Identification of pathogen responsible for angular leaf spot disease of *Momordica charantia* and evaluation of biological control system

M E Khan Chowdhury[1], M Shah Alam[1], Md. Asadul Islam[2], Biswanath Sikdar[2] and Md. Faruk Hasan[3],*

Professor Joarder DNA and Chromosome Research Lab., Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh, [1]MS Thesis Student, [2]Professor, [3]Assistant Professor.

***Corresponding Author Email:** faruk_geb@yahoo.com

DOI: 10.29322/IJSRP.8.12.2018.p8410
http://dx.doi.org/10.29322/IJSRP.8.12.2018.p8410

Abstract

The present investigation was conducted for morpho-physiological and molecular detection of the causal agent responsible for angular leaf spot disease of *Momordica charantia* and its biological control technique. The pathogen of the devastating disease was isolated and cultured on Lauria and Bertani (LB) agar medium. The pathogen was characterized by a series of biochemical and molecular methods. Isolated bacteria showed positive and negative responses in biochemical tests. Molecular detection of the isolated bacteria was performed by polymerase chain reaction (PCR) using specific primers which amplified approximately 1400bp size of DNA fragment clear cut band. 16S rDNA gene sequencing of the bacterial isolate showed 86% similarities with the original sequence of *Pseudomonas syringae*. In antibiotic assay, cefixime showed highest 17.0±0.5mm diameter zone of inhibition against the isolated bacteria. Methanolic extracts of *Azadirachta indica* and *Allium sativum* revealed the highest 10.0±0.6mm diameter zone of inhibition against the isolated pathogenic bacteria. The antagonistic activity of soil bacteria, *Rhizobium* from *Vigna mungo* showed prominent zone of inhibition against the isolated bacterial strain. The present investigation would be helpful for detection and control of the devastating disease.

Key words: Momordica charantia, Angular leaf spot disease, Detection, Control system

1. Introduction

Momordica charantia, a member of the Cucurbitaceae family, commonly known as bitter gourd, or bitter melon thrives in humid and subtropical regions around the world [1]. Although this massive beneficial vegetable is well adapted to a wide range of climates, there are two varieties of the bitter gourd available in Bangladesh. The small sized variety is called “Ucche”, while the large sized variety is locally known as “Korolla”. Nutritionally, bitter gourd fruits are good source of carbohydrates, proteins, vitamins, and minerals and have the highest nutritive value among cucurbits [2, 3]. The active constituents of bitter melon are not definitively determined, but numerous studies have confirmed that it contains alkaloids, glycoside, peptides, acids, cucurbitins, charantin, cucurbitacins, momordine, momorcharins and proteins [4]. In Ayurvedic medicine, it has been used for indigestion, intestinal gas, menstrual stimulation, wound healing, inflammation, fever reduction, gonorrhea, hypertension, as a laxative and emetic [5, 6]. Along with therapeutic usages, the revolutionary plant has also versatile applications in the food industry [7]. However, direct yield losses caused by pathogens, animals and weeds, are altogether responsible for losses ranging between 20% and 40% of global agricultural

productivity [8-11]. Like other living organisms, bitter gourd plants are also susceptible to numerous devastating diseases. The diseases of vegetables involve the harmful deviation or alteration from the normal functioning in the physiological processes. Several bacterial, fungal, and viral diseases adversely affect the worldwide cucurbit production. Among the diseases, angular leaf spot, caused by the gram-negative bacterium *Pseudomonas syringae*, is one of the most prominent disease in bitter gourd [12]. The small, round, water-soaked spots appear on leaf tissue, and expand until they are confined by veins, giving them the characteristic angular look. It spreads rapidly when conditions are moist and ambient temperatures are between 75 and 82°F (24-28°C). The angular leaf spot disease may cause significant yield losses in both greenhouse and field production [13] which results in severe economic loss to farmers of cucurbits [14]. In order to protect the crop from the yield reducing disease and increase the production level, it has become a burning issue to find out appropriate control measure so that the farmer can manage the deleterious disease properly. Hence, the present research work was aimed to study the isolation, characterization and evaluation of its suitable control systems.

2. Materials and methods

2.1 Collection of plant material

In the present investigation, disease infected leaves of bitter gourd plant were collected carefully from farmers' field in Naogaon district, Bangladesh. The samples were placed in sterile polyethylene bags, properly labeled, transported to the lab and analyzed immediately thereafter. The symptoms were identified on the basis of diseased symptoms by Bangladesh Council of Science and Industrial Research (BCSIR), Binodpur, Rajshahi.

2.2 Isolation of causal organism

Disease infected leaves with spot symptoms were washed with sterilized distilled water, and then treated with 0.5 % solution of hypochlorite [15] for 1-2 min to remove the contaminants, rinsed with sterile distilled water. Then the samples were cut with sterile scalpel and weighed approximately 1gm that was immersed in sterilized saline buffer and vortexed strongly. Then it was serially diluted 10 folds and 100µl of diluted sample was plated on LB agar plates and incubated at 37°C. The bacterial single colonies were further streaked on fresh LB agar plates to procure isolated colonies.

2.3 Morphological and biochemical characterization

The isolated bacteria were identified based on colony characteristics, gram staining methods and by various biochemical tests as given by Bergey's [16] Manual of Determinative Bacteriology.

Gram staining test: Gram staining is the most common staining technique used diagnostically within both the research laboratories and in clinical setting to differentiate between Gram positive and Gram negative bacteria [17, 18] based on the physical properties of their cell walls [19]. The cell wall of Gram-positive organisms retain s crystal violet-iodine complex after treatment with alcohol and appears purple, whereas gram-negative organism decolorize s following such treatment and appears pink.

SIM (Sulphide-Indole-Motility) medium test: SIM medium enables differentiation of bacteria on the basis of hydrogen sulfide production, indole production, and motility [20, 21]. In the medium, peptonized iron and sodium thiosulphate are the indicators of H_2S production. This H_2S reacts with peptonized iron to form black precipitate of ferrous sulphide [22, 23]. Casein peptone, another component of SIM Medium, is rich in tryptophan. The organisms possessing the enzyme tryptophanase degrade tryptophan to indole. Indole is detected upon the addition of Kovac's reagent. SIM medium is a semi-solid that extensively used in the determination of bacterial motility [24]. The semi-solid nature of this medium allows easy visual determination of motility which appears as growth extending outward from the original line of inoculation.

Simmon's citrate test: Simmon's citrate agar is used for differentiating gram-negative bacteria on the basis of citrate utilization [25]. Initially, the citrate medium was developed by Koser [26] containing ammonium salt as the only nitrogen source and citrate as the

only carbon source. Later on Simmons [27] modified Koser's formulation by adding agar and bromothymol blue [28]. The citrate test is performed by inoculating microorganism into the above mentioned synthetic media, where bromothymol blue is used as an indicator. Use of citrate results in the creation of carbonates and bicarbonates as byproducts, thus increasing the pH of the medium [29] responsible for the change of the medium colour from green to blue and this is considered as positive test.

Kligler iron agar (KIA) test: Kligler iron agar is a combination of the lead acetate medium described by Kligler [30] and Russels Double Sugar Agar [31]. It is used for the differentiation of microorganisms on the basis of dextrose and lactose fermentation and hydrogen sulfide production in a laboratory setting. At first, we suspended 52 g of the medium in one liter of distilled water that was heated with frequent agitation and boiled for one minute to completely dissolve the medium into water. Then it was distributed into test tubes and autoclaved for 15 minutes at 121°C. After autoclaving, the medium containing test tubes were kept into laminar air flow hood in a slanted position. After cooling and solidifying the medium, isolated colony was taken from the pure culture plate and stabbed it into the butt of the medium. Then it was streaked back on the surface of the agar slant and incubated these test-tubes with loosened caps for 18-24 hours at 35±2°C in an aerobic atmosphere.

Tween 80 hydrolysis test: The test was performed by inoculating purified bacterial culture according to Sierra [32] method. In the procedure, Tween 80 was added to the molten media followed by pouring it into petri dish. Then the isolate was streaked on a medium and incubated at 37ºC for seven days to observe opaque milky precipitate/milky crystal formation.

Triple sugar iron (TSI) agar test: The triple sugar iron (TSI) test is one type of biochemical test that is commonly used to determine the ability of the microorganism to ferment sugars and the production of hydrogen sulfide. The media used contains agar, phenol red, 1% lactose, 1% sucrose, 0.1% glucose, as well as sodium thiosulfate and ferrous sulfate or ferrous ammonium sulfate. All of these ingredients were mixed together and allowed to solidify in the test tube at a slanted angle to prepare TSI slant. After that a sterilized straight inoculation needle was used to touch the top of a well-isolated bacterial colony and inoculated the bacteria by stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. After inoculation, the test tubes were loosely capped and incubated at 37°C in ambient air for 18 to 24 hours.

Urease test: The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease. Two units of ammonia are formed resulting alkalinity in the presence of the enzyme, and the increased pH is detected by a pH indicator. The medium was cooled to 50 to 55^{o}C and aseptically added 100 ml of filter-sterilized urea base to the cooled agar solution and mix thoroughly. Finally the medium were distributed to sterile tubes and slanted the tubes during cooling until solidified.

MacConkey agar test: MacConkey agar is a selective and differential culture media designed to isolate Gram-negative and enteric bacteria, particularly members of the family Enterobacteriaceae and the genus *Pseudomonas*, based on the ability of lactose fermentation. In the experiment, the components (peptone, proteose peptone, lactose, bile salts, sodium chloride, neutral red, crystal violet and agar) of MacConkey agar were suspended into appropriate amount of purified water and mixed thoroughly. Then it was heated with frequent agitation and boiled for 1 minute to completely dissolve the components. Then it was autoclaved at 121°C for 15 minutes. After solidifying the medium into laminar air flow hood, the experimental bacterial single colony was streaked on the plate of MacConkey's agar and incubated it at 37°C.

Potassium hydroxide (KOH) test: For the KOH solubility test, one or two bacterial colonies were aseptically taken from petri-dish of pure cultured bacteria with a sterilized tooth pick or an inoculating wire loop and placed the colony onto a clean microscopic slide, mixed with 3% KOH solution for 1 minute and observed for the formation of a thread-like mass. The test was performed according to Halebian *et al.,* [33].

Mannitol salt agar test: Mannitol salt agar is a commonly used selective and differential growth medium in microbiology. If an organism can ferment mannitol, an acidic byproduct is formed, causes the phenol red in the agar to turn yellow [34].

Catalase test: Catalase is a ubiquitous antioxidant enzyme that degrades hydrogen peroxide into water and oxygen [35]. Several pathogens produce catalase in order to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by the host's immune system, in addition to oxidative stress. At first, a clear and sterilized microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacterial colonies from 24-hour pure culture was placed onto the microscopic slide. Then, 3% of hydrogen peroxide ($H_2O_{2)}$ solution was added to the slide containing isolated bacteria and mixed with it. Production of bubbles indicated the presence of catalase enzyme in the bacteria [36].

2.4 Antibiotic susceptibility testing

In microbiology laboratories, agar disk diffusion is routinely used for testing common, rapidly growing bacterial pathogens [37]. The susceptibility of bacterial isolate against antibiotic was determined by the disc diffusion method which uses antibiotic impregnated wafers (disk) to test whether the particular bacteria is susceptible to specific antibiotic or otherwise [38]. In this method, 0.1 ml of a 10^{-2} dilution of each bacterial culture was smeared evenly onto the surface of the Petri-plate containing about 25 ml LB agar medium. After that the disk of filter paper impregnated with a standard concentration of an antibiotic was placed to the plate surface [39]. Plates were incubated for 16–18 hours in ambient air at 37°C before the results determined. The bacterial strains resistant to a specific antibiotic grow up to the margin of disk. The diameter of zone of inhibition must be measured and result read from the Kirby Bauer chart as sensitive, intermediate or resistant. Total seventeen types of commercially available and frequently prescribed standard antibiotics namely, amoxycillin, ampicillin, azithromycin, cefixime, cefotaxime, clarithromycin, doxycycline erythromycin, gentamycin, kanamycin, nalidixic acid, neomycin, penicillin, rifampicin, streptomycin, tetracyclin and vancomycin were used to assess antibiotic susceptibility against the isolated bacterial strain.

2.5 Screening for the antimicrobial potential of the plant extracts

Different plant extracts are commonly used which are considered natural sources of antimicrobial agents, regarded as safe and easily degradable [40]. The present study was aimed at evaluating the *in vitro* antimicrobial activity of plants extracts such as- *Allium sativum*, *Azadirachta indica*, *Cassia alata*, *Coccinia grandis*, *Hibiscus rosa-sinensis*, *Ficus racemosa*, *Psidium guajava* and *Moringa oleifera* against the isolated bacterial strain. The specific plant parts (10 grams of powdered sample) were dissolved in 100 ml of methanol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190–220 rpm for 24 hours. The supernatants were collected slowly and evaporated in wide mouthed evaporating bowls at room temperature for 2–3 days till the total final volumes were reduced to one fourth of the original volumes of the solvent that was used which giving the concentration of 400 mg/ml [41]. Finally the extracts were stored at 4°C refrigerator in airtight bottles. In the study, leaves were used to prepare plant extracts for the plant samples neem, ringworm shrub, scarlet gourd, common fig, guava and drumstick whereas bulb and flower were used in case of garlic and China rose, respectively. Antibacterial activity of the selected plants parts was carried out by the disc diffusion method [42]. At first, the different plant extracts were dissolved in methanol at a concentration of 100 mg/mL and filtered through 0.45 µm sterile filter membranes. Then, 100 µL of bacterial broth culture were spread over plates containing LB agar media. After that discs (6 mm in diameter) impregnated with 30 µL of the extracts solutions (1 mg/disc) were placed on the surface of the LB agar plate. Two control discs were also used containing methanol and kanamycin (30 µg/disc) as negative and positive controls, respectively. The plates were incubated for 24 hours at 37 °C, and the zone of inhibitions were measured with the help of mm scale.

2.6 Screening of antagonistic activity of soil bacteria

The demand to discover newer and safer bio-control agents with lesser side effects are increasing day-by-day [43]. Soil is a natural reservoir for the microorganisms with their antimicrobial products and provides an excellent resource for the isolation and identification of therapeutically important products [44]. Agar well diffusion method [45] was used to assess the antagonistic activity of soil bacteria against the phyto-pathogenic bacteria. Tolal four types of soil bacteria namely, *Brevibacillus*, *Pseudomonas*,

Rhizobium from *Cicer arietinum* and *Rhizobium* from *Vigna mungo* were used as antagonistic microorganisms. Agar well diffusion method is widely used to estimate the antagonistic activity of microbial extracts [46]. Firstly, the pathogenic bacterial isolate was incubated in LB broth at appropriate temperature for 24 hrs. Petri dishes containing 20 ml of LB agar medium were prepared previously and inoculated with 0.1 ml of 24 hours incubated bacterial broth culture. Then, three wells were made into the agar medium with a sterile cork borer or a tip and filled with different amounts of soil bacterial culture broth like 20 µl, 30 µl, and 40 µl. After the incubation, Petri dishes were incubated at 37°C for 24 hrs. The diameter of inhibition zone was observed and measured manually with the help of mm scale which determined the antagonistic activity of the soil bacteria.

2.7 Molecular characterization of isolated bacteria

After studying the morphology and biochemical characters, the molecular techniques are applied for the better classification and identification of the isolated bacterial strain.

DNA extraction: For DNA isolation, a single colony was inoculated in LB broth and grown for overnight at 37ºC. The genomic DNA was isolated from these fresh cultured colonies by using the CTAB (N-cetyl-N, N, N trimethyl ammonium bromide) method described by Murray [47] and Thompson [48]. After that the DNA was suspended into TE buffer. The spectrophotometer was then used to quantify the isolated DNA followed by electrophoresis on 1% agarose gel.

PCR analysis: The 16S rDNA sequence was amplified from the isolated genomic DNA by PCR reaction in a thermo cycler (Nyx, Technic, Inc., USA), using the two universal primers 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1492R (5'-TACCTTGTTACGACTT-3'). The total volume of components for PCR was 25 µL containing nuclease free ddH_2O 15 µL, dNTP mix 1.0 µL, forward primer 1.0 µL, reverse primer 1.0 µL, DNA template 1.5 µL, $MgCl_2$ 2.5 µL, Taq buffer B 2.5 µL and Taq polymerase 0.5 µL. Thermocycling parameters were 95°C for 5 min, 35 cycles of 95°C for 40 s, 65°C for 1 min, and 72°C for 2 min; a final extension step at 72°C was added for 10 min, followed by cooling to 4ºC until the sample was recovered.. The length of amplified PCR products were then examined by 1% gel electrophoresis where 0.5x TBE (Tris-borate-EDTA) running buffer was used in agar gel and finally visualized under a UV trans-illuminator.

Purification of PCR products, sequencing and phylogenetic analysis: The amplified DNA was then purified by agar gel electrophoresis method using AccuPrep® Gel Purification, Bioneer kits. After that the purified products were sequenced in sequencing service laboratory, National Institute of Biotechnology (NIB), Bangladesh, using bacterial gene specific primers 27F (5′-AGAGTTTGATCCTGGCTC-3′).

The sequences was compared with their respective type strains using the BLASTN program in the GenBank nucleotide database (http://www.ncbi. nlm.nih.gov/BLAST). Finally, phylogenetic tree was constructed based on the Maximum Likelihood method.

3. Results

3.1 Isolation and purification of bacteria

The infected leaves samples produced turbid condition in LB liquid media after 16 hours of incubation at 37ºC indicated the bacteria were grown. From liquid culture, subculture were done by streaking onto the LB agar medium in 90 mm petri dishes. The bacterial isolate was partially identified based on colony morphology and purification done by further streaking method. The colonies were creamy white in color. The size and shape of colonies were found to be small to medium, convex, and mucoid.

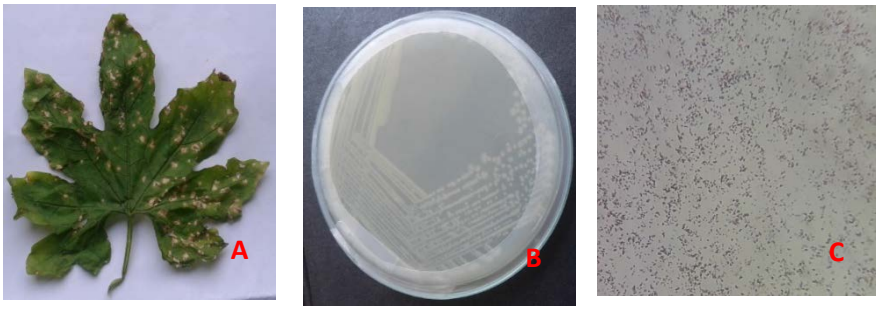

Figure 1: Showing the plant sample, isolation of causal organism and gram staining (A) angular leaf spot disease of bitter gourd, (B) Isolated bacterial pure colonies and (C) Gram negative bacteria

3.2 Morphological and biochemical characterization of isolated bacteria

In gram staining test, the isolate bacterial strain was rod shaped and pinkish in color when stained with counter-stained by the safranin that was the indication for gram negative bacterium (Figure: 1 C). In SIM-medium (Sulphide-Indole-Motility medium) test, the isolated bacteria did not produce any red or pink color band on the top of tube containing SIM medium after adding Kovac's reagent and no black precipitation formed as no H_2S was produced. Finally, the isolated bacterial strain showed motility in the medium after 48 hours of incubation. As a part of the biochemical test for the identification of bacteria, citrate test was performed based on the citrate metabolism ability of the strain. The isolated bacteria showed positive result in Simmon's citrate agar, because the colour of medium changed from green to royal blue, which indicated the isolated bacteria were capable to utilize citrate. Kligler iron agar is a complex medium that contains a large amount of lactose and a very small amount of glucose; a pH indicator (yellow in acid and red in base); and iron, which is precipitated as a black sulfide if H_2S is produced. In the study, no H_2S was produced, but the resulting yellow colour showed that the isolated pathogen was lactose and glucose fermenting bacteria. In tween 80 hydrolysis test, the inoculating medium showed crystal precipitation by hydrolyzing tween 80. Esterase hydrolyzed the tween and subsequent precipitation with the calcium chloride appeared as a turbid zone around the bacterial colonies. Change in the colour of TSI medium pointed out towards the positive result where yellow colour of both slant and butt indicated the isolated bacteria were glucose and lactose fermented microorganism. It produced a large amount of acid which turned the phenol red indicator into yellow colour in both butt and slant, but no H_2S was formed in the medium. The bacterial isolate was incubated into the urease media at 37℃ and the result was observed after 48 hours of incubation. The agar slant remained yellow and the isolated bacteria did not hydrolyze urea. So, urease test was negative for the isolated bacteria. In MacConkey agar test, the isolated bacteria were grown on MacConkey agar medium which indicated that they were gram-negative enteric bacteria. It also showed that it was capable of fermenting lactose producing pink color around the colony. In potassium hydroxide (KOH) test, the bacterial smear became a viscous, stringy, sticky mess within 15 seconds when picked up with a loop that confirmed positive result. In mannitol salt agar test, the bacteria fermented mannitol and resulting acidic byproduct responsible for the phenol red in the agar to turn yellow. The catalase test was performed to identify organisms that produce the enzyme- catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. After inoculating the bacteria with hydrogen peroxide on the clean slide, the bubbles resulting from production of oxygen gas clearly indicated that the strain produced positive result for catalase production.

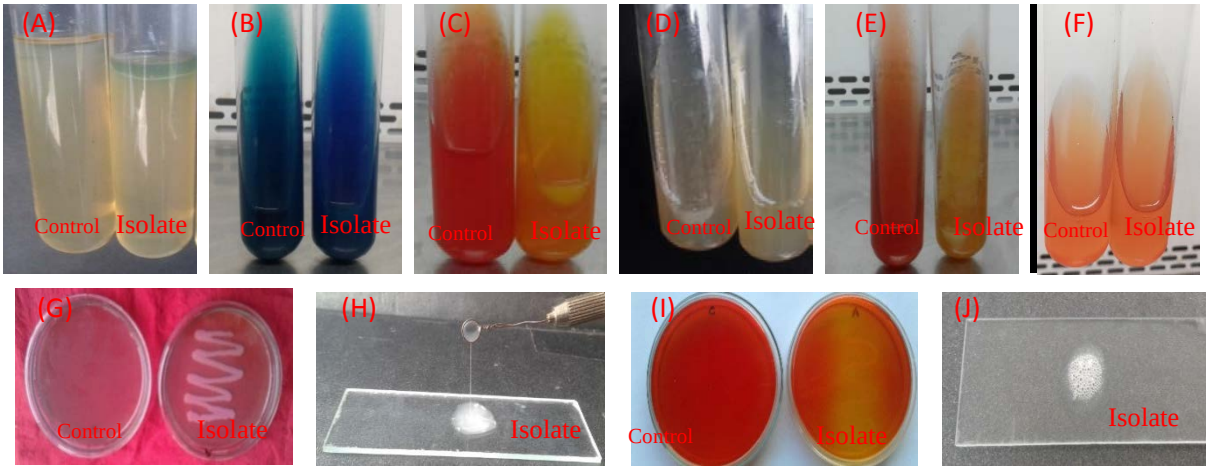

Figure 2: Showing biochemical characterization of isolated bacterial strain; (A) SIM medium test, (B) Simmon's citrate test, (C) Kligler Iron agar, (D) Tween 80 test, (E) Triple sugar iron test, (F) Urease test, (G) MacConkey agar test, (H) KOH solubility test, (I) Mannitol salt agar test and (J) Catalase test.

Table 1: Biochemical tests of isolated bacteria in different media

Name of the test	**Response**	**Appearance**	**Remarks**
Gram straining	-(ve)	Small, rod shaped, pink color colony	Pink color confirmed it was gram negative bacteria
SIM-medium test	+(ve), -(ve)	Motile, no H_2S and indole production	The bacteria was motile and did not produce any indole and H_2S
Simmon's citrate test	+(ve)	Royal blue color was produced	Isolated bacteria was capable to utilize citrate
Kligler iron agar test	+(ve)	Color changed from red to yellow	Acid was produced in test tube
Tween 80 test	+(ve)	Turbid zone around the colonies	The bacterial isolate was capable to hydrolyze tween 80
Triple sugar iron test	+(ve)	Yellow slants and butt	Isolated bacteria fermented glucose and lactose, but no gas and H_2S were formed
Urease test	-(ve)	Slant was yellow	It did not hydrolyze urea to ammonia
MacConkey agar test	+(ve)	Pink colour around the colony	Capable to ferment lactose
Potassium hydroxide test	+(ve)	Viscous and thread like slime	Isolated bacteria was gram negative
Mannitol salt agar test	+(ve)	Yellow colour around the colony	Mannitol was utilized
Catalase test	+(ve)	Presence of oxygen bubbles	Isolated bacteria produced catalase enzyme

3.3 Molecular characterization of isolated bacteria

Genomic DNA revealed sharp high molecular weight bands of DNA (Figure 3A). The sequence of 16S rDNA region of the bacterial strain was then amplified using universal bacterial specific primers 27F and 1492R through PCR. 16S rDNA amplified length was around 1400bp (Figure 3B). The data analysis revealed that the 16S of rDNA sequence of isolated bacterial strain showed 86% similarity with the original sequence of *Pseudomonas syringae*.

3.4 Phylogenic analysis

The 16S rDNA sequences of related bacterial strains were downloaded in FASTA format from GenBank. After that the downloaded data were aligned to construct phylogenetic tree (Figure 3c).

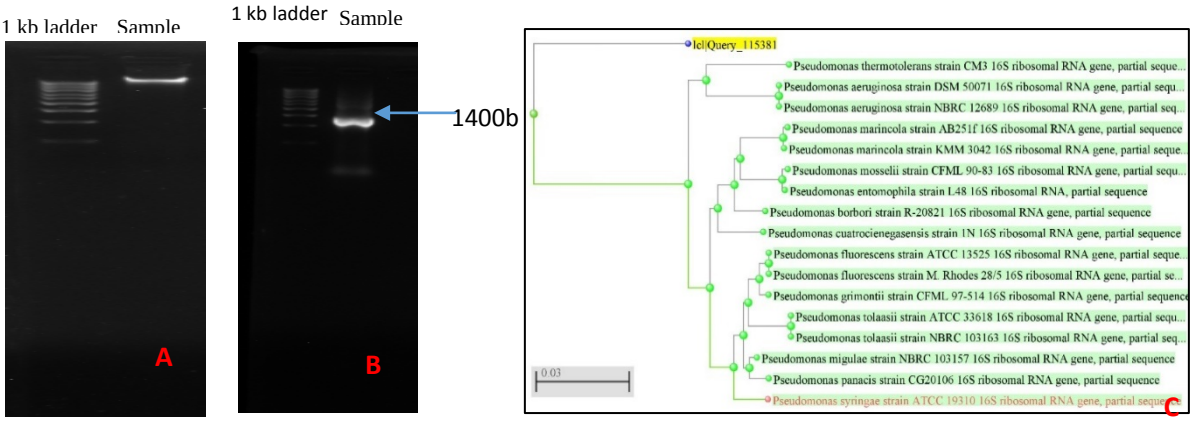

Figure 3: PCR amplification and phylogenetic tree, A) bacterial genomic DNA B) PCR product using agarose gel electrophoresis and C) Phylogenetic tree of isolated bacteria

3.5 Antibiotic susceptibility testing

The result showed that the pathogenic isolate was susceptible to cefixime, gentamycin 30 and intermediate resistant to nalidixic acid, cefotaxime, gentamicin 10, streptomycin, but resistant to amoxycilin, ampicillin, azithromycin, clarithromycin, doxycycline, erythromycin, kanamycin, neomycin, penicillin, rifampicin, tetracyclin and vancomycin.

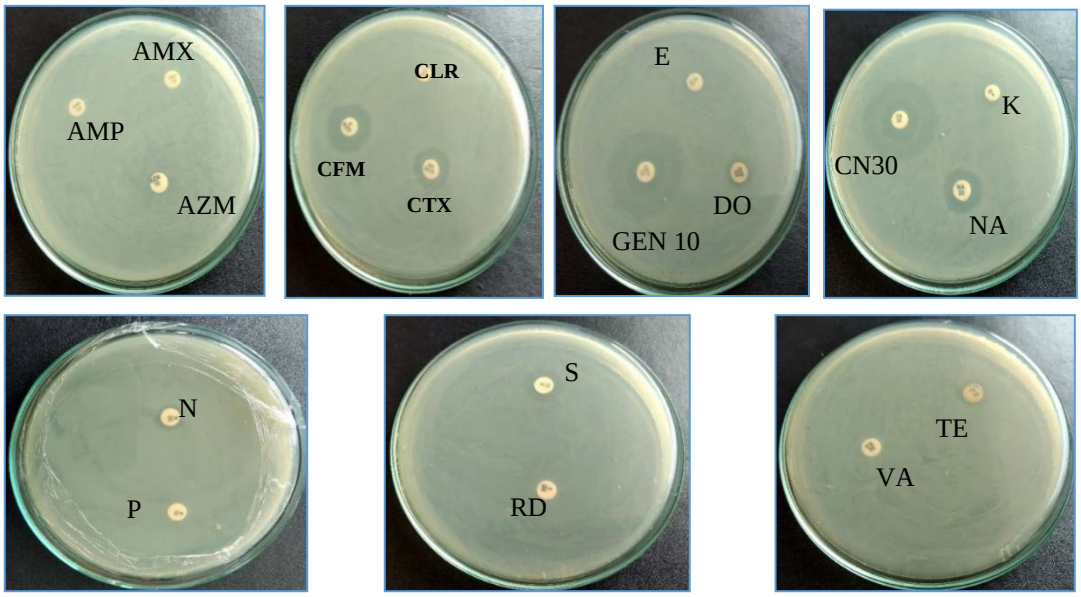

Figure 4: Antibiotic susceptibility testing against bacterial isolate

Table 2: Antibiotic susceptibility test of isolated bacteria

Name of antibiotic	Disc potency (µg/disc)	Zone of inhibition (mm)	Response
Amoxycillin	10µg	6±0.0	Resistant
Ampicillin	10 µg	6±0.0	Resistant
Azithromycin	15 µg	6±0.0	Resistant
Cefixime	5 µg	17±0.5	Susceptible

Cefotaxime	30 µg	13±0.4	Intermediate
Clarithromycin	15 µg	6±0.0	Resistant
Doxycycline	30 µg	8±0.2	Resistant
Erythromycin	15 µg	6±0.0	Resistant
Gentamycin	10 µg	13±0.3	Intermediate
	30 µg	15±0.4	Susceptible
Kanamycin	30 µg	8±0.2	Resistant
Nalidixic acid	30 µg	14±0.6	Intermediate
Neomycin	30 µg	8±0.3	Resistant
Penicillin	10 µg	6±0.0	Resistant
Rifampicin	5 µg	6±0.0	Resistant
Streptomycin	10 µg	12±0.3	Intermediate
Tetracyclin	30 µg	8±0.2	Resistant
Vancomycin	30 µg	6±0.0	Resistant

3.6 Screening for the antimicrobial potential of the plant extracts

The highest antimicrobial activity was found 10±0.5mm diameter of inhibition zone at 30 µl/disc concentration by methanolic extract of *Azadirachta indica* and *Allium sativum* respectively, against the isolated bacteria. Besides that *Moringa oleifera* and *Coccinia grandis* showed 8±0.4mm and 8±0.3mm diameter zone of inhibition at the concentration of 100µl/disc. On the other hand, *Cassia alata*, *Hibiscus rosa-sinensis*, *Ficus racemosa* and *Psidium guajava* showed no anti-bacterial activity against the isolated bacterial strain (Table not given).

3.7 Screening of antagonistic activity of soil bacteria

In antagonistic activity of soil bacteria, the *Rhizobium* from *Vigna mungo* showed 8.0±0.2mm zone of inhibition at 40µl/well concentration but others did not show any remarkable activity.

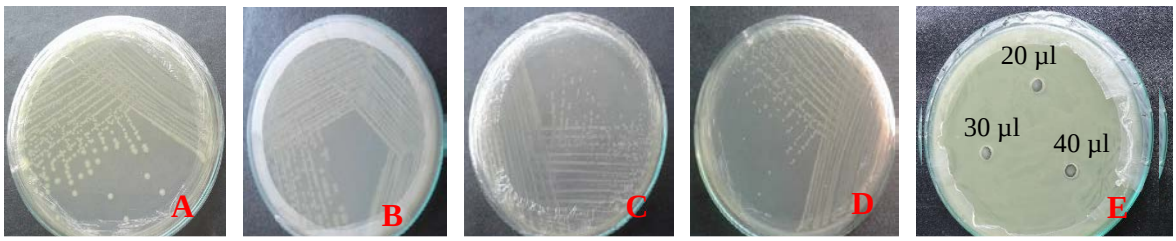

Figure 5: Showing the soil bacteria and antagonistic activity A) *Pseudomonas*, B) *Rhizobium* from *Cicer arietinum*, C) *Rhizobium* from *Vigna mungo*, D) *Brevibacillus*, E) co-culture of *Rhizobium* from *Vigna mungo* against isolated bacteria

4. Discussion

Bitter gourd, *Momordica charantia* L. is one of the most popular vegetable of Cucurbitaceae family in the tropical and subtropical countries [49, 50]. Bitter gourd is commonly attacked by a number of diseases that are of national importance and cause important economic losses in this vegetable [51, 52]. Angular leaf spot of bitter gourd is a world widely distributed disease generally

considered to be caused by *Pseudomonas syringae* [53, 54] responsible for 80 to 100% disease incidence in previous epidemics [55] causing severe damage to the yield of bitter gourd [56]. The symptoms of angular leaf spot include small, round to irregular, water-soaked lesions which may or may not have a halo. The foliar lesions of disease infected plant became angular as they grow, and tan to brown in color in matured stage. These symptoms were similar described by Zitter *et al*., [61].

The bacteria isolated from the disease infected leaves showed similar characteristics of the genus *Pseudomonas* and confirm the characteristics for the inclusion in the group of *Pseudomonas* bacteria [58]. The phenotypic description of the isolated bacteria is the same as genus *Pseudomonas.* The bacterial Colonies found from the angular leaf spot of bitter gourd plant were creamy white, small to medium, convex, and mucoid on LB agar medium. Aksoy, [59] reported that the *Pseudomonas spp.* isolates were able to produce round or circular domed shaped colonies that supports our findings. In our investigation, the isolated bacteria was rod-shaped and showed negative result in Gram staining test which in line with the study of Shila *et al*., [60] and Scortichini *et al*., [61]. The concerned bacterial strain didn't produce any indole or H_2S, but showed motility in the SIM medium which was also reported by Hirano and Upper [62].

According to Brown *et al.,* [63], utilization of citrate changes the Simmon's citrate agar medium from green to royal blue, which indicated the isolated bacteria was positive for the test. Our bacteria showed similar result that confirmed the isolated bacteria was capable to use citrate. Kligler iron agar recommended by ISO committee for identification of *Pseudomonas* species [64] showing positive result enabled the differentiation of species of the enteric bacteria. In tween 80 hydrolysis test, the inoculating medium produced crystal precipitation by bacterial isolate through hydrolyzing tween 80 [65]. In TSI agar medium, the isolated bacteria fermented the carbohydrates and its byproduct changed the colour of medium [66, 67]. The colour of medium used for the urease test was not changed after 48h of bacterial inoculation which indicated negative result that was similar with the findings of Bailey and Scott, [68]. In MacConkey agar test, the bacteria were grown indicated that they were gram-negative enteric bacteria. In case of the KOH solubility test, the bacterial colonies produced thread-like mass when mixed with 3% KOH solution [69]. The isolate was capable to utilize mannitol which was similar with the previous study [70]. The isolate was able to produce catalase enzyme in catalase test whereas the study of Carpena *et al*., [71] proved our finding.

Bactericidal antibiotics induced bacterial cell death by inhibiting synthesis of bacterial cell wall, DNA or RNA, proteins, competitive inhibition of folic acid or act as membrane disorganizing agents [72, 73]. The antibiotic susceptibility assay was examined where the result showed that the pathogenic isolate was highly susceptible to cefixime and gentamycin 30. Akinbowale *et al*., [74] also observed *Pseudomonas spp.* were sensitive to gentamycin. Our finding was also supported by Hossain *et al*., [75] where cefixime inhibited the bacterial growth to a great extent [76].

The pathogen was resistant to some antibiotic such as doxycycline, tetracyclin, vancomycin etc. The findings of the present study have been supported by Basak *et al*., [77], Akter [78] and Chowdhury [79]. *Pseudomonas syringae* showed resistance against doxycycline in their investigations. Tetracycline also didn't have negative effect on the growth of the pathogenic bacteria [80].

The antimicrobial activity exhibited by various plant extracts against harmful bacteria has been demonstrated by several researchers [81-84]. Recently, there have been many reports stated that some plant extracts and safe chemicals become a necessary to control many diseases of vegetable plants [85-88]. In the antibacterial assay, *Azadirachta indica* (neem) and *Allium sativum* (garlic) revealed the highest zone of inhibition against the pathogen among all plant extract used. According to Nahak and Sahu [89], neem extract showed significantly better performance over control of *Pseudomonas syringae* by 41.93%. The study of Guo *et al*., [90] also showed the antibacterial effect of garlic against *Pseudomonas syringae* which supported our findings.

Antagonistic bacteria play an important role in the suppression of plant diseases, and can be used as biological control agents [91, 92]. In the study, the antagonistic activity of *Rhizobium* from *Vigna mungo* were evaluated as highest antibacterial effects, 8.0±0.2mm zone of inhibition at 40µl/well concentration. Various strains of *Rhizobium spp.* and *Trichoderma spp.* have been described as effective biological control agents antagonistic to many plant pathogens [93, 94]. Various traits expressed synchronously, or in a controlled sequence, are considered responsible for the action of *Rhizobium* strains as biological control agents [95, 96]. Sequencing of 16S rDNA is a molecular technique for characterization of bacteria and the tool involved is to analyze the phylogenetic relationship of an organism**.** The nucleotide BLAST search in GenBank using 1389bp of 18S rDNA sequence revealed that the isolated bacteria showed 86% similarity with the original sequences of *Pseudomonas syringae*, as previously observed from partial 16S rDNA sequencing. The phylogenetic tree generated from GenBank proved that this organism genetically related with other organisms.

5. Conclusion

The disease, angular leaf spot is one of the most prevalent disease in bitter gourd responsible for huge economic loss of the farmers. The devastating bacterial disease of bitter gourd caused by *Pseudomonas syringae* is a Gram-negative, rod shaped motile bacteria. The present study involved isolation, biochemical and molecular characterization of the pathogen followed by assessment of some eco-friendly control measurement through antibiotics, plant extracts and soil bacteria. The antibiotic, especially cefixime, and the medicinal plant extract, neem had benign effect against the bacteria in the study. Besides that *Rhizobium* from *Vigna mungo* also showed prominent inhibitory effects on the phyto-pathogen in the antagonistic test. These biological prevention will ultimately favour safer management of the detrimental bacterial disease.

Acknowledgements

The authors are grateful to the Dean, Faculty of Life and Earth Science, University of Rajshahi, Bangladesh for providing financial support during the research works.

Author contributions

MEKC, MAI, BS and MFH designed the experiments, developed the methodology and prepared the manuscript. MEKH, MSA, BS and MFH collected the data and carried out analysis. MSA, MAI and BS assisted with manuscript preparation.

Author statement

All authors read, reviewed, agreed and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

6. Reference

[1]. Anjum, F., Shahid, M., Bukhari, S. A., Anwar, S., & Latif, S. (2013). Study of quality characteristics and efficacy of extraction solvent/technique on the antioxidant activity of bitter gourd seed. *J Food Process Technol*, *4*(2).

[2]. Miniraj, N., Prasanna, K. P., & Peter, K. V. (1993). Bitter gourd *Momordica spp. Kalloo G, Bergh BO (edns) Genetic improvement of vegetable plants. Pergamon Press, Oxford*.

[3]. Desai, UT., and A.M. Musmade. (1998). Pumpkins, squashes and gourds. pp. 273-298. In:D.K. Salunkhe and S.S. Kadam (eds.), Handbook of vegetable science and technology: Production, composition, storage and processing. Marcel Dekker, New York.

[4]. Torres, W. (2004). *Momordica charantia* Linn. Chemistry and pharmacology. *American Academy of Anti-Aging Medicine*.

[5]. Nadkarni KM (1993). *Indian Materia Medica*. India: Popular Prakashan.

[6]. Kumar, D. S., Sharathnath, K. V., Yogeswaran, P., Harani, A., Sudhakar, K., Sudha, P., & Banji, D. (2010). A medicinal potency of *Momordica charantia*.

[7]. Ali, M. A., Sayeed, M. A., Reza, M. S., Yeasmin, M. S., & Khan, A. M. (2008). Characteristics of seed oils and nutritional compositions of seeds from different varieties of *Momordica charantia* Linn. cultivated in Bangladesh. *Czech J Food Sci*, *26*(4), 275-83.

[8]. Teng, P. S., & Krupa, S. V. (1980). Assessment of losses which constrain production and crop improvement in agriculture and forestry. In *EC Stakman Commemorative Symposium (1980: University of Minnesota)*. Dept. of Plant Pathology, University of Minnesota.

[9]. Teng, P. S. (1987). *Crop loss assessment and pest management*. Aps Press.

[10]. Oerke, E. C., Dehne, H. W., Schönbeck, F., & Weber, A. (2012). *Crop production and crop protection: estimated losses in major food and cash crops*. Elsevier.

[11]. Oerke, E. C. (2006). Crop losses to pests. *The Journal of Agricultural Science*, *144*(1), 31-43.

[12]. Smith, E. F., & Bryan, M. K. (1915). Angular leaf spot of cucumbers. *Journal of Agricultural Research.*, *5*, 465-476.

[13]. Bhat, N. A., Masoodi, S. D., Mushtaq, A., & Zargar, M. Y. (2007). Occurrence and severity of angular leaf spot of cucumber in Kashmir. *Annals of Plant Protection Sciences*, *15*(2), 410-413.

[14]. Ozaktan, H., & Bora, T. (1994). Antagonistik bakterilerin hıyar köşeli leke (*Pseudomonas syringae* pv. *lachrymans*) hastalığının biyolojik savaşımında kullanılma olanakları üzerinde araştırmalar. In: Türkiye 3. Biyolojik Mücadele Kongresi, İzmir, pp 221–229.

[15]. Cotter, J. L., Fader, R. C., Lilley, C., & Herndon, D. N. (1985). Chemical parameters, antimicrobial activities, and tissue toxicity of 0.1 and 0.5% sodium hypochlorite solutions. *Antimicrobial agents and chemotherapy*, *28*(1), 118-122.

[16]. Bergey, DH; Holt, JG & Noel, RK, (1994). Bergey's Manual of Systematic Bacteriology. Vol.1, 9th Eds (Baltimore, MD: Williams & Wilkins).

[17]. Baron, E. J., Miller, J. M., Weinstein, M. P., Richter, S. S., Gilligan, P. H., Thomson Jr, R. B. & Robinson-Dunn, B. (2013). A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2013 recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM) a. *Clinical infectious diseases*, *57*(4), e22-e121.

[18]. Woods, G. L., & Walker, D. H. (1996). Detection of infection or infectious agents by use of cytologic and histologic stains. *Clinical microbiology reviews*, *9*(3), 382-404.

[19]. Madigan, M.T.; Martinko J & Parker J. *(*2004). Brock Biology of Microorganisms (10th ed.). Lippincott Williams & Wilkins.

[20]. MacFaddin, J. F. (1985). Media for Isolation-Identification-Cultivation-Maintenance of Medical Bacteria. *Vol. I Williams and Wilkins, Baltimore*.

[21]. Ewing, W. H. (1986). *Edwards and Ewing's identification of Enterobacteriaceae*. Elsevier Science Publishing Co. Inc.

[22]. Sulkin, S. E., & Willett, J. C. (1940). A triple sugar-ferrous sulfate medium for use in identification of enteric organisms. *Translational Research*, *25*(6), 649-653.

[23]. Sosa, H. (1943). Dr. Carlos G. Malbran'. *Rev. Inst. Bad*, *11*, 286.

[24]. Tittsler, R. P., & Sandholzer, L. A. (1936). The use of semi-solid agar for the detection of bacterial motility. *Journal of bacteriology*, *31*(6), 575.

[25]. Difcol (2009). (PDF) (2nd ed.). Sparks, Maryland: Difco Laboratories, Becton Dickinson and Company. p. 508.

[26]. Koser, S. A. (1923). Utilization of the salts of organic acids by the colon-aerogenes group. *Journal of bacteriology*, *8*(5), 493.

[27]. Simmons, J. S. (1926). A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolation of certain fungi. *The Journal of Infectious Diseases*, 209-214.

[28]. MacFaddin, J. F. (1985). Media for Isolation-Identification-Cultivation-Maintenance of Medical Bacteria. *Vol. I Williams and Wilkins, Baltimore*.

[29]. Koser, S. A. (1923). Utilization of the salts of organic acids by the colon-aerogenes group. *Journal of bacteriology*, *8*(5), 493.

[30]. Kligler, I. J. (1917). American Journal of Public Health, 7:1041.

[31]. Russell, F. F. (1911). The isolation of typhoid bacilli from urine and feces with the description of a new double sugar tube medium. *The Journal of medical research*, *25*(1), 217.

[32]. Sierra, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek*, *23*(1), 15-22.

[33]. Halebian, S., Harris, B., Finegold, S. M., & Rolfe, R. D. (1981). Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. *Journal of clinical microbiology*, *13*(3), 444-448.

[34]. Bachoon, Dave S., Dustman, & Wendy A. (2008). "Exercise 8: Selective and Differential Media for Isolation". In Michael Stranz. Microbiology Laboratory Manual. Mason, OH: Cengage Learning

[35]. Loewen, P. C., Switala, J., & Triggs-Raine, B. L. (1985). Catalases HPI and HPII in *Escherichia coli* are induced independently. *Archives of Biochemistry and Biophysics*, *243*(1), 144-149.

[36]. Rahman, S. S., Siddique, R., & Tabassum, N. (2017). Isolation and identification of halotolerant soil bacteria from coastal Patenga area. *BMC research notes*, *10*(1), 531.

[37]. Reller, L. B., Weinstein, M., Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical infectious diseases*, *49*(11), 1749-1755.

[38]. Jorgensen, J. H., & Turnidge, J. D. (2015). Susceptibility test methods: dilution and disk diffusion methods. In *Manual of Clinical Microbiology, Eleventh Edition* (pp. 1253-1273). American Society of Microbiology.

[39]. Duffy, C. F., & Power, R. F. (2001). Antioxidant and antimicrobial properties of some Chinese plant extracts. *International journal of antimicrobial agents*, *17*(6), 527-529.

[40]. Berahou, A., Auhmani, A., Fdil, N., Benharref, A., Jana, M., & Gadhi, C.A., (2007). Antibacterial activity of *Quercus ilex* bark's extracts. J. Ethnopharmacol. 112, 426–429.

[41]. Harborne, J. B. (1973). The terpenoids. In *Phytochemical Methods* (pp. 89-131). Springer, Dordrecht.

[42]. Gulluce, M., Sahin, F., Sokmen, M., Ozer, H., Daferera, D., Sokmen, A & Ozkan, H. (2007). Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. longifolia. *Food chemistry*, *103*(4), 1449-1456.

[43]. Gupte, M., Kulkarni, P., & Ganguli, B. (2002). Antifungal antibiotics. *Applied microbiology and biotechnology*, *58*(1), 46-57.

[44]. Dancer, S. J. (2004). How antibiotics can make us sick: the less obvious adverse effects of antimicrobial chemotherapy. *The Lancet infectious diseases*, *4*(10), 611-619.

[45]. Valgas, C., Souza, S. M. D., Smânia, E. F., & Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian journal of microbiology*, *38*(2), 369-380.

[46]. Valgas, C., Souza, S. M. D., Smânia, E. F., & Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian journal of microbiology*, *38*(2), 369-380.

[47]. Human Microbiome Jumpstart Reference Strains Consortium. (2010). A catalog of reference genomes from the human microbiome. *Science*, *328*(5981), 994-999.

[48]. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic acids research*, *25*(24), 4876-4882.

[49]. Kumar, V., Kerketta, A., Sahu, A., Teta, A., & Khare, C. P. (2018). Management of prevalent diseases of bitter gourd (*Momordica charantia* L.). *Journal of Pharmacognosy and Phytochemistry*, 26-35.

[50]. Win, N. K. K., Kim, Y. H., & Jung, H. Y. (2014). Bitter gourd little leaf disease associated to 'Candidatus Phytoplasma asteris'. *Tropical Plant Pathology*, *39*(1), 82-88.

[51]. Mathew, A. V., & Alice, K. J. (2002). Transmission, host range and etiology of mosaic disease of bitter gourd. *Indian Phytopathology*, *55*(2), 219-220.

[52]. Khan, F. M., Amin, M., Ullah, Z., Rehman, S., Amir, M., & Ali, I. (2014). Distribution of Alternaria Leaf Spot of bitter gourd in district Peshawar and Nowshera, Khyber Pakhtunkhwa, Pakistan. *Journal of Pharmacognosy and Phytochemistry*, *3*(2).

[53]. Bradbury, J. F. (1986). *Guide to plant pathogenic bacteria*. CAB international.

[54]. Zitter, T. A., Hopkins, D. L., & Thomas, C. E. (1996). *Compendium of cucurbit diseases* (No. 635.62 C737). American Phytopathological Society, St. Paul, Minn.(EUA).

[55]. Harighi, B. (2007). Angular leaf spot of cucumber caused by *Pseudomonas syringae* pv. *lachrymans* in Kurdistan. *Plant Disease*, *91*(6), 769-769.

[56]. Ozaktan H, Bora T (1994) Antagonistik bakterilerin hıyar köşeli leke (*Pseudomonas syringae* pv. *lachrymans*) hastalığının biyolojik savaşımında kullanılma olanakları üzerinde araştırmalar. *In: Türkiye 3. Biyolojik Mücadele Kongresi, İzmir, pp 221–229*Google Scholar

[57]. Zitter, T. A., Hopkins, D. L., & Thomas, C. E. (1996). *Compendium of cucurbit diseases* (No. 635.62 C737). American Phytopathological Society, St. Paul, Minn.(EUA).

[58]. Misaghi I, Grogan RG. Nutritional and biochemical comparison of plant pathogenic and saprophytic fluorescent pseudomonads. Phytopathology. 1969; 59:1436-1450.

[59]. Aksoy, H. M. (2006). Occurrence of *Pseudomonas syringae* pv. *lachrymans* [(Smith and Bryan) Young, Dye and Wilkie] at Bafra Province Greenhouses. *Plant Pathology Journal*, *5*(1), 80-82.

[60]. Shila, S. J., Islam, M. R., Ahmed, N. N., Dastogeer, K. M. G., & Meah, M. B. (2013). Detection of *Pseudomonas syringae* pv. *lachrymans* associated with the seeds of cucurbits. *Universal Journal of Agricultural Research*, *1*(1), 1-8.

[61]. Scortichini, M., Marcelletti, S., Ferrante, P., Petriccione, M., & Firrao, G. (2012). *Pseudomonas syringae* pv. *actinidiae*: a re-emerging, multi-faceted, pandemic pathogen. *Molecular plant pathology*, *13*(7), 631-640.

[62]. Hirano, S. S., & Upper, C. D. (1990). Population biology and epidemiology of *Pseudomonas syringae*. *Annual review of phytopathology*, *28*(1), 155-177.

[63]. Brown, A. and Smith, K., 2015. Benson's Microbiological Applications, *Laboratory Manual in General Microbiology*. 13th Ed. pp. 277.

[64]. International Organization for Standardization (ISO), 1995, Draft ISO/DIS 13720.

[65]. Surinenaite, B., Bendikiene, V., Juodka, B., Bachmatova, I., & Marcinkevichiene, L. (2002). Characterization and physicochemical properties of a lipase from *Pseudomonas mendocina* 3121–1. *Biotechnology and applied biochemistry*, *36*(1), 47-55.

[66]. MacFaddin, J. F. (1985). Media for Isolation-Identification-Cultivation-Maintenance of Medical Bacteria. *Vol. I Williams and Wilkins, Baltimore*.

[67]. Forbes, B. A., Sahm, D. F., & Weissfeld, A. S. (2007). *Study guide for Bailey & Scott's diagnostic microbiology*. USA: Mosby.

[68]. Bailey, W. R., & Scott, E. G. (1966). *Diagnostic Microbiology: A Textbook for the Isolation and Identification of Pathogenic Microorganism*. CV Mosby.

[69]. Shila, S. J., Islam, M. R., Ahmed, N. N., Dastogeer, K. M. G., & Meah, M. B. (2013). Detection of *Pseudomonas syringae* pv. *lachrymans* associated with the seeds of cucurbits. *Universal Journal of Agricultural Research*, *1*(1), 1-8.

[70]. Schaad, N. W., Jones, J. B., & Chun, W. (2001). *Laboratory guide for the identification of plant pathogenic bacteria* (No. Ed. 3). American Phytopathological Society (APS Press).

[71]. Carpena, X., Perez, R., Ochoa, W. F., Verdaguer, N., Klotz, M. G., Switala, J., ... & Loewen, P. C. (2001). Crystallization and preliminary X-ray analysis of clade I catalases from *Pseudomonas syringae* and *Listeria seeligeri*. *Acta Crystallographica Section D*, *57*(8), 1184-1186.

[72]. Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era?. *Archives of medical research*, *36*(6), 697-705.

[73]. Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, *8*(6), 423.

[74]. Akinbowale, O. L., Peng, H., Grant, P., & Barton, M. D. (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *International journal of antimicrobial agents*, *30*(2), 177-182.

[75]. Hossain, I., Hossain, M. D., & Khan, M. A. H. (2012). Leaf blight of litchi and mango and their management with molecular characterization of its pathogen, BAU Res

[76]. Hoque, M. M., Ahmad, M., Khisa, S., Uddin, M. N., & Jesmine, R. (2016). Antibiotic Resistance Pattern in *Pseudomonas Aeruginosa* Isolated from Different Clinical Specimens. *Journal of Armed Forces Medical College, Bangladesh*, *11*(1), 45-49.

[77]. Basak, R. P., Hossain, I., Kashem, M. A., Mondal, M. M. A., Rafii, M. Y., & Latif, M. A. (2014). Effect of antibiotics and BAU-Biofungicide in controlling bacterial leaf blight of litchi. *Research on Crops*, *15*(2), 389-393.

[78]. Akter, H. (2011). *Management of nursery diseases of Banana and Mango* (Doctoral dissertation, MS thesis).

[79]. Chowdhury, M. S. M. (2009). *Seed and seedling diseases of some selected fruits of Bangladesh* (Doctoral dissertation, Ph. D. Thesis. Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh).

[80]. Chambers HF. General Principles of antimicrobial therapy. In: Brunton LL, Lazo JS, Parker KL, editors. Goodman and Gilman's The Pharmacological Basis of Therapeutics. 11th ed. Mc-Graw Hill: Medical Publishing Division; 2006. pp. 1095–110.

[81]. Delgado, B., Palop, A., Fernández, P. S., & Periago, P. M. (2004). Combined effect of thymol and cymene to control the growth of *Bacillus cereus* vegetative cells. *European Food Research and Technology*, *218*(2), 188-193.

[82]. Alzoreky, N. S., & Nakahara, K. (2003). Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *International journal of food microbiology*, *80*(3), 223-230.

[83]. Verma, V., Singh, R., Tiwari, R. K., Srivastava, N., & Verma, A. (2012). Antibacterial activity of extracts of Citrus, Allium & Punica against food borne spoilage. *Asian Journal of Plant Science and Research*, *2*(4), 503-509.

[84]. Akinpelu, D. A., Aiyegoro, O. A., Akinpelu, O. F., & Okoh, A. I. (2014). Stem bark extract and fraction of *Persea americana* (Mill.) exhibits bactericidal activities against strains of *Bacillus cereus* associated with food poisoning. *Molecules*, *20*(1), 416-429.

[85]. Alkhail, A. A. (2005). Antifungal activity of some extracts against some plant pathogenic fungi. *Pak. J. Biol. Sci*, *8*(3), 413-417.

[86]. Deepak, S. A., Oros, G., Sathyanarayana, S. G., Shetty, N. P., Shetty, H. S., & Sashikanth, S. (2005). Antisporulant activity of leaf extracts of Indian plants against *Sclerospora graminicola* causing downy mildew disease of pearl millet. *Archives of Phytopathology and Plant Protection*, *38*(1), 31-39.

[87]. Deepak, S.A., Oros, G., Sathyanarayana, S.G., Shetty, H.S. and Sashikanth, S. (2007). Antisporulant activity of watery extracts of plants against *Sclerospora graminicola* causing downy mildew disease of pearl millet. *American Journal of Agricultural and Biological Sciences*, 2 (1):36-42.

[88]. Mandal, S., Mallick, N., & Mitra, A. (2009). Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. *Plant Physiology and Biochemistry*, *47*(7), 642-649.

[89]. Nahak, G., & Sahu, R. K. (2010). Antioxidant activity in bark and roots of Neem (*Azadirachta indica*) and Mahaneem (*Melia azedarach*). *Continental Journal of Pharmaceutical Sciences*, *4*, 28-34.

[90]. Guo, J. J., Kuo, C. M., Hong, J. W., Chou, R. L., Lee, Y. H., & Chen, T. I. (2015). The effects of garlic-supplemented diets on antibacterial activities against *Photobacterium damselae* subsp. *piscicida* and *Streptococcus iniae* and on growth in Cobia, *Rachycentron canadum*. *Aquaculture*, *435*, 111-115.

[91]. Whipps, J. M. (1997). Ecological considerations involved in commercial development of biological control agents for soil-borne diseases. *Modern soil microbiology*, 525-546.

[92]. Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., & Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual review of phytopathology*, *40*(1), 309-348.

[93]. Chao, W. L. (1990). Antagonistic activity of *Rhizobium* spp. against beneficial and plant pathogenic fungi. *Letters in applied Microbiology*, *10*(5), 213-215.

[94]. Ganesan, S., Kuppusamy, R. G., & Sekar, R. (2007). Integrated management of stem rot disease (*Sclerotium rolfsii*) of groundnut (*Arachis hypogaea* L.) using *Rhizobium* and *Trichoderma harzianum* (ITCC-4572). *Turkish Journal of Agriculture and Forestry*, *31*(2), 103-108.

[95]. Hafeez, F. Y., Naeem, F. I., Naeem, R., Zaidi, A. H., & Malik, K. A. (2005). Symbiotic effectiveness and bacteriocin production by *Rhizobium leguminosarum* bv. *viciae* isolated from agriculture soils in Faisalabad. *Environmental and experimental botany*, *54*(2), 142-147.

[96]. Nirmala, J., & Gaur, Y. D. (2000). Detection of bacteriocinogenic strains of Cicer-*Rhizobium* by modified simultaneous antagonism method. *Current Science*, *79*(3), 286-287.