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Biological control of anthracnose disease on banana var 'Namwa Mali-Ong' by *Neosartorya* species

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Abstract The anthracnose disease caused by *Colletotrichum musae* on banana was controlled by biological control agents. Seven isolates of *C. musae* were collected from banana growing area in Suphanburi, Nakhonpathom, Ratchaburi and Nonthaburi province, Thailand. Pathogenicity was tested on fresh banana, and proved that isolates CMDC-01 and CMNM-01 were most aggressive isolates. These isolates were molecular phylogeny confirmed species. *In vitro* test was done by dual culture tecnique, two isolates of pathogen were tested against ten isolates of antagonistic fungi (*Neosartorya hiratsukae, N. pseudofischeri, N, aureola, N. spinosa, N. fenneliae, Talaromyces muroii* and *T. trachyspermus*). The result showed the best antagonistic isolate was *N. pseudofischeri* EU13 which inhibited *C. musae* CMDC-01 of 51.23% and *N. fenneliae* CHA03-A11 inhibited *C. musae* CMNM-01 of 59.85%.

Keywords: Biological control, Anthracnose, 'Namwa Mali-Ong' banana, *Neosartorya* and *Talaromyces*

Introduction

Most of the people in Thailand are farmers. So, the agricultural industry especially, the fruit and vegetable industry are important business that contributes to the economy. However, agricultural production is severely damaged by pests and plant diseases. In particular, plant diseases caused by fungi can quickly and severely damaged crops. Farmers are used chemicals for their control because chemicals are easier, faster and more effective to control the diseases (Mari *et al.*, 2009; Schirra *et al*., 2011; Zhu *et al.*, 2015).

Anthracnose disease caused by *Colletotrichum musae*, is one of disease that damages the banana. The pathogen is infested with unripe banana showing green and signs when the fruits is riped it become yellow. Symptoms of the disease are more severe as the fruit ripens because of the amount of sugar.

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Leison are small sunken black spot and extended wide. The size and shape of the lesion is uncertain. In optimum condition, orange spots are visible on the lesion. The famers often use chemicals such as thiabendazole, carbendazim and prochloraz sprayed or coated at the surface of banana fruit to inhibit the growth of pathogen. But excessive use of chemicals negatively affects to the environment, farmers and toxic residue in the product (Khan *et al.*, 2001; Mehdi, 2010; Alizadeh *et al.*, 2015; Zhu *et al.*, 2015).

The disease can be controlled without using chemical fungicides that has been reported as biological control. Recently, the successful measure has been achieved using antagonistic microorganism to control plant disease. There are many reseasches reported that in the biocontrol activity of antagonistic microorganism such as competition for space and nutrient, activation of host defenses, and production of substance to inhibit the pathogens (Soytong and Quimio, 1992). Many researches reported that using antagonistic fungus to control anthracnose disease in various fruits such as *Neosartorya fischeri, N. glabra, N. hiratsukae, N. takakii,* and *N. tatenoi* to control anthracnose disease in chilli caused by *Colletotrichum capsici* (Eamvijarn *et al.*, 2009). *Talaromyces trachyspermus* EU09 and *T. muroii* EU04 can inhibit growth of *C. coffeanum* cause of anthracnose disease in coffee (Soytong and Poeaim, 2015). The objective was to isolate *Colletotrichum musae* from banana growing area in Suphanburi, Nakhonpathom, Ratchaburi and Nonthaburi province, Thailand. The pathogen was isolated, morphological and molecular phylogenetic confirmation. The potent antagonistic fungi *Neosartorya* and *Talaromyces* were tested to control the anthracnose pathogen.

Materials and methods

Isolation and identification of fungal pathogens

Colletotrichum musae was isolated from anthracnose lesions of banana fruits by tissue transplanting technique. Banana fruits with disease symtoms were collected from growing banana area in Suphanburi, Nakhonpathom, Ratchaburi and Nonthaburi province, Thailand. Infected areas were washed with sterile distilled water, cut to 5 mm diameter and surface sterilized with 1% sodium hypochlorite for 30 second and washed tree times with sterile distilled water. The washed tissue was transferred to Potato Dextose Agar (PDA) and incubated at room temperature. After incubation, cut the fungal hyphal tip from infected tissue transferred to new PDA and purified by tranfered single spore to new PDA. Identify species of pathogen isolates by morphological observation and molecular data (Sutton, 1992; Nuangmek *et al.*, 2008; Ismet *et al.*, 2012).

The pathogens were identified by morphological observation on character of lesion on fruits, colony, conidiomata, shape and size of conidia followed by Alizadeh *et al.* (2015). The pathogens were confirmed species by molecular data, dna extraction was modified the CTAB protocol from Doyle and Doyle (1990) and Suksiri (2018). The DNA templates were performed by sequencing the internal transcribed spacer (ITS) region and amplified by polymerase chain reaction (PCR) which modify the condition followed by White *et al*. (1990). The PCR products were performed to sequencing analysis by Bioneer Company, Korea. The sequences were identified species by aligning the sequence from database of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tools (BLAST).

Pathogenicity test

Pathogenicity test modified from Ismet *et al.* (2012), was done on healthy green 'Namwa Mali-Ong var.' banana fruits. Fruits were surface sterillized with sterile distilled water and four wounds were made on fruits by needle. The mycelium plug was corked by 0.5 mm diameter of cork borer and placed on each wound, use pure PDA plug for control. Three replications were made in each treatment (Figure 2A). The inoculated fruits were incubated in moisture chamber at room temperature for 5 days. *C. musae* was reisolated to PDA for further experiment.

Antagonist fungus

Neosartorya and *Talaromyces* are soil-brone fungi, isolated by soil dilution technique, heat treatment and alcohol treatment from soil. Species of antagonistic isolates were identified by morphological and molecular data. Ten isolates of *Neosartorya* and *Talaromyces* were offered by Mayamor Soytong, Supanan Suksiri and Supattra Poeaim which deposited in our laboratory. Antagonistic isolate *Neosartorya hiratsukae* EU06, *Talaromyces muroii* EU07, *N. pseudofischeri* EU13, *T. muroii* EU18, *T. trachyspermus* EU23 and *Neosartorya* sp. EU35, were isolated from Chiangmai province, Thailand. *N, aureola* CHA01-A01, *T. muroii* CHA03-A03, *N. fenneliae* CHA03-A11 and *N. spinosa* CHA09-A01, were isolated from Chumpon province, Thailand.

Dual culture

The isolates of antagonistic fungi were selected from *in vitro* test by dual culture technique (Nuangmek *et al.*, 2008; Sakunyarak and Satithorn, 2014). The mycelium plugs, 0.5 mm diameter of *C. musae* from colony incubated at

25 ℃ for 10 days were dual culture with 0.5 mm diameter mycelium plug of antagonist isolates. The distance between pathogen and antagonist were approximately 4.5 cm. Four replications were made for each treatment. Dual culture plates were incubated at 25 ℃ for 25 days. After incubation, colony of *C. musae* was measured and compared with control. The percentage of growth inhibition (GI) was calculated by using the equation (1). Data were statistical analysed and means were compared by Duncan Multiple Range Test (DMRT) at *p*-values less than 0.05 significantly level.

$$GI\ (\%) = [(A - B)/A] \times 100 \qquad (1)$$

(A) *C. musae* colony in control plate, (B) *C. musae* colony in treatment plate

Results

Isolation and identification of pathogen

Seven 'Namwa Mali-Ong var.' banana samples were collected from Suphanburi, Nakhonpathom, Ratchaburi and Nonthaburi province, Thailand. The pathogen was isolated from anthracnose lesion (Figure 1A). Banana samples were isolated and yielded seven isolates of CMKP-01, CMKP-02, CMKP-03, CMNM-01, CMRM-01, CMNS-01 and CMDC-01. All isolates were identified by mycelium and colony morphology, conidia shape and size and development of conidiomata which observed after 10 days incubation at room temperature. The morphological characteristics of *C. musae* isolate CMDC-01 which is shown in Figure 1. All isolates were produced orange conidiomata on the lesions after 3 days incubation in moisture chamber (Figure 1B). The colony were white mycelium and turned to pinkish orange in color with age (Figure 1D and 1E). Conidia were hyaline cylindrical shape, size of conidia ranged from 3.45-5.32 × 10.12-14.60 µm (Figure 1C and 1F). All of seven isolates were morphogically identified as *Colletotrichum musae.*

The DNA sequences of each isolate was amplified with ITS regions. PCR product were sequenced and compared with *C. musae* sequences in NCBI databases. The nucleotide sequences were identified as *C. musae* based on molecular analysis and comfirmed with morphological identification. BLAST analysis of all isolates complied with 99% identity with reference of *C. musae* accession number AJ301904, JN121212, NR120132 and JN943076 in Genbank database.

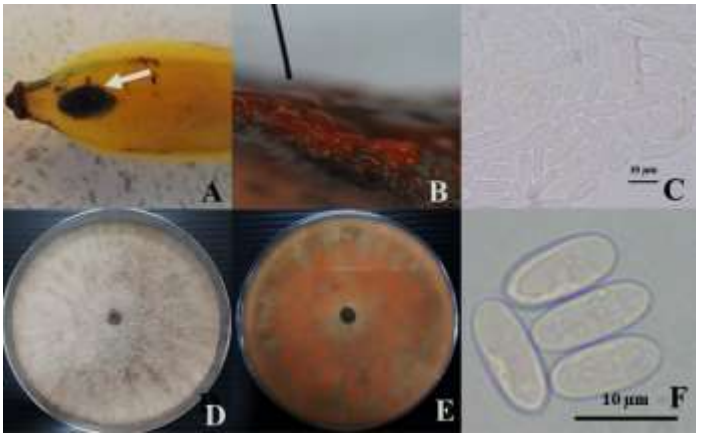

Figure 1. Morphological characters of *C. musae* isolate CMDC-01, (A) lesion on banana fruit, (B) conidiomata, (C and F) conidia and (D and E) colony on PDA after 10 days incubation

Pathogenicity test

Pathogenicity test was confirmed the pathogenic isolates which tested on 'Namwa Mali-Ong var.' banana fruits. The fruits were inoculated by *C. musae* isolate CMKP-01, CMKP-02, CMKP-03, CMNM-01, CMRM-01 CMNS-01 and CMDC-01. The fruits were showed symptoms of anthracnose after 3-5 days incubation in moisture chamber. Lesions were black, oval and sunken. After that, the lesions showed white mycelium and produced orange color of conidial masses (Figure 2B-2H). *C. musae* isolates of CMNM-01 and CMDC-01 made a largest lesion on fruits which were 24.18±0.98×33.81±2.96 mm and 23.46±2.24×30.66±3.32 mm, respectively. However, there was no significantly different in lesions on fruits which inoculasted CMNM-01 and CMDC-01 isolates, the lesions were 817.36 and 719.21 mm^2, respectively (Table 1).

Table 1. Lesion areas on 'Namwa Mali-Ong' banana fruits inoculated with seven isolates of *C. musae* after 5 days of incubation

Isolates	Lesion		
	Width (mm)*	Length (mm)*	Area (mm^2)
CMKP-01	16.57±3.34	24.35±4.09	403.45^{c}
CMKP-02	17.60±1.72	27.21±0.90	478.86^{c}
CMKP-03	18.02±1.99	26.19±2.59	471.97^{c}
CMNM-01	24.18±0.98	33.81±2.96	817.36^{a}
CMRM-01	15.41±2.48	28.81±2.25	443.81^{c}
CMNS-01	17.75±0.56	32.56±2.74	577.88^{bc}
CMDC-01	23.46±2.24	30.66±3.32	719.21^{a}

*Values expressed are mean ± SE

a-c means with different letters in the same column were significantly by ANOVA at P<0.05

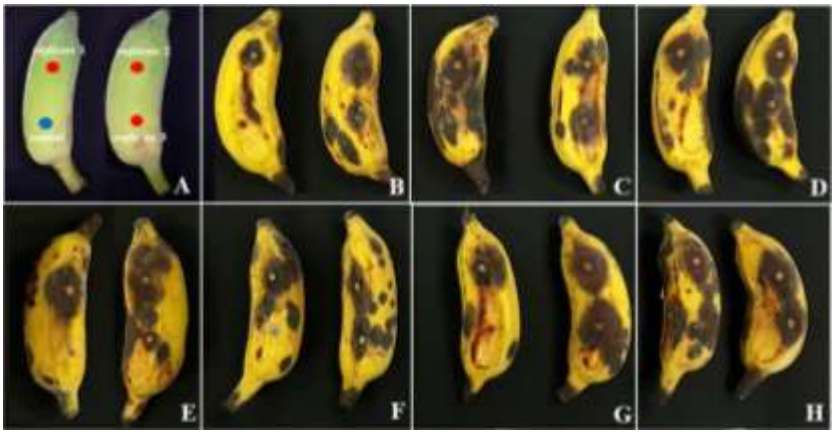

Figure 2. Lesion areas on banana fruits after pathogenicity test by isolates of *C. musae* (A) set of treatment in three replicates ● and control ● , (B) CMKP-01, (C) CMKP-02, (D) CMKP-03, (E) CMNS-01, (F) CMRM-01, (G) CMNM-01 and (H) CMDC-01

Effect of antagonist to control C. musae by dual culture test

The isolates CMNM-01 and CMDC-01 proved to be aggressive pathogenic isolates to banana. Dual culture was tested with ten antagonistic isolates. The colony diameter was masured after 20 days incubation. Result showed that antagonistic isolates EU13, EU06 and CHA03-A11 were strongly inhibited *C. musae* CMDC-01 of 51.23, 47.32 and 46.62%, respectively (Figure 3). The antagonistic isolates of CHA03-A03, EU06 and EU13 showed the highest effective isolates to control *C. musae* CMNM-01 which were 59.85, 50.33 and 46.92%, respectively (Figure 4, Table 2).

Table 2. Dual culture test of antagonistic fungi and pathogen

Isolates of antagonist	*C. musae* CMDC-01		*C. musae* CMNM-01	
	Colony diameter (mm)	Growth inhibition (%)	Colony radius (mm)	Growth inhibition (%)
Control	45.00	0.00	45.00	0.00
EU06	23.71	47.32	22.35	50.33
EU07	28.88	35.82	30.33	32.59
EU13	21.94	51.23	23.89	46.92
EU18	30.63	31.94	29.71	33.98
EU23	37.01	17.76	35.53	21.05
EU35	27.00	40.01	29.60	34.21
CHA01-A01	25.36	43.65	27.91	37.97
CHA03-A03	29.06	35.42	29.38	34.71
CHA03-A11	24.02	46.62	18.07	59.85
CHA09-A01	28.71	36.20	29.19	35.14

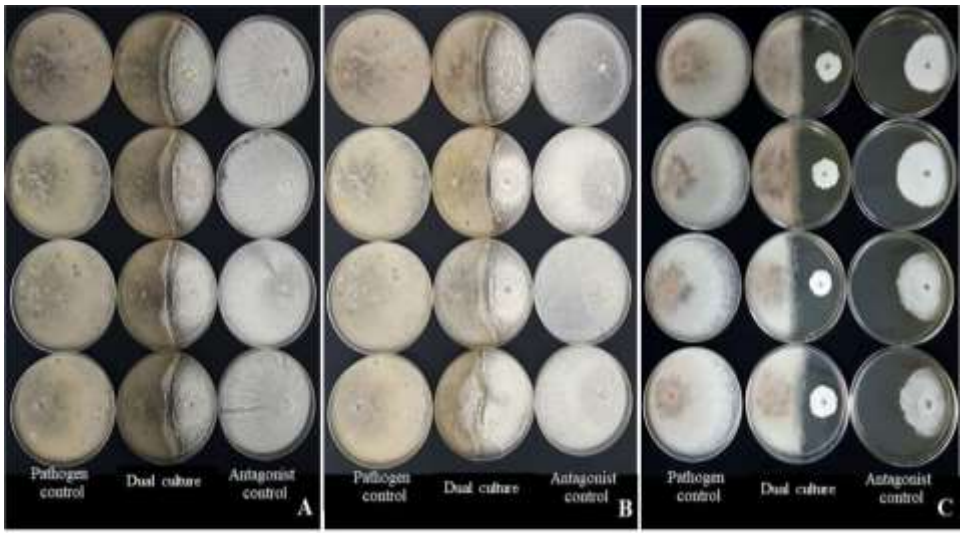

Figure 3. Antagonistic activity in dual culture test on PDA after 20 days incubation of *C. musae* CMDC-01 dual culture with (A) EU13, (B) EU06 and (C) CHA03-A11

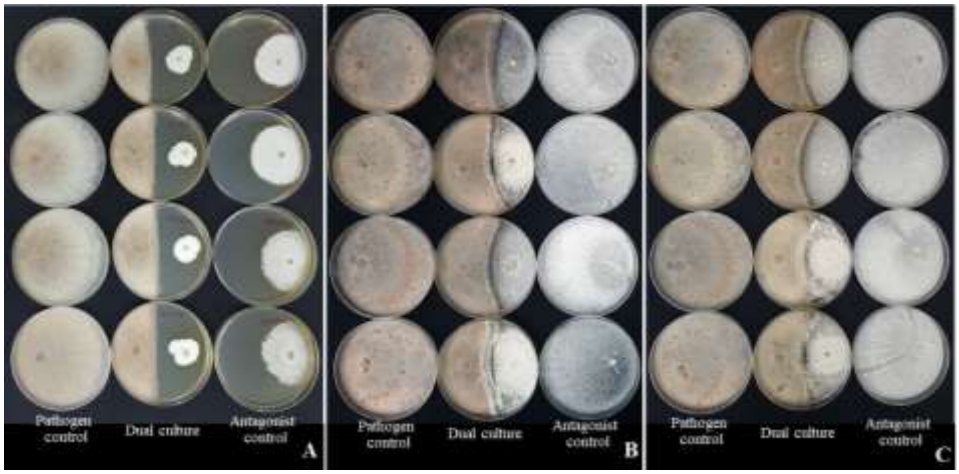

Figure 4. Antagonistic activity in dual culture test on PDA after 20 days incubation of *C. musae* CMNM-01 dual culture with (A) CHA03-A11, (B) th EU06 and (C) EU13

Discussion

Anthracnose of banana caused by *C. musae* that isolated from symptom showing black and sunken lesions and covered with orage conidiomata (Thangamani *et al.*, 2011). All the seven isolates showed hyaline cylindrical

conidia. The conidia size ranged in 3.45-5.32 × 10.12-14.60 µm and there were made a lesion was black and sunken on pathogenicity test. These characters agreed with Mehdi *et.al.* (2010) and Alizadeh *et.al.* (2015) also reported in the conidia size as 4.5-6 × 9.5-15 µm. The other species of *Colletotrichum* was a different shape and size of conidia. The average spore size of C. *capsici* causing anthracnose in chilli was 3.50 × 21.00 µm and sickle-shaped (Ghosh *et al.*, 2016). *C. gloeosporioides* were isolated from various host and conidia size was 3.5-6.0 × 12-17 µm, cylindrical shaped.

In this study, the antagonistic isolates EU13, EU06 and CHA03-A11 were strongly inhibited *C. musae* CMDC-01 as 51.23, 47.32 and 46.62%, respectively. The antagonistic isolates CHA03-A03, EU06 and EU13 showed highest anifungal activity against *C. musae* CMNM-01 which were 59.85, 50.33 and 46.92%, respectively. The result revealed that *N. hiratsukae* EU06, *N. pseudofischeri* EU13 and *N. fenneliae* CHA03-A11 gave the good control of both of tested isolates. Isolates EU06 and EU13 were actively against tested pathogenic isolates. Similar result from previous reports comfrimed *N. hiratsukae* were inhibited *Bipolaris maydis*, *C. capsici*, *C. gloeosporioides* and *Fusarium oxysporum* (Eamvijarm *et al.*, 2009). Crude ethyl acetate extract from *N. pseudofischeri* KUFA 0060 at 100 ppm was strongly inhibited *Phytophthora palmivora* and *C. capsici* (Boonsang *et al.*, 2014). The reserch finding, it is obiviously showed that *N. hiratsukae* EU06, *N. pseudofischeri* EU13 and *N. fenneliae* CHA03-A11 reported as the new antagonists to control banana anthracnose caused by *C. musae*. This study was similar to the report of *Chaetomium* spp. effectively against *Fusarium oxysporum* f.sp. *lycopersici*, stated that the control mechanism as antibiosis which produced *Chaetomium* produced antibiotic substance eg. chaetoglobocin-c to kill the pathogen cells (Soytong *et al*., 2001).

It is concluded that the biological control of banana anthracnose caused by *C. musae* using the effective antagonistic fungi. Isolates CMDC-01 and CMNM-01 were the highest pathogenic to infect the babana fruits. The antagonistic isolates *N. hiratsukae* EU06, *N. pseudofischeri* EU13 and *N. fenneliae* CHA03-A03 were storngly inhibited *C. musae* isolates CMDC-01 and CMNM-01.

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