World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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Identification and biological control of bacterial leaf spot disease of cucurbits

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World Journal of Advanced Research and Reviews, 2024, 23(01), 2480–2491

Publication history: Received on 05 June 2024; revised on 22 July 2024; accepted on 25 July 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.23.1.2139

Abstract

Objective: The present study was conducted for isolation and detection of the phyto-pathogen responsible for bacterial leaf spot disease of cucurbits as well as evaluation of its biological control techniques.

Methods: The pathogen of the disease was isolated from infected leaf of bitter gourd and cultured on Luria-Bertani (LB) growth medium. The morphological tests and biochemical tests revealed the isolated bacteria as gram negative. Furthermore, advanced molecular technique was applied to facilitate proper detection of the isolated bacteria. The PCR of the bacterial genomic DNA using specific primers generated clear band of approximately 1450 bp in gel electrophoresis.

Results: In gram staining test, the bacterial strain was found to be rod shaped and pinkish in color and Gram-negative. All the biochemical test showed positive without mannitol salt agar. The nucleotide sequencing of 16S rDNA gene of the bacterial isolate showed 86.02% similarities with the original sequence of *Xanthomonas cucurbitae*. In antibiotic sensitivity assay, the antibiotic cefixime showed highest 28.0±0.7 mm diameter of inhibition zone against the tested bacteria. The highest antibacterial activity was 8.0±0.5 mm zone of inhibition by of Allium sativum.

Conclusion: The present findings may be benevolent for the detection, characterization and development of biological control techniques to prevent the leaf spot disease of cucurbits crops in Bangladesh. It may help to find out the specific signaling pathway techniques for plant microbes relationship for disease formation in future.

Keywords: Cucurbits; Bacterial leaf spot; Characterization; Biological control

1. Introduction

Plant disease, any physiological or structural abnormality caused by microorganism, has been a major factor influencing food production and human societal development over thousands of years (Palmgren et al., 2015). Some diseases can devastate the plants on considerably large areas causing production unprofitable for many years and on a global scale, plant diseases cause an estimated $38 billion in annual losses (Goto, 2012). Disease causing organisms include bacteria, fungi, oomycetes, viruses, nematodes, phytoplasmas, and parasitic seed plants. Although most bacteria that are associated with plants are actually saprotrophic and do no harm to the plant itself, a small number- around 100 known species are able to cause disease (Jackson, 2009).

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The Cucurbitaceae family, also known as cucurbits, ranks among the highest of plant families for number and percentage of species used as human food (Bermejo and León, 1994). Particularly, cucumber, pumpkin, different types of gourds, melons, squash and zucchini are among the important edible plants of the family. It is attacked by several diseases that lower the nutritive and market value of the crop. These vegetables are susceptible to phyto-pathogens and the total loss of vegetable on this account has been estimated up to 20-30% but if the pathogens are allowed to develop, this loss may increase up to 80-90% (Glazebrook, 2005; Iqbal et al., 1996).

Among the all diseases, bacterial leaf spot disease of cucurbits, caused by *Xanthomonas cucurbitae*, has become a serious threat to cucurbit production around the world (Bradbury, 1986; Maringoni et al., 1988). They are reported as a seed-borne pathogen and can survive in plant debris (Babadoost and Zitter, 2009; Williams, 1996). Symptoms of bacterial spot on cucurbit leaves have been described by other investigators (Blancard et al., 1994; Koike et al., 2007). In the disease, lesions on leaves are small, dark, and angular and the center of the lesions becomes translucent. Plant diseases which caused by microorganisms are difficult to dominate due to their populations are changeable in genotype, space, climate and time. The use of bio-extracts in disease control has generated interest in developing countries due to high cost of synthetic pesticides and their hazardous effects on the environment (Salako, 2002; Tovignan et al., 2001). Many plant products contain anti-bacterial and anti-fungal constituents that have the potentials to control plant diseases (Bdliya and Dahiru, 2006; Enikuomehin and Peters, 2002; Opra and Wokocha, 2008).

There are no suitable report of the devastating pathogen causing disease in Bangladesh. Hence, the study was aimed to isolation, detection, and biological control of bacterial leaf spot disease of cucurbits.

2. Materials and methods

2.1. Plant material collection

In our study, disease infected leaf pieces were isolated from the symptomatic plant of cucurbit at the field located in Rajshahi district, Bangladesh and the disease was identified by a scientific officer of Bangladesh Council of Scientific and Industrial Research (BCSIR), Rajshahi, Bangladesh. The infected leaves were used as plant material for the present investigation.

2.2. Isolation and purification of bacteria

The disinfection and isolation were performed according to Araujo et al. (2002) with minor modifications. In brief, the infected leaf pieces were excised with sterile scalpel and then disinfected superficially through the following protocol: 70% alcohol for 1 min, sodium hypochlorite (2.5% Cl^-) for 4 min, ethanol for 30 s, and finally rinses of three times in sterile, distilled water. The sample was then placed in 100 ml of LB liquid medium and incubated at 37 °C for overnight. After incubation into LB liquid medium, a sterile loop was used to streak the bacteria onto a fresh LB agar plate and incubated again for 16 hours at 37 °C. After that a single colony was picked up by loop and streaked on another new media plate for pure culture. The pure cultures were preserved on LB slant at 4 °C for longer duration.

2.3. Morphological and biochemical characterization

2.3.1. Gram staining test

Gram-positive bacteria retain the crystal violet dye, and thus is stained violet, while the gram-negative bacteria don't. When the safranin is added, it stains the gram-negative bacteria a pink color. Both gram-positive and negative bacteria pick up the counterstain. But in case of Gram-positive bacteria, the counterstain can't be seen due to its crystal violet stain.

2.3.2. Biochemical characterization

The SIM medium contains peptonized iron that is used as the indicators of H_2S production. Moreover, the motility of bacteria was observed through the media, if they were motile. In the experiment, Kovac's reagent was added after 30 hours of bacterial incubation to detect the presence of indole which might be produced through the degradation of tryptophan by the enzyme tryptophanase.

The medium contains ammonium ion and other inorganic ions needed for growth. It also contains bromothymol blue, a pH indicator, which becomes green at pH below 6.9, and then turns blue at a pH of 7.6 or greater. Use of citrate results in the creation of carbonate and bicarbonate as byproducts, thus increasing the pH of the medium responsible for the change of the medium colour from green to blue and this is considered as positive test (Sierra, 1957).

The biochemical test- KIA combines the features of Kligler's lead acetate medium and Russell's double sugar agar which contains casein, meat peptones, lactose, dextrose as well as phenol red. The fermentation of lactose and/or dextrose results in the production of acid which changes the colour of the pH indicator (phenol red) and helps in the identification of the bacteria. The test was performed by inoculating bacterial culture according to Sierra method (Wilson, 2001). The medium (100 ml) consists of peptone (1 g), NaCl (0.5 g), $CaCl_2.2H_20$ (0.01 g) and agar (1.5 g). The pH of the medium was adjusted at 7.4 and after autoclaving, 1 ml of Tween 80 was added. The colony of isolated bacteria was streaked on the medium and incubated at 37 °C for seven days to observe turbid zone around the colonies. In the test, a color change from reddish salmon to pale yellow indicates carbohydrate fermentation whereas the acid production is detected by the phenol red. The prepared medium was sterilized at 121 °C for 20 minutes and after cooling, the overnight culture of bacterial isolate was stabbed into the butt and streaked on the surface of the medium into the test tube, then incubated aerobically at 37 °C for 16 hours. In order to prepare the urea base, the ingredients were dissolved in 95 ml of distilled water. Then it was sterilized by autoclaving at 121 °C for 20 minutes and cooled followed by the addition of 5 ml (40%) urea solution. After mixing thoroughly, it was stab and streaked with bacterial inoculum and incubated at 37 °C. In our test, the medium was autoclaved at 121 °C for 20 minutes. After autoclaving, it was poured into Petri plates and cooled to 50 to 55 °C until solidification. Then the isolate was inoculated on the medium and incubated at 37 °C for 16 hours. In order to conduct the test, one drop of 3% potassium hydroxide solution was placed on a clean microscope slide. Subsequently, a few bacterial colonies were emulsified to the drop of potassium hydroxide to make a dense suspension and stirred continuously for 60 sec and then gently pulled the loop away from the suspension. It consists of mannitol (1%), salt (7%), enzymatic digest of casein, animal tissue, beef extract, phenol and agar. In our experiment, the medium was sterilized through autoclaving at a temperature of 121 °C for 20 minutes and allowed to cool down. It was then mixed well before putting onto a sterile Petri dish. Mannitol salt agar was inoculated with bacteria using streak plate technique and incubated the Petri dish at 37 °C for 16 h. During our study, a small amount of bacterial colony was transferred on a clean glass slide by the use of sterile loop. Then a drop of hydrogen peroxide was place on bacterial colony. The production of oxygen can be seen by the formation of bubbles. The result of the test can be seen with the naked eye and without any aid of instruments.

2.4. Molecular characterization of isolated bacteria

2.4.1. DNA extraction

Bacterial isolate was subcultured on LB liquid medium and incubated at 30 °C for 48 hours. The total genomic DNA of the bacteria was isolated by N- Cetyl- N, N, N trimethyl-ammonium bromide (CTAB) method (Jorgensen and Turnidge 2015). The isolated DNA was then suspended into TE buffer. After that the isolated DNA was quantified followed by electrophoresis on 1% agarose gel.

2.4.2. PCR analysis

The isolated genomic DNA was subjected to PCR procedure to amplify 16S rDNA sequence by using two universal primers named, 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1492R (5'-TACCTTGTTACGACTT-3'). The total volume of the components for PCR procedure was 25 µL containing master mix 12.5 µL, T DNA (concentration 25-65 ng/µl) 1 µL, forward primer (concentration 10-20 pMol) 1 µL, reverse primer (concentration 10-20 pMol) 1 µL and nuclease free ddH_2O 9.5 µL. Thermo-cycling parameters were 95 °C for 2 min, 32 cycles of 95°C for 30 s, 48 °C for 30 s, and 72 °C for 90 s; a final extension step at 72 °C was added for 5 min, followed by cooling to 4ºC overnight. The PCR products amplified were then qualitatively analysed on 1% agarose gel where 0.5 x TBE (Tris-borate-EDTA) running buffer was used in agar gel and finally visualized under a UV trans-illuminator.

2.4.3. Purification of PCR products, sequencing and phylogenetic analysis

The amplified DNA was then purified by agar gel electrophoresis method using AccuPrep® Gel Purification, Bioneer kit according to the manufacturer's instruction. After that the purified product was sequenced in sequencing service laboratory, National Institute of Biotechnology (NIB), Bangladesh, using two universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1492R (5'-TACCTTGTTACGACTT-3'). The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (http//www.ncbi.nlm.nih.gov/blast). Finally, phylogenetic tree was constructed based on the Maximum Likelihood method.

2.4.4. Antibiotic susceptibility testing

Antibiotic susceptibility test was performed to produce a result of susceptible, intermediate (or indeterminate) or resistant for the phyto-pathogenic bacteria. The simple and practical disk diffusion technique, also known as Kirby Bauer disk method, was applied where antibiotic-impregnated wafers (disk) were used to test whether the isolated

bacterial strain was susceptible to specific antibiotic or otherwise (Duffy and Power, 2001). The isolated bacterial strain was grown overnight in LB liquid medium through shaker at 37 °C temperature and 160 rpm in order to conduct the antibiotic sensitivity test. A serial dilution technique was made for the test respective. In the method, 0.1 ml (10^{-2} dilution) of bacterial culture was smeared evenly onto the surface of the Petri-plate containing about 20 ml of LB agar medium. After that the disk of filter paper impregnated with a standard concentration of an antibiotic was placed to the plate surface (Reller et al., 2009). The diameter of the zone of clearance around the disc was measured and the result was read from the Kirby Bauer chart to determine if the organism was susceptible, intermediate or resistant against the antibiotic agents tested (Angaji and Angaji, 2009). Total eighteen types of commercially available and frequently prescribed standard antibiotics namely, Amoxycillin 10, Ampicillin 15, Azithromycin 5, Cefixime 30, Cefotaxime 30, Doxycycline 15, Erythromycin 10, Gentamycin 10, Gentamycin 30, Kanamycin 30, Nalidixic acid 30, Neomycin 30, Oxytetracyclin 30, Penicillin 10, Rifampicin 5, Streptomycin 10, Tetracyclin 30, and Vancomycin 30 were used to evaluate the antibiotic sensitivity pattern against the isolated bacterial strain.

2.4.5. In vitro antimicrobial screening of the plant extracts

The present study was designed to find out the effective biocontrol agent against the phyto-pathogenic bacteria where *in vitro* antimicrobial activities of black cardamom, black pepper, cardamom, cinnamon, clove and garlic were investigated. In order to conduct the experiment, the spices were collected from the local market of rajshahi, then washed with distill water followed by air drying. They spices were powdered in a grinding machine and stored in an airtight polybag in refrigerator at 4 °C.

Extracts were prepared by adding 150 ml of sterilized distilled water in 15 g powder of spice which were heated below the boiling point and stirred for 2-3 h. Finally, the extract was filtered by muslin cloth, then by filter paper (Whatman No 1). When half quantity of prepared extract was evaporated to dryness, it was ready to use in the experiment. The output extract and fraction were collected to glass vials and preserved in a refrigerator at 4 °C for further use (Al-Neemy and Shkh, 2006). Antibacterial activity of the selected plants parts was carried out by the disc diffusion method (Gulluce et al., 2007). In this procedure, firstly, the bacteria cultures were grown in LB liquid medium at 37 °C. After overnight of incubation, the bacteria were inoculated on the surface of fresh LB agar plates. Subsequently, the discs of filter paper (6 mm in diameter) saturated with the extract in different concentrations (30 µl, 50 µl and 100 µl) were placed on surface of each inoculated plate. A standard kanamycin antibiotic disc (30 µg) was used as a positive control. The plates were incubated at 37 °C for 24 h. After this period, it was possible to observe inhibition zone that was measured with the help of millimeter scale.

2.5. Statistical analysis

All the above experiments of the present study were conducted in triplicate for the consistency of results and statistical purpose. The data were expressed as mean±SE and analyzed by using Microsoft Excel software of 2013 version. $P<0.05$ was considered statistically significant.

3. Results

3.1. Isolation and purification of bacteria

The disease infected leaf pieces (Figure 1A) were placed on LB liquid medium resulting in turbid culture medium which indicated that the bacteria had grown into LB liquid medium. Then the bacteria were streaked onto a fresh LB agar plate and incubated again at 37 ^{o}C for overnight and eventually, single and pure colonies were found. In our study, the isolated strain showed yellow colored and round shaped colonies (Figure 1B).

3.2. Morphological characterization of bacteria

In gram staining test, the bacterial strain was found to be rod shaped and pinkish in color when observed under the light microscope at 100X using oil immersion. According to the result, it was evident that the isolate was gram-negative type of bacteria (Figure 1C).

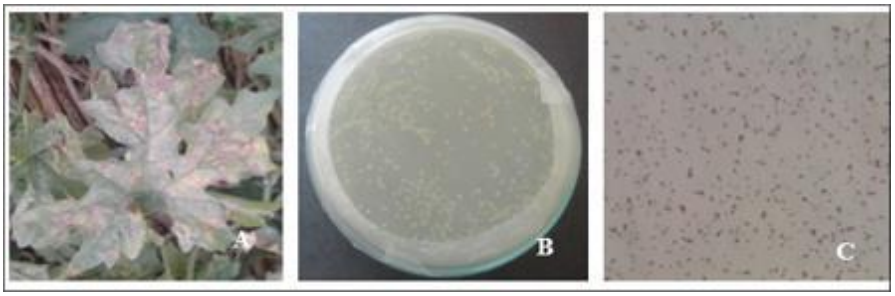

Figure 1 The disease infected leaf sample and isolation of causal organism; (A) angular leaf spot disease of bitter gourd, (B) bacterial pure colonies and (C) Gram negative bacteria

3.3. Biochemical characterization

Sulphide-Indole-Motility (SIM) test, the bacteria did not produce any band of red or pink color on the top of the medium when Kovac's reagent was added. Besides that no black precipitation was observed in the test. The test result indicated that they didn't produce any sulfide and H_2S whereas the extended growth proved that they were motile in nature (Figure 2A). In Simmon's citrate test, the colour of bacterial inoculated test tube changed from deep forest green to royal blue which proved the ability of the isolated bacteria to utilize citrate compound (Figure 2B). In Kligler iron agar (KIA) test, the medium turned into yellow coloured as well as no black precipitation was observed that ensured- the bacterial strain was lactose and glucose fermenting bacteria which couldn't produce H_2S (Figure 2C). Turbid zone was observed in Tween 80 hydrolysis test which indicated that the bacteria can produce esterase (Figure 2D). In case of triple sugar iron agar test, the medium became yellow colored pointed out towards the positive result (Figure 2E). In urease test, the bacterial isolate was grown into the urease media and the result was observed after 48 h of incubation at 37 °C. The bacterial strain was negative to the test as it showed no color change (Figure 2F). The bacterial sample was characterized as MacConkey agar positive, which confirmed that the isolated bacteria were capable of fermenting the sugar, lactose (Figure 2G). The KOH test is a confirmation test of the Gram staining. The isolated bacteria was Gram negative, because the cells displayed a viscous, stringy, sticky mess (Figure 2H). Mannitol salt agar test showed negative result as no growth or color change in the medium (Figure 2I). In catalase test, bubbles were produced due to breakdown of hydrogen peroxide into water and oxygen and confirmed that the bacterial strain produced the enzyme, catalase (Figure 2J).

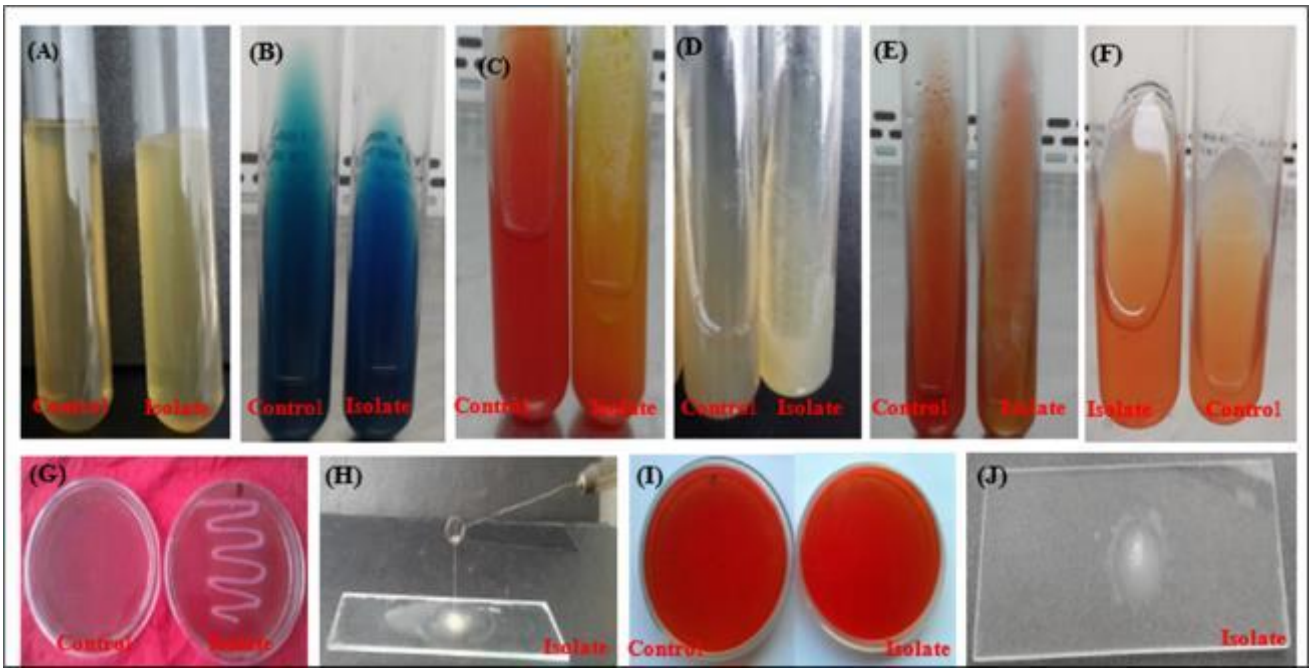

Figure 2 The biochemical characterization of the isolated bacterial strain; (A) SIM medium test, (B) Simmon's citrate test, (C) Kligler iron agar, (D) Tween 80 test, (E) Triple sugar iron test, (F) Urease test, (G) MacConkey agar test, (H) KOH test, (I) Mannitol salt agar test, (J) Catalase test

Table 1 Morphological and biochemical test of isolated bacteria

Name of the test	Response	Appearance
Gram straining	-(ve)	Small, rod shaped, pink colored colony
SIM-medium test	+(ve), -(ve)	Motile, no H_2S and indole production
Simmon's citrate test	+(ve)	Colour changed from deep forest green to royal blue
Kligler iron agar test	+(ve)	Color changed from red to yellow
Tween 80 test	+(ve)	Turbid zones were observed
Triple sugar iron test	+(ve)	yellow slants and butt
Urease test	-(ve)	The colour of the slant remained unchanged
MacConkey agar test	+(ve)	Bacteria grew well and retained pink colored
Potassium hydroxide test	+(ve)	Thread like viscous appearance
Mannitol salt agar test	-(ve)	Colour of medium was not changed
Catalase test	+(ve)	Bubbles were produced

3.4. Molecular characterization of isolated bacteria

3.4.1. PCR amplification

Electrophoretic analysis of the DNA isolated from the bacterial strain revealed sharp high molecular weight of DNA band which indicated that the DNA was of good quality and suitable for PCR analysis. The 16S rDNA region of the bacterial genome was then amplified by using bacteria specific universal primers 27F and 1492R. After that, the amplified PCR product was again subjected to electrophoretic analysis by using 1% agarose gel followed by observation on gel documentation system (Alpha Innotech). From the electrophoretic analysis it was observed that approximately 1450 bp of the 16S rDNA gene were amplified where 1 kb DNA ladder (Invitrogen) was used as marker (Figure 3).

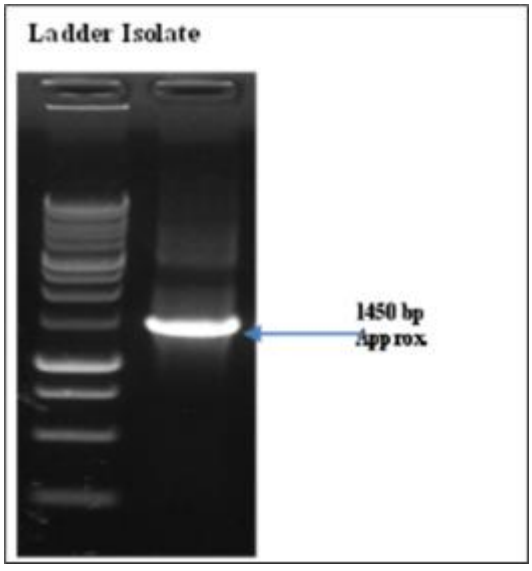

Figure 3 PCR amplification of isolated bacterial DNA using 27F and 1492R primers

3.4.2. Sequencing and phylogenetic tree construction

The sequenced data (data not shown) of the purified PCR product revealed 86% similarity with the original sequence of *Xanthomonas cucurbitae.* Then the 16S rDNA sequences of some related bacterial strains were downloaded in FASTA format from GenBank which were aligned to construct phylogenetic tree using Clustal Omega software (Figure 4).

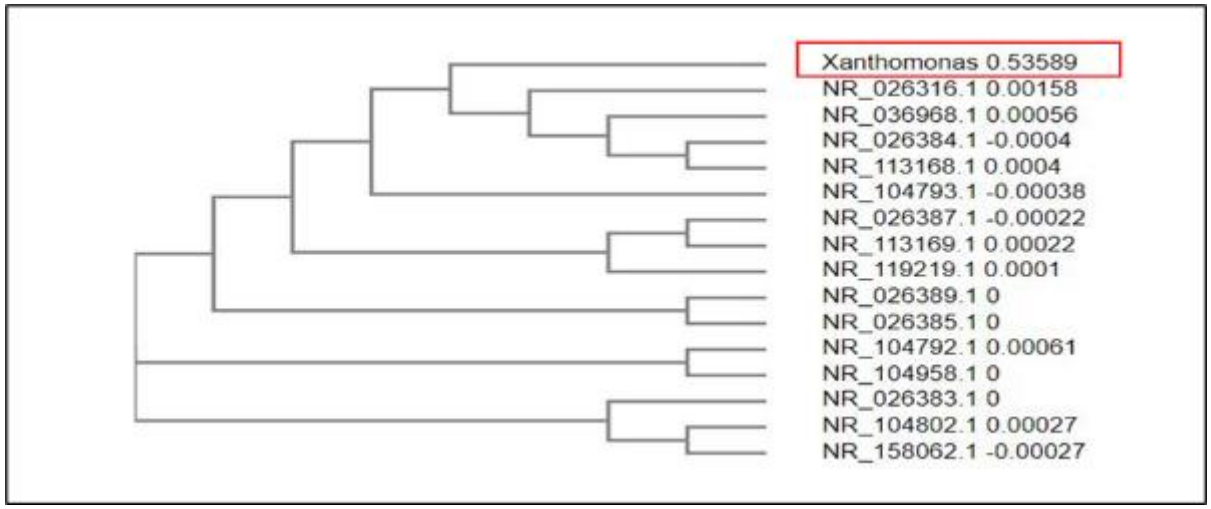

Figure 4 Phylogenetic tree for sequenced data of isolated bacteria

3.4.3. Antibiotic susceptibility testing

In this study, total eighteen types of different standard antibiotic discs were used to evaluate their sensitivity against the bacterial isolate. The result showed that isolated strain was susceptible to cefixime, nalidixic acid, azithromycin, gentamicin 30, kanamycin, gentamycin 10, cefotaxime whereas intermediate resistant to penicillin, oxytetracyclin, streptomycin, tetracycline, but resistant to amoxicillin, ampicillin, doxycycline, erythromycin, neomycin, rifampicin and vancomycin (Figure 5, Table 2).

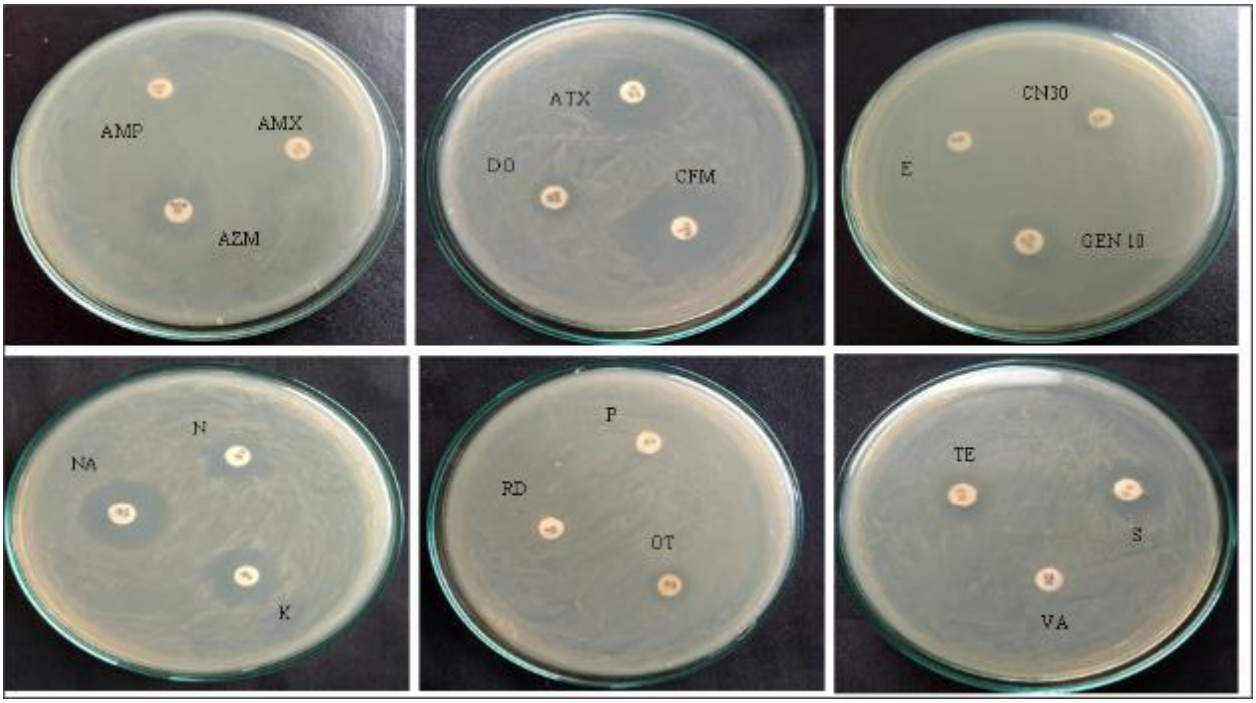

Figure 5 Antibiotic susceptibility testing against bacterial isolate

Table 2 Antibiotic susceptibility test against the isolated bacteria

Antibiotics	Disc potency (µg/disc)	Zone of inhibition (mm)	Response
Amoxycillin	10	6.0±0.0	Resistant
Ampicillin	10	6.0±0.0	Resistant
Azithromycin	15	18.0±0.8	Susceptible
Cefixime	5	28.0±0.9	Susceptible
Cefotaxime	30	15.0±0.7	Intermediate
Doxycycline	30	10.0±0.4	Resistant
Erythromycin	15	6.0±0.0	Resistant
Gentamycin 10	10	16.0±0.6	Susceptible
Gentamycin 30	30	17.0±0.4	Susceptible
Kanamycin	30	17.0±0.8	Susceptible
Nalidixic acid	30	19.0±0.7	Susceptible
Neomycin	30	6.0±0.0	Resistant
Oxytetracyclin	30	13.0±0.6	Intermediate
Penicillin	10	13.0±0.5	Intermediate
Rifampicin	5	7.0±0.2	Resistant
Streptomycin	10	12.0±0.7	Intermediate
Tetracyclin	30	11.0±0.6	Intermediate
Vancomycin	30	6.0±0.0	Resistant

3.5. *In vitro* antimicrobial screening of the plant extracts

The aqueous extract of *Allium sativum* showed highest antimicrobial activity that was 8.0±0.5 mm diameter of inhibition zone at 100 µl/disc concentration (Figure 6). The extracts of remaining spices didn't show any prominent antibacterial activity.

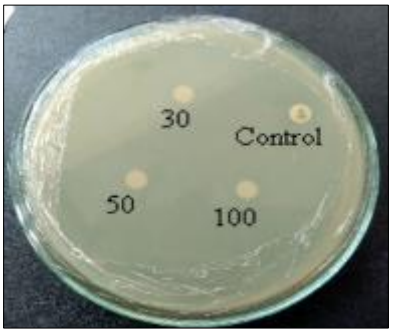

Figure 6 The inhibition zone against the isolated bacteria induced by extract of *Allium sativum*

4. Discussion

Cucurbits, members of the family Cucurbitaceae, represent one of the most important vegetables for human consumption whereas the production of cultivated cucurbits in the world is nearly 151, 212, and 210 tons per year throughout the world. Unfortunately, the cucurbit crops are often threatened by many diseases caused by various fungal, bacterial and viral agents. These pathogens along with numerous pests are serious constraints to the cultivation of the vegetables (Hans Petersen et al., 2010) that become serious limitations to cucurbit production through the

extensive damage they made, but also through the transmission of the detrimental diseases (Morse and Hoddle, 2006; Powell et al., 2006).

Bacterial leaf spot, incited by *X. cucurbitae* (Vauterin et al., 1995) has become one of the most important diseases of cucurbits which infects leaves and fruits of cucurbit crops (Babadoost and Ravanlou, 2012). The incidences of the disease are observed in all cucurbit-growing areas including Asia, Australia, Europe, and North America (Dutta et al., 2013). Incidentally, similar symptoms had been described in details (Lamichhane, 2010). In our study, we found rod shaped, yellow coloured gram negative bacteria that was consistent with the findings of Tjou-Tam-Sin et al. (2012).

In order to identify and characterize the pathogen responsible for the noxious disease, several biochemical tests were performed. In SIM test, some bacteria may show the capability to produce sulfide as well as may breakdown the amino acid tryptophane into indole compound. But in case of our concerned bacteria, it showed negative result (Ding et al. 2008) whereas positive result was noted in case of motility. Citrate utilization test was found positive for our bacterial isolate that also supported the result of the experiment conducted by Swings et al. (1990). From the following biochemical tests KIA, triple sugar iron and MacConkey agar, it was found that the bacterial strain fermented glucose and lactose whereas no gas and H_2S were formed or produced by the inoculated microbe. The results of these biochemical tests carried out on the pathogenic isolates indicated that the isolates was likely *Xanthomonas spp.* (MacFaddin, 2000). The pathogenic isolate was also capable to produce esterase enzyme in Tween 80 hydrolysis test and the previous study (Arshad et al. 2015) proved our findings. In case of urease test, the colour of the medium wasn't changed indicated that- the incubated bacterial isolate could not generate urease enzyme that was also reported by Trébaol et al. (2000). Along with these test, we also performed KOH test to accurately characterize Gram negative bacteria and the result was similar with the experiment of Suslow et al. (1982) conducted in case of wheat. According to Schaad et al. (2001), *Xanthomonas cucurbitae* produced positive result in catalase test. Our present study revealed that the enzymatic activity of *catalase* broke down hydrogen peroxide into water and oxygen and the resulting bubbles confirmed that the bacterial strain showed similar characteristic described by Schaad et al. (2001).

After performing biochemical tests, it's important to use molecular approaches to identify the microorganism precisely. Nowadays molecular detection technique have been developed to provide more rapid and accurate identification of bacteria using 16S rRNA gene sequence (Janda and Abbott, 2007). Regarding to our bacterial isolate, the query sequence showed 86.02% similarity with the *Xanthomonas cucurbitae* in NCBI BLAST and in addition, phylogenetic relationship of test organism was analyzed with other partial 16S rDNA sequence of similar microorganisms.

However, bacterial diseases of plants are usually very difficult to control and toxicity of pesticides is now well known. In fact around 40 antibiotics were screened for plant disease control in early days which depended mostly on resistance of host plant, quarantine, sanitation and cultural practices (Goodman, 1959). In some cases, chemical bactericides and biological control agents are effectively integrated into the disease management program. In our experiment, total seventeen types of antibiotics including two doses of gentamycin (10 µg and 30 µg) were used where cefixime, nalidixic acid, gentamycin etc. displayed prominent inhibitory effects against the bacterial isolate.

Our finding was also supported by the experiment of Jones (1995) where cefixime inhibited the growth of the bacteria *Xanthomonas* spp. to a great extent. Another study curried out by Pruvost et al. (2005) revealed that gentamycin exhibited highest inhibitory effect against the member of the genus, *Xanthomonas*. These results were almost similar with the findings of our investigation. Along with antibiotics, the extracts of various plants may be used to treat diseases and are considered as an important source of new antimicrobial agents (Cowan, 1999). In our study, total six types of spice extracts were used and among them, the aqueous extract of garlic showed highest zone of inhibition. The magnificent antibacterial effect of garlic was also stated by previous reports (Deresse, 2010; Kuda and Yano, 2004) that supported our findings.

5. Conclusion

The detrimental disease, bacterial leaf spot reduces the production and quality of the cucurbits and cause catastrophic or chronic losses to the farmers and ultimately to any country. The morphological and biochemical test performed in the study revealed various characteristics of the phyto-pathogen responsible for the exaggerated disease. The advanced molecular approaches along with previous morphological and biochemical characterization confirmed the identity of the bacteria as *Xanthomonas cucurbitae.* In order to uncover appropriate biological control measure of the noxious disease, various antibiotics and extracts of different spices were applied where cefixime and garlic produced promising results, respectively. So, it can be concluded that the findings of the study may favor in the thorough detection of the pathogenic bacteria as well as finding out proper biological control managements of the bacterial disease in near future. In the present study there are some limitations. Here we identified and characterized by biochemical and molecular

approaches but we didn't performed pathogenicity test for in vitro and in vivo. Also we need to check the couch postulate test and pathogen pacific marker for plant–microbe's interaction detection. So, we need to more study for detection of this devastating disease in future.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study

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