CHARACTERIZATION OF INDIGENOUS BIO-CONTROL AGENTS AGAINST WILT COMPLEX PATHOGENS OF TOMATO

PhD Thesis

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CHARACTERIZATION OF INDIGENOUS BIO-CONTROL AGENTS AGAINST WILT COMPLEX PATHOGENS OF TOMATO

A Dissertation Submitted in the Department of Microbiology under the University of Dhaka in partial Fulfillment of Requirements for the Degree of

Doctor of Philosophy

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Dedicated to my beloved parents

DECLARATION

I do hereby declare that the work submitted as a thesis entitled "**Characterization of indigenous bio-control agents against wilt complex pathogens of tomato**" to the Department of Microbiology, University of Dhaka for the degree of Doctor of Philosophy are the results of my own investigations and was carried out under the supervision of Professor Dr. Md. Abdul Malek and Professor Dr. M. Majibur Rahman, Department of Microbiology, University of Dhaka. Some of the experiments were also carried out in the Laboratory of Plant Pathology Department and Research field of Sher-e-Bangla Agricultural University, Dhaka. The research work has not previously been submitted anywhere for any degree.

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CERTIFICATE

This is to certify that Nazneen Sultana worked as a PhD fellow under our guidance. We are pleased to forward her thesis entitled "**Characterization of indigenous bio-control agents against wilt complex pathogens of tomato**" which was carried out in the Department of Microbiology, University of Dhaka and Department of Plant Pathology and Research field of Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. This work is original and has not been submitted so far in part or in full, for the award of any degree or diploma by any other University. It is to be mentioned that she has fulfilled all the requirements of the regulations and prescribed period of research for submission of thesis for the award of Doctor of Philosophy.

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The author

ABSTRACT

The main objective of the study was to find out potential indigenous bio-control agents active against tomato wilt complex pathogens like *Ralstonia solanacearum* and *Fusarium oxysporum* and various strains of the pathogens were first isolated from wilted tomato plants grown in different areas in Bangladesh. Then different indigenous strains of bacteria and fungi, known for their antagonistic activity against wilt complex pathogens, were isolated and tested against the pathogens. It was found that twenty strains of *R. solanacearum* and three strains of *F. oxysporum* demonstrated infectivity in tomato plants. Based on the ability to infect and severity of infections, one strain of each organism was then selected for further study.

Bacillus spp., *Pseudomonas* spp. *Rhizobium* spp. and *Trichoderma* spp. are known to have potential antagonistic effect against *R. solanacearum* and *F. oxysporum*. To determine their ability and efficacy as bio-control agents, all these organisms were isolated from rhizosphere except *Rhizobium* spp. which were isolated from root nodule of lentils.

In vitro screening test carried out for antagonistic property revealed that five *Bacillus* strains- *Bacillus subtilis* 1, *Bacillus subtilis* 2, *Bacillus pumilus*, *Bacillus licheniformis*, *Paenibacillus polymixa,* one strain of *Pseudomonas fluorescens*, one strain of *Rhizobium leguminosorum* and two strains of *Trichoderma harzianum* were effective against *R. solanacearum* and *F. oxysporum* f. sp. *lycopersici* as bio-control agents.

The findings indicated that *R. leguminosorum* inhibited the maximum growth of *F. oxysporum* f. sp. *lycopersici* in dual culture method with 65.56% inhibition while in case of fungi versus fungi, *T. harzianum* and *T. harzianum* (BAU) showed strong antagonism against *F. oxysporum* f. sp. *lycopersici* pathogen inhibiting 78.37% and 83.78% of radial mycelial growth, respectively after 7 days of incubation. On the other hand, *B. subtilis*, and *P. fluorescens* were found to be more potent against *R. solanacearum*. Four different methods of inoculation viz. collar region, root dipping,

soil drenching and sick bed were evaluated to determine the degree of pathogenicity of *R. solanacearum* and *F. oxysporum* f. sp. *lycopersici*. It was found that collar region inoculation method was best for evaluation of pathogenicity of both pathogens.

In the field experiment *B. subtilis* 2, *P. fluorescens* and *R. leguminosorum* showed similar effect against bacterial wilt when seedling roots were treated separately before transplanting. In case of fungal wilt, *B. subtilis* 2 and *T. harzianum* BAU were found to be most effective in reducing wilt severity. However, when two pathogens were combined together with the same root treatments, *B. subtilis* 2, *P. fluorescens*, *R. leguminosorum* and *T. harzianum* showed better results.

It results indices that the optimal temperature and pH for the growth of the indigenous bacterial bio-control agents were 35^0C and pH 7, respectively. Growth of the bacterial strains seemed to be affected by varying the temperatures and pHs.

From the present study, it suggests that *B. subtilis* 2, *P. fluorescens*, *R. leguminosorum* and *T. harzianum* (BAU) have great potential as bio-control agents not only active against the tested tomato wilt complex pathogens but also they hold a promising future in the development or formulation of an effective bio-control strategy against other plant pathogens.

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CHAPTER 1

INTRODUCTION

Tomato (*Lycopersicon esculentum*), often red fruit of the nightshade commonly known as tomato plant, is recognized as the world's healthiest fresh produce and is consumed in diverse ways, including raw, as ingredient of many dishes, sauces, salads and drinks. The fruit is rich in many important nutrients and vitamins including phosphorus, potassium, vitamins B, lycopene, vitamin C, etc. Because of its low calorie and absence of cholesterol, it is one of the recommendations of diets needing low cholesterol. It also contents various antioxidants and important components that act against breast and prostate cancers (Ajilogba and Babalola, 2013). It is grown mainly in winter season and limited in summer season. It is one of the most popular vegetables for its acid taste. More than five varieties of tomato are cultivated in 58854 acres of land in Bangladesh and the total production is 190213 thousands metric tons per year (BBS, 2010). About 162 million tons of tomato produced in the world and is important commercially for fresh market and processing.

In Bangladesh, congenial atmosphere remains for tomato production during October to March. Tomato is usually plants in November-December High temperature decreases flower production and /or to bud and flower drop. Went (1984) assured that fruit set was abundant only when night temperature was between 15^0C and 20^0C. Seedlings of 25-30 days and planted crops are ready for harvest in 80-90 days. Oxheart, Marglobe, Sunmargino, Roma VF, Pusa Rubi are some popular tomato varieties. Recently BARI developed 10 varieties of tomato namely, BARI Tomato 2 (Ratan), Manik, BARI Tomato-3, BARI Tomato-4, BARI Tomato-5, Chaiti, Apurba, Shila, Lalima and Anupama. BARI Tomato-4, BARI Tomato-5. The Tomato is winter crops but some varieties i.e. Lalima and Anupama (hybrid) can be grown in warm season (Anon., 2015). The average yield of tomato in Bangladesh is 6.92 metric ton per hector (BBS, 2011) which is marked as low yield compared to other leading tomato producing countries (FAO, 1999).

This economically important vegetable crop suffers from several soil borne diseases. (Babalola and Glick, 2012). Such diseases include Bacterial wilt, root knot nematodes disease, early blight, late blight and Fusarium wilt. Wilts, both bacterial and fungal caused by *Ralstonia solanacearum* (*Pseudomonas solanacearum*) and *Fusarium oxysporum* f. sp. *lycopercsici*, respectively are the most serious soil borne diseases in Bangladesh. In Bangladesh, the cultivations of tomato, eggplant, potato, and tobacco are greatly affected by bacterial wilt caused by *R. solanacearum* (Miah and Hoque, 1987). It is necessary to understand the nature of microorganisms caused wilt of tomato and their possible prevention by bio controlling agents.

1. 1 Bacterial Wilt of Tomato

Bacterial wilt caused by *Ralstonia solanacearum* is a devastating disease of crops (Hayward, 1991). It occurs widely in tropical and subtropical regions of the world causing severe losses in yield (Kelman, 1998). The disease affects crops such as tomato, eggplant, potato, tobacco and pepper as well as other important crops like banana, peanut and ginger.

Bacterial wilt of tomato caused by *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995), causes a considerable amount of damage to tomatoes and many other crops in tropical, subtropical and warm temperate regions of the world (Ji *et al*., 2005), that limits production of diverse crops such as potato, tomato, eggplant, pepper, banana and peanut (Williamson *et al*., 2002). The pathogen is a widespread and economically important bacterial plant pathogen (Horita and Tsuchiya, 2001). Bacterial wilt is also known as brown rot and is caused by *Ralstonia* (*Pseudomonas*) *solanacearum,* which is a soil borne bacterial species. It is one of the most destructive and prevalent diseases of solanaceous crops in tropical, sub-tropical and some warm temperate regions (Shekhawat *et al*., 1992; Buddenhagen, 1985). The pathogen infects roots through wounds and multiplies in the vascular system, causing xylem plugging, leaf wilting and hence, affect water transport in the host (Hartman and Datnoff, 1997). The bacterium can maintain infectious populations in soil over several years. The long term survival of the bacterium could also be due to

its ability to enter a dormant-like 'viable but not culturable state**,** like many other soil microbes (Grey and Steck, 2001). It possesses a wide variability in environments, geographic spread and its host range. Approximately 450 crop species were reported as hosts of this pathogen (Swanson *et al*., 2005). Highly susceptible crops are potato, tomato, egg plant, chili, bell pepper and peanut. (Hayward and Hartman, 1994; Hayward, 1992; Shekhawat *et al*., 1992). The disease has limited both commercial and domestic level production (Somodi *et al*., 1993).

An early crop of tomato is very profitable to farmers but its early production is badly hampered due to bacterial wilt. The loss in yield in tomato in India ranges from 11 to 91 percent while the plant mortality ranges from 10 to 100 percent (Ramkishun, 1987). In Bangladesh hundred percent crop failure may occur due to bacterial wilt when flowering stage and favorable condition coincide (Mew and Ho, 1977). In Bangladesh the disease was first recorded in tobacco from Rangpur district (Butler, 1918; Hutchinson, 1913) and after 28 years it was found to attack also tomato (Hedayetullah and Saha, 1941).

1.2 Fusarium Wilt of Tomato

Wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (sacc.) is one of the most destructive diseases all over the world (Suárez-Estrella *et al*., 2007; Beckman, 1987) and typical wilt disease resulting in 70% to 95% yield loss around the world (Lukyanenko, 1991). This pathogen invades through wounds on roots. Infected plants become stunted, chlorotic and wilt (Jones *et al.*, 1991). Symptoms begin as gradual yellowing and wilting of the lower leaves (Khan and Khan, 2002) which is brought about by the growth of the microconidia inter-cellularly in the xylem of the stem and root. As a result of the failure of the infected xylem of the plant to meet the water requirement of the plant, death of the tomato plant is inevitable (Burgess *et al*. 2008). Spores from the conidia are released into surrounding tissues as the plant dies. They later form chlamydospores that fall back into the soils (Jones, 2000). These spores can remain in the soil for as long as 30 years until favourable conditions are available and they can re-infect plants (Thangavelu *et al.*, 2004). *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a highly destructive pathogen of

both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and minimal or absent crop yield. Tomato yield is significantly reduced by *F. oxysporum* f. sp. *lycopersici* because it can destroy roots of tomatoes at growth stages. There may be a 30% to 40% yield loss (Kirankumar *et al*., 2008).

1.3 *Ralstonia solanacearum* the Causal Agent of Bacterial Wilt of Tomato

Ralstonia solanacearum (Yabuuchi *et al*., 1995), causal agent of wilt of potato and solanaceous crops including other host plants is formerly known as *Pseudomonas solanacearum* EF Smith. The pathogen is also identified as *Burkholderia solanacearum* (Yabuuchi *et al.*, 1992). *Ralstonia solanacearum* is a highly heterogeneous bacterial pathogen that causes severe wilting of many important plants (Smith *et al*, 1995). The disease is also called Southern bacterial blight, *Ralstonia solanaceous* wilt, Southern bacterial wilt and many other common names in countries where it occurs (Buddenhagen and Kelman, 1964). It is an aerobic obligate organism, strains of the pathogen have minimum, optimum and maximum temperature of 10, 35 and 41, respectively (Kelman, 1953). *Ralstonia solanacearum* is a Gram negative, non- spore forming rod, about 0.5 - 0.7µm ×1.5 – 2.0µm with a single polar flagellum (Sneath *et al*., 1986). The bacterium is aerobic and its colonies on solid media are small, irregularly round, white in reflected light and tan in transmitted light (Hayward, 1991). Four races and five biovars were classified based on oxidation of sugar and sugar alcohol (Strider *et al.*, 1981). Five pathogenic races and five biovars have been discriminated and Race 1 occurs in tropical areas all over the world (Janse, 2009). Different races of *Ralstonia solanacearum* have different host range. For example race 1 can affect many flowering crops, race 2 can affect Banana plant and race 3 can affect potato, tomato and some other Solanaceae, race 4 can affect ginger and some wild plant (Denny and Hayward, 2001). *Ralstonia solanacearum* have variation in metabolic activity into 5 to 6 different biovars. It is a complete species with considerable diversity, although workers have variously divided the species into ‘group, strains, pathovars, biotype and race’ (Strider *et al.,* 1981).

Classification of *Ralstonia solanacearum*:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Betaproteobacteria

Order: Burkholderiales

Family: Burkholderiaceae

Genus: *Ralstonia*

Species: *R. solanacearum*

Recently amore phylogenetically meaningful system has classified *R. solanacearum* into four major genetic groups called phytotypes that reflect the geographical origin and ancestral relationship between strains (Fegan and Prior, 2005). *R. solanacearum* race 3 biovar 2 is a soil-borne pathogen that persists in wet soils, depth soil layers (>75 cm) and reservoir plants. The tetrazolium medium (TZC), described by Kelman and Person (1954) is the best for culturing *R. solanacearum.* The organism produces two easily distinguishable types of colonies; one is small, flat, red and butyrous (Chen and Echandi, 1982) while the other colony is large elevated, mostly white with light pink centers and full of fluid using Casamino Acid Peptone Glucose (Cuppels *et al.*, 1978).

1.4 *Fusarium oxysporum* f. sp. *lycopersici* the Causal Agent of Fungal Wilt of Tomato

Fusarium is a large class of filamentous fungi widely distributed in soil and in association with plants. Most species are harmless saprophytes and are relatively abundant members of the soil microbial community. The fungus belongs to the sub system of the Fungi Imperfecti (Deuteromycotina). A large diversity of formae speciales of *Fusarium oxysporum* causes vascular wilt diseases affecting a great number of hosts (Martyn and Gordon, 1996; Nelson, 1981) and producing serious economic problem. These pathogens can exist for a long time among susceptible crops and in soil with their persistence mainly attributed to the production of long-lived chlamydospores (Garret, 1970; Newcombe, 1960) for many years. Mycelia enter the epidermal tissues invading through roots, extend to the vascular bundles

and form spores in plants (Chehri *et al*., 2010). The optimal soil temperature for penetration and infestation the host is 18-27°C. At higher soil temperature the pathogen can penetrate into the host without causing wilting (Martyn and Gordon, 1996). Fusarium pathogen penetrates the plant in most cases through wounds in the roots zone. Afterwards it proceeds to grow towards the xylem tubes. While in resistant tomato and cotton plants the fungi stop penetrating a few millimeters up to three centimeters from penetrating point, in sensitive plants the fungi succeed in growing and spreading systematically in the plant by disturbing or delaying the resistance mechanisms (Beckman, 1987). In the first stages of the disease the fungi develop slowly by mycelium, which passes from one xylem tube to the next through pits. Later, fungal conidia are carried by the transpiration stream in the xylem tubes. In the next stage the fungi spread into neighboring tissues of the xylem tubes (Nelson, 1981). The disease symptoms are wilting with or without yellowing (Mas *et al*., 1981). In the two cases, vascular browning can appear in the tubes which the pathogen has penetrated. The browning is a result of oxidation of phenols which are released into the tube as a part of the resistance mechanism against the fungal penetration (Beckman, 1987).

1.5 Biological Approach for Management of Wilt of Tomato

1.5.1 Suppression of soil-borne plant pathogens

Suppressiveness towards soil-borne plant pathogens was defined by Baker and Cook (1974). According to their definition, suppressive soil is a soil in which "the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil". Conducive soil is a soil in which the disease occurs and progresses. Disease suppression can occur in natural soils or can develop in soils or growing media as a result of growing management. Though some argue for a limiting use of the term disease suppressiveness to situations involving a clear biological component (Bruehl, 1987), there are ample evidence of the role of both biotic and abiotic elements of the soil in disease suppression. Chemical and physical attributes of soil, including pH, organic matter and clay content can operate in the suppression of plant diseases directly or indirectly through their impact on soil

microbial activity. Although these abiotic characteristics of soil can contribute to disease suppression. Soil suppressiveness is, directly or indirectly, a function of the activity of soil microorganisms or microbial metabolites (Mazzola, 2002).

Most soils have some natural ability to suppress plant pathogens, this ability is nullified after sterilization, due to the importance of the presence and activity of microorganisms (Mazzola, 2002; Baker and Cook, 1974). Plant protective microorganisms, mainly fungi and bacteria, are often isolated from suppressive environments. In other words, these beneficial microorganisms are generally obtained from aerial or underground parts of plants that are naturally less or not at all affected by a pathogen that devastates a neighboring group of the same plant species (Ryan *et al.*, 2009; Cook and Baker, 1983). The efficacy of several strains of bio-control agents isolated from a wilt suppressive soil in controlling wilt diseases of tomato, brinjal and other vegetable crops in green house tests have been proved (Larkin and Fravel, 1998). Selected isolates were found equally effective in reducing wilt of tomato and other vegetables in repeated tests, with reductions in disease incidence of 60% to 80% relative to the pathogen infested controls (Larkin and Fravel, 1998).

The concept of disease suppressive soil has been described in terms of general suppression and of specific suppression. General suppression is generated by the overall activity of the microbial biomass, while specific suppression is generated by the activity of one or a few populations (Weller *et al*., 2002; Hoitink and Boehm, 1999; Cook and Baker, 1983). General suppression is related to the level of microbial activity at critical stages of the pathogen's development, such as germination and pre-penetration growth in the host rhizosphere. The total microbial biomass competes with the pathogen for carbon and nitrogen and possibly causes inhibition through more direct forms of antagonism (Cook and Baker, 1983). "Specific suppression operates against a background of general suppression but is more qualitative, owing to more specific effects of individual or select groups of microorganisms antagonistic to the pathogen during some stage in its life cycle" (Cook and Baker, 1983). The microorganisms operating in pathogen suppression do

so via diverse mechanisms, including competition for nutrients, antibiosis and induction of host resistance. Non pathogenic *Fusarium* spp. and fluorescent Pseudomonas spp. play a critical role in soils that are naturally suppressive towards Fusarium wilt (Mazzola, 2002).

1.5.2 Bio-control agents

Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means (Harman, 1991) and is known to be a cheap and effective eco-friendly method for the management of crop diseases (Cook and Baker, 1983). The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Members of the genus *Pseudomonas* and *Trichoderma* have long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms (Pant and Mukhopadhyay, 2001). According to Beattie (2006), bacteria that reduce the incidence or severity of plant diseases are often referred to as bio-control agents whereas those that exhibit antagonistic activity toward a pathogen as defined as antagonists. Tomato disease control agents are *Trichoderma, Pseudomonas* and *Bacillus* species. *Bacillus*-based bio-control agents are quite important in the management of pests and plant diseases (Jacobsen *et al*., 2004).

1.5.3 Plant Growth Promoting Rhizobacteria (PGPR) as bio-control agents

The rhizosphere is the narrow zone of soil specifically influenced by the root system (Dobbelaere *et al*., 2003). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Gray and Smith, 2005). This situation is reflected by the number of bacteria that are found around the roots of plants, generally 10 to 100 times higher than that in the bulk soil (Weller and Thomashow, 1994). The rhizosphere is populated by a diverse range of microorganisms and the bacteria colonizing this habitat are called rhizobacteria (Schroth and Hancock, 1982).

Plant-associated bacteria can be classified into beneficial, deleterious and neutral groups on the basis of their effects on plant growth (Dobbelaere *et al*., 2003). Beneficial free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria (Kloepper *et al*., 1989). Bacteria of diverse genera have been identified as PGPR, of which *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore, 2006). The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth promoting bacteria to act as antagonistic agents against phytopathogens (Glick *et al.*, 2007).

Deleterious microorganisms living in the rhizosphere and interacting with the plant roots may cause development of plant diseases. PGPR, which exert a beneficial effect on the plant they colonize, on the other hand, interact with the plant roots as well as with other microorganisms in the rhizosphere. Some of the PGPR are antagonists to recognized root pathogens and may result in prevention of development of plant diseases (Cook *et al*., 1995). Bio-control is the use of the disease-suppressive PGPR to keep the level of deleterious microorganisms under control or below a threshold limit. This suggests the introduction of biocontrol agents from outside in the rhizosphere to achieve disease suppression.

Several studies have been made to control bacterial wilt of tomato with exogenous application of PGPR (Nguyen and Ranamukhaarachchi, 2010; Xue *et al.*, 2009; Aliye *et al.*, 2008; Hass and Defago, 2005). It is important to evaluate PGPR antagonistic to the pathogen and incorporate them into successful disease management as bio-control agent. A key feature of such organisms is their ability to adjust to the rhizosphere and to aggressively colonize the host roots (Dunne *et al.,* 1997). It is recommended that indigenous bio-control agents should be isolated and characterized to achieve greater efficiency at which it would be required to function (Cook, 1993) and the native isolates of certain bio-control agents showed superiority over other isolates for the management of crop diseases (Dubey and Patel, 2001).

Varieties of *Bacillus* and *Paenibacillus* help to promote the health of crops and control diseases by producing antibiotic metabolites, suppressing plant pathogens,

others antagonize plant pathogens by competing for nutrients like iron and phosphate, others indirectly fix nitrogen which they make available to the plants and help stimulate plant nutrient uptake (Gardener, 2004). Among these the bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence hence, *P. fluorescens* have been successfully used for biological control of several plant pathogens (Ramamoorthy *et al*., 2002). Biological control using PGPR strains especially from the genus *Pseudomonas* is an effective substitute for chemical pesticides to suppress plant diseases (Compant *et al*., 2005). Their applicability as bio-control agents has drawn wide attention because of the production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Nagarajkumar *et al*., 2004). Fluorescent *Pseudomonas* is uniquely capable of synthesizing many of these antibiotics, not only to enhance its own fitness but also to help in the maintenance of soil health and bioprotection of crops from pathogens (Gaur *et al*., 2004).

1.5.4 Soil microbes as bio-control agents

Plants are surrounded by diverse types of mesofauna and microbial organisms, some of which can contribute to biological control of plant diseases. Microbes that contribute most to disease control are most likely those that could be classified competitive saprophytes, facultative plant symbionts and facultative hyperparasites. These can generally survive on dead plant material, but they are able to colonize and express bio-control activities while growing on plant tissues.

A numerous soil microorganisms have demonstrated activity in the control of various soilborne plant pathogens. Fusarium wilt of chickpea caused by *F. oxysporum* f. sp. *ciceris* has been controlled by antagonistic microorganisms such as *Trichoderma harzianum, Bacillus* spp*., Pseudomonas fluorescens* and *Pseudomonas* sp. NBRI9926P3 (Ratul *et al.,* 2003). Fluorescent *Pseudomonas* spp. and the fungus *Trichoderma* spp. were found the major bio-control agents which reduced soilborne diseases of various crops (Lumsden and Locke, 1989). Utilization of antagonistic rhizosphere bacteria such as *Bacillus* spp., *P. fluorescens* and *P. putida* significantly increased the survival rate of tomato by 60–90% against bacterial and fusarium wilt

disease, respectively (Gamliel and Katan, 1993; Anuratha and Gnanamanikam, 1990).

Bacillus, *Pseudomonas* and recently the *Rhizobium* group were isolated from soil and found to effectively control various soil-borne plant pathogenic fungi under greenhouse and field conditions. As compared to the other bio-control agents, Rhizobia offer the great advantage of symbiotic nitrogen fixation by association with legumes (Peoples *et al.,* 1995). Among the *Rhizobium* group, *Rhizobium leguminosarum* have been used successfully against fungal pathogens (Ozkoc and deliveli, 2001). Rhizobia have several mechanisms of action that allow them to control pathogens. These mechanisms include competition for nutrients (Essalmani and Lahlou, 2002), production of antibiotics (Ehteshamul-Haque and Ghaffar, 1993; Chakrabortu and Purkayastha, 1984), promotion of plant growth, in terms of better shoot height, root length, dry weight and root nodulation (Siddiqui and Mahmoud, 2001), and induction of plant defense mechanisms (Abdelaziz *et al.*, 1996). Two of the major bio-control agents which reduce soilborne diseases of various crops include isolates of the bacterium fluorescent *Pseudomonas* spp. and the fungus *Trichoderma* spp. (Lumsden and Locke, 1989), specially *Trichoderma* species are the most-promising bio-control fungi against many fungal plant pathogens.

1.6 Background and Objectives of the Study

Developing an initial strategy to combat these devastating plant diseases generally include the use of cultural, physical and chemical controls. None of these strategies have been able to give the efficient results of completely ameliorating the situation except for the cultural method which is mainly preventive. A good knowledge of the nature, behaviour and environmental conditions of growth of the disease causing agent is very important to controlling the disease development in that case. Biological control has been shown to be an environmentally friendly alternative. It makes use of indigenous rhizospheric and endophytic microorganisms that can survive and compete favorably well with wilt pathogens. They include plant growth-promoting rhizobacteria (PGPR) such as *Bacillus* spp. and *Pseudomonas* spp. For PGPR to control or inhibit the growth of the wilt pathogens, they make use of mechanisms such as indole acetic acid production, siderophore production,

phosphate solublilization, systemic resistance induction and antifungal volatile production.

Interest in biological control has increased recently fuelled by public concerns over the use of chemicals in the environment in general, and the need to find alternatives to the use of chemicals for disease control. Biological control of wilt diseases has shown potential as an alternative disease management strategy (Alabouvette *et al*. 1998; Datnoff *et al*., 1995). Antagonist organisms have successfully reduced the incidence of wilt in numerous crops in greenhouse and field trials (Datnoff *et al*., 1995; Alabouvette *et al*., 1993; Alabouvette and Couteaudier, 1992; Paulitz *et al*., 1987). The key to achieving successful, reproducible biological control is the gradual appreciation that knowledge of the ecological interactions taking place in soil and root environments is required to predict the conditions under which bio-control can be achieved (Whipps, 1997) and, indeed, may be part of the reason why more bio-control agents are reaching the market-place (Whipps and Lumsden, 2001). However, for biological control to be implemented commercially on a practical level, it is necessary to more understand the ecology of these bio-control organisms and their interactions with the pathogen, host plant, and surrounding soil and rhizosphere microbial communities (Larkin *et al.*, 1998; Handelsman and Stabb, 1996; Cook, 1993). So, Characterization of the bio-control agent is imperative. Considering the above facts the present study was therefore undertaken with the following objectives:

1. To isolate and characterize the indigenous wilt complex pathogens of tomato plant.
2. To isolate suitable indigenous biocontrol agents against the wilt complex pathogens of tomato.
3. To determine in impact of the indigenous biocontrol agents on wilt causing pathogens and yield of tomato.

CHAPTER 2

MATERIALS AND METHODS

The laboratory and field experiments were carried out in the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka and Department of Microbiology, University of Dhaka, Bangladesh.

2.1 Collection of Wilted Tomato Plants

Bacterial and fungal wilt infected tomato plants were collected from different experimental fields of Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh Agricultural University (BAU), Mymensingh, and Sher-e-Bangla Agricultural University (SAU), Dhaka. Diseased plant samples were selected based on visible symptoms of bacterial and fungal wilts as described by Agrios (2006) and the Oozing test was performed for confirmation of bacterial wilt (Shekhawat *et al.*, 1992).

2.2 Isolation of *Ralstonia solanacearum* from Wilted Tomato Plants

The selected wilt infected tomato plants were prepared for isolation of bacterial strains caused bacterial wilt (Shekhawat *et al*., 1992). For isolation of *Ralstonia solanacearum* from wilt infected plant specimens, streak plate technique was followed using a selective medium, Tetrazolium chloride (TTC) agar (Appendix A.2) as described by Kelman (1954). Diseased stems of tomato were washed under tap water and cut into small pieces ((2-3cm) from the base. The pieces of infected stem were surface sterilized with 5% chlorox for 1 minute and 70% ethanol for 1 minute and rinsed with sterilized distilled water. The pieces were then immersed in 5 ml of sterilized distilled water in a test tube for standard isolation (Hildebrand *et al*., 1988). The bacterial ooze released from the infected stem was thoroughly mixed in sterilized water after discarding the stem pieces. One loopful of suspension was streaked on the TTC agar medium in petri-plates and incubated at 30^0C for 48 hrs. The single colony of *Ralstonia solanacearum* showing virulent, fluidal, irregular

and creamy white with pink at the center was selected and multiplied in Casamino-acid peptone glucose (CPG) agar medium (Appendix A.2).

The virulent colonies were selected based on color for further investigation. The isolated fluidal colonies were re-streaked on CPG (without the stock solution of TTC) plates for the growth of some strains sensitive to formazan pigment produced from TTC. Two loopful of bacteria from a composite of about six individual of 48-72 hrs old colonies were transferred to screw capped test tubes containing 6 ml of sterilized distilled water for storage at room temperature ((25-30^{0}C) (Kelman and Person, 1961). The isolates preserved in sterile water were re-cultured on TTC medium and checked at every three-month.

2.3 Isolation, Identification, and Purification of *Fusarium oxysporum* f. sp. *lycopersici* from Wilted Tomato Plants

The collected diseased plant samples were washed in running tap water and the infected portion along with the healthy portion of the plants were cut into small pieces (0.5-1 cm), and surface sterilized with 1% clorox for 2-3 minutes. The plant pieces were then rinsed with sterilized water and placed on filter paper to remove excess water adhering to the pieces, and plated onto wet blotter disc via standard blotter protocol (ISTA, 2003). The plates were incubated at 22^{0}C for 7 days with alteration of light and darkness at 12 hrs interval. The fungi developed on each root and stem samples were grown for at 25^{0}C for 7 days and isolated as pure cultures on potato dextrose agar (PDA) medium (Appendix A.19). *Fusarium oxysporum* was purified by using PDA (Begum *et. al.*, 1998) and was identified following the key outlined by Booth (1971). PDA slants were inoculated by mycelial block of *Fusarium oxysporum* f. sp. *lycopersici* and kept in incubator for 7 days at 22^o C and under 12 hrs alteration of light and darkness. Then pure culture of *F. oxysporum* f. sp. *lycopersici* was preserved in PDA slants at 5-8^{0}C in the refrigerator as stock culture for future study.

2. 4 Cultural and Biochemical Tests for Identification of *Ralstonia solanacearum*

Ralstonia solanacearum, formerly known as *Pseudomonas solanacearum* (Section1.3), was grown on cetrimide agar (Appendix A.4) as selective medium. All the isolates of *R. solanacearum* were inoculated on cetrimide agar plates and incubated at 37^0 C for 48 hrs. The isolates of *R. solanacearum* were also inoculated on NA plates and incubated at 40^0C for 7 days for comparative study. For characterization of the isolates of *Ralstonia solanacearum* a series of biochemical tests including Gram differentiation (Suslow *et al*., 1982) and gram reaction, catalase test (Hayward, 1992), oxidase test (Kovacs, 1956), motility test, nitrate reduction, arginine dehydrolase, pectolytic activity, etc. were conducted.

2.4.1 Gram differentiation test

The Gram differentiation method was performed for rapid detection of plant pathogenic bacteria without staining (Suslow *et al*., 1982). Two drops of 3% KOH solution were placed at the centre of a clean glass slide. Fresh culture of *R. solanacearum* (grown on TTC medium) was transferred from culture media aseptically with a flat wooden toothpick and placed into the drop of KOH with a rapid circular agitation for about 10 seconds, and the toothpick was alternatively raised and lowered just off the slide surface to detect a stringing effect. The 3% KOH test was considered positive if drop viscosity increased and stringing occurred within 15 seconds. This method utilizes the rapid disruption of the cell wall of gram negative bacteria in alkaline solution releasing deoxyribonucleic acid which causes the viscous threading. Gram positive bacteria do not lyse in 3% KOH.

2.4.2 Oxidase test

A strip of filter paper (Whatman No. 2) was soaked with 3 drops of 1% aqueous solution of freshly prepared tetramethyl-p-phenylene-diamine dihydrochloride (color indicator). A loopful of fresh bacterial culture (TTC medium) was rubbed separately on the impregnated surface of the filter paper stripe by a platinum loop. Purple color develops within 10 seconds, which indicated positive reaction of oxidase test.

2.4.3 Catalase test

Twenty four hours old culture of *R. solanacearum* and 3% hydrogen peroxide solution were used to observe production of gas bubbles, which indicates positive reaction (Hayward, 1992). A loop-full of bacterial culture (24 hrs old on TTC medium) of each isolate was placed on a clean glass slide. A drop of H_2O_2 solution (3%) was added and mixed with the culture. Production of gas bubbles indicated positive reaction.

2.4.4 Motility test

Motility agar was prepared in tubes without slanting. Sterile semisolid medium was inoculated by stabbing the centre of the medium without touching the bottom. The tube was incubated at 30^0C for 18 to 24 hrs. Motility of organism was shown by a diffused zone of the entire medium or only from one or two points.

2.4.5 Nitrate reduction test

Nitrate reduction test was carried out in the nitrate broth (Appendix A.12). The freshly prepared cultures were inoculated in sterile nitrate broth containing tubes and incubated at 30^0C for 24 hrs. At the end of incubation 0.1 ml solution of A was added followed by solution B in equal volume. The appearance of deep pink color showed the positive results.

2.4.6 Pectolytic activity

For this purpose potato slices (7-8mm thick) were washed and a depression cut was made on each. These slices were surface sterilized with alcohol. Potato slices were placed into Petri dishes containing a sterile moistened filter paper. A bacterial cell suspension was placed into the depression cut of the potato and kept at 22^0C for 24 hrs. Pectolysis that is potato soft rotting beyond the point of inoculation indicates a positive response.

2.4.7 Arginine dihydrolase activity

Arginine dihydrolase activity was performed for the presence of two enzymes that permits certain pseudomonads to grow under anaerobic conditions. The enzymes

generate ATP by the degradation of arginine to ornithine with the generation of CO_2 and NH_3. The two enzymes are arginine desmidase which degrades arginine to citrulline+NH_3. It is the alkaline reaction of NH_3 production that is detected by the test. A fresh culture was stabbed into a soft agar tube of Thornley's medium (Appendix A.6) and sealed with sterile mineral oil (1ml) and incubated at 28^0C. A color change from faint pink to red within four days indicated a positive reaction.

2.4.8 Tobacco Hypersensitivity reaction (HR)

Bacterial suspension (10^8-10^9CFU/ ml) of the isolate in sterile distilled water was prepared and infiltrated the lower surface of a mature tobacco leaf by pressing a syringe containing the suspension against the leaf, forcing the suspension into the leaf (Klement, 1963). Distilled water was used as negative control. Complete collapse of the tissue after 24 hrs indicated a positive reaction.

2.5 Growth Patterns and Conidial Characteristics of *Fusarium oxysporum* f. sp. *lycopersici* on PDA

The three isolates of *F. oxysporum* f. sp. *lycopersici* were grown on PDA plates. The mycelial block of the isolates were inoculated at the centre of the PDA plate and incubated at 25^0C for 7 days. Then the results were recorded as: a) Radial growth (mm), b) Colony color, c) Colony shape, d) Compactness of the colony, e) Number of conidia/ cm and f) Average size of the conidia.

2.6 Pathogenicity of *Ralstonia solanacearum*

Pathogenicity of the bacterial isolates were performed by inoculating the susceptible tomato cultivar BARI tomato 2 (Ratan). Seedlings of tomato were planted directly in polybag containing sterilized soils (well decomposed cow dung and silty soil at 1:1). Soil sterilization was done with formalin (40%) at the rate of 5ml/4 kg soil (Dasgupta, 1988). The formalin treated soil was covered with polythene sheet for 48 hours and then exposed for 48 hrs aeration before setting the experiment. Fertilizers were added @ 50g of TSP and 50g of MP per polybag. Bacterial isolates were grown on CPG agar medium for two days at 30°C, suspended in sterile distilled water and an optical density of 0.1 at 600 nm wavelength,

approximately 10^8 cfu ml^{-1} was adjusted. Inoculation was made at the three to four true leaf stages by puncturing the stem at the axils of the third fully expanded leaves from the apex with a needle dipped in inoculum (Winstead and Kleman, 1952). Five tomato plants were used for each isolates. Plants inoculated with sterile water served as negative control. Inoculated plants were kept in a net house. Plants were watered well, without wetting the foliage for 24 hrs (Williamson *et al*., 2002). The experiment was undertaken with completely randomized design and repeated twice. Wilt incidence has been calculated using the following formula:

$$\text{Percent wilted plants} = \frac{\text{Number of wilted plants}}{\text{Total number of plants}} \times 100$$

Data were recorded on percent wilted plants and days required for symptoms expression. On the basis of pathogenecity one virulent isolate of *R. solanacearum* was selected for further study.

2.7 Pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici*

The pathogenicity of three isolates of *Fusarium oxysporum* f. sp. *lycopersici* were tested by inoculating the tomato plants (var. BARI tomato 2). Seedlings of tomato were planted directly in polybag containing sterilized soils (well decomposed cow dung and silty soil at 1:1). Fertilizers were added @ 50 g of TSP and 50 g of MP per polybag. The spore suspension of actively growing fungal pathogen of each isolate was prepared in sterile distilled water. The concentration of spore was standardized and optimized (spores/ ml) using haemocytometer. Under net house conditions the young seedlings were inoculated by collar region inoculation method and plants were grown under transparent polythene covers for a period of 48 hrs. Five seedlings were inoculated for each isolate of *F. oxysporum* f. sp. *lycopersici.* Periodic observations were monitored for one week for symptom and development of the disease (Zaidi *et al*., 2010; Marlatt *et al.*, 2008). On the basis of pathogenicity one isolate of *F. oxysporum* f. sp. *lycopersici* was selected for further study.

2.8 Evaluation of Inoculation Methods

Inoculation methods were evaluated against *F. oxysporum* f. sp. *lycopersici* and *R. solanacearum*. Four methods of artificial inoculation viz. root dipping, soil drenching, collar region inoculation and sick bed methods were compared to determine their efficacy to cause fungal and bacterial wilt of tomato. In case of *Fusarium* the concentration of conidia used in all methods was 10^7spore/ml and for *Ralstonia* concentration was 10^8 cfu/ml.

2.8.1 Root dipping

25 days old seedlings were uprooted carefully and the roots were washed thoroughly under running tap water. The seedlings were dipped in fungal spore suspension and bacterial cell suspension for 10 minutes and transplanted in sterilized soil (Begum, 2007).

2.8.2 Soil drenching method

The spore and cell suspension of *F. oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum,* respectively were sprinkled around 25 days old seedlings so that the suspension reached at a depth of at least 10-15 cm of soil (Gangopathayay, 1984).

2.8.3 Collar region inoculation method

The sterilized needles were soaked in fungal / bacterial cell suspension the needles were used for making punctures around the collar region of the seedlings (Babar, 1999).

2.8.4 Sick bed inoculation method

Sterilized soil has been inoculated with fungal cultures (Purwati *et al.*, 2008) and bacterial cell suspension. Soil in each tray (50×35 cm) was inoculated with 500 ml suspension of *Ralstonia solanacearum*. In order to prepare soil inocula, agar plugs containing actively growing mycelium tips of *F. oxysporum* f. sp. *lycopersici* were used to inoculate slightly pressed sterilized corn seeds in 500 ml Erlenmeyer flasks. This mixture was incubated at room temperature for 10 days. In these experiments 10 g of infested corn seeds were added to 3 kg of soil. The mixing of fungal

preparation and bacterial cell suspension with the soil were conducted 7 days before transplanting of seedlings.

2.9 Isolation and Characterization of Indigenous Bio-control Agents

2.9.1 Isolation and preservation of *Trichoderma harzianum* from rhizosphere of tomato

Trichoderma isolates were isolated from rhizosphere and rhizoplane soils of tomato from SAU research plot by soil dilution plate technique and root washing methods (Tuite, 1969). The isolates of *Trichoderma* were purified in acidified agar (pH 4.5) using hyphal tip culture technique. Pure culture of *T. harzianum* was made. Before preservation *Trichoderma* sp was purified and identified as *T. harzianum* following Rifai (1969) and Kamal (1992). Pure culture was maintained as stock culture in PDA slants at 4^0C and preserved following the same procedure of preservation of *Fusarium oxysporum* f. sp. *lycopersici* for future use.

2.9.2 Collection of *Trichoderma harzianum*

Pure culture of *Trichoderma harzianum* (BAU) was collected from Plant Disease Diagnostic Clinic (IPM Laboratory), Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh.

2.9.3 Isolation of *Bacillus* spp. from rhizosphere of tomato

Sixty isolates of *Bacillus* spp. were isolated on nutrient agar plates from rhizosphere of tomato crop collected from different location of Bangladesh. The bacteria were isolated by serial dilution agar plate technique (Aneja, 2004). After purification gram reaction was performed for each isolate and then catalase test was performed to separate *Bacillus* spp. from *Clostridium* spp.

2.9.4 Isolation of *Pseudomonas fluorescens* from rhizosphere of tomato

Twenty isolates of *P. fluorescens* were isolated on King's B (KB) medium from rhizosphere soil of tomato collected from BAU, BARI and SAU campus of Bangladesh. The bacteria were isolated by serial dilution agar plate technique following the method of Vlassak *et al.*, (1992). 1 g of each soil sample was mixed

by shaking for 2 hrs on a rotary shaker at 200 rpm in 100 ml of Phosphate Buffered Saline (PBS). PBS (Appendix B.5) diluted extracts were then plated on medium which were made selective for isolation of *P. fluorescens* by adding cychlohexamide (100µg ml^{-1}), chloramphenicol (13 µg ml^{-1}) and ampicillin (50 µg ml^{-1}) (Simon and Ridge, 1974). After incubation at 28^0C for 24 hrs representative types of colonies were further purified on KB agar medium (Appendix A.3) and pure isolates preserved on KB slant and stored at 4^0C.

2.9.5 Isolation of *Rhizobium leguminosorum* from root nodules of lentil

A total of ten *R. leguminosorum* isolates were isolated from root nodules of lentil plants. Healthy lentil plants with root systems were collected from the field at the flowering stage. Roots were washed in running tap water to remove soil particles. Plum, healthy and rosy or fleshes colored nodules were removed from the roots by washing with sterile water. The washed nodules were surface sterilized in 95% alcohol followed by washing in sterile water and then in mercuric chloride solution (1:1000) for 2-3 minutes. After surface sterilization three times washed in sterile water. Then nodule were transferred to a culture tube and crushed with a sterile glass rod and added sterile water to obtain a milky bacterial suspension. After serial dilution suspension was spread over Yeast Mannitol Agar (YMA) (Appendix A.15) plates and incubated for 2 days at 28^0C. Semi-translucent, raised and mucilaginous colonies were re-streaked on YMA plates to get pure culture.

2. 10 Screening of Indigenous Bacterial Isolates Against *Ralstonia solanacearum*

All of the isolated bacteria were screened to determine their effect on the growth of *Ralstonia solanacearum* following perpendicular streak method (Egorov, 1985). For this purpose an agar plate (Nutrient Agar) was inoculated with a streak of the probable antagonistic bacteria (producer bacteria). After the microorganism grew enough, isolates of *Ralstonia solanacearum* were seeded in perpendicular streaks. Petri plates were placed in a thermostat at 30^0C and kept there for 20-24 hrs and observed the zone of inhibition. The results were recorded for every.

2.11 Screening of *Trichoderma harzianum* Against *Ralstonia solanacearum*

In order to check the efficacy of *Trichoderma harzianum* against *Ralstonia solanacearum*, paper disc method was followed (Gupta *et al.*, 1998). Sterilized discs (5 mm diameter) of Whatman's filter paper was dipped into the conidial suspension of *T. harzianum* culture, excess quantity of the solution was drained off by touching the disc to the side of the Petri-plates. Treated discs were then placed at the centre of the petriplates seeded with *R. solanacearum* under aseptic conditions and incubated at 28 ± 2^0 C. The plates without *Trichoderma* served as control.

2.12 Screening of Indigenous Bacterial Isolates Against *Fusarium oxysporum* f. sp. *lycopersici*

In vitro antagonism tests between *F. oxysporum* f. sp. *lycopersici* and isolated bacteria were performed on NA in 9 cm petri plates by applying a dual culture technique (Sadfi *et al*., 2001). Bacterial isolates were streaked at one side of the plate and 5 mm in diameter mycelial block of *F. oxysporum* was placed at the centre of the plate. Three replications were used for testing each bacterial isolate. The distance between the two microorganisms was 2.5 cm. Plates were incubated at 25^0C for one week. Percent growth inhibition (GI) of *F. oxysporum* f. sp. *lycopersici* (GI) after 7 days was calculated by the formula of Whipps (1987): $(R_1-R_2)/R_1\times100$, where, R_1 is the fungal radial growth (measured in mm) in direction opposite to the antagonist and R_2 is the radial growth toward the antagonist.

2.13 Screening of *Trichoderma harzianum* Against *Fusarium oxysporum* f. sp. *lycopersici*

In vitro screening of *T. harzianum* isolates against *F. oxysporum* f. sp. *lycopersici* was conducted on PDA following dual culture technique (Dhingra and Sinclair, 1985). Discs of mycelium (5 mm diameter) of each of the selected fungal isolates were cut from the edge of an actively growing fungal colony with a cork borer. Test plates were prepared by pouring 20 ml of PDA per plate. After solidification, one mycelial disc of *T. harzianum* and one disc of test fungal pathogen i.e. *F. oxysporum* f. sp. *lycopersici* was placed simultaneously on the edge of the each PDA plate at opposite direction maintaining equal distance from the peripheral zone. Three

replicated plates were used for each isolate of *Trichoderma* and test pathogen. The plates were arranged on the laboratory desks following completely randomized design. The plates received only mycelial discs of the test pathogens served as control. The plates were incubated in the laboratory having ambient temperature of 25±3^{0}C until mycelium of the test pathogens *F. oxysporum* f. sp. *lycopersici* cover the whole control plate. Thereafter inhibition percentages of *F. oxysporum* f. sp. *lycopersici* was calculated based on the growth of the pathogen on PDA plates following the formula as suggested by Sundar *et al.* (1995). Inhibition of radial growth was computed based on colony diameter on control plate using the following formula:

% Inhibition = (X-Y/X) × 100

Where,

X= Growth of control plate

Y= Growth of *Trichoderma* treated plate

2.14 Identification and Characterization of Isolated Indigenous Bio-control Agents

2.14.1 Identification and characterization of *Trichoderma harzianum*

A bit of mycelium was taken on a clean glass slide from the peripheral zone of *T. harzianum* culture and mounted with cotton blue and a semi permanent slide was prepared. The characteristics was studied under compound microscope and recorded.

2.14.2 Identification and characterization of *Bacillus* spp.

2.14.2.1 Growth on vessal medium

Selected isolates of *Bacillus* spp. were inoculated on Vessal medium (Appendix A.11) which is selective for *Bacillus* spp. culture. Then the plates were incubated for 7 days at 30^{0}C and growth was observed.

2.14.2.2 Microscopic observation of the isolated *Bacillus* strains

a. Gram staining was performed by standard gram staining method and observed under the microscope.

b. Spore shape and position study by light Microscopy (Chun and Vidaver, 2000): Spores observed by staining. On a clean and dry slide bacteria was spread over in a drop of saline water to make a bacterial smear and air dried. Then flooded the slide with 5% malachite green and stain for 10 minutes and washed under running water. Counter stain was made with 0.5% aqueous safranin for 15 seconds. Then rinsed with water and blot dried and observed under oil immersion objective. Bacterial bodies were red and spores were green.

2.14.2.3 Biochemical characterization of *Bacillus* spp.

Each of the selected bacterial bio-control agents was sub-cultured on nutrient agar plate and incubating at 30^0C for 24 hrs. Several biochemical tests were performed to observe the physiochemical activities of the organisms in order to confirm on the basis of their identity of the isolated organisms. Isolated strains were identified on the basis of their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). Oxidase test, catalase test, citrate utilization test, indole production test, starch hydrolysis, Methyl-red and Voges- Proskaur test, Motility test and carbohydrate fermentation test were performed to check the biochemical characteristics of producing strain (Bergey and Holt, 1994).

2.14.2.4 Indole production

One loop-full fresh bacterial culture was inoculated in peptone broth and incubated at 30^0C for 24 hours. Kovac's reagent was added and shaken vigorously for one minute. A red color ring formation was an indication of positive result.

2.14.2.5 Starch hydrolysis

Starch agar plates were inoculated with the test bacteria and incubated plates for 5 days. After 5 days of incubation plates were flooded with Lugol's iodine. Clear colorless zones indicated positive (Schaad *et al.*, 2001).

2.14.2.6 Methyl red and Voges-Proskaur

A sterile MR-VP broth was inoculated with the test organism and following incubation at 30^0C for 24 hrs, few drops of methyl red solution was added. A

distinct red color indicated MR positive test, while yellow or orange color indicated a negative result. A sterile MR-VP broth was inoculated with the test organism and following incubation at 30^0C for 24 hrs. 5 drops of (α) napthol solution and 5 drops of KOH solution were added. The development of a bright red or pink red color was recorded as a positive result.

2.14.2.7 Carbohydrate fermentation

Phenol red broth having different carbohydrates containing inverted Durham's tubes was inoculated with the test organism. Following incubation at 30^0C for 24 hrs, change in color indicated the acid production while formation of bubbles in Durham's tube indicated the gas production.

2.14.3 Characterization and identification of *Pseudomonas fluorescens*

The following morphological features such as colony type, bacterial shape and gram reaction of the selected isolate of *P. fluorescens* was determined using King's B agar medium. To identify species of fluorescent Pseudomonalds LOPAT schemes (Lelliott *et al.*, 1966) including Levan production on sucrose medium, Oxidase reaction, Pectiolytic activity on potato slices, Arginine dihydrolase activity and Hypersensitivity reaction (HR) on Tobacco leaves were done. Other biochemical tests also done including catalase, motility, nitrate reduction and growth at 41^0C, gelatin liquefaction, salt tolerance tests were performed following the method of Goszczynska *et al*. (2000), Pickett *et al.* (1991) and Arnow (1937).

2.14.3.1 Levan test

Nutrient agar medium added with 5% sucrose was inoculated with different isolates of Pseudomonalds. The plates were placed in an incubator at 30^0C for 3 to 5 days. White mucoid, dome shaped colonies indicated a positive reaction. Oxidase test, Pectolytic activity, Arginine dihydrolase activity and tobacco hypersensitivity were described in section 2.4.

2.14.3.2 Growth at 41^0C

Tubes containing yeast extract (5 g/ l) inoculated with fresh culture and kept in incubator at 41^0C for 48 hrs.

2.14.3.3 Nitrate reduction

Test tubes containing medium (Appendix A.12) was inoculated with the isolates and plugged each tube with 3% Nobble agar. Incubated at 27^0C for 5 days. Growth indicates positive reaction that is de-nitrification.

2.14.4 Characterization and identification of *Rhizobium leguminosorum*

R. leguminosorum was identified on the basis of physiological and biochemical characteristic such as growth at 40^0C, Growth in presence of 2% NaCl, Anaerobic growth, Motility, H_2S production, congo red test, growth on glucose peptone agar etc.

2.14.4.1 H_2S production

Test tubes containing the medium (Appendix A.14) were inoculated with *Rhizobium leguminosorum* and a lead acetate strip was suspended over the medium. These tubes were incubated for 14 days. Black discoloration of the strip means positive reaction.

2.14.4.2 Congo red test

The purity of the isolate was detected by adding congo red (0.25g/100ml of EtOH; 10ml stock/ litre of YMA) in YMA medium.

2.14.4.3 Growth on glucose peptone agar

Glucose peptone agar medium (Appendix A.16) was used to differentiate rhizobia from agrobacterium. Plates containing glucose peptone agar medium were streaked with *Rhizobium leguminosorum* and incubated for 48 hrs.

2.14.4.4 Growth on YDC agar

YDC (Appendix A.13) plates were streaked with *Rhizobium leguminosorum* and were incubated at 30^0C.

2.14.4.5 Salt tolerance test

Nutrient broth containing 1%, 2%, 3%, 4%, 5%, 6% and 7% salt were inoculated with *Rhizobium leguminosorum* and incubated at 30^0C and observed daily up to seven days for growth and recorded.

2.15 Carbohydrate Fermentation Test for Bacterial Bio-control Agents

Phenol red broth having different carbohydrates containing Durham's tubes were inoculated with the selected bio-control agents and incubated at 30^0C for 24 hrs. Change in color indicated the acid production while formation of bubbles in the Durham's tube indicated the gas production.

2.16 Interaction of Bacterial Bio-control Agents with *Fusarium oxysporum* f. sp. *lycopersici*

Different bacterial isolates were grown in Nutrient broth and 0.1 ml culture broth was taken in Nutrient agar plate and was spread over that. Five mm mycelial block of *F. oxysporum* f. sp. *lycopersici* was placed in the middle of NA plates following Chen *et al.* (2003). Three replications were used for each isolates of bio-control agent. In case of control, only the mycelial block of *F. oxysporum* f. sp. *lycopersici* was paced in the middle of the NA plates without spreading antagonist that grew in broth. The plates were then incubated at room temperature for 7 days and then their interactions were determined by observing the inhibitory effect of antagonist on mycelial growth of *F. oxysporum* f. sp. *lycopersici.* After 7 days of incubation the results were compared with streaking method.

2.16.1 Effect of temperature on growth of selected bio-control agents

For the determination of optimum temperature selected bacteria were inoculated in nutrient broth (pH 7.0) and incubated at different temperatures viz. 25^0C, 30^0C, 35^0C and 40^0C in shaking incubator at 150 rpm for three days. Optical density was measured every day at 600 nm of each samples.

2.16.2 Effect of pH on growth of selected bio-control agents

For the determination of optimum pH selected bacteria were inoculated in nutrient broth having different pH viz. pH 6, 7, 8 and 9 and incubated at 30^0C in a shaking incubator at 150 rpm for three days. Optical density was measured every day at 600 nm of each samples.

2.17 Antagonism Assay

Antagonism test of selected bacterial isolates were done by disc diffusion assay following methods explained in Vaseeharan and Ramasamy (2003). Colony of candidate bio-control agent was isolated on nutrient agar plates. Candidate bio control agent of *Ralstonia solanacearum* was inoculated in 5 ml nutrient broth and grown at 30^0C on a shaking incubator at 150 rpm for 72 hrs. After incubation cells were removed by centrifugation at 10000×g for 10 minutes and culture supernatant was sterilized by passage through 0.45 µm pore size filters (Millipore). Isolated colony of *R. solanacearum* was pre-cultured in nutrient broth, incubated at 30^0C for 48 hrs and 50µl of this culture was spread over CPG (Appendix A.2) agar plates. 80µl of three days old culture filtrate of candidate bacterial cultures were impregnated on 4 mm diameter sterile disc (Oxoid) and air dried. In one disc, nutrient broth was added as negative control to determine possible inhibitory activity of the medium. These discs were placed on CPG agar plates previously swabbed with the *R. solanacearum* isolates. This preparation was incubated at 30^0C for 24 to 48 hours. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the discs.

Antagonism assay was further evaluated by agar well diffusion method to check the production of antimicrobial metabolites (Sen *et al.*, 1995). CPG agar plates were swabbed with *Ralstonia solanacearum* after it was grown at 30^0C for 2 days in nutrient broth. Wells 5 mm in diameter were made with the help of sterilized borer in the agar plates and 80 µl culture filtrate of the producing organism was poured in the well and incubated for 24 hours at 30^0C. Zone of inhibition around the wells was measured for the activity of antimicrobial compounds.

2.18 Effect of Indigenous Bio-control Agents on Bacterial Wilt Disease Severity and Yield of Tomato in Field

2.18.1 Experimental site

The experiments were carried out in the experimental field of Sher-e-Bangla Agricultural University (SAU), Dhaka. The site of the experimental plot is in $23^0$74

N latitude and $90^0$35E longitudes with an elevation of 8.2 meter from sea level (Anonymous, 1989).

2.18.2 Experimental period

The experiments were carried out during the period of November, 2009 to February, 2010 and November, 2010 to February 2011.

2.18.3 Characteristics of the soil

The soil of the experimental area was non calcarious dark grey and belongs to the Madhupur Tract (UNDP, 1988) under AEZ 28. The selected plot was medium high land and the soil series was Tejgaon (FAO, 1988). The pH of the soil was 5.6. The characteristics of the soil under the experimental plot was analyzed in the SRDI, Soil testing laboratory, Khamarbari, Farmgate, Dhaka and details of the soil characteristics were presented in Appendix C.

2.18.4 Weather condition of the experimental site

The geographical situation of the experimental site was under the subtropical climate characterized by three distinct seasons, the monsoon of rainy season from November to February, the pre-monsoon period of hot season from March to April and monsoon period from May to October (Edris *et al*., 1997) the total annual rainfall of the experimental site was 218mm and average monthly maximum and minimum temperature were 29.45^0 and 13.86^0, respectively. In first and second cropping seasons, the minimum and maximum temperature were 9.6 °C -33.9 °C and 8.2 °C - 33.2 °C, respectively (Appendix C.3). In case of rainfall, only 1-3 mm rainfall recorded in November 2009 and 48 mm rainfall recorded in February 2010 while rainfall was only recorded in December 2010 that was 20-61 mm (Appendix C.4).

2.18.5 Collection of seed samples

Seeds of BARI Tomato 2 (Ratan) were used throughout the investigation and seeds were collected from Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur, Bangladesh.

2.18.6 Design and layout of the experiment

The experiments were laid out in randomized complete block design (RCBD) with three replications. Ten treatments were used in this experiment.

2.18.7 Preparation and sterilization of soil of nursery bed

Soil and cowdung mixed in 2:1 ratio and were thoroughly treated with commercial formalin (40%) at the rate of 5ml formalin diluted with 20ml of water and mixed with 4 kg of soil (Dasgupta, 1998). At the time of adding formalin the soils were constantly shuffled for mixing. The mixed soils were then covered by polythene sheet in order to fumigate the soil. After 48 hrs. the polythene sheets were removed for air drying and escaping of formaldehyde gas. The soil were shuffled again and kept open for 7 days for complete removal of the gas. Finally, the sterilized soil was used for nursery bed.

2.18.8 Growing of tomato seedlings

Tomato seedlings were grown in nursery bed of SAU farm. Sterilized soil was used in nursery for raising seedlings. Seeds of tomato were sown on 16th November, 2009 and 20th November, 2010.

2.18.9 Field preparation

The experimental field was properly ploughed to obtain a good tilth. Manures and fertilizers were used as per recommendation (Rashid, 1999). Cowdung (15ton/ha) was applied during final land preparation. Urea, triple super phosphate (TSP) and muriate of potash (MP) were used at the rate of 300 kg, 250 kg and 200 kg per hectare, respectively. At the time of final land preparation half of Urea, total TSP and MP were mixed with the soil. Two weeks after transplanting rest of the urea was applied in two splits at 15 days interval.

2.18.10 Seedling root treatment

Twenty five days old tomato seedlings were removed from the nursery bed and shaken gently to remove adhering soil. Roots were then washed under running tap

water for 10 minutes. Before planting roots were treated by dipping in suspensions of isolated biocontrol agents containing 10^8 cfu/ml bacterial cells and incase of *Trichoderma harzianum* 10^7cells/ml for 24hrs dipped in sterilized water served as control.

Treatments:

T_1= *Trichoderma harzianum* treated and *Rs* inoculated

T_2= *Trichoderma harzianum* (BAU) treated and *Rs* inoculated

T_3= *Bacillus subtilis* 1treated and *Rs* inoculated

T_4= *Bacillus subtilis* 2 treated and *Rs* inoculated

T_5= *B. licheniformis* treated and *Rs* inoculated

T_6= *Bacillus pumilus* treated and *Rs* inoculated

T_7= *Paenibacillus polymixa* treated and *Rs* inoculated

T_8= *Rhizobium leguminosorum* treated and *Rs* inoculated

T_9= *Pseudomonas fluorescens* treated and *Rs* inoculated

T_{10} = Sterile water treated and *Rs* inoculated

2.18.11 Transplantation of treated tomato seedlings in the field

Treated seeedlings were transplanted in the field on 7th December, 2009. For each treatment seedlings were transplanted in 2m×2m unit plot in which 60 cm row to row and 50 cm plant to plant distance were maintained.

2.18.12 Inocula production and inoculation of *Ralstonia solanacearum* (Rs) in the field

R. solanacearum pre-cultured overnight at 30^0C in CPG medium was inoculated into PS broth and grown 48 hrs with shaking. Cells were collected by centrifugation at 4000×g for 20 minutes, suspended in sterilized distilled water and adjusted to a concentration of approximately 10^8cfu/ml. The spore suspension of *Ralstonia solanacearum* was sprinkled around the transplanted treated plants so that suspension reached at the depth of at least 10-15 cm (Gangopadhay, 1984).

2.18.13 Intercultural operations

The proper growth and development of the plant were ensured by irrigation, weeding and doing other necessary intercultural operations as and when required.

2.18.14 Calculation of wilt intensity

Wilt intensity has been calculated after inoculation by 30, 45 and 60 days according to Winstead and Kelman (1952), using the following formula:

$I = [\Sigma (ni \times vi) \div (V \times N)] \times 100$

Where, I = wilt intensity (%); ni = number of plants with respective disease rating; vi = disease rating (following scale: 1 = no symptoms; 2 = one leaf wilted; 3 = two to three leaves wilted; 4 = four or more leaves wilted; 5 = whole plant wilted); V = the highest disease rating; and N = the number of plants observed.

2.18.15 Harvesting of fruit and data collection

Data were collected on: No. of fruits per plant, Yield per plant (kg), Yield per plot (kg) and Yield per hectare (ton).

2.19 Effect of Indigenous Bio-control Agents on Fungal Wilt Severity (*Fusarium oxysporum* f. sp. *lycopersici*) and Yield of Tomato

2.19.1 Experimental site

As described in 2.18.1. including the following treatments:

T_1= *Trichoderma harzianum* treated and FOL inoculated

T_2= *Trichoderma harzianum*(BAU) treated and FOL inoculated

T_3= *Bacillus subtilis* 1 treated and FOL inoculated

T_4= *Bacillus subtilis* 2 treated and FOL inoculated

T_5= *B. licheniformis* treated and FOL inoculated

T_6= *Bacillus pumilus* treated and FOL inoculated

T_7= *Paenibacillus polymixa* treated and FOL inoculated

T_8= *Rhizobium leguminosorum* treated and FOL inoculated

T_9= *Pseudomonas fluorescens* treated and FOL inoculated

T_{10} = Sterile water treated and FOL inoculated

The experimental site, experimental period, characteristics of the soil, weather condition of the experimental site, collection of seed samples, design and layout of the experiment, preparation and sterilization of soil of nursery bed, growing of

tomato seedlings, field preparation and transplantation of treated tomato seedlings were similar as described in 2.18.1 to 2.18.9 and 2.18.11, respectively.

2.19.2 Inocula production and inoculation of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) in the field.

Fusarium oxysporum f. sp. *lycopersici* was grown on PDA (Potato Dextrose Agar) medium at 25 ^{0}C temperature. After sporulation (15-20 days), added 5-ml/plate sterile water and the spore masses scraped away with sterile needle/scalpel. The conditional suspension thus made with additional water was then blended in a Moulinex blender for 2 minutes in medium speed and filtered through sterile cheesecloth, adjusted concentration 1.2×10^7 conidia/ml solution. Then inoculation done at the root zone of plant by drenching of spore suspension @ 250ml/plant with the help of compressed air hand sprayer following pulverized the soil to mix up the *Fusarium oxysporum* f. sp. *lycopersici* spores thoroughly to the soil.

2.19.3 Intercultural operations: As described in section 2.18.13.

2.19.4 Calculation of wilt intensity: Followed the same procedure described in section 2.18.14.

2.19.5 Harvesting of fruit and data collection: As described in section 2.18.15.

2.20 Effect of Indigenous Bio-control Agents on Severity of Wilt Complex (*Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *lycopersici*) and Yield of Tomato

The experiment was carried out during 2010-2011.

2.20.1 As described in 2.18.1. including the following treatments:

T_1= *Trichoderma harzianum* treated and *Rs*+*FOL* inoculated

T_2= *Trichoderma harzianum*(BAU) treated and *Rs*+*FOL* inoculated

T_3= *Bacillus subtilis* 1 treated and *Rs*+*FOL* inoculated

T_4= *Bacillus subtilis* 2 treated and *Rs*+*FOL* inoculated

T_5= *B. licheniformis* treated and *Rs*+*FOL* inoculated

T_6= *Bacillus pumilus* treated and *Rs*+*FOL* inoculated

T_7= *Paenibacillus polymixa* treated and *Rs*+*FOL* inoculated

T_8= *Rhizobium leguminosorum* treated and *Rs+FOL* inoculated

T_9= *Pseudomonas fluorescens* treated and *Rs+FOL* inoculated

T_{10} = Sterile water treated and *Rs+FOL* inoculated

2.20.2 As described in 2.19.2.

2.20.3 Inocula preparation and inoculation of *Fusarium oxysporum* f. Sp. *lycopersici* and *Ralstonia solanacearum*

Inocula preparation and method of inoculation of *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum* as described in section 2.19.3. and 2.18.12. In case of *Fusarium oxysporum* f. sp. *lycopersici*, inocula mixed with the soil 7 days before transplanting.

2.20.4 Intercultural operations: As described in section 2.18.13.

2.20.5 Calculation of wilt intensity: Followed the same procedure described in section 2.18.14.

2.20.6 Harvesting of fruit and data collection: As described in section 2.18.15.

2.21 Data Analysis

Data were analyzed using MSTAT computer programs. Means were compared following Duncun's Multiple Range Test (DMRT).

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF WILT COMPLEX PATHOGENS OF TOMATO

3.1 Symptoms of Wilted Tomato (*Lycopersicon esculentium)* Plants

The typical symptoms of wilt are loss of rigidity and drooping of plant parts, generally caused by insufficient water in the plants. Several reasons including microbiological cause of wilting of plants are also described earlier (Section 1.1 and Section 1.2).

3.1.1 Symptoms of bacterial (*Ralstonia solanacearum*) wilt

The disease occurred in scattered tomato plants in the field. Characteristic symptom was wilting of the entire tomato plant with no leaf yellowing (Figure 3.1.A). The infected tomato plants showed sudden wilting. The leaves of the infected plants lost turgidity, became flaccid, droop and finally died (Figure 3.1.B). In cross sections of infected stems brown discoloration was found with whitish bacterial ooze (Figure 3.2). Bacterial pockets were developed around the vascular bundles in the pith and in the cortex, and the roots especially stem was found rotten and disintegrated.

3.1.2 Symptoms of fungal (*Fusarium oxysporum* f. sp. *lycopersici*) wilt

The tomato plants showed slight vein clearing on outer leaflets and drooping of leaf petioles. Later the lower leaves beccme wilted, turned yellow and died. In many cases, a single shoot showed wilt symptoms or on only one side of the stem was found affected first (Figure 3.3.A) and progressed upward until the foliage was killed and the stem died (Figure 3.3.B). When the main stem was cut, dark chocolate brown streaks were seen running lengthwise through the stem (Figure 3.4). The discoloration was found often extended upwards for some distance and was especially evident at the point where the petiole joined the stem.

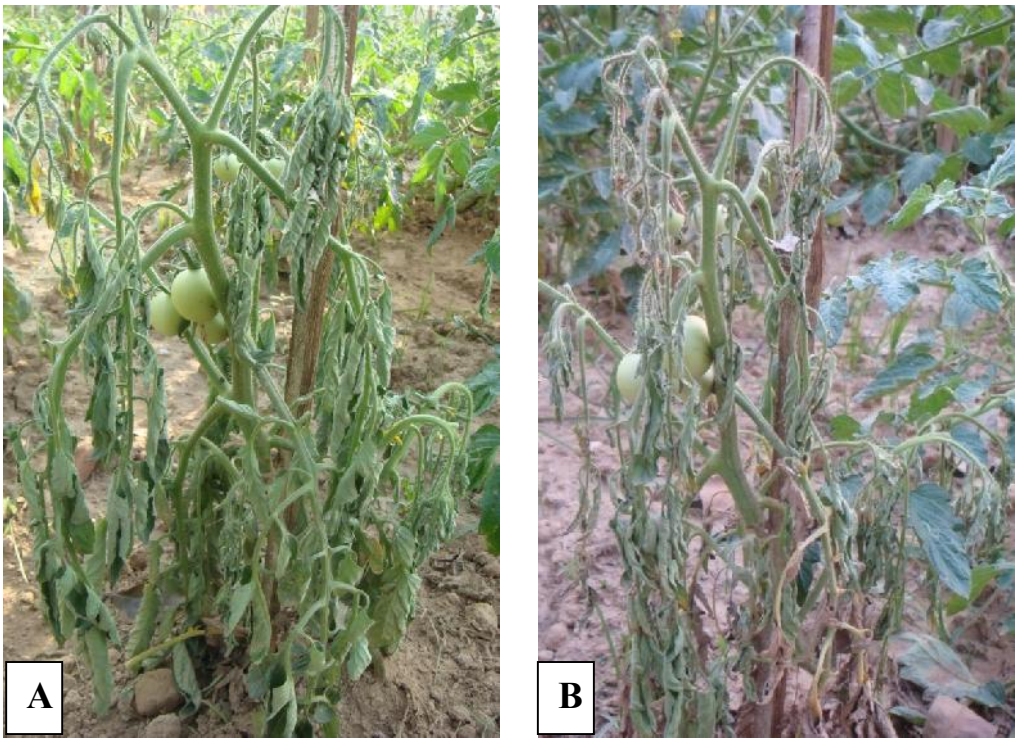


Figure 3.1 Bacterial wilt of infected tomato plants. A, initial stage; B, later stage.

Figure 3.2 Longitudinal section of the stem of tomato showing reddish lesion on vascular bundle.

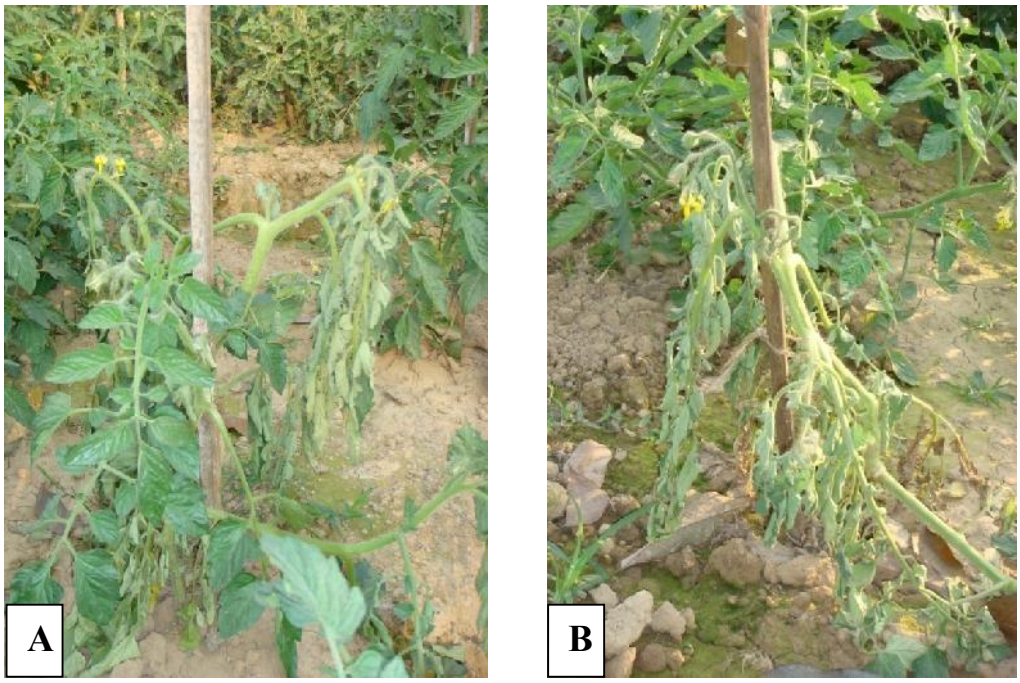


Figure 3.3 *Fusarium* wilt infected tomato plants. A, Earlier stage; B, Later stage.

Figure 3.4 Longitudinal section of *Fusarium* wilt infected tomato stem showing chocolate brown discoloration of vascular bundle.

3.2 Isolation and Characterization of *Ralstonia solanacearum* from Wilted Tomato Plants

3.2.1 Cultural characteristics of *Ralstonia solanacearum*

Twenty strains of *Ralstonia solanacearum* were isolated from wilted plants collected from SAU, BARI and BAU campus. The colonies of all strains of *R. solanacearum* produced highly fluidal, slightly raised and creamy white colonies with light pink or pinkish red centre and irregular margin after 48 hrs of incubation at 30^0C on TTC medium (Figure 3.5.A-B). Creamy white colonies were appeared during multiplication on CPG medium (Figure 3.6). All the strains grew over cetrimide agar (selective for *Pseudomonas s*pp.) and did not grow at 40^0C except isolate SAU5.

3.2.2 Biochemical test of *Ralstonia solanacearum*

Biochemical test results of *R. solanacearum* is presented in Table 3.1. The *R. solanacearum* expressed positive response in oxidase, catalase, motility, nitrate reduction, tobacco hypersensitivity tests (Figure 3.7.A-B) and pectolytic activity tests (Figure 3.8), but the isolate showed negative response in arginine dehydrolase activity.

3.3 Isolation and Identification of *Fusarium oxysporum* f. sp. *lycopersici* from Wilted Tomato Plants

Three strains of *Fusarium oxysporum* f. sp. *lycopersici* were isolated from wilt infected tomato plants (Figure 3.9.A-C). Evaluation of the infected part of tomato plants resulted in isolation of fungal pathogen which was identified and confirmed by observation under different magnifications of a stereomicroscope. The growth characteristics of the isolated fungus from root and stem samples and the morphological characteristics of micro-conidia, macro-conidia and chlamydospores, were observed under a compound microscope. Based on microscopic studies, the fungal pathogen was identified as *Fusarium oxysporum* on the basis of presence, shape and size of macro- and micro-conidia (Leslie and Summerel, 2006). Further confirmation for identification of the pathogen was performed following the standard methods of Burgess and coworkers (1994). Further confirmation was carried out via pathogenicity test.

Table 3.1. Morphological and Biochemical tests for identification of different strains of *Ralstonia solanacearum*.

Strains of *R. solanacearum*	**Gram differentiation**	**Gram reaction**	**Oxidase**	**Catalase**	**Motility**	**Nitrate reduction**	**Arginine dehydrolase activity**	**Tobacco hypersensitivity**	**Pectolytic activity**
SAU 1	+	-	+	+	+	+	-	+	+
SAU 2	+	-	+	+	+	+	-	ND	+
SAU 3	+	-	+	+	+	+	-	+	+
SAU 4	+	-	+	+	+	+	-	ND	+
SAU 5	+	-	+	+	+	+	-	ND	+
SAU 6	+	-	+	+	+	+	-	ND	+
SAU 7	+	-	+	+	+	+	-	ND	+
BARI 1	+	-	+	+	+	+	-	ND	+
BARI 2	+	-	+	+	+	+	-	+	+
BARI 3	+	-	+	+	+	+	-	+	+
BARI 4	+	-	+	+	+	+	-	ND	+
BARI 5	+	-	+	+	+	+	-	ND	+
BARI 6	+	-	+	+	+	+	-	ND	+
BARI 7	+	-	+	+	+	+	-	ND	+
BAU 1	+	-	+	+	+	+	-	ND	+
BAU 2	+	-	+	+	+	+	-	ND	+
BAU 3	+	-	+	+	+	+	-	+	+
BAU 4	+	-	+	+	+	+	-	ND	+
BAU 5	+	-	+	+	+	+	-	ND	+
BAU 6	+	-	+	+	+	+	-	ND	+

ND, Not determined; SAU, Sher-e-Bangla Agricultural University; BARI, Bangladesh Agricultural Research Institute; BAU = Bangladesh Agricultural University.

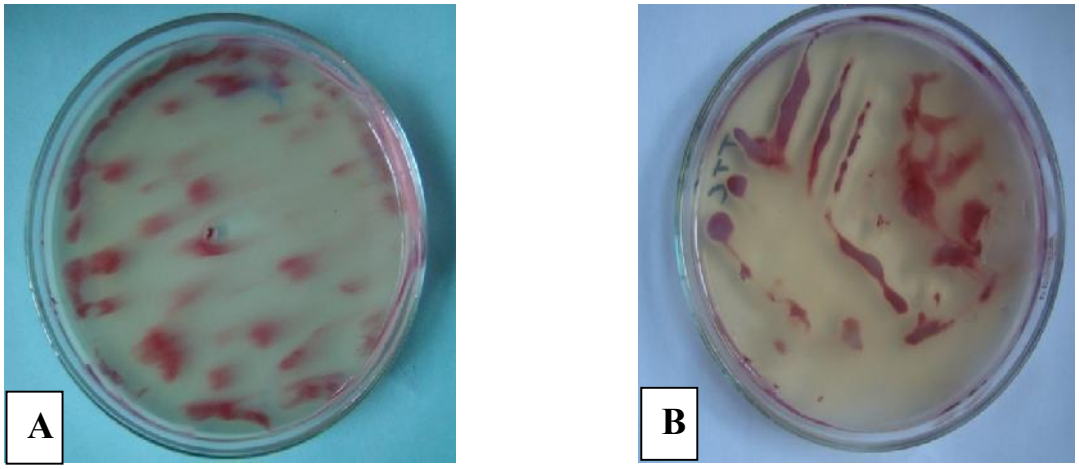


Figure 3.5 Growth of *Ralstonia solanacearum* on TTC medium. (Streaking method). A, Spread plate B, Streak plate

Figure 3.6 Growth of *Ralstonia solanacearum* on CPG medium.

Figure 3.7.A Tobacco hypersensitivity test (control).

Figure 3.7.B Tobacco hypersensitivity test (positive).

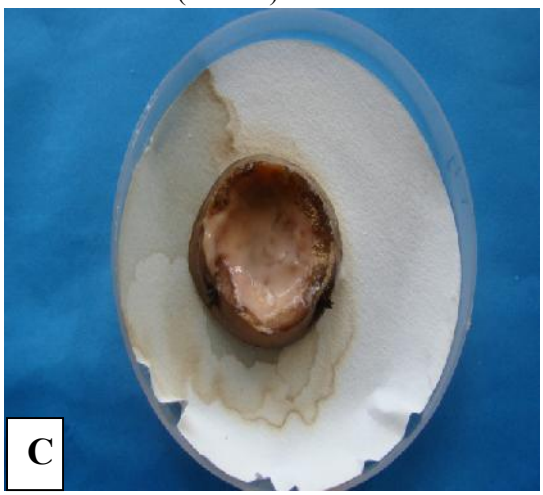


Figure 3.8 Pectolysis test of potato (positive).

Figure 3. 9.A SAU *Fusarium oxysporum* f. sp. *lycopersici.*

Figure 3.9.B BARI *Fusarium oxysporum* f. sp. *lycopersici.*

Figure 3.9.C BAU *Fusarium oxysporum* f. sp. *lycopersici.*

Figure 3.10.A Tomato seedling before inoculation of *Ralstonia solanacearum*.

Figure 3.10.B Bacterial wilt infected tomato seedling after *Ralstonia* inoculation.

3.4 Pathogenecity of *Ralstonia solanacearum*

All the strains of *R. solanacearum* were found to be pathogenic on tomato plants and produced typical symptoms of wilt (Figure 3.10.A-B). The strains SAU 2, SAU 7, BARI 5 and BAU 2 and BAU 6 exhibited the highest disease incidence (100% wilting) followed by SAU 1, SAU 6, BARI 1, BARI 3, BARI 4, BAU1 and BAU 5 where wilt incidence were recorded 80%. The lowest percentage (40% wilting) was recorded with BARI6. All the strains of *R. solanacearum* produced characteristics symptoms after 3-12 days of inoculation depending on the strains (Table 3.2). Strain SAU2 required minimum time (3.33days) for symptoms expression which was statistically similar with BARI 3 and BARI 4. And the maximum days required (11.33 days) for symptoms expression in strain BAU 2 which was statistically identical BARI 6. The variation of incubation period to manifest wilt symptoms among the strains varied possibly due to variation in virulence. According to the above results strain SAU 2 was selected and used for further study.

3.5 Pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici*

Tomato seedlings were inoculated with *F. oxysporum* f. sp. *lycopersici* using spore suspension with conidial concentration of 10^5 conidia/ml by collar region inoculation method, were found to be an effective and high virulent, where, and inoculated plants expressed severe infection with the typical sign of symptom like leaf chlorosis. The diseased leaves wilted and dried up. Dropping and wilting of the stem tip was also observed. The diseased plants wilted down and dried up completely. Their roots were necrotic and rotten, and the necrosis spread to the lower stem. In contrast, control plants were completely free from disease (Figure 3.11A-B).

3.6 Morphological and Conidial Characteristics of Different Strains of *F. oxysporum* f. sp. *lycopersici*

Morphological and conidial characteristics of the strains of *Fusarium oxysporum* f. sp. *lycopersici* were observed and shown in Table 3.3 and Table 3.4. The radial mycelial growth of *F. oxysporum* f. sp. l*ycopersici* was found different from one

strain to another. Radial mycelial growth varied from 65 to 74 mm after 7 days of incubation, where the lowest and the highest value counted from BARI *Fu* and SAU Fu, respectively. Colony color was recorded as pinkish white in BARI Fu and BAU Fu and purplish white in SAU Fu. In SAU Fu consistency was found compact, in BARI *Fu* it was medium fluffy and in BAU *Fu* was fluffy.

Fusarium oxysporum f. sp. *lycopersici* produced microconidia in abundance (Figure 3.12A). They were oval shaped, hyaline, usually non-septate but one septate conidia also found. It also produced few number of macro-conidia, which were hyaline, thin walled 3 septate, falcate to almost straight. At later stage *Fusarium* produced huge number of chlamydospores (Figure 3.12B). Length and breadth of micro conidia at 7 days of incubation were found different from one strain to another. Length of micro-conidia varied from 7.93 to 8.38 (µm) where the highest and the lowest value were recorded from BAU Fu and BARI Fu, respectively. Breadth of micro conidia varied from 2.10 to 2.40 µm, where the highest and the lowest value recorded from BAU Fu and BARI Fu, respectively. Total numbers of conidia were found different from one strain to another. Total number of conidia was recorded maximum in BAU Fu (252412.77).

3.7 Evaluation of Inoculation Methods of *Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *lycopersici*

Significant variations were observed among different methods of inoculation of wilt causing pathogens of tomato (Table 3.5 and Figure 3.13-3.16). In case of bacterial wilt the highest number (17.67%) of wilted plants was counted when seedlings were inoculated following collar region inoculation method followed by soil drenching (14.0%) and the lowest number of wilted plants (8.0%) was recorded from root dipping method of inoculation. In case of Fusarium wilt the maximum number of wilted plants (12.67%) was recorded when seedlings were inoculated following sick bed inoculation method which was statistically identical (11.33%) with the result of collar region inoculation method. The minimum number of wilted plants (6.33%) was counted from root dipping method of inoculation.

Table 3.2. Pathogenicity tests of *Ralstonia solanacearum*.

Strains of *Ralstonia solanacearum*	Wilted Plants (%)	Days required for symptoms expression
SAU 1	80	5.33fg
SAU 2	100	3.33i
SAU 3	60	6.33cd
SAU 4	60	6.33cd
SAU 5	60	8.33b
SAU 6	80	6.67c
SAU 7	100	6.00de
BARI 1	80	5.33fg
BARI 2	60	4.33h
BARI 3	80	3.67i
BARI 4	80	3.67i
BARI 5	100	5.33fg
BARI 6	40	11.0a
BARI 7	60	5.67ef
BAU 1	80	5.33fg
BAU 2	100	11.33a
BAU 3	60	3.67g
BAU 4	60	5.00g
BAU 5	80	5.00g
BAU 6	100	5.00g
LSD (0.5%)	ND	0.4149
SE	ND	

ND, Not determined; SAU, Sher-e-Bangla Agricultural University; BARI, Bangladesh Agricultural Research Institute; BAU, Bangladesh Agricultural University.

Table 3.3. Morphological characteristics of different strains of *Fusarium oxysporum* f. sp. *lycopersici*.

Fusarium strains	Morphological characters at 7 DAI				
	Radial mycelial growth (mm)	Colony color		Colony margin	Consistency
		Font	Reverse		
SAU Fu	74	Purplish white	Purple	Regular	Compact
BARI Fu	65	Pinkish white	Pink	Regular	Medium fluffy
BAU Fu	71	Pinkish white	Pink	Regular	Fluffy

SAU Fu, *Fusarium strain* collected from Sher-e- Bangla Agricultural University; BARI Fu, *Fusarium strain* collected from Bangladesh Agricultural Research Institute; BAU Fu, *Fusarium strain* collected from Bangladesh Agricultural University.

Figure 3.11.A Tomato seedling before *Fusarium oxysporum* f. sp. *lycopersici i*noculation.

Figure 3.11.B Wilted plant of tomato after inoculation of *Fusarium oxysporum* f. sp. *lycopersici.*

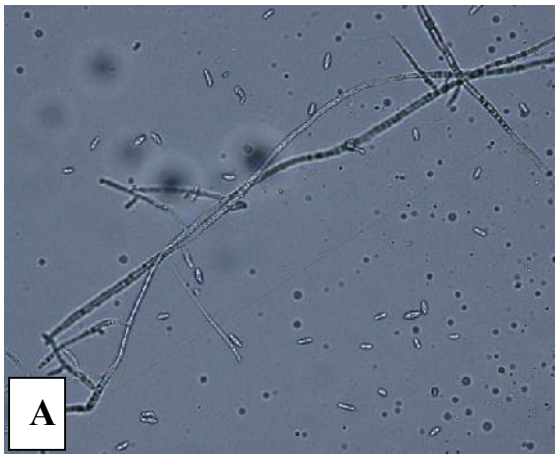


Figure 3.12.A Mycelia, Conidia , conidiophores of *Fusarium oxysporum* f. sp. *lycopersici.*

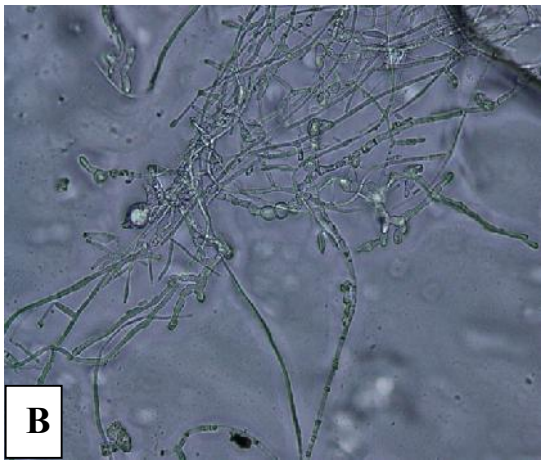


Figure 3.12.B Chlamydosores of *Fusarium oxysporum* f. sp. *lycopersici.*

Figure 3.13 Transplanted tomato seedling in *Ralstonia solanacearunm* inoculated soil at early stage.

Figure 3.14 Transplanted tomato seedling in *Ralstonia solanacearunm* inoculated soil at later stage showing wilted seedlings.

Figure 3.15 Transplanted tomato seedling in *Fusarium oxysporum* f. sp. *lycopersici* inoculated soil at early stage.

Figure 3.16 Transplanted tomato seedling in *Ralstonia solanacearunm* inoculated soil at later stage showing wilted seedlings.

Table 3.4. Conidial characters of strains of *Fusarium oxysporum* f. sp. *lycopersici*.

Fusarium strains	Conidial characters at 7 DAI						
	Average size of the conidia (µm)				No. of conidia/cm^2		
	Micro		Macro		Micro	Macro	Total
	Length	Breadth	Length	Breadth			
SAU Fu	8.25	2.23	10.58	6.10	240805.41	1020.22	241825.63
BARI Fu	7.93	2.10	10.18	5.12	250602.39	1810.38	252412.77
BAU Fu	8.38	2.40	12.38	6.22	212308.78	2544.33	214853.11

Table 3.5. Evaluation of inoculation methods of *Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *lycopersici*.

Methods of inoculation	Wilted plants (%)	
	Bacterial wilt	Fusarium wilt
Collar region	17.67 a	11.33 ab
Root dipping	8.00 d	6.33 c
Soil drenching	14.0 b	11.0 b
Sick bed	11.67 c	12.67 a
LSD (0.050)	1.216	1.631

3. 8 Discussion

Plants showing typical symptoms of bacterial wilt (Kelman, 1954) were collected from 3 different areas of Bangladesh. Isolation of the bacterium was made and tentative diagnosis of the diseased plants was made by water streaming test (Danks and Barker, 2000) bacterial ooze was seen from the bits of infected plants. Characterization of strains of *R. solanacearum*, the causal agent of tomato bacterial wilt disease was performed based on pathogenecity, biochemical and physiological tests. The bacterial ooze was subjected to morphological studies revealed that the strains were Gram negative rod shaped, non-capsulated and non-spore forming. *R. solanacearum* produced highly fluidal, slightly raised and creamy white colonies with light pink or pinkish red centre and irregular margin on TTC medium. The colony characters indicated that all strains were virulent. Similar colony characters for virulence of *R. solanacearum* were described by Hayward (1964), Shekhawat *et al.* (1992) and Mehan and Mcdonald (1995). Twenty pure cultures of pathogen strains have typical cultural characteristics and were proving to be pathogenic and

causing wilt symptoms to tomato plants were identified as *Ralstonia solanacearum* according to their morphological and physiological characteristics as reported by Hayward (1964) and Krieg and Holt (1984).

Characterization of three strains of *F. oxysporum* f. sp. *lycopersici* was performed based on pathogenecity, colony morphology, shape and size of macro and micro condia etc. The straind fungus identified on the basis of its morphological characteristics, which is the most difficult step in the process of identification (Rahjoo *et al.,* 2008). In the present study, following the growth characteristics on PDA the fungus formed hyaline, branching mycelium that was white, gray to light pink in color. All tomato fusarium strains rendered colonies with conidia and mycelia with morphological characteristics typical for *F. oxysporum* (Burgess *et al*., 1994). The aerial mycelium appears white and may subsequently change in color ranging from gray to violet and dark purple depending on the strain (or special form) of *F*. *oxysporum* (Smith *et al.*, 1988).

Among the four inoculation methods tested collar region inoculation showed the highest percent wilted plants in case of bacterial wilt followed by soil drenching method and sick bed inoculation method. In collar region method bacteria was able to infect directly into the vascular bundle of the plant, as a result more plants became wilted within a short period of time. Higher wilting due to stem inoculation as compared to root inoculation was also reported by French and Lindo (1982). Hanson *et al.* (1996) also found that the soil drenching method of inoculation was ideal in getting maximum percent of wilt incidence in tomato. In case of Fusarium wilt sick bed method of inoculation was found superior to other method of inoculation. Similar observation also made by Purwati *et al*. (2008).

.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF INDIGENOUS BIO-CONTROL AGENTS

4.1 Isolation and Identification of *Trichoderma harzianum*

Trichoderma harzianum produced dark green mycelial colony on PDA and growth rate was faster (Figure 4.1.A-B). Microscopic study revealed that it produced hyaline conidiophores, which was upright, much branched, not verticillate, phialides were found in groups, conidia were hyaline, single celled, ovoid and was borne in small terminal clusters (Figure 4.2.A-C).

4.2 Isolation and Identification of *Bacillus* spp.

Out of sixty strains of *Bacillus* spp., only five strains (Table 4.1) showed antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum* and those strains grew over Vessal medium which is selective for *Bacillus* spp. (Figure 4.3.A). Also, Morphological and biochemical characteristics showed that these strains belong to *Bacillus* spp. The traditional approach to the identification of *Bacillus* was based on the morphological groups mentioned. The bacterial strains were identified to the genus and species level using a panel of physiological and biochemical tests (Table 4.2, Figure 4.4.A-B) according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). Among five strains of *Bacillus* two strains were of *B. subtilis* which were coccobacilli shaped (Figure 4.3.B) others shapes were bacilli shaped. These *Bacillus* spp. were identified as *Bacillus subtilis* 1, *B. subtilis* 2, *B. licheniformis*, *B. pumilus* and *Paenibacillus polymixa.*

4.3 Isolation and Identification of *Pseudomonas fluorescens*

Out of Twenty Pseudomonas *fluorescens* only one showed antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum.* Pseudomonalds produced diffusible yellow, green or blue fluorescent pigments on KB medium after 24 to 48 hrs of growth (Figure 4.5). *Pseudomonas fluorescens* was identified on the basis of LOPAT (described in 2.14.3) tests. *P. fluorescens* showed

positive reaction in Levan, oxidase and arginine dehydrolase activity and negative in pectiolytic and tobacco hypersensitivity reaction. Biochemical tests results is shown in Table 4.3.

4.4 Isolation and Identification of *Rhizobium leguminosorum*

Rhizobium leguminosorum identified on the basis of its morphological and biochemical tests results (Figure 4.6 and Table 4.4). It produced semi-translucent, raised and mucilaginous colonies on YMA plates. Out of ten strains of *R. leguminosorum* only one showed antagonistic against *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum* and selected for the experiment. *Rhizobium leguminosorum* showed positive reaction in motility and negative in H_2S production, growth in anaerobic growth, growth in 2% NaCl, and growth at 40^0C and congo red test.

4.5 *In-vitro* Screening of Indigenous Bacterial Bio-control Agents against *R. solanacearum*

All the straind bacteria were tested against *Ralstonia solanacearum* following perpendicular streak method (Egorov, 1985). This test revealed that five strains of *Bacillus* namely *B. subtilis* strain 1, *B. subtilis* strain 2, *B. licheniformis*, *B. pumilus* and *Paenibacilus polymixa* (Syn*: Bacillus polymixa*), one strain of *Pseudomonas fluorescens* and one strain of *Rhizobium leguminosorum* produced inhibition zone against *R. solanacearum* (Figure 4.7.A-B).

4.6 *In-vitro* Screening of *Trichoderma harzianum* against *Ralstonia solanacearum*

In paper disc method *T. harzianum* and *T. harzianum* (BAU) produced 15 mm and 12 mm diameter inhibition zone against *R. solanacearum,* respectively (Figure 4.8).

4.7 *In-vitro* Screening of *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *lycopersici*

Interaction between *F. oxysporum* f. sp. *lycopersici* and *T. harzianum* (Table. 4.5) revealed that *T. harzianum* inhibited the growth of *F. oxysporum* f. sp. *lycopersici* (TIF) which exhibited 78.37% inhibition over control and another *T. harzianum*

(BAU) grew over *F. oxysporum* f. sp. *lycopersici* (TGF) which exhibited 83.78% inhibition over control (Figure 4.9.A-B and Figure 4.10).

Table 4.1. Morphological tests for identification of *Bacillus* spp.

Strains	Gram staining	Shape	Spore formation	Motility
Bacillus subtilis 1	+	Coccobacilli	+	+
Bacillus subtilis 2	+	Coccobacilli	+	+
B. licheniformis	+	Bacilli	+	+
Bacillus pumilus	+	Bacilli	+	+
Paenibacillus polymixa	+	Bacilli	+	+

Table 4.2. Biochemical test for identification of *Bacillus* spp.

Tests	*Bacillus subtilis* 1	*Bacillus subtilis* 2	*Bacillus licheniformis*	*Bacillus pumilus*	*Paenibacillus polymyxa*
Utilization of citrate	+	+	+	+	-
Oxidase	+	+	+	-	+
Catalase	+	+	+	+	+
Starch hydrolysis	+	+	+	-	-
Nitrate reduction	+	+	+	-	+
Indole production	-	-	-	-	+
Gas production from glucose	-	-	+	-	+
Methyl red	-	-	-	+	+
Voges Proskaur	+	+	+	+	+

Table 4.3. Biochemical test for identification of *Pseudomonas fluorescens*.

Tests	*Pseudomonas fluorescens*	Pathogenic *Pseudomonas*
Levan	+	-
Oxidase	+	-
Pectiolytic activity	-	+
Arginine dehydrolase	+	-
Tobacco hypersensitivity	-	+
Motility	+	+
Nitrate to N_2	+	-
Gelatin liquefaction	+	ND

Table 4.4. Biochemical test for identification of *Rhizobium liguminosorum*.

Characteristics	*Rhizobium liguminosorum*
Motility	+
H_2S Production	-
Anaerobic growth	-
Growth in presence of 2% Nacl	-
Growth at 40^0C	-
Congo red	-
Growth on glucose peptone agar	-

Table 4.5. Interaction studies between *Fusarium oxysporum* f. sp. *lycopersici* and *Trichoderma harzianum.*

Strains of *T. harzianum*	Interaction	% growth inhibition
T. harzianum	TIF	78.37
T. harzianum (BAU)	TGF	83.78

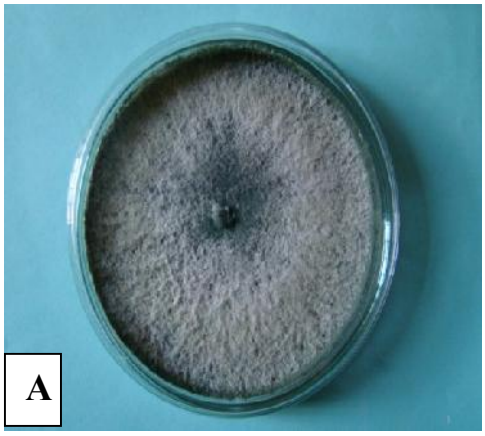


Figure 4.1.A Pure culture of *Trichoderma harzianum.*

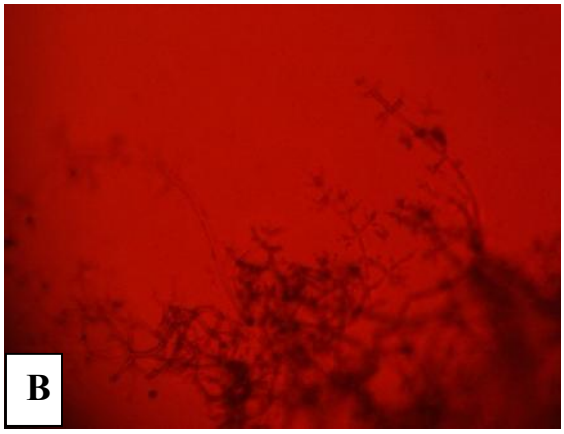


Figure 4.1.B Mycelia, conidiophores and conidia of *Trichoderma harzianum* under compound microscope at 10x stained with safranin.

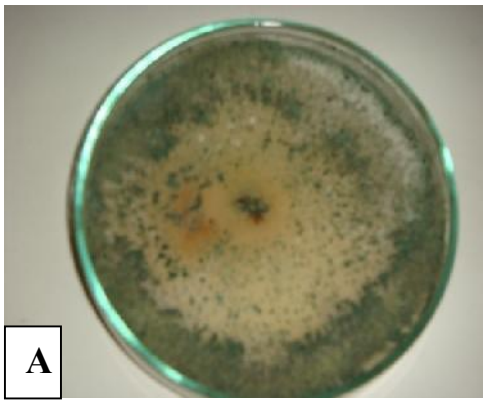


Figure 4.2.A Pure culture of *Trichoderma harzianum* (BAU).

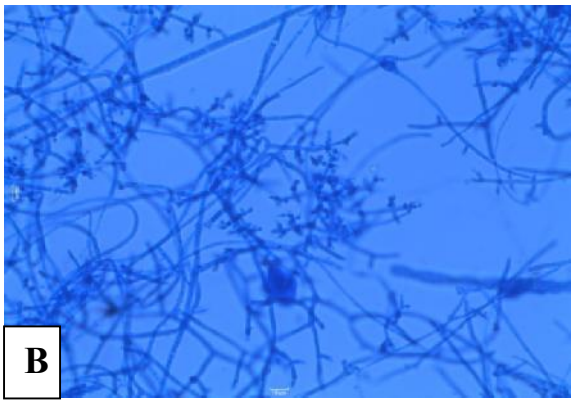


Figure 4.2.B Mycelia, conidiophores and conidia of *Trichoderma harzianum* (BAU) under compound microscope at 10x stained with cotton blue.

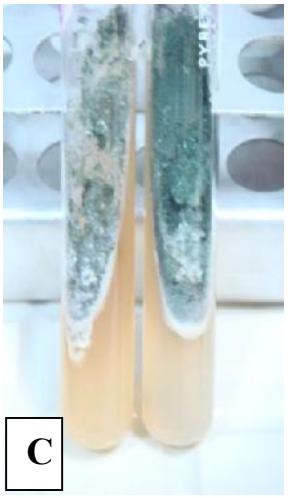


Figure 4.2.C Stock cultures of *Trichoderma harzianum.*

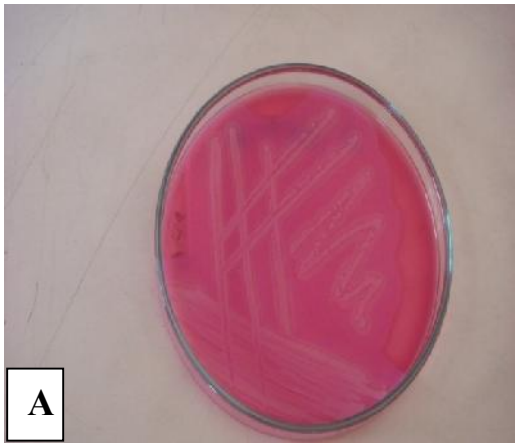


Figure 4.3.A Growth of *Bacillus* on vessal medium.

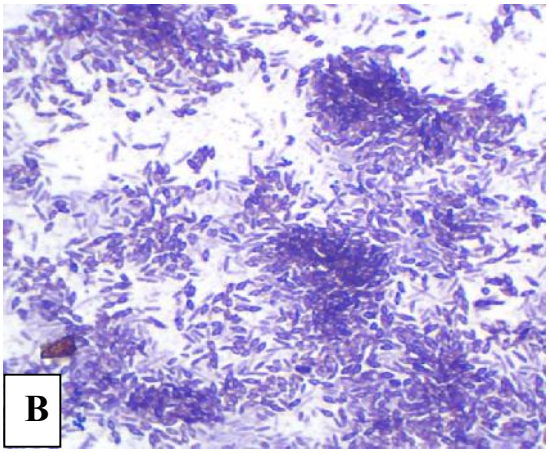


Figure 4.3.B *Bacillus subtilis* under compound microscope showing coccobacilli shaped cell.

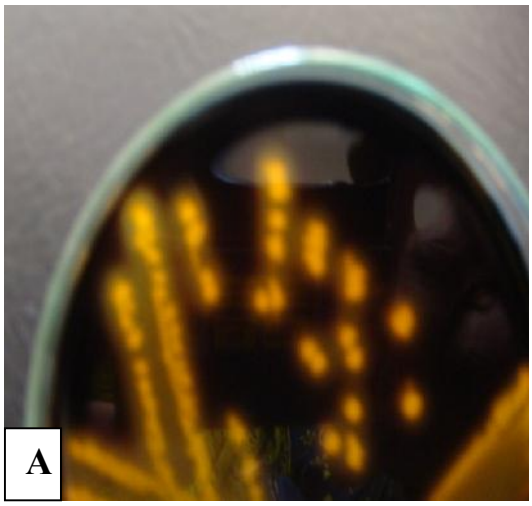


Figure 4.4.A Starch hydrolysis test. (positive)

Figure 4.4.B Citrate utilization test.

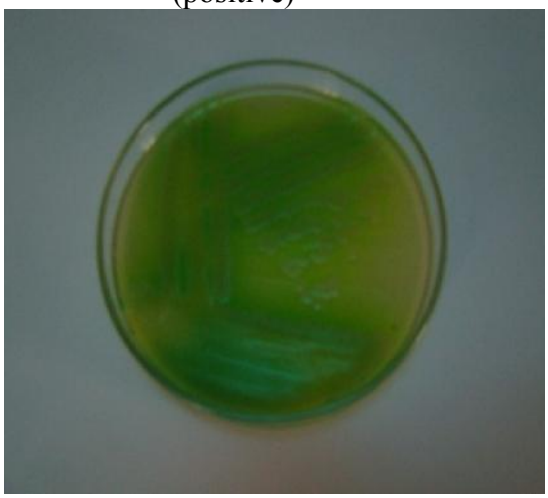


Figure 4.5 *Pseudomonas fluorescens* on KB medium.

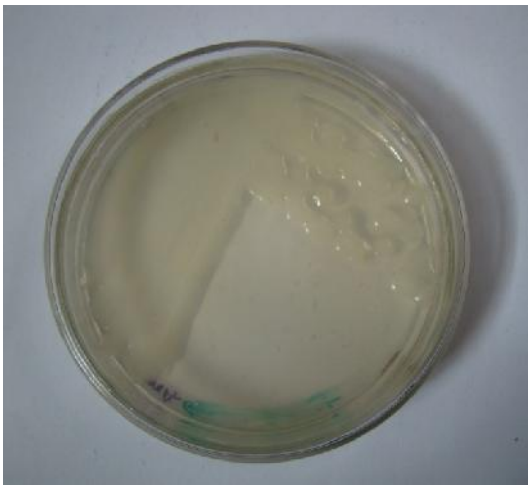


Figure 4.6 Growth of *Rhizobium leguminosorum* on YMA medium.

Figure 4.7.A Screening of *Bacillus licheniformis* against *R. solanacearum.*

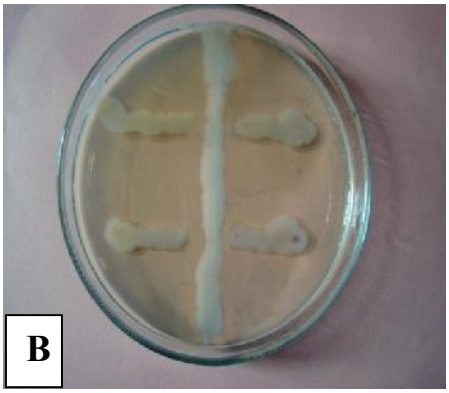


Figure 4.7.B Screening of *Bacillus subtilis* against *R. solanacearum*.

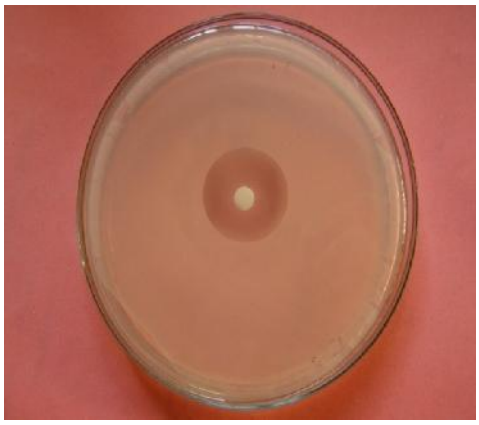


Figure 4.8 Screening of *Trichoderma harzianum* against *Ralstonia solanacearum.*

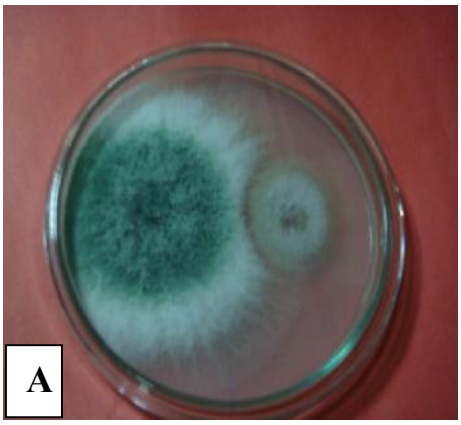


Figure 4.9.A Dual culture of *Trichoderma harzianum* and *F. oxysporum* f. sp. *lycopersici* after 3days after incubation.

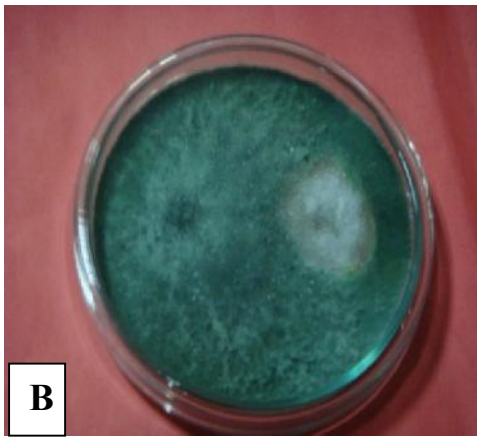


Figure 4.9.B Dual culture of *Trichoderma harzianum* and *F. oxysporum* f. sp. *lycopersici* after 7days after incubation.

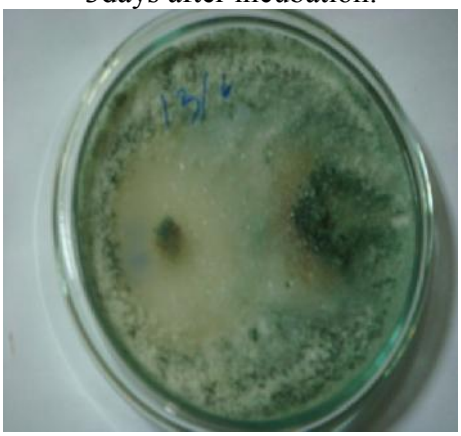


Figure 4.10 *Trichoderma harzianuim* (BAU) grew over *Fusarium oxysporum* f. sp. *lycopersici* after 7 days of incubation.

4.8 *In-vitro* Screening of Indigenous Bacterial Bio-control Agents against *Fusarium oxysporum* f. sp. *lycopersici*

The study conducted revealed that seven bacterial strain significantly inhibit the growth of *F. oxysporum* f. sp. *lycopersici* in dual culture method after different days of incubation (Table 4.6 and Figure 4.11.A-G). After 4 days of incubation *B. subtilis* 1 produced the highest (36.81%) zone of inhibition followed by *Paenibacillus polymixa* (33.33%) which was statistically simillar with *B. licheniformis* and *P. flourescens* where the zone of inhibition were 32.64% and 31.94%, respectively. At 4 days after incubaton *B. pumilus* produced lowest inhibition zone (18.06%) against *F. oxysporum* f. sp. *lycopersici.* After 7 days of incubation *Rhizobium leguminosorum* produced the highest (65.56%) zone of inhibition followed by *B. subtilis* 1 (20.74%) and lowest (12.22%) zone of inhibition was produced by *B. pumilus.*

Table 4.6. Effect of indigenous bacterial bio control agents against *F. oxysporum* f. sp. *lycopersici* through dual culture method.

Bacterial strains	Zone of inhibition (%) over control at different hours of time exposure	
	4 days	7 days
B. subtilis 1	36.81 a	20.74 b
B. subtilis 2	27.78 c	17.78 b c
B. pumilus	18.06 e	12.22e
B. licheniformis	32.64 b	17.04 c
Paenibacillus polymixa	33.33 b	14.44 d
P. flourescens	31.94 b	19.15b
Rhizobium leguminosorum	24.31 d	65.56 a
LSD $_{(0.01)}$	2.532	2.074
CV (%)	3.76	3.66
SE	0.60	0.49

Values in the column are the means of three replicates. In a column, means with same letters are statistically similar with each other.

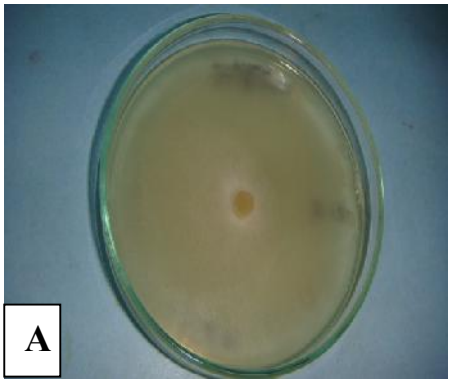


Figure 4.11.A Growth of *Fusarium oxysporum* f. sp. *lycopersici* on NA medium (Control

Figure 4.11.B *Bacillus subtilis* 2 and *F. oxysporum* f. sp. *lycopersici.*

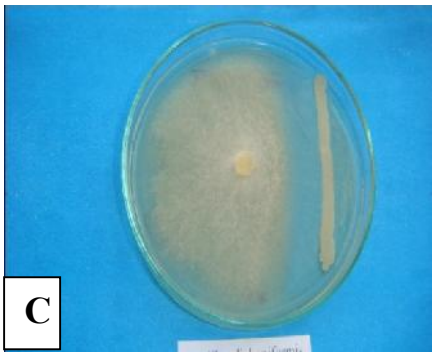


Figure 4.11.C *Bacillus licheniformis* and *F. oxysporum* f. sp. *lycopersici.*

Figure 4.11.D *Bacillus pumilus* and *F. oxysporum* f. sp. *lycopersici.*

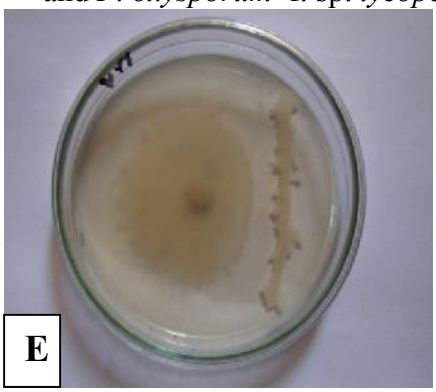


Figure 4.11.E *Paenibacillus polymixa* and *F. oxysporum* f. sp. *lycopersici.*

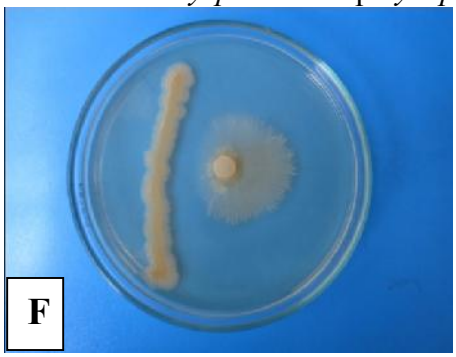


Figure 4.11.F *Rhizobium leguminosorum* and *F. oxysporum* f. sp. *lycopersici.*

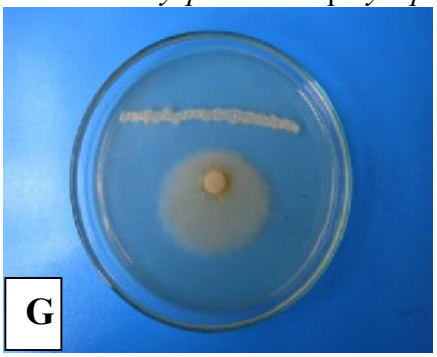


Figure 4.11.G *Pseudomonas fluorescens* and *F. oxysporum* f. sp. *lycopersici*

4.9 Comparative Effectiveness of Dual Culture in Streaking and Spreading Method on Zone of Inhibition (%) over Control of Bacterial Antagonists against *Fusarium oxysporum* f. sp. *lycopersici* after 7 Days of Incubation

A significant difference was observed in streaking and spreading method on zone of inhibition (%) over control of bacterial antagonists against *Fusarium oxysporum* after 7 days of incubation (Figure 4.12). In case of dual culture in sttreaking method zone of inhibition varied from 12.22 %to 65.56% in which the lowest and the highest value counted from *B. pumilus* and *R. leguminosorum*, respectively. In case of spreading method zone of inhibition varied from 56.08% to 64.27%, where the lowest and the highest value counted from *B. pumilus* and *B. licheniformis,* respectively. Comparatively in streaking method bacterial strains inhibited less than spreading method except *Rhizobium leguminosorum* (Figure 4.13.A-F).

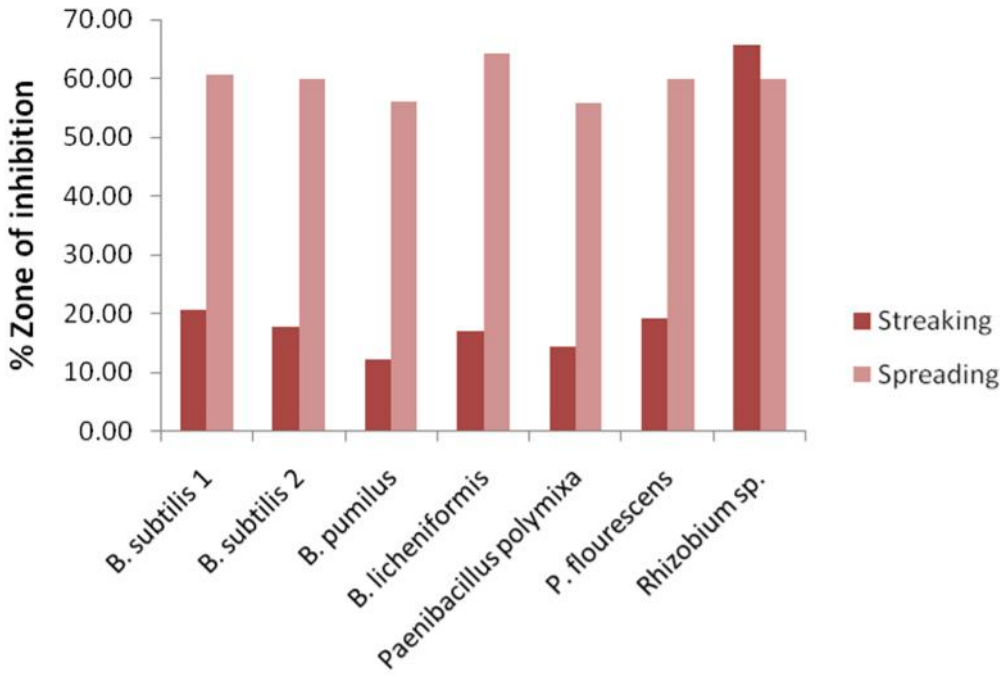


Figure 4.12 Comparative effectiveness of streaking and spreading method on zone of inhibition (%) over control produced by bacterial antagonist against *F. oxysporum* f. sp. *lycopersici* after 7 days of incubation.

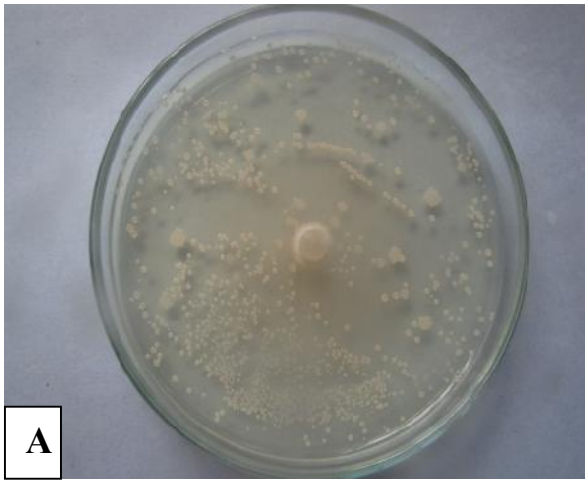


Figure 4.13.A *Bacillus subtilis* 2 and *F. oxysporum* f. sp. *lycopersici.*

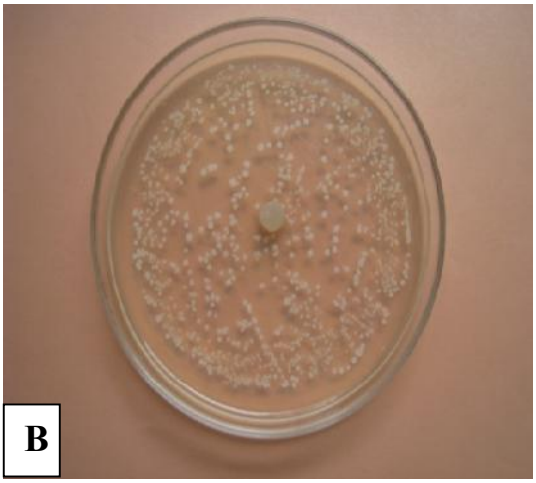


Figure 4.13.B *Bacillus licheniformis* and *F. oxysporum* f. sp. *lycopersici.*

Figure 4.13.C *Bacillus pumilus* and *F. oxysporum* f. sp. *lycopersici.*

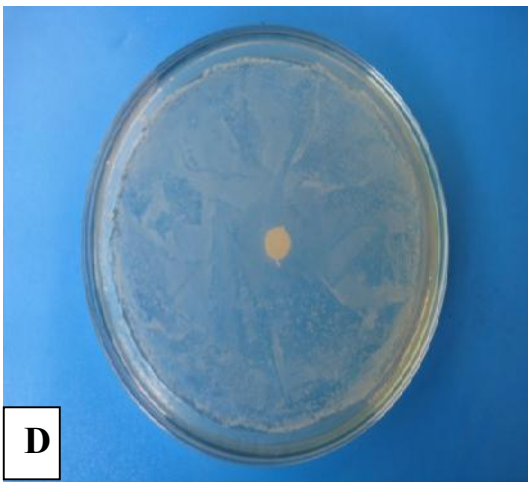


Figure 4.13.D *Paenibacillus polymixa* and *F. oxysporum* f. sp. *lycopersici.*

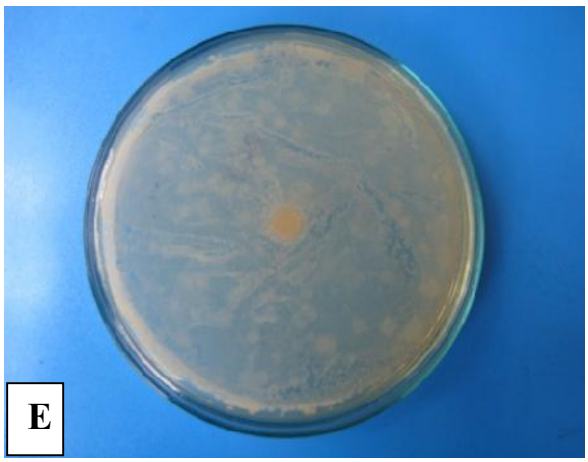


Figure 4.13.E *Rhizobium leguminosorum* and *F. oxysporum* f. sp. *lycopersici.*

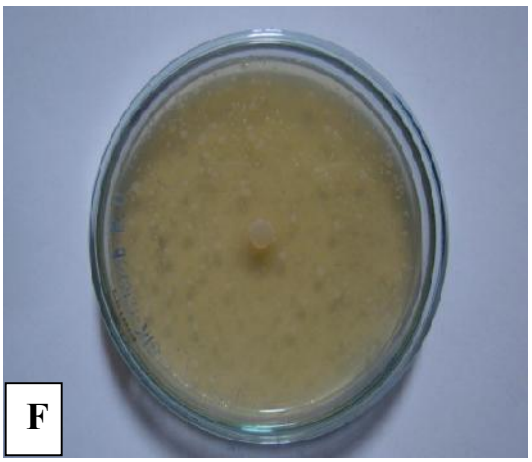


Figure 4.13.F *Pseudomonas fluorescens* and *F. oxysporum* f. sp. *lycopersici*.

4. 10 Carbohydrate Fermentation Test of Bacterial Bio-control Agents

Carbohydrate fermentation test results of different bio control agents were shown in Table. 4.7.

Table 4.7. Carbohydrate fermentation test results of different indigenous bio-control agents.

Bio-control agents	Arabinose	Sucrose	Maltose	Lactose	Dextrose
B. subtilis 1	+	+	+	+	+
B. subtilis 2	+	+	+	+	+
B. pumilus	+	+	+	+	+
B. licheniformis	+	+	+	-	+
Paenibacillus polymixa	+	+	+	+	+
P. flourescens	+	+	+	+	+
Rhizobium leguminosorum	+	+	+	+	+

4.11 Antagonism Assay of Indigenous Bio-control Agents against *Ralstonia solanacearum*

Disc diffusion analysis (Figure 4.14) revealed that culture filtrates of *Bacillus subtilis* 1, *Bacillus subtilis* 2 and *Pseudomonas fluorescens* produced clear zone of inhibition against the growth of *Ralstonia solanacearum* (Figure 4.15.A-B) and significant variation was observed. Culture filtrates of other strains did not show any inhibition against *R. solanacearum.*

At 24 hrs the highest zone of inhibition (22.67mm) recorded in *B. subtilis* 1 followed by *B. subtilis* 2 and the lowest (19.33mm) in *P. fluorescens*. At 48 hrs of exposure time no change of inhibition zone was observed in case of *B. subtilis* 1 and *Pseudomonas fluorescens* but a bit decrease was observed in case of *B. subtilis* 2. A gradual decreased of inhibition zone were observed with time.

In well diffusion method the highest zone of inhibition (27.67mm) produced by *B. subtilis* 1 and the lowest (25.0mm) in *P. fluorescens* at 24 hrs of exposure time (Figure 4.16.A-B). At 48 hrs of time effect of B. subtilis 1 was identical (27.67mm) with 24 hrs of time. A bit increase of inhibition was observed in case of *B. subtilis* 2 which was 26.33mm against *Ralstonia solanacearum*.

4. 12 Effect of Incubation Period on Zone of Inhibition

Relationship between exposure time and zone of inhibition produced by culture filtrates of *Bacillus subtilis* 1, *Bacillus subtilis* 2 and *Pseudomonas fluorescens* against *Ralstonia solanacearum* were studied (Figure 4.17-Figure 4.20). The study revealed that a negative correlation was observed between time and inhibition zone means that zone of inhibition decreased over time.

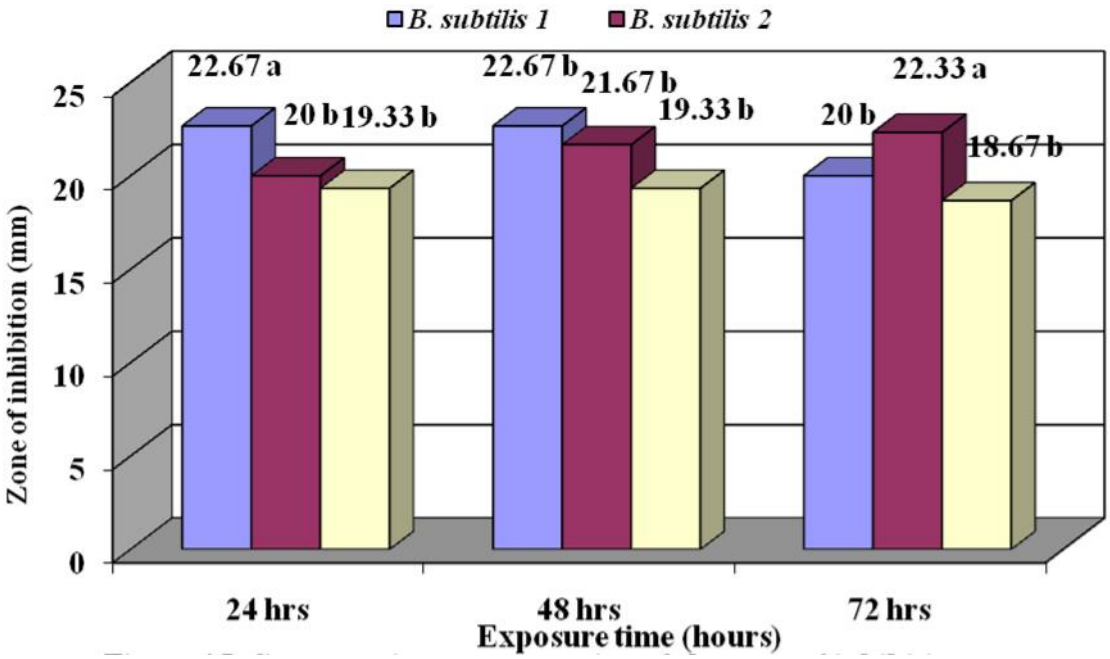


Figure 4.14 Comparative representation of the zone of inhibition produced by bacterial strains against *Ralstonia solanacearum* at different exposure time by disc diffusion method.

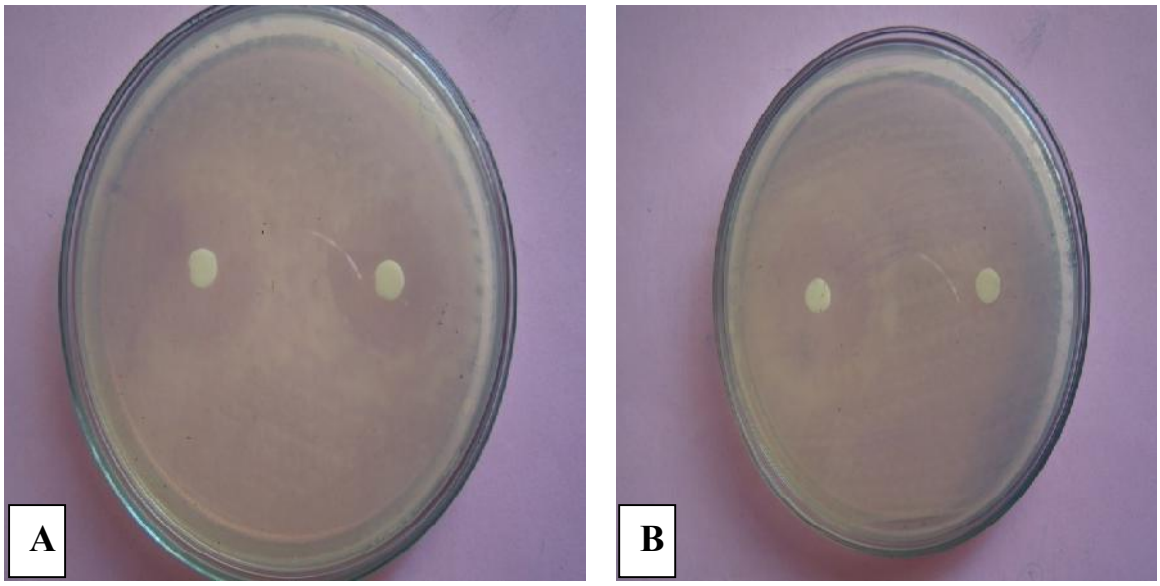


Figure 4.15 Culture filtrate of *Bacillus subtilis* 1. (A) and *Bacillus subtilis* 2 (B) against *Ralstonia solanacearum* (Disc method).

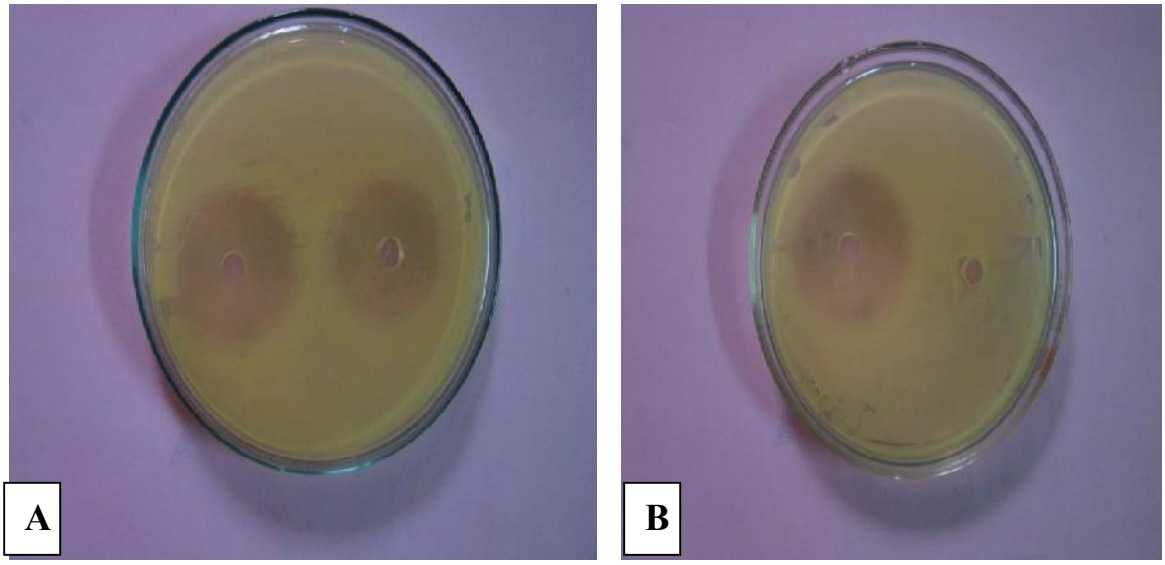


Figure 4.16 Culture filtrate of *Bacillus subtilis* 1 (A) and *Bacillus subtilis* 2 (B) against *Ralstonia solanacearum* (Well method).

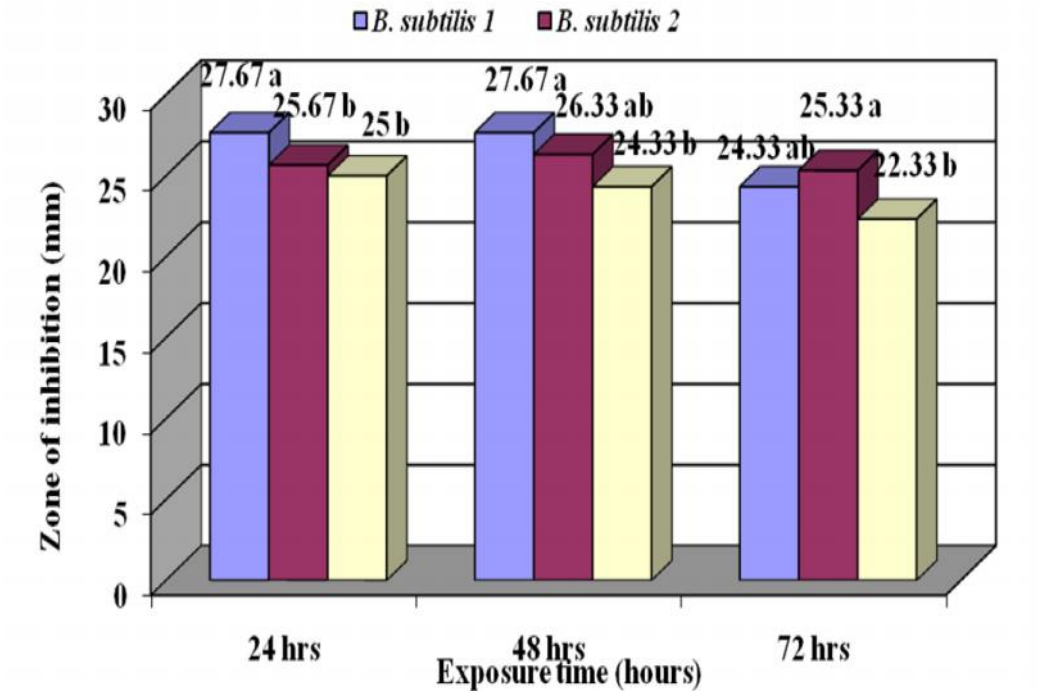


Figure 4.17 Comperative representation of the zone of inhibition produced by bacterial strains against *Ralstonia solanacearum* at different exposure time through well diffusion method.

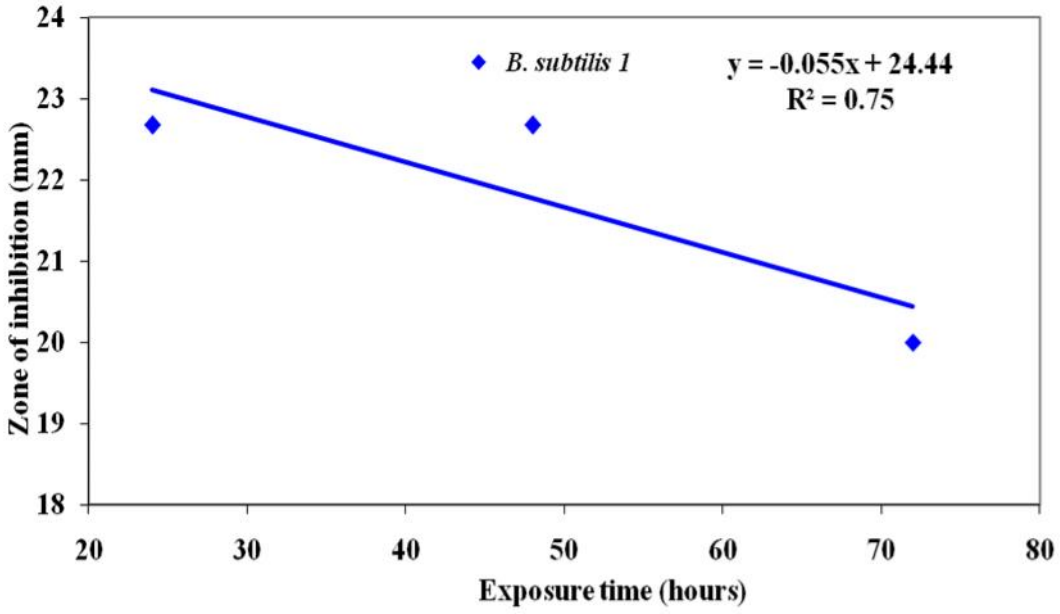


Figure 4.18 Relationship between exposure time and zone of inhibition produced by *Bacillus subtilis* 1 against *R. solanacearum* by disc diffusion method.

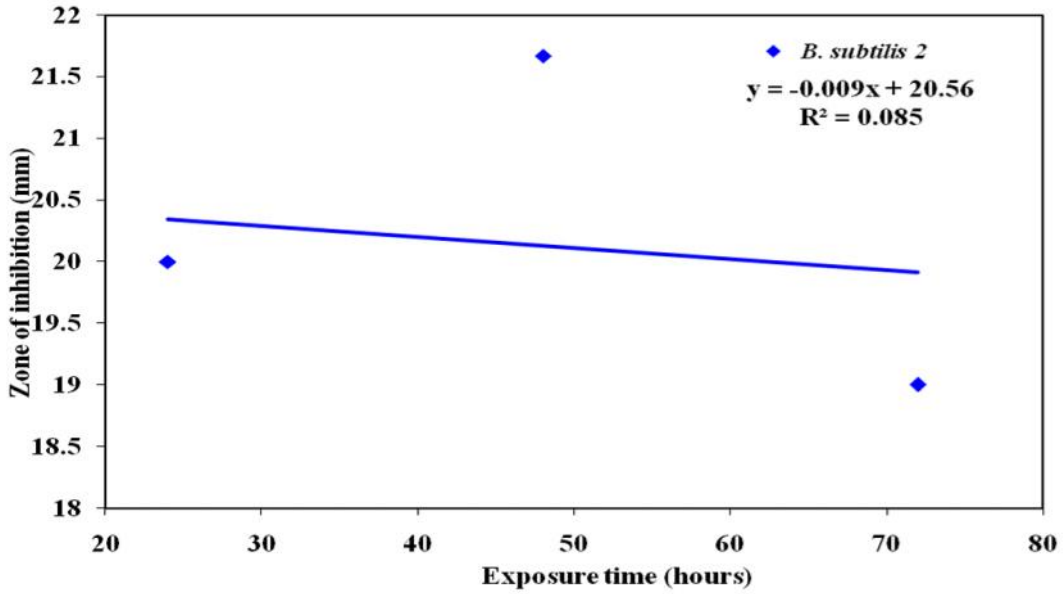


Figure 4.19 Relationship between exposure time and zone of inhibition produced by *Bacillus subtilis* 2 against *R. solanacearum* by disc diffusion method.

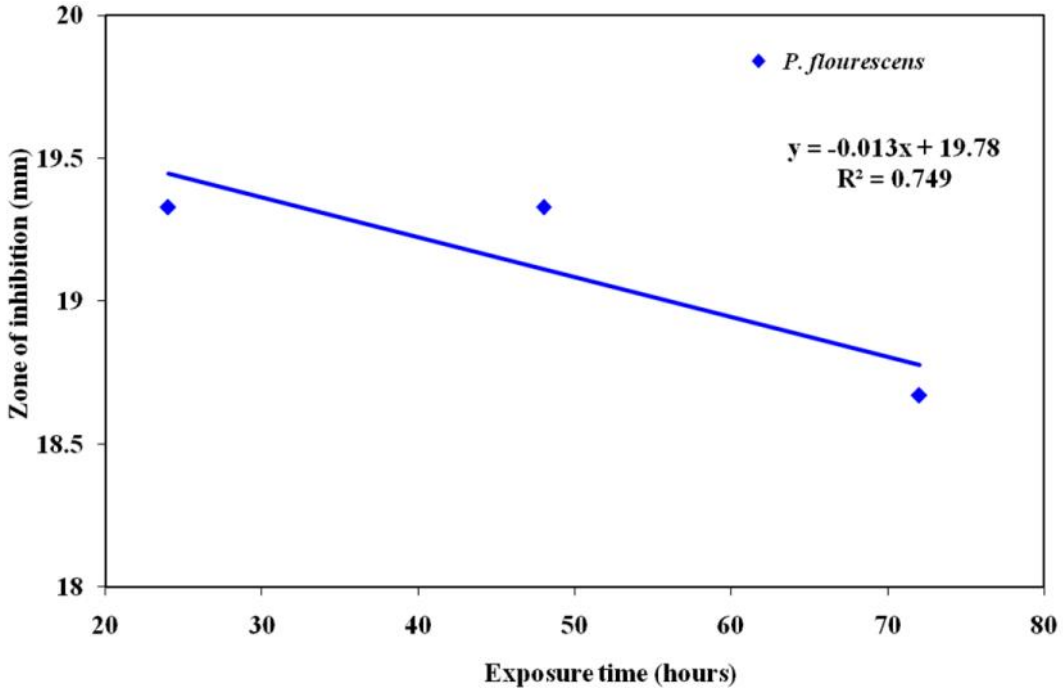


Figure 4.20 Relationship between exposure time and zone of inhibition produced by *P. fluorescens* against *R. solanacearum* by disc diffusion method.

4. 13 Effect of pH on Growth of Indigenous Bio-control Agents

Effect of pH on Growth of selected bio-control agents evaluated after different time of incubation period (Figure 4.21-4.23). Bacterial growth was measured by mesuring optical density at 600 nm. At pH 7 The highest optical density value were measured in all bacterial strains except *Rhizobium leguminosorum* in which the highest OD was measured at pH 6 in all counting. A gradual increase of optical density were observed upto 72 hrs of exposure time.

4. 14 Effect of Temperature on Growth of Indigenous Bio-control Agents

Effect of temperature on growth of selected bio-control agents were determined by measuring the optical density (OD) of each broth culture bio-control agents at different incubation period (Figure 4.24-4.26). The temperature range 30-35^{0}C was found to be optimum for the growth of bacterial bio-control agents. A gradual increase of optical density (OD) value were found over time upto 35^{0}C and then a gradual decrease was observed.

4. 15 Discussion

Bacillus spp. *Pseudomonas fluorescens* and *Trichoderma harzianum* were straind from rhizosphere soil of tomato plant and *Rhizobium leguminosorum* was straind from nodules of lentil plant. Seven bacterial strains and two species of *T. harzianum* (BAU) were found effective against wilt complex pathogens of tomato (*F. oxysporum* f. sp. *lycopersici* and *R. solanacearum*). Among them five were *Bacillus* spp., one was *Pseudomonas fluorescens* and one was *Rhizobium leguminosorum*. Diverse populations of aerobic endospore forming bacteria occur in agricultural fields and have been identified as potential biological control agents as they produce wide range of cyclic lypopeptide antibiotics active against various microorganisms (Batinic *et al.*, 1998; Berger *et al.*, 1996; Podile and Prakash, 1996).

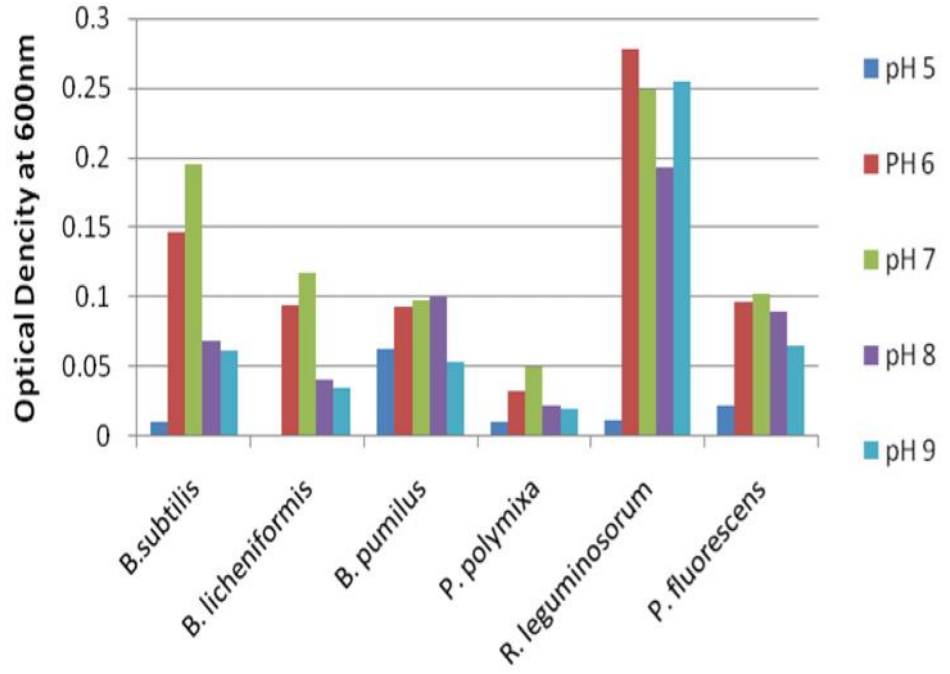


Figure 4.21 Effect of pH on growth of bio-control agents after 24 hrs of incubation.

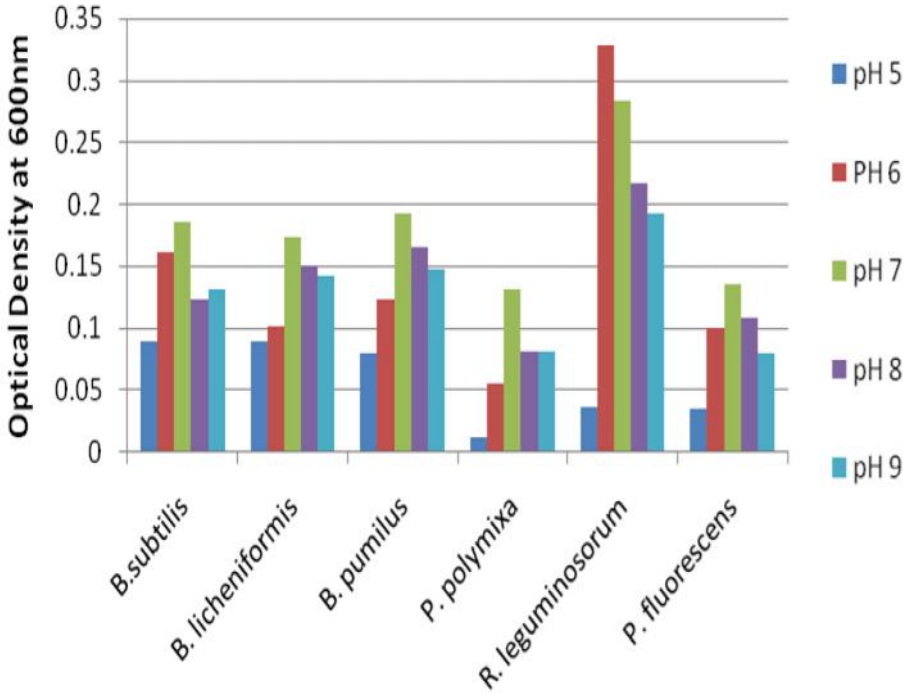


Figure 4.22 Effectof pH on growth of bio-control agents after 48 hrs of incubation.

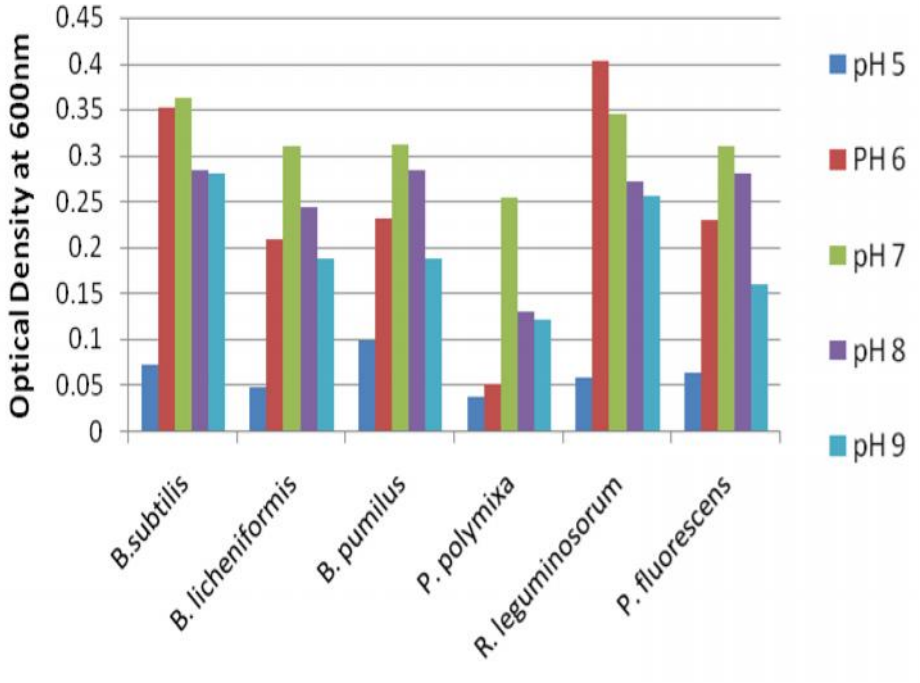


Figure 4.23 Effect of pH on growth of bio-control agents after 72 hrs of incubation.

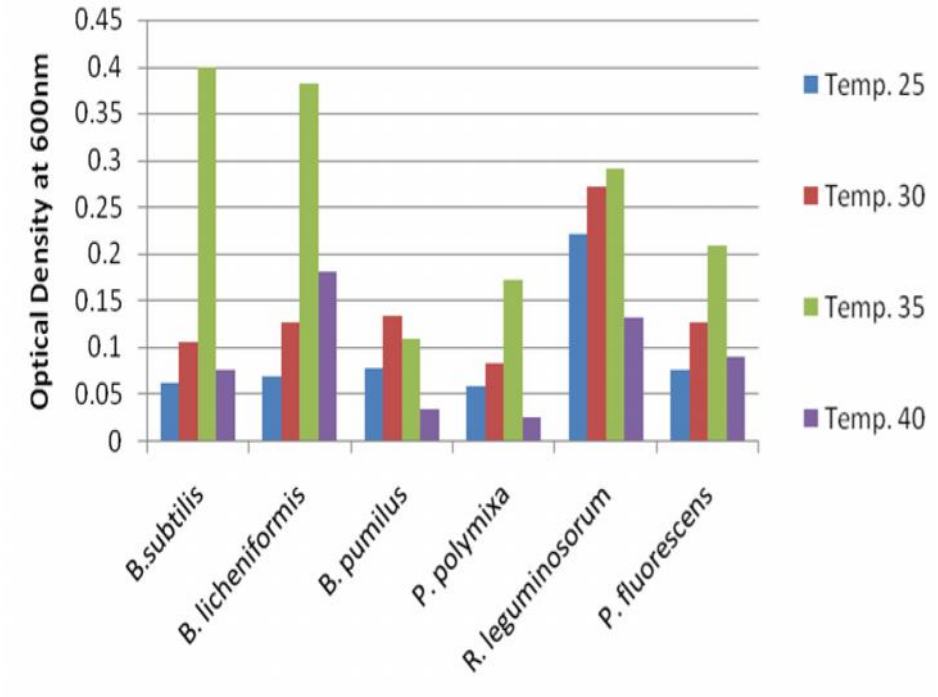


Figure 4.24 Effect of temperature on on growth of bio-control agents after 24 hrs of incubation.

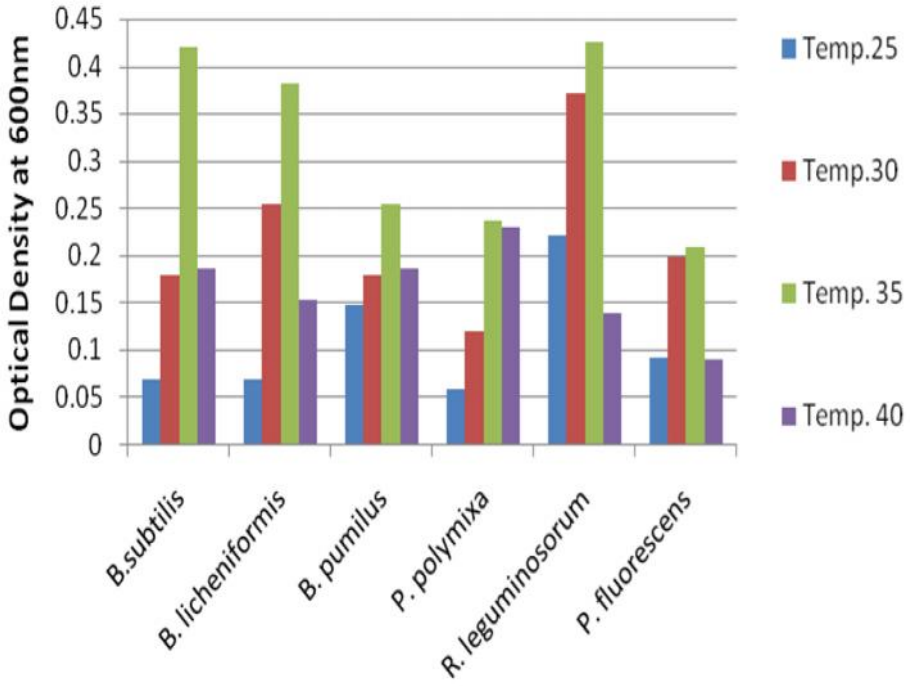


Figure 4.25 Effect of temperature on on growth of bio-control agents after 48 hrs of incubation.

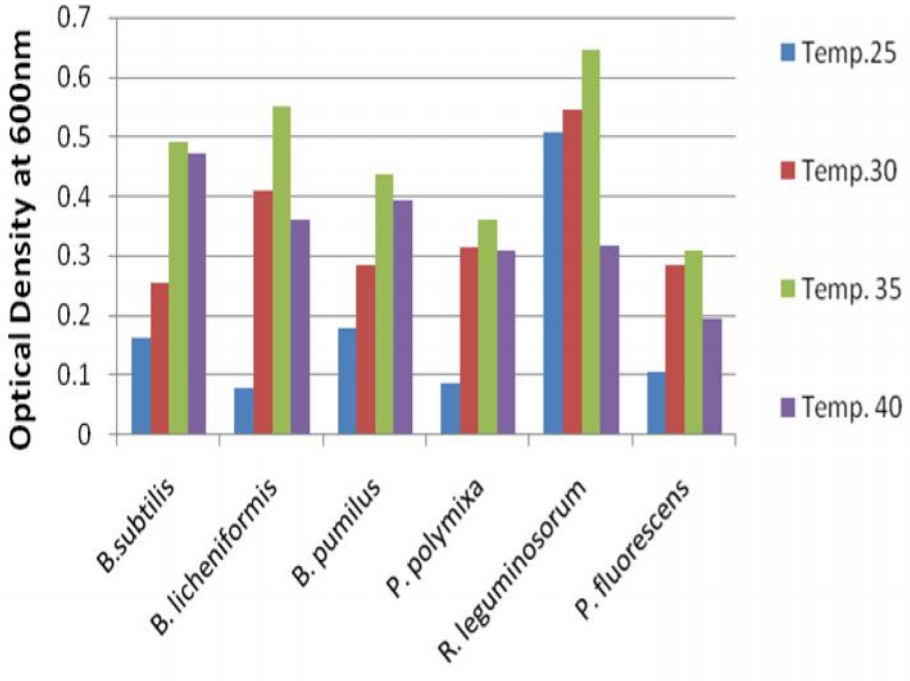


Figure 4.26 Effect of temperature on on growth of bio-control agents after 72 hrs of incubation.

In dual culture seven bacterial strains showed significant variation in inhibition of mycelial growth of *F. oxysporum* f. sp. *lycopersici.* The highest inhibition of radial mycelial growth of *F. oxysporum* f. sp. *lycopersici* was recorded against *Rhizobium leguminosorum* followed by *Bacillus subtilis* 1 and *Pseudomonas fluorescens*. Several workers reported similar effects (Ozkoc and Deliveli, 2001; Khot *et al.,* 1996). Fluorescent Pseudomonads and other plant growth promoting antagonistic rhizobacteria represent a diverse but dominant bacterial group in soil and their influence on plants varies from beneficial to deleterious although some behave neutral behavior. Kumar *et al.* (2002) confirmed that *Pseudomonas fluorescens* has a strong antifungal activity against *Fusarium oxysporum*, mainly by the production of the antifungal metabolites.

Rate of growth of the strains at different temperature ranges was also investigated and it was discovered that as the temperature increases above 35^0C the rate of growth of the strains were decreased. The temperature studied included 25, 30, 35 and 40^0C. Optimum temperature was determined 35^0 C. This implies that the bacterial strains will ultimately grow and metabolize best at room temperature (Stanley and Morita, 1968). The survival of the strains at different pH was monitored using spectrophotometer at a wave length of 600 nm and the optical density readings showed that as the pH was tending from acidic to neutral the growth rate of all the strains increased except *Rhizobium leguminosorum*. The pH value of these bacteria were within the range reported by Okonko *et al.* (2008). The optimum pH required by bacteria varies and sensitive to changes, thus a fluctuation in optimum pH may lead to a change in the metabolism of bacteria. (Edema *et al.,* 2001). The neutral and/or alkaline pH gave a better inhibition than the acid one. Rosenzweig and Stotzky (1979), in a similar way, observed an antagonistic effect against fungi and bacteria in soil when the pH increases.

In *in-vitro* antagonism assay of culture filtrate of *Bacillus subtilis* 1, *B. subtilis* 2 and *P. fluorescens* reaveled that *P. fluorescens* found less effective than *Bacillus subtilis* against *Ralstonia solanacearum.* Members of multiple *Bacillus* species especially *B. subtilis*, *B. amyloliquefaciens*, *B. cereus, B. licheniformis, B. megaterium, B.*

mycoides, and *B. pumilus* are producers of antibiotic molecules. *Bacillus subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). Antibiotics such as polymyxin, circulin, and colistin produced by the majority of *Bacillus* spp. are active against gram positive and gram negative bacteria as well as many pathogenic fungi (Maksimov *et al*., 2011). Savithiry and Gnanamanickam (1987) and Anuratha and Gnanamanickam (1990) obtained 2.5 to 4 cm and 1.0 to 2.8 cm inhibition zones on KB agar medium by *P. fluorescens* against *Rhizoctonia. solani* and *Ralstonia solanacearum,* respectively. Previous workers have established that antimicrobial compound are produced by *P. fluorescens* against *Ralstonia solanacearum* (Anuratha and Gnanamanickam, 1990; Sarvithiry and Gnanamanickam, 1987).

CHAPTER 5

EFFECT OF INDIGENOUS BIO-CONTROL AGENTS ON WILT DISEASE SEVERITY AND YIELD OF TOMATO IN FIELD

5.1 Effect of Indigenous Bio-Control Agents on the Disease Severity of Bacterial Wilt of Tomato at Different Days after Transplanting

Significant effect of indigenous bio-control agents on disease severity of bacterial was observed (Table 5.1 and Figure 5.1) in *Ralstonia solanacearum* inoculated field. At 30 days after transplanting the highest wilt disease severity in terms of PDI (1.1%) was recorded from control plots which were statistically similar with *Bacillus pumilus* treated plants. Disease severity were found 0% in *B. subtilis* 1, and *B. subtilis* 2, *B. licheniformis, Pseudomonas fluorescens and Rhizobium leguminosorum* treated plants followed by *Trichoderma harzianum* treated plants and *Paenibacillus polymixa* treated plants. At 45 days after transplanting the highest severity (3.89%) in terms of PDI was recorded in control plants and PDI was 0% in *P. fluorescens* treated plants followed by *B. subtilis* 2 treated plants. At 60 days after transplanting PDI was highest in control plants (6.11%) and severity was lowest (0.56%) in *B. subtilis* 2 treated plants. In case of percent decrease of disease severity at 60 days after transplanting, maximum decrease over control was observed in *B. subtilis* 2 treated plants (991.07%) and minimum decrease (37.61%) recorded in *B. pumilus* treated plants.

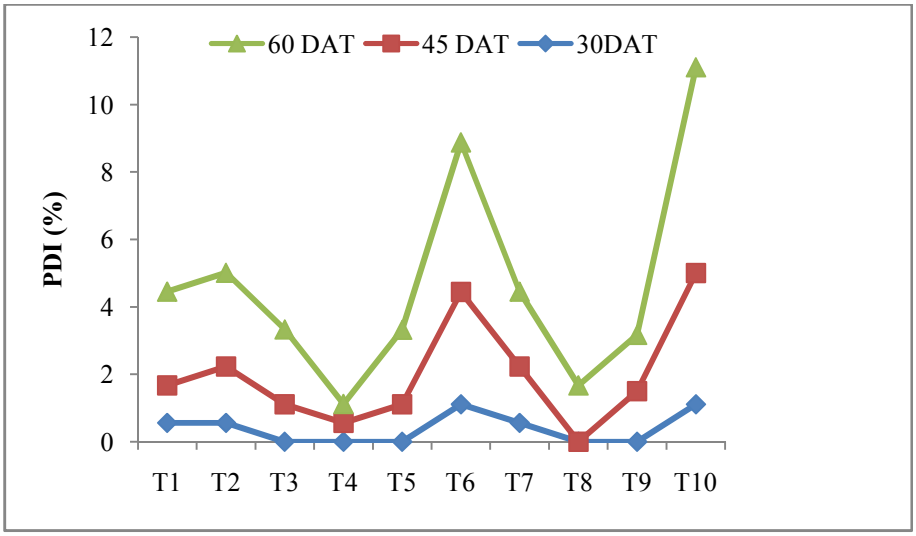
5.2 Effect of Indigenous Bio-Control Agents on the Yield Performance of Tomato Grown on *Ralstonia solanacearum* Inoculated Soil

Effect of indigenous bio-control agents on the yield performance of tomato in *R. solanacearum* inoculated soil was found significant (Table 5.2). Number of fruits per plant varied from 17.33 to 34.33 where the highest (34.33) and the lowest (17.33) number of tomato were recorded from *Pseudomonas fluorescens* treated plants and control plants, respectively. The highest yield per plant (3.69 kg) was obtained from *B. subtilis* 1 treated plants which was statistically identical with *B. subtilis* 2 and *B. licheniformis* treated plants.

Table 5.1. Effect of indigenous bio-control agents on the disease severity of bacterial wilt of tomato caused by *R. solanacearum* at different days after transplanting.

Treatment	Disease Severity (PDI)			
	30DAT	45 DAT	60 DAT	% decrease over control at 60DAT
T_1	0.56 b	1.11 d	2.78 c	119.78
T_2	0.56 b	1.67 c	2.78 c	119.78
T_3	0.00 c	1.11 d	2.22 d	175.23
T_4	0.00 c	0.56 e	0.56 f	991.07
T_5	0.00 c	1.11 d	2.21 d	176.47
T_6	1.11 a	3.33 b	4.44 b	37.61
T_7	0.56 b	1.67 c	2.22 d	175.23
T_8	0.00 c	0.00 f	1.67 e	265.87
T_9	0.00 c	1.50 c	1.67 e	265.87
T_{10}	1.11 a	3.89 a	6.11 a	
$LSD_{(0.01)}$	0.07432	0.2230	0.07432	-
CV (%)	1.77	5.82	0.55	-
SE	0.01826	0.05477	0.01826	-

DAT, Days after transplanting; Values in the column are the means of three replicates; In a column, means with same letters are statistically similar with each other.



T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

Figure 5.1 Effect of indigenous bio-control agents on the disease severity of bacterial wilt of tomato at different days after transplanting.

Table 5.2. Effect of indigenous bio-control agents on the yield performance of tomato grown on *R. solanacearum* inoculated soil.

Treatment	Fruit yield				
	Fruit (No./plant)	Yield (kg/plant)	Yield (kg/plot)	Yield (ton/ha)	% yield increase over control
T_1	24.08 ab	2.89 bcd	34.76 bcd	86.90 bcd	100.69
T_2	26.33 ab	2.62 d	31.52 d	78.80 d	81.99
T_3	33.33 a	3.69 a	44.32 a	110.8 a	155.89
T_4	30.42 a	3.45 ab	41.40 ab	103.5 ab	139.03
T_5	30.67 a	3.24 abc	38.92 abc	97.30 abc	124.71
T_6	28.42 ab	2.91 bcd	35.00 bcd	87.50 bcd	102.08
T_7	28.33 ab	2.68 cd	32.24 cd	80.60 cd	86.14
T_8	27.42 ab	2.58 d	30.96 d	77.40 d	78.75
T_9	34.33 a	2.93 bcd	35.24 bcd	88.10 bcd	103.46
T_{10}	17.33 b	1.44 e	17.32 e	43.30 e	-
$LSD_{(0.01)}$	10.12	0.5512	6.623	16.56	-
CV (%)	15.34%	8.25%	8.25	8.25	-

Values in the column are the means of three replicates; In a column, means with same letters are statistically similar with each other.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

The lowest yield per plant (1.44 kg) was obtained from control plots. The highest fruit yield per plot (44.32 kg) was recorded under *B. subtilis* 1 and the lowest yield per plot was obtained from control treatments. Fruit yield in ton per hectare was calculated, which varied from 43.32 ton/ ha to 110.8 ton/ha where the highest yield obtained from *B. subtilis* treated plants and the lowest in control treatments. The maximum yield increase (155.89%) over control was recorded from *B. subtilis* 1 treated plants and the minimum (78.75%) yield increase over control was found in *P. fluorescens* treated plants.

5.3 Effect of Indigenous Bio-control Agents on the Disease Severity of Fungal Wilt of Tomato at Days after Transplanting

Significant effect of indigenous bio-control agents on disease severity of fungal wilt were observed (Table 5.3 and Figure 5.2) in *F. oxysporum* f. sp. *lycopersici* inoculated field.

Table 5.3. Effect of indigenous bio-control agents on the disease severity of fungal wilt of tomato at different days after transplanting.

Treatment	Disease Severity (PDI)			
	30DAT	45 DAT	60DAT	% decrease over control at 60DAT
T_1	0.00 e	1.67 d	2.78 c	139.93
T_2	0.00 e	0.56 f	1.11 f	500.90
T_3	0.00 e	1.11 e	1.67 e	299.40
T_4	0.00 e	0.00 g	1.11 f	500.90
T_5	1.67 c	2.22 c	2.78 c	139.93
T_6	2.22 b	3.89 b	4.44 b	50.23
T_7	2.22 b	1.67 d	4.44 b	50.23
T_8	0.56 d	1.67 d	2.22 d	200.45
T_9	0.00 e	1.67 d	2.78 c	139.93
T_{10}	3.32 a	4.44 a	6.67 a	139.93
$LSD_{(0.01)}$	0.07432	0.07432	0.07432	-
CV (%)	0.94	0.47	0.57	-
SE	0.01826	0.01826	0.01826	-

DAT, Days after transplanting; Values in the column are the means of three replicates; In a column, means with same letters are statistically similar with each other.

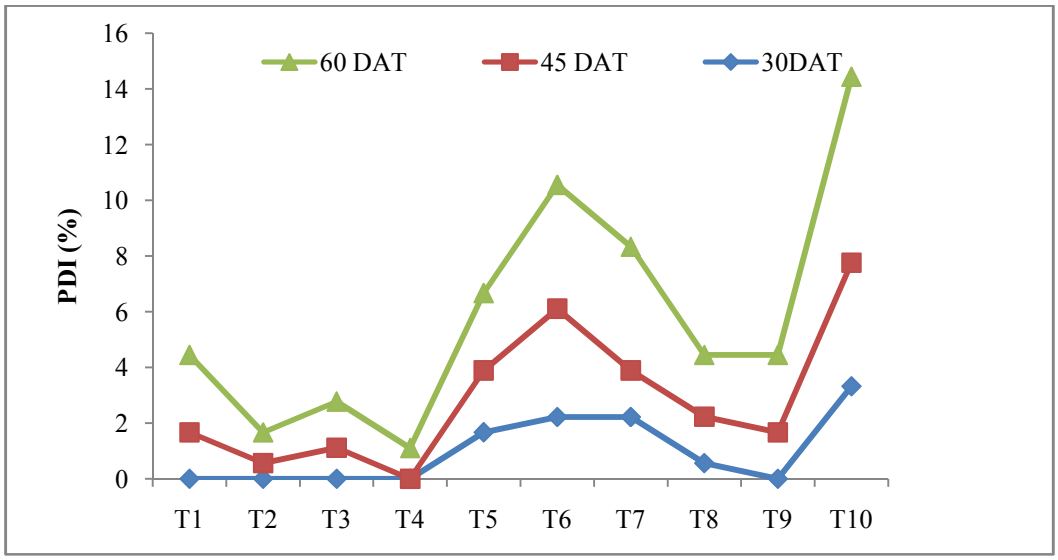


Figure 5.2 Effect of indigenous bio-control agents on the disease severity of fungal wilt of tomato at different days after transplanting.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

At 30 days after transplanting the severity in terms of PDI were 0.0% in *T. harzianum*, *R. leguminosorum* and both strains of *B. subtilis* treated plants and the maximum PDI (3.32%) was observed in control plots. At 45 days after transplanting fusarium wilt disease severity was recorded highest (4.4%) in control plants followed by *B. pumilus* (3.89%) and *B. licheniformis* (2.2%) treated plants. At 60 days after transplanting fusarium wilt disease severity was found maximum (6.67%) in control plants and minimum PDI value (1.1%) were recorded in *T. harzianum* and *B. subtilis* 2 treated plants. Percent decrease of severity over control varied from 50.23% to 500.99%, where the highest values were found in both *T. harzianum* and *B. subtilis* 2 treated plants and the lowest value was counted from both *B. pumilus* and *P. polymixa* treated plants.

5.4 Effect of Indigenous Bio-control Agents on the Yield Performance of Tomato Grown on *F. oxysporum* f. sp. *lycopersici* Inoculated Soil

Significant effects of indigenous bio-control agents on the yield performance of tomato under *F. oxysporum* f. sp. *lycopersici* inoculated soil were observed among different parameters measured (Table 5.4 and Figure 5.3-5.4). Number of fruits per plant varied from 16.67 to 31.17, where the highest (31.17) and the lowest (16.67) value were counted from *Paenibacillus polymixa* and control treatments, respectively. Effect of bio-control agents on yield per plant did not differ significantly and maximum yield (2.77 kg/ plant) obtained from *B. licheniformis* treated plants. Similar trends also found in terms of yield in kg/ plot and Fruit yield in ton per hectare varied from 51.40 to 83.20, where the maximum value counted from the plants treated with *B. licheniformis.* In case of percent yield increase over control the highest increase (61.87%) was found in *Bacillus licheniformis* treated plants and the lowest (41.63%) in *Rhizobium leguminosorum* treated plants.

Table 5.4. Effect of indigenous bio-control agents on the yield performance of tomato grown on *F. oxysporum* f. sp. *lycopersici* inoculated soil.

Treatment	Fruit yield				
	Fruit (No./plant)	Yield (kg/plant)	Yield (kg/plot)	Yield (ton/ha)	%increase over control
T_1	27.42 a	2.563 a	30.76 a	76.90 a	49.61
T_2	27.08 a	2.547 a	30.56 a	76.40 a	48.64
T_3	26.25 a	2.560 a	30.72 a	76.80 a	49.42
T_4	26.83 a	2.610 a	31.32 a	78.30 a	52.33
T_5	27.58 a	2.773 a	33.28 a	83.20 a	61.87
T_6	27.08 a	2.633 a	31.60 a	79.00 a	53.70
T_7	31.17 a	2.640 a	31.68 a	79.20 a	54.09
T_8	24.67 ab	2.707 a	32.48 a	81.20 a	57.98
T_9	25.75 a	2.427 a	29.12 a	72.80 a	41.63
T_{10}	16.67 b	1.713 b	20.56 b	51.40 b	-
$LSD_{(0.01)}$	8.682	0.6730	8.065	20.16	-
CV (%)	14.18%	11.36%	11.36	11.36	-

Values in the column are the means of three replicates; In a column, means with same letters are statistically similar with each other.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

Figure 5.3 Experimental field of tomato.

Figure 5.4. A portion of experimental field showing a replication of treatment T_3, *B. Subtilis* 1.

5.5 Effect of Indigenous Bio-control Agents on the Disease Severity of Wilt Complex of Tomato at Days after Transplanting

Different bio-control agents showed significant effect on disease severity of wilt complex (Figure 5.5.A-B) of tomato plants when Ralstonia and Fusarium both organisms inoculated in the field (Table 5.5 and Figure 5.6). At 30 days after transplanting disease severity were % in *B. subtilis*, *P. fluorescens* and *R. leguminosorum* treated plants and severity was maximum (2.78%) in untreated control plants. At 45 days after transplanting wilt severity was highest in untreated control plots and the lowest (1.11%) was found in *B. subtilis* treated plots, the effect was statistically identical with *P. fluorescens* treated plants. And severity of wilt complex was recorded 5% (highest) in untreated control plants. At 60 days after transplanting all the treatments showed significant effect in reducing wilt disease severity complex compared to control. Maximum PDI (6.67%) recorded in control plants and minimum PDI (1.67%) recorded in *B. subtilis* 1 treated plants. *B. subtilis* 2*, T. harzianum P. fluorescens* and *R. leguminosorum* showed similar effect on wilt disease severity. At 60 days after transplanting the highest (299.40%) decrease of severity over control was recorded in *B. subtilis* 1 treated plants and the lowest decrease (33.40%) over control was observed in *B. pumilus* treated plants.

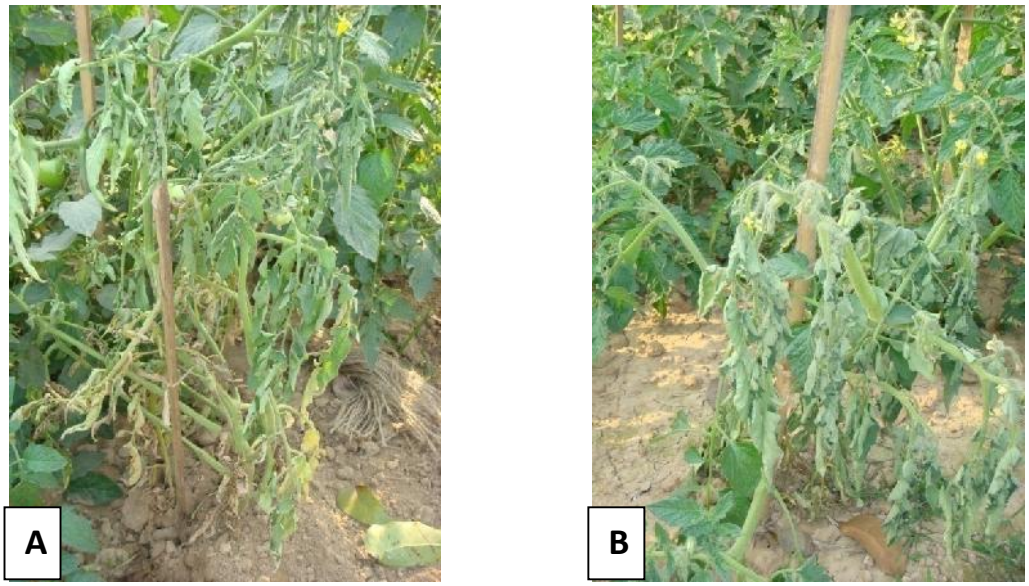


Figure 5.5 Wilted plants in the experimental field. A, Bacterial wilt B, Fusarium wilt.

Table 5.5. Effect of indigenous bio-control agents on the disease severity of wilt complex of tomato at different days after transplanting.

Treatment	Disease Severity (PDI)			
	30 DAT	45DAT	60DAT	% decrease over control at 60DAT
T_1	1.11 c	2.22 c	4.44 c	50.23
T_2	0.56 d	1.67 d	2.78 f	139.93
T_3	0.00 e	1.11 e	1.67 g	299.40
T_4	0.00 e	1.11 e	2.78 f	139.93
T_5	1.11 c	2.78 b	3.33 e	100.30
T_6	2.22 b	2.22 c	5.00 b	33.40
T_7	2.22 b	2.78 b	3.89 d	71.47
T_8	0.00 e	1.11 e	2.78 f	139.93
T_9	0.00 e	1.67 d	2.78 f	139.93
T_{10}	2.78 a	5.00 a	6.67 a	-
$LSD_{(0.01)}$	0.07432	0.07432	0.07432	-
CV (%)	0.92	1.45	0.97	-
SE	0.01826	0.01826	0.01826	-

DAT, Days after transplanting; Values in the column are the means of three replicates; In a column, means with same letters are statistically similar with each other.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

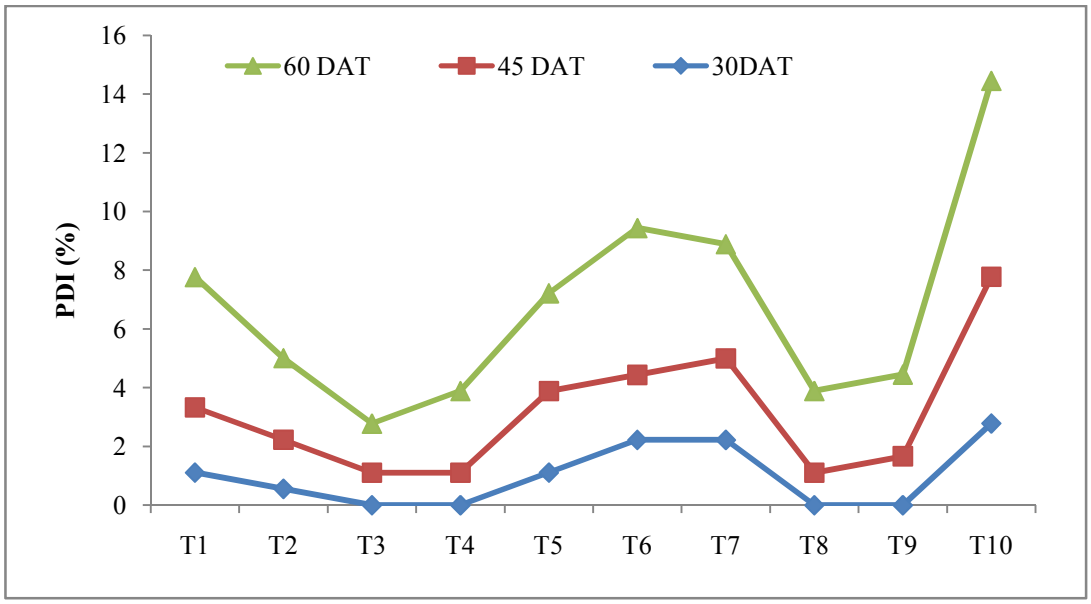


Figure 5.6 Effect of indigenous bio-control agents on the disease severity of wilt complex of tomato at different days after transplanting.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

5.6 Effect of Indigenous Bio-control Agents on the Yield Performance of Tomato Grown on *Ralstonia* and *F. oxysporum* f. sp. *lycopersici* Inoculated Soil

Significant effect of bio-control agents on the yield performance of tomato (Figure 5.7-5.8) grown on *Ralstonia* and *F. oxysporum* f. sp. *lycopersici* inoculated soil were observed (Table 5.6). Number of fruits per plant under different treatments varied from 18.08 to 33.0 where the highest number obtained from *B. subtilis* 1 treatment and the lowest from control treatment. The highest yield (2.93 kg/plant) obtained from *B. subtilis* 1 which was statistically identical with other treatments except *T. harzianum* (BAU). The lowest yield was obtained from control treatments. Similar trends also found regarding yield (kg/plot) and yield (ton / ha). The highest yield increase over control (91.50%) was measured from *B. subtilis* 1 treatments and the lowest yield increase over control (42.48%) was measured from *Trichoderma harzianum* (BAU) treatment.

Table 5.6. Effect of bio-control agents on the yield performance of tomato grown on *Ralstonia* and *Fusarium* inoculated soil.

Treatment	Fruit yield				
	Fruits (No./plant)	Yield (kg/plant)	Yield (kg/plot)	Yield (ton/ha)	%increase over control
T_1	27.83 abc	2.530 ab	30.36 ab	75.90 ab	65.36
T_2	24.58 abc	2.180 b	26.16 b	65.40 b	42.48
T_3	33.00 a	2.93 a	35.16 a	87.90 a	91.50
T_4	30.50 ab	2.92 a	35.04 a	87.60 a	90.85
T_5	22.08 bc	2.40 ab	28.84 ab	72.10 ab	57.08
T_6	28.67 ab	2.59 ab	31.16 ab	77.90 ab	69.72
T_7	30.50 ab	2.84 a	34.16 a	85.40 a	86.06
T_8	30.33 ab	2.75 a	33.04 a	82.60 a	79.96
T_9	32.25 ab	2.67 ab	32.12 ab	80.30 ab	74.95
T_{10}	18.08 c	1.530 c	18.36 c	45.90 c	-
$LSD_{(0.01)}$	9.369	0.5149	6.189	15.47	-
CV (%)	14.35%	8.65%	8.65	8.65	-

Values in the column are the means of three replicates; In a column, means with same letters are statistically similar with each other.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

Figure 5.7 A bunch of green tomato (BARI tomato 2) in the field.

Figure 5.8 Harvested tomato (BARI tomato 2).

5.7 Relationship among the Disease Severity of Bacterial Wilt, Fungal Wilt and Wilt Complex of Tomato Plant at 60 Days after transplanting (DAT)

Relationship among the disease severity of bacterial wilt, Fusarium wilt and Ralstonia-Fusarium wilt complex of tomato plant were recorded and compared at 60 days after transplanting (Figure 5.9). The severity of wilt was increased gradually with the progress of time after the first appearance of the disease under all treatments. In case of bacterial wilt the lowest disease severity (0.56%) observed under *B. subtilis* 2 treated plants followed by *P. fluorescens* and *R. leguminosorum* where severity were recorded 1.67%. In case of Fusarium wilt the lowest disease severity (1.11%) observed under *B. subtilis* 2 and *Trichoderma harzianum* (BAU) treated plants followed by *B. subtilis* 1. In case of wilt complex the lowest disease severity was observed under *B. subtilis* 1(1.67%) followed by *B. subtilis* 2, *P. fluorescens*, *R. leguminosorum* and *Trichoderma harzianum* (BAU) treated plants where the severity were recorded 2.78%. At all experiments wilt disease severity was found minimum in treated plants compared to untreated control.

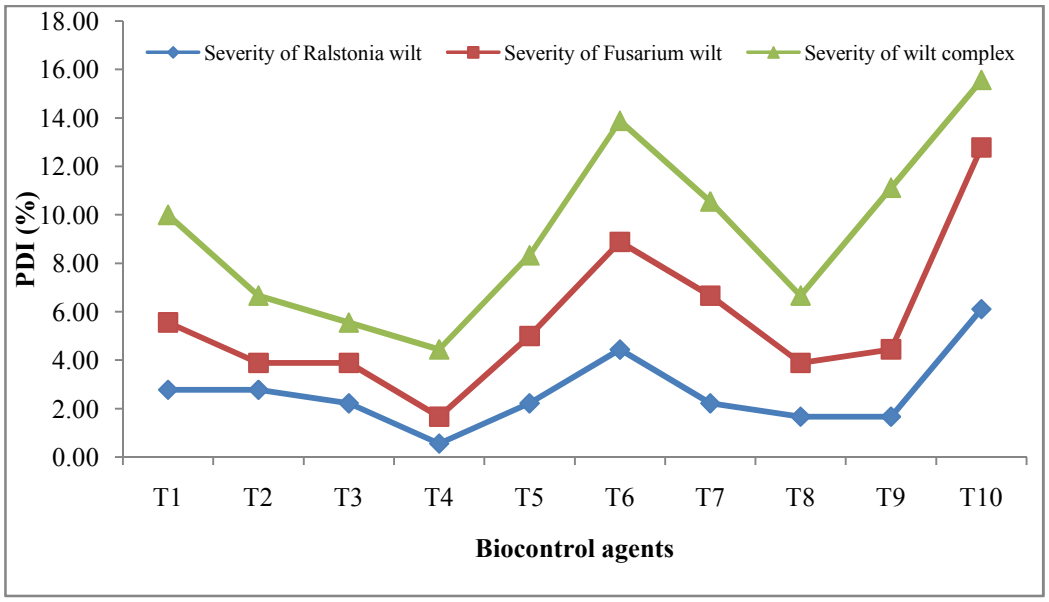


Figure 5.9 Relationship among the disease severity of *Ralstonia*, *Fusarium* wilt and *Ralstonia-Fusarium* wilt complex of tomato plant at 60 days after transplanting.

T_1, *Trichoderma harzianum*; T_2, *T. harzianum* (BAU); T_3, *B. Subtilis* 1; T_4, *B. Subtilis* 2; T_5, *B. licheniformis*; T_6, *B. pumilus*; T_7, *Paenibacillus polymixa*; T_8, *P. fluorescens*; T_9, *R. leguminosorum*; T_{10}, Untreated control.

5. 8 Discussion

Different bio control agents showed significant effect in reducing bacterial wilt disease severity. *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosorum* gave the best result in reducing bacterial wilt disease severity and increased the yield of tomato. Several biological control agents such as *Pseudomonas fluorescens, Bacillus licheniformis, B. cereus, B. subtilis* and Mycorrihiza were found very effective in delaying and reducing the wilt development (Mallikarjun *et al.,* 2008). Ramesh *et al.* (2008) reported that *Pseudomonas* is the major antagonistic endophytic bacterium of eggplant which has potential to be used as a biocontrol agent against *Ralstonia solanacearum* in eggplant.

In case of Fusarium wilt it was observed that *Trichoderma harzianum* (BAU), *Bacillus subtilis* and *Pseudomonas fluorescens* showed better performance and reduced the severity and increased the yield of tomato. A number of plant

associated microbes are free living and strongly beneficial to plants. This results partially supported by Alwathnani and Perveen (2012) who observed 44.4% control of Fusarium wilt with *Trichoderma harzianum* treated plants as compared to *Fusarium oxysporum* f. sp. *lycopersici* inoculated plants. Fungi in the genus of *Trichoderma* (Harman *et al*., 2004) and rhizobacteria in the genera of *Pseudomonas, Bacillus, Streptomyces, Enterobacter* and others (Belimov *et. al*., 2001; Pieterse *et al.,* 2001; Wang *et al.,* 2000) have evolved multiple mechanisms that result in improvements in plant resistance to disease and plant growth and productivity. Larkin *et al.* (1998) reported that strains of *Gliocladium virens*, *Trichoderma hamatum, Pseudomonas fluorescens* and *Bacillus cepacia* significantly reduced (30-65%) Fusarium wilt compared to control. Rhizobacterial strains of *Pseudomonas, Burkholderia* and *Bacillus* spp. also have been used to reduce disease caused by a variety of soil borne pathogens (Weller, 1998; Weller and Cook, 1983) including *Fusarium* spp. In recent years, there has been much success in obtaining effective control of plant pathogens using beneficial bio-control agents such as strains of *Pseudomonas* species have been used extensively for plant growth promotion and disease control because of many properties such as efficient colonization of underground plant organs, utilization of a large number of organic substrates commonly found in root exudates and production of a variety of secondary toxic to fungi and bacteria. Gamliel and Katan (1993) found that inoculation of fluorescent pseudomonas decreased the incidence disease caused by Fusarium wilt of tomato. The genus *Pseudomonas* is a very large and important group of non-fermenting, gram negative bacteria, living as saprophytes in soils, sediments and fresh water (Bossis *et al.,* 2000). Some of the species in this genus are already known to improve plant growth and health and are implicated in the natural suppression of certain soils to various soil borne diseases like bacterial wilt of potato, fusarium and verticilium wilt of vegetable crops whereas others are involved in the biodegradation of natural or manmade toxic chemical compounds (Bossis *et al.,* 2000).

The effecacy of selected biocontrol agents against wilt complex pathogens (both Ralstonia and Fusarium) was evaluated in filed condition when soil was inoculated with both pathogens and their effect on yield of tomato also evaluated. The results

revealed that *Trichoderma harzianum* (BAU), *Bacillus subtilis*, *Pseudomonas fluorescens* and *Rhizobium leguminosorum* gave the best result by reducing wilt complex severity as well as incrased the yield compared to control. A number of microorganisms such as *Trichoderma harzianum* (Mohiddin *et al.*, 2010; Khan *et al.,* 2001), *Pseudomonas fluorescens* (Peighami-Ashnaei *et al.*, 2009) and *Bacillus subtilis* (Dawar *et al*., 2010) have demonstrated antagonism against diseases caused by *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp., *Sclerotium* spp., and so forth, leading to enhancement in plant growth or yield.

CHAPTER 6

GENERAL DISCUSSION

Wilt caused by both bacteria and fungi is the most devastating disease of tomato that result significant yield losses of tomato. Farmers in general still rely on the use of synthetic chemicals to control diseases. These pathogens are soil borne, thus, difficult to control with chemicals and cultural practices especially bacterial wilt disease. Fusarium wilt can be controlled by using some fungicides. But fungicides have some limitations such as loss of soil fertility, contamination of ground and surface water, biomagnifications, health hazards etc. which are reported to have deleterious effect on health of all living organism of biosphere. Therefore alternative strategies are being widely employed. One such practice is use of bio-control agents. Research on bio-control agents have expanded in recent past as eco-friendly management of targeted crops. Therefore, the study was designed and executed towards isolation of indigenous bio-control agents from rhizosphere of tomato as the performance of the introduced bio-control agent may not be always favorable because of competition for space and resources with the already established microorganism in the microcosm.

Twenty strains of *Ralstonia solanacearum* and three strains of *Fusarium oxysporum* f. sp. *lycopersici* were isolated from wilted tomato plants collected from Research fields of Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur, Bangladesh Agricultural University (BAU), Mymensingh, and Sher-e-Bangla Agricultural University (SAU), Dhaka. Among them one *Ralstonia solanacearum* and one *Fusarium oxysporum* f. sp. *lycopersici* was selected and used for further study. *Ralstonia solanacearum* is a highly heterogeneous bacterial pathogen that causes severe wilting of many important plants (Smith *et al*., 1995). The disease is also called Southern bacterial blight, *Ralstonia* solanaceous wilt, Southern bacterial wilt and many other common names in countries where it occurs (Buddenhagen and Kelman, 1964).

Pure cultures of *Ralstonia solanacearum* are not difficult to identify. Cultural and physiological tests can quickly rule out related organisms (Anon., 2004). Cultural, physiological, and biochemical tests revealed that all strains identified as *Ralstonia solanacearum* biovar 2. All strains of *R. solanacearum* produced fluidal and irregular colonies with pink centre on TTC medium at 30^0C after 48 hrs of incubation. They were positive in oxidase, catalase, nitrate reduction, growth on Cetrimide agar (Selective for *Pseudomonas*) and negative in Gram reaction and was positive in hypersensitive reaction. *Ralstonia* is an aerobic obligate organism, strains of the pathogen have minimum, optimum and maximum temperature of 10, 35 and 41^0C, respectively (Kelman, 1953). *Ralstonia solanacearum* is a gram negative, non-spore forming rod, about 0.5 -0.7 µm×1.5-2.0µm with a single polar flagellum (Sneath *et al*., 1986). The bacterium is aerobic and its colonies on solid media are small, irregularly round, white in reflected light and tan in transmitted light (Hayward, 1991). Pathogenicity results showed that strain SAU 2 exhibited the highest disease incidence with 100% wilting. Such results agreed with previous workers, who mentioned that the different strains of *R. solanacearum* were varied in their pathogenicity (Abo-Elyousr and Asran, 2009; El-Ariqi *et al.*, 2005; Galal *et al.*, 2003). A variety of pathogens can cause wilting, so symptoms alone are not definitive for bacterial wilt causing by *R. solanacearum.* *R. solanacearum* (strain SAU 2) induce a hypersensitive response when infiltrated into tobacco leaves (Robertson *et al.,* 2004). Mohamed *et al.* (2014) straind 15 strains of *Ralstonia solanacearum* on triphenyl tetrazolium chloride medium and fifteen strains shown typical morphological and cultural characteristics were confirmed as *Ralstonia solanacearum* biovar 2 race 1and pathogenicity tests showed that all strains proved to be pathogenic to tomato plants which resulted 52 to 97% wilting.

Tomatoes are parasitized by a number of pathogens, including *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen, the causal agent of fusarium wilt of tomato (Ignjatov *et al.*, 2012), which is one of the most important species as tomato pathogen (Agrios, 2006). Browning of the vascular tissue is strong evidence of fusarium wilt (Snyder and Hans, 2003). The isolated fungus identified on the basis of its morphological characteristics (Rahjoo *et al.*, 2008; Burgess *et al*.,

1994) and the pathogenecity. Majority of Fusarium strains causing vascular wilts on different crops are morphologically identical and cannot be differentiated from non-pathogenic and saprophytic strains. Hence a huge morphological diversity exists. Further identification of the species has traditionally involved the pathogenecity testing with a set of host differentials appropriate for the formae specials in question. From a diagnostic point of view the separation of species into formae specials has important diagnostic and quarantine implications (Brett *et al*., 2003). Strains of *F. oxysporum* are often highly host specific. Virulence has been an extremely useful characteristic for differentiating strains of *F. oxysporum* into formae specials. The pathogen is distinct in symptomology, epidemiology and cultivar susceptibility (Vakalounakis, 1996).

Inoculation methods play an important role in pathogenesis. Thus an attempt was made to evaluate different methods of inoculation on pathogenecity of *Ralstonia solanacearum* and *F. oxysporum* f. sp. *lycopersici.* Four different methods of inoculations were evaluated viz. collar region, soil drenching, sick bed and root dipping method. In case of bacterial wilts, collar region method performed the best followed by soil drenching method. Most researchers used collar region inoculation method to test pathogenecity of *R. solanacearum*. *R. solanacearum* is soil borne bacterium and enter into the host through natural opening or artificial wounds created by nematodes or mechanical injury. In collar region inoculation method the bacterium directly get a chance to enter in to the xylem vessels and established infection rapidly. Araud-Razou *et al*. (1998) and Vasse *et al*. (1995) reported that the bacterium infects through root wounds or at sites of secondary root emergence, then colonizes the xylem vessels and spread rapidly to aerial parts of the plant through the vascular system. In xylem vessels the bacterial population can multiply extensively and rapidly reach very high levels ($>10^{10}$ cell/ cm of stem in tomato). The best method of inoculation for screening tomato, brinjal and chilli is the soil drenching method, which is less cumbersome, reliable and effective as it exactly simulates the natural infection as the bacterium is soil borne and enters the plant through roots under field condition (Vasse *et al*., 1995; Schmit, 1978; Kelman and Sequeira, 1965). Experimenting with tomato for the development of bacterial wilt

resistant varieties, Hanson *et al.* (1996) also found that the soil drenching method of inoculation was ideal in getting maximum percent of wilt incidence in tomato.

In case of *Fusarium* wilt sick bed method of inoculation was found best which resulted, the highest number of wilted plants and performance of collar region inoculation method was found statistically similar. The results differed from *R. solanacearum*, because the infection process of *Fusarium oxysporum* is different from *R. solanacearum.* Many of the economically important plant pathogenic fungi are soil borne and attack their hosts through the root or the hypocotyl. The early steps involved in fungal root infection have been studied considerably less than the corresponding stages in pathogens attacking the aerial parts of the plant, mainly because of methodological limitations. Generally, the morphogenetic events preceding penetration in root pathogens are viewed as less complex than those occurring in foliar pathogens, because the former usually lack fully differentiated infection structures such as appressoria (Mendgen *et al*., 1996). The fungus *Fusarium oxysporum* enters the roots directly through penetration hyphae and colonizes the cortex by intra and intercellular growth (Di Pietro *et al*., 2001; Fuchs *et al*., 1997; Rodríguez-Gálvez and Mendgen, 1995). Once it reaches the vascular tissue, *F. oxysporum* spreads rapidly upwards through the xylem vessels provoking the characteristic wilt symptoms (Beckman, 1987). Healthy plants can become infected by *F. oxysporum* if the soil in which they are growing is infested with the pathogen (Farr *et al*., 1989). However, pathogenic fungi of the genus *Fusarium* that is the causal agents of tomato wilt cause root and basal stem deterioration and result in the wilting of vegetable plants. Some strains of this fungus are pathogenic only to specific plant species (forma specialis) and there is also a large number of physiological races within each of these specialized forms, all of which make the selection for resistance to this pathogen more difficult (Armstrong and Armstrong, 1981). Comparing the above methods, collar region inoculation method gave best performance to evaluate the pathogenecity of both pathogens.

Plant diseases may be suppressed by the activities of one or more plant associated microbes, therefore an attempt was made to strain indigenous bio-control agents

from rhizophere of tomato and root nodules of lentil and characterize these organisms involved in biological control of wilts causing pathogens of tomato. *Trichoderma hazianum* and plant growth promoting rhizobacteria viz. *Bacillus* spp, *Pseudomonas fluorescens* were isolated from rhizosphere of tomato and *Rhizobium leguminosorum* were isolated from root nodule of lentil. The rhizosphere is a place of intense microbial activity with important exchanges between ground, root and microflora. The microbial density is high compared to that of a naked ground (Stengel, 1998). The studies of *in vitro* antagonism make it possible to select a great number of effective agents of bio-control (Edwards and Seddon, 2001). Sixty strains of *Bacillus* spp., 20 strains of *Pseudomons fluorescens*, 10 strains of *Rhizbium leguminosorum* and 2 strains of *Trichoderma harzianum* (including one BAU pesticide) were screen out against *Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *lycopersici.* Among the bacterial strains 5 strains of *Bacillus* spp., one strain of *Pseudomonas fluorescens* and one strain of *Rhizobium leguminosorum* and both *Trichoderma harzianum* were found effective against *Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *lycopersici* in *in-vitro* test. *Bacillus* spp. were inoculated on vessal media that are specific for *Bacillus* spp. and selected 5 strains produced pink color colony on vessal medium after 48 hrs of incubation. Further identified on the basis of morphological characteristics and finally confirmed by the biochemical tests (Krieg and Holt, 1984). The strains of *Bacillus* those were found effective against fungal and bacterial wilt pathogens were identified viz. *Bacillus subtilis* 1, *Bacillus subtilis* 2, *Bacillus licheniformis*, *Bacillus pumilus* and *Paenibacillus polymixa.* Pseudomonas fluorescens was isolated on KB medium which was made selective by adding some antibiotics. Pseudomonalds produced diffusible yellow, green or blue fluorescent pigments on KB medium after 24 to 48 hours of growth. Biochemical reactions such as fluorescien production, levan formation, oxidase, catalase, certain carbohydrate utilizations and morphological features of the strains obtained in this study were similar to the results reported by (Bossis *et al.*, 2000). According to Todar (2004), more than half of the *Pseudomonas* bacteria produce pyocyanin which is a blue-green pigment, while the nonpathogenic saprophyte *P. fluorescens* produces fluorescent pigment that is soluble and greenish. In this study, identified gram-negative *Pseudomonas*

strains that produced green fluorescent pigment on King's B medium under ultraviolet light at 365 nm. *Rhizobium leguminosorum* produced semi translucent, raised mucilaginous colony on YMA after 2-3 days of incubation which were similar to the result reported by (Johnston *et al*., 1978; Vincent, 1970). Colony formed by *T. harzianum* was found darker on PDA with sufficient conidiation, *Trichoderma harzianum* showed much pigmented fluppy mycelial growth and exhibited faster growth rate (Gams and Bissett, 1998).

Bacillus spp., *Pseudomonas* spp. *Rhizobium* spp. and *Trichoderma* spp. are known to have potential antagonistic effect against *R. solanacearum* and *F. oxysporum*. Thus *in-vitro* study was done to determine their ability as bio-control agents. In dual cultures all indigenous bio-control agents showed significant variations by reducing the mycelial growth of *F. oxysporum* f. sp. *lycopersici.* In *in-vitro* screening, *R. leguminosorum* was found most effective which inhibited the maximum radial mycelial growth of *F. oxysporum* f. sp. *lycopersici*. *Bacillus subtilis* 1 and *P. fluorescens* also performed better against *F. oxysporum* f. sp. *lycopersici. Bacillus licheniformis*, *P. polymixa* and *B. pumilus* also showed good effect and inhibited radial mycelial growth of *F. oxysporum* f. sp. *lycopersici.* Among two strains of *T. harzianum* the strain of *T. harzianum* (BAU) performed best which inhibited the growth of *F. oxysporum* f. sp. *lycopersici* and was found grew over *Fusarium.* This over growth may be due to its fast growing nature, rapid sporulation or secretion of cell wall lytic enzymes in dual culture. Thus it acts as both as a competitor and as an antagonist. *Trichoderma* spp. has been widely used against a number of phytopathogens (Rini and Sulochana, 2006; Bell *et al.*, 1982). Alippi and Monaco (1994) reported that *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin which have an inhibitory effect on fungal pathogens. Also, Sarhan *et al.* (2001) and Montealegre *et al. (*2005) pointed that the cell free culture filtrate of *B. subtilis* inhibited the mycelial growth, radial growth, spore germination and germ-tubes length of *F. oxysporum*. These findings are in harmony with those obtained by Montealegre *et al.* (2005) who reported that *Trichoderma* spp. secreted chitinase and B 1,3 glucanase in supernatants. There are reports that *Bacillus* spp. especially *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B.*

licheniformis, have been found effective against plant and fruit diseases caused by soil borne, aerial, or post-harvest fungal diseases (Broggini *et al.*, 2005; Szczech and Shoda, 2004; Yoshida *et al.*, 2002). *Trichoderma* spp. has proved to be useful in the control of phytopathogens affecting different crops (Soytong *et al.*, 2005; Benitez *et al.*, 2004).

Environmental factors are very important for the growth of microorganisms. The environment affects the survival and the activity of the agents of bio-control (Benbow and Sugar, 1999). Abiotic factors (temperature, pH, relative humidity) in which the air part of the plant develops act on the development of pathogenic and antagonist agents. Before biological control by any antagonistic agent can be practically implemented, it is essential to determine how biological control may be affected by changing environmental conditions. Overall, it is important to learn as much as possible regarding the ecology of these bio-control organisms and their interactions with the pathogen, host plant and their surrounding environments (Larkin *et al*., 1998; Handelsman and Stabb, 1996; Cook, 1993). A good bio-control agent must have a capacity of survival and adaptation to the various environmental conditions (Lepoivre, 2003). The antagonistic activity and growth of microorganisms are greatly influenced by the culture temperature and pH. So, the effects of temperature and pH on growth of different bacterial strains were studied. All bacteria showed highest growth at pH 7 except *Rhizobium leguminosorum* in which the highest growth was observed at pH 6. Gauri *et al*. (2011) were found optimum pH for Rhizobia was between 5.5 and 7.5. In accordance with Claus and Berkely (1986) the optimum growth of *P. fluorescens* is between 5.7 and 6.8. In addition it is important to mention that pH values, where the highest growth of these bacteria was obtained are coincident with the optimum pH for tomato development which between pH 5.5 and 7.0 (Nuez, 1995). All the bio-control agents showed the highest growth at 35^0C and a gradual decreased was observed after 35^0C. More than 86% Rhizobial strains grew well at 36^0C (Gauri *et al*, 2011). In general, the bacteria prevail in the neutral or slightly alkaline soils (Davet, 1996). Their relative abundance in the acid soils is only due to the difficulty for majority of the bacteria to develop at pH lower than 6.5 (Davet, 1996). This study shows that the antagonistic

bacteria behave differently according to environmental parameters. Biocontrol activity of antagonists may be influenced by the specific pathogen, host commodity and particularly by environmental conditions (Tian *et al.*, 2002; Spotts *et al.*, 1998).

In-vitro antagonism of these bacterial strains and *Trichoderma harzianum* revealed that culture filtrates of *Bacillus subtilis* and *Pseudomonas fluorescens* inhibited the growth of *Ralstonia solanacearum. Bacillus* species produce a variety of antimicrobial agents that were proposed to be important in plant bio-control since these molecules could inhibit growth of certain pathogenic soil microorganisms (Nagorska *et al*., 2007; Stein, 2005; Bais *et al*., 2004). Anuratha and Gnanamanickam (1990) and Xue *et al*. (2009) straind *Bacillus* species and *Pseudomonas fluorescens* from soil, those were antagonistic against *Ralstonia solanacearum.*

Three field experiments were carried out at Research field of Sher-e-Bangla Agricultural University, Dhaka during the period of 2009 and 2010 in rabi season to evaluate the efficacy of bio-control agents viz. *Trichoderma harzianum*, *T. harzianum* (BAU), *Bacillus subtilis* 1, *B. subtilis* 2, *B. licheniformis*, *B. pumilus*, *Paenibacillus polymixa, P. fluorescens* and *R. leguminosorum* on wilt severity and yield of tomato. In each experiment ten treatments were used including one control. BARI tomato 2 variety was used in those experiments. The experimental designs were RCBD with three replications. Land preparation and fertilization was done following recommended dose used for tomato cultivation. Seedlings were raised on sterilized soil and before transplanting roots of seedlings were treated by dipping the root in cell or spore suspension of selected bio-control agents for 24 hrs. Seedlings were then transplanted in the main field. Simultaneously root zone soil was inoculated with the pathogen either *Rastonia* (bacterial wilt) or *Fusarium* (fusarial wilt) or *Ralstonia* and *Fusarium* (wilt complex), respectively in three field experiments. Disease severity was measured three times viz. 30, 45 and 60 days after transplanting. Disease severity in terms of PDI was calculated following Winstead and Kelman (1952).

Effect of bio-control agents on bacterial wilt disease severity and yield of tomato was evaluated in *Ralstonia solanacearum* inoculated field during the period of 2009-2010. The results revealed that lowest wilt severity in terms of PDI was counted from *Bacillus subtilis* 2 treated plants and *P. fluorescens* and *R. leguminosorum* treated plants showed similar effect which was found better than other treatments. *Bacillus subtilis* 1, *B. subtilis* 2 and *B. licheniformis* treated plants showed statistically insignificant effect in terms of yield. But the highest percent yield increase over control was obtained from *B. subtilis* 1 treated plants. This result agreed with Van Loon (2007) who reported that some species of *Bacillus* suppress plant pathogens and insect pests by producing antibiotic metabolites, while other stimulate plant host defenses prior to pathogen infection, which indirectly contributes to increase crop production. Some of the naturally antagonistic microorganisms isolated successfully against *Ralstonia solanacearum* those were *Bacillus* species and *Pseudomonas fluorescens* (Anuratha and Gnanamanickam, 1990). Sutanu Maji and Chakrabartty (2014) assessed five strains of *P. fluorescens* from rhizosphere of tomato for their potential to bio-control and the manifestation of the pathogen on tomato by evaluating seedling emergence, vigour of the germinated seedlings and survivability of the seedlings following their transplantation. Among the strains *Pseudomonas* sp. BH25 was found to be promising to combat the pathogenic affect of *Ralstonia solanacearum* the causal agent of bacterial wilt of tomato in bioassays. They observed that the pathogen *Ralstonia solanacearum* caused only 40% seedling emergence as compared to 76% in the control, while combination of the antagonist BH25 with the pathogen improved the percentage of the seedling emergence and the value (75%) was almost similar to that of the control and they concluded that bio-control organisms could be isolated from rhizosphere and applied to bacterial wilt infested field to combat the disease infestation**.** Members of multiple *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus, B. licheniformis*, *B. megaterium*, *B. mycoides* and *B. pumilus* are known as very efficient producers of antibiotic molecules. Some more specific pathogen–bio-control strain interactions leading to pathogen restriction were reported such as interference with bio-film formation, inactivation of pathogen germination factors and degradation of pathogenicity factors such as toxins (Cawoy *et al*., 2011). Seleim

et al. (2011) found that *Pseudomonas fluorescens* caused the highest disease reduction percentage of tomato bacterial wilt disease while *P. putida* exhibited the lowest disease reduction percentage. Field results clearly pointed out that application of PGPR as potential bio-agents in controlling tomato bacterial wilt under field condition. Present results were agreed with those reported by Guo *et al*. (2004), who reported that *Bacillus* spp. and fluorescent pseudomonads reduced tomato wilt disease caused by *Ralstonia solanacearum* and increased yield of tomato when plants were treated with *Bacillus* spp. and fluorescent pseudomonads. It was reported that some strains of *P. fluorescens* actively suppressed disease occurrence of tomato bacterial wilt when introduced to plant rhizosphere by root dipping (Aino *et al*., 1993). It was also suggested that some of the *P. fluorescens* strains were incorporated into roots of tomato seedlings, probably through physically injured sites or normal openings and colonized in the tissues.

Effect of indigenous bio-control agents on fungal wilt and yield of tomato was evaluated and it was found that *B. subtilis* 2 and *Trichoderma harzianum* (BAU) treated plants caused the highest disease reduction percentage of fungal wilt and all bio-control agents increased the yield of tomato compared to control. The natural control of several phyto-pathogens is based on the presence of suppressive soils where several bio-control microorganisms belonging to *Trichoderma*, *Pseudomonas* and *Bacillus* genera were detected (Huang *et al.* 2005; Guo *et al.*, 2004; Weller *et al.*, 2002). Tomato plants treated by *Bacillus subtilis* only and/or *Trichoderma harzianum* have shown bio-control activity against damping off and root rot disease and gave high yield of tomato (Zaghloul *et al.*, 2007; Morsy, 2005). A number of strains of root-colonizing microbes have been identified as potential elicitors of plant host defenses. Some bio-control strains of *Pseudomonas* sp. and *Trichoderma* sp. are known to strongly induce plant host defenses (Haas and Defago, 2005; Harman *et al.*, 2004). Pea root bacterization with *Bacillus pumilus*, PGPR strain SE 34, triggered a set of plant defense reactions that resulted in the elaboration of permeability barriers and in the accumulation of fungitoxic compounds which provided enhanced protection against the pea root pathogen named *Fusarium oxysporum* f. sp. *pisi* (Benhamou *et al.*, 1996). Arfaouli *et al.* (2006) found that seed

treatment with Rhizobial strain reduced the percentage of wilted plants of chickpea. Several other workers have noticed the beneficial effects of rhizobia on plant growth and reduction of disease incidence (Hussain and Ghaffar, 1990). Siddiqui and Singh (2004) reported better plant growth, higher transpiration, lower wilting index in chickpea plants infected with *Fusarium oxysporum* f. sp. *ciceris* which inoculated with rhizobia. *T. harzianum* and *B. subtilis* were effective against *Fusarium oxysporum* (Getha *et al.*, 2005) and Soil inoculation with *B. subtilis* only gave higher records of yield and yield components than treated by *T. viride* (Ebtsam *et al.*, 2009). Sivamari and Gnanamanickam (1988) found an increased plant height and biomass of banana seedling by 62.17% and 61.54%, respectively due to bacterization with the suspension of *P. fluorescens* prior to planting in *F. oxysporium* f. sp. *cubense* infested soil. This is due to the fast growth rate followed by their aggressive root colonization nature that results in displacement of the pathogen and also high competitive and wide metabolic capability of the fluorescent pseudomonas strains.

Effect of indigenous bio-control agents on wilt complex pathogens and yield of tomato was assessed and *B. subtilis* 2, *P. fluorescens, R. leguminosorum* and *Trichoderma harzianum* (BAU) showed the best results, which reduced the highest percent of wilt severity and increased the yield of tomato over control. None of the study found related to bio-control of wilt complex pathogens. Several workers worked on bio-control of either fungal wilt or bacterial wilt of tomato. Potential agents for bio-control activity are rhizosphere competent fungi and bacteria, which in addition to their antagonistic activity are capable of inducing growth responses by either controlling minor pathogens or by producing growth stimulating factors. The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteria (PGPR) and primarily *Pseudomonas fluorescens* is identified as an important organism with ability for plant growth promotion and effective disease management properties (Mazzola *et al*., 1992). It is well established that only 1 to 2 % of bacteria promote plant growth in the rhizosphere (Antoun and Kloepper, 2001) Bacteria of diverse genera have been identified as PGPR, of which *Bacillus a*nd *Pseudomonas* spp. are predominant

(Podile and Kishore, 2006). Manikandan *et al.* (2010) applied liquid formulation of *P. fluorescens* strain Pf1 against Fusarium wilt increased the tomato fruit yield compared to untreated control under glass house and field conditions. Anuratha and Gnanamanikam (1990) and Gamliel and Katan (1993) reported that utilization of antagonistic rhizosphere bacteria such as *Bacillus* spp., *Pseudomonas fluorescens* and *P. putida* significantly increased the survival rate of potato, tomato, eggplant and cotton by 60-90%, 90% and 84-90%, respectively against bacterial and fusarium wilt disease. Chen *et al.* (2010) confirmed that among the 158 PGPR strains, *B subtilis* B579 suppressed the cucumber rot causing pathogen *F. oxysporum* f. sp. *cucumerium* by production of hydrolytic enzymes. *Bacillus subtilis* B579 increased the seedling vigor and growth of plants.

From the findings of the above studies it can be concluded that *B. subtilis*, *P. fluorescens*, *R. leguminosorum* and *T. harzianum* (BAU) are potential bio-control agents against wilt complex pathogens of tomato. Further research on the mechanism of action of these bio-control agents including structure further relationship will help us to develop an effective bio-control mix or formulation against wilt complex disease caused by bacteria and fungi.

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APPENDIX A

PREPARATION OF MEDIUM

1. Nutrient agar

Peptone	5.0 gm
NaCl	5.0 gm
Beef extract	3.0 gm
Agar	15.0 gm
Distilled water	1000ml

Sterilized at 121^0c under 15 Ibs/in pressure for 15 minutes

2. CPG and TTC media

Casamino acid(casein hydrolysate)	1.0 g
Peptone	10.0 g
Glucose	5.0 g
Agar	17.0 g

CPG contains the four ingredients shown. To make TTC medium, the medium was cooled to 55^0C and added 5 ml of a 1% stock solution of 2, 3, 5-triphenyl tetrazolium chloride. The stock was filter sterilized and stored at 4^0C in a refrigerator.

3. King *et al.*'s medium B agar [KB]

Proteose peptone#3 (Difco)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Glycerol	15.0 ml
Agar	15.g

4. **Cetrimide agar (CA)**

Pancreatic digest of gelatin	20.0 gm
Magnesium Chloride	1.4 gm
Potassium sulfate	10.0 gm
Agar	13.6 gm
Cetyl trimethyl ammonium bromide	0.3 gm
Glycerin	10.0 ml
Water	1000ml
pH after sterilization	7.2

Sterilized at 121^0c under 15 Ibs/in pressure for 15 minutes

5. Motility Indole Urease Agar (MIU)

Peptone	30.0 gm
NaCl	5.0 gm
Urea	20.0 gm
Monopotassium phosphate	2.0 gm
Phenol red	0.005 gm
Agar	4.0 gm
Distilled water	1000ml
pH	7.0

All ingredients except urea were dissolved in 900ml distilled water and sterilized at 121^0 C under 15Ibs/in pressure for 15 minutes. Urea solution 20% was filter-sterilized using mili-pore filter and was added to the autoclaved medium

6. Arginine dihydrolase medium (Thornley's medium)

Peptone	1.0 g
NaCl	5.0 g
K2HPO4	0.3 g
Agar	3.0 g
Phenol red	1.0 mg
Arginine HCl	10.0 g

Adjust pH to a faint pink color (pH 7.2).

7. MR-VP

Peptone	7gm
Dextrose	5 gm
Dipotassium phosphate	5 gm
Distilled water	1000ml
pH	6.9 gm

Sterilized at 121^0c under 15 Ibs/in pressure for 15 minutes

8. Phenol red carbohydrate broth

Trypticase	10.0 gm
NaCl	5.0 gm
Sugar(lactose/dextrose/glucose)	5.0 gm
Phenol red	0.02 gm
Distilled water	1000ml
pH	7.3

Sterilized at 121^0c under 15 Ibs/in pressure for 15 minutes

9. Simmon's citrate agar

Magnesium sulfate	0.2 gm
Sodium citrate	2.0 gm
NaCl	5.0 gm
Dipotassium phosphate	1.0 gm
Monopotassium phosphate	1.0 gm
Bromothymol blue	0.08 gm
Distilled water	1000ml
Agar	20.0 gm

Sterilized at 121^0c under 15 Ibs/in pressure for 15 minutes

10. MIO

Peptone	30 gm
KH_2PO_4	2.0 gm
NaCl	5.0 gm
Agar	4.0 gm
Phenol red	0.025 gm
Distilled water	1000ml
pH	6.8

Sterilized at 121^0C under 15 Ibs/in pressure for 15 minutes

20% urea solution was added.

11. Vessal medium

A. Composition of Vessal medium (selective for *Bacillus* spp.):

Meat extract	1.0 g
Bacto Peptone	10.0 g
D(-) Mannitol	10.0 g
NaCl	10.0 g
Phenol Red	0.025 g
Agar	15.0 g

The above (A) ingredients were added to 900ml distilled water and were heated to boiling to dissolve the medium completely. Then autovlave PH=7.1. The medium was then cooled to 49^0C and the following requirements were added.

a. 100 ml of a 50% Egg-yolk emulsion warmed to 50^0C

b. Polymyxin-B-Sulfate after sterile filtration in amount 5ml to 100 ml of the medium (Polymyxin-B-sulfate 50mg dissolve in 50ml Distilled water then filter sterilized store at 4^0C)

B. Ringer's solution

NaCl	9.0 g
KCl	0.42 g
$CaCl_2$	0.24 g
Sodium bi Carbonate	0.20 g
Distilled Water	1000ml

Preparation of Egg-yolk emulsion:

Fresh hens eggs were washed and placed in 70% ethanol for several hours. Then taken 50ml of egg-yolk and 50 ml of sterile ringer's (B) solution and mixed.

Note. 1 part of above solution was taken to 3 part of Distilled water and then autoclaved.

12. Medium for Nitrate reduction

Yeast extract	5.0 gm
KNO_3	3.0 gm
Nobble agar	1.0 gm
$NH_4H_2PO_4$	1.0 gm
KCl	0.2 gm
$MgSO_4$-$7H_2O$	0.2 gm
Distilled water	1000ml

Dispensed medium into tubes, autoclaved and cooled.

13. Yeast extract- dextrose- CaCO3 (YDC)

Yeast Extract	10.0 gm
Dextrose	20.0 gm
Calcium Carbonate, USP light powder	20.0 gm
Agar	15.0 gm
Distilled water	1000 ml

To obtain an even milky white medium finely ground $CaCO_3$ was used otherwise it will be precipitated to the bottom. All the ingredients autoclaved at 10 PSI for 1 hour and the autoclaved medium was cooled to 500C in a water bath and $CaCO_3$ suspended by swirling before pouring the plates.

14. H_2S production medium

$NH_4H_2PO_4$	0.5 gm
KH_2PO_4	0.5 gm
$MgSO_4$-$7H_2O$	0.2 gm
NaCl	5.0 gm
Yeast extract	5.0 gm
Peptone	0.5 gm
Distilled water	1000ml

Dispensed medium into tubes, autoclaved and cooled.

Lead acetate strips

Strips (1x 10 cm) were prepared by cutting the filter paper and then the strips were immersed in 5% lead acetate solution. Air dried and then autoclaved and stored at room temperatue.

15. YMA

Yeast extract	0.5 gm
Mannitol	10.0 gm
Agar	18.0 gm
K_2HPO_4	0.5 gm
$MgSO_4$-$7H_2O$	0.2 gm
NaCl	0.1 gm
Distilled water	1000ml

pH was adjusted 7 by adding 2 N HCl and autoclaved 20 minutes.

16. Glucose Peptone Agar (Vincent,1980)

Glucose	5.0 gm
Peptone	10.0 gm
Bromocresol purple (1% solution in alcohol)	10 ml
Agar	20.0 gm
Distilled water	1000ml

17. Peptone Sucrose broth

Sucrose	20.0 gm
Peptone	10.0 gm
Distilled water	1000ml

18. Peptone broth

Peptone	10.0 gm
NaCl	5.0 gm
Distilled water	1000ml

19. PDA medium

Potato	200 gm
Dextrose	20 gm
Agar	17 gm
Distilled water	1000ml

pH was adjusted 5.6 by adding 2 N HCl and autoclaved 20 minutes.

APPENDIX B

PREPARATION OF REAGENTS

1. Preparation of Kovac's reagent

4-Dimethyl amino benzaldehyde	5.0 g
Isoamyl (or amyl) alcohol	75. 0 ml
Hydrochloric acid(Concentrated)	25.0 ml

The aldehyde was dissolved in the alcohol. The concentrated acid was then added with care. The reagent changed light yellow to light brown. Protect from light and store at 40C.

2. Staining reagents

a) Gram's Crystal violet (hucker's modification):

Solution A:

Crystal violet(90% dye content)	2.0 g
Ethyl alcohol	20.0 ml

Solution B:

Amlonium oxalate	0.8 g
Distilled water	80.0 ml

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

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

Iodine	1.0 g
Potassium iodide(Kl)	2.0 g
Distilled water	300ml

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

c) Gram's alcohol (decolorizing agent)

Ethyl alcohol(95%)	98 ml
Acetone	2 ml

d) Safranin (counter stain)

Safranin (2.5%) solution in 95% ethanol)	10.ml
Distilled water	100ml

3. Oxidase reagent

1% aqueous solution of N, N,N,'N-tetramethyl-p-phenylene dihydrechloride.

4. Catalase reagent

3% aqueous solution of H2O2 was prepared from the H2O2 absolute solution.

5. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44g of Na_2HPO_4 and 2 g of KH_2PO_4 in 800 ml of distilled water. pH was adjusted to 7.4 with HCL. The final volume was adjusted to one litre by distilled water. The solution was sterilized by autoclaving for 20 minutes and stored at room temperature.

APPENDIX C

1. Characteristics of AEZ-28.

Land type	Medium to high land
General soil type	Non Calcareous dark gray flood plain soil
Soil Series	Tejgaon
Topography	Upland
Elevation	8.45
Location	SAU Research Farm
Field level	Above flood level
Drainage	Fairly good
Compactness (Firmness)	Compact to friable when dry

2. Result of mechanical and chemical analysis of soil of experimental plot.

Mechanical analysis

Constituents	Percent(%)
Sand	33.45
Silt	60.25
Clay	6.25
Textural class	Silt clay

Chemical analysis

Soil properties	Amount
Soil pH	6.12
Organic Carbon	1.32
Total Nitrogen (%)	0.08
Available P(ppm)	20
Exchangeable K	0.2

3. Minimum, maximum and average temperature during cultivation period.

Year	Month	Minimum Temperature °C	Maximum Temperature °C	Average Temperature °C
First cropping season				
2009	November	15.2-25.2	26.9-33.9	20.3-28.7
2009	December	11.4-18.4	21.1-29.0	17.3-22.2
2010	January	9.6-16.0	16.1-29.0	13.0-21.3
2010	February	12.0-20.8	26.1-31.2	19.1-25.1
Second cropping season				
2010	November	16.6-24.5	27.3-33.2	21.8-27.6
2010	December	11.0-20.8	22.8-29.7	17.1-23.0
2011	January	8.2-16.4	14.2-27.8	11.5-21.3
2011	February	13.0-21.6	26.0-31.0	20.4-25.4

Source: Bangladesh Meteorological Department, Climate Division, Agargaon, Dhaka-1207.

4. Minimum, maximum and average temperature during cultivation period.

Year	Month	Minimum Rainfall (mm)	Maximum Rainfall (mm)
First cropping season			
2009	November	1.0	3.0
2009	December	0.0	0.0
2010	January	0.0	0.0
2010	February	0.0	48.0
Second cropping season			
2010	November	0.0	0.0
2010	December	20.0	61.0
2011	January	0.0	0.0
2011	February	0.0	0.0

Source: Bangladesh Meteorological Department, Climate Division, Agargaon, Dhaka-1207.