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Bio-Priming with *Trichoderma* spp. to Suppress *Aspergillus flavus*, the Causal Agent of Damping-off Disease in Peanuts

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Abstract

Background: *Aspergillus flavus* is a fungus that produces aflatoxin, a toxic compound that can contaminate food products, especially nuts. The impact caused by *A. flavus* results in significant losses for peanut-producing countries in international trade. *A. flavus* infection in peanuts causes physical changes, quality, and a decrease in seed germination. Environmentally friendly control efforts for *A. flavus* can be implemented by utilizing biological agents, such as *Trichoderma* spp. This study aims to evaluate the ability of *T. harzianum* and *T. asperellum* to inhibit the growth of *A. flavus*. This study also evaluates *bio-priming* methods enriched with *Trichoderma* spp. to optimize peanut seed germination. **Method:** The research was conducted using a factorial Completely Randomized Design (CRD) consisting of 2 factors. The first factor is the isolate species *Trichoderma* spp. namely *T. harzianum* (T1), *T. asperellum* (T2), and Control (T0), namely *A. flavus* without biological control treatment. The second factor is the application method, which includes the bio-priming (B1) and bio-matrix *priming* (B2) methods. Each treatment combination was repeated 4 times, and the number of seeds used was five seeds in each unit. **Result:** *T. harzianum* showed the highest inhibitory ability against *A. flavus* in the in vitro test, namely 49%. *Bio-priming* treatment containing the active ingredient *T. harzianum* is the best treatment in increasing peanut germination, namely by 95%. **Conclusion:** The research results showed that *bio-priming* treatment with the active ingredient *T. harzianum* was able to increase the germination rate significantly compared to other treatments.

Keywords: *Aspergillus flavus*; Antagonist; Germination power; *Trichoderma* spp.

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Introduction

Peanuts are a food-crop commodity that is a superior product on the European continent, especially in the Netherlands. During the period 2018 to 2022, European peanut imports increased by 1.7% or 1.24 million tonnes annually (CBI, 2023). To meet the demand for peanuts, Europe establishes product quality standards and implements several regulations that importing countries must fulfill. General rules that must be complied with in peanut export activities include being free from mycotoxin contaminants, free from microbiological organisms, and free from pesticide residues. One of the strict regulations that causes serious problems for peanut-producing countries is mycotoxin contamination, especially aflatoxin. Aflatoxin is a toxic compound produced by the fungus *Aspergillus flavus,* which can cause health problems for livestock and humans if consumed in large doses. WHO (2023) reports that the maximum limit for aflatoxin levels set by the Codex Alimentarius Commission (CAC) in nuts is 15µ /kg. One of the most significant cases, due to high aflatoxin levels and resulting in export rejection, occurred in African

countries and was exported to Europe in 2000, with a product rejection percentage of 64% (Gbashi *et al*., 2019).

Aspergillus flavus is an aflatoxin-producing pathogen that frequently infects oil-containing crops. The *A. flavus* fungus will infect agricultural plants that contain oil, such as corn, peanuts, and cotton seeds, during planting, harvesting, storage, and processing (Amare & Keller, 2014). The presence of *A. flavus* in peanut seeds can cause changes in the physical shape of the seeds, the surface of the seeds being covered with spores, decreased seed viability, stunted plant growth, and further attacks, causing the shoots to fall off (Bulele, 2019; Pinaria & Assa, 2017). An infection percentage of 0.27% is the tolerance limit for infection because it can cause a reduction in peanut germination to 71% (Begum *et al.*, 2013).

Information on the control of aflatoxin-producing *A.flavus* is currently still minimal, but prevention efforts continue to be made. Prevention efforts currently being implemented include using varieties that are tolerant to Aspergillus sp. drying, gamma ray irradiation, spacing, seed coating, and the application of synthetic pesticides (Nurtjahja *et al*., 2018; Rahayuningsih *et al*., 2022; Sembiring *et al*., 2020). A control measure often employed to overcome this problem is the use of pre-planting seed treatment with fungicides. However, pesticide application is not the best choice because it can disrupt the environmental balance and promote the growth of virulent pathogens. An alternative control strategy for the presence of *A. flavus* and aflatoxin contamination that is effective, efficient, and environmentally friendly can be implemented by utilizing biological agents.

Trichoderma spp. is a biological control agent that employs a complex array of antagonistic mechanisms, including competition for space and nutrients, parasitization, and antibiosis, to inhibit the growth of pathogens. Research results Sari (2022) indicate that *Trichoderma* spp. Effective in inhibiting the growth of *Ralstonia solanacearum* wilt bacteria with an antibiosis mechanism, which is characterized by the presence of a clear zone, and is also known to have a competition mechanism for space and nutrients. Apart from acting as a controlling agent, Trichoderma is a fungus that serves as a PGPF (Plant Growth-Promoting Fungi), stimulating plant growth. *Trichoderma* can produce auxin in the form of *Indole Acetic Acid* (IAA), which optimizes the potential of roots to absorb nutrients and water, thereby increasing growth (Fitria *et al.,* 2021). Although *Trichoderma* spp. have been widely studied for plant disease control, studies on their specific role *in bio-priming* against *A. flavus* in peanut seed remain limited, particularly in relation to seed germination dynamics and antagonistic mechanisms *in vitro* and *in vivo* conditions.

The application of Trichoderma to pre-planting seeds can be done using the priming method. Priming is a method of seed hydration that can activate enzymes in the seeds so that they can then give rise to embryos and produce uniform germination. *Bio-priming* is a method of applying beneficial microorganisms that can enter into symbiosis with plants, both in supporting plant growth and protecting plants by activating plant hormones in the seed germination phase (Forti *et al*., 2020; Yadav *et al*., 2018). (Ferrigo *et al.*, 2020)-reported that the application of *T. harzianum* using the *bio-priming* method was able to reduce the symptoms and severity of disease in corn plants infected with *F. verticilioiders* by 69-84%.

Bio-priming treatment containing the active ingredient *Trichoderma* spp. on seeds aims to stimulate the seeds before they grow from *A. flavus* infection in the early germination period. Unlike previous studies that focused solely on antifungal activity, this research integrates *bio-priming* technology, morphological analysis, and seed vigor evaluation to explore a holistic approach to biological control in peanut cultivation. This study investigates explicitly the comparative effectiveness of *T. harzianum* and *T. asperellum* in controlling *A. flavus* and promoting seed germination through *bio-priming* techniques.

Methods

Research Design

This research used a Completely Randomized Factorial design consisting of 2 factors. The first factor is the isolate species *Trichoderma* spp. as a controlling agent for the pathogen *A. flavus*. Each treatment unit consisted of a 15×15cm polybag filled with 600 g of sterilized media. Observations were recorded on day 4 for in vitro antagonism, and at 7 days after sowing for germination tests. The *Trichoderma* species used were *T. harzianum* (T1), *T. asperellum* (T2), and control (T0), namely *A. flavus* without control agent treatment. The second factor is the application method, which includes the bio-priming (B1) and bio-matrix *priming* (B2) methods. Based on these treatments, six treatment combinations were obtained, and each treatment was repeated four times. Data were analyzed using Analysis of Variance (ANOVA). If it is known that there is a real effect from the treatment, then a further test is carried out using the Honestly Significant Difference Test (BNJ) at the 5% level.

Preparation of Fungal Isolate

The *Aspergillus flavus* fungus isolate is the result of a collection from BALITKABI and the *Trichoderma* spp. Isolate collection from the Plant Health Laboratory, Faculty of Agriculture, UPN Veteran, East Java. The isolate to be used is first rejuvenated in PDA medium. Rejuvenation and multiplication of fungi are carried out by taking one piece of the parent colony using a 5 mm cork borer, then culturing it in new PDA media, and then incubating it again for 7 days at room temperature.

Preparation and inoculation of the Pathogen Aspergillus flavus

Pathogen inoculation was carried out in the planting media 7 days before planting. The planting medium used is a 1:1 ratio of soil and compost. Before pathogen inoculation, the soil is first sterilized with high-pressure hot water steam. Next, the sterile soil is placed into a 15×15 polybag until it is full, approximately 600 g per polybag.

Making the *A. flavus* suspension begins by taking pieces of the *A. flavus* isolate, then placing them in 10 ml of sterile distilled water and homogenizing. Next, the spore density was calculated using an *Improved Neubauer Hemocytometer* until the desired density was obtained, namely 10^5 spores/ml. After obtaining the desired density, inoculation can be carried out in the planting media with a ratio of 120 ml to 600 g (Ayu *et al*., 2012). After inoculation, the planting medium can be incubated for up to ± 7 days.

Preparation and Application of Bio-priming and Bio-matrix priming in Peanuts

Making suspensions as *bio-priming* and *bio-matrix priming* application media was created using SPE (Sugar Potato Extract) media. Then, the SPE media that had been prepared were supplemented with pieces of each Trichoderma species. Isolate until a density of 10^6 spores/ml is obtained. To achieve the desired density, the suspension was made using the multistage dilution method. Preparation of *bio-matrix priming application media is done by mixing a suspension of Trichoderma spp. that has been made into sterile husk charcoal powder, passing it through a 0.5 mm sieve, with a ratio of 4 g of husk charcoal powder to* 7 ml of suspension. Furthermore, stirring can be continued until the mixture is well mixed.

The seeds to be used as test material are surface-sterilized using 1.5% sodium hypochlorite (NaOCl) for 6 minutes, then rinsed three times with sterile distilled water (Al-Amodi, 2016). Seed treatment with the *bio-priming* method is carried out by mixing sterile seeds into *bio-priming* media in a ratio of 10:25 (seeds/ml). After that, it can be shaken for 24 hours at a speed of 150 rpm. *Bio-matrix priming* treatment is carried out by mixing sterile seeds into the application medium evenly over the entire surface of the seed in a ratio of 10:4:7 (seed: g: ml). Furthermore, it can be incubated for 24 hours in a

tightly closed jar and stirred after 12 hours. After that, the treated seeds can be dried by aerating in a Laminar Air Flow.

Antagonist Test

Antagonistic testing of *Trichoderma* spp. against *A. flavus in vitro* was done by the diffusion technique (pits) with the dual culture method on PDA media. The first step is to create two wells on PDA media using a 5 mm diameter cork borer. Then the suspension of *Trichoderma* spp. and *Aspergillus flavus* suspension of as much as ± 100 µL was inoculated into the media pits face to face with a distance of 3 cm. As a control treatment, it can be done by growing each fungus in a different Petri dish. After that, incubation can be done at room temperature for 4 days (Darmayasa & Oka, 2016). After 4 days, the inhibition ability can be observed by measuring the colony area.

The inhibition ability produced by *Trichoderma* spp. Biological agents against *A. flavus* fungi in the in vitro test can be calculated based on the area of inhibition. The calculation of the inhibition area can be performed using millimeter paper, after which the measurement results are substituted into the Percentage Inhibition of Radial Growth (PIRG) formula (Tasik *et al*., 2015).

$$C\ (\%) = \frac{a-b}{a} \times 100\%$$

Description:
C = inhibition
, a = control area (*A. flavus* colony).
b = colony area of *A. flavus* with *Trichoderma* spp.

The percentage of inhibition produced is used to determine the classification of antifungals, according to Zivcovic et al. (2010).

Table 1. Categories of antifungal activity

Barriers (H) (%)	Antifungal Activity
0	Very weak
0 < H ≤ 25	Weak
25< H ≤ 50	Medium
50 < H ≤ 75	Strong
>75	Very Strong

Seed Germination Test

Seed germination tests can be carried out by planting seeds that have been given the previous treatment. Planting is done in a planting medium that has previously been inoculated with pathogens. Each unit of groundnut planting was done with as many as five seeds. The calculation of the percentage of seed germination is done by calculating the seed cotyledons that appear on the surface of the soil. According to Ali & Sonia (2021), the calculation of seed germination percentage can be calculated using the following formula:

$$x = \frac{y}{z} \times 100\%$$

Description:
x = germination rate (%)
y = number of germinated seeds
z = number of all germinated seeds

Result and Discussion

Isolate Rejuvenation

*The Trichoderma harzianum and Trichoderma asperellum isolates exhibit distinc*t morphological characteristics. The macroscopic morphological characteristics of *T.*

harzianum are that it has dark green colonies and a circular shape with clear colony boundaries. This is by the statement of Sandy *et al.* (2015) that *T. harzianum* has green, round-shaped colonies that form 1-2 concentric rings when the mycelium has filled the cup.

Microscopically, *T. harzianum* has oval-shaped phialids that are short and thick, branched conidiophores, and round conidia (Figure 1). This is in line with the statement of Sriwati & Chamzurni (2014) that *T. harzianum* has microscopic characteristics, namely having branched conidiophores, short phialid stalks with conical ends, while the conidia are globular (round), and there are also oval-shaped ones.

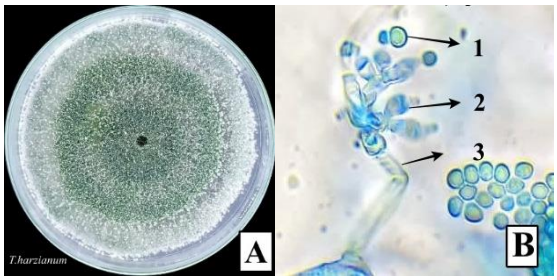

Figure 1. Colonies of the fungus *T. harzianum*: A) on PDA media, B). microscopic characters of *T. harzianum* 1) Conidia, 2) Fialid, 3) Conidiophore (1000× magnification).

The morphological characteristics of *T. asperellum* are macroscopically evident in colonies with an unsymmetrical shape at the edges, a light green to slightly yellowish center, and a white edge (Figure 2). This is the opinion of Oszako *et al.* (2021). *T. asperellum* has colonies with a circular shape forming concentric rings with smooth edges, and light green colonies in the middle with white edges. Microscopically, *T. asperellum* has leafy hyphae, round conidia, branched conidiophores, and phialids, which have a cylindrical shape with sharp ends and short stalks. This is in line with the statement of Oszako *et al.* (2021) that *T. asperellum* has branched conidiophores, cylindrical phialids resembling bottles, and round conidia.

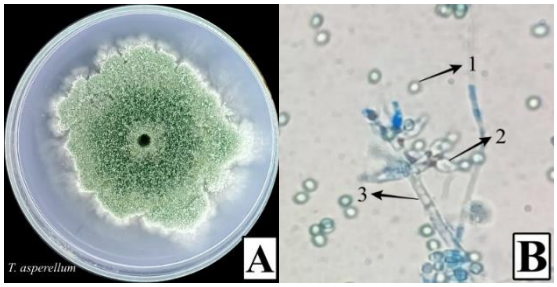

Figure 2. Colonies of *T. asperellum*; A) on PDA media, B). Microscopic characters of *T. asperellum*, 1) Conidia, 2) Fialid, 3) Conidiophore (1000× magnification).

A. flavus has the characteristics of compact, round colonies with a granular texture. It has a yellowish-green color in the middle of the colony and a white color in the young colonies at the edges of the colony. From microscopic observations, it can be seen that *A. flavus* has oval-shaped sterigmata, round-shaped conidia, and thick and long conidiophores (Figure 3). This is in line with the statement (Okayo *et al.*, 2020; Putra *et al.*, 2020; Putri *et al.*, 2022) that *A.flavus* has a characteristic yellowish-green colony with white edges, the surface of the colony is flat and thickened in the middle. Meanwhile, the microscopic characteristics include conidiophores, metulae, phialids, and vesicles, which are round to oval in shape.

The results of the in-vitro test of *T. harzianum* and *T. asperellum* antagonists against *A. flavus* with the dual culture method are known to inhibit the growth of *A. flavus*. Based on the observation on day 4, there was a significant difference in the ability of *T. harzianum* and *T. asperellum* to inhibit the growth of *A. flavus*. The *T. harzianum*

treatment showed higher results than the *T. asperellum* treatment in suppressing the growth of *A. flavus*. Based on these results, it can be seen that the *T. harzianum* treatment has a greater inhibitory ability compared to the *T. asperellum* treatment (Table 2).

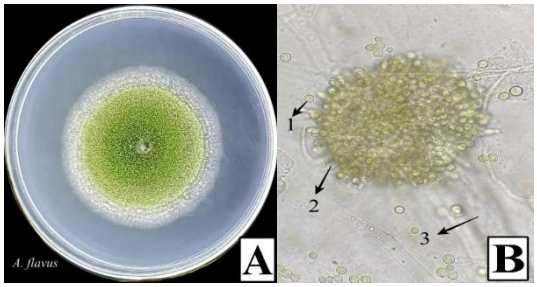

Figure 3. Morphology of *A. flavus* A) Colony of *A. flavus* on PDA media, B) Microscopic characters of *A. flavus*, 1) Conidia, 2) Sterigmata, 3) Conidiophores (1000× magnification).

Table 2. Results of the antagonistic test of *Trichoderma*spp. Against the average area of *A. flavus in vitro*

Treatment	Average Pathogen Area Day- (cm^2)				Inhibition (%)
	1	2	3	4	
T. harzianum	1,69 a	6, 38 a	9,94 a	12,94 a	49
T. asperellum	1,88 a	6,88 a	10,38 b	15,38 b	39
A.flavus (control)	2,44 a	7,81 b	13,94 b	25,25 c	0

Information: Numbers followed by the same letter in the same column show no significant difference based on the 5% BNJ test.

The difference in inhibition power possessed by each *Trichoderma* species is possible due to differences in the living environment, morphology, genetics, amount, and quality of antibiotics or substances produced (Herliyana *et al.*, 2013; Rizali & Sari, 2023). Sriwati & Chamzurni (2014) reported that *T. harzianum* species can survive and compete in obtaining space and nutrients that are relatively high when in the same growing environment as the pathogen.

The results of the antagonist test observations showed that the isolates of *T. harzianum* and *T. asperellum* were both able to inhibit the growth of *A. flavus,* as seen in Figure 4. The antagonist ability of *Trichoderma* spp. can be known based on the development of *Trichoderma* spp., which fills the petri dish so that it can inhibit the growth of *A. flavus* fungus. In addition, there is also an empty zone between the two isolates (Figure 4). *Trichoderma* can hinder the growth of pathogens through three mechanisms: competition for space and nutrients, hyperparasitism, and antibiosis, which can work alone or synergize to inhibit pathogens (Mukhopadhyay & Kumar, 2020).

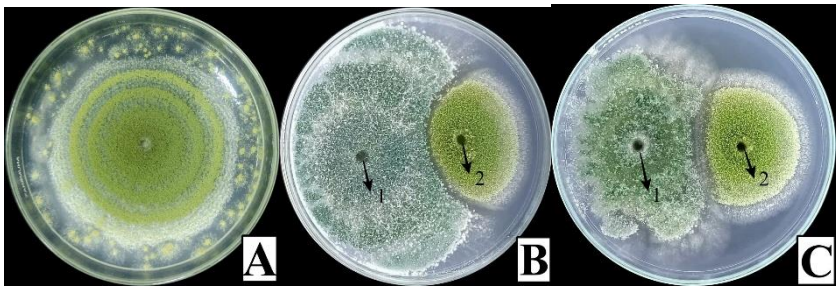

Figure 4. Inhibition of *Trichoderma*spp. Against *A.flavus* A). *A.flavus* control, B). Treatment (1) *T. harzianum*, (2) *A. flavus*, 3). Treatment (1) *T.asperellum*, (2) *A.flavus*

The results showed that the growth of *Trichoderma* spp. grew fast and dominated the petri dish space, indicating competition with pathogenic fungi to obtain growth space and nutrients. According to the opinion of Sood *et al.* (2020), *Trichoderma* spp. can utilize

nutrients from synthesized carbohydrates such as cellulose, chitin, glucan, and glucose, available in the growth medium. In addition, there is the growth of *Trichoderma* sp. and *A. flavus* pathogens that are not mixed, or there is an empty zone between the two isolates (Figure 4). According to Muhibuddin *et al.* (2021), the formation of this inhibition zone occurs due to antibiotic compounds produced by *Trichoderma* spp. The antibiotic compounds produced by *Trichoderma*spp. are harmonic acid, alamethicins, tricholin, peptaibols, 6-penthyl-α-phyrone, massoilactone, viridian, gliotoxin, glycoproteins, peptidic acid, trichodermin, dermadin, and others (Ningsih *et al.*, 2016).

Observation of the inhibitory power of *Trichoderma*spp. Microscopically, it can be seen that the hyphae of *Trichoderma*spp. are wrapped around the conidiophores of the fungus *A. flavus.* This shows that Trichoderma spp. also performs a mycoparasitic mechanism against pathogenic fungi (Figure 5). This is supported by the opinion of (Halifu et al. (2020) that one of the antagonistic mechanisms of *Trichoderma* sp. against pathogens is by mycoparasites. The ability of *Trichoderma* spp. to wrap around or make links with pathogen hyphae can cause abnormal pathogen hyphae, such as broken, swollen, and lysis.

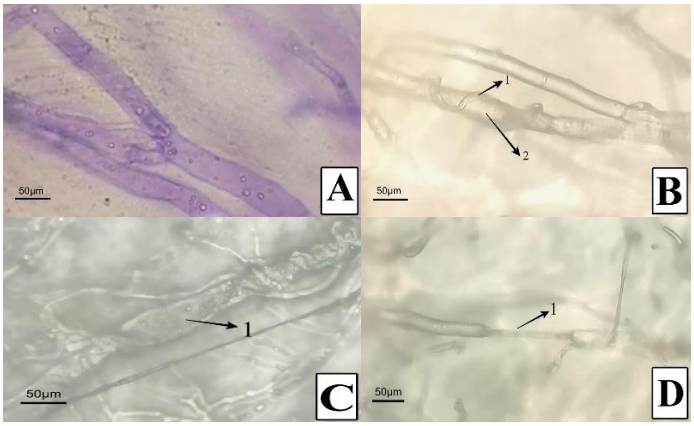

Figure 5. Microscopic observation of the effect of antagonist test A). Typical hyphae of *A.flavus*, B). Mycoparasitism (1) *Trichoderma* hyphae (2) *A. flavus* hyphae, C). Swollen hyphae of *A.flavus*, D). Lysed hyphae of *A. flavus* (1000x magnification)

According to Halifu *et al.* (2020), Trichoderma's ability to parasitize pathogens can occur due to its ability to identify lectins (proteins) secreted by pathogens and its ability to produce enzymes such as chitinase, cellulose, and xylanase. The enzymes produced by *Trichoderma* sp. play a role in degrading the cell wall of the pathogen, which supports the process of penetration of Trichoderma hyphae into pathogen hyphal cells to take nutrients, causing inhibition of growth and death of the pathogenic fungi.

Germination

Bio-priming and *bio-matrix priming* treatments of *Trichoderma* spp. showed significantly different results on seed germination compared to the control treatment. *Bio-priming* treatment containing active ingredients of *T. harzianum* showed the best results on seed germination ability of 95% (Table 3).

Table 3. Percentage of Seed Germination

Treatment	Germination (%)
Bio-priming + T.harzianum	95.00 b
Bio-priming +T. asperellum	90.00 b
Bio-matrixpriming + T. harzianum	90.00 b
Bio-matrixpriming +T. asperellum	85.00 ab
Priming (control)	60.00 a
Matrixpriming (control)	70.00 ab

Information: Numbers followed by the same letter in the same column show no significant difference based on the 5% BNJ test.

The results of the analysis of variance showed that the *bio-priming* treatment with the active ingredient *T. harzianum* was able to grow optimally and had resistance to the presence of *Aspergillus flavus*. The minimum value of seed germination is 80% (Tustiyani *et al.*, 2016). This ability demonstrates that the bio-priming method serves as a medium for inoculating the biological agent *Trichoderma* spp. demonstrated its ability to protect and induce seed germination resistance from *A. flavus* infection. The microbial population applied using the *bio-priming* method will be available and active on the seed surface in sufficient quantities to protect the seed during the germination process before it interacts with pathogens (Yadav *et al.*, 2018). Many researchers have stated that *bio-priming* treatment can be effectively utilized in the application of biological agents because it maintains a sufficient presence of biological agents on the surface and inside the seed, which can affect plant growth and development (Roy *et al.*, 2022).

During the priming process, there is a slow hydration process by the seed that allows *Trichoderma* to repair seed cells to increase the potential for normal growth and reduce the potential for abnormal sprouts. Priming treatment can produce beneficial interactions, allowing microbes to utilize cell leakage material in seeds during treatment to produce specific hormones that support seed germination (Zulueta-Rodriguez *et al.*, 2015). *Trichoderma* spp. can produce hormones that can increase plant growth, including auxins in the form of Indole Acetic Acid (IAA), which can increase plant growth and development, including root elongation to increase the role of roots in absorbing nutrients and water from the soil optimally (Fitria *et al.*, 2021).

Conclusions

The results of the study indicate that the in vitro treatment of Trichoderma spp. with the antagonist can inhibit the growth of *A. flavus*. The inhibition mechanism of *Trichoderma* spp. against *A. flavus* involves competition for nutrients and space, antibiosis, and mycoparasitism. The highest inhibition occurred in the *T. harzianum* treatment, which amounted to 49%. *Bio-priming* treatment containing *T. harzianum* active ingredients optimized germination up to 95%.

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Declaration statement

The authors reported no potential conflict of interest.

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