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Isolation of indigenous glyphosate degrading microbes from selected agro ecological zones of Malawi

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Abstract

Weed infestation is one of the remarkably causes for the decrease in yield and yield components for all type crops in Malawi. To minimize losses herbicides control like glyphosate is used because it surpasses other methods employed by farmers since it's supported by the current strategy of green revolution techniques, timely and cheap. The residuals of glyphosate compounds have an impact and effect on immunity, bio argumentation, bio-magnification, and environment. It is therefore important to investigate synchronized strategies that can degrade of glyphosate because no study has been performed showing prospect microbes in bioremediation study in Malawi. Microbes were isolated for their capacity to utilize glyphosate as sole carbon and phosphorous source complimented by presence of laccase gene. Biochemical test and molecular characterization using 16S rDNA and 18S rDNA genes for bacteria and fungus respectively were used in identification and this was supplemented by testing for the presence of Plant Growth Promoting (PGP). Several fungi and bacteria were found to degrade glyphosate; Mucor irregularis, Fusarium oxysporum, Meyerozyma caribbica, Aspergillus parasiticus for fungus and genus Achromobacter and Enterobacter and for bacteria. 90 % of strains had PGP traits besides potential in bioremediation. The study also adds new strains that can be used in degradation of glyphosate.

Keywords: Pesticide, Glyphosate, Soil microbes, Bio-degradation, Malawi.

Introduction

Weed infestation is one of the serious and remarkably causes for decrease in yield and yield components for all crops in Malawi. To minimize losses several methods of weed control are used such as mechanical, cultural, biological and chemical control methods. Chemical control using glyphosate a broad-spectrum systemic herbicide surpasses others in plantation crops because its supported by current strategy of green revolution techniques which base on usage of inorganic chemicals to improve and maintain productivity[1]. Chemical control method is also advantageous because it's quick, more effective, time and labor saving method than others (mechanical and biological)[2,3].

However, regardless of benefits these synthetic organic chemicals like glyphosate contain xenobiotics which have a negative impact on beneficial microbes, bio-magnification[45] and ecosystems due loss of biodiversity[6,7]. They have direct impact to yield and yield components because they make the environment not conducive for soil beneficial organism including PSM, and interfere with plant growth and nutrition[8,9].

There are several clean-up mechanisms for pesticides xenobiotics; volatilization, chemical treatment methods and incineration. These methods have not been accepted by public due to association with large volumes of acids and alkalis which create a problem of disposal and emissions of toxic chemicals by Chemical treatment and Incineration respectively[10,11]. These methods are also inefficient, not practical and expensive because the polluted soilscan't be extracted, transported, treated and replaced[12].

It's for these reasons that biological methods areused in biodegradation of pesticide xenobiotics[10,13,14]. This involves the use of microbes, for degradation of xenobiotics by a process called bioremediation[10]. To the advantage is that some microbes involved in bioremediation may also act as Bio fertilizers by using natural processes of nitrogen fixation, solubilizing phosphorus, and production of phytohormones[15]. Bioaugmentation, in which biostimulation of indigenous microflora, is of paramount importance when decontaminating historically and/or heavily polluted fields is also another promising technique. The use of filamentous fungi may be advantageous over bacterial in bioremediation however most studies prefer combination of both.

Several studies have documented that indigenous microbes have an important impact in bioremediation of pesticide xenobiotics residues in the environment. Many reports have documented bioremediation of glyphosate by various fungus and bacteria but there is no research describing isolation, diversity and

characterization of indigenous microbes involved in degradation of glyphosate in Malawi.

The aim of the study was to identify the potential microbial strain that degrade glyphosate from the polluted sitesin Malawi. In this study, isolation and characterization of glyphosate degrading microbes is examined with the hope of creating an environment free from xenobiotics which have an effect on contaminated farms and aquatic environments.

Materials and methods

Soil samples collection: The procedure followed Eman et al. with some modification where by Soil samples were collected from sites having long history of pesticides application except Chasatha farm which had 1 year history of pesticide application. Soil samples were collected late December 2016 from top layer (3-15 cm). The sites were Chasatha farm in Karonga district, Nkhozo farm (Exagris Estate) in Rumphi district and Khongoloni Tea Estate in Mulanje district. Sterile zipper polyethylene plastic bags were used to collect samples and transported in iced cooler boxes and subjected 4 ^{0}C storage environments before processing.

Pesticides: The pesticides glyphosate, used in this study was purchased from the Farmers Organization Limited shop by the guidance of Pesticide Control Board (Malawi).

Soil sampling technique: 3 places were identified for sampling from each sampling site namely; 500 m outside area of farm where no history of application of pesticide (upstream of drainage and wind movement), inside the farm with long history of pesticide, and downstream the drainage system of the farm.

Isolation of microorganisms: Microorganisms from collected soil samples were isolated using enrichment culture technique. 5 gm of soil sample was put into a 250 ml glass flask which had 50 ml of Mineral Salt Medium (MSM) with 100 ppm of glyphosate was used for isolating degrading microbes. MSM contents were (g/l) KH_2PO_4 (1.5), Na_2HPO_4 (0.6), $CaCl_2$ (0.01), NaCl (0.5), NH_4SO_4 (2), $MgSO_4.7H_2O$ (0.2) and $FeSO_4.7H_2O$ (0.001) was used. Microbe isolation was carried out at different concentrations of pesticides (100, 200, 1000 and 10000 ppm) on Czapek Dox agar[16,17]. Microbes that tolerated pesticide concentration up to 1000 and 10000 ppm were considered for further studies.

Pesticide utilization patterns of the different isolates: Later individual microorganisms from previous step was transferred into three 250-ml flask which had 50 ml MSM 1, MSM 2 and MSM 3 with the addition of 20 mls of pesticides as sole carbon, phosphorous sources or utilization both for 40 days 28°C. Controls had no microbe inoculation. This was modifications of Shamsuddeen and Inuwa and Akbar et al. studies with the following composition of MSM-1: KH_2PO_4 (1.5),$MgSO_4.7H2O$ (0.2), Na_2HPO_4 (0.6), NaCl (0.5), NH_4SO_4 (2), $CaCl_2$ (0.01) and $FeSO_4.7H_2O$ (0.001) in grams dissolved in 1 liter of water, pH 7.0. MSM-2 had no phosphate source and targeted pesticides to be sole P source, and had the following composition: glucose (10), Tris buffer (12), $CaCl_2$ (0.01), NaCl (0.5), NH_4SO_4 $CaCl_2$ (0.01) and $FeSO_4.7H_2O$ (0.001) in grams dissolved in 1 liter of distilled water, pH 7.0. MSM-3 was used for isolating microbes using pesticides as sole P and carbon source with the following composition: NaCl (0.5), $MgSO_4.7H_2O$ (0.2), KCl (0.5) NH_4SO_4 (2). After 40 days 1 ml was inoculated on Czapek Dox agar plates at 28°C. Growth was observed and recorded after 5 days.

Laccase gene presence: Isolates from previous step were streaked on Sabouraud Dextrose Agar (SDA), amended with 1% ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) by streak method. Development of dark green to purple color around the colonies indicated the presence of laccase products being produced by the microbes[18].

Biochemical tests: All isolated microbes were subjected to the following biochemical tests.

Production of Ammonia: Broth cultures of isolated microbes with $1*10^9$ CFU or 4 mm diameter of fungus were dropped on 10 mL tube of peptone water (incubation at 36±2°C for 48-72 hrs) followed by addition of Nessler's reagent (0.5 mL). Positive test for ammonia production is shown by development of yellow or brown Color[8].

Indole Acetic Acid Production (IAA): IAA traits by isolated microbes was determined as documented by Ahmad et al., 2008 with modification of not measuring the quantity. Isolates were grown in NB amended with tryptophan (100µg/ml) set at 30°C (lower limit 29 and upper limit 31) for 48 hours on orbital incubator (120 rpm). This was followed by 30 minutes centrifuging at 3000 rpm and 2 ml supernatant was added with 2 drops of o-phosphoric acid and four mls of Salkowski reagent. Salkowski reagent was formed by mixture of 50 mls of 35% perchloric acid, 1 ml of 0.5 M FeCl3 solution. Pink color development indicate IAA traits.

Catalase production: This was done by addition 2 drops of 3% hydrogen peroxide to grown culture of isolated microbes on a slide using wire loop in a biosafety cabinet. The effervescence indicated catalase activity.

Hydrogen cyanide production: Isolated microorganisms were tested in-vitro for hydrogen cyanide production by method documented by Ahmad et al., (2008) with some modifications. Isolates grown in nutrient broth and Sabouraud Dextrose broth amended with glycine 4.4g /L were inoculated on modified NA and SDA plates for bacteria and fungi respectively. Sterile Whatman filter paper (No.1) was dipped 0.5% picric acid solution (2.5% sodium carbonate) and later placed on grown cultures on agar plate. Agar Plates were tightly sealed by parafilm and incubated at 36°C (lower limit 35 and upper limit 37) for four days. HCN production was observed by color

change from orange-red colour or yellow on Whatman filter paper.

Siderophores production: Qualitative Production of Siderophore by microbes was done using universal chrome azurol S (CAS) agar plate assay as documented by Liu et al., (2016). This was done using CAS agar plates, because siderophore producing microbes forms orange halo around the colonies. This is so because Fe is changed from its original blue CAS–Fe (III) complex during the production of siderophore. After 7 days incubation (28 °C) plates were observed for the production of halo[8].

Nitrogen-Fixing Ability: Nitrogen fixing ability of microbes was assessed using Liu et al., 2016 with some modifications where isolates were streaked onnitrogen deficient modified Ashby's agar medium (0.2 g NaCl, 0.1 g $CaSO_4 \cdot 2H_2O$, 10 g sucrose (for fungus dextrose), 5 g $CaCO_3$, 0.2 g KH_2PO_4, 0.2 g MgSO4·7H2O and 15 g agar in 1 L distilled water; pH 7.0). Plates were incubation at 28 °C (lower limit 27 and upper limit 29) for 7 days to check for growth. Growth of strain was considered as an ability to fix atmospheric nitrogen.

Phosphate solubilization: All isolates that showed clear halo zone were screened for phosphate solubilization on Pikovskaya's medium. Isolates were inoculated at the Centre Pikovskaya medium plate and incubated at 28^0C but successively measuring diameter of clearance zone and colony up to 7 days. PSE is the ratio of clearance zone (which also includes bacterial growth) and the colony diameter. Experiment was set up in a CRD manner with 2 replicates. PSI (Phosphate Solubilization Index) was measured by taking Colony diameter plus Halo zone diameter divided by Colony diameter. Those with SI of greater than 1.5 were selected for further testing. Commercial strain was also used as control and for comparison.

Cell morphology and colonies: The pure isolates in new plates were preliminary observed for colony morphology using magnifying glass. Later morphological characters like colony surface texture, margins, elevation, pigmentation and shape, were observed using microscope. Gram staining was used to study cell structure, shape and size. The following processes were performed; grams staining smear from the isolated culture on microscopic glass slide, heat fixed and stained. This was observed on oil immersion lance-100 X of microscope. Fungi characterization was based on colony characteristics on PDA plates supplemented by microscopic analysis of colony on slide using lacto phenol blue stain.

Molecular characterization: Characterization of isolates were done by sequencing 16S rDNA gene and ITS (internal transcribed sequences) of 18S ribosomal DNA gene for bacteria and fungus respectively. Genomic DNA was extracted and purified using the ZR-kit following manufacturer's manual and amplified using Polymerase Chain Reaction (PCR) of the 16S rDNA and 18S rDNA all supplied by Inqaba http://www.inqababiotec.co.za. The primers were 907R (5'-CCGTCAATTCMTTTRAGTTT-3') and 1492R (5'-TACGG Y TACCTTGTTACGACTT-3') for bacteria and ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTT A TTGATATGC -3') for fungus**.** Sequencing of the isolates 16S rDNA and 18S rDNA genes was done by Inqaba Biotech Ltd in South Africa using Sanger sequencing. Consensus sequences of two PCR products of 16S rDNA and 18S rDNA sequence data was done using BioEdit software. The consensus sequence obtained in BioEdit was analyzed by BLAST algorithm for comparison of a nucleotide query sequence against public nucleotide sequence database to find the closely related strains**.** The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database, Sequences with high similarity scores were downloaded from the NCBI database. Based on maximum identity score first sequence was selected and aligned with isolate sequences using multiple alignment software program MUSCLE Distance matrix was generated using RDP database. First step the Neighbour Joining method was used for defining dataset because it establishes relationships between sequences according to their genetic distance (a phenetic criterion) alone, without taking into account an evolutionary model. Later Maximum Likelihood was used to because investigates the space of all possible phylogenetic trees, trying to identify those that are best its consideration of all possible trees to identify the best ones (i.e., exploring the space of all possible trees). Phylogenetic tree was constructed using Seaview. GenBank sequence database was used for depositing sequences and the accession numbers were obtained.

Results and discussion

Microbes involved in bioremediation: The use of microorganisms for xenobiotic degradation requires synergistic understanding of all biochemical, physiological, ecological, microbiological, and molecular aspects involved in pollutant degradation[13]. The study found out that some microbes can utilize glyphosate as sole carbon or Phosphorous (P) source or both which concur with other reports[16,20]. All microbes that were able to utilize glyphosate as sole carbon or P source or both had shown laccase production ability using SDA amended with ABTS Table-1. Laccase is a very potent enzyme with ability to act on a number of substrates. Since then, several laccase have been studied with respect to their biological function, etc. Results concur with other studies that document potential applications of laccases are related to bioremediation and waste treatment like degradation and detoxification of pollutants (EDCs, chlorophenols, PAhs, pesticides and others)[21,22]. Laccase also plays important roles in, lignolytic degradation, detoxification studies, plant pathogenesis, odor control in decomposition of wastes and pigment production[22]. The expression/efficacy of laccase in is influenced by several factors including nature and concentration of carbon source, nitrogen source, temperature, pH etc.[18,22].

Figure-1: Identified sites with long previous history of pesticide application; Left side weed infestation regardless of herbicides in peanuts (Nkhozo estate), Right tea plantation that used glyphosate to control weeds.

Table-1: Results of isolated pesticide degrading microbes accompanied by PGR traits.

LAB NO	Microbe	Site	Pesticide	Type	SI	IAA	Gram stain	Ammonia	Catalase	Siderophore	Shape	sole source			Laccase
												C	P	C&P	
1104	*Enterobacter cloacae*	K	G	B	1.0	+	-	+	+	+	R	-	+	-	+
1107	*Achromobacter sp*	M	G	B	1.1	+	-	+	+	+	R	-	+	-	+
1103	*Enterobacter aerogenes*	N	G	B	1.2	+	-	+	+	+	R	+	+	+	+
1105	*Enterobacter ludwigii*	M	G	B	1.13	+	-	+	+	+	R	+	+	+	+
6106b	*Mucor irregularis*	M	G	F	1.13	+		+	+	+		-	+	+	+
6106	*Fusarium oxysporum*	N	G	F	1.13	+		+	+	-		-	+	-	+
6103	*Fusarium oxysporum*	N	G	F	1.13	+		+	+	-		+	+	+	+
6102	*Fusarium oxysporum*	N	G	F	1.13	+		+	+	-		-	+	-	+
6101b	*Meyerozyma caribbica*	N	G	F	1.13	+		+	+	+		+	+	+	+
6100	*Aspergillus parasiticus*	N	G	F	1.13	+		+	+	+		+	+	+	+

G= Glyphosate, B= bacteria, F=Fungus, M=Mulanje, K=Karonga, N=Nkhozo, SI=solubilization index, Y= coccobacilli, R=Rod shaped.

Table-2: Distribution of pesticide degrading microbes within the application catchment area.

Karonga			Rumphi (Nkhozo)			Mulanje		
Outside the farm	Inside the farm	Downstream (drainage system)	Outside the farm	Inside the farm	Downstream (drainage system)	Outside the farm	Inside the farm	Downstream (drainage system)
	1104		6100	6103 6102 6101b 6100 1103	6102 6101b	1105 6106	1107 1105 6106	1107 1105 6106

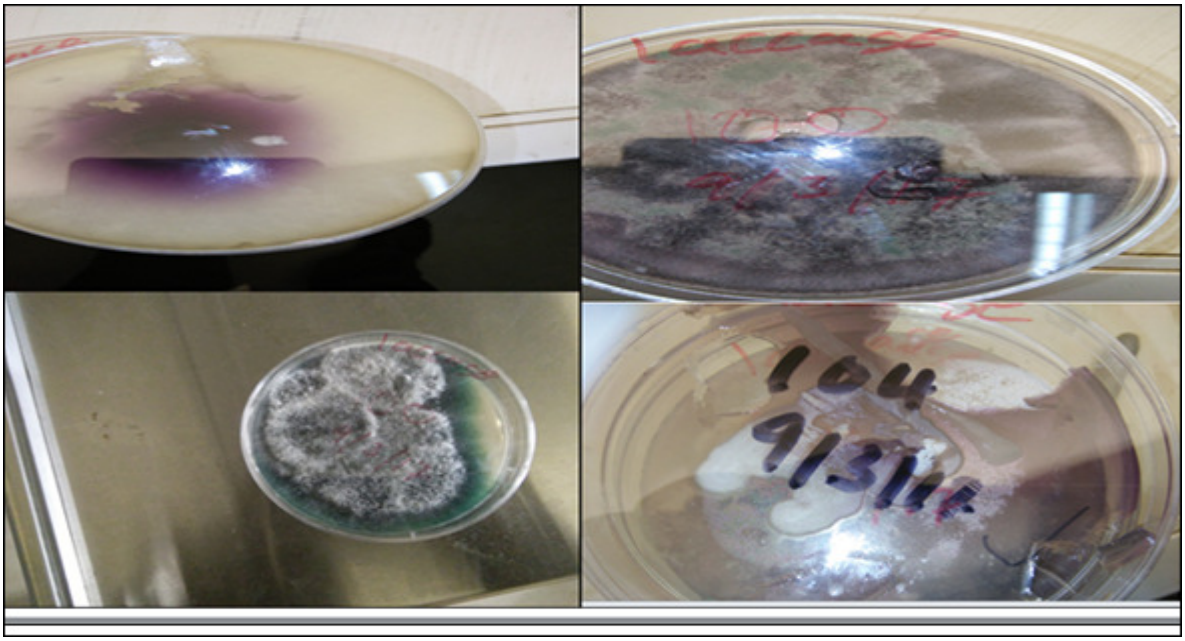

Figure-2: microbes showing presence of laccase enzymes by changing color when inoculated on Sabouraud Dextrose Agar (SDA), amended with 1% ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)).

Table-3: Blast related species and GenBank deposit accession numbers.

Lab No.	Related Species	Identity NCBI	Accession Number
6106b	*Mucor irregularis*	84	MF991235
6106	*Fusarium oxysporum*	100	MF974394
6103	*Fusarium oxysporum*	100	MF974394
6102	*Fusarium oxysporum*	92	MF974393
6100	*Aspergillus parasiticus*	100	MF983813
6101b	*Meyerozyma caribbica*	99	MF983800
1104	*Enterobacter cloacae*	99	MG031167
1107	*Achromobacter sp*	88	MG031169
1103	*Enterobacter aerogenes*	99	MG031163
1105	*Enterobacter ludwigii*	88	MG031168

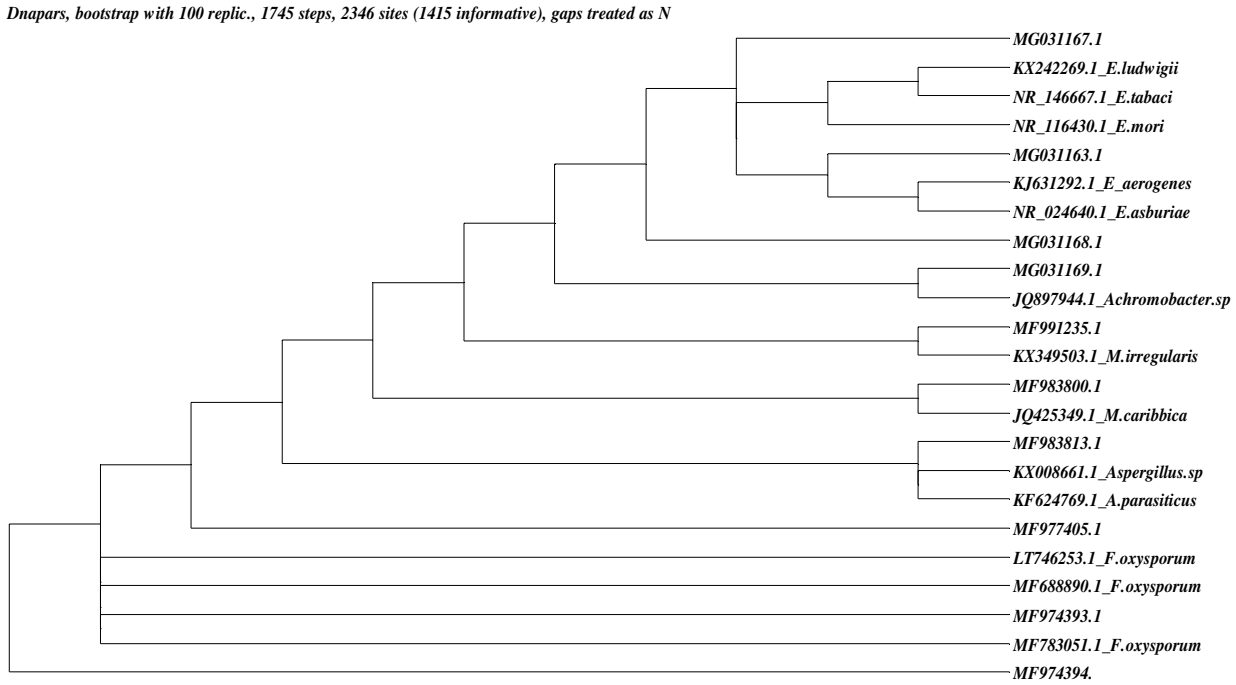

Figure-3: Phylogenetic tree derived from maximum parsimony analysis showing the position of glyphosate degrading microbes isolated from selected agro ecological zones of Malawi.

Heavy metals and xenobiotics induce laccase production in receptors of promoter regions of the genes encoding for laccase[23]. Concentration increase of certain inducers can lead to production of new isoforms of the enzyme which can be beneficial to remediation. The presence of laccase gene means the microorganism is able to degrade the xenobiotics of where it is found.

Bioremediation requires just isolating microbes without in-vitro quantitative analysis because real degradation is a factored by several synergistic biotic and abiotic complications [24,25]. In this study fungus and bacteria were found to degrade glyphosate.

Micro-organisms involved in bioremediation of glyphosate have 2 pathways to degradation. One is responsible for production of aminomethylphosphonic acid (AMPA) which has less interference to plant growth while the other pathway is responsible for production of sarcosine. Microbes use enzymes to utilize glyphosateas source of P and carbon. Diversity of isolated strains that utilize glyphosate as sole carbon or P source, is in line with study by Weaver et al., (2007) which showed no significant microbial population reduction when glyphosate is applied. The results fail to comply with facts that Glyphosate makes the microbes not to synthesize essential aromatic amino (Basics and Pont, 2000). 9 strains (5 fungus and 4 bacteria) were isolated to degrade glyphosate (Aspergillus parasiticus, Meyerozyma caribbica, 2 strains of Fusarium oxysporum, Mucor irregular is as fungus while *Achromobacter sp* and 3 strains of Enterobacter as bacteria) from 3 sites Table-1.

The study also found that natural selection is responsible for diversity of glyphosate degrading microbes shown by a lot of diversity in Nkhozo and Mulanje on Table-2 which has long history of pesticide application compared to CHASATHA farm in Karonga which had a year of application[26]. The results also show that aerial application, drainage and runoff has an impact to non-target sites shown by diversity of microbes responsible for degrading xenobiotics outside the farm downstream. Genus *Enterobacter* domination in bioremediation is in line with other studies[7,27,28]. The study also puts proposition that pest infestation in fields where pesticide application is a result of abundance of xenobiotic degrading microbes due to natural selection pressure not pesticide resistance of the pest Figure-1.

The study has found diversity of *Fusarium oxysporum* as shown that the other one utilize carbon as sole carbon source while the other P as only carbon source. This also supports other studies which associate glyphosate with complicated re-emergence of plant pathogens[9]. The results also concur with other studies which found that different strains of *Enterobacter*, *Aspergillus* and *Fusarium* that degrade glyphosate[27,29] but does not reflect other studies which puts *Pseudomonas sp* as best biodegrading microbe[30,31]. Study findings adds some unique strains of glyphosate degrading microbes from tropical soils that can be used in further studies like *Meyerozyma caribbica*. Our findings indicate that bioremediation can be done using bioargumentation but strains care must be considered because of disease causing microbes which are also bio degraders.

It is therefore probable that these indigenous glyphosate degrading microbes can also be used to support growth and

development of crop plants because of production of multiple PGP like IAA, Siderophore, Catalase etc. table 1 besides degradation which is also in support with other studies[32].

Production of IAA has an effect, root system increase in size and number of adventitious roots thus increasing large surface area for absorption of plant nutrients[33]. IAA production by PGPR vary because it is also influenced abiotic and biotic factors in the rhizosphere and also vary between species and strains[34,35].

All isolates can exert plant growth because they produce siderophores which are low molecular weight iron chelating compounds. The iron sequestered by microbial siderophores cannot be scavenged by pathogens. Siderophore producing microorganisms can protect plants either by limiting the growth of pathogenic microbes or by manipulating plant's defensive metabolism. These isolates have exhibited traits which were also found by other researchers[33]. Some of these PSM produce organic acids which also solubilize mineral K e.g. Pseudomonas putida[36].

Phylogenetic analysis based on ML method revealed that diversified divergent genera and species are involved in degradation of glyphosate. Genus *Enterobacter* and *Fusarium* is dominating in terms of diversity at species level and strain level in degradation of glyphosate respectively. Diversity in strains of *Fusarium oxysporum* was observed based on an outgroup isolate MF977405 which also was also depicted by the fact that other one utilize glyphosate as sole carbon source while as sole P source. *Fusarium oxysporum* isolate MF974394 was able to utilized glyphosate as P and C source. This indicates that these strains are of different phylotypes so that may be the reason diversity of microbes in clade.

Conclusion

The research met its objective in identifying indigenous strains involved in glyphosate degradation which can protect the environment from glyphosate xenobiotics.

The study also revealed new strain of microbes involved in degradation of glyphosate and the study puts proposition that pest infestation in fields is a result of abundance of xenobiotic degrading microbes due to natural selection pressure not pesticide resistance of the pest.

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