PREVALENCE OF BACTERIAL WILT PATHOGEN *Ralstonia solanacearum* OF POTATO IN SELECTED DISTRICTS OF NORTHERN BANGLADESH AND ITS *in vitro* MANAGEMENT BY SOME AGROCHEMICALS

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CERTIFICATE

This is to certify that the thesis entitled **"Prevalence of bacterial wilt pathogen *Ralstonia solanacearum* of potato in selected districts of northern Bangladesh and its *in vitro* management by some agrochemicals"** 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 Registration No. **08-03039** 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: 26.06.2016
Dhaka, Bangladesh

(Prof. Dr. M. Salahuddin M. Chowdhury)
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DEDICATED TO

MY

BELOVED PARENTS,
TWO SISTERS &
DR. SALMAN MAHMOOD

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ABSTRACT

Several varieties of potato viz. cardinal,granola,diamant,asterix,laura,cumbica were collected from selected districts of northern Bangladesh viz. Rangpur, Naogaon, Lalmonirhat, Kaunia through different potato producing companies viz. Tradelink International Ltd., Ashmina, Shahan Trade International, Hay Agro during December, 2014 to August, 2015 to evaluate prevalence of bacterial wilt of potato (*Ralstonia solanacearum*) for developing suitable *in vitro* agrochemical management. *Ralstonia solanacearum* was isolated from collected potato tubers and it's morphological, biochemical and cultural features were studied. The bacterium was gram negative, rod shaped and showed positive result in KOH solubility test, catalase test and pectolytic test and negative result in oxidase test, turbidity test and for some samples in pectolytic test. Some produced largely unmixed mucoid magenta deep red coloured colonies and some produced pink coloured with whitish margins on TTC medium. On NA medium, circular, mucoid, convex, lucid coloured colonies of bacterium were formed. After observing the features, pathogenic and non pathogenic bacteria were identified. *In vitro* efficacy of different agrochemicals was observed against pathogenic bacteria identified. *In vitro* evaluation of agrochemicals exhibited that among the agrochemicals Deoxymethasone (Neomycin) in 0.5% concentration and Streptomycin in 0.5% concentration were highly effective against *Ralstonia solanacearum* causing bacterial wilt of potato. Copper compounds (Cuprofix), Mancozeb (Indofil M-45 and Ridomil gold) showed satisfactory efficacy in 0.5% concentration against the pathogen.

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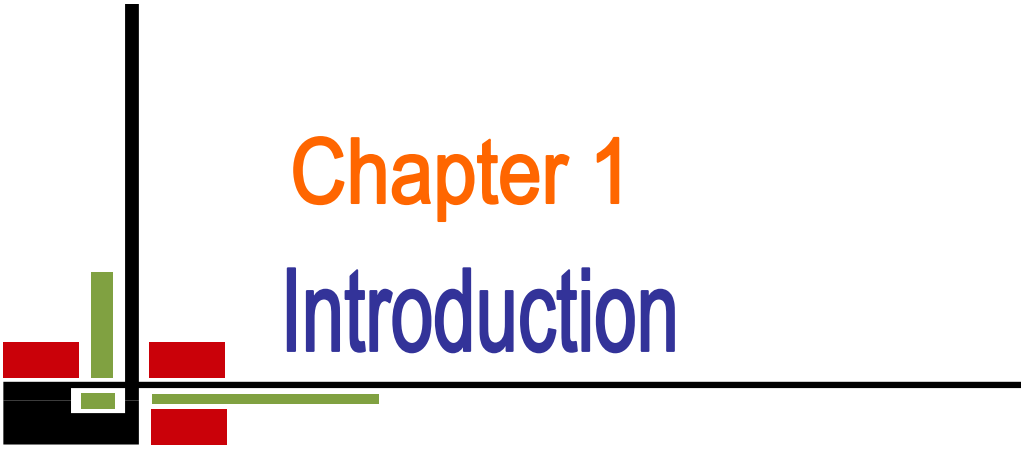
LIST OF SYMBOLS AND ABBREVIATIONS

No.	=	Number
%	=	Percentage
et al.	=	And others
^{o}C	=	Degree Celsius
@	=	At the rate
WP	=	Wettable Powder
EC	=	Emulsifiable Concentrate
etc.	=	Etcetra
J.	=	Journal
Viz.	=	Namely
Cm	=	Centimeter
Cfu	=	Colony forming unit
df.	=	Degrees of freedom
&	=	And
ppm	=	Parts per million
Kg	=	Kilogram
G	=	Gram
ml	=	Milliliter

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

hr	=	Hour (s)
i.e.	=	That is
T	=	Treatment
cv.	=	Cultivar (s)
var.	=	Variety
mm	=	Millimeter
µl	=	Microliter
µm	=	Micrometer
SAU	=	Sher-e-Bangla Agricultural University
BBS	=	Bangladesh Bureau of Statistics
USA	=	United States of America
NA	=	Nutrient Agar (media)
NB	=	Nutrient Broth (media)
TTC	=	Triphenyl Tetrazolium Chloride
ANOVA	=	Analysis of Variances
LSD	=	Least Significant Difference
CV%	=	Percentages of Co-efficient of Variance



Chapter 1

Introduction

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum*) is a starchy tuberous crop. It is the world's fourth largest and third largest food crop in Bangladesh and has recently occupied an important place in the list of major food and cash crops in Bangladesh (Ali and Haque, 2011). It belongs to the solanaceae or nightshade family. Potatoes are the swollen portion of the underground stem which is called a tuber and is designed to provide food for the green leafy portion of the plant. The potato was introduced in this subcontinent in the sixteenth century. It was grown then in small plots as a vegetable. Today potato has emerged as a major crop in Bangladesh. It is being cultivated on an area of 520 thousands ha and the total production is 89,50,024 metric tonnes with an average yield of 19.371 metric tonnes/ha (BBS, 2014). Potato is a staple food in the developed countries which accounts for 376,452,524 tonnes of the total crop production in the world (FAO, 2014). Potato is a popular and important vegetable in Bangladesh. For the whole year it is used as a main vegetable. Annual consumption of potato has been growing rapidly, from around 34 kg per capita (FAO, 2014). Considering the increased demand for the food in the country potato is likely to play a very important role in the future.

There are 27 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 local conditions. Through constant evaluation of the traits, varietal performance and consideration of other characteristics, 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, Diamant, Kurfi shindhury* etc. The Tuber Crops Research Centre of BARI has collected many new varieties from the International Potato Research Centre. The centre has

already made good contribution towards the development of some high yielding potato varieties (Anonymous, 2006).

In Bangladesh, so far as many 12 diseases in potato have been recorded (PRA, 2015). Among them late blight, stem rot/sclerotium rot, wilt, common scab, potato leaf roll and mosaic are the most important diseases (Ahmed *et al*., 2000). Bacterial wilt of potato caused by *Ralstonia solanacearum* is an important disease. It is one of the most destructive pathogens identified because it induces rapid and fatal wilting symptoms in the host plants. Bacterial wilt caused by *Ralstonia solanacearum* (Smith, 1986) formerly known as *Pseudomonous solanacearum* (Yabuuchi *et al*., 1995) is highly challenging and one of the most destructive diseases of solanaceous crops worldwide (Hayward, 2005). The disease can be found in all tropical,sub tropical and warm temperate regions of the world (Hayward, 2005). Bringing about severe crop losses worldwide,the disease is now receiving global profile (Allen *et al*., 2005). *Ralstonia solanacearum* has exceptionally wide diversity having strains originating from different geographical origins and hosts (Buddenhagan and Kelman, 1964; Hayward, 1991). The host range of the bacterium is unusually broad together with hundreds of plant species (Hayward, 1991). Worldwide major hosts are : *Solanum tuberosum* (potato), *Lycopersicon esculentum* (tomato), *Capsicum annuum* (sweet pepper), *Solanum melongena* (aubergine), *Nicotiana tabacum* (tobacco), *Arachis hypogaea* (groundnut), *Musa paradisiaca* (banana and plantain) and *Heliconia* spp. (Wang and Lin, 2005a). The most economically important crop affected by bacterial wilt in European countries is potato (Annon, 1998).

Ralstonia solanacearum is a soil-borne aerobic, rod shaped, non spore forming, non capsulate, gram-negative bacterium. The bacterium is oxidase positive and arginine dihydrolase negative (Kelman, 1981). It is an important soil borne pathogen globally. It causes devastating wilt on over 450 plant species belonging to 54 families, covering both monocots and dicots (Wicker *et al*., 2007, Hayward, 1991). The bacterium normally invades plant roots from soil

through wounds or natural openings. It colonizes the intercellular space of the root cortex and vascular parenchyma, and eventually enters the xylem vessel and spreads up into the stem and leaves. Affected plants suffer chlorosis, stunting, wilting and usually die rapidly. Losses caused by the disease are known to be enormous but cannot be accurately estimated because of abandonment of wilt susceptible crops in many parts of the world.

Wide host range and broad geographical distribution have made *Ralstonia solanacearum* an economically significant pathogen. 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).

Difficulties are associated with controlling *Ralstonia solanacearum* due to its abilities to grow endophytically, survive in soil, especially in the deeper layers. The management of bacterial wilt with physical, chemical, biological and cultural methods has been investigated for decades. Elphinstone extensively reviewed bacterial wilt in 2005, and many studies have since been conducted on this topic. Elphinstone reported that over 450 studies had been published on *R. solanacearum* since the second International Bacterial Wilt Symposium was held in Guadaloupe in 1997. A broad classification on these studies showed that 24% were concerned with breeding and selection for resistance, while the remainder investigated the diversity, distribution, and host range of the pathogen (22%), disease management and control (18%), pathogenicity and host-pathogen interactions (17%), biological control (10%), detection and diagnosis of the pathogen (4%), and epidemiology and ecology (3%). Cultural practices, crop rotation and host resistance may provide limited control of *Ralstonia solanacearum* (Kucharek, 1998; Pradhanang *et al*., 2003). Several plant essential oils and their component showed that some essential oils have significant efficacy against *Ralstonia solanacearum in vitro* and under

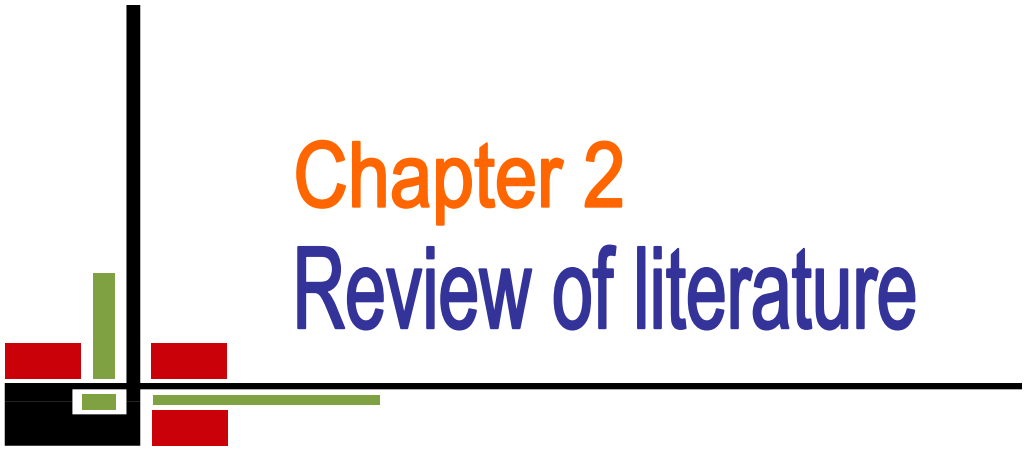
glasshouse (Momol *et al*., 1999). Rapid early detection of bacterial wilt is not only in tubers or plant debris but also in soil or soil related habitats is essential for disease management in the field to prevent losses and further pathogen spread (Janse *et al*., 1998). The common control measures used against bacterial wilt include the use of resistant varieties,healthy seed, crop rotation, agronomic practices, biological control and integrated management (Elphinstone and Aley, 1993). However there are no universal control measures which are effective across the wide host range of the pathogen (Cook *et al*., 1989).

Various control strategies are developed to suppress bacterial wilt of potato. Chemical control through antibiotics (streptomycin, neomycin, ampicillin, tetracycline, penicillin) has shown little suppression of *Ralstonia solanacearum* (Murakoshi & Takahashi, 1984). *In vitro* and *In vivo* investigations by some researchers have confirmed the antimicrobial potential of some plant species of some *Ralstonia solanacearum* (Cowan, 1999). Bacterial wilt is difficult to manage due to the genetic diversity and aggressiveness of the pathogen, its ability to survive in the varied and adverse environmental conditions, its modes of dissemination and large number of weed hosts (Ramesh and Phadke, 2012). Chemical bactericides such as copper compounds and antibiotics have limited impact (Hartman and Elphinstone, 1994).

Bacterial wilt disease of potato is difficult to manage. There is no single mean that would totally eradicate the disease, provide an absolute cure or fully protect host plants against the pathogen. However, only a few reports are available in respect of prevalence, isolation, identification and 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 programme has been designed with following objectives :

1. To assess the prevalence of bacterial wilt disease of potato caused by *Ralstonia solanacearum* in selected potato growing areas of Bangladesh.
2. To determine the *In vitro* efficacy of some selected agrochemicals against *Ralstonia solanacearum* causing bacterial wilt of potato.



Chapter 2
Review of literature

CHAPTER II
REVIEW OF LITERATURE

Bacterial wilt of potato caused by *Ralstonia solanacearum* is considered as a disease of major importance, becomes a serious threat for potato production. The disease assumed it's severity in all the growing areas of the world resulting severe yield losses both in terms of quality and quantity. The information are 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 reviewed 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.

Bacterial wilt caused by *Ralstonia solanacearum* has been described on a wide range of hosts in many tropical and subtropical regions by Agrios (2005).

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

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.

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

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.

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.

Nadia *et al*. (2013) 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).

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.

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

2.3. Symptomology

Gota (1992) characterized that *Ralstonia solanacearum* as sudden wilting of foliage where the young plant wass affected more. The symptoms occured 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, 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 (2005) 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* ingressing a plant through roots, penetrate the xylem, systematically colonizes the stem and causes wilt symptoms.

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

2.4. Isolation and identification of the pathogen and it's 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 *et al*. (1996), 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 ImmunoStrips, and a polymerase chain reaction (PCR) assay using *R. solanacearum* species complex-specific primers 759/760.

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

2.5. Evaluation of disease management strategy

It is difficult to control bacterial wilt disease in the soil (Jones, 1997). The use of disease free-seed, reliable and early detection of the pathogen, quarantine measures on infected fields and farms, sufficient crop rotation, control of weed hosts, volunteer plants and nematodes where present, avoidance of surface water for irrigation are key factors in control (Janse, 1996).

2.5.1. Chemical management

Some scientists reported that there are no bactericides available for chemical control of the bacterial wilt disease (Hartman et al., 1993); while others reported that it is difficult to control bacteria with chemicals (Grimault *et al*., 1992).

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

(Cowan, 1999) stated that *In vitro* and *In vivo* investigations by some researchers have confirmed the antimicrobial potential of some plant species of *Ralstonia solanacearum*.

Wilson *et al*. (1997) observed that essential oils and their components have been recognized as having fungicidal effects, but their efficacy as a biofumigant on *Ralstonia solanacearum* has not been studied prior to 1999.

Posas *et al*. (2007) found that the compounds that showed the most suppressive effect were glucose, proline, glutamine, serine, arginine and lysine.

Lemessa and Zeller, (2007) described that on the basis of *In vitro* screening, APF1 strain was found to be the most beneficial strain in disease suppression and also growth promotion resulting in 63% dry weight increase compared to untreated control.

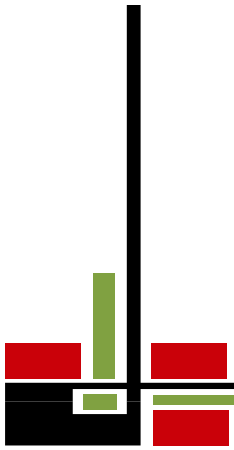
Stockwell and Duffy (2012) reported that antibiotics are essential for controlling bacterial diseases of plants.

Chaudhary *et al*. (2012) tested the effectiveness of some fungicides and antibiotics for the control of bacterial diseases.

Ferrante and Scortichini (2010) tested all copper-based compounds at different doses to show the bactericidal activity by inhibiting the growth.

Kennelly *et al*. (2007) recommended that copper compounds are the standard bactericides for controlling many bacterial diseases.

Pawar *et al*. (2004) tested the efficacy of different fungicides like mancozeb, copper oxychloride, copper hydroxide in controlling bacterial diseases.



Chapter 3

Materials and Methods

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 :

I. Survey on the prevalence of *Ralstonia solanacearum* causing bacterial wilt of potato.

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

III. *In vitro* evaluation by different agrochemicals concentration against *Ralstonia solanacearum* causing bacterial wilt of potato.

3.1.Experiment I. Survey on the prevalence of *Ralstonia solanacearum* causing bacterial wilt of potato

3.1.1. Collection of potato tuber

To determine the bacterial wilt, potato tubers were collected from Rangpur, Naogaon, Lalmonirhat and Kaunia. The collected potato tubers were of one (1) month old.

3.1.2. Selection of area and variety

The six varieties of potato tubers were collected from four companies. The varieties of potato tubers are the working samples of the study. The samples are of one month old. The potato tubers were collected from four districts as shown in table 1.

Table 1. Name of the company, area and potato variety collected

Name of the company	Area	Variety
Tradelink International Ltd.	Rangpur	Cardinal
		Granola
Ashmina	Naogaon	Diamont
		Asterix
Shahan Trade International	Mondolerhat,Lalmonirhat	Cardinal
		Laura
		Asterix
		Cumbica
		Granola
Hay Agro	Charuvadro,Kaunia	Granola
		Cardinal

3.1.3. Observation of the symptoms

Symptoms of the disease were studied by visual observation as per standard procedure by Champoiseau *et al*. (2009). Samples were visually observed for the symptoms of bacterial wilt by cutting the tuber at its stem end by sharp sterile knife. Sometimes hand lens were used for critical observation of the disease. Identification of the disease was finally confirmed through isolation and different biochemical tests.

3.2.Experiment II : Isolation and identification of *Ralstonia solanacearum* in the laboratory through biochemical tests

3.2.1.Collection of diseased specimens

Potato tubers were collected of six varieties from four districts. The specimens were collected from four companies. The specimen were kept in the refrigerator at 4^{o}c to preserve the diseased specimens until isolation was made.

3.2.2.Preparation of Nutrient Agar (NA)

Nutrient agar media (Appendix-I) was prepared according to the method followed by Schaad (1988). For the preparation of 1 liter NA medium at first 15g bacto agar was taken in an Erlenmeyer flask containing 1000 ml distilled water. 5 g peptone and 3 g beef extract were then added to it. For mixing properly the nutrient agar was shaken thoroughly for few minutes. It was then autoclaved at 121^{o}C under 15 PSI pressure for 15 minutes.

3.2.3. Preparation of Nutrient Broth (NB)

Nutrient broth (Appendix-I) was prepared according to method followed by Schaad (1988). For the preparation of 1 liter nutrient broth 5g peptone and 3 g beef extract were taken in an Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^{o}C under 15 PSI pressure for 15 minutes.

3.2.4. Preparation of Triphenyl Tetrazolium Chloride (TTC)

Triphenyl tetrazolium chloride (Appendix-I) was prepared according to method 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.

3.2.5. Method of isolation and identification of causal organism

3.2.5.1. Isolation and purification of bacterial wilt pathogen of potato

The collected potato tubers were washed under running tap water. Then the diseased portions were cut into small pieces. Surface sterilization were done by dipping them in 5% sodium hypochlorite solution for 2-3 minutes. Then washed them three times with sterile 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. One ml of this stock solution was transferred 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 spreaded over NA plate thrice to remove excess surface moisture as described by Goszczynska and Serfontein (1998). Spreading was done with the help of a glass-rod. The inoculated NA plates were kept in incubation chamber at 30^{o}C. The plates were observed after 24 hours and 48 hours. Then single colony grown over NA plate was restreaked on another plate with the help of a loop to get pure colony.

3.2.5.2. Preservation of bacterial wilt pathogen of potato

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

3.2.5.3. 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).

3.2.5.3.1. 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).

3.2.5.3.1.a. Gram's staining

A small drop of sterile water was 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 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. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then it was flooded with lugol's iodine solution for 1 minute. After that it was decolorized with 95% ethanol for 30 seconds and again rinsed with running tap water and air dried. Then it was counterstained with 0.5% safranine for 10 seconds. 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.

3.2.5.3.1.b. 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 and 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).

3.2.5.3.2.Biochemical characters

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

3.2.5.3.2.a. Turbidity test

Small pieces of 1gm potato tuber were cut and taken into a test tube containing 9ml sterile distilled water to observe the turbidity formed by the bacteria.

3.2.5.3.2.b. Pectolytic test

Potato tubers were washed and peeled. 1 cm 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 1 day. After 24 hours a loop was run across the potato slice.

3.2.5.3.2.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.

3.2.5.3.2.d. Oxidase test

At first oxidase disk containing 1 ml 1% aqueous w/v solution of NNN'N-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.

3.2.5.3.3.Cultural characters

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

3.2.5.3.3.a. Growth on 2,3,5 triphenyl tetrazolium chloride (TTC) media

One hundred fifty grams of tuber were mixed in sterile distilled water and was spread in TTC (2,3,5 triphenyl tetra zolium chloride) media after serial dilution and was incubated for 24 hours. Then it was observed for the presence of the colony.

3.2.5.3.3.b. Growth on nutrient agar (NA) media

Nutrient agar (NA) medium was poured into a sterile petri dish 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.

3.3. Experiment III : *In vitro* evaluation by different agrochemicals against *Ralstonia solanacearum* causing bacterial wilt of potato

3.3.1. Experimental site

The study relating to determine the *In vitro* efficacy by different agrochemicals against *Ralstonia solanacearum* causing bacterial wilt of potato was carried out in the disease diagnostic laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207.

3.3.2. Experimental period

The experiment was carried out from December 2014 to August 2015.

3.3.3. *In vitro* evaluation

3.3.3.1. Evaluation of different agrochemicals

3.3.3.1.1. Preparation of sterile swabs

A supply of cotton swabs on wooden applicator sticks was prepared. The Swabs were held in a dry tube and were sterilized in the autoclave (Vandipitte *et al.*, 1991).

3.3.3.1.2. Bioassay of different agrochemicals against the bacteria

Bioassay of antibacterial chemicals against the bacteria was done by well diffusion method measuring the inhibition zone (Annon, 1996). At first, two test tubes each containing 10 ml nutrient broth were taken which were inoculated with 48 hours old pure culture of bacteria grown on NA plate and another test tube containing only 10 ml nutrient broth was taken as control. Then the test tubes were transferred in incubating shaker machine maintaining 30^{o}C temperature and 150 rpm for 24 hours. Four (4) holes of 4 mm in diameter were punched into the same NA plate maintaining equal distance. After shaking, the broth culture was spreaded uniformly on it with the help of sterile cotton swabs. Then different volumes of chemical suspension at different concentration (0.1%, 0.3%, 0.5%, 1%) were added into the each hole each at three replications (Table 2). In case of control only sterile water was used instead of chemical. The plates were then incubated at 30^{o}C in incubation chamber, Zone of inhibition around the holes were measured and recorded after every 12 hours for 3 days.

Table 2. Name of different agrochemicals used in bioassay

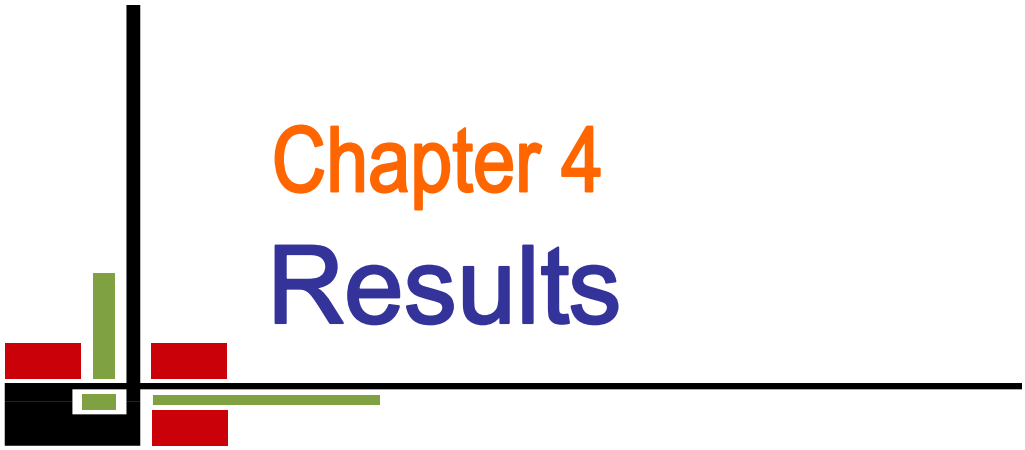
Trade name	Active ingredient	Chemical name	Concentration (%)		
Streptomycin	Streptomycin sulfate	D-Streptomycin	0.1	0.3	0.5
Neomycin	Dexamethasone	Neomycin	0.1	0.3	0.5
Cuprofix 50 WP	Copper oxychloride	Copper chloride oxide hydrate	0.1	0.3	0.5
Tilt 250 EC	Propiconazol	1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1H-1,2,4-triazole	0.1	0.3	0.5
Ridomil gold	Mancozeb	N-(2,6 dimethyl phenyl)-alanine methyl ester	0.1	0.3	0.5
Indofil M-45	Mancozeb	N-(2,6 dimethyl phenyl)-alanine methyl ester	0.1	0.3	0.5
Companion	Mancozeb	N-(2,6 dimethyl phenyl)-alanine methyl ester	0.1	0.3	0.5

3.3.3.2. Experimental design and layout

The experiment was laid out in Completely Randomized Design (CRD).

3.4. Statistical analysis

Data on different parameters were analyzed in two factor Completely Randomized Design (CRD) through computer software MSTAT-C (Anonymous, 1989). To determine the level of significant differences and to separate the means within the parameters, Duncan's Multiple Range Test (DMRT) and Least Significant Difference (LSD) test were performed.



Chapter 4
Results

CHAPTER IV

RESULTS

The results of the investigations undertaken on 'Prevalence of *Ralstonia solanacearum* causal agent of bacterial wilt of potato in Bangladesh : *In vitro* management by different agrochemicals during the study period are presented as below.

4.1. Symptomatology

Six varieties of potato were collected from four different potato producing companies from four districts. The samples were of one month old. There were no visible symptoms shown from the potato tubers. (Plate. 1).

Table 3. Symptoms shown from the potato variety collected

Name of the company	Area	Variety	Symptoms
Tradelink International Ltd. .	Rangpur	Cardinal	No visible symptoms
		Granola	No visible symptoms
Ashmina	Naogaon	Diamant	No visible symptoms
		Asterix	No visible symptoms
Shahan Trade International	Mondolerhat, Lalmonirhat	Cardinal	No visible symptoms
		Laura	No visible symptoms
		Asterix	No visible symptoms
Shahan Trade International	Mondolerhat, Lalmonirhat	Cumbica	No visible symptoms
		Granola	No visible symptoms
Hay Agro	Charuvadro,Kaunia	Granola	No visible symptoms
		Cardinal	No symptoms

4.2. Isolation and purification of bacterial wilt pathogen of potato

The causal organism was isolated from the infected tubers showing no visible symptoms of bacterial wilt (Plate. 1). 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 and some yielded pink coloured with whitish margin colonies of bacterium on TTC medium after 48 hours of incubation at 30^{o}C (Plate. 2). Well separated, typical, lucid, convex, mucoid colonies of bacterium on nutrient agar medium was yielded after 48 hours of incubation at 30^{o}C (Plate. 3). Colonies were purified by restreaking the isolated colony on nutrient agar plate.

4.3. Preservation of bacterial wilt pathogen of potato

Purified bacterium was kept in refrigerator at 4^{o}C in small screw-cap test tubes on NA slant, which served as a stock culture for further studies.

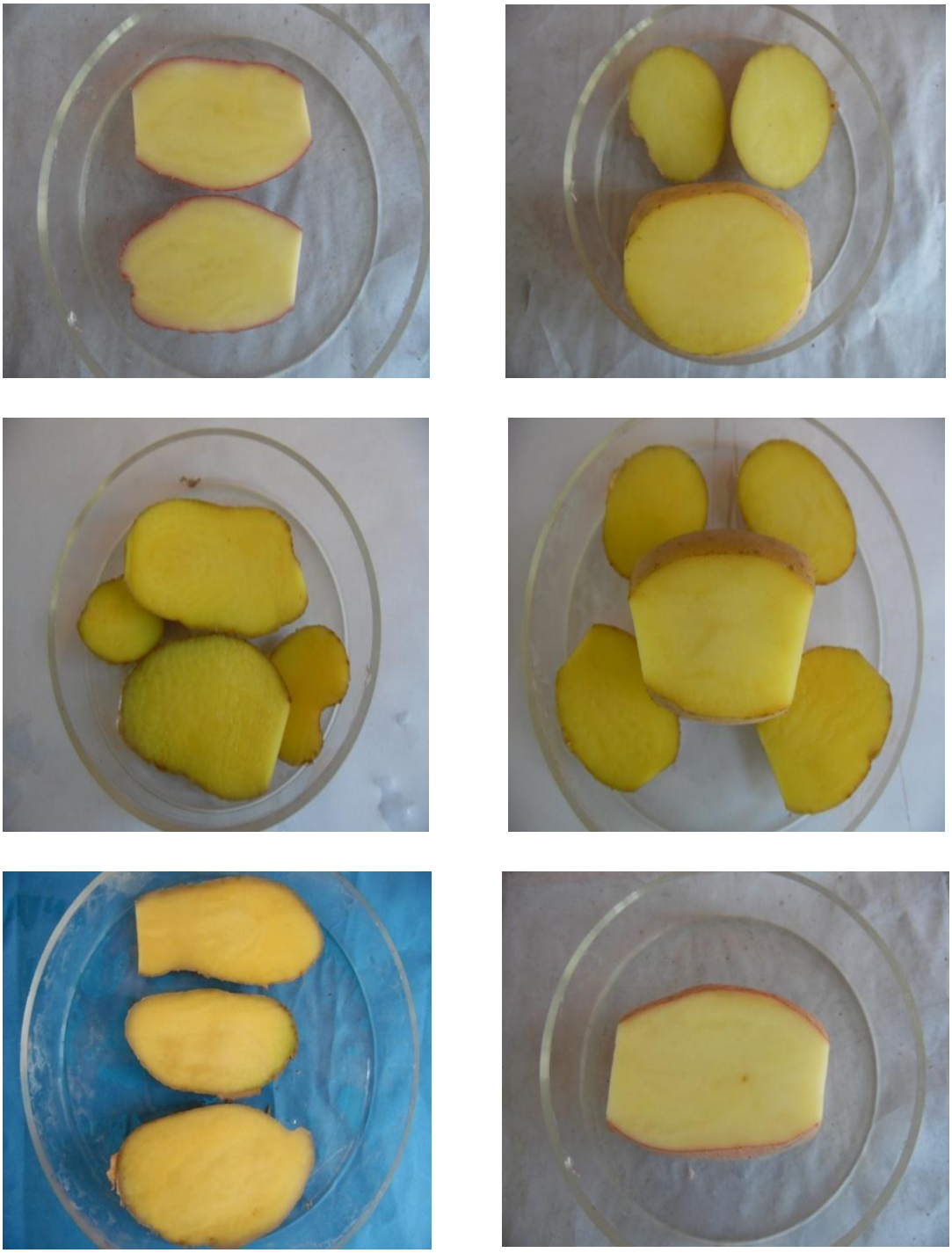


Plate. 1. Infected potato tuber slice with symptoms of *Ralstonia solanacearum*

4.4. Identification of the pathogen

Identification of the pathogen causing bacterial wilt of potato was done by conducting studies on biochemical and cultural features of the pathogen as per standard microbiological procedures.

4.4.1. Morphological characters

Under the compound microscope at 125x magnification with oil immersion, 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. 4A).

In KOH solubility test, a mucoid thread was lifted with the loop (). Therefore the test was positive i.e., the bacterium was gram negative that supports the result of gram's staining test. (Plate. 4B).

4.4.2. Biochemical characters

In turbidity test, no turbidity were formed by the bacteria in slightly warm water. (Fig. 1).

In pectolytic test, some samples showed the result as negative (avirulent) and some samples showed the result as positive (virulent). (Plate. 5).

In catalase test, after adding 3% H_2O_2 onto the colony of the bacterium bubbles were formed within a few seconds, which revealed that the test was positive.(Plate. 6A).

In oxidase test, after rubbing the bacterium onto the moistened oxidase disk, which revealed that the test was negative. (Plate. 6B).

Table 4. Biochemical test of the bacteria isolates from NA media

Biochemical test	Result
Turbidity test	-ve
Pectolytic test	+ve
Catalase test	+ve
Oxidase test	-ve

[(-ve) means negative, (+ve) means positive]

4.4.3. Cultural characters

4.4.3.1. Efficacy of different media in supporting the growth of the pathogen

Of the various media tested for the efficacy to support the growth of *Ralstonia solanacearum* TTC and NA medium were found significantly superior in promoting the luxurious growth of the pathogen.

4.4.3.2. Colony morphology on different growth media

Colonies of *Ralstonia solanacearum* of some samples on TTC medium appeared as largely unmixed mucoid magenta red coloured colonies (avirulent) and some samples appeared as pink coloured with whitish margins (virulent). (Table. 5, Plate. 2).

Circular, mucoid, convex, lucid coloured colonies were found on NA medium in respect to some samples (virulent) and some showed no colonies (avirulent) (Plate. 3).

The pink coloured with whitish margins colonies of bacterium on TTC media and circular, mucoid, convex, lucid colonies of bacterium on NA medium which were considered as virulent bacterium identifying by colour were then further studied for the *In vitro* management by different agrochemicals.

Table 5. Number of colonies of *Ralstonia solanacearum*

Name of the company	Area	Variety	cfu/g potato tuber
Tradelink international Ltd.	Rangpur	Cardinal	14
		Granola	5
Ashmina	Naogaon	Diamont	11
		Asterix	8
Shahan trade international	Mondolerhat,lalmonirhat	Cardinal	4
		Laura	5
		Asterix	16
		Cumbica	9
		Granola	15
Hay Agro	Charuvadro,Kaunia	Granola	6
		Cardinal	10

cfu/g potato tuber = colony forming unit/gram potato tuber

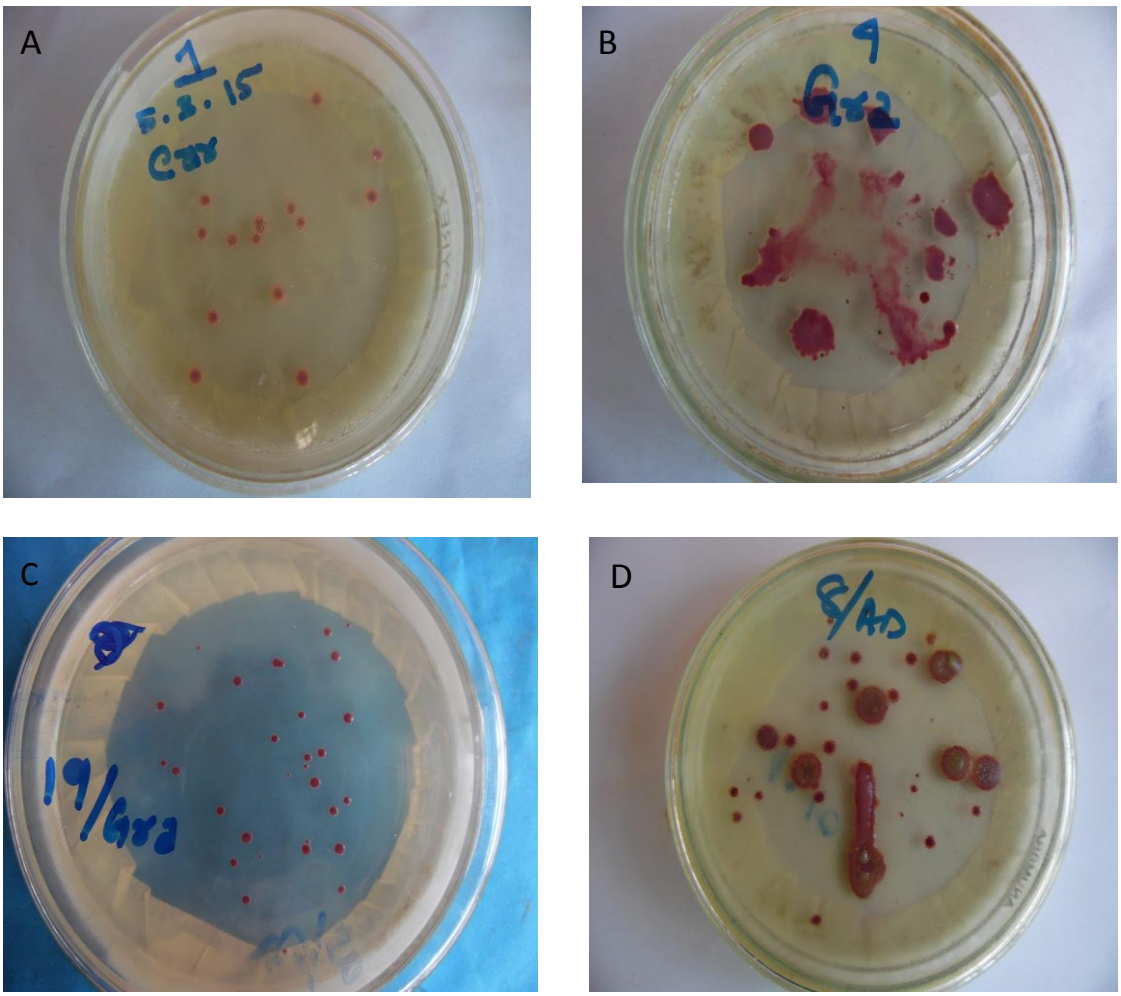


Plate. 2. Growth of *Ralstonia solanacearum* on TTC media

A. Virulent colony
B. Avirulent colony
C. Virulent colony
D. Avirulent colony

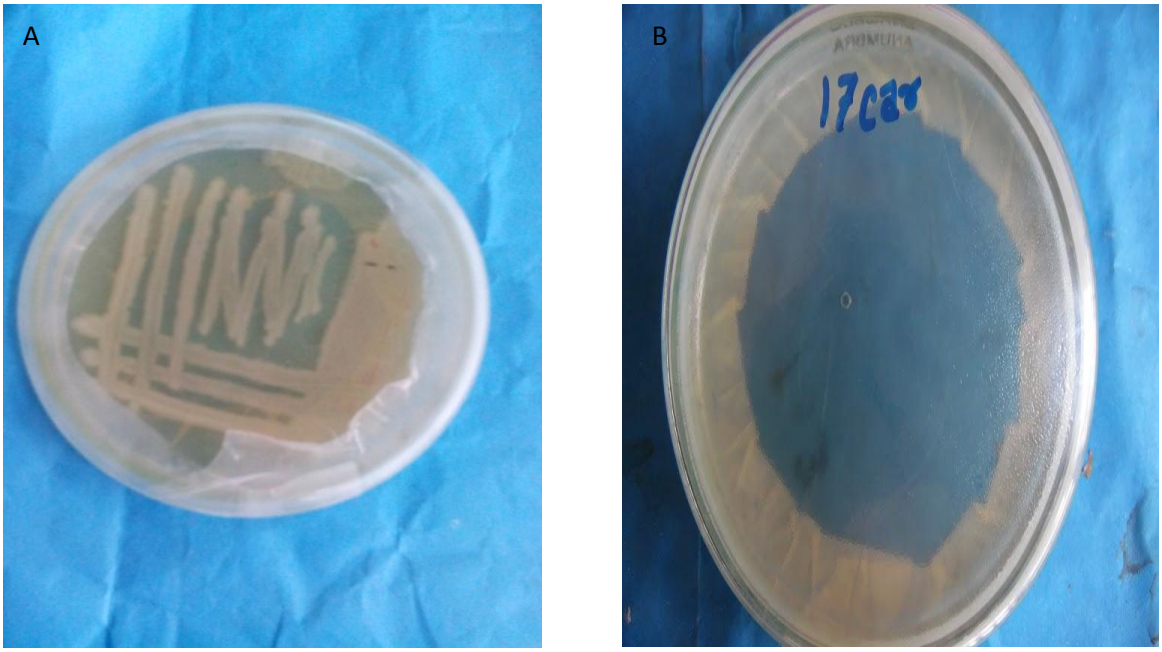
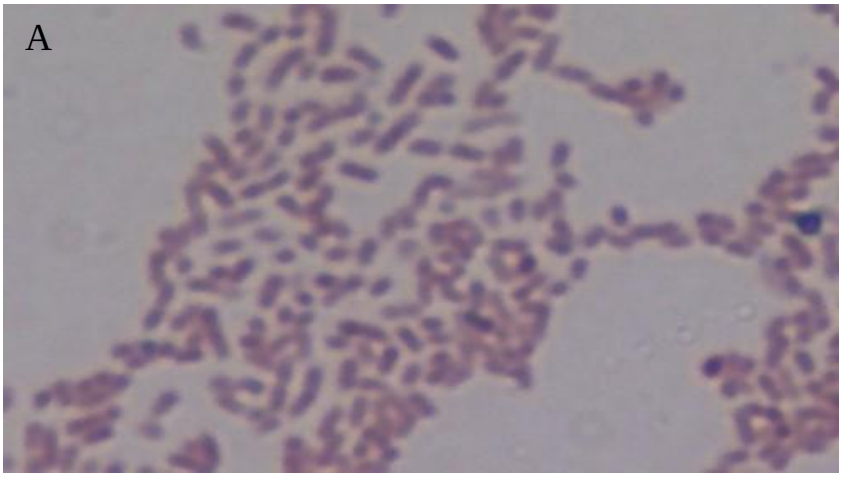


Plate. 3. Growth of *Ralstonia solanacearum* on NA media

A. Virulent colony.
B. Avirulent colony



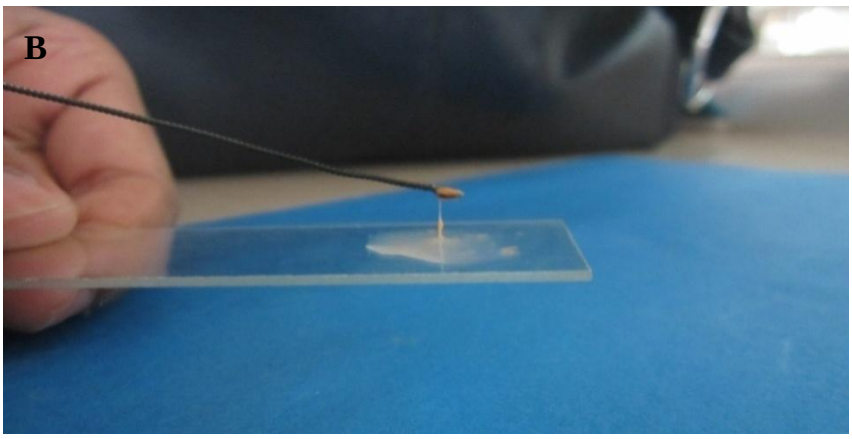


Plate. 4. Morphological characteristics of *Ralstonia solanacearum*

A. Microscopic view of *Ralstonia solanacearum* after gram's staining at 125x magnification

B. KOH solubility test for *Ralstonia solanacearum*

Fig. 1. Biochemical characteristics (Turbidity test) (Negative)

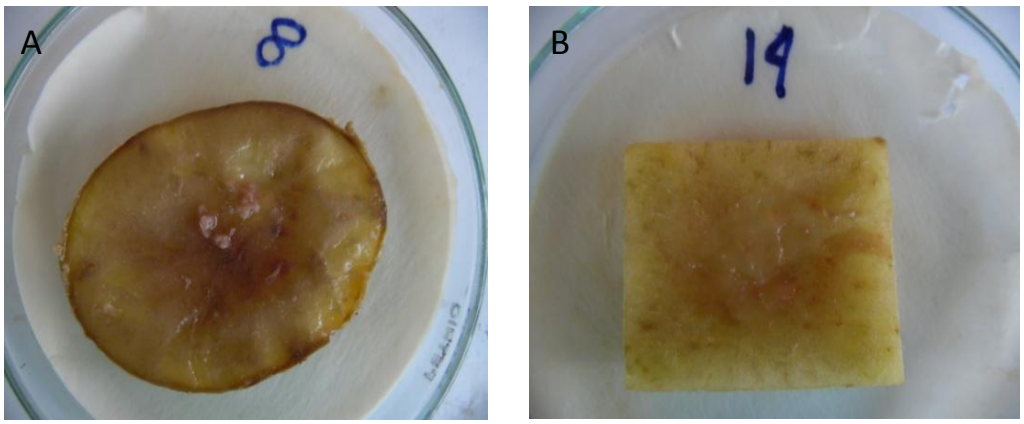


Plate. 5. Biochemical characteristics (Pectolytic test)

A. Positive
B. Negative

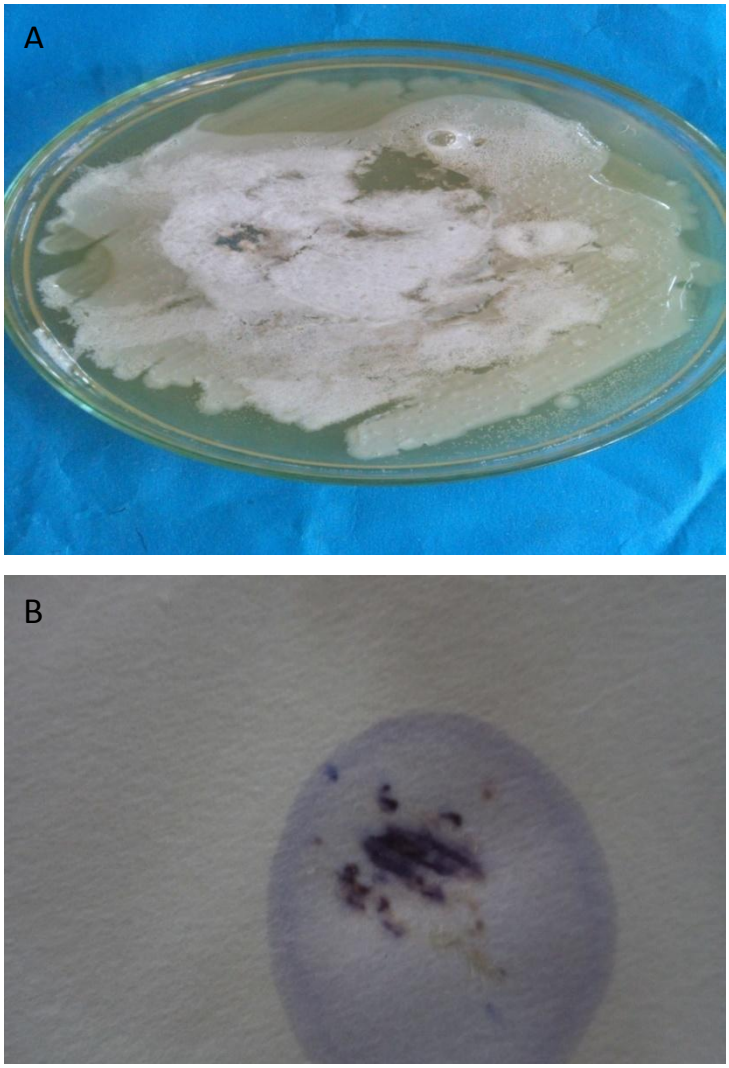


Plate. 6. Biochemical characteristics of *Ralstonia solanacearum*

A. Catalase test. (Positive)
B. Oxidase test. (Negative)

4.4.4. *In vitro* evaluation

4.4.4.1. *In vitro* evaluation of different agrochemicals

For the *In vitro* evaluation of different agrochemicals, two factors were considered for assessing the efficacy against *Ralstonia solanacearum*. The factors were the agrochemicals concentration taken and the other was the different agrochemicals used.

4.4.4.1.1. Efficacy of different concentration of agrochemicals against *Ralstonia solanacearum* at different days after incubation

In Table 6, treatment T_1 indicates the concentration 0.1%, T_2 for 0.3%, T_3 for 0.5% and T_4 stands for control water.

In case of 0.1% concentration, the highest inhibition zone (3.33 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (2.01 mm) was observed after 12 hours of incubation. On the other hand, no inhibition zone was observed in control condition (T_4). (Table 6).

In case of 0.3% concentration, highest inhibition zone (4.85 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (3.32 mm) was observed after 12 hours of incubation. On the other hand, no inhibition zone was observed in control condition (T_4). (Table 6).

In case of 0.5% concentration, highest inhibition zone (6.28 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (4.78 mm) was observed after 12 hours of incubation. On the other hand, no inhibition zone was observed in control condition (T_4). (Table 6).

Among the three agrochemicals concentration, the highest inhibition zone (6.28 mm) was shown in 0.5% concentration of agrochemicals and the lowest inhibition zone (2.01 mm) was shown in 0.1% concentration of agrochemicals. So, 0.5% concentration of agrochemicals was considered as the superior to other concentration, 0.3% as the moderate and 0.1% as low in consideration of efficacy.

4.4.4.1.2. Efficacy of different agrochemicals against *Ralstonia solanacearum* at different days after incubation

In table 7, treatment T_1 indicates the chemical Streptomycin, T_2 for Neomycin, T_3 for Cuprofix 50WP, T_4 for Tilt 250 EC, T_5 for Ridomil gold, T_6 for Indofil M-45, T_7 for Companion.

In case of Streptomycin, highest inhibition zone (7.33 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (5.33 mm) was observed after 12 hours of incubation, 6.33 mm was in 24 hours and 7.00 mm inhibition zone was shown in 36 hours of incubation. (Table 7).

In case of Neomycin, highest inhibition zone (9.00 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (7.00 mm) was observed after 12 hours of incubation, 7.66 mm was in 24 hours and 8.66 mm inhibition zone was shown in 36 hours of incubation. (Table 7).

In case of Cuprofix 50 WP, highest inhibition zone (5.00 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (3.33 mm) was observed after 12 hours of incubation, 4.33 mm was in 24 hours and 4.66 mm inhibition zone was shown in 36 hours of incubation. (Table 7).

In case of Tilt 250 EC, highest inhibition zone (3.66 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (1.66 mm) was observed after 12 hours of incubation, 3.00 mm was in 24 hours and 3.33 mm inhibition zone was shown in 36 hours of incubation. (Table 7).

In case of Ridomil gold, highest inhibition zone (4.33 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (3.00 mm) was observed after 12 hours of incubation, 3.00 mm was in 24 hours and 4.00 mm inhibition zone was shown in 36 hours of incubation. (Table 7).

In case of Indofil M-45, highest inhibition zone (4.33 mm) was observed after 48 hours of incubation, where the lowest inhibition zone (3.00 mm) was observed after 12 hours of incubation, 4.00 mm was in 24 hours and 4.00 mm inhibition zone was shown in 36 hours of incubation. (Table 7).

In case of Companion, highest inhibition zone (0.30 mm) was observed after 12 hours of incubation, where the lowest inhibition zone (0.11 mm) was observed after 24 hours, 36 hours and 48 hours of incubation. (Table 7).

From the above result, it was observed that the highest inhibition zone (9.00 mm) was made by the neomycin. So, it was evident that neomycin worked higher than the other agrochemicals used in the study for *In vitro* evaluation. The lowest inhibition zone was 0.11 m and it was made by companion. So, it had the lowest efficacy in relation to other agrochemicals. Streptomycin and cuprofix 50 WP had the high efficacy,tilt, ridomil gold and Indofil M-45 were the moderate in consideration of efficacy against *Ralstonia solanacearum*.

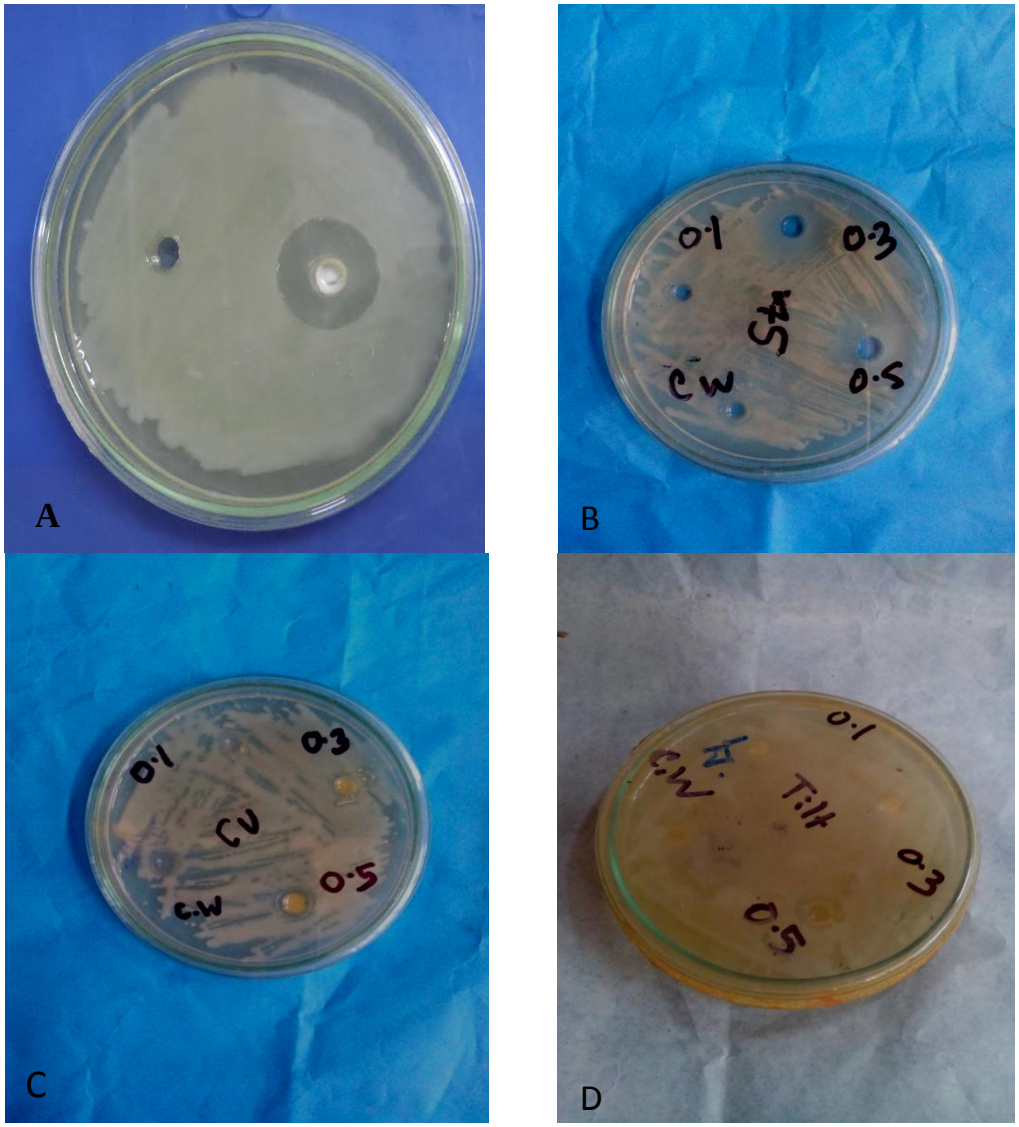


Plate. 7. *In vitro* evaluation of agrochemicals against *Ralstonia solanacearum* .

A. Neomycin.
B. Streptomycin.
C. Cuprofix 50 WP.
D. Tilt 250 EC.

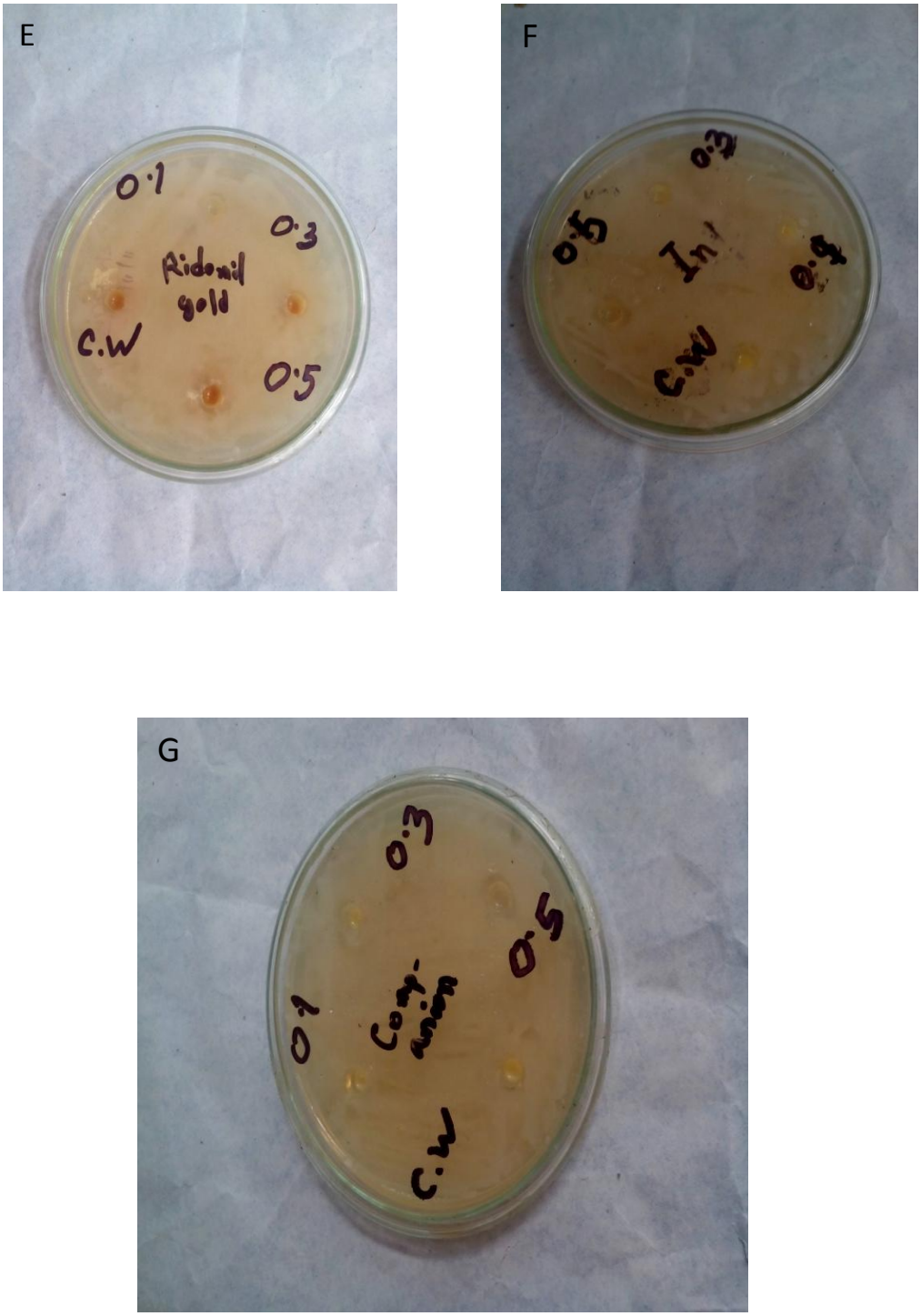


Plate. 8. : In vitro evaluation of agrochemicals against *Ralstonia solanacearum.*

E. Ridomil gold.

F. Indofil M-45.

G. Companion.

Table 6 : Efficacy of different agrochemicals concentrations used against *Ralstonia solanacearum*

Treatment	Inhibition zone (mm)			
	12hr	24hr	36hr	48hr
T_1	2.01 c	2.61 c	3.19 c	3.33 c
T_2	3.32 b	4.00 b	4.42 b	4.85 b
T_3	4.78 a	5.57 a	6.00 a	6.28 a
CV (%)	2.21%	3.10%	2.78%	2.61%
LSD	0.282	0.252	0.257	0.259
Level of significance	**	**	**	**

Each data represents the mean value of three replications

Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Here, the different concentration of agrochemicals were,

T_1 = 0.1%

T_2 = 0.3%

T_3 = 0.5%

Table 7 : Efficacy of different agrochemicals against *Ralstonia solanacearum* at different days after incubation

Treatment	Inhibition zone (mm)			
	12hr	24hr	36hr	48hr
T_1	5.33 b	6.33 b	7.00 b	7.33 b
T_2	7.00 a	7.66 a	8.66 a	9.00 a
T_3	3.33 c	4.33 c	4.66 c	5.00 c
T_4	1.66 e	3.00 e	3.33 e	3.66 e
T_5	3.00 d	3.00 e	4.00 d	4.33 d
T_6	3.00 d	4.00 d	4.00 d	4.33 d
T_7	0.30 f	0.11 f	0.11 f	0.11 f
CV (%)	2.21%	3.10%	2.78%	2.61%
s_ x	0.0816	0.0730	0.0733	0.0738
Level of significane	**	**	**	**

Each data represents the mean value of three replications

Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Here, T_1 = Streptomycin

T_2 = Neomycin

T_3 = Cuprofix 50 WP

T_4 = Tilt 250 EC

T_5 = Ridomil Gold

T_6 = Indofil M-45

T_7 = Companion

(Efficacy of different agrochemicals in relation to concentration against *Ralstonia solanacearum*)

Table 8. Efficacy of Streptomycin against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	3.00 f	4.00 f	4.00 f	4.00 g
0.3%	5.00 d	6.00 d	8.00 c	8.00 d
0.5%	8.00 b	9.00 b	9.00 b	10.00 b
CV (%)	2.20 %	3.0%	2.62%	2.54%
LSD	0.278	0.248	0.253	0.257
Level of significance	**	**	**	**

Each data represents the mean value of three replications

Table 9. Efficacy of Neomycin against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	4.00 e	4.00 f	6.00 d	6.00 e
0.3%	7.00 c	8.00 c	8.00 c	9.00 c
0.5%	10.00 a	11.00 a	12.00 a	12.00 a
CV (%)	2.23%	3.30%	2.82%	2.64%
LSD	0.283	0.254	0.259	0.261
Level of significance	*	*	*	*

Each data represents the mean value of three replications

Table 10. Efficacy of Cuprofix 50 WP against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	2.00 g	3.00 g	4.00 f	4.00 g
0.3%	3.00 f	4.00 f	4.00 f	5.00 f
0.5%	5.00 d	6.00 d	6.00 d	6.00 e
CV (%)	1.90%	3.12%	2.78%	2.62%
LSD	0.275	0.243	0.248	0.254
Level of significance	*	*	*	*

Each data represents the mean value of three replications

Table 11. Efficacy of Tilt 250 EC against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	1.00 h	2.00 h	2.00 h	3.00 h
0.3%	2.00 g	3.00 g	3.00 g	3.00 h
0.5%	2.00 g	4.00 f	5.00 e	5.00 f
CV (%)	1.61%	2.70%	2.41%	2.32%
LSD	0.232	0.215	0.218	0.236
Level of significance	*	*	*	*

Each data represents the mean value of three replications

Table 12. Efficacy of Ridomil gold against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	2.00 g	2.00 h	3.00 g	3.00 h
0.3%	3.00 f	3.00 g	4.00 f	4.00 g
0.5%	4.00 e	4.00 f	5.00 e	6.00 e
CV (%)	1.63%	2.74%	2.46%	2.34%
LSD	0.234	0.221	0.220	0.237
Level of significance	*	*	*	*

Each data represents the mean value of three replications

Table 13. Efficacy of Indofil M-45 against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	2.00 g	3.00 g	3.00 g	3.00 h
0.3%	3.00 f	4.00 f	4.00 f	5.00 f
0.5%	4.00 e	5.00 e	5.00 e	5.00 f
CV (%)	1.59%	2.68%	2.37%	2.29%
LSD	0.228	0.218	0.214	0.234
Level of significance	*	*	*	*

Each data represents the mean value of three replications

Table 14. Efficacy of Companion against *Ralstonia solanacearum*

Conc. (%)	Inhibition zone (mm)			
	12 hr	24 hr	36 hr	48 hr
0.1%	0.10 j	0.33 i	0.33 i	0.33 i
0.3%	0.30 ij	0.00 j	0.00 j	0.00 j
0.5%	0.50 i	0.00 j	0.00 j	0.00 j
CV (%)	1.47%	1.18%	1.18%	1.18%
LSD	0.118	0.106	0.106	0.106
Level of significance	*	*	*	*

Each data represents the mean value of three replications

4.4.4.1.2. Comparative efficacy of different agrochemicals against *Ralstonia solanacearum* at different concentrations at different days after incubation

After 12 hours of incubation, among seven agrochemicals Neomycin showed the highest inhibition zone (10.00 mm) at 0.5% concentration where Companion showed the inhibition zone (0.10 mm) at 0.1% concentration (Table 15).

After 24 hours of incubation, among seven agrochemicals Neomycin showed the highest inhibition zone (11.00 mm) at 0.5% concentration where Companion showed the inhibition zone (0.00 mm) at 0.3% concentration (Table 15).

After 36 hours of incubation, among seven agrochemicals Neomycin showed the highest inhibition zone (12.00 mm) at 0.5% concentration where Companion showed the inhibition zone (0.00 mm) at 0.3% concentration (Table 15).

After 48 hours of incubation, among seven agrochemicals Neomycin showed the highest inhibition zone (12.00 mm) at 0.5% concentration where Companion showed the inhibition zone (0.00 mm) at 0.3% concentration (Table 15).

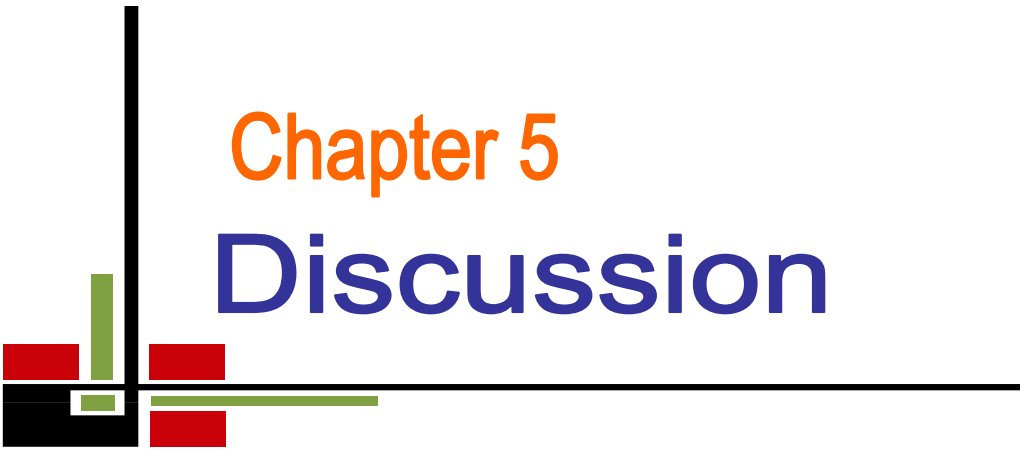
In vitro bioassay of agrochemicals revealed that highest inhibition zone was showed by Neomycin at 0.5% concentration than other agrochemicals used in the experiment, Streptomycin and cuprofix 50 WP had the high efficacy, tilt, ridomil gold and indofil M-45 was moderate and companion was the lowest in relation to efficacy against *Ralstonia solanacearum*. (Table 7).

Table 15 : Effectiveness of interaction between different fungicides and their concentration against *Ralstonia solanacearum* at different days after incubation

Treatment	Inhibition zone (mm)			
	12hr	24hr	36hr	48hr
$T1T_1$	3.00 f	4.00 f	4.00 f	4.00 g
$T1T_2$	4.00 e	4.00 f	6.00 d	6.00 e
$T1T_3$	2.00 g	3.00 g	4.00 f	4.00 g
$T1T_4$	1.00 h	2.00 h	2.00 h	3.00 h
$T1T_5$	2.00 g	2.00 h	3.00 g	3.00 h
$T1T_6$	2.00 g	3.00 g	3.00 g	3.00 h
$T1T_7$	0.10 j	0.33 i	0.33 i	0.33 i
$T2T_1$	5.00 d	6.00 d	8.00 c	8.00 d
$T2T_2$	7.00 c	8.00 c	8.00 c	9.00 c
$T2T_3$	3.00 f	4.00 f	4.00 f	5.00 f
$T2T_4$	2.00 g	3.00 g	3.00 g	3.00 h
$T2T_5$	3.00 f	3.00 g	4.00 f	4.00 g
$T2T_6$	3.00 f	4.00 f	4.00 f	5.00 f
$T2T_7$	0.30 ij	0.00 j	0.00 j	0.00 j
$T3T_1$	8.00 b	9.00 b	9.00 b	10.00 b
$T3T_2$	10.00 a	11.00 a	12.00 a	12.00 a
$T3T_3$	5.00 d	6.00 d	6.00 d	6.00 e
$T3T_4$	2.00 g	4.00 f	5.00 e	5.00 f
$T3T_5$	4.00 e	4.00 f	5.00 e	6.00 e
$T3T_6$	4.00 e	5.00 e	5.00 e	5.00 f
$T3T_7$	0.50 i	0.00 j	0.00 j	0.00 j
$T4T_1$	0.00 k	0.00 j	0.00 j	0.00 j
$T4T_2$	0.00k	0.00 j	0.00 j	0.00 j
$T4T_3$	0.00 k	0.00 j	0.00 j	0.00 j
$T4T_4$	0.00 k	0.00 j	0.00 j	0.00 j
$T4T_5$	0.00 k	0.00 j	0.00 j	0.00 j
$T4T_6$	0.00 k	0.00 j	0.00 j	0.00 j
$T4T_7$	0.00 k	0.00 j	0.00 j	0.00 j
CV (%)	2.21%	3.10%	2.78%	2.61%
$s_{\bar{x}}$	0.0816	0.0730	0.0733	0.0738
Level of significane	**	**	**	**

Each data represents the mean value of three replications

Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test



Chapter 5
Discussion

CHAPTER V

DISCUSSION

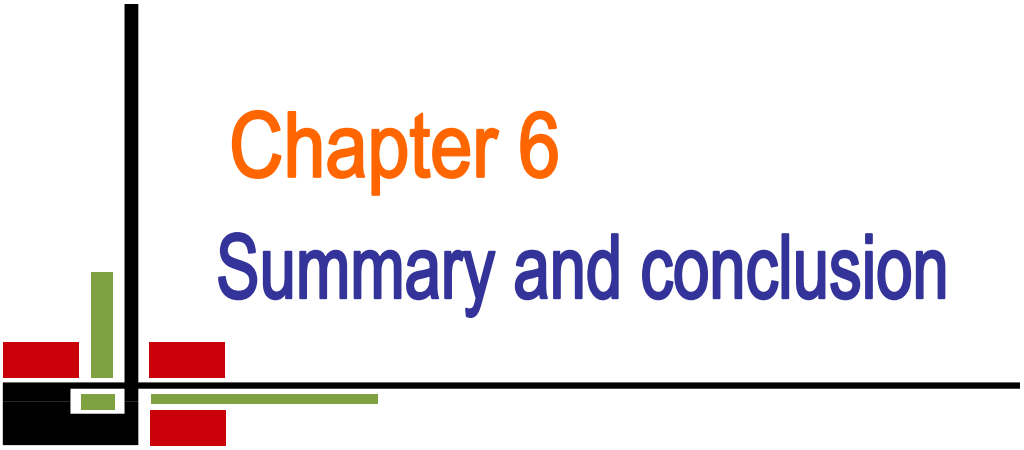
The diseased potato tubers of varieties cardinal, granola, diamant, asterix, laura and cumbica were collected from four potato distributing companies viz. Tradelink International Ltd., Ashmira, Shahan Trade Internetional and Hay Agro from four districts of Bangladesh viz. Rangpur, Naogaon, Lalmonirhat, Kaunia during the period of December 2014 to August 2015. An amount of one (1) kg potato per variety was used as working samples. The collected tuber samples did not show visible symptoms in the present study. The samples for the study collected from the potato distributing companies were just of one (1) month old. The potato tubers were so young to show the symptoms. In case of bacterial wilt of potato, minimum two (2) months require for the visible symptoms shown for the potato tuber (Champoiseau *et al*., 2009). In the present study the causal organism of bacterial wilt of potato (*Ralstonia solanacearum*) was isolated from diseased specimens following standard dilution plating technique using nutrient agar medium. Repeated isolation from the infected tubers yielded well separated, typical, lucid, convex, mucoid colonies of bacterium on nutrient agar medium after 48 hours of incubation at 30^{o}C. Isolation and identification of *Ralstonia solanacearum* was done using the protocol described by Wang (1998) and Hayward (1991). The causal agent of bacterial wilt of potato (*Ralstonia solanacearum*) was identified by conducting studies on it's morphological, biochemical and cultural features as per standard microbiological procedures.After the gram's staining under the compound microscope at 125x magnification with oil immersion, the bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red colour) and capsulated. (Murray *et al*., 2007). A mucoid thread was lifted with the loop in KOH solubility test that supports the result of gram's staining test i.e., the bacteria was gram negative. After the biochemical test, turbidity test was found as negative. As the samples were collected of one

(1) month old, so the test was found as negative in the bacterial streaming or turbidity test. In pectolytic test the causal agent of bacterial wilt of potato (*Ralstonia solanacearum*) was confirmed as avirulent strain as it was found negative for some samples and some samples showed positive result and confirmed as virulent strain. It was confirmed on the basis of colony morphology (Kelman, 1954). In the present study it was observed that *Ralstonia solanacearum* formed a satisfactory number of colonies on TTC after 48-72 hours of incubation. *Ralstonia solanacearum* also formed a large amount of colonies on NA medium after 48-72 hours of incubation.

In TTC medium, largely unmixed mucoid magenta deep red coloured colonies of *Ralstonia solanacearum* were developed for some samples collected and others developed pink coloured with whitish margins colonies of bacterium. Pink coloured with whitish margins colonies were identified as virulent and others were as avirulent. Circular, mucoid, convex, lucid coloured colonies of the bacterium were developed on NA medium for some samples and were identified as virulent. Some formed no colonies of bacterium and were identified as were identified as avirulent. It was confirmed according to the description of (Kelman and Person, 1954). It revealed that some can be identified as virulent and some were avirulent on the basis of morphological, biochemical and cultural features. Then the further study for the *In vitro* evaluation was carried on with the pathogenic bacteria.

The present study showed comparative efficacy of different agrochemicals at different concentration at different days of incubation. Among the evaluated agrochemicals in *In vitro* investigation, Neomycin [Dexamethasone] (12.00 mm) and Streptomycin [Streptomycin sulfate] (10.00 mm) exhibited superior efficacy at 0.5% concentration at 48 hours of incubation in inhibiting the growth of *Ralstonia solanacearum*. Stockwell and Duffy (2012) stated antibiotics are essential for controlling bacterial disease. They observed Streptomycin inhibits protein synthesis by binding to the bacterial ribosome. Cuprofix 50WP [Copper oxychloride) (6.00 mm), Ridomil gold [Mancozeb]

(6.00 mm), Indofil M-45 [Mancozeb] (6.00 mm), Tilt 250EC [Propiconazol] (5.00 mm) exhibited moderate efficacy in inhibiting the growth of *Ralstonia solanacearum*. Chaudhary *et al*. (2012) used copper oxychloride 50% and streptomycin in combination with each other to test the effectiveness of controlling bacterial disease. Ferrante and Scortichini (2010) used copper oxychloride, copper hydroxide, copper sulphate to observe the *in vitro* effectiveness and bactericidal activity of those agrochemical and found they completely inhibit the growth of test bacteria. Kennelly *et al*. (2007) recommended that copper compounds are the standard bactericides for controlling many bacterial diseases. In the experiment Companion [Mancozeb] failed to inhibiting the growth of *Ralstonia solanacearum*. Pawar *et al*. (2004) tested the efficacy of mancozeb in controlling the bacterial disease and found that application no inhibition zone over untreated control which corroborated with the result of present study. Based on the findings of the present study it may be concluded that antibiotics and copper based fungicides are effective against *Ralstonia solanacearum* which further should be tested *in vivo*.



Chapter 6

Summary and conclusion

CHAPTER VI

SUMMARY AND CONCLUSION

Potato (*Solanum tuberosum*) belongs to the family Solanaceae occupies the third important food crop in Bangladesh and the world's fourth largest food crop. It provides balanced source of starch, vitamins and minerals. Though It has the year round demand in Bangladesh, the production in terms of area and yield is not satisfactory due to lack of knowledge about the disease of potato. Tubers of potato are prone to attack by various diseases especially bacterial wilt. The least information are available regarding to the distribution, incidence, severity, epidemiology and management of the disease. Therefore, the present study was designed to the prevalence of bacterial wilt of potato and to study the effective management by different agrochemicals of the disease.

Bacterial wilt of potato is caused by *Ralstonia solanacearum*. It is a destructive pathogen for reducing the yield of potato. Even the fresh looked potato tuber can contain the pathogen in itself. In the present study, six varieties of potato tubers were collected from four districts of Bangladesh. As the potato tubers were of one (1) month old, those showed no visual symptoms. Even it contains no visible symptoms after the cutting of tuber. In consideration to the tender age of the potato tubers, they showed no visible symptoms but they had the possibilities of containing the pathogen *Ralstonia solanacearum*, the causal agent of bacterial wilt. So, isolation of *Ralstonia solanacearum* was done.

Causal organism of bacterial wilt, *Ralstonia solanacearum* was isolated from the infected tuber by following dilution plating technique using nutrient agar medium. Causal organism of bacterial wilt was purified by restreaking on nutrient agar medium with the colonies. The pathogen was identified by its morphological, biochemical and cultural features as per standard microbiological processes. The bacterium was gram negative, rod shaped with rounded ends. It showed positive result in catalase test and negative result in

oxidase test, turbidity test for some samples. Positive results were shown in pectolytic test for some samples and negative results for some samples. The bacterium developed largely unmixed mucoid magenta deep red coloured colonies (avirulent) and pink coloured with whitish margins colonies (virulent) on TTC medium. The bacterium appeared as circular, mucoid, convex, lucid colour colonies (virulent) and no colonies (avirulent) on NA medium.

In vitro of different agrochemicals were evaluated by measuring the inhibition zone at different concentration at different days of incubation. Among the seven different agrochemicals, *In vitro* evaluation of agrochemicals indicated that Deoxymethasone (Neomycin) at 0.5% concentration at 48 hours of incubation and D-Streptomycin (streptomycin) at 0.5% concentration at 48 hours of incubation were highly effective against *Ralstonia solanacearum*.

Present study revealed that the prevalence of *Ralstonia solanacearum* causal agent of bacterial wilt of potato is significant in four different districts of the country. Management of *Ralstonia solanacearum* responsible for bacterial wilt by different agrochemicals has the effective control significance against the pathogen. Neomycin, streptomycin, cuprofix 50WP at different concentration should be applied for the effective management of *Ralstonia solanacearum* causing bacterial wilt of potato. But regarding the management of destructive pathogen, *Ralstonia solanacearum*, causal agent of bacterial wilt of potato, detailed study is highly needed to be carried out.

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Appendices

APPENDICES

Appendix I. Preparation of culture media

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

Nutrient Agar (NA)

Beef extract (Difco)	3.0 g
Peptone (Difco)	5.0 g
Bacto agar	15.0 g
Distilled water	1000 ml

Nutrient Broth (NB)

Beef extract (Difco)	3.0 g
Peptone (Difco)	5.0 g
Distilled Water	1000 ml

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)	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 NNN'N-tetramethyl-p-phenylene-diamine dihydrochloride was prepared from the absolute solution.