Assessment of the Effect of Paraquat and Glyphosate Herbicides on Soil Microorganisms

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Abstract: Herbicides are chemicals used to control weeds' growth. The improper application of herbicides poses a challenge to agricultural sector as non-target organisms are directly affected and residues are left in soil. This study was aimed at assessing the effect of paraquat and glyphosate on soil microorganisms. Fifteen kilograms of composite soil from an Organic Vegetable Garden was prepared into 15 microcosms; containing 1 kg soil each. Five different treatments were prepared in triplicates. The treatments include recommended field rate (RFR) (Paraquat-3.62g/kg; Glyphosate-5.63g/kg), twice the recommended field rate (TRFR) and a control without herbicide. Soils were serially diluted and cultured on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) for the isolation and enumeration of both bacteria and fungi groups at 1, 5, 10, 15, 20, 25 days. Isolates were characterised and identified. Herbicides residues were determined using Gas Chromatography (GC) after 50 days of exposure. Data collected were subjected to analysis of variance at P = 0.05. The results obtained indicate that herbicides significantly inhibited bacteria population at 10 and 15 days with the highest inhibitions of 44.18% and 51.12% by paraquat and 46.00% and 56.92% by glyphosate at the RFR and TRFR respectively. Fungi populations were significantly inhibited within 5 to 20 days with the highest inhibitions of 22.50% and 80.43% by paraquat and 53.93% and 63.57% by glyphosate at the RFR and TRFR respectively. Eighty-four bacteria isolates were isolated and identified to the genera of *Bacillus* (35), *Klebsiella* (30), *Enterobacter* (6), *Micrococcus* (3), *Pseudomonas* (7), *Proteus* (1), *Serratia* (1) *and Acinetobacter* (1). Thirty-two fungal isolates obtained were identified as: *Aspergillus flavus* (7), *Aspergillus fumigatus* (2), *Aspergillus aculeatus* (2), *Aspergillus tamarii* (4), *Aspergillus candidus* (3) and *Fusarium* spp. (14). From the GC-Pulsed Flame Photometric Detector analyses, no significant difference was observed between the herbicide-treatments and the residues but there was significant difference between herbicide-treatments and controls. This study showed exposure of soil to herbicides reduces its microbial population and the isolates obtained, have potentials for use in herbicides biodegradation in the environment.
Keywords: *Herbicides, paraquat, glyphosate, microcosm*

INTRODUCTION

Microbial communities play vital roles in soil fertility, ecosystem, and other activities in the terrestrial ecosystem (Rajendhran and Gunasekaran, 2008; Schulz *et al*., 2013). Such organisms contribute immensely in the decomposition of nutrients and their cycling processes (Chauhan *et al*., 2006; Pandey *et al*., 2007).

Herbicides are a group of pesticides which include substances that inhibit the growth of weeds. As farmers realised the effectiveness of such herbicides, they kept increasing its application to meet their production target without paying much attention to the negative effects it exhibits in the soil ecosystem (Haney *et al*., 2000; Ayansina and Oso, 2006; Riaz *et al*., 2007). Soil microorganisms are affected by the application of herbicides which are targeted at promoting optimum crops yield (Pampulha *et al*., 2007; Zabaloy *et al*., 2008). If microorganisms are sensitive to a particular herbicide, its application will interfere with vital metabolic activities of the microorganisms and, therefore affect the availability of nutrients in the soil (Nautiyal, 2006; Oliveira and Pampulha, 2006).

Some microorganisms have the ability to degrade herbicides, while some others are adversely affected, depending upon the application rate and type of herbicides used

(Ayansina and Oso, 2006; Sebiomo *et al.,* Thus, the effect of herbicides on soil microorganisms may be either stimulating or depressive depending on the agrochemicals formulation, mode of application, group of microorganisms and environmental conditions (Subhani *et al*., 2000; Zain *et al*., 2013). Studies on pesticide residual effects are often done in soil microcosm small-scale experiment which can be interpreted accurately at larger scales (Benton *et al*., 2007). Therefore, this study was carried out to assess the effect of herbicides on soil microorganisms.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from the organic vegetable garden, University of Ibadan Research Farm, Oyo State. The top soil of 0 – 15 cm depths were sampled from the scattered points using cleaned trowel and mixed together to form a composite mixture. The dried samples were sieved in 2 mm metal sieve to remove stones and plant debris and transported for processing at the Soil Laboratory, Department of Agronomy, University of Ibadan. The composite mixture was used to prepare the microcosms.

Physicochemical Analysis of Soil Sample

Physicochemical parameters involving pH, moisture content, organic carbon, total nitrogen, available phosphorus, exchange acidity, exchangeable bases, and particle size distribution were determined using standard analytical procedures described by AOAC (1990).

Preparation of Soil Microcosm

Fifteen kilograms (15 kg) of the bulked soils with determined moisture content were mixed together and 122 mL of sterile distilled water was added to achieve the moisture level of 24.19% which was 50% of its maximum water holding capacity. The soil was placed in 15 sterile glass bottles each containing 1 kg of soil. Each bottle was loosely fit with a cap to allow for gas exchange and incubated at room temperature, for 10 days to allow time for adaptation of microorganisms before treatment with herbicides.

Herbicide selection and Soil treatments

Two commonly used herbicides (Paraeforce® (a sulfonylurea) and Force up® (an organophosphate)) were purchased from a local agrochemical shop in Ibadan which contained paraquat dichloride (276 g/L) and glyphosate (360 g/L) active ingredients. Two different concentrations each of paraquat (3.62 and 7.32 mg/g of the soil) and glyphosate (5.63 and 11.25 mg/g) were used as treatments. The treatments represent 1.0 and 2.0 times the recommended field rates of application of paraquat dichloride (200 g/ha) and glyphosate (480 g/ha). Fifty millilitres of glyphosate treatments and 50 mL of paraquat treatment were sprayed to twelve out of 15 labelled soil treatments using hand sprayer. The treatments represent the recommended glyphosate (3 out of 12), twice recommended glyphosate (another 3 treatments), recommended paraquat (3 out of the 12) and twice recommended paraquat (the last 3). Other 3 treatments from the total of 15 served as control where they received no herbicide treatments except being sprayed with 50 mL of distilled water only (Zain *et al*., 2013). The microcosms were incubated in the dark at constant moisture content over an interval of 5 days for 25 days exposure period. The rates of application of herbicides were calculated using the following formula as described by Pasaribu *et al*. (2013):

$$\text{Xmg/g of soil} = \frac{\text{desired field rate (g.a.i / ha)} \times 1000\ \text{mg}}{\text{amount of a.i. in formulation (g.a.i./L)} \times \text{spray volume (L/ha)} \times 1\ \text{g}}$$

Bacterial Isolation

Bacteria were isolated from the soil treatments using serial dilutions and plating techniques in nutrient agar at 5 days intervals. Pure isolates were preserved in glycerol stock for further identification (Olutiola *et al*., 2000).

Isolation of Fungi

Fungi were also isolated by plating technique using the potato dextrose agar at the incubation period of 5 days and subsequently identified based on their morphological and microscopic features as described by (Cowan and Steel, 1974; Barnett and Hunter, 2000).

Determination of Residual Herbicides

The herbicides residues in soils were analysed after 50 days using Gas Chromatography where the control soils were freshly-spiked with herbicides to provide baseline for comparison. Residual herbicides were analysed by the modified method of Perez *et al*. (1998). Ten grams of sample for extraction was transferred to the extracting bottle that was corked with TFE-fluorocarbon. Fifty millilitres of phosphate buffer was added followed by the pH measurement with the addition of sulphuric acid (1:1) for adjustment to less than 2. One gram of sodium chloride salt was added to the sample, sealed and shook so as to dissolve the salt. Fifty millilitres of analytical grade of ethyl ether was measured and poured into the sample. The sample was extracted with a sonicator for 30 minutes and filtered into Erlenmeyer flask. Extraction was repeated twice with fresh solvent and the filtrate was combined. The combined extract was free of water by pouring through a dry column containing a 10-cm anhydrous sodium sulphate (previously rinsed with methylene chloride). The filtrate was concentrated in the concentrator flask with a stream of nitrogen. The wall of the concentrator flask was rinsed with methyl tert-butyl ether so as to bring the final volume of the extract to 5 mL.

The cleanup of the concentrated extract was followed by packing the column with fluorisil. The eluent was eluted with methyl tert-buthyl ether and later concentrated to the final volume. Before each run, the instrument was standardised with external standards.

Statistical Analysis

The experiment was carried out using completely randomised design (CRD) with three replicates. Data from soil microcosm expressed as percentage inhibition of microbial population relative to their controls were analysed following a one-way Analysis of Variance (ANOVA) between herbicide treatments and each exposure time. Herbicides residues in soil after treatment days were also compared with their controls. Separations of obtained means were carried out by Duncan's Multiple Range Test (DMRT) using Statistical Analytical System (SAS). Results were expressed as significant difference at probability level ($p < 0.05$).

RESULTS

Physicochemical Parameters of Soil Sample

The soil physicochemical analysis shows the soil is loamy-sand. Soil physicochemical parameters are shown in Table 1.

Table 1. Physicochemical parameters of soil

Properties			Soil Samples/ Soil Parameters
pH			6.7
Organic Carbon (g/kg)			11.71
Moisture Content (%)			9.01
Organic Carbon (g/kg)			11.71
Total Nitrogen (g/kg)			1.68
Available Phosphorus (mg/kg)			209.60
Exchangeable Bases	Calcium		9.86
(cmol/kg)		Magnesium	2.81
		Potassium	1.37
		Sodium	1.22
Exchange Acidity		(cmol/kg)	0.8
Particle Size (g/kg)	Sand		846
	Silt		114
	Clay		40

Mg = milligram g= grams kg=kilograms cmol=centimole

Inhibition Percentages of Herbicides on Soil Bacterial Population

The inhibition percentages of herbicides showed glyphosate had its highest inhibitory effect on the microbial population at day 10 with 56.92% and 46% inhibitions on bacterial population relative to their controls at twice the recommended field rate (TRFR) and the recommended field rate (RFR) respectively. Similarly, paraquat herbicide had the highest inhibitory effect on microbial population at day 5 with 56.52% inhibition of bacterial population relative to their controls at TRFR. The effect of herbicide on bacterial population in herbicide-treated soil, expressed as percentage inhibition of their population relative to their controls is shown in Tables 2.

Table 2. Percentage Inhibition of Bacterial Population Relative to their Controls in Soil Treatments at Different Exposure Days

Days	Percentage Bacterial Inhibition Relative to Control Mean ± Standard Error					
	PAR X 1.0	PAR X 2.0	GLY X 1.0	GLY X 2.0	CONTROL	P VALUE
Day1	11.48 ± 1.56^{a}	0.42 ± 0.42^{a}	6.11 ± 1.15^{a}	11.60 ± 11.60^{a}	0.00 ± 0.00^{a}	0.4266
Day5	33.31 ± 19.02^{a}	56.52 ± 15.57^{a}	33.99 ± 20.66^{a}	40.75 ± 15.05^{a}	0.00 ± 0.00^{a}	0.2866
Day10	44.18 ± 4.82^{a}	51.12 ± 3.25^{a}	46.00 ± 5.06^{a}	56.92 ± 13.30^{a}	0.00 ± 0.00^{b}	0.0110
Day15	30.79 ± 4.70^{cb}	42.68 ± 5.72^{ab}	25.42 ± 2.84^{c}	47.58 ± 2.42^{a}	0.00 ± 0.00^{d}	0.0016
Day20	5.67 ± 2.30^{ab}	11.93 ± 0.57^{ab}	8.81 ± 2.56^{ab}	21.59 ± 9.09^{a}	0.00 ± 0.00^{b}	0.1061
Day25	12.52 ± 3.00^{ab}	8.05 ± 4.88^{ab}	3.45 ± 3.45^{ab}	4.03 ± 2.31^{a}	0.00 ± 0.00^{b}	0.1920

values in the same row followed by superscript with similar letter(s) are not significantly different by DMRT ($P > 0.05$). Data are presented as mean values (standard error) of three replicates at each exposure period.

PAR X 1.0 = Paraquat at the recommended field application rate; PAR X 2.0 = Paraquat at twice the recommended field application rate

GLY X1.0 =Glyphosate at the recommended field application rate; GLY X 2.0 = Glyphosate at twice the recommended field application rate

P Value = Probability Value

Inhibition Percentages of Herbicides on Soil Fungal Population

The inhibition percentages of herbicides showed glyphosate had its highest inhibitory effect on the microbial population at day 10 with 63.57% and 53.93% inhibitions on fungal population relative to their controls at twice the recommended field rate (TRFR) and the recommended field rate (RFR) respectively. Paraquat herbicide had the highest inhibitory effect on microbial population at day 5 with 80.43% and 23.42% inhibitions on fungal population relative to their controls at TRFR and RFR respectively. The effect of herbicide on fungal population in herbicide-treated soil, expressed as percentage inhibition of their population relative to their controls is shown in Tables 3.

Table 3. Percentage Inhibition of Fungal Population Relative to their Controls in Soil Treatments at Different Exposure Days

Days	Percentage Fungal Inhibition Relative to Control Mean ± Standard Error					
	PAR X 1.0	PAR X 2.0	GLY X 1.0	GLY X 2.0	CONTROL	P VALUE
Day1	8.56±1.89^{a}	6.72± 6.72^{a}	6.32 ±0.35^{a}	1.50±1.50^{a}	0.00±0.00^{a}	0.3766
Day5	23.42+2.91^{c}	80.43±6.75^{a}	33.74±2.16^{bc}	41.50±4.66^{a}	0.00±0.00^{d}	0.0003
Day10	22.50±2.50^{c}	80.00±0.00^{a}	53.93±8.93^{b}	63.57± 6.43^{ab}	0.00±0.00^{d}	0.0005
Day15	6.51+2.59^{bc}	9.76±3.88^{b}	4.68 ±0.13^{bc}	44.30±1.16^{a}	0.00±0.00^{c}	0.0001
Day20	3.17±1.38^{b}	9.01±0.08^{a}	2.68±2.68^{b}	4.06±0.49^{ab}	0.00±0.00^{b}	0.0405
Day25	8.10±1.43^{a}	3.49 ±1.27^{a}	7.15±7.15^{a}	6.98 ±2.54^{a}	0.00±0.00^{a}	0.5163

values in the same row followed by superscript with similar letter(s) are not significantly different by DMRT (*P > 0.05*). Data are presented as mean values (standard error) of three replicates at each exposure period.

PAR X 1.0 = Paraquat at the recommended field application rate; PAR X 2.0 = Paraquat at twice the recommended field application rate

GLY X1.0 =Glyphosate at the recommended field application rate; GLY X 2.0 = Glyphosate at twice the recommended field application rate

P Value = Probability Value

Bacterial Isolation and Biochemical Characterisation

A total of 84 bacterial isolates were obtained from soils to which glyphosate and paraquat herbicides were applied at the recommended field rate. Forty-two bacteria isolates were randomly obtained from paraquat-treated soils and glyphosate-treated soils respectively. The biochemical characterisation of the isolates showed that 41.67% of the bacteria isolates were Gram positive rods, 4.76% of the bacteria isolates were Gram positive cocci and 53.56% were Gram negative rods. Bacteria obtained from each treatment are highlighted in Table 4.

Table 4: Bacterial Isolates Obtained from the Herbicide-Treated Soils

S/N	PAR X 1.0	PAR X 2.0	GLY X 1.0	GLY X 2.0
1.	*Klebsiella oxytoca*	*Bacillus spp.*	*Bacillus cereus*	*Klebsiella pneumonia*
2	*Klebsiella pneumonia*	*Enterobacter aerogenes*	*Klebsiella pneumonia*	*Bacillus* spp.
3.	*Bacillus coagulans*	*Micrococcus* spp.	*Bacillus* spp.	*Klebsiella oxytoca*
4.	*Bacillus* spp.	*Klebsiella pneumonia*	*Klebsiella oxytoca*	*Enterobacter cloacae*
5.	*Enterobacter aerogenes*	*Klebsiella oxytoca*	*Proteus vulgaris*	*Bacillus subtilis*
6.	*Bacillus subtilis*	*Pseudomonas* spp.	*Bacillus marcerans*	*Bacillus polymyxa*
7.	*Pseudomonas* spp.		*Enterobacter aerogenes*	*Pseudomonas* spp.
8.	*Sarratia liquefaciens*		*Acinetobacter* sp.	
9.	*Bacillus insolitus*		*Bacillus subtilis*	
10.			*Enterobacter cloacae*	
11.			*Pseudomonas* spp.	

GLY X1.0 =Glyphosate at the recommended field application rate; GLY X 2.0 = Glyphosate at twice the recommended field application rate; PAR X 1.0 = Paraquat at the recommended field application rate; PAR X 2.0 = Paraquat at twice the recommended field application rate

Fungal Isolation and Characterisation

Thirty-two fungal isolates were obtained from soils to which glyphosate and paraquat herbicides were applied at the recommended and twice the recommended field rate. Sixteen fungal isolates were randomly obtained from paraquat-treated soils and glyphosate-treated soils respectively. Fungi obtained from the treatments are shown in Table 5.

Table 5. Fungal Isolates Obtained from the Herbicide-Treated Soils

S/N	PAR X 1.0	PAR X 2.0	GLY X 1.0	GLY X 2.0
1.	*Aspergillus aculeatus*	*Aspergillus flavus*	*Aspergillus flavus*	*Aspergillus tamarii*
2	*Fusarium oxysporun*	*Aspergillus candidus*	*Aspergillus fumigatus*	*Aspergillus aculeatus*
3.	*Fusarium* spp.	*Fusarium oxysporum*	*Aspergillus candidus*	*Aspergillus fumigatus*
4.	*Aspergillus candidus*	*Fusarium* spp.	*Aspergillus tamarii*	*Fusarium* spp.
5.	*Aspergillus flavus*		*Fusarium* spp.	

PAR X 1.0 = Paraquat at the recommended field application rate; PAR X 2.0 = Paraquat at twice the recommended field application rate

GLY X1.0 =Glyphosate at the recommended field application rate; GLY X 2.0 = Glyphosate at twice the recommended field application rate

Herbicides Residues Determination

Soils had less than 1% residual herbicides after 50days. Soils treated with twice the recommended field rate of herbicides retained more herbicides than soil samples treated with the recommended field rate. Table 6 shows the summary of the residual herbicides.

Table 6: Herbicides Residues in Soil after 50 Days

Herbicides Treatment	rfr	Retention time (min)	Amount/area (ppm/area)
Paraquat	X1.0	17.72	0.000593578 [b]
Paraquat	X2.0	17.71	0.000643306 [b]
Paraquat	Control	18.00	14.2870[a]
Glyphosate	X1.0	11.37	0.00474360[b]
Gyphosate	X2.0	11.37	0.00553787 [b]
Glyphosate	Control	11.37	68.12059[a]

rfr = Recommended Field Rate; X1.0 = Treatment at the recommended field rate; X2.0 = Treatment at twice the recommended field rate

Values followed by the same superscript are not significantly different (P > 0.05) by DMRT.

DISCUSSION

The effect of herbicide treatments on soil microbial population determined based on the inhibition percentages of the growth of bacterial and fungal population in each treatment relative to their controls increased with increased herbicide concentrations. Soils treated with two times the recommended field rate of application resulted in lower microbial counts when compared to soils treated with the recommended field rate which is in conformity with the works of (Ayansina and Oso, 2006; Sebiomo *et al*., 2011). Glyphosate and paraquat had their highest inhibitory effect on bacterial population and fungal population respectively.

These findings are in contrast with the work of Zain *et al*. (2013) who assessed the effects of selected herbicides on soil microbial populations from an oil palm plantation in Malaysia and observed paraquat and glyphosate had their highest inhibitions on bacterial population and fungal population respectively.

Soil microbial population showed varying sensitivity to herbicides at exposure days. Bacteria population showed significant inhibition ($P<0.05$) with herbicide treatments relative to their control at day 10 and day 15 after treatment with herbicides. Beyond 15days, no significant inhibition ($P>0.05$) was observed. This may be as a result of microorganisms acquiring adaptive mechanisms, and their ability to use the herbicides as sources of Carbon, Nitrogen and Phosphorus as opined by (Qui *et al*., 2009; Bera and Ghosh, 2013). Fungal population was significantly inhibited ($P< 0.05$) at day 5 to day 20. Adhikary *et al.* (2014) assessed the effect of herbicides on transplanted chilli and observed no significant inhibition of microbial population beyond day 15. These contrast maybe due to varying active ingredients of the herbicides.

This study revealed that diversity in the species of soil microorganisms decreased with an increase in the concentration of herbicide. *Proteus* sp., *Acinetobacter* sp., *Serrratia* spp. were isolated from soils with herbicides treatment at the recommended field rate but not isolated from soils with herbicide treatments at twice the recommended field rate. This suggests that higher concentrations of herbicides results in disappearance of some species of microorganisms as suggested by Singh and Walker (2006). *Bacillus* spp. had the highest percentage occurrence of 41.6% (n=35) which is line with the work of Oyeleke *et al.* (2011) who reported *Bacillus* spp. to be the most frequently isolated bacteria from herbicide-treated soil. It was also observed that *Bacillus* spp. was the most frequently isolated bacteria from glyphosate-treated soils and increased with increased concentration which showed a similar trend with the work of Ubuoh *et al.* (2012) and Ella *et al.* (2013). *Klebsiella* spp. occurred most frequently in paraquat-treated soil and this finding was in line with the work of Martin *et al*. (2007) who isolated and observed *Klebsiella* to be the best degrader of s-metachlor herbicide. The predorminant occurrence of *Klebsiella* species is in contrast with the work of Obuotor *et al.* (2016) who observed *Pseudomonas* species to have the highest occurrence during the biodegradation of paraquat-treated soil, although they were isolated also as degraders of paraquat.

Aspergillus spp. had the highest occurrence in glyphosate-treated soil and increased with an increased concentration of herbicide. This is in agreement with the findings of Ubuoh *et al.* (2012) who observed *Aspergillus* spp. to demonstrate a high tolerance to glyphosate and dominated even at higher treatments in soil. *Fusarium* spp. showed dominance in paraquat-treated soil which is in line with the findings of Lipsa *et al.* (2010) who assessed the effect of s-metachlor on soil microorganisms and observed that an increase in herbicide treatment resulted in an increase in the number of isolated fungus genus *Fusarium*.

Herbicides residues analysis using Gas Chromatography showed that after 50 day exposure of soil to herbicides at room temperature and the activities of indigenous microorganisms, there was a significant difference between herbicides treatments and their controls (which contained soils freshly-spiked with herbicides prior to analysis). The herbicides residues in soil treatments were observed to have reduced drastically compared with the initial concentrations of treatments added. These findings contradict the work of Mohamed *et al.* (2011) who analysed the residues of oxyfluorfen herbicide in soil and observed it to have been better degraded at higher temperature. Although, it is logical to expect a higher residue in soil treated with twice the recommended field rate, statistical analysis of residues of herbicides in soils treated with the recommended field rate and twice the

recommended field rate showed that concentration of herbicides applied to the soil did not significantly affect the residues of these herbicides in the soil after 50 days as shown by this study.

CONCLUSION

This study showed that exposure of soil to herbicides can reduce soil microbial population which in turn will affect the proper functioning of soil ecosystem. Using manufacturers' recommended field rate of herbicides could reduce the detrimental effect of herbicides on soil microbial population and microbial diversity in the soil. These microorganisms that persisted in soils treated with herbicides over a period of time have the potential to reduce the herbicide contamination in the soil as shown by a decrease in soil residual herbicides over time and thus can be beneficial as biodegraders in herbicide-contaminated soils.

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