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Cu Tolerance and Accumulation by *Centrosema Pubescen* Benth and Mucuna *Pruriens* Var Pruriens

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Abstract

In order to assess their practical capability for the absorption and accumulation of Cu, two common crop plants, i.e. Centro (Centrosema pubescens Benth) and Mucuna plants (Mucuna pruriens var pruriens) were tested in pot experiments using simulated crude oil polluted soil in the concentrations of 2, 4, 6, 8, and 10 % (v/w). A range of amendments of various types was tested for increasing the copper uptake with the test species and these included UREA fertilizer, NPK fertilizer and Chicken manure. Cu concentrations of the soil ranged from 201.1 to 271.5 mg/kg after spiking. Cu uptake and translocation into the shoots of Mucuna and Centro plants were 91 mg/kg and 6.25 mg/kg respectively, in the un – amended treatments at the highest contaminant dose of simulated spill. Amendments further took the observed levels to 90.1, 63 and 117 mg/kg Cu and 8, 23, and 10.92 mg/kg Cu for NPK, UREA and POULTRY amendments in Mucuna and Centro plants respectively. Cu root concentrations were markedly higher than those of the shoots for all Centro plants and the reverse for Mucuna species respectively. While POULTRY MANURE – assisted phytoextraction with Mucuna plants by all indices proved efficient, our study showed that Centro plants were not feasible to remediate the heavily or moderately contaminated soils simulated in order to achieve the target total metal soil concentrations irrespective of the amendments employed. Tests with NPK and UREA fertilizers indicated detrimental effects on copper uptake, biomass yield, and the translocation of copper from roots to shoots in Mucuna species.

Key Words: Copper; Phytoextraction; Toxicity; Amendments; Contamination; Bioconcentration factor (BCF).

INTRODUCTION

Petroleum is a complex mixture of hydrocarbons that form from the partial decomposition of biogenic materials [1]. Elemental analysis has revealed that spectra due to metal elements such as Ca, Fe, Mg, Cu, Zn, Na, Ni, K and Mo in crude oil were recorded using Laser induced breakdown spectroscopy (LIBS) technique [2] in crude oil. Crude oil is used for the production of fuel and lubricants for transportation and energy needs and as a raw material for the petrochemical industry [3]. The severity of oil spills on plant and microbial life depends on the type of hydrocarbons, toxic metals, amount of oil involved, type of habitat, degree of weathering, sensitivity of affected organisms, topography of the land and adequacy of response [4]. Crude oil spills lead to insufficient aeration, a reduction in the level of available plant nutrients and a rise in toxic levels of certain elements such as cadmium, copper, manganese and iron [5]. Remediation options currently applicable to heavy metal-contaminated soils are frequently expensive, environmentally invasive and do not make cost-effective uses of existing resources. These techniques are based upon civil engineering methodologies, involving either the excavation and removal of contaminated soil (dig and dump) or an ex situ remediation treatment that drastically alters soil structure, biological activity and subsequent function. There is a clear need for cost-effective, durable and validated alternative remediation strategies to those that are in current use [6]. The focus of much recent experimental work has been directed towards these ends, developing techniques that exploit biological (plant and micro-organisms) and chemical (use of metal-binding agents) processes to reduce the inherent risk associated with metal-contaminated soils. Strategies of this nature are classified under the generic heading of phytoremediation. Metals, radionuclides and other inorganic contaminants are among the most prevalent forms of environmental contaminants, and their remediation in soils and sediments is rather a difficult task [7]. Sources of anthropogenic metal contamination include oil spills, smelting of metalliferous ore, electroplating, gas exhaust, energy and fuel production, the application of fertilizers and municipal sludges to land, and industrial manufacturing [7] [8].

Use of soil amendments such as synthetics (ammonium thiocyanate) and natural zeolites have yielded promising results. Synthetic cross-linked polyacrylates, hydrogels have protected plant roots from metals toxicity and prevented the entry of toxic metals into roots. Hyperaccumulators accumulate appreciable quantities of metal in their tissue regardless of the concentration of metal in the soil, as long as the metal in question is present. Soil metals should also be bioavailable, or subject to absorption by plant roots [9]. Chemicals that are suggested for this purpose include various acidifying agents, fertilizer salts and chelating materials. The retention of metals to soil organic matter is also weaker at low pH, resulting in more available metal in the soil solution for root absorption. It is suggested that the phytoextraction process is enhanced when metal availability to plant roots is facilitated through the addition of acidifying agents to the soil [9]. Chelates are used to enhance the phytoextraction of a number of metal contaminants including Cd, Cu, Ni, Pb, and Zn. Researchers initially applied hyperaccumulators to clean metal polluted soils. Several researchers have screened fast-growing, high-biomass-accumulating plants, including agronomic crops, for their ability to tolerate and accumulate metals in their shoots. Genes responsible for metal hyperaccumulation in plant tissues have been identified and cloned [10]. Centro has good resistance to stressful conditions and experiences die-back from its shoot [11]. In many parts of the world Mucuna is used as an important forage, fallow and green manure crop [12]. As a member of the legume family (peas and beans), and with the help of nitrogen fixing bacteria, Mucuna takes nitrogen gas from the air and combines it with other chemical compounds producing fertilizer and improving the soil [13].

To date, studies on the tolerance and uptake of copper by Centro plants and Mucuna as affected by various types of amendments are limited. In this study, we investigated the efficacy of Mucuna and Centro plants to accumulate Cu from a crude-oil polluted soil and thereby potentially be relevant for remediating crude oil polluted soils with respect to metals. Specie choice was informed by the natural occurrence in the local environment and especially stressed type. Modifications with NPK (23:13:13) fertilizers, UREA fertilizers, and POULTRY MANURE were also investigated and choice of amendments was dependent on their semblance for natural plant root exudates.

MATERIALS AND METHOD

Description of Study Area

Soil sample consists of 18 individual soil cores from Choba East of Port Harcourt, Nigeria and thoroughly mixed. Choba is one of the Ikwerre speaking communities in Obio-akpor Local Government Area of Rivers state. The area is located on plain land rain forest belt of Nigeria. There are naturally occurring vegetation around the surrounding as part of which has given way to agricultural activities. Choba community is slightly metropolitan although communal life of the people seems intact with commercial trading and farming as their major occupation and has no history of oil spill.

Soil Sampling and Materials Sourcing

Surface Soil samples (0-20 cm depth) were collected using a clipped quadrat technique in a stratified random sampling design (randomized block design) for effective sample representation. Ca, Mg, Na and K, the four ‘base cations’ and other relevant parameters were characterized in the air-dried, gently crushed and sieved (2mm nylon screen) soils. The legumes, *Centrosema pubescen* Benth and Mucuna *pruriens* var pruriens were sourced from University of Port Harcourt Botanical garden and Nsirimo in Abia state both of Nigeria respectively. Viability test was run to confirm the seed quality. Also, prior to planting, scarification and imbibition of the seeds were carried out to improve germination. Chemical fertilizers were obtained from the Agricultural Development Project (ADP) Port Harcourt while fresh poultry manure were obtained from ‘De Peoples poultry’ in Idu local government area of AkwaIbom state all in Nigeria while Bonny light crude oil (see character in table 7) was sourced from the Nigerian National Petroleum Corporation (NNPC, PHRC).

Soil treatment/ Planting

Polythene bags measuring 45 cm x 45 cm were perforated with 3-5 holes to allow easy drainage and to it was added 2 kg soil each in triplicates for every treatment group. For soil preparation, 0.8 g of amendments were added according to the method of Akobundu [13], $C = (R \times A)/Q$ (where C = amount of amendment, $R = 2$ = a constant, A = weight of soil, and Q = product weight of substance as it is bagged). Preliminary range toxicity test was done to arrive at 5-concentrations of contamination (2, 4, 6, 8, and 10 % v/w) for each treatment option. Soil devoid of crude oil served as a control. Crude oil treated soil (topdressing) was left for one week and moistened for another one week to enable it to settle. Thereafter, amendments were applied as topdressed UREA, NPK AND POULTRY MANURE according to treatment groups to mimick natural homogenizing for two weeks. Deionized water was added to all bags when necessary in order to keep the soil moist for nutrient transport. To prevent losses of lightweight hydrocarbons by leaching, plants were not excessively watered but were provided with sufficient gravimetric water, resulting in approximately 75 % of soil capacity as measured by a soil tensiometer (Irrometer Company, Riverside, CA. Seeding date, rate, and variety were the same for each treatment group. Ten (10) Seeds each were germinated in the actual treatment soils and thinned down to three (3) after a week of growth in three (3) replicates. The experiment were set up in three replicates and monitored for twelve (12) weeks of growth. The growth indices collected at 2-weeks intervals were leaf area (LA), which was calculated from the method of Irwin [15] [16] and plant height. Available crop residues/remains, including straw, and organic wastes were put back to maintain the humus content of their soils by incorporation. Visual inspection of plant responses to treatment was carried out.

Post - harvest study

At the end of the 12 weeks study period, harvested plants were separated into shoots and roots. The plants were washed first with tap water and then with deionized water. Roots were immersed in a solution of 20 mmol L^{-1} Na_2 – EDTA for 30 min to remove extracellular metals before washing with tap water and deionised water. The washed root and shoot samples were dried at 80^oC for 48 h, then their dry weights were recorded and were ground and digested with aqua-regia (HCl and HNO_3 in the ratio of 3:1). Cu was determined by atomic absorption spectrophotometry (Varian SpectrAA 220 FS, Varian, Palo Alto, CA, USA). The quality assurance of Cu concentrations was determined with a certified reference material and blanks in each batch for quality control. Experimental unit was average values per pot of plants.

Statistical Analysis

Results are reported as means of at least two independent experiments with three replications. Statistical Package for Social Sciences for Windows version 10.0 (SPSS Inc., Chicago, IL) was used to perform one – or two – way analyses of variance and the pearson correlation. The standard error of means and significant differences according to the least significant difference (LSD) test at a significance level of $P < 0.05$ were calculated with the same software.

RESULTS AND DISCUSSION

Phytoremediation experiments carried out over the last decade, based upon the above principles, have shown promise as viable soil treatment techniques. Such “soft” technology can be subdivided into two possible alternative approaches, both reliant upon a reduction in the biologically-active soil metal pool. These are a) phytostabilization or phytorestoration with metal inactivation arising as a result of revegetation either with or without the use of metal immobilizing agents and b) phytoextraction, based upon accelerated metal removal from soil by cultivation of metal-hyperaccumulating plants. In both approaches, the potential role of micro-organisms often is underestimated. The potential toxic effects of the contaminant and amendments were assessed by visual observations of the root and tissue during the growth period, in addition to measurements of the root and shoot biomass production after exposure. Observations were made of toxicity symptoms such as wilting, discoloration, and leaf necrosis.

From visual inspections, Centro plants were less sensitive to treatment below 10 % contaminant concentration and amendment treatments.

In consonance with the findings of [9], root growth (data not shown) was more significantly affected than shoot growth due to the addition of chemical amendments for UREA – amended treatment. There was no statistical significant differences in average plant height among the contaminated, un- amended plants, contaminated, NPK – amended plants and POULTRY MANURE – amended plants. UREA – amended, contaminated plants gave stunted growth and less bunch growth above 2 % concentration of pollution. This could be due to an imbalance of nutrients in the soil. Tang et al. [17] reported similar observations.

Shoot biomass (Figures 1 and 2) of POULTRY MANURE treatment, PM was approximately 2 times larger than those of UREA and NPK at 12 weeks after germination, WAG and shoot biomass is a more reliable indicator of plant growth performance than plant height. Markedly increased was plant height and shoot biomass compared with UREA. Therefore, in the pot experiment, PM treatment had the largest effect on plant height and shoot biomass of Mucuna plants.

Copper translocation from root to shoot in Centro and Mucuna plants differed widely by amendment and contaminant concentrations (Tables 2, 3, 5, and 6). The addition of UREA fertilizer caused the most noticeable reduction of copper translocation to shoots in Mucuna plants (Table 3). Compared to low and high contaminant concentrations, the application of POULTRY MANURE proved to be most effective for enhancing copper translocation to Mucuna shoots. For both species, influences of the NPK fertilizer on copper translocation were not significant. There were no observed injures for POULTRY MANURE treatments for both plants. There are clear differences between the two species tested in terms of tolerance and translocation. Observations and measurements of toxicity and biomass production revealed that Mucuna showed greater tolerance to the amendments trialed, potentially making Mucuna a more suitable species for the phytoremediation of copper (Tables 1, 2 and 3). Although UREA fertilizer proved to be ineffective for enhancing metal accumulation by the plants (Tables 3 and 6), it may still be useful for enhancing metal mobilization from soil particles. Centro plants were more sensitive to the amendments than Mucuna, with nearly all amendment applications resulting in decreased dry matter accumulation (Figures 1 and 2).

Soil Cu removal proceeded with 40.15, 51.7, 41.58 and 52. 11 % and 48.21, 51.12, 50.28 and 59.85 % for Centro and Mucuna plants respectively while the unplanted un-amended contaminated soil gave 23.35 % soil Cu removal. Phytotoxicity symptoms [18] evident in high Cu levels in Centro plants resulted in wilting [5], and eventually death of plants at 12WAG. This levels falls within the critical Cu content for toxicity (20 – 30mg/kg) in most plants [17]. Their bioconcentration factor (BCF; the ratio of metal concentration in shoots to that of the soils was 0.65, 0.67, 0.47, and 1.1 and 0.04, 0.06, 0.15, and 0.1 for Mucuna and Centro plants respectively. The BCF obtained for POULTRY MANURE – amended treatment with Mucuna is comparable to that of cockscomb [19]. A range of 112 to 122 mg/kg soil Cu were referred to as low [19] and thus Poultry manure - assisted phytoextraction could be said to have reduced the soil Cu levels to low 109.3 mg/kg levels and is thus recommended. Also, the accumulation capacity of Mucuna plants (117 ± 36 mg/kg) was equivalent to those of cockscomb (117 ± 39) as reported by Lai and Chen [19].

Table 1 Mean levels of Cu (mg/kg) in Soil at 12WAG Mucuna Harvest

TRMT	PRE-P	CON	NPK	UREA	PM	UNPLANTED
CTRL	24.3a±7	24.33a±7	24.3a±7	24.3a±7	24.3a±7	24.3a±7
2	201.1d±70	118c±6	100.5g±35	96.2h±16	82h±25	162h±40.4
4	209.5b±54	123.1d±5	107.2g±33	98.6h±25	77.2h±23	162h±35
6	222.4b±44	128.7e±5	113d±29	114.0i±25	82.4h±17	183.2h±25
8	236.5b±39	137e±6	116.2p±34	119.5ci±26	98.1mh±15	189.5mh±20
10	271.5bc±88	140.6fe±4	132.7pd±24	135g±47	109.3m±8.9	208.1mh±18

Values denote mean ± SEM (n = 3). Different letters within each column indicate difference by least significant digit ($p < 0.05$). TRMT, CTRL, CON, PREP –P, PM, UREA, NPK and WAG represent treatment type, control experiment, contaminated treatment devoid of amendment, pre-plant soil i.e. 2weeks after contamination and before planting, and poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

Table 2 Mean levels of Cu (mg/kg) found in Mucuna root at 12WAG

TRMT	CON	NPK	UREA	PM
CTRL	11.5b±4.09	11.5b±4.09	11.5b±4.09	11.5b±4.09
2	30c±4.7	36nj±6	42o±6	37jk±6
4	32.10c±5	38.3j±11.6	42oj±6.4	391k±5
6	36.10cd±8.4	48j±15	58j±13.4	53l±5.7
8	40.05cd±15	51kj±12	67k±6.8	49.2kl±5.9
10	43.8cd±9.2	51.8k±9	69l±14	55.4l±4

Values denote mean ± SEM (n = 3). Different letters within each column indicate difference by least significant digit ($p < 0.05$). TRMT, CTRL, CON, PM, UREA, NPK and WAG represent, treatment type, control experiment, contaminated treatment devoid of amendment at 12WAG, poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment at 12 WAG, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

Table 3 Mean levels of Cu (mg/kg) found in Mucuna shoot at 12WAG

TRMT	CON	NPK	UREA	PM
CTRL	12.3b±3	12.3b±3	12.3b±3	12.3b±3
2	52.8c±5	60.3o±15	54.4o±16	82.1kl±13
4	50.6c±4	62.5jo±21	58.13j±9	82.08kl±9
6	54.01gh±6	65ko±26	50.4k±12	87.2k±25
8	55.9g±6	70.1l±19	50.1l±8.9	89.45h±16
10	91gij±6	90.1l±5	63hi±11	107hkl±36

Values denote mean ± SEM (n = 3). Different letters within each column indicate difference by least significant digit ($p < 0.05$). TRMT, CTRL, CON, PM, UREA, NPK and WAG represent, treatment type, control experiment, contaminated treatment devoid of amendment at 12WAG, poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment at 12 WAG, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

Table 4 Mean levels of Cu (mg/kg) in Soil at 12WAG Centro Harvest

TRMT	**PRE-P**	**CON**	**NPK**	**UREA**	**PM**	**UNPLANTED**
CTRL	28.78a±4.76	28.78a±4.76	28.78a±4.76	28.78a±4.76	28.78a±4.76	28.78a±4.76
2	201.1d±70	129.50b±27.66	110.22d±40	126±41.9	108.75i±5.87	162h±40.4
4	209.5ab±54	130.00c±35.44	119±38.7	128.5±33	118.75i±8.22	162h±35
6	222.4b±44	146.25c±4.96	122.1±43.4	143.9±40	120g±6.88	183.2h±25
8	236.5b±39	155.00d±5.34	124±30.6	150±35.6	123.25g±6.23	189.5mh±20
10	271.5bc±88	162.50d±5.45	130.03±29	158.6±34	131.25g±6.23	208.1mh±18

Values denote mean ± SEM (n = 3). Different letters within each column indicate difference by least significant digit ($p < 0.05$). TRMT, CTRL, CON, PREP –P, PM, UREA, NPK and WAG represent treatment type, control experiment, contaminated treatment devoid of amendment, pre-plant soil i.e. 2weeks after contamination and before planting, and poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

Table 5 Mean levels of Cu (mg/kg) found in Centro Root at 12WAG

TRMT	**CON**	**NPK**	**UREA**	**PM**
CTRL	3.6b±0.9	3.6b±0.9	3.6b±0.9	3.6b±0.9
2	15.00e±5.67	6.78cd±1.9	16.8v±1.1	6.9c±1.7
4	15.08e±5.23	7c±1.23	21.6w±2.3	9.8d±1.88
6	15.70e±6.02	9.22d±2.5	25.1w±3.4	10.4dc±2.4
8	16.63ef±4.67	9.8d±2.1	25.8wx±3.6	11.6edc±3
10	18.25f±6.23	10.1de±2.33	27.75x±3.91	11.8c±3.3

Values denote mean ± SEM (n = 3). Different letters within each column indicate difference by least significant digit ($p < 0.05$). TRMT, CTRL, CON, PM, UREA, NPK and WAG represent, treatment type, control experiment, contaminated treatment devoid of amendment at 12WAG, poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment at 12 WAG, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

Table 6 Mean levels of Cu (mg/kg) found in Centro shoot at 12WAG

TRMT	**CON**	**NPK**	**UREA**	**PM**
CTRL	1.51g±0.6	1.51g±0.6	1.51g±0.6	1.51g±0.6
2	5.00e±5.67	6a±1.4	15.1w±3.4	6.8a±1.1
4	5.00e±5.23	6.1ab±1.6	15.8wx±3.6	9.6bc±2.3
6	5.00e±6.02	7.2c±3	17.75x±3.91	10b±2.5
8	5.63e±4.67	7.91cd±2.9	20.1yx±4.2	10.6b±1.9
10	6.25f±6.23	8cd±3.02	23z±5.6	10.92bc±3

Values denote mean ± SEM (n = 3). Different letters within each column indicate difference by least significant digit ($p < 0.05$). TRMT, CTRL, CON, PM, UREA, NPK and WAG represent, treatment type, control experiment, contaminated treatment devoid of amendment at 12WAG, poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment at 12 WAG, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

Table 7 Composition of the Bonnylight crude oil used in this study

S/N	Parameter	Amount	S/N	Parameter	Amount
1	API gravity at 60F	38.1	11	Viscosity @ 100F Deg fsu	37.8
2	Specific gravity	0.84	12	Sediment and Water (bsw) v/v%	Trace
3	Characterization factor	11.75	13	Organic Chlorides	8.0
4	Colour	Brownish-green	14	Copper wt (ppm)	0.7
5	Acid number	0.39	15	Carbon residue w/w%	0.92
6	Pour point deg F.	35	16	Iron wt (ppm)	1.0
7	Salt Content Lbs/1000bbl	77.9	17	Vanadium wt (ppm)	2.0
8	Reid water pressure	4.9	18	Nickel wt (ppm)	4.0
9	Sulphur w/w%	0.18	19	Crude volume v/v%	32.7
10	Viscosity @ 60F Deg fsu	54.7	20	Density @ 15^{o}C	0.89

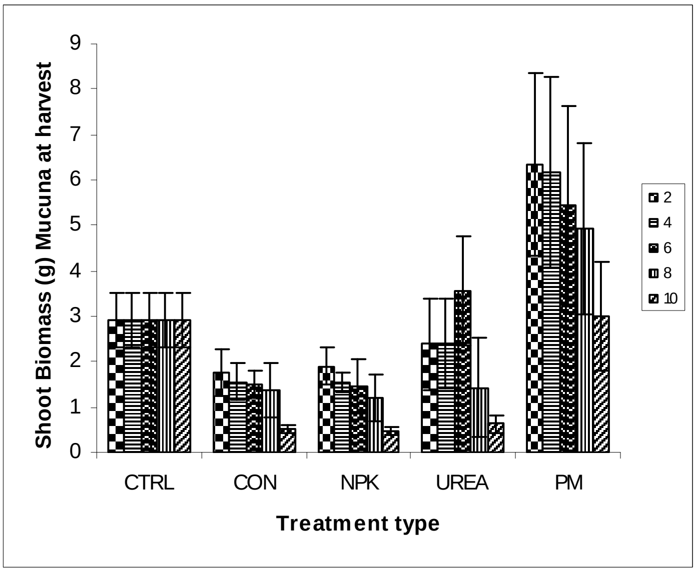

Fig 1 Shoot Biomass by Mucuna plants at 12 weeks after germination (12WAG). Values denote means ± standard error of the means (n = 3). CTRL, CON, PM, UREA, NPK and WAG represent, control experiment, contaminated treatment devoid of amendment at 12WAG, poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment at 12 WAG, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

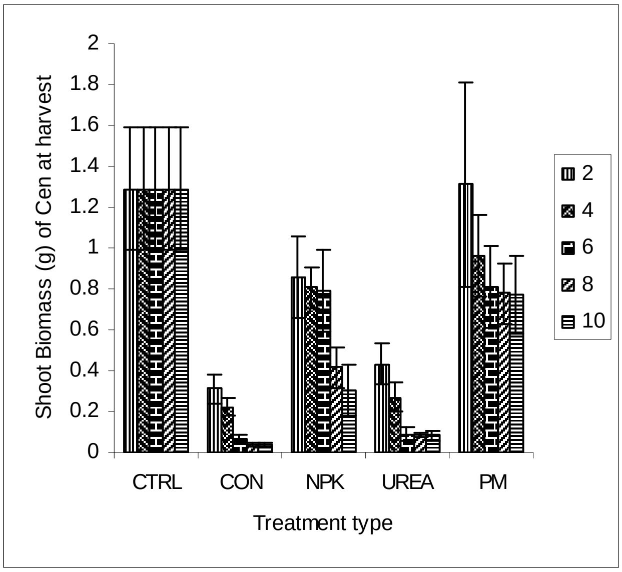

Fig 2 Shoot Biomass by Centro plants at 12 weeks after germination (12WAG). Values denote means ± standard error of the means (n = 3).). CTRL, CON, PM, UREA, NPK and WAG represent, control experiment, contaminated treatment devoid of amendment at 12WAG, poultry manure amended contaminated treatment, UREA fertilizer - amended contaminated treatment at 12 WAG, NPK fertilizer - amended contaminated treatment, and Weeks after germination. 2, 4, 6, 8, and 10 represent different spill concentrations (%v/w) simulated.

CONCLUSION

Observations and measurements of toxicity and biomass production revealed that Mucuna showed greater tolerance to the amendments trialed, potentially making Mucuna a more suitable species for the phytoremediation of copper. Accumulation of Cu was higher in the shoots compared to the roots or Mucuna, which is advantageous at green remediation. Although, there was no measurable uptake of contaminants with UREA amendments, it could be useful for enhancing contaminant mobilization from soil particles.

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