International Journal of Trend in Scientific Research and Development (IJTSRD)
Volume 6 Issue 7, November-December 2022 Available Online: www.ijtsrd.com e-ISSN: 2456 – 6470

Bioremediation of Glyphosate Polluted Soil using Fungal Species

Orji, M. U.*; Agu, K. C.; Ikele, M. O.; Uwanta, L. I.; Ugwuoke, G.

Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria

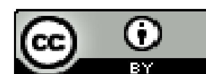
ABSTRACT

Glyphosate is an organophosphate compound used as herbicide in agricultural farms. It partly contributes to xenobiotic contamination of soil samples. Issues of toxicity and persistence have given rise to the need for its control in soils. The present study sought to bio-remediate glyphosate soil contamination using fungal cultures. Fungal isolates were obtained from glyphosate polluted soil and were screened for glyphosate utilization capacity. Isolates were identified using microscopy and molecular typing of the sequenced ITS-region. The best two degraders were made choice isolates used for the bioremediation study. They were used singly and in consortium for the bioremediation process monitored over a four-week period. Isolates obtained from the polluted soil sample are *Aspergillus niger, Aspergillus terreus, Aspergillus tamari, Fusarium oxysporium* and *Aspergillus flavus*. *Aspergillus terreus* and *Fusarium oxysporium* were the best glyphosate degraders and gave residual glyphosate values of 21.17 ug/ml and 17.22 ug/ml respectively. Their bi-culture degradation gave a residual glyphosate value of 8.87 ug/ml with 91.41 % degradation. This study thus shows that glyphosate soil pollution can be controlled with fungal cultures and can thus be said to be amenable to bioremediation process.

KEYWORDS: *Glyphosate, herbicide, pollution, xenobiotics*

How to cite this paper: Orji, M. U. | Agu, K. C. | Ikele, M. O. | Uwanta, L. I. | Ugwuoke, G. "Bioremediation of Glyphosate Polluted Soil using Fungal Species" Published in International Journal of Trend in Scientific Research and Development (ijtsrd), ISSN: 2456-6470, Volume-6 | Issue-7, December 2022, pp.726-732, URL: www.ijtsrd.com/papers/ijtsrd52396.pdf

Copyright © 2022 by author (s) and International Journal of Trend in Scientific Research and Development Journal. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0) (http://creativecommons.org/licenses/by/4.0)



INTRODUCTION

Herbicides are substances used for the control of weeds (Ifediegwu *et al.,* 2015). They could be broad based or specific in activity, basically classified as organochlorines, organophosphates, carbamates and pyrethrins (Godinez et al., 2021). The most common group of herbicides used by most farmers are the organophosphates being that they are milder in human toxicity compared to the organochlorine compounds. By principle it is believed that these herbicides are not toxic to man because they primarily attack the schikimic acid pathway in plants, which is a pathway not found in the metabolism of humans. However, studies have shown that persistence in soil and leaching into water bodies are main sources by which these herbicides find their way up the food chain to man, where they bioaccumulate free radicals believed to trigger various types of cellular cancers in humans. It thus becomes pertinent to see that these herbicides are degraded so as not to serve as chemical food hazards to humans.

Glyphosate is an organophosphate broad spectrum herbicide (Isopropylamine salt of N-Phosphono-methyl-glycene) used to kill weeds' predominantly annual broadleaf weeds as well as grasses competing with commercial crops grown around the world (Coup *et al*., 2012). Glyphosate's mode of action is the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, resulting in the depletion of essential aromatic amino acids needed for plant survival (Zablotowicz and Reddy, 2004).

Although this low-cost herbicide is able to effectively kill weeds, its extensive use has been negatively linked to human health due to its toxicity (Goldstein *et al*., 2002). This then elicits the need for control measures towards the persistence of this herbicide in the agricultural environment, of which bioremediation has been reported as a potent means. Bioremediation is the use of living organisms to minimize or eliminate the environmental hazards resulting from accumulation of chemicals and other hazardous wastes (Nawaz *et al*., 2011; Agu *et al.,* 2014; Mbachu *et al.,* 2014). Biological decomposition of herbicides is the most important and effective way to remove these compounds from the environment without producing toxic intermediates (Diez, 2010; Farukawa, 2003). Microorganisms provide a potential wealth in

biodegradation (Porto *et al.*, 2011; Agu et al., 2015; Anaukwu *et al.,* 2016; Okafor *et al.,* 2016). Microorganisms have the ability to interact, and utilize substances leading to structural changes or complete degradation of the target molecules (Raymond *et al.,* 2001; Anaukwu *et al.,* 2016, Ojiagu *et al.,* 2018; Agu and Odibo, 2021).

This study thus investigates the ability of various fungal organisms to degrade glyphosate polluted soil obtained from school farm at Nnamdi Azikiwe University, Awka, Nigeria.

Methods

Collection of Samples

Soil samples were collected from herbicide treated farms at Nnamdi Azikiwe University, Awka, Anambra State, Nigeria as a source of glyphosate degrading fungi.

Glyphosate herbicide (Round up) was purchased from the Ministry of Agriculture sales outlet Amawbia, Nigeria and was used for the study.

Isolation of Fungi

One gram (1g) of each soil sample was added into 10ml of sterile distilled water and shaken thoroughly. Two fold serial dilutions were carried out on the soil samples (5ml in 5ml). The diluted soil samples were plated out from dilution five on Sabourand Dextrose Agar (SDA) using pour plate method and incubated for seven days at room temperature ($30^oC \pm 3^oC$). The isolated fungi species were subcultured repeatedly on Sabourand Dextrose Agar and incubated for 7days at room temperature until pure cultures were made. Pure isolates were stored in Sabourand Dextrose Agar slants for further uses. Chloramphenicol (25µg/ml) were added into the media.

Characterization of the Fungal Isolates

Characterization of the fungal isolates was done by preparing slide cultures of the fungal isolates and their microscopic features were observed using the slide culture technique as described by Agu and Chidozie (2021). Their colony morphology and microscopic features observed were then compared with various standard atlases; atlas of clinical fungi (de Hoog *et al*., 2000), description of medical fungi (Ellis *et al*., 2007) and a colour atlas of pathogenic fungi (Frey *et al*., 1979).

Screening of Fungal Isolates for Glyphosate Utilization

Fungal screening was carried out by monitoring the growth capability of the fungi isolates at different concentrations of the herbicide (Glyphosate) (0.25ml, 0.5ml, and 1ml) in 50ml Czapek Dox broth medium ($NaNO_3$ 3.0g, K_2HPO_4 1.0g, KCl 0.5g, $MgSO_4$ 0.1g, $FeSO_4.2H_2O$ 0.1g, Sucrose 30g, pH 6.5). The sterilized media were inoculated with agar plugs of molds, and then incubated in a rotary shaker (Stuart orbital incubator S150) operated at 150 rpm for 15 days at room temperature ($30^oC \pm 3^oC$) (Eman *et al*., 2013).

Identification of Choice Isolates

The two fungal species which utilized the glyphosate-enriched medium the most were made choice isolates for further degradation studies. They were further identified using ITS region sequencing and molecular typing from the gene bank at Inqaba Biotech, South Africa.

Bio-degradation Study

Soil unpolluted with herbicides was collected from Nnamdi Azikiwe University, Awka and sieved through a mesh with 2mm diameters. A 2.5 kg portion of sieved soils was sterilized and poured into eight plastic containers. A 130 ml aliquot of prepared herbicide solution was used to pollute the soil samples and the polluted soils were allowed to stand for 2 hours.

Bioremediation was carried out by seeding 100 g of polluted soil samples with 30 ml of fungal suspension. The soil samples were allowed for biodegradation of the herbicides for four weeks. Soil samples were collected immediately after pollution and at the end of degradation for Gas Chromatographic (GC) analysis. The biodegradation set up is as follows:

Soil Sample A = No fungal isolate inoculated

Soil Sample B = Soil sample inoculated with *Aspergillus terreus*

Soil Sample C = Soil sample inoculated with *Fusarium oxysporium*

Soil Sample D = Soil sample inoculated with *Aspergillus terreus* and *Fusarium oxysporium*

Determination of Residual Glyphosate

The GC-MS technique was used for the determination of residual glyphosate from each biodegradation set-up according to methods described by AOAC 1990.

Statistical Analysis

Mean values of data obtained were determined and their analysis of variance was carried out at 95% confidence interval. Mean partitioning was done with the Tukey test.

Results

Isolation and Identification of Fungal Isolates from Polluted Soil Samples

Five fungal isolates were obtained from the herbicide polluted soil samples and their presumptive identification are shown in Table 1.

Biodegradation Screening of the isolates

The five obtained fungal isolates were screened for glyphosate utilization capacity and their results are shown in Table 2. Isolates A and D gave the least residual glyphosate values and were designated as choice isolates for further biodegradation studies.

Biodegradation Study using the Best Isolates

The four weeks biodegradation study using the choice isolates and a control experiment showed that the control experiment (sample A) had more residual glyphosate as it was left for natural attenuation. However, soil sample D which had a bi-culture inoculation of *Aspergillus terreus* and *Fusarium oxysporium* had the least residual glyphosate concentration with a degradation percentage of 91.41% as shown in Table 3.

Table 1: Identification of Fungal Organisms from Glyphostae Polluted Soil

Isolates	Cultural characteristics	Microscopic characteristics	Partial ITS rDNA sequencing analyses	Identity
4	On SDA, colonies were powdery, flat, white at first, then turned yellowish-brown with age. Colour on the reverse side was yellow. Colonies were incubated at 30 ^{o}C for 5 days	Conidiophores appeared blue and terminated in a vesicle of uniseriate phialides. Conidia were one-celled, rough-walled and produced in long chains which were divergent.	The sample was identified as a member of the genus Aspergillus from examination of morphology. DNA extracted from the sample consistently failed to amplify despite a number of attempts. It was therefore not possible to sequence the isolate in order to provide full species level identification. Impurity in the original sample was suspected.	*Aspergillus* terreus
6	On SDA, colonies were powdery, flat with radial grooves, yellow at first, but later turned to bright to dark yellow-green with age. Colour on the reverse side was creamy. Colonies were incubated at 30 ^{o}C for 5 days.	Conidial heads were radiate, splitting to form loose columns (300- 400 µm in diameter). Thus, conidiophores stipes was hyaline and coarsely roughened, noticeable near the vesicle and non-septate. Conidia were globose to subglose (3-6 µm in diameter), pale-green and conspicuously echinulate.	A BLAST search of the GenBank sequence data base showed 100 % Identity to multiple ITS sequences reported from *A. oryzae* and *A. flavus.* The best matches included sequences reported in peer-reviewed literature (Yin, Y. et al. (2009). Molecular characterization of toxigenic and atoxigenic *A. flavus* isolates collected from peanut fields in China. *Journal of Applied Microbiology* 107 (6): 1857- 1865). This sample was distinguished from *A. oryzae* based on its smaller conidia (up to 6 µm, whereas those of *A. oryzae* are larger, up to 8 µm).	*Aspergillus flavus*
27	Colonies had rapid growth, 4.5cm in 4 days. Aerial mycelium was white to cream with orange-	Conidiophores were hyaline, simple, bearing spore masses at the apexes. As tall as the length of macroconidia by		*Fusarium oxysporium*

	yellow pigmentation. Colour on the reverse side was dark-yellow. Colonies were incubated at 30 °C for 5 days.	a few times. Conidia were hyaline and phialosporous. 3- 5 macroconidia were present, which were fusiform, cylindrical, moderately curved with an indinstinctly pedicellate foot cell and a short blunt apical cell, 28-42 x 4-6 µm. Microconidia were abundant, which were cylindrical to oval, one to two-celled borne on lateral phialides, 8-16 x 2-4.5µm. Chlamydospores were hyaline, globose, smooth to rough-walled, borne singly and in pairs on short lateral branches, 6-10 µm.		
28	On SDA, colonies were colourless at first, then gradually turned orange-yellow to brown or light brownish-olive with age, but often presenting a suggestion of green that was transient and limited to areas of young heads. The reverse side was colorless. Growth rate was rapid (3 days)	Conidial heads varied greatly in size in the same fruiting area, from more or less columnar to nearly, but not completely, globose and up to 300m in diameter, with radiating chains and columns of conidia. Conidiophores arising from submerged hyphae, were up to 1 to 2 mm in length, colorless, with walls becoming abruptly thinner at the base of the vesicle. Vesicles were globose to subglobose, 25 to 50m in diameter with fairly thin walls which frequently crush in mounts, fertile over almost the entire surface. Sterigmata,were in one series in small heads and in two series in large heads.Conidia ranged from more or less pyriform, through sub-globose to globose, conspicuously roughened from prominent tubercles and bars of orange-yellow coloring matter deposited between the loose outer wall and the firm inner	A blast search using the GenBank sequence database, showed that the sequence from this sample showed 100 % identity to multiple ITS sequences reported from A. tamari. Best matches included sequences reported in peer-reviewed literature (Rakeman, *et al*. (2005). Multilocus DNA sequence comparisons rapidly identify pathogenic molds). Journal of Clinical Microbiology **43**: 3324-3333).	*Aspergillus tamari*

		wall, commonly ranging from 5.0 to 6.5mm in diameter, occasionally up to 8mm. Sclerotia were purplish, globose with apex white.		
29	On SDA, colonies had rapid growth rate. However, colonies were flat and compact with yellow basal felt covered by a dense layer of black conidial heads with powdery texture. The colour on the reverse side was pale yellow. Colonies were incubated at 30 ^{o}C for 5 days.	Conidiophores were hyaline or pale-brown, erect, simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial heads (up to 3 mm by 15 to 20 µm in diameter), split into over 4 loose conidial columns with over 4 fragments apically composed of catenulate conidia (over 15 conidia/ chain) borne on uniseriate and biseriate phialides on pale-brown, globose vesicles and phialides acutely tapered at apex. Conidia (3.5-5 µm in diameter) are phialosporous, brown, black in mass globose and minutely echinulate		*Aspergillus niger*

Table 2: Glyphosate Utilization Capacities of Fungal Isolates

Isolates	Glyphosate 0.25ml	Glyphosate 0.5ml	Glyphosate 1ml
A	2.41±0.01^{b}	3.72±0.01^{a}	6.84±0.01^{a}
B	2.81±0.01^{b}	5.03±0.01^{c}	10.09±0.01^{c}
C	3.58±0.01^{c}	4.68±0.01^{b}	8.31±0.01^{b}
D	2.09±0.01^{a}	3.8±0.01^{a}	6.86±0.01^{a}
E	3.97±0.01^{c}	6.75±0.01^{d}	11.42±0.01^{d}

Mean values along same column with different affixes are significantly different ($p<0.05$); initial glyphosate concentration = 103.24 ug/ml

Table 3: Biodegradation of Glyphosate Polluted Soil Samples using Choice Fungal Isolates

Soil Samples	Residual Glyphosate Concentration (ug/ml)	Percentage Degradation (%)
A	92.21	10.68
B	21.17	79.49
C	17.22	83.32
D	8.87	91.41

Soil Sample A = No fungal isolate inoculated; Soil Sample B = Soil sample inoculated with *Aspergillus terreus*; Soil Sample C = Soil sample inoculated with *Fusarium oxysporium*; Soil Sample D = Soil sample inoculated with *Aspergillus terreus* and *Fusarium oxysporium*

Discussion

The present study showed that several fungal isolates inhabiting the soil possess the capacity for glyphosate degradation. Some researchers have reported herbicide degradation by fungal species and posited that they have the capacity to degrade xenobiotics (Diez, 2010; Kanekar *et al.*, 2004; Nawaz *et al.*, 2011; Porto *et al.*, 2011). The fungal species isolated from this study corresponds with that of Eman *et al.* (2013) and Njoku *et al.* (2020).

Evaluation of glyphosate degradation capacity of the retrieved isolates showed that *Aspergillus terreus* and *Fusarium oxysporium* were the most glyphosate utilizers based on the preliminary screening. This finding also corresponds with that of Eman *et al*. (2013) and Godinez *et al*. (2021).

The four-week glyphosate degradation showed that leaving the polluted soil without any form of bioremediation amendment may take a longer time for the pollutant to be degraded, as seen by the residual glyphosate values of the control experiment (Group A) in Table 3. It was also observed that *Fusarium oxysporium* had a better degradation capacity more than *Aspergillus terreus*. However, a bi-culture amendment of the polluted soil sample had the best glyphosate degradation within the monitoring period with percentage degradation value of 91.41%. According to Godinez *et al*. (2021) microorganisms that possess the capacity for glyphosate utilization and degradation usually possess enzyme complexes that aid their degradation capacities. It is then possible that the bi-culture degradation which gave the best residual glyphosate values was enzyme mediated. Considering from the enzyme-substrate complex standpoint, it could be inferred that both fungal organisms possibly contributed higher enzyme concentrations which catalyzed the reaction rate faster than that of the individual organism.

Conclusion

Mycoremediation of glyphosate polluted soil samples is an efficient method of environmental herbicide pollution control and can as well be considered as a sustainable process. Sustainable in the sense that these molds are ubiquitous and are spore formers, which aid their ease of production and proliferation on industrial capacity.

Acknowledgement

I want to acknowledge in a very special way the efforts of Tertiary Education Trust Fund (TETFund) National and that of Nnamdi Azikiwe University for providing us with the Institution Based Research (IBR) Grant for the execution of this research; as well as the Vice Chancellor of Nnamdi Azikiwe University, for providing an enabling environment for this research to be conducted.

REFERENCES

[1] **Agu, K. C***. and Chidozie, C.P. (2021). An Improved Slide Culture Technique for the Microscopic Identification of Fungal Species. International Journal of Trend in Scientific Research and Development, **6** (1): 243-254, URL: www.ijtsrd.com/papers/ijtsrd45058.pdf

[2] **Agu, K.C.***, Bassey, E.E., Iloanusi, C.A., Awah, N.S., Okeke, B.C., Anaukwu, C.G., Ezenwa, C.U., Orji, M.U. and Okafor, A.C. (2015). Isolation and Characterization of Microorganisms from Oil Polluted Soil in *Kwata*, Awka South, Nigeria. *American Journal of Current Microbiology*, **3**: 46-59.

[3] Agu, K.C*. **and Frederick J. C. Odibo (2021). Biodegradation Potentials of** *Aspergillus Flavipes* **Isolated from Uburu and Okposi Salt Lakes. International Journal of Trend in Scientific Research and Development, 5 (5): 1160-1170. URL:** www.ijtsrd.com/papers/ijtsrd44949.pdf

[4] Anaukwu, C.G*., Ezemba, C.C., Anakwenze, V.N., **Agu, K.C.**, Okeke, B.C., Awah N.S., Ekwealor, I.A. (2016). Effect of biosurfactant produced by Citrobacter murliniae AF025369 and a synthetic surfactant on degradation of crude oil. *Edorium Journal of Microbiology,* **2**: 1–6

[5] Anaukwu, C.G.*, Ezemba, C.C., Anakwenze, V.N., **Agu, K.C.**, Amechi, S.N., Okeke, B.C., Awah, N.S. (2016). Influence of Anionic, Cationic And Non-Ionic Surfactants on Growth of Hydrocarbon Utilizing Bacteria. *American Journal of Current Microbiology*, **4**:10-16.

[6] Coup, R., Kalkhoff, S., Capel, P. &Gregoire, C. (2012). Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe. P5877.

[7] De Hoog, G. S., Guarro, J., Gene, J. and Figueras, M, J. A. (2000). Atlas of Clinical Fungi. 2nd edn. Centraalbureau voor Schimmelcultures. Utrecht, The Netherlands.

[8] Diez, M. C. (2010). Biological aspects involved in the degradation of organic pollutants. *Journal of SoilScience* and *PlantNutrition*. 10(3): 244-267.

[9] Ellis, D., Davis, S., Alexiou, H., Handke, R., and Bartley, R. (2007). Descriptions of Medical Fungi. 2nd edn. Nexus Print Solutions, Adelaide, Australia.

[10] Eman, A., Abdel-Megeed, A., Suliman, A., Sadik, M. W., and Sholkamy, E. N. (2013). Biodegradation of glyphosate by fungal strains isolated from herbicides polluted-soils in Riyadh area. *International Journal of Current Microbiology and Applied Sciences.* 2(8):359-381.

[11] Frey, D., Oldfield, R. J. and Bridger, R. C. (1979). A Colour Atlas of Pathogenic Fungi. Wolfe Medical Publications Ltd, London.

[12] Furukawa, K. (2003). 'Super bugs' for Bioremediation. *Trends in Biotechnology*. 21:187-190.

[13] Godinez, M.L.C., Sanchez, E.T., Cuevaz, L.V., Ramirez, M.E.R., Rodriguez, A. and Galante, P.M. (2021). Glyphosate Pollution Treatment and Microbial Degradation Alternatives, A Review. *Microorganisms*, **9**:2322.

[14] Goldstein, D.A., Acquavella, J. F., Mannion, R.m. & Farmer, D.R. (2002). An analysis of glyphosate data from the California Environmental Protection Agency. Pesticide illness Surveillance Program. *Journal of Toxicology and Clinical Toxicology* 40:885-892.

[15] Kanekar, P. P., Bhadbhade, B., Deshpande, N. M. and Sarnaik, S. S. (2004). Biodegradation of organophosphorus pesticides. *Proceedings of the Indian National Science Academy*. B70 (1):57-70.

[16] Mbachu, A.E., Onochie, C.C., Agu, **K.C*.,** Okafor, O.I. and Awah, N.S. (2014). Hydrocarbon Degrading Potentials of Indigenous Bacteria Isolated from Auto-Mechanic Workshops at *Mgbuka*-Nkpor, Nigeria, *Journal of Global Biosciences,* **3** (1): 321-326

[17] Nawaz, K. K., Choudary, N., Majeed, A., Ilyas, U., Ghani, A., Lin, F., Ali, K., Afghan, S., Raza, G., and Lashari, M.S. (2011). Eco-friendly role of biodegradation against agricultural pesticides hazards. *African Journal of Microbiology Research*. 5(3):177-183.

[18] Njoku, K.L., Eludini, P.O., Adesuyi, A.A., Ude, E.O. and Oyelami, A.O. (2020). Physiological and Molecular Characterization of Active Fungi in Pesticides Contaminated Soils for Degradation of Glyphosate. *Research Square*, https://doi.org/10.21203/rs.3.rs-27821/v2

[19] Okafor, U.C., Orji, M.U., Nwankwegu, A.S. Anaukwu, C.G., Onuorah, S.C., Archibong, E.J., Obika, I.E. and **Agu, K.C.** (2016). Effect of Chicken droppings amendment on bioremediation of crude oil polluted soil. *European Journal of Experimental Biology*, **6** (4): 62-68.

[20] Ojiagu, K.D., Odibo, F.J.C, Ojiagu, N.C., **Agu, K.C.,** Okafor, A.C. (2018). Biosorption of Hexavalent Chromium by *Pseudomonas aeruginosa* Strain ANSC: Equilibria Isothermic, Kinetic and Thermodynamic Studies. *Bioengineering and Bioscience*, **6**(1): 1-10. DOI: 10.13189/bb.2018.060101

[21] Porto, A. L. M., Melgar, G. Z., Kasemodel, M. C. and Nitschke, M. (2011). Biodegradation of pesticides. In: Pesticides in the Modern World-Pesticides Uses and Management. Stoytcheva, M. (Dr), Eds. ISBN: 978-953-307-459-7. InTech. pp. 407-438. Available form: http://www.intechopen.com/books/pesticides-in-the-modern-world-pesticides-use-and-manegement/biodegradation-of-pesticides.

[22] Raymond, J., Rogers, T., Shannard, D., and Kline, A. (2001) A review of structure-based biodegradation estimation methods. *Journal* of *HazardousMaterials*. 84:189-215.

[23] Zablotowicz, R.M. & Reddy, K.N. (2004). Impact of Glyphosate on the *Bradyrhizotium japonicum* symbiosis with Glyphosate Resistant Transgenic Soybean: *A minireview Journal of Environmental Quality,* **33**:825-831.