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Secondary Metabolites of *Trichoderma* sp. as Antifungal Against Rice Seed-borne Pathogen Fungi

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Abstract

The use of quality seeds is necessary to increase rice productivity. The existence of pathogenic fungi infections causes the quality of rice seeds to decrease and impacts the ability to grow plants. Trichoderma sp. is known to produce secondary metabolites that can inhibit the growth of pathogens. This study aimed to examine the ability of the secondary metabolites of Trichoderma sp. at various concentration levels and immersion time to suppress pathogenic fungi infections carrying rice seeds. The research was carried out by giving seed immersion treatment. Then identify the pathogenic fungi the seeds carry for further testing with the blotter test method. The parameters tested were infection rate, infection suppression, and seed viability. The identification results showed types of pathogenic fungi carried by rice seeds included Fusarium sp., Alternaria sp., Aspergillus sp., Penicillium sp., and Rhizopus sp. Research also shows that the secondary metabolites of Trichoderma sp. are effective in improving the quality of rice seeds. The test results showed that the secondary metabolites of Trichoderma sp. can suppress the level of pathogenic fungi infection. There was no interaction between the treatment of metabolite concentrations and immersion time on all test parameters. Seed immersion treatment with secondary metabolites in the 5-20% concentration range has effectively reduced fungi infection rates and increased seed viability. Meanwhile, various levels of soaking time did not affect reducing the level of fungi infection but instead affected the viability of the rice seeds.

Keywords: *Antifungal, Rice seeds, Secondary metabolites, Seed-borne pathogens, Trichoderma sp.*

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INTRODUCTION

Rice (*Oryza sativa* L.) is the most important agricultural commodity, and rice has become the primary source of carbohydrates for the majority of the population in Indonesia. According to (Badan Pusat Statistik 2023), rice production for national food consumption in 2022 is estimated to be around 32.07 tons. According to the OECD-FAO (2022), the level of rice consumption in Indonesia is the highest in Southeast Asia and ranks third in the world after China and India. The need for high rice consumption in Indonesia also demands high rice production.

Quality rice seeds are the first step in increasing rice production. However, some rice farmers still use rice seeds harvested from the previous period for replanting. This causes the seeds used to have low quality to be susceptible to attack by plant-disturbing organisms, including fungi. Seed-borne pathogenic fungi have an essential meaning for rice cultivation because the losses incurred are enormous, including causing physical damage, namely changes in the shape and color of the seeds, decreasing germination ability, causing disease in the field, and being able to increase the death of seedlings and young plants. Seed-borne fungi infections can reduce the quality of rice seeds and potentially cause a decrease in rice production in Indonesia. Several pathogenic fungi carried by rice seeds include *Fusarium, Rhizoctonia, Curvularia, Rhizopus, Aspergillus, Alternaria, Penicillium, Drechslera,* and *Tilletia* (Amteme & Tefa, 2018; Nurdin et al., 2022; Ramdan & Kalsum, 2018; Sobianti et al., 2020).

Trichoderma sp. can produce secondary metabolites as volatile organic compounds that act as antifungals. One of these volatile organic compounds is 6-pentyl-α-pyrone or 6-pentyl-2h-pyran-2-one (Kamaruzzaman et al., 2021). The impact of exposure to volatile organic compounds produced by *Trichoderma* sp. is the abnormal growth of pathogenic fungi hyphae structures (Intana et al., 2021). Zani & Anhar (2021), added that Trichoderma produces growth regulators (ZPT), which can stimulate the formation of phytohormones that will work actively so that seed growth and physiological processes can occur. The ZPT content of Trichoderma, which can stimulate growth, includes IAA (Indole Acetic Acid), cytokinins, and gibberellins. The presence of secondary metabolites of *Trichoderma* sp. This is expected to improve the quality and quality of rice seeds to minimize seed-borne pathogenic fungi infections. Therefore, it is necessary to test the potential of secondary metabolites of the endophytic fungi *Trichoderma* sp. against rice seed-borne pathogenic fungi.

METHOD

Metabolite Extraction of *Trichoderma* sp.

Secondary metabolites of *Trichoderma* sp. were isolated using Potato Dextrose Both (PDB) media by preparing five fungal isolates of *Trichoderma* sp. with a diameter of 0.5 cm, aged seven days, with a spore density 108. Furthermore, the fungal isolates were inoculated into 150 mL of PDB media in an erlenmeyer with a capacity of 250 mL. Then it was shaken using a circular shaker (orbital shaker) at 150 rpm at room temperature for seven days (Soesanto, 2020). Metabolite results *Trichoderma* sp., which was shaken and then transferred into a 2 ml centrifuge tube. The separation process between the supernatant and the pellet was carried out at a speed of 10,000 rpm for 10 minutes. The supernatant obtained was then filtered using a syringe filter 0.2 µm in size (Sukapiring et al., 2016).

Seed Treatment

The rice seeds were soaked in 100 ml of the metabolite suspension *Trichoderma* sp. using several concentrations of metabolites, including concentrations of 5% (5 ml solution of secondary metabolites in 100 ml of sterile distilled water); concentration of 10% (10 ml solution of secondary metabolites with 100 ml of sterile distilled water); and 20% concentration (20 ml solution of secondary metabolites with 100 ml of sterile distilled water). As a negative control, soaking seeds used sterile istilled water, and for positive control, seeds used a fungicide with an active ingredient Tricyclazole at a dose of 2.5 ml/kg of seed.

Blotter test

The blotter test is a method for detecting seed-borne pathogenic fungi. The test was conducted by placing ten rice seeds on three sheets of sterile filter paper moistened with sterile distilled water into a petri dish. The distance between the rice seeds is arranged so that the seeds do not touch each other. The petri dish containing the seeds then incubated. Incubation lasted seven days (Badan Karantina Pertanian, 2007).

Research design

This study used a factorial completely randomized design with two factors. The first factor was the concentration of immersion treatment (including control), namely: P1 = sterile distilled water (control -); P2 = tricyclazole fungicide (control +); P3 = secondary metabolite concentration of 5%; P4 = secondary metabolite concentration of 10%; P5 = secondary metabolite concentration of 20%. The second factor is the length of immersion time with three levels, namely, L1 = 6 hours, L2 = 12 hours, and L3 = 24 hours. When these two factors are combined, there will be 15 combination treatments, three replications, and 45 experimental units.

Research Parameters

Identification of Seed-borne Pathogenic Fungi

The morphology of seed-borne pathogen fungi was observed macroscopically using a stereo microscope and microscopically using a compound microscope.

Infection Rate and Infection Rate Emphasis

The following formula calculates Infection Rate (IR):

$$\text{IT} = \frac{Number\ of\ Infected\ seed}{NBumber\ of\ Observed\ seeds} \text{ x } 100\% \quad (1)$$

The following formula calculates Infection Rate Suppression (IRS):

$$\text{IRS} = \frac{(Control\ Negative\ Infection\ Rate - Treatment\ Infection\ Rate)}{Control\ Negative\ Infection\ Rate} \text{ x } 100\% \quad (2)$$

Seed Viability

Observations on the viability and vigor of rice seeds according to (ISTA (International Seed Testing Association) (2010), which has been modified by La & Sutariati (2021), include: Germination Power (GP), describes the potential viability of seeds, calculated based on the percentage of the number of Normal Sprouts (NS) on day five as count I (NS I) and day seven as count II (NS II), percentage germination power (GP) with the following formula.

$$GP = \frac{\Sigma NSI + \Sigma NSII}{\Sigma Planted\ Seeds} \times 100\% \quad (3)$$

Maximum Growth Potential (MGP), describes the total viability of seeds, calculated based on the percentage of seeds that were able to germinate on the last day of observation (7th day); the percentage of MGP is calculated using the following formula.

$$MGP = \frac{\Sigma Growing\ Seeds}{\Sigma Planted\ Seeds} \times 100\% \quad (4)$$

Vigor Index (IV), is calculated based on the percentage of usual sprouts on day five (NSI)

$$VI = \frac{\Sigma NSI}{\Sigma Planted\ Seeds} \times 100\% \quad (5)$$

Data analysis

Quantitative data were analyzed using Analysis *of Variance* (ANOVA) at 5% ($P \leq 0.05$), then continued with Duncan's test on R Studio 4.2.1 software. Quantitative and qualitative data are displayed in the form of tables and figures.

RESULTS AND DISCUSSION

Identification of Rice Seed-Borne Fungi

***Fusarium* sp.**

Macroscopic observation of rice seeds infected with *Fusarium* sp. shows that the entire surface of the source is covered by mycelium with a cotton-like texture and white color (Figure 1A). *Fusarium* sp infection seeds also cause symptoms in the form of changes in seed color to brown as the fungus grows. The fungus *Fusarium* sp. microscopically shows morphological characteristics in the form of microconidia, which are round, oval to elliptical, with one or two partitions (Figure 1B). The macroscopic and microscopic characteristics of *Fusarium* sp. This is in accordance with research by Sidik (2022), which states that seeds infected with *Fusarium* sp. show the presence of white to black mycelia covering the entire seed. Based on microscopic observations, *Fusarium* sp. has Microconidium as a one or two-celled conidium with an oval or egg-shaped shape. Fusarium can form many microconidia with a hyaline color; the microconidia are created in chains or false heads (clustered) on the lateral part of the conidiophore.

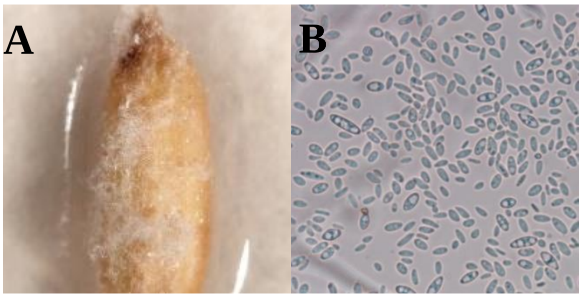

Figure 1. Morphology of *Fusarium* sp. (A) Colony of *Fusarium* sp. on the seed surface (B) Microscopy of *Fusarium* sp. (1000x magnification)

Fusarium sp. belongs to seed-borne fungi that infect seeds since they are in the field. This is because this fungus is a rice plant pathogen that can cause wilting and drooping of rice plant seedlings. According to Riskiya et al. (2022), symptoms of *Fusarium* sp. in rice plants are yellowish-green leaves that dry quickly, and if they attack the nursery, they can cause plant death. Although the Fusarium genus includes field fungi, the Fusarium genus will continue to damage seed materials in storage.

***Alternaria* sp.**

Alternaria sp. colonies that appeared macroscopically on the infected seeds had grayish-white and blackish colors on the filter paper (Figure 2A). This follows the statement of Amteme & Tefa (2018) that *Alternaria* sp. mushroom colonies are grayish-white to black. The direction of mycelium growth is sideways, and the mycelium structure is rough. *Alternaria* sp. can grow thick colonies that are usually black or gray. These fungi are parasitic on living plants or saprophytes on organic substrates. The range of Alternaria host pathogens is extensive. It is easy to recognize *Alternaria* sp. with large conidia morphology, formed in chains or solitary, usually oval, pale brown to brown, multicelled, and muriform. Microscopic observation, *Alternaria* sp. has slightly branched hyphae, which are insulated and dark in color. The shape of the conidia is elongated, insulated with brown color (Figure 2B). According to Waliha et al. (2022), *Alternaria* sp. conidiophores are dark in color, short or long in size, conidia are dark in color and sometimes colorless with a conical shape and long at the ends; conidia have 3-5 septa.

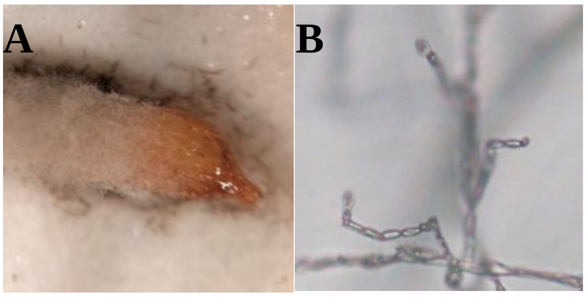

Figure 2. Morphology of *Alternaria* sp. (A) Colonies of *Alternaria* sp. on the seed surface (B) Microscopic *Alternaria* sp. (1000x magnification)

Altenaria sp. is a seed-borne fungus because it is a rice plant pathogen. *Alternaria* sp. fungus is the cause of Stacburn disease in rice plants. According to Defitri (2017), the symptoms of this disease attack are large, oblong, or round spots on the leaves, with brown, narrow, and clear edges that encircle the ring-like spots. The center of the spot is initially pale brown, gradually becoming almost entirely white, with many black dots consisting of fungal sclerotia.

***Aspergillus* sp.**

Two types of Aspergillus genus fungi infect rice seeds: *Aspergillus flavus* and *Aspergillus niger*. The results of macroscopic observations on rice seeds infected with the fungus *Aspergillus flavus* were characterized by fungal colonies with long, erect conidiophores and greenish-round conidial heads on top (Figure 3A). Meanwhile, microscopically it can be seen that the structure of the *Aspergillus flavus* fungus is in the form of a long, unbranched conidiophore, and at the top end, there is a round head of conidia, and around it, there is a conidium which is also round (Figure 3B). This follows the research of Sobianti et al. (2020) that this seed-borne fungus forms green colonies, round in shape, and the edges of the colonies are flat. The morphology of the pathogenic fungus shows a tree-like shape, containing conidiophores, spherical vesicles, phialides, and colorless conidia, spherical in shape, measuring 800 x (15–20) µm, with vesicles in the shape of spherical to slightly spherical. Conidiophores are colorless, erect, and unbranched.

The morphological characterization of *Aspergillus niger* is almost the same as *Aspergillus flavus*, which is characterized by the presence of fungal colonies with long, upright conidiophores and round conidial heads above them, except that *Aspergillus niger* is black (Figure 3A). Meanwhile, based on microscopic observations, the conidia are round and black; the conidiophores are colorless and are not insulated. The conidia are brown to black, rough, and globular (Figure 3B). Following research by Sobianti et al. (2020), mushroom colonies appear round, have a soft texture, flat colony edges, and are black. Observation of pathogenic fungi under a microscope showed that conidia were round and black, long conidiophores measuring 400-3000 µm, soft, and not insulated. Conidium brown to black, very rough, spherical, 4-5 µm in diameter.

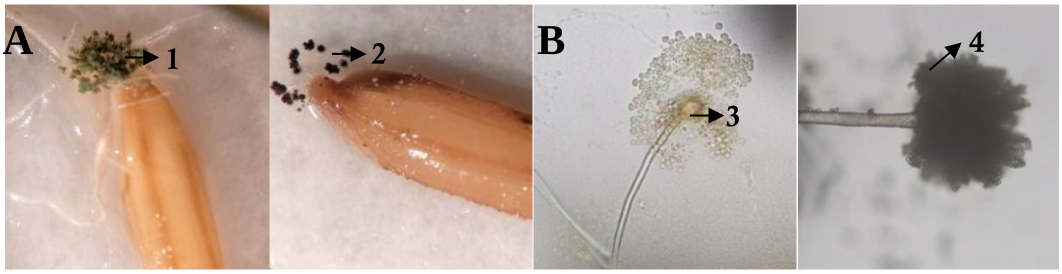

Figure 3. Morphology of *Aspergillus* sp. (A) Colonies of *Aspergillus* sp. on the seed surface (B) Microscopically *Aspergillus* sp. 1. *Aspergillus flavus* 2. *Aspergillus niger* (1000x magnification)

Aspergillus belongs to one of the seed-borne fungi and is almost always found in seeds because it is a facultative parasitic fungus. Aspergillus contamination starts from infection in plantings and is carried by seeds to storage areas, which can then cause damage to storage warehouses. According to Amteme & Tefa (2018), Aspergillus is one of the pathogenic fungi that infect rice seeds. This fungal infection resulted in a decrease in germination and seed quality. The main species of fungi that can become food contaminants include *Aspergillus flavus* and *Aspergillus niger*, with their ability to produce toxic substances, namely mycotoxins, which cause damage to food.

***Penicillium* sp.**

Macroscopic observations showed that rice seeds infected with this fungus were characterized by powdery greenish-grey colonies (Figure 4A). This is to research by Pemuda et al. (2022) that *Penicillium* sp. colonies that infect rice seeds have a greenish color. Based on microscopic observations, *Penicillium* sp. has twig-shaped and branched conidiophores, bottle-shaped fialids, round conidia and form chains (Figure 4B). These microscopic characteristics are by research by Zahara & Pamekas (2022) that *Penicillium* sp. has yellowish green colonies, branched conidiophores, cylindrical metulae with 3-6 bottle-shaped phialids, conidia are semi-round to elliptical in shape, hyaline or slightly greenish, have smooth walls and form chains.

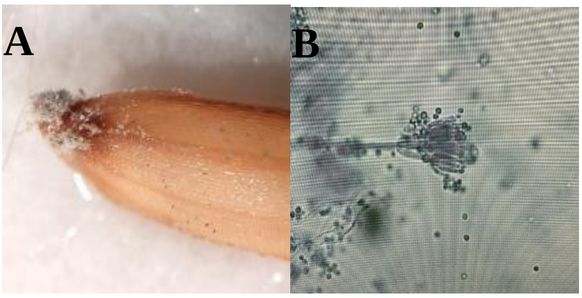

Figure 4. Morphology of *Penicillium* sp. (A) Colonies of *Penicillium* sp. on the seed surface (B) Microscopic *Penicillium* sp. (1000x magnification)

Penicillium sp. is classified as a seed-borne fungus that infects when in storage. This fungus is saprophytic and can damage agricultural products in storage. According to Zahara & Pamekas (2022), *Penicillium* sp. It is often found in rice seeds and causes symptoms in the form of changes in the color of the sources, rotting and causing low viability of the rice seeds.

***Rhizopus* sp.**

Index symptoms of seeds attacked by *Rhizopus* sp. macroscopically it is characterized by the covering of the entire surface of the seed by white, cottony-fibrous colonies with black spots on the top of the colonies (Figure 5A). Based on microscopic observations, *Rhizopus* sp. is round and black. The sporangiophore has a stalk that grows upright, is not insulated, branched, and is brownish-yellow in color (Figure 5B). According to Sobianti et al. (2020), *Rhizopus* sp. has white colonies covering the entire seed and extends to the blotter paper. The nature of the fungus spreads quickly. Colonies are white and turn gray with the increasing age of the culture. Observation of morphology under the microscope there are columella, sporangium, and sporangiophore. The spores are black, and the shape of the spores resembles round to oval. The sporangiophore grows upwards and contains a sporangium. The sporangium is black, and the columella is round.

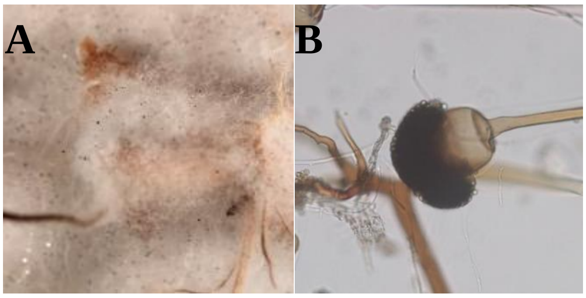

Figure 5. Morphology of *Rhizopus* sp. (A) Colonies of *Rhizopus* sp. on the seed surface (B) Microscopic *Rhizopus* sp. (1000x magnification)

Rhizopus sp. is a type of warehouse fungus that infects many seeds in plants, including rice. *Rhizopus* sp. causes disease in panicles or grains. The affected grain has brown to black spots and becomes hollow. Spores from Rhizopus can spread with the help of air and have hyphae that produce pectinolytic enzymes that damage the middle lamellae, infect the tissues, and make the plant soft, rotten, and eventually dry out and turn black (Semangun, 2010).

Infection Rate and Infection Rate Suppression

The analysis of variance showed no interaction between the treatment factor and immersion time on the infection rate or suppression of the infection rate. Meanwhile, the single treatment factor had a significant effect, while the soaking time did not. The average infection rate and suppressed infection rate are shown in Table 1.

Table 1. Test Results for the Effect of Secondary Metabolites on the Infection Rate and Infection Rate Suppression

Treatment	Infection Rate and Infection Rate Suppression	
	IR(%)	**IRS(%)**
P1 (Control -)	80.00a	-
P2 (Control +_)	21.11d	58.33a
P3 (Concentration 5%)	53.33b	33.33c
P4 (Concentration 10%)	50.00b	37.50c
P5 (Concentration 20%)	42.22c	47.22b
Immersion Time		
L1 (6 hours)	51.33a	52.08a
L2 (12 hours)	50.00a	46.87a
L3 (24 hours)	46.66a	44.79a
Interaction	not real	not real

The testing results with the blotter test method showed that the percentage of infection rates and the suppression of infection rates on the treatment factors gave significantly different results. Treatment with the addition of secondary metabolites gave significantly different results from the adverse control treatment. Treatment of secondary

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metabolite concentrations of 20% had significantly different levels of treatment with concentrations of 5% and 10%. The treatment of 20% concentration of metabolites had an infection rate of 42.22%, while the treatment of 10% concentration was 50% and 5% concentration was 53.33%. Treatment with a concentration of 20% also has a higher suppression of infection rates compared to the other two concentrations. The 20% concentration treatment had an emphasis level of 47.22%, followed by a 10% concentration of 37.50% and a 5% concentration of 33.33%.

Based on the results, it is known that there is a decrease in the infection rate and suppression of the infection rate in the seed immersion treatment using *Trichoderma* sp. secondary metabolite solution. This follows the research by Putri et al. (2022), that giving *Trichoderma* sp. filtrate. can reduce the population of *Aspergillus flavus* on the surface of Arabica coffee beans. The suppression of the level of pathogen infection is thought to occur due to the mechanism of antibiosis by secondary metabolites of *Trichoderma* sp. contained in the seed. According to Kumar & Khurana, (2021), the antibiosis mechanism of *Trichoderma* sp. is a method of fungi to induce the death of pathogenic fungi by releasing chemical compounds that are antagonistic to their environment; these chemical compounds consist of toxins, antibiotics, and enzymes that can inhibit the growth of pathogens.

The types of compounds contained in the secondary metabolites of the endophytic fungus *Trichoderma* sp. in this study have not been tested or analyzed. However, based on research by Kamaruzzaman et al. (2021), *Trichoderma* sp. may be able to produce secondary metabolites in the form of volatile organic compounds that act as antifungals. One of these volatile organic compounds is 6-pentyl-α-pyrone or 6-pentyl-2h-pyran-2-one. The impact of exposure to volatile organic compounds produced by *Trichoderma* sp. is the abnormal growth of pathogenic fungal hyphae structures (Intana et al., 2021).

The percentage of infection rate and the suppression of the infection rate in treating secondary metabolites with a concentration of 20% had significantly different results against concentrations of 5% and 10%. So the increasing concentration of secondary metabolites is in line with the reduced level of infection of seed-borne pathogenic fungi. The effect of the level of metabolite concentration on the suppression of pathogens has also been proven by Pamekas (2020), based on testing on PDA media, the diameter growth of *F. oxysporum* mushroom colonies is increasingly inhibited along with the increasing concentration of secondary metabolites of *Trichoderma* sp. In addition, Rahmadanty et al. (2021) also proved that treating *Trichoderma* sp. with high concentrations indicates failure in forming pathogenic fungal spores. The increased concentration of the filtrate treatment caused the number of secondary metabolites, both enzymes and antibiotics excreted, also to increase.

Rice Seed Viability and Vigor

The analysis of variance showed no interaction between treatment factors and soaking time on all parameters of seed viability, namely germination power, maximum growth potential, and vigor index. Meanwhile, the single factor of treatment and immersion time had a significant effect. The average germination power, maximum growth potential, and vigor index are shown in Table 2.

Table 2. Test Results for the Effect of Secondary Metabolites on Seed Viability

Treatment	Seed Viability		
	GP(%)	**MGP(%)**	**VI(%)**
P1 (Control -)	30.00c	34.44c	30.00c
P2 (Control +_)	53.33ab	56.66ab	53.33ab
P3 (Concentration 5%)	48.88b	55.55b	48.88b
P4 (Concentration 10%)	60.00ab	63.33ab	60.00ab
P5 (Concentration 20%)	63.33a	67.77a	63.33a
Immersion Time			
L1 (6 hours)	40.00c	45.33c	40.00c
L2 (12 hours)	50.00b	53.33b	50.00b
L3 (24 hours)	63.33a	68.00a	63.33a
Interaction	Not real	Not real	Not real

The test results showed that differences in the concentration of secondary metabolites did not significantly affect the percentage of seed germination power, maximum growth potential, and seed vigor index. Based on the study's results, it was found that the administration of secondary metabolites had a higher percentage of seed viability than without the administration of metabolites (negative control). Seed viability is the germination power of the seed, which shows the viability of the seed. Germinated seeds show that the metabolic processes in the seeds are active. Meanwhile, metabolism is influenced by endogenous enzymes and growth regulators (hormones) found in seeds (Tikafebrianti et al., 2019). Seed treatment using secondary metabolites of *Trichoderma* sp. is thought to be a stimulator so that metabolic processes in the seed become active and the seed can germinate usually. According to Lahati et al. (2021), they were stated that giving *Trichoderma* sp. can provide a good influence on the viability of cocoa seeds. Trichoderma, apart from being a biological agent, can also be a stimulator of plant growth both in plantations and food crops during the seed increase, as well as in rice seeds (food); Trichoderma can have a significant effect on the viability of rice seeds.

The increase in viability of rice seeds with secondary metabolite treatment is thought to occur due to the secondary metabolites of *Trichoderma* sp. containing compounds that can stimulate seed growth. According to Zani & Anhar (2021), Trichoderma produces growth regulators (ZPT), which can stimulate the formation of phytohormones that will work actively so that seed growth and physiological processes can occur. The ZPT content of Trichoderma, which can stimulate growth, includes IAA (Indole Acetic Acid), cytokinins, and gibberellins. The IAA hormone produced by Trichoderma can accelerate root growth. The hormone gibberellin can encourage seed development. Meanwhile, cytokinin hormones can encourage cell division and urge seeds to germinate (Asra et al., 2020).

CONCLUSION

The identification results showed that various types of pathogenic fungi carried by rice seeds that infected the blotter test method included *Fusarium* sp., *Alternaria* sp., *Aspergillus* sp. *Penicillium* sp., and *Rhizopus* sp. Research also shows that the secondary metabolites of *Trichoderma* sp. are effective in improving the quality of rice seeds.

Regarding the health quality of seeds, the effectiveness of secondary metabolites is demonstrated by their ability to suppress the level of fungal infection carried by rice seed pathogens at various levels of concentration. The treatment of 20% concentration of metabolites had an infection rate of 42.22%, while the treatment of 10% concentration was 50% and 5% concentration was 53.33%. Treatment with a concentration of 20% also has a higher suppression of infection rates compared to the other two concentrations. The 20% concentration treatment had an emphasis level of 47.22%, followed by a 10% concentration of 37.50% and a 5% concentration of 33.33%. In addition, the secondary metabolites of *Trichoderma* sp. also proved effective in increasing seed viability, namely germination power, maximum growth potential, and seed vigor index.

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