Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Antagonistic activity of *Streptomyces thermocarboxydus* to *Fusarium oxysporum*: The cause of leaf rot disease on Aloe Vera (*Aloe barbadensis* Mill.) in Bali, Indonesia

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Received 4 May 2017; Received in revised form 21 May 2017; Accepted 29 May 2017

ABSTRACT

Aims: Aloe vera (*Aloe barbadensis* Miller) has been cultured in Bali, Indonesia since 2006. Its cultivation area is 170 ha including 5 regencies *i.e.* Buleleng, Karangasem, Bangli, Badung and Gianyar. This study was conducted to isolate and identify the *Streptomyces* sp. that potentially can be used to inhibit the growth of *F. oxysporum* and control the leaf rot disease on aloe vera with and to study the structural responses of *F. oxysporum* to the treatment of *Streptomyces* culture filtrate.
Methodology and results: Samples were collected from Serokadan, Kerobokan, and Saba, Bali, Indonesia. The isolate of *Streptomyces* sp. that resulted in the highest antifungal activity was further observed on its morphological and ultrastructure characteristics using SEM and TEM. Identification was done by using 16S rRNA sequencing techniques. A greenhouse experiment was conducted to evaluate the effectiveness of the filtrate of *Streptomyces* sp. to control the leaf rot disease on aloe vera. The *Streptomyces* GYRRK was identified to be *S. thermocarboxydus* and the filtrate inhibited the growth of *F. oxysporum* by damaging cell wall and plasma membrane of macro conidia cell, micro conidia, and hypha. Treatment with the filtrate of *S. thermocarboxydus* with four sprays (one spray equal to 0.5 mL) over inoculated leaves of aloe vera reduced the leaf rot disease by 68%.
Conclusion, significance and impact of study: This result suggests that filtrate of *S. thermocarboxydus* potentially can be used as an alternative control agent against leaf rot disease on aloe vera in Bali.

Keywords: Aloe vera, *Fusarium oxysporum,* leaf rot disease, *Streptomyces thermocarboxydus*.

INTRODUCTION

Aloe vera (*Aloe barbadensis*) has been planted intensively in Bali, Indonesia since 2006. The cultivation area is 170 ha including five regencies *i.e.* Buleleng, Karangasem, Bangli, Gianyar and Badung which are cultivated by 139 farmers. This plant originally came from Mexico and introduced to Bali, Indonesia in 2006. A leaf rot disease was found in 2010 on several areas of aloe vera plantation in Bali. The symptom of the disease was observed on the leaf, where the leaf becomes rotten and dry with brown color crescent type. The causal pathogen of the disease has been identified as *Fusarium oxysporum* based on morphological characteristics and analysis of 18S rDNA (Kawuri *et al*., 2012).

Synthetic fungicides are currently utilized as disease control agent for diseases caused by pathogenic fungi. These synthetic fungicides may cause a number of problems such as environmental contamination and health problems. Moreover, synthetic fungicide can cause pathogen resistance and affect the non target organisms (Brimer and Boland, 2003). Based on this reason, an alternative safe way to control fungal diseases on plants must be found. Microorganisms can be used as biocontrol agent for plant disease, as they are more friendly to the environment and safe to the non target microorganism. Prapagdee *et al*. (2008) reported that many genus of *Streptomyces* have been used as anti fungal agents to control several pathogenic fungi on plants. The capability of *Streptomyces* in inhibiting growth of pathogenic fungi is due to its capability to produce both antifungal agent and extracellular hydrolytic enzymes those are able to degrade fungal cell wall.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

This study was conducted to isolate and identify the *Streptomyces* sp. that potentially can be used to inhibit the growth of *F. oxysporum* and control the leaf rot disease on aloe vera as well as to study the structural responses of *F. oxysporum* to the treatment of *Streptomyces* culture filtrate.

MATERIALS AND METHODS

Isolation and identification of *Streptomyces*

Streptomyces was isolated from the soil around aloe vera plants which did not show any disease symptom. Samples were collected from three locations in Bali *i.e.* Serokadan Village, Bangli Regency; Kerobokan Village, Badung Regency and Saba Village, Gianyar Regency. Macroscopic (morphology colony), microscopic (Gram staining, Ziehl Neelsen staining) and biochemical tests (catalase test) were observed based on the determination key book of Guide to the Classification and Identification of the Actinomycetes and their antibiotics written by Lechevalier and Waksman (1973). The *Streptomyces* sp. isolates obtained was examined on its inhibition capability over *F. oxysporum* that causing leaf rot disease on aloe vera plant using dual culture method. The most potential *Streptomyces* sp. in inhibiting *F. oxysporum* was further observed on its morphological characteristics using scanning electron microscope (SEM), while spore and hypa ultrastructure of *Streptomyces* sp. was examined using transmission electron microscope (TEM). Molecular identification of *Streptomyces* sp. was done based on 16S rRNA analysis with specific primer, i.e. 20f (5'-GAGTTTGATCCTGGCTCAG) and 1500r (5'-GTTACCTT GTTACGACTT) (Nishizawa *et al*., 2010).

Preparation of culture filtrate

Filtrate preparation of *Streptomyces* sp. was conducted by growing *Streptomyces* sp. on Yeast Extract Malt Extract (ISP2) medium for 14 days on incubator shaker at temperature of 28±2°C at 125 rpm. Filtrate from culture was then collected, followed by centrifugation at 11,000 rpm for 15 min and was filtered using filter paper 0.45 µm. Filtrate partition was done by pouring the filtrate into a 1 L separation bottle and then n-butane solvent was added at ratio of 1:1 (v/v), followed by evaporation to separate n-butane with filtrate using vacuum rotary evaporator (Buchan Motivator R-210, Japan). This filtrate was used for antifungal activity test against *F. oxysporum*. Minimum inhibitory concentration (MIC) of this filtrate was determined on PDA medium on Petri dish using diffusion well method. Two diffusion wells (5 mm in diameter) were made on each Petri dish and filled with 20 L filtrate each. The filtrate concentrations (%, v/v) tested on this test were 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and control (sterile distilled water).

Ultra structural response of *F. oxysporum*

Ultra structural response of *F. oxysporum* against treatment of filtrate of *Streptomyces* sp. was examined under scanning electron microscope (SEM) and transmission electron microscope (TEM). A 20 L filtrate of *Streptomyces* sp. at concentration 100% was into Petri dish and then added by melted PDA medium and shaken horizontally to mix the filtrate evenly with PDA. A mycelia plug taken from the edge of *F. oxysporum* colony (5 mm diam.) was put in the center of Petri dish and then incubated in the dark at 25 °C for 3 days (Picman *et al*., 1990). Fungal colony on the colony edge was cut 3 mm and 1 mm in size, followed by the sample preparation process for SEM (JSM-6701F, JEOL) and TEM (JEM-2100, JEOL) analyses (Hall, 1978; Hayat, 1981).

Greenhouse experiment

A greenhouse experiment was conducted to evaluate the effectiveness of the filtrate of *Streptomyces* sp. to control the leaf rot disease on aloe vera. The seedlings of aloe vera (2.5 mos old) were planted in pots filled with cultural medium (fertile soil and compost, 3:1) and adapted in a greenhouse for two weeks. These aloe vera plants were then inoculated with spores suspension of *F. oxysporum* in sterile water with 1% Tween-80. Prior to inoculation, three leaves of each plant were artificially injured with sterile needle as inoculation point. One inoculation point was made per leaf. Inoculation was done by spraying 0.5 mL spores suspension (10^6 spores /mL) of *F. oxysporum* over inoculation point. The next day, a 0.5 mL *Streptomyces* sp. filtrate was sprayed on leaves that already infected by *F. oxysporum*. The sprays were conducted 1 time, 2 times, 3 times and 4 times for STR1, STR2, STR3, STR4 treatment respectively. Six treatments *viz*. treatment with sterile distilled water (control), treatment with 2% Mancoseb (w/v) a synthetic fungicide (MnC), treatment with one spray of *Streptomyces* sp. filtrate (STR1), treatment with two sprays of filtrate (STR2), treatment with three sprays of filtrate (STR3), and treatment with four sprays of filtrate (STR4). Each treatment were replicated four times, thus there were 24 experimental units of 10 aloe vera plants per unit. Treatments allocation was done based on randomized block design. Disease incidence was determined based on the percentage of inoculation points that developed into lesion six week after inoculation. All data were subjected to the analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (SPSS software 17. 2009).

RESULTS

Characteristic of *Streptomyces* sp. isolate GYRRK

There were 24 isolates of *Streptomyces* sp. obtained in this study, and among of them 14 isolates showed antagonistic activity against *F. oxysforum*. Isolate GYRRK showed the highest inhibitory activity (93.4%). Based on

PCR analysis the size of the 16S rRNA of isolate GYRRK was 1,480 bp and indicated in Figure 1.

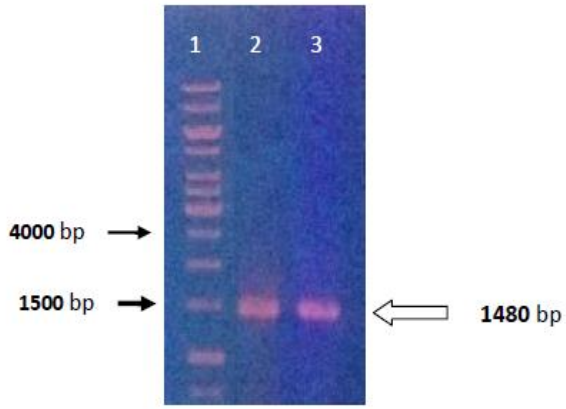

Figure 1: Agarose gel electrophoresis of 16S rRNA *Streptomyces* isolate GYRRK. Lane 1. Marker gen 1 kb DNA ladder (Fermentas). Lane 2 and 3 Fragment of 16S rRNA *Streptomyces* isolate GYRRK. (1480 bp)

Comparison of 16S rRNA homologous gene level of *Streptomyces* sp. isolate GYRRK with a number of sequences at GenBank using BLAST program showed top 10 of results of isolate GYRRK similarity compared to 16S rRNA sequence that are available at Gene bank / NCBI (*National Center for Biotechnology Information*). It was found that GYRRK isolate demonstrated similarity level of 96% with *Streptomyces thermocarboxydus* strain Hd 16S ribosomal RNA gene with total score of 2137.

Morphological characteristics observed by SEM indicated that *S. thermocarboxydus* had curvy aerial hypha and wavy structure with spore on the tip of hypha (Figure 2A). Conidia had rounded shape with uneven surface with chain shape or in cluster (Figure 2B). Observation by TEM showed that conidia's cell ultrastructure consisted of cell wall, cytoplasmic membrane, thick cortex and nucleus without membrane (prokaryot), as shown in Figure 2C. Ultrastructure hypha of *S. thermocarboxydus* consist of ribosome, mesosome and nuclear substance (Figure 2D).

Antifungal activity of *S. thermocarboxydus*

The filtrate of *S. thermocarboxydus* culture showed a strong antifungal activity against *F. oxysforum* on PDA with the diameter of inhibition zone by 16.53 mm. Minimum inhibitory concentration of this filtrate against *F. oxysforum* was 60% (v/v). This result showed that filtrate of *S. thermocarboxydus* effectively suppressed the growth of *F. oxysporum.* Morphological macroconidia of *F. oxysporum* on control showed crescent shape (Figure 2E). Observation on macroconidia under SEM and TEM showed that treatment with the filtrate of *S. thermocarboxydus* resulted in the damage of cell wall and cytoplasm membrane of macro conidia as shown in Figures 2F and 2G. Observation under SEM showed that microconidia has rough surface (Figure 2K). However after treatment with filtrate showed microconidia become broken (Figure 2L).

The surface of hypha of *F. oxysporum* on control was not curvy (Figure 2H), while the hypha treated with filtrate of *S. thermocarboxydus* was broken, with curvy surface as shown in Figure 2I. Observation under TEM showed that the cell wall was perforated and plasma membrane, organelle under pressure and wider vacoule causing cell became lysis and died (Figure 2J).

Effectiveness of filtrate of *S. thermocarboxydus* to to control leaf rot disease

Treatment with the filtrate of *S. thermocarboxydus* significantly (p<0.05) suppressed the incidence of leaf rot disease on aloe vera. The disease incidence of control was 86.1% that significantly higher than those of treatments MnC (synthetic fungicide): 19.4%, STR3 (three sprays of filtrate): 38.9%, and STR4 (four sprays of filtrate): 27.8% as presented in Table 1. The suppressive activity of these three treatments were 77%; 55% and 68% for MnC, STR3 and STR4, respectively.

Table 1: *In vivo* examination of *S. thermocarboxydus* culture filtrate over *F. oxysporum* isolate on Aloe vera plant (*A. barbadensis* Mill.).

No	Treatment	Disease incidence (%)*	Suppressive activity (%)
1	Control	86.1 a	-
2	MnC	19.4 b	77
3	STR1	68.9 ab	26
4	STR2	61.1 ab	29
5	STR3	38.9 b	55
6	STR4	27.8 b	68

Means followed by the same letters are not significantly different according to the Duncan's multiple range test at 5% level.

DISCUSSION

Streptomyces sp. isolate GYRRK was found to be a potential antagonist against *F. oxysforum* the cause of leaf rot disease on aloe vera (*A. barbadensis*) in Bali. The filtrate of *Streptomyces* sp. showed a strong antifungal activity against *F. oxysforum* on PDA medium with MIC at concentration of 60%. This result suggested that *Streptomyces* sp. isolate GYRRK released substances into cultural medium and inhibited the growth of *F. oxysforum*. This phenomenon has been known as antibiosis as described by Agrios (2005) that antibiosis is an antagonistic activity resulted from the capability of a microbe to produce secondary metabolites such as antibiotics, bacteriocyn, enzymes and volatile compounds.

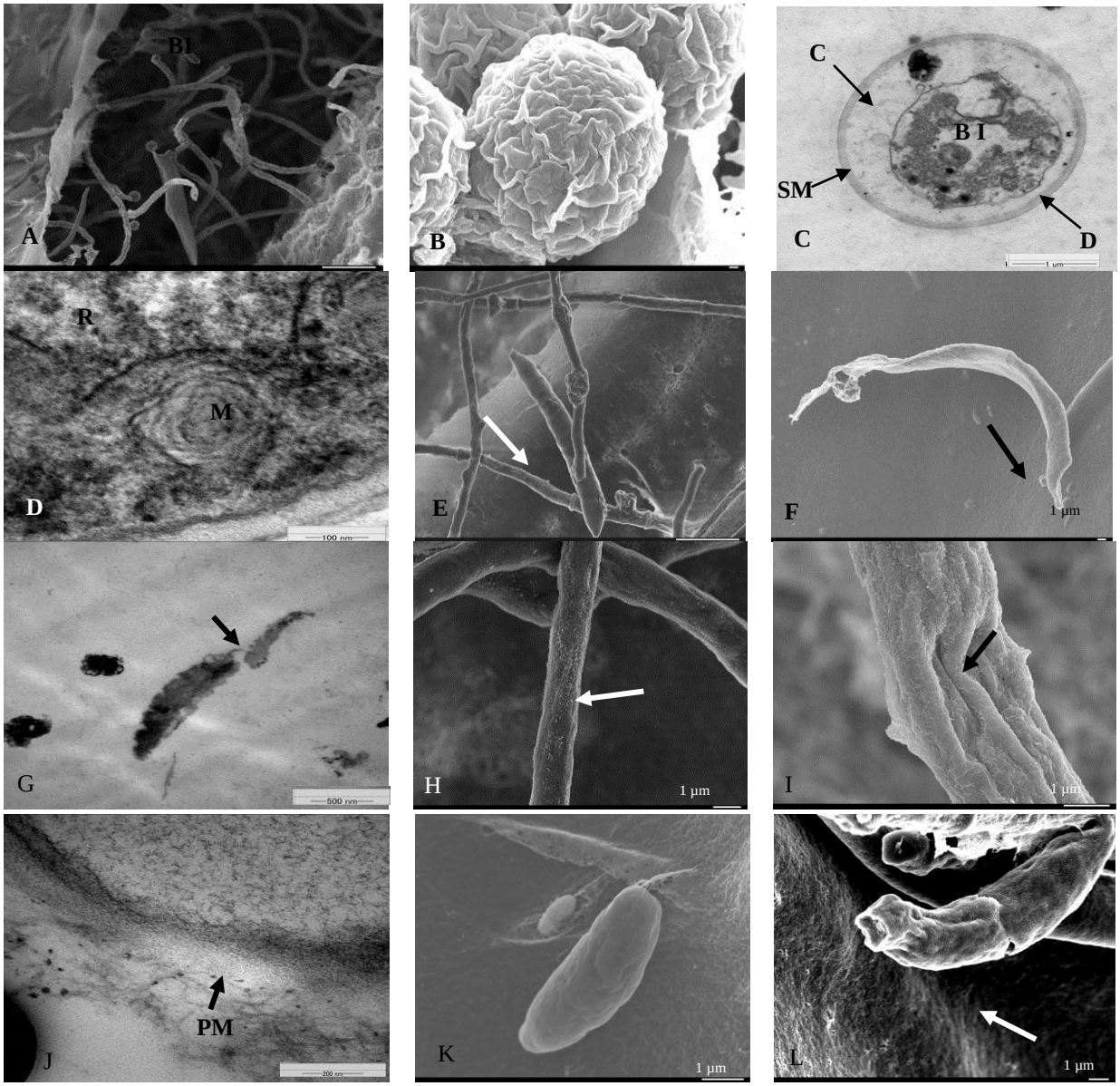

Figure 2: Scanning and transmission electron micrographs of *S. thermocarboxydus* and *F. oxysporum*. A: *S. thermocarboxydus* aerial hypha and conidia (SEM); B: Conidia's surface (SEM); C: Conidia *S. thermocarboxydus* (TEM. C: cortek, BI: nuclear substance, D: cell membrane, SM: cytoplasmic membrane); D: Ultrastucture hypha of *S. thermocarboxydus* (TEM. R: ribosome, M: mesosome, BI: nuclear substance); E: Macroconidia of *F. oxysporum* without treatment (SEM); F: Flatted macroconidia of *F. oxysporum* with filtrate treatment (arrow) (SEM); G: Damaged macroconidia's cytoplasmic membrane of *F. oxysporum* with filtrate treatment (arrow) (TEM); H: Hypha of *F. oxysporum* without filtrate treatment (arrow)(SEM); I: Damaged hypha of *F. oxysporum* with filtrate treatment (arrow) (SEM); J: Damaged hypha's plasma membrane of *F. oxysporum* with filtrate treatment (TEM. PM: plasma membrane); K: Microconidia of *F. oxysporum* without treatment (SEM), L: Broken microconidia of *F. oxysporum* with filtrate treatment (arrow)(SEM).

This is in agreement with Getha and Vilkineswary (2002) that growth inhibition could be caused by the production of enzymes and antifungal that are able to degrade hypha and cell wall of *Fusarium* sp. Narayana *et al*. (2007) reported that *Streptomyces* sp. has the capability to produce antibiotics with wide variation of chemical structures. *Streptomyces* sp. ANU 6277 produce 8-hydroxyquinoline that posses antifungal and antibacterial properties. Khamna *et al*. (2009) reported that MIC of *Streptomyces* spp. filtrate extracted with n-butanol against *Alternaria brassicola*, *A. porri*, *Colletrotrichum gloeosporioides*, *Fusarium oxysporum, Pinicillium digitatum* and *Sclerothium rolfsii*, were at a range of 0,781- 6,250 mg/mL. Study by Narayana *et al*. (2007) revealed that MIC from bioactive components (AFI –AF4) produced by *Streptomyces* sp. ANU 6277 against *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum*, *F. udum* and *Pinicillium citrinum* were at a range of 2-10 µg/mL (AF3), 10–50 µg/mL (AF1), 100-1000 µg/mL (AF2) and 250-1000 µg/mL (AF4) respectively.

Streptomyces sp. isolate GYRRK has the closest genetic relationship with *Streptomyces thermocarboxydus* strain Hd and thus, it is identified as *Streptomyces thermocarboxydus.* The morphology of *S. thermocarboxydus* observed under SEM showed a curvy aerial hypha and wavy structure with spore on the tip of hypha. The filtrate of this isolate strongly inhibited the growth of *F. oxysporum*. Based on the observation under SEM and TEM showed that this filtrate caused the damage of cell wall, plasma membrane of hypha, macro conidia, and micro conidia. These damage may responsible for the growth inhibition of *F. oxysforum*.

Similar study on *Fusarium* was done by Liyong *et al*. (2009) who found that chitosan treatment at concentration 0.5% effectively inhibited the growth of *Fusarium sulphureum*, the cause of dry rot disease on potato roots. Observation under SEM showed that the morphology of hypha was changed and become tangled, swollen and branching. TEM results showed that the cytoplasm distribution was not normal with thickened hypha membrane, formation of new septa on broken hypha, but micro conidia and macro conidia were not damaged. Domínguez *et al.* (2011) showed an ultra structure of hypha of *Alternaria alternata* treated with chitosan, and revealed that the cell membrane and conidia were damaged.

In this study we proved that the application of filtrate of *S. thermocarboxydus* isolate GYRRK, with three or four sprays significantly (p<0.05) suppressed the incidence of leaf rot disease on *A. barbadensis* grown under green house condition. These treatments were able to reduce the leaf rot disease incidence by 55 and 68% when compared to control. Study by Gopala *et al*. (2011) revealed that five isolates of actinomycetes *viz*. CAI-24, CAI 221, CAI129,KAI32 and KAI90 showed an antifungal activity against *F. oxysporum,* the cause of *Fusarium* wilt on broad bean. Likewise, these isolates suppressed the disease incidence by 45-76% in the glass house experiment. Diamond and Cooke (2003) reported the use of filtrate culture of *Phoma betae* was able to inhibit white spot disease on wheat caused by *Fusarium culmorum* by 60% compared to control after 25 days.

The filtrate of *S. thermocarboxydus* obviously suppressed the incidence of leaf rot disease on *A. barbadensis* under green house condition. The suppressive activity of this filtrate was comparable to mancozeb, a synthetic fungicide that commonly used for fungal disease control. This result suggested that filtrate of *S. thermocarboxydus* is a potential alternative safe agent that can be used to control the leaf rot disease on *A. barbadensis* instead of using synthetic fungicide

ACKNOWLEDGEMENT

A high appreciation goes to the Directorate General of Higher Education, Ministry Education and Culture of the Republic of Indonesia for partly supported this study under Sandwich-like program at the Collage of Agriculture, Ibaraki University, Japan.

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