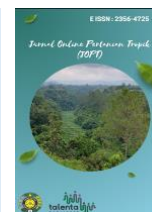




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Rhizosphere microbial resistance test to fungicide dimethomorph *in vitro*

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ABSTRACT

Dimethomorph is a systemic fungicide utilized within Indonesian agricultural sector. Inappropriate application of dimethomorph fungicide may result in environmental contamination and the development of resistance among rhizosphere microorganisms. This study aims to ascertain the responses of rhizosphere microbial isolates to dimethomorph fungicide, thereby providing critical insights for evaluating the efficacy of this fungicide against rhizosphere microbes. Such information is essential for formulating plant pest and disease management strategies that utilize more suitable and sustainable dosages. The investigation involved testing bacterial isolates MR-B01, MR-B02, MR-B03, MR-B04, and fungal isolate MR-F01 against dimethomorph fungicide employing the agar diffusion method with disc paper. The experimental design incorporated a treatment factor with five variation concentrations of dimethomorph fungicide (0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L), executed with ten repetitions. The findings indicated that varying concentrations of dimethomorph fungicide exhibited distinct effects on the resistance of the tested microbial isolates, specifically bacteria MR-B01, MR-B02, MR-B03, MR-B04, and fungal isolate MR-F01.

Keyword: compatibility, dimethomorph fungicide, pesticide, resistance, rhizosphere microbes, sensitivity



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1. Introduction

Indonesia is characterized as an agricultural nation, with a significant proportion of its populace engaged in the agricultural sector (Hasibuan *et al.*, 2022). Within this sector, pesticides serve a critical function as agents for pest control (Ula and Mizani, 2022). On a global scale, the predominant contributor to environmental pollution within agriculture arises from the application of chemical agents, including fungicides. Data from the Food and Agriculture Organization of the United Nations (FAO) (2023) indicate that the utilization of fungicides in Indonesia experienced an increase from 38,491.12 tons in 2019 to 39,736.1 tons in 2021. Fungicides comprise chemical compounds that inhibit the growth of fungi (Sari *et al.*, 2014). A notable active

ingredient in fungicides that demonstrates efficacy in combating fungal pathogens is dimethomorph, which is categorized within the morpholine group. Dimethomorph exerts its action by interfering with the synthesis of membranes and lipids in oomycete pathogens as well as the formation of their cell walls (Wulandari, 2020).

Excessive use of fungicides may also diminish the population of microorganisms in the soil, adversely impacting soil quality, particularly within the rhizosphere. The rhizosphere, defined as the region surrounding plant roots, is characterized by a high nutrient density that supports microbial growth. The presence of microorganisms in rhizosphere soil is instrumental in the mineralization and decomposition of organic matter, influencing the physical and chemical properties of the soil. Consequently, the rhizosphere environment is notable for its rich diversity of microorganisms, collectively referred to as rhizosphere microbes. These microbes contribute to nutrient availability for plants, nitrogen fixation, phosphate solubilization, and the stabilization of soil structure (Ginting *et al.*, 2024).

Groups of rhizosphere microbes include bacterial genera such as *Bacillus* sp., *Azotobacter* sp., *Azospirillum* sp., *Lactobacillus* sp., *Rhizobium*, *Pseudomonas* sp., *Nitrosomonas* sp., and *Nitrococcus* sp. Among the fungal genera are *Trichoderma* sp., *Aspergillus* sp., *Ectomycorrhiza*, and *Endomycorrhiza*. Rhizosphere microbes play a pivotal role in nutrient availability in the soil, which is intrinsically linked to soil fertility and health (Saraswati *et al.*, 2004). Sangkala *et al.* (2021) identified several genera of microorganisms that are abundant in the rhizosphere and serve essential functions for plants, including the provision of minerals such as nitrogen and phosphorus, as well as the production of amino acids, auxins, and gibberellins. Continuous fungicide application can lead to the development of resistance among microorganisms, particularly within the rhizosphere.

Research has demonstrated that certain rhizosphere microbes exhibit varying degrees of sensitivity to fungicides. Findings by Sanam *et al.* (2021) indicated that the fungicide mancozeb, at concentrations of 0.05%, 0.01%, 0.1%, 0.2%, 0.5%, 1%, 1.5%, and 2%, inhibited the growth of rhizosphere bacteria with codes B365 and AZB31. The Relative Inhibition Level (THR) recorded for bacteria with codes B365 was as follows: (0 mm, 6 mm, 7 mm, 8 mm, 9 mm, 10 mm, 10 mm, 10 mm), indicating that this bacterium is sensitive to mancozeb. In contrast, the THR for bacteria with code AZB31 was (0 mm, 0 mm, 0 mm, 0 mm, 7 mm, 8 mm, 9 mm, 10 mm). Additionally, research by Ruiz Tello (2021) demonstrated that the fungicide dimethomorph at a concentration of 10 ppm inhibited the growth of rhizosphere bacteria, with an average growth inhibition value of 52.75%.

This study presents findings that contrast with those of Archana *et al.* (2012), who reported that the fungicide azoxystrobin was effective in inhibiting the growth of rhizosphere bacteria at concentrations of 100 ppm, 150 ppm, 200 ppm, 250 ppm, and 300 ppm, yielding absorbance values of 2.19, 2.18, 2.18, 2.19, 2.19, and 2.20, respectively. These values did not exhibit statistically significant differences ($p\text{-value} = 0.05$), indicating an absence of inhibition of *Bacillus subtilis* growth by azoxystrobin. The measurements employed in this study utilized the turbidometry method, which assesses absorbance at a wavelength of 610 nm as an indicator of microbial growth.

Khulillah *et al.* (2021) reported that it was established that the fungicide propineb, at treatment concentrations of 50 ppm, 100 ppm, and 200 ppm, effectively inhibited the growth of rhizosphere bacteria, resulting in a Relative Inhibition Level (THR) ranging from 40% to 80%, suggesting that rhizosphere bacteria is sensitive to propineb fungicide. According to the results from Sanam *et al.* (2021), mancozeb fungicide at concentrations of 0.05%, 0.01%, 0.1%, 0.2%, 0.5%, 1%, 1.5%, and 2% did not inhibit the growth of rhizosphere bacteria with code ASP25, recording an inhibition zone value of 0 mm.

Furthermore, Ajith *et al.* (2024) demonstrated that the 50% dimethomorph fungicide at concentrations of 50 ppm, 100 ppm, 200 ppm, and 400 ppm also failed to inhibit the growth of rhizosphere fungi, yielding an average inhibition zone value of 0 mm. Conversely, Saha *et al.* (2023) reported that the 50% dimethomorph fungicide successfully inhibited the growth of the rhizosphere fungus *Trichoderma asperelloides*, with an inhibitory value of 75.50 mm, indicating a high sensitivity of the fungus to the dimethomorph fungicide. The aforementioned study employed a concentration treatment of 0.50 g or ml of the dimethomorph fungicide.

Resistance is defined as the capacity of a microbial strain to adapt to adverse conditions (Hajijah *et al.*, 2022). Resistance in microorganisms may be inferred from a decline in fungicide efficacy in the field as well as alterations in the microorganisms' response to the growth of colonies in vitro (Fauziah, 2023). The emergence

of microbial resistance within the rhizosphere to fungicides disrupts biological processes, such as organic matter decomposition, nutrient solubilization, and the synthesis of plant growth hormones. The objective of this study is to evaluate the response of rhizosphere microbial isolates to dimethomorph fungicide, which will yield critical insights for assessing the fungicide's effectiveness against rhizosphere microbes, thereby aiding in the development of a more appropriate and sustainable plant pest and disease control strategy.

2. Method

2.1. Type of research

This investigation constitutes experimental research employing the Complete Randomized Design (CRD) method. The treatment factors involved include various concentrations of fungicides containing dimethomorph active ingredients, administered in five variations (0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L), with ten repetitions. The test microorganisms utilized comprised rhizosphere bacteria (MR-B01, MR-B02, MR-B03, MR-B04) and a fungal isolate (MR-F01).

2.2. Tools and materials

The equipment utilized in this study includes an autoclave, analytical balance, precision balance, biosafety cabinet, microscope, UV-Vis spectrophotometer, vortex, petri dishes, test tubes, test tube racks, Erlenmeyer flasks, measuring cups, volumetric pipettes, dropper pipettes, ose needles, tweezers, Bunsen burner, micropipettes, tips, spatulas, beakers, hot plates, cuvettes, hole punches, callipers, and label paper. The materials employed comprise pure microbial isolates, including four rhizosphere bacterial isolates (MR-B01, MR-B02, MR-B03, MR-B04) and one rhizosphere fungal isolate (MR-F01), along with culture media such as MRS Agar, PDA, NB, MRS Broth, potatoes, sugar, sterile distilled water, 0.85% NaCl, dimethomorph (Acrobat 50 WP), 70% alcohol, 95% alcohol, plastic wrap, disc paper, aluminium foil, spirits, and paper.

2.3. Research Procedure

2.3.1. Sterilization of Tools

The tools utilized in this study are thoroughly washed with running water and subsequently dried. Glassware, including petri dishes, Erlenmeyer flasks, glass stirrers, and test tubes, undergo sterilization. Petri dishes, glass stirrers, and test tubes are placed in heat-resistant plastic containers, while Erlenmeyer flasks are covered with aluminium foil. The items are then autoclaved at a temperature of 121°C and a pressure of 1.5 atm for 15 minutes.

2.3.2. Preparation of Culture Media and Resistance Test Media

Culture media such as NB, MRSB, NA, MRSA, and PDA are measured using an analytical balance and transferred into a 1000 mL beaker. Subsequently, 1000 mL of distilled water is added, and the mixture is stirred until homogeneous using a stirrer. The prepared solution is then poured into a 100 mL Erlenmeyer flask, which is covered with aluminium foil and sealed with rubber. Following this, the media is sterilized in an autoclave at 121°C and 1.5 atm for 15 minutes, after which it is allowed to stand. For PDB media, 100 grams of potatoes are weighed with a precision scale and placed in a 500 mL beaker, followed by the addition of 500 mL of distilled water and heating on a hot plate. Ten grams of sugar is incorporated, and the mixture is stirred until homogeneous and brought to a boil. The resultant potato juice or water is transferred into a 100 mL Erlenmeyer flask in a volume of 50 mL, which is then covered with aluminium foil and sealed with rubber. Sterilization is performed using an autoclave at 121°C and 1.5 atm for 15 minutes, after which the media is allowed to stand.

2.3.3. Rejuvenation of Microbial Isolates

The rejuvenation of bacterial isolates (MR-B01, MR-B02, MR-B03) and fungal isolate (MR-F01) is conducted to activate these microbial isolates and optimize their growth. The rejuvenation of bacterial isolates (MR-B01, MR-B02, MR-B03) involves the transfer of a single colony from NA to slant NA media using a sterile loop. The rejuvenation process for MR-B04 isolates consists of transferring a single colony from MRSA to slant MRSA media utilizing the streak method. This is followed by incubation at room temperature (28°C) for a duration of 24 hours. In contrast, for fungal isolate MR-F01, one loop of a single colony is inoculated onto fresh PDA media using the dot method and incubated for 35 days at room temperature (28°C).

2.3.4. Preparation of Fungicide Stock Solution

The preparation of the fungicide stock solution involves weighing active ingredients of dimethomorph at quantities of 0.5 g, 1 g, 1.5 g, and 2 g, which are each dissolved in 1000 mL of distilled water and stirred until homogenous.

2.3.5. Preparation and Testing of Microbial Suspensions

The creation and testing of microbial suspensions involve the inoculation of bacterial isolates (MR-B01, MR-B02, MR-B03) by taking one loop of bacterial culture and introducing it into 50 mL of nutrient broth (NB) media, subsequently homogenizing the mixture. The bacterial isolate MR-B04 is treated similarly but is inoculated into 50 mL of de Man, Rogosa, and Sharpe broth (MRSB) media. The suspension of bacterial isolates is then agitated at room temperature (28°C) at a speed of 120 rpm for a duration of 24 hours. Following incubation, 1 mL of bacterial suspension is transferred to a test tube containing 5 mL of sterile 0.85% NaCl solution. Calibration of the spectrophotometer is performed using a blank solution (sterile 0.85% NaCl). As noted by Rosmania and Yanti (2020), the turbidity of the bacterial suspension is assessed by placing the test bacterial suspension into a cuvette and measuring with a UV-Vis spectrophotometer at a wavelength of 625 nm until an Optical Density (OD) value of 0.1 is achieved, which corresponds to a McFarland turbidity standard of 0.5. Should the Optical Density (OD) exceed 0.1, dilution is executed with 0.85% NaCl until an Optical Density (OD) of 0.1 is reached.

For the MR-F01 fungal isolate, one loop of fungal culture is inoculated into 50 mL of potato dextrose broth (PDB) media and homogenized. The resultant fungal isolate suspension is agitated at room temperature (28°C) at a speed of 120 rpm for five days. Following incubation, 1 mL of fungal suspension is placed into a test tube containing 5 mL of sterile 0.85% NaCl. Calibration of the spectrophotometer is reiterated with a blank solution (distilled water). According to Novianty et al. (2020), the turbidity of the fungal suspension is quantified by inserting the suspension into a cuvette using a UV-Vis spectrophotometer at a wavelength of 660 nm until an Optical Density (OD) range of 0.677-0.687 is achieved. If the Optical Density (OD) of the test fungal suspension exceeds 0.687, dilution is performed using 0.85% NaCl until an Optical Density (OD) within the 0.677-0.687 range is obtained.

2.3.6. Test of Microbial Isolate Resistance to Dimethomorph Fungicides

To assess microbial isolate resistance to the dimethomorph fungicide, 100 mL of the bacterial and fungal isolate suspensions subjected to turbidity testing via UV-Vis spectrophotometry is transferred to a petri dish. Nutrient agar (NA), potato dextrose agar (PDA), and mannitol salt agar (MRSA) media are then added, and the pour plate technique is applied until a homogeneous mixture is achieved, followed by a resting period of 20 minutes to allow the medium to solidify. Microbial resistance assessments against fungicides containing dimethomorph are conducted aseptically within a Laminar Air Flow (LAF) environment.

The resistance test, each dose of fungicide is prepared at 10 µL, which is dropped onto a paper disc and allowed to settle for 30 seconds. The utilized dosages for each active ingredient are 0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L. The paper discs containing respective doses of fungicide are placed onto the NA, MRSA, and PDA media pre-homogenized with microbes. After applying all treatments, the incubation of bacterial samples (MR-B01, MR-B02, MR-B03, MR-B04) is maintained for 24 hours, and the MR-F01 fungal sample is incubated for 5 days at room temperature (28°C). Throughout the incubation period, daily observations are conducted to measure the diameter of inhibition zones surrounding the fungicide discs using a caliper. Sensitivity of microbial colonies to fungicides is indicated by the presence of inhibition zones characterized by a clear diameter surrounding the fungicide disc.

2.3.7. Observation of microbial resistance to dimetomorphic fungicides

Observation of microbial resistance to dimethomorph fungicides was predicated on the formation of inhibition zones, quantifiable by measuring the clear zones surrounding the disc paper. Measurement was executed at 24 hours intervals using a caliper. Pursuant to Magvirah *et al.* (2019), the inhibition zone can be calculated using the following formula:

$$\text{Inhibition Zone Diameter} = \frac{(D_v - D_c) + (D_h - D_c)}{2} \quad [1]$$

Note: D_v = Vertical diameter
 D_h = Horizontal diameter
 D_c = Disc diameter
 Z_b = Clear zone

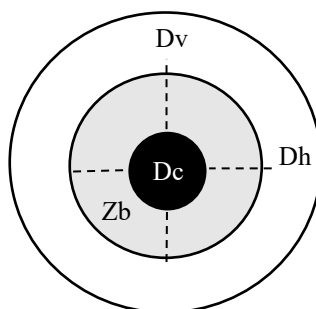


Figure 1. The formation of inhibition zones

Table 1. Relative Barrier Response Category

Inhibition Zone Diameter	Relative Barrier Respons
≥ 20 mm	Very Strong
10 – 20mm	Strong
5 – 10 mm	Medium
≤ 5 mm	Weak

2.3.8. Data Analysis

The calculation of the diameter of the inhibition zone was subjected to *One-Way Anova* statistical testing, followed by the *Mann Whitney* test. Initially, a normality test was conducted to ascertain the normal distribution of the data, followed by the homogeneity test. If both criteria were satisfied, the *One-Way Anova* test was performed. In cases where the *One-Way Anova* test did not meet the requisite conditions, the *Kruskal Wallis* nonparametric test was utilized to discern differences among treatments regarding the average diameter of the inhibition zone. This was succeeded by the *Mann Whitney* test to evaluate whether significant differences existed among the treatment outcomes.

3. Results and Discussion

Microbial resistance tests to dimethomorph-containing fungicides were conducted using concentration treatments of 0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L, with observations recorded over 1×24 hours for bacteria and 5 days for fungi at a temperature of 37°C. The bacterial resistance tests against the dimethomorph 50% fungicide treatment resulted in varying outcomes for each isolate, as illustrated in Figure 2. The inhibition zones observed in each isolate were a consequence of the interaction between the dimethomorph fungicide and the bacterial cells. The measurement of the inhibition zones was performed utilizing a caliper.

The treatments encompassing 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L yielded different inhibition zone values for each microbe. According to the average values represented in Table 1, three bacterial isolates MR-B01, MR-B03, and MR-B04 demonstrated a response to the fungicide administration. The classification of inhibition response established by Davis and Stout (1971) categorizes inhibition zone diameters as follows: ≥ 20 mm as very strong, 10-20 mm as strong, 5-10 mm as moderate, and ≤ 5 mm as weak. Utilizing this classification, the inhibitory capacity of the fungicide against bacterial isolate MR-B01 at concentrations of 0.5 g/L (0.25 mm), 1 g/L (0.5 mm), 1.5 g/L (0.55 mm), and 2 g/L (0.7 mm) fell within the weak category.

The fungicide's inhibitory capacity against bacterial isolate MR-B03 at concentrations of 0.5 g/L (0.8 mm), 1 g/L (1.15 mm), 1.5 g/L (1.3 mm), and 2 g/L (1.6 mm) similarly categorized as weak. In contrast, bacterial isolate MR-B04 produced a fungicide inhibition response with concentrations of 0.5 g/L (0.55 mm), 1 g/L (0.9 mm), 1.5 g/L (1 mm), and 2 g/L (2.45 mm), also classified as weak. Meanwhile, bacterial isolate MR-B02 and fungal isolate MR-F05 exhibited an average inhibition zone diameter of 0 mm across the concentrations of 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L.

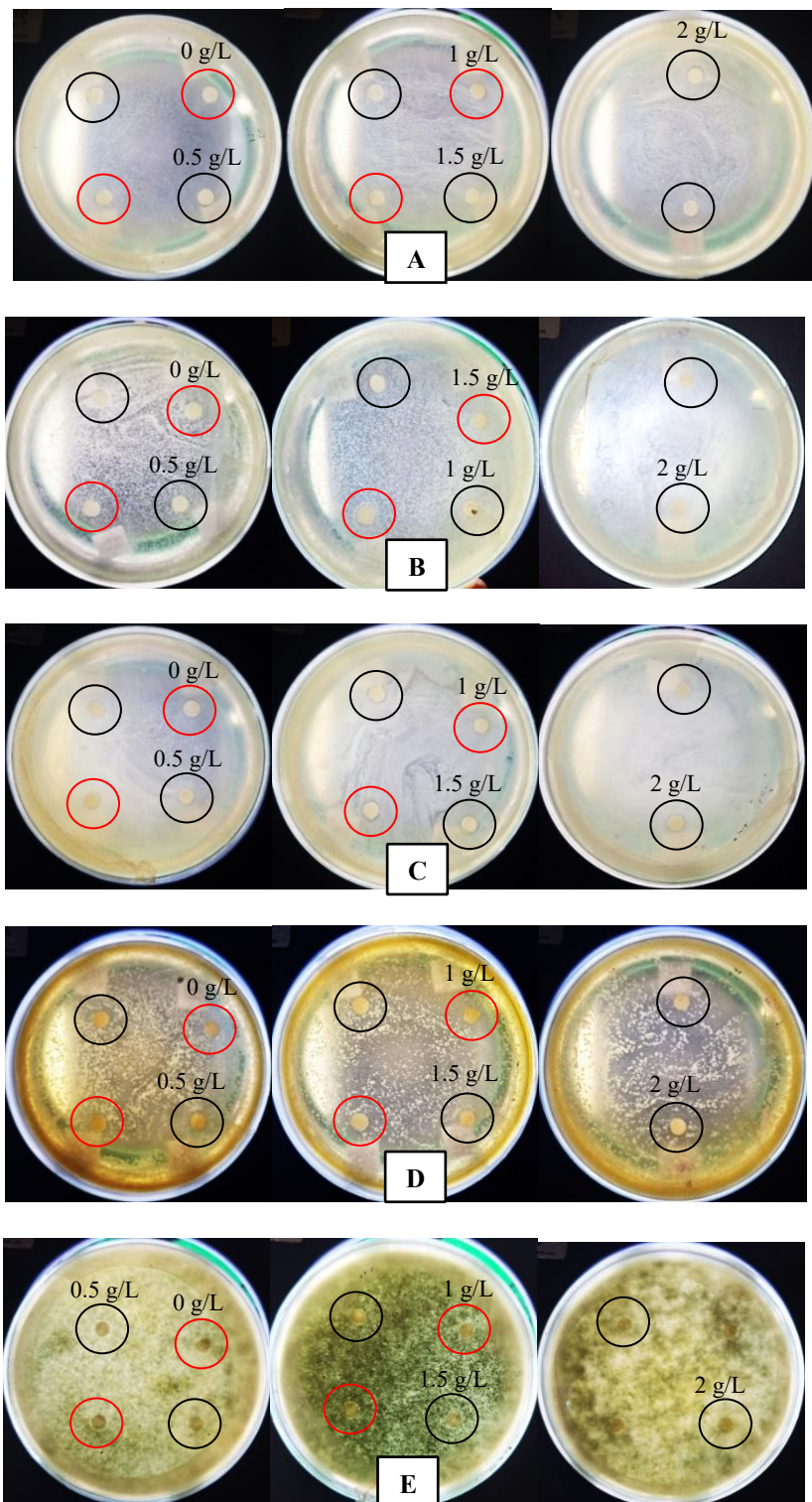


Figure 2. Inhibition zone in treatments 0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, dan 2 g/L. (a) MR-B01; (b) MR-B02; (c) MR-B03.; (d) MR-B04; (e) MR-F01

Table 2. Results of Measuring the Average Diameter of the Inhibition Zone in Treatments 0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, dan 2 g/L

Treatment	Microbial Inhibition Zone Diameter (mm)	Mean Rank (Kruskal Wallis)	Category
	Average $\pm$ SD		
MR-B01 P1	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B01 P2	0.25 $\pm$ 0.26 ^b	125.25	Weak
MR-B01 P3	0.5 $\pm$ 0.47 ^{bc}	145.60	Weak
MR-B01 P4	0.55 $\pm$ 0.49 ^{cd}	148.50	Weak
MR-B01 P5	0.7 $\pm$ 0.63 ^{bcd}	157.30	Weak
MR-B02 P1	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B02 P2	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B02 P3	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B02 P4	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B02 P5	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B03 P1	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-B03 P2	0.80 $\pm$ 0.42 ^{cd}	174.70	Weak
MR-B03 P3	1.15 $\pm$ 0.33 ^{def}	204.90	Weak
MR-B03 P4	1.30 $\pm$ 0.34 ^{ef}	213.75	Weak
MR-B03 P5	1.60 $\pm$ 0.61 ^f	220.80	Weak
MR-B04 P1	0.00 $\pm$ 0.00 ^a	81.50	Tidak ada
MR-B04 P2	0.55 $\pm$ 0.55 ^{bc}	148.55	Weak
MR-B04 P3	0.9 $\pm$ 0.87 ^{bcd}	168.65	Weak
MR-B04 P4	1 $\pm$ 0.88 ^{cdef}	174.50	Weak
MR-B04 P5	2.45 $\pm$ 1.99 ^{def}	195.50	Weak
MR-F05 P1	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-F05 P2	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-F05 P3	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-F05 P4	0.00 $\pm$ 0.00 ^a	81.50	Resistance
MR-F05 P5	0.00 $\pm$ 0.00 ^a	81.50	Resistance
Kruskal Wallis Significance Value			<0.001

Note :

P1 : concentration treatment 0 g/L

P2 : concentration treatment 0,5 g/L

P3 : concentration treatment 1 g/L

P4 : concentration treatment 1,5 g/L

P5 : concentration treatment 2 g/L

Symbol (*) = indicates a significant difference (p value = <0,05) in the *Kruskal Wallis* testa,a = the same notation denotes no significant difference in *Mann Whitney* test

Results from the *Kruskal Wallis* test indicated a significant value <0.001 (<0.05), resulting in the rejection of H0 and acceptance of H1, thereby confirming significant differences among treatment groups regarding the diameter of the inhibition zone formed. The mean rank represents the average value assigned to each treatment group based on the inhibition zone size of a microbe. This study elucidated that a higher mean rank value correlates with a larger average inhibition zone, indicating increased sensitivity of the microbe to the dimethomorph fungicide. Conversely, lower mean rank values signify smaller average inhibition zones, suggesting resistance to the fungicide.

Table 2 showed the highest mean rank values correspond to MR-B01 at 2 g/L (157.30), MR-B03 at 2 g/L (220.80), and MR-B04 at 2 g/L (195.50). The lowest mean rank values pertain to bacterial isolate MR-B01 at 0.5 g/L (125.25), MR-B03 at 0.5 g/L (174.50), MR-B04 at 0.5 g/L (148.55), alongside bacterial isolate MR-B02 and fungal isolate MR-F05, which consistently presented values of 81.50 across all concentrations (0.5 g/L, 1 g/L, 1.5 g/L, 2 g/L). The subsequent analysis employed the *Mann Whitney* test to ascertain any significant differences across each concentration of dimethomorph fungicide within the tested microbes.

The results of the *Mann Whitney* test presented in Table 1 indicate distinct variations in the inhibition zone diameter across different treatments. Notably, treatments exhibiting no significant differences in the average inhibition zone diameter include the 0 g/L treatment (P1) of each microbe in conjunction with the treatments (P2–P5 MR-B02 dan P2–P5 MR-F05). Similarly, the P2 treatment (MR-B01 0.5 g/L) demonstrated no significant variation in average inhibition zone diameter compared to treatments (P3 and P5 MR-B01, P2–P3 MR-B04). Treatments P3 and P4 (MR-B01) showed no significant differences with other treatments P4–P5 MR-B01 and P2– P4 MR-B04.), while treatment P5 (MR-B01) exhibited no significant difference when compared to treatments (P2–P3 MR-B03 and P2–P5 MR-B04) treatment groups. Additionally, treatment P2 (MR-B02) demonstrated no significant difference in average inhibition zone diameter relative to treatments (P3 MR-B03, and P2–P5 MR-B04), and treatment P3 (MR-B03) similarly showed no significant differences with treatments (P4–P5 MR-B03 and P3–P5 MR-B04).

In the P4 and P5 (MR-B03) treatments, no significant difference was observed in the average value of the inhibition zone when compared with the P3-P5 MR-B04 treatment. In the P2 (MR-B04) treatment, significant differences in the average diameter of the inhibition zone were noted in conjunction with the P3-P4 MR-B04 treatment. Notable treatments that exhibited significant differences in average diameter for the inhibition zone included P1 (MR-B01) in relation to the (P2-P5 MR-B01, P2-P5 MR-B03, and P2-P5 MR-B04) treatments. Furthermore, the P2 (MR-B01) treatment displayed significant differences with nearly all other treatments, as well as the P3-P5 MR-B01 treatment in relation to the (P1-P5 MR-B02, P1 MR-B03, P1 MR-B01, and P1-P5 MR-F05) treatments. In the P1-P5 MR-B02 and P1 MR-B03 treatments, significant differences in the average diameter of the inhibition zone were observed when compared with other treatment groups (P2-P5 MR-B03 and MR-B01).

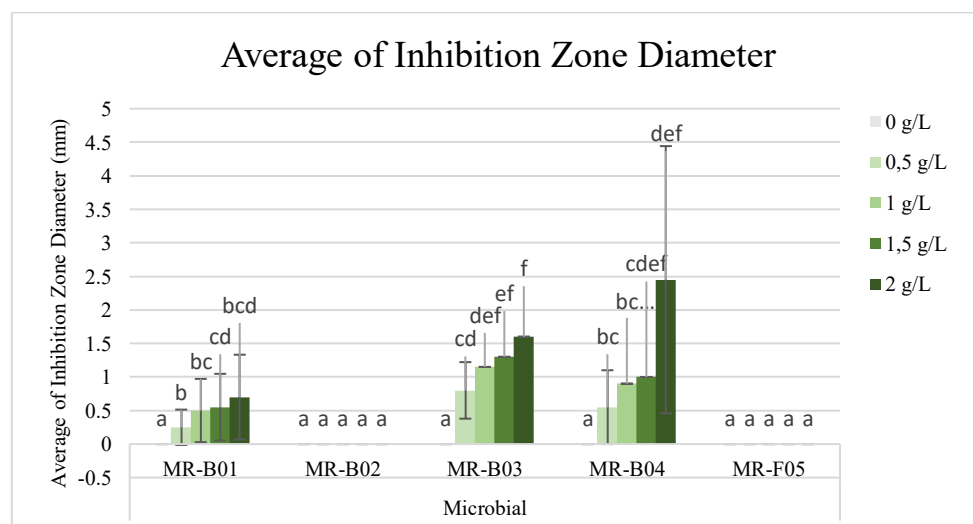


Figure 3. Graph of Average Diameter Zone Across each Concentration Treatment

Figure 3 illustrates the variability in average inhibition zone diameter across each microbe, which may be attributable to the concentrations of the fungicide Dimethomorph. The data presented in Figure 2 suggest that with increased concentrations of dimethomorph fungicide, the average inhibition zone diameter also increased for bacteria MR-B01, MR-B03, and MR-B04. This observation aligns with the findings of Dotulong *et al.* (2019), which state that higher fungicide concentrations result in increased toxicity and correspondingly larger inhibition zones.

Furthermore, these results correlate with research conducted by Sanam *et al.* (2021), which indicated that rhizosphere bacteria with codes B365 and AZB31 responded to chemical fungicides such as mancozeb. In their study, the inhibition zone values associated with bacteria code B365 against mancozeb at concentrations ranging from 0.05% to 2% varied from 0 mm to 10 mm, while bacteria code AZB31 exhibited inhibition zone values from 0 mm to 10 mm within the same concentration range. Conversely, observations regarding MR-B02 bacteria and MR-F05 fungi indicated the absence of inhibition zones around the disc paper across all concentrations tested.

The average diameter recorded for MR-B02 bacterial isolates and MR-F05 fungi was 0 mm at concentrations of 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L. Additionally, research conducted by Sanam *et al.* (2021) found that the mancozeb fungicide showed no inhibition zone against rhizosphere bacteria with code ASP25 at 0 mm. Ajith *et al.* (2024) reported that rhizosphere fungi such as *Trichoderma* spp. displayed a mycelial inhibition value of 0 mm against 50% dimethomorph fungicide across concentrations of 50 ppm, 100 ppm, 200 ppm, and 400 ppm.

The observed variability in concentration produced by the test microbes can be attributed to the inherent characteristics of the microbial cell wall, which serves as the principal protective structure determining microbial interaction with their environment. According to Amin *et al.* (2023), bacteria can be classified into Gram-positive and Gram-negative categories based on cellular composition and wall structures. The MR-B01 and MR-B04 bacterial classifications are identified as Gram-positive. Specifically, Sanuddin *et al.* (2022) noted that the cell walls of Gram-positive bacteria consist of peptidoglycan, polysaccharides, and teichoic acids. Gram-positive bacteria typically exhibit heightened sensitivity to chemical agents such as antibacterials and fungicides due to their simpler cell wall structures, facilitating easier compound penetration (Darsono and Fajriannor, 2020).

Additionally, MR-B02 and MR-B03 bacteria are classified as Gram negative. The composition and thickness of the outer membrane of MR-B03 differ from those of MR-B02, rendering them more permeable to certain molecules, including dimethomorph fungicides. MR-B03 possesses an outer membrane resistance that is readily penetrated by small molecules such as fungicides (Wulandari *et al.*, 2025). Furthermore, MR-B03 bacteria have a biochemical defence mechanism that relies on the production of exopolysaccharides (Hindersah *et al.*, 2024). Nonetheless, this defence mechanism is ineffective against systemic fungicides such as dimethomorph fungicides, which have the capacity to penetrate plant tissue and potentially affect the diversity of microorganisms within the soil (Zhang *et al.*, 2020).

According to Madigan *et al.* (2018), MR-B02 bacteria are categorized as Gram negative. The cell wall of MR-B02 comprises a peptidoglycan layer situated between an inner membrane and an outer membrane. This outer membrane consists of phospholipids, lipopolysaccharides, and specific proteins often referred to as auto layers (Nikaido, 2003). The presence of an outer layer enriched with phospholipids and lipopolysaccharides forms a selective barrier that inhibits the penetration of chemical compounds, including fungicides. Generally, bacterial cell walls composed of polysaccharides exhibit a greater susceptibility to denaturation compared to those containing phospholipids, as seen in Gram-negative bacteria. Therefore, the structure of the cell wall contributes to the chemical resistance observed in MR-B02 against dimethomorph fungicides (Helmiyati and Nurrahman, 2010).

In contrast, the MR-F05 fungus exhibits a distinct cell wall structure compared to bacteria. According to Wendersteyt *et al.* (2021), fungi possess a complex cell wall structure comprised of crystalline polysaccharides such as chitin and β -glucan, in addition to a matrix of amorphous polysaccharides and protein-saccharide complexes. Chitin and β -glucan are essential for maintaining the integrity of the fungal cell wall, thus providing protection against specific fungicidal compounds such as dimethomorph. The disparity in cell wall structure between fungi and bacteria significantly influences microbial responses to antimicrobial compounds such as fungicides, which may contribute to microbial resistance.

Figure 3 illustrates that MR-B02 bacteria demonstrate resistance to dimethomorph fungicide, as evidenced by the absence of an inhibition zone surrounding the disc paper. The average measured inhibition zone was 0 mm across all tested concentrations. The inability of the fungicide to impede the growth of MR-B02 indicates a pronounced level of compatibility or tolerance to the dimethomorph fungicide. These findings align with research conducted by Sanam *et al.* (2021), which reported that rhizosphere bacteria exhibited resistance to the fungicide mancozeb, manifesting an inhibition zone diameter of 0 mm, suggesting that this bacterial type is largely unaffected by exposure to certain fungicides.

Moreover, MR-B02 bacteria are known to synthesize various phytohormones, including auxin (IAA), abscisic acid (ABA), gibberellins, and cytokinin's, which can promote plant growth and enhance plant resistance under extreme conditions, including exposure to toxic chemical compounds such as fungicides (Ni'mah and Yuliani, 2022). The biochemical defence mechanism of MR-B02 bacteria encompasses efflux and detoxification pumps. Efflux pumps operate by expelling toxic compounds such as dimethomorph fungicides from the cell

membrane of MR-B02 bacteria before they inflict damage. The detoxification mechanism employed by MR-B02. bacteria involves the alteration or breakdown of the chemical structure of the fungicide into an inactive form.

Figure 3 presents the average value of the inhibition zone diameter generated by the MR-F05 fungus, recorded at 0 mm. Research conducted by Ajith *et al.* (2024) indicated that rhizosphere fungi exhibit considerable compatibility (resistance) to 50% dimethomorph fungicide. In this study, mycelial growth was not inhibited, as evidenced by an inhibition zone value of 0 mm across various concentrations of dimethomorph. These findings contradict the research conducted by Saha *et al.* (2023), which demonstrated that a 50% concentration of dimethomorph fungicide effectively inhibited the growth of rhizosphere fungi, specifically *Trichoderma asperelloides*, yielding an inhibition value of 75.50 mm, indicative of the fungus' high sensitivity to the dimethomorph fungicide.

The MR-F05 rhizosphere fungi possess enzymes that confer protection against chemical compounds. According to Kubicek *et al.* (2019), several enzymes produced by rhizosphere fungi can degrade fungicide molecules into less toxic forms. This enzymatic capacity facilitates the survival and activity of rhizosphere fungi in environments laden with toxic chemical compounds. A key mechanism supporting this capability is the synthesis of hydrolytic enzymes, including chitinase, glucanase, and protease, which contribute to biomass degradation and the detoxification of foreign compounds such as fungicides.

Furthermore, the resistance of MR-F05 fungi to fungicides is influenced by the biological and morphological characteristics intrinsic to the fungi. A significant factor in this resistance is the ability to produce spores in substantial quantities. The prolific production of spores allows a portion of them to endure and evade the lethal effects of fungicides (Kubicek *et al.*, 2019). The emergence of strains resistant to various fungicides is attributed to a reduction in the cell permeability of the MR-F05 fungus, limiting the absorption of chemical compounds such as fungicides, along with the detoxification capabilities exhibited by MR-F05 fungal cells.

Sumardiyono (2013) indicates that the sensitivity of rhizosphere fungi to specific fungicides can be linked to intrinsic resistance mechanisms, including reduced cell membrane permeability. This diminished permeability prevents fungicides from penetrating the cells and reaching their sites of action. Research conducted by Escudero-Leyva *et al.* (2022) reveals that certain rhizosphere fungi possess the capability to tolerate and degrade various fungicides, demonstrating that these fungi not only endure environments contaminated by fungicide residues but also mitigate the negative consequences for other organisms and the soil ecosystem.

Figure 3 illustrates that the application of dimethomorph fungicide against MR-B01, MR-B03, and MR-B04 bacteria resulted in the formation of an inhibition zone surrounding the paper disc. MR-B01 exhibited an average diameter of the inhibition zone across the various concentrations tested. Several studies corroborate these findings. Notably, research by Ruiz Tello (2023) found that a 10 ppm concentration of dimethomorph fungicide successfully inhibited the growth of rhizosphere bacteria, yielding an average growth inhibition value of 52.75%. Moreover, findings from Sanam *et al.* (2021) indicated that the chemical fungicide mancozeb inhibited the growth of rhizosphere bacteria designated as B365, manifesting average inhibition zone values of 6 mm, 7 mm, 8 mm, 9 mm, 10 mm, 10 mm, and 10 mm, thereby confirming bacterial sensitivity to fungicides.

Tkach *et al.* (2021) also conducted a study aligned with these findings, demonstrating that the *in vivo* application of a combination of dimethomorph and cymoxanil fungicides led to a significant reduction in soil microbial biomass, including nitrogen-fixing rhizosphere bacteria, with an average decrease of 44%. Additionally, research by Khulillah *et al.* (2023) indicated that certain rhizosphere bacteria exhibited sensitivity to fungicides containing the active ingredient propineb, with growth inhibition values ranging from 40% to 80%. Wang *et al.* (2015) elucidated that the presence of a designated inhibition zone in bacterial test samples can also be attributed to the characteristics and mechanisms of action of the fungicide's active ingredient. The morpholine compound in dimethomorph fungicide is noted for its potential to compromise bacterial cell wall integrity by interfering with peptidoglycan synthesis.

Morpholine's weak basic properties can disrupt the enzymes essential for this synthesis, thus inhibiting the formation of peptide cross-links critical for cell wall stability (Wang *et al.*, 2015). Furthermore, morpholine may inhibit protein synthesis in bacterial cells by affecting ribosomes and RNA polymerase, disrupting both

transcription and translation processes, which impedes bacteria from producing vital proteins necessary for growth, metabolism, and survival. Nucleic acids, both DNA and RNA, also play crucial roles in the storage of genetic information and regulation of cellular activities (Alexandrova *et al.*, 2023).

According to Alexandrova *et al.* (2023), morpholine compounds possess the capacity to alter intracellular pH by disrupting the activity of critical enzymes within bacterial cells. This alteration in pH precipitates an imbalance in intracellular physiological conditions, thereby inhibiting bacterial cell metabolism and growth. Furthermore, morpholine compounds may interact with nitrogen bases in DNA, leading to structural damage, including DNA chain breaks or the formation of cross-links between DNA strands. Such DNA damage impedes the processes of replication and repair, consequently affecting microbial survival.

The research conducted indicates that the application of fungicides containing dimethomorph as the active ingredient in vitro at concentrations of 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L elicited responses from several rhizosphere microorganisms, including *Bacillus* sp., *Azotobacter* sp., and *Lactobacillus* sp., as demonstrated by the formation of inhibition zones. Higher concentrations of the fungicide resulted in larger inhibition zone diameters against microbial isolates. However, bacterial isolates *Azospirillum* sp. and fungal isolates *Trichoderma* sp. exhibited notable resistance to dimethomorph concentrations of 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L, with no inhibition zones detected. Therefore, it is advisable to implement dose adjustments and periodic evaluations to ensure the effectiveness of fungicides while preserving the sustainability of microbial populations, particularly within the rhizosphere.

4. Conclusions

The results of the conducted research indicate that varying concentrations of dimethomorph fungicide exert differential effects on the resistance of the microbial isolates tested, specifically bacteria MR-B01, MR-B02, MR-B03, MR-B04, and fungal isolate MR-F01. It was observed that as the concentration of dimethomorph fungicide increased, the extent of the inhibition zone also increased. Notably, a concentration of 0.5 g/L of dimethomorph fungicide demonstrated the greatest efficacy against the resistance of bacteria MR-B01, MR-B03, and MR-B04. Conversely, fungicides containing dimethomorph at concentrations of 0.5 g/L, 1 g/L, 1.5 g/L, and 2 g/L exhibited the highest effectiveness against the resistance of bacteria MR-B02 and fungi MR-F05. Furthermore, certain test microbes displayed a pronounced level of resistance across various concentrations of dimethomorph fungicide, specifically bacteria MR-B02 and fungi MR-F05, which exhibited an average inhibition zone diameter of 0 mm.

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Declaration of generative AI use

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

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