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Tolerance and Utilisation of glyphosate by Plant Growth Promoting Bacteria (PGPB) isolated from rhizosphere soil of maize (*Zea mays* l)

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Abstract

Glyphosate is widely used by farmers to control weeds. Its excessive use by farmers affects non-target organism, this study was designed to evaluate the abilities of rhizobacteria to utilize glyphosate. Plant growth promoting bacteria isolated from the rhizosphere of maize were assessed for tolerance of glyphosate. The ability of the selected glyphosate tolerant isolates (*Bacillus mojavensis*, *Pseudomonas aeruginosa*, *Alcaligenes feacalis*, *Pseudomonas syringae* and *Bacillus cereus* 20UMPNR) to utilize the carbon and phosphorus contents at concentration of 3.1 mg/ml, 7.2 mg/ml and 14.4 mg/ml were evaluated by measuring their optical density and cell biomass. . *mojavensis*, *P aeruginosa*, *A. feacalis* strain, *syringae* and *B cereus* had higher level of tolerance to 7.2, 50.0 and 100.0 mg/ml of glyphosate. The growth of the isolates at different time and concentrations were significantly different (P= 0.05). The results of utilization as phosphorus (P), carbon (C) and both C and P source showed that *B cereus* and *P aeruginosa* had higher P-utilisation at the end of incubation while the least was *B mojavensis*. Also, *P aeruginosa* showed the highest evidence of C-utilization and least by *B mojavensis*. The same trend was observed when glyphosate was used as phosphorus and carbon source. This study has revealed the ability of these isolates to tolerate and utilize glyphosate.

Key words: Carbon; phosphorus; Glyphosate; Rhizosphere;Tolerance; Utilization

Introduction

Glyphosate is a post-emergent and non-selective (or broad spectrum) herbicide used in both agricultural and non-agricultural areas (WHO, 1994).It is a weak organic acid consisting of glycine and phosphonomethyl moiety. Its chemical name is N-(phosphonomethly) glycine. It is formulated as the Isopropyl amine or trimethylsolfonium salt of glyphosate (David and Topsy, 2001). Glyphosate is used to kill all plant types including grasses, perennial, and woody plants (Cox, 2000; USDA 2000). It can be used in non-till (zero tillage) agriculture to prepare field before planting instead of depending on mechanical tillage to control weeds and reduce soil erosion (USDA, 2000; David and Topsy, 2001). Its mode of action is the inhibition of the enzyme 5- enolpyruval shikimate-3- phosphate (EPSP) synthase in the shikimic acid pathway which is important in the biosynthesis of aromatic amino acids (Moneke *et al.,* 2010). This pathway exists in higher plants and microorganisms but not in animals. By this mechanism, animals are believed not to be directly affected by glyphosate. However, the environmental consequences of the widespread use of the herbicide have been reported (Rissoli *et al*.,2016). The use of glyphosate in arable areas causes die-back in hedgerow trees. Glyphosate has as well been reported to inhibit the formation of

nitrogen fixing nodules on Clover for 120 days after treatment. The environmental exposure to glyphosate is extensive due to the vast quantities used annually all over the world. Exposure could occur from direct application, accidental release or spray drift and its release affects soil microbial population (Mayeetreye *et al.,* 2013). Monsanto claims that once glyphosate is introduced into the environment, it is inactivated through adsorption to soil or sediment particles and is rapidly degraded by microbial activity. The residues can accumulate in drinking and ground water causing threat to human and animal lives (Rendon-von and Dzul-caamal, 2017). Indeed, glyphosate on application adsorbs to clay particles and remain unchanged. However its elimination depends on some factors: size and activity of microbial population, soil structure, its adsorption ability, climate conditions, depth of motility in vertical soil profile, etc. (Shuskova *et al*., 2004). Similarly, the degradation product depends on the pathway for the degradation as microbial degradation of Glyphosate occurs in two forms: one route through the formation of aminomethgyl phosphoric acid (AMPA) and through the formation of glycine (Jacob *et al*, 1988). Amino methylphosporic acid (AMPA) has been found to be persistent in the soil more than glyphosate itself with half-life of between 119 and 958 days while glyphosate is immobilized in the soil (Monsanto, 1998) due to its adsorption to clay particles, the surface runoff can nonetheless wash the particles together with glyphosate attached to it into the surface water where it affects the aquatic ecosystem and lives like fishes, snails, plants etc. and as well endangers the lives of unsuspecting individuals that use the water as their domestic and drinking water source (WHO, 1994). Studies indicated that the shikimic acid pathway which glyphosate affects directly, is present only in higher plants and microorganisms, however, toxicity effects of glyphosate on fishes have been reported (Ayoola, 2008; WHO, 1994). Glysphosate on its own may be harmless to humans and animals, but its formulation component such as polyoxyethlene amine (POEA) is more toxic than glyphosate alone (Atkison, 1985). Many toxicological effects of glysphosate on human and laboratory animals have also been reported (Pesticides Trust, 1996; Roy et *al.,* 2016). According to the reports of Cox (1995), Glyphosate readily is broken down by water or sunlight once in water. Its presence in food has also been reported. European Union (EU) has set a limit for any pesticide in drinking water to be 0.1 mg/1 while WHO set the acceptable daily intake of glyphosate in food at 0.3 mg/day for an average of 60kg man (David and Topsy, 2001). In Nigeria, though glyphosate herbicide utilization is widespread but its intake by human and animals is not monitored. It is imperative to investigate the toxicity of glyphosate used in this location and as well as investigate more economical means of removing the herbicide from the environment.

The removal of glyphosate from contaminated environment is by microbial processes as chemical process is ineffective because of the presence of direct C-P bond which is highly resistant to physicochemical effect (Shuskova, 2004). Microorganisms known for their ability to degrade glyphosate in the soil and water include *Pseudomonas sp* strain LBr (Jacob *et al*., 1988), *Pseudomonas fluorescens* (Zboinska *et al.*, 1992), *Arthrobacter atrocyaneus*. Strain kg 16, and

Ochrobactrum anthropi strain GPK3 (Shuskova *et al*., 2004). According to Moneke *et al*., (2010), among all the bacterial strains tested for glyphosate degradation, *Pseudomonas fluorescens* proved to be most effective. Nevertheless, other ubiquitous bacterial strains with high nutritional versatility, enzyme production and high tolerance to environmental changes are still available for exploitation. Many organisms have been reported to utilize glyphosates but there are few reports on the utilization of glyphosate by plant growth promoting bacteria from the rhizosphere maize. Hence this study is designed to evaluate the ability of plant growth promoting bacteria to tolerate and utilize glyphosate as a means of removing it from the soil.

Materials and Methods

Microorganisms and culture condition

Twelve plant growth promoting bacteria initially identified as *Micrococcus* sp., *Pseudomonas* sp. *P. fluorescence, Bacillus cereus*., *Alcaligenes feacalis, Micrococcus* sp., *Proteus mirabilis*, *P. aeruginosa*, *Citrobacter freundii*, *Pseudomonas* sp., *Bacillus* sp isolated from rhizosphere soil of yellow and white maize were used for this work. The isolates were maintained on nutrient agar slants at refrigerating temperature of 4 °C.

Herbicide

The herbicide commonly known as Forceup manufactured by Zhejiang Xinan Chem Group Co. Ltd which contains 360 g active glyphosate per litre was purchased from Jubaili Agrotec Company, Ibadan.

Tolerance of the isolates to different concentrations of herbicides

An aliquot of 2.5 ml of inoculum of the isolates with multiple growth promoting abilities were transferred to 250 ml Erlenmeyer flask containing 50 ml of nutrient broth amended with glyphosate to give final concentrations of 7.2, 50 and 100 µg/ml. This is to evaluate the effect of the recommended dose of 7.2 mg/ml by Moneke *et al*. (2010) and other higher concentrations on the growth of the isolates. All experiments were carried out in triplicates. The incubation were carried out on a rotary shaker (Gallenkamp) at 180 rev/min and 30°C for 96 h. Culture samples were taken at 24 h intervals during the incubation period and analyzed for optical density at 600 nm with a spectrophotometer. Control experiments were set up in parallel at the same time with inoculated flasks without herbicides amendment.

Selection of isolates for further studies: Further selection of five (*B. mojavensis* (MY4)*, P. aeruginosa* (MW18)*, A. faecalis* (MY19), *P. syringae* (MY20) and *B. cereus* (MY25)) out of the twelve isolates for utilization studies was based on their response to different concentrations of glyphosates. The selected five isolates showed higher growth in the presence of different concentrations of glyphosate.

Inoculum preparation and standardization for utilization studies

Preparation and standardization of inoculum was done to ensure equal cell density of the isolates. Inocula for the study were prepared by inoculating isolates into nutrient broth and incubated at 30°C for 24 h.

Using sterile normal saline, the cells from the above cultures were re-suspended to a 0.5 McFarland nephelometer standard (optical density of 0.17 at 660 nm). This comparison was made easy by viewing the tube against a sheet of white paper on which sharp black lines were drawn. The turbidity standard was agitated on a vortex mixer immediately prior to use. The turbidity of the bacterial suspension was adjusted to the proper density (0.5 McFarland turbidity standards) by adding sterile normal saline or adding more bacterial cells.

Utilization of glyphosate as carbon, phosphorus source or both

The screening medium (150 ml) was prepared according to Moneke *et al*., (2010). Three (3.0) ml filter-sterilized concentrate (containing 7.2 mg/ml of glyphosate) was added as phosphorus or carbon source or both. The medium was inoculated with the isolates and incubated at 30°C for 72 h on a shaker at 120 rpm. Five milliliters (5 ml) of the culture medium were collected from each flask at 24 h intervals and assayed for growth by measuring the optical density at 660 nm using a spectrometer. The cell biomass was measured at the end of the experiment. Increase in turbidity as well as bacteria cell biomass indicates the level of utilization by the bacteria strain.

Data analysis

Data were subjected to analysis of variance (ANOVA) and significant means were separated using Duncan Multiple Range Test (DMRT) at 5% level of probability.

Results

Tolerance of the Selected Isolates to different concentrations of glyphosate

The selected isolates were tested for their ability to tolerate different concentrations (7.2, 50 and 100) of glyphosate. The results of the growth responses of the isolates were presented (fig.1 to 3). The results showed a progressive increase in the growth (optical density) of the isolates from 0h to 24h except few isolates whose growth declined at 72h of incubation. As the concentration of glyphosate increased, there was a corresponding decrease in the growth (optical density) of the isolates. The highest growth was observed at the concentration of 7.2 mg/ml. At this concentration, *Pseudomonas aeruginosa* (MW18) and *Bacillus cereus* (MY25) recorded the highest growth while the least growth was recorded by *Citrobacter freundii* (MY15). The growth of *Bacillus mojavensis* (MY4) declined after 48h of incubation. There was also a steady growth of the isolates at the concentration of 100mg/ml with *Micrococcus* sp. (MY2) and *Citrobacter freundii* (MY15) having the highest and least growth, respectively. The comparative effect of different concentration on the growth of the isolates is presented in fig.4.

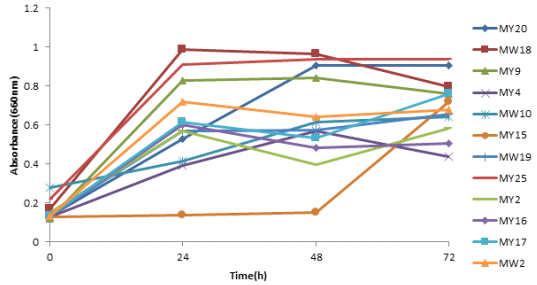

Fig 1: Growth of the twelve isolates on MSM amended with 7.2 mg/ml of glyphosate

**MY2 - *Micrococcus* sp., MY20 - *Pseudomonas* sp. MY9 - *P. fluorescence,* MY25 - *Bacillus cereus*.,
MY19 - *Alcaligenes feacalis.,* MY17 - *Micrococcus* sp, MW10 - *Proteus mirabilis*
MW18 - *P. aeruginosa*, MY15 - *Citrobacter sp*,
MY16 - *Pseudomonas* sp. MY4 - *Bacillus* sp

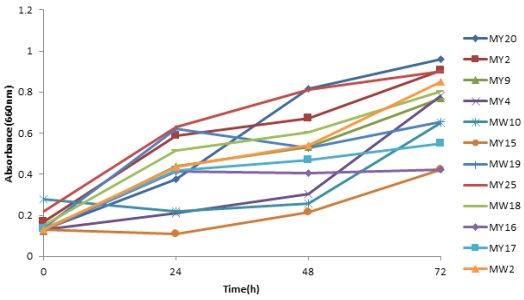

Fig 2: Growth of the twelve Isolates on MSM amended with 50mg/ml of glyphosate

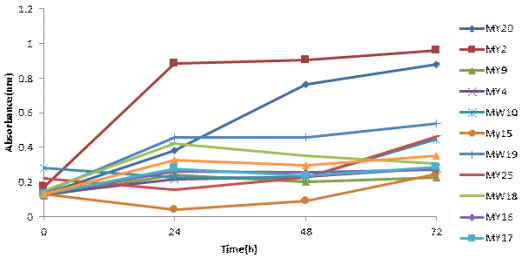

Fig 3: Growth of the Twelve Isolates on MSM Amended with 100 mg/ml of Glyphosate

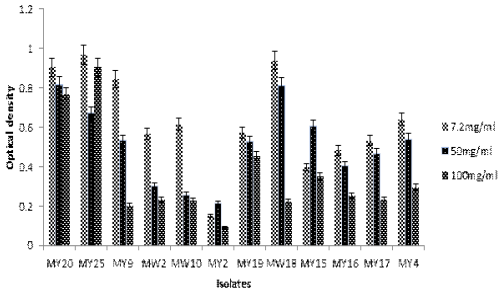

Fig 4: Comparative Effects of Different Concentrations on the Growths of the Twelve Isolates after 72 h of Incubation.

Key: MY2 - *Micrococcus* sp, MY20 - *Pseudomonas* sp. MY9 - *P. fluorescence,* MY25 - *Bacillus cereus*, MW19 - *Alcaligenes feacalis,* MY17 - *Micrococcus* sp, MW10 - *Proteus sp.* MW18 - *P. aeruginosa*, MY15 - *Citrobacter*, MY16 - *Pseudomonas* sp. MY4 - *Bacillus* sp. MW2 -*Pseudomonas sp.*

Utilization of Glyphosate as phosphorus, carbon source or both

Five isolates identified as *Bacillus mojavensis* (MY4), *Pseudomonas aeruginosa* (MW18), *Alcaligenes feacalis* (MY19), *Pseudomonas syringae* (MY20) and *Bacillus cereus* (MY25) were selected from the twelve isolates with multiple abilities based on their growth response to different concentrations of glyphosate, indole acetic acid (IAA) production and level of potassium and phosphorus solubilization index. The five bacterial isolates were screened for glyphosate utilization by measuring their growth turbidimetrically at 660 nm. Of the five bacterial isolates grown on the medium containing glyphosate as sole phosphorus source, *Bacillus cereus* significantly ($P < 0.05$) utilized glyphosate (mean OD 0.66). This was followed by *Pseudomonas aeruginosa*, *Alcaligenes feacalis and Pseudomonas syringae*. (mean OD 0.545,

0.31 and 0.10, respectively), *and least growth was recorded by Bacillus subtilis as shown in fig 6.The result of the utilization of glyphosate as carbon source showed that Pseudomonas aeruginosa* (*MW18) had highest utilization ability with* mean optical density and cell biomass of 0.79 and 0.039 mg / 20 ml, respectively while *Bacillus mojavensis* showed the least growth (Mean OD 0.14, cell biomass-0.011 mg / 20 ml). All the isolates showed ability to utilize glyphosate as both carbon and phosphorus source with *B. cereus* showing the highest ability to utilize glyphosate as phosphorus + carbon source and least by *B. mojavensis*. The results of the utilization of glyphosate as phosphorus, carbon source or both is presented in figs 5, 6 and 7 while the results of the cell biomass of the selected isolates is presented in Table 1.

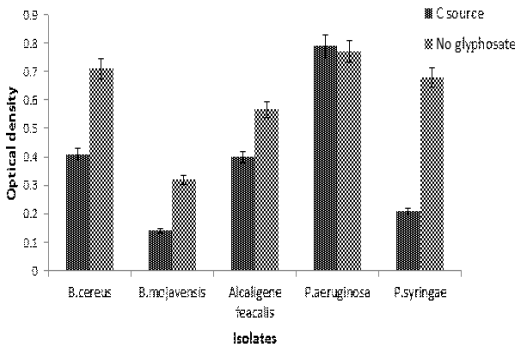

Fig 5: Utilization of Glyphosate as C-Source by the Five Selected Isolates

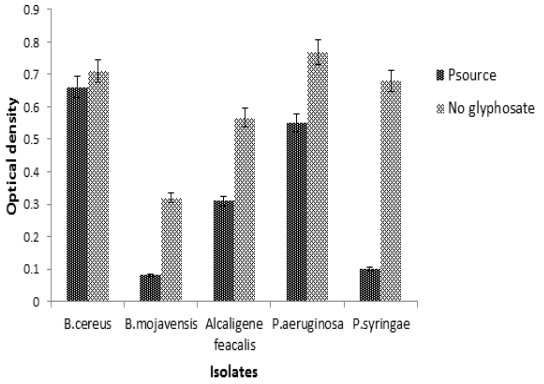

Fig 6: Utilization of Glyphosate as P-source by the Isolates

Discussion

The bacterial isolates from the rhizosphere of maize showed the ability to utilize glyphosate as source of nutrient. The result of the study showed a decrease in the number of bacteria species grown on the glyphosate amended solid media, this was in line with the report of Moneke *et al*.(2010) who reported a reduction in the number of bacterial species grown on

Table 1: Biomass of the Selected Strains in a Liquid MSM Amended with Glyphosate as P, C and P/C-Source

	Mean Biomass (mg/20 ml)			
Organism	**Control**	**P-source**	**C-source**	**P/C-source**
Bacillus mojavensis (MY04)	0.020	0.017	0.011	0.01
Pseudomonas syringae (MY20)	0.029	0.022	0.017	0.012
Alcaligenes feacalis (MY19)	0.038	0.025	0.028	0.022
Pseudomonas aeruginosa (MW18)	0.062	0.030	0.039	0.03
Bacillus cereus (MY25)	0.06	0.041	0.033	0.04

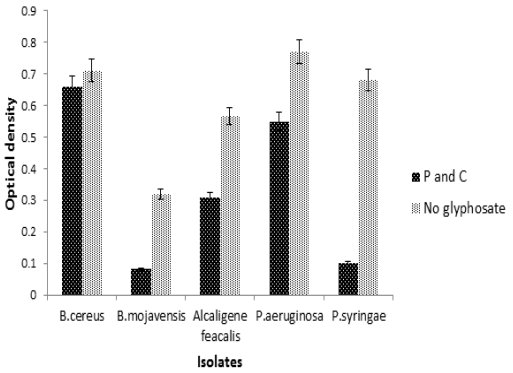

Fig 7: Utilization of Glyphosate as P+C-Source by the Isolates

glyphosate solid medium and it was also consistent with the report of Bussel *et al*.(2001) who showed that culturable bacteria and fungi are usually reduced in number or eliminated when extracted from soil or grown on the solid media containing glyphosate. These twelve isolates were tested for tolerance to different concentration (7.2, 50 and 100 mg/ml) of glyphosate, they showed varying degrees of tolerance to different concentrations of glyphosate. The differences observed in the growth of the isolates in the medium are indicative of the differences between the organisms in tolerating glyphosate. The results of this study showed a decrease in the growth of the isolates with an increase in the concentration of glyphosate. Our result agrees with the report of Moneke *et al*. (2010) who reported that an increase in glyphosate concentration led to a concomitant decrease in the growth of the isolates. This is in contrast with the report of Amoroso *et al*. (2007) who, while studying the effect of glyphosate at the concentrations of 50 and 100 mg/l observed an increase in Aeromonas counts in contrast with the control which contained no glyphosate. However, Some studies showed that glyphosate can either inhibit or stimulate soil microorganisms depending on the soil type or concentration of herbicide (Carlisle and Trevor (1988), Mayeetreyee *et al*.,2013). Although there was a decrease at higher concentration (100 mg/ml) the bacteria were still able to survive at such concentration, this may be as a result of the presence of degredative system that stabilizes the organism at such adverse condition (Moneke *et al.*2010).

The further selection of PGPB isolates identified as *Bacillus subtilis* (MY4),*Pseudomonas aeruginosa* (MW18), *Alcaligenes feacalis* (MY19), *Pseudomonas syringae* (MY20) and *Bacillus cereus*(MY25) for utilization studies were based on their significant level of tolerance of glyphosate at different concentrations and plant growth promoting abilities. Of the five bacterial isolates grown on the medium containing glyphosate as sole phosphorus source, *Bacillus cereus* significantly ($P < 0.05$) utilized glyphosate (mean OD 0.66). This was followed by *Pseudomonas aeruginosa*, *Alcaligenes* feacalis *Pseudomonas syringae* (mean OD 0.545, 0.31 and 0.10, respectively) and least growth was recorded by *Bacillus subtilis*. The result of the utilization of glyphosate as carbon source showed Pseudomonas aeruginosa having the highest utilization ability with no significant difference *(*$P < 0.05$) in the growth of the isolate (mean OD 0.79) and the control(mean OD 0.77) while *Bacillus subtilis* showed the least growth (mean OD0.14). These isolates were able to utilize glyphosate as carbon, phosphorus or both sources at different capacity however, the bacterial cell biomass was higher in the control when compared with that obtained when glyphosate was used as source of P or C, and this was in agreement with the finding of Kryuchkova

et al., (2014) who reported a decrease in cell biomass in a medium containing glyphosate when compared with control. Though, the results showed no significant difference in the utilization of glyphosate as P-source when compared to its utilization as C-source, the comparative role of glyphosate as carbon or phosphorus source revealed that glyphosate is better source of carbon than phosphorus source for all the isolates except *B. cereus* which utilized glyphosate as a better source of phosphorus than carbon. *P. aeruginosa* and *Bacillus cereus* showed greater efficiency for glyphosate utilization as source of phosphorus and carbon when compared to other isolates. The results showed no significant difference in the utilization of glyphosate as P-source when compared to its utilization as C-source. This was at variance with the result of Moneke *et al*. (2010) who reported glyphosate as better phosphorus source than carbon source for *Acetobacter* sp and *Pseudomonas fluorescence*. Many bacterial isolates have been reported to utilize glyphosate as phosphorus source (Motharasan *et al*., 2017). There have been several reports on the ability of microorganisms including some Pseudomonas species to effectively utilize glyphosate by naturally synthesizing appropriate enzymes or as results of genetic mutations (Jacob *et al*., 1988). The utilization of glyphosate by these isolates might be due to their previous contact with glyphosate in the soil from where they were isolated. It is also possible that the isolates have undergone mutation leading to the adaptability of the organisms to their microenvironment.

Conclusion

Excessive and indiscriminate use of herbicide in farming practices may cause the persistence of the chemical in the environment thereby affecting non-target and beneficial organisms. This study have identified five plant growth glyphosate tolerant bacteria namely *Bacillus subtilis* DMS10, *Pseudomonas aeruginosa* ZSL-2, *Alcaligenes feacalis* strain P156, *Pseudomonas syringae pv. Syringae* HS191, and *Bacillus cereus* 20UMPNR. These isolates successfully utilized glyphosate as carbon and phosphorus source. The ability of some of the isolates to exhibit plant growth promoting characteristics and utilize glyphosate effectively provides means of removing the compound from the environment as well enhance soil fertility. Thus, the ability of the isolates to withstand high concentration of glyphosate makes them very useful in bioremediation of glyphosate polluted soil.

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