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Screening of antagonistic potential bacteria from rhizosphere soil against phytopathogenic fungi related to selected vegetable crops

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ABSTRACT

Fungal phytopathogens cause serious losses of crop production worldwide, which causes serious losses of crop production. Bacteria play a role as the biocontrol agents for plant disease control. For this reason, the present study was conducted to determine the antagonistic potential of rhizosphere soil bacteria against selected phytopathogenic fungi. The screenings of potential antagonist isolated bacteria were applied by the dual culture technique with fungi *Fusarium oxysporum* and *Colletotrichum melongenae*. Molecular characterization was performed through 16S rDNA sequencing analysis. Sixteen (16) out of fifty (50) isolated bacteria showed different degrees of antagonism (25-67%) against both fungi *F. oxysporum* and *C. melongenae*. Among them, four (4) isolated bacteria (isolates A4, C1, C2, and E2) exhibited strong antagonism (more than 50% mycelial growth inhibition) against both fungi. The 16S rDNA sequences of the isolated bacteria A4, C1, C2 and E2 were 99-100% similar to *Providencia* sp. TT14, *Bacillus subtilis* 168, *Bacillus subtilis* RKP-2, and *Bacillus amyloliquefaciens* IBSDG-11, respectively. Based on the capability for the control of mycelial growth against both fungi, *B. subtilis* IUBTC2 was selected for optimization of growth characteristics and identification the bioactive metabolites which can enhance plant growth and disease control capacity of plants against the phytopathogenic fungi.

INTRODUCTION

Major crop production losses are caused worldwide by pathogenic microorganisms which is comparable to approximately $220 billion lost per year [1]. Worldwide, the crop production damage was also caused by different fungal phytopathogenic species as *Fusarium*, *Rhizoctonia* or *Colletotrichum* such [2-5]. *Fusarium* wilt of cucumber diseases was caused by *F. oxysporum*, which is a very common disease in cucumber worldwide and causes serious losses every year [6-8]. Until now, disease control mechanism mostly depends on the practice of artificial fungicides. This approach is very challenging, because fungal stains may become resistant to fungicides due to the accumulation of these artificial fungicide's in the ecosystem [9, 3]. Artificial fungicides are expensive. So, these are not sufficient for the disease control strategies [10].

In addition, public anxiety related to chemical pesticides has raised key attention to pursue additional control approaches [11]. Interactions are prevalent in nature between plant pathogens and antagonistic microorganisms. Similarly, plant pathogenic fungi and antagonistic microorganism interactions can employ to control or reduce diseases as biocontrol/biological agents which can suppress the plant pathogens [12]. It is one of the paths to reduce the usage of artificial fungicides in agriculture sector, where plant protection from diseases can be done by using microorganisms [13]. The biocontrol or

biological control mechanisms are the secretion of both small molecules and enzymes, which are extremely active against pathogens [14, 1, 3].

Antagonism is the phenomenon in which one microorganism inhibits the other interacting partner to ensure its own survival. The stalling products of microbes are preferably called antibiotic substances or metabolites. It was reported that antagonistic bacteria act as a biocontrol agent due to their ability of the different modes of action [12]. The antagonistic effect of microorganism is the result of interaction among the microbial populations. Several bacterial species have been tested as biocontrol/biological agents [15]. The bacterial antagonisms mechanisms are involved in competition for space or nutrients and antibiosis for enrichment of plant and root growth, and for introduction of plant inactivation and/or resistance of the pathogen's enzymes [16].

Antibiosis is the utmost significant machinery for the plant disease control. Cucumber (*Cucumis sativus* L.) is one of the key significant economical crops, which is grown either in the open field or greenhouses [17, 18]. *Fusarium* wilt of cucumber, caused by *F. oxysporum f. sp. cucumerinum*, is a very common disease of cucumber and oil palm in the world and causes serious losses every year [19, 6]. Eggplant (*Solanum melongena*) is a tropical, herbaceous, perennial plant, in the family Solanaceae, which is grown for its edible fruit [20]. The marketable eggplant production is negotiated due to affect by the insect which are generally recognized such as eggplant shoot and fruit borer (*Leucinodes orbonalis*) as well as various phytopathogenic fungi namely, *Fusarium* spp., *C. melongenae*, *Pythium* spp., *Leveillu lataurica, Rhizoctonia* spp., and *Verticillium* spp. [17]. Until now, disease control mainly deepens on the usage of synthetic fungicides. For this reason, the objective of this study was to screen the antagonistic potential of bacteria from rhizosphere soil against phytopathogenic fungi in selected vegetable crops in Bangladesh. At first, we isolated bacteria from rhizosphere soil at different sources and screened the antagonistic potential bacteria against phytopathogenic fungi. We also further investigated as morphological, biochemical and molecular characterization to determine the biocontrol/biological agents for the phytopathogenic fungus control.

MATERIALS AND METHODS

Fungal pathogens

Two fungal pathogens, namely, *F. oxysporum cucumerinum* and *C. melongenae* were used in this study, which were obtained from the Laboratory of Microbiology, Islamic University, Kushtia-7003, Bangladesh.

Isolation of soil bacteria

Rhizosphere soil was collected from 5 different agricultural and non-agricultural field crops of Shantidanga, Kushtia. The soil samples were brought to the laboratory in sterile polyethylene bags and stored at 4ºC for the isolation of microorganisms. Soil bacteria were isolated by serial dilution method from soil samples. In brief, it was suspended 5 g of soil in sterile distilled water and shaking at 120 rpm for 10 min in a rotary shaker. Then it was diluted with distilled water (1:9 ratio) up to10^7 fold. 100 µl aliquots from 10^4 to 10^7 dilutions was spread in the tryptone soya agar (TSA) media and mildly spread by a sterile glass spreader. Then it was incubated at 35ºC for 3 days, after it was sub-cultured onto the same medium plates based on morphological distinct colony and isolated single colonies from these plates.

Screening of antagonistic activities

Antagonistic activities were screened from the isolated bacterial strains through *In vitro.* Mycelial growth inhibition of *F. oxysporum* and *C. melongenae* was achieved through the dual culture method in potato dextrose agar (PDA) media. PDA media was organized and transferred in petri dishes (20 ml). Agar plug (5 mm) of vigorously rising culture of each fungus was positioned into the center of individual plate. Individual isolate was streaked 3 cm away and 1 cm away from the "edge of the" petri dish. Plates without bacterial inoculation was used a control. Then the plate was enclosed with parafilm and incubated at a temperature (27ºC) for 5 days till the fungal mycelia touched the control plate edge. All tests were done in triplicate for each isolate. Then circular mycelial development, inhibition percentage (%) was measured by the following formula [21]:

$$\text{Radial Inhibition (RI\%)} = \left(\frac{A_1 - A_2}{A_1}\right) \times 100$$

Where, A1 = radial growth of fungal mycelia without bacterial isolate, A2 = radial growth of fungal mycelia with bacterial isolate.

Morphological characterization and biochemical test of isolated bacteria

The morphological characterizations and physiological and biochemical tests were done on the selected isolates. In brief, morphological characterizations such as size, shape, motility and spore forming were observed. The biochemical tests such as Gram staining, catalase test, KOH test, lactose fermentation, sulfur reduction, and urease test of the isolated bacteria were done as described earlier [22, 23]. Determination of degree of growth of isolated bacteria, different types of media were used such as tryptone soya agar (TSA), lactose broth agar (LBA), yeast extract agar (YEA), potato dextrose agar (PDA), and King's medium B agar (KBA).

Molecular characterization of the isolated bacteria

Genomic DNA was extracted from the selected isolated bacteria according to the protocol designated by He [24] (phenol: chloroform: iso-amyl alcohol procedure). The sequence of 16S rDNA was amplified through polymerase chain reaction (PCR). Thermal cycles and other conditions were applied according to Rahman et al [25, 26]. The universal primer set of 63F (5-CAGGCCTAACACATGCAAGTC-3) [25, 26, 27] and 1389R (5-ACGGGCGGTGTGTACAAG-3) [25, 26, 28] was used for the amplification of 16S rDNA. Amplified PCR products were purified by purification kits (TaKaRa, Hot-Start Version). Then the purified PCR products were sequenced by DNA sequencer (Invent Technology, Dhaka, Bangladesh). The obtained sequences were analyzed through Chromas software. The similarity was observed with known sequences present in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) through the BLAST program. Multiple sequence alignment (MSA) of 16S rDNA sequences of the isolated bacteria was performed by using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The aligned sequences were then exposed to a Neighbor-Joining phylogenetic tree constructed by Clustal Omega. Then 16S r DNA sequences were deposited into the NCBI data bank. The sequences were submitted in the NCBI gene bank and accession numbers as MG237885 to MG237888 for *Providencia* sp. IUBTA4, *B. subtilis* IUBTC1, *B. subtilis* IUBTC2, and *B. amyloliquefaciens* IUBTE2, respectively.

RESULTS

Screening of antagonistic activities from isolated bacteria through an in vitro assay

Screening of antagonistic activity of each isolated bacterium was accomplished through an *in vitro* assay as shown in Figure 1. Fifty (50) bacterial isolates were obtained from five (5) different soil samples. Some isolates were antagonists against only one fungus. Among them, sixteen (16) isolates showed different degrees of antagonism (25-67%) against both fungi, *F. oxysporum* and *C. melongenae*. Four (4) isolates A2 (58% and 57%), C1 (50% and 63%), C2 (67% and 64%), and E2 (62% and 50%) showed a higher efficacy in inhibiting the radial growth fungal mycelia. The strong antagonism (more than 50% mycelial growth inhibition) was exhibited by the isolates A4, C1, C2, and E2 against both fungi (Figure 2).

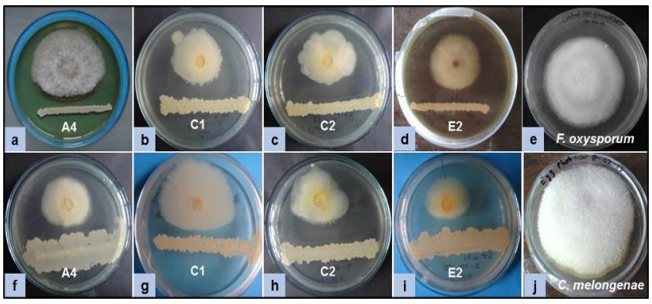

Figure 1. Antagonistic activity of isolated bacteria against *F. oxysporum* and *C. melongenae* in dual culture in PDA after 4 days of culture at 27ºC. (a-d and f-i). Fungal growth was inhibited towards the direction of isolated bacteria and (e and j) control plates inoculated with only the fungal isolate.

Morphological characterization of isolated bacteria

Morphological characterizations of isolated bacteria are shown in Table 1. Morphological studies showed that the isolated bacteria A4 was straight rods, non-spore former, and motile. The isolated bacteria C1, C2, and E2 were rods shape, spore former, and motile. The degree of growth characteristics of the isolated bacteria A4, C1, C2 and E2 was observed using different types of media as shown in Table 2 and the growth patterns were recorded as shown Figure 3. Four (4) isolated bacteria A4, C1, C2 and E2 showed the excellence growth and good growth on TSA and KBA media, respectively as compared with other

media. The growth patterns revealed that there was a distinct difference among the isolated bacteria.

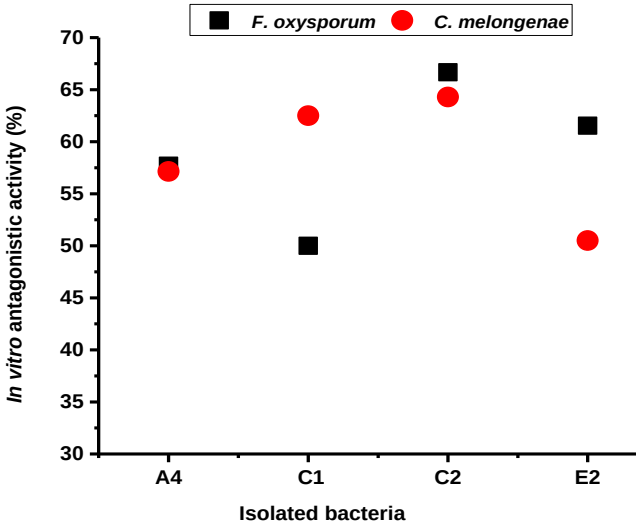

Figure 2. Antagonistic activities of potential isolated bacteria against *F. oxysporum* and *C. melongenae*. Selected bacteria, A4-*Providencia* spp. TT14, C1- *B. subtilis* 168, C2- *B. subtilis* RKP-2, and E2-*B. amyloliquefaciens* IBSDG-11.

Table 1. Morphological characteristics and the biochemical properties of the selected isolated bacteria

Properties	Isolates			
	A4	C1	C2	E2
Morphological characteristics				
Shape	Rod	Rod	Rod	Rod
Motility	+	+	+	+
Spore formation	-	+	+	+
Biochemical test				
Gram reaction	-	+	+	+
KOH test	-	+	+	+
Catalase test	+	+	+	+
Lactose fermentation	-	-	-	-
Sulphur reduction	-	-	-	-
Urease test	+	+	+	+

+ indicates positive, - indicates negative

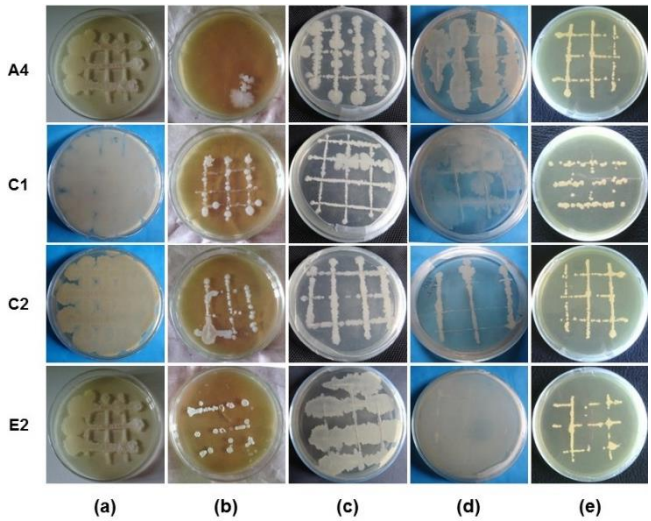

Figure 3. Growth patterns of the selected isolated bacteria. Selected bacteria, A4- *Providencia* spp. TT14, C1- *B. subtilis* 168, C2- *B. subtilis* RKP-2, and E2-*B. amyloliquefaciens* IBSDG-11in different culture media (a) Tryptone soya agar (TAS), (b) lactose broth agar (LBA), (c) yeast extract agar (YEA), (d) potato dextrose agar (PDA) and (e) King's medium B agar (KBA) after 66 h of incubation at 37ºC

Table 2. Degree of growth of the selected isolated bacteria in different culture media

Media	Isolated bacteria			
	A4	C1	C2	E2
TSA	Excellent growth	Excellent growth	Excellent growth	Excellent growth
LBA	Almost no growth	Good growth	Good growth	Almost no growth
YEA	Good growth	Good growth	Excellent growth	Excellent growth
PDA	Almost no growth	Excellent growth	Good growth	Excellent growth
KBA	Good growth	Good growth	Good growth	Good growth

TSA= tryptone soya agar, LBA= lactose broth agar, YEA= yeast extract agar, PDA= potato dextrose agar, and KBA= King's medium B agar

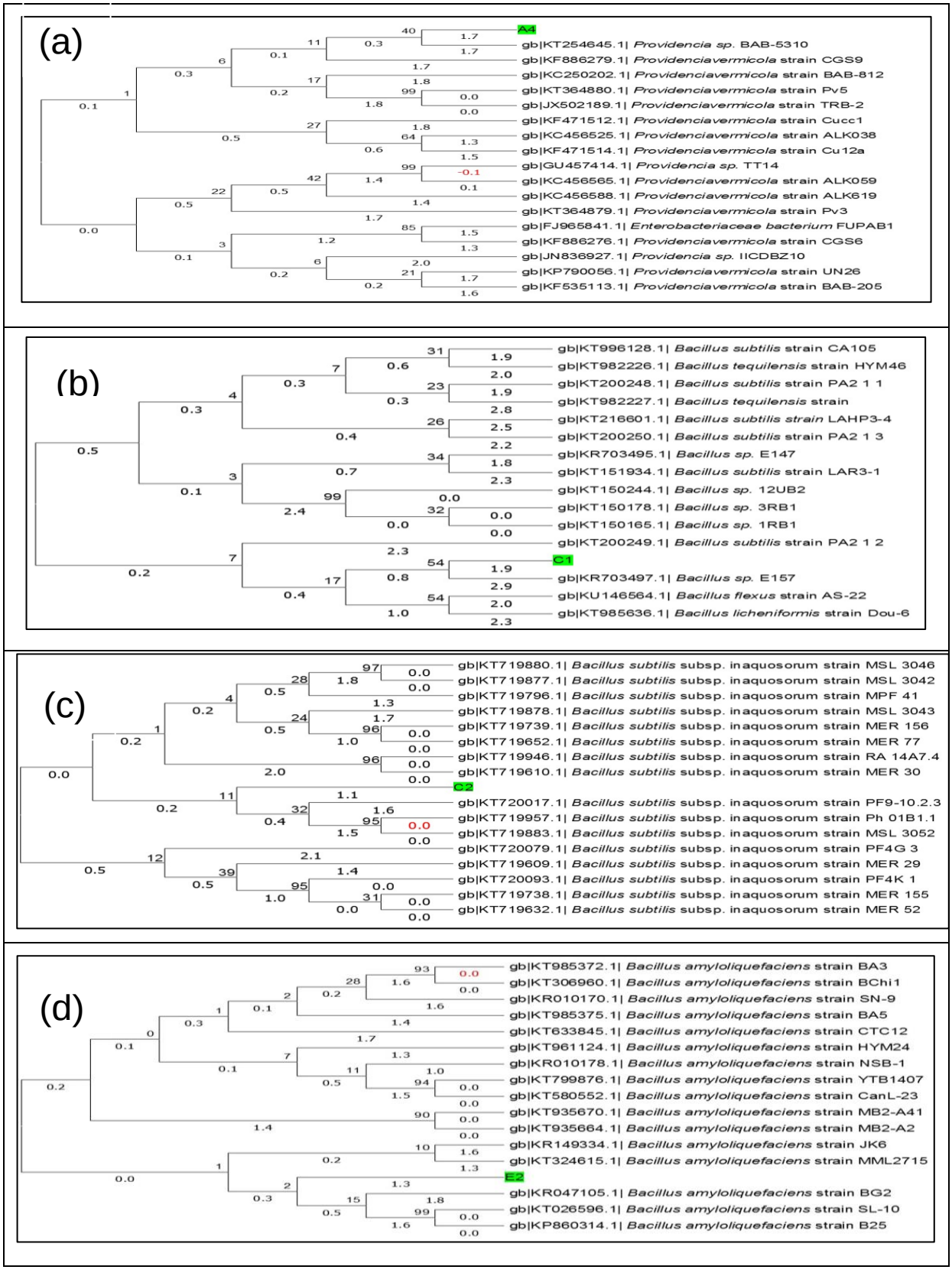

Figure 4. Neighbor-joining phylogenetic trees showing relationships of isolated bacteria a) A4- *Providencia* sp. TT14, b) C1- *B. subtilis* 168, c) C2- *B. subtilis* RKP-2, and d) E2-*B. amyloliquefaciens* IBSDG-11 with closely related strains based on their 16S rDNA sequences

Biochemical test of isolated bacteria

The results of biochemical test of isolated bacteria were shown in Table 2. The isolated bacteria C1, C2, and E2 were positive for the Gram staining and KOH test, respectively. Isolated bacteria A4 was negative against gram reaction and KOH test, respectively. Isolated bacteria A4, C1, C2, and E2 were positive in the catalase test and urease test. On the other hand, all of the four isolated bacteria showed negative for lactose fermentation and sulphur reduction (Table 2).

Molecular identification of selected bacterial isolated

Molecular identification was applied by 16S rDNA sequence analysis as shown in Table 3. The 16S rDNA sequences were received and analyzed in the Chromas software (version 2.6.4) showed good quality of the sequences. The similarity of the sequence of the isolates was exposed (100-99% similarity) through BLAST exploration and there was a close relationship presented between the isolated strains and known sequence of the gene bank NCBI. Isolated bacteria A4, C1, C2 and E2 were most closely related to *Providencia* sp. BAB-5310, *Bacillus* sp. E157, *B. subtilis* subsp. *inaquosorum* strain PF9 and *B. amyloliquefaciens* strain BG2. The sequences were submitted in the NCBI Gene Bank and accession numbers A4 (MG237885), C1 (MG237886), C2 (MG237887) and E2 (MG237888) were received for the isolated bacteria. Finally, the four isolates were identified as *Providencia* sp. IUBTA4, *B. subtilis* IUBTC1, *B. subtilis* IUBTC2 and *B. amyloliquefaciens* IUBTE2. Neighbor-joining phylogenetic trees were constructed of isolated bacteria as shown in Figure 4. Phylogenetic analysis indicated that isolated bacteria were represented in a distinct class, demonstrating that they are newly isolated bacteria as compared to NCBI Data bank.

DISCUSSION

The use of valuable microorganisms considers the greatest auspicious approaches for the harmless crop management performs [29]. In this respect, we tried to isolate and identify antagonistic bacteria with robust antifungal activities against *F. oxysporum* and *C. melongenae*. In our investigation, we found that among fifty (50) isolated bacteria, sixteen (16) isolated bacteria showed different degrees of antagonism (25-67%) against both fungi in dual culture. Four isolates with an inhibition rate above 50% were selected for further investigations (Figure 2). *In vitro* dual culture examination was widely applied to the preliminary screening of biocontrol/biological agents [21]. Effects of antagonistic are frequently established by the development of inhibition zones between fungal and isolated bacteria [21] or by calculating the radial mycelial growth inhibition in percentage towards the isolated bacteria [30]. The selected isolates were identified based on morphological, biochemical and molecular techniques.

Molecular characterization was established and approved almost 2 decades years before to identify isolated bacteria species. This identification is commonly based on some sole parts of their subunit of 30S ribosomal RNA, which is called 16S rDNA [31]. So, 16S rDNA- sequence based techniques were extensively applied to characterize the bacterial community structure in soils [32, 25, 26, 33]. The 16S rDNA sequences of isolateA4, C1, C2, and E2 revealed that they were closely matched with *Providencia* sp, *B. subtilis* 168, *B. subtilis* RKP-2, and *B. amyloliquefaciens*, respectively (Table 3).

Table 3. Closely related species of the four selected isolated bacteria based on the similarity of the partial 16S rDNA sequences in BLASTN

Isol-ate	Related species name	Strain no.	Accession number	Simil-arity (%)
A4	*Providencia* sp.	TT14	GU457414.1	100
C1	*Bacillus subtilis*	168	NC000964.3	100
C2	*Bacillus subtilis*	RKP-2	KR780236.1	99
E2	*Bacillus amyloliquefaciens*	IBSD G-11	KP036929	100

The *B. subtilis* are Gram positive, rod shaped, motile soil bacteria. Both *B. subtilis* strains showed the antagonistic activities in the dual culture. *B. subtilis* IUBTC1 showed the inhibition rates 50% and 63% against fungi *F. oxysporum* and *C. melongenae* respectively, whereas *B. subtilis* IUBTC2 showed the inhibition rates 67% and 64% against both fungi respectively due to produce more enzyme substances

(Figure 2). Similarly, Burhan et al. [34] demonstrated that different species of *Bacillus*, most notably, *B. subtilis* produced approximately 60% of commercially available enzymes. In previous studies, *Bacillus* spp. exhibited very broad spectra of action with an efficient antagonistic activity against *F. oxysporum* [35-37]. Several species of *Bacillus* have been recognized as plant-growth promoting bacteria (PGPB) and/or biocontrol agents (BCA) [37]. It was also reported that the genus *Bacillus* has become a dependable choice to discovery out novel and promising bacteria for the making of amylase and other extracellular enzymes [33]. According to the NCBI database, isolate E2 was identified as *B. amyloliquefaciens* IUBTE2. It exhibited inhibition rates 62% and 50% against *F. oxysporum* and *C. melongenae,* respectively (Figure 2). In previous, it was reported that the non-pathogenic bacilli, *B. amyloliquifaciens* showed an inhibition rate of 46% against *F. oxysporum* [38]. *Providencia* spp. are as one of the most common multi-drug resistant bacteria [39]. Similarly, Godebo et al [40] reported that 75% of *Providencia* isolates were multi-drug resistant. In this study, isolated bacteria A4 was identified as *Providencia* sp. IUBTA4 that showed 58% and 57% mycelial growth inhibition against *F. oxysporum* and *C. melongenae,* respectively, due to biocontrol potential elicited defense enzymes (Fig 2). Similarly, Rana et al. [41] reported that *Providencia* spp. act as PGPR with biocontrol potential elicited defense enzymes in wheat.

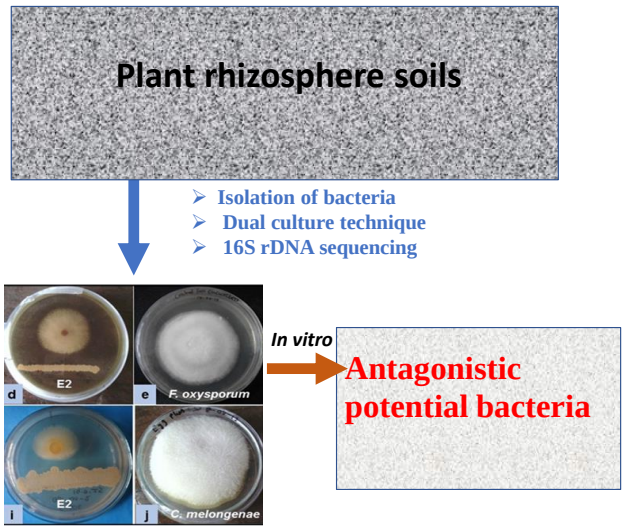

Figure 5. Schematic diagram of summary

CONCLUSION

The main ecological tasks facing microbiologists and plant pathologists are to produce the environment-friendly substitutions instead of chemical pesticides for the fighting of crop infections. Sixteen (16) isolates showed different degrees of antagonism (25-67%) against both fungi *F. oxysporum* and *C. melongenae*. Four (4) isolated bacteria, *Providencia* sp. TT14, *B. subtilis* 168, *B. subtilis* RKP-2, and *B. amyloliquefaciens* IBSDG-11, respectively were exhibited strong antagonism (more than 50% mycelial growth inhibition). *B. subtilis* IUBTC2 showed the highest capability for the control of mycelial growth against phytopathogenic fungi. Finally, it may conclude that newly isolated bacteria have a potential disease control capacity of the plant against the pathogenic fungi which can be enhanced plant growths and seed emergences as well as act as biocontrol agents for better green environmental management (Figure 5).

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AUTHOR CONTRIBUTIONS

MKA, MMR, and MRI comprehended and planned the study; MKA, and AHMJ carried out the analysis; MKA, and MMR wrote the manuscript; MMR prepared the graphs and illustrations; AHMJ and MRI contributed to the critical revision of the manuscript; MMR and MRI supervised the whole work; and all authors approved the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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