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Secondary Metabolites of *Bacillus sp*. as Antifungal of Seed-borne Pathogenic Fungi on Maize Seed Using Blotter Test Method

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Abstract

Background: Seeds are planting materials that must have high viability and quality, including being free from pathogens. One of the obstacles to the supply of quality maize seeds is seed-borne fungal pathogens, which can affect the quality of maize seeds and cause plant disease. Endophytic bacteria Bacillus sp. has been widely studied to be able to produce secondary metabolites as antifungals. This study aimed to determine the exact concentration of secondary metabolites of endophytic bacteria Bacillus sp. to decrease the infections of seed-borne pathogens fungal on maize seeds using the blotter test method. Methods: This study used a completely randomized design with four concentration levels (10%, 15%, 20%, 25%) and two strain codes of endophytic bacteria Bacillus sp. (Bth-31a and Bth-22). Results: The result of identifying seed-borne fungal pathogens on maize seed included Fusarium sp., *Aspergillus flavus*, Aspergillus niger, and Rhizopus sp. The Bth-22 treatment with a concentration of 25% could decrease the infection of seed-borne fungal pathogens and had the highest percentage until 50% compared to the control. The lowest percentage to reduce the infections of seed-borne fungal pathogens occurred in the Bth-31a treatment with a concentration of 10%, which was 32.1% compared to the control. Conclusions: All seed treatments that used secondary metabolites of Bacillus sp. decreased the infections of seed-borne fungal pathogens on maize seed compared to the control. In addition, treating secondary metabolites of *Bacillus sp.* can increase the germination of maize seeds.

Keywords: *Bacillus* sp.; Blotter test; Infection; Secondary metabolite; Seed-borne fungal pathogen.

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Introduction

Maize (*Zea mays* L.) is a vital food commodity as a source of carbohydrates besides rice and wheat. Maize production in Indonesia in 2016-2020 has increased and decreased consecutively to 23.58, 28.92, 30.25, 22.59, and 22.5 tonnes/year (Badan Pusat Statistik, 2021). According to the Badan Pusat Statistik (2021), the production of maize was 15.8 million tons. One of the causes of the decrease in maize production is seed-borne diseases. Seed-borne disease is essential because it is the primary source of inoculum that causes disease transmission and infection in plants.

Chemical control is considered the most effective and efficient method of controlling seed-borne fungal pathogens. The excessive use of chemicals will undoubtedly harm the environment and human and animal health, so an alternative control method that is effective, efficient, and environmentally friendly is needed. Hanif (2015) states that one way of controlling the decrease in infection rates on seed-borne pathogens is by soaking the seeds using secondary metabolite compounds produced by endophytic microbial biological agents.

Bacillus sp. is one of the microbes that can be used for biological control. Seed

treatment using endophytic bacteria *Bacillus* sp. can reduce the growth of seed-borne fungal pathogens (Fachrezzy, 2022). Munif *et al.* (2015) stated that endophytic bacteria effectively controlled several plant diseases even though the isolates of endophytic bacteria were obtained from plants in different families from the target plants. The results of previous research conducted by Fachrezzy (2022) proved that the endophytic bacteria from the eggplant plant, identified as *Bacillus* sp., has the potential as an antifungal for the seed-borne fungal pathogen in storage. Treating maize seeds using a suspension of endophytic bacteria *Bacillus* sp. from eggplant plant strains Bth-31a and Bth-22 decreased the infection rate of seed-borne fungal pathogens by 49.7% and 51%, respectively. The novelty of the research: (1) specific isolates of Bacillus sp Bth-31a and Bth-22 were isolated from eggplant and can decrease infection rate of maize seed-borne pathogenic fungi by 49.7% and 51% respectively using paper blotter test, (2) both of isolates can reduce the level of infection of 4 pathogenic fungi are *Fusarium* sp., *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus* sp. Research conducted by Hanif *et al.* (2016) stated that secondary metabolites of *Pseudomonas* sp. can reduce infection of *Fusarium* sp. by 65% using filter paper tests.

Pathogen control techniques using biological agents directly still have some drawbacks, namely having to consider the environment for the development of microorganisms, shelf life is not durable, preparation problems and deployment to several areas is less effective (Soesanto, 2017). Therefore, it is currently being developed to control seed-borne fungal pathogens using secondary metabolites produced by a microbe. The results of previous research by Fachrezzy (2022) will be the impetus for conducting this research by producing secondary metabolites of *Bacillus* sp. and then applied to the maize seed. So, it is expected that secondary metabolites of endophytic bacteria *Bacillus* sp. applied to maize seeds can be more effective and efficient than direct biological control agents.

The study aimed to obtain secondary metabolite concentrations from 2 Bacillus isolates, which can decrease the infection rate of maize seed-borne pathogenic fungi.

Method

Location and Time

This research was conducted from May to July 2023 at the Laboratory of Plant Health, Center for Seeds and Plantation Plant Protection (BBPPTP) Surabaya.

Tools and Materials

The tools used in this study included autoclaves, ovens, ose, tweezers, bunsen, analytical balances, laminar air flow, microscopes, test tubes, petri dishes, beaker glasses, erlenmeyer, tips, vortex, hot plates, magnetic stirrer, cell phone camera, tweezers, matches, label paper, ruler, pipette, glass funnel, slide, cover glass, IKA© KS 260 primary rotary shaker, TOMY MX-307 centrifuge, 0.2 µm syringe filter, Olympus CX31 microscope, and CKX41.

The materials used in this study included Merck KgaA NA (Nutrient Agar) media, Himedia M002 NB (Nutrient Broth) media, pure isolates of biological control agents for endophytic bacteria *Bacillus* sp. strains Bth-31a and Bth-22 from eggplant collection Dr.Ir. Arika Purnawati, MP., maize seed, Whatman No. filter paper. 42, fungicide propined 70%, alcohol 70%, spirits, distilled water, plastic wrap, cotton, rubber bands, tissue.

Extraction of Secondary Metabolites

Extraction of secondary metabolites of endophytic bacteria *Bacillus* sp. is a process to produce secondary metabolites from *Bacillus* sp. isolates strains Bth-31a and Bth-22 following the method of Elita *et al.* (2013), which has been modified. Bacterial isolates that were 48 hours old were harvested by adding 10 ml of distilled water. Then, the suspension was inoculated in 150 mL of NB (Nutrient Broth) media in each strain (Bth-22 and Bth-31a). NB media containing *Bacillus* sp. ensured the bacterial density was 1010 cfu/ml, then shaken using a shaker at 150 rpm for 48 hours. Furthermore, the method of separating the supernatant and the bacterial cell mass was followed by Utami (2011) namely, the bacterial culture on NB media was transferred into a centrifuge tube and then centrifuged at 3800

rpm for 20 minutes. The supernatant was filtered using a 0.22 µm millipore syringe filter. Then, the supernatant was diluted into 100 ml of sterile distilled water at each concentration (10%, 15%, 20%, and 25%) as a stock solution for seed treatment tests using the blotter test method.

Seed Treatment Test with Secondary Metabolite Compounds of Endophytic Bacteria Bacillus sp. using the Blotter Test Method

Testing of secondary metabolite compounds of endophytic bacteria *Bacillus* sp. with the blotter test method carried out on filter paper media. Maize seeds are sterilized first by soaking the seeds in a 1% Na-hypochlorite disinfectant solution for 1-2 minutes, then rinsed three times using sterile distilled water and dried using 3 or 4 sterile wipes in a petri dish (Badan Karantina Pertanian, 2007b). Seed treatment using secondary metabolites was carried out following Hanif (2015), namely seeds soaked in metabolites of endophytic bacteria *Bacillus* sp. with various concentrations, which were diluted in 100 ml of sterile distilled water. The seeds were soaked for 24 hours, after which they were dried. A total of 10 maize seeds were placed in a petri dish containing three sheets of sterile filter paper, which had been moistened using sterile distilled water. Each treatment was repeated three times and then incubated for seven days. Observations were made on the 7th day.

Data Collection Method

Data on maize seed-borne pathogenic fungi were collected by identifying the types of fungal pathogens that grow on the surface and around the seeds macroscopically and microscopically. Observations were made on the 7th day after incubation. The macroscopic and microscopic observations results were then matched with the identification book "Illustrated Genera of Imperfect Fungi 4th Edition" by Barnett and Hunter (1998).

Data on the infection rate is collected by counting the number of seeds grown by the fungal (Hanif, 2015). The formula for calculating the rates of fungal pathogen infections follows the Badan Karantina Pertanian (2007a).

$$\text{Infection rates (\%)} = \frac{n}{N} \text{ x } 100\%$$

n : Number of seeds infected with the pathogen
N : Total number of seeds observed

Data on decreased infection by fungal pathogens was collected using data from calculating the infection rate in each treatment and then comparing it with the control. The formula for calculating decreased infection by fungal pathogens follows:

$$\text{Infection-decreased (\%)} = \frac{C - N}{C} \text{ x } 100\%$$

C : Infection rate of control(%)
T : Infection rate of treatment (%)

Data on seed germination is collected by counting the number of seeds that germinate typically. The formula for calculating seed germination follows the Badan Karantina Pertanian (2007b).

$$\text{Seed germination (\%)} = \frac{g}{G} \text{ x } 100\%$$

g : Number of seeds germinate normally
G : Total number of seeds observed

Data analysis

Seed treatment testing using secondary metabolite compounds *Bacillus* sp. blotter test method using Completely Randomized Design (CRD). Research data derived from secondary metabolite tests were analyzed using R studio software with an ANOVA procedure. If the conclusion is obtained that the sig value < alpha (0.05), then the average difference between

treatments is tested using the DMRT test (Duncan Multiple Range Test) at a significant level of 5%.

Result and Discussion

Identification of Seed-borne Fungal Pathogen on Maize Seed

The results of the observation of the identification of seed-borne fungal pathogen on maize seed using the blotter test method were in the treatment of *Bacillus* sp., and fungicides contained colonies of *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium* sp. Meanwhile, the untreated maize seeds (control) contained colonies of *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* sp., and *Rhizopus* sp. According to Hausufa & Rusae (2018), the dominant fungal pathogen that infects a lot of maize seeds is *Fusarium* sp. and *Aspergillus* sp. Both can develop well in maize seeds' appropriate temperature and humidity storage.

Fusarium sp.

Observing the macroscopic characteristics of the Fusarium sp. shows that this fungi has a colony shape resembling cotton; the surface is rough, fibrous, wavy, and white. Colony diameter was about 7.5 cm on PDA media aged seven days (Fig. 1A). According to Sastrahidayat (2013), the morphological characteristics of *Fusarium* sp. that is, it has the form of insulated hyphae and creamy white colonies, under certain conditions it is pinkish to slightly purple. Morphological characteristics of *Fusarium* sp., according to Syaifudin (2020), are that it has white colonies on the top, the lower surface of the colonies is cream-colored, and the margins spread. Putra *et al.* (2020) also stated that *Fusarium* sp. has mycelium-like cotton.

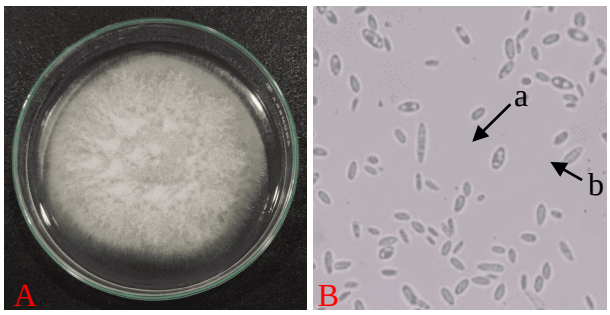

Figure 1. *Fusarirum* sp. (A) Macroscopic form on PDA media aged seven days; (B) Microscopic form (100x) (a) macroconidia (b) micronidia.

The results of observing the microscopic characteristics of *Fusarium* sp. with a 100x magnification microscope show that it has macroconidia and microconidia. Macroconidia are crescent and oval, with blunt apex and septate. The results of these observations are in line with Sastrahidayat's (2013) statement that *Fusarium* sp. has a structure consisting of microconidia, macroconidia, and chlamydospores. Macroconidia is long and curved, composed of 3-5 partitions. Meanwhile, 1-celled microconidia are round and not insulated. Chlamydospores are produced at the tips of old mycelium or in macroconidia, thick-walled, consisting of 1-2 cells. Based on the characteristics that have been observed, the fungi are suspected to be from the genus Fusarium.

Aspergillus flavus

The morphological form of the *Aspergillus* sp. has flat edges, hyphae septate, hyaline, and comprehensive. The conidia are erect, long, and accessible. The conidia are attached to the fialids, and the fialids are connected to the ends of the conidiophores, which experience swelling or are called vesicles (Hausufa & Rusae, 2018).

The results of observations of the macroscopic characteristics of *Aspergillus flavus* are that it has yellowish-green colonies with white edges and a rough, velvety, and granular colony surface (Figure 2A). According to Sukmawati *et al.* (2018), the morphological characteristics of *Aspergillus flavus* have green sporulation with white mycelium on the

edges. The texture of the colony is granular, and in the middle, the texture of the colony is floccose. It has a growing zone, does not have exudate drops, and has the color reverse of the gold colony.

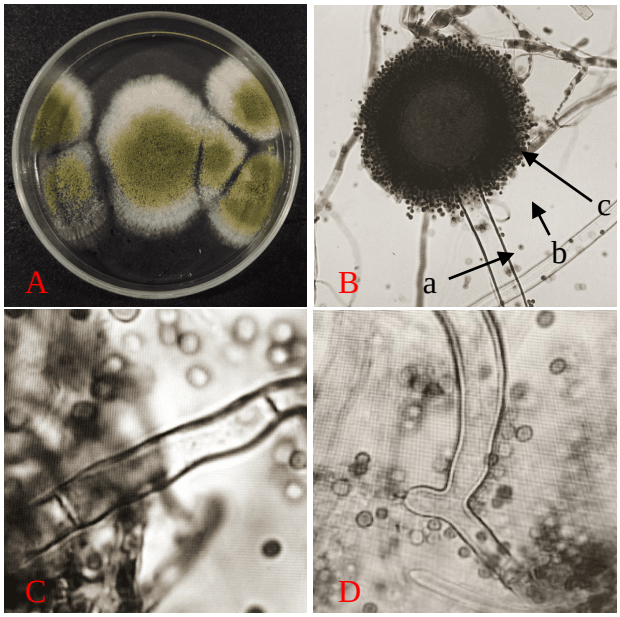

Figure 2. *Aspergillus flavus* (A) Macroscopic form on PDA media aged seven days; (B) Microscopic form (40x) (a) Conidiophores (b) Conidia (c) Vesicles; (C) septate hyphae; (D) Foot cells.

The results of observing the microscopic characteristics of *Aspergillus flavus* with a 40x magnification microscope showed that this fungus has long and cylindrical conidiophores, septate hyphae, round conidia, and round vesicles and belongs to the biseriate type (Figure 2B 2C 2D). *Aspergillus flavus* conidiophores can form two types of vesicles: biseriate and uniseriate (Cho *et al.*, 2022). Based on the characteristics that have been observed, the fungi is suspected to be *Aspergillus flavus*. According to Sukmawati *et al.* (2018), the microscopic characteristics of *Aspergillus flavus* are that it has a radial conidia head, the conidiophore looks upright and rough and has septa, there are cysts with a radial shape, the vesicles only look field, and the conidia are globose (round) with smooth edges, the conidia are 3.4 in size - 3.5µm.

Aspergillus niger

The results of observing the macroscopic characteristics of the *Aspergillus niger* on PDA media had black colonies, granular colony texture (Figure 3A). According to Sukmawati *et al.* (2018). The morphological characteristics of *Aspergillus niger* are black sporulation, white mycelium, no exudate granules, and granular-floccose colony texture.

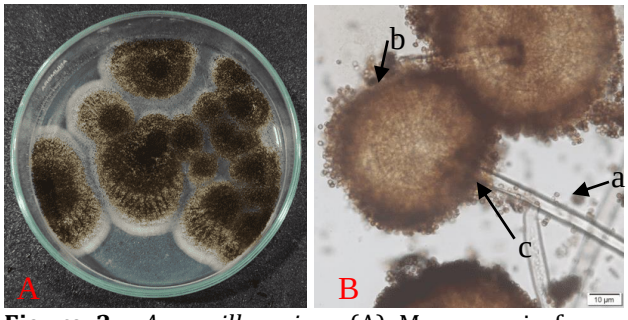

Figure 3. *Aspergillus niger* (A) Macroscopic form on PDA media aged seven days; (B) Microscopic form (40x) (a) Conidiophores (b) Conidia (c) Vesicles.

The results of observing the microscopic characteristics of the *Aspergillus niger* with a 40x magnification microscope show that this fungus has conidia heads that are spread out and round in shape, the entire surface of the vesicles is covered by metal, the cysts are round (Figure 3B). Based on the characteristics that have been observed, the fungi are suspected to be *Aspergillus niger*. According to Sukmawati *et al.* (2018), the microscopic morphology of *Aspergillus niger* is that it has conidial heads that radiate, vesicles globose (round), including Aspergillus type biseriate, metal covers the entire surface of the vesicles, and conidia are globose (round).

Rhizopus sp.

Results of observations macroscopic of *Rhizopus sp.* PDA media has characteristics such as white cotton covering the entire surface of the media and black spores (Figure 4A). According to Sobianti *et al.* (2020), the macroscopic characteristics of Rhizopus sp. are that it has white colonies and turns brown with the increasing age of the culture. *Rhizopus* sp. has black-brown sporangiospores, the mycelium color is white, and the texture of the sporangiophores is smooth (Stia & Aziz, 2011).

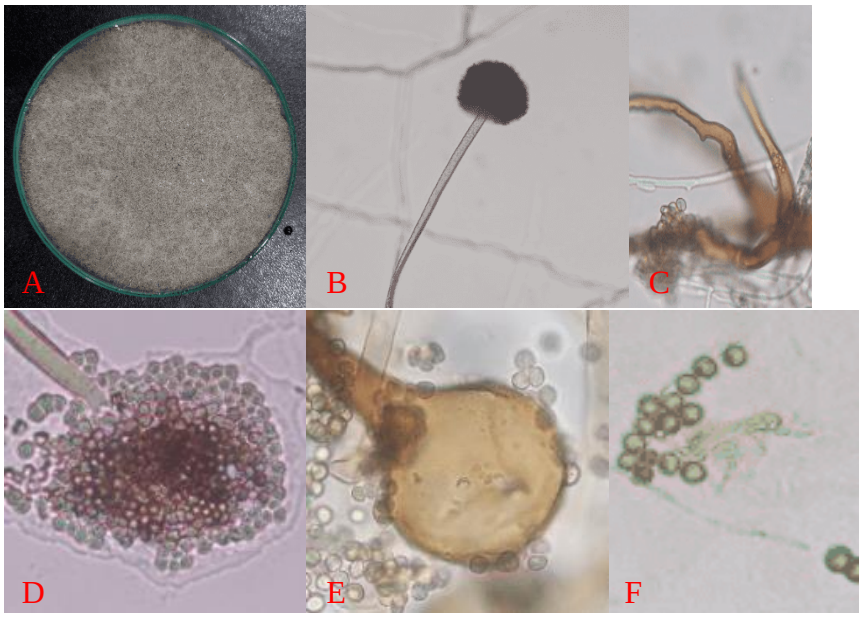

Figure 4. *Rhizopus* sp. (A) Macroscopic form on PDA media aged seven days (B) Sporangiophore (40x) (C) Branched rhizoid (40x) (D) Sporangium (100x) (E) Spherical columella (100x) (F) Sporangiospores (100x).

The results of observing the microscopic characteristics of *Rhizopus* sp. under the microscope, this fungi has a sporangiophore that grows opposite to the rhizoid, a round columella, a rounded sporangium, and round sporangiospores. Based on the characteristics that have been observed, the fungi are thought to belong to the genus Rhizopus. According to Sobianti *et al.* (2020), the microscopic characteristics of *Rhizopus oryzae* include columella, sporangium, and sporangiophore. The spores are black, and the shape of the spores resembles that of a round or oval. The sporangiophore grows upwards and contains a sporangium. The sporangium is black, and the columella is round.

Infection Rates (%)

The analysis of variance (ANOVA) showed that the treatment of secondary metabolites *Bacillus* sp. had a natural effect on the infection rates of the seed-borne fungal pathogen on maize seed using the blotter test method. Based on (Table 1) it can be seen that the rate of infection given by *Bacillus* sp. Bth-31a and Bth-22 strains showed no significant difference. However, both significantly differed from the control treatment, with an infection rate of 93.3%. Treatment of soaking maize seeds with metabolites of *Bacillus* sp. Bth-22 strain with

a concentration of 25% gave the lowest infection rate, namely 46.7%. Then, the treatment of secondary metabolites, *Bacillus* sp. Bth-31a concentration of 25% and Bth-22 concentration of 20% gave an infection rate of 50%, which is the same value as propined fungicide treatment. The treatment of secondary metabolites gave the highest infection rate, *Bacillus* sp. found in the Bth-31a treatment at a concentration of 10%, with an infection rate of 63.3%.

Table 1. Effect of Secondary Metabolites *Bacillus* sp. on Infection Rates

No.	Treatments	Infection rates (%)
1	Control	93.3a
2	Fungicide	50.0b
3	B-31a 10%	63.3b
4	B-31a 15%	60.0b
5	B-31a 20%	53.5b
6	B-31a 25%	50.0b
7	B-22 10%	56.7b
8	B-22 15%	53.3b
9	B-22 20%	50.0b
10	B-22 25%	46.7b

Note: Numbers followed by the same letter in the same column are not significantly different from Duncan's test at the 5% level.

The results of observing the symptoms of infection showed clearly visible growth of the fungal mycelium on the seed surface of the infected seeds (Figure 5). This observation was carried out on the 7th day after incubation.

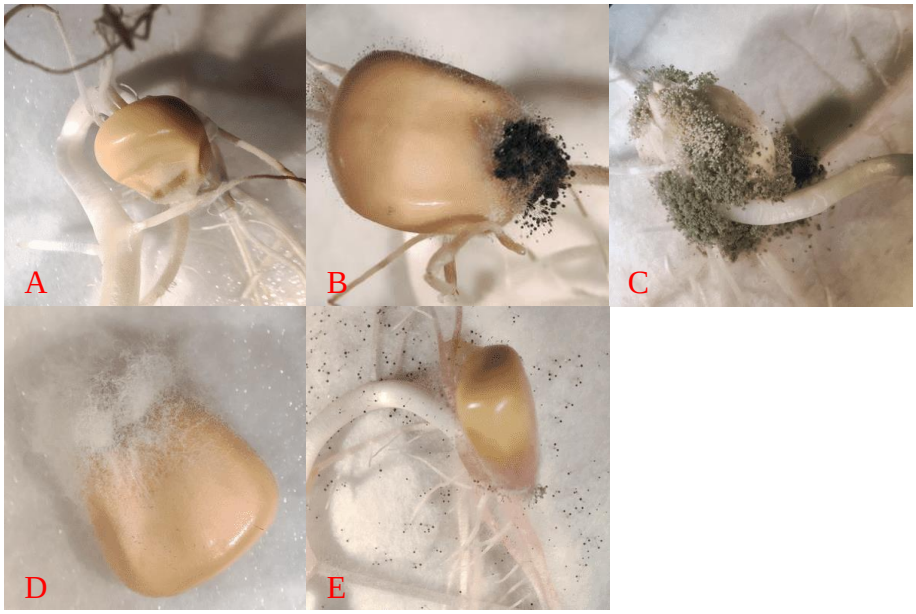

Figure 5. Symptoms of fungal pathogen infections in maize seeds (A) Normal seeds are not infected (B) *Aspergillus niger* (C) *Aspergillus flavus* (D) *Fusarium sp.* (E) *Rhizopus sp.*

Infection-Decreased (%)

The value of the infection rate in each treatment can be used as a calculation to decrease the infection rate compared to the control. Treatment of secondary metabolites *Bacillus* sp. and fungicides showed no significant difference in reduced infection to maize seed-borne pathogens (Figure 6). Maize seed immersion treatment using metabolite compounds *Bacillus* sp., the Bth-22 strain with a concentration of 25%, gave the highest percentage of decreased infection rate, which was 50% compared to the control. Then, the treatment of secondary metabolites *Bacillus* sp. Bth-31a at a concentration of 25% and Bth-22 at a concentration of

20% decreased the same infection rate with the fungicide treatment, which was 46.4% compared to the control. The lowest decrease in the infection rate was given by the secondary metabolite treatment of *Bacillus* sp. found in the BTH-31a treatment with a concentration of 10%, with an infection rate of 31.1%. The higher the decreased value of the infection rate, the more effective it is to reduce the infection rate of maize seed-borne pathogens. Zahara *et al.* (2021) proved that the growth of the fungal pathogen was increasingly inhibited as the concentration of the *Bacillus* sp. secondary metabolites used increased.

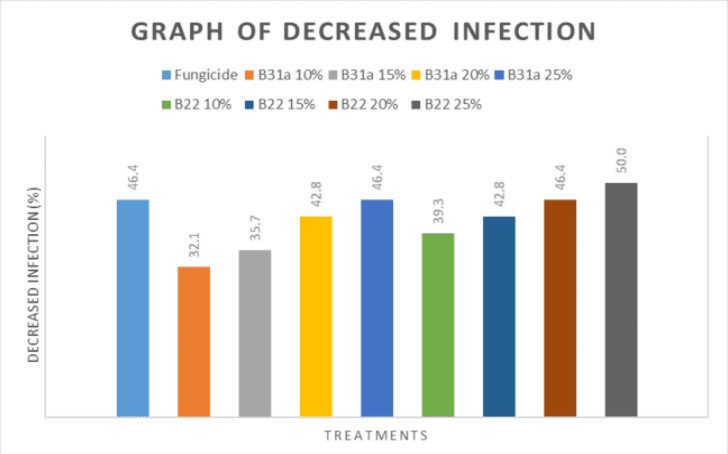

Figure 6. Graph of Effect Secondary Metabolites *Bacillus* sp. on Decreased-Infection

The decrease in infection seed-borne fungal pathogens on maize seed is thought to be due to antibiotic compounds from secondary metabolites of the bacterium *Bacillus* sp. The effect of using secondary metabolites *Bacillus* sp. with various concentrations not only inhibits the growth of fungi but also can cause abnormal growth of the fungal structure, namely hyphae. Hanif *et al.* (2019) stated that the fengisin compound produced by *Bacillus* sp. can damage *Fusarium graminearum* hyphae and change their shape to become thin and crooked. Some parts along the hyphal wall are broken. The bacillomycin-D compound can injure cell walls and cell membranes as well as hyphae and spores, then the cytoplasm and organelles inside the cell so that it can cause cell death (Gong *et al.*, 2014). The compound iturin a from *Bacillus amyloliquefaciens* can prevent the germination of fungal conidia, causing cell walls and cell membranes to be destroyed, causing cell dysfunction due to swelling of the mitochondria of *Fusarium oxysporum* (Wang *et al.*, 2022).

Seed Germination (%)

The analysis of variance showed that the treatment of secondary metabolites *Bacillus* sp. significantly influences the germination of maize seeds. Based on (Table 2) it is known that the treatment of secondary metabolites *Bacillus* sp. gave a percentage of germination between 70.0% to 86.7% and was very significantly different compared to the control with a germination rate of 56.7%. Secondary metabolites of *Bacillus* sp. strain Bth-22 concentration of 25% gave the highest germination percentage.

Bacillus sp.'s ability to increase germination percentage is due to *Bacillus sp.'s* capability to produce compounds that can stimulate seed germination. Thus, the germination of seeds treated with metabolites could be better than that of the control. According to Puspita *et al.* (2018), *Bacillus* sp. is a bacteria capable of producing growth hormones such as IAA.

Table 2. Effect of Secondary Metabolites Bacillus sp. on Seed Germination

No.	Treatments	Seed Germination (%)
1	Control	56.7a
2	Fungicide	76.7bc
3	B-31a 10%	70.0ab
4	B-31a 15%	73.3bc
5	B-31a 20%	80.0bc
6	B-31a 25%	83.3bc
7	B-22 10%	70.0ab
8	B-22 15%	76.7bc
9	B-22 20%	83.3bc
10	B-22 25%	86.7c

Note: Numbers followed by the same letter in the same column are not significantly different from Duncan's test at the 5% level.

As a plant growth-promoting agent, PGPR bacteria can produce growth-promoting hormones such as IAA, gibberellins, and cytokinins. Bacteria that can produce IAA will produce phytohormones that can accelerate plant growth (Herlina, Pukan, & Mustikaningtyas, 2016). The hormones auxin, gibberellins, and cytokinins are known to function in breaking seed dormancy. In addition, the presence of this growth hormone can accelerate the permeability of water entry into the seed so that the seed germinates more quickly. According to Puspita et al. (2013), IAA can stimulate cell division and accelerate the absorption of water and nutrients. So, it will affect plant growth.

Conclusions

All seed treatments used secondary metabolites of Bacillus sp. can decrease the rate of the seed-borne fungal pathogen on maize seeds compared to the control. In addition, treating secondary metabolites of Bacillus sp. can increase the germination of maize seeds. The secondary metabolite of the Bth-22 strain with a concentration of 25% was the best treatment, having the highest suppression percentage of 50% and germination of 86.7%.

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Declaration statement

The authors reported no potential conflict of interest.

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