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Original Research Article

Isolation and identification of endophytic fungi from cocoa plant resistante VSD M.05 and cocoa plant Susceptible VSD M.01 in South Sulawesi, Indonesia

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A B S T R A C T

The new prospective area on agriculture and forestry are the use of microorganisms to promote plant growth and to protect the plant hosts from pests and diseases. One group of the microorganisms is endophytic fungi. The research aims to isolate and to identify of fungal endophyte of clones cocoa resistant VSD M.05 and clones cocoa susceptible VSD M.01. A total of 10 isolates of fungal endophytewere isolated from clones cocoa resistent VSD M.05. The isolates belonged to 6 genera namely: *Curvularia* Sp., *Fusarium* Sp., *Geotrichum* Sp., *Aspergillus* Sp., *Gliocladium* Sp., *Colletotrichum* Sp., and 4 isolates that have not been identified as not showing conidia on media of PDA. The fungal endophyte were isolated of clones cocoa susceptible M.01, that as 4 genera identified as *Aspergillus* Sp., and *Gliocladium* Sp. and 2 isolates that have not been identified as not showing conidia on PDA media.

Keywords

Endophytic, fungi; resistant VSD M.05; susceptible VSD M.01

Introduction

The main Indonesian cocoa producing region is the island of Sulawesi which accounts for around 75 percent of Indonesia's total cocoa production. As Indonesia's cocoa productivity per hectare has been lagging behind that of other cocoa-producing countries, the government started a five-year cocoa revitalization program in 2009 to boost production through intensification, rehabilitation and rejuvenation activities, covering a total area of 450 thousand hectares. Factors that are hampering progress in the cocoa industry are aging trees (planted in the 1980s), insufficient improved planting materials and little farm maintenance. More investment in this sector is needed to reach the government's one million tonnes annual production target by 2013-2014. Main obstacle of cocoa growing is pest and disease infestations such as cocoa pod borer

(CPB), Vascular streak disease of cocoa (VSD) and black pod disease, resulting in reduction of yield producitivy about 40% (660 kg/ha/year) of 1100 kg/ha/year. Pest and disease infestation led to lose yield around 198,000 tonnes per year or it equaled to IDR 3.96 triliun per year. In addition, reduction of their infestations can cause poor bean quality so that cocoa bean export to United State of America faced potential loss around US$301.5 per ton.VSD cocoa disease known new disease was found in 1985 in Kolaka Regency (Southeast Sulawesi) and in 2002 in Polmandistrict (West Sulawesi) and Pinrang (South Sulawesi) (Rosmana, 2010). Although VSD is relatively new disease of cocoa, it spreads widely in the cocoa farmers in South Sulawesi. According to Data of State Crop Province of South Sulawesi that total cocoa areas infected VSD initially from about 7,000 ha in late 2003 to 20,607 ha end of July 2004 but it then doubled to 48,727 ha in 2009. Survey of Mars Incorporated in 2008 showed incidence of VSD disease in South Sulawesi around 21 – 68 % (Purung, 2008). Moreover, in 2010 Directorate general of State Crop Protection claimes that infestation of VSD disease in 6 provinces in Indonesia last three years reached 212,132.92 ha. Based on above control of VSD cocoa disease requires seriously and rapidly.

Plants are associated with many different organisms such as bacteria, insects, nematodes, protozoa or fungi (Sieber and Grünig, 2006; Müller and Döring, 2009), which can live endophytically within plant tissues. Endophytic microbes are a very diverse and common group of organisms that can be found in apparently healthy (including functioning but dying off or dead) plant tissue (Saikkonen *et al.*, 1998; Faeth and Fagan, 2002; Sieber, 2002; Vandenkoornhuyse *et al.*, 2002; Piercey *et al.*, 2004; Addy *et al.*, 2005; Porras-Alfaro and Bayman, 2011) and can be located in different plant organs such as leaves, needles, stems or roots (Sokolski *et al.*, 2007; Verma *et al.*, 2007; Grünig *et al.*, 2008b).

There are several definitions of the term 'endophyte'. De Bary (1866) was the first to define organisms invading and residing within healthy host tissue as endophytes. More than a century later, Carroll (1988) defined organisms causing asymptomatic infections within plant tissues as endophytes and excluded pathogenic fungi and special groups of mutualists such as mycorrhizal fungi. Petrini (1991) expanded Carroll`s definition to include all organisms which at certain times in their life inhabit plant organs without causing any harm. Endophytes have co-evolved for a very long period of time with their hosts and therefore usually show low virulence (Sieber, 2007). The behavior of fungal endophytes can range from mutualistic (Usuki and Narisawa, 2007; White and Torres, 2010) to pathogenic (Tellenbach, 2011; Tellenbach *et al.*, 2011), and endophytes can switch their behavior depending on environmental factors, described as the endophytic continuum (Schulz and Boyle, 2005). Arnold *et al.*(2003) could show that fungal leaf endophytes protect *Theobroma cacao* against *Phythophthora* diseases, and similarly Lee e*t al.* (2009) were able to show that the endophytic *Fusarium verticillioides* reduces disease severity of *Ustilago maydis* on maize.

Fungi ever isolated from cocoa plantation have potential as role of biological control agent for VSD cocoa disease. They also have identified morphologically and molecularly in genera: *Acremonium,*

Blastimyces, Botryspaeria, Cladosporium, Colletotrichum, Cordyceps, Diaporthe, Geotrichum, Gibberella, Gliocladium, Lasiodiplodia, Monilochoetes, Nectria, Pestalotiopsis, Phomopsis, Pleurotus, Pseudofusarium, Rhyzopyenis, Syncephalastrum, Trichoderma, Verticillium, and *Xylaria* (Rubini*et al*., 2005).

Relationship between endophytic fungi and the host is symbion mutualisms which both gain benefits each other to survive. Endophytic fungi gains substrate of nitrogent and carbohidrate from its host which substrate of host is poisonous compound and released afterwards it is consumed by endophytic fungi for life. The research aims to isolate and to identify of endophytic fungi of clones cocoa resistant VSD M.05 and clones cocoa susceptible VSD M.01.

Materials and Methods

Selection of cocoa clone

The clones of cocoa used were local of South Sulawesi clones such as M.05 and M.01 which had been tested against Vascular Streak Dieback disease of cocoa (VSD) in the field trial in 2010 conducted by Mars Inc. and Sasmono. The clone M.05 was resistant clone while M.01 was suscaptable.

Isolation of endophytic fungi from healthy branches

Cocoa branches chosen in this study were infected free, symptomless, healthy, and wooden tissue. The offshoots were hand carried to Laboratorium, washed by using drop water, and cutted into small pieces (4mm). Every offshoot was taken 5 pieces, peeled and sectioned in order to result in 10 pieces. Those pieces were sterilized surface with NaOCl 2.5% for 3 minutes and etanol 70% for 2 minutes, and then washed sterilized water for 1 minute. All pieces were laid into petridish covered by sterilized filter paper to dry out. They were then moved to Potato Dextrose Agar (PDA). Afterwards, they were incubated in the room temperature for several days. From 3 to 14 days after incubation, the endophytic fungi were growing and isolated to pure culture in another PDA.

Figure.1 The site of branch dissection (arrows)

Results and Discussion

Isolation and identification of fungal endophyte

10 isolates of endophytic fungi collected and growth from offshoot of clone resistance (M.05) against VSD were successfully detected as *Curvularia* Sp., *Fusarium* Sp., *Geotrichum* Sp., *Aspergillus* Sp., *Gliocladium* Sp., *Colletotrichum* Sp. and other 4 isolates have not been known as their conidia were absentin on the media of PDA. Meanwhile, in the suscaptable clones (M.01), there were only 2 isolates that have been successfully identified such as *Aspergillus* Sp., *Gliocladium* Sp., and other 2 isolates were unidentified due to none of conidia growing in the media PDA.

Typical endophytic fungi growing from clone resistance (M.05)

***1). Curvularia* Sp.**

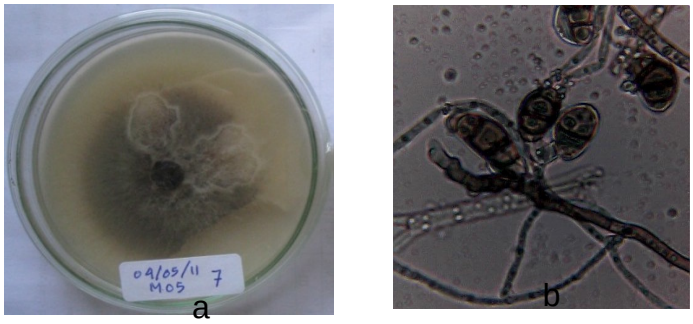

Figure.2 (a) Colony performance on the PDA. (b) Conidiophores and conidia

Macroscopic characterization: the colony is light brown and dark, micelliaare regularly growing with flat growth and thickness. The edge of mycelia shaps uneven and the color is cotton white partly and brown. Microscopic feature : in the hyphae, it has been proven that the septa are present with conidiophores are brown, conidia form spyriform, brown, multi septa dan cellular.

***2). Fusarium* Sp.**

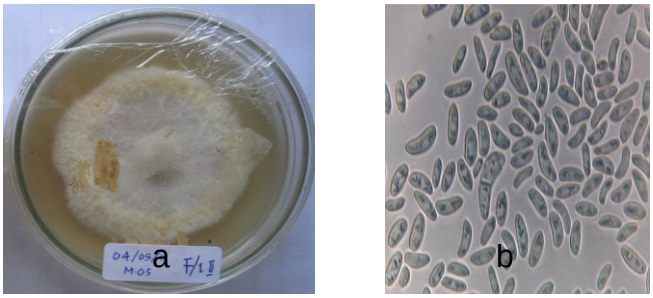

Figure.3 (a) Colony performance on the media PDA. (b) Macroconidia and microconidia

Macroscopic characterization: the color is white in the centre and orange in the edge, micellia are regular, growing with flat. Microscopic feature: there are hyaline microconidia, 1-2 cells, ovoid shape or formand arch. In the macroconidia, the hyphae are hyaline as well, forms up to 2 cells, appear curve orcanoe and the edge of peak appears hooked.

3). Geotrichum Sp.

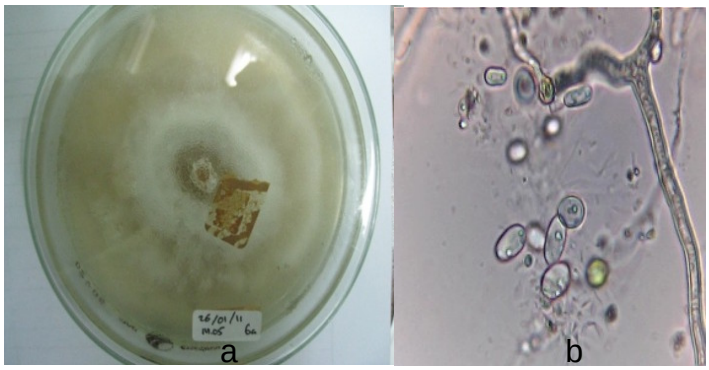

Figure.4 (a) Colony performance on the media PDA. (b) Macroconidia and microconidia

Macroscopic characterization: the colony was cotton white, micellium do not grow regular but flat colony, thickness, the edgeis not flat and the color is cotton white. Microscopic feature : Conodia are hyaline, forming diversity and producing sterigmata.

***4) Gliocladium* Sp.**

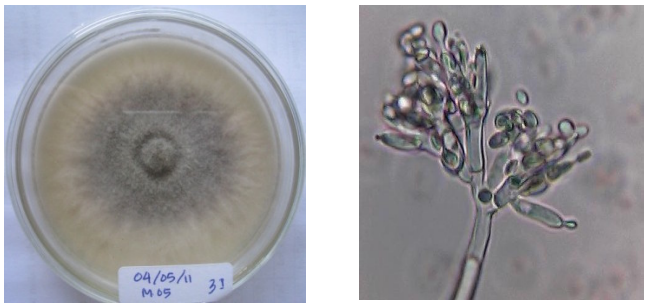

Figure.5 (a) Colony performance on the media PDA. (b) Macroconidia and microconidia

Macroscopic characterization : Colony is dark brown, producing irregular mycelia, growing flat colony, thickness, the edge of colony is not flatand produced dark cloud. Microscopic feature : hyphaeforms septae and both hyphae and conidiophores are hyaline, producing sterigmata. The shape of conidia isovoid, producing sterigmata and green. The top of conidiophores branches forms like a brush.

***5) Aspergillus* Sp.**

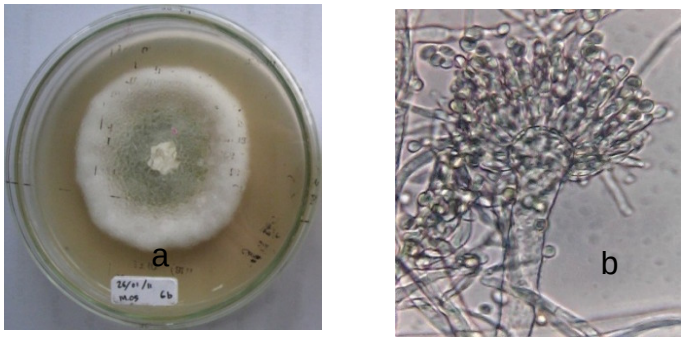

Figure.6 (a) Colony performance on the media PDA. (b) Macroconidia and microconidia

The characterization of macroscophy: the color of colony is green in the core of growth and white in the margin area, micellium are growing regular with flat colony, thickness and the margin of colony is flat as well. Feature of microscopy: hyphae are asepta, micellium have branches, conidiophores upright, prolong, but have branchless. The edge swells and produces vesicle. On the whole vesicle surface micellium are covered, forming phyalide. Conidia are formed subsequently in the phyalide. Conidia are spericle, hyaline, and 1 cell sterigmata.

***6). Colletotrichum* Sp.**

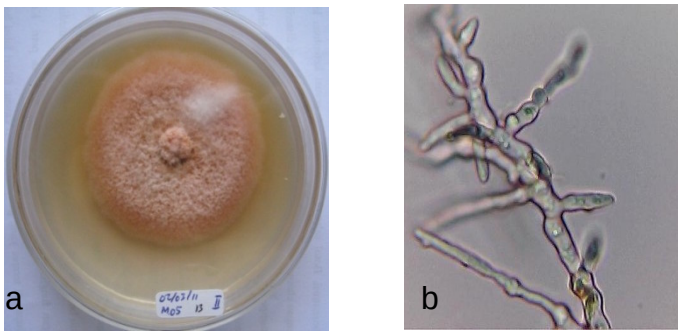

Figure.6 (a) Colony performance on the media PDA. (b) Macroconidia and microconidia

Macroscopic characterization : the colony is dark orange, micellium are regular, the gowth of colony is flat, thick, and the edge of colony is flat as well. Microscopic feature : conidiophores is short, and asepta. Conidia appear oblong and single cell sterigmata.

The characterization of macroscophy: the color of colony is green in the core of growth and white in the margin area, micellium are growing regular with flat colony, thickness and the margin of colony is flat as well. Feature of microscopy: hyphae are asepta, micellium have branches, conidiophores upright, prolong, but have branchless. The edge swells and produces vesicle. On the whole vesicle surface micellium are covered, forming phyalide. Conidia are formed subsequently in the phyalide. Conidia are spericle, hyaline, and 1 cell sterigmata.

The result of isolation and identification from clone resistance M.05 and suscaptable clone M.01 showed that there were 10 isolates of endophitic fungi from clone M.05 and 4 isolates from clone M.01. It seems that difference between clones has an effect on diversity and colony of endophytic fungi. In previous studies various endophytic fungi had been isolated from different plant hosts. Ten genera of endophytic fungi were isolated from root system of palm trees (Nur Amin, *et al* 2008). Two of those genera, *Aspergillus* sp and *Gliocladium* sp. were also found in the current study. *Fusarium* sp. had also been isolated from root systems of tomato and banana (Hallmann, 1994; Nur Amin, 1994). Nur Amin *et al*., (2008) points out the fungal colony of endophytic fungi influenced by biotic and abiotic components. Biotic component consists of varieties and specific host and abiotic includes climate, temperature,

humidity and ground water content along with culture practice. The various number of endophytic fungi showed high density level in the offshoot clone M.05 which related to have resistant level of clone against vascular disease such as VSD. Even though this hypothesis was too earlier to compare relationship between crop resistant level against the disease and the number of endophytic Microscopic feature : fungal endophyt isolated, it had a tendency to connect the existence of endophyt and plant nursery. Rubini *et al*., (2005) claim that endophytic fungi colony is very specific whitin the crop layer and may depend on the interaction between other crop patogens. Moreover, Pirttillä and Frank (2011) contend that diverse organism is needed to have long term stabization of biogeochemistry cycle in ecosystem. However, it has not been known yet how many beneficial species that reducting failure of ecosystem.

Regarding the result of macro and microscophic characterization for fungi found in the offshoot of clone M.05 were *Curvularia* Sp; *Fusarium* Sp., *Geotrichum* Sp., *Aspergillus* Sp., *Gliocladium* Sp., *Colletotrichum* Sp., and 4 isolates have not been identified yet as their spores or conidia did not appear and mycelium was steril in the media PDA. According to Rubini *et al*., (2005), fungal endophyte isolated from the offshoot were 25 genera including endophytic fungi being collected by the author except *Curvularia* Sp. and *Chetomium* Sp.

Based on macro and microscophic observation, *Curvularia* Sp. produces dark brown colony on the media, hyphae have septa and brown, conidiophore is brown, conidia form pyriform, brown, multi-septa, and multi cellular. Barnett and Hunter (1998) argue that *Curvularia*sp.have brown conidiophore with dark conidia and consist of 3-5 cells. Similar to Anonim (2011), *Curvularia*sp.have colony dark brown to black, hyphae septa, hyphae and conidiophore are brown, conidia are brown, pyriform and multi septa. Moving on to *Fusarium* Sp., its colony is white on the centre and the edge is orange, microconidia is hyaline and forms 1-2 cells, ovoid (the portion of tip narrows) or oval with slightly curved tip. Macroconidia is hyaline, up to 2 cells, and shapes like crescent or canoe with curved tip. Barnett and Hunter (1998) argue that microconidia have single cell, ovoid (the one of tip portions narrow) or oblong, macro conidia consist of multi cellular in particular shape like canoe. Turning to *Geotrichum* Sp., it has white colony, conidia is hyaline and formed by single cell. According to Anonim (2011) that *Geotrichum* Sp. produces white colony, conidia is hyaline, single cell, chain, and rectangle, spherical with portion of tip bubbles. Next, *Gliocladium* Sp. results in dark brown colony, hyphae have septa and hyaline, conidiophore has hyaline and branches. Conidia have the form ovoid, consist of single cell and greenish. On the tip of branches, conidiophore forms like a brush and compact. This fungal description is similar to Barnett dan Hunter (1998) that *Gliocladium* Sp. has conidiophore with hyaline, the portion of tip forms branches of penicillate like a brush and compact, conidia is single cell. In addition, Anonim (2011) shows that fungal hyphae belong to septa and hyaline, ovoid to cylindrical conidia forms. Furthermore, *Aspergillus* Sp. produce green in the centre of colony and white in the edge, hyphae asepta, micellium have branches with conidiophore is upright, long and branchless and the portion of tip swells and configurates vesikel. In entire vesicle surface, it is covered phyalid which shapes conidium chainly. Conidia

configure spherical, hyaline, and consist of single cell. As Barnett dan Hunter (1998) claim *Aspergillus* Sp. belong to upright conidiophore, simple, and its tip swells and shapes globose or clavate, entire surface is covered by phyalid, conidia have single cell, forming globose. Last, *Colletotrichum* Sp., produces dark orange colony, conidiophoreis short andnon septa. Conidia shape oblong, and consist of single cell. Dugan (2006) argue that *Colletotrichum* Sp. belongs to simple conidiophore, long, and conidia is hyaline, single cell, forming ovoid or oblong.

As endophytic fungi identified in this study, some of them are commonly crop parasites such as *Curvularia* Sp., *Fusarium* Sp., *Colletotrichum* Sp. *Aspergillus* Sp. It indicates that those fungi survived in the offshoot are both endophyte and pathogen. It has been proven by Backman and Sikora (2008) that endophyte is defined as all of living organism that survive whitin crop layer and have neutral, beneficial or parasitic roles.

The diversity of endophytic fungi that was investigated in the clone resistance against VSD (clone M.05) was much higher than suscaptable clone (M.01). Endophytic fungi were found in clone resistance such as *Curvularia* Sp., *Fusarium* Sp., *Geotrichum* Sp., *Aspergillus* Sp., *Gliocladium* Sp., *Colletotrichum* Sp., isolate KR 1, isolate KR 2, Isolate KR 3, and Isolate KR 4, while there were *Aspergillus* Sp., *Gliocladium* Sp., Isolate KS 1, and Isolate KS 2 in the clone M.01

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