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# Characterization of Actinobacteria with Antifungal Potential against Fusarium Crown-Rot Pathogen

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## ABSTRACT

Actinobacteria isolates recovered from soil were investigated for their antifungal properties towards *Fusarium* spp., the causal agent of Fusarium crown rot disease in bananas. We isolated 33 isolates of Actinobacteria from soil and established that all of the isolates produced antifungal compounds able to inhibit the growth of *Fusarium* spp. *in vitro*, although with varying degrees of inhibition. Characterization based on cultural, morphological and biochemical characteristics revealed that 16 different genera of Actinobacteria were isolated. The most dominant genus from our diverse sample pool was *Streptomyces* with 8 isolates, followed by *Nocardiopsis*, *Actinomadura* and *Saccharothrix*, with 4, 3 and 3 isolates, respectively. Isolates with the most antifungal potential belonged to *Streptomyces* spp. (isolate 20C1), followed by another isolate of *Streptomyces* spp. (isolate 15Br2) and *Saccharomonospora* spp. (isolate 21A2), with 96, 90 and 78% inhibition, respectively. These isolates can be potentially developed as biocontrol agents for field application as crown rot disease is initiated in the field. Future applications may also include investigations into harvesting and applying the metabolites produced by these isolates as biofungicide introduced at the post-harvest stage.

**Keywords:** antagonistic, antibiotics, biological control
**Abbreviations:** **ISP2**, International *Streptomyces* Project 2; **PDA**, potato dextrose agar; **PIRG**, Percentage of Inhibition of Radial Growth; **R1**, radial growth in control plate; **R2**, radial growth upon treatment with Actinobacteria

## INTRODUCTION

Fusarium crown rot disease of bananas is caused by a variety of *Fusarium* spp., such as *F. oxysporum*, *F. solani*, *F. roseum*, *F. moniliformae*, *F. pallidoroseum*, and *F. proliferatum* (Anthony *et al*. 2004; Ogundero 2007). Although infection is initiated in the field, symptoms of the disease are rarely seen until the banana fruits are harvested. The spores of the fungi, which are already present on the surface of the banana bunch and fruits, enter the wounded tissues from the cut surface of the crown (Krauss and Johanson 2000). The infection then spreads from the pedicels into the finger necks and eventually into the fruits. In severely infected fruits, white, grey or pink mould appears on the surface of the crown tissues (Win *et al.* 2007).

Attempts to control the crown rot disease were first implemented through proper cultural methods, by deflowering, defingering, delatexing and usage of clean knives (Krauss and Johanson 2000). The success of the cultural methods however, varies depending on the ripening period of the bananas and climate changes (deLapeyre *et al*. 2000). At the post-harvest stage, reports of the use of fungicides such as thiabendazole and imazalil have shown effectiveness in controlling crown rot disease (Johanson and Blazquez 1992; Krauss and Johanson 2000). However, prolonged usage of these chemicals risks the development of fungicide resistance and a shift in microbial population, which may result in opportunistic pathogens becoming more aggressive and difficult to control (Reyes *et al.* 1998). As an alternative, biological control approach is investigated as this method is more environmental-friendly, sustainable and poses less health risk when applied correctly. In biological control, microbial antagonists with antifungal and antibacterial properties replace the usage of chemicals. In the last decades, a number of bacteria, filamentous fungi and yeasts have shown potential to protect fruits from post-harvest pathogens (Janisiewicz 1987; Chan and Tian 2005; Guijarro *et al*. 2007). Disease suppression by these antagonists was mainly attributed to antibiosis (Pal and Gardener 2006).

In our study, we investigated the possible role of Actinobacteria as antagonists for the control of crown rot disease of banana. Actinobacteria are known to produce a variety of antibiotics (Sun *et al.* 2002; Liu *et al.* 2008; Prapagdee *et al.* 2008) which can be manipulated as biofungicides. Actinobacteria are also mostly saprophytic in nature compared to other microbial antagonists, thus may have a wider application in disease control strategies. The Actinobacteria isolates in this study were isolated from soil samples and screened for their antifungal properties against the crown rot pathogen. The isolates were then characterized based on their cultural, morphological and biochemical characteristics to identify the isolates to their presumptive genera.

## MATERIALS AND METHODS

### Isolation of Actinobacteria from soil samples

Thirty four soil samples were sampled from several locations in Kuala Lumpur, Malaysia. For isolation, 10 g of soil was first suspended in 100 ml of sterile distilled water in a 250 ml conical flask, and agitated (120 rpm) for 30 min at 27 ± 2°C. A six-fold dilution was then performed and a 0.1 ml aliquot from each dilution was pipetted and plated on arginine-glycerol agar (Difco™). Plates were then incubated at room temperature (30 ± 2°C) for 5 days. After 5 days of incubation, the morphology of cultures formed on the agar plates was observed under a stereo-microscope and the suspected Actinobacteria isolates were picked and inoculated onto yeast extract-malt extract agar (ISP2) (containing 4 g yeast extract, 10 g malt extract, 4 g glucose and 20 g agar for 1 L, adjusted to pH 7.3) to establish pure cultures.

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Research Note

## Characterization of Actinobacteria isolates

Actinobacteria isolates were identified to their genera based on their cultural, morphological and biochemical characteristics derived from various observations and tests. The cultural characteristics observed include the colony appearance on different agar medium, pigmentation on different agar medium and the general growth rate of the Actinobacteria isolates. Morphological characteristics evaluated include their Gram staining profile and the microscopic structures of the Actinobacteria were observed using the inclined coverslip method. The biochemical tests performed consisted of the following examinations: casein hydrolysis, starch hydrolysis, urea hydrolysis, citrate utilization, catalase activity, xanthine degradation, carbohydrate fermentation, temperature tolerance, salt tolerant test, aerobic and anaerobic determination tests. These observations were conducted based on methodologies listed by Cappuccino and Sherman (2005) and Bergey and Holt (1994).

## Antifungal assay

Each Actinobacterial isolate was first streaked vertically on separate potato dextrose agar (PDA) (Pronadisa®, Spain) plates, at a distance of 3 cm from the periphery of the plate and incubated for 14 days at room temperature (30 ± 2°C). A mycelial plug (0.5 cm diameter) of the crown rot pathogen, *Fusarium* spp. isolated by Chee and Ting (2008), was then inoculated 3 cm away from the Actinobacteria streak and the other periphery end of the plate. A control plate was also established similarly by streaking sterile distilled water instead of Actinobacteria. Two replicates were prepared for each tested isolate. All inoculated Petri plates were incubated at room temperature (30 ± 2°C) for 7 days. The percentage of inhibition of radial growth (PIRG%) of the pathogen (*Fusarium* spp.) by Actinobacteria was calculated based on the following equation (Ting *et al*. 2009):

$$\text{PIRG (\%)} = \frac{R1-R2}{R1} \times 100\%$$

where R1 is the radial growth of the fungal pathogen in the control plate and R2 is the radial growth of fungal pathogen in plates challenged with Actinobacteria. The experiment was conducted in a complete randomized design. The antifungal assay was repeated once.

# RESULTS AND DISCUSSION

## Recovery of Actinobacteria from soil samples

In our study, we isolated a relatively small number of Actinobacteria compared to the number of soil samples used as only 33 isolates of Actinobacteria were recovered. Our poor recovery rate (less than 1%) was contrary to many studies which have shown that Actinobacteria population is one of the major groups of soil population. We speculate that this could be due to the type of soils used in this study which were mostly light brown in colour and sandy, compared to dark top-soil containing humus which has more Actinobacteria.

## Characterization of Actinobacteria isolates

The characterization technique adopted in this study, although conventional and subject to improvements, has been used effectively by Oskay *et al.* (2004) and Remya and Vijayakumar (2008), and is extremely beneficial prior to sequencing efforts using 16SrDNA gene sequences (Taechowisan and Lumyong 2003). The isolates were characterized into 16 presumptive genera based on their collective results from cultural, morphological and biochemical observations. Although morphological observations may suggest several genera having the same characteristics, their subsequent biochemical results help to determine their most relevant genera. The genera identified were *Actinobispora*, *Actinomadura*, *Dactylosporangium*, *Glycomyces*, *Kitasatosporia*, *Microbispora*, *Micromonospora*, *Nocardia*, *Nocardiodes*,

**Table 1** Cultural and morphological characteristics of Actinobacteria isolates on ISP2 agar.

| Isolate | Pigmentation of aerial mycelium | Pigmentation of substrate mycelium | Colony diameter[a] (cm) | Colony morphology |
|---|---|---|---|---|
| 13B1 | Copper brown with white specks | Golden brown/Orange | 0.50 | Flat |
| 15B1 | White | Dark cream | 0.10 | Raised, powdery |
| 15Br2 | White | Cream | 0.90 | Flat, wrinkled |
| 20Ar1 | Light grey | Dark golden brown/Orange | 0.50 | Raised, dense |
| 20Ar4 | White | Creamy brown | 0.10 | Flat |
| 20Ar5 | Light grey | Grey | 0.25 | Raised centre |
| 21Ar1 | Dark grey | Greyish cream | 0.30 | Raised, dense |
| 21Ar2 | Greyish brown | Greyish cream | 0.10 | Raised |
| 21Ar3 | Dark grey | Greyish brown | 0.85 | Raised, dense |
| 21Ar11 | Greyish brown | Light grey | 0.20 | Raised, dense |
| 21Ar14 | White, sparse | Dark cream | 0.40 | Raised, leathery |
| 20C1 | White | Dark cream | 0.40 | Raised, wrinkled, powdery |
| 21A2 | Dark grey | Light grey | 1.90 | Flat, wrinkled |
| 21A8 | Brownish grey | Dark greyish cream | 0.35 | Raised |
| 21B2 | White/ transparent white | Cream | 0.70 | Raised |
| 21Br2 | Blackish grey | Brownish grey | 0.35 | Slightly raised, dense |
| 22Ar1 | White, powdery | Cream | 0.50 | Flat, powdery |
| St1 | Light grey to white | Cream | 0.40 | Flat, powdery |
| St4 | Light grey, white margins | Cream | 0.40 | Fuzzy, powdery |
| St5 | Light grey | Cream | 0.50 | Flat, powdery |
| St7 | White | Cream | 0.50 | Raised, leathery, fuzzy |
| St10 | (aerial mycelium absent) | Cream to opaque white | 0.80 | Glistening surface, raised |
| St11 | Pale pink, white margins | Cream | 0.50 | Powdery, raised |
| St12 | Light grey to white | Yellow | 0.40 | Fuzzy, raised |
| St14 | Pale pink, white margins | Pink | 0.80 | Raised, fuzzy |
| St17 | Light grey to white | Cream | 0.30 | Raised |
| St19 | Pale pink, white margins | Cream | 0.50 | Raised |
| St20 | Tan to white | Brownish | 0.50 | Powdery, flat |
| St21 | Grey to brown | Cream | 0.80 | Raised, powdery |
| St22 | White to light grey | Cream | 0.50 | Raised, fuzzy |
| St23 | White to pale brown | Cream | 0.40 | Flat, powdery |
| St29 | White | Grey | 0.40 | Aerial mycelium sparse |
| St30 | White to pale grey | Cream | 1.00 | Flat, fuzzy |

[a] : The colony size is measured from the average colony size of the isolate.

**Table 2** Spore-shape and branching patterns (microscopic morphology) of Actinobacteria isolates and their presumptive genera.

| Microscopic morphology | Isolates | Presumptive genera |
|---|---|---|
| Rod-shaped fragmentation | 20Ar4, 20Ar5, 21Ar1 | *Nocardiodes*, *Promicromonospora*, *Streptomyces* |
| Closed spirals | 13B1, 15B1, 15Br2, 22Ar1, St10, St11 | *Streptomyces*, *Dactylosporangium*, *Glycomyces*, *Micromonospora* |
| Open spirals | 21Ar11, 20C1, 21A8, St20, St23 | *Streptomyces*, *Nocardiopsis* |
| True branching | 21Ar2, 21Ar3, St4, St7, St17, St19, St22 | *Nocardia*, *Glycomyces*, *Nocardiopsis*, *Microbispora* |
| Long branching with spores | 20Ar1, 21A2 | *Micromonospora*, *Saccharomonospora* |
| Fragmentation into V,Y and rod-like shape | 21B2 | *Promicromonospora* |
| Sporangia and spiral formation | 21Ar14 | *Streptosporangium* |
| Straight filaments with spores on branches | 21Br2 | *Streptoverticillium* |
| Coccoid-shaped fragmentation | St1, St30 | *Actinomadura* |
| Fragmentation, spores on tips | St5, St12, St14, St21, St29 | *Saccharothrix*, *Kitasatosporia*, *Actinomadura* |

**Table 3** Biochemical test results for the tested Actinobacteria isolates.

| Isolate | Casein hydrolysis | Starch hydrolysis | Xanthine degradation | Urea hydrolysis | Catalase activity | $H_2S$ production | Indole production | Motility | Citrate degradation | Metabolism of glucose |
|---|---|---|---|---|---|---|---|---|---|---|
| 13B1 | - | + | - | + | + | - | - | + | - | F |
| 15B1 | + | + | - | - | + | - | - | + | - | F |
| 15Br2 | + | + | - | - | + | - | - | - | - | F |
| 20Ar1 | - | + | + | - | + | - | - | - | - | F |
| 20Ar4 | + | + | - | + | + | - | - | + | - | F |
| 20Ar5 | + | + | + | + | + | - | - | + | - | F |
| 21Ar1 | + | + | - | - | - | - | - | + | - | F |
| 21Ar2 | + | + | + | + | + | - | - | - | - | F |
| 21Ar3 | - | + | - | + | + | - | - | + | - | F |
| 21Ar11 | + | + | - | - | + | + | - | - | - | F |
| 21Ar14 | + | + | - | + | + | - | - | + | - | F |
| 20C1 | - | + | + | + | + | - | - | - | - | F |
| 21A2 | + | + | - | + | + | - | - | - | - | F |
| 21A8 | + | + | - | + | + | + | - | + | - | F |
| 21B2 | + | + | + | - | - | - | - | + | - | F |
| 21Br2 | + | + | - | + | - | - | - | + | - | F |
| 22Ar1 | + | + | - | - | - | - | - | - | - | F |
| St1 | - | + | - | - | - | - | - | + | - | F |
| St4 | - | + | + | - | - | - | - | + | - | F |
| St5 | + | + | + | - | - | - | - | + | - | F |
| St7 | + | - | - | - | + | - | - | + | - | F |
| St10 | + | + | + | + | + | - | - | - | - | F |
| St11 | - | + | + | + | - | - | - | + | - | F |
| St12 | + | + | - | + | + | - | - | + | - | F |
| St14 | - | + | - | + | + | - | - | + | - | F |
| St17 | + | + | - | - | + | - | - | + | - | F |
| St19 | + | + | + | - | - | - | - | + | - | F |
| St20 | + | + | + | - | + | - | - | + | - | O |
| St21 | + | + | - | + | + | - | - | + | - | F |
| St22 | - | + | + | - | - | - | - | + | - | F |
| St23 | - | + | + | + | + | - | - | - | - | F |
| St29 | + | + | - | + | + | - | - | + | - | O |
| St30 | + | + | - | - | + | - | - | + | - | F |

+: Positive results; -: Negative results; F: Utilization of glucose fermentatively; O: Oxidation of glucose

*Nocardiopsis*, *Promicromonospora*, *Saccharomonospora*, *Saccharothrix*, *Streptomyces*, *Streptosporangium* and *Streptoverticillium.*

We found that the most dominant species of Actinobacteria recovered from soil samples used in this study was the *Streptomyces* spp., with 8 isolates, as also observed by Grigorova and Norris (1990). However, several of the species found commonly in their study such as *Streptosporangium* spp., *Streptoverticillium* spp. and *Micromonospora* spp., were only isolated as single isolates in this study. We attribute the differences observed to their possible clustered growth at certain soil environments which may not have been sampled in the sampling exercise. The genus *Nocardiopsis* has four isolates, *Actinomadura* and *Saccharothrix* with three isolates each, and subsequently *Glycomyces*, *Nocardia* and *Promicromonospora*, with two isolates each. The remaining genera each consisted of a single isolate. The cultural characteristics of isolates in each genus are summarized in **Table 1**, while **Tables 2** and **3** summarize their morphological and biochemical observations, respectively.

## Antifungal potential by Actinobacteria

All 33 isolates screened against the pathogen *Fusarium* spp. for antifungal activity, showed positive results. Fifteen of the isolates were grouped as weak antagonists as they produced PIRG values less than 50%. The lowest PIRG value recorded was 8% by isolate 22Ar1. Moderate antagonists with PIRG values of 50 to 74%, were detected in 13 of the 33 isolates. Only five isolates were considered as strong antagonists as they produced PIRG values of more than 75%. The isolate with the most antifungal activity as indicated by the highest inhibition percentage was recorded by isolate 20C1 with 96%. The other four strong antagonists were isolates 15Br2, 21A2, St 5 and St 11, with 90, 78, 78, and 77% PIRG values, respectively. On agar plates, the inhibitory effect by 20C1 compared to a weak antagonist can be clearly observed (**Fig. 1**).

Despite the low number of Actinobacteria isolated, results from our antifungal assay revealed that 100% of the isolates have antifungal potential towards the crown rot pathogen (*Fusarium* spp.). This high percentile reaffirms the antibiotic-producing nature of Actinobacteria (Hirsch

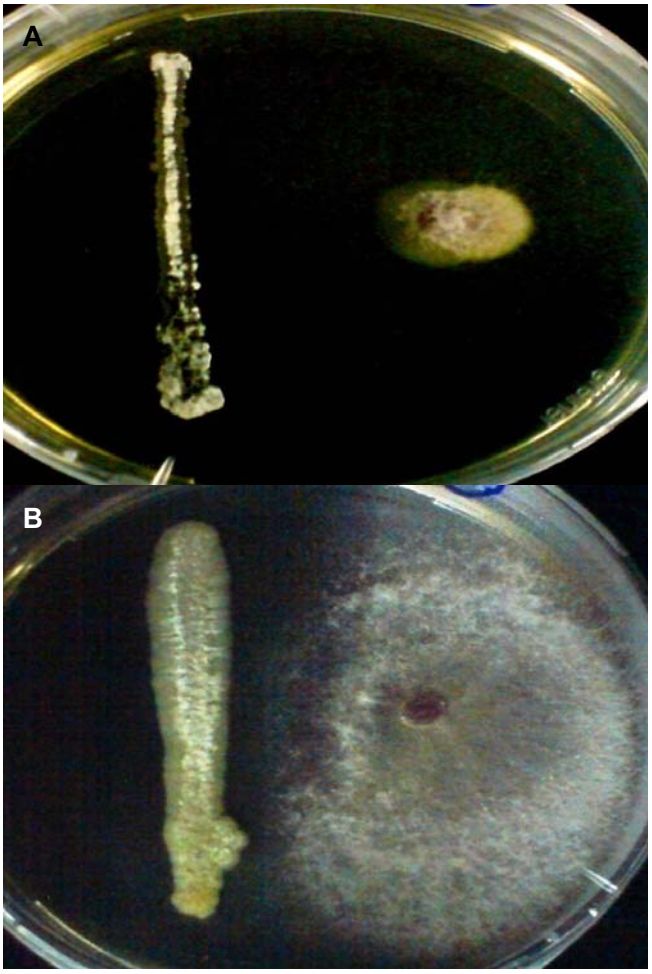


**Fig. 1** Isolate 20C1 showing strong inhibition towards *Fusarium* spp. (**A**) compared to isolates 22Ar1 with weak inhibition towards *Fusarium* spp. (**B**). Observation was conducted on day seven after co-incubation.

and Christensen 1983; Prapagdee *et al*. 2008), such that almost every single isolate recovered has the potential to produce beneficial inhibitory compounds. This also suggested that the recovery of beneficial Actinobacteria can be achieved successfully from sampling of common soils found ubiqui-tously in the environment.

The isolates with the highest values in PIRG, indicating strong antifungal potential, were identified as *Streptomyces* spp. (20C1, 15Br2), *Saccharomonospora* spp. (21A2), *Saccharothrix* spp. (St5) and *Glycomyces* spp. (St11). Although we did not investigate the type of antifungal compounds produced by these isolates, comparisons to literature search provided some explanations on the antifungal potential of the isolates. The antifungal potential of *Streptomyces*, *Saccharothrix* and *Glycomyces* are relatively more established than *Saccharomonospora*. *Streptomyces* are known to produce biologically active secondary metabolites such as chiti-nases and β-1,3-glucanases that lyses the cell walls of the fungal pathogen (Hopwood 2007; Prapagdee *et al*. 2008). This explains the high PIRG values derived from *Strepto-myces* spp. 20C1 (96%) and 15Br2 (90%), where mycelial growth of the *Fusarium* pathogen was severely inhibited. This showed that *Streptomyces* spp. also have immense pot-ential as biological control agents against agriculturally im-portant fungal pathogens such as the crown rot pathogen, despite the fact that they are more established as clinically important antibiotic producers.

Similarly, the antifungal potential of *Glycomyces* is attributed to the production of chitinases (Kawase *et al.* 2004). The antifungal potential of *Saccharothrix* spp. has also been documented, with reports suggesting several of its species such as *S. algeriensis* and *S. espanaensis* are capable of producing dithiolopyrrolone antibiotics (Zitouni *et al.* 2004) and oligosaccharide antibiotics such as saccharomicins A and B (Berner *et al.* 2006), respectively. Nevertheless, since we did not classify the species type of our *Saccharothrix* spp. St5, we can only confirm the antifungal potential of this isolate but unable to identify the antifungal compounds produced. Contrary, *Saccharomonospora* has been associated to pathogens causing lung infection in mushroom workers (Moore *et al*. 2004) thus may not be a suitable biocontrol agent for agricultural use despite the high PIRG value (78%) achieved in this study against the *Fusarium* pathogen.

To conclude, our study revealed that all the Actinobacteria isolates recovered from our soil samples were antagonistic towards the *Fusarium* crown-rot pathogen. Isolates *Streptomyces* spp. 20C1 and *Streptomyces* spp. 15Br2, has the most potential for future investigations and applications as biocontrol agents as both these isolates have high antifungal activity towards the pathogen. The nature of *Streptomyces* as well, ubiquitous in the environment, with good antibiotic-producing ability, favours the development of these isolates as biocontrol agents.

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