STUDY ON PHENOTYPIC CONVERSION, VIRULENCE AND BIOVAR EXPRESSION OF *RALSTONIA SOLANACEARUM*CAUSING POTATO WILT AND ITS MANAGEMENT (*In vivo*)

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This is to certify that the dissertation entitled **"STUDY ON PHENOTYPIC CONVERSION, VIRULENCE AND BIOVAR EXPRESSION OF *RALSTONIA SOLANACEARUM* CAUSING POTATO WILT AND ITS MANAGEMENT (*In vivo*)"** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN PLANT PATHOLOGY**, embodies the result of a piece of *bona fide* research work carried out by Zinnat Karim, Registration No. **14-06370,**under my supervision and guidance. No part of the thesis has been submitted any where for any other degree or diploma.

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

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ABSTRACT

BY

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Infected samples from different sources of inoculum viz. plant, soil, seed, weed, other crops, irrigation water etc. were collected from major potato growing districts of Bangladesh viz. Munshiganj, Chandpur, Tangail, Narayanganj, Jamalpur, Domar, Patuakhali, Rangpur, Bogra, Shariatpur, Meherpur, Joypurhat and Dinajpur to investigate the presence of *R. solanacearum*. Cross cut of plant samples showed bacterial ooze streaming in clear water and browning of the vascular bundle region of seed tuber were observed. Standard methods viz. Kelman's tetrazolium chloride (TZC) medium (semi-selective) for isolation, race test, biovar test, different biochemical tests, phylotype test by phylotype specific PCR amplification were used for detection and identification for *R. solanacearum* including latent infection. A total of 133 samples were tested on TZC solid medium out of which 94% (ie. 125) found positive for *R. solanacearum* presence. Among the isolates, thirty nine isolates were tested for race, biovar and phylotype study. It was observed that all thirty nine tested isolates expressed as race 3 while in biovar test thirty seven showed as biovar III and the rest two showed biovar I. Phylotype detection test was done through phylotype specific PCR amplification and all exhibited to originate as phylotype I (Asiatic type). All of the isolates showed PC (phenotypic conversion or VBNC) in aerated and non-aerated water medium. In case of virulence expression, all isolates showed a range of heterogeneity both in colony counts (virulent and avirulent in different dilution in water medium) and DSS (disease severity score) within 2-7 days after inoculation by isolates of *R. solanacearum*. The study revealed that disease severityof*R.solanacearum* was reduced in a good moist soil (where moisture level >5.5 ie. >55%). Change in biovar expression were observed where a range of heterogeneity viz. 13 isolates as biovar I, 6 isolates as biovar II, 15 isolates biovar III and 5 isolates as biovar IV were found while tested at 22^0C and 28^0C temperature. Again, under the influence of temperature avirulent colony of *R. solanacearum* showed VBNC state at 10^0C and over 40 ± 2^0C. *In vitro* and *in vivo* performance test with ten bioactive compounds against *R. solanacearum* were conducted as compared to control, bactericide and farmers practice to design an effective management tool. Propolis (@ 6mg/ml), turmeric powder (@ 10%) and cow dung (well dried) (@ 25%) were found effective in reducing the DSS of bacterial wilt (*R. solanacearum*) on potato plant, both seed and soil treatment compared to farmer's practice (stable bleaching powder- T13) and control (T1). Ten bioactive compounds were evaluated *in vitro* to find out the effectiveness against bacterial wilt of potato caused by *R. solanacearum* and it was found that all the selected compounds showed larger inhibition zone as compared to control which indicated the antibacterial effectiveness of test compounds against the wilt pathogen. The compounds were also evaluated for effectiveness in reducing the virulent colony count and it was observed that treatment cow dung followed by turmeric powder showed promising result. Again, several *in vitro* and *in vivo* evaluation was conducted in seed and soil (sterilized and unsterilized) and cow dung, propolis and turmeric powder treatments were found to promising in virulence reduction of *R. solanacearum* as compare to control and farmers practice (stable bleaching powder) especially in natural field soil condition. Again, propolis, turmeric powder and cowdung were evaluated against *R. solanacearum* and cow dung was found to perform best in all parameters which was followed by propolis and turmeric powder. Result of the study showed that the lowest disease severity score and the lowest colony count in cfu/ml was found to occur in cow dung which was followed by propolis and turmeric powder in all the cases in reduction of virulence of *R. solanacearum*. However, application of well dried cow dung (@ 25%) with proper soil moisture (>5.5ie.>55%) can be used as an effective management of the bacterial wilt disease of potato in the farmers' field.

Key words: Bacterial wilt disease, Bangladesh, heterogeneity in virulence and biovar(s), *Ralstonia solanacearum*; management, bioactive compounds.

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Chapter I
Introduction

Potato (*Solanum tuberosum* L.) is a tuber crop belongs to the family Solanaceae. 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. It is a crop of great economic significance, because it isanimportant source offood,employment andincome generating activities indeveloping countries. The easeofproductionhas also madeitanimportantcomponentofurbanagriculture whichprovidesjobsandfoodsecurityto some800million peopleglobally(HofflerandOchieng,2008). Thus, it is the 4th important crop after wheat, rice and maize in the world (FAOSTAT, 2015). Bangladesh is the 7th producer country in the world by producing 86.03 lakh tons of potato and the area of production is still in increasing from 4.44 to 4.62 lakh hectares in the country (Ahmed and Talukder, 1978; FAO, 2008; and FAOSTAT, 2017). The major potato growing areas of Bangladesh are Rangpur, Dinajpur, Joypurhat, Bogra, Nilphamari, Jamalpur, Sherpur, Meherpur, Chuadanga, Jessore, Patuakhali, Shariatpur, Chandpur, Faridpur, Munshigonj, Narayanganj, Manikganj and Tangail (Rahman, 1990; World Food Security Atlas, 2008; Ahmed *et al.,* 2013). However, the yield of potato is quite low in the country as compared to the major potato growing countries like- Ireland and India (FAOSTAT, 2017). The reasons behind the lower yield of potato includes lower soil fertility, inadequate supply of certified seeds, use of low yielding varieties, different pests and diseases etc. Among which soil borne diseases are considered to cause a yield loss of as much as 10–20% annually (USDA, 2003). *Ralstonia solanacearum* (Smith, 1896) formerly called *Pseudomonas solanacearum* (Yabuuchi *et al*., 1995) is the most destructive soil-borne pathogen (Yuliar *et al.*, 2015) that affects potatoes in temperate, subtropical and tropical regions throughout the world by causing bacterial wilt or brown rot disease (CABI, 2017; Champoiseau *et al.,* 2009). Its world-wide distribution, destructive nature and ability to host asymptomatically over 450 plant species to survive long in the environment (Kelman, 1998; Prior *et al.,* 1998) has resulted it to be the most important bacterial plant pathogen (Kelman, 1998). The bacterial wilt pathogen is very diversified, widely distributed and has an extensively wide host range (over 200 species) with major host crops like potato, tomato, *Musa* spp. etc. and some minors like groundnuts (*Arachis hypogaea*), brinjal (*Solanum melongena*) and ginger (*Zingiber officinalis*) (Eddins, 1941;Denny, 2006; Hayward, 1991). Yield losses due to the disease varied from 33 to 90% in the potato in different potato growing areas of the world (Elphinstone, 2005). It is responsible

for an estimated losses in US $1 billion each year and, the disease has been estimated to affect three million farm families for about 1.7 million hectares of potatoes in approximately 80 countries, which accounts for global damages over USD 950 million per annum and thereby, contributing to yield losses in potatoes of about 75% at medium to high altitudes (1500-2800 m) (Champoiseau *et al*., 2009). Yield losses continue during storage and transit due to rotting and decay leading to even more revenue losses. Seed borne wilt or latent infection in potato has often been resulted in severe out breaks of bacterial wilt (French, 1986). The total value of Egyptian potato exports fell from a peak of US$ 102.12 million in 1995 to $US 7.7 million in 2000 mainly due to brown rot quarantine, imposed by the European Union (EU) (Kabeil *et al.,* 2008). In India, this disease causes 50% crop loss in potato in a regular manner (Mukherjee and Dasgupta, 1989) and up to 75 % losses as reported in some areas of Karnataka (Gadewar *et al.*, 1991). Reports from Bangladesh quote some regions as having more than 30% of potato crops affected by *R. solanacearum,* with over 14% reduction in yield (Elphinstone, 2005). Nonetheless, Russia imposed a temporary ban on the entry of the potatoes from Bangladesh in May 2015 on food safety grounds after detecting this organism (Parvez, 2017).

Bacterial wilt caused by *R. solanacearum* is a vascular disease (Horton, 1987), which is fatal in infected plant and has been ranked as one of the most important bacterial plant pathogens identified to date, commonly known as bacterial wilt (in case of infected plant) and brown rot (in case of infected tubers). The disease appears as rapid and fatal wilting symptoms in host plants (Yuliar *et al.*, 2015) and infected potato plants die rapidly within 3-4 days. Older plants first show wilting of the young leaves, or partial one sided wilting of the plant and stunting, and finally the plants wilt permanently and die. The disease can be easily detected in the wilted plant stem by streaming the milky white oozes within clear water (Allen *et al.,* 2001). The bacterial wilt is primarily tuber-borne, but infested soil also serves as a source of infection. Tuber may carry the pathogen in vascular tissues, on the tuber surface and within lenticels (Ghosh and Mandal, 2009; Martin and French, 1985; EPPO, 2004). *R. solanacearum* is gram-negative, rod-shaped bacterium measuring 0.5-0.7 × 1.5-2.0 µm in size.Itgrowswellat28to32°Cinaerobicconditions(Hayward,1991). Nishat *et al.* (2015) reported the variation in *R.solanacearum* isolates of potato which was observed among different growing areas of Bangladesh. It was showed that the isolates were belonged to race 3 biovar III. Biovars I and II are predominant in the Americas. In Australia, however, biovar III predominates, biovars III and IV occurring to a lesser extent. Biovars II, III and IV also occur in India, Indonesia, Papua New Guinea, Sri Lanka and China (together with biovar 5). Only

Philippines have all of biovars 1-4 and here as elsewhere in Asia, biovar 3 predominates in the lowland regions (Hayward, 1991; CABI, 2017). However, report from Ahmed *et al.*(2013) showed that the *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh were belonging to Biovar III and Race 3. Prior and Fegan (2005) reported the diversity equivalences among phylotypes, biovars and races of *R. solanacearum* where it showed that race 1, 4 and 5 equivant to biovar 3, 4 and 5 which was originated from Asia belonged to phylotype I; and race 1, 2 and 3 equivant to biovar 2T, 1 and 2 which was originated from America belonged to phylotype II; but phylotype III (originated from Africa) and phylotype IV (originated from Indonesia) did not get any race equivqlence to biovar(s). Another study described by EPPO (2004), Champoiseau(2008) and Sullivan *et al*.(2013) the characteristics of races and their relationship to biovars of *R. solanacearum* and it reported that race 1 hosts all solanaceous crops and many other plants which was available in Asia, Australia, America, China, India, Japan, Nepal, Pakistan, Sri Lanka etc. and belonged to biovar III, IV, and I; race 2 hosts triploid bananas, other *Musa* spp. was available in Caribbean, Brazil, Philippines which was belonged to biovar I; race 3 hosts only potato and tomato which was available worldwide except US and Canada and belonged to biovar II or IIA; race 4 hosts ginger and unknown hosts available in Australia, China, Hawaii, India, Japan, Mauritius, South Asia, India and belonged IV and III; and race 5 hosts mulberry tree in China which belonged to biovar V. However, typical race 3 strains are sometimes referred to as biovar IIA and new race 3 strains from the Amazon basin have been placed in a new biovar designed as IIT or NII and their relation to races is unclear (Champoiseau, 2008; and Sullivan *et al*., 2013). It was historically subdivided into the five races based loosely on host range and, five biovars based on their ability to produce acids from a panel of 5 to 8 carbohydrate substrates viz. three hexose alcohols, namely mannitol, sorbitol and dulcitol and, three disaccharides, lactose, maltose and cellobiose (Hayward, 1994). Fegan and Prior (2005) and Prior and Fegan (2005)established a phylogenetically meaningful system that has been classified *R. solanacearum* into four major genetic groups called phylotype(s) (I, II, III and IV), which reflect the geographical origin and ancestral relationships of the strain(s) which, were detected and analyzed based on DNA sequence analysisare further subdivided into sequevars based on the sequence of the endoglucanase (*egl*) gene (Fegan and Prior, 2005; Prior and Fegan, 2005). But, He (1983) showed that within each of the races or biovars there are numerous subtypes that can be associated with certain geographical regions and this, together with *R. solanacearum* enjoys a world-wide distribution. However, several attempts have been made to find a suitable classification system for the isolates of *R. solanacearum* as they are considered

a "species complex" for their significant variation within the groups (Fegan and Prior, 2005) which were often differed in geographical distribution, pathogenicity etc. in the form of race(s), biovar(s) and/or phylotype(s). The geographic distributions of the pathogen are highly influenced by different factors like suitability of the climatic conditions and abundance of suitable host(s) etc. Moreover, the bacterium is known to enter in VBNC state (viable but not culturable) under unfavorable conditions (Elsas *et al.,* 2001). Such biological phenomena of the pathogen helps it to build up the inoculum potential which lead it to induce a destructive economic impact (Kelman, 1998).

Therefore, *R. solanacearum* is an important plant pathogen as it possesses some especial biological features viz. their abilities to grow endophytically (tending to grow inward into tissues), could survive long in the environment, having PC (phenotypic conversion) and/or VBNC or latency (VBNC- viable but non culturable) phenomena, and their relationship with weeds as asymptomatic hosts (Wang and Lin, 2005) etc. allow them to threaten the production and export of potato. The pathogen hosts crops only where a number of environmental factors conducive to disease expression coincide, such as temperature regime, rainfall, soil type, inoculum potential, and other biological factors such as nematode populations (Sitaramaiah and Sinha, 1984; Hayward, 1991; Hayward, 1994). Environmental temperature and moisture are two major factors favouring *R. solanacearum* survival in the field are permissive soil temperatures and high moisture contents (Kelman, 1953; Hayward, 1991; Swanepoel, 1990). When temperature is >40°C, it cannot survive, become severe between 35~24°C, no visible symptom show at <16°C (Ciampi and Sequeira, 1980; Seneviratne, 1988), and can survive long in lower temperature even at 4°C, which make it capable of dispersal and survival in the soil/plant materials for long period (Granada and Sequeira, 1983b). It can survive up to one year in agricultural soil even after treatment with an herbicide to eliminate the hosts (Elsas *et al*., 2000; Elsas *et al*., 2005), can be detected up to two years after crop removal (Shamsuddin *et al*., 1979), and withstand a four-year intercropping period keeping wilting capacity (Graham *et al*., 1979). Persistence is likely to be enhanced in deeper soil layers, because of less temperature fluctuation and/or less competition by the indigenous microbiota (Graham and Lloyd, 1979). Moderate differences in moisture do not drastically affect *R. solanacearum* populations contrarily to severe drought (Kelman, 1953; Elsas *et al*., 2000; Elsas *et al*., 2005). *R. solanacearum* has repeatedly displayed ability for survival in aquatic environments (Álvarez *et al*., 2007; Álvarez *et al*., 2010), and for being able to multiply in pure water in the absence of nutrients. It was reported in freshwater for variable periods (Álvarez *et al*., 2007), with populations surviving up until four years keeping pathogenic even by watering (Álvarez,

2009). The persistence in water was favoured at permissive temperatures and by the presence of sediment but, it was unfavoured in sediment subjected to drying and by levels of seawater salts similar to those of water in coastal areas, due to osmotic tensions (Álvarez *et al*., 2007). Populations in environmental water indicated a seasonal variation over years, consisting of relatively high levels during spring and summer, and unsuccessful recovery of cells in autumn and winter (Hong *et al*., 2005). Although the pathogen coped efficiently with abiotic factors acting simultaneously (Álvarez *et al*., 2010), biotic factors like water microbiota affected *R. solanacearum* survival (Álvarez *et al*., 2007). The bacteria can be disseminated with the irrigation water in the environment and can make the disease level increased and affected synergically while in the optimum temperature (Shekhawat *et al*., 1992). As a soil-borne pathogen, it can survive in various types of soils worldwide and show the ability of changing state [from virulent to avirulent termed as "PC" phenomena (phenotypic conversion) by reduced production of EPS (extracellular polysaccharides)] which make them to remain withstand and viable for a very long periods like 2 to 10 years (Denny *et al*., 1994; Poussier *et al*., 2003; Nesmith and Jenkins, 1985). It exists as a strategy of survival of the bacteria in energy-deficient system when inoculum level is decreased due to unfavorable environment, such as exposure to temperature stress, desiccation, anaerobiosis etc. Under those conditions, the colony spontaneously undergo from fluidal to afluidal morphology which is linked to a greater reduction in disease-inducing capacity or virulence as well as EPS production as a result of mutation in *phcA* genes. This phenomenon includes afluidal colony or asymptomatic or latent infection or VBNC state (viable but non culturable) which might complicate the culture-based diagnostic methods (Buddenhagen and Kelman, 1964; Devi *et al*., 1982; Brumbley and Denny, 1990; Shekhawat and Perombelon, 1991; Hayward, 1994; Morita *et al*., 1997; Elsas *et al*., 2001; Álvarez *et al*., 2010). In the process of virulence expression, main transcriptional regulator is PhcA (Phc, phenotype conversion) which is autoregulated by 3-OH PAME (3-hydroxypalmitic acid methyl ester) by acting in the quorum sensing system of *R. solanacearum* (Brumbley *et al*.,1993; Flavier *et al*., 1997b; Álvarez *et al*., 2010). At low bacterial densities PhcA regulator remains inactive due to the level of 3-OH PAME. When bacterial cell densities become high in the xylem vessels, 3-OH PAME accumulates extracellularly and promots the activation of PhcA regulatory network resulting in the activation of EPS synthesis which is repressed in non-host environments (Schell, 2000; Genin and Boucher, 2004). Extensive multiplication and EPS production taking place in the water-conducting system lead to wilting of the host due to clogging of the vessels. The plant collapses and dies and *R. solanacearum* is released (Kelman and Sequeira, 1965) to a

saprophytic life in the soil or other environments where it should survive until contact with a new host. As a result, life cycle of *R. solanacearum* includes life inside and outside the host. The bacterium moves to the host roots, attaches to the epidermis, infects the cortex, and colonizes the xylem, resulting in host wilting. After death of the plant, the bacterium is released into the environment, where it seems to survive in reservoir plants, soil and/or water, through diverse strategies, such as the VBNC state, the PC process, or the biofilm formation, until contact with a new host (Álvarez *et al*., 2010). PC-type variants can be easily observed by prolonged culture on agar plates, and when the organism is grown in a non-aerated liquid medium (Kelman, 1953; Buddenhagen and Kelman, 1964; Álvarez *et al*., 2010).

R. solanacearum is a very successful plant pathogen and due to the biological features, several difficulties are created in effective management through traditional practices. The pathogen created much more problems in controlling with chemicals, which was nearly impossible to apply; antibiotics showed hardly any effect (Murakoshi and Takahashi, 1984; Farag *et al.*, 1982); and adaptability problems of resistant varieties occurred due to the strain diversity and latent infection of the pathogen. However, biocontrol agents showed some effectiveness in the controlled condition which is still in its infancy (CABI, 2017). Therefore, it is listed as a quarantine organism (Champoiseau*et al.,* 2009). However, the pathogen is highly influenced by different factors like availability and abundance of the host(s), and suitability of the climatic conditions etc. The difficulties with traditional management practices challenged to search for alternative management including higher bioactivity andeffectivityinpresenceof organic matter. During the recent decades, many bioactive compounds have been extensively tested for their antimicrobial effectiveness and a good number of reports showed effective in inhibition of many dangerous strains of phytopathogenic bacteria (Leksomboon *et al.,* 2000). In the field of nutrition "bioactive compounds" are distinguished as extranutritional constituents (not essential nutrients) that typically occur in foods in small quantities. Precisely, it is a compound that has an effect on a living organism, tissue /cell and exhibits a great numbers of diverse and versatile biological effects among which, first of all is antimicrobial activities (Shukla, 2015). A wide range of pharmacological attributes of curcumin from turmeric has been well documented for antimicrobial and protective properties (Nagabhushan and Bhide, 1992). The chemical composition, medicinal and antibacterial activity of propolis from bees have been reported by Velikova *et al.* (2000a, b). Honey and propolis have been found to be very effective against both Gram negative and positive type bacterial pathogen management because of their phenolic substances include cinnamic acid derivatives, some

flavonoids which have been verified as antibacterial applicant (Miorin *et al.*, 2003). Cow dung and urine have been used as insecticides and have been reported that they contain antibiotic agents (Waziri and Suleiman, 2013). Oyarzua *et al.* (2014) showed that the magnesium salts in the microbiological experiments are typically associated with positive effects. It focuses on the usefulness of magnesium (in form of $MgCl_2$) as a stress enhancer against *Escherichia coli*. The reduction of wilt has been notedbyChellemi*etal.*(1992)with natural and organicamendments. Two traditional aromatic rice genotypes, viz. Kalijira and Chinigura, effectively inhibit the Gram negative type *Agrobacterium tumefaciens* (Mannan *et al.*, 2014). Iodine (mixed with a transporter known as iodofore) could successfully inhibit aerobic Gram positive and Gram negative bacteria (Estrela *et al.*, 2006). Sodium bicarbonate has shown antibacterial properties against different types of bacterial and fungal pathogens (Kelly and Kristin, 2005; Malik and Goyal, 2006; Arslan *et al.,* 2009). However, little works have been performed to investigate the antibacterial properties of such bioactive compounds against bacterial wilt pathogen (*Ralstonia solanacearum*) as an alternative approach of management.Therefore, the study emphasizes the effectiveness of such bioactive compounds in managing bacterial wilt disease (*R. solanacearum*) of potato with the following objective(s)-

Objective(s)-

1. To know the present status of the pathogen (*Ralstonia solanacearum*) in relation to race(s)/ biovar(s)/ phylotype(s) from major potato growing regions of Bangladesh.
2. To understand the influences of moisture and temperature on heterogeneity within species in relation to PC (Phenotypic conversion /VBNC), virulence and biovar expression by different isolates of *Ralstonia solanacearum* which is critical in effective management.
3. To find out the effectiveness of bioactive compounds in reducing the disease severity and virulence of the wilt pathogen (*Ralstonia solanacearum*) in potato.

Chapter II
Review of Literature

2.1. Distribution & host range

Denny(2006) described that bacterial wilt pathogen is widely distributed and has an unusually broad host range. Moreover, because of being a soil-borne pathogen, host resistance is limited (Hayward, 1991a; Saddler, 2005). *R. solanacearum* is one of the most destructive plant pathogens identified to date because it possesses some especial biological features to survive long in the environment and created difficulties in traditional management. The pathogen possesses extensively wide (over 200 species) and worldwide major host crops like potatoes, tomatoes, *Musa* spp. and tobacco with some minors like- groundnuts (*Arachis hypogaea*), *Capsicum annuum*, cotton (*Gossypium hirsutum*), rubber (*Hevea brasiliensis*), cassava (*Manihot esculenta*), castor beans (*Ricinus communis*), brinjal (*Solanum melongena*) and ginger (*Zingiber officinalis*) with many weeds as asymptomatic alternate hosts to induce a destructive economic impact (Kelman, 1998). Bacterial wilt affects crops of economic importance in almost all the tropical, subtropical and warmer temperate regions of the world. Biovar 2 presumed to have originated in South America (presumed site of origin of the potato) now has a wide spread distribution which can be transmitted as latent infections in potato seed tubers. In many countries of Southern Europe such as Portugal, biovar 2 is the sole biovar. This is also true for the Mediterranean area, Argentina, Chile and Uruguay (Hayward, 1991a). Biovars 1 and 2 are predominant in the Americas. In Australia, however, biovar 3 predominates, biovars 2 and 4 occurring to a lesser extent. Biovars 2, 3 and 4 also occur in India, Indonesia, Papua New Guinea, Sri Lanka and China (together with biovar 5). Only Philippines have all of biovars 1-4 and here as elsewhere in Asia, biovar 3 predominates in the lowland regions (Hayward, 1991a; CABI, 2017).

Ahmed *et al.,*(2013) reported that the *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh were belonging to Biovar III and Race 3.

Girard *et al.,* (1993) showed that *R. solanacearum* is known to have a very extensive host range including not only economically important crop plants such as potato, tomato, tobacco and banana, but also ornamental plants, trees and weeds. Species from more than 44 plant families have been identified by Hayward (1991a) and more hosts are being recognised and described. Some of the reports included onion, *Allium cepa*; custard apple, *Annona* spp.,

(Mayers and Hutton, 1987); florist geranium, *Pelagornium hortorum,* (Strider *et al.,* 1981); strawberry, *Fragaria* spp., (Hsu, 1991) and radish, *Raphanus sativus* L., (Hsu, 1991) etc. Cassava is cultivated in many countries where bacterial wilt is endemic, yet the disease on this host appears to be confined to Indonesia. Similarly bacterial wilt on sweet potato has only been reported in China (Hayward, 1991a). *R. solanacearum,* biovar 3, has also been noted on cashew in Indonesia and the Alexandra palm in Queensland, Australia (Hayward, 1991a). An alternative theory is that the pathogen hosts such crops may only where a number of environmental factors conducive to disease expression coincide, such as temperature regime, rainfall, soil type, inoculum potential, and other biological factors such as nematode populations (Hayward, 1991a; Hayward, 1994b).

Shekhawat *et al.,*(1992) however showed that all hosts of *R. solanacearum* do not necessarily express symptoms and can serve as symptomless carriers. The slow rate of colonisation and disease progress in symptomless hosts allows the bacteria to stay viable longer, serving as an inoculum source for susceptible crops or wild hosts. Studies conducted by Shekhawat *et al.,*(1992) indicated that *R. solanacearum* can even survive symptomless in roots of weed-hosts and in plants considered to be non-hosts in more than 450 species which have been reported as hosts or symptomless carriers (Prior *et al.,* 1998) of certain strains of *R. solanacearum.*

2.2. Yield loss

Elphinstone(2005) stated that *R. solanacearum* is the most serious pathogen of potato plants in tropical regions and can cause serious losses in temperate regions. A review of the older literature can be found in Kelman (1953). It is responsible for an estimated $1 billion US in losses each year and globally, the disease has been estimated to affect about 1.7 million hectares of potatoes in approximately 80 countries, with global damage estimate of over USD 950 million per annum thereby contributing to yield losses in potatoes of about 75% at medium to high altitudes (1500-2800 m) (Champoiseau *et al*., 2009). Seed borne wilt or latent infection in potato has often been resulted in severe out breaks of bacterial wilt (French, 1986). Yield losses continue during storage and transit due to rotting and decay leading to even more revenue losses. The disease has been estimated to affect three million farm families, which accounts for about 1.5 million Ha) in around 80 countries. In addition to causing yield losses in field crops, management efforts for prevention, eradication, and control

of *R. solanacearum* are extremely costly, which contribute heavily to economic losses (IPDN, 2014).

IPDN(2014) showed that different yield loss status has been reported in several countries. In Bolivia, potato yield loss at harvest ranged from 30- 90% and losses during storage were as high as 98% (Coelho and Nutter, 2005). In Nepal, tuber rotting occurred in an average of 10 % of stored potato, with a maximum of 50%. Crop losses in small farms in the Nepalese hills were up to 100%, mainly due to poor cultural practices, such as keeping seed from infected crop (IPDN, 2014; Elphinstone, 2005). Complete crop losses in small holdings in Nepal resulted from poor cultural practices including using seed from affected crops for subsequent plantings (CABI plantwise, 2017). In Venezuela, in the period 1992-1996, *R. solanacearum* was found in most localities between 1100 and 3000 m above sea level, but was not found in localities at altitudes greater than 3000 m. (CABI plantwise, 2017). The potato production and yield losses due to bacterial wilt as high as 100 per cent have been reported in parts of tropical Africa (Biology Discussion, 2016). In Kenya, the potato industry is threatened by bacterial wilt (BW) because soils in most production areas are infested with the wilt causing bacterium and over 50% yield losses have been reported (IPDN, 2014). The farmers reported experiencing yield losses ranging from 5% to 80% due to bacterial wilt. According to some recent studies, the disease is found in all the potato growing areas of Kenya and the country is affecting 77% of potato farms which had been introduced with tuber seeds imported from Europe (Kaguongo *et al*., 2010). Various reports from Kenya have indicated that there was an increase in the incidence of brown rot of potato due to the spread and build-up of the disease in the majority of the potato growing zones (IPDN, 2014; Ajanga, 1993; Barton *et al*., 1997; Ateka *et al*., 2001; IPDN, 2014). Potato yield losses in Uganda estimated about 30% (IPDN, 2014; Alacho and Akimanzi, 1993), with more severe losses being 100% (IPDN, 2014). In Burundi, losses of 64.1% were reported in seed potato (IPDN, 2014; Berrios and Rubirigi, 1992). Heavy losses of potato due to this disease were reported from the South Atlantic and Gulf Coast states of the USA (Kelman, 1953). Extensive losses of potato were reported in Greece (Zachos, 1957). In Israel, losses were heavier in the spring potato crop than the autumn crop, because of the higher growing temperatures in spring (Volcani and Palti, 1960). Kabeil*et al.,* (2008) reported that potatoes were one of the largest exported crops in Egypt. Yet, the total value of Egyptian potato exports fell from a peak value of US$ 102.12 million in 1995 to $US 7.7 million in 2000 mainly due to this organism related quarantine restrictions imposed by the European Union (EU) which used to account for about

70-90% of Egyptian potato exports and it represented a drop from approximately 419,000 metric tons to 48,500 tons. Multiplication by cutting seed potato seriously increases the risk of high losses. Cut seed potato increased disease incidence by 250% and reduced yield by 40% (CABI plantwise, 2017). In India, a yield loss study with one cultivar of tomato showed 10-100% mortality of plants and 0-91% yield loss (Elphinstone, 2005). In India, this disease causes 50% crop loss in potato in a regular manner and up to 75 % losses as reported in some areas of Karnataka (Mukherjee and Dasgupta, 1989). Reports from Bangladesh quote some regions as having more than 30% of potato crops affected by *R. solanacearum,* with over 14% reduction in yield (Elphinstone, 2005).

Chakraborty and Roy(2016) reported that in Bangladesh, *R. solanacearum* incidence was recorded 9.07% in Jamalpur area, 19.98% in Nilphamari area and 22.65% in Munshigonj area. During the fiscal year (2014-2015), exports of the produced potato had been hindered because of the embargo imposed by Russia due to the infection of the pathogen. So, the potato growers and businessmen of Bangladesh had experienced much problems on the disease especially in case of export to other countries like- Malaysia, Indonesia, Sri Lanka, Thailand, Hong Kong, Vietnam, Maldives etc.

2.3. Description of the Pathogen

Shekhawat *et al*., (1992) described that pathogen as non-spore forming (spores in bacteria terminology are survival structures rather than units of reproduction as in fungi), Gram-negative, rod-shaped bacterium 0.5-0.7 × 1.5-2.0 µm in size which is nitrate-reducing, ammonia-forming and grows wellinaerobicconditions(Hayward,1991a).Optimum growth temperatures for the bacteria ranging from 27-37°C, depending on the strain. Maximum temperature for growth is about 39°C and the minimum between 10-15°C. Populations within this genus and species can be further divided into races and biovars based on differing host ranges, biochemical properties, and serological reactions. The shape and size of the causal organism was first described as a small rod with one polar flagellum with rounded ends. The size of the bacterium vary according to different growing conditions (Kelman, 1953). Bacteria isolated from infected tissues were appeared as very short rods (0.3-0.6x 0.4-1.2µm) and those taken from young broths or cultures tend to be longer (ranging from 0.4-0.6x 1.0-1.8µm), whereas those from old cultures have a short coccus-like form (Kelman, 1953). Yabuuchi *et al.*,(1995) reclassified *Burkholderia solanacearum* as *Ralstonia solanacearum* which was based on the studies involving phenotypic characterization, rRNA-DNA hybridization,

phylogenic analysis of 16SrDNA nucleotide sequences, and analysis of cellular lipids and fatty acids.

Race(s) and Biovars of the pathogen

Nishat *et al*., (2015) reported the variation in *R.solanacearum* isolates of potato which was observed among different growing areas of Bangladesh. It was showed that the isolates were belonged to race 3 biovar III. Biovars I and II are predominant in the Americas. In Australia, however, biovar III predominates, biovars III and IV occurring to a lesser extent. Biovars II, III and IV also occur in India, Indonesia, Papua New Guinea, Sri Lanka and China (together with biovar 5). Only Philippines have all of biovars 1-4 and here as elsewhere in Asia, biovar 3 predominates in the lowland regions (Hayward, 1991a; CABI, 2017). However, report from Ahmed *et al.,*(2013) showed that the *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh were belonging to Biovar III and Race 3. Prior and Fegan (2005) reported the diversity equivalences among phylotypes, biovars and races of *R. solanacearum* where it showed that race 1, 4 and 5 equivant to biovar 3, 4 and 5 which was originated from Asia belonged to phylotype I; and race 1, 2 and 3 equivant to biovar 2T, 1 and 2 which was originated from America belonged to phylotype II; but phylotype III (originated from Africa) and phylotype IV (originated from Indonesia) did not get any race equivqlence to biovar(s). Another study described byEPPO (2004), Champoiseau (2008) and Sullivan *et al*., (2013)the characteristics of races and their relationship to biovars of *R. solanacearum* and it reported that race 1 hosts all solanaceous crops andmany other plants which was available in Asia, Australia, America, Bangladesh, China, India, Japan, Nepal, Pakistan, Sri Lanka etc. andbelonged to biovar III, IV, and I; race 2 hosts triploid bananas, other *Musa* spp. was available in Caribbean, Brazil, Philippines which was belonged to biovar I; race 3 hosts only potato and tomato which was available worldwide except US and Canada and belonged to biovar II or IIA; race 4 hosts ginger and unknown hosts available in Australia, China, Hawaii, India, Japan, Mauritius, South Asia, India and belonged IV and III; and race 5 hosts mulberry tree in China which belonged to biovar V. However, typical race 3 strains are sometimes referred to as biovar IIA and new race 3 strains from the Amazon basin have been placed in a new biovar designed as IIT or NII and their relation to races is unclear (Champoiseau, 2008; and Sullivan *et al*., 2013). It was historically subdivided into the five races based loosely on host range and, five biovars based on their ability to produce acids from a panel of 5 to 8 carbohydrate substrates viz. three hexose alcohols, namely mannitol, sorbitol and dulcitol and, three disaccharides, lactose, maltose and cellobiose (Hayward, 1994b). However, several attempts have been made to find a suitable classification system for the isolates of *R. solanacearum* as they often differ in host range, geographical distribution, pathogenicity, physiological properties etc. in the form of race(s), biovar(s) and/or phylotype(s). Considering

such subspecific classification, *R. solanacearum* is considered a "species complex" due to significant variation within the groups (Fegan and Prior, 2005). Thus, Fegan and Prior (2005), and Prior and Fegan(2005)established a phylogenetically meaningful system that has been classified *R. solanacearum* into four major genetic groups called phylotype(s) (I, II, III and IV),which reflect the geographical origin and ancestral relationships of the strain(s) which,were detected and analyzed based on DNA sequence analysisare further subdivided into sequevars based on the sequence of the endoglucanase (*egl*) gene (Fegan and Prior, 2005; Prior and Fegan, 2005). But, He (1983) showed that within each of the races or biovars there are numerous subtypes that can be associated with certain geographical regions and this, together with *R. solanacearum* enjoys a world-wide distribution. Consequently, it could affect crops of economic importance in tropical, subtropical and warmer temperate regions of the world.

2.4. Symptoms and diagnosis of the pathogen

Kelman(1953) described that *R. solanacearum* causes potato infection in two ways- i) premature wilting and plant death symptoms namely ‘bacterial wilt’ leading to total loss of yield; and ii) tuber rotting symptoms namely ‘brown rot’ occurs in the transit or storage. On potato plants, symptoms due to the blocking of the vessels caused by the bacteria is the major cause of wilting. The symptom starts with slight wilting of the leaves at the ends of the branches during the heating of the day which recovers at night; eventually, plants fail to recover which is soon followed by total wilting and if the base stem of the affected plant is cut transversely, the bacterial oozes comes out as milky white threads when kept in a beaker with water. Such threads are not formed by other bacterial pathogens of potato. In advanced stage, epinasty of the petioles may occur and die. However, under cool growing conditions, wilting and other foliar symptoms may not occur.

Symptoms in tubers, mostly occurs as vascular browning and rot and pitted lesions (Shekhawat *et al.,* 1992). In vascular rot, the vascular tissues looks like a water soaked circle, which subsequently may turn brown. A cross section will show a brown vascular bundle ring. As the tuber is pressed, slimy drops will be out of the ring. The lesions on tuber are produced due to infection through lenticels (skin pores) (Ghosh and Mandal, 2009; Martin *et al*., 1981; Martin and French, 1985; EPPO, 2004). If potato tubers are formed in the infected plants those will possibly show the symptoms. On tubers, external symptoms may or may not be

visible, depending on the state of disease development. *R. solanacearum*can be distinguished by the bacterial ooze that often emerges from the eyes and stolon-end attachment of infected tubers. Soil may adhere to the tubers at the eyes (OEPP/EPPO, 2004).

2.5. Detection

Fegan and Prior(2005) described the bacteria,*R. solanacearum*to be considered as a "species complex" due to significant variation within the group. Itcan be identified from either symptomatic or asymptomatic plants and from water or soil samples by means of several microbiological and molecular methods (Priou *et al.,* 2006; Schaad, *et al.,* 2001; Weller, *et al.,* 2000). Screening tests can facilitate early detection of *R. solanacearum* in plants or contaminated soil and water samples, but they cannot be used to identify the race or biovar. These screening tests include bacterial streaming, plating on a semi-selective medium, such as TZC medium etc. (Elphinstone *et al.,* 1996), polymerase chain reaction (PCR) with specific primers, and pathogenicity tests using susceptible hosts, such as tomato seedlings (Elphinstone *et al.,* 1996; Schaad *et al.,* 2001; Weller *et al.,* 2000). Commercially available immunostrips can be used for the rapid detection of *R. solanacearum* in the field or lab. Isolation from symptomatic material can easily be performed using Kelman's tetrazolium chloride (TZC) medium. In some cases when secondary infections were present, the isolation of the pathogen on selective media was necessary. According to Hayward (1994b), biovar test is a biochemical assaywhich can be identified from a panel of disaccharides and sugar alcohols based on their ability to utilize three hexose alcohols, namely mannitol, sorbitol and dulcitol; and to produce acids from the three disaccharides, lactose, maltose and cellobiose requires specialized media and it may take several days to several weeks. The strains of *R. solanacearum* can be sub-classified into phylotypes and then into sequevars using PCR and gene sequence analysis (Champoiseau *et al*., 2009). Many standard methods for the detection (of latent infection), identification and preparation of media for *R. solanacearum* has been suggested by EU testing schemes in EU (1998); Lelliott and Stead (1987); and OEPP/EPPO (1990, 2004, 2017a & 2017b). Detection of latent infection is performed by an immuno-fluorescence test and/or selective plating on SMSA medium eventually combined with optional PCR assays, ELISA or fluorescent *in situ* hybridization tests which can be performed for added sensitivity (OEPP/EPPO, 2004). A combination of at least two different complementary tests is required to identify the species and biovar unambiguously. Unequivocal identification of R3bv2 must rely on at least two distinct methods of screening and biovar test (Champoiseau *et al*., 2009). SMSA medium as modified by Elphinstone *et al*., (1996) has been used successfully in

Europe for latent infection (Elphinstone *et al*., 1998). A presumptive test in the field can be the water streaming test as described under disease symptoms or a serological agglutination test using a field kit in the form of a lateral flow device (Danks and Barker, 2000).

Colonycharacter

Kelman(1954) showed that on solid agar media, individual bacterial colonies are usually visible after 36 to 48 hours growth at 28°C, and colonies of the normal or virulent type are white or cream-colored, irregularly shaped, highly fluidal, and opaque. A tetrazolium chloride (TZC) medium can differentiate the virulent and non-virulent colony types by appearing as white with pink centers of virulent colonies and dry, uniform round and dark red of non-virulent/ mutant colonies. However, it has the ability of changing state from virulent to avirulent, termed as “phenotypic conversion” (PC) by reduced production of extracellular proteins & polysaccharides due to some environmental stress (Shekhawat and Perombelon, 1991).

2.6. Biologicalespecialtyof *R. Solanacearum*behind long-term survival in the environment

Kelman (1953) showed that bacterium could enter into host through the wounds created by nematodes or other organisms(Johnson and Schaal, 1952; Kelman, 1953); and the points of secondary root emergence(Buddenhagen and Kelman, 1964). However, Kelman and Sequeira(1965) and Álvarez *et al*., (2010) described that when relatively large numbers of bacteria were available around, it could enter into the host and caused disease.

Shekhawat *et al*., (1992) showed the sources of inoculum and dispersal in- i) infected plant materials (seeds, plant, tuber etc.); ii) infected debris, alternate hosts & weeds; iii) infested soil, irrigation water, equipments etc.; iv) plant parts (eg. tubers) with no visible symptom. When bacterial masses adhere to soil particles enhancing its survival, tubers can carry the bacteria in three manners, namely externally on tuber surfaces, in lenticels and in the vascular tissues (Shekhawat *et al*., 1992); plant parts (eg. tubers) with no visible symptom ensure the uninterrupted dispersal of the pathogen; infected host debris show an important short-term shelter for *R. solanacearum* in soil (Lloyd, 1978; andGraham *et al*., 1979) allowing survival between growing seasons and serve as a transmission agent; weeds serving as alternate hosts are available and these are more than 450 species which are symptomless carriers (Hayward, 199la; Prior *et al*., 1998). Association of *R. solanacearum* with either reservoir plants or plant debris has been frequently suggested to promote survival of the pathogen in soil and water,

and favour overwintering in temperate regions (Hayward, 1991a; Elsas *et al*., 2000). Reservoir plants would group together resistant/tolerant hosts and some of the non-hosts for the pathogen. Tolerant hosts have been described as those whose xylems can be invaded in the roots and weakly colonized at stem level, whereas non-hosts would not be invaded in plant xylem but there would be in them occasional presence of the pathogen in root cortex or on surface (Álvarez *et al*., 2008).

Ciampi and Sequeira(1980) and Seneviratne (1988) showed the influence(s) expression due to environmental temperature and moisture favouring *R. solanacearum* survival in the field are permissive soil temperatures and high moisture contents (Kelman, 1953; Hayward, 1991a). When temperature is >40°C, it cannot survive, become severe between 35~24°C, no visible symptom show at <16°C, and can survive long in lower temperature even at 4°C, which make it capable of dispersal and survival in the soil/plant materials for long period (Granada and Sequeira, 1983b). It can survive up to one year in agricultural soil even after treatment with an herbicide to eliminate the hosts (Elsas *et al*., 2000; Elsas *et al*., 2005), can be detected up to two years after crop removal (Shamsuddin *et al*., 1979), and withstand a four-year intercropping period keeping wilting capacity (Graham *et al*., 1979). Persistence is likely to be enhanced in deeper soil layers, because of less temperature fluctuation and/or less competition by the indigenous microbiota (Graham and Lloyd, 1979).

Elsas *et al*., (2005) showed moderate differences in moisture do not drastically affect *R. solanacearum* populations contrarily to severe drought (Kelman, 1953; Elsas *et al*., 2000). *R. solanacearum* has repeatedly displayed ability for survival in aquatic environments (Álvarez *et al*., 2007; Álvarez *et al*., 2010), being able to multiply in pure water in the absence of nutrients. It was reported in freshwater for variable periods (Álvarez *et al*., 2007), with populations surviving up until four years keeping pathogenic even by watering (Álvarez, 2009). The persistence in water was favoured at permissive temperatures and by the presence of sediment but, it was unfavoured in sediment subjected to drying and by levels of seawater salts similar to those of water in coastal areas, due to osmotic tensions (Álvarez *et al*., 2007). Populations in environmental water indicated a seasonal variation over years, consisting of relatively high levels during spring and summer, and unsuccessful recovery of cells in autumn and winter (Hong *et al*., 2005). Although the pathogen coped efficiently with abiotic factors acting simultaneously (Álvarez *et al*., 2010), biotic factors like water microbiota affected R. solanacearum survival (Álvarez *et al*., 2007). The bacteria can be disseminated with the

irrigation water in the environment and can make the disease level increased & affected synergically while in the optimum temperature (Shekhawat *et al*., 1992).

Denny *et al*., (1994)and Álvarez *et al*., (2010) described the “PC” phenomena (phenotypic conversion) andas a soil-borne pathogen, it can survive in various types of soils worldwide and show the ability of changing state [from virulent to avirulent termed as “PC” phenomena (phenotypic conversion) by reduced production of EPS (extracellular polysaccharides)] which make them to remain withstand and viable for a very long periods like 2 to 10 years (Poussier *et al*., 2003; Nesmith and Jenkins, 1985).Álvarez *et al*., (2010) described that latency and virulence expression by EPS production was existed as a strategy of survival of the in energy-deficient system when inoculum level is decreased due to unfavorable environment, such as exposure to temperature stress, desiccation, anaerobiosis etc. Under those conditions, the colony spontaneously undergo from fluidal to afluidal morphology which is linked to a greater reduction in disease-inducing capacity or virulence as well as EPS production as a result of mutation in phcA genes. This phenomenon includes afluidal colony or asymptomatic or latent infection VBNC state (viable but non culturable) which might complicate the culture-based diagnostic methods (Buddenhagen and Kelman, 1964; Devi *et al*., 1982; Brumbley and Denny, 1990; Shekhawat and Perombelon, 1991; Hayward, 1994b; Morita *et al*., 1997; Elsas *et al*., 2001; Álvarez *et al*., 2010). In the process of virulence expression, main transcriptional regulator is PhcA (Phc, phenotype conversion) which is autoregulated by 3-OH PAME (3-hydroxypalmitic acid methyl ester) by acting in the quorum sensing system of *R. solanacearum* (Brumbley *et al*.,1993; Flavier *et al*., 1997b; Álvarez *et al*., 2010). At low bacterial densities PhcA regulator remains inactive due to the level of 3-OH PAME. When bacterial cell densities become high in the xylem vessels, 3-OH PAME accumulates extracellularly and promots the activation of PhcA regulatory network resulting in the activation of EPS synthesis which is repressed in non-host environments (Schell, 2000; Genin and Boucher, 2004). Extensive multiplication and EPS production taking place in the water-conducting system lead to wilting of the host due to clogging of the vessels. The plant collapses and dies and R. *solanacearum* is released (Kelman and Sequeira, 1965) to a saprophytic life in the soil or other environments where it should survive until contact with a new host. Therefore, life cycle of R. *solanacearum* includes life inside and outside the host. The bacterium moves to the host roots, attaches to the epidermis, infects the cortex, and colonizes the xylem, resulting in host wilting. After death of the plant, the bacterium is released into the environment, where it seems to survive in reservoir plants, soil and/or water,

through diverse strategies, such as the VBNC state, the PC process, or the biofilm formation, until contact with a new host (Álvarez *et al*., 2010). PC-type variants can be easily observed by prolonged culture on agar plates (Kelman, 1953; Buddenhagen and Kelman, 1964; Álvarez *et al*., 2010).

Zhang *et al.,*(1993) showed moreover, disease severity mostly increases by changing the physiology of the plants and increases the susceptibility if *R. solanacearum* is found in association with root nematodes (Chen, 1984), as pathogen may be carried in the lenticel and/or in the tuber. Additionally, it cannot be detected in seeds with a water content of less than 10%. Therefore, seed-borne latent infection may result in severe out-breaks of bacterial wilt and/or brown rot of potato.

2.7. Difficulties in traditional management of *R. solanacearum*

McCarter(1969),Akira*etal*., (2009) and Mbaka*etal*.,(2013) described the long term survival strategies of the pathogen in the environment which successfully created difficulties and limited the success through traditional management practices, viz. i) preventive measures, ii) cultural measures, iii) chemical measures, and iv) biological measures. Controlling wilt through preventive options is not applicable in infested location (McCarter,1969). Cultural options hasshown limited success(Mbaka*etal*.,2013) due to its ability tosurvive inthesoilover a longtime with asymptomatic weed hosts and avery widehost range (Saddler, 2005), and the complexitiesofhost-pathogen-environmentinteraction make breeding forresistanceextremelydifficult(Tung*etal.,*1990). Use of chemicals against the pathogen remained achallengebecausethe bacterialocalize insidethexylemand thereare noknown eradicationbactericidesavailableforchemicalcontrolof the bacterialwiltdisease (Hartman and Elphinstone,1994). So, chemical control is ineffective. Antibiotics, viz. streptomycin, ampicillin, tetracycline and penicillin show hardly any effect (Farag *et al.,* 1982); in fact, streptomycin application has increased the incidence of bacterial wilt in Egypt (Farag*et al*., 1986;CABI, 2017). Biological control has been gainedpopularityin recentyearsdue toenvironmentalconcerns, but efficacious biocontrol agents have yet to be developed. However, positive results have been achieved in laboratory experiments with the antagonistic bacteria *Bacillus amyloliquefaciens, Ralstonia pickettii, Pseudomonas mallei* etc. (Yuliar *et al.,* 2015). But difficulties created with application procedure, inconsistentcolonization and

survival in the field, suppression etc. are sometimes too complex to useon a commercial scale (Whippsand Gerhardson, 2007; Akira*etal*., 2009). In discussion of success and limitations of preventive management practices against bacterial wilt pathogen quarantine, phytosanitary practices, disease free certified seeds, disinfected equipment, controlleduseoffloodirrigationand avoiding overheadirrigation etc. were considered as preventive measures and it was only successfulwherethepathogen wasnotpresent. Thus, not applicable in infested location (McCarter,1969). In success and limitations discussion of cultural management practices against bacterial wilt pathogen- i) Use of resistant cultivars- It is reported to be themosteffective andpracticalmethodto control bacterialwilt (Black*etal*., 2003;Grimault*etal*., 1994). Unfortunatelythe complexitiesofhost-pathogen-environmentinteraction make breeding forresistanceextremelydifficult(Tung*etal.,*1990). Because- i.*R. solanacearum*isa "heterogeneous species complex"with a wide hostrange (Kelman and Person, 1961; Álvarez *et al.,* 2010); ii. highvariability initsbiochemicalproperties(Cuppels*et al.,*1978;Hayward,1964), serological reactions(Schaad*etal*.,1978),membraneproteins(Dristig andDianese,1990)andphase susceptibility (OkabeandGoto,1963)confirming the challengesinbreedingforresistance. ii) Application oftheorganicamendments had been reported toreducethe disease(Chellemi*et al*., 1997). iii) Disinfected equipment, controlleduseoffloodirrigationand avoiding overheadirrigation use of crop rotation etc. are considered as good cultural measures. But the successis limited(Mbaka*etal*.,2013) as- i. thepathogenisabletosurvive inthesoilover a longtime; ii. it can exist in a very wide range of weedsandvolunteer crops (FajinmiandFajinmi,2010). The pathogenis also abletosurvive inthesoilover a longtime in asymptomatic weed hosts within avery widehost range (Saddler, 2005). In the discussion of success and limitations created by chemical management practices against bacterial wilt pathogen, it was difficulttocontrolwith chemicals(Grimault*etal*.,1994). Because- i. pathogenlocalize insidethexylemandit is able tosurviveinthesoil; ii. there is noknown eradicativebactericidesavailableforchemicalcontrolof the bacterialwiltdisease (Hartman and Elphinstone, 1994). However, there are following options- A) Use of Fumigants- Chloropicrin istheonlyformulation thatprovides significantcontrolthroughouttheseason among others [methyl bromide, DD• MENCS (amixture of methyl isothiocyanate,dichloropropane anddichloropropene) andmetham](Enfinger*et al.,* 1979). But, fumigant pesticides pose serious health risks and degrade soil health. One hundred years ago, chloropicrin was used during World War I as tear gas and "vomiting gas." Scientists have concluded that chronic exposure to chloropicrin results in "very high" cancer risks (Froines, 2010) and those are prohibited in

some countriesduetotheriskspposedtopesticide operatorsandaquaticorganisms,birds,andbees. B) Use of some antibiotics (Penicillin,Ampicillin,Tetracycline and Streptomycin)- Not successful when testedinbothgreenhouse andfield conditions (Hartman and Elphinstone,1994). C) Applicationofstablebleachingpowder (Saddler,2005)- It reduces bacterialpopulationsanddiseaseseverity onasmallscale(Saddler,2005). Sodium hypochlorite (4-6%) may produce skin and ocular irritation or gastric burns, inactivation by organic matter, and release of toxic chlorine gas when mixed with ammonia or acidic condition (Kennedy and Bek., 1998). In case of discussion of success and limitations of biological management practices against the bacterial wilt pathogen from 2005 to 2014 was reported by Yuliar *et al.,* 2015 and showed- i) 1. *Bacillus amyloliquefaciens* SQR-7 (a) and SQR-101 (b) and *B. methylotrophicus* SQR-29 (c) respectively @ Pouring, 6.8×10^{10}cfu plant^{-1} of (a), 7.5×10^{10}cfu plant^{-1}of (b) and 8.2×10^{10}cfu plant^{-1} of (c) yield increased 25–38% through production of indole acetic acid and siderophores; ii) 2. *Ralstonia pickettii* QL-A6 (a) @ stem injection, 10 µL of 10^{7}CFU mL^{-1} of (a) reduced the disease by competition which was not practically applicable; iii) *Pseudomonas monteilii* (a) + *Glomus fasciculatum* (b), Stem cuttings dipping in 9.1×10^{8}mL^{-1} of (a) →53 infective propagules of (b) addition to each cutting → then pouring again of (a) to each cuttings, increased plant nutrient uptake (N, P, K) and reduced the pathogen population 54%; iv) *Brevibacillus brevis* L-25 (a) + *Streptomyces roche* L-9 (b) + organic fertilizer- Mixing with soil at a density of 7.3×10^{7} cfu g^{-1}soil of (a) and 5.0×10^{5}cfu g^{-1} soil of (b), decreased root colonization by the pathogen >87%; v) *Bacillus amyloliquefaciens* + bio-organic fertilizer (BIO23) (a) *B. subtilis* +bio-organic fertilizer (BIO36) (b)- Mixing with soil at a density of 5.5×10^{6}cfu g^{-1} soil of (a) and 7.0×10^{6}cfu g^{-1}soil of (b), plantgrowthpromotion occurs 64–65% etc. created difficulties due to the application method of such inoculation rate and procedure and ensuring their survibility in large scale fields was practically complex to be maintained properly.

2.8. Risk Category of *R. solanacearum*

OEPP/EPPO(2017b)reported*R. solanacearum* is listed as a regulatory pathogen in A2 group (the group of quarantine pest which can be present in the location but cannot be widely distributed there and has to be officially controlled) quarantine organism under those circumstances and is listed by Asia and Pacific Plant Protection Commission (APPPC) and

International Association of Professional Security Consultants (IAPSC). The occurrence of different races and strains of the pathogen with varying virulence under different environmental conditions presents a serious danger to European and Mediterranean potato and tomato production. Therefore, the absence of the bacterium is an important consideration for countries exporting seed potatoes (Lambert, 2002;Champoiseau*et al.,* 2009; CABI, 2017).

2.9. Natural bioactive compounds as successful antibacterial agents

Leksomboon *et al.,* (2000) showed awareness about residual effects of pesticides and development of pesticide resistance has challenged to search for environment friendly alternatives in managing the constraints of traditional management practices. Important characteristicsofgood management techniques include higher bioactivity andeffectivenessinthepresenceof organic matter,lowertoxicityandeaseof use. During the recent decades, many bioactive compounds have been extensively tested and a good number of reports has been documented the effects of those compounds as effective inhibitors of phytopathogenic bacteria. A bioactive compound is a compound that has an effect on a living organism, tissue or cell. In the field of nutrition, "Bioactive compounds" are distinguished from essential nutrients as extranutritional constituents that typically occur in small quantities in foods. Bioactive compounds exhibit a great numbers of diverse and versatile biological effects, first of all antimicrobial activities. Different pathogenic and other microbes (Gram-positive, Gram-negative bacteria, fungi, yeasts, etc.) are described as test organisms in the direct activity-based screenings (Shukla, 2015). Such compounds could be an important consideration against the very successful bacterial pathogen *R. solanacearum.* The chemical composition, medicinal and antibacterial activity of propolis from bees have been reported by Velikova *et al.* (2000a, b). Honey and propolis are found to be very effective against both Gram negative and positive type of bacterial pathogen management because of their phenolic substances include cinnamic acid derivatives, some flavonoids which have been verified as antibacterial applicant (Miorin *et al.,* 2003). Honey contains the antioxidants and flavonoids that might function as antibacterial agents. Propolis, a flavonoid-rich product of honey comb, exhibit antibacterial properties (Bosio *et al.,* 2000) against both Gram negative and positive type bacteria. Honey inhibits the growth of dangerous bacteria from both Gram negative and positive type(s) such as *Escherichia coli, Staphylococcus aureus, Salmonella* etc. (Zumla and Lulat, 1989). Reductionin wiltwasnotedbyChellemi*etal.*,(1992)with natural and organicamendments. Turmeric (*Curcuma longa* L.) is a medicinal plant extensively used in

Ayurveda, Unani and Siddha medicine as home remedy for various diseases (Eigner and Scholz,1999). Turmeric is used as a food additive (spice), preservative and coloring agent in Asian countries, including China and South East Asia (Khattak *et al.,* 2005) which is also effective against different virulent strains of *R. solanacearum* in India (Narasimha *et al.,* 2015). Shrivastava*et al*., (2014) evaluated cow dung extract for antibacterial properties against *E. coli*, *Pseudomonas* and *Staphylococcus aureus.* It shows that cow dung is highly effective against both of those Gram positive and Gram negative type microbes. In a study, Oyarzúa *et al.*, (2014) shows that the magnesium chloride ($MgCl_2$) in the microbiological experiments typically associated with positive effects against Gram negative type *E. coli*. According to Mannan *et al.*, (2014) report, the fluids of unpolished rice grain of two traditional aromatic rice genotypes, viz. Kalijira and Chinigura, effectively inhibit the Gram negative type *Agrobacterium tumefaciens*. Iodine dissolved in aqueous potassium iodide, alcohol or mixed with a transporter (known as iodofore) are classified as disinfectants (Secorand Gudmestad, 1993). Jarvis *et al.,* (2001) found that cattle manure could be treated with sodium carbonate to eliminate *E. coli* and Corral *et al.,* (2006) found sodium bicarbonate (SB) to inhibit the growth of different bacterial pathogen in agar media. Besides, sodium bicarbonate is mostly used to formulate toothpaste and cosmetic products for its antibacterial and acid-neutralizing properties and there are evidences that exist for its antimicrobial activity against different types of bacterial and fungal plant pathogens (Kelly and Kristin, 2005; Malik and Goyal, 2006; Arslan *et al.,* 2009). Such bioactive compounds with their successes as antibacterial agents which could be considered to test the effectiveness against *R. solanacearum* are as follows:

2.9.1. Propolis

Bosio *et al.*,(2000)described propolis or bee glue as a resinous mixture produced by honey bees consisting of approximately 50 constituents, primarily resins (50%), waxes (30%), essential oils (10%), pollen (5%) and other substances (5%). It is used to prevent diseases and parasites from entering the hive, and to inhibit fungal and bacterial growth. Propolis is a flavonoid-rich product of honey comb, exhibiting antibacterial properties.

Rahman *et al.,*(2010)showed that the higher the concentration of propolis the greater the inhibition zones against Gram negative type *Escherichia coli* and Gram positive type *Staphylococcus aureus* by disc diffusion method. Because, it is a very powerful natural

antibiotic (Miorin *et al.,* 2003). Agar diffusion method is also used for antibacterial activity of beewax and propolis suggests that propolis has inhibitory activities against bacterial pathogens (Azevedo *et al*., 1963). The antibacterial activity of propolis may be related to the presence of flavonoids (Bosio *et al.,* 2000). Takaisi-Kikuni and Schilcher (1994) andNieva Moreno *et al.*, (1999) have shown that propolis extracts have antibacterial properties against some micro-organisms. Miorin *et al.*, (2003) suggests that the extent of effectiveness of honey or propolis and their chemical composition varies depending on bee species and geographic region.

2.9.2. Honey

Zumla and Lulat (1989) and Gheldof *et al.,*(2002) showed honey has a long tradition of use within various medical systems and over the past decade several research groups have focused their attention to the product (Allen *et al.,* 1991; Greenwood, 1993; Molan, 1992; and Moore *et al.,* 2001). The antibacterial activity of honey varies significantly depending on the floral source of the honey. The most important antibacterial factor in honey is hydrogen peroxide which is produced by the action of glucose oxidase and added to the honey by the bee. However, some other antibacterial activity of honey occurs due to the substances which are derived from the flowers (Allen *et al.,* 1991).

Balan *et al.*, (2016) reported that greater effectiveness of manuka honey (MH) against a range of serious bacterial pathogens both are Gram positive and Gram negative types and the higher the concentration of honey (from 2.5 to 20%) the greater the inhibition was observed. The manuka honey, produced in New Zealand, is produced from the flowers of two plants. These are the manuka and kanuka trees but are generally referred to as manuka trees (*Leptospermumscoparium*). The greater quality of New Zealand MH is because of the occurrence of higher amounts of methylglyoxal (MGO) and it is well documented. This MGO is identified as a bioactive compound which is responsible for the antibacterial activity of MH samples (Mavric *et al.,* 2008).

2.9.3. Turmeric

Khattak *et al.,* (2005) reported turmeric (*Curcuma longa* L.) as a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine as home remedy for various diseases (Eigner and Scholz,1999). It is used as a food additive (spice), preservative and coloring agent in Asian countries, including China and South East Asia. It is the source of curcumin (diferuloyl methane), a yellow lipid-soluble polyphenolic dietary compound, produced as the rhizome of

turmeric. It is widely used in foods (Aggarwal and Harikumar, 2009; Akram *et al*., 2010) and useful in numerous medicinal benefits and pathological research on curcumin (Gupta *et al.,*2012). A wide range of pharmacological attributes of curcumin, such as antioxidative, antimicrobial and wound-healing-protective properties, have been well documented (Nagabhushan and Bhide, 1992; Aggarwal and Harikumar, 2009; Frenkel *et al.,*2013). Biological activities of curcumin depend on its bioavailability and metabolism.

Narasimha*et al.*, (2015) reports that 10% (w/v) turmeric powder extract shows an inhibition zone ranged from about 15 to 25 mm against several virulent strains of *R. solanacearum*. Another study reports that curcumin hss tested for their antimicrobial activities against both Gram positive (*Bacillus subtilis* NCTC 6276, *Staphylococcus aureus* NCTC 8530) and Gram negative bacteria (*Escherichia coli* NCTC 10863, *Escherichia coli* O157:H7CDC strain G5244, *Salmonella typhimurium* CDC AMO 3398). Curcumin @ 100 mg/ml against Gram positive type and @ 250 mg/mL against Gram negative type are required to inhibit 100% growth of those strains (Balan *et al.,* 2016). However, fat soluble extracts of turmeric and its curcumin component exhibit strong antioxidant activity. Because the curcuminoids of turmeric are highly fat soluble and a little water soluble. If it is applied with water, they tend to arrive at cell walls in the form of relatively large, undissolved particles rather than as induvidual molecules. Since all cell walls are made of lipids.

2.9.4. Magnesium chloride

Oyarzúa *et al.,* (2014) showed magnesium as an element essential for life and is found ubiquitously in all organisms. It is a salt from Great Salt Lake, Utah, USA (Oren *et al*., 2009), where Mg2+ and Cl- are the most abundant cation/anion. It has the importance in microbiological context with healing and antiseptic properties. The different cations play important roles as enzymatic co-factors, as signaling molecules, and in stabilizing cellular components. Oyarzúa *et al.,* (2014) showed that magnesium salts in the microbiological experiments typically associated with positive effects. It focuses on the usefulness of magnesium (in form of MgCl2) as a stress enhancer against *Escherichia coli*(K-12). $MgCl_2$ did not affect bacterial viability at near-neutral pHs, but it shows a strong compromising ability against culturability and activity when cell suspensions are exposed to the salt at acidic pH. The principle is confirmed with a number of Gram-negative and Gram positive species.

2.9.5. Cow dung

Khanuja(2002) showed folkloric medicine developed over centuries within various countries and societies and have been used for the treatment of illnesses and infections far before the era of modern medicine. Different parts of plants and oils, animal's wastes have been used by traditional healers in the treatment of different categories of diseases with great success. Dung has been used as organic fertilizer and in the production of biogas to generate electricity and heat. Cow dung and urine are used as insecticides and contain antibiotic agents (Singh*et al.*, 2012; Khanuja, 2002). The use of cow dung in the bioremediation of toxicants in the environment has also been reported (Randhawa and Kullar, 2011). Cow dung has been identified by different names; it is referred to as cow chips or cow pit in British English while a deposit of the dung is referred to as cow pie in American English (Perry and Morton, 2009). Furthermore, a large number of microorganisms which have biological activities and presently in use as antibiotics and antitumor agents have been reported (Waziri and Suleiman, 2013). However, the success of such products is believed to depend on the active ingredients as well as method of preparation.The antibacterial and antifungal properties of cow dung extract in distilled water and ethanol have been evaluated against *E. coli*, *Pseudomonas* and *Staphylococcus aureus* and cow dung is highly effective against both of those Gram positive and Gram negative type microbes Shrivastava *et al*., (2014). The study has shown that cow dung extract possesses antimicrobial properties, which can be used to fight against certain pathogenic diseases and other ailments. Another study has revealed that the cow dung extract shows antibacterial activity against *Bacillus subtillis, Staphylococcus aureus* which is helpful in establishing the antibiotic property of the extract (Waziri and Suleiman, 2013). The levels of elements like k, Na, Mg etc were present in the extract and higher levels of these elements in the extract was responsible for the activity against the tested bacteria. Because, the role of metals in biochemical processes by acting as cofactors for various enzymes has been revealed.

2.9.6. Aromatic rice

Kawakami *et al.,* (2006) showed how natural medicinal compounds have attracted more and more interests because of their safety properties in recent years (Lewis, 1993; Hatate *et al*., 1990). Use of plant-based compound including vegetables, cereals, etc. plays a pivotal role in disease prevention. They can contribute to both the causes and prevention of diseases. Therefore, a great deal of recent research has been focused on the development of new bioactive agents from cereals (Wenzig *et al*., 2005; Chung *et al.,* 2006; Saikia and Deka, 2011). It is remarkable that rice possesses special dietary importance and availability in Asia (WCRF and AICR, 1997). Ishizone *et al.,* (2007) and Kawakami *et al.,* (2006) showed that

rice-fluid does show an antibiotic effect on Gram negative type *Helicobacter pylori* and its effect. Mannan *et al.,* (2014) reported that the methanol extract of unpolished grain of two traditional aromatic rice genotypes, viz. Kalijira and Chinigura were assayed for their activity on the growth and initiation of crown-gall tumors caused by Gram negative type three *Agrobacterium tumefaciens* strains (*A. tumefaciens*- AtSl0105, AtTa0112, and AtAc0114) on potato disks. The results demonstrated a high correlation between the ability of aromatic rice to inhibit the initiation and growth of *A. tumefaciens* strains on potato disks. Both unpolished grains show significant effect (Kalijira 57.43%, Chinigura 55.53%) to inhibit the tumor causing effect of the *A. tumefaciens* strains. It is also observed that tumor inhibition was maximum at higher concentrations (1,000 ppm) of Kalizira and Chinigura rice. Fang *et al.,* (2004),Kawakami *et al.,* (2006), Ishizone *et al.,* (2007),Chakuton *et al.*, (2012),and Deng *et al.*, (2013) suggested that the pure rice phytochemicals viz. oryzanol, anthocyanin, amino acid, essential oils, phenolics etc. are more potent against the pathogenic activity. Rice contains anthocyanins which are regarded as important nutraceuticals mainly due to their antioxidant effect, which provide a potential to prevent various diseases (Duthie *et al.*, 2000; Kong *et al.,* 2003).

2.9.7. Iodine

Estrela *et al.,* (2006) described the compound that contain iodine are employed for infection control. Iodine can be dissolved in aqueous potassium iodide, alcohol or make an assembly with a transporter (known as iodofore) and they are classified as disinfectants (Secorand Gudmestad, 1993). In a report, Estrela *et al.,* (2006) shows that iodoform’s action in releasing iodine gives a higher level of reactivity by precipitating proteins and oxidizing essential enzymes. In direct exposure test different pastes with and without iodoform (iodine releasing) shows effective inhibition of different bacterial colonies from both Gram positive and Gram negative type except for *B. subtilis*.

2.9.8. Sodium bicarbonate

McCombs *et al.,*(2001) and Barnes(1999) showed sodium bicarbonate, $NaHCO_3$, as for its significance in ever-growing use for its safety, low cost, low abrasivity, water solubility, acid buffering properties, and, antibacterial properties (McCombs *et al.,* 2001; Barnes, 1999). Because of its alkalinity, or buffering capacity, sodium bicarbonate has the ability to neutralize acids produced by microbes. Another factor in sodium bicarbonate's bacteriocidal abilities comes from changes in osmotic pressure. The hypertonic (with greater osmotic

pressure) sodium bicarbonate solution causes the more hypotonic microbial cell to lose water, consequently dehydrating and eventually killing the cell (Lawrence and Block, 1968). Although these are all desirable outcomes, some studies have shown that the sodium bicarbonate must be allowed to interact at least 30 minutes with the bacteria cell to be fully effective. Fletcher *et al.,* (1984) shows that sodium bicarbonate had no effect on the viability of *S. mutans* when exposed only for a short time. In many cases, sodium bicarbonate was found to be effective against different microorganisms.Although sodium bicarbonate has been used mostly to formulate toothpaste and cosmetic products for its antibacterial and acid-neutralizing properties, there are evidences of its antimicrobial activity against different types of plant pathogens (Kelly and Kristin, 2005; Malik and Goyal, 2006; Arslan *et al.,* 2009). It has been reported to be virucidal and inhibited the growth of several fungi (Malik and Goyal, 2006; Arslan *et al.,* 2009). Sodium bicarbonate has also been shown to enhance the effectiveness of other agents for controlling mould growth (Hang and Woodams, 2003; Wan *et al.,* 2003, Yao *et al,* 2004; Palou *et al.,* 2009; Casals *et al.,* 2010). Kelly and Kristin (2005) experimented on the growth of *Streptococcusmutans* where, row A was filled with distilled water without *S. mutans*; row B had 10% sucrose without *S. mutans*; row C had 10% sucrose and *S. mutans* alone; row D had 10% sucrose, sodium bicarbonate and *S. mutans*; row E had 10% sucrose, sodium bicarbonate, 3% hydrogen peroxide and *S. mutans*; row F had 10% sucrose, 3% hydrogen peroxide and *S. mutans*; and row G had *S. mutans* alone. Rows A (without *S mutans*), B (without *S mutans*), C (with *S mutans*), and G (with *S mutans*) were used as experimental controls and rows D, E, and F were the experimental groups. Significant statistical differences were observed in Rows D (sodium bicarbonate), E (sodium bicarbonate and hydrogen per oxide), and F (hydrogen per oxide) as compared to row C (*S. mutans* in succrose) and G (*S. mutans* alone) in the study. However, Rams *et al*., (1985) showed a five-minute exposure to sodium bicarbonate quickly immobilized the motile rods and also reported that higher concentrations of sodium bicarbonate may not only suppress harmful bacteria, but also lead to increase in healthy bacteria. Barnes (1999) shows *S. mutans* to be susceptible against 4% sodium bicarbonate. Additionally, a four-week study by Legier-Vargas *et al.,* (1995) establishes that using a concentration of 65% sodium bicarbonate lowered the level of *S. mutans*.

Chapter III

Materials and Methods

3. Experimental Site and period of the study

The study was carried out in the Molecular Disease Diagnostic Laboratory of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka and Honey Bee Pest and Disease Diagnostic Laboratory of Department of Entomology, Sher-e-Bangla Agricultural University, Dhaka from November, 2015 to March, 2018. To fulfil the objectives of the study three different experiments were carried out which are described below.

3.1. Experiment 1

Detection and characterization of race(s), biovar(s) &/or phylotype(s) of *Ralstonia solanacearum*causing potato wiltin major growing regions of Bangladesh

3.1.1. Sample(s) collection for study

Selection of location(s)

Major potato growing districts of Bangladesh viz. Munshiganj (Manikpur), Chandpur, Tangail (Nagarpur), Narayanganj (Sonargaon), Jamalpur, Domar (Dimla), Patuakhali, Rangpur, Bogra, Shariatpur (Jajira), Meherpur (Gangni), Joypurhat (Akkelpur) and Dinajpur (yellow spotted locations of the map showing in Fig. 1 & Table 1) were selected for collection of the infected samples from different sources of inoculum (plant, seed, soil,weed, other crop- chilli, water etc.) during the period of December'2015 to January'2016.

Symptom study

For collection of different samples symptoms were studied properly. Plant showing wilting of younger leaves, even under adequate soil moisture condition, were to suspect to have *R. solanacearum* infection(IPDN, 2014). The symptoms were to start with slight wilting of the leaves at the ends of the youngest branches (early symptom) during the warmest time of the day which were soon to show total wilting (Vanitha *et al*., 2009). Milky or cloudy threads like streaming were to signify the presence of *R. solanacearum* of bacterial wilt in the vascular

bundles blocking the vessels specifically in the xylem but lower populations of the pathogen may not be visible to the naked eye. Such threads are not formed by other bacterial pathogens of potato (Kelman, 1953). In advanced stage, epinasty (downward curvature of infected stem) or stunting may occur (late symptom) and die. Infected symptomatic tuber were to show browning of vascular bundle region. However, under cool growing conditions, wilting and other foliar symptoms may not occur which is called latent infection (Shekhawat *et al.,* 1992).

Samples collection

Random sampling technique was adopted for samples collection from different sources of infection viz. seeds, plants, infested soil, weeds and water etc. (Momol *et al*., 2006). During samples collection different divisions and AEZs (agro-ecological zones) (Table 1, Fig. 1) were considered consultinglocal farmers and local quality control/ crop protection officials from BADC (Bangladesh Agricultural Development Corporation)/ DAE (Department of Agricultural Extension).Field diagnosis of diseased plant samples were done critically by observing the symptoms of bacterial wilt and by performing some presumptive streaming test of the suspected plant samples. The test was carried out of infected potato stem both at sampling site and laboratory, just after collection of samples and before storing the samples to test the streaming of bacterial oozes.Soil samples were collected from plant streaming positive locations and other samples viz. weed, other crops, water etc. were also collected from the nearest infection positive locations. In order to increase the likelihood for detection, each site were sampled at several points and at a given site, sampling was made at least three places at about 10 feets intervals following Momol *et al*., (2006). Infection positive locations was surveyed at least three times (vegetation, late vegetation and harvest) depending on the infection availability to get different type of infection (early and late infection and tuber oozing) (Fig. 2) during the potato growing season. Labeling of each sample was done with the sample location, date, and sample identification number. Test of samples were carried to laboratory within 24 -36 hours after collection and collected samples were stored in the refrigerator between 4°C and 10°C (Momol *et al*., 2006; Sullivan *et al*., 2013) (Fig. 3). However, from each sample at least three replicates were made for advanced diagnosis which were was conducted following Goszczynska *et al*., (2000), Sullivan *et al*., (2013), IPDN (2014),Guido*tet al*., (2007) and Sagar *et al*., (2014).

Streaming test

The suspected stems were tested by dipping in a beaker/glass containing clean &/or warm water at least for 30 min after cutting longitudinal sections at the collar region to observe the milky/ cloudy oozing from vascular tissues of the diseased plants and, for oozing test in tubers, infected seeds were cut longitudinally to observe if there was oozing or browning in the vascular bundle region of the tuberrespectively as described by Danks and Barker (2000) and,IPDN (2014).

Table 1. Location of samples collection, related division and AEZ (Agro-ecological zones) of the studied samples

Sl. No.	Location of sample collection	Name of Division	AEZ covered in the region
1.	Dinajpur, Rangpur, Nilphamari	Rangpur	AEZ 1, 2, 3, 27
2.	Bogra, Joypurhat	Rajshahi	AEZ 4, 5, 6, 26
3.	Jamalpur, Tangail	Mymensingh	AEZ 7, 8, 9, Part of 28
4.	Meherpur	Khulna	Major part of AEZ 11, 14
5.	Patuakhali	Barishal	AEZ 13
6.	Chandpur	Comilla	AEZ 16, 30
7.	Shariatpur, Munshigonj, Narayangonj	Dhaka	Major part of AEZ 12, 14, AEZ 15 &part of 7, 8, 28
Quddus, 2009.			

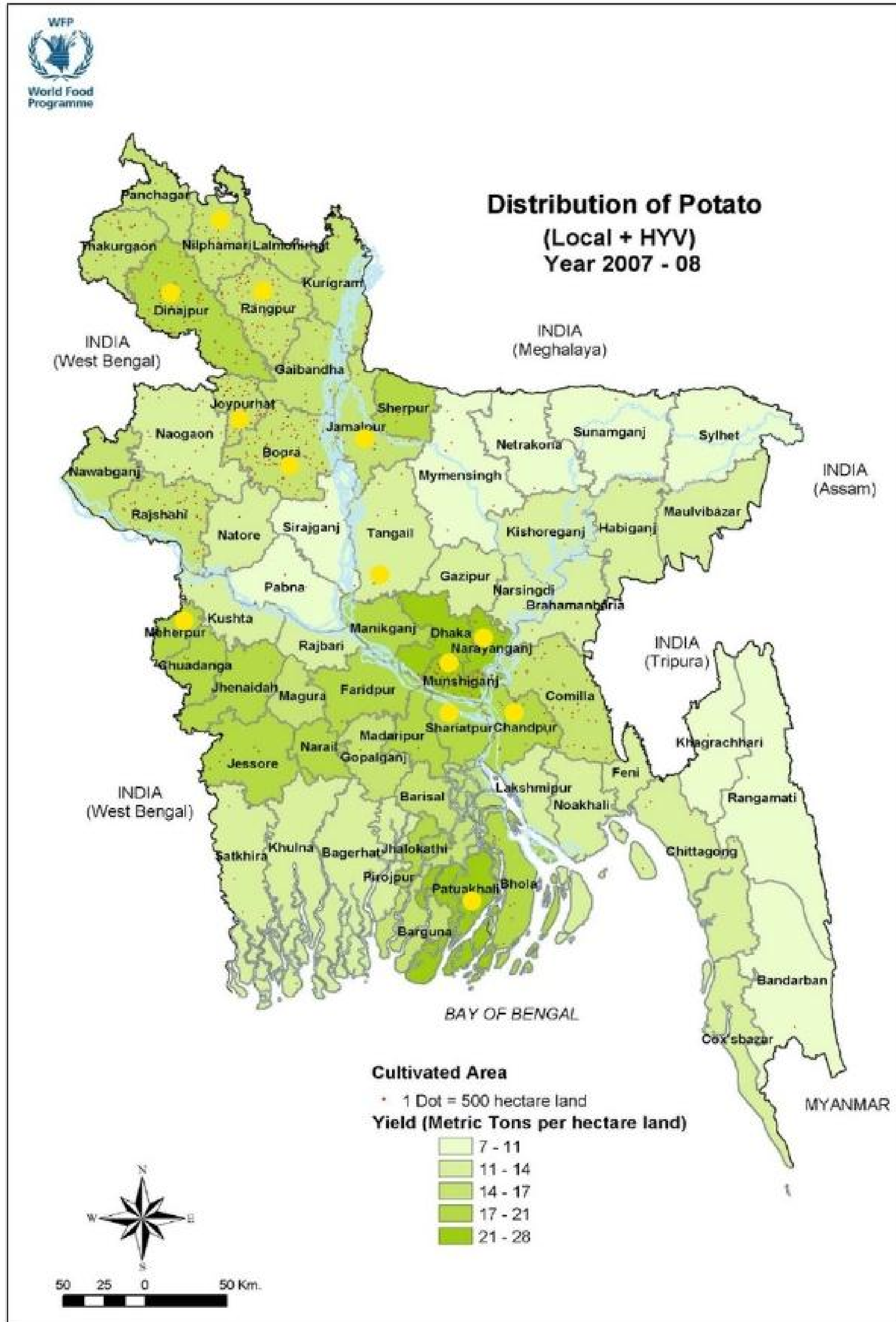


Fig. 1. Map showing location of bacterial wilt sample(s) collection from major potato growing regions of Bangladesh.

Fig. 2.a. Disease free potato field; and b.bacterial wilt infected potato field.

Fig. 2.c. Bacterial wilt infection in early vegetative stages of plants; and d. wilt infection in late vegetative stages of plantin potato.

Fig. 3.a. Different samples of bacterial wilt from plants, tubers, weeds, chilli and water in the laboratory for storing of the collected samples.

Fig. 3.b. Different steps of samples preparation infected by *R. solanacearum*to isolate and preserve the pathogen in the laboratory.

3.1.4. Isolation and preservation of *R. solanacearum*

Media preparation

To prepare TZC media 1 liter of CPG medium was prepared by using the following amount of compounds. The media was autoclaved at 121˚C for 20 minutes and5 ml of TZC (1% 2, 3, 5-tripheny tetrazolium chloride solution) was addedto the cooled the medium at 50-55˚C. It was then poured into the petri plates and stored at 4˚C. The media was prepared following Goszczynska *et al.*, (2000); Campoiseau (2008); Sullivan *et al.*, (2013); and IPDN (2014).

Casamino acid (casein hydrolysate) 1g/Litre (L)

Peptone 10g/L

Glucose/ Dextrose 5g/L

Agar (for solid media) 17g/L

1%2, 3, 5-tripheny tetrazolium chloride (TZC) 5ml/L

Isolation from stem samples

Wilted stems were collected from stem and collar regions were rinsed with sterilized distilled water after washing with 70% ethanol and/or 1% Clorox and then segmented and prepared stock solution in 10ml sterilized water (Fig. 3.b). The tubes were then stirred or vortexed and turbid bacterial suspensions were obtained. Dilutions were then carried out by adding 1 ml of sample stock to 9 ml of sterilized distilled water upto a dilution of 10^7 for stem samples. After that 100µl per sample was suspended on TZC media. It was then incubated at 28±1°C for 24 to 48 hours. Pink fluidal colonies of bacteria were collected and preserved as described later.

Isolation from seed/tuber samples

Seeds with vascular browning regions were rinsed,segmented, vortexed to prepare the stock solutionand dilutions upto 10^7were made by the same procedure as described above and were directly placed on sterilized TZC plates, incubated at 28-30°C for 2-3 days. Pink fluidal colonies of bacteria were collected and preserved as described later.

Isolation from soil samples

Soil samples (1g) and water sample (1ml) were dissolved in 10 ml of sterilized distilled water. The samples were agitated for 20 minutes and dilutions upto 10^7 were then carried out by the same procedure as described above and were directly placed on TZC plates, incubated at 28-

30°C for 2-3 days. Pink fluidal colonies of bacteria were collected and preserved as described later.

Isolation from weed and other samples

Weed and other crop (chilli) collar and root regions were rinsed, segmented, vortexed to prepare the stock and dilutions of upto 10^7 were made by the same procedure as described above and were directly placed on sterilized TZC plates, incubated at 28-30°C for 2-3 days. Pink fluidal colonies of bacteria were collected and preserved as described later.

Preservation

Virulent (pink color at the centre with fluidal in nature) colonies were isolated from the TZC media plates and then suspended in 1ml of sterilized distilled water in EP (Eppendrof tube) (@ 5-6 loops/ml water per isolate) and stored at room temperature as described by Sullivan *et al*., (2013) and IPDN (2014).

3.1.5. Hypersensitivity test of *R. solanacearum* isolates

Preparation of inocula

Strain from preserved cultures were randomly streaked on plates containing TZC media and incubated at the optimum temperature. Fluidal virulent colonies from the streaked plates were transferred to 10 ml falcon tubes containing TZC broth @ 5-6 loops/ ml for counting at 10^7 and 10^8 level of dilution. Then, culture in sterile water (@5 loops/ml) and incubated for 3 to 4 hrs. For the ease of work, it was studied and observed that a count of 5-6 loops of virulent colonies/ml could produce a good count at 10^8 level of dilution which measured following Janse (1953) and *Goszczynska et al., (2000*). After checking the count, the culture was used for infiltration or inoculation on the other (dorsal) side of leaves. During preparation for inoculation, TZC media plates and broth were kept ready in freeze at 6^0C to use in checking the colony count.

Inoculation

Hypersensitive reaction was performed on the leaf of *Euphorbia*following Shahbaz*et al*., (2015) and Zhang*et al*., (2015). Raised plants in pots were inoculated on the other side of leaves. Isolates of *R. solanacearum* for inoculations were multiplied on TZC medium for 48 h prior to inoculation. Bacterial suspensions were prepared in distilled sterilized water as described in preparation of inocula. Using 1 mL plastic disposable syringe, cell suspensions

were infiltrated into abaxial side of the mature leaves of *Euphorbia* and were observed up to 7 days after inoculation.

3.1.5.4. Race determination test by root inoculation

To determine the race of different isolates, seed bed soil was prepared by integration of farm yard manure, sand and clay in 1:1:1 ratio. This mixture was sterilized with 37% formalin by 1:9 ratio (1 part formalin and 9 parts soil). Soil mixture was covered with polyethylene sheet and placed for 3-4 days in sun light. Then in this soil potato, tomato and brinjal seeds were grown in pots for 4-5 leaf stage and seedlings were used for performing pathogenicity tests. Races were assigned based on the study of EPPO (2004)(Table 2) through artificial inoculation on potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum)* and brinjal (*Solanum melongena*). The isolates showing positive hypersensitive reaction were tested for race determination. The seedlings were inoculated with *R. solanacearum* by trimmed/wounded root dipping following Singh*et al*., (2014) and Kumar *et al*., (2017) in bacterial suspension for 30 minutes following Klement *et al.,* (1990)and *Goszczynska et al., (2000)*. About 10 mL of bacterial suspension (10^8 cfu/mL through colony counting of each isolate was used for the study. Inoculated plants were regularly watered and kept at temperature range from 28 to 30°C (Winstead and Kelman, 1952).The inoculated plants were then kept in observation until the symptoms were developed. To determine therace(s) of *R. solanacearum* thefollowing chart was followed (EPPO, 2004).

Table 2. Characteristics of races and their relationship to biovars of *R. solanacearum*

Race	Biovars	Hosts	Location
1	1,3,4	**All *Solanaceous* crops & many others hosts)**	Lowland tropics (Asia, Americas and Australia)
2	1,3	Banana and other *Musa* species	American and Asian tropics (Caribbean, Brazil, Philippines)
3	2A	**Potato and tomato**	Cool climate worldwide
4	3,4	Ginger	Asia
5	5	Mulberry	China
Not known	2T	Numerous	Amazon basin

3.1.5.6. Biochemical characterization of *R. solanacearum*

All of the 39 isolates were studied for specific biochemical tests of *R. solanacearun* which were routinely subcultured on TTC agar (aerobic, 30 °C) and on casamino acids-peptone-glucose (CPG) agar (Smith *et al*., 1995) and then tested for Gram staining test (Schaad, 1980), potassium hydroxide test (Suslow *et al.,* 1982), PHB test (poly-beta-hydroxybutyrate granules) (*Goszczynska et al., 2000*), catalase test (Schaad, 1980), kovacs oxidase test,

pectolytic test, temperature sensitivity test and sugar utilization test following Janse (1953), Hayward (1964), and *Goszczynska et al.,(2000).*

a. Gram staining

After purification gram-staining of bacterial isolates were performed. Young actively growing cultures (24hrs) were used for this study. A loop full of the bacterium was spreaded on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for 30 seconds and then washed with running tape water for one minute. It was then flooded with iodine for one minute, rinsed in tape water and decolorized with 95% ethanol until colorless runoff. After washing the specimen was counter-stained with safranin for approximately 10 seconds, washed with water, dried and observed microscopically at 10X, 40X and 100X using oil imersion (Schaad,1980).

b. Potassium hydroxide test

Isolates of *R. solanacearum* were aseptically removed from 24-36hrs old culture plates with a sterile tooth pick or an inoculating wire loop, placed on glass slide in a drop of 3% KOH solution, stirred for 10 seconds and observed for the formation of slime threads (Suslow *et al*., 1982).

c. PHB test (poly- -hydroxybutyrate granules)

A loop full of freshly grown (24-36hrs old) isolates of *R. solanacearum* was spreaded on a glass slide and fixed by heating on a very low flame. An ethanol based solution of Sudan black (0.3%) was spread over the smear for 5-15 min and then it was drained off and dried. It was then flooded with xylene for 10 seconds and dried. Then the specimen was counter-stained with safranin (0.5% aqueous solution) for approximately 5-10 seconds, washed with water, dried and observed microscopically at 40X and 100X using oil immersion(*Goszczynska et al., 2000*).

d. Catalase test

Young agar cultures (18-24 hrs) and 3% hydrogen peroxide (H_2O_2) were used to observe production of gas bubbles. A loop full of bacterial culture was mixed with a drop of H_2O_2 on a glass slide and observed for the production of gas bubbles with naked eye and under a dissecting magnification of 25X (Schaad, 1980).

e. Kovac's oxidase test

Oxidase reagent (1% tetramethyl-p-phenyl diamine dihydrochloride) solution (100 ml) was prepared and kept in rubber-stopper dark bottle. A drop of reagent was added to a piece of filter paper, placed within a glass Petri dish. Small quantities of the inoculum were rubbed on the filter paper impregnated with 1% (w/v) oxidase reagent solution. Bacteria were then noted for the development of purple color in 10-60 seconds (*Goszczynska et al., 2000*).

f. Pectolytic 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 following Janse (1953), Hayward (1964) and *Goszczynska et al.,(2000*). 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.

g. Temperature sensitivity test

The ability of bacterial isolates to grow at different selected temperature at 10±2°C, 28±2°C and 40±2°C were tested by initially growing isolates on TZC medium for 24-48 hrs. Bacterial suspensions from different isolates were prepared from 24hrs TZC cultures and 2-3 drops of suspension were plated on TZC medium at those selected temperatures (*Goszczynska et al., 2000*).

h. Sugar utilization test

The ability of bacterial isolates were tested to utilize different sugar/ carbohydrates (glucose, sucrose and maltose @ 10%) in basal medium which were incubated in 28±2°C for 24-48 hrs.After preparing the carbohydrate medium, it was brought to boil with constant stirring and raised the pH of the medium to 7.0 – 7.1 by drop wise addition of 1.0 N sodium or potassium hydroxide. When the medium turned green, the medium was then autoclaved at 121°C, 15 psi for 20 minutes.The carbohydrate medium was prepared with following amount of chemicals for per liter of media by following IPDN (2014) for sugar utilization test.

Ammonium dihydrogen phosphate (NH4H2PO4) 1.0 g

Potassium chloride (KCl) 0.2 g

Magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 0.2 g

Peptone 1.0 g

Bromothymol blue 0.03 g

Agar 3.0 g

Water 1.0 L

Bacterial suspensions from different isolates were prepared from 24hrs TZC cultures and 2-3 drops of suspension were placed on the basal medium containing those sugars in falcon tube (Hayward, 1964; Hayward, 1976;*Goszczynska et al., 2000*).

3.1.5.7. Determination of biovars

Carbohydrate medium was preparation

Carbohydrate medium was prepared with following amount of chemicals for per liter of media by following IPDN (2014) for biovar determination test. The medium was brought to boil with constant stirring and raised the pH of the medium to 7.0 – 7.1 by drop wise addition of 1.0 N sodium or potassium hydroxide. When the medium turned green, the medium was then autoclaved at 121°C, 15 psi for 20 minutes.

Ammonium dihydrogen phosphate ($NH_4H_2PO_4$) 1.0 g

Potassium chloride (KCl) 0.2 g

Magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 0.2 g

Peptone 1.0 g

Bromothymol blue 0.03 g

Agar 3.0 g

Water 1.0 L

Sugar/alcohol solution preparation

Cellobiose, lactose, maltose, dulcitol, mannitol and sorbitol was taken 1g from each of the charbohydrates and was dissolved in 10 ml distilled water separately and was filter-sterilized using 0.22µm filters.Preparation of media containing containing 10% of each disaccharides and sugar alcohol to determine the biovars of isolates of *R. solanacearum* were done following Hayward (1954) and He *et al.,* (1983). After melting the medium, it was then dispensed into the wells of microtitre plate.

***R. solanacearum* isolates preparation**

Isolates of *R. solanacearum* were grown on CPG solid media to test the biovar expression following and IPDN (2014) on the basis of carbon utilization in disaccharides and hexose alcohols following Hayward (1964), Denny and Hayward (2001) and Sullivan *et al*., (2013) which were incubated in 28^0C temperature and observed from 4-7 days in 96-well plates following IPDN (2014). To prepare CPG media following compounds were required.

Casamino acids (Difco) 1.0g
Bacto-Peptone (Difco) 10.0g
Glucose 10.0g
Bacto-Agar (Difco) 18.0g
Distilled water 1000ml

It was autoclaved the above ingredients at 121ºC for 15 minutes and cool to about 40-45ºCbefore pouring. Then, poured the homogenized medium into sterile petri dishes and let dry for 30 min. Isolates of *R. solanacearum* were then taken from pure culture to dilute @10 microliters/ml and spread plated and incubated at optimum temperature. Inocula for each isolates was prepared by adding several loopful of the bacteria from 24-48h old cultures to distilled water to make the suspension.

Inoculation and biovar determination

Semi-solid basal medium was melted in water bath and cooled to 60 to 70^oC. Then, 9 ml of basal medium was added in each 1ml of prepared sugar or alcohol solutions. Lactose, maltose, cellobiose, mannitol, sorbitol and dulcitol (previously made) @150 µL quantities were dispensed into pre-labeled ELISA plates (90 wells). These medium plates were kept at room temperature for solidification. Then ~7 µl of bacterial suspension per isolate was added to the wells of microtitre plate and incubated at 28°C. Then those were examined for 4-7 days after inoculation for change in pH by color change from olivaceous green to yellow color at the surface of the medium color change (Hayward, 1964; Schaad *et al.,* 2001) (Table 3). For control 10 mL of distilled sterilized water instead of sugar solution was used into the basal medium.

Table 3. Biovars of *R. solanacearum* based on the utilization of certain carbohydrates

Charbohydrate		Biovar 1		Biovar 2		Biovar 3		Biovar 4		Biovar 5	
Mannitol	Hexose alcohol	-	ie. do not utilize hexose alcohol	-	ie. do not utilize hexose alcohol	+	ie. utilize all of three hexose alcohol	+	ie. utilize all of three hexose alcohol	+	ie. do not utilize 2 of the hexose alcohol
Sorbitol		-		-		+		+		-	
Dulcitol		-		-		+		+		-	
Lactose	Disaccharide sugar	-	ie. do not produce acid	+	ie. produce acid	+	ie. produce acid	-	ie. do not produce acid	+	ie. produce acid
Maltose		-		+		+		-		+	
Cellobiose		-		+		+		-		+	

3.1.5.8. Phylotype detection of *R. solanacearum*through PCR test

Isolation of genomic DNA

Total genomic DNA was extracted as described by Favorgen Biotech Corporation (2017) and Sagar *et al.* (2014).A well separated bacterial colony on TZC agar was used to inoculate 1.5 ml of CPG broth (Peptone 10 g; glucose, 5 g; Casamino acid, 1 g; distilled water 1 l; pH 7.0-7.1) in 2.0 ml Eppendorf tubes. The cultures were grown at 28±2 °C for 48 h with vigorous shaking.**Favorprep DNA Extraction kit (*www.favorgen.com*)**were used to extract the DNA which was performed by adding 200µl FATG 1 bufferin each EP tube containing 100µl cell pellet (centrifuged broth cultures without fluids) of *R. solanacearum* isolates, then vortexed and incubated @ 65^0C for 3 hrsrespectively. Then, 200µl of FATG 2 buffer per samplewas added, vortexed and incubated @70^0C for 10 min.Ethanol (96%) @200µl per samplewas added and pulse vortexed thereafter and the mixtures were poured and centrifuged (@ 14,000 for 1 min) within FATG collection column and transferred to a new EP tube. Addition of 400µl of W-1 buffer per sample and centrifugation of the samples were done and flow-through were discarded. Then, addition of 750µl wash buffer per sample and centrifugation were done and flow-through werediscarded respectively. At that point 100µl pre-heated elution buffer per sample was added, let sit (for 3 min.) and centrifuged to get the genomic DNA from the isolates of *R. solanacearum*.

Table 4. List of primers used for PCR amplification targeted partial endoglucanase (*egl*) and transcriptional regulator (*hrpB*) genes

Sl. No.	Primer specificity	Primer sequence	Expected band size (bp)
1.	Forward primer for Phylotype 1	Nmult21:1F(5'-CGTTGATGAGGCGCGCAATTT-3')	**144**
2.	Forward primer for Phylotype 2	Nmult21:2F(5'-AAGTTATGGACGGTGGAAGTC-3')	**372**
3.	Forward primer for Phylotype 3	Nmult23:AF(5'ATTACSAGAGCAATCGAAAGATT -3')	**91**
4.	Forward primer for Phylotype 4	Nmult22:InF(5'-ATTGCCAAGACGAGAGAAGTA -3')	**213**
5.	Reverse primer for all Phylotype	Numult22:RR(5'TCGCTTGACCCTATAACGAGTA -3')	**NA**
6.	Species Specific forward primer	Rs759 (5'-GTCGCCGTCAACTCACTTTCC-3'),	**NA**
7.	Species Specific forward primer	Rs760 (5'-GTCGCCGTCAGCAATGCGGAATCG-3') 280 bp	**280**

Phylotype determination in PCR

Phylotype identification of each isolate was done as described by Fegan and Prior (2005) and, Prior and Fegan (2005). Phylotype specific PCR was carried out in 25 µl final volume of reaction mixture, containing 1×Taq Master Mix (PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 50 mM KCl, 10 mM Tris–HCl and 1.25U of Taq DNA polymerase), genomic DNA, distilled water and specific forward and reverse primers of targeting specific bands. The following cycling program was used in a thermal cycler: 96 °C for 5 min and then cycled through 30 cycles of 94 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s, followed by a final extension period of 10 min at 72 °C. A 5 µl aliquot of each amplified PCR product was subjected to electrophoresis on 2 % agarose gel, stained with ethidium bromide and bands were visualized on a UV-trans-illuminator. The PCR amplification was targeted 280-bp "universal" *R. solanacearum* specific reference band and following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372- bp amplicon from phylotype II strains; a 91-bp amplicon from phylotype III strains; and a 213-bp amplicon from phylotype IV strains (Sagar, *et al.*, 2014). However, determination of phylotype by using the phylotype specific primers (Table 4) were used in the study to understand the present subspecific diversity of *R. solanacearum*in major potato growing regions of Bangladesh.

3.2. Experiment 2

Study on the influence of moisture and temperature on heterogeneity within species in relation to PC (Phenotypic conversion /VBNC), virulence and biovar expression of *Ralstonia solanacearum* isolates

3.2.1. Determination of heterogeneity in PC (phenotypic conversion/VBNC) and virulence expression in relation to moisture

3.2.1.1.Determination of heterogeneity in PC expression (phenotypic conversion /VBNC)

Thirty nine *R. solanacearun* isolateswere selected for the study which were cultured on TZC agar (aerobic, 30 °C) and stored as pure culture following Smith *et al*.,(1995). Those isolates were preserved in different water medium (non-aerated and aerated) for different period to observe the phenotypic conversion as described by Alvarez *et al*., (2010)which has been described below.

Isolate preservation in non-aerated water medium

A set of pure culture of the isolates of *R. solanacearum* was preserved for 18 months in non-aerated (capped in EP tube) sterile water medium to observe the PC (phenotypic conversion/ VBNC) phenomena following Lemessa and Zeller (2007),Alvarez *et al*., (2010)and Liu *et al*., (2012).

Isolate preservation in aerated water medium

A set pure culture of isolates of *R. solanacearum* was preserved in aerated (air blown/fanned on uncapped EP tube) sterile water medium containing 10% dextrose (filter sterilized with 0.22µm pored filter)for just 36-72 hrs from the samenon-aerated pure cultures following Lemessa and Zeller (2007); Alvarez *et al*., (2010)and Liu *et al*., (2012).

3.2.1.2. Determination of the heterogeneity in virulence expression

Determining the pure culture concentration in broth and colony count of pure culture

Growth in TZC broth culture was determined by following Lemessa and Zeller (2007). Freshly cultured cells from pure culture were stabbed into 5 mL NA broth and incubated at 28°C for 18 h. The optical absorbance at 600 nm of RS cultures was adjusted to approximately 0.57 with fresh TZC broth under aseptic operation. The absorbance after 8 h of

incubations was determined using spectrophotometer and each isolate was repeated three times. The concentration of pure culture (cfu/ml) was measured on solid media during preservation of the isolates of *R. solanacearum* following*Goszczynska et al.,(2000).* The absorbance (optical density-OD at 600nm) and concentration (cfu/ml) of the pure culture was measured to check and compare the heterogeneity within species of *R. solanacearum* by correlation study.

Determining virulent and avirulent and/or interim colony count (cfu/ml)

Isolates of *R. solanacearum* were taken directly from broth cultures @ 10 microliter/ml to prepare the culture at 10^7, 10^8 and 10^9 level of dilution in sterile water medium containing 10% dextrose following Alvarez *et al*., (2010). Then isolates were plated on TZC solid media by following *Goszczynska et al.,(2000)* to observe the maximum count of virulent colony and incubated for 36-48 hrsand each isolate was replicated three times. During quantifying the colonies, t*he* virulent, and avirulent&/or interim colonies at 10^7 and 10^9 level were counted following Liu *et al*., (2004) and Zheng *et al.,*(2014). The number of virulent and/or avirulent colonies were counted by using colony counter (Fig. 4) and, for the ease of work, media plates and broth following proper autoclaving were kept ready in freeze at 6^0C to use in checking for colony count.

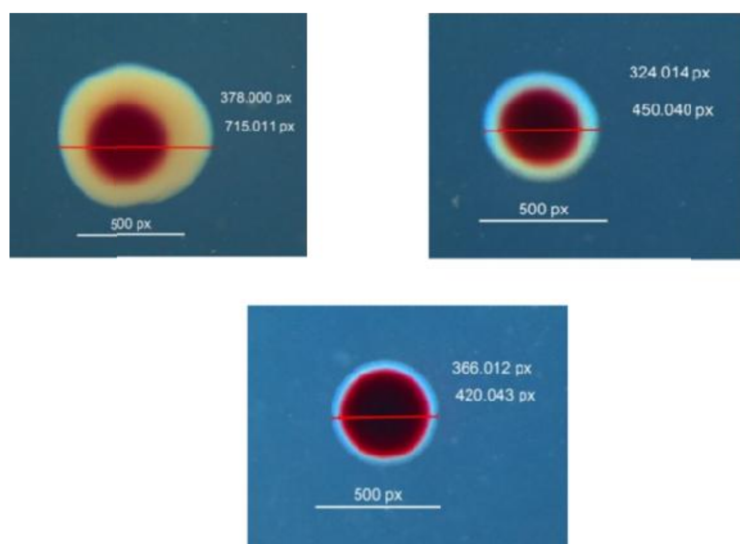


Fig. 4. Virulent, avirulent and interim colony of *R. solanacearum* counted in the study suggested by Liu *et al*., (2004) and Zheng *et al.,*(2014).

Virulence test in potato plants

Virulence assays were performed on potato plants (*Solanum tuberosum* cv. Diamant) using the root inoculation following Singh*et al*.,(2014),Zhang *et al*., (2013 & 2015) as described previously. For the inoculation, 2– 3-week-old plants were inoculated with bacteria at a final concentration of 10^8 cfu /ml. Plants were inspected for wilt symptoms daily upto 7-10 days after inoculation. Each test for one isolate included at least 9 plants per treatment and each assay was repeated at least three independent trials. The mean values were averaged with SD. Each test included at least three samples from different plants and mean values from trials were averaged with SD. Statistical significance was determined by t-test by following Zhang *et al*., (2015).

Preparation of inocula androot inoculation

Isolate(s) fromphenotypically converted culture(s) were**streaked** on plates containing TZC media and incubated at the optimum temperature. Then,each culture wasdiluted in 10ml sterile water medium with 10% dextrose in falcon tubes following Alvarez *et al*., 2010 and poured the virulent colonies @ **5loops/ml in falcon tube and incubated** for 36-48hrs which was then used in inoculation for virulence test which was **checked at 10^8 dilution** in pour plate to make sure of the colony counts which was then used to**root inoculate** in virulence test. It was observed that 4-5loops colony per ml could produce a good number of colony at 10^8 level of dilution. Cell density adjusted to 10^8cfu/ml through colony counting was measured as described by Janse (1953) and*Goszczynska et al.,(2000).* During such observation, set of 5-8 isolates were used at a time.*R. solanacearum* was used for artificial inoculation in seedlingswounds (root trimming with inoculated scissors)followingMichel *et al*., (1997) and Singh*et al*., (2014)after the pathogen was prepared before as described. The inoculum suspension was used to trim rootswith inoculated scissors of previously grown healthy potato seedlings and scored by observing the symptoms between 2-7 days after inoculation followingAyana *et al*., (2011) and Zheng *et al*., (2014).

Disease measurements

Symptom development was evaluated at a six point rating scale (0–5) followingSwanson *et al*., (2005); Ayana*et al*., (2011)and Zheng *et al*., (2014) modified from Winstead and Kelman (1952) which suggested-

0 = no wilt symptoms,

1 = one/few leaves or one third of whole leavesshowed wilt symptoms,

2 = several/more leaves or half of whole leavesshowed wilt symptoms,

3 = most leaves or two third of whole leavesshowed wilt symptoms,

4 = whole plant showed wilt symptoms, and

5 = death (collapse) of the whole plant.

3.2.1.3. Influence of soil moisture on virulence expression in potato plant and on colony count

The study was performed to assess the consequence of changing dilution level (by watering /irrigation) in soil on virulence expression (both on wilt disease severity and onvirulent & avirulentcfu/ml) of a least virulent strain of *R. solanacearum*. It was to understand the level of soil moisture that could upshot the disease severity of potato plant in relation to the expression of virulent colony count of *R. solanacearum* which was conducted by following method.

Preparation of inocula for soil inoculation

For the study, an isolate ($PS1_{1}$-STR08) was selected randomly from the least virulence (DSS 2.6) showing isolates of *R. solanacearum* based on previous study for soil inoculation following the method of French *et al*.,(1995) and was streaked on plates containing TZC media and incubated at the optimum temperature. Following French *et al*.,(1995) the inoculum suspension was prepared @ 10^8 colony forming unit per milliliter (CFU/ml) and cell density adjusted to 10^8cfu/ml through colony counting was done as described by *Goszczynska et al.,(2000)*. As it was needed to apply in pot soil, it was required in liters and therefore, it was cultured in 2 streak plates and transferred the fluidal virulent colonies with pink center (@ 2plates /litre) to the conical flask containing 1000 ml of TZC broth media and incubated. That culture was then checked at 10^8 dilution on TZC solid media plate and inoculated. The inoculum suspension was inoculated as a soil drench as described by Michel *et al*., (1997) on same day of transplanting.*R. solanacearum* used for artificial inoculation in pot after the pathogen was cultivated on TZC agar medium following the method of French *et al*.,(1995); Ayana*et al*., (2011) and Zheng *et al*., (2014).

Soil inoculation and moisture management

To observe the effect of different level of moisture, three cages of soils were inoculated @ 500 ml inoculum per cage of soil and mixed thoroughly by following French *et al*., (1995); Ayana *et al*., (2011) and Zheng *et al*., (2014). Then potato plants were grown in that soil which were termed as- a) T1 where irrigation was applied @ 2 per 7days, showed the moisture level >5.5; b) T2 where irrigation was applied @ 1 per 7days, showed the moisture

level 3-5.5; and c) T3 where irrigation was applied @ 1 per 10days, showed the moisture level <3. The level of moisture was measured by using moisture meter in the inoculated soils and colony counts present in soil dilution was done by dilution plate methods at both 10^6 (to compare & visualize the differences among the treatments) and 10^8 (to count the colonies clearly) level. *The experiment was* replicated four times *both in pot and* on TZC solid media to score the disease severity and to get the colony count (for virulent, interim and avirulent colony per square cm in colony counter) following Liu *et al*., (2004) and Zheng *et al.,*(2014) indexing.

Moisture measurement of *R. solanacearum* inoculated soil

The level of moisture was measured by using moisture meter in the inoculated soils before collecting the soil for colony counts present in soil dilution by dilution plated methods. The moisture meter was scored from 1-8 scale which was equivalent to the range of 10%-80% of soil moisture.

Disease measurements

Disease scoring was performed in a six point rating scale (0–5) followingSwanson *et al*., (2005); Ayana*et al*., (2011)and Zheng *et al*., (2014) modified from Winstead and Kelman (1952) which suggested-

0 = no wilt symptoms,
1 = one/few leaves or one third of whole leavesshowed wilt symptoms,
2 = several/more leaves or half of whole leavesshowed wilt symptoms,
3 = most leaves or two third of whole leavesshowed wilt symptoms,
4 = whole plant showed wilt symptoms, and
5 = death (collapse) of the whole plant.

As, all plants showed disease due to artificial soil inoculation, it was 100% incidence in all cases. Percent severity index (PSI) was calculated using the method described by Cooke (2006). PSI = (scores × 100)/ (number of plants rated × maximum scale of the scores) for each scoring date.

3.2.2. Determination of heterogeneity in biovar expression in relation to different incubation temperature

3.2.2.1.Determination of biovar(s) of *R. solanacearum* isolatesin different temperatures

Biovar determination

Isolates of *R. solanacearum* were grown on CPG solid media to test the biovar expression in two different incubation temperatures (22^0C and 28^0C) following and IPDN (2014) on the basis of carbon utilization in disaccharides and hexose alcohols as described earlier on section 3.1.by following Hayward(1964), Denny and Hayward (2001) and Sullivan *et al.*, (2013) which were incubated in two different temperatures (22^0C and 28^0C) and observed from 4-7 days in 96-well plates following IPDN (2014) for determination of the reaction as well as the biovar(s).

3.2.2.2. Effect of different temperature on avirulent colony of *R. solanacearum* in colony virulence and biovar expression

The study was performed to understand the effect of different temperature on an avirulent colony dilution of *R. solanacearum*. For this study, five avirulent colony forming unit of *R. solanacearum* was randomly isolated and diluted in 1000 microliter sterile water and dilution was made upto 10^6 and 10^7 level and spread plated onto TZC agar medium and incubated as 0 ± 2^0C (T1), 10 ± 2^0C (T2), 20 ± 2^0C (T3), 28 ± 2^0C (T4) and 40 ± 2^0C (T5) and observed 36-72 hrs. Nonetheless, cultures those were showing VBNC, were further incubated in optimum temperature 28 ± 2^0C to observe the growth status. Then, virulent, avirulent and/or interim colonies per square cm were counted by using colony counter as earlier following Liu *et al.*(2004) and Zheng *et al.*(2014).

For evaluating the biovar reaction of *R. solanacearum* cultures of T1, T2, T3, T4 and T5, clonieswere incubated at 22^0C and 28^0C to test the carbon utilization in case of disaccharides and hexose alcohols as described earlier on section 3.1. following Hayward(1964) and, Denny and Hayward (2001) and observed from 4-7 days in 96-well plates following IPDN (2014).

3.3. Experiment 3

Study on the management of bacterial wilt of potato caused by *Ralstonia solanacearum* by using selected bioactive compounds

3.3.1. Determination of effectiveness of selected bioactive compounds against *R. solanacearum*

Treatments dosage and preparation

During the study on the management of bacterial wilt of potato caused by *R. solanacearum,*thirteen treatments of bioactive compounds (Table 5) including a negative control (distilled water), a standard control (stable bleaching powder) and a chemical control [Krosin AG-a bactericide containing 9% streptomycin sulphate (w/w) and 1% tetracyline hydrochloride (w/w), marketed by Krishi Rasayan Export Pvt. Ltd., India] was evaluated to figure out their effectiveness by different methods (*in vitro, in vivo* and pot test using unsterilized field soil condition).

Table. 5. Treatments selected for study on the effectiveness against *R. solanacearum*following different review of their antibacterial properties and doses as bioactive compounds

Dosage of treatment prepared by ioactive compounds	Bioactive antimicrobial compound of treatment	Reference
T1= Control (sterile water)	-	
T2= Propolis solution (@ 6mg/ml)	Phenolics & flavonoids	Rahman *et al.,*(2010).
T3= Honey solution (@20%)	Hydrogen peroxide (H2O2), Methylglyoxal (MGO)& different enzymes	Majtan*et al.*, (2014); Balan *et al.,*(2016).
T4= Turmeric oil solution (@25%)	Curcumin (diferuloyl methane)	Balan *et al.,*(2016).
T5= Turmeric powder solution (@25%)	Curcuminoids which are fat soluble	Narasimha *et al.,*(2015).
T6= Magnesium chloride solution (@3%)	Mg+2 as enzymatic co-factors, as signaling molecules	Oyarzúa *et al.,*(2014).
T7= Rice extract solution (@1000ppm ie. 1g/liter)	Rice-fluid contains rice phytochemicals viz. oryzanols, anthocyanins, amino acids, essential oils, phenolics, etc.	Ishizone *et al.*, (2007); Kawakami *et al.*, (2006);Chakuton*et al.*, (2012); Deng *et al.*, (2013); Mannan *et al.*,(2014).
T8= Rice extract + Iodine (@2 drops of iodine per 100ml of 1000ppm ie. 1g/liter)	Phytochemicals oryzanols, anthocyanins, amino acids, essential oils, phenolics etc. & disinfectant	Mannan *et al.*,(2014);Estrela *et al.*,(2006).

Dosage of treatment prepared by ioactive compounds	Bioactive antimicrobial compound of treatment	Reference
T9= Cow dung powder solution (@25%)	Large number of microorganisms produce metabolites, like k, Na, Mg etc in higher levels acting as cofactors for various enzymes.	Shrivastava*et al.*,(2014); Waziri and Suleiman (2013).
T10= Krosin AG (Bactericide @ 0.5 g per liter water)	Streptomycin sulphate 9.0 % & Tetracyline hydrochloride 1.0 %, broad spectrum antibiotic bacteride.	Company packet
T11= Honey + Iodine (@2 drops of iodine per 100g of 20%)	Hydrogen peroxide (H2O2), Methylglyoxal (MGO)& different enzymes; disinfectants	Secorand Gudmestad (1993); Estrela *et al.,*(2006); Majtan *et al.*, (2014); Balan *et al.*,(2016).
T12= Sodium bicarbonate (@10%)	Nahcolite, acid neutralizing andchanges osmotic pressure which causes microbes to lose water and dehydrate	Kelly and Kristin (2005).
T13= Stable bleaching powder (@30 kg/ha).	Active chlorine 20-70% compromises the lipid membrane of bacteria	Sharma and Kumar(2000).

Treatment preparation by using the selected bioactive compounds was performed as following methods.

a.) T1/ Control (sterile water)

It was applied as spray in seed and soil treatments.

b.) T2/ Propolis solution (@ 6mg/ml)

Propolis was collected from Honey Bee Pest and Disease Diagnostic Laboratory of Department of Entomology, Sher-e-Bangla Agricultural University, Dhaka. It was weighed and soaked @ 6g/liter of preparation in 30% ethanol for 24hrs. Then, mixed with some sterilized soil(@ 1:1 ratio) and stored in room temperature. This was applied as seed and soil treatment to be applied in different experiments.

c.) T3/ Honey solution (@20%)

Honey was also collected from Honey Bee Pest and Disease Diagnostic Laboratory of Department of Entomology, Sher-e-Bangla Agricultural University, Dhaka. It was then 200g

honey was mixed per liter of distilled water and stored in room temperature to be applied as seed and soil treatment.

d.) T4/ Turmeric oil solution (@25%)

Commercial turmeric powder and mustard oil was mixed @ 1:1 and 250g per liter of distilled water and stored in room temperature to be applied as seed and soil treatment.

e.) T5/ Turmeric powder solution (@25%)

Commercial turmeric powder was mixed @ 250g per liter of distilled water with some sterilized soil (@1:1 ratio) and stored in room temperature to be applied as seed and soil treatment.

f.) T6/ Magnesium chloride solution (@3%)

Magnesium chloride salt was collected from market and @ 30g per liter of distilled water and stored in room temperature to be applied as seed and soil treatment.

g.) T7/ Rice extract solution (@100 times of 1000ppm ie. 1g/liter (as to be applied as soil and seed treatment)

Fresh kalojira aromatic rice was bought from market and after cleaning 100 g of the rice was boiled to a thick soup. After straining the soup was mixed @ 100ml per liter of distilled water and stored in 6^0C temperature to be applied as seed and soil treatment.

h.) T8/ Rice extract + Iodine (@2 drops of iodine per 100ml in 100times of 1000ppm ie. 1g/liter)

It was prepared in the same manner as T7 and just added 20 drops of iodine solution bought from market.

i.) T9/ Cow dung powder solution (@25%)

Well decomposed and dried cowdung was weighed for 250g and was brought to powder form by using morter and pestle. It was then mixed with some sterilized soil (@ 1:1 ratio) and distilled water by vigorous shaking and stored in room temperature to be applied as seed and soil treatment.

j.) T10/ Krosin AG (Bactericide @ 0.5 g per liter water)

It was bought from market and applied as suggested by the company.

k.) T11/ Honey + Iodine (@2 drops of iodine per 100g of 20%)

Honey solution was made as previous and just 20 drops of iodine solution was added as previous and well shaken and stored in room temperature to be applied as seed and soil treatment.

l.) T12/ Sodium bicarbonate (@10%)

It was brought from market and weighed 100g and added per liter of distilled water and stored in room temperature to be applied as seed and soil treatment.

m.) T13/ Stable bleaching powder (@30 kg/ha)

It was also bought from market and weighed for 11.5 g to mix per liter of distilled water and stored in room temperature to be applied as seed and soil treatment.

3.3.1.1. *In vitro*evaluation of selected bioactive compoundsin producing inhibition zone and reducing colony virulence of against *R. solanacearum*

Screening out the effectiveness of selected bioactive compounds in producing inhibition zone in disk diffusion method

Bioactive compounds were screened for their effectiveness in producing the inhibition zone against *Ralstonia solanacearum* by disk diffusion method by following Bauer*et al.*, (1966) and Yenjerappa (2009). Inhibition diameter at every plate under each treatment was measured by scale as described by Bauer *et al.*, (1966) and Bonev *et al.*,(2008).

Screening out the effectiveness of selected bioactive compounds in producing the avirulent colony counts in disk diffusion method

Bioactive compounds were screened for their effectiveness in producing the avirulent counts of *Ralstonia solanacearum* in disk diffusion method by following Bauer *et al.*, (1959, 1960 &1966),Liu *et al.*, (2004),Liu *et al.*, (2012),Zhang *et al.*,(2014) and Kumar *et al.*, (2017) as it shows phenotypic conversion phenomena. *R. solanacearum* were taken directly from bacterial suspension @ 10 microliter/ml to prepare the culture at 10^7 and 10^9 level of dilution in sterile water medium containing 10% dextrose following Alvarez *et al.*, (2010). The inoculum suspension was plated on TZC solid media by following *Goszczynska et al.,(2000)* to observe the maximum count of virulent colony and incubated for 36-48 hrsand each isolate was replicated three times. The individual colony of virulent, and avirulent&/or interim type as described by Liu*et al.*, (2004) and Zheng *et al.*,(2014) (as shown in Fig. 4) and TZC plated

colonies produced by avirulent *R. solanacearum* as described by Kumar *et al*.,(2017) (as shown in Fig. 5) were taken into consideration to count the virulent and avirulent and/or interim colonies by using colony counter (Fig. 6). For ease of work, media plates and broth following proper autoclaving were kept ready in freeze at 6^0C to use in checking for colony count.

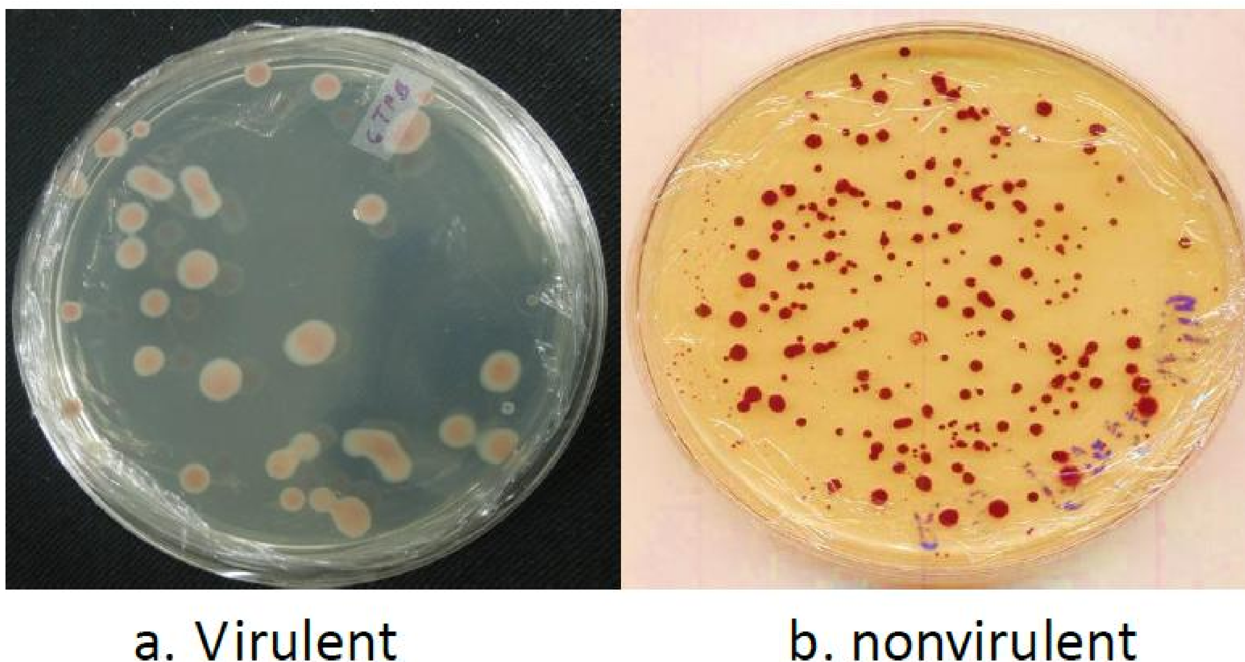


Fig. 5. Plate showing virulent and avirulent/nonvirulent colony of *R. solanacearum*suggested by Kumar *et al*., (2017).

Fig. 6. Colony counterused to count different colony of *R. solanacearum*.

Petri plate inoculation method

Inoculation of petri plates for evaluation of antimicrobial activity of selected bioactive compounds were performed by disk diffusion method according to Bauer *et al*., (1966). In this procedure, agar plates were inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test compounds (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12 and T13) at a desired

concentration, were placed on the agar surface. Then, petri dishes were incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits growth of the test microorganism and then the diameters of inhibition growth zones are measured (Balouiri *et al*., 2016). Disk diffusion was performed @ four to five colonies of the organism per 4 ml of sterile water and incubated for 3 to 4 h. Then, that suspension of *R. solanacearum* was suspended on the test petri plates by using sterile cotton swabs. After that, the disks of whatman no. 1 filter paper impregnated with selected bioactive compounds @ 10 microlitres at the prepared doses were placed on to the petri plates and incubated at 30^{0}C for 2 days (Bauer *et al*., 1966; Balouiri *et al*., 2016). After the incubation of 24-36hrs the measurement of inhibition zones and virulent & avirulent/interim colony counts (per square cm in colony counter) were taken following Fig. 5 from Kumar *et al*., (2017).

3.3.1.2. *In vivo*evaluation of effectiveness of selected bioactive compounds as seed treatments

Germinated potato seeds were treated to evaluate the selected bioactive compounds in micro plots to observe the performance of those compounds against the wilt pathogen, *R. solanacearum*. By following the study of Ayana *et al*., (2011),Singh *et al*., (2014) and Zheng *et al*., (2015), treated seeds (with bioactive compounds) were planted and pinch inoculated at 3rd week ie. 2weeks later treatment application on potato and seedlings were observed up to 10 days post inoculation for effectivity of those compounds in disease virulence expression.

Seed inoculation

R. solanacearum was used for artificial inoculation ingerminated seed wounds following Janse(1953), Hayward (1964),Lemessa and Zeller (2007) and Singh *et al*., (2014) after the pathogen was cultivated on triphenyl tetrazolium chloride (TZC) agar medium. The inoculum suspension was prepared @ 10^8 colony forming unit per milliliter (CFU/ml). The inoculum suspension was inoculated followingMichel *et al*., (1997) and Singh *et al*., (2014) on same day of planting.

Disease measurements

Disease measurementperformed by observing the prominent symptoms from 3rd to 4th week after inoculation in a six point rating scale (0–5) followingSwanson *et al*., (2005); Ayana *et*

al., (2011) and Zheng *et al*., (2014) modified from Winstead and Kelman (1952) which was described earlier.

3.3.1.3. *In vivo* evaluation of effectiveness of selected bioactive compounds as soil (sterilized) treatments

Three weeks old potato seedlings were planted in the selected bioactive compounds treated soils (sterilized) in micro plots to observe the performance of those compounds against the wilt pathogen, *R. solanacearum*. By following the study of Ayana *et al*., (2011),Singh *et al*., (2014) and Zhang*et al*., (2015),potato seeds were planted in treated soils of micro plots which were treated with the bioactive compounds two weeks before planting the potato seedlings. The soils were inoculated with pouring the pathogen suspension @10^8 cfu/ml by soil soak inoculation following Zhang *et al*., (2015) just before planting the seedlings of 3rd week and were observed up to 2 weeks (4th and 5th week) post inoculation to measure the reponse of those compounds in disease virulence expression.

Preparation of inocula

In case of management study, before inoculation, TZC media plate and broth media were kept ready in freeze at 6^0C to use in checking the colony count. Then, the isolate to be used for inoculation was gown in solid media by streak plate method and cultured @5loops of virulent colony/ml in 10 ml broth in falcon tube and incubated. While needed for soil inoculation, it was cultured again in 500ml or 1000ml broth in the same rate.That culture was then diluted upto 10^8 level for checking the count. Cell density adjusted to 10^8cfu/ml through colony counting was done as described by Janse (1953),*Goszczynska et al., (2000*) and Zhang*et al*. (2015).

Soil inoculation

R. solanacearum was used for inoculation of sterilized soil (121^0C at 15 for 1 hr) in pot after the pathogen was cultivated on triphenyl tetrazolium chloride (TZC) agar medium following the method of French *et al*.,(1995),Ayana *et al*., (2011),Zhang*et al*., (2015). The inoculum suspension was inoculated as a soil drench as described by Michel *et al*., (1997) on same day of transplanting.

Disease measurements

Disease measurementperformed by observing the prominent symptoms from 4th to 5th week after inoculation in a six point rating scale (0–5) followingSwanson *et al*., (2005),Ayana*et al*.,

(2011) and Zheng *et al*., (2014) modified from Winstead and Kelman (1952) which was described earlier.

3.3.1.4. *In vivo*evaluation of effectiveness of selected bioactive compounds as soil treatments in unsterilized soil

Seeds treated with selected bioactive compounds were planted in unsterilized (just solarized) soil which is an economic alternative to soil fumigation suggessted by Agrios (2005),Hardy and Sivasithamparam (1985),Chellemi *et al*., (1997), Pinkerton *et al*., (2000)and were inoculated at five weeks by root trimming inoculation with pathogen suspension following Kumar *et al*., (2017)which evaluatedto observe the performance of those compounds as in natural field condition by allowing the natural microbial community against the wilt pathogen, *R. solanacearum*.The seedlings were observed up to 2 weeks (6th and 7th week) post inoculation to record the disease response of those compounds in virulence expression.

Root inoculation

R. solanacearum was used for artificial inoculation in seedling wounds (root trimming with inoculated scissors) in unsterilized soil (just solarized to allow the natural soil microbes)following Janse (1953), Hayward (1964),TanakaandNoda (1973), Singh *et al*., (2014)and Kumar *et al*., (2017) after the pathogen was cultivated on triphenyl tetrazolium chloride (TZC) agar medium. The inoculum suspension was prepared @ 10^8 colony forming unit per milliliter (CFU/ml) as described earlier. The inoculum suspension was inoculated followingMichel *et al*., (1997) and Singh *et al*., (2014)and treatments on same day of planting.

Disease measurements

Disease measurementperformed by observing the prominent symptoms from 4th to 5th week after inoculation in a six point rating scale (0–5) followingSwanson *et al*., (2005),Ayana*et al*., (2011) and Zheng *et al*., (2014) modified from Winstead and Kelman (1952) which was described earlier.

3.3.2. Evaluation of effectiveness of propolis, turmeric powder and cowdung as both soil and seed treatment

Better preforming bioactive compounds (in all evaluation especially in unsterilized soil condition) viz. propolis, turmeric powder and cow dung were evaluated as both seed and soil treatment against *R. solanacearum*. Germinated potato seeds and soils (different cages) were

treated with propolis, turmeric powder and cow dung along with control and stable bleaching powder (as farmers practice) at the specific doses (as described earlier) to observe and compare the performance of those compounds against the wilt pathogen, *R. solanacearum* following the study of Lemessa and Zeller (2007),Ayana *et al*., (2011),Singh*et al*., (2014),Zhang*et al*., (2015).Treated seeds (with bioactive compounds) were pinch inoculated following Lemessa and Zeller, (2007) and treated soil was soil-soak inoculated 2weeks later treatment application following Zhang *et al*., (2015) and were observed up to 7th weeks after inoculation for disease measurement in virulence expression.

Seed inoculation

R. solanacearum was used for artificial inoculation ingerminated potato seeds treated with propolis, turmeric powder and cow dung following Lemessa and Zeller (2007) and Singh *et al*., (2014) after the pathogen was cultivated on triphenyl tetrazolium chloride (TZC) agar medium. The inoculum suspension was prepared @ 10^8 colony forming unit per milliliter (CFU/ml). The inoculum suspension was inoculated followingMichel *et al*., (1997) on same day of planting as described earlier.

Soil inoculation

R. solanacearum was used for soil-soak inoculation of treated unsterilized soils in different cages after the pathogen was cultivated on triphenyl tetrazolium chloride (TZC) agar medium by following the method of French *et al*.,(1995),Ayana*et al*., (2011) andZhang *et al*., (2015). The inoculum suspension was inoculated following Michel *et al*., (1997) on same day of transplanting as a soil drench as described earlier.

Disease measurements

Disease measurementperformed by observing the prominent symptoms from 4th to 5th week after inoculation in a six point rating scale (0–5) followingSwanson *et al*., (2005),Ayana *et al*., (2011) and Zheng *et al*., (2014) modified from Winstead and Kelman (1952) which was described earlier.

3.3.2.1. Determination of virulent and avirulent colony count of propolis, turmeric powder and cow dung treated soil

Virulent and avirulent colony counts (cfu/ml) of propolis, turmeric powder and cow dung treated soil as compared to control and stable bleaching powder were measured from soil dilution (@ 10^5 and 10^7) on TZC solid media to observe the avirulent colony count at 7th (last) week by following Zheng *et al*., (2014) and Kumar *et al*., (2017) as previous.

3.4. Statistical analysis

Each test (virulence, colony count and effectiveness) included at least three samples and mean values from trials were averaged with SD. Data were compiled, tabulated and subjected to statistical significance analysis by using MSTAT-Cand *t*-test following Gomez and Gomez (1984) and Zhang*et al*.,(2015). For correlation study, to figure out the heterogeneity expressed in t*he* virulent, and avirulent &/or interim colony counts at different dilution level within the species of *R. solanacearum* were performed by using data analysis toolpak software in windos 10.

Chapter IV

Results

4.1. Experiment 1

Detection and characterization of race(s), biovar(s) &/or phylotype(s) of *Ralstonia solanacearum*causing potato wiltin major growing regions of Bangladesh

4.1.1.a. Sample(s) collection

Symptom studyand streaming test

Plant showing wilting of younger leaves, even under adequate soil moisture condition, were suspected to have *R. solanacearum* infection. The symptom started with slight wilting of leaves at the ends of the youngest branches (early symptom) during the warmest time of the day and eventually, plants failed to recover followed by total wilting. Milky or cloudy threads like streaming signified the presence of *R. solanacearum* of bacterial wilt disease which was observed.In advanced stage, epinasty (downward curvature of infected plant parts/stem) or stunting observed to occur (late symptom) and died. Infected symptomatic tuber showed browning of vascular bundle region (Fig. 7.a,b,c&d).

In streaming test it was observed that,$PS3_2STM01$ and $PS3_2STM02$ no. samples from Munshiganj; $PS8_{2-}STC01$ and $PS8_{2-}STC02$ no. samples from Chandpur, $PS7_{1-}STN01$, $PS7_{1-}STN02$ and $PS7_{1-}STN03$ no. samples from Tangail; $PS9_{1-}STNG02$ and $PS9_{1-}STNG04$ no. samples from Narayanganj; $PS2_{3-}STJ03$, $PS2_{3-}STJ04$ and $PS2_{3-}STJ05$ no. samples from Jamalpur; $PS10_{1-}STD01$, $PS10_{1-}STD04$, $PS10_{1-}STD05$ and $PS10_{1-}STD06$ no. samples from Domar; $PS4_{3-}STP07$ no. samples from Patuakhali; $PS1_{1-}STR07$ and $PS1_{1-}STR08$ no. samples from Rangpur; and $PS11_{1-}STB08$ and $PS11_{1-}STB09$ no. samples from Bogra showed bacterial ooze streaming in clear water and based on this response other samples (soil, weeds, other crop (chilli) and water) were selected. In case of potato seeds no oozing was found in the samples except browning of the vascular bundle region of seed tuber. Besides, the weed plant samples did not show any streaming in any case (Table 6).

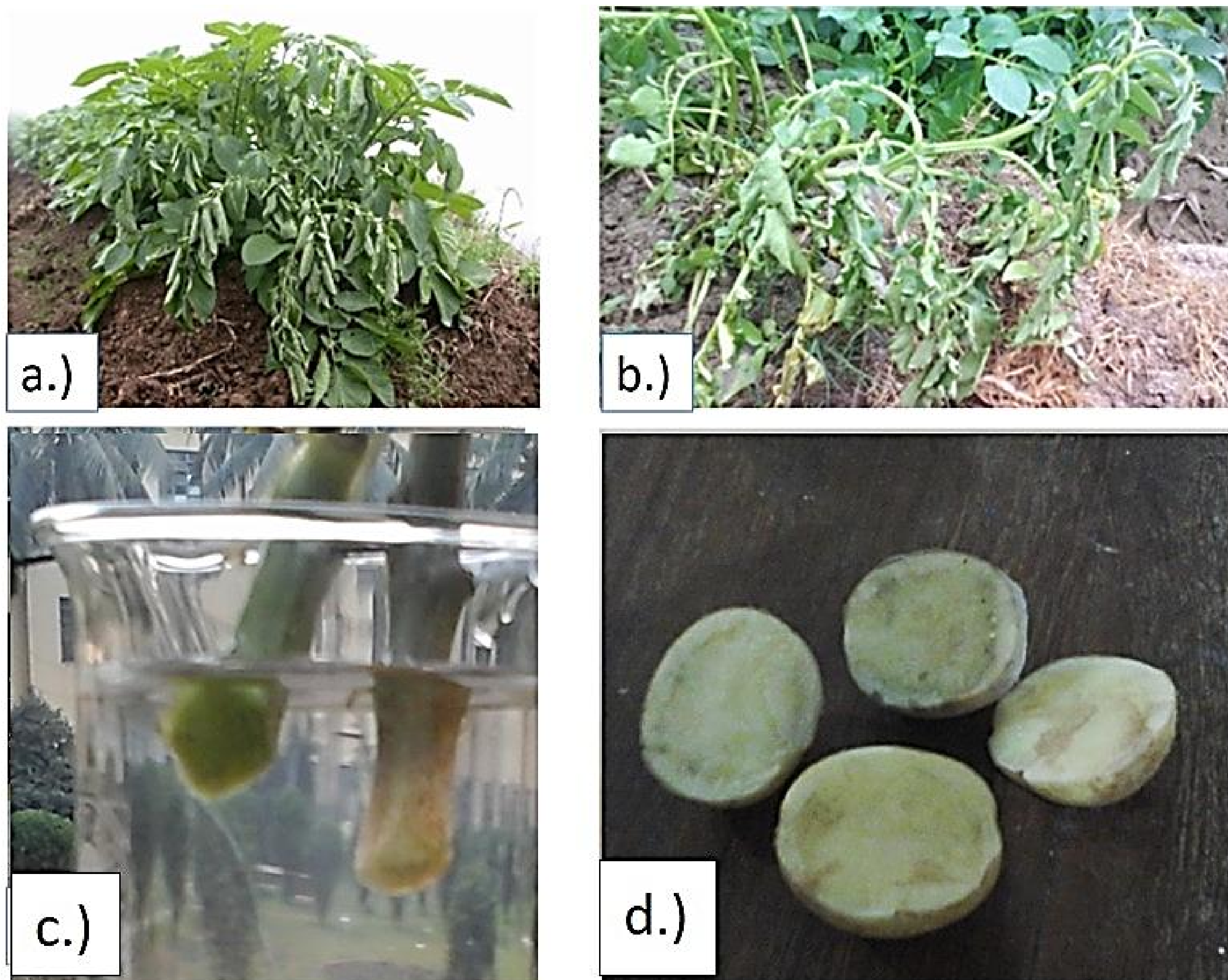


Fig. 7.a.Early symptoms of infection; b. late symptoms (epinasty/ downward curvature) of infection; c. bacterial ooze streaming out of the infected potato stem; and d. infected potato seed with vascular browning of bacterial wilt disease caused by *R. solanacearum* in potato plants.

Table. 6. List of samples used in study based on reaction to streaming test from different sources of infection and their location

Sl. No.	Location (source)	Source of infection/ inoculum	Isolate	Streaming test
1.	Munshiganj (Manikpur)	Plant	PS3$_2$STM01 PS3$_2$STM02 PS3$_2$STM03	+ ve + ve - ve
2.	Chandpur	Plant	PS8$_2$.STC01 PS8$_2$.STC02 PS8$_2$.STC03	+ ve + ve - ve
3.	Tangail (Nagarpur)	Plant	PS7$_1$.STN01 PS7$_1$.STN02 PS7$_1$.STN03	+ ve + ve + ve
4.	Narayanganj (Sonargaon)	Plant	PS9$_1$.STNG01 PS9$_1$.STNG02	- ve + ve

Sl. No.	Location (source)	Source of infection/ inoculum	Isolate	Streaming test
			$PS9_1$-STNG03 $PS9_1$-STNG04	- ve + ve
5.	Jamalpur	Plant	$PS2_3$-STJ03 $PS2_3$-STJ04 $PS2_3$-STJ05	+ ve + ve + ve
6.	Domar (Dimla)	Plant	$PS10_1$-STD01 $PS10_1$-STD04 $PS10_1$-STD05 $PS10_1$-STD06	+ ve + ve + ve + ve
7.	Patuakhali	Plant	$PS4_3$-STP01 $PS4_3$-STP02 $PS4_3$-STP07	- ve - ve + ve
8.	Rangpur	Plant	$PS1_1$-STR01 $PS1_1$-STR06 $PS1_1$-STR07 $PS1_1$-STR08	- ve - ve + ve + ve
9.	Bogra	Plant	$PS11_1$-STB01 $PS11_1$-STB03 $PS11_1$-STB08 $PS11_1$-STB09	- ve - ve + ve + ve
10.	Narayanganj (Sonargaon)	Seed	SS20-STNG01 SS20-STNG03 SS20-STNG04	- ve - ve - ve
11.	Munshiganj (Manikpur)	Seed	SS11b-STM01 SS11b-STM02 SS11b-STM03	- ve - ve - ve
12.	Meherpur (Gangni)	Seed	SS16-STG01 SS16-STG02 SS16-STG03	- ve - ve - ve
13.	Bogra	Seed	SS28av-STB02 SS28av-STB04 SS28av-STB07 SS28av-STB08 SS28av-STB09	- ve - ve - ve - ve - ve
14.	Rangpur	Seed	SS1-STR03 SS1-STR04 SS1-STR05 SS1-STR06 SS1-STR07 SS1-STR08	- ve - ve - ve - ve - ve - ve
15.	Patuakhali	Seed	SS12-STP03 SS12-STP06 SS12-STP07	- ve - ve - ve
16.	Shariatpur (Jajira)	Seed	SS14-STJ9 SS14-STJ10 SS14-STJ11	- ve - ve - ve
17.	Bogra	Seed	SS32-STB1 SS32-STB5 SS32-STB8 SS32-STB10	- ve - ve - ve - ve
18.	Jamalpur	Seed	SS8-STJ01 SS8-STJ02 SS8-STJ04 SS8-STJ05	- ve - ve - ve - ve
19.	Domar (Dimla)	Seed	SS21-STD02 SS21-STD04 SS21-STD05 SS21-STD06 SS21-STD07	- ve - ve - ve - ve - ve
20.	Chandpur	Seed	SS19-STC01 SS19-STC02 SS19-STC03	- ve - ve - ve
21.	Joypurhat (Akkelpur)	Soil	DS21-STJH01 DS21-STJH02	- -

Sl. No.	Location (source)	Source of infection/ inoculum	Isolate	Streaming test
			DS21-STJH03	-
22.	Bogra	Soil	DS03-STB01 DS03-STB02 DS03-STB03	- - -
23.	Rangpur	Soil	DS24-STR01 DS24-STR02 DS24-STR03	- - -
24.	Dinajpur	Soil	DS25-STD01 DS25-STD03 DS25-STD04	- - -
25.	Rangpur	Soil	S1-STR4 S1-STR9 S1-STR10 S1-STR11	- - - -
26.	Jamalpur	Soil	S2-STJ03 S2-STJ05 S2-STJ08	- - -
27.	Domar (Dimla)	Soil	S3-STD01 S3-STD02 S3-STD08 S3-STD09	- - - -
28.	Munshiganj (Manikpur)	Soil	S4-STM01 S4-STM02 S4-STM03 S4-STM05	- - - -
29.	Tangail (Nagarpur)	Soil	S5-STN03 S5-STN06 S5-STN07	- - -
30.	Shariatpur (Jajira)	Soil	S6-STJ11 S6-STJ12 S6-STJ13	- - -
31.	Bogra	Soil	S7-STB1 S7-STB11 S7-STB12	- - -
32.	Meherpur (Gangni)	Soil	S18-STG10 S18-STG12 S18-STG14	- - -
33.	Patuakhali	Soil	S9-STP8 S9-STP9 S9-STP10	- - -
34.	Chandpur	Soil	S10-STC04 S10-STC05 S10-STC06	- - -
35.	Titbegun (*Solanum nigrum*), Nagarpur, Tangail, Farmer	Weed	**W27-SN02** W33-SN01 W31-SN05	- ve - ve - ve
36.	Shaknote (*Amaranthus viridis*), Chandpur, Comilla, Farmer	Weed	**W29-AV01** W28-AV02 W21-AV03	- ve - ve - ve
37.	Durba (*Cynodon dactylon*), Narayanganj, Dhaka, Farmer	Weed	W30-CD01 W32-CD01 **W30-CD03**	- ve - ve - ve
38.	Chilli (*Capsicum annum* -other crop), Bogra, Farmer	Other crop	35-CA01 35-CA02 **39-CA04**	- ve - ve - ve
39.	Water sample (Domar), Farmer	Water	41-W01 42-W05 **47-W05**	- - -

'+ve' = positive reaction in streaming test; '-ve' = negative reaction in streaming; '-' = not applicable for streaming.

4.1.1.b.Isolation and preservation of *R. solanacearum*

Virulent and avirulent colonies

The virulent (colonies with pink or light red center with white colored wider fluidal zone) and avirulent (colonies with dark pink center with white colored thinner fluidal zone) from different isolates of *R. solanacearum* were identified on TZC media plates. The differentiation was done based on their typical fluidal, irregular growth in colony morphology with a characteristic pink centre (Fig. 8).

Fig. 8. Morphological differences of the virulent and avirulent colonies of *R. solanacearum*based on their typical fluidal colony with pink centre on TZC medium.

A total of 133 samples were prepared for isolation in TZC solid media. Among those 31 were plant samples, 42 were seed samples, 45 were soil samples, 9 were weed samples, 3 were other crop samples and 3 were water samples. Considering location, 36 samples of different sources from Rangpur division (Dinajpur, Rangpur, Nilphamari), 28 samples of different sources from Rajshahi division (Bogra, Joypurhat), 19 samples of different sources from Mymensingh division (Tangail, Jamalpur), 6 samples of different sources from Khulna

division (Meherpur), 9samples of different sources from Barisal division (Patuakhali), 12 samples of different sources from Comilla division (Candpur) and 23 samples of different sources from Dhaka division (Munshiganj, Narayanganj, Shariatpur) were plated for isolation in TZC media for the study (Table 6, Fig. 9).

Among those, all plant, seed and soil samples and,5 out of 9 weed samples, 1 out of 3 other crop (chilli) samples and 1 out of 3 of water samples showed positive on TZC solid media. However, some of the isolates got contaminated (* marked isolates) and discarded. The rest samples were preserved properly for further characterization of *R. solanacearum* (Table 6).

Table. 7. Reaction of different isolates of *R. solanacearum* to TZC test and hypersensitivity test collected from different AEZ(s) of Bangladesh

Sl. No.	Location (source)	Source of inoculum	Isolate	Isolation on TZC	HR (Hypersensitive Reaction) test
1.	Munshiganj (Manikpur)	Plant	PS3$_2$STM01	+ ve	++ ve
			PS3$_2$STM02	+ ve	- ve
			PS3$_2$STM03	+ ve	- ve
2.	Chandpur	Plant	PS8$_2$-STC01	+ ve*	-
			PS8$_2$-STC02	+ ve	++ ve
			PS8$_2$-STC03	+ ve	- ve
3.	Tangail (Nagarpur)	Plant	PS7$_1$-STN01	+ ve*	-
			PS7$_1$-STN02	+ ve*	-
			PS7$_1$-STN03	+ ve	++ ve
4.	Narayanganj (Sonargaon)	Plant	PS9$_1$-STNG01	+ ve*	-
			PS9$_1$-STNG02	+ ve*	-
			PS9$_1$-STNG03	+ ve	- ve
5.	Jamalpur	Plant	PS2$_3$-STJ03	+ ve*	-
			PS2$_3$-STJ04	+ ve*	-
			PS2$_3$-STJ05	+ ve	++ ve
6.	Domar (Dimla)	Plant	PS10$_1$-STD01	+ ve*	-
			PS10$_1$-STD04	+ ve	- ve
			PS10$_1$-STD05	+ ve	- ve
7.	Patuakhali	Plant	PS4$_3$-STP01	+ ve*	-
			PS4$_3$-STP02	+ ve*	-
			PS4$_3$-STP07	+ ve	++ ve
8.	Rangpur	Plant	PS1$_1$-STR01	+ ve*	-
			PS1$_1$-STR06	+ ve	- ve
			PS1$_1$-STR07	+ ve*	-
9.	Bogra	Plant	PS11$_1$-STB01	+ ve*	- ve
			PS11$_1$-STB03	+ ve	- ve
			PS11$_1$-STB08	+ ve	- ve
10.	Narayanganj (Sonargaon)	Seed	SS20-STNG01	+ ve	- ve
			SS20-STNG03	+ ve*	-
			SS20-STNG04	+ ve	+++ ve

Sl. No.	Location (source)	Source of inoculum	Isolate	Isolation on TZC	HR (Hypersensitive Reaction) test
11.	Munshiganj (Manikpur)	Seed	SS11b-STM01 SS11b-STM02 SS11b-STM03	+ ve + ve + ve	++ ve - ve - ve
12.	Meherpur (Gangni)	Seed	SS16-STG01 SS16-STG02 SS16-STG03	+ ve + ve + ve*	++ ve - ve -
13.	Bogra	Seed	SS28av-STB02 SS28av-STB04 SS28av-STB07	+ ve + ve* + ve	- ve - - ve
14.	Rangpur	Seed	SS1-STR03 SS1-STR04 SS1-STR05	+ ve + ve* + ve*	- ve - -
15.	Patuakhali	Seed	SS12-STP03 SS12-STP06 SS12-STP07	+ ve* + ve + ve	- - ve ++ ve
16.	Shariatpur (Jajira)	Seed	SS14-STJ9 SS14-STJ10 SS14-STJ11	+ ve* + ve + ve	- - ve +++ ve
17.	Bogra	Seed	SS32-STB1 SS32-STB5 SS32-STB8	+ ve + ve* + ve	- ve - - ve
18.	Jamalpur	Seed	SS8-STJ01 SS8-STJ02 SS8-STJ04	+ ve + ve* + ve	- ve - - ve
19.	Domar (Dimla)	Seed	SS21-STD02 SS21-STD04 SS21-STD05	+ ve* + ve + ve	- - ve - ve
20.	Chandpur	Seed	SS19-STC01 SS19-STC02 SS19-STC03	+ ve + ve + ve*	- ve +++ ve -
21.	Joypurhat (Akkelpur)	Soil	DS21-STJH01 DS21-STJH02 DS21-STJH03	+ ve + ve + ve*	+++ ve - ve -

Sl. No.	Location (source)	Source of inoculum	Isolate	Isolation on TZC	HR (Hypersensitive Reaction) test
22.	Bogra	Soil	DS03-STB01 DS03-STB02 DS03-STB03	+ ve + ve + ve	+++ ve - ve - ve
23.	Rangpur	Soil	DS24-STR01 DS24-STR02 DS24-STR03	+ ve* + ve + ve	- - ve +++ ve
24.	Dinajpur	Soil	DS25-STD01 DS25-STD03 DS25-STD04	+ ve + ve* + ve	- ve - ++ ve
25.	Rangpur	Soil	S1-STR4 S1-STR9 S1-STR10	+ ve + ve* + ve	- ve - - ve
26.	Jamalpur	Soil	S2-STJ03 S2-STJ05 S2-STJ08	+ ve* + ve + ve	- - ve +++ ve
27.	Domar (Dimla)	Soil	S3-STD01 S3-STD02 S3-STD08	+ ve + ve* + ve	- ve - - ve
28.	Munshiganj (Manikpur)	Soil	S4-STM01 S4-STM02 S4-STM03	+ ve* + ve + ve	- - ve - ve
29.	Tangail (Nagarpur)	Soil	S5-STN03 S5-STN06 S5-STN07	+ ve + ve* + ve	- ve - ++ ve
30.	Shariatpur (Jajira)	Soil	S6-STJ11 S6-STJ12 S6-STJ13	+ ve + ve* + ve	- ve - +++ ve
31.	Bogra	Soil	S7-STB1 S7-STB11 S7-STB12	+ ve* + ve + ve	- - ve ++ ve
32.	Meherpur (Gangni)	Soil	S18-STG10 S18-STG12 S18-STG14	+ ve* + ve + ve	- - ve ++ ve

Sl. No.	**Location (source)**	**Source of inoculum**	**Isolate**	**Isolation on TZC**	**HR (Hypersensitive Reaction) test**
33.	Patuakhali	Soil	S9-STP8 S9-STP9 S9-STP10	+ ve + ve* + ve	- - ve +++ ve
34.	Chandpur	Soil	S10-STC04 S10-STC05 S10-STC06	+ ve + ve + ve	- ve - ve +++ ve
35.	Titbegun (*Solanum nigrum*), Nagarpur, Tangail, Farmer	Weed	**W27-SN02** W33-SN01 W31-SN05	+ ve + ve - ve	++ ve - ve - ve
36.	Shaknote (*Amaranthus viridis*), Chandpur, Comilla, Farmer	Weed	**W29-AV01** W28-AV02 W21-AV03	+ ve - ve - ve	+++ ve - ve - ve
37.	Durba (*Cynodon dactylon*), Narayanganj, Dhaka, Farmer	Weed	W30-CD01 W32-CD01 **W30-CD03**	- ve + ve + ve	- ve - ve +++ ve
38.	Chilli (*Capsicum annum* -other crop), Bogra, Farmer	Other crop	35-CA01 35-CA02 **39-CA04**	- ve - ve + ve	- ve - ve ++ ve
39.	Water sample (Domar), Farmer	Water	41-W01 42-W05 **47-W05**	- ve - ve + ve	- ve - ve +++ ve

‘+++ve’ = strongly positive ie. HR within 2-3 days after inoculation; ‘++ve’ = weakly positive ie. HR after 3-5 days after inoculation; ‘+ve’ = positive reaction inTZC test; ‘-ve’ = negative reaction in TZC test; ‘*’ = contaminated & rejected.

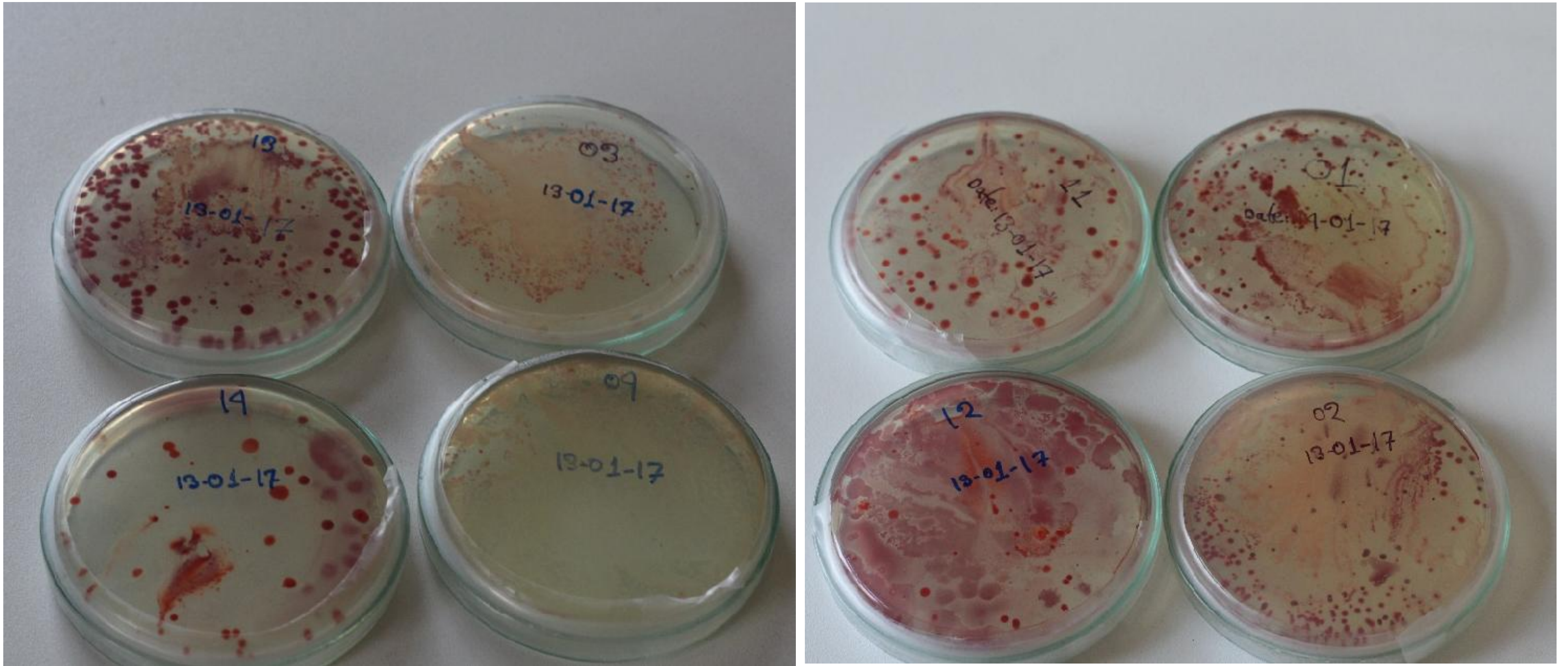


Fig. 9. Isolates of *R. solanacearum* on TZC media plates collected from different potato growing regions of Bangladesh.

4.1.2. Hypersensitivity test

The TZC positive uncontaminated isolates (from plant, seed, soil, weed, other crop-chilli and water) were tested for their pathogenicity level in hypersensitivity test and result of this study showed that 9 out 31 plant samples, 11 out of 42 seed samples, 14 out of 45 soil samples, 3 out of 9 weed samples, 1 out 3 other crop samples and 1 out of 3 water samples showed that reaction 2-5 days after inoculation (Table 7). The TZC positive uncontaminated isolates (from plant, seed, soil, weed, other crop-chilli and water) viz. PS32STM01 no. isolate from Munshiganj (Manikpur), PS82-STC02 no. isolate from Chandpur, PS71-STN03 no. isolate from Tangail (Nagarpur), PS91-STNG04 no. isolate from Narayanganj (Sonargaon), PS23-STJ05 no. isolate from Jamalpur, PS101-STD06 no. isolate from Domar (Dimla), PS43-STP07 no. isolate from Patuakhali, PS11-STR08 no. isolate from Rangpur, PS111-STB09 no. isolate from Bogra, SS20-STNG04 no. isolate from Narayanganj (Sonargaon), SS11b-STM01 no. isolate from Munshiganj (Manikpur), SS16-STG01 no. isolate from Meherpur (Gangni), SS28av-STB09 no. isolate from Bogra, SS1-STR08 no. isolate from Rangpur, SS12-STP07 no. isolate from Patuakhali, SS14-STJ11 no. isolate from Shariatpur (Jajira), SS32-STB10 no. isolate from Bogra, SS8-STJ05 no. isolate from Jamalpur, SS21-STD06 no. isolate from Domar (Dimla), SS19-STC02 no. isolate from Chandpur, DS21-STJH01 no. isolate from Joypurhat (Akkelpur), DS03-STB02 no. isolate from Bogra, DS24-STR03 no. isolate from Rangpur, DS25-STD04 no. isolate from Dinajpur, S1-STR11 no. isolate from Rangpur, S2-STJ08 no. isolate from Jamalpur, S3-STD09 no. isolate from Domar (Dimla), S4-STM05 no. isolate from Munshiganj (Manikpur), S5-STN07 no. isolate from Tangail (Nagarpur), S6-STJ13 no. isolate from Shariatpur (Jajira), S7-STB12 no. isolate from Bogra, S18-STG14 no. isolate from Meherpur (Gangni), S9-STP10 no. isolate from Patuakhali, S10-STC06 no. isolate from Chandpur, W27-SN02 no. isolate from Titbegun (*Solanum nigrum*), W29-AV01 no. isolate from Shaknote (*Amaranthus viridis*), W30-CD03 no. isolate from Durba (*Cynodon dactylon*), 39-CA04 no. isolate from Chilli (*Capsicum annum* -other crop) and 47-W05 no. isolate from Water sample (Domar) expressed hypersensitive reaction on *Euphorbia*(Fig. 10, Table 7).

Fig. 10. Lesions produced due to hypersensitive reaction by isolates of *R. solanacearum* on leaves of *Euphorbia*.

4.1.3. Race determination of *R. solanacearum*

Race determination were conducted following EPPO, 2004 as described in methodology. Thirty nine isolates out of 133 isolates of *R. solanacearum*obtained from plant, seed, soil, weed etc. were further characterized for race determination (Table8, Fig. 11).It was found that all the 39 isolates showed wilting symptoms on potato and tomato except brinjal seedlings which showed just chlorosis against some isolates.

The study revealed that, all of the isolates viz. PS32STM01 no. isolate from Munshiganj (Manikpur), PS82-STC02 no. isolate from Chandpur, PS71-STN03 no. isolate from Tangail (Nagarpur), PS91-STNG04 no. isolate from Narayanganj (Sonargaon), PS23-STJ05 no. isolate from Jamalpur, PS101-STD06 no. isolate from Domar (Dimla), PS43-STP07 no.

isolate from Patuakhali, PS11-STR08 no. isolate from Rangpur, PS111-STB09 no. isolate from Bogra, SS20-STNG04 no. isolate from Narayanganj (Sonargaon), SS11b-STM01 no. isolate from Munshiganj (Manikpur), SS16-STG01 no. isolate from Meherpur (Gangni), SS28av-STB09 no. isolate from Bogra, SS1-STR08 no. isolate from Rangpur, SS12-STP07 no. isolate from Patuakhali, SS14-STJ11 no. isolate from Shariatpur (Jajira), SS32-STB10 no. isolate from Bogra, SS8-STJ05 no. isolate from Jamalpur, SS21-STD06 no. isolate from Domar (Dimla), SS19-STC02 no. isolate from Chandpur, DS21-STJH01 no. isolate from Joypurhat (Akkelpur), DS03-STB02 no. isolate from Bogra, DS24-STR03 no. isolate from Rangpur, DS25-STD04 no. isolate from Dinajpur, S1-STR11 no. isolate from Rangpur, S2-STJ08 no. isolate from Jamalpur, S3-STD09 no. isolate from Domar (Dimla), S4-STM05 no. isolate from Munshiganj (Manikpur), S5-STN07 no. isolate from Tangail (Nagarpur), S6-STJ13 no. isolate from Shariatpur (Jajira), S7-STB12 no. isolate from Bogra, S18-STG14 no. isolate from Meherpur (Gangni), S9-STP10 no. isolate from Patuakhali, S10-STC06 no. isolate from Chandpur, W27-SN02 no. isolate from Titbegun (*Solanum nigrum*), W29-AV01 no. isolate from Shaknote (*Amaranthus viridis*), W30-CD03 no. isolate from Durba (*Cynodon dactylon*), 39-CA04 no. isolate from Chilli (*Capsicum annum* –as other crop) and 47-W05 no. isolate from Water sample (Domar) expressed wilting on potato and tomato within 2-7days after inoculation, but no wilting on brinjal except some chlorosis within 4-5 days after inoculation (Fig. 11, Table 8).

Table 8. Reaction of race determination test after inoculation on potato, tomato and brinjal seedlings by different isolates of *R. solanacearum* collected from different AEZ(s) of Bangladesh

Sl. No.	Isolate	Location (source)	Source of inoculum	Race determination			Inference
				Potato	Tomato	Brinjal	Race
1.	PS3$_2$STM01	Munshiganj (Manikpur)	Plant	+++ ve	+++ ve	- ve	3*
2.	PS8$_2$-STC02	Chandpur	Plant	+++ ve	+++ ve	- ve	3*
3.	PS7$_1$-STN03	Tangail (Nagarpur)	Plant	+++ ve	+++ ve	- ve	3*
4.	PS9$_1$-STNG04	Narayanganj (Sonargaon)	Plant	+++ ve	+++ ve	- ve	3*
5.	PS2$_3$-STJ05	Jamalpur	Plant	+++ ve	+++ ve	- ve	3*
6.	PS10$_1$-STD06	Domar (Dimla)	Plant	+++ ve	+++ ve	- ve	3*
7.	PS4$_3$-STP07	Patuakhali	Plant	+++ ve	+++ ve	- ve	3*
8.	PS1$_1$-STR08	Rangpur	Plant	+++ ve	+++ ve	- ve	3*
9.	PS11$_1$-STB09	Bogra	Plant	++ ve	+++ ve	- ve	3*
10.	SS20-STNG04	Narayanganj (Sonargaon)	Seed	++ ve	+++ ve	- ve	3*
11.	SS11b-STM01	Munshiganj (Manikpur)	Seed	+++ ve	+++ ve	- ve	3*
12.	SS16-STG01	Meherpur (Gangni)	Seed	+++ ve	+++ ve	- ve	3*
13.	SS28av-STB09	Bogra	Seed	++ ve	+++ ve	- ve	3*
14.	SS1-STR08	Rangpur	Seed	++ ve	+++ ve	- ve	3*
15.	SS12-STP07	Patuakhali	Seed	+++ ve	+++ ve	- ve	3*
16.	SS14-STJ11	Shariatpur (Jajira)	Seed	++ ve	+++ ve	- ve	3*
17.	SS32-STB10	Bogra	Seed	+++ ve	+++ ve	- ve	3*
18.	SS8-STJ05	Jamalpur	Seed	++ ve	+++ ve	- ve	3*
19.	SS21-STD06	Domar (Dimla)	Seed	+++ ve	+++ ve	- ve	3*
20.	SS19-STC02	Chandpur	Seed	++ ve	+++ ve	- ve	3*
21.	DS21-STJH01	Joypurhat (Akkelpur)	Soil	++ ve	+++ ve	- ve	3*
22.	DS03-STB02	Bogra	Soil	++ ve	+++ ve	- ve	3*
23.	DS24-STR03	Rangpur	Soil	++ ve	+++ ve	- ve	3*
24.	DS25-STD04	Dinajpur	Soil	+++ ve	+++ ve	- ve	3*
25.	S1-STR11	Rangpur	Soil	+++ ve	+++ ve	- ve	3*
26.	S2-STJ08	Jamalpur	Soil	++ ve	+++ ve	- ve	3*

Sl. No.	Isolate	Location (source)	Source of inoculum	Race determination			Inference
				Potato	Tomato	Brinjal	Race
27.	S3-STD09	Domar (Dimla)	Soil	+++ ve	+++ ve	- ve	3*
28.	S4-STM05	Munshiganj (Manikpur)	Soil	++ ve	+++ ve	- ve	3*
29.	S5-STN07	Tangail (Nagarpur)	Soil	+++ ve	+++ ve	- ve	3*
30.	S6-STJ13	Shariatpur (Jajira)	Soil	++ ve	+++ ve	- ve	3*
31.	S7-STB12	Bogra	Soil	+++ ve	+++ ve	- ve	3*
32.	S18-STG14	Meherpur (Gangni)	Soil	+++ ve	+++ ve	- ve	3*
33.	S9-STP10	Patuakhali	Soil	++ ve	+++ ve	- ve	3*
34.	S10-STC06	Chandpur	Soil	++ ve	+++ ve	- ve	3*
35.	W27-SN02	Titbegun (*Solanum nigrum*), Nagarpur, Tangail, Farmer	Weed	+++ ve	+++ ve	- ve	3*
36.	W29-AV01	Shaknote (*Amaranthus viridis*), Chandpur, Comilla, Farmer	Weed	++ ve	+++ ve	- ve	3*
37.	W30-CD03	Durba (*Cynodon dactylon*), Narayanganj, Dhaka, Farmer	Weed	++ ve	+++ ve	- ve	3*
38.	39-CA04	Chilli (*Capsicum annum* -other crop), Bogra, Farmer	Other crop	+++ ve	+++ ve	- ve	3*
39.	47-W05	Water sample (Domar), Farmer	Water	++ ve	+++ ve	- ve	3*

'+++ve' = wilting symptoms within 2-4days after inoculation; '++ve' = wilting symptom after 4-7 days; and 3* = race 3 hosts only potato and tomato, not all solanaceous plants which occurs in worldwide according to EPPO, 2004.

Fig. 11. Race determination test of isolates *R. solanacearum* on a.) potato, b.) tomato and brinjal leaves.

4.1.4. Biochemical test of *R. solanacearum*

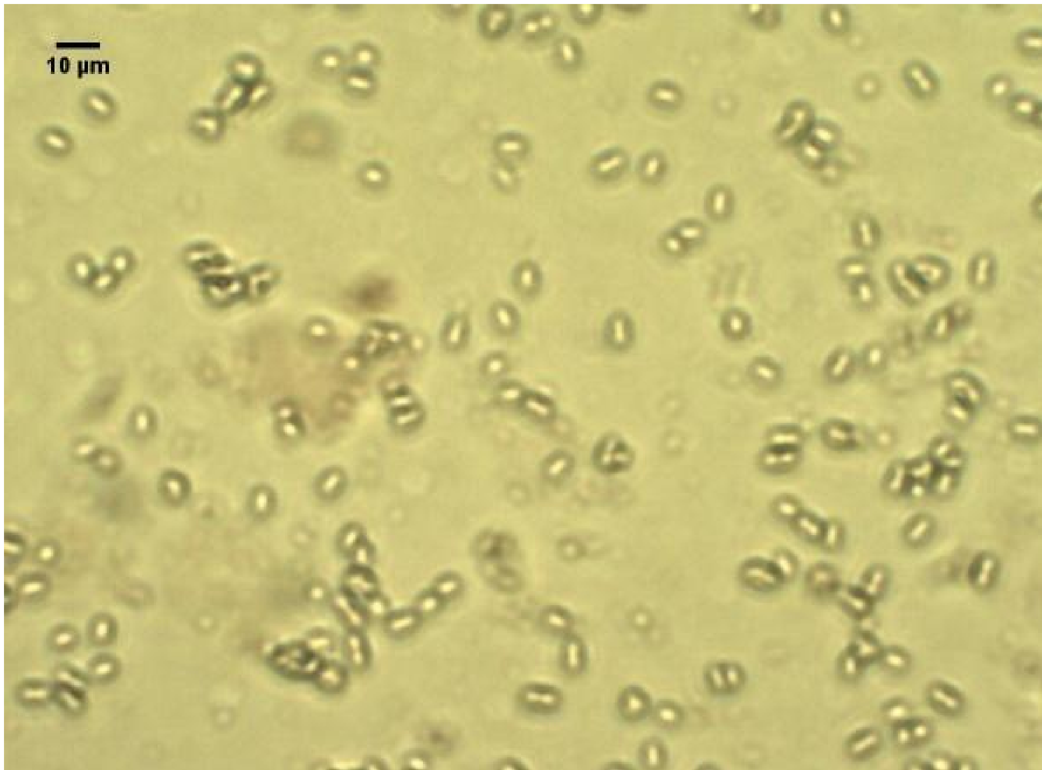


Fig. 12. Microscopic view of *R. solanacearum* in 100X magnification.

Different biochemical tests were carried out following Janse, 1953; Hayward, 1964; and *Goszczynska et al.,(2000) in case of 39 isolates of R. solanacearum* (from plant, seed, soil, weed, chilli and water).In case of biochemical tests, all the 39 isolates collected from plants, seeds, soils, weeds, water, chilli and water showed positive reaction in Gram staining test, KOH test, PHB test, catalase test, oxidase test and pectolytic tests and showed similar reaction to temperature sensitivity test. But, in different sugar utilization tests (glucose, sucrose and maltose), those showed almost similar results by all *R. solanacearum* isolates except two (1 from weed and 1 from water) in oxidizing maltose(Table 9& Fig. 12-20).

Table 9. Reaction of different biochemical test for characterization of different isolates of *R. solanacearum* collected from different AEZ(s) of Bangladesh

Sl. No.	Isolate No.	Gram Reaction Test	KOH Test	PBH Test	Catalase Test	Kovac's Oxidase Test	Potato soft rot/ pectolytic test	Temperature sensitivity test			Sugar Fermentation/ oxidative/ Hugh-Leifson test or sugar utilization test			Inference
								4 ^{0}C	28 ^{0}C	43 ^{0}C	G	S	M	
1.	PS3$_2$.STM01	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
2.	PS8$_2$.STC02	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
3.	PS7$_1$.STN03	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
4.	PS9$_1$.STNG04	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
5.	PS2$_3$.STJ05	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
6.	PS10$_1$.STD06	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
7.	PS4$_3$.STP07	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
8.	PS1$_1$.STR08	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
9.	PS11$_1$.STB09	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
10.	SS20-STNG04	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
11.	SS11b-STM01	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
12.	SS16-STG01	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
13.	SS28av-STB09	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
14.	SS1-STR08	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
15.	SS12-STP07	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
16.	SS14-STJ11	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
17.	SS32-STB10	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
18.	SS8-STJ05	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
19.	SS21-STD06	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
20.	SS19-STC02	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
21.	DS21-STJH01	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*

Sl. No.	Isolate No.	Gram Reaction Test	KOH Test	PBH Test	Catalase Test	Kovac's Oxidase Test	Potato soft rot/ pectolytic test	Temperature sensitivity test			Sugar Fermentation/ oxidative/ Hugh-Leifson test or sugar utilization test			Inference
22.	DS03-STB02	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
23.	DS24-STR03	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
24.	DS25-STD04	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
25.	S1-STR11	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
26.	S2-STJ08	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
27.	S3-STD09	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
28.	S4-STM05	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
29.	S5-STN07	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
30.	S6-STJ13	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
31.	S7-STB12	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
32.	S18-STG14	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
33.	S9-STP10	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
34.	S10-STC06	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
35.	W27-SN02	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
36.	W29-AV01	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
37.	W30-CD03	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	- ve	*R. solanacearum*
38.	39-CA04	- ve	+ ve	+ ve	+ ve	+ ve	+++ ve	- ve	+ ve	- ve	+ ve	+ ve	+ ve	*R. solanacearum*
39.	47-W05	- ve	+ ve	+ ve	+ ve	+ ve	++ ve	- ve	+ ve	- ve	+ ve	+ ve	- ve	*R. solanacearum*

G= Glucose, S= Sucrose, M= Maltose; '+ ve' means positive reaction; '- ve' means negative reaction; '+++ ve' means strongly positive reaction; and '++ ve' means weakly positive reaction.

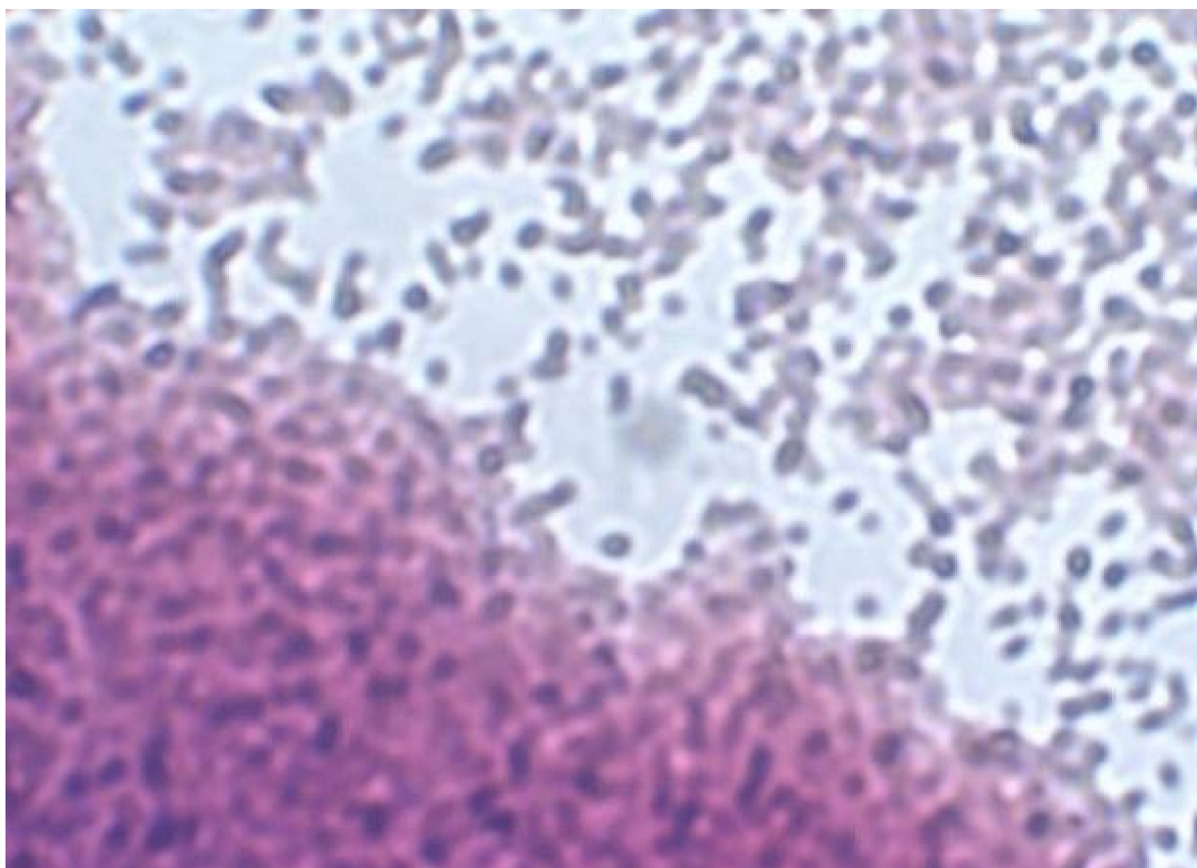


Fig. 13. Microscopic view of pink color restaining chemical of cells of *R. solanacearum* in Gram reaction test.

a. Gram staining test

All the 39 isolates showing positive hypersensitive reaction, exhibited reddish pink color which indicated that those were Gram negative type (Table 9; Fig. 13) as described by Schaad (1980). Bacteria retaining reddish pink colony color showed that those were gram –negative (G -ve) which were parallel with the findings of Suslow, *et al*. (1982).

b. Potassium hydroxide test

All of the isolates showed a sticky thread was observed which presented a positive reaction of the test. It is a rapid method to distinguish between Gram-positive and Gram-negative type bacteria by testing the solubility of the isolate in 3% KOH. It was formed when the loop was raised some millimeters from the bacterial solution on potassium hydroxide solution on the microscope slide in all the cases (Table 9& Fig. 14).

Fig. 14. Potassium hydroxide solubility test showing positive reaction as indicated by sticky thread formed from a loop full of *R. solanacearum* colony.

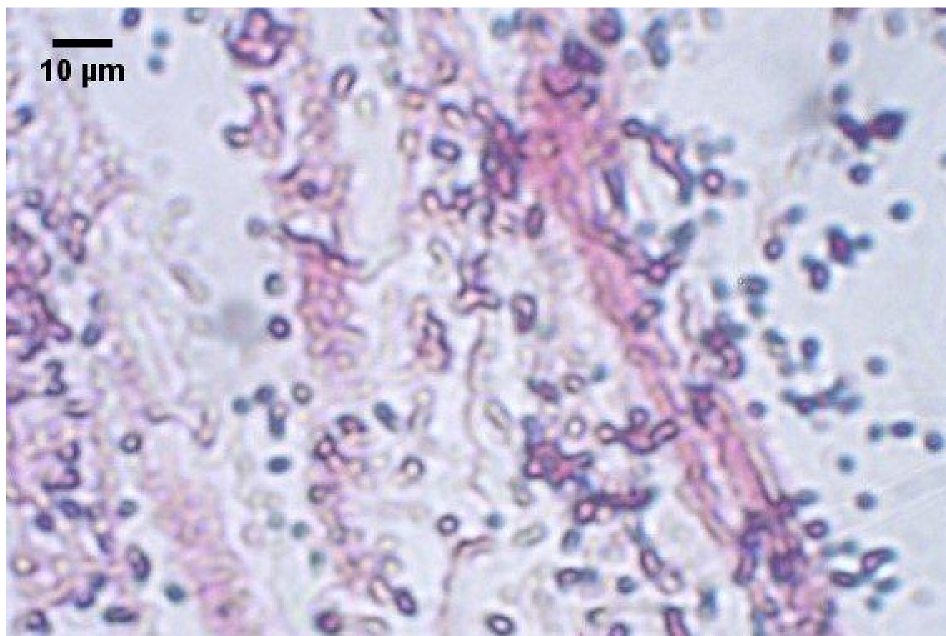


Fig. 15. Microscopic view of production of black/dark colored organic reserve materials in cells of of *R. solanacearum*in 100X magnification in PHB test.

c. PHB test (poly- -hydroxybutyrate granules)

Isolates of *R. solanacearum* showed positive reaction and produced black or dark color while stainig with PHB reagent as a result of production of organic reserve materials in the form of polyesters of -hydroxy butyric acid which could be observed microscopically by PHB staining. (Table 9& Fig. 15).

Fig. 16. Colonies from *R. solanacearum*isolate(s) producing bubbles in presence of 3% H_2O_2in catalase test.

d. Catalase test

In catalase test, all the isolates of *R. solanacearum* showed positive reaction of produced bubbles within 30 seconds of mixing with 3% H_2O_2(Table 9& Fig. 16).

e. Kovac's oxidase test

In Kovac's oxidase test, all the isolates of *R. solanacearum* showed positive reaction produced dark purple or purple blue color within a few seconds when a mass of bacterial growth was rubbed on the moistened filter paper (whatman no. 1) impregnated with the oxidase reagent (Table 9& Fig. 17).

g. Pectolytic test

In potato pectolytic test, all the isolates of *R. solanacearum* showed positive reaction in producing soft rot in inoculated potatoes (Table 9& Fig. 18).

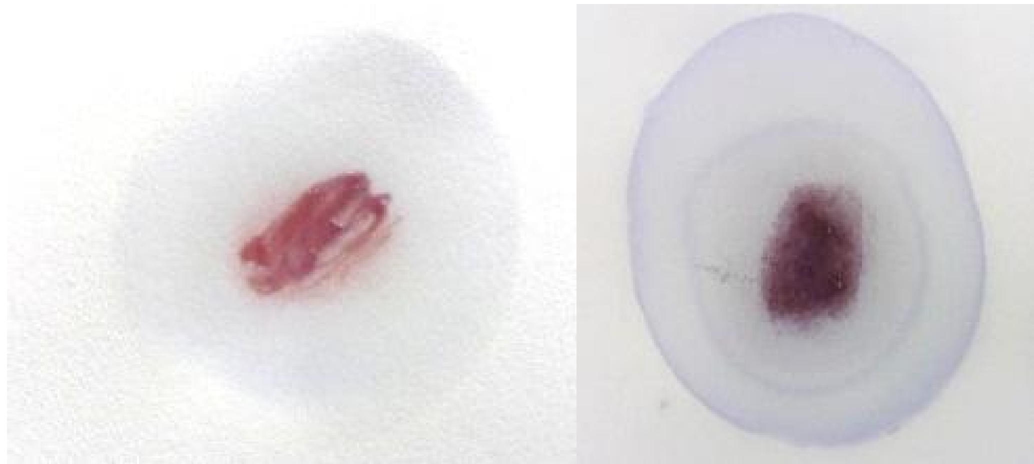


Fig. 17. Colonies from*R. solanacearum* isolate(s) showing dark purple or purple blue color in Kovac’s test.

Fig. 18. Potato tubers showing soft rotof tubers inoculated by *R. solanacearum* isolate(s) inpectolytic test.

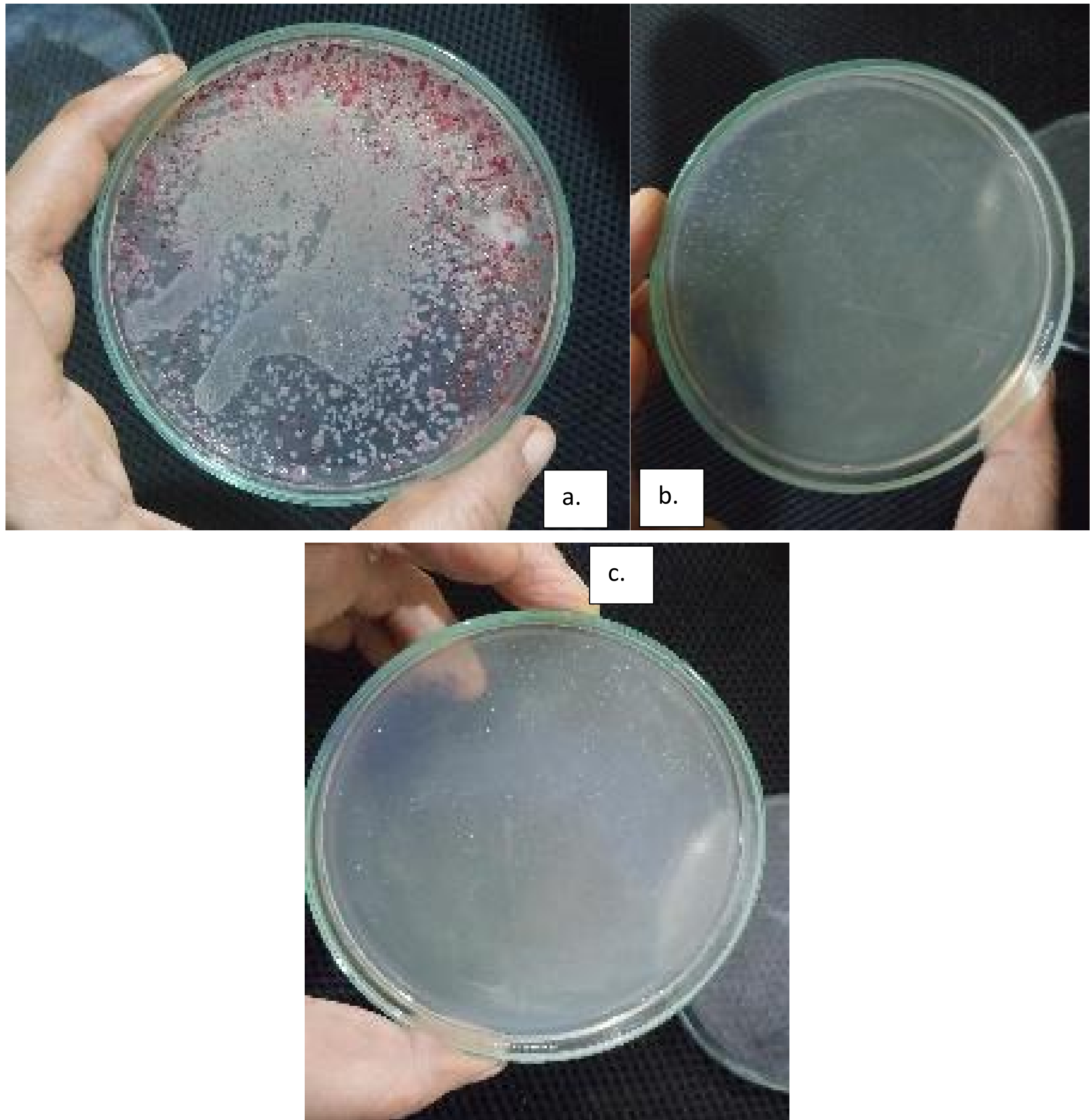


Fig. 19. Isolates of *R. solanacearum*showing positive and typical growth at 28^0(a), but no potential growth at4^0(b), and 40^0(c) C.

h. Temperature sensitivity Test

The test isolates showed positive and typical growth at 28 deg centigrade but did not show any potential growth at 4 deg and 43 deg centigrade (Table 9& Fig. 19).

i. Sugar utilization test

Isolates of *R. solanacearum*viz. PS32STM01-Manikpur, Munshiganj; PS82-STC02-Chandpur, Comilla; PS71-STN03-Nagarpur, Tangail; PS91-STNG04-Narayanganj, Dhaka;

PS23-STJ05-Jamalpur; PS101-STD06-Domar (Dimla), Nilphamari; PS43-STP07-Patuakhali; PS11-STR08-Rangpur; PS111-STB09-Bogra; SS20-STNG04-Narayanganj, Dhaka; SS11b-STM01-Munshiganj; SS16-STG01-Gangni, Meherpur, Kustia; SS28av-STB09-Bogra; SS1-STR08-Rangpur; SS12-STP07-Patuakhali; SS14-STJ11-Jajira, Shariatpur; SS32-STB10-Bogra; SS8-STJ05-Jamalpur; SS21-STD06-Domar, Nilphamari; SS19-STC02-Chandpur, Comilla; DS21-STJH01-Joypurhat(Kalai); DS03-STB02-Bogra; DS24-STR03-Rangpur; DS25-STD04-Dinajpur; S1-STR11-Rangpur; S2-STJ08-Jamalpur; S3-STD09-Domar, Nilphamari; S4-STM05-Munshiganj; S5-STN07-Nagarpur, Tangail; S6-STJ13-Jajira, Shariatpur; S7-STB12-Bogra; S18-STG14-Gangni, Meherpur, Kustia; S9-STP10 - Patuakhali;S10-STC06-Chandpur, Comilla,; W27-SN02-Titbegun (*Solanumnigrum*), Nagarpur, Tangail; W29-AV01-Shaknote (*Amaranthusviridis*), Chandpur, Comilla; and 39-CA04-Chilli (*Capsicumannum* -other crop), Bogra; except W30-CD03-Durba (*Cynodon dactylon*), Narayanganj, Dhaka, and 47-W05-Water sample, Domar, had shown to oxidize the maltose sugar. But, in case of glucose and sucrose, all of the 39 isolates oxidized those sugars (Table 9 & Fig. 20).

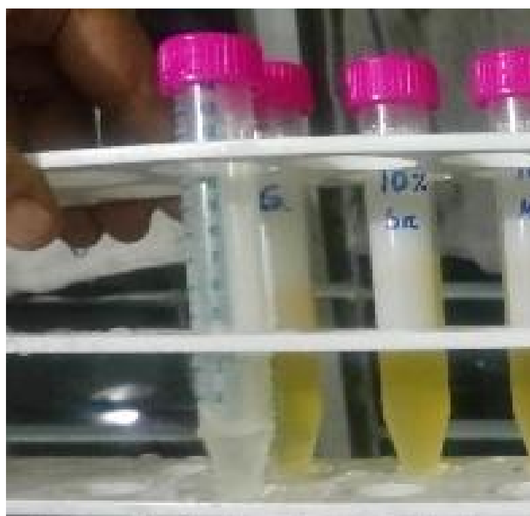


Fig. 20. Utilization of glucose, sucrose and maltose shown by isolate(s) of *R. solanacearum.*

4.1.5. Biovar(s) test

In biovar test, isolates of *R. solanacearum*viz. PS32STM01-Manikpur, Munshiganj; PS82-STC02-Chandpur, Comilla; PS71-STN03-Nagarpur, Tangail; PS91-STNG04-Narayanganj, Dhaka; PS23-STJ05-Jamalpur; PS101-STD06-Domar (Dimla), Nilphamari; PS43-STP07-Patuakhali; PS11-STR08-Rangpur; PS111-STB09-Bogra; SS20-STNG04-Narayanganj,

Dhaka; SS11b-STM01-Munshiganj; SS16-STG01-Gangni, Meherpur, Kustia; SS28av-STB09-Bogra; SS1-STR08-Rangpur; SS12-STP07-Patuakhali; SS14-STJ11-Jajira, Shariatpur; SS32-STB10-Bogra; SS8-STJ05-Jamalpur; SS21-STD06-Domar, Nilphamari; SS19-STC02-Chandpur, Comilla; DS21-STJH01-Joypurhat(Kalai); DS03-STB02-Bogra; DS24-STR03-Rangpur; DS25-STD04-Dinajpur; S1-STR11-Rangpur; S2-STJ08-Jamalpur; S3-STD09-Domar, Nilphamari; S4-STM05-Munshiganj; S5-STN07-Nagarpur, Tangail; S6-STJ13-Jajira, Shariatpur; S7-STB12-Bogra; S18-STG14-Gangni, Meherpur, Kustia; S9-STP10 -Patuakhali; S10-STC06-Chandpur, Comilla,; W27-SN02-Titbegun (*Solanumnigrum*), Nagarpur, Tangail; W29-AV01-Shaknote (*Amaranthusviridis*), Chandpur, Comilla; and 39-CA04-Chilli (*Capsicumannum* -other crop), Bogra; except W30-CD03-Durba (*Cynodon dactylon*), Narayanganj, Dhaka, and 47-W05-Water sample, Domar, reacted to both disaccharidesugars (lactose, maltose and cellobiose) and hexose alcohols (dulcitol, mannitol and sorbitol) by changing green color into yellow color which was indicating the oxidation of both carbon sources. But isolates, W30-CD03(*Cynodon dactylon*)and 47-W05neither changed green color for disaccharides nor for alcohols which indicated that those were not able to utilize either of the carbon sources. Thus, these 37 isolates were expressed as biovar III and the remaining 2 were expressed as biovar I(Table 10,11& Fig. 21).

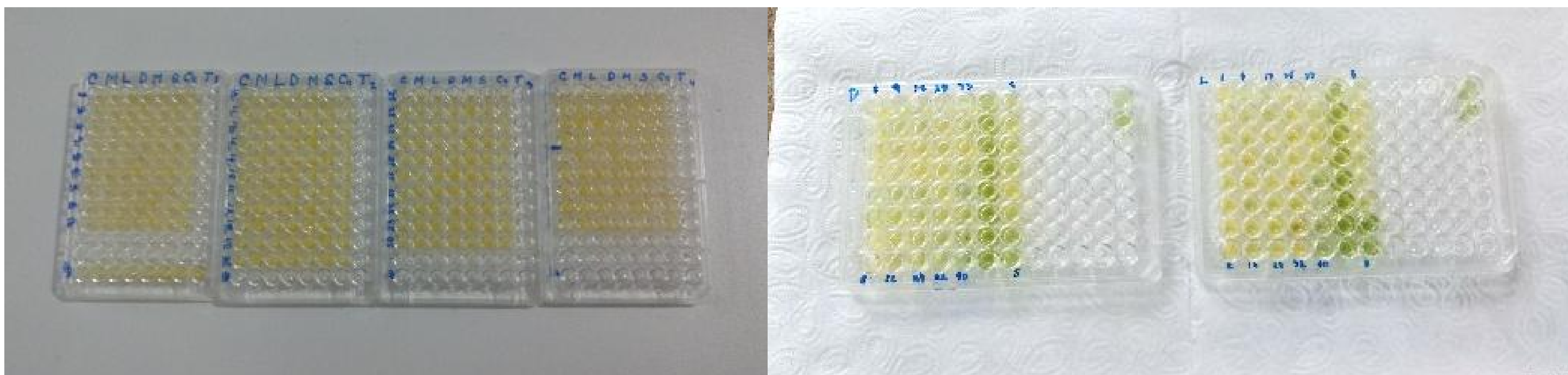
Table 10. Reaction of different carbon utilization test (different disaccharide sugar& hexose alcohols) for characterization of different isolates of *R. solanacearum* collected from different AEZ(s) of Bangladesh

Sl. No.	Isolate	Location	Hexose alcohol			Disaccharidesugar		
			Dulcitol	Sorbitol	Mannitol	Cellobiose	Maltose	Lactose
1.	PS3_2STM01	Manikpur, Munshiganj, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
2.	PS8_2-STC02	Chandpur, Comilla, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
3.	PS7_1-STN03	Nagarpur, Tangail, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
4.	PS9_1-STNG04	Narayanganj, Dhaka, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
5.	PS2_3-STJ05	Jamalpur, BARI	+ve	+ve	+ve	+ve	+ve	+ve
6.	PS10_1-STD06	Domar (Dimla), Nilphamari	+ve	+ve	+ve	+ve	+ve	+ve
7.	PS4_3-STP07	Patuakhali, BADC	+ve	+ve	+ve	+ve	+ve	+ve
8.	PS1_1-STR08	Rangpur, BADC	+ve	+ve	+ve	+ve	+ve	+ve
9.	PS11_1-STB09	Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
10.	SS20-STNG04	Narayanganj, Dhaka, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
11.	SS11b-STM01	Munshiganj, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
12.	SS16-STG01	Gangni, Meherpur, Kustia, BADC	+ve	+ve	+ve	+ve	+ve	+ve
13.	SS28av-STB09	Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
14.	SS1-STR08	Rangpur, BADC	+ve	+ve	+ve	+ve	+ve	+ve
15.	SS12-STP07	Patuakhali, BADC	+ve	+ve	+ve	+ve	+ve	+ve
16.	SS14-STJ11	Jajira, Shariatpur, Farmer	+ve	+ve	+ve	+ve	+ve	+ve

Sl. No.	Isolate	Location	Hexose alcohol			Disaccharidesugar		
			Dulcitol	Sorbitol	Mannitol	Cellobiose	Maltose	Lactose
17.	SS32-STB10	Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
18.	SS8-STJ05	Jamalpur, BARI	+ve	+ve	+ve	+ve	+ve	+ve
19.	SS21-STD06	Domar, Nilphamari, BADC	+ve	+ve	+ve	+ve	+ve	+ve
20.	SS19-STC02	Chandpur, Comilla, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
21.	DS21-STJH01	Joypurhat (Kalai), Farmer	+ve	+ve	+ve	+ve	+ve	+ve
22.	DS03-STB02	Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
23.	DS24-STR03	Rangpur, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
24.	DS25-STD04	Dinajpur, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
25.	S1-STR11	Rangpur, BADC	+ve	+ve	+ve	+ve	+ve	+ve
26.	S2-STJ08	Jamalpur, BARI	+ve	+ve	+ve	+ve	+ve	+ve
27.	S3-STD09	Domar, Nilphamari, BADC	+ve	+ve	+ve	+ve	+ve	+ve
28.	S4-STM05	Munshiganj, TCRSC	+ve	+ve	+ve	+ve	+ve	+ve
29.	S5-STN07	Nagarpur, Tangail, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
30.	S6-STJ13	Jajira, Shariatpur, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
31.	S7-STB12	Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
32.	S18-STG14	Gangni, Meherpur, Kustia, BADC	+ve	+ve	+ve	+ve	+ve	+ve
33.	S9-STP10	Patuakhali, BADC	+ve	+ve	+ve	+ve	+ve	+ve
34.	S10-STC06	Chandpur, Comilla, Farmer	+ve	+ve	+ve	+ve	+ve	+ve

Sl. No.	Isolate	Location	Hexose alcohol			Disaccharidesugar		
			Dulcitol	Sorbitol	Mannitol	Cellobiose	Maltose	Lactose
35.	W27-SN02	Titbegun (*Solanum nigrum*), Nagarpur, Tangail, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
36.	W29-AV01	Shaknote (*Amaranthus viridis*), Chandpur, Comilla, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
37.	W30-CD03	Durba (*Cynodon dactylon*), Narayanganj, Dhaka, Farmer	-ve	-ve	-ve	-ve	-ve	-ve
38.	39-CA04	Chilli (*Capsicum annum* -other crop), Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve
39.	47-W05	Water sample (Domar), Farmer	-ve	-ve	-ve	-ve	-ve	-ve

Y= Yellow color produced due to change in pH; G= Green color remained as pH did not change.



(in presence of positive control) (in presence of negative control)

Fig. 21. Reactions showed bydifferent isolates of *R. solanacearum*to different disaccharide sugar and hexose alcohols in determination of biovar(s) test.

4.1.5.8. Phylotype detection of *R. solanacearum*through PCR test

4.1.5.6. Determination of phylotype(s)

During performing the phylotype(s) test,all 39 isolates of *R. solanacearum*viz. PS32STM01-Manikpur, Munshiganj; PS82-STC02-Chandpur, Comilla; PS71-STN03-Nagarpur, Tangail; PS91-STNG04-Narayanganj, Dhaka; PS23-STJ05-Jamalpur; PS101-STD06-Domar (Dimla), Nilphamari; PS43-STP07-Patuakhali; PS11-STR08-Rangpur; PS111-STB09-Bogra; SS20-STNG04-Narayanganj, Dhaka; SS11b-STM01-Munshiganj; SS16-STG01-Gangni, Meherpur, Kustia; SS28av-STB09-Bogra; SS1-STR08-Rangpur; SS12-STP07-Patuakhali; SS14-STJ11-Jajira, Shariatpur; SS32-STB10-Bogra; SS8-STJ05-Jamalpur; SS21-STD06-Domar, Nilphamari; SS19-STC02-Chandpur, Comilla; DS21-STJH01-Joypurhat(Kalai); DS03-STB02-Bogra; DS24-STR03-Rangpur; DS25-STD04-Dinajpur; S1-STR11-Rangpur; S2-STJ08-Jamalpur; S3-STD09-Domar, Nilphamari; S4-STM05-Munshiganj; S5-STN07-Nagarpur, Tangail; S6-STJ13-Jajira, Shariatpur; S7-STB12-Bogra; S18-STG14-Gangni, Meherpur, Kustia; S9-STP10 -Patuakhali; S10-STC06-Chandpur, Comilla,; W27-SN02-Titbegun (*Solanumnigrum*), Nagarpur, Tangail; W29-AV01-Shaknote (*Amaranthusviridis*), Chandpur, Comilla;W30-CD03-Durba (*Cynodon dactylon*), Narayanganj, Dhaka; 39-CA04-Chilli (*Capsicumannum* -other crop), Bogra; and 47-W05-Water sample, Domarproduced the bands of 280bp size specific for *R. solanacearum* and the bands of 144 bp size specific for phylotype I when amplified in PCR with specific primers which meant that, those isolate(s) were belonged to Asiatic origin(Table 11& Fig. 22, 23).

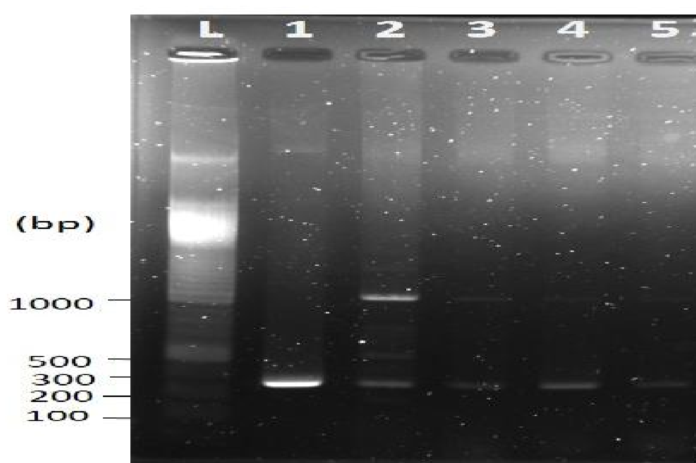


Fig. 22. PCR amplification of 280 bp fragment specific to *Ralstonia solanacearum*; L= DNA ladder, 1= PS32-STM01, 2= PS82-STC02, 3= PS71-STN03, 4= PS91-STNG04 & 5= PS23-STJ05.

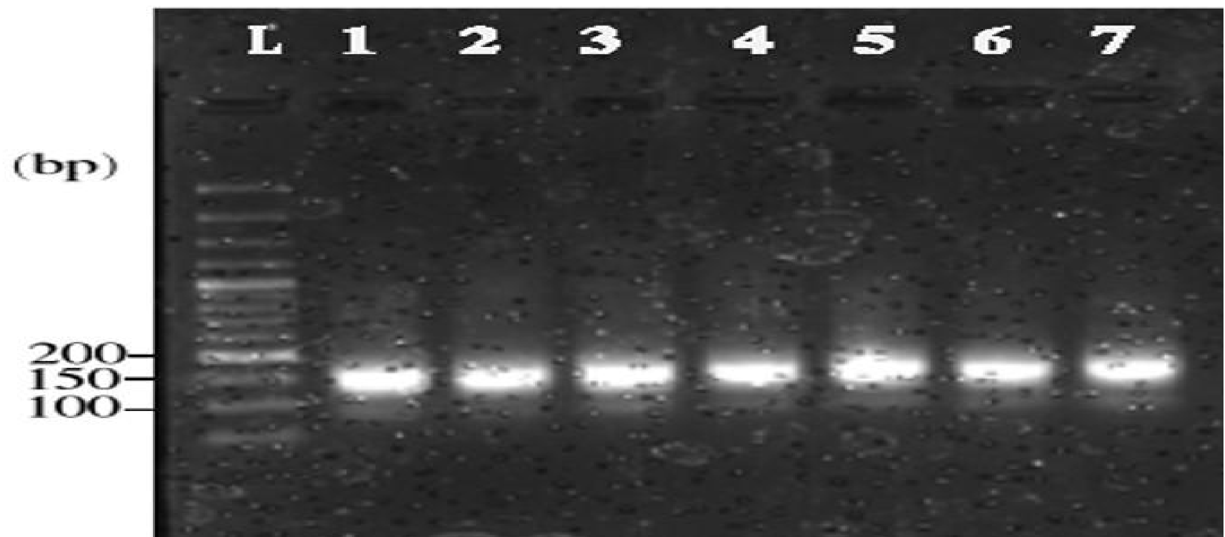


Fig. 23. PCR amplification of 144 bp product for characteristic band of phylotype I of *R. solanacearum*; L= DNA ladder, 1= PS32-STM01 (Munshiganj), 2= PS82-STC02 (Comilla), 3= PS71-STN03 (Tangail), 4= PS91-STNG04 (Narayanganj), 5= PS23-STJ05 (Jamalpur), 6= PS101-STD06 (Domar) & 7= PS43-STP07 (Patuakhali).

Table. 11. Status of race(s), biovar(s), *R. solananacearum* specific primer response and phylotype(s) of different isolates of *R. solanacearum* collected from different AEZ(s) of Bangladesh

Sl. No.	Isolate	Source of isolation	Location	Race	Biovar'	PCR 759R/760F	Phylotype
1.	PS3_2STM01	Plant	Manikpur, Munshiganj, Farmer	3	III	+ ve	I
2.	PS8_{2-}STC02	Plant	Chandpur, Comilla, Farmer	3	III	+ ve	I
3.	PS7_{1-}STN03	Plant	Nagarpur, Tangail, Farmer	3	III	+ ve	I
4.	PS9_{1-}STNG04	Plant	Narayanganj, Dhaka, Farmer	3	III	+ ve	I
5.	PS2_{3-}STJ05	Plant	Jamalpur, BARI	3	III	+ ve	I
6.	PS10_{1-}STD06	Plant	Domar (Dimla), Nilphamari	3	III	+ ve	I
7.	PS4_{3-}STP07	Plant	Patuakhali, BADC	3	III	+ ve	I
8.	PS1_{1-}STR08	Plant	Rangpur, BADC	3	III	+ ve	I
9.	PS11_{1-}STB09	Plant	Bogra, Farmer	3	III	+ ve	I
10.	SS20-STNG04	Seed	Narayanganj, Dhaka, Farmer	3	III	+ ve	I
11.	SS11b-STM01	Seed	Munshiganj, Farmer	3	III	+ ve	I
12.	SS16-STG01	Seed	Gangni, Meherpur, Kustia, BADC	3	III	+ ve	I
13.	SS28av-	Seed	Bogra, Farmer	3	III	+ ve	I
14.	SS1-STR08	Seed	Rangpur, BADC	3	III	+ ve	I
15.	SS12-STP07	Seed	Patuakhali, BADC	3	III	+ ve	I
16.	SS14-STJ11	Seed	Jajira, Shariatpur, Farmer	3	III	+ ve	I
17.	SS32-STB10	Seed	Bogra, Farmer	3	III	+ ve	I
18.	SS8-STJ05	Seed	Jamalpur, BARI	3	III	+ ve	I
19.	SS21-STD06	Seed	Domar, Nilphamari, BADC	3	III	+ ve	I
20.	SS19-STC02	Seed	Chandpur, Comilla, Farmer	3	III	+ ve	I
21.	DS21-STJH01	Soil	Joypurhat (Kalai), Farmer	3	III	+ ve	I
22.	DS03-STB02	Soil	Bogra, Farmer	3	III	+ ve	I
23.	DS24-STR03	Soil	Rangpur, Farmer	3	III	+ ve	I
24.	DS25-STD04	Soil	Dinajpur, Farmer	3	III	+ ve	I
25.	S1-STR11	Soil	Rangpur, BADC	3	III	+ ve	I
26.	S2-STJ08	Soil	Jamalpur, BARI	3	III	+ ve	I

Sl. No.	Isolate	Source of isolation	Location	Race	Biovar'	PCR 759R/760F	Phylotype
27.	S3-STD09	Soil	Domar, Nilphamari, BADC	3	III	+ ve	I
28.	S4-STM05	Soil	Munshiganj, TCRSC	3	III	+ ve	I
29.	S5-STN07	Soil	Nagarpur, Tangail, Farmer	3	III	+ ve	I
30.	S6-STJ13	Soil	Jajira, Shariatpur, Farmer	3	III	+ ve	I
31.	S7-STB12	Soil	Bogra, Farmer	3	III	+ ve	I
32.	S18-STG14	Soil	Gangni, Meherpur, Kustia, BADC	3	III	+ ve	I
33.	S9-STP10	Soil	Patuakhali, BADC	3	III	+ ve	I
34.	S10-STC06	Soil	Chandpur, Comilla, Farmer	3	III	+ ve	I
35.	W27-SN02	Weed	Titbegun (*Solanum nigrum*), Nagarpur, Tangail, Farmer	3	III	+ ve	I
36.	W29-AV01	Weed	Shaknote (*Amaranthus viridis*), Chandpur, Comilla, Farmer	3	III	+ ve	I
37.	W30-CD03	Weed	Durba (*Cynodon dactylon*), Narayanganj, Dhaka, Farmer	3	III	+ ve	I
38.	39-CA04	Other crop	Chilli (*Capsicum annum* -other crop), Bogra, Farmer	3	I	+ ve	I
39.	47-W05	Water	Water sample (Domar), Farmer	3	I	+ ve	I

The genomic DNA was amplified in PCR specific for *R. solanacearum*which produced the 280 base pair (bp) products (Fig. 22) by using 759R and 760F primers specific for *R. solanacearum*. It proved that genomic DNA were from *R. solanacearum*.After that, DNA from those 39 isolates were again amplified in PCR by using phylotype specific primers sequentially which resulted the characteristic band of 144 bp product (Fig. 23) specific for phylotype I. That meant all those 39 isolates, tested in the studycollected from different regions of Bangladesh, were belonged to Asian origin.

So, it was analyzed that out of collected 133 samples from different soureces, 94% samples showed positive on TZC test. Among those, 9 out of 34 from Rangpur, 8 out of 26 from Rajshahi, 6 out of 18 from Mymensingh, 2 out of 6 from Khulna, 3 out of 9 from Barisal, 4 out of 10 from Comilla and 7 out of 22 from Dhaka showed race 3; biovar III except 1 from each of Rangpur and Dhaka which showed biovar I; and all those were originated as phylotype I/Asiatic type.

4.2. Experiment 2

Study on the influence of moisture and temperature on heterogeneity within species in relation to PC (Phenotypic conversion' /VBNC), virulence and biovar expression by different isolates of *Ralstonia solanacearum*

4.2.1. Determination of the heterogeneity in phenotypic conversion'/ VBNC and virulence expressionin relation to moisture

4.2.1.1. Determination of the heterogeneity in PC expression (phenotypic conversion/ VBNC)

It was observed that all 9 plant samples showed phenotypic conversion (PC) from avirulent to virulent state on TZC culture medium due to shift from non-aerated to aerated growth medium(water)(Table 12, Fig. 24). Similarly, 3 isolates from 15 soil samples, 8 isolates from 10 seed samples, 3 weed samples 1 chilli sample and 1 water samples showed the VBNC (viable but non culturable) state along with phenotypic conversion (PC) from avirulent to virulent state due to the shift of the medium.

Table 12. Heterogeneity within the species in PC expression (phenotypic conversion/ VBNC) in aerated and non-aerated water mediumby isolates of *R. solanacearum*

Sl. No.	Location (source)	Isolate	Culture from non-aerated water medium	Culture from aerated water medium
1.	Munshiganj (Manikpur)	PS3$_2$STM01	PC (av)	PC (v)
2.	Chandpur	PS8$_2$-STC02	PC (av)	PC (v)
3.	Tangail (Nagarpur)	PS7$_1$-STN03	PC (av)	PC (v)
4.	Narayanganj (Sonargaon)	PS9$_1$-STNG04	PC (av)	PC (v)
5.	Jamalpur	PS2$_3$-STJ05	PC (av)	PC (v)
6.	Domar (Dimla)	PS10$_1$-STD06	PC (av)	PC (v)
7.	Patuakhali	PS4$_3$-STP07	PC (av)	PC (v)
8.	Rangpur	PS1$_1$-STR08	PC (av)	PC (v)
9.	Bogra	PS11$_1$-STB09	PC (av)	PC (v)
10.	Narayanganj (Sonargaon)	SS20-STNG04	VBNC, PC (av)	PC (v)
11.	Munshiganj (Manikpur)	SS11b-STM01	PC (av)	PC (v)
12.	Meherpur (Gangni)	SS16-STG01	PC (av)	PC (v)
13.	Bogra	SS28av-STB09	VBNC, PC (av)	PC (v)
14.	Rangpur	SS1-STR08	PC (av)	PC (v)
15.	Patuakhali	SS12-STP07	VBNC, PC (av)	PC (v)
16.	Shariatpur (Jajira)	SS14-STJ11	PC (av)	PC (v)
17.	Bogra	SS32-STB10	VBNC, PC (av)	PC (v)
18.	Jamalpur	SS8-STJ05	VBNC, PC (av)	PC (v)

Sl. No.	Location (source)	Isolate	Culture from non-aerated water medium	Culture from aerated water medium
19.	Domar (Dimla)	SS21-STD06	VBNC, PC (av)	PC (v)
20.	Chandpur	SS19-STC02	PC (av)	PC (v)
21.	Joypurhat (Akkelpur)	DS21-STJH01	PC (av)	PC (v)
22.	Bogra	DS03-STB02	PC (av)	PC (v)
23.	Rangpur	DS24-STR03	PC (av)	PC (v)
24.	Dinajpur	DS25-STD04	PC (av)	PC (v)
25.	Rangpur	S1-STR11	VBNC	PC (v)
26.	Jamalpur	S2-STJ08	VBNC	PC (v)
27.	Domar (Dimla)	S3-STD09	VBNC, PC (av)	PC (v)
28.	Munshiganj (Manikpur)	S4-STM05	PC (av)	PC (v)
29.	Tangail (Nagarpur)	S5-STN07	PC (av)	PC (v)
30.	Shariatpur (Jajira)	S6-STJ13	VBNC, PC (av)	PC (v)
31.	Bogra	S7-STB12	VBNC, PC (av)	PC (v)
32.	Meherpur (Gangni)	S18-STG14	VBNC	PC (v)
33.	Patuakhali	S9-STP10	VBNC	PC (v)
34.	Chandpur	S10-STC06	VBNC	PC (v)
35.	Titbegun (*Solanum nigrum*), Nagarpur, Tangail	W27-SN02	VBNC	PC (v)
36.	Shaknote (*Amaranthus viridis*), Chandpur, Comilla	W29-AV01	VBNC	PC (v)
37.	Durba (*Cynodon dactylon*), Narayanganj, Dhaka	W30-CD03	VBNC	PC (v)
38.	Chilli (*Capsicum annum* -other crop), Bogra	39-CA04	VBNC	PC (v)
39.	Water sample (Domar)	47-W05	VBNC	PC (v)

VBNC = (viable but non-culturable state/ potentially no visible colony growth) no colony; PC (av) = Phenotypic conversion virulent to avirulent state; PC (v) = Phenotypic conversion avirulent into virulent state.

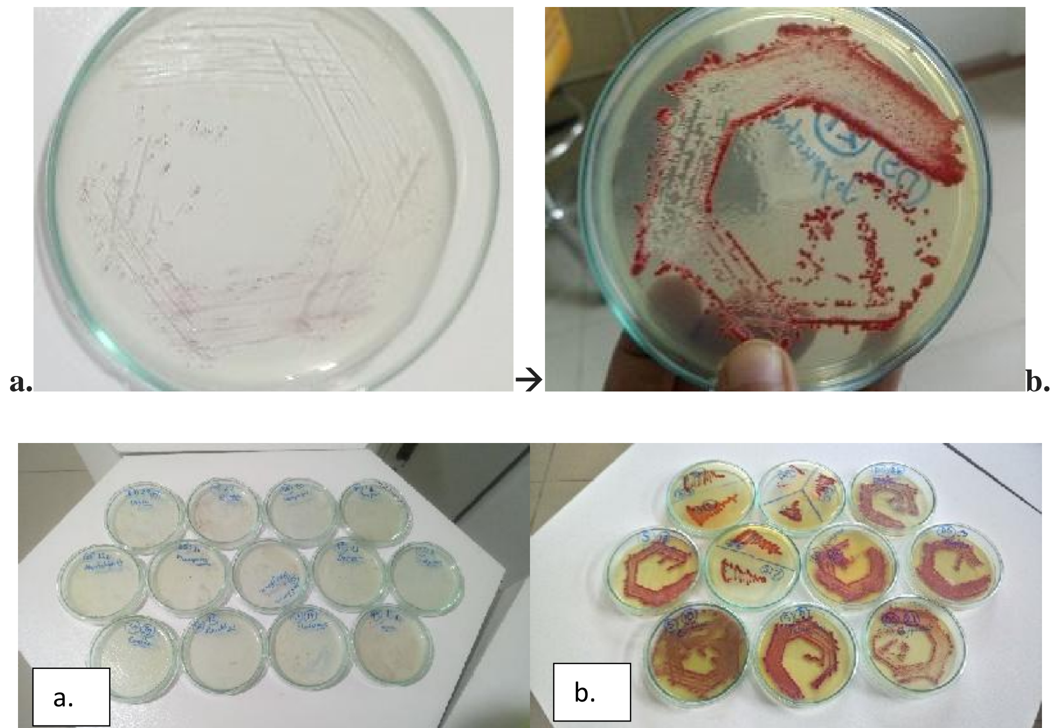


Fig. 24. A representative image showing PC (phenotypic conversion), VBNC ('viable but non culturable' state) of different isolates of *R. solanacearum*grown from a. non-aerated and b. aerated sterile water medium.

4.2.1.2. Determination of the heterogeneity in virulence expression

Inassessing the heterogeneity in virulence expression (in colony count) & disease scoring) by isolates *R. solanacearum*from phenotypically converted cultures in different dilutions (10^7 & 10^9) of water medium, it was found that different ranges of cfu/ml was counted in virulent and avirulent/or interim type colony in both the cases of dilution. Among them, both the dilutions showed heterogeneity and more or less positive correlation between avirulent &/or interim colony count (cfu/ml) and absorbance value (at 600nm) within isolates of*R. solanacearum*. But, in case of virulent colony, no correlation was observed between virulent colony count (cfu/ml) and absorbance value (at 600nm) at either of the dilutions except heterogeneity within isolates of*R. solanacearum*. In case of virulence expression (disease scoring) in potato plants, all the isolates produced symptoms of the disease and showed a range of heterogeneity within 2-7 days after inoculationby isolates of*R. solanacearum* which was measured as DSS (disease severity score) (Table 13, Fig. 25-30).

Table 13. Heterogeneity within the species in virulence expressionby isolates of *R. solanacearum* in water mediumat two different level of dilution (10^7 & 10^9)and in potato plants

Sl. No.	Isolate	Location (source)	Turbidity of pure culture in TZC broth of 8hrs old biomass	Cfu/ml of pure culture of 36 hrs old biomass	Phenotypically converted			Phenotypically converted			Virulence expression	Virulence expression
			OD$_{600nm}$	cfu/ml	Colony (mean(cfu/ml) at 10^7 dilution			Colony (mean(cfu/ml) at 10^9 dilution			10^9 dilution	10^9 dilution
					Virulent	Avirulent &/or interim	Stdev	Virulent	Avirulent &/or interim	Stdev	Symptom	DSS
1.	PS3$_2$STM01	Munshiganj (Manikpur)	0.988	29X10^7	28.1	52.36	7.52	17.5	22.24	7.67	+ ve	3.2
2.	PS8$_{2-}$STC02	Chandpur	0.647	45X10^6	13.0	21.25	9.95	11.0	12.33	9.67	+ ve	2.8
3.	PS7$_{1-}$STN03	Tangail (Nagarpur)	0.653	46X10^6	14.9	26.25	9.36	9.0	17.23	4.10	+ ve	2.6
4.	PS9$_{1-}$STNG04	Narayanganj (Sonargaon)	1.965	40X10^8	34.2	72.50	4.42	7.5	39.60	16.07	+ ve	3.8
5.	PS2$_{3-}$STJ05	Jamalpur	2.001	29X10^8	38.6	77.52	15.32	22.5	36.25	5.66	+ ve	2.8
6.	PS10$_{1-}$STD06	Domar (Dimla)	0.942	94X10^7	42.5	58.13	17.01	18.0	22.67	1.70	+ ve	3.4
7.	PS4$_{3-}$STP07	Patuakhali	1.861	137X10^8	45.0	88.75	22.92	30.5	43.83	16.51	+ ve	5
8.	PS1$_{1-}$STR08	Rangpur	0.813	39X10^6	15.0	23.75	4.60	5.0	15.00	8.16	+ ve	2.6
9.	PS11$_{1-}$STB09	Bogra	2.038	36X10^8	32.7	75.76	15.08	19.0	35.46	3.92	+ ve	4.2
10.	SS20-STNG04	Narayanganj (Sonargaon)	2.070	23X10^8	23.0	69.64	2.19	19.0	38.98	13.41	+ ve	3.8
11.	SS11b-STM01	Munshiganj (Manikpur)	1.989	35X10^8	33.2	73.89	10.94	19.5	32.30	3.61	+ ve	2.8
12.	SS16-STG01	Meherpur (Gangni)	1.457	42X10^7	24.4	53.13	2.41	17.5	23.06	6.84	+ ve	3.4

Sl. No.	Isolate	Location (source)	Turbidity of pure culture in TZC broth of 8hrs old biomass	Cfu/ml of pure culture of 36 hrs old biomass	Phenotypically converted			Phenotypically converted			Virulence expression	Virulence expression
			OD_{600nm}	cfu/ml	Colony (mean(cfu/ml) at 10^7 dilution			Colony (mean(cfu/ml) at 10^9 dilution			10^9 dilution	10^9 dilution
					Virulent	Avirulent &/or interim	Stdev	Virulent	Avirulent &/or interim	Stdev	Symptom	DSS
13.	SS28av-STB09	Bogra	0.708	32X10^6	18.7	39.17	3.42	17.0	13.44	2.17	+ ve	2.8
14.	SS1-STR08	Rangpur	0.909	72X10^7	25.3	54.19	7.52	34.0	22.19	11.66	+ ve	2.8
15.	SS12-STP07	Patuakhali	1.830	30X10^8	25.9	75.27	5.07	18.0	39.29	16.67	+ ve	3.8
16.	SS14-STJ11	Shariatpur (Jajira)	1.913	68X10^8	33.3	76.14	14.39	18.0	38.85	12.48	+ ve	4
17.	SS32-STB10	Bogra	1.894	48X10^8	35.1	68.59	22.44	13.5	31.57	11.06	+ ve	3.8
18.	SS8-STJ05	Jamalpur	2.016	70X10^8	9.4	72.16	16.99	22.0	37.62	14.49	+ ve	4.2
19.	SS21-STD06	Domar (Dimla)	1.923	49X10^8	17.0	78.29	15.97	23.5	30.18	5.52	+ ve	4.2
20.	SS19-STC02	Chandpur	1.674	51X10^8	31.3	72.19	16.74	16.0	38.86	4.77	+ ve	3.4
21.	DS21-STJH01	Joypurhat (Akkelpur)	1.721	15X10^8	12.8	70.50	5.04	22.5	31.68	28.19	+ ve	3.8
22.	DS03-STB02	Bogra	1.458	30X10^7	28.0	55.00	8.75	15.5	20.17	21.10	+ ve	4.2
23.	DS24-STR03	Rangpur	1.970	18X10^8	36.7	70.72	7.27	25.0	38.79	20.21	+ ve	4
24.	DS25-STD04	Dinajpur	1.992	37X10^8	31.3	70.59	3.16	18.0	31.51	21.26	+ ve	3.8
25.	S1-STR11	Rangpur	1.813	42X10^8	31.4	72.56	16.86	21.5	34.44	8.35	+ ve	2.6
26.	S2-STJ08	Jamalpur	1.961	101X10^8	45.6	80.56	25.82	43.0	39.37	20.25	+ ve	5
27.	S3-STD09	Domar (Dimla)	1.864	20X10^8	38.2	71.54	30.33	13.5	30.28	3.37	+ ve	3.6

Sl. No.	Isolate	Location (source)	Turbidity of pure culture in TZC broth of 8hrs old biomass	Cfu/ml of pure culture of 36 hrs old biomass	Phenotypically converted			Phenotypically converted			Virulence expression	Virulence expression
			OD_{600nm}	cfu/ml	Colony (mean(cfu/ml) at 10^7 dilution			Colony (mean(cfu/ml) at 10^9 dilution			10^9 dilution	10^9 dilution
					Virulent	Avirulent &/or interim	Stdev	Virulent	Avirulent &/or interim	Stdev	Symptom	DSS
28.	S4-STM05	Munshiganj (Manikpur)	2.102	33X10^8	14.2	73.66	7.24	18.0	37.47	5.08	+ ve	4.2
29.	S5-STN07	Tangail (Nagarpur)	1.966	32X10^8	17.9	70.27	2.92	11.5	31.45	5.25	+ ve	3.2
30.	S6-STJ13	Shariatpur (Jajira)	1.969	30X10^8	18.4	71.35	6.98	16.5	38.11	19.69	+ ve	4.2
31.	S7-STB12	Bogra	1.754	37X10^8	37.1	73.45	11.16	14.5	31.21	3.13	+ ve	2.8
32.	S18-STG14	Meherpur (Gangni)	1.681	152X10^8	42.8	92.97	40.30	51.5	44.67	26.06	+ ve	5
33.	S9-STP10	Patuakhali	1.666	72X10^8	42.5	75.43	20.95	20.0	32.34	16.36	+ ve	3.8
34.	S10-STC06	Chandpur	1.730	163X10^8	45.7	95.02	35.15	53.0	46.13	20.53	+ ve	5
35.	W27-SN02	*Solanum nigrum*, Nagarpur, Tangail,	1.622	58X10^8	30.8	77.71	11.95	20.5	37.72	5.11	+ ve	3.2
36.	W29-AV01	*Amaranthus viridis*, Chandpur, Comilla,	1.816	24X10^8	32.9	76.49	2.14	11.5	31.45	11.68	+ ve	3.8
37.	W30-CD03	*Cynodon dactylon*, Narayanganj, Dhaka,	1.914	33X10^8	35.4	71.17	13.39	12.5	36.79	3.87	+ ve	4
38.	39-CA04	*Capsicum annum*, Bogra,	1.887	55X10^8	37.9	71.20	6.97	21.0	31.60	10.70	+ ve	3.8
39.	47-W05	Water sample (Domar), Farmer	1.960	51X10^8	35.5	73.93	7.50	19.5	39.52	2.06	+ ve	3.8
			**P < 0.01	**P < 0.01	**P < 0.01	**P < 0.01	**P < 0.01	**P < 0.01	**P < 0.01	**P < 0.01		**P < 0.01

DSS= Disease severity score.

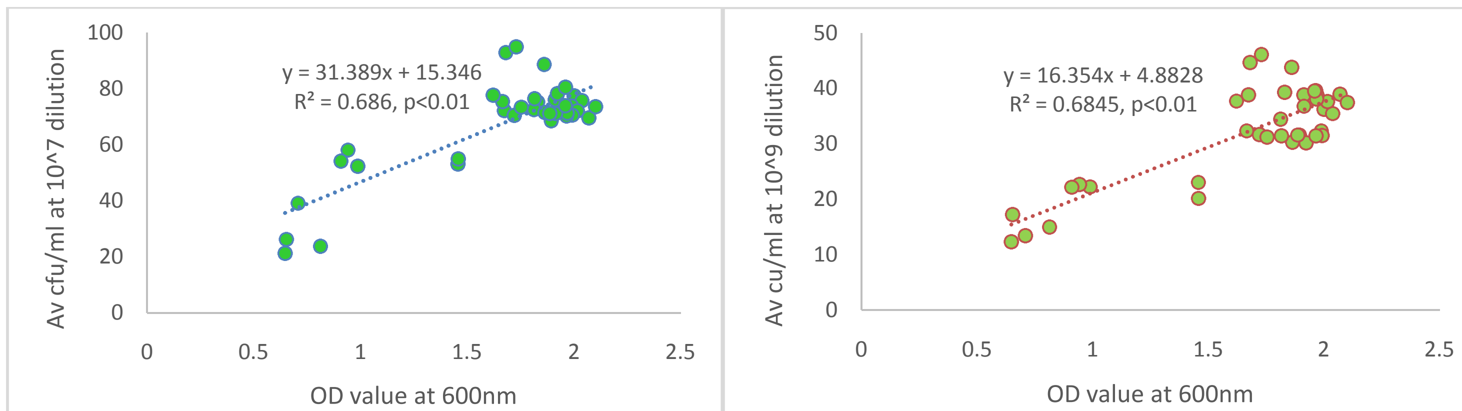


Fig. 25. Correlation between avirulent cfu/ml and absorbance (OD at 600nm) of*R. solanacearum*isolates in 10^7 & 10^9 dilution.

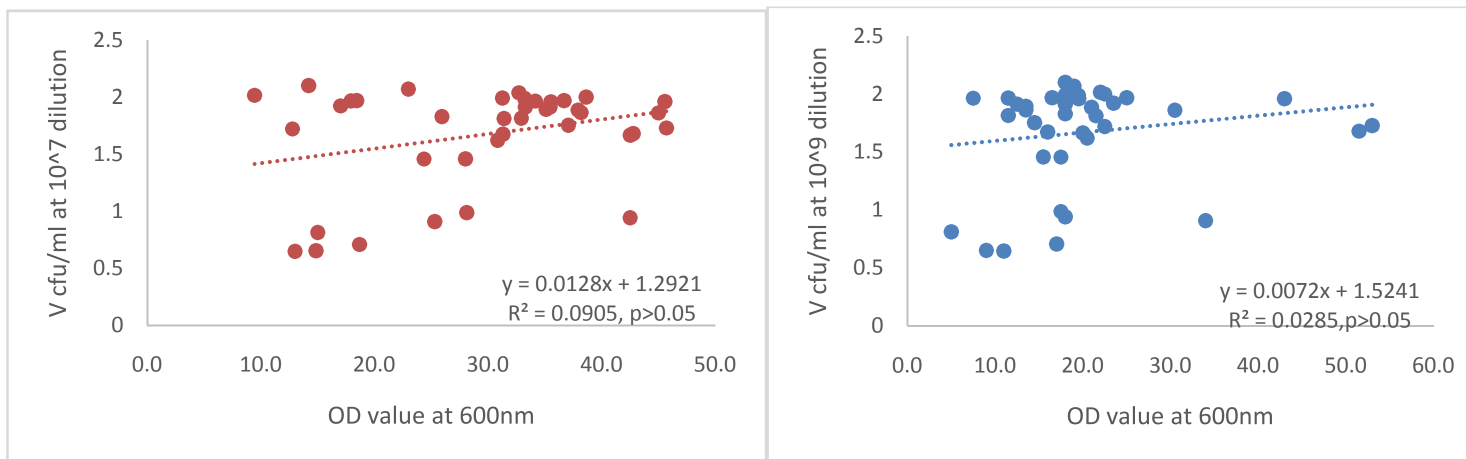


Fig. 26. Correlation between virulent cfu/ml and absorbance (OD at 600nm) of*R. solanacearum*isolates in 10^7 & 10^9 dilution.

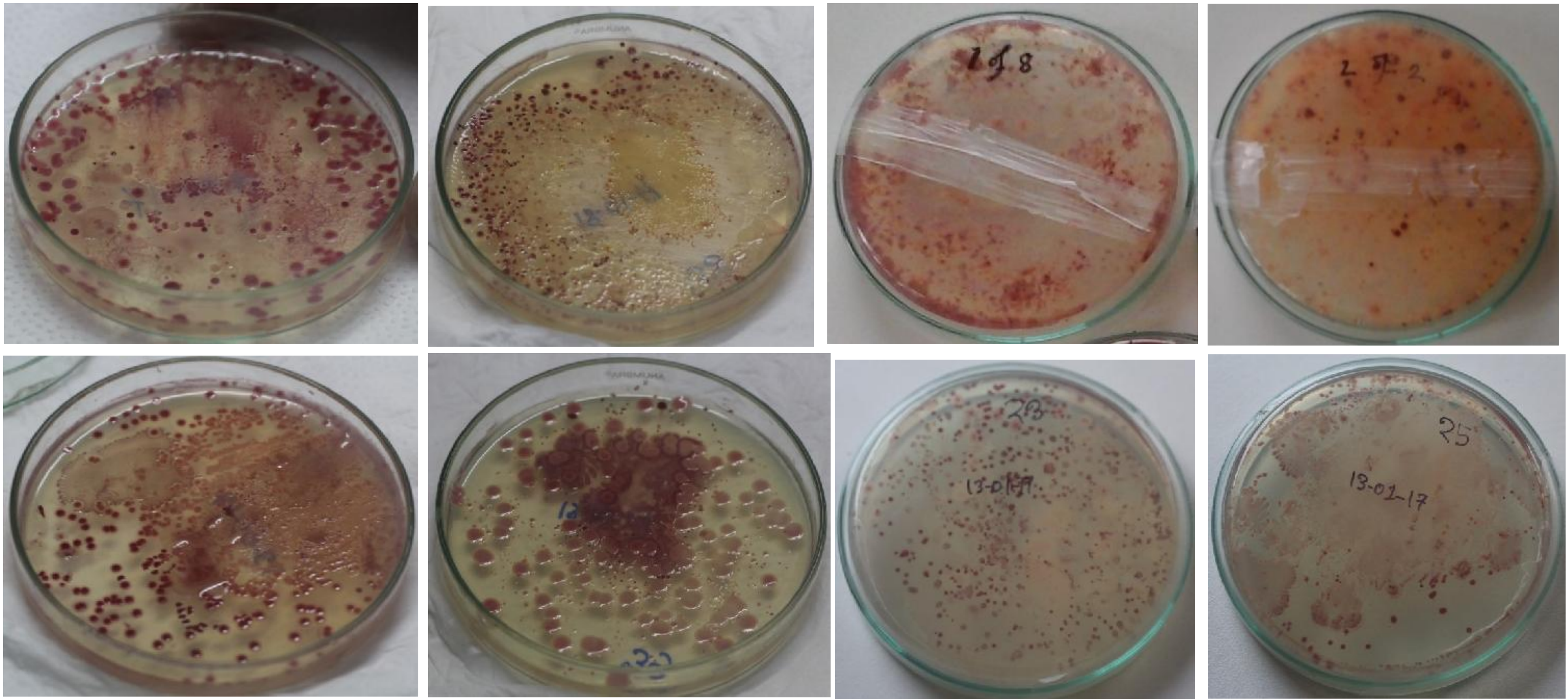


Fig. 27. Isolates of *R. solanacearum* showing heterogeneityin colony type expressing virulent and/or avirulent colonies on TZC solid media during collection and pure culture preservation.

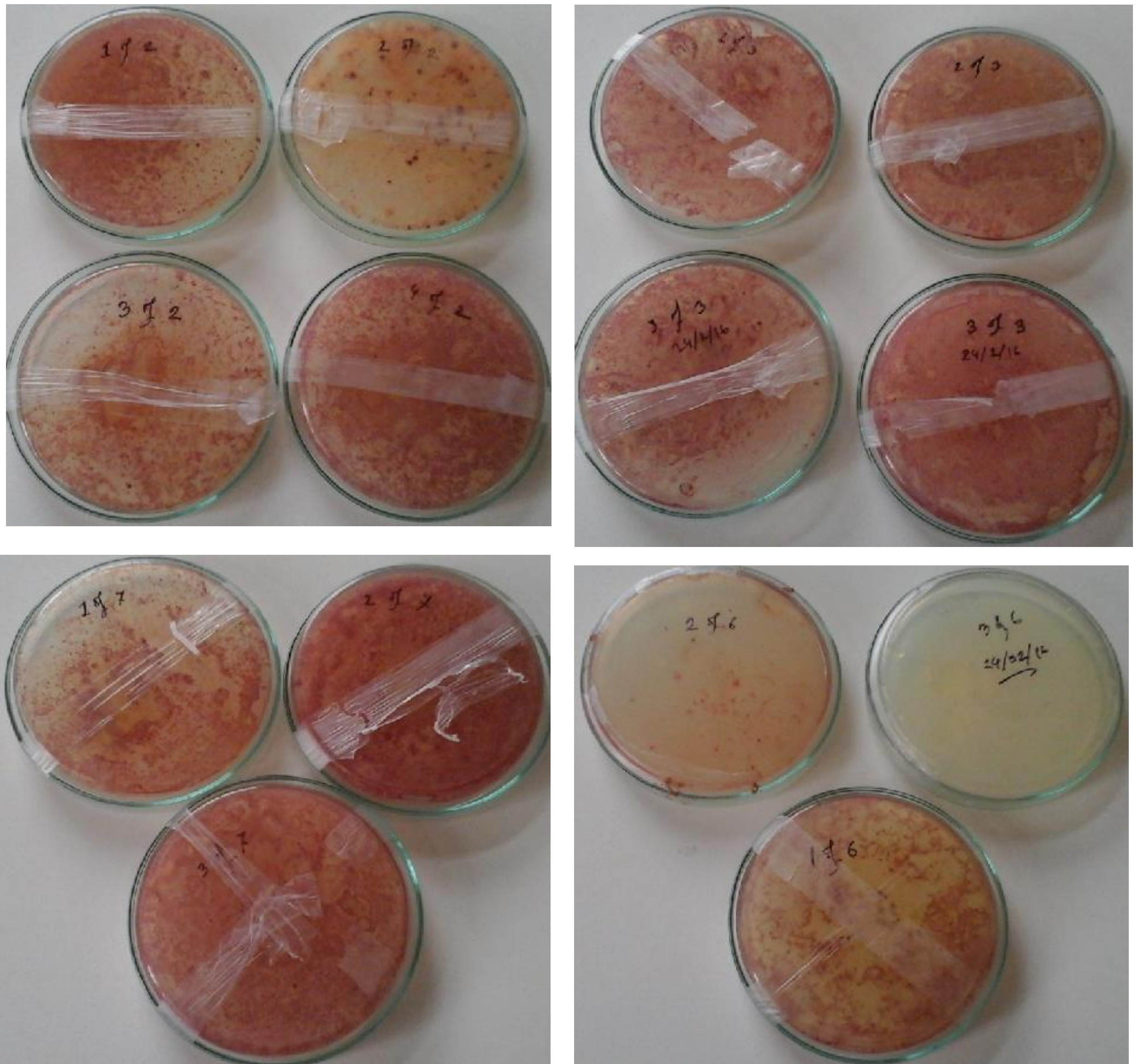
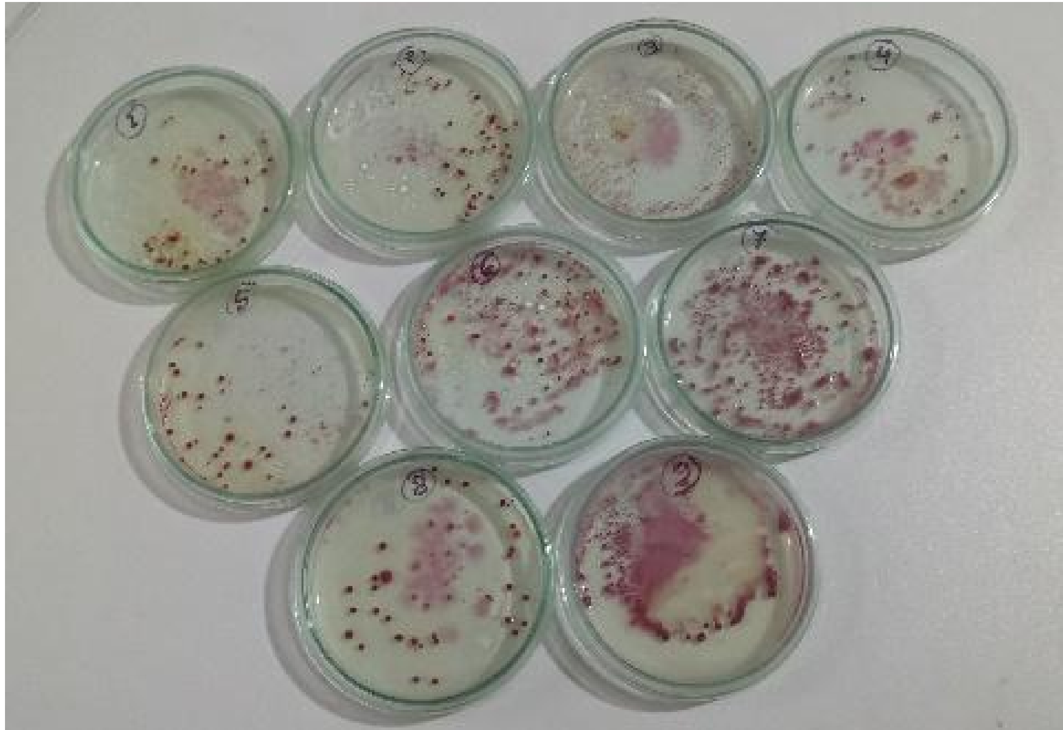


Fig. 28.*R. solanacearum* isolates from pure cultures at different dilutions showing the heterogeneity in concentration within isolates of *R. solanacearum* on TZC plates.



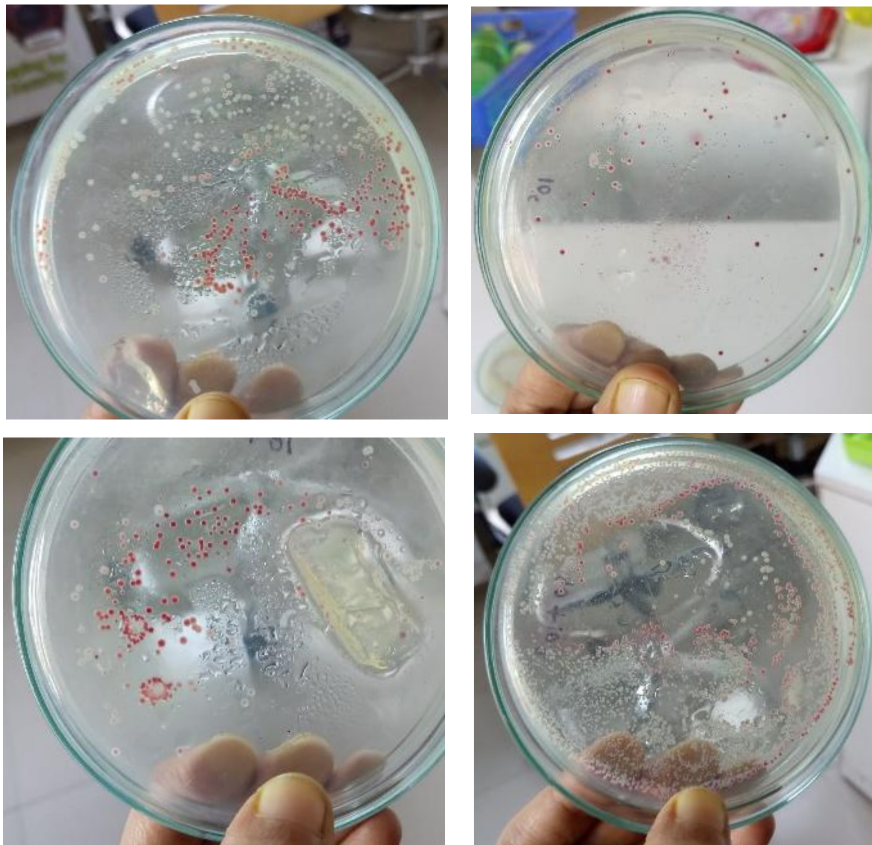


Fig. 29. A representative image showing counts of virulent, avirulent &/or interim colonies of phenotypically converted isolates of *R. solanacearum* at 10^7 (top) and 10^9 level (bottom) of dilution.

Fig. 30. Helathy potato seedlings (top), diseased seedlings at 3rd day (middle) and diseased seedlings at 7th day (bottom) after inoculationin virulence test by isolates of *R. solanacearum* at 10^9 dilution.

Thus, the study revealed that, condition (aerated and non-aerated) and level of dilution (10^7 &10^9) in water mediumplayed an important role in expression of heterogeneity between isolates of the pathogen in terms of influence of moisture. It was observed that isolateswere showed to shift into phenotypic conversion due to change in water medium aeration and,also showed to vary in colony counts (cfu/ml) as compared to pure culture cfu/mldue to change in dilution level in water medium. It was also observed that avirulent counts of different isolates of *R. solanacearum* varied more or less accordingly or positivelycorrelated with the absorbance except with virulent colonies which were showed weak or no correlation with absorbance.So, to understand the influence of soilmoistureprecisely in disease expression on potato plants by*R. solanacearum*the next study was performed.

4.2.1.3. Influence of different level of moisture in inoculated soil on virulent expression in potato plant andin colony count

Different level of moisture was applied in inoculated soil for evaluating the consequence of soil moisture level (by watering /irrigation) on virulence expression in potato plant (both on wilt disease severity and onvirulent & avirulentcfu/ml) by a least virulent strain of *R. solanacearum*it was observed that all the disease parameters viz.-

Table 14. Effect of different level of moisture on virulent/avirulent colony count in inoculated soil and wilt disease severity caused by *R. solanacearum*

Moisture level of inoculated soil	Disease severity score	PSI (Percent Severity Index) (%)	virulent colony (cfu/m/sq.cm)	avirulent & interim colony (cfu/m/sq.cm)	Stdev
			At 10^8 dilution	At 10^8 dilution	
T1- moisture level >5.5 (@ 2 irrigation per 7days	2.60 ± 0.89	52~61	31.45	68.55	±6.56
T2- moisture level 3-5.5 (@ 1 irrigation per 7days)	3.20 ± 0.84	64~72	54.75	45.25	±7.9
T3- moisture level <3 (@ 1 irrigation per 10days)	3.60 ± 0.55	72~78	70.40	29.60	±6.93
	**P < 0.01	**P < 0.01	*P < 0.05	*P < 0.05	

disease severity score, PSI (percent severity index) and virulent colony (cfu/ml) count were highest and, avirulent &/or interim colony (cfu/ml) count was lowest in T3 (moisture level <3 as irrigation @ 1 per 10days) as compared to T2 (moisture level 3~5.5 as irrigation @ 1 per

7days) and T1 (moisture level >5.5 as irrigation @ 2 per 7days). The highest disease reduction was observed in T1in all the cases (Table 14, **$p < 0.01$; Fig. 31). It was also observed thatmoisture level of soil changed by different level of irrigation influenced the disease severity and colony virulence level of *R. solanacearum* on same inoculated soil. In fine, a good moist soil (moisture level >5.5) could reduce the virulence highest and otherwise it could upshot the disease severity and virulent count in soil by *R. solanacearum*.

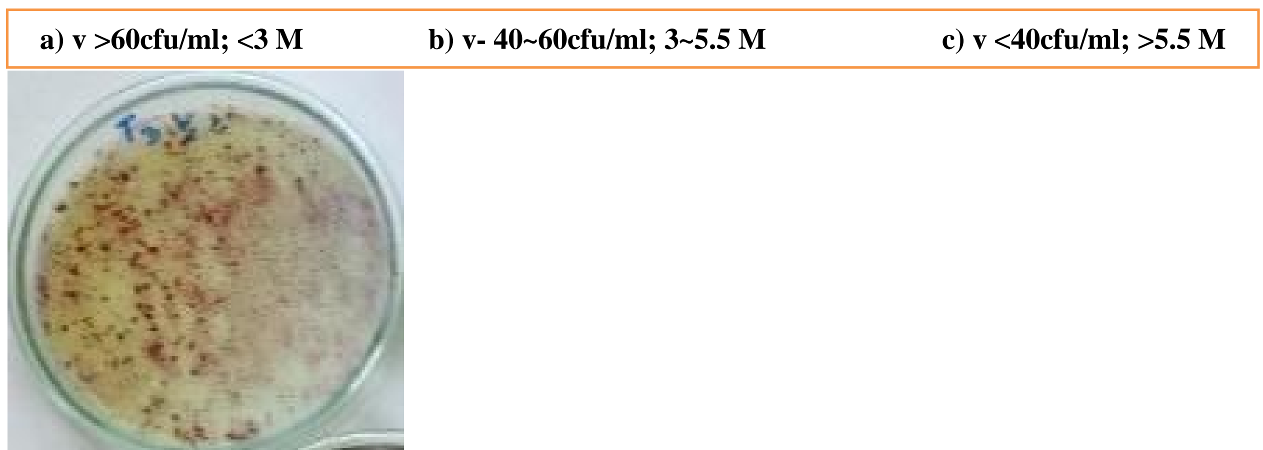


Fig. 31. Effect of different level of moisture in same inoculated (@10^8 cfu/ml) soil on wilt disease severity and virulence expression by *R. solanacearum* at 10^6 dilution plates.

4.2.2. Determination of heterogeneity in biovar expression in relation to different incubation temperature

4.2.2.1. Determination of biovar(s)of *R. solanacearum* isolatesin different temperatures

The heterogeneity within species of *R. solanacearum*isolates inrelation to different disaccharides and sugar alcohols utilizaionin two different incubation temperature (22^0C& 28^0C) were studied. It was observed that, all the isolates except W30-CD03- Durba (*Cynodon dactylon*)and 47-W05reacted to both disaccharides and sugar alcohols positively in developing yellow colorsat 28 ^{0}C and it indicated the oxidation of both carbon sources. But isolates W30-CD03 (*Cynodon dactylon*)and 47-W05 (Water sample) neither changed the color for disaccharides nor for sugar alcohols, which indicated those were not able to utilize either of the carbon sources at 28^0C temperature. On the other hand, different types of reactions were showed by the same isolates in utilizingdisaccharides and sugar alcohols at 22 ^{0}C viz.-

PS111-STB09-Bogra; SS20-STNG04-Narayanganj; SS28av-STB09-Bogra; SS1-STR08-Rangpur, SS8-STJ05-Jamalpur; SS19-STC02-Chandpur, Comilla; DS21-STJH01-Joypurhat (Kalai); S2-STJ08-Jamalpur; S9-STP10-Patuakhali; S10-STC06-Chandpur, Comilla; W29-AV01-Shaknote (*Amaranthusviridis*), Chandpur, Comilla; 39-CA04-Chilli (*Capsicumannum* - other crop), Bogra; and 47-W05-Water sample, Domarwere not able to utilize either of the carbon sourcesas such 13 isolates were observed as biovar I.

PS32STM01-Manikpur, Munshiganj, PS82-STC02-Chandpur, Comilla; PS71-STN03-Nagarpur, Tangail; PS91-STNG04-Narayanganj, Dhaka; PS43-STP07-Patuakhali; PS11-STR08-Rangpur; SS11b-STM01-Munshiganj; SS12-STP07-Patuakhali; SS32-STB10-Bogra; SS21-STD06-Domar, Nilphamari; DS25-STD04-Dinajpur; S1-STR11-Rangpur; S3-STD09-Domar, Nilphamari; S5-STN07-Nagarpur, Tangail; and W27-SN02-Titbegun (*Solanumnigrum*), Nagarpur, Tangail were able to utilize both of the carbon sources.So, total 15isolates were observed as biovar III.

Isolate, PS23-STJ05-Jamalpur; PS101-STD06-Domar, Nilphamari; SS16-STG01-Gangni, Meherpur; S7-STB12-Bogra; S18-STG14-Gangni, Meherpur; and W30-CD03 (*Cynodondactylon*), Narayanganj, Dhaka were able to utilize disaccharides but not the sugar alcohols. Thus, 6isolates were observed as biovar II,

SS14-STJ11-Jajira, Shariatpur; DS03-STB02-Bogra; DS24-STR03-Rangpur; S4-STM05-Munshiganj; S6-STJ13-Jajira, Shariatpur were able to utilize only sugar alcohols except disaccharides. Hence,total 5isolates were observed as biovar IV (Table 15, Fig. 32-35).

Table 15. Biovar reaction of different isolates of *R. solanacearum* to different disaccharides and hexose alcohols in different incubation temperature

Sl. No.	Isolate	Location	Hexose alcohol at 22^{0}C			Disaccharide at 22^{0}C			Biovar	Hexose alcohol at 28^{0}C			Disaccharide at 28^{0}C			Biovar
			D	Mn	S	L	M	C	At 22^{0}C	D	Mn	S	L	M	C	At 28^{0}C
1.	PS3$_2$STM01	Manikpur, Munshiganj, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
2.	PS8$_2$.STC02	Chandpur, Comilla, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
3.	PS7$_1$.STN03	Nagarpur, Tangail, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
4.	PS9$_1$.STNG04	Narayanganj, Dhaka, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
5.	PS2$_3$.STJ05	Jamalpur, BARI	-ve	-ve	-ve	+ve	+ve	+ve	II	+ve	+ve	+ve	+ve	+ve	+ve	III
6.	PS10$_1$.STD06	Domar (Dimla), Nilphamari	-ve	-ve	-ve	+ve	+ve	+ve	II	+ve	+ve	+ve	+ve	+ve	+ve	III
7.	PS4$_3$.STP07	Patuakhali, BADC	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
8.	PS1$_1$.STR08	Rangpur, BADC	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
9.	PS11$_1$.STB09	Bogra, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
10.	SS20-STNG04	Narayanganj, Dhaka, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
11.	SS11b-STM01	Munshiganj, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
12.	SS16-STG01	Gangni, Meherpur, Kustia, BADC	-ve	-ve	-ve	+ve	+ve	+ve	II	+ve	+ve	+ve	+ve	+ve	+ve	III
13.	SS28av-STB09	Bogra, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
14.	SS1-STR08	Rangpur, BADC	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
15.	SS12-STP07	Patuakhali, BADC	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
16.	SS14-STJ11	Jajira, Shariatpur, Farmer	+ve	+ve	+ve	-ve	-ve	-ve	IV	+ve	+ve	+ve	+ve	+ve	+ve	III
17.	SS32-STB10	Bogra, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
18.	SS8-STJ05	Jamalpur, BARI	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III

19.	SS21-STD06	Domar, Nilphamari, BADC	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
20.	SS19-STC02	Chandpur, Comilla, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
21.	DS21-STJH01	Joypurhat (Kalai), Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
22.	DS03-STB02	Bogra, Farmer	+ve	+ve	+ve	-ve	-ve	-ve	IV	+ve	+ve	+ve	+ve	+ve	+ve	III
23.	DS24-STR03	Rangpur, Farmer	+ve	+ve	+ve	-ve	-ve	-ve	IV	+ve	+ve	+ve	+ve	+ve	+ve	III
24.	DS25-STD04	Dinajpur, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
25.	S1-STR11	Rangpur, BADC	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
26.	S2-STJ08	Jamalpur, BARI	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
27.	S3-STD09	Domar, Nilphamari, BADC	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
28.	S4-STM05	Munshiganj, TCRSC	+ve	+ve	+ve	-ve	-ve	-ve	IV	+ve	+ve	+ve	+ve	+ve	+ve	III
29.	S5-STN07	Nagarpur, Tangail, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
30.	S6-STJ13	Jajira, Shariatpur, Farmer	+ve	+ve	+ve	-ve	-ve	-ve	IV	+ve	+ve	+ve	+ve	+ve	+ve	III
31.	S7-STB12	Bogra, Farmer	-ve	-ve	-ve	+ve	+ve	+ve	II	+ve	+ve	+ve	+ve	+ve	+ve	III
32.	S18-STG14	Gangni, Meherpur, Kustia, BADC	-ve	-ve	-ve	+ve	+ve	+ve	II	+ve	+ve	+ve	+ve	+ve	+ve	III
33.	S9-STP10	Patuakhali, BADC	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
34.	S10-STC06	Chandpur, Comilla, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
35.	W27-SN02	Titbegun (*Solanum nigrum*), Nagarpur, Tangail, Farmer	+ve	+ve	+ve	+ve	+ve	+ve	III	+ve	+ve	+ve	+ve	+ve	+ve	III
36.	W29-AV01	Shaknote (*Amaranthus viridis*), Chandpur, Comilla, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
37.	W30-CD03	Durba (*Cynodon dactylon*), Narayanganj, Dhaka, Farmer	-ve	-ve	-ve	+ve	+ve	+ve	II	-ve	-ve	-ve	-ve	-ve	-ve	I
38.	39-CA04	Chilli (*Capsicum annum* -other crop), Bogra, Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	+ve	+ve	+ve	+ve	+ve	+ve	III
39.	47-W05	Water sample (Domar), Farmer	-ve	-ve	-ve	-ve	-ve	-ve	I	-ve	-ve	-ve	-ve	-ve	-ve	I
			D	Mn	S	L	M	C		D	Mn	S	L	M	C	

Y= Yellow color produced due to change in pH; G= Green color remained as pH did not change; D= Dulcitol, Mn= Mannitol, S= Sorbitol, L=Lactose, M=Maltose, C=Cellobiose.

Fig. 32. Isolates of *R. solanacearum* on CPG medium to be tested for biovar expression.

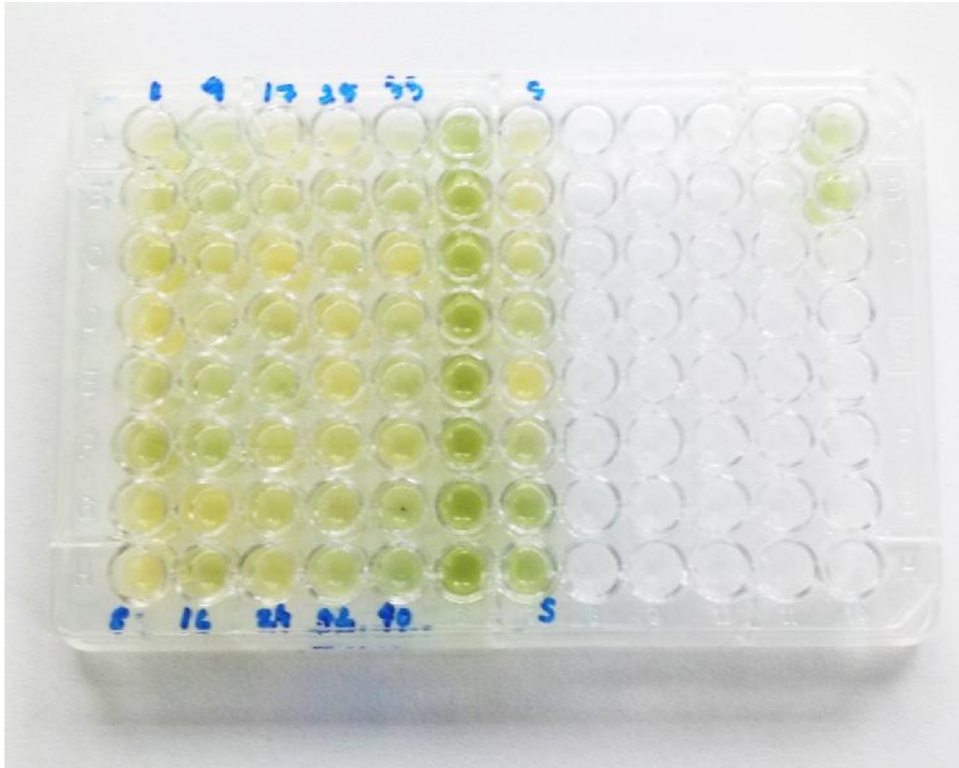


Fig. 33. Representative reaction to sugar alcohol by different isolates of *R. solanacearum* at 22^0C incubation.

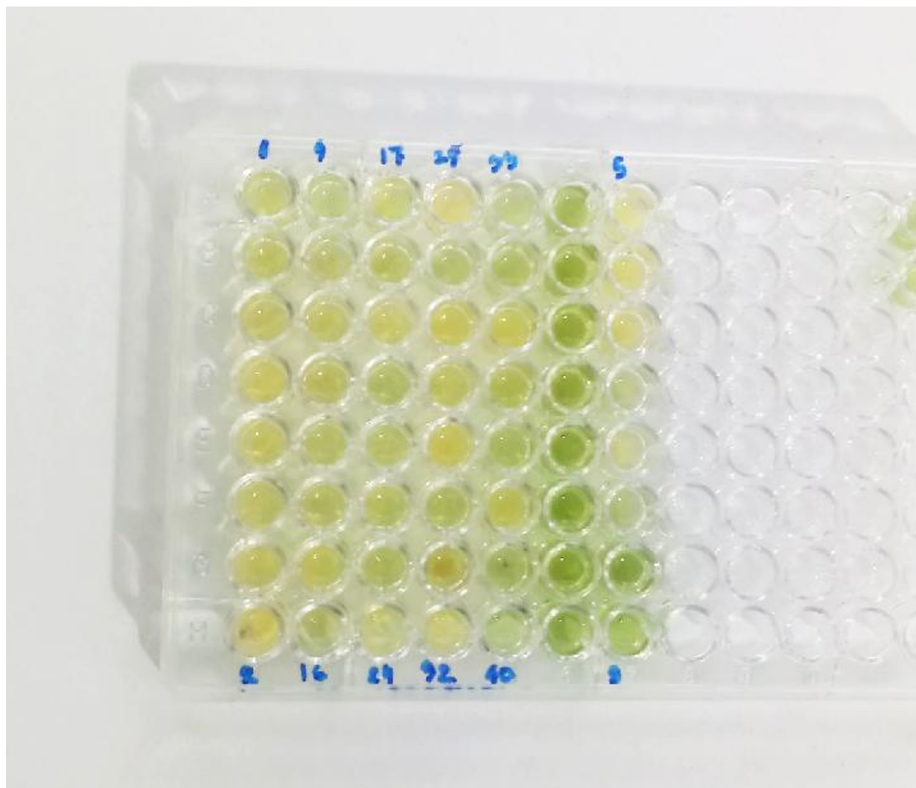


Fig. 34. Representative reaction to disaccharides by different isolates of *R. solanacearum* at 22^0C incubation.

Fig. 35. Representative reaction to sugar alcohol and disaccharides by different isolates of *R. solanacearum* at 28^0C incubation.

4.2.2.2. Effect of different temperature onavirulent colony of *R. solanacearum* in colony virulence and biovar expression

To understand the influence of temperature precisely on avirulent colony dilution of *R. solanacearum*, the study was performed. The influence of different incubation temperature on avirulent colony in biovar expression of *R. solanacearum*were studied and observed that culture T1 in 0 ± 2^0C, T2 in 10 ± 2^0C and culture T5 in 40 ± 2^0C showed no visible growth ie.VBNC (‘viable but non culturable’ state). But, T4 culture in 28 ± 2^0C showed the highest growth of avirulent colony (77.58 cfu/ml) whereas culture T3 in 20 ± 2^0C showed the highest virulent colony (49.72 cfu/ml) at 10^7 level of dilution within 72 hrs. However, when those VBNC cultures (T1, T2, and T5) were incubated at 28 ± 2^0C, the same T1, T2 and T5 cultures showed potential and visible growth of both virulent and avirulent colony (Fig. 36&37, Fig. 38-43) within 36 hrs.

From the study it was found that *R. solanacearum* colony showed VBNC state in below10^0C and over40 ± 2^0C. Both virulent and avirulent &/or interim colonies were produced from avirulent colony and from VBNC state in presence of favorable temperature.

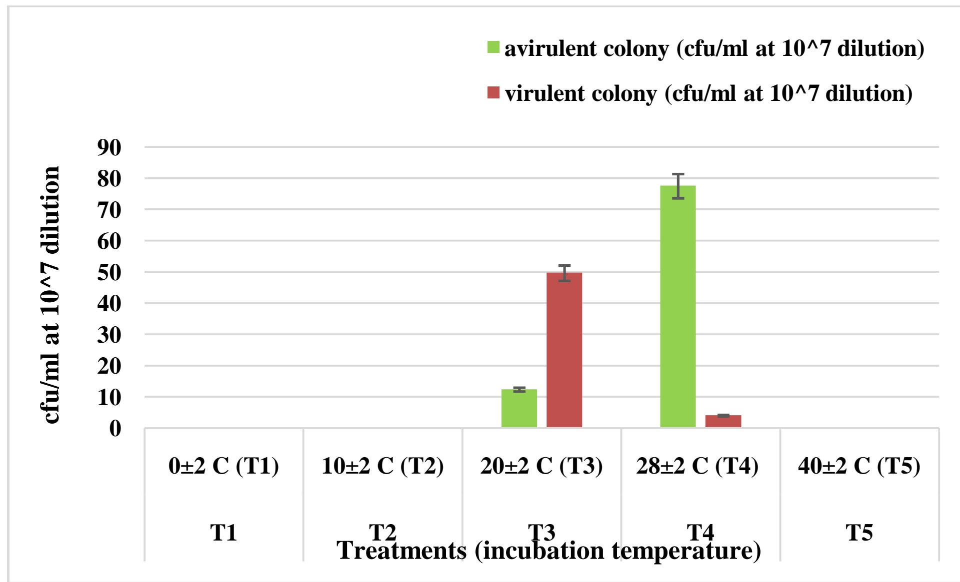


Fig. 36. Effect of different incubation temperature on avirulent colony of *R. solanacearum* in expression of colony count (**p < 0.01 for virulent colony; *p< 0.05 for virulent colony).

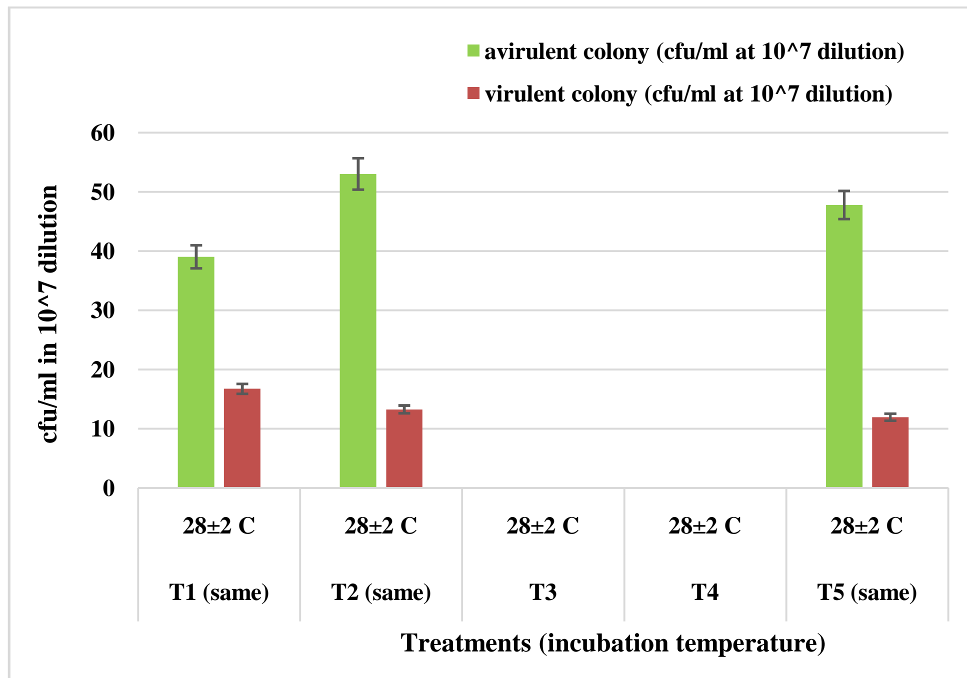


Fig. 37. Effect of different incubation temperature on avirulent colony of *R. solanacearum* in expression of colony count (**p < 0.01 for virulent colony; *p< 0.05 for virulent colony).

a.

b.

c.

d.

e.

Fig. 38. Avirulent colony dilution (@10^6) of *R. solanacearum* incubated as showed in- a. T1 in 0 ± 2^{0}C, b.T2 in 10 ± 2^{0}C, c.T3 in 20 ± 2^{0}C, d. T4 in 28 ± 2^{0}C ande.T5 in 40 ± 2^{0}C.

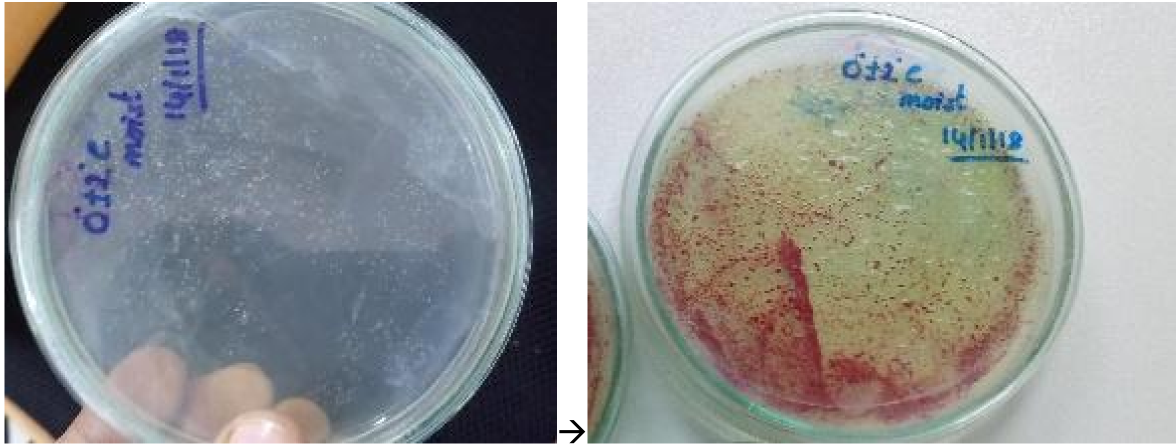


Fig. 39.T1 (culture) showed VBNC &/or latency in 0^0C and produced visible colony (both virulent and avirulent) in 28^0C (from left 1&2).

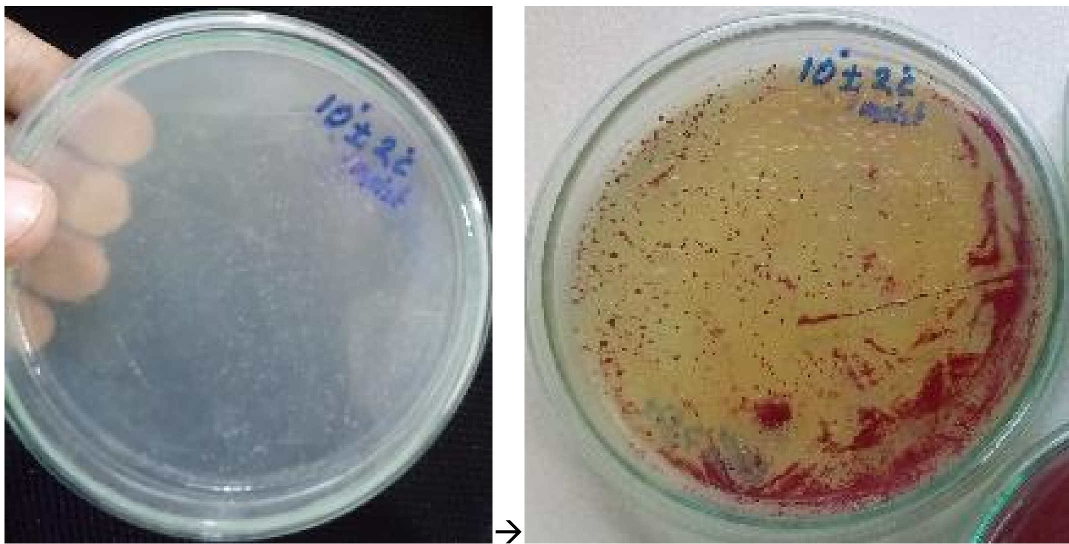


Fig. 40. T2 (culture) showed VBNC &/or latency in 10^0C and produced visible colony (both virulent and avirulent) in 28^0C (from left 1&2).

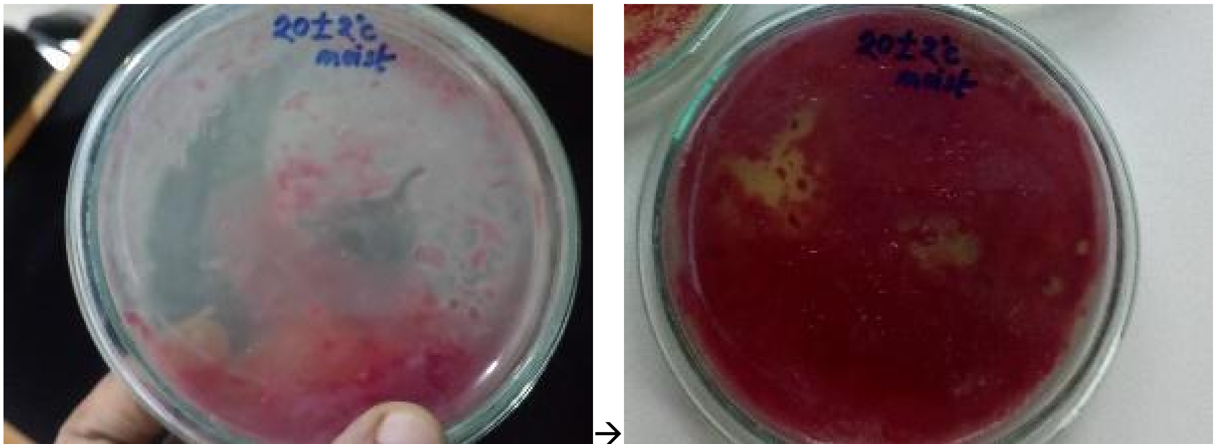


Fig. 41. T3 (culture) showed fluidal growth (both virulent and avirulent) in 20^0C and mostly turned into avirulent in 28^0C (from left 1&2).

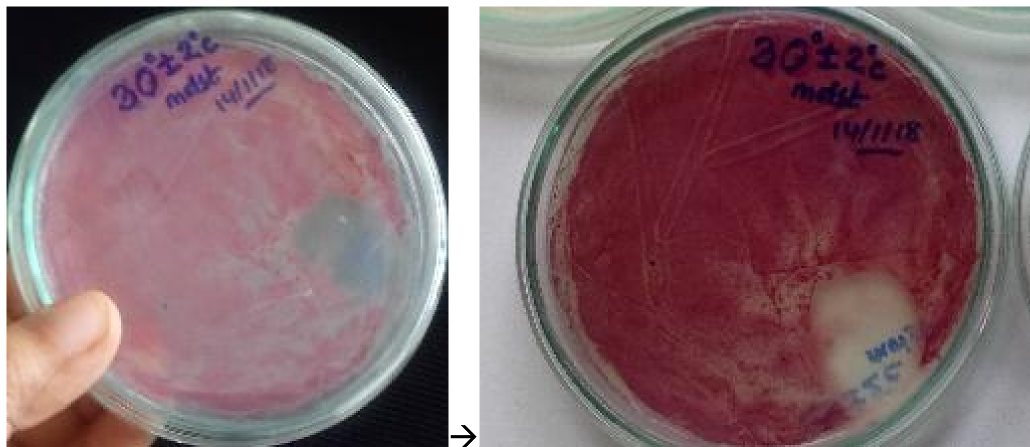


Fig. 42. T4 (culture) showed fluidal growth (both virulent and avirulent) in 30^0C and mostly turned into avirulent in 28^0C (from left 1&2).

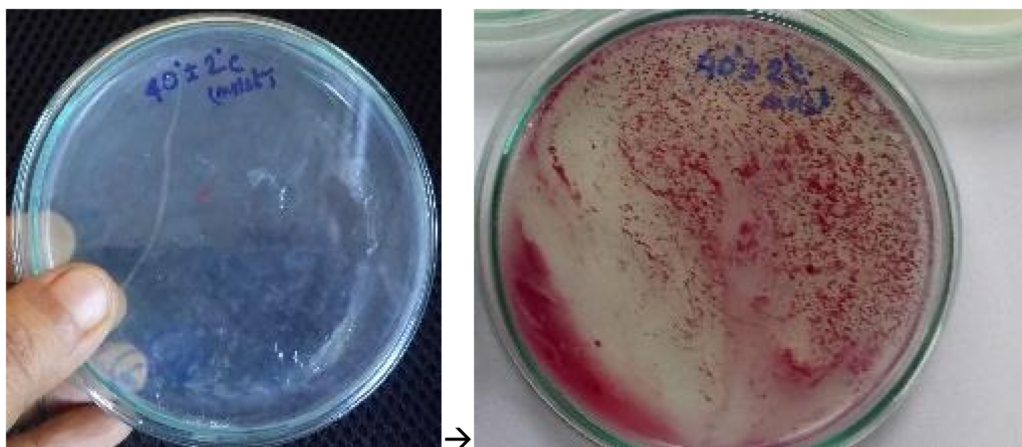
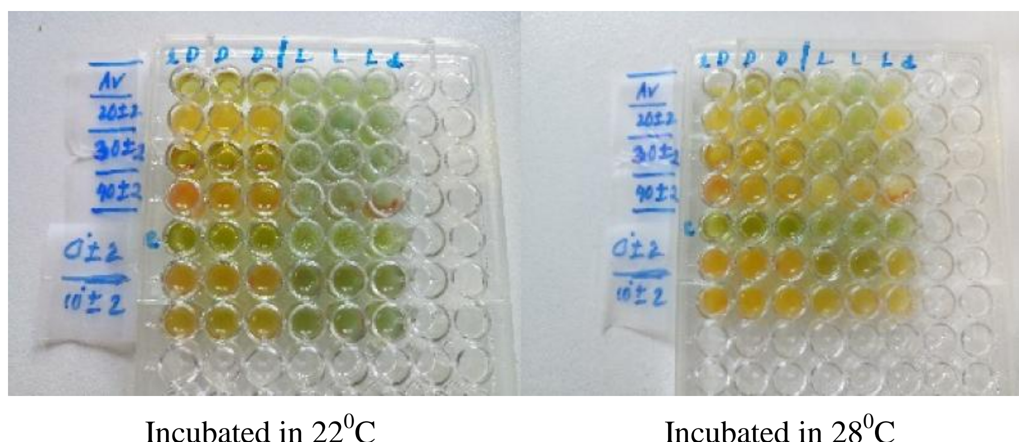


Fig. 43. T5 (culture) showed VBNC &/or latency in 40^0C and produced visible colony (both virulent and avirulent) in 28^0C (from left 1&2).

For determination of biovar expression, T1, T2, T3, T4 and T5 cultures of *R. solanacearum*(grown from avirulent colony) incubated at different temperatures showed that among the cultures all were biovar 2 except T4 when incubated in 22^{0}C whereasthe same cultures ie.T1, T2, T3, T4 and T5 expressed biovar 3 when incubated in 28^{0}C (Table 16, Fig. 44). It was also observed that biovar type reaction of same culture was changed while exposed/ incubated to different temperature (22^{0}C and 28^{0}C).

Table 16. Effect of different incubation temperature on avirulent colony cultures of *R. solanacearum* in biovar expression

	at 22^{0}C temperature						at 28^{0}C temperature						Biovar	Biovar
	L Disaccharides			D Sugar alcohol			L Disaccharides			D Sugar alcohol			at 22^{0}C	at 28^{0}C
Avirulent colony culture (av)	G	G	G	G	G	G	Y	Y	Y	Y	Y	Y	I	III
0±2^{0}C culture (T1)	Y	Y	Y	G	G	G	Y	Y	Y	Y	Y	Y	II	III
10±2^{0}C culture (T2)	Y	Y	Y	G	G	G	Y	Y	Y	Y	Y	Y	II	III
20±2^{0}C culture (T3)	Y	Y	Y	G	G	G	Y	Y	Y	Y	Y	Y	II	III
30±2^{0}C culture (T4)	G	G	G	G	G	G	Y	Y	Y	Y	Y	Y	I	III
40±2^{0}C culture (T5)	Y	Y	Y	G	G	G	Y	Y	Y	Y	Y	Y	II	III



Incubated in 22^{0}C Incubated in 28^{0}C

Fig. 44. Biovar expression reaction of avirulent colony cultures of *R. solanacearum*fromT1 as‘0±2’^{0}C, T2 as ‘10±2’^{0}C, T3 as‘20±2’^{0}C, T4 as‘28±2’^{0}C, T5 as‘40±2’^{0}C and avirulent source culture as ‘av’ (from left 1&2).

4.3. Experiment 3

Study on the management of bacterial wilt of potato caused by *Ralstonia solanacearum* by using selected bioactive compounds

4.3.1. Determination of effectiveness of selected bioactive compounds against *R. solanacearum*

4.3.1.1. *In vitro*evaluation of selected bioactive compoundsin producing inhibition zone and reducing colony virulence of against *R. solanacearum*

Table 17. *In vitro*response of the selected bioactive compounds in producing inhibition zone and reducing colony virulence of against *R. solanacearum*

***In vitro* Treatment**	**Mean (IZ) Inhibition zone**		**Mean cfu/ml(per square cm)**	**Mean cfu/ml(per square cm)**	
Dosage		**Stdv**	**Virulent**	**Avirulent**	**Stdv**
T1= Control (sterile water)	**8.48**	**0.53**	**53.10**	**4.62**	5.49
T2= Propolis solution	14.46	0.58	8.23	13.43	4.03
T3= Honey solution	16.24	0.62	37.69	9.42	17.30
T4= Turmeric oil solution	17.39	2.20	33.59	42.75	5.61
T5= Turmeric powder solution	20.19	1.64	11.72	16.86	9.58
T6= Magnesium chloride solution	15.07	0.69	29.68	9.89	13.16
T7= Rice extract solution	17.89	0.56	27.66	5.66	12.85
T8= Rice extract + Iodine	14.84	0.77	30.21	15.56	7.66
T9= Cow dung powder solution	20.69	1.10	12.21	64.12	10.30
T10= Krosin AG	17.18	0.86	47.83	22.51	7.73
T11= Honey + Iodine solution	17.73	1.25	36.55	10.31	7.97
T12= Sodium bicarbonate	13.34	0.24	39.29	4.86	11.50
T13= Stable bleaching powder	14.21	1.21	21.85	25.65	7.04
	**P value <0.01		**P value<0.01	**P value<0.01	

Evaluation of antibacterial effectiveness of some bioactive compounds viz. T1 (control), T2 (propolis solution @ 6mg/ml), T3 (honey solution @20%), T4 (turmeric oil solution (@25%), T5 (turmeric powder + oil solution (@25%), T6 (magnesium chloride solution (@3%), T7 (rice extract solution (@1000ppm ie. 1g/liter), T8 (aromatic rice extract + iodine@2 drops of iodine per 100ml of 1000ppm ie. 1g/liter), T9 (cow dung powder solution @25%), T10 (Krosin AG @ 0.5 g per liter water), T11 (honey + iodine @2 drops of iodine per 100g of 20%), T12 (sodium bicarbonate @10%) and T13 (stable bleaching powder @30 kg/ha) were performed *in vitro* to assess the antibacterial effectiveness against *R. solanaceatum* and significant (**p < 0.01) difference was observed. It was observed that the highest significant

inhibition zone was created by T9 which was followed by T5, T7 and T10 whereas significant lowest zone was found in T1 (control)(Table 17; Fig. 45, **p<0.01; Fig. 47-50).

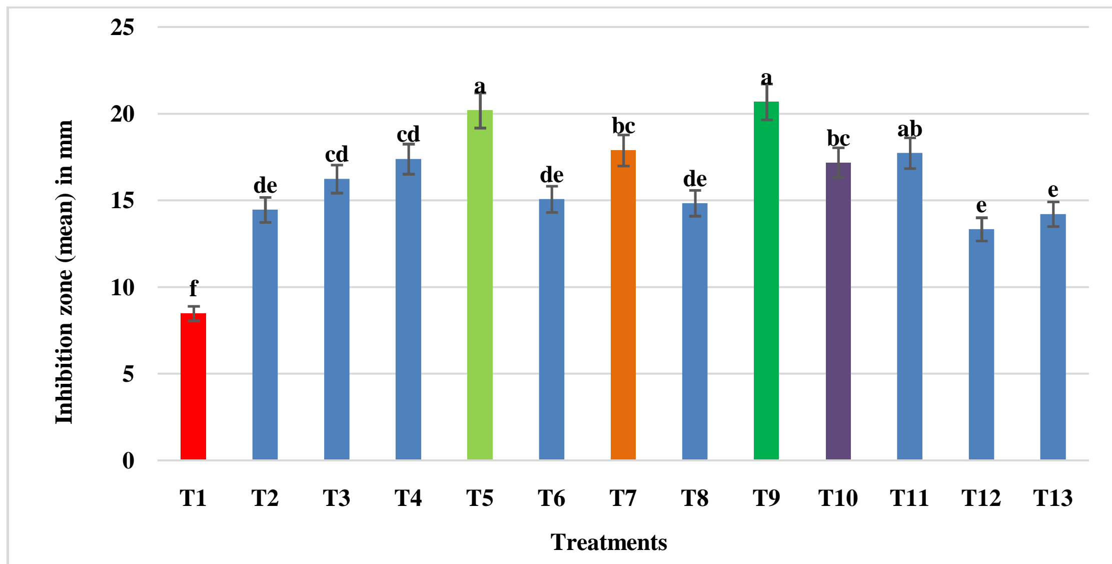


Fig. 45. Effectivenessof the selected bioactive compounds in showing inhibition zone against the bacterial wilt pathogen, *R. solanacearum* (**p < 0.01).

Performance of the selected bioactive compounds in suppressingcolony virulence were recorded by countingthe virulent and avirulent/interim colony in cfu/ml inper square cm of the petriplate (from 10^{-5} dilution stock inoculation) against the test pathogenand it was observed that the lowest significant virulent colony was produced by T2 which was followed by T5, T9 whereas significant highest was observed in T1 (control) and the rest were laid significantly inbetween during *in vitro*evaluation (Fig. 46, **p<0.01;&Fig. 47-50).

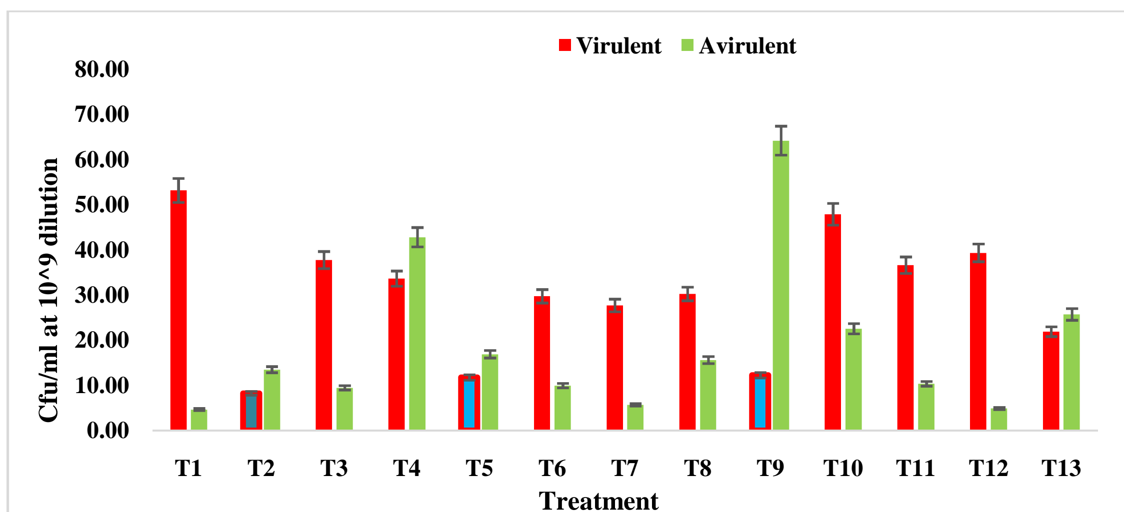


Fig. 46. Efeectiveness of the selected bioactive compounds in suppressing the virulent colony counts (*in vitro*) against *R. solanacearum* (**p<0.01)

T1

T2

T3

Fig. 47. Effectiveness of the selected bioactive compounds against the bacterial wilt pathogen, *R. solanacearum* in showing inhibition zone (mm) and avirulent colony counts (cfu/ml) in T1, T2 and T3.

T4

T5

T6

Fig. 48. Effectiveness of the selected bioactive compounds against the bacterial wilt pathogen, *R. solanacearum* in showing inhibition zone (mm) and avirulent colony counts (cfu/ml) in T4, T5 and T6.

T7

T8

T9

Fig. 49. Effectiveness of the selected bioactive compounds against the bacterial wilt pathogen,*R. solanacearum* in showing inhibition zone (mm) and avirulent colony counts (cfu/ml) in T7,T8 and T9.

T10

T11

T12

T13

Fig. 50. Effectiveness of the selected bioactive compounds against the bacterial wilt pathogen, *R. solanacearum*is showing inhibition zone (mm) and avirulent colony counts (cfu/ml) in T10, T11, T12 and T13.

4.3.1.2. *In vivo*evaluation of effectiveness of selected bioactive compounds as seed treatments

Selected bioactive compounds were used *in vivo*as seed treating agent in inoculated ptato seeds (germinating) against the bacterial wilt pathogen, *R. solanacearum*andit was observed that the lowest significant DSS (disease severity score) was produced by T9 which was followed by T2, T7 whereas significant highest of that was showed by T1 (control) which was identical to T4, T5, T6, T10, T11 and the rest were laid significantly in between during evaluation against the bacterial pathogen, *R. solanacearum (in vivo)* (Fig. 51, **p<0.01).

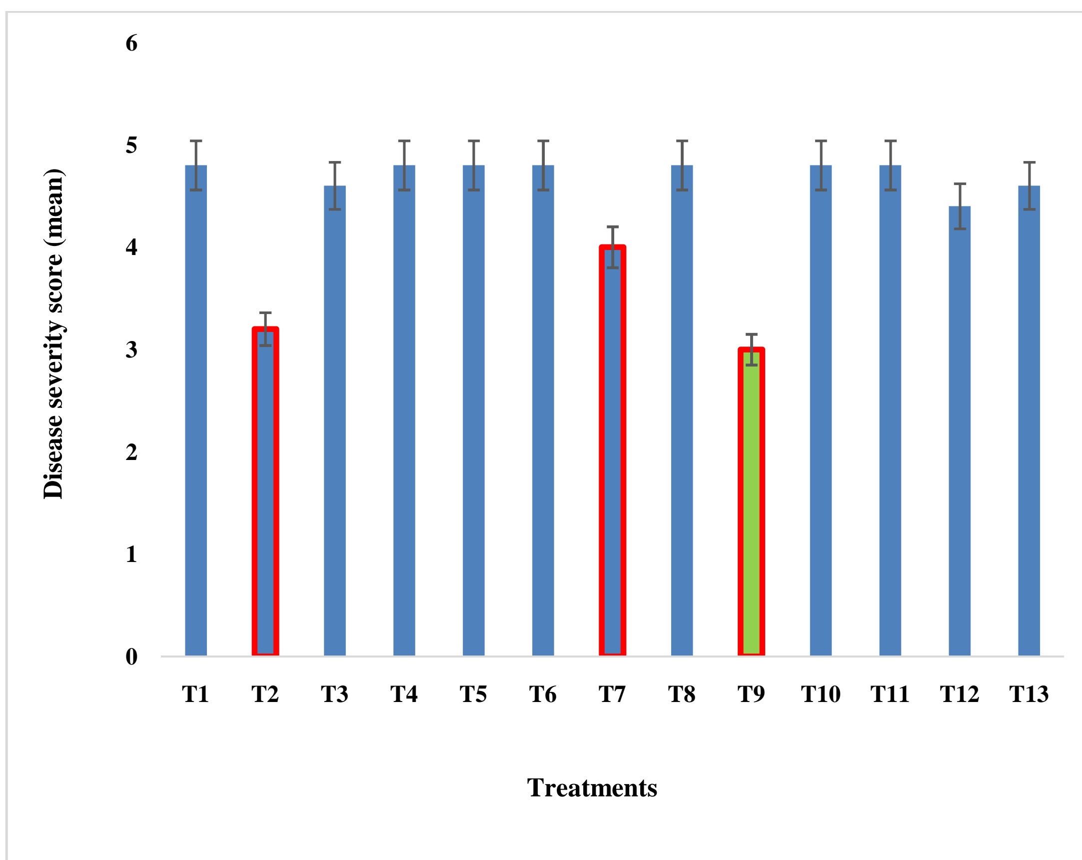
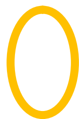


Fig. 51. Disease severity score (DSS) of the selected bioactive compounds as seed treating agent against bacterial wilt pathogen, *R. solanacearum* in germinated potato seeds (**p<0.01) (*in vivo*).

T1T2T3T4T5T6 **at 3rd week** **T7T8T9T10**

T9T10T11T12 T13 at 3rd week

Fig. 52. Germinated potato seeds 2 days after inoculation at 3rd week inevaluation of selected bioactive compounds as seed treatment against *R. solanacearum* (*in vivo*).



T1T2T3T4T5T6 **at 4th week** **T7T8T9T10T11T12**

Fig. 53. Disease severity at 4th week in selected bioactive compounds as seed treatment against bacterial wilt pathogen, *R. solanacearum* in germinated potato seeds (*in vivo*).

In the study, it was observed thatthe lowest significant PSI (percent severity index) was produced by T9 which was followed by T2, T7 whereas significant highest of that was showed by T1 (control) which was identical to T4, T5, T6, T10, T11 and the rest were laid significantly in between during evaluation against the bacterial pathogen, *R. solanacearum (in vivo)*(Fig. 54, **p<0.01).

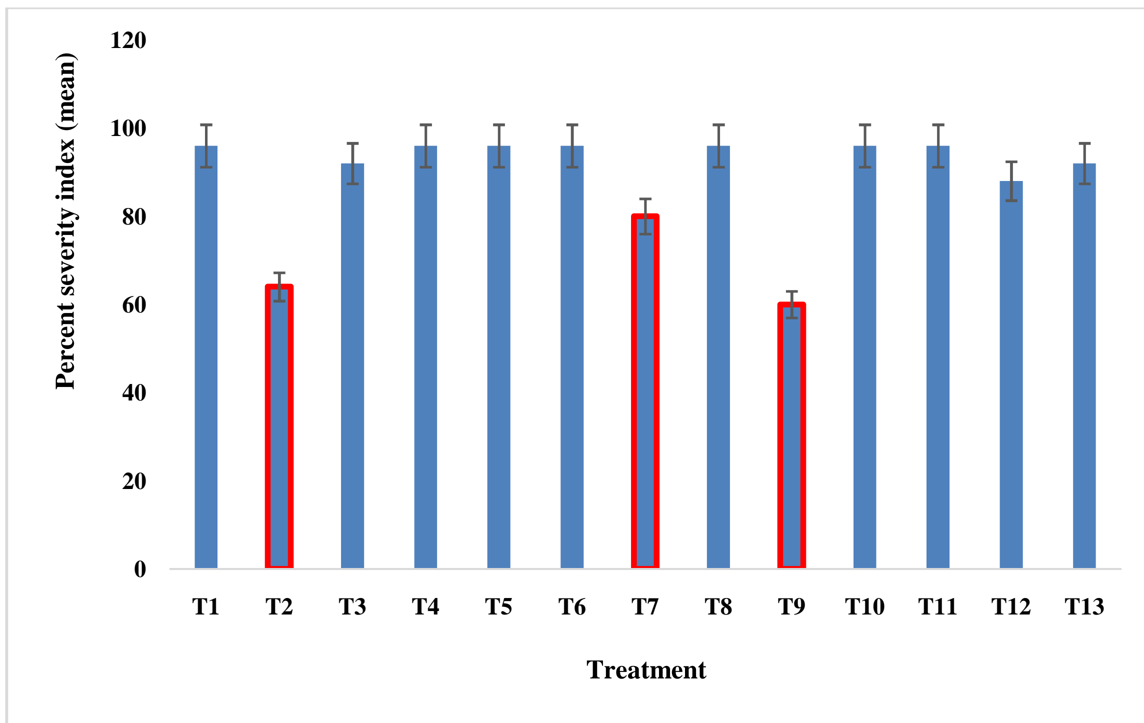


Fig. 54. Percent severity index (PSI) of the selected bioactive compounds as seed treatment (*in vivo*) against *R. solanacearum* in germinated potato seeds (**p<0.01).

The highest significant reduction in PSI (percent severity index) was produced by T9 which was followed by T2, T7 whereas significant lowest of that was showed by T1 (control) which was identical to T4, T5, T6, T10, T11 and the rest were laid significantly in between during evaluation against the bacterial pathogen, *R. solanacearum (in vivo)*(Fig. 52-53 &Fig. 55, *p<0.05).

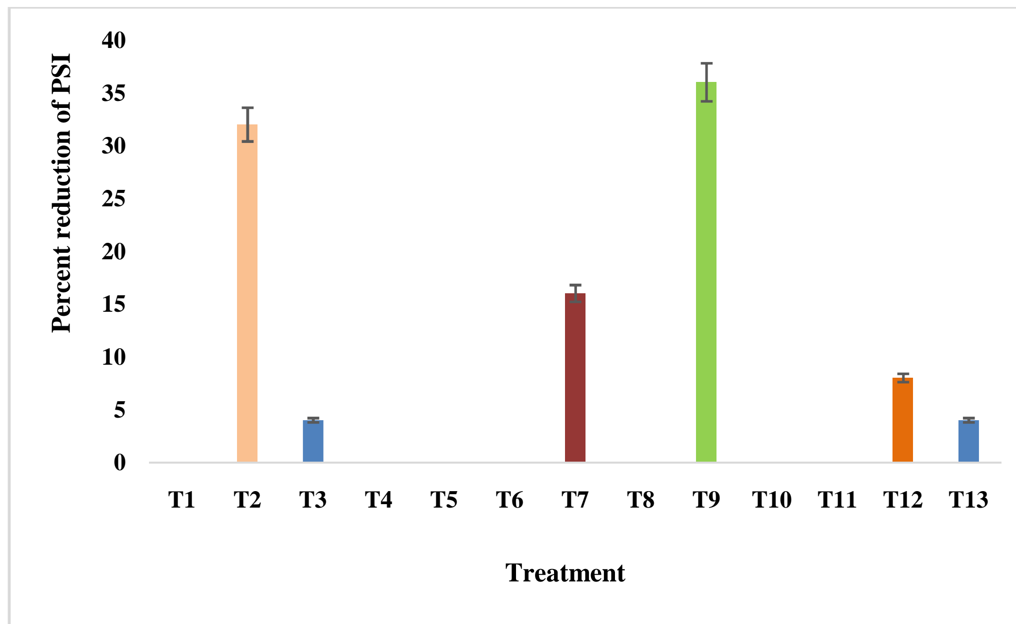


Fig. 55. Reduction of PSI (percent severity index) in selected bioactive compounds as seed treatment (*in vivo*) against *R. solanacearum*at 4th week after inoculation (*p<0.05).

4.3.1.3. *In vivo* evaluation of effectiveness of selected bioactive compounds as soil treatments treatments (in sterilized soil)

Selected bioactive compounds were evaluated in sterilized soilagainst *R. solanacearum (in vivo)*and it was observed at 5th (last) WAI (week after inoculation) that the lowest significant DSS (disease severity score) was produced by T7 which was followed by T13, T9 and T3 whereas significant highest of that was showed by T1 (control) which was identical to T2 and the rest were significantly found in between during evaluation against the pathogen, *R. solanacearum (in vivo)* (Fig. 56, **p<0.01; Fig. 58).

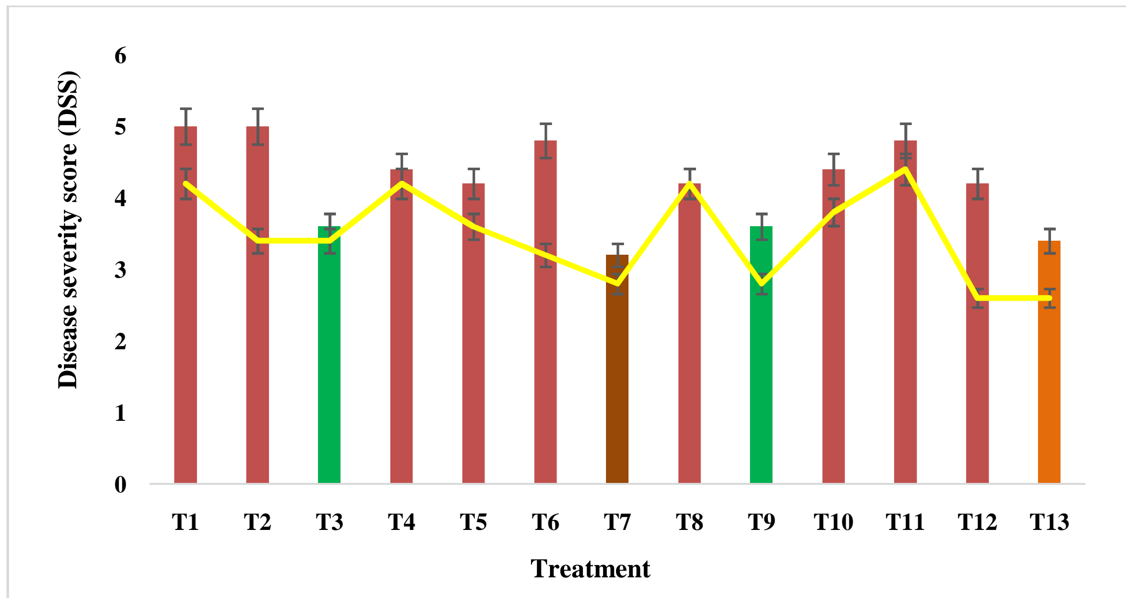


Fig. 56. Disease severity score (DSS) in potato seedlings of selected bioactive compounds as soil treatment in sterilized soil against *R. solanacearum* at 4th and 5th week (**p<0.01).

At 5th (last) WAI (week after inoculation) the lowest significant PSI (percent severity index) was observed to produce by T7 which was followed by T13, T9 and T3 whereas significant highest of that was showed by T1 (control) which was identical to T2 and the rest were significantly found in between during evaluation against the pathogen, *R. solanacearum (in vivo)* (Fig. 57, **p<0.01; Fig. 58).

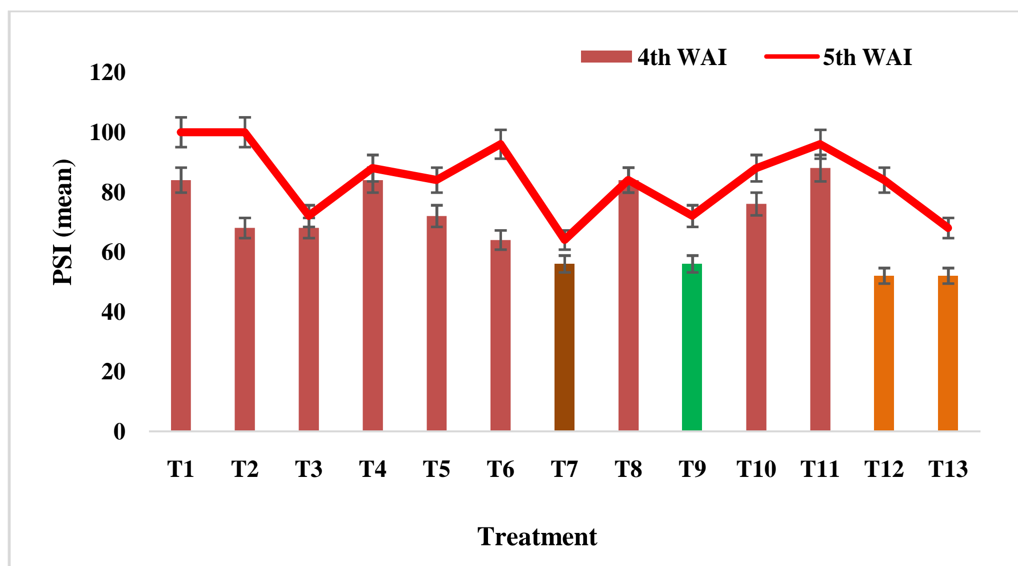
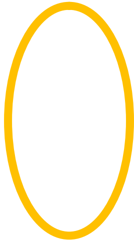


Fig. 57. Percent severity index (PSI) of selected bioactive compounds in potato seedlings as soil treatment(in sterilized soil) against*R. solanacearum* at 4th and 5th week (**$p < 0.01$).



T1, T2, T3, T4 & T5 at 4th and 5th (last) week

T5, T6, T7, T8, T9 & T10 at 4th and 5th (last) week

T9, T10, T11, T12 & T13 at 4th and 5th (last) week

Fig. 58. Disease severity showing potato seedlings at 4th and 5th week in selected bioactive compounds as soil treatment in sterilized soil against *R. solanacearum*.

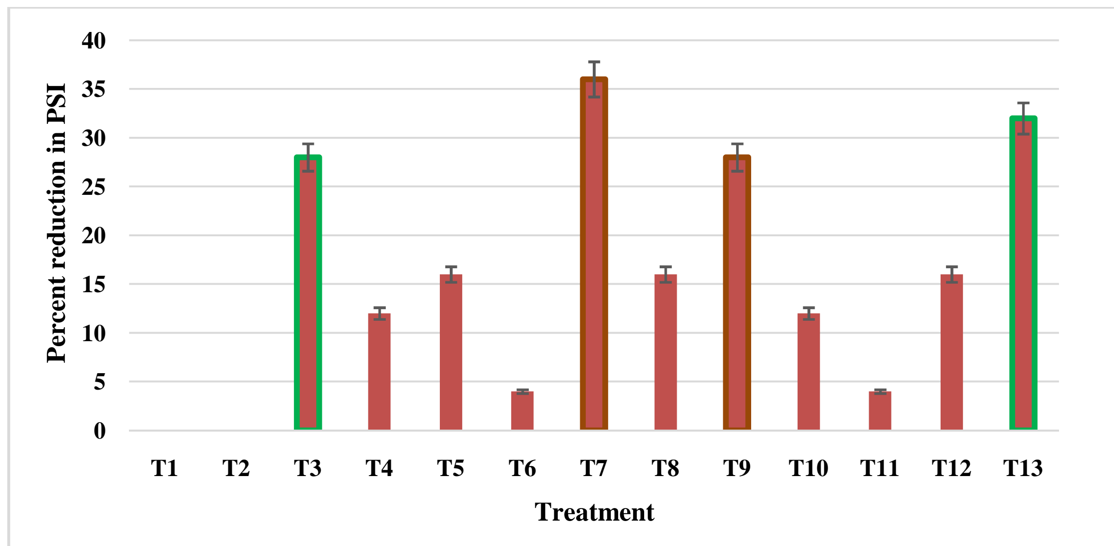


Fig. 59. Percent severity index (PSI) of selected bioactive compounds as soil treatment in potato seedlings against *R. solanacearum* at 5th week (*p<0.05).

In the study, it was also observed at 5th (last) WAI (week after inoculation) that the highest significant reduction of PSI (percent severity index) was occurred at T7 which was followed by T13, T9 and T3 whereas significant lowest of that was showed by T1 (control) which was identical to T2 and the rest were significantly found in between during evaluation against the pathogen*R. solanacearum (in vivo*(Fig. 59, *p<0.05).

4.3.1.4. *In vivo*evaluation of effectiveness of selected bioactive compounds as soil treatments (in unsterilized soil)

Selected bioactive compounds were evaluated in unsterilized soil (just solarized)to observe the performance of those compoundsin natural field soil against the wilt pathogen, *R. solanacearum*and it was found at 7th (last) week that the lowest significant DSS (disease severity score) was occurred at T9 which was followed by T2 and T5 whereas significant highest of that was showed by T1 (control) which was followed by T4, T7, T10, T11, and the rest were significantly found in between of those treatments during the evaluation in natural field soil against the pathogen, *R. solanacearum*(Fig. 60, **p<0.01).

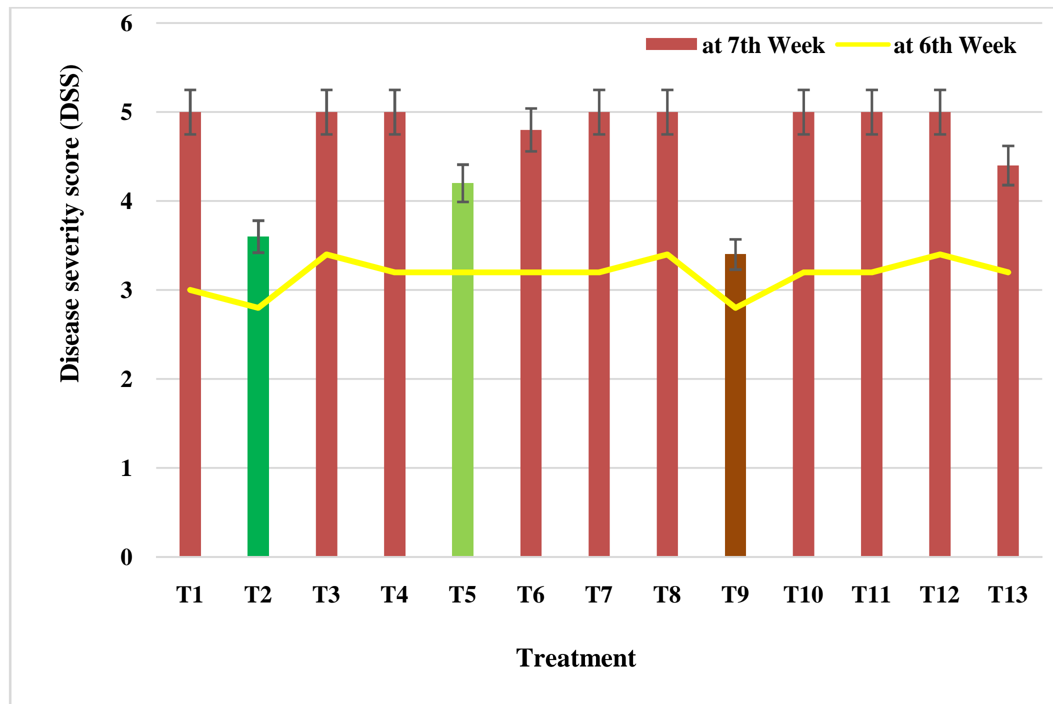


Fig. 60. Disease severity score (DSS) of potato plants in selected bioactive compounds in unsterilized (solarized) soil at 6th and 7th week against bacterial wilt pathogen, (*R. solanacearum)* (**p<0.01).

In 7th (last) week the lowest significant DSS (disease severity score) grwoth was occurred at T9 which was followed by T2 and T5 whereas significant highest of that was showed by T1 (control) which was followed by T4, T7, T10, T11, and the rest were significantly laid in between of those during the evaluation in natural field soil against the pathogen, *R. solanacearum*. So, it was observed in the study that T2, T5 and T9 showed most promising response in natural soil condition against the wilt pathogen, *R. solanacearum*. Therefore, it was selected for further evaluation in natural field soil condition as compared to farmer's practice (stable bleaching powder) and control to figure out the performance as seed treating and soil treating agents (Fig. 61, **p<0.01).

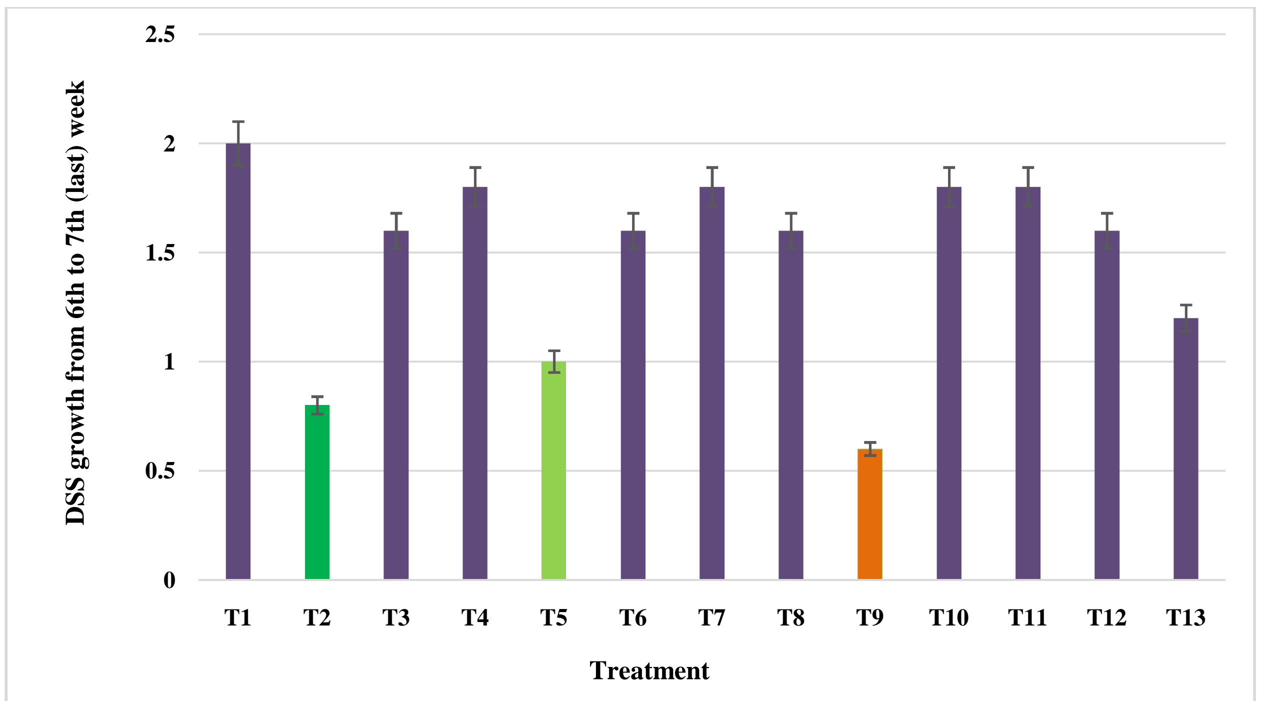


Fig. 61. Effectiveness of selected bioactive compounds in reducing DSS growth in unsterilized (solarized) soil against bacterial wilt (*R. solanacearum*) (**p<0.01).

6th week **7th week**

Fig. 62. Effectiveness of some selected bioactive compounds in potato plants in reducing DSS growth in unsterilized (solarized) soil against bacterial wilt (*R. solanacearum*).

4.3.2. Evaluation of effectiveness of propolis, turmeric powder and cowdung as both soil and seed treatment

As best performing treatments in most of the evaluation, propolis, turmeric powder and cowdung were evaluatedas both seed and soil treatment to figure out the performance against *R. solanacearum* in potato.It was observed at 7th (last) WAI (week after inoculation) that the lowest significant DSS (disease severity score) was occurred at T4 (3.44 out of 5.00 in cowdung @ 25%) which was followed by T2 (3.56 out of 5.00 in propolis @ 6mg/ml) whereas significant highest of that was showed by T3 (4.44 out of 5.00 in stable bleaching powder @ 1.15%) which was followed by T1 (4.22 out of 5.00 in control). T5 (3.89 out of 5.00 in turmeric powder @ 25%) was significantly laid in between during the evaluation in natural field soil against the pathogen, *R. solanacearum* (Fig. 63, **$p < 0.01$).

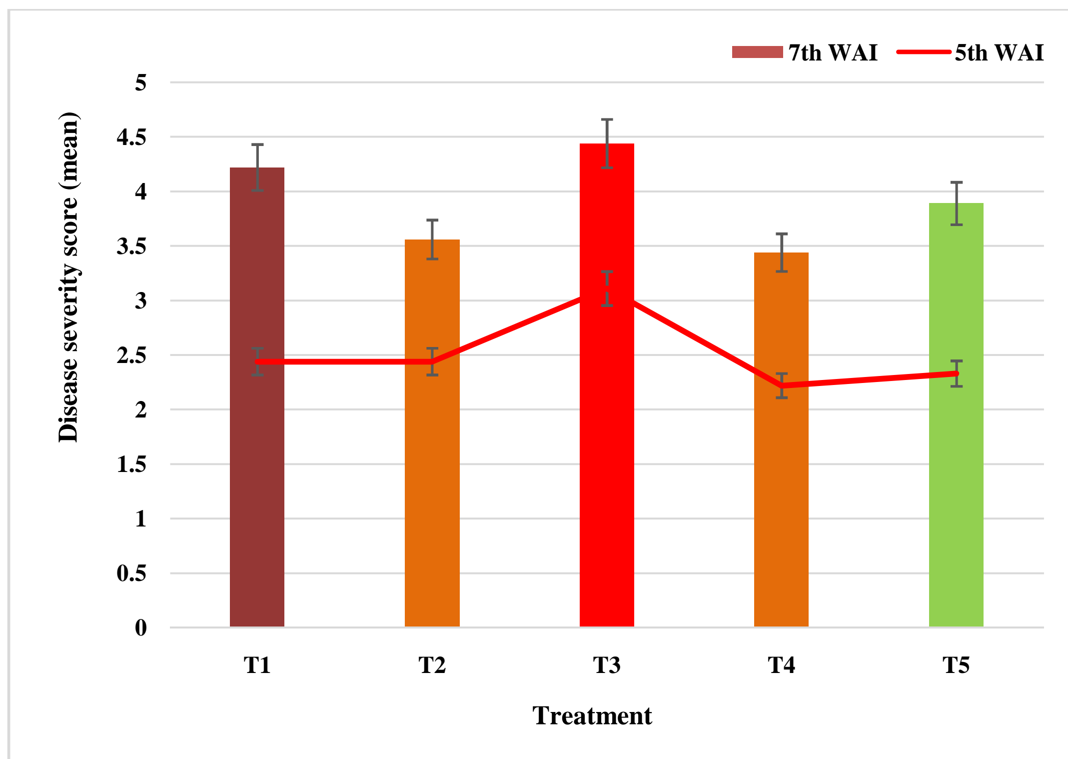


Fig. 63. Disease severity score (DSS) of propolis, turmeric powder and cowdungas both seed and soil treatment against bacterial wilt pathogen (*R. solanacearum*) in 5th and 7th week after inoculation (WAI) (**$p < 0.01$).

It was observed at 7th (last) WAI (week after inoculation) that the lowest significant PSI (percent severity index) was occurred at T4 (60.00% in cowdung @ 25%) which was followed by T2 (62.22% in propolis @ 6mg/ml) whereas significant highest of that was showed by T3 (88.89% in stable bleaching powder @ 1.15%) which was followed by T1 (84.44%in control). T5 (66.67% in turmeric powder @ 25%) was significantly laid in between during the evaluation in natural field soil against the pathogen, *R. solanacearum* (Fig. 64, **p<0.01).

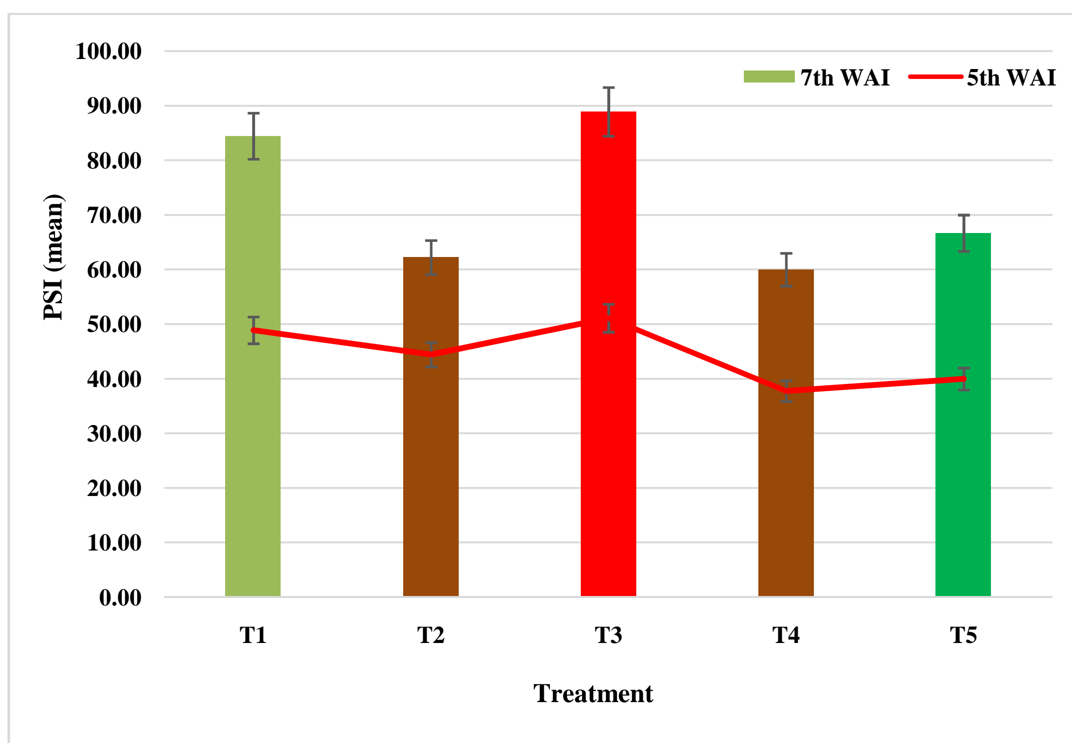


Fig. 64. Percent severity index (PSI) of propolis, turmeric powder and cowdungas both seed and soil treatment against bacterial wilt pathogen (*R. solanacearum*) in 5th and 7th week after inoculation (WAI) (**p<0.01).

It was also found at 7th (last) WAI (week after inoculation) that the highest significant reduction in PSI (percent severity index) over the lowest T3 (stable bleaching powder @ 1.15%) occurred at T4 (28.89% in cowdung @ 25%) which was followed by T2 (26.67% in propolis @ 6mg/ml) whereas significant lowest of that was observed in T1 (84.44%in control). T5 (22.22% in turmeric powder @ 25%) was significantly laid in between during the evaluation against wilt causing *R. solanacearum* (Fig. 65; *p<0.05).

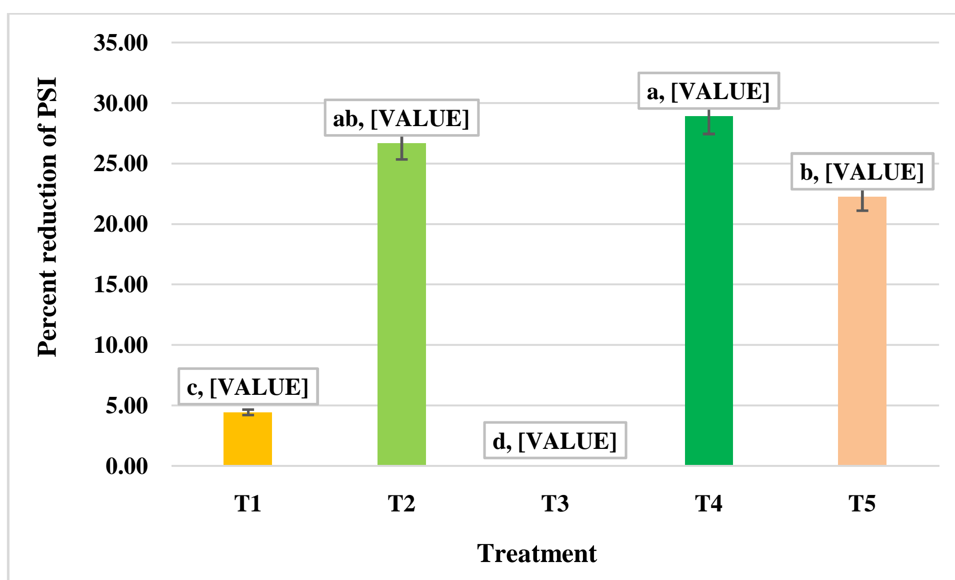


Fig. 65. Effectiveness of propolis, turmeric powder and cowdungas both seed and soil treatment in reducing PSI (percent severity index) of bacterial wilt pathogen (*R. solanacearum*) in 7th week after inoculation (WAI) (*p<0.05, lsd value=4.22).

4.3.2.1. Determination of virulent and avirulent colony count of propolis, turmeric powder and cowdung treated soil

As treatments showed positive effects in reducing the wilt severity index, it was to understand whether there is any effect in soil colonization by the pathogen. Thus, virulent and avirulent colony counts (cfu/ml) were measured at 7th (last) WAI (week after inoculation) in dilution plates (@ 10^7 dilution)from propolis, turmeric powder and cowdung treatment andit was found that the significant lowest virulent colony count was found in T4 (26.48 cfu/ ml in cowdung @ 25%) which was followed by T2 (29.44 cfu/ ml in propolis @ 6mg/ml) with comperatively dry colonies and T5 (33.54 cfu/ ml in turmeric powder @ 25%) whereas the lowest was found in case of T3 (65.71 cfu/ ml in stable bleaching powder @ 1.15%) which was followed by T1 (55.10 cfu/ ml in control) (Fig. 44.a, & b). On the other hand, the significant highest avirulent colony count was found in T5 (52.46 cfu/ ml in turmeric powder @ 25%) which was followed by T4 (49.18 cfu/ ml in cowdung @ 25%) and T2 (31.89 cfu/ ml in propolis @ 6mg/ml) and whereas the lowest was found in case of T3 (8.96 cfu/ ml in stable bleaching powder @ 1.15%) which was followed by T1 (39.90 cfu/ ml in control) (Fig. 66, **p<0.01; Fig.67, 68).

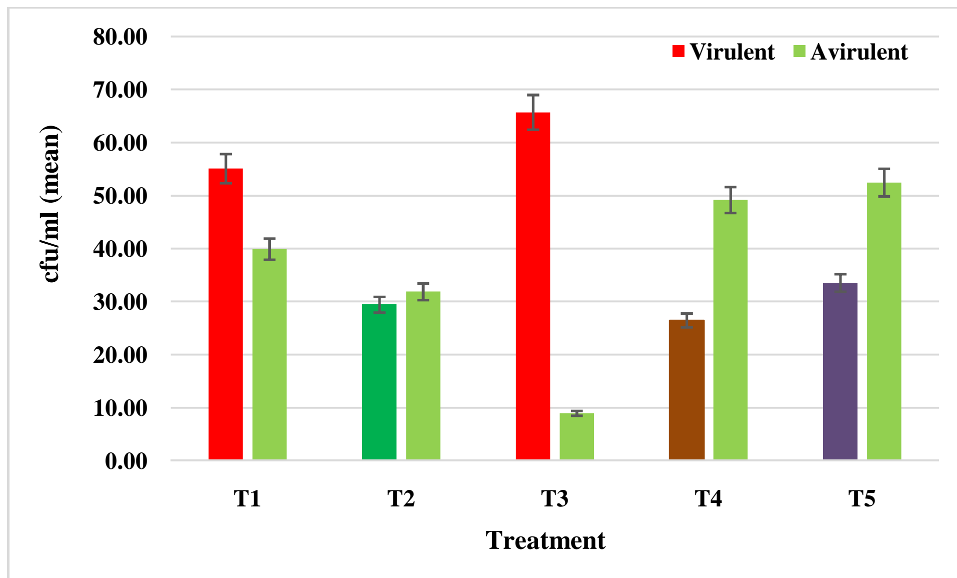


Fig. 66. Virulence expression of *R. solanacearum* in colony count plates from soil dilution (@ 10^7 cfu/ml)inpropolis, cowdung and turmeric powder treatment in 7th week (**p<0.01).

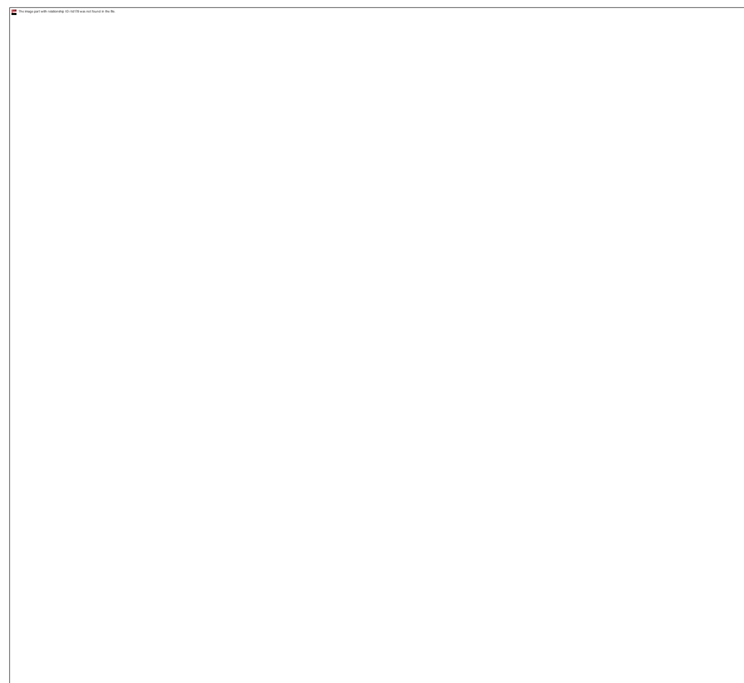


Fig. 67. Virulent and/or avirulent colony expression of *R. solanacearum* in 10^5 level soil dilution of propolis, cowdung and turmeric powder treatment in 7th week.

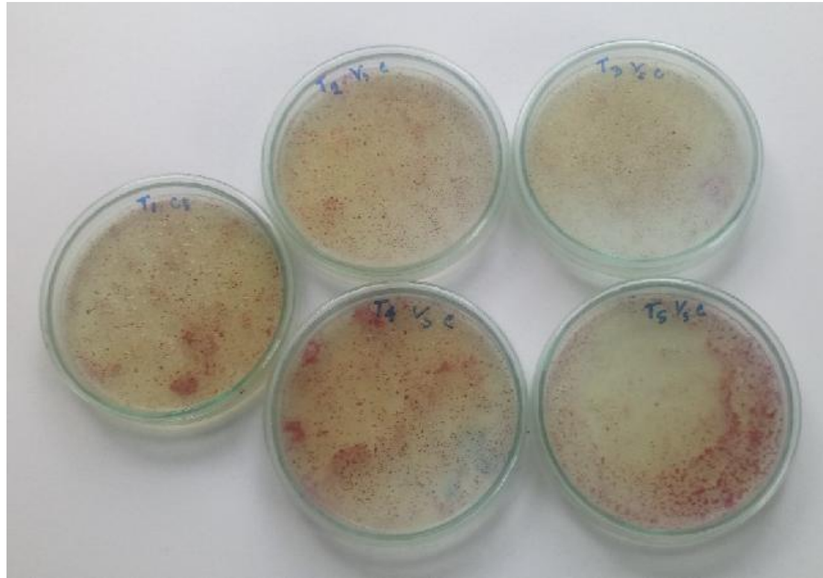


Fig. 68. Virulent and/or avirulent colony expression of *R. solanacearum* in 10^7 level soil dilution of propolis, cowdung and turmeric powder treatment in 7th week.

In the study,the highest significant reduction of percent virulent colony over lowest (T3 in stable bleaching powder @ 1.15%) was found in T4 (39.22 cfu/ ml in cowdung @ 25%) which was followed by T2 (36.27 cfu/ ml in propolis @ 6mg/ml) and T5 (32.17 cfu/ ml in turmeric powder @ 25%) whereas the lowest was found in case of T1 (10.61 cfu/ ml in stable bleaching powder @ 1.15%) (Fig. 69, *p<0.05; Fig. 70-78).

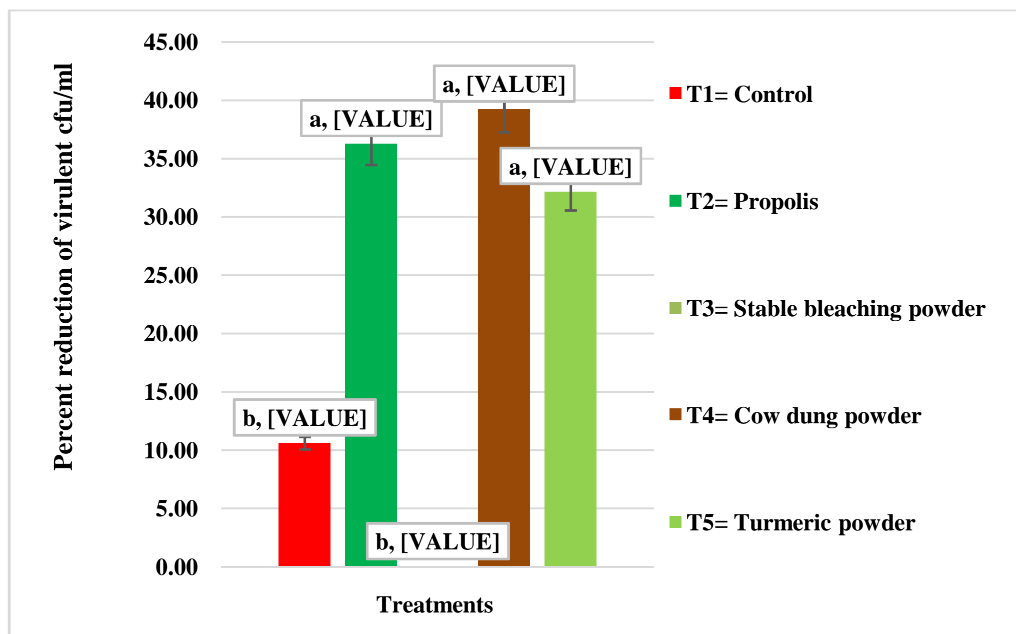


Fig. 69. Percent reduction of virulent count (cfu/ml) of *R. solanacearum* in soil dilution of propolis, cowdung and turmeric powder treatment in 7th week (*p<0.05, lsd value=19.30).

T5-turmeric powder T4-cowdung T3-bleaching powder T2-propolis T1-control

Fig. 70. Disease expression of soil and seed treated potato plants in propolis, cowdung and turmeric powder treatment against*R. solanacearum*at4thweek.

T5-turmeric powder T4-cowdung T3-bleaching powder T2-propolis T1-control

Fig. 71. Disease expression of soil and seed treated potato plants in propolis, cowdung and turmeric powder treatment against*R. solanacearum*at5thweek.

T5-turmeric powder T4-cowdung T3-bleach powder T2-propolis T1-control

Fig. 72. Disease expression of soil and seed treated potato plants in propolis, cowdung and turmeric powder treatment against*R. solanacearum*at 7thweek.

Fig. 74. Disease expression of soil and seed treated potato plants in propolis, cowdung and turmeric powder treatment against*R. solanacearum* at8th week.

Fig. 75. Wilt disease expression (epinasty/ downward curvature) of potato plants caused by *R. solanacearum* in propolis, cowdung and turmeric powder treatment pots at9thweekafter inoculation (WAI).

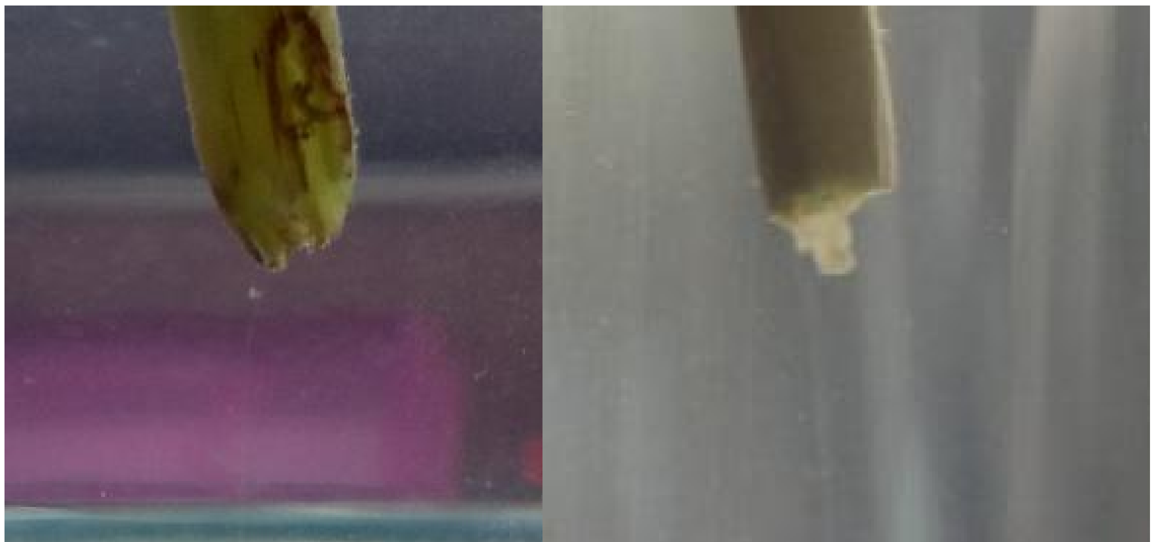


Fig. 76. Potato plant oozing from epinasty (downward curvature) showing potato plants caused by *R. solanacearum*in propolis, cowdung and turmeric powder treatment at9th weekafter inoculation (WAI).

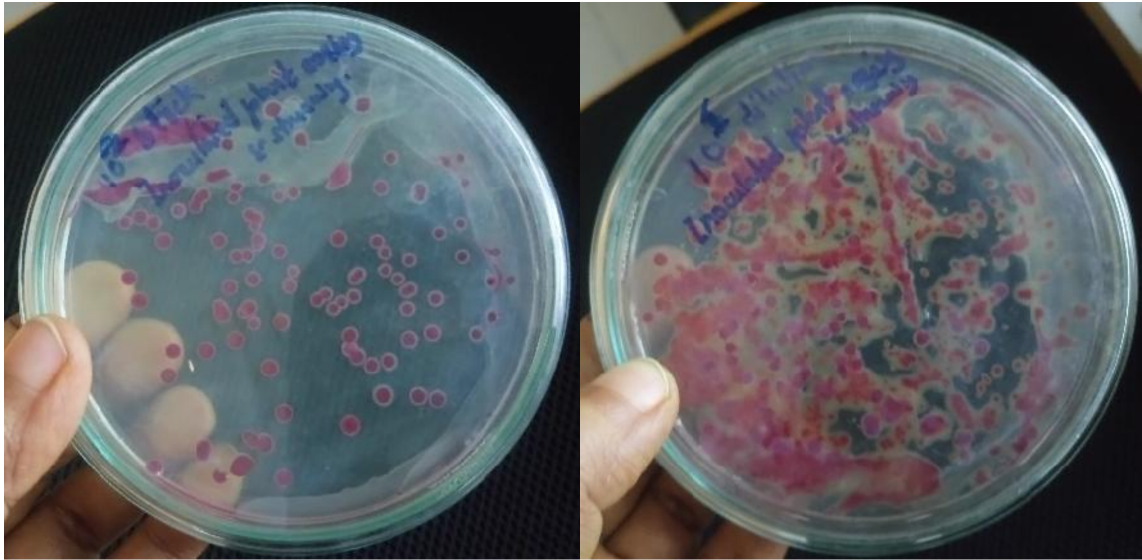


Fig. 77.*R. solanacearum*colonies at 10^6 dilution on TZC solid media from potato plants oozing in propolis, cowdung and turmeric powder treatmentat9th week after inoculation.

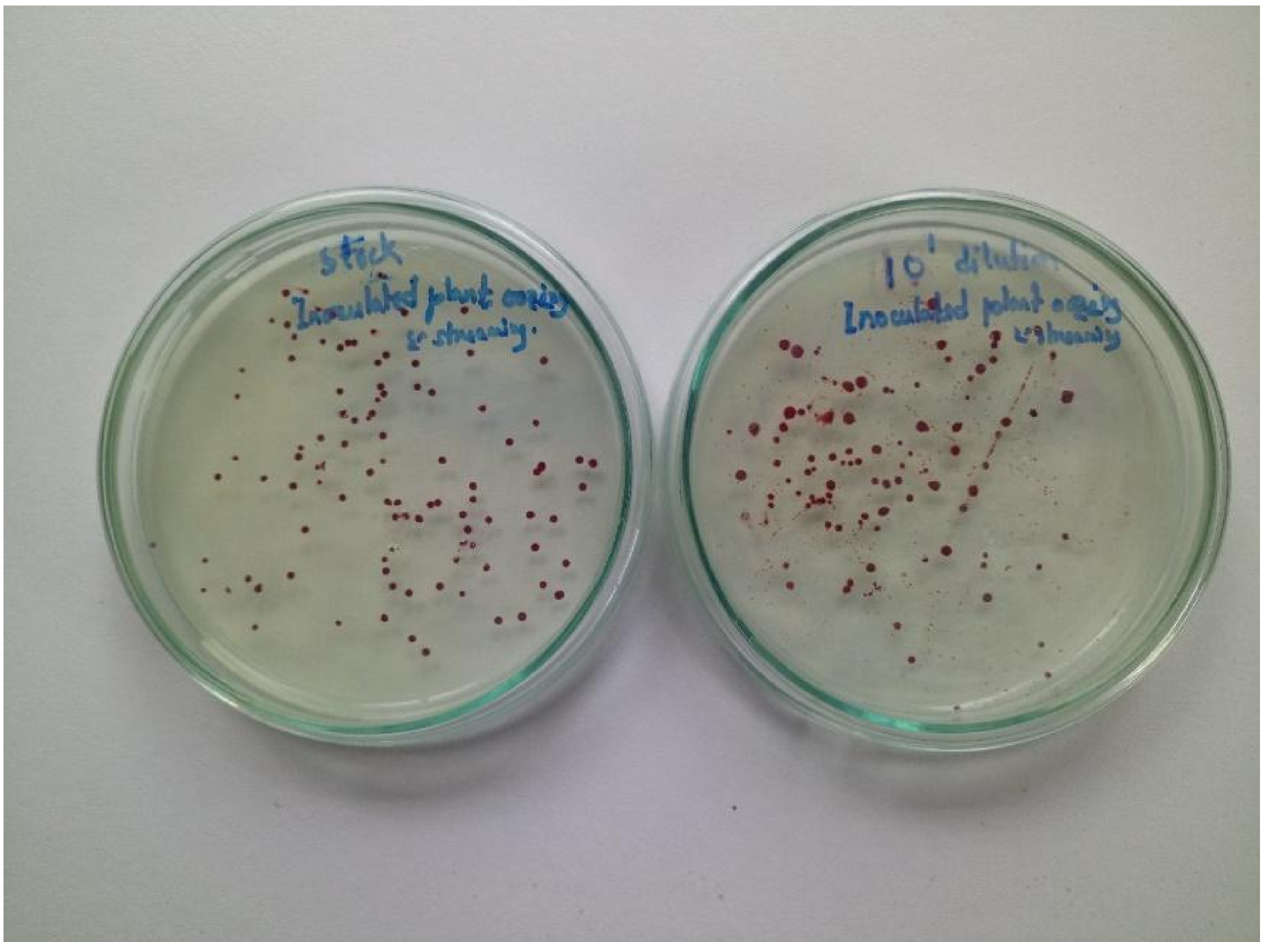


Fig. 78. *R. solanacearum*colonies at 10^8 dilution on TZC solid media from potato plants oozing in propolis, cowdung and turmeric powder treatmentat9th week after inoculation.

Chapter V

Discussion

Infected samples from different sources of inoculum viz. plant, soil, seed, weed, other crops, irrigation water etc. were collected from major potato growing districts of Bangladesh viz. Munshiganj, Chandpur, Tangail, Narayanganj, Jamalpur, Domar, Patuakhali, Rangpur, Bogra, Shariatpur, Meherpur, Joypurhat and Dinajpur to investigate the presence of *R. solanacearum*. The bacterial wilt disease has been described and the causal agent isolated from plants all around the world (EPPO, 2004; Elphinstone, 2005; Champoiseau *et al.,* 2009; Sullivan *et al*., 2013; Kumar *et al*., 2017), tuber (EPPO, 2004; Elphinstone, 2005; Kabeil *et al.,* 2008; Ghosh and Mandal, 2009), soil (Sequeira and Averre, 1961;McCarter*et al*., 1969;Elsas *et al*., 2000; Yuliar *et al*., 2015; Kumar *et al*., 2017), asymptomatic weeds (Sunaina *et al*., 1989;Shekhawat *et al*.,1992; Prior *et al*., 1998; Swanson *et al*., 2005), other crops (chilli) (Kelman, 1998; Sullivan *et al*., 2013) and water (Milling *et al*., 2009; Swanson *et al*., 2007; Marco-Noales *et al*., 2008; Sullivan *et al*., 2013) and found in associated micro environment by many researchers around the world (Kelman, 1998; Yabuuchi *et al*., 1995; Champoiseau *et al.,* 2009; CABI, 2017). Cross cut of plant samples showed bacterial ooze streaming in clear water that corroborates with the result of Allen *et al.,* 2001; Champoiseau *et al.,* 2009 and Kumar *et al*., 2017. This presumptive test, namely streaming test, after collection of the freshly wilted plant was also suggested by Danks and Barker, 2000. In potato seeds no oozing was found but browning of the vascular bundle region of seed tuber were observed. Allen *et al.,* 2001 and Champoiseau *et al*., 2009 also described grey-brown discoloration of vascular tissues, commonly called a vascular ring and/or bacterial ooze in infected potato tubers. Standard methods viz. Kelman's tetrazolium chloride (TZC) medium (semi-selective) for isolation (Elphinstone *et al*.,1996), race test (Dhital*et al*., 2001;EPPO, 2004), biovar test (Hayward, 1964; Hayward, 1994b; Schaad*et al*.,2001; Floyd, 2008;IPDN, 2014), different biochemical tests (Janse, 1953; Hayward, 1964 and Janse, 1996; *Goszczynska et al., 2000*), phylotype test by phylotype specific PCR amplification (Fegan and Prior, 2005; Prior and Fegan, 2005), were used for detection and identification for *R. solanacearum* including latent infection. Champoiseau *et al*., 2009 recommended a combination of at least two different complementary tests to identify the species unambiguously. Fegan and Prior, 2005 stated *R. solanacearum*as a "species complex," due to significant variation within the groups in

subspecific classification. A total of 133 samples were used for isolation on TZC solid medium out of which 94% (ie. 125) found positive for *R. solanacearum* presence. Among the isolates, thirty nine isolates were tested for race, biovar and phylotype study based on a preliminary hypersensitive reaction test on *Euphorbia* leaves suggested by Shahbaz *et al*., 2015. Race and biovar of the test pathogen were determined following standard procedure described by EPPO (2004) andKumar *et al*.,(2017); and *Goszczynska et al., (2000)and*IPDN (2014) respectively. It was observed that all thirty nine tested isolates expressed as race 3 while in bovar test thirty seven showed as biovar III except two showed biovar I. EPPO(2004)clearly showed that race 1 hosts all solanaceous and a wide range of plants (solanaceous and nonsolanaceous weeds, diploid bananas, groundnut, olive, ginger, strawberry, geranium, Eucalyptus, some other plants) which were available in Asia, Australia and America while race 3 infects only potato and tomato worldwide. This result also supported by Ahmed *et al*., (2013) and Nishat *et al*., (2015) who found Bangladeshi *R. solanacearum* isolates of potato belonging to race 3 biovar III. They also observed that, biovar III oxidizes both disaccharide sugar and hexose alcohols, biovar II oxidizes only hexose alcohols whereas Biovar I oxidizes none of disaccharide sugar and hexose alcohols, and biovar IV oxidizes only disaccharide sugar which was parallel to IPDN (2014). The study of EPPO (2004) also supported the result found in race study with 39 isolates of *R. solanacearum.* However, in the present study Race 2, Race 3 and Race 5 were not detected since race 2 is restricted to triploid banana and Heliconia; race 4 infects ginger; and race 5 is pathogenic on mulberry (Buddenhagen *et al.,* 1962; Aragaki and Quinon, 1965; He *et al.,* 1983; Begum, 2005; Rahman *et al.,* 2010; Chandrashekara *et al.,* 2012, Popoola *et al.,* 2015). Five races are also different in geographical distribution and ability to survive under different environmental conditions (French, 1986). *R. solanacearum* has beengrouped into four biovars on the basis of utilizing and/or oxidizing three hexoses (mannitol, dulcitol and sorbitol) and three disaccharides (lactose, maltose and cellobiose) (Hayward, 1954; He *et al.,* 1983; Begum, 2005; Rahman *et al.,* 2010; Chandrashekara *et al.,* 2012, Popoola *et al.,* 2015). In phylotype detection test through phytotype specific PCR amplicaiton and all exhibited to originate as phylotype I (Asiatic type). The phytotype detection was conducted following Sagar *et al*., (2014) and it stated that, phylotype I shows Asian origin, the phylotype II shows American origin; the phylotype III shows African origin and the phylotype IV shows to be Indonesian origin. However, phylotype I represent that no race 3 biovar II detected in any of the thirty nine isolates of *R. solanacearum* from different potato growing regions of Bangladesh. The

study was also conducted for detection *R. solanacearum* by many researchers around the world (Fegan *et al*., 1998; Fegan and Prior, 2005; Prior and Fegan, 2005; Kumar *et al*, 2017).

In the heterogeneity determination study phenotypic conversion and or VBNC of different isolates *R. solanacearum* in aerated and non-aerated water medium were observed. Nine plant samples showed phenotypic conversion (PC) from avirulent to virulent state due to shift from non-aerated to aerated sterile water growth medium. However, 3 strains from a total of 15 soil samples, 8 strains from a total of 10 seed samples and all of the 3 weed, 1 other crop (chilli) and 1 water samples showed the VBNC (viable but non culturable) state along with phenotypic conversion (PC) from avirulent to virulent state due to the shift of the medium. This observed phenomenon of phenotypic conversion is also in accordance to the findings of Alvarez *et al*., (2010). Alvarez *et al*., (2010), Kelman (1954), Buddenhagen and Kelman (1964), Brumbley and Denny(1990), Denny *et al*., (1994) and Poussier*et al*., (2003) stated a phenomenon that was termed as "phenotypic conversion" (PC) which was explained as a spontaneously shifting of *R. solanacearum* colonies from fluidal to afluidal morphology. Brumbley and Denny (1990) and Alvarez *et al*., (2010) found it to occure in in most *R. solanacearum*. Kelman(1954), Buddenhagen and Kelman (1964), and Kelman and Hruschka (1973) found it easily to occur in prolonged culture on agar plates when the organism is grown in a non-aerated liquid medium with glucose. Shekhawat and Perombelon (1991) described the longevity of *R. solanacearum*to be affected by the oxygen status of growing media in which anaerobic condition favoured a shift from virulence to avirulence state of the pathogen. Thus, findings of the study related to moisture condition (aerated and non-aerated) of growing media to contribute on virulent/avirulent colony and hence this expression is agreed with the findings of many others.

In virulence test on potato seedlings by the same isolates at 10^9 dilution, all isolates produced symptoms of the disease and showed a range of heterogeneity in DSS (disease severity score) which ranged from 2.6 to 5 within 2-7 days after inoculation. It directed that, colony count (cfu/ml) on solid media was influenced and shifted from pure culture to phenotypically converted culture which were grown in glucose water media and played a role in terms of moisture influence. Virulent colony type and pathogenicity was studied by Liu *et al*., (2004), Zheng *et al.,*(2014), Kumar *et al*., (2017), Liu *et al*., (2004) and Zheng *et al.,*(2014) showed the three significant pathogenic types of *R. solanacearum* strains based on the colony morphology on TTC medium- (a) virulent type, in which the colony shape was

irregular, highly mobile and fluidal, and displayed a pink spot in the middle of the colony and a large white edge (EPS), (b) interim type, in which the colony was immobile, humid at the surface, and displayed a dark red spot in the middle of the colony and a narrow white edge (EPS) and (c) avirulent type, in which the colony was round, immobile, dry and displayed a dark red spot in the middle of the colony and a narrow or no white edge (EPS). Parallel study was also explained by Kumar *et al*., (2017) in which it was clearly differentiated the colony plates containing virulent and avirulent colonies. Buddenhagen and Kelman(1964), Devi *et al*., (1982), Brumbley and Denny (1990), Shekhawat and Perombelon(1991), Hayward (1994b), Morita *et al*., (1997), Elsas *et al*., (2001) and Álvarez *et al*., (2010) described that, morphologically fluidal and afluidal *R. solanacearum* colonies can be typically observed on agar plates which exists as a strategy of survival of the in energy-deficient system due to unfavorable environment, such as exposure to stress condition, desiccation, anaerobiosis etc. (Buddenhagen and Kelman, 1964; Devi *et al*., 1982; Brumbley and Denny, 1990; Shekhawat and Perombelon, 1991; Hayward, 1994b; Morita *et al*., 1997; Elsas *et al*., 2001; Álvarez *et al*., 2010). Alvarez *et al*., (2010) also explained how phenotypic conversion is related to glucose in the growing medium along with Kelman (1954), Buddenhagen and Kelman (1964) and Kelman and Hruschka (1973) which was described earlier. Thus, findings of this part of the study was parallel to findings of others. In case of virulence expression, disease scoring (DSS) in potato seedlings showed by *R. solanacearum* isolates was ranged from 2.6 to 5 DSS in potato seedlings and it was similar to the study of Lemessa and Zeller (2007). They also observed the disease severity in potato and tomato of 62 isolates of the pathogen ranged from 2.6 to 5 but did not showed isolate wise. However, it was similar to the findings ofLemessa and Zeller (2007).

The influence of soil moisture in disease expression on potato plants by *R. solanacearum* were studied precisely. Potato plant grown by applying different level of irrigation in same inoculated soil and it was observed that all disease parameters viz.- disease severity score (DSS), PSI (percent severity index) and virulent colony count (cfu/ml) were highest and avirulent &/or interim colony count (cfu/ml) was lowest in T3 (moisture level <3 as irrigation @ 1 per 10days) as compared to T2 (moisture level 3~5.5 as irrigation @ 1 per 7days) and T1 (moisture level >5.5 as irrigation @ 2 per 7days). So, the best disease reduction in all cases was showed by T1. It specified that, moisture level changed by different level of irrigation application influenced the colony virulence level of *R. solanacearum* on same inoculated soil and the virulence was reduced in good moist soil (moisture level >5.5). Okabe (1971),Moffett*et al*., (1983),Shekhawat andPerombelon(1991),Shekhawat *etal*., (1992) and

TanakaandNoda (1973) also studied sensitivity of the pathogen related to soil moisture. Okabe (1971)found that*R.solanacearum* grewmoreactivelyindrysoilof15-20%watercontent (WC)thaninmoist soil(40-50%WC)andreasoned thatthispathogenhadthe specific nature to utilisesmall amountsof capillary water heldamong soilparticleswhilegrowthofothermicro-organismswasdelayed.Shekhawat andPerombelon(1991)reported thatpopulation declinewas atitslowestinsoil moistureat 60% of water holding capacity (WHC). AccordingtoShekhawat*etal*.,(1992) soil moisture and temperaturehavea synergistic effect on diseasedevelopment and high temperatures orhigh soilmoisturealonewillnot inducesymptoms. They found inIndia,potato wilt washigherafteronsetofthe monsoon, even though high temperatures prevailed earlierin theseason. However, in a study of factors governing the survival of tobacco wilt disease by TanakaandNoda (1973), it wasfoundthatgrowthrateof*R.solanacearum*insterilesoil washigherat highsoilmoistures(80-100%watercontent)thanin low moisture soil (40%ofwater content). However, it was found that the moisture level tested by TanakaandNoda (1973) were higher than in Okabe (1971). Thus, it was revealed that growthrateof*R.solanacearum* was declined in a good moist soil which supported the findings of the study.

In heterogeneity study for biovar of *R. solanacearum* isolates in different incubation temperature, it was observed that, the same isolates expressed as different biovar due to incubation in different temperature and*R. solanacearum* colony showed VBNC state in below 10^0C and over 40^0C. Influence of temperature was studied by Ciampi and Sequeira, 1980; and Seneviratne 1988 showed that when temperature is >40°C, it cannot survive; become severe between 35~24°C; andshows no visible symptom at <16°C. Granada and Sequeira (1983b) observed that the pathogen can survive long in lower temperature even at 4°C, which make it capable of dispersal and survival in the soil/plant materials for long period. Kelman (1953) and Hayward(1991a) showed that environmental temperature and moisture are two major factors favouring *R. solanacearum* survival in the field which was agreed by Hayward(1991a) and Hayward(1994b).Kelman (1954),Katayama and Kimura (1984) and Champoiseau*et al*., (2009) studied with race 3 biovar II and described the subspecies race3biovarII as the most virulent biovar on plants which was detected as two different type, the virulent type appeared as highly fluidal (more EPS) colonies with appearance of avirulent type dry or less flidal colonies (less EPS) those could be differentiated on TZC medium. This race3biovarII type decrease in aggressiveness when temperatures exceed 35°C or fall below 16°C and, it mean that biovar expression of *R. solanacearum* influences by temperatures fluctuation which was

in support of the study. Álvarez *et al*., (2010) also supported those findings which has been described earlier. Hence, findings of the study was found in accordance with others.

Realizing problems with traditional management practices in designing effective management, thirteen bioactive compounds were tested against *R. solanacearum*. Scientists around the world worked with several management strategies hitherto, no single management practice controlled the disease successfully. Mbaka*etal*., (2013) and Saddler (2005) showed cultural options to have limitions in controllingwilt in infested location due to its ability tosurvive inthesoilover a longtime with asymptomatic weed hosts and avery widehost range which was agreed with McCarter (1969) findings about controllingwilt with preventive options viz. quarantine, use of disease free seeds etc. to be successful only wherethepathogen wasnotpresent. Nonetheless, the pathogen was detected in some potato growing locations and in exported potatoes to Russia during 2014-15 (Chakraborty and Roy, 2016; Parvez, 2017). Use of chemicals against *R. solanacearum* researched by Farag *et al.* (1982) and Hartman and Elphinstone (1994). They found the application of chemicals as achallengebecausethe bacterialocalize insidethexylemand thereare noknown eradicationbactericidesavailableforchemicalcontrolof the bacterialwiltdisease. Farag *et al.,* (1982) observed that chemical control with antibiotics, viz. streptomycin, ampicillin, tetracycline and penicillin showed hardly any effect, in fact, streptomycin application has increased the incidence of bacterial wilt in Egypt (CABI, 2017). Although, applicationofstablebleachingpowderreduced bacterialpopulationsanddiseaseseverity onasmallscale by Saddler (2005), it was observed to be inactivated by organic matter, and released toxic chlorine gas when mixed with ammonia or acidic condition which was shown by Kennedy and Bek. (1998). Enfinger*et al*.,(1979) showed chloropicrin (trade name- Terr-o-gas, Tri-chlor, Metapicrin etc.) istheonlyformulation [methyl bromide, DD.MENCS [amixture of (methyl isothiocyanate,dichloropropane anddichloropropene) andmetham]thatprovides significantcontrolthroughouttheseason among others. Still, it is a fumigant pesticides and exposure of it pose “very high” cancer risks to human (Froines, 2010). Nonetheless, it was used as tear gas and “vomiting gas” during World War I and also degrade soil health. So, those are prohibited in some countriesduetotheriskstohuman, birds,bees and friendly organisms. Though, biocontrol agents had shown some positive results in laboratory experiments with antagonistic bacteria which was reviewed by Yuliar*et al.,*(2015). But difficulties created with application procedure, inconsistentcolonization and survival in the field, suppression etc. were found too complex to useon a commercial scale

which was supported by Whippsand Gerhardson (2007) and Akira*etal*., (2009). Use of resistant cultivars is reported to be themosteffective andpracticalmethodto control the bacterialwilt (Black*etal*., 2003;Grimault*etal*., 1994). Unfortunately, Tung*etal.,* (1990) observed that the complexitiesofhost-pathogen-environmentinteraction in case of *R. solanacearum*which isa “heterogeneous species complex”, make breeding forresistanceextremelydifficult due to having a wide hostrange (Kelman and Person, 1961; Álvarez *et al.,* 2010); and high variability in biochemicalproperties(Cuppels*et al.,*1978;Hayward,1964) etc. However, application oftheorganicamendments had been reported toreducethe disease by Chellemi*et al*., (1997) and Leksomboon *et al*.,(2000) showed many natural bioactive compounds as effective inhibitors against many dangerous pathogenic strains. Shukla (2015) showed that bioactive compounds exhibit a diverse biological effects first of which is antimicrobial activities. Considering the facts discussed, ten bioactive compounds viz. propolis, honey, tumeric powder, $MgCl_2$, aromatic rice extract, iodine, cow dung and sodium bicarbonate were selected to be tested as comared to control, bactericide and farmers practice against *R. solanacearum*. The chemical composition, medicinal and antibacterial activity of propolis from bees were studied by Velikova*et al.*, (2000a, b) and Miorin *et al*.,(2003). Miorin *et al*.,(2003) showed honey and propolis to be very effective against both Gram negative and positive type of bacterial pathogen management because of their phenolic substances include cinnamic acid derivatives, some flavonoids and have been verified as antibacterial applicant. Bosio *et al*.,(2000) showed propolis and honey to contain the antioxidants and flavonoids that function as antibacterial agents against both Gram negative and positive type bacteria. The growth of dangerous bacteria from both Gram negative and positive type(s) such as *Escherichia coli, Staphylococcus aureus, Salmonella* etc. were studied by Zumla and Lulat (1989) which was showed to inhibits by honey. Turmeric (*Curcuma longa* L.) from medicinal context was studied by Eigner and Scholz(1999) and Narasimha *et al*., (2015) and found to be effective against different virulent strains of *R. solanacearum* in India (Narasimha *et al.,* 2015). Several researcher, viz. Singh (2001), Khanuja (2002),Waziri and Suleiman (2013) and Shrivastava *et al*., (2014) have studied the antibacterial properties of cow dung which is generally used as fertilizer and found it to contain antibiotic agents in cow dung extract which was effective against both of those Gram positive and Gram negative type bacterial microbes. A large number of microorganisms were found in cow dung by Waziri and Suleiman (2013) which shows those organisms having biological activities and was in use as antibiotics agents. In a microbiological study, Oyarzúa *et al*., (2014) showed that magnesium chloride ($MgCl_2$) typically associated with the positive

effects against Gram negative type bacteria. According to Mannan *et al*., (2014) report, the fluids of unpolished rice grain of two traditional aromatic rice genotypes, viz. Kalijira and Chinigura, effectively inhibit the Gram negative type bacteria. Iodine dissolved in aqueous potassium iodide, alcohol or mixed with a transporter were classified as disinfectants by Secorand Gudmestad (1993). Growth inhibition of different bacterial pathogen in agar media was observed by Corral *et al.,* (2006) by using sodium bicarbonate (SB) and existence of antimicrobial activity against different types of bacterial and fungal plant pathogens was observed by Kelly and Kristin(2005), Malik and Goyal (2006) and Arslan *et al.,* (2009). In view of the findings, those bioactive compounds were evaluated *in vitro* to find out the effectiveness against bacterial wilt of potato caused by *Ralstonia solanacearum* and it was found that all of the selected compounds showed larger inhibition zone as compared to control indicating the antibacterial effectiveness against the wilt pathogen. The compounds were also evaluated for effectiveness in reducing the virulent colony count and it was observed to perform the best in both parameters by cowdung treatment which was followed by turmeric powder treatment. Thus, several *in vitro* and *in vivo* evaluation was performed by using those treatments as seed and soil treatments (sterilized and unsterilized) and cow dung, propolis and turmeric powder treatments were found to perform best in virulence reduction against *R. solanacearum* as compare to control and farmers practice (stable bleaching powder) especially in natural field soil condition. Consequently, propolis, turmeric powder and cowdung were selected for performance test as both seed and soil treatment for precise evaluation as compared to farmer's practice and control against the wilt pathogen. During evaluation of those three bioactive compounds, cowdung was found to performe best in all parameters which was followed by propolis, turmeric powder against *R. solanacearum*. Result of the study showed that the lowest disease severity score and the lowest colony count in cfu/ml was found to occure in cow dung which was followed by propolis and turmeric powder in all the cases in reduction of virulence of *R. solanacearum*. Studies were conducted by Shrivastava *et al*., (2014) and Waziri and Suleiman (2013) to reveal the antibacterial properties of cow dung and it was showed by Shrivastava*et al*., (2014)that cow dung extract was highly effective against both of Gram negative and Gram positive type bacteria viz. *E. coli*, *Pseudomonas* and *Staphylococcus aureus*. In another study by Waziri and Suleiman (2013), ithas revealed the antibacterial activity of cow dung against *Bacillus subtillis* and*Staphylococcus aureus*due to having the elements like k, Na, Mg etc in higher levels. The role of those metals present in cow dung to act as cofactors for various enzymes in different biochemical processes in relation to antibacterial activity has also been revealed in the study which was similar to the

findings of Gupta *et al*., (2016). Traditionally, cow dung is used asorganic fertilizer in farmingfor centuries which increases themineral status of soil, enhances resistance of plantagainst pests and diseases; stimulate plant growthand other beneficial activities such as sulphuroxidation and phosphorous solubilization. Generally, composition of cow dung is about 80% water andit supports a matrix of undigested plant material that isrich in nutrients, micro-organisms, and theirbyproducts (Sethuraman and Ray, 2003). In a study of *in vitro* inhibition of blight pathogen, Muhammad and Amusa (2003) found that cow dung micro flora contains abundantnumber of bacilli, lactobacilli and cocci and someunidentified fungi and yeasts. According to Ware *et al.,* (1988), lower part of the gutof the cow contains various microorganismsincluding *Lactobacillus plantarum*, *Lactobacilluscasei*, *Lactobacillus acidophilus*, ***B. subtilis***,*Enterococcus diacetylactis*, *Bifido bacterium* andyeasts (commonly *Saccharomyces cerevisiae*) with having a probiotic (beneficial microorganisms) activity.Furthermore, a rich microbial diversity was found to harbor in cow dung by Nene (2003), Randhawa and Kullar (2011) and Sawant*et al*., (2007).Propolis, which is rich in flavonoids and phenolics, exhibited antibacterial properties in a study conducted by Bosio *et al*., (2000) and Miorin*et al*., (2003). Several studies have been verified that phenolics from propolis including cinnamic acid derivatives and flavonoids to exhibit antibacterial properties by Zumla and Lulat (1989), Martos *et al*., (1997), Marcucci *et al*., (2001), Takaisi-Kikuni and Schilcher (1994) and Nieva Moreno *et al*., (1999). Rahman *et al*.,(2010) studied with the concentration of propolis as antibacterial agent and found, the higher the concentration of propolis the greater the inhibition zones against Gram negative type *Escherichia coli* and Gram positive type *Staphylococcus aureus* by disc diffusion method. However, Miorin *et al*., (2003) suggested that the extent of effectiveness of propolis and their chemical composition varies depending on bee species and geographic region. Eigner *et al*.,(1999) and Gupta *et al*., (2012) reported turmeric (*Curcuma longa* L.) as a medicinal plant extensively used in numerous pathological research for presence of curcumin (diferuloyl methane) which shows a wide range of pharmacological attributes, such as antioxidative, antimicrobial and wound-protective properties which were also documented by Nagabhushan and Bhide (1992), Aggarwal and Harikumar (2009), Frenkel *et al.,*(2013) and Moghadamtousi*et al*., (2014). In a study, Narasimha *et al*., (2015) reported that 10% (w/v) turmeric powder extract shows an inhibition zone ranged from about 15 to 25 mm against several virulent strains of *R. solanacearum*. But none of the study was found to study on the effectiveness of those compounds in reducing the virulent colony counts and/or to compare those compouds together for their antibacterial activity against *R. solanacearum* or any other bacterial pathogen.

Nevertheless, findings of the management study against *R. solanacearum* were found in support as those reports of different researchers.

Chapter VI

Summary and conclusion

To fulfil the objectives of the study, infected samples from different sources of inoculum viz. plant, soil, seed, weed, other crops, irrigation water etc. were collected from major potato growing districts of Bangladesh viz. Munshiganj, Chandpur, Tangail, Narayanganj, Jamalpur, Domar, Patuakhali, Rangpur, Bogra, Shariatpur, Meherpur, Joypurhat and Dinajpur considering different divisions and AEZs. Field screening and selection of diseased plant samples were done by critically observing the symptoms of bacterial wilt which were done at 3 stages of plant growth viz. early vegetative, late vegetative and harvest to get different type of infection (early infection, late infection and tuber oozing). Sampling of *R. solanacearum* infected plant sources were done randomly based on streaming test, some at sampling sites and the rest in laboratory just after arriving. Seed/ tuber and soil samples were collected from the from the streaming positive plant locations and other samples viz. weed, other crops, water etc. were collected from the nearest infection positive locations. The presumptive test (streaming) was carried out of infected stem by cutting longitudinal sections of the collar portion in a beaker containing clean warm water at least for 30 min to observe the milky/cloudy oozing from vascular tissues of the diseased plants. Total 21 plant samples out 31 showed bacterial ooze streaming in clear water and in case of potato seeds no oozing was found in the samples except browing of the vascular bundle region of seed tuber. Besides, the weed plant samples did not show any streaming in any case. So, a total of 133 samples were prepared for isolation in TZC solid media. Among those 31 were plant samples, 42 were seed samples, 45 were soil samples, 9 were weed samples, 3 were other crop samples and 3 were water samples. Considering location, 36 samples of different sources from Rangpur division (Dinajpur, Rangpur, Nilphamari), 28 samples of different sources from Rajshahi division (Bogra, Joypurhat), 19 samples of different sources from Mymensingh division (Tangail, Jamalpur), 6 samples of different sources from Khulna division (Meherpur), 9samples of different sources from Barisal division (Patuakhali), 12 samples of different sources from Comilla division (Candpur) and 23 samples of different sources from Dhaka division (Munshiganj, Narayanganj, Shariatpur) were plated for isolation in TZC media for the study. For isolation and maintenance, pathogenic isolatesof *R. solanacearum* were cultured on TZC (2,3,5- triphenyltetrazolium chloride) media, asTZC medium can differentiate the virulent and

non-virulent colony types by appearing as white with pink centers of virulent colonies.. The media was steam sterilized at 121°C and 15-psi pressure for 30-40 minutes. The samples were dilution plated and incubated at 28±1°C for 24 to 48 hours which was stored as pure culturein sterile water in room temperature. Out of 130 isolates 122 isolates of *R. solanacearum* from different location and different sources of inoculum (plant, seed, soil, weed, other crop-chilli and water) showed positive reaction on TZC agar medium in producing typical virulent colonies of *R. solanacearum*. Among those, all plant, seed and soil samples and, 5 out of 9 weed samples, 1 out of 3 other crop (chilli) samples and 1 out of 3 of water samples showed positive on TZC solid media. However, some of the isolates got contaminated and discarded. The rest samples were preserved properly for further characterization of *R. solanacearum.*

The TZC positive uncontaminated isolates of different location from different sources (plant, seed, soil, weed, other crop-chilli and water) were further characterized for their pathogenicity level by performing hypersensitivity test. Considering the pathogenicity in *Euphorbia milli*of hypersensitive reaction, total thirty nine different isolates of *R. solanacearum* out of 122 TZC positive isolates were further characterized on the basis of different subspecies determination test viz. race determination test, different biochemical reaction tests, biovar test, *R. solanacearum* specific PCR test and phylotype test. Races were assigned based on the host range study on potato (*Solanum tuberosum),* tomato (*Solanum lycopersicum)* and brinjal (*Solanum melongena*). The study revealed that, all of the 39 isolates expressed wilting on potato and tomato but no wilting on brinjal except some chlorosis on leaves. Thus, 9 out 31 plant samples, 11 out of 42 seed samples, 14 out of 45 soil samples, 3 out of 9 weed samples, 1 out 3 other crop samples and 1 out of 3 water samples showed that reaction within 2-7 days after inoculation which were showed to be race 3 from different potato growing regions of Bangladesh. In case of biochemical tests, all of the 39 isolates collected from plants, seeds, soils, weeds, water etc. showed positive Gram reaction, KOH reaction, PHB reaction, catalase, oxidase and pectolytic tests and showed similar reaction to temperature sensitivity test. But, in sugar utilization tests (glucose, sucrose and maltose) all *R. solanacearum* isolates showed similar results ie.positive oxidizing of glucose, sucrose and maltose, but twoisolates (1 from weed and 1 from water) showed similar positive result in oxidizing glucose and sucrose except maltose. In case of bivar test, 37 out of 39 isolates of *R. solanacearum* had shown to oxidize the different carbon sources from both disaccharides and sugar alcohols and changed the green color of the medium into yellow which was indicating the oxidation of both carbon

sources expressing those as biovar III. But, the remaining two isolates neither changed the color for disaccharides nor for sugar alcohols which meant that those were not able to utilize either of the carbon sources and were biovar I. In case of phylotype test in PCR, all of the thirty nine isolates produced the bands (280bp) specific for *R. solanacearum* and the bands (144 bp) specific for phylotype I. So, it was observed that from a total of 133 collected samples from different soureces, 94% samples showed positive on TZC test. Among those, 9 out of 34 from Rangpur, 8 out of 26 from Rajshahi, 6 out of 18 from Mymensingh, 2 out of 6 from Khulna, 3 out of 9 from Barisal, 4 out of 10 from Comilla and 7 out of 22 from Dhaka showed race 3; biovar III except 1 from each of Rangpur and Dhaka which showed biovar I; and all those were originated as phylotype I/Asiatic type.

In the study of determination of the heterogeneity within species in relation to PC (phenotypic conversion or VBNC and/or latency) of different isolates *R. solanacearum* in aerated and non-aerated water medium, it was observed that all of 9 plant samples showed phenotypic conversion (PC) from avirulent to virulent state as a result of shift from non-aerated to aerated sterile water growth medium which was observed on TZC culture medium. However, 3 strains from a total of 15 soil samples, 8 strains from a total of 10 seed samples and all of the 3 weed, 1 other crop (chilli) and 1 water samples showed the VBNC (viable but non culturable) state along with phenotypic conversion (PC) from avirulent to virulent state due to the shift of the medium.Inassessing the heterogeneity in virulence expression (in colony count) & disease scoring) by isolates *R. solanacearum*from phenotypically converted cultures in different dilutions (10^7 & 10^8) of water medium, it was found that different ranges of cfu/ml was counted in virulent and avirulent/or interim type colony in both the cases of dilution. Among them, both the dilutions showed heterogeneity within, but more or less positive correlation between avirulent &/or interim colony count (cfu/ml) and absorbance value (at 600nm) within isolates of*R. solanacearum*. But, in case of virulent colony, no correlation was observed between virulent colony count (cfu/ml) and absorbance value (at 600nm) at either of the dilutions except heterogeneity within isolates of*R. solanacearum*. In case of virulence expression (disease scoring) in potato plants, all the isolates produced symptoms of the disease and showed a range of heterogeneity within 2-7 days after inoculation by isolates of*R. solanacearum* which was measured as DSS (disease severity score).In case virulence expression in potato plants, all the isolates showed a range of heterogeneity in virulence measured as DSS (disease severity score). Thus, the study revealed that, level of dilution in

water medium in terms of level of moisture played an important role in expression of heterogeneitybetween isolates of the pathogen which showed to vary accordingly within avirulent &or interim colonies except with virulent colonies. That meant, in water medium change dilution level could randomly change the count of virulent colony (cfu/ml) of the same isolates of *R. solanacearum* which might in turn contribute to influence the virulence expression of isolates of *R. solanacearum*. So, to understand the influence of soil moisture precisely in disease expression on potato plants by *R. solanacearum* the next study was performed.So, it was studied by applying different level of irrigation in same inoculated soil and it was observed that all the disease parameters viz.- wilt severity score, PSI (percent severity index) and virulent colony (cfu/ml) count, and were highest and avirulent & interim colony (cfu/ml) count was lowest in T3 (moisture level <3 as irrigation @ 1 per 10days) as compared to T2 (moisture level 3~5.5 as irrigation @ 1 per 7days) and T1 (moisture level >5.5 as irrigation @ 2 per 7days). The best in disease reduction in all cases was showed by T1. Therefore, it was revealed that, moisture level changed by different level of irrigation influenced the colony virulence level of *R. solanacearum* on same inoculated soil and virulence was reduced in good moist soil (moisture level >5.5). Thus, it was revealed that disease severityof*R.solanacearum* was declined in a good moist soil which supported the findings of the study.In case of determination of biovar expression in different incubation temperature by *R. solanacearum* isolates, it was found at 28 ^{0}C that all of 39 isolates reacted to both disaccharides and sugar alcohols positive/ yellow color developed indicating the oxidation of both carbon sources except 2 isolates indicating those were not able to utilize either of the carbon sources at 28^{0}C temperature. On the other hand, at 22 ^{0}C, different types of reactions were observed and out of 39 isolates of *R. solanacearum*, total 13 isolates were observed as biovar I, 6isolates were observed as biovar II, 15isolates were observed as biovar III and 5isolates were observed as biovar IV at 22^{0}C. It also observed that, the same isolates, which shown only biovar I & III in 28^{0}C, had been shown biovar I, II, III & IV by different isolates of *R. solanacearum* in 22^{0}C temperature. Hence, it was to understand the influence of temperature on avirulent colony in production of virulent and/or avirulent colony and their biovar expression in different temperatures. To study the influence of different incubation temperature on avirulent colony dilution in biovar expression of *R. solanacearum,*it was observed that culture T1 in 0±2^{0}C, T2 in 10±2^{0}C and culture T5 in 40±2^{0}C showed no visible growth that means VBNC (‘viable but non culturable’ state). But, T4 culture in 28±2^{0}C showed the highest growth of avirulent colony (77.58 cfu/ml) whereas culture T3 in 20±2^{0}C showed the highest virulent colony (49.72 cfu/ml) at 10^7 level of dilution. However, when

those VBNC cultures (T1, T2, and T5) were incubated at 28±2^{0}C, the same T1, T2 and T5 cultures showed potential and visible growth of both virulent and avirulent colony. From the study it was found that *R. solanacearum* colony showed VBNC state in below10^{0}C and over40±2^{0}C. And, it was also revealed that both virulent and avirulent &/or interim colonies could be produced from avirulent colony and from VBNC state in presence of favorable temperature. For evaluation of biovar expression of the T1, T2, T3, T4 and T5 cultures grown at different incubation temperatures from avirulent colony of *R. solanacearum*, it was found that all the cultures(T1, T2, T3 and T5) showed biovar 2 except T4 when incubated in 22^{0}C. But the same cultures of T1, T2, T3, T4 and T5 expressed biovar 3 while incubated in 28^{0}C. It did mean avirulent colony is capable of expressing any biovar type depending on the favorable temperature condition.

To design an effective management against *R. solanacearum*some natural bioactive compounds wereselected to find out effectiveness against bacterial wilt of potato caused by *Ralstonia solanacearum* and at first, *in vitro* evaluation of those compounds were performedagainst *R. solanacearum*. During evaluation *(in vitro),* it was observed that the highest significant inhibition zone was created by T9 which was followed by T5, T7 and T10 whereas significant lowest zone was showed by T1 (control) and the rest were laid significantly in between in the study of effectiveness of the selected bioactive compounds in showing inhibition zone against the bacterial wilt pathogen, *R. solanacearum*. It was also observed that the lowest significant virulent colony was produced by T2 which was followed by T5, T9 whereas significant highest of that was showed by T1 (control) and the rest were laid significantly in between. In the evaluation*(in vivo)* of the bioactive compounds against bacterial wilt (*R. solanacearum*) as seed treating agent it was observed that the lowest significant DSS (disease severity score) was produced by T9 which was followed by T2, T7 whereas significant highest of that was showed by T1 (control) which was identical to T4, T5, T6, T10, T11 and the rest were laid significantly in between during evaluation against the bacterial pathogen, *R. solanacearum (in vivo).* It was also observed thatthe lowest significant PSI (percent severity index) was produced by T9 which was followed by T2, T7 whereas significant highest of that was showed by T1 (control) which was identical to T4, T5, T6, T10, T11 and the rest were laid significantly in between during evaluation against the bacterial pathogen, *R. solanacearum.* Consequently, the highest significant reduction in PSI (percent severity index) was produced by T9 which was followed by T2, T7 whereas significant lowest of that was showed by T1 (control) which was identical to T4, T5, T6, T10, T11 and the rest

were laid significantly in between during evaluation against the bacterial pathogen, *R. solanacearum (in vivo).*During the evaluation of effectiveness *(in vivo)* of the selected bioactive compounds as soil treating agent in sterilized soil against the bacterial wilt pathogen (*R. solanacearum*)in potato seedlings, it was observed at 5th (last) WAI (week after inoculation) that the lowest significant DSS (disease severity score) was produced by T7 which was followed by T13, T9 and T3 whereas significant highest of that was showed by T1 (control) which was identical to T2 and the rest were significantly found in between against the pathogen, *R. solanacearum*. It was also found at 5th (last) WAI (week after inoculation) that the lowest significant PSI (percent severity index) was produced by T7 which was followed by T13, T9 and T3 whereas significant highest of that was showed by T1 (control) which was identical to T2 and the rest were significantly found in between against the pathogen. So, it was observed at 5th (last) WAI (week after inoculation) that the highest significant reduction of PSI (percent severity index) was occurred at T7 which was followed by T13, T9 and T3 whereas significant lowest of that was showed by T1 (control) which was identical to T2 and the rest were significantly found in between during evaluation against the pathogen, *R. solanacearum (in vivo).*Evaluation of the effectiveness of the selected bioactive compounds in unsterilized soil against *R. solanacearum* was performed to figure out the effectiveness of the compounds in unsterilized soil (to allow the good microbial community as in natural good field condition) where inoculation was done in 5th week old potato seedlings(root trimming inoculation) and then root dipping in the treatment and it was observed at 7th (last) week that the lowest significant DSS (disease severity score) was occurred at T9 which was followed by T2 and T5 whereas significant highest of that was showed by T1 (control) which was followed by T4, T7, T10, T11, and the rest were significantly found in between. The lowest significant DSS (disease severity score) grwoth was occurred at T9 which was followed by T2 and T5 whereas significant highest of that was showed by T1 (control) which was followed by T4, T7, T10, T11, and the rest were significantly laid in between of those during the evaluation in natural field soil against the pathogen, *R. solanacearum*. Based on better performance in all those evaluation especially in presence of unsterilized soil condition, evaluation of effectiveness of propolis, turmeric powder and cowdung were performed against the bacterial wilt pathogen (*R. solanacearum*), as both seed treating and soil treating agent for precise evaluation as compared to farmer's practice (stable bleaching powder- T13) and control (T1). So, evaluation of effectiveness of propolis, turmeric powder and cowdung were performed against*R. solanacearum* as both seedand soil treatment and it was observed at 7th (last) WAI (week after inoculation) that the

lowest significant DSS (disease severity score) was occurred at T4 (3.44 out of 5.00 in cowdung @ 25%) which was followed by T2 (3.56 out of 5.00 in propolis @ 6mg/ml) whereas significant highest of that was showed by T3 (4.44 out of 5.00 in stable bleaching powder @ 1.15%) which was followed by T1 (4.22 out of 5.00 in control). T5 (3.89 out of 5.00 in turmeric powder @ 10%) was significantly laid in between during the evaluation. It was also observed at the 7th week that the lowest significant PSI (percent severity index) was occurred at T4 (60.00% in cowdung @ 25%) which was followed by T2 (62.22% in propolis @ 6mg/ml) whereas significant highest of that was showed by T3 (88.89% in stable bleaching powder @ 1.15%) which was followed by T1 (84.44%in control). T5 (66.67% in turmeric powder @ 10%) was significantly laid in between. As a result, the highest significant reduction in PSI (percent severity index) over the lowest T3 (stable bleaching powder @ 1.15%) was found to occurre at T4 (28.89% in cowdung @ 25%) which was followed by T2 (26.67% in propolis @ 6mg/ml) whereas significant lowest of that was observed in T1 (84.44%in control). T5 (22.22% in turmeric powder @ 10%) was significantly laid in between during the evaluation. As treatments showed positive effects in reducing the wilt severity index, it was to understand whether there was any effect in soil colonization by the pathogen. Thus, determination of virulent and avirulent colony count (cfu/ml) of inoculated soil with *R. solanacearum*at 7th (last) week in propolis, turmeric powder and cowdung treatment was conducted and, it was observed at 7th (last) WAI (week after inoculation) in dilution plates @ 10^9 dilution that the significant lowest virulent colony count was found in T4 (26.48 cfu/ ml in cowdung @ 25%) which was followed by T2 (29.44 cfu/ ml in propolis @ 6mg/ml) with comperatively dry colonies and T5 (33.54 cfu/ ml in turmeric powder @ 10%) whereas the lowest was found in case of T3 (65.71 cfu/ ml in stable bleaching powder @ 1.15%) which was followed by T1 (55.10 cfu/ ml in control) (Fig. 44.a, & b). On the other hand, the significant highest avirulent colony count was found in T5 (52.46 cfu/ ml in turmeric powder @ 10%) which was followed by T4 (49.18 cfu/ ml in cowdung @ 25%) and T2 (31.89 cfu/ ml in propolis @ 6mg/ml) and whereas the lowest was found in case of T3 (8.96 cfu/ ml in stable bleaching powder @ 1.15%) which was followed by T1 (39.90 cfu/ ml in control). The highest significant reduction of percent virulent colony @ 10^9 dilutionover the lowest (T3 in stable bleaching powder @ 1.15%) was found to occur in T4 (39.22 cfu/ ml in cowdung @ 25%) which was followed by T2 (36.27 cfu/ ml in propolis @ 6mg/ml) and T5 (32.17 cfu/ ml in turmeric powder @ 10%) whereas the lowest was found in case of T1 (10.61 cfu/ ml in stable bleaching powder @ 1.15%).

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