

**Citral-based amide derivatives improve the antifungal activity of
Kresoxim-methyl against *Rhizoctonia solani*.**

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Abstract

BACKGROUND: *Rhizoctonia solani*, the fungal pathogen causing rice sheath blight, threatens global food security. Prolonged use of Kresoxim-methyl (KM), a common fungicide, has led to resistance and reduced effectiveness. This study investigates the synergistic effects of citral-based amide derivatives with KM and explains the underlying mechanisms.

RESULT: Among the tested derivatives, d25 exhibited the most pronounced synergistic effect, achieving a peak synergistic ratio ($SR = 2.66 \pm 0.67$) at a molar ratio of 25% in the mixture. At a molar ratio of 10%, the mixture (Mix) demonstrated significant antifungal activity both *in vitro* and *in vivo*, effectively reducing mycelial dry weight and enhancing rice leaf protection. Mechanistic studies revealed that Mix disrupted the tricarboxylic acid (TCA) cycle, thereby inhibiting energy metabolism. Additionally, d25 interfered with arachidonic acid metabolism, impairing cell membrane repair mechanisms and enhancing the fungicidal efficacy of KM. Toxicity assessments revealed that d25 exhibited minimal cytotoxicity against human cell lines and low acute toxicity toward *Apis mellifera* L. and zebrafish.

CONCLUSION: The findings elucidate the synergistic mechanisms of d25 and Mix, underscoring their potential as effective fungicide synergists. This study establishes a robust theoretical and practical foundation for enhancing the efficacy of chemical control strategies against *Rhizoctonia solani*, providing new insights into the development of safe and efficient fungicide formulations.

Keywords: Citral-based amide derivatives; Kresoxim-methyl; *Rhizoctonia solani*; Cell

membrane; Synergistic antifungal activity

1 INTRODUCTION

Rice is one of the most important food crops globally, and guaranteeing the safe cultivation of rice holds crucial significance for global food security¹. However, the rice sheath blight caused by *Rhizoctonia solani* has a severe impact on the normal growth and yield of rice². When rice sheath blight occurs severely, it can result in a reduction of rice production ranging from 10% to 50%^{3,4}. Consequently, the management of rice sheath blight has emerged as a critical issue in contemporary rice cultivation⁵.

Presently, the application of fungicides continues to be the predominant method for managing rice sheath blight². Kresoxim-methyl (KM), being the first methoxyacrylate fungicide to enter the market, possesses the merits of a broad fungicidal spectrum, low toxicity, and a prolonged residual period, and is extensively employed in the prevention and control of rice sheath blight^{6,7}. The antifungal effect of KM is achieved by inhibiting the cytochrome c reductase activity of plant pathogenic fungi⁸. KM has been used for more than 25 years, and a variety of plant pathogenic fungi have developed strong resistance⁹⁻¹². Therefore, it is of great value to reduce the drug resistance of plant pathogenic fungi to KM and improve the antifungal effect of KM.

In recent years, considerable attention has been directed towards mitigating the escalation of fungicide resistance through the utilization of synergists to enhance fungicide efficacy¹³. The incorporation of synergists not only amplifies the efficacy of fungicides but also diminishes the quantity of fungicides required, consequently mitigating environmental impact and curbing the development of resistance¹⁴. Bao *et*

al. demonstrated that the incorporation of surfactants improved the wettability, adhesion, penetration, and absorption of pyraclostrobin on hydrophobic leaf surfaces, thereby enhancing its bioactivity and reducing potential environmental risks¹⁵. Nevertheless, the majority of documented fungicidal synergists are surfactants, which can enhance the efficacy of fungicides by optimizing their penetration properties and improving their resistance to rainwater erosion^{16,17}.

In addition to surfactant-based agents, specific synergists can augment fungicidal efficacy by disrupting the physiological processes or metabolic pathways of pathogens via multi-target mechanisms. This enhances the bioavailability of the active ingredient, thereby optimizing its antifungal performance^{18,19}. In a study conducted by Rahman *et al.*, it was demonstrated that the application of chitosan significantly enhanced the inhibitory effect of commercial fungicides against *Botrytis cinerea*, reducing spore germination by 90%²⁰. Wang *et al.* found that the combination of eugenol and citral (CEC) disrupted *Aspergillus niger* cell membranes through a synergistic effect²¹. Camiletti *et al.* found that combining essential oils with iprodione had a synergistic effect in managing garlic white rot. Essential oils from *Tagetes filifolia* L. and *Laurus nobilis* L., when combined with iprodione, significantly reduced the density of mycorrhizal fungi and the incidence of *Sclerotium cepivorum* Berk²². However, there is currently a lack of research on the development and utilization of synergistic fungicidal agents to enhance the efficacy of KM. This gap in the existing literature warrants further investigation.

In our previous studies, we synthesized a series of citral-based amides and

observed their antimicrobial activity against *R. solani*. These citral-based amide derivatives demonstrated inhibitory effects on *R. solani* by affecting cell membrane function, antioxidant enzyme activity, and tricarboxylic acid cycle-related enzyme activity. They also enhanced the degradation of exogenous metabolites²³. These findings suggest that citral-based amide derivatives possess a multi-site mode of action, making them promising candidates as synergists in combination with single-site fungicides such as KM, potentially reducing resistance risk. Given the pronounced inhibitory impact of these citral-based amide derivatives on *R. solani* (preliminary EC₅₀ values ranging from 0.015 to 112.21 µg/mL), they have the potential to act as synergistic fungicidal agents for KM. The objective of this study is to investigate the potential of citral-based amide derivatives to enhance the fungicidal effect of KM by affecting the cell membrane function of *R. solani*. The findings may provide a theoretical basis for the research and development of novel fungicidal synergists.

2 MATERIALS AND METHODS

2.1 Materials and Strains

The *R. solani* strain was generously provided by the Laboratory of Plant Pathology, College of Agriculture, Jiangxi Agricultural University, China, and has been used in previous studies²³. The KM was provided by Shanghai Yuanye Biotechnology Co., Ltd. Potato Dextrose Agar (PDA) was purchased from Huankai Microbial Sci. & Tech. Co., Ltd., and Potato Dextrose Water (PDW) was obtained from Qingdao Haibo Biotechnology Co., Ltd. All other chemicals used were of analytical grade and were supplied by Xilong Scientific Co., Ltd. Citral-based amide derivatives are pre-prepared

by our research group. Note: The designation of compounds (d1 – d28) in this study refers to a curated subset of previously synthesized derivatives and may not align with the numbering system employed in our earlier publication (Table S1)²³.

2.2 Determination of *in vitro* Antifungal Activity

Citral-based amide derivatives (**d1** to **d28**) were mixed with KM according to a 10% molar ratio ($\mu\text{mol}:\mu\text{mol}$), respectively. The EC_{50} (median effect concentration) of these mixtures were subsequently determined in preliminary screening experiments. Based on the lowest EC_{50} value, compound **d25** was selected for further analysis. In subsequent experiments, the compound **d25** was formulated into combinations with KM at varying molar ratios (5%, 10%, 15%, 20%, and 25%). Dimethyl sulfoxide (DMSO) was used as a solvent for the preparation of compounded solutions. A 50 μM solution of the compound **d25** and a KM solution containing a mixture of the two (for example, a 10% **d25**/KM compound solution corresponds to 5 μM **d25** and 45 μM KM) were separately prepared with DMSO and used as stock solutions. Subsequently, a medium containing drug concentrations of 50, 25, 12.5, 6.25, 3.125, and 1.5625 μM was prepared using the stock solutions.

The mycelial growth rate method was used to assess the *in vitro* antifungal activity of citral-based amide derivatives and KM complexes²⁴. Activated *R. solani* mycelial clumps (0.5 cm diameter) were inoculated into the drug-containing medium, with an equal amount of DMSO as a blank control. Petri dishes were incubated at 28 °C for 2 days. Each concentration was tested in triplicate. Colony diameters were measured, and EC_{50} values were calculated for each treatment. The Wadley method was used to

calculate the synergistic ratio (SR)²⁵. The formula for its calculation is as follows:

$$X_1 = \frac{P_A + P_B}{P_A/A + P_B/B} \times 100 \quad (1)$$

$$SR = \frac{X_1}{X_2} \quad (2)$$

In the formula, X_1 is the theoretical EC_{50} value, P_A is the percentage of component A (citral-based amide derivative), P_B is the percentage of component B (KM), A and B are their respective EC_{50} values, and X_2 is the actual EC_{50} value of the mixture. Synergism is indicated when $SR > 1.5$; additivity when $0.5 \leq SR \leq 1.5$; and antagonism when $SR < 0.5$.

2.3 Determination of *in vivo* Antifungal Activity

According to Peng *et al.*, the *in vivo* antifungal activities of citral-based amide derivatives, ether esters, and compounds were investigated using potted rice leaves as experimental materials²⁶. The rice variety used in this study is E'huxiangnuo. KM, compound **d25** and the combination Mix were each tested at a concentration of 500 μ M. Deionized water was used as the blank control. In the curative activity assay, rice leaves were punctured with a sterile disposable syringe and inoculated with *R. solani* on day 1. Twenty-four hours after inoculation, the treated leaves were sprayed with the pre-prepared test sample solution. In the protective activity assay, the test samples were uniformly sprayed on rice leaves on day 1. Twenty-four hours later, the leaves were punctured with a sterile syringe and inoculated with *R. solani*. All plants were maintained at the same growth stage and cultivated under strictly controlled conditions to minimize environmental variability. Treatments were randomly assigned to experimental groups, and each group included three independent biological replicates

to ensure data reliability. Samples were incubated for 7 days at 28 ° C and 90% relative humidity, with each treatment replicated three times. At the end of the incubation period, the infection level was evaluated by calculating the lesion area using the following formula:

$$A(\%) = [A_{CK} - A_{treat}]/A_{CK} \times 100\% \quad (3)$$

Where A (%) represents the inhibition rate or efficacy (%), A_{CK} is the lesion area in the blank control group, and A_{treat} is the lesion area in the treatment group.

2.4 Determination of Dry Weight of Mycelium of *R. solani*

According to the method provided by Mo *et al.*, *R. solani* mycelial clumps with a diameter of 0.5 cm were inoculated into PDW containing 2 µM compound **d25**, 18 µM KM, and 20 µM **Mix** (comprising 10 mol% **d25** and 90 mol% KM), respectively²⁷. The inoculated PDW was placed on a shaker at 28 °C, 140 rpm for 3 days. The mycelium was then collected and dried at 60 °C for 24 hours. The dry weight of the mycelium was determined. Each treatment group had three independent replicates.

2.5 Cytotoxicity Analysis

The cytotoxicity of compound **d25** against LO₂ (human normal liver cells) and HEK293T (human embryonic kidney 293T cells) was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method described²⁸. LO₂ and HEK293T cells were inoculated into 96-well plates at a density of 1.5 × 10⁴ cells per well and treated with **d25** at a concentration of 10 µg/mL for 24 hours. Untreated cells served as controls. Then, 20 µL of 5 mg/mL MTT was added to each well and incubated for 4 hours. After incubation, the supernatant was removed,

and 150 μL of DMSO was added to dissolve the crystals. Absorbance was measured at 492 nm using an enzyme marker to assess cell viability. Each treatment was replicated three times.

2.6 Acute Oral Toxicity Test in *Apis mellifera* L.

The acute oral toxicity of **d25** to honey bees was evaluated using the method described by Orčić *et al.*²⁹. The bees were provided by the Honey Bee Research Institute of Jiangxi Agricultural University and were maintained in a dark environment at a temperature of 25 ± 2 °C and relative humidity of 60-70%. They were fasted for 2 hours. **D25** was dissolved in a minimal volume of DMSO, and a 50% (w/v) sucrose aqueous solution was subsequently added to prepare the stock solution. Bees were administered a 50% sucrose solution containing 2 μL of the active ingredient at concentrations of 100, 50, 25, 12.5, and 6.25 μg per bee, diluted in 200 μL of water. Dimethoate served as the positive control, while an equimolar solution of sucrose in DMSO functioned as the blank control. Each treatment group comprised three replicates, with each replicate consisting of ten honeybees (*Apis mellifera* L.). After 24 hours, the mortality rate of the bees was observed and recorded.

2.7 Acute Toxicity Test in Zebrafish

By the previously described protocols, the acute toxicity of compound **d25** to zebrafish was assessed³⁰. Groups of ten zebrafish (3.0 ± 0.1 cm in length) were exposed to **d25** concentrations of 2, 4, 6, 8, 10, and 20 mg L^{-1} for 96 hours under static conditions (with $n = 3$ replicates per concentration). The experiment was conducted at a controlled temperature of 28 ± 1 °C. Mortality was recorded daily, and the 96-hour

LC₅₀ value was determined using probit regression analysis performed with IBM SPSS Statistics 25.

2.8 Assessment of Cell Membrane Permeability

The cell membrane permeability of *R. solani* hyphae was evaluated using the method described by Kang *et al*³¹. A total of 0.5 g of mycelium was suspended in 20 mL of deionized water containing the following test samples: 2 µM **d25**, 18 µM **KM**, and 20 µM **Mix**. An equal volume of deionized water served as the blank control. The conductivity of the mycelial solution was measured at 0 (E₀), 20, 40, 60, 90, 120, and 180 minutes (E₁). Subsequently, the conductivity was determined after boiling the mycelium in the solution and allowing it to cool (E₂). The relative conductivity was calculated using the following equation:

$$\text{Relative conductivity} = \frac{E_1 - E_0}{E_2 - E_0} \times 100\% \quad (4)$$

2.9 Evaluation of Soluble Protein Content

Active *R. solani* mycelium disks (0.5 cm in diameter) were inoculated into PDW and incubated at 28 °C on a shaker at 140 rpm for 3 days. Subsequently, various test samples were added to the PDW to achieve final concentrations of 2 µM **d25**, 18 µM **KM**, and 20 µM **Mix**. A blank control was established by adding an equal volume of DMSO to the PDW. After 24 hours of treatment, the soluble protein content of the mycelia under different conditions was determined using the protocol provided by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Each treatment was performed in triplicate.

2.10 Detection of Mycelial Morphology and Microscopic Structure

With reference to the method described by Cao *et al.*, activated *R. solani* mycelium pieces (0.5 cm in diameter) were inoculated in PDW and cultured on a shaking table at 28°C and 140 rpm for 3 days³². Subsequently, different test samples were added to the PDW to achieve final concentrations of 2 µM **d25**, 18 µM **KM**, and 20 µM **Mix**, respectively. A blank control was established by adding an equal volume of DMSO to the PDW. After 24 hours of treatment, the surface morphology of the hyphae was examined using a scanning electron microscope (SEM, Hitachi Regulus 8100), and ultrastructural changes in the hyphae were observed using a transmission electron microscope (TEM, Hitachi HT-7800).

2.11 Metabolomics Analysis

Active *R. solani* mycelium disks (0.5 cm in diameter) were inoculated into PDW and incubated at 28 °C on a shaker at 140 rpm for 3 days. Subsequently, different test samples were added to the PDW to achieve final concentrations of 2 µM **d25**, 18 µM **KM**, and 20 µM **Mix**. A blank control was established by adding an equal volume of DMSO to the PDW. Each treatment was repeated six times. After 24 hours of treatment, fungal hyphae were collected from the different treatment samples. After three washes with phosphate-buffered saline (PBS), the sample was immediately frozen in liquid nitrogen for subsequent metabolomics analysis. The extraction, identification, and quantitative analysis of metabolites were performed by Genedenovo Biotechnology Co., Ltd. (Guangzhou, China; www.genedenovo.com). Metabolites were extracted and analyzed by LC-MS/MS according to the service provider's standard protocol. Liquid chromatography was carried out on an Agilent 1290 Infinity LC system coupled with a

TripleTOF 6600 mass spectrometer (AB SCIEX). Data acquisition was performed in both positive and negative ionization modes. Peak detection, alignment, and quantification were achieved using XCMS software. Metabolite identification was performed using publicly available databases: HMDB (<http://www.hmdb.ca/>), MassBank (<http://www.massbank.jp/>), and METLIN (<http://metlin.scripps.edu/index.php>). Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were conducted on the metabolic data of all samples using the R software package (<http://www.r-project.org/>). Using the variable importance in projection (VIP) values from OPLS-DA and the *p*-values from t-tests, a screening criterion for differentially accumulated metabolites (DAMs) between different comparison groups was established: a fold change (FC) of ≥ 2 or ≤ 0.5 , $VIP \geq 1$, and $p < 0.05$. The differences in metabolic pathways were identified through the KEGG database (<https://www.kegg.jp/kegg/pathway.html>).

2.12 Enzyme Activity Assay

Activated *R. solani* mycelial clumps (0.5 cm in diameter) were inoculated into PDW and incubated on a shaker at 28 °C and 140 rpm for 3 days. Subsequently, various test samples were sequentially added to the PDW, resulting in final concentrations of 2 μM **d25**, 18 μM **KM**, and 20 μM **Mix**, respectively. After 24 hours of treatment, the mycelia were collected via vacuum filtration, washed three times with pre-cooled physiological saline, and homogenized in pre-cooled physiological saline. The homogenate was centrifuged at $8000 \times g$ and 4 °C for 10 min, and the resulting supernatant was collected for enzyme activity assays. The activities of malate

dehydrogenase (MDH), succinate dehydrogenase (SDH), and ATPase in the mycelium were determined using assay kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Specifically, MDH activity was determined by monitoring the decrease in absorbance at 340 nm, SDH activity was assessed by measuring the reduction of 2,6-dichlorophenol-indophenol at 600 nm, and ATPase activity was evaluated by detecting the release of inorganic phosphate at 660 nm. Each treatment was performed in triplicate.

2.13 Statistical analysis

Statistical analysis was conducted using SPSS 25.0 (IBM, Armonk, NY, USA) based on three independent biological replicates. Data obtained from physiological experiments were presented as mean $\pm$ standard deviation (SD). A one-way analysis of variance (ANOVA) was performed, and significant differences among treatment groups were assessed using Duncan's multiple range test, with a significance level set at $p < 0.05$.

3 RESULTS

3.1 *In vitro* Evaluation of Antifungal Activity

The antifungal effects of citral-based amide derivatives, KM, and their combination on *R. solani* were evaluated using the mycelial growth rate method. As shown in Table S2, at the molar ratio where the citral-based amide derivative constitutes 10% of the mixture, the EC₅₀ value for **d25** is the lowest, indicating the highest antifungal efficacy among all tested combinations. Based on this result, the antifungal effects of **d25** mixed with KM at different molar ratios (5%, 10%, 15%, 20%, and 25%)

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were further studied, and the SR was calculated. As shown in **Figure 1(A)**, the actual EC₅₀ of the **d25** + KM combination at the 10% molar ratio was significantly lower than that of **d25** alone, KM alone, and the theoretical EC₅₀ of **d25** + KM. As shown in **Figure 1(B, C)**, derivative **d25** exhibited a synergistic effect with KM at various molar ratios, with SR values exceeding 1.5 in the assay. As the molar ratio of **d25** in the mixture increases from 5% to 25%, the SR of the **d25** + KM combination also increases. When the molar ratio of **d25** reaches 25%, the EC₅₀ of the **d25** + KM mixture decreases to 6.79 μ M, and the SR attains its maximum value of 2.66 ± 0.67 .

3.2 *In vivo* Evaluation of Antifungal Activity

Further experiments were conducted on rice leaves to evaluate the enhancement effect of **d25** on KM. As shown in **Figure 2** and **Table 1**, in the preventive trial, both the CK group and the **d25** group exhibited severe lesions, while the lesion area in the KM group was reduced. Notably, the Mix group demonstrated the smallest lesion area, with a control efficiency of 80.26%. In the curative trial, similar trends were observed, with the Mix group achieving a control efficiency of 98.16%. The aforementioned results indicate a synergistic enhancement effect between compound **d25** and KM.

3.3 Effect on Mycelial Dry Weight

Significant differences were observed in the effects of various treatments on *R. solani* growth. As illustrated in **Figure 3**, the dry weight of hyphae was 0.1525 g in the control (CK) group and 0.1543 g in the **d25**-treated group, indicating that **d25** alone did not exert a significant inhibitory effect. In contrast, treatment with KM significantly reduced mycelial dry weight to 0.0674 g. Notably, the Mix treatment further decreased

mycelial dry weight to 0.0475 g, representing a 29.5% reduction compared to KM alone. These results indicate that the mixture of **d25** and KM is more effective in inhibiting *R. solani* compared to KM alone.

3.4 Evaluation of Cytotoxicity and Acute Toxicity on Non-target Organisms

The cellular toxicity of compound **d25** was evaluated using the MTT assay on LO₂ and HEK293T cell lines. As shown in **Figure 4**, the survival rates of LO₂ and HEK293T cells treated with 10 µg/mL of compound **d25** were nearly 100%, indicating that compound **d25** does not exhibit significant toxicity to these two cell lines.

To evaluate the ecological safety of compound **d25** for non-target organisms, acute toxicity tests were conducted on honeybees and zebrafish. As presented in **Table 2** and **3**, the median lethal dose (LD₅₀) value of **d25** for honeybees was determined to be 65.87 µg/bee, indicating low toxicity. Additionally, the 96-hour median lethal concentration (LC₅₀) value for zebrafish was calculated as 15.98 mg/L (**Table 3**). Based on the environmental safety assessment guidelines for chemical pesticides, compound **d25** was classified as safe for both honeybees and zebrafish^{33,34}.

3.5 Effect of the Mix on Membrane Permeability of *R. solani*

The effects of different treatments on the permeability of the *R. solani* cell membrane were evaluated using the relative conductivity method. As illustrated in **Figure 5(A)**, under the **Mix** treatment, the relative conductivity of *R. solani* was consistently higher compared to that of the other treatment groups, with the most pronounced difference observed at the 180-minute mark. The relative conductivity in the **Mix** group was the highest, reaching 24.47%, which was significantly higher than

that observed in the KM treatment group (18.90%), the **d25** treatment group (13.91%), and the control group (13.40%). This result indicates that the **Mix** treatment induces the most significant damage to the cell membrane of *R. solani*.

3.6 Effect of the Mix on Soluble Protein Content in *R. solani*

By measuring the soluble protein content, the effects of different treatments on the cell membrane of *R. solani* were investigated. As shown in **Figure 5(B)**, the soluble protein content of the mycelium following **Mix** treatment was significantly lower than that of the other treatment groups. Specifically, the soluble protein content in the **Mix** treatment group was 3.859 g/L, which was 36.01%, 34.95%, and 34.95% lower than that of the CK, **d25**, and KM treatment groups, respectively. These findings indicate that the **Mix** treatment significantly reduces the soluble protein content in *R. solani*, resulting in cell membrane disruption.

3.7 Effect of the Mix on the Morphology and Microstructure of *R. solani* Mycelia

As shown in **Figure 6(A)**, the hyphae in the CK group appeared light yellow, were evenly distributed, and exhibited an intact structure. The mycelial morphology of the **d25** treatment group was not significantly different from that of the control group. However, in the KM treatment group, the mycelial volume was reduced, the color turned deep yellow, and there was a noticeable degree of adhesion. Notably, the mycelial morphological changes in the **Mix** treatment group were the most pronounced, characterized by significant contraction, deeper pigmentation, and more severe adhesion. SEM analysis further revealed ultrastructural damage. As shown in **Figure 6(B)**, the mycelium surface following KM treatment exhibits marked wrinkling and

depression, whereas the mycelium surface after **Mix** treatment displays the most extensive damage, characterized by deep wrinkling, collapse, and fracture. These results demonstrate that the mycelium morphology of *R. solani* was fundamentally altered following **Mix** treatment, indicating that **Mix** significantly inhibited the normal growth of *R. solani* mycelium.

Following the application of various treatments, the ultrastructure of *R. solani* mycelia was examined using TEM. As illustrated in **Figure 7**, the *R. solani* cells in the control group displayed a complete cellular structure and normal organelle morphology. No discernible ultrastructural changes were observed in the **d25** treatment group. Furthermore, examination of *R. solani* cells under KM treatment revealed evidence of cytoplasmic contraction, mitochondrial damage, and vacuolization. It is noteworthy that *R. solani* cells treated with **Mix** exhibited the most severe damage, as evidenced by the thinning of the cell wall, blurring of the cell membrane boundary, and the formation of numerous vesicles. These observations suggest that **Mix** induced irreversible damage to the organelles of *R. solani*.

3.8 Comprehensive Metabolomics Analysis

The application of non-targeted metabolomics in conjunction with Liquid chromatography-mass spectrometry (LC-MS/MS) technology has facilitated the analysis of metabolite changes exhibited by *R. solani* under varying treatment conditions (Table **S3**). This approach has elucidated the mechanism underlying the synergistic effect of the **Mix** treatment on *R. solani*. The results of the PCA are presented in **Figure 8(A)**, where samples from the same treatment group are distinctly

clustered, whereas samples from different treatment groups are not well separated. As illustrated in **Figures 8(B, C, D)**, the results of the OPLS-DA analysis and hierarchical clustering analysis indicate that the classification is effective, highlighting significant disparities in metabolite profiles among the distinct treatment groups. Metabolites exhibiting significant differences ($p < 0.05$), high VIP values (> 1), and substantial fold changes ($FC > 2$ or $FC < 0.5$) were selected according to the established screening criteria. Between the control group and the **Mix** treatment group, 539 DAMs were identified, of which 268 were significantly upregulated and 271 were significantly downregulated following **Mix** treatment. Meanwhile, 334 DAMs were identified between the KM treatment group and the **Mix** treatment group, with 122 being significantly upregulated and 212 significantly downregulated following **Mix** treatment. As previously mentioned, the results demonstrate that the **Mix** treatment induces severe metabolic disorders within *R. solani* cells.

3.9 Metabolic Pathway Analysis

The KEGG database was utilized for the annotation and mapping of differential metabolites. Subsequently, KEGG pathway enrichment analysis was performed to investigate the potential synergistic mechanisms underlying the **Mix** treatment on *R. solani*.

As shown in **Figure 9(A)**, significant changes in DAMs were observed in the "Biosynthesis of Amino Acids" metabolic pathway ($p < 0.05$) when comparing the control group with the **Mix** treatment group. Among these metabolites, those associated with the TCA cycle, including alpha-ketoglutarate and pyruvate, have undergone

significant alterations. This suggests that **Mix** inhibits the growth of *R. solani* by interfering with its energy metabolism. The synergistic mechanism of **Mix** was further elucidated by comparing the **Mix**-treated group with the KM-treated group. As illustrated in **Figure 9(B)**, the DAMs in this comparative group were significantly enriched in arachidonic acid metabolism ($p < 0.05$). KEGG analysis revealed significant alterations in metabolites associated with the regulation of cell membrane lipid stability, including arachidonic acid and 20-hydroxy arachidonic acid. These findings indicate that **Mix** exerts a synergistic effect by modulating the stability of *R. solani* cell membranes.

3.10 Effects on SDH and MDH Enzyme Activities

By quantifying the activities of SDH, MDH, and ATPase in *R. solani* hyphae, this study investigated the effects of various treatments on energy metabolism. As shown in **Figure 10**, compared with the control group, there was no significant change in the activities of SDH, MDH, and ATPase in the **d25** treatment group. In contrast, the KM treatment group exhibited a significant decrease in the activities of these enzymes. Notably, the **Mix** treatment group showed the most substantial reduction, with decreases of 47.96%, 38.62%, and 53.09% in SDH, MDH, and ATPase activities, respectively. These results indicate that **Mix** significantly affects the energy supply of *R. solani* by reducing the activity of key enzymes in the TCA cycle, thereby inhibiting its growth and reproduction.

4 DISCUSSION

Plant pathogenic fungi pose a significant threat to global food security³⁵. Notably,

rice sheath blight caused by *Rhizoctonia solani* substantially reduces rice yield and quality³⁶. Prolonged reliance on chemical fungicides has resulted in the development of resistance and raised environmental concerns³⁷. Although citral-based amide derivatives have shown inhibitory effects against *R. solani*, their potential for synergistic interactions remains underexplored²³. This study aims to evaluate the synergistic effects of citral-based amide derivatives on the fungicidal activity of Kresoxim-methyl, a methoxyacrylate fungicide, and to elucidate its potential mechanisms of action. The *in vitro* antifungal experiment revealed that the citral-based amide derivative **d25** exhibited the most significant synergistic effect when combined with KM. The EC₅₀ value of the KM + d25 combination at a 10% molar ratio (10% d25 and 90% KM), as demonstrated in **Figure 1**, was significantly lower than both the theoretical additive EC₅₀ and the individual EC₅₀ values of KM or d25 alone ($p < 0.01$). Moreover, the SR increased progressively with increasing proportions of **d25**, reaching a maximum of 2.66 at 25% **d25**, which supports the existence of a synergistic interaction. These findings indicate that **d25** enhances the antifungal activity of KM against *R. solani* in a concentration-dependent manner, demonstrating a synergistic effect. The results of the antifungal experiments on rice leaves demonstrated that the preventive and control efficiencies of the **Mix** treatment were superior, achieving 80.26% and 98.16%, respectively. These findings suggest that **d25** not only significantly enhances the antifungal efficacy of KM but also reduces the required dosage of KM, potentially mitigating the development of fungicide resistance by decreasing the selection pressure associated with high-dose monotherapy³⁸.

To further elucidate the synergistic mechanism between **d25** and KM, we subsequently examined the morphological alterations in *R. solani* following treatments with **d25**, KM, and **Mix**. To investigate whether **d25** enhances the activity of KM under an equivalent KM dosage, the **Mix** group in these assays was designed to contain the same amount of KM as the KM-alone group, supplemented with a small amount of **d25**. Physiological and biochemical analyses revealed that the cell membrane permeability of *R. solani* in the **Mix** treatment group increased, while the soluble protein content significantly decreased, suggesting compromised cell membrane integrity^{39,40}. These observations indicate compromised cell membrane integrity and function. This finding provides critical evidence for the subsequent metabolomics analysis.

Amino acids are essential for protein synthesis, gene expression, and the maintenance of cellular osmotic stability^{41,42}. Metabolomics analysis demonstrated that, compared to the control group, the **Mix** group exhibited significant alterations in the amino acid biosynthesis metabolic pathway. Among these metabolites, those associated with the TCA cycle and ATP synthesis, including α -ketoglutarate and pyruvate, exhibited notable down-regulation^{43,44}. It indicates that **Mix** potentially inhibits the growth and reproduction of pathogenic fungi by interfering with their energy metabolism and ATP synthesis. Since the TCA cycle plays a central role in ATP production, any disruption to this pathway can directly compromise fungal biosynthesis, inhibit cell growth, and weaken stress resistance⁴⁵. The resultant energy deficit is likely a critical factor contributing to the suppression of fungal viability and ultimately leading to cell death⁴⁶. SDH and MDH are key enzymes in the TCA cycle. SDH catalyzes the

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oxidation of succinate to fumarate, transferring electrons to FAD, whereas MDH catalyzes the oxidation of malate to oxaloacetate, generating NADH for energy production^{47,48}. ATPase plays a crucial role in the synthesis and supply of cellular energy⁴⁹. The reduction in SDH, MDH, and ATPase activities in *R. solani* following **Mix** treatment suggests that **Mix** may inhibit the growth and reproduction of *R. solani* by disrupting its energy metabolism and ATP synthesis pathways. Specifically, KM, a methoxyacrylate fungicide, primarily inhibits the function of mitochondrial complex III, leading to an inadequate energy supply for pathogenic fungal cells and consequently suppressing their proliferation⁵⁰. Considering that the predominant component of the **Mix** group remains KM, this outcome aligns with our expectations.

Further analysis revealed that, in comparison to the KM group, the **Mix** group exhibited markedly significant alterations in the arachidonic acid metabolic pathway. Following **Mix** treatment, the metabolic products of *R. solani* exhibited significant upregulation of arachidonic acid and 20-hydroxy arachidonic acid. Arachidonic acid and its metabolites are crucial for maintaining cell membrane stability, facilitating signal transduction, and modulating cellular stress responses^{51–53}. When the cell membrane is damaged or subjected to stress, arachidonic acid may be released from membrane phospholipids, participating in membrane repair or the generation of various biologically active compounds⁵⁴. 20-Hydroxyarachidonic acid, a principal metabolic product of arachidonic acid, plays a crucial role in regulating the stability of cell membrane lipids⁵⁵.

In this study, following **Mix** treatment, the metabolites of *R. solani*, including

Prostaglandin I₂, Prostaglandin J₂, and Prostaglandin A₂, were significantly downregulated. Prostaglandins are significant metabolic products of arachidonic acid and are closely associated with the repair and stabilization of cell membranes⁵⁶. It suggests that **Mix** inhibits critical steps in arachidonic acid metabolism, consequently impacting the stability and repair capability of cell membranes. Impaired arachidonic acid metabolism may result in the accumulation of free fatty acids and inadequate prostaglandin synthesis, both of which are critical for membrane repair and homeostasis⁵⁷. This metabolic disruption compromises the structural integrity of fungal cell membranes, thereby increasing permeability and enhancing susceptibility to fungicide action. This finding aligns with the research conclusions of Bao *et al.*, suggesting that the synergist augments the antifungal efficacy of the pesticide either by enhancing its permeability or through direct interaction with the cell membrane¹⁵. Collectively, our findings indicate that the synergistic antifungal effect of the **Mix** treatment is attributed to a dual mechanism in which the **Mix** formulation disrupts energy metabolism in *R. solani*, and **d25** enhances membrane permeability, thereby promoting the cellular uptake of KM. This combined action impairs both the energy supply and membrane integrity of the pathogen, ultimately leading to fungal growth inhibition and cell death.

The safety profile of synergists is a critical parameter in evaluating their application value. This study systematically assessed the cytotoxicity of **d25** and its potential effects on non-target organisms. The research findings indicate that **d25** exhibits low toxicity, thereby highlighting its substantial potential for the development

and utilization of environmentally friendly fungicides. In addition to exhibiting negligible cytotoxicity in mammalian cell lines, compound **d25** demonstrated low acute toxicity toward both honeybees and zebrafish. These results indicate its favorable ecological safety profile³⁰. Future research should further explore the interaction mechanisms between d25 and KM, evaluate synergistic proportions under a variety of environmental conditions, and assess their persistence and safety in complex ecosystems. In particular, long-term studies are essential to comprehensively evaluate the impact of synergists on resistance evolution in field populations.

This study has elucidated the synergistic mechanism of **d25** in conjunction with KM, thereby providing robust theoretical support for the application of synergists and establishing a foundation for the development of highly efficient, low-environmental-impact pesticide formulations. Future research should aim to optimize the molecular structure of synergists to enhance their agricultural utility and address the challenge of drug resistance.

5 CONCLUSION

In summary, this study demonstrates that the citral-based amide derivative **d25**, when used in conjunction with compound KM, exhibits potent synergistic antifungal activity against *R. solani*. Compared to individual treatments, the Mixture of **d25** and KM (**Mix**) exhibited a more pronounced inhibitory effect against *R. solani* in both *in vitro* and *in vivo* experiments. In addition, compound **d25** exhibited relatively low cytotoxicity toward human cell lines, and its acute oral toxicity to honeybees as well as its acute toxicity to zebrafish were both negligible, suggesting its potential biosafety for

non-target organisms. From a mechanistic perspective, the **Mix** results in a significant increase in cell membrane permeability and severe morphological damage to *R. solani* hyphae, as well as enhanced protein leakage. Metabolomics analysis demonstrates that **Mix** exerts antifungal effects on *R. solani* by disrupting its energy metabolism and exhibiting synergistic effects through cell membrane damage. Enzyme activity analysis demonstrates that **Mix** reduces the activity of key enzymes involved in energy metabolism, including SDH, MDH, and ATPase, thereby disrupting the critical energy metabolism pathway essential for fungal survival. These results indicate that **d25** can serve as a booster to enhance the utilization efficiency of KM, and merits further development. Further studies are necessary to validate the identified molecular targets and comprehensively assess the long-term risk of resistance development associated with the use of synergists under field conditions.

CONFLICT OF INTEREST DECLARATION

The authors declare no competing financial interest.

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Captions for Figures and Tables

Table 1. *In Vivo* Preventive and Curative Effects (%) of **d25**, KM, and the **Mix** against *R. solani*.

Table 2. Acute Oral Toxicity to Honeybees (*Apis mellifera* L.) of Compound **d25** and dimethoate.

Table 3. Acute Toxicity to zebrafish of Compound **d25**.

Table S1. Molecular formula and structural formula of Citral-based amide derivatives and Kresoxim-methyl.

Table S2. Antifungal activity of the mixture (Molar Ratio: 10% citral-based amide derivatives) against *R. solani*.

Table S3. Qualitative and quantitative results of metabolites.

Figure 1. Synergistic effect between **d25** and KM against *R. solani* (A); EC₅₀ values (B) and SR (C) of the mixtures containing different concentrations of **d25**. * and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively.

Figure 2. Preventive and curative control of **Mix** against *R. solani* on rice leaves at 500 µM.

Figure 3. The effect of **Mix** on the dry weight of mycelium.

Figure 4. *In vitro* cytotoxicity of **d25** against LO₂ and HEK293T cells.

Figure 5. The effect of **Mix** on the Relative conductivity (A) and soluble protein (B) of *R. solani*.

Figure 6. Morphological observation of *R. solani* on PDW (A) and hyphal ultrastructure under SEM (B) after different treatments.

Figure 7. TEM Observation of *R. solani* Treated with DMSO (A, B), **d25** (C, D), **KM** (E, F), and **Mix** (G, H). cell wall (CW); plasma membrane (PM) and mitochondria(M).

Figure 8. Metabolomic Profiling and Heatmap Clustering of *R. solani* under **Mix**, **KM**, and **d25** Treatments. (A) PCA score plot; (B) OPLS-DA score plot; (C) Heatmap of differential metabolites. Different colors represent different relative abundances of metabolites, where orange represents higher intensity and blue represents lower intensity.

Figure 9. Integrated KEGG enrichment and differential metabolite analysis. Top 20 KEGG pathways with the lowest *P*-values in the **CK vs Mix** (A) and **KM vs Mix** (B) comparisons.

Figure 10. Effect of Mix on ATPase (A), MDH(B), and SDH(C) of *R. solani*.

Table 1. *In Vivo* Preventive and Curative Effects (%) of **d25**, KM, and the **Mix** against *R. solani*.

	d25	KM	Mix
Preventive	6.2%±1.73%	40.16%±1.07%	80.26%±3.61%
Curative	4.85%±0.77%	38.52%±1.57%	98.16%±1.26%

Table 2. Acute Oral Toxicity to Honeybees (*Apis mellifera* L.) of Compound **d25**^a and dimethoate.

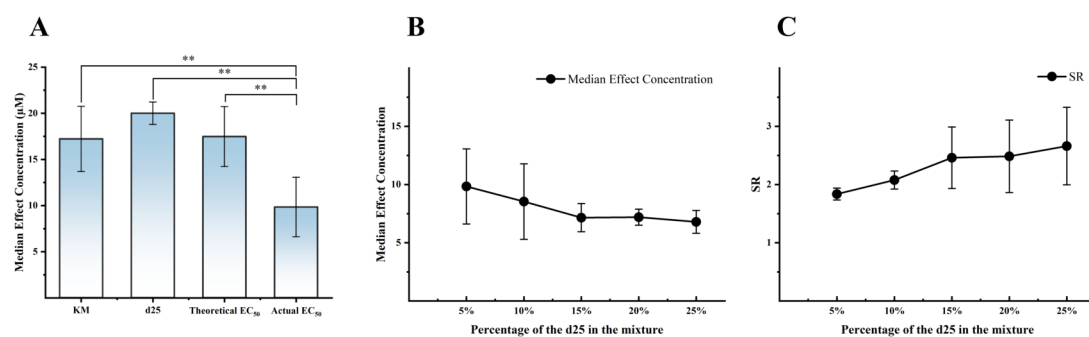
Compound	LD₅₀ (µg a.i./bee) (CI, 95%)	R²	Toxic regression equation	Honeybee -toxicity
d25	65.87 (35.56~435.94)	0.913	y=-4.49+2.45*x	low
Dimethoate	0.213 (0.099~0.407)	0.921	y=1.73+2.52*x	high

^aLD₅₀, acute oral median lethal dose; CI, confidence interval; classified as highly toxic (LD₅₀ < 2 µg a.i./bee), moderately toxic (2.0 < LD₅₀ ≤ 11 µg a.i./bee), and low toxic (LD₅₀ > 11 µg a.i./bee).

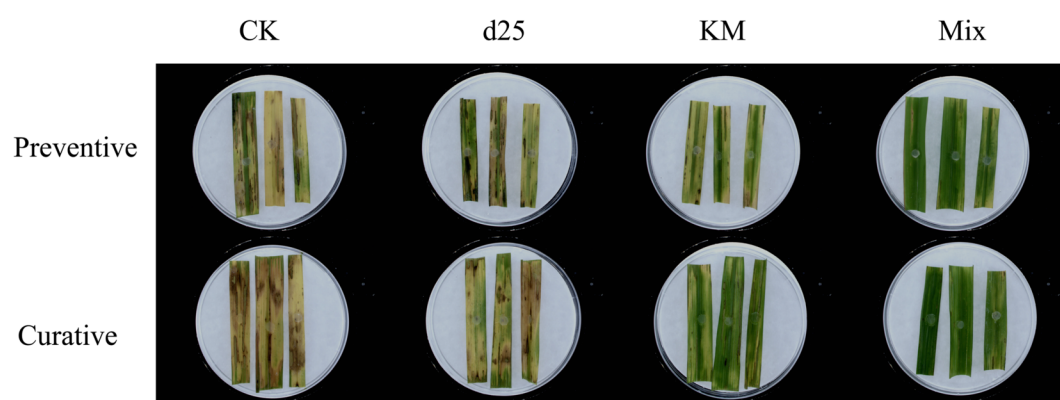
Table 3. Acute Toxicity to zebrafish of Compound **d25**^b

Compound	LC₅₀ (96 h) (mg a.i./L) (CI, 95%)	R²	Toxic regression equation	Zebrafish -toxicity
d25	15.98 (13.825~18.533)	0.961	y=-5.19+4.26*x	low

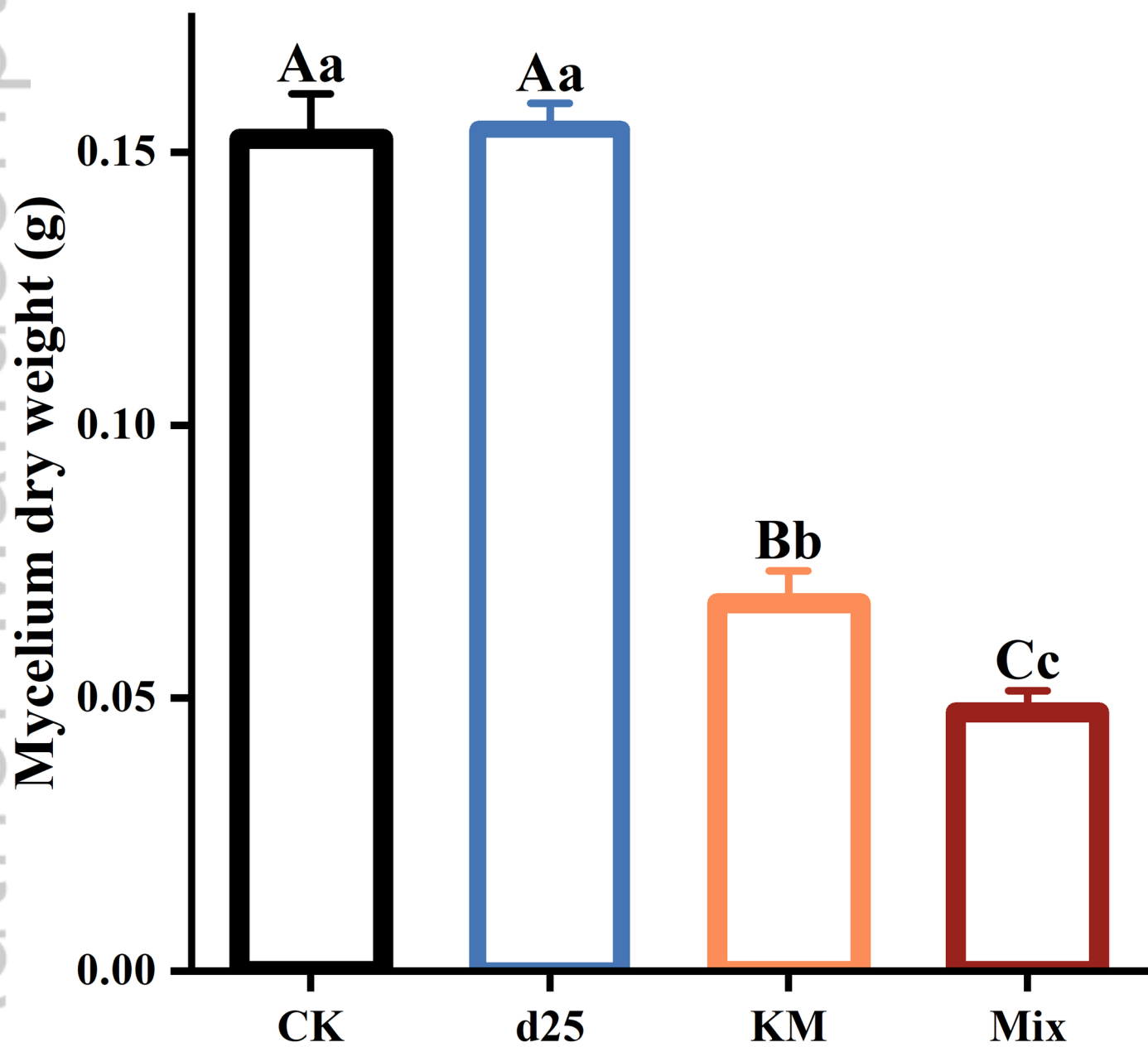
^bLC₅₀, acute median lethal concentration (96 h); CI, confidence interval; classified as extremely toxic (LC₅₀ ≤ 0.1 mg a.i./L), highly toxic (0.1 < LC₅₀ ≤ 1.0 mg a.i./L), moderately toxic (1.0 < LC₅₀ ≤ 10 mg a.i./L), and low toxic (LC₅₀ > 10 mg a.i./L).



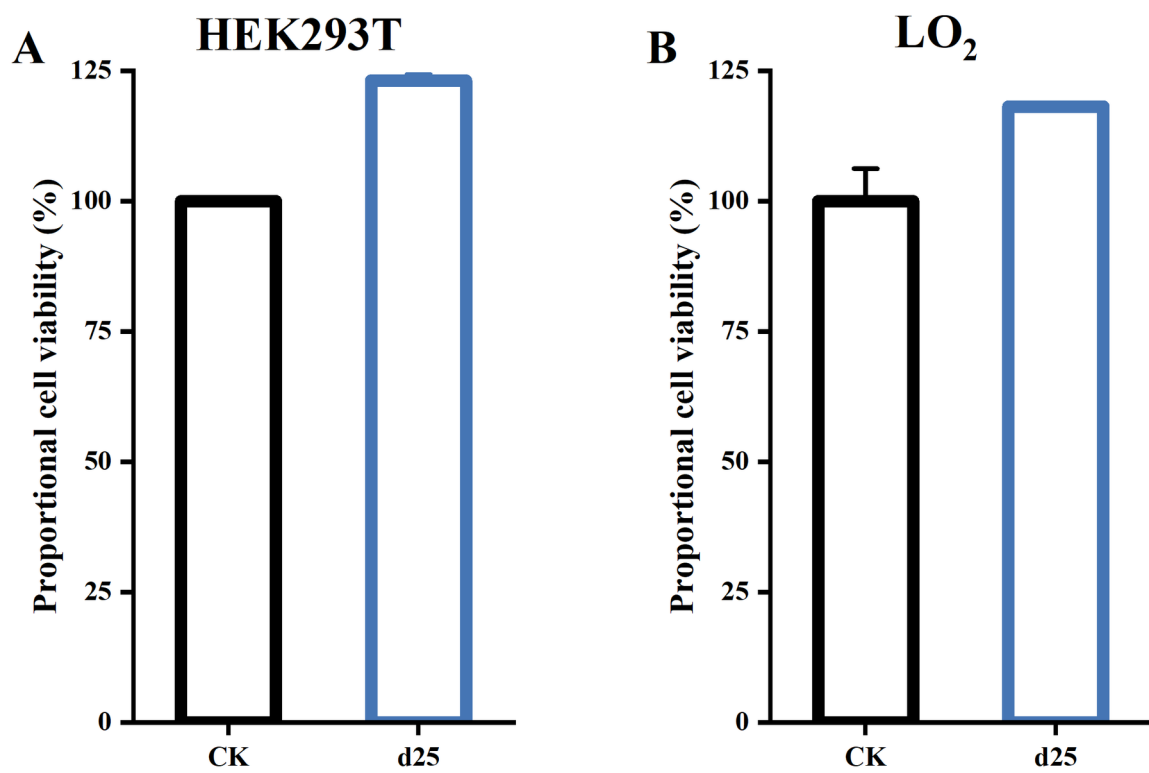
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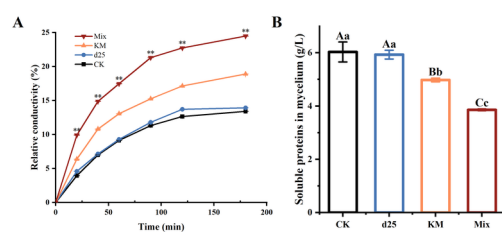
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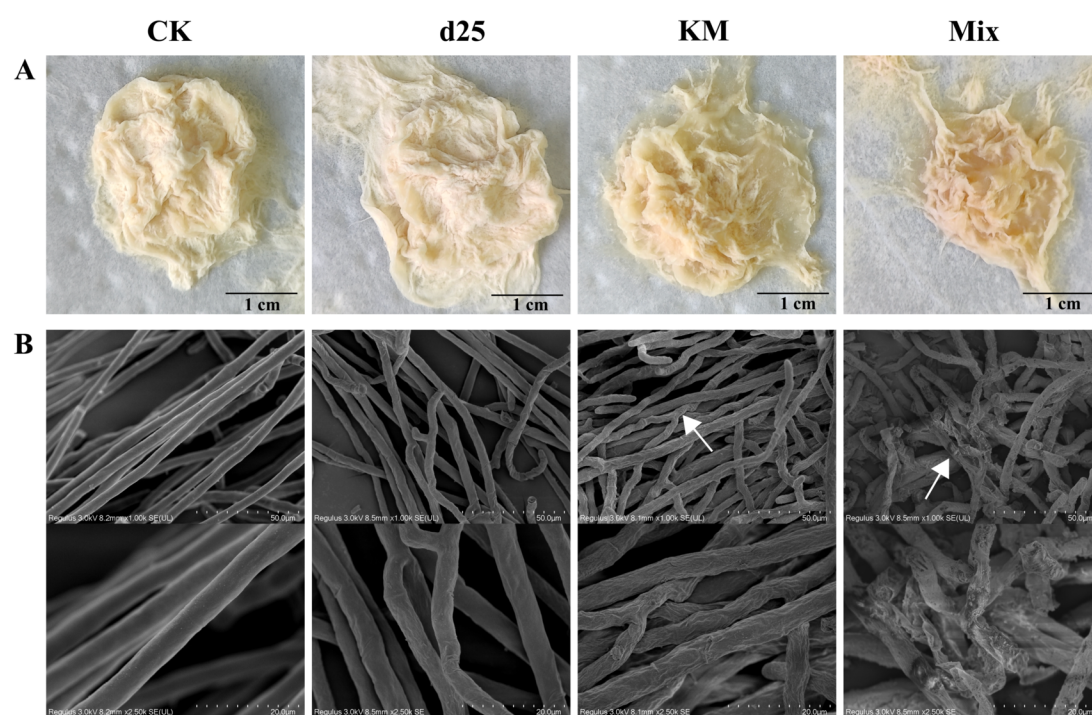
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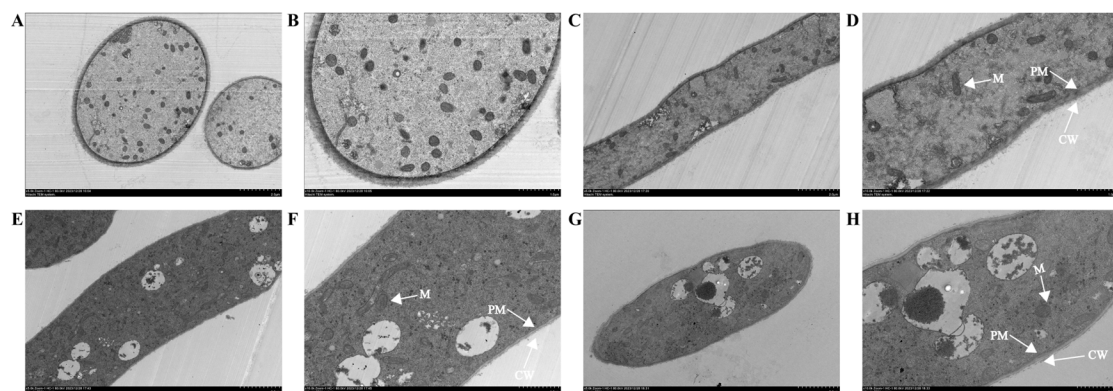
PS_8931_f4_Figure_4.png



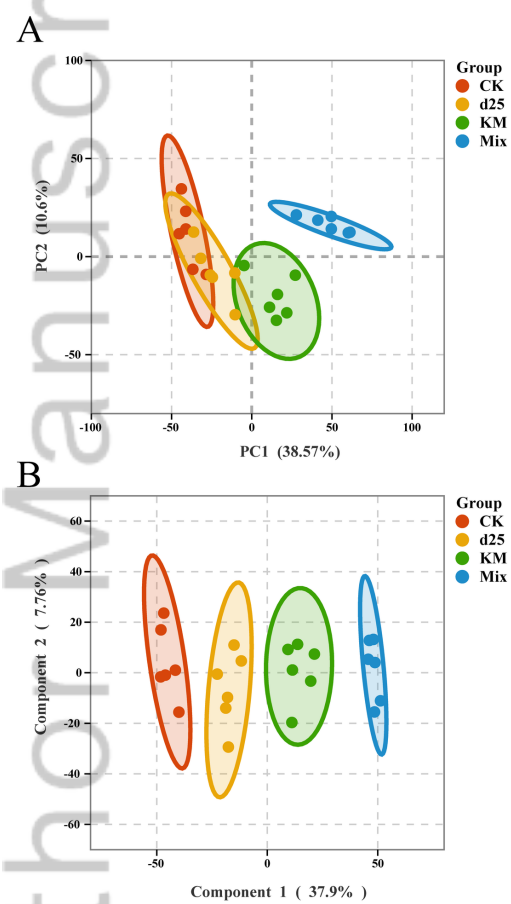
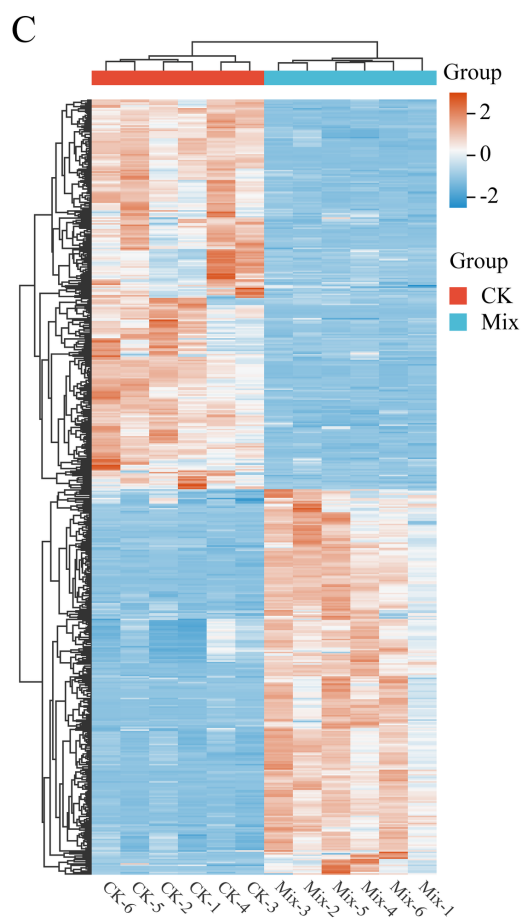
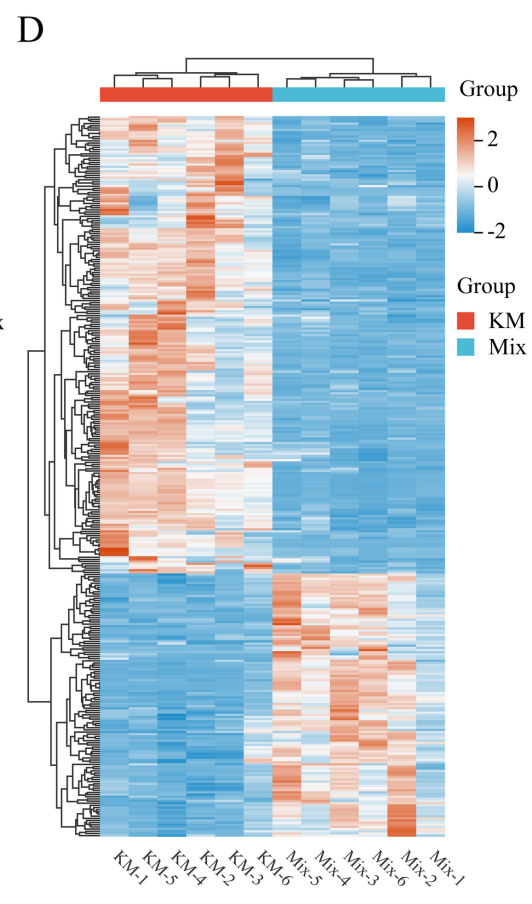
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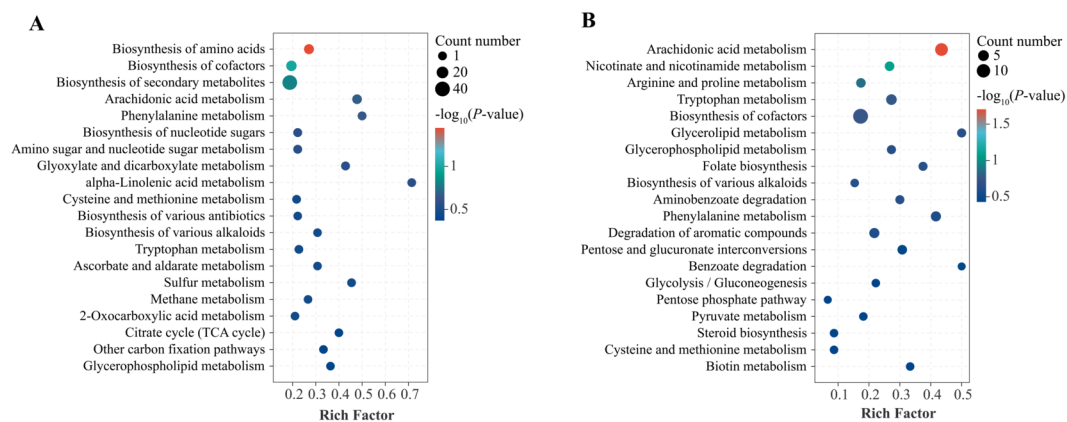
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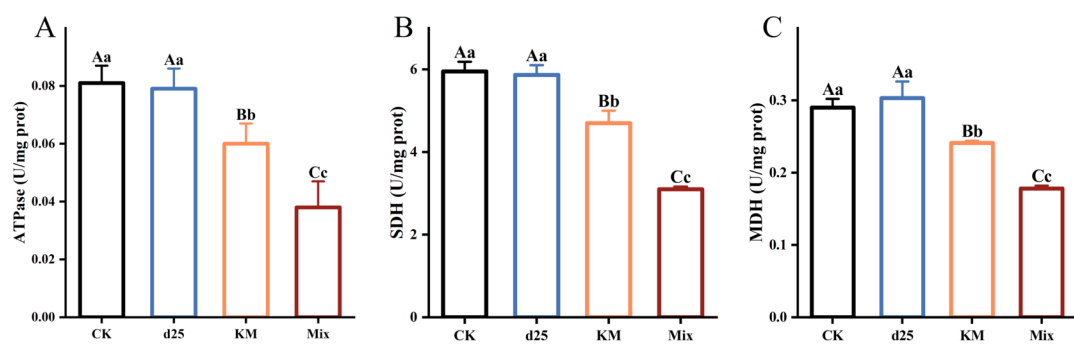
PS_8931_f7_Figure_7.tif



PS_8931_f8_Figure_8.tif



PS_8931_f9_Figure_9.tif



PS_8931_f10_Figure_10.tif