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Rhizoremediation activities of *Vigna unguiculata* (L.) in lead polluted soil under *Rhizobium*-symbiotic intervention

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Abstract

Potentials of Cowpea (*Vigna unguiculata)* to bio accumulate Lead (Pb) from soil after treatment with poultry manure were assessed to provide phytoremediation of Pb pollutant in contaminated soil. Soil samples were spiked at lead concentrations of 0 mg kg^{-1} Pb, 250 mg kg^{-1} Pb, 50mg kg^{-1} Pb, 750 mg kg^{-1} Pb and 1000 mg kg^{-1} Pb in a completely randomised design with poultry manure added at 0.0 g kg^{-1} and 12.5 g kg^{-1}. The setup was simulated thrice. Values of soil parameters were obtained using standard methods. At 12 weeks, the concentrations of lead were assessed in soil and plants using analytical methods. Data obtained were analyzed statistically using descriptive and inferential models. The mean concentrations of Pb in *Vigna unguiculata* treated with poultry manure were approximately 4-folds higher than the concentration of Pb ions reported in unfertilized ones. The mean concentrations of Pb in the root and shoot were 313 $mgkg^{-1}$ and 46 mg kg^{-1} respectively with bioaccumulation and Transfer factor below 1.0. However, the rhizobium counts decreased as the pollution strength of the Pb ion increased. This study concluded that *Vigna unguiculata* immobilized Pb contaminants from the soil environment and that leguminous plants could be model plants for the treatment of Pb contaminated soil.

Keywords: Lead, Nitrogen-fixing bacteria, Phytoremediation, Rhizostabilization, *Vigna unguiculata*

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Introduction

In many countries, pollutants such as heavy metals released from anthropogenic activities such as transportation and industrial activities affect all the components of the ecosystem. As one of the components of the environment, the soil ecosystem is significantly affected because it is a natural sink for most pollutants. Studies have revealed that the conventional or traditional methods for soil remediation are expensive with a requirement for technical staff to handle the methods (Saquing et al., 2016; Guerra et al., 2021). The use of plant species to remove pollutants from the environment termed phytoremediation has been suggested as an environmental-friendly and most cost effective biotechnology (Cristaldi et al., 2020; Yang et al., 2022). To efficiently remove toxic substances, plants use diverse phytoremediation techniques such as phytostabilization, phytoextraction, rhizoremediation,and phytovolatilization among others to remove hazardous substances. Many plants have been identified to be metalliferous or hyperactive-accumulators of metals from polluted sites (Krämer, 2010; Abdulkadir et al., 2019; Yang et al., 2020). Out of these plant species, the majority are exotic species that may not thrive successfully for phytoremediation of Lead (Pb) contaminants in the tropics. Thus, there is a need for identifying local plant species that could be used for improving the quality of the soil environment.

Some research conducted on selected tropical plant species revealed that tropical plants are promising bioagents for ecosystem restoration (Gómez-Sagasti & Marino, 2015; Oseni et al., 2015; Ojuederie & Babalola, 2017; Dada, 2019 a,b). In phytoremediation technology, the use of legumes employed in this study for restoration of Pb contaminated soil is termed rhizoremediation. This phytoremediation technique (rhizoremediation) is innovative and cost-effective because the method combines both rhizoremediation and phytoextraction methods (Gervais-Bergeron et al., 2022). In rhizoremediation techniques, rhizosphere organisms such as nitrogen-fixing bacteria are involved in the process of metal removal along with the legume hosting the rhizosphere organisms. Naturally, legumes are angiosperms that exceptionally thrive in a wide range of habitats as well as improve soil fertility through the activities of soil microbiota that depend on the organic compounds at their rhizosphere zone (Yan et al., 2020; Lázaro & Ana, 2021). Examples of these microbiota are rhizobium and mycorrhizal species (Awotoye et al., 2011).

Over the years, the use of leguminous crops to remove metal pollutants has been receiving public attention because soil nitrogen fixing bacteria in the root of legumes assist in the uptake of pollutants especially in nitrogen deficient ecosystems (Lasat, 2002). Therefore, bioaccumulation of metals in the roots of legume indicate metal phytostabilization traits (Yang et al., 2020). Among legumes, cowpea has distinct features for rhizosphere technology. This is because cowpea possesses appreciable biomass and longer root length than grasses. This

type of root system is an advantage for its use in remediation of soil with high concentrations of metal loadings. This is because plants with lengthy roots enable the movement of soil microbiota easily to the site of lead contamination (Ojuederie & Babalola, 2017**)**. Pollutants that migrate beyond the root zones of plants in soil can be removed more easily by plants with extensive root length than plants with short root length such as grasses (Li et al., 2007; Wang et al., 2022).

Thus, there is a need not to only carry out studies on the phytoremediation potentials of tropical leguminous plants, but to also improve their phytoremediation strategies. Consequently, in most phytoremediation technology, augmentation is one of the strategies to improve the efficiency of plants to remove metal such as Pb from the soil (Wang et al., 2010). Augments are substances introduced during the phytoremediation process to assist plants to remove pollutants from the environment. There are two types of augments. These are bio augment and chemo augment. Fertilizers and biological agents are examples of both chemoaugment and bioaugment respectively. In this study, the rhizobium is the bioagent that assists the test plant to uptake lead ion; as well as alleviate the stress of metal toxicity in the test plants. The activities of rhizobium as bioaugment and beneficial symbionts are necessary because one of the characteristics of plants growing on metal contaminated soils is poor growth. This may involve changes in physiological and biochemical processes in plants in response to Pb toxicity (Azubuike et al., 2016). Therefore, the augmentation of plants by adding organic manure is important (Wang et al., 2020).

Augmentation protects plants from toxicity, but also influences the absorption of metals from nature. In a study conducted by Dada (2013), the phytoremediation potentials of augmented tropical plants namely *Euphorbia heterophylla Linn* (spurge weed), *Tithonia diversifolia* (Mexican sunflower) and *Synedrella nodiflora gaertn* (Synedrella) were improved than the unaugmented ones. Besides, application of organic manure increases the bioavailability of metals in soil environment as the pH of the soil reduces after soil manuring (Dada, 2013; Beiyuan et al., 2017). In this study, *Vigna unguiculata* amended with organic manure was exposed to Pb toxicity under Legume–*Rhizobium* Symbiotic technology in an attempt to assess the potentials of *Vigna unguiculata* to remove Pb pollutants from Pb contaminated soil under legume–*Rhizobium* partnership for bioremediation purposes.

Materials and Methods

Study area

The study was carried out at the research site of Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria. The study area is a tropical environment with wet and dry seasons. The mean temperature recorded was about 30 °C with daily minimum and maximum temperature of approximately 21^{o}C and 32^{o}C respectively. However, the North-easterly winds are brought by harmattan from December to February, thus lowering the effect of dry season high temperatures. Besides, average daily temperature of about 21^{o}C (minimum) and 32^{o}C (maximum) respectively were reported. The site recorded a gradation from weakly acidic to moderately basic pH while the average photoactive radiation of 825 µmol m-2 s^{-1} was reported at the screen-house.

Experimental design

The screen-house experiment was replicated thrice at five levels of lead concentrations of 0, 250, 500, 750 and 1000 mgkg^{-1} with a plant species and two levels (0 and 12.5 gkg^{-1}) of poultry manure in a completely randomized design. This gives a total of 30 experimental pots.

Sampling procedures/Pre-planting operations

Collection of soil samples

Soil auger was used to collect soils from a forest area that is free from anthropogenic activities within Joseph Ayo Babalola University. Top soil was obtained and sieved to remove debris using sieve of size 2 mm pore size.

Collection of the seeds of *V. unguiculata*

The variety seed of the *V. unguiculata* cultivated was Ife Brown Beans. The seeds were obtained from the International Institute of Tropical Agriculture, Ibadan, Oyo State.

Collection of organic manure

Poultry manure was collected from the agricultural farm of Joseph Ayo Babalola University; and air-dried for two weeks. The manure was further crushed to powdery form and sieved through 2 mm sieve pore size.

Preparation of lead solution

Following the standard procedure to prepare 1000 ppm of Pb solution, 1.6 g of Pb nitrate salt was weighed using analytical balance in 1000 mls of deionized water. For 750 ppm Pb nitrate solution, 1.2 g of Pb nitrate salt was weighed into 1000 mls of deionized water while 0.8 g of lead nitrate salt was weighed and dissolved in 1000 mls of deionized water to prepare 500 ppm of Pb nitrate solution. Similarly, 0.4 g of Pb nitrate salt was dissolved in 1000 mls of distilled water to prepare 250 ppm of Pb nitrate solution. Each solution was vigorously shaken before use to allow the Pb nitrate salt to dissolve completely.

Preparation of the experimental plots

Plastic pots of 15 cm diameter with 23 cm height were perforated in order to avoid water-logging. Each of

the plastic pots was filled with 5 kg of soil and placed in a 25 cm diameter tray to accommodate excess water that drains after wetting and allow cooling.

Pollution of soil with heavy metals

Five kilograms of soil were placed in each of the 30 experimental pots. The respective pots were spiked separately by pouring the solution of Pb nitrate salts according to the four levels of lead nitrate solution prepared: 250 mg kg^{-1}, 500 mg kg^{-1}, 750 mg kg^{-1} and 1000 mg kg^{-1}. Each of these treatments were replicated three times. Thereafter, the soil was left for seven days in order to achieve equilibration.

Application of organic amendment to *Vigna unguiculata*

After pollution with Pb ion solution, air dried poultry organic manure with nitrogen content of 0.82% was added following the method of Oseni et al. (2015). Plants were harvested 12 weeks after planting (WAP) when morphological features of plant maturity such as seeds were produced by the plants.

Planting of *Vigna unguiculata*

Six viable seeds of *Vigna unguiculata* were aseptically planted in each pot, thinned to three stands and allowed to be exposed naturally to the rhizobium in soil for 12 WAP. At 12 weeks, most physiological features found on matured plants were observed on the test plants.

Method of watering

Adequate moisture content was maintained in order to avoid waterlogging effect as the plants were watered at the required field capacity. For subsequent wetting, excess water drained into the tray was used to wet the plants. However, if the required field capacity was not reached, extra moisture was added.

Pre-harvest bio-monitoring analyses

After two weeks of planting, the number of leaves were counted visually while agronomic parameters such as plant height and the leaf breadth were measured using meter rule. The method of Osei-Yeboah et al. (1983) was used to estimate the area of the leaves of the test plants. However, the stem girth of each test plant was measured using vernier calliper.

Area of leaf = leaf length × leaf breadth × correction factor (CF)

For *Vigna unguiculata* the CF used was 0.7.

These parameters were determined fortnightly.

Harvesting of *Vigna unguiculata* plant

At 12 weeks, test plants were harvested and separated into roots and shoots. Soil samples were also prepared for laboratory analyses.

Hyperaccumulating activity of *Vigna unguiculata*

Root bioaccumulation factor (BCFr)

The root bioaccumulation factor (BCFr) is the ratio of metal concentrations in plant roots to that of the environment as presented by Cui et al. (2011).

Bioaccumulation factor (BCFsh)

The Shoot bioaccumulation factor (BCFsh) is the ratio of metal concentrations in plant shoots to that of the environmental concentration as indicated by Yoon et al. (2006).

Transfer factor (TF)

The TF for each weed species was reported as the ratio of metal concentrations in plant shoots to that of the metal concentrations in the root of plants.

Microbiological analyses

The analyses were determined according to the procedure of Vincent (1970). Isolation of microorganisms from roots of the *V. unguiculata* as well as direct microscopic examination of the isolates. Using the methods of Arora & Arora (2008), biochemical identification such as Gram's staining, Lysine decarboxylase test, and VogesProskauer among others were carried out.

Preparation of the root nodules of *Vigna unguiculata* L. for the isolation of Rhizobium

The pink nodules of *Vigna unguiculata* plants were selected and sterilized using agents of 0.1% mercuric chloride and 3-5% hydrogen peroxides for 4 to 5 minutes. Then, the nodules were washed with distilled water and 70% ethyl-alcohol; and later with sterile distilled water.

Isolation of Rhizobium following Vincent (1970) standard procedure

The isolation of rhizobium by serial dilution method was carried out following standard procedure described by Vincent (1970). Nodular extracts of *Vigna unguiculata* plants were obtained. One ml of the nodular extract suspension was prepared using the serial dilution method and thereafter cultured on Yeast Extract Mannitol Agar (YEMA) plates. The sample of test isolate was streaked throughout the YEMA plates and inoculated petri- dishes were incubated for 5 days at 37^{o}C.

Biochemical test

Gram staining

A smear was made on a grease-free slide and heat fixed, then flooded with crystal violet for 60 seconds. Slides were rinsed with flowing tap water. Lugol's iodine as mordant was added in drops and left for about 60 seconds and then washed off with distilled water. Ethanol was added in drops for 5 seconds and washed off immediately with distilled water. Safranin was added and allowed to stay for about 60 seconds after which it was rinsed off with distilled water and blotted dry. The stain was covered with a drop of immersion oil and later viewed under the light microscope using the oil immersion objective lens (x100).

Gelatin hydroxylase test

The gelatin medium in the tube is inoculated with 5 drops of a 24-hour broth medium. The inoculated tubes are incubated at 37°C in air for 48 hours. After the first incubation, the tubes were placed at 4°C for another 24 hours.

Voges-proskauer (VP) test

The Medium was allowed to equilibrate to room temperature. Isolate from 24-hour pure culture was lightly inoculated into the medium and incubated aerobically at 37^0C for 24 hours. Then, aliquot of 2 ml of the broth was added to a clean test tube and re-incubated the remaining broth for an additional 24 hours. Six drops of 5% alpha-naphthol were added and mixed well to aerate. A few drops of 40% potassium hydroxide were added and mixed well to aerate. Within 30 mins, the tube was shaken vigorously to observe for a pink-red color at the surface.

Citrate utilization test

Simmons citrate agar was inoculated with the fresh test organism and incubated at 37^0C for 24 hours. A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using a loop, then a drop of 3% peroxide (H_2O_2) was placed on the slide, mixed and examined for the evolution of bubbles within 10 seconds.

Coagulase test

A drop of distilled water was placed on a clean slide; test colony from nutrient agar was picked with a sterile loop and emulsified with the distilled water to form a suspension.. A loopful of anticoagulated plasma was prepared by adding EDTA to fresh plasm. A loopful of anticoagulated plasma was gently mixed with the suspension containing the test colony. Clumping within 10 seconds indicated positive test while no clumping indicated negative test.

Oxidase test

A filter paper was soaked with tetramethyl-p-phenylenediamine dihydrochloride and moisten the paper with sterile distilled water. The colony was tested with a platinum loop and the colony was smeared in the filter paper. Inoculated area of paper for a color change to deep blue or purple within 30 seconds.

Lysine decarboxylase test

Inoculum from a pure culture was transferred aseptically to a sterile tube of lysine decarboxylase broth. The inoculated tube is incubated at 35^0C -37^0C for 24 hours. Then, the preliminary results are determined with a change from purple to yellow. The culture was incubated for an additional 24 hours at 35-37 C to allow the microbe to use the lysine. The final results are then obtained by observing the tube at 48 hours.

Arginine dihydrolase test

The reagents of Arginine dihydrate were mixed in sterile distilled water. The pH was adjusted to a light orange-pink color and later dispensed into tubes to solidify. Inoculated tubes were stabbed with bacteria from a fresh culture and later covered the tube with a few mls of sterile mineral oil. The setting was incubated at 27°C for 4 days.

Tryptophan deaminase test

A sterilized test tube containing 4 ml of tryptophan broth was inoculated with inoculum by taking the growth from 18 to 24 hrs culture and incubating the tube at 37°C for 28 hours. Later, 0.5 ml of Kovac's reagent to the broth culture for the presence or absence of a ring.

Cell motility test

A drop of 18 hours peptone medium culture of the test organism was placed on a clean grease-free slide with the aid of Pasteur pipette. The slide was then covered with a cover slip and viewed under the microscope using x40 objective lens. A Brownian motion movement into various directions of the bacteria particles indicated the motility of the organism while the stagnancy of bacteria particles in the suspension shows non-motile organisms.

Sugar fermentation tests

Pure isolates from an 18-24 hour culture were obtained and inoculated in duplicate. Each test organism was inoculated by stabbing the agar containing each sugar respectively to approximately 0.25 inches from the bottom. Sterile melted petrolatum were added to the duplicate tubes. The cap of the overlaid tube was tightened while the cap of the non-overlaid was loosened. Then, both tubes were incubated aerobically at 35ºC for up to 14 days and each tube was observed for change in color every day. This procedure was repeated for all the

sugars which are glucose, sucrose, mannose, arabinose, rhamnose, inositol, melibiose, adonitol, sorbitol, accordingly.

Chemical analysis

Soil analyses

The particle size of the soil was obtained using the hydrometer method (Bouyoucos, 1962) and the soil pH was determined using the pH meter. The soil organic matter was determined using the Walkley and Black (1934) method while the total nitrogen (N) was estimated using the modified macro-Kjeldahl method.

Determination of exchangeable bases

The clear supernatant of prepared soil samples was carefully decanted into 100 mls of volumetric flasks. This procedure was repeated twice with the soil and the supernatant transferred into the volumetric flask and made up with the 1N (NH_4OAc) solution. Amount of K, Na, and Ca were measured on flame photometer at 768 nm, 589 nm and 471 nm respectively while Mg was determined at 285.2 nm on atomic absorption spectrophotometer (Jackson, 1958).

Determination of Cd, Pb, and Cu in soil.

The soil samples were digested and the nitrogen in the sample was determined using the steam distillation technique (Keenly and Bremner, 1966). The Wavelengths for atomic absorption spectrometric determination of Cd, Pb, and Cu were estimated on Perkin-Elmer Spectronic Version 20. The concentrations of Cd, Pb, and Cu were determined at 228.8 nm, 283.3 nm, and 324.7 nm wavelengths respectively while concentrations of Fe and Zn were determined at 248 nm and 213.8 nm wavelengths.

Determination of available phosphorus

Available phosphorus was extracted using the Bray-1 method (Bray & Kurtz, 1945) and concentrations of phosphorus were estimated using atomic absorption spectrophotometry (AAS) set at 600-nm wavelength.

Plant analysis

The concentrations of Pb in plant samples separated into roots and shoots were digested with concentrated H_2SO_4 and 50 % (v/v) H_2O_2 at 90º C by micro-Kjeldahl method (Jackson, 1962). The Wavelengths for atomic absorption spectrometric determination of Cd, Pb, and Cu were estimated on Perkin-Elmer Spectronic Version 20. The concentrations of Cd, Pb, and Cu were determined at 228.8 nm, 283.3 nm, and 324.7 nm wavelengths respectively while concentrations of Fe and Zn were determined at 248 nm and 213.8 nm wavelengths.

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) to test for treatment effects using statistical analytical software (SAS) mixed model systems version 8.0.

Results

Soil characteristics

The values of the soil parameters were presented in Table 1. The result revealed that the soil was moderately acidic and the pH value was 6.30 while after harvesting the test plants, the pH of soil increased to 8.19. This may be due to the addition of an organic amendment. The soil total nitrogen and organic matter contents were 0.75 and 1.46. These values increased to 1.02 and 2.05, respectively after the experiment.

Table 1 Parameters of soil used for the experiment

Property	Values before experiment	Values after experiment
pH (1 :1) Soil-water	6.30	8.01
Organic matter (%)	1.46	2.05
N (g/kg)	0.75	1.02
Available P (mg/kg)	0.13	0.37
Exchangeable acidity (c mol kg^{-1})	2.46	3.05
Effective cation exchange capacity	13.08	15.02
Exchangeable cations (c mol kg^{-1})		
Ca	15.00	18.90
Mg	0.04	0.13
Na	0.64	0.34
K	7.00	9.01

Extractable micronutrient (mg kg^{-1})		
Fe	10.00	14.11
Zn	0.26	0.55
Heavy metals (mg kg^{-1})		
Pb	0.06	0.04
Cd	0.02	0.01
Cu	0.50	0.22
Clay (%)	5.00	4.98
Silt (%)	45.00	45.00
Sand (%)	50.00	50.01
Particle size	Sand loamy	Sand loamy

For the exchangeable cations such as calcium, magnesium, sodium and potassium their respective values before the experiment were 15.0. 0.04, 0.64 and 7.00. However, these values for calcium, magnesium and potassium improved by 21 %, 69 %, and 22 % after harvesting of the test plants. Contrarily, the concentration of sodium was reported to reduce by 88 %. In contrast, the concentrations of heavy metals obtained from the soils of the experiment were low. The exchangeable acidity value of soil used in the greenhouse before the commencement of the experiment was 2.46 while after the experiment it was 3.07. The initial effective cation exchangeable capacity was 13.08 cmol Kg^{-1} while after harvesting the value increased to 15.02 cmol Kg^{-1}. The result of the physical characteristics showed that the soils used were sandy-loam (Table 1). Increased mean values observed in the soil characteristics after harvest may be due to the application of poultry manure as organic amendment.

Effect of lead on the growth of *Vigna unguiculata*

The total number of leaves of *Vigna unguiculata* were presented in Fig. 1 to 5. In non-contaminated soils, the number of leaves of the plant were enhanced significantly by poultry manure applications. In soil spiked with Pb pollutants at varied concentrations, the highest number of leaves was reported in *Vigna unguiculata* L. grown in 250 mgkg^{-1} Pb contaminated soils under poultry manure applications with 83% increments when compared with unfertilized ones (Fig. 2). Moreover, for *V. unguiculata* grown in 750 mg kg^{-1} Pb, the number of leaves reduced by 28% (Fig. 4).

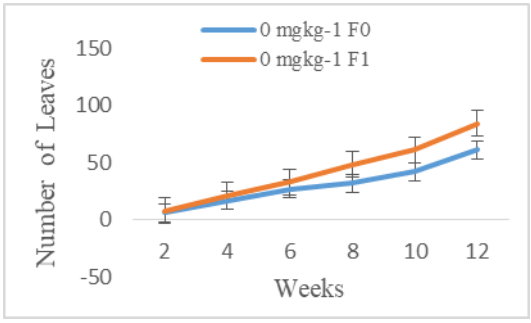

Fig. 1 Number of leaves produced by *V. unguiculata* in control with and without Poultry manure amendment

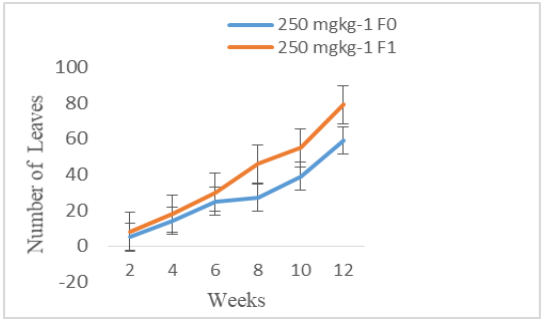

Fig. 2 Number of leaves produced by *V. unguiculata* grown in 250 mgkg^{-1} Lead with and without Poultry manure amendment

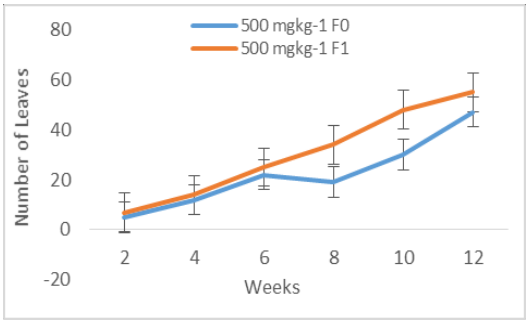

Fig. 3 Number of leaves produced by *V. unguiculata* in 500 mgkg^{-1} Lead with and without Poultry manure amendment

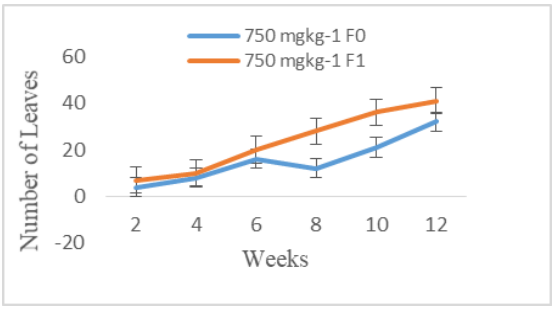

Fig. 4 Number of leaves produced by *V. unguiculata* in 750 mgkg^{-1} Lead with and without Poultry manure amendment

However, without fertilizer application, least number of leaves were reported in *Vigna unguiculata* grown in soil spiked with 1000 mgkg^{-1} Pb as 72% reduction occurred when compared with fertilized ones (Fig. 5). Similarly, as the concentration of the contaminants increased, significant reductions were observed in the number of leaves produced across the test plants. At 1000 mg kg^{-1} Pb, there were no leaves to be counted as none of the V. *unguiculata* survived in the absence of poultry organic manure. The rate at which leaves dropped was significant and chlorotic expressions were evident in *V. unguiculata* grown under poultry organic manure amendment.

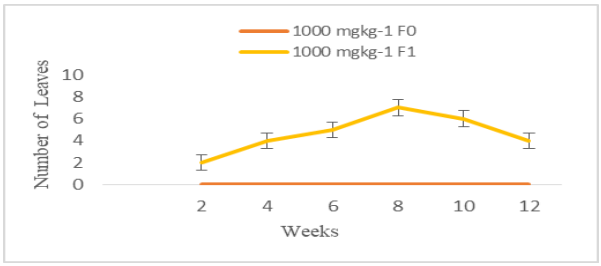

Fig. 5 Number of leaves produced by *V. unguiculata* in 1000 mgkg^{-1} Lead with and without Poultry manure amendment

Legend: F_0---- Without Poultry manures application, F_1-----With Poultry manures application, I -------Standard Error bar

The plant heights of *Vigna unguiculata* were presented in Fig. 6 to 10. Comparing the plant heights of fertilized plants without contaminants (Control) (Fig. 6), the control was higher than the fertilized ones spiked with contaminants. The plant heights ranged from 2.0 cm to 45.6 cm at 0 to 250 mgkg^{-1} Pb levels of contaminants. However, at 500 mgkg^{-1} Pb to 750 mgkg^{-1} Pb contaminations, plant heights were higher than those of unaugmented plants. At all levels of Pb contamination, the heights of the plants at 6 to 12 weeks were enhanced by poultry organic manure (Fig. 6 to 10).

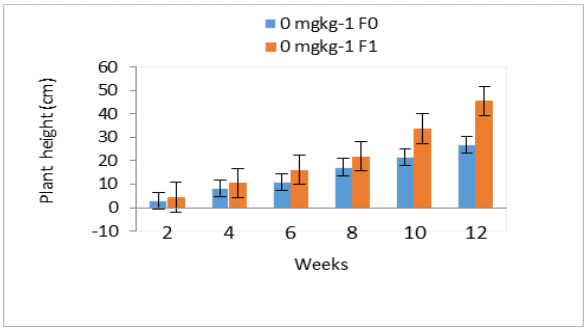

Fig. 6 Plant height of *V. unguiculata* grown in control with and without Poultry manure amendment

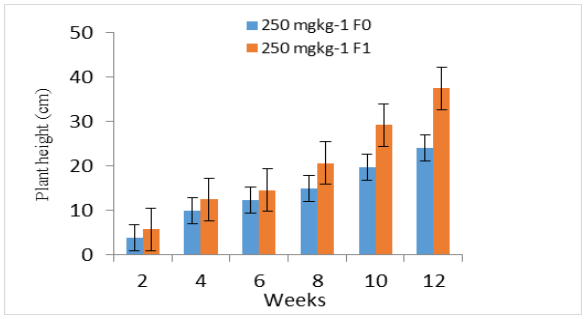

Fig. 7 Plant height of *V. unguiculata* grown in 250 mgkg^{-1} Lead with and without Poultry manure amendment

The height of 37.4 cm was highest under the treatment of poultry organic manure fertilizer (OMF) (Fig. 7) and the lowest height (2 cm) was found at 2 weeks in untreated *V. unguiculata* (Fig. 9). Compared with plants grown in soil contaminated with 250 mgkg^{-1} Pb, the highest height of the plants grown in soil polluted with the highest (1000 mgkg^{-1} Pb) metal concentration under poultry manure reduced by 76%. From 8 to 12 weeks, the influence of poultry organic manure fertilizer on the plant height became more apparent than when the plants were not fertilized.

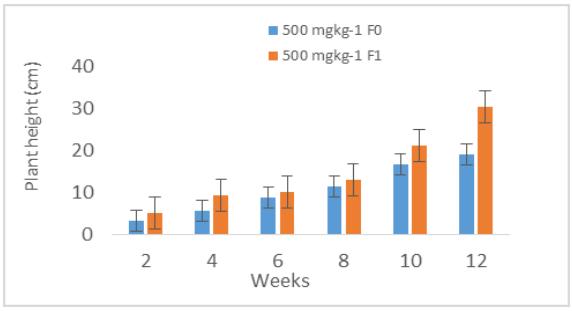

Fig. 8 Plant height of *V. unguiculata* grown in 500 mgkg^{-1} Lead with and without Poultry manure amendment

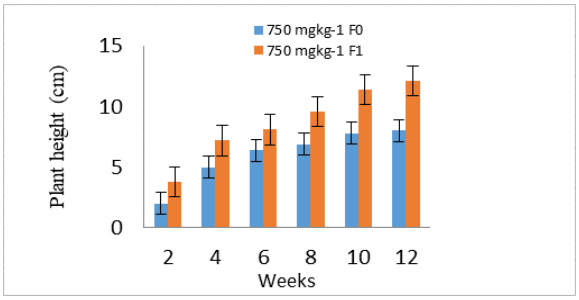

Fig. 9 Plant height of *V. unguiculata* grown in 750 mgkg^{-1} Lead with and without Poultry manure amendment

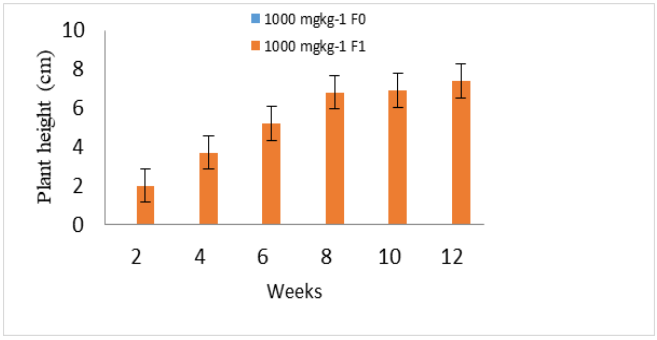

Fig. 10 Plant height of *V. unguiculata* grown in 1000 mgkg^{-1} Lead with and without Poultry manure

Legend: F_0---- Without Poultry manures application, F_1-----With Poultry manures application, I -------Standard Error bar

Fig. 11 to 15 represent the results of leaf area measurement of *Vigna unguiculata*. In Pb contaminated soils, the leaf area of the plants at 2 weeks were similar. With fertilizer application, the highest (156.8 cm^2) leaf area was observed at 12 weeks (Fig. 12) while the lowest (4.1 cm^2) leaf area was found at the first week (Fig. 15).

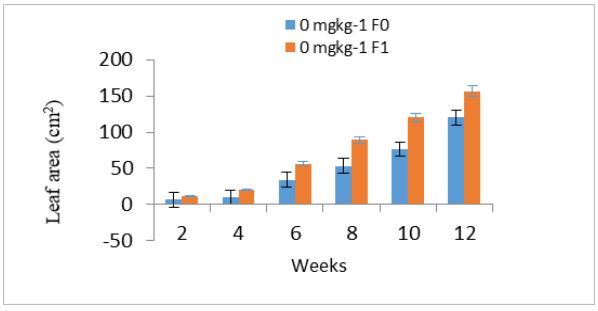

Fig. 11 Leaf area of *V. unguiculata* in control with and without Poultry manure amendment

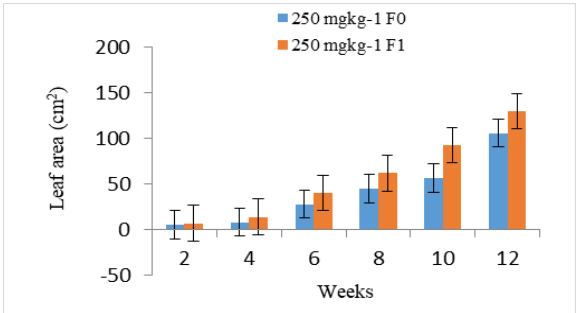

Fig. 12 Leaf area of *V. unguiculata* in control with and without Poultry manure amendment

At 12 weeks, the highest (156.8 cm^2) leaf area was different from that without fertilizer application. However, the leaf areas at 8 week were improved from the leaf areas of other plants at 2 week to 4 week across the plants. The stem girths of *Vigna unguiculata* were shown in Fig. 16 to 20. In 0 $mgkg^{-1}$ Pb spiked soils with poultry OMF application, there was a slight difference in the stem girth of the plants at 2 weeks. The plant had the highest (4.9 cm) stem girth in 250 $mgkg^{-1}$ spiked soil. The lowest (0.5 cm) stem girth was recorded at 2 week in 750 $mgkg^{-1}$ spiked soil (Fig. 19). Increased Pb concentration in soil, were observed to reduce the rate at which stem girth of the plant increased in sizes.

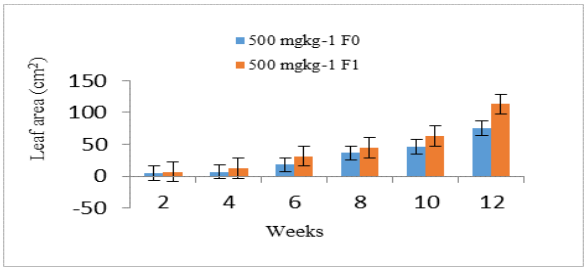

Fig. 13 Leaf area of *V. unguiculata* grown in 500 $mgkg^{-1}$ Lead with and without Poultry manure amendment

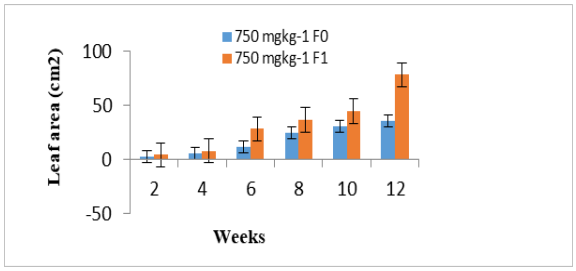

Fig. 14 Leaf area of *V. unguiculata* grown in 750 $mgkg^{-1}$ Lead with and without Poultry manure amendment

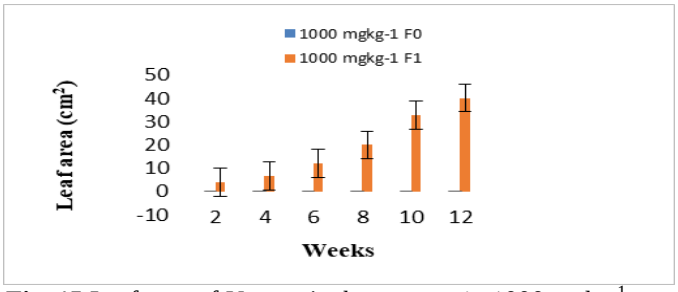

Fig. 15 Leaf area of *V. unguiculata* grown in 1000 $mgkg^{-1}$ Lead with and without Poultry manure amendment

Legend: F_0---- Without Poultry manures amendment, F_1-----With Poultry manures amendment, I -------Standard Error bar

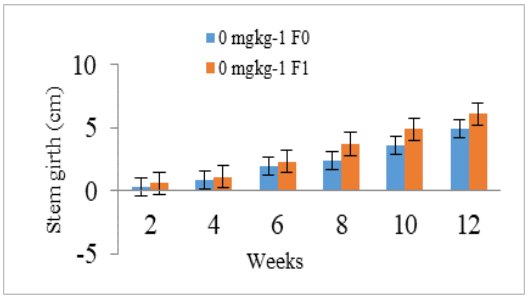

Fig. 16 Stem girth of *V. unguiculata* grown with and without Poultry manure amendment

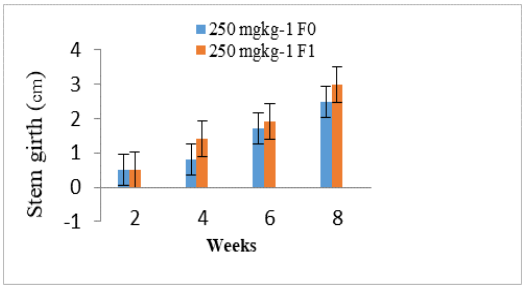

Fig. 17 Stem girth of *V. unguiculata* grown in 250 $mgkg^{-1}$ Lead with and without Poultry manure amendment

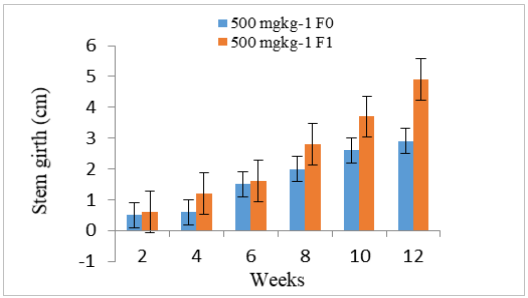

Fig. 18 Stem girth of *V. unguiculata* grown in 500 mgkg^{-1} Lead with and without Poultry manure amendment

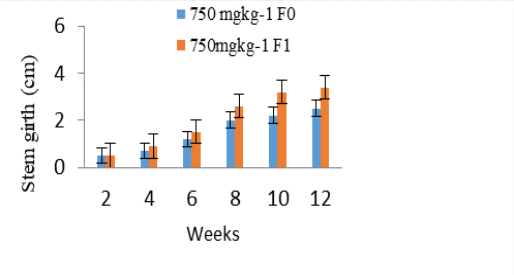

Fig. 19 Stem girth of *V. unguiculata* grown in 750 mgkg^{-1} Lead with and without Poultry manure amendment

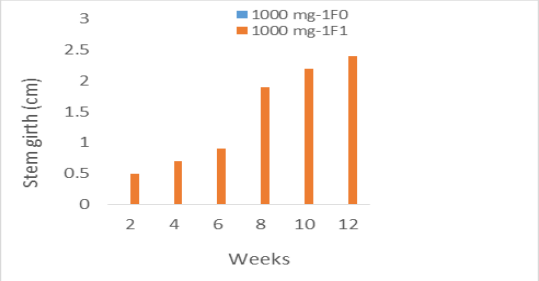

Fig. 20 Stem girth of *V. unguiculata* grown in 1000 mgkg^{-1} Lead with and without Poultrv manure amendment

Legend: F_0---- Without Poultry manures application,
F_1-----With Poultry manures application, I -------Standard Error bar

Hyperaccumulation potentials of *Vigna unguiculata* grown in lead contaminated soils at 12 weeks

Table 2 presented hyperaccumulation potentials of *Vigna unguiculata* grown in Pb polluted soil by revealing the concentrations of lead in the root and shoot of *Vigna unguiculata*. In 250 mgkg^{-1} Pb without OMF application, the concentration of Pb in the root of *Vigna unguiculata* (72.61 mg kg^{-1}) was more than that of the shoot (5.11 mgkg^{-1}) . Similarly, in *Vigna unguiculata* cultivated in 250 mgkg^{-1} Pb under OMF application, the concentrations of Pb in the roots of *Vigna unguiculata* were four-folds higher than the concentrations of Pb in the shoots. Among the plants with and without OMF application, as the pollution strength of Pb increased, the amount of Cd and Pb reported in the shoot and the root increased. Without OMF application, at highest concentration of 1000 mgkg^{-1} Pb, *Vigna unguiculata* plants did not survive the stress of lead ion toxicity. Although, at the same concentration with OMF application, highest (553.50 mgkg^{-1}) and (87 mgkg^{-1}) elemental deposition of lead ion was reported in the root and shoot respectively. Across the plants, fertilization of *Vigna unguiculata* with OMF significantly ($p \leq 0.05$) enhanced the accumulations of Pb in both the shoots and roots. In lead spiked soil at different levels of pollution, non-augmented *Vigna unguiculata* had BCF values of below 1.00 for shoot and root. The BCF values for both shoot and root were observed to increase significantly (Table 2) as pollution strength increased.

Table 2 Hyperaccumulation potentials of *Vigna unguiculata* grown in lead contaminate soil at 12 weeks

Level of pollutant (Pb) (ppm)	Root	Soil	Root BCF	Shoot	Soil	Shoot BCF	Transfer factor
Control + F0	5.00^{d}	9.61^{d}	0.52^{b}	0.00^{d}	9.61^{d}	0.00^{c}	0.00^{c}
250 ppm + F0	72.61^{c}	120.12^{c}	0.60^{b}	5.11^{c}	120.12^{c}	0.04^{b}	0.07^{b}
500 ppm + F0	112.42^{b}	296.57^{b}	0.38^{c}	14.16^{b}	296.57^{b}	0.05^{ab}	0.13^{a}
750 ppm + F0	276.50^{a}	431.35^{a}	0.64^{a}	30.5^{a}	431.35^{a}	0.07^{a}	0.11^{ab}
1000 ppm + F0	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Control + F1	9.00^{e}	11.2^{d}	0.80^{e}	1.74^{d}	11.2^{d}	0.16^{d}	0.19^{a}

250 ppm + F1	139.62[d]	37.8[c]	3.69[b]	29.18[c]	37.8[c]	0.77[a]	0.21[a]
500 ppm + F1	307.25[c]	109.64[b]	2.80[d]	40.59[b]	109.64[b]	0.37[c]	0.13[c]
750 ppm + F1	441.79[b]	130.12[a]	3.39[c]	80.37[a]	130.12[a]	0.62[b]	0.18[a]
1000 ppm +F1	553.50[a]	131.56[a]	4.21[a]	87.1[a]	131.56[a]	0.66[b]	0.16[b]

F0 = without fertilizer; F1 = with fertilizer; Means with different letters are significantly different ($p \leq 0.05$)

At 250 mg kg^{-1} metal pollution the shoot BCF value (0.04) of fertilized *Vigna unguiculata* increased by 19% while the root BCF (3.68) value increased by 62% when compared with unfertilized *Vigna unguiculata* grown at the same concentrations. Highest BCF values for both shoot and root were reported for *Vigna unguiculata* fertilized with poultry OMF. The transfer factors of across all the plants were below 1.0 showing the phytostabilization strength of *Vigna unguiculata*. Table 3 revealed the microbiological analyses of the rhizobium species isolated from the rhizosphere of *Vigna unguiculata*.

Table 3 Characteristics of *Rhizobium species* isolated from the *Vigna unguiculata* grown in lead contaminated soil

Identification Tests	Characteristics of *Rhizobium species* isolated from the roots nodules of *Vigna unguiculata* grown in Lead Contaminated Soil			
Colony	*Rhizobium species* 1 Round, transparent, non-mucilaginous, smooth, mucoid	*Rhizobium species* 2 Round, transparent, non-mucilaginous, smooth, mucoid	*Rhizobium species* 3 Round, transparent, non-mucilaginous, smooth, mucoid	*Rhizobium species* 4 Round, transparent, non-mucilaginous, smooth, mucoid
Cell shape	Rods	Rods	Rods	Rods
Cell motility	Non-motile	Non-motile	Non-motile	Non-motile
Gram staining	-	-	-	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
Identification Tests	**Characteristics of *Rhizobium species* isolated from the roots nodules of *Vigna unguiculata* grown In Lead Ion Contaminated Soil**			
	Rhizobium species 1	*Rhizobium species* 2	*Rhizobium species* 3	*Rhizobium species* 4
Sodium citrate	-	-	-	-
Lysine decarboxylase	+	+	+	+
Arginine dihydrolase	-	-	+	-
Tryptophan deaminase	+	+	+	-
Voges proskauer	+	+	+	+

Gelatin hydrolysis	-	-	-	-
Acid from glucose	+	-	+	-
Identification Tests	Characteristics of *Rhizobium species* isolated from the roots nodules of *Vigna unguiculata* grown In Lead Ion Contaminated Soil			
	Rhizobium species 1	*Rhizobium species* 2	*Rhizobium species* 3	*Rhizobium species* 4
Acid from sucrose	-	-	+	+
Acid from mannose	+	+	+	+
Acid arabinose	-	+	+	-
Acid from rhamnose	+	+	-	+
Acid from sorbitol	+	+	+	+
Acid from adonitol	-	+	+	+
Acid from melibiose	+	+	+	+
Acid from inositol	+	-	+	-

Rhizobium counts (cfu/g) in root of *Vigna unguiculata* in relation with pollutant levels

Table 4 presents the rhizobium counts (cfu/g) in the root of *Vigna unguiculata* in relation with pollutant levels. The rhizobium counts ranged from $1.41x10^2$ to 2.10×10^2 in soil without OMF fertilizer application. At 250 ppm, when compared with the control, the microbial count reduced by 18 % while at 500 ppm, rhizobium counts reduced by 24%. Similarly, at 750 ppm, the rhizobium counts were reduced by 33%. However, for soils with OMF fertilizer application, the rhizobium counts ranged from $4.10x10^3$ to 4.09×10^2 when compared with unaugmented *Vigna unguiculata*, *Vigna unguiculata*, grown at 250 ppm had improved rhizobium counts of up to 87% while rhizobium counts increased by 81% in the roots of *Vigna unguiculata* grown in 500 ppm soil. However, at 1000 ppm soil treated with OMF fertilizer, the rhizobium counts were 4.09×10^2 while the *Vigna unguiculata* were not treated with OMF fertilizer the *Vigna unguiculata* plants did not survive the pollution stress at 1000 ppm. As the pollution stress increased, rhizobium counts gradually reduced.

Table 4 Rhizobium counts in the root of *Vigna unguiculata* grown in lead contaminated soil at 12 week

Treatments	Rhizobium counts (cfu/g)
Control + F0	2.10×10^2
250 ppm + F0	1.72×10^2
500 ppm + F0	1.60×10^2
750 ppm + F0	1.41×10^2
1000 ppm + F0	Nil
Control + F1	4.101×10^3
250 ppm + F1	1.32×10^3

500 ppm + F1	8.34×10^2
750 ppm + F1	5.39×10^2
1000 ppm + F1	4.09×10^2

F0 = without fertilizer; F1= with fertilizer

Discussion

Rhizoremediation process is an environment-friendly method that combines the activities of nitrogen-fixing bacteria (NFB) with the absorptive remediation capabilities of plants to remove contaminants from the environment. In this study, rhizobial activities at the root zone of *Vigna unguiculata* and the addition of poultry manure suggested to contribute to the uptake of Pb ion from polluted soil. This is because improved plant growth parameters were observed in the amended plants. The agronomic parameters of amended *Vigna unguiculata* were improved when exposed to heavy metal pollution stress. This may be as a result of nitrogen, phosphorus and potassium absorbed from the amended soil and the improved activities of nitrogen-fixing bacteria. This result corroborated with the reports of Wei et al. (2020) in which the addition of different fertilizers influenced the phytoremediation capability of *S. nigrum* exposed to heavy metals as a legume was investigated. In their study, under a pot-culture system, the input of organic manure reduced the heavy metal loadings in shoots of *S. nigrum*. Meaning that poultry manure might be a better fertilizer for strengthening rhizoremediation processes by immobilizing lead in the root of leguminous plants. Moreso, rhizobia as beneficial rhizosphere microbes are known to confer protection to plant exposed to metal pollution stress as well as enhance the uptake of Nitrogen (N) through nitrogen fixation which is later transported to the host plants (Ojuederie & Babalola, 2017; Shikha et al., 2021).

Other factors that were considered in assessing the rhizoremediation potentials of *V. unguiculata* in Pb contaminated soils are bioaccumulation factors (BCFs) and Transfer factors (TFs) (Wang et al., 2020). The root bio accumulation factor (BCF) of *Vigna unguiculata* grown in soil contaminated with lead ion was higher than those reported in the shoots of *Vigna unguiculata*. This shows that *Vigna unguiculata* acted as a phytostabilizer of lead pollutants. These findings are corroborated with the result presented by Wang et al. (2022) and Sharma *et al.* (2020). Outcome of phytoremediation research revealed that roots of photostabilizers possess traits to uptake significant quantities of pollutants and bioaccumulates the metal in the host below ground biomass as shown in this study with low TF and high root BCF value reported across all plants. Here, the effect of Pb toxicity was suggested to have affected the rhizosphere organism that would have been responsible for the uptake of soil nutrients to enhance phytoremediation processes (Oseni et al., 2016; Dada, 2019b; Shikha et al., 2021; Wei et al., 2022).

The outcome of the rhizobia counts also revealed that as the lead pollution strengths increased approximately 52 % reduction in rhizobia counts occurred when compared with those from the control pot-culture due to metal toxicity. Moreover, the drastic reduction to zero level of microbial load and absolute zero tolerance to metal stress was evident in *V. unguiculata* grown in soil with highest lead ion concentration without amendment. However, *V. unguiculata* amended with poultry manure survived with chlorotic expressions on the leaves. This result obtained from this study supported the study carried out by Sharma et al. (2020) on plant-microbe interactions in polluted environments. This revealed the toxicity effects of heavy metal agents uptaken by plants from the background soil in the polluted site. Microbial agents assessed by these scientists indicated that metal toxicity affected activities of bioauguments.. It is suggested that inhibition of microbial growth and activities had occurred due to metal toxicity stress. Similarly, in another study carried out by Dada (2013); Dada, 2019a, other rhizosphere soil microbes such as mycorrhizas were also observed to decrease as pollutant strength increased after inoculating upland weeds grown in lead ion contaminated soils. However, in pots spiked with lead ion pollutants augmented with organic manure fertilizer (OMF), the beneficial effects of the combined use of both the rhizobia and the amendment (OMF) were obvious from the reports obtained on the growth parameters of the plants. Highest mean values were reported at 5% level of significance in *Vigna unguiculata* exposed to 1000 mg kg^{-1} Lead compared to results obtained when soil was not amended. This shows that the influence of rhizobia on the plant parameters assessed as well as both the wet and dry biomass of the plants also depend on the OMF applications. Although, decrease in rhizobia counts were noticed as the level of pollutants increased. However, increased elemental deposition of lead was evident as significant amounts were bioaccumulated in the plants root tissues than in the soil-environment with root BCF above 1.0. Although, with TF below 1.0. This shows that along with OMF amendment, rhizobia enhanced the phytostabilization potentials of *Vigna unguiculata*.

Moreover, only *Vigna unguiculata* growing in soils contaminated with lead without amendment showed significantly reduced growth parameters and diverse grades of chlorosis and leaf deformation. Those growing in soils spiked with Pb contents above 250 ppm between 2 weeks and 8 weeks showed practically no significant morphological differences but variables such as height and vigour barely reached 50% of those shown by controls growing in non-polluted soil. However, above 8 weeks until 12 weeks. *Vigna unguiculata* showed chlorotic expressions and increased leaf drops. Although, *Vigna unguiculata* grown under augmentations by OMF showed chlorotic expressions at the 12 weeks. This can be attributed to lead toxicity as *Vigna unguiculata* grown in 1000 mg/kg Pb pollution without augmentation did not

germinate. Here, the tolerance level of *Vigna unguiculata* was exceeded.

Conclusion

In this study, *Vigna unguiculata* was observed to rhizoremediation Lead with and without augmentations by acting as phytostabilizers when exposed to metal contamination. Therefore, *Vigna unguiculata* is a prospective entrant for phytostabilization of lead pollutants in soils polluted by metals. A major advantage of using *Vigna unguiculata* is the ability to thrive in various wastelands or habitats around the world which increases the chances of commercial application in tropical and subtropical environments. However, the study recommended that leguminous plants are efficient in remediation of soil polluted with heavy metal such as lead. Besides, isolated rhizobia strains also displayed a resistance to heavy metals toxicity and could be commercially formulated and used to promote plant growth during phytoremediation processes.

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