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Identification of *Colletotrichum acutatum* and Screening of Antagonistic Bacteria Isolated from Strawberry in Chiang Mai, Thailand

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Abstract Strawberry anthracnose is a major disease of cultivated strawberry in the highland area in Chiang Mai, Thailand. This major disease of strawberry fruit is caused by *Colletotrichum* spp. The symptoms appear as water soaked lesions, which are covered with salmon−colored spore masses. Ten isolates of *Colletotrichum* spp. were collected from the field at Nonghoi Royal Project, Maehae Royal Project, Samoengs district and Suthep Royal Project Marketing store in Chiangmai, Thailand. The isolates were identified as *Colletotrichum acutatum* based on morphological characteristics and PCR analysis using specific primers. Pathogenicity tests on fresh strawberry fruit in the laboratory revealed that all the fungal isolates were pathogenic, but *C. acutatum* isolate CK21 resulted in the most severity symptoms. The isolate CK21 were effuse, first white later becoming orange, then turning into greenish grey as the cultures aged and later become cover with orange to salmon conidial masses and conidia were fusiform. In this study, a total of 105 microbial strains were isolated from fresh strawberry leaves and fruit. They were tested for growth inhibition of *C. acutatum* isolate CK21 by the dual culture technique on PDA. It was shown that the antagonistic bacterium isolate K27 was found to be the most effective in inhibiting the development *C. acutatum* isolate CK21 (66.25%). Isolate K27 was identified as *Bacillus subtilis*. The biocontrol was tested on strawberry leaves by using fresh cells of the bacterial antagonist in greenhouse experiments. The results showed that spaying 1 d before or after the potential the pathogen inoculation significantly suppressed anthracnose compared to the non-treated control. This study suggests of developing bacteria isolate K27 as a biological control of strawberry anthracnose disease.

Keywords: strawberry, anthracnose disease, antagonistic bacteria, *Colletotrichum acutatum*, *Bacillus subtilis*

Introduction

Strawberry (*Fragaria×ananassa* Duch.) belongs to the Rosaceae family is an important horticultural crop in many countries and also in the northern part of Thailand. Chiang Mai province is the center of the country's strawberry

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production (Doymaz, 2008). The Royal Project in Thailand developed the strawberry cv. Pharachatan 80 for commercial purposes (Narongchai *et al*., 2008). In 2005 to 2010, strawberry produced in Chiang Mai and Chiang Rai provinces had a high economic value of more than 200 million baht per year (Panid n.d.).

Anthracnose disease of strawberry fruit is caused by three species Colletotrichum: *C. acutatum* (Simmonds), *C. gloeosporoides* (Penz) and *C. fragariae* (Brooks) (Denoyes Rothan *et al*., 2003). Anthracnose of strawberry has been shown to be caused by: *C. acutatum* and *C. gloeosporoides* in Egypt (Embaby and Amany, 2013), Sorbia (Mirko *et al*., 2007); *C. acutatum*, *C. fragariae* and *C. gloeosporoides* in China (Xie *et al*., 2010); and *C. acutatum* in Thailand (Than *et al*., 2010). Anthracnose is a major disease of cultivate strawberry in China (Xie *et al.*, 2010). The pathogens cause yield losses in strawberry production in the field and post-harvest (Sreenivasaprasad and Talhinhas, 2005). The symptoms appear as water soaked sports, which become covered with pink or orange salmon–colored spore masses under humid conditions (Smith and Black, 1990).

At present, farmers heavily use fungicides in strawberry production. The misuse and overuse of such toxic compounds is hazardous to humans and environment. Biological control seems to be the best alternative to controlling of plant disease (Svetlana *et al*., 2010). Antagonistic bacteria have been shown to be efficacious as biological control agents (Irtwange, 2006, Sirinunta and Akarapisan, 2015). The mechanisms of biological control may be divided into antibiosis, enzymatic degradion, competition and induced resistance (Chaur, 1998). Antagonistic bacteria efficacious as biological control agents include the *Bacillus* group, which was found to be effective in preventing mycelial development of fungal pathogens (Donmez, 2011). Most of the literature on biocontrol activity of Bacillus has been analyzed separately. *Bacillus amyloliquefaciens* strain DGA14 produced extracellular metabolites in solid and liquid media that suppressed the growth of *C. gloeosporioides* causing anthracnose in mango cv. Carabao. The antagonistic bacteria were observed adjacent to the pathogen affecting its spore germination and mycelium development (Dionisio and Miriam, 2015). Nineteen *Bacillus* isolates were obtained from the rhizoplane and rhizosphere of wild and cultivated castor bean plants, to control the fungus *Macrophomina phaseolina*. These isolates were reported to produce siderophores and chitinase (Igor, 2015). Antagonistic strains such as *Bacillus lentimorbus*, *B. megaterium*, *B. pumilis* and *B. subtilis* were found to be effective in inhibiting the development of *Botrytis cinerea* on

strawberry fruit (Donmaz *et al.*, 2011). Therefore, the main purpose of this study was to identify isolates of *Colletotrichum* that cause anthracnose on strawberry fruit and select antagonistic bacteria for control of the pathogen.

Materials and methods

Fungal Isolation

The anthracnose pathogens were isolated from strawberry cv. Pharachatan 80 in the highland area in Chiang Mai, Thailand in 2014. The pathogen isolates were collected from fields at the Nonghoi Royal Project, Mae Hae Royal Project, Sa Moengs district and Su Thep Royal Project Marketing store. The pathogen was isolated from spore masses with the single spore isolation technique. Pure culture were cultivated on potato dextrose agar (PDA) at 25ºC.

Morphological examination

The pathogen isolates were identified as *Colletrotrichum* spp. based on conidia morphology by Than *et al*. (2008) and Xie *et al*. (2010). Colony diameter of culture was recorded on PDA at 25ºC. After 10 d, colony size and color of the conidial masses were recorded. For conidial measurements, the length and width were measured after 7 d on PDA at ×400 magnification (10× ocular, 40× objective) using bright field compound microscope with thirty conidia replications.

DNA extraction

Fungal isolates were cultured in potato dextrose broth (PDB) at room temperature (30±2ºC) for 7 d. DNA was extracted from all isolates using a modification of the protocol of Stewart and Via (1993). About 0.5 g of mycelia of fungal isolates were ground with 12 µl of 2–mercaptoethanol and 2400 µl of DNA extraction buffer (2% w/v CTAB, 1.42M NaCl, 20mM EDTA, 2% w/v polyinylpyrrolidone, 5mM citric acid and 100mM Tris-HCl pH 8.0. 500 µl of extracted solution was removed into a sterile 1.5 ml tube. Then 500 µl of a chloroform and isoamyl alcohol (24:1) solution was added and mixed. The solution was centrifuged at room temperature at 5000 rpm for 5 min. The upper aqueous phase containing the DNA was removed into a new sterile 1.5 ml tube.

The genomic DNA was precipitated using 0.7X isopropanol by centrifugation under room temperature at 14000 rpm for 20 min. Genomic DNA in TE buffer was visualized in 1% (w/v) agarose gels after ethidium bromide staining. DNA concentration was determined by gel electrophoresis.

PCR amplification

DNA amplification and sequencing were performed by Polymerase chain reaction (PCR). PCR primer for detection of the pathogen included the ITS4 primer (5′–TCCTCCGCTTATTGATATGC–3′) coupled with the specific primer for *C. acutatum* (*Ca*Int2) (5′–GGGGAAGCCTCTCGCGG-3′) and the ITS4 primer coupled with the specific primer for *C. gloeosporoides* (CgInt) (5′–GGCCTCCCGCCTCCGGGCGG–3′) (Sreenivasaprasad *et al*., 1996; White *et al*., 1990). *C. acutatum* and *C. gloeosporoide* specific PCR reactions were performed in a total volume of 25 µl, containing 50 ng of genomic DNA, 10X of PCR buffer, 10 mmol/L of each dNTP, 50mmol/L $MgCl_2$, 1 U of *Taq* DNA polymerase and 0.5 mmol/L of each primer. The reaction mixtures were incubated in a Peltier-based Thermal Cycler A100/A200 (LongGene Document Version 1.4). Following an initial denaturation at 95 °C for 4 min, the DNA templates were amplified for 35 cycles consisting of 1 min at 95 °C, 30 s at 58 °C, and 1 min at 72 °C. Amplification products were separated in 1% (w/v) agarose gels, and viewed under UV light by gel electrophoresis**.**

Pathogenicity Test

Fresh strawberry fruits were inoculated with a spore suspension of each *Colletrotrichum* isolate. Conidial concentration was determined using a haemocytomete. Fruit were inoculated by applying 20µl of a 1×10^6 conidia per ml spore suspension in sterile distilled water. Fruit were incubated at room temperature in a moist chamber with four replications. Control fruit were inoculated with 20 µl of sterile distilled water. A 1×10^6 conidia per ml suspension of the pathogen was applied to pin-pricked wounds in strawberry leaves and stalks. The most virulent Colletrotrichum isolate was used for further study.

Isolation of Antagonistic bacteria

The antagonistic bacteria were isolated from symptomless strawberry leaves and fruit by a washing technique using fresh leaves and fruit immersed in 100 ml sterile distilled water agitated on a shaker at 200 rpm for 2 h. The suspension were serially diluted to 10^{-1}, 10^{-2} and 10^{-3}, spread on nutrient agar (NA), and incubated at room temperature for 48 h. A single bacterial colony was selected and grown as a pure culture on NA.

Screening and Dual Culture Inhibition Assay

Initial screening of bacteria for maximum inhibitory activity against the mycelial growth of *Colletrotrichum* sp. was carried out using the dual culture technique on PDA. A 6 mm–diameter mycelial plug was obtained from the periphery of a 5–day–old colony of the fungus. Potential antagonistic bacteria were streaked 5 cm apart from the fungal pathogen and incubated at a temperature of 25ºC. The control plate consisted of a streak of sterile distilled water. The diameter of fungal growth were measured compared with the control and the experiment was repeated four times. The experiment was arranged using a complete randomized design (CRD) with four replications**.** The percentage inhibitions of the diameter of growth (PIDG) values were determined according to the following equation (Rahman, *et al*. 2007; Sariah, 1994):

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

Where, R1 = Radial growth of pathogen in control plate.
R2 = Radial growth of pathogen interacting with antagonistic bacteria.

Greenhouse Experiment

The experiment used a complete randomized design which was divided into five treatments (T1-T5). Fresh cells (1×10^8 cell per ml) of the most inhibitory bacterial antagonist were spayed 1 d before and after pathogen inoculation. A 40 µl drop of a 1×10^6 conidia per ml suspension of the pathogen

was applied to the wounds on strawberry leaves. The experiment was incubated at room temperature in moist chamber with four replications.

Results

Fungal Isolates

Ten isolates of *Colletrotrichum* spp. were isolated from strawberry cv. Pharachatan 80 in the highland area in Chiangmai, Thailand in 2014. This isolates were collected as follows: from fields at Nonghoi Royal Project (isolates CN1 and CN2), Maehae Royal Project (isolates CM1 and CM2), Samoengs district (isolates CS1 and CS2) and Suthep Royal Project Marketing store (isolates CK11, CK12, CK21 and CK22). The isolated were collected from plants exhibiting water soaked lesions, which they were covered with orange conidial massed on lesions under humid condition (Figure 1A).

Morphological examination

The colonies of *Colletotrichum* spp. were effuse, first white later becoming orange, then turning into greenish grey as the cultures aged and later became covered with orange to salmon conidial masses (Figure 1B). Conidia were fusiform, with dimension of 10.00–13.75 × 2.50–3.75 µm (Figure 1C). Therefore, *Colletotrichum* isolates was identified as *Colletotrichum acutatum*. Similar results spore masses. Conidia were fusiform, with dimensions of 13.00 × 3.5 µm.

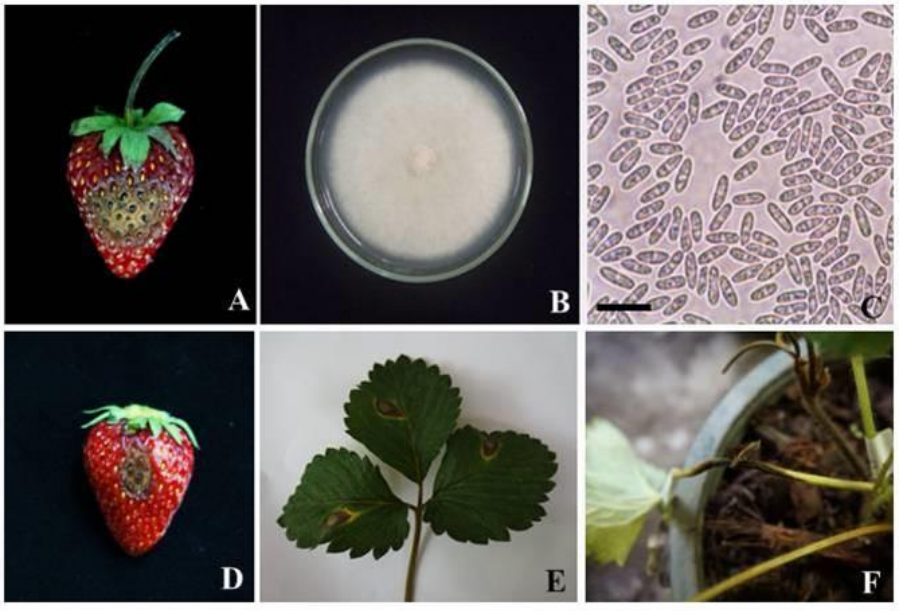

Figure 1. Antracnose symptoms and morphology of *Colletotrichum acutatum* on strawberry cv. Pharachatan 80 (A) Anthracnose symptom on strawberry fruit, (B) The colony of *Colletotrichum acutatum* isolate CK21 at 25^{o}C on PDA, (C) The conidia of *Colletotrichum acutatum* isolate CK21 under light microscope at 40X, Scale bar = 20 µm, (D, E and F) Symptoms that appeared after inoculation on strawberry stems fruit, leaves and stalks.

PCR amplification

The 10 isolates of *Colletotrichum* spp. from strawberry fruit were identified by using the *C. acutatum*–specific primer CaInt2 with the ITS4 primer about a 490–bp fragment and *C. gloeosporoides*–specific primer CgInt with the were obtained by Than *et al*. (2008), they isolated *C. acutatum* from strawberry from a local market in Chaingmai, Thailand. Colonies of *C. acutatum* produced white to pale gray colonies, sometimes with pinkish ITS4 primer about a 450–bp fragment, with the control *C. gloeosporoide*s isolated from coffee. This species-specific PCR results confirmed that the 10 isolates of *Colletotrichum* from strawberry fruit were identified as *C. acutatum*,

consistent with the morphological identification. About a 490–bp fragments were amplified from the genomic DNA of the *C. acutatum* isolates (Figure 2).

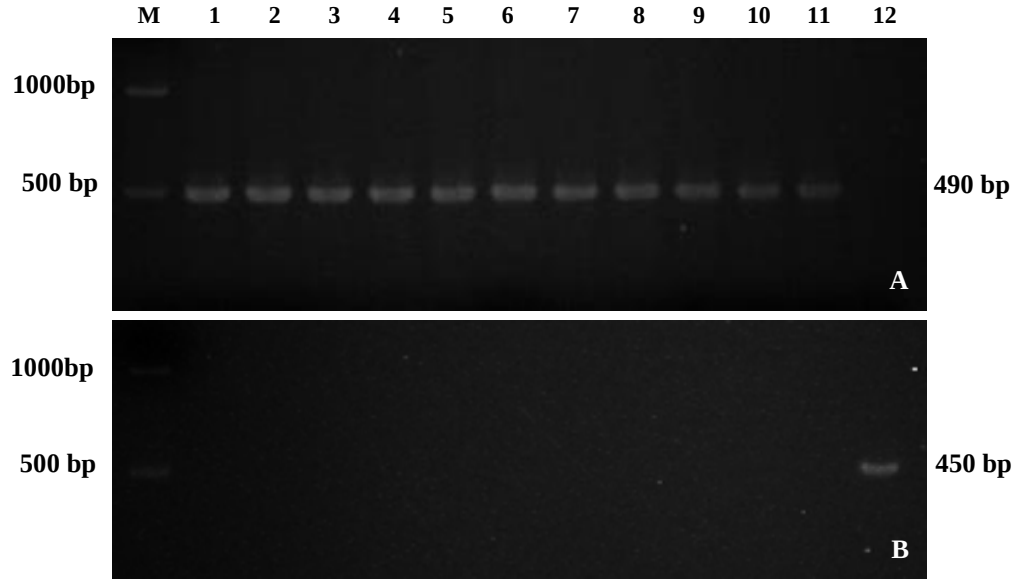

Figure 2. PCR amplification of a specific fragment from *Colletotrichum* species (A) using the *C. acutatum* specific primer CaInt2 in conjunction with the primer ITS4, (B) using the *C. gloeosporoides* specific primer CgInt in conjunction with the primer ITS4. Lanes 1-10 are ten isolates of *C. acutatum* from strawberry fruit (isolate CN1, CN2, CS1, CS2, CK11, CK12, CK21, CK22, CM1 and CM2, respectively); Lane 11 is *C. acutatum* from leaf strawberry; Lane 12 is *C. gloeosporoides* control from coffee; Lane M corresponds to the 250-10000 bp molecular weight marker 1Kb sharp (50 µg/ 500 µl).

Pathogenicity Test

Inoculation of the *Colletotrichum* isolates into strawberry fruits was done in the laboratory. The results showed that *C. acutatum* isolate CK21 caused the most severe symptom because it produced the widest lesion in the moist chamber at room temperature (30ºC±2). Therefore, the isolate was selected for further study. Inoculation with all isolates of *Colletotrichum* on wounded strawberry leaves and stalk produced typical anthracnose symptoms. The

symptoms on strawberry fruit appeared as water soaked lesions, covered with orange conidial masses under humid conditions. The symptoms on strawberry stalks appeared as water soaked lesions which turned brown (Figure 1D, E and F).

Screening and Dual Culture Inhibition Assay

A total of 105 bacterial isolates from fresh strawberry leaves and fruit were initially screened on NA using the dual culture technique. Inhibition assays found that five antagonistic bacteria isolates K18, K27, S15, S16 and S17, showed the most inhibition of the mycelial growth of *C. acutatum* isolate CK21. Bacterial isolate K27 was the most antagonistic producing a 66.25% inhibition of mycelial growth (Figure 3). Isolates S16, S17, K18 and S15 inhibited mycelial growth by 37.50%, 33.75%, 33.75% and 31.25%, respectively. K27 was identified as *Bacillus subtilis* using the Biolog bacterial databases.

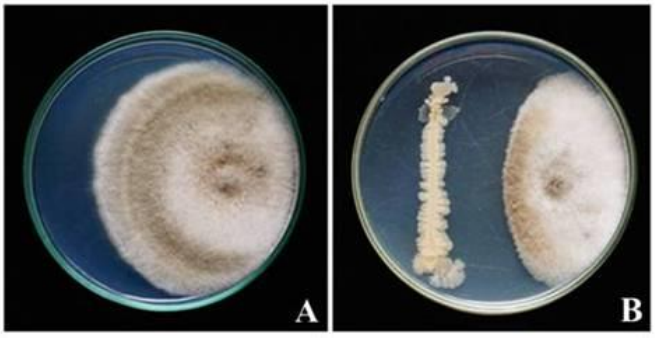

Figure 3. The effect of antagonistic bacterium *Bacillus subtilis* K27 inhibiting mycelial growth of Colletotrichum acutatum isolate CK21 in the dual culture technique at room temperature (30ºC±2) after incubation for 8 d on PDA. (A) control, (B) antagonistic isolate K27.

Greenhouse Experiment

The biocontrol was tested on strawberry leaves by using fresh cells of the bacterial antagonist in greenhouse experiments. Fresh cells of

isolate K27 spayed 1 d before and after pathogen inoculation (T4 and T5, respectively) reduced the severity of the anthracnose disease; and the diameters of the wounds were about 8.10 and 32.09 mm^2 respectively, when compared with the positive control of pathogen inoculation were found to be wound measuring 193.10 mm^2 in diameter (T3). The result obtained upon the treatment of the strawberry leaves had significance (p=0.05) In addition, the negative control of antagonistic bacteria suspension isolate K27 only (T2) did not effect of strawberry leaves when compared with the negative control (T1), which there were found to be wound measuring 0.00 mm^2 in diameter (Table 1).

Table 1. Effectiveness of *Bacillus subtilis* isolate K27 in reducing anthracnose disease on strawberry leaves at 10 d after inoculation

Treatment		**Diameter of wound (mm^2)***
T1	Negative control (applied with sterile distilled water only)	0.00^c
T2	Negative control (applied antagonistic bacteria K27)	0.00^c
T3	Positive control (pathogen inoculation only)	193.10^a
T4	Antagonistic bacterium K27 applied 1 d before pathogen inoculation	8.10^b
T5	Antagonistic bacterium K27 applied 1 d after pathogen inoculation	32.09^b
	LSD (p=0.05)	29.50
	CV (%)	36.68

*The average was calculated using data from four replications.
**The values within the table with different superscripts are significantly different (p=0.05).

Discussion

This study was conducted during 2014 and focused on strawberry anthracnose. Strawberry is cultivated extensively in some areas of Thailand including Chiang Mai and Chiang Rai provinces. Strawberry anthracnose has been prevalent for many years. In this study, we identified the *Colletotrichum* sp. causing strawberry anthracnose in northern Thailand as *C. acutatum* based on morphological characteristics and DNA sequence analyses using a species–specific (PCR). The colonies of *Colletotrichum* isolates were effuse, first white later becoming orange, then turning into greenish grey as the cultures aged and

later became covered with orange to salmon conidial masses. Conidia were fusiform. Similar results were obtained by Than *et al*. (2008), Colonies of *C. acutatum* produced white to pale gray colonies, sometimes with pinkish spore masses. Conidia were fusiform. Xie *et al*. (2010) found *C. acutatum* on anthracnose–affected strawberry in China. Conidia were elliptic to fusiform. The colony was white for 4–5 d and later became gray-brown. Embaby and Amany (2013) identified *colletotrichum* species by morphology; they found that *C. acutatum* and *C. gloeosporoides* are the major cause of fruit rot and yield losses on strawberry in Egypt from 2010 to 2012. Conidia were cylindrical and attenuated at both ends, with dimensions of 12.6 (11.8×15.4) × 4.1(3.3–5.1) µm. Moreover, in this study 10 isolates of *Colletotrichum* spp. from strawberry fruit were identified as *C. acutatum* based on detection of a 490-bp fragment by PCR.

Many authors have identified *Colletotrichum* causing strawberry anthracnose based on the morphological, pathological characteristics and PCR analyses using specific primers. Species specific PCR was performed by using two primers, primers: CaInt2 specific for *C. acutatum* and CgInt specific for *C. gloeosporioides*, each in combination with the conserved primer ITS4. The first species identified in Egypt was *C. gloeosporioides* and the second *C. acutatum* (Embaby and Amany, 2013). In addition, Xie *et al*. (2010) identified 31 isolates of *Colletotrichum* spp. which cause strawberry anthracnose in Zhejiang Province and Shanghai City, China. Eleven isolates were identified as *C. acutatum*, 10 as *C. gloeosporioides* and 10 as *C. fragariae* based on morphological characteristics, phylogenetic and sequence analyses. Species-specific PCR and enzyme digestion further confirmed the identification of the *Colletotrichum* spp. They used the specific primer CaInt2 with the ITS4 primer which amplified a 490–bp fragment, and the restriction enzyme *MvnI* for identification *C. gloeosporioides*.

The results of screening antagonistic bacteria isolated from fresh leaves and fruit of strawberry. Indicated that isolates K18, K27, S15, S16 and S17 were found effective in inhibiting the growth of *C. acutatum* isolate CK21. Bacterial isolate K27 was found to be the most inhibitory (66.25%). The biocontrol was tested on strawberry leaves by using fresh cells of the bacterial antagonist in greenhouse experiments. The results showed that spaying 1 d before or after the pathogen inoculation significantly suppressed anthracnose compared to the non–treated control. Similar results were obtained by Rahman *et al*. (2007), screening 27 antagonistic bacteria isolated from the fructosphere

of papaya by dual culture. They found that four isolates, B23, B19, B04 and B15, had high antagonistic activities against *C. gloeosporoides* from papaya. Svetlana *et al*. (2010) found that five biocontrol agents such as *Trichoderma harzianum, Gliocladium roseum, Bacillus subtilis, Streptomyces noursei* and *Streptomyces natalensis* inhibited *Colletotrichum* isolates in fruit crops. Nam *et al*. (2014), reported that *Bacillus velezensis* (NSB–1) isolated from the leaves strawberry cultivar Seolhyang in Korea had high antagonistic activities against *C. gloeosporoides* causing crown rot of strawberry. Kuenpech and Akarapisan (2014) reported that the yeast isolate *Pichia* sp. Y2 showed high biocontrol efficacy against *C. gloeosporioides* causing anthracnose of orchid, and was used to formulate a liquid bioproduct. The *Pichia* sp. Y2 could inhibit the growth of the pathogen after application either 1 h or 1 d before pathogen inoculation when compared with the control. In addition, other research screened antagonistic microorganisms for their ability to inhibit the growth of *C. musae*, the causal agent of anthracnose disease on banana fruits. It was found that 11 isolates produced zones of inhibition against *C. musae* on PDA. The antagonistic microorganisms used were *Pantoea agglomerans* and *Enterobacter* sp. (Khleekorn and Wongrueng, 2014). This present study suggests the potential of developing antagonistic bacteria for the biological control of strawberry anthracnose disease.

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