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Full Length Research Paper

Isolation, characterization and identification of potential actinobacteria with antifungal activities towards chilli anthracnose

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Actinobacteria from the genus of Streptomycetes have been regarded as the most potent producers of bioactive compounds in the world. In this study, a total of 132 isolates of actinobacteria were isolated from rhizospheres of various plant species planted at MARDI Langkawi Agro Technology Park, Malaysia. These isolates were screened for the ability to inhibit the growth of pathogenic fungi, *Colletotrichum capsisi* and *Colletotrichum gloeosporioides* isolated from chilli fruit. From these screening it revealed that 45 isolates of actinobacteria were able to produce antifungal activity towards *C. capsici,* while 67 isolates produced antifungal activity towards *C. gloeosporioides.* Out of these 132 isolates, 2 of the best antifungal-producer were selected and identified as *Streptomyces* spp. strain PM2 and PM4. Observation using scanning electron microscope (SEM) showed that the spore surface for both *Streptomyces* spp. strain PM2 and PM4 were rough and spiky. Physiological characterization of both strains showed their ability to grow in 1 to 4% of NaCl, growth temperature of 17 to 35°C and pH of 5 to 11. The ability of these *Streptomyces* spp. to secrete antifungal compounds may have been related to the availibility of the carbon sources. These findings suggest that *Streptomyces* spp. strain PM2 and PM4 are potential candidate for biocontrol against anthracnose disease.

Keywords: Actinobacteria, *Colletotrichum capsici*, *Colletotrichum gloeosporioides*, anthracnose, antifungal activity.

INTRODUCTION

Colletotrichum capsici and *Colletotrichum gloeosporioides* have been known to be the casual agents for the anthracnose disease on chilli fruits world wide (Manandhar et al., 1995; Sangchote, 1999). *C. gloeosporioides* was the most dominant anthracnose causing agent with its ability to infect other crops and fruits such as yam and mango. Anthracnose attack on chilli fruits causes the fruits to be blemishes and thus, making it unmarketable. The typical symptoms of anthracnose attack on chilli fruit are characterized by the formation of sunken necrotic lession, with concentric rings of acervuli.

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Soil borne actinobacteria had been known to possess the ability to produce bioactivities such as enzymes, pesticides, herbicides and also antibiotics (Parungao et al., 2007). Currently, fungicides were sprayed to the fruits to inhibit the disease, but the used of chemical fungicides would caused the development of resistance by the fungal and also the risk of polluting the environments. The used of biological control agents had been suggested as an alternative way of controlling plant diseases (Compant et al., 2005). Jeffrey (2008) has suggested that actinobacteria belonging to the genus of Streptomyces can be used as biocontrol agents for several plant diseases. Study by Johnson (1954) shows that *Streptomycetes* spp. gave significant control towards pythium root rot of corn and sugarcane. While Lee and Hwang (2002) showed the production of antifungal activity by actinomycete isolated from Korean soil

towards *Alternaria mali, C. gloeosporioides*, *Fusarium oxysporum* f.sp. *cucumerinum* and *Rhizoctonia solani*. In Malaysia, the used of biological agents especially from actinobacteria had not been well documented for tagricultural purposes.

This study describes the findings obtained in the quest for antifungal activity produced by actinobacteria towards *C. capsici* and *C. gloeosporioides*.

MATERIALS AND METHODS

Sampling

Soil samples were collected from several plants planted in the vicinity MARDI Langkawi Agro Technology Park at the depth of about 15 cm from the top soil surface. Collected soil samples were put into a double ziplock bag and kept in ice while transportation back to the laboratory.

Isolation of actinobacteria

Soil actinobacteria were isolated by mixing 10 g of soil with 100 ml of sterile distilled water. The soil suspension was shaken with an orbital shaker at 250 rpm for approximately 1 h. After 1 h, 150 µl of the suspension was pipeted and lawned onto starch casien agar (Küster and Williams, 1964). The plates were incubated at 28 ± 2°C for 14 days prior selection of the actinobacteria colonies (Ndonde and Semu, 2000). Selected actinobacteria were subcultured onto fresh starch casien agar (SCA) plates and further incubated for another 7 days. Serial dilution from 10^2 to 10^7 was done to estimate the total colony forming unit of actinobacteria from each soil samples. The colonies were observed for its purity before being used for the test and kept as stock in 20% (v/v) glycerol stock

Preparation of pathogen inoculum

C. capsici and *C. gloeosporioides* were isolated from infected red chili fruits using surface sterilization method. In this method, infected chili fruits were cut at the infected site and dipped into 2% sodium hypochloride for 2 min. After which the infected sites were washed with sterile distilled water before being submerged into 75% ethanol for 30 s. The pieces of cut chili fruits were then left to dry in laminar flow for about 30 min before being placed onto fresh potato dextrose agar plates (PDA). The plates were then incubated for about 21 days for the emerging of *Colletotrichum* spp. The emerging fungal colonies were pick and grown on fresh PDA for another 8 days to determine the purity of the isolates. The pure isolates of *Colletotrichum* spp. were identified based on their conidia morphology using research microscope (Meiji MX 5000) and also molecular method using the ITS 1 and ITS 4 primers (White et al., 1990), before proceeding to screening for the antigonistic activity by the actinobacteria.

Antifungal testing of actinobacteria towards *C. capsici and C. gloeosporioides*

Isolated actinobacteria were tested for their inhibitory activity against *C. capsici* and *C. gloeosporioides* by performing a dual-culture *in vitro* assay. Potato dextrose agar plate was used as the media for the test. Each plate of PDA containts two test strain agar plugs, a positive control disc impregnated with 50 mg/ml of cycloheximides, a negative control agar plug and an agar plug of either *C. capsici* or *C. gloeosporioides* at the middle of the plate. All the plugs were made using sterile borer. The plates were later incubated at room temperature (28±2°C) for about 5 days before the inhibition zones were measured. All tests were conducted in a triplicate manner.

Actinobacteria identification

DNA extraction method as describes by vivantis was followed (http://www.vivantis.com) with little modification where the final elution volume was reduced to 30 µl instead of 50 µl of sterile distilled water added onto the membrane and left to stand for 2 min before centrifugation at 10,000 x *g* for 2 min. The eluted DNA was later used for polymerase chain reaction (PCR) according to the PCR parameter and conditions stated by Jeffrey (2008).

The PCR products were later subjected to purification using vivantis GF-1 Gel DNA recovery kit (http://www.vivantis.com). The purified PCR products were sequenced using ABI PRISM ® 377 DNA Sequencer (Applied Biosystems). Results obtained from the sequencing were blast thru NCBI database (Altschul et al., 1990).

Physiological characterization of *Streptomyces* spp. strain PM2 and *Streptomyces* spp. strain PM4

Streptomyces spp. strain PM2 and *Streptomyces* spp. strain PM4 were characterized for their physiological (temperature;17, 28 and 35°C, salinity; 1 to 4% and pH; 5, 7, 9 and 11), morphological and biochemical properties. In the physiological test, selected isolates were grown in a 250 ml of Erlenmeyer flask containing 100 ml of starch casien broth (SCB) for 3 days with shaking of 200 rpm. Each of the tests were performed in triplicate manner. Otherwise stated all the tests were conducted under pH 7.

Morphology characterization of *Streptomyces* spp. strain PM2 and *Streptomyces* spp. strain PM4

Streptomyces spp. strain PM2 and PM4 morphology were viewed using a research microscope (Meiji MX 5000) and scanning electron microscope (XL 30) to observe their spore chain arrangement and their spore surface.

Biochemical characterization of *Streptomyces* spp. strain PM2 and *Streptomyces* spp. strain PM4

Both selected *Streptomyces* spp. were tested for their carbon sources utilization pattern using Gen III microplate obtained from Biolog. Protocols used were as stipulated by the manufacturer (http://www.biolog.com).

RESULTS

Isolation and enumeration of actinobacteria

A total of 132 isolates of actinobacteria were isolated from a total of 7 soil samples collected from MARDI Langkawi Agro Technology Park. Colony forming unit per gram (cfu/g) of soil obtained varies from 2.0×10^5 to 7.5×10^7. The aerial mycellium observed were in the colour range of white, grey, brown, red and yellow. It was also observed that higher count of actinobacteria were found

Table 1. Numbers of actinobacteria isolated from different rhizosphres of plant species and the colony forming unit.

Plant species	cfu g^{-1} of dry soil	Number of actinobacteria isolated
Citrus maxima	3.2×10^7	18
Artocarpus heterophyllus	2.4×10^7	18
Cocos nucifera	7.5×10^7	15
Nephelium mutabile Blume	2.0×10^5	5
Orthosiphon stamineus	1.0×10^7	20
Hylocereus spp	1.2×10^6	28
Phoenix spp.	3.0×10^6	28

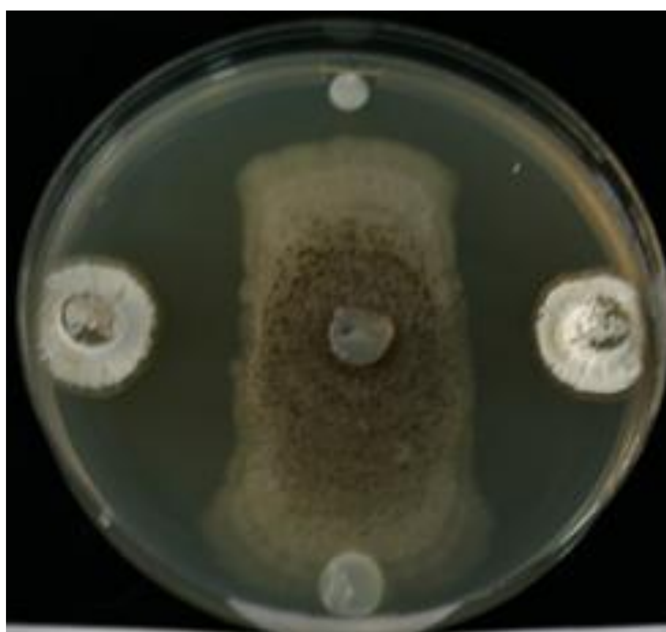

Figure 1. Antifungal screening of actinobacteria with *C. capsici*.

within rhizosphere of *Cocos nucifera* (7.5×10^7), while from rhizosphere of *Nephelium mutabile* Blume gave the lowest count (2.0×10^5) of actinobacteria. Table 1 showed the summarized results of colony forming unit per gram of dry soil for actinobacteria from different rhizospheres.

Identification of *Colletotrichum* spp.

Isolated fungi were identified as *C. capsici* and *C. gloeosprioides* from their conidia and also BLAST results from NCBI. *Colletotrichum capsici* conidia was described to be one- celled, smooth walled, hyaline, falcate with sometime fusiform, tapered towards both ends and acute at the apex (Shenoy et al., 2007). Meanwhile, *C. gloeosprioides* conidia was known to be singly borne, cylindroidal with rounded ends, nonseptate, hyaline and smooth walled (Pitt and Hocking, 2007).

Screening of actinobacteria towards *C. capsici* and *C. gloeosporioides*

A total of 45 and 67 isolates of actinobacteria showed antifungal activity toward *C. capsici* and *C. gloeosprioides,* respectively, by the formation of inhibition zone around them (Figure 1). From this, only 10 isolates of actinobacteria showed antagonistic activity towards both the *C. capsici* and *C. gloeosprioides*. Inhibition zone produced by these 10 isolates varies from 6 to 12 mm (Figure 2). Results showed that most of the actinobacteria gave higher activity towards *C. gloeosprioides* compared with *C. capsici*.

Although, S*treptomyces* spp. PM2 and PM4 gave the highest antifungal activity but actinobacteria isolated from the rhizosphere of *Cocos nucifera* showed the highest number of actinobacteria with antifungal activity (K16, K24, K27). Isolates K27 gave the highest antifungal activity towards *C. capsici* with the inhibition zone of 12 mm

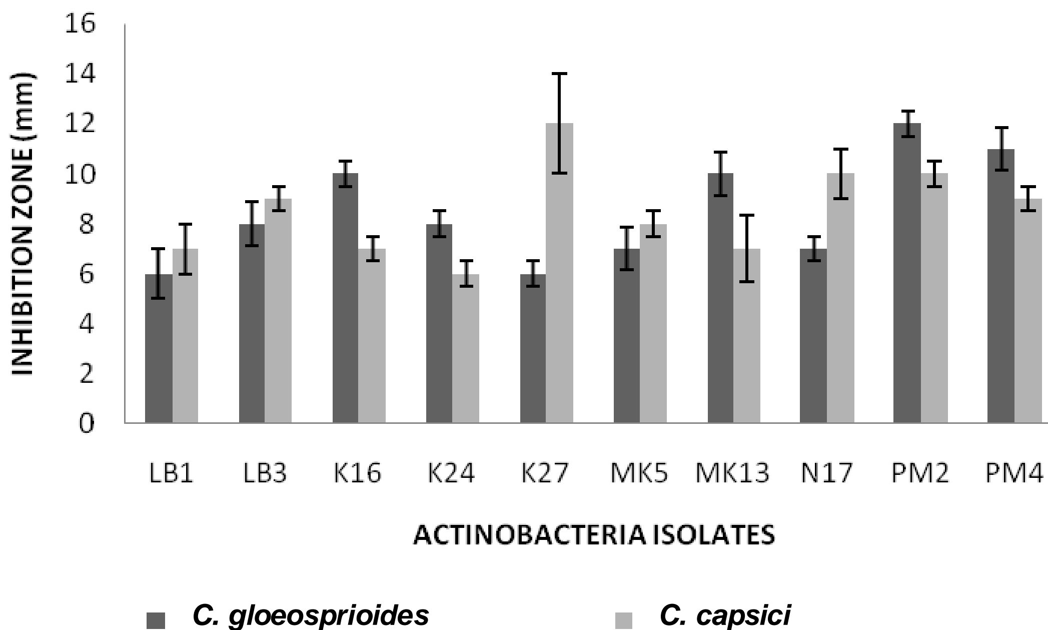

Figure 2. Comparison of antifungal activity by selectes actinobacteria against *C. capsici* and *C. gloeosprioides.* Mean ± standard deviation.

but it was not significant for *C. gloeosprioides* with the inhibition zone of only 6 mm. While PM2 gave the highest antifungal activity towards *C. gloeosprioides* (inhibition zone of 12 mm), LB1 gave the lowest antifungal activities towards both *C. capsici* and *C. gloeosprioides* with the inhibition zone of 7 and 6 mm, respectively.

Identification of the best isolates

From BLASTing our sequences with the NCBI databases, we identified both PM2 and PM4 to be *Streptomyces* spp. Both sequences had been deposited with the NCBI databases with the accession number of HM598070 and HM598071, respectively. Apart from the BLAST results, morphological identification with reference to the Bergey's Manual showed that both isolates contained spiral spore chain arrangement using a research microscope (Figures 3 and 4) and rough and spiky surface using SEM (Figures 5 and 6) which are typical for the actinobacteria from the genus of *Streptomyces* (Holt et al., 1994).

Characterization of best isolates

It was observed that both PM2 and PM4 were able to grow under pH 5, 7, 9 and 11. Both isolates were observed to grow in the concentration of NaCl ranging from 1 to 4% and temperature of 17 to 37°C. Metabolite analysis of PM2 and PM4 showed that both of the isolates have the ability to utilize gentiobiose, α-D-glucose, D-fructose, D-galactorunic acid and α-hydroxy-D,L-butyric acid for their growth (Table 2).

DISCUSSION

From the total of 132 isolates of actinobacteria isolated, majority of the actinobacteria show the aerial mycellium to be in the grey colour group. This results were also noted in the study done by Jeffrey (2008). From this study, we noticed that the cfu/g for actinobacteria isolated was lower compared with the study done by Jeffrey (2008). In his study, Jeffrey (2008) recorded the highest cfu/g count as 8.0×10^7, while the lowest was 9.8×10^6. Both results were higher compared with the results we obtained in this study. Study by Lee and Hwang (2002) and Takizawa et al. (1993) showed that cfu/g obtained from both Chesapeake Bay (1.8×10^2 to 1.4×10^5) and Korean vegetative soil (1.17 to 4.20×10^6) respectively, were lower than the findings of this paper. All these findings may suggest that differences in the type of soil and the agriculture practices may influence the population of the actinobacteria. This statement is supported by Athalye et al. (1981) and Oskay et al. (2004), where they found that the growth rate of microorganisms were influenced by the humidity and pH of the soil. Merckx et al. (1987) stated that soil rhizospere had a diverse saprophytic microorganisms due to the organic material derived from the plant roots and its exudates. This statement is further supported by Tewtrakul and Subhadhirasakul (2007) study, where they obtained a correlation relationship between the diversity of actinobacteria with the type of plants and the soil organic matter content.

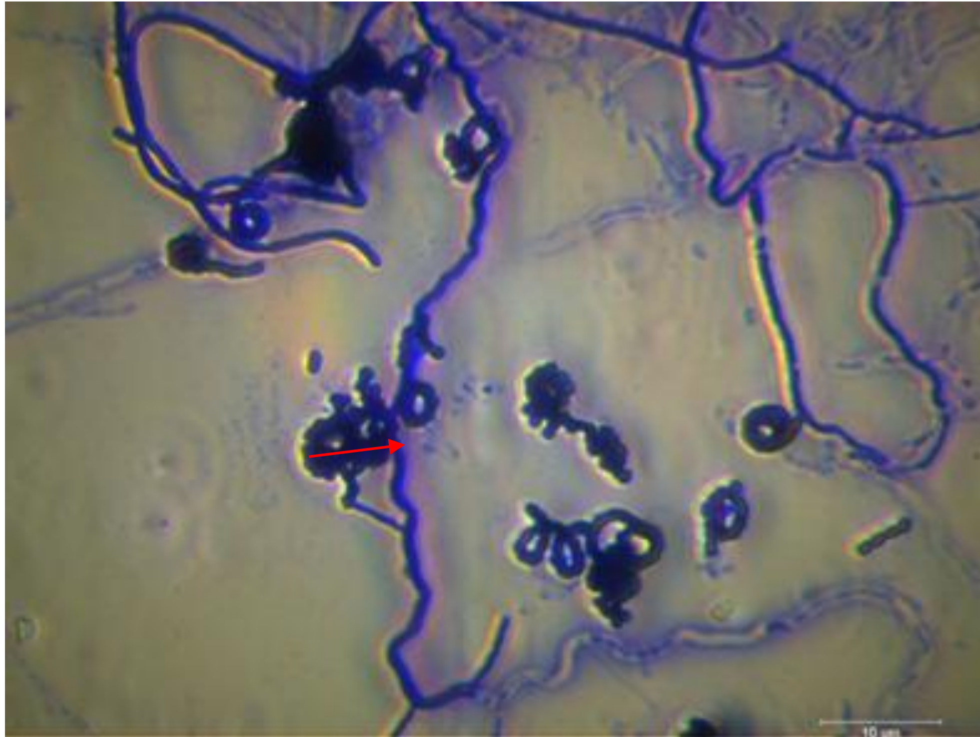

Figure 3. Spore chain arrangement of PM2 observed at the magnification of 1000X using research microscope. Red arrow showing the spiral spore chain.

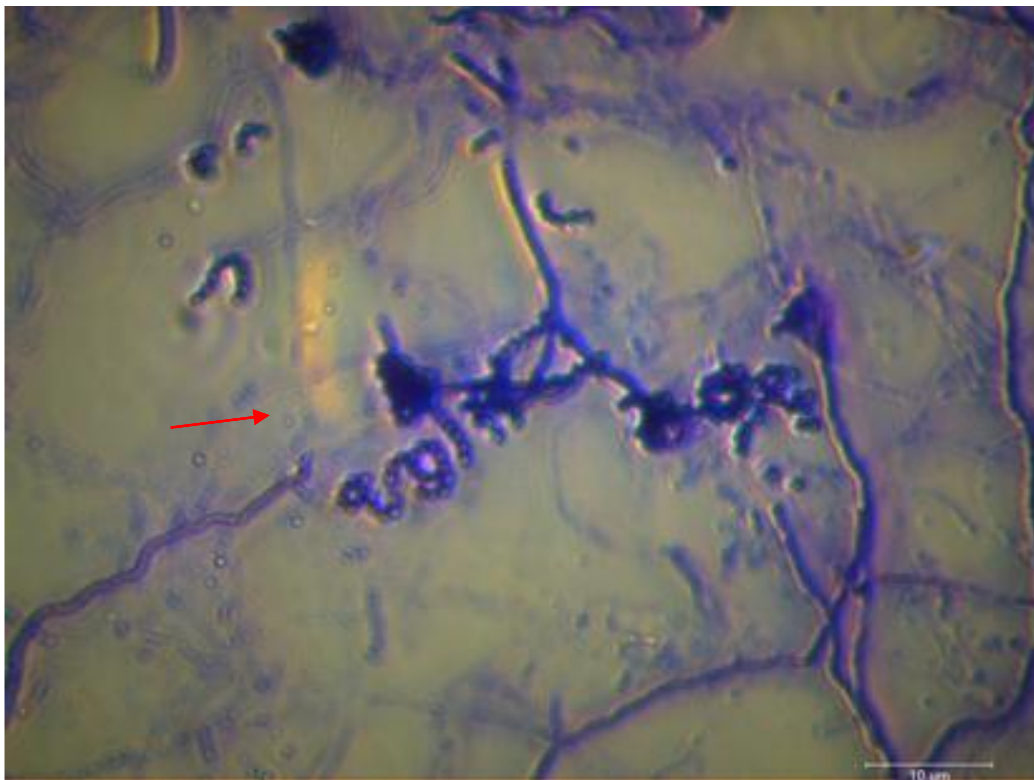

Figure 4. Spore chain arrangement of PM4 observed at the magnification of 1000X using research microscope. Red arrow showing the spiral spore chain.

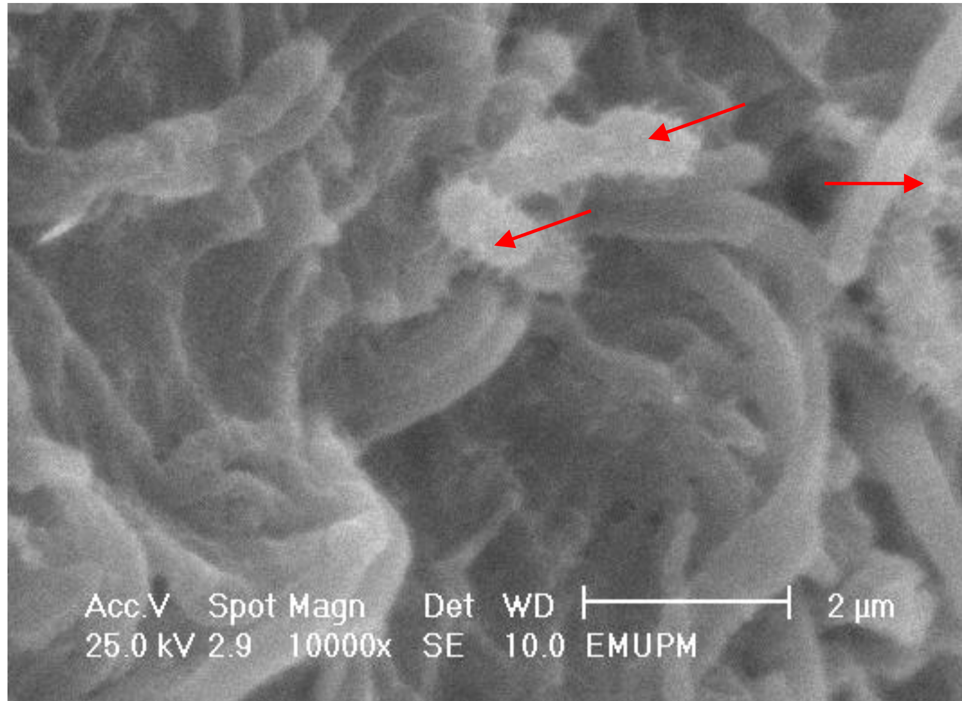

Figure 5. SEM micrograph of *Streptomyces* spp. strain PM2. The spores are rough and spiky. Red arrows showed the spikes on the surface of the strain.

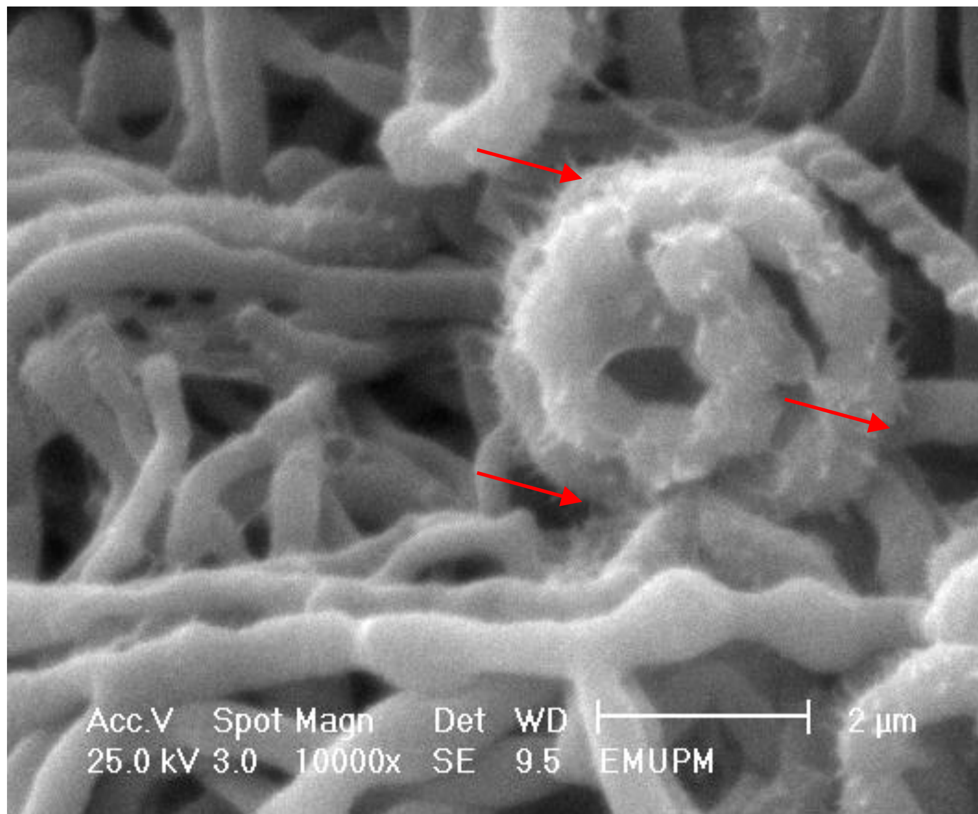

Figure 6. SEM of *Streptomyces* spp strain PM4. The spore chain was spiral and the spore surface was rough and spiky. Red arrows indicate the spikes from the spore's surface.

Table 2. Characterization of *Streptomyces* spp. strain PM2 and PM4.

Parameter	Selected actinobacteria isolate	
Strain number	**PM2**	**PM4**
Strain identity	***Streptomyces* spp.**	***Streptomyces* spp.**
Morphology characterization		
Colour of substrate mycelium	Dark grey	White
Colour of aerial mycelium	Dark brown	Grey
Spore chain arrangement	Spiral	Spiral
Spore surface	Rough and spiky	Rough and spiky
Pigment production	+	+
Enzymatic activity		
Lipase activity	+	+
Cellulase activity	+	+
Protease activity	+	+
Mannanase activity	-	+
Xylanase activity	+	+
Hydrolysis of starch	+	+
Hydrolysis of casein	+	+
Physiological properties		
Grow on:		
Starch casein agar	+	+
Nutrient agar	+	+
1% NaCl	+	+
4% NaCl	+	+
17°C	+	+
28°C	+	+
35°C	+	+
pH 5	+	+
pH 7	+	+
pH 9	+	-
pH 11	+	-
Carbon source utilization		
Dextrin	-	-
D-Maltose	-	-
D-Trehalose	-	-
D-Cellobiose	+	-
Gentiobiose	+	+
Sucrose	-	-
D-Turanose	+	-
α-D-Glucose	+	+
D-Raffinose	+	-
α-D-Lactose	-	-
D-Mannose	+	-
D-Fructose	+	+
D-Galactose	+	-
3-Methyl Glucose	+	-
L-Rhamnose	+	-
D-Sorbitol	-	-
D-Mannitol	-	-
D-Arabitol	-	-

Table 2. Contd.

Pectin	-	-
Glycerol	-	-
Gelatin	-	-
L-Histidine	+	-
L-Serine	-	-
D-Galactonic acid	+	+
D-Glucuronic acid	+	-
α-Keto-Butyric acid	+	+
Propionic acid	-	+
Acetic acid	-	+
Acetoacetic acid	-	-
L-Lactic acid	-	-

+ Mean positive reaction; - mean negative reaction.

In the quest for the best antifungal compounds, a large pool of actinobacteria were needed. Thus, sampling of soil samples from MARDI Langkawi Agro Technology Park has been considered due to the hypothesis that the diversity of actinobacteria may be influenced by the diversity of plants. According to Oskay et al. (2004), the adaptation of actinobacteria with its surrounding would affect their ability to secrete certain types of secondary metabolites.

The differences in the numbers of actinobacteria producing antimicrobial activities towards both *C. capsici* and *C. gloeosporioides* shows that most of the actinobacteria isolates tested produce narrow range antifungal compounds, while only some (10 isolates) produces the wide range antifungal compounds. The ability of actinobacteria to secrete antifungal compounds were also dependent on the type of plant where they were isolated (Crawford et al., 1993; Yuan and Crawford, 1995). Apart from the type of plants, the usage of carbon sources had also been related with the optimum production of antibiotics (Pandey et al., 2005; Vasavada et al., 2006). This is supported by the study done by Pandey et al. (2005), where by the production of antibiotic was at its optimum for *Streptomyces kananmyceticus* M27 when dextrose was used. Another study by Bhattacharyya et al. (1998) showed that the production of antibiotics was optimum when glycerol was added to the media as the carbon source.

Apart from carbon and nitrogen sources, salinity was also suggested as a factor in the production of antibiotics. Vasada et al. (2006) showed that *Streptomyces sannanensis* isolated gave the best antimicrobial activity when the NaCl concentration was 3%. What we obtained in this study was both *Streptomyces* spp. (PM2 and PM4) and were able to survive in the 4% NaCl but no antifungal activity was done. The ability of both *Streptomyces* spp. PM2 and PM4 to grow under temperature of 17 to 37°C suggest that they belongs to the mesophilic group of bacteria. The ability of both *Streptomyces* spp. PM2 and PM4 to secrete lipases, cellulases, proteases and xylanases would allowed both strains to be utilized for composting purposes other than being used as biological control agents.

Conclusion

Streptomyces spp. (PM2 and PM4) isolated were not much different in their morphology but both species were diversed in their carbon sources utilization and their antifungal abilities. The antifungal properties of *Streptomyces* spp. strain PM2 and PM4 towards *C. capsisi* and *C. gloeosporioides* observed in this study, are potential for future field application as biocontrol agents against antrhracnose disease.

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REFERENCES

Altschul SF, Jenkins A, Kristiansen BE, FrØholm CO (1990). Basic local alignment search tool. J. Mol. Biol., 215: 403-410.

Athalye M, Lacey J, Goodfellow M (1981). Selective ioslation and enemeration of actinomycetes using rifampicin. J. Appl. Bacteriol., 51: 289-297.

Bhattacharyya BK, Pal SC, Sen SK (1998). Antibiotic production by *Streptomyces hygroscopicus* D1.5: Cultural effect. Rev. Microbiol., 29: 36-41.

Biolog (2008) Gen III microplate, Instruction to used. BIOLOG Inc. http://www.biolog.com (2008). Access on 5th June 2008.

Compant DL, Duffy B, nowak J, Clément C, Barka EA (2005). Use of growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action and future prospect. Appl. Environ. Microbiol., 71(9): 4951-4959.

Crawford DL, Lynch JM, Whipps JM, Ousley MA (1993). Isolation and characerization of actinomycetes antagonists of a fungal root pathogen. Appl. Environ. Microbiol., 59: 3899-3905.

Holt JG, Krieg NR, Sneath PHA, Stacey JT, Williams ST (1994). Bergey's Manual of Determinative Bacteriology (9th edition). Baltimore, Willams and Wilkins.

Jeffrey LSH (2008). Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak. Afr. J. Biotechnol., 7(20): 3700-3705.

Johnson LF (1954). Antibiosis in relation to Pythium Root Rot of sugarcane and corn. Phytopathology, 44: 69-73.

Küster E, Williams ST (1964). Selection of media for isolation of Streptomyces. Nature, 202: 928-929.

Lee JY, Hwang BK (2002). Diversity of antifungal actinomycetes in various vegetative soils of Korea. Rev. Can. Microbiol., 48: 407-417.

Manandhar JB, Hartman GL and Wang TC (1995). Conidial germination and appressorial formation of *Colletotrichum capsici* and *Colletotrichum gloeosporioides* isolates from pepper. Plant Dis., 79:361-366.

Merckx R, Dijkra A, Hartog AD, Veen JAV (1987). Production of root-derived material and associated microbial growth in soil at different nutrient levels. Biol. Fertil. Soils, 5: 126-132.

Ndonde MJM, Semu E (2000). Preliminary characterization of some Streptomyces species from four Tanzanian soils and their animicrobial potential against selected plant and animal pathogenic bacteria. World J. Microbiol. Biotechnol., 16: 595-599.

Oskay M, Usame Tamer A, Azeri C (2004) Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. Afr. J. Biotechnol., 3(9): 441-446.

Pandey A, Shukla A, Majumdar SK (2005). Utilization of carbon and nitrogen sources by *Streptomyces kanamyceticus* M27 for the production of an antibacterial antibiotic. Afr. J. Biotechnol., 4: 909-910.

Parungao MM, Maceda EBG, Villano AF (2007). Screening of antibiotic producing actinomycetes from marine, Brackish and terrestrial sediments of Samal Island, Philippine. J. Res. Sci. Com. Eng., 4(3): 29-38.

Pitt JJ, Hocking AD (2007). Primary keys and miscellaneous frungi; Genus *Colletotrichum* corda. In: Fungi and food spoilage. Springer. pp. 81-82.

Sangchote S (1999). Anthracnose Resistant in Chilli. Progress report at the 20th Anniversary of Kamphaeng saen campus, Kasetsart University, 29th November – 5th December.

Shenoy BD, Jeewon R, Lam WH, Bhat DJ, Than PP, Taylor PWJ, Hyde KD (2007). Morpho-molecular characterization and epitypification of *Colletotrichum capsici* (*Glomerellaceae*, *Sordariomycetes*), the causative agent of anthracnose in chili. Fungal Diversity. 27: 197-211.

Takizawa M, Colwell RR, Hill RT (1993). Isolation and diversity of actinomycetes in the Chesapeake Bay. Appl. Environ. Microbiol., 59: 997-1002.

Tewtrakul S, Subhadhirasakul S (2007). Anti-allergic activity of some selected plants in Zingiberaceae family. J. Ethnopharmacol., 109: 535-538.

Vasavada SH, Thumar JT, Singh SP (2006). Secretion of a potent antibiotic by salt –tolerent and alkaliphilic actinomycete *Streptomyces sannanensis* strain RJT-1. Curr. Sci., 91: 1393-1397.

Vivantis (2009). GF-1 Gel DNA Reocovery Kit. Vivantis. http://www.vivantis .com (2009). Access on 21st February.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: PCR protocols. Eds. Innis MA, Gelfand DH, Sninisky JJ, and White TJ. Academic Press, San Diego. pp. 315-322.

Yuan WM, Crawford DL (1995). Characterization of *Streotomyces lydicus*WYEC108 as a potential biocantrol agent againstfungal root and seed rots. Appl. Environ. Microbiol., 61: 3119-3128.