IN VITRO MANAGEMENT OF FOOT AND ROOT ROT OF BETEL VINE (*Piper betle* L.) INCITED BY *Sclerotium rolfsii*

A THESIS

BY

Shipra Mondol

STUDENT ID. MS-170802

A Thesis (Course No. PLP-6104) Submitted to Agrotechnology Discipline in Partial Fulfillment of the Requirements for the Degree of

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IN

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Approved as to Style and contents

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DEDICATED TO

MY BELOVED PARENTS

ABSTRACT

Betel vine (*Piper betle* L.) is largely grown as an important cash crop throughout the tropical and subtropical regions in Bangladesh. Disease damage to the crop is one of several known limiting factors. Foot and root rot of betel vine caused by *Sclerotium rolfsii* is the most overwhelming disease which decreases the production of betel leaf to a great extent. The present study was carried out in the Plant Protection Laboratory of Agrotechnology Discipline, Khulna University, Khulna to evaluate *in vitro* the efficacy of different fungicides, botanicals, antagonist microorganisms and organic amendments against *Sclerotium rolfsii*. To fulfill the objectives four fungicides, four plant extracts/botanicals, two bioagents and four organic amendments at different concentration was considered against *Sclerotium rolfsii*, causal agent of foot and root rot disease of betel vine. The experiment was laid out in Completely Randomized Design (CRD) with four replications. Radial growth and formation of sclerotia were recorded. Radial growth inhibition was calculated thereafter. In all the cases, the growth inhibition was found to be increased with the increase of concentration against *Sclerotium rolfsii*. Four fungicides *viz.*, Unisaaf 75WP, Dithane M-45, Exzole 50 SC and Provax-200 at different concentrations (100, 150, 200, 250 and 500 ppm), were tested. Among the tested fungicides, Exzole 50 SC and Provax-200 showed complete growth inhibition of *S. rolfsii* at all the concentrations and consequently no sclerotia were formed. Four botanicals viz., Neem leaf, Tulsi leaf, Marigold leaf and Allamanda leaf extracts at different concentrations (10, 15, 20, 25 and 30%), were tested. Neem leaf extracts at 30% concentration showed highest percent inhibition (63.20%) and lower number of sclerotia formation (200.00) followed by tulsi leaf extract, marigold leaf and allamanda leaf extracts respectively. Two antagonist microorganisms *T. harzianum* and *Bacillus subtilis* strain (ATCC 6633) was evaluated against *S. rolfsii*. *T. harzianum* showed promising effect against mycelial growth inhibition (88.91%) of *S. rolfsii* and subsequently no sclerotia was formed. Secondary metabolites of *Bacillus subtilis* strain (ATCC 6633) was evaluated at different concentrations (1:2, 1:4 and 1:6) against *S. rolfsii*. Secondary metabolites at 1:6 concentrations showed highest percent of mycelial growth inhibition (89.54%) and consequently lowest number (34.00) of sclerotia was formed. Four organic amendments viz., Neem cake, Wood ash, Mustard cake and Coconut cake at different concentrations (10, 15, 20, 25 and 30%), were tested. All tested amendments at 30% concentrations showed highest percent inhibition and lower number of sclerotia formation.

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ACRONYMS

BARI	: Bangladesh Agricultural Research Institute
ppm	: Parts per million
et al.	And others
i.e.	: that means
CRD	: Completely Randomized Design
° C	: Degree Celsius
%	: Percent
CV (%)	: Percentage of coefficient of variance
viz.	: Videlicet (namely)

CHAPTER I

INTRODUCTION

Disease damage to the crop is one of several known limiting factors. The betel vine is highly susceptible to diseases, pests and natural climates (Sayeeduzzaman, 1988). Humid and moist shaded conditions are favorable for betel vine growth and also favor a variety of root and foliage disease development (Goswami et al., 2002). Betel vine is subjected to a number of diseases. Among the diseases of betel vine, foot and root rot caused by *Sclerotium rolfsii* is the most devastating diseases which decrease the production of betel vine to a great extent. In 2004, sixty percent betel vine was damaged due to foot rot disease in three upazillas of Rajshahi districts (Islam, 2005).

Sclerotium rolfsii 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 omni-pathogenic 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). The pathogen infects the vine resulting quality of leaf reduces and for which the farmers are deprived of usual market price.

As the fungus is soil inhabitant and omni-pathogenic, so it is very difficult to control. Of the various methods like as chemicals, botanicals biological and organic were used to control *S. rolfsii*, but the use of chemical fungicides is very common and effective to control *S. rolfsii* (Johnson and Subramanyam 2000; Palaiah, 2002).

Scientists are now searching for natural products, instead of the agro-chemicals, for the management of fungal diseases in plants. Biological control could be successful alternative to control the pathogens. Different biological control methods such as the use of different plant extracts, antagonist microorganisms and organic amendments etc are becoming popular to control *S. rolfsii* (Kanwal et al., 2011and Javaid et al., 2012).

Plant extracts are biologically degradable (Devlin and Zettel, 1999) and their application in crop protection is a nearly sustainable substitute. Plant extracts and their essential oils were found to be effective against a wide range of fungi (Abd-Alla et al., 2001). Yield and quality of betel leaf can also be increased through proper nutrient management (Fahim et al., 2017) by using organic amendment.

Biocontrol agent *Trichoderma* spp. was found to be more effective against different sclerotia forming fungi (Khalid, 2013). *B. subtilis* is also the antagonist microorganism in the control of *S. rolfsii*, recorded sufficient antagonism due to its good inhibitory performance (Rajkumar et al., 2018). Botanical and biological control measures are unique and easily available for the farmers and small industries.

Considering the above facts, the present study has therefore, been undertaken with the following objectives:

- To evaluate *in vitro* the efficacy of different fungicides, plant extracts/botanicals, antagonist microorganisms and organic amendments against *S. rolfsii*

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II
REVIEW OF LITERATURE

Betel vine (*Piper betle* L.) is prone to attack of many diseases at all stages of growth. In Bangladesh the diseases of betel vine were studied to a limited extent. Foot and root rot of betel vine caused by *S. rolfsii* is an omnivorous, soil borne fungal pathogen, causes disease on a wide range of agricultural and horticultural crops and very much difficult to control. So, it is very essential to work on it to find out an effective measure to control this pathogen which will reduce the cultivation cost ensuring sound environment.

In this chapter attempt made to review the available literature about the effect of some chemical fungicides, botanical extracts, antagonist microorganisms and organic amendments on the mycelial growth and sclerotia formation of *S. rolfsii* are given below.

2.1. *In Vitro* Evaluation of Fungicides against *S. rolfsii*

There are several published reports in controlling *S. rolfsii* of different crops by the application of fungicides. Some of the important ones are listed here.

Mundhe (2005) studied *in vitro* the efficacy of fungicides against *S. rolfsii* causing foot rot of nagli. They reported that the fungicides *viz.*, Metalaxyl + Mancozeb (0.1%), Difenconazole (0.05 %), Propiconazole (0.05%), Hexaconazole (0.05%) and Metiram (0.1%) caused 100 percent inhibition of mycelial growth of the test fungus. Rest of the fungicides caused inhibition in the range of 31.88 to 81.55 per cent, except Carbendazim, copper hydroxide and Thiophanate methyl where the inhibition was only 3.77, 7.77 and 17.11 per cent, respectively.

Yaqub and Shahzad (2006) evaluated *in vitro* six fungicides *viz.*, Benomyl, Mancozeb, Dhiovit, Dithane M-45, Carbendazim and Topsin-m against *S. rolfsii*. They reported Mancozeb as most effective with 60 percent reduction in growth of *S. rolfsii* (@ 100 ppm), followed by Dithane M-45 @ 100 ppm (more than 50 % reduction). Benomyl and Carbendazim caused more than 50 per cent reduction @ 1000 ppm.

Patil and Raut (2008) tested *in vitro* efficacy of 11 fungicides and one antibiotic against *S. rolfsii* causing collar rot of betelvine. They reported that the fungicides *viz.*, Metalaxyl + Mancozeb (1000 ppm), Difenconazole (500 ppm), Propiconazole (500 ppm), Hexaconazole (500 ppm) and Metiram (1000 ppm) caused 100 per cent inhibition of the test pathogen, followed by Chlorothalonil (81.55 %), Propineb (80.44 *%)* and antibiotic Kasugamycin (59.33 %). Fungicides *viz.*, Carbendazim, Copperoxy chloride and Thiophanate methyl were least effective against the test pathogen.

Singh et al. (2008) examined *in vitro* 11 fungicides, one antibiotic and three botanical extracts (bio-pesticides) against *Sclerotinia sclerotiorum* causing blight of brinjal. They reported that the fungicides *viz.*, Vitavax, Bavistin, Copperoxy chloride and Topsin- M caused 100 per cent inhibition of mycelial growth of the test pathogen. This was followed by the biopesticides Nimbicidine / *A. indica* (97.32 %), fungicides Chlorothalonil (95.00 %), Kitazin (90.10 %), Zineb (89. 93 %), Captafol (85.87 %), botanical, garlic bulb extract (82.26 *%)* and antibiotic Streptocycline (75.38%).

Bindu and Bhattiprohi (2011) evaluated *in vitro* the efficacy of nine fungicides at their recommended dosages against *S. rolfsii* causing dry root rot of chilli. They reported that of the fungicides tested, significantly highest mycelial growth inhibition (94.10 %) and sclerotial inhibition (94.10 %) were recorded by the fungicides Tebuconazole (1500 ppm) and Carbendazim + Dithane M-45 (2000 ppm). This was followed by the fungicides *viz.*,

Difenconazole @ 500 ppm (93.3 and 91.1 %), Hexaconazole @ 2000 ppm (84.4 and 91.5 %), Propineb @ 2000 ppm (92.60 and 88.50 %) and Propiconazole @ 1000 ppm (82.50 and 94.10 %) for mycelial growth inhibition and Sclerotial germination inhibition, respectively.

Bhuiyan et al. (2012) tested six fungicides namely Provax-200, Bavistin, Ridomil, Dithane M-45, Rovral 50 WP and Tilt were screened at 100, 200 and 400 ppm concentration for their efficacy against the radial colony growth of *S. rolfsii*. The complete inhibition was obtained with Provax-200 at all the selected concentrations. Complete inhibition also obtained at the highest concentration of Tilt. The highest concentration of Rovral 50WP inhibits 93.88% radial growth and significantly superior to Dithane M-45 at the highest concentration.

Rather et al. (2012) experienced *in vitro* nine fungicides against the pathogenic fungi *(S. rolfsii, F. oxysporum* and *R solani*) causing wilt complex disease in bell pepper (Simla mirch). They reported that of the fungicide tested *In vitro,* significantly highest mycelial growth inhibition in *S. rolfsii* was caused with the fungicides *viz.*, Captan @ 500 ppm (79.30 %), followed by Captan @ 250 ppm (74.80 %), Mancozeb + Metalaxyl @ 500 ppm (70.00 %), SAFF @ 500 ppm (68.80 %), Copper Oxychloride @ 100 ppm (64.4 %), Mancozeb @ 500 ppm (68.50 %) and Copper Oxychloride @ 500 ppm (61.80 %). Rests of the fungicides tested were found less effective with mycelia growth inhibition in the range of 12.60 to 41.10 per cent.

Khan and Javaid (2015) reported that four fungicides tegula (tebuconazole), thiophanate methyl, ridomil gold (metalaxyl+mancozeb) and mancozeb significantly inhibits the radial growth of *S. rolfsii* under *in vitro* condition. Besides it, two fungicides thiophanate methyl and mancozeb substantially control the growth of *S. rolfsii* under *in vivo* condition responsible for causing collar rot disease in Chickpea.

Sarita et al. (2018) evaluated four fungicides against *Sclerotium rolfsii* on Groundnut. Among the fungicides Tebuconazole and Hexaconazole inhibited mycelial growth maximum at both 500 and 750 ppm concentrations. In field condition the minimum per-cent incidence was found in Tebuconazole (250, 500 and 750 ppm) which is most effective treatment among all the treatments respectively.

2.2. *In Vitro* Evaluation of Plant Extracts/ Botanicals against *S. rolfsii*

During the last few years, a number of investigators isolated and identified several phytochemical compounds from leaves and seeds of many plant species as growth inhibitors (Jacobson et al., 1975; Menn, 1970). Some of the works regarding to the control of plant pathogenic fungi including *S. rolfsii* by botanical extracts are outlined here.

Suryawanshi et al. (2007) studied *(in vitro* and pot culture) six plant extracts against *S. rolfsii* infecting pigeon pea. They reported that all the aqueous leaf extracts (@ 10 %) significantly inhibited mycelial growth of the test pathogen; however, highest growth inhibition was recorded with neem (43.63 %), followed by eucalyptus (42.24 %), custard apple (36.36 %), castor (27.63 %) and parthenium (21.38 %). In pot culture studies, all the plant extracts tested significantly increased the seed germination and it was maximum with neem extract (88.33 %), followed by eucalyptus (85.00 %).

Patil and Rant (2008) considered *in vitro* seven botanicals aqueous extracts (each @ 10 %) against *S. rolfsii* causing collar rot of betel vine. They reported that the bulb extracts of *A. sativum* caused 100 percent inhibition of mycelial growth of the test pathogen, followed by *A. indica* (78.22 *%)* and C. *roseus* (77.55 %). The extracts of the botanicals *viz, A. cepa, O. sanctum* and *B. spectablis* were found least effective against the test pathogen.

Mundhe et al. (2009) evaluated *in vitro* the efficacy of plant extracts against *Sclerotium rolfsii,* causing foot rot of finger millet They reported that maximum mycelial growth inhibition was caused by Sarphagandha (87.77 %), followed by Neem (85.88 %) and Sadafu (83.33 %). However, Glyricidia (25.22 %), Tulsi (24.44 %), Karanj (14.11 %) and Bougainvillea (12.22 %) were less effective.

Mandhare and Suryawanshi (2009) evaluated *in vitro* the efficacy of botanical extracts (@ 10 %) against *R. bataticola* causing dry root rot of chickpea. They reported that the minimum mycelial growth and its maximum inhibition were caused by garlic (20 mm and 77.77 %), followed by neem (32 mm and 64.44 %). The extract of Tulsi, Nilgiri, Kanher and ginger were ineffective against the test pathogen. There was 100 and 87.50 per cent inhibition of *S. rolfsii* with the extracts of *A. indica* and *A. sativum*, respectively.

Sultana et al. (2012) evaluated *in vitro* four plant extracts (each @ 5, 10 and 20 %) against *S. rolfsii* causing root rot of chilli. They reported that of the botanicals tested, garlic bulb extract caused maximum mean mycelial growth inhibition (67.78 and 75.18 %), followed by neem extract (58.52 and 69.90 %), onion bulb extract (50.74 and 61.85 %) and ginger rhizome extract (48.52 and 53.33 %), respectively at 10 and 20 per cent concentrations.

Amin et al. (2013) tested the effect of different plants extracts and namely rhizome of turmeric, rhizome ginger, neem leaf, tobacco leaf, tobacco leaf extract in water, tobacco leaf extract in cow's urine, and cow's urine at different concentrations (70%, 60%, 50%, 40% and 30%) on the growth and sclerotia formation of *Sclerotium rolfsii*, causal agent of foot and root rot disease of betel vine. No growth of the tested fungi was observed in all concentrations of tobacco leaf extract in cow's urine, 70%, 60%, 50%, 40% concentration of cow's urine alone, and 70% and 60% concentration of tobacco leaf extracts in water and consequently no sclerotia were formed. In other concentration of tobacco leaf extracts in

water (50%, 40% and 30%) growth inhibitions were 92.7%, 69.87% and 47.28% respectively. Considerable growth inhibitions were observed in all concentrations of turmeric rhizome which were 47.16%, 50.04%, 55.97%, 56.43% and 57.13% at 30%, 40%, 50%, 60% and 70% concentrations respectively. Few sclerotia were formed in the plates treated with tobacco leaf extract in water at 50% and 40% (14 and 53 respectively). Less than 50% sclerotia were formed at all concentration of turmeric rhizome extract.

Butt et al. (2016) reported that two important indigenous plants like *Alstonia scholaris* and *Azadirachta indica* leaf extract were more effective against *S. rolfsii* under *in vitro* condition at different concentrations (1%, 2%, 3%, 4% and 5%). Different concentrations of *A. indica* leaf extract appreciably reduced the fungal biomass growth up to 76% as compared to the control. In the same manner, various concentrations of the leaf extract of *A. scholaris* significantly decreased fungal biomass up to 70% as compared to the control. Higher fungal growth was reduced by a 2% concentration of both plants.

2.3. *In Vitro* Evaluation of Antagonist Microorganisms against *S. rolfsii*

2.3.1. *In Vitro* Evaluation of *T. harzianum* against *S. rolfsii*

Manu et al. (2012) reported that maximum inhibition of mycelia growth (61.88%) was noticed in *T. harzianum,* followed by *T. viride* (57.77 %). *Pseudomonas fluorescens* (0.00 %) and *Bacillus subtillis* (0.00 %) did not show any inhibition of mycelial growth of *S. rolfsii.*

Patro and Madhuri (2013) found that *T. harzianum* inhibits mycelial growth of *S. rolfsii* under *In vitro* condition which cause foot rot in finger millet. A total of 5 bioagents were screened *In-vitro* against *Sclerotium rolfsii* causing foot of ragi. Among the bioagents *Trichoderma harzianum* – 2 isolate was found to be effective (82.11%) than other biogents.

Darvin et al. (2013) selected three species of *Trichoderma* (*T. viride, T. harzianum* and *T. longibrachiatum*) for inhibition of radial growth of *S. rolfsii. T. viride* and *T. harzianum* have highest radial growth inhibition and *T. longibrachiatum* has lowest radial growth inhibition of *S. rolfsii* using dual culture technique under *in vitro* condition.

Sab et al. (2014) studied eight bio-agents tested against *S. rolfsii* in dual culture technique, all bio-agents inhibit growth of *S. rolfsii* causal agent of collar rot of chickpea, but *Trichoderma harzianum* recorded maximum growth inhibition of *S.rolfsii,* compared to *Pseudomonas fluorescens* and *Bacillus subtilis. Trichoderma harzianum* and *Trichoderma viride* act as biocontrol agent and inhibits 53.8% to 83.1% growth of *Sclerotium rolfsii* and *Rhizoctonia solani.*

Swathi et al. (2015) reported that *T. harzianum* and *T. virens* were more active against *S. rolfsii* with 100% inhibition under *in vitro* condition.

Basumatary et al. (2015) have selected six fungal species viz. *Penicillium* sp, *Curvularia sp Aspergillus niger, Trichoderma harzianum, Trichoderma viride* and *Fusarium* spp as bio-agents against *Sclerotium rolfsii.* The maximum percentage of growth inhibition of *Sclerotium rolfsii* was observed with *Trichoderma harzianum* (77.39%) and *Trichoderma viride* (76.54%), while *Penicillium* sp (29.05%), *Aspergillus niger* (30.48%), *Curvularia* sp (13.57%) and *Fusarium* sp. inhibited the growth by 3.02% under *in vitro* condition.

Kushwaha et al. (2018) evaluated three biocontrol *agents viz., Trichoderma viride, T. virens* and *T. harzianum* against *Sclerotium rolfsii* under In vitro conditions. The rate of inhibition was fastest in *T. harzianum* (63.60%) followed by *T virens* (51.5 %). Least inhibition was recorded in *T. viride* (50.85%) after 72 hours of incubation. However, *T. viride* showed the highest (91.31%) reduction in sclerotia formation followed by *T. harzianum* (84.92%) and *T. virens (*84.29%) after 15 days of incubation. The volatile compounds from *Trichoderma*

viride were found most effective in suppressing the mycelial growth (51.11%) and sclerotia production (95.90%) of the target pathogen. The culture filtrate from both *T. harzianum* and *T. viride* (15% concentration) was found very effective in inhibiting the radial growth (57.46 and 49.62%) and sclerotia formation (98.20 and 99.83%) of *Sclerotium rolfsii.* The antagonists such as *T. harzianum* and *T. viride* can be used as a bio-control agent against *S. rolfsii* under field condition.

2.3.1. *In Vitro* Evaluation of *Bacillus subtililis* Strain (ATCC 6633) against *S. rolfsii*

Khalid (2013) isolated four bioagents viz, *Bacillus subtilis, Pseudomonas fluorescens,* yeast and *Trichoderma viride* which inhibited damping-off disease of bean caused by *S. rolfsii. Bacillus subtilis, T. viride,* and *P. fluorescens* control 88.7%, 83.7% and 86.3% respectively as bioagents against *S. rolfsii* and plants survived by 90.3%, 86.1% and 87.6% respectively.

Shifa et al. (2015) a total of seven biocontrol agents with known antifungal activity against other soilborne fungal pathogens were screened for their antagonistic potential against *Sclerotium rolfsii* , the causal agent of stem rot disease of groundnut (*Arachis hypogaea* L.) by dual culture assay. Among the various biocontrol agents tested *Bacillus subtilis* strain G-1 was the most effective in inhibiting the mycelial growth of *S. rolfsii* and recorded an inhibition of 28%.

Ramzan et al. (2016) studied 15 bio-agents against *S. rolfsii* responsible for root rot of mungbean in which *Trichoderma harzianum, Bacillus cereus, B. subtilis, T. virens, Pseudomonas fluorescens* and *Micrococcus varians* were effective bio-agents but *Bacillus subtilis* and *Trichoderma harzianum* were more effective.

Rajkumar et al. (2018) screened thirty *B. subtilis* isolates *in vitro* against *S. rolfsii*. The isolates showed different levels of inhibition of mycelia growth of *S. rolfsii*. Among different isolates BS16 inhibited maximum mycelial growth 64.04 per cent followed by BS 30 (11.98 %) and minimum inhibition of mycelial growth was observed in case of BS17 (11.98 %) compared to check isolate with 47 per cent inhibition of mycelial growth of *S. rolfsii*.

2.4. *In Vitro* Evaluation of Organic Amendments against *S. rolfsii*

Gurjar et al. (2003) reported about the collar rot of chilli caused by *Sclerotium rolfsii* in relation to the effect of organic amendments like FYM, vermicompost, cotton oil, mustard oil, castor oil, neem oil and groundnut oil against the disease. All these amendments were found to be significantly superior compared to control. Neem cake was found to be the most effective with the least incidence of the disease (i.e. 18.50 per cent).

Mathur et al. (2006) experimented on the management of fenugreek wilt (*F. oxysporum*) with organic amendments under field conditions for three years. They reported that on the basis of three years pooled data, significantly minimum mean wilt disease incidence (26.47%) was recorded with the Vermicompost (@ 10 q / ha), followed by the treatments *viz.*, mustard oil cake and sesamum oil cake @ 25 q / ha (each 34.97%), cotton oil cake (36.97%) and FYM (37.88 %), as against 42,15 per cent wilt incidence in untreated control.

Lahre et al. (2012) studied efficacy of bioagents and organic amendments against *S. rolfsii* in chickpea. They reported that under natural condition, all the organic amendments significantly increased seed germination and reduced the collar rot incidence over untreated control. However, among all the treatments, seed treatment of bioagents *Trichoderma* + Neem seed cake soil application was the most effective with maximum seed germination (93.05 %) and minimum total mortality (8.32 %), followed by *Trichoderma* + Mustard cake (85.03 and 9.55 %) and *Trichoderma* + Karanj cake (80.86 and 11.41 %).

Thakkar et al. (2018) evaluated six organic amendments (castor cake, cotton cake, FYM (Farm Yard Manure), mustard cake, neem cake and vermicompost) were tested by poison food technique in vitro to know their fungitoxic effect on the growth and sclerotial production of *S. rolfsii* a causal agent of stem rot of cluster bean. All the six organic amendments at different concentrations (10, 20 and 30%) were found inhibitory to the fungal growth. Significantly maximum growth inhibition of the fungus was recorded in castor cake at 10, 20 and 30 per cent concentrations (76.74, 91.71 and 98.55%, respectively) followed by neem cake at 10 (38.11%), 20 (55.52%) and 30 per cent (80.97%). All the six amendments significantly inhibited the sclerotial production of *S. rolfsii*. Significantly highest the inhibition of the sclerotial production was recorded in castor cake at 10, 20 and 30 per cent concentrations (68.67, 18.33 and 0.00, respectively) followed by neem cake at 10, 20 and 30 per cent (149.00, 114.67 and 56.00, respectively)

Latha and Rajeswari (2019) tested the efficacy of oilcakes viz., Neem cake, Jatropha cake, Sesamum cake, Mahua cake, Groundnut cake, and Castor cake at 10 per cent concentration against mycelial growth of *S. rolfsii*. All the oilcake extracts at 10 per cent concentration were effective in inhibiting the radial mycelial growth of *S. rolfsii*. However, Mahua oilcake extracts more inhibitory (83.07 percent) effect than others (15.23 mm). This was followed by castor cake (31.70 mm). The other oilcakes were found to be less inhibitory effect.

CHAPTER III

METERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

All experiments were conducted in the Plant Protection Laboratory of Agrotechnology Discipline, Khulna University, Khulna Bangladesh, during June to November 2019 to evaluate the effect of fungicides, plant extracts, antagonist microorganisms and organic amendments against *S. rolfsii*. The details of the materials used and methods followed for various experiments are described here in the following chapter.

3.1. Collection of the Fungal Isolate

An isolate of *S. rolfsii* was isolated from infected stem of betel vine. After isolation it was collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

Plate 1. Pure culture of *S. rolfsii* showing mycelial growth and immature sclerotia

3.2. Preparation Method of Potato Dextrose Agar (PDA) Medium and Multiplication of Fungal Isolates

The basic Potato Dextrose medium was prepared by following the standard procedure (Anonymous, 1968). At first 200 g potato was cut into slice and then boiled in 1000 ml water. After boiling, it was sieved in a 1000 ml beaker and 15 g dextrose was mixed with it. After few minutes 20 g agar was mixed with it in the water bath. Prepared PDA was poured in 250ml Erlenmeyer flask plugged with cotton and then sterilized in autoclave at 121°C temperature for about 20 minutes. Then it was used as medium. The fungal isolate was multiplied on PDA medium for further use at pH 6.0.

3.3. Treatments and Design of the Experiment

All experiments were conducted in Completely Randomized Design (CRD) with four replications

3.4. Different Experiments of Disease Management

To test efficacy of different agents against *S. rolfsii*, four *in vitro* experiments were conducted.

3.4.1. Experiment 1. *In vitro* Evaluation of Fungicides against *S. rolfsii*

Four fungicides viz. Unisaaf 75 WP, Dithane M-45, Exzole 50 SC and Provax-200 were tested following poisoned food technique (Grover and Moore, 1962) *in vitro* at five concentrations (@ each 100, 150, 200, 250 and 500 ppm) to evaluate their effect on mycelial growth and sclerotia formation of *S. rolfsii* (Table 1).

3.4.1.1. Collection of Fungicides

Fungicides were collected from different pesticide and fertilizer stores/shops of Khulna city

Table1. List of fungicides and concentrations used in the experiment

Common Name	% Active ingredient	Concentrations (ppm)
Unisaaf 75WP	Mancozeb 12% + Carbendazim 63%	100, 150, 200, 250, 500
Dithane M-45	80% Mancozeb	100, 150, 200, 250, 500
Exzole 50 SC	Hexaconazole	100, 150, 200, 250, 500
Provax-200	Carboxin 17.5% + Thiram 17.5%	100, 150, 200, 250, 500
Control	--	--

3.4.1.2. Preparation of Media having Fungicides

Based on active ingredient, the requisite quantity of each test fungicide was calculated and mixed thoroughly with autoclaved and cooled (50°C) PDA medium separately in conical flasks (250 ml) to obtain desired concentrations of 100, 150, 200, 250 and 500 ppm. Fungicide amended PDA medium was then poured (20 ml plate^{-1}) aseptically in glass Petri plates (90 mm dia.) and allowed to solidify at room temperature. After solidification of the medium, all the plates were inoculated aseptically with 5 mm culture disc obtained from a 5 days old actively growing pure culture of *S. rolfsii.* The culture disc was placed on PDA in inverted position in the centre of the petriplate and plates were incubated at 25±2 °C. Petriplates filled with plain PDA (without any fungicide) and inoculated with the culture disc of *S. rolfsii* were maintained as control plate.

3.4.2. Experiment 2. *In vitro* Evaluation of Plant Extracts/ Botanicals against *S. rolfsii*

Aqueous leaf extracts of four botanicals viz., Neem, Tulsi, Marigold and Allamanda were evaluated each with five concentrations i.e. 10, 15, 20, 25 and 30% *in vitro* against the mycelia growth and sclerotia formation of *S. rolfsii* (Table 2 and Plate 2).

3.4.2.1. Collection of Botanicals

Leaves of botanicals were collected from Khulna University campus.

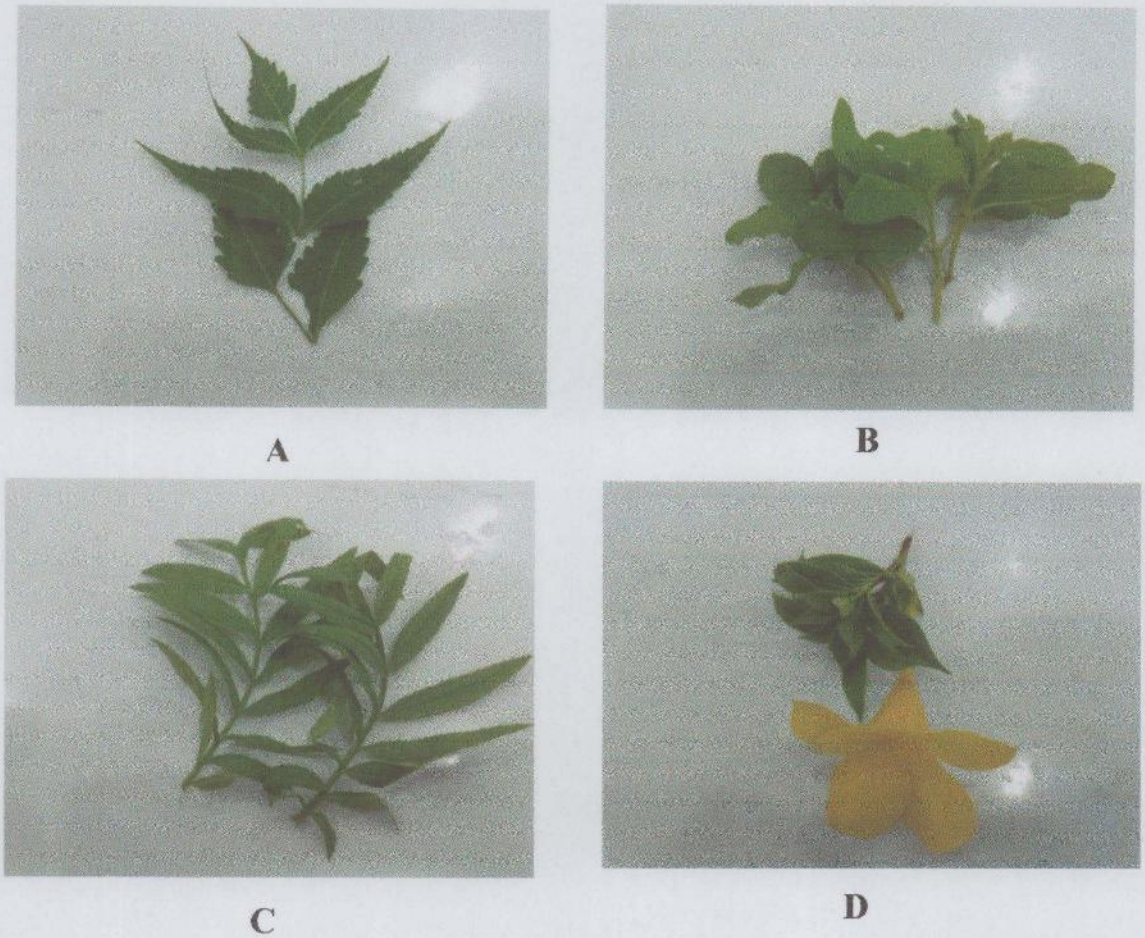


Plate 2. Plant parts used to test antifungal activity against *S. rolfsii* A. Neem (*Azadirachta indica*) B. Tulsi (*Ocimum tenuiflorum)* C. Marigold (*Tagetes erecta*) and D. Allamand (*Allamanda cathertica*)

Table 2. The parts of the plants and concentrations used in the experiment

Common Name	Scientific Name	Family	Plant Parts Used	Concentration (%)
Neem	*Azadirachta indica*	Meliaceae	Leaves	10, 15, 20, 25, 30
Tulsi	*Ocimum tenuiflorum*	Lamiaceae	Leaves	10, 15, 20, 25, 30
Marigold	*Tagetes erecta*	Asteraceae	Leaves	10, 15, 20, 25, 30
Allamanda	*Allamanda cathertica*	Apocynaceae	Leaves	10, 15, 20, 25, 30
Control	--	--	--	--

3.4.2.2. Preparation of Media having Botanical Extracts

Botanical extracts were prepared by following the Poison Food Technique (Grover and Moore, 1962). At first healthy leaves (Neem, Tulsi, Marigold and Allamanda) were collected, cleaned, weighted and made extract by using electric blender. For getting extracts of 1:1 (v/v) ratio, 100 ml of distilled water was mixed with 100g plant parts (Mahato et al., 2014). The pulverized mass was squeezed through 3 folds of fine cotton cloth. For evaluation, desired concentration (10%, 15%, 20%, 25% and 30%) was obtained by adding appropriate amount of standard solution of plant extract to 100 ml PDA medium in 250 ml conical flasks. After thorough mixing of each extracts separately with PDA medium approximately 20 ml of that of each was poured into separate petriplate. After solidification, the plates were inoculated by placing 5 mm mycelial disc of *S. rolfsi* from the periphery of 5 days old culture. The mycelial discs were placed at the centre of the petriplate and incubated at 25±2°C till the control plates were fully covered with mycelial growth of the test pathogen.

3.4.3. Experiment 3. *In vitro* Evaluation of Antagonist Microorganisms against *S. rolfsii*

Bioagents *T. harzianum* and secondary metabolites of *Bacillus subtilis* Strain (ATCC 6633) were evaluated against *S. rolfsii.*

3.4.3.1. Collection of *Trichoderma* Isolate and *Bacillus subtilis* Strain (ATCC 6633)

An isolate of *Trichoderma harzianum* was collected from plant protection laboratory of Agrotechnology Discipline, Khulna University, Khulna and *Bacillus subtilis* strain (ATCC 6633) was collected by personal contact with pharmacologist.

3.4.3.2. Dual Culture Method for Screening *T. harzianum* against *S. rolfsii*

Bioagent *T. harzianum* were evaluated against *S. rolfsii* following dual culture method (Dennis and Webster, 1971). Discs of mycelia of fungi was cut from the edge of an actively growing fungal colony with a 5 mm diameter cork borer and three disc of *Trichoderma harzianum* were placed near the edges of each PDA plate (90 mm). One disc of pathogenic fungus was placed in the center. The plate only with the disc of pathogenic fungi in the center was used as control plate. Plates were incubated in growth chamber for 25±2°C until the mycelia of pathogenic fungi was observed. Area of the colony of tested fungus was calculated by ½×base ×height. The area of the colony of control plate was also measured by $\pi \times r^2$. The shapes of the tested fungus colony become triangular shape and that's why this formula is used. Thereafter inhibition percentages of the pathogenic fungi were calculated by following the formula suggested by Naz et al., (2006).

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

Where X= Mycelial growth of pathogen alone (control)

Y= Mycelial growth of pathogen along with antagonist

3.4.4. Experiment 4. *In Vitro* Evaluation of Organic Amendments against *S. rolfsii*

A total of four organic amendments viz., Neem cake, Wood ash, Mustard cake and Coconut cake were tested following poisoned food technique (Grover and Moore, 1962) *in vitro* at five concentrations (@ each 10, 15, 20, 25 and 30%) to evaluate their effect on mycelial growth and sclerotia formation of *S. rolfsii* (Table 4 and Plate 3).

3.4.4.1. Collection of Organic Amendments

The organic amendments were collected from different pesticide and fertilizer stores/shops of Khulna city.

A

B

C

D

Plate 3.Organic amendments used to test antifungal activity against *S. rolfsii* A. Neem cake B. Wood ash C. Mustard cake and D. Coconut cake

Table 4. Organic amendments and concentrations used in the experiment

Name of Organic Amendments	Concentrations (%)
Neem Cake	10, 15, 20, 25 and 30
Wood ash	10, 15, 20, 25 and 30
Mustard Cake	10, 15, 20, 25 and 30
Coconut Cake	10, 15, 20, 25 and 30
Control	---

3.4.4.2. Preparation of Media having Organic Amendments

Organic amendments were prepared by following the Poison Food Technique (Grover and Moore, 1962). At first the organic amendments were collected, weighted and mixed with water at the rate of following proportion- Neem cake : water = 1:4, Wood ash : water = 1:3, Mustard cake: water = 1:3 and Coconut cake : water = 1:4 until fully saturated and kept for overnight (Thakkar et al. 2018). Then each mixture was blended in electric blender and squeezed through 3 folds of fine cotton cloth. For evaluation, desired concentration (10%, 15%, 20%, 25% and 30%) was obtained by adding appropriate amount of standard solution of organic amendments to 100 ml PDA medium in 250 ml conical flasks. Then about 20 ml amendments mixed with PDA medium was poured in sterilized petriplates. Discs of 5mm diameter was cut out from actively growing culture of the isolate (5 days old) and transferred to the centre of petriplates. The inoculated plates were incubated at 25±2 °C till the control plates were fully covered with mycelial growth of the test pathogen.

3.5. Data Collection

The data were collected on the following parameters;

I. Radial mycelial growth was measured after 4 days of inoculation

II. Number of sclerotia after 30 days of inoculation

3.5.1. Measurement of Radial Mycelial Growth Inhibition

The radial growth of mycelium of each plate was measured by taking average of the two diameters taken right angles for each colony in mm. and then these plates were kept for 30 days for sclerotia formation. Inhibition of radial growth was computed based on colony diameter on control plate using the following formula (Naz. et al., 2006) shown below:

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

Where,

X= Average radial growth (mm) of *S. rolfsii* in control plate

Y= Average radial growth (mm) of *S. rolfsii* in each fungicide, plant extract, antagonist microorganism and organic amendment treated plates

3.5.2. Counting of Sclerotia

After 30 days the sclerotia of each petriplate was separated by using camel hair brush and number of sclerotia of each petriplate was counted manually. Here a petridish was maintained as control to compare it with others (Amin et al., 2013).

3.6. Data Analysis

Data were analysed by using STAR (Statistical tools for agricultural research program, version-02, IRRI, Losbaños, Philippines). Arc sine and square root transformation were done as necessary.

CHAPTER IV

RESULTS

CHAPTER IV
RESULTS

This chapter includes the experimental results. Effects of different management (fungicides, botanicals, antagonist microorganisms and organic amendments) in controlling foot and root rot disease of betel vine caused by *S. rolfsii* were assessed *in vitro*. The results of the experiments are given below which were prepared based on the inhibition of radial mycelial growth and number of sclerotia formation.

4.1. *In Vitro* Evaluation of Fungicides against *S. rolfsii*

Results indicated that all the fungicides tested significantly by inhibiting mycelial growth and sclerotia formation of *S. rolfsii* over control at all the tested concentrations and it was found that the redial mycelial growth and sclerotia formation of test pathogen decreased with increasing the concentrations of the tested fungicides.

4.1.1. Effect of Fungicides on Mycelial Growth inhibition percentage and Sclerotia Formation of *S. rolfsii* at Different Concentrations

The results are presented in Table 5. Effects of fungicides on the mycelial growth inhibition of *S. rolfsii* were found significantly ($p< 0.01$) different at all concentrations. Complete inhibition was found by using Exzole 50 SC and Provax-200 against *S. rolfsii* i.e. no growth was observed at any concentration.

In case of Unisaaf 75 WP, highest growth inhibition was found at 500 ppm (61.41%) concentration against *S. rolfsii* which was statistically differed from all other concentrations. Dithane M-45 at 500 ppm inhibited the growth of *S. rolfsii* (56.79%) which was statistically differed from all other concentrations. But, the effect of Unisaaf 75 WP at 200 ppm (50.43%) and 150 ppm (42.05%) statistically similar with Dithane M-45 at 250 ppm (50.43%) and 200 ppm (43.31%) respectively. In case of Unisaaf 75 WP, lowest inhibition was observed at 100 ppm (34.67%) which was statistically similar with Dithane M-45 at 100 ppm (33.82%) and 150ppm (35.84%) concentrations (Plate 4 and 5).

Performance of all the four tested fungicides significantly ($p<0.01$) inhibited the sclerotia formation of *S. rolfsii* whereas number of sclerotia formation decreased with increases of concentrations. Among tested four fungicides, no sclerotia were formed by applying Exzole 50 SC and Provax-200 fungicides i.e. 100% reduction of sclerotia was found at all concentrations (Table 5).

The maximum number of sclerotia formation (800.00) was recorded in control plate. In case of Unisaaf 75WP, highest number of sclerotia formation was found at 100 ppm (704.00) which was statistically differed with all other concentrations. Then Unisaaf 75Wp at 250ppm (306.67), 200ppm (340.00) and 150ppm (424.00) statistically similar with Dithane M-45 at 500ppm (285.33), 250ppm (340.00), 200ppm (416.00) and 150ppm (448.00) concentrations respectively. Significantly lowest number of sclerotia was recorded in Unisaaf 75WP at 500 ppm (200.00) concentration (Plate 6).

Table 5. Effect of fungicides at different concentrations on mycelial growth inhibition and sclerotia formation of *S. rolfsii*

Fungicides	Concentration (ppm)	% Inhibition	No. of Sclerotia
Control	0	0.00	800.00a
Unisaaf 75WP	100	34.67 gh (36.08)	704.00 b
	150	42.05 f (40.42)	424.00 de
	200	50.43 e (45.24)	340.00 f
	250	53.33 d (46.91)	306.67 g
	500	61.41 b (51.60)	200.00 h
Dithane M-45	100	33.82 h (35.56)	504.00 c
	150	35.84 g (36.77)	448.00 d
	200	43.31 f (41.16)	416.00 e
	250	50.43 e (45.25)	340.00 f
	500	56.79 c (48.91)	285.33 g
Exzole 50 SC	100	100 a (90.00)	0.00 i
	150	100 a (90.00)	0.00 i
	200	100 a (90.00)	0.00 i
	250	100 a (90.00)	0.00 i
	500	100 a (90.00)	0.00 i
Provax-200	100	100 a (90.00)	0.00 i
	150	100 a (90.00)	0.00 i
	200	100 a (90.00)	0.00 i
	250	100 a (90.00)	0.00 i
	500	100 a (90.00)	0.00 i
CV(%)		1.02	3.45
Level of significance		**	**

**= 1% level of significance

Means with the same letter are not significantly different

Figures in the parentheses are arcsine transformed values

4.1.2. Functional Relationship between Concentration and Mycelial Growth Inhibition Percentage of *S. rolfsii* at Different Concentrations of Fungicides

Regression equation (Fig.1) between concentrations and inhibition percentages of Exzole 50 SC and Provax-200 exposed that the inhibition percentage was 100% i.e. no variation in inhibition percentage was found by the increase of concentration.

Regression equation (Fig.1) between concentrations and mycelial growth inhibition percentages of Unisaaf 75 WP and Dithane M-45 revealed that more than 85% and 82% of the variation in increased inhibition percentage could be explained by the increase of concentration.

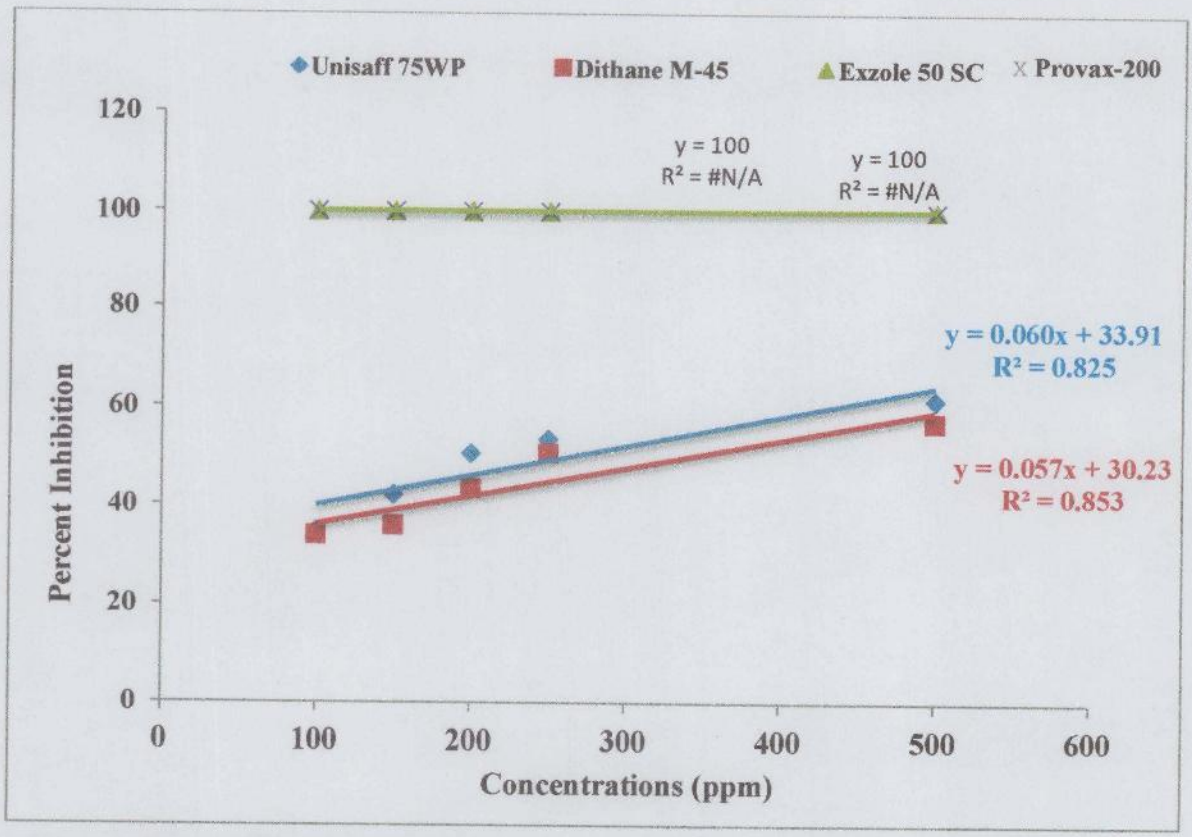


Figure 1. Functional relationship between concentrations of fungicides and mycelial growth inhibition percentage of *S. rolfsii*

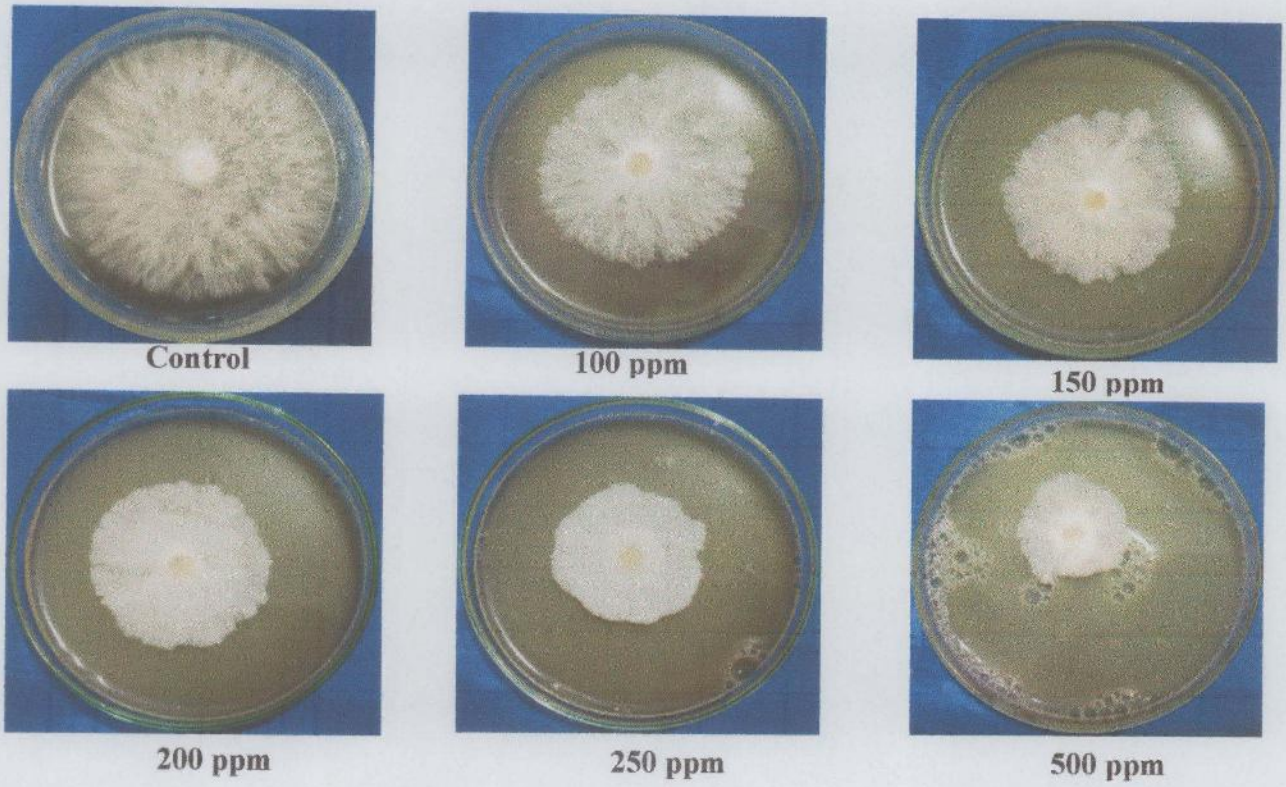


Plate 4. Mycelial growth of *S. rolfsii* at different concentrations of fungicide Unisaaf 75wp

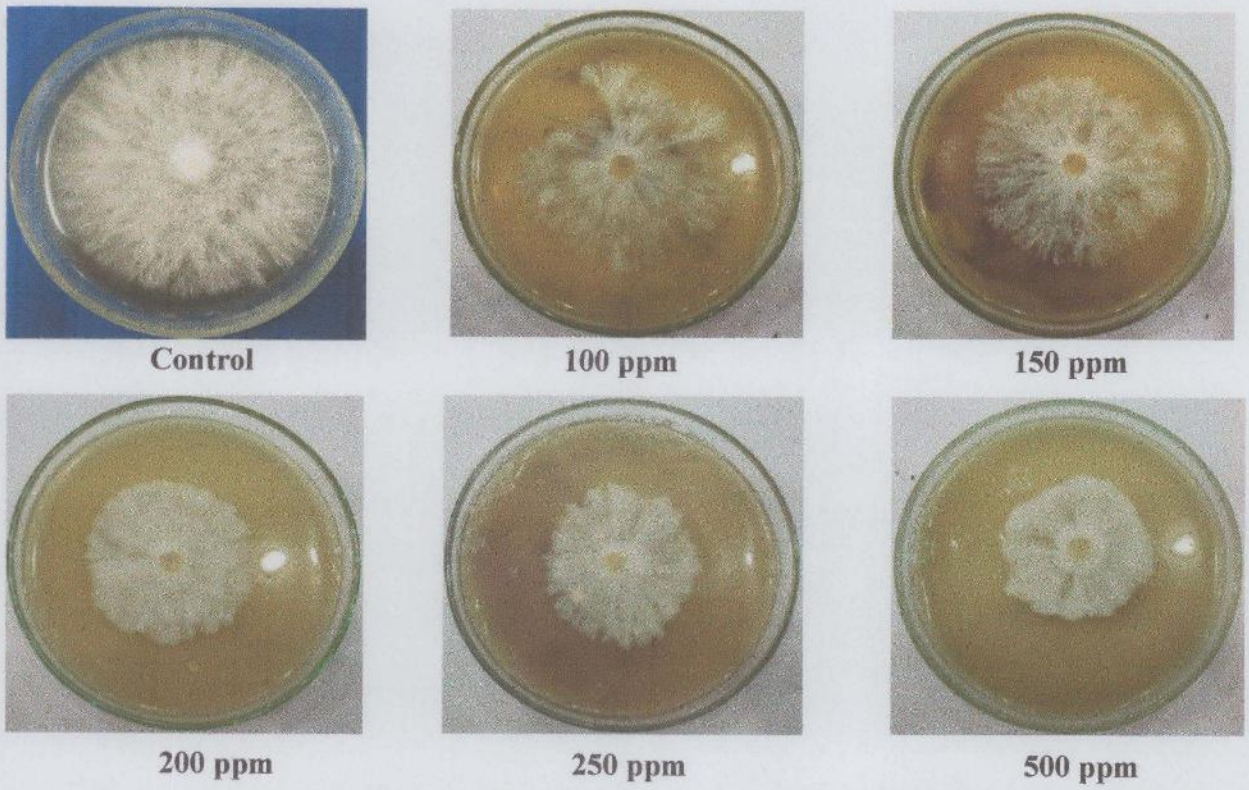


Plate 5. Mycelial growth of *S. rolfsii* at different concentrations of fungicide Dithane M-45

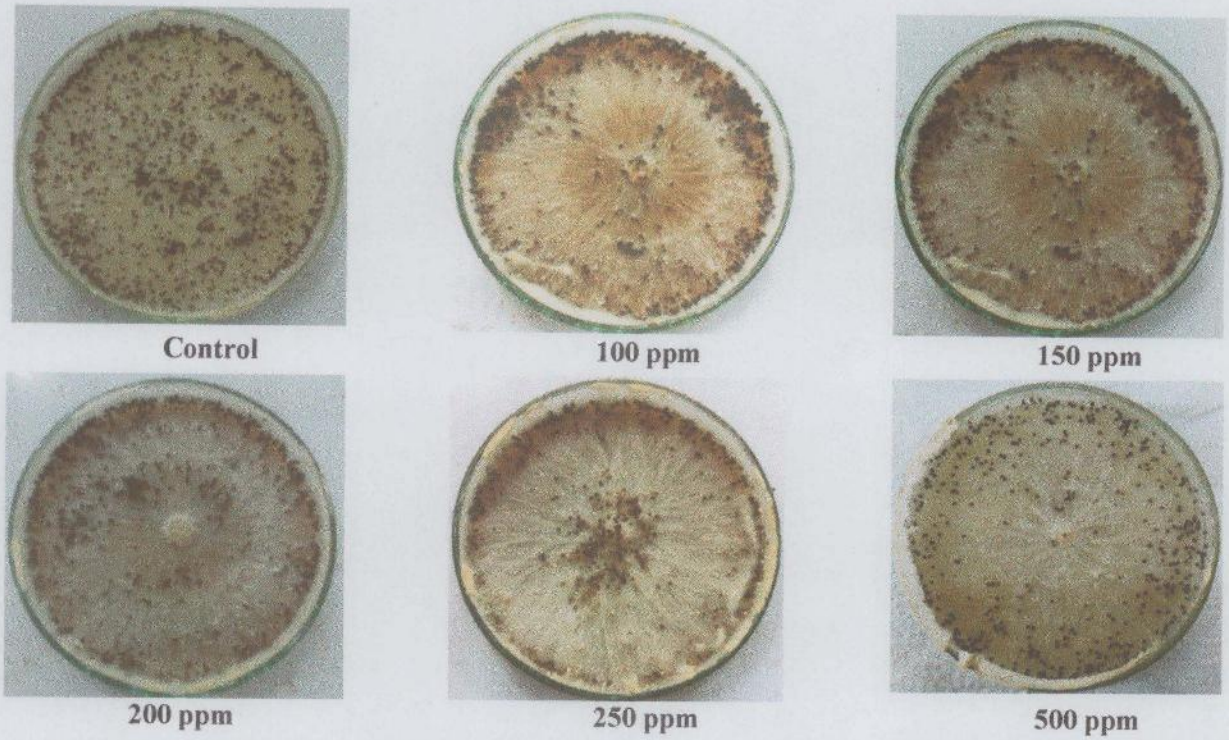


Plate 6.Sclerotia formation of *S. rolfsii* at different concentrations of fungicide Unisaaf 75wp

Plate 7.Sclerotia formation of *S. rolfsii* at different concentrations of fungicide Dithane M-45

4.2. *In vitro* Evaluation of Plant Extracts/ Botanicals

4.2.1. Effect of Plant Extracts/Botanicals on Mycelial Growth Inhibition and Sclerotia Formation of *S. rolfsii* at Different Concentrations

Effect of botanicals extracts on mycelial growth inhibition and sclerotia formation of *S. rolfsii* differed significantly ($p< 0.01$) compared to the control. In all concentrations, the growth inhibition due to the application of different extracts was found to be increased and the number of sclerotia formation was decreased with the increase of concentration (Table 6 and Figure 2).

The highest inhibiton percentage of mycelial growth was observed in case of neem leaf extracts at 30% concentration (63.20%) which was statistically similar with neem 25% (59.26%), tulsi 25% (58.82%) and tulsi 30% (56.79%) but differed from all other concentrations. The lowest percentage of inhibition was recorded in case of allamanda at 20% (7.73%) concentrations which was statistically similar with 25% (8.22%) concentrations of allamanda. Moderate inhibition was observed at 20% concentration of neem which was statistically similar with 20% concentration of tulsi leaf extracts. No inhibition was observed at 10% and 15% concentrations of marigold and allamand leaf extracts which was statistically similar with the control (Plate 8).

In case of sclerotia formation, highest number of sclerotia (824.00) was produced on control plate which was statistically similar at 10% and 15% concentrations of marigold and allamanda leaf extracts and the lowest on the neem extracts containing media (200.00) at a concentration of 30% which was statistically differed with all other concentrations. In tulsi leaf extracts, significantly lowest number of sclerotia formation was recorded at 30% concentration (221.33) which was statistically similar with the neem 25% (237.33), tulsi 25% (250.67) and 20% (285.33) concentrations (Plate 9).

Table 6. Effect of plant extracts at different concentrations on mycelial growth inhibition and sclerotia formation of *S. rolfsii*

Botanical extracts	Concentrations (%)	% Inhibition	No. of Sclerotia
Control	0	0.00	824.00a
Neem	10	16.78 gh (24.18)	577.00 e
	15	28.90 e (32.51)	432.00 h
	20	52.40 c (46.38)	306.66 i
	25	59.26 ab (50.34)	237.33 jkl
	30	63.20 a (52.65)	200.00 l
Tulsi	10	46.25 d (42.84)	513.33 fg
	15	50.36 cd (45.21)	467.67 gh
	20	55.76 bc (48.30)	285.33 ij
	25	58.82 ab (50.08)	250.67 jk
	30	59.26 ab (50.34)	221.33 kl
Marigold	10	0.00 j (0.00)	824.00 a
	15	0.00 j (0.00)	824.00 a
	20	13.86 h (21.85)	696.00 cd
	25	21.02 fg (27.33)	664.00 d
	30	22.77 f (28.50)	526.67 ef
Allamanda	10	0.00 j (0.00)	824.00 a
	15	0.00 j (0.00)	824.00 a
	20	7.73 i (15.89)	778.67 ab
	25	8.22 i (16.62)	736.00 bc
	30	13.86 h (21.86)	661.33 d
CV (%)		4.53	2.93
Level of Significance		**	**

**= 1% level of significance

Means with the same letter are not significantly different

Figures in the parentheses are arcsine transformed values

4.2.2. Functional Relationship between Concentration and Mycelial Growth Inhibition Percentage of *S. rolfsii* at Different Concentrations of Plant Extracts

From regression equation (Fig.2) between concentrations and mycelial growth inhibition percentages of neem leaf, tulsi leaf marigold leaf and allamanda leaf revealed that more than 95%, 93%, 91% and 90% of the variation in increased inhibition percentages could be explained by the increase of concentrations.

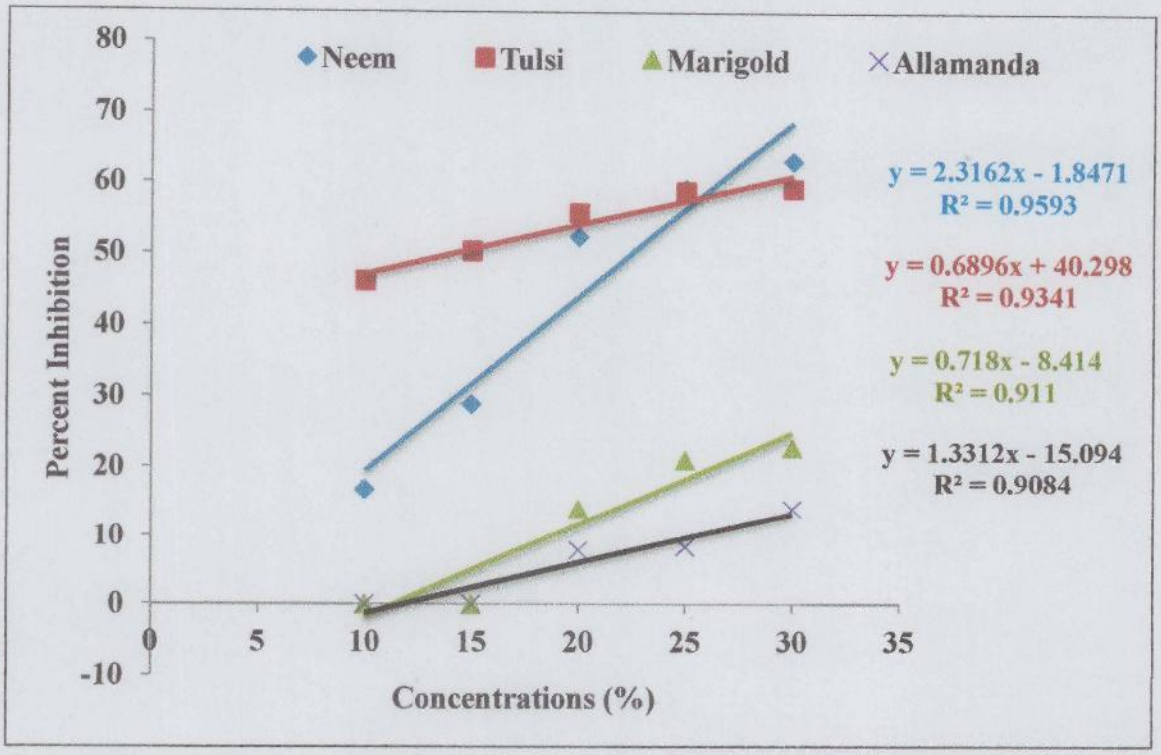


Figure 2. Functional relationship between concentrations of plant extracts and mycelial growth inhibition percentage of *S. rolfsii*

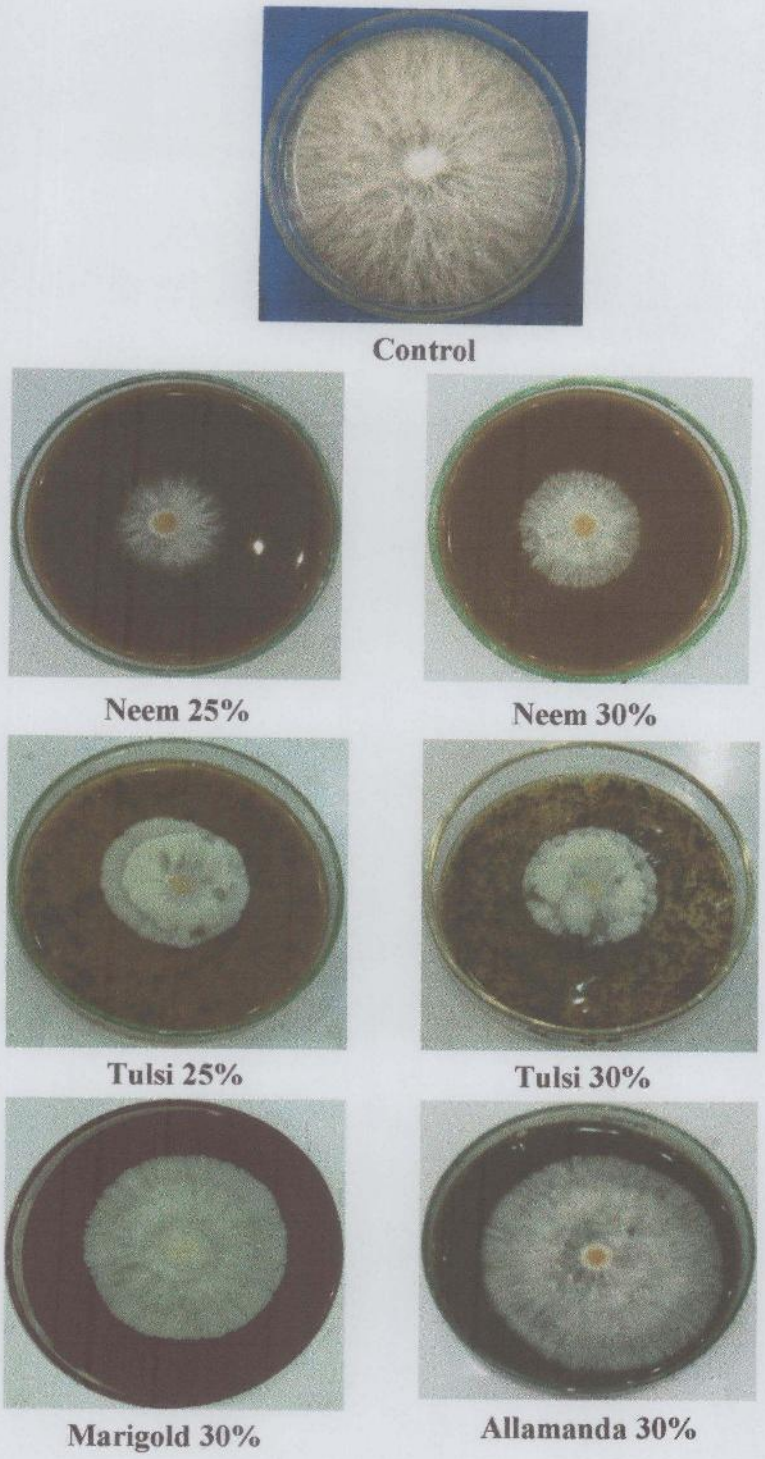


Plate 8. Mycelial growth of *S. rolfsii* at different concentrations of plant extracts

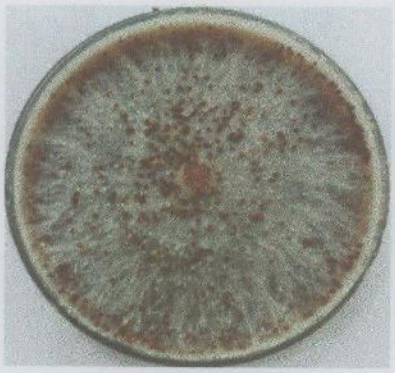
Control

Neem 25%

Neem 30%

Tulsi 25%

Tulsi 30%



Marigold 30%

Allamanda 30%

Plate 9. Sclerotia formation of *S. rolfsii* at different concentrations of plant extracts

4.3. *In vitro* Evaluation of Antagonist Microorganisms against *S. rolfsii*

4.3.1. Effect of *T. harzianum* on Radial Mycelial Growth Inhibition and Sclerotia Formation of *S. rolfsii*

Antagonistic effect of *T. harzianum* aganist *S. rolfsii* was tested on PDA in dual culture technique. It was found that, 88.91% mycelial growth of *S. rolfsii* was inhibited and no sclerotia were formed (Plate 10).

Control **Dual Plate Culture**

Plate 10. *In vitro* effect of *T. harzianum* on mycelia growth of *S. rolfsii*

4.3.2. Effect of Secondary Metabolites of *Bacillus subtilis* (ATCC 6633) on Radial Mycelial Growth Inhibition and Sclerotia formation of *S. rolfsii* at Different Concentrations

The secondary metabolites of *Bacillus subtilis* (ATCC 6633) was tested to evaluate their antifungal activity against *S. rolfsii* by poison food technique (Dennis and Wester, 1971).The results obtained on mycelial growth inhibition percentages and number of sclerotia formation of *S. rolfsii* are presented in the Table 7.

From these results, it was shown that significantly highest mycelial growth inhibition (89.54%) was recorded at 1:6 concentration which was statistically different from other treatments. The lowest mycelial growth inhibition was observed at 1:2 (72.90%) concentration whereas no inhibition was recorded in control plate (Table 7 and Plate.11).

The result obtained on the sclerotia formation showed significantly lowest number of sclerotia (34.00) was recorded at 1:6 concentrations. These were followed by the concentrations *viz.*, 1:4 (103.33) and 1:2 (312.00) respectively. Whereas the highest number of sclerotial (800.00) was recorded in control plate (Table 7 and Plate 12).

Table 7. Effect of secondary metabolites of *B. subtilis* at different concentrations on mycelial growth inhibition percentage and sclerotia formation of *S. rolfsii*

Treatment	Concentrations	Inhibition (%)	No. of Sclerotia
Control	0	0.00	800.00 a
	1:2	72.90c	312.00 b
Secondary metabolites of	1:4	83.50b	103.00 c
B. subtilis (ATCC 6633)	1:6	89.54a	34.00 d
CV(%)		1.22	3.14
Level of significance		**	**

**= 1% level of significance

Means with the same letter are not significantly different

4.3.2.1. Functional Relationship between Concentration and Mycelial Growth Inhibition Percentage of *S. rolfsii* at Different Concentration of Secondary Metabolites of *B. subtilis* (ATCC 663)

From regression equation (Fig.3) between concentrations and inhibition percentages of secondary metabolites of *B. subtilis* revealed that more than 97% of the variation in increased inhibition percentage could be explained by the increase of concentration.

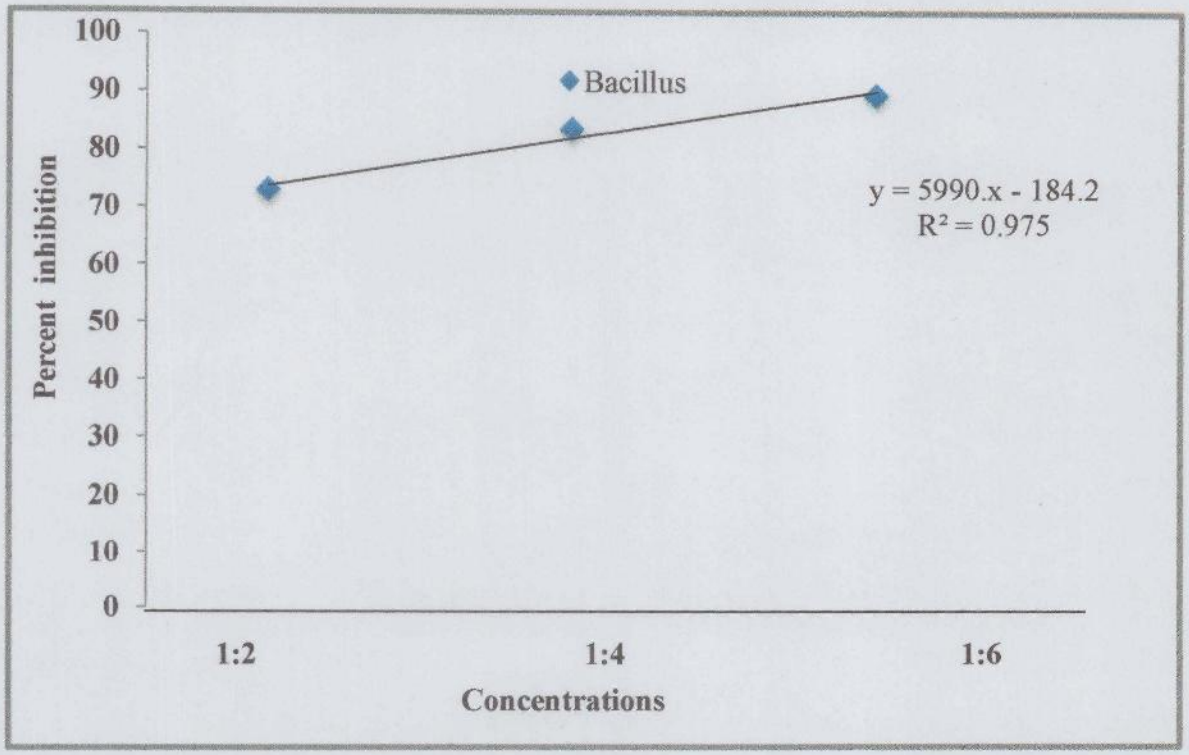
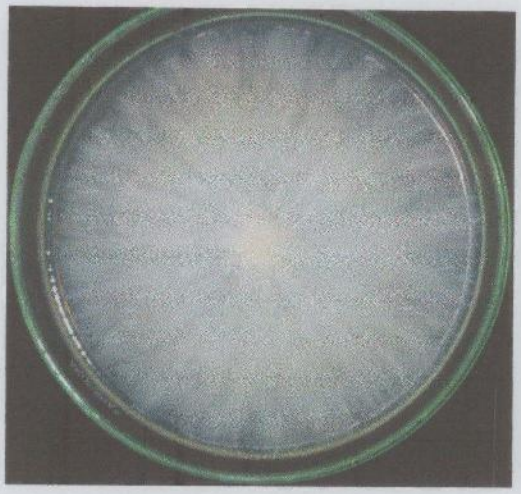
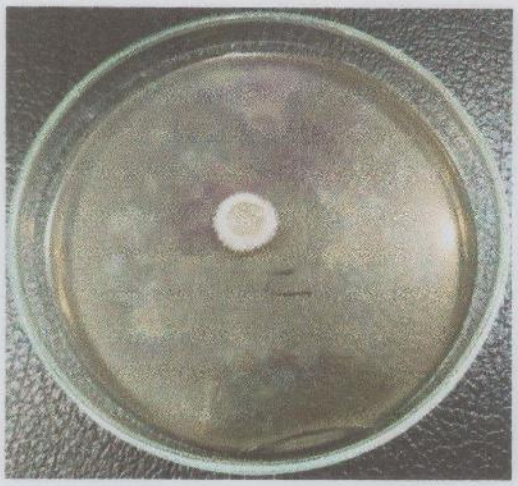


Figure 3. Functional relationship between concentrations of secondary metabolites and mycelial growth inhibition percentage of *S. rolfsii*

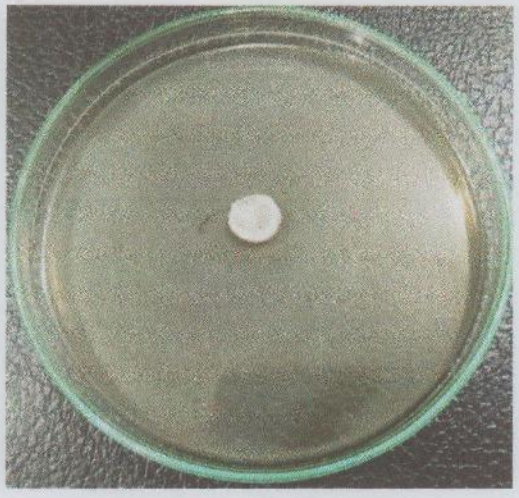


Control

1:2 Concentration



1:4 Concentration



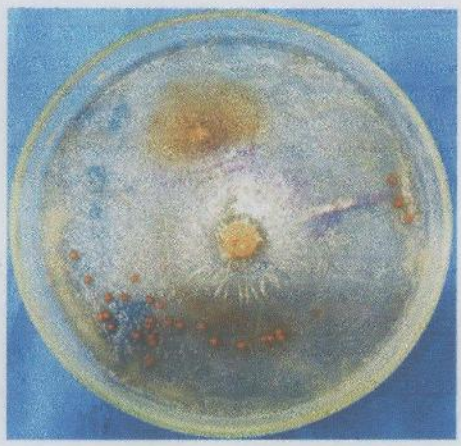
1:6 Concentration

Plate 11. Mycelial growth of *S. rolfsii* at different concentrations of secondary metabolites of *Bacillus subtilis*(ATCC 6633)

Control

1:2 Concentration

1:4 Concentration



1:6 Concentration

Plate 12. Sclerotia formation of *S. rolfsii* at different concentrations of secondary metabolites of *Bacillus subtilis*

4.4. *In vitro* Evaluation of Organic Amendments against *S. rolfsii*

A total of four organic amendments were evaluated *in vitro* (each @ 10, 15, 20, 25 and 30%) against *S. rolfsii.* Results of Table 8 revealed that all of the tested four amendments were fungistatic to *S. rolfsii,* which exhibited comparatively less mycelial growth and sclerotia formation by comparing untreated plate.

4.4.1. Effect of Organic Amendments on Mycelial Growth Inhibition and Sclerotia Formation of *S. rolfsii* at Different Concentrations

The organic amendments in different concentrations inhibited the mycelial growth and reduced the number of sclerotia formation of *S. rolfsii* significantly ($p<0.01$). In all concentrations, mycelial growth inhibition was increased and number of sclerotia formation decreased with increasing concentration (Table 8 and Figure 4).

Significantly maximum growth inhibition of *S. rolfsii* was recorded in neem cake at 30 % concentration (71.75%) which was statistically differed with all other concentrations. Secondly wood ash at 30% concentration showed maximum growth inhibition of *S. rolfsii* (64.42%) which was statistically similar with the 25% concentrations of neem cake (66.32%), 25% of wood ash (63.25%) and 20% of neem cake (63.98%). Minimum percent of inhibition was found at 10% concentration (33.52%) of coconut cake which was statistically similar at 10% concentrations of neem cake (34.25%) and wood ash (33.38%) respectively (Plate 13).

All the used amendments at different concentrations significantly ($p< 0.01$) reduced the number of sclerotia formation of *S. rolfsii.* Among tested amendments, neem cake and wood ash were found to be more effective than the other amendments against sclerotia formation of *S. rolfsii.* Significantly lowest number of sclerotia was recorded in neem cake at 30% concentration (205.33) which was statistically similar with 30% concentration (209.33) of

wood ash. But effect of neem cake on the number of sclerotia formation of *S. rolfsii* at 25% (266.67) and 20% (285.33) concentrations was statistically similar with mustard cake 25% (265.33), coconut cake 25% (266.67) and 30% concentrations (269.33) respectively. The highest number of sclerotia was recorded in control plate (800.00) which was followed by coconut cake at 10% (704.00) and mustard cake at 10% (664.00) concentrations respectively (Plate 14).

Table 8. Effect of organic amendments at different concentrations on mycelial growth inhibition and sclerotia formation of *S. rolfsii*

Organic amendments	Concentration (%)	% Inhibition	No. of Sclerotia
Control	0	0.00	800.00 a
Neem Cake	10	34.25 j (35.82)	426.67 ef
	15	52.13 f (46.22)	340.00 g
	20	63.98 bc (53.12)	285.33 h
	25	66.32 b (54.53)	266.67 hi
	30	71.75 a (57.89)	205.33 j
Wood Ash	10	33.38 j (35.29)	577.00 c
	15	48.02 g (43.86)	477.33 de
	20	58.56 d (49.93)	424.00 f
	25	63.25 c (52.69)	256.00 hij
	30	64.42 bc (53.38)	209.33 j
Mustard Cake	10	39.38 i (38.87)	664.00 b
	15	44.07 h (41.59)	504.00 d
	20	45.09 h (42.18)	448.00 ef
	25	54.61 ef (47.65)	265.33 hi
	30	57.98 d (49.59)	233.33 ij
Coconut Cake	10	33.52 j (35.38)	704.00 b
	15	43.92 h (41.51)	608.00 c
	20	46.41 gh (42.94)	456.00 def
	25	54.91 ef (47.82)	266.67 hi
	30	55.78 de (48.32)	269.33 hi
CV (%)		2.18	4.00
Level of Significance		**	**

**= 1% level of significance

Means with the same letter are not significantly different

Figures in the parentheses are arcsine transformed values

4.4.2. Functional Relationship between Concentration and Mycelial Growth Inhibition Percentages of *S. rolfsii* at Different Concentrations of Organic Amendments

From regression equation (Fig.4) between concentrations and inhibition percentages of Neem Cake, Wood ash, Mustard cake and Coconut cake revealed that more than 82%, 91%, 85% and 88% of the variations in increased inhibition percentages could be explained by the increase of concentrations.

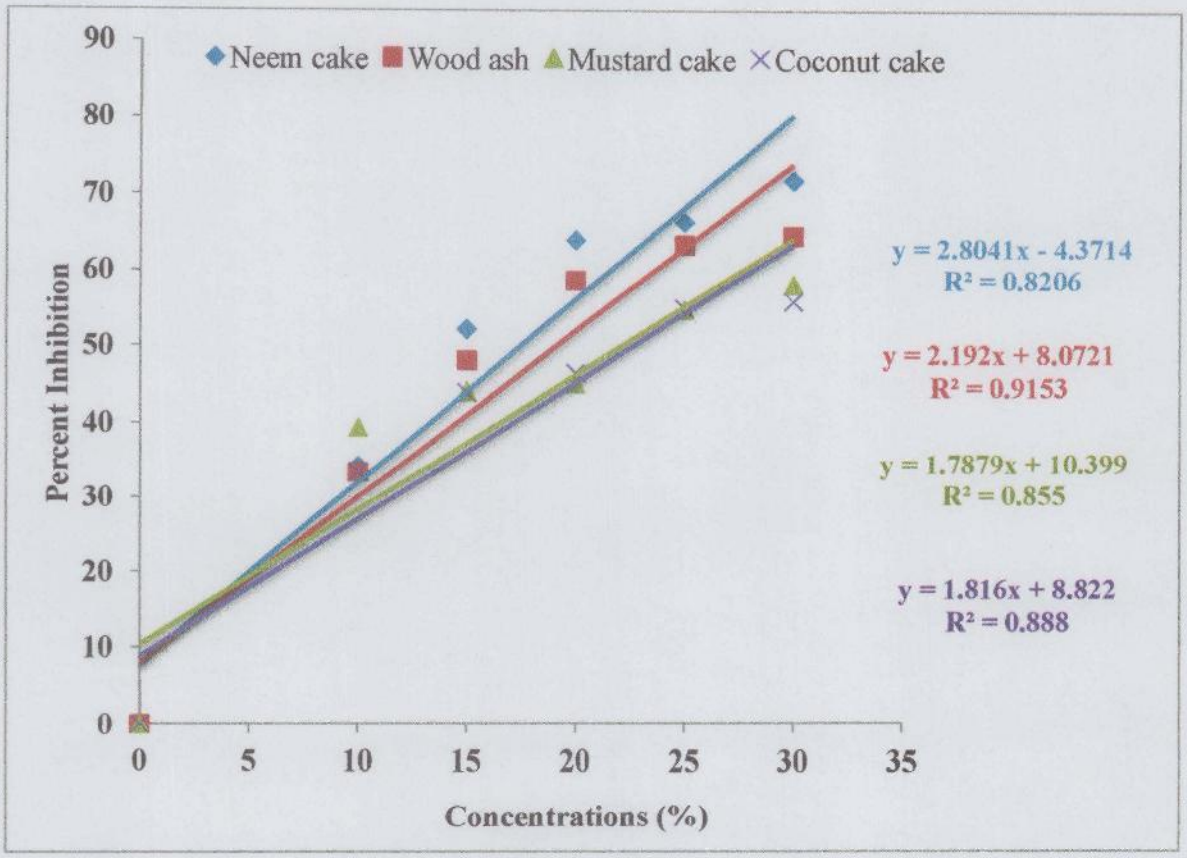
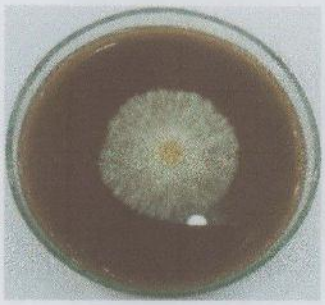


Figure 4. Functional relationship between concentrations of amendments and mycelial growth inhibition percentage of *S. rolfsii*

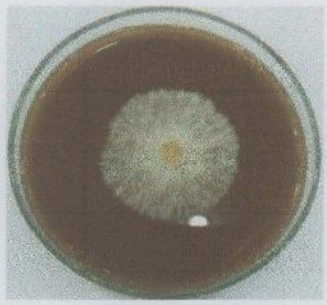
Control

Neem Cake 25%

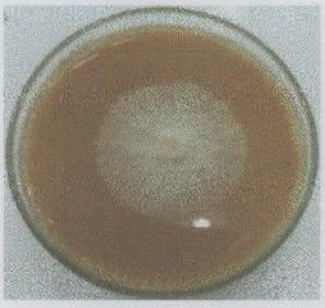
Neem Cake 30%



Wood Ash 25%

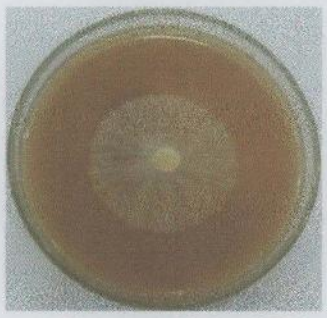


Wood Ash 30%



Mustard Cake 25%

Mustard Cake 30%



Coconut Cake 25%

Coconut Cake 30%

Plate 13. Mycelial growth of *S. rolfsii* at different concentrations of organic amendments

Control

Neem Cake 25%

Neem Cake 30%

Wood Ash 25%

Wood Ash 30%

Mustard Cake 25%

Mustard Cake 30%

Coconut Cake 25%

Coconut Cake 30%

Plate 14.Sclerotia formation of *S. rolfsii* at different concentrations of organic amendments

CHAPTER V
DISCUSSION

CHAPTER V

DISCUSSION

Betel vine (*Piper betle* L) is an important cash crop grown on a commercial scale in Bangladesh. The Major Constraint to cultivation of betel vine is different diseases that severely damage foot, stem, root and foliage. Betel vine plants are cultivated under shady and humid conditions that also favor the development of many diseases (Chattopadhyay and Maiti, 1990). Among various diseases foot and root rot caused by *S. rolfsii* is one of the most important soil borne diseases of the betel vine inflecting heavy yield losses in betel vine. To control these foot and root disease of betel vine caused by *S. rolfsii*, the present experiments were undertaken. The results obtained during these experiments are being discussed here in the following paragraphs.

5.1. *In Vitro* Evaluation of Fungicides against *S. rolfsii*

Results indicated that all the tested fungicides significantly inhibited mycelial growth and sclerotia formation of *S. rolfsii* at all the concentration over control treatment. Regression equation revealed that the redial mycelial growth inhibition was increased with increase in the concentration of the fungicides. Among tested four fungicides, performance of Exzole 50 SC and Provax-200 was better compared to other fungicides. Similar fungistatic effects of the fungicides (Carboxin, Carbendazim, Hexaconazole, Tebuconazole+Trifloxystrobin Mancozeb, Propiconazole, Tebuconazole and Dithane M-45) against the mycelia growth and sclerotia formation of *S. rolfsii* infecting were reported earlier by several workers (Arunasari et al., 2011; Bindu and Bhattiprolu, 2011; Rather, 2012; Mahato et al., 2014; Banakar et al., 2017 and Sarita et al., 2018).

5.2. *In Vitro* Evaluation of Plant Extracts/Botanicals against *S. rolfsii*

Efficacy of botanicals was evaluated for their antifungal property against *S. rolfsii* at 10, 15, 20, 25 and 30% concentrations by using poison food technique. However, all the extracts showed significantly highest percentage of inhibition when used in higher concentration. In the present experiment, we found that neem leaf and tulsi leaf extracts were more effective at higher concentration. Neem leaf was superior among the extracts followed by tulsi, marigold and allamanda leaf (Banakar et al., 2017). Leaf and bark extracts of neem contain antioxidant activity (Ghimeray et al., 2009). Many active phytoconstituents of *A. indica* (azadirachtin, salanin, meliantriol, nimbin, etc.) have been identified. The most active component is reported as azadirachtin that inhibit the growth of the fungal pathogen (Koul, 1990). Tulsi leaf extract also has a substantial effect on the growth and sclerotia formation of *S. rolfsii* at higher concentration (30 and 25% concentrations). Leaves of tulsi contain different phytochemicals such as tannins, saponins, proteins, alkaloids, steroids and terpenoids that act antifungal activities to control fungal diseases (Saranya et al., 2019). Botanicals, marigold and allamanda leaf were found comparatively less effective with lowest mycelial growth inhibition at higher concentration than other extracts. Similar effect of the test botanicals against *S. rolfsii* causing foot and root rot of betel vine. *S. rolfsii* infecting many other crops were reported earlier by several workers. But different botanicals *viz.*, Onion, Garlic, Periwinkle, Tulsi, Neem, Marigold, Allamanda, Bougainvillea, Giripushpa and Nilgiri were reported to cause significant mycelia growth inhibition and number of sclerotia reduction of *S. rolfsii* (Islam and Faruq, 2012; Banakar et al., 2017; Mundhe et al., 2009; Mandhare and Suryawanshi 2009 and Sultana et al., 2012) which were agree with that of present study.

5.3. *In Vitro* Evaluation of Antagonist Microorganisms against *S. rolfsii*

Biocontrol agents, *T. harzianum* was evaluated *in vitro* for their efficacy against *S. rolfsii* by applying dual culture technique (Dennis and Wester, 1971) and using PDA as basal medium. In the present study, it has been found that *T. harzianum* have strong effect to inhibit mycelial growth and sclerotial formation of *S. rolfsii* in culture media. Similar findings were reported earlier by several workers (Samsuzzaman et al., 2012; Manu et al., 2012; Kushwaha et al., 2018 and Basumatary et al., 2015). It is known that the Trichoderma-based biocontrol mechanisms mainly rely on mycoparasitism, production of antibiotic and/or hydrolytic enzymes, competition for nutrients, as well as induced plant resistance; numerous secondary metabolites produced by *Trichoderma* species could directly inhibit the growth of several plant pathogens (Benítez et al., 2004). *Trichoderma harzianum* excreted β-1, 3-glucanase and chitinase on the cell walls of the pathogen *Sclerotium rolfsii*, as sole carbon source and inhibit the growth (Gajera et al., 2013).

The secondary metabolites of *Bacillus subtilis* strain (ATCC-6633) were also evaluated in *vitro* for their efficacy against *S. rolfsii* by applying poision food technique (Dennis and Wester, 1971). The secondary metabolites of *Bacillus subtilis* strain inhibited the mycelial growth and sclerotia formation of *S. rolfsii* at all concentrations. Similar findings were reported earlier by several workers (Shifa et al., 2015; Rajkumar et al., 2018 and Ramzan et al., 2016). *Bacillus subtilis* strain (ATCC-6633) regarded as safe biological agents and their potential is higher to control plant disease. Antifungal peptides produced by *Bacillus* species include mycobacillins, surfactins, mycosubtilins and fungistatins (Majumdar et al., 1958; Kluge et al., 1988; Peypoux et al., 1976 and Phae et al., 1990) for this reason, it act as bacterial antagonists. It can also produce a wide range of other metabolites, including chitinases and other cell wall-degrading enzymes, volatiles, and compounds that elicit plant resistance mechanisms (Frändberg et al., 1994 and Sadfi et al., 2001).

CHAPTER VI

SUMMARY AND CONCLUSION

CHAPTER VI

SUMMARY AND CONCLUSION

Betel vine (*Piper betle* L.), is one of the important perennial, evergreen crop which suffers from substantial damage with foot and root rot disease incited by *Sclerotium rolfsii*. The pathogen is soil borne with wide host range and almost of worldwide distribution. The disease seems very difficult to control, due to its wide host range, prolific growth and ability to produce large number of sclerotia that may persist in soil for several years. Therefore, an integrated disease management approach that encompass the use of chemicals, plant extracts/botanicals, antagonist microorganisms and organic amendments, could be the most economical and effective strategy to manage foot and root rot and other soil borne plant diseases.

Keeping in view, economic importance of the crop, losses caused by *S. rolfsii* in betel vine and possible management options; the present experiment was carried out in the Plant Protection Laboratory of Agrotechnology Discipline, Khulna University to evaluate the effect of some selected fungicides, plant extracts, antagonist microorganisms and organic amendments at different concentrations on the mycelial growth and sclerotia formation of *S. rolfsii*, causal agent of foot and root disease of betel vine. The results obtained during present experiment are being summarized in the following paragraphs.

Results (Table. 5) obtained on *in vitro* evaluation of fungicides indicated that all the tested fungicides significantly inhibited mycelial growth and sclerotial formation of *S. rolfsii* over control at all the tested concentrations. Among the tested fungicides, no growth i.e.100% inhibition was found at every concentration of Exzole 50 SC and Provax-200. In case of Unisaff 75WP, highest percentage of inhibition (61.41%) was recorded at 500 ppm concentration followd by Dithane M-45 at 500 ppm concentration (56.79%).

Performance of all the tested four fungicides significantly ($p<0.01$) inhibited the sclerotia formation of *S. rolfsii* whereas number of sclerotia formation decreased with increases of concentrations. Among tested four fungicides, no sclerotia were formed by applying Exzole 50 SC and Provax-200 fungicides i.e. 100% reduction of sclerotia was found against *S. rolfsii* at all concentrations. Then significantly lowest number of sclerotia was recorded in Unisaaf 75WP at 500 ppm concentration (200.00) followed by Dithane M-45 at 500 ppm concentration (285.33).

The results of Table 6 showed that the effect of different plant extracts at different concentrations on percent inhibition of mycelial growth of *S. rolfsii* was substantially affected ($p<0.01$). In all concentrations, the growth inhibition due to the application of different extracts was found to be increased with the increase of concentration. The highest percentage of mycelial growth inhibition (63.20%) was observed by the application of neem extract at 30% concentration followed by tulsi at 30% concentration (59.26%). However botanicals, marigold leaf (22.77% only at 30% concentration) and allamanda leaf (13.86% only at 30% concentration) were found comparatively less effective with lowest inhibition percentage. The lowest growth inhibition was observed with the application of different extracts at lower concentration.

Effect of plant extracts/botanicals on sclerotia formation also differed significantly ($p< 0.01$) compared to the control. In all concentrations, number of sclerotia formation was decreased with increasing concentration. Neem and tulsi were found to be more effective than the other extracts against sclerotia formation of *S. rolfsii* (Table 6). The highest number of sclerotia formation (824.00) was recorded in untreated plate and lowest number of sclerotia was recorded in neem leaf extracts at 30% concentration (200.00).

From the experiments on antagonist microorganisms, it was found that the *T. harzianum* exhibited fungistatic activity against *S. rolfsii*. The highest percentage of mycelial growth inhibition (88.91%) was recorded with *T. harzianum*.

Results obtained on sclerotia formation, the highest number of sclerotia (800.00) was produced in untreated plate and no sclerotia were formed in media containing *T. harzianum*.

Effect of secondary metabolites of *Bacillus subtilis* strain (ATCC 6633) at different concentrations on percent inhibition of mycelial growth of *S. rolfsii* was substantially affected ($p<0.01$). In all concentrations, the growth inhibition was found to be increased with the increase of concentration. However, significantly highest mycelial growth inhibition (89.54%) was recorded at 1:6 concentration. These were followed by the concentrations *viz.*, 1:4 concentration (83.50 %) and 1:2 concentration (and 72.90 %), respectively (Table 7).

The result on the formation of sclerotia at different concentrations was differed significantly ($p<0.01$) compared to the control. The lowest number of sclerotia (34.00) was recorded at 1:6 concentration. These were followed by the concentrations *viz.*, 1:4 (103.33) and 1:2 (312.00) respectively.

From the (Table 8), it was revealed that all the tested organic amendments in different concentrations significantly ($p< 0.01$) inhibited the mycelial growth of *S. rolfsii*. In all concentrations, mycelial growth inhibition was increased with increasing concentration. Among different tested organic amendments, neem cake at 30% concentration showed highest percentage (71.75%) of mycelial growth inhibition followed by wood ash at 30% concentration (64.42%) which was significantly differed from other amendments. The lowest growth inhibition was observed with the application of different extracts at lower concentration.

Effect of organic amendments on sclerotia formation also differed significantly ($p < 0.01$) compared to the control. In all concentrations, number of sclerotia formation was decreased with increasing concentration. However, signaficantly lowest number of sclerotia (205.33) was recorded with neem cake at 30% followed by wood ash (209.33) at 30% concentration.

CONCLUSION

Thus from the results obtained on various aspects during present experiments on *Sclerotium rolfsii*, causing the foot rot of betelvine, it can be concluded that;

I. All the fungicides, evaluated *in vitro* significantly reduced mycelial growth and sclerotia formation of *S. rolfsii*. However, the fungicides Exzole 50 SC and Provax-200 were most effective at all concentrations.

II. Aqueous leaf extracts of all the four botanicals evaluated *in vitro* were found fungistatic to *S. rolfsii* at different concentrations. Neem and Tulsi leaf extracts at 30% were found to be more effective against mycelial growth and sclerotia formation.

III. The bioagent *T. harzianum* showed potential antagonistic activity against *S. rolfsii* in dual culture plate technique.

IV. The secondary metabolites of *Bacillus subtilis* strain (ATCC 6633) evaluated *in vitro* and proved to be potential antagonists against mycelial growth and sclerotia formation of *S. rolfsii* at all concentrations.

V. All the four organic amendments evaluated *in vitro* were found to be more effective against mycelia growth and sclerotia formation of *S. rolfsii* at higher concentration. Among them, neem cake and wood ash showed highest percentage of growth inhibition and lowest number of sclerotia formation at 30% concentration.

CHAPTER VII
REFERENCES

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REFERENCES

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