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Isolation and Identification of Endophytic Fungi in Cocoa Seedlings (*Theobroma cacao* L.)

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ABSTRACT

Endophytic fungi are organisms that infect healthy plant tissue without causing visible symptoms. This study aimed to determine the presence of endophytic fungi in cocoa plants following the application of boron and sodium hypochlorite. The research was conducted at the Screen House of the Faculty of Agriculture at Hasanuddin University and continued at the Disease Laboratory within the Department of Pest and Plant Disease Science, also at Hasanuddin University, from October 6, 2013, to March 15, 2014. Boron was applied to the plants in three treatments: Treatment A received 1 gram of boron, Treatment B received 0.5 grams of boron, and Treatment C served as the control group with no boron applied. Additionally, the plants were treated with sodium hypochlorite, which was sterilized for 5 minutes before the identification process to characterize the fungi. The results indicated that three genera of endophytic fungi were isolated from the cocoa plant stems following the application of boron: *Aspergillus sp., Trichoderma sp.,* and *Colletotrichum sp.* Furthermore, one endophytic fungus, *Rhizoctonia sp*., was isolated from the cocoa stems after applying sodium hypochlorite.

Keywords: Boron, Cocoa, Endophytic Fungi, Growth, Sodium Hypochlorite

1. INTRODUCTION

Cocoa is the only species among the 22 in the Theobroma genus of the Sterculiaceae family that is commercially cultivated. Cocoa plants thrive in soil with a pH of 6.0 to 7.5. Groundwater is crucial in aeration and nutrient exchange, significantly impacting cocoa plant growth. A minimum groundwater depth of 3 meters is required for optimal cocoa plant growth. Additionally, groundwater depth is influenced by the slope of the land; areas with a slope exceeding 40% are unsuitable for cocoa cultivation. The ideal conditions for planting cocoa include regions with annual rainfall between 1,100 and 3,000 mm and an optimal temperature of 27°C (Carr et al. 2011).

The natural habitat of cocoa plants consists of tropical wet forests, which thrive under the shade of other vegetation. In optimal cultivation practices, several characteristics of their natural habitat are preserved, particularly by ensuring adequate shade. During the early stages of growth, a high level of shade is provided, which is gradually reduced as the plants mature, depending on various growth factors available (Indonesian Coffee and Cocoa Research Center, 2010).

Endophytic fungi live in plant tissue for a specific period and can live by forming colonies in plant tissue without harming their hosts. The ability of endophytic fungi to produce secondary metabolite compounds according to their host plants is a vast and reliable opportunity to produce secondary metabolites from endophytic microbes isolated from their host plants. Of the approximately 300,000 types of plants spread across the earth, each contains one or more endophytic microbes consisting of bacteria and fungi (Sriwati, 2012).

Endophytic fungi can be found in both agricultural plants and grasses. Colonization of endophytic fungi can increase phenolic compounds in the host; Phenolic compounds can inhibit pathogens directly or with their oxidation products and increase complex metabolic changes such as compounds that can form defense barriers. The inhibition of endophytic fungi against pathogens can be directly related to antagonistic mechanisms and indirectly associated with induced resistance mechanisms. Plant protection with induced resistance is based on stimulating resistance mechanisms by metabolic changes that allow plants to be more effective in their resistance. It is estimated that induced resistance can develop if plant cells can produce new enzymes that activate plant genes responsible for the plant's resistance mechanism (Fitria, 2012).

Boron is present in soil solution at very low levels as boric acid and is adsorbed by soil particles as borate (Degryse, 2017). Boron, the only nonmetal among micronutrient elements, has a constant valence of 3+ and the smallest ionic radius. In the geological environment, B solutions are primarily contained in H_3BO_3 and $H_2BO_3^-$ (Mortvedt et al., 1991). H_3BO_3 is a simple inorganic compound that plants use as a raw material for synthesizing other compounds (Lakitan, 2007).

The provision of microelements of boron can increase the B available in the soil and the concentration and absorption of B in the shoots. Boron is one of the essential micronutrients for plants because it develops new cells in the meristematic tissue, flowering, and fruit development (Syukur, 2005).

Boron affects cell development by controlling sugar transport and polysaccharide formation. Other functions of boron in plants include playing a role in the integration and structure of cell walls and the metabolism of nucleic acids, carbohydrates, proteins, phenols, and auxins. In addition, it also plays a role in cell division, elongation, differentiation, membrane permeability, and pollen germination (Azhari, 2008).

Previous research has been conducted on red chili plants (*Capsicum* *annuum* L.), affecting their growth (Yermiyahu et al. 2008). However, there is no specific information about the effect of Boron administration that is correlated with endophytic fungi.

Based on the description above, it is necessary to conduct research to determine the presence of endophytic fungi in cocoa plants after boron and Sodium Hypochlorite administration.

This study aimed to determine the presence of endophytic fungi in cocoa plants after the administration of boron and Sodium Hypochlorite elements. The usefulness of this study is that it provides information about the presence of endophytic fungi in cocoa plants after the administration of boron and sodium hypochlorite elements.

2. MATERIAL AND METHOD

2.1 Place and Time

The research was conducted at the Screen House, Faculty of Agriculture, Hasanuddin University, and continued at the Disease Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, Hasanuddin University 5.1306° S, 119.4850° E, 10 ASL from October 6, 2013 to March 15, 2014.

2.2 Research methods

2.2.1 Treatment Using Boron

Provision of Treatment

Preparing the seeds to be used, namely, 21 seeds ready to plant. After the cocoa plants are 2 weeks old, they are separated into three groups: treatment A, treatment B, and treatment C. Each treatment consists of 7 cocoa plants of the same height. Cocoa plants are treated with boric acid-containing boron elements. Boric acid is then applied to the plants by dissolving it. In treatment A, 1 gram of boric acid is dissolved in 1000 ml of distilled water; in treatment B, 0.5 grams of boric acid is dissolved in 1000 ml of distilled water; in treatment C, no boric acid is given (control). Then, the boron solution is put into a hand sprayer and sprayed onto the cocoa plants. Spraying is only done once.

Isolation and Characterization

After the plants were 2 months old, the research was continued in the Disease Laboratory of the Department of Plant Pests and Diseases, Faculty of Agriculture, Hasanuddin University. In each treatment, random plant samples were taken, 3 plants each, and then the stems were isolated. Stems are cut into 1-2cm and then split into 2 parts. Then, they are placed on filter paper sterile in a petri dish. Surface sterilization was done with immersion in sodium hypochlorite, 70 % ethanol, and distilled water, which were sequentially sterilized before being placed on sterile filter paper. To obtain pure culture, a fungus that grows in the stem section is transferred to media PDA in Petri dish and then identified to determine the characterization of the fungus.

2.2.2 Treatment Using Sodium Hypochlorite

Provision of Treatment

Prepare the seeds to be used, namely 50 seeds ready to plant. 25 seeds were first sterilized using sodium hypochlorite for 5 minutes, while the other 25 were not treated (control). Then, the seeds are planted in polybags.

Isolation and Characterization

After the plants were 3 weeks old, the research was continued in the Disease Laboratory of the Department of Plant Pests and Diseases, Faculty of Agriculture, Hasanuddin University. In each treatment, random plant samples were taken, 3 plants each, and then the stems were isolated. Stems are cut into 1-2cm and then split into 2 parts And placed on filter paper sterile in petri dish. Surface sterilization was done with immersion on sodium hypochlorite, ethanol 70 %, and distilled water, are sequentially sterilized before placed in on sterile filter paper. To obtain pure culture, fungus which grows in stem part transferred to media PDA in Petri dish and then identified to determine the characterization of the fungus.

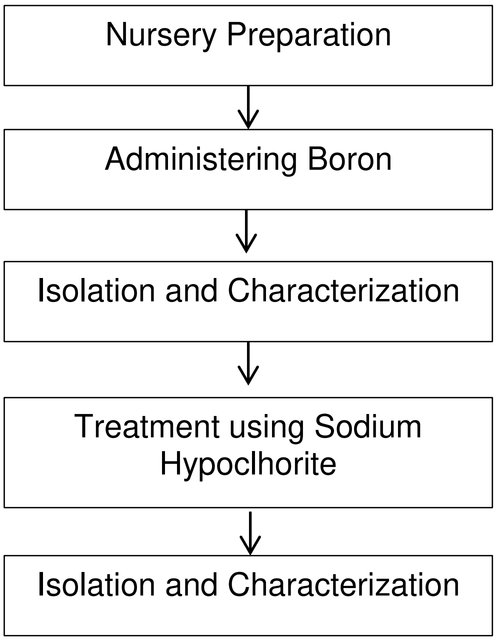

Figure 1. Research Flow Diagram

3. RESULT AND DISCUSSION

3.1 Results

3.1.1 Identification of Endophytic Fungi in Cocoa Plants Isolated on Stems with Boron Treatment

The identification results of cocoa stem isolation using boron treatment obtained 6 isolates. The identified fungi were Colletottrichum sp., Aspergillus sp., Trichoderma sp., and 2 isolates whose genus was unknown, while the identification results of cocoa stem isolation using sodium hypochlorite treatment obtained 2 isolates, the identified fungi were Rhizoctonia sp. and 1 isolate whose genus was unknown. The morphological forms of the fungi found can be seen in Table 1 and Table 2.

Table 1. Characteristics of endophytic fungi of cocoa plants isolated from the stem with Boron treatment

Treatment	Isolate	Identification						Genus
		Macroscopic		Microscopic				
		Top colony color	Color of the lower colony	Conidia/ Conidophore	Hyphae			
					Asepta	Septa		
Boron 1 g	A3	Transparent white border chocolate	Dark white	Oval and hyaline		✓		*Colletotrichum,* sp
	A4 S1	Black	Black	Round and Conidiophores long, *hyaline,* on the end grow bigger to form circle		✓		*Aspergillus,* sp
	A4 S2	Green	Green	The shape of the conidiophore is branched in many forms, 2 – 3 at the end		✓		*Trichoderma,* sp.

				branching, and on each tip forms vialid in groups			
Boron 0.5 g	B5 S2	Black	Black	Round and Conidiophores long, *hyaline,* on the end grow bigger to form circle		✓	*Aspergillus,* sp
	BI 21	White	White	-		✓	Not yet known
Control	C1.2	Black with white edges like cotton	Black with white edges like cotton	Round	✓		Not yet known

Colletotrichum sp.

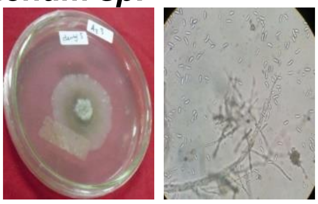

Figure 2. Macroscopic and microscopic morphology of endophytic fungus *Colletottrichum sp.* isolate A3.

Colletottrichum sp. fungus has the following characteristics: Hyphae are not septal, conidia are oval and hyaline, macroscopic characteristics are the color of the upper colony is brown with a transparent white edge, and the color of the lower colony is dark white.

Aspergillus sp.

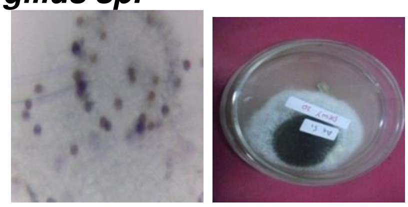

Figure 3. Microscopic and macroscopic morphology of endophytic fungi Aspergillus sp. isolates A4 S1 and B5 S2

Aspergillus sp., as in Table 1. has a round conidia shape, long conidiophores, and hyaline, and the tip of the conidiophores is enlarged to form round, septate hyphae and black colony color with white edges.

Trichoderma sp.

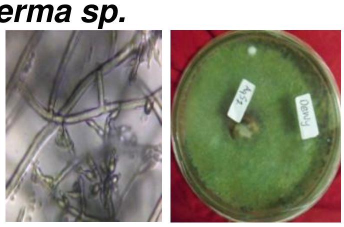

Figure 4. Macroscopic and microscopic morphology of endophytic fungus *Trichoderma sp.* isolate A4 S2

Trichoderma sp. has a green colony color, septate hyphae shape, conidiophore shape with many branches, 2-3 shapes. At the end, branching, and on each tip, there are clustered vialids.

I pray B1 21

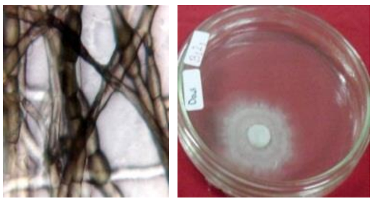

Figure 5. Macroscopic and microscopic morphology of isolate BI 21, an unknown fungus.

Isolate B1 21 is an unknown fungus because only hyphae are visible in isolate B1 21.

I pray C1.2

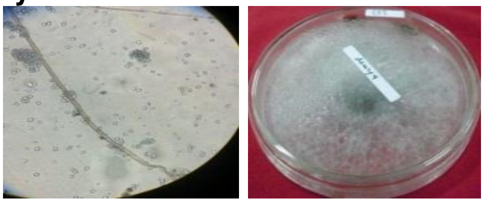

Figure 6. Macroscopic and microscopic morphology of the unknown fungal isolate C1.2.

Isolate C1.2 is an unknown fungus because only spores are seen in isolate C1.2.

3.1.2. Identification of Endophytic Fungi in Cocoa Plants Isolated on Stems with Sodium Hypochlorite Treatment

Table 2. Characteristics of endophytic fungi of cocoa plants isolated from the stem with sodium hypochlorite treatment.

Treatment	Isolate	Identification					Genus
		Macroscopic		Microscopic			
		Top colony color	Color of the lower colony	Conidia / Conido for	Hyphae		
					Asepta	Septa	
Sodium hypochlorite	A222	Dark Grey	Black		✓		Not yet known
	B21	Blackish gray	Blackish gray	-		✓	*Rhizoctonia,* sp

Rhizoctonia sp.

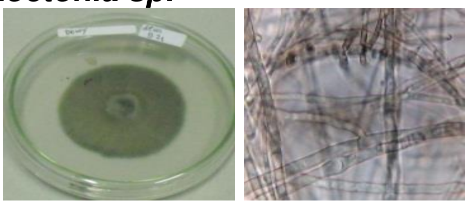

Figure 7. Macroscopic and microscopic morphology of endophytic fungus *Rhizoctonia sp.* isolate B21

Rhizoctonia sp. does not have conidia and long or straight hyphae and septate. Macroscopic characteristics are blackish-gray colony color.

Isolate A222

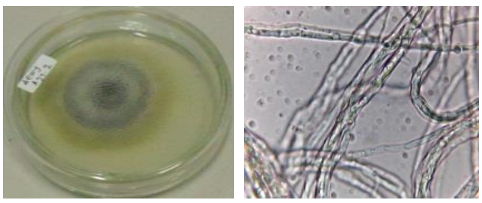

Figure 8. Macroscopic and microscopic morphology of the unknown fungus isolate A222

I pray A222 is a fungus that is not yet known because it is only visible in the presence of conidia; there are only hyphae.

3.2. Discussion

Based on the identification results, it was found that there were endophytic fungi isolated from the stem of the cocoa plant, namely *Aspergillus sp., Trichoderma sp., Colletottrichum, sp., and Rhizoctonia, sp.* Based on research conducted by Hanada et al. (2010), several endophytic fungi are often found in cocoa plantations, such as Pestalotiopsis spp. The frequency of occurrence is 12, *Fusarium spp.* The frequency of occurrence is 11, *Penicillium spp. Trichoderma spp.* The frequency of occurrence is 9, *Acremonium spp. Aspergillus spp.* T. Stromaticum frequency of occurrence is 6, Colleotetrichum gloeosporioides frequency of occurrence is 2.

In the 1 g boron treatment, more endophytic fungi were obtained than 0.5 g boron and control. This is influenced by the nutrient content because the higher the boron element given to plants, the higher the function of boron in providing carbohydrates and proteins so that endophytic fungi in plant tissue get a lot of food supply. This follows the opinion of Azhari (2008) that giving micronutrient boron to plants plays a role in the combination and structure of cell walls, nucleic acid metabolism, carbohydrates, proteins, phenols, and auxins. Boron affects cell development by controlling sugar transport and polysaccharide formation. The provision of micronutrient boron can increase B available in the soil and the concentration and absorption of B in the shoot. Boron is one of the essential micronutrients for plants because of its role in developing and growing new cells in the meristematic tissue, flowering, and fruit development. In addition, it also plays a role in cell division, elongation, differentiation, membrane permeability, and pollen germination.

Colletottrichum sp has the following characteristics: The hyphae are not septate, and the conidia are oval and hyaline. This is following the opinion (Semangun, 2000) that Colletotrichum generally has hyaline conidia, single-celled, measuring 9-24 x 3-6 µm, not septate, elongated, formed at the tip of a simple conidiophore.

Aspergillus sp, as in Table 1, has a round conidia shape, long conidiophores, and hyaline, and the tip of the conidiophores is enlarged to form round, septate hyphae and black colony color. This follows Barnet's opinion and Hunter's (1972) that *Aspergillus sp.* has septate and branched hyphae, at the end of the hyphae, especially at the upright part enlarges to form conidiophores. Conidiophores at the end are rounded into vesicles. In the vesicles, there are short rods called sterigmats. Aspergillus colonies are gray, black, brown, and greenish.

Trichoderma species exhibit a green colony color, characterized by septate hyphae and branched conidiophores. The conidiophores typically display 2-3 branches at their tips, each bearing a cluster of vesicles. This observation aligns with the findings of Barnett and Hunter (1972), who noted that Trichoderma colonies on agar media initially appear white. Over time, the mycelium transitions to a greenish hue, with the most intense green color observed in the colony's center, surrounded by white mycelium. Eventually, the entire medium becomes green. Trichoderma fungi possess upright and branched conidiophores with hyaline conidia that are unicellular, oval in shape, and measure approximately 3-5 µm in diameter. These conidia are typically clustered at the tips of the vesicles. Trichoderma species can be easily identified due to their rapid growth and widespread occurrence. Reason: Improved clarity, vocabulary, and technical accuracy while maintaining the original meaning.

Rhizoctonia sp. does not produce conidia and possesses long, straight, septate hyphae. This fungal isolate is characterized by the absence of visible conidia, with only hyphae present. One identified species is Rhizoctonia sp., which exhibits microscopic features such as perpendicular hyphal branching and septa. Macroscopically, it is characterized by a blackish-gray colony color. This aligns with the observations of Barnett and Hunter (1998), who noted that the characteristics of Rhizoctonia sp. include mycelium, which can be clear in some types and dark in others. The mycelial cells are typically elongated, with septa forming at the branches of the main body. Notably, Rhizoctonia sp. lacks conidia and other reproductive cells, but it produces sclerotia that can range from light brown to black. Additionally, Alexopoulos et al. (1996), as cited in Ridho Pratomo (2006), noted that *Rhizoctonia sp.* features a perpendicular or nearly perpendicular arrangement of hyphal branching, the presence of porous septa (deliport septa), the absence of clamp connections, and a narrowing of the hyphae near the branching points.

4. CONCLUSION

Based on the conducted research, it can be concluded that the application of boron elements resulted in the identification of three species of endophytic fungi in cocoa plants isolated from the stem: *Aspergillus sp., Trichoderma sp., and Colletotrichum sp.* Additionally, the administration of sodium hypochlorite led to the isolation of one endophytic fungus from the cocoa stem, specifically *Rhizoctonia sp.*

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