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Metabolic pathway of tebuconazole by soil fungus *Cunninghamella elegans* ATCC36112

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Abstract Tebuconazole is the most widely used fungicide in agriculture. Due to its long half-life, tebuconazole residues can be found in the environment media such as in soil and water bodies. Here, the metabolic pathway of tebuconazole was studied in *Cunninghamella elegans* (*C. elegans*). Approximately 98% of tebuconazole was degraded within 7 days, accompanied by the accumulation of five metabolites. The structures of the metabolites were completely or tentatively identified by gas chromatography-mass spectrometry (GC–MS) and ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). To identify representative oxidative enzymes that may be involved in the metabolic process, treatment with piperonyl butoxide (PB) and methimazole (MZ) was performed. PB had a strong inhibitory effect on the metabolic reactions, while MZ had a weak inhibitory effect. The results suggest that cytochrome P450 (CYP) and flavin-dependent monooxygenase are involved in the metabolism of tebuconazole. Based on the results, we propose a metabolic pathway for the fungal metabolism of tebuconazole. Data are of interest to gain insight into the toxicological effects of tebuconazole and for tebuconazole bioremediation.

Keywords Biodegradation · *Cunninghamella elegans* · Fungal metabolism · Inhibitor · Tebuconazole

Min Meng, Zhaochi Zhai and Zhenxing Zhang contributed equally to this work.

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Introduction

The fungicidal effect of triazoles is very efficient compared to other fungicides and thus they are widely used in agriculture and industry. In addition, these chemicals have been used as drugs for the treatment of a variety of fungal infections, including neonatal vaginal fungal disease and oral thrush (Cui et al. 2018). Tebuconazole belongs to the group of triazole fungicides which act by inhibiting the biosynthesis of ergosterol. It is used for the control of fungal pathogens in a range of vegetables, fruits and

crops. (Azhari et al. 2018; Dong et al. 2018; Hou et al. 2017). Currently, tebuconazole has become the most widely used fungicide in agriculture. Due to its long half-life, tebuconazole resides in various environmental media such as the atmosphere, soil, environmental water samples, fruits and vegetables (You et al. 2017). This may well impact mammals. For example, they show a strong ability to interfere with CYP enzymes, leading to endocrine disrupting effects (Yang et al. 2018). Moreover, tebuconazole induces developmental disorders, immune abnormalities, reproductive dysfunction, nephrotoxicity and hepatotoxicity. Metabolic studies are important for understanding the toxicity and safety of pesticides (Madrigal-Matute and Cuervo 2016; Franco et al. 2020; Shen et al. 2019; Castro et al. 2018). Currently, studies on tebuconazole metabolism have focused on mice, birds and other mammals (Bellot et al. 2022; Hillebrands et al. 2020). In addition, the metabolism and toxicity of tebuconazole in vitro (HepG2 cells and human liver microsomes, etc.) and bacteria (*Pseudomonas fluorescens* and *Pseudomonas putida*, etc.) have also been reported (Kwon et al. 2021; Hillebrands et al. 2020). However, there are few reports on the metabolism of tebuconazole in fungi. Biodegradation by microorganisms is one of the most important steps in the dissipation and detoxification of toxic chemicals in the natural environment. Among the microbiota in the soil environment, fungal biomass is usually much higher than other species (Coombes et al. 2015; Keum et al. 2009). Therefore, their contribution to external biological dissipation is important. *C. elegans* is widely used as a microbial model for mammals to study the biotransformation of drugs, pesticides and the environmental decontamination of pollutants (Zhu et al. 2010). The main objective of this study was to biotransform tebuconazole and identify metabolites using *C. elegans* (Palmer-Brown et al. 2019). The oxidases involved in the metabolic reactions were identified by using selected inhibitors.

Materials and methods

Chemicals

Tebuconazole (98% of purity) was received from DANO (http://m.haonongzi.com). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Aladdin Reagents Ltd (https://www.aladdin-e.com). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA + TMCS, 99:1, sylon BFT) was purchased from Merck (https://www.merck.com). Piperonyl butoxide (PB) and methimazole (MZ) were from Macklin Corporation (http://www.macklin.cn). Ethanol, acetonitrile, acetone and dichloromethane used in the experiments were purchased from Tianjin Fuyu Chemical Co Ltd (https://www.chembk.com). Anhydrous sodium sulfate and sodium chloride were from Aladdin. All other reagents are analytical or chromatographic grade.

Microorganism

Cunninghamella elegans ATCC36112 was acquired from the American Type Culture Collection (Manassas, VA). Stock cultures of *Cunninghamella elegans* ATCC36112 were maintained on PDA plates at 27 °C. Spores and mycelia from several plates were used to inoculate on PDB medium. Fungal cultures were typically maintained on PDA at 27 °C while liquid cultures were grown on PDB at 27 °C and 170 rpm.

Metabolic response of *C. elegans* to tebuconazole

For the metabolic reaction system, *C. elegans* was cultured on PDB for 3 days. Cultured mycelium (approximately 2 g) was added to fresh PDB (500 mL, pH = 7.0), supplemented with tebuconazole (5 mg), and incubated at 27 °C (170 rpm). Negative and blank control experiments were carried out through systems using only *C. elegans* and only tebuconazole, and three parallel experiments were done for each level. All media throughout the experiment were sterilized before use (15 min, 121 °C).

Metabolic pathway analysis of tebuconazole by *C. elegans*

At each sampling time (0, 1, 2, 3, 5 and 7 days after treatment), 50 mL of the sample was transferred to a separatory funnel and then 10 g of NaCl was added and extracted twice with 50 mL of dichloromethane. The dichloromethane phases was dried through anhydrous sodium sulfate. After evaporation of the solvent, 0.5 mL pyridine was added, followed by

derivatization with BSTFA + TMCS (99: 1,300 µL) at 70 °C for 35 min, and analyzed by GC–MS.

Enzyme inhibitor assay for tebuconazole by *C. elegans*

PB and MZ (5 and 50 mg/L, respectively) were added to the culture medium to investigate the effects of cytochrome P450 (CYP) and flavin-dependent monooxygenase (FMO) metabolism, respectively. Tebuconazole (10 mg/L) was added after 12 h of culturing *C. elegans*. Samples were collected at 2 h, 1, 2, 3, 5 and 7 days, respectively.

Isolation and identification of tebuconazole metabolites by large-scale culture

Four 500 mL cultures (maximal metabolite formation) were extracted after 3 days of incubation, combined and concentrated as described above. The major metabolites M1 and M2 were separated in the extracts by preparing the liquid phase. The isolated samples were analyzed by UPLC-MS/MS.

Instrumental analysis methods

Tebuconazole and its metabolites were detected by GC–MS (QP 2010, Shimadzu, Japan). The GC–MS was equipped with an RTX-5MS column (30 m, 0.25 m film thickness, 0.25 mm inner diameter; Agilent Technologies, USA). GC–MS was performed with helium as carrier gas at a flow rate of 7.0 mL/min. The programmed warming methods used in the experiments were 80 °C (1 min), 7 °C/min–240 °C (2 min), 7 °C/min–290 °C (30 min). The injection port and interface were set to 260 and 280 °C, respectively. The mass spectrometer of the GC–MS was used in electron impact (EI) mode at 70 eV during sample detection. Preparative liquid phase analysis was performed using a column equipped with a Sino-Chrom ODS-BP (5 µm, 10 mm × 250 mm); mobile phase A: methanol; mobile phase B: distilled water; mobile phase composition: 30–100% A and 7–0% B (gradient elution); flow rate: 3 mL/min; injection volume: 2 mL; UV detection wavelength: 230 nm; M1 eluted at 29.1 min, M2 eluted at 28.4 min. M1 eluted at 29.1 min and M2 eluted at 28.4 min.

Results

Metabolism of tebuconazole by *C. elegans*.

Analysis of the GC–MS results showed that tebuconazole can be rapidly converted into a variety of metabolites (Fig. 1). For example, the degradation rate of tebuconazole in the culture 72 h after treatment was about 85%. M1 and M2 were the major

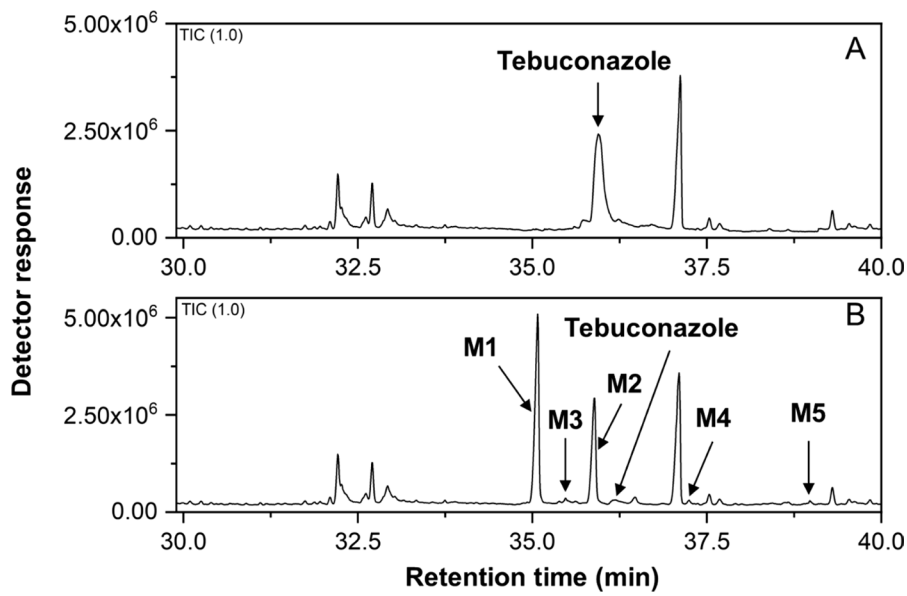

Fig. 1 Ion chromatograms (TIC) of tebuconazole and its TMS-derivatized metabolites by *Cunninghamella elegans* at 3 days (**B**); negative control: no fungus (**A**)

metabolites (Fig. 2A). M1 was detected at 24 h and reached its maximum concentration at 3 days. Another metabolite, M2 was similarly detected at 24 h and the highest accumulation of this metabolite was observed after 5 days. M1 was the most abundant metabolite throughout the experiment. Identification was performed by large scale culture. There was no substantial degradation of tebuconazole identified in sterilization control trials, and no metabolite peaks were observed. (Fig. 1A).

Effects of PB on the degradation of tebuconazole

In contrast to the control experiments, a significant amount of tebuconazole was still present in the PB-treated culture. For instance, the amount of tebuconazole that remained after 3 days was roughly 28–67% of the initial dose and after 7 days of tebuconazole was about 13–63% of the initial dose (Fig. 2). In all the incubations containing PB, minor metabolites (M3-M5) were not detected or found to accumulate in very small amounts. The concentration of M1 in PB-treated cultures (5 mg/L) was approximately 4–12-fold less than that of the control (Fig. 2A, B). Its concentration reached a maximum at 72 h. M2 concentrations found in PB-treated cultures (5 mg/L) were approximately 5–10 times lower than in the control cultures (Fig. 2B), and its concentration started to decrease after reaching a maximum at 5 days. At higher levels of PB treatment (50 mg/L), the concentrations of metabolites M1 and M2 were only found at trace levels and thereby being much lower than in the control (Fig. 2C).

Effects of MZ on the degradation of tebuconazole

Tebuconazole was rapidly degraded in both concentrations of MZ-treated cultures (5, 50 mg/L). In cultures treated with high concentration of MZ (50 mg/L), residual tebuconazole was approximately 10% of the initial dose after 7 days (Fig. 3C). The overall characteristics of the metabolites of tebuconazole and low levels of MZ (5 mg/L) were not different from the control (Fig. 3B). In PB-treated cultures, M1 was the major metabolite, and its kinetic response in MZ-treated cultures was almost similar to that of the control.

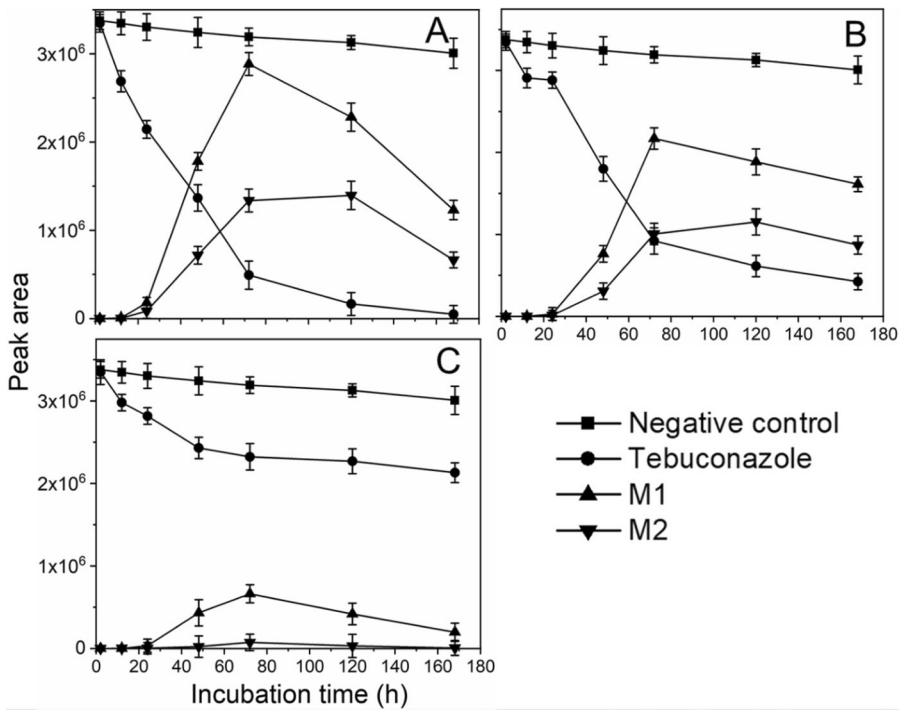

Fig. 2 Degradation and accumulation pattern of tebuconazole and metabolites by *C. elegans* in piperonyl butoxide-treated cultures (0, 5, and 50 mg/L piperonyl butoxide for **A**, **B**, and **C**)

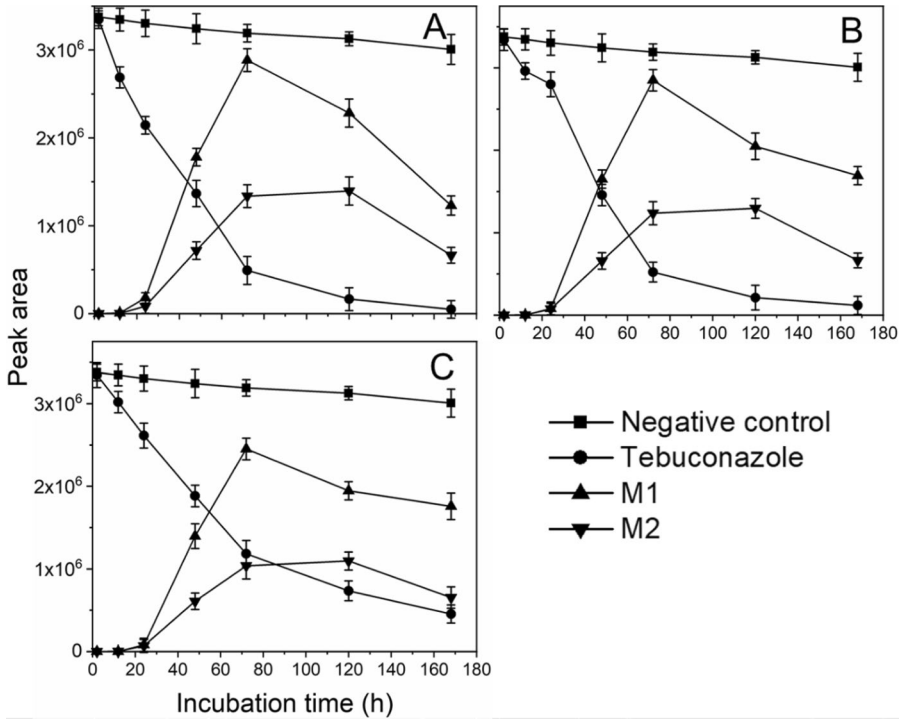

Fig. 3 Degradation and accumulation pattern of tebuconazole and metabolites by *C. elegans* in methimazole-treated cultures (0, 5, and 50 mg/L methimazole for **A**, **B**, and **C**)

Identification of tebuconazole metabolites by *C. elegans*

Throughout the experiment, five tebuconazole metabolites were identified, with two major metabolites (M1–M2). The mass spectral details of these metabolites are shown in Table 1. The GC–MS results showed retention times of 35.067 min and 35.858 min for M1 and M2, respectively. The molecular ion peak of the TMS derivatives of M1 and M2 was *m/z* 395, which was 88 Da (-OTMS) more than tebuconazole, indicating that the metabolites M1 and M2 were monohydroxylated tebuconazole. M1 and M2 have the same fragment ion peaks (*m/z* 338, *m/z* 313, *m/z* 248, *m/z* 213). The fragment ions *m/z* 338, *m/z* 313, *m/z* 213 of M1 and M2 were 88 Da (-OTMS) more than the fragment ions *m/z* 250, *m/z* 225, *m/z* 125 of tebuconazole, respectively, and presumably the

Table 1 GC–MS retention times and mass fragmentations of tebuconazole and the TMS-derivatized metabolites

	Retention time(min)	Molecular and fragment ions
Tebuconazole	35.942	307 (M^+,4.04), 250 (100), 213(13.98), 163 (17.29), 125 (91.96), 83 (59.72), 70 (74.67), 57 (57.66)
M1	35.067	395 (M^+, 3.3), 338 (3.2), 313(1.64),248 (80.72), 213 (36.48), 163 (5.11), 125 (11.42), 73 (84.45), 70 (40.3), 57 (100)
M2	35.858	395 (M^+, 2.04), 338 (1.46), 313(1.62),248 (53.35), 213 (44.66), 125 (10.69), 73 (78.64), 70 (39.67), 57 (100)
M3	35.450	483 (M^+, 1.24), 342 (58.19), 316(6.87), 248 (41.11), 225(19.71),213 (4.27), 125 (67.31), 85 (61.7), 73 (68.64)), 57 (100)
M4	37.257	409 (M^+, 1.68), 313 (14.3), 248 (29.12), 225 (6.03), 179 (16.87), 125 (27.06), 83 (37.86), 73 (82.46), 57 (100)
M5	38.975	395 (M^+, 1.66), 313 (27.45), 250 (13.71), 213 (5.99), 188 (41.62),147 (13.32), 125 (85.33), 103 (7.04), 83 (51.5), 73 (100)

[a]Values in parenthesis are relative abundance of specified fragment ions

fragment ions of tebuconazole (*m/z* 250, *m/z* 225, *m/z* 125) all contained the structure of benzene ring, so it was concluded that M1 and M2 were hydroxylated on the benzene ring. To further determine their structures, M1 and M2 were separated using a preparative HPLC. The separated M1 and M2 were analyzed by UPLC-MS/MS, and the results showed that the molecular weight of M1 was 324.14682 ($[M+H]^+$) (Fig. S1) and M2 was 324.14713 ($[M+H]^+$) (Fig. S2). Their structure was deduced from the molecular weight, and the chemical formula was $C_{16}H_{23}ClN_3O_2$ ($[M+H]^+$). The order of the peaks of M1 and M2 in the liquid phase enabled the determination of the polarity of both M1 and M2, and thus the position of the hydroxyl groups of the two metabolites in the benzene ring. Metabolite M2 has a greater polarity than M1. Therefore, it is presumed that M1 is 5-(3-((1*H*-1,2,4-triazol-1-yl)methyl)-3-hydroxy-4,4-dimethylpentyl)-2-chlorophenol and M2 is 2-(3-((1*H*-1,2,4-triazol-1-yl)methyl)-3-hydroxy-4,4-dimethylpentyl)-5-chlorophenol.

Additional metabolites (M3-M5) were initially identified by mass spectrometry patterns and relevant references. The TMS-derivatized molecular ion peaks for M3 and M4 were *m/z* 483 and *m/z* 409, respectively. M3 and M4 have the same characteristic ion peaks as M1 and M2 (*m/z* 248). The characteristic ion peaks *m/z* 248 of the metabolites M1 and M2 are the loss of hydroxyl and tert-butyl groups and further loss of TMS-derivatized trimethylsilane, indicating that both M3 and M4 change at the tert-butyl position. The molecular ions of M3 are 178 Da more than tebuconazole, indicating that M3 is possibly a dihydroxylated tebuconazole. In addition, M3 has the same fragment ions as M1 and M2 (*m/z* 248, *m/z* 225, *m/z* 163), indicating that M3 has one hydroxyl group added to the benzene ring and another hydroxyl group added to the tert-butyl group, but the position of the hydroxyl group on the benzene ring is uncertain. The molecular ion of M4 is 74 Da higher than that of tebuconazole, and the molecular ion of M4 is 14 Da larger than that of M1 and M2. The fragment ion of M4 has the same fragment ion as the metabolites M1 and M2 (*m/z* 313, *m/z* 248, *m/z* 213, *m/z* 125), indicating that M4 underwent hydroxylation on the benzene ring. M4 has the fragment ion *m/z* 248, indicating that on the tert-butyl change has occurred. Therefore, it is speculated that M4 is a ketone structure formed on the basis of M1 and M2. The TMS-derivatized molecular weight of M5 is *m/z* 395. M5 has the same characteristic fragment ion *m/z* 250 as tert-butyl alcohol. The fragment ion *m/z* 250 is formed by the loss of tert-butyl, so it is speculated that M5 is formed by the addition of hydroxyl group at the tert-butyl position. On this basis, we speculated the metabolic pathway of *C. elegans* to tebuconazole (Fig. 4).

Discussion

C. elegans and other strains of the same genus are capable of metabolizing a wide range of compounds. There is a considerable similarity between their xenobiotic metabolism and that of animals, which will aid us in determining the metabolic destiny of the organic compounds in mammalian hepatocytes and studying the biotransformation of medications, pesticides, and pollutants in the environment. (Schocken et al. 1997; Palmer-Brown et al. 2017; Chen et al. 2017).

The current studies on the metabolism of tebuconazole found that the main metabolic pathways are hydroxylation and hydrolysis reactions. For example, in plants, tebuconazole is first hydroxylated and then continues to undergo glycosylation at the hydroxylated site (Obanda and Shupe 2009; Stoll et al. 2006; Hillebrands et al. 2021). Mold (*Trichoderma harzianum*), soft rot (*Chaetomium globosum*), white rot (*Phanerochaete* chrysosporium), and brown rot (Meruliporia. incrassata) can metabolize tebuconazole, and the main metabolic modes of tebuconazole are hydroxylation and hydrolysis reactions, and the carboxylation reaction also occurred after hydroxylation (Ge et al. 2021; Teng et al. 2019; Han et al. 2021; Theron et al. 2019). In this study, only hydroxylation reactions were found, and no glycosylation reactions as in plant metabolism or hydrolysis reactions as in microbial metabolism were found. It may be due to the lack of enzymes for these metabolic reactions in *C. elegans*. In terms of degradation rate, *C. elegans* could metabolize 40% of tebuconazole within 2 days, which is 7–10 times faster than the rate of microbial metabolism in previous studies. During this rapid degradation process, it is possible that some of the metabolites were rapidly converted to other compounds or metabolites that were difficult to detect and therefore not observed in the assay system. To study the oxidases involved in

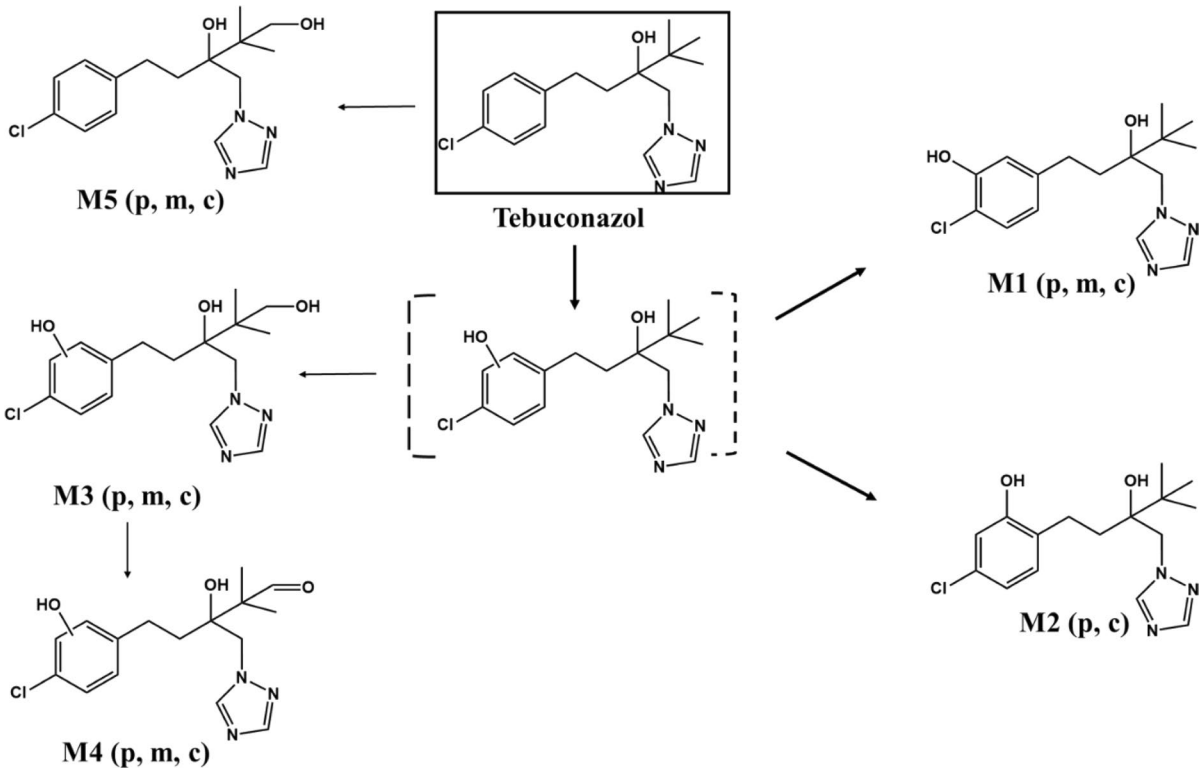

Fig. 4 Proposed metabolic pathways of tebuconazole in *C. elegans* (c). Major pathways are expressed as thick arrows. Abbreviations: m, microorganism; p, plants

the metabolism of tebuconazole, PB and MZ were added to the cultures, as PB is a well-known CYP inhibitor and MZ is also a well-known FMO inhibitor. (Greule et al. 2018). The results of this study showed that PB greatly inhibited tebuconazole dissipation and the production of the five metabolites, but MZ only produced significant inhibition at the highest dose. The research results indicated that CYP plays a major role in the transformation of tebuconazole by *C. elegans* and FMO may play a lesser role in tebuconazole metabolism. However, the effect of FMO on the metabolism of tebuconazole cannot be ignored (Than et al. 2008; Zhang et al. 2020). The good metabolism of tebuconazole by *C. elegans* can help to remove tebuconazole from the environment. In addition, the metabolic pattern of *C. elegans* is similar to mammalian metabolism so it can provide the toxicity study and evaluation of metabolites during the metabolism of exogenous compounds in mammals (Mueller-Eigner et al. 2022). For other metabolic function enzymes in *C. elegans* remains to be studied.

Conclusions

In this study, the metabolism of tebuconazole by *C. elegans* is reported. Five metabolites were totally or tentatively identified with GC–MS and UPLC-MS/MS. The metabolism of tebuconazole by *C. elegans* was demonstrated to involve CYP and FMO. The experimental results revealed that the degradation of tebuconazole by *C. elegans* was superior to that of previously reported microorganisms. These findings will aid in the understanding of tebuconazole fungal degradation and its possible application in tebuconazole bioremediation.

Author contributions YZ contributed to the study conception and design. MM, ZZ and ZZ performed the experiments

and analysis. Material preparation, data collection, and analysis were performed by MM, ZZ and ZZ. The manuscript was written by ZZ, and edited by MM. All authors read and approved the manuscript.

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Data availability The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no competing interests.

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