**INTRODUCTION**

Rice (Oryza sativa L.) dirty panicle is one of the most important diseases in rice production in Thailand. This disease is caused by several fungal species including Curvularia lunata, Bipolaris oryzae, Fusarium incarnatum, Sarocladium oryzae, Trichoconis padwickii and Cercospora oryzae. They are distributed in different geographical areas in Thailand. This disease causes a risk to rice production and significant yield losses especially in the case of severe infection. The quality and quantity of rice yield is reduced by fungal pathogen infection of the panicle. For the prevention of disease outbreaks, the paddy fields are intensively sprayed with fungicides by the farmers. However, the causal agents of this disease include various genera and species, therefore specific and broad-spectrum fungicides are applied to control these fungal species. Using various fungicides can result in environmental contamination and cause residues in food. The alternative method of using biocontrol agents is ecosystem friendly, reduces the disease distribution, and induces resistance to suppress fungal infection. In Thailand, there has been little

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**ABSTRACT**

Rice dirty panicle disease is one of the most important problems in Thailand. The fungal pathogens were reported to be many species including Curvularia lunata, Bipolaris oryzae, Fusarium incarnatum, Sarocladium oryzae, Trichoconis padwickii and Cercospora oryzae. Biological control is an alternative method for controlling this disease and reducing the application of fungicides. Therefore, this study aimed to evaluate the antagonistic potential of bacteria isolated from peat swamp forests in Thailand. A total of 513 bacterial isolates were collected and screened in the laboratory using the dual culture method. The results revealed that three of the 513 bacterial strains (IBK-4, IBK-8 and IBK-5) were highly inhibitory to the fungal pathogens including C. lunata, B. oryzae and F. incarnatum. These three strains were identified as Bacillus (IBK-4 and IBK-8) and Brevibacillus (IBK-5) based on 16S rRNA sequencing. Then, these three strains were evaluated on a susceptible rice variety by inoculation with three fungal pathogens. The results indicated that Bacillus strain IBK-8 had the highest efficiency to control the disease development as observed in the disease severity and index. The results of this study indicated that bacterial strains from peat swamp forests have the potential to be antagonistic to plant pathogens.

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research to identify effective microorganisms as biocontrol agents for this disease such as *Trichoderma* species (Charoenrak & Chamswarng, 2016). The peat swamp forests are tropical moist forests, consisting of waterlogged soil containing leaves and wood. This generates a thick layer of acidic peat overtime. In Thailand, the peat swamp forests are located in the Southern and Eastern regions. A previous report indicated that the highest bacterial populations were observed in organic soil. Most of them were identified as *Proteobacteria* and *Acidobacteria*. These bacteria have various activities in the peat swamp soil such as lignocellulose, phosphate, sulfate, and methane digestion. Moreover, they can produce plant hormones, induce disease resistance in plants and fix nitrogen. There have been many reports on biological control using bacteria from swamps such as *Brevibacillus formosus* DSM 9885 and *B. brevis* NBRC 15304 to control *Alternaria alternata* the causal agent of potato brown leaf spot (Ahmed, 2017), acid-resistant purple non sulfur bacteria (PNSB) from peat swamp forest (PSF), *Rhodopseudomonas palustris* KTSSR54 to control rice fungal pathogens (*Bipolaris oryzae* and *Curvularia oryzae*) (Nookongbut et al., 2019), peat soil bacteria against non ESBL-producing *Escherichia coli* ATCC25922, ESBL-producing *E. coli* ATCC35218, methicillin susceptible *Staphylococcus aureus* ATCC29213 and MRSA ATCC43300 (Mahdiyah et al., 2020). The mechanism for biocontrol of bacterial strains was reported to be the activities of metabolites released from bacteria. Certain secondary metabolites were reported such as zwitermicin, bacillomycin, fongycin, bacilysin, and difficidin (Athukorala, Fernando, & Rashid, 2009; Chen et al., 2009), putative antimicrobial compounds (Mahdiyah et al., 2020) or volatile compounds such as 1,3,5-heptatriene (Cordero, Principe, Jofré, Mori, & Fischer, 2014). These metabolites can inhibit the mycelial growth of fungal pathogens. Therefore, this study aimed to evaluate the efficacy of bacterial strains isolated from peat swamp soil as biocontrol agents against three species of rice dirty panicle fungi to reduce the application of fungicides.

**MATERIALS AND METHODS**

**Survey and Sample Collection**

Surveys and soil sampling were conducted in 2017-2018 in four areas: Khan Thuli Mangrove Natural Learning Center, primary peat swamp forest (PF Khandhlu) (5 samples), and secondary peat swamp forest (SF Khandhlu) (5 samples) at Surat Thani, Khuang Khrang peat swamp forest at Nakhon Sri Thammarat, secondary peat swamp forest (SF Khuankhrung) (6 samples) and Rayong Botanical Garden at Rayong, secondary peat swamp forest (SR) (6 samples). From each sampling site, a 5 kg peat sample was taken from a 30-50 cm depth using an auger. After mixing, approximately 2 kg were brought to the laboratory in an icebox and kept at 8°C until used for bacterial isolation and identification. (Fig. 1).

**Isolation and Identification of Acid-Tolerant Bacteria (ATB)**

Total plate count using the spread plate technique was conducted to isolate acid-tolerant bacteria in soil from peat swamp forests. Ten grams of soil was suspended in 90 ml of sterile normal saline in 250 ml Erlenmeyer flasks, shaken on a rotary shaker at 150 rpm for 30 minutes, and then subjected to a ten-fold serial dilution with sterile normal saline. Each dilution was spread on R2A agar (Merck, Germany) adjusted to pH 4.5, and plates were incubated at 35°C for 2-5 days. Based on the differences in colony morphology, ATBs were selected and further purified by cross streaking on tryptic soy agar (TSA) plates. Pure cultures were maintained on TSA slants as working cultures and in 30% (v/v) glycerol at -80°C as stock cultures.

Bacterial isolates were identified based on molecular and phylogenetic taxonomy. DNA was extracted according to the method of Vingataramin & Frost (2015) and used as a DNA template for PCR amplification of 16S rRNA gene sequences using 27F (5’-AGAGTTTGGATCMTGGCTCAG-3’) and 1492R (5’-CGGTTACCTTGTTACGACTT-3’) primers. The sequences were analyzed for the similarity with reference strains by using Nucleotide Blast program (Altschul et al., 1997) and aligned with related species by Multiple Alignments using ClustalW in MEGA 7.0 (Kumar, Stecher, & Tamura, 2016). Phylogenetic analysis was clustered by Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap value was calculated with 1000 heuristic replicates to estimate support for the clade stability of the consensus trees (Felsenstein, 1985).
Antagonistic Efficiency of Bacterial Isolates Against Rice Dirty Panicle Fungi

Five hundred and fifteen isolates of bacteria from peat swamp forests were investigated against three isolates of rice dirty panicle fungi including *B. oryzae* (NPT0508) *C. lunata* (SPB0627) and *F. incarnatum* (SKA06131) by the dual culture technique using PDA medium (Fig. 2). One disc (0.5 cm in diameter) of the fungal isolate was placed on the center of a sterile petri dish containing PDA. For testing the antagonistic effect of bacterial isolates, each of them was streaked on both sides of the fungal colony after culturing for 1 day. The control treatment was the petri dish with fungal culture and without bacterial isolates. Each treatment was done with four replications. The ability of bacterial isolates to inhibit the fungal pathogen was calculated as percent inhibition of colonial fungal growth by using the following formula:

\[
\text{Inhibition} \, (\%) = \left(\frac{R_1 - R_2}{R_1}\right) \times 100
\]

Where:  
\[ R_1 = \text{control plates (only fungi)} \]  
\[ R_2 = \text{Growth of fungal pathogen in dual culture plates} \]

Following ANOVA, Duncan’s New Multiple Range Test (P=0.05) was used for means separation.
Inoculation of Susceptible Rice Variety with Three Fungal Pathogens and Antagonistic Bacteria

The most aggressive pathogen causing rice dirty panicle, *C. lunata* (SPB0627) was cultured on PDA plates and incubated at room temperature (25-28°C) for 7 days. The bacteria isolates IBK-8, IBK-4 and IBK-5 were cultured in nutrient agar broth for 24 hours and bacterial concentrations were measured by spectrophotometer at 600 nm then adjusted to O.D. 1.1-1.2 with $10^8$ CFU/ml (Schaad, Jones, & Chun, 2001). The fungal strain (SPB0627) was inoculated on a susceptible variety, RD47 in the booting stage of rice by applying spore suspension ($10^5$ spores/ml) after inoculation with antagonistic bacteria such as IBK-8; IBK-4; IBK-5. The experiment was conducted by Completely Randomized Design (CRD), with inoculation onto three panicles/plant of five plants. The disease evaluation was done by bulking grain seeds from 15 panicles and then randomly selecting 400 seeds five times. The evaluation of disease severity was conducted at 21 days after inoculation (DAI) (Table 1). The Rx64 3.4.1 (R-language and environment for statistic computing and graphics) was used to analyze the data.

**Fig. 2.** Colonial and conidial characteristics of *B. oryzae* NPT0508 (A, B), *C. Lunata*, (C, D) SPB0627 and (E, F) *F. incarnatum* SKA06131
Table 1. Disease index and phenotype of rice dirty panicle disease caused by fungi in greenhouse conditions (McMaugh, 2005)

| Description                                                                 | Score |
|------------------------------------------------------------------------------|-------|
| No symptom                                                                   | 0     |
| Show spot lesions less than 0.1 mm, not more than 5 % of seed area           | 1     |
| Show spot lesions larger than 0.1 mm, not more than 25 % of seed area        | 2     |
| Show black lesions not more than 50 % of seed area                           | 3     |
| Show black lesions larger than 50 % of seed area                             | 4     |
| Show black lesions larger than 50 % of seed area and undeveloped kernels     | 5     |

Remarks: Disease severity was observed at 21 DAI with \( C. \ lunata \), then the disease index was calculated by the following formula: Disease index = \( \frac{\text{na} \times 0 + \text{nb} \times 1 + \text{nc} \times 2 + \text{nd} \times 3 + \text{ne} \times 4 + \text{nf} \times 5}{\text{N} \times 5} \) \times 100; Where \( \text{na}, \text{nb}, \text{nc}, \text{nd}, \text{ne}, \) and \( \text{nf} \) are number of seeds with disease scores are 0, 1, 2, 3, 4, and 5, respectively and \( \text{N} \) from 400 random seeds following the procedures of the International Seed Testing Association.

RESULTS AND DISCUSSION

Survey and Sample Collection

The soil sampling and bacterial isolation were done from peat swamp forests including 122 isolates from PF Khan Thuli, 132 isolates from SF Khan Thuli, 138 isolates from SF Khuan Khreng, and 121 isolates from SR Rayong. The bacterial concentration in swamp peat soil at primary and secondary peat swamp forest were \( 1.5 \times 10^5 \) – \( 4.8 \times 10^6 \) CFU/g and \( 2.6 \times 10^4 \) – \( 4.0 \times 10^6 \) CFU/g respectively. Most bacterial isolates were gram-positive, rod-shaped while gram-negative and coccoid bacteria were very few. The gram-positive bacteria were observed at 67% (PF Khatnuli), 82% (SF Khanthuli), 96% (SF KhuanKhreng), and 76% (SR Rayong).

Antagonistic Efficiency of Bacterial Isolates Against Rice Dirty Panicle Fungi

The antagonistic effect of isolated bacteria against \( B. \ oryzae \) NPT0508, \( C. \ lunata \) SPB0627 and \( F. \ incarnatum \) SKA06131 was studied. All bacterial isolates (513 isolates) were evaluated for their effects on the mycelial growth of the three rice dirty panicle fungi using the dual culture technique. In dual culture for evaluation of bacterial strains, the rate of inhibition (%) observed was 0-34.55% (\( B. \ oryzae \)), 0-38.78% (\( C. \ lunata \)) and 0-34.97% (\( F. \ incarnatum \)). However, only three isolates of bacteria caused high inhibition of the three pathogens including IBK-8, IBK-4 and IBK-5. The percent inhibition was 28.88% (IBK-8), 22.12% (IBK-4) and 23.45% (IBK-5) for \( B. \ oryzae \), 28.88% (IBK-8), 22.12% (IBK-4) and 23.45% (IBK-5) for \( C. \ lunata \), and 31.12% (IBK-8), 21.49% (IBK-4) and 23.14% (IBK-5) for \( F. \ incarnatum \) (Fig. 3 and Fig. 4).

Inoculation of Susceptible Rice Variety with Three Fungal Species and Antagonistic Bacteria

According to the screening in the laboratory, the potential antagonistic bacterial isolates selected were IBK-8, IBK-4, and IBK-5. These isolates were identified based on partial molecular 16S rRNA gene (~1500 bp) sequencing profiles. The phylogenetic tree based on the Neighbor-Joining method is presented in Fig. 5. Isolate IBK-4 (GenBank accession number: MN428231) and IBK-8 (MN428235) exhibited a homology of 100% with \( B. \ saefensis \) NBRC 1008220 (NR_113945.1) and 96.2% with \( B. \ shackletonii \) LMG 18435 (NR_025373.1), respectively, while IBK-5 (MN428232) demonstrated a homology of 99.6% with \( Brevibacillus \) formosus DSM 988 (NR_040979.1). The results revealed that bacterial isolate IBK-8 could control rice dirty panicle caused by \( B. \ oryzae \) NPT0508, \( C. \ lunata \) SPB0627 and \( F. \ incarnatum \) SKA06131 with the lowest disease indices of 27.6% (44.60% in control treatment), 22.6% (45.40% control treatment), and 19.40% (32.0% in control treatment), respectively (Table 2; Fig. 6).
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Fig. 3. The graph of inhibition percentages against three rice dirty panicle fungi: *B. oryzae* NPT0508, *C. lunata* SPB0627, *F. incarnatum* SKA06131 by three strains of bacteria (IBK-8, IBK-4, IBK-5) in dual culture tests.

Fig. 4. Effectiveness of antagonistic bacteria on the mycelial growth of rice dirty panicle fungi A: IBK-8; B: IBK-4; C: IBK-5 and D: control (fungi); 1: *B. oryzae* NPT0508; 2: *C. lunata* SPB0627 and 3: *F. incarnatum* SKA06131.
Fig. 5. Phylogenetic tree of bacteria isolated from peat swamp forests and their closely related taxa obtained from the GenBank database based on the sequence analysis of the 16S rRNA using neighbor joining method in MEGA 7. Numbers on branches indicate percentage of bootstrap sampling (1000 replications).

| Treatment                          | Disease severity (%) | Disease index (%) |
|------------------------------------|----------------------|-------------------|
| B. oyzae NPT0508 + IBK-4          | 32.4                 | 30.60             |
| B. oyzae NPT0508 + IBK-5          | 36.2                 | 32.60             |
| B. oyzae NPT0508 + IBK-8          | 30.6                 | 27.60             |
| B. oyzae NPT0508                  | 45.0                 | 44.60             |
| C.V. (%)                           |                      | 4.2259            |
| C. lunata SPB0627 + IBK-4         | 28.2                 | 23.60             |
| C. lunata SPB0627 + IBK-5         | 34.7                 | 33.20             |
| C. lunata SPB0627 + IBK-8         | 25.1                 | 22.00             |
| C. lunata SPB0627                 | 46.3                 | 45.40             |
| C.V. (%)                           |                      | 4.8175            |
| F. incarnatum SKA06131 + IBK-4    | 30.8                 | 28.20             |
| F. incarnatum SKA06131 + IBK-5    | 27.7                 | 25.00             |
| F. incarnatum SKA06131 + IBK-8    | 23.8                 | 19.40             |
| F. incarnatum SKA06131            | 33.9                 | 32.00             |
| C.V. (%)                           |                      | 5.7962            |
In this research, the effectiveness of bacteria isolated from the four areas of peat swamp forests was evaluated as biocontrol agents against rice dirty panicle fungi including *B. oyzae* NPT0508, *C. lunata* SPB0627, and *F. incarnatum* SKA06131. The best three isolates, IBK-4, IBK-5, and IBK-8 were selected and identified as *Bacillus safensis*, *Brevibacillus formosus*, and *Bacillus* sp., respectively. They were effective against all three fungal pathogens. The results revealed that these three isolates inhibited the mycelial growth of the three rice dirty panicle fungi. Previous research applied the in vitro dual culture method to evaluate the efficiency of biocontrol activities. Ahmed (2017) reported that the growth inhibition of *Alternaria alternata* is the causal agent of potato brown leaf spot disease by *Brevibacillus formosus* strain DSM 9885 and *B. brevis* strain NBRC 15304. In dual cultures with assayed bacterial strains, evident inhibitory action (clear zone of mycelial...
inhibition) was observed. The highest inhibitory effect of bacteria was recorded in the 6-7 days after cultivation with fungal isolates. It was suggested that *Brevibacillus* strains released metabolites that act as an obstacle between the fungal colony and bacteria; the mycelial development was restricted by the antifungal activity surrounding colonies. The secondary metabolites produced by bacteria were discussed in previous research, Dihazi et al. (2012) reported that the inhibitory activity of *B. amyloliquefaciens* occurred as the result of antifungal compounds or metabolites released into the PDA. Moreover, it has been reported by Athukoral, Fernando, & Rashid (2009) and Chen et al. (2009) that *B. amyloliquefaciens* strains showed the ability to inhibit the growth of fungal pathogens because they can produce a vast array of antibiotics such as zwittermicin, bacillomycin, fongycin, bacilysin, and difficidin. Marroni & Germani (2011) reported that a strain of *Bacillus* can produce and release volatile metabolite to control *Macrophomina phaseolina*, the causal agent of castor stem rot. While, Cordero, Príncipe, Jofré, Mori, & Fischer (2014) evaluated the ability of volatile compounds such as 1,3,5-heptatriene produced by *Pseudomonas fluorescens* to inhibit *F. proliferatum* mycelial growth. The three isolates of peat soil bacteria (25PS, 26PS and 27PS) were potentials sources of new antimicrobials with strong activities against ESBL-producing *E. coli* and reference strains (Mahdiyah et al., 2020).

This research also evaluated the efficiency of bacteria against the rice dirty panicle fungi by inoculation of a susceptible rice variety under greenhouse conditions. The results revealed that the three bacterial strains tested suppressed the development of the disease. *Bacillus* sp. IBK-8 showed the highest ability to inhibit the three fungal rice pathogens. This result was correlated with the observation of in vitro experiments by the dual culture method. The in vitro experiment was conducted for screening a large number of collected bacterial strains. However, it is important to investigate the potential of bacterial strains using plants grown in fungal-infested soil (Rocha et al., 2017). Many researchers have evaluated the potential of antagonists to control disease incited by subsequent inoculation of plants with the pathogens. The mixture of antagonistic microorganisms has been evaluated, for example, *Bacillus paenibacillus* strains have been used with *Trichoderma* and *Pseudomonas* to control *Fusarium* wilt in tomato plants with the experimental successes of up to 65% (Raza et al., 2017). Yanti, Warnita, Reflin, & Busniah (2018) reported that all *Bacillus* strains obtained from previous research could both control *Ralstonia* and *Fusarium* wilt and induce the increased growth rate of chili pepper in field conditions. This bacterial strain produces various effective substances that cause growth promotion in chili peppers. This result agrees with Adebayo & Ekpo (2004) because *B. subtilis* inhibited fungal growth and also promoted the growth of tomato plants in screen house trials. Moreover, *Bacillus subtilis* has shown a broad spectrum of antimicrobial activities against various fungal and bacteria pathogens (Grover, Nain, & Saxena, 2009). Khalid, Arshad, & Zahir (2004) suggested that *Bacillus* species have a high potential in plant root colonization and production of phytohormones that improve crop yield. Our results revealed that bacteria isolated from peat swamp forests, especially *Bacillus* strain IBK-8, were potential biocontrols for rice dirty panicle fungi including *C. lunata, B. oryzae* and *F. incarnatum*.

**CONCLUSION**

The present study revealed that bacterial strains from peat swamp forests are potential and effective as biological control agents. Their ability was evaluated by inhibition to rice dirty panicle fungi including *B. oryzae, C. lunata* and *F. incarnatum* under laboratory and greenhouse conditions. *Bacillus* strain IBK-8 had the highest effectiveness to control the fungal pathogen and disease development. The results indicated that bacterial strains from peat swamp forest could be considered antagonistic to plant disease.

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