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A promising microbial use on cocoa: decomposing cocoa waste and controlling *Lasiodiplodia theobromae* in-vitro

A promising microbial use on cocoa: decomposing cocoa waste and controlling *Lasiodiplodia theobromae* in-vitro

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Abstract. This study aimed to test the effectiveness of number of potential microbes to decompose cocoa pod husk and to control the pathogen *Lasiodiplodia theobromae* in-vitro. This research consisted of several activities; investigating the ability of microbes to decompose cocoa pod husk while to test its effectiveness in controlling *L. theobromae* in both solid and liquid medium. The findings suggest that *Trichoderma sp*., *Trametes* sp., *Pleurotus ostearotus*, and bacterial consortium Microbat shown to perform an effectiveness in decomposing cocoa pod husk and in limiting filamentous growth of *L. theobromae* on both medium. Amongst trials, only isolate *Trichoderma sp*. shown to have a much higher restriction (66,84%) and performed a more considerable complete interaction i.e. antibiosis, competition for space and nutrients, mycoparasitism and lysis before other Microbial isolate shown to restrict filamentous growth of pathogen on both medium.

1. Introduction

Cocoa (*Theobroma cocoa L.*) is one of the most global exported commodities and has become an engine of rural economic growth wherever it is grown. In terms of global bean supply, the only three main bean producer countries is consistently to supply over 400,000 tonnes annum i.e. Cote Ivory, Ghana and Indonesia [1]. In Indonesia, about 65% of area and national production is from Sulawesi [2, 3]. However, behind tremendous pod resource, most of farmers still see that only beans are valuable part of pod while others remain as waste and unfortunately this constraint begins. As the greatest component of pod consisting of 70% weight after placenta and beans, husk and shell can press environment. The farmer collects pods, separates beans before discharges the husk and shell on the ground and this way seems to be impractical and pest and pathogen take opportunity to complete their life cycle. To reduce the waste and to cut the life cycle, microbial decomposer was potentially used driven by native group of fungi whose capable of decomposing lignin, cellulose and hemicellulose compounds and of controlling the pathogen. Fungi produce extracellular enzymes to depolymerize large-sized compounds into small water solutions (substrate for microbes), and the microbes transfer the subtract into cells through the cytoplasmic membrane to complete the process of decomposition [20]. *Trichoderma sp., Trametes sp*. oyster and *Pleurotus ostreatus* are potential fungal decomposer [23, 36].

In addition, together with waste issue in cocoa orchards, cocoa disease caused by fungal pathogens is another critical constrain and one of them is *Lasiodiplodia theobromae* [4-7]. *L. theobromae* has a wide-range of host [6, 8, 9] and in Indonesia and Latin America, its role is a new and non-prominent

pathogen [6, 7, 10]. In Sulawesi, the pathogen was found to associate with the cause of vascular streak dieback symptoms, *Ceratobasidium theobromae* [11]. While dealing with waste issue on cocoa farm is important, controlling *L. theobromae* is not apart and therefore, to manage both issues this research is proposed.

2. Research Methods

This research was carried out at the Lab Agricultural Biotechnology of Research Centre and Plant and Pest disease Department, Faculty of Agriculture Hasanuddin University, Makassar, started from August 2013 to January 2014.The lab instruments included autoclave, oven, petri-dish, laminar air flow, shaker, hot plate, incubator, analytical scales, scales, microscopes, Erlenmeyer, basins, cameras, jam bottles, measuring cups, goblets, flame, sprayers, rods stirrers, carbohydrates, needles, glass preparations, haemocytometers, filters, spatulas, plastic cups, Eppendorf pipettes and stationery. The materials used were cocoa husk, bran, agricultural lime, baglog plastic wrap, rubber bands, infected cocoa, *Trichoderma* sp., PCK_9, *Pleurotus ostreatus, Microbials*, potatoes, jelly, granulated sugar, chloramphenicol, distilled water, 70% alcohol, wrapping plastic, tissue, filter paper, aluminium foil, spirits and label paper. The cocoa husk was collected from Soppeng South Sulawesi Province and the isolate effectivity test was carried out after the cocoa pod husk being processed into a substrate of organic material. For more detail as follows:

a. Preparation and isolation
   6.5 kg cocoa husk was washed and grounded before it was dried. Grounded husk was mixed with 1.3 kg and 5.6 g lime (with ratio 5: 1: 0.05). All mixture was added water until glued tightly. The mixture was put into a 100-gram culture bottle and covered with plastic coated with aluminium foil, then tied using rubber bands and autoclave 2x for 2 hours. For fungal other microbe isolation, *Trichoderma sp., Trametes sp., P. ostreatus* and *microbes* were grown on the PDA culture. The microbials with a total concentration of 10% were dripped on the substrate of organic matter and the 20% concentration were used on the effectiveness test of isolates against pathogens. Microbials with 20% concentration were dropped on culture then flattened with a spatula before being incubated at room temperature (28°C - 31°C) for 5 days.
b. Inoculation and dual culture test
   The trials consisted of inoculating *Trichoderma sp., Trametes sp., P. ostreatus* and *microbials*, 10% with 11 treatments and four replications including control. In each treatment, five isolates were used, then being put in a bottle containing sterilized organic material before being incubated at room temperature and observed in a three days observation for a month. Combination trial consisted of Control; *Trichoderma* sp.; Trametes sp.; *P. ostreatus;* 10% microbial; *Trichoderma* sp. + *Trametes* sp.; *Trichoderma* sp. + *P. ostreatus; Trichoderma* sp. + 10% microbial; *Trichoderma* sp. + 10% mikrobat; *Trametes* sp. + *P. ostreatus; Trametes* sp. + 10% microbial; and *P. ostreatus* + mikrobat 10%.
   In dual culture test, trials were by growing altogether fungi and mikrobat and pathogen (*L. theobromae*) on PDA culture, which was inhibition zone was main parameter on this test. Also, interaction pathway between antagonist and pathogen was undertaken by looking at physical appearance on dual culture (Figure 1).

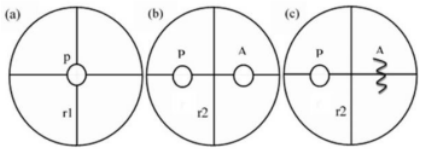


Figure 1. Matrix of dual culture test in liquid phase

Information:

(a)	Control (r1)	P:	Pathogen
(b)	Pathogenic fungus isolates with antagonist fungus isolates	A:	Antagonists
(c)	Isolates of pathogenic fungi with microbial antagonists	r1:	Diameter of pathogenic colonies on control
		r2:	Diameter of pathogenic colonies in multiple cultures

For the use of experimental design, a completely randomized design was applied consisting of five combination isolate trials with four replications including Control (*L. theobromae*); *L. theobromae* + *Trichoderma* sp.; *L. theobromae* + *Pleurotus ostrearus*; *L. theobromae* + *Trametes* sp.; and *L. theobromae* + Microbials. Inhibitory role of isolates against the pathogen *L. theobromae* was formulated as following:

$$P = \frac{r1 - r2}{r1} x\ 100\%$$

Information:
P: percentage of inhibitor r1: Diameter of pathogenic colonies in the control r2: Diameter of pathogenic colonies in dual culture

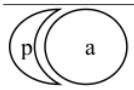
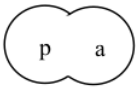
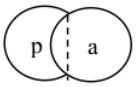
For making liquid media, the procedure was almost the same with PDA by adding jelly as compactor. materials which were mixed and heated, were poured into a 100 mL bottle of culture and covered by aluminium foil. It was then sterilized in an autoclave for 2 hours before being stored into culture bottles then shackled for a week. A complete randomized design was used consisting of 9 combination isolate trials with 3 replications. combination trial consisted of Control (*L. theobromae*); *Trichoderma* sp.; *Pleurotus ostreatus; Trametes* sp.; Mikrobat; *L. theobromae* + *Trichoderma* sp.; *L. theobromae* + *Pleurotus ostreatus;*and *L. theobromae* + *Trametes* sp. The observation commenced in the 7th and 9th day after inoculation with amount of conidia/ ml as parameter. Subsequently, spore concentration was calculated after stored by using a set of haemocytometer squares as following [15]:

$$C = \frac{t}{n\ .\ 0{,}25}\ x\ 10^6$$

Information:
C : spore density per ml of solution
t : total number of spores in every set of squares
n : number of spores in the squares
0.25 : a correction factor for using Haemocytometer squares

In addition, mass mycelium measurement was subsequently undertaken by counting dry and wet filaments using the thermogravimetric method, ovening for overnight at 105°C and cooling down for a day to obtain a constant weight. The experimental design used was a randomized design consisting of 9 trials with 3 replications. Trial combination was shown Control (*L. theobromae*); *Trichoderma* sp.; *Pleurotus ostreatus*; *Trametes* sp.; *L. theobromae* + *Trichoderma* sp.; *L. theobromae* + *Pleurotus ostreatus*; and *L. theobromae* + *Trametes* sp. For antagonistic mechanism, interaction biocontrol and fungal pathogen was illustrated as follows;

Table 1. Matrix of interaction between potential biocontrol agent and fungal pathogen in vitro; a = biocontrol agent and p = pathogen

Interaction	Role	Information
	Antibiosis	There is a distance in the resistance area. In the area, pathogenic hyphae are seen to enlarge and experience lysis
	Competition for nutrients and space	Pathogenic colonies are covered by fungal test. In the contact area, hyphal pathogens undergo lysis.
	Mycoparasitism	In the contact area of hyphae, the test fungus convolves pathogenic hyphae, then pathogenic hyphae enlarge and experience lysis. In addition, they are capable of producing enzymes that can degrade cell walls. Pathogens then enter the lumen of the target fungus.

Antibiosis such as alametichin, paracelsin, and trichotoxin can destroy fungal cells by damaging the permeability of cell membranes, chitinase, and laminarinase lysing cell wall [12]. Competitions for nutrients and space refer to the ability to compete with pathogens, especially in terms of extracting nutrients in the soil such as carbon, nitrogen, macro elements and other microelements. The ability of such competition causes inhibition of pathogen to grow. Meanwhile, mycoparasitism is the mechanism of parasitizing other fungus mycelium by penetrating the cell wall and entering the cell to take food substances causing the death of fungus [12].

3. Results and Discussion

One of parameters to examine effective isolates is structure of compost maturity including color and presence of mass mycelium performed to decompose organic waste of cocoa. Table 2 depicts that mycelium growth of all trials varied to respond to growth culture. Mikrobat alone and mixed with *Trichoderma* sp. seemed to grow faster to cover the culture from the beginning to the end of observation while other mycelium trials were somewhat slow to grow. In terms of mycelium appearance by the time, only *Trametes* sp. mixed with *P. ostearotus* and microbat trials and *P. ostearotus* combined with microbat trial underwent consistently color transformation from white brownish to dark brown appearance. The observation with intervals of three days basis shown that *Trichoderma* sp. alone and combined with other trials were able to grow and played its role in a better decomposing cocoa husk, containing enormous nutrients [36]. Once *Trichoderma* sp. encounters appropriate nutrient on growth medium, it can perform a better penetration and lysis of the cell wall [12,22]. *Trichoderma* sp. is a superior fungal decomposer since it is equipped with enormous enzymes i.e. cellulase and chitinase as well as *Trametes sp*. and *Pleurotus ostreatus* [22,23,26, 29, 32, 35,36].

Table 2. Morphological appearance of compost maturity made from cocoa husk and decomposed by isolate *Trichoderma* sp., PCK_9, *Pleurotus ostearotus* and microbials in culture bottles

Trials	Parameter of compost maturity							
	Week 1		Week 2		Week 3		Week 4	
	Mycelium	Color	Mycelium	Color	Mycelium	Color	Mycelium	Color
Control	-	-	-	-	-	-	-	Brown
Trichoderma sp.	**	White	***	White	****	White, brownish	****	White, brownish
No Trametes sp.	**	White	***	White	****	White, brownish	****	White, brownish
Pleurotus ostearotus	*	White	***	White	****	White, brownish	****	White, brownish

Mikrobat	***	White	***	White	****	White, brownish	****	White, brownish
Trichoderma sp. + *Trametes* sp.	*	White	**	White, brownish	****	White, brownish	****	White, brownish
Trichoderma sp. + *Pleurotus ostearotus*	*	White	***	White	****	White, brownish	****	White, brownish
Trichoderma sp. + Mikrobat	***	White	***	White	****	White, brownish	****	White, brownish
Trametes sp. + *Pleurotus ostearotus*	*	White	***	White, brownish	****	White, dark	****	Dark brown
Trametes sp. + Mikrobat	*	White	***	White	****	White brownish	**** .	Dark brown
Pleurotus ostearotus + Mikrobat	**	White	***	White	****	White brownish	****	Dark brown

Information:
- : No mycelium growth and no discoloration
* : 1%-25% mycelium growth in culture
** : 26%-50% mycelium growth in culture
*** : 51%-75% mycelium growth in culture
**** : 76%-100% mycelium growth in culture

Table 3. Compost performance was made from cocoa husk

Trial	Aroma	Color	Texture	Weight (g)
Control	Cocoa	Brown	Hard	7.25
Trichoderma sp	Fragrant	Brownish white	Mild	4.25
Trametes sp.	Fragrant	Blackish	Mild	11.25
Pleurotus ostearotus	Fragrant	Brownish white	Mild	5.75
Mikrobat	Fragrant	Brownish white	Mild	9.75
Trichoderma sp. + *Trametes* sp.	Fragrant	Darken	Mild	9.00
Trichoderma sp.+ *Pleurotus ostearotus*	Fragrant	Blackish white	Mild	2.00
Trichoderma sp. + Mikrobat	Fragrant	Blackish white	Soft	3.25
Trametes sp.+ *Pleurotus ostearotus*	Fragrant	Blackish	Mild	0.75
Trametes sp.+ Mikrobat	Fragrant	Blackish white	Soft	1.75
Pleurotus ostearotus + Mikrobat	Fragrant	Blackish white	Soft	9.50

In addition, aroma, color, texture, weight of grounded cocoa husk (initial - final weight) are important parameters of determining the level of maturity and success of composting process. Table 3 suggests that all parameters to determine maturity of husk compost decomposed by trials varied. No different trials were to perform aroma. Besides color and soft texture, a better composting process can be also examined from its weight. Once the weight of cocoa husk is big change to origin during fermentation process, the maturity level of compost seems to complete. The finding shows that *Tricoderma* sp., *Trametes* sp. and *Mikrobat* shown a considerable change to original weight of cocoa husk (6.5 kg) and these trials could decompose a better cocoa husk. Especially, *Trichoderma* sp. produces cellulase consisting of β-1.4 *gluchidrolase* and β-glucosidase [12] which lyses cellulose, starch, lignin, gum and soluble organic compounds. *Trametes sp*. is capable of lysing lignin more rapidly and breaks up of chains between cellulose and hemicellulose with lignin. *Lignocellulose* bonds can be broken by ligninase, such as *lignin peroxidase* and *manganese peroxidase* produced by PCK_9 [22, 24]. *P. ostreatus* causes structure loss by lysing lignin [31-32] and this fungus is not merely capable of lignin break up, but also able to penetrate the substrate such as wood chips.

3.1. Testing isolates against L. theobromae in dual culture test

Dual culture is one of important part of understanding isolate achievement to restrict pathogen in vitro. Inhibitory activity of isolates against to *L. theobromae* was shown in Figure 2.

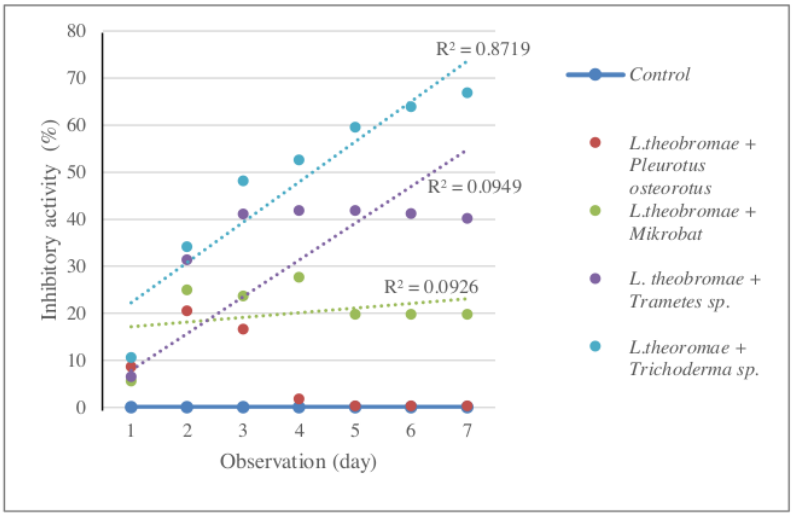


Figure 2. Inhibitory activity of isolates against *L. theobromae* in vitro

It is noted that figure 2 shows a better restriction to mycelium growth of *L. theobromae* in vitro with Trichoderma sp. trial. According to a trendline analysis during observation that the only *Trichoderma* sp. isolate was nearly to 1.0 value of R square coefficient (or up to 80% of inhibitory activity) while the other trendlines of *Tramestes* sp., Mikrobat and *P. osteorotus* performed farer from 1.0 value of R square coefficient, indicating a slow restricted activity of mycelium pathogen. No inhibitory activity in Control for entire observation.

Table 4. Matrix of antagonistic role of isolates

Trial	Antagonistic mechanism			
	Competition	Antibiosis	Mycoparasitism	Lysis
L. theobromae + *Trichoderma* sp.	√	√	√	√
L. theobromae + *Trametes* sp.	-	√	-	-
L. theobromae + *Pleurotus ostearotus*	-	-	-	-
L. theobromae + Mikrobat	-	√	√	√

Interaction between fungal trials and *L. theobromae* shows that a greater inhibitor to pathogen mycelium occurred in the trial of Trichoderma sp. which performed all parameters of competition, antibiosis, mycoparasite and lysis. Only *P. ostearotus* shown mutualism interaction to *L. theobromae* and *Trametes* sp. seemed to have less capable of antagonism. In addition, Mikrobat trial was capable of expressing antibiosis and parasitic and it was able to lyse pathogen mycelium even if competition of nutrient abstained in vitro. The role of *Trichoderma* sp. in controlling crop pathogen with parasitism pathway while boost yield was undoubted. The evolutionary biocontrol convinced with scientific evidence and changed in knowledge about biocontrol [12] and since then, the study on biocontrol agents become more and more developed. *Trichoderma* sp. has been widely tested to control fungal pathogens in many crop orchards and shown to have effectiveness [18,21]. The study of Hakkar et al., [13] revealed that *Trichoderma asperellum* was able to reduce disease incidence up to 40% compared without trial (80%) for 12 weeks observation. Concentration also affects to achieve a successful control of pathogen, the more diluted volume, the lesser effective suppressing the black pod pathogen.

3.2. Testing isolates in dual culture against L. theobromae

Another two parameters connecting to perform the ability of isolates to control pathogen is by examining formation of spore density and mass mycelium (dry and fresh weight). For more detail as follows;

Table 5. Formation of spore density of *L. theobromae* per ml solution and mass mycelium (dry and wet weight)

Trial	Average spore density	Weight (g)
L. theobromae	$4{,}91\times10^{6}$ a	24.69
L. theobromae + *Trichoderma* sp.	0 c	4.97
L. theobromae + *Trametes* sp.	$5{,}33\times10^{5}$ c	3.26
L. theobromae + *Pleurotus ostearotus*	$9{,}6\times10^{5}$ c	18.6
L. theobromae + Mikrobat	$2{,}93\times10^{6}$ b	0.87

Table 5 shows that the spore density formation in each trial varied. In this case, the smaller spore density of pathogen performed, which means that, the better inhibitory pathogen formation occurred. Therefore, trial of *Trichoderma* sp. isolate shown to have much greater spore suppression of *L. theobromae* as spore pathogen failed to develop. The finding suggests that the parameter of spore density formation of pathogen considerably associates with a complete interaction role of antagonism being performed by *Trichoderma* sp. (Table 3). The success of *Trichoderma sp*. as biocontrol has been wider reports [12-15] due mainly to producing hydrolytic enzymes β-1,3 glucanase, chitinase and cellulase, which these enzymes functions to lyse cell walls consisting of β-1,3 *glucan* (linamirin) and chitin polymers. The study on several isolates of *Trichoderma* sp. applying on the cocoa flower and pod layers was undertaken and the result evidenced that β-1,3 glucanase and chitinase were released to vary in environmental condition [16]. In contrast to *Trichoderma* sp. isolate, most of isolates shown to have less effective to restrict spore development.

The final parameter to examine the effectiveness of trials to limit fungal pathogen is by assessing dry and fresh weight. Table 5 depicts that combination between Pathogen and trials varied to respond mass mycelium of pathogen. If combination of pathogen and trials performed to have less mass mycelium, the trials were likely to capable of reducing mass mycelium of pathogen [14] and this happen to most of isolates showing much lesser weight than *L. theobromae* alone.

Conclusion

Based on the results of the study, it concludes that all isolates such as *Trichoderma* sp., *Trametes* sp., *Pleurotus ostearotus* and Microbat shown to be capable of decomposing cocoa husk but not all trial performed to restrict pathogen mycelium in vitro. In dual culture test, a much more effective restriction of filamentous *L. theobromae* in vitro was performed by *Trichoderma* sp. (66.84%). Furthermore, the only *Trichoderma sp*. performed a complete interaction role; antibiosis, competition for nutrients and space, mycoparasitism and lysis before mikrobat isolate.

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