Effect of Selected Pesticides on Survivability of Exserohilum Longirostratum, A Bioherbicide for Itchgrass

Potential of Pseudomonas sp. (UniSZA-MKB10) and Bacillus spp. (UniSZA-BK3, UniSZA-BK4 and UniSZA-DA) as Biological Control Agent for Controlling Anthracnose Disease of Carica papaya L.

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Keywords:
Antracnose,
Carica papaya,
Plant disease,
Pseudomonas sp.,
Bacillus sp.,
Biological control
**ABSTRACT**

*Colletotrichum gleosporioides* is known as the causal agent of anthracnose disease of *Carica papaya* in Malaysia. Economic losses are mostly reported during postharvest where the disease incidence and severity was recorded as 90-98 % and 25-38 % respectively. *Colletotrichum* sp. were isolated from lesions of infected *C. papaya* that showing the typical anthracnose disease symptoms. Three types of fungal cultures were obtained and identified as *Colletotrichum* sp., *Fusarium* sp. and *Rhizoctonia* sp. One of the fungal obtained that was confirmed to be *C. gleosporioides* is based on morphological characteristics on PDA and Koch’s Postulate has confirmed *C. gleosporioides* as causal agent of papaya anthracnose disease. The pathogen under study varied in its ability to grow under different environmental conditions. However, isolate preferred temperature range of 20°C to 30°C for the growth on PDA media. *C. gleosporioides* isolates grew well at pH values within range of pH 6 to pH 7. Four bacterial strains, UniSZA-MKB10, UniZA-BK4, UniSZA-BK3 and UniSZA-DA, were tested in this study and proved to have antifungal properties against *C. gleosporioides*. Dual culture assay, double layer and double plate method were conducted to test their metabolites as well as diffusible and volatiles antibiotic compound of bacteria. Out of four, three bacterial were found to show inhibition ability with MKB10 showed highest percentage of inhibition on radial growth with 62.08 ± 3.64% PIRG as well as produced greater diffusible and volatiles substances that significantly (P<0.05) inhibited the mycelial growth of *C. gleosporioides* with PIRG of 85.48 ± 1.53 % and 31.03 ± 2.56 % respectively, and followed by UniSZA-DA. Culture filtrate of BK4 also showed highest significance effect towards inhibition of spore germination where the germ tube produced is the shortest (0.9055 μm).

**Keywords:** Antracnose, *Carica papaya*, Plant disease, *Pseudomonas* sp., *Bacillus* sp., Biological control

**ABSTRAK**

*Colletotrichum gleosporioides* dikenali sebagai agen penyebab penyakit antraknos (bintik berpusar) *Carica papaya* (betik) di Malaysia. Kerugian dilaporkan berlaku pada peringkat lepas tuai yang mana kehadiran penyakit serta keterukan penyakit adalah sekitar 90-98% dan 25-38% bagi setiap satunya. *Colletotrichum* sp. telah berjaya dipencilkun dari bahagian berpenyakit pada *C. papaya* yang menunjukkan simptom penyakit antraknos. Tiga jenis kultur kulat telah berjaya diperolehi iaitu, *Colletotrichum* sp., *Fusarium* sp. and *Rhizoctonia* sp. Satu daripada kulat yang diperolehi telah berjaya dikenal pasyi sebagai *C. gleosporioides* berdasarkan kriteria morfologi yang ditunjukkan di atas media PDA. Manakala Koch’s Postulate telah membuktikan bahawa *C. gleosporioides* adalah agen penyebab kepada antraknos betik. Patogen yang dikaji menunjukkan pelbagai keupayaan di bawah keadaan yang berbeza. Walau bagaimana pun, patogen menunjukkan kebolehan yang baik pada suhu antara 20°C ke 30°C di atas media PDA. *C. gleosporioides* juga menunjukkan pertumbuhan yang baik pada pH antara pH6 ke pH7. Empat strain bakteria iaitu UniSZA-MKB10, UniZA-BK4, UniSZA-BK3 and UniSZA-DA telah diuji dalam kajian ini dan terbukti memiliki kandungan anti-kulat terhadap *C. gleosporioides*. Ujian dua kultur, dua lapisan, dan dua piring berkingting telah dilakukan untuk menguji metabolit juga kompuan antibiotic yang resap dan merupak pada bakteria. Daripada empat kultur bakteria, tiga darinya menunjukkan keupayaan perencatan, dengan MKB10 menunjukkan peratusan paling tinggi terhadap jejari pertumbuhan dengan 62.08 ± 3.64% PIRG. MKB10 juga merupakan pengeluar terbanyak kandungan resap dan merupak yang telah secara beerti (P<0.05) merencakat pertumbuhan *C. gleosporioides* dengan nilai PIRG sebanyak 85.48 ± 1.53% dan 31.03 ± 2.56% bagi setiap satunya, diikuti UniSZA-DA. Manakala kultur turasan menunjukkan perbezaan beerti terhadap perencatan spora kulat dengan penghasilan tiub percambahan paling pendek (0.9055 μm).

**Kata Kunci:** Antraknos, *Carica papaya*, Penyakit tumbuhan, *Pseudomonas* sp., *Bacillus* sp., Kawalan biologi
INTRODUCTION

Anthracnose is a well-known disease among farmers especially those who cultivated fruit tree. Anthracnose may infect wide range variety of plant, from tropical to subtropical crops. There are also cases where anthracnose disease in a crop is caused by several Colletotrichum species such as strawberry anthracnose caused by *C. acutatum*, *C. fragariae*, *C. gleosporioides* (Smith and Black, 1990). *Colletotrichum gloeosporium* is well-known as a causal agent for anthracnose disease in most of fruits the tropics such as papaya, mango, guava and capsicum while banana is caused by *C. musae*. On papaya, anthracnose appears primarily as water-soaked spots that become sunken, turn brown to black, and enlarge to 5 centimeters or more in diameter. Pinkish-orange areas are formed by the conidial masses that cover the lesion center. As the fruit ripens, these spots rapidly enlarge up to 20 mm in diameter, to form the characteristic circular sunken lesions. Infected fruit is of much low quality and of course reduced the price. Most significant economic losses are reported occur during post-harvest (Freeman et al., 1998). Geographically, the climate of Malaysia is highly conducive to maintain and cause outbreaks of anthracnose all year round, thus, the development of management recommendations will be inevitable for anthracnose control (Mahmodi et al., 2013). This study was conducted primarily to determine the most suitable biological control agent of anthracnose disease of papaya caused by *C. gleosporioides*. Biocontrol approach is necessary to be developed as an alternative control methods to chemical. It is generally more safe and environmental friendly but these potential bio control agents need to be tested.

MATERIALS AND METHODS

Isolation and Identification of *Colletotrichum* sp.

Sampling of Anthracnose Fungi

Causal agent of anthracnose disease, *Colletotrichum* sp. was isolated from lesions of infected *Carica papaya* L. from a supermarket in Jerteh, Terengganu, Malaysia. The infected fruits were taken to the laboratory and isolated using the protocol as outlined by Cai et al., (2009).

Pathogen Isolation

Infected part of fruit was cut into small pieces of 1 cm² along with some healthy tissue. Then soaked into 10% sodium hypochlorite for 30 sec, 70% ethanol for 30 – 60s and washed with distilled water for 60s. Dried with sterile filter paper and immediately placed on PDA. Mix colonies of fungi isolates were then re-isolated again to obtain pure culture for each plate. Plates incubated at 30°C in incubator. The observation on colony morphology was done by naked eyes and mycelium and conidia was viewed under light microscope.

Pathogenicity Test

Koch’s Postulate was conducted twice. Healthy and uninjured fruits were undergoes surface sterilized by using 70 % ethanol for thirty seconds and rinsed thrice in sterile distilled water. The inoculated fruits were kept along with wetted tissue at the bottom of the container, to maintain humidity and incubate placed at dark place. The pathogen was re-isolated from the diseased portion and compared with original cultures to prove Koch’s postulates. The process of Koch’s postulate consists of three steps where the pathogen must be isolated from infected fruits and grown in pure culture. Next, the culture pathogen should cause anthracnose when introduced into healthy fruits. Then, the pathogen from inoculation will be re-isolated and the identity of original specific causative agent can be identified (Agrios, 2005).

Preparation of Pathogen and Treatment Sample
Preparation of Conidial Suspension of C. gloeosporioides
Spores of C. gloeosporioides that was grown on PDA at 30°C for 7 days were subsequently harvested by flooding the surface of media with sterile distilled water and gently agitating the plate with a bent glass rod to dislodge the spores. The resulting suspension was then filtered through two layers of sterile muslin cloth and kept in the sterile falcon tube. The concentration of conidia in the filtered suspension was adjusted to $5 \times 10^5$ conidia per mL with sterile distilled water by using haemacytometer (Sariah, 1994; Obagwu and Korste, 2003).

Preparation of Aqueous Antagonist Suspension
To prepare the aqueous antagonist suspension, overnight culture of all antagonistic bacterial were grown on NA agar at 30°C. Two loops of each culture was then transferred to a 250 mL conical flask containing 50 mL of nutrient broth (NB, Difco) and incubated on rotary shaker at 120 rpm for 48 h at room temperature. The isolates were kept in freezer at 5 °C for further used.

Preparation of Filter Sterilized Culture Filtrate
Culture filtrates of all isolates were prepared by using NB medium following the procedure as described by Yoshida et al. (2001) and used to determine their effect on fungal growth. NB contained isolates were centrifuged at 9000 rpm for 10 min. Pellets obtained were discarded. Supernatant was filtrated by using 0.45 mm syringe filter and kept in sterile falcon tube at 5°C.

Screening of Antagonist Activity
Dual Culture Assay
Dual culture plates were prepared as described by Salhah et al., (2016) and Sariah et al., (1994). 7 mm fungal disc was inoculated at center of petri dish and antagonists bacteria (MKB10, B40, B43 and DA) were inoculated 2.5 Cm away from the center. Plates were incubated for seven days at 25 °C. Radial growth of the pathogen was measured and compared to control, where the bacterial suspension was replaced by sterile distilled water. Results are expressed as the means of the percentage inhibition of radial growth (PIRG) based on following formula (Rahman et al. 2007; Sariah, 1994):

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

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

Each treatment was carried out with four replicates with two trial. The experiment was arranged according to completely randomized design.

Double Plate
PDA plates were inoculated at center of petri dish with a 0.5 Cm diameter of mycelia and NA plates were streak with antagonistic bacteria. Fungal isolates and antagonistic bacteria were separately cultivated. The lids of two plates that contained each bacterial and fungal respectively were removed. Plate contained bacteria was inverted and placed on top of the other plate. The two plate bases were seal with a double layer of parafilm. All plates were incubated at 25 °C for seven days. Controls were prepared with sterile water instead of the antagonist culture. All treatment was conducted in four replicates. Radial growth of fungus was recorded every day. PIRG of antagonist against pathogenic fungus was measured after incubation period and statistical analyses were performed.

Double Layer
To carry out this test, both of the PDA and NA were poured on the same plate. After both of the media prepared and autoclaved, NA was poured as a first layer and left to cool. After PDA was solidified,
A sterile filter paper was put on top of the PDA and NA was poured as a second layer. Then, antagonistic bacteria were streak on NA layer and incubated for 48 hours at 25 °C. After 48 hours, filter paper with NA layer on it was removed. A 0.5 cm fungal disc was inoculated on PDA layer and incubated for 7 days at 25 °C. Each treatment was performed in four replicates. Radial growth of fungi, PIRG and statistical analysis were measured and performed.

**Study on Hyphal Morphology**
A 0.5 cm diameter of mycelial plug from C. gloeosporioides culture was transferred to the center of petri dish, containing 15 mL of PD broth and incubated at 25 °C for 48 h. After 48 h of incubation period, 100 µL of bacteria suspension was pipetted into the plate and incubate again at same temperature for 3 days. Hyphal strands at the end of fungal colony were removed and examined under a microscope for abnormalities (Sariah, 1994). Cultures of C. gloeosporioides in PD broth without bacterial inoculation served as control. The experiment was done with four replications for each treatment.

**Spore Germination Test**
Two experiments were conducted to determine the effect of antagonistic bacteria on the spore germination of C. gloeosporioides. Spore suspension of C. gloeosporioides and bacterial suspension were prepared. Fungi suspension was adjusted to 5x10^5 spores/mL. Then, 100 µL of mixed suspension was spread onto PDA plates. The treated plates were incubated at 25°C for 24 h. As for the control plate, sterile water was used instead of bacterial suspension. Treated spores were examined under light microscope after 8 h. According to Sariah (1994), spores were considered germinated when the length of germ tube was half of the length of the spore.

In another experiment, filter-sterilized culture filtrates of antagonist bacteria were used instead of bacterial suspension to determine their effect on spore germination. 100 µL of spore suspension was pipetted onto PDA and spread over the plates. Then two sterile paper discs were placed 3 cm apart on the agar and 50 µL of filter sterilized culture filtrates of antagonist bacteria were pipetted onto each of paper disc. Disc pipetted with received 50 µL NB as control. After 24 h of being incubated at 25°C, spore occurring within 10 mm around each disc were examined under microscope for germination.

**Data analysis**
Data were statistically analyzed using SPSS Statistic 17.0 software, by one way ANOVA and the significance of treatments was determined using Tukey’s-b (p<0.05)

**RESULTS AND DISCUSSION**

**Isolation of Fungal Pathogen**
Three types of pure culture fungi consistently obtained after undergoing several series of subculture. The pure cultures obtained were identified as Colletotrichum sp., Fusarium sp. and Rhizoctonia sp. respectively. Only Colletotrichum sp. isolate used for further study as it showed the most similarity and matched with characteristic of C. gloeosporioides (Fig.1).

**Fungal Pathogen Identification**
The phenotypic characterization of fungus from sample F1 was identified and selected as C. gloeosporioides based on several characteristics. The colony of C. gloeosporioides isolates that grew on PDA showed a dense, white mycelial growth that turning into orange (Fig. 2). After 4 – 5 days, colony shows ring-like shaped and rounded center with darker orange color. Conidia observed were one – celled, straight and oblong with obtuse ends. Size of spores obtained is in between 8.74 µm to 11.95 µm (Fig. 2). The shape and characteristic of colony and conidia are consistent to previous descriptions by Dickman, (1993).
Figure 1 Cultures of sample from primary isolation: *Colletotrichum* sp. (A), *Rhizoctonia* sp. (B) and *Fusarium* sp. (C).

Figure 2 Pure Culture of pathogenic fungus isolated, 7-day-old colony grown on PDA at 25 °C (A), Fungi spores (B), and Hyphal of *C. gloeosporioides* (C).
Koch’s Postulate
Parts of the papaya fruits were inoculated with *C. gloeosporioides* spore suspension. Symptom started with spot was appeared after 48 h of inoculation and it was highly similar to typical symptoms of anthracnose disease in the fields or markets. The symptom showed a round, water soaked and sunken spot on the surface of fruit (Fig. 3). Meanwhile two others fungi isolated was not show any symptom or disease.

![Figure 3](image)

**Figure 3** Healthy of *Carica papaya* as a control (A), infected sampling fruit (B), and Papaya after inoculation with fungus isolates on 4th days (C).

Screening of Antagonist Activity
Production of Metabolites: Diffusible and Volatile Antibiotics
Three tests that have been carried out to determine diffusible as well as volatile antibiotics production of all bacteria. All bacterial shows inhibition ability towards mycelial growth of *C. gloeosporioides* with significantly different compared to control (p < 0.05). Out of all bacterial, two isolates, MKB10 showed significantly (P<0.05) antagonistic activity against *C. gloeosporioides* in dual culture assay (Figure 4). Both *Pseudomonas* sp. (UniSZA-MKB10) and *Bacillus* sp. (UniSZA-DA) were strongly inhibited the fungal growth by an average of 62.08 ± 3.64 % and 58.89 ± 2.72 % at 7 days after incubation with respect to control (Table 1).

<table>
<thead>
<tr>
<th>Antagonistic bacterial</th>
<th>Percentage inhibition of radial growth (%)</th>
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</thead>
<tbody>
<tr>
<td>MKB10</td>
<td>62.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BK4</td>
<td>48.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BK3</td>
<td>18.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>DA</td>
<td>58.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
</tr>
</tbody>
</table>

For double layer method, three isolates showed highest percentage of inhibition on radial growth which were *Pseudomonas* sp. (UniSZA-MKB10) with 85.48 ± 1.53 % PIRG, followed by *Bacillus* spp. (UniSZA-DA) with 82.62 ± 0.69 % PIRG and *Bacillus* spp. (UniSZA-BK4) with 71.05 ± 1.83 % PIRG (Table 2). These three bacterial strongly inhibited the fungal growth at 7 days after incubation with respect to
control. All of bacterial isolates showed significantly (P<0.05) different with each other (Table 5). Meanwhile *Bacillus* spp. (UniSZA-BK3) showed the lowest inhibition rate with average of 42.78 ± 1.37 % of PIRG after 7 days of incubation.

Double plate test shows MKB10 is the most ability to inhibited *C. gloesporioides* radial growth with only 3.25 cm with significant different (p<0.05) compared to control. All bacteria tested were significantly successful inhibited the *C. gloesporioides* (Table 6). The weakest ability to inhibit the *C. gloesporioides* is BK3 with 5.20 cm (Fig. 7).

![Figure 4](image)

**Figure 4.** Antagonist test of *C. gloesporioides* by several bacteria. *C. gloesporioides* without any bacteria treatment as a control (A), *Pseudomonas* sp. (UniSZA-MKB10) (B); *Bacillus* spp. (UniSZA-DA) (C); *Bacillus* spp. (UniSZA-BK4) (D); *Bacillus* spp. (UniSZA-BK3) (E).

<table>
<thead>
<tr>
<th>Antagonistic bacterial</th>
<th>Percentage inhibition of radial growth (%)</th>
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</thead>
<tbody>
<tr>
<td>MKB10</td>
<td>85.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BK4</td>
<td>71.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BK3</td>
<td>42.78&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>DA</td>
<td>82.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
</tr>
</tbody>
</table>

![Figure 5](image)

**Figure 5** Effect of antagonist bacteria on *C. gloesporioides* radial growth of fungi on dual layer methods test.
Table 3 Means of *C. gloesporioides* radial growth treated with bacteria on PDA media.

<table>
<thead>
<tr>
<th>Antagonistic bacterial</th>
<th>Radial growth (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKB10</td>
<td>3.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BK4</td>
<td>4.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BK3</td>
<td>5.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DA</td>
<td>4.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>6.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Figure 6 Radial growth of *C. gloesporioides* in the presence of antagonists by dual layer method.

Study on Hypha Morphology

A mycelia growth of *C. gloesporioides* was observed after introduction of isolates MKB10, BK4, BK3 and DA. Hyphae tips become malformed and hyphae were thickened and vacuolar compared with hyphae in control plate. It was same to the result by Rahman *et al.*, (2008). Most swellings occurred in the hyphae or at the tips of hyphal compared to normal hyphal walls with no swelling or vacuolation (Fig. 7). The length of septate was also reduced compared to un-inoculated *C. gloesporioides*. Meanwhile figure 7c shows no different in hyphae character compared to control.

Figure 7 Hyphal morphology of *C. gloesporioides* as affected by B43 (A), MKB10(C), B40 (D) & DA (D). (E) Normal hyphae. Observed under light microscope with 40x magnification.
Spore Germination test
As the spores of *C. gleosporioides* was spread equally on the PDA media. The germination of the spores was proved by the mycelium growth which then will formed a white mycelium. A clear zone was occurred around the paper discs in the presence of both *Pseudomonas* sp (UniSZA-MKB10) and *Bacillus* sp (UniSZA-DA) shows that no mycelium present (Fig. 8A & B). It was proved that the spores was not germinate. Result also proved that *Bacillus* sp (UniSZA-BK4) and *Bacillus* sp (UniSZA-BK3) did not inhibit the germination of spores (Fig. 8C).

**Figure 8** Clear zone in presence bacterial suspension of: *Pseudomonas* sp (UniSZA-MKB10) (A), *Bacillus* sp (UniSZA-DA) (B), and Control (C) on third days of inoculation on PDA media.

For culture filtrate test, result observed was contradict to the present of bacteria. Spores germination was inhibited in the presence culture filtrate of *Bacillus* sp. (UniSZA-BK4). Culture filtrate of *Pseudomonas* sp. (UniSZA-MKB10) did not inhibit the spore germination, but the process of spore germination was slower compared to control. Result of this study also showed that culture filtrate of *Bacillus* spp. and *Bacillus* sp (UniSZA-BK3) did not give any effect on spore germination and extended hyphal of *C. gleosporioides* could be observed. A clear zone around the paper disc inoculated with culture filtrate of *Bacillus* sp. (UniSZA-BK4) could be spotted (Fig. 9).

**Figure 9** Clear zone in presence bacteria culture filtrate of: Control (A), MKB10, DA, B43 (B) and B40 (C).

**Screening of Antagonists Characteristics**
*Pseudomonas* sp (UniSZA-MKB10) was found to be a gram-negative and others were found to be gram-positive bacterial (Table 4). In another test, *Bacillus* spp (UniSZA-BK3 and UniSZA-BK4) and *Pseudomonas* sp (UniSZA-MKB10) were spotted to produce a clear zone when inoculated on Skimmed Milk Agar (Table 4). This indicated that these bacterial were able to produce exoenzyme to catalyze casein in the SMA. For antibiotic test, there were clear zone surround the paper discs with antibiotic as an indicator that all bacterial were inhibited by the antibiotic inoculated on same agar.
Table 4 Morphology of the potential effective bacteria.

<table>
<thead>
<tr>
<th></th>
<th>MKB 10</th>
<th>DA</th>
<th>BK 04</th>
<th>BK 03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Shape</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>-Ve</td>
<td>+Ve</td>
<td>+Ve</td>
<td>+Ve</td>
</tr>
<tr>
<td>Colour</td>
<td>Light – Green</td>
<td>White</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Odour</td>
<td>No</td>
<td>Odour</td>
<td>Odour</td>
<td>Odour</td>
</tr>
<tr>
<td>Grow</td>
<td>Very Fast</td>
<td>Very Fast</td>
<td>Very Fast</td>
<td>Very Fast</td>
</tr>
<tr>
<td>Casein Test</td>
<td>+Ve</td>
<td>+Ve</td>
<td>+Ve</td>
<td>+Ve</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>-Ve</td>
<td>-Ve</td>
<td>-Ve</td>
<td>-Ve</td>
</tr>
</tbody>
</table>

Positive reaction: +. Negative reaction: - : Resistant S: Susceptible

*Very fast colonies appeared after 24 hours incubation. Fast colonies appeared after 48 hours incubation. Slow colonies appeared after 72 hours incubation. + = positive; - = negative

Figure 10 Gram staining of effective bacteria; Gram negative UniSZA-MKB 10 (A), Gram positive; UniSZA-DA (B), UniSZA-BK4 (C) and UniSZA-BK3 (D).

DISCUSSION

Results of present study clearly showed the potential of using isolates antagonistic bacteria to control C. gloeosporioides, a pathogen of anthracnose disease of papaya. *Pseudomonas* sp (UniSZA-MKB10) was identified as the most efficacious biocontrol agent among all bacteria tested on the ability to inhibit mycelial growth of pathogenic fungus. It also found that *Pseudomonas* sp (UniSZA-MKB10) produced volatile and non-volatile substances as well as diffusible substances that significantly inhibited the mycelial growth of *C. gloeosporioides*.

*Pseudomonas* spp. has been reported to produce volatile ammonia that has been indicated as possible mechanism to control soil borne pathogens (Rahman *et al.*, 2008; Baligh *et al.*, 1996; Conway *et al.*, 1989). This finding was consistent the results of this study that showed *P. aeruginosa* might produce volatile substances that eventually suppress and slowing down the growth of pathogen. Koomen and Jeffries (1993) also reported that *Pseudomonas fluorescence* were chosen as the most potential candidates based on a series of screening of biological control agent (BCA) from in vitro to a
field trial conducted in the Philippines. Pseudomonas strain produces numerous compounds that are responsible for disease control. Deshwal, (2011) also stated that *Pseudomonas* sp. reported to be very effective inhibitors towards *sclerotiorum*, mycelial growth and suppresses germination of sclerotia and ascospores. During dual culture assay and dual layer test, these substances produced by *Pseudomonas sp* (UniSZA-MKB10) were suspected to released and diffused to the layer of agar and inhibit the growth of pathogen.

*Bacillus* sp. (UniSZA-DA) also gives second highest effect on inhibiting mycelial growth. *Bacillus* spp. is known as one of the promising BCAs and some have been produced as commercial products (Stewart, 2001). Study by Rungiindamai, (2016) stated that the two most effective BCAs were identified as *Bacillus* spp. Alamri *et al.* (2012) showed that *Bacillus* sp. were able to inhibit pathogens with more than one mode of action such as secreting protease enzymes and producing antibiotics. The results of present study demonstrated that the isolate of *Bacillus* spp. produced antibiotic substances in vitro that inhibit mycelial growth and spore germination of *C. gloeosporioides*.

The inhibition of radial growth of mycelium in this study is considered to be antibiosis, in which the antibiotic metabolites may be penetrates in cell and inhibited its activity by chemical toxicity. Antibiosis plays a crucial role for the bioactivity of *Bacillus* spp. Rungiindamai, (2016) also stated that various bioactive substances produced by *Bacillus* spp. were reported for their inhibitory effect against *C. gloeosporioides*. In present study indicates that bioactives compounds might have been produced, released and diffused to the PDA medium to inhibit the growth of pathogen.

The failures of spores of *C. gloeosporioides* to germinate after 24 h exposure to culture filtrate of *Bacillus* sp. (UniSZA-BK4) indicates that secondary metabolites produced did inhibit the growth of fungi. *Bacillus* sp. (UniSZA-BK4) is a gram-positive bacterium and produce exoenzyme. Result of this study was supported with a study conducted by Ebrahim et al. (2014) stated that the antimicrobial compound produced by the *Bacillus* sp. (UniSZA-BK4) was broad-spectrum antibiotic, exhibited heat stability, and was active under both acidic and basic conditions.

This is a valuable feature, since for the effective protection of fruit from post-harvest pathogens the antagonists must act at the earliest stage of fruit infection (Bryk *et al.*, 1998). However, less is known about the volatiles produced by *Bacillus* sp. (UniSZA-BK4) in the biocontrol of post-harvest pathogens. Further study on the potential of an application on field needs to be investigated.

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