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Rapid Inoculation Technique and Biological Control of Leaf Spot Disease in Oil Palm

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ABSTRACT

Oil palm (*Elaeis guineensis*) is one of the most efficient oil-producing crops in the world. However, fungal diseases such as basal stem rot (BSR) caused by *Ganoderma* sp. causes significant loss to the yield of adult palms. Apart from BSR, leaf spot disease caused by *Curvularia* sp. also causes significant loss during nursery stages. In this study, *Curvularia* sp. was isolated from a diseased palm seedling and a rapid and reproducible artificial inoculation method was developed. The technique has bioassay to determine the level of success of the control of leaf spot disease in a glasshouse setting. A natural, organic cyclic peptide fungicide and living cells of a bacterial strain *Paraburkholderia* sp. CP01 were tested for their efficacy to control leaf spot in oil palm seedlings. The severity of leaf spot disease in oil palm seedlings treated by organic cyclic peptide fungicide and CP01 was significantly lower than untreated control, indicating potential biological control agents. The results presented here provide technical reference and novel approach to controlling leaf spot disease of oil palm.

Keywords: *Curvularia*, *Elaeis guineensis*, organic fungicide

INTRODUCTION

Cultivated oil palm (*Elaeis guineensis*) was originated from West Africa and it is one of the most efficient oil-yielding crops in the world, in terms of utilization of plantation area, efficiency, and productivity of the plants (Sumathi *et al*. 2008). Palm oil contributes to about 36% of the total world vegetable oil production with main producers include Indonesia, Malaysia, Thailand, Nigeria, and Colombia which together provide around 93% of total world palm oil production (Sharma *et al*. 2012).

In Indonesia, the oil palm industry has grown significantly over the years and palm oil has become a major export commodity with a total production of 34.5 million tons of crude palm oil in 2017 (BPS 2018). However, despite the exciting growth, diseases are becoming serious limiting factors given effective control is still absent.

BSR caused by *Ganoderma boninense* is an obvious threat that reduces yield of productive palms. Another important disease that somehow is rather overlooked is leaf spot (synonymous

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to brown or blight spot) caused by *Curvularia* sp. The disease attacks oil palm seedlings at pre- and main-nursery stages (Susanto & Prasetyo 2014). It has been considered to be a minor problem but the spreading of the disease in oil palm growing areas is quite rapid (Kittimorakul *et al*. 2013). Through contact from infected leaves to another, the possibility of disease expansion to spear leaf and growth point can eventually kill young palms (de Franqueville 2003).

Until now, chemical fungicides are still applied to manage leaf spot disease, which can lead to the development of resistance pathogens and long-term impact on the environment. An emerging alternative to the chemical fungicide is using biological control mainly employing antagonistic microbes to fend-off the pathogens. Another approach, which may not fit well in the category of biocontrol nor chemical agents, is to use paraprobiotics, which by definition is non-viable, inactivated probiotics (de Almada *et al*. 2016). The objectives of this research were to confirm *Curvularia* sp. is the causal agent of leaf spot disease and to develop rapid inoculation technique that reproduce consistent disease in palm seedlings. Secondly, to evaluate a possible control of the disease through the use of a biocontrol agent and an organic fungicide that is a paraprobiotic.

MATERIALS AND METHODS

Isolation and Identification of *Curvularia* sp.

Diseased oil palm leaves were collected from the nursery facility at PT AMP, Pasaman, West Sumatera, Indonesia. Isolation of *Curvularia* sp. was conducted using a method as described by Zhu & Qiang (2004) with several modifications. Infected leaves were cut and sections of cut leaves (1-2 cm^2 cuttings) were made so that the edges of the cuttings spanned the necrotic and healthy parts or regions on the lesions. The cut leaf sections were rinsed in sterile distilled water and dabbed on sterile tissue paper to absorb excess water. The leaf sections were then placed on 1/5-strength potato dextrose agar (PDA) (Difco™, Becton, Dickinson & Company, USA) and incubated for 4-7 days at 28 °C. Mycelia of putative *Curvularia* sp. were isolated from growing mycelia around the leaf section. The mycelia were transferred onto 1/5-strength PDA and incubated for at least 7 days at 28 °C.

Genomic DNA was isolated from mycelia of pure cultures of fungal colonies isolated from the infected leaves. Around 50 mg of mycelia were frozen using liquid nitrogen and ground on a mortar with a pestle into powder. The mycelia were ground again using liquid nitrogen into finer powder. DNA was isolated using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Molecular identification was performed by analysing the DNA sequences in 5.8S rRNA gene and within the internal transcribed spacer (ITS) region. DNA sequences were amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 primers (5'-TCCTCCGCTTATTGATATGC-3'). The amplified DNA fragments were then sequenced (3130 Genetic Analyzer, Thermo Scientific) and verified. The DNA sequence was queried for similarity using non-redundant BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). DNA sequences from BLAST hits were then aligned using MUSCLE (Edgar 2004). Phylogenetic tree was constructed by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). The bootstrap consensus tree inferred from 1 000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Evolutionary analyses

were conducted in MEGA6 (Tamura *et al*. 2013).

Rapid Inoculation Technique

We sought to test whether the isolated *Curvularia* sp. is pathogenic and thus causing leaf spot disease in oil palm seedlings. Germinated oil palm seedlings (tenera, dura × pisifera) cultivar TS3 were grown in a glasshouse for 2.5 months. *Curvularia* sp. was grown on PDA for 3 weeks until spores were produced in abundance. A quick inoculation method was done by attaching 1x1 cm^2 mycelial plugs of *Curvularia* sp. cultures on PDA onto the abaxial-side of oil palm leaves. After inoculation, the palm seedlings were covered with transparent plastic bags for 3 days to maintain humidity. Incubation was continued for one week in a glasshouse until necrotic spots on leaves were visible. After a week, disease was assessed based on the presence and absence (disease incidence) of necrotic spots on the inoculated area of leaves.

The second method of inoculation was conducted by spraying spore suspension onto leaves. Spores of *Curvularia* sp. were prepared by scrapping colonies of the fungus on PDA plates and the spores were then resuspended in sterile water supplemented with 2% (w v^{-1}) sucrose and 0.02% (v v^{-1}) Tween 20. Concentration of spores was adjusted to 10^5 spores mL^{-1}. The pathogen was inoculated by spraying the spore suspension onto both abaxial and adaxial sides of the leaves. Leaves of the inoculated seedlings were then covered with transparent plastic bags for 3 days, then plastics were removed and incubation was continued until 30 days.

***In Vitro* Antagonism Assay and Biological Control of Leaf Spot Disease in Oil Palm**

Once the isolated *Curvularia* sp. was confirmed to be virulent, *in vitro* antagonism and disease control assays were attempted using our collection of a biological control agent i.e. a bacterial strain namely CP01, and a cyclic peptide-based organic fungicide with commercial name of ProPlant C®. For antagonism assay, a 1×1cm^2 mycelial plug of *Curvularia* sp. was inoculated on PDA at the centre of the Petri plate. At the same time, a single colony of CP01 grown on King's B agar was streaked on the *Curvularia*-inoculated PDA around 2.5 cm-away from the mycelial plug. Another colony of CP01 was streaked at the opposite end, thus creating parallel lines flanking the mycelial plug. As an untreated control, only mycelial plugs were inoculated on PDA without inoculation of bacterial cells. The plates were then incubated at 28 °C for 14-21 days until the mycelia of *Curvularia* sp. (untreated control) had grown and covered the PDA completely. Each treatment (with or without CP01) was repeated in 6 Petri plates. The degree of inhibition was measured as a percentage of ratio of growth radius as follow:

$$\text{Inhibition} = \left(1 - \frac{\text{radius of mycelia challenged with biocontrol agents}}{\text{radius of untreated mycelia}}\right) \times 100\%$$

An isogenic line (TC21) of 2.5-month-old tissue culture-generated oil palm seedlings (ramets) were used for the biological control assay. CP01 was grown for 24 h in King's B broth (28 °C, 200 rpm). After 24 h, cells were harvested by centrifugation and resuspended in phosphate-buffered saline solution (0.1 M, pH 7.0). Concentration of cells was adjusted to OD_{600} = 0.5, which is equivalent to 10^9 cells mL^{-1}. ProPlant C® was used at its original concentration (without dilution) according to the product's label. Three days prior to inoculation with *Curvularia* sp., CP01 cell suspension and ProPlant C® were applied by spraying onto abaxial and adaxial sides of leaves of oil palm. As a control without treatment, water was sprayed 3 days

prior to inoculation with *Curvularia* sp. Three days after application of CP01 and ProPlant C®, the pathogen was inoculated by spraying the spore suspension onto both abaxial and adaxial sides of leaves as described above. At 14 days post inoculation (dpi), CP01 and ProPlant C® were applied again on the leaves, thus during 30 days of incubation, CP01 and ProPlant C® were applied twice. Eight plants were used for each treatment, and for each plant there were normally 3 leaves. The experiment was repeated 2 times.

Disease Assessment and Statistical Analysis

Disease progression was scored and evaluated by assessing the percentage of disease severity index (% DSI). The disease severity index was measured on a scale of 0-4 according to the symptoms of the disease observed (Susanto & Prasetyo 2014), where 0 = 0% of leaf area infected, 1 = 1-25% of leaf area infected, 2 = 26-50% leaf area infected, 3 = 51-75% of leaf area infected, 4 = 76-100% of leaf area infected. After the disease assessment, the % DSI was calculated by the following equation:

$$ABCF = \frac{\Sigma(Ax0)+(Bx1)+(Cx2)\ldots(Fx4)x100}{\Sigma(A+B+C+\ldots+F)x4}\ \%$$

Where A, B, C and F is the number of plants to be multiplied by the 0-4 score (Nur & Abdullah 2008).

The disease ranking is an ordered categorical (ordinal) variable that has non-Gaussian distribution, thus the statistical model applied was non-parametric. Statistical test was conducted using a Kruskal-Wallis test to compare significance of scores between treatments and probabilities were adjusted further by using a Dunn's multiple comparison post hoc test. All statistical analyses were conducted using the R statistics program (R Core Team 2016).

RESULTS AND DISCUSSION

Isolation and Identification of Pathogens Causing Leaf Spot of Oil Palm

A fungal isolate was isolated from necrotic oil palm leaves. The colony of the fungus resembled *Curvularia* sp. as characterized by its cottony, greyish colour of the mycelia that turned to dark grey or black as the colony grew older. Molecular identification based on the ITS region identified the fungal isolate (designated as *Curvularia* AMP isolate) to be most closely related to *C. ravenelii* (77% bootstrapped similarity) (Figure 1). A number of independent colonies were isolated from several necrotic spots but molecular analysis of ITS regions suggested that they were the same strains (data not shown).

Pathogenicity Test Showed that *Curvularia* sp. is the Causal Agent of Leaf Spot Disease in Oil Palm

We sought to test whether the isolate of *Curvularia* sp. was the causal agent of the leaf spot disease in oil palm. A rapid inoculation assay was therefore developed by directly attaching the mycelial plugs onto the abaxial side of leaves. Symptoms of leaf spots were observed at 7 dpi. Foliar symptoms marked by necrotic lesions with yellow to brown borders were visible at the inoculated sites both at abaxial and adaxial sides of the oil palm leaves (Figure 2). The disease progressed gradually from 7 until 30 dpi. The symptoms were similar to the leaf spot disease symptoms observed on the oil palm seedlings in the nursery where they were originated (Figure 2). We noticed, however, that when the pathogen was inoculated on the adaxial side of leaves, infection did not occur and thus there was no disease (data not shown). On the other hand, infection always occurred when the pathogen was inoculated on the abaxial side.

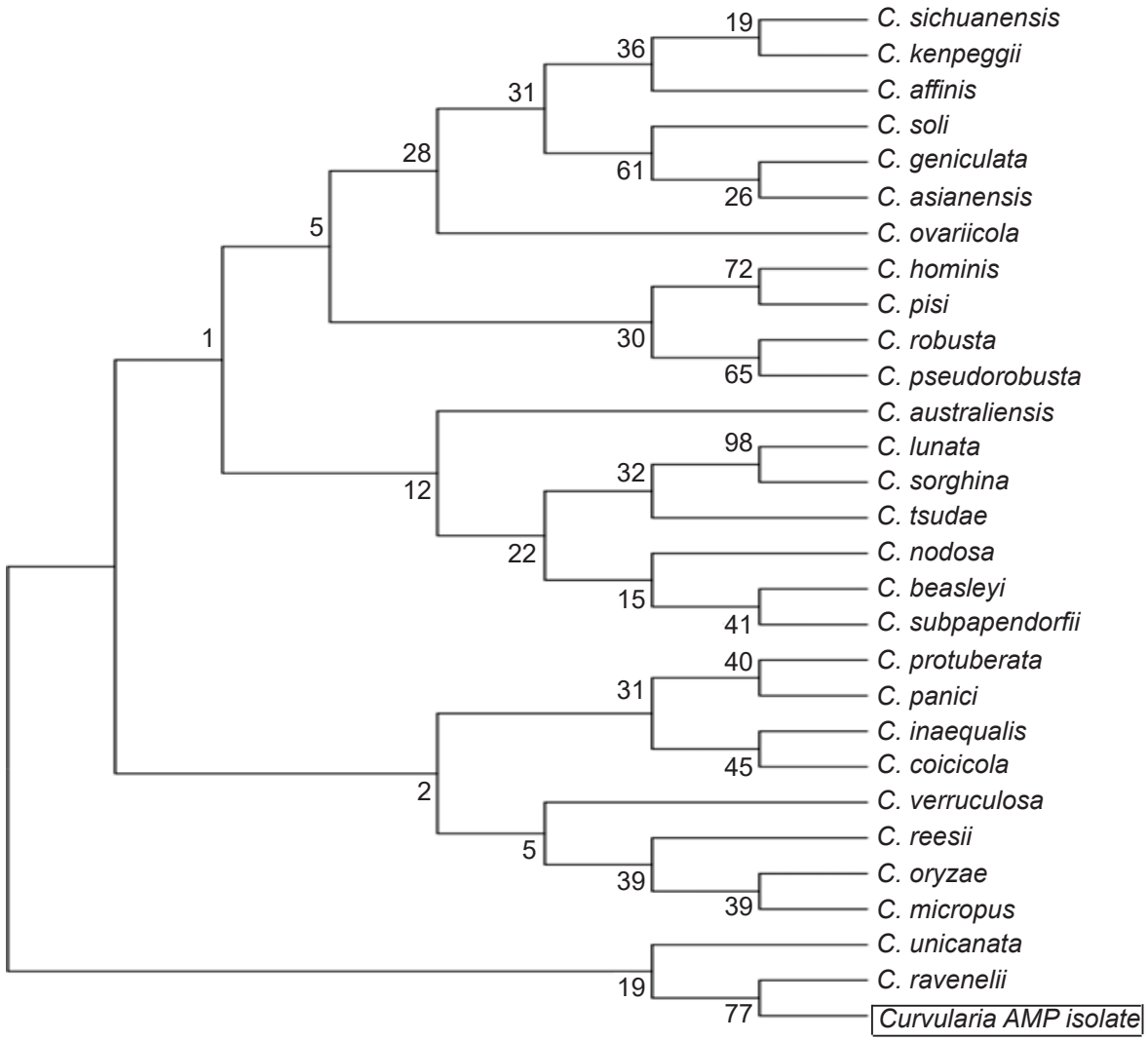


Figure 1 Phylogenetic tree showing the position of isolate *Curvularia* sp. originated from PT AMP Plantation (*Curvularia* AMP isolate) relative to other *Curvularia* species. The 5.8S rRNA and internal transcribed spacer sequences were retrieved from GenBank and aligned using MUSCLE (Edgar 2004) and tree was constructed using the Neighbor-Joining method. Tree topology was inferred with the Tamura-Nei correction model (Tamura and Nei 1993) with 1 000 bootstrap replications based on the best substitution model.

The second method of infection was by spraying spore suspension onto leaves on both adaxial and abaxial sides. Using this method, the disease was produced consistently with typical symptoms resembling natural leaf spot disease as observed in the field (Figure 2b, c). However, using this method, the disease progression took longer to develop than inoculation through attaching mycelial plugs.

CP01 Inhibited the Growth of *Curvularia* sp. Through Secretion of Antifungal Metabolites

In vitro antagonism assay showed that CP01 inhibited the growth of *Curvularia* sp. strongly (Figure 3a, b). On average, the percentage of inhibition of *Curvularia* sp. by CP01 was 68% (±1.44%). The average radius of mycelia of *Curvularia* sp. that were challenged by CP01 was 1.4 cm (±0.06 cm), whereas the

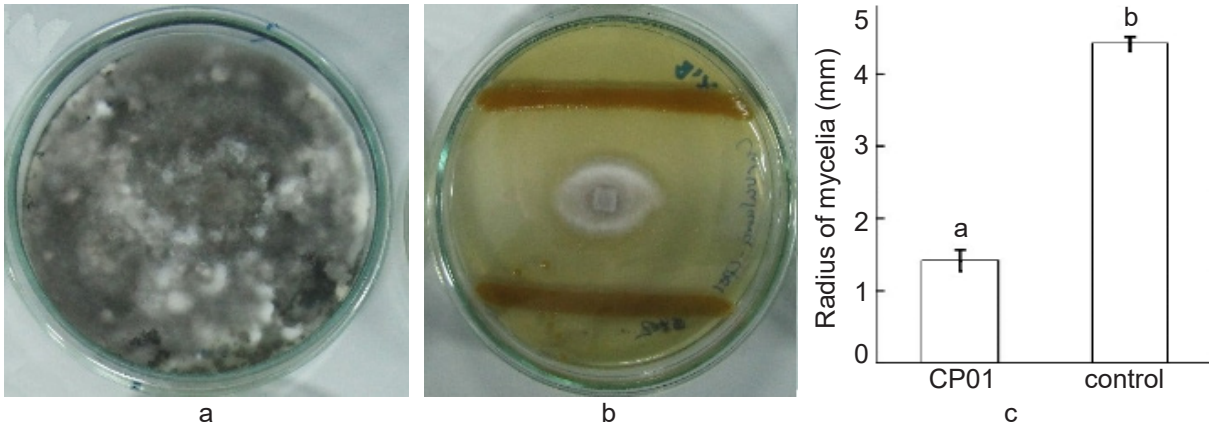
radius of growth of unchallenged mycelia could reach a full growth of 4.5 cm (±0.04 cm) (Figure 3c). No direct physical contact occurred between CP01 and *Curvularia* sp. since the beginning of the co-culture until the end of observation (Figure 3b).

Evaluation of Biological Control of Leaf Spot Disease in Oil Palm

Pathogenicity test has confirmed that our isolate of *Curvularia* sp. was virulent. We then sought to attempt to control the disease using CP01 and ProPlant C®. After 7-14 dpi, symptoms of early stage of leaf spot were observed, i.e. brown spots surrounded by yellowing or chlorotic edges, in all inoculated leaves both treated and untreated with biocontrol agents (Figure 4). However, the extent to which the symptoms progressed and expanded, differed significantly between untreated leaves and leaves treated with CP01 and ProPlant C®. The symptoms in the treated leaves were significantly less severe than the untreated leaves as shown by highly reduced spot lesions (Figure 4) and thus lower disease severity index (DSI) (Figure 5). Between the two control

a b c

Figure 2 Pathogenicity test of isolated *Curvularia* sp. in oil palm seedlings. a Symptoms of leaf spot disease observed on leaves caused by infection of *Curvularia* sp. through attachment of mycelial plug and b spraying of spore suspension. c Symptoms of natural leaf spot disease observed in the field.



a b c

Figure 3 *In vitro* antagonism assay of bacterial isolate CP01 against *Curvularia* sp. a Colony of *Curvularia* sp. in the absence of CP01 (control); b Colony of *Curvularia* sp. that was inhibited by CP01; c Radius of mycelial growth of *Curvularia* sp. in the presence or absence of CP01. Bars topped by different letters are statistically significant at $P<0.05$ (n=6). SE=standard error of the means.

agents, ProPlant C® prevented leaf spot disease slightly better than CP01, although the difference was not significant (DSI of 27% and 30%, respectively). On the other hand, untreated leaves were severely diseased (DSI of 45%) and several leaves eventually died at 30 dpi. Not only that the symptoms in untreated leaves were more severe, but the symptoms also appeared earlier than the treated leaves.

In addition to BSR disease, leaf spot disease caused by *Curvularia* sp. is also a common disease found in oil palm. In contrast to BSR disease that primarily attacks

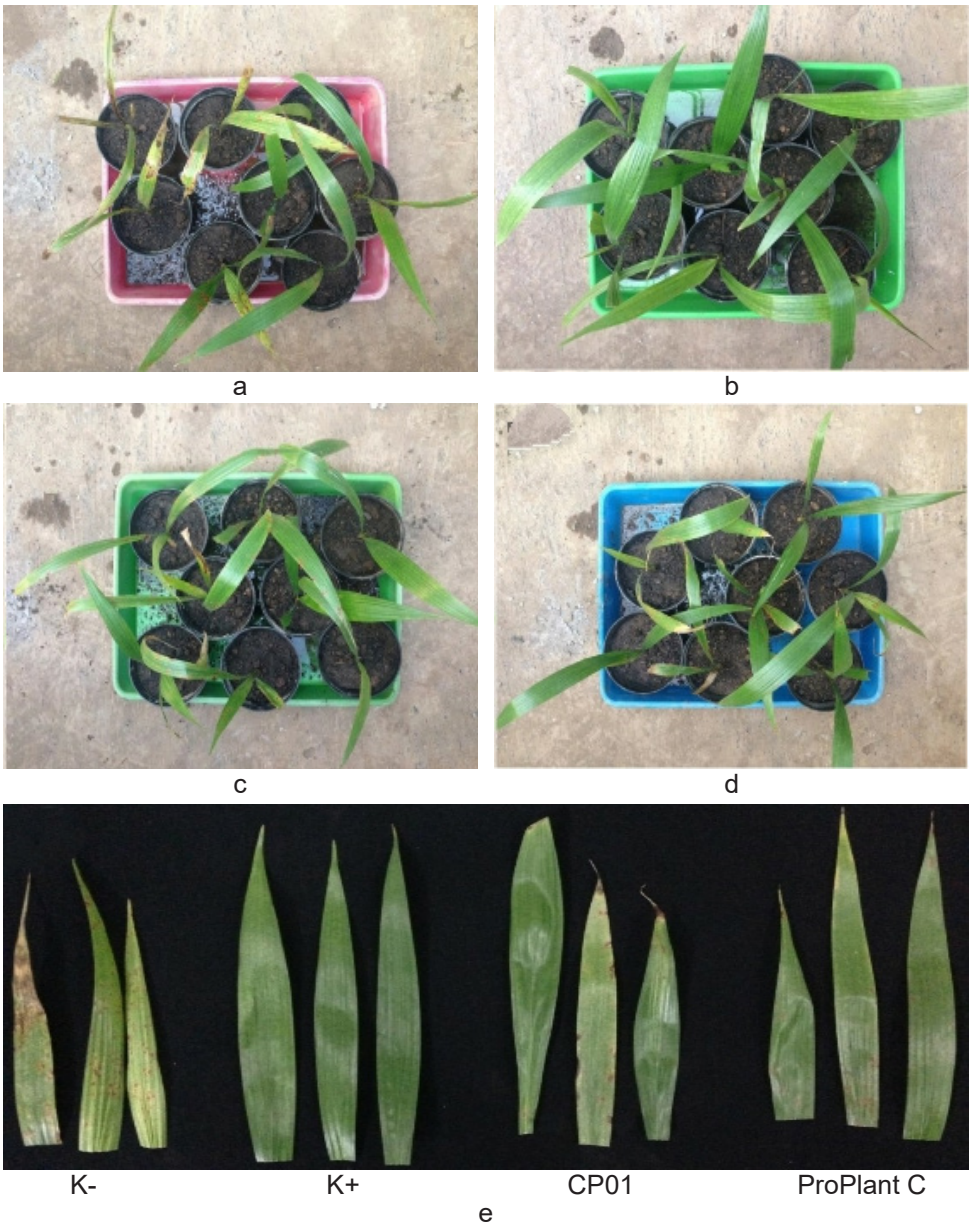


Figure 4 *In planta* bioassay of a bacterial isolate CP01 and organic fungicide ProPlant C®. a Seedlings inoculated with *Curvularia* sp. without treatment of any biocontrol agents; b Untreated and uninoculated seedlings; c Seedlings inoculated with *Curvularia* sp. and treated with cells of CP01; d Seedlings inoculated with *Curvularia* sp. and treated with ProPlant C®. e Samples of leaves that show symptoms of leaf spot: K- Inoculated with *Curvularia* sp. without treatment of biocontrol agents; K+ Untreated and uninoculated; CP01 Inoculated with *Curvularia* sp. and treated with CP01; ProPlant C Inoculated with *Curvularia* sp. and treated with ProPlant C®.

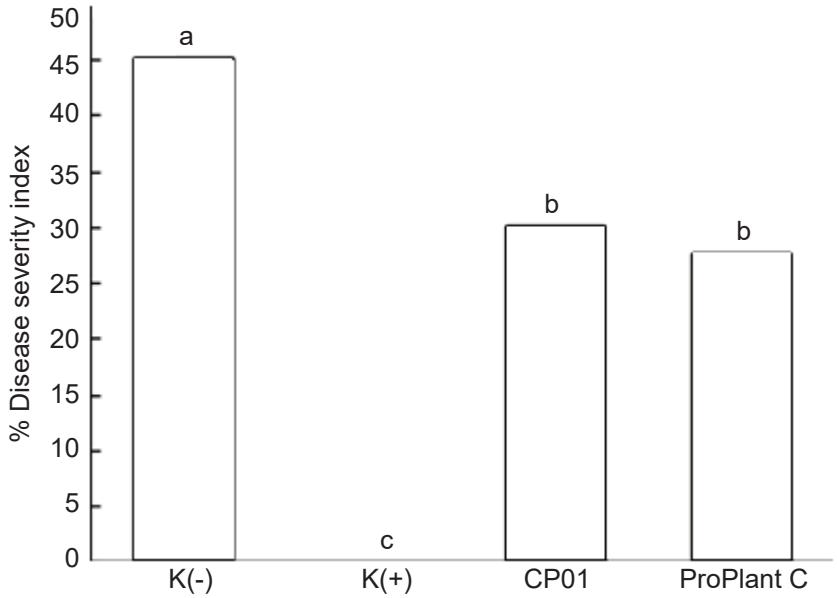


Figure 5 Percentage of disease severity index of leaf spot disease on tissue culture-generated seedlings of oil palm (ramets) in green house condition following treatment with biological control agents CPO1 bacterial suspension and ProPlant C®. K(-) inoculated with *Curvularia* sp. but without any treatments with biocontrol agents; K(+) untreated and uninoculated; CP01 inoculated with C*urvularia* sp. and treated with CP01; ProPlant C inoculated with *Curvularia* sp. and treated with ProPlant C®. Bars topped by different letters are statistically significant at $P<0.05$ (n=2).

adult, productive palms, leaf spot is found mainly in seedling and young oil palms at the nursery stages (Pornsuriya *et al*. 2013). Our study shows and confirms that leaf spot disease is caused by *Curvularia* sp. and thus fulfilled the Koch's postulate. Furthermore, this study also has enabled assay of biological control of leaf spot using a biocontrol agent (*Paraburkholderia* sp. CP01) and an organic cyclic peptide fungicide (ProPlant C®), and it was found that they were effective to reduce severity of leaf spot disease in oil palm seedlings in glasshouse.

A number of studies found that *Curvularia* sp. may not be the only pathogen causing the disease (Kittimorakul *et al*. 2013; Suwannarach *et al*. 2013; Sunpapao *et al*. 2014). However, so far *Curvularia* sp. is the only genus that has been shown to be associated with the disease (Sunpapao *et al*. 2014; Sunpapao *et al*. 2018). Our study further confirms that infection of only *Curvularia* sp. is sufficient to establish leaf spot disease in oil palm.

To enable the study of plant diseases and to be able to conduct experiments to control the diseases, a workable artificial inoculation needs to be established. We found that to artificially inoculate *Curvularia* sp. onto oil palm seedlings and to establish leaf spot disease was quite straightforward, requiring only virulent isolates or strains and appropriate levels of humidity. Nonetheless, infection occurred mainly when *Curvularia* sp. was inoculated on the abaxial side of leaves. In contrast, infection seldom occurred when *Curvularia* sp. was inoculated on the adaxial side of leaves. This could be due to the difference in the number of stomatal openings between adaxial and abaxial sides of leaf and this would affect penetration sites for

the fungus to infect the leaves. In rice, stomatal openings are natural route for infection of *C. oryzae* (de Luna *et al*. 2002). Like many other leaf pathogens, infection of *C. oryzae* on leaf of rice is initiated by the formation of appressoria, which infect leaf through either stomatal openings or epidermal cells (de Luna *et al*. 2002).

The methods for infection were done in two ways, i.e. attachment of mycelial plugs and spraying of spores onto leaves. Both methods resulted in consistent and robust disease assessment. This has enabled a trial on biological control to prevent leaf spot disease. Both living CP01 cells and ProPlant C® were effective to control leaf spot disease. Application of these control agents was aimed at prevention by spraying or coating the leaves with the agents prior to inoculation of the pathogen. The severity of the disease was significantly reduced in leaves applied with the control agents although the effect on prevention was not complete. The mechanism of control was likely due to direct inhibition of the growth of the pathogen as shown by inhibition of fungal growth *in vitro* (Figure 3).

Biocontrol approach towards leaf spot disease in oil palm has been reported using chitinolytic bacteria (Asril 2014) and a mixture of antagonistic microbes such as *Streptomyces* and *Trichoderma* (Sunpapao *et al*. 2018). In the fields however, chemical fungicides are still the major means to control leaf spot disease although only few systematic studies have been conducted to assess whether chemical fungicides were effective (Sunpapao *et al*. 2018). Several other biocontrol agents have been used to control some important foliar diseases in rice (Mahmud *et al*. 2016; Tann & Soytong 2016). However, unlike ProPlant C®, those biocontrol agents in the forms of biofungicides utilise living cells as the agents and thus can be categorised as probiotics. ProPlant C® on the other hand, is a paraprobiotic (Prihatna *et al*. 2017, 2019). Paraprobiotics have been utilised mainly as supplements or foods that can modulate immune system in human (de Almada *et al*. 2016; Murata *et al*. 2018; Taverniti & Guglielmetti 2011). To our knowledge, there have been no reports on the use of paraprobiotics in agriculture. Thus, ProPlant C® might be the first paraprobiotics that can be utilised as an organic fungicide in agriculture. The reduction of disease through application of both CP01 cells and ProPlant C® prior to inoculation of the pathogen suggests that preventive measure is effective to control leaf spot disease. For instance, in peanut, Sclerotinia blight was suppressed more effectively by fungicides when they were applied preventatively (Woodward *et al*. 2015).

CONCLUSION

This study confirmed *Curvularia* sp. as the causal agent of leaf spot disease in oil palm and laid out protocol for rapid inoculation of *Curvularia* sp. and biological control assay to suppress the disease. Both biological control agents used in this study i.e. CP01 cells and ProPlant C® were able to suppress leaf spot, thus suggesting that preventive application using natural, organic biological control agents can be an effective and environmentally friendly measure to control leaf spot disease.

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