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Evaluation of antagonistic bacteria inhibitory to *Colletotrichum musae* on banana

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This aim of this study was to screen antagonistic microorganisms for their ability to inhibit the growth of *Colletotrichum musae*, the causal agent of anthracnose disease on banana fruits. Two hundred ninety-five isolates of antagonistic microorganisms were collected from healthy banana fruits and leaves. Fifty-six isolates showed some antagonistic activity in dual culture on PDA against *C. musae*. Eleven isolates produced zones of inhibition against *C. musae* on PDA. *In vivo* studies indicated that isolate MD1 and SPX showed the highest antagonistic activity. Percent anthracnose severity was reduced to 6% and 11% by isolates MD1 and SPX, respectively, compared to disease severity percentage of 100% for the respective controls. Comparisons of the ribosomal RNA sequences from the isolates MD1 and SPX against the nr-NCBI database showed a high level of homology with *Pantoea agglomerans* and *Enterobacter* sp., respectively.

Keywords: antagonistic microorganism, anthracnose, banana, *Colletotrichum musae, Enterobacter* sp., *Pantoea agglomerans*, biological control

Introduction

Banana (*Musa sapientum* Linn.) is a common plant in tropical and warm temperate regions with high levels of rainfall. Banana species are involved not only in traditional culture but also in the daily life of Thai people. The banana is regularly exported to many countries; the export value of banana 2012 for Thailand was 57.2 million bath (Center for Agriculture Information Office of Agricultural Economic, Ministry of Agriculture and Co-0perstive, Thailand, 2012). The world's demand for banana increases annually (Food and Agriculture Organization of the United Nation, 2006). The control of damaging fungal disease in banana fruit, especially anthracnose, relies mainly on the use of synthetic fungicides. The frequent use of these fungicides may ultimately result in fungicide-resistant pathogens.

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In addition, there is also a potential problem of fungicide-contamination of both fruits and the environment. Thus, there is an obvious and increasing need for alternative control strategies.

Research on the use biological controls as alternatives to synthetic fungicide treatments has been conducted for decades (Pal and McSpadden, 2006). Recently, considerable success has been achieved using preharvest application of antagonistic microorganisms to control and postharvest disease in citrus (Laifeng *et al*., 2013). A variety of microbial antagonists have been reported to control several different postharvest pathogens on various fruits and vegetables (Chanchaichaovivat *et al*., 2007). Mechanisms which have been reported to play a role in the biocontrol activity of antagonistic microorganisms include competition for space and nutrient, activation of host defenses, and production of extracellular depolymerases which putatively act on pathogen cell walls (Pal and McSpadden, 2006). Competition for space and nutrients has been demonstrated as a major mechanism in the antagonism of biocontrol against postharvest fungal pathogens (Wojciech and Lise, 2002). The objective of the current research was to identify antagonistic microorganisms capable of reducing postharvest anthracnose in banana caused by *Colletotrichum musae*. Potential antagonistic microorganisms were isolated from banana fruit and studied for antagonistic activity against the pathogen.

Materails and methods

Experimental design

Microbial isolation

Banana fruits and leaves were harvested from the organically managed orchards in Chiang Mai province. Washings were conducted by shaking fruit or leaves in a flask of sterile distilled water (100 ml water per 10 g fruit or leave) on an orbital shaker for 10 min at 200 rpm. The washings were used for the isolation of microorganism antagonistic to *C. musae* by plating on potato dextrose agar (PDA). Serial tenfold dilutions of the washings were made and 0.1 ml of each dilution was spread on the medium. The plates were incubated at 25^{o}C for 48 h and the colonies were tentatively identified, selected and transferred to PDA slants and stored at 4 ^{O}C for further experimentation.

In vitro Screening of antagonistic microorganism by dual culture

Screening of the antagonistic microorganism was carried out by inoculating each isolates in dual culture with *C. musae* in Petri dishes containing PDA (Rahman *et al*., 2009). The dual culture method is shown in Figure 1. The microbial isolate was streaked on the medium in 90- mm-diameter Petri dishes, 20 mm from the dish edge. An 8-mm-diameter mycelial disk of *C. musae* was placed 30 mm from the opposite edge of the dish. The radial growth of the mycelium towards the microbial isolate strip was measured after incubation at room temperature (30 ± 2 °C) for 7 d. Three Petri dishes per treatment (isolate) were used and the percent inhibition of radial growth (PIRG) was recorded. PIRG was defined using the equation indicated below (Skidmore and Dickinson, 1976).

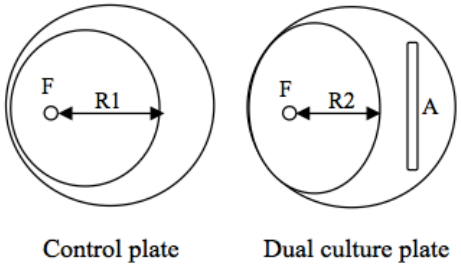


Fig. 1. Dual culture method illustrating measurement of radial growth of *C. musae* mycelia: A.- Putative antagonistic isolate F.- Fungal plug R1.- Control colony of radius R2.- Antagonist-treated colony radius

$$PIRG = \frac{R1 - R2}{R1} \times 100 \quad (1)$$

R1= average of colony radius of pathogen in control plate
R2= average of radial colony of fungus in dual culture plate

The microbial isolates with the highest percentage inhibition were selected as candidates for the *in vivo* study.

In vivo biocontrol efficacy of microbial antagonists

The biocontrol activity of selected isolated against *C. musae* was tested on fruit. Cultures of microbial antagonists were grown on PDA for 48 h at 25 °C. The banana fruits were surface sterilized with 70% ethanol and wounded 3 mm deep three times at each of three sites with sterilized needles (Figure 2). Twenty ml of a 10^8 CFU ml^{-1} sterile water suspension of selected antagonistic microorganisms or sterile distilled water (control) were applied to each wound

site. After 24 h, 20 ml of an aqueous suspension of conidia of the pathogen *C. musae* (1 x 10^6 conidia ml^{-1}) were used to challenge-inoculate each wound site.

Treated banana fruits were placed in paper boxes at room temperature (30° ± 2 °C) under humid conditions. The percentage of infected wounds and lesion diameter were determined 7 d after inoculation. Severity percentage was recorded using the equation below.

$$\% \text{ Severity} = \frac{\text{LdA}}{\text{LdC}} \times 100 \qquad (2)$$

LdA = Average lesion diameter in wounds treated with antagonist, prior to inoculation with a pathogen
LdC = Average lesion diameter in control inoculated wounds

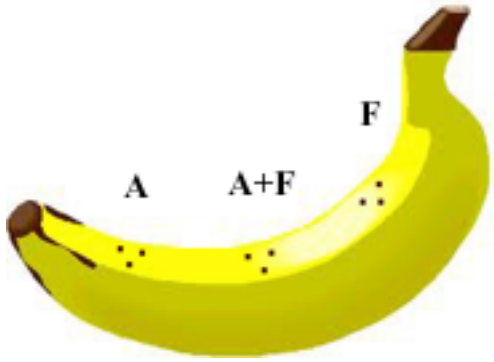


Fig. 2. Three sets of three puncture wounds were made on banana fruit: A. Antagonist A+F. Co-inoculation of antagonist and fungus (*Colletotrichum musae*) F. Fungus

Antagonistic microorganism identification

The potential antagonistic micro-organisms were identified by conventional morphological characteristic and molecular techniques. Antagonistic bacteria was sent to Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology, *Faculty of Science*, *Mahidol University*, Bangkok, Thailand for DNA identification.

Results and discussions

Screening microbial antagonists in vitro by dual culture

Two hundred ninety-five microbial isolates were collected from healthy banana fruits and leaves. All these epiphytic isolates were screened against *C.*

musae (data not shown). Fifty-six of these isolates showed some antagonistic activity against *C. musae* in dual culture (data not shown). Forty-five isolates exhibited rapid growth on the medium which blocked the growth of the fungal pathogen (Figure 3A). Eleven isolates produced distinct zones of inhibition against *C. musae* (Figure 3B). The five most effective antagonists are listed in Table 1.

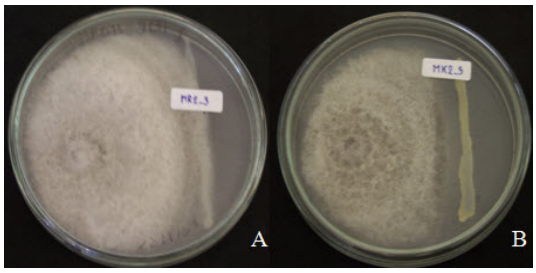


Fig. 3. Antagonistic activity in dual culture on PDA: A. Microbial antagonist blocked the growth of *Colletotrichum musae.* B. Microbial antagonist produced a zone of inhibition against the pathogen.

Table 1. Effect of antagonistic bacteria on the growth of *Colletotrichum musae* in dual PDA culture

Isolate	Interaction pattern		PIRG[1]
	Growth blocked	Zone of inhibition	
MR		+++++	58.22
SPX		+++++	56.89
DSK		++++	53.17
MD1	+++		35.34
SS4	+++		32.76

[1]PIRG = Percent inhibition of radial growth

*Inhibition zone: - no inhibition zone; + (very weak),0-10 mm; ++ (weak), 10-20 mm; +++ (moderate), 20-30 mm; ++++ (strong), 30-40 mm; +++++ (very strong), > 40 mm

In vivo biocontrol efficacy of microorganisms against Colletotrichum musae

The microbial isolates, which showed antagonistic activity were further tested in biocontrol experiment against *C. musae* in banana fruit 7 d after incubation. Isolates MD1 and SPX showed the highest antagonistic activity (Figure 4). Anthracnose symptoms were clearly detected 4 d after incubation. The percentage of severity of disease controlled by MD1 and SPX were 6% and 11% by isolates MD1 and SPX, respectively, compared to disease severity percentage of 100% and 100% for the respective control as shown in Table 2.

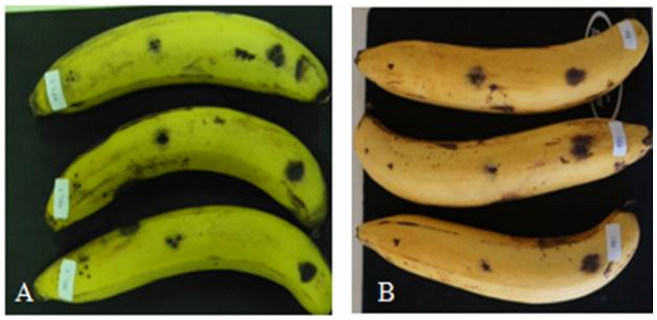
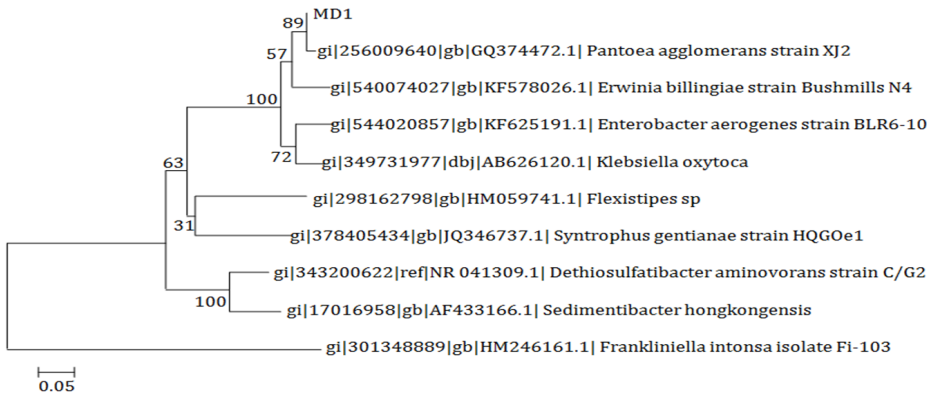


Fig. 4. Bacteria isolates SPX (A) and MD1 (B) showed the highest disease suppressive activity 7 d after inoculation with *Colletotrichum musae*

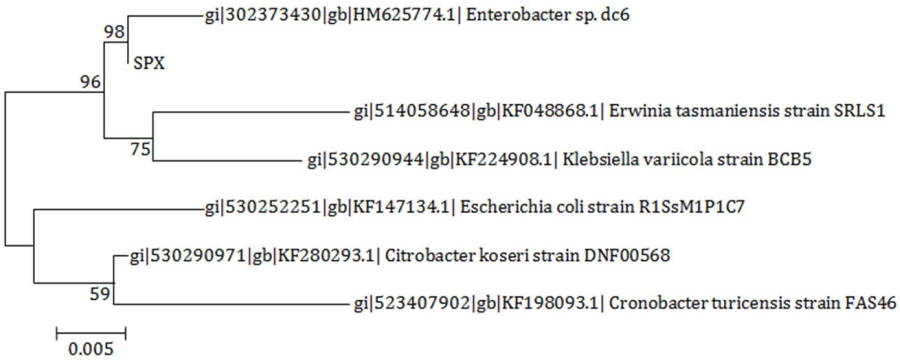
Table 2. Effect of bacterial isolates on anthracnose in banana.

Isolate	Disease severity (%)
MD1	6.3[a]
SPX	10.8[b]
MR1	26.6[c]
MT4	31.1[d]
DSK	39.4[e]

(Statistic: Anova $p<0.05$)



A



B

Fig. 5. A. Alignment of the 1405 bp amplified sequence from the isolated mcroorganism show 99% similarity with the 16S ribosomal RNA gene from *Pantoea agglomerans* strain XJ2 (GeneBank accession no. GQ 374472.1) B. Alignment of the 1394 bp amplified sequence from the isolated microorganism show 100% similarity with the 16S ribosomal RNA gene from *Enterobacter* sp. dc6 (GeneBank accession no. HM 6257741)

Identification of antagonistic microorganisms

BLAST comparisons of the ribosomal RNA sequences from the isolates MD1 and SPX against the nr-NCBI database showed a high homology with *Pantoea agglomerans* and *Enterobacter* sp. respectively. The 1405 bp amplified sequence of MD1 was compared with the 16S ribosomal RNA gene from *Pantoea agglomerans* strain XJ2 (GeneBank accession no. GQ 374472.1), showing 99% similarity (Fig. 5A). The 1394 bp amplified sequence of SPX was compared with the 16S ribosomal RNA gene from *Enterobacter* sp. dc6 (GeneBank accession no. HM 6257741), showing 100% similarity (Fig. 5B).

Discussion

The initial *in vitro* screening of isolated microorganisms provided information on possible modes of action which lead to control of *C. musae*. An efficient microbial antagonist ideally operates through different control mechanisms, which may include mycoparasitism, antibiosis, competition for space and nutrients, and ability to induce resistance in their hosts (Wilson and Wisniewiski, 1995). We selected and tested a number of antagonistic bacteria, which proved to be effective against the most important post-harvest pathogen of banana fruit. Two effective antagonists against *C. musae* were *Enterobacter* sp. and *Pantoea agglomerans*. However, some reports indicated that *Pantoea agglomerans* can be a disease agent; additional research is necessary to confirm that the bacterium is safe to use as a biocontrol agent in banana. On the other hand, the results of the *in vitro* and *in vivo* assays showed that *Enterobacter* sp.

also caused a significant reduction in the progress of the anthracnose disease in banana. Future studies will investigate the biological control activities and the antagonist mechanisms of *Enterobacter* sp. in inhibiting the growth of *C. musae.*

Acknowledgements

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