

Inhibitory effects of *Trichoderma asperellum* culture filtrates on pathogenic bacteria, *Burkholderia pseudomallei*

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ABSTRACT

Background. *Burkholderia pseudomallei* is a soil- and water-dwelling bacterium that causes the life-threatening infection melioidosis. Patients typically acquire this infection through environmental exposure, so reducing *B. pseudomallei* levels in the environment could mitigate the risk of infection. *Trichoderma asperellum* is a biological control agent that synthesizes a diverse range of antimicrobial substances targeting other microorganisms. This study therefore examined the antibacterial and anti-biofilm activities of *T. asperellum* culture filtrate against *B. pseudomallei*.

Methods. The antibacterial activities of *T. asperellum* culture filtrates, collected at various time intervals, were assessed against *B. pseudomallei* using the agar well diffusion method. Subsequently, the minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), and anti-biofilm activities of the culture filtrate exhibiting the highest inhibitory effect were determined. Bactericidal efficacy was further evaluated *via* a time-kill assay. The mechanisms underlying inhibition were then investigated using scanning electron microscopy and crystal violet uptake assays.

Results. Filtrate collected from 7-day old cultures of *T. asperellum* (TD7) exhibited the strongest inhibitory effect on *B. pseudomallei*, with an inhibition zone of 30.33 ± 0.19 mm. The MIC of TD7 against *B. pseudomallei* was 7.81 ± 0.00 mg/mL and the MBC ranged from 7.81 ± 0.00 to 11.72 ± 1.75 mg/mL. Time-kill studies with TD7 confirmed its bactericidal activity, with complete elimination of *B. pseudomallei* occurring within 30 min treatment at 62.48 mg/mL (8xMIC) and 24 h treatment at 7.81 mg/mL (1xMIC). At a concentration of 7.81 mg/mL, TD7 also significantly reduced *B. pseudomallei* biofilm formation. Scanning electron microscopy revealed surface roughening and cell shrinkage of TD7-treated *B. pseudomallei*. TD7-treated bacteria were also found to absorb more crystal violet dye than untreated cells, indicating that TD7 might inhibit and kill *B. pseudomallei* by disrupting cell membrane permeability.

Conclusions. Our findings demonstrate that *T. asperellum* culture filtrates possess bactericidal activity and effectively disrupt biofilm formation by *B. pseudomallei*. This suggests that *T. asperellum* could potentially be used to reduce the presence of *B. pseudomallei* in the environment and, consequently, lower the incidence of melioidosis.

Submitted 7 October 2024
Accepted 4 February 2025
Published 28 February 2025

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Academic editor
Nagendran Tharmalingam

Additional Information and
Declarations can be found on
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DOI 10.7717/peerj.19051

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OPEN ACCESS

Subjects Microbiology, Mycology, Natural Resource Management

Keywords *Trichoderma asperellum*, Culture filtrate, *Burkholderia pseudomallei*, Antibacterials, Anti-biofilm

INTRODUCTION

Burkholderia pseudomallei is a Gram-negative bacterium that causes melioidosis, a serious human and animal infection that can manifest as bacteremia, pneumonia, hepatosplenic abscesses, septic arthritis, and skin or soft tissue infections (Cheng & Currie, 2005; Currie, Dance & Cheng, 2008; Limmathurotsakul et al., 2012; Currie, 2015). This pathogen is widely distributed in soil and water and is endemic in Southeast Asia and Northern Australia (Cheng & Currie, 2005; Currie, Dance & Cheng, 2008; Wuthiekanun et al., 2009; Limmathurotsakul et al., 2016; Hinjoy et al., 2018; Gee et al., 2022; Currie, Meumann & Kaestli, 2023). The majority of melioidosis patients are rice farmers who have been exposed to *B. pseudomallei* present in wet soil (Suputtamongkol et al., 1999; Limmathurotsakul et al., 2010; Rattanavong et al., 2011; Limmathurotsakul et al., 2013). Reducing *B. pseudomallei* levels in soil and water may therefore mitigate the risk of exposure and infection.

Several studies have proposed that modification of the physiochemical or biological properties of soil could reduce *B. pseudomallei* in the environment (Ngamsang et al., 2015; Boottanun et al., 2017; Sermswan & Wongratanacheewin, 2017). *B. pseudomallei* is known to form biofilms though (Vorachit, Lam & Costerton, 1995), an important survival strategy for adaptation to harsh environmental conditions (Davey & O'toole, 2000; Hall-Stoodley, Costerton & Stoodley, 2004; Kamjumphol et al., 2013). Biofilm formation enables bacteria, including *B. pseudomallei*, to survive in the presence of chemical agents such as herbicides, pesticides, and disinfectants (Inglis & Sagripanti, 2006; Lima et al., 2020; Freitas et al., 2021) and against host immune responses as well as antibiotic treatments (Sawasdidoln et al., 2010; Duangurai, Indrawattana & Pumirat, 2018; Khamwong et al., 2022). Thus, eliminating bacteria within biofilms is challenging. Two methods have been proposed for eradicating biofilm-forming bacteria in the environment—the first disrupts the biofilm matrix to enhance chemical penetration and the second transitions the bacteria to the more susceptible planktonic form (Davies, 2003; Pakkulnan et al., 2019; Jalil & Ibrahim, 2021; Pakkulnan, Thonglao & Chareonsudjai, 2023).

Trichoderma is a genus of filamentous fungi belonging to the family Hypocreaceae, commonly found in soil and decomposing organic matter. *Trichoderma* species, including *T. asperellum*, produce primary and secondary metabolites that promote plant growth (Wu et al., 2017; Herrera-Téllez et al., 2019), inhibit bacterial pathogens of humans (Phupiewkham et al., 2015; Santos et al., 2018; Zhang et al., 2021) and demonstrate antagonistic activity against phytopathogenic fungi (Vizcaino et al., 2005; Vinale et al., 2014; Zeilinger et al., 2016; Herrera-Téllez et al., 2019; Khan et al., 2020). Given that *T. asperellum* is already mass-produced and distributed to farmers in Thailand for controlling phytopathogenic fungi (Kamkha, 2019; Chamswarn, 2020; Unartngam et al., 2020), it could potentially be repurposed for *B. pseudomallei* control if it is found to have sufficient activity against this species. To our knowledge, information regarding the

effects of *Trichoderma* on *B. pseudomallei* is very limited. In this study, we investigated the bactericidal and anti-biofilm properties of *T. asperellum* culture filtrates against *B. pseudomallei*. We also investigated the mechanism of action of *T. asperellum* culture filtrate using scanning electron microscopy to examine cell morphology and a crystal violet uptake assay to assess membrane permeability. These findings provide insights into the potential application of *T. asperellum* or its culture filtrates as biological or biochemical control agents against soil- and water-dwelling *B. pseudomallei*.

MATERIALS & METHODS

Fungal and bacterial strains

The *Trichoderma asperellum* strain used in this study was obtained from Khon Kaen Agricultural Technology Promotion Center (Plant Protection) in Khon Kaen Province, Thailand. The two bacterial strains, *Burkholderia pseudomallei* K96243 and *B. pseudomallei* H777 ([Taweekaisupapong et al., 2005](#); [Kunyanee et al., 2016](#); [Khamwong et al., 2022](#)), were provided by the Melioidosis Research Center at Khon Kaen University Faculty of Medicine.

Fungal cultivation and culture filtrate preparation

T. asperellum was cultured on potato dextrose agar (PDA) at 30 °C for 5 days. Thereafter, mycelial discs were cut and placed into 50 mL of potato dextrose broth (PDB), incubated at 30 °C in an incubator shaker at 125 rpm. The culture supernatants were collected on days 3, 5, 7, 9, 11 and 14 by centrifugation at 4,000 rpm for 10 min at 4 °C and filtered through a 0.2 µm filter before freeze-drying. The dried culture filtrates were weighed and dissolved in sterile distilled water to achieve a final concentration of one g/mL and then stored at 4 °C as stock solutions for further use ([Sangdee et al., 2016](#); [Chaianunporn et al., 2018](#)).

Bacterial culture conditions

B. pseudomallei K96243 and H777 strains, obtained from glycerol stocks, were cultured on Ashdown's agar at 37 °C for 48 h ([Kunyanee et al., 2016](#); [Pakkulnan et al., 2019](#); [Khamwong et al., 2022](#); [Pakkulnan, Thonglao & Chareonsudjai, 2023](#)). A single colony of *B. pseudomallei* was inoculated into three mL of tryptic soy broth (TSB), Mueller-Hinton broth (MHB) or Luria-Bertani (LB) broth and incubated at 37 °C at 200 rpm for 18 h, until the culture reached a concentration equivalent to a 0.5 McFarland turbidity standard ($OD_{600} = 0.08-0.1$). Thereafter, the bacterial cultures were diluted to approximately 10^6-10^8 colony forming units (CFU)/mL for the subsequent experiments ([Sangdee et al., 2016](#); [Pakkulnan et al., 2019](#); [Chaianunporn, Chaianunporn & Chareonsudjai, 2020](#); [Pakkulnan, Thonglao & Chareonsudjai, 2023](#)).

Preliminary screening of *T. asperellum* culture filtrates for antibacterial activity by the agar well diffusion method

The antibacterial activity of *T. asperellum* culture filtrates were examined against *B. pseudomallei* K96243 using the agar well diffusion method ([Sangdee et al., 2016](#); [Chaianunporn et al., 2018](#)). The *B. pseudomallei* K96243 inoculum in TSB (10^6 CFU/mL) was spread on Nutrient agar (NA) plates before punching them with a sterile cork borer.

One hundred microliters (μL) of *T. asperellum* culture filtrates from days 3, 5, 7, 9, 11 and 14 at a concentration of 400 mg/mL, along with sterile distilled water (negative control) and 30 $\mu\text{g/mL}$ ceftazidime (CAZ –positive control), were added to each well and incubated at 37 °C for 18 h. The diameters of the zones of inhibition around the culture filtrates and controls were then measured. Six replicates of each filtrate were tested. The culture filtrates with the largest inhibition zone were used for subsequent experiments.

Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *T. asperellum* culture filtrate against *B. pseudomallei*

Two-fold serially diluted *T. asperellum* culture filtrate with concentrations ranging from 500 to 0.24 mg/mL were prepared in 96-well microtiter plates. Subsequently, an inoculum of *B. pseudomallei* K96243 and H777 in MHB (10^6 CFU/mL) was added to each well and incubated at 37 °C for 18 h. Growth control wells were included on each plate. The MIC values were documented as the lowest concentration at which no visible bacterial growth was observed. The MBC values were determined by transferring suspension from wells that showed no visible growth onto NA plates. These plates were then incubated at 37 °C for 24 h (Chaianunporn, Chaianunporn & Chareonsudjai, 2020). Six replicates were performed for each *B. pseudomallei* isolate.

Evaluation of bactericidal activity of *T. asperellum* culture filtrate by time-kill assay

B. pseudomallei K96243 suspension (10^6 CFU/mL) was inoculated into TSB tubes containing different concentrations of *T. asperellum* culture filtrate at 7.81 mg/mL ($1\times\text{MIC}$), 15.62 mg/mL ($2\times\text{MIC}$), 31.24 mg/mL ($4\times\text{MIC}$), and 62.48 mg/mL ($8\times\text{MIC}$) along with a growth control tube without the culture filtrate were compared in this study. These tubes were incubated at 37 °C. Bacterial enumeration was performed at specified time intervals (0, 2, 4, and 24 h) using the drop plate method (Herigstad, Hamilton & Heersink, 2001). The number of bacteria remaining in each sample was plotted over time to determine the rate of killing. A three \log_{10} reduction in bacterial counts was used as the criterion for bactericidal activity (Sangdee et al., 2016; Thammawat, Sangdee & Sangdee, 2017). Each concentration was tested in triplicate.

Anti-biofilm activity of *T. asperellum* culture filtrate against *B. pseudomallei* biofilm

Biofilm formation of *B. pseudomallei* strain H777 was assessed using crystal violet staining in 96-well microtiter plates (Pakkulnan et al., 2019; Allkja et al., 2020; Pakkulnan, Thonglao & Chareonsudjai, 2023). Two hundred microliters (μL) of *B. pseudomallei*-inoculated Luria-Bertani (LB) broth (10^7 or 10^8 CFU/mL) was dispensed into each well of a 96-well flat-bottomed polystyrene plate. *T. asperellum* culture filtrate at 3.91 mg/mL ($0.5\times\text{MIC}$) and 7.81 mg/mL ($1\times\text{MIC}$) was then added to the cell suspension plates either at 0 h (adhesion stage treatment) or at 24 h (biofilm formation stage treatment). Each treatment plate was further incubated at 37 °C under static conditions. After incubation for 48 h, biofilms were carefully washed three times with 200 μL per well of sterile distilled water.

The biofilm in each well was fixed with 99% methanol for 15 min and air dried. Next, the biofilms were stained with 2% w/v crystal violet (the working solution was prepared at a ratio of Solution A (20 g of 85% crystal violet dye dissolved in 100 mL of 95% (v/v) ethanol), Solution B (one g of ammonium oxalate dissolved in 100 mL of distilled water), and distilled water as 1:8:1) for 5 min. Excess stain was gently washed with running tap water until clear. After air-drying, adherent crystal violet stain was dissolved in 200 μ L of 33% (v/v) glacial acetic acid. Finally, the solutions were transferred to a new 96-well plate and subsequently diluted five-fold with additional acetic acid. The optical density of each sample at 620 nm (OD_{620}) was measured using a microplate reader (TECAN Safire, Port Melbourne, Australia). The values were calculated by multiplying by 5 to determine the original optical density. Each concentration was tested eight times across three independent experiments.

Use of scanning electron microscopy to detect *T. asperellum* culture filtrate-induced changes in *B. pseudomallei* cell morphology

T. asperellum culture filtrate-treated *B. pseudomallei* K96243 were observed for morphological alterations using a scanning electron microscope. *B. pseudomallei* cells were suspended in TSB (10^6 CFU/mL) and treated with 3.91 mg/mL ($0.5\times$ MIC) and 7.81 mg/mL ($1\times$ MIC) of *T. asperellum* culture filtrate at 37 °C for 16 h. The treated samples were then fixed with 2.5% glutaraldehyde at 4 °C for 12 h, before being washed three times with phosphate-buffered saline (PBS). Bacterial cell dehydration was achieved using increasing concentrations of ethanol (30%, 50%, 80% and 100%). After this, the dried samples were mounted onto stubs, coated with 40–60 nm of gold, and observed using a desktop scanning electron microscope (MiniSEM; SEC model SNE-4500M, South Korea) ([Chaianunporn et al., 2018](#)).

Detection of *B. pseudomallei* membrane damage by crystal violet uptake assay

Alterations in the membrane permeability of *T. asperellum* culture filtrate-treated *B. pseudomallei* were assessed using the crystal violet uptake assay ([Devi et al., 2010](#); [Khan et al., 2017](#)). Suspensions of *B. pseudomallei* K96243 in TSB (10^6 CFU/mL) were harvested by centrifugation at 10,000 rpm for 5 min, then washed twice and resuspended in PBS. The cell suspensions were treated with 3.91 mg/mL ($0.5\times$ MIC) and 7.81 mg/mL ($1\times$ MIC) of the *T. asperellum* culture filtrate at 37 °C for 30 min. Cells treated with EDTA (0.25 M) were used as a positive control, and untreated cells were used as a negative control. After that, the bacterial cells were centrifuged at 10,000 rpm for 5 min and then resuspended in PBS containing 10 μ g/mL of crystal violet. The cell suspensions were then incubated at 37 °C for 10 min and centrifuged at 10,000 rpm for 5 min. Subsequently, the optical density of the supernatants at 590 nm (OD_{590}) was measured using a UV–VIS spectrophotometer (Naondrop2000C; Thermo Fisher Scientific, Waltham, MA, USA). Each concentration and control were tested in at least four replicates. The absorbance value of the initial crystal violet solution used in the assay was considered as 100%. The percentage of crystal violet uptake was calculated using the following formula:

$$\% \text{Crystal violet uptake} = (OD_{590} \text{ of the sample} / OD_{590} \text{ of crystal violet solution}) \times 100.$$

Table 1 Antibacterial activity of *T. asperellum* culture filtrates collected at different time points against *B. pseudomallei* K96243 assessed by zone of inhibition measurements.

<i>T. asperellum</i> culture filtrates	Inhibition zones (mm)
Day 3	19.33 ± 0.56 ^{ab}
Day 5	25.83 ± 1.09 ^c
Day 7	30.33 ± 0.19 ^d
Day 9	22.50 ± 1.31 ^{ab}
Day 11	17.83 ± 1.48 ^b
Day 14	21.00 ± 0.47 ^a
Ceftazidime (30 µg/mL)	37.67 ± 0.19

Notes.
 The data are represented as the mean ± standard error (SE) from six replicates.
 Different superscript letters a, b, c, d indicate statistically significant differences as determined by Tukey–Kramer test at $p < 0.05$.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by the Tukey–Kramer test at a significance level of 0.05 for comparing differences between mean values by using R version 4.4.0 (*R Core Team, 2024*).

RESULTS

Antibacterial activity of *T. asperellum* culture filtrates against *B. pseudomallei* by the agar well diffusion method

The *T. asperellum* culture filtrates collected on days 3, 5, 7, 9, 11 and 14 could all inhibit *B. pseudomallei* K96243, though the inhibition zone sizes varied significantly among the collection times (one-way ANOVA: $F_{5,30} = 26.58$, $p = 3.53\text{e}^{-10}$). Notably, the culture filtrate collected on day 7 (TD7) produced the largest zone of inhibition ([Table 1](#)). TD7 was therefore used for determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in subsequent experiments.

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *T. asperellum* culture filtrate against *B. pseudomallei*

The MIC and MBC values of TD7 against *B. pseudomallei* K96243 were both 7.81 ± 0.00 mg/mL. For *B. pseudomallei* H777, the MIC was 7.81 ± 0.00 mg/mL and the MBC was 11.72 ± 1.75 mg/mL.

Bactericidal activity of *T. asperellum* culture filtrate measured by time-kill assay

The relationship between culture filtrate concentration and antibacterial activity could be assessed over time using a time-kill assay. Treatment with 62.48 mg/mL (8×MIC) of TD7 demonstrated a potent bactericidal effect, completely eradicating *B. pseudomallei* K96243 within 30 min ([Fig. 1](#)). Reducing the concentration to 31.24 mg/mL (4×MIC) and

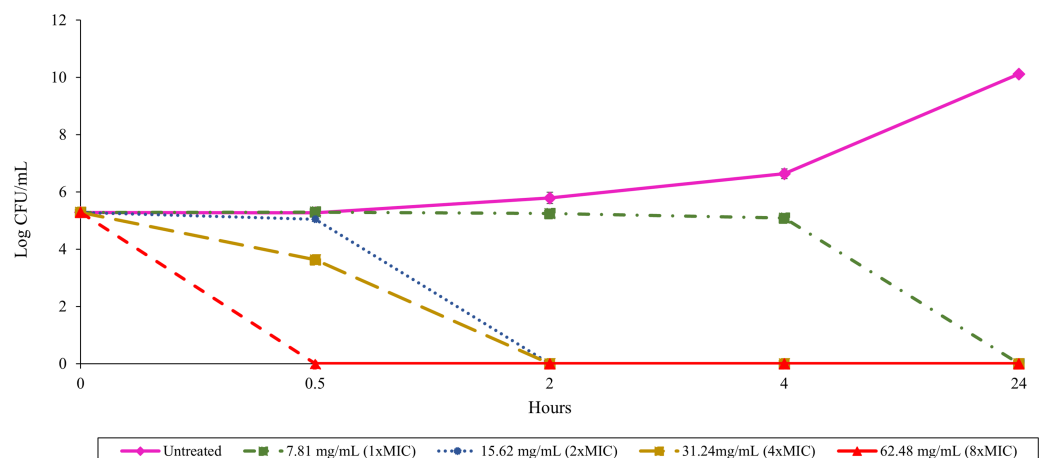


Figure 1 Time-kill assay with *B. pseudomallei* K96243 and varying concentrations of TD7. Different lines with symbols show results from different TD7 concentrations: untreated control (pink solid line with diamonds), treated with 7.81 mg/mL (green dash-dotted line with squares), 15.62 mg/mL (blue dotted line with circles), 31.24 mg/mL (yellow dashed line with squares) and 62.48 mg/mL (red dashed line with triangles) of TD7. The data are presented as the means \pm standard error (SE) from three replicates.

Full-size [DOI: 10.7717/peerj.19051/fig-1](https://doi.org/10.7717/peerj.19051/fig-1)

15.62 mg/mL ($2\times$ MIC) led to a slower bactericidal effect, with complete loss of bacterial viability occurring within 2 h (Fig. 1). TD7 maintained its bactericidal activity against *B. pseudomallei* K96243 at a concentration of 7.81 mg/mL ($1\times$ MIC) but took up to 24 h to kill bacteria (Fig. 1).

Anti-biofilm activity of *T. asperellum* culture filtrate against *B. pseudomallei* biofilm

After treating *B. pseudomallei* H777 with 3.91 ($0.5\times$ MIC) and 7.81 mg/mL ($1\times$ MIC) of TD7 at the adhesion stage (0 h) and biofilm formation stage (24 h), we observed that the higher concentration of TD7 was more effective in controlling biofilm formation compared to the lower concentration at both stages. At a concentration of 7.81 mg/mL, TD7 significantly reduced biofilm formation by 6-fold during the adhesion stage (Fig. 2A) and by 3-fold during the biofilm formation stage (Fig. 2B) compared to the control (one-way ANOVA at adhesion stage: $F_{2,69} = 801.7$, $p < 2e-16$, at biofilm formation stage: $F_{2,69} = 578.9$, $p < 2e-16$).

T. asperellum culture filtrate-induced morphological changes in *B. pseudomallei*

Scanning electron microscopy of untreated control cells of *B. pseudomallei* K96243 showed that they had smooth surfaces and intact membranes (Fig. 3A). In contrast, some *B. pseudomallei* K96243 cells treated with *T. asperellum* culture filtrate at 3.91 mg/mL ($0.5\times$ MIC) exhibited a shrunken appearance and a roughened surface (Fig. 3B). The higher the concentration of *T. asperellum* culture filtrate at 7.81 mg/mL ($1\times$ MIC), the greater the number of cells exhibiting this shrunken appearance and roughened surface (Fig. 3C).

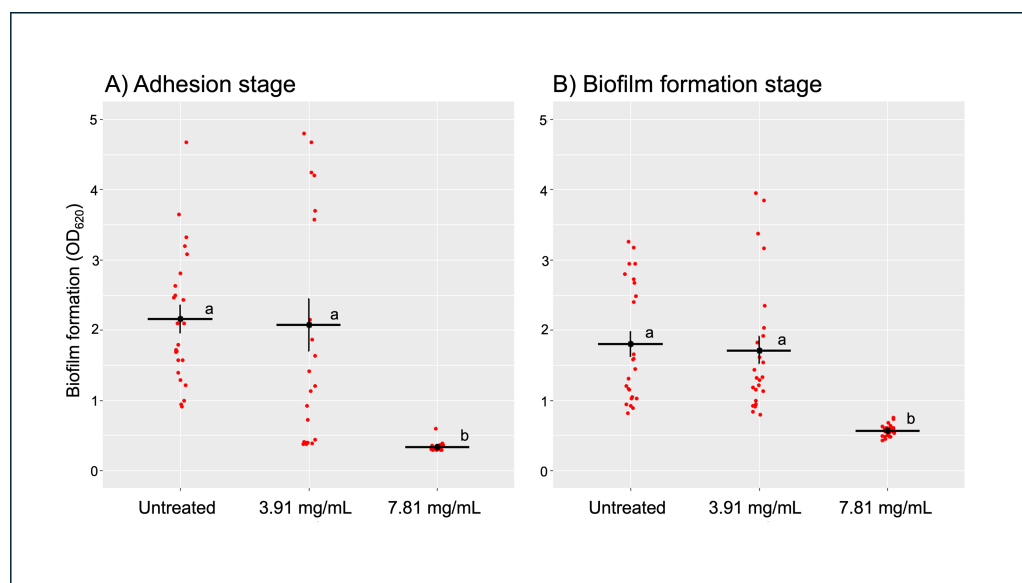


Figure 2 Quantitative analysis of *B. pseudomallei* H777 biofilm formation following treatment with TD7 during the adhesion stage (A) and biofilm formation stage (B). Each treatment included 24 replicates, comprising eight technical replicates from three independent experiments. Each point in the scatter plot represents an individual replicate, while horizontal lines indicate the mean, and vertical lines represent the standard error (SE). Statistically significant differences determined using the Tukey–Kramer test at $p < 0.05$, were denoted by different letters (a, b).

Full-size [DOI: 10.7717/peerj.19051/fig-2](https://doi.org/10.7717/peerj.19051/fig-2)

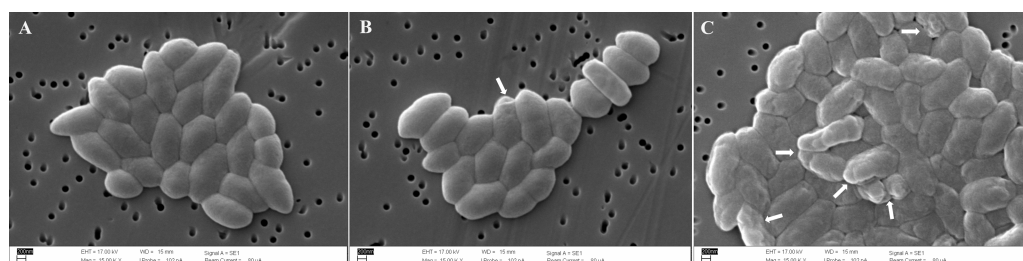


Figure 3 Scanning electron micrographs (15,000x) of untreated *B. pseudomallei* K96243 (A), *B. pseudomallei* K96243 treated with 3.91 mg/mL (0.5xMIC) (B) and with 7.81 mg/mL (1xMIC) TD7 (C). White arrowheads point to the locations of damaged cells.

Full-size [DOI: 10.7717/peerj.19051/fig-3](https://doi.org/10.7717/peerj.19051/fig-3)

T. asperellum culture filtrate-induced alterations in *B. pseudomallei* membrane permeability

Membrane permeability in treated and untreated cells of *B. pseudomallei* K96243 was evaluated by the crystal violet uptake assay (Fig. 4). In untreated (negative control) cells, the uptake of crystal violet was 14%. Following treatment with 3.91 mg/mL (0.5xMIC) and 7.81 mg/mL (1xMIC) of TD7, the uptake increased to 18% and 27%, respectively. In comparison, the uptake of crystal violet in EDTA-treated (positive control) bacteria was 32% (Fig. 4). We found that the crystal violet uptake for the bacteria treated with

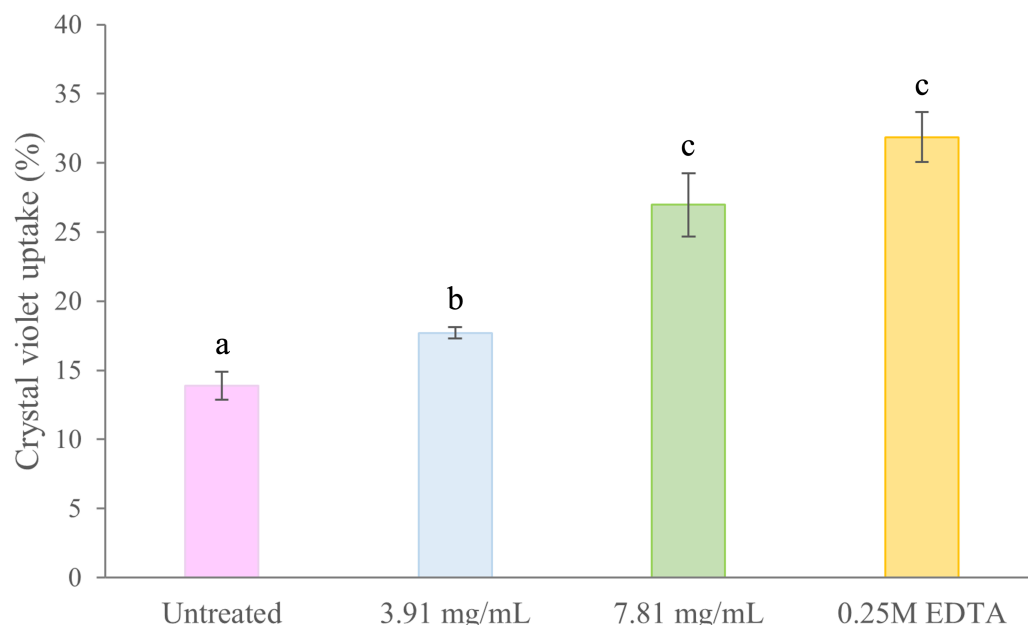


Figure 4 Crystal violet uptake (%) by *B. pseudomallei* K96243 cells treated with different concentrations of TD7. Two different concentrations of TD7 (3.91 mg/mL, blue bar, 7.81 mg/mL, green bar) were tested. Untreated cells (pink bar) were used as a negative control and 0.25M EDTA-treated cells (yellow bar) were used as a positive control. The data are presented as the means \pm standard error (SE) from at least four replicates. Different letters (a, b, c) indicate statistically significant differences by Tukey–Kramer test at $p < 0.05$.

Full-size [DOI: 10.7717/peerj.19051/fig-4](https://doi.org/10.7717/peerj.19051/fig-4)

3.91 mg/mL and 7.81 mg/mL of TD7 was significantly different from the untreated control (one-way ANOVA: $F_{3,8} = 45.24$, $p = 2.31e-05$).

DISCUSSION

Trichoderma species are fungi that are widely used in biocontrol due to their ability to promote plant growth (Wu et al., 2017; Herrera-Téllez et al., 2019), and inhibit microbial growth via mycoparasitism, antibiotic production, and competition for nutrients (Benítez et al., 2004; Vizcaino et al., 2005; Zeilinger et al., 2016; Zhang et al., 2021). This study highlights the antibacterial and anti-biofilm activity of *T. asperellum* culture filtrates against *B. pseudomallei*, the etiological agent of melioidosis. As rice farmers are typically at high risk of *B. pseudomallei* exposure (Limmathurotsakul et al., 2013), and both *T. asperellum* and *B. pseudomallei* are microorganisms naturally found in soil, *T. asperellum* might hold potential as a control agent against *B. pseudomallei* in endemic areas such as northeastern Thailand. This fungal strain benefits plants and combats phytopathogenic fungi; therefore, farmers may be particularly motivated to adopt its use. By integrating *T. asperellum* into their agricultural practices, the risk of *B. pseudomallei* infection could potentially be reduced. Consequently, this practice may contribute to broader public health benefits in regions prone to melioidosis.

We found that *T. asperellum* culture filtrates collected at different time points exhibit different levels of inhibitory activity against *B. pseudomallei*, with the filtrate obtained on day 7 (TD7) being the most potent. Our results are consistent with previous studies demonstrating that *Trichoderma* species exhibit the highest antibiotic production during the log phase of growth, typically between days 3 and 7 (Benítez et al., 2004; Vinale et al., 2014; Zeilinger et al., 2016).

The time-kill assay was used to determine whether *T. asperellum* culture filtrate components have a bacteriostatic or bactericidal effect against *B. pseudomallei*. Our results indicate that TD7 exhibits bactericidal activity against *B. pseudomallei* in both a time- and concentration-dependent manner, i.e., within 30 min at a concentration of 62.48 mg/mL (8×MIC), within 2 h at a concentration of 31.24 mg/mL (4×MIC) and 15.62 mg/mL (2×MIC), and within 24 h at a concentration of 7.81 mg/mL (1×MIC). This finding is consistent with previous studies indicating that metabolites with antimicrobial activity, secreted within culture filtrates, effectively kill pathogens in both a time- and concentration-dependent manner (Sangdee et al., 2016; Boottanun et al., 2017; Thammawat, Sangdee & Sangdee, 2017).

Biofilm formation is a significant factor contributing to the antibiotic resistance of *B. pseudomallei* and this presents a challenge for effective treatment (Sawasdidoln et al., 2010; Sirijant, Sermswan & Wongratanacheewin, 2016). We found that TD7 significantly reduces the ability of *B. pseudomallei* to establish biofilms during both the adhesion stage (0 h) and the biofilm formation stage (24 h). This effect may be attributable to TD7 inhibiting or killing bacteria before they can adhere to surfaces during the adhesion stage, thereby preventing subsequent biofilm development (Stoodley et al., 2002; Chung & Toh, 2014; Pakkulnan et al., 2019; Pakkulnan, Thonglao & Chareonsudjai, 2023; Shobha et al., 2023; Li et al., 2024). Given that TD7 also inhibits biofilm formation at the 24-hour time point, it may be that TD7 contains substances that disrupt the formation of early biofilm architecture. Disruption of biofilm architecture during the maturation process is an important strategy for eradicating biofilms (Stoodley et al., 2002). Our observation is in accordance with the study of Papaianni et al. (2020) which reported that *Trichoderma* extracts reduced biofilm formation by the plant pathogenic bacterium *Xanthomonas campestris*. Their research identified 6-pentyl- α -pyrone (6PP), a metabolite commonly found in various *Trichoderma* species (Khan et al., 2020), as the key component of an extract that could dissolve biofilms and inhibit their development and maturation. Further research on *T. asperellum* culture filtrate should be conducted to identify the active components effective against *B. pseudomallei* and its biofilm.

The morphological alterations of *B. pseudomallei* observed through SEM in our study suggests that TD7 may affect the bacterial cell wall and cell membrane. The membrane surface irregularities observed in this study closely resemble the alterations in *B. pseudomallei* treated with metabolites produced by *Bacillus amyloliquefaciens* KKU1 (Potisap et al., 2018). In the study of *B. amyloliquefaciens*, the cell wall abnormalities of *B. pseudomallei* were attributed to protein substances with antibacterial properties, specifically bacteriocins. Bacteriocins possess hydrophobic or amphiphilic properties, targeting bacterial membranes and causing pore formation or complete disintegration

of the cell wall (Baker, 1968; Potisap et al., 2018; Wu et al., 2017). The effect of TD7 on membrane permeability was evidenced by the higher uptake of crystal violet dye compared to untreated control cells. Therefore, *T. asperellum* culture filtrate might contain some lipophilic/hydrophobic substances that preferentially partition from an aqueous phase into bacterial membrane structures, resulting in membrane disruption, increased membrane fluidity and increased permeability (Devi et al., 2010; Khan et al., 2017).

CONCLUSIONS

Our findings show that *T. asperellum* culture filtrates have bactericidal activity and can reduce biofilm formation. This suggests that *T. asperellum* could be used to reduce the presence of *B. pseudomallei* in the environment and, consequently, lower the incidence of melioidosis. Thus, *T. asperellum* is one of potential biological control agents against *B. pseudomallei* in soil and water environment besides *Bacillus amyloliquefaciens* KCU1 (Potisap et al., 2018). In recognition that soil is a complex mixture containing diverse microbial communities, it is important to test the efficiency and impact of these biological control agents in real environment. Future studies by our group will thus assess the inhibitory effects of *T. asperellum* in co-culture with *B. pseudomallei* in soil. This will aid in evaluating the potential of *T. asperellum* as a biological control agent against *B. pseudomallei* in the natural environment.

ACKNOWLEDGEMENTS

We would like to thank the Melioidosis Research Center for providing *Burkholderia pseudomallei* strains K96243 and H777. We also extend our gratitude to the Khon Kaen Agricultural Technology Promotion Center (Plant Protection), Khon Kaen Province, for providing *Trichoderma asperellum*.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This research was funded by “The research capability enhancement program through graduate student scholarship” Faculty of Science, Khon Kaen University to Thotsapol Chaianunporn and Naritsara Roopkhan with grant number SCG-2022-2 and a research grant from Faculty of Medicine, Mahasarakham University to Kanokporn Chaianunporn. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Faculty of Science, Khon Kaen University: SCG-2022-2.
Faculty of Medicine, Mahasarakham University.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Naritsara Roopkhan conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Thotsapol Chaianunporn conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Soruksiri Chareonsudjai conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Kanokporn Chaianunporn conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data sets used for analysis, providing detailed information on the results of the study, are available in the [Supplementary Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.19051#supplemental-information>.

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