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Laccase-Producing Fungal Strains from Pesticide-Farmland Soil: Evaluating Their Heavy Metal Tolerance

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Abstract

Indiscriminate pesticide applications allow metals to accumulate in soils and crops, endangering human health, environment and food security. This study examines the metal removal potential by laccase-producing fungi. Five inorganic soil samples were collected from farmland. Soil aliquot was inoculated into sterile potato dextrose agar plates and fungi were identified based on their macroscopic, microscopic and molecular techniques. Laccase production was screened using a tannic acid indicator, and the guaiacol assay measured laccase activities. Metal tolerance was assessed for lead (Pb) and arsenic (As) at concentrations of 5 to 20 g/L. Fourteen isolates that belong to the genera of Candida (14%), *Trichoderma* (21%), *Trichosporon* (22%), *Aspergillus* (36%) and *Rhodotorula* (7%) were obtained. Only *Aspergillus niger* and *Aspergillus japonicus* demonstrated laccase production. *A. niger* exhibited highest enzyme activity (0.8026U/mL) while the least laccase activity (0.1453U/mL) was produced by *A. japonicum.* The optimal As and Pb tolerances of *A. niger* are 5 g/L and 10 g/L, respectively. *A. japonicus* showed stronger tolerance up to 15 g/L for Pb, whereas that of As remains 5 g/L. These results demonstrate that *A. niger* and *A. japonicus* hold promise as valuable bio-resources due to their effective laccase production and notable metal tolerance, underscoring their potential applications in bioremediation.

Keywords: Heavy metals, Laccase, Metal tolerance, *Aspergillus*, Pesticides

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Introduction

Rising urbanization and the resulting growing need for food have led to the adoption of several techniques and the widespread use of chemical pesticides for improving crop yields (Magnoli, et al., 2023). Fertilizers and pesticides might be useful resources to boost agricultural output and guarantee food security, but only 0.1 to 5% of all pesticides applied to the soil ultimately reach their intended pests while the remainder ends up in the soil and water (Bisht and Chauhan, 2021). Pesticide improper use, overuse, buildup and improper handling have a substantial negative impact on the environment, human and animal health (Rathna et al., 2018). Metal-containing substances in pesticides enhance their durability and efficacy. Heavy metals can be non-biodegradable and accumulate in soil, water and food chains which makes it one of the most urgent global issues (Gautam et al., 2014).

Globally, accidental acute pesticide poisoning affects 385 million people annually (Boedeker et al., 2020). Lead, copper, arsenic and zinc are a few of the common metals found in pesticides (Briffa et al., 2021). The precise pesticide formulation and its intended usage depends on the type and presence of metals in it (Abubakar et al., 2020). Furthermore, because of their detrimental effects on both human health and environs, WHO has classified Pb, Hg, Cd, As, Cr and Ni as priority metals and have been reported to cause neurological disorders, cancer and organ damage, among other health dangers (Tchounwou et al., 2012).

Effective remediation techniques are necessary to lessen the resulting burdens caused by the indiscriminate application of pesticides containing metals widely used in the agricultural sector. The safest and most effective way to convert toxic substances into simpler, benign chemicals have been through biological processes (Bharadwaj, 2018). Kavamura and Esposito (2010) have reported that some microorganisms are capable of using heavy metals and exhibit tolerance mechanisms as well as generating chelated compounds which reduce metal toxicity. Fungi possess a vast array of excellent enzymes, metabolic pathways and regulatory mechanisms that have evolved to break down pesticides, making them biological recycling agents and a promising solution to the issue of contamination (Spina et al., 2018). Catalysis of phenolic substrates by laccase fungi uniqueness have garnered interest from researchers (Kumar et al., 2014). Laccase is a feasible remediation strategy for removal of pesticide-contaminated materials because of its phenolic ring structure and substrate specificity including the capability for non-phenolic molecules with mediators (Bilal et al., 2019).

The WHO has identified lead (Pb) and arsenic (As) as parts of priority hazardous metals that are present in the environment and pesticides that have an adverse effect on all living forms. Previous studies have focused on the biological application of possible microorganisms in heavy metal-contaminated soil from industrial settings. However, limited information have been reported on inorganic soil of fungi-producing laccase. Moreover, metal-microbe tolerance is also of immense significance to researchers and industry on account of the quest for novel heavy metals tolerance strains under the WHO priority metals. Therefore, this study aimed to isolate fungi from inorganic pesticide farmland soil and to investigate the fungi capabilities to produce laccase and to detoxify heavy metals (Pb and As).

Materials and Methods

Sample Collection

Five pesticides inorganic soil samples were collected from agricultural farm, Federal University of Agriculture, Abeokuta Ogun State. Corn cobs and potato peels were obtained from Osiele. Abeokuta.

Chemicals

As and Pb powder salts of 99.0 % and 99.99 % purity, respectively were purchased from Libertas Laboratory Services Limited, Abeokuta, Ogun State, Nigeria

Enrichment and isolation of fungal isolates

Soil samples (10g) were each transferred into sterile bottles containing 90 mLs of sterilized Potato Dextrose Broth (PDB) and incubated for 3 days under shaking condition (150rpm) at 30°C. On the third day, 10mLs of the PDB containing the soil samples were withdrawn, re-introduced into fresh 90mLs of the broths and incubated for another 3 days. Each broth medium (1mL) was serially diluted to 10^{-6}, 0.1 mL of 10^{-6} dilution was plated on PDA and incubated for 3-5 days at 28°C. The pure cultures were maintained on agar slants (Usman et al., 2019).

Identification of fungal isolates

This was carried out using macroscopic and microscopic characteristics, including colony morphology (Beemrote et al., 2023).

Qualitative screening for laccase production

Sterile PDA plates infused with 0.5 % tannic acid and fungal mycelium were incubated for 12 days at 25°C and appearance of reddish-brown halo around the fungi showed the ability for laccase synthesis (Adiveppa and Basappa, 2015).

Laccase production using corncob and potato peels

This was carried out by introducing 2 mLs spore suspension into sterile 100 mLs mineral salt medium containing (g/L): KCl, 0.5; MgSO4.7H2O, 0.5; (NH4)2HPO4, 0.5; NaH2PO4, 0.5; CaCl.2H2O, 0.01; FeSO4.7H2O, 0.001; ZnSO4.7H2O, 0.002 and each of 10 g corn cob and 10g of potato peel. The pH was adjusted to 5.0 and incubated at 25°C in a rotatory shaker at 150 rpm for 9 days (Sanjeeviravar et al., 2015).

Determination of laccase activity using Guaiacol assay method

The reaction mixture contains 1 mL of 2 mM Guaiacol, 3 mLs of 10 mM Sodium acetate buffer, 1 mL of fungal supernatant which serves as enzyme source and a blank which contains 1 mL of distilled water instead of the enzyme. The mixture was incubated at 30°C for 15mins and the absorbance was read at 450 nm using UV spectrophotometer (Abd El Monssef et al., 2016).

Assessment of heavy-metals tolerance by laccase-producers

Fungal cell mass (2% w/v) was added to cooled PDA medium and 0.1 mL metal solutions (5 to 20 g L^{-1}) of As and Pb were added appropriately into 6mm diameter wells on the agar plates. Incubation was done for 96 hours at 28 °C and observe for zones of inhibition (Omeike et al., 2014).

Molecular characterization of metal tolerance and laccase-producing strains

Genomic DNA of potential heavy metal tolerance and laccase fungal producers were carried out using CTAB method as described by Zhang et al. (2010). Forward primer (ITS 1)- 5′ TCCGTAGGTGAACCTGCGG -3′ and reverse primer (ITS 4)- 5′ TCCTCC GCT TATTGATATGC -3′ were employed and the purified PCR products were subjected to sanger sequencing using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit (Gandarilla-Pacheco *et al.,* 2021). Nucleotide sequences obtained were analyzed using National Center for Biotechnology Information (NCBI) database and MEGA 11.0 software was used for the construction of phylogenetic tree, using Neighbour joining tree statistical method and Kimura-2 parameter model (Beemrote *et al.,* 2023).

Statistical analysis

Statistical Package for Social Sciences (SPSS) was used to analyse the data. Duncan multiple range test was used for descriptive statistics and one-way analysis of variance.

Results

Characterization of fungal isolates

Table 1 indicates the macroscopic and microscopic characteristics of the fungal isolates. *Aspergillus* species were the dominant species. Isolates F1, F6 and F14 were identified as *Aspergillus niger*. Isolates F2 and F9 were identified as *Candida* sp. Three isolates (F3, F11 and F13) were *Trichoderma hariazum* and (F5, F7 and F12) were *Trichosporon sp.* Isolates F4, F8 and F10 were identified as *Rhodotorula* sp**,** *Aspergillus flavus* and *Aspergillus japonicus* (Table 1).

Table 1: Macroscopic and microscopic characteristics of the fungal isolates

Isolates	Macroscopy characteristics	Microscopy characteristics	Inference
F1	Black mycelium; pale yellow reverse	Brownish conidiophore, globose vesicle, globose conidia	*Aspergillus niger*
F2	Colonies are small, smooth, creamy and moist	Oval elongated chains of yeast cell	*Candida sp*
F3	Dark green producing tufts by mycelium. Reverse color is dull yellowish.	Branching conidiophores, subglobose to obovoid conidia	*Trichoderma hariazum*
F4	Slightly pinkish red, moist, yeast-like colonies	Spherical to elongate budding yeast like cells.	*Rhodotorula sp*
F5	Filamentous creamy-white small colonies with a distinctive odor.	Ellipsoidal yeast-like cells, clusters with regular septa wall.	*Trichosporon sp*
F6	Black mycelium; pale yellow reverse	Brownish conidiophore, globose vesicle, globose conidia	*Aspergillus niger*

F7	Medium size smooth white colony, presence of fluffy on colony.	Irregular septation, true hyphae yeast-like cells.	*Trichosporon sp*
F8	Large green colonies, colonies enclosed in white mycelium	Non-septate hyphae, rough globose conidiophores.	*Apergillus flavus*
F9	Creamy, round, mucoid and large colonies	Pseudohyphae, round yeast cells with bud.	*Candida sp*
F10	White to creamy colonies and fluffy.	Septate hyphae, unbranched conidiophores, globose or ellipsoidal conidia.	*Aspergillus japonicus*
F11	Dark green producing tufts by mycelium. Reverse color is dull yellowish.	Branching conidiophores, subglobose to obovoid conidia	*Trichoderma hariazum*
F12	Medium size smooth white cottony colony	Irregular septation, true hyphae, yeast-like cells.	*Trichosporon sp*
F13	Dark green producing tufts by mycelium. Reverse color is dull yellowish.	Branching conidiophores, subglobose to obovoid conidia	*Trichoderma harizanum*
F14	Black mycelium; pale yellow reverse	Brownish conidiophore, globose vesicle, globose conidia	*Aspergillus niger*

Occurrences of fungal isolates

Figure 1 shows the percentage occurrences of the fungal species. *Candida* spp were 14%, *Trichoderma* spp (21%), *Trichosporon* spp were 22%, *Aspergillus* spp (36%) and *Rhodotorula* sp had the least occurrence of 7%**.**

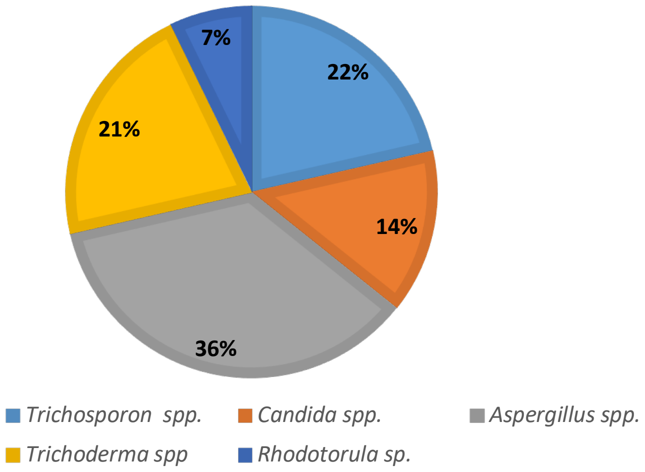

Fig 1: Occurrences of fungal species from inorganic soil

Qualitative screening for laccase production by the fungal isolates

Table 2 depicts the laccase abilities of the 14 identified strains. The appearance of reddish-brown halo around the fungi showed the ability for laccase synthesis while the absence of the reddish-brown indicated the non-laccase producers. Two isolates; F6 (*Aspergillus niger*) and F10 (*Aspergillus japonicus*) were only positive for laccase production.

Table 2: Screening for laccase synthesis by fungi

Isolates code	**Isolate identity**	**Laccase production**
F1	*Aspergillus niger*	Negative
F2	*Candida sp*	Negative
F3	*Trichoderma hariazum*	Negative
F4	*Rhodotorula sp*	Negative
F5	*Trichosporon sp*	Negative
F6	*Aspergillus niger*	Positive
F7	*Trichosporon sp*	Negative
F8	*Apergillus flavus*	Negative
F9	*Candida sp*	Negative
F10	*Aspergillus japonicus*	Positive
F11	*Trichoderma hariazum*	Negative
F12	*Trichosporon sp*	Negative
F13	*Trichoderma harizanum*	Negative
F14	*Aspergillus niger*	Negative

Laccase production by fungi using corn cob and potato peels

Asp niger and *Asp japonicum* exhibited higher enzyme activities in potato peel than corn cob (Figure 2). *Asp niger* recorded higher laccase activity using potato peel substrates (0.8026U/mL) while the least laccase activity was produced by *Asp japonicum* (0.1453U/mL) when corn cob was utilized as substrate.

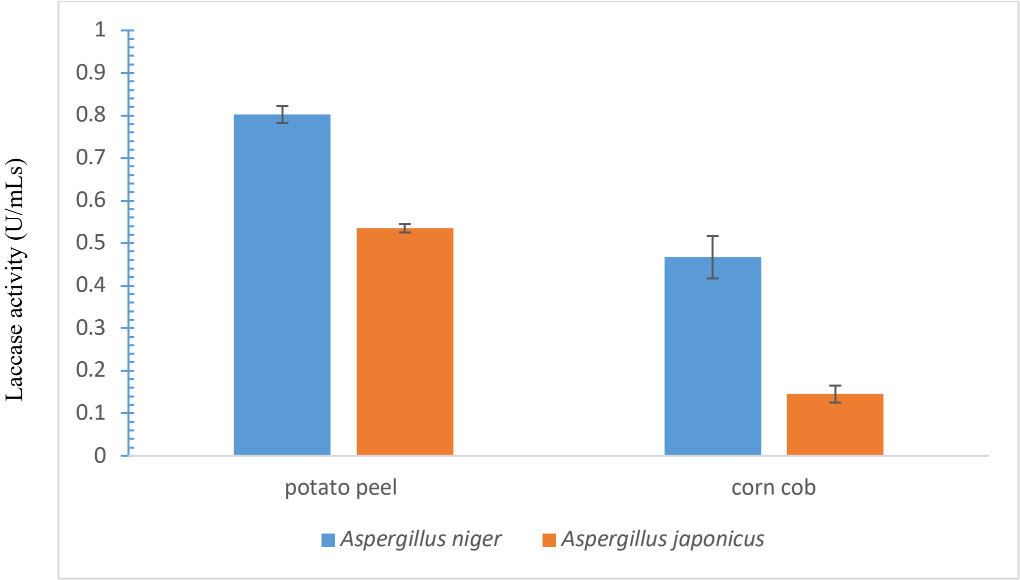

Fig 2: Laccase activity produced by *Asp niger* using local substrate (corn cob and potato peel)

Metal tolerance (lead, arsenic) by fungal strains

It was observed that both *Aspergillus niger* and *Aspergillus japonicus* tolerated Pb than As in all the tested concentrations. Pb at 10g was highly tolerated by *Aspergillus niger* while 5g of As had better tolerance among the tested concentrations. Similar observation was seen in the tolerance of *Asp japonicus* to As and Pb. The results indicated that *A. japonicus* was tolerant to As (5g) and 15g to Pb than *Aspergillus niger* (Table 3). This observation indicates that the growth and clear zone devoid by the fungal isolates at higher concentration of the heavy metals was due to the fungi tolerance.

Table 3: Heavy-metal tolerance of *A. niger* and *A. japonicus* at different concentrations

Metal concentrations (g/L)	*Aspergillus niger* (cm)		*Aspergillus japonicus* (cm)	
	As	Pb	As	Pb
5	1.7±0.2	2.6±0.1	2.0±0.1	2.2±0.3
10	1.1±0.0	3.9±0.2	1.4±0.4	2.0±0.6
15	0.8±0.1	2.1±0.6	1.6±0.2	3.2±0.4
20	0.5±0.0	1.7±0.3	1.0±0.0	2.4±0.1

Molecular characterization of the laccase-producing fungi

The two potential metal tolerance and laccase producers were *A. niger* and A. *japonicus*. Figure 3 depicts the gel electrophoresis and Figure 4 shows the evolutionary distances among the strains.

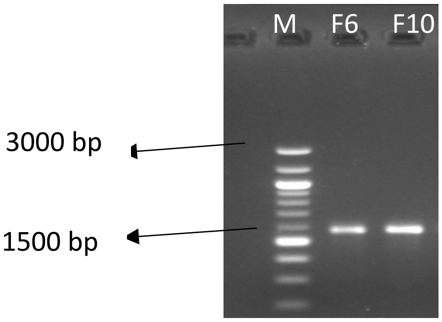

Fig 3: ITS gene amplification of the fungal isolates

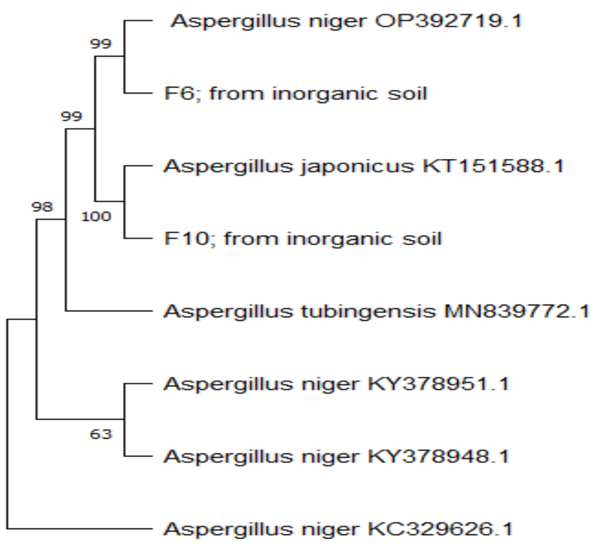

Fig 4: Phylogenetic relatedness of metal tolerance fungi with other sequences from NCBI database.

Discussion

Heavy metal pollution is one of the primary environmental contaminants that poses a major risk to human health and the ecosystem (Sukmana et al., 2021). The health of humans, animals and ecosystems can all be compromised by pesticides, heavy metals and organic pollutants (Briffa et al., 2020). Fungi have successfully coexisted with soil because they may take on multiple forms in response to extreme circumstances and can create a wide range of extracellular enzymes (Frac et al., 2018). *Candida, Trichoderma, Trichosporon, Aspergillus,* and *Rhodotorula* were the five genera of fungi that were isolated and are among the prevalent in soil. Similar findings were reported by Norjmaa et al. (2019); and Raja et al. in (2017).

The inorganic content of the soil may be responsible for the limited distribution of the fungal isolates and this has also been confirmed by Ishaq and Khan, (2011) where the fungal species from organic field was quite rich in microbial flora than inorganic soil. The most prevalent fungi, *Aspergillus* (*A.niger*) indicated that it adapts well to a variety of environments. Saini et al. (2016) stated that the density and diversity of microorganisms in the soil are influenced by various factors, including organic matter, pH and moisture content. In this study, *A. niger* (4) and *A. flavus* (1) were obtained. Abdallah et al. (2015) have reported on the natural fungicidal properties and biocontrol potential of several *Aspergillus* species and Beemrote et al. (2023) also documented that *Aspergillus* was the richest genera in their findings. The abundance of *Aspergillus* species may be attributed to their capacity to produce a wide variety of enzymes and antibiotics that both protect them from other soil organisms and may impede the growth of other fungal species.

Laccase's stability, catalytic efficiency and substrate specificity render it an effective tool for environmental cleanup especially heavy metals, including zinc, nickel, cadmium, copper and lead (Wang et al., 2021; Zhao and Yi, 2010). Among the isolated fungi, our findings indicated that two distinct species of *Aspergillus* (*A. niger* and *A. japonicus*) produced laccase and this could likely be due to strain diversity within the same species. This, however, disagrees with Abd El Monssef et al. (2016) conclusion, who claimed only T. harzianum to be laccase producer. The observed laccase activity suggests that *A. niger* and *A. japonicus* produced laccase using potato peel and corn cob.

The use of mediated degradation by laccase is a useful method that has several benefits for the environment and produces no harmful consequences (Nguyen et al., 2016). During laccase-mediated oxidation, the heavy metal ions are transformed from their soluble and poisonous form to their insoluble and non-toxic form (Alengebawy et al., 2021; Hussain et al., 2021). The maximum enzyme activity in this findings was 0.8026 U/mL, which was greater than the 0.718 U/mL generated by Marasmius sp. in the report of Adiveppa and Basappa (2015) and lower (2.2280 U/mL) than the value reported by Nuhu et al., 2020. Amanpreet et al. (2017) reported a greater laccase activity of 28.2 U/mL, which is in contrast to our finding. The reason for this significant variation was because Amanpreet et al. (2017) introduced $MgSO_4$ as enzyme inducer under optimized conditions.

The increased activity shown in this study by *A. niger* (F6) in comparison to *A. japonicus* (F10) may be as a result of variations in the capacity to produce enzymes amongst strains belonging to the same genus and substrates affinity. The abilities of the two strains to utilise potato peels and corn-cob substrates for laccase production suggests the isolates employ corn cob and potato peel as carbon sources. The nutrients included in these substrates function as a carbon source and are crucial for the development of microbes as well as the metabolic release of laccase for breakdown (Sullivan et al., 2015).

The results obtained from different concentrations of Pb and As also showed the stability of the organism in the presence of heavy metals. It was noted that 10g and 15g of Pb by *A. niger* and *A. japonicus* gave better tolerance, it can be observed that when As concentration was 5g, the diameter of growth increased and further increase in As concentration led to a constant decrease of the degradative activity. This suggests that different fungal strains exhibited different metabolism and desire for metal salt tolerance. The phylogenetic tree revealed that *A. niger* (F6) and *A. japonicus* (F10) could be identified as *A. niger* OP392719.1 and *A. japonicus* KT151588.1, respectively and have an evolutionary lineage and linkage with the nucleotides sequences retrieved from the NCBI database.

Conclusion

Fungi are useful microorganisms that are abundant in the ecosystems of soil and water. The characterization of the different species showed that the agricultural soil harbors a variety of fungal communities and the inorganic chemical pesticides nature of the soil samples could be responsible for the moderate fungi diversity. Among the isolated fungi, *A. niger* and *A. japonicus* exhibited excellent performance in terms of laccase abilities and metal tolerance of WHO priority metals (Pb and As). These laccase-producing microorganisms can be used for As and Pb removing agents. Further research on optimization studies for improved laccase activities and the molecular mechanisms causing the emergence of metals resistance in soil microorganisms are recommended.

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