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A Study on Inhibitory Effect of *Trichoderma* sp. TKD on *Aspergillus flavus* FNCC6109 and Its Molecular Identification

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ABSTRACT

Aspergillus flavus is one type of fungus that often cause problems in the field of agriculture and animal husbandry. Biological control is necessary in order to reduce the use of synthetic fungicides which have good side effect on humans and the environment. Exploratory research that has been done shows a candidate isolate potential to inhibit the growth of Aspergillus flavus. These isolates, isolated from the rhizosphere of corn and is named Trichoderma sp. TKD. The purpose of this study was to determine the species of Trichoderma sp. TKD and its ability in inhibiting the A. flavus FNCC6109. Identification of Trichoderma sp. TKD was done macroscopically, microscopically and molecularly using internal transcribed spacer region (ITS 5 F: 5 '- ¬GAAGTAAAAGTCGTAACAAGG-- 3' and ITS 4 R: 5 '- TCCTCCGCTTATTGATATGC - 3'). The test of the inhibitory power used dual culture method. The results showed Trichoderma sp. TKD isolated from the rhizosphere of corn was identified as Trichoderma asperellum and had the potential to inhibit the growth of A. flavus FNCC6109 at 98.849±1.100%.

Keywords: *Aspergillus flavus FNCC6109, Trichoderma sp.TKD, Aflatoxin*

INTRODUCTION

Aspergillus flavus is one type of fungus that often cause problems in the field of agriculture and animal husbandry. This fungus is able to produce aflatoxin noxious plants, animals and humans. In conditions of relative humidity of 85% and a temperature of 25°C to 35°C, *A. flavus* excellent growth to produce aflatoxin[1]. Many aflatoxins have been known, but this fungus only produces aflatoxin B1 and B2[2]. Aflatoxin B1 is the most potent aflatoxin poison power[1]. In high concentrations aflaktoxin B1 can cause death. Whereas at low concentrations in the long run can cause necrosis of the liver and kidneys[3,4].

This fungus is believed to have a very strong resistance in extreme conditions and very easy to compete with other organisms. While control this fungus has done is to use chemicals such as sodium calcium aluminosilicate, a combination of propionic acid and nisin[5,6]. Chemical control, besides the costs, is also feared that residue can interfere with human health posed.

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Based on this, it is necessary to control the biological agent by searching for potential candidates isolate microorganisms to suppress the growth of fungi *A. flavus*. Results of preliminary research that has been done, gained 5 isolates isolated from samples of tempe, yeast tape brands NKL, saliva of Bali cattle, fresh milk and rizospher potency as an antagonist against *A. flavus* FNCC6109. Early identification is obtained *Trichoderma* sp. TKD most potential to inhibit the growth of *Aspergillus flavus* FNCC6109. *Trichoderma* sp. was believed to have the ability to inhibit other microorganisms, so widely used as biocontrol[7]. Soesanto *et al*.[8] reported some isolates of *Trichoderma* sp. same grown on different media (PDA and MEA) gave colonies of different morphological characters[8]. And if identified to a level *Trichoderma* species based solely on morphological characteristics of the colony alone, can lead to errors in identify it. Therefore, the analysis of genes is necessary, thus standing between *Trichoderma* species can be seen clearly.

MATERIALS AND METHODS

Isolation of *Trichoderma* sp. TKD on Rhizosper of Corn

Trichoderma sp.TKD isolates obtained from rhizosper of corn were isolated using platting through serial dilution method[9]. The steps taken is to weigh as much as 10 g soil sample put in 90 mL of sterile saline to obtain 10^{-1} dilution. then homogenized with a vortex. 5 prepared test tube containing 9 mL of saline and 6 sterile Petridish. To get a 10^{-2} dilution is done by taking 1 mL of 10^{-1} dilution put in 9 mL of normal saline so forth until dilution 10^{-4}. Each dilution was taken 1 mL inserted into each sterile Petridish which have been labeled from 10^{-1} to 10^{-4} and then pour the MEA medium and incubated at 28^{o}C for 4 days. Fungal colonies growing on media observed morphological traits, further purification by taking one loop on the fungal colonies. Early identification of macroscopic and microscopic fungi, referring to the determination book Pit and Hocking[2].

Identification of *Trichoderma* sp. TKD

Pure culture isolates of *Trichoderma* sp. TKD identified macroscopically and microscopically based on morphological characteristics and pigmentation on MEA media. While the molecular basis of *Trichoderma* sp. TKD workmanship using internal transcribed spacer region (ITS), which consists of ITS 1 and ITS2 and 5.8S rRNA which aims to identify *Trichoderma* to the level of species[10].

Molecular Identification of *Trichoderma* sp. TKD

Isolation of genomic DNA

The isolation of genomic DNA from *Trichoderma* sp. TKD was done by following the methods of Sambrook *et al.*[11], that is, the fungus *Trichoderma* sp. TKD growing on MEA media its hyphae was taken then suspended in 2 mL of sterile water. A total of 1.5 mL suspension of the fungus was put into a 1.5 mL Eppendorf then it was centrifuged at a speed of 8000 rpm for 10 minutes, the supernatant was discarded and the pellet formed was washed with STE buffer (composition: 0.3 M sucrose; 25 mMTris-HCl; 25 mM EDTA.2Na pH 8), then centrifuged at a speed of 8000 rpm for 10 minutes. The pellets were washed 3 times repeatedly. Subsequently the supernatant was discarded and the pellet obtained was added 200 mL STE buffer and 45 mL lysozyme (20 mg/mL) slowly inverted and incubated at 55^{o}C for 1 hour to form protoplasts. A total of 20 µL proteinase-K (20 mg/mL) was added to the mixture and incubated at 55ºC for 60 minutes. After it was added 400 µL of 10% CTAB in 0.7 M NaCl solution and then incubated at 65^{o}C for 30 minutes. Then to the solution was added 1 time the volume of phenol: chloroform (25:24) and centrifuged at the speed of 12000 rpm for 10 minutes. The clear phase was transferred to a new tube and added 0.6 time the volume of isopropanol and 20 mL of sodium acetate with a concentration of 50 mM, and incubated at a temperature of -20ºC for 12 hours. Then,it was centrifuged at 12,000 rpm for 10 minutes. Part of supernatant was discarded while the pellet was washed using 70% alcohol of 1 mL. DNA was dried for 1 hour to dispose of the alcohol and then dissolved in 50 µL of ddH2O (doubledestilation H_2O) sterilized then stored at 4^{o}C or -20^{o}C[11].

DNA Amplification by PCR (Polymerase Chain Reaction)

Identification of fungal isolates was conducted molecularly based on genetic analysis using internal transcribed spacer region (ITS), which consists of ITS 1 and ITS2. PCR amplification using ITS 5 F: 5`-3` GGAAGTAAAAGTCGTAACAAGG- and ITS 4 R: 5`-3` TCCTCCGCTTATTGATATGC-[12]. Amplification was done by machine Polymerase Chain Reaction (Perkin Elmer GeneAmp PCR System 2400, Germany) on a volume of 25 µL with reaction composition: 10 µL nuclease free water, Go taqgreen mastermix^{TM} 12.5 µL, ITS5 and ITS4 respectively 0.5 µL, 0.5 µL DMSO and 1 µL DNA template. PCR amplification of ITS region consists of: pre-denaturation 95ºC for 90 seconds, then 95ºC for 30 seconds with 35 cycles, annealing 55ºC in 30 seconds, extension 72ºC in 90 seconds, and a final extension 72ºC for 5 minutes. The resulting product was purified to continue sequencing stages.

Sequencing Analysis

Analysis of the base sequence of nitrogen readings used the services of PT. Macrogen, Korea by means of automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The raw data of sequencing results were then trimmed and assembled using Chromas Pro program version 1.5. Data that had been assembled were subsequently BLASTed with genomic data that had been registered with NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Some sequence data of the blast result of that are the closest species and Type Strains of each species are taken from the data bank of genes in the NCBI. Then the data were analyzed again by aligning these sequences using the program MEGA v. 5.0[13] and bootstrap used was 1000 replications[14].

Inhibitory Activity Test of *Trichoderma* sp. TKD

Inhibitory activities of *Trichoderma* sp. TKD against *A. flavus* FNCC6109 were done using dual culture method[15]. The procedure is to prepare a sterile Petridish filled with 15 mL of MEA media. Right in the middle of a Petridish that already contains the media is inoculated with colonies of *A. flavus* FNCC6109 with a diameter of 5 mm. Prepared with two colonies of *Trichoderma* sp. TKD with a diameter of 5 mm. Each colony of *Trichoderma* sp. TKD was inoculated with a distance of 2 cm on the left and right of the center of the colony of *A. flavus* FNCC6109. As for control, a Petri dish contained only MEA media and colonies of *A. flavus* FNCC6109. All Petridishes were incubated at 28ºC for 4 days. The percentage of inhibition can be determined by measuring the area of *A. flavus* fungus colony FNCC6109 in the control and treatment. Then it is plugged in the following formula:

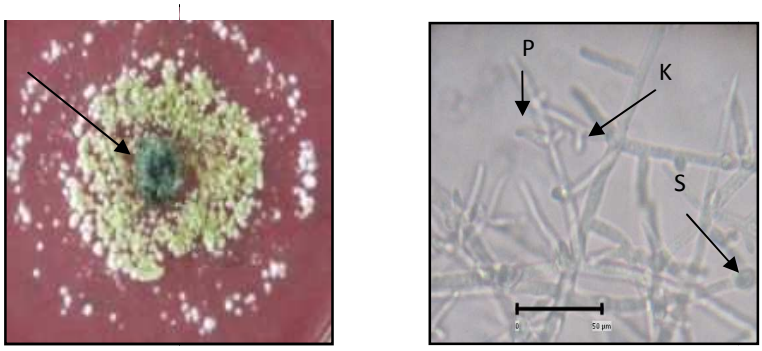
$$P = \frac{L1-L2}{L1} \times 100\%$$

Description:
P = Inhibition percentage
L1= Colony area of *A. flavus* incontrol
L2= Colony area of *A. flavus* in treatment

RESULTS AND DISCUSSION

Macroscopic and microscopic characteristics of *Trichoderma* sp. TKD

Trichoderma sp. TKD isolated from rhizosper of corn grown on MEA medium with an incubation period of 4 days showed the characteristic colony morphology as follows: growth of irregular colonies with colony reaches a diameter of 4.6 cm, mycelia quickly spread to look like piles, originally color greenish white colonies and transformed into a dark green after an extended incubation period (Figure 3.1a). The same thing also delivered by Nurhayati[16] and Subash *et al*.[17] that in order for the medium *Trichoderma* sp. look colony white color, further mycelium will turn into greenish and looks most of the middle colonies are green but still surrounded by white mycelium. If the incubation period is extended, the entire colony becomes greenish. Next Tandion[18] reported a *Trichoderma* sp. colony can reach a diameter of more than 5 cm within the incubation period of 9 days, which initially colored colonies hyalin, then white and then green greenish dim, especially on the part that shows there are many conidia[18].



A **B**

Fig. 3.1 . A. The colony morphology of *Trichoderma* sp . TKD (arrows) on MEA Medium with incubation period of 4 days at a temperature of 28^{o} C . B. Microscopic structure of *Trichoderma* sp. TKD at 400x magnification (arrows S = conidia ; P = sterigma (phyaled) ; K = conidiophores).

Results of microscopic observation of *Trichoderma* sp. TKD with 400x magnification, the structure obtained in the form of hypa, conidiophores, and conidia phialed, more details are presented in Figure 3.1b . Conidiophores appear branched and difficult to separate, on the lateral part of conidiophores are branches in pairs or not, at the end of a branch of conidiofor are phialed as bearing on the emergence of conidia. Pitt and Hocking[2] states in general conidia of Trichoderma green[2]. Results of the study were presented by Soesanto *et al.*[8] that the same Trichoderma isolates grown in different medium (MEA and PDA) provide the same color characteristics conidia are colorless hyalin[8].

Molecular Identification of *Trichoderma* sp. TKD

ITS primer amplification result with a 5 F: 5`-` GGAAGTAAAAGTCGTAACAAGG-3' and ITS 4 R: 5`-TCCTCCGCTTATTGATATGC-3' indicate a pattern of fragment separation as presented in figure 3.2. ITS tape label after the sequence with a nucleotide sequence of results is shown in Table 3.1. Followed by a sequence of results trimmed and assembled using the program Chromas Pro version 1.5. It was known that *Trichoderma* sp. TKD had the closest kinship with *Trichoderma asperellum* strain DWG3 (1), *T. asperellum* strain 2630 and *T. asperellum* strain CEN768, (Table 4.1) with the percentage of similarity respectively 99%. The same percentage of similarity of *Trichoderma* sp. TKD with 3 strains of *T. asperellum* was caused by the alignment of isolate base sequence starting from the base no. 10 aligned with *T. asperellum* strain DWG3 (1) starting from the base sequence no. 41. Genetic Relationship on phylogeny tree provided information that *Trichoderma asperellum* TKD was included in *Trichoderma asperellum* with bootstrap value of 99% (Figure 3.2). Dharmayanti[19] pointed out that the bootstrap values> 70% describing the ramifications are significant and permanent. Lopes *et al.*[20] found 21 isolates of *Trichoderma* sp. isolated from beans in the area Gerrado Brazil, once identified molecularly using internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2) obtained totaling 42.86% was *Trichoderma asperellum* and the rest was Trichoderma of the other types. It was further explained that from antagonist test results with dual culture method it was found out that *Trichoderma asperellum* has the highest ability to inhibit the growth of *Sclerotinia sclerotiorum* (> 50%). Villalobos *et al.*[21] *Trichoderma* isolates from three different places of the mango producer in mexico (Chalapas , Oaxaca and Michoacan). From his research by using transcribed spacer 1 (ITS1) primary forward and 2 reverse primer ITS find the 21 strains of Trichoderma , 3 of which are isolates of *T. asperellum* (T7 , T8A , and T8c) . *In vivo T. asperellum* T8A by 80 to 100 percent were able to suppress the growth of *Colletotrichum gloeosporioides* ATCC MYA456 on fruit manggo[21]. Furthermore Shahid *et al.*[22] found *T. asperellum* in farmland Kanpur, Uttar Pradesh India by identifying the use of universal primers 18SrRNA21 gene fragment.

Table 3.1 Complete results of BLAST-N sequence of *Trichoderma* sp. TKD

Description	Similarity	Accession Number
T. asperellum strain DWG3(1)	99% (590/591)	KM268676.1
T. asperellum strain 2730	99% (590/592)	EU272534.1
T. asperellum strain CEN768	99% (588/589)	KC576729

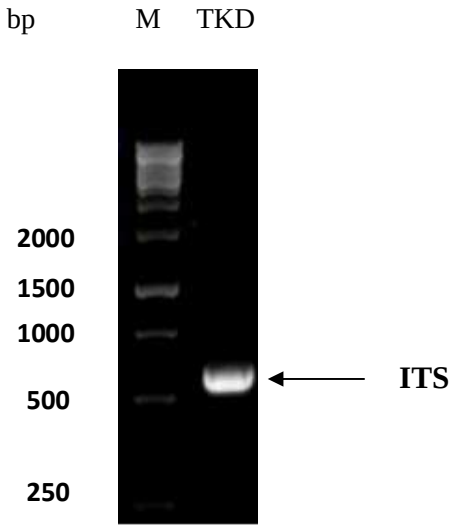


Fig. 3.2 PCR products Trichoderma sp. TKD using Primer _ITS_5F dan_ITS_4R . Lane M = marker . I leadder Kb DNA (Fermentas) . TKD lanes is Ribbons arrow.

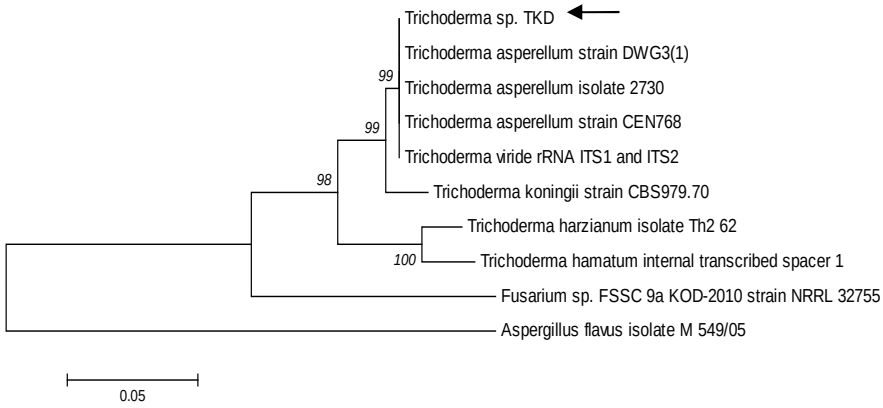


Fig. 3.3. Phylogenetic trees showing the proximity of *Trichoderma* sp. TKD (arrow) to some other species based on bootstrap 1000x

Inhibitory Activities of *Trichoderma asperellum* TKD. Against *Aspergillus flavus* FNCC6109 *in vitro*

Confirmatory test of inhibitory activities of *Trichoderma asperellum* TKD against *Aspergillus flavus* FNCC6109 with dual culture method obtained an average yield of the colony area of *A. flavus* FNCC6109 amounted to 0.196±0.079 cm2. When compared with the average area of the control colonies of *A. flavus* FNCC6109 which was not given antagonist (*T. asperellum* TKD) during the 3 day incubation period was much bigger i.e. 17.049±0,445cm^2 (Table 3.2.). Table 4.2 showed the average percentage of inhibition of *T. asperellum* TKD on the growth of *A. flavus* FNCC6109 reaching 98.849±1.100%. The ability of *T. asperellum* TKD in inhibiting the growth of *A. flavus* FNCC6109 the MEA media is presented in Figure 3.4.

Table 3.2 The inhibition of *T. asperellum* TKD against the growth of *A. flavus* FNCC6109

Treatment	colony area of *A. flavus* FNCC6109 (cm^2)					Total Average	
	1	2	3	4	5	(cm^2)	(cm^2)
Control	17,341	16,611	16,611	17,340	17,342	85,245	17,049±0,445
*T. asperellum*TKD	0,197	0,195	0,196	0,197	0,195	0,98	0,196±0,079
Inhibition (%)	98,864	98,826	98,820	98,863	98,875	494,247	98,849±1,100

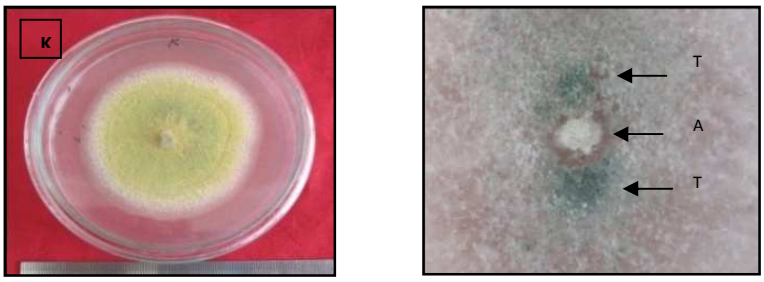


Fig. 3.4. Inhibition test of *Trichoderma asperellum* TKD against *A. flavus* FNCC6109 in MEA medium with incubation period of 3 days at 28°C (K. control; P. treatment. The arrow shows A= *A. flavus* FNCC6109 and T= *T. asperellum* TKD).

The amount of ability inhibition of the of *T. asperellum* TKD was caused by the mechanisms that inhibit the growth of *A. flavus* FNCC6109. Figure 3.5 showed there were clear differences in terms of size and appearance of colonies of *A. flavus* FNCC6109 that grew between the control and treatment. In the control, colonies of *A. flavus* FNCC6109 seemed to have a larger size compared with the treatment. Similarly, the color appearance of colonies on the control appeared to have more vivid colors than the treatment. Colony color of *A. flavus* FNCC6109 in treatment appeared to be more white and dull. This means that the growth of colonies of *A. flavus* FNCC6109 in treatment got pressures from antagonist given, so that there was a failure in the formation of pigments as well as areal expansion of the media in a Petri dish.

Through metabolites produced by *T. asperellum* TKD assumed to have fungicidal properties could inhibit the growth of *A. flavus* FNCC6109. The ability of the rapid growth of *T. asperellum* TKD in competition over nutrient resources within the media in Petri dishes was also the cause of its competitors cannot grow well. This is consistent with the statement of Rahman *et al.*[23] saying that *Trichoderma* sp. had the ability to live better and the competing power was high enough on various conditions. Furthermore Chaube[24] said that in addition to having a high competitive power for nutrients and space, *Trichoderma* sp. could produce antibiotics that inhibit or kill other microbes.

This study also demonstrated that *T. asperellum* TKD produced secondary metabolites that could inhibit *A. flavus* FNCC6109. It can be seen in Figure 3.5, where around colonies of *T. asperellum* TKD there was a clear zone which indicated the formation of metabolites or active compounds excreted by *T. asperellum* TKD. It is probable that *T. asperellum* TKD which was isolated successfully from rhizosphere of corn could produce enzymes or antibiotics that could damage or disrupt the physiological process of *A. flavus* FNCC6109. Although *A. flavus* FNCC6109 had a strong competitive edge by producing secondary metabolites to inhibit competitors but in fact with *T. asperellum* TKD, *A. flavus* FNCC6109 showed no significant resistance. Hyphae and spores of *A. flavus* FNCC6109 failed to undergo growth around the clear zone of the colony *T. asperellum* TKD. Even it seems that aflatoxin produced by *A.*

flavus FNCC6109 could not do much to inhibit the growth of *T. asperellum* TKD. *In vitro* have also demonstrated the ability of the filtrate *T. asperellum* TKD has the ability to inhibit both against fungi and against bacteria [25]. *Trichoderma* genus are well known producers of secondary metabolites with a direct
activity against phytopathogens and compounds that substantially affect the metabolism of the plant[26].The potential of Trichoderma are known as microbial antagonists against several plant pathogens, including *Trichoderma koningii* (57.70%) and *Trichoderma harzianum* (54.40%) in vitro was known to inhibit *A. niger*. Some pathogens in *Manil karazapota* L. (*Rhizoctonia solani* and *Geotrichum candidum*) plants under laboratory conditions can be inhibited by *T. koningii* and *T. pseudokoningii*[27].

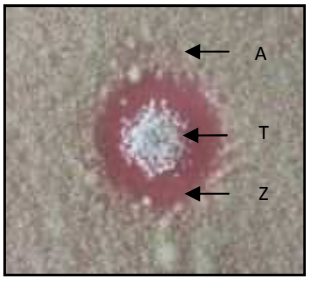


Fig. 3.5. Clear zone around the diameter of 5 mm colony of *T. asperellum* TKD in antagonistic test against *A. flavus* FNCC6109 on PDA medium with an incubation period of 4 days the temperature of 28^{o}C (arrows indicate T = *T. asperellum* TKD; A = *A. flavus* FNCC6109 and Z = clear zone).

CONCLUSIONS

Trichoderma sp. TKD which isolated of corn rhizospere is *Trichoderma asperellum* and *in vitro* have the ability to inhibit the growth of *A. flavus* FNCC6109 amounted to 98.849 ± 1.100%.

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