EFFICACY OF SOME BIOCONTROL AGENTS IN CONTROLLING *Ralstonia solanacearum* CAUSING BROWN ROT OF POTATO

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CERTIFICATE

This is to certify that the thesis entitled **"EFFICACY OF SOME BIOCONTROL AGENTS IN CONTROLLING *Ralstonia solanacearum* CAUSING BROWN ROT OF POTATO"** submitted to the department of Plant Pathology, faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka-1207 in partial fulfillment of the requirements for the degree of **Master of Science (MS) in PLANT PATHOLOGY**, embodies the result of a piece of bona fide research work carried out by Registration No.: **14-05947,** 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.

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Dedicated To

My Beloved Family

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EFFICACY OF SOME BIOCONTROL AGENTS IN CONTROLING *Ralstonia solanacearum* CAUSING BROWN ROT OF POTATO

ABSTRACT

An experiment was conducted to investigate symptom, detection of pathogen and find out the efficacy of some BCAs on disease suppression as well as agronomical performance of potato. The experiment was carried out in a potato field in Gowalkhali of Sirajdikhan, in Munshiganj district and the *in vitro* research had been done at Disease Diagnostic Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka from November 2019 to October, 2020. Four BCAs namely Greenstree, Neutrase, ACI Bamper Trico , Biocoa which contained microbial agent called *Bacillus subtilis, B. amyloliquefaciens, Trichoderma* sp. and mixer of microbes respectively, were used as treatment. Each BCAs were used as soil application and foliar spray. Significant pathogen *Ralstonia solanacearum* was identified by morphological, biochemical and cultural test. Field application of the BCAs as spray and soil treatments significantly reduced wilting and the brown rot incidence. The brown rot incidence was in the range from 2.8 % to 35%. Application of *Bacillus subtilis* soil application showed the lowest incidence. The BCAs treatments improved the vegetative growth parameters such as plant height, leaf number/plant and average tuber weight compared to the control plants. The maximum number of leaves per plant was found in *Bacillus subtillis* soil application (19.86). Plant height was also high on this treatment. Again, the average weight of tuber were found in *Bacillus subtillis* in soil application(39.3g) while highest number of tuber per hill was found in foliar spray of *Trichoderma*(8.18*).* This experimental results suggested that the soil application of BCAs were the best to protect the potato tubers against bacterial brown rot disease and gave significant effect on the physiological growth and development of potato tubers. The soil applications of *B. subtilis is* the most promising BCA whose antibacterial effect reduced brown rot and promote tuber growth and development.

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LIST OF SYMBOLS AND ABBREVIATIONS

% = Percentage

et al. = And other

spp. = Species

J. = Journal

No. = Number

viz. = Namely

df. = Degrees of freedom

℃ = Degree Celsius

cm = Centimeter

cfu = Colony forming unit

ppm = Parts per million

NaCl = Sodium chloride

Kg = Kilogram

g = Gram

ml = Milliliter

WP = Wettable Powder

T = Treatment

ft = Feet (s)

pv. = Pathovar

var. = Variety

mm = Milimiter

µl = Microliter

µm = Micrometer

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a tuber crop belongs to the family Solanaceae. It is the 4th important crop after wheat, rice and maize in the world and Bangladesh is the 7th producer in the world for more than 98 lakh tons of potato production (BBS, 2021). This crop considers one of the most important vegetable either for local consumption and exportation (Gado, 2013). The area of potato production is still in increasing from 4.61 to 4.68 lakh hectares in Bangladesh (BBS, 2021).Average yield rate of potato has been estimated 21.096 metric ton per hectare (BBS, 2021). It is nutritionally considered a super vegetable as well as a versatile food item and it produces more carbohydrates per unit amount than either rice or wheat (Zinnat *et al.*, 2018).With comparison to cereals, potatoes have more protein, minerals, and dry matter per unit area. Some basic nutrients provided by the potato include minerals, dietary fiber, carbohydrates, and several minerals (Kadiri *et al.,* 2021).One of the major problems faced by developing countries in general and Bangladesh in particular, is the ever increasing population. In order to increase agricultural production further, the only option is to grow high productivity crops, like potato (Azimuddin *et al.,* 2019). The major potato growing areas of Bangladesh are Munshigonj, Jamalpur, Nilphamari, Jessore, Bogra, Pabna, Rangpur and Panchagorh. It contributes alone as much as 54% of the total annual vegetable production of Bangladesh (BBS, 2015).

Potato diseases are caused by different groups of pathogenic microorganisms. Twenty nine fungal, Thirty nine viral, seven bacterial and six nematode, thirteen physiological disorder, three phytoplasmic diseases have been recorded in potato. In Bangladesh potato suffer from various post-harvest diseases, but very few record are available of it. Brown rot of potato disease is caused by soil-borne bacterium *Ralstonia solanacearum* which is causing bacterial wilt in a very wide range of potential host plants (Prior *et al*., 2013; Agrios, 2008; Paret *et al.,* 2008 and Andersona and Gardner, 1999). The bacterium affects more than 30 plant species, the most susceptible crops being potato, tomato, eggplant, pepper, banana and groundnut (Priou *et al.,* 1999). It is one of the most destructive pathogens identified because it induces rapid and fatal wilting symptoms in the host plants. Potato brown rot caused by *Ralstonia solanacearum* (Saddler, 2005).It formerly known as *Pseudomonous solanacearum* is highly

challenging and one of the most destructive diseases of solanaceous crops worldwide (Hayward, 2005). Bringing about severe crop losses worldwide, the disease is now receiving global profile, *Ralstonia solanacearum* has exceptionally wide diversity having strains originating from different geographical origins and hosts (Hayward, 1991). *R. solanacearum* is responsible significantly in yield losses where about 450 plant species are recorded as hosts for this pathogen (Maji and Chakrabartty, 2014).

The disease is known to spread very quickly through furrow irrigation as well as rain water (Taylor *et al.*, 2011). Initially, leaf drooping occurs, followed by wilting. Wilted leaves may turn yellow and plants become stunted. Wilting may be expressed in one side of a leaf or branch. Plant symptoms are generally followed by tuber symptoms. Distinct browning of the vascular tissue of the tubers is evident. The roots and stolons may also show brown vascular discoloration. Infected plants may be localized or sparsely distributed in the field (Ali, 1995). The pathogen is able to colonize the exudation sites such as root extremities and axils of secondary roots. Thereafter it inter-cellularly infects the inner cortex and the vascular parenchyma and then invades the protoxylem vessels causing degradation to cell walls (Janse, 1996 and Tan *et al.*, 2016). In Bangladesh twelve diseases occurred in potato in which bacterial wilt is most important bacterial disease (DAE, 2015). Although the actual loss due to this bacterium in Bangladesh is still not reported. However potato export from Bangladesh to Russia was halted due to the presence of this bacteria in stored potato, which caused a loss about $9 million in 2014 (EPB, 2015).Currently, it is difficult to find an effective way to control the disease, because the pathogen is transmitted by irrigation water, soil, surface water, agricultural machines and infected organic material and can survive for a long time.

Chemicals are usually used to control plant diseases. However, in addition to the environmental pollution resulted by chemical pesticides and the induction of resistant strains of the pathogen, the agricultural chemical pesticides are ineffective in controlling the soil-borne bacterium (Li *et al*., 2016). Also, once the disease is established in the field, it cannot be controlled by chemical mean. The most widely used chemical treatment is fumigation by methyl bromide and disinfection of the infested farm areas using sodium hypochlorite, they are expensive, tedious and cannot be used extensively (Verma *et al.*, 2014). Up till now, no effective chemical product is available for controlling potato brown rot caused by *R. solanacearum*.

Excessive use of pesticides to control plant diseases is an important problem in the agricultural fields, so it is a priority study for biological control, because the current production systems demand the crop protection by innovative and environmentally methods compatible with sustainable agriculture as an alternative to chemical application (Kuc, 2001). Among the several methods of disease management, bio control plays an important role in disease control. Bio control may help development of alternative management measures or being integrated with other practices for effective control and for minimizing the environmental pollution due to use of chemical pesticides (Lwin and Ranamukhaarachchi, 2006 and Achari and Ramesh, 2014). This disease is almost incurable, some cultural, chemical and biological practices were found effective against brown rot disease of potato (Patrice, 2008; Anonymous, 2004; Basan, 2002 and Aspiras *et al.,* 1985). Many fungal and bacterial pathogens have been examined over a period of time for their potential as bio control agents (Bonev *et al.*, 2008). The biological control approach was successfully used to control many potato diseases. Antagonists belonging to the genus *Trichoderma* are among the most commonly isolated soil fungi. Due to their ability to protect plants and contain pathogen populations under different soil conditions, these fungi have been widely studied and commercially marketed as bio pesticides, bio fertilizers and soil amendments (Vinale *et al.* 2008). The Gram positive bacteria Bacillus subtilis and the Gram-negative Pseudomonas are widely used as biological agents against soil-borne pathogens. *Trichoderma* sp. have played a considerable role as bio-control agent (Papavizas, 1985) and is recognized as an effective bio-control agent against soil-borne plant pathogen (Gomez *et al.,* 1997).

Therefore, considering the above facts and points this research work was designed to achieve the following objectives:

Objectives:

- ✓ To isolate and identify the bacteria from potato.
- ✓ To evaluate the efficacy of some BCAs against *Ralstonia solanacearum* in field.
- ✓ To find out the effect of BCAs on the growth and yield of potato.

CHAPTER II
REVIEW OF LITERATURE

Ralstonia solanacearum constitutes a serious obstacle to the cultivation of many solanaceous plants in both tropical and temperate regions. The greatest economic damage has been reported on potatoes, tobacco and tomatoes. It can sometimes cause total crop losses. Hence, the literature pertaining to the brown rot of potato along with information on related crops disease and pathogen are reviewed here as under.

2.1. Symptoms of Brown Rot disease of potato

According to Karem and Hossain (2018), a plant showing wilting can be suspected to have *R. solanacearum* infection. The symptom starts with slight wilting of the leaves at the ends of the branches during the day which recovers at night; eventually, plants fail to recover which is soon followed by total wilting. Milky or cloudy threads like streaming signifies the presence of *R. solanacearum* of brown rot disease.

Chakraborty and Roy (2016) observed in their work that the earliest symptom is slight wilting of the leaves at the ends of the branches during the heating of the day which recovery at night; eventually, plants fail to recover and die which is soon followed, by total wilting. In advanced stage, as the disease develops, a streaky brown discoloration of the stem maybe observed on stems up to 2.5 cm or more above the soil line and the leaves turns into a bronze tint. On potato tubers, if infested plants have formed tubers, those will possibly also show symptoms. Two types of symptoms are produced in tubers, vascular rot and pitted lesions.

Kabeil *et al*., (2008) reported that the pathogen enters the vascular system of the plant and under favorable conditions cell numbers increase and spread up the stem and to tubers. In warmer regions, where transpiration rates the disease usually manifests itself as a general wilting of the shoot system (bacterial wilt).

Symptom expression occurs at different rates in different varieties and is favored by warm temperatures (above 15°C with optimum around 25°C) and other environmental conditions (especially high soil moisture). When the bacteria can latently infect tubers without causing noticeable symptoms, the pathogen can survive seed tubers during storage and cause disease at planting in the next season. (Annonymous, 2008).

2.2. Characteristics of Causal Organism

Van der Wolf and de Boer (2007) reported in their work that the bacterium is often considered a soil-borne vascular pathogen. Under favorable conditions, the pathogenic bacterium rapidly develops and causes economic damage to tuber yield. No effective control measure is available yet to control the brown rot pathogen. In general, bactericides are ineffective as crop production agents and their usefulness for disinfecting seed tubers is limited.

Elphinstone,(2005) described *R. solanacearum* as one of the world's most important phytopathogenic bacteria due to its lethality, persistence, wide host range, and broad geographic distribution.

Denny and Hayward,(2001) Dhital *et al*., (2001) reported that, the bacterium (*Ralstonia solanacearum*) showed positive results in starch hydrolysis test, catalase test, levan test, pecteolytic test and gelatine liquefaction test and negative result in oxidase test.

From the work Hayward (1991) described that, Typical, whitish, watery convex, mucoid, colonies of bacterium are produced on nutrient agar medium after 48 hours of incubation at 30˚C. The bacterium is rod shaped with rounded ends gram negative (red color) and capsulated, after gram's staining under the compound microscope at 100x magnification with oil immersion.

Kishun and Chand, (1991); and Celino *et al*., (1952) showed in KOH solubility test that a mucoid thread produce in KOH solubility test that supports the result of gram's staining test.

2.3. Efficacy of BCAs against *Ralstonia solanacearum*

Ceballos *et al.,* (2014) reported that In vitro, crude extracts of two strains and two commercial products of *Trichoderma* spp. inhibited 100% of *Ralstonia solanacearum. T. viride* and Ecoterra treatments showed low levels of disease severity by *R. solanacearum* in plants (0.63 and 1.88% respectively).

Murthy *et al.*, (2013) reported that *Trichoderma asperellum* was used as a biological control agent against bacterial wilt disease caused by *Ralstonia solanacearum*. Two isolates of *Trichoderma asperellum* (T_4 and T_8) exhibiting high antagonistic activity against a virulent strain of *Ralstonia solanacearum* (RS. Seed treatment with *T. asperellum* isolates significantly improved the quality of seed germination and seedling vigor. Higher accumulation of phenolics was noticed in plants pre-treated with T_4 and T_8 challenged with *Ralstonia solanacearum.*

Abd-El-Khair, H. and Seif El-Nasr. H. I. (2012) were used three biocontrol agents (BCAs) namely *Bacillus subtilis, Trichoderma album* and *Trichoderma hamatum,* isolated from commercial potato field and identified in the Department of Plant Pathology, National Research Centre in Egypt. *T. hamatum* completely protected the potato tubers against brown rot, than *T. album* and *B. subtilis* as well as the control. The BCA as soil treatments were more effective than tuber treatments for decreasing the BRI in potato and enhancing the growth and tuber yield parameters. This study revealed that *B. subtilis, T. hamatum* and *T. album* are promising as BCAs which are effective under field conditions for controlling potato brown rot.

Two bio control agents *Bacillus subtilis AP-10 and Trichoderma harzianum AP-001* alone or in combination were investigated in controlling three tobacco diseases including *R. solanacearum* .Neither *Bacillus subtilis* nor *Trichoderma harzianum* alone could control the bacterial wilt, but when combined, their controlling capabilities were as effective as a chemical treatment (Maketon *et al*., 2008).

Posas *et al.* (2007) reported that bacterial wilt caused by *R. solanacearum* is a serious threat for agricultural production. In this study, *Bacillus amyloliquefaciens* strains CM-2 and T-5 were found antagonistic to *R. solanacearum*. The possible mechanism of resistance inducement by the antagonistic bacteria was also evaluated .

Koller *et al.* (2006) reported that the natural control of several phyto-pathogens is based on the presence of suppressive soils where several bio-control microorganisms belonging to *Trichoderma, Pseudomonas* and *Bacillus* genera are detected.

2.4 Efficacy of BCAs on the growth and yield of potato cultivation

Elazouni *et al. (*2019) were revealed that *P. fluorescens* and *B. subtilis* were the highest for their activities against infection, followed by *P. aeruginosa* and then *Trichoderma* spp. They used three different potato cultivars were planted in soil infested with two virulent strains of *R. solanacearum* race 3 biovar 2. The results indicated that the soil treated with tested biological agents significantly stimulated the plant height, fresh weight, number of branches, dry weight, tuber number and potato weight/plant, up to 75.0 cm, 96.0 g, 6.0, 25.0 g, 10.0, 103.0 g, respectively, compared with control (plant only). Treatment with bio-control agents gives protection to the infected plants, resulting to an increase in growth parameters and yield of potato cultivars compared to pathogen control (infected plant).

Vinale *et al.* (2008) Showed that the BCAs produce numerous biologically active compounds including cell wall degrading enzymes and secondary metabolites for suppressing various pathogen.

Verma *et al.* (2007) reported that *Trichoderma* spp. have been widely used as antagonistic fungal agents against several pathogen as well as plant growth enhancers, namely cellulases, hemicllulases, proteases and *b*-1,3-glucanase.

According to Abd-El-Ghafar (2004) application of *B. subtilis*, *P. fluorescens*, *P. solanacearum* (avirulent strain) and *Streptomyces griseovirids*, as single or combination treatments, under artificial inculcation or naturally infested conditions decreased the wilt disease severity under greenhouse and field conditions and also significantly increased the potato yield in field applications.

Bustamante and Ciampi (1989) reported that use of the biocontrol agents successfully reduced brown rot and wilts disease in potatoes. *B. subtilis* and *B. amyloliquefaciens* have significant effect on the physiological growth and development of potato tubers.

CHAPTER II

MATERIALS AND METHODS

3.1. Experimental Site

The experiment was carried out in a potato cultivation land in Gowalkhali village at Sirajdikhan upzilla in Munshiganj district and the *in vitro* research had been done at Molecular Disease Diagnostic Laboratory of the Department of Plant Pathology in Sher-e-Bangla Agricultural University, Dhaka. Details experimental location has been given in Figure-1.

3.2. Period of Experiment

Field experiment was conducted during November 2019 to May 2020 and the laboratory research had been done from January-October-2020.

3.3. Experiments and Design of the experiment

Three experiments were carried out viz.

i. Study on wilt symptom, detection and isolation of causal organism.

ii. Study on effect of BCAs against *Ralstonia solanacearum* .

iii. Study on effect of BCAs on growth parameters of potato plant and tuber.

The experiment was conducted in randomized complete block design (RCBD) with three (3) replications and nine (9) treatments.

3.4. Land preparation

20 Decimal of medium high land with well drainage system was selected. The experimental field was first ploughed on 10th November 2019. The land was ploughed thoroughly with a power tiller and then laddering was done to obtain a desirable tilt. The clods of the land were pulverized to make the soil into small pieces. Weeds, stubbles and crop residues were cleaned from the land. The final ploughing and land preparation was done on 25th November, 2019.

3.5. Layout of Experimental Land

The field layout was done as per experimental design on 30th November, 2019. The field was divided into three blocks each of which representing a replication. The unit plot size was 2.5m × 1.8m and plot to plot distance was 0.5m and block to block distance was 0.75 meter.

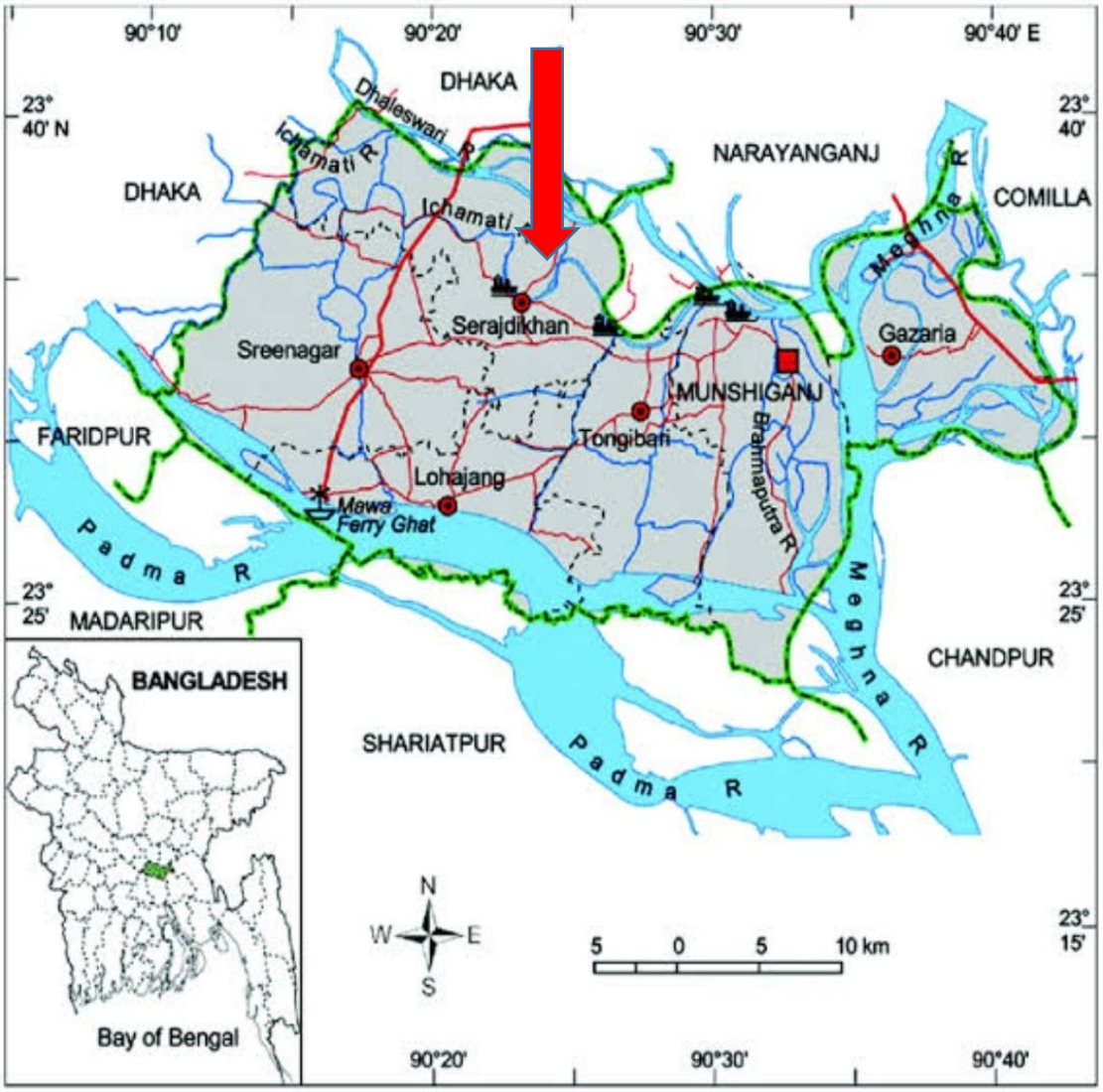

Figure 1. Map of the Experimental Site.

3.6. Variety selection and collection

The selected seed tuber variety was collected from cold storage of Bangladesh Agricultural Development Corporation (BADC), Munshiganj and the variety was Diamond.

3.7. Collection of Bio Control agents

Greenstree ,Neutrase and Biocoa powder were imported from China. ACI Bamper Trico- Powder was collected from Siddikbazar market, Dhaka.

3.7.1. BCA'S Used in the Experiment

Four BCA's were used. Their trade name and active ingredients are presented in Table-1.

Table 1 . BCAs used in the Experiment.

Sl. No	Trade name	Active Ingredients / BCA
1	Biocoa	Mixer of 50% *Bacillus subtilis* + 50% *Trichoderma sp*. 22 billion cfu/g WP
2	ACI Bamper Trico- Powder	*Trichoderma harzianum* 100 billion cfu/g WP
3	Greenstree	*Bacillus subtilis* 100 billion cfu/g WP
4	Neutrase	*Bacillus amyloliquefaciens* 50 billion cfu/g WP

Figure.2. Bio control agents used against *Ralstonia solanacearum*

A- Biocoa

B- ACI Bamper Trico

C- Greenstree

D- Neutrase

3.7.2. Application Method of BCAs

Two methods of BCAs application were used in this study; in first application method potato seed tubers were treated with each antagonist as seed dressing. Potato seed tubers (35-45 mm size) were mixed with each BCA mixture with recommended dose for five minutes and then spraying on soil before seven days of sowing. Secondly, BCAs were sprayed 30,45 and 60 days after planting. This application method discussed by Abd-El-Sayed *et al*.,(2003); Kabeil *et al.*,(2008). There were used nine (9) treatments, four (4) treatments were used at the time of seed dressing after then applied in soil before seven (7) days of planting and another four (4) treatments were used as foliar spray to evaluate their efficacy against *Ralstonia solanacearum* .

Table. 2. Application method and Dose of Treatments

Treatments	Application Method	Dose
T_1	Application of Biocoa on soil	5.0gm / 1L water
T_2	Foliar spray of Biocoa	5.0gm / 1 L water
T_3	Application of ACI Bamper Trico on soil	3.0gm / 1L water
T_4	Foliar spray of ACI Bamper Trico	3.0gm / 1L water
T_5	Soil application of Greenstree	2.0gm / 1L water
T_6	Foliar spray of Greenstree spray	2.0gm / 1L water
T_7	Soil application of Neutrase Soil	2.5gm / 1L water
T_8	Foliar spray of Neutrase	2.5gm / 1L water
T_0	Control	

3.8. Symptomological Study (Visual assessment)

Symptomological study was done during the time of cultivation. The diseased plant parts (potato) were carefully examined visually to observe the disease symptom development and, sign of the pathogen. Idea about causal organisms (fungi, bacteria, nematode and virus) was taken from these information (Pernezny *et al.*, 2008; Mullen, 2007).

3.9. Collection of Specimen

During the time of harvesting diseased potato tubers were collected from research field. The samples were preserved temporarily in air tight zip locked poly bags and tagged for later convenience. Then the samples were carried to the Plant Disease Clinic of SAU. The collected samples were preserved in the laboratory following standard procedure.

3.10. Isolation and Detection of causal organism of brown rot of potato

3.10.1. Preparation of Nutrient Agar (NA)

Nutrient agar media was prepared according to the method followed by Schaad *et al.*, (2001). At first 15 g 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 the preparation of 1 liter NA medium. For mixing properly the nutrient agar was shaken thoroughly for few minutes. It was then autoclaved at 121°C under 15 PSI pressure for 15 minutes.

3.10.2. Isolation of causal organism on NA media

The causal organism of brown rot of potato was isolated by dilution plate method. The diseased potatoes were washed under running tap water. And then was cut into small pieces. Surface sterilization was done by dipping them in 5% sodium hypochlorite solution for 2-3 minutes. It was then 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. 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. Then 0.1 ml of each dilution was spread over NA plate previously dried (to remove excess surface moisture) at three replications as described by Goszczynska and Serfontein (1998). Glass-rod was used for spreading. The inoculated NA plates were kept in incubation chamber at 30˚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.

3.10.3. Isolation on TTC medium

For isolation of causal organism from infected potato specimens, streak plate technique was followed using a selective medium, Tetrazolium chloride agar (TZC) as described by Kelman (1954). The medium contained peptone 10 g, casein hydrolysate 1 g, glucose 5 g, and agar 20 g in 1000 ml of distilled water. The mixture was cooked, pH was adjusted to 7.0 using 0.1N KOH and autoclaved at 121˚C under 1.1 kg/cm2 pressure for 20 minutes. Aqueous solution of 2, 3, 5- triphenyltetrazolium chloride (TTC) was prepared by dissolving 1g of the chemical in 100 ml of distilled water in an Erlenmeyer flask. The 1% stock solution of TTC solution was separately sterilized by passage through 0.45µm pore size filters (Millipore). The sterilized TTC solution was poured into the sterilized medium at the rate of 5 ml/1000 ml before solidification and mixed thoroughly. The medium was poured into petri plates (9 cm) at the rate of 20 ml/plate.

The TTC was kept in a colored bottle and was wrapped with aluminum foil to avoid light and preserved in a refrigerator at 40˚C for future use. The surface sterilized pieces of potato tuber were immersed in 5 ml of sterilized distilled water in a test tube for oozing. The bacterial ooze released from the infected tuber was thoroughly mixed in water after discarding the tuber pieces. One loopful of suspension was streaked on the TZC agar medium in Petri plates and virulent colonies were identified on the basis of characteristic colony characters on TZC medium (Kelman, 1954).

3.11. Morphological and Biochemical tests

3. 11.1. Gram's staining

A small drop of sterile water was placed on a clean microscope slide. Part of a young colony (18-24 hrs 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 spread bacterial film was air dried. Underside of the glass slide was heated by passing it four times through the flame of a sprit lamp for fixing the 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 at 40x and 100x magnification using oil immersion.

3.11.2. KOH solubility test

It is a rapid method for gram differentiation of plant pathogenic bacteria without staining (Suslow *et al.*, 1982). Two drops of 3% KOH solution were placed at the centre of a clean glass slide. One loopful colonies of bacterial pathogen (grown NA medium) were added to the KOH solution and homogenized with a nichrome loop with rapid circular movement of about 10 seconds. Viscous strand formation was observed and on drawing it with a loop it formed a fine thread of slime, 0.4 to 2.5 cm in length.

3.11.3. Oxidase test

This test is particularly valuable for differentiating Pseudomonalds from certain other gram negative rods (Shekhawat *et. al.*, 1992). Aerobic or facultative anaerobic bacteria, i. e., those with respiratory activity are divisible into two groups, those which are oxidase positive and those which are oxidase negative. An oxidase positive reaction transport is indicative of the presence of a cytochrome- C-Oxidase in the respiratory electron chain. Among Pseudomonalds, the test has important differential value because isolates of R. solanacearum give positive reaction. Tetramethyl-p- 15 phenyl

diamine is oxidised by the cytochromecytochrome oxidase system of the bacterium to a purple compound. Aqueous solution of (1%) of tetramethyl-pphenylenediamine is used as test reagent. A strip of Whatman filter paper (NO-2) was soaked with 3 drops of 1% aqueous solution of freshly prepared tetra methyl- pphenylene- diamine dihydrochloride (color indicator). A loopful of young bacterial culture (TTC medium) of each isolate was rubbed separately on the impregnated surface of the filter paper stripe by a platinum loop. Purple color develops within 10 seconds, which indicated positive reaction of oxidase test.

3.11.4. Pecteolytic test

Potato tubers were disinfected with 99% ethanol, cut up into slices of about 7-8 mm thick, and then placed on moistened sterile filter paper in sterile Petri dishes. Bacterial cell suspension was pipetted into a depression cut in the potato slices. One potato slice pipetted with sterile water was treated as control. Development of rot on the slices was examined 24–48 h after incubation at 25 °C. Examination was done for 5 days after inoculation. Two slices were inoculated for each isolate.

3.11.5. Gelatine liquefaction test

One loop-full bacterial culture was stub inoculated into the tube containing 12% (w/v) gelatine with the help of a sterile transfer loop. Then it was incubated at 30℃ for 24 hours. Gelatin liquefied microorganism was determined by the formation of liquid culture after keeping it at 5˚C in refrigerator for 15 minutes.

3.11.6. 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˚C for 24 hours to observe whether levan is produced or not.

3.11.7. 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.

3.12. Determination of Brown rot disease incidence

Harvesting was done on 21th March 2020. At harvesting time, tubers yield of ten (10) potatoes were randomly collected from each replicate and marked separately for each treatment. The effect of BCAs treatments on the incidence of brown rot disease was calculated as the percentage of infected potato tuber in relation to the total tuber yield.

$$Disease\ incidence\ (DI) = \frac{\text{No.of infected tubers}}{\text{Total no.of tuber observed}}\ \text{X}\ 100$$

3.13. Vegetative growth and Potato tuber yield parameters

Ten potato plants were randomly chosen from each replicate, for each treatment as well as control, after 60 days of sown. The effect of BCA treatments on the average leaves per plant , plant height, and number of stems per pit were determined following Abou-Hussein *et al.* (2002). At harvest time data on the average total potato tuber yield (g) per plant and the average tuber weight (g) at each BCA treatment were determined.

3.14. Statistical analysis

Data collected during experimental period were compiled and tabulated in Microsoft Excel 2013 and analyzed with Statistical package program STATISTIX 10.0.Treatment means were compared with Least Significance Difference Test (LSD) (Gomez and Gomez, 1984). The value of LSD at the significance level of 5% was used for comparison between the data mean.

CHAPTER IV

RESULTS

This chapter includes the experimental results. During growing season symptoms of wilted plant and data on growing plants including effect of BCSs on potatoes were assessed.

4.1. Detection of infected plant and Symptom study

Typical symptoms of wilting, yellowing and rapid death of the plant were observed. Plant showing wilting of younger leaves were suspected to have *R. solanacearum* infection. The symptom started with slight wilting (Figure 3A) of the leaves at the ends of the branches which later fall to total wilting. In streaming test milky or cloudy threads like (Bacterial ooze) streaming came out that indicated the presence of *R. solanacearum* (Figure 3B). Infected potato tubers formed vascular rot and pitted lesions (Figure 3C).

Figure 3.Brown rot symptom of potato: (A) Early symptom (B) Bacterial ooze in stem (C) Vascular rot in tuber.

4.2. Isolation of brown rot pathogen from potato tuber

Ralstonia solanacearum isolated from infected tuber which yielded well separated, typical, white, convex, mucoid, irregular watery colonies of bacterium on nutrient agar medium after 48 hours of incubation at 30ºC (Figure 4A.). Colonies were purified by re-streaking the isolated colony on nutrient agar plate. The bacterial pathogen 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 30ºC on TTC medium (Figure 4B). Colonies were purified by re-streaking the isolated colony on TTC plate. Typical, whitish, watery convex, mucoid, colonies of bacterium were produced on nutrient agar medium after 48 hours of incubation at 30˚C (Schaad *et al*., 2001). The bacteria produced small whitish with pink centered colony on TTC medium (Kelman, 1954) those are supportive to our research work findings.

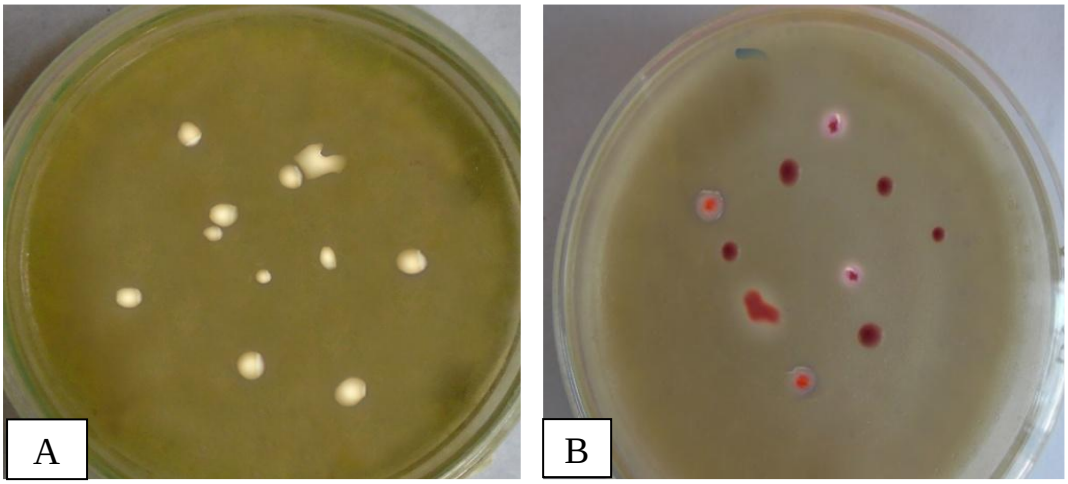

Figure 4. Culture of *Ralstonia* on (A) Nutrient Agar medium; (B) TTC medium

4.3. Identification of the pathogen

Brown rot pathogen was identified by studying morphological, biochemical and cultural features of the pathogen as per standard microbiological procedures.

4.3.1. Morphological Characteristics of *Ralstonia solanacearum*

The bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red color) and capsulated under the compound microscope at 100x magnification with oil immersion. The cells were readily stained with common stains such as crystal violet (Figure-5A) in gram staining test.

In KOH solubility test, a mucoid thread was produced by the bacteria (Figure-5B). Therefore the test was positive i.e., the bacterium was gram negative that supports the result of gram's staining test. 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. Similar result in KOH solubility test was found by Schaad, (1992), Kishun and Chand, (1991); and Celino *et al*., (1952).

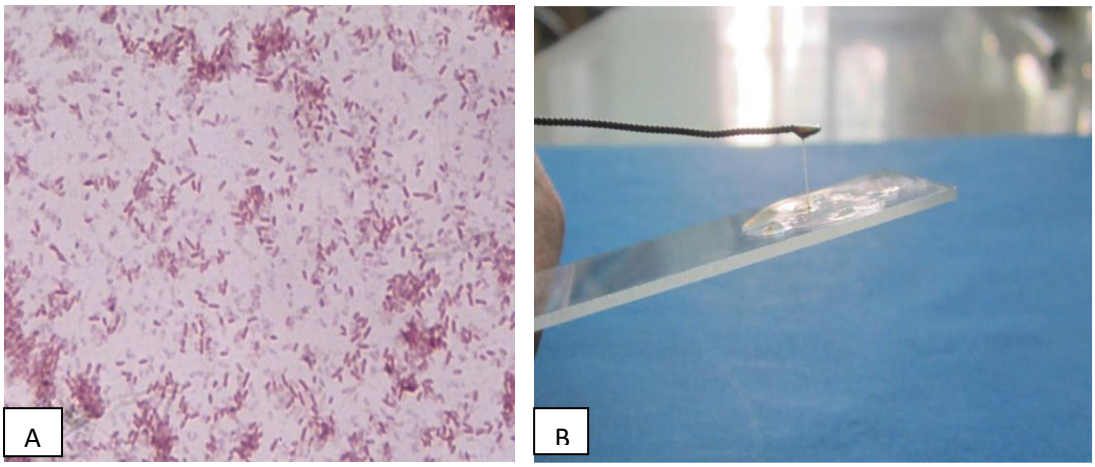

Figure 5. Morphological identification of *Ralstonia solanacearum*

(A) Gram staining test; (B) KOH solubility test

4.3.2. Biochemical Characteristics of *Ralstonia solanacearum*

Biochemical characteristics of isolates were studied in order to check similarity of biochemical features with genus *Ralstonia* by subjecting to various biochemical tests as shown in Table 3.

Table 3. Responses of the Isolated Bacteria in Different Biochemical test Media

Biochemical tests	**Results**
Catalase test	Positive
Oxidase test	Positive
Starch hydrolysis test	Positive
Gelatine liquefaction test	Positive
Levan test	Positive
Pecteolytic test	Positive

In Oxidase test, after rubbing the bacterium onto the moistened whatman filter paper ,Purple color develops within 10 seconds, which indicated that test result was positive (Figure 6A). In Starch hydrolysis test, a clear zone was formed after adding lugol's iodine around the bacterial colony indicated starch hydrolysis (amylase activity) i.e., the test was positive (Figure 6B).In Catalase test, bubbles were formed after adding 3% H_2O_2 onto the colony of the bacterium within a few seconds (Figure 6C), which revealed that the test was positive. In Pecteolytic test the bacteria showed positive result. After incubation for 48 hours the bacterium was able to rot the potato (Figure 6D).In Gelatine liquefaction test, after 15 minutes of refrigeration at 5˚C, gelatin was liquefied (Figure 6E). Thus the bacterium showed the positive result. In Levan test after incubated at 30˚C the bacteria produced levan thus the bacterium showed positive result (Figure 6F).

On the basis of morphological, biochemical and cultural characteristics the causal organism of brown rot of potato was identified as *Ralstonia solanacearum*

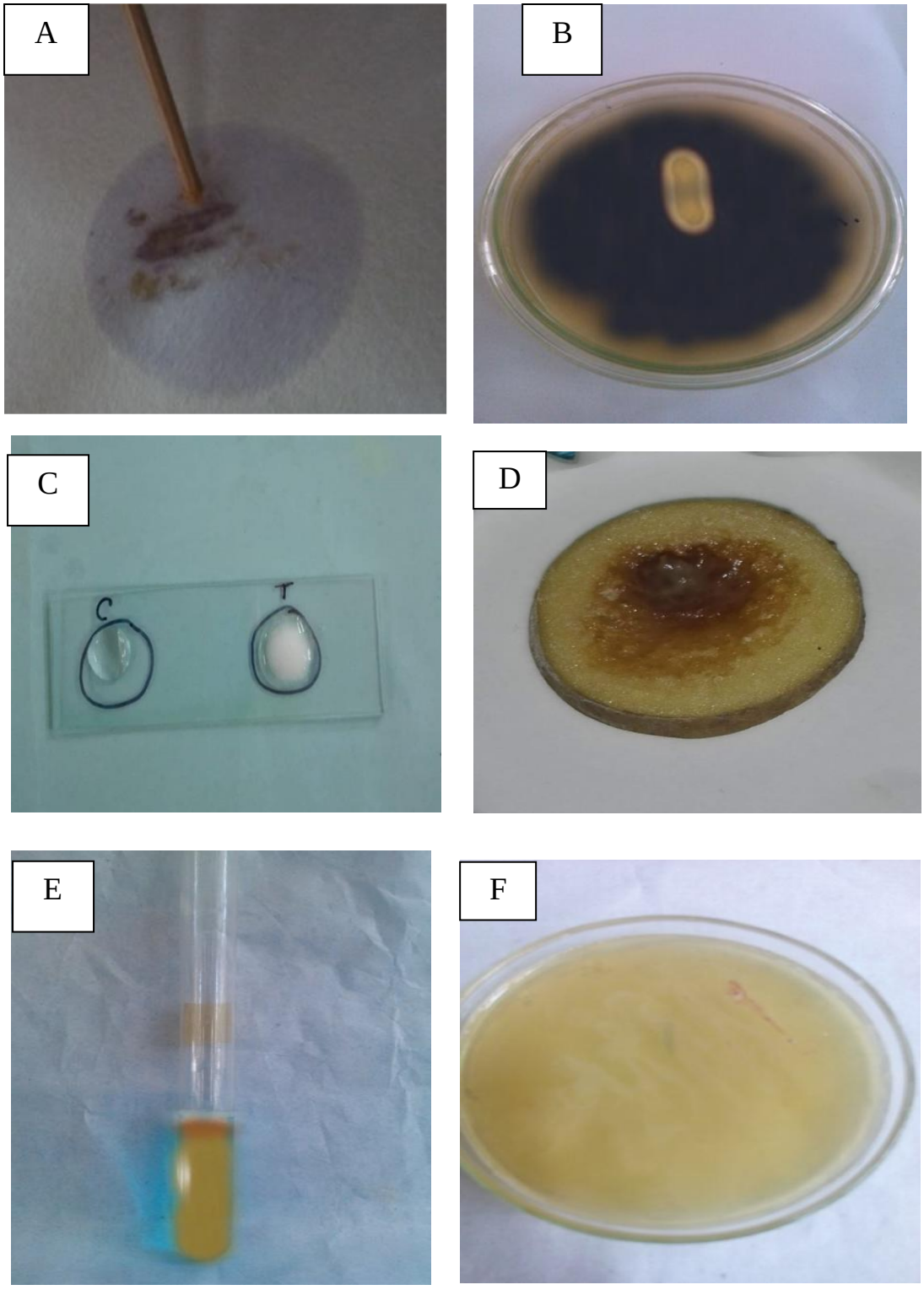

Figure 6. Biochemical test result for identification of causal organism;

A. Oxidase test B. Starch hydrolysis test C. Catalase test

D. Pecteolytic test E.Gelatin liquefaction test F. Levan test

4.4. Efficacy of biocontrol agents (BCAs) on brown rot incidence of potato tuber

Efficacy of biocontrol agents (BCAs) on bacterial wilt incidence of potato were tested by applying different treatments (Figure.7). A total 100 plants were observed in the experimental field which contained approx.1000 plantations.

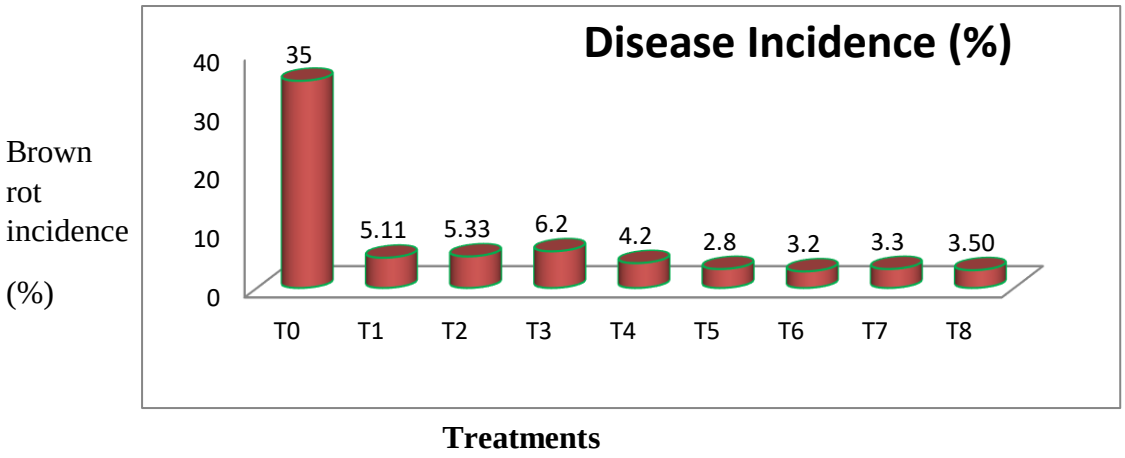

Figure 7. Effect of Biocontrol Control agents (BCAs) on brown rot incidence of potato tubers

T_0= Control ,T_1= Application of Biocoa on soil, T_2= Foliar spray of Biocoa, T_3= Application of ACI Bamper Trico on soil, T_4= Foliar spray of ACI Bamper Trico, T_5=Application of Greenstree on soil , T_6= Foliar spray of Greenstree spray, T_7= Application of Neutrase on soil, T_8= Foliar spray of Neutrase

Result showed that disease incidence of brown rot of potato varied between BCAs and ranged from 2.8% to 35% (Figure.7). The highest incidence 35% of brown rot of potato was found in T_0 (35%) followed by T_3 (6.2%), T_2(5.3%). Lowest incidence was found in T_5 (2.8%), followed by T_6 (3.20%) and T_7 (3. 30%).So, Due to use the treatments of bio control agents in the field of potato cultivation the disease incidence became lower than control conditions.

4.5. Effect of bio-control agents on vegetative growth and yield parameters under field conditions

Table 4. Effect of different treatments on vegetative growth

Treatment	Leaves/Plant	Plant Height	Tuber / Hill
T_0	**16.66c**	**28.86b**	**5.49g**
T_1	19.46ab	35.63ab	7.45bc
T_2	18.06abc	29.26ab	6.17efg
T_3	17.60abc	29.26ab	6.45def
T_4	18.40abc	32.06ab	**8.40a**
T_5	**19.86a**	**36.53a**	8.18ab
T_6	19.33abc	32.73ab	7.14cd
T_7	18.33abc	33.93ab	7.00cde
T_8	16.80bc	30.20ab	5.89fg
CV (%)	8.78	13.21	7.51
LSD (5%)	2.7773	7.33	0.9605

(Each data represents the mean value. Values followed by the same letter within a column are not significantly different ($p \leq 0.05$))

T_0= Control ,T_1= Application of Biocoa on soil, T_2= Foliar spray of Biocoa, T_3= Application of ACI Bamper Trico on soil, T_4= Foliar spray of ACI Bamper Trico, T_5=Application of Greenstree on soil , T_6= Foliar spray of Greenstree spray, T_7= Application of Neutrase on soil, T_8= Foliar spray of Neutrase

Result showed that number of leaves per plant, plant height and tuber/hill varied with different BCAs treatment.

The number of leaves per plant varied ranged from 16.66 to 19.86. The highest number of leaves was found in T_5 (19.86) followed by T_1 (19.46), T_4 (18.40) and T_7 (18.33).The lowest number of leaves plant was found in T_0 (16.66) followed by T_7 (16.80), T_3 (17.60) and T_2 (18.06).The height of plant varied with 28.86cm to 36.53cm. The tallest plant was found in T_5 (36.53cm) followed by T_1 (35.63cm), T_7 (33.93cm), and T_6 (32.73cm).The shortest plant was was found in T_0 (28.86cm) followed by T_3 (29.26), and T_8 (30.20).

The number of tuber per hill varied from 5.49 to 8.40.The highest number *o*f tuber per hill was found in T_4 (8.40) followed by T_5 (8.18), T_1 (7.45) and T_6 (7.14).The lowest tuber per hill which was found in T_0 (5.49) followed by T_2 (6.17), T_3 (6.45), T_7 (7.00).Results of the experiment showed that BCAs were significantly increased leaves per plant, plant height and tuber/hill of potato.

4.6. Effect of bio-control agents on Yield

Table 5. Effect of different treatments on average weight of tuber

Treatment	Average weight of tuber
T_0	**21.75e**
T_1	34.33bcd
T_2	34.06cd
T_3	36.06abcd
T_4	36.50abc
T_5	**39.03a**
T_6	32.53d
T_7	37.80ab
T_8	37.56abc
CV (%)	2.31
LSD (5%)	2.01

(Each data represents the mean value. Values followed by the same letter within a column are not significantly different ($p \leq 0.05$))

T_0= Control ,T_1= Application of Biocoa on soil, T_2= Foliar spray of Biocoa, T_3= Application of ACI Bamper Trico on soil, T_4= Foliar spray of ACI Bamper Trico, T_5=Application of Greenstree on soil , T_6= Foliar spray of Greenstree spray, T_7= Application of Neutrase on soil, T_8= Foliar spray of Neutrase

Result showed that, average weight of tuber varied with the application of different BCAs treatments. The average weight of tuber varied from 21.75gm to 39.03gm.The highest average weight of tuber of potato was found in T_5 (39.03gm), followed by T_7(37.80gm), T_8 (37.56gm) and T_4(36.50kg).The lowest average weight of tuber was T_0 (21.75gm) followed by T_6 (32.53gm*)* and T_2 (34.06gm).Due to the application of BCAs average weight of tuber potato was higher than control. Results of the experiment showed that all tested BCAs, when applied as treatment, significantly increase average weight of tuber as well as increase yield of potato.

CHAPTER V

DISCUSSION

The experiment was conducted to investigate symptom, detection of pathogen and find out the efficacy of BCAs on disease suppression as well as agronomical performance of potato. Typical symptoms were observed on experimental site. The symptom started with slight wilting of the leaves at the ends of the branches during the day which recovers at night; eventually, plants failed to recover which was soon followed by total wilting. Milky or cloudy threads like streaming signifies the presence of *R. solanacearum* of brown rot disease. The symptoms observed were similar to the observation of Karem and Hossain (2018).

In the present study, the causal organism of brown rot of potato (*Ralstonia solanacearum*) was isolated from infected potatoes collected from experimental site following standard dilution plating technique using nutrient agar medium and TTC selective medium. Typical, whitish, watery convex, mucoid, colonies of bacterium were produced on nutrient agar medium after 48 hours of incubation at 30◦C. 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 with oil immersion. Isolation and identification of *R. solanacearum* was done using the protocol described by Hayward (1991*)*. 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. Similar result in KOH solubility test was found by Schaad, (1992), Kishun and Chand, (1991); and Celino *et al*., (1952). In the present study the bacterium (*Ralstonia solanacearum*) showed positive results in starch hydrolysis test, catalase test, levan test, pecteolytic test and gelatine liquefaction test and negative result in oxidase test.The result of the present study was in agreement with the report Denny and Hayward, (2001) Dhital *et al*., (2001) and Christ, (1998).Field application of the BCAs as spray and soil treatments significantly reduced the brown rot incidence and bacterial wilt severity. The use of BCAs in controlling potato diseases had also been studied by many workers around the world (Abd-El-Khair, H. and Seif El-Nasr, H. I. (2012), Alabouvette *et al*., 2006, Jacobson 2002).In the present study the brown rot incidence was in the range from 2.8 % to 35%. *Bacillus subtillis* soil application showed the lowest incidence. Abd-El-Khair, H. and Seif El-Nasr. (2012) also studied use of *Trichoderma and Bacillus subtilis* soil application reduced the brown rot incidence from 15.3 to 21.3%

in the untreated plants and reported that incidence was *B. amyloliquefaciens* application on soil completely protected the potato tubers against brown rot compared with *Bacillus subtilis* soil application as well as control. BCAs significantly reduced the incidence of wilt in treated potato plants. These results are in agreement with Abd-El-Ghafar (2004) who reported that the BCAs used successfully reduced brown rot and wilts disease in potatoes. Lemessa and Zeller (2007) also showed that using antagonistic isolates like *B. subtilis* and *B. amyloliquefaciens* has potential in potato bio protection or as a part of an integrated disease management package for bacterial diseases. It is clear that BCAs have promising antagonistic effects on controlling brown rot disease. Bustamante and Ciampi (1989) reported that the biocontrol agents used successfully reduced brown rot and wilts disease in potatoes. BCAs gave significant effect on the physiological growth and development of potato tubers. Experimental result showed that, the number of leaves per plant, number of tuber per hill, average weight of tuber, yields per plant were varied with different bio agents. The most number of leaves per plant was found in *Bacillus subtillis* soil application (19.86.) Plant height was also high on this treatment .The average weight of tuber were also found high in *Bacillus subtillis* soil application(39.3gram) while highest number of tuber per hill was found in spraying application of *Trichoderma* . These results are in agreement with those recorded by Verma *et al.* (2007). They reported that *Trichoderma* spp. have been widely used as antagonistic fungal agents against several pathogen as well as plant growth enhancers. These results are in agreement with those recorded by Ryan *et al.* (2001) who reported that *B. subtilis* ,*Trichoderma* and *B. amyloliquefaciens* were able to promote plant growth directly. He also reported that the best control of potato brown rot disease was achieved with *B. subtilis*, and *Trichoderma spp*. compared to controls. Vinale *et al.* (2008) also reported that the BCAs produce numerous biologically active compounds including cell wall degrading enzymes and secondary metabolites for suppressing various pathogen. Verma *et al.* (2007) reported that *Trichoderma* spp. and *B. subtilis* have been used in a wide range of commercial enzyme productions, namly cellulases, hemicllulases, proteases and *b*-1,3-glucanase.This experimental results suggested that the soil application of BCAs were the best to protect the potato tubers against bacterial brown rot disease and gave significant effect on the physiological growth and development of potato tubers. The soil applications of *Bacillus subtilis* is the most promising BCAs whose antibacterial effect reduced bacterial wilts and promote tuber growth and development.

CHAPTER VI

SUMMARY AND CONCLUSION

Potato belongs to the family Solanaceae is an important tuber crop grown all over the world. It is a staple food in the developed countries and which account for 37% of the total potato production in the world. and Bangladesh is the 7th producer of potato in the world. Though the demand of potato is increasing day by day, it's production in terms of area and yield is not satisfactory due to different diseases of potato. Tuber of potato is vulnerable to attack by various diseases in Bangladesh especially brown rot. However least concrete information regarding their distribution, incidence, severity and management is available. Therefore, the present study was conducted to investigate symptom, detection of pathogen and find out the efficacy of some BCAs on disease suppression as well as agronomical performance of potato. The experiment was carried out in a potato cultivation land in Gowalkhali village at Sirajdikhan upzilla in Munshiganj district and the *in vitro* research had been done at Molecular Disease Diagnostic Laboratory of the Department of Plant Pathology in Sher-e-Bangla Agricultural University, Dhaka. Typical symptoms were observed on experimental site. The symptom started with slight wilting of the leaves at the ends of the branches during the day which recovers at night; eventually, plants failed to recover which was soon followed by total wilting. Milky or cloudy threads like streaming signifies the presence of *R. solanacearum* of brown rot disease. The causal organism was isolated from infected potatoes collected from experimental site following standard dilution plating technique using nutrient agar medium and TTC selective medium. Typical, whitish, watery convex, mucoid, colonies of bacterium were produced on nutrient agar medium after 48 hours of incubation at 30˚C. 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 with oil immersion.

BCAs produce numerous biologically active compounds including cell wall degrading enzymes and secondary metabolites which reduce the activity of pathogen. Field application of the BCAs as spray and soil treatments significantly reduced the brown rot incidence.The brown rot incidence was in the range from 2.8 % to 35%. *Bacillus subtillis* soil application showed the lowest incidence.BCAs significantly reduced the incidence of wilt in treated potato plants.

Use of BCAs in the field of potato cultivation the morphological and physiological growth and development of potato is higher. The number of leaves per plant, number of tuber per hill, average weight of tuber, yields per plant were varied with different BCAs. The most number of leaves per plant was found in *Bacillus subtillis* soil application .Plant height was also high on this treatment. Again, the average weight of tuber were found in *Bacillus subtillis* soil application while highest number of tuber per hill was found in spraying application of *Trichoderma*.

This experimental results suggested that the soil application of BCAs were the best to protect the potato tubers against bacterial brown rot disease and gave significant effect on the physiological growth and development of potato tubers. The soil applications of *Bacillus subtilis* is the most promising BCAs whose antibacterial effect reduced bacterial wilts and promote tuber growth and development. Therefore, further study is required to develop effective management strategies.

CHAPTER VII

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APPENDIX

Preparation of culture media and reagents

The compositions of the media used in lab work are given below, mentioned all media were autoclaved at 121˚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

Potato Dextrose Agar (PDA)

Peeled potato	200 g
Dextrose	20 g
Agar	17 g
Distilled water	1000 ml

Potato Dextrose Broth

Peeled potato	200 g
Dextrose	20 g
Distilled water	1000 ml

Gelatine Liquefaction Media

Beef extract	3.0 g
Peptone	5.0 g
Gelatine	120 g
Distilled water	1000 ml

Starch hydrolysis media and reagent

Culture medium Nutrient broth	8.0 g
Soluble potato starch	10.0 g
Bacto agar	15.0 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

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-diaminedihydrochloride was prepared from the absolute solution.