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Ecological study on the effects of Dizam fungicide on useful *Rhizobium* sp. bacteria

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Abstract

Nitrogen-fixing beneficial bacteria improve soil fertility but can be adversely affected by fungicides, which may reduce their numbers or survival. To assess the effects of Dizam 50% SC as a fungicide on useful root nodule bacteria from *Rhizobium* sp. (collected from roots of *Vigna* spp. black-eyed peas), bacteria were isolated from the plant's root system and exposed to varying concentrations of the fungicide (1,000–12,000 mg/L). The findings revealed that the average number of bacterial colonies decreased with increasing fungicide concentrations, while the percent mortality of the bacteria also increased. There also appeared to be some variability among the tested concentrations (2,000, 6,000, and 7,000 mg/L) in terms of both the mean bacterial count per mL and percent mortality compared with the other concentrations tested. Furthermore, the findings revealed a median lethal concentration (LC_{50}) of approximately 9,359.54 mg/L; there was a statistically significant inverse relationship between fungicide concentration and bacterial colonization, and there was a positive association between fungicide concentration and percent mortality of the bacteria. Lastly, based on the collected data, it was shown that when the previously described two strains were re-cultured in media containing various concentrations of the fungicide (range: 1,000–12,000 mg/L), their respective LC_{50} values were determined to be 75.19 and 57.36 mg/L. Therefore, these findings suggest a dose-dependent reduction in *Rhizobium* sp. colony formation. However, these data do not confirm genetic resistance to Dizam 50% SC, which requires verification using molecular techniques.

Keywords: Fungicide; LC_{50}; *Rhizobium* sp.; Ecosystem; Mortality

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1. Introduction

Fungicides are broadly utilized to prevent fungal infections in a variety of crops, with the purpose of maximizing crop productivity. However, when fungicides are overused or continuously sprayed onto soil, they generate chemically active residues in the soil. These residues can then adversely affect soil quality and the microbial equilibrium of soil ecosystems. Chemical products like fungicides can be introduced into ecosystems without being controlled, which could allow foreign substances to enter ecosystems. Consequently, there exists a possibility for these chemical products to negatively impact the physical, chemical, and biological properties of soil. One of the negative impacts on the biological properties of soil is the negative influence on the growth, activity, and

survival of naturally occurring microorganisms that have beneficial characteristics.

Rhizobium sp. is a Gram-negative, aerobic, rod-shaped, non-spore–forming soil-dwelling bacterium that belongs to the class *Alphaproteobacteria* and the order Rhizobiales. In addition to fixing atmospheric nitrogen in association with legumes, *Rhizobium* sp. plays an important role in promoting plant productivity and maintaining soil fertility. *Rhizobium* sp. develops a symbiotic relationship with the roots of leguminous plants through the formation of root nodules. Within these root nodules, nitrogenase enzymes convert atmospheric nitrogen into a biologically available form of nitrogen. Rhizobia are among the most important and diverse groups of soil-dwelling microorganisms. As such, they play critical roles in promoting soil fertility, plant growth promotion, nutrient cycling, and the translocation of essential nutrients to plants. Thus, disruptions in their growth or survival will likely result in a disruption in both the functionally relevant processes within the microbial community of the soil and sustainable crop production.

Chemical fungicides accumulate at abnormally high levels in soils and can cause significant alterations to the types and quantities of beneficial soil microorganisms. Studies have shown that fungicides can alter microbial respiration, enzyme activity, nitrogen transformations, and populations of beneficial soil bacteria. Additionally, since many fungicides are slowly broken down in the environment or produce toxic compounds upon breakdown, it is possible for them to disrupt the functionally relevant processes associated with beneficial soil microorganisms. Numerous reports indicate that chemicals such as pesticides can affect nitrogen-fixing bacteria[1], but few studies have explored the non-target effects of fungicides on nitrogen-fixing bacteria, especially at concentrations that reflect either field exposure or accidental buildup.

Dizam 50% SC is a systemic fungicide product that can be utilized for both preventive and curative control of fungal diseases. It is absorbed by the roots and/or shoots of plants and works by inhibiting the hyphal growth of fungi. Dizam 50% SC can be applied to a wide range of crops, including cereals, oilseeds, sugarbeet, grapes, tomato, apple, cotton, barley, millet, oats, rice, sorghum, wheat, peanut, and stone fruits.[2] The product is typically utilized in a liquid format or as a suspension concentrate. Although Dizam 50% SC is intended for controlling fungal diseases in crops, there is evidence suggesting that its use could also influence beneficial microorganisms that reside in the soil. Such microorganisms play a direct role in enhancing soil fertility. Based on available data, Dizam's fungicide properties do not include carcinogenic or mutagenic effects unless certain metabolites are present.[3] It is noted that Dizam 50% SC is broken down in soil via enzymatic reactions initiated by microorganisms found in soils and water over long periods of time (e.g., months).[4,5] Dizam 50% SC is considered to be highly toxic to aquatic organisms. Its environmental hazards are likely linked to its mobility and ecological effects.[6]

Based on its agricultural uses as a broad-spectrum systemic fungicide and possible residual effects in soils following applications, Dizam 50% SC was chosen as the focus of the present study. To assess acute toxicity responses to varying degrees of fungicide-induced stress, selection criteria included a fungicide concentration range established from recommended application rates provided by the manufacturer, with additional extended exposure concentrations. Limited investigations have been conducted on assessing toxicity responses in *Rhizobium* sp., which are beneficial nitrogen-fixing bacteria associated with legume plant growth and soil fertility, following exposure to Dizam 50% SC using assessments such as colony growth evaluations, percentage mortality determinations, and median lethal concentration (LC_{50}) estimations. A knowledge gap thus existed regarding how beneficial nitrogen-fixing bacteria would respond to increased concentrations of this fungicide under stress conditions.

This study assessed the toxicity effects of Dizam 50% SC on beneficial *Rhizobium* sp. obtained from root nodules of black-eyed peas (*Vigna* sp.). Specific objectives included determining the effects of various concentrations of Dizam 50% SC on bacterial colony growth, estimating percent mortality, and calculating LC_{50} values. Assessments were also made to compare the ability of exposed strains to regrow under conditions of stress caused by fungicide exposure. The hypothesis for this study was that increases in the concentration of Dizam 50% SC would significantly decrease *Rhizobium* sp. colony size while significantly increasing bacterial mortality in a dose-dependent fashion.

2. Materials and methods

2.1. Isolation of root-nodulating bacteria

Samples of root nodule bacteria were obtained from cultivated *Vigna* sp. plants in agricultural areas of the north of Hilla City, Babylon Province, Iraq (Figure 1A). Root samples were extracted from each plant at a depth of 10–25 cm and placed into sterile nylon bags. Samples were maintained aseptically during transportation to the laboratory and examined immediately upon arrival.

The process used in this study closely follows the methods reported by Somasegaran and Hoben[7] and Kaur *et al.*[8] but has been slightly modified. The roots of the

black-eyed pea plants were washed thoroughly with tap water and then with distilled water, and dried completely before approximately 50 random root nodules were removed from the root system in a separate clean Petri dish. The 50 selected nodules were then added to a bottle containing 95% ethanol for 5–10 s; they were rinsed with sterile distilled water, and then submerged in 1% sodium hypochlorite solution for 6 min. Each of the nodules was washed more than six times with sterile distilled water to ensure all sterilizing chemicals were removed. The final distilled water wash was also cultured on a solid nutrient agar or yeast extract mannitol agar plate and incubated to verify the absence of visible microbial growth, thereby confirming the effectiveness of the surface sterilization.

The sterilized nodules were quickly dipped into 95% ethanol and flamed to evaporate the ethanol while ensuring additional surface sterilization. The nodules were subsequently crushed in a clean Petri dish under aseptic conditions using sterilized forceps with tapered tips. Then, 25 mg of each crushed nodule was suspended in a nutrient broth in a separate Petri dish, shaken vigorously, and centrifuged prior to being diluted serially and plated onto yeast extract mannitol medium for the purpose of isolating pure cultures of the bacteria associated with the nodules.

2.2. Identification and maintenance of root nodule bacteria

Bacteria that had been isolated as single colonies were first assessed through colony morphology, Gram stain results, and a variety of other biochemical tests. All isolates were then tested with a computerized identification system called the VITEK 2 (bioMérieux, France) to assist in identifying the bacteria and to obtain pure culture isolates of the bacteria for the toxicology studies.[9] Isolates exhibiting characteristics of *Rhizobium* sp.—such as a Gram-negative staining, rod-shaped morphology, and the ability to grow on yeast extract mannitol media—were used in subsequent studies.

Because 16S ribosomal RNA gene sequencing was not performed, final identification was based on colony morphology, Gram staining, biochemical tests, and VITEK 2 analysis. Yeast extract mannitol agar slant cultures of each isolate were grown, stored at 4 °C, and sub-cultured every few weeks to maintain bacterial viability and culture purity before performing toxicity testing.

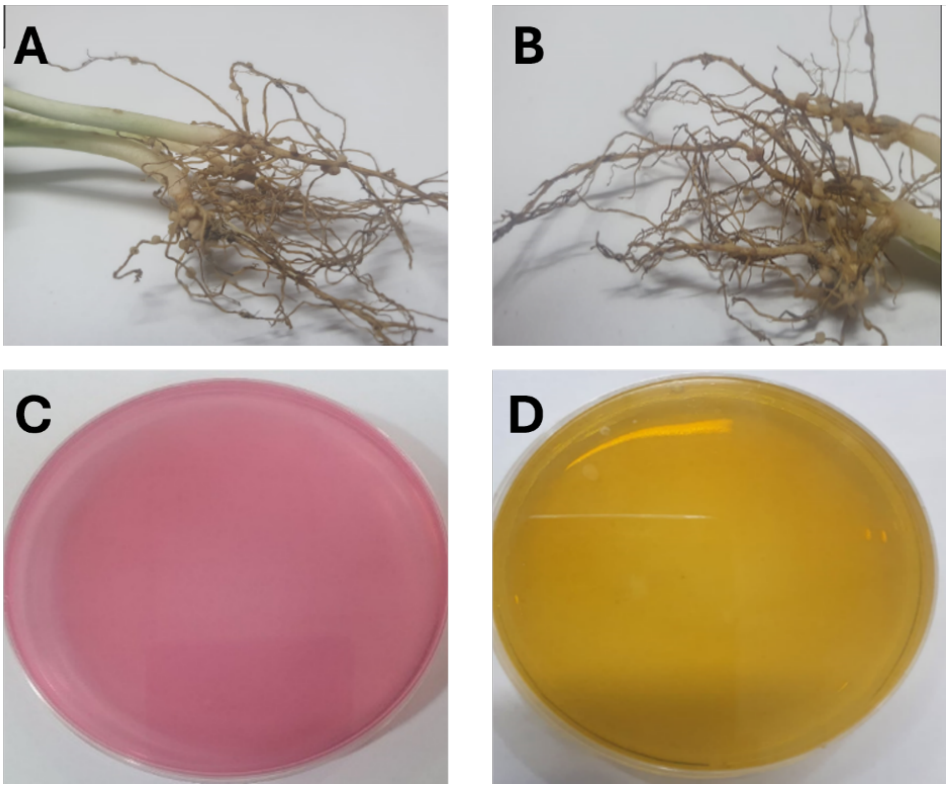

Figure 1. Root nodule bacteria and yeast extract mannitol agar. (A, B) *Vigna* sp. roots with nodules. (C) Yeast extract mannitol agar. (D) Yeast extract mannitol agar showing color change.

2.3. Preparation of culture media and fungicide-treated agar

Yeast extract mannitol agar (Figure 1C) was used to culture the bacterial isolates. This medium was sterilized by autoclaving at 121 °C for 15 min. Once the autoclave process was completed and the medium had reached a temperature of approximately 45–50 °C, the fungicide was added to the medium.

Following the addition of the fungicide to the cooled, sterilized agar, the medium was poured into separate Petri dishes. Each container was then treated with different amounts of Dizam 50% SC fungicide by supplementing the medium with different concentrations.

Before adding the fungicide to the agar, the fungicide solutions were sterilized using a 0.22 µm membrane filtration under vacuum. The use of a membrane filter to sterilize the fungicide solutions eliminated the need for heat sterilization of the fungicides via autoclaving, which would have caused potential degradation of the chemicals.

Once the fungicide-treated media were uniformly distributed throughout each plate and the media were fully solidified, they were examined for subsequent bacterial growth assessment.

A total of twelve fungicide-treated concentrations were tested, ranging from 1,000 to 12,000 mg/L, along with a fungicide-free control. These values were determined based on a combination of recommendations provided by Dizam and extended laboratory exposure levels designed to evaluate acute toxic effects at elevated fungicide concentrations. The highest concentrations were beyond typical field exposure rates and should therefore be considered laboratory stress concentrations rather than representative environmental exposure levels. As controls for comparison purposes, identical preparations containing fungicide-free controls were made and processed in the same manner as those with fungicides.

The method used by Hossain *et al.*[10] was slightly modified. Following the preparation of a series of dilutions of the bacterial stock, 0.1 mL of the second (10^{-2}) dilution was selected as it provided the most discrete/isolated colonies. This bacterial stock was then plated onto separate Petri dishes that contained yeast extract mannitol agar amended with varying concentrations of Dizam 50% SC fungicide (1,000–12,000 mg/L).

Using the pour plate technique, the Petri dish contained the yeast extract mannitol agar that had been treated with fungicide and a known amount (0.1 mL) of the previously prepared bacterial stock. In addition to the treated agar samples, a control sample devoid of fungicide was also prepared to enable comparison of bacterial growth.[11] Both components were thoroughly mixed, after which the Petri dishes were allowed to set.

Following solidification, both plates were inverted and incubated at 28 °C for 24 h and 48 h. Once the incubation period had elapsed, visual inspection revealed the presence of bacterial colonies, which were subsequently enumerated. As each treatment was replicated three times, and the trial was performed independently, the mean number of colonies per plate enabled the calculation of colony-forming units (CFU) and mortality percentages.

2.4. Calculation of growth and mortality percentage

The percentage of bacterial growth and mortality has been measured previously through an equation that has been adapted slightly for this study.[12] To determine average mortality, the number of colonies in the fungicide treatment was subtracted from the number of colonies in the control group, as follows:

$$\begin{aligned} Average\,mortality = {} & Average\,growth\,in\,control\,sample \\ & - average\;\;growth\;\;in\;\;treated\;\;sample \end{aligned} \quad (1)$$

Equation 2 was used to calculate percentage mortality:

$$Percentage\;\;mortality\,(\%) = \frac{Average\,mortality\,in\,treated\,sample}{Average\,growth\,in\,control\,sample} \times 100 \quad (2)$$

This equation allows expression of the reduction in bacterial colony growth due to fungicide application relative to the untreated control. Using the same logic, the percentage increase in bacterial colonies was calculated using **Equation 3**:

$$Percentage\;\;of\;\;bacterial\;\;colony\;\;increase\,(\%) = \frac{Average\,increase\,in\,treated\,sample}{Average\,increase\,in\,control\,sample} \times 100 \quad (3)$$

2.5. Testing of possible resistant or surviving colonies

After observing bacterial development under various fungicide concentrations, colonies developed at 1,000 mg/L and 12,000 mg/L were isolated for evaluation. These colonies were selected to determine whether they are able to develop into active colonies after previous exposure to both low (1,000 mg/L) and high (12,000 mg/L) fungicide levels.

The selected colony samples were cultured in a mixture of nutrient broth and yeast extract mannitol broth at a 1:1 ratio to create a defined medium; this medium allows the

selection of surviving *Rhizobium* sp. cultures from the 1,000 mg/L and 12,000 mg/L treatments. The selected colonial isolates were then inoculated into media containing Dizam 50% SC fungicide at concentrations ranging from 1,000–12,000 mg/L, utilizing the most probable number (MPN) methodology to assess the ability of previously exposed isolates to grow under repeated fungicide stress.

This experiment was designed to assess the likelihood of colony development under conditions of continued fungicide stress following initial exposure. Additional studies may be needed to further define the genetic mechanisms involved in confirmed resistance.

2.6. Calculation of colony-forming unit and most probable number

Following the appearance of bacterial colonies on yeast extract mannitol agar, colonies that grew on fungicide-treated culture media were enumerated. As explained by Gronewold and Wolpert,[13] the number of CFU/mL can be estimated by the following formula:

$$CFU/mL = \frac{Number\ of\ colonies\ grown\ on\ culture\ medium \times dilution\ factor}{Volume\ of\ cultured\ sample\ (mL)} \quad (4)$$

The *Rhizobium* sp. cultures obtained through activation and multiplication of cultures from the 1,000 mg/L and 12,000 mg/L treatment groups were then diluted to 10^{-2}. A dilution series of the activated and multiplied samples was then incubated in tubes containing various levels of fungicides. Growth assessment of bacteria was performed using the MPN method in three tubes per fungicide concentration. Turbidity and color change were indicative of successful bacterial growth. Therefore, the percentage of growth in relation to fungicide concentration could be determined. Due to the limitations of turbidity-based MPN estimates relative to direct enumeration of bacterial colonies, interpretation of MPN data should be limited to comparative assessments of bacterial growth potential rather than absolute determination of bacterial quantity.

2.7. Data analysis

A completely randomized design was used. Data were analyzed statistically using least significant difference at the 0.05 significance level following analysis of variance (ANOVA) (SPSS, version 29.0, IBM Corporation, USA).[14]

3. Results and discussion

3.1. Detection of some fungicides

Based on Table 1, numerous pesticide residues were identified in the soil from which *Rhizobium* sp. was isolated. The sampling site was located in the agricultural areas in the north of Hilla City, Babylon Province, Iraq—an area with a significant proportion of land used for agriculture. Identified pesticide residues (e.g., insecticides, herbicides, and fungicides) included compounds that contain a fungicide similar to Dizam 50% SC as identified in the present study. These findings suggest that the environment used for isolation of root nodule bacteria was likely contaminated due to multiple previous applications of agrochemicals.

Data from pesticide residue analysis should be interpreted with caution, as the primary focus of this study is to assess the effects of Dizam 50% SC on *Rhizobium* sp. growth under laboratory conditions. The identification of pesticide residues provides useful background information regarding the agricultural soil; however, a comprehensive risk assessment based on residue levels would require a separate, validated analytical methodology (e.g., sample extraction procedure, calibration curve, limit of detection, limit of quantitation, recovery percentage, and quality control parameters). Thus, the results of liquid chromatography–mass spectrometry in this study can be considered supporting environmental background information rather than primary experimental evidence.

Table 1. Pesticides detected in agricultural soil from Babylon Province, Iraq, used as the source of *Rhizobium* sp.

Pesticide	Type	Concentration (mg/kg)
Emamectin benzoate	Insecticide	0.492
Diazinon	Insecticide	0.604
Difenconazole	Fungicide	0.732
Imidacloprid	Insecticide	0.588
Metolachlor	Herbicide	0.768
Malathion	Insecticide	0.552
Thiophanate-methyl	Fungicide	0.436
Azoxystrobin	Fungicide	0.488
Abamectin	Insecticide	0.42
Acetamiprid	Insecticide	0.516
Atrazine	Herbicide	0.412
Carbendazam	Fungicide	0.728
Glyphosate	Herbicide	0.572

3.2. The average growth of *Rhizobium sp.* colonies

Growth of *Rhizobium* sp. bacterial colonies (average) per mL on agar medium following treatment with fungicide containing different concentrations of Dizam 50% SC

(from 1,000 to 12,000 mg/L), at two time points (after 24 h and 48 h), was compared to untreated control samples. On average, there was a decrease in colony numbers for all treatments when compared to untreated controls.

Colony counts decreased with increasing concentrations of Dizam 50% SC (1,000–12,000 mg/L) at both 24 h and 48 h. At 24 h, values ranged from 8 to 76 colonies/mL, while at 48 h, higher overall colony counts were observed, ranging from 20 to 89 colonies/mL. In general, lower colony numbers were recorded at higher fungicide concentrations, indicating a concentration-dependent inhibitory effect. It has been suggested that the decrease in colony growth could be due to toxic effects of fungicide exposure on the bacteria, which resulted in a reduced ability to multiply and develop into colonies. The detailed growth, CFU, and inhibition values are presented in Table 2, and the average colony-growth trend is shown in Figure 2.

Previous research has shown that certain fungicides are capable of reducing populations of beneficial soil microorganisms. Previous studies have also demonstrated that this reduction in population size is often influenced by several factors, including the type of fungicide used, the concentration applied, the microbial strains affected, whether the strains originated from soil, and the duration of exposure to the fungicide. A few instances were observed at concentrations of 2,000, 6,000, and 7,000 mg/L, where relative increases in colony counts were observed compared with adjacent concentrations. Although these findings suggest potential evidence of possible resistance to the fungicide, it is difficult to draw conclusions regarding resistance based solely on increased growth rates. There are other explanations for these observations, including temporary tolerance of bacterial cells during exposure to the fungicide, variability among bacterial strains, and random variation among colonies exposed to the same fungicide concentrations.

Thus, caution should be taken when interpreting these apparent anomalies, and future experiments would be required to confirm whether these observations reflect true resistance mechanisms. The term "inhibition of growth" rather than "mortality" is preferred here since the experiment measured bacterial colony formation on

Table 2. Growth response and inhibition of *Rhizobium* sp. after 24 and 48 hours of exposure to Dizam 50% SC

Concentration (mg/L)	Average growth colonies after 24 hours (colony/mL)	Average growth colonies after 48 hours (colony/mL)	CFU/mL after 48 hours	Percentage of growth after 48 hours (%)	Average reduction in colonies after 48 hours (colony/mL)	Percentage inhibition after 48 hours (%)
Control	62 ± 3.12	85 ± 27.45	$8.5 \times 10^4 \pm 0.71 \times 10^4$	100 ± 8.36	0 ± 0	0 ± 0
1,000	51 ± 5.34	76 ± 11.12	$7.6 \times 10^4 \pm 2.11 \times 10^4$	89 ± 24.68	9.00 ± 1.06	11 ± 3.05
2,000	76 ± 2.78	89 ± 6.34	$8.9 \times 10^4 \pm 2.46 \times 10^4$	105 ± 29.12	1.00 ± 0.02	0 ± 0
3,000	33 ± 3.34	73 ± 3	$7.3 \times 10^4 \pm 2.02 \times 10^4$	86 ± 23.85	12 ± 3.24	14 ± 3.88
4,000	29 ± 30.34	58 ± 24.78	$5.8 \times 10^4 \pm 1.61 \times 10^4$	68 ± 18.85	27 ± 2.35	32 ± 8.87
5,000	27 ± 2.12	50 ± 6.78	$5.0 \times 10^4 \pm 1.38 \times 10^4$	59 ± 16.36	35 ± 7.73	41 ± 11.37
6,000	34 ± 5.45	79 ± 7.12	$7.9 \times 10^4 \pm 2.19 \times 10^4$	93 ± 25.79	6.00 ± 1.41	7 ± 1.94
7,000	46 ± 5.45	86 ± 17.34	$8.6 \times 10^4 \pm 2.38 \times 10^4$	101 ± 28.01	3.00 ± 0.62	0 ± 0
8,000	31 ± 7.45	49 ± 4.78	$4.9 \times 10^4 \pm 1.35 \times 10^4$	58 ± 16.08	36 ± 2.67	42 ± 11.64
9,000	31 ± 3.45	46 ± 7.45	$4.6 \times 10^4 \pm 1.27 \times 10^4$	54 ± 14.97	39 ± 1.91	46 ± 12.75
10,000	28 ± 3.45	32 ± 5.45	$3.2 \times 10^4 \pm 0.88 \times 10^4$	38 ± 10.53	53 ± 3.5	62 ± 17.19
11,000	16 ± 1.34	28 ± 3.12	$2.8 \times 10^4 \pm 0.77 \times 10^4$	33 ± 9.15	57 ± 2.17	67 ± 18.58
12,000	8 ± 0.78	20 ± 2.12	$2.0 \times 10^4 \pm 0.55 \times 10^4$	24 ± 6.65	65 ± 4.23	76 ± 21.07

Abbreviation: CFU: Colony-forming unit.

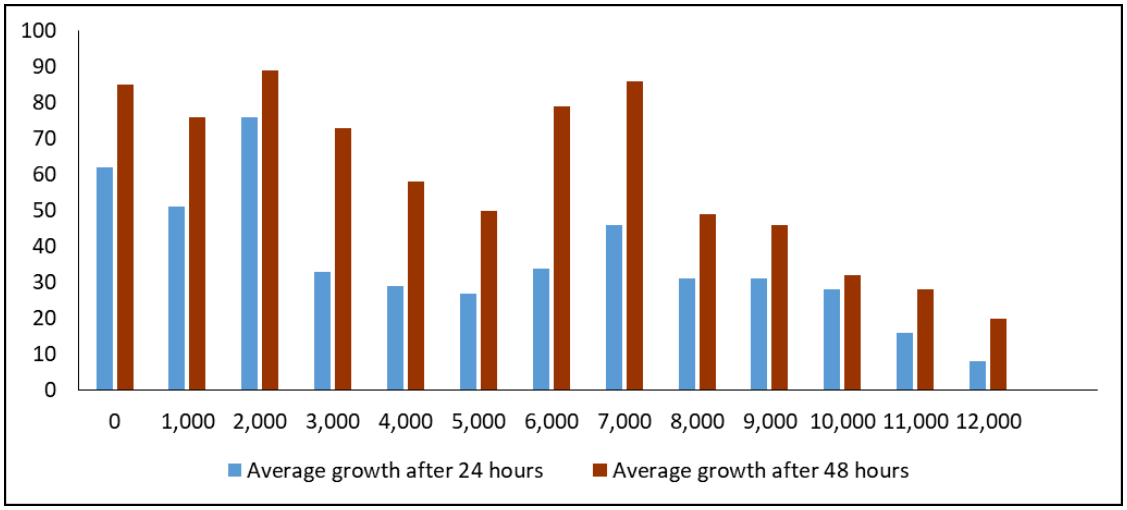

Figure 2. Average colony growth of *Rhizobium* sp. after 24 and 48 hours of exposure to Dizam 50% SC

agar media rather than direct cell death. Because viability assays (e.g., live/dead staining, measurement of cellular membrane integrity) were not performed, it is not possible to directly correlate reductions in colony numbers with cell death.

The one-way ANOVA results for average growth after 24 and 48 hours of exposure are presented in Table 3.

3.3. Percentage of mortality of *Rhizobium sp.* colonies

Table 2 presents a decreasing trend in percentage growth of bacteria when treated with increasing concentrations of Dizam 50% SC, indicating fewer colonies formed per area compared to the untreated control sample. In this study, the decrease in the number of colonies was considered to be an inhibition of growth as opposed to a killing effect on the bacteria. Since this experiment evaluated bacterial colony development, it could not evaluate the viability of individual bacterial cells through viability tests. As shown in Figure 3, there was a general increase in colony inhibition with increasing concentrations of Dizam 50% SC, with a few exceptions at 2,000, 6,000, and 7,000 mg/L, where colony inhibition values were lower than those found at all other concentrations.

As the colonies developed less rapidly, there was a greater percentage of growth inhibition relative to the control sample. These reductions in percentage growth may reflect the inhibitory effects of the fungicide on bacterial growth and reproduction. Several studies have demonstrated that certain chemicals can disrupt signaling pathways among *Rhizobium* sp. bacterial cells, resulting in decreased growth rates and numbers.[15] Additionally, Zilli *et al.*[16] demonstrated that the toxicity of fungicides toward *Rhizobium* sp. is often associated with disruption of normal cellular function, while Hamid and Nadarajah[17] and Wekesa *et al.*[18] suggest that toxicants may disrupt structural and functional integrity within bacterial cells. However, since this study did not evaluate biochemical signaling, electron transport, polysaccharide synthesis, membrane permeability, or viable bacterial cells, these potential mechanisms for the inhibitory effects noted here are based solely on interpretations made from previous research and should not be considered to be proven by this study.

Table 3. One-way ANOVA summary for average growth after 24 and 48 hours of Dizam 50% SC exposure

Response variable	Degrees of freedom (between, within)	*F*	*p*	*F* critical	Interpretation
Average growth after 24 h	4, 60	30.482	7.45×10^{-14}	2.525	Significant difference among treatments
Average growth after 48 h	4, 60	30.246	8.69×10^{-14}	2.525	Significant difference among treatments

There appears to be an anomalous growth response at 2,000, 6,000, and 7,000 mg/L, as presented in Table 2 and Figure 3. There are several reasons why this might occur. Bacterial cultures may exhibit a short-term tolerance or a physiologic adaptation to fungicide exposure, or different *Rhizobium* sp. strains used in this study may have had varying levels of sensitivity to each of the three concentrations tested. Previous studies have addressed mechanisms of resistance, including gene-mediated resistance,[19] emergence of resistant strains,[20] and mutations leading to survival during periods of stress.[21] Schmeisser *et al.*[22] suggested that environmental factors within soil may either make rhizobia more susceptible to fungicides or enhance their ability to survive when exposed to fungicides. However, since this study did not include molecular, genetic, or biochemical assessments for resistance to fungicides, the observed anomalous growth should be viewed with caution and should not be construed as evidence of acquired or permanent resistance. Instead, it may represent tolerance or variation in responses among the various bacterial colonies studied. The corresponding 48-hour growth-inhibition trend is shown in Figure 3, while the ANOVA summary is presented in Table 4, and the correlation matrix is presented in Table 5.

3.4. The exceptional growth of *Rhizobium sp.* colonies

Figures 4 and 5 show that there was a positive growth in some of the *Rhizobium* sp. bacterial colonies that were grown on yeast extract mannitol agar treated with various concentrations of Dizam 50% SC fungicide. In particular, when comparing the relative growth of the cultures to the untreated control culture, it was evident that higher concentrations of the fungicide produced unexpectedly higher relative growth. At 2,000, 6,000, and 7,000 mg/L, the percentages of growth were 105%, 93%, and 101%, respectively.

Based on the findings, it can be inferred that the *Rhizobium* sp. responded differently to the Dizam 50% SC fungicide across all concentrations. Although there was an unusual amount of growth at the three concentrations listed above, caution is required when interpreting these results due to the lack of molecular, genomic, transcriptomic, and biochemical methods used in this study to support claims of resistance mechanisms.

Several previous studies have proposed that certain chemicals applied at specific levels could serve as stressors affecting the way a bacterium responds to its environment

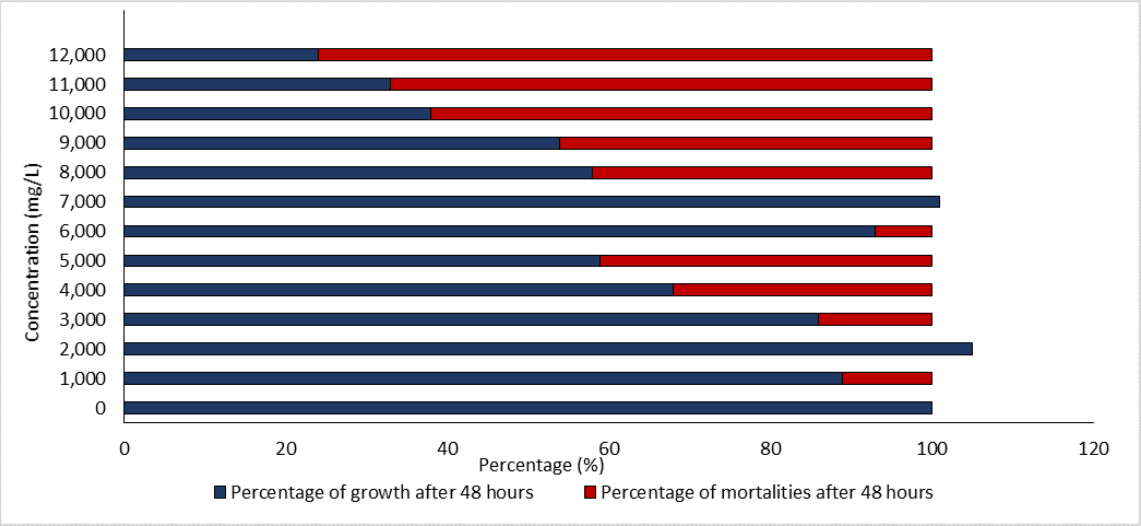

Figure 3. Percentage growth and inhibition of *Rhizobium* sp. after 48 hours of exposure to Dizam 50% SC

Table 4. One-way ANOVA summary for average colony-growth inhibition after 48 hours of Dizam 50% SC exposure

Response variable	Degrees of freedom (between, within)	*F*	*p*	*F* critical	Interpretation
Average colony-growth inhibition after 48 h	4, 60	30.579	6.99×10^{-14}	2.525	Significant difference among treatments

Table 5. Simplified correlation summary among key treatment variables

Variable pair	Correlation coefficient (*r*)	Direction of relationship	Interpretation
Fungicide concentration vs. growth after 24 h	−0.786	Negative	Higher concentration was associated with lower colony growth
Fungicide concentration vs. growth after 48 h	−0.831	Negative	Higher concentration was associated with lower colony growth
Fungicide concentration vs. CFU/mL after 48 h	−0.831	Negative	Higher concentration was associated with lower CFU/mL
Fungicide concentration vs. percentage growth after 48 h	−0.830	Negative	Higher concentration was associated with lower percentage growth
Fungicide concentration vs. colony-growth inhibition after 48 h	0.846	Positive	Higher concentration was associated with greater colony-growth inhibition
Fungicide concentration vs. percentage inhibition after 48 h	0.833	Positive	Higher concentration was associated with greater percentage inhibition

Abbreviation: CFU: Colony-forming unit.

or how toxic a fungicide is toward *Rhizobium* sp.[23] Other studies have found that fungicides tend to selectively impact some strains of *Rhizobium* sp. over others,[8] and that the effect of fungicides on *Rhizobium* sp. can depend on many factors including crop type, the strain of bacteria studied, and the environmental source from which the *Rhizobium* sp. was isolated.[24]

Previous studies have discussed the potential for *Rhizobium* sp. to undergo stress-related modifications, mutations, or adaptations to their physiological state when exposed to high levels of chemicals,[25] whereas other researchers have linked the ability of bacteria to survive in environments rich in toxic chemicals with resistance-related traits or genes responsible for antibiotic resistance.[26,27] Similar to these studies, Hamuda[28] stated that environmental pressures imposed by high levels of fungicides may allow for only those *Rhizobium* sp. strains most tolerant to fungicides to continue growing.

Although the growth rates at 2,000, 6,000, and 7,000 mg/L of Dizam 50% SC fungicide were significantly greater than those of the control group, there is insufficient molecular evidence to describe this phenomenon as genetic resistance, as a result of mutation, or as a plasmid-based adaptation. The findings, therefore, cannot be classified as such until further molecular characterization and/or marker-assisted resistance-testing methods are employed.

3.5. Percentage of growth of *Rhizobium* sp. colonies after 24 and 48 hours

Table 2 and Figures 5 and 6 present the growth response of *Rhizobium* sp. colonies following exposure to increasing concentrations of Dizam 50% SC for 24 and 48 h. Overall, the results demonstrate a non-linear, concentration- and time-dependent growth response pattern across treatments. At 24 h, growth values varied across concentrations from 1,000 to 12,000 mg/L. At 48 h, a similar variation in growth values was observed across the same concentration range.

The presence of growth across different concentrations of the Dizam fungicide may be related to the fact that there are varying degrees of sensitivity among *Rhizobium* sp. bacterial strains to different fungicides, depending on the particular strain.[4] There have been several reports indicating that some fungicides can lead to increased numbers of *Rhizobium* sp. and/or increased numbers of root nodules,[29] while other studies have found that some fungicides decrease the number of root nodules but do not affect the total number of *Rhizobium* sp. bacteria.[24]

Several studies refer to the described changes as possible examples of evolutionary responses exhibited by *Rhizobium* sp. strains possessing plasmids that possess flexible pili, allowing for adaptation to hostile environments.[30] Other studies view these as temporary adaptations until the environmental condition causing stress is alleviated.[31]

In this study, since no plasmid analysis or gene expression studies were conducted, nor were resistance marker tests performed or complete genomic sequences generated, all potential mechanisms previously discussed in the literature can be offered only as possible interpretations and not as validated mechanisms based upon this experiment.

The statistical comparisons (Table 5) indicated statistically significant differences among treatment groups and a negative correlation coefficient between fungicide concentration and colony growth. Correlation

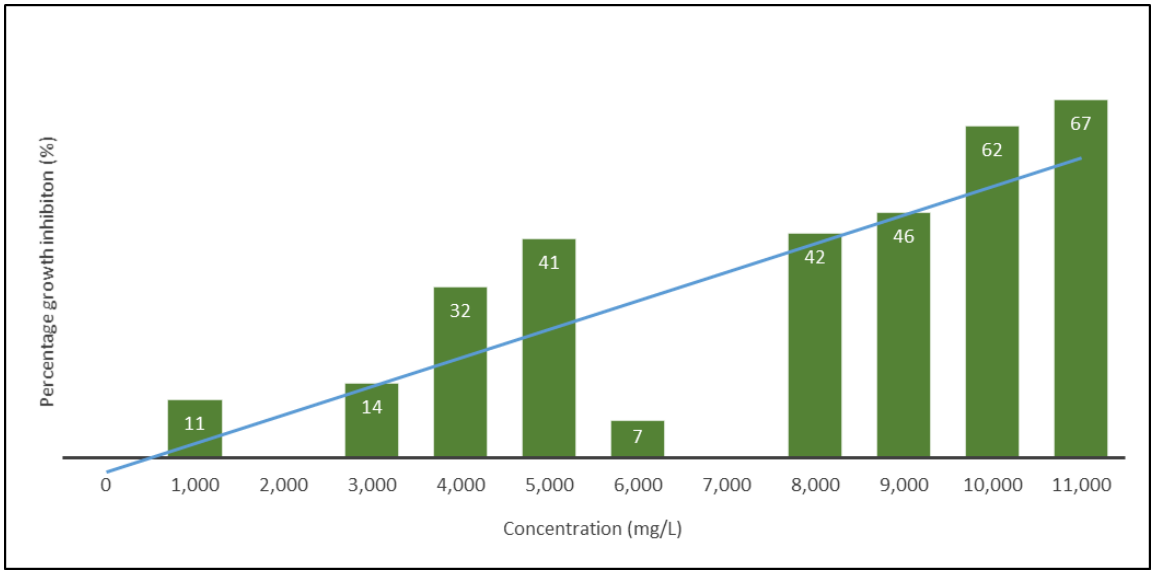

Figure 4. Percentage growth inhibition of *Rhizobium* sp. after 48 hours of exposure to Dizam 50% SC

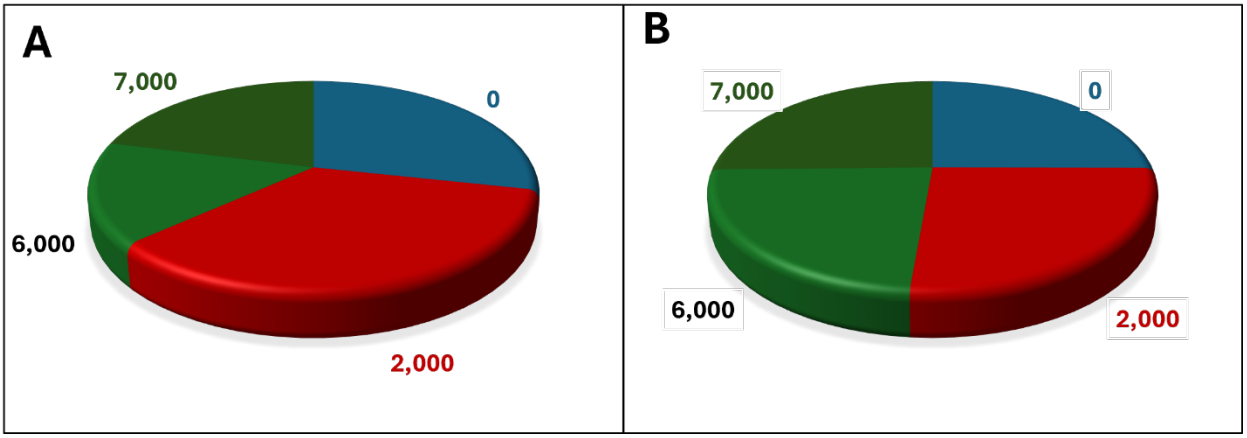

Figure 5. Percentage growth response of *Rhizobium* sp. after (A) 24 h and (B) 48 h exposure to different concentrations of Dizam 50% SC

relationships were interpreted conservatively because of the mathematical inter-relationship among some variables, such as percent growth and percent inhibitory effect. Representative agar observations are shown in Figure 7, and the 48-hour percentage-growth response is presented in Figure 8.

3.6. Effects of Dizam 50% SC on colony-forming unit/mL of *Rhizobium* sp.

In contrast to Table 1, Table 2 and Figure 9 show the effects of the Dizam 50% SC fungicide on CFU/mL of *Rhizobium* sp. cultured on yeast extract mannitol agar at different concentrations. In general, there was an increase in the inhibitory effect of the fungicide against *Rhizobium* sp. as the concentration increased. This is clearly evident in the decrease in CFU values from 8.5×10^4 CFU/mL in the untreated control group to 2.0×10^4 CFU/mL at the highest concentration.

The findings suggest that Dizam 50% SC inhibited the colony formation of *Rhizobium* sp. across all concentrations tested, but this is likely related to its capacity to inhibit or reduce the metabolic activity required for colony formation rather than directly causing bacterial cell death. Although the lower numbers would suggest less ability to form colonies and therefore reduced potential for population increases during colonization processes in plants, this interpretation should be made with caution, as viability was not directly assessed.

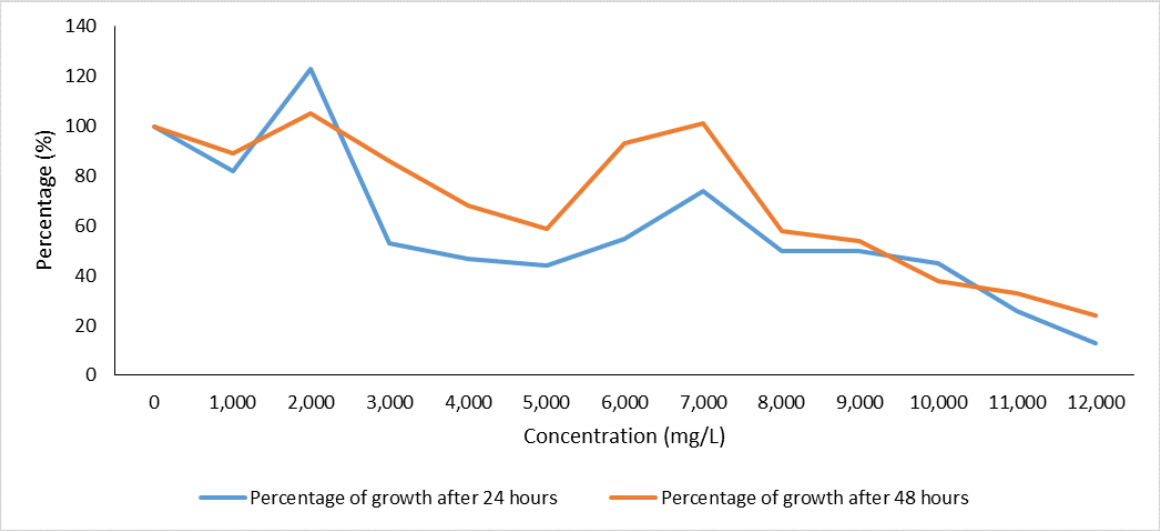

Figure 6. Percentage growth of *Rhizobium* sp. compared with the control after 24 and 48 hours of exposure to Dizam 50% SC

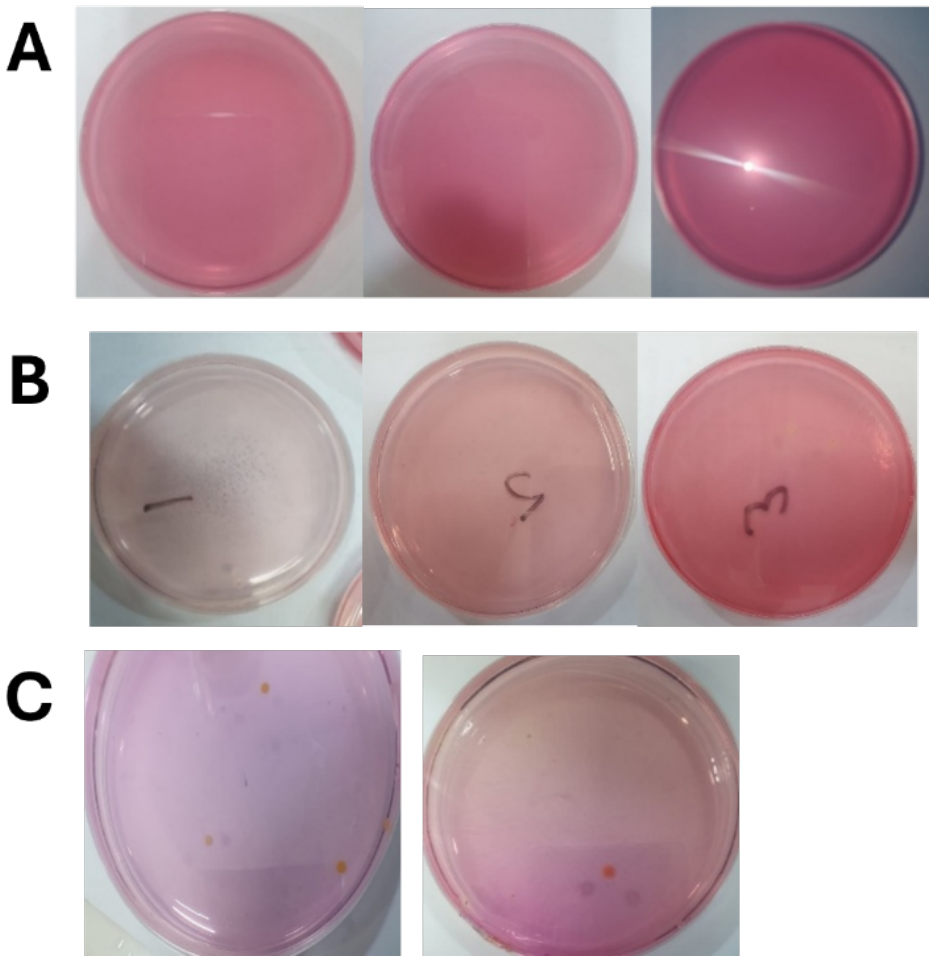

Figure 7. Yeast extract mannitol agar treated with Dizam 50% SC fungicide. (A) Control plates. (B) Plates showing reduced colony growth. (C) Plates showing visible colonies after exposure.

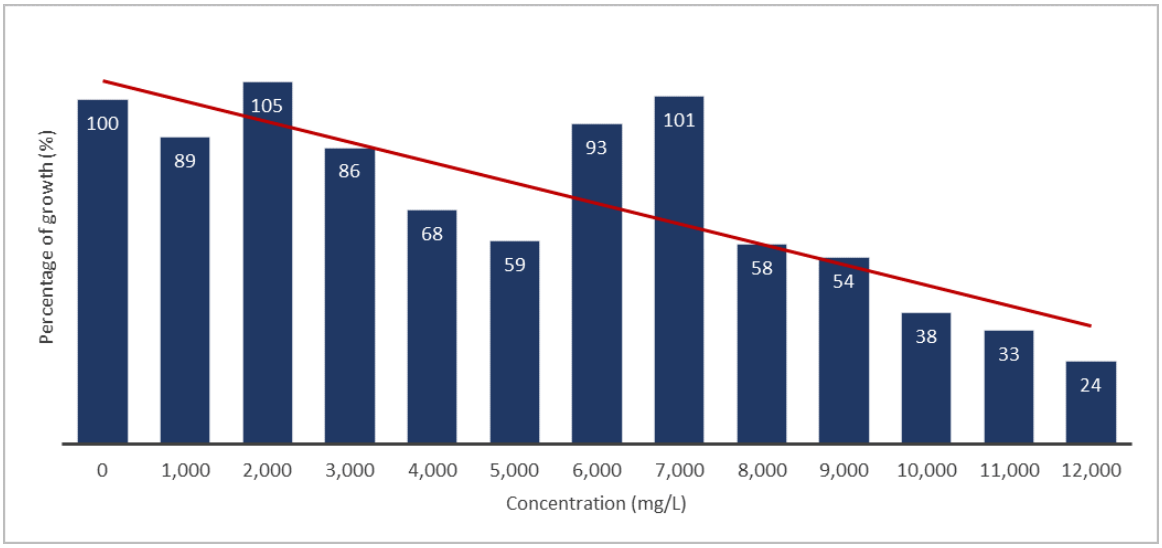

Figure 8. Percentage growth of *Rhizobium* sp. after 48 hours of exposure to Dizam 50% SC

Variations were observed within each treatment group, such as the CFU values obtained after treatments at 2,000, 6,000, and 7,000 mg/L, which were relatively greater than their adjacent treatment groups. Such variations could be due to differences among strains tested, temporary tolerance mechanisms in certain populations, and physiological responses varying among individuals. Regardless of what caused these variations, none of them can be concluded as conferring resistance since no tests for molecular, biochemical, or genetic resistance were performed.

3.7. Effects of Dizam 50% SC on LC_{50} of *Rhizobium* sp.

Figure 10 presents the LC_{50} for Dizam 50% SC fungicide after 48 h of exposure to *Rhizobium* sp. across different concentrations. The LC_{50} value was 9,359.54 mg/L, representing the concentration of fungicide needed to reduce the colony-forming capability of *Rhizobium* sp. by approximately 50% during laboratory experiments. While the LC_{50} is useful in identifying how much fungicide is required to reduce colony formation by 50% after 48 h of exposure, its interpretation should be made with caution, as this study measured reductions in visible colony formation rather than direct bacterial cell death. Therefore, it best represents a 50% reduction in growth, unless viability is proven through additional viability testing.

There are several reports of fungicides becoming either more toxic or having increased activity when they interact directly with bacteria isolated from root nodules.[16] Several studies have also reported differences in toxicity responses between laboratory settings and natural soils due to environmental influences on fungicide persistence and degradation, as well as microbial responses.[32-34] Thus, while the LC_{50} is a useful indicator of potential toxicity under controlled laboratory conditions, it should not be assumed from these values alone that Dizam 50% SC is environmentally safe. LC_{50} values are most commonly used as indicators of acute lethal effects of substances on test species under defined laboratory conditions.

However, ecological safety cannot be inferred solely from LC_{50} values, as numerous variables influence outcomes under field conditions, including soil type, organic matter content, microbial biodiversity, frequency of fungicide exposure, degradation rates, and chemical interactions among agrochemicals.

Accordingly, variations in experimental conditions such as the source of *Rhizobium* sp., exposure duration, culturing methods, and environmental parameters are likely to alter LC_{50} values.

3.8. Growth response of *Rhizobium* sp. at 1,000 mg/L

To assess whether colonies exposed to Dizam 50% SC during the initial treatment continued to grow upon re-exposure, *Rhizobium* sp. isolates obtained from cultures grown at 1,000 mg/L were plated on yeast agar and subsequently transferred into broth media containing different concentrations of Dizam 50% SC (1,000–12,000 mg/L).

The findings show that the colonies were more appropriately described as "surviving" rather than "resistant," since no biochemical, molecular, or genetic tests were performed to confirm true resistance. The corresponding growth response is shown in Figure 11, and the summary of the ANOVA analysis of the broth data is summarized in Table 6.

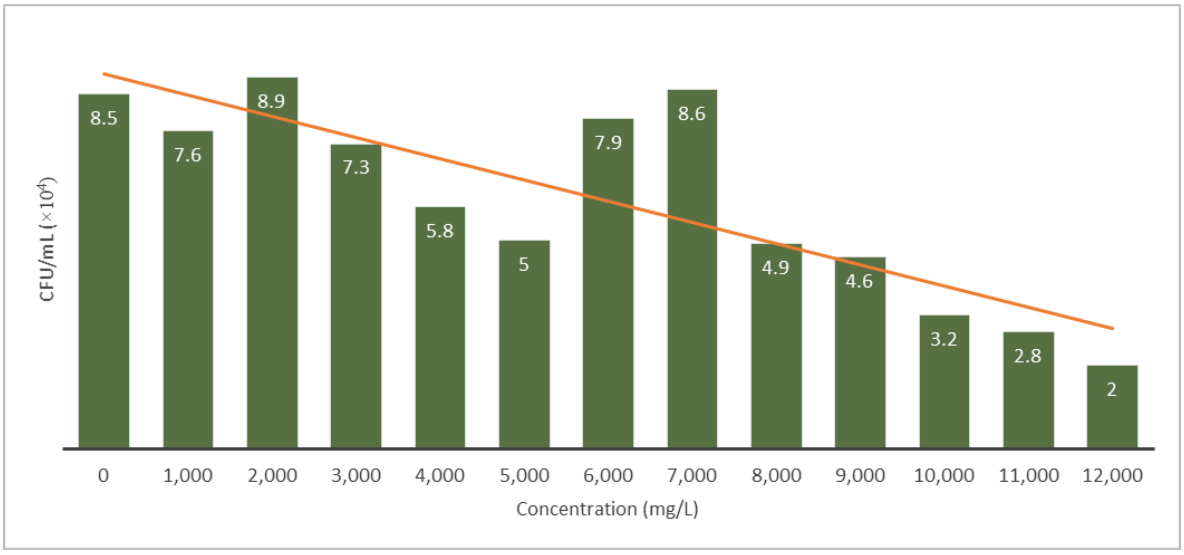

Figure 9. CFU/mL (×10⁴) of *Rhizobium* sp. after 48 hours of exposure to Dizam 50% SC. Abbreviation: CFU: Colony-forming unit

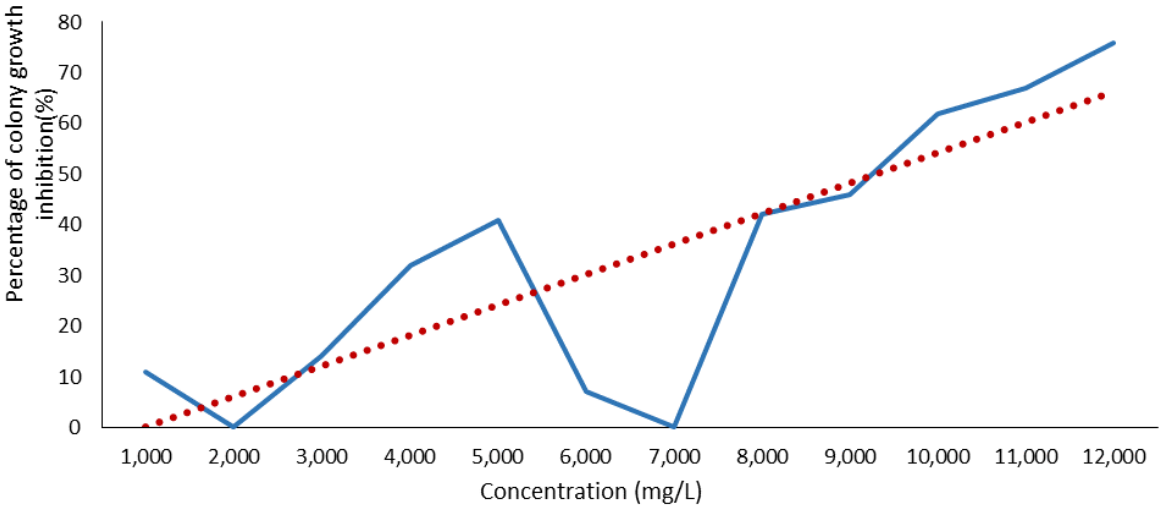

Figure 10. LC_{50} of *Rhizobium* sp. exposed to Dizam 50% SC exposure after 48 hours

The average MPN/mL and CFU/mL values of *Rhizobium* sp. recovered from the 1,000 mg/L treatment group after exposure to broth containing different concentrations of Dizam 50% are presented in Table 7. A progressive decrease in percentage growth was observed with increasing fungicide concentration. A concentration-dependent reduction in percentage growth was observed, decreasing from 45.02% at 1,000 mg/L to 2.52% at 12,000 mg/L.

These findings suggest that the *Rhizobium* sp. colonies isolated from the 1,000 mg/L treatment group exhibited a limited capacity for regrowth upon re-exposure to high fungicide concentrations. Thus, the results did not support the development of resistance; instead, they suggest either a reduced growth capacity or transient tolerance in response to fungicidal stress. Growth was observed as turbidity in the culture medium or, in some cases, as color changes in certain cultures. Turbidity and/or color change should be considered indicators of potential bacterial growth and not equivalent to CFU values unless confirmed by direct plate counts or an appropriate MPN estimation method.

Several studies, such as Unay and Perret,[35] have also described this form of tolerance as a transient condition that may diminish following multiple rounds of cell division. This interpretation is consistent with the findings of the present study, as evidenced by the progressive decrease in growth percentage with increasing fungicide concentrations. The results demonstrated significant

variability among treatment groups, as well as a strong negative correlation between fungicide concentration and growth percentage (Tables 6 and 8).

3.9. Growth response of *Rhizobium* sp. at 12,000 mg/L

The growth response of the 12,000 mg/L isolate is shown in Figure 12. Table 9 presents the average MPN/mL and CFU/mL values for *Rhizobium* sp. isolated from this treatment following initial growth on yeast extract mannitol agar and subsequently re-exposed in broth media containing different concentrations of Dizam 50% SC (1,000–12,000 mg/L). These colonies are described as "surviving" rather than "resistant," as no molecular, genetic, or biochemical confirmation of resistance was performed. Representative broth-tube observations are shown in Figure 13.

While *Rhizobium* sp. colonies isolated from 12,000 mg/L could survive repeated exposure to the fungicide, their growth percentage decreased markedly with increasing fungicide concentration. The percentage growth values ranged from 37.20% to 0.60% across concentrations of 1,000 to 12,000 mg/L, indicating a progressive reduction in growth capacity. The findings suggest that the ability of the surviving colonies to regrow after subculture decreased significantly with successive exposure and increasing fungicide concentration.

While evidence of colony survival at 12,000 mg/L initially suggests a degree of tolerance to acute levels of Dizam 50% SC, the observed decline in growth during subsequent subculture indicates that no stable resistance to Dizam 50% SC was demonstrated under the conditions of this study. Rather, the findings likely reflect reduced

Table 6. One-way ANOVA summary for MPN broth data obtained from 1,000 and 12,000 mg/L treatments

Isolate source	Degrees of freedom (between, within)	F	*p*	*F* critical	Interpretation
Obtained from 1,000 mg/L treatment	6, 84	30.678	3.10×10^{-19}	2.209	Significant difference among concentration groups
Obtained from 12,000 mg/L treatment	6, 84	30.678	3.11×10^{-19}	2.209	Significant difference among concentration groups

Table 7. Number of positive MPN tubes for *Rhizobium* sp. isolated from the 1,000 mg/L treatment after 48 h exposure to Dizam 50% SC

Concentration (mg/L)	Positive tube pattern (three replicate MPN codes)	MPN (middle inoculum set)	CFU/mL (MPN equivalent)	Growth (%)
Control	331, 332, and 331	6.73	6.73 ± 1.47	100
1,000	331, 323, and 313	3.03	3.03 ± 0.84	45.02
2,000	330, 322, and 321	2	2 ± 0.55	29.71
3,000	313, 321, and 313	1.57	1.57 ± 0.43	23.32
4,000	312, 313, and 312	1.33	1.33 ± 0.36	19.76
5,000	303, 311, and 320	0.88	0.88 ± 0.24	13.07
6,000	302, 310, and 311	0.61	0.61 ± 0.16	9.06
7,000	223, 310, and 233	0.46	0.46 ± 0.12	6.83
8,000	232, 233, and 310	0.47	0.47 ± 0.13	6.98
9,000	301, 223, and 222	0.39	0.39 ± 0.1	5.79
10,000	222, 230, and 301	0.34	0.34 ± 0.09	5.05
11,000	212, 131, and 123	0.24	0.24 ± 0.06	3.56
12,000	112, 123, and 111	0.17	0.17 ± 0.04	2.52

Abbreviations: CFU: Colony-forming unit; MPN: Most probable number.

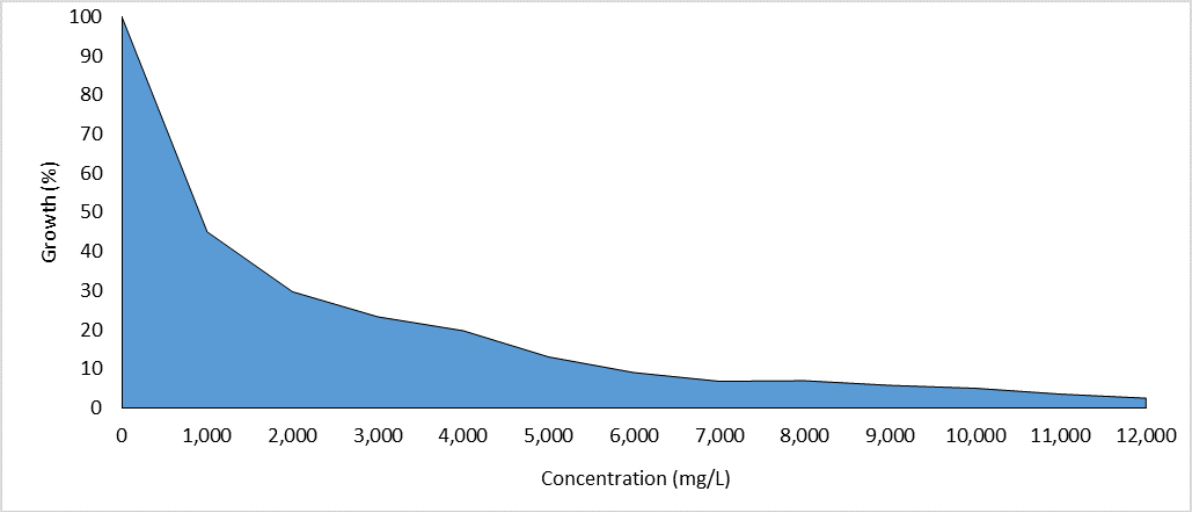

Figure 11. Percentage growth of isolated *Rhizobium* sp. colonies from the 1,000 mg/L treatment after 48 hours of exposure to Dizam 50% SC

growth capacity, transient tolerance, or physiological responses to Dizam 50% SC exposure. Decreased membrane permeability has previously been proposed as a potential resistance mechanism in some *Rhizobium* sp. Strains,[36] and environmental adaptation has been suggested as possible tolerance response under adverse physiological conditions.[37] However, these mechanisms remain speculative in the present study, as no molecular, biochemical, or physiological assays were conducted to confirm them.

Additionally, several studies have reported that certain bacterial species exhibit partial detoxification or reduced fungicide toxicity through gene-mediated pathways or partial degradation under controlled laboratory or field conditions.[38,39] Environmental factors may also influence both the growth of *Rhizobium* sp. colonies and fungicide toxicity under natural soil conditions.[6,40] However, potential fungicide degradation, gene-mediated pathways, environmental interactions, and molecular resistance markers were not investigated in this study. Therefore, any inference regarding inheritable resistance genes or transfer of resistance traits to progeny cells cannot be supported and should be avoided.

Based on the decreasing growth percentages at higher concentrations, it is likely that increased growth inhibition or loss of culturable cells among surviving colonies occurred due to fungicide-induced stress. Additional hypotheses proposed in previous studies include secondary mutations,[41] loss of gene expression,[42] and deactivation of resistance-associated genes.[43] However, these mechanisms cannot be evaluated in the present study due to the absence of gene expression analysis, plasmid profiling, mutation analysis, or whole-genome sequencing.

The comparative inhibition profile is shown in Figure 14, with correlation and two-way ANOVA results presented in Table 8 and 10, respectively.

3.10. Growth in a mixture of nutrient broth, yeast extract, and mannitol broth

Significant differences were observed between the surviving *Rhizobium* sp. isolates obtained from 1,000 mg/L and 12,000 mg/L treatments when re-cultured in a mixture of nutrient broth and yeast extract mannitol broth and challenged with different concentrations of Dizam 50% SC fungicide. For both groups of isolates, the percentage growth decreased significantly with increasing fungicide concentration.

Since all isolates had been previously exposed to Dizam 50% SC at their respective treatment concentrations, the findings suggest that repeated exposure reduced their growth capacity.

Isolates obtained from the 1,000 mg/L treatment exhibited higher growth percentages than those obtained from the 12,000 mg/L treatment at most applied fungicide concentrations tested. These findings suggest that previous exposure to higher fungicide concentrations reduced the regrowth capacity of these isolates, resulting in greater growth inhibition upon subsequent exposure. The results do not support the development of resistant strains but instead suggest transient tolerance, physiological stress responses, or reduced growth capacity following repeated fungicide exposure.

Previous studies have suggested that fungicide concentration is an important factor influencing survival responses in *Rhizobium* sp. under stressful conditions.[44]

Table 8. Simplified correlation summary of isolated grown under 1,000 and 12,000 mg/L Dizam 50% SC treatment

Isolate source	Variable pair	Correlation coefficient (*r*)	Direction	Interpretation
1,000 mg/L isolate	Concentration vs. average MPN growth after 48 h	−0.778	Negative	Growth decreased as concentration increased
	Concentration vs. percentage growth after 48 h	−0.778	Negative	Percentage growth decreased as concentration increased
	Concentration vs. percentage inhibition after 48 h	0.778	Positive	Percentage inhibition increased as concentration increased
12,000 mg/L isolate	Concentration vs. average MPN growth after 48 h	−0.755	Negative	Growth decreased as concentration increased
	Concentration vs. percentage growth after 48 h	−0.755	Negative	Percentage growth decreased as concentration increased
	Concentration vs. percentage inhibition after 48 h	0.755	Positive	Percentage inhibition increased as concentration increased

Abbreviation: MPN: Most probable number.

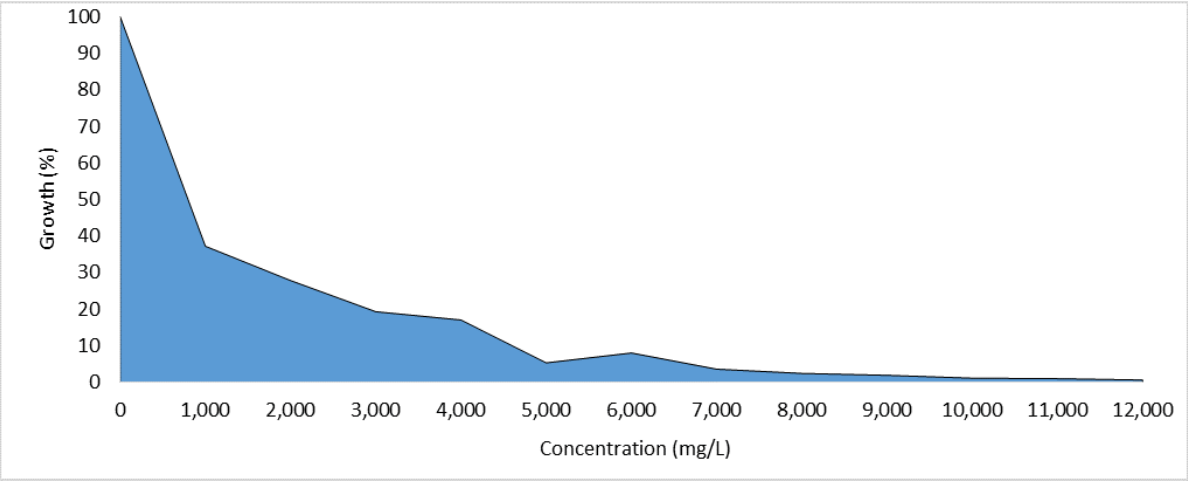

Figure 12. Percentage growth of isolated *Rhizobium* sp. colonies from the 12,000 mg/L treatment after 48 hours of exposure to Dizam 50% SC

Other studies have shown that soil type and environmental conditions can affect the sensitivity or tolerance of root nodule bacteria to toxic substances.[28] In addition, several studies have indicated that biotic and abiotic components can influence the survival and competitive ability of *Rhizobium* sp. under natural soil conditions.[44,45]

Although the present study was conducted under controlled laboratory broth-culture conditions, soil interactions, resistance gene expression, fungicide degradation, or long-term adaptations were not investigated. Therefore, these factors should be regarded as possible explanations for the observed findings rather than confirmed mechanisms. The results suggest that the survival and re-growth of *Rhizobium* sp. are likely influenced by multiple factors, including fungicide concentration, previous fungicide exposure, the physiological status of the bacteria, and culture conditions.

Finally, it is important to recognize that CFU and MPN are based on distinct methodologies. While CFU measures cultivable colonies on solid agar, MPN provides a statistical

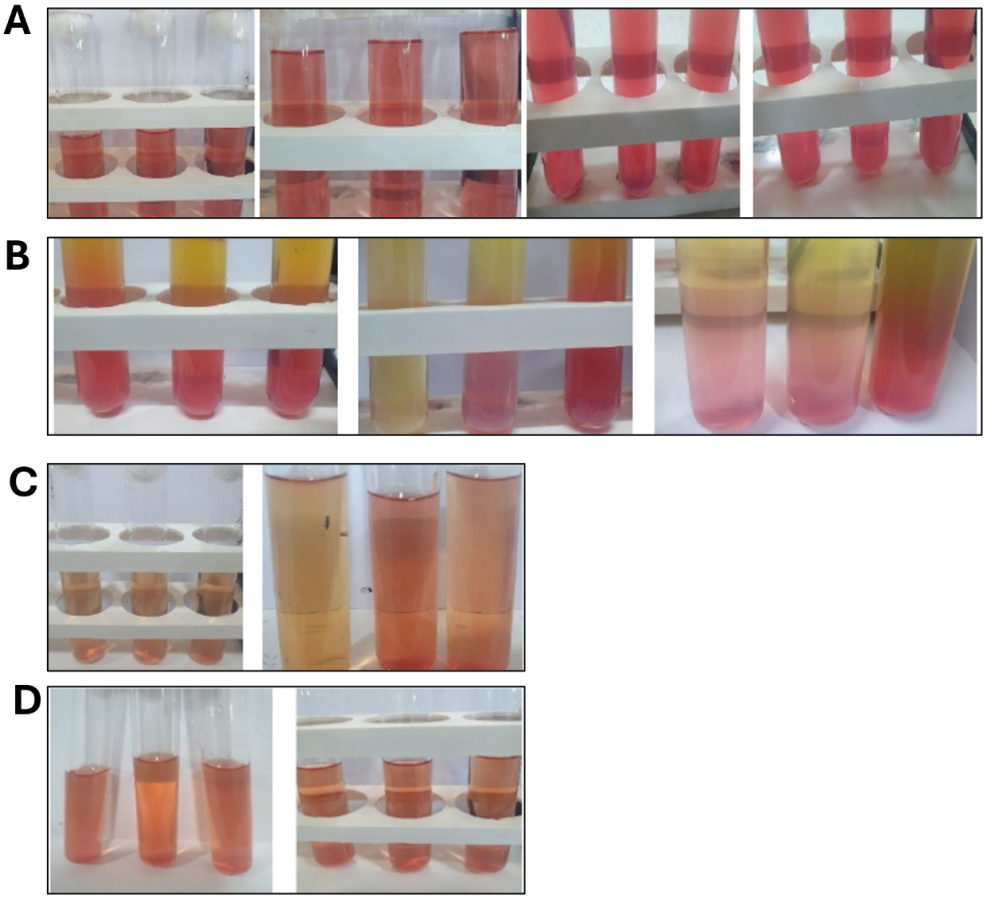

Figure 13. Broth culture observations after 48 h. (A) Control tubes. (B) Positive turbidity. (C, D) Color changes in mixed broth for re-cultured isolates from (C) 1,000 mg/L and (D) 12,000 mg/L treatments.

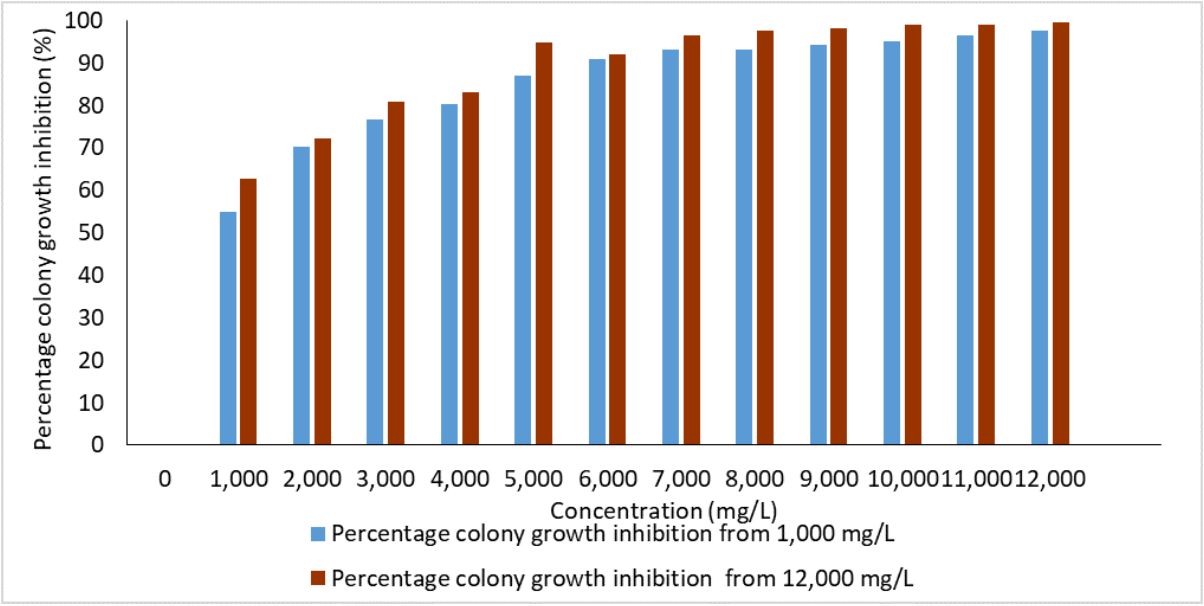

Figure 14. Percentage growth inhibition of isolated R*hizobium* sp. after 48 hours of Dizam 50% SC treatment

Table 9. Positive tubes containing *Rhizobium* sp. from the 12,000 mg/L treatment after 48 hours of exposure to Dizam 50% SC

Concentration (mg/L)	Positive tube pattern (three replicate MPN codes)	MPN (middle inoculum set)	CFU/mL (MPN equivalent)	Percentage of growth (%)
Control	332, 331, and 332	8.87	8.87 ± 2.03	99.96
1,000	331, 330, and 323	3.3	3.3 ± 0.91	37.20
2,000	323, 330, and 322	2.47	2.47 ± 0.68	27.81
3,000	322, 320, and 322	1.71	1.71 ± 0.47	19.28
4,000	321, 322, and 320	1.51	1.51 ± 0.41	17.02
5,000	233, 232, and 232	0.47	0.47 ± 0.13	5.30
6,000	310, 311, and 303	0.71	0.71 ± 0.19	8.00
7,000	213, 212, and 213	0.32	0.32 ± 0.08	3.57
8,000	132, 131, and 131	0.21	0.21 ± 0.05	2.41
9,000	121, 122, and 121	0.17	0.17 ± 0.04	1.88
10,000	102, 102, and 101	0.1	0.1 ± 0.02	1.10
11,000	30, 21, and 101	0.09	0.09 ± 0.02	0.97
12,000	20, 100, and 11	0.05	0.05 ±0.01	0.60

Abbreviations: CFU: Colony-forming unit; MPN: Most probable number.

Table 10. Two-way ANOVA summary comparing the growth responses of isolates under 1,000 and 12,000 mg/L Dizam 50% SC treatment

Source of variation	Degrees of freedom	*F*	*p*	*F* critical	Interpretation
Concentration (1,000 vs. 12,000 mg/L)	11	0.9999	0.4618	2.014	No significant effect of concentration on growth response
Growth response (across groups)	4	38.407	8.04×10^{-14}	2.584	Significant differences among growth response groups
Residual (error)	44	–	–	–	Residual variation

estimate of bacterial growth in liquid broth. Consequently, MPN values should not be interpreted as equivalent to CFU counts unless validated experimentally. Furthermore, the criteria used to identify positive broth tubes for MPN calculations should be clearly defined in the table footnote, and the probability table or statistical model used to estimate MPN values should be appropriately referenced.

3.11. LC_{50} of re-cultured *Rhizobium* sp. colonies

The LC_{50} values of re-cultured *Rhizobium* sp. colonies, shown in Figures 15 and 16, were 75.19 mg/L and 57.36 mg/L for colonies initially isolated from the 1,000 mg/L and 12,000 mg/L treatments, respectively. As noted earlier, these colonies are more appropriately described as re-cultured surviving colonies rather than resistant colonies, because resistance was not confirmed through molecular or biochemical analyses. The results indicate that the re-cultured colonies remained highly sensitive to fungicide exposure.

The percentage growth inhibition increased with increasing fungicide concentration, as shown in Figure 14. These findings suggest that the sensitivity of *Rhizobium* sp. to Dizam 50% SC increased following re-culture, as reflected by the marked decline in growth percentage at higher fungicide concentrations.

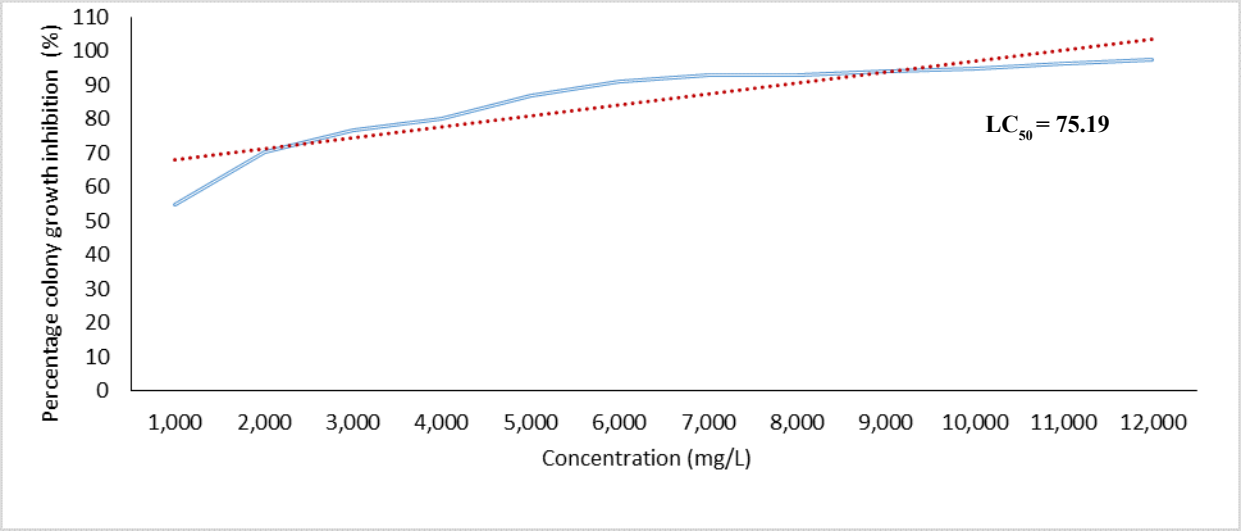

Figure 15. LC_{50} of *Rhizobium* sp. isolated from the 1,000 mg/L treatment after 48 hours of Dizam 50% SC exposure

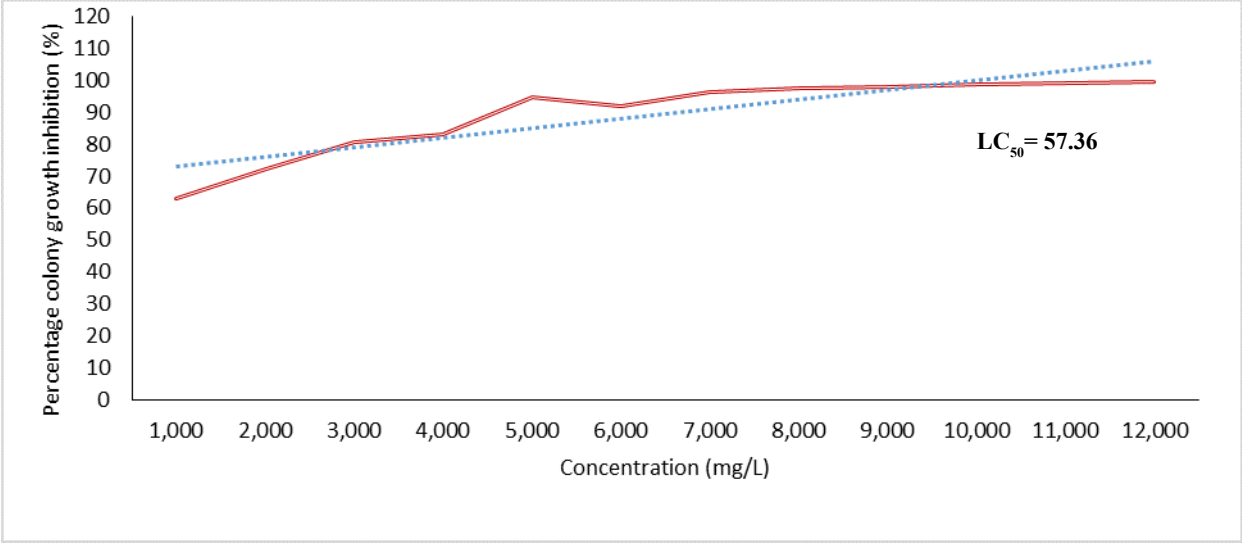

Figure 16. LC_{50} of *Rhizobium* sp. isolated from the 12,000 mg/L treatment after 48 hours of Dizam 50% SC exposure

A comparison of the LC_{50} values showed that colonies isolated from the 12,000 mg/L had a lower LC_{50} than those isolated from the 1,000 mg/L treatment, suggesting that prior exposure to the higher fungicide concentration was associated with greater sensitivity during subsequent re-culture. Accordingly, the findings do not support the development of stable resistance but instead suggest increased susceptibility or reduced growth capacity following repeated exposure to Dizam 50% SC.

Several studies have proposed alternative hypotheses to explain variations in bacterial susceptibility, including symbiotic genes, cellular osmolarity, and alterations in bacterial physiology.[46,47] Others have reported the potential reversibility of mutations or modification of resistance-related traits under stressful environmental conditions.[48,49] However, the present study did not assess symbiotic genes, osmolarity, mutational changes, plasmid profiles, or gene expression; therefore, these mechanisms are discussed only as literature-based possibilities rather than experimentally verified outcomes.

Lower LC_{50} values may indicate a greater toxic effect of Dizam 50% SC on the re-cultured colonies. LC_{50} values are commonly interpreted as an inverse measure of sensitivity to toxic agents,[50] with lower values indicating higher

sensitivity. Accordingly, the re-cultured colonies did not maintain a tolerant response throughout the re-culture process. Overall, the findings suggest increased sensitivity rather than the development of resistance.

To enhance the credibility of toxicity assessment, the methodology used for LC_{50} estimation should be clearly reported. Ideally, LC_{50} values are derived using probit analysis or dose–response regression, together with confidence limits, regression equations, and goodness-of-fit statistics. In the absence of these statistical parameters, LC_{50} values should be interpreted as preliminary estimates of toxicity rather than definitive indicators of resistance or environmental safety.

Collectively, the LC_{50} results obtained for re-cultured *Rhizobium* sp. colonies indicate that prior exposure to Dizam 50% SC did not result in the development of confirmed resistant strains. Instead, the lower LC_{50} values observed in previously exposed colonies suggest increased sensitivity and reduced growth capacity under continued fungicide exposure.

4. Conclusions

Rhizobium sp. is a bacterium that resides in the nodules of legume roots (*Vigna* sp.) in a symbiotic relationship with the host plant. In this study, the impact of Dizam 50% SC on *Rhizobium* sp. was evaluated using different fungicide concentrations. The experimental results showed that increasing concentrations of Dizam 50% SC were associated with a reduction in bacterial density per unit area as well as a decrease in viable cell counts (CFU) after 24 and 48 hours of exposure.

In addition, the original isolate exhibited an LC_{50} of 9,359.54 mg/L. Re-cultured colonies obtained from the 1,000 mg/L and 12,000 mg/L treatments showed markedly lower LC_{50} values (75.19 mg/L and 57.36 mg/L, respectively). The findings indicate that previously exposed colonies were more sensitive to the fungicide compared with the non-exposed isolate. This evidence does not support the development of stable resistant mutants but instead suggests increased susceptibility and reduced growth capacity following prior exposure to Dizam 50% SC. Therefore, this study suggests that Dizam 50% SC may also negatively affect beneficial rhizobia, particularly at higher concentrations.

Recommended agricultural rates for Dizam 50% SC should be adhered to in order to minimize adverse effects on beneficial soil microorganisms. Future studies could incorporate field-relevant concentrations, along with molecular identification (i.e., 16S ribosomal RNA sequencing) and viability assays, to determine whether Dizam inhibits bacterial growth or induces cell death. LC_{50} values should be analyzed in relation to dose–response models, with appropriate confidence intervals reported. Claims regarding resistance should be supported by molecular evidence, including plasmid profiling, expression of resistance-associated genes, or genomic sequencing data. Long-term field studies are also recommended to provide insight into how repeated application affects soil microbial diversity, nitrogen fixation in legumes, and overall legume productivity.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: May Hameed Mohammad Al-Dehamee
Investigation: All authors
Methodology: Abdulwahhab Jasim Mahdi
Writing–original draft: May Hameed Mohammad Al-Dehamee
Writing–review & editing: Abdulwahhab Jasim Mahdi

Availability of data

Data are available from the corresponding author upon reasonable request.

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