PRINT ISSN 1119-8362
Electronic ISSN 2659-1502

Full-text Available Online at
https://www.ajol.info/index.php/jasem
http://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 27 (6) 1047-1056 June 2023

Rhizoremediation of a Cadmium-polluted Soil using *Pseudomonas aeruginosa* and *Eleusine indica*

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ABSTRACT: Rhizoremediation involves the use of plants and plant growth promoting rhizobacteria (PGPR) for remediation of contaminated soil. This study evaluated the use of *Pseudomonas aeruginosa* and *Eleusine indica* to remediate cadmium-polluted soil. Soil samples were collected around the roots of *E. indica* and PGPR were isolated and evaluated for their capacity for plant growth promotion. *P. aeruginosa* was selected for Cd remediation experiment which was based on treatments T1 (control soil), T2 (soil + cadmium), T3 (soil + plant + Cd + *Pseudomonas* applied 2x monthly) T4 (Soil + Plant + Cd + *Pseudomonas* applied once). T5 (soil + plant + Cd + *Pseudomonas* applied 1x monthly) T6 (soil + plant + Cd) and T7 (soil and plant). Cd concentration in soil treatments was equivalent to 8 mg/kg and treatments were monitored for 2 months. Bacterial count, physicochemical parameters, nutrient analyses, and Cd removal were subsequently evaluated. T3 had higher bacterial count and pH values ($p < 0.05$) relative to other treatments and this could be due to the inoculum addition. Conversely, reduction in electrical conductivity, organic carbon and organic matter observed in T3 could be attributed to improved rhizospheric activities. Again, rhizoremediation was highest ($P < 0.05$) in T3 (90 %) and this suggests *P aeruginosa* made bio-absorption of Cd easier for *E. indica* relative to treatments that do not have the inoculum. This study showed that *E. indica* together with P. aeruginosa are suitable candidates for phytoremediation of Cd contaminated soils.

DOI: **https://dx.doi.org/10.4314/jasem.v27i6.1**

Cite this paper as: OBUEKWE, I. S; ILECHUKWU, O. E (2023). Rhizoremediation of a cadmium-polluted soil using *Pseudomonas aeruginosa* and *Eleusine indica*. *J. Appl. Sci. Environ. Manage.* 27 (6) 1047-1056

Dates: Received: 17 April 2023; Revised: 18 May 2023; Accepted: 06 June 2023
Published: 30 June 2023

Keywords: Rhizoremediation; cadmium pollution; *E. indica*; *P. aeruginosa*; bio-inoculant.

Heavy metals are well-known environmental pollutants due to their toxicity, persistence in the environment, and bio-accumulative nature (Obuekwe and Semple, 2013a; Ali *et al.*, 2021). Their natural sources include weathering of metal-bearing rocks and volcanic eruptions, while anthropogenic sources are mining, and various industrial and agricultural activities. Mining and industrial processing for extraction of mineral resources and their subsequent applications for industrial, agricultural, and economic development has led to an increase in the mobilization of these elements in the environment (Hazrat *et al.*, 2019; Haider *et al.*, 2021). Cadmium (Cd) is a heavy metal of concern because it is ubiquitous in the environment. Development in agriculture and industries has culminated in high concentration of Cd in agricultural soils. The pollution of Cd in the soil environment has been rapidly increasing in recent years, as a result of the use of agrochemicals such as fertilizers and herbicide (Amir *et al*, 2016; Bojorquez *et al.*, 2016). Phosphate fertilizers are widely regarded as being the major sources of Cd contamination to the agricultural soil. Phosphate fertilizer contains a relatively high amount of Cd (up to 500 mg kg^{-1}) from its manufacturing process (Loganathan *et al.*, 2003). Hence, the intensive use of phosphate fertilizer could result in high Cd contamination in the soil (Zeng *et al.*, 2007; Tijani, 2008). The use of organic fertilizer, such

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as farmyard manure is also considered as source of Cd contamination to the soil (Tijani, 2008). Large annual application of farmyard manure (35 mg of fresh manure ha^{-1}) has been reported to contain significant amount of Cd (0.3 - 1.8 $mgkg^{-1}$) that can contaminate the soils (Alloway, 1995). Looking at potential soil contamination with Cd, it is important that farmers are starting to apply the appropriate soil remediation techniques to minimize the damage to plant and soil. One of the proposed soil remediation techniques is phytoremediation of Cd by using the indigenous hyperaccumulator plants (Amir *et al.*, 2016). The indigenous hyperaccumulator plants could reduce the migration of Cd through the soil medium using phyto-stabilisation mechanism (Robinson *et al.*, 2009). A study by Amir *et al.* (2016) reported the potential of using indigenous hyperaccumulator plants to stabilize heavy metals and remediate contaminated soil to be used as agricultural soil again. Again, in the last few decades, it has been reported that some types of plant growth promoting rhizobacteria (PGPR) are of high resistibility to Cd, and can diminish Cd bioaccumulation through precipitating or by absorption (Haider *et al.*, 2021). These Cd resistant PGPR strains with high Cd immobilizing ability include *Pseudomonas* (Li *et al.*, 2018), *Delftia* (Liu *et al.*, 2020), *Enterobacter* (Mitra *et al.*, 2018; Pramanik *et al.*, 2018), *Arthrobacter* (Bafana *et al.*, 2010), and *Bacillus* (Jiang *et al.*, 2009; Wang *et al.*, 2014). Hence this study investigates the use of rhizoremediation (*Pseudomonas* sp obtained from root zones of *E. indica* and *E. indica*) for remediation of Cd contaminated soil.

MATERIALS AND METHODS

*Collection of samples***:** Soil samples and *E. indica* plant were obtained from metal scrap yards within Benin City. The soil samples were collected using a shovel by digging around the roots of the selected hyper accumulator plant (*E. indica*). The soils were transferred into polymer bags and taken to the laboratory for further investigation. The plant was identified by a Taxonomist in Plant Biology and Biotechnology (PBB) Department of University of Benin.

*Soil sample Preparation and media preparation***:** The soil samples were sun-dried, homogenized and passed through a 2 mm sieve and stored in a polybag until required. All used media were prepared following manufacturers' instructions and sterilization was done at 121°C for 15 mins at 15 atm (pressure).

Rhizobacterial isolation and identification: Soil samples obtained from the respective locations were analyzed in replicates using a ten-fold dilution after a stock was prepared by mixing 30 g of soil in 270 mL of sterile saline water for the isolation and enumeration of bacterial isolates. The stock solution was then serially diluted after which 1 mL aliquot from appropriate tubes were introduced into nutrient agar plates supplemented with fluconazole. The plates were incubated at room temperature 28 ± 2°C for 24 h. Bacterial colonies were counted using a colony counter and results were recorded. Further confirmation of bacterial identity was carried out using morphological, biochemical and microscopic characteristics (Bridson, 2006)

Screening for Plant Growth Promoting Rhizobacteria (PGPR): Screening for Indole Acetic Acid (IAA) production: This was determined by reaction of liquid culture of rhizobacterial isolates grown in 500 mg/L L-Tryptophan (the precursor for IAA biosynthesis) placed in tryptic soy broth (1 g/L MES hydrate, pH 6) and Salkowki's reagent. Inoculated broth was incubated at 30°C for 72 h in a rotary shaker. After incubation broth was centrifuged at 3000 rpm for 15 min. Then 1.0 mL of the supernatant was mixed with 2.0 mL of Salkowski reagent (50 mL of 35% Perchloric acid + 1 mL of 0.5 M $FeCl_3$ solution), and the mixture was then incubated at room temperature for 25 mins. Development of pink color after incubation at room temperature indicated IAA production (Kumar *et al.*, 2012; Ngoma *et al.*, 2013).

Screening for ammonia production: Freshly grown bacterial cultures were inoculated in 10 mL nutrient broth and incubated at 30°C for 48 h in a rotatory shaker. After incubation, 0.5 mL of Nessler's reagent was added to each tube. The development of a yellow to brown color indicated a positive reaction for ammonia production (Kumar *et al.*, 2012).

Screening for Nitrogen fixation activity: A day-old culture of bacterial isolates grown on nutrient agar was streaked on a Jensen's Nitrogen free medium otherwise known as NFM (formulated via the addition of 20 g/L sucrose, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L NaCl, 0.1 g/L $FeCl_3$, 0.005 g/L $Na_2MoO_4.2H_2O$, 2 g/L $CaCO_3$, 15 g/L agar). Plates were incubated at 28°C for 1-7 days. Growth on nitrogen deficient medium confirms the ability to fix nitrogen (Weselowski *et al.*, 2016).

Screening for Phosphate Solubilization activity: Isolated bacteria were cultured in replicates on prepared Pikovskya's agar (Micromaster) plates and incubated at 30°C for 3 days. A zone of clearing around the colonies after 1-3 days was scored as positive for phosphate solubilization. The diameter of the halo zone and its bacterial colony from individual

isolates was measured. The data obtained was used to calculate solubilization index (SI) (Doilom *et al.*, 2020).

Bioinoculant (Pseudomonas aeruginosa) Development: This choice of bacteria was based on its high phosphate solubilizing capacity. A loopful of freshly prepared agar plates of *Pseudomonas aeruginosa* was inoculated into 15 mL nutrient broth (NB) and was incubated at 30^{o}C for 24 h. The cell density in each culture was standardized to 0.5 McFarland's solution with approximate cell density of 1.5 x10^{8} cfu/mL

Soil samples amendment with Cd: The soil used in the study was obtained from the botanical garden, Faculty of Life Sciences, University of Benin. Due to the ecological benchmark for cadmium in the soil which is set at 4 mg/kg, the soil was contaminated with double the ecological benchmark (8 mg/kg) via the addition of 40 mg of cadmium chloride monohydrate (98% purity) in 500 mL of water before it was finally added to 5 kg of the soil in each treatment. This was done with proper consideration of the water holding capacity of the soil. The seven treatments employed in this study is shown in Table 1.

Table 1: Different treatments employed in the study

Treatment codes	Composition/Constituents
T1	Control soil alone
T2	Soil + Cadmium
T3	Soil + *E. indica* + Cadmium + Inoculum (2x monthly)
T4	Soil + *E. indica* + Cadmium + Inoculum (one off)
T5	Soil + *E. indica* + Cadmium + Inoculum (1x monthly)
T6	Soil + *E. indica* + Cadmium
T7	Soil + *E. indica*

Heterotrophic bacterial count: This was done in the different treatments before and after Cd amendments using standard technique (Bridson, 2006).

Physicochemical analyses and nutrient composition of different Soil Treatments: The physicochemical properties of the different soil treatments were determined using standard methods (Denning *et al*., 2011; FAO, 2020). Parameters and nutrients determined were: pH, electrical conductivity, organic matter, organic carbon, potassium, available phosphorous and nitrate.

Cd determination in soil: Soil samples (1 g) from different treatments were mixed with ten (10) mL of nitric acid- perchloric acid, ratio 2:1 and boiled at 105^{o}C. Subsequently, five (5) mL of HCl was added, this was digested for 30 mins. The cooled digest was washed into a 100 mL standard volumetric flask and was made up to 100 mL mark with distilled water. Cadmium was determined with the Atomic Absorption Spectrometer (ASS) PG 550 model (Adelekan and Abegunde, 2011).

Evaluation of Residual Cadmium Concentration in Soil (rhizoremediation efficiency): The residual concentration of cadmium in soil was evaluated using the formula below

$$Cd\ Residual = \left(100 - \left(\left({}^{Fc}/_{Ic}\right)x\ 100\right)\right)$$

Where Fc = final concentration of Cd obtained from pre-treatment; Ic = initial concentration of Cd concentration in the system

Data analysis: Data collected were analyzed using Microsoft Excel (2019) and graph pad prism 5. Descriptive statistics using mean and standard deviation were employed in this study. One-way analysis of variance (ANOVA) using Turkey's test of honest significance and least significant difference (LSD) was used to check for the statistical difference at $p < 0.05$. The cadmium removal efficiency results were in percentages (Ogbeibu, 2005).

RESULTS AND DISCUSSION

This study investigated plant-microbial interactions in the rhizoremediation of a cadmium-polluted soil. Soil samples from the rhizosphere of *E. indica* were analyzed for PGPR and subsequently, *P. aeruginosa* because of its high phosphate solubilization capacity was used as the test organism for Cd rhizoremediation in different soil treatments. Isolated heterotrophs from *E. indica* rhizosphere were identified and were further subjected to plant growth promoting tests, and this showed that *B. subtilis*, *P. aeruginosa* and *E. coli* had 100% plant growth promoting properties. Conversely *P. mirabilis* and *S. marcescens* had 50 % PGP properties (Table 2). Generally, other Authors have reported some active genera of PGPR (*Bacillus*, *Pseudomonas* and *Serratia*) amongst other isolates in the rhizosphere of *E. indica* (Welbaum *et al.*, 2004; Raaijmakers *et al.*, 2010). *P. aeruginosa* had the highest phosphate solubilization capacity relative to other isolated PGPR and this further necessitated its use for Cd rhizoremediation (Figure 1). This finding is consistent with the report of Weller, (2007) who reported that *Pseudomonas* possess the ability to colonize and multiply in the rhizosphere and intercellular spaces of plant tissue and produce a wide spectrum of bioactive metabolites such as antibiotics, siderophore, and growth promoting substances. Marques *et al.* (2010) also reported that *Pseudomonas* also play a decisive role in plant growth promotion

through reduction of ethylene production by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This further suggests that plant growth promotion by *Pseudomonas* occur via direct and indirect mechanisms.

Table 2: Plant growth promoting properties of bacterial isolates obtained *E. indica* rhizosphere.

Isolates	NFM	NH_3	IAA	PSI	% PGP
Bacillus subtilis	+	+	+	+	100
Pseudomonas aeruginosa	+	+	+	+	100
Escherichia coli	+	+	+	+	100
Proteus mirabilis	+	+	-	-	50
Serratia marcescens	-	-	+	+	50

Key: NFM = Nitrogen Free Medium; NH_3 = Ammonia production; IAA = Indole acetic acid; PSI = Phosphate Solubilization Index; PGP = Plant Growth Promotion

Seven treatments (T1, T2, T3, T4, T5, T6 and T7) were adopted in the rhizoremediation of Cd polluted soil which was evaluated using *E. indica* with *P. aeruginosa* for 2 months. These treated soils were assessed for heterotrophs (Figure 2), physicochemical parameters and nutrients (Figures 3 - 9) and Cd concentration (Table 3) after 2 months of experiment. Total heterotrophic bacterial count of the control soil (T1) showed $\log_{10}$ 4.10 ± 0.03 through-out the period of the study. However, treated soils had significant ($p<0.05$) increases in heterotrophs after 8 weeks of the experiment (Figure 2). The highest count was observed in T3 with a value of $\log_{10}$ 5.09±0.03 cfu/g and the least count in T2 with a value of $\log_{10}$ 4.10±0.03 cfu/g after 8 weeks of experiment. Generally, it was observed that all treatments containing *P. aeruginosa* (T3, T4 and T5) had higher bacterial counts compared to treatments without the inoculum. Relative to other treatments, the increase of heterotrophs in the aforementioned treatments were statistically significant ($p<0.05$). This could be attributed to the inoculum addition in the treatments. This would undoubtedly scale up the bacteria population present in the treatments. Most studies that employ the use of bioinoculants usually follow this trend of higher bacteria density for treatment containing bioinoculants as was reported by Bashan and Levanony, (1990) where inoculants of PGPR (*Azospirillum*) were applied to soils. Physicochemical parameters and nutrient levels assessed after 8 weeks of treatment revealed that the soil pH was highest in the treatment containing T3 (8.6±0.05) and least in treatment T7 containing soil and plant only (6.75±0.20). However, pH values of other treatments were found to be within the range of neutrality (Figure 3) and there was a significant difference ($p<0.05$) between the pH values inT3 and T7 treatments.

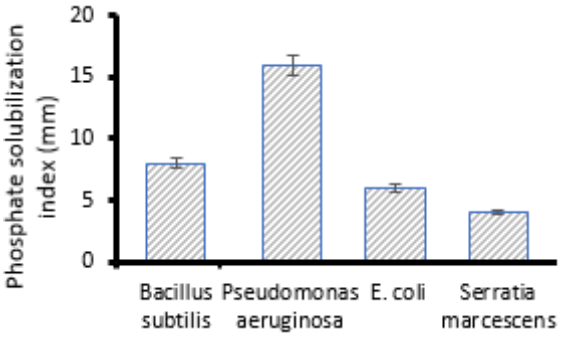

Fig 1. Phosphate solubilization capacity of bacterial isolates from rhizosphere of *E. indica*

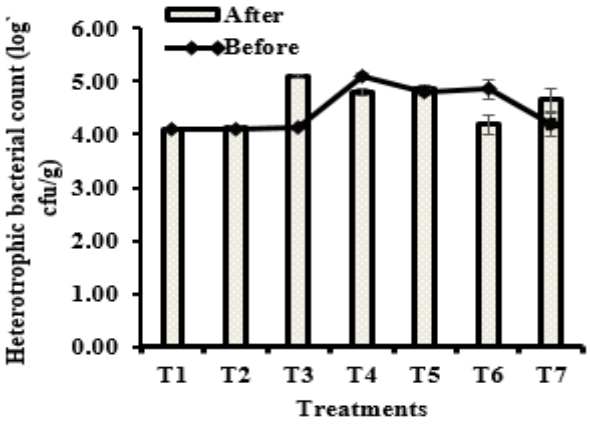

Fig 2. Total heterotrophic bacterial count before and after 2 months treatment

Composition/Constituents	Code
Control soil alone	T1
Soil + Cadmium	T2
Soil + *E. indica* + Cadmium (2X monthly)	T3
Soil + *E. indica* + Cadmium (one off)	T4
Soil + *E. indica* + Cadmium (once a month)	T5
Soil + *E. indica* + Cadmium	T6
Soil + *E. indica*	T7

The neutral pH values are inherent soil characteristics of the studied soil however, increase in pH values in T3 relative to other treatments could be attributed to the addition of more inocula in this treatment. Xu *et al.* (2020) attributed high pH values during bioremediation of Cd by both heterotrophic and autotrophic strains (Co-sys) to the presence of heterotrophic isolates. Although phytoremediation capacity of *E. indica* in a crude oil polluted soil had been reported in slightly acidic soil (Ochekwu *et al.* 2020), it could be that variations may be as a result of the inherent differences in soil characteristics and other factors. Interestingly, low concentration of H^+ ions in alkaline soils make cations less susceptible to leaching (Jackson and Meetei, 2018).

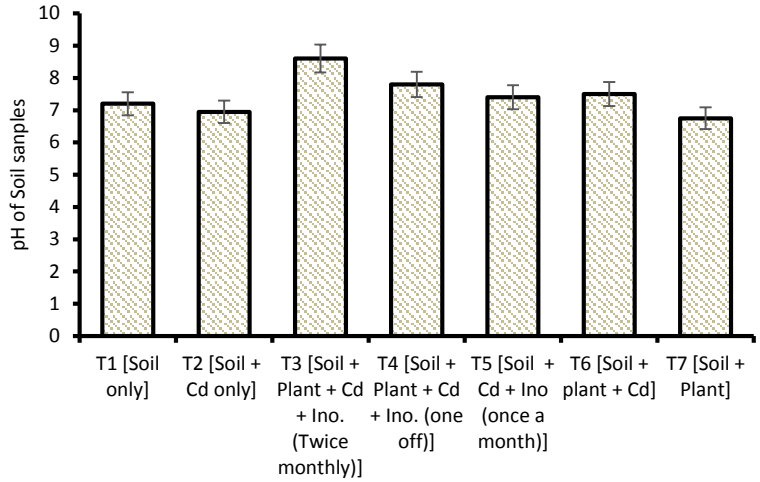

Fig 3. pH of soil samples of different treatments after 2 months

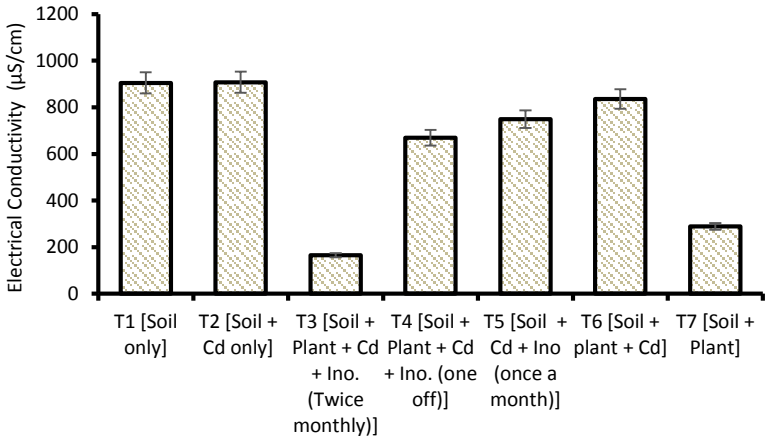

Fig 4. Electrical conductivity of soil samples of different treatments after 2 months

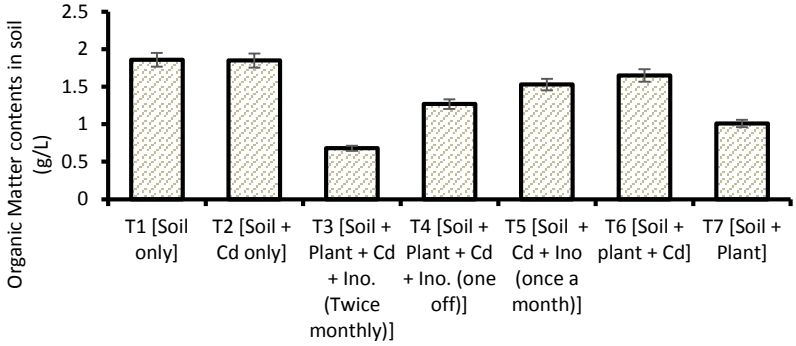

Fig 5. Organic matter contents of soil samples of different treatments after 2 months

The less availability of Cd with increasing pH could be because cations are more available in acidic conditions. Increase in pH causes strong binding of cations to the soil thus, making them not readily exchangeable. More so, it was revealed that electrical conductivity was highest ($p<0.05$) in treatment T2 (905.70±20.50 µS/cm) closely followed by T1 (905.00±25.00). Meanwhile samples having the inoculum had lower EC values compared to those treatments without the inoculum (Figure 4). The difference in organic matter contents was statistically significant between treatment T3 and T1 ($p<0.05$; Figure 5). Similarly, organic carbon content was highest in treatments T1 and T2 but was least (0.4±0.10 g/L) in treatment T3 (Figure 6).

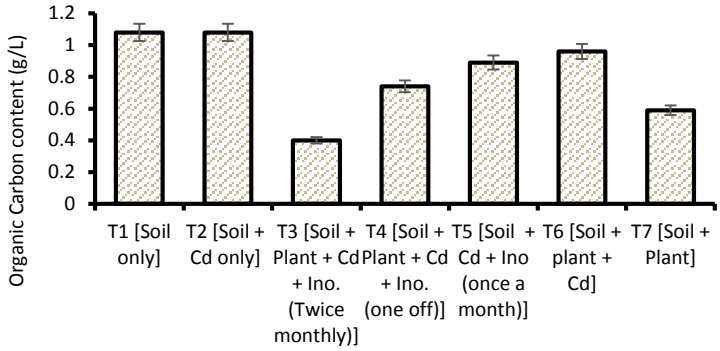

Fig 6. Organic carbon contents of soil samples of different treatments after 2 months

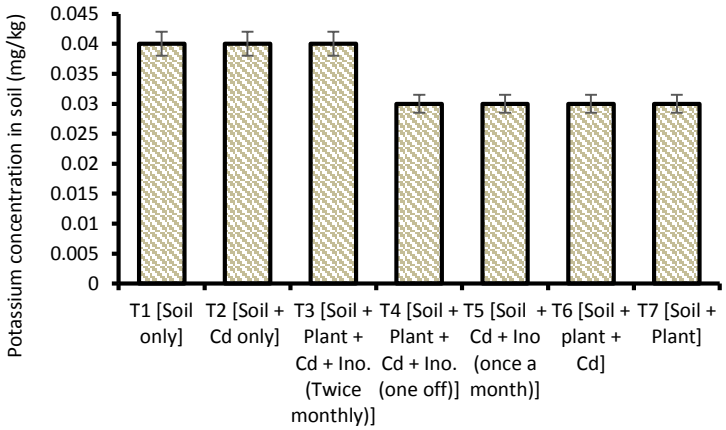

Fig 7. Potassium contents of soil samples of different treatments after 2 months

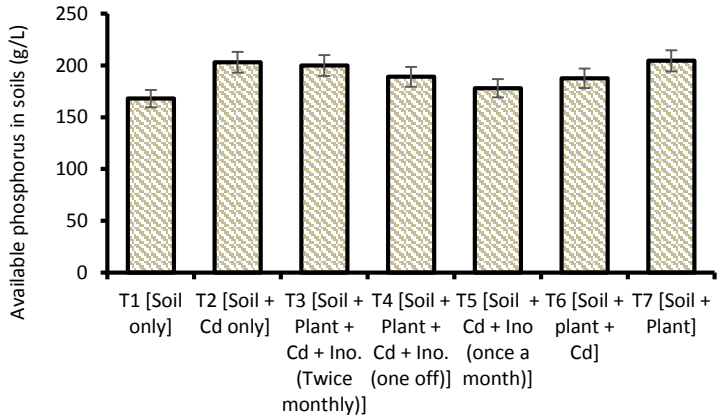

Fig 8. Available phosphorus of soil samples of different treatments after 2 months

Also noteworthy is that the treatments containing *P. aeruginosa* and *E. indica* (T3, T4, and T5) showed lower organic carbon contents when compared to control soils and the difference was also significant ($p<0.05$). Reduction in electrical conductivity, organic carbon, organic matter, and nitrate observed in PGPR amended treatments could be attributed to improved rhizospheric activities as a result of addition of *Pseudomonas aeruginosa*. Nonetheless, the potassium contents of soil samples of different treatments after 2 months of experiment revealed that treatments T1, T2 and T3 had the highest values (0.04±0.01 mg/kg) while other treatments had 0.03±0.01 mg/kg as values for potassium contents. However, there was a significant difference ($p<0.05$) between T3 and T7. The available phosphorus content of the samples was higher in T3 relative to T1 (control soil; Figure 7). There was however no significant difference in the available phosphorus contents obtained between T3 and T1 ($p= 0.133$; Figure 8). The nitrate content was highest in the control soil (T1) but was least in the treatment T7 (Figure 4.7). There was a significant difference in the

nitrate contents ($p<0.05$) between T1 and other treatments in the study. The high potassium content found in the different treatments could be attributed to it been readily available in a pH range of 6.5 - 8 that was observed in this study (Jackson and Meetei, 2018). Potassium has been reported to be one of the very important micronutrients needed for plant growth and development. Again, the high Phosphorous content in the PGPR treatments could be because of phosphate solubilizing ability of the inoculum. Similar values of these parameters were reported by Ochekwu *et al.* (2020) and Ikhajiagbe *et al.* (2020) also reported similar findings following remediation performance of *E. indica* in oil and Cd polluted soil.

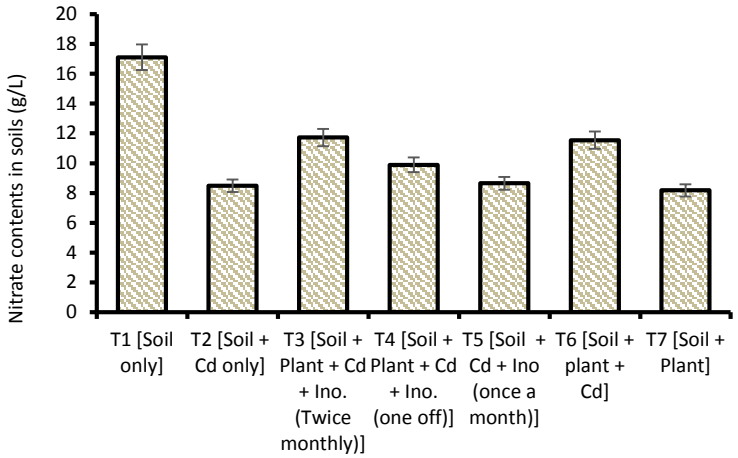

Fig 9. Nitrate content of soil samples of different treatments after 2 months

Table 3: Residual soil concentration (rhizoremediation efficiency) of cadmium after 2 months of treatment

Treatments	Cd concentration at termination	Rhizoremediation efficiency (%)
T2 [Soil + Cd only]	3.79	52.6
T3 [Soil + Plant + Cd + Ino. (Twice monthly)]	0.80	90.0
T4 [Soil + Plant + Cd + Ino. (one off)]	2.10	73.8
T5 [Soil + Cd + Ino (once a month)]	2.66	66.8
T6 [Soil + plant + Cd]	2.82	64.8

Start concentration = 8 mg/kg

It was generally observed that all treatments containing *P. aeruginosa* as well as *E. Indica* had lower concentration of Cd compared to the control treatment (Table 3). The rhizoremediation of cadmium by the *P. aeruginosa* and *E. indica* was found to be highest (90%) in the treatment containing the inoculum applied twice in a month relative to treatments without the inoculum (Table 3). There was a significant difference in the cadmium contents between treatment T3 and other treatments in the study ($p<0.05$). The least remediation efficiency (52.6 %) was observed in the control soils. Treatment T6 containing Soil + Plant + Cd had remediation efficiency of 64.8 %. These findings suggest transport of soluble Cd by *Pseudomonas* sp into plant roots thereby, facilitating its uptake by *E. indica* (Ikhajiagbe *et al.*, 2020). Therefore, the inoculum made bio-absorption of Cd easier for *E. indica* relative to treatments that do not have the inoculum. Rhizosphere microorganisms advance plant growth by improving nutrient acquisition through atmospheric nitrogen and/or liberating phosphate from organic compounds (Ikhajiagbe *et al.*, 2020). Again, remediation of cadmium was found to be slightly better in the treatments containing inoculum alone as compared to that of plant alone. Several Authors have reported the use of microorganisms or plants in the remediation of heavy metal polluted soil (Garba *et al.*, 2012; Obuekwe and Semple 2013b; Amir *et al.*, 2016; Ma *et al.*, 2020; Ochekwu *et al.*, 2020; Ikhajiagbe *et al.*, 2020). Ma *et al.* (2020) showed significant decreases in acetic acid-extractable Cd when Biochemical Composites Materials (PGPB strain YTZ5 and Biochar) were used to bioremediate Cd polluted soil. Similarly, Obuekwe and Semple, (2013b) showed enhanced phenanthrene bioremediation in soil amended with 500 mg/kg aged and fresh Fe because Fe was acquired for microbial growth. Again, Bojorquez *et al.* (2016) reported similar findings to the results obtained in the study using both *P. aeruginosa* and *Enterobacter cloacae* for the removal of cadmium and lead from contaminated soils. Their experiment mimicked T5 used in the study which had 66.8% cadmium removal efficiency. Furthermore, Cd

remediation in T6 suggests that *E. indica* without the assistance of the bioinoculant is capable of carrying out remediation of cadmium with efficiency above 50%. Amir *et al.* (2016) evaluated the phytoremediation of cadmium-contaminated agricultural land using indigenous plants and reported that *E. indica* along with other plants are capable of carrying out remediation of Cd polluted soils. The Authors showed that *E. indica* (KB-1) had highest soil Cd reduction as compared to *Ageratum conyzoides* (KB-2) and *Euphorbia hirta* (KB-3) during Cd remediation by these plants. Reduction in Cadmium, chromium, manganese, iron and zinc in *E. indica* sown soils implied bioaccumulation of these metals by the plant (Ikhajiagbe *et al*., 2020). In the same vein Garba *et al.* (2012) reported the assisted-phytoremediation capacity of *E. indica* with the aid of ethylenediaminetetraacetate (EDTA) in a soil contaminated with cadmium, chromium, cobalt and lead. Again, Gudusu *et al*. (2019) revealed the ability of *E. indica* to effectively absorb zinc, copper, lead, nickel, cadmium and cobalt from contaminated soils. The results of their study demonstrated that the elevated concentrations of these metals in roots, as well as their translocation to the aerial parts of the grasses suggest their suitability for phytoremediation. Furthermore, Ochekwu *et al.* (2020) revealed significant reduction in crude oil contamination using *E.indica* with *Panicum maximum* Jacquin and *Lablab purpureus* L. which further suggests that *E. indica* is not just capable of phytoremediation of heavy metals but also of organic environmental contaminants. The results obtained in this study further demonstrates the importance of bacterial activity and hyperaccumulator plant in rhizoremediation of Cd polluted soil.

Conclusion: In this study an assessment of remediation of Cd contaminated soil was done using *E. indica* in combination with *P. aeruginosa. E. indica* demonstrated improved ability to absorb and accumulate cadmium from polluted soils and this was indicative of Cd bioavailability which was made possible by *P. aeruginosa*. The results of this study showed that *Pseudomonas aeruginosa* and *E. indica* are suitable candidates for rhizoremediation of Cd contaminated soils.

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