WALAILAK JOURNAL
http://wjst.wu.ac.th Agricultural Technology and Biological Sciences

Evaluation of Antifungal Activity of Antagonistic Bacteria against Butt Rot Disease Pathogen of Pineapple

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Received: 30 May 2017, Revised: 7 December 2017, Accepted: 26 December 2017

Abstract

Butt rot disease, caused by *Thielaviopsis paradoxa* (De Seynes) Hohn., is one of the major diseases in pineapple cultivation in Malaysia. The objectives of this study were to evaluate the antifungal effect of antagonist bacteria against *T. paradoxa*, a causal agent of butt rot disease, and to observe the mechanism of antifungal activity of tested antagonist bacteria microscopically. In this study, *in vitro* antifungal potential of 5 antagonist bacteria, namely B1, B2, B3, B4, and B5, were isolated from infected and non-infected soil samples and evaluated using dual culture method against *T. paradoxa*. The mechanisms of antifungal activities of antagonist bacteria against the pathogen were microscopically observed. All of the bacteria showed inhibitory effects against the pathogenic fungi. B1 bacteria showed the highest inhibitory potential, with 73 % inhibition, followed by B2, B3, B4, and B5, with 71, 57, 56, and 48 % of inhibition compared to control, respectively. The results also showed that B2, B3, and B4 bacteria exhibited positive inhibition towards the pathogen, with more than 50 % percentage inhibition. The development of a new product for use as a biocontrol agent, used as an additional control or used in combination with existing ones, may reduce dependency on chemical control and increase antagonistic activity efficiency.

Keywords: *Thielaviopsis paradoxa*, antifungal activity, antagonist bacteria, biocontrol agent, butt rot

Introduction

The pineapple, or *Ananas comosus*, belongs to the family of Bromeliaceae, and is an industrial crop that has been planted widely in the south of the Peninsular Malaysia, Johor, over an area of 9,093 hectares, producing the highest yield of pineapple, with 202,183 metric tons [1]. Pineapple is rich in micronutrients and antioxidants like vitamin C, polyphenols, flavonoids, and phytochemicals. It is consumed either fresh, for desserts, jellies or pastries, or processed, as purees, jams, or beverages [2]. The symptoms of butt rot disease can be found in planting materials like suckers, crowns, and slips, prior to or right away after planting. The primary infection commonly occurs in planting materials when the environment is wet and warm, which is the favored environmental condition for the fungi to grow in. The infection can occur through the broken end of planting materials. The soft base tissue turns into a gray and black rotting color, leaving a cavity and stringy fiber at the basal of the stem tissue. This disease is caused by a soil-borne pathogen fungus called *T. paradoxa* [3-5]. Pesticides are used to prevent or reduce damage caused by pests or pathogens, but these chemical substances may lead to various adverse effects in humans. The production of food can be increased by using naturally resistant varieties of crops, pesticides, or good cultural practices. However, negative trends in using chemical fungicides will have a

negative influence on the future of food security, increased disease outbreaks, and pathogen resistance. The applications of pesticides contribute to contamination of the environment and also harm to human health [6]. It is effective to use pesticides in controlling pest or pathogen infestation; however, this chemical substance has long-term toxicity effects on other living organisms, such as animals, humans, and crops [7]. The losses of crop production not only affect food security, but also the income of farmers and others who are dependent upon agricultural sectors. Disease problems must be solved before they reach economically damaging levels [8,9]. In sustainable agricultural practices, Integrated Disease Management (IDM) encourages farmers to minimize the use of pesticides and emphasize other control methods, like cultural control, resistance crops, and biological control. It can conserve the environment and natural resources, making it more beneficial to humans and non-target organisms. For biological control, antagonistic bacteria can be used as biocontrol agents to control disease infection, and have a broad spectrum of control on pathogenic fungi [10]. *Bacillus subtilis* has been previously reported against *Rhizoctonia solani*, showing a positive reduction in disease incidence caused by the pathogenic fungi [11]. Antagonistic bacteria will compete for nutrients with pathogens, especially with soil-borne pathogens, since endobacteria live inside the fungal cell, within the hypha, and will absorb nutrients from the cytoplasm of fungi to survive. Extracellular bacteria can affect spore production and fungal development, leading to inhibition of fungi growth [12,13]. This is in accordance with a previous study, where an antagonist bacteria, *Pseudomonas aeruginosa*, produced antibiosis as the main antifungal mechanism, causing malformation and swelling of the hypha, which significantly inhibited the growth of the fungi [14]. In another study, *Klebsiella* sp. and *Pseudomonas* sp. were found to have strong antifungal activities against 3 types of pathogenic fungi, *Aspergillus niger*, *A. flavus*, and *Fusarium oxysporum* [15]. The responsiveness towards environmental conditions, and the decrease in the efficiency of synthetic pesticides, over a period of time from previous usage of pesticides has encouraged people to develop new pesticides, by minimizing the contents of chemical substances or not using any [16]. In this study, research was carried out to evaluate the antifungal effect of antagonist bacteria against *T. paradoxa*, a causal agent of butt rot disease. Furthermore, this research highlighted the mechanism of antifungal activity of each antagonist bacteria against the pathogen microscopically.

Materials and methods

Isolation of bacteria

Soil samples were collected from infested and non-infested pineapple plants at Sungai Miang, Pekan, Pahang. The bacteria were isolated by using serial dilution method. About 1 g of soil sample was mixed with 9 ml sterile distilled water in a vial (10^{-1}). Then, about 1ml of each dilution (10^{-1}) was transferred to the next serial transfer (9 ml of diluent) until reaching 10^{-8} dilution. Then, about 0.5 ml of suspension of the final 4 dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) was put into Nutrient Agar (NA) plates. A flame sterilized hockey stick glass rod was used to spread suspensions. All of the plates were incubated for 24 h at room temperature. After 24 h incubations, the bacterial colonies that appeared on the NA plate were subcultured on the new NA plates to get pure cultures of the bacteria. Pure culture bacteria isolates (B1, B2, B3, B4, and B5) were grown and maintained in the NA, Tryptic Soy Agar (TSA), and selective media for *Pseudomonas* sp. culture.

Identification of bacteria: Gram Staining

The isolated bacterial were gram stained to determine their gram reactions. The bacteria specimens were mounted on a glass slide and air-dried. The slides containing bacteria specimens were heat fixed by using a Bunsen burner. The fixed specimens on slides were flooded with crystal violet for one minute, and rinsed with tap water. Then, the slides were flooded with iodine solution for one minute, and rinsed again with tap water. The slides were decolorized with 90 % alcohol, until the slides were clear from stains, and rinsed again with tap water. Lastly, the slides were flooded with safranin for 30 s, then rinsed with tap water. The slides were air-dried and observed under a light microscope.

Isolation of fungal pathogen

T. paradoxa was isolated from infected pineapple that showed typical symptoms of butt rot, collected from the farmers' field, Pekan, Pahang. The samples were put in moisturize plastic bags, to maintain freshness, and brought to the laboratory. The samples were cut into small pieces and surface sterilized by soaking in 10 % sodium hypochlorite for 5 min. After that, the samples were rinsed twice with sterile distilled water. The samples were then blotted dry with sterile tissue paper. Each of the samples were then put in fresh Potato Dextrose Agar (PDA) and incubated at room temperature for 48 h. After 48 h incubation, mycelium appeared and was subcultured in fresh PDA. The isolated fungus was then identified, based on morphological characteristics. The morphological characteristics were observed and compared to references [17,18]. The fungal pathogen was then maintained on the PDA medium until further use.

Pathogenicity test

The isolated fungi were tested for pathogenicity by using pineapple plants in the laboratory. Six mm diameter plugs from 7-day-old cultures of *T. paradoxa* that were grown on PDA were placed in contact onto wounds at the basal stem of plants, created by using a sterilized knife. Moist sterilized cotton was placed on the agar plugs and wrapped with plastic tape to encourage the growth of fungi and to prevent desiccation. Three pineapple plants were inoculated with the isolated fungi and were maintained at room temperature for 10 days incubation. The pathogens was re-isolated from diseased tissues to determine their morphological characteristics. The fungi were observed under a light microscope for their morphological characteristics and compared with previous isolated fungi.

Antifungal activity assay of antagonistic bacteria

The isolated bacteria (B1, B2, B3, B4, and B5) were tested against *T. paradoxa* using dual culture method. Bacterial strain was streaked on TSA (Tryptic Soy Agar) media at 2.5 cm distance from mycelia plugs of fungi on both sides. For a negative control, mycelia plugs of fungi were placed at the center of a TSA agar plate, without any bacteria streaked, and commercial fungicide (Benomyl) was used as a positive control in this study. The experiment was set up in a completely randomized design, with 10 replications for each treatment. All of the plates were incubated at room temperature, and data was collected by measuring radial growth of fungi toward bacteria colonies daily, up to 6 days of incubation. Percentage of inhibition was calculated by using PGI formula [19];

$$\text{Percent Growth Inhibition (PGI)}: \frac{(\text{Dc}) - \text{Dt}}{\text{Dc}} \times 100 \quad (1)$$

where Dc is Distance of radial growth of fungi, and
Dt is Distance of fungal growth from inoculation to bacteria colony on treated plate.

Microscopic observation of the effects on antifungal activity by antagonist bacteria

The observation of antifungal activity by biocontrol agents was made at the edge of the inhibition zone of fungal growth. All of the prepared slides were observed from 10× to 40× magnification under a light microscope. The normal growth of *T. paradoxa* was represented by the healthy growth of hyphae. The change of structures and morphologies of fungi were observed and compared with healthy structures.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and one way ANOVA was used to determine significant differences among each treatment at a 5 % ($p < 0.05$) level of significance.

Results and discussion

Isolation of disease and identification of fungal pathogen

Butt rot disease caused by *T. paradoxa* initiates infection through wounds occurring when vegetative planting materials have been removed from parent plants. The wounds open up a way for the pathogen to penetrate into the plant. Infected samples exhibiting symptoms of black rot at the basal stem of the pineapple, in which the internal tissues had turned from brown to black [5,20-22], were collected. **Figure 1** shows the symptoms of butt rot disease on pineapple basal stems. Infected plants exhibit soft rot with a black color of tissue due to the black color of chlamydospores at the pineapple basal stem [23]. Isolations of diseased samples resulted in several fungal growths, and each of the fungi were sub-cultured into fresh new PDA plates. Each of the isolated fungi was then observed for morphological characteristics to identify *T. paradoxa*. The identification of fungi was through microscopic examination. The morphological characteristics of this fungus were identified based on references [17,20,21,24], which stated that conidiosphores were straight, colorless to light brown color, septated in form at the base, and can growth up to 250 µm long; conidia were in cylindrical to slightly ellipsoidal shapes, ranging from 4 - 21 × 2 - 6 µm, colorless to light brown color, smooth, and in chains in side branches of hyphae; and chlamydospores were brown to brownish color, oval shaped, smooth, thick-walled, in terminal chains in hyphal branches, and ranged from 9 - 25 × 5 - 15 µm. These characteristics are shown in **Figure 2**. Seven-day-old cultures of *T. paradoxa* on PDA could be recognized visually based on the development of black color culture on petri plates, as seen in **Figure 2**. Mycelium of these fungi was white in color, then turned to black when the culture matured [25]. Based on the morphological characteristics, it can be confirmed that the isolated fungal pathogen was *T. paradoxa*.

Figure 1 Infected samples of pineapple caused by *T. paradoxa*, pathogen of butt rot disease

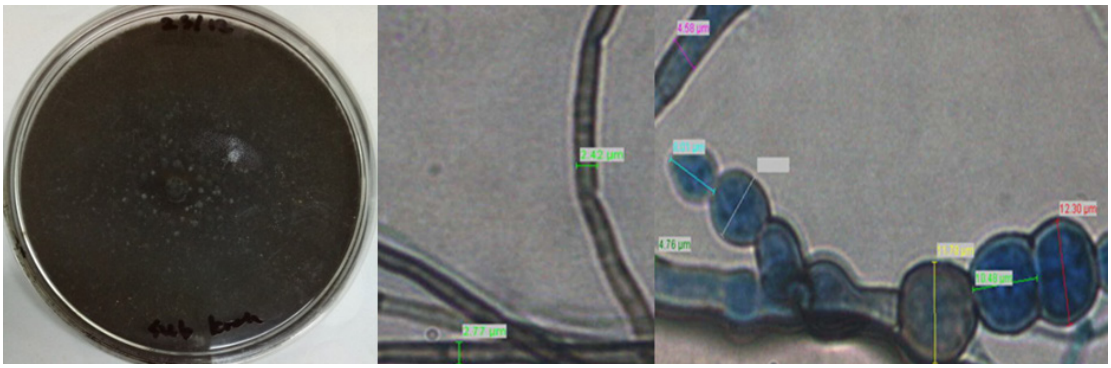

Figure 2 The black colour of *T. paradoxa* culture on petri plate and morphological characteristics of the pathogen.

Isolation and identification of bacteria: Gram Staining

The antagonistic bacteria were isolated from infected and non-infected soil in the pineapple field. Isolation of antagonistic bacteria resulted in 6 isolates, of which 5 were isolates from an infected area, and one was from a non-infected area. However, the isolate from the non-infected area showed the same characteristics as one of the isolates from the infected area. Thus, the total number of isolates were only 5. The isolates were designated as B1, B2, B3, B4, and B5. Pure cultures of the bacterial isolates were maintained in Nutrient Agar (NA), Tryptic Soy Agar (TSA), and Pseudo-F media, until further use.

The characteristics of the bacteria were determined morphologically, as shown in **Table 1**. The bacteria B1, B3, B4, and B5 were gram negative bacteria, which exhibited different colors of colonies, such as blue-green, orange, dark purple, and pink-peach. The bacteria B2 was gram positive, exhibiting a milky white colony color. All isolated bacteria showed a rod-shaped appearance of morphology, except for bacteria B3, which showed a round-shaped morphological appearance, as per **Table 1**.

Table 1 Morphological characteristics and gram staining of isolated bacteria (B1, B2, B3, B4, and B5).

Bacteria	Origin	Colony color	Appearance morphology	Gram staining
B1	Soil	Blue-green	Rod-shaped	Gram negative
B2	Soil	Milky white	Rod-shaped	Gram positive
B3	Soil	Orange	Round-shaped	Gram negative
B4	Soil	Dark purple	Rod-shaped	Gram negative
B5	Soil	Pink-peach	Rod-shaped	Gram negative

Bacteria are abundant, and are some of the important microorganisms present in soil [26]. Soil-borne microbes have the potential be used in antagonist actions against some pathogenic microorganisms [27]. According to [28], a total of 342 microorganisms were isolated from soil and, out of them, only 22 bacterial were identified, which resulted in 2 types of bacteria, namely *Bacillus* sp. and *Pseudomonas* sp. Another study by [29] found that, from 29 isolated soil bacteria, there were 6 types of bacteria identified, namely *Bacillus* sp. *Sphingomonas* sp., *Kocuria rhizophila*, *Microbacterium ginsengisoli*, and *Variovorax paradoxus*.

Based on morphological observation, in bacteria B1, probably *Pseudomonas* sp. according to [30], the *Pseudomonas* colonies exhibited blue-green color on media, had rod-shaped morphological appearance, and were gram negative bacteria. Bacteria B2, which could have been *Bacillus* sp. according to [31,32], was gram positive, with rod-shaped morphology, and exhibited a white colony color. Furthermore, bacteria B4 and B5 could have been *chromobacterium* sp. and *serratia* sp., based on morphology stated by [33], who reported that *chromobacterium* were gram negative, rod-shaped bacteria, and exhibited a purple colony color; meanwhile, [34] stated that *Serratia* were gram negative and rod-shaped bacteria, and exhibited dark red to pale pink colony colors.

In vitro* assay of antagonist bacteria against *T. paradoxa

Antagonistic activity was studied by using soil-borne bacteria in order to select effective biocontrol agents that were capable of inhibiting the development of pathogenic fungi on pineapple. Five isolated bacteria were screened for their antifungal activity against *T. paradoxa*. The results of this antifungal activity screening are presented in **Table 2** and **Figure 3**. Four of the isolated bacteria (B1, B2, B3, and B4) showed positive inhibition against fungal growth, exceeding 50 % of inhibition after 6 days of incubation. **Table 2** shows that B1 was the most effective in inhibiting the growth of *T. paradoxa*, with 73 % radial growth inhibition. B2, B3, B4, and B5 bacteria gave 71, 57, 56, and 48 % radial growth inhibition, respectively. The tested fungal pathogen showed significant inhibition of radial growth after being exposed to the tested bacteria strains. However, comparing them to the positive control showed that the positive control gave the highest inhibition, at 85 %.

Antagonist bacteria have a broad spectrum of antifungal activity on different plant pathogen species. The results of the study showed that all isolated antagonist bacteria exhibited an antifungal activity against *T. paradoxa*, the causal agent of butt rot disease on pineapple, to various degrees of percentage inhibition, **Figure 4**. Idris *et al.* [35] reported that, of 78 strains of bacteria isolated from soil, 23 of the isolates showed various antifungal activity. ranging from 30 to 66 % mycelial growth inhibition. against *F. oxysporum*. The highest inhibition (66 %) turned out to be *Chromobacterium violaceum*.

The ability of bacteria to multiply rapidly and produce certain antibiosis and enzymes can be an advantage for them in suppressing the growth of pathogenic fungi. *Pseudomonas* sp. produces siderophore, and some of the *Bacillus* sp. produce chitinase, during antagonistic activity [36]. In another study, antagonist bacteria were also found to parasitize the mycelium, and caused vacuolation and malformation of pathogenic hyphae [14]. In yet another study, antagonist bacteria were found to produce glycoproteins and other compounds like lipopeptides [37]. Moreover, Wang *et al*. [38] found that *Serratia marcescens* produce aflatoxin and chitinase as its antagonistic mechanisms for suppressing growth of other microorganisms. It also stated that the bacteria showed high mycelial growth inhibition against *Aspergillus parasiticus*, with more than 95 % inhibition. The same results were found by [39], who also studied *Serratia marcescens* as an antagonistic bacteria against *Rhizoctonia solani*. The study stated that the bacteria inhibited the growth of fungus pathogen through the production of antibiosis. In agar-well diffusion testing, the growth of the tested fungus was reduced to 30 %, compared to the normal growth of bacteria; however, the pouring test showed better antagonist activity, by reducing it up to 65.6 %. Bacteria that had been tested under greenhouse conditions reduced disease incidence up to 100 % for stem canker disease and 36.47 % for black scurf disease. The results of this study showed the highest inhibition for *in vitro* study at 73 % inhibition, higher than the percentage inhibition recorded in the previous study. This showed there is a potential for the bacteria tested in this study to reduce disease incidence in the field. Altinok *et al*. [40] reported that most of the 7 strains of *Pseudomonas* sp. showed high inhibition toward *F. oxysporum*, ranging from 39 to 72 % mycelial growth inhibition. Meanwhile, 3 *Bacillus* sp. showed more than 50 % inhibition, ranging from 52 to 55 % inhibition of mycelial growth.

Table 2 Inhibition percentage of antagonist bacteria against *T. paradoxa* on TSA after 6 days of incubation by dual culture method.

Types of Bacteria isolate	Radial growth (cm)	Percentage of inhibition (%)
B1	1.08 ± 0.1317	73
B2	1.17 ± 0.1636	71
B3	1.71 ± 0.2885	57
B4	1.75 ± 0.3810	56
B5	2.10 ± 0.4450	48
Negative Control	4.00 ± 0.0000	0
Positive Control	0.62 ± 0.1269	85

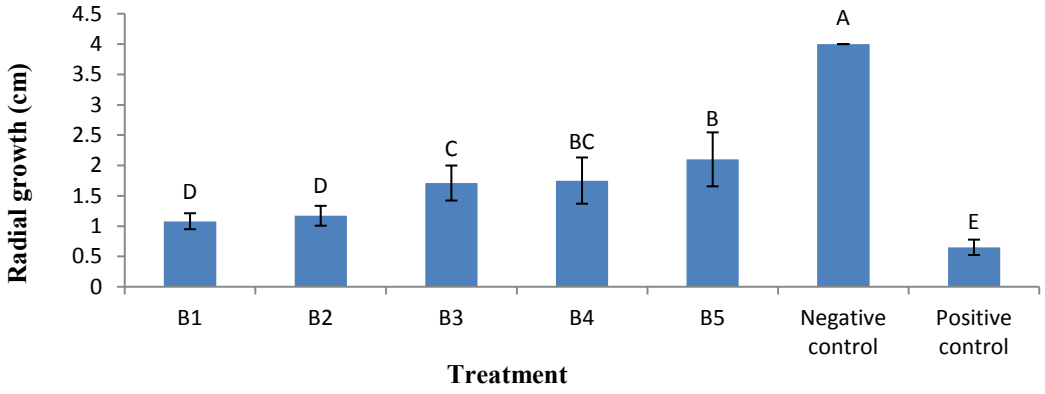

Figure 3 Antifungal activity of antagonistic bacteria (B1, B2, B3, B4, and B5) against *T. paradoxa* in dual culture after 6 to 7 days of incubation.

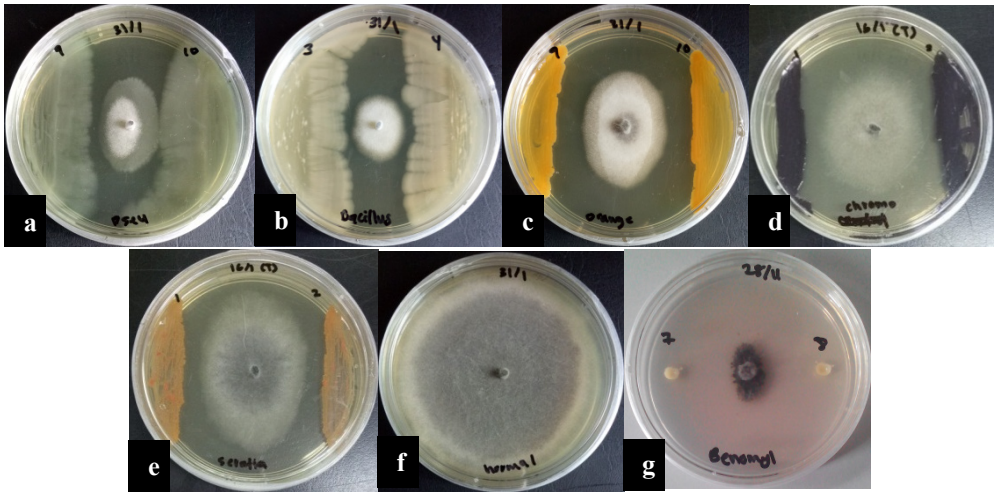

Figure 4 Antifungal activities of 5 isolated bacteria against *T. paradoxa* with positive control (Benomyl) and negative control (normal growth).
Legend: (a) B1, (b) B2, (c) B3, (d) B4, (e) B5, (f) negative control, (g) positive control.

Microscopic observation of antifungal activity by antagonist bacteria

The mechanism of antifungal activities by 5 antagonist bacteria was observed by taking the edge of the inhibition zone of fungal growth. The antifungal mechanism caused abnormalities in hyphae; all of the isolated bacteria caused hyphae to become coagulated, coiled, destructed, malformed, and retarded after 7 days of incubation. This fungus usually takes 7 to 10 days to mature. As seen in **Figure 5**, the observation of antifungal mechanisms revealed that B2 bacteria caused thorn-like structures of hyphae, while B2, B3, and B5 bacteria inhibited the growth of isolated pathogens through breakage of hyphae. *Bacillus subtilis* as antagonist bacteria have been reported to possess significant antifungal activity against fungal plant pathogens like *Fusarium equiseti*, *F. verticilloides*, and *Rhizoctonia solani* by exhibiting antibiosis and production of specific cellular enzymes which could be colorless metabolites that spread into the agar as antifungal mechanisms [41]. In addition, B3, B4, and B5 bacteria have the same

mechanisms, like the formation of dark spots on hyphae and swelling in hyphae, whereas healthy hyphae have smooth and clean surfaces, as seen in **Figure 6**. The antifungal mechanisms of antagonist bacteria may involve induction of host resistance, parasitism, production of antibiosis, and direct competition for space and limited resources. Multiple mechanisms were used by antagonist bacteria to suppress pathogen growth, such as mycoparasitism, which directly attacks the pathogen, antibiosis suppression, which is a production of toxic and poisonous substances that are efficient at low concentration, and enzymes, which degrade the cell wall and which involve the production of enzymes that can hydrolyze the proteins, hemicellulose, cellulose, and chitin of pathogens [42]. Lastly is direct competition, which is the rapid colonization of bacteria, giving an advantage to consuming the available space and limited nutrients, compared to the pathogen [43].

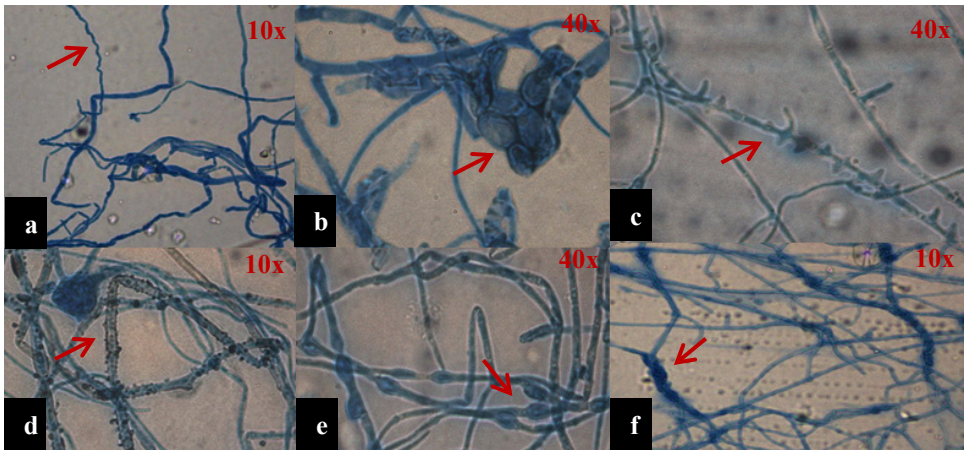

Figure 5 Microscopic observation of antifungal mechanisms of antagonistic bacteria (B1, B2, B3, B4, and B5) under 10× and 40× magnification of digital light microscope.
Legend: (a) retarded and shrunken hyphae, (b) coagulated and malformed hyphae, (c) thorn-like structures of hyphae, (d) dark spot on hyphae, (e) swollen hyphae, (f) coiling of hyphae.

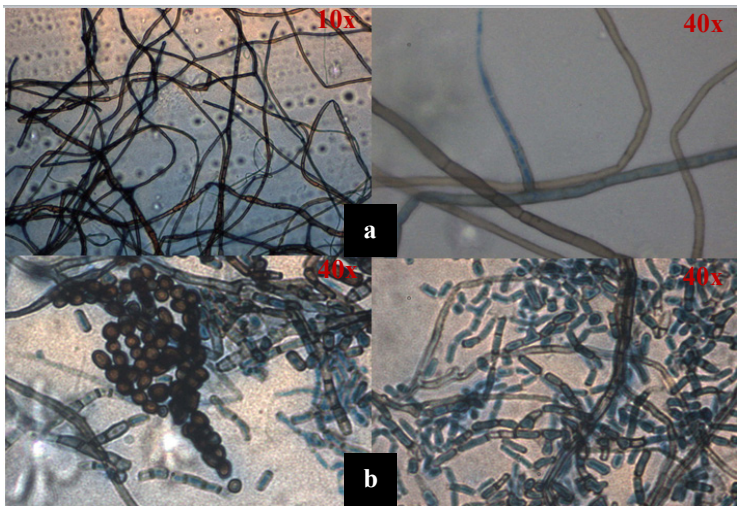

Figure 6 Microscopic observation of healthy hyphae and spores of *T. paradoxa* under 10× and 40× magnification of digital light microscope.
Legend: (a) hyphae of fungi (above,) (b) the conidia, conidiosphores, and chlamydospores (below).

Conclusions

The findings in this study conclude that antagonist bacteria can effectively control the pathogen of butt rot disease. All isolates of soil-borne bacteria, namely, B1, B2, B3, B4, and B5 bacteria, showed strong antifungal activity by exceeding 50 % of radial growth inhibition percentage, except for B5 bacteria, with 48 % of radial growth inhibition, using dual culture tests. This study suggests that bacteria may produce inhibitory substances and enzymes that are toxic to isolated pathogens and may change the morphology of those pathogens. A potential biocontrol agent, like antagonist bacteria, can be improved by continual improvement in the isolation, antifungal screening, formulation, and application methods that are effective in controlling the disease *in vitro* and *in vivo*.

Acknowledgements

This work was financially supported by Universiti Teknologi Mara (UiTM). I would like to express my special gratitude to my supervisor and co-supervisor, as well as to other lecturers and laboratory assistants who guided and helped me a lot while doing this study. Also, thank to my parents and friends for their support and help in finishing this study.

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