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Evaluation of local beneficial microorganisms as biological control agents against grey leaf spot disease of coconut

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Keywords

Streptomyces spp., grey leaf spot disease, coconut

Abstract

Grey leaf spot is one of the major coconut leaf diseases in nursery caused by a fungus *Pestalotiopsis* sp. In this study, an approach by using local beneficial microorganism from *Streptomyces* spp. as a biological control agent were evaluated as *in vitro* and *in vivo* to control *Pestalotiopsis* sp. Double petri dishes assay was evaluated in vitro. While for in vivo, glasshouse trial was conducted. Coconut seedlings were inoculated with *Pestalotiopsis* sp. suspension and subsequently treated with *Streptomyces* spp. one month after pathogen inoculation. The study was carried out using complete randomized design with three replications. The result showed, *S. fumigatiscleroticus* have higher suppression towards *Pestalotiopsis* sp. colony compared to *S. seoulensis* with an average inhibition at 60.5% and 53.3% respectively. Glasshouse evaluation also recorded significant reduction of disease percentage on coconut seedling treated with both *Streptomyces* spp. *S. fumigatiscleroticus* was observed to have the highest control on grey leaf spot disease with 81.24% differences in disease percentage compared to the control. Therefore, these *Streptomyces* spp. have been determined as the potential and promising biological control agents to control grey leaf spot disease of coconut in the future.

Introduction

Coconut palm (*Cocos nucifera* L.) is belongs to family Arecaceae. It is an important multipurpose perennial crop and was recognized as source of wealth for the nation. Malaysia was recorded amongst the top coconut producers in the world. Total coconut planted area are 82000.5 ha which are mainly in Sabah, Sarawak, Johor, Perak and Selangor (DOA, 2015).

The long-life span and high cost of production involved in establishing a coconut plantation, necessitates the selection of good quality seed nuts and seedlings (Nair *et al*., 2008). Because of the performance of coconut tree only can be evaluated after 10-15 years of planting, selection of high quality and disease tolerant planting materials are the key factors for the successful of coconut cultivation.

Unfortunately, coconut seedling diseases have not been thoroughly investigated in Malaysia. Little information is available on the incidence, prevalence, epidemiology and disease management. Hence, this study was aimed to evaluate the potential of local beneficial microorganisms as biological control agents for controlling major coconut foliar diseases especially in nursery stage. This approach is believed to be a potential control approach and will become an alternative to the fungicide usage.

Materials and Methods

Sampling and pathogen isolation

Diseased palm leaves were collected from several coconut nurseries in Perak and Johor in ` snap-lock ' plastic bags and examined as soon as possible for associated fungi in laboratory. Isolations were made by cutting small pieces of tissue from the edge of lesions, surface sterilizing them in 96 percent (%) alcohol for 30 seconds, rinsed three times with sterile distilled water and blotted dry onto sterile filter paper. The leaf fragments were plated onto Potato Dextrose Agar (PDA) and incubated in day light at room temperature (22±25 °C) until discrete colonies developed.

Isolation and screening of beneficial microorganisms

Soil samples were collected from eight States in Peninsular Malaysia (Pahang, Selangor, Perak, Negeri Sembilan, Melaka, Terengganu, Pahang and Johor). Isolation of *Streptomyces* spp. strains using Actinomycetes Isolation Agar (AIA) from soil samples were carried out in the laboratory. Successfully isolated Streptomyces species were kept at 4 degree Celsius (°C) for short term storage.

Rapid screening using dual culture test *in vitro* against *Pestalotiopsis* sp. were conducted. Briefly, a 0.65cm diameter mycelial disc was cut from the white perimeter of *Pestalotiopsis* sp. colony that had been cultured on PDA plates for seven days. The disc was placed 1.5cm from the edge of a fresh PDA plate, and the test soil microorganism was streak into the same plate, 1.5cm from the opposite edge. Control plates without soil microorganisms were prepared simultaneously. Percentage inhibition (%) of the fungal growth then was measured and evaluated.

Molecular Identification of microorganisms

The isolates which showed strong antimicrobial activity were subjected to further evaluation by molecular methods. Genomic DNA was extracted and purified by using a commercial kit, DNeasy Blood and Tissue Kit (QIAGEN, Germany). Extraction method was according to the manufacturer's instruction. DNA was stored at –20 °C until required. To amplify the fragment of 16S rDNA gene, primer pair fD1/rP2 (forward: 5´-AGAGTTTGATCCTGGCTCAG-3´ and reverse: 5´-ACGGCTACCTTGTTACGACTT-3´) was used. PCR amplification was performed using Thermo Scientific- DreamTaq Green PCR Master Mix (2×), 1 µM or each primer, 1 µg of DNA template and the final volume was reached to 50 µl. The PCR amplification was performed using the thermal cycler (Bio Rad, USA) program as follows: 94 °C for 10 min as a primary denaturation step, 40 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min and final extension was 72 °C for 10 min. All PCR products were analyzed on a 1% agarose gel at 80 volts for 90 min in Tris-borate EDTA (TBE) buffer. Gel was stained with Florosafe DNA stain and visualized by Compact Digimage System UVDI (Major Science), together with 1 kb ladder (Thermo Scientififc). PCR products of high yield isolates were purified using PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. The purified products were sequenced by 1st Base Laboratories (Selangor, Malaysia). Furthermore, using BLAST software, the determined sequences were compared with the sequences deposited in NCBI GenBank as 16S rDNA gene of different Streptomyces species.

Glasshouse evaluation

Glasshouse evaluation was conducted at MARDI Hilir Perak Research Station using complete randomize design (CRD) with 3 replications. Coconut seedlings were inoculated with fungal suspension at 1x 10^6 CFU per mL one month before *Streptomyces* spp. application. 100ml of *S. fumigatiscleroticus* inoculum at 1x 10^6 CFU per mL was then applied in each replicate in Treatment 1, *S.seoulensis* inoculum in Treatment 2 and control (without inoculum) as Treatment 3.The seedlings were kept in glasshouse and data collection was carried out on weekly basis throughout this evaluation period.

Statistical data analysis

Glasshouse evaluation was performed using complete randomized design (CRD) with three replications. Percentage leaf infected were recorded and data was then analysed using analysis of variance (ANOVA) followed by comparison of means separation using Duncan multiple range test (DMRT).

Results and Discussion

Sampling and pathogen identification

Pestalotiopsis sp. is the major pathogen to be associated with leaf spot disease of coconut and normally occurred in nursery stage and newly establish coconut plantation. Observation in surveyed coconut nurseries was found that the size of the spot appeared to be large and the spots sometimes combined together to form larger lesion. This observation also reported by Azlan *et al*., (2018) that under severe conditions, spots caused by this pathogen will coalesce to form larger lesions. Pornsuriya *et al*., (2013) also found that the symptom of leaf spot caused by *Pestalotiopsis* sp. showed the large lesion, irregularly shaped and orange-red in color. This infection also can greatly reduce the photosynthesis activity of the plant and eventually unfit for field planting (Palomar and Betanio, 1982).

Observation of the fungal growth in laboratory was found; the colony of *Pestalotiopsis* sp. is white in colour on the surface of agar (figure 1a), yellowish on the reverse side and produces abundant of spores in culture conditions. The spores (conidia) are oblong in shape with a thick posterior end completed with a pair of flagella. It is also found to be septate as shown in Figure 1b.

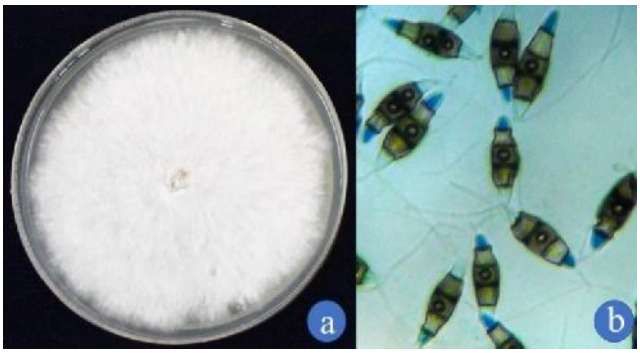

Figure 1: Pathogen of grey leaf spot disease; *Pestalotiopsis* sp. (a) colony morphology (b) and conidia of *Pestalotiopsis* sp.

Isolation and screening of beneficial microorganisms

Four (4) selected strain of Streptomyces from previously study namely as S1, S5, S7 and S12 were used in this evaluation. These isolates were screened for their antagonistic activity against *Pestalotiopsis* sp. using dual culture tests based on the percent inhibition of radial growth (PIRG). From the study, Streptomyces S12 and S5 have significantly showed antagonistic response towards the growth of *Pestalotiopsis* sp. in dual culture test (Figure 2) with an average inhibition at 60.5% and 53.3% respectively (Figure 3). However, Streptomyces S1 and S7 did not show any antagonistic activities. These two strains of Streptomyces (S12 and S5) was then subjected to molecular identification and selected for screening in glass house evaluation.

Figure 2: Screening the potential *Streptomyces* spp. towards *Pestalotiopsis* sp. using dual culture technique *in vitro*. (a) S12: *S. fumigatiscleroticus* (b) S5: *S. seoulensis* (c) Control.

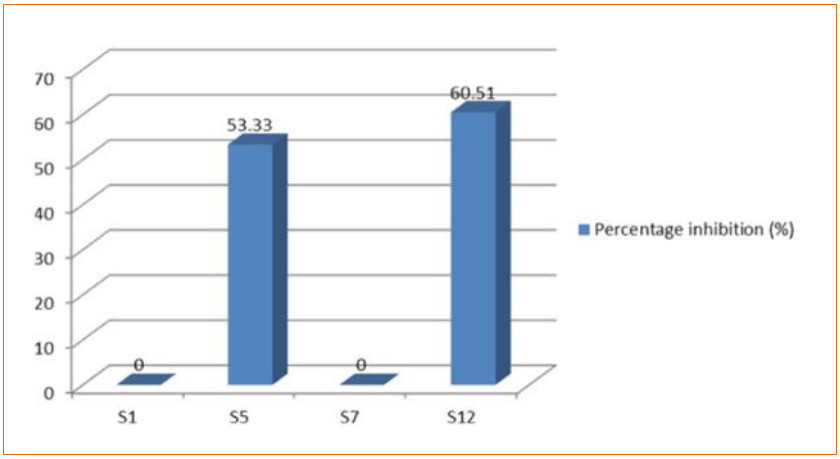

Figure 3: Percentage of growth inhibition of *Pestalotiopsis* sp. in dual culture test *in vitro*

PCR amplification and sequencing of 16S rDNA gene

Approximately 1500-bp product (Figure 4) was obtained using the 16S primer. The partial 16S rDNA gene sequences obtained was then compared with gene sequences of known strains in GenBank database. Sequencing of the isolates S5 and S12 revealed similarity to *Streptomyces seoulensis* (AB249970) and *Streptomyces fumigatiscleroticus* (AB184248) with degree of similarity of 99.7% and 99.8% respectively. Application of 16S rDNA gene is simpler and more efficient, in identification of new Streptomyces strains (Anderson and Wellington, 2001). It is worth noting that although 16S rDNA gene has less changes and transformation through evolution, it is deemed to be a superior candidate for taxonomic studies because of 5' variable areas including α, β, δ, ε, and particularly variable γ part which shows relatively high polymorphism at the 5′ end of its structure (Shirling and Gottlieb, 1968; Stach *et al*., 2003) which could be exploited for studying the genetic diversity of various Streptomyces species (Nimnoi and Pongsilp, 2009). Identification of new strains of Streptomyces have been frequently described in the literature using amplification of hyper variable regions that can provide strain specific signature (Goodfellow, 1988).

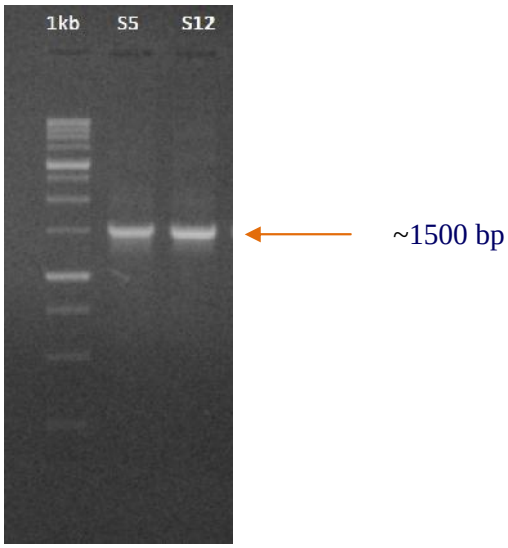

Figure 4: Amplification of the 16S rDNA gene displaying 1500-bp amplification product of the soil isolates S5 and S12.

Glasshouse evaluation

Biological control approach for controlling *Pestalotiopsis* sp. of coconut was reported in several studies. Khan and Hossain (2013) reported that BAU-Biofungicides incorporated with Bavistin (chemical control) are capable to reduce disease incidence of grey leaf spot up to 60.12%. Local biological agents are also believed to have more adaptation to the environment and will be perform better. It is also in supported by Chowdhury (2009) who also found that environmental factors influenced the effectiveness of disease control.

Because of that, these two (2) potential local soil microorganisms were selected and screened *in-vivo* towards *Pestalotiopsis* sp. Glass house evaluation recorded significant differences in week 6 of the observation (Table 1). Both *S. fumigatiscleroticus* and *S. seoulensis* were observed capable to significantly reduce the disease percentages on coconut seedlings after 6 weeks of application (Figure 5). *S. fumigatiscleroticus* also was observed to have the highest effectiveness to control grey leaf spot disease with 81.24% differences in disease percentage compared to the control.

Table 1: Mean Square ANOVA for evaluation of *S. fumigatiscleroticus* and *S. seoulensis* as biological control agents toward grey leaf disease of coconut.

Sources of Variances	Disease evaluation			
	0 week	2 weeks	4 weeks	6 weeks
Trt	212.3	16.4	277.1	396.3*
Grand Mean	49.4	52.88	45.31	34.94
C.V. (%)	12.16	4.76	17.23	9.61

Note: Mean followed by * is significantly difference at 0.05

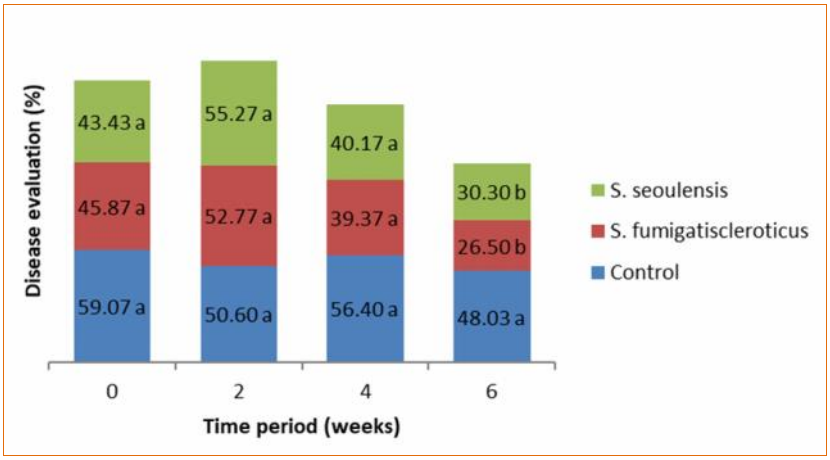

Figure 5: Effect of different treatment towards percentage of disease evaluation.

Therefore, these two species of Streptomyces were identified to have the potential as biological control agents for controlling grey leaf spot disease of coconut. Further study needs to be carried up on these two local beneficial microorganisms especially on identification of stable and effective formulation in the future.

Conclusion

Two (2) strains of Streptomyces identified as *S. fumigatiscleroticus* and *S. seoulensis* were identified significantly capable on controlling grey leaf spot disease as *in vitro* and *in vivo* evaluation. This positive result has encouraged Malaysian Agricultural Research and Development Institute (MARDI) research team to be conducted further research on the production of bio-based fungicide using suitable and effective formulation from Streptomyces as an eco-friendly approach in controlling grey leaf spot disease of coconut seedling in the future. This effort also eventually will help to reduce the usage of agricultural chemical pesticide inputs in our food supply to achieve safer food production.

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