PREVALENCE OF *Ralstonia solanacearum* AS LATENT INFECTION IN POTATO TUBERS AT SELECTED COLD STORAGES OF NARAYANGANJ DISTRICT

MOURY KABIR

DEPARTMENT OF PLANT PATHOLOGY

FACULTY OF AGRICULTURE

SHER-E-BANGLA AGRICULTURAL UNIVERSITY DHAKA-1207

December, 2018

PREVALENCE OF *Ralstonia solanacearum* AS LATENT INFECTION IN POTATO TUBERS AT SELECTED COLD STORAGES OF NARAYANGANJ DISTRICT

BY

MOURY KABIR

REGISTRATION NO.: 17-08236

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: JULY- DECEMBER, 2016

Approved by:

Dr. M. Salahuddin M. Chowdhury **Professor** **Department of Plant Pathology** **Sher-e-Bangla Agricultural University** **Supervisor**	**Dr. F. M. Aminuzzaman** **Professor** **Department of Plant Pathology** **Sher-e-Bangla Agricultural University** **Co-Supervisor**

Professor Dr. Khadija Akhter
Chairman
Examination Committee
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka

Department of Plant Pathology

Sher-e-Bangla Agricultural University

Sher-e -Bangla Nagar, Dhaka-1207

CERTIFICATE

This is to certify that the thesis entitled **"Prevalence of *Ralstonia solanacearum* as Latent Infection in Potato tubers at Selected Cold Storages of Narayanganj District"** submitted to the Department of Plant Pathology, Sher-e- Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in Plant Pathology**, embodies the result of a piece of bona fide research work carried out by **MOURY KABIR Registration No. 17-08236** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

Dated: 02.12.2019

Dhaka, Bangladesh

(Prof. Dr. M. Salahuddin M. Chowdhury)
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Dhaka-1207
Supervisor

DEDICATED TO
MY
BELOVED PARENTS,
TWO SISTERS &
BROTHER

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, sincere appreciation and heartfelt in debtedness to her 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, boundless gratitude and best regards to her respectable co-supervisor ***Dr. F. M. Aminuzzaman****, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for his valuable advice, constructive criticism and factual comments in upgrading the research work and the documents.*

The author would like to express her deepest sense of respect to Professor Dr. ***Dr. Khadija Akhter ,*** *chairman , Department of Plant Pathology, for his valuable suggestion during the entire study period.*

The author would like to express her 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.

The author is deeply indebted to her respectful father, mother, two sisters and beloved brother for their moral support, encouragement and unquantifiable love with cordial understanding. The author would like to expresses cordial thanks to her friends well wishers Ferdousn Jahan Urmi, Sadia Tisha, Rakibul Hasan Nitol, Md. Sourav, Sumona Rahman who helped her with their valuable suggestions and directions during the 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 her during the period of study.

PREVALENCE OF *Ralstonia solanacearum* AS LATENT INFECTION IN POTATO TUBERS AT SELECTED COLD STORAGES OF NARAYANGANJ DISTRICT

ABSTRACT

An experiment was conducted in the Molecular Phytobacteriology Laboratory of Department of Plant Pathology at Sher-e-Bangla Agricultural University, Dhaka during the period of May, 2018- October, 2018 to observe the prevalence of *Ralstonia solanacearum* in stored potato tuber collected from Narayanganj District of Bangladesh viz- Hazi Rahmatullah cold storage, Sahin cold storage and Ma cold storage. *Ralstonia solanacearum* was isolated from collected potato tubers and it's morphological, biochemical and cultural features were studied. On NA medium, circular, mucoid, convex, lucid coloured colonies of bacterium were formed. It produced highly fluidal, slightly raised and creamy white colonies with light pink or pinkish red centre and irregular margin after 48 hrs of incubation at 30^{o}C on TTC medium. The bacterium was gram negative, rod shaped and showed positive result in KOH solubility test, levan production test, catalase test, oxidase test and pectolytic test. Incidence of *Ralstonia solanacearum* is significantly influenced by temperature, relative humidity, ammonia supply and O_2 supply & CO_2 removal. Incidence varied significantly from one cold storage to another cold storage with increase of temperature. Maxmium incidence was found in the month of October (26.21%) at temperature 4.44^{o}C, relative humidity 90%, ammonia supply 9 hours and O_2 supply & CO_2 removal 1hour at Ma cold storage. Lowest incidence was found 12.42% in May at temperature 1.67^{o}C, relative humidity 86%, ammonia supply 13 hours and O_2 supply & CO_2 removal 1hour at Sahin cold storage. By correlation regression analysis confirmed that Maximum incidence was occurred at highest temperature (4.44^{o}C).

LIST OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF SYMBOLS AND ABBREVIATIONS

No.	=	Number
%	=	Percentage
et al.	=	And others
^{o}C	=	Degree Celsius
^{o}F	=	Degree Fehrenheit
@	=	At the rate
etc.	=	Etcetra
J.	=	Journal
Viz.	=	Namely
&	=	And
Kg	=	Kilogram
G	=	Gram
ml	=	Milliliter
hr	=	Hour (s)
i.e.	=	That is
Tm	=	Temperature
RH	=	Relative Humidity
NH_3	=	Ammonia
O_2	=	Oxygen
CO_2	=	Carbon-Di-Oxide

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

SAU	=	Sher-e-Bangla Agricultural University
BBS	=	Bangladesh Bureau of Statistics
USA	=	United States of America
NA	=	Nutrient Agar (media)
CPG	=	Casamino Peptone Glucose
TTC	=	TriphenylTetrazolium Chloride
ANOVA	=	Analysis of Variances
LSD	=	Least Significant Difference
CV%	=	Percentages of Co-efficient of Variance

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the main root and tuber crop and the third most important food crop in the world after rice and wheat. It is grown in over 125 countries and is consumed by over a billion people (CIP, 2008). Nutritionally, the tuber is rich in carbohydrates or starch and is a good source of protein, vitamin C and B, potassium, phosphorus, and iron (Ensminger *et al*., 1983). The world total production of potato was 388,191,000 tons where China ranks first while Bangladesh ranks position (FAO, 2019). The area of potato production is still in increasing from 4.44000 to 4.6200 hectares in Bangladesh (FAO, 2019). However, the yield of potato per unit area is quite low in the country as compared to the major potato growing countries like- Ireland and India (FAO, 2019).

Potato production and export is being hampered due to many diseases. In Bangladesh twelve diseases attack in potato of which brown rot is caused by *Ralstonia solanacearum* is very destractive (DAE, 2015). The pathogen is an aerobic, non-sporing, gram-negative plant pathogenic bacterium. It is soil-borne and motile with a polar flagellar tuft. It colonizes the xylem, causing bacterial wilt in a very wide range of potential host plants (Terblanche and Villiers, 2013; Agrios, 2008; Paret *et al*., 2008 and Andersona and Gardner, 1999). It is a vascular disease which is fatal in infected plant and has been ranked as one of the most important bacterial plant pathogens identified to date, commonly known as bacterial wilt (in case of infected plant) and brown rot (in case of infected tubers (Prior *et al*., 1998). Although there are reports of the pathogen being able to survive at relatively low temperatures for long periods of time (Milling *et al*., 2009 and Van *et al*., 2000), most of the *R. solanacearum* strains identified to date are

non pathogenic at below 20°C (Ciampi and Sequeira, 1980). Seed borne wilt or latent infection in potato has often been resulted in severe outbreaks of bacterial wilt (French, 1994).

Yield losses of potato due to bacterial wilt disease are estimated to be as high as 75 (%) in seed potato and 50 (%) in ware house potato (Ajanga, 1993). Potato export from Bangladesh to Russia was halted due to the presence of this bacteria in stored potato, which caused a loss about $9million in 2014 (EPB, 2015).

Yield losses due to the disease varied from 33 to 90% in the potato in different potato growing areas of the world (Elphinstone, 2005). The total value of Egyptian potato exports fell from a peak of USD 102.12 million in 1995 to USD 7.7 million in 2000 mainly due to brown rot quarantine, imposed by the European Union (EU) (Kabeil *et al.,* 2008).

Reports from Bangladesh quote some regions as having more than 30% of potato crops affected by *R. solanacearum* with over 14% reduction in yield (Elphinstone, 2005). However potato export from Bangladesh to Russia was halted due to the presence of this bacteria in stored potato, which caused a loss about $9million in 2014 (EPB,2015).The trend to increase production of potato in Bangladesh is retarded mainly by post-harvest problems among which storage is an important one. A report indicated that 0.187 million tons of potatoes were lost in Bangladesh due to post harvest diseases (Anonymous, 2006). Potatoes which are ultimately used for the seeding purposes are stored at lower temperature at about 4°C and those potatoes which are used for the processing purpose are stored at 8°C (Van Elsas *et al.* 2000). A few studies on economic aspect of cold storage have been conducted so far in Bangladesh. In a preliminary survey of the diseases of potatoes in cold storage in Bangladesh it was found

that 2-9 percent of cold stored potatoes were lost in every year due to disease (Fakir, 2009). An amount of Taka eight crores approximately was lost annually due to storage disease. (Khalil *et al*., 2013).Temperature, humidity, carbon dioxide and air movement are the most important factors during storage (Maldegem, 1999).The optimum range of temperature and relative humidity of the air inside cold stores are 2-4^{o}Cand 85-90% respectively for long term storage (Van't Oostar, 1999).

Chakraborty and Roy (2016) found in their study *R. solanacearum* incidence was 9.07% in Jamalpur, 19.98% in Nilphamari and 22.65% in Munshigonj in Bangladesh. So, the potato growers and businessmen of Bangladesh had experienced much problems on the disease especially in case of export potato to other countries like- Malaysia, Indonesia, Sri Lanka, Thailand, Hong Kong,Vietnam, Maldives etc. Losses have been reported to be serious due to ability of the seed tuber to harbor latent infection. This type of infection is considered the main source of disease outbreaks, ensuring carryover of the disease into subsequent growing seasons and new regions (Ajanga, 1993).Considering the above facts, the present research programme was undertaken with following objectives:

1. To find out the prevalence of *Ralstonia solanacearum* as latent infection of potato collected from selected cold storages in Narayanganj district of Bangladesh .
2. To isolate, identify and characterize *Ralstonia solanacearum* from potato tuber.
3. To ascertain the effect of storage internal condition that induce the diseases and to determine the disease incidence and severity in storage.

CHAPTER II
REVIEW OF LITERATUR

2.1. Prevalence of *Ralstonia solanacearum* of cold storages potato tubers

EPB (2015) reported that potato export from Bangladesh to Russia was halted due the presence of *Ralstonia solanacearum* bacteria in stored potato which caused a loss about 9 million doller in 2014.

Dale (2014) observed that in the United Kingdom, approximately half of the total harvested tubers are stored for up to 11 months.

Kohli (2009) stated that storage facilities need to be carefully designed to keep the potatoes alive and slow the natural process of decomposition, which involves the breakdown of starch. It is crucial that the storage area is dark, well ventilated and for long-term storage maintained at temperatures near 4°C (39°F). For short-term storage before cooking, temperatures of about 7 to 10°C (45 to 50°F) are preferred.

Elphinstone (2007) reported the higher incidence of bacterial wilt disease in all seed storage methods unlike certified seed could be attributed to latent infections in tubers before storage which led to pathogen accumulation during storage and faster spread in the field.

Sagar and Bakade (2007) observed that the pathogen can cause damage at two stages, the first being killing the standing plants by causing wilt and by causing rot of infected tubers in storage, and another indirect loss is spread of the disease through planting of healthy looking tubers harvested from infested fields.

Elphinstone (2005) reported that brown rot was mainly tuber-borne as it could latently infect tubers and survived in seed tubers during storage, causing disease when planted in the next season. The bacterium was spread on machinery and in irrigation water. The disease was persisting in fields where infected ground keepers were present.

Kleinkopf and Olsen (2003) found that, potatoes are usually cured after harvest to improve skin-set. Curing allows the skin to fully set and any wounds to heal. Wound-healing prevents infection and water-loss from the tubers during storage. Curing is normally done at relatively warm temperatures 50 to 60°C (122 to 140°F) with high humidity and good gas-exchange if at all possible.

Ajanga (1993) experimented that yield losses due to bacterial wilt disease are estimated to be as high as 75 % in seed potato and 50 % in ware house potato. Such losses have been reported to be serious due to ability of the seed tuber to harbour latent infection.

Janse (1988) reported that the most effective means of spread of brown rot worldwide was through distribution and planting of infected seed potatoes. In some case the bacteria could latently infect tubers without causing noticeable symptoms and could survive in seed tubers during storage and caused disease when planted in the next season. Symptom expression was occurred at different rates in different varieties and was favored by warm temperatures (above 15^{o}C with optimum around 25^{o}C) and other environmental conditions (especially high soil moisture).

Martin and French (1985) stated that infected seed potato tubers are the most common source of inoculums especially latent infection by *Ralstonia solanacearum*.

2.2. Isolation and identification of the *Ralstonia solanacearum* and it's Pathogenicity study

Rahman *et al*. (2010) reported that virulent isolates produce pink or light red colour colonies or colonies with characteristic red centre and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation.

Champoiseau (2008) observed that *Ralstonia solanacearum* developed two types of colonies on tetrazolium chloride (TZC) medium on which virulent colonies appear as white with pink centers and non-virulent colonies appear as small off-white colonies. On this medium, typical bacterial colonies appear fluidal, irregular in shape, and white with pink centers after 2 to 5 days incubation at 28ºC.

Hossain *et al.* (2008) described several biochemical tests viz. Gram staining reaction, Potassium hydroxide solubility test, Kovac's oxidase test, Levan test and Sugar fermentation test were performed for confirmation of *R. solanacearum* isolates.

Janse (2006) found that small, red colored cells (Gram stain) of *Ralstonia solanacearum* in a spiral vessel (smallest element in vascular tissue) of brown rot affected potato tuber.

Priou *et al.* (2006) found that ELISA test is able to detect very low concentration of *Ralstonia* as far as 108 bacteria/ml if enriched.

Mulder and Trukensteen (2005) observed that symptoms in the tuber were very specific: brownish-grey areas were seen on the outside, especially near the point of attachment of

the stolon. Cut tubers showed pockets of white to brown pus or browning of the vascular tissue which exuded dirty white globules of bacteria. As the disease progresses bubbly globules of bacteria were exuded through the eyes; soil were adhered to the exuded bacteria, hence the name 'sore eyes' or 'jammy eyes'.

Denny and Hayward (2001) reported that *Ralstonia* was positive in H_2S production, starch hydrolysis, KOH solubility, gelatin liquefaction, sucrose utilization, indole production, Levan Test and Kovac's Oxidase Test and was able to utilize Dextrose, Maltose, Lactose, Sorbitol, Manitol, Dulsitol.

Hayward (2001) discovered *Ralstonia solanacearum* is a Gram-negative, rod-shaped, strictly aerobic bacterium that is 0.5-0.7 × 1.5-2.0 μm in size. For most strains, the optimal growth temperature is between 28 and 32°C.

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

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.

Janse *et al*. (1998) observed that symptoms were most obvious in the tuber; initially a brown staining of the vascular ring (hence brown rot) started at the stolon end, with

further disease progression the vascular tissue were rot away completely and a pale colored sticky ooze was appeared at the eyes lenticels and/or stolon end of the tuber.

Kucharek (1998) observed that under hot humid condition disease complete wilting occurs and the plant dies. The brown discoloration was shown in the lower stem of vascular tissues.

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

Kelman (1997) 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.

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.

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

Yabuuchi *et al.* (1995) discovered that it is a gram negative, aerobic, motile and rod shaped bacterium belonging to genus *Rasltonia*.

Engelbrecht (1994) developed 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.

Seal *et al.* (1993); Seal and Elphinstone, (1994); Anon, (1997) advised that biochemical tests, fatty acid analysis, RFLP and protein analysis can be used for the identification of *Ralstonia solanacearum*.

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.

Hayward (1991) found that *Ralstonia solanacearum* as aerobic and its colonies on solid media were small, irregularly round, white in reflected light and tan in transmitted light.

Hildebrand *et al*. (1988) found that a single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center was selected and multiplied ina TTC (without adding TZC) medium.

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

Schaad (1980) and Suslow *et al*. (1982) described that isolates were studied according to specific biochemical tests for *R. solanacearun* i.e., gram staining, potassium hydroxide test, catalase test and kovacs oxidase test.

Cuppels *et al.* (1978) stated 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 and Sequeira (1965) conducted an experiment that a loop full culture was taken from the bottom of the beakers and streaked on to paradises which contained sterilized tetrazolium chloride (TZC) agar medium.

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*.

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

Kelman (1953) observed that after 48-72 h of incubation at 28^{o}C *Ralstonia* gave circular, smooth, convex and viscous bacterial colonies with pink center and whitish margin on 2,3,5 Triphinyl Tetrazolium Chloride (TTC) medium. On NA medium the bacteria produce watery whitish or off white or cream color irregular colonies.

2.3. Effect of storage condition on prevalence of *R. solanacearum*

Chakraborty and Roy (2016) carried out a survey in some selected potato growing districts in Bangladesh to know the status of bacterial wilt disease caused by *Ralstonia solanacearum* in terms of its incidence and severity. The results showed that the highest wilt incidence was recorded in Munshigonj (22.65%); followed by Nilphamari (19.98%) and the lowest incidence was recorded in Jamalpur (9.07%). The highest bacterial wilt severity was recorded in Munshigonj (3.80), while the lowest wilt severity was recorded in Jamalpur (2.90).

Zgorska and Grudzinska (2012), Grudzinska *et al*.(2016) estimated that losses of potato tubers due to improper storage conditions can reach even up to 40% of tuber weight, while losses caused by periderm injuries (resulting in excessive transpiration and respiration) can account for further 10% loss.

Hirpa (2010) described that storage methods had a significant effect on incidence of *Ralstonia solanacearum* in potato tubers. Seed tubers stored under diffused light and in jute bags resulted in the highest infection levels with *Ralstonia solanacearum* in potato tubers while the certified seed tubers resulted in the least bacterial wilt incidence caused by *Ralstonia solanacearum*.

Nourian *et al*. (2003) stated that appropriate storage environment should maintain potato tubers in their edible and marketable conditions by preventing large moisture losses, spoilage by pathogens, quality deterioration and sprout growth.

Western Potato Council (2003) observed that, potato should be stored at (3-5^{o}C) with 95% RH. The condition of the potato piles should be checked periodically to ensure temperature and relative humidity are maintained. This is important to minimize disease development. Access to the storage should be restricted to reduce potential for introducing diseases.

Chourasia and Goswami (2001) pointed out that low-temperature storage can cause freezing injuries which cause potato tubers to become soft and unusable. However, high storage temperature produces greater quality loss and increases respiration activity and thus lowered shelf life.

Van't Oostar (1999) stated the optimum range of temperature and relative humidity of the air inside cold stores are 2-4 ^{0}C and 85-90% respectively for long term storage.

French (1994) described that disease development occurred at different rates in different varieties of potato tubers, but was favored by warm temperatures (above 15^{0}C with optimum of 27^{0}C) and high soil moisture levels. Brown rot was primarily spread by the planting of infected seed potatoes, but can also spread in soil and in irrigation water.

Shekhawat *et al*. (1992) stated temperature has an influence on the symptom expression of the brown rot disease. In general it may be accepted that symptoms are more intensely

expressed with an increased temperature. At lower temperatures a larger extent of latent infection occurs, whilst plants and tubers show no visual symptoms.

Janse (1988) published that methods used to detect *R. solanacearum* in latently infected potato tubers were based on the recommended quarantine procedure.

Harbenburg *et al*. (1986) and Maldegem (1999) stated that temperature, humidity, carbon dioxide and air movement are the most important factors during storage of potato tubers.

Ciampi *et al.* (1980) and Ciampi-Panno *et al*. (1981) reported that storage at 4°C did not eliminate the pathogen from inoculated tubers of potato even after 40 days.

Ciampi *et al.* (1980) found that the bacterial population declined drastically after one day in potato tubers stored at 4°C, (compared to those stored at higher temperatures) and reached a level of only a few or no viable cells after three weeks.

Nielsen (1963) found that *R. solanacearum* survives in potato tubers and in culture at low temperature

CHAPTER III

MATERIALS AND METHODS

This chapter described the methodologies that followed in conducting research work under the following experiment:

I: Isolation and identification of *Ralstonia solanacearum* and

II: Survey on prevalence of latent infection of potato tubers by *Ralstonia solanacearum.*

3.1. Experiment I: Isolation and identification of *Ralstonia solanacearum*

3.1.1. Sample collection

Potato tuber samples were collected from three different cold storage of Narayanganj districts viz- Hazi Rahamatullah cold storage, Sahin cold storage and Ma cold storage. In total ninety potato tubers were randomly collected from three cold storages. Twenty medium sized stored potatoes were selected randomly for working sample from each cold storage.

3.1.2. Duration of Experiment

The experiment was conducted from May, 2018 to October, 2018.

3.1.3. Experimental Site

The experiment was carried out in the Molecular Phytobacteriology Laboratory of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3.1.4. Observation of the symptoms

Visual observation of the samples were carried out as per standard procedure following Champoiseau *et al.* (2009). Samples were visually observed for the symptoms of brown rot by cutting the tuber at its stem end by sharp sterile knife. Finally, Identification of the pathogen was confirmed through isolation and different biochemical tests.

3.1.5. Preparation of Nutrient Agar (NA)

Ingredient	Amount
Nutrient Agar	28g
Agar	15g
Water	1000ml

Nutrient agar media was prepared according to the method given by Schaad (2001). At first Nutrient Agar (28g) and Agar (15g) was taken in an Erlenmeyer flask which contained 1000ml distilled water and shaken to mix them well for few minutes. Then the mouth of the flask was covered with aluminum foil paper and tight with a thinrope. It was then autoclaved at 121^0C at 15 Ibs pressure for 15 mins. Then the media was taken to laminar air flow to cool down. When the media become near about 40-50^0C, then it was poured on sterilized glass petridish for making NA plates. The plates were kept for futher use in refrigeator.

3.1.6. Isolation of causal organism on NA media

Isolation was done by dilution plate method. The diseased potatoes were washed under running tap water. And then was cut into small pieces. Surface sterilize were done by dipping them in 5% sodium hypochlorite solution for 2-3 minutes. Then it was washed three times with sterile water. After surface sterilization the 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. With the help of sterile pipette 1ml of this stock solution was transferred 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}. Then 0.1 ml of each dilution was spread over NA plate at three replications as described by Goszczynska and Serfontein (1998). Glass-rod was used for spreading of bacterial solution. The inoculated NA plates were kept in incubation chamber at 30^{o}C. The plates were observed after 24 hrs and 48 hrs. Then single colony grown over NA plate was restreaked on another plate with the help of a loop to get pure colony.

3.1.7. Preparation of Triphenyl Tetrazolium Chloride (TTC)

At first 1% aqueous solution of 2, 3, 5- triphenyl tetrazolium chloride (TTC) was prepared in an Erlenmeyer flask by dissolving 1g of the chemical in 100mlof distilled water. Then it was shaken thoroughly for few minutes. Then 1% stock solution of TTC medium was separately sterilized by passage through 0.45µm pore size filters (Millipore). It was then autoclaved at121^{o}C under 15 PSI pressure for 15 minutes. The TTC was kept in a colored bottle and was wrapped with aluminum foil to avoid light and preserved in a refrigerator at 4°C for future use (Schaad, 1988).

3.1.8. Preparation of Triphenyl Tetrazolium Chloride (TTC) media

The following ingredients and the proportion were used to prepare the TTC media:-

Ingredient	Amount
Peptone	10g
Casamino acid	1g
Glucose	5g
Agar	17g
Water	1000ml

The mixture is taken in an erlenmeyer flask and was then autoclaved for 20 minutes at 121℃ under 15 PSI pressure to make CPG media. Then media was then taken to laminar air flow. The sterilized TTC solution was poured into the sterilized CPG medium before solidification which was prepared previously by dissolving 1g 2,3,5 triphenyl tetrazolium chloride powder in 100ml distilled water and it was mixed thoroughly. For solidification, the CPG media with TTC was poured in to several petridish. TTC media was prepared by method of Schaad (1988).

3.1.9. Preservation of bacteria of potato

After purification of bacteria on NA plate, it was kept in refrigerator at 4^{o}C in C in small screw-cap test tubes on NA slant for future use.

3.1.10. Identification of the bacteria in the laboratory

Identification of the bacteria of potato tuber was done by cultural features, morphological features and biochemical tests of the pathogen as per standard microbiological procedures by Hayward (1991).

3.1.10.1. Cultural characters

Growth characteristics of the pathogen were studied by using selective media as per the standard procedure by Kelman and Person (1954).

3.1.10.1.a. Growth on nutrient agar (NA) media

Nutrient agar (NA) medium was poured into a sterile petridish and after cooling, pure colony of bacterium was streak inoculated on the plate with the help of a sterile transfer loop. Then it was incubated at 30^{o}C for at least 24 hours in incubation chamber and observed the colony characters (Kelman and Person, 1954).

3.1.10.1.b. Growth on 2,3,5 Triphenyl Tetrazolium Chloride (TTC) media

The bacteria from NA media were transferred to TTC (2,3,5 triphenyl tetrazolium chloride) media with the help of sterile loop through streaking method. Then it was kept in incubation chamber for 24 hours at 30^{o}C. Then it was observed for the presence of the colony (Kelman and Person, 1954).

3.1.10.2. 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) and Bradbury (1970).

3.1.10.2.a. Gram's staining

A small drop of sterile water was taken and placed on a clean microscope slide. Part of a young colony (18-24 hours old) was removed with a cold, sterile loop from the nutrient agar (NA) medium and the bacteria were smeared on to the slide that was very thin. The thinly spreaded bacterial film was air dried. Undesirable of the glass slide was heated by

passing it four times through the flame of a spirit lamp for fixing bacteria on it. Then the slide was flooded with crystal violet solution for 1 minute. Then It was rinsed under running tap water for a few seconds and excess water was removed by air. Again it was flooded with lugol's iodine solution for 1 minute. After that it was decolorized with 95% ethanol (Appendix I**)** for 30 seconds and again rinsed with running tap water and air dried. Then it was counterstained with 0.5% safranine for 10 seconds. Again it was rinsed under running tap water for a few seconds and excess water was removed by air. Then the glass slide was examined at40x and 100x magnification using oil immersion. The Gram-negative bacteria appeared red in color and Gram-positive bacteria appeared violet in color (Pastor *et al*., 2010).

3.1.10.3. Biochemical test

Different biochemical tests such as, pectolytic test, catalase test and oxidase test were studied as per the standard procedures described by Kelman (1954).

3.1.10.3.a. KOH solubility test

A drop of 3% KOH (aqueous) was placed on a glass slide. Part of a single colony (18-24 hours old) was removed from the NA plate using a cooled, sterile loop. Then it was mixed with KOH solution until an even suspension was obtained. The loop was raised a few centimeters from the glass slide and repeated strokes to have strands of viscid materials as described by Suslow *et al.* (1982).

3.1.10.3.b. Levan test

One loop-full bacterial culture was streak inoculated into NA plate containing 5% (w/v) sucrose with the help of a sterile transfer loop. Then it was incubated at 30^0C for 24 hours to observe whether levan is produced or not.

3.1.10.3.c. Catalase test

A few drops of freshly prepared 3% H_2O_2(Hydrogen peroxide) was added with 48 hours old pure culture of bacterium grown on NA plate and observed whether it produced bubbles within a few seconds or not.

3.1.10.3.d. Oxidase test

At first oxidase disk containing 1 ml 1% aqueous w/v solution of tetramethyl-p-phenylene-diamine-dihydrochloride solution was soaked in sterile water and placed on a petri dish. Then a part of a colony was removed with a sterile toothpick and smeared onto the moistened oxidase disk and observed up to 60 seconds whether it changed colour to dark purple or not (Kovacs, 1956).

3.1.10.3.e. Pectolytic test

Potato tubers were washed and peeled. Before testing one centimeter slices were made and the slices were dipped in alcohol. A slice was placed in a petri dish with sterile water to a depth of 3-4 mm. A nick in the center of potato was made with a sterile tool and inoculated with a loopful of fresh culture (24 hr). Then it was incubated at 27^{o}C for 24-48 hours. After 24 hours a loop was run across the potato slice. Examination was done for 5 days after inoculation.

3.2. Experiment II: Survey on the cold storages and prevalence of latent infection by *Ralstonia solanacearum* in potato

3.2.1. Survey on cold storages in Narayanganj Districts

Three selected cold storages of Narayanganj district viz- Hazi Rahamatullah cold storage, Sahin cold storage and Ma cold storage were surveyed in every month from

May, 2018 to October, 2018. Data on physical condition of cold storages viz- year of eastablishment, cold storage facility and storage capacity were recorded.

3.2.2. Observation Internal conditions of cold storages

The cold storages were equipped with machinery and mechanism to control temperature, relative humidity, ammonia supply and O_2 supply & CO_2 removal. Data on the stated parameters were collected from the record of cold storage. Data on the following parameters were recorded from the cold storage authority in every month such as-

- Temperature (^oC),
- Relative humidity (%),
- Ammonia supply (Hr),
- Oxygen supply and Carbon di-oxide removal (Hr)

3.2.3. Determination of Incidence of latent *Ralstonia solanacearum* with visible and invisible symptoms

One month old potato samples were selected for the study. The collected samples were then kept in a polythene bag which was further placed inside the gunny sack. Within a day, the samples were brought to plant pathology laboratory of Sher-e-Bangla Agricultural University. Collected potato samples were observed visually for tuber infection symptoms. Twenty medium sized one month stored potatoes from each sample were cut to observe the symptoms. Collected potatoes showed visible and invisible symptoms of brown rot disease of potato. The potatoes were cut at three locations viz. stem end portion, middle portion and seed end portion to observe the symptoms. On the basis of symptom development incidence were calculated.

Every potato with visible and invisible symptoms was counted in the laboratory for calculating the incidence of the pathogen and then it was expressed in percentage. Disease incidence of latent pathogen of potato was determined by the following formula (Rai and Mamatha, 2005).

$$\text{Percent potato tuber Infection} = \frac{\text{Number of infected potato tuber}}{\text{Number of total potato tuber observed}} \times 100$$

The following information were considered to calculate and estimate the loss of potato tubers in cold storage -

a) Number of room in each cold storages

b) Weight of potato tuber of potato in each bag

c) Types of symptoms

3.2.4. Statistical analysis

The data were statistically analyzed using computer package program of statistrix 10 sowftware. Treatment means were compared by LSD at 0.05 level of significance. The data obtained in the present investigation for various parameters were subjected to ANOVA for a completely randomized design for in vitro studies.

CHAPTER IV

RESULTS

Potatoes were collected from three different cold storage to identify *Ralstonia solanacearum* as latent infection of potato. Results were compiled based on disease incidence at different storage period.

4.1. Isolation and Identification of *Ralstonia solanacearum* in the Laboratory

4.1.1. Isolationof *Ralstonia solanacearum*

The causal organism was isolated from the infected tubers showing visible and invisible symptoms of bacterial wilt. Isolation was done by employing the dilution plate technique using nutrient agar medium. Repeated isolation from the infected tubers some yielded well separated, deep red coloured, largely unmixed mucoid magenta colonies of bacterium on TTC medium after 48hours of incubation at 30^{o}C(Plate. 1B). Well separated, typical, lucid, convex, mucoid colonies of bacterium on NA medium was yielded after 48hours of incubation at 30^{o}C (Plate.1A). Colonies were purified by restreaking the isolated colony on nutrient agar plate.

4.1.2. Identification of the pathogen

Cultural, morphological and biochemical characteristics of bacteria were observed through isolation of bacteria are discribed below:

4.1.2.1. Identification of *Ralstonia solanacearum* by cultural characters

Isolated bacteria culture were observed morphologically. Bacteria were grown on NA and TTC media. On NA medium the bacteria showed creamy white color after 24-48 hrs incubation at 30^{0}C in incubation chamber. Circular, mucoid, convex, lucid coloured

colonies were found on NA medium (Table 2 & Plate. 1A).To obtain pure culture, streaking was done again on NA media.

Table 1. Cultural characterization of *Ralstonia solanecearum* on NA plates

Cultural characteristics	Observation
Colony size	Moderate
Form	Circular
Pigmentation	Creamy white
Elevation	Convex

On TTC medium colonies of *Ralstonia solanacearum* appeared as largely unmixed, mucoid, magenta red coloured colonies (Plate.1C) after 24-48hrs incubation at 30^0C.

4.1.2.2. Identification of *Ralstonia solanacearum* by Morphological characters

Under the compound microscope, the bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red colour) and capsulated. The cells were readily stained with common stains such as crystal violet (Plate.2A).

4.1.2.3. Identification of *Ralstonia solanacearum* by Biochemical characters

In KOH solubility test, a mucoid thread was lifted with the loop. Therefore the test was positive (Plate.2B). The bacterium was gram negative that supports the result of gram's staining test.

In catalase test, the colony of the bacterium formed bubbles within a few seconds after adding 3% H_2O_2, which revealed that the test was positive (Plate. 2C).

In oxidase test, tetra methyl- p- phenylene- diamine dihydrochloride produce purple color smeared develops within 10 seconds, which indicated positive reaction of oxidase test.

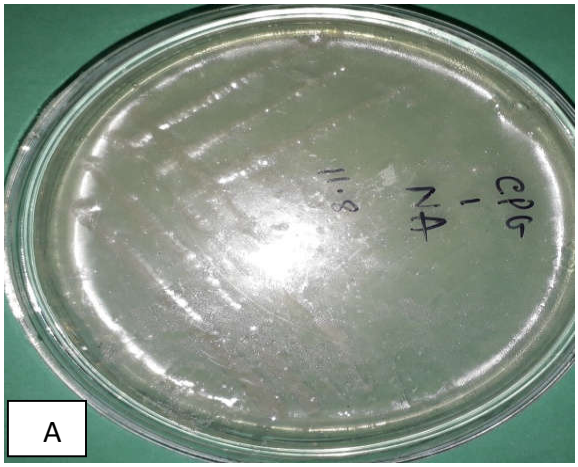
In levan test after incubated at 30^{o}c the bacteria produced levan thus the bacterium showed positive result.

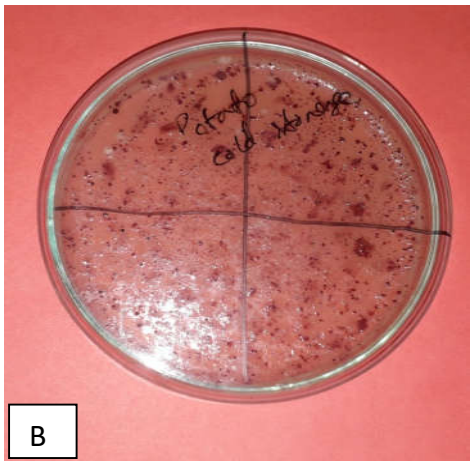
In pecteolytic test, the bacteria showed positive result. After incubation for 48 hours the bacterium was able to rot the potato.

Table 2. Biochemical characteristics of isolated *R. solanecearum* by different tests

Name of tests	Reaction
Gram staining	Negative
KOH solubility test	Positive
Catalase test	Positive
Oxidase test	Positive
Levan test	Positive
Pectolytic test	Positive

Based on the colony morphology, biochemical and morphological test, the pathogen was identified as *Ralstonia solanecearum.*





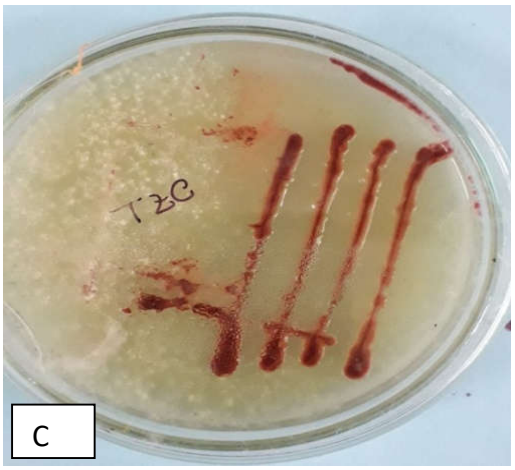
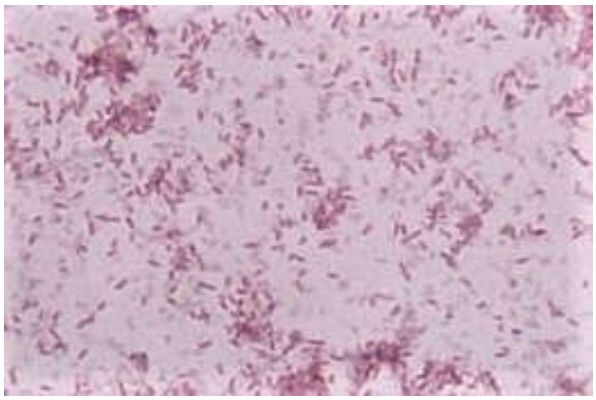
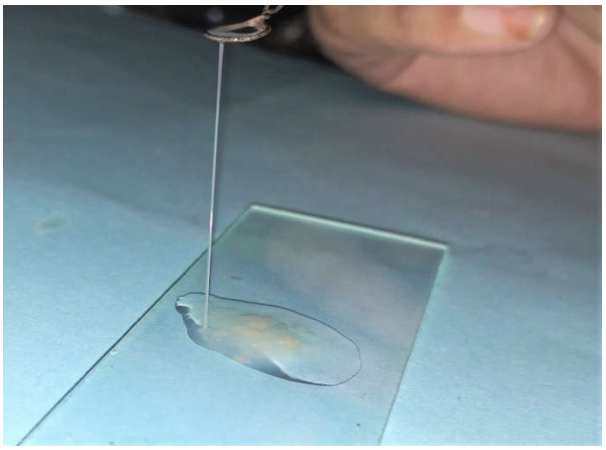


Plate 1: Cultural Characteristics of *Ralstonia solanacearum*

A. Streaking on NA media
B. Colony on TTC media
C. Streaking on TTC media



A. Gram staining Test



B. KOH Solubility test

C. Catalase test

Plate 2: Biochemical characteristics *R. solanecearum*

4.2. Survey on three cold storages internal condition

Physical condition of three cold storages viz- Hazi Rahamattullah cold storage, Sahin cold storage and Ma cold storage at Narayanganj district were surveyed from May, 2018 to October, 2018 and result described below:

At first, survey was done at Hazi Rahamattullah cold storage. Hazi Rahamattullah cold storage was established in 1968. The storage capacity of potato was 50000 ton where hundred percent place is occupied by potato from March to November (Plate 3).

Secondly, survey was done at Sahin cold storage. It was established in 1985. Storage capacity of potato was 30000 ton which is fully occupied in season by potato (Plate 4A).

Final survey was done at Ma cold storage. Ma cold storage was established in 1992. Storage capacity was 40000 ton where hundred percent placed was occupied by potato (Plate 4B). Every cold storage has some characteristics of industries such as fixed establishment, machinery, workers, administration, input (potato).

Cold storages internal conditions such as- temperature, relative humidity, ammonia supply and oxygen supply& carbon di-oxide removal were recorded in every month that shown in table (1 to 4). There is no significant variation in internal temperature, relative humidity, ammonia supply and oxygen & carbon di-oxide supply in three cold storages.

4.2.1. Temperature recorded at different cold storages of Narayanganj district from May, 2018 - October, 2018

Internal temperature of three cold storages of Narayanganj was recorded in every month from May to October. Temperatures were fluctuated in each month at three cold storages viz- Hazi Rahamattullah Cold Storage, Sahin cold storage and Ma cold storage. Internal temperature of three cold storages was ranged from 1.67^{o}C - 4.44^{o}C. Highest temperature (4.44^{o}C) was found in the month of October at Hazi Rahamattullah cold storage, Sahin cold storage and Ma cold storage respectively. Lowest temperature (1.67^{o}C) was

recorded in the month of May at Hazi Rahamattullah cold storage and Ma cold storage (Table 3).

Table 3. Temperature (^{o}C) recorded at different cold Storage from May-October, 2018

Name of Month	Temperature (^{o}C)		
	Hazi Rahamattullah cold storage	Sahin cold storage	Ma cold storage
May, 2018	1.67	2.22	1.67
June, 2018	2.22	2.22	2.78
July, 2018	2.78	2.78	2.78
August, 2018	3.33	2.78	3.33
September, 2018	4.44	3.33	3.33
October, 2018	4.44	4.44	4.44

4.2.2. Relative Humidity recorded at different cold storages of Narayanganj district from May, 2018 - October, 2018

Relative humidity of three cold storages of Narayanganj district was different from one another. Relative humidity was ranged from 85-90%.Highest relative humidity was recorded at 90% in the month of June, July, September and October at Hazi Rahamattullah cold storage, Sahin cold storage and Ma cold storage respectively. Lowest relative humidity was found 85% in the month of May at Hazi Rahamattullah cold storage and Ma cold storage respectively (Table 4).

Table 4. Relative Humidity recorded (%) at different cold storages from May-October, 2018

Name of Month	Relative Humidity (%)		
	Hazi Rahamattullah cold storage	Sahin cold storage	Ma cold storage
May, 2018	85	86	85
June, 2018	90	90	88
July, 2018	88	88	90
August, 2018	88	90	88
September,2018	90	88	90
October, 2018	90	90	90

4.2.3. Ammonia Supply recorded at different cold storages of Narayanganj district from May, 2018 - October, 2018

Supply of ammonia at three cold storages was not significantly different from one cold storage to another. Supply of Amonia was ranged from 9 to 13 hour. Highest supply of Ammonia was found 13 hour in May, June, July at three cold storages respectively. Therefore lowest supply of ammonia was recorded in October at Sahin cold storage and Ma cold storage (Table 5).

Table 5. Ammonia Supply (hr) recorded at different cold storages from May-October, 2018

Name of Month	Ammonia Supply (hr)		
	Hazi Rohamattullah cold storage	Sahin cold storage	Ma cold storage
May, 2018	13	13	13
June, 2018	13	12	13
July, 2018	12	12	13
August, 2018	12	10	12
September, 2018	10	10	10
October, 2018	10	9	9

4.2.4. O_2 Supply and CO_2 Removal recorded at different cold storages of Narayanganj district from May, 2018 - October, 2018

Supply of O_2 and CO_2 removal of three cold storages was ranged from 0.8 to 1hour. Highest supply (1hour) was recorded indifferent month of May to October at Hazi Rahamattullah cold storage, Sahin cold storage and Ma cold storage respectively. Lowest supply of O_2 supply & CO_2 removal (0.8 hour) was found in July and October month at Ma cold storage and Hazi Rahamattullah cold storage respectively (Table 6).

Table 6. O_2 Supply and CO_2 Removal (hr) recorded at different cold storages from May- October, 2018

Name of Month	O_2 Supply and CO_2 Removal (hr)		
	Hazi Rahamattullah cold storage	Sahin cold storage	Ma cold storage
May, 2018	1	1	1
June, 2018	1	1	1
July, 2018	1	1	0.8
August, 2018	0.9	1	0.9
September, 2018	1	0.9	1
October, 2018	0.8	1	1

Front Side

Office Room

Sorting and Drying Area

Storage Room

Plate 3: Pictorial view Hazi Rahmattullah cold storage

Storage Room

Sorting Area

Drying Area

A. Sahin cold storage

Sorting Area

Storage Room

B. Ma cold storage

Plate 4: Pictorial view of Sahin and Ma cold Storage

4.2.5. Incidence of *Ralstonia solanacearum* at three cold storages of Narayanganj District from May to October, 2018

The survey results that latent infection of *Ralstonia solanacearum* pathogen was found in potato tuber collected from three cold storages of Narayanganj districts in different months of storage period (Plate 5).

Result showed that incidence of *Ralstonia solanacearum* varied from cold storages to cold storages and ranged from 12.42% to 26.21 % at different storage period. In the month of May, incidence was found 14.42%, 12.42% and 13.66 % at Hazi Rahmatullah cold storage, Sahin cold storage and Ma cold storage respectively. In June, incidence was recorded 17.43%, 16.41%amd 16.44 % at Hazi Rahmatullah cold storage, Sahin cold storage and Ma cold storage respectively. For the month of July, incidence was found 19.57%, 18.17% and 18.47% at Hazi Rahmatullah cold storage, Sahin cold storage and Ma cold storage respectively. Incidence was found 21.00%, 20.92% and 21.02% at Hazi Rahmatullah cold storage, Sahin cold storage and Ma cold storage respectively in the month of August. In next month September, incidence was found 22.66%, 23.08% and 23.75% at Hazi Rahmatullah cold storage, Sahin cold storage and Ma cold storage. Finally, in the month of October, incidence was found 24.63%, 25.64% and 26.21 % at Hazi Rahmatullah Cold Storage, Sahin cold storage and Ma cold storage respectively. Among all six months, maximum incidence was found (26.21%) in the potato sample collected from Sahin cold storage in the month of October and minimum incidence was found (12.42%) at Sahin cold storage. Maximum incidence was found in October and minimum disease incidence found in May.

Table 7: Incidence *Ralstonia solanacearum* at three cold storages in Narayongonj Districts from May to October, 2018

Name of Month	Latent infcetion (%)		
	Hazi Rahmatullah cold storage	Sahin cold storage	Ma cold storage
May, 2018	14.42 e	12.42 f	13.66 f
June, 2018	17.43 d	16.41 e	16.44 e
July, 2018	19.57 c	18.17 d	18.47 d
August, 2018	21.00 bc	20.92 c	21.02 c
September, 2018	22.66 ab	23.08 b	23.75 b
October, 2018	24.63 a	25.64 a	26.21 a
CV (%)	5.75	4.01	4.50
$LSD_{0.05}$	0.94	0.63	0.73

Each data represents the mean value. Values followed by the different letter within a column are significantly different ($p \leq 0.05$) according to Least Significant Difference test.

A.Visible Symptom

B. Apparently healthy but latent infection found

C. Latent infection found

Plate 5: Collected potato samples from three cold storages

4.2.6. Effect of cold storage internal condition on Incidence of *Ralstonia solanacearum* pathogen at Hazi Rahmatullah cold storage in Narayanganj, 2018

Effect of different internal condition viz- temperature, relative humidity, ammonia supply and O_2 supply & CO_2 removal on incidence of *Ralstonia solanacearum* pathogen at Hazi Rahmatullah cold storage in Narayanganj were observed. Variation of the incidence of *Ralstonia solanacearum* due to variation of temperature, relative humidity, ammonia supply and O_2 supply & CO_2 removal were observed in every six month from May to October, 2018 (Fig. 1). Incidences were recorded 14.42 %, 17.43%, 19.57%, 21.00%, 22.66% and 24.63% during the month of May, June, July, August, September and October respectively. Highest incidence was recorded in the month of October (24.63%) at temperature 4.44^{o}C, relative humidity 90%, ammonia supply 10 hours and O_2 supply & CO_2 removal 0.8 hour. On the other hand, lowest disease incidence (14.42%) was recorded in May at temperature 1.67^{o}C, relative humidity 85%, ammonia supply 13 hours and O_2 supply & CO_2 removal 1hour.

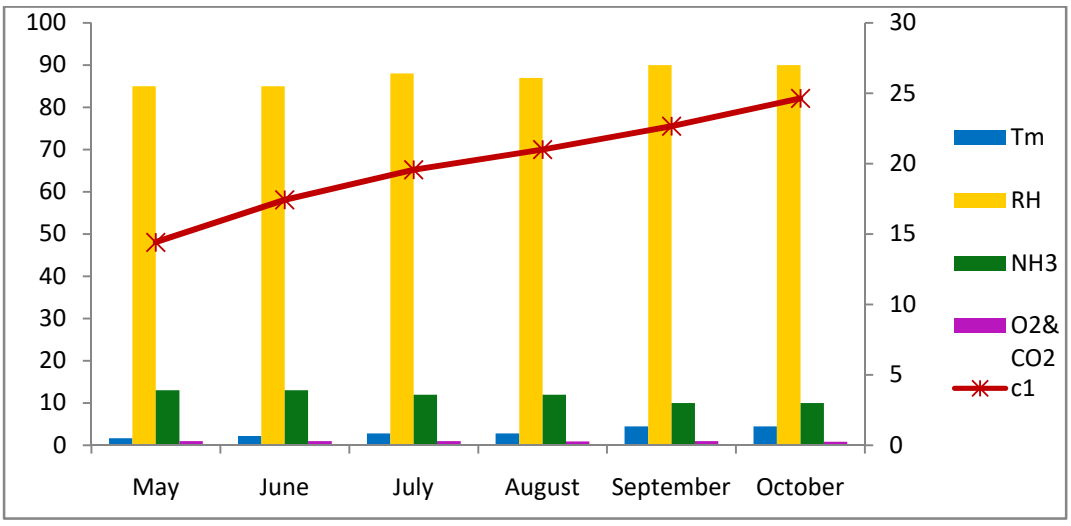


Figure 1: Disease Incidence of *Ralstonia solanacearum* at Hazi Rahmatullah cold storage

N.B. Tm =Temperature (^{o}C), RH= Relative Humidity (%), NH_3= Amonia Supply (hr), O_2 supply & CO_2 removal (hr), c1= Incidence of *R. solanacearum* (%)

4.2.7. Effect of cold storage internal condition on Incidence of *Ralstonia solanacearum* pathogen at Sahin Cold Storage in Narayanganj, 2018

Effect of different internal condition viz- temperature, relative humidity, ammonia supply and O_2 supply& CO_2 removal on incidence of *Ralstonia solanacearum* pathogen at Sahin cold storage in Narayanganj were observed. Variation of the incidence of *Ralstonia solanacearum* due to variation of temperature, relative humidity, ammonia supply and O_2 supply & CO_2 removal were observed in every six month from May to October, 2018 (Fig. 2). Incidences were recorded 12.42%, 16.41%, 18.17%, 20.92%, 23.08% and 25.64% during the month of May, June, July, August, September and October respectively. Highest incidence was recorded during the month of October 2018 (25.64%) at temperature 3.33^{o}C, relative humidity 90%, ammonia supply 10 hours and O_2 supply & CO_2 removal 0.8 hour. On the other hand, lowest disease incidence (12.42%) was recorded in May at temperature 2.22^{o}C, relative humidity 85%, ammonia supply 13 hours and O_2 supply & CO_2 removal 1hour.

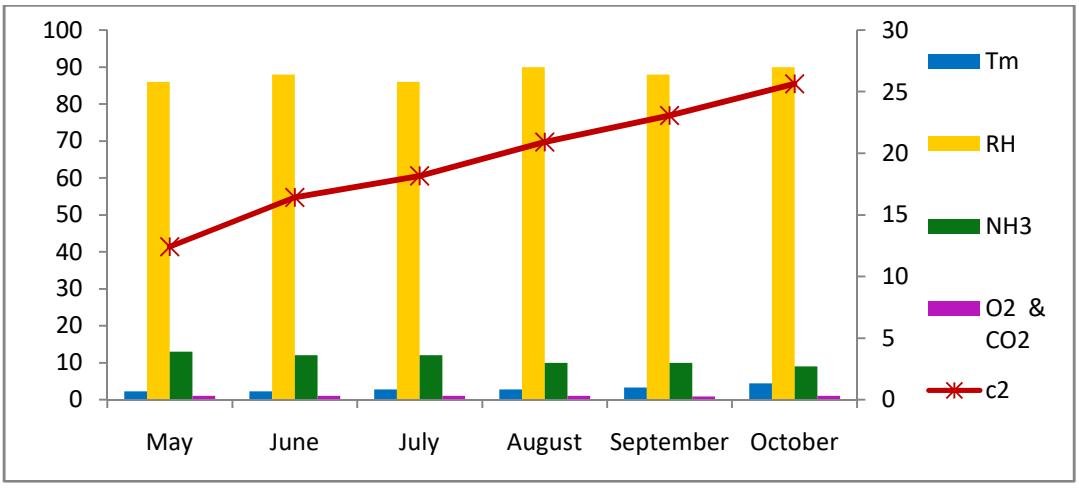


Figure 2: Disease Incidence of *Ralstonia solanacearum* at Sahin cold storage

N.B. Tm =Temperature (^{o}C), RH= Relative Humidity (%), NH_3= Amonia Supply (hr), O_2 supply & CO_2 removal(hr), c2 = Incidence of *R. solanacearum* (%)

4.2.8. Effect of cold storage internal condition on Incidence of *Ralstonia solanacearum* pathogen at Ma Cold Storage in Narayanganj, 2018

Effect of different internal condition viz- temperature, relative humidity, ammonia supply and O_2 supply& CO_2 removal on incidence of *Ralstonia solanacearum* pathogen at Ma cold storage in Narayanganj were observed. Variation of the incidence of *Ralstonia solanacearum* due to variation of temperature, relative humidity, ammonia supply and O_2 supply & CO_2 removal were observed in every six month from May to October, 2018 (Fig. 3).Then incidences were recorded 13.66%, 16.44%, 18.47%, 21.02%, 23.75% and 26.21% during the month of May, June, July, August, September and October respectively. Highest incidence was recorded during the month of October 2018 (26.21%) at temperature 4.44^{o}C, relative humidity 90%, ammonia supply 10 hours and O_2 supply & CO_2 removal 0.8 hour. On the other hand, lowest incidence (13.66%) was recorded in May at temperature 1.67^{o}C, relative humidity 85%, ammonia supply 13 hours and O_2 supply & CO_2 removal 1hour.

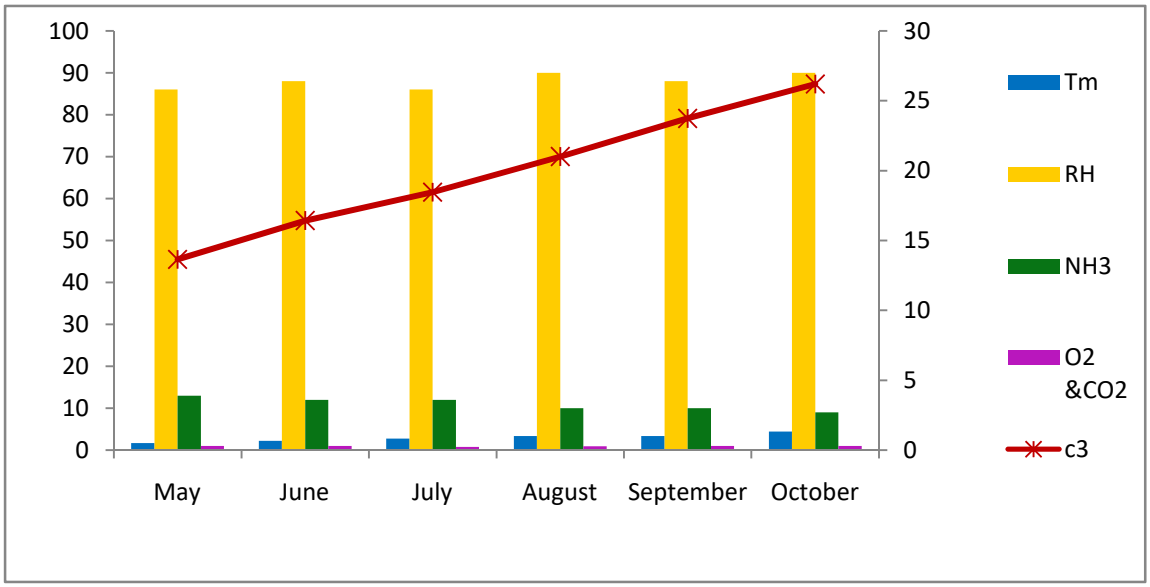


Figure 3: Disease Incidence of *Ralstonia solanacearum* at Ma cold storage

N.B. Tm =Temperature (^{o}C), RH= Relative Humidity (%), NH_3= Amonia Supply (hr),O_2 supply & CO_2 removal (hr), c3= Incidence of *R. solanacearum*

4.2.9. Relationship between internal conditions of cold storages and incidences of *Ralstonia solanacearum*

Correlation and linear regression analysis were performed to determine the relationship between internal conditions (temperature, relative humidity, ammonia supply and oxygen supply & carbon di-oxide removal) and incidence of *Ralstonia solanacearum* (Fig. 4). From the correlation studies it was revealed that internal conditions (temperature, relative humidity, ammonia supply and oxygen supply & carbon di-oxide removal) were positively and negatively correlated to incidence of *Ralstonia solanacearum* at three cold storages. Among all these temperature (R^2 =0.829) was very positively correlated to the incidence of *Ralstonia solanacearum* (Fig. 4). On the other hand, relative humidity was poorly (R^2= 0.478) was very poorly but positively correlated to incidence of *Ralstonia solanacearum* (Fig. 5).In case of ammonia supply (R^2 =0.807) of cold storages was negatively correlated to incidence of *Ralstonia solanacearum* (Fig.6). Similarly oxygen supply & carbon di-oxide removal also negatively and very poorly (R^2= 0.069) correlated to incidence of *Ralstonia solanacearum* (Fig.7).

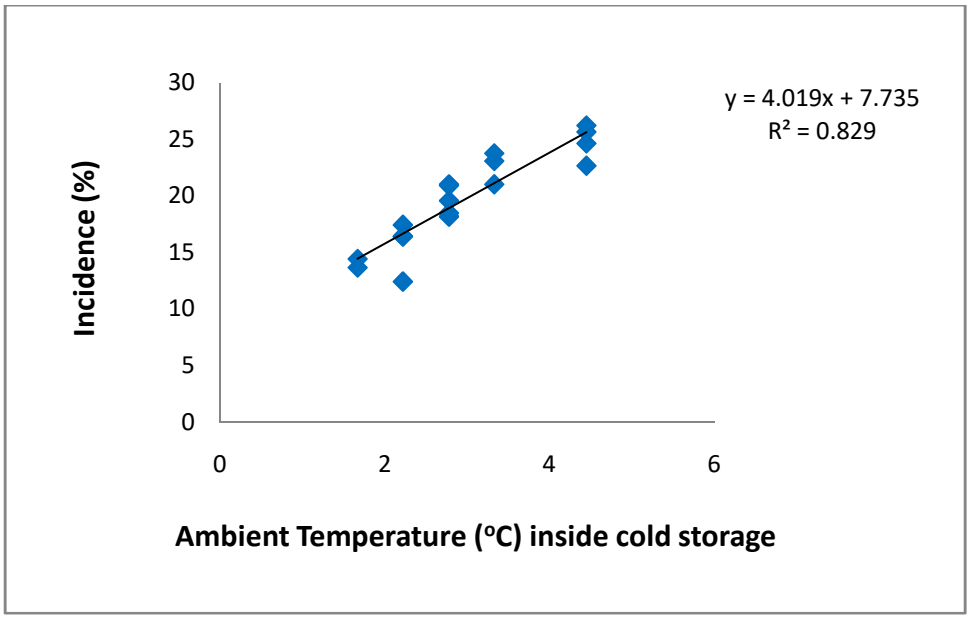


Figure 4: Relationship between Temperature (^{o}C) and incidence (%) of *Ralstonia solanacearum*

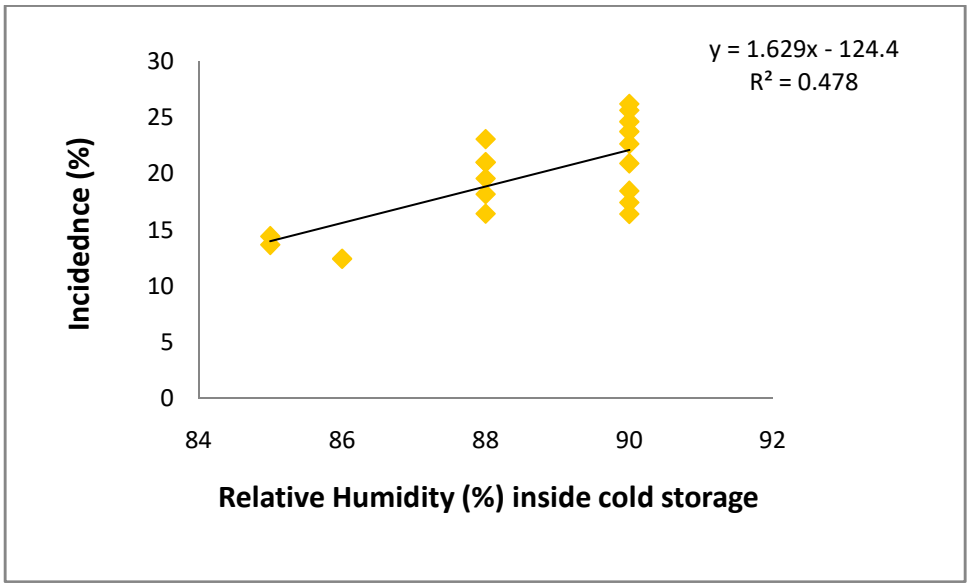


Figure 5: Relationship between Relative Humidity (%) and incidence (%) of *Ralstonia solanacearum*

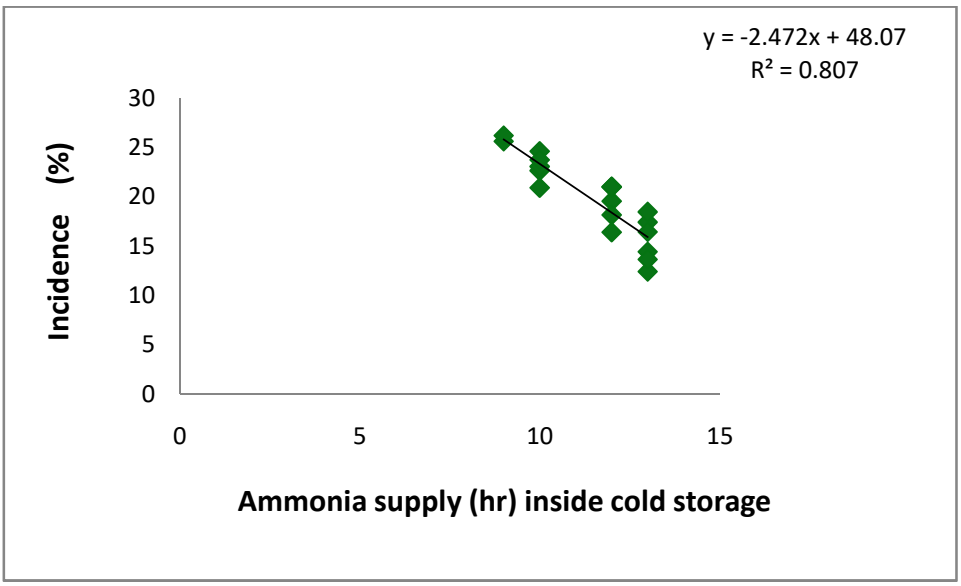


Figure 5: Relationship between ammonia supply (hr) and incidence (%) of *Ralstonia solanacearum*

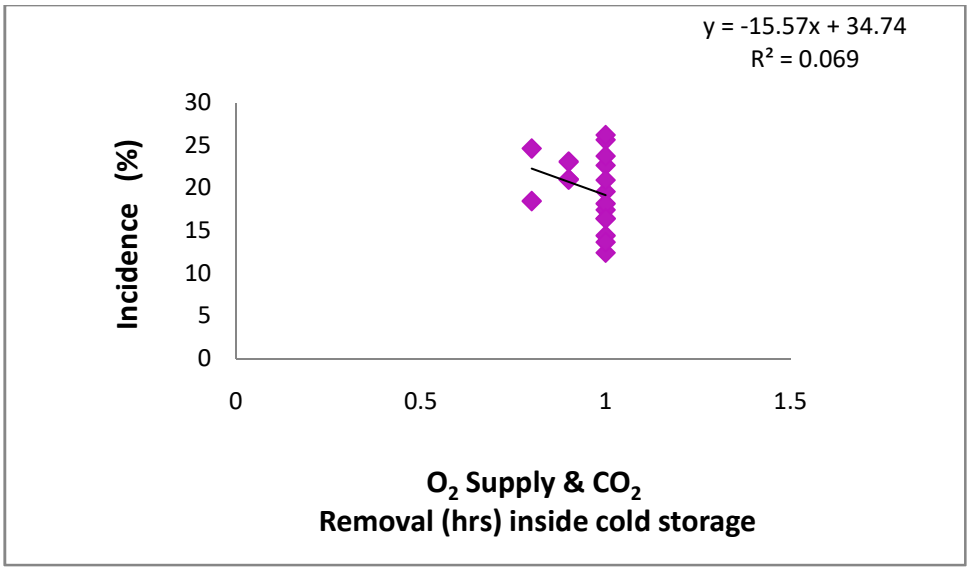


Figure 7: Relationship between O_2 Supply & CO_2 Removal (hr) and incidence (%) of *Ralstonia solanacearum*

CHAPTER V
DISCUSSION

The study was conducted in selected cold storage of Narayanganj district to observe the prevalence of *Ralstonia solanacearum* as latent infection in stored potato. Potato samples were collected from three different cold storages viz. Hazi Rahamatullah cold storage, Sahin cold storage and Ma cold storage during the period of May 2018 to October 2018. In total 360 samples were analysed during six months of the study period. The internal conditions of cold storages were also studied and data were recorded on temperature (^{o}C), relative humidity (RH%), ammonia supply (hr), O_2 Supply & CO_2 removal (hr) and their effect on incidence of *Ralstonia solanacearum*.

In the present study isolation of *Ralstonia solanacearum* were done using the protocol described by Hayward (1991). Repeated isolation from the infected tubers yielded well separated, typical, lucid, convex, mucoid colonies of bacterium on nutrient agar medium (NA) after 48 hours of incubation at 30^{o}C. A satisfactory number of colonies were formed on TTC after 48-72 hours of incubation of *Ralstonia solanacearum*. Cultural characteristics also observed as diagnostic features of this pathogen. *Ralstonia solanacearum* formed a large amount of colonies on NA medium after 48-72 hours of incubation. Circular, mucoid, convex, lucid coloured colonies of the bacterium were developed on NA medium. In TTC medium, largely unmixed mucoid magenta deep red coloured colonies of *R. solanacearum* were developed with whitish margins colonies of bacterium. It was confirmed according to the description of Kelman and Person, (1954). By using TTC and NA media Schaad *et al*. (2001), Champoiseau (2008) and Rahman *et al*. (2010) also identified *R. solanacearum.*

The bacterium was rod shaped with rounded ends gram negative (red color) and capsulated, after gram's staining under the compound microscope at 100x magnification (Murray *et al.,* 2007). A mucoid thread was produced in KOH solubility test that supports the result of gram's staining test i.e., the bacteria was gram negative. It was confirmed on the basis of colony morphology (Kelman, 1954). In levan test after incubated at 30^0c the bacteria produced levan thus the bacterium showed positive result. Smear culture with a drop of hydrogen peroxide (H_2O_2) produced bubbles indicating positive for catalase tests. In oxidase test, tetra methyl phenylene diaminedi hydrochloride produce purple color smeared develops within 10 seconds (Kovacs, 1956). It showed positive result in levan test and pectolytic test, they are unable to utilize starch what was reported by Bradbury (1986). By using following biochemical test Denny and Hayward (2001) and Hossain *et al.* (2008) confirmed *R. solanacearum.*

In the survey study of three cold storages internal condition showed no significant difference among them where temperature 1.67^oC-4.44^oC, relative humidity 85-90%, ammonia 9 to 13 hours and oxygen supply & carbon di-oxide removal0.8 to 1 hours. Harbenburg *et al*. (1986) and Maldegem (1999) stated that temperature, humidity, carbon dioxide and air movement are the most important factors during storage. Hirpa (2010) described that storage methods had a significant effect on incidence of *Ralstonia solanacearum*. Incidence of *Ralstonia solanacearum* is significantly influenced by average temperature, relative humidity, ammonia supply and O_2 & CO_2 supply. Variation of the incidence of *Ralstonia solanacearum* were observed due to variation of temperature, relative humidity, ammonia supply and O_2 supply and CO_2 removal in cold storages. Van't Oostar (1999) stated the optimum range of temperature and relative humidity of the air inside cold stores are 2-4 ^oC and 85-90% respectively for long term

storage. Western Potato Council (2003) observed that potato should be stored at (3-5^{o}C) with 95% RH.

The study showed that incidence was also varied with the increase of storage period. The highest incidence was found after six month of storage. Long term storage increased this incidence significantly. The pathogen infection was recorded in the present study based on visible and invisible symptoms following the description of Brunings and Gabriel (2003). Elphinstone (2005) reported that brown rot was caused by *R. solanacearum* mainly tuber-borne as it could latently infect tubers and survived in seed tubers during storage, causing disease when planted inthe next season. French (1994) stated that seed borne wilt or latent infection of *R. solanacearum* in potato has often been resulted in severe outbreaks of bacterial wilt. In this study maximum incidence was recorded 26.21% at Ma cold storage in October at highest temperature (4.44^{o}C) and lowest incidence was found 12.42% at Sahin cold storage in May at lowest temperature (2.22^{o}C). By following this method Chakraborty and Roy (2016) was recorded incidence of *R. solanacearum*. Nielsen (1963) found that the pathogen survives in potato tubers and in culture at low temperature. Ciampi *et al.* (1980) and Ciampi-Panno *et al*. (1981) reported that storage at 4°C did not eliminate the pathogen from inoculated tubers even after 40 days. Prevalence of *Ralstonia solanacearum* in cold storage potatoes were varied in respect of infection. So we can say incidence was increased slowly from May to October month in three cold storages of Narayanganj district.

From the correlation studies it was revealed that temperature of cold storages mainly responsible for increased of incidence of *Ralstonia solanacearum* and relative humidity was poorly responsible for this. On the other hand ammonia supply function as a refrigerant to absorb large quantities of heat without changing its temperatures and

decreased incidence. Similarly O_2 supply & CO_2 removal has no significant effect on incidence of *Ralstonia solanacearum.*

The present study was based on only the prevalence of *Ralstonia solanacearum* in stored potato and effect of storage internal condition on incidence of *Ralstonia solanacearum* From the result, it could be concluded that highest incidence of *Ralstonia solanacearum* was found at highest temperature (4.44^oC).

CHAPTER V
SUMMARY AND CONCLUSION

Tuber of potato is vulnerable to attack by various diseases in Bangladesh especially by the pathogen *Ralstonia solanacearum*. Even the fresh looked potato tuber can contain the pathogen in itself. Therefore, the present study was designed to study the prevalence of *Ralstonia solanacearum* as latent infection in potato tuber samples collected from three cold storages of Narayanganj districts viz- Hazi Rahmatullah cold storage, Sahin cold storage, Ma cold storage during the month of May 2018 to October 2018.

In the present study, potatoes samples were collected in every month from three cold storages in the month of May to October. Then isolation and identification was done in the Phytobacteriology Laboratory at Sher-e-Bangla Agricultural University, Dhaka. *Ralstonia solanacearum* was isolated from the infected tuber by following dilution plating technique using nutrient agar medium. The bacterium appeared as circular, mucoid, convex, lucid colour colonies on NA medium. The bacteria produced small whitish with pink centered colony on selective TTC medium. The pathogen was identified by its morphological, biochemical and cultural features as per standard microbiological processes. It showed positive result in KOH solubility test, Levan test, Catalase test, Oxidase test, Pectolytic test and negative result in Gram staining test.

The study revealed that incidence of *Ralstonia solanacearum* varied from cold storage to cold storage. Variation of the incidence of *Ralstonia solanacearum* due to variation of temperature, relative humidity, ammonia supply and CO_2 and O_2 supply in cold storages. Highest incidence was found in the month of October (26.21%) at temperature 4.45^{o}C, relative humidity 90%, ammonia supply 9 hours and O_2 supply & CO_2 removal 1hour at

Ma cold storage. Lowest incidence was found 12.42% in May at temperature1.67^{o}C, relative humidity 86%, ammonia supply 13 hours and O_2 supply & CO_2 removal 1hour at Sahin cold storage. From the correlation studies it was revealed that temperature (^{o}C) temperature of cold storages mainly responsible for increased of incidence of *Ralstonia solanacearum* and relative humidity was poorly responsible.

The present study result revealed that the latent pathogen *Ralstonia solanacearum* can stay longer time in potato tuber and completely destroy vascular bundle in severe stage. Incidence was increased gradually for long term storage and increase with temperature. Therefore, Incidence of *Ralstonia solanacearum* is significantly influenced by average temperature and relative humidity. Maxmium disease incidence was found 26.21% at Ma cold storage in October at highest temperature (4.44^{o}C). Minimum incidence was found 12.42% at Sahin cold storage in May at lowest temperature (1.67^{o}C). Maximum incidence was observed at highest temperature (4.44^{o}C).

References

Agrios, G. N. (2008). Plant Pathology, Amsterdam. Elsevier Academic Press, pp. 647-649.

Ajanga, S. (1993). Status of Bacterial Wilt of Potato in Kenya; In Hartman G. L; Hayward E C (Eds): Bacterial Wilt, ACIAR proceedings.**45**:338-340.

Andersona, R.C. and Gardner, D.E. (1999). An Evaluation of the Wilt-Causing Bacterium *Ralstonia solanacearum* as a Potential Biological Control Agent for the Alien Kahili Ginger (*Hedychiumgard nerianum*) in Hawaiian Forests.*Biol. Control*. **15**(2)**:** 89-96.

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*. **206**: 36-106.

Bradbury, J. F. (1970). Isolation and preliminary study of bacteria from plants.*Rev. Pl. Path*.**49:** 213-218.

Brunings, A. M. and Gabriel, D. W. (2003). *Ralstonia solanacearum:* Breaking the surface. *Mol. Plant Pathol*. **4**(3): 141–157.

Chakraborty, R. and Roy, T. S. (2016). Threats faced by brown rot of potato in Bangladesh. *Microbiology Re-search.***7**:8–12. doi: 10.4081/mr.2016.6258.

Champoiseau, G. (2008). *Ralstonia solanacearum*race 3 biovar 2: detection, exclusion and analysis of a Select Agent Educational modules. The United States Department of Agriculture National Research Initiative Program (2007-2010).

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.

Chourasia, M.K. and Goswami, T.K. (2001). Losses of potatoes in cold storage types, mechanism and influential factors. J*ournal of Food Science Technology Mysore*, **38** (4): 301-313.

Ciampi, L., Sequeira, L. and French E.R (1980). Latent infection of potato tubers by *Pseudomonas solanacearum. Am. potato J.* **57**: 377-386.

Ciampi-Panno, L., Sequeira, L. and French E.R (1981). *Pseudomonas solanacearum.* Distribution in plants; Establishment of latent infections. In/; Proceedings of the 5th Conference on Plant Pathogenic Bacteria. Cali, pp 148-161**.**

CIP, (2008). Potato fact sheets. [ftp://ftp.fao.org/docrep/fao/012/1127e/i1127e03.pdf] site visited on 28.11.2019.

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

DAE. (2015). Department of Agriculture Extension, Ministry of Agriculture, Khamarbari, Farm Gate, Dhaka.

Dale, F. (2014). Breeding for Storage.

Denny, T. P. and Hayward, A. C. (2001). Gram-negative bacteria: *Ralstonia*. In Schaad, N. W., Jones, J. B. and Chun, W.(Eds.), Laboratory guide for identification of plant pathogenic bacteria (pp. 151-174, 3rd edn). APS Press, St.

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

Elphinstone, G. J. (2007). The Canon of potato science: Bacterial pathogens. *Potato. Res*. **50:** 247-249.

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

Ensminger, A. H., Ensminger, M. E., Kondale, J. E. and Robson, J. R. K. (1983). Foods & Nutrition Encyclopedia. Pegus Press, Clovis, California, USA.

EPB (2015). Export Promotion Bureau. Ministry of Commerce, Government of the Peoples Republic of Bangladesh.

Fakir, G.A. (2009).Storage Diseases of Potato: Proceedings of the 2nd Workshop of Potato Research Workers, BARI, Joydebpur, Bangladesh.

FAO. (2019). Food and Agricultural Organization of the United Nations.Rap. Pub.71 p.

French, E. R. (1994). Strategies for integrated control of bacterial wilt of potato. In. Hayward, A. C and Hartman, G. L. (Eds.), *Bacterial wilt: The disease and its causative agent, Pseudomonas solanacearum*. pp. 199–207.

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

Goszczynska, T. and Serfontein, J. J. (1998). Milk Tween agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. phaseoli. *J. Microbiol. Method*.**32**(1): 6572.

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

Grudzinska, M., Czerko, Z. and Borowska-Komenda, M. (2016). Changes of organoleptic quality in potato tubers after application of natural sprout inhibitors. *Agricultural Engineering.***20** (1): 35-43.

Harbenburg, R.E., Watada, A.E. and Wang, C.Y. (1986). The commercial storage of fruits, vegetables and florist and nursery stocks.U.S. Department of Agriculture, 66-68 pp.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol*. **29**: 65–87.

Hildebrand, D.C, Schroth, M.N. and Sands, D.C. (1988). Laboratory Guide for identification of plant pathogenic bacteria. In: *Pseudomonas* (NW Schaad, ed). The American Phytopathological Society, St. Paul, Minnesota. Pp. 60-81.

Hirpa, P., Miranda, T., Agajie, M.,Lommen, A., Lansik, C.,Struik, and Tsegaye, A. (2010). Analysis of seed potato systems in Ethiopia.*Am. J. Potato Res*. **87**. pp. 537-522.

Hossain, M. A., Hasan, M. K. and Naher, Q. (2008). Assessment of technical efficiency of potato producers in some selected areas of Bangladesh. *J Agric Rural Dev*. **6**(1&2): 113-118.

Janse, J. D. (1988). A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. *Bulletin OEPP/EPPO.*18:343–351.

Janse, J. D. (2006). Potato brown rot in Western Europe-history present occurrence and some remarks on possible origin, epidemiology and control strategies. *Bulletin OEPP*. **26**(3/4): 679-695.

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. and L. H. Person, (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., (1997). One hundred and one years of research bacterial wilt. *Bacterial Wilt Dis*. **14**: 1-5.

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.

Kleinkopf, G.E. and Olsen, N. (2003). Storage Management, in: Potato Production Systems, Stark, J. C. and Love, S. L. (eds), University of Idaho Agricultural Communications, 363–381.

Kohli (2009). Potato storage and value Preservation: The Basics. Cross Tree techno-visors. http://www.crosstree.info/Documents/POTATO%20STORAGE.pdf.

Kovacs, N. (1956). Identification of *Pseudomonas solanacearum* by the oxidase reaction, Nature, 178: 703.

Kucharek, T. (1998). Bacterial Wilt of Row Crops in Florida. Cooperative Extension Service, Institute of Food and Agricultural Sciences, Florida.

Maldegem, J. P. V. (1999). State of the art techniques for the potato storage. Abstract, Global Conference on Potato, New Delhi, December, pp 6-11.

Martin, C and French, E.R. (1985).Bacterial Wilt of Potato. *Ralstonia solanacearum Technical Information bulletin* No 13.C/P.

Milling, A., Meng, F. H., Denny, T. P., and Allen, C. (2009). Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* race 3 biovar 2. *Phytopathology.* **99**:1127-1134.

Mulder, A. and Trukensteen, L. J. (2005). Potato diseases, 2508 AC Den Haag the Netherlands.

Nielsen, L.W. (1963). Longevity of *Pseudomonas solanacearum* in potato tibers and culture in cold storage. *Am Potato J.* **40**:344-348.

Nourian, F., Ramaswamy, H. S. and Kushalappa, A. C (2003). Kinetics of quality change associated with potatoes stored at different temperatures. Lebensm. Wiss. U. Technol, 36: 49-65.

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.

Paret, M. L., Silva, A. S., Criley, R. A. and Alvarez, A. M. (2008). *Ralstonia solanacearum* Race 4: Risk Assessment for Edible Ginger. *Hort. Technol*. **18**:90-96.

Pastor, N.A., Reynoso, M.M., Tonelli, M.L., Masciarelli, O., Rosaso, S.B., Tonelli, M.L., Masciarelli, D., Rosas, S.B. and Rovera, M. (2010). Potential bio control *Pseudomonas* sp. Pc12 against damping-off of tomato caused by *Sclerotium rolfsii*. *J. Plant Pathol*. **92**: 737– 745.

Prior, P., Bart, S., Leclercq, S., Darrasse, A. and Anais, G. (1998). Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas* (*Burkhoderia*) *solanacearum* in the stem tissues. *Plant Pathol.* **45**: 720-726.

Priou, S. (2006). DAS-ELISA kit for the detection of *Ralstonia solanacearum* in potato stems. Instructions for use.

Rahman, M. F., Islam, M. R., Rahman, T. and Meah, M. B. (2010). Biochemical characterization of *Ralstonia solanacearum* causing bacterial wilt of brinjal in Bangladesh. *J. Progres Agric*. **21**(1&2): 9-19.

Rai, V. R. and Mamatha, T. (2005). Seedling diseases of some important forest tree species and their management. In working papers of the Finish Forest Research Institute.p. 11.

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; 36ref.

Sagar, V. and Bakade, R . (2007). Bacterial diseases and their management in potato production. [14.139.61.86/E-Book.../lecture-notes/Bacterial diseases Vinay Sagar. pdf]. site visited on 14/06/2019.

Schaad, N.W. (1980). Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd edition, p: 164. The Bacteriology Committee of the American Phytopathological Society.

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. (2001). Biochemical characterization of *Ralstonia solanacearum* causing brown rot of potato in Bangladesh. *Journal of Progressive Agriculture,* **21**(1&2): 9-19.

Schaad, N. W., Jones, J. B., and Chun, W. (2001). Laboratory Guide for the Identification of Plant Pathogenic Bacteria, 3rd Edn. American Phytopathological Society, St. Paul, MN.

Seal, S. E., Jackson, L. A., Young, J. P. W. and Daniels, M. J. (1993). Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas picketti* and blood disease bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. *J. Gen. Microbiol*. **139**:1587-1594.

Shekhawat, G. S., Chakraborti, S. K and Gadewar, A. V. (1992). Potato bacterial wilt in India. ICAR Tech. Bull. No.38. Central potato Research Institute (CPRI), Shimla, H.P. India p. 52.

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 R. N. Smith, (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.

Terblanche, J. and Villiers, D. A. (2013). Prior, Philippe, Allen, Caitilyn, Elphinstone,John, eds. Bacterial Wilt Disease: Molecular and Ecological Aspects (1st ed.).Paris: Springer Science and Business Media. P. 326.

Van Elsas, J. D., Kastelein, P., Van Bekkum, P., Van Der Wolf, J. M., De Vries, P. M., and Van Overbeek, L. S. (2000). Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathology.* **90**:1358-1366.

Van Elsas, J. D., Kastelein, P., de Vries, P. M. and van Overbeek, L. S. (2001). Effects of ecological factors on the survival physiology of *Ralstonia solanacearum*bv.2 in irrigation water.*Canadian Journal of Microbiology.* **47**: 1-13.

Van't Oostar, A. (1999). Storage of potatoes, In: F. W. Bakker-Arkema (Ed.), CIGR Handbook of Agricultural Engineering. ASAE, Vol. IV: 93-125.

Western Potato Council.(2003). Guide to commercial potato production on the Canadian Priaries. http://www.gov.mb.ca/ agriculture/crops/potatoes/bda04s11.html (accessed on April 1, 2010).

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta and Nishiuchi Y. (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia*gen. proposal for *Ralstonia pickettii*, *Ralstonia solanacearum* and *Ralstonia eutropha*. *Microbiol.Immunol*.**39**: 897-904.

Zgorska, K. and Grudzinska, M. (2012). Changes in selected quality parameters of potato tubers during storage, Acta Agrophysica **19:** 203-214.

APPENDICES

Appendix I. Biochemical reagents

Gram's Crystal violet (Hucker's modification)	
Solution A : Crystal violet (90% dye content)	2.0 g
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 tetramethyl-p-phenylene-diaminedihydrochloride was prepared from the absolute solution.