

Physiological and molecular characterization of active fungi in pesticides contaminated soils for degradation of glyphosate

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Research

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Abstract

Understanding the physiological and molecular characteristics of naturally occurring fungi in glyphosate pesticide contaminated environment is crucial to managing its contamination. The study was aimed at isolating and characterizing soil fungi for their physiological roles towards glyphosate degradation. Pure cultures of fungi were isolated from soil contaminated with glyphosate at farms in Lagos, Nigeria. The cultures were grown on minimal salt agar media amended with glyphosate. The best isolates exhibiting good tolerance to the glyphosate were characterized using molecular techniques. The BLAST search indicated that the fungi belong to four *Aspergillus* species (*Aspergillus flavus* strain JN-YG-3-5, *Aspergillus niger* strain APBDSF96, *Aspergillus fumigatus* strain FJAT-31052 and *Aspergillus flavus* strain APBSWTPF130, *Trichoderma gamsii* and *Penicillium simplicissimum*. The biodegradation study of the glyphosate by the selected fungi species showed the presence of Aminomethylphosphonic Acid (AMPA) except for *Aspergillus fumigatus* strain FJAT-31052. Annotation analysis of the partial gene sequence shows that the strains possess protein coding gene clusters for glyphosate utilization and other physiological activities. The GhostKOALA output confirmed that CYP2W1 gene (Cytochrome P450, fungi type) was present in *Aspergillus fumigatus* strain FJAT-31052 which was absent in genome of other fungi. The physiological and molecular characteristics of *Aspergillus fumigatus* strain FJAT-31052 clearly show that this isolate is a useful organism for managing contamination by glyphosate pesticide.

Introduction

Glyphosate (N-(phosphonomethyl) glycine) pesticide is a widely used pesticides against broad spectrum of pests such as weeds, insects, fungi, nematodes and rodents in agriculture [1, 2]. The intensive use of pesticides has led to an increased level and risk of contamination of the ecosystem and harmful effects on biodiversity, food security, water and other resources [3, 4, 5]. Glyphosate is a highly effective pesticide because it prevents biosynthesis of some notable proteins that are needed for plant growth and it also inhibits a specific enzyme pathway known as the shikimic acid pathway. This pathway is important for plants and some microorganisms. Studies have shown that the inhibitors of the shikimate pathway enzymes are potential herbicidal because an inhibitor of a key enzyme of plant metabolism might be herbicidal without being toxic to animals. Also, this pathway operates only in plants and microorganisms [6].

The high solubility in water and strong binding capacity to soil organic matter by glyphosate is the reason for its fast and easy distribution in ecosystems compartments [7]. The half-life of glyphosate ranges from 8.3 to 141.9 days, and it has been reported to be up to 1 year in some extreme cases [8]. The differences in rates of glyphosate degradation might be due to the changing microbial activity and extent of soil-binding. The ability of microorganisms to degrade glyphosate assumes the occurrence of enzymes cleavages by utilization of glyphosate as sources of energy. According to Sviridov *et al.* [9] two pathways have been proposed for glyphosate degradation, which are the AMPA pathway and the C-P lyase pathway. In the AMPA pathway, glyphosate is cleaved into aminomethylphosphonic acid (AMPA) and glyoxylate by the presence of glyphosate oxidoreductase, whereas in the C-P lyase pathway, degradation of glyphosate is catalyzed by C-P lyase with the formation of sarcosine as an intermediate product, which in the end forms formaldehyde and glycine in a reaction catalyzed by sarcosine oxidase [9].

Microorganisms known to degrade glyphosate by way of glycine comprise of *Arthrobacter sp.* strain GLP-1 and *Pseudomonas sp.* (strain PG2982) [10]. Researches have also shown that the cleavage of the C-P bond of glyphosate to produce sarcosine and finally to glycine is mediated by sarcosine oxidase-dehydrogenase [11, 12, 13]. It has also been reported that some group of bacteria, represented by a *Flavobacterium sp.* (Strain GDI) as well as the earlier-reported mixed bacterial cultures from soil degrade glyphosate by cleaving its carboxymethyl carbon-nitrogen bond to produce AMPA [10]. Some of the AMPA generated in this way if not further metabolized is a great concern to the environment because of their potential toxicity. This has led to mounting concern of extensive contamination of the environment resulting to likely potential risks to non-target organism due to entry into the food chain.

Despite the imminent toxicity posed by environmental threats, some organisms can still withstand glyphosate. The ability of some organisms to survive and live in a polluted environment and remediate it depends on physiological, molecular, genetic and ecological traits possessed by such organisms. Fungi are extremely important for many physiological functions in environmental assessment and protection. Their physiological and molecular determination can be useful in ecotoxicity studies and ecosystem management. Based on this, we selected two farms in Lagos Nigeria where glyphosate is used as herbicides, isolated the fungi in pure culture, grow them in the medium using glyphosate as energy and study their physiological and molecular roles towards glyphosate degradation.

Materials And Methods

2.1 Soil samples collection

The soil samples used for this study were obtained from two farms: Abeto Farm and Igbalu Farm in Ikorodu, Lagos, Nigeria. The selected farms have a history glyphosate organophosphorus herbicide for the past 5-6 years to control pests. Glyphosate is effectually still used to control pests at the farms. Soil samples were collected from four different points in each farm's location at 100 metres apart as described by Asef [14]. The soils were collected using a spatula at a depth of 15 cm and transferred to sterile containers. The soils samples were transported to the laboratory and stored at 4 °C until further analysis. Soil samples were air dried 24 hours and sieved through a 10mm mesh prior to screening.

2.2 Quantification of glyphosate content and metabolites

Pesticides and their metabolites were measured and quantified using Gas chromatography as described by Moye and Deyrup [15]. Samples (about 2.0 g) were extracted with 10 mL of an aqueous solution containing 0.25 M NH₄OH and 0.1 M KH₂PO₄. The samples were shaken on a mechanical shaker for 60 min. A 2-mL aliquot of the supernatant was withdrawn using a 0.45 µm syringe filter. This extract was stored in GC vials, before being derivatized. Aliquots (1.6 mL) of the derivatization reagent mixture (by mixing 1 volume of Heptafluoro butanol (HFB) to 2 volumes of Trifluoroacetic anhydride (TFAA) were added to 2 mL GC vials. The vials were sealed using screw caps with septa. The vials were then cooled to about -4 °C before proceeding. A variable volume Eppendorf pipet was used to add a 36 µL aliquot of sample extract (or a dilution of the extracts), or standard solution to the derivatizing reagent. Analyte derivatization was then performed by heating the reaction vials to 95 °C for 1 h; and then cooled to room temperature. The excess derivatization reagents were evaporated under a gentle stream of nitrogen. The residue was dissolved in 200 µL of ethyl acetate, capped and stored for GC analysis.

Chromatographic analysis was performed using an Agilent GC/MS, equipped with a split/splitless injector, and an autosampler. The analytes (glyphosate and AMPA) were quantitated using standard method with calculations based on peak area. Analyte quantitation was carried out with Agilent Mass-Hunter GC software [15]. Also, aminomethylphosphonic acid (AMPA), a primary degradation product of glyphosate in the samples were assayed using the method of Ermakova *et al.* [16]. Glyphosate calibration stock solution was made by diluting aliquots of the herbicide concentrate to known amounts of the analyte in water. Working calibration solutions of 1, 10 and 100 µg/mL were made by serially diluting the stock solution as needed.

2.3 Soil incubations, isolation and colony characteristics

The isolation of glyphosate degraders from soil samples was done to screen for strains that could degrade glyphosate in liquid enrichment medium. Ten grams of soil sample was weighed on analytical balance. One gram of the sample was transferred into 90 ml of sterile Minimum Salt Medium (MSM) broth in a 250 ml of Erlenmeyer flask respectively and incubated at 30 °C in a gyratory shaker for seven days. After the incubation period, 1.0 ml of sample was withdrawn and was serially diluted using 9 ml amount of sterile distilled water up to 10⁵ dilution. All the replicates of each soil type and treatment were treated separately for isolation purposes. Pesticide degraders were enriched in the dark on a shaker at 180 rpm for seven days. The colony characteristics were examined. A second enrichment was done thereafter as described below by transferring pre-grown culture from each of the replicates to the fresh media.

2.4 Enrichment of glyphosate degraders

The fungi isolated from the soil samples were afterward used for the enrichment of potential glyphosate degraders. These media were further treated separately with glyphosate to enrich for glyphosate degraders. The enrichment of glyphosate degraders was carried out in liquid media by dissolving the components in 1000 ml of distilled water and adjusting the pH of the basal medium to 6.0 using 1 M NaOH Solution. 150 ml of the basal medium was dispensed into 250 ml Erlenmeyer flask and the pesticide substrate was introduced into each flask at 100 ppm after sterilization in an autoclave at 121 °C for 15 minutes. 1.0 ml aliquot of diluted broth culture of each isolate (10⁴ cells/ml) were seeded into each flask and incubated in a gyratory shaker incubator at 150 rev/min at 30 °C for a period of 32 days. The growth and enrichment ability were monitored at four-day intervals. The utilization of the pesticide fractions by the fungal isolates was evaluated by monitoring the fungal growth measured by viable count on PDA, the Optical Density (OD) at 620nm wavelength with 770 UV/Visible Light PG Spectrophotometer and changes in ionic concentration pH was determined with pH meter (model P2II) [17].

2.5 Degradation of glyphosate

The medium was supplemented with glyphosate (100 ppm). The bacterial isolates were first grown in the medium supplemented with glyphosate to determine activity of fungi in the degradation of glyphosate.

The percentage loss of the pesticide was calculated as:

See formula 1 in the supplementary files.

While the efficiency of biodegradation was calculated using the formulae: $LT - LC$ (Where LT is % loss of pesticides in treatments and LC is % loss of pesticides in control).

2.6 Molecular characterization of isolates

2.6.1 Colony DNA extraction and amplification by polymerase chain reaction (PCR)

Genomic DNA was extracted from the isolates using a Zymo Quick-DNA Fungal/Bacteria Micro-prep kit. After extraction, the DNA concentration and purity were checked using a ThermoScientific Nanodrop, model 2000. The extracted DNA were amplified with primers for genes used for identifying fungi. The Internal Transcribed Spacer (ITS) gene for characterization of fungi, ITS universal primer set which flanks the ITS4, 5.8S and ITS5 region was used:

- ITS4 TCCTCCGCTTATTGACATGS
- ITS5 GGAAGTAAAGTCGTAACAAGG

Polymerase chain reaction (PCR) conditions was set on an initial denaturation temperature of 94 °C for 5 minutes, followed by 35 cycles of 30 seconds denaturation at 94 °C, 30 seconds annealing of primer at 55 °C, 1½ minutes extension at 72 °C and a final extension for 7 minutes at 72 °C [18].

The amplified fragments were purified by ethanol in order to remove the PCR reagents, before they were sequenced using Applied Biosystems Genetic Analyzer 3130xl sequencer and Big Dye terminator v3.1 cycle sequencing kit. Bio-Edit and MEGA 6 were used for all genetic analysis.

The classification/identification of organisms were performed by a local nucleotide BLAST search against the non-redundant version of the NCBI ref database [19]. Phylogenetic relationship analysis was performed on the sequences of isolated microorganisms using Molecular Evolutionary Genetics Analysis version 6 [19]. The sequences were prepared using FASTA format and aligned using ClustaW option of the program [19]. The phylogenetic dendrogram was constructed using the maximum likelihood with 1000 bootstrap.

2.6.2 Genome sequence annotation

The annotation of protein-coding genes was provided by FGENESB; further gene prediction and functional annotation were performed by [GhostKOALA](#). The pathways of selective compounds were interpreted using KEGG pathway chart.

2.7 Statistical analysis

Data sets were analyzed using origin software and GraphPad prism 8.04. Comparative of means were done using descriptive statistics. Significant means were compared using Tukey's multiple T-test and two-way analysis of variance (ANOVA). All graphical presentations were done using origin software. Correlation analysis was done to compare the relationship between pesticide degradation, colony count, optical density and pH. Biodegradation study of glyphosate was expressed as percentages, evolution of AMPA and glyphosate and as co-efficient of degradation using descriptive statistics. Molecular identification and characteristics were done using e-values and percentage identity.

Results

3.1 Characterization of the soil from the locations

All the locations had evidence of glyphosate contamination though the contamination levels varied depending on location (Figure 1) and they were significant ($p < 0.05$). Location 1 had the highest contamination comprising 319.1 mg/kg glyphosate and 194.2 mg/kg AMPA. Location 3 showed increased glyphosate level without transformation product (AMPA). This could suggest the active state of the organisms present. Among the locations, the lower level of glyphosate was observed more rapid in location 4 with only traces of glyphosate present (6.98 mg/kg).

Correspondingly, the fungal density varied within the location (Figure 2). There was high enumeration of fungal count in locations where AMPA concentration was high and with significant concentrations of glyphosate. However, other locations where AMPA concentration was low or absent had low fungal count. This implies active metabolic state of the fungi. As a result, this study evaluates the relationship between the fungal density, glyphosate and AMPA. The Pearson correlation shows that fungal load is significantly related to AMPA ($r = 0.94965$; $p \leq 0.05$) in comparison to glyphosate.

3.2 Selection and isolation of glyphosate degraders

A total of 14 isolates were obtained from the farms (Table 1). The fungal inoculum from soils enrichment were plated on MSM agar plates to check for their ability to grow in the presence of glyphosate on solid media. It was observed that S1b, S1c, S2.3, S3.2, S3.3, S4.1 and S4.4 isolates did not grow from any of the enriched soils on MSM agar when glyphosate was added externally, during the set incubation time. They displayed poor (+) clear zone, therefore, no further analysis was conducted on them. The isolates that showed good (++) clear zone (S1a, S1d, S2.1, S3.1, S4.1 and S4.3) were further analyzed for their ability to degrade glyphosate. A total of six (6) potential degraders were obtained after successive sub-culturing from the soils (Table 2).

3.3 Enrichment of glyphosate degraders

We use optical density (OD) at a wavelength of 600nm (OD₆₀₀) in addition to colony forming units (cfu) to evaluate microbial enrichment and growth in liquid fungal culture (figure 3). Several distinct growth phases were observed within the fungal growth curve, and these were the lag phase, the exponential or log phase, the stationary phase, and the death phase. Each of these phases represents a distinct period of growth that is associated with typical physiological changes in the cell culture.

Six fungal isolates were stimulated to grow in the presence of glyphosate. The glyphosate mixed with MSM showed enhanced growth of the fungal isolates as shown by their optical density and cfu. The logarithmic growth phase for OD₆₀₀ lasted for 8 days in all the fungal isolates while for cfu, it lasted for 24 days. The stationary phase commenced after day 8 in growth curves for OD₆₀₀ till end of the experiment whereas the cfu had a very sharp stationary phase 24 to day 28. The exponential growth (log phase) for cfu was observed to be biphasic with a transition from an initial to a subsequently slower rate of growth leading to the stationary phase. The first exponential phase was between 8 days and 12 days, while the second exponential phase was between 16 day and 24 days. The OD₆₀₀ growth did not have death phase while cfu had a death phase after 28th day of incubation until the end the experiment. This discrepancy between OD₆₀₀ and cfu could be from the hypothesis that optical density measurement is the quantity of viable and non-viable cells in a sample. The colony forming unit measures only the viable cells in a sample. An OD 600nm is an approximation CFU/ml and cannot fully take into account the non-viable cells the OD may be reading. It may also depends on the microorganism that is being researched.

Consequently, the specific growth rate estimated as a function of first order kinetics revealed *Trichoderma gamsii* P2-18, *Aspergillus flavus* JN-YG-3-5, *Aspergillus niger* APBDSDF96, *Aspergillus fumigatus* FJAT-31052, *Aspergillus flavus* EFB01 and *Penicillium simplicissimum* SNB-VECD11G have 0.47, 0.40, 0.57, 0.49, 0.41 and 0.46 rate respectively. From these findings, the growth of fungal isolates, *A. fumigatus* FJAT-31052 and *A. flavus* EFB01 were promoted and can grow in glyphosate whereas, *T. gamsii* P2-18 had the lowest growth promotion compared to other fungal isolates. Hence, specific growth values at the two selected points were calculated for both phases of growth and are given in Table 3 below. The data shows that the specific growth rates are comparable for the two different exponential phases. From these findings, the order of the growth of the isolates as estimated from slope of cfu growth curve are as follows: *P. simplicissimum* SNB-VECD11G > *A. fumigatus* FJAT-31052 > *A. flavus* EFB01 > *A. niger* APBDSDF96 > *A. flavus* JN-YG-3-5 > *T. gamsii* P2-18 (0.23 cfu/day, 0.2282 cfu/day, 0.2257 cfu/day, 0.2234 cfu/day, 0.2195 cfu/day and 0.2171 cfu/day) respectively. The growth of fungal isolates, *A. fumigatus* FJAT-31052 and *A. flavus* EFB01 were more promoted in glyphosate compared to others whereas, *T. gamsii* P2-18 had the lowest growth promotion. Therefore, we hypothesize that these isolates biodegradable ability to glyphosate will differ.

In this study, the initial pH for all the isolates were weakly acidic (within 6.0). Thereafter it declined gradually and became more acidic at the end of the experiment. The change in pH was more obvious in *A. flavus* EFB01 (22.06%) and lowest in *A. flavus* JN-YG-3-5 (19.21%). From this, we can hypothesize that fungal growth are inversely proportional with the pH. This suggest that fungal are more active in slight acidic environment compared to basic environment.

3.4 Degradation of glyphosate

The potential ability of the six fungal strains for glyphosate biodegradation were observed for 32 days (Figure 4). Our hypothesis is that these selected isolates ability to degrade glyphosate will differ because their growth in glyphosate differed. The strain *T. gamsii* P2-18 sp. degraded 91.45% of glyphosate leaving 930.81mg/kg of AMPA. In addition, it was observed that there was 92.07% glyphosate degradation when inoculated with *A. niger* APBDSDF96 leaving 113.53 mg/kg AMPA . Interestingly, *A. flavus* JN-YG-3-5 utilized 92.86% without accumulation of AMPA; this had the highest extent of degradation.

Overall, an analysis of the degradation efficiency of the fungi strains in glyphosate degradation showed that the isolates were efficient degraders with percentage degradation above 90% (Figure 5). However, *A. flavus* EFB01 had the poorest percentage degradation (27.17%) indicating poor metabolism of glyphosate. The degradation efficiency of *A. flavus* JN-YG-3-5 was the most efficient fungi (85.6%).

3.5 Molecular characteristics

The molecular characteristics of these promising isolates are shown in Table 5. BLAST analysis (ITS gene sequence) carried out through NCBI GenBank showed that the first two bacterial sequences were identified as strains of *T. gamsii* P2-18 (94.57% similarity) and *A. flavus* JN-YG-3-5 (99.28% similarity), respectively. Other isolates were identified as *Aspergillus niger* APBDSDF96 (95.22% similarity), *A. fumigatus* FJAT-31052 (99.30%) similarity, *A. flavus* EFB01 (99.29%) similarity and *P. simplicissimum* SNB-VECD11G (89.91) similarity. The isolates had high level of GC contents ranging from 53.54% in *P. simplicissimum* SNB-VECD11G to 58.66% in *Aspergillus flavus* JN-YG-3-5 suggesting their potential for environmental management.

The ITS gene sequence showed that all the six isolates clustered into three group (*Penicillium sp.*, *Trichoderma sp.* and *Aspergillus sp.*) (Figure 6) for phylogeny analyses of the isolates. *Aspergillus flavus* JN-YG-3-5 clustered with genus *Aspergillus flavus* EFB01 showing similarity, they distantly clustered with *Aspergillus niger* APBDSDF96 and *Aspergillus fumigatus* FJAT-31052. However, *Trichoderma gamsii* P2-18 and *Penicillium simplicissimum* SNB-VECD11G out clustered.

3.6 Bacteria genome annotation

Automated annotation identified several genes using a statistical significance threshold (Table 6). The genome sequences of the fungi were compared to those of several organisms (*Archae generic*, *C. pefringes*, *B. subtilis* and *P. putida*) known to function in metabolic processes. Validation of the sequence annotation using the FGENESB database yielded the following result: *Rhizobium huautilense* comprises 5 potential protein coding genes, 1 operon and 4 transcription units. *Pseudomonas aeruginosa* strain MZ4A contains 11 protein genes, 1 operon and 7 transcriptional units. *Pseudomonas aeruginosa* strain 22ABUH7 had 5 protein genes, 1 operon and 3 transcriptional units. *Bacillus subtilis* strain VBN01 had 8 protein genes, 1 operon and 5 transcriptional units. *Pseudomonas aeruginosa* strain HS-38 sequence was made up of 6 potential protein coding genes, 1 operon and 5 transcriptional units. *Pseudomonas aeruginosa* strain MZ4A and *Pseudomonas aeruginosa* strain HS-38 had potential protein coding genes similar to *Pseudomonas putida* while others did not. A search of the identified proteins for specific functions revealed that the genes are distributed in different functional categories majorly protein metabolism and respiration (Table 7). Numerous genes associated with pesticide degradation were identified.

Discussion

Our findings showed that the topsoil from the farms in various locations contain residues of herbicide chemical glyphosate and its metabolite, AMPA. This can be attributed to the over-reliance of this chemical in agricultural practices. However, the concentration of this glyphosate in the field was found to be relatively higher than published work on Environmental Health Criteria 159 under the sponsorship of United Nations Environment Programme, the International Labour Organisation, and the World Health Organization [7]. Therefore, this calls for serious remedial action to be taken as the accumulation of glyphosate is likely to pose serious danger to ecological receptors.

Notwithstanding the diversity of organisms present in the contaminated site, our interest was majorly on fungi as little is known on their role for biodegradation of glyphosate. There was high enumeration of fungal count in location where AMPA level was remarkably high as well as relatively high level of glyphosate compared to lower levels. This was also supported by the pearson correlation which shows high correlation with AMPA. A range of bacterial strains have been implicated to be abundant in glyphosate contaminated environment either because of their capability of using the compound as sole source of phosphorus, carbon or nitrogen [20]. As such they play a role in degradation. Therefore, investigation of the role of diversities of fungi in the degradation of glyphosate can be remarkably interesting.

In order to isolate potential fungi to degrade glyphosate we observed the enhanced growth of these microbes from the four soil locations. Only six isolates from all the location demonstrated enhanced growth in the presence of glyphosate. This shows their ability to use glyphosate as an energy source. However, the inability of the other isolates to survive could be the toxic effect of glyphosate on the organisms. Eman *et al.* [21] noted that application of pesticides has the possibility to exert some effects on non-target organisms, plus the soil microorganisms. Presence of pesticides makes some microorganisms to lyse while other microorganisms may be resistant and tolerant to a pollutant, hence, increase in their numbers and biomass due to decreased competition [21].

In studying the effect of glyphosate on the activities of the fungi in the enriched medium supplemented with glyphosate, the pure isolates induced changes in the medium such as changes in pH, optical density, and fungal counts. The decrease in the pH levels of the culture medium may be as a result of microbial metabolism and production of secondary metabolites. Analysis of supernatants by Montserrat *et al.* [22] also demonstrated a decrease in pH resulting from rapid production of lactic, acetic, pyruvic and citric acids. The implication is that such changes in pH can influence bacteria growth. For instance, Yang *et al.* [23] found out that pH level of culture medium was one of the key factors influencing the growth of four bacteriocinogenic strains. Furthermore, LeBlanc *et al.* [24] stated that the growth of *Lactobacillus*

fermentum CRL 722 was noticeably slower at pH 4.5 ($\mu_{\max} = 0.78 \text{ h}^{-1}$) than at other pH values including pH 5.0, 5.5, and 6.0 ($\mu_{\max} = 1.15 - 1.25 \text{ h}^{-1}$). This is in agreement with results obtained in this study which shows that optimum growth of fungi under glyphosate was obtained at approximately 5.0. This might also influence bioremediation activity. Therefore, changes in metabolic state of the fungal can be the driving force of the pH suggesting active state of the fungal cells. Longer lag phase (16 days after inoculation) could suggest that fungal enrichment in glyphosate treated medium may be slower than bacteria. However, efficiency in transformation of glyphosate need to be evaluated. The short exponential phase could suggest active state of the fungi towards glyphosate remediation. Stratton and Stewart [25] observed a small rise in microbial biomass but no negative or positive effects in respect to the number of microorganisms. In addition, Haney *et al.* [26] and Busse *et al.* [27] assessed the effect of glyphosate on soil's microbial community and their findings concluded that microbial activity was stimulated even in the presence of this herbicide. Therefore, it is prospective that the glyphosate provided nutrients for fungal growth, as shown by the significant growth and increase in microbial population.

Glyphosate is a nonselective, broad-spectrum, post-emergence herbicide that is widely used in agriculture; hence, degradation of the compound will be a positive obligation in agricultural practices. It was evident that the isolates of fungal strains degraded glyphosate. The growth ability of the fungal strains could ascertain a significant assimilation of glyphosate. One reason could be that the fungal strains exhibited optimal growth rates, in order to potentially adapt to the glyphosate concentration and to assimilate it. Also, it is possible that they possess enzymes capable of cleaving the C-P bond. It is remarkable to mention that this assimilation took place without enhancement by sucrose, nitrogen (N), and phosphorus source. For example, Eman *et al.* [21] reported that a concentration of 1% sucrose was important for the initial stimulation of fungal strains in glyphosate degradation. Studies reported that the development of enhanced degradation of xenobiotics or pollutants depends on multiple factors such as nutrient composition, chemical structure, soil properties including the presence of degrading microbes with appropriate metabolic functions [28, 29]. Our aim was to isolate the key player of glyphosate degrading fungi from soils which demonstrated rapid degradation capability. The intensity of glyphosate biodegradation with the indigenous microbial pure strain was highest in *A. flavus*JN-YG-3-5 which utilized 92.86% without accumulation of AMPA. Thus, this makes it so interesting for environmental application. Other strains had high capability but produced AMPA which might be detrimental to the environment.

Aspergillus sp have received tremendous interest for their suitability in bioremediation [30]. This could be the reason scientists and environmentalist are interested to develop various strategies for the use of *Aspergillus sp.* in bioremediation. This species will be useful in pesticide contaminated soil. Different species of fungi were identified using BLAST analysis. The high abundance of *Aspergillus* species in the samples may be due to their ability to tolerate and degrade pesticides. Similar studies have been conducted by Asef, [14] and have revealed the isolation, molecular characterization and pesticide degradation by *Aspergillus* species. Thus the reason *Aspergillus sp* have received tremendous interest for suitability to remediate wide range of xenobiotic compounds. The identified fungal strains observed in this study have high GC contents. This is likely to have made them tolerant to pesticide. One imperative property of the GC base pair is its higher thermal stability than the AT base pair. An increase in GC content correlates with a broader tolerance range of species [31].

The range of GC contents in the fungi suggests characteristics of microbe from soil. *Aspergillus flavus* JN-YG-3-5 can be a particularly important tools for use in biotechnology because it yielded high pure DNA quantity and has a GC content similar to well-known GC in soil for active physiological functions. The works of Smarda *et al* [31] and Njoku *et al.* [18] reported that GC-rich genes facilitate the response to environmental stress. In addition, it can also facilitate complex gene regulation. Thus, improved responses to environmental conditions might be enabled by GC-rich genes. The fungi having higher GC contents were better in glyphosate degradation thus giving a beneficial advantage to be utilized in a wide range of environmental applications. This could have also been an added advantage to *Aspergillus flavus* JN-YG-3-5 for complete mineralization of glyphosate pesticide.

Phylogenetic analysis explicitly showed that the polluted soil sheltered diverse fungi population belonging to three clusters of orthologous groups with *Aspergillus flavus* JN-YG-3-5 clustering together suggesting their similar ancestry. It can also be due to combination of selective factors, proximity and functional capacity [32]. The different groups that they belong to does not necessarily mean that they degraded the contaminant through different processes. It has been hypothesized that phylogenetically distant lineages might share mutual functions and functional features. The work agreed with the work of Ning and Beiko [32] who reported that functional similarities exist between operational taxonomic units (OTUs) that belong to different high-level taxonomic groups for fungi.

Automated annotation identified several proteins within the genome of fungal strains to include ABC transporters, these are members of a protein superfamily known to be involved in the efflux of drugs from the cells of target organisms. Also, the Zinc finger protein gene and zinc finger chimera 1, were discovered along with many oxidoreductase genes. A search of the identified proteins for specific functions revealed that the genes are distributed in different functional categories majorly protein metabolism and respiration. Numerous genes associated with pesticide degradation were identified.

Interestingly, The GhostKOALA output identified CYP2W1 gene (Cytochrome P450, fungi type) present in *Aspergillus fumigatus* strain FJAT-31052 which was absent in genome of other fungi. The cytochrome P450 enzymes are monooxygenases which catalyze many active reactions involved in the metabolism of wide variety of xenobiotics [33]. Previous studies have reported the activities of human CYPs involved in the metabolism of pesticides [34, 35]. CYP-pesticides interactions are by either the induction or inhibition of the metabolizing enzymes. In a study by Khaled *et al.* [33] HepaRG cells express a large panel of liver-specific genes including several CYP enzymes, which contrasts with HepG2 cell lines. Both immunoblotting and reverse transcription polymerase chain reaction (RT-PCR) techniques have been used to examine the pesticide-CYP induction [33, 36, 37].

Although fungi have received tremendous interest for their suitability in detoxifying a variety of contaminants, its ability to degrade glyphosate is a new area of research interest. Two different routes have been proposed to be utilized by soil microorganisms to metabolize glyphosate: The C-P lyase and AMPA pathways [9]. To demonstrate the valid pathway, identification of AMPA even to a significant amount shows that AMPA pathway is valid. This mechanism involves the oxidative cleavage of the C-N bond on the carboxyl side catalyzed by glyphosate oxidoreductase (GOX) which results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate. The mechanism for detoxification of glyphosate was suggested by activities of certain enzymes that catalyze the reaction such as: oxidoreductases that cleave C-N with stoichiometric formation of glyoxylate and aminomethylphosphonic acid (AMP) [6]. Aminotransferase which catalyzes the conversion of AMP to phosphonoformaldehyde. Phosphonatease which catalyzes the cleavage of phosphonoacetaldehyde C-P bond to form acetaldehyde. It was evident and validated from the annotated gene results that dehydrogenase/oxidoreductase related pathway is valid by the presence of dehydrogenase related protein (alcohol dehydrogenase) discovered in their genome.

Conclusions

In this study, novel glyphosate-degrading fungi strains were isolated from farm soils in Nigeria. All strains used for enhanced biodegradation grew in the presence of glyphosate and were able to degrade glyphosate. This is the first report to show fungal degradation of glyphosate from Nigerian soil. The use of these indigenous fungal strains promises to be effective in practical application of bioremediation of glyphosate since the microbes have already adapted to the localized habitat conditions. The essence of this is that isolated strains can also be added to other soils as microbial inoculants for their potential to degrade pesticides by improving soil quality for sustainable agriculture and environment. This study has provided strains with biodegrading genes, enzymes and pathways to be harnessed for a range of biotechnological and bioremediative applications. It provides novel insights into specialised organisms for active bioremediation. The physiological and molecular characteristics shows that *Aspergillus* species are useful organism for managing contamination by glyphosate pesticide.

Declarations

Availability of data and materials

All generated or analysed data during this study are included in this published article and are also available from the corresponding author upon request.

Competing Interest

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' Contribution

KLN, EOP and AAA carried out the design and development of the research idea. EOP and EOU carried out the biological and technical experiments. AOO, AAA and KLN carried out formal analyses and review. All the authors read, agreed and approved the final manuscript made by AAA.

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Tables

Table 1: Summary of the fungal culture characteristics

Isolate code	Colour/pigment	Growth of Isolate on AGAR+ Herbicide	Substrate specificity (Optical Density at 620nm)		
			Day 0	Day 4	Day 7
S1a	Greenish Yellow	++	0.056	0.261	0.569
S1b	White	+	0.034	0.133	0.166
S1c	Green	+	0.042	0.158	0.386
S1d	Black	++	0.063	0.246	0.399
S2.1	Yellow	++	0.052	0.258	0.421
S2.2	Brown	++	0.076	0.274	0.481
S2.3	Green	+	0.039	0.144	0.383
S3.1	Green	++	0.048	0.261	0.493
S3.2	Green	+	0.035	0.147	0.171
S3.3	White	+	0.041	0.166	0.352
S4.1	Green	++	0.059	0.241	0.581
S4.2	Grayish Green	+	0.033	0.193	0.236
S4.3	White	++	0.038	0.186	0.311
S4.4	Brown	+	0.061	0.263	0.461

Key: + poor clear zone, ++ good clear zone; S1 - Soil 1; S1a, S1b and S1c (1st, 2nd and 3rd isolates from soil 1); S2 - Soil 2; S2.1, S2.2 and S2.3 (1st, 2nd and 3rd isolates from soil 2); S3 - Soil 3; S3.1, S3.2 and S3.3 (1st, 2nd and 3rd isolates from soil 3); S4 - Soil 4; S4.1, S4.2, S4.3 and S4.4 (1st, 2nd, 3rd and 4th isolates from soil 4).

Table 2. The selected isolates, locations and identities.

Selected isolate	Source	Organisms Name
S1a	Location 1	<i>Trichoderma gamsii</i> P2-18
S1d	Location 1	<i>Aspergillus flavus</i> JN-YG-3-5
S2.1	Location 2	<i>Aspergillus niger</i> APBSDSF96
S3.1	Location 3	<i>Aspergillus fumigatus</i> FJAT-31052
S4.1	Location 4	<i>Aspergillus flavus</i> EFB01
S4.3	Location 4	<i>Penicillium simplicissimum</i> SNB-VECD11G

Table 3: Specific Growth of Fungal Isolates grown on PDA at 30C

Organisms	Initial	Subsequent
<i>Trichoderma gamsii</i> P2-18	0.66	0.61
<i>Aspergillus flavus</i> JN-YG-3-5	0.67	0.62
<i>Aspergillus niger</i> APBSDSF96	0.62	0.65
<i>Aspergillus fumigatus</i> FJAT-31052	0.63	0.63
<i>Aspergillus flavus</i> EFB01	0.61	0.60
<i>Penicillium simplicissimum</i> SNB-VECD11G	0.65	0.61

Table 4: Summary of pH changes with respect to days

Sample code	Day 0	Day 4	Day 8	Day 12	Day 20	Day 24	Day 28	Day 32	% change in pH (Initial - Final OD)
<i>T. gamsii</i> P2-18	6.06	6.00	5.00****	5.76	5.30**	5.00****	4.86****	4.75****	21.62
<i>A. flavus</i> JN-YG-3-5	6.04	5.96	5.80	5.66	5.44	5.24**	5.06***	4.88****	19.21
<i>A. niger</i> APBSDSF96	6.06	5.99	5.84	5.74	5.33**	5.16***	4.92****	4.79****	20.96
<i>A. fumigatus</i> FJAT-31052	6.05	6.01	5.81	5.63	5.22**	5.03****	4.89****	4.73****	21.82
<i>A. flavus</i> EFB01	6.03	5.96	5.82	5.50	5.10***	4.96****	4.83****	4.70****	22.06
<i>P. simplicissimum</i> SNB-VECD11G	6.04	5.95	5.78	5.46	5.18****	5.00****	4.90****	4.82****	20.20

Values with asterisk have significant difference between the day of observation and day 0. * = p<0.05; **= p<0.001; ***= p<0.0001; ****= p<0.0000. % change in pH is from difference between day 0 vs day 32

Table 5: Molecular characteristics of the isolates

Organisms Name	Identity (%)	E-Value	Sequence length (Bp)	% Guanine-Cytosine
<i>Trichoderma gamsii</i> P2-18	94.57	0.0	589	56.71
<i>Aspergillus flavus</i> JN-YG-3-5	99.28	0.0	612	58.66
<i>Aspergillus niger</i> APBDSDF96	95.22	0.0	590	58.31
<i>Aspergillus fumigatus</i> FJAT-31052	99.30	0.0	583	57.98
<i>Aspergillus flavus</i> EFB01	99.29		588	56.8
<i>Penicillium simplicissimum</i> SNB-VECD11G	89.91	2e-147	579	53.54

Table 6: Gene Statistics using FGENESB

1	Closest organism	No of Predicted protein coding genes	No. of Operons	No of Transcript units	Predicted proteins
P2-18	<i>C. pefringes</i>	4	1	2	MPVRASFQPSNPPGRRWGSQTPQTGSRPRNTVAVSPQPLLRSSLHNSHREERGASTCRKTP MTLGQACPEYWRAQCAFKDSMIH MPARILAGAMCVQRFDDSLNSAIHITYRISLRSSSMPEPRDPLLKVLHFDFLLRAVKIT SARGLQ LVGPSAAPGSGLRRTTRGVTTPRAQFGMFTWVGVTNRNDPSAGSQRNLYIFYF
JN-YG-3-	<i>C. pefringes</i>	2	0	2	LRPLVFRGACLSERHCCPSSTACVLGRRPLSGGDGPQRQRHRVRSSSVWGFVTRSVGPA GAARTQINLTT MGSNDARTGMPPGIPGGAMCVQRLDDSRNSAIHTSYRISLRSSSMPEPRDPLLKVLTD TINSDFTSRDRVGVSGGRGPGAESPRRP
3SDSF96	<i>C. pefringes</i>	2	1	1	MVGIRRQAPANPTEHVTKPHTLEDTRTRCRRCLSGPSRRGGRRPNTQAGLEGSNDARTGM PPGIPGGAMCVQRLDDSLNSAIHISYRISLRSSSMPEPRDPLLKVLTD VFVLSGSPAGTGGGQRRP LGPKGPGTRVMILPQGSPTETLGYDF
tus 52	<i>C. pefringes</i>	2	0	2	MGLCHLLCRPGRRQPTPNFIY VTKPHTLEDTRTRCRRCLSGPSRGRGGRPNTQAVLEGSNDARTGMPPGIPGGAMCVQRLD DSLNSAIHITYRISLRSSSMPEPRDPLLKVLTDYDNQLRLHFRFRTAFMLGSSAGAGPGAQ GLPGGRRNGGPAEATRYDRHGWEVGTGPSLGNTPSAGSPTETLLRFFTS
EFB01	<i>C. pefringes</i>	2	0	2	LRPLVFRGACLSERHCCPSSTACVLGRRPLSGGDGPQRQRHRVRSSSVWGFVTRSVGPA GACRTQINLST MGSNDARTGMPPGIPGGAMCVQRLDDSRNSAIHTSYRISLRSSSMPEPRDPLLKVLTD TINSDFTSRDRVGVSGGRGPGAESPRRP
simum D11G	<i>C. pefringes</i>	5	2	3	LLYNRLIGPTERTVNTIRSRTRGRGAAAFRARPAGGRSPTHKPA VPPLPFGPAPREPGGEAQHTSRLEGSNDARTGMPPEIPGGAMGVQRLDVSLISDIQLRID FAAFFIEAEQKTVEV VLSYLCGAPSRPPGASAPGPCREDTNNSVEDAVEQIS MPPAAVRGPPNTGTITRGRWPRGLPECPSSRLDGCTNGIPPRGPIF MHARPTGVQRFRCPLQAPAFRGCHCLVPVFEFAAPPVRAAIMRRL

Table 7: Gene predictions

<i>T. gamsii</i> P2-18	<i>A. flavus</i> JN-YG-3-5	<i>A. niger</i> APBSDSF96	<i>A. fumigatus</i> FJAT-31052	<i>A. flavus</i> EFB01
NMS; neuromedin S	Granulins isoform	guanylate kinase	glycosyltransferase	A-kinase anchor protein
NUDIX hydrolase	Phosphoinositide phosphatase	metastasis suppressor protein	Zinc finger protein	Histone-lysine N-methyltransferase
modular polyketide synthase	Prestalk protein	glutamate-rich protein	Hydroxytryptamine receptor	L-sorbose dehydrogenase
Protein transport protein	Tyrosine-protein kinase	patatin-like phospholipase	Intestine-specific homeobox isoform	Major facilitator superfamily
Peptidase	Clc chloride channel	beta-1,2-xylosyltransferase	Thyroglobulin	Phosphatidylinositol 4,5 biphosphate kinase
formin-1	transmembrane protein	Cardiomyopathy-associated protein	Cytochrome P450	
WD repeat containing protein	Senescence-associated protein	Zinc finger protein	Epoxide hydrolase	
anaphase-promoting complex	Sphingomyelin phosphodiesterase	NLR family CARD domain protein	Peptide synthetase	
Multiple epidermal growth factor	Phosphatidylserine decarboxylase	Cysteine proteinase	Peptidyl-prolyl cis-trans isomerase	
leukocyte receptor cluster member	Putative LacI family transcriptional regulator	Galactoside	ATR-interacting protein	
amino acid transporter	MFS transporter	Myocilin	Molybdenum cofactor	
Xenobiotic-transporting ATPase	EglN1; egl-9 family hypoxia inducible factor 1	Lactate dehydrogenase	Histone-lysine N-methyltransferase	
Radical SAM superfamily enzyme	dachshund homolog 2 isoform X1	Senescence-associated protein	Diguanylate cyclase	
serine carboxypeptidase	tyrosine-protein phosphatase non-receptor	sugar kinase	Metabotropic glutamate receptor	
amino acid adenylation domain containing protein	NusB antitermination factor	Pyridine nucleotide-disulfide oxidoreductase	A-factor biosynthesis protein	
tyrosine-protein kinase receptor	Mandelate racemase/muconate lactonizing protein	collagen alpha-1	Metalloproteinase	
carboxypeptidase D	Cytochrome P450	SGNH hydrolase	Tyrosine-protein kinase	
NADPH dehydrogenase	MATN4; matrilin-4 isoform X1	Reductases	ATR-interacting protein	
N-acetylglucosamine-1-phosphodiester	slitrk3; SLIT and NTRK-like protein 3	oxidoreductase neomenthol dehydrogenase	Sulfatase protein	
o-succinylbenzoate--CoA ligase	zinc finger protein 575	Exopolyphosphatase	Sulfatase protein	
cation-independent mannose-6-phosphate receptor	collagen alpha-1(I) chain-like	AMP-dependent synthetase and ligase	Bifunctional DNA primase/polymerase	
auxin efflux carrier component	A-kinase anchoring protein 8	VPS10 domain-containing receptor SorCS2 isoform	SPARC related modular calcium binding	
auxin response factor	telomere-associated protein RIF1 isoform X1		Senescence-associated protein	
auxin efflux carrier component	MORN1; MORN repeat-containing protein 1			
NAD epimerase/dehydratase	CSPG5; chondroitin sulfate proteoglycan 5			
Mitogen-activated protein kinase	LMOD3; leiomodoin-3			
Dual oxidase	Myelin protein zero			
Small conductance calcium-activated potassium channel protein 2	hypoxia-inducible factor 3-alpha			
FxsA cytoplasmic membrane protein	LOW QUALITY PROTEIN: caskin-1-like			
Ubiquitin protein ligase	A-kinase anchoring protein 8			
phosphatidylinositol 4-kinase	telomere-associated protein RIF1 isoform X1			
	FSIP2; fibrous sheath-interacting			

	protein 2			
	IQSEC2; IQ motif and SEC7 domain-containing protein 2 isoform X1			
	single-stranded DNA-binding protein 4-like			
	tbc1d5; LOW QUALITY PROTEIN: TBC1 domain family member 5			
	cyclin-dependent kinase C-1			
	subtilase family protease			
	putative AraC family transcriptional regulator			
	FSIP2; fibrous sheath-interacting protein 2			
	IQSEC2; IQ motif and SEC7 domain-containing protein 2 isoform X1			
<i>P. simplicissimum</i> SNB-VECD11G				
Tensin-4-like isoform				
WASL-interacting protein				
Peptide ABC transporter substrate-binding protein				
Membrane-associated serine protease				
TOG array regulator				
opioid growth factor receptor				
Alpha kinase				
TMV resistance protein				
GTP-binding protein				
Formin-like protein				
Cactin				
Thiosulfate sulfurtransferase				
Putative tyrosine-protein phosphatase				
Zinc finger protein				
Mercaptopyruvate sulfurtransferase				
TRAP dicarboxylate transporter				
Deoxyloganetic acid glucosyltransferase				
BCCT family transporter				
ATP-dependent valine adenylyase				
Paxillin				
Membrane-bound lytic murein transglycosylase				
Putative transport protein				
Amino acid adenylation protein				
protein KHNYN				
Beta hydrolase				
ABC transporter ATP-binding protein				
Protein-tyrosine-phosphatase				
putative rRNA methyltransferase				
Probable dioxygenase				
acetolactate synthase catalytic				
tyrosine-protein phosphatase non-receptor type 11-like isoform X1				
ATP-binding cassette				
Acyltransferase				

Figures

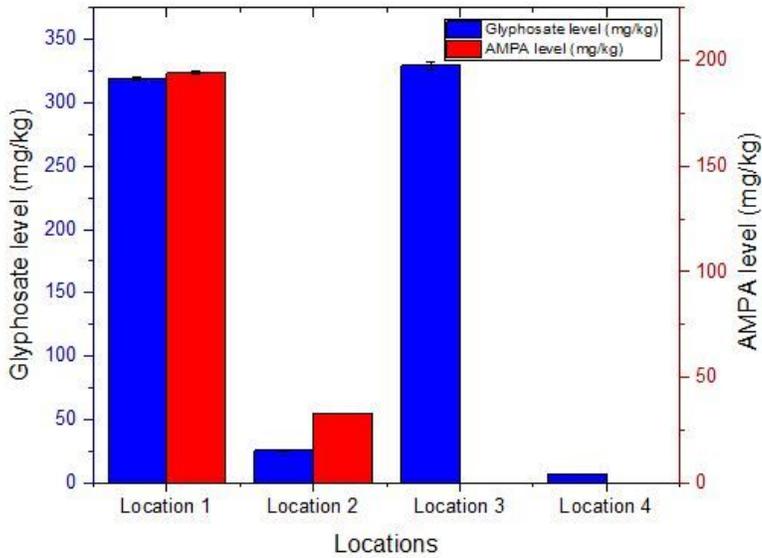


Figure 1

The initial glyphosate residue of the different soil locations (left) and transformation product AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) (right). Values are mean levels with standard errors of the pollutants in the locations where fungi were isolated.

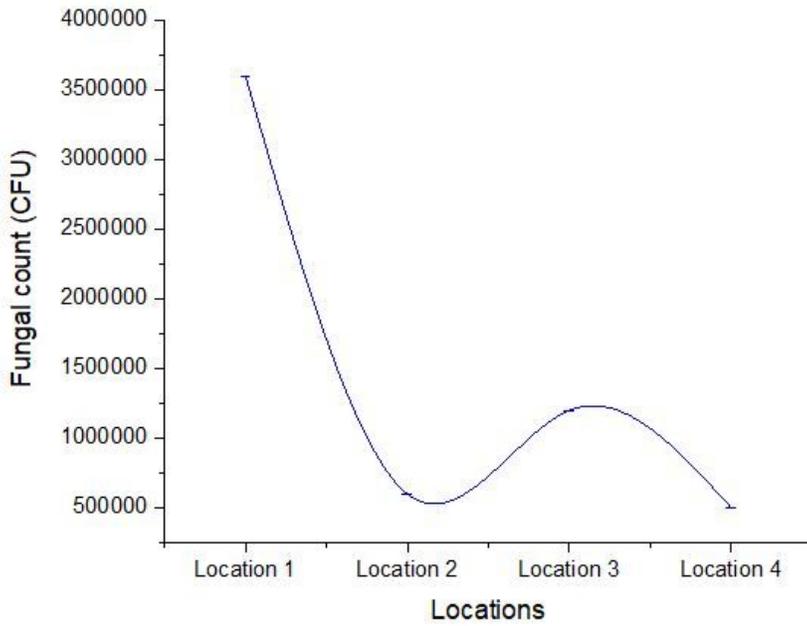
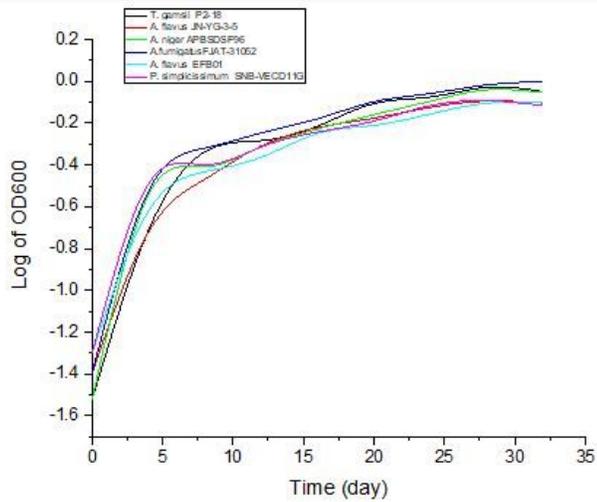
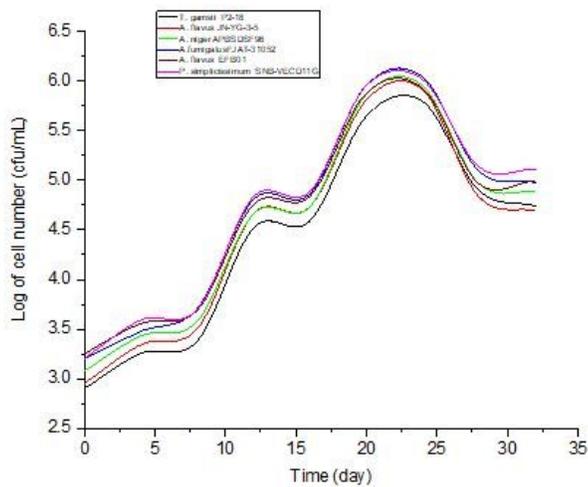


Figure 2

Fungal counts (CFU) of soil samples from the different locations. The values are means of the fungal counts with standard errors isolated from the farms where glyphosate is used as pesticide.



a.



b.

Figure 3

Time dependent changes in microbial growth of the different fungal isolates after 32 days of incubation. (a) OD600 of 1 mL aliquots of fungal cultures were measured using a spectrophotometer. (b) Cell number (cfu/mL) was done microscopically every four days for 32 days.

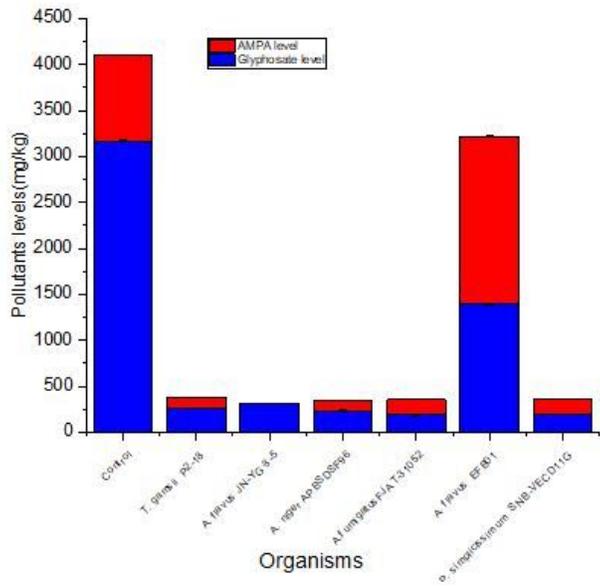


Figure 4

Glyphosate biodegradation by the various organisms

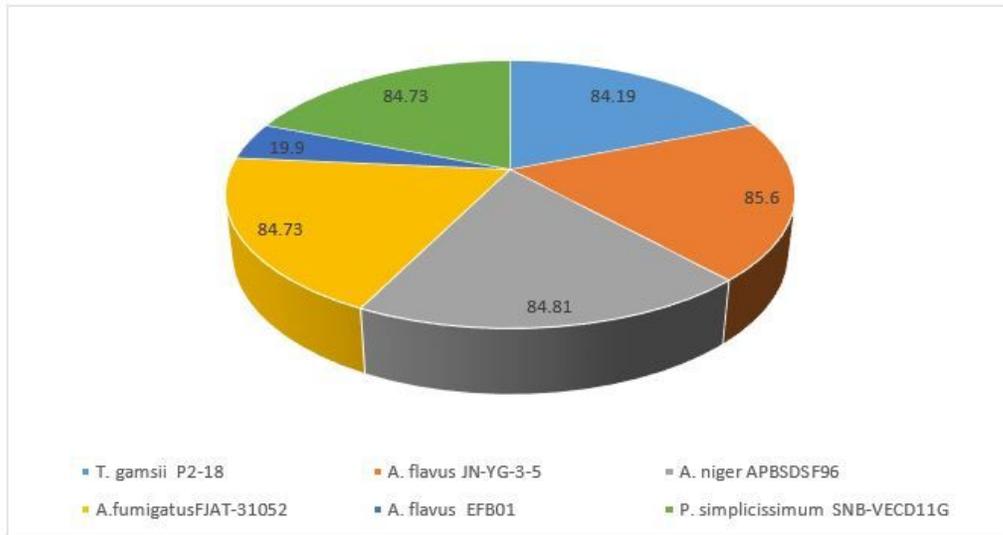


Figure 5

Degradation coefficient of glyphosate by the various organisms

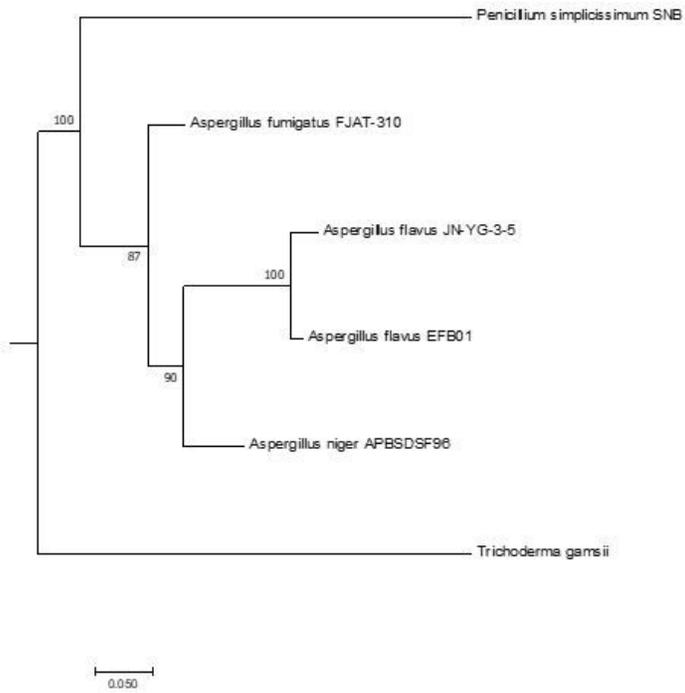


Figure 6

Phylogenetic relationship of bacterial isolates

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