



Study on Interactive Effects of Different Levels of Lead and Mercury on Nitrogen Fixation of Some Diazotrophs

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Authors' contributions

This work was carried out in collaboration between both authors. Author KUA collected the data, wrote the manuscript, performed the statistical analysis and literature searches. Author JUJ conceived and designed the study. Both authors read and approved the final manuscript.

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ABSTRACT

Researchers have studied the effects of addition of metal elements in combination with nitrogen fixing organisms as inoculants on the plants (growth) predominantly in legumes, however there is a major gap because responses and effects of these proposed micronutrients on the nitrogen fixation activity of these microbes both free living and symbiotic remains sketchy at best. Therefore, the effect of supplementation of lead and Mercury (bioaugmentation) on the nitrogen fixation potential of two (2) diazotrophs was evaluated in this study.

Aims: To evaluate the interactive effects of different levels of Lead and Mercury on Nitrogen fixation of both *Rhizobium* and *Xanthobacter* spp *in-vitro*.

Place and Duration of Study: Sample organisms were collected from Groundnut rhizospheric soil of a farm in Cross River state, Nigeria. The microorganism isolation and nitrogen fixation analysis was further carried out at MacCliff General services Laboratory, Owerri, Nigeria for a duration of 3 months.

Study Design: The interactive plots serve to show the effect of one variable (lead) on the value of mercury (the other) and is derived by selecting high and low values for lead (Pb) and entering them into the equation along with the range of values for Mercury (Hg). The values of independent

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variables (lead and mercury levels) used in the plots were selected by observing the highest concentration (+1) and lowest concentration (-1) values which are able to support nitrogen fixation independently in *Rhizobium* and *Xanthobacter*.

Methodology: The soil samples were collected from groundnut rhizosphere at a 20 cm depth using sterile soil corer (sterilized with 95% ethanol) and matured Groundnut plants were uprooted with care. From these samples, both *Rhizobium* and *Xanthobacter* spp were isolated. The isolated organisms were re-vitalized in Jensen's nitrogen free broth and standardized to 0.5 McFarland standards. To determine nitrogen fixation, the broth cultures were examined for nitrate nitrogen ($\text{NO}_3\text{-N}$) and amino nitrogen (Amino-N) levels after ten days of the experiment under continuous airflow using the Jensen's nitrogen free broth containing the metal salts, Mercury (II) chloride HgCl_2 and Lead (II) acetate trihydrate $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$. Nitrate nitrogen and amino Nitrogen was obtained using cataldo and ninhydrins methods respectively. The data obtained was made in triplicates and reported as mean values. Interactive effect plots and statistical analysis were done using Minitab 17 software at 5% level of significance ($p < 0.05$).

Results: The main effect plots illustrate that to maximize nitrogen fixation in *Xanthobacter* sp through the utilization of the selected metals as micronutrient, we should use lead at 6.25 mg/L and mercury at 25mg/L yielding 0.508 mg/L for nitrogen fixation response. The plot also suggests that if lead metals are used at a higher concentration than stated nitrogen fixation will decline. On the interaction plots, the slopes indicate that an interference or antagonistic interaction effect (crossed lines) exist between lead and mercury in the nitrogen fixation activity of *Xanthobacter*. The R-squared adjusted value suggests that 70.87% of the variations in nitrogen fixation response is explained by the interaction of lead and mercury, hence the model likely fits the data. However, the P-value was not significant at 0.102. For *Rhizobium* sp. mercury also has a higher fixation magnitude than Lead but relatively at 0.554 mg/L. However, the interaction plot showed parallel lines indicating that there was no interaction effect. Therefore, one can say that the relationship between lead and nitrogen fixation does not depend on the concentration of mercury and vice versa. The model was also statistically insignificant at 0.981.

Conclusion: Interactive effect only occurred in the nitrogen fixation of *Xanthobacter* sp. This raises a need for further study combination of metal elements which could be utilized to stimulate nitrogen, phosphorus and potassium production in Diazotrophs both in the field and *in-vitro*.

Keywords: Interactive effects; lead; mercury; diazotrophs; Nitrogen fixation.

1. INTRODUCTION

Free-living and symbiotic nitrogen fixing microorganisms known as Diazotrophs, fix nitrogen by conversion of atmospheric nitrogen (N_2) into ammonium, NH_4^+ [1]. The most commonly researched group of nitrogen-fixers are; Asymbiotic bacteria and the symbiotic bacteria generally termed "Rhizobia", which have the ability to form organs known as nodules, that aid nitrogen-fixing in leguminous plants [2]. Symbiotic Plant Growth Promoting Rhizobacteria (PGPR) are host-specific and fix nitrogen from the atmosphere to plants in exchange for carbon source, the most widespread PGPRs are *Rhizobium* and *Bradyrhizobium* [3]. Non-symbiotic PGPR (Asymbiotic) are free-living species that also carry out biological nitrogen fixation either by living within the soil rhizosphere as endophytes or colonizing root surfaces, these include; *Xanthobacter*, *Azotobacter*, *Achromobacter*, *Acetobacter*, *Azospirillum*, *Bacillus*, *Clostridium*, *Enterobacter* and

Pseudomonas spp [4]. These beneficial organisms can also be called Biofertilizers, they are required for high yield in farming practices and to supply nutrients through an environmentally friendly process known as Biological Nitrogen Fixation, BNF [5].

Heavy metals are contained in chemical fertilizers, herbicides and pesticides used continuously in agriculture especially the metals lead and mercury and their repeated use tends to have such adverse effects as overtime accumulation in the soil, plants and consequently in the harvested crops. Although most heavy metals are toxic, some are important micronutrients which promote plant growth, there is however a line between toxicity and beneficial micronutrient to crop health [6]. Mercury and Lead (lead arsenate) are introduced to the environment by toxic and persistent application of chemicals like pesticides, herbicides and fertilizers [7]. Diverse studies disclosed that when present or accumulated in agricultural soils, they

impede agricultural output and fertility of the soil due to their harmful effects to organisms and soil ecosystem [8]. Intensive farming systems require the addition of high amounts of fertilizers especially in supplying sufficient Nitrogen, Phosphorus and Potassium for crop growth and with continued fertilizer application trace amounts of heavy metals are incorporated [9]. These agrochemicals particularly fertilizers are composed of metals like Zn, Hg, Cd, Pb, Cu, Cr etc. as their constituents, they invariably annihilate non-target bacteria or soil microorganisms with a resultant effect of lowering their residual concentrations, nitrogen fixation capacity and raise heavy metal levels in the soil solution [10,11,12].

Many soils suffer from multiple nutrient deficiencies including macronutrients as well as micronutrient deficiency [13], hence replacement of fertilizers with microbes (biofertilizers) will be environmentally friendly and cost effective especially using nitrogen fixing organisms which aid the supplementation of nitrogen which is a major soil nutrient. Many nitrogen fixing organisms are tolerant to these heavy metals which may prove a useful factor in improving their efficiency. However, as a lot of studies agree on the negative effect of heavy metals on microbiological activities of the soil, other experiments display that there is no evidence of correlation between these soil properties and heavy metal contamination [14]. Hence, for bio-augmentation and optimization of these biofertilizers nitrogen fixation, it is beneficial to study the concentrations at which the selected heavy metals would improve known diazotrophs nitrogen fixation. To be useful to sustainable agriculture in the presence of heavy metal stress, the key mechanisms used by nitrogen fixers such as *Rhizobium* for tolerating heavy metals toxicity are; metal ion accumulation inside the cells, bio-reduction of the toxic metals, methylation, precipitation, chelation and evidently reduction via phytoremediation process [15]. *Xanthobacter* however, possesses DNA composing of mer genes, these genes are responsible for the resistance to mercury metal of the bacteria and necessitate the toxicity of mercury entering food chains [16]. These properties make this nitrogen fixing bacteria a potential candidate for biofertilizers which aid soil fertilization without applying hazardous chemical fertilizers [17].

There is stunted understanding of the influence of micronutrients (heavy metals) on soil bacterial population both singular or in combinations, so

far little is obtainable about their roles and interactive influences on Biological nitrogen fixation [18,19]. Of late, the emphasis of research has been placed majorly on inhibitory effects of trace metals on plant responses especially for symbiotic nitrogen-fixing associations rather than on interactions with the microorganisms in the rhizosphere [14]. Reasonable effort has been dedicated to using metals as catalysts and diazotrophs to enhance the conversion of nitrogen to usable forms such as ammonia, NH₃ [15]. To this end, Mercury and Lead of which little information is known about how it affects free-living and symbiotic diazotrophs [2] would be studied due to their presence in agricultural soils from fertilizers and pesticides. Attention was paid to understanding how the metals in interaction could affect the nitrogen-fixing ability of *Rhizobia* as a model symbiotic organism and *Xanthobacter* sp as a free-living organism.

2. MATERIALS AND METHODS

2.1 Test Organisms Isolation

To isolate the free-living organism, soil samples were collected from groundnut (*Arachis hypogaea*) rhizosphere at a 0-30 cm depth. Plant residues, gravel and other debris were gently removed from the soil. The samples were then serially diluted and 0.1 ml streaked on a petri plate using Jensen's nitrogen free media as selective media to grow this nitrogen fixing bacteria. The streaked plates were incubated at 28°C and observed after 48 hours. The samples were further analyzed for microbiological characterization [21].

Mature Groundnut plants were uprooted and their roots cleaned. Healthy nodules were cut from the roots and sterilized using 90% ethanol, they were further crushed in sterile distilled water to release the bacteria and form a suspension. Ten-fold serial dilution method was utilized for dilution of the suspension and the 10³ part smeared on Yeast Extract Mannitol Agar, YEMA petri plates [17]. At 29 °C, the plates were incubated and after 4 days translucent, elevated and entire colonies observed on the medium's surface. Congo red dye served as an indicator and Jensen's medium used for further authentication of the samples. The isolates were identified using morphological and biochemical methods [22].

2.2 Microbial Analysis

Colonies were observed on the YEMA and Jensen plates at 32°C, to establish the colony characteristics. Viewing the plates with a microscope ascertained the morphological characteristics of the isolates. Several biochemical tests viz; Gram staining, Motility test, Oxygen utilization test, Catalase test, Oxidase, Indole tests, Growth in peptone water and spore staining determined the biochemical characteristics of the isolates. The derived morphological and biochemical characteristics were compared to the schemes of Bergey's Manual of Determinative Bacteriology as a guide for identification of *Rhizobium* and *Xanthobacter* species.

2.3 Preparation of Inoculum

Cultured samples of *Rhizobium* and *Xanthobacter* spp were re-vitalized in Jensen's broth. Sample organisms from the Jensen's agar slant were introduced to sterile Jensen's (nitrogen free) broth and incubated at room temperature for 7 days under continuous airflow. Standardization of the inoculum was done using 0.5 McFarland standards [23].

2.4 Estimation of Metals Effects on Nitrogen Fixation

Effect of these metal salts in interaction on the Diazotrophs were investigated. 6.25mg/L and 25 mg/L concentrations of Mercury (II) chloride $HgCl_2$ and Lead (II) acetate trihydrate $Pb(CH_3COO)_2 \cdot 3H_2O$ salts in Jensen's broth (10ml) was prepared based on the highest and lowest levels at which there was nitrogen fixation response (6.25mg/L, 12.5 mg/L, 25 mg/L, 50 mg/L and 100 mg/L).

A loop full of bacterial culture was inoculated separately into each flask containing Jensen's diluted broth medium amended with the different salt concentrations including the control without metals. The inoculated flasks were then placed at 28 ± 1 on a shaker at speed 150 rpm and incubated for 10 days at 30°C. According to Orji [24], the broth cultures were examined for nitrate nitrogen (NO_3-N) and amino nitrogen (Amino-N) levels after ten days of the experiment under continuous airflow. Nitrate nitrogen was tested using Cataldo's method, the procedure involves: 2.5 μ l of the sample solution put into a 1.5 ml Eppendorf tube, 10 μ l of salicylic acid-sulfate solution mixed in and kept for 20 minutes. After

which, 250 μ l of 2M NaOH solution was added and kept for 20 minutes. 200 μ l of this reaction solution was put in a 722 visible spectrophotometer and the absorption at 410 nm recorded. Standard solution was made by disintegrating 42.5 mg of $NaNO_3$ in 100 ml of water, which comprises 5 mM nitrate (70 mg N l⁻¹).

For Amino nitrogen, ninhydrin method was used entailing; 2.5 μ l of sample solution added to a 1.5 ml Eppendorf tube and 75 μ l of citrate buffer mixed in. Afterwards, 60 μ l of the solution was dissolved in 3.2 ml of water and methoxyethanol added to bring up to 100 ml in a flask. The flask was covered and heated for 20 minutes in a hot air oven at 100°C. 300 μ l ethanol was introduced and left to sit at room temperature for 10 minutes. Then, 200 μ l of the reaction solution put in a 722 visible spectrophotometer for absorption readings to be taken at 570 nm. Standard solution was made by dissolving 66.1 mg of asparagine and 73.1 mg glutamine in 100 ml of water, already containing 5 mM asparagine + 5 mM glutamine (280 mg N L⁻¹).

2.5 Statistical Analysis

The triplicate measurements of the data were recorded as mean values and presented using Graphs, ANOVA and Pearson's correlation analysis from the Minitab 17 software. Significant difference was taken at 5% level of significance ($p < 0.05$).

3. RESULTS AND DISCUSSION

One of the goals of present agricultural study is the selection of Bacteria which are highly effective nutrient (nitrogen) fixers. After 10 days of incubation, concentrations of lead were detrimental to the nitrogen fixation of *Xanthobacter* and *Rhizobium* spp, every mgL^{-1} of lead also diminished the nitrogen yields as seen in the plots. These findings correlated with Oijagbe, Abubakar & Edogbanya (2018) report, stating that there was a negative effect or inhibition of microbial nitrogen after application of lead at different concentrations and combinations.

Mercury concentration on the other hand, was beneficial to nitrogen fixation activity of *Xanthobacter* from the control 0 mgL^{-1} up to 25 mgL^{-1} (0.382 – 0.491 mgL^{-1}) before the nitrogen yields dwindled, this was better than the activity of lead on the same organism. Thus,

mercury may be utilized improvement of the organism's nitrogen yield but not above 25mgL⁻¹. *Rhizobium* maintained a negative relationship with the mercury concentrations from control to 100mgL⁻¹ (i.e. 0.925 - 0.297 mgL⁻¹). There was an immediate decline with addition of mercury, probable inhibition of nitrogenase enzyme is associated with Mercury, which leads to reduction in amount of nitrogen available especially nitrate which is an important macronutrient for bacteria cell formation (Hindersah *et al.*, 2019). Interestingly, mercury bio-stimulation of *Xanthobacter* nitrogen fixation may be associated with the presence of mer genes in the DNA, which induces the resistance to mercury to this genus of bacteria [16]. Other heavy metals such as Copper (CuSO₄) and Zinc (ZnSO₄) have also exhibited bio-stimulation of nitrogen fixation in free-living diazotrophs e.g. *Azotobacter* spp but at low concentration of 12.5 mgL⁻¹ respectively. Whereas, higher concentrations 25 mgL⁻¹, 50 mgL⁻¹, 100 mgL⁻¹ and 200 mgL⁻¹ caused progressive reductions in amounts of nitrogen fixed [23].

For model complexity and correctness, we studied the main effects and the interaction of both independent variables on each other, we indicate that the third variable (mercury) influences the relationship between the first independent variable (lead) and dependent variable (nitrogen fixation). This is also because these metals exist together in the environment and not independently and may likely interact

with each other affecting or determining the final nitrogen fixation by these organisms. The interactive plots serve to show the effect of one variable (lead) on the value of mercury (the other) and is derived by selecting high and low values for lead (Pb) and entering them into the equation along with the range of values for Mercury (Hg). The values used in the plots were the lowest concentration, 6.25 mg/L (-1) and highest concentration, 25 mg/L (+1) able to support nitrogen fixation of these diazotrophs. From these plots, one can decipher whether changing the concentration of lead during nitrogen fixation of Biofertilizer organisms in the presence of mercury will affect the overall nitrogen fixation activity and vice versa. In other words, does the relationship between lead and nitrogen fixation change based on mercury concentrations. Although these plots interpret the interaction effects, the P-value (0.05) from the analysis of variance will be used to determine whether the effects are statistically significant.

The interaction plot of Lead and Mercury on *Xanthobacter* sp, shows the presence of main effects as the lines are not horizontal, this means that nitrogen fixation in *Xanthobacter* can be maximized through the utilization of the selected metals as micronutrients. The plot also suggests that if lead metals are used at a higher concentration than stated nitrogen fixation will decline. It also shows that mercury could be a lot more beneficial to *Xanthobacter* sp nitrogen fixing activity than lead. Contrarily, the highest

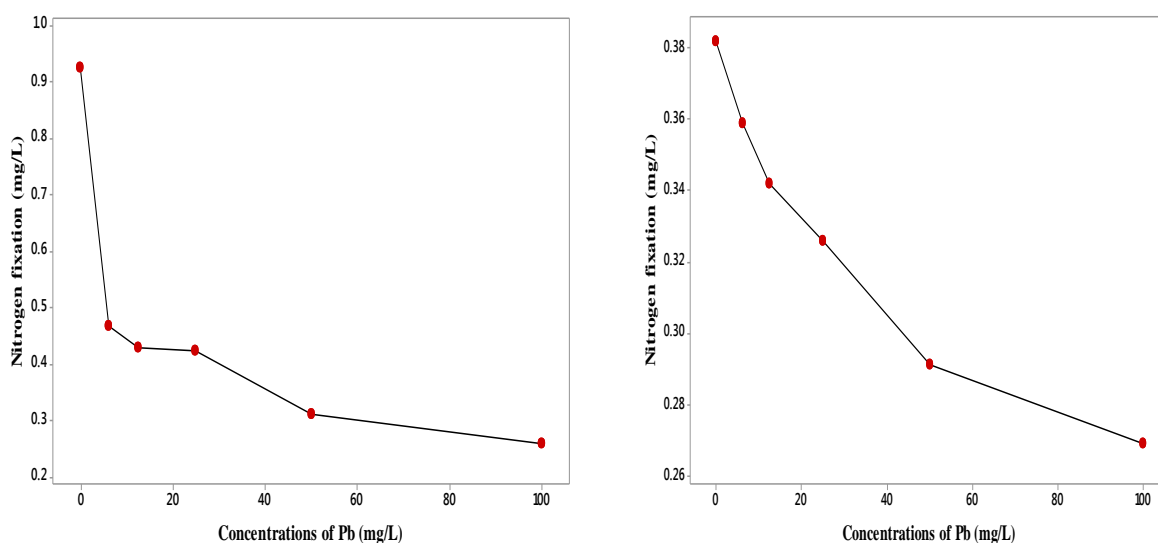


Fig. 1. *Rhizobium* and *Xanthobacter* Nitrogen fixation capacity in the presence of Lead respectively

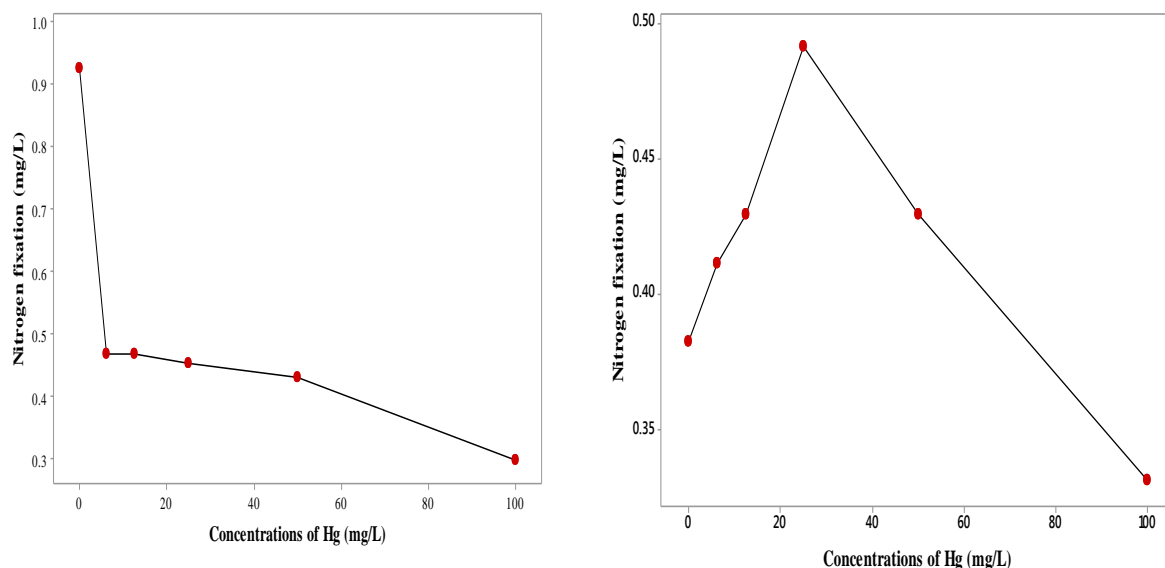


Fig. 2. *Rhizobium* and *Xanthobacter* Nitrogen fixation capacity in the presence of Mercury respectively

nitrogen mean is produced by the lead 6.25 mg/L concentration. On the interaction plots, the slopes indicate that an interference effect (crossed lines) exist between lead and mercury in the nitrogen fixation activity of *Xanthobacter* sp. This interaction suggests that the relationship between lead and nitrogen fixation depends on the level of mercury because the higher the value of mercury (25mg/L) the greater the nitrogen content provided while the concentration of lead (6.25) is kept at minimum. The statistics indicate (R-squared adjusted value) suggests that 70.87% of the variations in nitrogen fixation response is explained by the interaction of lead and mercury, hence the model likely fits the data. However, the P-value of the interaction term “Levels of Pb * Levels of Hg” shows that the effect is not significant at 0.102 which is greater than the alpha level. Notably, the highest nitrogen yield was 0.508 mg/L and was obtained during the interaction of mercury and lead rather than when these metals were introduced singly.

In the case of *Rhizobium* sp, there is a main effect and Lead and Mercury seems to affect the nitrogen fixation response differently because the lines are not horizontal. Mercury has a higher fixation magnitude than Lead but relatively, higher mercury concentration appears to be beneficial to nitrogen fixation unlike its lead counterpart which is detrimental. The parallel lines indicate that there is no interaction effect. Therefore, one can say that the relationship between lead and nitrogen fixation does not depend on the concentration of mercury and vice versa. Additionally, the R-adjusted value indicates that the model does not fit the sample data or predict new observations. The P-value which is greater than the 0.05 confidence level also agrees that the effect is not statistically significant. This means that the nitrogen fixation means for the level of lead does not depend on the value of mercury levels. The findings also indicate that in combination, the metals promote higher nitrogen yields for *Rhizobium* at 0.554mg/L.

Table 1. Readings of interactive effects of lead and mercury levels on *Xanthobacter* sp

RUNS 2 ²	LEVELS (mg/L)		pH	TEMP. °C	NH ⁴ -N	NO ³ -N	NH ⁴ -N + NO ³ -N
	Pb	Hg					
A	25	6.25	8.51	26.8	0.089	0.305	0.394
B	6.25	25	8.52	27	0.115	0.393	0.508
C	6.25	6.25	8.58	26.7	0.067	0.23	0.297
D	25	25	8.77	26.3	0.058	0.199	0.257

Where; -1= 6.25 mg/L, +1 = 25 mg/L

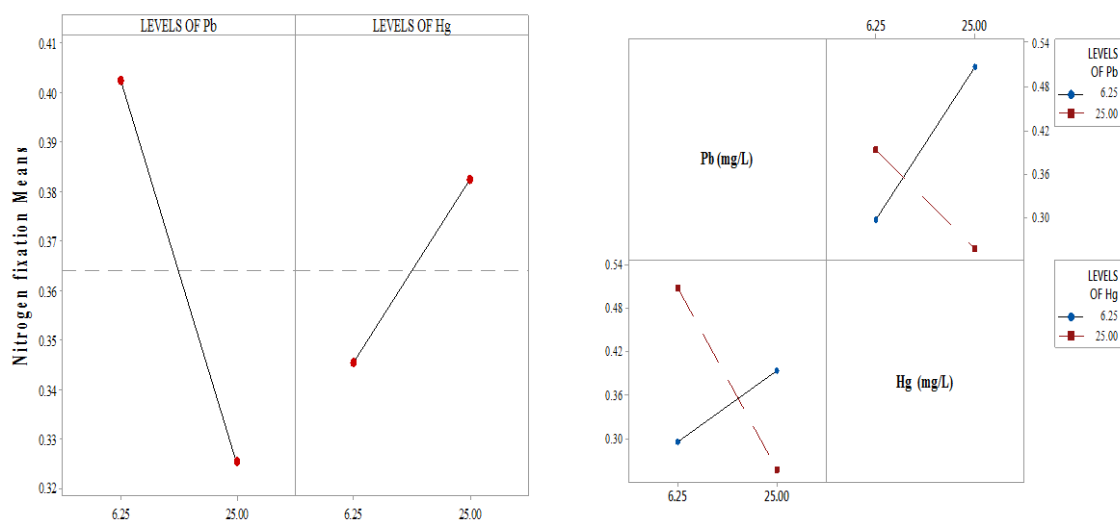


Fig. 3. Main and Interactive effects plots of lead and mercury levels on *Xanthobacter* sp



Plate 1. Groundnut rhizosphere and uprooted root nodules

Table 2. Readings of interactive effects of lead and mercury levels on *Rhizobium* sp

RUNS 2 ²	Levels (mg/L)		pH	TEMP. °C	NH ⁴ -N	NO ³ -N	NH ⁴ -N + NO ³ -N
	Pb	Hg					
A	25	6.25	8.49	26.4	0.083	0.283	0.366
B	6.25	25	8.57	26.8	0.125	0.429	0.554
C	6.25	6.25	8.49	26.7	0.102	0.349	0.451
D	25	25	8.48	26.5	0.107	0.367	0.474

Where; -1= 6.25 mg/L, +1 = 25 mg/L

In combination these elements at the stated concentrations may bio-stimulate nitrogen fixation since the already improve nitrogen fixation in this organism solely, however this

interaction was not statistically significant. Agreeably, the study by Ahmad et al. [25] reported that when metals were combined (K₂Cr₂O₇, CdCl₂, CuCl₂), the tolerance levels

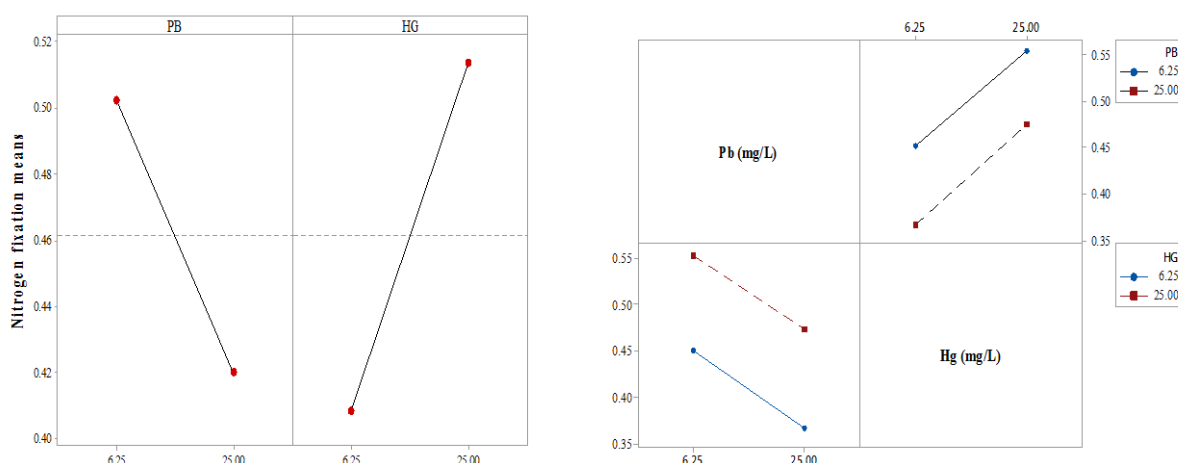


Fig. 4. Main and Interactive effects plots of lead and mercury levels on *Rhizobium sp*

considerably rose in the isolates probably as a result of synergistic effects of one metal on another metal ion. Cu^{2+} (copper) in combination with Cr^{6+} (Chromium) increased resistance level from less than $25\mu\text{g mL}^{-1}$ to less than $100\mu\text{g mL}^{-1}$ for one of the isolates of Rhizobia (*Rhizobium leguminosarium*) in comparison to single application of the metals.

4. CONCLUSION

Based on the findings of this study, it can be recommended that minute concentrations of these heavy metals be incorporated in biofertilizers as catalyst for nitrogen production but higher concentrations avoided since they trigger toxicity. Essentially, for being tolerant to these heavy metal concentrations, these diazotrophs are promising for the production of inoculants for cultivation and nutrient supply in areas of stress.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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