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Isolation, Screening and Characterization of Effective Microbes with Potential for Biological Control of Fusarium wilt of Rock Melon

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Abstract Effective microbes are a group of microorganisms that can be found in the rhizosphere, in association with plant roots which can suppress soil-borne plant pathogens directly or indirectly. A large number of bacteria including species of *Alcaligenes, Arthrobacter, Bacillus, Pseudomonas, Rhizobium* and *Serratia* have been reported to suppress the soil borne plant pathogens. In this study, Fusarium wilt symptomless Rock melon rhizospheric soil samples were collected from three locations (Malaysia). A total of seventy two effective bacteria were isolated by the dilution method. These isolates were firstly dually cultured *in vitro* on PDA medium with *F. oxysporum f.s. melonis,* the causal agent of Fusarium wilt of Rock melon. Isolates with inhibitory characteristics against the test fungus were selected for further screening by means of extracellular metabolite test. Seven isolates which showed >60% inhibition of the fungal growth was further identified on the basis of colony morphology, biochemical tests and Biolog® System. These isolates were identified as *Pseudomonas sp., Bacillus sp., Serratia sp.,* only one isolate could not be identified. MKB04 and MKB10 gave the best suppression of *Fom* mycelial growth. Further molecular identification of these two isolates identified them as *Bacillus amyloliquefaciens*, and *Alcaligenes faecalis* respectively. Effective microbes are environmental friendly and *in vitro* antagonistic activities they manifested against *F. oxysporium f. s. melonis* in this study suggest that they can be used as an effective biological control agent.

Keywords: *biological control, effective microbes (EM), fusarium wilt, rock melon*

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1. Introduction

Rock Melon is one of the most important cultivated cucurbits worldwide. They are grown primarily for their fruit [1]. It is threatened by many diseases including *Fusarium oxysporum* which causes *Fusarium* wilt. This pathogen is the most widely diverse of the *Fusarium* species [2]. *Fusarium* wilt of Rock melon is caused by *Fusarium oxysporum f. sp melonis* (Fom) a seed and soil born fungus that is specific to melon. It is one of the most destructive vascular diseases of Rock melon leading to serious economic losses, especially when grown without rotation in the field [3,4]. Soil borne fungus is one of the factors that limit the production of different crops. Cultural practices and chemical control are the most used control methods, though they caused various problems such as environmental pollution and humans' hazards, as well as resistance of certain pathogens to these chemicals. The difficulty in controlling *Fusarium* wilt with excessive use of chemical has stimulated renewed interest in biological control as a disease management alternative [5]. Application of effective microbes EM technology in agriculture makes it possible to increase crop productivity and play an important role in inhibiting or suppressing the soil-borne pathogens through their antagonistic activity [6,7]. The microorganisms that are able to colonize the rhizosphere and show bio-control potential play important roles in using of bio-control inoculants for protection of crops against soil borne plant pathogens [8]. A large group of bacteria of different species in rhizosphere have been considered as important in sustainable agriculture because of their biocontrol potentials and plant growth promotional activities [9,10].

Biological control using effective microbes has been reported as an attractive alternative due to their ability to antagonize the pathogen by a combination of modes of action, and to effectively colonize plant rhizosphere [11]. A significant role of antagonistic effective microbes in biocontrol of *Fusarium* and improving crop productivity in controlled laboratory or greenhouse conditions has been reported in many studies [12,13,14].

The objective of this study was the *in vitro* isolation; screening and identification of promising effective microbes from the rhizosphere for biological control of *F.*

oxysporum f. s. melonis, the specific causal pathogen of Fusarium wilt disease in Rock melon.

2. Materials and Methods

2.1. Pathogen

The pathogen *F. oxysporum* f. s. *melonis*, isolated from naturally infected Rock melon plants, was used for inoculations. Samples of infected plants were provided by the Department of Plant Protection- Faculty of Agriculture- University Putra Malaysia. Fungal cultures of the pathogen were purified and kept on potato dextrose agar (PDA) slants and stored in the laboratory at 4°C.

2.2. Isolation and Purification of Effective Microbes

Effective microbes' bacteria were isolated by the serial dilution plating technique from rhizosphere soil of healthy rock melon from three locations in Malaysia. Ten grams of rhizosphere soil were soaked in 100 mL of sterilized water and rotary shaking for 24 hrs at room temperature (28±2°C). A ten folds serial dilution (10^{-1}-10^{-10}) of suspension was made in test tubes. Then 50µl suspension from each test tube was spread on nutrient agar (NA) medium with bent glass rod and incubated for 24-48 hrs at 28±2°C. Purification of bacterial isolates was carried out by sub-culturing thrice, where single colonies of bacteria were then plated onto Petri dishes containing nutrient agar (NA) [15,16].

2.3. *In vitro* Screening of Effective Microbes

Seventy two isolates of Effective microbes bacteria were obtained by the serial dilution plating technique from rhizosphere soil of healthy rock melon. These isolates were first dually cultured with *F. oxysporum f.s. melonis* on PDA medium. Isolates with inhibitory characteristics against *F. oxysporum f. s. melonis* were selected for further screening by means of dual culture test and extracellular metabolites (culture filtrate) test based on the percentage inhibition of radial growth (PIRG).

2.4. Dual Culture Test for Effective Bacterial Isolates

A 5-mm diameter agar disc was taken from the edge of 7 days old culture of Fom and then placed in centre of a 9 cm culture plate containing PDA and incubated at an ambient temperature 25°c ±2 for 48 hours, a loop full of bacteria from 48 hours of NA culture was taken and streak at 3-cm away from the pathogen disc on the same plate and incubated for 7 days. Five replicates of each test antagonistic pairings was adopted; Antagonistic properties were quantified by measuring the radius of pathogen colony in the direction to the antagonist colony and calculated as the % of inhibition of radial growth (PIRG) in relation to the radius of uninhibited pathogen colony in control plate using the following formula [17].

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

Where: R_1: Radial growth (control), R_2: Radial growth (treated).

2.5. Culture Filtrate Test

The effective bacterial isolates that gave high PIGR results in the dual culture test were inoculated in 250 ml nutrient broth at 28±2°C for 72 hrs. The culture was then centrifuged at 10000 rpm for 10 min, supernatant was collected and pellets were discarded. The supernatant was filtered through a 0.22 µm membrane filter in sterile conditions. The filtrates were incorporated into sterilized PDA in 20% (v/v) concentration; 20 ml of the amended agars were poured into each Petri plate and allowed to solidify. *Fom* mycelial disc of 5mm was centrally inoculated in each plate. Non-amended PDA was used as the control. The diameter of the mycelial growth of *Fom* was measured over seven days. The antagonistic activity was expressed as PIRG in relation to the mycelia growth of *Fom* in the control plate [16].

2.6. Characterization and Identification of Effective Bacteria

2.6.1. Morphological, Physiological and Biochemical Characterization

Seven effective bacterial isolates showing efficacy in inhibition of fungal growth were selected for morphological, physiological and biochemical characterization by conventional bacteriological methods, and the ability to use different carbon sources was tested using Biolog® GN III Microplates method as well. Morphological cell shape, colony colour and cell motility, Physiological characterization were done on NA. The biochemical tests such as catalase, indole Acetic acid (IAA), nitrate reduction (NR), gelatin liquefaction, urease production were completed following the standard methods of Bergey's Manual of Determinative Bacteriology [18].

2.6.2. Identification of Selected Effective Microbes' Bacteria

The effective bacteria were then identified using Biolog® (Biolog Inc., Hayward, CA, USA) identification system with the software Micro-station System followed the Biolog procedures as described by [4,19] where the procedures for identification utilized 96 wells of microplate containing 95 different dried carbon sources plus control.

16S rRNA gene amplification and sequencing

Total genomic DNA of MKB04 and MKB10 was extracted and purified according to the CTAB method [20] with slight modifications. 16S rRNA genes were amplified from each of MKB04 and MKB10 genomic DNA with the primers 27F: 5-AGA GTT TGA TCC TGG CTC AG-3 and 1492R: 5-CGG TTA CCT TGT TAC GAC TT-3. PCR amplification was performed as follows: 5 min at 94°C, 94°C for 1 min, 1 min at 55°C, followed by 32 cycles of 90 s at 72°C, and 5 min at 72°C, 4°C 15 min save. DNA sequence homology searches were performed using the online BLAST search engine in GenBank (available at: http://www.ncbi.nlm.nih.gov). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [21].

2.7. Statistical Analysis

The experiments were conducted in Completely Randomized Design (CRD) with five replicates. Recorded data were analyzed with SAS® Software. Statistical significant data were determined using Duncan's Multiple Range Test ($p<0.05$). The percentage data were transformed into Arcsine transformation before subjected to ANOVA [22].

3. Results

3.1. Isolation and Purification of Effective Bacteria

Seventy two of effective bacteria were successfully isolated from the symptomless Rock melon rhizosphere soil. The separation of effective bacteria was carried out depending on morphological characteristics such as colony colour, elevation, the margin of colony and colony surface. Pure culture of each isolate was maintained at 4°C for screening test against *Fom.* All the effective bacterial isolates were coded as MKB01 to MKB72. The dual culture test - an established method used to distinguish isolates with antagonistic potential from large populations was adopted. Preliminary screening results showed that from the 72 effective bacterial isolates tested, only 42% (30 isolates) showed antagonistic activity towards *Fom* with the PIRG > 50%. The remaining of twenty-five percent (18) and 33% (24) of the tested bacteria isolates were weak inhibitors with PIRG values less than 50% and 35% respectively (Figure 1). The bacterial isolates with a significantly higher inhibition percentage were further evaluated for their antagonistic activity toward *Fom.*

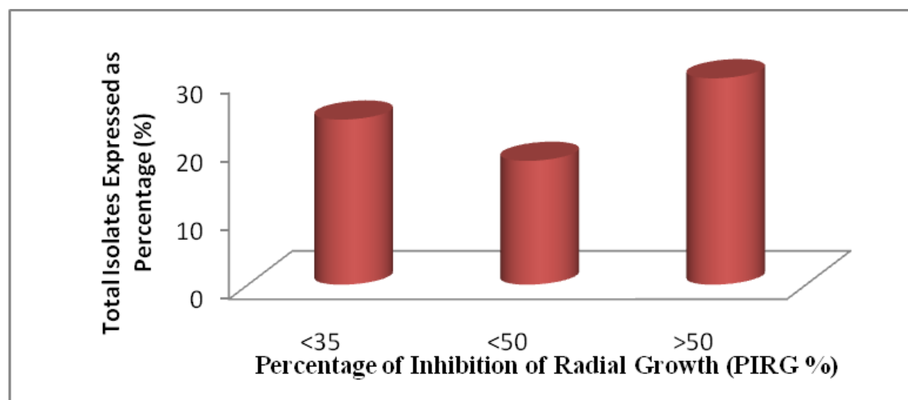

Figure 1. Percentage of EMs with antagonistic potential against *Fom* based on their respective PIRG values in dual culture test

3.1.1. *In vitro* Screening of Microbial Bacteria against *F. oxysporum f. s. melonis* in Dual Culture Assay Test

Effective bacteria isolates in preliminary screening that showed higher inhibition in the mycelial growth rate were further re-evaluated by dual culture test and isolates with antagonistic activity towards *Fom* with the PIRG > 60% were selected. From the statistical analysis, the selected seven isolates showed significantly different inhibitory activity; where the isolates MKB10, MKB15, and MKB04 had the maximum inhibitory effect against *Fom* respectively followed by MKB09, MKB12, MKB37 and MKB24 (Table 1).

Table 1. Activity of effective bacteria against *F. oxysporum f. s. melonis in vitro*

Isolate	DC (%)	EM (%)
MKB04	70.45b	84.88 b
MKB09	68.18b	82.56c
MKB10	75 a	87.20 a
MKB12	68.18bc	81.4d
MKB15	71.59ab	80.23e
MKB24	64.77c	80.23e
MKB37	67.05bc	80.23e

Data are means of five replicates. Means within a column followed by different letters are significantly different at P = 0.05 according to DMRT. DC = % growth inhibition in dual culture test, EM= & growth inhibition in extracellular metabolites test.

3.1.2. Extracellular Metabolites Efficacy Test

The result showed that the pathogen could not grow well in the presence of effective bacterial extracellular metabolites. There was a significant difference between the isolates and the % inhibition of fungal radii. Growth of *Fom* was significantly inhibited by the effective bacteria tested. Isolates MKB10, MKB04 and MKB09 were the most efficient with 87.20, 84.88 and 82.56 % inhibition of fungal growth, respectively as presented in (Table 1). The effective bacteria MKB04, MKB09, MKB10, MKB12, MKB15, MKB24 and MKB37 were selected on the basis of this test. Whereas among them MKB04 and MKB10 were the most promising isolates to inhibit the fungal growth.

3.2. Characterization and Identification of Effective Bacteria

Morphological, physiological and biochemical tests were performed for the identification of the selected effective bacteria. Gram staining and light microscopic studies showed that all the effective bacterial isolates selected were Gram-negative and rod-shaped except MKB04 which was gram positive and rod-shaped as well. All isolates were motile and may belong to different genera. Isolates recorded toleration to 1% NaCl and 4% NaCl and pH level 5-6 (Biolog). The biochemical test (Table 2) results indicated that all the isolates were positive to catalase, urease production and nitrate reduction, and starch hydrolysis, and indole production. Five Isolates were positive to gelatine hydrolysis while isolates MKB10 and MKB15 were negative to that. In Biolog result isolates showed positive reaction for utilization of glucose, Maltose, Sucrose, Mannitol and lactose.

Table 2. Biochemical and morphological properties of Effective Bacteria (7 isolates)

Tests	MKB04	MKB09	MKB10	MKB12	MKB15	MKB24	MKB37
Cell Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+
Gram reaction	+	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	+
Urease Production	+	+	+	+	+	+	+
HCN Production	-	+	-	+	+	-	+
Indol production	+	+	+	+	+	+	+

Positive reaction: +. Negative reaction: **-.**

All selected isolates for biocontrol effectiveness towards *Fom* were identified using Biolog ® identification system to species level except MKB10 which could not be identified. MKB04 was identified as *Bacillus amyloliquifaciens,* MKB09, and MKB37 as *Serratia sp*, MKB12 *Serratia marcescens ss marcescens,* MKB15 as *Pseudomonas aeruginosa* and MKB24 as *Pseudomonas sp.* (Table 3).

Table 3. Identification of potential EMs using Biolog® Identification System

Isolate	Gram Test	Oxidase Test	Species
MKB04	Gram positive	Non-enteric	*Bacillus amyloliquefaciens*
MKB09	Gram negative	Non-enteric	*Serratia marcescens ss marcescens*
MKB10	Gram negative	Non-enteric	No ID
MKB12	Gram negative	Non-enteric	*Serratia marcescens ss marcescens*
MKB15	Gram negative	Non-enteric	*Pseudomonas aeruginosa*
MKB24	Gram negative	Non-enteric	*Pseudomonas spp*
MKB37	Gram negative	Non-enteric	*Serratia marcescens ss marcescens*

MKB04 and MKB10 were further Identified using *16S rDNA* as they showed the highest PIRG and were selected to study their antifungal metabolite production and plant growth promotional activity.

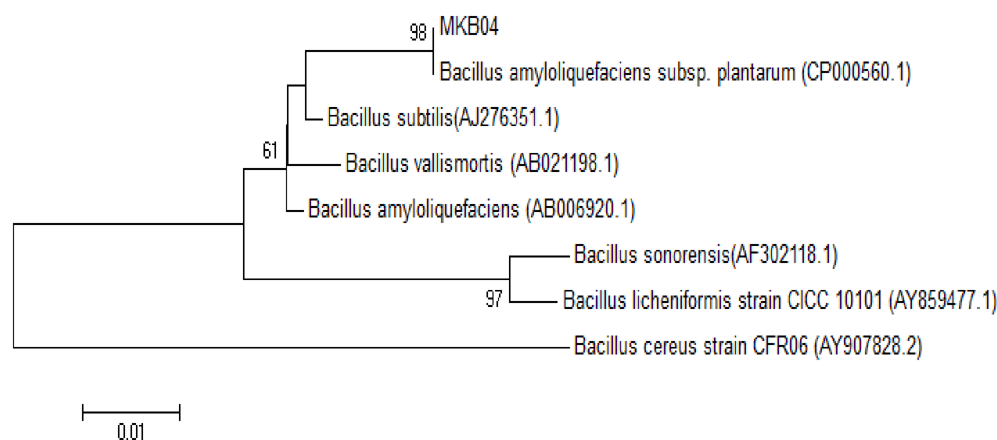

Figure 2. Phylogenetic tree of MKB04 constructed using neighbour joining method

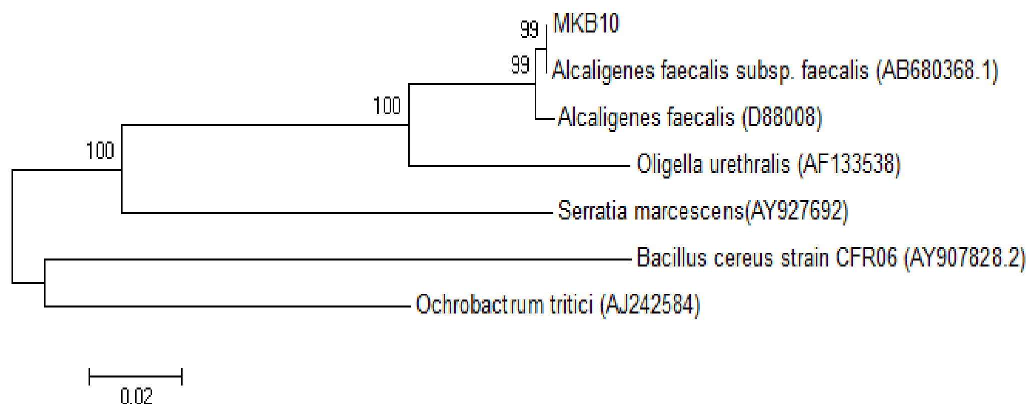

Figure 3. Phylogenetic tree of MKB10 constructed using neighbour joining method

PCR products of approximately 1380 and 1270 bp were generated for the two antagonistic bacteria MKB04 and MKB10 respectively. The partial 16S rDNA sequences of isolates MKB04 and MKB10 were determined and

aligned to other known sequences database in GenBank. The sequence similarity between MKB04 bacteria and other known species of *Bacillus* strains was 100% and 100% for MKB10 with *Alcaligenes* strain too. The aligned sequences of the two isolates were determined and deposited in GenBank under accession number (KM220771) and (KM220772) for MKB10 and MKB04 respectively. In the phylogenetic analysis based on these sequences, MKB04 should be identified as *Bacillus amyloliquefaciens*, and MKB10 *as Alcaligenes faecalis.* With 98% and 99% similarity to type species *Bacillus amyloliquefaciens subsp plantarum* and *Alcaligenes faecalis subsp faecalis* respectively (Figure 2 and Figure 3).

4. Discussion

Rhizosphere soil antagonistic microorganisms have been reported to suppress a great number of soil-borne pathogens. This was demonstrated by [11,23,24,25]. Various studies related to biological control of Fusarium wilts by effective microbes have helped to reduce the incidence of these diseases in different plant, banana, black paper, radish, tomato, and cucurbits including rock melon as well [26-31]. This study was conducted to isolate effective microbes from the rhizosphere soil of the symptomless Rock melon plant and to evaluate their potential capabilities for biocontrol of Fusarium wilt disease. The effective microbes' isolates were isolated depending on morphological characteristics such as colony colour, colony surface, margin of surface. These seventy two isolates were coded from MKB01 to MKB72, 30 isolates showed inhibitory effect >50% towards *Fom* in confrontation test, only seven of them gave PIRG >64%. According to culture filtrate test these isolates recorded higher inhibitory effect >80% where MKB10 recorded the highest % (87%) followed by MKB04 (84%). These results are in agreement with those of [32] showing that microorganisms inhabiting the rhizosphere of the plant have remarkable effect on controlling and protecting the plants from soil-borne pathogens. The production of extracellular metabolites by all these isolates is one of the important mechanisms to suppress the fungal growth directly. Similar results were demonstrated in other studies [33].

The efficacious bio-control agents were identified using Biolog ® identification system though the isolate MKB10 could not be identified as such[34]. This might be due the limitation of the methods used or may be this is a new species. No data concerning this species (MKB10) was available in Biolog data base list. Three isolates were identified successfully to species level in three different genera; *Bacillus* and *Pseudomonas* and *Serratia*, while for three more isolates only genus was identified. In most cases, effective bacteria used as biocontrol agents of plant diseases belong to the genera *Bacillus, Pseudomonas* and *Serratia* as reported by many investigators [35-40].

Results in this study demonstrated the activity of species from Bacillus, Serratia and Pseudomonas toward *Fom* and the ability of these species to produce many types of antimicrobial compounds to be used as biological control agents, as well as their different combination of mechanisms of action. These findings are similar to those reported by previous studies [41,42,43,44,45].

In this study results obtained from all the seven effective microbes *in vitro* proved to be effective biocontrol agents against *Fom*, with MKB04 and MKB10 giving the best suppression of the *Fom* mycellial growth. Further identification of these two isolates using PCR protocol identified them as *Bacillus amyloliquefaciens*, and *Alcaligenes faecalis* respectively. The ability of *B. amyloliquefaciens* and *A. faecalis* to produce many types of antimicrobial compounds and *B. amyloliquefaciens* endospores production as well as mode of actions merits considering them for use as biological control agents.

5. Conclusion

In conclusion, *Fusarium* wilt disease caused by *Fom* is one of diseases that seriously threaten the production of Rock melon. Soil-borne pathogens occur in dynamic environments that complicate the control of the disease. Although effective microbes' bacteria showed biological control potential against *Fusarium*, in this study it represents the first step towards the implementation of biocontrol at the practical level**.** For this reason further studies and information regarding antagonistic mechanisms of action, interaction, requirement and conditions for biological control are going on.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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