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Isolation and Identification of Endophytic Fungi on Cocoa Plant (*Theobroma cacao* L.) and Antagonist Activity against *Colletotrichum gloeosporioides*

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A B S T R A C T

Anthracnose disease which is caused by *Colletotrichum gloeosporioides* fungi becomes a resistor factor in cocoa production. Infect on young leaves creates leaf blight, consequently those leaves can die completely or only several parts starting from leaf blade and then fall, but infection at mature leaves spots appear in random border form and leave hole in the same location. Branches with infected leaves will experience die back and look like a broom, in the other side, infection at young fruit turns fruit to be dried and wrinkled while at mature fruits showed dried and rotten tips. This research is aimed at finding potential endophytic fungi from cocoa plant that can resist the growth of pathogenic fungi, *C. gloeosporioides* which cause anthracnose on cocoa plant (*Theobroma cacao* L). Isolation and identification of endophytic fungi on cocoa plant from Banggai district in Central Sulawesi results showed 10 isolates of endopphytic fungi: 2 isolates from leaves organ isolation; 4 isolates from stem; and 4 isolates from fruit pod. They both identified morphologically and macroscopically and found to have similarity to *Trichoderma* sp, *Penicillium* sp, *Aspergilus* sp that has resistance capability against *Colletotrichum gloeosporioides* colony through antagonist mechanism marked by the presence of resistance zone. *Trichoderma*sp demonstrated the highest percentage at isolate G in 56,92%.

Introduction

Cocoa is one of main commercial plants in many tropical countries. Cocoa (*Theobroma cacao* L) is developed in all tropical low land in the world processed seeds produce chocolate and chocolate fat. Indonesia considers Cocoa as one of prominent farming commodities as it boosts national economy. One of disease attacks that is assumed to be

resistor factor in the reduce of Cocoa production is anthracnose by *Colletotrichum gloeosporioides.* Colletotrichum is a main pathogen plants that let anthracnose to develop many life inhabit plants including amnosperm, angiosperm, decorative plants, fruits, vegetables, plants and even grass. Its Inocolum is cosmopolitic, spreaded through wind and rain, consequently it becomes significant economical problem for plant production in worldwide. Chemical method in controlling disease is thought to be expensive, less effective. Furthermore, it gives negative impact to the environment and human as well. Effective plant treatment strategy should be focusing on ecologically safe method, minimizing side effect from product use treatment since this is the best alternative for pathogen microorganism control for todays. (Oerke, 2006) and one of the ways might be applied is utilizing bio control agent (Larran *et al.,* 2016; Vinale *et al.,* 2008). Classic biological control is where natural enemy evolutes together and offers the biggest potency as management strategy for sustainable control of pathogen organism (Evans *et al.,* 2003). Endophyte is microorganism which occupies inside the high level of plant tissue but symptomless. Endophytic fungi are various in taxonomy and biology but all of them have characteristic that likely to take over plant internal tissue with no obvious damage to their life inhabit plants Wilson, 1995).They are able to produce new bioactive and chemical compounds which are potential to be exploited in many medical and agriculture fields (Strobel *et al.,* 2005). The interaction between endophyte and their life inhabit plants is running a mutual symbiotic, endophytic microbes receive nutrients supply and protection from unfriendly environment along the production and colonization (Schulz *et al.,* 2006), in the opposite, their life inhabit plants can promote growth and get protection from abyotic stress through induction of plant resistance response by endophyte that produce phytohormone such as auxin and giberelin to promote access toward mineral, nutrients and antagonistic synthetical metabolite (Dutta *et al.,* 2014; Jeffrey *et al.,* 2008). In several cases, their presence is helpful for the life inhabit plants as some of bioactive compounds are alcohol such as esther, ceton and others, those can interact with another compounds and kill pathogen microorganism (Specian *et al.,* 2012). This research covers a few steps: endophytic fungi isolation, identification of endophytic fungi and antagonist test against isolates with resistance percentage > 50%.The purpose of this research is to obtain potential endophytic fungi resistant *C. gloeosporioides* from cocoa plant (*Theobroma cacao* L) since this pathogenic fungi causes anthracnose.

Materials and Methods

Location and Time of the Research

This research was conducted In the Disease Labolatory of Agrotechnology Departement, agriculture faculty, Hasanuddin University in January 2021. Endophytic fungi were isolated from Cocoa plants from farming field belongs to society in Batui region, Banggai district, Central Sulawesi. Isolation was performed by using the cut of leaves, stems and healthy fruit. Leaves, stems and fruit pod were washed under fauced water for 5 minutes to remove dirts and dry out by wind for 30 minutes, the stems were cut in 0,5 cm length while the leaves and fruit pod are in 0,5 × 0,5 cm2. All of those cuts were dipped into 0,525% sodium hyphochlorite for 3 minutes and alcohol 70% for 2 minutes then moved twice to sterile distilled water for 2-3 minutes (Mejía *et al.,* 2008). Then, get dried on sterile filter paper and moved to petridish that filled with sterilized potato dextrosa agar media. There were 5 per one petri dish. All petri dishes were kept at temprature25°C for 5 days. Pure culture of endophyte isolate from these petri dishes is pured further.

Materials and Research Tools

Materials used are the culture of *Colletotrichum gloeosporioides* from disease laboratory collection of Agriculture faculty, Hasanuddin University, 10 endophytic fungi isolates from Cocoa plant in Batui Region, Banggai district, Central Sulawesi, laminar air flow, otoklaf, oven, petri dishes, digital camera, ruler, pinset, cutter, microscope, ose needle, scapel, besturi no. 13 and 18, plastic wrapping, bunsen, digital scale, strain paper, label, ballpoint, black and red snowman marker alcohol 70 %, sodium hypoclorite 0,525 %, water one and Potato Dextrosa Agar (PDA).

Research Method

Isolation and Identification of Endophyte Isolate

Identification was carried out based on guidance (Barnett & Hunter, 1998)by macroscopic observation that covers color and form of colony inside petri dishes (concentric and non concentric) colony texture and growth(cm/day) and by microscopic including the presence of septa on hyphae (insulated or non insulated), hyphae growth (furcated or non furcated), hypae and conidia color (dark or transparent),the presence of conidia (exists or no) and conidia form (round, oval, chained or random).

Observation Variable

The observation variable was determined by performing resistance percentage test of endophytic fungi from Cocoa plant (*T. cacao* L.) against micelium growth of *C. gloeosporioides* through dual culture.

Resistance test was conducted by putting the 10 endophyte fungi isolates of Cocoa plant that have been already growth on PDA media in3 mm for 7 days against micelium cuts of *C. gloeosporioides* in 3 cm from 9 cm diameter petridish border. The measurement was taken once in two days and in the third, it was repeated for 3 times. Resistance percentage observation was counted by using formula:

$$P = \frac{R1-R2}{R1} X10$$

(Fokkema, 1973).

P = InhibitionZone

R1 = mycelium growth of *C. gloeosporioides* moved away from endophyte.

R2 = mycelium growth of *C. gloeosporioides* moved closer to endophyte.

Then, antagonist mechanism observation was continued based on five valuation criterias: 1 = Antagonist dominated pathogen fully and covered whole media surface 2 = Antagonist grew at least more than two third of medium surface. 3 = Each antagonist and pathogen took over around half of media surface and showed no other dominating organism. 4 = pathogen colonialized at least two third of media surface and survived from antagonist disturbance. 5 = Pathogen fully dominated antagonist, grew over and occupied whole medium surface (Bell *et al.*, 1982).

Data Analysis

This research applied qualitative data Analysis technique where observation results data are presented in sentences, figures and tables. Resistance treatment effect test by 10 endophytic fungi isolates was performed using complete random design. Datas were processed through variant analysis, if they show obvious difference thus will continue with Tujey test.
Applied linear model is as following:

$$Yij = \mu + Ti + \varepsilon ij$$

i = treatment of endophyttic fungi isolation.

j = repetition

i, j = 1, 2, 3,...,n

Yij = observation at treatment to I and repetition to j

μ = general average

Ti = the effect of endophyte fungi to i.

εij = the error experiment of endophytic fungi

Isolate to I repetition to j

Results and Discussion

Isolation and identification of Endophyte Isolation

Endophytic fungi diversity was macroscopically classified based on color variation, colony texture, radial or round line of colony concentric on PDA media.

Isolation and identification result of endophytic fungi from Cocoa plant located in Banggai district, Central Sulawesi found 10 endophytic fungi isolates: 2 was isolated from leaves organ, 4 from stems and 4 from fruit pod. They were identified morphologically and microscopically and resulted to have similarity to *Trichoderma* sp, *Penicillium* sp, *Aspergilus* sp genus respectively. There were several microscopic fungi from the same genus, hence to differ the word "isolate" was added following by alphabet code thus to clear the application name of the next fungi. Variety of isolation source and endophytic fungi genus of Cocoa plant in Batu region of Banggai district, Central Sulawesi was presented in table 2.

Endophyte contributes to life inhabit plants by producing many active compounds providing protection and impact on plant life sustainability. A large number of new and potential natural products that have anti microbial activity have been isolated from endophyte and exploited to overcome resistant chain treat from plant and human pathogen. Isolated anti microbial metabolites from endophyte posess miscelanious structural classes, for instance alkaloid, peptide, steroid, terpenoid, fenol, quinine and flavonoid (Yu *et al.,* 2010). Endophyte has fungi attributed with exoenzyme that needed to exploit their life inhabit plants. They grew well in apoplastic washing liquid of life inhabit plants providing mutualistic association, promoting life inhabit plants growth, supplying mycobiont with enough nutrients to exploit life inhabit plants roots extensively (Schulz *et al.,* 2002).

Aspergillus (Isolate A, IsolateB, Isolate Cand Isolate F)

The four morphology and microscopic characters of *Aspergillus* genus are presented in the figure 1:

The four endophytic fungi isolates are similar to *Aspergillus* in their morphology and microscopic characteristic. *Aspergillus* taxonomy focuses on Aspergillum morphology that come with spore bearing structure or conidiophore, they have long stipe that grow bigger at the tip.

On the extended apical surface, a set of cells bringing spore that so called phialide (Wisdawati *et al.,* 2019).

Repeated Mytosisfission inside phialide nucleus producing a sexual spore called conidiospore or conidia, they varied in forms, some showed round shape and even long with smooth texture or connected with random borders. Conidia is very hidrofobic and able to

spread easily through air (Samson *et al.*, 2006). Generally basic important tool to identify Aspergillus species is macroscopic characteristic, such as conidia color, exudate, upside colony and microscopic characters including conidiophore, vesicle, metulae, pialide and conidia (Afzal & Shahzad, 2015; Diba *et al.*, 2007; Wisdawati *et al.*, 2021).

Penicillium Fungi Genus (Isolat E, IsolatI, and IsolatJ)

The three morphology and microscopic characters of genus *Penicillium* are described in figure 2 as following:

Endophytic fungi morphology from cocoa plant is similar to *Penicillium* genus marked by a huge amount of dried conidio spore production which are usually grey to green until blue to green.

Microscopic conidia seems like chain, has furcated fialid and metulae, looks like tooth brush with cilindric conidiophore. Monoverticillate conidiophore has terminal circle in fialida and in certain species, terminal cell of conidiophore get little swollen or has vesicle.

Biverticillat conidiophore owns 3 or more circles of metulae between stipe end and phialides, terverticillate conidiophore has another furcation level between stipe and metulae, quarter verticilate conidiophore was produced only by certain species and has one level of extra furcation beside terverticilat pattern. Terverticillate conidiophore and Quarter verticillate tend to be asymmetric (Samson *et al.*, 2014).

Trichoderma Genus Fungi (Isolate D, Isolate G, and isolate H)

The three morphology and microscopic characteristics of *Trichoderma* are demonstrated in the figure 3 below:

Colony morphology of this genus seems to have well arranged concentric line structure when it grows on PDA medium. Those three isolates showed color surface dominated by green mixed white they grew very fast and only in 3 days, colony had already filled 9 cm diameter petri dishes, this is assumed as *Trichoderma* genus. Microscopic conidiais round with furcated conidiophores and has fialid.

Species from *Trichoderma* could create many active biological compounds and secondary metabolites including decomposer enzyme of cell wall, capable of controlling pathogen population under competitive environment (Schuster & Schmoll, 2010; Vinale *et al.*, 2008), excrete elements that disturb fitopathogen organism life cycle as one of antagonist mechanism forms (Busby *et al.*, 2016).

Resistance Percentage of endophyte from cocoa plant against *C.gloeosporioides*

Observation toward resistance percentage of endophytic fungi isolates was done by measuring mycelium of *C. gloeosporioides* that both moved closer to micelium growth direction of endophytic fungi or away. Measurement started in the 2^{nd} until 6^{th} day when the two colonies already have met each other. Percentage data is shown at table 3.

Table.1 Diversity of endophytic fungi of cocoa plant from Banggai district, Central Sulawesi

Plant Tissue	Genus	Isolate amount
Leaf	*Trichoderma* sp	1
	Penecilium sp	1
Stem	*Aspergilus* sp	4
Fruit	*Trichoderma* sp	2
	Penecilium sp	2

Table.2 Percentage of resistance capability of entophytic fungi isolate against *C. gloeosporioides* micelium growth and antagonist activity based on valuation criteria

Treatment	Resistance Percentage	Antagonist Classes
A	43,29	Class 2
B	46,53	Class 2
C	27,62	Class 2
D	52,18	Class 1
E	25,12	Class 4
F	46,39	Class 2
G	56,92	Class 1
H	49,93	Class 1
I	30,37	Class 4
J	30,91	Class 4

Exp :Class 1 = Antagonist fully dominated pathogen and covered whole media surface Class 2 = Antagonist grew at least more than two third of media surface Class 3 = Antagonist and pathogen took half of media surface respectively and showed no another dominant microorganism, class 4 = pathogen colonialized two third of media surface and survive from antagonist disturbance class 5 = Pathogen fully dominated, antagonist grew over and occupied whole media surface (Bell et al., 1982).

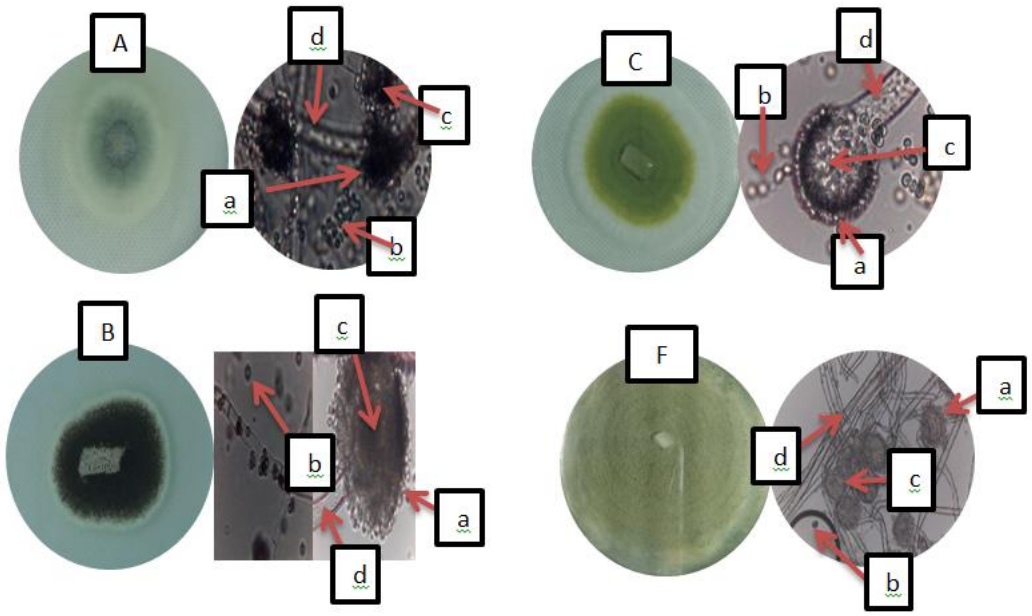
Table.3 Resistance Variant Analysis of endophytic fungi isolates cocoa plant against mycelium growth *of gloeosporioides* by invitro

Tests of Between-Subjects Effects					
Dependent Variable: Colletotrichumcolony					
Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	3506.299[a]	9	389.589	6.619	.000
Intercept	50248.943	1	50248.943	853.672	.000
Perlakuan	3506.299	9	389.589	6.619**	.000
Error	1177.242	20	58.862		
Total	54932.484	30			
Corrected Total	4683.541	29			
a. R Squared = .749 (Adjusted R Squared = .636)					

Table.4 Continued Tujey test of endophytic fungi isolates resistance of cocoa plant against micelium growth of *C. gloeosporioides* by invitro

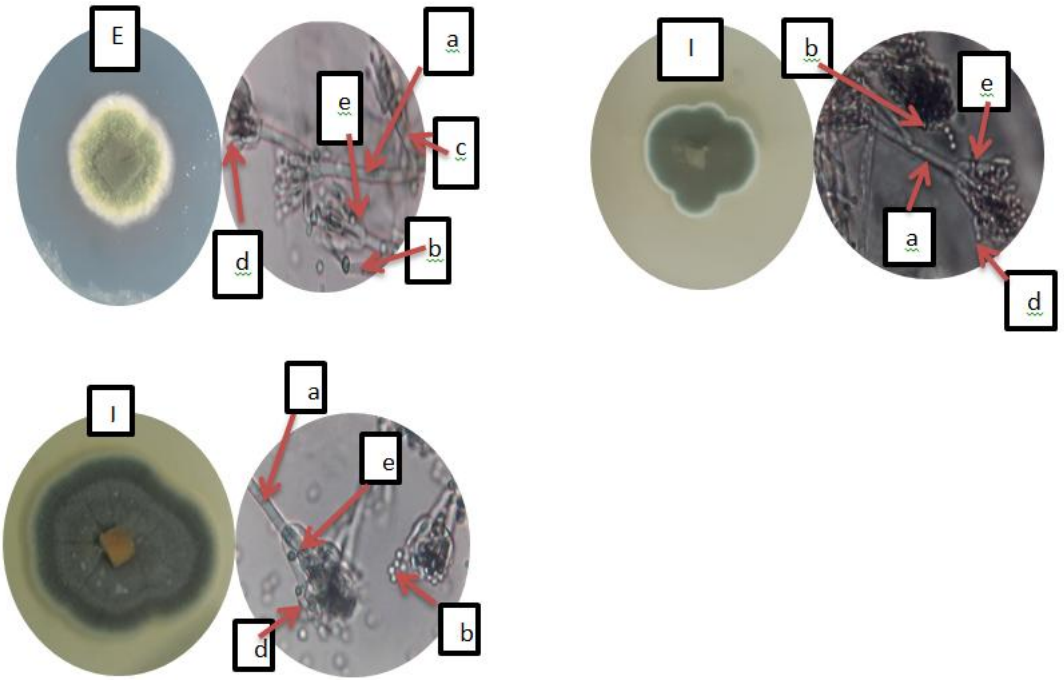
Miselium*C. gloeosporioides*				
Tukey HSD[a,b]				
Treatment	N	Subset		
		1	2	3
E	3	25.1200[a]		
C	3	27.6167[a]		
I	3	30.3733[a]	30.3733[ab]	
J	3	30.9167[a]	30.9167 [ab]	
A	3	43.2933[a]	43.2933 [ab]	43.2933[abc]
F	3	46.3900[a]	46.3900 [ab]	46.3900[abc]
B	3	46.5267[a]	46.5267 [ab]	46.5267[abc]
H	3		49.9300 [b]	49.9300[bc]
D	3		52.1800 [b]	52.1800[bc]
G	3			56.9167[c]
Sig.		.064	.056	.502
Means for groups in homogeneous subsets are displayed. Based on observed means. The error term is Mean Square(Error) = 58.862.				
a. Uses Harmonic Mean Sample Size = 3.000.				
b. Alpha = ,05.				

Fig.1 Morphology and microscopic characters of *Aspergillus* in the 7th day



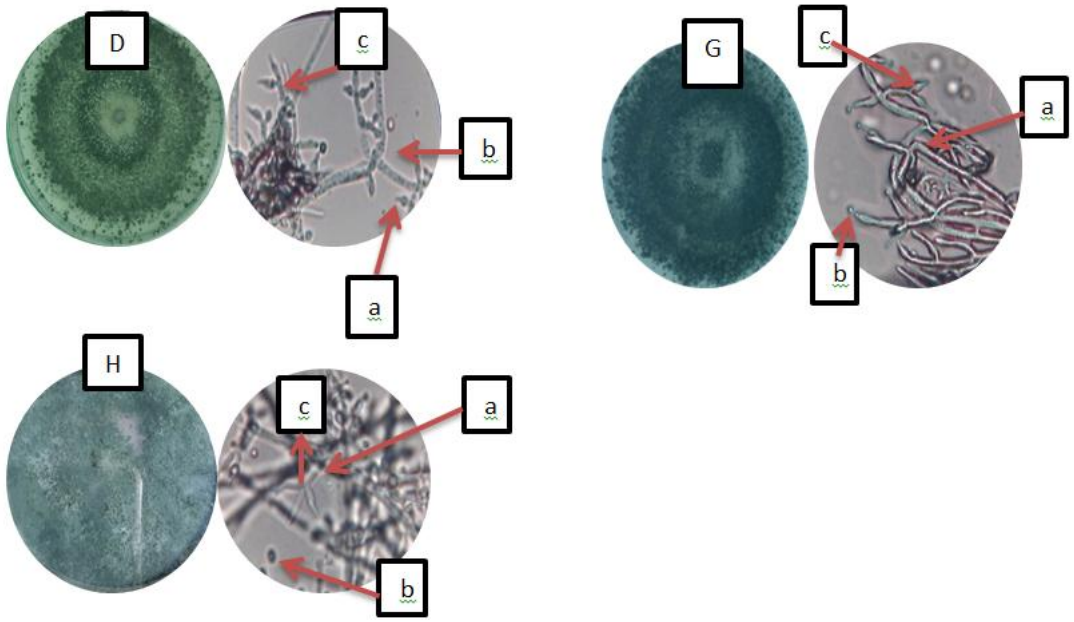
Exp: Morphology characters of endophytic fungi of Aspergillus from Isolat A, Isolat B, Isolat C and Isolat F; miscrocopic characteristic 100x magnificance. (a. Fialid, b. Conidia, c. Vesicle d. Conidiophore)

Fig.2 Morphology and Microscopic Characteristic of *Penicillium* in 7th day.



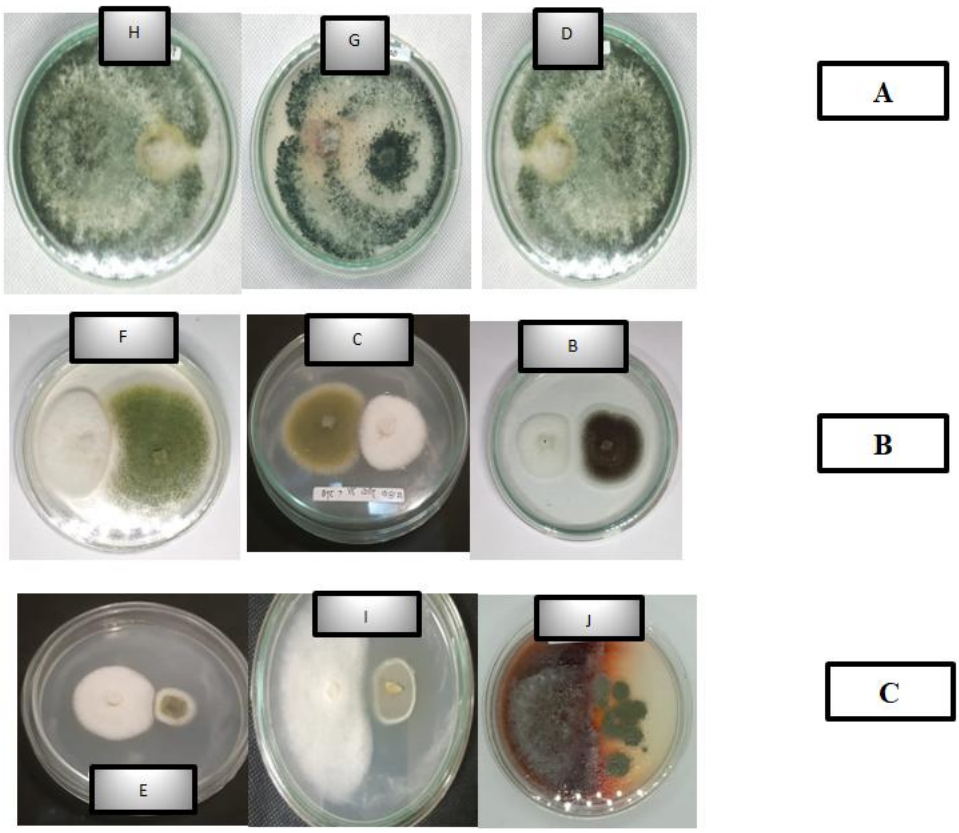
Exp: morphology characteristic of endophytic fungi of Penicillium genus, Isolate E, Isolate I and Isolate J Microscopic characteristic with 100xmagnificance. a:conidiophore, b:conidia, c: conidiophore furcation d: fialid, e: metuale

Fig.3 Morphology and Microscopic Characteristic of *Trichoderma* in the 7th day.



Exp: Morphology Characteristic of endophytic fungi of Trichoderma genus, Isolate D, Isolate G and Isolate Hmicroscopic Characteristic is magnifief100 X (a: conidiophore, b: conidia, c: fialid)

Fig.4 Resistance zone of 10 endophytic fungi isolates against *C.gloeosporioides* colony growth



Exp : A= resistance of endophytic fungi isolates from *Trichoderma* genus against *C.gloeosporioides*, B= resistance of endophytic fungi isolates from *Aspergillus* genus against *C.gloeosporioides*, C= resistance of endophytic fungi Isolates from *Penicillium* genus against *C.gloeosporioides.*

The result of resistance capability percentage count of 10 endophytic fungi isolates of cocoa plant toward colony growth of *C.gloeosporioides* on PDA media demonstrated various increase of resistance during the count from beginning to end. Average percentage respectively was around 27,41 – 56,92 % and mostly the highest values were 56,92 %, for treatment D, 52,18 %of H and 49,93 % of G from *Trichoderma* then followed by another endophytic isolates; 46,53 % of B, 46,39 % of F and dan 41,32 % of A from *Aspergillus* genus, 29,97 % of I, 27,41 %, of J, 25, 15 % of E from *Penicillium* and 27,61 % of C from *Aspergillus*. Colony resistance growth of *C.gloeosporioides* by 10 endophytic fungi isolates could be seen by the presence of resistance zone, assuming that all endophytic fungi isolates from Cocoa plant were potential antagonist agent to reduce pathogen colony growth of *C.gloeosporioides*. Valuation criteria on antagonist activity owned by 10 endophytic fungi isolates which started from class 1 until 4 was based on endophyte capability in colonilizing growth space. The resistance of endophytic fungi isolated was provided in figure 4 below:

The highest resistance was dominated by endophytic fungi isolates from Trichoderma. The percentage was 49,93-56,92 %, following the activity valuation criteria of resistance at class 1(figure 4). Trichoderma genus isolates filled almost whole petri dishes dominating

C.gloeosporioides. In general, microorganism death are caused by malnutrients and *Trichoderma* is safrofit soil that can take over the nutrients and space faster. (Montero-Barrientos *et al.*, 2011) commit physical contact and synthesis of hydrolitic enzyme as well as toxic or antibiotic compound which work sinergically with enzyme (Benítez *et al.*, 2004).

Biotrophic interaction of Trichoderma through penetrating into another fungi hypae. It might be production of poisoned secondary metabolite that finally kill and turn them into prey (Druzhinina & Kubicek, 2016; Harjono dan Widyastuti, 2001).

Analysis result by in vitro of resistance variant of 10 endophyte fungi isolates against *C.gloeosporioides* is shown at table 4. Anove test result showed F < 0,05 which means there was obvious difference for each treatment to micelium growth of *C.gloeosporioides* and to find out its significancy level, it was continued to Tujey test as presented in table 5 below:

Based on research result, conclusion could be drawn that here were 10 endophytic fungi isolates obtained from cocoa plant isolation. They emerged from 4 genera: *Aspergillus*(4 isolates); *Penicillium* (3 isolates), and *Trichoderma* (3 isolates).

Each has different morphology and microscopic characteristic as well as resistance capability against *C. gloeosporioides*. *Trichoderma* demonstrated effective resistance growth against *C.gloeosporioides* colony at treatment G.

It holds the highest resistance percentage with value 56,92% compared to *Aspergillus* and *Penicillium*. Resistance process happened through antagonist mechanism marked by resistance zone presence.

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