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EEE conceived and designed the study. JIO and CVA did literature review. All the authors were involved in the write-up, and statistical analysis; JIO and MNI revised the paper.

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***Correspondence**

Joel Inya Odo
Email: odojoel@gmail.com

Effect of Glyphosate on Nitrogen Fixing Rhizobium isolated from Root Nodules of Cowpea

Esther Eneyi Ebah[1], Joel Inya Odo*[2], Charles Vershima Atule[1], Muhammad Naeem Iqbal[3,4]

[1]Department of Microbiology, Joseph Sarwuan Tarka University, P M B 2373, Makurdi, Benue State, Nigeria.
[2]Department of Fisheries and Aquaculture, Joseph Sarwuan Tarka University, P M B 2373, Makurdi, Benue State, Nigeria.
[5]Pacific Science Media, United Kingdom.
[6]Association of Applied Biomedical Sciences, Narowal, Pakistan.

Abstract:
This study was carried out to investigate the effect of glyphosate on *Rhizobium* spp. isolated from soil in farm land cultivated with cowpeas at Joseph Sarwuan Tarka University, Makurdi. Glyphosate was obtained from Wurukum Markets, Makurdi Metropolis. Standard Microbiological and biochemical laboratory tests were carried out to identify Rhizobium sp. The experimental setup was done by crushing the root nodules and mixing it with the soil sample. The mixture was further divided into 100g in five places in duplicate and labeled as A, B, C, D and E. Samples A, B, C, and D were treated with 10 ml each of 100%, 50 %, 25%, and 12.5% of glyphosate concentration respectively while sample E was used as positive control without treatment. The study results showed that glyphosate impacted the colonies of *Rhizobium* at different concentrations. *Rhizobium* sp. colony counts decreased with increased concentration of glyphosate in descending order of 1.53 x 10^7 CFU/g, 2.12 x10^6 CFU/g, 2.33 x10^5 CFU/g, and 2.61 x10^3 at 12.5%, 25%, 50% and 10% concentration of Glyphosate respectively. The higher the concentration of glyphosate, the significant reduction in the total colony count of *Rhizobium* as compared to the control. The colonies were very sensitive to 100% concentration of glyphosate with drastic cell reduction. This study indicates that the effects of Glyphosate on bacterial populations are dose-dependent and highly temporal and this could lead to the rapid enrichment of opportunistic bacteria that use the compound as a nutrient or as a source of Carbon. Glyphosate should be applied in moderate and diluted quantities on farmland.

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INTRODUCTION

Rhizobium is a genus of Gram-negative soil bacteria that fix nitrogen (Kennedy *et al.*, 2015; Siyar *et al.*, 2019). The nitrogenase enzyme found in rhizobium fixes atmospheric nitrogen into ammonia (NH_3), a form of nitrogen that plants can use when they partner with legumes that are compatible with them. It could be introduced via seed inoculation therapy or exist naturally in the soil (Lindström and Mousavi, 2020). *Rhizobium* sp. can fix nitrogen, which can lower the expense of artificial nitrogen fertilizers. For this reason, it is crucial to inoculate legumes with *Rhizobium* to encourage nitrogen fixation (Fahde *et al.*, 2023; ul Islam, 2018).

Herbicides and other agricultural chemicals are among the many factors that impact the ecology of soil microbes (Meena *et al.*, 2020). Glyphosate, also known as N-(phosphonomethyl) glycine, is a broad-spectrum herbicide that is particularly effective against biennial, annual, and deep-rooted perennial grasses, sedges, and broadleaved weeds (Kanissery *et al.*, 2019). Glyphosate prevents the synthesis of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan, and as a result, it causes a number of metabolic disruptions. These include the inability to produce proteins, the biosynthesis of secondary products, and a general disruption of the phenyl propanoid pathway (Orcaray *et al.*, 2011; Zulet-González *et al.*, 2020). Assessing the effects of pollutants on soils seems to be a valuable method, based on the patterns and measurement of the enzymatic activities of the entire microbial population (Edwards, 2002). Crop yields will suffer if there is a disruption to symbiotic nitrogen fixation (Mus *et al.*, 2016). Herbicides are one type of possible constraint that may impact nitrogen fixation in symbiotic relationships. Herbicides, as opposed to other pesticides, were found to have the biggest effect on symbiotic fixation disruption in earlier investigations (Burul *et al.*, 2022).

The extensive application of herbicides, such glyphosate, poses a threat to the environment due to its possible negative impacts on beneficial soil microbes and biological processes in the soil. Ecosystem function greatly depends on activities in soil that are mediated by biology and biochemistry. A variety of soil processes, such as the conversion of organic matter, plant nitrogen fixation, nutrient release, and xenobiotic degradation, are primarily powered by soil bacteria (Gandhi *et al.*, 2021). The paucity of data concerning the effects of glyphosate on nitrogen fixing bacteria (*Rhizobium*) has gain research focus. This study will be to Isolate and identify *Rhizobium* spp from Glyphosate treated Root nodules of Cowpea plants and Soil, determine the total *Rhizobium* spp counts from Glyphosate treated Root Nodules of Cowpea plants and soil and to determine the effect of Glyphosate on *Rhizobium* spp at different time intervals.

MATERIALS AND METHODS

Sample collection

Root nodules of cowpea infected with Rhizobium were collected in farms at Joseph Sarwuan Tarka University, Makurdi. The roots nodules were collected by cutting 0.5 cm of the root on each side. Soil will also be collected from 20 to 30 cm depth within the root. Glyphosate were obtained from Wurukum Markets, Makurdi Metropolis. The samples were transferred into a separate polyethylene bag, labelled and were taken to Microbiology Laboratory, Joseph Sarwuan Tarka University for analysis.

Preparation of Different Concentrations of Glyphosate

Liquid glyphosate (MONSANTO) was taken in full strength concentration of 500pmm. *Further* dilutions of 250pmm, 125pmm and 62.5pmm were prepared by addition of appropriate amount of distilled water.

Experimental Set-Up

The root nodules crushed and mixed with the soil sample. The mixture was further divided into 100g in five places in duplicate and labelled as A, B, C, D and E. The mixture was further divided into 100g in Five places in duplicate and

labelled as A, B, C, D and E. Sample A was treated with 10mL of 100% of glyphosate, Sample B was treated with 10mL of 50 % concentration of glyphosate, Sample C was treated with 10mL of 25% of glyphosate concentration, Sample D was treated with 10mL of 12.5% while sample E was used as positive control without treatment.

Preparation of Serial Dilution

A stock solution was prepared by transferring One (1) gram of each of the sample treatment into 9 ml of distilled water and mixed thoroughly. Ten-fold serial dilutions of the sock solution were done using sterile water as blank. Nine milliliter (9 mL) of the blank was dispensed into 5 sterile test tubes which were labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. One milliliter (1 mL) from the stock were transferred into the first test tube labeled 10^{-1} and were mixed thoroughly to homogenize. 1 mL of the 10^{-1} fold homogenate was transferred to a test tube marked 10^{-2} dilution in order to further dilute the sample. The same procedure was used to prepare the serial dilutions for 10^{-3}, 10^{-4}, and 10^{-5}. Duplicate sterile petri dishes were filled with one millilitre each of the 10^{-2} and 10^{-4} dilutions.

Determination of Rhizobia Load

The pour plate method was used for the enumeration of rhizobia. Using a pipette, each of the diluted samples were taken from 10^{-2} and 10^{-4} diluted tube and were inoculated on the Yeast Extract Mannitol Agar (YEMA) medium (DIFCO) and incubated at 28 °C for 5 to 7 days (Rasool *et al.*, 2019; Somasegaran and Hoben, 2012). The total rhizobium presents in each sample treatments were expressed as colony forming units per millimeter (CFU/g) and were calculated as follows;

$$\text{Cfu/g} = \frac{\text{colonies counted}}{\text{Actual volume of sample plated}} \text{x dilution factor}$$

Isolation and Characterization of *Rhizobium* spp

Discrete colonies obtained were isolated and purified by streaking repeatedly on the Yeast Extract Mannitol Agar (YEMA) medium (DIFCO) and incubated at 28 °C for 5 to 7 days (Somasegaran and Hoben, 2012). The pure cultures were subsequently characterized and tentatively identified on the basis of their cultural morphology, microscopic and biochemical characters (Cheesbrough, 2018; Hussain *et al.*, 2016; Iqbal *et al.*, 2016; Mohammad *et al.*, 2021).

Statistical Analysis

Data were statistically analysed using SPSS (20). One-way analysis of variance (ANOVA) was used to statistically evaluate mean comparisons. P-values, or probability values, less than 0.05 were regarded as significant.

RESULTS

Table 1 shows the Physicochemical Analysis of soil samples from different sampling point in Joseph Sarwuan Tarka University, Makurdi. The pH ranges from 6.25 – 7.15, Percentage Moisture ranges from 35.40 – 37.00. Temperature ranges from 28.50 – 29.70 ^{o}C, Sand ranges from 88. 40 – 91.10, Silt ranges from 6.25 -9.65 while Clay ranges from 1.85 – 2.65.

Table 2 shows the Total Viable Count (TVC) and Total Coliform Count (TCC) of the samples isolated from the different sampling points at Joseph Sarwuan Tarka University, Makurdi laballed as JOSTUM A, B, C and D. JOSTUM C had the highest TVC of 261.00×10^{5} cfu/g compared to the other sampling points and JOSTUM A had the least (153.00×10^{5} cfu/g). Also JOSTUM C had the highest TCC of 114.50×10^{5} cfu/g whereas JOSTUM (59.50×10^{5} cfu/g) had the least.

Table 3 shows the Cultural, Morphological and Biochemical Characteristics of the Bacteria Isolate from Soil. *Rhizobium* was identified based on its distinct microbial characteristics. It has Creamy/Whitish colony color, Convex elevation, Rod shape, Gram negative, Catalase Positive, Citrate negative, Indole Positive, Urease Positive and Hydrogen Sulphide Negative.

Table 1: Physicochemical Analysis of Soil Sample from Different Sampling Point in Joseph Tarka University, Makurdi.

Sample Location	pH	Percentage Moisture	Temperature (^oC)	Sand	Silt	Clay
JOSTUM A	6.30 ± 0.00	36.35 ± 0.49	29.70 ± 0.00	91.10 ± 0.00	6.25 ± 0.07	2.65 ± 0.07
JOSTUM B	7.15 ± 0.07	37.00 ± 0.00	28.90 ± 0.00	88.40 ± 0.28	9.65 ± 0.07	1.95 ± 0.35
JOSTUM C	6.25 ± 0.07	35.40 ± 0.57	28.50 ± 0.00	91.05 ± 0.49	7.15 ± 0.07	1.80 ± 0.42
JOSTUM D	6.50 ± 0.00	36.25 ± 0.35	29.20 ± 0.14	91.30±0.00	6.85 ± 0.07	1.85 ± 0.07
P value	0.00	0.08	0.00	0.01	0.00	0.10

Table 2. Total Viable and Coliform Count of soil samples.

Sample Location	**TVC(×10^5 cfu/g)**	**TCC(×10^5 cfu/g)**
JOSTUM A	153.00 ± 2.83	76.50 ± 0.71
JOSTUM B	212.00 ± 5.66	97.50 ± 0.71
JOSTUM C	261.00 ± 7.07	114.50 ± 2.12
JOSTUM D	233.50 ± 2.12	59.50 ± 9.19
P value	0.00	0.01

df=1, P< 0.05

NOTE: Means on the same row with different superscript differ significantly

Table 3. Cultural, Morphological and Biochemical Characteristics of the Bacteria Isolate from Soil.

Colony colour	**Colony shape**	**Elevation**	**Morphology (Shape)**	**Grams reaction**	**Catalase**	**Citrate**	**Indole**	**Urease**	**H_2S Production**	**Motility**	**Bacteria species**
Creamy/ Whitish	Circular	Convex/ Raised	Rod	Negative	+	-	+	+	-	Motile	*Rhizobium*

Table 4 shows the activity of glyphosate on *Rhizobium* at different concentrations (100%, 50%, 25% and 12.5%). At 100% concentration, more inhibition was observed and this decreased with decrease in the concentration.

The Effects of Glyphosate on *Rhizobium* Total Viable Count at Different Concentrations are presented in Table 5. The total viable colony counts of *Rhizobium* decreased with increased in Glyphosate concentration as compared with the control The highest *Rhizobium* Colony counts (1.53 x 10^7 (CFU/g)was recorded at 12.5% concentration of Glyphosate followed by 25% (2.12 x10^6). 100% concentration of Glyphosate had the lowest viable count of the *Rhizobium* isolates.

Table 4. Activity of Glyphosate on Rhizobium Isolate at Different Concentrations.

SAMPLES	Concentrations(%) and Corresponding Zones of Inhibition(mm)			
	100%	50%	25%	12.5%
Replica A	36	32	21	16
Replica B	40	31	22	13
Replica C	37	29	25	14
Mean	37.67 ± 2.08	30.67 ± 1.53	22.67 ± 2.08	14.33 ± 1.53

Table 5. Effects of Glyphosate on Rhizobium Total Viable Count at Different Concentrations.

Samples	Concentrations (%) and Corresponding Bacteria Viable Counts (CFU/g)			
	100%	12.5%	25%	50%
Rhizobium Spp	$1.53 \times 10^{7} \pm 2.83$	$2.12 \times 10^{6} \pm 5.66$	$2.33 \times 10^{5} \pm 2.12$	$2.61 \times 10^{3} \pm 7.07$
Control	$7.65 \times 10^{6} \pm 3.83$	$6.150 \times 10^{6} \pm 2.12$	$7.95 \times 10^{6} \pm 9.19$	$7.75 \times 10^{6} \pm 0.71$

P= 0.00 DF=1 P< 0.05

DISCUSSION

Since glyphosate is not intended to suppress microorganisms, it is possible that it will have some unintended effects on their activity if the microbial population contains sensitive individuals. In this study the effect of glyphosate was investigated on *Rhizobium*. *Rhizobium* sp was isolated from soil in farm land. Glyphosate had impact on the colonies of *Rhizobium* at different concentration of 100%, 50%, 25% and 12.25%. The result of this finding is in line with a previous study that report the effects of Glyphosate at different concentration on soil microorganisms (King *et al.*, 2001).

In this study, *Rhizobium* spp. colony counts decreased with increased concentration of glyphosate. At lower concentration the colony counts of *Rhizobium* sp. was higher than the control. The fact that the bacterial population increased significantly at lower concentrations suggests that the glyphosate offered nutrients for bacterial development. This is in agreement with a previous study that reported an increase in heterotrophic bacteria of a soil with history of glyphosate application (Ashraf *et al.*, 2006).

The higher the concentration of glyphosate caused significant reduction in the total colony count of *Rhizobium* as compared to the control. The colonies were very sensitive with drastic cell reduction. This is in conformity with the previous study that reported that rhizobial isolates reduced with higher concentration of herbicide (King *et al.*, 2001). The toxic effect could be due to higher dose of glyphosate which can interrupt microbial cell wall and cell membrane functions which could further impair the cell's ability to take in nutrients and by extension its ability to reproduce. However, the mechanism of the toxic effects of glyphosate on microorganism is not well understood (King *et al.*, 2001). It has been proposed that its action against the microbes may be due to the inhibition of cell wall formation in the cell resulting in a leakage of cytoplasmic content, stop membrane synthesis, and inhibit spore germination and cellular respiration (Duke and Powles, 2009).

CONCLUSION

It has been concluded from the results that Glyphosate had effects on *Rhizobium* sp. isolated from soil. *Rhizobium* spp. colony counts decreased with increased concentration of glyphosate. The higher the concentration of glyphosate, the significant reduction in the total colony count of *Rhizobium* as compared to the

control. The colonies were very sensitive to 100% concentration of glyphosate with drastic cell reduction. The results of this study show that glyphosate has dose-dependent and highly temporal impacts on bacterial populations, which may hasten the enrichment of opportunistic bacteria that utilize the substance as a carbon source or food.

RECOMMENDATION

In line with the findings of this study the following recommendation are made:

Glyphosate should be applied in moderate and diluted quantity in farm land

Further studies should be carried out on the mechanisms of actions of glyphosate on soil microorganisms

CONFLICT OF INTEREST

Authors hereby declare that they have no conflict of interest.

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