 3-10 days.

The medium for the isolation of *Acidobacterium* spp. was adopted from Campanharo et al. (2016). It comprised (g/L): sucrose 30; KH_2O_4 1.8; $MgSO_4.7H_2O$ 0.2; yeast extract 1.0; agar 15 and pH 5.0. Additionally, *Azospirillum* spp were sourced using the method prescribed by Haiyambo and Chimwamurombe (2018). One gram of rhizosphere soil was introduced into 9 mL of enrichment broth contained in a test tube. This was incubated under

microaerophilic condition, at 30°C for ten days; after which, 0.1 ml was aseptically withdrawn and sub-cultured into Yeast Extract Mannitol Agar (YEMA) using the pour plate method. Further incubation under microaerophilic condition, at 30°C for five to ten days was done. Distinct, pinkish colonies suggestive of *Azospirillum* were sub-cultured for biochemical characterization. The enrichment broth for the isolation of *Azospirillum* spp. as compounded by Bashan et al. (2011) comprised (g/L) the following: gluconate 5; trypton 5; yeast extract 5; glucose 5; NaCl 1.2; $MgSO_4.7H_2O$ 0.25; K_2HPO_4 0.13; $CaCl_2$ 0.22; K_2SO_4 0.17; Na_2SO_4 2.4; $NaHCO_3$ 0.5; Na_2CO_3 0.09 and Fe (III) EDTA 0.07. Characterization of bacterial isolates from rhizosphere of elephant grass was based on morphological, cultural and biochemical properties in line with Beygey's Manual of Determinative Bacteriology John et al., 1994; Gurrity et al., 2005).

Determination of isolates response to toxicity of Cadmium

The short-term shake flask method for testing toxicity of chemicals described by Oranusi and Ogugbue (2002) was adopted for testing bacterial isolates response to cadmium toxicity. Cadmium concentrations of 0.01, 0.1, 1.0 and 10 mg/L were prepared using $CdSO_4$ salt in deionized water. These were then dispensed in 9 ml amounts into test tubes. After which, 1 ml of standardized inoculum of each isolate (ranging from 1.34 to 2.00 × 10^6 CFU/ml) was introduced into the respective metal concentrations. Incubation under shaken condition (150 rpm) at 30°C was carried out immediately for 24 h. At the end of the incubation period, 0.1 mL was withdrawn and inoculated into nutrient agar and YEMA (for *Azospirillum* spp.) using the pour plate method. The plates were further incubated at 30°C and examined for growth at intervals of 24 h for a maximum of 7 days. Additionally, YEMA plates were given microaerophilic condition. Growth at all tested concentrations was taken as resistance or cadmium tolerant.

Determination of bacterial isolates' capacity to sorb Cadmium

Isolates that were cadmium tolerant were selected for this experiment. Again, a standardized inoculum of each isolate (ranging from 1.34 to 2.00 × 10^6 CFU/ml) was introduced into 9 ml of each of the various cadmium concentrations [0.01, 0.1, 1.0 and 10 (mg/L)]. Incubation at 30°C under shaken condition (150 rpm) for 24 h followed immediately. At the end of the incubation period, cells were harvested by centrifugation and residual cadmium concentration in the supernatant was determined using atomic AAS. Cadmium percent biosorbed was calculated as shown below:

$$\% \text{ Cd uptake} = (C_i - C_f / C_i)100$$

Where, C_i = Initial cadmium concentration, C_f = Final cadmium concentration

Determination of the effect of augmentation with rhizospheric bacteria on the removal of cadmium from soil

The method described by Ubogu et al. (2019b) was adopted. Having confirmed the presence of cadmium (6.118 mg /Kg) in the baseline physicochemical analysis of soil employed in this study, soil sample was distributed in 4000 g amounts into five sets of new, clean, pre-sterilized plastic pots in duplicate. Then, each cadmium tolerant rhizobacteria isolate as determined above (*Pseudomonas* sp., *Bacillus* sp. and *Azospirillum* sp.) was scaled up to 1.58 - 2.71 × 10^9 CFU/mL and inoculated into the first, second and third pots respectively. However, the fourth pot received a consortium of the three isolates while the fifth was uninoculated to serve as control. The contents of each pot were mixed thoroughly and were all left to stand for 7 days to enable organisms for augmentation to adequately acclimatize.

Thereafter, fresh stem cuttings of elephant grass with two or three nodes and about 15 cm in length were cultivated into each pot. Soils in the various pots were watered about 2-3 times a week to maintain necessary moisture content (but not to saturate). At intervals of 30, 45 and 60 days, soil samples were collected from various pots for the determination of residual cadmium which was done using atomic absorption spectrophotometer. Also, on each analysis day, elephant grass in each pot was uprooted to obtain rhizosphere soil which was used for the determination of total viable

counts of the bacterial isolates introduced. Nutrient agar, Centrimide agar, and YEMA were used to monitor populations of *BacilIus* sp , *Pseudomonas* sp. and *Azospirillum* sp. respectively. However, rhizosphere soil obtained from pot augmented with *BacilIus* sp. was pre- treated by heating to 80^{o}C for selective isolation of *BacilIus* sp. Thereafter, roots of harvested grasses were thoroughly washed in several changes of sterile deionised water to completely remove entire soil particles and then the whole plant was dried, crushed, digested and assayed for Cadmium uptake using Atomic absorption spectrometry (AAS).

Statistical analysis

Data obtained were analysed using mean, analysis of variance (two way) and t-test.

RESULTS

The result of the physicochemical analyses of soil sample, as presented in Table 1, indicated that the soil is slightly acidic and contaminated with cadmium at a concentration of 6.118 mg/kg. Several bacteria isolates belonging to four genera including *Pseudomonas*, *Bacillus*, *Citrobacter* and *Azospirillum* were observed to be associated with the rhizosphere of elephant grass. However, *Citrobacter* sp. was obtained from only two of thirty elephant grass samples. Also, it was observed that *Acidobacterium* spp. were absent in all tested rhizosphere samples. The frequency of occurrence of the isolates in the grass samples analysed is as shown in Table 2. Additionally, some of these bacteria were found to be cadmium tolerant. It was observed that all isolates of *Pseudomonas* (100%) demonstrated resistance to Cd. Twenty- seven out of 30 isolates of *Bacillus* obtained (90%) and six of nine *Azospirillum* isolates obtained (66.67%) were resistant to the toxicity of cadmium (Table 2).

The results of the determination of the isolates' ability to sorb cadmium *in vitro* are as presented in Figure 1. It was noticed that *Pseudomonas* sp. and *Bacillus* sp. were able to absorb Cd significantly at all tested concentrations but *Azospirillum* sp. could not efficiently absorb the heavy metal at all tested concentrations. The aforementioned isolates displayed the capacity to remove cadmium but to varying degrees. Although, percent cadmium uptake declined as initial cadmium concentration increased, there was a significant difference in the amount of cadmium absorbed by each isolate at the various initial cadmium concentrations (at $p \leq$

Table 1. Baseline physicochemical characteristics of soil.

Parameter	Cadmium contaminated soil
pH	6.1
Cd(mg/kg)	6.118
Sand (%)	68.5 ± 0.01
Silt (%)	20.1± 1.5
Clay (%)	4.6 ± 0.5
Nitrogen (%)	0.08± 1.0

Table 2. Frequency of occurrence/cadmium resistance profile of elephant grass rhizobacteria.

Isolate	Number of rhizosphere samples harbouring isolate	Cd-resistant isolates in rhizosphere (%)
Pseudomonas sp.	30	100 (n = 30)
Bacillus sp.	30	90 (n = 27)
Azpspirillum sp.	9	66.67 (n=6)
Citrobacter sp.	2	0 (n = 0)
Acidobacterium sp.	ND	-

ND = Not detected; n= number of isolates that were Cd resistant.

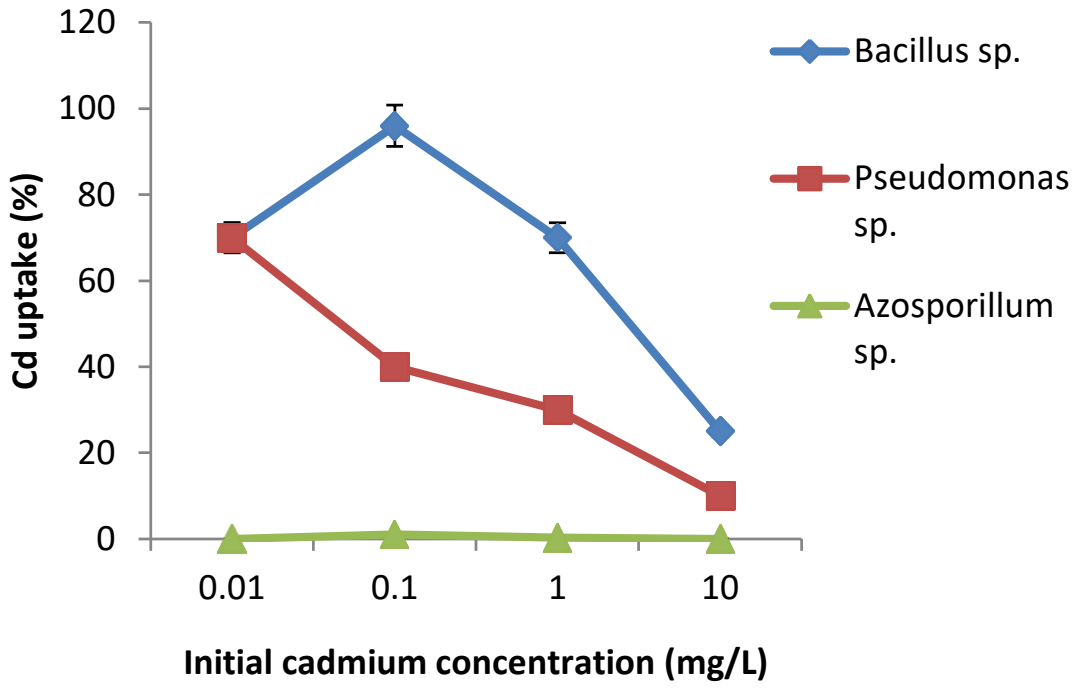

Figure 1. Effect of initial cadmium concentration on uptake by cadmium-resistant isolates.

0.05). Again, analysis of soil in all set-ups for residual cadmium revealed a progressive drop in cadmium concentration as the study period increased. Residual cadmium was lowest in soil augmented with the bacteria consortium and highest in uninoculated soil or soil that did not receive any form of augmentation as presented in Figure 2. Consistently, at all exposure durations, residual cadmium concentrations in soils were lower in augmented soils. There was a significant difference (at $p \leq 0.05$) in the loss of Cd from the various set- ups as presented in Figure 2.

Furthermore, results of uptake of Cd by elephant grass cultivated in both augmented and uninoculated soils are presented in Figure 3. Higher cadmium uptake was observed in grasses from soils with prior inoculation than grass from the uninoculated set-up. The trend noticed is as follows; cadmium uptake by elephant grass from soil augmented with bacteria consortium > cadmium uptake by elephant grass from soil augmented with *Pseudomonas* sp. ≥ Cadmium uptake by elephant grass from soil augmented with *Bacillus* sp. > Cadmium uptake by elephant grass from soil augmented with *Azospirillum* sp. > Cadmium uptake by elephant grass from soil unaugmented. Also, at $p \leq 0.05$, there was significant difference in cadmium uptake by elephant grass in all set ups at all exposure periods.

Finally, the determination of total rhizospheric population of each organism used in augmentation revealed quite a significant increase in the population of each organism as duration of the study increased. The rhizosphere population of each organism used for augmentation was higher than its counterpart from elephant grass rhizosphere from unaugmented soil as represented in Figures 4 to 6.

DISCUSSION

Although very little information exists on the kinds of microorganisms indigenous to the rhizosphere of elephant grass, many reports have it that diazotrophic bacteria such as *Azospirillum* spp. and *Acidobacteria* spp. are common in the rhizosphere of grasses (Dubeux et al., 2014; Trognitz et al., 2016). The complete absence of *Acidobacterium* spp. in all the rhizosphere samples analysed may be due to the chemical composition of the soil from which the grass obtained. Similar observation has been made by Cahyan et al. (2019). It is likely that *Acidobacter* spp. are unable to tolerate cadmium toxicity. The cadmium contamination may also account for the

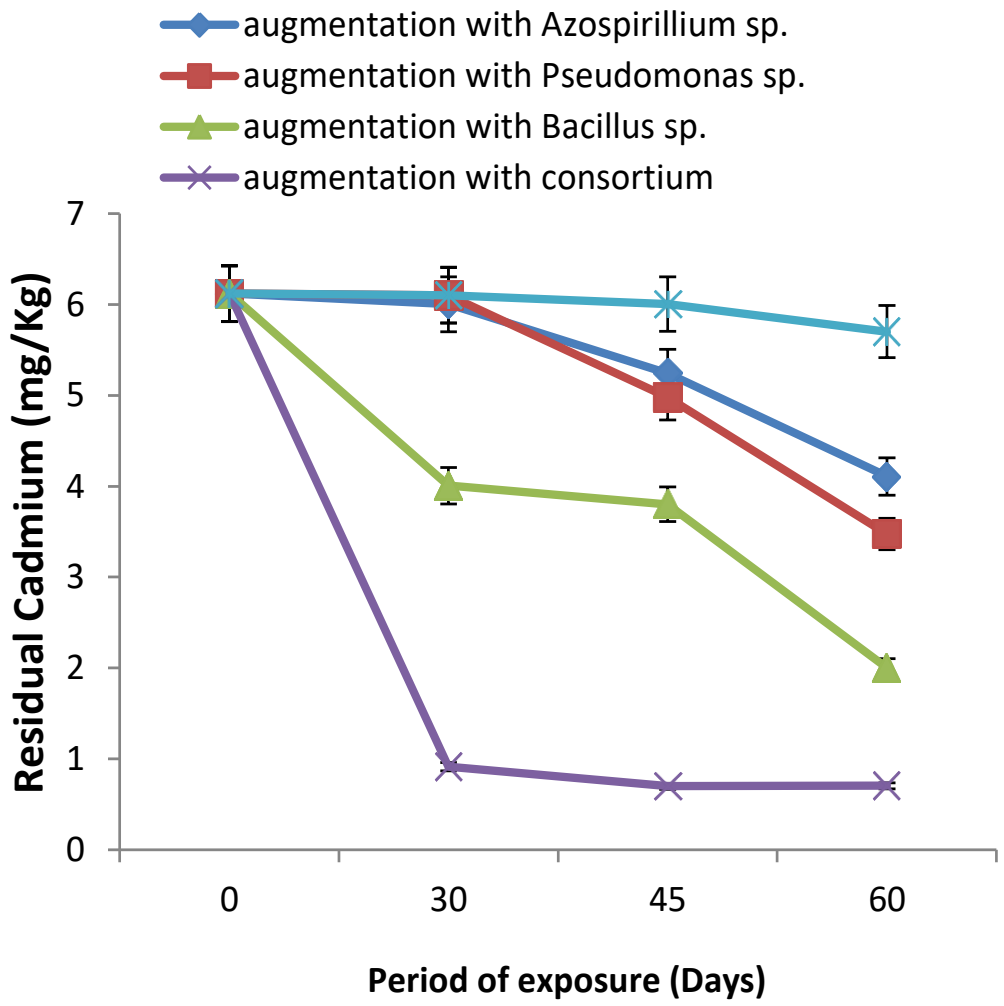

Figure 2. Effect of bioaugmentation on cadmium removal from soil.

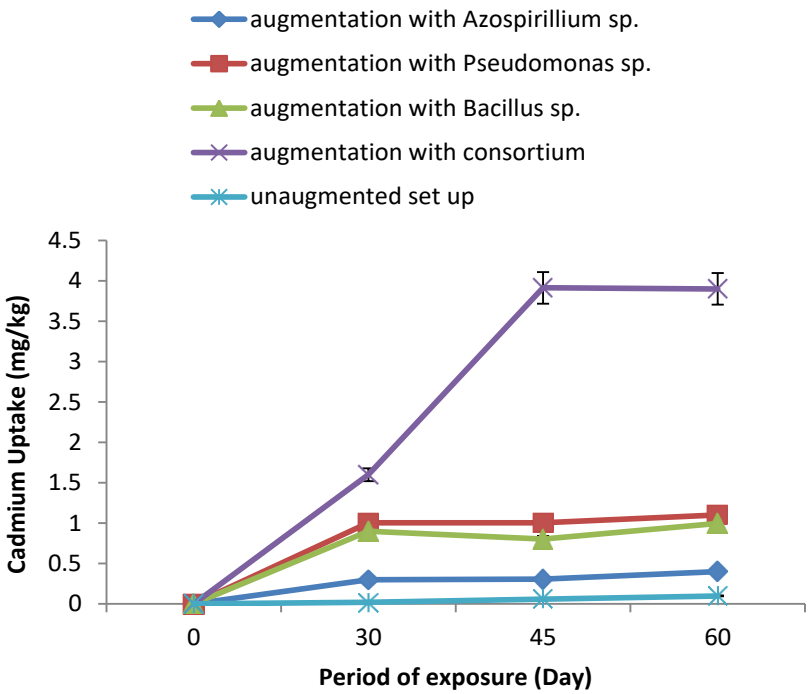

Figure 3. Effect of bioaugmentation on cadmium uptake by elephant grass.

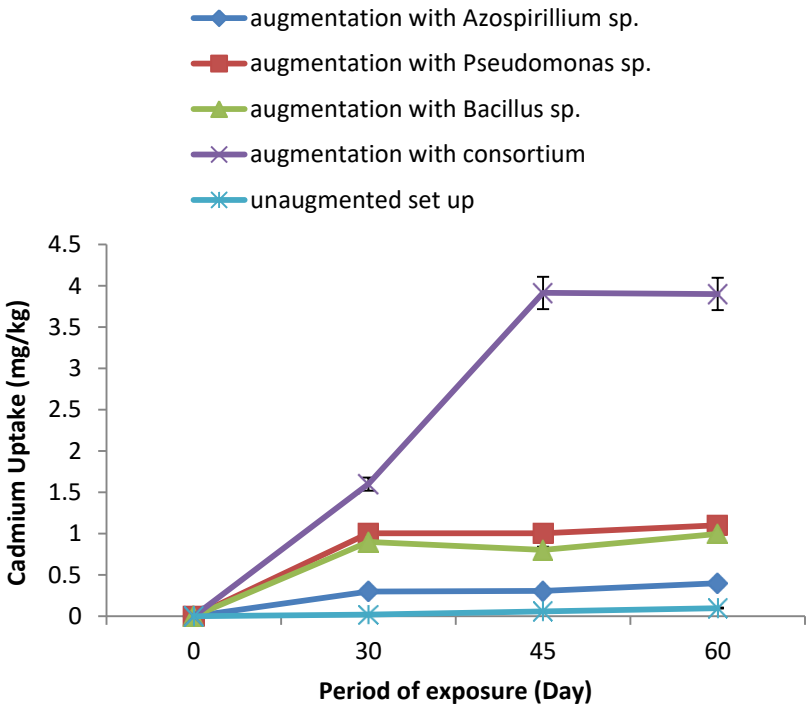

Figure 3. Effect of bioaugmentation on cadmium uptake by elephant grass.

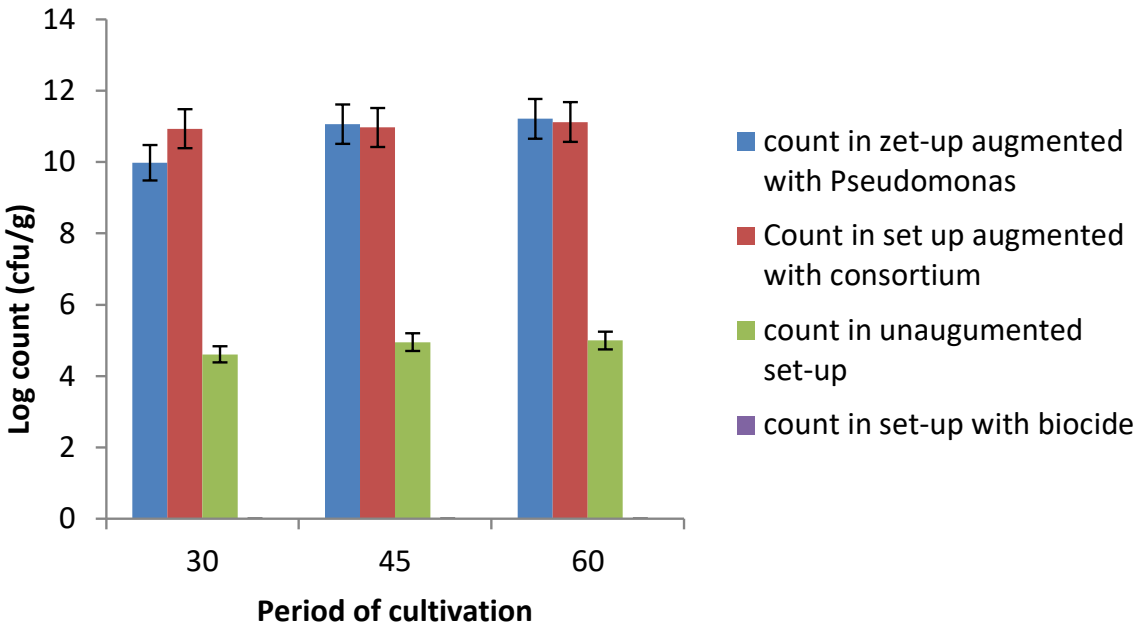

Figure 4. Total *Pseudomonas* count in in various set-ups.

occurrence of Citrobacter in only two of thirty elephant grass rhizosphere screened. However, the other isolates obtained including species of *Pseudomonas*, *Bacillus* and *Azospirillum* displayed potentials to withstand Cadmium toxicity. Similar results had been reported previously (Kunito et al., 2001; Oves and Zaidi, 2013; Seneviratne et al., 2015; Ojudeirie and Babalola, 2017). The resistance displayed may be due to the occurrence of heavy metal resistant genes in the organisms. It might also be due to the presence of one or more detoxification mechanisms within the cell cytoplasm (Oves and Zaidi, 2013). The isolates could have also acquired resistance to cadmium as a result of selective pressure arising from prolonged

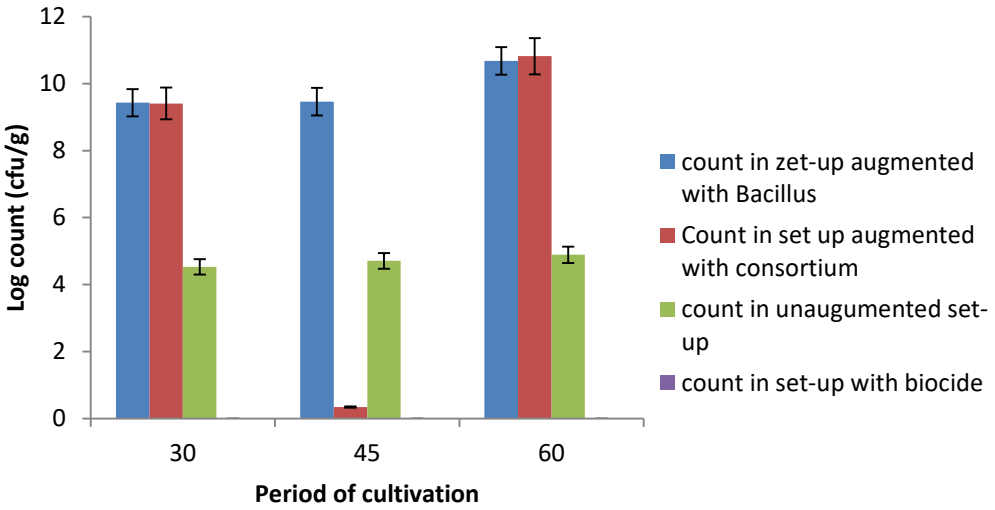

Figure 5. Total Bacillus count in in various mecocosms.

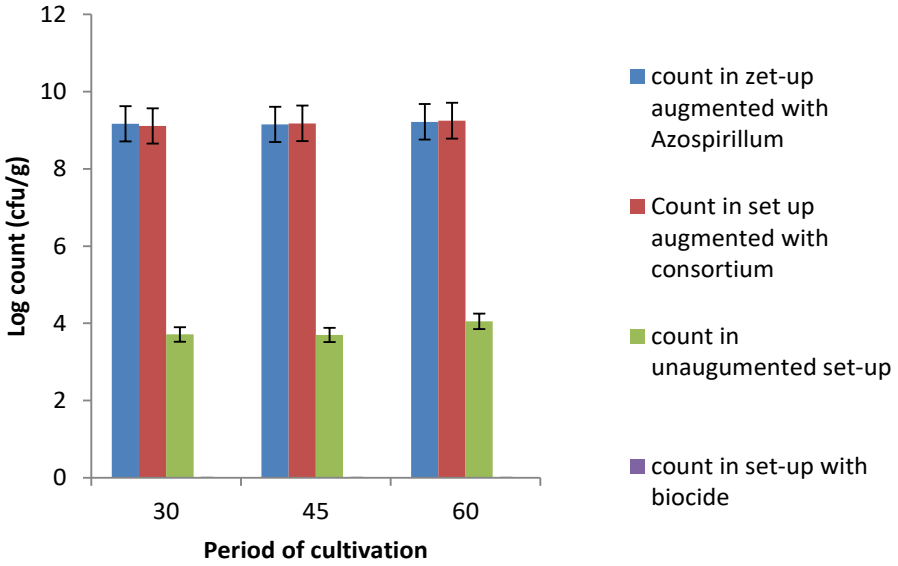

Figure 6. Total *Azospirillum* count in various mecocosms.

exposure to sub-lethal concentrations in the natural environment from which they were obtained. Incidental acquisition of antibiotics and Cd resistant factors have been previously reported in isolates exposed to Cd contaminated environments (Abbas et al., 2014). Furthermore, only some isolates of *Azospirillum* (66.67 % of total *Azopirillum*) isolated from elephant grass were resistant to the toxicity of Cd. The resistance to Cd demonstrated by *Azospirillum* sp. may be linked to transformation processes that probably occurred in its ecological niche. This natural phenomenon could have enabled the delivery of mobile heavy metal resistant genes borne on plasmid from other rhizobacteria to it.

Biosorption of Cd *in vitro* followed previously established pattern in which uptake increased as initial concentration increased. The mechanisms associated with this might be by bioconcentration in/on the cell walls, cytoplasm or spores (Odokuma and Akponah, 2009; Odokuma and Akponah, 2010). In this study, augmentation with the rhizobacteria consortium resulted in the highest uptake of Cd into the elephant grass. Probably, this could be related to the fact that the consortium comprised three different plant growth promoting - heavy metal tolerant bacteria. Similar reports had been made in which a mixture of heavy metal tolerant bacteria biomass and

fungal spores successfully removed Cr and Fe ions (Migahed et al., 2017). The consortium probably tackled the problem by modulating plant growth as well as by altering physicochemical properties of soil to enhance metal bioavailability. This might have triggered Cd uptake by the grass in a greater dimension in comparison to the activity of single bacterial augmentation. The overall effectiveness of Cd removal from soils may be due to synergy between the grass and its rhizobacteria. Inoculation with *Bacillus* sp., *Pseudomonas* sp., and *Azospirillum* sp. showed great promise in the removal of Cd. It can be thought that these organisms which have been hitherto established as plant growth promoting bacteria (Zaidi et al., 2006*;* Mishra et al., 2017) could probably have increased growth and biomass yield of the grass and this might have favoured uptake and translocation of the heavy metal in the plant. This mechanism is exemplified in the way *Azosporillium*, which though was not efficient in the biosorption of Cd *in vitro*, resulted in a high rate of Cd removal when used in soil augmentation. The resistance of *Azospirillum* enabled its persistence in Cd contaminated soil where it possibly stimulated the grass growth through nitrogen fixation. Increased grass biomass may therefore have resulted in additional ability of the plant to:

(i) Accumulate Cd
(ii) Secrete greater amounts of exudates rich in nutrients that stimulated the growth of the rhizobiota and hence increased capability of removing Cd by all biosorbemts present within the rhizosphere.

The nitrogen fixed in soil could also have increased the proliferation of other bacteria in the consortium as well as other members of the elephant grass rhizobiome and thus increasing the metal mobilizing activities and hence increasing uptake into both bacteria and the plant. (Ojuedeire and Babalola, 2017).

Residual cadmium concentrations that were consistently lower in soils that received augmentation than uninoculated soil clearly demonstrates the role played by bioaugmentation in the uptake of cadmium by elephant grass. Mishra et al. (2017) established that plant growth promoting bacteria not only contribute to plant growth enhancement, but also, accelerate the biosorption of heavy metals from soils. The high levels of Cd removed from soil that was augmented with *Pseudomonas* sp and *Bacillus* sp. may be as a result of the production of siderophores, exopolysaccharides and organic acids. There has been report attributing the production of siderophore by Pseudomonas and Indole Acetic Acid (IAA) by Bacillus to the stimulated growth of *Brassica juncea* and enhanced uptake of Ni from Ni contaminated soils (Zaidi et al., 2006). Furthermore, organic acids secreted by microorganisms have been shown to solubilize heavy metals such as Cd, Cu, Pb and Zn hence increasing uptake into plants. Also, IAA production, siderophore producers and nitrogen fixers have been shown to assist in the alleviation of heavy metal stress to plants by either biotransformation or increasing the bioavailability of heavy metals (Wu et al., 2010; Gupta et al., 2014; Pinter et al., 2017). Similar to the results obtained in this study was the report by Luo *et al*. (2012). These co-authours attributed increased Cd uptake from the rhizosphere of sorghum to rhizospheric *Bacillus* sp producing siderophore. Additionally, Pb and Cr uptake by Z*ea mays* was increased by siderophore producing *Pseudomonas auroginosa* (Braud et al., 2009 and Luo et al., 2012); the siderophores complexes with the metal, thereby increasing its bioavailability. Another factor that has been affiliated to the increased biosorption of heavy metal in rhizospheres is the production of exopolymeric substances (Seneviratne et al., 2015) which contain several anionic functional groups (carboxyl, phosphoric, amino and hydroxyl) enabling it to sequester heavy metals. Exopolysaccharide production has been known to be important in the biosorption of metals. More so, rhizospheric bacteria produce more exopolymeric substances than non rhizosphereic organisms (Kunito et al., 2001). *Pseudomonas* sp produces quorum sensing signals which enable the production of exopolymeric substances. This could have been the strategy employed by the isolates used for bioaugmentation in this study to enable elephant grass biosorb Cd more efficiently. It has been established that rhizospheric microorganisms secrete anionic

substances such as glycoproteins, lipopolysaccharides and polysaccharides which act as useful palliative measures in ameliorating metal toxicity around the root zone (Miransari, 2011). In a study, Murthy et al. (2011) found an increase in the metallothione biosynthesis in *Bacillus cereus* when exposed to increased Pb concentration. Metallothione is a metal binding peptide which possibly facilitates the deposition of metals into cells including microbial, animal and plant cells (Kovarova et al., 2009). Additionally, the fibrous root system of the grasses grants them good ability to provide a large surface area for the production of nutrient-rich exudates and adherence of the isolates (Ubogu et al., 2019a). This may account for the increase in bacterial counts obtained in the rhizosphere within the study period. Therefore, the study strongly demonstrates that elephant grass possesses rhizobacteria efficient not only in biosorption of cadmium, but also in elevating the ability of the grass to accumulate Cd especially when the turnout of biomass around the root zone is enlarged through bioaugmentation. Thus, the method holds a high promise in the rhizoremediation of cadmium contaminated soils. This biotechnology can be of immense use in reclaiming heavy metal contaminated soils and as such, increasing the quality and yield of such soils.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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