Investigating Antibiotic Effects on *Trichoderma* sp. Growth and Virulence: A Basis for CRISPR-Cas9 Preparation

Hari Purnomo[1], *Ali* Wafa[1], *Himmatul* Hasanah[1], *Putri* Nawangsari[1], *Dita* Megasari[2], *April Hari* Wardhana[4], *Syaiful* Khoiri[4*]

[1]Universitas Jember, Jember, East Java, Indonesia, 68121.
[2]Universitas Pembangunan Nasional "Veteran" Jawa Timur, Surabaya, East Java, Indonesia, 60294.
[3]National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia, 16114.
[4]Universitas Trunojoyo Madura, Bangkalan, East Java, Indonesia, 69162

Abstract. *Trichoderma* sp. is an antagonistic fungus that is used as a biological control. Understanding how antibiotics affect *Trichoderma* sp. growth and virulence is critical for developing effective CRISPR-Cas9 gene editing strategies. However, implementing CRISPR-Cas9 technology in *Trichoderma* requires a comprehensive understanding of how external factors, such as antibiotic exposure, affect the fungus's growth and virulence over successive generations. Antibiotics long-term effects on fungal physiology remain unclear. This study addresses this gap by evaluating the impact of antibiotic treatment on *Trichoderma* sp., laying the groundwork for effective and precise genetic modifications using CRISPR-Cas9. Five types of antibiotics used for this test are Chloramphenicol, Gentamicin sulphate, Kanamycin Meiji, Penicillin Meiji, and Tetracycline HCI. The study found that several antibiotics accelerated the growth of *Trichoderma* sp., allowing the fungus to fill a petri dish in under 5 days. However, this rapid growth was delayed in subcultures over five generations. Although the color of the fungus did not vary much across treatments, subtle changes in density and brightness were detected in each generation using the "color grab" application, with these attributes diminishing over time. Spore density in the fourth generation, particularly under chloramphenicol and gentamicin treatments, differed from other antibiotics. Germination rates were initially high (≥70%) but decreased with each generation. The fungus maintained strong virulence against *Fusarium* sp., with inhibition rates ≥50%, and exhibited mycoparasitism, characterized by hyphal growth at the *Fusarium* sp. colony edges. This research contributes to understanding how antibiotic exposure affects the long-term growth, spore density, and virulence of *Trichoderma* sp., providing essential insights for optimizing its use in biocontrol and genetic engineering applications.

1 Introduction

The expanding field of sustainable agriculture has driven considerable interest in the use of biological control agents as an alternative to chemical pesticides [1–4]. Among these agents, *Trichoderma* sp. has emerged as a key player due to its multifaceted benefits, which include promoting plant growth, enhancing soil fertility, and controlling a wide range of plant pathogens [5,6]. The genus *Trichoderma* is particularly noted for its mycoparasitic activity, which allows it to suppress plant pathogenic fungi through mechanisms such as the production of hydrolytic enzymes, secondary metabolites, and the induction of systemic resistance in plants [7–11]. These attributes have positioned *Trichoderma* sp. as a cornerstone in integrated pest management strategies, contributing to the reduction of chemical inputs and the promotion of sustainable agricultural practices [3].

However, the application of genome editing for *Trichoderma* sp. in biotechnology in agriculture is not without challenges. The widespread use of antibiotics, both in the laboratory and in agricultural practices, raises concerns about their impact on non-target organisms, including beneficial fungi like *Trichoderma* sp. [12]. Antibiotics are routinely used to prevent bacterial and fungal contaminations in culture media, yet their effects on the physiology, growth, and functional capabilities of *Trichoderma* sp. have not been thoroughly investigated. This lack of understanding is particularly problematic given the growing interest in leveraging *Trichoderma* sp. for advanced biotechnological applications, such as CRISPR-Cas9 mediated gene editing. For these technologies to be effectively implemented, it is crucial to ensure that

* Corresponding author: syaiful.khoiri@trunojoyo.ac.id

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Trichoderma sp. maintains its genetic stability and functional integrity in the presence of antibiotics [13].

The influence of antibiotics on *Trichoderma* sp. extends beyond simple growth inhibition. Antibiotics can induce stress responses that may alter the fungus's virulence, sporulation, enzyme production, and secondary metabolism. These changes can have significant implications not only for the efficacy of *Trichoderma* sp. as a biocontrol agent but also for its use in biotechnological applications [13].

Given the importance of *Trichoderma* sp. in agriculture and biotechnology, a deeper understanding of how antibiotics affect its growth, virulence, and genetic stability is essential. This study aims to fill this gap by systematically evaluating the effects of various antibiotics on *Trichoderma* sp. across multiple generations, specifically, to assess how antibiotics influence the growth rate, sporulation, enzyme activity, and virulence of *Trichoderma* sp., as well as their impact on the stability of genetic modifications introduced through CRISPR-Cas9 technology. By elucidating these interactions, it can provide valuable insights that will inform the safe and effective use of *Trichoderma* sp. in both agricultural and biotechnological contexts. The findings of this research will have broad implications for the use of *Trichoderma* sp. as a biocontrol agent and as a platform for genetic engineering and understanding the interplay between antibiotics and *Trichoderma* sp. to optimization of culture conditions and the development of strategies to mitigate any adverse effects.

2 Research methods

2.1 Time and place

This research was conducted at the Applied Molecular and Microbial Biotechnology Laboratory (AM2B Laboratory), University of Jember, this research was carried out from January - April 2024.

2.2 Materials and tools

2.2.1 Materials

The materials used in this study are *Trichoderma* isolates, SDA Hi-media, antibiotics Kanamycin Meiji (PT. Meiji Indonesian), Microtina Chloramphenicol (PYRIDAM), Penicillin-G Meiji (PT. Meiji Indonesian), Gentamicin sulphate, Tetracycline HCI (PT. Zenith Pharmacy Factory), 70% alcohol, distilled water, *Fusarium* isolates collection.

2.2.2 Tools

The tools used in this study are B-ONE MINI Laminar Air Flow (Model Mini LAF-50-VAD), TOMY autoclave ES-215, micropipettes (Nichipet EX II Volume 2-20 µL (J18700601), Nichipet EX II Volume 20-200 µL (J18707131), Accucare Volume 100-1000 µL), ANUMBRA petri dish (90 x 15 mm), test tubes, Erlenmeyer flask 1000 mL; 250 mL, DURAN lab bottle (250 mL), tweezers, knife blades, thermometer, cork drill, disposable inoculation loop BIOLOGIC 1 µL (65-0001), 10 µL (65-0010), BIOLOGIC disposable cell spreaders (65-1010), Vortex Mixer MX2500, Compact Digital Mini Rotator Thermo Scientific (I9KT26013), Nikon microscope (Model Eclipse E100 LED Binocular MV R), Sartorius Analytical Scales, tray, Bunsen burner, measuring cup, rack, scissors, aluminum foil, cotton/PHIL M-4, plastic wrap, ruler, ice box (Marina cooler 6S) capacity 5.5 L, Ice Gel GABAG, Haemocytometer, preparation glass, alcohol spray, counter, name label, stationery, and gloves.

2.2.3 Experimental Design

This study was conducted using a completely randomized design (CRD) with 5 treatments and 5 replicates, using 5 successive generations inoculated with *Trichoderma*, then on antibiotics (Kanamycin Meiji, Chloramphenicol, Penicillin Meiji, Gentamicin sulphate, Tetracylcin HCI) retailed in a ratio of 1:1000 or 1 gram:10 mL. In the 5th generation, *Trichoderma* was tested for virulence using *Fusarium*.
C=*Trichoderma* sp. + Chloramphenicol
G=*Trichoderma* sp. + Gentamicin Sulphate
K=*Trichoderma* sp. + Kanamycin Meiji
P=*Trichoderma* sp. + Penicillin Meiji
T=*Trichoderma* sp. + Tetracyclin HCI

Table 1. Randomization of antibiotic treatment on *Trichoderma* sp. with 5 replicates.

K3	G2	C1	K3
T5	C1	G2	G2
P4	P4	T5	P4
G2	K3	P4	C1
C1	T5	K3	T5

2.3 Research procedure

2.3.1 Trichoderma sp. isolate

Trichoderma collection isolates were then rejuvenated and isolated by taking 1 gram of soil dissolved in 10 mL of sterile water. do serial dilutions up to 10^{-3}. In the 10^{-3} dilution, take about 0.1-0.2 mL to be dropped on media without antibiotics. Then flatten using disposal L-cell spreaders then incubate for 5-7 days at room temperature.

2.3.2 Antibiotic preparation

Antibiotic includes Chloramphenicol 250 mg per capsule, Gentamicin 1000 mg, Kanamycin 1000 mg, Penicillin 1000 mg, and Tetracycline 500mg per capsule. All antibiotic-type content is made at 1000 mg. How to make this antibiotic Chloramphenicol 250 mg per capsule needed as many as 4 capsules, Gentamicin 1000 mg in the form of paste weighed as much as 1 g,

Kanamycin 1000 mg in the form of powder weighed as much as 1 g, Penicillin 1000 mg in the form of powder weighed as much as 1 g, and Tetracycline 500 mg per capsule needed as much as 2 capsules. Then each antibiotic that has been equalized to 1000 mg is dissolved in sterile distilled water and then vortexed. Calculated using the formula:

$$\text{PPM} = \frac{Dissolved\ mass\ (mg)}{Volume\ (L)} = \frac{1000}{0{,}01} = 100.000\ \text{PPM}$$

2.3.3 Making SDB-Agar Media

Making media using Sabouraud Dextrose Broth (SDB) weighed as much as 30 g using analytical scales, making media following packaging instructions [14]. Heat the media with a microwave, stirring occasionally so that the media does not clot. After boiling the SDB agar, it is then put into an autoclave for the media to be sterilized first (temperature 121°C with a pressure of 1 atm for 15 minutes) and antibiotics are added with a media temperature of 45-60°C. then each media is given an antibiotic stock solution (Kanamycin Meiji, Chloramphenicol USP Grade, Penicillin Meiji, Gentamicin sulphate, Tetracyclin HCI) as much as 200 µL per 200mL, then the platting process is carried out in Petri dishes in laminar air flow cabinet.

2.3.4 Preparation of Trichoderma sp isolate

The 5-day-old *Trichoderma* sp isolate is taken using a loop inoculation, then placed at one point in the center of a petri dish containing SDA media that has been prepared. The Petri dish was closed and isolated with plastic wrap. Incubation of treatment fungi is carried out using antibiotic treatment. Then the isolate is placed back and observations of colony growth will be made every day for 24 hours. Observations of spore density, spore viability, and antagonistic power were carried out on day 5 after application.

2.3.5 Spore Density and Viability Test

Trichoderma sp. After 5 days of age do the calculation under the microscope Prepare 10 mL sterile distilled water in a test tube. Put 5 ml of sterile distilled water into the fungal isolate in SDA media. Scrape the fungus using a loop inoculation until all fungal spores are released from the surface of the SDA media. Put the suspension into a new test tube, Add the remaining sterile distilled water (5 ml) to clean the remaining fungal scrapings on the SDB-agar medium, and mix the spore suspension into the initial suspension until the total suspension becomes 10 mL (stock solution) Homogenize the spore suspension using a vortex for 3 minutes. Perform multilevel dilutions by taking 1 ml of suspension and putting it into 9 ml of sterile distilled water in a test tube, then homogenizing for 3 minutes (10-1 dilution). Perform these steps until dilution 10-3.

Observation of spore density was carried out under a binocular microscope by taking a suspension of *Trichoderma* isolates at a dilution of 10^{-3} as much as 0.1 mL and then placing it on a haemocytometer. Observe under a counting microscope using a hand-tall counter. A portion of *Trichoderma* isolates at 10^{-3} dilution was used for the viability test.

2.3.6 Antagonist test

The test procedure with *Trichoderma* sp. isolates in each 5th generation was taken at the edge of the colony that was 5 days old using a 50 mm diameter cork borer. *Trichoderma* sp. isolates that have been cut are placed on a 20 mL SDB-agar media per petri dish. The isolate was placed on the Petri dish at a distance of 2 cm from the edge and then marked for *Trichoderma* sp. (T). Pathogen isolates aged 5-7 days were taken using a 50 mm diameter cork borer from the edge of the colony. *Fusarium* isolates were placed on SDA media at a distance of 2 cm from the edge of the petri dish on the opposite side of the BCA's isolate and then marked for Fusarium pathogen (F). The growth of each colony was observed for up to 7 days and the colony contact was measured (Fig. 1).

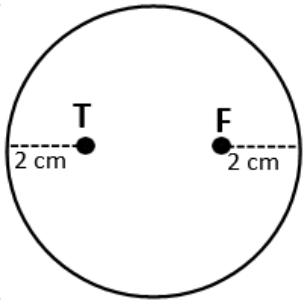

Fig. 1. Dual culture *Trichoderma* sp (T) and *Fusarium* spp (F).

2.4 Observation variable

2.4.1 Average colony growth (Colony radius)

Colony measurement on *Trichoderma* sp. is done every day after the mycelium is seen growing by measuring using a ruler. The purpose of this measurement is to determine the growth rate of the effect of each antibiotic on *Trichoderma* growth.

2.4.2 Colony color

Trichoderma sp. has a dark green color with a yellowish tint, the colony shape of the isolate spreads irregularly and has uneven colony edges. To find out the changes that occur after isolation given a mixture of antibiotics on the media to see changes in the color of the fungus directly using the Color grap application, by photographing *Trichoderma* that is 5 days old. This photo uses a mini studio photo box measuring (22.5cm × 21.8cm × 27cm) with a light intensity of ≤1400 nm, using an OPPO A96 cellphone with a rear camera resolution of 50 MP.

2.4.3 Spore density calculation

Count the density of spores in each treatment under a binocular microscope observed at 100x magnification, to get the counting field on the haemocytometer. Perform multilevel dilution by taking 1 ml of suspension and putting it into 9 mL of sterile distilled water in a test tube, then homogenized for 3 minutes dilution 10^{-1}. Take the fungal suspension at 10^{-3} dilution. Homogenize the spore suspension using a vortex for 3 minutes. Take 0.2 mL of spore suspension using a syringe (syringe) drop it in a haemocytometer, and observe under a microscope. The spore density data were analyzed using the formula of Gabriel & Riyatno (1989) as follows [15]:

$$S = \frac{X}{L\,(mm)^2 \times t\,(mm) \times d} 10^3 \quad (1)$$

Note:
S = Spore density per ml of solution
X = Total number of spores in the box a, b, c, d
L = Area of the counting box 0,04 mm^2
T = Depth of count field 0.1 mm
d = Dilution factor
10^3 = Calculated suspension volume (1 mL = 10^3 mm^3)

2.4.4 Conidia Viability Test

The viability or germination test is calculated from the number of conidia that germinate divided by the total number of conidia observed. The spore viability test was tested by taking a spore suspension from a 10^{-3} dilution (10 mL test tube) that had been shaken with a shaker (speed of 500 rpm per minute), then do it by mashing *Trichoderma* spores on an SDA medium disc (without antibiotics) with a diameter of 0.5 cm give 1 drop using a syringe (±5 µL) placed on a glass object, then incubate for 12 hours. After 12 hours count the number of spores that germinate and do not germinate in the field of view under a binocular microscope with a magnification of 400x [16]. Spore viability was calculated using the following formula:

$$VK = \frac{\Sigma KB}{\Sigma\, KB+KTB}\, x100\% \quad (1)$$

Note:
VK = Spore germination (viability)
KB = Number of spores that germinate
KTB = Number of spores that do not germinate

2.4.5 Dual Culture Test

Dual culture testing was carried out 7 Days after application. The minimum requirement for inhibition of BCA's fungi against pathogens in general is ≥50%. Calculation of antagonistic ability:

$$Z = \frac{R1-R2}{R1}\, 100\% \quad (1)$$

Note:
H = Inhibition ability
R1= radius of *Fusarium* colony in the control plate
R2= radius of *Fusarium* colony in a dual culture plate

2.5 Data analysis

The data obtained will be analyzed using descriptive analysis of quantitative data and analyzed using ANOVA (Analysis of Variance), if the results show very different and significantly different, it will be continued with the Tukey test.

3 Result and discussion

3.1 Result

Microscopically, the *Trichoderma* used is a collection isolate, namely *Trichoderma* sp. strain MJDI. It had the shape of the conidia, phialid, conidium, and hyphal insulation microscopically. The Treatment of several types of antibiotics on 5-day-old *Trichoderma* sp. for 5 generations macroscopically (Fig. 2).

Based on the observation of samples from the 1st to 5th generations of the 4th generation, it is known in Table 2 that in the 2nd and 3rd generations growth is very fast, this is the case when the comparison of each replicate is significantly different. However, the 4th and 5th generations experienced a slow average growth but the results of each replicate were not significantly different.

The effect caused by the addition of antibiotics to *Trichoderma* growth media is very different in each treatment and generation, this is evident macroscopically by taking data using the "Color grab" application. The following data results are obtained. Based on the observation of antibiotic treatment, each generation experienced a color change, the average color produced from yellowish green to grayish green in the 5th generation. Treatments that experienced good color changes occurred in the treatment of Chloramphenicol and Gentamicin. The treatment that is considered the least significant occurs in the Tetracycline treatment (Fig. 3). Color detection using the Color Grab application has 3 color interpretations, namely Hue (°), Saturation (%), and Value (%). The following is the calculation data that shows the difference in each treatment and gene (Table 4-6).

Based on the spore density of each antibiotic treatment, there was not a very far difference, but in the 4th generation, the treatment of Chloramphenicol and Gentamicin experienced a difference from the others. However, in each generation, it can be seen from the number that the spore density has decreased (Table 7).

Based on the viability test of each antibiotic treatment, there is no difference, but in each treatment in the generation of uppercase notations the viability test has decreased and the difference is not significant (Table 8).

Based on Table 9, shows that all of these treatments have a percentage ≥ 50%, which means that the inhibition of *Trichoderma* is very good. The high treatment was shown in the Chloramphenicol treatment, while the low treatment was shown in the Tetracycline treatment.

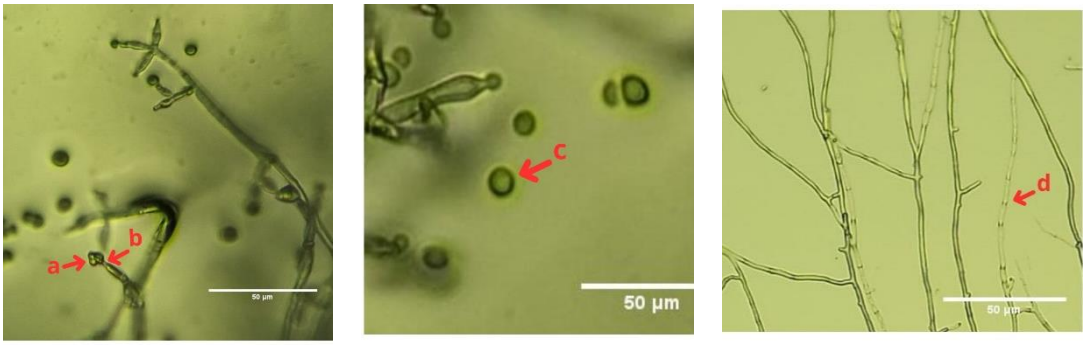

Fig. 2. Microscopic characteristics of *Trichodema* sp. strain MJD1 (a=conidia, b=phialid, c=conidium, d=hyphal septae).

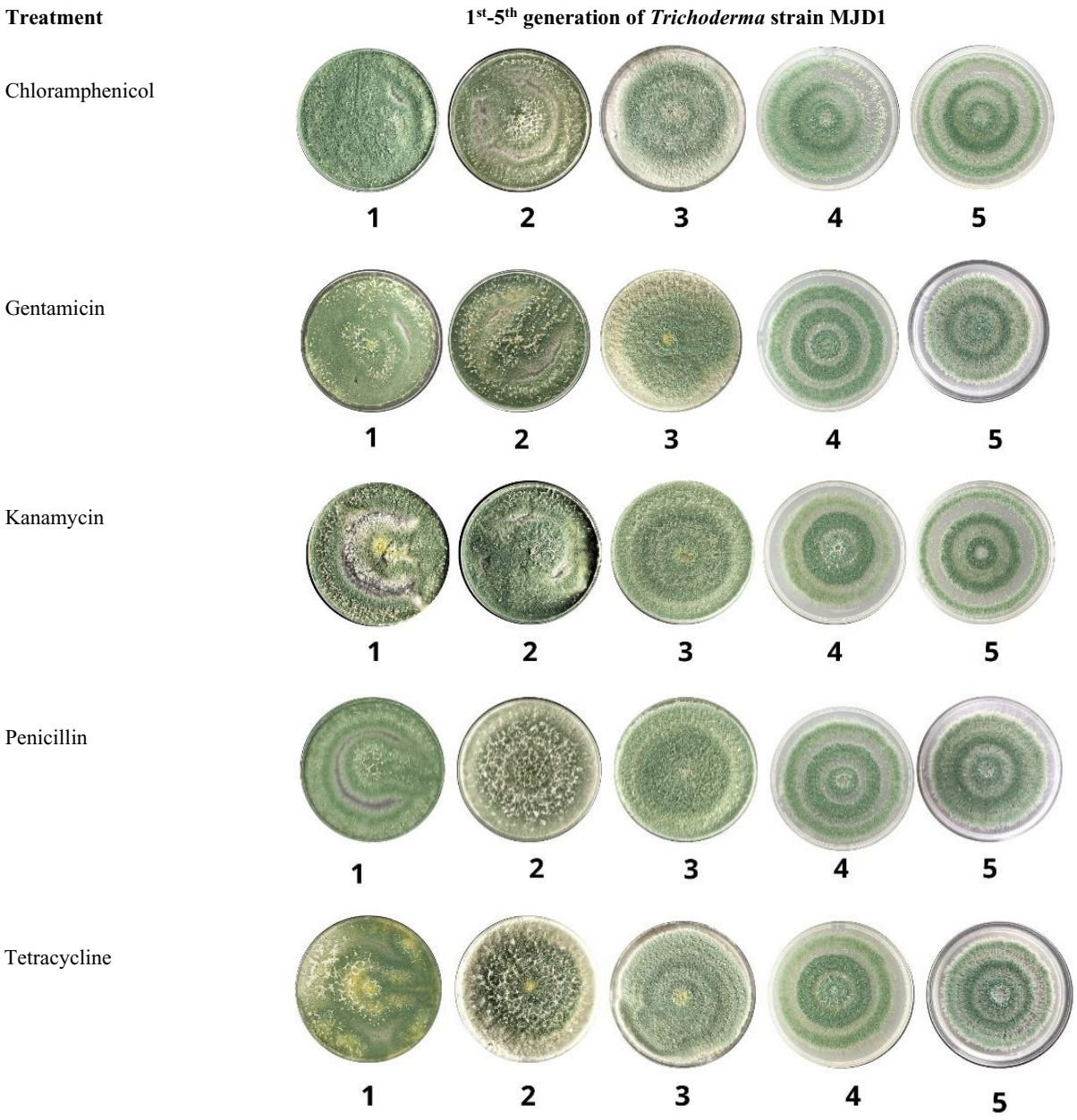

Fig. 3. Effect of antibiotic addition to in-vitro growth media on the growth of *Trichoderma* colony radius for 5 generations.

Table 2. Effects of adding antibiotics to in-vitro growth media on the average growth radius of *Trichoderma* colonies for 5 generations.

Treatment	Average colony radius growth (cm)				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	2,48 ±0,12a A	2,53±0,09b A	2,82±0,04b A	2,44±0,11a A	2,31±0,06a A
Gentamicin	2,50 ±0,16a B	2,63±0,06bc BC	2,79±0,07b C	2,45±0,08a B	2,27±0,13a A
Kanamycin	2,37±0,13a A	2,71±0,08c A	2,82±0,04b A	2,41±0,10a A	2,18±0,39a A
Penicillin	2,35±0,09a AB	2,26±0,09a A	2,81±0,11b C	2,47±0,03a B	2,19±0,12a A
Tetracycline	2,53±0,13a B	2,76±0,06c C	2,55±0,07a B	2,48±0,06a B	2,25±0,10a A

Note: Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. uppercase symbols of treatment differences between generations.

Table 3. The effect of adding antibiotics to in-vitro growth media on the color of Trichoderma colonies for 5 generations.

Treatment	Colony Color				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	Deep Green	Green-Yellow	Deep Green	Green-Grey	Green-Grey
Gentamicin	Green-Yellow	Green-Yellow	Green-Yellow	Green-Grey	Green-Grey
Kanamycin	Green-Yellow	Green-Yellow	Green-Yellow	Green-Grey	Green-Grey
Penicillin	Deep Green r	Green-Yellow	Green-Yellow	Green-Grey	Green-Grey
Tetracycline	Green-Yellow	Green-Yellow	Green-Yellow	Green-Grey	Green-Grey

Table 4. Effect of adding antibiotics to in-vitro growth media on Hue Color (°) of Trichoderma for 5 generations.

Treatment		HUE (°)			
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	89±13,1b AB	75,0±12,5a B	119,6±13,0b D	97,2±5,8a BC	109,6±4,0ab CD
Gentamicin	78,2 ± 3,2b A	77,2±12,2a A	78,6 ±26,5a A	96,0±9,3a A	123,8±4,4c B
Kanamycin	57,2 ± 1,0a A	79,2±10,6a BC	73,0±15,7a AB	95,8±6,7a CD	98,0±8,7a D
Penicillin	86,6 ± 4,2b A	74,8±1,3a A	76,6± 16,1a A	90,0±13,5a A	121,6±9,20bc B
Tetracycline	62,2±11,0a A	68,2±5,0a A	85,6±8,2a B	90,4±13,7a BC	103,2±4,08a C

Note: Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. Uppercase symbols of treatment differences between generations.

Table 5. Effect of adding antibiotics to in-vitro growth media on Saturation Color (%) of Trichoderma for 5 generations

Treatment	Saturation (%)				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	20,6±3,4a A	22,6±7,0a A	23,2±1,3a A	23,4±2,8a A	18,4±2,4a A
Gentamicin	23,6±2,9a BC	25,6±1,6a BC	28,4±6,5a C	20,4±0,8a AB	18,8±1,4a A
Kanamycin	24,2±5,7a AB	21,0±2,1a A	30,4±4,8a B	19,8±1,6a A	21,4±3,5a A
Penicillin	23,8±3,0a A	26,2±4,0a A	23,0±3,0a A	21,6±3,9a A	20,0±3,5a A
Tetracycline	25,6±6,8b B	27,6±1,6a A	23,8±1,3a A	24,2±3,9a A	23,0±2,2a A

Note: Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. Uppercase symbols of treatment differences between generations.

Table 6. Effect of adding antibiotics to in-vitro growth media on Value Color (%) of *Trichoderma* for 5 generations.

Treatment	Value (%)				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	65,8±7,0a AB	68,6±2,3b A	67,6±5,0a B	55,6±5,1a AB	52,4±2,3a A
Gentamicin	69,8±5,8a A	63,6±5,5ab A	66,6±11,8a A	62,0±3,0 a A	59,4±2,1a A
Kanamycin	83,8±10,8a B	63,4±5,9ab AB	67,0±7,5a AB	64,0±3,3a AB	57,2±8,1a A
Penicillin	66,4±5,4a B	53,6±3,2a A	62,0±4,0a AB	55,4±5,1a A	55,0±1,8a A
Tetracycline	81,4±15,8a B	63,4±3,2ab AB	54,6±2,2a A	57,6±7,6a AB	58,4±3,9a AB

Note : Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. Uppercase symbols of treatment differences between generations.

Table 7. Effects of adding antibiotics to in-vitro growth media on Trichoderma spore density for 5 generations.

Treatment	Spore Density (×10 ^{9})				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	2,48±0,12 a A	2,16±0,15 a AB	2,15±0,11 a AB	2,01±0,80 b A	1,54 ±0,22 a A
Gentamicin	2,50±0,16 a C	2,53 ±0,35 a C	1,89±0,07 a B	2,04±0,17 b B	1,33±0,28 a B
Kanamycin	2,37±0,13 a B	2,37± 0,51 a B	1,81±0,24 a AB	1,60±0,18 a A	1,40±0,15 a A
Penicillin	2,35±0,09 a B	2,55±0,57 a B	1,85±0,42 a A	1,49±0,19 a A	1,30±0,13 a A
Tetracycline	2,53±0,13 a B	2,37±0,70 a B	2,03±0,35 a AB	1,74±0,27a A	1,40±0,18 a A

Note : Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. Uppercase symbols of treatment differences between generations.

Table 8. Effect of adding antibiotics to in-vitro growth media on Trichoderma viability test for 5 generations.

Treatment	Spore Viability (%)				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	87,4±4,1a C	86,0±3,8a BC	80,0±3,3a AB	81,8±4,7a A	77,2±3,4a BC
Gentamicin	81,6±1,8a AB	86,4 ±2,5a B	81,0±7,1a AB	82,8±4,4a A	76,8±2,1a AB
Kanamycin	80,2±7,0a A	81,4±8,5a A	80,2±3,9a A	79,4±5,4a A	77,0±2,9a A
Penicillin	86,4±5,8a A	80,6±4,5a A	81,2±5,1a A	79,4±5,1a A	81,2±1,3a A
Tetracycline	85,0±4,8a B	83,8±2,7a AB	77,0±5,1a AB	76,8±3,7a AB	82,0±4,1a A

Note : Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. Uppercase symbols of treatment differences between generations.

Table 9. Effects of adding antibiotics to in-vitro growth media on virulence of Trichoderma for 5 generations.

Treatment	Inhibition (%)				
	1st Generation	2nd Generation	3rd Generation	4th Generation	5th Generation
Chloramphenicol	87,4±3,2 a A	77,8±5,4 a A	79,6±8,5 a A	74,6±6,4 a A	71,2±17,0 a A
Gentamicin	74,0±15,7 a BC	72,0±9,2 a AB	81,2±6,0 a A	71,0±4,2 a A	76,8±12,9 a A
Kanamycin	78,4±17,0 a BC	71,6±7,0 a AB	74,8± 6,1 a A	70,8±4,5 a A	69,4±7,6 a A
Penicillin	70,2±11,4 a AB	75,0±12,9 a A	80,4±7,8 a A	77,6±6,0 a A	74,0±14,6 a A
Tetracycline	68,0±13,8 a A	73,6±10,4 a A	72,8±8,1 a A	70,4±13,4 a A	59,6±11,3 a A

Note : Numbers followed by lowercase letters in the same column indicate significantly different treatments at the 5% Tukey test level. Uppercase symbols of treatment differences between generations.

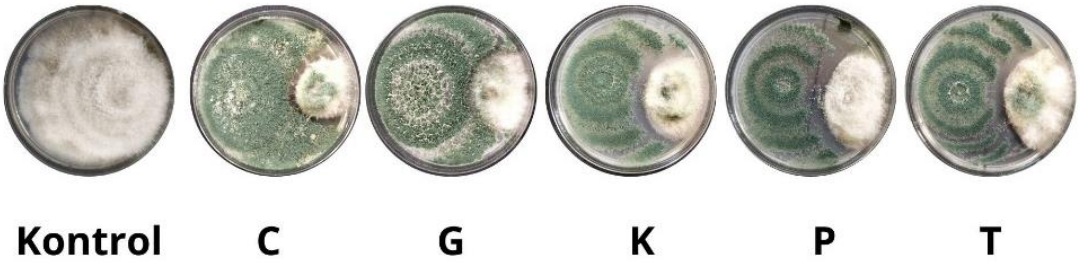

Fig. 4. Effects of antibiotic addition to in-vitro growth media on virulence of *Trichoderma* with *Fusarium* for 5 generations (C=Chloramphenicol, G=Gentamicin, K=Kanamycin, P=Penicillin, T=Tetracycline).

3.2 Discussion

The effect of adding antibiotics to *Trichoderma* growth media on its growth rate is very good because all types of antibiotics can inhibit contamination. So *Trichoderma* can grow faster, but *Trichoderma* during subculture there is a possibility of mutation or adaptive changes after several generations of subculture. Subculture 5th generations of each generation of growth slightly decreased and different, this happened because the inoculum conditions were too low or too high which resulted in an influence on the growth of fungi. *Trichoderma* grew for more than 3 days and filled the media in Petri dishes. The initial growth of hyphae is white but after more than 3 days the hyphae will be solid green to faded green as the subculture is carried out. The micro-morphological descriptions usually used as morpholgical identification [17].

The effect of antibiotics on each generation's colony color of Trichoderma is measured using the "Color Grab" application, which provides color indication and interpretation in terms of Hue (°), Saturation (%), and Value (%). Hue (°) represents the color's degree range (0-360°) on the color wheel, Saturation (%) indicates color density from gray to dense (0-100%), and Value (%) shows color brightness from dark to white (0-100%) [18]. The addition of five types of antibiotics over five generations significantly influences the colony color of *Trichoderma*. These changes are attributed to the different mycelial growth in each antibiotic-treated growth media or potential biochemical changes in the fungus after specific treatment durations [19].

The changes in colony color during each treatment and generation of *Trichoderma* are attributed to enzyme activity in each subculture generation. This fungus biosynthesizes secondary metabolites that manifest as pigmentation [20]. Essentially, fungi produce various pigments, including metabolites like melanin. The color pigment changes caused by antibiotic addition affect the appearance of fungal colonies in the growth medium, influencing their color, texture, and shape. This can also be due to environmental stress induced by antibiotics or changes in fungal metabolism [21]. Some antibiotics may react with media components or fungal metabolites, leading to color changes. These chemical reactions can alter the visual appearance of fungal cultures. Changes in mycelial growth identified due to antibiotic administration or biochemical alterations include pigment production in the growth environment. Color pigment changes in colonies are often due to oxidation. Oxidation reactions of phenolic compounds cause color changes or the formation of melanin and new pigments through enzyme reactions encoded by fungal genes [21].

Fungi commonly utilize carbon sources such as carbohydrates (polysaccharides, disaccharides, and monosaccharides), organic acids, amino acids, and lignin. The availability of appropriate nutrients can significantly accelerate the growth of fungal mycelium. Fungi require simple compounds as nutrient sources to ensure easy absorption by the mycelium. In each generation, the mycelium of *Trichoderma* exhibits different colors. In the first generation, the mycelium appears solid green to yellowish. However, by the fourth and fifth generations, the color changes to a grayish-faded green. This change occurs because the fungal filaments have the capacity or ability to produce various substances that generate pigments. These pigment-producing substances are influenced by the available nutrients and environmental conditions, leading to the observed color variations in different generations [22–24].

Antibiotic treatment of *Trichoderma* does not result in a significant difference in spore density. However, antibiotics can induce environmental stress in fungi, inhibiting essential metabolic processes and ultimately reducing the fungi's ability to produce spores during subculturing. This stress can significantly decrease both the density and viability of *Trichoderma* spores on SDB (Sabouraud Dextrose Broth) media. As subculturing progresses, the energy stored in the spores diminishes, impacting their ability to germinate and develop into sprout tubes by utilizing proteins, carbohydrates, and fats [25,26]. The decrease in spore energy is attributed to the nutritional content of SDB media, which consists mainly of chitin and has very low protein levels. Spore viability is classified as good if it is between 80-100%, moderate if between 70-85%, and poor if between 55-70%. Spores will germinate if the nutrients are suitable for growth. Subculturing leads to a decline in spore viability due to the depletion of carbon sources, which are essential for hyphal growth [23–25].

The growth at the germination rate of *Trichoderma* in each treatment showed no significant effect after subculturing for 5th generations, though each generation experienced a decrease and difference. The addition of antibiotics during subculturing can affect the expression

of genes involved in sporulation and germination processes. These changes in gene expression can lead to a decrease in the efficiency or ability of spores to germinate under conditions that normally favor germination. The results of the spore viability test over 8 hours of observation indicated that antibiotics had a significant impact on treatment C, which showed a high percentage of viability, particularly in genes 1 and 3. In contrast, the lowest percentage of viability was observed in treatment K. This difference is likely related to the presence of enzymes that trigger the germination of fungal conidia. The impact of antibiotics on these enzymes can vary, leading to differences in spore germination rates and overall viability across different treatments [27].

Subculturing entomopathogenic fungi like *Metarhizium anisopliae* for more than five generations can significantly reduce spore density and virulence [28]. However, for *Trichoderma* fungi, subculturing for five generations in each antibiotic treatment also reduces spore density but does not affect virulence due to their antagonistic properties, making them easy to grow and spread. *Trichoderma* virulence tests showed no significant differences across generations. Observations indicate that interactions between colonies occur because Trichoderma strains can compete for growth space and exhibit mycoparasitism, with hyphae growing at *Fusarium* colony points [29]. The antagonistic mechanism involves several enzymes that support *Trichoderma's* antagonistic ability, which are produced as secondary metabolites. These enzymes include protease, cellulase, cellobiase, chitinase, and 1,3-β-glucanase [30].

The stability of the *Trichoderma* spores is essential for maintaining consistent gene editing outcomes, as variations in spore viability could lead to inconsistent or unpredictable results, which in turn could affect the efficiency of gene editing processes like those employed in CRISPR-Cas9. The effectiveness of CRISPR-Cas9 relies on the precision with which genetic modifications can be introduced and maintained. *Trichoderma harzianum* developed with CRISPR/Cas9-based system using pNOM102 plasmid resistant to hygromycin phosphotransferase gene (hyg) is used as selecttable markers [31]. Furthermore, the expression of genes related to sporulation and germination could be affected by antibiotic-induced stress, potentially altering the outcomes of CRISPR-Cas9 interventions. This underscores the need for careful consideration of antibiotic use in *Trichoderma* cultures intended for genetic engineering applications.

The findings of this study suggest that while *Trichoderma* sp. can tolerate and even thrive in the presence of antibiotics, the long-term effects of such treatments, particularly when combined with CRISPR-Cas9 technology, warrant further investigation. It is crucial to ensure that the genetic and physiological integrity of *Trichoderma* is preserved to maintain its utility as a biocontrol agent and as a reliable platform for gene editing.

4 Conclusion

The impact of antibiotics on *Trichoderma* growth in vitro demonstrated that all tested antibiotics successfully prevented contamination and accelerated hyphal growth. The colony color remained relatively consistent across treatments, although it gradually shifted from solid green in the 1st generation to a yellowish-green by the 4th generation, and finally to a grayish-green by the 5th generation. Treatments Chloramphenicol and Penicillin were notable for maintaining better color consistency. While spore density and viability remained stable initially, they decreased after five generations of subculturing. *Trichoderma* virulence testing cannot reduce the antagonistic ability, but best treatment is shown in treatment Chloramphenicol. These findings are critical for applications involving CRISPR-Cas9, where the genetic stability and consistent performance of *Trichoderma* are essential for reliable and accurate gene editing.

The authors sincerely acknowledge to the National Research and Innovation Agency, Indonesia (BRIN) for their generous support and funding of this research under contract number 146/IV/KS/11/2023.

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