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Biochemical characterization and biological control measures of citrus variegated chlorosis (CVC) bacteria

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Abstract
The present investigation was undertaken to isolate and characterize citrus variegated chlorosis (CVC) bacteria as well as evaluation of its control measures through standard antibiotics and medicinally important plant extracts. The isolated bacteria was characterized by different morphological and biochemical test methods. Standard antibiotics and antibacterial sensitivity of plant extracts were tested against the isolated bacteria using disc diffusion method. In biochemical tests isolated bacteria was found to be positive in carbohydrate fermenting, catalase, Simmons Citrate, Methyl red, Kligler Iron Agar test, tween 80 test and potassium hydroxide test, while it showed urease test negative. The extract of *Allium sativum* showed highest antibacterial activity with 10±.5mm diameter of zone of inhibition. Erythromycin showed highest antibiotic activity with 32±0.5mm diameter of zone of inhibition against isolated bacteria. The present investigation would be helpful for further detection and biological management of CVC disease caused by *Xylella fastidiosa* pathogen.

Keywords: *Citrus* spp., Citrus Variegated Chlorosis (CVC), *Xylella fastidiosa*, Biochemical test, Biological control

Introduction
The present investigation was undertaken to isolate and characterize citrus variegated chlorosis bacteria as well as evaluation of its control measures through standard antibiotics and medicinally important plant extracts. *Citrus* species are evergreen, subtropical important fruit crops all over the world, belonging to the family of Rutaceae. It was originated in tropical and subtropical Southeast Asia. Citrus lemon is most important fruit in all over world (Mohanapriya *et al.,* 2013; Chaturvedi *et al*., 2016) [1, 2]. Citrus fruit contain zero saturated fats or cholesterol, but are good source of vitamin and dietary fiber (7.36% of RDA). It is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (viz., leaves, stem, root and flower) against clinically significant bacterial strains (Kawaii *et al.*, 2000) [3]. Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Burt, 2004; Ortuno *et al.,* 2006) [4, 5]. *Xylella fastidiosa* is a xylem-limited gram negative bacterium, which affects a large number of species, including monocotyledon and dicotyledon woody plants (Chagas *et al.,* 1992) [6]. The development of CVC disease affects the host plant metabolism and induces morphological alterations in leaves and fruits (Silva Stenico *et al.,* 2009) [7]. These younger trees become systemically affected by *X. fastidiosa.* Affected trees show foliar chlorosis resembling zinc deficiency with interveinal chlorosis. The chlorosis appears on young leaf as they mature and may also occur on older leaves and fruit size is greatly reduced. It is well known that citrus plants have been infected by *Xylella fastidiosa* display nutritional deficiencies, probably caused by production of extracellular polymers by the bacteria that block normal nutrient flow through the xylem. It can spread rapidly and results in significant economic losses. There are many reports on citrus plant diseases like citrus canker (Mubeen *et al.,* 2015) [8], citrus greening (Hosokawaa *et al*,. 2015) [9] and others. As far we know, there is no suitable report on this devastating disease detection and their control management. That is why, thepresent research work was aimed to characterize the CVC bacteria through different morphological and biochemical test and to evaluate its biological control measures by different antibiotics and some medicinally important plant extracts.

Material and methods
Collection of plant materials
The present investigation was conducted during the period of 2015-2016 at Professor Joarder

DNA and Chromosome Research Laboratory in the Department of Genetic Engineering and Biotechnology, University of Rajshahi. Infected leaves from different sections of the same citrus plant were collected from Rajshahi University area, Rajshahi, Bangladesh and were identified the disease by a scientific officer of Bangladesh Council of Scientific and Industrial Research (BCSIR), Rajshshi, Bangladesh. Leaves were taken from symptomatic trees and used as plant material. Typically, irregular chlorosis evolves in mature leaves recognized by interveinal yellowing on the upper side of leaf and corresponding brownish gum like material over the side**.** Later on, brown spots coalesce and necrosis becomes evident, eventually leading to leaves dropping from branches.

Isolation of bacteria from infected leaves

Symptomatic leaves were surface-sterilized with 10% bleach for 5 min, followed by seven rinses in sterile distilled water. Midribs and petioles are aseptically excised with a sterile scalpel and placed onto sterile petridishes. Then sample was crushed with sterile mortar and pestle. The crushed tissue was placed in a LB liquid medium and incubated at 37 ^{0}C overnight (Bertani *et al.,* 1951) [10]. After that a sterile loop was used to streak the bacteria onto a solid agar medium. The bacteria were allowed to grow for at least 16 hours at 37 ^{0}C and examined periodically for colony growth. Both solidified and liquid media were used for the present study. Plating was done to get single colony from bacterial culture. In order to identify bacteria, it is necessary to obtain a pure culture. This was done by using the streak-plate method. Isolation, plating, streaking and subculture were done manually. At each time of plating and streaking precaution was taken to minimize cross contamination of samples.

Morphological and biochemical characterization of the isolated pathogen

Isolated bacteria were characterized by some morphological and biochemical tests as described by Diagnostician's Cookbook and Bergey's Manual of Systematic Bacteriology (Bergey *et al.,* 1994) [11]. Colony morphology of the isolated bacteria on the agar plate was recorded after 16 h of growth on LB agar plate at 37^{0}C. Gram negative test was done on the basis of their physiological and chemical properties of cell wall of the test pathogen. *X. fastidiosa* can also be isolated onto suitable selective media (Raju *et al.,* 1982) [12]. Biochemical test including, SIM-medium test (Sulphide-Indole-Motility medium), Indole test, Citrate test (Difco *et al.,* 1998) [13], Catalase test, MacConkey agar test (MacConkey, 1905) [14], Kligler Iron Agar test,Urease test, KOH test (Suslow *et al.,* 1982) [15], Triple Sugar Iron (TSI) test, Tween 80 test and Methyl Red tests were carried out against the isolated bacteria following the company manual instructions. Different biochemical test chemicals were collected from Oxido Ltd. Basingstoke, Hampshire, England.

Antibacterial activity screening of different plant extracts

Antimicrobial activity test was performed by moderate agar disc diffusion method (Alzoreky and Nakahar, 2003) [16]. Four different plant extracts namely *Allium sativum*, *Allium cepa*, *Ocimum basilicum*, *Zingiber officinale* were used against the isolated bacteria. Different parts of four plant species were harvested from Rajshahi University campus, Rajshahi, Bangladesh. Collected parts of the plants were cut, air-dried powdered in a grinding machine and stored in an airtight polybag. Powdered dried plants were extracted (cold) in flat bottom conical flask, through occasional shaking and stirring for 10 days using distilled water. The content was pressed through the markin cloth to get maximum amount of extract. The whole mixture was then filtered by Whatman filter paper No. 41 and the remaining filtrate was dried (Hussain *et al.,* 2010) [17] in vacuo to afford a blackish mass. The output extract and fraction were collected to glass vials and preserved in a refrigerator at 4°C. The isolated bacterial strains were grown overnight in nutrient broths medium that were placed in the shaker at 37^{0}Cwith 150rpm.Sterile Whatman no. 41 filter paper was used as disc (6mm).The antimicrobial activity of the plant extracts was evaluated and the results of diameter of zone of inhibitions were measured in millimeter (mm) scale.

Antibiotic sensitivity test

Antibiotic sensitivity was performed by moderate disc diffusion method (Hussain *et al.,* 2010) [17] against the isolated bacteria, which is a qualitative to semi quantitative test. Twelve standard antibiotics namely Doxycycline, Streptomycin, Kanamycin, Azithromycin, Ampicillin, Carbenicillin, Tetracyclin, Erythromycin, Neomycin, Gentamycin, and Chloramphenicol, Cefotaxime were used against isolated bacteria. Briefly, 20 ml quantities of nutrient agar were plated in petri dish with 0.1 ml of a 10^{-2} dilution of each bacterial culture. Antibiotics discs (6mm in diameter) impregnated with the concentration of antibiotics discs on isolated organism-seeded plates. The activity was determined after 16 h of incubation at 37°C. The diameters of zone of inhibition produced by the antibiotics were then measured in mm scale. All the test were performed manually and enough care was taken for plating, streaking and handling of the test pathogen.

Statistical analysis

All the above investigations of the present study were conducted in triplicate and repeated threes for consistency of results and statistical purpose. The data were expressed as mean and standard error (mean ±SE) using Microsoft Excel 2010 version. $P<0.05$ was considered statistically significant.

Results and Discussion

Isolation and purification of bacteria

The leaves samples were collected and bacterial liquid culture was obtained after overnight incubation at 37^{0}C. Mixed culture showed yellow and white colonies**.** From the mixed culture, pure culture was isolated and the bacteria was partially identified based on colony morphology. The size and shape of colonies were found to be small to medium, convex and mucoid.

Morphological and biochemical characteristics of isolated bacteria

Gram Staining Test: Gram-negative bacteria have a thinner layer (10% of cell envelope), and are stained pink by the Safranin. Here, the isolated bacteria was gram-negative which showed the pink color**.** *Xylella fastidiosa* is a xylem-limited gram negative bacterium. *Xylella fastidiosa* is a slow-growing, gram-negative, xylem-limited, fastidious, leafhopper-transmitted bacterial plant pathogen (Wells *et al.,* 1987) [18] that causes many economically important plant diseases.

Sulphide-Indole-Motility (SIM) Test: SIM medium is recommended for the differentiation of gram negative enteric

bacilli on the basis of sulfide production, indole formation and motility. The medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production is detected when ferrous sulfide, a black precipitate, is produced after inoculating the bacteria. After adding kovac's reagent, bacteria did not produce red/pink color band on the top of tube and H_2S was not produced as no black precipitation formed and isolated bacteria showed motility in SIM medium

Simmons Citrate Agar Test: As a part of the microbiological and biochemical test for the identification of bacteria, citrate test w
as performed based on the citrate metabolism ability of the strains. When the bacteria metabolize citrate,the ammonium saltsare broken down to ammonia**,** which increases alkalinity**.** The shift in pH turns the bromthymol blue indicator in the medium from green toblue above pH 7.6.The uninoculated medium had the deep forest green color. The test tubes containing media inoculated with the strains changed from green to the royal blue color as the bacteria metabolized citrate**.**

Catalase Test: This test was used to identify organisms that produce the enzyme, catalase**.** This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The presence of bubbles resulting from production of oxygen gas clearly indicates a catalase positive result in this study.

MacConkey agar Test: The sample (Isolate) and control strain were grown on MacConkey agar plates for the identification of gram-negative enteric bacteria. As the sample grown well on the MacConkey agar, they were gram-negative enteric bacteria. The lactose fermenting capability of the strain was also detected from the MacConkey agar test. The isolated strains were grown on the media and it was gram negative. The isolate produce pink color around the colony so it was lactose fermenting**.**

Kligler Iron Agar Test: This 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. Lactose positive organisms yield a yellow slant and lactose negative organisms yield a red slant. Cracks, splits, or bubbles in the medium indicate gas production.The isolated strains were grown on the media and it was lactose fermenting because it yields yellow slants**.**

Urease Test: This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease. If organisms produce urease, the color of the slant changes from yellow to pink. If organisms do not produce urease, the agar slant remains yellow.In this study, isolated bacteria did not hydrolyze urea. So, urease test was negative for isolated bacteria.

Kovac's Oxidase Test: After inoculating bacteria in the petridish containing filter paper and oxidizing agent Kovac reagent was added,if purple color is produced then it is positive to the Kovac reagent. Our test bacteria did not produce any color, so it was negative to Kovac reagent.

KOH Test: The KOH test is a confirmation test of the Gram stain, especially with difficult bacterial species. Gram-positive cells with their tough thick cell walls of peptidoglycan do not lyse whereas gram-negative cells with their thinner more porous cell walls lyse and DNA comes out of the cells. As a result, the bacterial smear becomes a viscous, stringy, sticky mess, when picked up with a toothpick during KOH test which is absent in gram positive bacteria.

Triple Sugar Iron Test: The sample organism and control strain were grown on TSI agar plates for the identification of gram-negative enteric bacteria. As the sample grown well on the TSI agar, they were considered as gram-negative enteric bacteria. Lactose or sucrose or glucose fermentation was observed. Since these substances are present in higher concentrations, they serve as substrates for continued fermentative activities with maintenance of an acid reaction in both the slant and the butt.

Tween 80 Hydrolysis Test: In this study, isolated bacteria did not produce hydrogen sulfide and was lactose, glucose or sucrose fermenting.The inoculating medium showed crystal precipitation by hydrolyzing Tween 80. Esterase hydrolyzed the tween and subsequent precipitation with the calcium chloride appears as a turbid zone around the colonies

Methyl Red Test: In the Methyl Red test, the test bacteria was grown in a broth medium containing glucose**.** The bacterium had the ability to utilize glucosewith production of a stable acid, the color of the methyl red changes from yellow to red, when added into the broth culture. Bacteria showed positive result against Methyl Red test**.** The results of the morphological and biochemical test are represented in Table 1. Wells *et al.,* (1987) [18] reported that, the result of the different biochemical tests were carried out on the pathogenic isolates from Citrus Variegated Chlorosis indicated that the isolates are likely *Xylella* spp. Findings were also likely similar with the report in fastidious plant and phony disease of peach, respectively (Wells *et al.,* 1987; Wells *et al.,* 1983) [18, 19]. Biochemical and physiological test of the isolated bacteria were performed. The isolated bacteria showed negative response in Urease test. Positive results were recorded in Catalase test, Simmons citrate test, Methyl Red test, Tween 80 test and Klinger iron agar tests. TSI test indicated that the bacteria was carbohydrate fermenting. Gram staining result showed it was a gram-negative bacterium. Similar results of biochemical tests were reported by Wells *et al.,* (1987) [18] in fastidious plants and Wells *et al.,* (1983) [19] in phony disease of peach which support our findings. SIM test confirmed the motility of the gram-negative bacteria without indole production and H_2S gas formation (Edmondson and and Sanford, 1967) [20].

Table 1: Effect of the isolated bacteria in morphological and different biochemical test media

Name of the Test	Reaction	Appearance	Remarks
Gram staining	-ve	Small, rod shaped, pink color colony	Gram staining is negative, showing confirmation of gram negative bacteria
SIM	+ve,(-ve)	Motile, no H_2S and indole production	Gram negative bacteria showed motility and did not produce any indole and H_2S
Simmons citrate agar	+ve	Color changed from green to the royal blue	Citrate metabolizing gram negative bacteria
Catalase	+ve	Presence of bubbles	Gram negative bacteria formed bubbles resulting from production of O_2 gas
MacConkey agar	+ve	Pink color around the colony	Gram negative bacteria showed pink color confirming lactose fermenting
Kligler Iron Agar	+ve	Yields yellow slants	Gram negative bacteria yields yellow slants confirming lactose fermenting
Urease	-ve	Slant remains yellow	Gram negative Bacteria did not hydrolyze urea
Kovac's oxidase	-ve	No color	Gram negative bacteria did not produce any characteristic color
KOH	+ve	Thread like slime	Gram negative bacteria formed thread like slime
TSI	+ve	Color changed from red to yellow	Gram negative bacteria did not produce H_2S and confirming lactose fermenting
Tween 80 Hydrolysis	+ve	Turbid zone around the colonies	Gram negative bacteria positively hydrolyzed the tween 80
Methyl Red	+ve	Color changed from yellow to Red ring	Gram negative bacteria had the ability to utilize glucose

Antibacterial activity screening test using different plant extracts

In vitro antibacterial activity of four plant extracts were evaluated against the isolated bacteria. The obtained results showed that the bacteria were resistant to *Allium sativum* and *Allium cepa*. The extract of *Allium sativum* showed highest antibacterial activity with 10.0±0.5mm diameter of zone of inhibition at 20µl/disc concentration followed by 8.0±0.5mm diameter of zone of inhibition at 15µl/ disc concentration. On the other hands, the extract of *Ocimum basilicum* and *Zingiber officinale* showed no inhibition zone against the isolated bacteria. The results are shown in Table 2. Lacava *et al.,* (2004) [21] reported that the growth of *Xylalla fastidiosa* was inhibited by endophytic *Curtobacterium flaccumfaciens in vitro*, and Lacava *et al.,* (2007) [22] demonstrated that *Curtobacterium flaccumfaciens* reduced the severity of disease symptoms when co-inoculated with *Xylalla fastidiosa* in periwinkle (*C. roseus*) plants, which supports our present finding in antibacterial activity screening tests.

Table 2: Effect of some plant extracts against isolated bacteria

Name of plant extract	Dose of plant extract (zone in mm)			Sensitivity pattern
	10µl/disc	15µl/disc	20µl/disc	
Allium sativum	6.0±0.5	8.0±0.5	10.0±0.5	Resistant
Allium cepa	4.0±0.5	5.0±0.5	7.0±0.5	Resistant
Ocimum basilicum	0.0±0.0	0.0±0.0	0.0±0.0	Resistant
Zingiber officinale	0.0±0.0	0.0±0.0	0.0±0.0	Resistant

Legend: R = Resistant (5-10 mm), I = Intermediate (11-15 mm), S = Susceptible (16 mm ≥)

3.3.11 Antibiotic sensitivity test against isolated bacteria

The antibacterial activities of twelve standards antibiotics against isolated bacteria were determined. The standard erythromycin revealed highest antibacterial activity with 32.0±0.5mm diameter of zone of inhibition at 15µg/disc concentration followed by streptomycinwith 29.0±0.5mm diameter of zone of inhibition at 10µg/disc concentration. On the left hands, the standard ampicillin revealed lowest activity with 6.0±0.5mm diameter of zone of inhibition at 10µg/disc concentration against isolated bacteria**.**The result of antibiotic sensitivity test is summarized in Table 3. No major differences were found in sensitivity to antibiotics among the strains of *Xylella* that were isolated from grape, oleander, and almond, although genetic variability has been reported between the strains from these hosts (Hendson *et al.,* 2001) [23]. Growth inhibitionby tetracycline and streptomycin, which was observed in our *X. fastidiosa* isolates, agreed with the findings of Darjean *et al.,*(2000) [24]. Control has been attempted by antibiotic treatment of grapevines against *Xylella fastidiosa* and by insecticide treatment against its vectors, but with only partial success (Lacava *et al.,* 2004) [21]. Kuzina *et al.,* (2006) [25] reported that the antibiotics with the lowest minimum inhibitory concentration (MIC) for *Xylella fastidiosa* strains responsible for Pierce's disease were gentamicin, tetracycline, ampicillin, kanamycin, and novobiocin, chloramphenicol, and rifampin, which support our findings. Resistance to penicillin in some *X. fastidiosa* isolates was described by Simpson *et al.,* (2000) [26] and Lacava *et al.,* (2001) [27]. Dayakar and Gnanamanickam, (1996) [28] reported that, kanamycin, tetracycline and chloramphenicol are susceptible to *Xylella* spp.,which are similar to our present findings.

Table 3: Effect of some standard antibiotics against isolated bacteria

Name of antibiotic	Disc concentrations (µg/disc)	Diameter of zone of inhibition (in mm)	Sensitivity pattern
Doxycycline	30	19.0±0.5	Susceptible
Streptomycin	10	29.0±0.5	Susceptible
Kanamycin	30	18.0±0.5	Susceptible
Azithromycin	15	26.0±0.5	Susceptible
Erythromycin	15	32.0±0.5	Susceptible
Neomycin	30	27.0±0.5	Susceptible
Gentamycin	15	28.0±0.5	Susceptible
Chloramphenicol	30	23.0±0.5	Susceptible
Cefotaxime	30	14.0±0.5	Intermediate
Ampicillin	10	6.0±0.5	Resistant
Carbenicillin	100	13.0±0.5	Intermediate
Tetracycline	30	12.0±0.5	Intermediate

Legend: R = Resistant (5-10 mm), I = Intermediate (11-15 mm), S = Susceptible (16 mm ≥)

Conclusion

Citrus Variegated Chlorosis is a devastating disease of *Citrus* spp. Disease severity results in defoliation, dieback, premature fruit drop and blemished fruit which consequently decrease fruit production and market value. It was concluded that biochemical and molecular characterization of *Xylella fastidiosa* is necessary for the identification and biocontrol measures of citrus Variegated Chlorosis disease.

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Conflict of interest

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

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