BIO-EFFICACY OF MICROBIAL ANTAGONISTS AGAINST FOOT AND ROOT ROT PATHOGEN(S) OF LENTIL

SANZIDA HOQUE

DEPARTMENT OF PLANT PATHOLOGY,
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
SHER-E-BANGLA NAGAR, DHAKA -1207

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SANZIDA HOQUE
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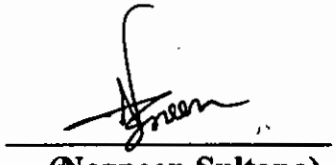
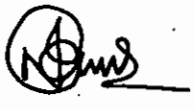


(Nazneen Sultana)
Professor
Supervisor



(Abu Noman Faruq Ahmmed)
Assistant Professor
Co-Supervisor

Dr. F. M. Aminuzzaman
Chairman
Examination Committee
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka

Department of Plant Pathology
Sher-e-Bangla Agricultural University
Dhaka-1207, Bangladesh

Fax:: +88028155800
Web site: www.sau.edu.bd

CERTIFICATE

This is to certify that thesis entitled, **"BIO-EFFICACY OF MICROBIAL ANTAGONISTS AGAINST FOOT AND ROOT ROT PATHOGEN(S) OF LENTIL"** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN PLANT PATHOLOGY,** embodies the result of a piece of bona fide research work carried out by **SANZIDA HOQUE** bearing Registration No. **06-01914** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

31st December, 2013
Dhaka, Bangladesh

.....
(Nazneen Sultana)
Professor
Department of Plant Pathology
Supervisor

DEDICATED TO
MY
BELOVED PARENTS

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The Author.

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Sanzida Hoque

ABSTRACT

The experiment was conducted in the Disease Diagnostic Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka during the period of November 2011-February 2013 to determine the bio-efficacy of microbial antagonists against foot and root rot pathogen(s) of lentil. Two fungal genera namely *Fusarium oxysporum* and *Sclerotium rolfsii* were isolated from foot and root rot infected lentil plants. Seven seed treatment agents, viz., *Rhizobium leguminosarum* isolate 1, isolate 2, isolate 3, isolate 4, *Pseudomonas fluorescens, Trichoderma harzianum* and Control (sterile water) were used in this experiment. In dual culture method, highest zone of inhibition of *F. oxysporum* (57.37%) was measured against *R. leguminosarum* isolate 3 and isolate 4. In case of *S. rolfsii,* 80.00% and 37.85% zone of inhibition were measured against *P. fluorescens* and *T. harzianum,* respectively. In rolled paper towel method, the highest germination (91.00%) and lowest dead seeds (9.00%), no abnormal and diseased seedlings were counted from seeds treated with *R. leguminosarum* isolate 1. The maximum vigor index (1390.00) was observed in *P. fluorescens* treated seeds. In water agar test tube method, the highest germination (88.00%) and lowest dead seeds (11.67%) were counted from *R. leguminosarum* isolate 4 treated seeds. The maximum number of normal seedlings (84.56) and minimum number of abnormal seedlings (2.00) were counted from seeds treated with *R. leguminosarum* isolate 1 and isolate 2, respectively. The minimum number of diseased seedlings (0.67) were counted from *T. harzianum* treated seeds.

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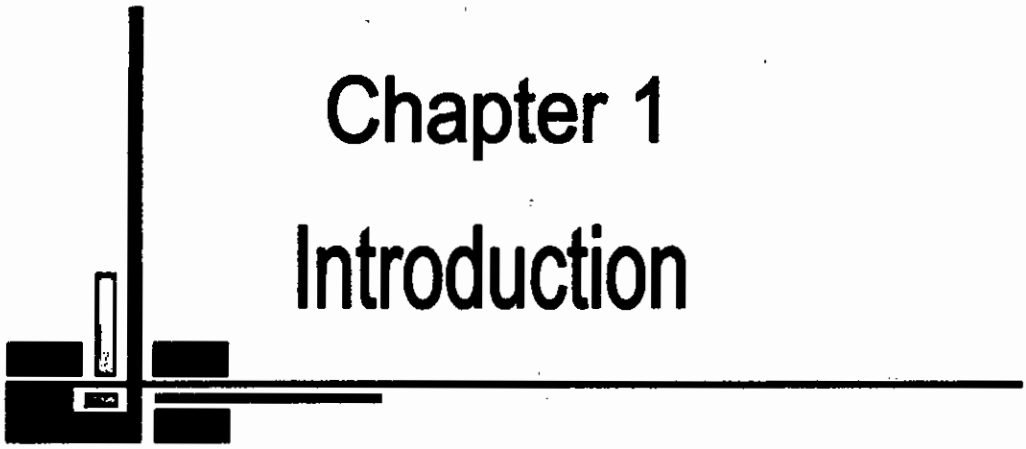


Chapter 1
Introduction

CHAPTER I

INTRODUCTION

Lentil (*Lens culinaris*) is an edible pulse. Bangladesh grows various types of pulse crops. Among them grass pea, lentil, mungbean, blackgram, chickpea, field pea and cowpea are important. It is an important food crops because it provides a cheap source of easily digestible dietary protein. Pulse protein is rich in amino acids like isoleucine, leucine, lysine, valine etc. According to FAO (1999) a minimum intake of pulse by a human should be 80 g per head per day, whereas it is only 14.19 g in Bangladesh (BBS, 2006). This is because of the fact that national production of the pulses is not adequate to meet the national demand.

Lentil contains 25.78% protein (Erskine and Witcombe, 1984) and 59% carbohydrate (Bakhsh *et al.*, 1991). Proteins include the essential amino acids isoleucine and lysine. Lentil is an essential source of inexpensive protein in many parts of the world, especially in West Asia and the Indian subcontinent, which has large vegetarian populations. Lentils are deficient in two essential amino acids, methionine and cysteine. However, sprouted lentils contain sufficient levels of all essential amino acids, including methionine and cysteine. Lentils also contain dietary fiber, folate, vitamin B_1, and minerals. Red (or pink) lentils contain a lower concentration of fiber than green lentils (11% rather than 31%). It has some anti-nutritional factors, such as trypsin inhibitors and relatively high phytate content. Trypsin is an enzyme involved in digestion, and phytates reduce the bio-availability of dietary minerals. The phytates can be reduced by soaking the lentils in warm water overnight.

In Bangladesh lentil is cultivated in an area of 1,54,655 ha and the yearly production is about 1,22,000 mt (BBS, 2009). However, the yield of lentil is much lower in Bangladesh compared to that of other lentil growing countries like Syria, Turkey, Canada, USA and Ethiopia (Hossain *et al.*, 1999).

There are various reasons associated with low yield of lentil in Bangladesh, where diseases are considered as the major constraints. Various diseases may cause 30-

40% yield loss in lentil (Begum, 2003). Among the major diseases of lentil, foot and root rot is the most important one. The disease is caused by soil borne fungi like *Fusarium oxysporum, Sclerotium rolfsii* and *Rhizoctonia solani*. It may cause 100% seedling mortality in the field under monoculture and conducive weather conditions (Begum, 2003). As there is no effective fungicide or resistant variety for the management of this disease, the farmers can not maintain the optimum plant population in the field and the yield of the crop is drastically reduced.

In order to control the diseases of crop plants in Bangladesh farmers have to use chemicals. Indiscriminate use of chemicals for controlling diseases of crop plants causes environmental pollution and health hazards all over the world. Not only this, the costly chemicals are being imported to our country from aboard and farmers have to pay a high price. Moreover, huge foreign currency goes out of the country every year for purchasing plant protection chemicals. As an alternative means of avoiding these problems, biological control agents are now being used in many developed countries for combating the disease with the aim of increasing food production.

The biological management of soil-borne diseases is increasingly gaining stature as a possible practical and safe approach. The potential of the antagonistic micro-organisms in reducing the intensity of crop damage by the soil-borne plant pathogens has been reported (Lewis and Larkin, 1997). Several strains of *Trichoderma* spp. have been found to be effective as biocontrol agents of various soil-borne plant pathogenic fungi such as *Fusarium, Pythium, Rhizoctonia* and *Sclerotium* (Papavizas, 1985). *Trichoderma* spp. are found in almost all tropical and temperate soil. The suppression of disease by *Trichoderma* is based on hyperparasitism, antibiosis, induced resistance in the host plant and competition for nutrients and space (Harman *et al.*, 2004).

The use of rhizosphere resident microbial antagonist specifically the *Pseudomonas fluorescens* is noted as a promising control agent. The rhizosphere is a habitat in which several biologically important processes and interactions takes place which is primarily due to the influx of mineral nutrients from plant

roots through mass flow and diffusion (Sorensen, 1997; Bias, 2004). Among the rhizosphere organisms fluorescent *Pseudomonas* strains are often selected for biological control agents because of their ability to utilize varied substrates under different conditions; short generation time and motility and assist colonization of roots. Moreover, they produce active extra cellular compounds such as siderophores responsible for the biological suppression of several soil borne plant pathogens (Bagnasco *et al.*, 1998).

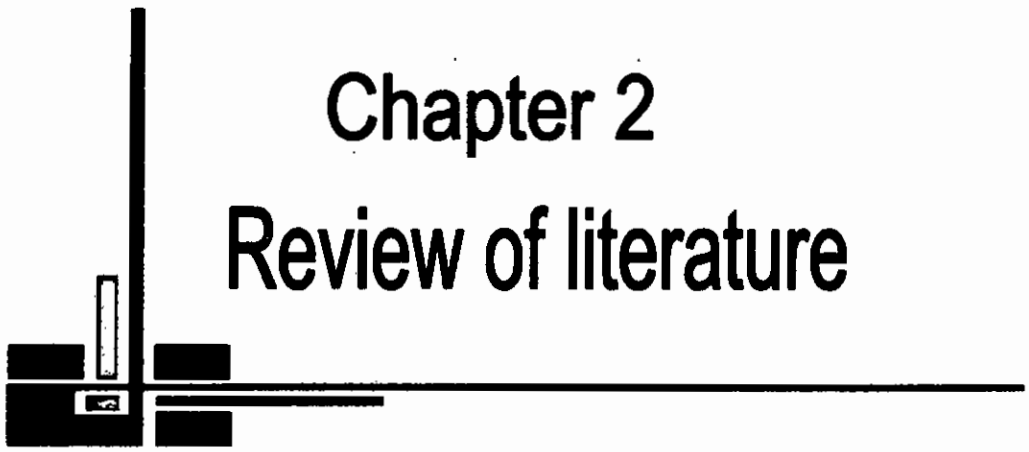
The use of antagonistic bacteria as biological control method may provide a treat challenge to combat the seed and soil borne pathogens. Strains of several *Rhizobium* spp. have been reported to inhibit in varying degrees the *in vitro* growth of several root- infecting fungi (Antoun *et al.*, 1977; Drapeau *et al.*, 1973; Gray and Sackston, 1980). It inhibits the growth of foot and root rot causal pathogens, decrease foot and root rot disease and increase yield of leguminous and nonleguminous crops (Kibria and Hossain, 2002). *Rhizobium* produces some volatile substances, fluorescent pigment, enzymes, chitinase, beta-1, 3-glucanase, siderophore, hormone, indol acetic acid and gibbeellins which inhibits and suppresses the growth and sporulation of pathaogens under cultural conditions and in the field (Kumar *et al.*, 2001). If *Rhizobium* can successfully be used for controlling foot and root rot of lentil, this may help saving the foreign currency as well as may reduce the environmental pollution. In addition, *Rhizobium* would enrich soil nitrogen level through BNF (Biological Nitrogen Fixation) and crop plants would be immensely benefited. The farmers of Bangladesh may enjoy dual benefits. Firstly, disease management without chemicals and thus without environmental pollution and secondly, extra benefit of nitrogen addition to the soil through nodulation in the root system of lentil by using microbial antagonist.

No such research work in this area of controlling foot rot of lentil by microbial antagonist means in Bangladesh has so far been conducted. Therefore, the present research programme was undertaken to achieve the following objectives:

i. to isolate the causal organisms of foot and root rot of lentil.

ii. to isolate and identify probable antagonists against foot and root rot pathogen(s) of lentil.

iii. to determine the efficacy of antagonistic microorganisms against foot and root rot pathogen(s) of lentil.



Chapter 2
Review of literature

CHAPTER II

REVIEW OF LITERATURE

Drapeau *et al.* (1973) studied antifungal activity of *Rhizobium* and found that *Rhizobium* isolates showed antifungal activity against *Fusarium* spp., *Colletotrichum destructivum, Phytophthora caetocum* and *Coniothyruium* sp..

Antoun *et al.* (1977) carried out an experiment on the effect of volatile substances produced by *Rhizobium* on radial growth of some plant pathogenic fungi. They observed that volatile metabolites produced by *R. meliloti* and *R. trifolii* affect the radial growth of *F. avenaceum, F. oxysporum, Phoma medicaginis* and *Stemphylium sarcinaeforme.*

Panwar *et al.* (1977) studied response of gram, lentil and field peas to inoculation of *R. meliloti* and levels of nitrogen and phosphorus and reported that inoculation of lentil and peas increased the yield up to 11.0%.

Sekhon *et al.* (1978) found that effective symbiosis with *Rhizobium* strain and consequent increase of grain yield in lentil. Inoculation with a superior strain produced 23-32% more grain than no inoculation on loamy-sand soil and 46-90% more on sandy loam soil.

Tu (1978) conducted an experiment to prevent soybean nodulation by using tetracyclin and observed its effect on soybean root rot caused by *F. oxysporum.* He observed that inoculation with *Bradyrhizobium* reduced the severity of root rot in soybean.

Bhargava *et al.* (1979) correlated the relation between the number of root nodules and incidences of root rot and wilt diseases of pulses. They observed that plants with numerous nodules, collected in the Allahabad area, had less or no infection by root rot and wilt pathogens (*Fusarium* spp.). Plants of *Phaseolus aureus* (*Vigna radiata*) and *V. mungo*, inoculated with *Rhizobium* alone, remained healthy; those inoculated with *Rhizobium* + a pathogen and with the pathogen alone had 20-30% and 90% infection, respectively.

Rai and Singh (1979) studied response of strains of *Rhizobium* on grain yield, protein and amino acid content of chickpea and recorded that inoculation with *Rhizobium* bacteria led to a significant increase in seed yield of chickpea compared with the uninoculated control.

Tu (1979) conducted an experiment on differential tolerance of four species of fungi to parasitism by *R. japonicum in vitro* in terms of fungal sporulation. Fungal sporulation was generally reduced by rhizobial inoculated colonies. The percentages of reduction were 75, 65, 35 and 47% for *Phytophthora megasperma, Pythium ultimum, Ascochyta imperfecta* and *F. oxysporum*, respectively when compared with uninoculated controls.

Tu (1980) studied incidence of root rot and over wintering of alfalfa as influenced by *Rhizobia* and observed that higher levels of *Rhizobia* reduced root rot caused by *Phytophthora megasperma* and *F. oxysporum*. Maximum protection was obtained when *Rhizobia* were added at the same time as the fungal inoculum. *Rhizobia* have been found to protect the plants by reducing rot severity and increasing total N content.

Kerr and Antoun (1980) studied interaction effect of *Rhizobium* and soil borne fungi and reported that the growth of *F. avenaceum* was inhibited by *Rhizobium*.

Sawada (1982) studied interaction between rhizobial nodulation of alfalfa and root rot caused by *F. oxysporum in-vitro* and observed that hyphal growth of *F. oxysporum* was suppressed by multiplication of *R. meliloti* in a mixed culture *in vitro*.

Chary *et al.* (1983) carried out an experiment on interaction of seed-borne fungi and *Rhizobium* on nodulation and plant growth of mungbean *(Vigna radiata)* and reported that inoculation with *F. oxysporum, Phoma exiqua* and *R. solani* completely inhibited nodule formation of mungbean in sterilized soil which was improved when inoculated simultaneously with *Rhizobium*.

Harnadez and Hill (1983) studied the effect of plant population and inoculation on yield and yield component of chickpea and observed that inoculation with *Rhizobium* strain CC 1192 increased seed yield of chickpea by 29% with significant increase in branch and pod number.

Konde *et al.* (1984) conducted an experiment to study the effects of some microorganisms on *Rhizobium* and seed germination in chickpea. *In-vitro* study showed that growth of the antagonists (*Aspergillus flavipes, Penicillium funiculosum* and *Bacillus* sp.) and of the pathogens (*F. oxysporum f.* sp. *ciceri* and *Rhizoctonia solani*) was suppressed by *Rhizobium* sp. by 9-11 and 20-28%, respectively. An increase in seed germination of 3.57-15.47% and plumule length of 3.77-12.75% were recorded when antagonists were inoculated together with *Cicer arietinum rhizobia.*

Sandhu (1984) carried out an experiment on the effect of sowing date, phosphorus level and herbicides on the response of *Rhizobium* inoculation in lentil and reported that lentil seed inoculation with *Rhizobium* culture improved yield by 8-22%.

Patil (1985) conducted an experiment to determine the effects of fungal infection on nodulation and nitrogen fixation in soybean. Soybean cv. Bragg seeds were inoculated with *R. japonicum* and the seedlings were subsequently inoculated with *F. udum* f. sp. *crotalariae, Rhizoctonia solani* or *Sclerotium rolfsii* and observed that infection caused a reduction in the number of effective nodules/plant.

Buonassisi *et al.* (1986) studied the effect of *Rhizobium* spp. on *Fusarium solani* f. sp. *phaseoli* in snap bean in dual culture. *Rhizobium* strains isolated from nodules of commercial snap beans (*Phaseolus vulgaris*) in the Lower Fraser Valley of British Columbia inhibited the radial growth of strains of *F. moniliforme, F. oxysporum* and *F. solani* f. sp. *phaseoli.*

Pal and Ghosh (1986) studied response of lentil and chickpea to inoculation with different strains of *R. leguminosarum* and found that seed inoculation with *R. leguminosarum* strain L 25 and L 20 increased nodulation and enhanced seed yield

by 59.8% in lentil. *Rhizobium* inoculation also increased up to 38.87% seed yield in chickpea.

Sesan (1986) carried out an experiment on biological control of fungul diseases of crop plants. Compatibility was shown between treatments with *Trichoderma viridae* and with the nitrogen-fixing bacterium *R. phaseoli.* Good results were obtained with bio-preparations applied in soil and seed treatments against *Pythium debaryanum, R. solani, Fusarium* spp. and *Sclerotinia sclerotiorum* on sunflower and leguminous plants and against *Botrytis cinerea* on grapevine.

Zaki and Ghaffar (1987) conducted greenhouse experiments on cotton, mungbean, okra and sunflower to determine the antagonistic activity of *Rhizobium* against *Macrophomina* root rot caused by *Macrophomina phaseolina*. They reported that, *Rhizobium* isolates from pea, luceme and soybean showed significant reduction in severity of *Macrophomina* root rot of mungbean, okra and sunflower.

Ordentlich *et al.* (1987) isolated two hundred and three different strains of bacteria from the rhizosphere soil of bean, peanut and chickpea plants from *S. rolfsii* infested soil. A bacterium, identified as *Serratia marcescens*, was found to be the best biocontrol agent of the pathogen, under greenhouse conditions (up to 75% disease reduction). This bacterium significantly reduced damping off incidence of bean, caused by *R. solani*.

Balasundaram and Sarbhoy (1988) conducted inhibition study on seven soybean pathogens (*S. rolfsii, F. semitectum (F. pallidoroseum), F. solani, Fusarium* spp., *Rhizoctonia bataticola (Macrophomina phaseolina), R. solani* and *Xanthomonas campestris* pv. *glycines*) by 20 *R. japonicum* strains *in vitro*. Several *R. japonicum* strains, particularly fast growing strains, prevented the growth of *Corticium rolfsii* and *Macrophomina phaseolina*. Combined inoculation of *M. phaseolina* with a rhizobial strain (SB 118) resulted in significantly higher dry weight of shoot/plant compared with fungal inoculation alone.

Satter and Podder (1988) conducted a field experiment on the effectiveness of *Rhizobium* isolates of lentil with and without nitrogen fertilizer and found that

Rhizobium isolate L5, L1 and LN12 increased the grain yield of lentil 11.57, 14.41 and 9.75%, respectively, over the uninoculated control.

Bhattacharya and Mukherjee (1988) studied *R.-Sclerotium* interaction in groundnut and observed that plants inoculated with both the *Rhizobium* symbiont and *S. rolfsii* developed less disease than plants inoculated with the pathogen alone in pot experiments. Nodule numbers and volume were higher in double inoculated plants than in those with *S. rolfsii* alone. In field experiments, *Rhizobium* inoculated groundnuts yielded twice as much as control plants and nearly 3 times as much as plants inoculated with the pathogen. Inoculation with both organisms gave higher yields than in the controls.

Gupta *et al.* (1988) showed the effect of seed treatment with Bavistin and *Rhizobium* on wilt incidence, nodulation and yield of chickpea. They reported that the highest number of nodules/plant and maximum grain yield in *Cicer arietinum* were obtained in case of 0.1% Bavistin (carbandazim) treated seed against *F. oxysporum* f. sp. *ciceris* was followed by *Rhizobium* inoculated in field trials over two seasons.

Chakraborty and chakraborty (1989) studied interaction of *R. leguminosarum* and *F. solani* f. sp. *pisi* on pea and reported that bacterization of seed with *R. leguminosarum* biovar *viceae* was highly effective against the severity of foot rot of pea.

Lalande *et al.* (1989) carried out *in-vitro* inhibition studies between *R. leguminosarum* and mycelial growth of root infecting fungi. Superimposed dual culture tests showed that *F. solani* and *F. equiseti* were slightly inhibited and *F. oxysporum, F. acuminatum* and *F. avenaceum* were strongly inhibited by some isolates of *Rhizobium*.

Hilal *et al.* (1990) evaluated five seed dressing fungicides alone or in combination with *R. lupine* for control of soil-borne groundnut diseases. Seed or soil application of *Rhizobium* also increased plant survival. All treatments increased pod yield, with the highest yields when plants were grown with *Rhizobium*.

Hussain *et al.* (1990) conducted an experiment on biological control of charcoal rot of sunflower and mungbean (*M. phaseolina*) and reported that infection by *M. phaseolina* was significantly reduced by seed treatment with *R. meliloti.*

Nagar *et al.* (1990) studied the effect of seed inoculation with *R. lupine* for controlling crown rot disease. They reported that incidence of crown rot of groundnuts in soil caused by *Aspergillus niger* was significantly reduced when the seed was inoculated with *R. lupine.*

Bhattacharya and Mukherjee (1990) found that the presence of *S. (Corticium) rolfsii* reduced the population of *Rhizobium* sp. in the rhizosphere of groundnut. Similarly soil inoculation with *Rhizobium* reduced the population of *Corticium rolfsii.* In field, *C. rolfsii* reduced root nodulation and reduced crop yields. *Rhizobium* increase yields by 121 to 129%, but when *C. rolfsii* was also present, yield increased by only 48 and 13% compared with a uninoculated control. Disease incidence in groundnut was increased in inoculation with C. *rolfsii* but the incidence was reduced when *Rhizobium* was also present. They suggested that *Rhizobium* could be used to reduce incidence of *C. rolfsii* on groundnut.

Farzana *et al.* (1991) reported that seed treatment with *R. meliloti* was significantly effective in reducing the disease infection of soybean plant caused by root infecting fungi (*R. solani, M. phaseolina* and *Fusarium* spp.). The effectiveness of the antagonists decreased with time.

Blum *et al.* (1991) evaluated 49 *Rhizobium* strains against *R. solani.* Among them six were inhibitory and six were stimulatory. They inoculated two inhibitory *Rhizobium* strains (573-127K14 and 576-2535) with *Phaseolus vulgaris* seeds in green house and found that the strains decreased the incidence of damping off and the index of disease severity.

Perveen *et al.* (1991) treated tomato seeds with *T. harzianum, Gliocladium virens, Paecilomyces lilacinus, R. meliloti* or *Streptomyces* sp. and obtained complete control of *F. oxysporum* on 30 days and 120 days old tomato plants. The effectiveness of the biocontrol agents against *M. phaseolina* decreased with time, and only *G. virens* could reduce *M. phaseolina* infection after 120 days. All

treatments resulted in higher seed germination rates, and increased fresh weight, shoot length and plant height compared with an untreated control.

Haque *et al.* (1992) studied the efficiency of *R. meliloti* for controlling root rot of fenugreek and found that seed dressing and soil drench with *R. meliloti* reduced *M. phaseolina* infection by > 50% on 30 days old fenugreek seedlings. Seed dressing or soil drench with *R. meliloti* completely controlled infection by *R. solani* on 30 days and 60 days old plants.

Jadhav *et al.* (1993) studied response of chickpea cultivars to inoculation of different strains of *Rhizobium* and noted that soil inoculation with *Rhizobium* strains increased nodulation, plant dry matter (DM) and N-content of chickpea with strain CR-1 having the greatest effect.

Haque and Ghaffar (1993) used *Rhizobium* in the control of root rot diseases of sunflower, okra, soybean and mungbean. They found that *R. meliloti, R. leguminosarum* and *Bacillus japonicum* used either as a seed dressing or as a soil drench reduced infection of *M. phaseolina, R. solani* and *Fusarium* spp. in both leguminous (soybean and mungbean) and non-leguminous (sunflower and okra) plants and gave increases in shoot length and fresh weight as compared with untreated controls in field.

Dawar *et al.* (1993) conducted an experiment to evaluate the effects of seed pelleting with the microbial antagonists *Stachybotris atra, Memnoniella echinata* and *R. meliloti* on the colonization of mungbean (*Vigna radiata*) and cowpea to control *M. phaseolina, R. solani* and *Fusarium* spp.. *Stachybotrya atra* combined with *R. meliloti* was more effective in suppressing colonization by *M. phaseolina* and *Fusarium* spp. on mungbean (*Vigna radiata)* and *R. solani* on cowpea roots than their separate use. Colonization of cowpea roots by all three pathogens and of mungbean rots by *Fusarium* spp. was greatly suppressed by combined use of *Macropomina echiata* and *R. meliloti* compared with their separate use.

Hoque (1993) used *Rhizobium* inoculants as a source of nitrogen for grain legumes and found that the inoculants markedly increased nodule number, nodule mass,

shoot weight and yield of crops compared with control and urea-N treatment. For lentil, inoculation increased yield by 30%, over control and 13% over urea treatment.

Parveen *et al.* (1994) reported that *Bradyrhizobium japonicum* and *R. meliloti* significantly controlled infection of *M. phaseolina, F. oxysporum* and *F. solani* on tomato and okra of both *F. oxysporum* infested and uninfested natural soil.

Chandra (1995) studied response and economics of *Rhizobium*, phosphorus and zinc application in gram (*Cicer arietinum* L.) and reported that seed inoculation with *Rhizobium* in gram produced significantly more number and dry weight of nodules than the uninoculated control during early crop growth period with highest cost benefit ratio (BCR).

Toth *et al.* (1995) conducted both pot and field experiments to find out interactions between *Rhizobium* inoculation, *Fusarium* infection and nutrient supply of alfalfa. The study showed that *Fusariom* infection was reduced by *R. meliloti* inoculation.

Haque *et al.* (1995) conducted an experiment on the efficacy of *Bradyrhizobium* sp. and *Paecilomyces lilacinus* with oil cakes in the control of root rot of mungbean and reported that *Bradyrhizobium* sp. alone significantly reduced infection by *M. phaseolina, R. solani* and *Fusarium* spp. on roots of mungbean.

Haque and Gaffar (1995) significantly controlled soil borne infection of 30 days old seedlings by *M. phaseolina, R. solani* and *Fusarium* spp. by soybean seed treatment with *B. japonicum*. But greater grain yield was recorded when *B. japonicum* (TAL 102) was used with *T. hamatum*.

Izhar *et al.* (1995) observed that combined use of *Bradyrhizobium* sp. (Tal 480) and *Pseudomonas aeruginosa* completely controlled infection of *R. solani* and *F. oxysporum* in chickpea. The combined treatment gave better control of *F. solani* infection than when used singly.

Sharma *et al.* (1995) obtained that the mungbean (*Vigna radiata* L.) seed bacterization with *Rhizobium* increased nodulation (in terms of their number and

dry weight), biomass and grain yield over the treatments where seeds were disinfected with fungicides alone.

Khot *et al.* (1996) isolated *Rhizobacteria* from the rhizosphere of chickpea plants. Seed inoculation with these bacteria caused remarkable reduction of wilt disease in crops in a wilt sick soil under net house conditions. In field condition, two isolates, CRS M8 and CRS M18 reduced wilt disease by 48.6 and 31.5% respectively, in a wilt sick nursery.

Muthamilan and Jeyarajan (1996) studied integrated management of *Sclrotium* root rot of groundnut involving *T. harzianum, Rhizobium* and carbendazim and reported that seed and soil inoculation with *Rhizobium* strain reduced groundnut root rot caused by *S. rolfsii.*

Namdeo *et al.* (1996) carried out an experiment on the influence of rhizobial inoculation on nodulation and yield of lentil genotypes under rainfed conditions and stated that *Lens culinaris* (Lentil) inoculated with *Rhizobium* strains increased seed yield by 17.5-23.2% compared with no inoculation.

Xi *et al.* (1996) applied formulated rhizobacteria against root rot of field pea and suggested that *Rhizobium* is compatible with biological control agents for controlling root rot of pea.

Beigh *et al.* (1997) studied *in-vitro* interaction between *F. solani* and *R. leguminosarum.* They observed that interaction between *F. solani* and *R. leguminosarum* pv. *phaseoli* caused a reduction of 35 to 52% in mycelia biomass production during 14 days of incubation at 28±1°C *in- vitro.* There was also a remarkable decrease in fungal sporulation.

Gupta and Samaddar (1997) studied interaction between soil organisms and *Rhizobium* and found that many soil organisms including fungi, bacteria and actinomycetes were inhibitory to the growth of *Rhizobium* spp. in dual culture on agar plates. The root pathogens of legumes were most inhibitory to the lentil *Rhizobium* isolate.

Nautiyal (1997) used *Pseudomonas* sp. and *Rhizobium* sp. for the suppression of chickpea pathogenic fungi. They obtained 256 bacterial strains from chickpea, representing different morphological types and studied their bio-control activity against *F. oxysporum* f. sp. *ciceri*, *R. bataticola* and *Pythium* sp. under *in-vitro* conditions. *Pseudomonus* sp. NBRI9926 and *Rhizobium* sp. NBRI9513 were selected because of their unique ability to inhibit all three fungi.

Sattar (1997) reported that yield was increased by using Rhizobial biofetilizers in lentil (15-40%), chickpea (20-45%), Mungbean (18-35%), yard long bean (25-45%), grasspea (20-30%) and groundnut (20-40%). He also stated that biofertilizers increased protein (%) in the crops and decreased diseases of pulse crops.

Pawar *et al.* (1997) showed field reaction of chickpea and inoculation of *Rhizobium* strains to nodulation and grain yield and found that seed inoculation with *Rhizobium* strains increased the number of nodule/plant, nodule dry weight/plant and seed yields in chickpea. They obtained highest seed yields from seed inoculation with *Rhizobium* strains CO-Be-12 (4.06 t/ha), SG-8-87 (4.01 t/ha) and SG-3-87- (3.84 t/ha).

Bhattacharyya (1998) studied the effect of simultaneous infection of Pigeonpea (*C. caja)* with its pathogen *F. oxysporum* and the symbiont JCC/C3 strain of cowpea *Rhizobium* on nodulation, wilt disease intensity and yield for two consecutive years in India. The crop grown from seeds inoculated with *Rhizobium* produced significantly higher yield.

Omar and Abd-Alla (1998) tested 21 *Rhizobium* and *Bradyrhizobium* strains against the mycelial growth of three pathogenic fungi on solid and liquid media. All tested *Rhizobia* and *Bradyrhizobium* significantly suppressed the growth of *F. solani, M. phaseolina* and *R. solani in vitro.*

Siddiqui *et al.* (1998) carried out an experiment to determine the effect of fungicides on the efficacy of *R. meliloti* and *Bradyrhizobium* sp. to control the root infecting fungi of chickpea. They reported that *Bradyrhizobium* sp. with benomylor carbendazim and *R. meliloti* isolates with captan or thiophanate-methyl showed

better control of *F. solani* on chickpea roots than when used separately. *Bradyrhizobium* sp. (mungbean isolate) with carbendazim showed complete control of *M. phaseolina* infection, whereas captan was more effective against *R. solani* when used alone or with rhizobia. The maximum number of nodules/plant was produced by *Bradyrhizobium* sp. (TAL-620, chickpea isolate) used with benomyl or carbendazim. Some rhizobial isolates significantly increased plant height when used with benomyl, carbendazim or thiophante-methyl.

Sukhovitskaya *et al.* (1998) selected promising rhizobacterial for inoculating agricultural plants. Some 75 *Rhizobium* isolates from the rhizobacteria of various grasses and cereals were examined for nitrogenase activity, colonizing ability (on wheat, rape and mustard), and antimicrobial properties (against the phytopathogens *F. oxysporum, F. sporotrichiella, A. alternata* and *P. syringae*). Four isolates were inhibitory.

Khan *et al.* (1998) carried out a pot experiment on biological seed treatment with *Rhizobial* inoculants to control foot and root rot of lentil and reported that up to 62.5% and 73.3% reduction of Fusarial and Sclerotial foot and root rot, respectively. Under field condition, up to 87.43% reduction of foot and root rot of lentil was obtained in Rhizobial inoculants treated seeds. Seed yield increased significantly in Rhizobial inoculants treated seed.

Hossain *et al.* (1999a) tested Bavistin (Carbendazim) at 0.1 and 0.2% by dry weight and *R. leguminosarum* (BAUR 303) inoculants at 30, 50, and 70 g/kg of seeds as seed treating agents to control foot and root rot of lentil (Variety Utfafa) caused by *F. oxysporum*. Seeds treated with Bavistin at 0.2% showed the highest germination percentage (92%). Bavistin at 0.2% and Rhizobacteria at 30 g/kg increased seed germination by 39 and 23% over control, respectively. The highest grain yield was obtained at 0.2% Bavistin and 50 and 70 g/kg inoculants. Treatments with Bavistin at 0.2% and Rhizobial inoculants at 50 g/kg resulted in the best control of seed-borne *F. oxysporum.*

Hossain *et al.* (1999b) conducted an experiment on seed treatment with *Rhizobium* in controlling *F. oxysporum* and *S. rolfsii* of lentil in pot and under field

conditions. They reported that seed treatment with *Rhizobium* inoculants showed excellent effect in protecting attack of both *F. oxysporum* and *S. rolfsii* resulting 16.9 and 31.4% higher seed germination, respectively in pot soil artificially inoculated with these pathogens. In field, Rhizobial inoculants significantly decreased the number of foot and root rot plants and increased germination, shoot length, dry weight of plants, pods/plants and seed yield up to 74.3, 7.3, 111.6, 42.1 and 118.7%, respectively, over the untreated control.

Hossain (2000) carried out an experiment on biocontrol of *F. oxysporum* and *S. rolfsii* infection in lentil, chickpea and mungbean. He used peat based biofertilizer and Rhizobial strains for controlling *F. oxysporum* and *S. rolfsii* causing foot and root rot of lentil, chickpea and summer mungbean in pot soil. Rhizobial strains viz. L 4, L 634 and L 640 were used for lentil, while C 220, D 620 and C 1140 were applied for chickpea and THA 301, M 169 and SP 10 were employed for summer mungbean. Promising results have been found by treating seeds either with biofertilizer or Rhizobial strains in controlling infection of *F. oxysporum* and *S. rolfsii* causing foot and root rot and death of plants of lentil, chickpea and summer mungbean. Treatment of seeds with biofertilizer resulted 85.19 and 73.08% reduction in death of plants due to infection by *F. oxysporum* in lentil and chickpea, respectively. Treatment of seeds with biofertilizer also showed 76.67, 86.96 and 72.72% reduction in death of plants due to infection by *F. oxysporum* in lentil and chickpea, respectively. In case of infection by *S. rolfsii* it has been observed that Rhizobial strains decreased up to 93.33, 84.78 and 84.85% death of plants in lentil, chick pea and summer munbean, respectively.

Siddiqui *et al.* (2000) evaluated *Rhizobia* as biocontrol agent of root-infecting fungi in okra. Nine rhizobial strains isolated from the root nodules of *Cicer arietinum, Vigna radiata, V. mungo, Samanea saman, Sesbania sesban, Leucinia (Leucanea)* sp., *Prosopis cineraria* and *Medicago sativa* were used to study their effects on root-infecting fungi viz., *M. phaseolina, F. solani* and *R. solani*. In dual culture plate assay, strains of *Bradyrhizobium* sp. and *R. meliloti* were found to inhibit

radial growth of *M. phaseolina*, *F. solani* and *R. solani*, producing zones of inhibition.

Kumar *et al.* (2001) studied potentiality for improving pea production by co-inoculation with fluorescent *Pseudomonas* and *Rhizobium* and observed that antagonist bacteria produced siderophores which were shown to express antifungal and antibacterial activity. They also reported that potential use of these bacteria induced plant growth and disease suppression in sustainable agriculture production systems.

Ozkoc and Deliveli (2001) studied inhibition of the mycelial growth of some isolates of root rot fungi (*R. leguminosarum* bv. *phaseoli*). The effects of 23 *R. leguminosarum* bv. *phaseoli* isolates on the mycelium development of three phytopathogenic fungi (*F. oxysporum*, *P. ultimum* and *R. solani*) were tested *in vitro*. Most of the *rhizobia* inhibited fungal development. The inhibitory effect of the *rhizobia* occurred mostly on the isolates of *F. oxysporum* and *F. ultimum* and least on those of *R. solani*. Percent inhibitions were 14.58- 29.75% for *F. oxysporum* isolates, 14.62- 30.35% for *F. ultimum* isolates and 14.65- 16.03% for *R. solani* isolates.

Siddiqui *et al.* (2001) conducted an experiment and thirty-two isolates of *P. aeruginosa* and a *B. subtilis* strain were isolated from rhizosphere and rhizoplane of four wild and 15 cultivated plants. Biocontrol and growth-promoting potentials of the bacterial isolates were tested under laboratory, greenhouse and field conditions. The bacterial isolates not only exhibited nematicidal activity by killing the second stage larvae of *Meloidogyne javanica* to a varying degree but also produced inhibition zones by inhibiting the radial growth of *M. phaseolina*, *F. solani* and *R. solani*. Strain IE-2 and IE-6 of *P. aeruginosa* also inhibited the fungal mycelium. *P. a.* and *B. subtilis* used as seed dressing or as soil drench significantly suppressed root rot–root knot infection and nematode population densities under greenhouse and field conditions and thereby enhanced plant growth and yield in mungbean.

Kibria and Hossain (2002) studied the effect of biofertilizer and *Rhizobium* on foot and root rot disease and seed yield of mungbean and reported that seeds of mungbean cv. BINA Mung-5 treated with biofertilizer and Rhizobial inoculates resulted significant effect in reducing the severity of foot and root rot of mungbean. Biofertilizer and Rhizobial strains resulted up to 70.16% and 80.83% reduction of foot and root rot of mungbean cv. BINA Mung-5, respectively. Biofertilizer and Rhizobial strains increased shoot length up to 28.46%, number of nodules/plant by 64.4% and seed yield/plant up to 61.17% over untreated control.

Hossain and Mohammed (2002) conducted a field experiment on seed treatment with biofertilizer to control mungbean diseases. They inoculated seeds with biofertilizers (BARI biofertilizers and BINA Biofertilizers) for controlling the diseases of mungbean (var. BINA Mung-3 and BINA Mung-4). They observed BARI and BINA biofertilizer increased seed germination 1.25 and 5.67% over the untreated control, respectively and in BINA Mung-4 and BINA Mung-3, 7.78 and 10.81% seed germination, respectively. Treating seeds of BINA Mung-3 with biofertilizers of BARI and BINA exhibited reduction of yellow mosaic incidence up to 75.31 and 76.43% over control, respectively at 80 days after sowing (DAS), but in case of BINA Mung-4 the reduction were up to 57.42 and 58.12% over control, respectively. Treated seeds of BINA Mung-3 with biofertilizers of BARI and BINA reduced yellow mosaic disease severity up to 6.6 and 13.4% over control at 80 DAS, respectively and in case of BINA Mung-4 yellow mosaic severity reduction up to 8.99 and 17.50%, respectively over control at 80 DAS. Biofertilizers of BARI and BINA profoundly increased number of nodules/plants by 38.90 and 50.69% in BINA Mung-3, respectively and in case of BINA Mung-4, number of nodules/plant increased 10.46 and 12.79%, respectively, in case of BINA Mung-4, yield increased 9.72 and 20.83% yield (t/ha), respectively.

Bandopadhyay (2002) conducted an experiment on management of root diseases in bast fibre plants with conservation of natural and microbial agents. He observed that rhizosphere microorganisms and biofertilizer application prevented root-infecting fungi. Seed inoculation with *R. japonicum* significantly reduced sunhemp

wilt (*F. udum* f.sp. *crotalariae*) and jute (*Corchorus olitorius)* root rot (*M. phaseolina),* influencing root nodulation in sunhemp and biomass promotion in both.

Chahal and chahal (2002) investigated the effects of *Meloidogyne indica* and *F. oxysporum* on mungbean cv. SML-134 inoculated with *Rhizobium strain* R-2. They inoculated roots with *M. incognita* juveniles (2000 per pot), *F. oxysporum* (200 mats), or both after seven days of sowing of *Rhizobium* treated seeds. They found that seed inoculation with *Rhizobium* significantly increased nodule number, dry weight and nodule nitrogenase activity.

Mahajan (2007) studied the antagonistic effect of 13 bacterial isolates by employing dual culture technique on media. Results indicated that all the test organisms inhibited growth of *F. udum* as compared to control.

Siddiqui and Akhtar (2007) conducted an experiment to evaluate the effects on chickpea (*Cicer arietinum*) of the phosphate- solubolizing microorganisms *Aspergillus awamori, Pseudomonas aeruginosa* (isolate Pa28) and *Glomus intraradices* in terms of growth and content of chlorophyll, nitrogen, phosphorus and potassium and on the root-rot disease complex of chickpea caused by *Meloidogyne incognita* and *M. phaseolina* were evaluated. Application of these phosphate- solubolizing microorganisms alone and in combination increased plant growth, pod number, and chlorophyll, nitrogen, phosphorus and potassium contents and reduced galling, nematode multiplication and root-rot index of chickpea. *P. aeruginosa* reduced galling and nematode multiplication followed by *A. awamori* and *G. intraradices*. Combined inoculation of these microorganisms caused the greatest increase in plant growth and reduced the root-rot index more than individual inoculations. Pathogens adversely effected root colonization by *G. intraradices*. However, root colonization and root nodulation were increased when co-inoculated with *P. aeruginosa* and *A. awamori* whether in the presence or absence of pathogens.

Sunitha and Kurundkar (2007) evaluated the efficacy of *Trichoderma* isolates against *F. oxysporum* f. sp. *ciceri* under laboratory condition by employing dual

culture technique on potato dextrose agar. Results indicated that in general *Trichoderma* isolates inhibited growth of that pathogen.

Zote *et al.* (2007) reported that soil / seed application of *T. viridae* was found most effective and recorded lowest wilt incidence (19.04 to 33.33%) with highest wilt reduction (66.67 to 80.86%) and maximum seed germination (86.73 to 90.00%) over untreated control.

Khalequzzaman and Hossain (2008) conducted an experiment during 2002—03 in the infested plot of the BINA sub-station at Ishwardi, Pabna to evaluate the effectiveness of *Rhizobium* strains and biofertilizers in controlling the foot and root rot disease and to increase green pod yield of Bush bean under field condition. The highest germination (91.6%) was recorded with BINAR P36, which was statistically similar to BAUR 107, BARIR 7029, BARIR 1000, BINAR P6 and BINA biofertilizer. The lowest germination (77.9%) was found in untreated control. The highest foot and root rot (24.5%) was observed in untreated control and the lowest (10.00%) was in BINAR P36. Biofertilizers and Rhizobial strains decreased foot and root rot up to 59.2% over untreated control. The highest green pod yield (20.8 t/ha) was obtained for using BINAR P36, which was followed by BARIR 7029, BARIR 892, BARIR 1000, BINAR P6 and BINA biofertilizer. The lowest green pod yield (13.6 t/ha) was found in untreated control. *Rhizobium* strain BINAR P36 may be used as seed treatment for controlling foot and root rot and increasing green pod yield of Bush bean.

El-Mohamedy and Abd El-Baky (2008) evaluated the efficacy of different types of seed treatments i.e., bio-priming, seed coating with bio-control agents (*T. harzianum, B. subtilis* and *P. fluorescens*), seed priming and seed dressing with these antagonistic micro-organisms enhanced their effectiveness in control of root rot disease incidence compared to other treatments.

El-Mougy and Abdel-Kader (2008) evaluated the effect of bio-priming of faba bean seeds against root rot pathogens (*R. solani, F. solani* and *S. rolfsii*). They noticed that bio-primed faba bean seeds showed a highly significant effect causing

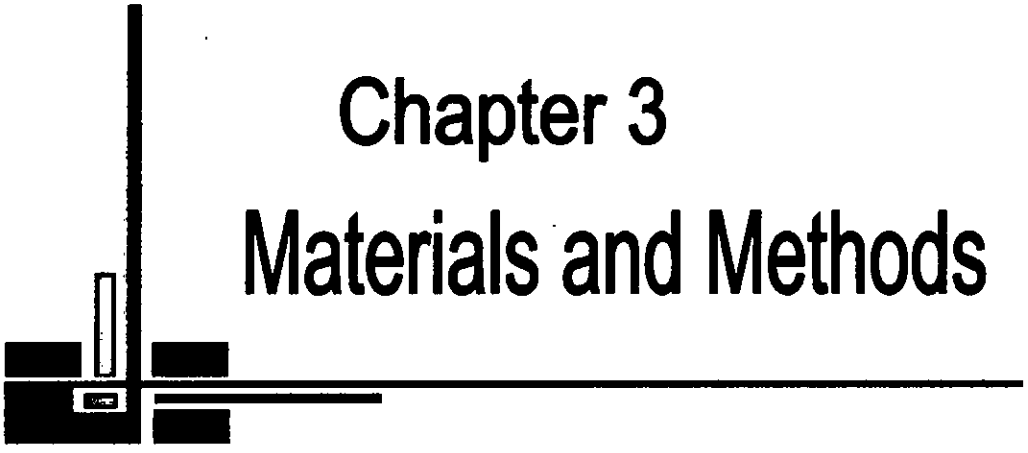
complete reduction of root rot incidence at both pre and post emergence stages of plant growth compared with the control treatment.

Hemissi *et al.* (2011) conducted a study to observe the antagonistic activity of different *Rhizobium* strains against *R. solani* that caused foot and root rot disease in chickpea (*Cicer arientinum* L.) in dual culture technique *in vitro* and under greenhouse conditions. The benefits of Rhizobial inoculant in nitrogen fixation, phosphorous uptake and on plant growth promotion were demonstrated with 42 *Rhizobium* strains. Among the 42 strains tested, 24 isolates effectively controlled *R. solani in vitro*. The results showed that 10 strains were able to solubolise phosphorus and 13 strains produced volatile compounds. In pot trials, the percentage of chickpea plants inoculated with different *Rhizobium* strains showed significant reduction of root rot disease compared to the control growing in uninoculated soil. Among these *Rhizobium* strains, the strain S27 proved efficient against the soil borne pathogen *in vitro* and in pot experiments. Their study suggested that inoculation with specific *Rhizobium* strain significantly suppressed *R. solani* in soil.

Yaqub and Shahzad (2011) found that *S. rolfsii* had significant negative effect on plant growth due to severe root colonization, whereas, presence of the microbial antagonists showed significant positive effect on plant growth by reducing the colonization of roots by *S. rolfsii*. Highest root colonization by *S. rolfsii* and significant reduction in plant growth were observed in sunflower and mungbean plants growing in soil artificially infested with sclerotia of *S. rolfsii*. Use of biocontrol agents in *S. rolfsii* infested soil showed significant reduction in root colonization index (RCI) accompanied by increase in plant growth. *Bradyrhizobium* sp., was found most effective ($p<0.01$) followed by *Rhizobium* sp., *T. harzianum, T. pseudokoningii, T. polysporum* and *T. virens*. Efficacy of biocontrol agents was comparatively suppressed in *S. rolfsii* infested soil as compared to non-infested soil. There was no significant difference in plant length in infested and non-infested soils where biocontrol agents were used. However, plant weight was less in infested soils as compared to non-infested soils. The

microbial antagonists persisted in the soil and produced similar results when mungbean and sunflowers were re-sown in the same plots.

Kashem *et al.* (2011) conducted an experiment to assess the effect of 14 isolates of *Trichoderma* spp. (*T. harzianum* and *T. viridae*) for control of foot and root rot of lentil (*Lens culinaris*) caused by *F. oxysporum*. The pathogenecity of 12 isolates of *F. oxysporum* and the mass production of an isolate of *T. harzianum* on 25 substrates were also studied. The isolates of *Trichoderma* spp. and *F. oxysporum* were collected from different locations of Bangladesh. *Trichoderma* isolates inhibited the growth of *F. oxysporum* from 45.87 to 92.07 % at 7 days after inoculation on agar plates. The isolate TG-2 of *T. harzianum* showed the highest inhibition of the pathogen in field condition. The lowest foot and root rot incidence (6.9%), highest seed germination (82.08%), maximum plant stand (93.12%) and the highest seed yield (3726.67 kg ha^{-1}) were recorded in plots where the isolate TG-2 was applied. In pathogenecity test, the highest foot and root rot incidence and the lowest plant stand of lentil were recorded in the pots where soil inoculation with the isolate FBg-1 of *F. oxysporum* was done. The study concluded that the isolate TG-2 of *T. harzianum* can be used to control foot and root rot disease of lentil in Bangladesh and chickpea bran is a useful substrate for mass production of that isolate for soil application.



Chapter 3
Materials and Methods

Chapter III

MATERIALS AND METHODS

3.1. Experimental site

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

3.2. Experimental period

This study was conducted during the period of November 2011 to February 2013.

3.3. Collection of seed samples

Seeds of lentil variety BARI Masur-4 were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

3.4. Isolation, identification, purification and preservation of foot and root rot pathogens

3.4.1. Collection of diseased plants from the field

Diseased plant specimens having typical foot and root rot symptoms (Fig. 1 A and B) were collected in polythene bags from Agronomy Research Field of Sher-e-Bangla Agricultural University, Dhaka. The collected specimens were brought to the Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for isolation of foot and root rot causal pathogens.

3.4.2. Preparation of Potato Dextrose Agar (PDA)

PDA was prepared and poured in 500 ml glass bottles and sterilized in an autoclave at 121^{0}C under 15 PSI pressure for 15 minutes. The medium was acidified with 30 drops of (50%) lactic acid per 250 ml medium.

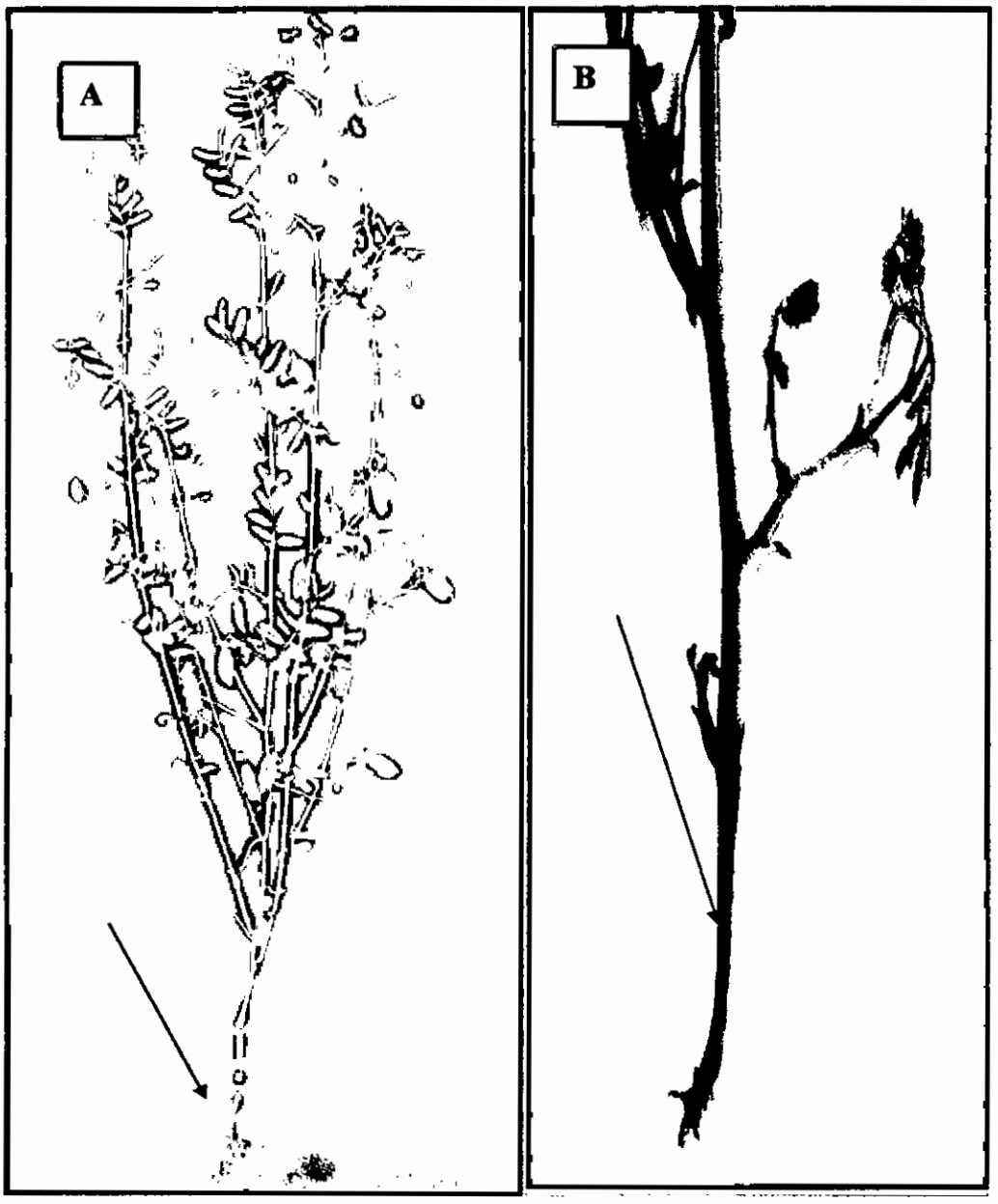


Fig. 1 A. Healthy plant of lentil

B. Foot and root rot infected lentil plant

3.4.3. Isolation, identification, purification and preservation of the fungi

The collected diseased plant specimens were first washed in tap water to make free from sand and soils. The infected portion along with the healthy portion of the plants were cut into small pieces (0.5-1.0 cm) and surface sterilized with 1% clorox for 2-3 minutes. Then the plant pieces were washed with sterilized water thrice and placed on sterilized filter paper to remove excess water adhering to the pieces. Three pieces were plated in acidified PDA plates and also in moist chambers aseptically maintaining equal distance. The plates were incubated for 7 days at 25±1°C. After incubation period, the fungal mycelia that grew over PDA were taken with the help of a sterile needle and transferred on new PDA plates in three replications. Then the plates were incubated for 7 days in 12 hours alternating cycles of light and darkness at 25±1°C temperature. After incubation pure culture of the fungus was identified following the key outlined by Booth (1971) and Singh (1982). *Fusarium oxysporum* and *Sclerotium rolfsii* were purified by using PDA (Begum *et al.*, 1998) and shown in Fig. 2 and 3. The pure culture of the pathogens was preserved in PDA slants at 5±1°C in refrigerator as stock culture for future use.

3.5. Isolation, identification and purification of *Rhizobium leguminosarum*

Legume plants viz; Sesbania and Mungbean were collected from the Agronomy Research Field of Sher-e-Bangla Agricultural University. Each plant sample was kept in polythene bags with proper labeling. After collection, the bags were kept in Plant Pathology Laboratory. The roots were cut from the plants and washed in tap water to remove sand and soils. Then nodules were separated from the root and collected in a petridish. Then they were surface sterilized with 1% clorox for one minute. The nodules were washed with sterilized water thrice and placed on filter paper to remove excess water adhering to those. The nodules were then crushed in sterilized petriplate with few drops of sterilized water and the bacterial ooze that came from the nodules was streaked on petriplates containing Yeast Mannitol Agar (YMA) medium.

For preparing 1000 ml YMA medium, Mannitol 10.0 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.2 g, NaCl 0.1g, Yeast extract 0.4 g, Agar 12.0 g were mixed well in 1000 ml distilled water. It was then autoclaved at 121^0 C under 15 PSI pressure for 15 minutes.

Fifteen millilitre warm melted YMA medium (approx. 55^0C) was poured in each sterile petriplate and kept for setting. Bacterial ooze was streaked with a sterile loop on the plates containing YMA medium. The inoculated YMA plates were kept in incubation chamber for 2-3 days at 30^0 C. After incubation, bacteria appeared with crystal colony color. Then the bacterial colonies were isolated and restreaked on YMA medium separately for pure culture (Fig. 6). Again bacterial colonies were streaked on YMA medium and incubated for 2 days (Fig. 6).

3.6. Collection of *Trichoderma harzianum* and *Pseudomonas fluorescens*

Pure culture of *T. harzianum* (Fig. 4) and *P. fluorescens* (Fig. 5) were collected from the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

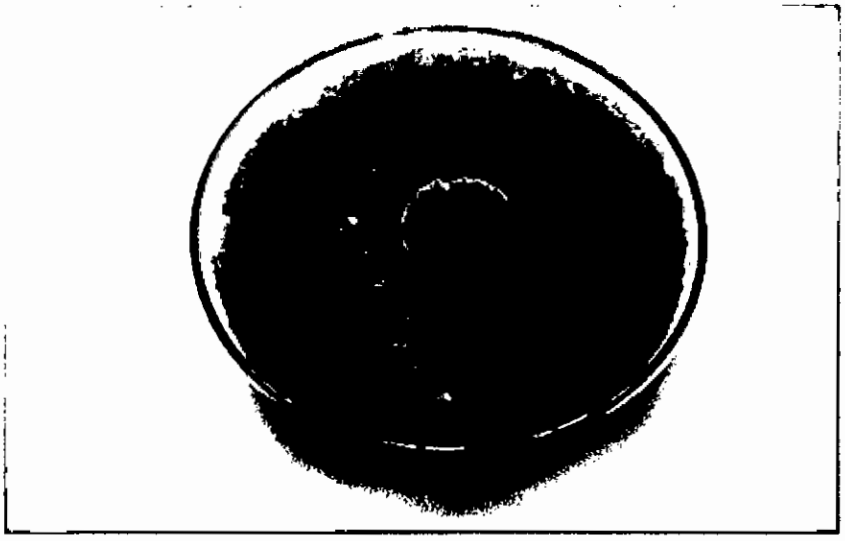


Fig. 4. Pure culture of *Trichoderma harzianum* on PDA medium

Fig. 5. Pure culture of *Pseudomonas fluorescens* on NA medium

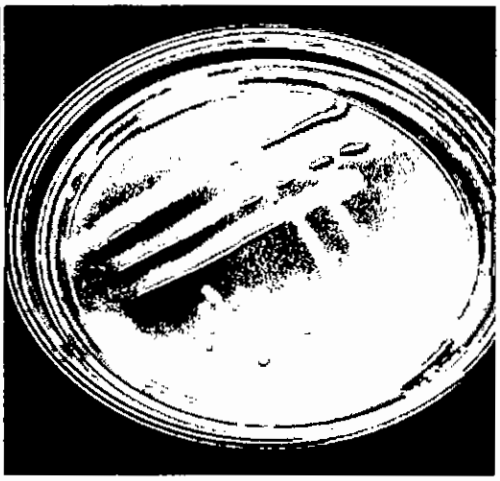


Fig. 6. Pure culture of *Rhizobium leguminosarum* on YMA medium

3.7. Characterization of the bacteria

3.7.1. Morphology of the bacteria

A total of twenty isolates of *R. leguminosarum* were isolated from the nodules of Mungbean and Sesbania plants. Among them only four isolates were found effective against *F. oxysporum*.

3.7.1.1. Study of colony color

For morphological characters, colony color, shape and surface textures were carefully studied and recorded as one bacterial isolate developed after 24 hrs. of incubation in NA medium for *P. fluorescens* and another on YMA medium for *R. leguminosarum*.

3.7.1.2. Gram's staining

A small drop of sterile water was placed on a clean slide. Part of a young colony (18-24 hrs old) of the bacteria was removed with a cold, sterile loop from the culture plate and the bacteria were smeared on to the slide that was very thin. The thinly spreaded bacterial film was air dried. Underside of the glass slide was heated by passing it four times through the flame of a spirit lamp for fixing the bacteria on it. Then the smear was flooded with crystal violet solution for 1 minute. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then it was flooded with lugol's iodine solution for 1 minute and rinsed under running tap water for few seconds and excess water was removed by air. After that it was decolorized with 95% ethanol for 30 seconds and again rinsed under running tap water and air dried. Then it was counterstained with 0.5% safranine for 10 seconds and rinsed briefly under tap water and excess water was removed by air. Finally it was examined under microscope at 100x .ie., oil immersion objective.

3.7.1.3. KOH solubility test

On glass slide a loop-full of bacteria from a well grown colony was mixed with a drop of 3% aqueous KOH. Mixing was continued for less than 10

seconds. Then the loop was raised a few centimeters from the glass slide and repeated strokes to have strands of viscid materials as described by Suslow *et al.*, (1982).

3.7.2. Different biochemical tests

Different chemical tests were done for each antagonistic bacterial isolate such as starch hydrolysis test, citrate utilization test, catalase test, oxidase test and gelatin liquefaction test.

3.7.2.1. Starch hydrolysis

A nutrient agar plate containing 2% soluble starch was inoculated with the bacterial isolate to be tested. Then the plate was incubated at 30^0 C temperature for 48 hours. After incubation, the plate was flooded with lugol's iodine and observed.

3.7.2.2. Catalase test

One colony of the organism from the agar plate was taken on a slide onto which one drop of 3% H_2O_2 (Hydrogen Peroxide) was added and observed.

3.7.2.3. Oxidase test

A portion of the test organism was picked up from the agar plate with a sterile wooden toothpick onto the wet oxidase disk containing tetramethyl-p-phenylene-diamine dihydrochloride. Formation of a dark purple color developed within 5-10 seconds indicted a positive test for oxidase.

3.7.2.4. Citrate utilization test

A portion of the test organism was picked up from the agar plate with a sterile inoculating loop and streaked into Simmon's citrate agar slants. Following incubation at 30°C for 24 hours changing of the green bromothymol blue indicated positive results.

3.7.2.5. Gelatin liquefaction test

One loop-full bacterial culture was inoculated with a sterile straight wire stabbed into the media and incubated at 30°C for 24 hours. Gelatin liquefied microorganisms is detected by the formation of liquid culture at 4° C in a refrigerator.

3.8. *In-vitro* antagonism of isolated bacteria against foot and root rot pathogens of lentil

In-vitro antagonism test of bacteria was done to check their antagonistic activity. Bacteria were screened out for their antagonistic activity against isolated fungi by following dual culture method.

3.8.1. Dual culture method

Dual culture method was carried out as described by Azadeh *et al.*, (2010). A plug of mycelium of isolated pathogen (5 mm diameter) was plated at the centre of the petridish containing 25 ml PDA, then the bacterium was streaked 2 cm away from the agar plug at both sides towards the edge of the plate by a loop loaded with 48 hrs old bacterial culture grown in incubation chamber on YMA medium for *Rhizobium leguminosarum* and NA medium for *Pseudomonas fluorescens*. All the tested plates were incubated at 28°C for 7 days and antagonistic effect was determined by measuring the longest and shortest free growth zone between the bacteria and fungi. In case of control, only a plug of mycelium of isolated pathogen (5 mm diameter) was plated at the centre of the petridish containing 25 ml PDA, without streaking of bacteria in the plate.

Inhibition percentage of the isolated pathogen was calculated based on the growth of the pathogen on PDA plates in absence of antagonistic bacteria following the formula as suggested by Sundar *et al.* (1995).

$$\% \text{ Growth inhibition} = \frac{X-Y}{X} \times 100$$

Where,

X = Mycelial growth of the pathogen in absence of antagonistic bacteria (control)

Y = Mycelial growth of the pathogen in presence of antagonistic bacteria

3.9. *In-vitro* antagonism of *Trichoderma harzianum* against foot and root rot pathogens of lentil

An *in-vitro* study was conducted to find out the antagonistic effect of *Trichoderma harzianum* against the isolated pathogen on PDA by dual culture technique (Sundar *et al.*, 1995). Discs of mycelium (5 mm diameter) of each of the selected fungal cultures were cut from the edge of an actively growing fungal colony with a cork borer (5 mm diameter). One 5 mm blocks of *T. harzianum* culture (7 days old) was transferred to the plate containing PDA one side and another block of same size of the isolated pathogen (7 days old) was placed at the other side of the plate. The plate only with the disc of the isolated pathogen in the center was used as control plate. The plates were then incubated at room temperature (25±2°C) until the mycelium of the isolated pathogen in control plate covered the whole plate. After then the diameter of the colonies were measured.

Inhibition percentage of the isolated pathogen was calculated based on the growth of the pathogen on PDA plates in absence of antagonistic fungal cultures following the formula as suggested by Sundar *et al.* (1995) which is described in 3.8.1.

3.10. Efficacy of seed treatment with different microbial antagonists in prevalence of *Fusarium oxysporum* and *Sclerotium rolfsii*

Treatments of the experiment:

The treatments were-

T_1= Seed treatment with *R. leguminosarum* isolate 1

T_2= Seed treatment with *R. leguminosarum* isolate 2

T_3= Seed treatment with *R. leguminosarum* isolate 3

T_4= Seed treatment with *R. leguminosarum* isolate 4

T_5= Seed treatment with *P. fluorescens*

T_6= Seed treatment with *T. harzianum*

T_7= Control (untreated)

3.10.1. Seed treatment with bacterial antagonists

The seeds were first treated with microbial antagonists in a specific way. First of all, 24 hrs. aged *R. leguminosarum* culture was made on YMA media and same aged *P. fluorescens* culture on NA media. Then bacterial cultures were scraped out and 10^8 cfu/ml suspensions were made in sterile distilled water in a beaker for each treatment. Then two hundred seeds for each beaker were dipped for two hours. In case of control, two hundred seeds were surface sterilized with 3% clorox and dipped in sterile water for two hours.

3.10.2. Seed treatment with *Trichoderma harzianum*

T. harzianum was collected from the Laboratory of Plant Pathology Department of Sher-e-bangla Agricultural University and multiplied in PDA medium in 9 cm petridish. Spore suspension was made by scraping the 10-15 days old culture substrate with the help of blender @ one petriplate in 250 ml water to adjust the concentration 10^7 conidia/ml solution. Collected lentil seeds were dipped in the spore suspension of bio-agent (*T. harzianum*) for two hours.

3.11. Effect of seed treatment with different microbial antagonists on seedling vigor of lentil (rolled paper towel method)

Seedling infection and seedling vigor test was done in rolled paper towel method (Warham, 1990). In this method, two hundred seeds were randomly taken from each treatment and were placed uniformly between a pair of moist paper towels. The towels were rolled and the two ends were closed with rubber band as the moist could not remove easily. Then the rolled papers containing seeds were placed in an upright position for 7 days at room temperature under normal 12/12 light and darkness cycle. Length of shoot was measured from the base of the stem up to the growing point of the youngest leaf. Similarly, length of root was measured from starting point of the root to the largest available lateral root apex. Data on germination percentage, abnormal seedlings, normal seedlings, diseased seedlings, dead seeds and seedling weight were also recorded. Vigor of the seedlings was calculated by using the formula of Baki and Anderson (1973) as shown below:

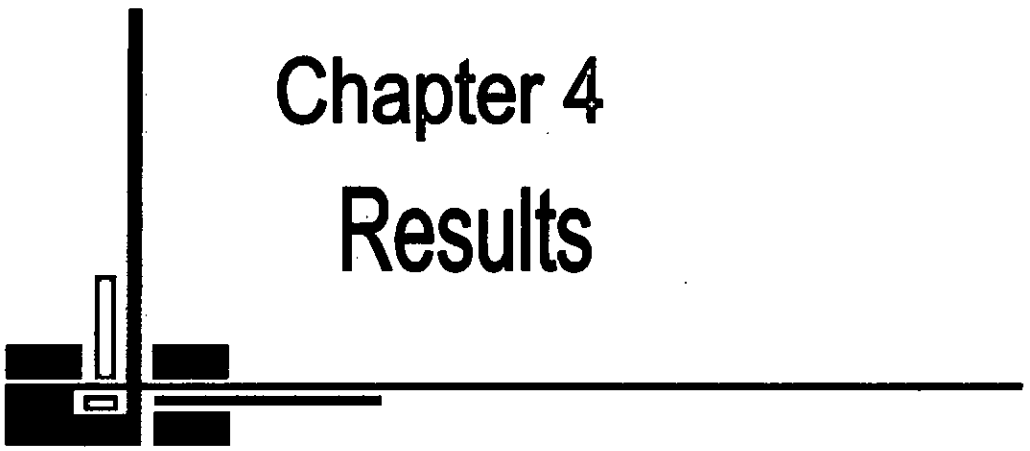
Vigor Index (VI) = (Mean shoot length + Mean root length) × Germination (%)

3.12. Effect of seed treatment with different microbial antagonists against foot and root rot pathogens of lentil (water agar test tube method)

The water agar test tube seedling symptom test was used in the present evaluation. In this technique, test tubes were prepared by pouring 10 ml of 1% water agar in each test tube (2 cm in diameter and 15 cm in length) and then sterilized in autoclave for 15 minutes under 15 PSI at 121°C temperature. The water agar in the test tube was solidified at an angle of 60°. Two hundred treated seeds for each treatment *i.e.* T_1, T_2, T_3, T_4, T_5, T_6 were taken. Seeds were then dried and one seed per slant was placed on solidified water agar. Thereafter, a mycelial block of the isolated pathogen was placed one cm away from the seed in test tube slant following Begum *et al.* (1999). In case of control, two hundred seeds dipped in sterile water, drained off and shade dried and one seed was placed in the slant along with a mycelial block of the isolated pathogen. Then all the test tubes were plugged with cotton and incubated at

erect condition in an air cooled room (22°C) under fluorescent day light. The cotton plugs were removed when the seedlings reached the rim of the test tubes. Then the following data were recorded -

a) Germination percentage,
b) Number of normal seedlings,
c) Number of abnormal seedlings,
d) Number of diseased seedlings,
e) Number of dead seeds



Chapter 4
Results

CHAPTER IV

RESULTS

This chapter comprises the presentation and discussion of the results from the experiment. The experiment was conducted to determine the bio-efficacy of microbial antagonists against foot and root rot pathogen(s) of lentil. Some of the data have been presented and expressed in table for discussion, comparison and understanding. A summary of all the parameters have been shown in possible interpretation wherever necessary.

4.1. Morphological characteristics of antagonistic microorganisms

Bacterial colony of *R. leguminosarum* appeared as crystal color on Yeast Mannitol Agar (YMA) medium. On Nutrient Agar (NA) medium the bacterial colony of *P. fluorescens* appeared as light cream color. Cultures of *T. harzianum* are typically fast growing at 25-30°C. Colonies are at first white on richer media such as potato dextrose agar (PDA). Conidia typically form within one week in compact or loose tufts in shades of green or yellow or less frequently white. Conidiophores are highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the aerial hyphae. Typically the conidiophore terminates in one or a few phialides and bear conidia in a drop of clear green liquid at the tip of each phialide. Phialides are typically enlarged in the middle. Conidia are typically smooth.

Under the compound microscope *Rhizobium leguminosarum* was rod shaped and Gram negative bacteria; therefore it appeared pink under the microscope due to the safranin stain and the two layer cell wall. *Pseudomonas fluorescens* was straight to curved rod and Gram negative (red color).

In KOH solubility test, a mucoid thread was lifted with the loop in case of both *R. leguminosarum* and *P. fluorescens*. The test was positive i.e., both the bacteria were Gram negative that supports the result of Gram's staining test (Fig. 7).

4.2. Biochemical characteristics of antagonistic bacteria

Results obtained on various biochemical tests for the antagonistic bacteria were presented in Table 1.

Table 1. Biochemical characteristics of antagonistic bacteria

Biochemical tests	*Rhizobium leguminosarum*	*Pseudomonas fluorescens*
Starch hydrolysis test	+	+
Catalase test	+	+
Oxidase test	+	+
Citrate utilization test	+	+
Gelatin liquefaction test	+	+

In starch hydrolysis test, after adding lugol's iodine a clear zone was formed around the bacterial colony which indicated starch hydrolysis (amylase activity) (Fig. 8 A) i.e., the test was positive (Table-1).

In catalase test, after adding 3% H_2O_2 (Hydrogen Peroxide) onto the colony of the bacterium bubbles were formed within a few seconds (Fig. 8 B) which revealed that the test was positive (Table-1).

In oxidase test, after rubbing the bacterium onto the moistened oxidase disk, dark purple color was formed in the disk (Fig. 8 C) which revealed that the test was positive (Table-1).

In citrate utilization test, after 24 hours of incubation green color of Simmon's citrate agar slant changed into a bright blue color which indicated that the test was positive i.e., the bacterium used citrate as a carbon source for their energy (Fig. 8 D, Table-1).

In gelatin liquefaction test, gelatin was liquefied after 15 minutes of refrigeration at 4°C (Fig. 8 E). Thus the bacterium showed the positive result (Table-1).

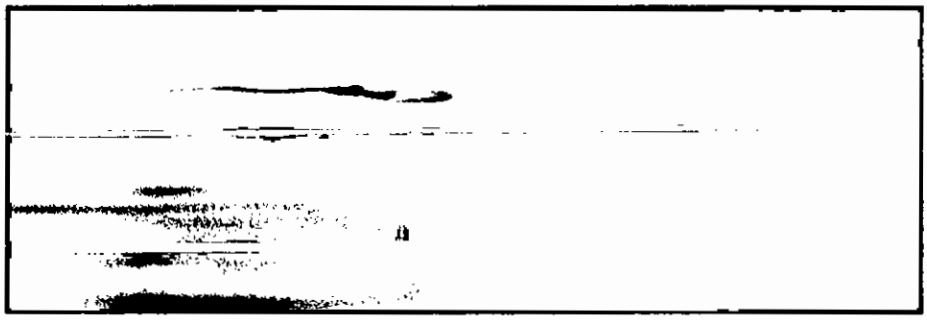


Fig. 7. KOH solubility test

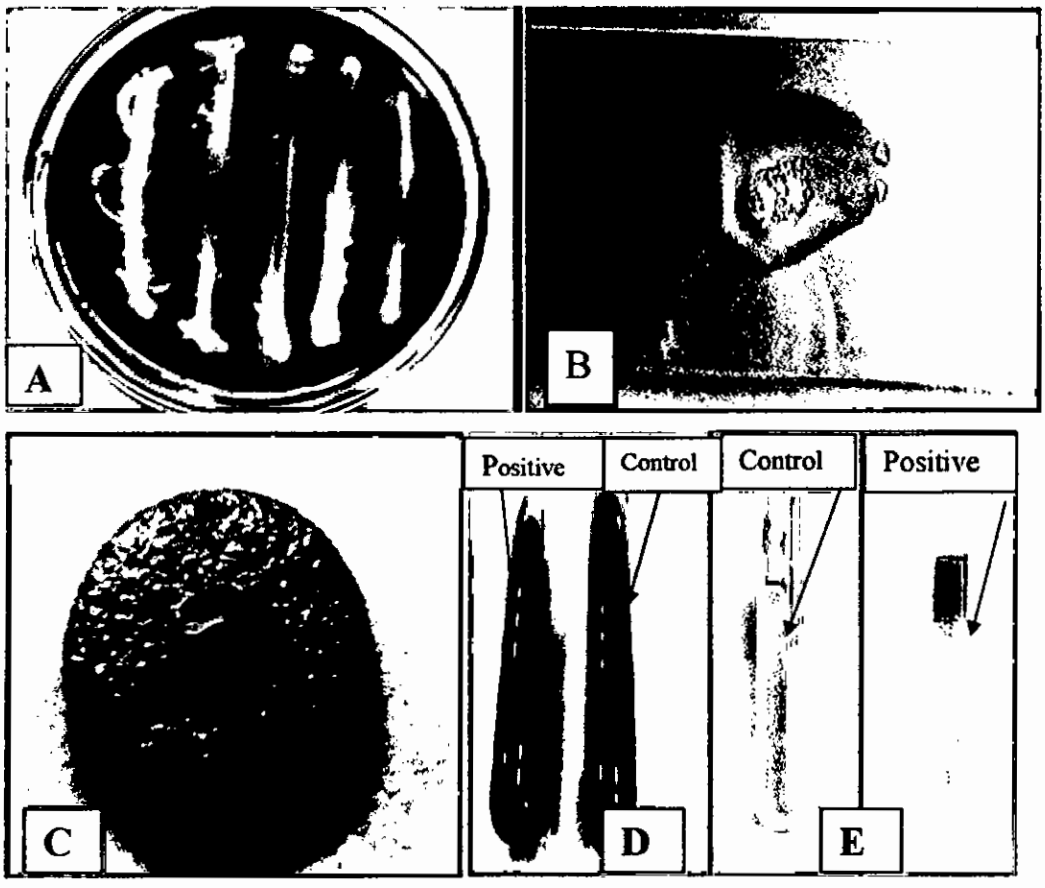


Fig. 8. Different biochemical tests
A. Starch hydrolysis test (positive)
B. Catalase test (positive)
C. Oxidase test (positive)
D. Citrate utilization test
E. Gelatin liquefication test

4.3. Effect of different microbial antagonists on *in-vitro* growth of *Fusarium oxysporum* and *Sclerotium rolfsii.*

4.3.1. Radial mycelial growth of *Fusarium oxysporum*

Significant effect of different microbial antagonists were found in radial mycelial growth of *F. oxysporum* in dual culture method. Radial mycelial growth of *F. oxysporum* varied form 4.77-2.03 cm, where the highest radial mycelial growth (4.7 cm) was recorded in control (without antagonist) and lowest radial mycelial growth (2.03 cm) was recorded in dual culture with *R. leguminosarum* isolate 3 and isolate 4 that were statistically identical with *R. leguminosarum* isolate 1, isolate 2, *T. harzianum* and *P. fluorescens* (Table-2, Fig. 9).

4.3.2. Percent growth inhibition of *Fusarium oxysporum*

Percent growth inhibition of *F. oxysporum* significantly varied due to the effect of different microbial antagonists. The highest zone of inhibition (57.37%) was recorded in dual culture with *R. leguminosarum* isolate 3 and isolate 4 that were statistically identical with *R. leguminosarum* isolate 1, isolate 2, *T. harzianum* and *P. fluorescens* and no zone of inhibition (0.00) was observed in control (Table-2, figure. 9).

4.3.3. Radial mycelial growth of *Sclerotium rolfsii*

Significant effect of different microbial antagonists were observed in radial mycelial growth of *S. rolfsii* but isolates of *R. leguminosarum* did not show any effect. The highest radial mycelial growth (9.00 cm) was recorded in control (without antagonist) followed by dual culture with *T. harzianum* (5.59 cm) and lowest radial mycelial growth was recorded 1.80 cm by dual culture with *P. fluorescens* (Table-2, Fig. 10).

4.3.4. Percent growth inhibition of *Sclerotium rolfsii*

Percent growth inhibition of *S. rolfsii* significantly varied due to the effect of different microbial antagonists. The highest zone of inhibition (80.00%) was recorded against *P. fluorescens* followed by dual culture with *T. harzianum* and no zone of inhibition (0.00) was observed in control (Table-2, Fig. 10).

Table 2. Effect of different microbial antagonists on *in-vitro* growth of *Fusarium oxysporum* and *Sclerotium rolfsii.*

Microbial antagonists	*Fusarium oxysporum*		*Sclerotium rolfsii*	
	Radial mycelial growth (cm)	% growth inhibition (cm)	Radial mycelial growth (cm)	% growth inhibition (cm)
R. leguminosarum isolate 1	2.10 bc	55.97 ab	0.00 d	0.00 c
R. leguminosarum isolate 2	2.18 bc	54.23 ab	0.00 d	0.00 c
R. leguminosarum isolate 3	2.03 c	57.37 a	0.00 d	0.00 c
R. leguminosarum isolate 4	2.03 c	57.37 a	0.00 d	0.00 c
Pseudomonas fluorescens	2.20 bc	53.88 ab	1.80 c	80.00 a
Trichoderma harzianum	2.25 b	52.83 b	5.59 b	37.85 b
Control (without antagonists)	4.77 a	0.00 c	9.00 a	0.00 c
$LSD_{(0.05)}$	0.16	3.37	1.35	1.32
CV(%)	5.57	4.99	9.34	4.39

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

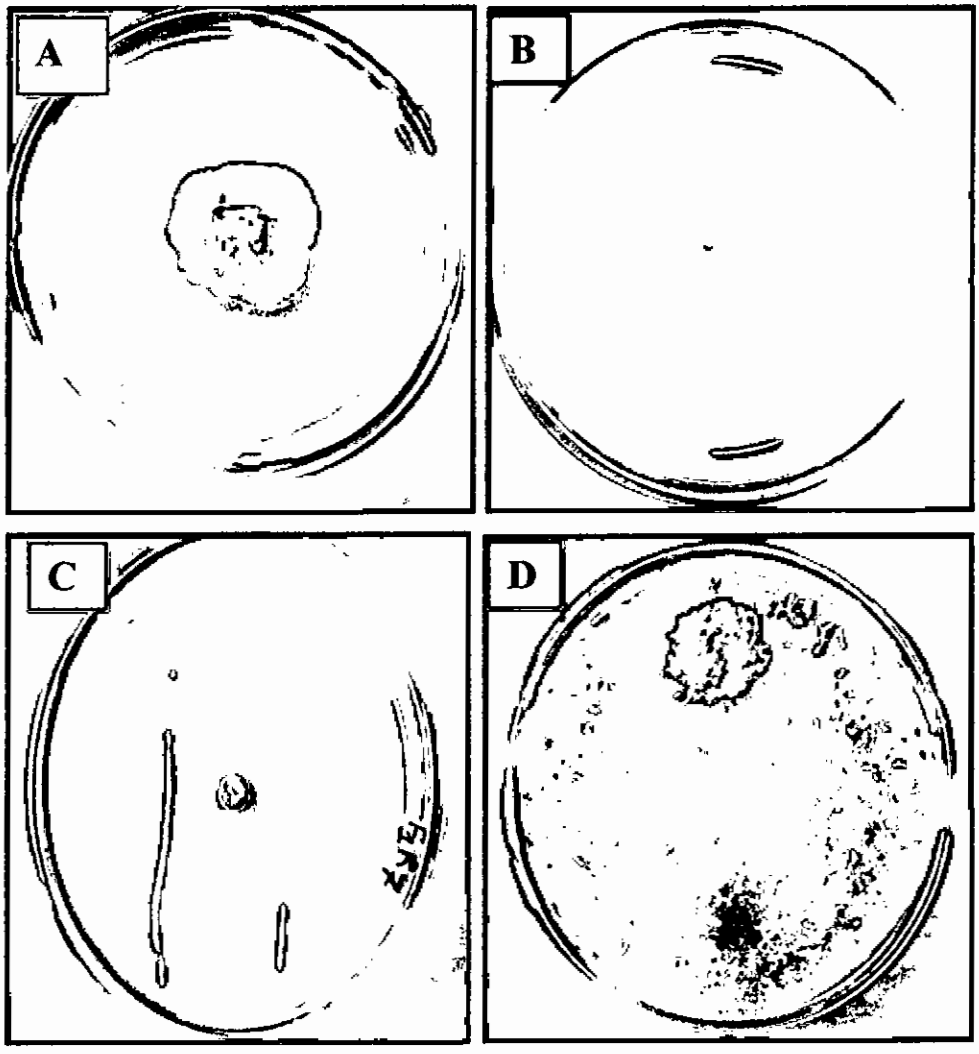


Fig. 9. A. Growth of *Fusarium oxysporum* on PDA medium without antagonists (control)

B. Dual culture of *F. oxysporum* with *Pseudomonas fluorescens*

C. Dual culture of *F. oxysporum* with *Rhizobium leguminosarum*

D. Dual culture of *F. oxysporum* with *Trichoderma harzianum*

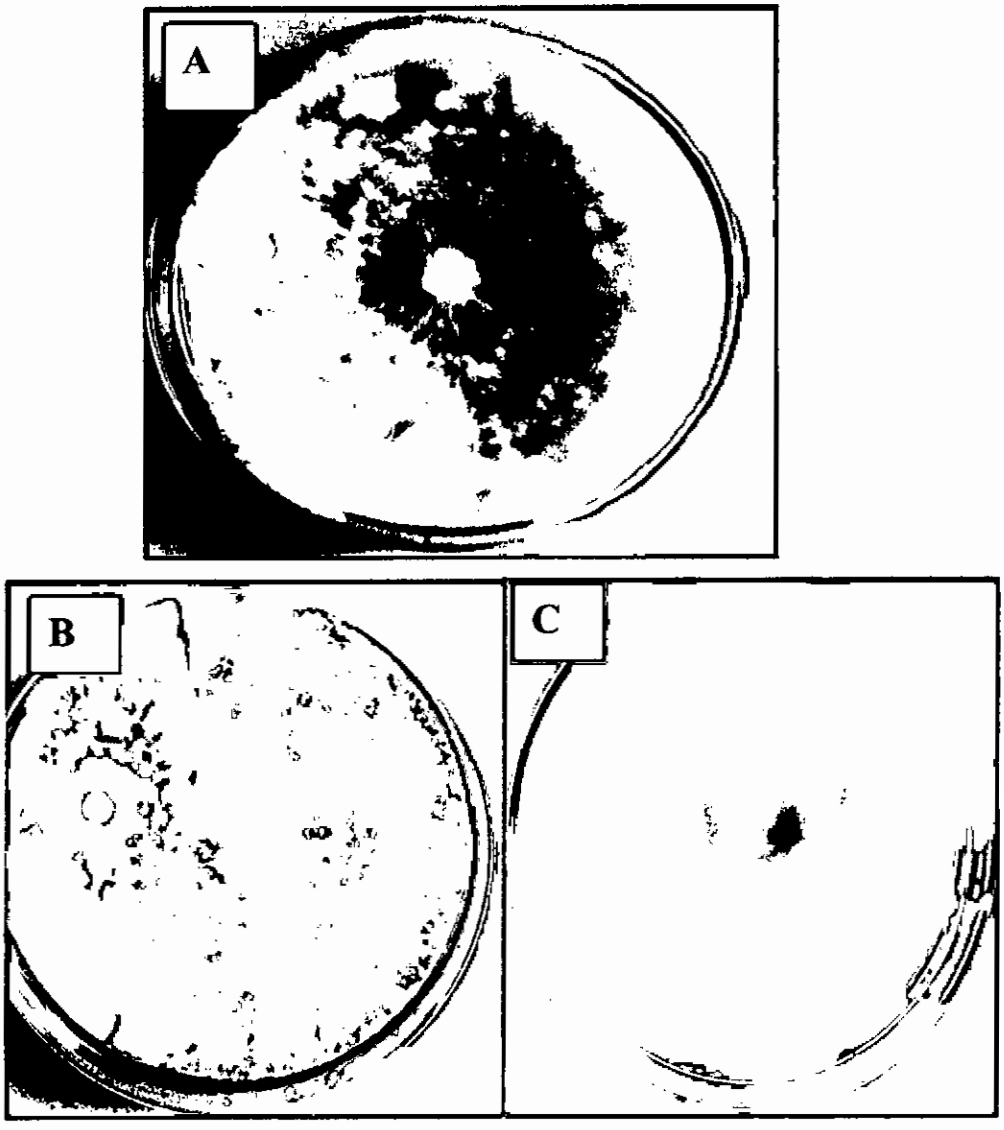


Fig. 10. A. Growth of *Sclerotium rolfsii* on PDA medium without antagonist (control)
B. Dual culture of *S. rolfsii* with *Trichoderma harzianum*
C. Dual culture of *S. rolfsii* with *Pseudomonas fluorescens*

4.4. Effect of seed treatment with different microbial antagonists on seedling vigor of lentil (rolled paper towel method).

Effect of seed treatment with different microbial antagonists on seedling vigor of lentil were recorded and significant difference were observed regarding the following parameters (Table-3, Fig. 11).

4.4.1. Seed germination

Significant effect of different treatments were found or seed germination. Germination varied form 91.00-82.00%, where the highest germination (91.00%) was recorded in seeds treated with *R. leguminosarum* isolate 1 that was statistically identical with *R. leguminosarum* isolate 2, isolate 3, isolate 4 and *T. harzianum* treated seeds and lowest germination (82.00%) was recorded in control (Table-3).

4.4.2. Shoot length (cm)

Shoot length was significantly varied in different treatments. The maximum shoot length (6.96 cm) was measured in seeds treated *with P. fluorescens* which was statistically identical with seeds treated with *R. leguminosarum* isolate 1, isolate 2, isolate 3, isolate 4 and *T. harzianum*. The minimum shoot length (4.83 cm) was measured in control (Table-3).

4.4.3. Root length (cm)

Significant effect of different treatments on root length was observed. The maximum root length (9.01cm) was measured in *P. fluorescens* treated seeds which was statisticaly similar with seeds treated by *R. leguminosarum* isolate 1, isolate 2, isolate 3 and isolate 4. The minimum root length (4.99 cm) was measured in control (Table-3).

4.4.4. Vigor index

Significant effect of different treatments were found in seedling vigor. The maximum vigor index (1390.00) was observed in *P. fluorescens* treated seeds that was statistically identical with *R. leguminosarum* isolate 2 treated seeds. The minimum vigor index (851.70) was observed from control (Table-3).

4.4.5. Abnormal seedling

Significant effect of different treatments on abnormal seedlings (Fig. 12 C) was observed and presented in Table 3. The maximum number of abnormal seedlings (5.39) was counted from control (untreated). No abnormal seedling (0.00) was found from seeds treated with *R. leguminosarum* isolate 1, isolate 3, isolate 4.

4.4.6. Diseased seedling

Significant effect of different treatments on diseased seedling was observed and presented in Table 3. The maximum number of diseased seedlings (4.22) was counted from control (untreated) and the minimum number of diseased seedlings (1.00) was counted from *T. harzianum* treated seeds. No diseased seedling (0.00) was found in *R. leguminosarum* isolate 1, isolate 2 and *P. fluorescens* treated seeds.

4.4.7. Dead seed

The variation in the dead seed (Fig. 12 A) in different treatments was statistically significant. The maximum dead seeds (18.33%) was observed in control (untreated). The minimum dead seeds (9.00%) was observed in seeds treated with *R. leguminosarum* isolate 1 that was statistically identical with *R. leguminosarum* isolate 2, isolate 3, isolate 4 and *T. harzianum* treated seeds (Table-3).

Table 3. Effect of seed treatment with different microbial antagonists on seedling vigor of lentil (rolled paper towel method).

Treatments	Germination (%)	Shoot length (cm)	Root length (cm)	Vigor index	Abnormal seedling	Diseased seedling	Dead seed	Weight of seedlings (gm)
R. leguminosarum isolate 1	91.00 a	5.83 ab	7.15 ab	1182.00 b	0.00 d	0.00 d	9.00 c	11.70 a
R. leguminosarum isolate 2	89.00 ab	6.46 a	8.44 a	1326.00 a	2.00 bc	0.00 d	11.00 bc	11.68 a
R. leguminosarum isolate 3	89.00 ab	5.90 ab	7.35 ab	1189.00 b	0.00 d	1.40 bc	11.00 bc	10.56 ab
R. leguminosarum isolate 4	90.00 ab	6.14 ab	7.36 ab	1147.00 b	0.00 d	1.50 b	10.00 bc	10.65 ab
Pseudomonas fluorescens	88.00 b	6.96 a	9.01 a	1390.00 a	2.33 b	0.00 d	12.33 b	10.22 ab
Trichoderma harzianum	89.00 ab	5.65 ab	6.55 bc	1145.00 b	1.50 c	1.00 c	10.33 bc	9.50 b
Control(untreated)	82.00 c	4.83 b	4.99 c	851.70 c	5.39 a	4.22 a	18.33 a	5.09 c
$LSD_{(0.05)}$	2.55	1.30	1.73	77.69	0.54	0.44	2.55	1.87
CV(%)	6.67	10.52	9.60	8.49	4.56	3.34	3.21	4.56

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

Fig. 11. Seedlings raised on paper towel.

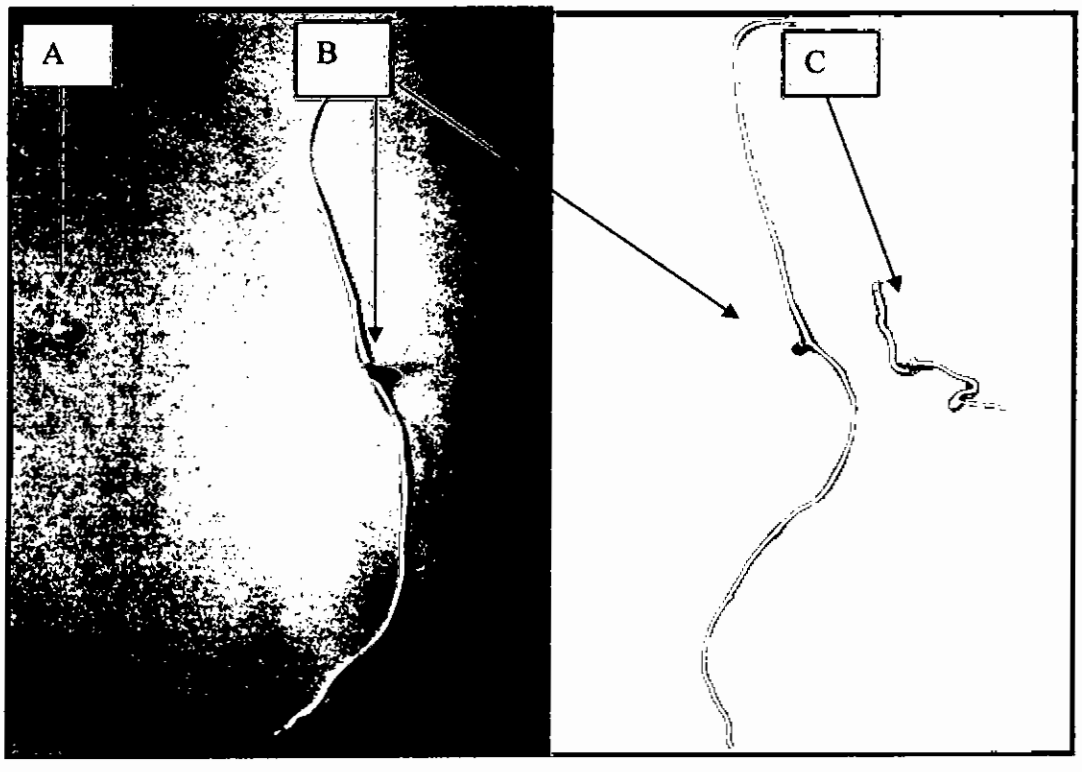


Fig. 12. Seedlings raised on paper towel

A. Non germinated seed (dead seed)

B. Normal seedling

C. Abnormal seedling

4.4.8. Weight of seedling

Significant effect of different treatments on weight of seedlings was observed and presented in table 3. The maximum weight of seedlings (11.70 gm) was recorded from *Rhizobium leguminosarum* isolate 1 treated seeds that was statistically similar with *Rhizobium leguminosarum* isolate 2, isolate 3, isolate 4 and *Pseudomonas fluorescens* treated seeds. The minimum weight of seedlings (5.09 gm) was recorded from control.

4.5. Effect of seed treatment with different microbial antagonists against foot and root rot pathogens of lentil (water agar test tube method)

Effect of seed treatment with different microbial antagonists against foot and root rot pathogens of lentil was recorded and significant difference were observed regarding the following parameters (Fig. 13).

4.5.1. Seed Germination

Significant effect of different treatments was found in seed germination. Germination varied form 88.00-76.00%, where the highest germination (88.00%) was recorded in seeds treated with *R. leguminosarum* isolate 2, isolate 3, isolate 4 and *T. harzianum* that was statistically identical with *R. leguminosarum* isolate 1 treated seeds. The lowest germination (76.00%) was recorded in control (Table-4).

4.5.2. Dead seed

The variation in the dead seeds (Fig. 14 E) in different treatments was statistically significant. The highest dead seeds (24.33%) was recorded in control. The lowest dead seeds (11.67%) was recorded in seeds treated with *R. leguminosarum* isolate 4 that was statistically similar with *R. leguminosarum* isolate 1, isolate 2, isolate 3 and *T. harzianum* treated seeds (Table-4).

Table 4. Effect of seed treatment with different microbial antagonists against foot and root rot pathogens of lentil (water agar test tube method).

Treatments	Germination (%)	Dead seed	Normal seedling	Abnormal seedling	Diseased seedling
R. leguminosarum isolate 1	87.00 ab	12.67 bc	83.83 a	2.00 c	1.50 b
R. leguminosarum isolate 2	88.00 a	12.00 c	84.56 a	2.44 c	1.00 d
R. leguminosarum isolate 3	88.00 a	12.33 c	84.00 a	2.33 c	1.33 bc
R. leguminosarum isolate 4	88.00 a	11.67 c	84.17 a	3.17 bc	1.00 cd
Pseudomonas fluorescns	85.00 b	15.33 b	80.67 b	3.00 bc	1.00 cd
Trichoderma harzianum	88.00 a	12.33 c	83.00 ab	4.00 b	0.67 d
Control(untreated)	76.00 c	24.33 a	57.67 c	11.00 a	7.00 a
$LSD_{(0.05)}$	3.07	2.68	2.71	1.24	0.32
CV(%)	5.69	15.33	6.16	12.59	6.33

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

4.5.3. Normal seedling

Significant effect of different treatments on normal seedlings (Fig. 14 A) was observed and presented in Table 4. The maximum number of normal seedlings (84.56) was counted from seeds treated with *R. leguminosarum* isolate 2 that was statistically similar with *R. leguminosarum* isolate 1, isolate 3, isolate 4 and *T. harzianum* treated seeds. The minimum number of normal seedlings (57.67) was counted from control.

4.5.4. Abnormal seedling

Significant effect of different treatments on abnormal seedling (Fig. 14 D) was observed and presented in Table 4. The maximum number of abnormal seedlings (11.00) was counted from control. The minimum number of abnormal seedling (2.00) was counted from seeds treated with *R. leguminosarum* isolate 1 that was statistically similar with *R. leguminosarum* isolate 2, isolate 3, isolate 4 and *P. fluorescens* treated seeds.

4.5.5. Diseased seedling

Significant effect of different treatments on diseased seedlings (Fig. 14 B) was observed and presented in Table 4. The maximum number of diseased seedlings (7.00) was counted from control. The minimum number of diseased seedlings (0.67) was counted from seeds treated with *T. harzianum* that was statistically similar with *R. leguminosarum* isolate 2, isolate 4 and *P. fluorescens* treated seeds.

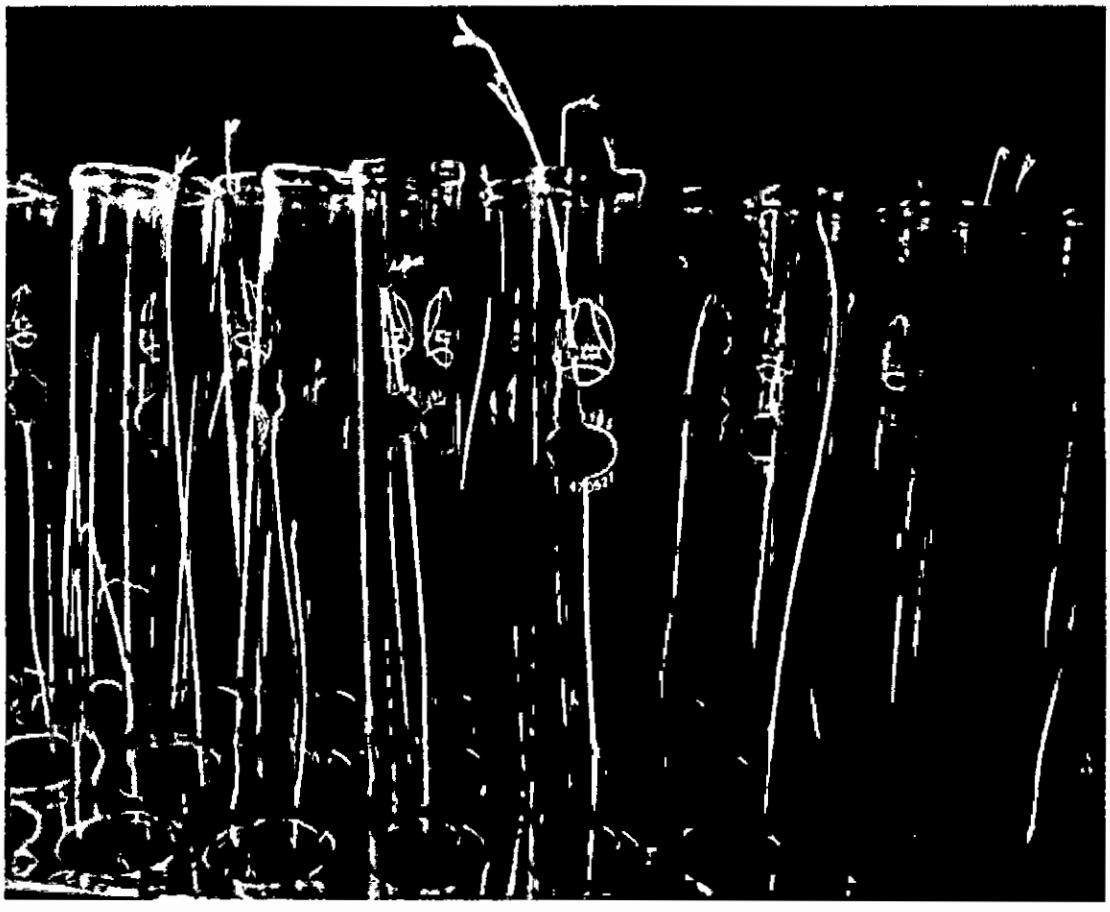


Fig. 13. Seedlings raised in water ager test tube method.

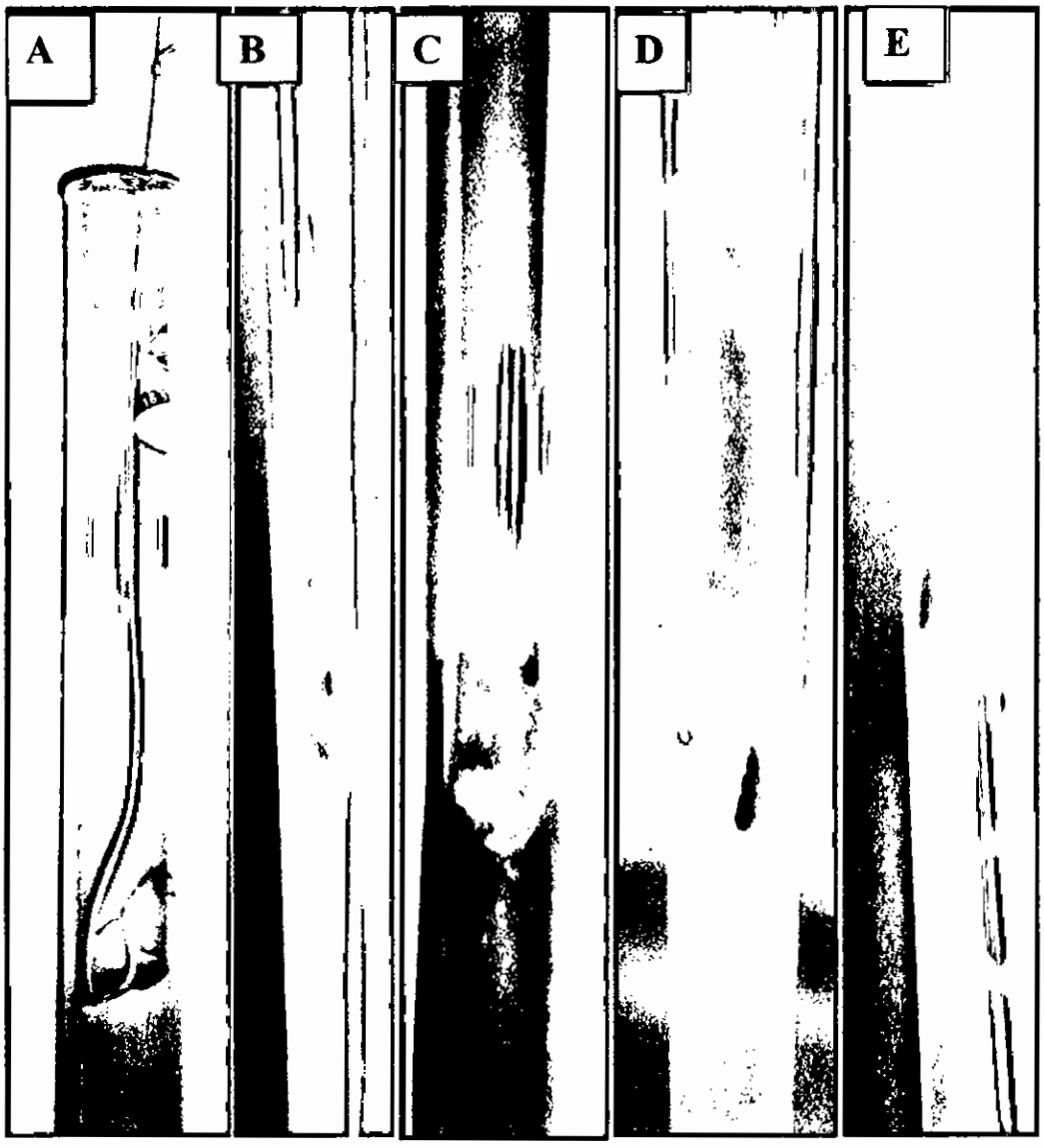


Fig. 14. Seedling symptoms on water ager test tube method

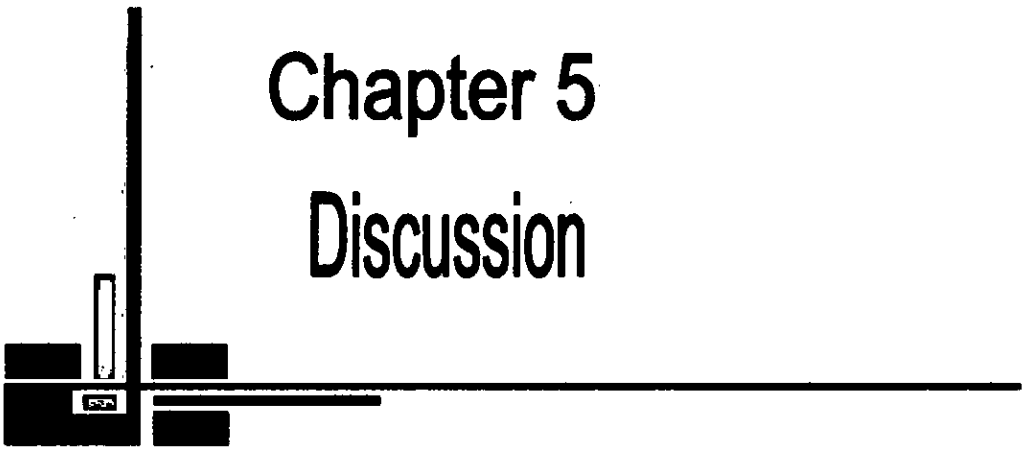
A. Normal seedling

B. Diseased seedling

C. Non germinated seed with fungal structures

D. Abnormal seedling

E. Non germinated seed (dead seed)



Chapter 5

Discussion

Chapter V

DISCUSSION

The experiment was carried out in the Disease Diagnostic Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka during the period of November 2011to February 2013 to determine the bio-efficacy of microbial antagonists against foot and root rot pathogen(s) of lentil. Two fungul genera *S. rolfsii* and *F. oxysporum* were isolated from foot and root rot infected seedlings. Padilla (1979) and Abo Ellil *et. al.* (1998) found *S. rolfsii*, *F. oxysporum* and *R. solani* as the causal agents of seedling wilt and root rot amongst 40 varieties of Franch bean. Wilt complex of French bean caused by *S. rolfsii*, *F. oxysporum* and *R. solani* was found as an important disease in several states of India (Mathew and Gupta, 1998).

In this experiment four isolates of *R. leguminosarum*, *P. fluorescens* and *T. harzianum* were used. *R. leguminosarum* were rod shaped, gram negative bacteria. *P. fluorescens* was straight to curved rod and gram negative bacteria. A mucoid thread was lifted with the loop incase of both *R. leguminosarum* and *P. fluorescens* in KOH solubility test that supports the result of gram staining test i.e. the bacteria were gram negative. Similar result in KOH solubility test was found by Kishun and Chand (1991), Schaad (1992), Gerhardt (1981), Bradbury (1970). Starr and Stephens (1964) reported *R. leguminosarum* as gram negative, rod shaped bacteria. *R. leguminosarum* and *P. fluorescens* showed positive results in starch hydrolysis, catalase, oxidase, citrate utilization and gelatin liquefaction test. Similar results has been reported by Yenjerappa (2009), Kishun and Chand (1991) and Chand and Pal (1982).

Effect of different microbial antagonists on *in-vitro* growth of *F. oxysporum* and *S. rolfsii* were determined and significant variation were observed regarding radial mycelial growth and percent growth inhibition. Radial mycelial growth of *F. oxysporum* varied from 2.03-4.77 cm. This finding is in aggrement with the findings of Mathur and Kongsdal (1994). Sharma *et al.* (1996) studied variability among five isolates of *F. solani* causing root rot of mulberry and found that five

F. solani isolates resulted 40.00-55.00 mm radial mycelial growth at 7 days after inoculation, while Mukherjee and Tripathi (2000) measured 30 mm radial growth of *F. oxysporum* f. sp. *phaseoli* after 72 hrs. old inoculation. The volatile metabolites produced by *R. meliloti* and *R. trifolii* affect the radial growth of *F. avenaceum, F. oxysporum, Phoma medicaginis* and *Stemphylium sarcinaeforme* (Antoun *et al.*, 1977). Effects of microbial antagonists were studied and highest zone of inhibition of *F. oxysporum* (57.37%) were measured against *R. leguminosarum* isolate 3 and isolate 4. Mahajan (2007) tested the antagonistic effect of 13 bacterial isolates by employing dual culture technique on PDA media and observed that all the test organisms inhibited the growth of *Fusarium udum* as compared to control. Buonassisi *et al.* (1986) found that *Rhizobium* spp. inhibited the radial growth of strains of *F. moniliforme, F. oxysporum* and *F. solani* f. sp. *phaseoli* in dual culture. Lalande *et al.* (1989) observed that *F. solani* and *F. equiseti* were slightly inhibited and *F. oxysporum, F. acuminatum* and *F. avenaceum* were strongly inhibited by some isolates of *Rhizobium in-vitro.* Sunitha and Kurundkar (2007) observed that *Trichoderma* isolates inhibited the growth of *F. oxysporum* f. sp.*ciceri* under laboratory condition in dual culture technique on potato dextrose agar. Strains of *Bradyrhizobium* sp. and *R. meliloti* were found to inhibit radial growth of *M. phaseolina, F. solani* and *R. solani,* producing zones of inhibition (Siddiqui *et al.*, 2000). Kashem *et al.* (2011) evaluated 14 isolates of *Trichoderma* spp. (*T. harzianum* and *T. viride*) for control of foot and root rot of lentil (*Lens culinaris*) caused by *F. oxysporum* and showed that *Trichoderma* isolates inhibited the growth of *F. oxysporum* from 45.87 to 92.07 % at 7 days after inoculation on agar plates. No zone of inhibition of *S. rolfsii* was found against *R. leguminosarum.* In this study only twenty isolates of *R. leguminosarum* were used and none of them were effective against *S. rolfsii.* It may be due to the genetical character of this pathogen. But there are evidence that *R. leguminosarum* has significant effects on *S. rolfsii.* Bhattacharya and Mukherjee (1988) studied *Rhizobium-Sclerotium* interaction in groundnut and observed that plants inoculated with both the *Rhizobium* symbiont and *S. (Corticium) rolfsii* developed 50-80% disease but less than plants inoculated with

the pathogen alone in pot experiments. Bhattacharya and Mukherjee (1990) found that soil inoculation with *Rhizobium* reduced the population of *S.(Corticium)rolfsii*. Incase of *S. rolfsii* 80.00% and 37.85% zone of inhibition were measured against *P. fluorescens* and *T. harzianum*, respectively. Yaqub and Shahzad (2011) found *Bradyrhizobium* sp., as most effective followed by *Rhizobium* sp., *T. harzianum, T. pseudokoningii, T. polysporum* and *T. virens*. In *S. rolfsii* infested soil these organisms showed significant reduction in root colonization index (RCI) accompanied by increase in plant growth.

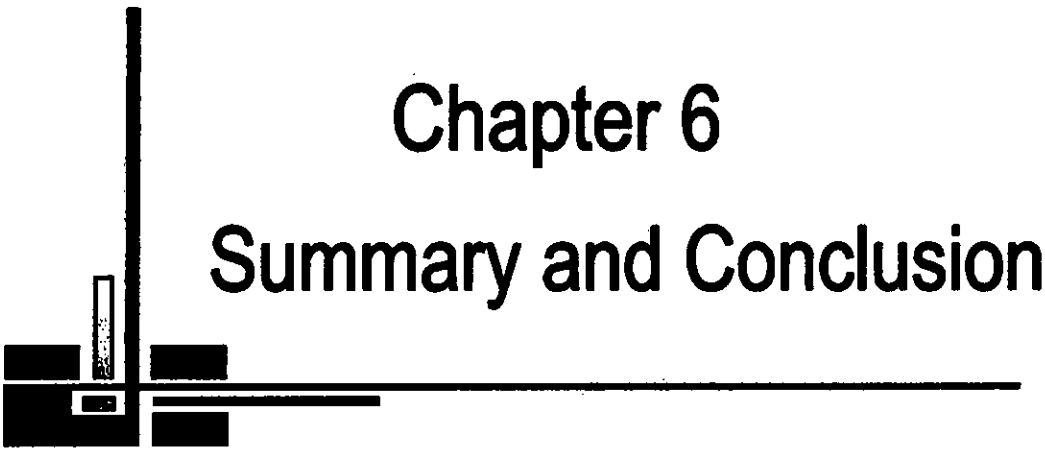
Effect of seed treatment with different microbial antagonists on seedling vigor of lentil (rolled paper towel method) were determined and significant variation were observed regarding germination percentage, shoot length, root length, vigor index, abnormal seedling, diseased seedling, dead seed and weight of seedlings. The highest germination (91.00%), lowest dead seeds (9.00%), no abnormal and diseased seedlings (0.00) and maximum weight of seedlings (11.70gm) were recorded from *R. leguminosarum* isolate 1 treated seeds. Haque (1993) found that lentil seedlings inoculated with *Bradyrhizobium* increased nodule number, nodule mass, shoot weight and yield of crops compared with control. This finding is supported by Sandhu (1984). He reported that lentil seed inoculation with *R.* isolates improved yield by 8-22%. Patil (1985) showed that infection of soybean plants by *F. udum* f. sp. *crotalariae, R. solani* or *S. rolfsii* were reduced when they were inoculated with *R. japonicum*. Pal and Ghosh (1986) studied the response of lentil and chickpea to inoculation with different strains of *R. leguminosarum* and found that seed inoculation with *R. leguminosarum* strain L 25 and L 20 increased nodulation and enhanced seed yield by 59.8% in lentil. The maximum vigor index (1390.00) was observed in *P. fluorescens* treated seeds. Izhar *et al.* (1995) observed that combined use of *Bradyrhizobium* sp. (Tal 480) and *Pseudomonas aeruginosa* completely controlled infection of *R. solani* and *F. oxysporum* in chickpea. Siddiqui *et al.* (2001) also found that *P. aeruginosa* and *B. subtilis* used as seed dressing or as soil drench significantly suppressed root rot infection and

nematode population densities under greenhouse and field conditions and thereby enhanced plant growth and yield in mungbean.

Effect of seed treatment with different microbial antagonists against foot and root rot pathogens of lentil (water agar test tube method) were determined and the highest germination (88.00%) was recorded from seeds treated with *R. leguminosarum* isolate 2, isolate 3, isolate 4 and *T. harzianum* . The lowest dead seeds (11.67%) were recorded from *R. leguminosarum* isolate 4 treated seeds. The minimum number of diseased seedlings (0.67) was counted from *T. harzianum* treated seeds. Chakraborty and chakraborty (1989) found that bacterization of seed with *R. leguminosarum* biovar *viceae* was highly effective against the severity of foot rot of pea. Seed treatment with *R. meliloti* was significantly effective in reducing the infection of 30 and 60 days old soybean plants by root infecting fungi namely *R. solani*, *M. phaseolina* and *Fusarium* spp. of soybean plant (Farzana *et al.*, 1991). Haque and Ghaffar (1993) found that *R. meliloti*, *R. leguminosarum* and *B. japonicum* used either as a seed dressing or as a soil drench reduced infection by *M. phaseolina*, *R. solani* and *Fusarium* spp. in both leguminous (soybean and mungbean) and non-leguminous (sunflower and okra) plants, and gave increases in shoot length and fresh weight as compared with untreated controls in field. Khan *et al.* (1998) determined the effect of biological seed treatment with *Rhizobium* inoculants to control foot and root rot of lentil and observed up to 62.5% and 73.3% reduction of *Fusarium* and *Sclerotium* foot and root rot, respectively. Hossain *et al.* (1999) observed excellent effect of *Rhizobium* inoculants in protecting attack of both *F. oxysporum* and *S. rolfsii* resulting 16.9 and 31.4% higher seed germination, respectively in pot soil artificially inoculated with these pathogens. Yaqub and Shahzad (2011) found that *S. rolfsii* had significantly negative effect on plant growth due to severe root colonization, whereas, presence of the microbial antagonists showed significantly positive effect on plant growth by reducing the colonization of roots by *S. rolfsii*. Highest root colonization by *S. rolfsii* and significant reduction in plant growth were observed in sunflower and mungbean plants grown in soil artificially infested with sclerotia of *S. rolfsii*. Use of biocontrol agents in *S. rolfsii* infested soil showed

significant reduction in Root Colonization Index accompanied by increase in plant growth. *Bradyrhizobium* sp., as found most effective ($p<0.01$) followed by *Rhizobium* sp., *T. harzianum, T. pseudokoningii, T. polysporum* and *T. virens*. Efficacy of biocontrol agents was comparatively suppressed in *S. rolfsii* infested soil as compared to non-infested soil. Bio-priming, seed coating with bio-control agents (*T. harzianum, Bacillus subtilis* and *P. fluorescens*), seed priming and seed dressing with these antagonistic micro-organisms enhanced their effectiveness in control of root rot disease incidence compared to other treatments (Mohamedy and Baky , 2008). Biofertilizers and Rhizobial strains decreased foot and root rot up to 59.2% over untreated control (Khalequzzaman and Hossain , 2008).

In this study antagonistic microorganisms were isolated and tested against the foot and root rot pathogens of lentil. Among the isolates *R. leguminosarum* isolate 1, *P. fluorescens* and *T. harzianum* showed potential effect against *F. oxysporum* and *S. rolfsii* causing foot and root rot of lentil. Further research works need to be conducted to test their efficacy in pot culture and as well as in the field condition.



Chapter 6

Summary and Conclusion

CHAPTER VI

SUMMARY AND CONCLUSION

Lentil is a highly nutritious and protein rich pulse crop which is liable to infection by different phytopathogenic organisms causing many diseases. Among the diseases, foot and root rot (*F. oxysporum*, *S. rolfsii* and *R. solani)* is a common one. The present research programme was undertaken to find out the bio-efficacy of microbial antagonists against foot and root rot pathogens of lentil. In vitro experiments were conducted in the Disease Diagnostic Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207 during the period of November 2011 to February 2013.

Foot and root rot infected plants of lentil were collected from Agronomy Research Field of Sher-e-Bangla Agricultural University, Dhaka and two pathogens were isolated and identified namely *F. oxysporum* and *S. rolfsii.*

In this experiment twenty Rhizobial isolates were isolated from nodules of sesbania and mungbean plant. Among them only four isolates were found effective against foot and root rot pathogens. These four Rhizobial isolates, *P. fluorescens* and *T. harzianum* were used as microbial antagonist. *P. fluorescens* and *T. harzianum* were collected from the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207.

The radial mycelial growth of *F. oxysporum* and *S. rolfsii* ranged from 2.03 - 4.77 cm and 1.80- 9.00 cm, respectively. Among twenty isolates of *R. leguminosarum*, only four isolates showed better performance in inhibiting mycelial growth of *F. oxysporum* but none of them inhabited mycelial growth of *S. rolfsii*. In case of *F. oxysporum*, the highest zone of inhibition (57.37%) was measured against *R. leguminosarum* isolate 3 and isolate 4. The highest zone of inhibition of *S. rolfsii* (80.00%) was recorded against *P. fluorescens* and no zone of inhibition (0.00) was observed against *R. leguminosarum*.

In seedling vigor (rolled paper towel method) test highest germination (91.00%), lowest dead seeds (9.00%), no abnormal and diseased seedlings

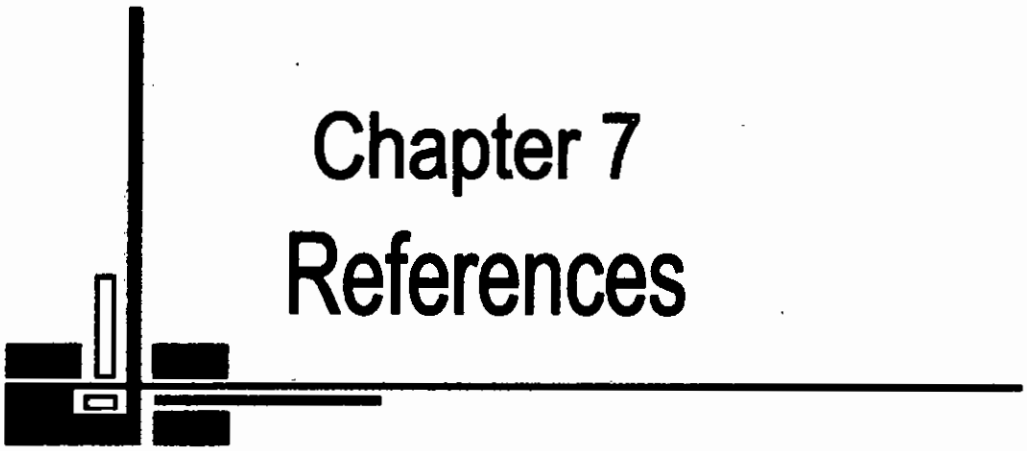
(0.00) were recorded from *R. leguminosarum* isolate 1 treated seeds. Maximum vigor index (1390.00) was observed in *P. fluorescens* treated seeds.

In water agar test tube method, the highest germination (88.00%) was recorded from seeds treated with *R. leguminosarum* isolate 2, isolate 3, isolate 4and *T. harzianum* . The lowest dead seeds (11.67%) were recorded from *R. leguminosarum* isolate 4 treated seeds. The maximum number of normal seedlings (84.56) were counted from *R. leguminosarum* isolate 3 treated seeds. The minimum number of diseased seedlings (0.67) were counted from *T. harzianum* treated seeds.

Overall results of the present study indicate that among the treatments *R. leguminosarum* isolates 3 and isolates 4 showed better performance in inhibiting the mycelial growth of *F. oxysporum* and *P. fluorescens* showed better performance in inhibiting the mycelial growth of *S. rolfsii*.

In seedling vigor test better performance in increasing germination and decreasing disease was recorded from *R. leguminosarum* isolate 1 treated seeds.

In water agar test tube method maximum germination percent, maximum number of normal seedlings and minimum number of abnormal seedlings and dead seeds were recorded from *R. leguminosarum* isolates treated seeds and minimum number of diseased seedlings were counted from *T. harzianum* treated seeds. Therefore it may be concluded that foot and root rot of lentil can be controlled by seed treatment with Rhizobial isolates, *P. fluorescens* and *T. harzianum*.



Chapter 7
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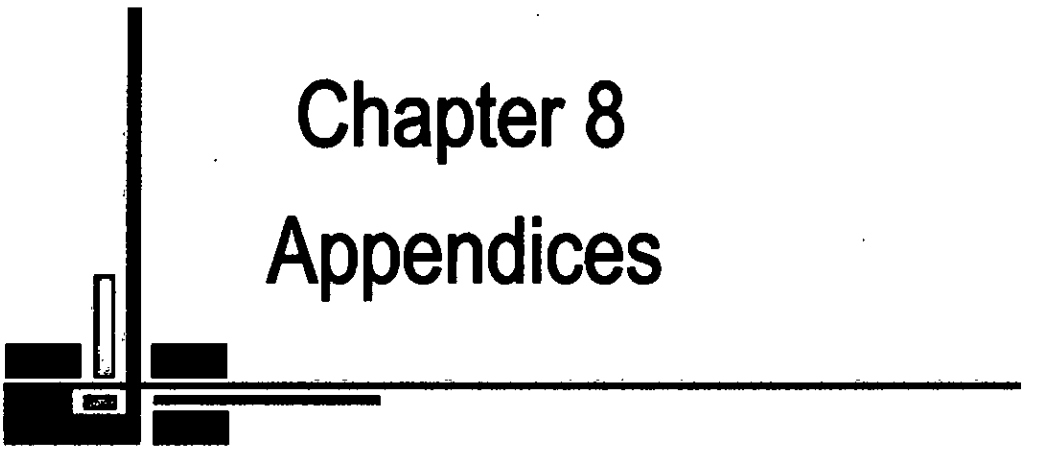

Chapter 8
Appendices

APPENDIX 1

PREPARATION OF MEDIA AND REAGENTS

Composition of Media:

The compositions of the media used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved t 121^0c for 15 minutes at 15 lbs pressure.

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Distilled water	1000 ml

Potato Dextrose Agar (PDA)

Peeled Potato	200g
Dextrose	20.0g
Agar	17.0g
Water	1000ml

Yeast Mannitol Agar (YMA)

Mannitol	10.0g
K_2HPO_4	0.5g
$MgSO_4$	0.2g
NaCl	0.1g
Yeast extract	0.4g
Agar	12.0g
Distilled water	1000ml

Gelatin liquefaction medium

Peotone	5.0g
Beef extract	3.0g
Gelatin	120.0g
Distilled water	1000 ml

Simmon's citrate agar medium

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

Starch hydrolysis media and reagent

Culture medium

Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1000 ml

Reagent (Lugol's iodine)

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	100 ml

Preparation of Reagents

Gram Stain Solution

a. Stock Crystal violet

Crystal violet	10.0g
Ethyl alcohol (95%)	1000ml

b. Stock oxalate

Ammonium Oxalate	1.0g
Distilled Water	1000ml

Crystal violet working solution: 20 ml of solution no. a mixed with 80 ml solution no. b. Additional dilution was made when desired.

c. Lugol's Iodine Solution

Iodine Crystal	1.0g
Potassium Iodide	2.0g
Distilled water	300.0 ml

Dissolved completely in 10 ml of distilled water, and then added to distilled water to make 300 ml. Stored in amber bottle.

KOH solubility Test

3% aqueous solution of KOH was prepared from the KOH granules.

Catalase test

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.

Oxidase Test

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