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Rapid communication

Isolation and evaluation of biocontrol agents in controlling anthracnose disease of mango in Thailand

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Abstract: The agricultural based economy is a core business in Thailand and food export is one of the main sources of income for the Thai population. However, pesticides are overused and misused. As a result there is an urgent need to reduce the use of synthetic chemicals. Biological control offers an alternative to the use of pesticides. Mango (*Mangifera indica* L.) is widely planted in Thailand and is one of the major cash crops for international export. However, mango suffers from various diseases especially anthracnose, a fungal disease caused by *Colletotrichum gloeosporioides.* One hundred and twelve isolates of epiphytic microbes were isolated from healthy leaves and fruits of mangoes; this included 93 and 19 isolates of epiphytic bacteria and yeasts, respectively. They were screened for bioactivity against a pathogenic strain of *C. gloeosporioides* isolated from diseased mangoes using a dual culture technique. Out of 112 isolates, eight isolates exhibited at least 60% inhibition. These isolates were further screened for their inhibition on mango using fruit inoculation. Two isolates reduced the lesion sizes caused by *C. gloeosporioides* compared to control treatment. These two isolates, based on phenotypical and biochemical tests, were identified as *Bacillus* sp. MB61 and *Bacillus* sp. LB72.

Key words: anthracnose, *Bacillus*, biocontrol, *Colletotrichum gloeosporioides,* epiphytes, mango

Introduction

Thailand is one of the world's major food exporters and Thai fruits and vegetables are mainly shipped to various countries such as the United States, the European Union, China and Japan. Therefore the agricultural sector plays an essential role in Thai economy. Agricultural production used 40% of the workforce and 40% of the land area (Panuwet *et al.* 2012) making it the main source of income for rural Thai people who rely heavily on this sector. To increase the product yield, pesticides are overused and misused in spite of health concerns, environmental contamination and export bans (Panuwet *et al.* 2008). There is an urgent need to find alternative ways to replace or reduce the use of synthetic chemicals. Biological control, using microbes to prevent and/or suppress plant diseases, offers an alternative to the use of pesticides (Bale *et al.* 2008; Holb 2009).

Mango (*Mangifera indica* L.) is widely planted in many parts of Thailand and it is one of the major fruits consumed domestically as well as exported internationally. The mango variety Nam Dokmai is the most popular variety for export due to its sugary flavor and soft texture. The export value of mango was 22.8 million USD per year which makes it one of the major cash crops in Thailand (OAE 2012). However mango suffers from various diseases caused by many phytopathogens. Anthracnose, caused by a fungal pathogen – *Colletotrichum gloeosporioides*, is a severe disease which can cause serious losses at various stages of mango production ranging from the blossom period to post-harvest. Although chemical treatment using various fungicides presented high efficiency against *C. gloeosporioides,* the emergence of fungicide-resistant isolates of this pathogen has been reported (Kongtragoul *et al.* 2011). Hence there is an urgent need to tackle this problem.

Epiphytic microbes that live on plant surfaces without causing any symptoms to the plants are well known for their efficacy to inhibit plant pathogens (McGrath and Andrews 2005; Janisiewicz *et al.* 2010; Janisiewicz *et al.* 2013). In this study bacteria and yeasts are focused on because they are able to produce diverse groups of bioactive substances and they can persist under a wide range of environmental conditions which is the key for future industrial application. *Bacillus* species are particularly favored because they are best known for producing secondary metabolites (Sharma *et al.* 2009; Amin *et al.* 2012).

Biological control is a crop treatment which potentially can be used individually or in combination with other types of treatment to reduce loss caused by plant pathogens. Various effective combination methods to control fruit decay were reviewed by Janisiewicz and Conway (2010). For example, *Metchnikowia pulcherrima,* an antagonistic yeast, was used in combination with heat and 1-methylcyclopropene (1-MCP) treatments to effectively

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control bitter rot caused by *Colletotrichum acutatum* on apples (Janisiewicz *et al.* 2003). Microbial biocontrol against *C. gloeosporioides* developed for mangoes has been reported in many countries such as China (Zheng *et al.* 2013), Taiwan (Senghor *et al.* 2007), Mexico (Bautista-Rosales *et al.* 2014), and the Philippines (Koomen and Jeffries 1993). However limited research on biocontrol of mangoes has been done in Thailand.

The overall aim of this work was to expand the knowledge of using biocontrol and to find new microbial biological control agents (BCAs) for anthracnose on mangoes in Thailand. The specific objectives of this work were (1) to isolate epiphytic microbes from mango, (2) to screen for their antagonistic activity against *C. gloeosporioides* and (3) to identify the most effective microbial isolates.

Materials and Methods

Isolation of epiphytic microbes

Microbes including bacteria and yeasts were isolated from leaves and fruits of mangoes collected from two orchards in suburbs of Bangkok, Thailand (Toongkaru and Ladkrabang Orchards). Plant materials were healthy and spotless and there were no symptoms present. They were washed with running tap water to remove dust and dirt from the plant surface and left to dry at room temperature under a flow cabinet for one hour. Swab technique was applied and then streaked onto isolation media with an antibiotic concentration of 50 $\mu g \cdot l^{-1}$. Nutrient Agar (NA) amended with nystatin and Potato Dextrose Agar (PDA) amended penicillin were used for bacterial and yeast isolation, respectively. The isolation media were incubated at 25°C for 5 days. Single colonies grown on the media were randomly picked and transferred to fresh media without antibiotics. The cultures of epiphytic microbes were maintained in 15% glycerol solution and kept at –80°C in a deep freezer.

Isolation of the pathogen – *Colletotrichum gloeosporioides*

Mango fruits were bought from local fresh markets in Bangkok, Thailand. Mangoes with tiny black spots showing signs of anthracnose were preferred. The fruits were kept in moist chambers and incubated at 25°C for three days. The areas showing black spots were cut by using a flame sterilized surgical blade. Pieces of surface cut (1×1 cm^2) were put onto PDA amended with penicillin ($50 \mu g \cdot l^{-1}$) to suppress bacterial growth and then the PDA plates were incubated at 25°C for 10–15 days. The fungal hyphal tips growing from the diseased plant materials were transferred to fresh PDA without an antibiotic. Cultures of the fungal pathogen – *C. gloeosporioides* were confirmed for their pathogenicity using Koch's postulate technique and the fungal cultures were maintained at –80°C in a deep freezer.

In vitro test

Two isolates of *C. gloeosporioides* isolated from diseased mangoes were used. They were maintained on PDA slants and stored at 4°C until needed. A dual culture technique was used to determine mycelium inhibition, in which the test bacterial or yeast isolates were inoculated 2.5 cm away from the margin of a Petri dish containing PDA. An agar plug cut from actively growing mycelium of *C. gloeosporioides* using a cork borer (no. 2: diameter 5 mm) was placed 1 cm away from the margin of the opposite site of the PDA. The plates were incubated at 25°C for a further 14 days. There were two replicate plates per treatment and per strain of *C. gloeosporioides*. Radius growth of *C. gloeosporioides* was measured and percentage of inhibition was calculated.

In vivo test

There were eight isolates of epiphytic bacteria selected from the *in vitro* test. The *in vivo* test on detached mango fruits was adapted from Rungjindamai *et al.* (2013). Single colonies of the candidate BCAs were transferred to 2 ml of nutrient broth (NB) and the cultures were incubated at 25°C for 18 h. Apparently healthy mangoes cv. Nam Dokmai were picked from orchards between March – May 2015. Fruits were stored at 4°C and used within one week of picking. Fruits were washed with sterile distilled water to remove dust and then the fruits were surface sterilized by wiping with sterile tissue paper soaked with 95% ethanol. After air-drying in a flow cabinet, the mangoes were wounded using a sterile 200 µl tip (2 mm diameter, 2 wounds per mango) and 5 µl of cell suspension of each candidate BCA (a concentration of candidate BCAs between 10^7–10^8 cfu $\cdot$ $\cdot$ ml^{-1}) was pipetted into the wounds. The treated mangoes were left to dry for 1 h and then an equal volume of spore suspension of *C. gloeosporioides* (concentration at 1×10^5 spores $\cdot$ ml^{-1}) was pipetted into the same wound. The numbers of BCAs and the pathogen were determined by using the spread plate method and direct counting with a haemocytometer, respectively. Sterile normal saline solution (0.85% NaCl) was used as a control.

The inoculated mangoes were placed into square plastic boxes (1 mango per box). The lids were put on and the mangoes were incubated at 4°C for one week. The experiments were carried out with three replicates (three mangoes per treatment and six wounds in total). Rot development was assessed within 7 days after the inoculation of the pathogen. The lesion diameter was measured using a Vernier caliper. The diameter of a lesion was the average of its vertical and horizontal diameters. The statistical analysis of Analysis of Variance (ANOVA) was performed using SPSS version 22.0 and the data are presented as means±SE.

Identification of candidate BCAs

All candidate BCAs are apparently bacteria. Therefore the bacterial BCAs were grown on NA and incubated at 25°C for 7 days without light. Bergey's Manual of Systematic and Determinative Bacteriology was used to identify the two isolates of bacterial BCAs (Logan and De Vos 2009). Phenotypic and biochemical characteristics were used in this study.

Results

Isolation of epiphytes

A total number of 112 epiphytic microbes were isolated in this study. Sixty-two and 50 isolates were from fruits and leaves of mangoes, respectively. The samples from two orchards including Ladkrabang and Toonkaru Orchards yielded similar numbers at 46 and 66 isolates, respectively. Out of 112 isolates, 93 and 19 isolates were bacteria and yeast, respectively. They were screened for their antagonistic activity against *C. gloeosporioides*.

In vitro test of antagonists

Figure 1 shows the number of epiphytic microbes according to their ability to control the pathogen, *C. gloeosporioides*. Nearly half of the epiphytic microbes (54 isolates) failed to inhibit the fungal pathogen (less than 20% inhibition). There were 34 and 16 isolates that moderately inhibited the growth of the fungal pathogen with 20–59% inhibition. There were eight isolates which were able to strongly inhibit *C. gloeosporioides* with more than 60% inhibition. However there were no isolates which could completely control the pathogen with 100% inhibition.

Out of 112 isolates, eight isolates exhibited strong inhibition against *C. gloeosporioides*. Wide inhibitory zones between the pathogen and antagonists were clearly present (Fig. 2), while *C. gloeosporioides* in control treatment (PDA without antagonist, Fig. 2, upper left) grew well and reached the other side of the test medium. These eight isolates with good bioactivity were chosen and further screened in the *in vivo* test on mango fruits.

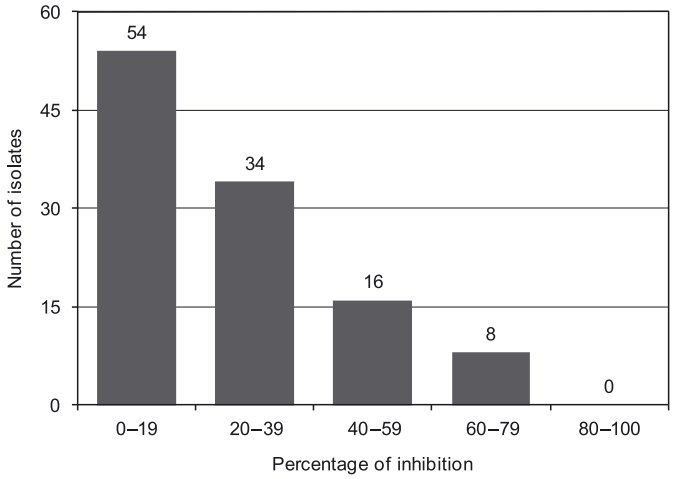

Fig. 1. A histogram of percentage of inhibition and number of epiphytic microbes tested against *Colletotrichum gloeosporioides*. The experiment was performed on PDA incubated at 25°C for 2 weeks

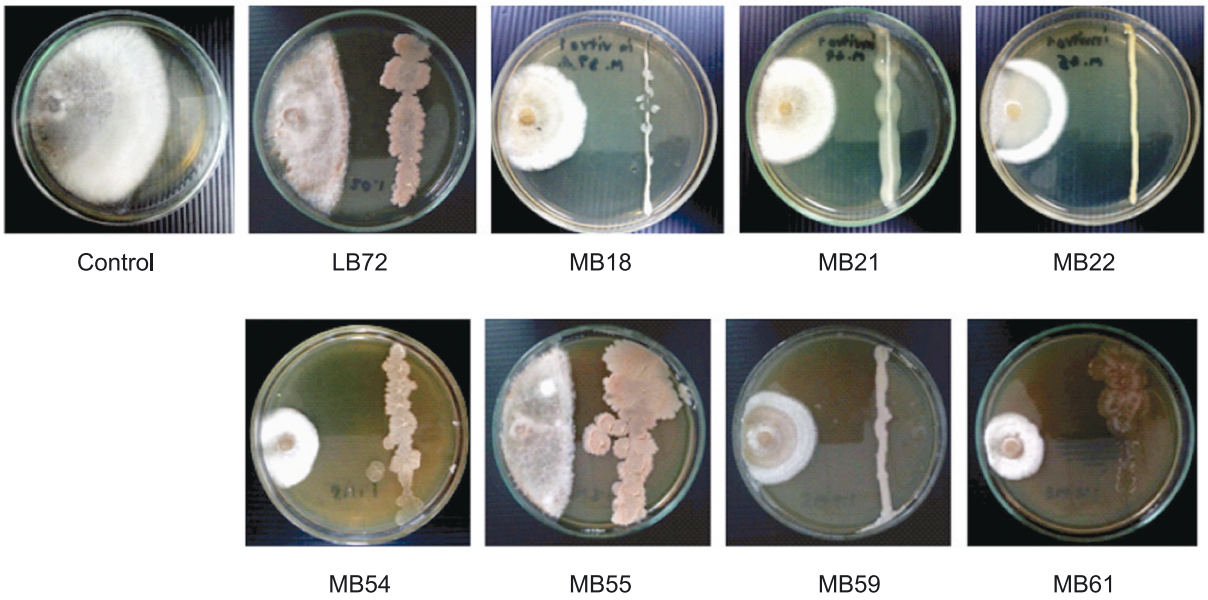

Fig. 2. Inhibitory effect of eight epiphytic microbes (inoculated on the right of medium) isolated from mangoes against the fungal pathogen, *Colletotrichum gloeosporoides* (inoculated on the left of medium). The PDA plates were incubated at 25°C for 2 weeks

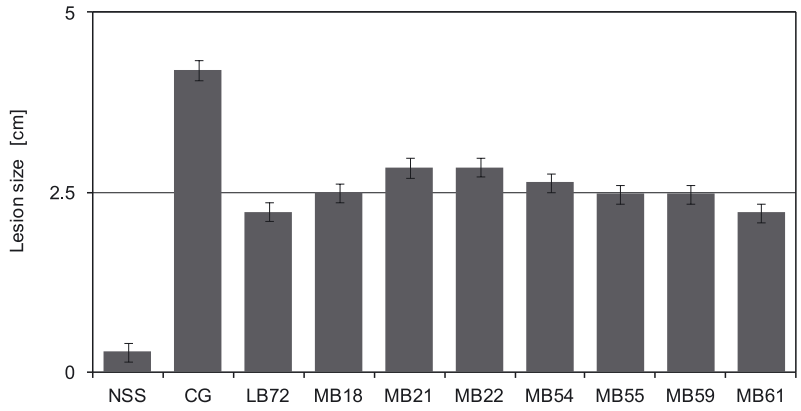

Fig. 3. Mean values of lesion size on mangoes caused by artificial inoculation of *Colletotrichum gloeosporioides* (1 × 105 spores · ml^{-1}), then the wounds were treated with cell suspension of eight candidate biological control agents (BCAs) (1 × 107–108 cfu · ml^{-1}). Control treatment was sterile normal saline solution inoculated on mangoes without the pathogen. The fruits were incubated at 4°C for one week. Bars represent standard error of the mean: NSS = normal saline solution, CG = *C. gloeosporioides*, LB and MB = = epiphytic microbes

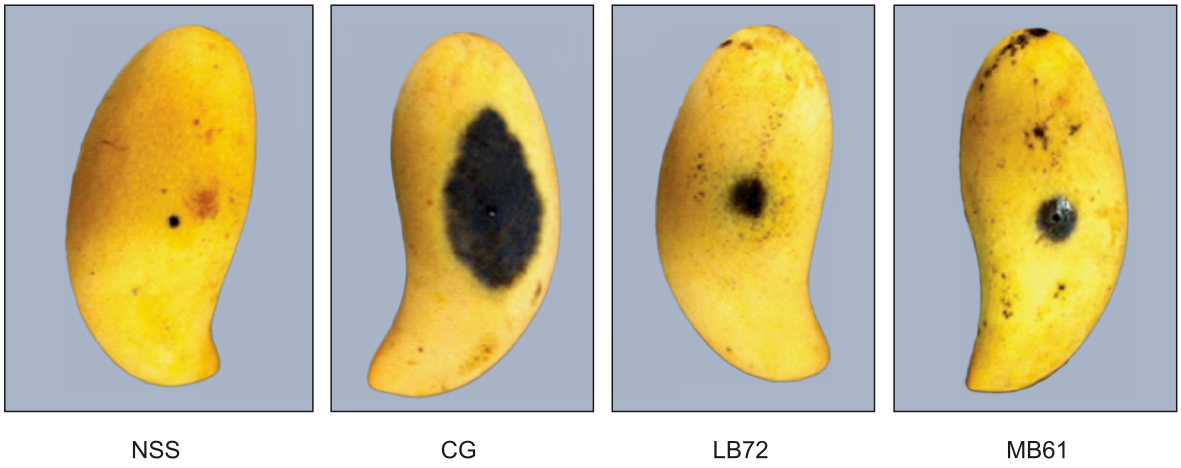

Fig. 4. Lesion development on mangoes treated with different treatments incubated at 4°C for one week: NSS = a wound treated with sterile normal saline solution, CG = a wound inoculated with spore suspension of *Colletotrichum gloeosporioides*, LB72 and MB61 = wounds treated with epiphytes LB72 and MB61, respectively then inoculated with spore suspension of *C. gloeosporioides*

In vivo test on mango fruits

Disease development on mango surface was expressed as the mean lesion size (in centimeters, Figs. 3 and 4). Lesion sizes of control treatments were 0.28 and 4.20 cm for no pathogen (sterile normal saline solution) and with the pathogen (*C. gloeosporioides*), respectively. All eight candidate BCAs significantly reduced lesion diameter on mangoes caused by *C. gloeosporioides*. Among these isolates, there were two isolates (LB72 and MB61) which demonstrated the best bioactivity by reducing the lesion sizes to 2.23 and 2.22 cm, respectively. Therefore these two isolates were subjected to further identification.

Identification of the potential BCAs

The two candidate BCAs were identified according to morphological and biochemical characteristics. Both of them were bacteria and the cells stained Gram-positive were rod-shaped, spore producing, facultative anaerobes. They produced similar types of colonies. When grown on NA and incubated at 25°C for 24 h, their colonies were creamy white to light yellow, opaque, smooth to slimy and formed circular colony. For biochemical characteristics, motile, catalase positive, oxidase positive. Results from triple sugar iron agar (TSI), produced acid at slant and butt, did not produce gas and hydrogen sulfide. According to the results these two bacterial BCAs were assigned to *Bacillus* sp. LB72 and *Bacillus* sp. MB61.

Discussion

Microbes with bioactivities have been reported from various habitats and diverse environments. Antagonistic microbes used to control plant diseases should be collected from similar ecological niches where the targeted plant disease is present because they are likely to have the potential to compete with plant pathogens (Janisiewicz and Korsten 2002). In this work, resident microbes that live epiphytically on mangoes were found to have an antagonistic effect against *C. gloeosporioides*. This confirms the finding that epiphytes are a good source of biocontrol for controlling plant diseases.

Colletotrichum gloeosporioides has been extensively studied because it can infect a wide range of crops and cause serious economic losses (Arzanlou *et al.* 2015). Although fungicides have been reportedly used to control *C. gloeosporioides*, the pathogen has become resistant to fungicides (Valero *et al.* 2010). Clearly the use of fungicides is not sustainable. Rather than the heavy use of fungicides alone, biocontrol could be used to reduce, or, together with chemicals, to avoid fungicide-resistance which may occur in plant pathogens. Biocontrol of *Colletotrichum* has been widely reported on various tropical fruit crops for example banana (Peeran *et al.* 2014), grape (Mochizuki *et al.* 2012) and papaya (Lima *et al.* 2013). However, mango has received much attention in biological control research because of its worldwide economic value (Admasu *et al.* 2014; Bautista-Rosales *et al.* 2014).

The *Bacillus* species has been in the spotlight for biocontrol research for decades due to its various modes of actions (Jacobsen *et al.* 2004; Jamalizadeh *et al.* 2009; Khiyami *et al.* 2014). Four modes of action in biocontrol include (1) competition for nutrients and space, (2) production of antibiotics, (3) direct parasitism and (4) inducing plant resistance (Sharma *et al.* 2009). Antibiosis plays a crucial role for the bioactivity of *Bacillus*. Various bioactive substances produced by *Bacillus* spp. were purified and reported for their inhibitory effect against *C. gloeosporioides*. Ruangwong *et al.* (2012) isolated and identified potential metabolites produced by *B. subtilis* which strongly inhibit spore germination of *C. gloeosporioides*. Alamri *et al.* (2012) showed that *Bacillus* were able to inhibit pathogens with more than one mode of action such as secreting protease enzymes and producing antibiotics.

Our *in vitro* results indicate that producing antibiotics could play an essential role for *Bacillus* species because wide, clear zones were present without any contact between the test epiphytes and pathogen. Bioactive compounds might have been produced, released and diffused to the test agar medium to inhibit the pathogen. In addition to mycelial inhibition in *in vitro* screening, our two potential BCAs were able to inhibit spore germination inoculated in artificially wounded mangoes. In this present work, both BCAs were *Bacillus* spp. Koomen and Jeffries also reported (1993) that *Bacillus cereus* and *Pseudomonas fluorescens* were chosen as the most potential candidates based on a series of screening of BCAs from *in vitro* to a field trial conducted in the Philippines. This shows that it may be possible to use *Bacillus* as biocontrol treatment to reduce the development of anthracnose lesions. Also it has potential for post-harvest treatment when synthetic fungicides are strictly prohibited.

Several kinds of microbes were able to effectively inhibit *Colletotrichum* species on mangoes including a filamentous fungus, *Trichoderma hazianum* (de los Santos-Villalobos *et al.* 2013), a yeast, *Cryptococcus* (Bautista-Rosales *et al.* 2014) and a filamentous bacterium, *Streptomyces* (Soares *et al.* 2006; Palaniyandi *et al.* 2013; Traquair *et al.* 2013). However, bacterial biocontrol has been proven to control *C. gloeosporioides* under various conditions such as *in vitro, in vivo*, greenhouse and in the field (Senghor *et al.* 2007; Mohammadipour *et al.* 2009; Mochizuki *et al.* 2012). In this present work, the two most effective BCAs were identified as *Bacillus* spp.. Undoubtedly *Bacillus* is one of the promising BCAs and some have been produced as commercial products (Stewart 2001). Rather than relying on importing foreign biocontrol products, searching for indigenous biocontrol should be encouraged in order to reduce costs of crop production, and to avoid the environmental impact of the introduced species as well as the complicated registration of foreign products. We have shown that two indigenous BCAs isolated from mangoes have the potential to be developed for further study.

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