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To cite this article: Muhammad Junaid *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **486** 012160

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2 Testing cutinase produced by native *Trichoderma* isolate and its persistence in pod and flower surfaces on cocoa tree in South Sulawesi

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Abstract. *Trichoderma* species is a powerful biocontrol agent against the agents of cocoa diseases. Although it is a typical soil fungus, it is associated with plant tissue as a symbiont. *Trichoderma* species has associated with cocoa as a living plant symbiont but understanding the main factor to help *Trichoderma* isolates in retaining in the plant cuticle is still scarce. While it is critical to understand its role in tissue surface, the study will reveal the ability of *Trichoderma* isolates obtained from South Sulawesi to produce cutinase in order to help spore's persistence in pod and flower surfaces before penetrating to control cocoa diseases.

1. Introduction

Cutin polymer is commonly found in the outer fruit tissues including cocoa pod which consists of a waxy substance physically characterized with a waterproof. Srivastava [1] argues that cutin polymer is primary alcohol ester linkages derivative from fatty acids with C16 and C18. As part of alcohol ester linkages, interaction of a tiny living creature and polymer cutin indicates *p-nitrophenol* (pNP). To break down cutin polymer in fruit skin, the need of enzyme to lyse the polymer which is known as cutinase is critical. Cutinase is produced by living microbes in different roles i.e. as plant symbiont [2], plant pathogen, mycoparasite [3], or decomposer [4]. In Plant symbiont, microbial fungus infects the tissue without expressing lesion due to its avirulent gene [5]. *Trichoderma* species is a better example of symbiotic mutualism to cocoa [6]. Once the avirulent fungus penetrates to tissue surface, the first barrier is a waxy layer and therefore producing cutinase is important to facilitate an initial host recognition. In plant pathogen role, similar to plant symbiont fungus, cutinase is produced by plant pathogen to address cuticle barrier and cutinase production is necessarily utilized to infect host tissue in advance. Producing cutinase also associates with germination and virulent fungus. The study of Koller and Kolattokudy [7] revealed that *Fusarium solani f.sp.pisi* which is characterized as virulent fungus produces much higher amounts of cutinase once its spore germinates. Therefore, this study was undertaken to understand cutinase produced *Trichoderma* isolates of interest and its spore persistence once applied in cocoa flower and pod in nature.



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2. Research methods

2.1. Culture and subculture of isolates

Trichoderma isolates obtained from Sulawesi were grown into solid medium, 2% (g/v) potato dextrose agar (PDA) as a initial culture. For subculture, 1000 mL Malt Extract (ME) medium consisted of malt extract pH 7.0, 1 g K₂HPO₄, 0.5 g KCL, 0.5 g MgSO₄.7H₂O, 0.01 g, FeSO₄.7H₂O. The mixture was homogenized while heated at 100 °C for 10 minutes and then the ME was autoclaved 1.5 bar with 121 °C temperature for 15 minutes. As inoculum source, 50 mL sterilized ME was prepared and loaded into a sterilized erlenmeyer and subsequently, the isolate was collected from original PDA medium and grown to ME medium. The medium was gently shaken 150 rpm for 4 days. And for production medium, 10 mL of colony was pipetted again and loaded into 100 mL ME and then shaken 150 rpm for seven days. Once the isolate was growing optimum within a week, separation between mass mycelium and rough cutinase was undertaken with 100 g of mycelium harvested and centrifuged at 3800 rpm for 15 minutes 29 °C. Substrate and waste was filtered to have candidate rough enzyme.

2.2. Subculture for cutinase activity

Trichoderma isolates of interest were grown in Yeast extract (YE). To obtain 1 Litre medium YE, reagent composition with pH 7 consisted of 1 g K₂HPO₄, 0.5 g KCl, 0.5 g MgSO₄.7H₂O, 0.01 g FeSO₄.7H₂O, and added 2 g cutin-modified obtained from red apple skin as source of cutinase (red apple was peeled, dried before extracted to have soft flour). The mixture was homogenised while boiled to 100 °C and it subsequently was autoclaved at 121 °C for 15 minutes before moving to a new sterilized erlenmeyer for initial inoculum and mass production medium purposes.

2.3. Rough cutinase determination

Once rough enzyme was collected, it was stored at -20 °C for several days. To measure every rough enzyme activity, the activity depended on the amount of *para-nitrophenyl-butyrate* (pNB) released by isolates of interest [9]. Nine reaction tubes were prepared and one was for control (without pNB). In every tube, buffer Tris HCl pH 8.0, 0.2 mL rough cutinase and PNB solution which was previously diluted with 0.1 mL acetonitrile 2.68 mM were loaded into reaction tube as stock solution. 0.1 mL the mixture was pipetted and added 4.7 mL Tris HCl pH 8.0 50 mM to reach 5 mL of total volume. Cutinase activity was heated to cease the activity with temperature 30 °C for 5 minutes. The mixture became a yellowish solution and from this cutinase activity was calculated with colour absorbance from spectrophotometer UV/VIS 400 nm. The colour absorbance appearance was a *para-nitrophenol*.

2.4. Testing enzyme production obtained from *Trichoderma* isolates

The concentration of pNP solution was divided into 20 ppm, 30 ppm, 40 ppm, 60 ppm and 80 ppm respectively and one was as control (without pNP). Every concentration consisted of 4.7 mL Tris HCl buffer 50 mM pH 8.0 mixed with 0.2 mL pNP and 0.1 mL pNB solution. The mixture was heated at 30 °C for 5 minutes. pNP activity as a result of mixture reaction was calculated with spectrophotometer UV/VIS 400 nm.

Table 1. Determination of enzyme activity obtained from *Trichoderma* isolates

Objective	Formulation of enzyme activity	Marker	Source
Cutinase	$\text{Cutinase activity} = \frac{\text{pNP concentration}}{\text{mass molecul of pNP}} \times \text{time incubation}$	<i>para-nitrophenol</i> (pNP)	[4]

Note: 1 unit of enzyme activity = 1 μmol p-nitrophenol (cutinase) per minute

Testing enzyme production by *Trichoderma* isolates were based on the amount of enzyme activity released on the media. To measure cutinase, the enzyme activity was determined by the amount of pNP performed from cutin hydrolysis (table 1).

2.5. Testing persistence of *Trichoderma* isolates in pod and flower surfaces

Known isolates producing enzyme activities were applied to pod and flower surfaces in the field in the dry season. 1 mL of 10^6 spores was pipetted and diluted with 10 mL sterilized water and the mixture was sprayed in the pod and flower surfaces and 3 days after spray the spores were harvested from the original tissues and examined its spore density by growing into pure culture. Pod and flower were collected and placed in different small bottles with 20 mL sterilized water. The bottle with pod or flower was gently shaken for 5 minutes and spores were pipetted 0.5 mL volume and grown into pure culture. Active spores indicated milk-spotted in the culture surface and subsequently accounted as following;

$$\text{Spore density} = \frac{0.5 \text{ mL} \times 2 \times 20 \text{ mL} \times \text{total spores on culture}}{\text{pod or flower}}$$

3. Result and discussion

3.1. Producing cutinase by *Trichoderma* isolates

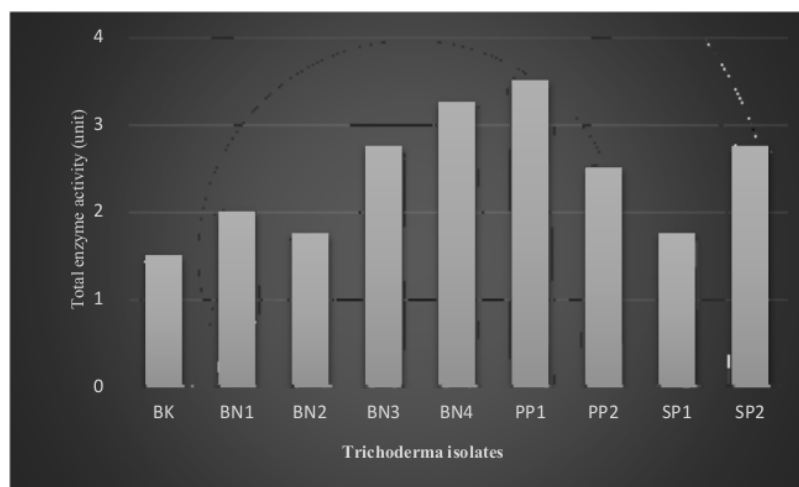


Figure 1. Cutinase activity varied released by *Trichoderma* isolates

Figure 1 depicts that overall all isolates of interest were able to produce important enzymes for retaining in surfaces of pod and flowers in nature. The enzyme activity generated by isolates varied depending on genetic variation [8, 9]. Isolates of BN4 and PP1 shown to have the highest enzyme production and the lowest enzyme production was in isolates of BK, BN2 and SP1.

3.2. Testing persistence of *Trichoderma* isolates to cocoa flower and pod surfaces

The importance of cutinase generated from *Trichoderma* species is due mainly to the need of persistent role in pod and flower surfaces. It is expected that once the isolate initially penetrates to pod and flower surfaces, the isolate of interest can retain addressing physical barriers. The most physical barrier faced by *Trichoderma* to retain in the pod layer is a waxy structure of pod tissue that can remove it prior to commencing to penetration activity. Of total isolates, BN3, BN4, PP1 and SP2 isolates shown to produce

much higher cutinase (figure 1) and those isolates can indicate to have a robust enzyme property addressing physical barrier of pod or flower before mycoparasite plant fungal pathogens in nature.

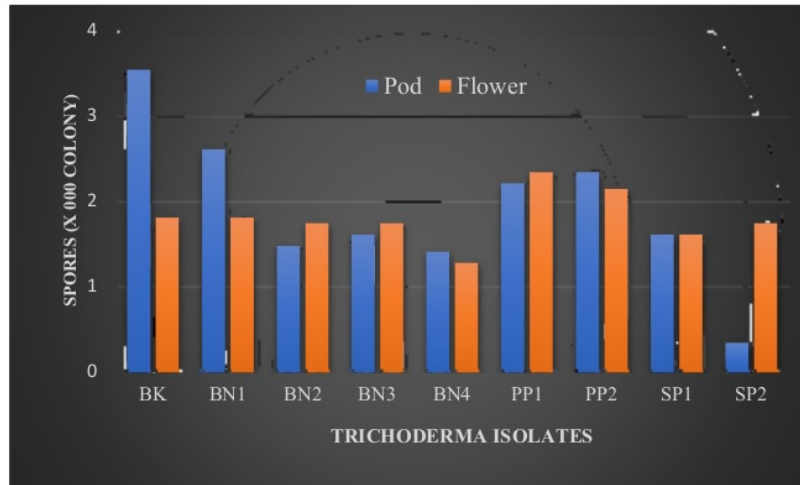


Figure 2. The average of spore density obtained from flower and pod surfaces after spray.

To examine persistence of *Trichoderma* isolates on flower and pod surfaces, a proper re-inoculation method to collect spores 3 days after spray was undertaken to check its spore concentration. It is seen that collecting spores in each isolate varied and there was a tendency of similar spore concentration obtained from flower and pod. Overall, the amount of spore concentration spray before and after faced a tripled spore reduction of all isolates in the beginning of 10^{-6} concentration. Isolates of BK and BN1 shown to have a greater retention in the pod surface and in the flower the spores reinoculated were almost alike.

3.3. Morphological character

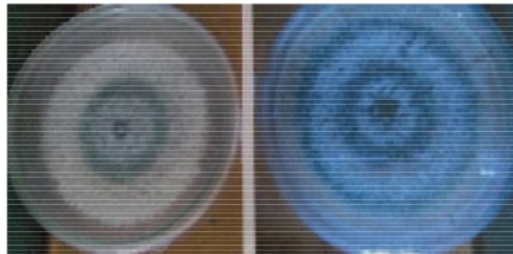


Figure 3. Colony performance of *Trichoderma* isolates grown in PDA medium with 20 mL (left) and 10 mL volume

The isolates of *Trichoderma* were tested in different culture volume and overall the isolates formed like cotton white and green mycelium. On the first day, the hyphae commenced to develop white transparent followed by dense mycelium like cotton before the mycelium transformed to be light and dark green (spore formation phase). The findings suggest that a rapid green hyphal development depends on nutrient sufficiency. The hyphae in a small amount of medium tended to grow faster.

4. Conclusion

All *Trichoderma* isolates generated to vary cutinase and after spray they still sustained into pod and flower surfaces. The isolates of BN4, and PP1 were the highest chitinase activity and BK, BN 2 and SP1 were the lowest activity. For persistence of spores, overall isolates could sustain onto the outer layer of flower and pod, but most isolates had more spore persistence in pod than flower surface.

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