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Potential of *Pseudomonas* sp. & *Bacillus* sp. for Controlling *Fusarium oxysporum,* A Causal Agent For Rockmelon Fusarium Wilt Disease

Mohammad Hailmi Sajili, Ainur Ainiah Azman, Noor Afiza Badaluddin, Aina Syaeirah Mohd Ghazali, Salmah Mohamed and Norhayati Ngah

Department of Agricultural Science,
Faculty of Agriculture and Biotechnology,
Universiti Sultan Zainal Abidin,
Besut Campus, 22200 Besut,
Terengganu Darul Iman, MALAYSIA.

Corresponding author: Mohammad Hailmi Sajili
Department of Agricultural Science,
Faculty of Agriculture and Biotechnology,
Universiti Sultan Zainal Abidin,
Besut Campus, 22200 Besut,
Terengganu Darul Iman, MALAYSIA.
Email: mhailmi@unisza.edu.my

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ABSTRACT

Fusarium sp. recognized as among main pathogen to the rockmelon. The disease was renown as Fusarium wilt disease (FWD). As to the FWD, objectives of this study were to obtain the pure culture of *Fusarium oxyporum* f. sp. *melonis (Fom),* and to control the Fom via biological control method using effective bacteria. Beside, the study was also screened the plant growth promoting properties of *Pseudomonas* sp. and *Bacillus* sp.. *Fusarium oxysporum* f. sp. *melonis* Snyder & Hans caused postharvest disease problem affecting melon production and loss almost 100 % due to this destructive disease. Effective bacteria like *Bacillus* sp. and *Pseudomonas* sp. has a good potential to suppress growth of pathogen. Based on the phenotypic identification and morphological characterization of fungus isolated was identified as *Fusarium oxysporum* f. sp. *melonis* and it was than confirmed with molecular methods with 99% similarity. Environmental factors that give the optimum growth of *Fusarium* were evaluated. Based on the result, the growth of fungus showed the best on PDA media (2.538 ± 0.095 cm), 30 °C (2.475 ± 0.096 cm), pH 4 (2.700 ± 0.216 cm) and under continuous dark condition (3.433 ± 0.115 cm). The *Bacillus* sp. (DP - 1) showed the highest antagonistic activity of fungus and bacteria with 70.68 % in dual culture assay and highest inhibition of fungus growth in double layer test with no ability to growth. As production of protease, all 7 bacteria tested showed positive result of by producing clear zone on PDA media except by B43. From several parameter tested, the result showed that *Bacillus* sp. has more potential as biological control agent to control the Fusarium wilt disease in rockmelon plant compared to *Pseudomonas* sp..

Keywords: *Fusarium oxysporum* f. sp. *melonis,* biological contol agents, *Bacillus* sp. , *Pseudomonas* sp. Melon, Rockmelon, Fusarium wilt

ABSTRAK

Fusarium adalah antara patogen utama dalam penyakit layu pokok tembikai susu (rockmelon). Ia dikenali sebagai Penyakit Layu Fusarium (PLF). Merujuk kepada penyakit (PLF) ini. objektif kajian ini adalah untuk mendapatkan kultur tulen *Fusarium oxyporum* f. sp. *melonis (Fom)* dan menjalankan kawalan biologi terhadap PLF dengan menggunakan ajen biologi menggunakan bakteria berfaedah. Selain itu, saringan terhadap keupayaan *Pseudomonas* sp. dan *Bacillus* sp. sebagai penggalak pertumbuan juga dilakukan. *Fom* juga mendatangkan masalah penyakit selepas tuai yang menjejaskan pengeluaran tembikai dan kerugian akibat penyakit ini boleh mencecah 100 %. Bakteria berfaedah seperti *Bacillus* sp. dan *Pseudomonas* sp. boleh bertindak sebagai agen kawalan biologi, dan menganggu pertumbuhan patogen. Berdasarkan ciri ciri morfologi, kulat yang teah dipencilkan adalah *Fusarium oxyporum* dan ianya kemudian disahkan dengan teknik molekular dengan 99% persamaan. Dalam kajian ini, faktor persekitaran yang memberi impak pada pertumbuhan optimum kulat *Fusarium* juga dikaji. Berdasarkan hasilnya, pertumbuhan kulat yang terbaik ialah pada media PDA (2.538 ± 0.095 cm), 30 °C (2.475 ± 0.096 cm), pH 4 (2.700 ± 0.216 cm) dan keadaan gelap berterusan (3.433 ± 0.115 cm). *Bacillus* sp. (DP - 1) menunjukkan aktiviti antagonistik tertinggi antara kulat dan bakteria dengan 70.68 % dalam ujian dwi kultur dan perencatan pertumbuhan kulat yang tertinggi dalam ujian dua lapisan media. Bagi potensi bakteria untuk menggalakan pertmbuhan tanaman. Kesemua 7 bakteria yang diuji menunjukkan hasil positif dengan menghasilkan zon jelas pada media. Keseluruhannya, *Bacillus* sp. adalah bakteria yang lebih berpotensi sebagai agen kawalan biologi untuk mengawal penyakit layu Fusarium pada pokok tembikai susu berbanding *Pseudomonas sp.*

Kata kunci: *Fusarium oxysporum* f. sp. *melonis,* agen kawalan biologi, *Bacillus* sp. , *Pseudomonas* sp. Melon, Tembikai Susu, Layu Fusarium

INTRODUCTION

Rockmelons are well - known as cantaloupe and it is widely cultivated in the tropics area for its highly nutritious fruits. It is one of the members of Cucurbitaceae family including watermelon and muskmelon. They are famous because of their fruit (Wehner & Maynard, 2003). *Fusarium oxysporum* f. sp. *melonis* Snyder & Hans. (*Fom*) is the causal agent of most infectious disease, which is Fusarium wilt disease in rockmelon plant (Suarez – Estella et al., 2004). Due to their infection activity, this disease caused main postharvest disease problem affecting melon production (Huang et al., 2000). Therefore, this destructive disease caused inflicts as a major yield loss throughout the world (Katan et al., 1994). It also gives the negative impact by reducing the value and number of the crop production (Sahi & Khalid, 2007; Wongpia & Lomthaisong, 2010). The losses of melon production can cost as high as 100 % because of this destructive disease (Wechter et al., 1995). Once this fungal introduced into the soil, it can persist in the soil even there is no host and after crop rotation (Banihashemi et al., 1975; Gordon

et al., 1989). Symptoms of the Fusarium wilt disease are quite changeable, but include combinations of vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission. The most leading internal symptom is browning of the vascular stem (MacHardy & Beckman, 1981).

Fusarium wilt disease can be managed through chemical soil fumigation and resistant cultivar. Unfortunately, the soil fumigation using the methyl bromide has been banned by the Montreal protocol (Djordjevic et al., 2010; Mao et al., 1998) and cause environment damaged (Groenewald et al., 2006). Therefore, the biological control is one of the best alternatives to substitute the chemical control of the disease (Anjaiah et al., 2003; Landa et al., 1997). The biological controls provide more advantages and reduce the environmental impacts (Reino et al., 2008) which are ecology conscious and eco - friendly. Plant growth promoting rhizobacteria (PGPR) are biological control agents, which is a group of bacteria with plant root habitat suitable for control of disease (Schmidt et al., 2004). PGPR to improve soil fertility, increase plant resistance and suppress the growth of the pathogen. PGPR such as *Pseudomonas* sp. and *Bacillus* sp. are major root colonizers (Manikandan et al., 2010; Joseph et al., 2012) and become plant protector (Kloepper et al., 2004).

MATERIALS AND METHODS

Isolation and Identification

Diseased melon plants showing symptoms were collected directly from the field. Infected parts of the roots, stems and leaves were cut about 1 - 2 cm using a sterilized scalpel along with some healthy portion. They were soaked in 10 % sodium hypochlorite solution for 30 seconds, 70 % ethanol for 30 to 60 seconds and followed by washed with sterile distilled water for 60 seconds. Then, the infected parts were dried with sterile filter paper and immediately placed on PDA. All of them were carried out in aseptic techniques. Mix colonies of fungi were isolated then re - isolated again the mix colonies of fungi to obtain pure culture for each plate. The plates were incubated at 25 °C for 3 to 5 days. Species of the *Fusarium* isolates were identified based on microscopic and macroscopic characteristics (totally morphological characteristics) of single - spored *Fusarium* isolates as described in other studies (Leslie & Summerell, 2006; Marasas et al., 2001; Schroers et al., 2009; Skovgaard et al., 2003).

Molecular Identification of Fungus

Molecular identification was conducted for identification confirmation to the phenotypic identification. In order to collect the mycelia, the cultures of *Fusarium oxysporum* from the culture of 7 days old cultures *Fom* were collected and were inoculated using a sterilized scalpel. The collected mycelia were grinded using the pestle to break the cell wall of fungus. Wizard Genomic DNA purification kit was used for extraction of bacteria and fungus.

The collected sample was transferred to a 1.5mL centrifuge tube and 200µL of cell lysis solution were added. The reaction mixture was vortex for 1 - 3 seconds and incubated at 65 ˚C for 15 minutes. Then, 600µL of nuclei lysis solution was added and followed by vortex the mixture for 1 - 3 seconds and incubated at 65 ˚C for 15 minutes. 3µL of RNase solution were added into the reaction mixture, inverted the tube 2 - 5 times and incubated the mixture at 37 ˚C for 15 minutes. Then, let it cool for 5 minutes before 200µL of protein precipitation solution was added and vortex for 20 seconds. The reaction mixture was centrifuged at 13000xg for 3 minutes. The supernatant was transferred to a new 1.5 mL of centrifuge tube that filled with 600µL of room temperature isopropanol. The mixture was inverted until thread like strands of DNA form a visible mass. The reaction mixture was centrifuged again at 13000xg for 1 minute. The supernatant was discarded and 600µL of 70 % of ethanol was used to wash the pellet. Then, it dried for 15 minutes at 37 ˚C. Lastly, 50µL of DNA rehydration solution was added into the mixture and was incubated at 65 ˚C for 1 hour.

The DNA fragments were separated according to their size by gel electrophoresis. The 5µL of DNA sample was mixed with 2µL loading dye. Then, it was loaded onto 1.0 % agarose gel. The sample was electrophoresed in 1 x TBE buffer at 80 V for 1 hour 40 minutes.

For Polymerase Chain Reaction (PCR) pereparation, Firstly, 20µL of PCR mixture was taken using Eppendorf Mastercycler Gradient. The PCR reaction mixture was composed of 0.5µL of template DNA, 10µL of MasterMix, 7.5µL of deionized distilled water, 1µL of forward primer and lastly 1 µL of reverse primer. The primer a pair was used to identify ITS ribosomal RNA gene was ITS1: 5' – TCC GTA GGT GAA CCT GCGG – 3' for forward primers and ITS4: 5' – TCC TCC GCT TAT TGA TAT GC – 3' for reverse primer.

Suarez - Estrella et al. (2007) reported the conditions for PCR amplification was conducted as follows, initial denaturation at 95 °C for 5 minutes, denaturation at 95 for 30 seconds, annealing temperature at 60 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 5 minutes. PCR run for 30 cycles to reach sufficient amplification. The PCR products separated by 1 % agarose gel and it stained with ethidium bromide to be visualized. The single band image with the predicted size without nonspecific bands or smear indicated that the PCR amplification process was a success.

Environmental Effects on Fungal Growth

Different pathogen needs a different level of environmental factor to express their pathogenicity ability. To understanding the environmental needs of each pathogen is a must, in order to find the best solution of control methods for specific pathogens.

Effect of Temperature on Growth of Fungi

Five millimeter of a plug was taken from the margin of an actively growing culture of 7 - day - old then, cultured and inoculated on PDA. The plug was placed at the center of a Petri dish and sealed with parafilm. After that, the fungi was incubated at four different temperatures which were of 20 °C, 25 °C, 30 °C and 35 °C in the incubator. Four replicates were repeated for each temperature. The radial growth of each fungus for seven days was measured until the fungi growth at a full plate.

Effect of pH on Growth of Fungi

A 100 mL of Difco's Nutrient Broth was prepared and used for each pH. The pH that was tested in this experiment were pH 4, 5, 6, 7 and 8. The pHs were adjusted accordingly by using HCl and NaOH. The correct amount of Difco's granulated agar was added to each pH solution. Then, the solution was autoclaved and poured into Petri dishes, a 5 mm fungal disc from the periphery of 7 day old culture was taken and transferred to the center of agar. After that, the Petri dishes were incubated at 25 °C and took the reading for 7 days. There were four replicates for each treatment.

Effect of Media on Growth of Fungi

Five millimeter of fungal disc was taken from the margin of an actively growing culture of 7 -day - old then transferred to the center of PDA, NA, Difco's Corn Meal Agar, and V8 Juice Agar. Then, the culture media was incubated for 7 days at 25 °C. There were four replicates for each treatment. The radial growth of each fungus was measured daily for 7 days.

Effect of Light on Growth of Fungi

Five millimeter of plug was taken from the margin of an actively growing culture of 7 – day - old then, cultured and inoculated on PDA. The plug was placed at the center of a Petri dish and was sealed with parafilm. After that, the plate was put in incubated condition under three different conditions which were 24 hours under dark condition, 24 hours under light condition, 12 hours under light or dark condition. Three replicates were used for each condition. The radial growth of each fungus was measured for 7 days.

Screening of Antagonist Activity

Antagonist test objective is to identify the ability of selected bacteria to suppress the fungal pathogen growth and pathogenicity potential.

Dual Culture Assay

For this test dual culture plates was prepared according to Suparman et al. (2002). A 5 mm fungal disc was took and inoculated from the edge of 7 days old culture of *Fom* and was placed in the center of a 8 cm culture plate containing PDA and incubated at an ambient temperature 25 °C for 48 hours, a loop full of bacteria from 48 hours of NA culture was taken and streaked at 3 cm away from the pathogen disc on the same plate and incubated for 7 days. The radial growth of the pathogen was measured and compared to control. Results were expressed as the means of the percentage inhibition of radial growth (PIRG) in relation to the radius of uninhibited pathogen colony. Each treatment was carried out with three replicates.

Double Plate Test

The PDA plates were inoculated in the center with a 5 mm diameter of mycelia and were streaked NA plates with antagonistic bacteria. The fungus was isolated and the antagonistic bacteria were cultivated separately per plate. The lids of two plates that contains each bacterial and fungal respectively were removed. The plate was streaked with bacteria, inverted and placed on top of the other plate. The two plate bases sealed with a double layer of parafilm. Then, all plates were incubated at 25 °C for seven days. Each treatment was carried out with three replicates.

Double Layer Test

To carry out this test, PDA and NA were poured on the same plate. The media was prepared then autoclaved; PDA was poured as a first layer and let it cooled. After PDA was solidified, a sterile filter paper was put on top of the PDA and NA was poured as a second layer. Then, the antagonistic bacterium was streak on NA layer and incubated for 48 hours at 25 °C. After 48 hours, filter paper was removed with NA layer on it with an assumption that metabolites produced by bacteria already diffused into the PDA layer. After that, a 5 mm fungal disc inoculated on PDA layer for 7 days at 25 °C. Each treatment had three replications.

Identification of Antagonistic Bacteria

Molecular Identification of Bacteria

The single colonies from the cultures of bacteria were transferred into nutrient broth (NB) media and left overnight. Wizard Genomic DNA purification kit was used for extraction of bacteria and fungus. 1 mL of overnight culture was transferred to 1.5 mL centrifuge tube and was centrifuged at 13000xg for 2 minutes. Then, the supernatant was removed. 600µL of nuclei lysis solution was added then resuspended the mixture gently and incubated at 80 ˚C for 15 minutes. 3µL of RNase solution was added into the reaction mixture and inverted the tube 2 - 5 times then incubated the mixture at 37 ˚C for 15 - 60 minutes. Then, let it cool for 5 minutes before 200µL of protein precipitation solution was added and vortex for 20 seconds. After that, the mixture was incubated on ice for 5 minutes. The mixture then was centrifuged at 13000xg for 3 minutes. The supernatant was transferred to a new 1.5 mL of centrifuge tube that filled with 600µL of room temperature isopropanol. The mixture was inverted until thread like strands of DNA form a visible mass. The reaction mixture was centrifuged at 13000xg for 2 minutes. The supernatant was discarded and 600µL of 70 % of ethanol was used to wash the pellet. It was centrifuged again at 13000xg for 2 minutes. The supernatant was removed and it died dry for 15 minutes at 37 ˚C. Lastly, 50µL of DNA rehydration solution was added into the mixture and was incubated at 65 ˚C for 1 hour.

The DNA fragments were separated according to their size by gel electrophoresis. The 5µL of DNA sample was mixed with 2µL loading dye. Then, it was loaded onto 1.0 % agarose gel. The sample was electrophoresed in 1 x TBE buffer at 80 V for 1 hour 40 minutes.

For Polymerase Chain Reaction (PCR) preparation, PCR was used to amplify the targeted DNA strands. Firstly, 25µL of PCR mixture was taken by using Eppendorf Mastercycler Gradient. The PCR reaction mixture was composed of 5µL of template DNA, 12.5µL of myTaq Red Mix, 5.5 µL of deionized distilled water, 1µL of forward primer and 1µL of reverse primer. The primer a pair was used to identify 16S ribosomal RNA gene were 16S1 - A, AGG GTT GAT AGG TTA AGA GC for forward primers and 16S2 - A, CCA ACA GCT AGT TGA CAT CG for reverse primer.

According to Klindworth et al. (2013), PCR amplification was conducted as follow, initial denaturation at 95 ˚C for 5 minutes, denaturation at 95 for 40 seconds, annealing temperature at 55 ˚C for 2 minutes, extension at 72 ˚C for 1 minute and final extension at 72 ˚C for 7 minutes. PCR run for 25 cycles to reach sufficient amplification. The PCR products were separated by 1 % agarose gel and it was stained with ethidium bromide to be visualized. The single band image with the predicted size without nonspecific bands or smear indicated that the PCR amplification process was a success.

Screening of Plant Growth Promoting Properties

Protease Production

Productions of protease by bacterial isolates were tested by growing them on skim milk agar (SMA) (Chantawannakul et al., 2002). The bacteria were streaked at the centre of the NA plate. The plates were left for overnight. An ability to clear the skim milk suspension in the agar was taken as evidence for the secretion of protease. Non - bacteria inoculated plates were used as the control.

Data Analysis

The data of experimental results were analyzed by using SPSS Statistic 25.0 software, by one way ANOVA and the significant difference of treatments were determined by using Tukey's - b ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation and Identification

Several fungi were consistently isolated from the diseased Fusarium wilt disease. However, only *Fusarium oxysporum f. sp. melonis* fulfilled Koch's postulate. This fungus was colony color on PDA was orange or violet; microconidia were abundant, single - celled, oval, elliptical to kidney - shaped; macroconidia were fusiform, 3 to 4 celled (2 – 3 - septate), apical cells either tapered, curved or both, basal cells typically foot - shaped or occasionally slightly curved; and chlamydospores with either smooth, rough or both. For *Fusarium oxysporum* f. sp. *melonis* was characterized by dense, whitish aerial mycelium that diffused into the agar (Gordon et al., 1989).

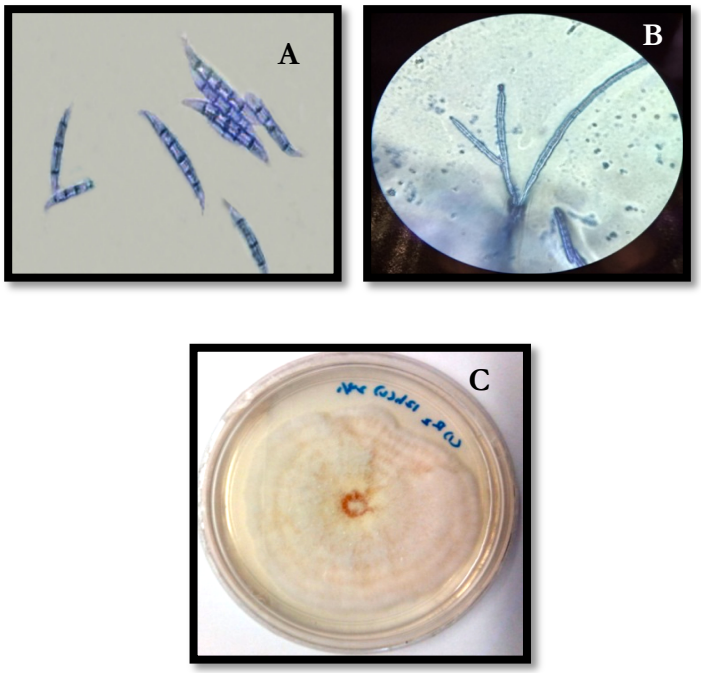

Figure 1 Pure culture of pathogenic fungus isolated from F_1R_2 sample: (A) Spores of fungus (B) Hyphae of *F. oxysporum* f. sp. *melonis,* (C) 7 – day - old culture grown on PDA at 25 °C.

Molecular Identification of Fungus

For molecular identification of fungus, ITS rDNA primers were used. PCR products of approximately ~500 bp for ITS rDNA primers were generated for the fungus F_1R_2. The partial ITS rDNA sequences of isolated bacteria aligned to other known sequences database in GenBank. The result showed that fungus isolates had successfully identified as *Fusarium oxysporum* sp.

Environmental Effects on Fungal Growth

Effect of Media on Fungal Growth

The *F. oxysporum* f. sp. *melonis* was tested on different type of environmental factors to ensure the suitable and optimum growth of fungal. Table 2 showed that *Fusarium oxysporum* f. sp. *melonis* exhibit greater colony growth on NA (3.450 ± 0.320 cm). Therefore, the radial of fungal growth in NA showed only the spore with very less visible mycelia. The table 1 also showed that there was significant different between the radial growth of fungus at all media tested ($p < 0.05$). Although the fungal growth of PDA media showed the lowest growth with 2.538 ± 0.095 cm but it showed the best morphology of *Fusarium oxysporum* f. sp. *melonis* according to colony on PDA media, it showed dense, whitish mycelium with and orange color rounded center.

Fungi were recognized and identified basically based on their phenotypes (Zain et al., 2009). PDA media showed the best fungal growth compared to other media. According to several researchers, PDA was said to be the best media for the optimum growth of fungus mycelial (Xu et al., 1984; Maheshwari et al., 1999; Saha et al., 2008). Therefore, this fact showed that PDA was the most suitable media for the growth of fungal. PDA reproduced most visible colony morphology and selected to be used throughout this study.

Table 1 Mean of radial growth of fungus on different media

Media	Mean of Radial Growth (cm)
PDA	2.538[b]
V8	3.300[a]
NA	3.450[a]
CMA	3.425[a]

*Significant value ($P < 0.05$) was indicated by different alphabet

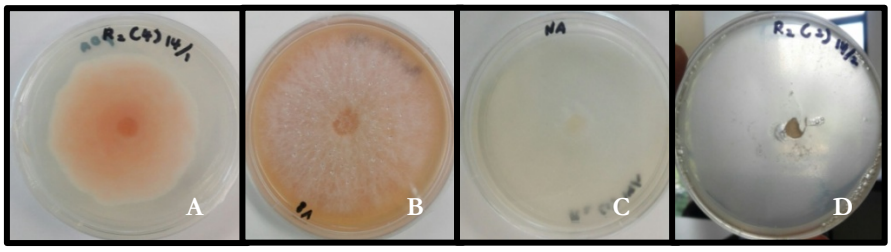

Figure 2 Morpho - cultural characteristics of *Fusarium oxysporum* f. sp. *melonis* on (A) PDA media, (B) V8 Juice Agar (C) NA media (D) CMA media under laboratory condition.

Effect of Temperature on Fungal Growth

Temperature was one of the important components to determine the environmental factors influenced on fungal growth. The results in Fig. 3 showed that 30 °C was the optimum temperature for *F. oxysporum* f. sp. *melonis* to grow. The temperature better suited for mycelial growth ranged from 25 to 30 °C. As the temperature increased, the mycelial growth increased but at 35 °C the growth started to decline. As shows in the Table 3, *Fusarium oxysporum* f. sp. *melonis* showed the best reading of radial growth at temperature of 30 °C with means 2.475 ± 0.096 cm.

Fungi grew at slowest rate at 35 °C (0.138 ± 0.075cm). All of the temperatures showed significantly different from each other (Table 3).

The result was consistent to the result by Mohsen et al. (2016) who reported that the temperature ranged between 15 - 30 °C were the best temperature condition of fungus. While the temperature below 100 °C or higher than 30 °C caused declined of *F. oxysporum* growth. Based on the Table 2, *Fusarium oxysporum* f. sp. *melonis* showed the best reading of radial growth at temperature of 30 °C with means 2.475 ± 0.096 cm. Fungi grew at slowest rate at 35 °C (0.138 ± 0.075cm). Overall *F. oxysporum* f. sp. *melonis* could tolerate and grow in temperature range from 20 °C to 30 °C but the most favorable temperature was 30 °C.

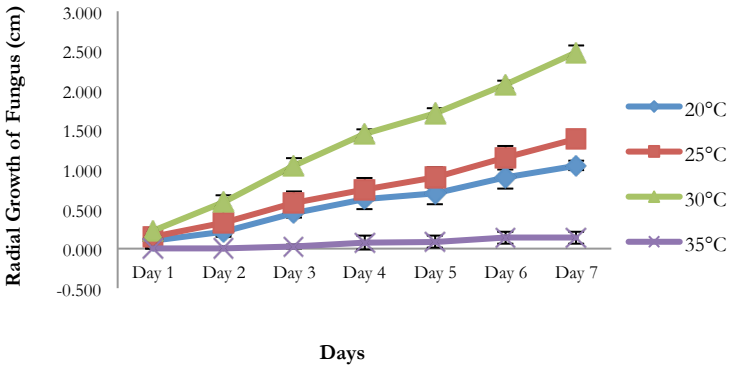

Figure 3 *Fusarium oxysporum* f. sp. *melonis* radial growth at different temperature condition

Table 2 Mean of radial growth of fungus at different temperature.

Temperature (°C)	Mean of Radial Growth (cm)
20 °C	1.050[c]
25 °C	1.388[b]
30 °C	2.475[a]
35 °C	0.138[d]

*Significant value (P < 0.05) was indicated by different alphabet

Effect of pH on Fungal Growth

The effect of various pH on the growth and sporulation of many fungi had been studied under *in vitro* condition. Overall, Table 3 showed that all of pH tested showed there was significant different (P <0.05) with each other (Table 3) except pH5 and pH4. pH 4 was found to be ideal and produced the maximum mycelial growth of 2.700 ± 0.216 cm followed by pH 5 (2.550 ± 0.252 cm) and pH 6 (2.375 ± 0.287 cm). pH 7 and pH 8 recorded the lowest mean mycelial growth at which were 2.300 ± 0.245 cm and 2.238 ± 0.275 cm.

Lilly and Barnett (1951) reported that pH of the medium effect the rate and amount of growth including many other life processes of fungi. A medium with a specific pH also said to appropriate for the fungal growth but be unfavorable for sporulation or other processes. Kumara and Rawal (2010) also reported that a medium that was suitable for sporulation in most fungi was medium with pH between range of 5 to 6.

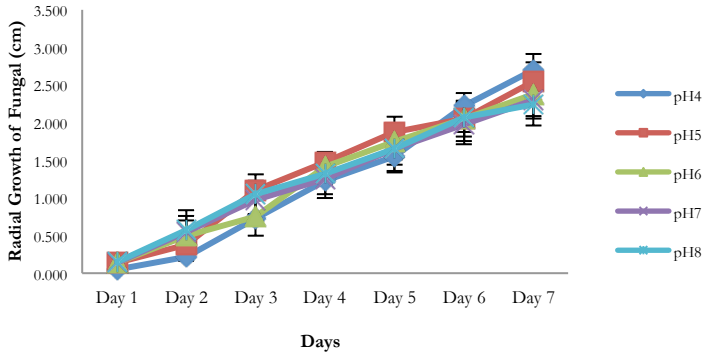

Figure 4 *Fusarium oxysporum* f. sp. *melonis* radial growth at different pH media

Table 3 Mean of radial growth of fungus at different pH.

pH	Mean of Radial Growth (cm)
4	2.700[a]
5	2.550[ab]
6	2.375[c]
7	2.300[c]
8	2.238[c]

*Significant value ($P < 0.05$) was indicated by different alphabet

Effect of Light on Fungal Growth

Based to the Table 4, even though there is no significant different between 24h dark and 12h dark and 12 light, the result showed that optimum light condition for fungal growth was found under the continuous dark condition, which was 24 hours dark condition with 3.433 ± 0.115 cm mean of radial growth of fungus at 30 °C, in 7 days. Then it followed by the radial growth under continuous light condition, 24 hours light condition (2.533 ± 0.115 cm) and 12 hours light/ dark condition with 2.900 ± 0.346 cm. There were significant different ($p < 0.05$) between 12 h dark and 12 light to the rest level of light on the radial growth of fungus under all the light condition (Table 4).

Griffin (1996) stated that the pre-exposure of media to light can result in inhibition of fungal growth. Hence, the light modulation of *in vitro* fungal growth must be clarified carefully. Based on the result, the evidence was showed that the inhibition may be due to the formation of peroxidase in the medium.

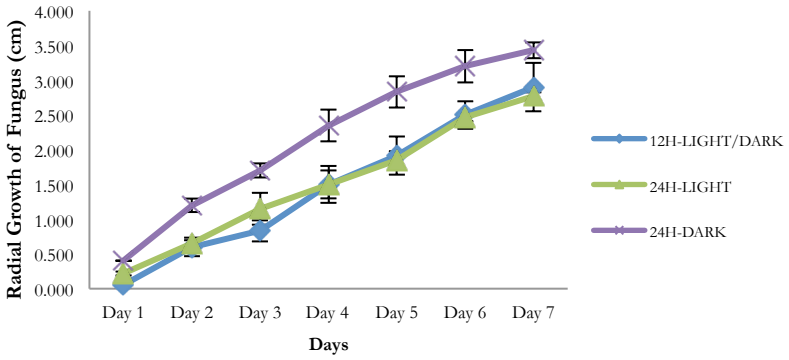

Figure 5 *Fusarium oxysporum* f. sp. *melonis* radial growth at different light condition

Table 4 Mean of radial growth of fungus at different light condition.

Light condition	Mean of Radial Growth (cm)
12 hours light/dark	2.900[ab]
24 hours light	2.533[b]
24 hours dark	3.433[a]

*Significant value ($P < 0.05$) was indicated by different alphabet

Molecular Identification of Bacteria

The result showed that MKB10 and BLH were *Pseudomonas* sp., UNISZA 2 was *Bacillus subtilis,* DP - 1 and DP - 3 were *Bacillus* sp while B40 and B43 were unreported. There were some previous studies stated the universal uses of 16S rRNA as the primer for identification of microorganisms. Srinivasan et al. (2015) stated that 16S rRNA was used as the gene sequencing for clinical microbiology.

Table 5 The identification result of bacteria

Bacteria/ Fungi	Primer	ID Name	Similarity
Bacteria – UNISZA 2	16S	*Bacillus subtilis*	99 %
Bacteria – MKB10	16S	*Pseudomonas* sp.	99 %
Bacteria - BLH	16S	*Pseudomonas* sp.	89 %
Bacteria – DP - 3	16S	*Bacillus* sp.	99 %
Bacteria – DP - 1	16S	*Bacillus* sp.	97 %
Bacteria - B40	16S	Unreported	-
Bacteria – B43	16S	Unreported	-

Screening of Antagonistic Activity

Three test were carried out to determine whether all bacterial tested produce a diffusible and as volatile compounds. All of the bacteria were shown inhibition effects towards mycelial growth of *Fusarium oxysporum* f. sp. *melonis*, which showed significantly different ($p < 0.05$) with control (Table 6).

Table 6 The percentage inhibition of radial growth of fungus against antagonistic bacteria

Antagonistic Bacteria	Percentage of Inhibition Radial Growth (%)
Control	100[a]
Bacillus sp. (DP - 3)	61.33[c]
Bacillus sp. (DP - 1)	70.68[b]
B40	36[e]
Pseudomonas sp.(BLH)	17[f]
B43	12[g]
Pseudomonas sp. (MKB10)	12[g]
Bacillus subtilis (UNISZA 2)	61[d]

Dual Culture Assay

Out of all bacterial, DP - 1 and DP - 3 showed significantly higher antagonistic activity against *Fusarium oxysporum* f. sp. *melonis* (Table 6). Both of them showed with high PIGR value for DP - 1 (70.68 %) followed by DP - 3 (61.33 %) while minimum inhibition were shown by B43 (12 %) and MKB10 (12 %). According to Fatima et al. (2009) reported that the reduction of fungal growth by bacteria and formation of inhibition zone were supposedly due to the production of antifungal substances or degrading enzymes released by the bacteria into the culture medium. The production of extracellular metabolites by all these isolates was one of the important mechanisms to suppress the fungal growth directly.

Double Plate Test

Based on the Table 8, MKB10 showed the lowest means of radial growth of fungal which was 1.333 ± 0.153 cm indicated the highest inhibition rate of fungal growth. . However, B40 showed the highest growth of fungus with 2.633 ± 0.153 cm. All of bacterial isolates showed significant different of radial growth of fungus with each other. The results supported by Romanenko and Alimov (2000) and Abo - Elyousr et al. (2009) stated that *Pseudomonas* sp. had the higher ability to produce volatile compounds more than *Bacillus* sp.

Table 7 Mean of radial growth of fungus against antagonistic bacteria

Antagonistic Bacteria	Mean of Radial Growth (cm)
Control	2.900^{e}
Bacillus sp. (DP - 3)	2.567^{de}
Bacillus sp. (DP - 1)	2.167^{bc}
B40	2.633^{de}
Pseudomonas sp.(BLH)	2.333^{bcd}
B43	2.033^{b}
Pseudomonas sp. (MKB10)	1.333^{a}
Bacillus subtilis (UNISZA 2)	2.483^{cd}

Double Layer Test

Based on the Table 9, DP - 1 and DP - 3 showed the highest inhibition of fungal growth with lowest radial growth of fungal with 0.000 ± 0.000 cm and 0.050 ± 0.050 cm respectively while B40 (2.433 ± 0.666 cm) showed the lowest inhibition of fungal growth with highest radial growth of fungal. The result showed there were significant different between all the means radial growth of fungus ($p < 0.05$). According to Abo-Elyousr et al. (2009) reported that *Bacillus* sp. produced more secondary metabolites that damaged the fungal cellular than *Pseudomona*s sp.

Table 9 Mean of radial growth of fungus against antagonistic bacteria

Antagonistic Bacteria	Mean of Radial Growth (cm)
Control	2.867^{d}
Bacillus sp. (DP - 3)	0.050^{a}
Bacillus sp. (DP - 1)	0.000^{a}
B40	2.433^{d}
Pseudomonas sp.(BLH)	1.467^{bc}
B43	2.333^{cd}
Pseudomonas sp.(MKB10)	2.400^{d}
Bacillus subtilis (UNISZA 2)	0.833^{ab}

Screening of Plant Growth Promoting Properties

Protease Production Test

This experiment was carried out to check the ability of bacteria to hydrolyse casein as the major protein component in milk was casein. Some microorganism had the ability to hydrolyse the protein casein through the production of exoenzyme called protease. Protease which had interferenced in cell wall degrading of fungal pathogen was produced by some of the isolated bacteria, especially *Bacillus* sp. (Ahmadzadeh & Tehrani, 2009). The protease activity was able to hydrolyse the casein by the formation of a clear zone surrounded the bacterial growth indicated a positive reaction while the medium surrounded the growth of bacteria remains opaque indicates a negative reaction. Based on Table 9 showed that only B43 give negative result indicated the bacteria do not have ability to hydrolyze casein.

Table 9 Protease production of bacteria

Bacteria	Result
MKB10	+
B40	+
B43	-
DP – 3	+
BLH	+
UNISZA 2	+
DP – 1	+

Conclusion

This study has proved the ability of some bacteria for controlling pathogenic fungi. Suppressing growth of the pathogenic fungi *Fusarium oxyporum* by *Bacillus* sp. were very promising application of biological control in controlling Fusarium wilt disease in Melon industry. Meanwhile, production of protease is an indicator of potential PGPR which will contributed to the plant growth enhancement. Further study needs to confirm the PGP potential of the bacteria tested.

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