Microbiology Research Journal International

26(6): 1-11, 2018; Article no.MRJI.47829
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Glyphosate Degradation by Two Plant Growth Promoting Bacteria (PGPB) Isolated from Rhizosphere of Maize

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2018/v26i630081
Editor(s):
(1) Dr. Laleh Naraghi, Plant Disease Research Department, Iranian Research Institute of Plant Protection, Iran.
Reviewers:
(1) Dr. V. Vasanthabharathi, Annamalai University, Tamil Nadu, India.
(2) Samia Mezaache-Aichour, UFA Sétif 1, Algeria.
Complete Peer review History: http://www.sdiarticle3.com/review-history/47829

Original Research Article

Received 18 November 2018
Accepted 02 March 2019
Published 12 March 2019

ABSTRACT

This study was aimed at evaluating the possible utilization of glyphosate tolerant plant growth promoting bacteria (*Pseudomonas aeruginosa* and *Bacillus cereus*) for bioremediation of glyphosate polluted soil. The soil samples were spiked with 3.1 mg/ml, 7.2 mg/ml and 14.4 mg/ml of glyphosate and then inoculated with *Pseudomonas aeruginosa* and *Bacillus cereus,* level of glyphosate pollution before and after inoculation with the bacteria were determined using Gas Chromatography-Mass Spectroscopy (GC-MS) after extraction with acetonitrile. The bacteria showed significant ability to degrade glyphosate. *Pseudomonas aeruginosa, Bacillus cereus*, their mixed culture and control recorded percentage degradation of 76.11, 85.8, 75.8 and 49%, respectively at 3.1 mg/ml of glyphosate while At the concentration of 7.2 mg/ml, the percentage degradation by *P. aeruginosa, Bacillus cereus*, mixed culture of the isolates and control was 84.9, 72.7, 66.4% and 39.2%, respectively. The isolates also showed significant rate of degradation at

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the concentration of 14.4 mg/ml. The GC-MS results showed a significant variation in the degradation products obtained when compared with control. This study revealed that substantial amount of glyphosate was degraded by *P. aeruginosa and Bacillus cereus*. Hence, they may have great potential in bioremediation of glyphosate polluted soil.

Keywords: Bioremediation; glyphosate; concentrations; PGPB.

1. INTRODUCTION

Soil is one of the most important natural resource on which lives of all plants, animals and microorganisms directly or indirectly depend on. In soil, different microorganisms thrive on nutrients therein and through various interactions play a pivotal role in cycling of nutrients and pedogenesis [1]. Alteration or disturbance in soil ecosystem by added pollutants leads to substantial changes in functional activities of these important soil microorganisms [2]. The excessive use of glyphosate to control weed contributes in altering the natural environment due to the pollution of the environment by this persistent chemical. The mode of action of glyphosate involves 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 [3]. This pathway exists in higher plant and microorganisms but not in animals [4]. 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 [5]. Several factors can affect the elimination of glyphosate in the environment, these factors includes 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). 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 [4]. Glyphosate alters natural ecosystem by altering different components of soil microbial community [6], it inhibits the growth and decreases the activities of soil organisms [7]. The main way of glyphosate degradation is by degradation by enzyme system of some microorganism Strange-Hansen et al. 2004. The utilization of plant growth promoting bacteria for biodegradation of glyphosate will not only reclaim the polluted soil but can as well enhance the fertility of the soil. These organisms enhance plant growth promotion through solubilization of insoluble nutrients in the soil and production of essential plant phytohormones. Plant associated bacteria, such as endophytic bacteria and rhizospheric bacteria have been shown to contribute to biodegradation of toxic organic compounds in contaminated soil [8]. Less attention has recently been paid to bioremediation of contaminated soils with Plant growth promoting rhizobacteria(PGPR), Promotion of plant growth by bacteria is well documented [9] and PGPR have been successfully used to reduce plant stress in contaminated soils. Some microbial communities have the ability to sequester some pollutants and therefore may also be useful in bio remediating contaminated soils Hallberg and Johnson, 2005. Studies have shown that some PGPR can tolerate herbicides; therefore, this study is designed to assess the ability of PGPR to remediate herbicide polluted soil.

2. MATERIALS AND METHODS

2.1 Microorganisms and Culture Condition

Two glyphosate tolerant plant growth promoting bacteria were initially identified as *Pseudomonas aeruginosa strain* ZSL-2 and *Bacillus cereus strain* 20UPMNR .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^{o}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.

2.2 Sample Collection

Soil samples were collected from research farm of the Institute of Agricultural Research and Training Moor plantation Ibadan.

2.3 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.

2.4 Spiking of Soil with Different Concentrations of Glyphosate

The soil to be used was weighed, sieved and 5 kg of soil were filled in perforated plastic pots, the herbicide (force up) was mixed with water and spiked on the soil samples until it reached the final concentration of 3.1, 7.2 and 14.4 mg/ml. All the samples were thoroughly mixed with metal spatula. All treatments were laid in Complete Randomized Design (CRD) with three replicate.

2.5 Physicochemical Analysis Soil Sample for Screen House and Field Studies

The physicochemical analysis such as moisture contents, pH, temperature, cation exchange bases, phosphorus,% total nitrogen, % total carbon, sodium, magnesium, potassium, sulphate, chloride etc were determined

2.6 Preparation of Bacteria Inoculum and Inoculation of the Spiked Soil

The isolates were inoculated in 50ml conical flask containing 25ml of prepared and sterilized luria broth and incubated at 30^{o}C in an orbital incubator shaker for 24 h. After incubation, the cultures were centrifuged at 4000 rpm for 20 mins.The cells were harvested and washed with normal cell. In other to ensure equal cell Size, the cells were diluted to 0.5 Mcfarlands Standard to give approximate cell density of 1.5 X10^{8}cfu/ml and 100 ml of the bacterial suspension were inoculated on the soil already spiked with glyphosate. Un-inoculated soil were used as control

2.7 Collection of Soil Sample for Analysis of Initial and Residual Glyphosate

Soil samples were collected from each pot immediately after application of herbicides and at the end of the experiment to determine the initial and residual herbicide. Initial and final soil samples were also collected in the field for analyses of initial and residual so as to validate the screen house studies.

2.8 Extraction of Glyphosate from Soil Samples

The extraction of glyphosate from the soil samples were carried out by the method described by Frimpong et al. [10] with slight modification from the Ghana Standard Authority (GSA) Herbicide Residues Laboratory Protocols. Ten grams (10 g) of the sub-soil samples were weighed and transferred into 250 ml separating flasks. A 10 ml of acetonitrile was added and the corked flasks sonicated (Grant XUB 18UK) for 5 min. An additional 10 ml of acetonitrile was added, and the separating flasks closed tightly. The content of the flasks were placed on a horizontal mechanical shaker (Ika-Werke HS 501 Digital), and was set to shake continuously for 30 min at 300 mot/min, and allowed to stand for 10 min to sufficiently separate the phases or layers. The supernatants (organic layers) were carefully transferred into 50 ml centrifuge tubes for centrifugation (Thermo/CR3i Multifunction) at 3000 rpm for 5 min. A 10 ml aliquot of the supernatants (organic phases/top layers) equivalent to 5.0 g soil weight were pipetted and dried/passed over 5 g anhydrous sodium sulphates through a filter paper into 50 ml round-bottom flasks. Then, 5 ml of acetonitrile was used to rinse the salt into the round-bottom flasks. The concentrates were then adjusted to about 2 ml using the rotary film evaporator (Buchi Ratovapor R-210, USA) at 35°C, and made ready for the analysis.

3. GC-MS ANALYSIS

GC-MS analysis was carried out on GCMS-QP2010 PLUS SHIMADZU. The column used was Perkin Elmer Elite - 5 capillary column measuring 30m × 0.25mm with a film thickness of 0.25 mm composed of 95% Dimethyl polysiloxane. The carrier gas used was Helium at a flow rate of 0.5 ml/min. One microliter of sample injection volume was utilized. The inlet temperature was maintained at 250°C. The oven temperature was programmed initially at 80°C for 4 min, then increased to 200°C and then programmed to increase to 280°C at a rate of 20°C. Total run time was 35 min and the MS transfer line was maintained at a temperature of 200°C. The source temperature was maintained at 180°C. GCMS was analyzed using electron impact ionization at 70eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the NIST 2005 GC-MS library.

3.1 Determination of Percentage Degredation

The percentage degradation of each treatment and control was estimated by considering the

products containing the active ingredient present in the herbicide (glyphosate). The percentage degradation was calculated using the method of Adeyemi et al. 2009.

3.2 Field Experiment

The field experiment were carried out in a plot of land at the Institute of Agriculture Research and Training More Plantation Ibadan during 2017 farming seasons, with the experiment laid out in a complete randomized block design with three replicates and plot size of 2x3m. The experimental site is a plot of land with sandy loam soil, located at a latitude $7^{o}22.701^{1}$N and longitude $3^{o}50.308^{1}$E. It is in the rainforest ecological zone of South west, Nigeria.

3.3 Land Preparation and Application of Herbicides/Bacterial Inoculants for Field Studies

The land were cleared and tilled prior to application of glyphosate(force up). Six hundred (600 ml) of the water containing 3.1, 7.2 and 14.4 mg/ml of glyphosate corresponding to half, field application rate and twice the field application rate were spiked on each of the 2x3 m plot. The plots were then spiked with 350 ml of the bacteria suspension of *Pseudomonas aeruginosa strain* ZSL-2, *Bacillus cereus strain* 20UPMNR and mixed culture of both isolates and the unspiked plots were used as control.

The method of Frimpong et al. [10] and GC MS were used for extraction and analysis of residual herbicides as earlier stated. Initial and final soil samples were also collected in the field for analyses of initial and residual glyphosate so as to validate the screen house studies. The residual herbicides were determined using GC MS after extraction. The percentage degradation was evaluated as earlier stated.

3.4 Data Analysis

Data obtained were analyzed using descriptive statistics and analysis of variance (ANOVA)

4. RESULTS

4.1 Physicochemical Parameters of the Soil

The result of the physicochemical analysis of the soil sample from the field is presented on Table 32. The results showed range of parameters before and after planting as between: pH-5.91-6.19, Ca(cmo/kg)-1.73-2.54, Mg(cmo/kg)-0.76-1.49, K(cmo/kg)-0.14-0.22, % total carbon-0.67-1.01, % total nitrogen-0.04-0.07, % Organic matter-2.42, particle size-clay-13.4, sand-70.76 and silt-15.84 (Table 1).

Table 1. Physicochemical parameters of the soil before and after planting

Parameters	Value
PH	5.93
Ca (cmo/kg)	1.73
Mg(cmo/kg)	0.96
K(cmo/kg)	0.97
Na(cmo/kg)	0.28
H^{+}	0.11
Electrical conductivity (µs)	62.37
P(ppm)	13.24
%Total carbon	1.41
% Total nitrogen	0.14
% Organic matter	2.42
Cupper(PPM)	1.89
Fe(PPM)	129.56
Mn(PPM)	75.03
Sulphur(PPM)	10.32
Boron(PPM)	0.10
Zinc(PPM)	3.03
CEE(Cmo/kg)	4.06
% Base saturation	97.20
Particle size-Sand	70.76
Silt	15.84
Clay	13.4

Table 2. Initial and degredative product of glyphosate in the soil

Before degredation	Mol.weight	Formular	After degredation	Mol. weight	Formular
pyridine	93	C_6H_7N	2-Butylene Himidazole	122	$C_7H_{10}N_2$
6methyl -picolinic acid	137	$C_7H_2NO_2$	4-pentyl-2-tuylamine	123	$C_8H_{13}N$
N N-isophthaloylbis	404	$C_{22}HN_{206}$	2-Propanamine N-methyl ethylidene	99	$C_6H_{13}N$
Dodecane(1-chlorodecylchloride)	176	$C_{10}H_{21}Cl$	Pyrolidine	157	$C_8H_{15}NO_2$
1-chlorononyl chloride	176	$C_9H_{19}Cl$	2-Propanomine	99	$C_6H_{13}N$
Dodecanol	186	$C_{12}H_{26}O$	3-Azonia 5-hexene- 1-ol	173	$C_8H_{17}N_{202}$
Dodecene	168	$C_{12}H_{24}$	4-piperidinone	155	$C_9H_{17}NO$
Tridecene	154	$C_{13}H_{26}$	2-pentanamine	129	C_8H1_9N
Hexanoic acid	158	$C_9H_{18}0_2$	2,2,5,5 tetrmethyl 4 ethyl imidazole	172	$C_9H_2ON_2O$
Heptanoic acid	144	$C_8H_{16}O_2$	Pyrolidinone	99	C_5H_9NO
Bipyrine	156	$C_{10}H_8N_2$	Pyridazine	204	$C_9H_{12}N_6$
NN-Isopropyl N-4-butyl guanidine	240	C13H28N4	Silane	144	$C_{22}H_{45}Cl_3SI$
			2H Pyrol-2-one	97	C_5H_7NO

4.2 Biodegredation of Glyphosate on the Soil

The ability of the two isolates to degrade glyphosate at different concentration was tested. The percentage of glyphosate degraded in the soil by the isolates and their Chromatogram from GC-MS analysis at different concentrations are presented in Figs. 1 and 2.

Fig.1 shows the percentage degradation of 3.1 mg/ml glyphosate by the isolate. The result showed that *P. aeruginosa, Bacillus cereus*, mixed culture of the isolates and control recorded percentage degradation of 76.11, 85.8, 75.8 and 49%, respectively. The GC-MS results showed a significant variation in the degradation products obtained when compared with control (Table 2).

At the concentration of 7.2 mg/ml, the percentage degradation by *P. aeruginosa, Bacillus cereus*, mixed culture of the isolates and control was 84.9, 72.7, 66.4% and 39.2%, respectively (Fig. 2), whereas the percentage degradation by *P. aeruginosa, Bacillus cereus*, mixed culture of the isolates and control at the concentration of 14.4 mg/ml was 47.15, 57.26, 55.7 and 27.4%, respectively. The result of degradation at 14.4 mg/ml is presented in Fig. 3. The rate of degradation by the isolates decreased with increase in concentration of glyphosate except *P. aeruginosa* that showed higher rate of degradation at 7.2 mg/ml when compared to 3.1 mg/ml. There was also a significant variation in the degradation products obtained when compared with control. The results of degradation products also showed transformation or total breakdown of some of the product found in the initial samples when compared with products recovered at the end of the experiment.

5. DISCUSSION

Two glyphosate tolerant plant growth promoting rhizo bacteria namely, *P. aeruginosa* and *B. cereus*were used singly and combined to bio remediate glyphosate polluted soil. These isolate exhibited high ability to degrade glyphosate at different concentration*. B. cereus* recorded highest ability at the concentration of 3.1mg/ml and 14.4 mg/ml while *P. aeruginosa* showed highest ability at the concentration of 7.2 mg/ml, the least % degredation was recorded by the control in all the concentration. The rate of degredation was lower when the isolates are mixed than when used singly. This might be as a result of antagonistic interaction between the two isolates which may have interfered with their metabolic activities. The ability of the isolates to degrade glyphosate may be connected with its ability to utilize glyphosate as C and P source since the degredation of glyphosate involves the lysis of C-P bond. The two pathways for glyphosate degredation involves cleaveage AMPA and glyoxylate by the presence of glyphosate oxidoreductase where as in the other pathway, degredation is catalyzed by C-P lyase with the formation of sarcorsine which eventually forms formaldehyde and glycine [11]. The findings of this research is line with Haoyu et al. [12] who reported the degradation of glyphosate by Pseudomonas sp., Jacob et al. [13] isolated a Pseudomonas strain which completely metabolized 3.21 g/l glyphosate with a degrading efficiency of about 2 gGp/g dry biomass. Two bacteria strains of bacteria were reported to be efficient degraders of glyphosate, these isolates are Ochro bacterium anthropic [11] and Bacillus cereus CB4 [14]. These two isolates were reported to degrade glyphosate through the two pathways mentioned earlier. Lufti et al. [15] reported glyphosate degradation by two plant growth promoting bacteria *Enterobacter* sp and *Pseudomonas fluoresces*. The isolates used in this study have two functions as plant growth promoting bacteria and degradation of glyphosate herbicide. These characteristics are quite beneficial to humans where bacteria can help to reduce levels of glyphosate that has high persistence and poisonous to plant and beside has plant growth promoting properties that can increase crop yield [15]. Glyphosate degradation also depends on the adaptation of bacteria to herbicides, phosphate status in bacteria cell and bacteria culture growth phase (Kryuchkova et al. 2014, [16]) reported that Pseudomonas and Azospirilum are capable of detoxifying glyphosate because the undergo longer stationary phase and delayed phase of death. The report of our findings is also in line with the findings of Inna et al. [6] who reported that high degradation of glyphosate using degraders belonging mostly to the genera Pseudomonas, Arthrobacter and Alcaligenes isolated from different source including soil. The report of the percentage degradation by the mixed culture of the isolates agreed with findings of Romeo and Hendawi 2014 who reported higher efficiency in herbicide degradation by *A. lipoferum* when used singly (48.3%) than when combined with *B. polymyxa* (46.8%). Yu et al. 2015 reported 17.65-66.97% degradation of glyphosate in sterile soil and 19.01-71.57% in unsterilized soil using *Bacillus subtilis.* The effectiveness of

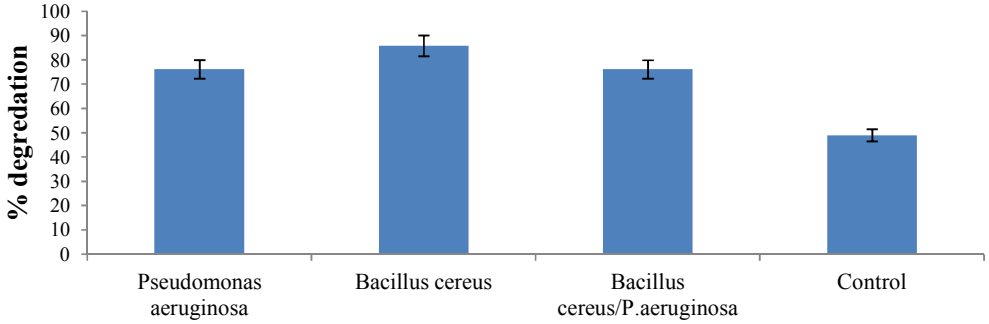

Fig. 1. Percentagedegredation of glyphosate by the isolates at concentration of 3.1 mg/g

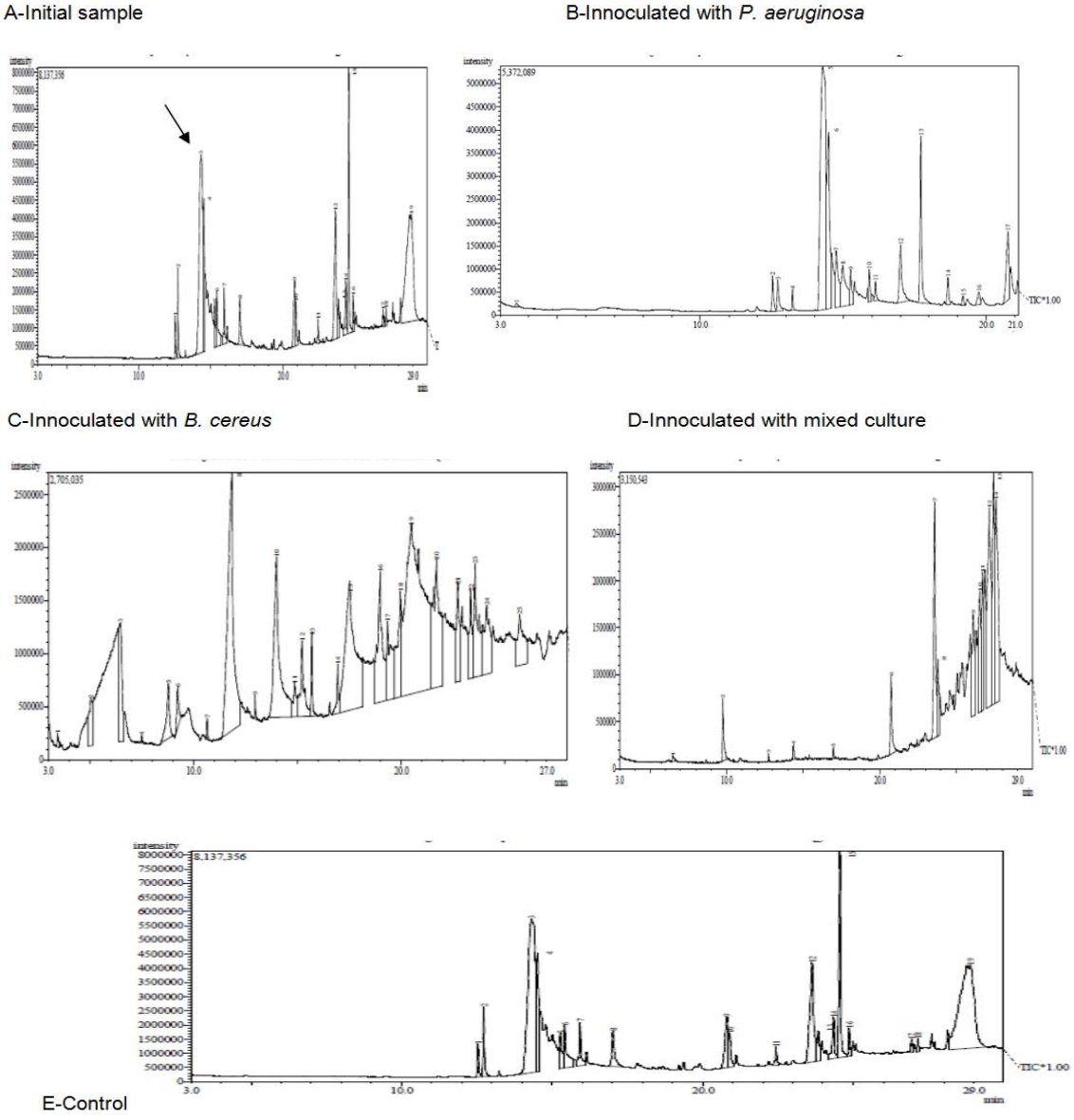

Fig. 2. Chromatogram of GCMS analysis of soil spiked with 3.1 mg/ml

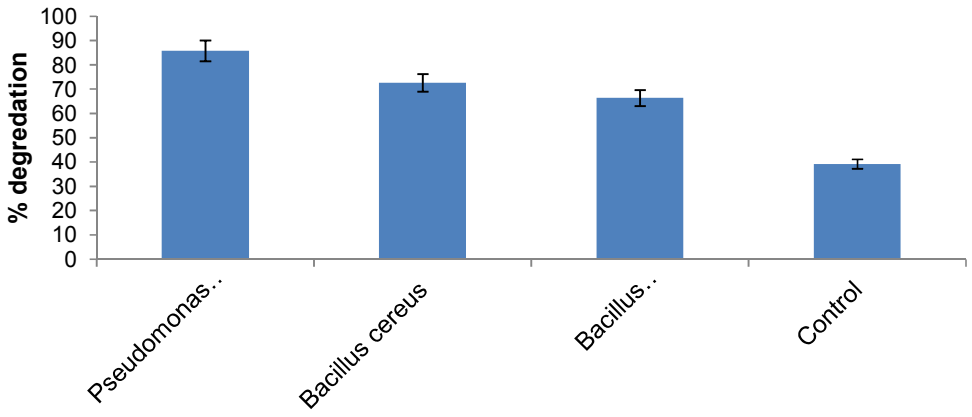

Fig. 3. Percentagedegredation of glyphosate by the isolates at concentration of 7.2 mg/g

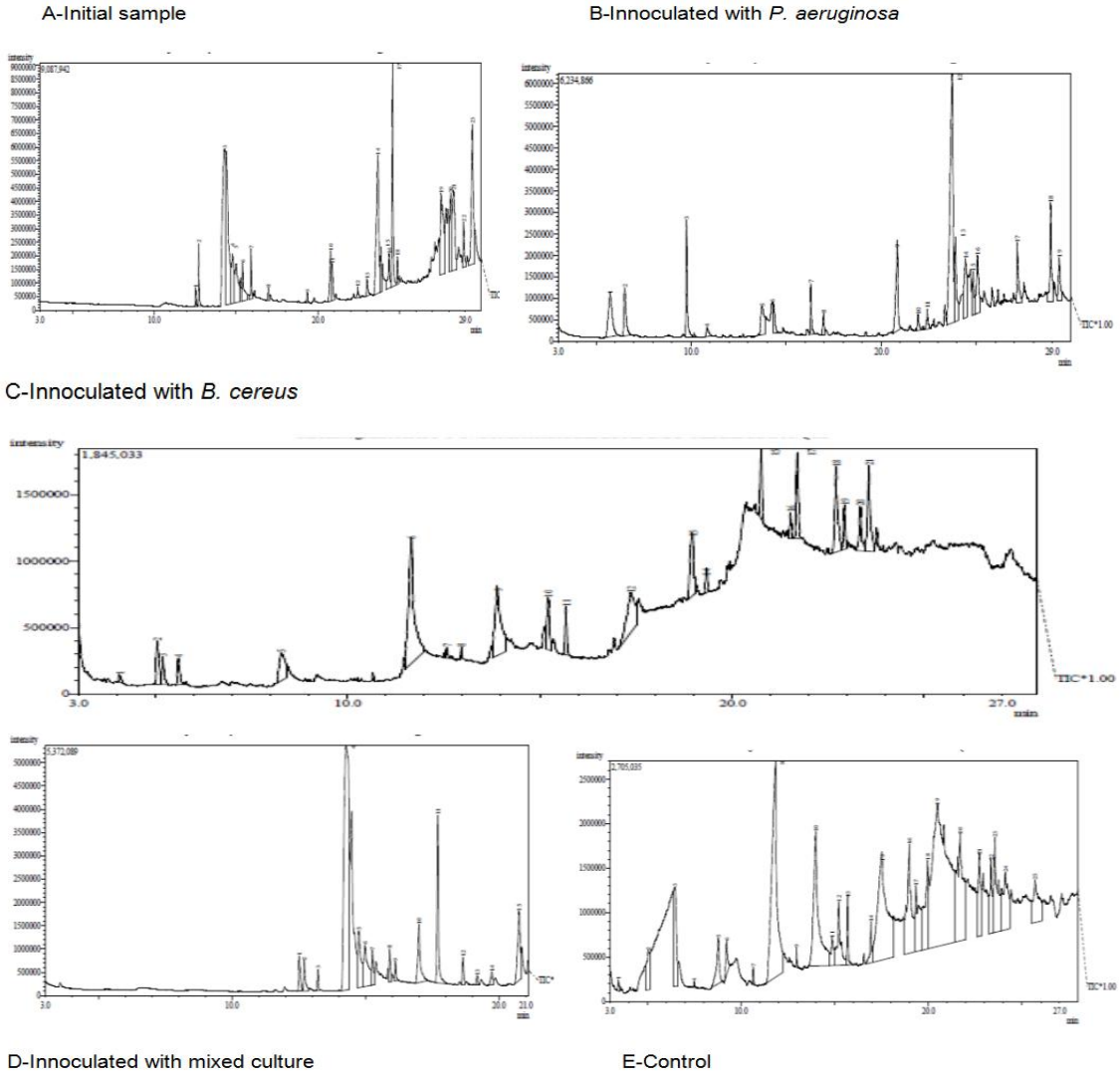

Fig. 4. Chromatogram of GCMS analysis of soil spiked with 7.2 mg/ml

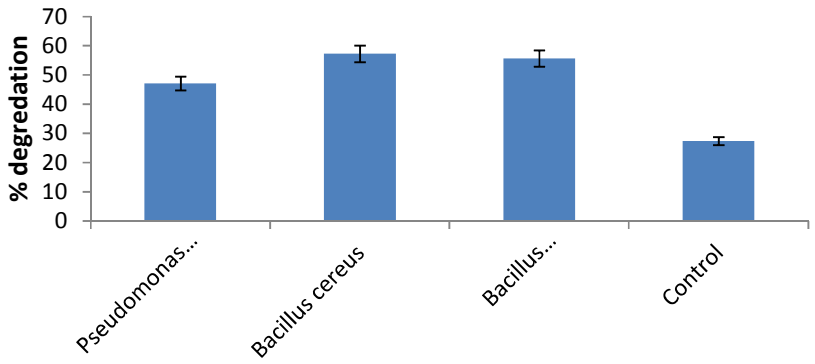

Fig. 5. Percentagedegredation of glyphosate by the isolates at concentration of 14.4 mg/g

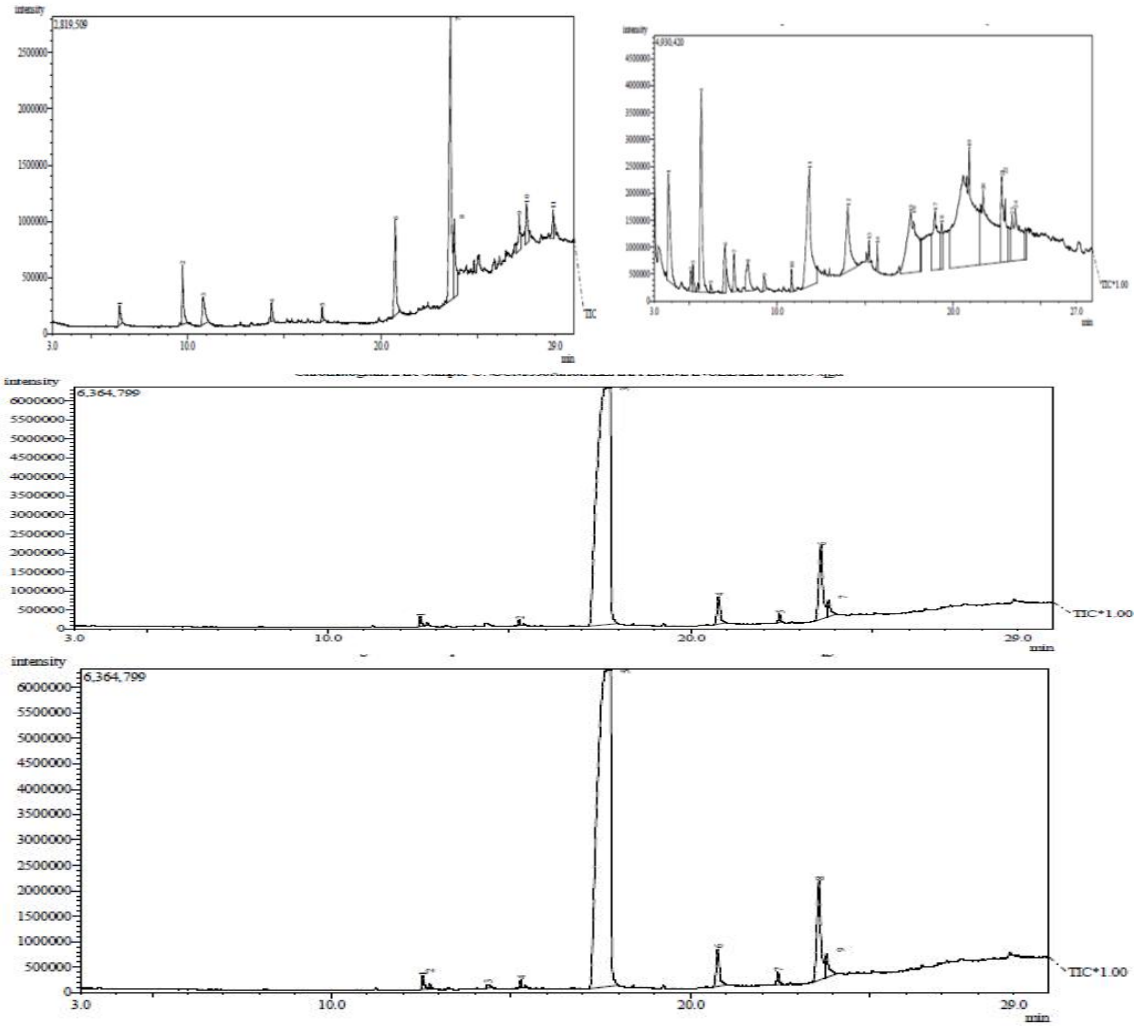

Fig. 6. Chromatogram of GCMS analysis of soil spiked with 14.4 mg/ml of glyphosate

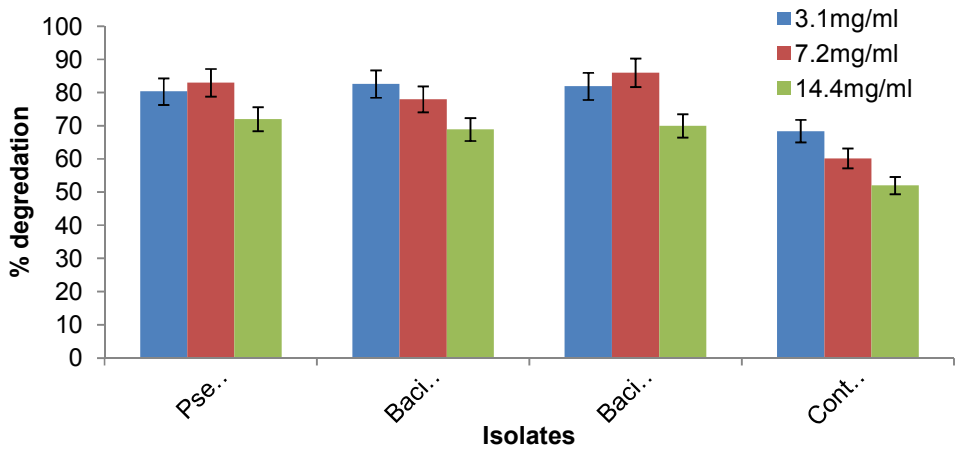

Fig. 7. Percentage degredation of glyphosate by the isolates at different concentration of glyphosate in the field

Bacillus sp., Citrobacter and *P. flourescens* to degrade glyphosate up to 50 mg/l concentration were also reported (Abubacker et al. 2016). The initial and final GC-MS analysis of the polluted soil showed transformation or total breakdown of the components of the herbicides. Most of the products of the initial samples were not found at the end of the experiment where as some new compound were seen at the end of the experiment were not in the initial sample. This is also in line with the findings of Abubacker et al. (2016) who reported the transformation of the components of glyphosate during its degradation at the end of his experiment. The transformation may be as a result of microbial action or plant enzymes. The simultaneous cleanup of herbicides using chemical and thermal methods are both technically difficult and expensive, these methods also destroys the biotic components of the soil. The utilization of plant growth promoting bacteria will enhance the biodegradation as well restore soil and biotic components [17].

6. CONCLUSSION

The result of this study showed that these isolates can be useful in the process of soil cleans up after glyphosate application to prevent its accumulation in the soil and the reduction of its toxicity. The results have also revealed the ability of the two isolates to effectively degrade glyphosate without accumulation of amino methyl phosphonic acid (AMPA) as a metabolic product. Hence this isolates can be employed in bioremediation of glyphosate polluted soil.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/47829