Influence of glyphosate on rhizosphere microorganisms and their ability to solubilise phosphate

ADEBANKE ADEORITE AGBOOLA[1], FATUYI OLANIPEKUN EKUNDAYO[2], ESTHER AANUOLUWA EKUNDAYO[3], AYODEJI AKINWANDE FASORO[4], KEHINDE JOSEPH AYANTOLA[5], ADEOYE JOHN KAYODE[6]

[1]Department of Medical Microbiology and Parasitology, Afe Babalola University, Ado-Ekiti, Nigeria; [2]Department of Microbiology, Federal University of Technology, Akure, Nigeriae; [3]Department of Microbiology, Afe Babalola University, Ado-Ekiti, Nigeria;[4]Department of Public Health, Afe Babalola University, Ado-Ekiti, Nigeria; [5]Department of Science Laboratory Technology, Ekiti State University, Ado-Ekiti, Nigeria; [6]Department of Biochemistry and Microbiology, University of Fortare, South Africa

Received 04 September 2018
Accepted 11 November 2018

ABSTRACT

Objective: To determine cowpea rhizosphere microorganisms with phosphate solubilizing potential in a soil treated with glyphosate. **Methods:** Four different concentrations of glyphosate herbicides (0.00, 0.50, 1.00, and 3.00) were assayed. Cowpea seedlings were planted and later harvested after 30 days of germination. Microorganisms from rhizosphere treated with glyphosate were cultured and identified using selective media Wakesman A and B agar. The activity of isolates were evaluated by Tricalcium phosphate solubilization and screening for phosphatase in a Pikovskaya medium..

Results: *Bacillus cereus, Bacillus subtilis, Micrococcus luteus, Pseudomonas aeruginosa, Proteus mirabilis, Bacillus pumilis, Staphylococcus aureus, Aspergillus flavus, Rhizopus nigrifican, and Aspergillus saprophyiticus* were obtained from the glyphosate treated soil. There was decrease in the bacterial and fungal population in the rhizosphere as glyphosate concentration increased. The concentration of the solubilized Tricalcium phosphate ranged from 42.48 to 515.78gl^{-1}. *Micrococcus luteus, Bacillus subtilis, Proteus mirabilis, Aspergillus flavus, Aspergillus saprophyticus* isolated at the different concentrations of glyphosate have increased phosphate solubilization with reduction in their pH. *Proteus mirabilis* showed the highest phosphatase activity (23.157mM/min/ml) at 30hrs and *Aspergillus saprophyticus* showed highest phosphatase activity (35.263 mM/min/ml) at 72hrs. *Bacillus subtilis* had the lowest protein concentration (3.034mg/ml) at 12hrs.

Conclusion: This study showed that these rhizosphere microorganisms when applied as inoculants into the soil at different concentration of glyphosate can help increase the availability of soluble phosphates.

KEY WORDS: Rhizosphere, Cowpea, Glyphosate, Fungi, Bacteria

Introduction

Cowpea *(Vigna unguiculata*), the most diverse of the cultivated subspecies and the widest distributed, is an important food legume and versatile crop [1]. Cowpeas are grown mostly for their edible beans, although the leaves, fresh peas and fresh pea pods can also be consumed. What the nitrogen fixing ability means is that as well as functioning as a sole-crop, it can be effectively intercropped with millet, sorghum, cassava, maize or cotton [2].

Glyphosate [N-(phosphonomethyl)glycine] is the most widely used herbicide in the world. Glyphosate is a broad-spectrum herbicide used for the control of weeds in glyphosate resistant crops. However, there is concern that the widespread use of glyphosate may be having unintended, undesirable consequences. These concerns include glyphosate interactions with plant nutrition, the effects on soil microbial communities including an increase in plant pathogens, and the emergence of glyphosate resistant weeds [3]. Studies indicate that soil microorganisms can use glyphosate as a source of Carbon [4]. In addition, some bacteria can use glyphosate as a source of phosphorus [5].

It has been suggested that the degradation kinetics of glyphosate indicate that glyphosate does not support microbial growth. The effect of glyphosate on soil microorganisms has been widely studied, with conflicting results. In

Correspondence to: Dr. Ayodeji Akinwande Fasoro
Email: : akinfasoro@abuad.edu.ng

part this may be because soil microbial communities are diverse and live in diverse soil ecosystems. A study found that glyphosate has no significant effect on microbial community activity and composition [6]. There is evidence of increase in fungal activity and populations in soil treated with glyphosate. Araujo and colleagues [7] in their study reported that culturable bacterial population was not affected by glyphosate amendment while fungal and actinomycetes populations increased. This effect was larger in soils that had greater previous exposure to glyphosate. Some studies have shown that glyphosate use is associated with an increase in the plant pathogens (*Fusarium* and *Pythium)* [8, 9].

Rhizosphere microorganisms can increase or decrease the availability of phosphorus to plants [10]. Phosphate solubilizing microorganisms can bring about mobilization of insoluble phosphates in the soil and also increase plant growth under conditions of poor phosphorus availability [11]. The main objective of this study was to determine cowpea rhizosphere microorganisms with phosphate solubilizing potential in a soil treated with glyphosate.

Materials and Methods

Collection of Samples and Sterilization of Materials

The cowpea (*Vigna unguiculata*) used in this research was obtained from Ministry of Agriculture, Akure, Ondo State, Nigeria. Powdered Tricalcium phosphate was used. Wakesman A and B medium used in this project were sterilized by autoclaving at 121°C for 15 min. Soil sample used for this experiment was analyzed to determine its physicochemical according to the Association of Official Analytical Chemists [12] and Gregorich and Carter [13]. The pH of the soil sample was determined by using the method of Halder and colleagues [14].

Planting of cowpea

A piece of land behind the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure (FUTA), Nigeria was used in carrying out a field experiment on cowpea seedling. The herbicide was sprayed and allowed to act on the soil for 14days before planting the cowpea while the remaining portion of soil was unsprayed to serve as control. Seeds were surface sterilized for 2min in 70% alcohol, rinsed twice in distilled water and two seeds were planted at a depth of 3cm and at 10cm distance from each other. The plants were watered regularly to maintain a good soil moisture condition. Harvesting of cowpea seedling after 30 days of planting was done by using hand trowel. Soil around roots of each cowpea seedling was collected by gently shaking the already uprooted cowpea plant root into a polythene bags for further investigation in the laboratory [15].

Experimental design

The experimental design for planting of cowpea was a complete randomized design with 2x4 factorial arrangement of treatment with 3 replications. The factors considered were herbicides application (glyphosate) and four concentrations of the herbicide. The concentrations used for each herbicide were untreated (0.00ml, glyphosate), low (0.05ml, glyphosate), moderate (1.00ml glyphosate) and high (3.00ml glyphosate) concentrations.

Isolation of rhizosphere microorganisms

Isolation of bacteria and fungi was done according to the method of Jahnel and colleagues [16] and biochemical test of rhizosphere microorganisms was according to the methods of Olutiola and colleagues [17].

Phosphate solubilization experiment and phosphatase activity of the rhizosphere microorganisms

The phosphate solubilization ability and phosphatase activity of the rhizosphere microorganisms were done using Apha [18] and Makoi and colleagues' [19] methods respectively.

Statistical analysis

Data obtained were analyzed using Statistical Package for Social Scientist (SPSS) version 20.0. The mean differences among the treatments were determined using Analysis of Variance (ANOVA). In cases where there were significant differences among the treatments considered, post- hoc analysis using Duncan's Multiple Range Test was conducted. Descriptive statistics (means and standard deviation) were used to present the data.

Results

The result of the chemical analysis of the soil sample used was 63.40% sand, 9.00% silt, and 27.60% clay. This indicated that the soil was clay loamy. The pH of the soil was

6.20, which indicated the slight acidity of the soil. The percentage of potassium, organic matter, organic carbon and nitrogen were 0.02±0.03%, 0.58±0.04%, 0.35±0.04% and 0.23±0.33% respectively while the amount of calcium, magnesium, sodium were 3.2±0.31cmol/kg, 1.7±0.02cmol/kg and 0.02±0.03cmol/kg respectively.

Total Plate Count of Fungi and Bacteria in the Rhizosphere

The plate count of bacteria found in glyphosate treated soil ranged from (3.37×10^4 to 1.03×10^4) cfu while fungal count ranged from (2.0×10^4 to 0.3×10^4) sfu. There was gradual decrease in both bacterial and fungal population in soil treated with glyphosate just as the concentration applied increased. This means the higher the concentration of the herbicides on the soil, the higher their effect on the rhizosphere microorganisms. Reduction in the bacterial and fungal population may be due to higher application of the herbicides. Three fungi and seven bacteria were isolated from the herbicide treated rhizosphere of cowpea as shown in Tables 1 and 2.

Amount of solubilized Tricalcium phosphate in isolated bacteria from glyphosate treated rhizosphere of cowpea in Pikovskaya medium

All the rhizospheric isolates showed variable phosphate solubilizing potentials. Table 1 shows the amount of solubilized Tricalcium phosphate found in each bacterium isolated from the rhizosphere of cowpea plant treated with glyphosate at different day's intervals and their pH per day.

Table 1. Amount and pH of solubilized Tricalcium phosphate (gl^{-1}) in pikoskvaya medium by rhizosphere bacteria of Cowpea treated with glyphosate at different days intervals.

	Amount of solubilized Tricalcium phosphate(gl^{-1})					PH of the solubilized Tricalcium phosphate				
Organisms involved in solubilization	Day1	Day2	Day3	Day4	Day5	Day1	Day2	Day3	Day4	Day5
B.subtilis SGO	377.69 ± 0.63^{d}	440.13 ± 126^{e}	214.98 ± 0.31^{b}	233.70 ± 0.20^{c}	146.64 ± 0.10^{a}	6.39	4.63	4.65	5.04	4.84
M. luteus SGO	243.40 ± 0.20^{b}	415.53 ± 0.31^{e}	106.58 ± 0.06^{a}	301.08 ± 0.23^{d}	238.22 ± 0.08^{c}	5.79	4.44	3.91	3.86	4.38
B .cereus SGO	515.78 ± 0.06^{e}	263.15 ± 0.05^{b}	260.475 ± 0.05^{a}	289.47 ± 0.04^{c}	294.79 ± 0.06^{d}	6.14	5.06	4.76	4.86	5.19
P.mirabilis SGO	216.85 ± 0.05^{c}	236.82 ± 0.06^{e}	218.60 ± 0.20^{d}	191.05 ± 0.03^{b}	156.85 ± 0.05^{a}	6.26	5.07	4.74	4.76	4.44
B.pumilus SGO	297.25 ± 0.10^{d}	289.492 ± 0.61^{c}	184.53 ± 0.31^{a}	299.58 ± 0.35^{e}	211.85 ± 0.10^{b}	6.49	5.6	4.6	4.97	5.54
M. luteus SGI	214.75 ± 0.03^{c}	255.47 ± 0.25^{d}	206.58 ± 0.55^{b}	311.85 ± 0.04^{e}	113.16 ± 0.04^{a}	6.65	4.67	4.84	4.64	5.1
S.aureus SG1	296.10 ± 0.60^{e}	270.30 ± 0.30^{d}	221.58 ± 0.55^{c}	206.39 ± 0.05^{b}	184.26 ± 0.07^{a}	5.8	4.74	4.33	4.52	5.69
P.aeruginosa SG2	485.27 ± 0.31^{e}	335.58 ± 0.06^{d}	288.45 ± 0.31^{c}	144.76 ± 0.04^{a}	206.85 ± 0.05^{b}	5.82	5.1	4.02	4.03	3.8
B. subtilis SG2	357.70 ± 0.20^{c}	412.23 ± 0.13^{d}	224.47 ± 0.04^{a}	443.47 ± 0.04^{e}	232.23 ± 0.15^{b}	6.74	5.73	5.56	5.35	5.4
B. subtilis SG3	236.15 ± 0.03^{b}	258.48 ± 0.05^{c}	396.36 ± 0.03^{e}	297.37 ± 0.05^{d}	211.81 ± 0.03^{a}	5.54	4.37	3.73	3.99	4.1
P.mirabilis SG3	360.58 ± 0.05^{d}	421.38 ± 0.05^{e}	301.847 ± 0.05^{c}	271.58 ± 0.07^{b}	194.48 ± 0.06^{a}	6.77	4.79	4.55	4.62	4.4
B. cereus SG3	461.15 ± 0.02^{e}	443.46 ± 0.04^{d}	209.40 ± 0.20^{c}	185.57 ± 0.05^{b}	121.08 ± 0.03^{a}	5.34	4.54	3.79	3.74	4.1
Control (no organism)	42.40 ± 0.15^{a}	42.45 ± 0.03^{a}	42.48 ± 0.05^{a}	42.45 ± 0.03^{a}	42.45±o.06^{a}	6.83	6.83	6.83	6.83	6.83

SG0: Soil treated with 0.00mg/ml of glyphosate, SG2: Soil treated with 1.00mg/ml of glyphosate,
SG1: Soil treated with 0.50mg/ml of glyphosate, SG3 Soil treated with 3.00mg/ml of glyphosate,
*Means with the same superscripts on the same column are not significantly different ($p>0.05$)

The amount of the solubilized phosphate (AMSP) was lower in the control experiment where no bacterium was inoculated than where inoculated. Among the bacteria isolated from the untreated rhizosphere soil of cowpea untreated, *Bacillus cereus* had the highest AMSP (515.78 ±0.06gl^{-1}) which was on the first day while *Micrococcus luteu*s had the lowest AMSP (106.58±0.06gl^{-1}) on the third day. Among all the organisms isolated from the untreated soil, only *Bacillus subtilis*, *Microccocus luteus* and *Proteus mirabilis* showed general increased AMSP on the second day with reduction in their pH. This indicates that only these microorganisms can solubilize phosphate among the organisms isolated from the untreated soil with the level of the AMSP and pH.

At concentration 0.50mg/ml (low concentration) of glyphosate application to the rhizosphere of cowpea, *Staphylococcus aureus* had the highest AMSP ($296.10\pm0.06gl^{-1}$) on the first day and *Microccocus luteus* had the lowest AMSP ($113.16\pm0.04gl^{-1}$) on the fifth day. Among all the organisms isolated from the rhizosphere treated low concentration, only *Microccocus luteus* showed increased AMSP on the second day with reduction in the pH. This implies only *Microccocus luteus* can solubilize phosphate among the organisms isolated from low concentration with the level of the AMSP and pH.

At concentration 1.00mg/ml (moderate concentration) of glyphosate application to the rhizosphere of cowpea, *Pseudomonas aeruginosa* had the highest AMSP ($485.27\pm0.31gl^{-1}$) on the first day and lowest AMSP ($144.76\pm0.04gl^{-1}$) on the fourth day. Among all the organisms isolated from the rhizosphere treated with moderate concentration, only *Bacillus subtilis* showed increased AMSP on the second day with reduction in the pH. This implies *Bacillus subtilis* can solubilize phosphate at moderate concentration of glyphosate application.

At concentration 3.00mg/ml (high concentration) of glyphosate application to the rhizosphere of cowpea, *Bacillus cereus* had the highest AMSP ($461.15\pm0.02gl^{-1}$) on the first day and lowest AMSP ($121.08\pm0.03gl^{-1}$) on the fourth day. Among all the organisms isolated from the rhizosphere treated at high concentration, only *Bacillus subtilis* and *Proteus mirabilis* showed increased AMSP on the second day with reduction in the pH. This implies *Bacillus subtilis* and *Proteus mirabilis* can solubilize phosphate even at high concentration of glyphosate.

Amount of solubilized Tricalcium phosphate in isolated fungi from glyphosate treated rhizosphere of cowpea in Pikovskaya medium

All the rhizospheric isolates showed variable phosphate solubilizing potentials. Table 2 shows the amount of solubilized Tricalcium phosphate found in each fungus isolated from the rhizosphere of cowpea plant treated with glyphosate at different day's interval and their pH per day.

Table 2. Amount and pH of Tricalcium phosphate (ug^{-1}) in Rhizosphere fungi isolated from cowpea plant in Pikovskaya medium at 24hours intervals**.**

Fungal isolates	Concentration of Tricalcium phosphate (gl^{-1}) in Rhizosphere fungi					pH of Tricalcium phosphate (ug^{-1}) in Rhizosphere fungi				
	DAY1	DAY2	DAY3	DAY4	DAY5	DAY1	DAY2	DAY3	DAY4	DAY5
Aspergillus flavus	127.67 ± 0.03^{c}	108.88 ± 0.08^{b}	191.08 ± 0.04^{e}	158.48 ± 0.06^{d}	101.33 ± 0.03^{a}	6.65	4.05	2.49	2.54	2.74
Rhizopus nigrifican	105.76 ± 0.05^{b}	122.68 ± 0.06^{c}	150.25 ± 0.03^{d}	181.67 ± 0.40^{a}	334.53 ± 0.31^{e}	4.55	3.05	5.51	3.33	3.06
Aspergillus saprophyiticus	87.18 ± 0.10^{a}	127.68 ± 0.05^{c}	400.53 ± 0.31^{e}	118.46 ± 0.33^{b}	$177.64\pm o.04^{d}$	4.79	2.89	6.45	3.34	3.21
Control (no organism)	48.30 ± 0.34^{a}	48.40 ± 0.05^{a}	48.50 ± 0.03^{a}	48.20 ± 0.03^{a}	48.15 ± 0.03^{a}	6.83	6.83	6.83	6.83	6.83

*Means with the same superscripts on the same column are not significantly different ($p>0.05$)

The AMSP ($48.15\pm0.03gl^{-1}$) was lower in the control experiment where no fungus was inoculated than where inoculated. Among the fungi isolated from the untreated rhizosphere soil of cowpea (0.00 mg/ml), *Aspergillus saprophyticus* had the highest AMSP ($400.53\pm0.31Ug^{-1}$) on the third day and the lowest AMSP $87.18\pm0.10gl^{-1}$ on the first day. Among all the fungi isolated from the untreated soil, only *Aspergillus flavus* showed increased AMSP on the third day with reduction in its pH. This indicates that this fungus can solubilize phosphate among the organisms isolated from the untreated soil.

At concentration 0.50mg/ml of glyphosate application to the rhizosphere of cowpea, *Aspergillus saprophyticus* had the highest AMSP ($400.53\pm0.31gl^{-1}$) on the third day and the lowest AMSP ($87.18\pm0.10gl^{-1}$) on the first day. Among all the fungi isolated from the rhizosphere treated at low concentration, only *Aspergillus saprophyticus* showed increased AMSP on the third day with reduction in the pH. This implies *Aspergillus saprophyticus* can solubilize phosphate at low concentration.

At concentration 1.00mg/ml of glyphosate application to the rhizosphere of cowpea, *Aspergillus flavus* had the highest AMSP ($191.08\pm0.04gl^{-1}$) on the third day and lowest AMSP ($101.33\pm0.03gl^{-1}$) on the fifth day. Among all the organisms isolated from the rhizosphere treated with moderate concentration, *Aspergillus flavus* showed increased

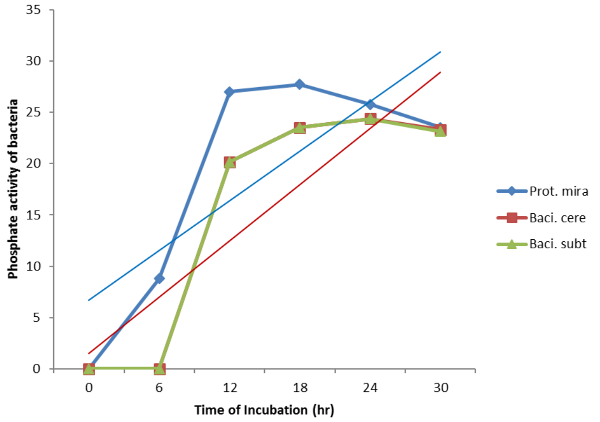
AMSP on the third day with reduction in the pH. This implies *Aspergillus flavus* can solubilize phosphate at moderate concentration of glyphosate.

At high concentration (3.00mg/ml) of glyphosate application to the rhizosphere of cowpea, *Aspergillus flavus* had the highest AMSP (191.08±0.04gl^{-1}) on the third day and lowest AMSP (101.33±0.03gl-1) on the fifth day. Among all the organisms isolated from the rhizosphere treated at high concentration, *Aspergillus flavus* showed increased AMSP on the third day with reduction in the pH. This implies *Aspergillus flavus* can solubilize phosphate at high concentration.

Phosphatase activity of rhizosphere microorganisms isolated from glyphosate treated soil of cowpea

Proteus mirabilis also isolated from glyphosate treated soil shows the highest phosphatase activity (23.157mM/min/ml) at 30hrs and shows lowest activity (8.771mM/min/ml) at 6hrs (Figure 1).

Figure 1. Phosphatase activity of rhizosphere bacteria isolated from cowpea (*Vigna unguiculata*) plant with glyphosate.



Highest protein concentration (9.93mg/ml) at 24hr was shown in *Bacillus cereus* and *Bacillus subtilis* has the lowest protein concentration (3.034mg/ml) at 12hrs according to Figure 2. Figure 3 shows that among the fungi isolated from glyphosate treated soil, *Aspergillus saprophyticus* had the highest phosphatase activity (35.263 mM/min/ml) at 72hrs and *Aspergillus flavus* had lowest activity (2.28Mm/min/ml) at 12hr. *Rhizopus nigrican* has highest protein concentration (33.931mg/ml) at 36hr and *Aspergillus flavus* has lowest protein 10.482mg/ml at 12hr (Figure 4).

Figure 2. Protein concentration of rhizosphere bacteria isolated from cowpea (*Vigna unguiculata)* plant treated with glyphosate.

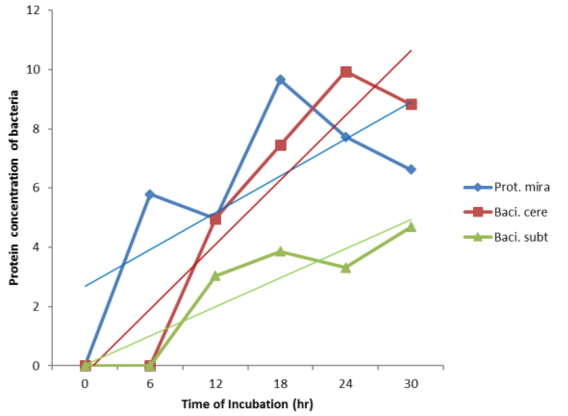


Figure 3. Phosphatase activity of the rhizosphere fungi isolated from cowpea plant (*Vigna unguiculata*) treated with glyphosate.

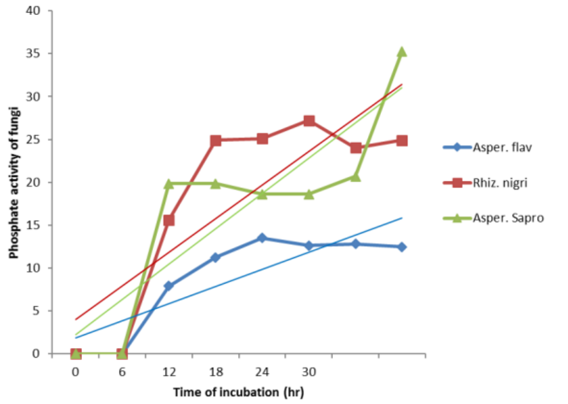
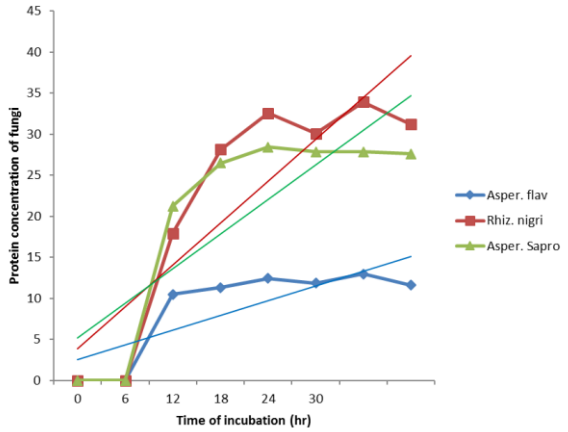


Figure 4. Protein concentration of the rhizosphere fungi isolated from cowpea plant (Vigna unguiculata) treated with glyphosate.



Discussion

The pH of the soil used for this research work fall slightly below the ideal pH range of a fertile soil of 6.3 to 6.8. The amount of phosphorus was 159mg/kg. The phosphorus level in this rhizosphere of cowpea plant areas is excessively higher than the values suggested by Landon [20] as high. This could be attributed to excessive use of phosphorus fertilizer during both rainy and dry season with the consequent manifestation of the residual effects of phosphorus. The percentage of potassium, sodium, organic carbon, organic matter and nitrogen are low to the percentage needed in an ideal soil. It could be said that sampled rhizosphere soil of cowpea plant is only very fertile, especially as most of the parameters for which more is better (organic matter, organic carbon, potassium and nitrogen), are only within the low-medium range when compared with the standard values given by Landon [20] It may be because of high rate at which organic matter is lost, high rate of leaching or low input agricultural practices.

The rhizosphere microorganisms when cultured selectively grew distinctively well on Wakesman agar A and B. There was gradual decrease in both bacterial and fungal population in both rhizosphere treated with herbicides (glyphosate) at different concentration and day's intervals. It has also been reported that glyphosate application results in substantial decrease in the abundance of soil microorganisms [6, 21, 22]. In general, herbicides affect microbes indirectly, causing physiological changes, increased enzymatic production or, when applied in high doses, death of susceptible groups of microorganisms [23].

This study has been able to show that rhizosphere microorganisms (*Bacillus subtilis Proteus mirabilis, Staphylococcus aureus, Micrococcus luteus, Aspergillus flavus, Apergillus saprophyiticus)* with phosphate solubilizing potentials when applied as inoculants into the soil at different concentration of glyphosate, can help increase the availability of soluble phosphates by solubilizing the inorganic phosphate thereby enhancing plant growth. Based on the physicochemical result of the rhizosphere soil of cowpea, the soil used in this study is not very fertile. It has low-medium minerals and organic matter content compared to the standards for soil nutrient requirements. To increase the soil quality, the plants nutrient needs should be supplemented with organic fertilizer. Besides, the addition of organic matter to the soil is necessary to lead to increase in the nitrogen content of the soil

The treatment of the rhizosphere soil with herbicides may negatively affect the beneficial microorganisms such as the nitrogen fixers, which can eventually hinder some activities like nitrogen cycling and decomposition. An effective method of herbicide application should always be employed to prevent any threat to rhizosphere microorganisms.

Conflict of Interest

We declare that we have no conflict of interest.

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