



Isolation and Characterization of Glyphosate–Degrading Bacteria from Agricultural Soil in Awka, Anambra State, Nigeria

Helen Obianuju Obiefuna*¹ and Samuel Chinedu Onuorah¹

¹Department of Applied Microbiology and Brewing, NnamdiAzikiwe University, Awka, Nigeria.

KEYWORDS

Glyphosate degradation
Pesticide
Bioremediation
Soil bacteria

ARTICLE HISTORY

Received 17 September 2022
Received in revised form
20 October 2022
Accepted 21 October 2022
Available online 22 October
2022

ABSTRACT

Glyphosate is a very effective pesticide; hence it has been excessively used in agricultural activities. This led to glyphosate residue accumulation in the soil and resulted in various adverse effects on humans and the ecosystem. This necessitates the need to reduce glyphosate accumulation in the soil and microbial bioremediation can be the solution. Thus, this study aimed to isolate and characterize glyphosate degrading bacteria from agricultural soils exposed to glyphosate weed control method. Bacterial organisms were isolated from glyphosate polluted agricultural soil and were characterized using routine microbiological biochemical methods. The isolates were screened for glyphosate utilization using a mineral salt medium containing glyphosate as a carbon source. Confirmed glyphosate degraders were identified using 16s rDNA sequencing. These isolates were assessed for their ability to degrade 106.16 µg/ml glyphosate in varied volumes (1 ml, 1.5 ml and 2 ml), singly and in consortium. Glyphosate degraders were identified as *Exiguobacterium alkaliphilum*, *Alcaligenes faecalis*, *Sinorhizobium fredii* and *Acinetobacter nosocomialis*. These organisms significantly ($p < 0.05$) degraded different glyphosate volumes as a consortium more than as individual organisms. This study has been able to show the different glyphosate degraders present in some glyphosate-polluted agricultural soils in Awka, Anambra State, Nigeria.

© 2022 The Authors. Published by Pentract Technology.

This is an open access article under the CC BY-NC 4.0 license (<https://creativecommons.org/licenses/by-nc/4.0/>).

1. INTRODUCTION

Pesticides are defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest or weed [1]. The excessive use of pesticides leads to its accumulation in the environment, thereby posing as a health hazard for the current and future generations due to uptake and accumulation of these toxic compounds in the food chain and drinking water [2]. Glyphosate (N-phosphono-methyl-glycine) is the most used herbicide worldwide [3]. It is a broad-spectrum, post-emergence non-selective herbicide that inhibits the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), blocking the synthesis of essential aromatic amino acids [4]. The intensive and excessive use of herbicide in agricultural and non-agricultural processes is a general practice and, thus,

a matter of environmental concern [5]. It has been negatively linked to human health due to its toxicity [6] and has been revealed to be associated with various health problems such as cancer, chronic kidney disease, hypothyroidism and birth defects [7, 8, 9].

On application, glyphosate remains unchanged in the soil for varying lengths of time, because of its absorption on clay particles and organic matter present in the soil [10]. The removal of glyphosate from the environment is commonly carried out using microbiological process, as chemical degradation is ineffective due to the presence of highly stable bonds (carbon-phosphorous bonds) in the compound [11, 12].

Many bacteria that are able to degrade glyphosate have been isolated from soil around the world [13]. They include: *Pseudomonas* sp. Strain LBr [14], *Pseudomonas fluorescence*

*Corresponding author:

E-mail address: Helen Obianuju Obiefuna <ujuokoye3@gmail.com >

2785-8901/ © 2022The Authors. Published by Pentract Technology.

This is an open access article under the CC BY-NC 4.0 license (<https://creativecommons.org/licenses/by-nc/4.0/>).

[15], *Achromobacter* sp. Strain kg16, *Ochrobactrum thropi* strain GPK3 [16], *Arthrobacter atrocyaneus* [17] and *Acetobacter* sp [5].

The aim of this present study was to isolate and characterize glyphosate-degrading bacteria from three glyphosate-contaminated agricultural soils in Awka, Nigeria, evaluate their degradation abilities at different concentrations of glyphosate and assess the growth response of the isolates. This study will add to the knowledge of the best bacteria species to be employed in the bioremediation of glyphosate contaminated soils.

2. MATERIALS AND METHODS

2.1 Chemicals and media

The isopropylamine salt of glyphosate known as Roundup (containing 450g active ingredient/L of glyphosate) was purchased from Agricultural Department Programme (ADP) Awka, Anambra State, Nigeria. Soil samples were collected from three different agricultural fields treated with glyphosate in Awka, Anambra State, Nigeria.

A modified Mineral Salts Medium (MSM) was used for the isolation of bacteria using glyphosate as a sole carbon and energy source. The MSM contained in gram per liter of distilled water: KH_2PO_4 (1.5), Na_2HPO_4 (0.6), NaCl (0.5), NH_4SO_4 (2), CaCl_2 (0.01), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.001).

2.2 Collection of soil samples

Glyphosate-contaminated soil samples were collected from three farms within Awka. Each farm was divided into 5 portions and triplicate soil cores were obtained from each portion from depth of 0-15cm. They were sieved and homogenized and immediately transferred to the laboratory using sterile transport polyethylene bags.

2.3 Isolation of glyphosate degrading strains

For the isolation of the bacteria from the contaminated soil. 5g of mixed soil sample was added to 95ml of MSM in 250ml conical flask and 1ml of glyphosate (Roundup®) was added, this was incubated at room temperature in a rotary shaker (150rpm) for 7 days. Serial dilution of the medium was prepared and 0.1ml was plated out on mineral salts medium containing agar and 1ml glyphosate. This was incubated for 3 days at 30°C. Morphologically distinct colonies were isolated and sub-cultured onto mineral salt agar MSA plates until pure isolates were obtained [18].

2.4 Characterization and identification of isolated bacterial strains.

The bacterial strains obtained were characterized and identified based on morphological and biochemical characteristics using the methods described by Cheesbrough [19], Fawole [20]. The biochemical tests carried out are: gram staining, oxidase, catalase, Voges-Proskauer, nitrate reduction, citrate, indole production, urease as well as sugar test. Further identification of the bacterial isolates was made via the 16S rDNA gene extraction, PCR amplification and DNA sequencing methods. The PCR amplification process was carried out with 12.5µl of one Taq Quick- Load 2x Master, Mix with standard Buffer (New England Bio Labs Inc.); 0.5µl

each of forward and reverse primers (27F: 5'-AGAGTTTGATCGTGGCTCAG -3' and 5'-1492RTACGGTTACCTTGTTACGACTT -3'); 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to thermal cycler. The thermal cycle consisted of Initial denaturation for 30sec at 94°C, followed by 35cycles of denaturation at 94°C for 20sec, primer annealing at 56°C for 1min. final extension at 68°C for 5min on an Eppendorf neux gradient Mastercycle (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with Ethidium bromide. Sequence chromatogram analysis was performed using FinchTV analysis software. [18]

2.5 Determination of bacterial biodegradation of glyphosate using GCMS analysis.

The bacterial glyphosate degradation ability was assessed using the gas chromatography – mass spectrophotometer (GC-MS) analysis of the residue from cultures that were grown for 14 days. Isolates and their consortium were inoculated into different test tubes containing 10ml of Nutrient broth and incubated for 24 h. A 0.1 ml aliquot of 10^{-2} dilution factor was collected from each culture tube and placed into different 250ml ErleneMeyer flasks containing the following mixtures: 99ml of MSM with 1ml (106.16µg/ml) of glyphosate; 98.5ml of MSM with 1.5ml of glyphosate; and 98ml of MSM with 2mls of glyphosate, individually. These flasks were incubated at 150rpm in a rotary shaker for 14 days at 30°C. At the end of the 14 days, the residual pesticides were determined using GC-MS according to AOAC (1990) [18].

2.6 Bacterial growth analysis

Bacterial growth in the mineral salt medium-glyphosate mixture was monitored by measuring the optical density (OD) using the spectrophotometer at 660 nm.

2.7 Statistical Analysis

Statistical data was analyzed using one- way Anova and mean partitioning was done using tukey test at 95% confidence interval. The statistical package for service solution (SPSS) software version 21 was used for the data analysis.

3. RESULTS AND DISCUSSIONS

3.1 Identification of bacterial strains

Morphological, biochemical characterization and molecular identification showed the isolates to be *Exiguobacterium alkaliphilum*, *Alcaligenes faecalis*, *Sinorhizobium fredii* and *Acinetobacter nosocomialis*. The biochemical test is shown in Table 1 and the phylogenetic mapping is shown in Fig 1-4. Firdous [24] reported the presence of *Alcaligenes* in glyphosate contaminated soil and this also agreed with the findings of the present study. Mussali-Gadante *eLebeau*, [21] also reported the presence of *Sinorhizobium* which corresponds with the findings in this study. *Acinetobacter* sp has been reported by Olawale et al, (2011) [21] as a glyphosate degrader while Moneke et al, (2010) [5] reported the presence of *Alcaligenes* sp. in glyphosate contaminated soil. Mussali et al. [22] also reported the presence of *Sinorhizobium* sp. *Exiguobacterium alkaliphilum* has been reported as a good crude oil and

beverage effluent degrader [22], however, this finding has also shown that this bacterium is a good glyphosate pesticide degrader. *Sinorhizobium fredii* is a known nitrogen fixing bacteria [23], thus, its ability to survive the presence of glyphosate, could be because of the fact that glyphosate pesticide is a nitrogen-based chemical. *Acinetobacter nosocomialis* is a well-known opportunistic pathogen that is usually implicated in acquired hospital infections [24]. This study, thus, B. Powleswn that *Acinetobacter nosocomialis* is not just a known hospital opportunistic pathogen, but also a glyphosate degrader.

3.2 Glyphosate residual analysis

Table 2 showed the ability of these glyphosate degraders to catabolize different concentrations of glyphosate in mineral salts medium, *in vitro*. All four bacterial isolates showed variations in their ability to degrade 1 ml, 1.5 ml, and 2 ml volumes of 106.16 ug/ml of glyphosate. There was significant (p<0.05) difference in their residual glyphosate concentrations, which showed that the tested bacterial organisms did not possess same capacity for glyphosate biodegradation. It was observed that all the isolates closely mineralized glyphosate up to 1.5 ml, while the consortium degraded the 2ml glyphosate better than the monoculture. *Acinetobacter nosocomialis* had the best degradation ability amongst the four isolates.

Table 1: Morphological and biochemical properties of the different bacteria isolates

Property	Isolate 1	Isolate2	Isolate3	Isolate4
Morphology	Rod shaped	Rod shaped	Rod shaped	Cocobacillus shaped
Motility	+	+	+	-
Biochemical tests				
Gram stain	-	+	-	-
Oxides	+	-	+	+
Catalase	+	+	+	+
Citrate	+	+	-	-
Urease	-	+	-	-
Voges-proskauer	-	-	-	-
Indole production	-	-	-	-
Gelatine hydrolysis	-	+	-	-
Nitrate reduction	-	+	+	-
Methyl red	-	+	-	-
Spore	-	-	-	-
Sugar use				
Glucose	-	+	-	+
Sucrose	-	+	+	-
Lactose	+	+	-	+
Maltose	-	+	+	+
Mannose	-	-	-	+
Xylose	-	-	-	+
SuspectedOrganism	<i>Alcaligenes sp</i>	<i>Bacillus sp</i>	<i>Rhizobium sp</i>	<i>Acinetobacter sp</i>

(+) positive reaction, (-) negative reaction

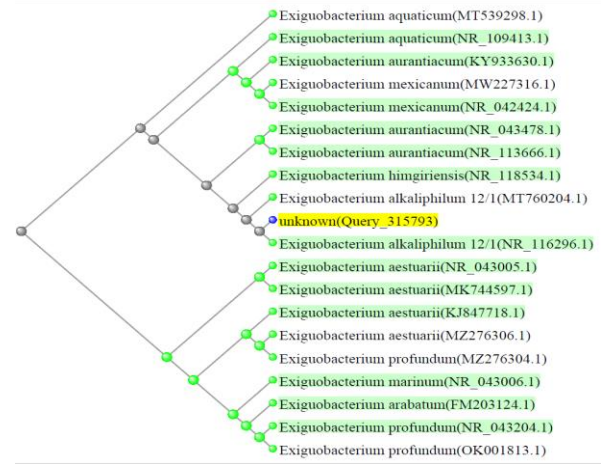


Fig. 1. Neighbour joining tree based on 16SrDNA sequences for Exiguobacterium alkaliphilum

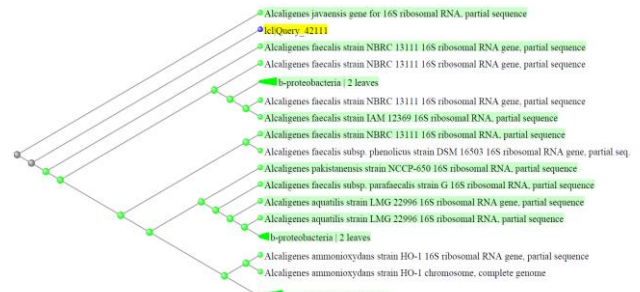


Fig. 2. Neighbour joining tree based on 16SrDNA sequences for Alcaligenes faecalis.

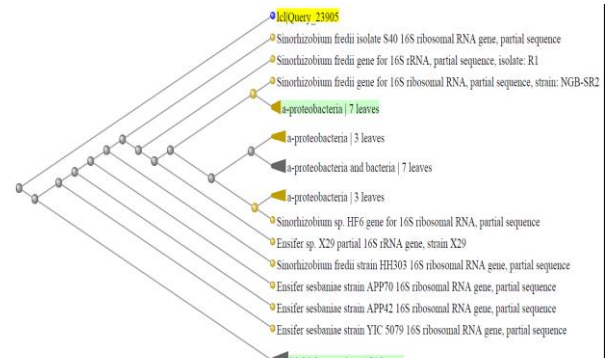


Fig. 3. Neighbour joining tree based on 16SrDNA sequences for Sinorhizobium fredii

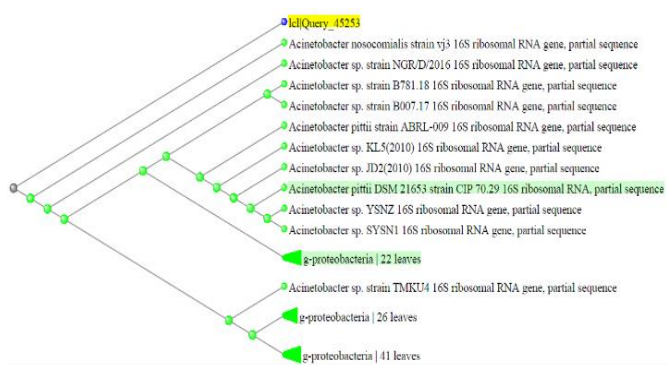


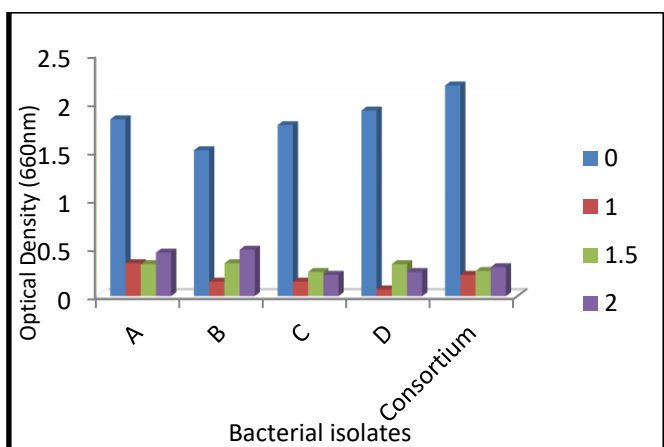
Fig. 4. Neighbour joining tree based on 16SrDNA sequences for *Acinetobacter nosocomialis*

Table 2. GC-MS result for Glyphosate degradation by the isolates

Isolates	Residual Glyphosate (µg/ml) 1ml vol	Residual Glyphosate (µg/ml) 1.5ml vol	Residual Glyphosate (µg/ml) 2ml vol
<i>Exiguobacterium alkaliphilum</i>	2.81±0.01 ^c	3.92±0.01 ^d	28.84±0.01 ^e
<i>Alcaligenes faecalis</i>	2.61±0.01 ^a	5.23±0.01 ^e	22.09±0.01 ^d
<i>Sinorhizobium fredii</i>	3.38±0.01 ^e	2.88±0.01 ^c	18.31±0.01 ^c
<i>Acinetobacter nosocomialis</i>	2.99±0.01 ^d	1.88±0.01 ^a	16.86±0.01 ^b
Consortium	2.57±0.01 ^b	2.75±0.01 ^b	10.42±0.01 ^a

Mean values along same column with different affixes are significantly different (p<0.05).

*Glyphosate concentration in Roundup = 106.16 ug/ml



A: *Exiguobacteriumalkaliphilum*
 B: *Alcaligenesfaecalis*
 C: *Sinorhizobiumfredii*
 D: *Acinetobacternosocomialis*

Fig. 5. Bacterial Growth at Different Concentrations of Glyphosate

3.3 Bacterial growth analysis in mineral salts medium

Mineral salts medium without glyphosate incorporation gave a notable microbial growth for all the isolates and their consortium, however, variations were noted amongst the isolates and their consortium with incorporation of different concentrations of glyphosate in the mineral salt’s medium, as shown in Fig.5.

It was observed that all the degraders had luxuriant growth in mineral salts medium without the incorporation of glyphosate (Fig.5). However, upon glyphosate incorporation, *Exiguobacterium* and *Alcaligenes* experienced growth with increase in glyphosate concentration, while the reverse was the case for *Sinorhizobium* and *Acinetobacter*. This corroborates the report of Singh *et al.* (2020) that *Exiguobacterium* (formerly a *Bacillus* member) and *Alcaligenes* are good pesticide utilizers but this study showed that though they had a luxuriant growth at high concentration they did not degrade the herbicide more than their counterparts that had a slower growth.

4. CONCLUSION

This study reports the isolation and identification of four bacterial isolates with the abilities to degrade glyphosate, two of which have been formerly proven to be good glyphosate degraders and two which has been confirmed by this study to have the abilities to degrade glyphosate and at higher concentrations. of glyphosate. The application of these bacteria can be used to remediate pesticide- contaminated soil.

ACKNOWLEDGEMENT

The authors acknowledge the research assistance obtained from the Microbiology Laboratory of Nnamdi Azikiwe University Awka, Nigeria.

REFERENCES

- [1]. TheR. Coup, S. Kalkhoff, P. Capel, and C. Gregoire, “Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe”, pp.5877, 2012.
- [2]. A.K. Olawale, and O.K. Akintobi, Biodegradation of glyphosate pesticide by bacteria isolated from agricultural soil. Report and opinion, vol. 3, issue (1), pp.124-128, 2011
- [3]. J.E. Franz, M.K. Mao, and J.A. Sikonski, “Glyphosate: A unique global herbicide. American chemical society monograph, American chemicals society, Washington DC. pp. 189, 1997.
- [4]. S.O. Duke, and S.B.Powles, “Glyphosate: a none-in-a-century herbicide”, Pest manage sci vol.64, issue (4), pp.319-325, 2008.
- [5]. A.N. Moneke, G.N. Okpala, and C.U. Anyanwu, “Biodegradation of glyphosate herbicide in vitro using bacterial isolates from four rice fields”, African journal biotechnology, vol.9, pp.4067-4074,2010.
- [6]. D.A Goldstein, J.F. Acquavella, R.M. Mannion, and D.R. Farmer, “An analysis of glyphosate data from the California environmental protection agency, pesticide illness surveillance Program”, Journal of Toxicology and clinical toxicology, vol. 40, pp. 885-892, 2002.
- [7]. C.Jayasumana, S. Gunatilake, and P. Senanyake, P,k, “Glyphosate, hard water and nephrotoxic metals: are they the culprit behind the epidemic of chronic kidney disease of unknown etiology in Sri Lanka?”,International journal of environmental research and public health.vol.11, pp. 2125-2147, 2014.
- [8]. R. Romano, P. Souza, M. Nunes, and M. Romano, “Perinatal exposure to a commercial formulation of glyphosate reduces the rRNA expression

and increases the protein content of beta TSH in the Pituitary of male offspring”, *Endocrinology: annala of surgical treatment and research*, vol. 29, pp. 753, 2012.

- [9]. R. Mesnage, C. Moesch, and R.L.G. Grand, “Glyphosate exposure in a farmer’s family”, *Journal of environmental protection*, vol. 03, pp. 1001-1003, 2012.
- [10]. A. Penalzoza-Vazquez, G.L. Mena, L. Herrera-Estrella, and A.M. Bailey, “Cloning and sequencing of the genes involved in glyphosate utilization by *Pseudomonas pseudomallei*”, *Appl. Environ. Microbiol.*, vol. 61, issue (2), pp.538-543,1995.
- [11]. A. Mendez-Villas, “Microbes in applied research: current advances and challenges”, *world scientific. Formatex Manchester, UK.* Pp. 27-30, 2012.
- [12]. A.L. Gimsing, O.K Borggard, and P. Sestoff, “modelling the kinetics of the competitive adsorption and desorption of glyphosate in soils”, *Environ sci. technol.*, vol.38, issue(17), pp. 18-1722, 2004.
- [13]. S. Bazot, and T. Lebeau, “Simultaneous mineralization of glyphosate and diuron by a consortium of three bacteria as free-and/or immobilized-cells formulation”, *Applied microbiology and biotechnology*, vol. 77, pp.1351-1358, 2008.
- [14]. G.S. Jacob, J.R. Garbour, and L.E. Hallas, “Metabolism of glyphosate in *Pseudomonas* sp. Strain LRr”, *Appl.environ microbial*, vol. 54, pp.2953-2958, 1988.
- [15]. E. Zboniska, B. Lejczak, and P. Kafarski, “Organophosphate utilization by the wild-type strain of *pseudomonas fluorescens*”, *Applied and environmental microbiology*, vol. 58, issue (9), pp. 2993-2999, 1992.
- [16]. I.T.Ermakova, N.I.Kiseleva, and T.Shushkova, “Bioremediation of glyphosate-contaminated soils”, *Applied microbiology and biotechnology*, vol.88, pp. 585-594, 2010.
- [17]. R. Pipke, and N. Amrhein, “Degradation of phosphonate herbicide: glyphosate by *Athrobacteratrocyaneus* ATCC 13752”, *Appl.environ.microbiol.*, vol. 54, pp.1293-1296, 1988.
- [18]. O.Benslama and A. Boulahrauf, “Isolation and characteristics of glyphosate degrading bacteria from different soils of Algeria”, *African journal of microbiology research*, vol.7 pp.5587-5595, 2013.
- [19]. M. Cheesbrough, “Medical laboratory manual for tropical countries”, 5th (ed) tropical health technology publications. U.K. Pp. 223-392, 2006.
- [20]. M.O. Fawole, and B. A. Oso, “Laboratory manual of micro- biology”, *Spectrum books limited, Ibadan*, pp.127, 2001.
- [21]. P.Mussali-galante, M. L.Castrejon-Godinez, G. E. Rosas- Ramenez, and A. Radroquez, “Glyphosate pollution treatment and microbial degradation alternatives:a review”, *Microorganisms*, vol. 9, issue (11), pp. 11-13, 2021.
- [22]. D. Yanina, Y. Kocharovskaya, A. Bogan, A. Sizova, V. Solomentsev, L. Iminova, N. Iyakhovchenko, A. Zinovieva, M. Goyanov, and I. Solyanikova, “Characterization and genomic analysis of *Exiguobacterium alkaliphilium* B-3531D, an efficient crude oil degrading strain”, *Biotechnology reports*, vol 32, pp. 1-7, 2021.
- [23]. J. G. Irene, F. Perez – Montano, C. Medina, F. J. Ollero, and F. J. Lopez-Baena, “The *Sinorhizobium* (*Ensifer*) *fredii* HH103 nodulation outer protein NOD1 is a determinant for efficient nodulation of soybean and cowpea plants”, *Applied and Environmental microbiology*, Vol. 83, issues (5), 2017
- [24]. S. Firdous, S. Iqbal, S. Anwar, and H. Jabeen. “Identification and analysis of 5-enolpyruvyl shikimate-3-phosphate synthesis (EPSPS) gene from glyphosate resistance *Ochrobactrum Intermedium* SQ 20”. *Pest management sci.*, vol 74, issue (5), pp 1184-1196, 2017.