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Metalaxyl Pesticide Degradation by *Aspergillus wentii* : Proposed Pathways and Degrading Enzymes

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Abstract

Metalaxyl (MY) is a water soluble synthetic acylanilide fungicide widely used in agriculture practices to control phytopathogenic fungi, but it elicits environmental pollution to underground water as well as human health. The safe and effective removal of agrochemical pollutants from contaminated field sites is performed by some soil and aquatic microorganisms. However, limited work has been done focusing on the intermediate metabolites' residues produced and enzymes involved in the biodegradation process of fungicides. The degradation ability of the isolated aquatic fungus *Aspergillus wentii* of the MY as a sole carbon source in only sterilized distilled water was evaluated *in vitro* after 15 days, reaching high levels of biodegradation at 68%. Furthermore, the possible catabolic degradation pathways of MY based on the detected metabolites beside the enzymes involved in the biodegradation process were established. Moreover, a TripleTOF 5600 + LC-MS/MS was employed to identify the enzymes involved in the biodegradation process of the fungicide. The results revealed that CYP450 is responsible for metabolism of MY. It could be concluded that *Aspergillus wentii* enzymes have a prospective potential to eliminate a wide spectrum of toxic environmental pollutants, such as MY. To our knowledge, this study is the first report that *Aspergillus wentii* CYP450 could catalyze the degradation of metalaxyl.

***keywords*:** Agrochemicals, *Aspergillus wentii*, Metalaxyl, Cytochrome P450

1. Introduction

The Egyptian population and agricultural sectors mainly depend on the Nile River as a water resource. Egypt's climate is classified as arid to semi-arid, there are a limited rainfall and ground water [1,2]. Some references reported that the shortage could even reach 21 BCM/yr in 2019. The annual water shortage was estimated to be seven billion cubic meters (BCM/yr) according to recent report published [3]. However, by 2050, the situation would be significantly worse, with a shortage of 26 billion cubic meters is expected [4] . The gap between the limited water resources and the demand is increasing due to rapid population growth and climate changes [5]. Further challenges regarding the Egyptian water resources come from the upper Nile basin countries amid the construction of Grand Ethiopian Renaissance Dam. The use of non-conventional water resources (such as seawater desalination and reusing drainage water) became crucial to compensate that gap [2,6,7]. The quality of recycled water produced from wastewater treatment plants must be monitored for toxic pollutants [8,9]. These toxic pollutants include high salt contents, heavy metals, recalcitrant and xenobiotic compounds such as fertilizers and pesticides [10–12]. Pesticides are organic chemicals that are designed to increase agricultural yield, crop quality, soil productivity, and reduce agricultural product losses caused by crop pests [13]. The residual pesticides in soil are a broad category of compounds that include insecticides, fungicides, herbicides, molluscicides, bactericides, rodenticides, nematicides, plant growth regulators and others. The overuse of pesticide in agricultural practices can cause severe water quality issues because of the persistence and dispersion of residual unmetabolized pesticides to non-target organism in wild life [14]. Pesticides are degraded into other chemical components, which sometimes become more hazardous than the original pesticides [15]. It was estimated that just ten percent of applied pesticides reach the target organism, and the remaining high percentage is settled on non-target areas such as soil, water, and sediments causing serious environmental pollution [14]. Pesticides undergo many different pathways once they enter the environment, including sorption/desorption, transformation/degradation, volatilization, uptake by plants, discharge into surface waters, and transport into groundwater [16]. The WHO sets a limit for pesticide residue in water at 0.1 µg/L for an individual and 0.5 µg/L for all pesticides [17].

The fungicide metalaxyl ([N-(2, 6-dimethylphenyl)-N-(methoxyacetyl) alanine methyl ester]) is an important systemic acylanilide compounds widely used against plant downy mildew disease caused by members of the family Oomycetes [18]. The metalaxyl fungicidal mechanism comes from interaction with the complex of RNA polymerase-I-template therefore inhibiting incorporation of RNA building unit (ribonucleotide triphosphates) into ribosomal RNA [19,20]. The metalaxyl is very soluble in water (8.4 g/L), characterized by long half-life values in soil, photostable and heat resistant. In addition, metalaxyl is characterized by the low vapor pressure (3.3 mPa at 25°C), low soil adsorption and high mobility hence it is very stable in water within a broad range of pH (1.0 - 8.5) and has the potential to contaminate groundwater [21–24].

Although metalaxyl was classified as a slightly hazardous pesticide (toxicity class III; WHO), cytogenetic effects of metalaxyl on human and animal chromosomes was demonstrated by *in vitro* studies [25,26] as well as cocarcinogenic and

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nephrotoxicity potential in mice [27]. Metalaxyl has the ability to activate human pregnane X receptor, which is highly related to breast cancer cell metabolism [28,29]. Exposure of human lymphocytes to metalaxyl *in vitro* resulted in micronucleus formation and sister-chromatid exchange [30]. In addition, adverse effects was reported on cardiac development in zebrafish embryos, and disruption of the transcription of genes related to cardiac development and function [31].

There are several remediation methods for metalaxyl and other pesticide residues from water such as advanced oxidations processes using different catalysts (zinc oxide, fenton oxidation, and titanium dioxide), phytoremediation, and adsorption [32]. However, these methods still have some disadvantages such as the production of toxic metabolites, high cost, consume more energy, and inability to eliminate a variety of pesticides. Therefore, bioremediation of pesticides is the most effective and important way to remove these compounds from the water [33].

Microorganisms can interact physically, and chemically with substances leading to structural changes or complete degradation of pesticides [34,35].

Metalaxyl is naturally degraded in soil by the cleavage of the methyl ester group, producing acidic metabolite, alternatively benzylic hydroxylation of methyl chain or aromatic hydroxylation could also occur. Another metabolite (N-methoxyacetyl-2,6-dimethyl-aniline) is formed either directly from metalaxyl or from the acidic metabolite by N-dealkylation [36]. Many attempts were performed for the accelerated degradation of metalaxyl *in vitro* using specific bacterial and fungal isolates, but the specific pathway and enzymes responsible for the metalaxyl degradation was not identified yet [32,37–39].

In this study, local isolate of *Aspergillus wentii* was selected for metalaxyl biodegradation based on the fungus ability to use the metalaxyl as the only nutrient source in a sterilized distilled water as well as the fungus tolerance to high concentration of metalaxyl reached to 260 mg/L. Furthermore, the enzyme involved in the biotransformation was identified and the pathway for the metalaxyl degradation was postulated based on the detected metabolites by LC/MS.

2. Material and Methods

2.1 Chemical

Metalaxyl analytical grade with a purity of >98 % and carboxymethyl cellulose (CMC) were purchased from (Sigma-Aldrich, Germany). Phenylmethylsulfonyl fluoride (PMSF) and NADPH were obtained from Biobasic Inc., Canada.

2.2 Water and soil samples collection

Different water and soil samples were collected from ten different sites along the Nile River in Northern Delta of Egypt (Fig. 1). Water samples were collected by submersing a sterile bottle below the water surface at mid-stream to fill it completely. Soil samples were collected from the upper layer (10–20 cm) of same sample sites but in the riverbank. Pesticide residues present in water samples were analyzed by the Central Laboratory of Residue Analysis of Pesticides and Heavy Metal in Food (Agricultural Research Centre, Egypt).

2.3 Isolation and purification of fungi

Aquatic and terrestrial fungi were isolated from all samples collected from water and soil sites utilizing solid Czapek's Dox medium g/L (sucrose, 30; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; Agar, 15) containing 100 mg/L of ampicillin to inhibit bacterial growth. Samples were diluted in series up to 10^{-6} and then 100 µL of each diluted sample was inoculated onto Czapek's Dox medium by spread plate method. The plates were incubated at 30 °C for 7 days or monitoring for the appearance of colonies. Single colonies were selected by picking it using sterile needle and were re-streaked on the same medium. Small leaves and branches of trees found in the collected water samples were used also to isolate aquatic fungi. Further purification was done by culturing single colonies on agar medium containing CMC as a carbon source to enhance the growth of the cellulose decomposing fungi which are adsorbed on the surface of leaves and wood.

Figure 1. Northern Delta of Egypt map demonstrate the water and soil samples collection sites along the Rashid branch of Nile River.

2.4 Screening of fungal isolates for degradation of pesticide

In order to select potential fungal isolates that have the ability to degrade the detected pesticide. Screening for pesticide-degrading fungal isolates were performed by inoculating one disc of each fungus (0.5 cm diameter) onto a sterilized Czapek's Dox agar medium supplemented with 10 mg/L of the detected pesticide as a sole carbon source. The detected pesticide was prepared by dissolving in distilled water then filtered through membrane filter (0.45µm in diameter; Millipore Corp., Burlington, MA, USA), before addition to the autoclaved medium. The incubation was at 30°C for 15 days, and the fungal growth diameter was monitored at different time points (5, 10, 15) days. The isolates with increasing growth diameter were

used for further screening at higher concentration of the detected pesticide including 30, 150, 300, 600, and 900 mg/L. The tolerant fungal isolate was identified by the Moubasher Mycological Center at Assiut University (AUMMC, Assiut, Egypt).

2.5. Optimization of pesticide concentration for biodegradation

To evaluate the optimum concentration of the detected pesticide for the tolerant fungal isolate growth in liquid media, one disc of the fungus (0.5 cm diameter) inoculated onto a sterilized Czapek's Dox broth medium supplemented with different concentrations (10, 50, 100, 150, 200, 250) mg/L of the detected pesticide as a sole carbon source. The incubation was at 30°C for 7 days at 120 rpm. The fungal mycelial mats were separated by filtration. Then, the mycelial mats were dried in the oven at 80°C until constant weight. A control one containing the fungus in the absence of pesticide was run in parallel under the same conditions.

2.6. Quantification of metalaxyl biodegradation

To confirm the metalaxyl degradation by the selected fungus, high-performance liquid chromatography (HPLC) was employed to calculate the degradation percentage of metalaxyl. Potato dextrose broth medium (PDB; Laboratories Conda SA, Madrid, Spain) was inoculated with a fungal disc (5 mm diameter) from a four-day-old fungal plate (previously grown on potato dextrose agar medium to activate the fungus). After incubation at 30 °C in a static condition for four days, the mycelial mat obtained from 50 mL of PDB culture was washed three times by sterilized 100 mM sodium phosphate buffer (pH 7), then the mycelial mat was added to 20 mL of sterilized distilled water containing filtered sterilized metalaxyl with a concentration of 100 mg/L. The mixture was further incubated at 30 °C for 15 days at 120 rpm. After 15 days of fungal incubation, the mycelial mat was separated from the solution by filtration. The cell-free supernatants of the sample were obtained by centrifugation at 10,000 rpm for 10 min then filtered using a 0.45 µm membrane filter. The obtained cell-free supernatants were extracted three times with equal volume of chloroform using separating funnel, transferred into screw-top vials, evaporated to dryness under reduced pressure using a rotary evaporator at temperatures of 45 °C [40]. Two control flasks were prepared under the same conditions, one inoculated with the mycelial mat without pesticide and other flask contained only pesticide to determine the abiotic degradation.

In order to confirm the capabilities of the selected fungus for biodegradation a high concentration of metalaxyl, the same experiment was performed utilizing a higher concentration of metalaxyl (260 mg/L) and procedures as described above.

The samples were re-suspended in methanol before injection into HPLC instrument to be analyzed for metalaxyl residues according to previously described method [41]. Briefly, the column (250 × 4.6 mm) packed with Kromasil C18 was utilized for chromatographic separation and isocratic elution system with a mobile phase consisting of acetonitrile–formic acid (50:50, v/v). The injected volume of sample was 10 µL and the flow rate of the mobile phase was adjusted to 1 mL/min at ambient column temperature. The run time of the analysis was 10 min and metalaxyl was detected at wavelengths of 220 nm. Identification of the pesticide in the samples was undertaken based on its retention time and by comparison between the UV spectrum of the pesticide in the standard solutions and the UV spectrum of the detected peak in the sample. Quantification was accomplished with a freshly prepared standard curve of the metalaxyl.

2.7. Detection of metalaxyl metabolites using (LC–MS) Analysis

The collected samples from both experiments were used to detect the metalaxyl metabolites by LC/MS. The samples were analyzed using a XEVO TQD instrument (Waters Corporation, Milford, MA01757 USA) equipped with Acquity UPLC-BEH C18 Column (1.7 µm particle size - 2.1 × 50 mm) coupled to a triple quadruple mass spectrometer. The elution solvent consisted of water containing 0.1 % formic acid and acetonitrile containing 0.1 % formic acid. The eluting solvent was applied at a flow rate of 0.2 mL/min. The metalaxyl metabolites identification was performed by matching the obtained mass spectra with National Institute Standard and Technology (NIST) database for authentic compounds as well as by comparison of their fragmentation pattern of the mass spectral data with those reported in literature. The obtained LC/MS results were analyzed for identification of metalaxyl biodegradation metabolites.

2.8. Preparation of fungal cell lysate

In order to evaluate the protein involved in the metalaxyl degradation process, the mycelial mat obtained from 50 mL of PDB culture was washed three times by sterilized 100 mM sodium phosphate buffer (pH 7). Then it was added to 20 mL of sterilized distilled water containing filtered sterilized metalaxyl with a concentration of 100 mg/L. After incubation at 30 °C for 15 days at 120 rpm, the mycelial mat was collected by filtration through a No.1 filter paper Whatman for further protein analysis. A control flask containing 20 mL of sterilized distilled water without metalaxyl was inoculated with a fungal mycelial mat and run in parallel.

To extract protein from fungal mycelia, the above mentioned collected mycelial mats were grinded in liquid nitrogen to a fine powder. One ml of cold lysis buffer (0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, and 10% glycerol) was used per gram of ground mycelial mat. Phenylmethyl sulfonyl fluoride (PMSF) was added at a final concentration of 2 mM immediately as described [42]. All samples were centrifuged at 21,000 *g* and 4 °C for 15 minutes and the protein containing supernatant was transferred to a new tube. Samples were stored at -20 °C for further analysis. Total protein content was determined with the Bradford protein assay [43], using bovine serum albumin (BSA) as a standard.

2.9. Detection of metalaxyl degradation enzymes

According to the LC/MS analysis for pesticide metabolites and the data from previous literature investigations, Cytochrome P450 enzyme was the proposed enzyme involved in the biodegradation of metalaxyl. The enzymatic activity was assayed according to (Abass et al., 2007) [26] by measuring the residual concentration of metalaxyl by HPLC after incubation with fungal lysate compared to control. The assay mixture, comprised of 200 µl of the filtered fungal lysate as an enzyme source, was added to 200 µl of a reaction mixture containing 0.1 M phosphate buffer pH 7.5, 10 mM NADPH, and 100 µM metalaxyl

as a final concentration. After overnight incubation at 37 °C, 200 µL of 20% trichloroacetic acid was added to stop the reaction. The Cytochrome P450 enzyme assay was carried out three times and the average reading was calculated.

Detection of Cytochrome P450 heme-content

To determine the Heme-content of Cytochrome P450, 20 µg of the extracted proteins from fungal mats were added to each lane to be separated using Native 8% Polyacrylamide Gel Electrophoresis (PAGE). After electrophoresis, gel was immediately transferred to sodium phosphate buffer (50 mM, pH 7.5) containing 0.25 µg/mL o-Dianisidine (Sigma Chemical Co., MO, USA) and 0.5% H_2O_2 [42]. The gel was incubated for 1 h at 37 °C till the development of brown color. To confirm the biodegradation of metalaxyl by proteins separated by native PAGE, unstained band from adjacent lane to the reactive band (size 2 x 0.4 cm) was cut out, transferred to a sterile vial and immersed in sodium phosphate buffer 0.1 M, pH 7.5 containing 100 µM of metalaxyl and 10 mM NADPH. The mixture was incubated overnight at 37°C. Followed by determination metalaxyl remaining by HPLC. Control one was done by immersing clean separating gel piece equivalent to the unstained band in an eppendorf with sodium phosphate buffer 0.1 M, pH 7.5 containing 100 µM of metalaxyl and 10 mM NADPH to detect if imbibition occurred causing decrease in metalaxyl concentration.

Gel digestion

The brown band formed was cut from the native gel and fixed by adding a fixing solution (50% methanol and 12% acetic acid) with overnight incubation at 4 °C after removing the de-staining solution. Gel pieces were washed using gel-wash buffer (50% acetonitrile in 50 mM ammonium bicarbonate) and dried using speed vacuuming. Reduction buffer (10 mM dithiothreitol in 50 mM ammonium bicarbonate) was added to the dried gel pieces and incubated at 60 °C for 30 min.

Alkylation buffer (55 mM iodoacetamide in 50 mM ammonium bicarbonate) was then added and incubated in dark at room temperature for 30 min. Gel pieces were washed using 25 mM ammonium bicarbonate before adding acetonitrile for 15 min. Digestion solution (50 µl Trypsin containing 10 ng/µl procaine enzyme) was added to gel pieces until gel hydration and incubated overnight at 37 °C at 600 rpm. Extraction buffer (66 acetonitrile: 33 milliQ water: 1 formic acid) was then added to extract digested peptides. Peptides were quantified using the Pierce BCA protein assay kit (23225, Thermo Fisher) [44].

Nano LC-MS/MS analysis was conducted using a TripleTOF 5600 + (AB Sciex, Canada) connected at the front end with an Eksigent nano-LC 400 auto-sampler with an Ekspert nano-LC 425 pumps. Peptides were injected into a peptide trap column CHROMXP C18-CL, 5 µm (10 × 0.5 mm) (Sciex, Germany).

The MS and MS/MS ranges were 400–1250 *m/z* and 170–1500 *m/z*, respectively. A mobile phase consisting of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid) was used. A 55-min linear gradient of 3–80% solvent B was applied. The raw MS files from the TripleTOF^{TM} 5600+ files were analyzed by Protein Pilot (version 5.0.1.0, 4895), Paragon Algorithm (version 5.0.1.0, 4874), and searched in the Uniprot *Aspergillus wentii* organism (Swiss-Prot and TrEMBL) database.

3. Results

3.1 Pesticides residues in water and soil samples

The analysis of the ten collected samples along the Nile River demonstrated that metalaxyl and carbendazim were the two detected pesticides. The concentration of metalaxyl was ranging from 0.07 to 0.09 µg/L. Table 1 listed the site of detection and the concentration of each pesticide.

3.2 Screening of fungal isolates for degradation of metalaxyl

Out of the 67 morphologically different fungi isolated from water and soil samples, only 18 isolates had the ability to grow on Czapek's Dox agar plates containing 10 mg/L metalaxyl as a sole carbon source. To select the most tolerant isolate in the biodegradation, the concentration of metalaxyl was gradually increased up to 900 mg/L.

The number of isolates capable of survival dropped from 18 isolates at 10 mg/L to only three isolates at 900 mg/L. It was noticed that prolonged incubation up to 15 days increases the growth of fungal isolates Gb2 even at the highest tested concentration 900 mg/L, while no significant increase in the growth was observed in the other isolates (Fig. 2).

The fungal isolates Ka1and Kh2 maintain growth on Czapek's Dox agar plates supplemented with 900 mg/L after 15 days of incubation but with high sporulation. Therefore, isolate Gb2 was selected for further identification and analysis.

3.3 Identification of the most tolerant fungal isolate

The morphological criteria of the prospective fungal isolate with code Gb2 showed that on Czapek's agar, colonies attain margin white, conidial areas floccose, yellow-orange shifting to brownish near to Greyed-Orange toward the center and the reverse petri dish of Czapek's Dox agar has greyed-yellow color (Fig. 3 A).

The microscopic examination showed that septate hyphae produced non-septate, unbranched and long conidiophore terminated with chains of conidia. The conidial heads are large, globose and radiated (Fig. 3 B, C).

The identity of the fungal isolate was confirmed by Assiut University Moubasher Mycological Centre (AUMMC, Assiut, Egypt) as *Aspergillus wentii* with AUMC No. 15221.

3.4. Optimization of pesticide concentration for biodegradation

After 7 days of incubation of *A. wentii* in Czapek's Dox broth medium containing different concentrations (10, 50, 100, 150, 200, 250) mg/L of metalaxyl as a sole carbon source, the fungal growth increased with the concentrations of metalaxyl until 100 mg/L, but for the concentration of 250 mg/L a decrease in the growth rate was observed (Fig. 4).

Table 1: The detected pesticides in water

Sample No.	Sample site	Detected pesticide	Concentration
1	El-Khedr Farm Moshtohor,Banha	Not detected	-
2	El-Hayania Canal Moshtohor,Banha	Not detected	-
3	El-Gharbia Canal, Banha	Metalaxyl Carbendazim	0.09 µg/L < LOQ
4	El-Ghafara Canal-Kafr Saad, Banha	Not detected	-
5	Kafr El-Zayat	Metalayl	0.07 µg/L
6	Dinshal, Damanhour	Not detected	-
7	Rasheed	Not detected	-
8	Izbat Al Jiddiyya, Rasheed	Carbendazim	0.06 µg/L
9	Idfina, Rosetta	Carbendazim	0.07 µg/L
10	Metoubes, Abyanah	Carbendazim	< LOQ

LOQ: limit of quantification

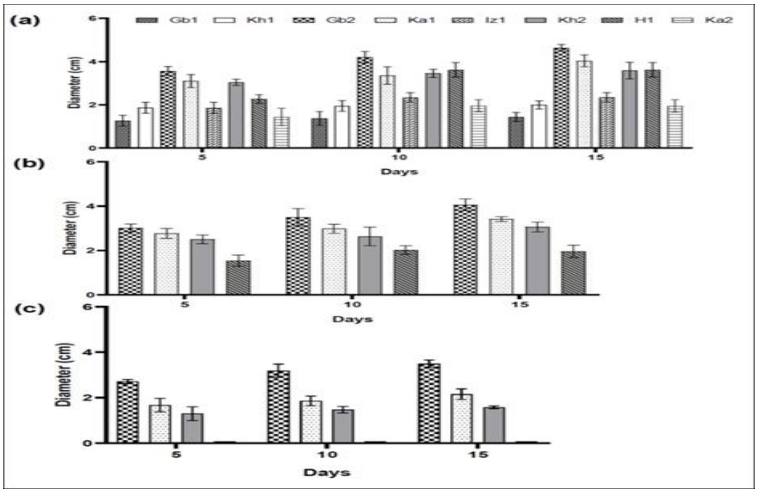
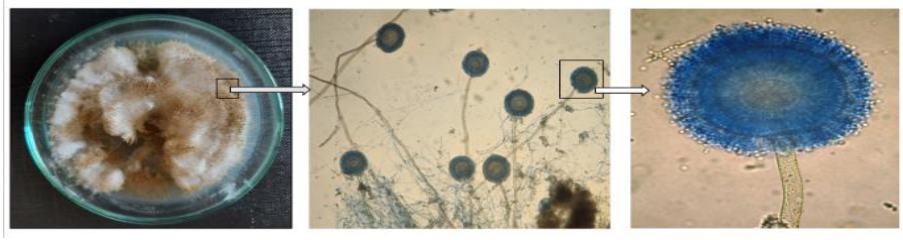


Figure 2. Growing of the fungal isolates represented by diameter of fungal colony on Czapek's Dox medium containing different concentration of metalaxyl as a sole carbon source. (a) 300 mg/L, (b) 600 mg/L and (c) 900 mg/L of metalaxyl.



A) B) C)

Figure 3. Morphological characterization of *Aspergillus wentii* isolate Gb2. (A) Growth on Czapek's Dox agar medium. (B) Bright field micrograph showing non-septate, unbranched conidiophore and, radiated conidial heads (arrows). (C) Bright field micrograph showing terminal conidial heads with chain of conidia.

3.5 Quantitative estimation of metalaxyl by HPLC

Further analysis of metalaxyl degradation by HPLC was performed to determine the efficiency of biodegradation by *A. wentii* Gb2. The residual concentrations were drastically reduced after 15 days of incubation of mycelial mat with a 100 mg/L metalaxyl initial concentration, which recorded a 68% reduction, whereas abiotic degradation was unnoticeable under the same incubation conditions. The degradation percentages were decreased to 18.5% when the fungus was grown with an initial 260 mg/L of metalaxyl concentration under the same conditions.

3.6 Identification of metalaxyl metabolites

In order to detect the biodegradation metabolites of metalaxyl, the LC/MS analysis was performed after 15 days of *A. wentii* Gb2 incubation on metalaxyl (100 mg/L) as a sole carbon source. (Fig. 5) showed the difference in the fungal growth after 15 days of incubation period. In the absence of metalaxyl, the fungal culture showed sporulation, and the fungal mat turned into yellowish color as a result of surviving extreme conditions. While in the presence of metalaxyl, no sporulation occurred and the fungal mat remained white in color indicating the ability of *A. wentii* to use metalaxyl as a nutrient.

The LC/MS analysis revealed that the spectrum patterns of metalaxyl showed three main biodegradation metabolites beside a significant peak of metalaxyl with molecular weight (m/z) of 280 and a retention time (RT) of 9.88 min (Fig. 6). The peak of metalaxyl was confirmed by comparing it with the authentic standard in the library databases and the pure compound (control sample). The first major compound with m/z 296 at RT 7.45 was identified as hydroxymetalaxyl. The hydroxylation could occur on the aromatic ring or on either methyl side chain of the ring. The second metabolite with m/z 267 at RT 8.90 was identified as N-(2,6-dimethylphenyl)-N-(hydroxyacetyl) alanine methyl ester. The third metabolite with *m/z* 206 at RT 11.13 was identified as N-(2,6-dimethylphenyl)-N-ethyl-2hydroxyacetamide. All detected compounds were identified according to the different libraries databases including WILEYREGISTRY 8E, MAINLIB and the mass spectral data with those reported in literature (Table 2). The analysis of fungal metabolite formed after metalaxyl biodegradation at 260 mg/L detected only hydroxymetalaxyl metabolite formation with m/z 296.

Finally, a proposed biotransformation pathway for metalxayl by *A. wentii* Gb2 has been postulated based on the identified intermediate compounds that have been detected in the two tested concentrations (Fig. 7).

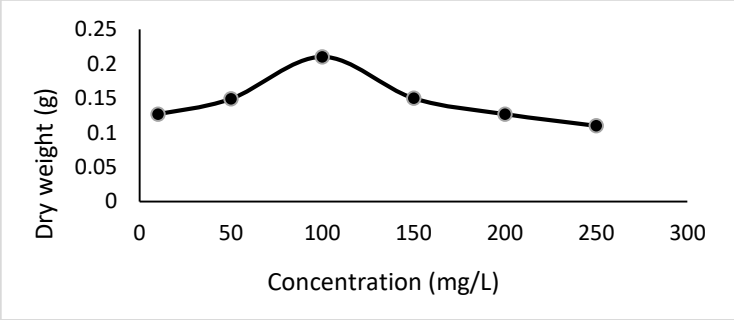


Figure 4. The growth of *A. wentii* in Czapek's Dox broth medium containing different concentrations of metalaxyl as a sole carbon source.

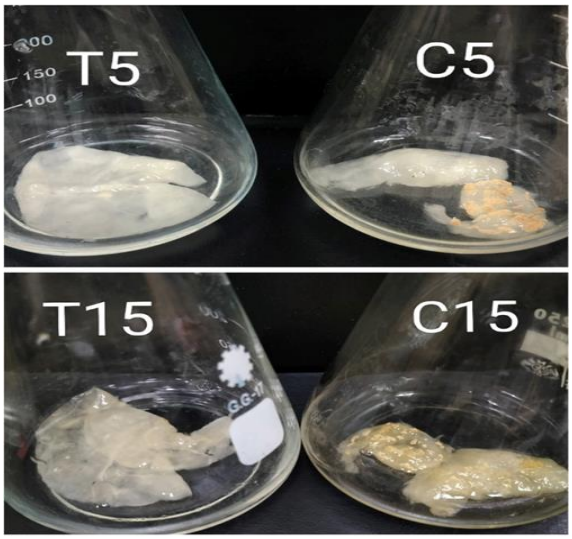


Figure 5. The fungal mat of *Aspergillus wentii* Gb2 in the presence (T) and absence (C) of 100 mg/L of metalaxyl in sterile distilled water. (A): After 5 days. (B): After 15 days of incubation.

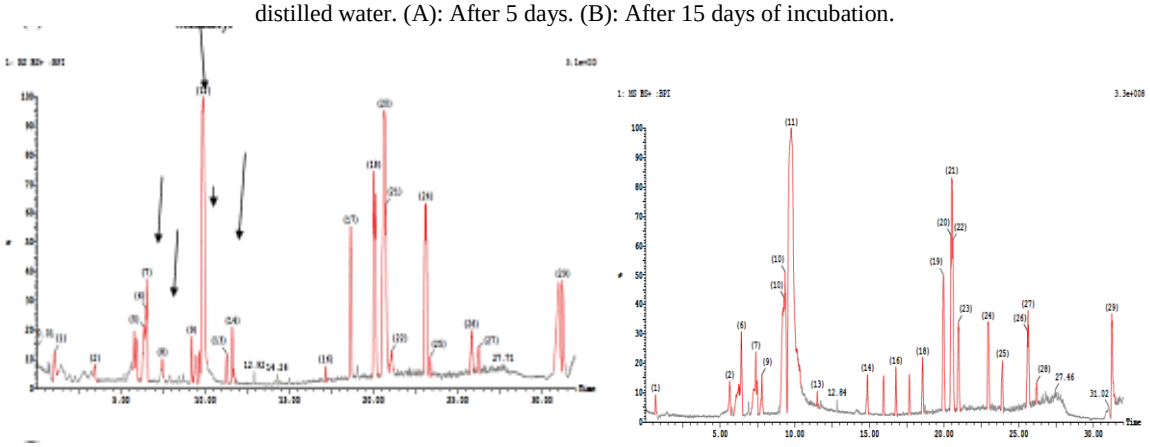


Figure 6. LC/MS spectrum of metalaxyl metabolites detected after 15 days of incubation of the fungal mat of *A. wentii* in (A): 100 mg/L and (B): 260 mg/L of metalaxyl in sterile distilled water.

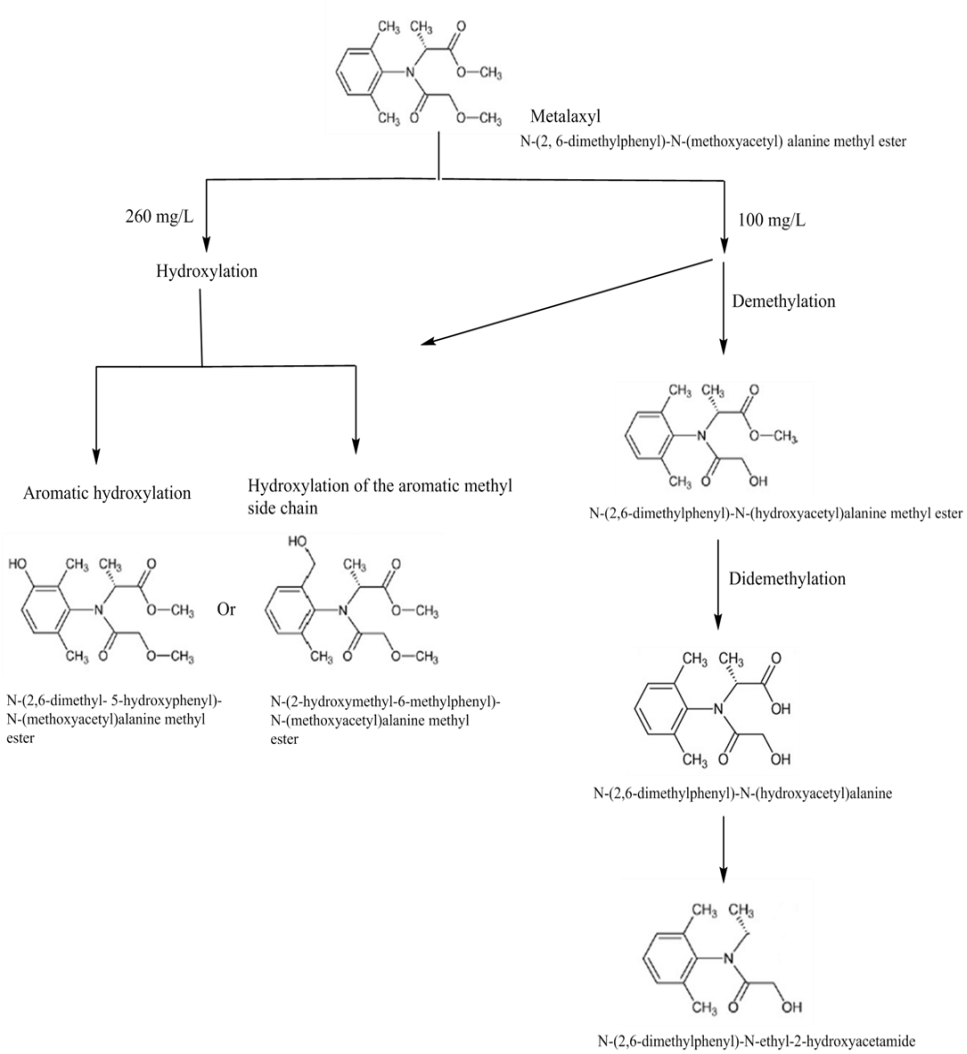


Figure 7. The proposed metalaxyl biodegradation pathway for *A. wentii* Gb2 based on the detected metabolites after incubation of the fungus in two different initial concentrations of metalaxyl (100 or 260 mg/L).

3.7 Detection of the fungal degradation of metalaxyl

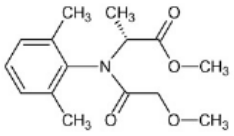
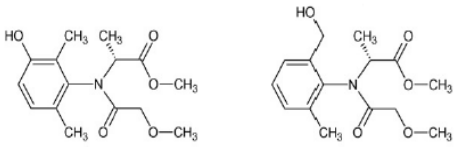
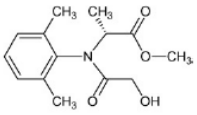
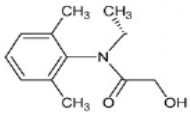
To reveal the metalaxyl *Aspergillus wentii* Gb2 degradation enzyme, the pesticide was incubated with fungal cell lysate previously grown in the presence of metalaxyl or in the control medium (absence of metalaxyl). The results revealed that a seven-percent reduction in the metalaxyl concentration was achieved in the *A. wentii* Gb2 cell lysate, compared to a two-percent reduction in the control using the same protein concentrations.

3.8 Detection of Cytochrome P450 heme-content

To confirm that the activity of Cytochrome P450 enzyme in the biodegradation of metalaxyl, the heme iron center bound to the enzyme was detected by separating the proteins of the fungal lysate by native-PAGE. The assay results revealed that an intense brown band was detected in the protein lysate previous grown in the presence of 100 mg/mL metalaxyl, in contrast to a much lighter band present in protein lysate prepared from mycelial mat grown in the absence of metalaxyl (Fig. 8). Furthermore, the incubation of the cut native band with metalaxyl as a substrate and NADPH as an electron donor showed a 40.6 % decrease in metalaxyl concentration compared to 2.7% in the control sample without the enzyme sample (equivalent clean gel piece).

The protein in the active gel band was extracted, digested by trypsin and identified by a TripleTOF 5600+ LC-MS/MS system. The results revealed the presence of Cytochrome P450, which was detected with molecular masses of distinctive peptide fragments (1065.52, 937.43, 937.43, 937.43) of sequences (KAFEEWTR, AFEETADR, AFEEWTR, VDEFSWR) respectively.

Table 2: A summary of the metabolites detected by LC/MS during the biodegradation of metalaxyl by *A. Wentii* Gb2 after 15 days.

NO	Rt(min)	m/z	Fragments	Compound
1	9.88	280 $[M+H]^+$	248,220,192	Metalaxyl 
2	7.45	296 $[M+H]^+$	278,264,236, 208,192,146	Hydroxymetalaxyl 
3	8.90	267 $[M+2H]^+$	235,207,179, 178,149,146	Demethylmetalaxyl 
4	11.13	206 $[M]^+$	178,146,105	Removal of ester group 

4. Discussion

The high utilization of chemically synthesized agrochemicals worldwide in the agriculture practice exerts a negative effect on the environmental equilibrium, contamination to underground water and food chain due to their persistence; hence, the search in the microbial diversity for new microbial isolates capable of the safe elimination of such pollutants should be encouraged [10]. Nonetheless, the isolated microorganisms should have an effective enzyme system to detoxify high concentration of pollutants to less toxic or nontoxic compounds [15]. In the current study, analysis of the water samples collected along the Nile River at the North Delta region was performed to select the persistent pesticide. Metalaxyl was the highest recorded pesticide in surface water which may be attributed to its chemical properties such as high-water solubility, low hydrophobicity, photolytic stability, tolerance to different pH and temperature, and resistance to chemical hydrolysis [21,23]. Under field experiment, successive and repeated application of metalaxyl to soil influenced the structure of microbial communities abundances as it inhibits transiently the sensitive soil fungi [45].

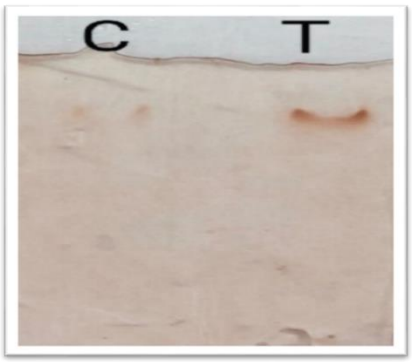


Figure 8. Native-PAGE for determination of heme-content of the separated protein determined by oxidation of o-Dianisidine. 100 mg/L of metalaxyl (T), control (C) without metalaxyl.

In literature, different species of microorganisms, including bacteria, yeast and filamentous fungi, were able to degrade metalaxyl with different potentialities depending on different factors such as initial metalaxyl concentrations. The transformation of metalaxyl in a liquid medium by eight fungal and six bacterial strains was reported before [46]. The

biodegradation process of metalaxyl with a single microorganism reported to be ranging from 36 to 52% after 25 days of incubation and up to 75% with mixtures of either fungi or bacteria in the presence of glucose (malt extract and casamino acids) as the microorganisms could not utilize metalaxyl as a carbon source. The highest degradation rate of metalaxyl by *Candida tropicalis* at optimum concentration of 5 mg/L of metalaxyl was studied by (Derbalah et al., 2020) [32]. Many studies were carried out to investigate the fungal biodegradation of metalaxyl. The ability of two mucorales strains, *Gongronella* sp. and *Rhizopus oryzae* to use metalaxyl as the main carbon source at 10 mg/L was decreased at 50 mg/L even in the presence of glucose and yeast extract [39]. Longer incubation time (42 days) was required for the white rot fungi including *Coriolus versicolor*, *Hypholoma fasciculare* and *Stereum hirsutum* with an initial concentration of 10 mg/L metalaxyl and it was recorded a maximum degradation of metalaxyl for less than 44% [47]. A study showed the ability of *Rhizopus stolonifer* and *Gongronella sp.* to tolerate and degrade the commercial metalaxyl fungicide (containing folpet 40% and metalaxyl (10% w/w)) (not pure metalaxyl) even in high concentrations up to 150 mg/L in malt extract broth medium [48]. Another study showed that *Aspergillus niger, Cladosporium herbarum* and *Penicilluim sp.* are able to degrade (100 µg/ml) of metalaxyl in aqueous system after 28 days of incubation with degradation percentages 52%, 28%, and 20% respectively [34]. Therefore, in this study, the increasing metalaxyl concentration after successive selection for most potential fungal isolates only led to three fungal isolates out of 67 isolates capable of growing in the presence of metalaxyl. But the isolate Gb2 was the only one selected as it was the most tolerant isolate with a high concentration of metalaxyl at 900 mg/L in Czapek's Dox agar medium as a sole carbon source. The isolated *Aspergillus wentii* Gb2 showed a promising degradation rate, reaching 68% of metalaxyl with an initial concentration of 100 mg/L as the only nutrient in sterile distilled water after 15 days of incubation. In addition, the fungus had the ability to tolerate high concentrations of metalaxyl up to 260 mg/L, with 18% degradation percentage using HPLC. This observation may be explained that the microbial enzyme system responsible for metalaxyl transformation was inhibited by a higher metalaxyl concentration [40].

Many studies were carried out to investigate the biodegradation metabolites of metalaxyl in soil systems, involving a wide range of microorganisms and detecting the formation of acid metabolite as the main metabolite by cleavage of the methyl ester group [36,38,49,50–52]. A second metabolite called (N-methoxyacetyl-2,6-dimethyl-aniline) was also detected [36,52], but without identifying the responsible specific microbial strains for metalaxyl degradation.

In this study, the LC/MS results detected the formation of the hydroxyl metabolite as the main metabolite, either at a metalaxyl concentration of (100 or 260) mg/L. Hydroxylation of metalaxyl also appeared by *Syncephalastrum racemosum* when incubated in a liquid medium supplemented with glucose, yeast extract and metalaxyl as the fungus could not use metalaxyl as the sole source of carbon. The transformation rates decreased as the concentrations of metalaxyl increased from 5 to 100 mg/L and no transformation was observed when the concentration of metalaxyl was higher than 100 mg/L [40]. Our study also detected the formation of further metabolites when using 100 mg/L of metalaxyl other than the hydroxyl metabolite. Demethylation from the ether group [N-(2,6-dimethylphenyl)-N-(hydroxyacetyl) alanine methyl ester] and the other metabolite was [N-(2,6-dimethylphenyl)-N-ethyl-2-hydroxyacetamide]. To our knowledge, this is the first record of detecting those two metabolites by microbial degradation of metalaxyl. Acidic metabolite, hydroxymetalaxyl, and demethylation from the ether group were also detected in the human, animals, and plants metabolism of metalaxyl [26,53,54]. Little has been published concerning the enzymes responsible for microbial metalaxyl degradation. Earlier study showed that there was no relation between the degradation of mono-aromatic pesticides (diuron, metalaxyl, atrazine) and the ligninolytic activity by the white rot fungi [47].

The role of cytochrome P450 has been shown to be important in the degradation of a variety of xenobiotics, including pesticides [55]. The involvement of P450s has been proven in the metabolism of aliphatic, alicyclic, and aromatic molecules in reactions resulting in hydroxylation, dealkylation and other reactions [56]. The metabolic diversity of fungal Cytochrome P450 is suggested as a mediator for the degradation of pesticides [57–60]. It was also reported that the metabolism of metalaxyl in human and animals by Cytochrome P450, giving hydroxylation, dealkylation, and didealkylation reactions [26,61]. Based on the reported results, Cytochrome P450 could be involved in the biodegradation of metalaxyl by *A. wentii* and it was confirmed by the protein analysis using a TripleTOF 5600+ LC-MS/MS system. Seven percentage reductions in the metalaxyl concentration were achieved in the *A. wentii* Gb2 cell lysate, compared to two percentage reductions in the control. This could mean that a constitutive enzyme was overexpressed when the fungus was grown in the presence of metalaxyl. Overexpression in the CYP family as detoxifying enzymes of pesticides was also reported [62]. The incubation of the cut native gel band with metalaxyl and NADPH showed a 40.6 % decrease in metalaxyl concentration compared to 2.7% in the control (without the enzyme) which is showing imbibition at 2.7%.

5. Conclusion

It could be concluded that this study showed the ability of *Aspergillus wentii* to use the metalaxyl pesticide as a sole carbon source in sterilized distilled water without adding any other nutrient supplements with a high degradation rate and can tolerate high concentrations of metalaxyl. The biodegradation pathway was postulated, and the enzyme involved in the biotransformation was identified. These findings highlight the potential of *Aspergillus wentii* enzymes in the elimination of various toxic environmental pollutants, including metalaxyl. Further study exploring the enzymatic mechanisms underlying metalaxyl degradation by the isolated *Aspergillus wentii*, as well as investigating its potential applications in bioremediation strategies for contaminated soil and water ecosystems are needed. Furthermore, studies investigating the ecological impact of metalaxyl degradation by *Aspergillus wentii* on microbial community dynamics and soil health would provide valuable insights for sustainable agricultural practices.

6. Conflicts

The authors declare no conflict of interest.

7. References

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