MANAGEMENT OF BACTERIAL WILT OF POTATO (*Ralstonia solanacearum*) BY SOIL AMENDMENTS

KAZI AKTAR SAMDANI

DEPARTMENT OF PLANT PATHOLOGY
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

JUNE, 2017

MANAGEMENT OF BACTERIAL WILT OF POTATO (*Ralstonia solanacearum*) BY SOIL AMENDMENTS.

BY

KAZI AKTAR SAMDANI

REGISTRATION NO. : 11-04435

A Thesis

Submitted to the Faculty of Agriculture

Sher-e-Bangla Agricultural University, Dhaka,

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

PLANT PATHOLOGY

SEMESTER: January-June, 2017

Approved by:

……	……
(Dr. M. Salahuddin M. Chowdhury)	**(Dr. Fatema Begum)**
Professor	Associate Professor
Department of Plant Pathology	Department of Plant Pathology
Sher-e Bangla Agricultural University	Sher-e Bangla Agricultural University
Supervisor	**Co- Supervisor**

…….

(Khadija Akhter)

Chairman

Examination Committee

Department of Plant Pathology

Sher-e Bangla Agricultural University

Department of Plant Pathology
Sher-e-Bangla Agricultural University
Dhaka-1207, Bangladesh

Fax: +88029112649
Web site: www.sau.edu.bd

CERTIFICATE

This is to certify that the thesis entitled, **"MANAGEMENT OF BACTERIAL WILT OF POTATO (*Ralstonia solanacearum*) BY SOIL AMENDMENTS''** submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in the partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) IN PLANT PATHOLOGY,** embodies the result of a piece of bonafide research work carried out by **KAZI AKTAR SAMDANI** bearing **Registration No. 11-04435** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: 16.09.2018
Place: Dhaka, Bangladesh

(Dr. M. Salahuddin M. Chowdhury)
Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Supervisor

ACKNOWLEDGEMENTS

All praises are due to the Almighty Allah, the great, the gracious, merciful and supreme ruler of the universe to complete the research work and thesis successfully for the degree of Master of Science (MS) in Plant Pathology.

The author expresses the deepest sense of gratitude, and heartfelt indebtedness to his reverend research supervisor, ***Dr. M. Salahuddin M. Chowdhury****, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for his scholastic guidance, innovative suggestion, constant supervision and inspiration, valuable advice and helpful criticism in carrying out the research work and preparation of this manuscript.*

The author deems it a proud privilege to acknowledge his gratefulness and best regards to his respectable co-supervisor ***Dr. Fatema Begum****, Associate Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for her valuable advice, constructive criticism and factual comments in upgrading the research work and the documents.*

The author would like to express his deepest sense of respect to Professor and Chairman, ***Khadija Akhter*** *for her valuable suggestion during the entire study period.*

The author would like to express his deepest respect and boundless gratitude to all the respected teachers of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for their valuable teaching, sympathetic co-operation, and inspirations throughout the course of this study and research work.

Finally the author appreciate the assistance rendered by the staffs of the Department of Plant Pathology and Plant Pathology Laboratory, Sher-e-Bangla Agricultural University, Dhaka who have helped him during the period of study.

The author

MANAGEMENT OF BACTERIAL WILT OF POTATO (*Ralstonia solanacearum)* BY USING SOIL AMENDMENTS

ABSTRACT

A survey on bacterial wilt of potato was conducted in different location of major potato growing upazilas namely Shipgonge, Bogra sadar, Kalai, Khatlal, Dinajpur sadar, Birol, Rangpur sadar and Pirgonge during the growing season of 2016-2017. Bacterial wilt incidence ranged from 5 to 15% was recorded in those upazillas. The survey results showed that the highest mean (15%) bacterial wilt incidence was observed in Shipgonge followed by Bogra sadar (12%). The isolated pathogen was *Ralstonia solanacearum* which was confirmed by different biochemical test viz. Gram staining reaction, Potassium hydroxide solubility test, Kovac's oxidase test, Levan test, Catalase test. Six treatments viz. Urea, Cow dung, Bleaching powder, lime, (Urea+Lime) and Streptomycin were evaluated in the pot experiment. Study revealed that an average (60%) percent reduction of bacterial wilt severity in amendment soils compared to unamendment soil. The DSS (diseases severity score) was recorded at the 25^{th} day against bacterial wilt (*R. solanacearum*) of potato. Results showed that the lowest DSS (diseases severity score) 2.075 was observed in case of T_5 (Streptomycin) which was followed by T_6 (cow dung) treatments (2.46). However, cow dung powder treated plants were showed the second lowest DSS which was followed by (Urea+Lime) treated plants. Results also showed that the lowest PDI (percent disease index) 40% was observed in case of T_5 (Streptomycin) which was same as T_6 (cow dung) treatments and T_2 (Urea+Lime) treatments. But, the lime and urea showed the highest PDI in artificially inoculated potato plants.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

% = Percentage

& = And

@ = At the rate

AEZ= Agro-Ecological Zone

ANOVA = Analysis of Variances

BBS = Bangladesh Bureau of Statistics

Cfu = Colony forming unit

Cm = Centimeter

CV% = Percentages of Co-efficient of Variance

cv. = Cultivar (s)

DAI=Days after inoculation

df. = Degrees of freedom

DSS=Diseases severity score

et al. = And others

etc. = Etcetra

G = Gram

H_2O_2 = Hydrogen peroxide

hr = Hour (s)

i.e. = That is

J. = Journal

LIST OF SYMBOLS AND ABBREVIATIONS

Kg = Kilogram

LSD = Least Significant Difference

ml = Milliliter

mm = Millimeter

NA = Nutrient Agar (media)

NB = Nutrient Broth (media)

No. = Number

^{o}C = Degree Celsius

PDI = percent diseases index

PSI=Pounds Per Square Inch

R=Replication

RCBD= Randomized Complete Block Design

SAU = Sher-e-Bangla Agricultural University

T = Treatment

TTC = Triphenyl Tetrazolium Chloride

USA = United States of America

var. = Variety

Viz. = Namely

µl = Microliter

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum*) is a tuber crop belonging to the family Solanaceae. Potatoes are the swollen portion of the underground modify stem which is called a tuber and is designed to provide food for the green leafy portion of the plant. The potato was familiarized in this subcontinent in the sixteenth century. Today potato has emerged as a major tuber crop in Bangladesh. It contributes alone as much as 54% of the total annual vegetable production of Bangladesh (Anonymous, 2006). Average yield rate of potato has been estimated 19.371 metric ton per hectare (BBS, 2014). Potato is a staple food in the developed countries and which accounts for 37% of the total potato production in the world (FAO, 2013). Potato is a common and important vegetable in Bangladesh. For the whole year it is used as a vegetable food. Annual consumption of potato has been growing rapidly, from around 34 kg per capita (FAO, 2013). It ranks third in area acreage after rice and wheat and is cultivated in almost all agro-ecological regions of Bangladesh.

Many local varieties of potatoes cultivated in the different parts of the country. In the last few decades, several dozens of high yielding varieties of potatoes were brought to Bangladesh and tried experimentally under native conditions. Through constant assessment of the traits, varietal performance and consideration of other features, about 10 HYV have been released for cultivation in the country (Khalil *et al*., 2013). Among the high yielding popular varieties, some notable varieties are: Cardinal, Diamond, Kurfi shindhury, lalpakry, etc. The Tuber Crops Research Centre of BARI has composed many new varieties from the International Potato Research Centre. The Centre has already made good role towards the development of some high yielding potato varieties (Anonymous, 2006). In Bangladesh, almost 12 diseases in potato have been recorded (PRA, 2015). Among them late blight, stem rot/sclerotium rot, bacterial wilt, common scab, potato leaf roll, early

blight, soft rot, dry rot and mosaic are the most important diseases (Ahmed *et al*., 2000). Diseases caused by different fungal and bacterial pathogens (Jones *et al*., 1991) are the major constraints of potato production. Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi *et al*., 1995) is one of the most important diseases that limit potato production. The disease also constitutes a serious damage to the cultivation of many other Solanaceous crops (potato, tobacco, pepper and eggplant) in tropical, sub-tropical and temperate regions (Hayward, 1991). It is one of the most damaging pathogens of potatoes and can persist in the soil for considerable periods of time. Bacterial wilt (*Ralstonia solanacearum*) disease attack over 200 plant species in 50 families (Hayward, 2000) and other biotic factor limiting growth and development of several important crops of family Solanaceae, including potato, tomato, eggplant, pepper and tobacco (French and Sequeria, 1970); (Anith *et al*., 2004) and Leguminosae (e.g. ground nut and French bean) and several tree and shrub hosts (Genin and Boucher, 2002).

Ralstonia solanacearum is a non-spore forming, non-capsulate, gram-negative bacterium that accumulates poly-B-hydroxybutyrate intracellularly. This bacterium is oxidase positive and arginine dihydrolase negative (Kelman, 1981; Denny, 2007). Most strains grow optimally at 30-32°C. They do not multiply at 4 or 40^{o}C and growth is highly inhibited in 2% NaCl. They produce fluidal, irregularly round, white colonies with pink centers on 2,3,5-triphenyltetrazolium chloride-amended (TZC) medium (Kelman, 1954). Avirulent mutants that can develop in culture are non-fluidal, uniformly round, butyrous and deep red in color. Such mutants are highly motile with the help of a polar flagellum (Kelman and Hruschka, 1973), (Husain and Kelman, 1958). Some factors such as susceptible host can revert the avirulent mutant to the virulent wild type (Denny *et al*., 1994; Poussier *et al*., 2005). Wide host range and broad topographical distribution have made *R. solanacearum* an economically significant pathogen. Potato, tomato, tobacco, eggplant, banana, groundnut, and ginger are some of the economically important hosts. Bacterial wilt of potato has been estimated to cause annual losses of more than $950

million globally (Walker and Collin, 1998). Bacterial wilt of potato has been estimated to cause annual losses of more than $1500 million globally (EPPO, 2014). Up to 100% loss of potato has been reported in certain parts of Nepal (Gurung and Vaidya, 1997) with approximately 14% annual losses reported in Bangladesh (Elphinstone, 2005).

Bacterial wilt is difficult to manage due to the genetic diversity and aggressiveness of the pathogen, its ability to survive in varied and adverse environmental conditions, its modes of dissemination, and the large number of weed hosts (Ramesh and Phadke, 2012; Saddler, 2005). The controlling of bacterial wilt with physical, chemical, biological and cultural methods has been investigated for decades. Elphinstone extensively reviewed bacterial wilt in 2005 and numerous studies have been conducted on this topic. Elphinstone described that over 450 studies had been published on *R. solanacearum* since the second International Bacterial Wilt Symposium was held in Guadaloupe in 1997.

Several plant essential oils and their component exhibited that some essential oils have significant efficacy against *Ralstonia solanacearum* in vitro and under glass house (Momol *et al*., 1999). Rapid early recognition of bacterial wilt is not only in tubers or plant debris but also in soil or soil related habitats is essential for disease controlling in the field to prevent losses and further pathogen spread (Janse *et al*., 1998). The common control measures used against bacterial wilt include the use of resistant varieties, healthy seed, crop rotation, agronomic practices, biotic control and integrated management (Elphinstone and Aley, 1993).

Soil amendments that enhance host plant resistance have been given due consideration (Datnoff *et al*., 2001). The use of soil amendments with organic matter has been adopted for controlling certain soil borne diseases including bacterial wilt. Studies have shown that one way of stimulating bio control in the soil is the use of organic matter. Efficient soil management generally improves the composition and activities of microorganisms, thereby enhancing

the biological control capacity of the soil. Inorganic soil amendments have sometimes shown promising results both in pot experiments and in the field but in other cases there was little effect. An increase or decrease in pH, reduced wilt in tobacco, tomato and eggplant when sulphur (acidification) or lime (neutralization) was added, especially in relatively acid, fine sandy soils (Kelman, 1953; Michel and Mew, 1998). A high nitrogen dose reduced bacterial wilt in sandy soils; nitrates were more effective than ammoniacal compounds (Kelman, 1953; Michel and Mew, 1998). Fertilization and amendment of soil with high inputs of urea and CaO or MgO also significantly decreased populations of *R. solanacearum* populations (Elphinstone and Aley, 1993; Michel *et al*., 1997).

Apart from inorganic amendments, organic amendments can affect both survival of the pathogen in soil and infection of the host. Amendment of soil with compost or manure (Scho¨nfeld *et al.*, 2003) or pig slurry reduced soil pathogen populations depending on soil type (Domo ˜nguez *et al*. 2001; Kelman, 1953; Michel and Mew, 1998). So far, information on the effect of organic matter on the suppressive of *R. solanacearum* is still limited.

In view of the potential suppressive effects of soil amendments, it would be interesting to know if organic management could lead to a reduction in bacterial wilt. Root diseases and wilts caused by fungi are frequently well controlled in organically managed soils (van Bruggen and Termorshuizen, 2003). However, only a few reports are available in management of this disease in the country. Therefore, attempt should be put forward to prevalence of this disease and its management.

Considering the above facts, the present research program has been designed with following objectives:

1. To determine the incidence and severity of wilt of potato tuber in selected locations of Bangladesh.

3. To isolate, identify and characterize of causal organism.

4. To evaluate soil amendments for controlling bacterial wilt disease of potato.

CHAPTER II

REVIEW OF LITERATURE

Bacterial wilt of potato caused by *Ralstonia solanacearum* is considered as major important diseases. It becomes a serious threat for potato production. The disease assumed it's severity in all the growing part of the world resulting severe yield losses both in terms of quality and quantity. The information is available on this disease, pathogen, management strategies. Hence, the literature pertaining to the bacterial wilt of potato along with information of disease and pathogen are studied here as under.

2.1. Survey and report on occurrence of bacterial wilt of potato

The first record of bacterial wilt caused by *Ralstonia solanacearum* (Smith, 1896) in the world was reported by Burril in 1890 in Japan. That was found to be on tuber rot of potato (Gota, 1992).

Kelman (1997); Denny (2006) observed five races of *Ralstonia solanacearum* having different host ranges and geographic distributions. Among them race 3 were distributed worldwide and has primarily been associated with potato.

Pitkethley (1981) observed biovar 1 as predominant in U.S.A. and biovar 3 in Asia, whereas biovar 2 and 5 occur in Australia and China. Biovar 4 was recorded in India and Indonesia.

Hayward (1991) said that the origin of *Ralstonia solanacearum* is not clear. He suggested that it predates the geological separation in the continents as the bacterium has been found in the virgin jungle in South America and Indonesia.

Agrios (1953) said that bacterial wilt caused by *Ralstonia solanacearum* has been described on a wide range of hosts in many tropical and subtropical regions.

OEPP/EPPO (1999) found that in Africa, the bacterial wilt disease caused by *Ralstonia solanacearum* was recorded in Egypt, Libya, South Africa, Zambia and Burundi.

Abdullah (1988) Observed bacterial wilt disease and the pathogen *Ralstonia solanacearum* was isolated from the infected crop plants at the farm of University Putra Malaysia, Selangor.

Anonymous, (1995).*Ralstonia solanacearum* is listed as quarantine organism in the European Union.

In EPPO regions bacterial wilt disease caused by *Ralstonia solanacearum* was found in Belgium, Spain, Netherlands, Germany, United Kingdom and Hungary (OEPP/EPPO, 1999).

Wicker *et al*. (2007) observed that *Ralstonia solanacearum* caused devastating wilt of 450 plant species belonging to 54 families, covering both monocots and dicots globally.

He *et al*., (1983); Hayward (1991) said that the strains of *R. solancearum* have been subdivided into five races on the basis of host range.

Gurung and Vaidya (1997) observed up to 100% loss of potato due to bacterial wilt has been reported in certain parts of Nepal.

Elphinstone (2005) reported approximately 14% annual losses of potato due to bacterial wilt caused by *Ralstonia solanacearum* in Bangladesh.

Annon (1998) found that the most important crop affected by bacterial wilt in European countries was potato.

Geddes (1989) observed bacterial wilt of potato disease in Pakistan which was then first reported.

Allen *et al*. (2005) stated that bringing about severe crop losses worldwide; the disease is now receiving global profile.

Danial *et al*. (2006) observed that RS strains (race 3/biovar 2A) were mainly responsible for outbreak of potato brown rot in Europe.

Lemessa and Zeller (2007) stated that biovar 2 strains had limited host range (only affecting potato) as compared to biovar 3 strains.

2.2. Disease causal agent

Smith *et al*. (1995) observed that *Ralstonia solanacearum* is a highly heterogeneous bacterial pathogen that causes severe wilting of many important plants.

Kelman (1953) observed that *Ralstonia solanacearum* is an aerobic obligate organism; strains of the pathogen have minimum, optimum and maximum temperature of 10, 35 and 41^{o}c respectively.

Sneath *et al.* (1986) said that *Ralstonia solanacearum* is a gram negative, non-spore forming rod, about 0.5-0.7 µm X 1.5-2.0µm with a single polar flagellum.

2.3. Symptomology

Gota (1992) characterized that *Ralstonia solanacearum* as sudden wilting of foliage where the young plant was affected more. The symptoms occurred as discoloration of vascular system from pale yellow to dark.

Kelman and Sequeira (1965) found that *Ralstonia solanacearum* entered roots through wounds caused by transplanting, cultivation, nematode, and insects and through natural wounds. Then it started to multiply rapidly in the vascular system, finally the xylem elements were filled with bacterial cell and slime.

Kelman and Sequeira (1965) observed the incidence of the disease infection may range from a very few scattered plants or loci of infection in fields where low or erratic natural infestations occur to the rapid death of the plants.

Agrios (1953) discovered that older plant leaves first show wilting before the youngest leaves or one sided wilting and stunting and finally the plant wilts permanently and dies.

Kelman (1953) found *Ralstonia solanacearum* engrossing a plant through roots, penetrate the xylem, systematically colonizes the stem and causes wilt symptoms.

2.4. Isolation and identification of the pathogen and its pathogenicity

Kelman and Person (1954) described that the tetrazolium medium (TZC) is the best for culturing *Ralstonia solanacearum*.

Cuppels *et al*. (1978) found that *Ralstonia solanacearum* produces two distinguishable types of colonies in tetrazolium medium (TZC). One is small, flat, red and butyrous while the other colony is large, elevated, mostly white with light pink centers and full of fluid using Casamino Acid Peptone Glucose (CPG).

Kelman (1954) found to grow the bacterium in the medium incubation should be done at 28^{o}c for at least 24 hours. After isolation, *Ralstonia solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TTC) plate.

Kelman (1954) identified the virulent (colonies with pink or light red colour or characteristic red center and whitish margin) and avirulent (smaller, off-white and non-fluidal colonies) strains of *Ralstonia solanacearum* were in TTC medium containing 0.005% TTC.

Rajeshwari *et al.* (1998) developed an ELISA test using polyclanal sera against the virulence exopolysaccharide component for detection of *Ralstonia solanacearum* in seed.

Kelman (1954) and Singh (1994) conducted an experiment where twenty-five potato seeds were ground and suspended in 1 ml sterile distilled water. 0.1 ml

of the suspension was plated on a semi-selective medium or placed directly on this medium. The development of the distinctive mucoid magenta- pigmented colonies indicated the presence of the pathoen, *Ralstonia solanacearum*.

Engelbrecht (1994) developed and was modified by Elphinstone, (1993), an effective selective medium SMSA that can be successfully used for isolation of *Ralstonia solanacearum* from seed and incubated at 25-27^{o}c for 2 days.

Schaad *et al*. (2001) observed that isolation is the best made for early infection stages, small pieces of tissue being excised from the margins preferably of the youngest lesions. These are comminuted in small quantities of sterile water and streaked on TTC medium.

Schaad *et al.* (2001) observed single colonies were sub-cultured onto nutrient agar for storage and confirmation of the identity of *Ralstonia solanacearum*.

Opina *et al*. (1997) confirmed that identity of bacteria was based on colony morphology on TZC medium, *Ralstonia solanacearum* specific Immuno strips, and a polymerase chain reaction (PCR) assay using *R. solanacearum* species complex-specific primers 759/760.

2.5. Evaluation of disease management strategy

2.5.1. Inorganic amendments

Murakoshi & Takahashi, (1984) found that chemical control through fumigation and antibiotics (streptomycin, ampicillin, tetracycline, penicillin) has shown little suppression of *Ralstonia solanacearum*.

Kelman (1953); Michel and Mew (1998) state that an increase or decrease in pH reduced wilt in potato, tomato and eggplant when sulphur (acidification) or lime (neutralization) was added, especially in relatively acid, fine sandy soils.

2.5.2. Organic amendments.

Kelman (1953) said that a high nitrogen dose reduced bacterial wilt in sandy soils; nitrates were more effective than ammoniacal compounds.

Lemaga *et al*, (2005) said that soil amendment with NPK at 100 kg /ha significantly decreased bacterial wilt incidence on potato and increased potato yield .

Elphinstone and Aley (1993); Michel *et al*. (1997) found that Soil fertilization and amendment of soil with high inputs of urea and CaO or MgO also significantly decreased populations of *R. solanacearum* populations.

Incorporation of household compost (Schönfeld *et al*., 2003), cow dung manure (Nishyama, *et al*., 1999) and pig slurry (Gorissen *et al.,* 2004) have been found to reduce bacterial wilt incidence and severity.

Lemaga *et al*. (2005) reported that the application of nitrogen (N) + phosphorus (P) + K and N + P (application rate of each fertilizer = 100 kg/ ha) reduced bacterial wilt by 29% and 50%, respectively.

Shekhawat *et al*. (1990) reported that amending infested soils with stable bleaching powder (SBP) at 25 kg ha^{-1} was effective and suitable for control of bacterial wilt under glass house and field condition. A combination of the use of pathogen-free seed tubers and application of SBP at 12 kg ha^{-1} along with 30 kg ha^{-1} additional nitrogen during the time of planting is effective against bacterial wilt.

Sharma *et al*. (2000) Soil amendment or the combination of organic matter with a non-pesticide chemical such as formaldehyde or bleaching powder appear to have effectively reduced the incidence of bacterial wilt and increased crop yield.

Vinh and Tung (2005) describe that in integrated disease management, soil amendments with 300 kg N and 1,500 kg CaO, together with soil solarization

using transparent plastic mulches, reduced the incidence of wilt in the tomato and potato by 20%.

Dhital *et al.* (1997) describe that bleaching powder at the rate of 25 kg/ha or urea at the rate 428 kg/ha and lime 5000 kg/ha or nitrogen 200 kg/ha and CaO 5000kg/ha or phosphoric acid (H_3PO_3) urea, fly ash and bleaching powder singly or in combination control of bacterial wilt of potato.

CHAPTER III

MATERIALS AND METHODS

Three experiments were carried out throughout the research period in order to study the disease of bacterial wilt of potato. The experiments were as follows:

1. To determine the incidence and severity of wilt of potato tuber in selected locations of Bangladesh.

3. To isolate, identify and characterize of causal organism.

4. To evaluate soil amendments for controlling bacterial wilt disease of potato.

3.1. Experiment I. Survey on the presence of *Ralstonia solanacearum* in plant and potato tuber in selected locations of Bangladesh

3.1.1. Location of survey area

A survey was carried out for the collection of infected samples of four major potato growing districts viz. Bogura, Joypurhat, Dinajpur, Rangpur during the potato growing season of 2016-2017.

3.1.2. Survey and sampling procedure

Survey was done on the field prevalence of the disease. Three infected fields from each Upazila were selected and as such total eight upazila were selected for survey. The infected samples were collected randomly from each infested field during investigation. Percent incidence of the disease in potato field was recorded based on their symptomatology. Infected potato plants with tuber were collected based on typical symptoms and put in a polyethylene bags and brought to the laboratory of Plant Pathology Department, Sher-e-Bangla Agricultural University for bacterial isolation, identification and confirmation for further study.

Table 1. Name of districts, upazila and collected potato tuber from wilt infected field

Districts	upazila	variety
Bogura	Shipgonge	Rumana
	Bogra sadar	Rumana
Joypurhat	Kalai	Granula
	Khatlal	Granula
Dinajpur	Dinajpur sadar	Granula
	Birol	Diamond
Rangpur	Rangpur sadar	Granula
	Pirgonge	Granula

3.1.3. Observation of the symptoms

Symptoms of the infection were studied by visual observation as per standard procedure by Champoiseau *et al.* (2009). Samples were visually observed for the symptoms of bacterial wilt. Identification of the disease was lastly confirmed through isolation and different biochemical tests.

3.2 Isolation and identification of *Ralstonia solanacearum* in the laboratory through biochemical tests

3.2.1. Preservation of diseased specimens

Potato tuber, potato plant and soil sample were collected from eight upazilas of four districts. The specimens were kept in the refrigerator at 4^{o}c to preserve the diseased specimens until isolation was made.

3.2.2. Preparation of Triphenyl Tetrazolium Chloride (TTC)

Triphenyl tetrazolium chloride was prepared according to technique followed by Schaad (1988). For the preparation of 1% TTC medium at first 0.1 g 2, 3, 5 triphenyl tetrazolium chloride was taken in an Erlenmeyer flask containing 10ml distilled water. Then it was shaken thoroughly for few minutes. It was then autoclaved at 121^{o}C under 15 PSI pressure for 15 minutes.

3.2.3. Method of isolation and identification of causal organism

3.2.3.1. Isolation and purification of bacterial wilt pathogen of potato

The collected potato tubers/plants were splashed under running tap water. Then the diseased portions were cut into small pieces. Surface sterilization were completed by dipping them in 5% sodium hypochlorite solution for 3-5 minutes and washed three times with sterilized water. After surface sterilization cut pieces were kept in a test tube containing 3-4 ml of sterile water and kept for 30 minutes for bacterial streaming and getting stock. 1 ml of this stock solution was shifted with the help of sterile pipette into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10^{-1} dilution. Similarly, final dilution was made up to 10^{-4}. After preparing different dilution, 0.1 ml of each dilution was spread over TTC media plate thrice to removed excess surface moisture as described by Goszczynska and Serfontein (1998). Spreading was done with the help of a glass-rod. The inoculated TTC media plates were kept in incubation chamber at 28- 30^{o}C. The plates were observed after 24-48 hours. Then single colony grown over TTC plate was transferred on another plate with the help of a loop to get pure colony.

3.2.3.2. Preservation of bacterial wilt pathogen

After purification of bacteria on TTC media plate, it was kept in refrigerator at 4^{o}C in small eppendorf tubes for future use (Kelman, 1954) .

3.2.3.3. Identification of the pathogen

Identification of the pathogen causing bacterial wilt of potato was done by biochemical tests and cultural features of the pathogen as per standard microbiological procedures by Wang (1998) and Hayward (1991).

3.2.3.4. Morphological characters

Morphological characteristics of the pathogen such as cell shape, Gram's reaction and pigmentation were studied as per standard procedures described by Gerhardt (1981).

3.2.3.5. Biochemical tests of isolated bacteria

3.2.3.6. Gram staining test

A loop full of bacteria was picked from maintained virulent cultures. Aqueous crystal violet solution of 0.5 % was then spread over the smear for 30 seconds, and then washed with running tape water for a minute. Iodine (95%) was then flooded for a minute followed by rinsing with tap water. Then the slides were decolorized with 95% ethanol until colorless runoff. The slides were then counter stained with safranin for 10 seconds and washed with water. The slides were dried under the laminar flow cabinet and placed under the light microscope at 10X, 40X and 100X for observation using oil (Schaad, 1980).

3.2.3.7. Potassium hydroxide solubility test

Bacteria were picked from petri-plates by wire loop and placed on glass slide containing a drop of 3% KOH solution, stirred for 10 seconds and observed for the formation of slime threads (Suslow *et al.*, 1982).

3.2.3.8. Catalase oxidase test

A loop full of bacterial culture obtained from young agar cultures of 18-24 h were mixed with a 3% hydrogen peroxide (H_2O_2) on a glass slide to observe

production of gas bubbles with a naked eye and under a dissecting magnification of 25X (Schaad, 1988)

3.2.3.9. Kovacs oxidase test

Oxidase reagent (1% tetra-methyl-p-phenyl diamine dihydrochloride) solution of 100ml was prepared and kept in rubber stopper dark bottle. A drop of reagent was added to a piece of filter paper placed within a glass Petri dish. Small quantity of inoculum was rubbed on the filter paper containing oxidase reagent solution. Bacteria were then observed for the development of purple color in 10-60 seconds.

3.2.4.1. Levan test

The bacterium (*R. solanacearum*) was inoculated on Nutrient agar with 5% sucrose and incubated at 30^0c for 48 hrs. Levan sucrase which catalyzes the synthesis of Levan form sucrose is produced by a number of bacteria including *R. solanacearum*. When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose. During the fermentation process, the bacteria also utilize sucrose for maintenance and growth.

3.3. In vitro evaluation of soil amendments for controlling Bacterial wilt of potato

3.3.1. Experimental site

The experiment was conducted in the net house of department of plant pathology, Sher-e-Bangla Agricultural University, Dhaka. The location of the site was 23^o 74 N latitude and 90^o 35 longitude with an elevation of 8.2 meter from sea level.

3.3.2. Experimental period

The experiment was carried out during the Rabi season from November 2016 to January 2018. Potato tubers were sown on 1st November 2017 in pots and data were collected on January 2018.

3.3.2.1. Soil Preparation

Soil collected from farm land, breaking clods and separated weeds, brick, plant debris from soil. Finally soil mixed with amendments and poured on medium size pots. 10 kg amendments soil was filled in each pot.

3.3.3. Soil type

The experimental site was situated in the subtropical zone. The soil that collected lies in agro-ecological regions of "Madhupur Tract" (AEZ No. 28). Its top soil is clay loam in texture and olive gray with common fine to medium distinct dark yellowish brown.

3.3.4. Variety

The potato (*Solanum tuberosum*) variety Diamond was used for the experiment. Seed was collected from local Market.

3.3.5. Treatments of the experiment

Multiple treatments were applied in the experiment. Name of different amendments used in experiment.

Table 2. Name of treatments, their active ingredients and concentration

Trade name	Active ingredient	Chemical name	Concentration (%)
Urea	Nitrogen	Urea	0.6
Cow dung	-	-	10
Bleaching powder	Chlorine	Calcium cloro-hypochloride	0.4
lime	Calcium	Calcium oxide	5
Urea +Lime	Nitrogen+ Calcium	-	5.6
Streptomycin	Streptomycin sulphate	D-Streptomycin	0.1

3.3.6. Preparation and inoculation of bacterial solution

A bacterial solution of 500 ml was prepared and allowed to multiply at 30^{o}C for 48 h. A ten-fold serial dilution was made from it and 0.1 ml from the 7th to the 10th dilutions were plated on PSA and incubated for 24 h. The numbers of living bacteria (or colony forming units, CFU) on the Petri dishes were used to determine the concentration of bacterial colonies in the stock solution according to following formula;

$$CFU = \frac{\text{Number of colonies per ml plated}}{\text{Total dilution factor}}$$

The inoculum suspension was inoculated as a soil drench to the pot transplanted seedlings, at a rate of 1 liter/m^2 as described by Michel *et al*. (1997).

3.3.7. Intercultural operation

All treatments were directly mixed with soil as per recommend concentration. Potato tubers were cut into small size with eye and 3-4 peaches were sowing in each pot. In this experiment four pots were use per treatments and 3 to 4 plants per pot. Intercultural operations such as weeding, irrigation were done uniformly in the pots. Post sowing irrigation was given after germination of seeds to bring proper moisture condition of the soil and ensure uniform growth of the plant. The first weeding was done at 30 days after sowing. During the same time thinning was done for maintaining proper distance.

3.3.8. Tagging and data collection

Plants were selected from each pot and tagged for data collection and mean values were determined to get rating score of each treatment.

3.3.9. Disease measurement in experimental pots

Symptom development was evaluated daily until the fourth day, then at 5, 10, 15, 20 and 25 days after inoculation. A six point rating scale (0–5) modified from Kelman (1954) was used, where:

0 = no wilt symptoms, 1 = 1-25% wilted,

2 = 26-50% wilted, 3 = 51-75% wilted, 4 = >75% wilted

5 = completely death (collapse)

$$PDI = \frac{\text{Sum of all rating}}{\text{Total no. of observation x highest scale}} \times 100$$

The status of bacterial wilt of potato was assayed based on wilt incidence. Data on wilt incidence were recorded at least three locations from farmer's field for each growing area. Then the percent wilt incidence and Percent severity index (PSI) were calculated by the following formula:

$$\% \text{ Wilt incidence} = \frac{\text{Number of wilted plant in each field}}{\text{Total number of plant in each field}} \times 100$$

$$\% \text{ PSI} = \frac{\sum (\text{scores} \times 100)}{(\text{Number of plants rated} \times \text{maximum scale of the scores})}$$

3.3.10. Experimental design and layout

The experiment was laid out in Randomized Complete Block Design (RCBD) with four replications. Analysis of variance was done with the help of computer package program M-STATC.

CHAPTER IV

RESULTS

3.4.1. Prevalence of bacterial wilt of potato in the farmer's fields of eight Upazilas of four districts

Prevalence of bacterial wilt in the farmer's fields was recorded from different infected potato fields of four districts. Bacterial wilt incidence in potato field was ranged from 5-15%. The highest (15%) bacterial wilt incidence was observed in Shipgonge followed by Bogra sadar (12%) (Table 1). The lowest bacterial wilt incidence was observed in Pirgonge (5-6%). Five potato varieties namely- Rumana, Granula, Diamond, and Cardinal etc. were found to be mostly cultivated (Table 3).The highest wilt incidence was found in Rumana variety (15%) and the lowest (5-6%) wilt incidence was observed on Diamond variety.

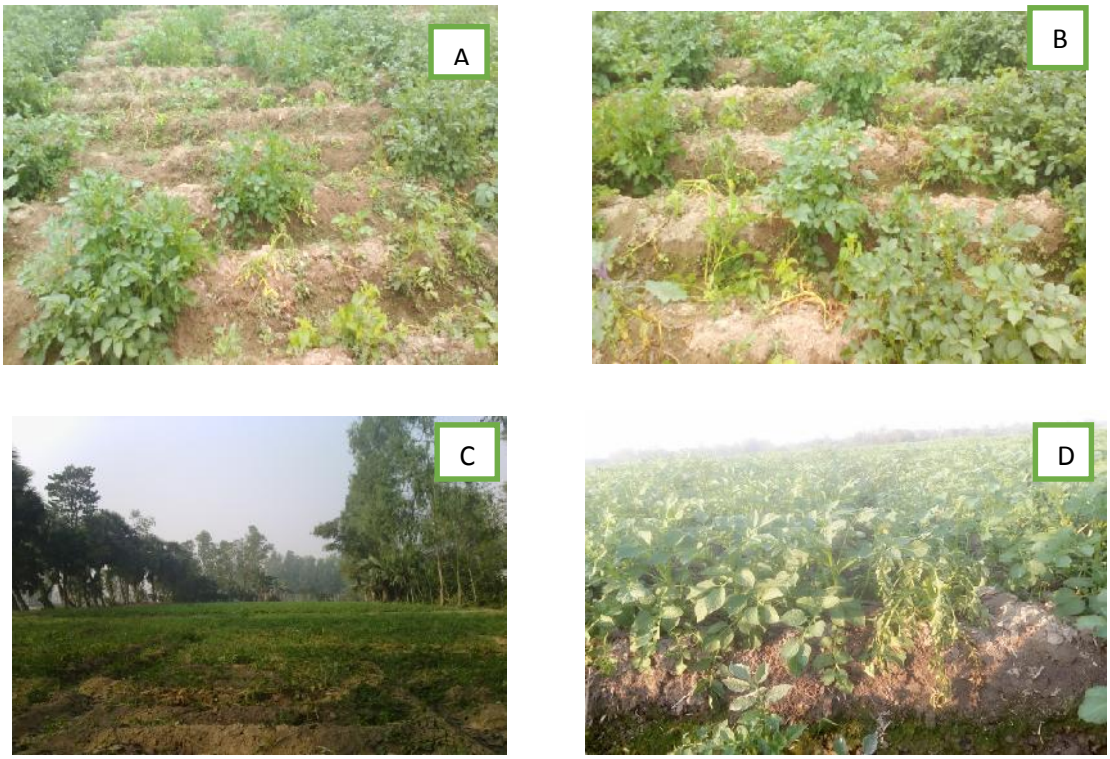

Figure 1. Field prevalence of bacterial wilt of potato in (A) Dinajrpur, (B) Rangpur, (C) Bogra, (D) Joypurhat districts.

Table 3. Condition of the potato fields and prevalence of wilt disease incidence during the survey of eight Upazilas of four districts

Location	Upazilas	AEZ	Cropping pattern	Variety	Incidence of the field
Bogra	Shipgonge	Karatoya-bangali floodplain	Boro-T. aman-Potato	Rumana	15%
	Bogra sadar			Rumana	12%
Joypurhat	Kalai	Tista meander floodplain	Boro-T. aman-Potato	Granula	9%
	Khatlal			Granula	11%
Dinajpur	Dinajpur sadar	Old himalayan piedmont plain	Boro-T. aman-Potato	Granula	6-7%
	Birol			Diamond	6-7%
Rangpur	Rangpur sadar	Active tista floodplain	Boro-T. aman-Potato	Granula	8-9%
	Pirgonge			Diamond	5-6%

3.4.1.1. Field Symptoms of bacterial wilt of potato caused by *Ralstonia solanacearum*

Typical symptoms (Figure 2) such as wilting, yellowing and rapid death of the plants was observed in the infected field. Wilting was first seen as drooping of the tip of some lower leaves. Symptoms of the disease were studied and confirmed by visual observation as per standard procedure describe by Momol (2003). In tuber brownish discoloration of the vascular ring and slight squeezing forces, a pus-like slime out of the ring was found when cut tubers. The vascular ring or the whole tuber disintegrated completely at advanced stages of necrosis development (figure no. 2.A). External symptoms on the tubers were seen at harvest when infection was severe.

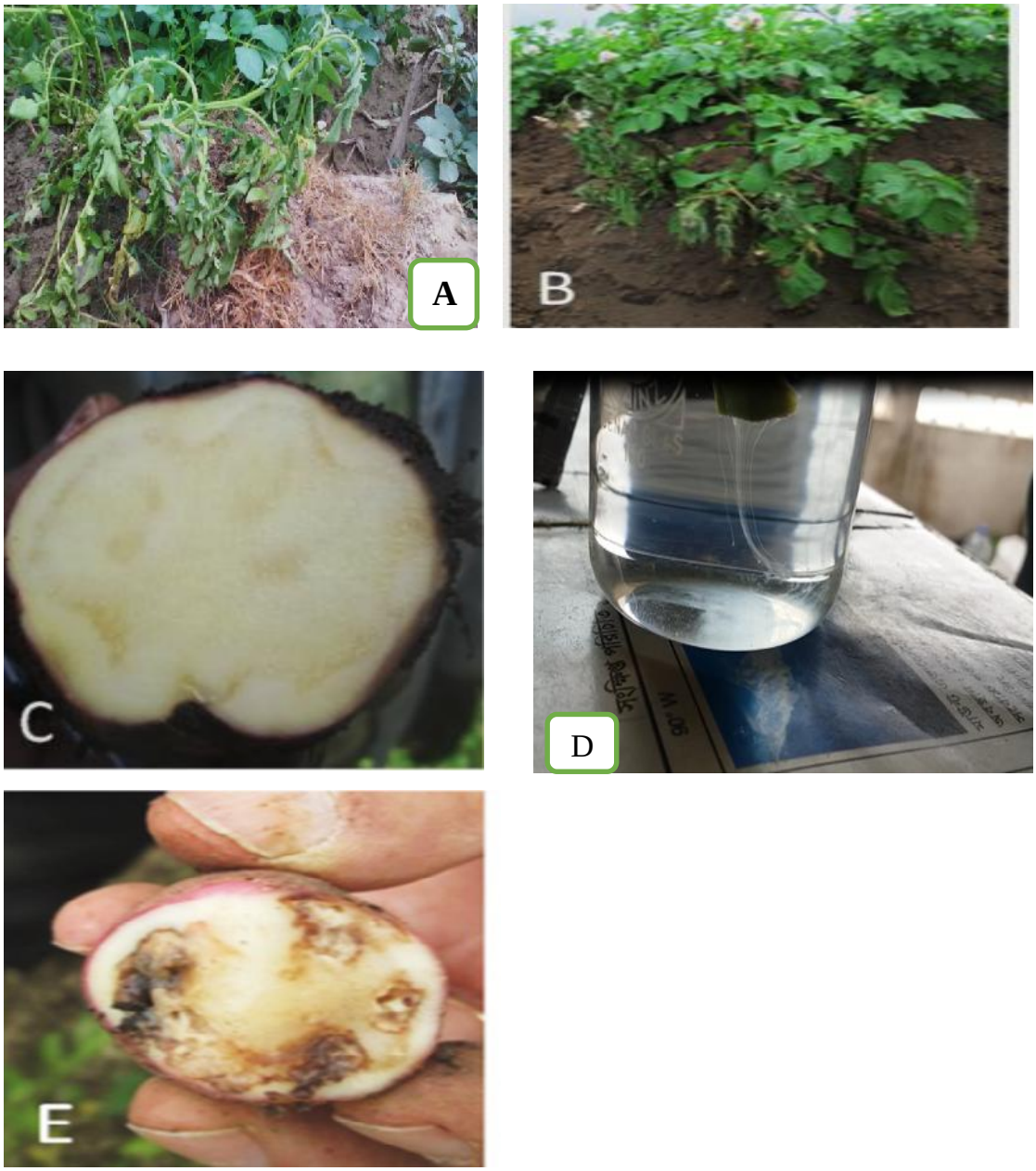

Figure 2. Bacterial wilt and brown rot symptoms caused by *R. solanacearum* on potato plants: A: Wilting of the whole plant; B: Wilting of a few stems of the potato; C: Oozing of the vascular tissues and brown rot; D: Bacterial streaming test; E: Further rotting of tubers infected with *R. solanacearum*.

3.4.1.2. Bacterial Streaming Test

Viscous white spontaneous slime streaming from the cut end of the stem was observed when the cut stem was placed in clear water as shown in (Photo 2. D). This streaming represented the bacterial ooze exuding from the cut ends of colonized vascular bundles.

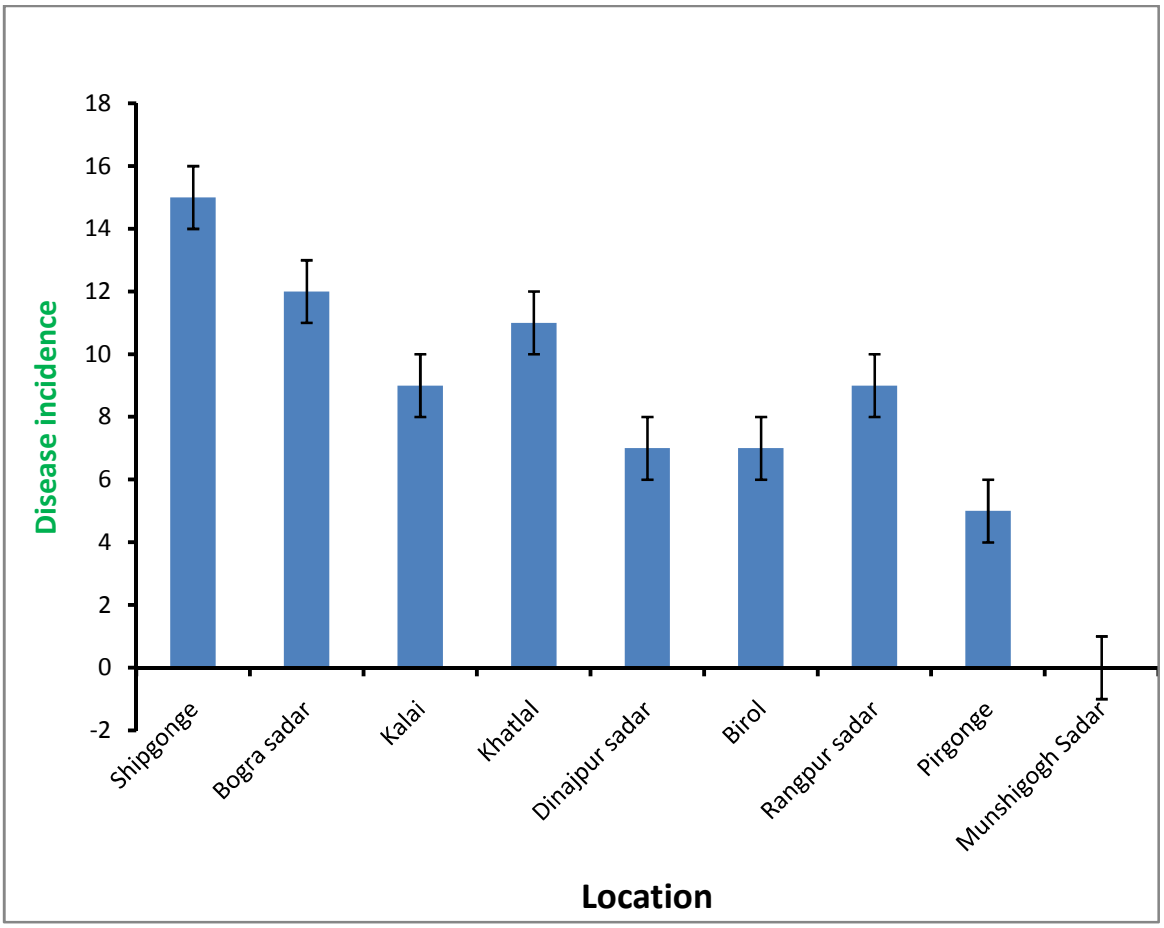

Figure 3. Disease incidence (%) of bacterial wilt in different locations of Bangladesh

3.4.2. Isolation and identification of the pathogen from collected samples

Total sixteen *R. solanacearum* isolates were obtained from the wilted potato plant and tuber that collected from different locations. Isolates that collected from Dinajpur sadar and Birol were grown on TTC media produced pink colony colour whereas isolates collected from Rangpur sadar, Pirgonge and khatlal porduced purple colony colour. Isolates collected from Shibgonj, Bogra sadar and Kalai formed both pink and purple colony on TTC medium (table 2).

Table 4. Isolates of *Ralstonia solanacearum* collected from major potato growing areas in Bangladesh

Districts	Upazila	No of isolates	Present in TTC	Colony colour
Bogra	Shibgonj	2	+ve	pink and purple colony
	Bogra sadar	2	+ve	pink and purple colony
Joypurhat	Kalai	2	+ve	pink and purple colony
	Khatlal	2	+ve	purple colony
Dinajpur	Dinajpur sadar	2	+ve	mostly pink
	Birol	2	+ve	pink colony
Rangpur	Rangpur sadar	2	+ve	purple colony
	Pirgonge	2	+ve	purple colony

3.4.3. Morphological characteristics of the isolates

The virulent and Avirulent isolates of *R. solanacearum* were differentiated by Tetrazolium choloride (TTC) agar test. The results showed that virulent isolates produce pink or light red color colonies with characteristic red center and whitish margin and Avirulent isolates produced smaller, off-white and non-fluidal on TTC medium after 24 hours of incubation.

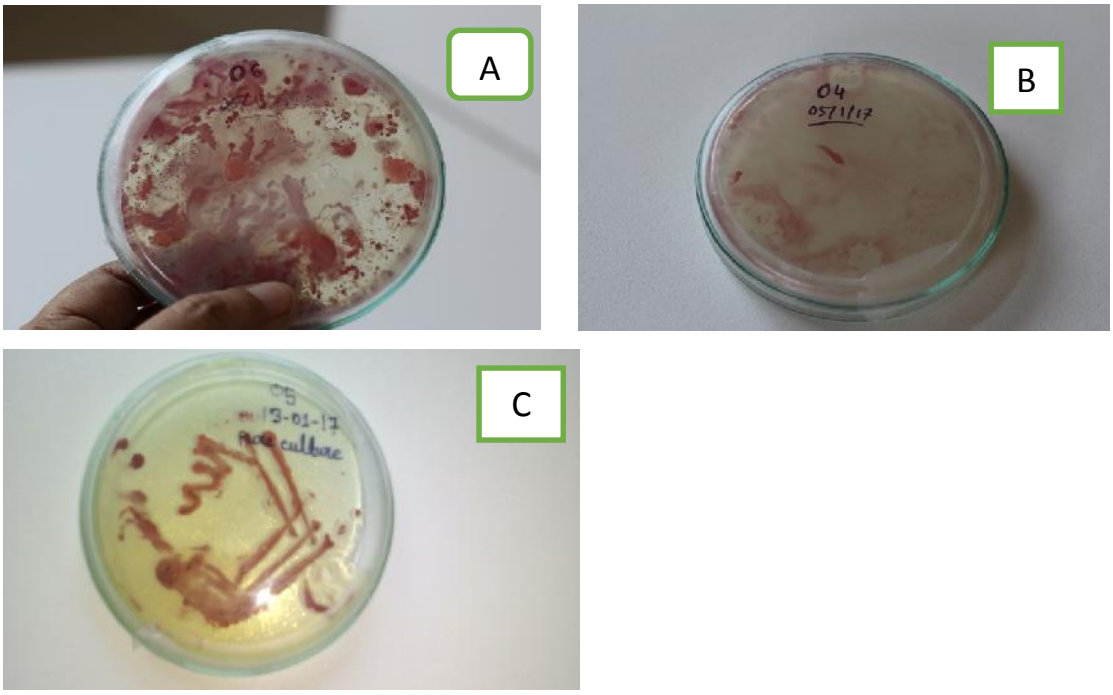

Figure 4. Virulent colony (A), Avirulent colony (B), Pure culture (C) of *R. solanacearum* on TTC medium.

3.4.4. Biochemical tests and characterization of bacteria isolated from different samples

A series of biochemical tests such as Gram staining test, Potassium hydroxide solubility test, Kovac's oxidase test, Levan test, catalase test and Biovar test especially selective for *Ralstonia solanacearum* were performed;

3.4.4.1. Gram staining test

All isolates gave negative response when tested for Gram staining. Bacteria retained reddish pink colony color when counter stained with safranin. This showed that they were Gram negative. In gram reaction as described by Schaad (1988) bacteria retaining reddish pink colony colour are Gram negative (G-ve) while Gram positive (G+ve) stain the blue violet colour.

3.4.4.2. Potassium hydroxide solubility test

The isolates tested were positive on KOH loop test as they formed slime threads when the bacterial cultures (48 h) were mixed with 3 % KOH solution. Gram negative bacteria had relatively fragile cell walls which were bounded by an outer membrane.

3.4.4.3. Catalase test

The bacterial isolates tested produced gas bubbles when mixed with a drop of H_2O_2 on a glass slide (Table 5). Production of gas bubbles was a tendency of all Gram negative bacteria and it gave a clue for presence of aerobic and facultative anaerobic bacteria (Schaad, 1988). Catalase was a hemi-enzyme capable of decomposing hydrogen peroxide to water and oxygen gas (Klement *et al.*, 1964).

3.4.4.4. Kovac's oxidase test

In Kovac's oxidase test positive isolates produced purple blue color when mass of bacterial growth was rubbed on filter paper impregnated with oxidase reagent. This test was used for difference between aerobic and anaerobic bacteria. In my studied, the tested isolates of all group of *Ralstonia solanacearum* isolates presented positive response in development of color within few seconds which indicated that the result of the test was positive for *Ralstonia solanacearum* isolates.

3.4.4.5. Levan test

The result showed that all group of *R. solanacearum* isolates were able to produced distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (Table 4). When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose.

Table 5. Results of Biochemical tests of *R. solanacearum* of potato collected from different locations of four districts

Isolate No.	Gram's staining test	KOH test	Kovac's oxidase test	Levan test	Catalase test
Isola5e 1	-	+	+	+	+
Isolate 2	-	+	+	+	+
Isolate 3	-	+	+	+	+
Isolate 4	-	+	+	+	+
Isolate 5	-	+	+	+	+
Isolate 6	-	+	+	+	+
Isolate 7	-	+	+	+	+
Isolate 8	-	+	+	+	+

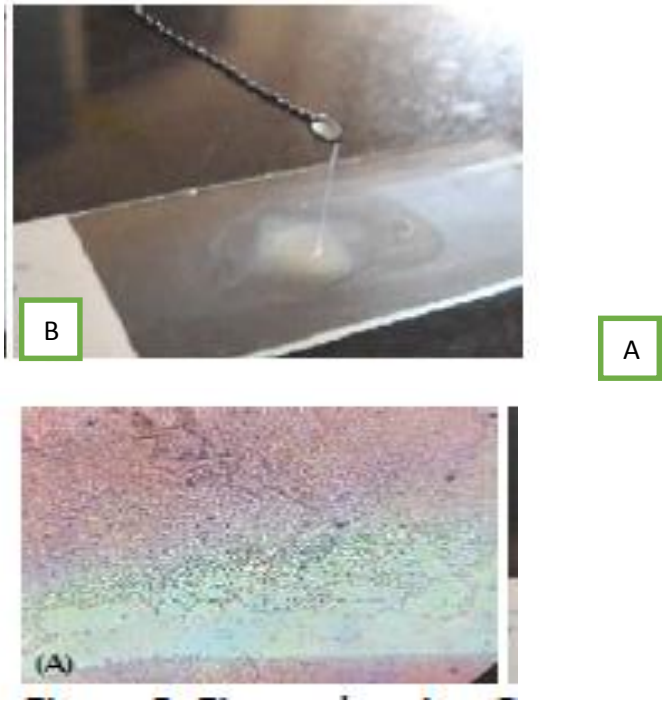

Figure 4.1. Gram staining reaction(A) and KOH test (B) of Gram (–)ve of *R. solanacearum*

3.4.5. Effect of treatments on the PDI at different DAI of bacterial wilt pathogen (*R. solanacearum*) on potato plants

The lowest PDI (20%) was obtained in T_5 (Streptomycin) and which was significantly different than rest of the treatments used in the experiment at 15^{th} and 20^{th} DAI respectively. However Cow dung, beaching power and lime treated plants were showed the second lowest PDI (20%, 40%) which were followed by Urea treated plants. The height PDI (40%, 55%) was obtained in T_7 (control) treatments at 15^{th} and 20^{th} DAI respectively.

3.4.6. Effect of different treatments on PDI at the 25^{th} DAI against bacterial wilt *(R. solanacearum)* of potato

Due to the effect of different treatments different PDI was recorded and the lowest PDI (percent disease index) 40% was observed in case of T_5 (Streptomycin) which was same as T_6 (cow dung) treatments and T_2 (Urea+Lime) treatments. The highest PDI (100%) was observed in case of control (T_7) treatments (Table 7).

3.4.7. Effect of different treatments on diseases severity score (DSS) at the 25^{th} day against bacterial wilt (*R. solanacearum*) of potato

Due the effect of different treatments applied different DSS was recorded and the lowest DSS (diseases severity score) 2.075 was observed in case of T_5 (Streptomycin) which was followed by T_6 (cow dung) treatments (2.46). Whereas, the highest DSS (5.00) was observed in case of T_7 (control) treatments.

3.4.8. Effect of treatments on the diseases severity score (DSS) at different DAI of bacterial wilt pathogen *(R. solanacearum)* on potato plants

The lowest DSS (1.02 and 1.83) were recorded in T_5 (Streptomycin) which was significant than rest of the treatments used in the experiment at 15^{th} and 20^{th} DAI respectively. The highest DSS (2.31, 3.03 and 4.02) was obtained in T_7 (control) treatments at 10^{th} 15^{th} and 20^{th} DAI respectively.

3.4.9. Effect of different treatments in percent reduction of disease severity of bacterial wilt *(R. solanacearum*) in potato plants at 25th day after inoculation

Significant differences were observed due to the effects of different treatments in case of percent reduction of disease severity of bacterial wilt (*R. solanacearum)* in inoculated potato plants at 25th DAI (day after inoculation). It was observed that percent reduction of disease severity highest in case of Streptomycin (T_5) (60%), cow dung (T_6) and (Urea+Lime) treated plants which were followed by Lime (40%) and bleaching powder (45%) treated plants at 25th day after inoculation.

Table 7. Effect of treatments on the PDI at different DAI of bacterial wilt pathogen (*R. solanacearum*) on potato plants

	PDI of the inoculated plant									
Treatment	5th DAI		10th DAI		15th DAI		20th DAI		25th DAI	
	DSS	PDI	DSS	PDI	DSS	PDI	DSS	PDI	DSS	PDI
T_1	0.05c	5	0.81 cd	20	1.688 c	20	2.45 c	40	3.075 c	55
T_2	0.06c	10	0.92c	20	1.46 cd	20	2.13 d	40	2.785 d	40
T_3	0.10 c	10	0.81 cd	20	1.16 de	20	2.66 c	40	3.200 c	60
T_4	1.05 b	20	1.56 b	25	2.500 b	40	3.02 b	55	3.860 b	60
T_5	0.00 c	0	0.33 d	20	1.028 e	20	1.83 e	20	2.075 f	40
T_6	0.00 c	0	1.00 c	20	1.612 c	20	2.01 de	40	2.460 e	40
T_7	1.51 a	20	2.31 a	40	3.033 a	55	4.025 a	75	5.000 a	100
LSD(.01)	0.2575		0.5385		0.3153		0.2654		0.2654	
CV (%)	21.44		14.85		8.66		4.96		4.01	
	PDI=PERCENT DISEASES INDEX; DAI=DAYS AFTER INOCULATION; DSS=DISEASES SEVERITY SCORE									

T_1 – Bleaching powder

T_2 - Urea+Lime

T_3 – Lime

T_4 – Urea

T_5 - Streptomycin

T_6 – Cow dung

T_7 - Control

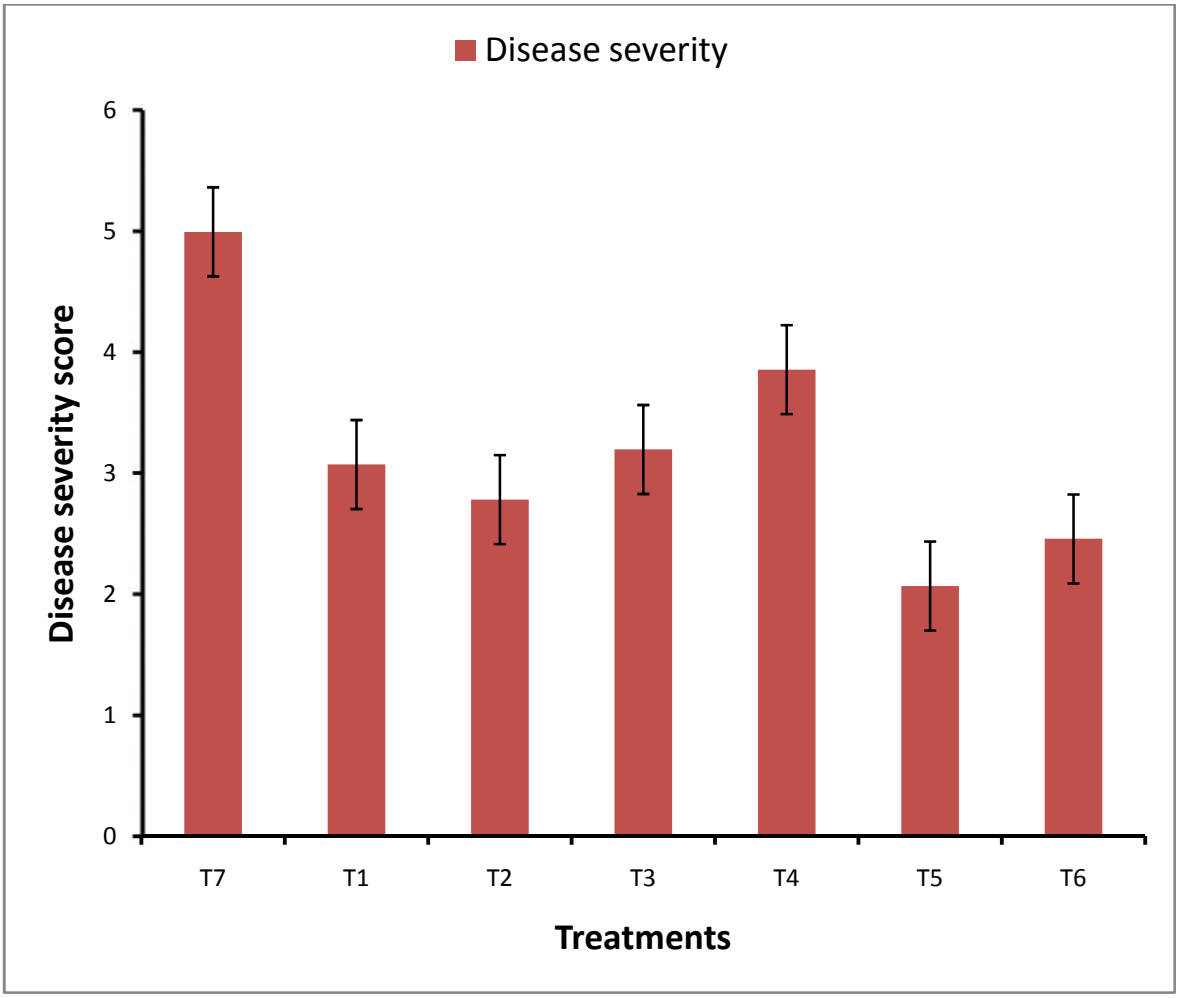

Figure 5. Effect of different treatments on the disease severity of bacterial wilt (*R. solanacearum)* of potato

T_1 – Bleaching powder

T_2 – Urea+Lime

T_3 – Lime

T_4 – Urea

T_5 – Streptomycin

T_6 – Cow dung

T_7 - Control

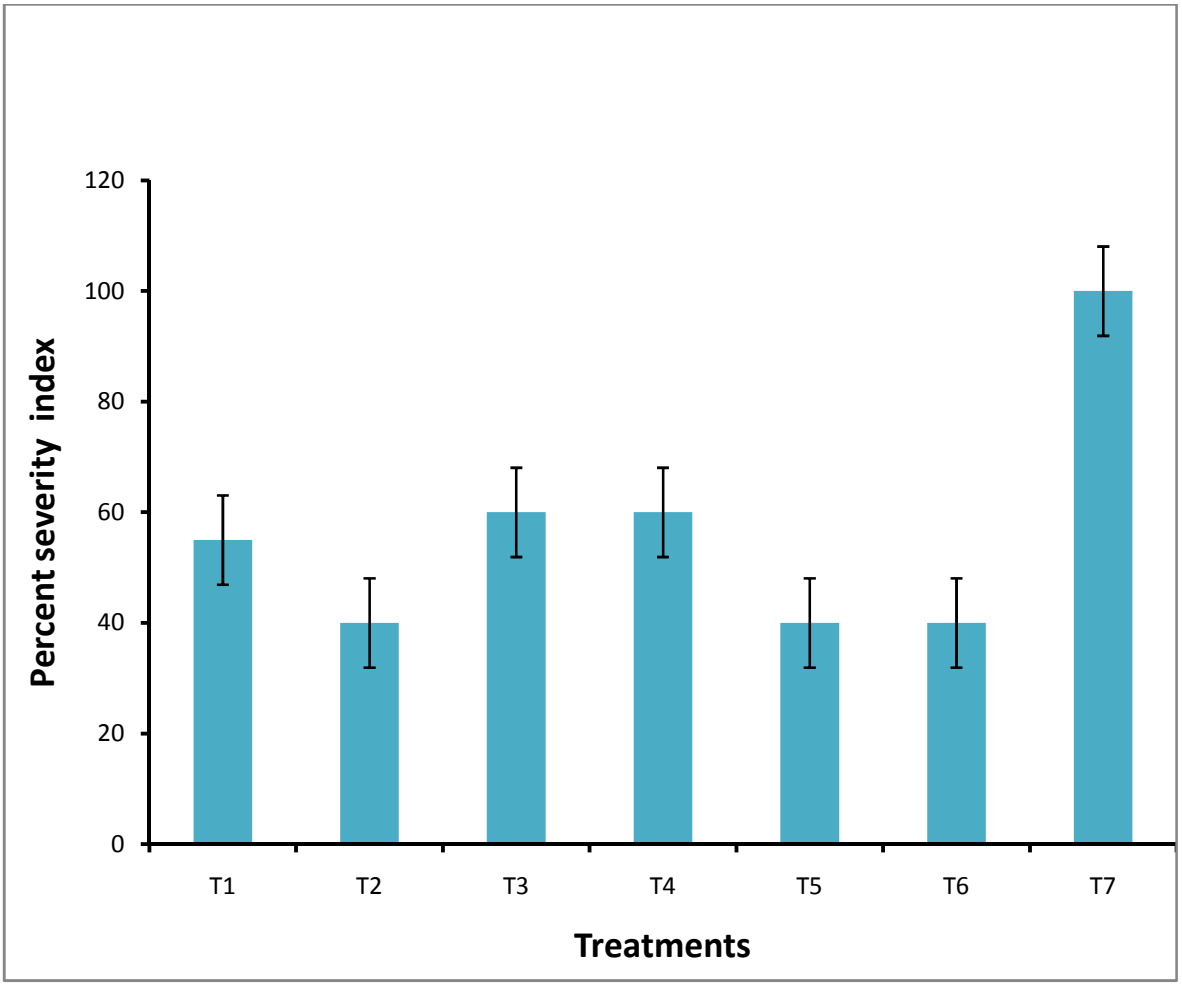

Figure 6. Effect of different treatments on the PDI (percent disease index) of bacterial wilt (*R. solanacearum*) of potato

T_1 – Bleaching powder

T_2 – Urea+Lime

T_3 – Lime

T_4 – Urea

T_5 – Streptomycin

T_6 – Cow dung

T_7 – Control

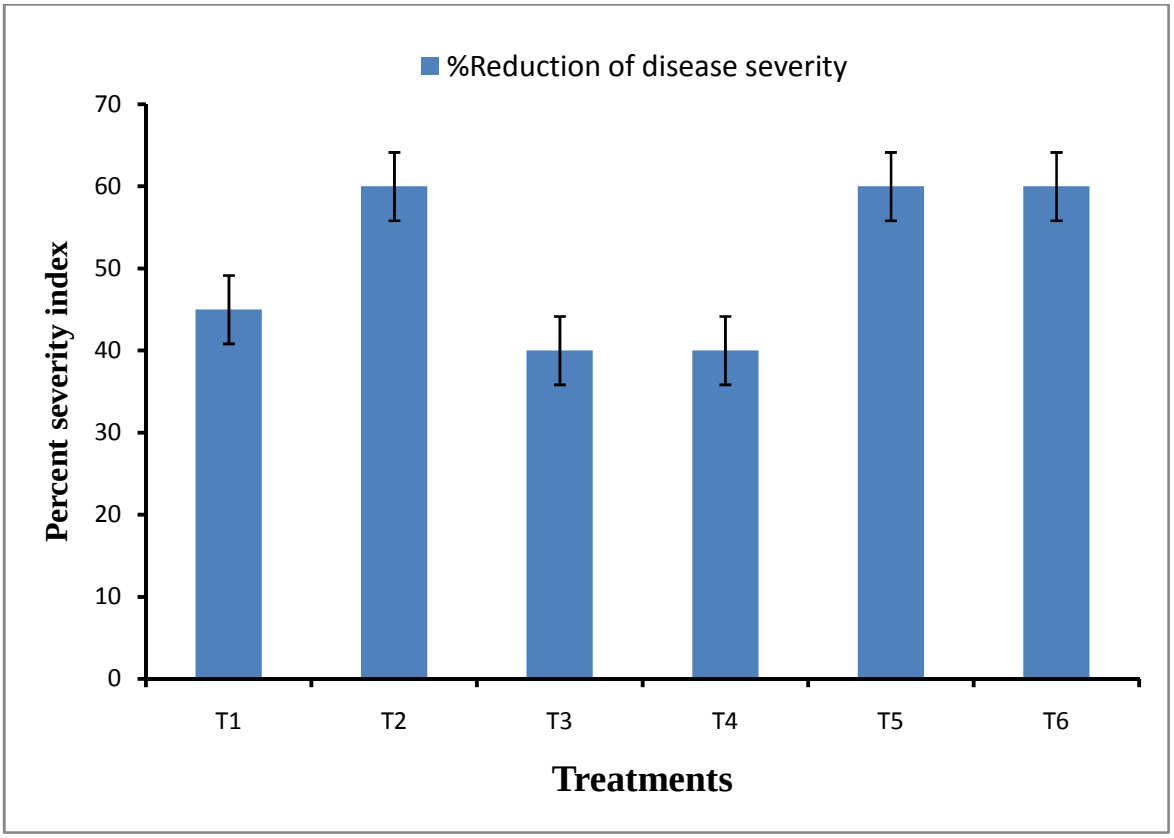

Figure 7. Effect of different treatments in reduction disease severity over control for bacterial wilt (*R. solanacearum)* in potato plants at 25th day after inoculation

T_1 – Bleaching powder

T_2 – Urea+Lime

T_3 – Lime

T_4 – Urea

T_5 – Streptomycin

T_6 – Cow dung

Figure 8. Symptoms of Seedling with the application , Bleaching Powder (A), Urea+Lime (B), Lime (C), Urea (D), Streptomycin(E), Cow dung (F)

Figure 9. Soil preparation (A), Experiment pots (B), artificial inoculation (C) after inoculation (D)

CHAPTER V

DISCUSSION

A survey was conducted in eight upazila under four districts of Bangladesh during the potato growing season of 2016-2017 to observe the Bacterial wilt incidence in farmer's fields in survey area. The survey results showed that the highest (15%) bacterial wilt incidence was observed in Shipgonge. The lowest bacterial wilt incidence was observed in Pirgonge (5-6%) which was support by Nadia *et al*. (2013). In a similar survey the wilt incidence was recorded maximum (22.52) in Rangpur followed by bogra (20.56) (Chatterjee *et al.*, 1997). The survey results indicated a regional variation in bacterial wilt incidence. Differences of wilt incidence were also reported due to the great diversity of host plants affected by this pathogen, phenotype and genotype of *R. solanacearum*, its wide geographical distribution and the range of environmental conditions conducive to bacterial wilt. The causal agent of bacterial wilt of potato (*Ralstonia solanacearum*) was identified by conducting studies on its morphological and biochemical features as per standard microbiological procedures. Virulence and avirulence of an isolate can be resolute on the basis of colony traits by using TTC medium. Fluidal colonies with reddish pink center and irregular margins are usually virulent while dark coloured non-fluidal colonies with darker red colours and smooth margins are normally avirulent. These results are in harmony with the findings of Rahman, (2010) and Shahbaz (2015).

KOH solubility test and gram reaction test suggested that the bacterium test was non-fluorescent bacterium. The *R. solanacearum* bacteria produced gas bubbles when these were mixed with a drop of H_2O_2 on glass slide (Hayward, 1964); (He *et al*., 1983) and (Kumar *et al*.,1993). Production of gas bubbles was a tendency of all Gram negative bacteria in Catalase oxidase test In Kovacs oxidase test positive isolates produced purple colour when mass of bacterial growth is rubbed on filter paper impregnated with oxidase reagent. Similar results found by (Kovacs, 1956). The *R. solanacearum* colonies of

Levan positive cultures produced colonies that were raised convex and mucoid (Schaad, 1980).

Our study revealed that an average (60%) percent reduction of bacterial wilt severity in amendment soils compared to unamendment soil. The DSS (diseases severity score) was recorded at the 25^{th} day against bacterial wilt (*R. solanacearum*) of potato. Results showed that the lowest DSS (diseases severity score) 2.075 was observed in case of T_5 (Streptomycin) which was followed by T_6 (cow dung) treatments (2.46). However, cow dung treated plants were showed the second lowest DSS which was followed by (Urea+Lime) treated plants. In this study cow dung and streptomycin was observed to show the highest reduction of wilt diseases and it was supported by Shrivastava and Pal (2014). Manure does not breakdown the pathogenicity of the pathogen. It might be due to improve the composition of soil and activities of soil microorganisms (Adebayo and Epko, 2001). In fact efficient soil management generally improves the composition and activity of soil microbiota thereby enhancing the biological control capacity of the soil (Djeugap *et al*., 2014). In case of urea and bleaching powder it was also observed to reduce the wilt disease which was supported by (Shekhawat *et al.,* 1990) and it was partially supported by (Danial *et al.,* 2006). Disease suppression may be result of higher ammonium concentrations at certain pH levels which are toxic *to R. solanacearum* (Michel and Mew, 1998). Lime was also found to be effective against the disease which was supported by the (Michel *et al.,* 1997). Both K and Ca are known for enhancing plant defense (Flego *et al.* 1997). High fertilizer applications cannot be recommended for control of brown rot, as these may enhance susceptibility to other diseases and would not be sustainable in the long run.

CHAPTER VI
SUMMARY AND CONCLUSION

The present study was conducted to find out the prevalence of bacterial wilt disease of potato in different potato growing locations and management by the soil amendments. The survey results showed that the highest mean (15%) bacterial wilt incidence was observed in Shipgonge followed by Bogra sadar (12%).

In the present study, sixteen samples were collected from farmer's field. The symptomatology was studied and samples were brought to the laboratory of Department of Plant Pathology at Sher-e-Bangla Agricultural University, Dhaka and isolates *Ralstonia solanacearum* on TTC. Different biochemical test viz. Gram's staining test, KOH test, Kovac's oxidase test, Levan test and Catalase test were carried out for the characterization and identification of the isolated bacteria. The findings of all biochemical tests is that all isolates were showed positive results and finally wilt disease were confirmed that it caused by *Ralstonia solanacearum.*

Six different treatments were conducted to know the effect of soil treatment on the management of bacterial wilt pathogen in a net house of Department of Plant Pathology at Sher-e-Bangla Agricultural University, Dhaka and isolates *Ralstonia solanacearum* on TTC. Results showed that the lowest PDI (20%) was found in T_5 (Streptomycin) and which was significant than rest of the treatments used in the experiment at 15^{th} and 20^{th} DAI respectively. The heights PDI 40% and 55% were obtained in T_7 (control) treatments at 15^{th} and 20^{th} DAI, respectively. Results showed that the lowest PDI (percent disease index) 40% was observed in case of T_5 (Streptomycin) which was same as T_6 (cow dung) treatments. The lowest DSS (1.02 and 1.83) were recorded in T_5 (Streptomycin) which was significant than other treatments used in the experiment. The highest DSS (2.31, 3.03 and 4.02) was obtained in T_7 (control) treatments at 10^{th} 15^{th} and 20^{th} DAI, respectively.

It also observed that percent reduction of disease severity highest in case of Streptomycin (T_5) (60%), cow dung (T_6) and T_2 (Urea+Lime) treated plants which were followed by Lime (40%) and bleaching powder (45%) treated plants at 25th day after inoculation.

Incidence and severity of potato could differ in the different locations of Bogra as well as the whole country. We recommend the use of cowdung as well as the urea+lime and stable bleaching powder solution could be the better treatments against *R. solanacearum.*

REFERENCES

Abdullah, H.B. (1988). Biology and survival of *Pseudomonous solanacearum* in Malaysia. Ph. D. Thesis, Universiti Putra Malaysia (UPM), pp: 210.

Anith, K.N., Momol, M.T., Kloepper, J.W., Marois, J.J., Olson, S.M., and Jones, J B. (2004). Efficacy of plant growth–promoting *rhizobacteria*, acibenzolar-S-methyl, and soil amendment for integrated management of bacterial wilt on tomato. Plant Dis. **88**:669-673.

Agrios, G.N. (1953). *Plant Pathology*. 5th Edn., Academic Press, New York, USA., ISBN-**13**: 9780120445653, Pages: 922.

Ahmed, H.U., Ali M.S. and Dey T.K. (2000). Tuber crop disease management. Poster presented in the International Conference on Integrated Plant Disease Management for Sustainable Agriculture. Mitra DK (ed), *Indian Phytopathological Society,* New Delhi, India. **3**: 1281 p

Allen, C P., and Hayward A.C. (2005). Bacterial wilt disease and the RS species Complex. The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, Minnesota, U.S.A. p1.

Annon, (1998). Council Directive of 20 July 1998 on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. official J. Eur. Commun. L**235**: 1-39.

Anonymous, (1995). Brown Rot (*Pseudomonous solanacearum*). The Scottish Agriculture and Science Agency Edinburgh, Scotland.

Anonymous, (2006). Commission Directive 2006/63/EC of 14 July 2006: amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* Official *Journal of the European Communities*, L**206**: 36-106.

BBS, (2014). Bangladesh Bureau of Statistics. Statistical Year Book of Bangladesh. Statistics Division. Ministry of Planning. Government of the Peoples Republic of Bangladesh. p. 375.

Champoiseau, P.G., Jones, J.B. and Allen, C. (2009). *Ralstonia solanacearum* race 3 biovar 2 causes tropical losses and temperature anxieties. Online. Plant Health Progress. doi: 10.1094/PHP-2009-0313-01-RV.

Cook, D., Barlow, E., Sequeira, L., (1989). Genetic diversity of *Pesudomonas solanacearum*: detection of restriction fragment length polymorphoisms with DNA probes that specify virulence and the hypersensitive response. *Molecular Plant-Microbe Interactions* 2, 113-121.

Cuppels, D.A., Hanson, R.S. and Kelman, A. (1978). Isolation and characterization of a bacteriocin produced by *Pseudomonous solanacearum*. *J. Gen. Microbiol*., **109**: 295-303.

Danial, J., McHugh R.C. and Saddler, G.S. (2006). Molecular characterization of the potato brown rot pathogen *Ralstonia solanacearum* race 3/biovar 2A. Proceedings of 4th International Bacterial Wilt Symposium. The Lakeside Conference Centre. United Kingdom.

Datnoff, L.E., Seebold, K.W., Correa, V.F.J. (2001). The use of silicon for integrated disease management: reducing fungicide applications and enhancing host plant resistance. p. 171–83. **In:** "*Silicon in Agriculture*" (L.E. Datnoff, G.H. Snyder, G.H. Korndorfer, eds.). The Netherlands, Elsevier, 424 pp.

Denny, T. (2007). Plant Pathogenic *Ralstonia* Species. Pages 573-644 **in:** Plant Associated Bacteria. S.S. Gnanamanickam, ed., Springer, The Netherlands.

Denny, T.P. (2006). Plant Pathogenic *Ralstonia* species. **In:** Plant-Associated Bacteria, Gnanamanickam, S.S. (Ed.). Springer Publishing, Dordrecht, The Netherlands, pp. 573-644.

Denny, T.P., Brumbley, S.M., Carney, B.F., Clough, S.J., and Schell, M.A. (1994). Phenotype conversion of *Pseudomonas solanacearum*: its molecular basis and potential function. Pages 137-147 **in:** Bacterial Wilt: the Disease and its Causative Agent, *Pseudomonas solanacearum*. A.C. Hayward, and G.L. Hartman, eds., Wallingford, UK: CAB International.

Dhital, S.P., Thaveechi, N., Kositratana, W., Piluek, K. and Shrestha, S.K. (1997). Effect of chemical and soil amendment for control of bacterial wilt of potato in Nepal caused by *Ralstonia solanacearum*. *Kasetsart Journal of Natural Sciences* **31**: 497-509.

Domo˜nguez, J., Negro˜n, M.A., & Rodro˜guez, C.M. (2001). Aggregate water-stability, particle-size and soil solution properties in conducive

and suppressive soils to Bacterial wilt of banana from Canary Islands (Spain). Soil Biology and Biochemistry, **33**: 449–455.

Elphinstone, J.G. & Aley, P. (1993). Integrated control of bacterial wilt of potato in the warm tropics of Peru. In Hartman, G.L. & Hayward, A.C. (Eds.) Bacterial wilt. ACIAR. Proceedings 45. ACIAR, (pp. 276–283) Canberra, Australia.

Elphinstone, J.G. (2005). The current bacterial wilt situation: A golobal overview. Pages 9-28 **in:** Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. The American Phytopathological Society, St. Paul, MN.

Elphinstone, J.G. and Aley, P. (1993). Integrated control of bacterial wilt of potato in the warm tropics of Peru. **In**: Hartman, G.L., Hayward, A.C. (Eds.), Bacterial wilt Proceedings of an International Conference held at Kaohsiung, 28-31 October 1992, Taiwan, ACIAR Proceedings, vol. 45,pp. 276-283.

Elphinstone, J.G. (2005). The current bacterial wilt situation: A global overview. **In:** Bacterial wilt disease and the *Ralstonia solanacearum* species complex. C. Allen, P. Prior, and A.C. Hayard, Eds., American Phytopathological Society, Saint Paul MN. pp. 9 – 28.

Engelbrecht, M.C. (1994). Modification of a semi-selective medium for the isolation and quantification of *Pseudomonous solanacearum*. Bacterial Wilt Newsletter, **10**: 3-5.

Elphinstone, J.G. (1996). The current bacterial wiltsituation: A global overview.In Bacterial wilt diseaseand the *Ralstonia solanacearum* species complex. C. Allen, P. Prior and A.C. Hayard, Eds., American Phytopathological Society, Saint Paul MN. pp. 9 – 28.

EPPO. (2014). PQR database. Paris, France: European and Mediterranean Plant Protection Organization.

FAO. (2013). Food and Agricultural Organization of the United Nations. Rap. Pub. 71 p.

Flego, D., Pirhonen, M., Saarilahti, H., Palva, T.K. & Palva, E.T. (1997). Control of virulence gene expression by plant calcium in the phytopathogen *Erwinia carotovora*. *Molecular Microbiology*, 25, 831–838.

French, E.R. and Sequeira, L. (1970). Strains of *Pseudomonas solanacearum* from central and South America: a comparative study. Phytopathology **60**: 506-512.

French, E.R. Ferrante, P. (1995). Multiple disease resistance in potato cultivars with Solanum phureja and Solanum demissum background. Phytopathology, **75**: 1288.

Geddes, A.M. (1989). Potato Atlas of Pakistan: Information of potato production by agroecological zones. Pak Swiss potato development Project, PARC, Islamabad, Pakistan. P. 77.

Genin, S., Boucher, C. (2002). *Ralstonia solanacearum*: secrets of a major pathogen unveiled by analysis of its genome. Molecular Plant Pathology **3**: 111-118.

Gerhardt, P. (1981). Manual of methods of General Bacteriology. American Society of Microbiology Washington, D. C. p. 127.

Gorissen, A., van Overbeek, L.S. & van Elsas, J.D. (2004). Pig slurry reduces the survival of *Ralstonia solanacearum* biovar 2 in soil. *Canadian Journal of Microbiology*, **50**:587–593.

Goszczynska, T. and Serfontein, J.J. (1998). Milk Tween Agar, a semiselective medium for isolation and differentiation of *Pseudomonous syringae pv. syringae, Pseudomonous syringae pv. Phaseolicola* and *Xanthomonas axonopodis pv. phaseoli*. J. Microbiol,. Method. **32**(1): 65-72.

Gota, M. (1992). Fundamentals of Bacterial Plant Pathology, Academic Press, San Diego, California, pp. 282-286.

Gurung, T.B., and Vaidya, A.K. (1997). Baseline study report of a community participatory bacterial wilt management program in Ulleri and Jhilibarang villages of western Nepal. Working paper no. 97. Lumle Regional Agricultural Research Centre, Nepal.

Gurung, T.B. and Vaidya, A.K. (1997). Baseline study report of a community participatory bacterial wilt management program in Ulleri and Jhilibarang villages of western Nepal. Working paper no. 97. Lumule Regional Agricultural Research Centre, Nepal.

Hayward A.C., (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Review of Phytopathology **29**: 65-87.

Hayward, A.C. (2005). Research on Bacterial Wilt: A perspective on International links and access to literature. **In**: Bacterial Wilt Disease and the *Ralstonia solanacearum* species Complex. eds. Allen, C., P. Prior and A.C. Hayward. The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, Minnesota, U. S. A. pp. 1-8.

Hruschka, J., Kelman, A. (1973). The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. J. Gen. Microbiol. **76**:177-188.

Prior D.R. and Hayward, A.C. (1952). The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, Minnesota, U. S. A. pp. 1-8.

He L.Y., Sequeria, L., Kelman, A. (1983). Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Disease **67**: 1357-1361.

Janse, J.D., Araluppan, J., Schans, M., Wenneker, F.A.X. and Westerhuis, W. (1998). Experience with Bacterial Brown Rot *Ralstonia solanacearum* Biovar 2, Race 3, Springer-Verlag, Berlin, Germany, The Netherlands.

Jones, J.B., Jone, J.P., Stall, R.E. and Zitter, T.A. (1991). Compendium of Tomato Diseases. The APS Press, St. Paul, MN, USA, 73 pp.

Kelman, A. (1953). The bacterial wilt caused by *Pseudomonas solanacearum*. North Carolina Agricultural Experimental Station Technical Bulletin **99**: 194 pp.

Kelman, A. (1953). The bacterial wilt caused by *Pseudomonous solanacearum*: A literature review and bibliography. North Carolina Agric. Exp. Station Tech. Bull., **99**: 194-194.

Kelman, A. (1954). The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology, **44**: 693-695.

Kelman, A. (1954). The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology, **44**: 693-695.

Kelman, A. and Person, L.H. (1954). Strains of *Pseudomonous solanacearum* differing in pathogenecity to tobacco and peanut. Phytopathology, **51**: 158-161.

Kelman, A. and Sequeira, L. (1965). Root to root spread of *Pseudomonous solanacearum*. Phytopathology, **55**: 304-309.

Kelman, A. and Hruschka, J. (1973). The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. J. Gen. Microbiol. **76**:177-188.

Kelman, A. (1981). The bacterial wilt caused by *Pseudomonas solanacearum*. North Carolina Agricultural Experimental Station Technical Bulletin 99.

Khalil M.I., Haque M.E. and Hoque M.Z. (2013). Adoption of BARI recommended potato (*Solanum tuberosum*) varieties by the potato farmers in Bangladesh. The Agriculturists 11(**2**): 79-86.

Lemaga, B., Kakuhenzine, R., Kassa, B., Ewell, P.T. and Priou S. (2005). Integrated control of potato bacterial wilt in eastern Africa: the experience of African highlands initiative. **In**: Allen, C., Prior, P., Hayward, A.C., editors. Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society Press; St Paul, MN: 2005. pp. 145–158.

Lemaga, B., Kakuhenzire, R., Kassa, B., Ewell, P.T. & Priou, S. (2005). Integrated control of potato bacterial wilt in Eastern Africa: The experience of African highlands initiative. In C. Allen, P. Prior, & A.C. Hayward (Eds.), Bacterial wilt disease and the *Ralstonia solanacearum* species complex (pp. 145–157). Minnesota, USA: The American Phytopathological Society.

Lemessa, F. and Zeller, W. (2007). Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. Biol. Control, **42**: 336-344.

Michel, V.V. & Mew, T.W. (1998). Effect of soil amendment on the survival of *Ralstonia solanacearum* in different soils. Phytopathology, **88**: 300–305pp.

Michel, V.V., Wang, J.F., Midmore, D.J. & Hartman, G.L. (1997). Effects of intercropping and soil amendment with urea and calcium oxide on the incidence of bacterial wilt of tomato and survival of soil-borne *Pseudomonas solanacearum* in Taiwan. Plant Pathology, **46**: 600–610.

Momol, M.T., Momol, E.A., Dankers, W.A., Olson, S.M., Simmons, J. A. and Rich, J.R. (1999). Evaluation of selected plant essential oils for suppression of *Ralstonia solanacearum* and *Meloidogyne arenaria* on tomato. Abstr. Phytopathol, **89**: 554-554.

Murakoshi, S. and Takahashi, M. (1984). Trials of some control of tomato wilt caused by *Pseudomonas solanacearum* bulletin of the Kanagawa. Hortic. Exp. Station, **31**: 50-56.

Nishiyama M., Shiomi Y., Suzuki S. and Marumoto T. (1999). Suppression of growth of *Ralstonia solanacearum*, tomato bacterial wilt agent, on/in tomato seedlings cultivated in a suppressive soil. Soil Science and Plant Nutrition **45**: 79-87.

OEPP/EPPO, (1999). EPPO standard PP 1/213(1). Resistance Risk Anal. EPPO Bull., **29**: 325-347.

Opina, N., Tavner, F., Hollway, G., Wang, J. F., Li, T.H., Maghirang, R., Fegan, M., Hayward, A.C., Krishnapillai, V. and Hong, W.F. (1997). A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia-Pac. J. Mol. Biol. Biotechnol. **5**:19-30.

Pitkethley, N. (1981). Host range and biotypes of *Pseudomonous solanacearum* in the northern territory. Aust. Plant Pathol., **10**: 46-47.

PRA. (2015). Pest Risk Analysis (PRA) of potato in Bangladesh.

Rajeshwari, N., Shylaja M.D., Krishnappa M., Shetty H.S., Mortensen C.N., Mathur, S.B. (1998). Development of ELISA for the detection of *Ralstonia solanacearum* in tomato: its application in seed health testing. *World Journal of Microbiology and Biotechnology*, **14** (5): 697-704; 36.

Ramesh, R., and Phadke, G.S. (2012). Rhizospere and endophytic bacteria for the suppression of eggplant wilt caused by *Ralstonia solanacearum*. Crop Prot. **37**:35-41.

Saddler, G.S. (2005). Management of bacterial wilt disease. Page 121-132 **in**: Bacterial Wilt Disease and *Ralstonia solanacearum* Species Complex. Allen, C., Prior, P., and Hayward, A.C. eds. St. Paul, M.N, APS Press.

Schaad, N.W. (1980). Laboratory guide for the identification of plant pathogenic bacteria. America Phytopathology,Society of Minneapolis Saint Paul.28-45.

Schaad, N.W. (1988). Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. American Phytopathological Society, St. Paul, Minnesota. pp. 185-197.

Schaad, N.W., Jones, J.B. and Chun, W. (2001). Laboratory guide for the identification of plant pathogenic bacteria. Laboratory guide: for the identification of plant pathogenic bacteria.

Schönfeld J., Gelsomino A., van Overbeek L.S., Gorissen A., Smalla K., van Elsas J.D. (2003). Effects of compost addition and simulated solarisation on the fate of *Ralstonia solanacearum* biovar 2 and indigenous bacteria in soil. FEMS Microbiology Ecology **43**: 63-74.

Sharma J.P, Kumar, S. (2000). Management of *Ralstonia* wilt through soil disinfectant, mulch, lime and cakes in tomato (*Lycopersicon esculentum*) Indian J Agr Sci. 2000;**70**:17–19.

Shekhawat, G.S, Bhal, B.K, Kishore, V., Sangar, R.B.S., Patel, R.L., Sinha, S.K., Pani, A.K. (1990). Control of bacterial wilt of potatoes by agronomic practices. *Journal of Indian Potato Association* **17** (1,2) : 52-60.

Singh, R., (1994). Seed transmission studies with *Pseudomonous solanacearum* in tomato and eggplant. ACIAR Bacterial Wilt Newsletter, **11**: 12-13.

Smith, E.F. (1896). A bacterial disease of the tomato, eggplant, and Irish potato (*Bacillus solanacearum nov. sp*.). Bulletin, Division of vegetable Physiology and Pathology. US Department of Agriculture, **12**: 1-28.

Smith, J.J., Offord, L.C., Holderness, M. and Saddler, G.S. (1995). Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonous solanacearum*) race 3 in Kenya. Applied and Environmental Microbiology, **61**(12): 4263-4268.

Sneath, P.H., Bread, R.S., Murray, E.G. and Smith, R.N. (1986). Bergeys Manual of Determination Bacteriology. William and Wilkins Co., London, pp: 232.

Suslow, T.V., Schorth, M.N. and Isaka, M. (1982). Application of a rapid method for gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathol. **72**: 917-918.

van Bruggen, A.H.C. & Termorshuizen, A.J. (2003). Integrated approaches to root disease management in organic farming systems. Australasian Journal of Plant Pathology, **32**: 141–156.

Vinh, MT, Tung, T.T., Quang, H.X. (2005). Primary bacterial wilt study on tomato in vegetable areas of Ho Chi Minch city, Vietnam. **In:** Allen, C.,

Prior, P., Hayward, A. editors. Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society Press; St Paul, pp. 177–184.

Walker, T. and Collin, N. H. (1998). Priority setting at CIP for the 1998-2000. Medium Term Plan International Potato Centre, Lima, Peru.

Wang, J.F and Lin, C.H. (2005). Integrated Management of Bacterial Wilt of tomatoes. Asian Vegetable Research Centre Publication 05-615.

Wicker, E., Grassart, L., Coranson-Beaudu, R., Mian, D., Guilbaud, C. and Fegan, M. (2007). *Ralstonia solanacearum* strains from martinique (French West Indies) Exhibiting a new pathogenic potential. Appl. Environ. Microbiol., **71**: 6790-6801.

Yabuuchi, E., Kosako,, Y., Yano I., Hotta, H. and Nishiuchi Y. (1995). Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. nov: Proposal of *Ralstonia picketii* (Ralston, Palleroni and Doudoroff 1973) comb. nov, *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. **39**: 897–904

APPENDIX

Preparation of culture media

The compositions of the media used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved at 121^0C for 15 minutes at 15 lb pressure.

Triphenyl Tetrazolium Chloride (TTC)

2, 3, 5 triphenyl tetrazolium chloride (Soluble)	10.0 g
Distilled water	1000 ml

Gram's staining reagents

Gram's Crystal violet (Hucker's modification)

Solution A: Crystal violet (90% dye content)	2g
Ethyl alcohol	20.0 ml
Solution B: Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B in equal volume to prepare crystal violate solution.

Gram's Iodine (Gram's modification of Lugol's solution).

Iodine	1.0 g
Potassium iodide (KI)	2.0 g
Distilled water	300.0 ml

Add iodine after KI is dissolved in water to prepare Gram's Iodine solution.

Gram's alcohol (decolorizing agent).

Ethyl alcohol (95%) 98 ml

Acetone 2 ml

Safranin (counter stain)

Safranin (2.5% solution in 95% ethanol) 10 ml

Distilled water 100 ml

KOH solubility reagent

3% aqueous solution of KOH was prepared from the KOH granules.

Catalase reagent.

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.

Oxidase reagent

1% aqueous solution of NNN'N-tetramethyl-p-phenylene-diamine dihydrochloride was prepared from the absolute solution.

Levan reagent

Nutrient agar with 5% sucrose

Nutrient agar

Beef extract (Difco) 3.0 g

Peptone (Difco) 5.0 g

Bacto agar 15.0 g

Distilled water 1000 ml