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Controlling Anthracnose Disease of Locally Chili in Marginal Wetland using Endophytic Indigenous Microbes and Kalakai *(Stenochlaena palustris)* Leaf Extract

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ABSTRACT

The research aims were to get the indigenous endophytic microbial consortium and to test the potency of kalakai leaf extract as biopesticides and biofertilizer on chili plant specific to wetlands (i.e., var. Hiyung). The microbes capable of inhibiting the growth of anthracnose have been performed on in-vitro test in pairs method. It was found that 12 isolates have the ability to inhibit the growth of pathogens. However, based on the results of a confirmatory endophytic test only three isolates had positive role as endophytic in chili plants, namely *Trichoderma* sp DN3, *Trichoderma* sp AK2, and *Trichoderma* sp BT1. The results of the effectiveness of each treatment on chilli plants in the greenhouse and the field shows that the application of endophytic could inhibit the development of anthracnose and spur the growth of plants. It could be concluded that the applications of *kalakai* leaf extract at the rate of 30 mL/plant can function as biopesticides and biofertilizer.

Key words: Endophytic, Anthracnose, Chili, and wetland

INTRODUCTION

The utilization of marginal wetlands must be intensified because the fertility of land in South Kalimantan continues to shrink due to land conversion. The success of land use depends on the ability to improve the poor soil of nutrients (Annisa et al., 2012), and to reduce the disruption of plant diseases that become severe recently (Budi et al., 2013).

The use of synthetic pesticides and fertilizers in tidal wetlands is ineffective because the water is not remained on the land sometimes, but some time rather move out the land. The use of indigenous microbes as biopesticides and biofertilizer is to be a right solution (Budi and Mariana, 2013).

The indigenous biological agents in tidal land could inhibit the progression of stem rot disease of rice (*Rhizoctonia solani*) nearly 87% (Budi and Mariana, 2013) and could inhibit the disease progression of stem rot (*Ganoderma* sp) of palm oil by reducing the severity of the disease up to 96.45% (Budi, 2015). The appropriate biological agents is not only to inhibit the development of pests and diseases, but also capable to promote plant growth. The application in the field is facing constraint because difficulties in microbes propagation and plant application level.

The kelakai (*Stenochlaena palustris*), a tidal swamp typical weed, grows abundantly in the tidal area of South Kalimantan. The local residents already use it as skin medicine, though it is yet not for economical purposes. Previous studies has shown that the leaves of kalakai contain many alkoloid, the compounds being capable of inhibiting the growth of microbes (Suhartono et al., 2012).

Current study tried to advantage the endophytic microbes with *kalakai* leaf extract as carrier material and media for antagonist microbes propagation. This research is expected to formulate the indigenous antagonist and a carrier material applicable to farmers, specially to chili (*Capsicum annum* L.) farmer in South Kalimantan.

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METHODS

The isolation of antagonist microbes (fungi) was collected from healthy plants that were among the sick plants in chilli garden in Tapin regency, South Kalimantan. The exploration and isolation of antagonistic microbes used PDA media (Homby procedure) (Tuite, 1969). Pathogenic microbes was obtained from previous study by Budi et al (2013). The isolated antagonist was tested to determine the antagonistic power, those that have a high ability to inhibit the growth of pathogens. The test was performed on a PDA media in pairs using the direct opposition means (Fokkema et al., 1973) (Figure 1).

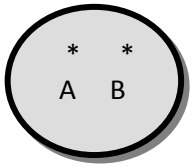

Figure 1. Placement of the pathogen and antagonist in Petri dish "A" = pathogenic; B = antagonists

The calculation of inhibiting antagonist against pathogens is conducted using the formula (Fokkema et al., 1973):

$$I = (r_1 - r_2)(r_1)^{-1} \times 100$$

Where: I is the inhibiting percentage; "r_1" is the colony radian of A that comes approaching B; and "r_2" is the colony radian of A that appears toward B

The chili was planted with a spacing of 10 cm x 10 cm in plastic box measuring one m^2. The pathogens formula (i.e., combination of microbes and kalakai compost) was sprinkling at the rate of 30 kg/ha in the soil surface around the chili stem in three weeks old. Sterile plot was also prepared as comparison. The observation of effectiveness, efficiency, and survival was based on counting the number of plants that is withered or that is asymptomaticly infected at three weeks from the time of treatment applications.

Kalakai leaf was extract with method described by Pimenta *et al*. (2003). In this method, kelakai leaves were washed using flowing water, then were dry in an oven at 45^{o}C for 48 hours. Dried leaves were than blended in a blender until it becomes powder. The powder was macerated with methanol for 7 days. Finally, the powder was filtered through Whatman No 1 filter paper and the methanol was evaporated, resulting in *kelakai* extracts.

The antagonistic fungi was mixed with *kalakai* extract to a density of 2.3 x 10^7 spore/g. The formulas were applied to chili pant in pot at the rate of 0, 10, 20 or 30 ml/plant. Sterile plot was also prepared as comparison. The observation of effectiveness, efficiency, and survival was based on counting the number of plants that is withered or that is asymptomaticly infected at three weeks from the time of treatment applications.

The intensity of the disease was observed since the appearance of anthracnose symptoms being characterized (i.e., the appearance of patches of necrotising form of concentric circles on chilies). The chili fruits are positively stricken with anthracnose if having a diameter of $\geq$ 4 mm spotting necrosis.

The rate of fruit rot disease attack was observed by counting the number of fruits attacked and the number of healthy fruits per sample plot (Shuseela, 2012):

$$P = \frac{A}{N} \times 100\ \%$$

Where : P is the rate of plant rot (%); A is the number of of fruits attacked per sample plot; and N is the number of observed fruits per sample plot

The test of chili growth is based on the fresh fruit weight per crop by weighing the

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fresh fruit per plant. Total plant fresh weight (grams) was calculated by weighing all parts of plants (fruits, leaves, stems, and roots). The calculation of total dry weight (grams) was calculated by weighing all parts of plants (fruits, leaves, stems and roots) after the plant had been dried using an electric oven at a temperature of 60^0C for 24 hours. The data were analyzed for analysis of variance (ANOVA) and Duncan multiple range test (DMRT) at the level of $\alpha = 0.05$.

RESULT AND DISCUSSION

The exploration of microbes from the leaves, fruit flesh, roots, stems, and seeds of chilli resulted 12 isolates. Five isolates were found on leaves, thee isolates on flesh, two isolates on the root, one isolate from seeds, and one isolate from the base of the stem.

The test result of endophytic inhibition toward isolates of pathogens causing anthracnose disease based on the analysis of variance were proven that all isolates antagonists influenced significantly in suppressing the growth of anthracnose pathogen *Colletotrichum* sp. The DMRT showed that the inhibition was seen in isolates being derived from the leaves (*Trichoderma* sp DN3) with inhibition of 48.5%, followed by isolates being derived from the root (*Trichoderma* sp AK2) of 42.3%, and isolates from the base of the stem (*Trichoderma* spp BT1) of 29.7%.

The test result of antagonistic mechanism toward pathogenic isolates showed that there were some having more than two antagonistic mechanisms. The isolates DN3 has the antagonistic mechanisms, such as competition and antibiosis space, isolates AK2 with competition mechanism and mycoparasit space, and BT1 isolates with antibiosis mechanism. The test result of endophyte confirmation of the three isolates states that they are inoculated in the plant tissue with visible presence of fungal hyphae inoculated after being re-isolated to get the same isolates (Figure 2).

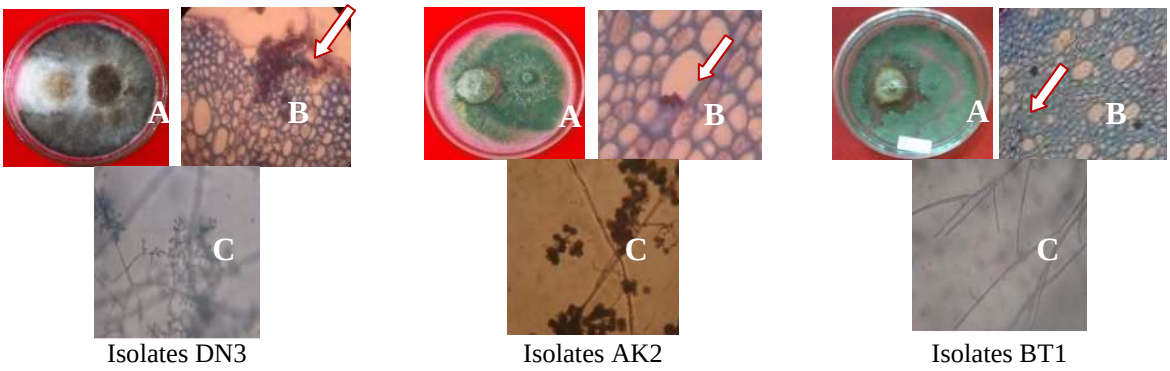

Figure 2. The characteristics of three selected endophytic Fungi isolates
Note: A is the mechanism of inhibition test results; B is the result of confirmatory test of endophyte in plant tissue, and C is the morphology of fungi Endophytic at 400x magnification

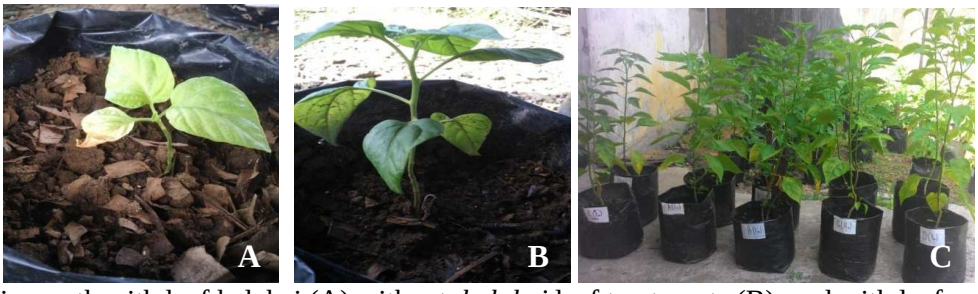

Figure 3. Chili growth with leaf kalakai (A),without *kalakai* leaf treatments (B), and with leaf extract (C)

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The test result of giving kelakai leaves as basal fertilizer was proven a failure because its leaves did not undergo decomposing. Although it was even during storage over 6 months, the kalakai leaf did turn rotten. The application of kalakai leaf on soil as a base fertilizer resulted in inhibition of chilli plant growth (Fig. 3). Although the result of application of kalakai leaf with endophytic promote the plant growth, the application of kalakai leaf extract was proved to be able of acting as a biopesticide and biofertiliser (Table 1).

Table 1. Effect of endophytic and *kalakai* leaf treatments toward the disease intensity and chili growth

Dose of *kalakai* extract	Desease Intensity (%)	Branch Number	Fruit Weight	Wet Plant Weight	Dry Plant Weight
0 ml/plant	53 ^{c}	3,20 ^{a}	0,40 ^{ab}	45,55 ^{b}	21,18 ^{b}
10 ml/plant	26 ^{ab}	3,60 ^{a}	0,11 ^{a}	48,24 ^{b}	21,27 ^{b}
20 ml/plant	33 ^{b}	3,60 ^{a}	0,65 ^{b}	57,23 ^{c}	24,28 ^{bc}
30 ml/plant	20 ^{a}	5,00 ^{b}	1,51 ^{c}	61,77 ^{d}	25,93 ^{c}
Control	68 ^{d}	2,20 ^{a}	1,56 ^{c}	39,47 ^{a}	16,85 ^{a}

The observational data of the number of plant branch indicate that the doses of *kalakai* solution influenced the diseases intensity and number of branches of chilli plants. The lowest debases intensity was observed at a dose of 30 ml/plant of kalakai leaf extract. The dose of 30 ml/plant of kalakai leaf extract gave the highest chili branch number. However, the observation data of fruit weight showed that the dose of kalakai solution did not influence the chilli plants significantly. Moreover, the observations of dry weight showed that the dose of kalakai solution did not influence the dry weight of chilli plants significantly so that it did not need the further test using DMRT at the level of 5% (Table 1).

Fungi from chili *hiyung* in fairly rotten plant can inhibit the growth of pathogenic anthracnose *Colletotrichum* spp in which three isolates (DN3, AK2, and BT1) were proven endophytic. According to Ramdan *et al* (2003), the plant root of chili has the wealth of highest endophytic fungus toward leaf and stem. Khalili et al. (2012) and Stone et al. (2004) states that *Trichoderma sp* associates with plant and commonly is found in the root and stem. According to Naemi et al. (2010), *Trichoderma* sp appears in the phyllosphere.

The isolates DN3, AK2 and BT1 were able to inhibit the growth of *Colletotrichum* spp and were endophytic because of having the different antagonist mechanisms. The antagonistic mechanism occurs in the form of space competition, antibiosis, mikoparasit, and non-pathogenic to plants. The mechanism of biological control *Trichoderma* spp against fungal pathogens are generally divided into three kinds, namely parasitism, antibiosis, and competition for places of growing and nutrition (Harman, 2006; Howell, 2013). Ghisalberti et al. (1991) states that one of the antagonistic microbes properties is the space competition so that it grows faster than the pathogen or by producing antibiotic compounds that can inhibit the growth of pathogens. According to Atanasova et al. (2013), a corpse is antagonistic towards other bodies when the barrier zone between the two colonies isolates were tested in pairs. interaction between *Trichoderma* spp with the target fungi is largely mikoparasitisme mechanism which is divided into two types, namely mikoparasit biotrop which does not cause the death of the host fungi and mikoparasit nekrotrop by killing the host after some time or shortly after contacting with the host. Mikoparasitism is the ability to utilize the fungal host directly as a nutrient to life with the lytic enzymes help. The antibiosis mechanism is the ability of an agent antagonist to produce the toxic metabolites or

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antibiotics as inhibitors of the host while the space competition mechanism is antagonist mechanism in the form of competition of growing space and nutrients so that the growth of the pathogen can be inhibited.

The *Trichoderma* spp fungi can position it self as the ideal antagonist because of producing a variety of active ingredients, such as glioviridin, sesquiterpenoids, trichothecenes, cyclic peptides, and the content of metabolites isocyanide (tricho viridin). It is also potential to inhibit the other microorganisms (Brewer et al., 1982; Howell, 2013). Elfina et al. (2001) states that the *Trichoderm*a spp fungus produces the toxin substance in the form of antibiotic compounds, such as suzukalin and alametisin that are anti fungal and bacterial. In addition, *Trichoderma* also produces the antibiotic substances in the form of Trichodermin that can control the *Colletotrichum lindemuthianum* fungi (Shentu et al., 2014). It is also in line with studies conducted by Alfizar et al. (2013) states the ability to inhibit the broadth of *Trichoderma* spp colony *Colletotrichum* sp on the seventh day of 1.6 cm^2, the *Fusarium* sp and *Sclerotium* sp, colonies width of each on the seventh day and 1.8 to 2.6 cm^2. This is assumed due to the antagonistic agent *Trichoderma* spp. which inhibits the growth of these three pathogens. Herliyana et al. (2013) in his study also said the slow growth of the pathogen colony diameter of *Ganoderma* sp in provision treatment of antagonistic fungi of *Trichoderma* spp. It is strongly assumed because of a reaction between the toxic compounds from fungal antagonist *Trichoderma* spp against pathogens in plants sengon *Ganoderma* sp.

The endophytic fungus as an antagonist agent is widely used because it can be associated with host plants mutually beneficial properties. The endophytic fungus from the group of *Trichoderma* spp and *Fusarium* sp can promote the growth of tomato plants (Azarmi et al. 2011). Besides, it is able to stimulate plant growth; endophytic fungi can improve the response of plants toward drought (Shukla et al., 2012). The endophytic fungi can also improve the crop harvest of chilli (Khan et al., 2013). The endophytic non-pathogenic fungus *Fusarium oxysporum* can promote the growth of banana plants on a laboratory scale (Nel et al., 2006). The fungus endophyte of grass and bamboo roots can promote the plant growth of broccoli (Bullis et al., 2014). The research result conducted by Suyoto (2009) also proves that the fungus of *Aspergillus niger* is endophyte and could increase the growth and production of rice and corn.

Application of Trichoderma with kelakai can increase the growth of plants. This is because trichoderma role in decomposing nutrients previously unavailable become available to plants (Howell, 2013)

CONCLUSSION

Based on the result and discussion above, it is found that there are three endophytic isolates that are able to control the anthracnose desease and drive the growth of chili from genus Trichoderma sp by using the antagonist mechanism in the form of a competition mechanism of space, antibiosis and it is non-pathogenic. The treatment applications of kalakai solution at a dose of 30 ml/plant can spur the growth and the best yield of chilli Hiyung.

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