IMPACTS OF GLYPHOSATE ON PLANT GROWTH PROMOTING ABILITIES OF RHIZOBACTERA ISOLATED FROM AGRICULTURAL SOIL

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ABSTRACT

The contamination of agricultural farm lands due to pollution arising from excessive and indiscriminate use of herbicide, such as glyphosate, leads to the reduction in densities of important soil microbial communities, which play several roles such as nutrient cycling and other key roles necessary for the maintenance of soil fertility. Due to the adverse effects of this chemical to non-target beneficial organism, this study was designed to evaluate the effect of different concentrations of glyphosate (3.1, 7.2 and 14.4 mg/ml) on plant growth promoting (PGP) ability of Bacillus mojavensis, Pseudomonas aeruginosa, Alcaligenes faecalis, Pseudomonas syringae and Bacillus cereus. The effects of different concentrations of glyphosate on Indole acetic acid (IAA) production, potassium and phosphorus solubilisation and nitrogen fixing abilities of the isolates were evaluated using Salkowsky, Atomic Absorption Spectroscopy (AAS), vanadomolybdate and microkjedhal method. The highest amount of IAA was recorded by Pseudomonas aeruginosa and least by Bacillus mojavensis. The results of P-solubilisation followed the same trends with Pseudomonas aeruginosa having the highest amount of P-solulisation while the least was recorded by Bacillus mojavensis. The results of K-solubilisation were significantly different (P≤0.05) at different concentrations of glyphosate. The nitrogen fixing ability of the isolates were also affected by the increase in concentration of glyphosate. All the isolates showed the least nitrogen fixed at the concentration of 14.4mg/ml. The result of this study has revealed the need for the farmer to stick to recommended or even below the recommended rate of glyphosate application to reduce its deleterious effects on the beneficial soil organism and to enhance sustainable soil quality.

Keywords: Glyphosate; PGP-abilities; Concentrations.

INTRODUCTION

Glyphosate is a non-selective herbicide, that is, it can kill all plant species, although there is variation between species with regard to levels of natural tolerance. Glyphosate (*N*-(phosphonomethyl) glycine) is a broad-spectrum systemic herbicide and crop desiccant. It is an organophosphorus compound, specifically a phosphonate. It is used to kill weeds, especially annual broadleaf weeds and grasses that compete with crops. It was discovered to be a herbicide by Monsanto chemist John E. Franz in 1970 (Franz, 1997). Monsanto brought it to market in 1974 under the trade name "Roundup" and Monsanto's last commercially relevant United States patent expired in 2000. Farmers quickly adopted glyphosate, especially after Monsanto introduced glyphosate-resistant Roundup Ready crops, enabling farmers to kill weeds without killing their crops. Glyphosate has

little or no herbicidal activity in soil and, thus, is used only with foliar spray applications. Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Saharan and Nehra, 2011). PGPR are applied as plant inoculant or soil amendment to enhance the growth and fertility of soil. Rhizobial inoculants as bio-fertilizers are therefore, applied to soils/seeds of legumes to ensure effective nodulation and subsequent N_2 fixation and consecutively, to increase the nitrogen pool of soils (Dudeja and Singh, 2008). The inoculants are often used together with agrochemicals, which besides containing essential nutrients also contain contaminants and toxic elements. The exposure of these chemicals to field-grown plants could be either intentional (e.g. by spraying the legumes with pesticides) or through residues remaining from previous applications (Khan *et al*., 2004). Of these chemicals, herbicides and their microbially degraded products interact with soils and rhizosphere microorganisms including rhizobia and cause DNA, protein, oxidative or membrane damage (Pham *et al*.,2004). In addition, the common use of herbicides in agricultural practices has been shown to affect N_2 fixation adversely, either directly by affecting the rhizobia (Mallik and Tesfai, 1985; Anderson*et al*., 2004) or disrupting the signaling between plant derived phytochemicals and Rhizobium Nod receptors (Fox *et al*., 2007) or indirectly by reducing photosynthate allocation to the nodules for N_2 fixation (Sprout *et al*., 1992;Koopman *et al*., 1995; Datta *et al*., 2009) or by restricting root growth and hence reduce the number of sites available for infection (Eberbach and Douglas, 1991). More so, herbicides that persist in soils may have a long-lasting impact on rhizobial survival and function (Eliason *et al*., 2004). An alternative to overcome the deleterious effects of pesticides on plants could be the treatment of seeds with rhizobia as a bio-inoculant which displays a wide range of tolerance to herbicides and exhibit PGP activities including their inherent N_2-fixing attribute under herbicide-stress (Ahemad and Khan, 2010). Therefore, herbicides tolerant rhizobia possessing multiple plant growth promoting activities will be useful in optimizing the yields of plant in stressed production systems (Ahemad and Khan, 2011).

Studies on the effect of various herbicides have largely been focused on changes in populations of soil microorganisms and less attention has been paid to the effect of these agrochemicals especially glyphosate on plant growth promoting (PGP) activities of rhizobia. This study was therefore, designed to evaluate the effects of three concentrations of glyphosate on PGP activities of bacteria.

MATERIALS AND METHODS

Microorganisms and culture condition

Five glyphosate tolerant plant growth promoting bacteria initially identified as *Bacillus mojavensis* **strain NBSL5** (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156 (MY19), *Pseudomonas syringaepv.syringae*HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25) isolated from rhizosphere soil of yellow and white maize were used for this work. These isolates had been screened and had shown

evidence of multiple plant growth promoting abilities. The isolates were maintained on nutrient agar slants at refrigerating temperature of 4°C. Each seed culture was prepared accordingly by inoculating a loop of the stock culture into 50ml of nutrient broth after which the bacteria cells were harvested, washed and re suspended in distilled water. To ensure equal cell population of each of the bacteria strain, their turbidity was adjusted to 0.5 McFarland standards(Anyanwu and Ezaka2011).

Herbicide

The herbicide Force Up® that contains 360g active ingredient per litre was used for the research work. The herbicide was purchased from agrochemical shop in Ibadan.

Effect of glyphosate on IAA production

The selected bacterial isolates were inoculated into 10ml peptone water and incubated on an orbital shaker for 24h. One ml of the bacterial culture was transferred into a fresh 50ml peptone water amended with 3.1, 7.2 and 14.4mg/ml of glyphosate(Force Up®) with the addition of 5ml of L-tryptophan as a precursor of indole acetic acid(IAA). Peptone water (without glyphosate) inoculated with the isolates served as control. A portion (10ml) of the bacterial culture was transferred into a sterile tube and centrifuged at 7000rpm for 7 min. The supernatant (1ml) was mixed with 2ml of Salkowsky reagent (2% of 0.5m $FeCl_3$ in 35% perchloric acid (Gorddon and Weber, 1951). The solution was allowed to stand for 25mins.The absorbance values were determined using a spectrophotometer at 535nm and compared to the standard curve to determine the IAA concentration.

Effect of glyphosate on potassium solubilization:

One ml of overnight culture of each isolatediluted to McFarland's standard were inoculated to 50ml of Aleksandrov broth amended with and without 3.1, 7.2 and 14.4mg/ml of glyphosate (Force Up®) and incubated for five days. At the end of the five days, the broth cultures were centrifuged at 10,000rpm for 10min to separate the supernatant from the bacterial cells and insoluble potassium. One ml of the supernatant was taken in a 50ml volumetric flask and the volume was made up to 50ml with distilled water and mixed thoroughly. The available K contents in the solution were determined using flame photometer (Tan *et al*., 2014).

Effects of glyphosate on phosphorus Solubilization by the isolates

One ml of overnight culture of each isolate diluted to McFarland's standard was inoculated to 50ml of Pikovskaya broth amended with and without 3.1, 7.2 and 14.4mg/ml and incubated for five days. At the end of the five days, the broth cultures were centrifuged at 10,000rpm for 10min to separate the supernatant from the bacterial cells and insoluble phosphorus. 2.5 ml of the supernatant was taken in a 25ml volumetric flask and 20ml of distilled was added, this was followed by addition of 2.5ml of Bartons reagent and was allowed for 10min for color development. The absorbance value was determined using spectrophotometer at 430nm. Standard curve was prepared using stock solution of KH_2PO_4 at different concentrations. The amount of phosphorus solubilized by the isolates was calculated from the standard curve (Tan *et al.*, 2014)

Effects of glyphosate on Nitrogen fixing abilities of the isolates

The effect of glyphosate on the efficiency of nitrogen fixing abilities by the isolates were made in semisolid Nfb medium containing 0.05% ofmalate as carbon source and amended with 3.1, 7.2 and 14.4mg/ml of glyphosate. The isolates were inoculated in Nfb medium and incubated at 30^{o}C for 10days. After 10 days of incubation, the total amount of nitrogen fixed was determined by Microkjedahl analysis (Kanimozhi and Panneerselvam,2010). In this method, the media containing the isolates were digested in the 100ml flask by adding the salt mixture (50:10:1 ratio of K_2SO_4, $CuSO_4$ and metallic selenium) and 3ml of concentrated H_2SO_4. After digestion, 100ml of distilled water was added and cooled. The digested samples were poured into the microkjeldahl distillation apparatus. For quick delivery, 10ml of 40% NaOH was added into the distillation apparatus. In a 20ml Erlenmayer flask, 10ml of 4% boric acid reagent and 3 drops of mixed indicator were added. The flask was placed under the condenser of the distillation apparatus and the tip of the condenser outlet was beneath to the surface of the solution in the flask. The solution, boric acid mixed with indicator containing the distilled off (NH_3) was titrated against standard HCl.

Statistical analysis: The data obtained were analyzed using analysis of variance, the means were separated using Duncan multiple range test.

RESULTS

The results of the effects of different concentrations of glyphosate on the abilities of the selected isolates to produce indole acetic acid (IAA), solubilize potassium and phosphorus were presented in Table 1 to 3. The results showed a decrease in the PGP abilities of the isolates with increase in the concentration of glyphosate. The highest amount of IAA was recorded by MW18 at the concentrations of 0 and 3.1mg/ml (25.61 and 20.77mg/l, respectively) followed by MY19 which produced 25.61, 20.77, 12.92 and 5.65mg/l of IAA at the concentration of 0, 3.1, 7.2 and 14.4mg/l, respectively (Table 1). Isolate MY20 recorded 15.65, 3.195, 2.63 and 2.99mg/l at the concentration of 0, 3.1, 7.2 and 14.4mg/l and Isolate MY25 recorded 13.77, 4.83, 4.41 and 4.19 mg/l of IAA at the concentration of 0,3.1,7.2 and 14.4mg/l, respectively while isolate MY4 recorded 12.65, 3.99,4.26 and 2.1 mg/l at the concentration of 0, 3.1, 7.2 and 14.4mg/l, respectively. The results of P-solubilised by the isolates at different concentrations of glyphosate are presented in Table2. The results showed that isolate MW18 recorded the highest amount of P-solubilization (1035.9, 993.86, 116.76 and 1231.75mg/l) at the concentrations of 3.1, 7.2, 14.4 and 0 mg/l, respectively while the least was recorded by isolate MY 20. The results also showed a decrease in the amounts of phosphorus produced with increase in the concentrations of glyphosate. Similar trends were observed on the effects of different concentrations of glyphosate on potassium solubilisation (Table 3). The results also showed a decrease in the amount of K-solubilised with increase in the concentration of glyphosate.

TABLE 1: MEAN AMOUNT OF IAA (MG/L) PRODUCTION BY THE ISOLATES AT DIFFERENT CONCENTRATIONS OF GLYPHOSATE

Isolates	Glyphosate concentrations(mg/ml)			
	Control(0)	3.1	7.2	14.4
		Amount of IAA produced(mg/l)		
MY4	12.65± 0.21^{d}	3.99±0.66^{d}	4.26±1.01^{c}	2.10±0.05^{c}
MW18	25.61±1.05^{a}	20.77±0.57^{a}	12.92±0.62^{b}	5.65±1.08^{b}
MY19	22.66± 0.33^{b}	15.27± 1.20^{b}	15.17±0.33^{a}	11.59±0.42^{a}
MY20	15.65±0.47^{c}	3.19 ± 0.77^{d}	2.63±0.15^{d}	2.99±1.30^{d}
MY25	13.77±0.06^{cd}	4.83±0.59^{c}	4.41±2.01^{c}	4.19±0.55^{b}

Means with the same letters within a column are not significantly different (P≤0.05).

Key: *Bacillus mojavensis* strain NBSL5 (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156 (MY19), *Pseudomonas syringae pv.syringae* HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25)

TABLE 2: P – SOLUBILIZATION (MG/L) BY THE ISOLATES AT DIFFERENT CONCENTRATIONS OF GLYPHOSATE

Isolates	Glyphosate concentrations(mg/ml)			
	Control(0)	3.1	7.2	14.4
		P-solubilized(mg/l)		
MY4	610.11±0.03^{b}	98.66±0.32^{d}	73.29± 0.52^{d}	70.16±0.31^{d}
MW18	1231.75±0.11^{a}	1035.90±0.11^{a}	993.86±0.20^{a}	116.76±0.04^{a}
MY19	530.71±0.02^{c}	541.18±0.04^{b}	101.83±0.26^{c}	103.49±0.18^{b}
MY20	510.65±0.41^{d}	82.27±0.41^{e}	64.05±0.15^{e}	62.66±0.30^{e}
MY25	408.15±0.33^{e}	278.42±0.28^{c}	182.54±0.32^{b}	84.71±0.31^{c}

Means with the same letters within a column are not significantly different (P≤0.05).

Key: *Bacillus mojavensis* strain NBSL5 (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156 (MY19), *Pseudomonas syringae pv.syringae* HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25)

TABLE 3: POTASSIUM – SOLUBILIZATION BY THE ISOLATES AT DIFFERENT CONCENTRATIONS OF GLYPHOSATE

Isolates	Glyphosate concentrations(mg/ml)			
	Control(0)	3.1	7.2	14.4
		K-solubilized(mg/l)		
MY4	101.66±1.07^{b}	72.09±1.52^{d}	84.22±0.33^{c}	70.21±0.60^{c}
MW18	325.63±0.42^{a}	184.01±0.21^{a}	119.83±1.01^{a}	83.21±2.55^{a}
MY19	106.03±0.51^{b}	93.03±0.07^{b}	78.67±0.17^{cd}	63.00±0.72^{bc}
MY20	92.57±1.31^{d}	87.60± 1.03^{c}	62.00±0.08^{d}	52.25±2.30^{d}
MY25	171.56±0.88^{c}	84.34± 0.15^{c}	103.21±0.33^{b}	79.90±0.41^{b}

Means with the same letters are not significantly different (P≤0.05)

Key: *Bacillus mojavensis* strain NBSL5 (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156 (MY19), *Pseudomonas syringae pv.syringae* HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25)

Effects of glyphosate concentration on the nitrogen fixing abilities of the isolates

The results of effect of glyphosate on nitrogen fixing abilities of *Bacillus mojavensis*strain NBSL5 (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156 (MY19), *Pseudomonas syringaepv.syringae* HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25) showed a decrease in the nitrogen fixing ability of the isolates at the concentration of 14.4mg/ml. The amount of nitrogen fixed at 3.1 mg/ml glyphosate was not significantly different when compared with the control(0 mg/ml). The result showed *Alcaligenes faecalis strain* P156 having the highest nitogen fixing ability(8.01mg N fixed/g) and least was recorded by *Pseudomonas syringae* (4.1 mg N fixed/g) at 3.1mg/ml of glyphosate. Similar trend was also observed at the concentration of 7.2mg/ml with *Alcaligenes faecalis strain* P156 showing highest amount of nitogen fixed followed by *Pseudomonas aeruginosa strain* ZSL-2 and least by *Pseudomonas syringaepv.syringae* HS191. At the glyphosate concentration of 14.4mg/ml, *Pseudomonas aeruginosa strain* ZSL-2 recorded highest amount of nitrogen fixed(5.3mg N fixed/g) while the least was observed in *Bacillus mojavensis*strain NBSL5(1.1Mg N fixed/g). The results of the effect of different concentration of glyphosate on the nitrogen fixing abilities of the isolates were presented in Fig1.

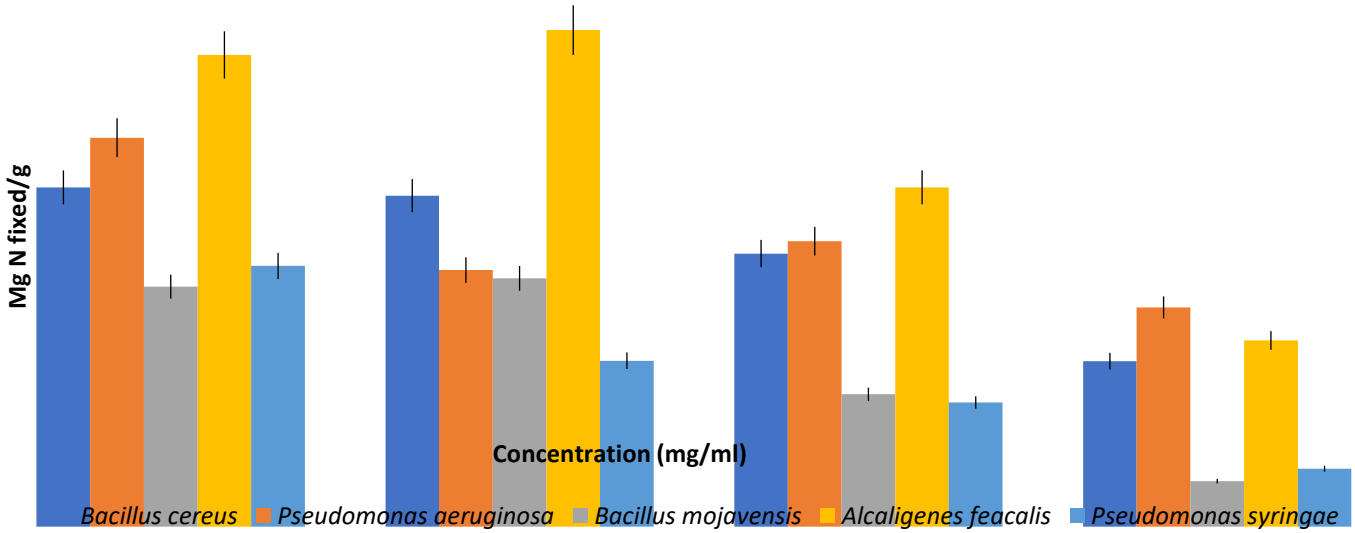

FIG 1: EFFECTS OF GLYPHOSATE CONCENTRATION ON THE NITROGEN FIXING ABILITIES OF THE ISOLATES

DISCUSSION

The excessive use of glyphosate beyond the recommended dose causes adverse effect to the non-target soil organisms. This also affects soil fertility due to reduction in the activities of the beneficial organism which contributes directly or indirectly in the fertility of soil as well as crop yield. In this study, *Bacillus mojavensis*strain NBSL5 (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156(MY19), *Pseudomonas syringaepv.syringae* HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25) exhibited plant growth promoting traits such as Potassium and phosphorus solubilisation, nitrogen fixation and indole acetic acid production in significant amount both in the absence and presence of different concentration of glyphosate. These PGB activities of these isolates can help in increasing soil fertility and yield. For instance, the phytohormone, the IAA produced by rhizobacteria promotes root growth by directly stimulating plant cell elongation or cell division. A low level of IAA produced by rhizosphere bacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibit primary root growth (Ma *et al*., 2009). The isolates showed a significant ability to produce IAA in absence and presence of glyphosate though with a decrease in the amount of IAA produced at higher concentration. Each PGP trait of bacteria is the result of sequential metabolic reactions mediated by various specific functional proteins (enzymes) along the defined metabolic pathway. The metabolic pathways for any specific PGP trait may be more than one depending upon the type of the PGP substances and bacterial genera/species.

Herbicides adversely affect protein synthesis and the metabolic enzymes (Kapoor and Arora, 1996; Boldt and Jacobsen, 1998). Therefore, it seems probable that herbicide(glyphosate) employed in this study might have inhibited the functioning of the enzymes participating in different metabolic pathways of PGP traits in the bacteria which resulted in the decrease of the amount of IAA produced at high concentration of herbicide. The results of IAA production at different concentrations of glyphosate agrees with the findings of Munnes and Mohamed (2011) which reported a decrease in IAA production by bacteria isolates due to pesticides stress. This also is at variance with the findings of Chennappa*et al*.(2014) which reported no significant effect on effect of glyphosate IAA production by bacteria isolates. Solubilization of P and K by the isolates also decreased at concentration higher than recommended (14.4mg/ml), this is also in line with the findings of Munnes and Mohamed (2011) which reported a decrease in P and K solubilization under glyphosate stress. It is also similar to the findings of Pharm *et al* (2004) which reported inhibition of PGP ability of Azotobacter due to pesticide stress. It is reported that pesticides not only damage structural proteins essential for the growth of the organism but also responsible for geno-toxicity (Pham *et al*., 2004), eventually leading to the decreased functioning and survival of organisms exposed to high concentration of pesticides (Kumar *et al*., 2010).Hence, the decline in the PGP abilities of the isolates. The results of Nitrogen fixing ability of the isolates followed the same trend. The results were not significantly different ($P \leq 0.05$) at lower concentration (3.1mg/ml) when compared with control. All the organisms showed a decrease in Nitrogen fixing ability at higher concentration (14.4mg/ml). Anderson *et al*. (2004) reported that the common use of herbicides in agricultural practices has been shown to affect N_2 fixation adversely, either directly by affecting the rhizobia or disrupting the signaling between plant derived phytochemicals (luteolin, apigenin) and Rhizobium Nod receptors (Fox *et al*., 2007). This study has revealed that the use of glyphosate above the recommended dose impact negatively on the IAA production, P and K solubilization as well as nitrogen fixing abilities of *Bacillus mojavensis*s train NBSL5 (MY4), *Pseudomonas aeruginosa strain* ZSL-2(MW18), *Alcaligenes faecalis strain* P156(MY19), *Pseudomonas syringaepv.syringae* HS191(MY20) and *Bacillus cereus strain* 20UPMNR(MY25). All the isolates showed decreased PGP abilities at higher concentrations of glyphosate.

CONCLUSION

This study has revealed the potentials of these isolates to solubilize Phosphorus and potassium, fixing nitrogen as well as produce indole acetic acid(IAA) at different rates of glyphosate. The results has also revealed that the application of glyphosate beyond the recommended dose affect plant growth promoting abilities of the isolates. This serves as vital information to farmers to stick or use below the recommended dose to reduce the deleterious effects of glyphosate to beneficial soil organism due to excessive use of glyphosate.

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