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BIOREMEDIATION OF PESTICIDE (MANCOZEB) BY *ASPERGILLUS* SPECIES

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ABSTRACT

Pesticides, which are the most effective way to prevent the main plant diseases that harm crops, are crucial for maintaining or increasing agricultural productivity. However, the majority of these compounds pose a serious risk to the environment and public health since they are extremely poisonous, persistent, and slowly biodegradable. Exposure to Mancozeb causes toxicity in both occupational and non- occupational contexts. The microbial metabolism of local microorganisms can be used for degradation since bioremediation is an environmentally acceptable, economically viable, and more effective method than physical and chemical ones. The relationship between mancozeb and soil fungus was studied with an eye toward environmental safety. It was found that the *Aspergillus niger* fungal species could thrive in media containing mancozeb at doses of 150 and 200 ppm. In the bioremediation process, the *Aspergillus niger* strain can be envisioned.

KEY WORDS : Mancozeb, *Aspergillus niger*, Pesticide, Bioremediation, Toxicity, Molecular identification

INTRODUCTION

The vast reservoir of microbial diversity found in soil serves a variety of purposes and provides a wealth of resources, including regulating geochemical cycles, cleaning up pollution, and producing new pharmaceuticals, among other things. Although thousands of soil microbial species can be found in a single gram of soil, little is known about the processes that give rise to this variety. This diversity has been attributed to the heterogeneity of soil particles and how they are arranged structurally, which both increase the variety of settings for organisms and isolate populations, fostering differentiation (Naranjo-Ortiz and Gabaldón, 2019).

The 3.8 million species of fungi that make up the fungal kingdom represent a huge diversity of life forms, feeding habits, and relationships with other organisms. Through the cycling of organic matter and the distribution of nutrients across trophic levels, fungi play a significant ecological role in both terrestrial and aquatic settings (Nilsson *et al.,* 2019). Different methods and ideas based on morphology, physiology, biochemistry, or responses to chemical tests have been used to distinguish various fungal species. Through the use of morphology and the integration of chemotaxonomy, phylogeny, genetics, ecology, and molecular biology, modern

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mycotaxonomy has advanced. Phylogenetic, biological, genetic, and evolutionary investigations using sequencing data have revealed information about the diversity and interactions between and within fungal taxa (Senanayake *et al.,* 2020).

Plant extracts, specifically pyrethrin, were utilized as insecticides, fungicides, and herbicides during the 19th and 20th centuries (Lücking *et al.,* 2020; Randhawa and Kullar, 2011). Compounds known as fungicides work by destroying parasitic fungus or their spores. They enable the management of fungal infections, particularly those that affect the entire food supply. Fungicides are used extensively in the agriculture sector (Seaton *et al.,* 2023; Raffa and Chiampo, 2021). The cytoplasm and mitochondria of fungi are home to several metabolic pathways that fungicides disrupt. They block several enzymes and proteins that are involved in processes like lipid metabolism, fungal respiration, and adenosine triphosphate synthesis (ATP) (Gajjar *et al.,* 2020; Prabha, 2017; Thind and Hollomon, 2018). Potatoes, grapes, citrus, apples, stone fruits, tomatoes, melons, and bananas are some of the main foods they used. Additionally, they are crucial for the disease control of ornamental plants, grains, corn, peanuṫs, onions, sugar beets, asparagus, almonds, and brassica vegetables (Randhawa and Kullar, 2011; Deshmukh, 2016).

Mancozeb is a fungicide that is widely used in agriculture. Ethylene thiourea, the primary metabolite of the active ingredient, has been linked to health problems (Raffa and Chiampo, 2021; Gupta and Crissman, 2013). Its presence in water must be quantified using effective, quick, and reliable methods for environmental and health concerns (McPartland and McKernan, 2017). Mancozeb, is also wide-range fungicide employed in horticulture, expert yard maintenance, and agriculture, received its initial registration in the United States in 1948. The fungicides in the ethylene bisdithiocarbamate (EBDC) class, which also contains the associated active elements Maneb and metiram together makeup mancozeb (Jaganathan, 2020). In its assessments, the Agency took into account ethylene thiourea (ETU), a common degradant in EBDCs. Two Special Reviews have been conducted on the EBDCs. The Agency started a Special Review for products containing EBDCs in 1977 after receiving evidence indicating that EBDCs and ETU, a byproduct, metabolite, and breakdown product of these pesticides, may be hazardous to human health and the environment (Jahin *et al.,* 2020).

The most widely used dithiocarbamate, mancozeb, was reported to have reduced sensitivity in field isolates of the fungi *Alternaria alternate* (tomato fruit rot), *Venturia inaequalis* (apple scab), and *Cercospora beticola* (sugar beet leaf spot). In the 1940s, thiocarbamate fungicides, also known as dithiocarbamates, were developed. This was a significant advancement because dithiocarbamates quickly became the most well-liked and often-used class of organic chemistry fungicides, protecting a variety of crops from fungal diseases. Maneb, also known as mancozeb (manganese ethylene bisdithiocarbamate and 2% zinc ion), was the most significant and frequently utilized fungicide of all the dithiocarbamates when it was first registered in 1962. The earliest broad-spectrum foliar fungicides were alkylene-bis-dithiocarbamates, also known as ethylene-bis-dithiocarbamates (EBDCs). Even more than 50 years after their introduction, EBDCs are still employed to control plant diseases, earning the moniker "old reliables" (Axelstad *et al.,* 2011; Shah *et al.,* 2022).

Numerous fungi in agricultural soils can be used to biodegrade pesticides. Molds, yeast, and filamentous fungi are all members of this class of microorganisms. The ability of fungi to synthesize several enzymes involved in deteriorative processes promotes fungal breakdown. Additionally, these microbes have the power to adjust the soil's permeability and ion exchange capacity. Due to their unique bioactivity, growth morphology, and great tolerance even at high concentrations of contaminants, fungi can be better degraders than bacteria (Park *et al.,* 2021; Pandey and Choudhury, 2020). Using fungi and bacteria to accelerate breakdown is a typical strategy because fungi can convert poisons into a palatable form for bacteria (Bianchi *et al.,* 2020). Bioremediation, a recently developed technique used for clean-up tactics, is the term used to describe the biological response to environmental abuse. Bio means biological, and remediation means to remedy or eliminate. Bioremediation is the process of using microbial flora, such as bacteria, algae, fungi, actinomycetes, and yeast, either as native organisms or used as a consortium under controlled conditions, to degrade environmental contaminants or organic waste into much fewer toxic compounds that are negatively affecting human health as well as an ecosystem (Cocco, 2022).

Most agrochemical dealers cannot afford to use

traditional remediation techniques for polluted soils since they are expensive and include the excavation, removal, and treatment of the contaminated soil (Hawkins and Fraaije, 2018). Compared to traditional physicochemical approaches, bioremediation is less disruptive and more effective at restoring soil functions. Recent developments in microbial consortium bioremediation technologies have been demonstrated to help treat pesticides in soil. With the significant release of anthropogenic toxins into the environment, bioremediation as a sustainable method has gained importance (Ahlawat *et al.*, 2010; Maqbool *et al.,* 2016; Nihana *et al.*, 2020). The aim of the present study is to investigate the efficiency or potential of *Aspergillus* species in degradation of Mancozeb.

MATERIALS AND METHOD

This study was conducted at the microbiology laboratory at JSS Academy of Higher Education and Research, Mysuru. Soil samples were collected from agricultural fields polluted by fungicides from a localized region on the outskirts of Mysuru, Karnataka. Unithane M-45 which contained 75% mancozeb was used for this study.

Isolation of fungi from the soil sample

For the isolation of fungi, Chloramphenicol was employed as an antimicrobial in potato dextrose agar medium (PDA) (Nining *et al.,* 2020). In order to isolate soil fungus, the dilution plate method was performed. 1g of soil was added to a test tube, along with 10 ml of sterile distilled water, and the test tube was then shaken until homogeneous. Transferring one mL of the homogeneous solution to the test tube, adding 9 ml of sterile distilled water to create 10-2 dilutions, and then taking one ml of the 10-2 dilution and adding 9 ml of sterile distilled water to create 10-3 dilutions. A petri dish containing medium was then filled with the desired dilution, and the mixture was flattened with an L-stick (Aimeur *et al.,* 2016). To avoid contamination, the experiment was conducted under laminar airflow, and the plates were incubated for 5-14 days at room temperature (27 °C).

Pure culture

A little amount of the inoculum was removed from each distinct fungal colony, regrown on a petri dish with solid PDA, and cultured for five to seven days at room temperature (27 °C).

Identification of soil fungi

To identify soil fungi, molecular levels and morphology were used. Macroscopic and microscopic observations were carried out for Morphological identification. Macroscopic morphology includes the shape, edges, and color of the colony growing on PDA media supplemented with 75% Mancozeb.

Mancozeb soil fungi inhibition test

Growing fungal isolates on PDA media containing a fungicide 75% Mancozeb with an active component of concentration 150 ppm and 200 ppm was used for the inhibition assay. On PDA media containing Mancozeb, one of the sub-cultured soil fungus isolates was cultivated. Ten to fourteen days after point inoculation, fungus development was detected. Fungicides were added in an effort to stop the development of soil fungi on particular types of medium.

Molecular Identification of fungus using ITS method

DNA isolation by CTAB Method

1000 µl of CTAB Extraction Buffer was added to 0.5 grams of the fungal sample, homogenized, and incubated at 30 minutes for 60 °C. Centrifuge the homogenate for 5 minutes at 14,000 x g after the incubation period. Adding equal parts of chloroform and isoamyl alcohol (1:24). To separate the phases, vortex the sample for 5 seconds, then centrifuge it for 5 minutes at 14,000 x g. Change the tube holding the aqueous top phase. Add 0.7 volume of cold isopropanol to the DNA to precipitate it, then let it sit at -20°C overnight. Then add 750 µl at a time to the DNA column and spin it for one minute at 12000 rpm. Spin for one minute at 12000 rpm after adding 750 µl of wash buffer. Do the wash again. Dry spin the DNA column at a speed of 12000 rpm for two minutes. Include 20 µl of elution buffer, allow it to sit at room temperature for 3 minutes, and then spin for 1 minute at 12000 rpm. Add 1 µl of RNase solution A, then wait 30 minutes at 37°C. Utilizing agarose gel electrophoresis, the DNA thus recovered was quantified (30).

PCR Conditions

Initial denaturation for two minutes at 95 °C. Final denaturation for 30 seconds at 95 °C Annealing at 50 °C for 30 seconds. 72 °C elongation for one minute. Repeat steps 2, 3, and 4 a total of 30 times (30). Ten

minutes for the final elongation at 72 °C (Fig 1 and Fig. 2). Maintain at 4°C forever (Table 1).

Fig. 1. 100 bp ladder comparison with genomic DNA from lichen.

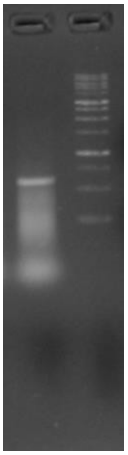

Fig. 2. ITS gene (600 bp) amplified from lichen, compared with 1000 bp ladder

Table 1. Primers with sequence of ITS 1 and ITS 4.

Primer	Sequence	Annealing temperature
ITS-1	5′-TCCGTAGGTGAACATGCGG-3′	53 ^{0}C
ITS-4	5′-TCCTCCGCTTATTGATATGC-3′	57 ^{0}C

Preparation of PCR reaction mixture

Each PCR reaction used to build multiplex PCR assays for DNA barcode primers and assess the efficiency of amplification contained 2 µl of 10X reaction buffer, 0.5 µl of MgCl2 (50 pM), 1 µl of DNA template (25 ng), 1 µl of forward primer (10 pM), 0.5 µl of Taq polymerase (5 For the amplification of the 18srRNA gene,1 µl of the dNTPs mix (10 mM), 1 µl of reverse primer (10 pM)(30), Standard primers are offered as primers.

Gel purification protocol

On the gel, cut the desired DNA band. Include 600 µL of a buffer for gel solubilization, and heat at 55 C until the gel is fully dissolved. After mixing 200 µL of isopropanol, add it to the column. Spin for one minute at 12000 rpm. Spin for one minute at 12000 rpm after adding 700 µL of wash buffer. Perform a 2-minute dry spin. Spin for one minute at 12000 rpm after adding 20 µL of elution buffer (Abadi *et al.*, 2022).

Sanger Sequencing PCR

Initial denaturation for two minutes at 95 °C. 30 seconds of final denaturation at 95 °C Annealing for 30 seconds at 50 °C. Stop at 60 °C after 4 minutes. Carry out actions 2, 3, and 4 for 30 times. Hold at 4°C indefinitelyc (Abadi *et al.*, 2022).

Post-Sequencing and PCR Purification

To each well, add 125 Mm and 2.5 µL of EDTA, then quickly spin. Utilizing a multichannel pipette, add 35 µL of ethanol. Centrifuge at 3510 rpm for 30 minutes after vertexing for 10 minutes at 2000 rpm. Decant ethanol using a tissue bed at 300 rpm (for 30 seconds invert the plate). Fill the wells with 40 µL of 80% ethanol and centrifuge them for 12 minutes at 3510 rpm. Repeat the above-mentioned inverted spin. Let the plate air dry for 30-45 minutes while covering it with a lint-free tissue. Add 13 µL of HiDiFormamide and give the mixture a quick spin. Denature for five minutes at 95 °C. Insert the plate into the sequencerc (Abadi *et al.*, 2022).

Data analysis

The obtained sequencing files will be. Software like FinchTV, BioEdit, Chromas Lite, Seq Scanner, etc. can be used to view files in the AB1 format. Electropherogram peaks can be used to determine the sequence's quality. Employ the BLAST server or servers connected to particular databases to analyze the sequencing data.

RESULTS AND DISCUSSION

To potentially colonize soil and outcompete the competition from natural microorganisms Fig. 3), the fungus utilized in bioremediation must have fast growth rates (Fig. 4)**.** In this study, 3 isolates were grown on potato dextrose agar (PDA) amended with 150 ppm and 200 ppm mancozeb (Fig. 6). Overall, 1 test isolate was tolerant to the pesticide treatments. After molecular identification, Phylogenetic tree, the test isolate was identified as *Aspergillus niger* (Fig. 5 and Fig. 7).

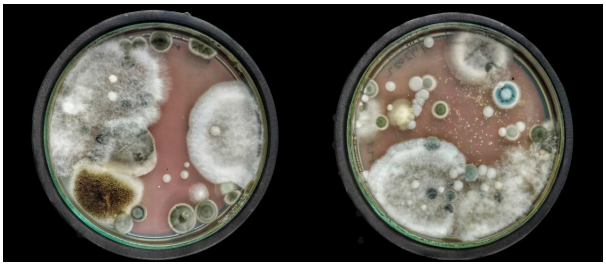

Fig. 3. Agricultural soil isolates on PDA after incubation at 27 ^{o}C for 7 days.

Fig. 4. *Aspergillus* species and *Penicillium* species after purification.

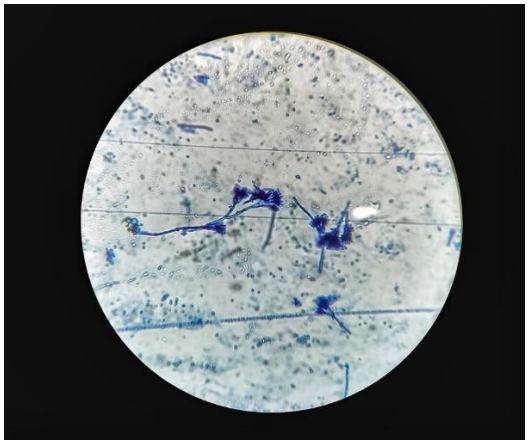

Fig. 5. *Aspergillus niger* stained with lactophenol cotton blue stain under 40 xmagnification

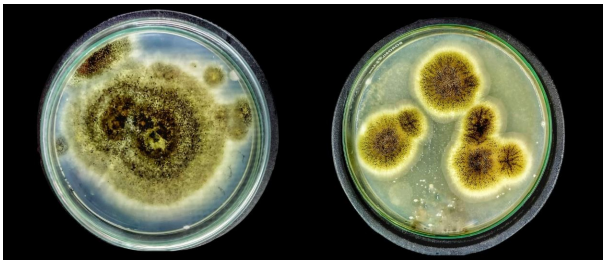

Fig. 6. *Aspergillus niger* grown on PDA with mancozeb at 150 and 200 ppm concentrations respectively.

Aimeur *et al.*, (2016) was also studied and concluded that *Aspergillus* speices isolated from the surface water contaminated by the pesticides. The catabolic power seems better removal rate exceeded 50%. The pesticide business in India has fostered the misconception that these chemicals are necessary to increase yield in the agricultural production system. government organizations that oversee agriculture. Chemical usage has not succeeded in getting through to farmers to monitor usage (Axelstad *et al.*, 2011). As a result, the majority of farmers are unaware of the harmful consequences of these poisonous chemical pesticides. As a result, they use pesticides irresponsibly and disregard the advised safety precautions (Shah *et al.*, 2022). Dithiocarbamates have remained effective and are among the most widely used contact fungicides. Several organic sulfur fungicides, including Mancozeb, Thiram, Maneb, Zineb, Ziram, Metiram, and Propineb, have helped farmers control a number of economically significant plant diseases (Park *et al.*, 2021; Rani *et al.*, 2014). Due to their multi-site mode of action and broad- spectrum disease control, they are among the most well-liked partners in combinations of a variety of single-site fungicides as part of resistance management approaches (Mishra *et al.*, 2021). The main metabolite of some of these fungicides, ethylene thiourea, is thought to affect animal endocrine and reproductive systems. (Gupta and Crissman, 2013). The carbamates function as endocrine disruptors and acetylcholinesterase inhibitors. respiratory conditions are brought on by carbamates exposure (Bianchi *et al.*, 2020). According to toxicological studies, prenatal and postnatal carbamate exposure can harm the hippocampus and have an impact on fetal and infant development (Cocco, 2022). It was noted that numerous fruits and drinks contain ethyl carbamate, a chemical known to cause cancer in several animal species and sometimes in humans (Hawkins and Fraaije, 2018). This insecticide can specifically cause DNA depurination, reactive oxygen species generation, and mitochondrial malfunction (Abadi *et al.*, 2022; Thind and Hollomon, 2018).

Mancozeb targets the thyroid, just like other EBDCs and ETUs do. Numerous studies across

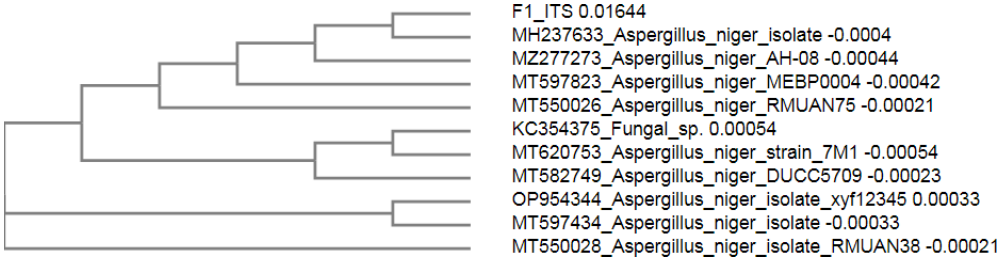

Fig. 7. Phylogenetictree based on 18S rRNA sequence

>F1_ITSGTGACGCTAGAGTGCGGTCCTTTGGGCCAAC
CTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGC
GGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCC
TCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCA
ACACGAACACTGTCTGAAAGCGTGCAGTCTGAGT
TGATTGAATGCAATCAGTTAAAACTTTCAACAATG
GATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC
GAAATGCGATAACTAATGTGAATTGCAGAATTCAG
TGAATCATCGAGTCTTTGAACGCACATTGCGCCCC
CTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCA
TTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGT
CGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAA A
GGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGT
ATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGG
CGCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTT
GACCT CGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATCAATAACGAGAGAGACTTCCGGAGGGA
GAACTCGCCGGATATACAACCAGCGGGGGGATTGC
GAGCTGTATGCTTTCGGGCTCTCGCTTGTGGTCGCG
GGGGGGGTGCTCTGCCCCCGGGCCGTGCCGCGGA
GACCAAACGACACTGTTTGAAAGCGTG

Fig. 8. *Aspergillus niger* partial sequence of the isolate A18 small subunit ribosomal RNA gene.

species have found thyroid impacts. Thyroid poisoning was indicated by changes in thyroid hormone levels, a rise in microscopic thyroid lesions (mainly thyroïd follicular cell hyperplasia), thyroid weight, and thyroid malignancies (Jahin *et al.,* 2020). EBDCs including zineb, Maneb, and mancozeb has been shown to be endocrine and reproductive disruptors in laboratory animals (Ahlawat *et al.,* 2010; Maqbool *et al.,* 2016). Mancozeb, zineb, and Maneb have antithyroid and hypothyroid actions that are linked to their metabolite ethylene thiourea (ETU) (Maqbool *et al.,* 2016). ETU has been identified by the United States Environment Protection Agency as a potential human carcinogen because of its propensity to cause cancer and birth defects in test animals (Mishra *et al.*, 2021). India is only the second country in Asia and the world behind China in terms of fungicide production, and only 2% to 3% of pesticide residues are used (Nihana *et al.,* 2020). The rest are left in the soil and water, where they harm biota and accumulate in human body parts like blood, adipose tissue, and lymphoid organs (Nayak *et al.,* 2020). They also cause chromosome mutations in both men and animals, which can result in lung and liver cancer when consumed through food. They can cause hepatotoxicity, neuropathy, nephropathy, and reproductive problems and are teratogenic and mutagenic (Abadi *et al.,* 2022).

CONCLUSION

To find fungi-based remedies for the treatment of mancozeb-contaminated soils, this research effort has clearly defined a direction for future research. An in-depth investigation of the potential of mycoremediation would be the focus of future work. The research gap between lab and pilot scale studies is too great to precisely pinpoint the factors that would ensure the success of a full- scale project. For the multiple-level sustainable management of waste and pollutants, bioremediation has a higher chance of success.

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