EFFECT OF HERBICIDE ON THE PLANT GROWTH PROMOTING PROTEOBACTERIA AND FIRMICUITES

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Abstract

Although the agricultural sector is the primary user of synthetic herbicides worldwide, however their effect on soil Proteobacteria and Firmicuites has resulted in loss of soil fertility and reduced plant growth. This study was carried out to evaluate the effect of six (6) synthetic herbicides (glyphosate, paraquat, atrazine, pendimenthalin, diuron and nicosulfuron) used in Nigeria agricultural environment at both the recommended concentration (1X RFD) and higher concentrations (2X and 3X RFDs) on the survival and growth of isolated Proteobacteria (*Kosakonia sacchari, Leclercia adecarboxylata, Enterobacter asburae, Pseudomonas putida*) and Firmicutes (*Bacillus nakamurai, Bacillus nitratireducens, Bacillus velezensis, and Bacillus subtilis*). The Proteobacteria and Firmicuites were isolated from maize (*Zea mays*) rhizosphere and identified following 16SrRNA sequencing. Agar diffusion and plate count method was used to determine the tested herbicides effect on isolates survival and growth. A progressive, herbicide-concentration-dependent effect was observed on the survival and growth of Proteobacteria and Firmicuites isolates. The effects differed significantly depending on the concentration and type of the herbicides applied. Herbicide type; paraquat, atrazine and glyphosate affected the survival of both Proteobacteria and Firmicuites at three times (150mg/100ml) the recommended concentration. Survival and growth of *L. adecarboxylata* and *B. subtilis* were significantly affected by paraquat and atrazine at all concentrations (50, 100, and 150mg/100ml). Herbicide types; diuron and nicosulfuron had no significant negative effect on both the Proteobacteria and Firmicutes at all concentrations tested. There were more growth observed at the lower concentrations (1X and 2X RFDs) of the herbicides tested, thus indicating a concentration-dependent effect. Therefore, the study concluded that the tested herbicides have fatal effect on the growth of soil Proteobacteria and Firmicuites at higher concentrations above the recommended rates.

Introduction

Widespread use and disposal of herbicides by farmers, institutions and the public in general provide many possible sources of agrochemicals and their metabolites into the atmosphere, soils and rivers thus threatening both human health and the ecosystem at large. Although herbicides constitute an important aspect of modern agriculture, their excessive and persistent use has resulted in damaged farmland causing serious environmental pollution (Cardozo et al., 2020). Previous studies estimated that herbicides had the potential to impact non-target organisms and become widely dispersed in the environment (Muturi et al., 2017; Sidhu et al., 2019; Xie et al., 2018). This has been attributed to lack of proper legislation, improper market regulations and ignorance shown by people, especially in developing countries like Nigeria where people are prone to experience high levels of herbicide metabolites in their environment (Mark et al., 2016). The overuse or misuse of herbicides is contributing adversely to the environmental health as well as to ecosystem sustainability (Deng et al., 2015; Hassan et al., 2017; Soo et al., 2020). Considering the ecological significance of plant growth promoting bacteria (PGPB) in maintaining crop and soil health through versatile mechanisms such as; nutrient cycling and uptake, suppression of plant pathogens, induction of resistance in plant host and direct stimulation of plant growth, PGPB responses to herbicides contamination provide relevant models for ecological study (Chaudhary et al., 2017; Soo et al., 2020). Previous studies showed that herbicides generally decreases the diversity and activity of soil bacterial leading to a reduction in the total microbial biomass, decrease in population and a shift in community structure (Deng et al., 2015; Li et al., 2023; Suleman et al., 2018). Study by Singh et al. (2015), on bacterial composition in pesticide-contaminated maize rhizosphere showed that gram-positive Firmicuites were more dominant

than gram-negative Proteobacteria and Bacteroidetes. Furthermore, Suleman et al. (2018) in their study attributed resistance (tolerance) against herbicide to physiological changes that induce microbial metabolism to form new metabolic pathway to bypass a biochemical reaction inhibited by a specific herbicide while permanent resistance, on the other hand, depends upon genetic modifications, inherited and transferred by subsequent generations of microbes. Their report provide valuable realizations into how herbicides can impact the communities of useful PGPB.

Soil ecosystems are typically exposed to varying concentrations of different kinds of herbicides, and understanding the response of PGPB to these agrochemicals can improve our ability to predict the full effect of chemical perturbations on microbial processes and community interactions (Kumari et al., 2018; Nyadar et al., 2019). Sustainable management of soils requires soil monitoring, which can provide many potentially interesting indicators for environmental monitoring in response to a range of stresses or disturbances (Cardozo et al., 2020; Vejan et al., 2016). The environmental protection in maintaining the ecological balance of the agro-ecosystem is sacrosanct, considering the effect in terms toxicity or relative safety profile of the amount or exposure of various herbicides. Studies involving effect of herbicides on plant growth promoting Proteobacteria and Firmicuites are complex, still poorly understood and are relatively limited. Thus, this study seeks to evaluate the concentration-based effects of herbicides commonly used in Nigeria agro-ecosystem on the growth of Proteobacteria and Firmicutes plant growth promoter.

Materials and Methods

Materials

All herbicides (paraquat, atrazine, glyphosate, pendimenthalin, diuron and nicosulfuron) used in the study were of the analytical grade (98% w/w) and purchased from Sigma Agrochemical, Amilengbe, Molecular biology grade chemicals were obtained from NEB, USA and Takara, Japan. Details of the herbicides used in the study is mentioned in Appendix 1. Modified mineral salt media (mMSM) (MgSO4.7H2O,0.25 g; FeSO4.7H2O,0.005 g; CuSO4,0.01 g; KH2PO4,7.5 g; K2HPO4, 1.25 g of; NH4NO3 1.0 g; Ca(NO3)2.2H2O, 0.04 g; pH 7.0, (adjusted by 0.1 N NaOH/HCl) was used in for bacterial growth and pesticide degradation study.

Collection of Soil Samples

Rhizospheric soil samples from maize (*Zea mays*) plants were collected at different locations from the research farm of the University of Ilorin (Lat 8°28[1]11.3N;Long 4°39[1]44.2E) according to the methods described by de Souza et al., (2015) with some modifications. Samples (20g) each were collected in Zip lock bag, labeled and transported immediately to the laboratory of the department of microbiology, University of Ilorin for analysis.

Isolation of Proteobacteria and Firmicuites

One gram of soil sample was weighed aseptically and homogenized in 9 ml distilled water (0.86% NaCl) by vortexing vigorously and spread plating 1ml of 10^{-3} and $10^{-4}$of soil suspension on Nutrient agar medium containing (per 1000mL) [peptone, 5g; Nacl 5g; Beef extract; Yeast extract 1.5g; agar 15g], for 24hours at 37^0C. Twenty (20) morphologically different pure colonies were selected after purification using quadrant streak method and stored as a stock on Nutrient agar.

Preliminary Identification and Screening of Isolates

Each selected isolate was streaked on Nutrient Agar (NA) and incubated for 24 h at room temperature. Preliminary identification of isolates was conducted by Gram staining to identify the cellular characteristics of bacterial cells following standard methods described by (Majeed et al., 2015).

Molecular Identification

Each pure bacteria isolates was grown in 50 mL Nutrient Broth (NB) and incubated at 37°C for 24 h. Bacterial cells then centrifuged at 9,500×g for 1 min. Selected bacterial genomic DNA was extracted using PrestoTM gDNA Bacteria Mini Kit (Genaid) . The 16S rRNA gene was amplified using Polymerase Chain Reaction (PCR) machine. Fifty microliters of PCR mix was created with composition: 25 µL GoTaq Green Master Mix 2 (Promega, Madison, WI, USA), 2.5 µL (100 pmol) for each primer: 16SF (5'AGAGTTTGATCCTGGCTCAG3) and 16SR (5' TACCTTGTTACGACTT3), 0.3 µL (64.4 mg µL) of DNA templates and 19.7 µL nuclease free water. Polymerase Chain Reaction (PCR) was performed under the following conditions: Pre-denaturation at 94°C for 5 min, denaturation at 92°C for 30 sec, annealing at 58°C for 30 sec, elongation at 72°C for 1.5 min and final extension at 72°C for 5 min with 35 cycles. Finally the temperature declined to 4°C for 10 min to stop PCR reaction. Polymerase Chain

Reaction (PCR) products were purified and sequenced by sending it to sequencing service company (IITA Ibadan). The sequences realized were analyzed using Bioedit and juxtaposed with BLAST program for semblance with bacteria species on NCBI GenBank at http://www.ncbinlm-nih.gov/. After molecular identification, 16S rRNA nucleotide sequences of each isolates was deposited at the NCBI GenBank with the following accession numbers: *Kosakonia sacchari* (OQ678004), *Enterobacter asburae* (OQ710322), *Pseudomonas putida* (OQ683798), *Leclercia adecarboxylata* (OQ683808), *Bacillus nakamurai* (OQ860818), *Bacillus nitratireducens* (OR098644), *Bacillus velezensis* (OQ860817), *Bacillus subtilis* (OQ836626).

Toxicological Effect of Herbicides on the Proteobacteria and Firmicutes Isolates

Toxicological study was conducted to assess isolates survival and growth at different concentrations of herbicides using agar plate dilution method as described by Majeed et al. (2015). Freshly prepared Mueller Hinton medium (Beef extract [2g], Acid hydrolysate of casein [17.50g], Starch [1.50g], distilled water 1000mL) agar plates were amended with increasing concentrations of selected herbicides (1X, recommended field dose; 2X, double dose; and 3X, three times of the recommended dose). Assessment without herbicides was considered as control. Thereafter, plates were spot inoculated with 0.1mL of 10^8 cfu/mL of selected strains and incubated at 28 ±2 °C for 72 h. Effect of herbicides were evaluated using plate count method every 24hours for 3days. Details of herbicides used in the present study along with their recommended field concentration are presented in Table 3.

Statistical analysis

SPSSv. 15.0 was employed for data analysis. After ANOVA significant means were separated with Tukey's post hoc test at a 0.05 level of significance.

RESULTS

Effect of Different Concentration of Herbicides on Proteobacteria

Proteobacteria growth responded differently depending on the type and concentration of the herbicide (Figure 1). Effects on growth differed significantly depending on the species of the isolate ($F = 2.399$, $p=0.008$; Appendix Table I1) and type of herbicide used ($F = 27.482$, $p<0.05$; Appendix Table I1). The herbicides; paraquat, atrazine and glyphosate had most inhibitory effect on the survival and growth of tested proteobacteria (Figure 1 [A]–[D]). This effect were most significant on survival at three times (150mg/100ml) the recommended concentration. At 3X RFDs concentration, atrazine significantly inhibited the survival of L. *adecarboxylata, K. sacchari, E. asburae and P. putida*, while glyphosate affected the survival of *K. sacchari and P.putida,* also, pendimenthalin affected the survival of *E. asburae.* However the herbicide paraquat affected the survival of *L. adecarboxylata* at all RFDs. Compared to other results, the herbicide type diuron and nicosulfuron had no significant negative effect on the survival and growth of the tested proteobacteria at all RFDs. Generally, there was more growth at the lower concentration of any of the herbicides, indicating a concentration-dependent effect of the herbicides on the growth inhibition or enhancement of the Proteobacteria (X1; 113.315^c, X2; 93.494^b, X3; 76.565^a).

Effect of Different Concentration of Herbicides on Firmicuites

A progressive, herbicide-concentration-dependent, decline in survival and growth of tested Firmicuites was observed when the isolates were grown in the presence of increasing concentration of the herbicides under study (Figure 2 [A]–[D]). Effects on firmicuites growth and survival differed significantly depending on the concentration and type of the herbicides used ($F = 12.941$, $p<0.05$; Appendix Table I1). At three times the recommended concentration, the herbicide type; paraquat and atrazine had significant inhibitory effect on the survival and growth of *B. nakamurai*, *B. nitratireducens*, *B. velezensis* and *B. subtilis,* while glyphosate had effect on the survival of only *B. nitratireducens.* Meanwhile, the herbicide type; pendimenthalin, diuron and nicosulfuron had no significant negative effect on tested firmicuites at all tested RFDs. Effect of herbicides under study were also significantly dependent on the tested firmicuite specie ($F = 2.400$, $p=0.023$; Appendix Table 11). *B. subtilis* survival and growth was severely affected by paraquat and atrazine at all RFDs/tested concentration. Generally, just as observed in proteobacteria, there was more growth observed at the lower concentration (1X and 2X RFD) of any of the herbicides under study, thus indicating a concentration-dependent effect. Finally, there was more growth at the lower concentration of any of the herbicides under study, indicating a concentration-

dependent effect of the herbicides on the survival and growth of tested firmicuites (X1; 105.830[a], X2; 96.071[a], X3; 86.580[a]).

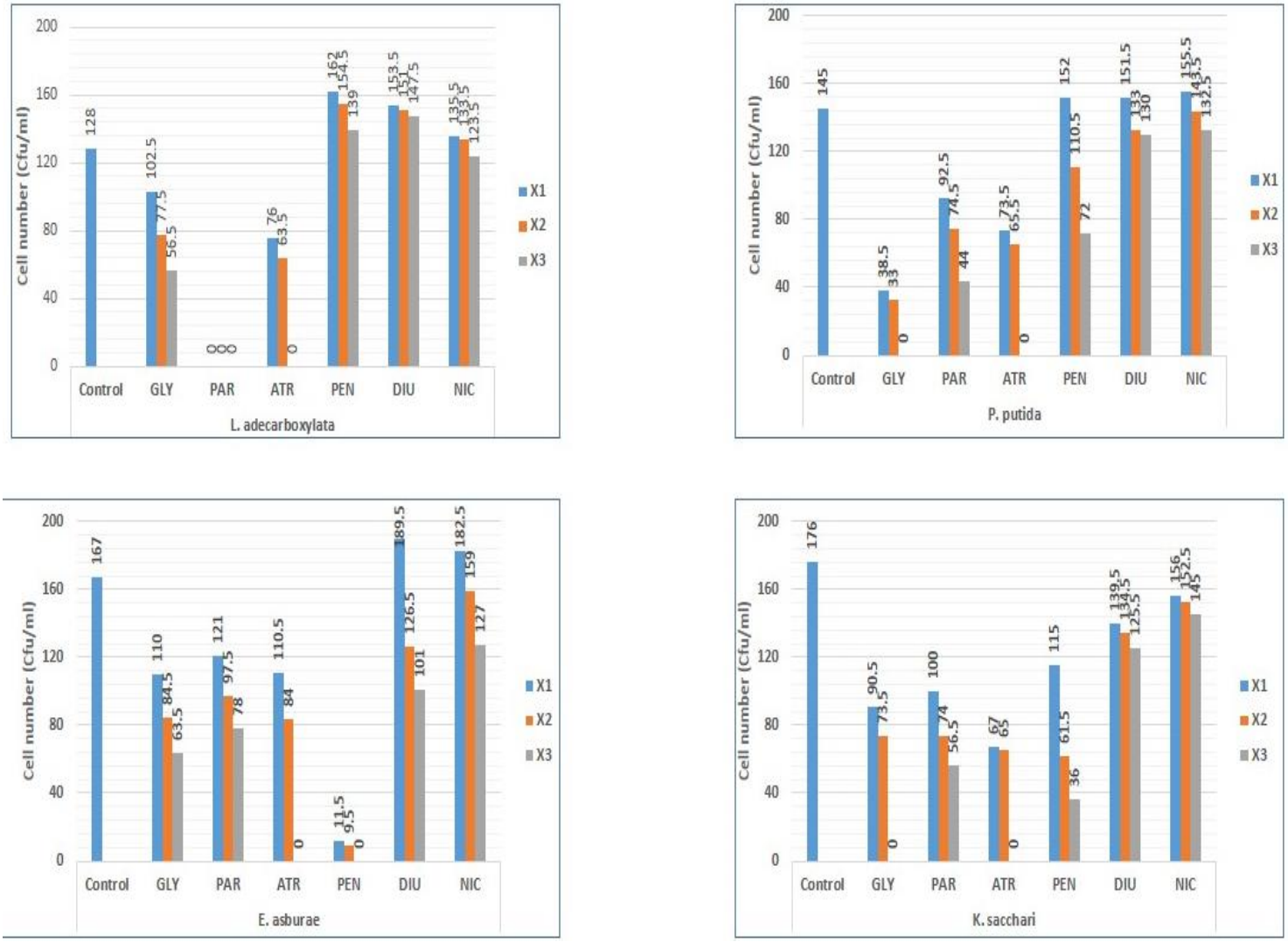

Figure 1: Effect of herbicide concentrations on Proteobacteria [A-D]

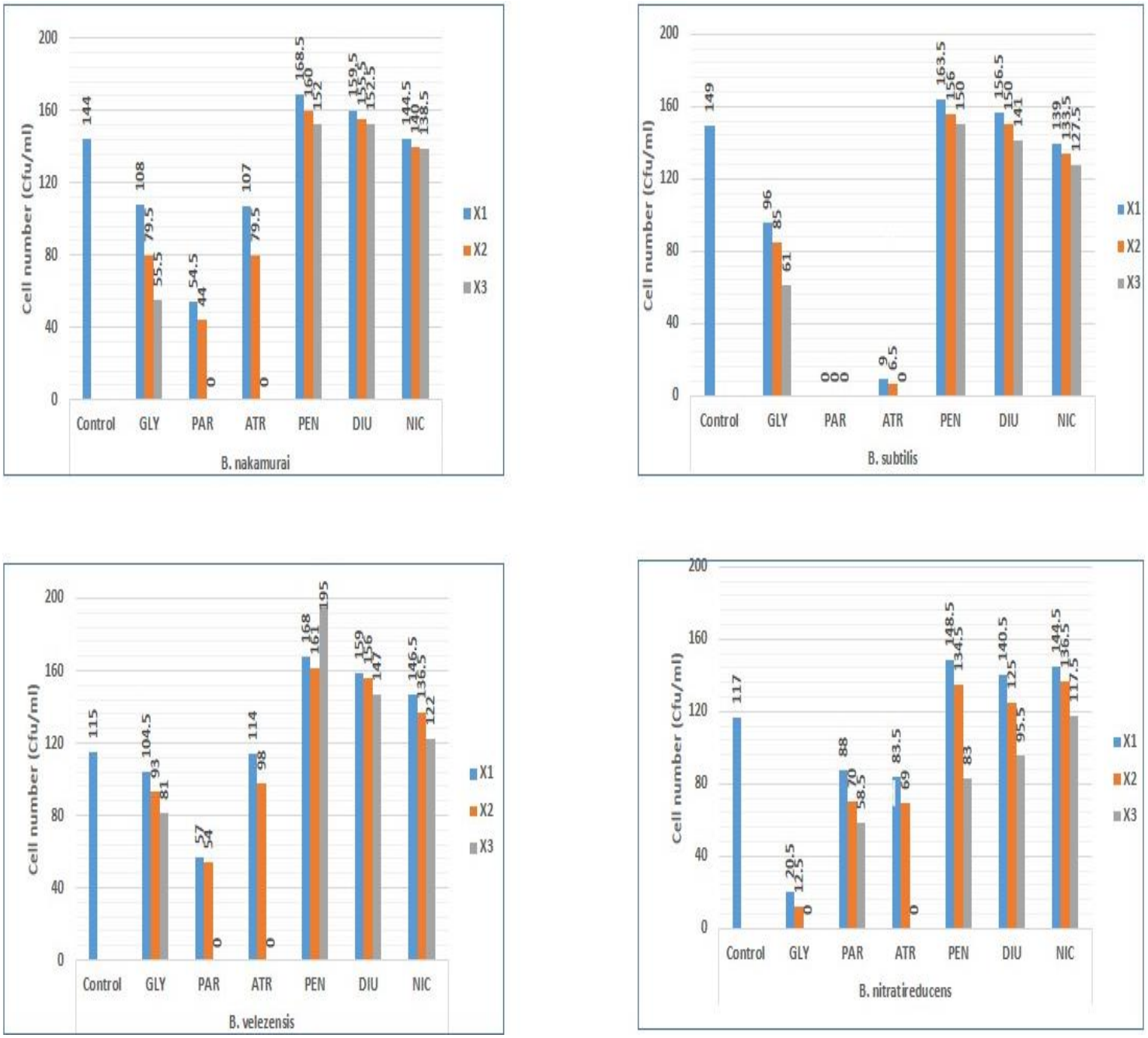

Figure 2: Effect of herbicide concentrations on Firmicutes [A-D]

Discussion

Sustainable management of soils requires monitoring, including biological indicators such as microbial communities, which provide many potential indicators for environmental response to a range of stresses or disturbances. This study was carried out to evaluate the toxicity of some agrochemical herbicides (paraquat, atrazine, glyphosate, pendimenthalin, diuron and nicosulfuron) at both the recommended concentration (1X) and higher concentration rates (2X and 3X) on the survival and growth of isolated Proteobacteria and Firmicutes in maize rhizosphere. The growth of Proteobacteria ($F = 2.399$, $p=0.008$) and Firmicuites ($F = 2.400$, $p=0.023$) responded differently depending on the type and concentration of the herbicides tested under study. Generally, our result showed progressive decline in Proteobacteria and Firmicuites survival and growth when environmentally relevant concentrations of herbicides were exceeded with few exceptions in, diuron, and nicosulfuron. Similar result was reported by Xie et al., (2018) on the toxicity of pesticides on soil microbiota. The effect of herbicides have been less consistent across the two bacteria phyla (Proteobacteria and Firmicuites) under study. Variations in the effects observed on both the survival and growth in comparison with the control across the two phyla suggested that microbial communities responded differently to varying concentrations of each herbicides which in in

agreement or in line with previous report by Ligor and Staneczko-baranowska, (2019).

Differences in responses of Proteobacteria and Firmicuites to varied concentration of herbicides can be attributed to their unique cell wall structures (Muturi et al., 2017). Proteobacteria have an outer membrane composed of lipopolysaccharides, which acts as barrier against various toxic substances, including herbicides. The outer membranes make it harder for herbicides to penetrate the cell wall and reach the target sites within the bacteria cell. Furthermore, Proteobacteria often possess efflux pumps and specialized transport proteins that actively pump out toxins from bacterial cell (Xie et al., 2018). Firmicutes on the other hand lacks this outer membrane and have a thick layer of peptidoglycan in their cell wall. This peptidoglycan layer is generally more permeable to molecules, allowing the herbicides to enter the bacterial cell more easily and attack the target sites (Soo et al., 2020). This assumptions therefore elaborate the scientific basis for understanding the varied responses of Proteobacteria and Firmicuites to herbicides tolerance under the present study.

While herbicides tend to produce generally negative effects in most microbes, some differences were observed among the selected herbicides under study. Herbicides (atrazine, paraquat and glyphosate) belonging to organochlorine and organophosphate pesticide group have more pronounced effects on survival and growth of isolates compared to other herbicides (nicosulfuron, diuron and pendimenthalin). The significant effects were not unexpected since previous studies by Li et al. (2023) had reported similar observation about organochlorine herbicide inducing shifts in microbial community structure. According to Kumari, 2018, effects of organochlorine herbicides on bacteria have generally been negative, while Sidhu et al. 2019 suggested that some organophosphate and urea-based herbicides have reduced effect at environmentally relevant concentrations. However, outcome of our study showed that the probability of herbicide producing a negative or no effect is dependent upon the Proteobacteria and Firmicuites species, and the concentration of the herbicide in use (Nyadar et al., 2019). Furthermore, microbial tolerance against herbicides is regulated by both physiological and genetic factors (Huang et al., 2018). Suleiman et al. (2018) in their study attributed temporary resistance (tolerance) against herbicide to physiological changes that induce microbial metabolism to form new metabolic pathway to bypass a biochemical reaction inhibited by a specific herbicide while permanent resistance, on the other hand, depends upon genetic modifications, inherited and transferred by subsequent generations of microbes. Our study therefore concluded that short-term exposure to low or high concentrations of herbicides may have profound effects on plant growth promoting Proteobacteria and Firmicuites depending on the bacteria species and the concentration of herbicides applied. Microbial communities are key mediators of critical ecosystem processes and any herbicide-mediated changes in microbial diversity and community structure can disrupt ecosystem functions if the herbicide-tolerant microorganisms are unable to compensate for loss in functions associated with herbicide-sensitive microbes. The study recommended proper education and monitoring to ensure strict compliance with herbicide guidelines, on use, exposure and concentration required by farmers.

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Appendix

Table I: Summary of Herbicides Effects on Isolates

Chemical Family	Brand Name	Chemical Name	Reccomended Dose
Organophosphate	Glyphosphate	N-(Phosphonomethyl) glycine	1680mg/L
Organochlorine	Paraquat	1-methyl-4-(1-methylpyridin-ium-4-yl)dichloride	1680mg/L
Anilide	Pendimenthalin	3,4-dimetyl-2,6-dinitro-N-pentan-3-ylaniline	5600mg/L
Organochlorine	Atrazine	6-Chloro-4-N-etyl-2-N propan-2yl-1,3,5-triazine-2,4-diamine	1000mg/L
Phenylurea	Diuron	3-(3,4-Dichlorophenyl)-1,1-dimethylurea	2000mg/L
Phenylamide	Nicosulfuron	2-(carbomoylsulfamoyl)-N,N-dimethylpyridine-3-carboxamide	3000mg/L

Table I1: Summary of Herbicides Effects on Isolates

Three way ANOVA

Source of variation	DF	Proteobacteria		Source of variation	DF	Firmicutes	
		F	P			F	P
Organism	11	2.399	0.008	Organism	7	2.400	0.023
Herbicide type	6	27.482	<0.05	Herbicide type	6	12.941	<0.05
Herbicide concentration	2	10.042	<0.05	Herbicide concentration	2	1.267	0.285
Residual	232			Residual	152		
Total	251			Total	167		

Proteobacteria Herbicide type		Firmicuites Herbicide type	
Control	129.667^{bc}	Control	137.125^{b}
Glyphosphate	47.833^{a}	Glyphosphate	49.583^{a}
Paraquat	52.625^{a}	Paraquat	44.021^{a}
Atrazine	41.958^{a}	Atrazine	48.021^{a}
Pendimenthalin	102.389^{b}	Pendimenthalin	141.479^{b}
Diuron	153.250^{c}	Diuron	142.583^{b}
Nicosulfuron	133.486^{bc}	Nicosulfuron	109.937^{b}
SEM	8.866		0.679
Herbicide concentration		Herbicide concentration	
X1	113.315^{c}	X1	105.830^{a}
X2	93.494^{b}	X2	96.071^{a}
X3	76.565^{a}	X3	86.580^{a}
SEM	5.804		8.550