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MYCOLYTIC BACTERIA AS POTENTIAL BIOCONTROL AGENT AGAINST PHYTOPATHOGENIC FUNGI OF PIPER NIGRUM

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Abstract— **In recent years, phytophthora rot, a slow decline and anthracnose disease are widely spread among black pepper (piper nigrum) cultivation in Sarawak, Malaysia. The infection has caused a tremendous decline in production. Mechanical control, crop management and chemical control are strategies traditionally used to manage the diseases. However, once the soil is infected with pathogens, it is hard and costly to remove it. Biological control is an alternative approach to control the phytopathogens. The strategy of disease management using biological control (BCA's) is an environmentally friendly approach in which it does not bring any negative effects toward the environment and human health. The five mycolytic enzyme producing bacteria, Acinetobacter calcoaceticus, Chryseobacterium indologenes, Serratia marcescens, Pseudomonas putida and Bacillus cereus were selected to evaluate their antagonistic activities against plant pathogenic fungi, Fusarium solani, Phytophthora capsici and Colletotrichum gloeosporioides by dual culture test, double plate assay and mycelial growth test. In dual culture test, S. marcescens had the highest PIRG value among all the mycolytic bacteria against F. solani, P. capsici and C. gloeosporioides with 33%, 73% and 34% respectively. Meanwhile, in double plate assay, C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 28%, 21%, 9%, 13% and 23% respectively in PIRG against phytopathogenic fungus P. capsici. C. indologenes and S. marcescens scored 10% and 12% respectively in PIRG against F. solani. Besides that, C. indologenes and S. marcescens scored both 16% in PIRG against C. gloeosporioides. In mycelial growth test, C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 100%, 100%, 89%, 79% and 73% respectively in PIRG against P. capsici. C. indologenes and S. marcescens scored 5% and 88% respectively in PIRG against F. solani. Apart from that, C. indologenes and S. marcescens scored 11% and 81% respectively in PIRG against C. gloeosporioides. In conclusion, the metabolites of Serratia marcescens showed the greatest potential as biocontrol agents against Fusarium solani, Phytophthora capsici and Colletotrichum gloeosporioides. Further study can be done by combining Serratia marcescens with other types of mycolytic enzyme producing bacteria to identify the effectiveness of inhibiting the fungal disease.**

Keywords— **Mycolytic Enzyme Producing Bacteria, PIRG, Dual Culture Test, Double Plate Assay, Mycelial Growth Test**

I. INTRODUCTION

Piper nigrum commonly known as black pepper is an important commercial spice crop which generates revenue for Sarawak in Malaysia. In recent years, phytophthora rot, a slow decline and anthracnose disease are widely spread among black pepper (piper nigrum) cultivation in Sarawak. The infection has caused a tremendous decline in production. Major diseases that infect Piper nigrum plantations in Malaysia specifically, regions such as Sarawak and Sabah due to their excessive moisture resulting from higher rainfall are Phytophthora rot, Slow decline and Anthracnose disease [15].

These diseases are caused by phytopathogenic fungi species, Phytophthora capsici, Fusarium solani and Colletotrichum gloeosporioides respectively [11]. The diseases can infect the black pepper plants in any stages of growth. In Sarawak, due to the climate condition, the disease can be easily spread among the plants through soil and water.

Mechanical control, crop management and chemical control are strategies traditionally used to manage the diseases. Since the pathogens favoured wet conditions, good drainage system is required in the cultivation. However, once the soil is infected with pathogens, it is hard and costly to remove it. Copper based fungicides is the current recommendation and has been being widely applied to combat Phytophthora in black pepper. Although it is effective in managing the disease, the residue remains are hazardous and can pollute the environment [1]. The prolonged usage could cause the chemical accumulated in the soil, affecting the soil quality, and human health. It is also toxic to plants and soil microbes which can lower their biological activities and eventually loss of soil fertility [8].

Biological control presents an alternative approach to control the phytopathogens. The strategy of disease management using biological control (BCA's) is an environmentally friendly approach in which it does not bring any negative effects toward the environmental and human health [13]. In previous study, Acinetobacter calcoaceticus, Chryseobacterium indologenes and Pseudomonas putida showed promising results in controlling Ganoderma boninense, a causative agent of Basal Stem Rot disease [18]. Study showed Bacillus subtilis possessed significant antifungal activity against Rhizoctonia solani dan Fusarium oxysporum. The volatile organic compounds (VOCs), outer membrane proteins (OMPs) and secondary metabolites of B. subtilis exhibited promising antifungal properties which can serve as a potential biocontrol agent against fungi [6]. In addition, the purified compound, 2,4-DTBP of Serratia marcescens BKACT depicted 75.5 ± 0.80% of mycelial inhibition against Fusarium foetens NCIM 1330 [9]. This present S. marcscens BKACT as a potential candidate against fungi.

In this study, five mycolytic enzyme producing bacteria, Acinetobacter calcoaceticus, Chryseobacterium indologenes, Serratia marcescens, Pseudomonas putida and Bacillus cereus from Wee [17] were studied for their effectiveness in inhibiting phytopathogenic fungi, Phytophthora capsici, Fusarium solani, and Colletotrichum gloeosporioides.

II. MATERIALS AND METHODS

2.1 Evaluating the effectiveness of in vitro antifungal activity of the bacterial isolates against plant pathogenic fungi, F. solani, P. capsici, and C. gloeosporioides.

The top five mycolytic enzyme producing bacteria, Acinetobacter calcoaceticus, Chryseobacterium indologenes, Serratia marcescens, Pseudomonas putida and Bacillus cereus from Wee et al. [18] were selected to evaluate their antagonistic activities against plant pathogenic fungi, Phytophthora capsici, Fusarium solani and Colletotrichum gloeosporioides by dual culture test [12], double plate assay [16] and mycelial growth test [16].

Three phytopathogenic fungi species F. solani, P. capsici, C. gloeosporioides and five mycolytic enzyme producing bacteria, A. calcoaceticus, C. indologenes, S. marcescens, P. putida and B. cereus were supplied by School of Chemical Engineering and Science of Swinburne University of Technology, Sarawak. Phytopathogenic fungi; P. capsici, F. solani and C. gloeosporioides were grown on Potato Dextrose Agar (PDA) (PDA, Himedia, Mumbai, India) plates at 25-28ºC for 1 week before conducting the assays. The agar plugs were cut from actively growing mycelia regions and transferred onto assay plates using sterile straws. Meanwhile, the mycolytic enzyme producing bacteria were inoculated on Nutrient Agar (NA) (Himedia, Mumbai, India) and were incubated for 3 days at 30ºC prior to start of experiment.

2.2 Dual Culture Assay

Three fungi were tested separately against 5 mycolytic enzyme producing bacteria. Each 7th days old fungus were grown side by side with each bacterium on NA plate and incubated for 1 week at 30ºC. Negative control plates contained only the fungus agar plugs. Five replicates were done per each treatment including for the control. After 1 week of incubation, the radial growth of fungi was measured and percentage inhibition of radial growth (PIRG) was calculated by using the following equation:

$$PIRG\ (\%) = ((R1-R2))/R1 \times 100$$

Where PIRG (%) is percentage inhibition of radial growth; R1 and R2 is the radial growth in control and radial growth in sample respectively.

Bacteria that had 25% PIRG value or more were chosen for double plate assay and mycelia growth test.

2.3 Double Plate Assay

For double plate assay, the effectiveness of volatile compounds produced by mycolytic bacteria were evaluated based on Toh et al. [16]. Effective bacteria whose PIRG value was 25% or more were streaked on a NA plate while fungi agar plug was place at the centre of another NA plate. The bacterial NA plate was inverted and placed on top of fungus plate without covers. The two plates were then sealed together. Negative control included fungi agar plugs only. Five replicates were done per each treatment including for control. The plates were incubated for 1 week at 30ºC, radial growth was measured at the end of incubation period and PIRG was calculated.

2.4 Mycelia Growth Test

Mycelial growth test was adopted based on Toh et al. [16] to evaluate the antagonistic interaction of secondary metabolites produced by mycolytic bacteria on pathogenic fungi. Briefy, the effective bacteria were inoculated in 30 mL of Nutrient Broth (NB) (Himedia, Mumbai, India) and incubated with shaking at 30ºC for 1 day prior to test. Fungal agar plugs were soaked in respective bacteria culture broth for 30 minutes, then removed, dried for 30 minutes in biosafety cabinet (BSC) and next transferred onto NA plates. Fungus agar plugs soaked in sterile water served as negative control. Five replicates were done per each treatment including for negative control. The plates were incubated for 1 week at 30ºC, radial growth was measured at the end of incubation period and PIRG was calculated.

2.5 Statistical Analysis

Data collected from the three bioassays were processed and analysed primarily using MS Excel and further statistical analysis was carried out using SPSS for two-way ANOVA and Tukey post-hoc test to determine the significance between results.

III. RESULTS AND DISCUSSION

3.1 Dual Culture Assay

The antagonistic activities between mycolytic enzyme producing bacteria and plant pathogenic fungi were evaluated based on dual culture assay of Oldenburg et al. [12]. Dual culture assay was carried out as a preliminary screening test for identifying the effective mycolytic bacteria which had high antagonistic effect by inhibit phytopathogenic fungi [12][16]. As shown from figure 1, mycolytic enzyme producing bacteria C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 27%, 33%, 13%, 14% and 20% respectively in PIRG against phytopathogenic fungus, F. solani. Furthermore, mycolytic enzyme producing bacteria, C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 48%, 73%, 50%, 26% and 48% respectively in PIRG against phytopathogenic fungus, P. capsici. Also, mycolytic enzyme producing bacteria C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 29%, 34%, 17%, 14% and 13% respectively in PIRG against phytopathogenic fungus, C. gloeosporioides. To conclude, S. marcescens had the highest PIRG value among all the mycolytic bacteria against F. solani, P. capsici and C. gloeosporioides with 33%, 73% and 34% respectively. This showed that S. marcescens is able to inhibit the growth of the three phytopathogenic fungi. The threshold of 25% PIRG was used in preliminary screening test and only the samples which met the threshold were used for further tests. In a previous study, the threshold of 30% PIRG was used, however after consideration about the media used in this study was different, sample size was smaller, and the period to collect data was also different, the threshold was set to 25% to identify more sample for further studies in order to confirm their antagonistic properties [16]. After the preliminary screening test, the sets of five mycolytic enzyme producing bacteria strains with P. capsici met the 25% PIRG threshold, all of them proceeded to double plate assay and mycelial growth test. However, for the sets with F. solani and C. gloeosporioides, only C. indologenes and S. marcescens met the 25% PIRG threshold and were chosen for double plate assay and mycelia growth test. The data was analysed with two-way ANOVA and Tukey post-hoc test. Each type of mycolytic enzyme producing bacteria strains had similar inhibition effect towards the growth of F. solani and C. gloeosporioides. All the bacteria strains had relatively high effectiveness to inhibit the growth of P. capsici. These bacteria also showed significant results towards P. capsici ($p \leq 0.05$).

Study showed that the novel chitinase from a native Serratia marcescens B4A was characterized and further monitored its chitinase activity by scanning electronic microscopy in which the chitin porosity show progressive changes upon treatment with chitinase. This enzyme possesses antifungal activity against a wide range pf phytopathogenic fungi, for example, Bipolaris sp., Alternatia brassicicola, Rhizoctonia solani, and Alternaria raphani which serves as a potential application for the industry with potentially exploitable significance [10].

In previous study, S. marcescens was evaluated for its biocontrol efficacy against nine different fungal strains, Pestalotiopsis theae, Lasiodiplodia theobromaein, Rhizoctonia solani, Curvularia eragrostidis, Colletotrichum camelliae, Ustulina zonata, Sphaerostilbe repens, Poria hypobrunae and Fomes lamaoensis causing several root and foliar disease in tea (Camellia sinensis). The bacterium was found to produce several hydrolytic enzymes, for example, protease, cellulase, chitinase, lipase and plant growth promoting metabolites like siderophore and IAA. The percent inhibition of pathogen by S. marcescens against Lasiodiplodia theobromaein, Rhizoctonia solani, Sphaerostilbe repens, Fomes lamaoensis, Ustulina zonata, Poria hypobrunae, Pestalotiopsis theae, Colletotrichum camelliae, and Curvularia eragrostidis were 51.5 ± 0.8%, 66.7±0.7%, 71.1±0.7%, 76.7±0.4%, 61.1±0.5%, 71.1±0.7%, 76.7±0.6%, 65.5±0.5%, and 81.5±0.5% respectively. In the study of the interaction zone between antagonistic bacterial isolate and pathogen, Rhizoctonia solani, severe deformities, like perforations, bulging and bursting in the hyphae at specific sites and surface irregularities were observed on the fungal mycelia in the scanning electron microscopic studies. Therefore, S. marcescens could be suggested to be used in fungal infections management of tea [5].

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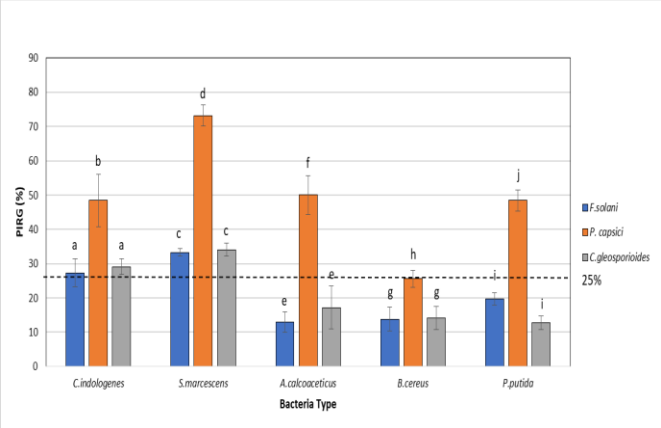

Fig. 1: The percentage inhibition of radial growth of five mycolytic enzyme producing bacteria strains, Acinetobacter calcoaceticus, Chryseobacterium indologenes, Serratia marcescens, Pseudomonas putida and Bacillus cereus against the three phytopathogenic fungi strains Phytophthora capsici, Fusarium solani and Colletotrichum gloeosporioides following 1 week of incubation at 30 ˚C obtained from dual culture assay. The value bars with corresponding letters are not significantly different from each other ($p \leq 0.05$) according to Tukey test. Bacteria with PIRG above threshold value of 25% were chosen for further tests.

3.2 Double Plate Assay to test for volatile compounds

Double Plate Assay was a screening test to detect the volatile metabolites produced by the isolated bacteria that effectively inhibit fungal growth [16]. In double plate assay, the fungus and bacterium were inoculated in separate NA plate, there was no diffusion of metabolites, only the volatile gas produced by bacterium can reach the fungus. Mycolytic enzyme producing bacteria, C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 28%, 21%, 9%, 13% and 23% respectively in PIRG against phytopathogenic fungus, P. capsici. Mycolytic enzyme producing bacteria, C. indologenes and S. marcescens scored 10% and 12% respectively in PIRG against phytopathogenic fungus, F. solani. Besides that, mycolytic enzyme producing bacteria, C. indologenes and S. marcescens scored both 16% in PIRG against phytopathogenic fungus, C. gloeosporioides. Overall, the PIRG value in double plate assay was lower than that in dual culture assay, it might be caused by the volatile metabolites not reaching the effective concentration within one week. Moreover, in the dual culture assay, not only volatile metabolites had the inhibitory effect, but also the non-volatile metabolites affect the growth inhibition of fungi. The data was analysed with two-way ANOVA and Tukey post-hoc test. C. indologenes produced the most effective volatile metabolites to inhibit the growth of P. capsici. However, P. putida, S. marcescens and B. cereus had no significant differences with C. indologenes against P. capsici in double plate assay. C. indologenes and S. marcescens had similar inhibition effect towards the growth of F. solani and C. gloeosporioides, the PIRG value had no significant differences between each other.

Rhizobacteria, Serratia plymuthica and Pseudomonas fluorescens produced volatile dimethyl disulfide (DMDS) able to suppress crown gall disease caused by Agrobacterium vitis in grapevines (Vitis vinifera). DMDS could efficiently suppress the growth of gall formation by reduction of three- to eight- fold in tumor mass on tomato seedlings [4].

According to Gong et al. [7], volatile dimethyl disulfide produced by Serratia marcescens Pt-3 depicted wide range and effective antifungal activity to seven important fungal pathogens. The compounds produced possess multiple functions which can be used as effective bio-active agents in increasing soil fertility and controlling plant disease.

Volatile compounds, such as, ketones, sulfides and benzenes produced by Pseudomonas and Bacillus inhibited the growth of C. gloeosporioides, Fusarium sp. and P. cinnamomi [2].

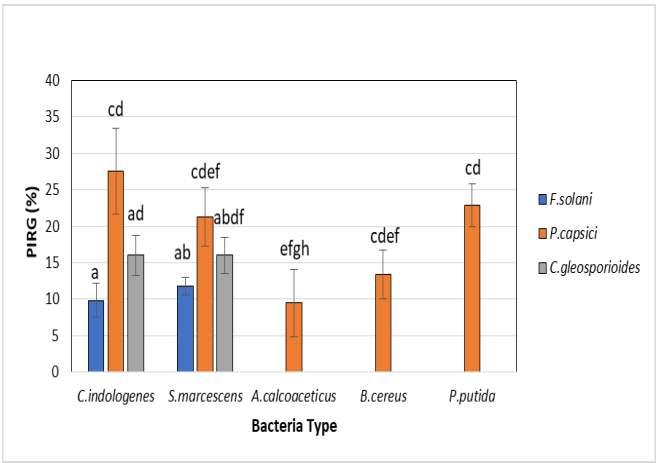

Fig. 2: The percentage inhibition of radial growth of five mycolytic enzyme producing bacteria, Acinetobacter calcoaceticus, Chryseobacterium indologenes, Serratia marcescens, Pseudomonas putida and Bacillus cereus against phytopathogenic fungi strain Phytophthora capsici and PIRG% of Chryseobacterium indologenes, Serratia marcescens, against Fusarium solani and Colletotrichum gloeosporioides strains following 1 week of incubation at 30 ˚C obtained from double plate assay. The value bars with corresponding letters are not significantly different from each other ($p \leq 0.05$) according to Tukey test.

3.3 Mycelial Growth Test for non-volatile compounds

Mycelial growth test was designed to study the non-volatile metabolites produced by the isolated bacteria in broth culture [16]. In mycelial growth test, the fungi agar plug was soaked into bacteria broth culture to allow direct contact, the non-volatile metabolites can directly inhibit the growth of fungi when the agar plug was inoculated on NA plate. Mycolytic enzyme producing bacteria, C. indologenes, S. marcescens, A. calcoaceticus, B. cereus and P. putida scored 100%, 100%, 89%, 79% and 73% respectively in PIRG against

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phytopathogenic fungus, P. capsici. Mycolytic enzyme producing bacteria, C. indologenes and S. marcescens scored 5% and 88% respectively in PIRG against phytopathogenic fungus, F. solani. Besides that, mycolytic enzyme producing bacteria, C. indologenes and S. marcescens scored 11% and 81% respectively in PIRG against phytopathogenic fungus, C. gloeosporioides. The mycolytic enzyme producing bacteria produced non-volatile metabolites which had very high inhibitory effect against P. capsici. However, the bacteria existed on the fungi agar plug might also affect the inhibition of fungi growth by competing for the nutrient and living space. Culture filtrate test was suggested to confirm only the effect of non-volatile metabolites.

The culture filtrate method had filtered out the bacteria, thus only the secondary metabolites were collected. The secondary metabolites would directly function on the fungi to test the antagonistic properties.

The data was then analysed by two-way ANOVA and Tukey post-hoc test. C. indologenes and S. marcescens produced the most effective non-volatile metabolites to inhibit the growth of P. capsici, both sets had no significant differences with each other. On the other hand, only S. marcescens produced the most effective non-volatile metabolites to inhibit the growth of F. solani and C. gloeosporioides, both sets had similar effectiveness of inhibition.

In a previous study, Serratia quinivorans KP32 exhibited biological activity against Colletotrichum dematium, Sclerotinia sclerotiorum, Rhizoctonia solani dan Fusarium avanaceum. Its broad spectrum of biocontrol features, for example, the production of a number of lytic enzymes (amylases, proteases and chitinases), salicylic acid, siderophores, N-acyl-homoserine lactones, volatile inorganic compounds and organic compounds mediate the inhibition of growth of those phytopathogens. Higher expression of chitinase (chiA) and genes involved in the biosynthesis of hydrogen cyanide (hcnC), acetoin (budA) and enterobaction (entB) upon exposure to fungal filtrates established that these factors could act in combination, leading to a synergistic inhibitory effect of the strain against phytopathogens. This biological activity indicates that Serratia quinivorans KP32 has the potential to be used as an active biopesticide [3].

In other study, Serratia marcescens showed the ability to degrade high concentrations of chitin completely. It produced large inhibition halos (>30 mm) toward Rhizoctonia solani fungi, Fusarium oxysporum, and Fusarium solani. It caused micromorphological alterations in the inocula, lead up to the inhibition of R. solani sporulation and spore germination. This showed that Serratia marcescens is a potential candidate as a biocontrol agent in agriculture [14].

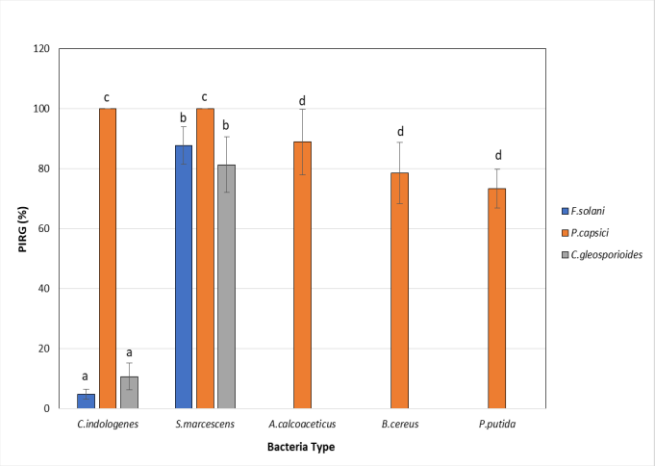

Fig 3: The percentage inhibition of radial growth of five mycolytic enzyme producing bacteria, Acinetobacter calcoaceticus, Chryseobacterium indologenes, Serratia marcescens, Pseudomonas putida and Bacillus cereus against phytopathogenic fungi strain, Phytophthora capsici and PIRG% of Chryseobacterium indologenes, Serratia marcescens, against Fusarium solani and Colletotrichum gloeosporioides strains following 1 week of incubation at 30 ˚C obtained from mycelial growth test. The value bars with the corresponding letters are not significantly different from each other ($p \leq 0.05$) according to Tukey test.

IV. CONCLUSION

In conclusion, the metabolites of Serratia marcescens showed the greatest potential as biocontrol agents against Fusarium solani, Phytophthora capsici and Colletotrichum gloeosporioides in cultivation. Further study can be done by combination of Serratia marcescens with other types of mycolytic enzyme producing bacteria to identify the effectiveness of inhibiting fungal diseases.

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