ISOLATION CHARACTERIZATION AND DIVERSITY OF INDIGENOUS PESTICIDE DEGRADING MICROBES FROM SELECTED AGRO ECOLOGICAL ZONES OF MALAWI

ABSTRACT

Pesticide xenobiotics have great impact on bio argumentation, bio-magnification, and environmentdegradation regardless of being adopted by green revolution technologies.Bioremediation is widely accepted becauseit'scheap, practicalandenvironmental friendly. Bioremediation advocates indigenous microbes use to degrade pesticide,therefore a study has been performed to show prospects of degrading microorganisms. The study isolated microbes from different agro ecological zones to assess their capacity to utilize some pesticide as sole carbon source complimented by the presence of laccase gene. Biochemical test and genetic characterization using 16S rDNA genes were used in identification. Diversified species and strains of genus Enterobacter, Klebsiella, Pseudomonas,Pantoea and Leclercia, were found to degrade cypermethrin and acetochlor but no microbe was found to degrade dimethoate. The study adds new strain of microbes involved in degradation of cypermethrin and acetochlor and also strains that that can degrade both. 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.

Keywords: Pesticide, Cypermethrin, Dimethoate, Acetochlor, indigenous soil microorganisms, Bioremediation, Malawi

1. INTRODUCTION

Pest infestation is one of the causes for decrease in yield and yield components in Malawi. Green revolution techniques prefer usage of pesticides backed by advantages of being quick, more effective, time and labor saving than , cultural, mechanical and biological methods[1,2]. Currently the use of different pesticides (herbicides and insecticides) in one field is essential to control diverse weeds and insects due to compatibility and effectiveness of pesticides[3].

Pesticides which include herbicides and insecticides are of paramount importance for controlling weeds and insectrespectively by farmers in Malawi[4]. However, regardless of benefits,pesticide contain xenobiotics which have a negative impact on bio-magnification,beneficial soil microbes, [5,6] and ecosystem[7]. They have directand indirect impact to yield components and yield because they interfere with soil beneficialorganism and plant 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 [10,11]. These methods are also inefficient, not practical and expensive because the polluted soils can't be extracted, transported, treated and replaced [12].

It's for these reasons that biological methodsinvolved in biodegradation are preferred [111314]. This involves the use of microorganisms to degrade xenobiotics by a process called bioremediation [11]. Its advantageous because some microbes involved in bioremediation can fix nitrogen fixation, produce phytohormones and solubilizing phosphorus[15]. Bioaugmentationby biostimulation of indigenous microbes is promising technique when dealing with heavily and/or historically contaminated sites.

Previous studies have documented that microbes play important roles indetoxificationand degradation of xenobiotics[16–20]. Thus far, many reports have described the biodegradation of cypermethrin and

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acetochlor by various microorganisms but there is no research describing isolation and characterization of indigenous microbes involved in degradation of these pesticides in Malawi and its neighboring countries except for glyphosate[21].

The research aim was to identify the potential microbial strainwith ability to degrade cypermethrin, dimethoate and acetochlor from the contaminated soil of Malawi. In this study, isolation and characterization of cypermethrin, dimethoate and acetochlor degrading microbes is examined with the hope of creating an environment free from xenobiotics which have an effect in contaminationof farms and aquatic environments.

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2. METHODOLOGY

2.1. Soil Samples Collection

The procedure was as described by Eman et al., (2013) with some modification whereby Soil samples were collected from sites having long history of pesticides application except Champhoyo farm which had 1 year history of pesticide application. Soil samples were collected from the 3-15 cm top layer of cultivated soil from several plots of the farms. The sites were Champhoyo farm in Karonga district, Nkhozo farm (Exagris estates) in Rumphi district and Tea Estate in Mulanje district. Samples were collected in polythene bags and placed in iced cooler boxes during transportation to the laboratory and stored at 4 ^{0}C. 3 places were identified for sampling from each sampling site namely; 500m outside area of farm where no history of application of pesticide (upstream of drainage and wind), inside the farm with long history of pesticide, and downstream the drainage system of the farm.

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2.2. Types of Pesticides Used

The pesticides used in this study were cypermethrin, Dimethoate and acetochlor which were purchased from the Farmers Organisation Limited shop.

2.3. Isolation of Microorganisms

Microorganisms were isolated from soil samples using enrichment culture technique. Firstly 5 gm of soil sample was put into a 250 ml flask containing 50 ml of sterile liquid Mineral Salt Medium (MSM) with 100 ppm of pesticide was used for isolating degrading microbes. Mineral Salt Medium (MSM) containing (g/l) KH_2PO_4 (1.5), Na2HPO$_4$ (0.6), NaCl (0.5), NH_4SO_4 (2), $MgSO_4.7H_2O$ (0.2), $CaCl_2$ (0.01) 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 [22,23]. Microbes that tolerated pesticide up to 1000 ppm were considered for further studies

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2.4. Determination of Pesticide Utilization Patterns

These were modifications of Shamsuddeen & Inuwa, (2013) and Akbar et al., (2015) where by individual microorganisms that were tolerant to pesticide were 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. Negative Controls were not inoculated. The composition of MSM-1 was KH_2PO_4 (1.5), MgSO4.7H2O (0.2), Na2HPO$_4$ (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 L 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), CaCl2 (0.01), NaCl (0.5), NH4SO4 $CaCl_2$ (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), MgSO4.7H2O (0.2), KCl (0.5) NH_4SO_4 (2). Growth of microorganism which is an indicator of degradation was observed by inoculating 1 ml of individual isolate in MSM after 40 days on Czapek Dox agar plates at 28 °C. Second growth was quantified by checking isolates periodically by measurement of the turbidity at 625 nm using a spectrophotometer but for fungus development of spores or hyphae on top of media.

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2.5. Determination of Laccase Enzyme

To determine the presence of laccase enzyme production isolates were streaked on Sabouraud Dextrose Agar (SDA), amended with 1% ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-

sulphonic acid)) by streak method. Positive test for production of laccase enzyme was confirmed by development of dark green to purple colour around the colonies [24].

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2.6. DETERMINATION OF PLANT GROWTH REGULATORY TRAITS

To determine if isolates had Plant Growth Regulatory Traits (PGRT) the following biochemical tests were conducted:

2.6.1. DETERMINATION OF AMMONIA PRODUCTION

Broth cultures of isolated microbes were inoculated in 10 mL tube of peptone water and incubated at 36 ± 2°C for 48-72 hrs. There after 0.5 mL of Nessler's reagent was added. Positive test for production of ammonia was confirmed by development of yellow or brown colour [8].

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2.6.2. DETERMINATION OF INDOLE ACETIC ACID PRODUCTION

The production of IAA by isolated microbes was determined as described by Ahmad et al., (2008) with slight modification. The isolates were grown in NB amended with tryptophan (100µg/ml) set at 30 °C for 48 hours in orbital incubator while shaking at 120 rpm. Broth media with isolates was centrifuged at 3000 rpm for 30 minutes. Thereafter 2 ml supernatant was recovered and 2 drops of o-phosphoric acid and 4 mls of Salkowski reagent were added in succession. Positive test for production of IAA was confirmed by development of pink.

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2.6.3. DETERMINATION OF 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. Positive test for production of catalase was confirmed by effervescence[25–30].

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2.6.4. DETERMINATION OF HYDROGEN CYANIDE PRODUCTION

Isolated microorganisms were tested in-vitro for Hydrogen Cyanide (HCN) production by method described by Ahmad et al., (2008) with some modifications. Isolates grown in nutrient broth and Sabouraud Dextrose broth amended with glycine 4.4 g /L were inoculated on modified NA and SDA plates for bacteria and fungi respectively. Sterile Whatman filter paper No. 1 was dipped in 2.5% sodium carbonate in 0.5% picric acid solution and later placed on top of the grown cultures on agar plate. Agar Plates were tightly sealed and incubated for four days. Positive test for production of HCN was confirmed by colour change from yellow to orange-red colour on Whatman filter paper.

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2.6.5. DETERMINATION FOR THE PRODUCTION OF SIDEROPHORES

Qualitative Production of Siderophore by isolates 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 microbesforms 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 zone [8].

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2.6.6. DETERMINATION FOR NITROGEN-FIXING ABILITY

Nitrogen fixing ability of microbes was assessed using Liu et al., (2016) with some modifications where isolates were streaked on modified nitrogen deficient 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

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g agar in 1 L distilled water; pH 7.0). Plates were incubation at 28 °C for 7 days to check for growth. Growth of isolates on the media was considered as an ability to fix atmospheric nitrogen.

2.7. CELL MORPHOLOGY AND COLONIES

The isolates 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.

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2.8. IDENTIFICATION OF THE MICROBES BY GENETIC ANALYSIS

Characterizations of isolates were done by sequencing 16S rDNA gene. 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 supplied by Inqaba http://www.inqababiotec.co.za. The primers were 907R (5'- CCGTCAATTCMTTTRAGTTT-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3') and Sequencing of the isolates 16S rDNA was done by Inqaba Biotech Ltd in South Africa using Sanger sequencing.A consensus sequence of two PCR products of 16S 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 (http://blast.ncbi.nlm.nih.gov/Blast.cgi. 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 investigate the space of all possible phylogenetic trees, trying to identify those that are best taking into consideration of all possible trees to identify the best ones. Phylogenetic tree was constructed using Seaview. All sequences were deposited in the GenBank sequence database, and the accession numbers were obtained.

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3. RESULTS

All microbes that were found to grow on 100 ppm of pesticide were the same microorganism tolerating pesticide up 1000 ppm and also same microbes which utilized pesticide as sole carbon source. Isolate 3106br (2106 r) was identified to degrade both cypermethrin and acetochlor as indicated in table one and figure 1.Nine strains of bacteria were found to degrade cypermethrin *(Serratia marcescens, Pseudomonas, Leclercia sp*., 2 strains of *Klebsiella pneumoniae* and 4 strains of *Enterobacter*) while 11 strains of bacteria were found to degrade acetochlor *(Pantoeaagglomeran,* and 10 strains of *Enterobacter*) from 3 sites as shown in table 1 and 2.

Table 1: List and biochemical tests of pesticide degrading microbes isolated from selected agro ecological zones of Malawi

Comment [U45]: pesticide -degrading

LAB NO	Microbe	Site	Pesticide	PS	IAA	Gram	Amm	Catal	Sider	Shap	sole source			Lacca
											Ca	P	Ca &P	
3106r	*Enterobacter*	M	C	+	+	-	+	+	+	R	+	-	-	+
3103	*Enterobacter*	N	C	+	+	-	+	+	+	R	+	-	-	+
3100 a	*Enterobacter cloacae*	N	C	+	+	-	+	+	+	R	+	-	-	+
3100 b	*Klebsiella pneumoniae*	N	C	+	+	-	+	+	+	R	+	-	-	+
3106b	*Enterobacter asburiae*	M	C	+	+	-	+	+	+	R	+	-	-	+

r														
3104 b	*Klebsiella pneumoniae*	K	C	+	+	-	+	+	+	R	+	-	-	+
3106b	*Leclercia sp.*	M	C	+	+	-	+	+	+	R	+	-	-	+
3102	*Klebsiella oxytoca*	N	C	+	+	-	+	+	+	R	+	-	-	+
3104 a	*Pseudomonas aeruginosa*	K	C	+	+	-	+	+	-	R	+	-	-	+
2101	*Enterobacter cloacae*	N	A	+	+	-	+	+	+	R	+	+	+	+
2106 r	*Enterobacter asburiae*	M	A	+	+	-	+	+	+	R	+	+	+	+
2100a	*Enterobacter asburiae*	N	A	+	+	-	+	+	+	R	+	+	+	+
2100B	*Enterobacter cancerogenus*	N	A	+	+	-	+	+	+	R	+	+	+	+
2103-2	*Enterobacter tabaci,*	N	A	+	+	-	+	+	+	R	+	+	+	+
2103	*Enterobacter asburiae*	N	A	+	+	-	+	+	+	R	+	+	+	+
2106a	*Enterobacter xiangfangensis*	M	A	+	+	-	+	+	+	R	+	+	+	+
2104-	*Enterobacter cloacae*	K	A	+	+	-	+	+	+	R	+	+	+	+
2106b	*Enterobacter xiangfangensis*	M	A	+	+	-	+	+	+	R	+	+	+	+
2107b	*Pantoeaagglomerans,*	M	A	+	+	-	+	+	+	R	+	+	+	+
2104	*Enterobacter cloacae*	K	A	+	+	-	+	+	+	R	+	+	+	+
2105	*Enterobacter tabaci,*	M	A	+	+	-	+	+	+	R	+	+	+	+

C=cypermethrin, A=Acetochlor, B=bacteria, M=Mulanje, K=Karonga, N=Nkhozo, SI=solubilization index, Y= coccobacilli, R=Rod shaped, PS=Phosphate solubilization, Ca=carbon, P=phosphorous IAA=Indole Acetic Acid

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

Pesticide	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)
C		3104b 3104a	3104 b		3103 3100a 3100b 3102	3100b 3102	3106 3106r 3106b	3106 3106r 3106b	3106 3106r 3106b
A		2104	2104		2101 2100a 2100B 2103-2 2103	2103-2 2103	2106r 2106a 2106b 2107b 2105	2106r 2106a 2106b 2107b 2105	2106r 2106a 2106b 2107b 2105

C= cypermethrin, A= Acetochlor

The study found that natural selection is responsible for diversity of xenobiotic degradation shown by a lot of diversity in Nkhozo and Mulanje which has long history of pesticide application compared to CHAMPHOYO farm in Karonga which had a year of application as shown in table 2. These results also show that aerial application has an impact to non-target sites shown by diversity of microbes responsible for degrading xenobiotics outside the farm (table 2).

Figure 1: Isolate 3106br, 2102r (*Enterobacter asburiae)* that degrade both cypermethrin and acetochlor on NA.

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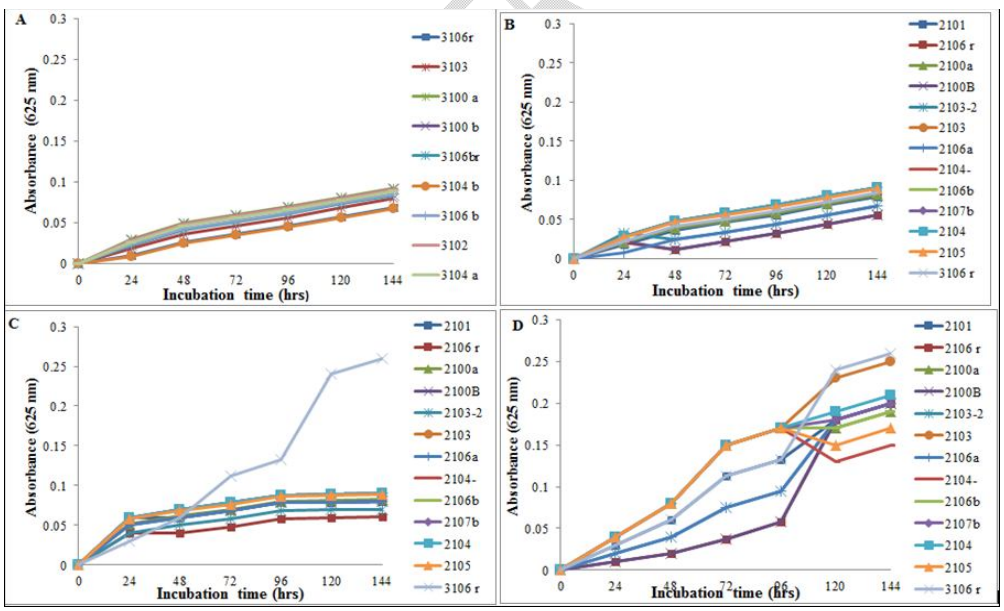

Figure 2: Growth kinetics of cypermethrin and acetochlor degrading bacteria through utilization of pesticides as sole carbon and phosphorous source for 144 hours. A: Growth kinetics of isolates using cypermethrin as sole carbon source, B: Growth kinetics of isolates using acetochlor as sole carbon and P

source, C: Growth kinetics of isolates in acetochlor as sole phosphorus source, D: Growth kinetics of isolates using Acetochlor as sole carbon source.

Results on growth kinetics show that different isolated microorganism showed different growth rates under different conditions. Microbes show high growth rate by utilisation of acetochlor as carbon source than Phosphorous source as shown in figure 2 B and 2 D. isolates showed slow growth rate in utilisation of cypemethrin than in acetochlor as carbon sources as shown in figure 2 A and 2 D. By utilising carbon from pesticide they are degrading the pesticide using laccase enzyme as shown in figure 3. All microbes that were able to utilize cypermethrin and acetochlor showed the presence of laccase enzyme as also indicated on table 1.

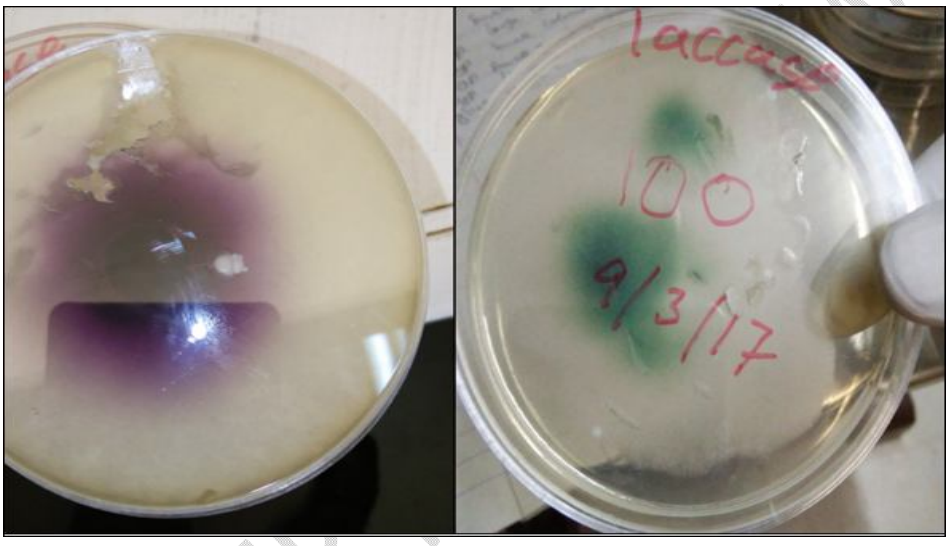

Figure 3: microbes showing presence of laccase enzymes by changing color when inoculated on Sabouraud Dextrose Agar (SDA), amended with 1% ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). All microbes that were able to degrade pesticides produced laccase enzyme

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Table 3: Isolates and their blast related species and GenBank deposit accession numbers

LAB NO	RELATED SPECIES	NUCLEOTIDE IDENTITY %	ACCESSION NUMBER
3106b	Enterobacter asburiae	99	MF979777
3106 br	Enterobacter asburiae	99	MF979662
2106b	Enterobacter cloacae	99	MF979810
2106A	Enterobacter cloacae	97	MF979821
2105	Enterobacter sp.	91	MF979964
2104-2	Enterobacter sp.	99	MF979876
2104-1	Enterobacter cloacae	99	MF979885
2103	Enterobacter sp.	99	MF980152
2103-2	Enterobacter sp.	99	MF980711
2101	Enterobacter sp.	99	MF980718

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3106B	Enterobacter sp.	98	MF980912
2107b	Pantoeaagglomerans,	96	MF980788
2100a	Enterobacter cloacae	99	MF980882
2100B	Enterobacter sp.	99	MF980911
3103	Enterobacter asburiae	98	MF980919
3100 a	Enterobacter cloacae	99	MF980916
3100 b	Klebsiella pneumoniae	99	MF980917
3106 r	Enterobacter asburiae	99	MF980922
3104 b	Klebsiella pneumoniae	98	MF980921
3102	Serratia marcescens	98	MF980918
3104 a	Pseudomonas aeruginosa	93	MF980920

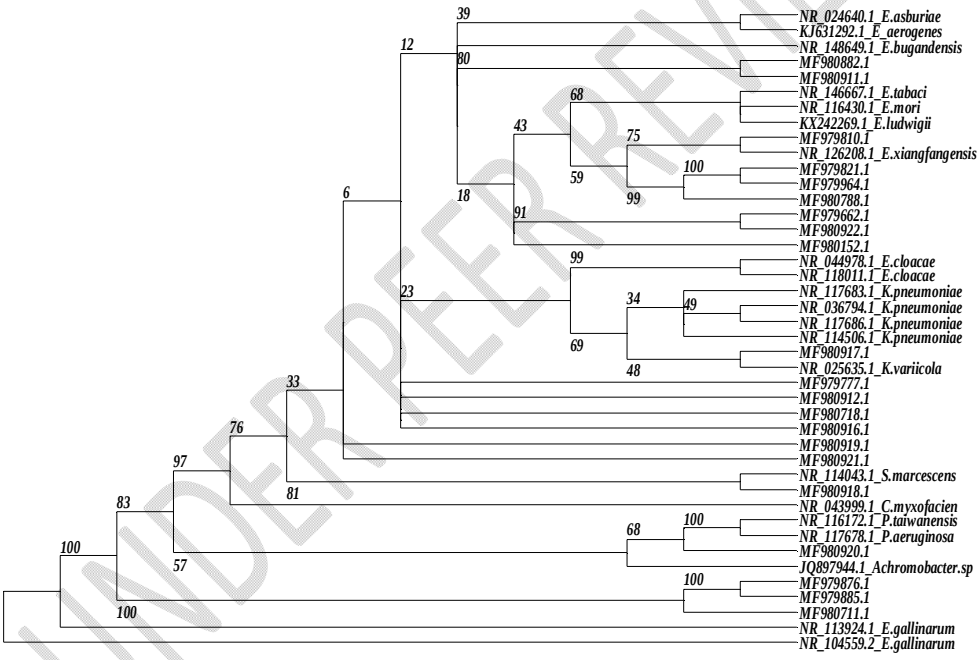

Figure 4: Phylogenetic tree based on 16S rDNA gene sequence derived from maximum parsimony analysis showing the position of cypermethrin and acetochlor degrading microbes isolated from selected agro ecological zones of Malawi and those of NCBI. Bootstrap values (%) are indicated at the nodes. Those with accession numbers with no names attached are from different agro ecological zones of Malawi as indicated on table 1, and 3 while the corresponding ones attached to the name are obtained from GenBank.

4. DISCUSSION

Potentiality of bioremediation require complex interaction knowledge of biochemical, physiological, ecological, microbiological, and molecular aspects involved in xenobiotics degradation[13]. The study found out that some microbes can utilize pesticides as sole carbon or P source or both as shown in table 1which is in line with other reports [22,32]. In this study only bacteria were found to degrade cypermethrin and acetochlor and some microbes were found to degrade both cypermethrin and acetochlor like Enterobacter asburiae table 1 and figure 1.Genus Enterobacter domination in bioremediation is in line with other studies [7,33,34].

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 by the pest table 2[35].

Higher hydrophobicity causes strong adsorption to soil of synthetic pyrethroid insecticides cypermethrin not leached [36]. Half-life of this pesticide vary from 4 days to 8 weeks and is significantly affected by soil characteristics and microbial activity [37]. The major degradation pathway of cypermethrin is 3-phenoxy benzyl alcohol and 3-phenoxy benzoic acid which acts hydrolytically by cleaving of an ester linkage. 3-phenoxy benzoic was observed by presence of layers on degrading tubes. Several microbes were isolated for degradation of cypermethrin by utilization of cypermethrin as sole carbon source which concur with other studies [22,38]; Serratia marcescens and Pseudomonas[39,40], Enterobacter [7,41,42] but no study has foundLeclerciasp and Klebsiella pneumoniae strains that can degrade cypermethrin. Thus, this finding adds strains of Leclerciasp, Klebsiella pneumoniae etc. to the list of cypermethrin degrading microbes.

However, there are reports that some of above isolated microbe can degrade other pesticides like glyphosate and others, but no study has documented that these strains degrade glyphosate in Malawi[21]. The study also found that all microbes were not able to utilize cypermethrin as sole P source. This could be because P is not active ingredient of cypermethrin and its adjuvants.

Strong mobility of Acetochlor poses an environmental risk to arable land, groundwater and surface water [43]. Acetochlor is also suspected to be endocrine disruptor and regarded as a probable human carcinogen. Half-lives of the acetochlor are 3.4 and 2.8 days in the bulk soil and rhizosphere respectively but residue of 0.02–0.07 µg/g can still be detected 40 days after its application in the soil but do not confer a long term impairment on viable bacterial groups [44]. All microbes were isolated for degradation of acetochlor by utilization of acetochlor as sole carbon source which is in support of other related studies of [22,38]. One strain *Enterobacter asburiae* was found to degrade both acetochlor and cypermethrin which was found in 2 sites which is in support of studies that associate the microbe with degradation of acetochlor [45,46].

All isolates had shown laccase production ability using SDA amended with ABTS. Results concur with other studies that document potential applications of laccases are related to bioremediation and waste treatment like degradation and detoxification of pollutants[47,48]. Laccase also plays important roles in, lignolytic degradation, detoxification studies, plant pathogenesis, odor control in decomposition of wastes and pigment production [48]. Xenobiotics induce laccase gene transcription because of its interaction with receptors in the promoter regions of the genes encoding for laccase [49].

Beside xenobiotics degrading these isolates can be used to support growth and development of crop plants because of production of multiple PGP like Phosphate solubilization IAA, Siderophore, Catalase etc.as shown in table 1 [50]. Some of these phosphate solubilising microbes (PSM) like, Klebsiella pneumonia have been documented antifungal activity towards Fusarium oxysporum, Sclerotium rolfsii, Alterneriaalternatae and Macrophominaphasiolina[51] while some might also enhance drought tolerance in plant.

Phylogenetic analysis based on the ML methods revealed that diversified divergent genera and species are involved in degradation of acetochlor and cypermethrin shown by bootstrap values as shown in figure 4. Genus Enterobacter is dominating in terms of diversity at species level and strain level in degradation of acetochlor and cypermethrin.

Phylogenetic tree shows diversity of isolates from Malawi forming unique clades separate from those of GenBank of NCBI. More than 95% of isolate unique clades were formed by those degrading Acetochlor herbicide based on agro ecological zone indicating that they have distant relationship. Many isolates formed single outgroup clades like isolate MF980152 or as group outgroup clade MF979876, MF979885 and MF980711.

5. Conclusion

The research met itsobjective in identifying indigenous strains involved in degradation of cypermethrin and Acetochlor. The study also revealed new strain of microbes involved in degradation of cypermethrin and Acetochlor 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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