FORMULATION OF *Trichoderma* BASED BIOPESTICIDES IN CONTROLING FOOT AND ROOT ROT OF BETELVINE

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FORMULATION OF *Trichoderma* BASED BIOPESTICIDES IN CONTROLING FOOT AND ROOT ROT OF BETELVINE

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This is to certify that the thesis entitled, "Formulation of *Trichoderma* based bio-pesticides in controlling foot and root rot of betel vine" Submitted to the Department of Plant Pathology, 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 Reg. No.: 13-05777 under my supervision and guidance. No part of the thesis has been submitted any where for any other degree or diploma.

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

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Dedicated to
My
Beloved Parents

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The Author

Formulation of *Trichoderma* based bio-pesticide in controlling foot and root rot of betel vine

ABSTRACT

An experiment was conducted at Sher-e-Bangla Agricultural University (SAU) during the period from September 2014 to December 2014 for Formulation and evaluation of *Trichoderma* based bio-pesticide for controlling foot and root rot of betel vine. The effect of nine *Trichoderma* based substrates *viz*., T_1 (*Trichoderma* + Peat soil + Rice bran + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water), T_4 (*Trichoderma* + Peat soil + Gram bran + Water), T_5 (*Trichoderma* + Peat soil + Black gram bran + Water), T_6 (*Trichoderma* + Peat soil + Mustard oil cake + Water), T_7 (*Trichoderma* + Peat soil + Grass pea bran + Water), T_8 (*Trichoderma* + Peat soil + Saw dust + Water), T_9 (Control) were evaluated for growth and sporulation of *Trichoderma harzianum* and acting against *Sclerotium rolfsii* for the management of foot and root rot of betel vine. The effect of the treatments varied significantly in terms of production of *Trichoderma* spore and reducing foot and root rot diseases in comparison to control. Among the treatments, soil application with T_1 (*Trichoderma* + Peat soil + Rice bran + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water), T_4 (*Trichoderma* + Peat soil + Gram bran + Water) and T_5 (*Trichoderma* + Peat soil + Black gram bran + Water) completely control foot and root rot of betel vine. No plants were infected in case of application of T_1, T_2, T_3, T_4 and T_5 while 100% plants were infected in control treatment.

LIST OF CONTENTS

LIST OF CONTENTS (Contd.)

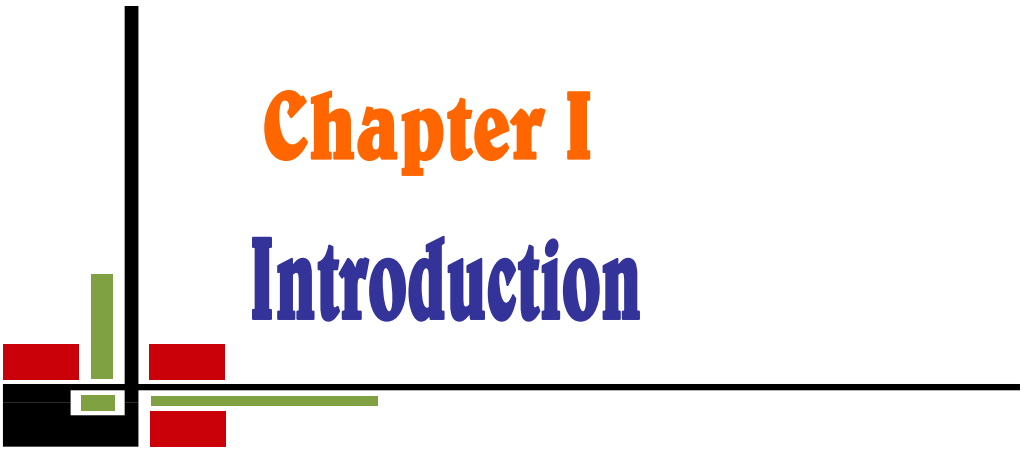
LIST OF TABLES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBRIVIATIONS

BARI	=	Bangladesh Agricultural Research Institute
cm	=	Centimeter
^{0}C	=	Degree Centigrade
DAI	=	Days after inoculation
DAS	=	Days after sowing
et al.	=	and others *(at elli)*
Kg	=	Kilogram
kg ha^{-1}	=	Kilogram per hectare
g	=	gram (s)
LSD	=	Least Significant Difference
MP	=	Muriate of Potash
m	=	Meter
P^H	=	Hydrogen ion conc.
RCBD	=	Randomized Complete Block Design
R^H	=	Relative humidity
TSP	=	Triple Super Phosphate
t ha^{-1}	=	ton per hectare
%	=	Percent



Chapter I

Introduction

CHAPTER I

INTRODUCTION

Betel leaf (*Piper betle* L.), is a perennial dioecious climber that climbs up trees or other supporting materials with the help of its adventurous roots. It is a popular mastication, usually chewed with slice of betel nut and lime, widely popular among people of Bangladesh, India, Pakistan, Nepal, Myanmar, Srilanka, Indonesia and Malaysia.

It is a climbing plant with shiny, green, heart-shaped leaves and white catkin. The stem is climbing by many short adventitious roots (Hassan and Shahadat, 2005). The leaf is widely used in social, cultural and religious events for hospitality and also has medicinal value.

Betel leaves are beneficial to the throat and remove viscidity in human beings. It is also good for the respiratory system and is used in treatment of bronchitis, cough and cold (Chopra *et al*, 1956). It increases digestive capacity when used with lime. Besides, it neutralizes the acidity and acts as blood purifier. Main constituents of betel leaves are vitamin B and C, carotene, and other elements. Paan contains some vitamins, enzymes, thiamine, riboflavin, tannin, iodine, iron, calcium, minerals, protein, essential oil and medicine for liver, brain and heart diseases (Chopra *et al*., 1956).

The deep green heart shaped leaves of betel vine are popularly known as *Paan* in Bangladesh. It is an important cash crop for country. It is also known as Nagaballi, Nagurvel, Saptaseera, Sompatra, Tamalapaku, Tambul, Tambuli, Vaksha Patra, Vettilai, Voojangalata etc in different parts of India (Guha and Jain, 1997). In Bangladesh, it is widely cultivated in Sylhet, Moulvibazar, Jessore, Khulna, Kustia, Bagerhat, Satkhira, Narail, Bhola, Barisal, Faridpur, Rajshahi, Rangpur, Gaibanda, Pabna, cox's Bazar and in greater Chittagong district.

There are about 100 varieties of betel leaf (paan) across the world of which 40 are encountered in India and 30 in west Bengal and Bangladesh (Samanta, 1994). In Sri Lanka, 18 species are found and three are endemic (Dassanayake and Fosberg 1981). Betel vine cultivars like, Desi Bangla, Bangla, Kali Bangla, Jhali, Sanchi, Bhabna, Mitha, Geso, Bonhoogly etc. are found in Bangladesh.

The betel vine cultivation is practiced in the south region of the country where humidity and temperature do not fluctuate abnormally and high humidity with moderate sunshine prevails throughout the year. In Bngladesh, Betel vine is cultivated mainly under an artificially erected structure, known as Boroj, Bareja or Bheet, which is a kind of hut which sides and roof are made of jute slaths or straw on a light frame work of bamboo. To cultivate the betel vine, low light intensity, mild temperature (10°C to 30°C), high humidity with moderate sunshine & 1450-1700 mm rainfall and frequent irrigation are needed throughout the year.

Bangladesh is the second largest grower of betel vine on about 14000 hectares of land. Total annual production of the crop in Bangladesh is about 72,500 tons. The average yield is 2.27 tons per acre (Anonymous, 2006).

It is an important economic crop in Bangladesh and exported to Middle East, Britain, Pakistan and some other African countries. In Bangladesh, most of the marginal farmers are involved in betel vine cultivation, as it is a continuous source of income.

Such a potential crop is known to suffer from different diseases. Among them foot and root rot caused by *Sclerotium rolfsii* has been treated as one of the major constrains of eggplant, tomato, betel vine etc. cultivation. The fungi *Fusarium oxysporum*, *Sclerotium rolfsii* and *Rhizoctonia solani* are soil inhabiting pathogen with wide host range and therefore, very difficult to control them (Rangswami, 1988; Martin and Torres, 1989).

The acreage of betel vine is decreasing gradually in our country because of some physical and socioeconomic barriers like unavailability of credit facilities, uncontrolled marketing system and infestation of diseases and pest (Islam, 2005). Disease is one of several imiting factors. The betel vine is highly susceptible to diseases, pests and some natural climates (Sayeeduzzaman, 1988). Among the diseases of betel vine, foot and root rot caused by *Sclerotium rolfsii* Sacc. are the most devastating diseases which decrease the production of betel vine to a great extent. In 2004, Sixty percent betel vine damaged due to foot rot disease in 3 upazilla of Rajshahi (Islam, 2005).

Sclerotium rolfsii Sacc. is a soil borne pathogenic fungus and harmful to many crops which are economically valuable in most of the tropical and subtropical region of the world (Aycock, 1966). It has a wide host range and has been referred as an almost omnipathogenic organism (Talukder, 1974). The fungus is a facultative parasite and can maintain continuity of generation under adverse situation by the formation of sclerotia (Ahmed, 1980). It is very difficult to control even by the use of chemical fungicide.

As a creeper crop the basal part of the betel vine stem to be kept in soil by folding and it's a continuous process as the part of the cultivation practice. The stem kept in the soil often affected by the soil borne fungus *Sclerotium rolfsii*. The basal part of the stem become rotten and caused a huge loss of the betel vine growers reduces the quality of betel leaf and hence the farmers deprived from the usual market price.

There are several methods for controlling foot and root rot disease of plants. Farmers try to overcome this problem through different cultural practices and use of chemical fungicides. But the control of soil borne pathogens with chemicals is very expensive and soil drenching by chemical fungicides is almost impractical in Bangladesh. In addition, indiscriminate use of chemicals in agriculture causes environment pollution and health hazards, destroying the natural balance and

beneficial micro-flora of the soil. Moreover, consumers are becoming increasingly concerned about chemical pollution of the environment and pesticide residues on food. Farmers are more often being faced with pathogen's resistance to chemical fungicides. Therefore, it is an urgent need for efficient alternative measures to combat the disease and inoculums buildup.

As alternative of chemical fungicides, bio-pesticides are now being considered eco-friendly component. In this case, living microorganisms act as antagonist, parasites and predators (Kwok *et al*., 1987). The antagonism of a biological agent reduces pathogen's ability to produce inoculums. In this context, future disease levels will eventually be reduced by such biological control measures (Fokkema, 1995). T*richoderma* spp. has played a considerable role as bio-control agent and is recognized as an effective bio-control agent against soil-borne plant pathogenic fungi such as *Fusarium*, S*clerotium*, *Rhizoctonia* etc. (Chet and Inbar, 1994). *Trichoderma* significantly destroys the sclerotia of *S. rolfsii* (Susceelendra and Schlosser, 1999) and it is antagonistic to *S. rolfsii*, overlaps the pathogen and suppresses their growth (Iqbal *et al*., 1995).

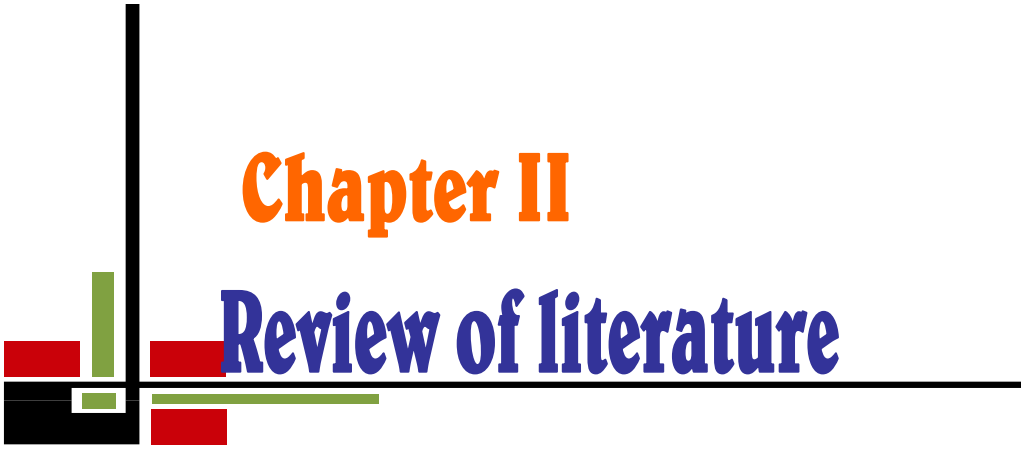
Trichoderma produces chemicals called trichodermin which is responsible for its antagonistic properties (Tverdyukov *et al*., 1994). *T. harzianum* may be used as an eco-friendly option to save many beneficial micro-organisms in the nature. This bio-control agent would be potential to protect seedlings against diverse soil borne pathogenic fungi. It is also reported that *Trichoderma* has promising contribution in plant growth (Burr *et al*., 1978 and Baker, 1988).

The major limitation of biological control by *Trichoderma* sp. is the production of inoculam in large scale. Many researchers have worked on mass production of *Trichoderma* inocula in the form of spore or other propagules (Papavizas and Lewis 1985). The viable inocula must be produced in an inexpensive medium and the cost of production for treatment of large areas must be competitive with that of the chemical pesticides. The economic mass production of antagonists could be

achieved by using readily available crude agricultural product. Various substrates like grain bran (Wells *et al*., 1972), celatom and molasses (Backman and Rodriguez-Kabana, 1975), wheat straw (Akhtar, 1977), wheat bran (Hader *et al*., 1979), cereal meal and sand (Mangenot and Diem, 1979), barley grain (Moity and Shatla, 1981), wheat bran and saw dust (Lewis and Papavizas, 1980), sand and corn meal (Lewis and Papavizas, 1980) and combination of different substrates have been used to produce inocula of *Trichoderma* spp (Dubey and Patel, 2002).

On the basis of above facts the present investigation was undertaken with the following objectives

1. Selection of effective substrates for growth and sporulation of *Trichoderma harziaum*
2. To determine the effect of soil based substrats in growth and sporulation of *Trichoderma harziaum*
3. To formulate *Trichoderma* based bio-pesticides using effective peat soil based substrats
4. Control of foot and root rot of betel vine by formulated *Trichoderma* based bio-pesticides



Chapter II

Review of literature

CHAPTER II

REVIEW OF LITERATURE

Pan (betel leaf, *Piper betel*) grown in tropical regions, is a kind of creeper leaf belonging to the pepper family of plants named *Piper betle*. Pan differs in shape of leaves, bleaching quality, softness, pungency and aroma. The stems are semiwoody, climbing by many short adventitious roots. Leaves are large, 15-20 cm, broadly ovate. Fruits sparingly produced, quite immersed in the fleshy spike, which is about 5 cm long and pendulous. *Cultivation'* In olden times, pan was produced in all parts of Bengal, though some districts like Dinajpur, Rangpur, Midnapur and Chittagong were particularly famous for its production. Pan (betel vine) seedlings are frequently attacked by damping off pathogen in vine ground. A number of soil born organisms like *Sclerotium rolfsii, Fusarium oxysporum*, *Pythium spp.* and *Rhizoctonia solani* are involved to cause this disease. This disease is great threat for production of betel vine in our country.

Sclerotium rolfsii, the causal agent of foot rot or collar rot of many crops having wider host range (Talukdar, 1974) attracted the attention of plant pathologist and professional researcher throught the world. The pathogen is known to cause diseases of cereals, pulses, oil crops, betel vine, potatoes, vegetables, ornamentals and nursery seedlings of fruits and forest trees. In Bangladesh, disease caused by *Sclerotium rolfsii* in different crops have been reported among many others by Meah and Khan (2003).

In this chapter attempt has been made to review the available literature about foot and root rot disease. Some important literatures supporting the symptom, effect of the diseases, method of inoculation of *Sclerotium roifsii,* host range of *Sclerotium roifsii,* histopathology are reviewed here.

Evidences of research work regarding management of damping off of betel vine are very limited. However, some available and important findings on various

aspects for management of damping off of seedlings has been compiled and presented in this chapter:

2.1 History

The foot and root rot of betel vine have been reported from almost all betel vine growing countries in the world including Indonesia, Myanmer, Srilanka and Bagladesh (Tuner, 1969) etc. in west Bengal, the highest intensity of foot and root rot have been recorded in Midnapore and Nadia district (Dasgupta and Sen, 1997). The extent of losses may vary from 30-100% in case of foot and root rot (Maiti and Sen, 1982; Dasgupta *et al.*, 2000).

Islam (2005) observed that farmers growing *Piper betel* in three upazilas of Rajshahi incurred a huge loss as foot rot disease damaged about 60% of the cultivation in the year of 2004.

2.2 Environmental factor

An epidemiological studies were reported that the maximum temperature, maximum relative humidity and rainfall played an important role in the development of both the diseases of betel vine *(Piper betel* L.) (Anonymous, 2006; Maiti and Sen, 1982).

According to Punja *et al.* (1988), temperature is the principal limiting factor in the geographic distribution of *Sclerotium roifsii.* The disease rarely occurs where average daily minimum winter temperatures are below freezing (0^{o}C). Maximum disease occurs at 25-35^{0}C which is also optimum range for mycelia growth and *sclerotia* germination of the fungus.

Farr *et al.* (1989) found that, fungus *Sclerotium roifsii* attacks all plant parts in the contact with the soil under favourable environmental conditions including stems, roots, and fruits. Chattopadhyay and Maiti (1990) observed that the plants of betel

vine are cultivated in conservatories under shady and humid conditions that also favor the development of many diseases.

According to Jana (1995), in the areas with lower rainfall (1500- 1700 mm) the crop is cultivated with small and frequent irrigations, i.e. every day in summer and every 3-4 days in winter, whereas adequate drainage is required during the rainy season.

CSIR (1969); Guha and Jain (1997) observed that vines grows best under the shaded, tropical forest ecological conditions with a rainfall of about 22504750 mm, relative humidity and temperature ranging from 40-80% and 15- 40°C, respectively. A well-drained fertile sandy or sandy loam or sandy clay soil with p^H range of 5.6 to 8.2 is considered suitable for its cultivation.

Mollah (2012) found that 29^0C and 85% R^H, the disease incidence and severity of foot and root rot of betel vine was the highest and was the lowest when the temperature laid around 18.7^0C and the R^H laid around 75%. Al-Askar *et al.* (2013) found that sclerotial diseases caused by *Sclerotium rolfsii* occur primarily in warm climates, especially at high temperatures.

2.3 Symptom and effect of the disease

Bertus (1929) stated that *Sclerotium rolfsii* possessed the ability to cause damping off of the seedlings of certain plants when the pathogen was brought in contact with stem of these plants. Conditions that appeared to be necessary for the fungus were its presence in the upper four inches of the soil, high temperature and humidity. On a number of other hosts like chilli, tomato, groundnut etc it caused collar rot of the plants.

Ramkrishan *et al.* (1930) found that *Sclerotium rolfsii* causing wilt, it was common on Irish potatoes, sweet potatoes and almost all kinds of vegetables and flowers in Jamaica.

Dastur (1935) observed that the foot and root rot of betel vine, the leaves and shoots turn yellow, wither and finally dry out to a pale brown colour. The fungus attack the roots and stem near the soil level. Black lesion develops following necrosis of the plant cells. The mycelium invades the stem and rots the affected portions. As a result, the plant wilts and gradually dies. Abundant white mycelium and small light brown Sclerotia form on the rotted plants.

Das *et al.* (2000) found that the disease symptoms of foot and tuber rot of tuberose caused by *Sclerotium rolfsii* is preceded by the appearance of prominent coarse mycelia masses on leaf surfaces at or near the soil surface. The infected leaves detached from the plant fall on the soil surface. More or less round sclerotia, brown in colour, are formed on and around the infected leaves. As a result the infected plants become weak and send out few or none of the flowering shoots in case of severe damage.

Aycock (1966) stated that host range of *Sclerotium rolfsii* is very wide and includes not only many important horticultural and agronomic crops. The soil borne plant pathogenic fungus *Sclerotium rolfsii* attacking more than 500 spp. of plants belonging to over 100 families.

Aycock (1966) reported that, stem rot disease familiarly known as *Sclerotium* wilt, *Sclerotium* blight and white mold which affects all part of the plant at any stage of crop growth. Formation of deep brown lesions around the meristem below the soil surface are the first characteristics symptom. The lesions become covered with radiating mycelium which encircles the affected portion of the stem, resulting in the development of yellowing and wilting of the whole or part of the plant.

Giganate (1950) stated that potatoes grown in northern and central Italy were occasionally attacked by *Sclerotium rolfsii.* The fungus affected collar region to cause collar rot, the plants turning yellow and rapidly wilting. Spherical, elliptical or irregular sclerotia of 1-3 mm in diameter were found in the affected part of tuber. The disease occurred mostly in sandy or compact clay soils and was

favoured by hot moist weather.

Choudhury (1967) stated that, foot rot disease of brinjal is caused by *Sclerotium* sp. Minute mustard like structure, adheres to the stem at ground level. These put out mycelia which enter the stem and choke the vessels. This disease is spread from one plant to the other by irrigation. It is difficult to control the disease as the fungus persists in the soil. Crop rotations with non-host crops and drenching some copper fungicides in the soil before planting helped to control the disease.

Mostofa (1973) stated that the presence of *Sclerotium rolfsii* in soil produce collar rot and cause ultimately wilting of betel vines. Dutta *et al.* (2002) observed that *Sclerotium rolfsii* initially attacks roots of tuberose plant and later advances to the tubers and petioles to cause disease.

According to Chet *et al.* (1994), *Sclerotium rolfsii* Sacc. causes the disease known as southern blight in wide variety of crops. *Sclerotium rolfsii* form brownish sclerotia that can survive in the soil for longer period of time.

Singh (1970) reported that *Sclerotium rolfsii* infects the base of stems producing a fan of silky white mycelium and round sclerotia which are initially white and gradually darken.

Punja *et al.* (1988) reported that root-rot disease caused by *Sclerotium rolfsii* being one of the most important diseases of crops. Ahmed and Hossain (1985) reported that collar rot, foot and root rot disease caused by *sclerotium rolfsii* caused considerable damage both in seedling and adult stages of Indian spinach, and there existed variations in the incidence of the disease in different parts of Bangladesh.

Chakravarty and Bhowmik (1983) studied on symptoms and techniques of inducing collar-rot of sunflower caused by *Sclerotium rolfsii* Sacc. The fungus caused pre- and post-emergence damping off of sunflower seedling and collar rot of adult plants. Disease development was highest in 60 day old plants, with maximum rotting in internal tissues and highest incidence in the field at the stage.

Hsiehh (1979) reported from Taiwan that *Sclerotium rolfsii* was found to cause rot of three ornamentals viz. *Saintpaulia, Jonantha gloxinia and Streptocarpus hybridus.* Bisth (1982) described that the pathogen infected the potato plants at collar region causing wilting of plants. White or brown *sclerotia* were developed at maturity in the root and collar regions of the infected plants. The infection spread within few days either by irrigated water or by farm implements used for cultural practices. The pathogen damaged either stem or root.

Gurkins and Jenkins (1985) reported that carrot became diseased by *Sclerotium rolfsii* about 90-100 days of planting when the plant canopies shade the soil surface and create a micro environment suitable for southern blight development in the North Carolina.

Yasmin-Ahmed *et al.* (1988) reported that *Sclerotium rolfsii* caused collar rot of maize. The pathogen was isolated from infected maize and pure culture was subsequently inoculated into Maize cv. Shaheen sown in soil infested with the pathogen. Within 15 days of seedling emergence, sclerotia were seen on the soil surface and around the seedling. Seedlings were killed within 10-15 days.

Wangihr *et al.* (1988) reported that an outbreak of collar and root rot was observed on Capsicum in Maharastra, India during the first week of October, 1985.The disease was most severe on cultivars Jurala and CA-960. The causal agent was identified as *Sclerotium (Coeticium) rolfsii.*

Montealegre and Esterio (1989) reported that outbreak of *Sclerotium rolfsii* affected more than 150 hectares of *Phaseolus vulgaris* occurred in Chile in January 1987.The disease was unequally distributed with large area of dead plants and patches of small plants showed wilt and chlorosis, Adult plants which were infected but did not die produced fewer fruits and smaller seeds than uninfected plants.

Mridha and Alamgir (1989) observed sclerotial wilt of betel vine in thirty selected

gardens in Chittagong. Plants showed decay at the collar region and below the soil level. It has been reported that infected plants lost luster, leaves turned yellow and the whole plant wilted and died. The infected portion of stem was covered with white cottony mycelia strands with small, light to deep brown sclerotia on the stem as well as adjacent soil surface.

Okoli *et al.* (1991) reported that *Sclerotium rolfsii* caused heavy infestation on sunflower, plants wilted and dried out with basal stem dry rot. Symptoms included an initial acropetel wilting of the entire plants. Affected plants gradually dry-out but remained green and attached to stem. They found that within 24 hrs of wilt onset, a white mat of mycelia formed around the discolored site on the stem base. Within 1-2 days, the mat had rounded off into small white balls and characteristics brown mature sclerotia within 24 hrs.

Sugha *et al.* (1991) reported that *Sclerotium rolfsii* caused collar rot of chickpea. A total of 210 lines and cultivars of chickpea tested by placing one wheat grain fully covered with mycelium of *S. rolfsii* at the collar rot of 7 days old seedling in pot of sterilized garden soil. All were find to be susceptible to collar rot.

Khanna and Jyotsama Sharma (1993) described the symptoms of *Sclerotium* rot of potato as dark brown lesions appearing on the stem just below the soil surface followed by wilting of lower leaves and gradually drying of the entire plant. Such wilted plants showed white cover of fungal threads, girdling the basal part of stem, which moved above and below to the stem and roots. Sclerotia resembling mustard seeds, developed on infected plant parts and also on soil.

Alexander and Stewart (1994) worked on *Sclerotium rolfsii* (Telemorph; *Athelia rolfsii)* and found it causes serious root and stem rots of a range of economically important fruit and vegetable crops. Sclerotia are the important propagules for the survival of this pathogen. Under favourable conditions, sclerotia may germinate to cause infection usually occurs at or just below the soil surface and symptoms includes yellowing, browning and wilting of entire plants.

Kulkarni *et al.* (1994) reported that, the pathogen affected either stem or root or tubers. The infected stem produced dark brown lesions at collar region causing wilting and ultimately plants dried up. Brownish sclerotia resembling mustard seed developed at later stages on the root and collar regions of the infected plants. After that, tubers get infected and rotten in the field.

The pathogens of sclerotial diseases cause damping-off of seedlings, stem canker, crown blight, root-rot, crown rot, bulb, tuber and fruit rots. Sclerotial diseases frequently affect a wide variety of plants, including most vegetables, flowers, legumes, cereals, forage plants and weeds (Agrios, 1997).

Mullen (2001) reported Southern blight, Southern stem blight, White mold caused by *Sclerotium rolfsii.* Anahosur (2001) observed the dark brown lesion on the stem just below the surface followed by drooping and wilting of infected leaves and gradually wilting of the entire plant. Such wilted plants showed whitish mycelia growth with sclerotial bodies resembling mustard seeds on collar region and also roots.

Lievens *et al.* (2004) studied that a severe rot and collar/foot rot was observed on two month old wilted tomato *(Lycopersicum esculentum)* plants in a large scale (2.5 ha) commercial green house setting in Belgium. Symptom development was restricted to lower plant parts with severe rotting of the entire root system and dark lesions girdling the stem base.

Yaqub and Shahzad (2005) proved *Sclerotium rolfsii* highly pathogenic on sunflower, and mildly pathogenic on tomato, lentil, sweet pumpkin and cabbage and non-pathogenic on cauliflower plant in a pot experiments. Increase in inoculum density of *Sclerotium rolfsii* caused gradual relation in growth parameters of sunflower and mungbean plants where as a positive correlation was observed between root colonization and population of *Sclerotium rolfsii* in soil.

Garibaldi *et al.* (2006) observed severe basal rot symptoms of potato *(Solanum*

tuberosum L.) in a commercial field near Alessandria (northern Italy). According to Daami-Remadi *et al.* (2007), potato tubers showing a fan-like mycelia growth at their surface and severe soft rot symptoms were observed in traditional potato storage at Essaida (North of Tunisia).

2.4 Method of inoculation of *Sclerotium rolfsii*

Islam (2008) inoculated the eggplants following soil inoculation technique using the barley culture of the pathogen *(Sclerotium rolfsii).* All the varieties were infected ranging from 66.66 to 100%. Varieties varied in percent mortality.

Kashem (2005) used soil infestation method for inoculation of *S. rolfsii.* He found that soil infestation with grain culture at the rate of 0.1% weight basis of dry soil before sowing seeds caused heavy infestation.

Babar (1999) used 10 g of colonized dried oat grains or pouring fungal suspension with soil near plant base for inoculation and covered with moist cotton. Older plants (60-90 days age) developed infections quicker (8-10 days) and larger sized lesions than that in younger ones (10-45 days age).

Hiremath *et al.* (1998) compared 5 methods of inoculation of *S. rolfsii.* Soil infestation technique was the most efficient for inducing infection of seedlings by *S. rolfsii.* Incorporation of 2% inoculum with soil was sufficient to produce high disease levels. Disease incidence on plants inoculated at 30 and 60 days by toothpick method increased with plant age.

Waraitch *et al.* (1986) used soil mixing method of inoculation of *S. rolfsii* (multiplied on sterilized sorghum seeds pre-soaked in 2% sucrose solution) was mixed in soil near the plants @ three 500 ml flask per 100 m .

Chakravarty and Bhowmik (1983) used both soil infestation and toothpick method for inoculation of *S. rolfsii.* Both techniques effectively induced the disease, soil: inoculums ratio of 50:1(w:w) caused heavy infection.

2.5 Pathogenicity

Sengupta and Das (1970) studied the cross inoculation of isolates of *Sclerotium rolfsii* from groundnut, wheat, potato, guava, and benglagram. They concluded that, benglagram was the most susceptible host for *Sclerotium rolfsii*.

Thammsak-Sommat *et al.* (1982) made an investigation on the pathogenicity of *Sclerotium rolfsii*, and reported that the pathogen could infect its host cotton severly; disease severity in average was 84%. The pathogen caused pre and post emergence damping off symptoms of cotton seedlings. They also found the soil amendment by crop refuses, nitrogen fertilizers and lime decreased disease intensity.

Palakshappa (1986) observed considerable foot rot infection when betel vine were inoculated with two and three percent inoculum of *S. rolfsii.*

Siddaramaiah (1988) confirmed the pathogenicity of *Sclerotium rolfsii* on *Desmodium uncimatum* Desv. and *Cotonoris ainesii* Eckl and Zeyh, two important forage legumes of hill zone by similar producer.

Siddaramaiah and Chandrapa (1988) proved the pathogenicity of *Sclerotium rolfsii* on cardamon in pot culture studies by inoculating 25 days old sclerotial cultures which was grown on sand corn meal medium and observed the symptoms a week inoculation.

Fakir *et al.* (1991) reported that sowing of lentil during third week of November was found to reduce the incidence of collar rot and root rot caused by *Sclerotium rolfsii and Fusarium oxysporum* compared to early sowing. Artificial inoculation often selected genotypes of lentil to collar rot pathogen, *Sclerotium rolfsii* showed that all the lines were susceptible to the test pathogen.

2.6 Incidence and severity of *Sclerotium rolfsii*

Meah (2007) tested the pathogenecity of 10 isolates of *Sclerotium rolfsii* on eggplant (var. Dohazari) and he found that all the isolates of *S. rolfsii* significantly affected the seed germination, pre-emergence death, damping off, foot rot and plant stand.

Palakshappa (1986) surveyed the incidence of *S. rolfsii* on *Piper betle* L. in different areas of Karnataka state during 1984-85 and recorded 35 to 39 percent disease incidence.

Meah (1994) reported the incidence and severity of collar rot of sunflower in fifteen (15) varieties which were grown at 2 (two) agro-ecological zones (AEZ) of Bangladesh. Survey was conducted during (Kharif-I) July, 1994. He observed that almost all the varieties were affected by collar rot. At Bangladesh Agricultural University (BAU), Mymensingh collar rot was prevalent throughout the crop season. All varieties at BAU were heavily affected with collar rot. Some 3.0-5.0% plants were killed at flowering stage.

Khan (1996) reported the incidence and severity of collar rot of sunflower in fifteen (15) varieties which were grown at 3 agro-ecological zones (AEZ) of Bangladesh. Survey was conducted during Rabi (1993-94) and Kharif-I (1994) at flowering and harvesting stages of the crop. He observed that young plants were more susceptible to collar rot. Incidence of the disease were minimum in Rabi season and a higher percentage of plants were killed in Kharif-I season.

Further report of Meah (1997) includes the incidence and severity of collar rot of sunflower in thirty (30) varieties which were grown at 2 (two) agro ecological zones (AEZ) of Bangladesh. Survey was conducted during March, 1997. He observed that collar rot affected almost all the sunflower varieties except MSFH-17 and MSFH-592.

Rahman and Sultana (2011) found that, in Jamalpur region, the incidence and severity of Sclerotial rot of betel vine is more or less highest and lowest throughout the year.

Mollah (2012) found that, in case of foot and root rot of betel vine in Satkhira district, highest disease incidence were found in August (12.50% to 32.50%) and lowest disease incidence were found in December (0% to 8.33%) in 2010. The highest disease incidence were found in August (18.75% to 50%) and the lowest disease incidence were found in December (0% to 2.08%) in 2011.

2.7 Management of foot and root by *Trichoderma* based bio-pesticide

Kumar (2013) reported that, *Trichoderma* is a genus of asexually reproducing fungi that is present in all types of soils. Recent reports show that they are opportunistic, avirulent plant symbionts, as well as being parasites on other fungi. A number of successful bio-control products based on different species of *Trichoderma* have been commercialized in India, USA and elsewhere in the world.

Kashem *et al*. (2011) conduct a series of experiments to assess the effect of 14 isolates of *Trichoderma* spp. (*Trichoderma harzianum* and *T. viride*) for controlling foot and root rot of lentil caused by *Fusarium oxysporum*. The pathogenecity of 12 isolates of *F. oxysporum* and the mass production of an isolate of *T. harzianum* on 25 substrates are also studied. *Trichoderma* isolates inhibited the growth of *F. oxysporum* up to 92.07 % on agar plates.

Pandya *et al*. (2011) reported that, soil-borne pathogens in fungi cause important losses, being the most aggressive. The distribution of several phytopathogenic fungi, such as Pythium, Phytophthora, Botrytis, Rhizoctonia and Fusarium have widely spreaded during the last few years due to change of intensive farming crops culture and environment. *Trichoderma* as a bio-control agent (BCAs) is well

recognized due to their high reproductive capability and show strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms.

Amin *et al*. (2010) carried out a study on six isolates of *Trichoderma* spp. for their ability to inhibit soil borne pathogens of different vegetables viz., *Rhizoctonia solani* (isolates from tomato), *Sclerotium rolfsii* (causing collar rot of tomato) and *Sclerotinia sclerotiorum* under in vitro conditions. Dual culture of pathogens and *Trichoderma* spp. revealed that *T. viride* highly inhibited (65.71%) mycelial growth of *Rhizoctonia solani* over control. In case of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, *T. viride* proved to be potential inhibiting mycelial growth of thet pathogens.

Hossain and Hossain (2010) formulate a *Trichoderma* based BAU-bio fungicide that was found very much effective agent several tikka disease of groundnut, foot and root rot of pulses and diseases of some vegetable crops. BAU-bio fungicide also helpful to control seed borne mycoflora, increasing seed germination and seedling vigour of some vegetables. Management of seedling diseases are successfully possible by using BAU-Biofungicide, biofertilizer and cowdung in blackgram, mungbean and lentil.

Tran (2010) conducted surveys on food crops, industrial crops, vegetable crops and fruit crops in the north and south of Vietnam and reported that *Trichoderma* can be isolated easily from soil, root and plant organic matters. *Trichoderma viride*, *T. harzianum*, *T. hamatum* were predominant species in Vietnam. Laboratory and field trials proved that *Trichoderma* species had ability to suppress growth of fungal plant pathogens and enhance plant growth and development. He reported that, *Trichoderma* products have been commercially developed by several companies, institutes and universities such as: BIMA, Trico-ĐHCT, Promot Plus WP, Vi DK, NLU-Tri, Bio – Humaxin and are available in markets. *Trichoderma*

product can be used in many ways including: seed treatment, applied direct to the soil before planting and added to organic fertilizers.

Chandrasehar *et al*. (2005) conducted lab and green house experiments to determine the antagonistic effect of *Trichoderma harzianum* against *S. rolfsii* that caused tomato collar rot. They found that *Trichoderma harzianum* in in vitro condition completely suppressed the growth of *S. rolfsii* and in green house condition in pot culture increased the percent survival of treated seedling applied as seed treatment and soil drenching.

Islam (2005) also reports while working on controlling of seedling diseases of eggplant that *Trichoderma harzianum* T22 effectively controlled damping off disease of seedlings.

Kashem (2005) used soil infestation method for inoculation of *Sclerotium rolfsii*. He found that soil infestation with grain culture at the rate of 0.1% weight basis of dry soil before sowing seeds caused heavy infestation.

Meah *et al*. (2004) reported that *Trichoderma harzianum* cp and *Trichoderma harzianum* T22 grown on peat soil based black gram bran was found effective in controlling nursery diseases like damping off, tip over and seedling blight of eggplant and promoted seed germination.

Meah (2003) listed a number of diseases of eggplant caused by fungi, bacteria, virus, nematode and mycoplasma. Of them, collar rot caused by *Sclerotium rolfsii* is damaging to the crop.

Shamsuzzaman *et al*. (2003a) studied for mass production of *Trichoderma harzianum*. Of them, rice straw chick pea bran, rice course with 3% chick pea powder, rice straw with 5% sucrose black gram bran, grass pea bran and peat based wheat bran supported best in mass production of conidia (42.93×10^7/g culture). Shamsuzzaman *et al*. (2003b) further reported that seed treatment with *Trichoderma harzianum* grown on black gram resulted up to 16.66% higher seed

germination, 266.33% fresh shoot weight, 157.14% fresh root weight and 98.55 vigor index of cucurbits over control.

Howlader (2003) reported that *Trichoderma harzianum* cp yielded good result against phomopsis blight and foot rot of eggplant in the field. Islam *et al*. (2002) evaluated nine organic substrates for their suitability for mass culture of an isolate (GT-1) of *Trichoderma harzianum*, a potential bio-control agent. They found that maize meal was the best substrate for maximum spore production also colony diameter, mycelial growth was fast compared to others.

Sultana *et al*. (2001) observed growth and storability of *Trichoderma harzianum* and its effect on germination of egg plant seeds. They found that *Trichoderma* treated seed resulted up to 48.62% germination than that of control (untreated).

Chowdhury *et al*. (2000) reported that seed treatment with *Trichoderma harzianum* and *Gliocladium viride* against *Sclerotium rolfsii* resulted up to 21.61% and 48.43% increase in germination in mungbean, black gram, pigeon pea and tomato, respectively and showed good effect on seed born mycoflora. Moreover, significant growth enhancements of mungbean, blackgram and tomato have been achieved by treating seeds with antagonists. The antagonists were found effective against *Sclerotium rolfsii*.

Rettinassababady and Ramadoss (2000) reported that *Trichoderma* spp. were mass multiplied in black ash, coir waste, farmyard manure, rice husk, spent straw from mushroom bed, sugarcane bagasse, talc and vermiculite. *Trichoderma* growth and spore production was maximum in farmyard manure and coir waste (474 x 105, 263×10^5 spore/g) in 3 weeks in culture.

Sultana and Hossain (1999) evaluated *Trichoderma harzianum* for controlling foot and root rot (*Fusarium oxysporum* and *Sclerotium rolfsii*) of Lentil cv. BARI Masur-1 under field condition. Seeds of lentil treated with *Trichoderma harzianum* contributed 47.85% to 112.49% reduced of foot and root rot diseased

plants over control. *Trichoderma harzianum* treated seeds increased germination up to 13.37% and resulted up to 3.69% more field emergence over control. *Trichoderma harzianum* treated seeds resulted yield up to 1783.33 kg/ha that accounted 81.60% higher seed yield.

Shamarao *et al*. (1998) tried mass multiplication and sporulation of *Trichoderma viride* using different substrates like oil cake, farmyard manure, wheat bran, poultry manure, dung, jaggery, groundnut cake, neem cake and pongamia. Wheat bran was the most suitable substrate for sporulation of the antagonists.

Begum (1997) selected four *Trichoderma* spp. and evaluated their antagonistic potential against the major soil-borne plant pathogens *Sclerotium rolfsii*, *Fusarium oxysporum* and *Macrophomina phaseolina*. Two induced mutants of *Trichoderma* spp. showed better performances than control strain in reducing the seedling mortality in chickpea and lentil caused by *Fusarium oxysporum* and *Sclerotium rolfsii* under glasshouse condition.

Das *et al*. (1997) screened five media (wheat bran, rice bran, maize meal, sand medium, potato dextrose agar and saw dust) for mass multiplication of *Trichoderma viride, T. harzianum* and *T. koningii* in vitro. Wheat bran proved to be more promising for the growth and sporulation of the fumgi. Growth and sporulation of *Trichoderma* spp. were significantly higher after 14 days, than after 7 days of inoculation.

Roberti *et al*. (1996) investigated the activity of *Trichoderma harzianum* 74 on bean (*Phaseolus vulgaris*) rot caused by *Scelorotium rolfsii* when applied to seeds. Trichoderma strains were active in bean root rot ensuring control of *Scelorotium rolfsii*. *Trichoderma harzianum* reduced the growth of *Scelorotium rolfsii* and parasitized *Scelorotium rolfsii*, hyphae by direct contact, forming coils, short contact branches and hook-shaped hyphal tips.

Mukherjee *et al*. (1995) observed that *Trichoderma harzianum* was effective in suppressing *Sclerotium rolfsii* and *Rhizoctonia solani*. *Trichoderma harzianum* was found to be effective in destroying the sclerotic of both fungi.

Mukhopadhyay (1995) stated that two bio-agents viz., *Gliocladium virens* and *Trichoderma harzianum* were used for treating seeds of various crops, like chickpea, lentil, groundnut, tomato and cauliflower for protection against wide range of soil brone pathogens viz., *Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium* spp. and *Fusarium oxysporum*. Such biological treatment was also integrated with suitable fungicide in view of the insensitivity of the bio-agents to some chemicals. The treatment was found highly effective and resulted in enhanced crop performance when compared with biological or chemical treatment alone.

Inber *et al*. (1994) used *Trichoderma harzianum* to cucumber seedlings as a peat-bran preparation incorporated into the propagation mixture in a commercial plant production nursery. Increase of 23.8% in seedlings height and 96.1% in leaf area were recorded. On marketing day (after 18 and 30 d) recorded significant DW compared with untreated control plants. *Trichoderma*-treated plants. However, significant reductions in damping-off by 67% and 52% were obtained in middle and border, respectively, during the 2^{nd} growing cycle compared with untreated controls.

Chet and Inbar (1994) studied on biological control of fungal pathogens and reported that *T. harzianum* as effective bio-control agent of soil-borne plant pathogenic fungi. Lectins were found to be involved in the recognition between *Trichoderma* spp. and its host fungi, where as Chitinase is involved in the degradation of the host wall.

Sugha *et al*. (1993) reported that conidial coating of the antagonistic *Trichoderma harzianum* and *T. viride* on seeds significantly reduce seedling mortality (47-65%) infected by *Sclerotium rolfsii* compared with untreated controls.

Sangeetha *et al*. (1993) found farmyard manures as the best for formulation of *Trichoderma viride* and *Trichoderma harzianum* followed by wheat bran and rice bran. Peat soil alone and rice straw were found as poor substrates.

Xu *et al*. (1993) observed that both isolates of *Trichoderma* T82 and NF9 inhibited hyphal growth of *Sclerotium rolfsii, Rhizoctonia solani, Pythium aphanidermatum, P. spinosum* and *Fusarium oxysporum*. In greenhouse experiments, soil treatment with 0.6 % (w/w) T82 bran culture (107CFU /g) reduced incidence of disease caused by *S. rolfsii, R. solani* and *P. aphanidermatum* by 46.5%, 28.4% and 81.2% respectively, 20 days after inoculation with the pathogens. Seed treatment with T82 or NF9 spore suspension (108 CFU /ml) increased emergence of cucumber seedlings by 14% and 20%, respectively, 11 days after inoculation with *S. rolfsii*.

Kaur and Mukhapadhyay (1992) reported that integrated use of *Trichoderma harzianum* with fungicidal seed treatments in the fields significantly reduced the incidence of chickpea wilt complex and increased crop yield. Seed treatment with vitavax-200 (Carboxin + Thiram) and Ziram resulted 29.9% disease control. This control increased to 63.3% when *Trichoderma harzianum* was added.

Monaco *et al*. (1991) used for treating seeds as bio-control agents of *Fusarium* and *Sclerotium*. They isolated *Trichoderma harzianum, Trichoderma koningii* and *Trichoderma aureoviride* from tomato fields in the horticultural area of Laplanta, Argentina, naturally infected with *Fusarium* spp. and *Sclerotium* (Corticum) *rolfsii*. All 3 species of *Trichoderma* were effective against *Fusarium* spp. and *Corticium rolfsii* in vitro and in subsequent field trials. Seedling emergence was significantly increased when *Trichoderma harzianum* were applied to seeds sown in soil infected with the pathogens. They also reported that each *Trichoderma* spp. was effective against *C. rolfsii*.

Haque *et al*. (1990) used *Trichoderma harzianum* as bio-control agent for controlling root rot diseases of okra, sunflower, soybean and mungbean.

Trichoderma harzianum used as seed treatments or as soil drenches for the control of root rot caused by *Macrophomina phaseolina, Rhizoctonia solani* of sunflower, soybean and *Vigna radiate* under field conditions. *Trichoderma* showed excellent inhibitory effect of controlling *Fusarium* and *Rhizoctonia*.

Kumar and Khare (1990) found *Trichoderma harzianum* as antagonistic to *Sclerotium rolfsii* when soybean seed were treated with *Trichoderma harzianum*, *Gliocladium virens, Bacillus subtilis* and *Streptomyces* spp. They also showed that Fusarium infection of sunflower was reduced by *Trichoderma harzianum*.

Krishnamoorthy and Bhoskaran (1990) reported that soil inoculation with *Trichoderma harzianum* and *Trichoderma viride* gave good control of *Sclerotium rolfsii* and in treated pots gave 78.2% and 72.2% egg plant seed germination respectively compared to 19.3% in the control.

Harman *et al*. (1989) reported on combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatments. They developed progeny strains (T12 and T95) by fusing two strains of *Trichoderma harzianum* and two of which were selected for further study. Seeds of cotton, cucumber, pea, snap bean, maize and wheat were also planted in soil infested with *Pythium ultimum* and *Rhizoctonia solani*. In all crop pathogen combinations, seed treatments with parental and progeny *Trichoderma* strs with or without solid matrix priming increased stands relative to the untreated control and were as effective as vitavax-200 (Carboxin + Thiram).

Shin *et al*. (1987) found that soil treated with *T. viride* reduces damping off of sesame seedlings. The sesame seedlings on beds treated with the antagonist grew better than seedlings in untreated soil. Soil and seed treated with *T. viride* reduced sunflowers infection (*S. sclerotiorum* and *B. cinerea*) in the glasshouse and prevented infection also in the field.

Jabos and Kamoen (1986) found that *Trichoderma harzianum* produced cell wall lysine enzymes which developed antagonism against plant pathogens and improved biological control.

Sivan and Chet (1986) prepared wheat bran / peat mixture (1:1v/v) adjusted to 40% moisture (w/w) autoclaved for 1hr at 1210C. The substrate mixture was inoculated with a conidial suspension of *Trichoderma* and incubated in an illuminated chamber at 30^0C. This preparation of *Trichoderma* was mixed with soil (5g/kg soil) before sowing seeds of the test plants.

Strashnow *et al*. (1985) reported that application of *T. harzianum* to soil or by coating tomato fruits was found to reduce *R. solani* fruit rot by up to 43% and 85% respectively under laboratory conditions. When it was mixed with naturally infested soil. *T. harzianum* reduced the *R. solani* inoculum potential of soil by 86% and fruit rot by 27-51%).

Sivan *et al*. (1984) used wheat bran/peat preparation of *Trichoderma harzianum* mixed with loamy sand (5g/kg soil) artificially infested with *Pythium aphanidermatum* significantly reduced disease incidence caused by this pathogen in cucumber, pea and tomato at 69,81 and 85% respectively.

Mirkova (1982) studied the antagonistic activity of *Trichoderma* spp. against some pathogens and reported that among 5 *Trichoderma* spp, 3 isolates of *Trichoderma harzianum* were most antagonistic.

Elad *et al*. (1982) studied on the prevention of plant infection by biological means. *Trichoderma harzianum* isolated from the soil showed on antibiotic activity against *Sclerotium rolfsii* when grown on cell walls of the pathogens. It produced extra cellular B (1-3) glucose and chitinase when applied in the form of wheat bran culture to soil infested with *Sclerotium rolfsii* in the glass house. *Trichoderma harzianum* effectively controlled damping off of eggplant.

Agrawal *et al*. (1977) found filtrates of *Trichoderma* inhibited the growth of *Sclerotium rolfsii* on PDA, in pot trial the antagonist controlled seedling death. Culture was more effective when applied to seed rather than soil.

Wells *et al*. (1972) found *Trichoderma harzianum* pathogenic to *Sclerotium rolfsii* in agar medium. They reported *Trichoderma harzianum* to effectively control *Sclerotium rolfsii* on peanuts, tomatoes and blue lupins under greenhouse condition and also under greenhouse condition and also under field condition when applied (1-3 times) over the plants on to the soil surfaces.

Chapter III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

In this chapter, the details of different materials used and methodology followed during the experimental period are described.

3.1 Experimental Site

The experiments were conducted at the central laboratory as well as in the nursery house, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, 1207. A temporary made betel vine boraj was used for evaluation of formulated *Trichoderma harzianum*.

3.2 Experimental Period

The experiments were carried out during the period from September 2014 to December 2014.

3.3 Isolation of *Trichoderma*

Trichoderma was isolated from rhizosphere soil of betel vine from four different Districts (Satkhira, Jessore, Barisal and Rangpur) in Bangladesh by soil dilution technique.

Trichoderma was isolated by dilution plate technique (Dhingra and Sinclair, 1985) as described below:

i. Disinfection of working area

Since the bacteria and fungi are always present as contaminants in the soil, it is important to exclude them as much as possible from the surface of the working area and the equipment to be used. The surface of the working area was disinfected with cotton soaked in methylated spirit (70%). The hands and equipments were disinfected by the same means. The glass wares (test tubes, petri dishes, pipettes, beakers etc.) were sterilized in dry oven.

ii. Preparation of working samples

For every soil samples, working sample was prepared from the composite sample that was made in immediate after collection of sample from rhizosphere root of betel vine.

iii. Making suspension (soil dilution)

a. 1gm of the soil was taken in test tube containing 9 ml of sterile water and stirred thoroughly for few minutes in order to obtain an uniform 1:10 soil suspension. This solution was used as stock suspension.
b. 1ml of that 1:10 stock suspension was transferred with the help of sterile pipette into the 2nd test tube containing 9 ml sterile water and shaken thoroughly to make 10^{-1}.
c. 1ml of the 10^{-1} dilution is transferred to 3^{rd} test tube containing 9 ml sterile water by sterile pipette to make 10^{-2} dilution. In this way dilution was made up to 10^{-4}

iv. Isolation of micro-organisms (*Trichoderma* sp.) from soil

a. 20 ml of warm (approx. 45^{o}C) melted PDA medium was poured in each sterile petri-plate.
b. 1 ml of diluted soil sample (10^{-4}) was placed at the center of PDA and spreaded. Four petri-dishes were inoculated with 1 ml of each diluted sample.
c. The inoculated PDA plates were incubated for 7-10 days at room temperature (25 ± 1^{0}C).
d. The colonies grown out on PDA were recorded after 3-5 days of incubation. Sub cultures were made by transferring a small colony to a new petri-dish on the basis of color and morphology of the colony. Further recultures were made for purification. The contaminated plates were discarded.

(a) TSD - *Trichoderma harzianum*, Sher-e-Bangla Nagar, Dhaka

(b) Conidia and mycelium of *Trichoderma harzianum*

Plate 1. Isolates of *Trichoderma harzianum* (a) TSD - *Trichoderma harzianum*, Sher-e-Bangla Nagar, Dhaka and (b) Conidia and mycelium of *Trichoderma harzianum*

3.4 Collection of peat soil

Peat soil was collected from Tungipara, Gopalgonj, Bangladesh.

3.5 Collection of substrates

Substrates were collected from local shops of Kawran Bazar, Dhaka. Substrates were kept in 4^0 C until use.

3.6 Variety used

Bangla paan variety was used in the experiment.

3.7 Treatments of the experiment

All together 8 peat soil based substrates along with a control were explored in the experiment stated below:

1. Peat soil + Rice bran + Water (1:1:2)
2. Peat soil + Wheat bran + Water (1:1:2)
3. Peat soil + Lentil bran + Water (1:1:2)
4. Peat soil + Gram bran + Water (1:1:2)
5. Peat soil + Black gram bran + Water (1:1:2)
6. Peat soil + Mustard oil cake + Water (1:1:2)
7. Peat soil + Grass pea bran + Water (1:1:2)
8. Peat soil + Saw dust + Water (1:1:2)
9. Control

3.8 Sterilization of Substrates and Inoculation of *Trichoderma* in the multiplication

The requisite amount of materials for each substrate was thoroughly mixed in a 1000 ml Erlenmeyer flask and autoclaved at 121^0C for 15 minutes for sterilization. The sterilized substrates allowed to cool down and then inoculated with 5 mm dia mycelia disc of 7 days old *Trichoderma* culture. Seven discs for each flask were used for inoculation. Inoculated flasks were then incubated at room temperature $(25\pm2)^0$C.

a) Sterilized substrates before inoculation

b) Sterilized substrates after 15 days of inoculation

Plate 2. Mass multiplication of *Trichoderma* in different substrates (a) sterilized substrates before inoculation (b) Sterilized substrates after 15 days of inoculation

3.9 Formulation and measurement of spore/g substrate

After incubation for 25 days, the contents were taken out from the flasks, air dried in laminar airflow cabinet and grinded in a blender. The grinded materials were kept in polythene bag with labeling and treated as formulated *Trichoderma*. The spores of *Trichoderma* per gram of formulated products were measured by Haemocytometer. The number of conidia per plate was determined with the help of Haemocytometer following the procedure of Ashrafuzzaman (1976).

3.10 Collection/isolation and Maintenance of *Sclerotium rolfsii*

The pathogen was isolated from naturally infected betel vine plant grown in the betel vine boroj of the Department of Plant Pathology, SAU, Dhaka. The typical foot root rot symptoms of betel vine plant was still alive with pale green, and reduced sized leaves. Numbers of round brown to black sclerotia were found.

The infected tissue of the collar region of the plant was collected and repeatedly washed in fresh water and surface was sterilized with 10% Clorox for 1 minute followed by three times washing in distilled water.

Then the pieces of infected tissue were placed on PDA acidified with one drop of 5% lactic acid and inoculated at 22± 2ºC for 7days. After incubation, white mycelia and sclerotia were formed (Plate 3). The pathogen was purified and multiplied subsequently through hyphal tip culture on PDA, for preparation of inocula.

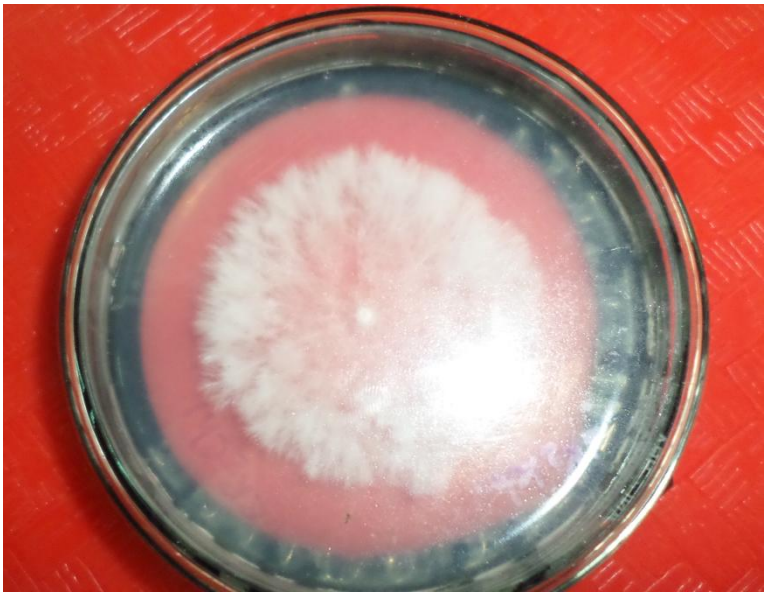


Plate 3. Culture of *Sclerotium rolfsii* (5 days old)

3.11 Preparation of inocula of *Sclerotium rolfsii*

Barley culture method was followed to culture and multiply *Sclerotium rolfsii*. Inocula of *Sclerotium rolfsii* were prepared in barley culture. Barley grains collected from market were throughly washed in water and kept soaked in fresh water for 24hrs. After decantation, barley grains were taken in 500 ml Erlenmeyer flask at the rate of 200g in each. The flasks were plugged with cotton followed by wrapping the mouth with brown paper. The flasks containing moist barley grains were sterilized in autoclave at 121ºC under 15lbs pressure for 15 minutes. The sterilized barley grains in the flask were cooled and inoculated aseptically with mycelial blocks (5mm) of pure culture of *Sclerotium rolfsii* on PDA and inoculated at room temperature for 7-8 days. The flasks were shaken periodically with hand for proper distribution of fungal mycelium throughout the entire mass of the inoculated barley grains (Plate 4).

The mycelial growth of the fungus covered entire barley mass in the flask when small round white sclerotia started to form. It was taken out of the flask after

fifteen days. The entire mass was spread on brown paper and air dried at room temperature. The colonized dried barley grains were used as inocula for inoculation of plants (Plate 5) (Babar, 1999).

Plate 4. Air drying of barley culture of *Sclerotium rolfsii*

Plate 5. Barley culture of *Sclerotium rolfsii* after inoculation of 15 days

3.12 Inoculation of betel vine with the *Sclerotium rolfsii*

Betel vine plants were individually inoculated by adding 10 grams of burley culture of *Sclerotium rolfsii* near the plant base and covered with soil. The inoculated root zone was kept moist by adding water as required.

Plate 6: Inoculation of betel vine with *Sclerotium rolfsii* at the base of plant

3.13 Application of *Trichoderma* based bio-pesticide

A 5×7 m^2 sized established boroj was selected for working area that was prepared in the nursery of the Department of Plant Pathology Sher-e-Bangla Agricultural University (SAU). *Sclerotium rolfsii* was inoculated in the field and also *Trichoderma* based bio-pesticide was used for the management of *Sclerotium rolfsii*.

3.14 Data collection

Data were recorded on the following parameters

1. % Disease incidence
2. % Disease severity

3.15 Assessment of disease incidence and severity

Disease incidence was calculated by the following formula:

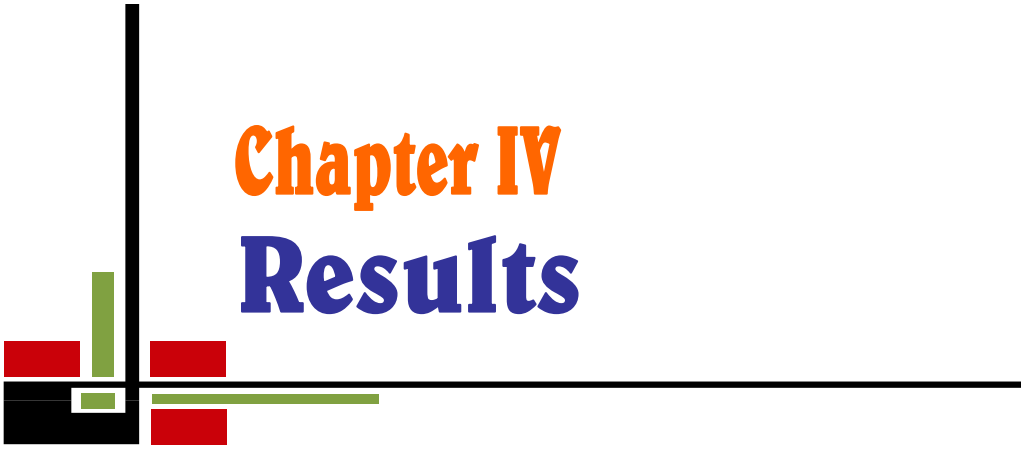
$$\% \text{ Disease incidence} = \frac{\text{Number of diseased plants}}{\text{Number of total plant inspected}} \times 100$$

Disease severity was calculated using the following formula:

$$\% \text{ Disease severity} = \frac{\text{Area of stem tissue infected}}{\text{Total stem area inspected}} \times 100$$

3.16 Statistical analysis of data

Completely Randomized Design (CRD) was followed for the laboratory and nursery experiment. The data was statistically analyzed by using computer package program (MSTAT-C). The significant difference of the treatment means was compared by Duncan's Multiple Range Test (DMRT).



Chapter IV
Results

CHAPTER IV

RESULTS

The experiment was conducted for the formulation of *Trichoderma* based biopasticides in controling foot and root rot of betelvine. The results obtained from the present study on the effect of nine different treatments *viz*. T_1 (*Trichoderma* + Peat soil + Rice bran + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water), T_4 (*Trichoderma* + Peat soil + Gram bran + Water), T_5 (*Trichoderma* + Peat soil + Black gram bran + Water), T_6 (*Trichoderma* + Peat soil + Mustard oil cake + Water), T_7 (*Trichoderma* + Peat soil + Grass pea bran + Water), T_8 (*Trichoderma* + Peat soil + Saw dust + Water), T_9 (Control) for the management of foot and root rot of betel vine seedlings. The results of the present investigation have been presented in this chapter.

4.1 Number of spore/mm^2 (×10^4) in different *Trichoderma* isolates

Nubmer of spores in different *Trichoderma harzianum* isolates was presented in Table 1. The highest number of spore/mm^2 was observed in isolate TS = *Trichoderma harzianum*, Satkhira (5.38×10^4) followed by isolate TJ = *Trichoderma harzianum*, Jessore (4.68×10^4). The lowest number of spore/mm^2 was observed in isolate TR = *Trichoderma harzianum*, Rangpur (4.18×104) preceded by isolate TB = *Trichoderma harzianum*, Barisal (5.30×104). Based on the higher spore production isolate TS = *Trichoderma harzianum*, Satkhira was considered to make the subsequent formulation.

Table 1. Inoculum potential (spore/mm^2 *Trichoderma* Isolate) by different *Trichoderma* isolates assayed in the experiment

Trichoderma Isolate	No. of spore/mm^2($\times 10^4$)
TS	5.38 a
TJ	4.68 b
TB	4.36 c
TR	4.18 c
$LSD_{(0.05)}$	0.34
CV (%)	4.12

TS = *Trichoderma harzianum*, Satkhira

TJ = *Trichoderma harzianum*, Jessore

TB = *Trichoderma harzianum*, Barisal

TR = *Trichoderma harzianum*, Rangpur

4.2 Effect of different substrates on mass multiplication of *Trichoderma harzianum*

Inoculum potentials in respect of number of spore of *Trichoderma harzianum* in different treatments was presented in Table 2. All the treatments differed significantly in terms of number of spores of *Trichoderma harzianum*. The highest number of spore/gm (25.18×10^7) was observed in T_1 where black gram bran and peat soil were mixed with water at 1:1:2 ratio followed by T_2 (23.12×10^7) where grass pea bran was mixed with peat soil and water at 1:1:2 ratio. The third highest number of spore/gm (21.23×10^7) was observed in T_3 (Gram bran + Peat soil + Water, ratio - 1:1:2) followed by treatment T_4 (Wheat bran + Peat soil + Water, ratio - 1:1:2) (19.57×10^7). The lowest number of spore/gm was observed in T_8 (Saw Dust + Peat soil + Water, ratio - 1:1:2) (10.23×10^7) preceded by treatment T_7 (16.44×10^7). In case of T_5 and T_6 number of spore/gm were 19.33×10^7 and 17.26×10^7.

Table 2. Effect of different substrates on mass multiplication of *Trichoderma harzianum*

Treatment	No. of spore/gm ($\times 10^7$)
T_1	25.18 a
T_2	23.12 b
T_3	20.68 c
T_4	19.57 d
T_5	19.33 d
T_6	17.26 e
T_7	16.44 f
T_8	10.47 g
$LSD_{(0.05)}$	0.44
CV (%)	3.29

T_1= *Trichoderma* + Black gram bran + Peat soil + Water

T_2= *Trichoderma* + Grass pea bran + Peat soil + Water

T_3 = *Trichoderma* + Gram bran + Peat soil + Water

T_4 = *Trichoderma* + Wheat bran + Peat soil + Water

T_5 = *Trichoderma* + Rice bran + Peat soil + Water

T_6 = *Trichoderma* + Lentil bran + Peat soil + Water

T_7 = *Trichoderma* + Mustard oil cake + Peat soil + Water

T_8 = *Trichoderma* + Saw dust + Peat soil + Water

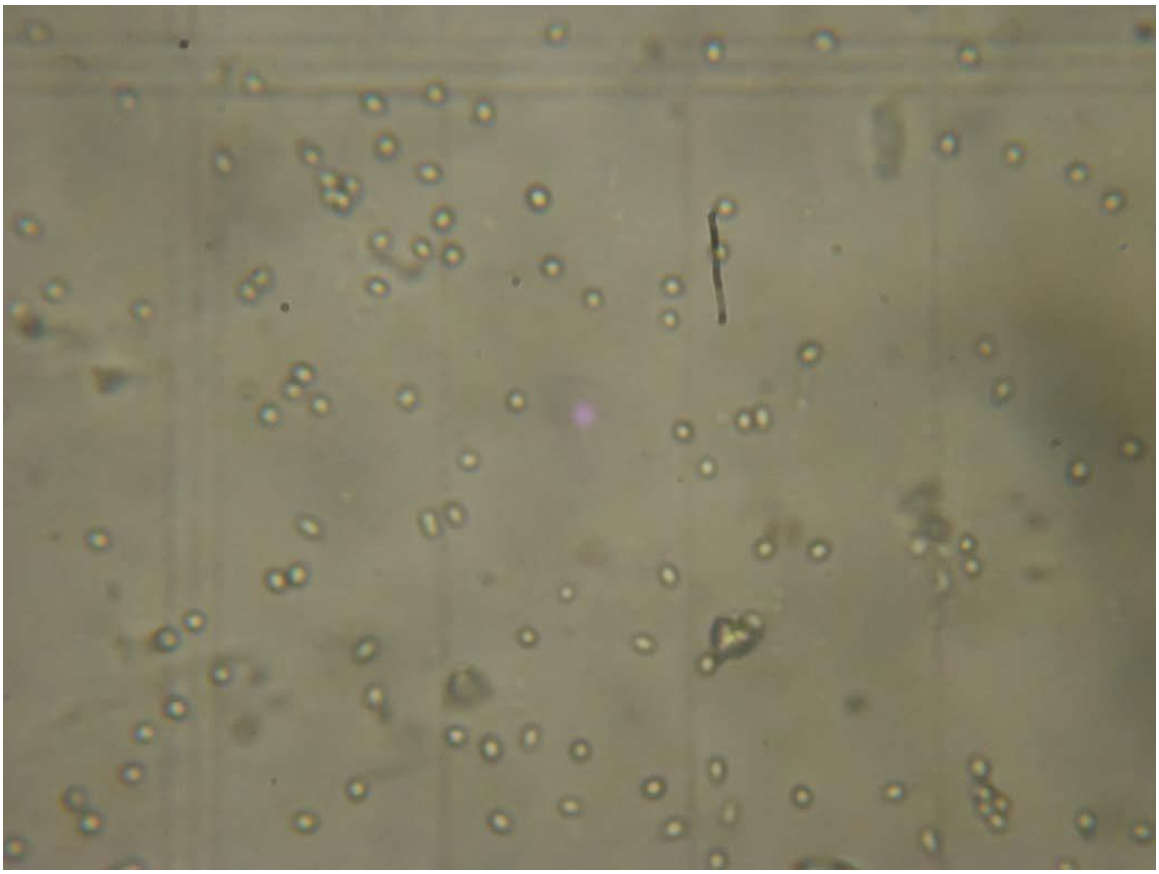


Plate 7. Spores of *Trichoderma* isolate TS (*Trichoderma harzianum*, Satkhira) in treatment T_5 (*Trichoderma* + Rice bran + Peat soil + Water) in haemocytometer observed under compound microscope

4.3 Evaluation of formulated *Trichoderma harzianum* based bio-pesticides in controlling foot and root rot of betel vine

Trichoderma isolate (TS - *Trichoderma harzianum*, Satkhira) cultured on different substrates were evaluated against *Sclerotium rolfsii* for controlling foot and root rot of betel vine. The peat soil based substrates found promising in production of spores viz., T_1 (*Trichoderma* + Black gram bran + Peat soil + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water), T_4 (*Trichoderma* + Peat soil + Gram bran + Water) and T_5 (*Trichoderma* + Peat soil + Black gram bran + Water) were considered for evaluation. The selected formulated *Trichoderma* based bio-pesticide showed promising effect in controlling foot and root rot of betel vine compared to control. No betel vine plant were died while applied *Trichoderma* based bio-pesticide even after 10 days of inoculation whereas 100% plant were died even after 7 days after inoculation in control treatment.

Table 3. Effect of *Trichoderma* based bio-pesticide in controlling *Sclerotium rolfsii*.

Treatment	% Plant died	
	7 DAI	10 DAI
T_1	Nil	Nil
T_2	Nil	Nil
T_3	Nil	Nil
T_4	Nil	Nil
T_5	Nil	Nil
T_9 (Control)	100	100

* DAI = Days after inoculation

T_1 = *Trichoderma* + Peat soil + Rice bran + Water

T_2 = *Trichoderma* + Peat soil + Wheat bran + Water

T_3 = *Trichoderma* + Peat soil + Lentil bran + Water

T_4 = *Trichoderma* + Peat soil + Gram bran + Water

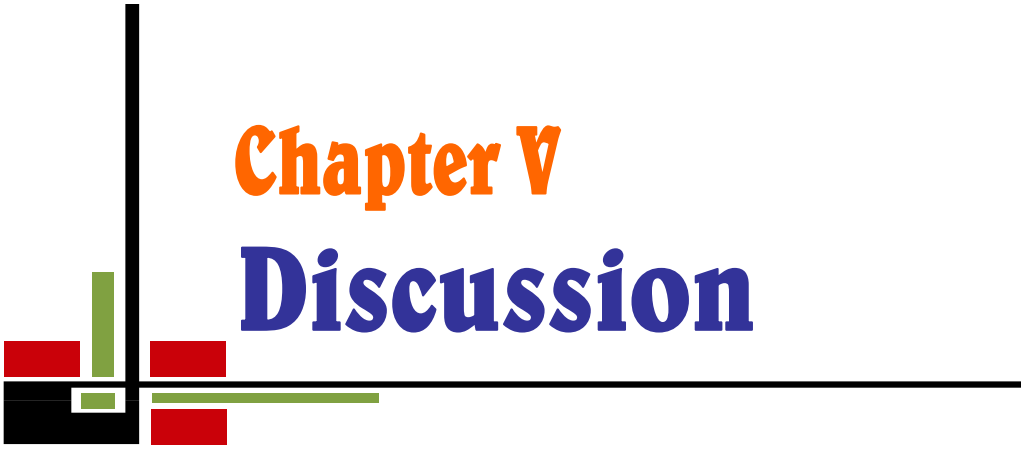
T_5 = *Trichoderma* + Peat soil + Black gram bran + Water

T_9 = Control

(a) Damage of betel vine plants in control treatment

(b) Non-affected plant with different *Trichoderma* based treatments

Plate 8. Field condition of betel vine showing (a) Damage of betel vine plants in control treatment and (b) Non-affected plant with different *Trichoderma* based treatments



Chapter V
Discussion

CHAPTER V

DISCUSSION

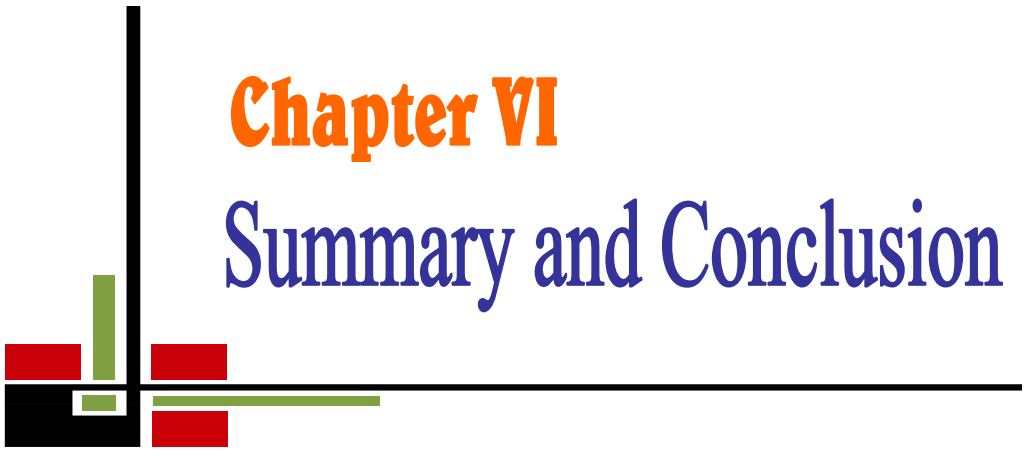
The present study was carried out with nine different treatments of *Trichoderma* based substrates *viz*., T_1 (*Trichoderma* + Peat soil + Rice bran + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water), T_4 (*Trichoderma* + Peat soil + Gram bran + Water), T_5 (*Trichoderma* + Peat soil + Black gram bran + Water), T_6 (*Trichoderma* + Peat soil + Mustard oil cake + Water), T_7 (*Trichoderma* + Peat soil + Grass pea bran + Water), T_8 (*Trichoderma* + Peat soil + Saw dust + Water), T_9 (Control) were evaluated for sporulations of *Trichoderma harzianum* and acting against *Sclerotium rolfsii* for the management of foot and root rot of betel vine plants.

Different isolates of *Trichoderma* evaluated in the experiment found to be differed in terms of number of spore production. The highest number of spore ($6.42\times10^4/mm^2$) was observed in isolate TS = *Trichoderma harzianum*, Satkhira that was used in the substrate to construct the treatment combinations. In mass multiplication of *Trichoderma* isolate TS = *Trichoderma harzianum*, Satkhira black gram brans combined with peat soil and water (1:1:2 w/w/v) proved to be suitable substrate for producing the highest CFU/g of *Trichoderma* sp. Earlier studies support the results of the present findings (Shamsuzzaman *et al*., 2003a, Islam, 2005). Shamsuzzaman *et al*., 2003 reported that the highest production of conidia ($42.93 \times 10^7/g$) was recorded in black gram based substrates while used for mass multiplication of *Trichoderma* isolates. Several other workers also used different agro-products for multiplication of *Trichoderma* sp. but they did not include peat soil based black gram brans in their study (Rettinassababady *et al*., 2000, Shamarao *et al*., 1998).

The peat soil based *Trichoderma* formulations were evaluated in the nursery house against *Sclerotium rolfsii* for the management of foot and root rot of betel vine. *Trichoderma* formulations prepared on peat soil based substrates showed

promising effect against *Sclerotium rolfsii* in controlling foot and root rot of betel vine. No plants were affected by *Sclerotium rolfsii* while applied *Trichoderma* formulation prepared on peat soil based different substrates. Hundred percent plant were died in case of control treatment.

A number of successful bio-control products based on different species of *Trichoderma* have been commercialized in India, USA and elsewhere in the world. Kashem *et al*. (2011) assessed 14 isolates of *Trichoderma* spp. (*Trichoderma harzianum* and *T. viride*) for controlling foot and root rot of lentil caused by *Fusarium oxysporum*. *Trichoderma* isolates inhibited the growth of *F. oxysporum* up to 92.07 % on agar plates. Pandya *et al*. (2011) reported that *Trichoderma* as a bio-control agent (BCAs) is well recognized due to their high reproductive capability and show strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms. Amin *et al*. (2010) made six isolates of *Trichoderma* spp. to inhibit soil borne pathogens of different vegetables viz., *Rhizoctonia solani* (isolates from tomato), *Sclerotium rolfsii* (causing collar rot of tomato) and *Sclerotinia sclerotiorum* under in vitro conditions. Dual culture of pathogens and *Trichoderma* spp. revealed that *T. viride* highly inhibited (65.71%) mycelial growth of *Rhizoctonia solani* over control. In case of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, *T. viride* proved to be potential inhibiting mycelial growth of that pathogen.



Chapter VI

Summary and Conclusion

CHAPTER VI

SUMMARY AND CONCLUSION

The experiments were conducted in the laboratory as well as in the nursery house of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, during the period from September 2014 to December 2014. The experiment was carried out with nine different treatments viz. T_1 (*Trichoderma* + Peat soil + Rice bran + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water), T_4 (*Trichoderma* + Peat soil + Gram bran + Water), T_5 (*Trichoderma* + Peat soil + Black gram bran + Water), T_6 (*Trichoderma* + Peat soil + Mustard oil cake + Water), T_7 (*Trichoderma* + Peat soil + Grass pea bran + Water), T_8 (*Trichoderma* + Peat soil + Saw dust + Water) and T_9 (Control). Collected data on different parameters were analyzed by using MSTAT statistical package program.

Trichoderma harzianum was isolated from different agro-ecological areas of Bangladesh. Among them TS = *Trichoderma harzianum,* Satkhira was the best isolate in respect of number spore production (6.42×10^4). Different substrates were used with peat soil and water at a ratio (1:1:2) to know the inoculum potentiality in respect of number spore of *Trichoderma harzianum*. The highest number of spore/gm (24.27×10^7) was observed in (Black gram bran + peat soil + water, at ratio - 1:1:2).

The peat soil based *Trichoderma* formulations were evaluated in the nursery house against *Sclerotium rolfsii* for the management of foot and root rot of betel vine. The data recorded on percent (%) disease incidence and severity.

Trichoderma isolate; TS = *Trichoderma harzianum,* Satkhira cultured on different substrates were evaluated against *Sclerotium rolfsii* for controlling foot and root rot of betel vine. The peat soil based substrates found promising in production of spores viz., T_1 (*Trichoderma* + Black gram bran + Peat soil + Water), T_2 (*Trichoderma* + Peat soil + Wheat bran + Water), T_3 (*Trichoderma* + Peat soil + Lentil bran + Water),

T_4 (*Trichoderma* + Peat soil + Gram bran + Water) and T_5 (*Trichoderma* + Peat soil + Black gram bran + Water) were considered for evaluation. The selected formulated *Trichoderma* based bio-pesticide showed promising effect in controlling foot and root rot of betel vine compared to control. No betel vine plant were died while applied *Trichoderma* based bio-pesticide even after 10 days of inoculation whereas 100% plant were died even after 7 days after inoculation in control treatment.

From the present experiment, it may be concluded that peat soil based substrates comprised with either black gram bran, grass pea bran or gram bran with water revealed promising in culture of *Trichoderma harzianum* for mass multiplication and these *Trichoderma* based bio-pesticides are effective in controlling foot and root rot of betel vine.

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Appendices

APPENDICES

Appendix 1. Composition of Potato Dextrose Agar (PDA) Media: The compositions of the media used in this thesis work are given below: This media were autoclaved at 121Oc for 15 minutes at 15 lb pressure.

Ingredients	g/L
Peeled Potato	200g
Dextrose	20g
Agar	17g
Water	1000ml

Appendix 2. Market prices of different substrates: Prices of different substrates used in this thesis work are given below:

Substrates	Price (Tk/kg)
Rice bran	15
Wheat bran	15
Lentil bran	16
Gram bran	15
Black gram bran	18
Grass pea bran	20
Mustard oil cake	22
Saw dust	15