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Isolation, Identification, *In Vitro* Antibiotic Resistance and Plant Extract Sensitivity of Fire Blight Causing *Erwinia amylovora*

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Abstract

Background: *Erwinia amylovora* is the causal organism of fire blight. The fire blight is widely spread in bacterial disease of plants from both epidemiological and economic points of view. Furthermore, the situation is worsening by the advent of increased antibiotic resistance in these bacteria. The study was aimed to determine the *in vitro* antibiotic and herbal sensitivity of *E. amylovora* isolated from plants available in Sylhet, Bangladesh.

Methods: In this study, bacterial isolates taken from five fire blight infected plants like apple, pear, lemon, orange and olive plants were identified based on morphological, cultural and biochemical characteristics. All the isolates were tested for antibiotic sensitivity against five commonly used antibiotics and herbal sensitivity against five plants extract.

Results: Morphological, physiological and biochemical study of pure culture of suspected organism revealed *E. amylovora* bacteria which was found 100% resistant to Cefotaxime and 81.89% to Bacitracin. Chloramphenicol was found most effective as all the isolates were sensitive to it. Besides that, most of the isolates were susceptible to plant extracts and found maximum sensitive to *Allium sativum* and *Syzygium cumini* whereas resistant to *V. amurensis*.

Conclusion: It can be concluded that the investigation of herbal treatment can be implicated for fire blight disease in contrast of antibiotic test in future.

Keywords: Fire blight; *Erwinia amylovora*; Antibiotic sensitivity and plant extract

Introduction

Most damaging disease in the fruit growing world is fire blight caused by *Erwinia amylovora* (Burrill) [1], a gram negative, facultative anaerobic, rod shaped bacterium belongs to Enterobacteriaceae family (EPPO, 2006) which generally infects plants from the Rosaceae family [1]. It was considered to be native to North America and later detected in New Zealand in 1920 is now present in 43 countries [2]. Although the life cycle of the bacterium is still not well understood, it is known that it can survive as endophyte or epiphyte for variable periods of time depending of environmental factors [3]. The development of fire blight symptoms follows the seasonal growth development of the host plant. It begins in the spring with the production of the primary inoculum and the blossoms infection, continuing on summer with the shoots and fruits infection. Economic importance of this disease is caused losses of 68 million dollars in North-West America, 10 million dollars in one region of New Zealand, and 500,000 trees were destroyed in Lebanon and in Italy [4]. Since the discovery of fire blight in Morocco in May 2006 [5] the disease spread to most of the pome fruit producing regions, inducing severe damage. There is no single control measure for fire blight that will totally eradicate the disease, provide an absolute cure, or fully protect an orchard. However, fire blight damage can be kept to a minimum by using large number of chemicals like copper compounds, antibiotics, carbamates and miscellaneous compounds.. But the main disadvantage of chemicals like copper compounds is their phytotoxicity on host plants, especially pears [6] whereas antibiotics have lead to the selection of resistant bacterial populations and therefore their use is strictly limited or even forbidden in a number of countries [7]. For this reason many researcher trying to establish alternative controlling pathway of the pathogen since 1989 by using plant extract instead of chemical [8,9]. Moreover, using of plant extracts is eco-friendly and may reduce cost of cultivation. Considering all these viewpoints, our objective of the research work was to identify the bacteria on the basis of morphological, physiological, biochemical test and make a comparative study *in vitro* between antibiotic and plant extract sensitivity of the organism.

Materials and Methods

Collection and processing of samples

Total number of 21 diseased plant samples was collected from different nurseries of Sylhet city according to standard pathological procedure. Then, 1 ml of fruits rinsed water and fruit juices sample was taken to a test-tube containing 9 ml of sterile water and thoroughly mixed to get a 10^{-1} dilution of the water sample. Again, 1 ml of 10^{-1} dilution was transferred again to another 9 ml of sterile water in another test-tube and thoroughly mixed to get a 10^{-2} dilution. In such way serial dilution of water samples were made up to 10^{-4}.

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Scientific name	Local name	Used parts	Used volume(µl)
Allium cepa	Onion	Bulb	50
Allium sativum	Garlic	Bulb	50
Syzygium cumini	Kalajam	Leaf	50
Vitis amurensis	Grape	Leaf	50
Litchi chinensis	Litchi	Leaf	50

Table 1: Herb samples used for *in vitro* herbal sensitivity experiment.

Samples Code	G	Growth at 39°C	Growth at 4% NaCl	Fl under UV	TSI		I	C	N	OF	U	Gel	Ma	Su
					Gas	H_2S								
F1	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F2	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F3	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F4	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F5	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F6	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F7	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F8	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F9	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F10	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+
F11	Rod -	-	+	-	+	-	-	+	-	F	-	+	+	+

G = Gram Test, Fl= Fluorescent, TSI = Triple Sugar Iron, I= Indole Test, C = Citrate Test, OF = Oxidation Fermentation Test, N= Nitrate Test, U= Urease Test, Gel= Gelatine Liquefaction, Ma= Mannitol Fermentation, Su= Sucrose Fermentation.

Table 2: Physiological & Biochemical tests for identification of bacterial isolates causing Fire Blight.

Isolation, purification and preservation of the isolates

Isolation of *E. amylovora* was done on Nutrient Agar (NA) or Leavan media which was prepared by dissolving 1g yeast extract, 2.5 g peptone, 2.5g NaCl, 25 g sucrose into 500ml of distilled water, pH adjusted to 7.0-7.2 and sterilized by autoclaving at 121^{0}C, 15 psi for 15 minutes. Then, transferred the suspected single colony from NA plate by sterile loop and inoculated on the King's medium agar B (KB) which was prepared by peptone 20g, glycerol 10 mL,K_2HPO_4 1.5g, $MgSO_4 \cdot 7 H_2O$ 1.5 g, agar 15 g, distilled water 1000 mL, pH adjusted to 7.0-7.2 and sterilized by autoclaving at 121° for 20 minutes [10]. The plates were then incubated at 27°C for 2-3 days and observed daily for bacterial growth. Suspected colonies of *E. amylovora* (white, circular, mucoid, and curved) were selected and further purified again on KB agar at 27°C. This operation was repeated three to four times to be sure that pure cultures were obtained for identification tests [11] and preserved it for next investigation.

Identification of the isolates

For identification of *E. amylovora*, colony morphology was studied when it appeared in KB and NA media. Moreover, Gram's staining was performed as described by [12] and growth of pure cultured isolates was measured at 39°C and 4% NaCl containing NA media. Besides that, fluorescent test was done and under UV light at 366 nm after 48 h of incubated plate.

Biochemical tests

Biochemical tests like oxidative-fermentative test, nitrate reduction test, citrate utilization test, urease test, sucrose fermentation test, TSI (Triple Sugar Iodine) test, mannitol fermentation test, gelatine hydrolysis were done.

Antibiotic susceptibility test

In antibiotic susceptibility test 250 µl of microbial inoculums of *E. amylovora* strain from cultured nutrient broth was spread on the surface of Mueller-Hinton agar (CM337-OXOID) by using a sterile L-shaped glass rod. Then *in vitro* five different commercially available antimicrobial discs Streptomycin (10 µg), Gentamycin (10 µg), Chloramphenicol (30 µg), Cefotaxime (5 µg), Bacitracin (10 µg) were applied on the inoculated plates according to the Kirby-Bauer [13]. During susceptibility test same bacterial density was maintained by using Spectrophotometer at OD_{600}. After incubation, the plates were examined and the diameters of the zone of complete inhibition were measured in mm.

Plant extracts sensitivity studies

The antimicrobial activity of five plants extracts were used in the herbal sensitivity experiments. Name and parts of the plants are given in Table 1. After cleaning the selected parts of plant by sterilized distilled water, extracts were collected in a falcon tube and centrifuged at 4000 rpm for few minutes. Besides that wells of 5 mm diameter were punched into the agar plates with the help of sterilized cork borer and 50 µl of the plant extracts were added by using a micropipette to the wells made in the agar plate along with well diffusion 0.1 ml of diluted inoculum of the freshly cultured experimental strains. Inhibitory response of the herbal extracts was recorded according to the normal growth response of the bacteria after incubation at 28°C for 24 hour and zone of inhibition was measured by mm.

Results and Discussion

Fire blight is alarming hazardous threat to citrus fruits and for economy. So, proper understanding of the pathogenic specialization of this pathogen is necessary. Bacteria were isolated from plant samples and identified using cultural, physiological and biochemical test.

Isolation and identification of bacteria

Morphological studies of *E. amylovora* colonies were done after incubating pure culture at 28°C for overnight on NA and KB media (Table 2 and Figure 1A). Physical appearance of isolates showed whitish, circular, domed, smooth, mucoid colonies on NA media (Figure 1B)

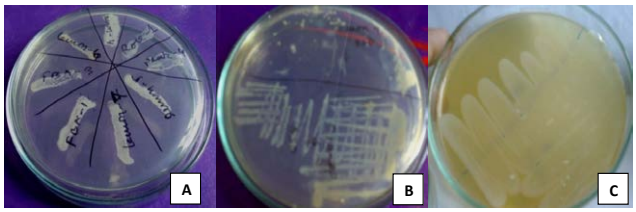

Figure 1: A. Pure culture of *Erwinia amylovora* from different fire blight infected plant, **B.** *E. amylovora* on NAS media and **C.** *E. amylovora* on KB media.

Name of Antibiotics	Disc Conc.	No. of isolate	Sensitivity pattern of *Erwinia amylovora*(11)		
			%R	%I	%S
Streptomycin (S)	10 µg	20	18.1	27.3	54.6
Bacitracin (B)	10 µg	20	81.89	18.1	-
Chloramphenicol (C)	30 µg	20	-	18.1	81.89
Cefotaxime (CTX)	30 µg	20	100	-	-
Gentamycin (GEN)	10 µg	20	9.99	54.6	36.4

R= resistant, I= intermediate and S= susceptible

Table 3: Antibiogram of isolated *Erwinia amylovora* causing Fire Blight.

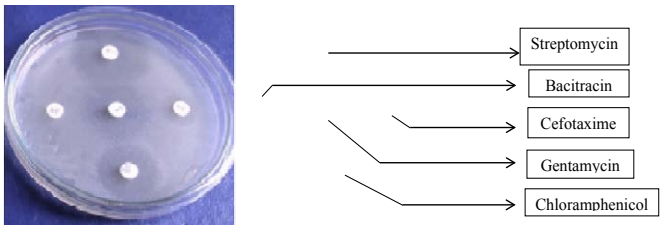

Figure 2: Antibiogram of *Erwinia amylovora*.

Plant samples	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
	Erwinia amylovora										
A.sativum	20	15	14	20	40	14	13	5	16	12	11
A. cepa	-	-	-	-	-	2	-	-	-	-	-
S. cumini	10	2	3	-	3	-	-	-	5	5	-
V. amurensis	-		-	-	-	-	-	-	-	1	-
L. chinensis	-	-	-	-	-	-	-	-	-	-	-

Table 4: Isolates with inhibition zone (mm) for different plant samples.

whereas creamy white, circular, intending to spread colonies was found on KB media (Figure 1C) which resemble with [14] and [15] respectively. Among 21 isolates, a total of 20 were indicated as gram negative rod shaped bacteria whereas no growth was observed at 39°C and 12 had growth and 9 isolates didn't grow on 4% salt concentration. Moreover, *E. amylovora* exhibited non-fluorescent under UV light at 366 nm after 48 h which allowed the distinction from fluorescent *Pseudomonads*.

Biochemical tests

For biochemical characterization, a series of tests were performed with the suspected gram negative bacteria and results are given in Table 3. After analyzing the results for all bacterial isolates, it was confirmed that 11 isolates were *E. amylovora*. Identification was done by morphological, physiological and biochemical tests according to the EPPO standards diagnostic protocol (EPPO/CABI). In this table, TSI positive means both the slant and butt turned into yellow due to bacterial fermentation and produce gas and some produce H_2S gas. Mannitol and Sucrose fermentation positive means both acid and gas were produced and OF result F means they are fermentative bacteria.

Antibiotic susceptibility test

Antibiotics were first used to control fire blight in the 1950's and quickly became an important tool for disease management. The sustainability of antibiotics for disease prevention has been threatened by emergence of antibiotic-resistant populations of *E. amylovora*, which has reduced the efficacy of some of the antibiotics in certain locations. It has been evident that bactericidal bind irreversibly to the bacterial ribosome and blocking the synthesis of proteins to make it inactive whereas mutation in chromosomal gene which encodes the production of ribosomal protein makes resistant [16,17]. In our study eleven isolates were screened for drug resistance profile which indicated the sensitivity pattern against commonly used antibiotics in fire blight (Figure 2). All the isolates offered high degree of resistance against the commonly used antibiotics. By comparing the zone created by the isolates with the standard zone of inhibition we found all isolates were 100% resistant to a single drug except Chloramphenicol (C) was the most sensitive. Among the antibiotics, resistant *E. amylovora* isolates were 9.99% , 81.89%,18.1%, 100% 0% against the antibiotic GEN,B, S, CTX and C (Table 3). So, study among the five antibiotics test low resistance showed in Streptomycin and Gentamycin which is in accordance with victoria et al. [18] and Spitkoin and Alvarado [19]. Besides that, tested pathogen revealed highly resistant against Bacitracin and Cefotaxime antibiotics and same results were also found by Kumar, Singh and Robert et al. [20,21] respectively. Moreover, highly susceptible was showed in Chloramphenicol antibiotics against *E. amylovora* in this research which is not resemblance with Weixin et al. [22] who concluded that hydrolysis of Chloramphenicol by *E. coli* conferred resistance.

Plant extracts sensitivity studies

Plants remain one of the main sources of natural products for new therapies particularly in poor countries, because most of them are cost less, affect a wide range of antibiotic resistant microorganisms, and another reason is there is an erroneous impression that herbal medicines have fewer adverse effects [23]. In present research antibacterial activity of aqueous extracts of all the five plants are presented in Table 4 in which highly significant antibacterial activity was observed in *A. sativum* and *S. cumini*, respectively against the tested pathogen. Our findings agree with other observations [24] who concluded that antimicrobial activity of allicin from garlic (*Allium sativum*) exhibit strong activity against *E. carotovora*. Even similar results was also found stated that methanol extract of *S. cumini* to be more effective on both gram positive and gram negative bacteria, and especially against gram positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis*.

Conclusion

It can be concluded that, present study showed all the isolates of *E. amylovora* were resistant to at least one or more of commonly used antibiotics. So, emphasis must be placed on the development of effective bactericides and their proper use with knowledge of the appropriate dosage. On the other hand, herbal sensitivity test has paved the way the viable introduction of plants for the treatment of disease causing microorganism in cheaper cost and eco-friendly way. Therefore, it will be more beneficial to put emphasis on biological control of *E. amylovora* through plant extracts instead of antibiotics. Moreover, further researches are necessary to find more plant species

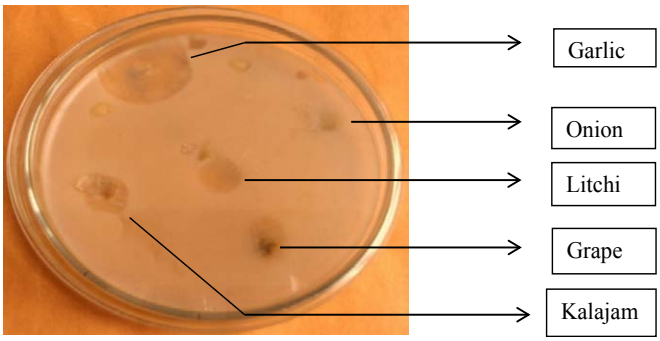

Figure 3: Antimicrobial activity of some plant species on *Erwinia amylovora*.

and purify the antimicrobial substances present in the crude plant extracts effective against *E. amylovora* (Figure 3).

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