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Control of the Banana Anthracnose Pathogen Using Antagonistic Microorganisms

Khleekorn, S.[1*], McGovern, R. J.[2] and Wongrueng, S.[1]

[1]Division of Biotechnology of Agro-Industry, Faculty of Graduated School, Chiang Mai University, Chiang Mai 50200, Thailand; [2]Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand.

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Abstract This study aimed to determine the biological control abilities of selected antagonistic microorganisms to inhibit the growth of *Colletotrichum musae*, the causal agent of anthracnose disease found in banana fruits. In previous studies, *Pantoea agglomerans* and *Enterobacter* sp. showed effectiveness in controlling the anthracnose disease. It was found that the maximal germination rate of *C. musae* was 93.35 % at 31 °C after 35 h. The inhibition of the conidial germination of *C. musae* by *P. agglomerans* and *Enterobacter* sp. occurred 1 h after incubation at 31 °C. At 50 % mixture of *P. agglomerans* or *Enterobacter* sp. with PDA showed effectiveness in inhibiting conidial germination by 97.23% and 88.05%, respectively, and the capability to inhibit the mycelial growth of the fungus by 99.61 % and 99.23 %, respectively. The study of direct parasitism showed evidence of antagonist cells around the fungal hyphae, but it did not show that the hyphae became irregular in shape or twisted. Based on the *in vivo* study, a selected *Enterobacter* sp. antagonist applied on a banana every week before harvest showed the most effectiveness in controlling anthracnose, with 87.6 % inhibition.

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

Introduction

The control of fungal diseases in banana fruits, especially anthracnose, still relies mainly on the use of synthetic fungicides. Thus, there is an obvious and increasing need for an alternative control strategy. Recently, a biological control was developed as an alternative to synthetic fungicide treatment. Among different biological approaches, the use of microbial antagonists like yeast, fungi, and bacteria is quite promising and gaining popularity (Droby, 2006, Korten, 2006, Wisniewski and Wilson, 1992). Several modes of action have been suggested to explain biological control activity against plant pathogens on fruits and vegetables including direct parasitism, induced resistance, competition, etc. (El-Ghaouth *et. al*., 2004, Janisiewicz *et al*., 2000). This study investigated the biological control activities of *Enterobacter* sp. for inhibiting the growth of *C. musae*.

Methodology

Fungus isolation

Colletotrichum musae was isolated selectively from anthracnose lesions of banana fruits in our previous work (Khleekorn and Wongrueng, 2014). Identification of the fungal isolate was carried out microscopically according to taxonomic keys (Sutton, 1980) with molecular confirmation (Jantaporn, 2004).

Antagonistic microorganism isolation

The selected microorganism antagonists in the previous study were the isolates MD1 and SPX, which showed the highest antagonistic activity. BLASTIN comparisons of the ribosomal RNA sequences from the two isolates against the nr - NCBI database showed significant homology with *P. agglomerans* and *Enterobacter* sp., respectively. The colonies were stored on nutrient agar (NA) slants at 4 °C for further experimentation.

Conidial germination of C. musae

The fungus conidial concentration was adjusted to 10^6 conidia/ml by haemocytometer. The percentage of conidial germination was determined microscopically by placing 20 µl of a spore suspension of *C. musae* and 20 µl of PDB (Potato Dextrose Broth) onto a slide covered by cover slip. The percentage of conidial germination was recorded every hour until the germination rate dropped. Three replications of one hundred conidia were used.

Antagonistic inhibition of conidial germination of C. musae

P. agglomerans and *Enterobacter* sp. were cultured in 100 ml of PDB for 48 h at 180 rpm. Twenty microliters of each sample were centrifuged at 6000 rpm for 10 min. Twenty microliter of supernatant were dropped onto a slide together with 20 µl of *C. musae* inoculum at 10^6 conidia/ml. There were three replicates of one hundred conidia. The percentage of conidial germination inhibition (PCGI) was recorded.

PCGI was defined as: $PCGI = \frac{C - T}{C} \times 100$

C = The number of germinated conidia in control treatment
T = The number of germinated conidia in the antagonist treatments

The efficiency test of antagonists by the poisoned agar technique

P. agglomerans and *Enterobacter* sp. were cultured in 250 ml of PDB at 180 rpm. After 48 h, each antagonist culture was poured into 75% and 50% of PDA when the temperature of PDA was less than 45 ^{o}C. A 15 ml mixture of PDA and antagonists was poured into petri dishes. The experiments tested the inhibitory activity of the antagonists on conidial germination and growth of fungus. Conidial germination was measured by spreading 0.1 ml of a 10^2 conidia/ml suspension on PDA containing 25% and 50% antagonist inocula in the petri dish. The growth of *C. musae* was measured by putting a 5-mm-diameter agar plug of the fungus on PDA containing 25% and 50% antagonist inocula in the petri dish; fungal growth was compared with the non-treated control. There were three replicates of five plates per treatment. The percentage of inhibition was recorded.

$$\text{The percentage of inhibition} = \frac{(A-B) \times 100}{A}$$

A = The diameter of the fungal colony in the control treatment
B = The diameter of the fungal colony in the antagonist treatments

Study of direct parasitism of C.musae in vivo by Enterobacter sp.

The banana fruit was inoculated simultaneously with *Enterobacter* sp. and *C. musae* and incubated at room temperature (30 ± 2 °C) for 7 d. Samples from fruit showing fungus-antagonist interaction were removed and observed by scanning electron microscopy (Jantaporn, 2004).

Effect of preharvest application of antagonistic bacteria in the field on anthracnose

Enterobacter sp. was applied in selected test banana fields during flowering to harvest in December 2012 – March 2013. Ten milliliters volume containing 10^8 CFU ml^{-1} of *Enterobacter* sp. was sprayed on banana flowers weekly, biweekly and monthly; sterilized distilled water provided the control. Five banana trees were used in each treatment. After harvesting, ten banana fruit samples were taken from each treatment and incubated in paper boxes at room temperature for 7 d for disease assessment. The experimental was analyzed by one-way ANOVA.

Results and discussion

In a previous *in vivo* study, isolates MD1 and SPX had the highest antagonistic activity (Khleekorn and Wongrueng, 2014). BLASTIN comparisons of the ribosomal RNA sequences from the isolates MD1 and

SPX against the database showed significant homology with *P. agglomerans* and *Enterobacter* sp., respectively.

Conidial germination of C. musae

Conidial germination was observed under a light microscope. It was found that the germination started at 1 h incubation. The maximal germination rate was 93.35 % after 35 h incubation, as shown in Figure 1. The percentages of conidial germination of *Colletotrichum gloeosporioides* and *Dothiorella aromatica* were 89% and 99% after 24 h at 30 °C, respectively (Denner *et al*., 1986).

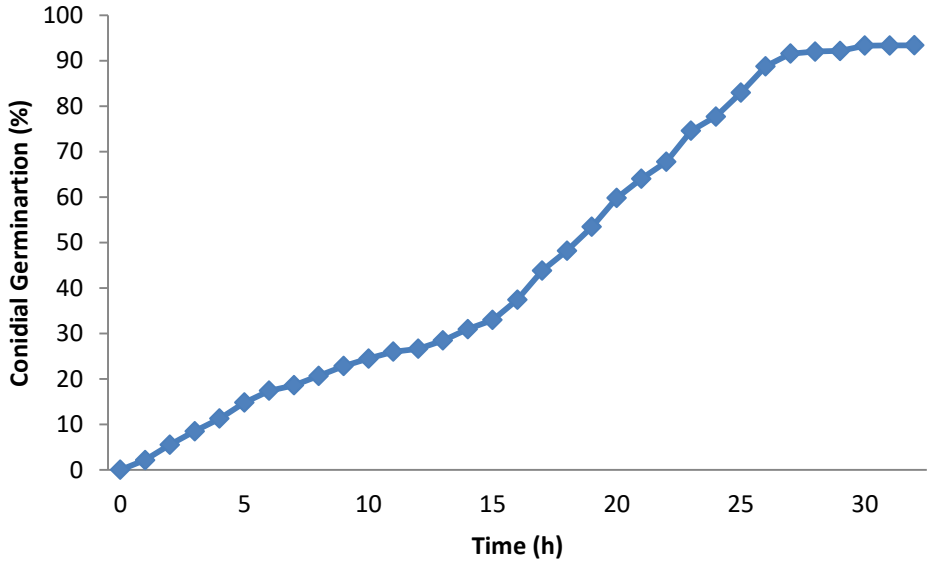

Figure 1. Percentage of conidial germination of *C. musae*

Antagonistic inhibition on conidial germination of C. musae by P. agglomerans and Enterobacter sp.

The inhibition of conidial germination by *P. agglomerans* and *Enterobacter* sp. began after 1 h inhibition and the inhibition rate increased until the maximum germination rate of fungus was reached. Both *P. agglomerans* and *Enterobacter* sp. were able to inhibit conidial germination of the pathogen. *Enterbacter* sp. was shown to produce the most rapid inhibition of conidial germination compared with *P. agglomerans* in the first 6 h. The maximum inhibition occurred at 6 h for *Enterobacter* sp., and at 24 h for *P. agglomerans*, as shown in Figure 2.

Acacia albida, *Prosopis juliflora*, and *Dovalis abyssinica* (plant extracts) showed potent antifungal activity in reducing the spore

germination of *C. musae* to 0.2, 0.5, and 0.3%, respectively (Bazie *et al*., 2014a). Hot-water treatments at 55 °C for a 5 to 17 min duration of exposure resulted in 100% inhibition of conidial germination of *C. musae* after 24 h incubation (Bazie *et al*., 2014b).

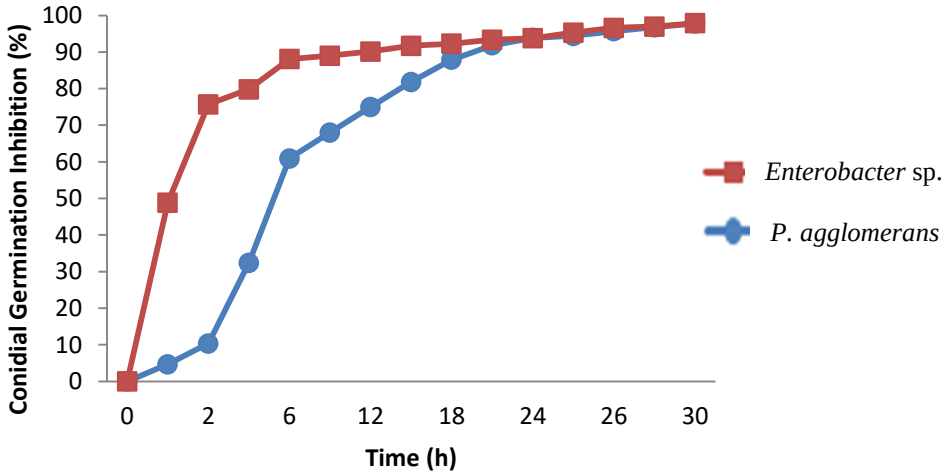

Figure 2. Antagonistic inhibition of the conidial germination of *C. musae* by *P. agglomerans* and *Enterobacter* sp.

Evaluation of antagonistic activity by the poisoned agar technique

At a rate of 50% *P. agglomerans* and *Enterobacter* sp. very effectively inhibited conidial germination of *C. musae* by 97.23% and 88.05%, respectively (Table 1). At the same rate both *P. agglomerans* and *Enterobacter* sp., were also effective in inhibiting the mycelial growth of the fungus by 99.61 % and 99.23 %, respectively. Figures 3 and 4 present images of conidial germination and mycelial growth inhibition of *C. musae* by the bacterial antagonists.

Crude Extracts of *Allamanda cathartica* 'Alba', *A. cathartica* 'Jamaican Sunset' and *A. blanchetti* showed potent inhibitory effects against *C. gloeosporioides* and they gradually suppressed mycelial growth of the fungus by up to 72%, 72%, and 70%, respectively as the concentration increased to 7 mg/ml (Haron *et al*., 2013). Control of anthracnose of banana using 10% Arabic gum with 1% chitosan which are edible composite coatings inhibited conidial germination up to 92.5% (Maqbool *et al*., 2010a). Cinnamon oil at a concentration of 0.4% inhibited conidial germination inhibition up to 83.2% (Maqbool *et al*., 2010b).

Table 1. Effect of *P. agglomerans* and *Enterobacter* sp. on the conidial germination and mycelial growth inhibition of *C. musae* using the poisoned food technique

Isolate	Percentage of conidial germination inhibition		Percentage of mycelium growth inhibition	
	25%	**50%**	**25%**	**50%**
P. agglomerans	72.14^{b*}	97.23^{a}	61.40^{c}	99.61^{a}
***Enterobacter* sp.**	64.69^{c}	88.05^{ab}	57.17^{d}	99.23^{a}

*Means within columns followed by the same letter are not significantly different by ANOVA (P<0.05) .

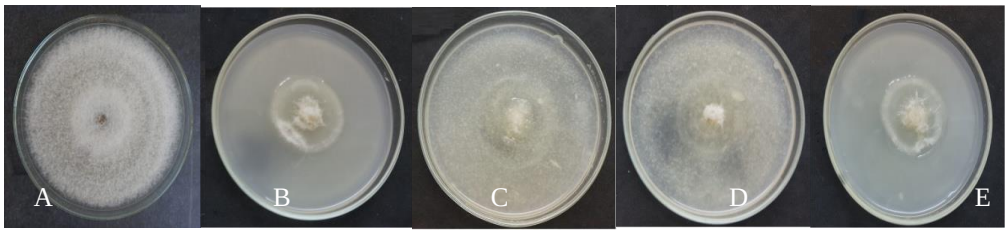

Figure 3. Inhibition of fungal mycelium on PDA containing 25% (B) and 50% (C) *P. agglomerans* and PDA containing 25% (D) and 50% (E) *Enterobacter* sp. inocula compared with the control (A)

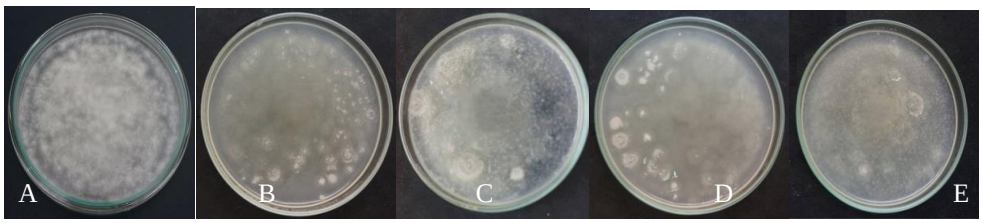

Figure 4. Conidial germination inhibition on PDA containing 25% (B) and 50% (C) *P. agglomerans* and PDA containing 25% (D) and 50% (E) *Enterobacter* sp. inocula compared with the control (A)

Study of direct parasitism of C. musae in vivo by Enterobacter sp.

After incubation for 7 d, electron microscopy revealed that the bacterial cells surrounded banana tissue and fungal hyphae, but it did not show that the fungal hyphae became irregular shaped or twisted (Figure 5). On the other hand, other researchers demonstrated by scanning electron micrographs that a crude extract of *Streptomyces* sp. strain CF-1 potent to *C. musae* caused mycelial deformation typified by thickened and bulbous structures (Ara *et al*., 2012). These researchers also indicated that potent antagonists inhibited *C. musae* by releasing extracellular diffusible

metabolites that inhibited hyphal growth. Scanning electron micrograph of antagonistic yeast cells of *Pichia anomala* Moh 93 interacting with hyphae of *Botryodiplodia theobromae* on PDA after 4 d incubation showed the yeast colonization around the hyphal tips of the pathogen which were totally killed by penetration of the antagonistic yeast (Hashem and Alamri, 2009).

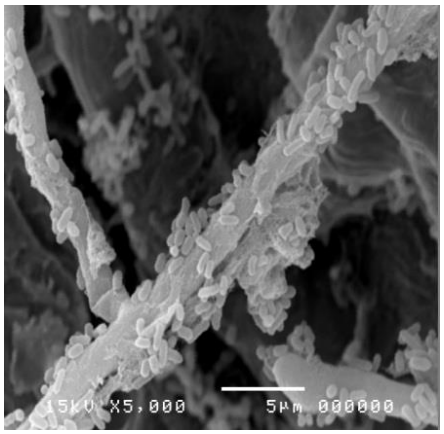

Figure 5. Interaction between *Enterobacter* sp. and *C. musae* in banana tissue observed under the electron microscope

Effect of preharvest application of antagonistic bacteria in the field on anthracnose

The effectiveness of *Enterobacter* sp. in controlling anthracnose in the field varied with the frequency of application. The antagonist applied preharvest on the banana fruit every week was the most effective treatment in controlling the anthracnose disease (Table 2).

Candida sake treatments of preharvest wounds significantly decreased both the incidence and lesion size of *Penicillium expansum* rot in apples compared to the untreated control. Severity and incidence of the disease were higher in untreated apples while the decay reduction on apples treated preharvest with *C. sake* was about 50% (Teixido *et al*., 1999). Sweet cherry sprayed with *Cryptococcus laurentii* before harvest showed effectiveness in controlling the postharvest decay of sweet cherry under storage conditions (Shi-Ping *et al*., 2004).

Table 2. The effect of applying *Enterobacter* sp. in the field on control of anthracnose disease

Frequency of antagonist applied	The percentage of inhibition
Control	72.9[b]
Applied every week	87.6[a]
Applied every 2 weeks	75.8[b]
Applied every 4 weeks	70.2[b]

Values followed by the same letter are not significantly different at $P < 0.05$, according to Tukey HSD and Scheffe's Test

Conclusion

Many post-harvest fungi initiate their infection cycle while the fruit are still in the field before harvest. In order to be effective in controlling a post-harvest disease, it is very important that the antagonist be applied to the fruit surface as early as possible, otherwise the fruit may be colonized either by post-harvest pathogens or resident microbial flora (Ippolito and Nigro, 2000). Therefore, it is very important to get the control agent established on fruit surfaces before the pathogen arrives at the infection site. Future studies will investigate the biological control activities and the antagonist mechanisms of *Enterobacter* sp. in inhibiting the growth of *C. musae*.

Acknowledgement

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