Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 752-757

Received: 14-03-2018
Accepted: 15-04-2018

Razia Sultana
Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Md. Samiul Islam
Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Md. Asadul Islam
Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Biswanath Sikdar
Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh

Correspondence
Biswanath Sikdar
Professor, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh

Identification of pathogen causing common bacterial blight (CBB) of bean through the biochemical and molecular pathway and their management system

Razia Sultana, Md. Samiul Islam, Md. Asadul Islam and Biswanath Sikdar

Abstract
The present laboratory assays were done in September 2017 to identify the causal agent of Common Bacterial Blight (CBB) disease in bean (*Phaseolus vulgaris* L.) and its management options. The disease caused by the gram-negative bacterial pathogen *Xanthomonas axonopodis* pv. *phaseoli*. The growth characteristics were determined on the basis of appearance, color, size, and shape of colonies. Among the different biochemical tests, some showed a positive reaction against the isolated bacteria while rest of showed negative results. An antibiotic sensitivity test was done to control the isolated bacteria where potential results were found. To control the isolated bacteria antagonistic assay has been done using four isolated soil bacteria where no potentiality was found. PCR was performed with the specific primer pairs, 27F and 1319R which direct the amplification of the 1250-bp DNA fragment. This study assists to confirm that the investigated strains belong to species *Xanthomonas axonopodis* pv. *phaseoli* and its management options.

Keywords: CBB, *Xanthomonas axonopodis* pv. *phaseoli*, characterization, management assay

1. Introduction
Common bean (*Phaseolus vulgaris* L) is an economically important grain legume food crop grown around all over of the world [1]. The total production of beans is exceeds 23 Tg (23 × 106 metric tonnes) of which 7 Tg are produced in Latin America and Africa [2]. Beans are a good source of protein and other minerals contents such as iron, phosphorus, potassium, calcium, zinc and vitamin B (folate) for the rural and urban households which contribute to a balanced healthy diet [3]. Regular intake of common beans has a medicinal significance which contributes to minimizing the risk of diabetes, cancer and heart diseases [4]. Due to attractive market price, the fresh pods and dry seeds of common beans are a very good source of income [5]. Despite the importance of common beans in developing countries, they are mostly produced by small-scale workers whose production is mainly dependent on wildwood local cultivars that have been selected over many years in their localities [6]. The production incommodity reported in the literature for common beans are impoverished agricultural practices, soil infertility, lack of technical and skilled cultivars, moisture content, weed emulation and damage or injury caused by pests and diseases [7, 8]. Rust (*Uromyces appendiculatusa*), anthracnose (*Colletotrichum lindemuthianum*), common bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*), angular leaf spot (*Phaeoisariopsis griseola*), web blight (*Rhizoctonia solani* pv. *phaseoli*), root rots (*Fusarium solani* pv. *phaseoli*) are the major bacterial disease of common beans [9]. Among these common bean diseases, common bacterial blight (CBB) is one the most considerable disease and economically important and is considered as a major constraint to the realization of high yields all over the world [10]. CBB is a significant seed-borne disease which is caused by gram-negative bacterial pathogen *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) and its fuscans variants *Xanthomonas fuscans* subsp. *fuscans* (*Xff*) [11, 12]. Both the strains caused distinguished symptoms but *Xanthomonas phaseoli* var. *fuscans* has been reported to be more invasive [13]. Knowing the general biology, epidemiology, and symptom of the disease is very important to manage and forecasting. Inappropriate agricultural practices and activities can influence CBB attack and epidemic under field condition [14]. Evaluation of host-pathogen interaction characterized the causal organism, use of resistant varieties with chemical seed treatment, biological management

assay, and proper agronomic practices could be the best alternative options in managing CBB of bean and reducing yield losses. Therefore, the objective of this study was to isolate and characterize the causal organism on CBB of bean and its management options.

2. Materials and methods

2.1 Samples collection: The leaves sample were collected from infected bean plants and confirmed by a scientific officer of Bangladesh Fruits Research Institute, Binodpur, Rajshahi, Bangladesh. The infected leaves sample was cut using a sterilized surgical blade, then washed with distilled water, and finally kept into a plastic bag and placed in a refrigerator.

2.2 Sterilization of infected sample: The leaves sample were sterilized with 75% ethanol for 1-2 minute and lastly the leaves sample washed with distilled water [15].

2.3 Dilution-plating assay and bacterial isolation: The dilution and isolation were done on LB media. The sterile leaves sample of bean plant was placed on mortar pestle and homogenized with distilled water and finally, the homogenizing sample put into LB liquid media. Then media was incubated overnight at 37 ºC. A tenfold-dilution series was prepared from leaves to extract and the liquid culture was spread on LB agar plate. Cultured samples were incubated for overnight at 28 ºC. All colonies typical of the *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) were examined and counted to calculate a number of the colony by plate counting method. After that, a single colony from the overnight spreading plate was taken to streak the bacteria on LB agar media plate and incubated overnight at 37 ºC. A pure culture is very important in order to identify the isolated bacteria [16].

2.4 Characterization and identification of the bacterial pathogen: Characterization of the presumptive pathogen was done by regarding the isolated bacterial colonies to various morphological and biochemical tests, e.g., gram staining, motility, Simmons citrate, urease, catalase, potassium hydroxide, triple sugar iron, Kligler iron agar, MacConkey agar, mannitol salt agar, King's medium B, tween 80 hydrolysis, carbohydrate utilization. Each test was repeated twice [17].

2.5 Molecular characterization: Bacterial DNA was extracted from bacterial cells using CTAB (Cetyl-trimethyl-ammonium-bromide) method [18]. The bacterial cells were resuspended in warm extraction buffer (20 mM EDTA, pH 8.0, 1.4 M NaCl, 10 mM Tris–HCl pH 8.0, 3% CTAB and 0.3% mercaptoethanol) and incubated at 65 ºC for 30 min. The suspension was extracted with chloroform/isoamyl alcohol (24:1) and DNA was precipitated by adding 0.6 volume of ice-cold isopropanol. The pellet was washed with 70% ethanol, dried and resuspended in sterile water and quantified using a spectrophotometer and then electrophoresed on 0.3% agarose gel in Tris–boric-EDTA (TBE) buffer [19].

2.6 PCR amplification: PCR amplification were conducted with two primer sets: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1319R (5′-GACGGGCGGTGTGTRCA-3′) respectively. Each reaction consisted of 15 µl Nuclease free ddH_2O, 1 µl of dNTP mix, 1 µl of forward primer, 1 µl of reverse primer, 1.5 µl DNA template, 2.5 µl of $Mgcl_2$, 2.5 µl of Taq buffer and 0.5 µl of Taq DNA polymerase were performed in an automated thermal cycler with an initial denaturation (95 ºC 5 min) followed by 30 cycles of denaturation (94 ºC 1 min), annealing (61 ºC 1 min) and extension (65 ºC 8 min) with a single final extension (72 ºC 5 min) [20]. PCR reactions were then electrophoresed directly on 0.3% agarose gel in Tris–borate-EDTA (TBE) buffer. The gel was stained with 0.5 µl ethidium bromide/ml and visualized. DNA patterns were visually analyzed; sizes of fragments were determined by comparison with 1 kbp standard DNA molecular marker.

2.7 Antibiotic sensitivity test: Antibiotic sensitivity test was done by disc diffusion method [16]. In this test, isolated bacteria were grown in nutrient broth medium, then take 1ml of overnight inoculum and transferred on the nutrient agar plate allowed for drying. Antibiotic discs Ampicillin, Amoxicillin, Azithromycin, Carbenicillin, Cefotaxime, Clarithromycin, Chloramphenicol, Doxycycline, Erythromycin, Gentamycin, Kanamycin, Neomycin, Streptomycin and Tetracycline respectively were placed in the center of the Petri plates and incubated overnight at 37 ºC for 16 hours [21].

2.8 Antagonistic test: In the antagonistic test, four different soil bacteria such as *Acetobacter*, *Bacillus*, *Bravibacillus*, and *E. coli*, were used for this study by the method of delayed antagonism in the solid nutrient medium [22]. Briefly, all the bacteria were inoculated as a line on the surface of a nutrient media. After overnight of growth at 30 ºC overnight test-cultures were inoculated as a perpendicular line to the all bacteria culture. The plates were incubated for 16 hours at 37 ºC. The antagonistic activity detected as a zone of inhibition [23].

2.9 Statistical analysis

Above tests were conducted in repeated triplicate for significant results. All the inhibition zone were revealed as a mean and standard error (M±SE). $P<0.5$ was considered statistically significant.

3. Results

3.1 Isolation and identification of the bacterial pathogens: Naturally affected leaves with *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) showed discoloration at the upper portion. The mixed culture showed yellow colonies on LB liquid media. In order to isolate single colony from the mixed colonies we partially identified based on colony morphology. The size and shape of colonies were found to be small to medium, convex and mucoid morphology.

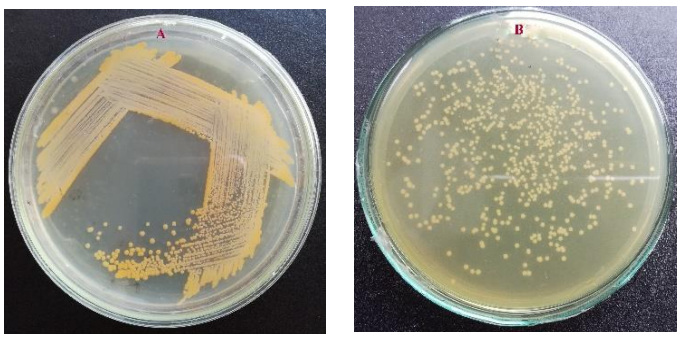

Fig 1: *Xanthomonas axonopodis* pv. *phaseoli* colonies on LB medium are typically straw yellow and glistening (A). Spreading plate of isolated bacteria (B).

In the dilution-plating assay, we calculate the number of the colony on the plate by plate counting method.
Sample was taken 100µl
Dilution time (-15)
Number of colony 51
We know,
Cell density = (number of colony × dilution time) / sample taken

$= (51\times10^{15})$ /100µl
$= 5.1\times10^{14}$ cells/µl

3.2 Biochemical test: For biochemical characterization, the isolated bacteria were usually gram-negative, motile, aerobic, had oxidative and fermentative metabolism and nonfluorescent in King's medium B (All morphological and biochemical characteristics see fig 2 and table 1).

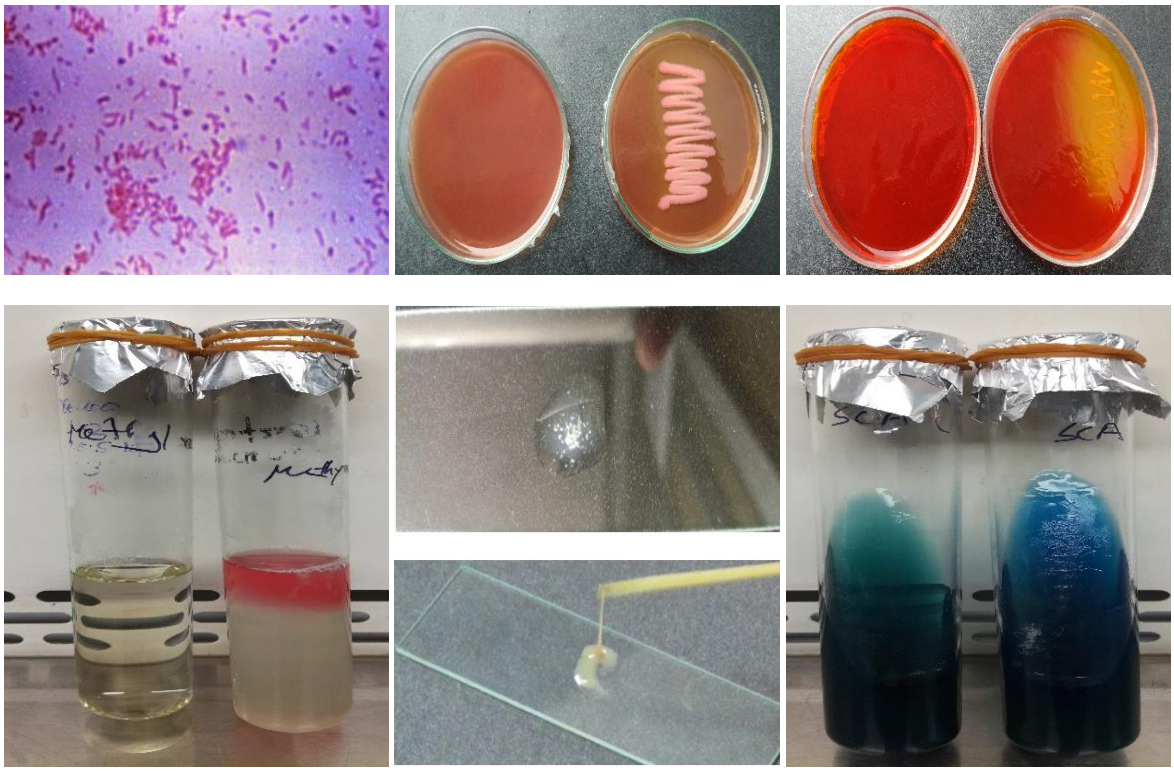

Fig 2: Positive result against the *Xanthomonas axonopodis* pv. *phaseoli* in staining (A), MacConkey agar test (B), Mannitol salt agar test (C), Simmons citrate agar test (D), catalase, KOH and methyl red test

Table 1: Biochemical characterization tests of the pathogen *Xanthomonas axonopodis* pv. *phaseoli* from common bean leaves

Biochemical tests	Results	Findings	
Gram staining	+	Pink color, rod-shaped	
Motility	+	Growth area extending away from the line of inoculation	
MacConkey agar	-	Lactose-fermenting	
Catalase	+	Oxygen bubbles	
KOH	+	Viscous and sticky slime	
KIA	+	Lactose-fermenting, but no gas form	
Simmons citrate	-	Capable to utilized citrate	
TSI	+	Lactose-fermenting, but no gas and H_2S form	
Urease	-	No color	
Tween 80 hydrolysis	-	No color	
SIM	-	No color, but motile	
MR	+	Utilize glucose	
Fluorescence under UV	-	No color	
Mannitol fermentation	-	Isolates were able to grow high salt condition area	
Carbohydrate utilization test	+	**Carbohydrate**	**OD**
		Sucrose	0.47
		Fructose	0.26
		Glucose	0.24
		Lactose	0.22
		Maltose	0.20

All tests were repeated twice; (+) – positive, (–) – negative reaction and/or result.

3.3 Molecular identification: For molecular identification, we isolate *Xanthomonas axonopodis* pv. phaseoli genomic DNA by using the CTAB method. We found clear DNA band in 0.3% agarose gel electrophoresis. A sensitive and specific assay was developed to detect common bacterial blight caused by *Xanthomonas axonopodis* pv. phaseoli in leaves of common beans. Primers *Xap* 27 F and *Xap* 1319 R were used to amplify a 1250 bp DNA fragment (Fig.3. a,b).

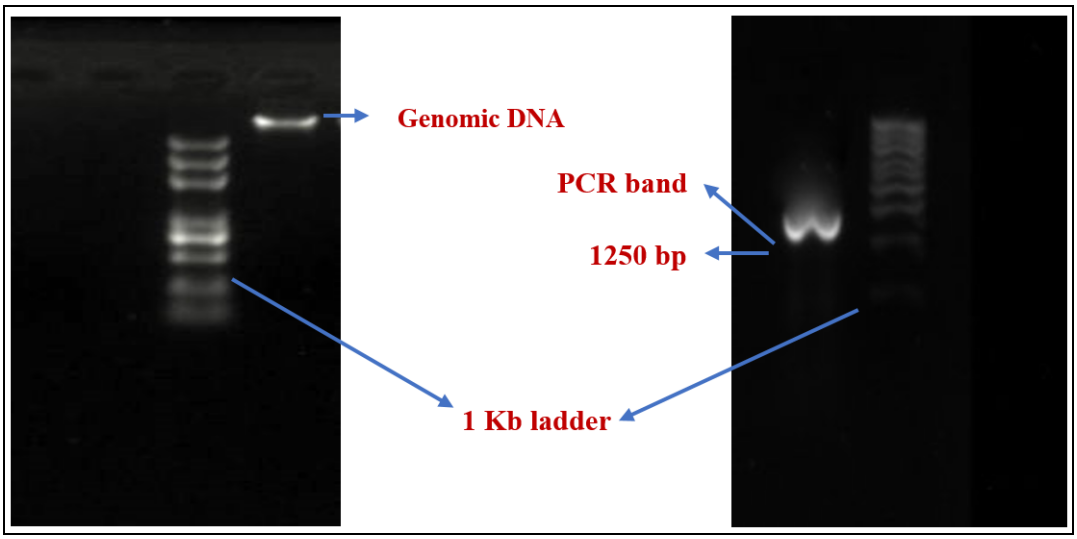

Fig 3 (a): Genomic DNA of bacteria

Fig 3 (b). Agarose gel electrophoresis of PCR products

3.4 Antibiotic sensitivity test: For antibiotic sensitivity test, fourteen different antibiotics disc were used against *Xanthomonas axonopodis* pv. *phaseoli*. Figure 4 showed the maximum inhibition zone pattern against the *Xanthomonas axonopodis* pv. *phaseoli* and table 2 provides all sensitivity result against the used antibiotics. Among all the antibiotics, Cefotaxime and Kanamycin showed the highest inhibition zone with 30 mm in diameter and Azithromycin showed lowest inhibition zone with 8 mm and rest of them showed moderate results against the isolated bacteria.

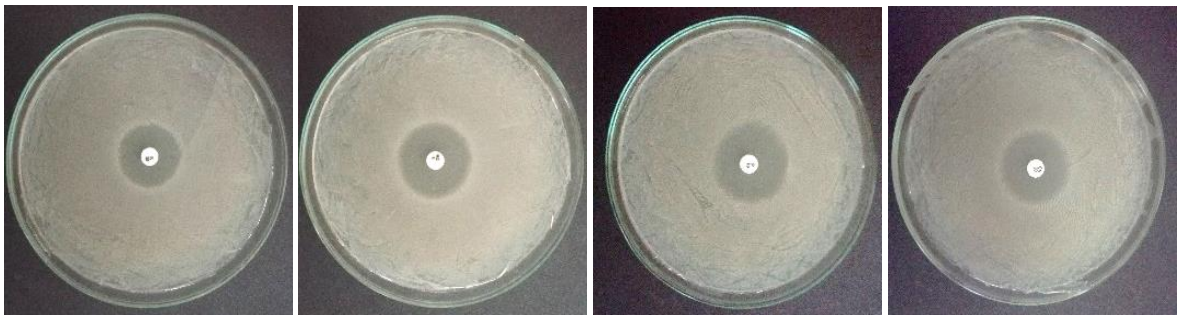

Fig 4: Maximum antibiotic sensitivity zone against the isolated bacteria (a) Cefotaxime, (b) Kanamycin, (c) Gentamycin, (d) Chloramphenicol

Table 2: Effects of antibiotics against the isolated bacteria

Antibiotic Name	Symbol	Disc Potency (µg/disc)	Zone of inhibition (M±SE)	Response
Ampicillin	AMP	10	12±0.5	I
Azithromycin	AZM	15	8±0.5	R
Amoxicillin	AML	10	10±0.5	R
Carbenicillin	CB	100	26±0.5	S
Cefotaxime	CTX	30	30±0.5	S
Clarithromycin	CLR	15	7±0.5	R
Chloramphenicol	C	30	28±0.5	S
Doxycycline	DO	30	14±0.5	I
Erythromycin	E	15	13±0.5	I
Gentamycin	GE	10	27±0.5	S
Kanamycin	K	30	30±0.5	S
Neomycin	N	30	16±0.5	S
Streptomycin	S	10	6±0.5	R
Tetracycline	TE	30	16±0.5	S
R= Resistant (5-10 mm) I= Intermediate (11-15 mm) S= Susceptible (16 mm ≥) [17]				

3.5 Antagonistic test: Four soil bacteria didn't show any inhibition zone against the isolated bacteria.

4. Discussion

Common bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *phaseoli* are one of the most destructive diseases of common bean worldwide. Several biochemical tests were done to characterize the isolated bacteria as gram-negative bacteria. Pink color and rod-shaped appearance indicated isolated bacteria were gram negative which previously described by Islam *et al*. (2017) [16]. The motility test confirmed the isolated bacteria was motile. *Xanthomonas axonopodis* pv. *phaseoli* showed pink color in the MacConkey agar media plate which indicates our bacterium were able to ferment lactose which observed by Brodsky and Nixon in *P. aeruginosa* bacterium [24]. The oxygen bubbles

clearly indicate isolated bacterium were positive to catalase test. In addition, Faqrul *et al*. (2017) performed KOH test in gram-negative bacteria of banana, where the isolated bacterium clearly showed positive results [25]. In case of KIA and TSI test, the isolated bacterium was able to ferment lactose but no gas was formed. According to Mustansar *et al*. (2015) [26] Simmons citrate, tween 80 hydrolysis, and methyl red test showed negative results in *Xanthomonas oryzae* pv. *oryzae* which showed similar results against the isolated bacteria [26]. In case of SIM test, *Xanthomonas axonopodis* pv. *phaseoli* showed negative to sulfur and indole production but motile which carried out by Lelliott and Stead (1987) in the book of Methods in Plant Pathology [27]. In case of King's B medium and mannitol fermentation test, *Xanthomonas axonopodis* pv. *phaseoli* bacteria were non-fluorescent under UV and able to ferment mannitol salt medium which showed similar results with Islam *et al*. (2014) [28]. Liu was done carbohydrate test in *P. aeruginosa* species, *Xanthomonas axonopodis* pv. *phaseoli* were also showed similar results against different carbohydrates such as sucrose, fructose, glucose, maltose, and lactose [29]. Molecular identification was applied to detect the pathogen in naturally or artificially infected leaves of citrus. Primers *Xap* 27 F and *Xap* 1319 R were used to amplify a 1250 bp DNA fragment in PCR. Most antibiotics showed great effectiveness *in vitro* against the isolated strain. This test possesses better emphasize of effective bactericides and gives us proper knowledge of the appropriate dosage.

5. Conclusion

To conclude, we found both positive and negative results in case of different tests and potential control system against the isolated bacteria. The above tests and analysis give us detailed information about the pathogen characteristics and its management system against *Xanthomonas axonopodis* pv. *phaseoli*.

6. Conflict of interest

None

7. Acknowledgment

The authors wish to thanks Professor Joarder DNA and Chromosome Research Laboratory, Dept. of Genetic Engineering and Biotechnology, the University of Rajshahi for providing all the lab facilities and guidance.

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