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Morphological and Molecular Characterization of Sclerotium ROLFSII SACC. and Integrated Management of Foot Rot Disease of Betel Vine (PIPER BETLE L.)

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University of Rajshahi

Ph. D. Thesis

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *SCLEROTIUM ROLFSII* SACC. AND INTEGRATED MANAGEMENT OF FOOT ROT DISEASE OF BETEL VINE (*PIPER BETLE* L.)

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A Thesis Submitted for The Degree of
Doctor of Philosophy
In The Department of Botany
University of Rajshahi, Bangladesh

SUBMITTED BY

NARGIS TANJILA

BSc (Hons), MSc

June 2018

Mycology, Plant Pathology & Microbiology Laboratory
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DECLERATION

I do hereby declare that this PhD thesis entitled "**Morphological and Molecular Characterization of *Sclerotium rolfsii* Sacc. and Integrated Management of Foot Rot Disease of Betel Vine (*Piper Betle* L.)"** has been originally carried out by me under the guidance and supervision of Professor Dr. Most Ferdousi Begum, Department of Botany, University of Rajshahi and it has not been submitted to anywhere for any other degree.

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উদ্ভিদ বিজ্ঞান বিভাগ
রাজশাহী বিশ্ববিদ্যালয়
রাজশাহী-৬২০৫, বাংলাদেশ।

CERTIFICATE

This is to certify that the thesis entitled "**MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *SCLEROTIUM ROLFSII* SACC. AND INTEGRATED MANAGEMENT OF FOOT ROT DISEASE OF BETEL VINE (*PIPER BETLE* L.)"** submitted by Nargis Tanjila Roll No.10503, Registration No. 2880, Session: 2010-2011, Department of Botany, University of Rajshahi, Bangladesh embodies original work done by her under my guidance and supervision for the degree of Doctor of Philosophy. The work or part of it has not been submitted before as candidature for any other degree.

Supervisor

_____

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DEDICATED
TO
MY BELOVED PARENTS
&
LOVING SON TANJIM

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The Author

ABSTRACT

Betel vine (*Piper betle* L.) is an important cash crop of Bangladesh but this crop suffers from several diseases and among them foot rot disease is the most devastatic. In the present research betel vine plantations were surveyed in different locations of Northern Parts of Bangladesh during June 2011 to December 2013. The highest disease incidence was recorded at Mohonpur area in Rajshahi district during 2011-2012. On the other hand in 2012-13 cropping season, the highest disease incidence was recorded in Baghmara area. Total twenty two isolates of *S. rolfsii* were isolated from foot rot affected portion of the betel vine boroj of different areas of Northern Parts of Bangladesh. These isolates were considered for pathogenesity test and morphological and molecular characterizations. The most virulence isolate was Baghmara-1 showed 23.42% disease severity. On the basis of mycelial growth rate the isolates were grouped as the fast grower BA-1, PA-1 and CH-2; slow grower MA-2, MO-2, PO-1, PO-2, DU-1, PU-2, N-2 and medium grower CH-1, BA-2, BA-3, BA-4, MA-1, MO-1, DU-2, PU-1, NA-1, PU-2, PA-2. For somatic compatibility test, total 22 isolates were tested in 122 combinations and only thirty eight combinations showed somatic compatible reaction and others showed somatic incompatible reaction. Combined morphological data of twenty two characters were analyzed by MVSP and the results showed six groups at 62% similarity level as cluster-1 BA-1, N-2, MO-2, BA-4, MA-2, MO-1, BA-5, DU-2, PU-1, BA-6, PO-2, PA-2`; cluster-2 DU-1 cluster-3 BA-3, PA-1,N-1; cluster-4 BA-2, CH-1; cluster-5 MA-1, PU-2 and PO-1 and cluster-6 CH-2. RAPD PCR was conducted using RAPD-1, RAPD-2, and RAPD-3 and RAPD-4 primer. Dendogram was constructed using PAST software which separated the isolates into two groups and each groups further divided by different sub groups. Representative isolates of betel vine were identified as *S. rolfsii* (teleomorph: *Athelia rolfsii*) based on phylogenetic analysis using rDNA gene sequicing. Based on the phylogenetic relationship and sequence alignment showed that there is variation exists among the *S. rolfsii* isolates in Bangladesh. So, the microclimates of different regions may influence the pathogen morphology and genetic diversity. In *in vitro* condition eight chemical fungicides namely secure, thiovit, ridomil, rovral, antracle, dithan M-45, cupravit, bavistin were evaluated. Among them the most effective chemical fungicides were bavistin and dithan M-45

which showed 100% inhibition at the concentrations of 100, 200, 400 and 800 ppm while no inhibition was exhibited in control. Twelve medicinal plants were evaluated against *S. rolfsii* using three solvents i.e. water, ethanol and acetone. Radial mycelial length (mm) and percent inhibition radial growth (PIRG) were measured. Out of twelve plants maximum antifungal activity (100%) were exhibited in *D. metel,* and *L. inermis* at all used solvents and concentrations while no inhibition was exhibited in *H. indicum*. Among the six antagonists, the highest inhibition was recorded (66.10%) in *T. harzianum* after 7 days of incubation in dual culture terchnique. On the other hand in poison agar technique, the highest inhibition (66.88%) was recorded in *T. harziunum*. Out of eleven soil amendments neem leaf dust amended soil was the most effective because hundred percent reduction of sclerotia germination was observed in this amendment. For management of foot rot disease different fungicides, plant extracts, bio-control agent and soil amendments were tested in boroj condition during 2014-15 cropping season. Out of three fungicides bavistin showed better performance and among the plants extracts, *A. indica* L. was the most effective. Among the antagonists the lowest percent disease incidence and highest yield was observed in *T. harzianum*. On the other hand, neem leaf dust showed better performance for every yield contributing characters. In integrated disease management the highest yield and the lowest disease incidence was recorded in treatment T_6 (bavistin + datura leaf extracts + *T. harzianum* + neem leaf dust). To study the biochemical changes within the treated betel vine leaves, HPLC analysis were conducted. The total number of phytocompounds were varied with the treatments and the maximum number (31) of compounds and the highest (35%) percentage area was detected in T_6 (T_1+T_2+T_3+T_4) treatment. Biochemical analysis of betel vine leaf also indicate that the integrated treatment enriched the amount of phytoconstituents of the plants. From the results it may be concluded that the isolates of *S. rolfsii* were morphologically and molecularly varied within the locations and integrated management will be fruitful means to control of foot rot disease of betel vine. Thus the present results will provide a valuable information to the farmers of Bangladesh for management of foot rot disease of betel vine and has scope to extend more research in this content in future.

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ABBREVIATIONS

%	:	Percent
@	:	At the rate of
°C	:	Degree Celsius
BBS	:	Bangladesh Bureau of Statistics
cm	:	Centimeter(s)
e.g.	:	(exempli gratia) for example
et. al.	:	and other people
etc	:	et cetra (and all the others)
F	:	Ratio between two mean squares
Fig	:	Figure
g	:	Gram
h	:	Hour
ha	:	Hectare
I	:	Liter
ie	:	(idiest) in the other words
m	:	Meter
mg	:	Milligrame(s)
ml	:	Millilitre(s)
mm	:	Millimetre(s)
mt	:	Metric ton(s)
pH	:	Negative logatrithm of hydrogen ion (-log H+)
PPM	:	Parts per million(s)
RH	:	Relative humidity
S	:	Susceptible
sp.	:	Species (Singular)
spp	:	Species (Plural)
sq	:	Square
t	:	Ton
T	:	Treatments(s)
viz.	:	Videli (namely).
AFLP	:	Amplified fragment length polymorphism
DI	:	Disease incidence

DNA	:	Deoxyribo nucleic acid
DS	:	Disease severity
EDTA	:	Ethylene di amine tetra acetic acid
ha	:	Hectare
ITS	:	Internal Transcribed spacer
LSD	:	Least significant difference
MVSP	:	Multi Variable Statistical Package
PCR	:	Polymerase Chain Reaction
PDA	:	Potato dextrose agar
PD	:	Potato dextrose
ppm	:	Parts per million
RAPD	:	Randomly amplified polymorphic DNA
RCBD	:	Completely randomized block design
SCG	:	Somatic compatibility group
SDS	:	Sodium dodecyl sulphate
SWD	:	Sterile distilled water
TAE	:	Tris- acetate – EDAT
TSP	:	Triple super phosphate
UPGMA	:	Unweighted pair group method with arithmetic mean
UV	:	Ultra violate

CHAPTER- 1

Introduction

INTRODUCTION

The betel vine (*Piper betle L.*) is an important cash crop of Bangladesh. Betel leaves cultivation areas are 55,417 acres with a total annually production of 136284 metric tons (BBS, 2012-13). It is used as a masticator and has a great medicinal significant. It is an important economic crop in Bangladesh and exported to Middle East, Britain, Pakistan and some other African countries Khaleque (1998). 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 (Fila *et al.* 2006 and BBS, 2010). The leaf is widely used in social, cultural and religious events for hospitality and also has medicinal value (Guha, 2006). Foot rot diseases caused by *S. rolfsii Sacc.* is one of the major disease of betel vine. Each year this disease is spreading very rapidly but still no such effective control measures has shown against the disease.

1.1 Details view on betel vine

1.1.1 Origin and history

Betel vine belongs to the dicotyledonous family Piperaceae which comprises of 12 genera and 13000 species (Chaurasia, 2001). Malayan Archipelago may be the home of betel vine (De Candolle, 1884) and considered that it had been under cultivation for more than 2000 years. Betel vine has become cultivated with in rather short period, subsequently spreading through tropical Asia and Malaysia. Much later it spread into Madagascar and East Africa and later on it has been transplanted to the West Indies. Betel vine is largely grown as an important cash crop through out the tropical and subtropical regions like India, Bangladesh, Srilanka, Malaysia and to some extent of Singapore, Thailand, Burma and Papua New Guinea. Betel vine has been refered in the Indian history, religion and culture dating back to 3000 B.C. It is geographically belongs to the region bounded by 68° E to 118° W longitudes and 30° N to 12° S latitudes.

1.1.2 Taxonomic description

Betel vine is a perennial, dioeciously (male and female plant separate), rod climber plant. In normal practice under close conservatories (Familiarly known as 'Baroj') plant does not go under flowering. However, under open system of cultivation in south Indian conditions both male and female plants start flowering after 2-3 years of planting (Maiti, 1999). The 'Ashami' cultivar, which is cultivated openly in betelnut garden in Nilphamari district of Bangladesh used to produce flowers. The vines under wild condition or when trained on areca nut of coconut plantations, are vigorous, grow as high as 20m and stem of 15 to 20 cm in diameter and produce dense, thick green leaves. In close conservatories that in 'Baroj' conditions pan grow about 3m high with poor branching but the leaves are comparatively larger in size (Balasubrahmanyam and 1981).

According to Hutchinson piperales in the 58th order of the phylum Angiospermae the systematic postion of betel vine is:

Phylum	:	Angiosparmae
Sub-phylum	:	Dicotyledonae
Division	:	Harbaceae
Order	:	Piperaceae
Genus	:	*Piper*
Species	:	*P. betle.*

1.2 Importance of betel vine

Betel vine is an important economic crop in Bangladesh and is grown mainly for its leaves, which are chewed. It is also used for its medicinal qualities; it has antibacterial and antifungal properties and is used against Sankebites, Catarrh, night blindness and cerebral congestion, Pan is an important cash crop extensively cultivated in different parts of Bangladesh covering an area of about 34,135 acres with a total production of 73,525 metric ton (Hossain, 1986). This crop has a great market value inside and outside of the country.

1.3 Traditional uses of betel leaves

Betel leaf has some traditional uses. It is a popular home remedy for headache. The betel leaf has analgesic and cooling properties. Its juice is credited with diuretic properties. Its juice, mixed with dilute milk and sweetened slightly, helps in easing urination. Betel leaves play a vital role in the treatment of nervous pains, nervous exhaustion and debility. The juice of a few betel leaves, with a teaspoon of honey, will serve as a good tonic. A teaspoon of this can be taken twice a day. Betel leaf is an excellent household remedy in the treatment of cough and sore throat and also useful in pulmonary affection in childhood and old age. The leaves, soaked in mustard oil and warmed, may be applied to the chest to relieve cough and difficulty in breathing. In the case of constipation in children, a suppository made of the stalk of betel leaf dipped in castor oil can be introduced in the rectum. The application of leaves smeared with oil is said to promote secretion of milk when applied on the breasts during lactation. Applied locally, betel leaves are beneficial in the treatment of inflammation such as arthritis and orchitis that is inflammation of the testes.

1.4 Therapeutic values of *Piper betle*

Aqueous and methanol extract of the leaves of *Terminalia catappa* L., *Manilkara zapota* L. and *Piper betel* L., for antibacterial activity against 10 Gram positive, 12 Gram negative bacteria and one fungal strain, *Candida tropicalis* were studied(Nair and Chanda 2008). Antihistaminic activity of *Piper betle* L. leaves for its antihistaminic activity were evaluated (Hajare *et al*. 2011). Anti-inflammatory effects of betel leaf has been reported to possess anti-inflammatory activities at non-toxic concentrations in the complete Freund's adjuvant-induced model of arthritis in rats (Dohi *et al*., 1989). Antioxidant effects of betel leaf constituent's eugenol, hydroxychavicol and alpha-tocopherol were also shown to enhance the levels of GSH in mouse skin and liver (Azuine *et al*. 1991 and Bhide *et al*.1991). Recently, Manigauha *et al*., (2009) observed that the methanolic extracts of the betel leaves possess reducing power, DPPH radical, superoxide anion scavenging and deoxyribose degradation activities.

Antimutagenic effects of the betel leaf is devoid of mutagenic activities in both prokaryotic and eukaryotic assay system and also to possess antimutagenic (Shirname *et al*., 1983) and anticlastogenic effects (Bhattacharya *et al*., 2005).

Anti-haemolytic activity was studied by (Chakraborty *et al*., 2011), using erythrocytes model *piper betel* leaf extracts and the extent of lipid peroxidation of the same was also determined.

Antibacterial activity were evaluated that the cold aqueous, methanolic, ethanolic and ethyl acetate extracts of dried leaves of all the four varieties of *Piper betel* at a final concentration of 500 mg/ml were tested against pathogenic microorganisms such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* using agar well diffusion method (Agarwal *et al*., 2012)

Antifungal activity of the hydroxychavicol, isolated from the chloroform extraction of the aqueous leaf extract of *Piper betel* L., (Piperaceae) was investigated for its antifungal activity against 124 strains of selected fungi (Ali *et al*., 2010). In vitro screening of antifungal activity of plants in Malaysia were studied by (Nazmul *et al.,* 2011 and Nazmul *et al.,* 2013).

Anti-diabetic activities were investigated the antidiabetic activity of *Piper betel* leaves, tested in normoglycaemic and strepozotocin (STZ)-induced diabetic rats using oral administration of hot water extract (HWE) and cold ethanolic extract (CEE) (Arambewela *et al*. 2005).

Inhibitory effects of *Piper betle* on production of allergic mediators by bone marrow-derived cells and lung epithelial cells anti-allergicactivity were studied (Wirotesangthong *et al.* 2008).

Oral hygiene and concluded that oral microbial population due to synergistic effect of the combination of betel leaf, cadamom and clove. Dental caries had a chronic endogenous infection caused by the normal oral commensally flora were examined for the oral hygiene (Bissa *et al.* 2007).

1.5 Cultivars of betel vine in Bangladesh

The betel leaf might have developed into a large number of ecotypes owing to their long presence in a particular environment. In different localities different varietal names have developed (Hossain *et al.* 1986). In respect of size, color and teste of leaf they are different from each other. Cultiver 'Mitha,' 'Sanchi' in Maheskhali Upazilla in Cox's Bazar district; Cultiver 'Deshi,' 'Ashame, 'Moohore,' in Nilphamari district; Cultiver 'Chaltabuti, 'Mahanali', 'Patabahar' are found to grow in Barisal district; Cultiver 'Shal,' 'Dooga, 'Sauchi' in Rajshahi district; Cultivar 'Bhola pan' in Bhola district; Cultivar 'Rangpuria pan' in Rangpur district; Cultivar 'Shatoshi' in Nawabganj district; Cultivar 'Xailo' in Bagerhat district; Cultivar 'Gach pan' in Ukhia and Teknaf Upazilla in Cox's Bazar and Sylhet district.

1.6 Diseases of betel vine

Although cultivation of betel vine is a lucrative profession, the crop is however, threatened almost every year by ravage diseases. The betel vine gardens (Baroj) are specially designed to maintain condition of moisture, humidity, temperature and light for growth and development of betel vine crop. Micro-environment are also ideal for the growth of pathogenic fungi, in certain cases nematodes, bacteria, insect pest, scale insect, betel vine bug, mealy bug, white fly and red spider mites are also involved (Chaurasia, 2001). The important disease which affect the plants are foot rot, leaf rot, root rot, leaf spot, marginal blight, sclerotial wilt, stem rot, powdery mildew, bacterial leaf spot, leaf blight and root rot nematode. Information on the disease of "Pan" in Bangladesh appears to be limited with the reports by (Anonymous, 1976; Alamgir, 1985). During the East Pakistan the sporulation of *Phytophthora parasitica* and occurance of leaf rot were reported. Some research works have been done on root rot and anthracnose disease of betel vine (Akhter *et al.,* 2002). They studied also some biological activities of the pathogen and developed control measure of these diseases.

Foot rot disease of Betel vine

Foot rot disease caused by *S. roflsii* is one of the major diseases of betel vine. Each year this disease is spreading very rapidly but still no such effective control

measurements has shown against the diseases. Root rot disease is observed throughout the season but severely in the rainy season and it spreads widely over all the borojes. Rooting of stems near the soil level produces black discoloration on the affected region. After rotting at the foots of stems the plants are wilt. In the wilted plants the disease caused following out of the pith of the first internodes. The fungus forms dense white colony mycelial mass around the base of stem. Among the diseases of betel vine, foot rot caused by *Sclerotium rolfsii* is the most overwhelming disease which decreases the production of betel leaf to a great extent. In Bangladesh, foot rot disease caused by *S. rolfsii* in different crops have been reported by many reserachers (Talukder, 1974, Meah 1994, and Khan, 2003).

Smptoms of foot rot disease

Foot rot disease is observed through out the season but severely in the rainy season and it spreads widely over all the barojes. The affected plants show general loss of tissue and dropping of leaves and tender shoots. Rotting of stems near the soil level producting black discoloration on the affected region. After rotting at the foots of stems the plants are wilt. In the wilted plants the disease causes hollowing out of the pith of the first internode. The cortex is found under a soft and somes times slimy rotting over about three nodes and internods. The fungus forms dense white colony mycelia mass around the base of stem. Tumerous sclerotic parts form on the affected area.

Environmental factor of foot rot disease

An epidemiological studies were reported that the maximum temperature, maximum relative humidity and rainfall played an important role in the development of the diseases of betel vine (Anonymous, 2000-2006; Maiti and Sen, 1982). According to Punja *et al*. (1988), temperature is the principal limiting factor in the geographic distribution of *S. roifsii*. The disease rarely occurs where average daily minimum winter temperatures are below freezing (0ºC). Maximum disease occurs at 25-350ºC, fungus *S. rolfsii* attacks all plant parts in the contact with the soil under favourable environmental conditions including stems, roots, and fruits.

1.7 Cultivation process of betel vine

In Bangladesh most of the betel vine cultivation takes places in close conservation called 'Baroj' but in Nilphamari, Rangpur, Dinajpur and Hilly area it grows in open field condition with the support of Areca nut or other tree plants. The betel leaf is also cultivated in most South and Southeast Asia. Since it is a creeper, it needs a compatible tree or a long pole for support. Fertile soil is best for betel vine cultivation. The farm yard is fenced with bamboo sticks and coconut leaves. The betel vine cuttings are planted after proper dressing in the months of May and June, at the beginning of the monsoon season. The plants are neatly arranged in parallel rows about two feet apart, and the saplings are twined around upright sticks of split bamboo and reeds. Proper shade and irrigation are essential for the successful cultivation of this crop. The plants are regularly watered in the hot months. The leaves of the plant become ready for plucking after one year of planting and the production lasts for several years from the date of planting. Betel needs constantly moist soil, but there should not be excessive moisture. Hence, frequent light irrigations are given. The quantity of irrigation water should be such that the standing water should not remain for more than half an hour in the bed. If water logging by heavy rains or excess irrigation occurs, drainage should be arranged immediately. The best time for irrigation is morning or evening.

1.8 Betel leaf production in Bangladesh

In Bangladesh betel vine largely grown in different region even in hilly area. This crop has a great market value inside and outside the country. Total cultivated area and production of betel vine leaves by regions from 2008-2009 to 2012-2013 in Bangladesh are shown in Table- 1.1 The important betel vine production districts are Rajshahi, Khulna, Barisal, Chittagong, Sylhet, Mymensingh, Chuadanga, Meherpur, Cox's Bazar, Kushtia, Jessore, Nilphamari, Bhola, Bagerhat and Satkira. In addition to the small scale production is also found in the district of Rangamati, Bandarban, Moulvibazar, habiganj, Netrokona, Jamalpur, Sherpur, Kishoregang, Gazipur, Munshiganj, Manikganj, Narsingdi, Narayanganj, Faridpur, Pabna, Gopalganj, Madaripur, Sariatpur, Rajbari, Jhenaidah, Magura, Narail, Pirojpur, Lalmonirhat and

Nawabganj (Roy, 1993). The massive betel vine production Upajilas are Babuganj, Uzirpur, Banaripara, and Gaurrad under Barisal district; Fatikchari under Chittagong district; Ukhia, Taknaf, Maheskhalis under Cox's Bazar district; Mahanpur, Bagmara and Paba under Rajshahi district; Chilahati under Nilphamari district and Sadar Upazilla of Bagerhat; Meherpur and Chuadanga district.

Table 1.1 Area and production of betel vine leaves by region during 2008-2013 in Bangladesh (area in acres and production in the metric tons).

	2008-09		**2009-10**		**2010-11**		**2011-12**		**2012-13**	
	Area	**prod**	**Area**	**Prod**	**Area**	**prod**	**Area**	**prod**	**Area**	**prod**
Bandarban	138	97	127	96	115	102	113	94	144	98
Chittagong	5088	23963	4152	8282	3259	4757	14292	30182	13063	17470
Comilla	582	1825	585	2010	546	1957	527	1589	569	1773
Khagrachari	95	137	102	153	108	158	108	163	108	161
Noakhali	1419	2090	1408	1998	1355	2979	1361	3015	1359	2998
Rangamati	165	128	167	131	168	135	183	148	176	142
Sylhet	801	837	342	739	435	726	444	735	440	731
Dhaka	843	2350	749	1933	714	1815	734	1899	724	1858
Faridpur	1653	4449	1852	3071	1805	2938	2015	3877	1912	407
Jamalpur	146	508	141	457	136	424	141	444	139	435
Kishorgonj	211	346	201	348	182	303	173	292	178	298
Mymensingh	333	1020	350	1291	354	1609	367	1415	361	1212
Tangail	46	293	72	247	71	230	45	172	58	201
Barisal	8025	7315	8798	10119	8088	10522	7912	13828	4301	12176
Jessore	7007	18914	7010	23529	7288	29714	7716	49519	7502	47405
Khulna	2736	8231	2823	6832	2745	5759	2993	6375	2870	6068
Kushtia	7507	16758	10408	18598	10674	23423	11005	20731	10839	22078
Patuakhali	1859	37255	1900	1620	1917	3960	1892	3906	1905	3934
Bogra	657	1531	661	411	687	1710	228	566	458	739
Dinajpur	233	410	218	133	216	430	215	436	216	433
Pabna	56	125	59	9392	61	132	64	158	63	145
Rajshahi	3306	9504	3367	91681	3410	10986	3466	11173	3438	11081
Rangpur	632	992	645	1187	636	1184	611	1097	624	1141
Total Bangladesh	43578	105448	44141	91681	45070	105953	56605	151814	55417	136284

Source: BBS (2008 to 2013) in Bangladesh.

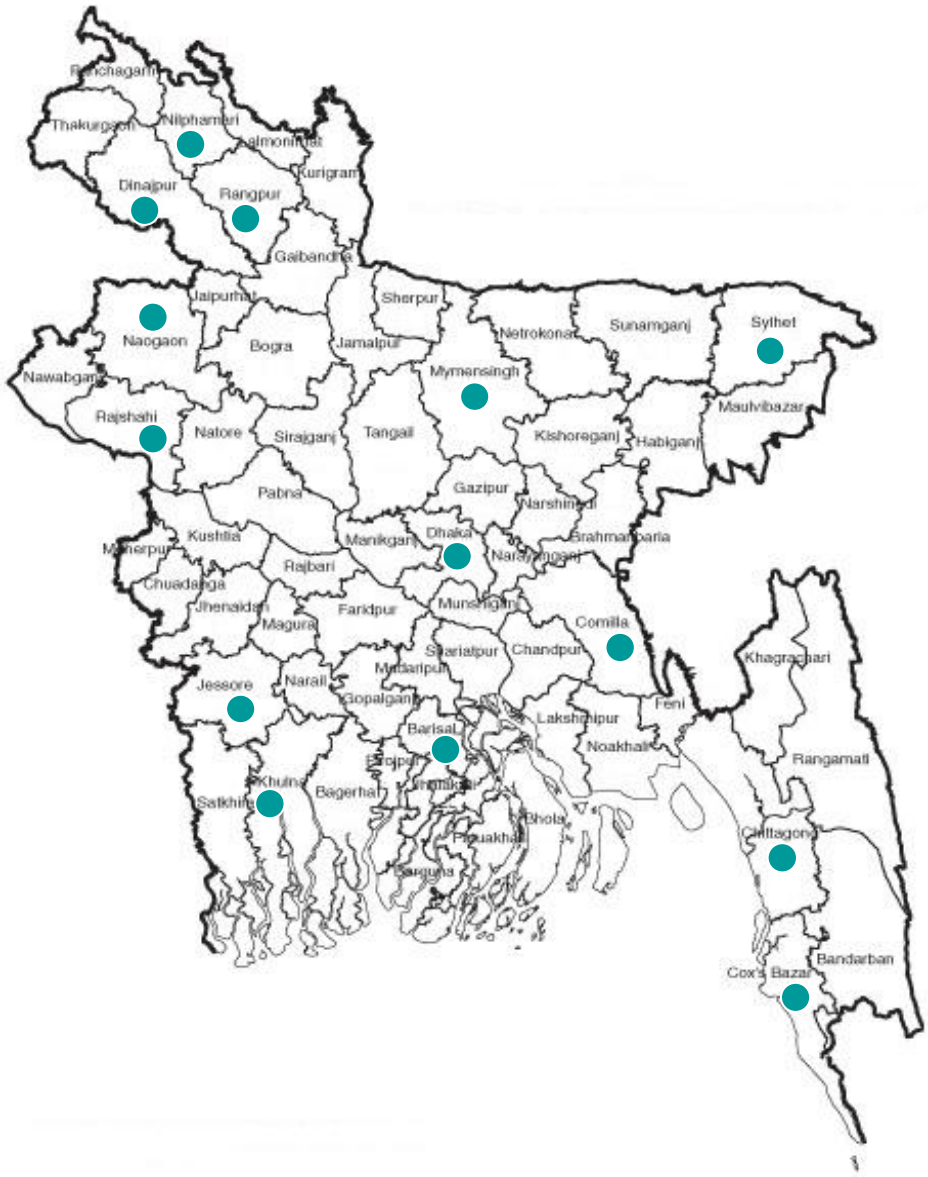


Fig 1. District Map of Bangladesh showing production areas.

1.9 Morphological characteristics of *S. rolfsii* causal agent of betel vine.

The mycelium of *S. rolfsii* highly variable in appearance depending on its age, composed at first of colorless hyphae, later becoming yellowish and then deep brown in color. The mycelial stage producing sclerotia is the commonly uncounted stage. In artificial culture media, the fungus produces a fast growing mycelium with septate hyphae. Young colonies may look white but older mycelium is invariably some shade of brown sclerotia may be formd. In some cases (primary stages), the sclerotia are

light white while in some they are so dark as to look black. The diameter of hyphae ranges between 5 and 14 microns. The septa are typically of dolipore type. The lateral branches from the main hyphae are invariably constricted at the point of origin and a septum occurs in the branch near the junction with the main axis. In young advancing hyphae the branching almost invariably occure near the distal septum but it may occur at any point in older hyphae. The mycelium produces barrel-shaped cells, which have been termed as chlamydospore.

The sclerotia of the fungus are distinct from many other fungi in that there is no differentiation of the scierotial tissue into a rind and internal medulla although outer cells may be darker and thicker walled. On the host, the mycelial stage of the fungus produces superfacial, irregular, scab-like, black sclerotia growing on the surface of the usually subterranean parts of the plant. The sclerotia are flattened on the lower side. This is preceded or accomparied by a superficial, dark colored, short-celled, abundantly branched, stout mycelium. This mycelium is entirely different in appearance form the mycelium inside the host tissues, which is slender, hyaline, and longer celled. No other spores are produced in the asexual stage of the fungus.

Taxonomic Position

Division : Eumycota
Sub: Division : Deuteromycotina
Form: class : Hlyphomycetes
Form: order : Agonomycetales/ Mycelia sterilia
Form: family : Agonomycetaceae
Form: genus : *Sclerotium*
Form: species : *S. rolfsii.*

1.10 Molecular variability of *S. rolfsii*

S. rolfsii is a soil born plant pathogen causing disease on a wide rang of agricultural and horticultural crops. The fungus was placed in the form genus *Sclerotium* by (Saccardo, 1913) as it formed differentiated sclerotia and sterile mycelium. The fungi included in this genus were characterized by production of small tan to dark brown or black spherical sclerotia with internally differentiated rind, cortex and medulla. *S. rolfsii* was reported as the best known member of the genus by Punja and Damiani (1996). The isolaes of *S .rolfsii* originating from various plant species and different geographical regions present

wide variation growth rate, morphological characteristics, mycelial compatibility and also exhibited genetic variability. Eight strains of *S. rolfsii* using randomly amplified polymorphic DNA (RAPD) analysis DNA was extracted from the mycelial mat of each strain grown on PDA plate for 7 days at 28°C. Mycelium (200 mg) was transferred to 1.5 ml micro centrifuge tubes containing liquid nitrogen.The data obtained under this experiment confirmed the efficiency of RAPD-PCR technique for determination and estimation of genetic similarities and differences among fungal strains Punja and Sun (2001). Therefore, RAPD analysis was found to be an informative DNA marker system to assess genetic relatedness and diversity among different strains (Tanwir *et al*., 2007; Asif *et al*., 2005). RAPD-PCR analysis had also been used by other worker to investigate genetic variation among isolates of *S. rolfsii* collected from different geographical regions. Punja and Sun, (1997) compared 128 isolates of *S. rolfsii* from 36 host species and 23 geographic regions by means of random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) which confirmed that many isolates from the same host belongs to the same mycelial compatibility group (MCG). The variation among 30 isolates of *S. rolfsii* from different hosts and regions of Brazil was studied Almeida *et al*., (2001) undertaking analysis of genomic DNA through random amplified polymorphic DNA (RAPD-PCR) techniques. These techniques confirmed that there was considerable variability among isolates in relation to the number, size and location of sclerotia on the surface of medium. Thus, the present study was undertaken to assess the significant genetic variability by RAPD techniques to distinguish different virulent isolates of *Sclerotium rolfsii* for durable management package against the disease.

1.11 Disease managment

Management of dfferent types of diseases is difficult and depends on a combination of cultural, biological and chemical methods. Good cultural practices include rouging, eliminating weed hosts, and avoiding crop injury during cultivation. A dense canopy increases disease incidence, thus increasing plant spacing's can help keep infection down. Because *S. rolfsii* has such a broad host range, crop rotation has less of a chance of being successful as there are few resistant crops.

Deep plowing (at least 20 cm) with a moldboard extension inverts soil so that organic matter, sclerotia, and plant debris are buried at least 10 cm beneath the surface. This helps to eliminate inoculum when plowing occurs just prior to planting. Buried soil must not be re-surfaced during the growing season. Compost, oat, or straw added to the soil has been shown to limit disease incidence. The addition of an amendment may increase populations of antagonistic soil microorganisms. This method may be reasonable for small-scale farms and green houses, but is probably not practical for large farms unless it is combined with crop rotation. Soil solarization or solar heating is a relatively recent method for controlling *S. rolfsii* inoculum Sclerotia grown in vitro are still viable after 12 hours at 45Cº, but are killed in 4-6 hours at 50ºC and in 3 hours at 55ºC. Covering soil with transparent polyethylene sheets during the hot season increases soil temperatures and kills sclerotia when the temperature under the sheets get hot enough for an appropriate length of time. Most field trials have achieved sclerotia degradation at 1 cm, but eradication at greater depths usually did not occur. In addition, this method requires immediate planting, which excludes crops that are planted in spring because temperatures are not high enough to affect sclerotia. Soil solarization combined with the addition of *Trichoderma harzianum* has been shown to decrease disease incidence more than either treatment alone. However, the practicaly of soil solarization is questionable. First, the length of time of solarization may be limited. Second, it is not known what affect solarization has on the existing soil microflora and what affect any microflora change would have on the crop. Third, it is not known what affect solarization has on non-target and/or temperature-tolerant pathogens. Mulching with black plastic has been shown to reduce disease incidence and perhaps provide greater crop yields. Black plastic mulch (BPM) prevents or

reduces the "bridge" of dead tissue between the soil and plant and may increase temperatures, conserve soil moisture, and help control weeds for a higher crop yield. BPM alone and BPM with floating row covers both provide better control than no treatment. Disease incidence can still be high, but significantly lower than no treatment. BPM alone and BPM with floating row covers combined with a chemical treatment (PCNB) provide even better control.

A number of antangonistic fungi have been shown to provide control against *S. rolfsii* in controlled experiments, though field trial results vary. Some of the commonly used organisms are: *Trichoderma harzianum, T. viride, Bacillus subtilis, Penicillium* spp., and *Gliocladium virens. Trichoderma* spp. are known mycoparasites of a number of plant pathogens. *T. harzianum* colonizes *S. rolfsii* hyphae, disrupts mycelial growth, and kills the organism. Field studies where effective control was obtained, involved application rates in the range of 140 to 600 to 1500 kg/ha. However, the populations of the pathogen and the antagonist were not monitored over time. *T. viride* has been shown to provide good control, especially when used in combination with an herbicide or pesticide. When combined with EPTC (an herbicide) in autoclaved soil, *S. rolfsii* activity was diminished, eventhough EPTC alone stimulated growth of the pathogen. In natural soil, the effectiveness of *T. viride* was reduced in the presence of EPTC, indicating the involvement of other soil microorganisms. *T. viride* in combination with PCNB has been shown to provide good disease control and better yield in artificially inoculated than in non-inoculated, untreated field plots. *T. viride* without PCNB provided statistically similar disease control but a lower yield. PCNB alone was less effective than PCNB with *T. viride* or *T. viride* alone.

Control measures include chemical disinfection of vegetative propagation material, adjustment of soil pH by liming, adjustment of fertilizer regime, and use of herbicides for weed control. Formalin, chlorobromopropene and methyl bromide are among the most promising fumigants for treatment of seed beds or fields for valuable crops.

Pre-plant chemicals and application techniques; fumigants such as metamsodium (Vapam), Vorlex, methyl bromide, and chloropicrin, when applied to soil, reduce southern blight incidence.

1.12 Aims and Objectives of the Present Research

S. 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 omnipathogenic organism (Talukder, 1974). Foot rot disease is one of the major diseases of betelvine which spread widely all over the year. The foot rot of betel vine have been reported from almost all betel vine growing countries in the world including Indonesia, Myanmer, Srilanka (Paul, 1939) and Bagladesh (Roy, 1948) etc. in west Bengal and the highest intensity of foot and root rot have been recorded in Midnapore and Nadia district (Dasgupta and Sen, 1997 and 1999). The extent of losses may vary from 30-100% in case of foot and root rot (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. For the favorable environmental condition foot rot disease increases rapidly day by day in Bangladesh. Rajshahi is one of the major betel vine growing district in Bangladesh. Most of the marginal farmers are involved in betel vine cultivation, as it is a continuous source of income, but still there no effective control measure has been undertaken. Thus keepping these in mind, the present project has been undertaken for the first time in Bangladesh for detail investigation on the biology of *S. rolfsii* specially about morphological and moleculer characteristics and its control with multidisciplinary approach with the following objectives:

1. Survey of betel vine plantations to study the incidence of foot rot disease caused by *Sclerotium rolfsii*.
2. Isolation and identification of the causal pathogen.
3. Pathogenicity test of different isolates of *S. rolfsii*.
4. Morphological characterization of different isolates of *S. rolfsii.*
5. Molecular characterization of different isolates of *S. rolfsii.*
6. *In vitro* evaluation of different fungicides, plant extracts, bio-control agents and organic amendments against *S. rolfsii.*
7. Development of an effective control measure of foot rot disease of betel vine.
8. Analysis of phytochemical compounds of experimental betel vine leaves through HPLC.

CHAPTER -2

Review of Literature

REVIEW OF LITERATURE

Foot rot disease of betel vine caused by *S. rolfsii* is the most destructive fungal disease in Bangladesh and causes 30-100% losses Dasgupta *et al.* (2000). The leaf rot, foot rot and root rot of betel vine have been reported from almost all betel vine growing countries in the world including Indonesia, Myanmar, Sri Lanka (Paul, 1939) and Bangladesh (Roy, 1948). But, very little research appears to have been done in Bangladesh on foot rot disease of betel vine caused by *S. rolfsii*. Few literatures are available on the control of foot rot disease of betel vine caused by *S. rolfsii* mainly from neighboring countries. But few literatures are available on the survey of foot rot disease of betel vine characteristic of the pathogen and temperature, pH and relative humidity effect on the growth and germination of fungul pathogens. On the other side, some of few literatures are available on the post inflectional (*S. rolfsii*) bio-chemical deficiencies of betelvine, such as chlorophyll, total sugar and vitamin C and deficiencies.

2.1 Survey

Jahan *et al*. (2016) evaluated investigation on foot and root rot disease of betel vine (*Piper betle* L.) in Kushtia district of Bangadesh and reported the maximum disease incidence were recorded in Mirpur upazilla where disease incidence ranged from 54.00% to 64.00% and the minimum disease incidence were recorded in Khoksha upazilla where disease incidence ranged from 28.00% to 34.00%.

Mollah (2012) found that 290ºC and 85% RH, 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 18.70ºC and the RH laid around 75% in Satkhira district.

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

Dutta *et al.* (2011) evaluated effect of organic and inorganic sources of N on growth, yield and disease incidence of betelvine (*Piper betle L.*) reported that incedence of diseases like foot rot, leaf spot, anthracnose and bactetial leaf spot was more in urea and less in neem cake treated plots.

Rahman and Sultana (2011) recorded that, in Jamalpur region, the incidence and severity of sclerotial rot of betel vines were more or less highest and lowest throughout the year.

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.

Das *et al*. (2000) found that the disease symptoms of foot and tuber rot of tuberose caused by *S. 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.

Dasgupta and Sen (1999) investigated about betel vine and reported the extents of losses of betel vine vary from 5 to 90 percent.

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.

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 per-cent disease incidence.

Hossain *et al.* (1985) survyed different betel vine gardens at Babugong and Gournadi areas of Barisal district.They reported four severe diseases of betelvine; *Colletotrichum* leaf spot, *Phytophthora* leaf rot, *Sclerotium rolfsii* stem rot and *Fusarium* foot rot.

Maithi and Sen (1982) surveyed three distrcts of west Bengal (India) and reported foot rot caused by *S.rolfsii* may cause losses up to 25-90%.

2.2 Pathogenicity

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.

Datar and Bindu (1974) proved the pathogenicity of *S. rolfsii* on sunflower by soil inoculation method under glass house conditions *S. rolfsii* grown on PDA. Seven discs per flask were added and flasks were incubated for three weeks at 28 ± 20ºC.

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

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

Yaqub and Shahzad (2005) proved *S. rolfsii* highly pathogenic on sunflower, and mildly pathogenic on tomato, lentil, sweet pumpkin and 16 cabbages and non-pathogenic on cauliflower plant in a pot experiments.

Siddique (1997) studied histopathology of foot rot and showed that *S. rolfsii* penetrated through cuticle and spread both intra and intercellularly and destroyed cortex most rapidly and then advanced towards vascular bundle.

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 *S. rolfsii* and *Fusarium oxysporum* compared to early sowing. Artificial inoculation often selected genotypes of lentil to collar rot pathogen, *S. rolfsii* showed that all the lines were susceptible to the test pathogen.

Siddaramaiah (1988) confirmed the pathogenicity of *S. 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 *S. 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.

Mirsha and Bais (1987) used 15 days old fungal culture grown on sand corn meal medium for proving pathogenicity of root rot of barley caused by *S. rolfsii* mixing upper 4-5 layer of soil with inoculums at the rate of one flask per pot.

Palakshappa (1986) observed considerable foot rot infection when betel vine were inoculated with two and three percent inoculum of *S. rolfsii*. They recorded percent infection at four pe rcent and above inoculum levels.

2.3 Morphological characteristics of *S. rolfsii*

Rasu *et al.* (2013) studied morphological and genomic variability among *S. rolfsii* populations and reported that out of 17 isolates tested for their cultural morphology, most of them were observed with compact colonies and few were fluffy colonies. Based on growth rate, they were categorized into three groups such as slow growing, fast growing and intermediate.

Kokub *et al.* (2007) evauated cparative growth, morphoogilacal and moleculer characterization of indigenous *S.rolfsii* strains isolated from different locations of Pakistan and reported that out of 8 strans Growth rate of 8 fungal strains of *Sclerotium rolfsii* on potato dextrose agar plates at 28ºC ranged from 0.86-1.35 mm hour-1. All strains produced round shaped sclerotia with average diameter of 0.5-2.0 mm.

Akram *et al*. (2007) reported mycelial incompatibility and morphological diversity between 12 isolates of *S. rolfsii* collected from different locations of Pakistan (Panjab) and reported that mycelial incompatibility and compatibility between different isolates of *S. rolfsii*.

Sarma *et al*. (2002) investigated the variability in growth and basidial stage production of 26 isolates of *S. rolfsii*. The isolates of *S. roifsii* varied in all of the charaters such as colony morphology, mycelial growth rate, sclerotial production, basidiocarp induction, sclerotial size and color.

Punja and Sun (2001) evaluated genetic divercity among mycelial compatibility groups of *S. rolfsii* (teleomorph *Athelia rolfsii*) and *S. delphini* reported that, the extent of genetic dversity among the isolates of *S. delphinii* was lower than that observed in *S. rolfsii.*

Zarani and Christias (1997) and Sarma *et al.* (2002) reported about morphology of *S. rolfsii* and observed on production of small, spherical and tan to dark brown and black colored sclerotia.

Punja and Sun (1997) observed recognition of non-self from self is the underlying basis of the incompatible reaction. The earlier workers also observed that when mycelia of different isolates belonging to the same species confront with one another, either on agar media or a suitable growth substrate, a distinct zone of demarcation (barrage or aversion zone) was developed between the colonies.

Punja and Damiani (1996) studied morphological diversity and recorded the average sclerotial diameter of 1.0 ± 0.2 mm at 20°C and greatest number of sclerotia (1043 ± 340) was produced by S. *rolfsii* on PDA plates at an incubation temperature of 35°C.

Punja (1988) reported that the large number of sclerotia produced by *S. rolfsii* Sacc. and their ability to persist in the soil for several years as well as the profuse growth rate of the fungus make it well studied facultative parasite and pathogen of major importance through the world.

Wheeler and Sharan (1965) and Zoberi (1980) investigated about morphology of *S. rolfsii* and reported that media which supported extensive growth also produced greatest number of sclerotia.

West (1947) described variations in sclerotial morphology among single basidiospore strains of *S. rolfsii* isolated from clambing fig, indicating that some strains resemble *S. delphini* rather than *S. rolfsii.*

2.4 Moleculer characterization of *S. rolfsii*

Genetic variability among the isolates of *S. rolfsii* was studied many researchers using molecular techniques like RAPD, ITS-PCR and RFLP some are presented here-

Rasu *et al.* (2013) studied morphological and genomic variability among *sclerotium rolfsii* populations and reported the result from UPGMA based dendrogram generated for the *S. rolfsii* isolates revealed that they were divided into two main clusters which were further divided into sub-clusters. Overall, all the isolates have about 54% similarity coefficient indicating that they were genetically varied by their unique banding patterns however, they shared more number of common bands and clustered together. Hence this study clearly indicated that genetic variability existed among *S. rolfsii* isolates.

Prasad *et al.* (2010) studed moleculer variability among the isolates of *S. rolfsii* causing stem rot of groundnut by RAPD, ITS-PCR and RFLP. The RAPD banding pattern reflected the genetic diversity among the isolates by formation of two clusters. A total of 221 reproducible and scorable polymorphic bands ranging approximately as low as 100 bp to as high as 2500 bp were generated with five RAPD primers. ITS region of rDNA amplification with specific ITS1 and ITS4 universal primers produced approximately 650 to 700 bp in all the isolates confirmed that all the isolates obtained are *S. rolfsii*. Studies by ITS-RFLP indicated that there was no polymorphism in restriction banding pattern among the isolates with the restriction endonucleases used.

Tanwir *et al*. (2007) and Asif *et al*. (2005) evaluated that RAPD analysis was found to be an informative DNA marker system to assess genetic relatedness and diversity

among different strains. RAPD-PCR analysis had also been used by other worker to investigate genetic variation among isolates of *S. rolfsii* collected from different geographical regions.

Kokub *et al.* (2007) evaluated comparative growth, morphological and molecular characterization of indigenous *S. rolfsii* strains isolated from different locations of Pakistan. Random amplified polymorphic DNA (RAPD) analysis revealed that strains D5 and D7 shared 94 % similarity while strains D3 and D8 were 83% similar.

Okabe and Matsumoto (2003) observed phylogenetic relationship of *S. rolfsii* (teleomorph *Athelia rolfsii*) and *S. delphini* based on ITS sequences and reported that the ITS regions were cloned and sequenced to identify three distinct ITS types and two different ITS types exist within S. *rolfsii* and *S.delphini.*

Almeida *et al*. (2001) reported variation among 30 isolates of *S. rolfsii* from different hosts and regions of Brazil was studied by undertaking analysis of genomic DNA through random amplified polymorphic DNA (RAPD-PCR) techniques. These techniques confirmed that there was considerable variability among isolates in relation to the number, size and location of sclerotia on the surface of medium.

Okabe *et al. (*2001) reported different ITS types in a strain of *S. rolfsii* reside in different nuclei. The ITS heterogeneity in *S. rolfsii* and *S. delphinii* strains may also originate from different nuclei, via hybridization between species or subgroups caring different ITS types. Both ITS types are present although somatic or sexual recombination events occurred in some ITS copies (e.g., 63TI103). The conclusion that some *S. rolfsii* strains are hybrids is also supported by the morphological diversity among strains.

Punja and sun (2001) assesed genetic similarities and differences among eight strains of *S. rolfsii* using randomly amplified polymorphic DNA (RAPD) analysis DNA was extracted from the mycelial mat of each strain grown on PDA plate for 7 days at 28^{o}C. Mycelium (200 mg) was transferred to 1.5 mL micro centrifuge tubes containing liquid nitrogen.

Harlton *et al.* (1995) screend a world wide collection of *S. rolfsii* which reaveled that variation in ITS regions of 12 sub-groups of *S. rolfsii.*

Okabe *et al*. (1998) divided 67 isolates of the southern blight fungus from Japan into five groups based on ITS-RFLP analysis of nuclear rDNA.Three groups were reidentified as *S. rolfsii* and two resembled *S. delphinii* in RFLP patterns. In that investigation, all the enzymes above have shown different restriction pattern but the each enzyme has the same restriction sites among all the isolates under the study. It is worth while to use more number of enzymes to study the polymorphism.

Punja and Sun (1997) compared 128 isolates of *S. rolfsii* from 36 host species and 23 geographic regions by means of random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) which confirmed that many isolates from the same host belongs to the same mycelial compatibility group (MCG).

Shokes *et al.* (1996) tested the pathogenic variability and pathogenic potential of *S. rolfsii* isolates on the groundnut variety TGCS888. The molecular variability among the isolates of *S. rolfsii* was studied by using ITS region of rDNA, Random Amplified Polymorphic DNA (RAPD) and Internal Transcribed Spacer-Restriction Fragment Length Polymorphism (ITS-RFLP).

Harlton *et al*. (1995) reported the variation in internal transcribed spacer regions (ITS) of ribosomal DNA of *S. rolfsii* and related fungi by RFLP (restriction fragment length polymorphisms) analysis. Punja and Damiani (1996) also demonstrated a close affinity between *S. rolfsii* and *S. delphinii* welch on a molecular basis, although they are morphologically distinct.

Harlton *et al*. (1995) also reported geographical variability was considered to exist in the *S. rolfsii* population because of its life cycle strategy; they tested 67 isolates from different hosts and various parts of Japan, which country was not included in the previous paper.

Chowdhury *et al*. (1993) and Punja and Damiani (1996) observed that ITS-RFLP analysis revealed the existence of five groups in Japanese isolates. Each group had its own morphological characteristics and RFLP patterns, corresponding to previous results, but often differed in growth temperature reactions. Groups 1 was typical of *S.*

rolfsii in sclerotial morphology and RFLP patterns and was identical to group II, one of the major groups of *S. rolfsii* in the U.S.A. and South Asia, whose mycelial growth and sclerotia formation occurred at 35ºC. Group 2, which has not been reported in other countries, was similar to group 1 in morphological characters. Groups 1 and 2 accounted for 73% of Japanese isolates, whereas groups I, III, and IV, the major groups in the U.S.A. were not found in Japan Morphological characterization of group 3 was difficult because the only isolates available did not produce sclerotia. However, its RFLP pattern demonstrated that it corresponded to group XI *(S. rolfsii*) in Nepal.

2.5 *In vitro* control of *S.rolfsii* in different plant disease

Control of *S. rolfsii* with chemical fungicides *(in vitro)*

Pervin *et al.* (2016) evaluated comparative efficacy of different fungicides against foot and root rot disease of betel vine caused by *Sclerotium rolfsii* and reported that fungicide bavistin proved to be the best for controlling the radial mycelial growth of *S. rolfsii.*

Fouzia Yaqub and Saleem Shahzad (2015) studied the effect of fungicides on *in vitro* growth of *S. rolfsii* and reported that six fungicides viz., benomyl, sancozeb, thiovit, dithane M-45, carbandazim and topsin-M were tested against *S. rolfsii* by food poison method. At low concentration, no fungicide inhibited the growth of *S. rolfsii*, however, at high concentration Dithane M-45 and Sencozeb significantly reduced the growth.

Rondon *et al.* (1995) used copper oxychioride, vinclozolin (as Ronilan), iprodione (as Rovral), metalaxyl (as ridomyl), chiorothalanil (as daconil), PCNB [Quintozene], captan, benomyl, carboxin + thiram and thiabendazole at five concentrations against the growth and sclerotia formation of *S. rolfsii.* Carboxin + thiram, copper oxychioride and quintozene were found to be most effective, both inhibitmg mycelial growth and scierotia formation at low concentration.

Shahid *et al.* (1990) evaluated ten fungicides (*in vitro)* and found ridomil [metalaxyl] was the most effective in inhibiting mycelia growth and sclerotial production of *S. rolfsii*. Benlate [Benamyl] and metalaxyl inhibited germination of sclerotia most

effectively. Metalaxyl and benomyl at 500 ppm applied as seed treatment and soil drench respectively gave 100% control of collar rot lentil seedlings.

Okonkwo (1988) investigated with chemicals against stem-rot fungus *S. rolfsii* and reported the effectiveness of formaldehyde at various concentrations against this pathogen.

Punja *et al*. (1982) found that, eruptive and hyphal germination of dried seed sclerotia of two isolates of *S. rolfsii* at 1% noble and bacto water agar was totally inhibited by carboxin, cycloheximide, oxycarboxin and experimental fungicides CGA-64251 in the agar @ 100 and 200 µg /ml.

Siddaramaiah *et al.* (1979) investigated with calixin against *S. rolfsii in vitro* and observed that calixin completely inhibited the mycelial growth of *S. rolfsii* they also found the inhibition of sclerotial germination by bayletan and panoram at 100 ppm.

Diomande and Beute (1977) used a soil plate method to evaluate seven fungicides for control of *S. rolfsii* in laboratory tests. In all tests arboxin and tryphenyltin hydroxide were effective in preventing mycelia growth of *S. rolfsii*.

Mahmood *et al.* (1977) observed that formaldehyde completely inhibited the fungal growth of *S. rolfsii* and formaldehyde was most effective in killing sclerotia of the test fungus.

Vyas and Joshi (1977) evaluated chemicals against *S. rolfsii* and they observed that out of tested fungicides carboxin was highly effective for controlling the tested pathogen *S. rolfsii*.

Pandav *et al.* (2014) evaluated with chemicals in controlling collar rot disease of gerbera caused by *S. rolfsii.* Among the various chemicals tested for their effect in resting the germination of sclerotia of *Sclerotium rolfsii* formaldehyde (5%) and ethyl alcohol (40%) showed complete inhibition of sclerotial germination irrespective of soaking period. Similarly, sclerotia exhibited viability for 150 days when stored at ambient temperatures.

Lilly and Barnett (1951) reported that there was no useful universal fungicide, because different species of fungi exhibit greater variation in their sensitivity to various fungicides. A fungicide, which in lethal or highly toxic to a particular fungus may be totally ineffective against another fungus.

Control of *S. rolfsii* with plant extracts *(in vitro)*

Enikuomehin *et al.* (1998) worked on the evaluation of ash from some tropical plants of Nigeria for the control of *S. rolfsii* on wheat (*Tritichum aestivum* L.). Nine tropical plants were screened for their abilities to inhibit mycelial growth and sclerotial germination of Nigerian isolate of *Corticium rolfsii* on agar and in soil. Of the 11 samples tested 10 showed some activity against mycelial growth of *C.rolfsii in vitro.*

Sharma and Basandrai (1997) evaluated with some plant extracts and that found leaf extract of *Azadirhicta indica* effective in reducing sclerotial viability of *Sclerotinia sclerotiorum* isolated from beans.

Pani and Patra (1997) utilized some phyto-extracts for controlling *S. rolfsii* during paddy straw mushroom (*Volvariellavo lvacea*) cultivation. *In vitro* and *in vivo* studied were conducted to determine the effect of extracts of *Azadirachta indica*, *Psidium guagava, Lantana* camara, *Sopindus trifoliate*, *Cynodon dactylon*, *Tamarindus indica*, *Echhornia crassipes*, *Adhatoda vasica*, *Pong amiaglabra* and *Tagetese rectaon* the mycelia growth of *Volvariella volvacea* and *S. rolfsii*. Paddy straw mushroom inoculated with *S. rolfsii* and treated with *A. indica* leaf extract resulted in the highest sporophore yield followed by *Sopindus trifoliate* seed extract and *Moringa aoleifera* root extract.

Arun and Chitra (1995) observed that extracts of garlic bulb were effective in suppressing radial growth of the pathogen *Fusarium* spp. and *S. rolfsii* and was more effective when added after sterilization.

Singh *et al.* (1989) reported that, out of six plant oils tested against *S. rolfsii*, leaf oil of *Azadirachta indica* was found most effective followed by that from *Eucalyptus globules* and *Ocimum camum.*

Jalal and Ghaffar (1992) studied antifungal characteristics of *O. sanctum* L. and found that its leaf extract completely inhibited the growth of *S. rolfsii* and other fungi. Ethanol extracts of *Aframomum melegueta* and *Oscimum gratissmum* at 3 to 5% concentration showed 100% inhibition on mycelial growth of *S. rolfsii*. Use of barks and corks of commonly grown trees and shrubs is also an economical and feasible way of controlling major plant diseases.

Singh and Dwivedi (1990) studied with *S.rolfsii* and observed that, the viability of sclerotia was reduced when treated with neem oil.

Shahzad and Ghaffar (1988) reported that *Paecilomyces lilacinnus* (a fungal parasite) was effective to inhibit growth of scierorial fungi i.e. *Macrophomina phaseolina, Rhizocotinia solani* and *Sclerotium oryzae*, causing root rot in many plants. Leaf extracts of some medicinal plants in our study also exhibited significant effect against *S roifsii*.

Sivakadacham (1988) reported that leaf extracts of *Adhatoda vasica* and *Cullen corylifolium* suppressed the mycelium growth of *S. rolfsii.*

Dutta and Deb (1986) evaluated with plant extracts and reported that, leaf extract of *Eupatorium adenophorum* reduced the pathogen (*S. rolfsii)* sporulation in the rhizosphere.

Singh and Dwivedi (1987) observed that, hyphal dry weight and sclerotial production of *S. rolfsii* were significantly reduced by bark extracts of *Acacia arabica*.They also tried bulb and leaf extracts of garlic and onion, leaf extracts of *Rauvolfia serpentine*, *Lawsonia alba*, *Datura stramonium*, *Solanum xanthicarpum*, *Calotropis procera*, *Eucalyptus globus* and *Azadirachta indica* fruit and leaf extracts, *Emblica officinalis* and rhizome extracts of turmeric, ginger extracts against *S. rolfsii* and found that those extracts were more or less effective for inhibit the fungus.

Parven *et al*. (2016) evaluated *in vitro* efficacy of some fungicides, plant extracts and bioagents against *S. rolfsii* and control of foot and root rot disease of betel vine. They observed out of five plants garlic showed better performance.

Arora and dwivedi (1979) found that *T. harzianum* significantly reduced the growth of *S. rolfsii*, the causal organism of root disease of lentil (*Lens esculenta*) on agar.

Darvin (2013) evaluated effect of plant extracts on radial growth of *S.rolfsii* Sacc. causing stem rot of groundnut and effectively control the mycelial growth of *S. rolfsi* and reported that among those plants extracts, clove extract of garlic was most effective and recording lowest mycelial growth (0.0 cm) and highest per cent inhibition (PI) (100%) in both sterilized and un-sterilized conditions.

Farooq *et al.* (2010) reported the maximum inhibition of mycelial growth of *S. rolfsii* causing southern sclerotium rot in sugar beet, was recorded by *A.indica* (73.8%), *Cassia fistula* (73.5%) *and Cannabis sativa* (67.1%).

Iqbal *et al*. (2006) evaluated *in vitro* efficacy of plant leaf extracts against *S. rolfsii* Sacc. and reported that minimum number of sclerotia was found at 2% leaf extracts concentration of all the plant species (*Datura alba, Calotropois procera* and *Cannabis sativa).*

Islam (2005, and Arun *et al*. 1995) evaluated with some plant extracts and reported that antifungal activities of garlic, neem and allamanda were effective against *S.rolfsii.*

Seshakiran (2002) reported that, *Eupatorium odoratum* L., *C. occidentalis* and *Azadrachta indica* were highly antifungal to mycelial growth of *S. rolfsii*. However, root extract of *Pathenium hysterophorus* L. exhibited maximum inhibition of mycelium growth of *S. rolfsii.*

Control of *S. rolfsii* with bio-control agents

Murtaza *et al*. (2012) studied the effect of *Trichoderma* culture filtrate with different concentrations grown and they observed 60 to 80% inhibition at 100% concentration and low inhibition observed at low concentration.

Amin *et al.* (2010) tested the ability of six isolates of *Trichoderma* spp. for their ability to inhibit soil borne pathogens of different vegetables viz., *Rhizoctonia solani* (isolates from tomato), *S. rolfsii* (causing collar rot of tomato) and *Sclerotinia*

sclerotium (causing web blight of beans) under *in vitro* conditions. Dual culture of pathogens and *Trichoderma* spp. revealed *T. viride* (Tv-2) highly inhibited the mycelial growth (71.41% over control) in case of *Rhizoctonia solani* followed by *T. viride* (Tv-1) and *T. harzianum* (Th-1) showing 65.71% and 60.51% inhibition over control, respectively. Similarly in case of *S. rolfsii* and *Sclerotinia sclerotiorum; T. viride* (Tv-1) proved to be best overall isolates in inhibiting mycelial growth of test pathogens (67.91% and 66.21% inhibition over control, respectively). Further, all *Trichoderma* isolates significantly inhibited the production of sclerotia in test pathogens. *T. viride* (Tv-1) was most effective in reducing sclerotial production (83.75% in *R. solani*, 80.18% in *S. rolfsi* and 70.15% in *S. sclerotium*).

Bari *et al.* (2000) evaluated *Trichoderma* spp. was verry effective for control of *S.rolfsii* and reported that significant reduction on radial growth of *S.rolfsii* by *Trichoderma spp*. in dual culture on PDA plate.

Biswas and Sen (2000) reported the dual culture of the 11 isolates of *T. harzianum* viz. T_8, T_{10} and T_{12} were effective against *S. rolfsii* and they over grew the pathogen up to 92%, 85% and 79% respectively *in vitro*. Both the T_8 and T_{10} isolates reduced stem rot incidence

Desai and Schlosser (1999) collected 44 isolates of *Trichoderma* belonging to eight species were tested for their ability to infect, macerate 15 and kill the sclerotia of *S. rolfsii* of them 14 isolates infected and killed the sclerotia of *S.rolfsii*.

Mondal (1999) studied fifty five isolates of *T. harzianum* against *S. rolfsii* and observed that out of tested fifty five isolates of *T. harzianum*, isolate TF-24 showed 93% inhibition of mycelia growth of *S. rolfsii* on PDA.

Sharma *et al.* (1999) reported that *T. harzianurn* were most effective in inhibiting mycelial growth of *Sclerotinia sclerotium* (Lib) de Bary causing chickpea stem rot in dual culture. They conducted the experiment in pot and field and reported that treatment of chickpea seeds with mycelia preparation of *T. harzianum* reduced seedling mortality in pot experiments and rescued the disease considerably in the

field. Pre-application *T. harzianurn* preferred as compared to the application at sowing time.

Kurucheve *et al*. (1998) tested *in vitro* evaluation of botanicals leaf extracts of *Lawsonia inermis* L. and *Piper betel* L. at four concentrations against *S. rolfsii*, the causal agent of wilt of peanut. They observed that the leaf extracts of two botanicals inhibited the mycelial growth and sclerotial production completely at 10% concentration.

Ellil *et al.* (1998) stated that, *T. harzianum* reduced root rot infection by 6.7-45.0% in bean. *Trichoderma* spp. obviously antagonized the effects caused by the pathogen *S. rolfsii* and *Fusarium solani*

Virupaksha *et al*. (1997) tested the antagonistic organisms against *S. rolfsii.* Among them, *T. harzianum* and *T. viride* were found to be effective in inhibiting the mycelial growth and reducing production of sclerotial bodies irrespective of inoculation periods. They also observed inhibition zone and reduction in size of sclerotial bodies in presence of antagonists.

Muthamilan and Jeyarajan (1996) reported that, 67.4% reduction of sclerotial production in *S. rolfsii* was observed in the presence of *T. viride*. Mature sclerotia from each dual culture plate were smaller than the control plate.

Mukherjee *et al.* (1995) observed that *T. harzianurn* was effective in suppressing *S. rolfsii* and *Rhizoctonia solani. T. harzianum* was found to be effective in destroying the sclerotia of both fungi.

Mukhopadhyay (1995) used *T. harzianum* for testing seeds of various crops like chickpea, lentil, groundnut, tomato and cauliflower for protection against a wide range of soil borne pathogen viz., *R. solani, S. rolfsii, Pythium* spp. and *F. oxysporum*.

Iqbal *et al*. (1995) tested the micro-organisms for antagonism to *S. rolfsii*. All the organisms viz., *Trichoderma koningii*, *Trichoderma viride*, *Gliocladium virens* Miller, *Aspergillus canditus* Link, *Paecilomyces lilacinus* (Thom), *Samson* and *Bacillus* spp. significantly inhibited the mycelial growth of *S. rolfsii*, *Trichoderma harzianum,*

Trichoderma koningii and *Trichoderma viride* overlapped the pathogen and suppressed growth by 63.6%, 54.9% and 51.89% respectively.

Mukherjee *et al*. (1995) compared antagonistic properties of *T. harzianum* and *Gliocladium virens* in suppressing *S. rolfsii* and *Rhizoctonia solani* in *in vitro*. They observed that *T. harzianum* was less effective than *G. virens*. Only *T. harzianum* parasitized the hyphae of *S. rolfsii* and the two antagonists were comparable in respect to antibiosis on the test pathogens.

Lim and Teh (1990) reported that isolates of *T. harzianum*, inhibited the growth of *S.rolfsii* up to 67% in dual culture on malt agar and up to 100% using acellophane overlay technique at 20 ± 1.5º C. Growth of the test organism was inhibited by the production of both diffusible and volatile metabolites and various hyphal interactions were observed; hyphal coiling, appressoria and hooks were produced by the *Trichoderma spp*.

Ikotun and Adekunle (1990) isolated *T. harzianum* from soils grown to cassava plants and observed that *T. harzianum* was an active hyper parasite which attacked the mycelia of target organisms (*S. rolfsii*) and prevented their continued growth.

Chamswarng and Sangkaha (1988) collected 147 isolates of *Trichoderma* and *Gliocladium* group. *In vitro* test of bio-control potential of all isolates indicate that 123 were antagonistic to *S.rolfsii*.

Ambra and Ferrata (1984) observed the reduction of mycelial growth, sclerotia formation, sclerotial germination and number of sclerotia of *S. rolfsii* when inoculated with different inoculum concentration of *T. harzianum*.

Elad *et al*. (1983) studied the parasitism of *T. harzianum* to the soil borne plant pathogen, *S. rolfsi*. They observed that hyphae of the parasites contact with their host either producing appressorium like bodies or coiling around the hyphae, enzymatically digest host cell walls.

Henis *et al*. (1983) reported that *Trichoderma* produced volatile and non-volatile antibiotics which are active against *S. rolfsii* and also inhibited the sclerotial germination.

Echeverria *et al*. (1982) studied the antagonistic effect of *Trichoderma* spp. to control *S. rolfsii* on *Phaseolus vulgaris* under field and laboratory conditions and found antagonism between *Trichoderma spp.* and *S. rolfsii*.

Almeida and Landim (1981) reported that an isolate of *Trichoderma spp*. was hyper parasitic on *S. rolfsii* of cowpea under field and laboratory condition.

Mathur and Sarbhoy (1978) observed that the comparative effectiveness of *T. viride* and *T. arzianum* under both *in vitro* and glass house conditions against root rot of sugar beet caused by *S. rolfsii*. Both species of *Trichoderma* appeared to be strongly antagonistic, causing 88% and 86% inhibition of the growth of *S. rolfsii* by *T. viride* and *T. harzianum*, respectively. While tested under glasshouse condition, *S. rolfsii* caused only 13.3% and 20% infection in presence of *T. viride* and *T. harzianum*, respectively compared with 100% infection recorded in absence of any of the antagonists.

Harder and Troll (1973) tested the antagonism of *Trichoderma* spp. to sclerotia of *Typhulain carnata* and observed that several *Trichoderma* spp. parasitized the sclerotia in culture on artificial media and on soil, greatly reducing the viability of the sclerotia.

Mostofa (1973) evaluated *S. rolfsii* causes wilt disease of betel vine. He observed that the presence of any amount of *S. rolfsii* would produce collar rot and ultimately wilting of betel vines.

Aycock (1966) reported that stem rot disease also known as southern blight, Sclerotium wilt, Sclerotium blight and white mold which is affected all part of the plant at any stage of crop growth but stem infection is most common. 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.

Control of *S. rolfsii* with soil amendments

Blum and Kabana (2004) evaluated effect of organic amendment on sclerotial germination, mycelial growth, and *S. rolfsii* induced diseases. Disease severity on tomato *(Lycopersicon esculentum*) plants was loWin soil treated with kudzu or velvet bean (30 and 35 g kg^{l}) and pine-bark (35 g kg'^{l}). Dried powders of kudzu, velvet bean, or pine-bark added to soil can reduce disease by reducing pathogen inoculum.

Chowdhury *et al*. (1992) studied the effect of growth and sclerotia productions *in-vitro* using a soil plate method of the fungus S. *rolfsii* on different textural soil and its response to different chemical. They found that maximum mycelial growth at 8.5 PH and sclerotia formation on sandy loam (448.33mm) and silty clay soil (373.33mm) where range of PH from 6.1- 6.5 respectively.

Hadar and Gorodecki (1991) found an inhibitory effect of grape marc compost on sclerotial germination and viability, and associated this effect with high numbers of *Penicillium* isolated from sclerotia.

Palakshappa (1986) found that the maximum suppression of the saprophytic activity of *S. rolfsii* causing foot rot of betelvine by ground nut and sunaflower oil cake and F.Y.M. paddy hulls and wheat bran were less effective.

Dutta and Deb (1986) studied the effect of organic and inorganic amendments on the soil and rhizosphere microflora in relation to the biology and control of *Sclerotium rolfsii*.

Punja (1985) reported that the addition of organic amendments such as compost, oats or corn straw to soil limited the disease caused by *S. rolfsii* possibly due to release of toxic ammonia or increased in the level of resident antagonistic soil microorganisms.

Chowdhury (1985) reported application of manure reduced the death of betelvine plants due to infection of *S. rolfsii*. Among the organic amendments tested the mustard oil cake was found to be effective.

2.6 *In vivo* control of *S. rolfsii* through different methods for disease management.

Control of *S. rolfsii* with chemical fungicides

Madhovi and Bhattiprolu (2011) evaluated *in vivo* soil drenching with nine fungicides and found tuberconazole and combination of carbendazim + mancozeb effective for controlling *S. rolfsii* at 1000-3000 ppm.

Mohanty *et al.* (2011) conducted field experiment for two consecutive years at the betel leaf garden (Pan Barejas) at Virus Research Farm, Kalyani and observed that integrated use of sanitation, Bordeaux mixture and *T. harzianum* gave the best control of foot rot and leaf rot of betel vine. The treatment yielded maximum leaf production (3.448 million ha/year) and the highest fresh weight of 100 leaves (351.85 g). The economic analysis showed that application of Bordeaux mixture along with T. *harzianum* and sanitation may be useful to obtain satisfactory control of both the diseases and economic benefit.

Rahman *et al*. (1994) demonstrated that the effect of vitavax-200, apron-TZ, dithane M 45, thiram, captan and baytan 100S [Triadimeno] on foot and root rot disease on cowpea (Vignaunguiculata) caused by *S. rolfsii*. Seeds of a susceptible variety were treated before sowing. Vitavax-200 was the best fungicides in respect to controlling seedling mortality.

Water field and Siler (1990) evaluated with chemicals and reported that propiconazole was found highly effective in inhibiting the growth of *S. rolfsii*.

Santosh *et al.* (1989) reported the use of rhizolex 50 WP, O-dimethyl-O (2, 6-dicloro-4-methyl-phenyl)-phosphorothioate, a new fungicide, against *S. rolfsii*, the causal organism of foot rot of betel vine (*Piper betle* L.). Two months old potted betel vine plants were inoculated with mycelial culture of *S. rolfsii* by soil mixing method. Fungicide was applied by drenching method in desired concentration and on desired date. Fungicide treated pots showed lower percentage of vine mortality than the check (no fungicide). There was no significant difference in percent mortality of vines under 0.2 and 0.4% fungicide applied at same date however higher dose (0.4%) of fungicide applied at 2 days after inoculation had shown lower percentage of vine mortality as

compared to the lower dose (0.2%) at 5 days after inoculation. Thus both concentration of Rhizoplex 50WP and time of its application play an important role in the control of foot rot disease of betel vine.

Pan and Sen (1987) demonstrated soil drenches with benodanil and seed treatments with campogram M and observed that highly effective in reducing wheat seedling mortality caused by *S. rolfsii*.

Patil *et al.* (1986) observed soil drenching of captan, copperpoxychloride and difoliton were found effective for controlling wilt disease of betel vine caused by *S. rolfsii.*

Patil *et al.* (1986) reported that in field trials against the foot rot disease of *piper betle* caused by *S. rolfsii* were control by soil drenches with copper oxychloride, cupravit, dithane M-45, difolatan and bordeaux mixture were very effective.

Wokocha and Ebeneb (1986) used six fungicides aatopam-N, aldrex-T, calixinM, PCNB [Quintozene], captan and captafol at 200 mg/litter in green house test against *S. rolfsii* on tomato completely suppressed the disease when applied as soil drenches up to 4 day before inoculation, but only quintozene was effective when applied 10 day before and post-inoculation treatments were effective.

Patil *et al.* (1986) reported the effectiveness of captan (ethyl mercaptan) another fungicide in reducing the mortality of *Piper betle* due to *S. rolfsii*. Soil drenching with dithane M-45, captan, difolatan and boredeux mixture were found highly effective in reducing the mortality of *Piper betle* due to *S. rolfsii.*

Fahim *et al*. (1984) observed, seed treating agent vitavax-200, homai-80, orthocide 75 and captan @ 3g/kg seed by dusting or glutting (modification or pelleting method) to reduce pre-emergence damping-off of sugar beet (Beta vulgaris) caused by *S. rolfsii* in infested soil. Post-emergence damping of was greatly reduced in soil infested before sowing after seed germination.

Anilkumar and Pandurangegowa (1984) evaluated the efficacy of vitavax as soil drenching fungicide against *S. rolfsii* in reducing the seedling mortality.

Dhamnikar and Peshney (1982) evaluated twenty fungicides against *S. rolfsii* on peanut by different methods (*in vivo*). Rovral, vitavax, brassicol, captan and dithane M-45 controlled the disease effectively as dry seed dresser. As soil drench, vitavax-200 was the most effective followed by rovral and brassicol controlling the disease.

Patil and Rane (1982) observed vitavax, ceresan wet proved to be effective in inhibiting the growth of the pathogen as well as affecting germination of sclerotia. These fungicides were also proved effective in reducing the incidence of seed borne and soil borne infection by seed and soil treatments.

Kulkarni (1980) found that in field trials foot rot of wheat caused by *S. rolfsii* was controlled effectively by seed treatment with panoram, brassicol, panoctine-35 guazatine] vitavax and calixin [tridemorph] were less effective. Seed treatment with 0.2% of these chemicals protects wheat seedlings for up to 35 days even in heavily infested soil.

Reddy *et al*. (1976) demonstrated that plantvax (oxycarboxin) and vitavax (carboxin) as seed treatments at 2g/kg gave effective control of *S. rolfsii* on heat up to 35 days after seedling.

Dutta (1975) found that soil application of fungicides such bavistin (0.5-0.7%), brassicol (0.1%), three times at 20 days interval has been effective in controlling foot and tuber rot disease of tuberose.

Khare *et al*. (1974) conducted an experiment with thirteen fungicides to control *Sclerotium rolfsii* from wilted lentil plant. Five fungicides namely benlate, thiram, dithane-M 45, captan and phaltan complex reported cease the growth of *S. rolfsii.*

Sen *et al*. (1974) found that, *Sclerotium rolfsii* on wheat could be controlled by seed treatment with 5g PCNB (Quintozene) per kg seed.

Control of *S. rolfsii* with plant extracts

Darvin (2013) evaluated effect of plant extracts on radial growth of *S. rolfsii* causing stem rot of groundnut and reported that among these plant extracts, clove extract of

garlic was most effective and recording lowest mycelial growth (0.0 cm) and highest per cent inhibition (PI) (100%) in both sterilized and un-sterilized conditions.

Amin e*t al.* (2013) evaluated effect of some botanical extracts and cow's urine on *Sclerotium rolfsii* causal agent of foot and root rot disease of betel vine and reported that 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 oncentration of turmeric rhizome extracts.

Okereke and Wokocha (2006) reported that the inhibition of damping off disease of tomato incited by *S. rolfsii* was the heighest with soil drenching with neem seed (62%) followed by ginger (57.4%).

Daynaram and Tewari (1994) evaluated the soil application of *Adhatoda vasica, Aegle marmelos, Anisomeles ovata, Azadirchca indica, Cymboposon flexuos leaves, Curcuma amada* rhizomes and *ferula foetida* resin for pre and post emergence collar rot of chickpea caused by *S.rolfsii.*

Dayaram and Tewari (1994) found that, the soil application of green leaves of *Adathoda vasica*, *Aegle marmelos*, *Anisomele sovata*, *Azadirchta indica*, *Cymbopogon flexuous*, rhizomes of *Curcuma amada* and resin of *Ferula foetida* at 2 to 5 per cent concentration reduced both pre and post emergence collar rot of chickpea caused by *S. rolfsii*. Five percent *Ferula foetida* resin applied 48 hours before sowing of seeds in artificial inoculation of soil provided nearly 100 per cent protection.

Control of *S. rolfsii* with bio -agents (*in vivo)*

Dasgupta *et al.* (2011) evaluated management of foot rot of betel vine caused by *Phytophthora parasitica* dastur and observed minimum foot rot disease by four application of *Trichoderma* at quaterly intervals.

Mohanty *et al.* (2011) conducted field experiment for two consecutive years at the betel leaf garden (Pan barejas) at Virus Research Farm, Kalyani, India, to test the efficacy of sanitation, two fungicides viz. Fosetyl-Al, Bordeaux mixture, biocontrol agent *T. hazianum* used in different combinations for the control of two major diseases namely foot rot and leaf rot of betelvine caused by *Phytophthora*.

Roy *et al.* (2005) reported that chemical fungicide like Bordeaux mixture was superior over bio-agent like *Trichoderma* in reducing disease incidence of betelvine.

D'Souza *et al.* (2001) investigated with some biocontrol agents and observed that significant reduction of foot rot disease of betel vine with the use of *T. harzianum.*

Muthamilan and Jeyarajan (1996) reported that, *T. harzianum* reduced groundnut root rot caused by *S. rolfsii*. Maximum number of plants survived when the antagonist was applied asseed treatment prior to sowing.

Samuels (1996) found that the use of *Trichoderma* isolates in combination with lower levels of fumigant gave improved control of root rot of individual trees of stem rot of tomato in Thailand. The increase in survival rates ranged from 6.7 to 26.7% in green house trials and from 17.3 to 38.1% in field trials.

Mukhopadhyay (1994) used *T. harzianum* for testing seeds of various crops like chickpea, lentil, groundnut, tomato and cauliflower for protection against a wide range of soil borne pathogen viz., *Rhizoctonia solani, S. rolfsii, Pythium* spp. and *Fusarium oxysporum* and obsreved that the treatment was quite in expensive and eco-friendly as compared to other method of disease control and can be successfully exploited for the control of a wide range of seed as well as soil borne diseases.

Kay and Stewart (1994) found that *T. viride* and *T. harzianum* were capable of reducing the incidence of onion white rot caused by *S. rolfsii* reduction was observed when the test fungi were applied as seed coating or incorporated into alginate pellets.

Chet and Inber (1994) reported that *S. rolfsii*. causes the disease known as southern blight in wide variety of crops. *S. rolfsii* from brownish sclerotia that can survive in the soil for longer period of time.

Abada (1994) reported that, the *T. harzianum* caused a great reduction in the infection level of damping off and root rot disease of sugar beet caused by *R.solani*, *S. rolfsii*, *Fusarium*, *Mucor*, *Alternaria*, *Pythium ebaryanumand* resulted in increased root weight both in pot and field experiments.

Sugha *et al*. (1993) reported that, conidial coating of the antagonists *T. harzianum* and *T.viride* on seeds significantly reduced seedling mortality (47-65%) infected by *S.rolfsii* compared with the untreated control.

Chamswamg (1992) reported that isolates of *Trichoderma* species increased the survival rate of tomato seedlings infected by *S. rolfsii* the causal pathogen.

Farzana *et al*. (1991) observed that infection of 30 and 60 days old soybean plants by root infecting fungi *(S.rolfsii*, *R. solani, Fusarium spp*.) was significantly reduced following seed treatment with *T. harzianum*.

Sharma and Sing (1990**)** reported that *T. harzianum* was effective against *Sclerotium sclerotiorum* when added to sterilized and unsterilized soils of pea in green house condition. Mycelial preparation of *T. harzianum* was the most effective against S. *sclerotiorum.*

Krutova (1987) reported from the laboratory and field experiment that *T. harzianurn* showed hyper parasitic activity on sclerotia of *Sclerotinia sclerotium* and were capable of destroying its sclerotia in soil.

Elad *et al*. (1980) reported that incorporation of a wheat bran inoculums of *T. harzianum* in pathogen infested soil significantly reduced bean (*Phaseolus vulgaris*) disease caused by *S. rolfsii* and *R. solani* under glasshouse condition.

Chet *et al*. (1979) stated that, when applied in the form of wheat bran culture to soil infested with *R. Solani* and *S. rolfsii* in the green house, *T. harzianum* effectively controlled damping off diseases of peanuts, beans, and eggplants caused by soil borne plant pathogens. Field experiments were carried out and a significant reduction in disease incidence was obtained. Application of PCNB at sub inhibitory doses improved control of disease when applied together with the *T. harzianum*.

Grinstien *et al*.(1979) demonstrated a wheat-bran preparation of antagonistic fungus, *T. harzianum* Rifaiaggr applied to fields at rates of 500-1500 kg/ha, reduced the incidence of diseases caused by *S. rolfsii* and *R. solani* and this control lead to increase yield in various crops.

Mehrotra and Tiwari (1976) showed that dipping of cutting in a *Trichoderma viride* cell suspension effectively reduced the foot rot disease of betelvine.

Backman and Rodriguez (1975) observed a diatomaceous earth granule impregnated with 10% of molasses solution was found suitable for growth and delivery of *T.harzianum* to peanut fields. *Trichoderma* was grown on sterile earth granules for 4 days and applied to 70 and 100 days after planting. Significant reduction in *S. rolfsii* in peanut fields was recorded over the 3-years test period.

Wells *et al.* (1972) stated that *T. harzianum* effectively controlled *S. rolfsii* on blue lupines, tomatoes and peanuts. Under natural field conditions, one to three applications of *T. harzianum* inoculums applied over the plants onto the soil surface was highly effective in reducing *S. rolfsii* damage to tomato.

Homer *et al*. (1971) showed that *T. harzianum* effectively controlled *S. rolfsii* on blue lupins, tomatoes and peanuts. Under natural field conditions one to three applications of *T.harzianum* inoculum applied over the plants onto the soil surface was highly effective in reducing *S. rolfsii* of the transplanted tomato

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.

Bertus (1929) stated that *S. 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.

Control of *S.rolfsii* with soil amendment

Rahman (2005) reported that application of mustard oil cake @ 3 ton/ha and half decomposed poultry manure @ 5 ton/hac weeks before sowing seeds performed better in reducing incidence of collar rot of Chickpea.

Anonymous (1997-98, 1998-99 and 1999-2000) evaluated four application of *T. harizanum* inoculated with oil cake in soil at quarterly interval significantly reduced the disease incidence as well as increase in leaf yield and fresh weight of 100 leaves but was less effective than Bordeaux mixture (1%) application at monthly intervals for four times starting at the onset of monsoon in all the AICRP centres of betelvine.

Jagadeesh and Geeta (1994) reported that a wheat bran and biogas manure mixture (1:1) stimulated growth and multiplication of the biological control agent *T. harzianum*, which suppressed *S. rolfsii* and increased seedling emergence of groundnut.

Dutta and Deb (1986) reported the effect of organic and inorganic amendments on the soil and rhizosphere microflora in relation to the biology and control of *S. rolfsii.*

Mukhopadhyay (1987) stated that, application of wheat bran saw dust preparation of *T. harzianum* and *T. koningii* brought an excellent control of damping-off of tomato and eggplant, wilt and foot rot of lentil (caused by *S. rolfsii*) under field conditions.

Sommat *et al*. (1982) made an investigation on the pathogenicity of *S. 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 decreased disease intensity e.g. crop refuses, nitrogen fertilizers and lime. Seed dressing with five fungitotoxicants showed that vitavax gave a complete protection when grown in infested soil.

Mehrotra and Tiwari, (1976) evaluated use of corn straw and til oil cake also reduced disease of the 2 approaches of biological control viz indirect through manipulation of associated microbiota by amendments and direct use of antagonist.

Integrated management of *S. rolfsii*

Till the year 1999 very much little information has been published about the Integrated Disease Management (IDM) of betelvine. Some of the report have published in country and abrood in this regards are given below-

Suryawanshi (2015) evaluated integrated management of collar rot (*S. rolfsii*) of brinjal (*Solanum melongena)* and observed that significantly highest reduction in pre-emergence (69.96%), post-emergence (55.43%) and average (62.37%) mortality were recorded with treatment of Vitavax +Thiram + *P. fluorescens* + NSC. Thus, it is concluded that brinjal collar rot can be managed effectively by seed treatment with fungicides (Vitavax, Thiram), bioagent (*P. fluorescens*) and soil amendment with neem seed cake.

Sultana *et al.* (2012) evaluated integrated approach of mitigating root rot of chilli caused by *S. rolfsii* and reported that significantly effective control of root rot of chilli was observed when both soil was inoculated with *S. rolfsii* treated with *T. harzianum* (Th1), *C. globosum* (Cg5), Vitavax-200 at 400 ppm and garlic extractat 5% combindly (T9) in comparison to any other intgegrated combinations and individual measure. Application of individual components of the above treatment combination (T9) was alsosignificantly effective over allother measures to minimize root rot of chilli.

Madhavi et *al*. (2011) reported integrated disease management of dry root rot of chilli incited by *S. rolfsii* (Sacc.) Integration of different treatments including seedling dip with carbendazinm +mancoxeb. addition of vermicopost, drenching with fungicide and application of *T. harziamum* (7%) were found to be effective in management of disease in comparison with individual treatments.

Brahankar *et al.* (2011) evaluated neem cake @2000kg/ ha, NPK@ 100:50:50 kg/ha, soil solarization, *Trichoderma* @ 10 kg/ha and drip irrigation system was effective for controlling wilt disease of betelvine caused by *S.rolfsii.*

Roy (2001) found that when Bordeaux mixture was used in combination with earthing-up, sanitation and biocontrol agent (*T. harzianum*) and found the leaf and

foot rot incidence of betelvine were reduced. The results showed that integrated use of sanitation, Bordeaux mixture and *T. harzianum* gave the best control of foot rot and leaf rot of betelvine. The treatment yielded maximum leaf production (3. 448 million ha-1year-1) and the highest fresh weight of 100 leaves (351.85 g). The economic analysis showed that application of Bordeaux mixture along with T. *harzianum* and sanitation may be useful to obtain satisfactory control of both the diseases and economic benefit.

Maiti (1999) has meticulously identified the component of IDM that are now available for the *Phytophthora* disease of betelvine, including disease resistance, plant nutrition sanitation, chemical and biological control. The constraints to IDM development are identified as lack of forecasting system. Non-availability of tools for breeding either conventionally or biotechnologically and a sketchy understanding of the biological control system.

Anonymous (1998, 1999, 2000) reported the efforts on IDM of betel vine were initiated under the AICRP Betelvine project at several centers and some prima facie improvement in disease management and yield increase were

Anandaraz and Sarma (1994) reported an integrated approach in which antagonists such as *Trichoderma* sp. and *Gliocladium* sp. Together with sprays of Bordeaux mixture or meaf metalaxyl or phosphoric acid and cultural measures against aerial infection, provides the best prospect for minimizing losses.

2.7 Phytochemical analysis of betel vine

Piper betel contains a wide variety of biologically active compounds whose concentration depends on the variety of the plant, season and climate. Chemical compositions of essential oil constitute safrole present in the leaf, stalk, stem, root and ß-phellandrene present in the fruit.

Syahidah *et al.* (2017) evaluated phytochemical analysis, identification and quantification of antibacterial active copounds in betel leaves, *Piper betle* methanolic extract and showed the presence of alkaloids, phenols, flavonoids, tannins, saponins, glycosides, terpenoids and steroids. Therefore, that HPLC method to quantify the

content of hydroxychavicol and eugenol in *P. betle* methanolic extract was employed. The linear regression for both analytes has showed good linearity in the investigated ranges with correlation coefficients of 0.9990 for hydroxychavicol and 0.9959 for eugenol. The average retention time of hydroxychavicol and eugenol was found at 4.02±0.002 and 7.61±0.005 min, respectively.

Foo *et al.* (2015) evaluated and reported that HPLC is an efficient method in terms of simplicity, precision, rapid and accurate for the simultaneous determination of bioactive compounds in the extracted sample.

Dwivedi *et al.* (2014) and Chahal *et al* (2011) reported that *Piper betel* leaves have an essential oil composing of terpinen4-ol, safrole, allyl rocatechol monoacetate, eugenol, eugenyl acetate, hydroxyl chavicol, ugenol, piper betol and the betel oil contains cadinene carvacrol, allyl catechol, chavicol, p-cymene, caryophyllene, chavibetol, cineole and estragol as the major components.

Singtongratana *et al.* (2013) evaluated hydroxychavicol and eugenol profiling of betel leaves from *Piper betle* L. obtained by liquid-liquid extraction and supercritical fluid extraction.

Periyanayagam *et al.* (2012) reported hydroxychavicol acetate, allylpyrocatechol piperbetol, isoeugenol, anethole, stearic acid, methyl eugenol, carvacrol, polyphenol, alkaloids, saponin, tannin, steroids and other compounds are also found in *Piper betle.*

Kumar *et al.*(2010) examined the antibacterial activity and quantitative determination of protein from leaf of *Datura stramonium* and four varieties of *Piper betle* at concentration of 500 mg ml−1 were tested against pathogenic microorganisms like *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* using agar well diffusion method. And discussed the antimicrobial screening on leaves of *Piper betle.*

Dwivedi and Mehta (2011) studied chemical investigation of *Piper betle* leaf stalk also and their structures were determined by spectroscopic and chemical methods.

Sugumaran (2011) reported the active ingredient of piper betel oil which is obtained from the eaves is primary a class of allyl benzene compounds, chavibetol, chavicol, estragole, eugenol, methyl eugenol and hydroxycatechol, piperol-A, piperol-B, methyl piper 22 betlol and they also have been isolated.

Rathee *et al.* (2006) reported antioxidant activity of *Piper betle* leaf extract and its constituents. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of the ethanol extracts of three varieties (Bangla, Sweet, and Mysore) of *Piper betle* revealed the Bangla variety to possess the best antioxidant activity that can be correlated with the total phenolic content and reducing powers of the respective extracts. Column chromatography of the extract of the bangla variety led to the isolation of chavibetol (CHV), allylpyrocatechol (APC) and their respective glucosides. The HPTLC analyses of the extracts revealed similar chemical properties of three *Piper betle* varieties.

Dasgupta *et al.* (2004) evaluated antioxident activity of *Piper betle L*. leaf extract *in vitro.* The data indicate that the antioxidant activities differed in varieties. The antioxidant activities of the three varieties are in the order Kauri > Ghanagete > Bagerhati. All three varieties of *P. betle* have more potential to prevent lipid peroxidation than does tea. Total antioxidant capacity (equivalent to gallic acid) of Kauri is also higher than tea. Total phenolic concentration, expressed as gallic acid equivalents showed correlation with the antioxidant activity, being highest in Kauri and lowest in Bagerhati.

Dasgupta *et al.* (2004), and Aliahmat (2012) reported antioxidant activities of *Piper betle* L. extracts with different solvents and extraction times were done by many scientists. Total phenolic content was evaluated according to the Folin Ciocalteu procedure. The polarity of the plant extract from various solvents was assessed by determining the oil-water partition coefficient by High Performance Liquid Chromatography (HPLC). *Piper betle* leaf phenolics were found to have less polarity than other phenolic antioxidants due to their high value of oil-water partition coefficient. The experiment indicated that the extraction solvent and time are important for the preparation of *betle* leaf extract for use as natural antioxidant.

CHAPTER-3

Morphological and Moleculer chatracterizations of *S.rolfsii* Sacc.

INTRODUCTION

Betel vine (*Piper betle* L.) is an important horticultural crop which has a lot of commercial values. 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 (Maity, 1989; Samanta, 1994; Guha 1997). Rajshahi is the one of the well known betel vine producing region in Bangladesh. The farmers of different Thana viz. Baghmara, Mahanpur, Poba, Durgapur etc of Rajshahi district cultivating betel vine regular basis and contribute a lot to meet up the national demand as well as exporting betel vine leaf abroad. The cultivation of betel vine is gradually decreasing because of some physical and socioeconomic barriers like unavailability of credit facilities, uncontrolled marketing system and infestation of diseases and pest (Islam, 2005). The betel vine is highly susceptible to diseases, pests and some natural climates (Sayeeduzzaman, 1988). Among the diseases of betel vine, foot rot caused by *S. rolfsii* are the most devastating diseases which decrease the production of betel vine to a great extent. Variability among *S. rolfsii* isolates from different geographical regions was demonstrated by earlier workers (Harlton *et al.*1995, Okabe *et al.,* 1998, Sarma and Singh, 2002). Welsh and McClelland (1990) described a modification of the PCR procedure referred to as the randomly amplified polymorphic DNA (RAPD) marker technique that can be used to detect genetic polymorphisms in fungi. RAPD analysis being used as a powerful tool for the investigation of genetic relatedness and diversity among closely related strains and was found to be a valuable method for differentiating the genetic variability of *S. rolfsii* isolates (Saude *et al*., 2004). On the otherhand, the internal transcribed spacer (ITS) is common genomic target used to measure fungal genetic diversity at the molecular level (Xie, 2012) and this approach now using different fungal species including *S. rolfsii*. The level of variability between species or strains or within the species examining by ITS rDNA sequences is low; however, this method shows improved sensitivity at the genus level (Harlton 1991). However, so far my knowledge there is no studies has performed to identifying the genetic diversity based on sequencing of ITS rDNA among *S. rolfsii* infecting betelvine in Bangladesh. Hence, the present study has been undertaken to evaluate disease occurrence on betelvine by *S. rolfsii*, analyze the molecular data by RAPD and ITS rDNA sequencing and morphological characteristics of the pathogen, and test their pathogenicity.

3.1 Survey

The survey of foot rot disease of betel vine has been done in Northern Parts of Bangladesh namely Baghmara, Mahonpur, Mougachi, Durgapur, Puthia, Chapai Nawabgonj and Pabna during June 2011 to December 2013. In order to survey the foot rot disease affected betel plants were detected in a boroj and several visits were made. Disease incidence was recorded by adopting the grading formula of Siddaramaiah *et al.*(1978). The percentage of infected betel vines was calculated by the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Total No. of Infected Plants}}{\text{Total No. of Plants}} \times 100$$

3.2 Isolation of different isolates of foot rot pathogen

3.2.1 Sampling

The foot rot infected parts of betel vine were collected from different boroj of Northern Parts of Bangladesh during June 2011- December 2013. After proper noting down disease affected plant sample were collected from the field and kept separately in polythene bags with proper labeling. Then the samples brought to the Plant Pathology, Mycology & Microbiology Laboratory, Depertment of Botany University of Rajshahi. Along with the infected samples, a large number of healthy samples were also collected separately for pathogenicity test.

3.2.2 Sterilization and Incubation

After collections the samples were water soaked and the portion of the lesion were cut into small sections (approximately 5x5 mm per section) and surface-sterilize was done by deeping in 5% commercial chlorox (5.25% sodium hypochlorite).Then these were given several washes in sterilized distilled water so as to remove sodium hypochlorite from them. Excess water from the surface of the materials were removed by gently pressing them between to flaps of previously sterilized filter papers. The materials were removed with a flamed forceps and placed within a humid chamber the sterilized materials were placed over the moist filter paper. 2-3 glass bars (90 mm

x 2 mm x 2 mm) were placed on the moist filter paper of the humid chamber, which prevented them from coming in contact with the moisture of the filter papers. The materials under this condition when incubated were found to maintain their good health for longer period to support profuse growth of mycelia and sclerotia of the fungus.

3.2.3 Isolation of *S. rolfsii*

Total 22 isolates of *Sclerotium rolfsii* were isolated from foot rot symptoms of betel vine plants which were collected from different locations of Northern Parts of Bangladesh (Table 3.1). The pathogen was isolated following either direct method or plate method.

Table 3.1: The list of locations and the isolates code.

Sl.No.	Name of location	Isolate code used
1.	Baghmara	BA-1, BA-2, BA-3, BA-4, BA-5, BA-6
2	Mahonpur	MA-1, MA-2
3	Mougachi	MO-1, MO-2
4	Durgapur	DU-1, DU-2
5	Puthia	PU-1, PU-2
6	Chapai nawabgonj	CH-1, CH-2
7	Pabna	P-1, P-2
8	Natore	N-1, N-2

(i) Direct Method of Isolation

The method consisted of sterilization and incubation of the diseased plant parts in humid chamber following the technique described before. The affected parts were incubated for 15 days at 28±2°C. During this period, the fungus was found to grow mycelia and sclerotia on the necrotic areas. The structure of sclerotia could be seen easily under steroscopic binocular microscope and the sclerotia were collected on a

very small agar block (about 2 mm in length), mounted at the tip of a flamed spearheaded tungsten needle. Sclerotia were collected by touching them with the agar block and could be seen lying attached to transparent surface of agar, which was then transferred to a freshly prepared PDA plate and incubated at 28ºC for development of fungal colonies.

(ii) Plating Method of Isolation

In this method, diseased parts were cut into small pieces, about 0.5 cm in length, in such a manner so as to include both healthy and necrotic tissues in each piece. The pieces were then washed in running tap water, sterilized in 5.25% sodium hypochlorite solution and washed repeatedly for several times in sterilized distilled water to remove sodium hypochlorite solution. Three pieces were taken out using flamed forceps and dried between filter papers and finally transferred to a PDA plate. Plates were incubated at 28±2°C for 15 days, during the period of the fungal colonies appeared on the PDA plates often the colonies were found to become contaminated due to unwanted growth of bacteria and other fungi. No doubt the pathogen was obtained in pure form but not so as it was with the direct method. Moreover, this method was found to be time consuming and it required further transfer to get pure culture. Where as in the direct method pure colony of *Sclerotium* could be obtained by single transfer. Unless other wise stated direct method has been used throughout this work for the isolation of the pathogen.

3.3 Identification of isolated fungus

The fungal isolates were isolated from infected tissues of stem of betel vine plant are described as above. The fungal isolates were transferred to PDA slants and subculture on PDA for the identification. The fungus was identified with the help of keys outlined by Subramanian (1971).

3.4 Pathogenicity test of *S.rolfsii*

(i) Planting of betel vine

For pathogenicity test, the betel vine cuttings were grown, in poly bag containing loam soils.Then the betel vine plantlet were inoculated with sclerotial suspension of *S. rolfsii* when two to three seedlings were raised in each polybag.

(ii) Preparation of sclerotial suspension

For preparation of sclerotial suspension of 22 isolates of *S. rolfsii* are grown on PDA plate, separately for 10-16 days. When the plate were fulled with sclerotia 20 ml distilled water was added per plate and shaken well to remove maximum number of sclerotia in it. Using about 4-5 plates a total of 100 ml of sclerotial suspension was prepared for each isolates.

(iii) Method of inoculation

Seedlings of the plants (in pan) were inoculated by spraying a sclerotial suspension with an atomizer. The inoculated plant was covered with polythene bags and incubated at 28±2°C for two days in boroj condition. Water was added every day to keep them under moist condition after removing the polythene bags. Observations were made after 10-12 days when the stem of the host plant had developed characteristic symptoms of foot rot and compared it with the matured-developed symptoms recorded before.

(iv) Reisolation of the pathogens

The artificially infected stems were collected and after sterilization, the pathogen was isolate following the usual procedure. In most cases pathogens were successfully isolated from the infected plants on PDA plates. The morphological characters of the isolated organisms were compared with the original isolates.

Measerment of disease severity

Percentage of disease severity of 22 isolates of *S. rolfsii* was measered. Total stem area and total infected area was measured by a meter scale and the disease severity were caluculated by the following fomula:

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

3.5 Morphological characterizations of the selected isolates *of S. rolfsii*

Measurment of mycelial growth of *S. rolfsii* isolates

Total twenty two isolates of *S. rolfsii* were used for this experiment (Table no-3.1). Mycelial discs (6mm) were transferred aseptically to PDA plates and incubated at room temperature. Radial mycelial growths were taken from the center of inoculated plug after 2-7 days of incubation. The colony diameter was measured using ruller scale and the diameter of sclerotia was measured by optica microscopes (Italy), with calibration slide.

Analysis of morphological diversity among the isolates of *S. rolfsii*

Twenty two *S. rolfsii* isolates were used for this experiment. Mycelial block of 6 mm diameter from the margin of 3 days old colonies were placed aseptically to PDA plates and incubated at room temperature for 25 days. Three replications were used for each isolate. Morphological characteristics of all the isolates were studied on the basis of appearance of the colony and the formation pattern, size, and shape of the sclerotia. The cultural characteristics and sclerotial character states are shown in Table-3.2. The isolates were grouped with the maximum similarities by MVSP (Multi Variable Statistical Package) program (version 3.2).

Table 3.2. Morphological characteristics used for description of *S. rolfsii* and their attributes (Banniza, 1997 and Ali, 2002)

Sl no.	Characters	Character state
1.	Mycelial color on PDA	1=White, 2=Off white, 3=White & off white.
2.	Arial mycelial quantity	1=Few, 2=Moderate,3=Abundant.
3.	Color of sclerotia	1=Brown, 2=off white, 3=White, 4=Light brown 5=Brown, Off white, white& light brown, 6=White, Off white & Brown, 7=Brown & light brown, 8=Off white& Brown.
4.	Quantity of sclerotia	1=Few , 2=Moderate , 3=abundant.
5.	Topography of sclerotia	1=Superficial 2=Immersed
6.	Shape of sclerotia	1=Round, 2=Oval, 3=Round &oval 4=Round, Oval& Irregular.
7.	Superficial sclerotia (ss) discrete(<1mm)	0=Absent, 1=Few, 2=Moderate, 3=Abundant.
8.	SS discrete (>1mm)	0=Absent,1=Few, 2=Moderate, 3=Abundant
9.	SS aggregated	0=Absent,1=few, 2=Moderate, 3=Abundant
10.	SS scattered	0=Absent,1=Few,2=Moderate, 3=Abundant
11.	SS near inoculums	0=Absent,1=Few, 2=Moderate, 3= Abundant
12.	SS near margin	0=Absent,1=Few,2=Moderate, 3=Abundant
13.	SS dispersed on whole colony	0=Present, 1=Absent
14.	Exudate droplets on sclerotium surface	0=Absent, 1=Present
15.	Fluffy mycelium	0=Absent,1=Present
16.	Quantity of fluffy mycelium	1=Few, 2=Moderate, 3=Abundant
17.	Colony reserve pigment	0=Absent, 1=Present.
18.	Pseudo sclerotia	1=Few, 2=Moderate, 3=Abundant
19.	Sclerotia on lid	0=Absent, 1=Present
20.	Zonation	0=Absent, 1=Present
21.	Zonation type	0=No zonation, 1=Cr. (central ring) weakly developed, 2=Cr. sharply developed, 3=Peripherally developed
22.	Mycelial growth on lid	1=Few, 2=Moderate, 3=Abundant

Somatic Compatibility test of the isolates

Total 22 *S. rolfsii* isolates were considered for somatic compatibility test. Mycelial disc having 5 mm diameter were taken from growing culture of 4 days old and placed on 85cm diameter petri plates were apparoximately 2 cm apart from each other. All plates were incubated at room temperature in the dark for 10 days. Twenty two isolates were tested in all possible combinations. The experiment included three replications and repeated once. Isolates that grew together and failed to show a barrage reaction at the colony junction were classified as same as somatic compatibility group (SCGs), while isolates exhibiting a barrage reaction were classified into different groups (DSCGs).

3.6 Data analysis

Whole experiment was repeated twice with four replications. Data were subjected to analysis of variance (ANOVA). The treatment means were compared by using Least Significant Difference (LSD) and Duncan's multiple range test (DMRT) with the help of SPSS Computer software programme (version 20). Combined morphological data of twenty two characters were analysed with Unweighted Pair-Group Method on Arithmetic mean (UPGMA) by MVSP (Multivariable Statistical Package).

3.7 Molecular Characterization of *S. rolfsii*

3.7.1 Raising of *S. rolfsii* culture for genomic DNA extraction

Preperation of culture

Culture of 22 isolates of *S. rolfsii* was grown on PDA. Hyphal tip of 4 days old pure cultures were transferred into 250 ml conical flask containg 100 ml potato dextrose (PD) broth and placed on electric shaker for four days at 120 rpm at 25°C. Four days old mycelia of *S. rolfsii* from potato dextrose broth were filtered with filter paper to remove water. The filtered mylcelial mate was blotted, dry to remove moisture as much as possible.The blotted; flattened mylcelium of *S. rolfsii* was folded into an aluminium foil paper and frozen at -20ºC.

Fungal genomic DNA extraction

DNA was extracted from 22 isolates of *S. rolfsii* following the method of Reader and Broda (1985). Frozen mycelia were grounded in to fine powder using mortar and pestle by liquid Nitrogen. An amount of 0.5 gm powdered mycelia were poured into 1.5 µl Centrifuge tube and homogenized with 600 µl of extraction buffer (250 mM NaCl, 100mM Tris-HCl,100m MEDTA, Sigma Aldrich) by stirring on vortex for lysis of the cells. 100 µl of 10% sodium dodecyle sulphate (SDS) was added in to centrifuge tube containing powdered mycelia and extraction buffer and then mixed by inverting the tube 5-6 times and incubated for 30 minutes at 65°C in water bath. After incubation, 264 of 3M sodium acetate (pH.5.2) was added into the centrifuge tube, mixed gently and kept in ice for 15 minutes.

The mixture was then centrifuge (Hawkd15/05) 13000 rpm at 4°C for 10 minutes and supernatant was transferred in to a new tube. After that, (500 µl) of Chloroform was added in to the collected supernatant and mixed thoroughly by vortex. The mixer was centrifuge at 12500 rpm at 4°C for 30 minutes to precipitate the protein and long chain polysaccharides from the DNA. The upper aqueous phase was collected into new tubes very carefully without disturbing the interface.

Precipitation and re-suspension of DNA

Precipitation of DNA was done following the procedure of Anon (2001). Two volumes of pure ethanol (99%) were added into the DNA suspension, kept on ice for 10 minutes to allow the DNA molecules to aggregate. The DNA was precipitated down to the bottom of the centrifuge tube as a pellet by centrifuging at 13000 rpm at 4°C for five minutes. Then the supernatant was discarded using a wide bore pipette and preserved the centrifuge tube with the DNA pellet. An amount of 800 µl of 70% ethanol was added into the centrifuge tube, stirred on vortex for washing the DNA pellet and centrifuge at 13000 rpm at 4°C for two minutes, ethanol was discarded using a micropipette. The centrifuged tube containing DNA pellet was dried into a vacuum desiccators for two minutes. The DNA pellet was re- suspended by 50 µlTE buffer (10mM Tris, pH-8.0, 1M EDTA, Sigma Aldrich) . The DNA solution was preserved at -20°C for further studies.

Quantification of genomic DNA

Prior to set of PCR reaction the extracted fungal genomic DNA was check by run on 2% agarose gel and then quantify by micro spectrophotometer (K2 800 nucleic acid analyzer, Chaina).

Selection of primers for PCR amplification of fungal genomic DNA using RAPD

Four primers were selected and used for PCR amplification using RAPD, primer name and sequences are listed in table-3.3.

Table 3. 3: Name of primers and their sequences

Name of primer	Sequence
RAPD-1	5'-AATCGGGCTG-3'
RAPD-2	5'-GGTCCCTGAC-3'
RAPD-3	5'-CAATCGCCGT-3'
RAPD-4	5'-TGCGCCCTTC-3'

3.7.2 PCR amplification of fungal genomic DNA using RAPD primers

For PCR amplification total 20µl of reactions mixture was prepard including 10µl of 10 X PCR Master mix (Promega, USA), 1 ng of fungal genomic DNA (2-4 µl), 1µl of 10pm of RAPD primer and remaining up to DEPC treated water. The PCR products were fitted into the blocks of PCR machine (Thermal Cycler, Labnet International Inc.) by pcr tube. The PCR machine was run with the following PCR profil intital denaturation at 94ºC for 2 minutes and then denaturation at 94ºC for 15 seconds, anneling at 29ºC 30 seconds, extention 72ºC 1min with a 35 cycle and then final extention 72ºC for 5 mins and hold at 4 ºC.

The tubes with the PCR amplified products (amplicon) were taken out from the PCR machine and DNA fragments were separated by agarose gel electropherosis by stained with ethidium bromide the gels were visualized under UV light by Gel Doc and photographed.

Preparation of 2% agarose gel

For preparation of 2% agarose gel 2g agarose was weighed in a conical flask. An amount of 100 ml of 1×TAE buffer was poured in to the conical flask having 1.8 gm agarose and melted by heating in a microwave oven until dissolved fully. The melted agarose gel was cooled down under flowing tap water. The plate of the gel tank was placed flat on the table. The gel was poured smoothly and continuously starting from one corner until reached to the other corner. Then comb was inserted halfway into the gel immediately for making well, allowing an hour for polymerization before removing the comb gently. The casted plate was assembled in the tank. The gel was submerged into in to the 1×TAE running buffers in gel tank.

Gel loading and running

PCR product (10µl) stained with 2µl of 6× loading dye was loaded in to the well of the agarose gel and electrophoresed for 25 minutes at 100 volts to move the negatively charged DNA towards the anode.

Visualization of the PCR product in 2% Agarose gel

The gel was taken out of the gel tank and stained with ethidium bromide solution (0.5 µl) for an hour. The stained gel was rinsed with water for distaining , illuminated on UV transilluminator and photographed by gel documentation (Bio-Rad) for measuring the bands of amplified DNA fragments . The selected RAPD primer was used for the rest of the studies with 22 selected isolates for amplification of DNA. The gel used to resolve the amplified DNA was stained, illuminated under UV light and the fingerprints photographed by gel documentation.

Analysis of RAPD results

The banding patterns were scored for RAPD primers in each *S. rolfsii* isolate starting from the large size fragment to small sized one. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908) using past software program and each RAPD pattern was compared with the other patterns and Euclidean distance matrix was calculated.

3.7.3 Amplification of ITS region of fungal genomic DNA using ITS 4 and ITS 5 primers

PCR was performed using Thermocycler (Peqlab, Germany). The universal primers for PCR were obtained from Invent Technology, Bangladesh. The primer pairs ITS5-5'-CGGATCTCTTGGTTCTGGCA-3'and ITS4-5'GACGCTCGAACAGGCATGCC-3' were used for rDNA amplification. The PCR amplification was carried out in 25µl reaction mixture containing 1ng of DNA sample, 5µl of 5× PCR buffer, 2.5mM MgCl2, 2.0µl of 2mM dNTPs (Promega, USA), 20pmol of each forward and reverse primer (1.0µl) and 0.2µl of Taq DNA Polymerase and made up to 25µl with nuclease free water. The PCR conditions include initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 30 s, primer annealing at 55ºC for 30 s, followed by primer extension for 30 s at 72ºC and final extension at 72ºC for 10min. The amplicon were gel pufified by PCR clean up kits (Promega, USA) and sending for sequencing to Aplical Scientific Sdn Bhd, Selangor, Malaysia. Based on the mrphologolocal characteristics of the fungi I have sequenced six isolates namely DU-1, BA-1, BA3, CH-1, CH-2 and MA-1.

Sequence analysis

The assembly of fungal genome were performed using the web service of CAP3 program (http://doua.prabi.fr/software/cap3) and Bio-Edit V5. ITS-rDNA sequences were aligned using the CLUSTALW2 program (Larkin et al., 2007). The assembled sequenced contain partial sequmce of 18s ribosomal RNA gene, ITS-1, 5.8s ribosomal RNA gene, ITS2 complete sequence and partial sequence of 28S ribosomal RNA

partial sequence were submitted to NCBI. The accession number of the Bangladesi isolates of *S. rolsii* include in this study were isolate DU-1 accession no MH513999; BA-1 for MH514000, BA-3 for MH514001; CH-1 accession no MH514002, CH-2 accession no MH514003; and isolate MA-1 accession number MH514004,respectively. The complete list of different species of *Sclerotium* and their host and GenBank accession number are listed (Appendix 8.4). Phylogenetic tree was constructed based on the Neighbour-joining [NJ] method as implemented in MEGA version 6 (Tamura *et al.* 2011) using Kimura-twoparameter model with 1000 bootstrap replications.

RESULTS

3.1 Survey

The incidence of foot rot disease of betel vine at different plantations were recorded from different areas of Northern Parts of Bangladesh during Jun 2011 to December 2013 and the results are presented in Table 3.4.

Table 3.4: The incidence of foot rot disease of betelvine recorded in Northern Parts of Bangladesh during June 2011 to December 2013

S N	L	TNB	TNP		TNIP		DI	
			2011-12	2012-13	2011-12	2012-2013	2011-12	2012-13
1	Baghmara	19	8151	13664	7525	7392	46.50	54.09
2	Mohanpur	15	3733	5431	2535	4231	51.90	49.20
3	Mougachi	20	9222	11253	8069	10433	45.11	40.33
4	Poba	9	6759	5529	2533	1527	37.47	27.61
5	Durgapur	10	3094	5983	1537	3152	49.45	52.68
6	Puthia	10	5537	8932	2575	4275	44.50	47.86
7	Nawabganj	11	2736	2736	1869	517	28.31	18.89
8	Natore	10	12672	11503	6575	4229	36.76	41.88
9	Pabna	8	2736	6064	1117	1921	31.67	40.82

[Key: L = Location, TNB. = Total No. of boroj, TNP = Total No. of plant, TNIP = Total No. of infected plants, DI = Disease incedence.]

From the survey of 2011-2012 cropping season it is evident that among the 10 betel vine plantations the highest disease incidence (51.90 %) was observed in Mohanpur areas followed by Durgapur (49.45%), Baghmara (46.50%), Mougachi (45.11%), Puthia (44.50 %), Poba (37.47%), Natore (36.76%) and Pabna (31.67%) areas and the lowest (28.31%) in Nawabgong areas (Table 3.4).

On the other hand in 2012-13 cropping season the incidence of foot rot disease of different betel vine plantations were recorded and the results are presented in Table (3.3). From the survey it is evident that among the ten betel vine plantation areas the highest disease incidence (54.09%) was recorded in Baghmara areas followed by Durgapur (51.90) Mahanpur (49.20%), Puthia (47.86%), Natore (41.88%), Pabna (40.82%), Mougachi (40.33%) and Poba (27.61%) areas and the lowest incidence of foot rot disease (18.89%) in Nawabgong areas.

Plate 3.1 Photograhs showing healthy boroj (A), affected boroj (B), and foot rot symptom of betel vine.

3.2 Isolation and identification of different isolates of the pathogen

In the present study total twenty two isolates were isolated from foot rot affected part of betel vine of different betel vine boroj in Northern Parts of Bangladesh.With proper note down each isolates were identified based on cultural and microscopical characteristics. The production of selerotia on white to pale-brown and mycelia on PDA after 2 weeks are also diagnosised. The isolates were finally identified as *S.*

rolfsii comparing with the standard reference of the key characteristics of *S. rolfsii* is given below:

Colonies of *S. roflsii* are readily distinguished on plant material or artificial media by gross morphological characteristics. Rapidly growing, silky-white hyphae tend to aggregated into rhizomorphic cords. In culture, the whole area of a petri plate is rapidly covered with mycelium, including aerial hyphae which may cover the lid of the plate. Both kin culture and in plant tissue, a fan-shaped mycelia expanse may be observed growing outward and branching acutely. At least two types of hyphae are produced. Coarse, straight, large cells (2-9 um x 150-250um) have two clamp connections at each septation, but may exhibit branching in place of one of the clamps. Branching is common in the slender hyphae (1.5-2.Sum in diameter) which tend to grow irregularly and lack clamp connections. Slender hyphae are often observed penetrating the substrate. Mustard like sclerotia (0.5-2.0mm diameter) begin to develop after 4-7 days of mycelial growth. Initially a felty white appearance, sclerotia quickly melanize to a dark brown coloration.

Taxonomic Position

Division : Eumycota

Sub-Division : Deuteromycotina

Form-class : Hyphomycetes

Form-order : Agonomycetales / Mycelia sterila

Form-family : Agonomycetaceae

Form-genus : *Sclerotium*

Form-species : *S. rolfsii*

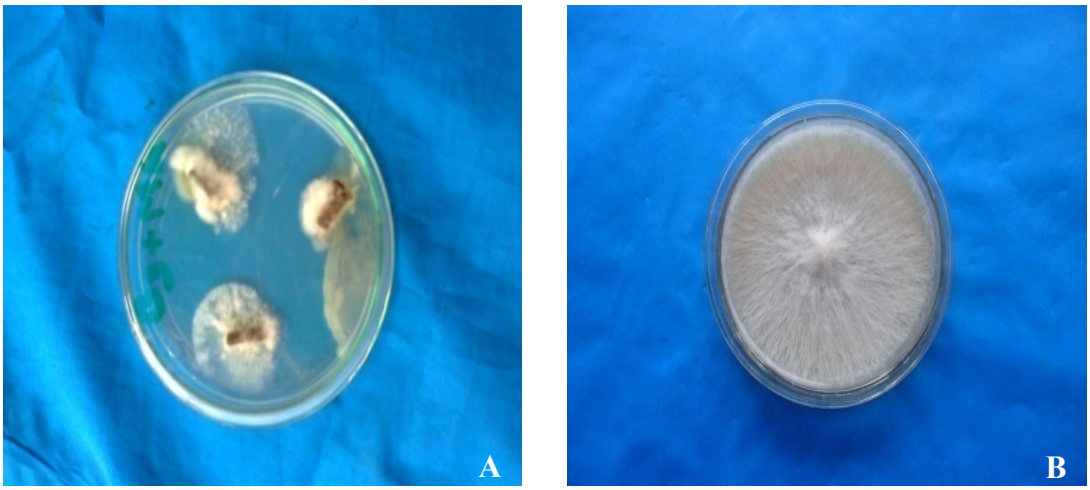


Plate 3.2 Photographs showing inoculating PDA plate (A) and Pure culture of *S. rolfsii* (B).

3.3 Pathogenisity test of the selected isolates

Inoculation and observations of the symptoms of foot rot disease

Artificial inoculation of selected isolates of *S. rolfsii* were conducted for pathogenisity test and the symptoms of foot rot of betel vine plants were produced after 30 days. *S.rolfsii* found to attack the stem near the soil level. At first the plants turned yellow and finally dried out to a pale brown color.

Re-isolation and comparison of the pathogen

Infected portion of the plants were collected and the pathogens were re-isolated. It was found that the pathogen showed the same characteristics on PDA culture medium as found earlier on isolation from naturally foot rot affected betel vine plant.

Plate 3.3 Photograph showing experimental betelvine cuttings which were inoculated with the isolates of *S. rolfsii* for pathogenicity test.

Plate 3.4 Photograph showing foot rot symptom which is produce after inoculation of 30 days.

Disease severity

Pathogenicity test was conducted with total 22 isolates of *S. rolfsii* and disease severity has been recorded. From the experiment it was observed that the highest percent disease severity (23.42%) of betel vine was recorded the in BA-1 followed by CH-1 (22.03%), BA-3 (20.98%), CH-2 (20.02%), MA-1 (19.51%), DU-1 (17.20%), BA-2(16.33) MA-2 (16.00%), MO-1, BA-5, and BA-6 (15.99%), PU-1(14.32%), PU-2 (14.00%), PO-2 (13.20), MO-2 (13.00%), BA-4 (12.00%), PO-1 (10.21%), DU-2 (8.0%), N-1 and N-2 (5.2%), PA-2 (4.55%) and the lowest (4.00%) was recorded in PA-1 isolate. So, most virulent isolate was BA-1 and this isolate was used for further experiments.

Table 3.5 Percentage of disease severity of 22 isolates of *S. rolfsii*

Code of the isolates	Disease severity (%)
BA-1	23.42a
CH-1	22.03a
BA-2	16.33h
BA-3	20.98b
BA-4	12.00e
BA-5	15.00c
BA-6	15.99c
MA-1	19.05b
MA-2	16.00c
MO-1	15.00d
MO-2	13.00d
PO-1	10.00f
PO-2	13.29d
DU-1	17.20c
DU-2	8.00g
PU-1	14.32d
PU-2	14.00d
N-1	5.09h
N-2	5.00h
PA-1	4.00h
CH-2	20.02b
PA-2	4.00h

[Values in a column having same letter do not differ significantly (p=0.05) according to DMRT]

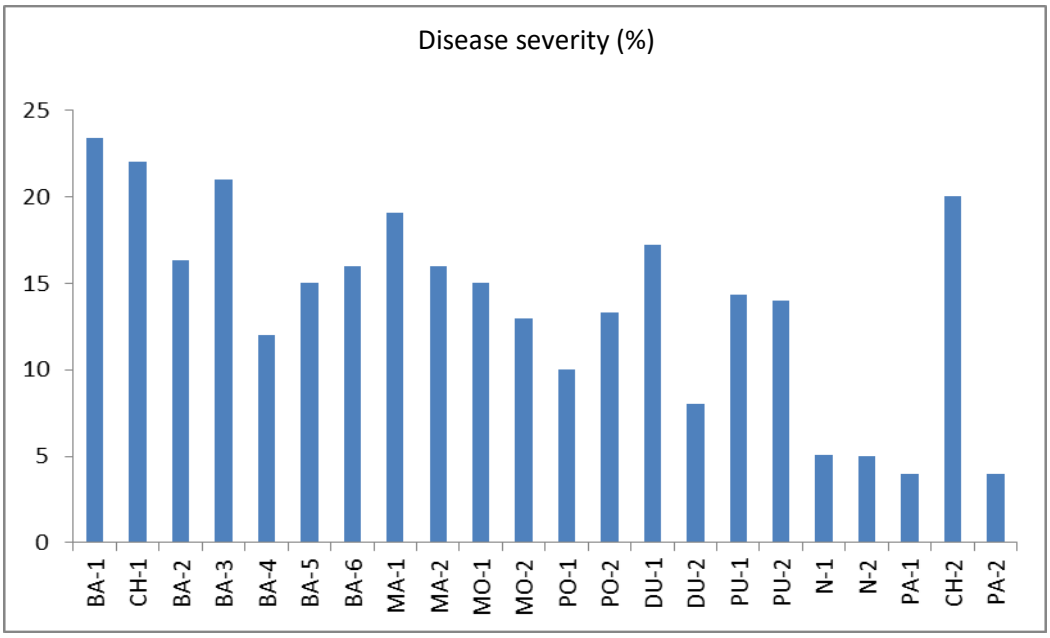
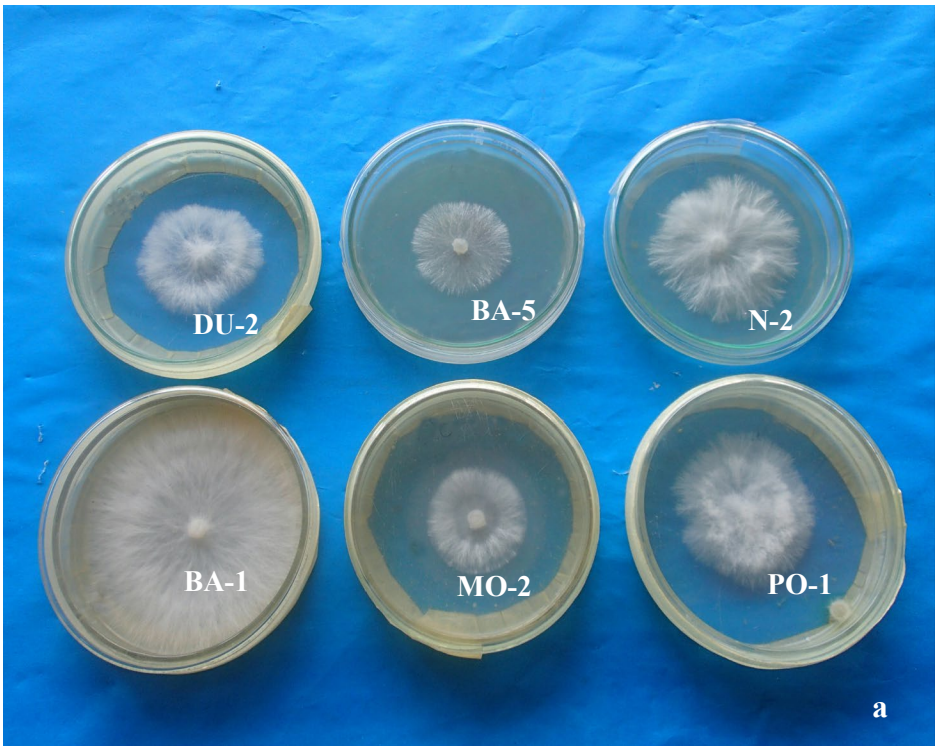


Fig. 3.1 Disease severity (%) of twenty two isolates of *S. rolfsii*.

3.4 Morphological characterization of selected *S. rolfsii* isolates.

3.4.1 Measurement of mycelial growth of *S. rolfsii* isolates.

From the results it was observed that mycelial growth rate was significantly varied (p=0.05) among the isolates (Table-3.6). The isolates were divided into three distinct groups according to growth rate such as slow, fast and medium growing isolates. Slow growing isolates ranged from 0.9-1.37 mm per day with an average of 1.14 mm per day while mycelial growth rate showed by the comparatively fast growing isolate ranged from 1.81-1.84 mm per day with an average of 1.83 mm per da. According to growth rate, slow growing isolates were MA-2, MO-2, PO-1, PO-2, DU-1, PU-2, N-2; medium growing isolates were BA-1, CH-1, BA-2, BA-3, BA-4, MA-1, MO-1, DU-2, PU-1, NA-1, PU-2, PA-2 and fast growing isolates were BA-1, PA-1, CH-2. Among the isolates, maximum growth rate (1.84 mm/day) showed BA-1 isolate at Baghmara thana in Rajshahi District. In contrast, minimum growth rate (0.9 mm/day) showed DU-1 isolate at Durgapur Thana. Variation in mean mycelia growth among the isolates were found within the locations and as well as with the different locations. Mycelial growth rate was rapidly increased up to 2 days after that it was slightly decreased until culture plates were completely filled up. In regard to mycelial growth intra district isolates variations were observed among the Baghmara, Mahonpur and Durgapur Thana in Rajshahi District. However, inter isolates variability in mycelial growth was also observed among Nator, Pabna , Nawabgang and Rajshah district.



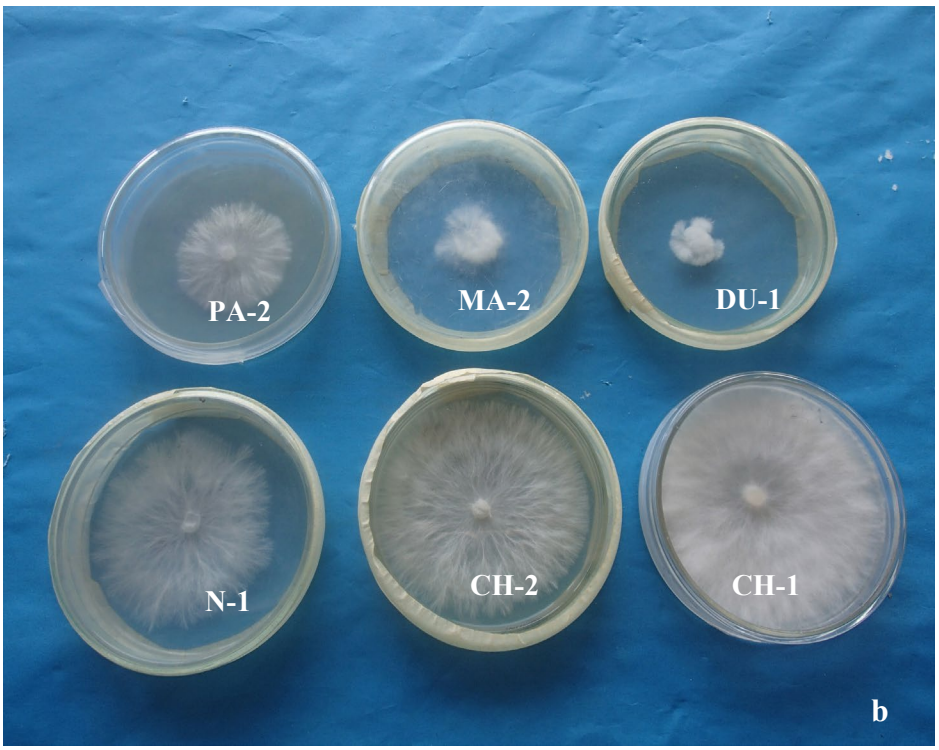


Plate 3.5 Photographs showing variation in mycelial growth of different isolates after 3 days of incubation (a and b).

Table 3.6 Mycelial growth of different *S. rolfsii* isolates on PDA plates during 5 days of incubation.

Isolates	Mycelial growth (mm) after different incubation periods (days)				Growth rate (mm/day)
	2nd	3rd	4th	5th	
BA-1	17.5f-i	36.5h	79f	90d	1.84k
CH-1	10j	34h	65d	89.5 ab	1.79i
BA-2	20.5c-f	46.75cd	68.5d	87bc	1.74h
BA-3	35.5a	65.25a	83.75a	85.3c	1.71h
BA-4	28.5b	54.25b	72.5c	86.3bc	1.73h
BA-5	10j	18m	41hI	76d	1.52g
BA-6	16d-I	24g,h	50.5e	76.5a	1.53g
MA-1	17.75efghi	41.5f	65.75d	89.3abc	1.79i
MA-2	14.5ghi	28.5j	44.25d	64.5h	1.29d
MO-1	20.0cdef	39.5j	59.25e	72e	1.44f
MO-2	10j	20.5m	35j	62h	1.24b
PO-1	15.5ghi	28.5j	45gh	68.3fg	1.37e
PO-2	13.25ij	28.75j	50f	68.5f	1.37e
DU-1	14.5hi	23.5li	40i	45i	0.9a
DU-2	18c-h	30.25j	47.5fg	70.3ef	1.41f
PU-1	21.75cd	43.75dc	67.0d	87.5bc	1.75i
PU-2	21cde	35h	45gh	62.5h	1.25c
N-1	17.25e-I	35.25h	57.5e	76.8d	1.54g
N-2	19.75c-g	33i	47.5fg	61h	1.22b
PA-1	22.5c	47.75c	77.5b	90ab	1.81j
CH-2	3.75a	57.25b	73.8c	80ab	1.81j
PA-2	26b	36.25h	60e	86.75bc	1.74h
± SE	0.90	1.44	1.64	1.56	0.03
LSD (P=0.05)	1.23	2.702	2.40	2.61	0.80

[Values in a column having same letter do not differ significantly (p=0.05) according to DMRT].

3.4.2 Studies of morphological diversity among the isolates of *S. rolfsii*

Morphological variation among the twenty two isolates of *S.rolfsii* was studied after 25 days of incubation. The isolates showed a high degree of morphological variation irrespective of all isolates mycelia were hyaline and produced white to brown sclerotia on PDA surface. Among the isolates number of sclerotia/plate were ranged from 55 to 950. The maximum number of sclerotia (950) was found in DU-1 isolate and the minimum number of sclerotia (55) was found in MA-2 isolate. On the other hand the diameter of sclerotia were ranged from 0.91mm to 2.0 mm.The maximum diameter of sclerotia (2.0 mm) was found in MA-2 isolate and the minimum diameter of sclerotia (0.91mm) was found in N-2 isolate.

Distribution or production pattern, quality, size and shapes of the sclerotia indicated that the variation was existed among the isolate. The color of sclerotia tended white to pale brown except a few. Three types of zones and six types of sclerotial production pattern were observed. Among the tested isolates BA-1, BA-2, MA-2, MO-1, MO-2, PO-2, PU-1, PU-2, BA-5, BA-6, CH-2 no. isolates group produced sclerotia near the inoculums while BA-1, BA-2, BA-3, BA-4, MA-2, PO-2, DU-1 isolates group produced scattered sclerotia all over the plate. The other isolates group as BA-1, CH-1, BA-3, BA-4, MA-1, MO-1, MO-2, PO-2, DU-2, PU-1, PU-2, BA-5 produced sclerotia as peripheral, aggregate on zonal area and BA2, BA-3, BA-4, MA-1, MA-2, MO-1, PO-1, PO-2, DU-2, N-1, PA-1 isolates group produced immerged or embedded in agar respectively. The other isolates produced sclerotia as like any one of the above mentioned patterns. Investigation on mycelial growth indicated that some isolates produced a moderate quantity of aerial mycelium on the colony surface as well as on the lid and produced few sclerotia on the lid results are presented (Table 3.2 and Appendix 8.1).

Plate 3.6 Photographs showing morphological variation of some isolates of *S. rolfsii* with various from of sclerotia

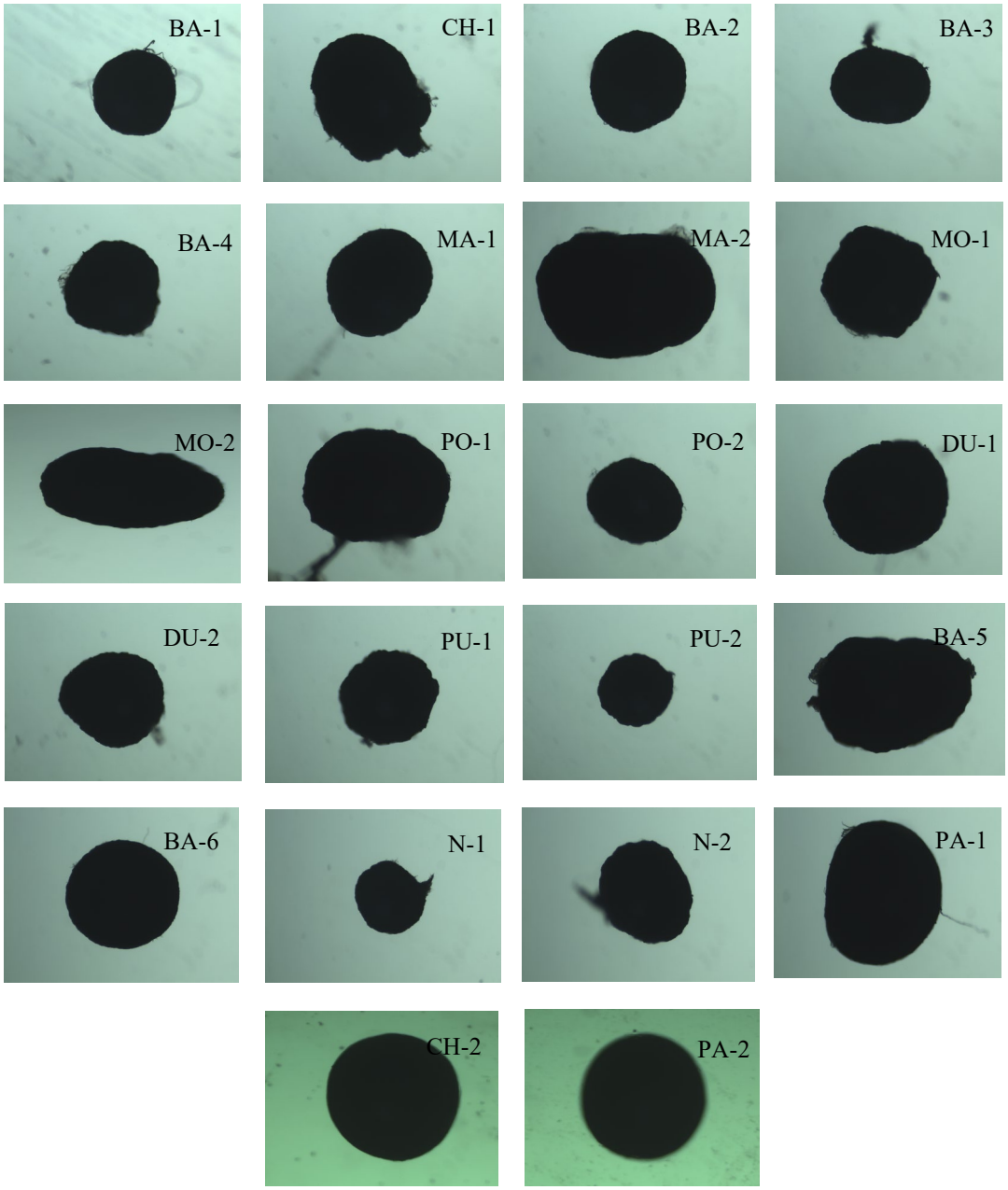


Plate 3.7 Photographs showing morphological variation of sclerotia of 22 isolates of *S. rolfsii* (40 x)

Table 3.7 Color, number, diameter, and weight of sclerotia of different isolates of *S. rolfsii* on PDA plates after 25 days of incubation.

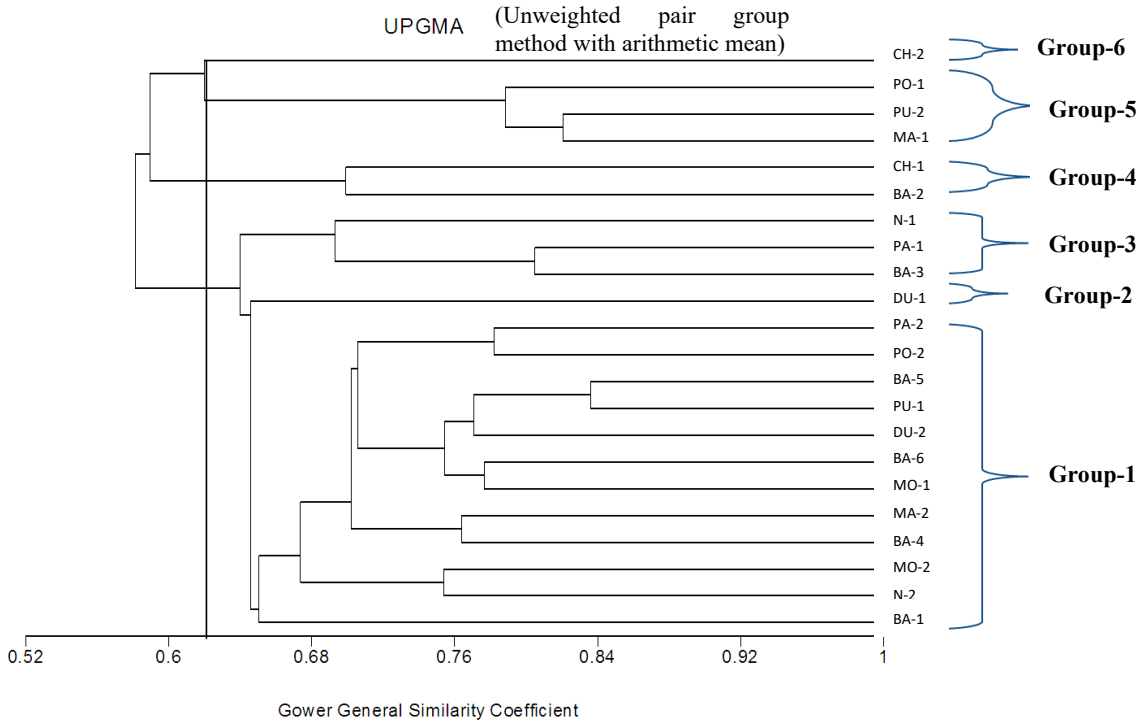
Isolate Code	Color of sclerotia	No.of sclerotia/plate	Diametre(mm) of sclerotia	Weight of per 100 sclerotia (mg)
BA-1	B., W	950a	1.30b	6abc
CH-1	O. W, B	350g	1.03c	1c
BA-2	B, W	280i	1.09e	2bc
BA-3	W, O. W, B	120n	1.13d	4abc
BA-4	B, O. W, W	110n	1.09c	3bc
BA-5	W,B	270d	1.36b	7a
BA-6	L. B, D. B	180l	1.03c	5abc
MA-1	B, O.W, W	160.00m	1.17c	5abc
MA-2	W, B	55.o	2.0a	4abc
MO-1	B	500e	1.11d	3b,c
MO-2	W,B,O. W	240j	1.03c	1c
PO-1	B, W	400f	1.25b	2bc
PO-2	B, O .W, W	65o	1.09c	5abc
DU-1	B	120n	1.31b	3bc
DU-2	B	200k	1.31b	3bc
PU-1	W, L. B, B	340h	1.31b	1c
PU-2	D. B, O. W, W	275i	1.18c	2bc
N-1	O.W ,B	270i	1.15c	2bc
N-2	L. B	630d	0.91d	4abc
PA-1	O. W	830b	1.21b	1c
CH-2	O.W	800c	1.47b	1c
PA-2	O.W, W, B	150m	1.25b	2bc

[Key: W=White, O.W=Off white [, B =Brown, L.B= Light brown, D.B=Dark Brown]

Values in a column having same letter do not differ significantly (p=0.05) according to DMRT.

3.4.3 Analysis of morphological diversity of *S. rolfsii* isolates with UPGMA using MVSP

Combined morphological data of twenty two characters were analyzed with UPGMA by MVSP and the results showed six groups at 62% similarity level. Twelve isolates were clustered in Group 1, which further constituted three sub-groups at 75% similarity level. Group 2 consisted of 1 isolate at 65% similarity level. Group 3 contained 3 isolates at 70% similarity level. Group 4 contained 2 isolates at 73% similarity level. Group 5 contained 3 isolates at 79% similarity level and Group 6 contained 3 isolates at 79% similarity. Group 1 and Group 3 was slower than the Group 2 and Group 4. However, Group 4 consisted of two isolates i.e.BA-3 and PU-2 contain light brown sclerotia and abundant fluffy type mycelia which was characteristically different from other cluster groups.

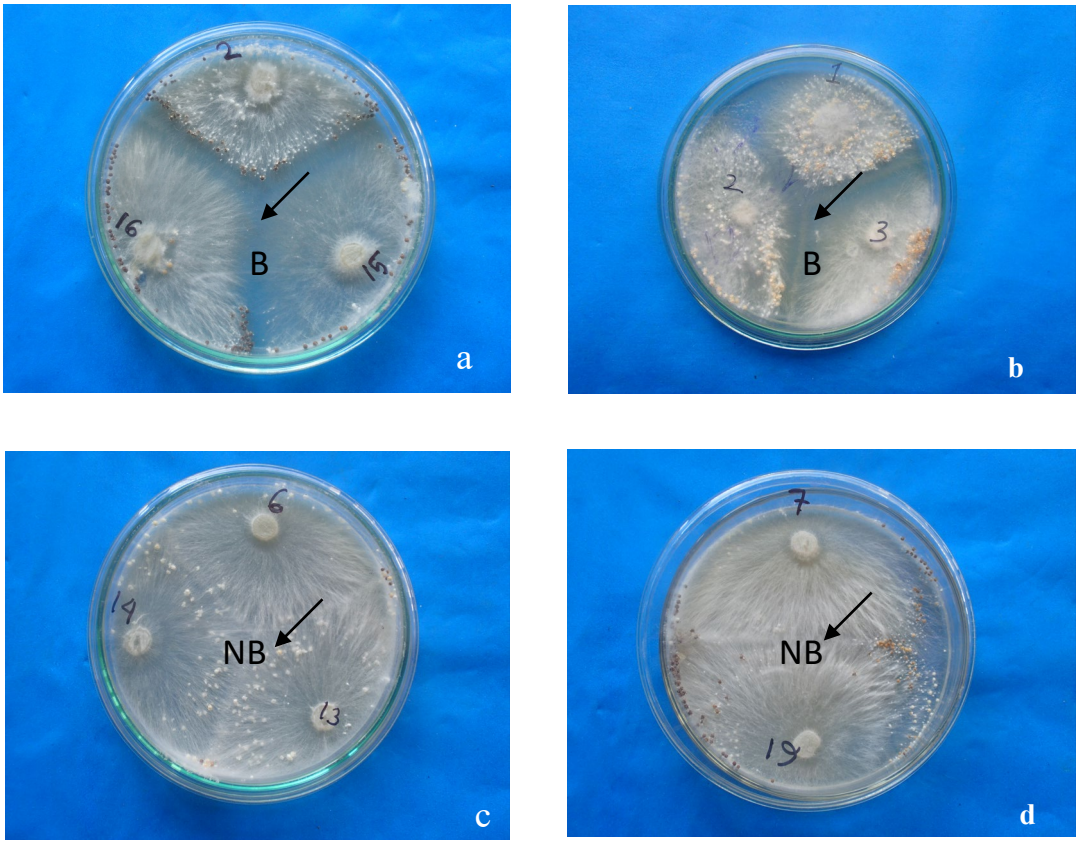


[Combined morphological data of twenty two characters were analyzed with MVSP showing six groups at 62% similarity level]

Fig 3.2 Relatedness of *S. rolfsii* isolates based on morphological characteristic by MVSP (Multivariable Statistical Package)

3.4.4 Somatic compatibility test

Somatic compatibility test was conducted to detect morphological diversity of 22 isolates of *S. rolfsii* in a petri plates. After inoculation and growth of three different isolates on one plate, it was observed that as the colony size increased, their mycelia came in contact with each other. Initially, intermingling of the mycelia of two incompatible isolate took place but later on lysis of mycelia of two isolates and development of a clear zone was observed at the region of interaction (mycelial contact). Among the isolates of barrage zone and no barrage zone was observed at the colony interface of two or three different isolates. Total 122 combinations were conducted wth 22 isolates of *S. rolfsii* and out of them 38 combinations showed somatic compatible reaction and rest of all showed somatic incompatible reaction (Apendix 8.2).



[B = barrage zones, NB= No barrage zones.]

Plate 3.8 Photographs showing somatic incompatibility (a & b) and compatibility (c & d) reactions among the isolates of *S. rolfsii* after 10 days of incubation.

3.5 Moleculer characterizations of *S. rolfsii* through RAPD PCR

3.5.1 DNA fingerprint analysis

Genetic variation was determine among twenty two isolates of *S. rolfsii* using RAPD primers i.e, RAPD-1, RAPD-2, RAPD-3 and RAPD -4. The fungal genomic DNA was amplified by the RAPD-4 primer and generated different sizes total (100 bp to10000 bp) distinct and clear badns but there was no amplification was obserbved by using other three primers (RAPD-1, RAPD-2, RAPD-3) (Fig.3.3). In this study it is indicates RAPD-4 primer showed hundred percent polymorphism.

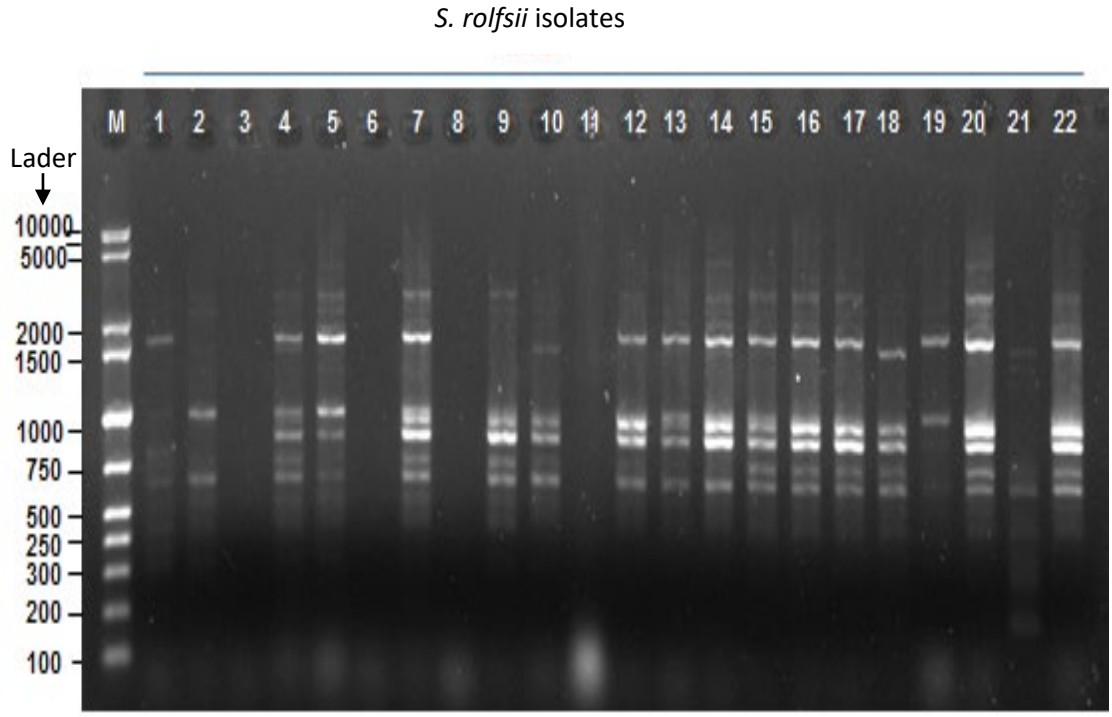
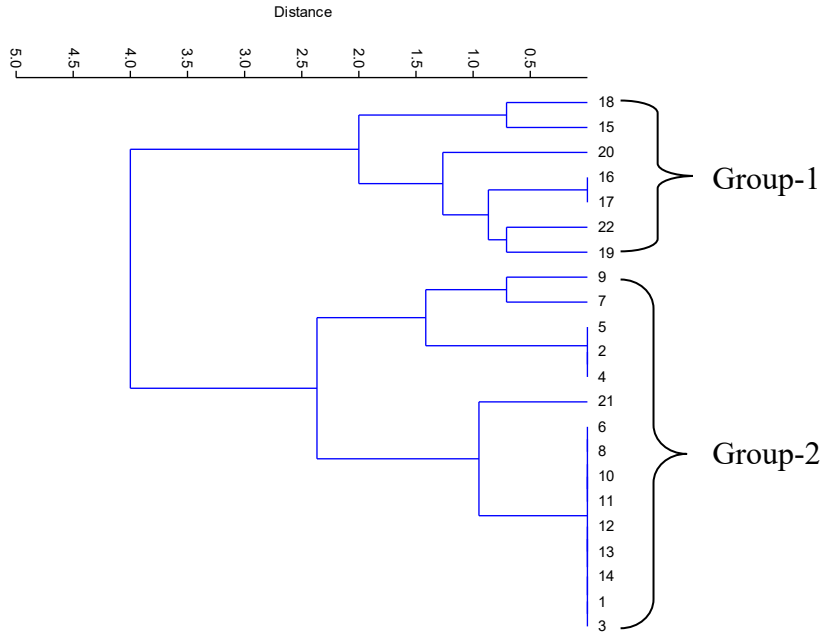


Fig. 3.3: DNA fingerprint profiles of 22 isolates of *S rolfsii* using RAPD-4 primer (5′-TGCGCCCTTC-3′). Lane M indicate the DNA marker and lane 1-22 funal genomic DNA of Isolate BA-1,CH-1, BA-2, BA-3, BA-4, MA-1, MA-2, MO-1, MO-2, PO-1, PO-2, DU-1, DU-2, PU-1, PU-2, BA-5, BA-6, N-1, N-2, PA-1, CH-2, PA-2 repectively.

Cluster analysis

Phylogenetic dendogram generated with the primer showed that the 22 isolates of *S. rolfsii* were categorized into 2 main groups which divided by further sub groups. On the basis of the average genetic distance 22 isolates of *S. rolfsii* were grouped as group -1 contained 8 Isolates and the distance of this group is 2.1unit and group -2 contained of 14 isolates and the distance of this group is 2.5 unit (Fig. 3.4). Jaccard's simmilarity coefficient matrix was calculated and the range of genetic distance were 0 to 2.5 unit the results are presented in (Appendix 8.3).



[Key: 1-22 serial number indicate the all isolates *S. rolfsii* i.e. BA-1,CH-1, BA-2, BA-3, BA-4, MA-1, MA-2, MO-1, MO-2, PO-1, PO-2, DU-1, DU-2, PU-1, PU-2, BA-5, BA-6, N-1, N-2, PA-1, CH-2, PA-2 repectively.]

Fig 3.4 Phylogenetic dendogram generated from the banding pattern for genomic DNA of 22 isolates of *S. rolfsii*.

3.5.2 PCR amplification of ITS region of *S rolfsii* isolates from betelvine in Bangladesh

The amplicon size 750 bp of the ITS region were amplified by using ITS4 and ITS 5 primers which include partial sequence of 18S ribosomal RNA gene, ITS-1, 5.8S ribosomal RNA gene, ITS2 complete sequence and partial sequence of 28S ribosomal RNA sequence Fig (3.5).

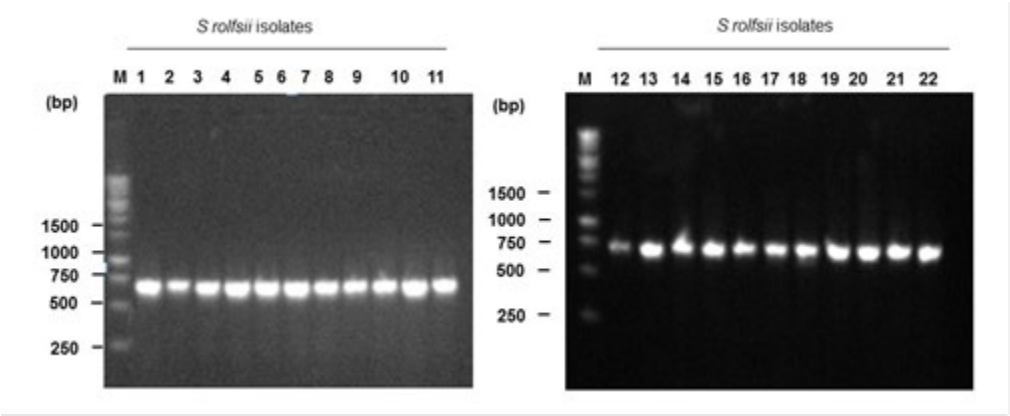


Fig. 3.5 Agarose gel electrophoresis showing the amplification of fungal DNA using ITS4 and ITS5 primers. Lane M= DNA Marker and lane 1-22 funal genomic DNA of Isolate BA-1,CH-1, BA-2, BA-3, BA-4, MA-1, MA-2, MO-1, MO-2, PO-1, PO-2, DU-1, DU-2, PU-1, PU-2, BA-5, BA-6, N-1, N-2, PA-1, CH-2, PA-2 repectively.

Phylogenetic placement of *S.rolfsii* isolates from Betel vine in Bangladesh

A total of six isolates ITS region were sequenced and all sequences showed 98-100% similarity with sequences of *Athelia rolfsii* (anamorph: *S. rolfsii*) from GenBank by nBLAST search analysis. Phylogenetic tree was constructed using MEGA V6 where *Rhizoctnia sonali* is an out group. For construction of a phylogenetic tree, sequences of *S. rolfsii* collected worldwide retriving from GenBank, along with *S. delphinii*, *S. coffeicola, S. Shydrophilum, S. rhiodes, S. cepivorum, S. denigrans and S. perniciosum. A rolfsii* (Anamorpph: *S. rolfsii)* is clustering with the *S. rolfsii* isolates from betelvine in Bangladesh produced a separate subgroup, supporting that the isolates identified in this study is *S. rolfsii* (Fig. 3.5). The neibourjoing (NJ) tree indicates that all sequences produced three major clusters designated as S_1, S_2 and S_3

(Fig. 3.6). *S. rolfsii S. delphinii* and *S. coffeicola* included in the S_3 cluster whear as *S. hydrophilum*, *S. rhizodes* in cluster S_2 and *S. cepivorum*, *S. rhizodes*, *S. perniciosum* clustering together with S_1 respectively.

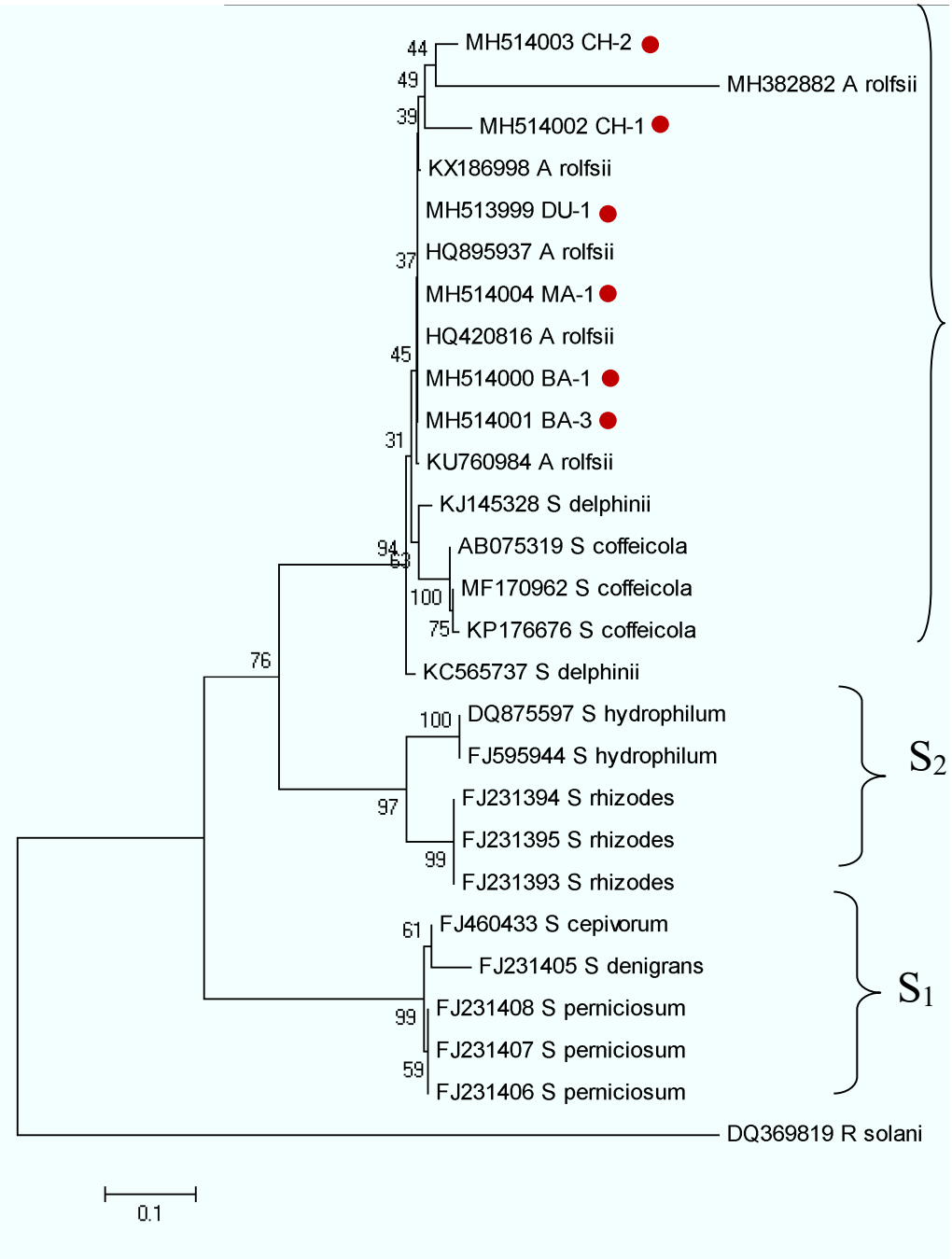
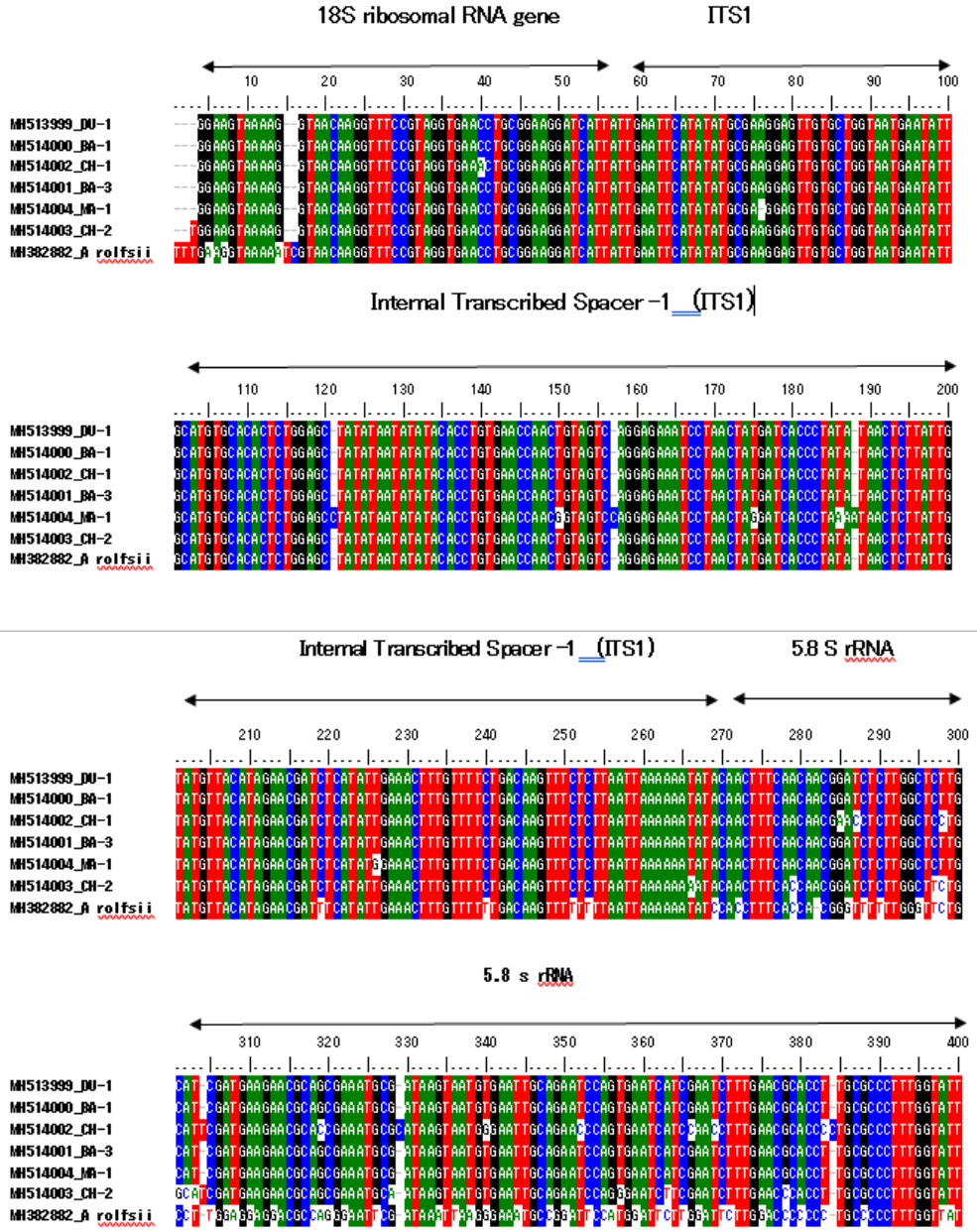


Fig. 3.6 The evolutionary history was inferred using the Neighbor-Joining method [Saitou N. and Nei M. (1987)]. The optimal tree with the sum of branch length = 2.25075645 is shown.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [Felsenstein J. (1985)]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method [Tajima F. and Nei M. (1984).] and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et *al* 2007).

Multiple sequence alignment of *S. rolfsii* isolates from Bangladesh

The multiple sequence alignment was performed using Bio-edit software V5. The sequence include in this study are all six newly sequencd Bangladeshi isolates and perivirusly sequenced isolates accession no MH382882. The results of the multiple sequenced alignments indicate that the partial sequence of 18S ribosomal RNA (18S rRNA) gene almost identical but two nucleotide (TC) missing at 15 nt position form the previously sequenced isolates (Fig. 3.7). There is not so much diversity found in internal transcribed spacer 1 (ITS1) except few nucleotides in MA-1 and the previous sequenced. Three additional nucleotides (2C and 1A) found in isolate BA-1 at 121, 157 and 188 nt position. In the nucleotide position 150nt, 175nt, 226 nt G Substitute T in isolate BA-1 but other reanaing isolates are similar but in the isolates CH-2 only one nucleotide (nt) found dissimilar at 26 nt A instead of T but four nucleotides at the position 219 nt, 240nt, 251nt and 253 nt found T instead of C in CH-2 isolates. More variability observed in 5.8S rRNA gene in isolate MH382882 but betelvine isolates are almost identical except a few nucleotide (nt) cahang in CH-1 and CH-2. One additional T was found in the isolates CH-1 and CH-2 at the position of 304 nt and another there more nucleotide (2C & 1G) found in the position 329 nt, 384nt and 413nt in the isolate CH-1. In the ITS2 region upstream region of MH382882 is more variable except a few nucleotide changes in CH-1 and Ch-2. The downstream region of the ITS 2 is almost identical of all Bangladehi *S. rolfsii* isolates. In the partial sequence of 28S rRNA the two nucleotide change in MA-1 but there is more variability found in MH382882 (Appendix 8.4).


18S ribosomal RNA gene
ITS1
Internal Transcribed Spacer -1 (ITS1)
Internal Transcribed Spacer -1 (ITS1)
5.8 S rRNA
5.8 s rRNA
MH513999_DU-1
MH514000_BA-1
MH514002_CH-1
MH514001_BA-3
MH514004_MA-1
MH514003_CH-2
MH382882_A rolfsii

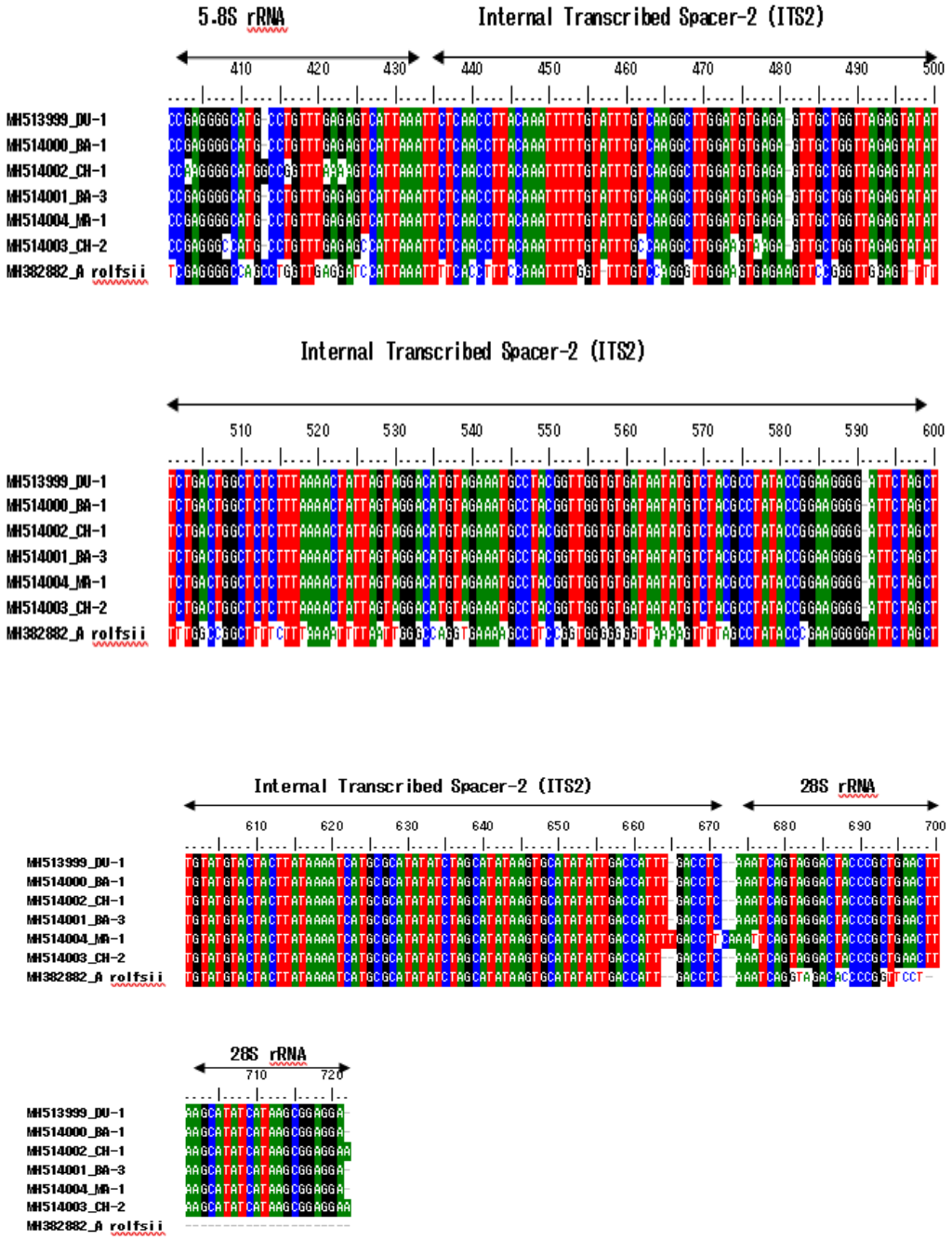


Fig. 3.7 The multiple sequence alignment of the Bangladeshi siolates of *S. rolfsii*

DISSCUSSION

The incidence of foot rot disease of betel vine at different boroj of Northern Parts of Bangladesh were survyed during June 2011-Dcember 2013. From the survey result it was observed that the highest disease incidence (51.90%) was recorded in Mohonpur areas and the lowest (28.31%) was in Nawabganj areas at 2011-2012 cropping season. On the other hand at 2012-13 cropping season, the highest disease incidence (54.09%) was recorded in Baghmara area and the lowest (18.89%) was in Nawabgonj area. In earlier study Mridha and Alamgir (1989) observed sclerotial wilt of betel vine in thirty selected gardens in Chittagong and the infected plants showed decay at the collar region, leaves turned yellow and the whole plant wilted and died. Palakshappa (1986) surveyed the incidence of foot rot disease of *Piper betle L*. in different areas of Karnataka state during 1984-85 and recorded 35 to 39 per-cent disease incidence. Meah (1994) reported the incidence and severity of collar rot of sunflower in fifteen (15) varieties. Mollah (2012) found that, in case of foot and root rot of betel vine in Satkhira district, the 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 while 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. Jahan *et al*. (2016) investigated on foot and root rot disease of betel vine in Kushtia district of Bangadesh and reported the maximum disease incidence were recorded in Mirpur upazilla where disease incidence ranged from 54.00% to 64.00% and the minimum disease ncidence were recorded in Khoksha upazilla where disease incidence ranged from 28.00% to 34.00%. The present result supports the findingings of Jahan (2016) and it is noted that foot rot disease incidence are increases in Northern Parts of Bangladeh compare to others.

Pathogenicity test of the *S. rolfsii* isolates were conducted by artificially inoculating of betel vine plantlet and disease severity (%) was measured. The heighest percent disease severity was recorded in BA-1 (Baghmara-1) and the lowest was in PA-1 (Pabna -1) so that the most virulence isolate was BA-1. Sengupta and Das (1970) studied the cross inoculation of the isolates of *S. rolfsii* from groundnut, wheat, potato, guava, and benglagram. They concluded that, benglagram was the most

susceptible host for *S. rolfsii*. Palakshappa (1986) observed considerable foot rot infection when betel vine were inoculated with two and three percent inoculum of *S. rolfsii*. They recorded percent infection at four percent and above inoculum levels.

Scientist develops their technologies which are beneficial to human being. With the development of science and technology the scientist of different region of the world tried their best to isolate and characterize *S. rolfsii.* In the present study total 22 isolates were isolated from different betel vine boroj of Northern Parts of Bangladesh. These isolates were identified following the standard refference. Morphological diversity was observed among the isolates by studing mycelial growth rate, number, shape, size and color of sclerotia. Sclerotia are considered to be extremely hardy and relatively resistant survival structures. Profuse mycelial growth and sclerotial production contribute to the considerable crop losses associated with *S. rolfsii* (Kokub *et al.,* 2007). Because of its basidial stage which is not easily observed, this fungus is characterized by the morphology of sclerotia in most cases (Punja and Damiani, 1996). Sclerotia forming on a host tend to have a smooth texture, whereas those produced in culture may be pitted or folded. Serving as a protective structure, sclerotia contain viable hyphae and serve as primary inoculum for disease development.

The isolates of *S. roifsii* varied in all of the characters such as colony morphology, mycelial growth rate, sclerotial production, sclerotial size and color. Out of twenty two isolates the heighest mycelal growth rate was recorded in BA-1 isolate and the lowest was in Du-1. The variability of twenty six Indian isolates of *S. rolfsii* were observed which were collected from different host (Sarma *et al*. 2002). Akram *et al*. (2007) reported mycelial incompatibility and morphological diversity between 12 isolates of *S. rolfsii* collected from different locations of Panjab in Pakistan. Sarma *et al*. (2002) investigated the variability in growth and basidial stage production of 26 isolates of *S. rolfsii*.

During the present study, it was observed that the sclerotia of these isolates were mostly round and oval in shape. The number of sclerotia produced by different

isolates were varied and ranged from 65-950 per plate. The sclerotial diameter of twenty two fungal isolates was ranged from 0.91-2.0 mm. However, the average sclerotial diameter of 1.0 ± 0.2 mm at 20°C was recorded by Punja and Damiani (1996). Color of sclerotia was also observed and five types color was noted and most of the sclerotia were dark brown. Production of small, spherical and tan to dark brown and black colored sclerotia was also reported by Zarani and Christias (1997) and Sarma *et al.* (2002). In another study done by Punja and Damiani (1996) reported that greatest number of sclerotia (1043 ± 340) was produced by S. *rolfsii* on PDA plates at 35°C. Rasu *et al* (2013) studied morphological and genomic variability among S. *rolfsii* populations and reported that out of 17 isolates tested for their cultural morphology, most of them were observed with compact colonies and few were fluffy colonies. Based on growth rate, they were categorized into three groups such as slow growing, fast growing and intermediate. In this study it was also observed that twenty two isolates were categorized into three groups according to growth rate; and compact and fluffy colonies were also observed. These findings were consistent with the earlier investigations made by Rasu *et al* (2013). Moreover, sclerotia of some isolates showed shiny appearance due to presence of gummy material on their surface. The presence of gummy material on the surface of sclerotia might be due to the production of extracellular polysaccharides by these isolates. Flieger *et al.* (2003) reported that filamentous fungi were very promising producer of 13 D-glucan as the hyphal cell wall and extracellular matrix contain more than 75% polysaccharides. Earlier studies on sclerotia formation by S. *rolfsii* revealed that a large number of factors were responsible for production of sclerotia such as some nutritional and non nutritional factors (Trevethick and Cooke, 1971), nutrient depletion (Hadar *et al.,* 1983), restriction of growth by a physical barrier (Wheeler and Waller, 1965), as well as by imposing a step down in the level of nitrogen following active growth (Punja, 1986). In this study, it was remarkly noticed that if a contaminant (bacterial or fungal) came into contact with S. *rolfsii* strain on agar plate, a number of sclerotia developed around the contaminant and blocked its further growth and proliferation. However, by producing large number of sclerotia, the fungal cells were protected. Therefore, some times sclerotia might be developed as a part of defense mechanism. Combined morphological data of twenty two characters were analyzed with UPGMA by MVSP

and the results showed six groups at 62% similarity level. Twelve isolates were clustered in Group 1, which further constituted three sub-groups at 75% similarity level. Group 2 consisted of 1 isolate at 65% similarity level. Group 3 contained 3 isolates at 70% similarity level. Group 4 contained 2 isolates at 73% similarity level. Group 5 contained 3 isolates at 79% similarity level and Group 6 contained 3 isolates at 79% similarity.

Somatic compatibility incompatibility reaction was conducted within 22 isolates of *S.rolfsii.* Among the twenty two isolates, seven pairs isolate showed mycelial compatibility and fiften showed mycelial incompatibility. Mycelial compatibility and incompatibility reaction by different species was also reported by Punja and Sun (2001) and Sarma *et al.* (2002). Punja and Sun (2001) evaluated genetic diversity among mycelial compatibility groups of *S. rolfsii* (teleomorph *Athelia rolfsii*) and *S. delphinii*. The earlier workers also observed that when mycelia of different isolates belonging to the same species confront with one another, either on agar media or a suitable growth substrate, a distinct zone of demarcation (barrage or aversion zone) was developed between the colonies. Recognition of non-self from self is the underlying basis of the incompatible reaction (Punja and Sun, 1997). Mycelial compatibility reaction was also used by Sarma *et al.,* (2002) to study the variability and relatedness among fungal species belonging to different geographical regions.

In moleculer characterization of 22 isolates of *S. rolfsii* was conducted using RAPD PCR technique and genetic variability was observed among the isolates of Northern Parts of Bangladesh. Genetic variability among the isolates of *Sclerotium rolfsii* was studied many reseachers using molecular techniques like RAPD, ITS-PCR and RFLP. Shokes *et al.* (1996) tested the pathogenic variability and pathogenic potential of *S. rolfsii* isolates on the groundnut variety TGCS888. The molecular variability among the isolates of *S. rolfsii* was studied by using ITS region of rDNA, Random Amplified Polymorphic DNA (RAPD) and Internal Transcribed Spacer-Restriction Fragment Length Polymorphism (ITS-RFLP). Okabe *et al*. (1998) divided 67 isolates of the southern blight fungus from Japan into five groups based on ITS-RFLP analysis of nuclear rDNA.Three groups were reidentified as *S. rolfsii* and two resembled *S. delphinii* in RFLP patterns. Punja and sun (2001) assesed genetic similarities and

differences among eight strains of *S. rolfsii* using randomly amplified polymorphic DNA (RAPD) analysis while DNA was extracted from the mycelial mat of each strain grown on PDA plate for 7 days at 28ºC. Mycelium (200 mg) was transferred to 1.5 mL micro centrifuge tubes containing liquid nitrogen. Tanwir *et al*. (2007) and Asif *et al*. (2005) evaluated that RAPD analysis was found to be an informative DNA marker system to assess genetic relatedness and diversity among different strains. RAPD-PCR analysis had also been used by other worker to investigate genetic variation among isolates of *S. rolfsii* collected from different geographical regions. Punja and Sun (1997) compared 128 isolates of *S. rolfsii* from 36 host species and 23 geographic regions by means of RAPD which confirmed that many isolates from the same host belongs to the same mycelial compatibility group (MCG). Almeida *et al*. (2001) reported variation among 30 isolates of *S. rolfsii* from different hosts and regions of Brazil was studied by undertaking analysis of genomic DNA through random amplified polymorphic DNA (RAPD-PCR) techniques.These techniques confirmed that there was considerable variability among the isolates in relation to the number, size and location of sclerotia on the surface of medium. Harlton *et al*. (1995) reported the variation in internal transcribed spacer regions (ITS) of ribosomal DNA of *S. rolfsii* and related fungi by RFLP (restriction fragment length polymorphisms) analysis. Nishihara, (1961); Punja and Damiani (1996) also demonstrated a close affinity between *S. rolfsii* and *S. delphinii* welch on a molecular basis, although they are morphologically distinct. Harlton *et al*. 1995) also reported geographical variability was considered to exist in the *S. rolfsii* population because of its life cycle strategy, they tested 67 isolates from different hosts and various parts of Japan, which country was not included in the previous paper. Chowdhury *et al*. (1993) and Punja and Damiani (1996) observed that ITS-RFLP analysis revealed the existence of five groups in Japanese isolates. Each group had its own morphological characteristics and RFLP patterns, corresponding to previous results, but often differed in growth temperature reactions. Groups 1 was typical of *S. rolfsii* in sclerotial morphology and RFLP patterns and was identical to group II, one of the major groups of *S. rolfsii* in the U.S.A. and South Asia, whose mycelial growth and sclerotia formation occurred at 35ºC. Group 2, which has not been reported in other countries, was similar to group 1 in morphological characters. Groups 1 and 2 accounted for 73% of Japanese isolates,

whereas groups I, III, and IV, the major groups in the U.S.A. were not found in Japan. However, its RFLP pattern demonstrated that it corresponded to group XI *(S. rolfsii*) in Nepal. Almedia *et al* (2001) studied variability among 30 isolates of *S. rolfsii* from different hosts of Brazil using RAPD profiles and identified 11 haplotipes. Harlton *et al* (1995) screend a world wide collection of *S. rolfsii* which reaveled that variation in ITS regions of 12 sub-groups of *S. rolfsii.* Punja and Sun (2000) evaluated genetic diversity among myceliial comatibiiliity groups of *S. rolfsii (teliomorph Atheia rolfsii)* and *S. delphini.* In earlier study Sarma *et al* (2002) evaluated variability in Indian isolates of *S. rolfsii.* Okabe and Matsumoto (2003) observed phylogenetic relationship of *S. rolfsii* (teleomorph *Athelia rolfsii*) and *S. delphini* based on ITS sequences. Asif *et al* (2005) and Tanwir *et al* (2007) found that RAPD ws an informative DNA marker to asses genetic relatedness and diversity among different strains. Kokub *et al.* (2007) evaluated comparative growth, morphological and molecular characterization of indigenous *S. rolfsii* strains isolated from different locations of Pakistan. Prasad *et al* (2010) studed moleculer variability among the isolates of *S.rolfsii* causing stem rot of groundnut by RAPD, ITS-PCR and RFLP. The present results also supports the above findings.

Phylogenetic analysis of all ITS sequences of the isolates from the present study revealed that the references sequences of *A. rolfsii* clustered together in a group and suggested that this species are closely related to each other. *S. delphinii* and *S. coffeicola* produced a separate subgroup in the same cluster, which separated these two species from *S. rolfsii.* Similar ITS phylogeny described earlier on *S. rolfsii* and explained the same as of the present study (Paul *et al.,* 2017, Mahadevakumar *et al.* 2016). They showed the similarity among the above mentioned three species of Sclerotium species and narrated slight differences of *S. rolfsii* with the others (Mahadevakumar *et al.* 2016). Harlton *et al.*1995 described that *S. rolfsii* and *S. delphinii* clustered together and *S. coffeicola* separated by different closely related cluster, whereas Adandonon *et al.* (2005) mentioned that the ITS sequences could separate *S. rolfsii* from the other two related species. Hence, all the isolates obtained in the present study were identified as *S. rolfsii* on the basis of ITS sequence analysis.

Based on the phylogenetic relationship and sequence alignment it is clear that there is variation exists among the *S. rolfsii* isolates in Bangladesh. From the phylogentic analysis the isolates CH-1 and CH-2 yielded a same group in a same cluster S_3. This two isolates collected from the same area but away from the other isolates. So, the microclimates of different regions may influence the pathogen morphology and genetic diversity. So, it is urgent dement to know the genetic variablility of *S. rolfsii* by sequencing others isolates form different hosts and agro-ecological zones from Bangladesh.

CHAPTER -4

In vitro control of*S. rolfsii* Sacc.

INTRODUCTION

Betel vine is an important cash crop of Bangladesh. It suffers from several diseases such as foot rot, root rot, wilt, anthracnose, stem rot and leaf spot. Out of them most sevear disease is foot rot caused by *S. rolfsii*. Every year foot rot causing the economic losses of betel vine. Some plant extracts also found to be most effective in reducing the growth and development of many pathogens (Raihan *et al*. 2003). Medicinal plants represent a rich source of antimicrobial agents. Many of the plant materials used in traditional medicine are readily available in rural areas as relatively cheap medicine. Plants are the sources of natural pesticides that make excellent lead for new pesticide development (Arokiyaraj *et al*., 2008; Shanmugavalli *et al*., 2009; Swarna Latha and N. Reddy, 2009. Plant metabolites are eco-friendly where botanicals place an important role (Sahayaraj *et al*., 2009). Biological control of plant diseases including fungal pathogens has been considered a viable alternative method to chemical control. In plant pathology, the term biocontrol applies to the use of microbial antagonists to suppress diseases. Some amendments suppress and others stimulate sclerotium germination in soil (Beute and Kabana, 1979). Germinated sclerotia are much more susceptible to antagonists than viable non-initiated ones (Smith, 1972). Non-germinated sclerotia can have their viability decreased by some amendments, thus becoming more sensitive to antagonistic action by other microorganisms (Hadar and Gorodecki, 1991) and volatile compounds on the germination of *S. rolfsii* sclerotia (Beute and Kabana, 1981; Maiti and Sen, 1988; Canullo *et al*.,1992).Thus plant extracts can be used as effective botanical fungicide in combination with chemical fungicide and a bio-control agent in integrated disease management practices that may be less harmful and eco-friendly.

MATERIALS AND METHODS

4.1 Effect of different fungicides against *S. rolfsii*

The effect of *in vitro* fungicidal effect was conducted according to Forsberg's, (1949) technique with slight modification. Eight different fungicides namely secure(fluazinam-Syngenta), Theovit (Sulpher containg/ Syngenta), Bavistin DF (Benzimidazole/ BASF), Ridomil Gold (mancozeb and metalaxyl-M / Syngenta), Cupravit (Copper oxychloride/ Bayer crop science), Rovral 50 WP(Iprodione), Dithane M-45(Mancozeb), Antracol (dithiocarbamate/ Bayer crop science)at different concentrations i.e. 100, 200, 400 and 800 ppm were used. Three replicated plates were conducted for each concentration and control was maintained with no fungicides. Sterilised PDA medium was poured into petri plates at the rate of 20 ml/plate. After solidification of the medium, a mycelial plug (5mm diameter) cut from 5 days old culture of *S. rolfsii*. The mycelial growth rate was measured after 2 to 7 days of incubation period. Percent inhibition radial growth (PIRG) was calculated using the following formula:

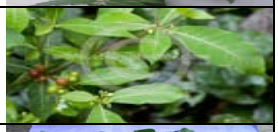
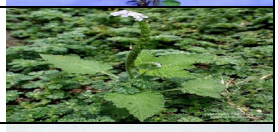
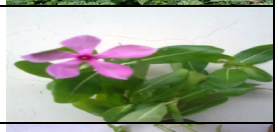
I=(C-T)/C×100, where, I = percentage of inhibition, C = growth of fungus in control plate, T = growth of fungus in treated plate .

4.2 Effect of plant extracts against *S. rolfsii*

Plant sample collection

To study the effect of plant extract against *S. rolfsii,* twelve medicinal plants were selected which were collected from Rajshahi University Campus, Rajshahi. Plant specimens were brought to the laboratory and stored in refrigerator at 4^0C for future use. The collected plants were identified by plant taxonomist Dr. Mahabub Rahman, Associate Professor, Department of Boany, R.U. These are shown in Table -4.1.

Table 4.1 List of selected medicinal plants common name, botanical name, family, and used parts with photographs.

Sl. No.	Common name	Botanical name	Family	Used part	Photograph
1	Mehendi	*Lawsonia inermis L.*	Lythyraceae	Leaf	
2	Dhutura	*Datura metel L.*	Solanaceae	Leaf	
3	Kalomegh	*Andrographis paniculata L.*	Acanthaceae	Leaf	
4	Bashok	*Adhatoda vasica L.*	Acanthaceae	Leaf	
5	Neem	*Azadirachta indica L.*	Meliaceae	Leaf	
6	Sarpogondha	*Rawuolfia serpentina L.*	Apocynaceae	Leaf	
7	Tulsii	*Ocimum sanctum L.*	Lamiaceae	Leaf	
8	Hatishur	*Heliotropium indicum L.*	Boraginaceae	Leaf	
9	Nayon Tara	*Vinca rosea L.*	Apocynaceae	Leaf	
10	Shojina	*Moringa oleifera L.*	Moringaceae	Leaf	
11	Poroshpiple	*Thespesia populnea L.*	Malvaceae	Leaf	
12	Holud	*Curcuma longa L.*	Zingiberaceae	Rhizom	

Preparation of plant extract

Plant extracts were prepared following the method of Bhatti (1988). At first 100 gm fresh plant materials were washed with tap water. Then plant tissues were cut into pieces (1-2 cm) and air dried. Then add 100 ml of distilled water or ethanol or acetone was added separately. It was then crushed thoroughly in a mortar with a pestle and filtered through Whitman No. 1 filter paper and centrifuged the filtrate properly. Then 5, 10, 15, 20 and 25 % of concentration of each plant extract was prepared with PDA medium.The Petri plates were gently swirled to ensure even distribution of the extracts. The agar extract mixture was allowed to solidify. Then the culture disc of the pathogen was put on the center of the petri plate and the mycelial growth was noted. In control plate no plant extract was mixed. Data on mycelial growth at 7 days (DAI) were recorded.

The efficacy of medicinal plant products were expressed with percent of radial mycelial growth over the control which will be calculated by using the following formula (Vincent, 1947)

$$\text{PIRG} = \frac{C - T}{C} \times 100$$

Where,

PIRG = Percent inhibition of radial growth

C = Radial growth in control

T = Radial growth in treatment

4.3 Effect of bioagents against *S. rolfsii*

Sources of bioagents

Six bio-agents namely *Trichoderma harzianum*, *T. virens, Botrytis* sp., *Aspergillus* sp., *Penicillium* sp. and *Fusarium oxysporum* were used in this study. *T. harzianum* (Rifai, IMI-392432) and *T. virens* (IMI- 392430) were obtained from the stalk culture of the Plant Pathology and Microbiology and Mycology

Laboratory, University of Rajshahi, Bangladesh which were identified by CABI Biosciences, Surrey, United Kingdom. Rest of the antagonists was isolated from the betel vine boroj soil following serial dilution technique. On the basis of cultural and morphological characteristics identification were confermed with the help of standerd manual (Booth, 1971).

Preparation and application of bioagents

Procedure

Selected six bioagents were subjected to in an *in vitro* screening by dual culture and poison agar technique (Skidmore and Dickinson, 1975) for detection of the most active antagonistic isolates against *S. rolfsii*.

(a) Dual Culture

Two parameters were observed in this test, the percent inhibition of radial growth (PIRG) and the number of days taken for the antagonist observed to totally cover the grow of *S. rolfsii* colony. For each strain 5 mm diameter agar disc was taken from the edge of 7 days old PDA culture and was placed at the periphery of 90mm diameter of PDA culture plates. Then another agar disc of the same size and age of the test antagonist was similarly placed at the periphery but on the opposition end of the same petridish. For control, *S. rolfsii* alone was placed in a similar on a fresh plate. All pairing were carried out in four replicates and incubated at 28 ± 2°C. Antagonistic activity was assisted at 7days after incubation by measuring the radius of the *S. rolfsii* colony in the direction of the antagonist colony (R_2) and the radius of the *S. rolfsii* colony in the control plate (R_1). The two reading were transformed into percent inhibition of radial growth (PIRG) using the following formula of (Skidmore and Dickinson, 1976)

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

Where, R_1 = The radius of the *S .rolfsii* colony in the control plate (R_1)

R_2 = The radius of the *S. rolfsii* colony in the direction of the antagonist colony

The number of days taken for the antagonist to overgrow the whole colony of *S. rolfsii* was recorded.

The degree of antagonisms between each bioagent and test pathogen in dual culture was scored on scale of 1-5 as proposed by Bell *et al*. (1982).

1 Antagonist completely overgrew the pathogen and covered the entire medium surface.
2 Antagonist overgrew at least two third of the medium surface.
3 Antagonist and the pathogen each colonized one half of the medium surface (more than one third and less than two third) and neigther organism appeared to dominate each other.
4 The pathogen colonized at least two third of the medium surface and appeared to with stand encroachment.
5 The pathogen completely overgrew the antagonist and occupied the entire medium surface.

(b) Poison agar technique using crude metabolites

For preperation of crude metabolites 200ml potato dextrose broth (PDB) medium was poured into 500ml conical flasks and autoclaved for 15 minute at 121ºC/1.05kg/cm pressure. Six pieces of agar discs (5mm) were cut out from the margin of 7days old culture of the antagonists and kept in per flask with 5 replications. Then the flasks were incubated at 28±1ºC for 7, 14 and 21 days. After incubation the liquid cultures were firstly filtered through Whitman No. 1 filter paper to remove hyphal fragments and prepared 30, 60 and 90% concentrations of metabolites with PDA medium. After preparing of poison agar medium at different concentrations of metabolites of each antagonist were plated. Then 7days old culture disc (5mm) of *S. rolfsii* was placed in the centre of the previously prepared plate and incubated at 28±2°C for 7days. Observation was made on radial extension of the mycelia on culture plate for both the treatment and control where no metabolites were used. Data were recorded on the mycelial extention of

colony diameter after 4 days to 7 days of incubation. The readings were calculated for the percent inhibition of radial growth (PIRG) using the following formula

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

Where, R_1 = the radius of the *S .rolfsii* colony in the control plate (R_1)

R_2 = the radius of the *S. rolfsii* colony in the treated plate.

4.4 Effect of soil amendments against *S. rolfsii*

4.4 .1 Preperation of soil amendments

To observe the germination of sclerotia and mycelial growth of *S. rolfsii* different kind of soil amendments were used. The experiment were conducted as T_1= (Soil + sawdust), T_2= (Soil + rice husk), T_3= (soil + ash), T_4= (Soil + cow dung), T_5= (Soil + mustard oil cake), T_6= (Soil + compost), T_7= (Soil + *T. harzianum), T_8*= (Compost + *T. harzianum),* T_9= (Soil + datura leaf dust), T_{10}= (Soil + neem leaf dust), T_{11}= (Soil + henna leaf dust) T_{12} = (Untreated control). After collection the samples were preserved in the laboratory at room temperature and the fresh leaf and *Trichoderma* cultures were preserved in the refrigerator. For preparation of plant leaf dust at first 40gm fresh leaves were (datura, henna and neem) taken and kept in the oven at 60^0 C for 28 hours. After oven dry plant patrs were cut into pieces and crused into powder. The leaf dust were mixed (10%) well with the collected boroj soil for each experiment. On the other hand dried cowdung, mustard oil cake was also crused with the mortar pestle.

4.4.2 Methods for detection of the effect of organic amendments on germination of sclerotia and mycelial growth of *S. rolfsii*

Two types of experiments were conducted as surface culture and pot culture. Both of the experiments were done for observing the germination of sclerotia and measuring the mycelial length of used pathogen.

Surface culture Technique

For surface culture 1000 gm of amended (10%) soil was taken in a tray and mixed up properly. Then transfer it separately in a plastic box and added 150 ml water to moist the soil. Then the surface was devided into 24 small squares and the each sclerotia were put on the surface of each amended soil (24 sclerotia/ box) on the center of each square. The distance was 12 mm for proper note down of germination of sclerotia and mycelial growth. The boxes were covered with polythene which has a lot of holes for passing air then the boxes kept in the growth chamber under controlled temperature. Observation was started after 24 hours up to 7 days. The germination number of sclerotia was counted every day and the length of mycelia was also measured. The number of germinated sclerotia and length of mycelia during experiment was noted timely. The temperature of each experiment was uniform.

Pot culture Technique

For pot culture, a portion of amended soil was infested with sclerotia (100 sclerotia /150gm soil). After proper mixing the amended soils were transferred to the pots. Fifteen pots were then covered with sterilized paper to maintain moisture. After 10, 20, 30, 40 days the soils from the pot were washed in running tap water with an 850 milimicrone-mesh sieve. Sclerotia were recovered with fine forceps from the residue remained sieve. Sclerotia germination was determined by plating @20 of sclerotia /1 plates and incubated at room temperature and germination of sclerotia were assessed visually after 36 hours. The germination rate was observed during the time of experiment. On the other hand for measuring the mycelial length single sclerotia put on the centre of PDA plates and measured the mycelial length upto 7 days.

RESULTS

4.1 Effect of different fungicides on mycelial growth of *S. rolfsii*

The results of the *in vitro* fungicidal effect on mycelial growth of *S. rolfsii* after 7 days of incubation at different concentrations are presented in the Table 4.2 and Plate 4.1. Radial growth of mycelium of *S. rolfsii* gradually inhibited with increasing of concentrations. From the results it was observed that two fungicides namely bavistin and dithan M-45 showed hundred percent inhibition of radial growth of *S. rolfsii* at all concentrations (100 to 800 ppm). On the other hand rovral (400 and 800 ppm) and antracol (only 800 ppm) showed hundred percent inhibition of radial growth of *S. rolfsii.* Next of inhibition 85.33% observed in secure at 800 ppm concentration. Rest of fungicides thiovit, ridomil and cupravit showed 66.11%, 43.33% and 26.66% inhibition of radial growth at 800 ppm concentration and the lowest inhibition (9.44%) was showed in antracol at 100 ppm concentration. There was no inhibition showed in untreated control at all concentrations and observed the maximum mycelial growth.

Table 4.2 Effect of different fungicides on mycelial growth of *S. rolfsii* after 7 days of incubation periods

Fungicide	Radial growth of mycelium (mm) in different concentrations (PPM)			
	100 (ppm con.)	**200 (ppm con.)**	**400 (ppm conc.)**	**800 (ppm conc.)**
Bavistin DF	0 (100*)e	0 (100*)e	0(100*)f	0(100*)f
Ridomil Gold	56(37.77)d	53(40.0)d	53(40.0)d	51(43.33)c
Cupravit	80(11.11)b	75(16.66)b	75(16.66)b	66(26.66)b
Rovral 50 WP	90(0)a	70(21.94)c	0(100)f	0(100)f
Thiovit	80(11.11)b	70(21.66)c	31(65.0)e	31(66.11)d
Secure	70(21.94)c	54(39.44)d	52(42.22)d	12(85.33)e
Antracol	81(9.44)b	74(16.88)b	61(32.22)c	0.0(100)f
Dithan M-45	0.0(100)e	0.0(100)e	0.0(100)f	0.0(100)f
Control	90(0)a	90(0)a	90(0)a	90(0)a
± SE	± 6.65	± 6.02	± 6.32	± 6.31
F value	33.27	21.01	40.95	98.04
LSD (p=0.05)	21.48	25.71	20.21	14.78

*=inhibition percentage

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

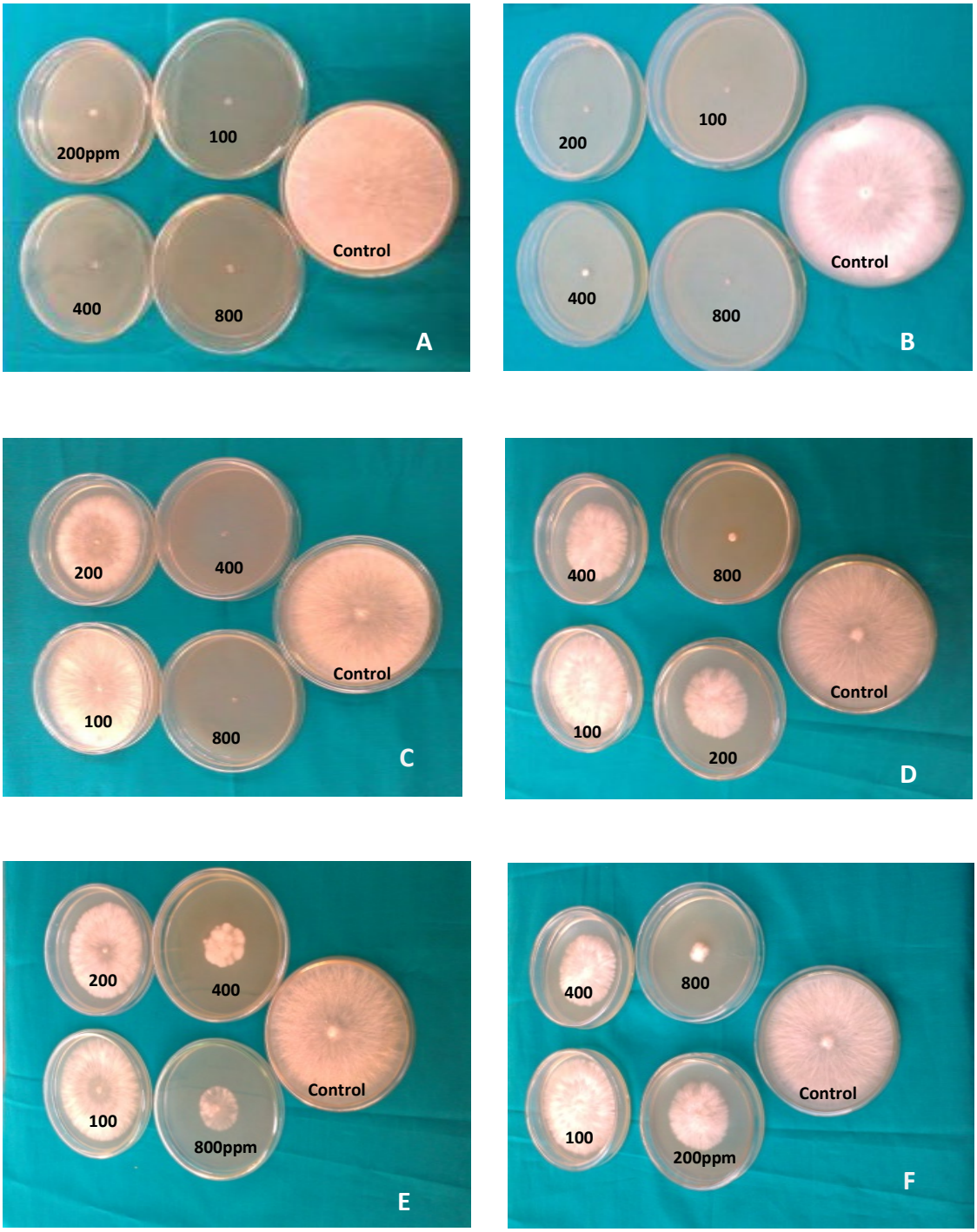


Plate 4.1 Photographs showing effect of Bavistin (A), Dithan M-45 (B), Rovral (C), Antracol (D), Thiovit (E) and Secure (H) on mycelial growth of *S. rolfsii* at different concentrations.

4.2 Effect of different plant extracts on mycelial growth of *S. rolfsii*

Effect of aqueous extracts on mycelial growth of *S. rolfsii*

Twelve medicinal plants extracts namely *Lawsonia inermis* L., *Datura metel* L., *Andrographis paniculata* L., *Adhatoda vasica* L., *Azadirachta indica* L., *Rawoulfia serpentina* L., *Ocimum sanctum* L., *Heliotropium indicum* L., *Vinca rosea* L., *Moringa oleifera* L., *Thespesia populnea* L., and *Curcuma longa* L.were evaluated against *S. rolfsii* the results are Presented in Table 4.3 and Plate 4.2. Aqueous extracts of *L. inermis* and *D. metel* were completely inhibited the pathogen at all concentrations. *A. indica* completely inhibited the pathogen at 15, 20 and 25% concentrations. On the other hand *A. paniculata, A. vasica, M. oleifera*, *T. populnea* and *C. longa* inhibited the pathogen completely at 20 and 25% concentrations. *V. rosea* showed no inhibition at 5% concentration.

Table 4.3 Effect of aqueous extracts of medicinal plants on mycelial growth of *S. rolfsii* on PDA medium recorded on 7 days of incubation.

Name of plants	Radial growth(mm) of mycelium of different concentrations				
	5%	10%	15%	20%	25%
L. inermis	0.00 (100*) g	0.0 (100*) i	0.0 (100*) i	0.0 (100*) f	0.0 (100*) f
D.metel	0.0 (100) g	0.0 (100) i	0.0 (100) i	0.0 (100) f	0.00 (100) f
A. paniculata	71.5 (18.75)d	53.0 (39.77) e	34.5 (60.79) e	0.0 (100) f	0.00 (100) f
A. vasica	85.0 (3.41) b	40.0 (3.98) f	12.0 (40.91)h	0. 0(100) f	0.00(100) f
A. indica	57.00 (36.66) e	35.32 (60.77) g	16.54(81.82) g	0.0(100) f	0.00(100) f
O. sanctum	78.0 (3.37) c	66.0 (7.87) c	60.0 (11.84) c	55.2 (29.77) d	50.0 (43.85) d
H. indicum	85.0 (5.55) b	85.0 (5.55) b	80.0 (11.11) b	80.0 (11.11) b	80.0 (11.11) b
V. rosea	90.0 (0.0) a	85.0 (5.55) b	80.0 (11.11) b	75.0 (16.66) c	75.0 (16.66) c
M. oleifera	59.5 (33.88) e	56.5 (37.22) d	35.0 (61.11) e	0.0 (100) f	0.0 (100) f
C. longa	59.5 (33.88) e	40.25 (55.27) f	25.0 (72.22) f	0.0 (100) f	0.0 (100) f
R. serpentina	57.0 (36.66) e	50.0 (44.44) e	45.5 (49.44) d	40.0 (55.55) e	40.0 (55.55) e
T. populnea	47.5 (47.22) f	25.0 (72.22) h	10.0 (88.88)h	0.0 (100) f	0.0 (100) f
Control	90.0(0.0) a	90.0(0.0) a	90.0(0.0) a	90.090.0) a	90.0(0.0) a
SE ±	± 4.69	± 4.58	± 4.88	± 5.67	± 5.62
F value	4.65	5.97	8.44	13.09	23.40
LSD (p=0.05)	22.43	22.43	22.43	21.23	1.89

*=Inhibition percentage.

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

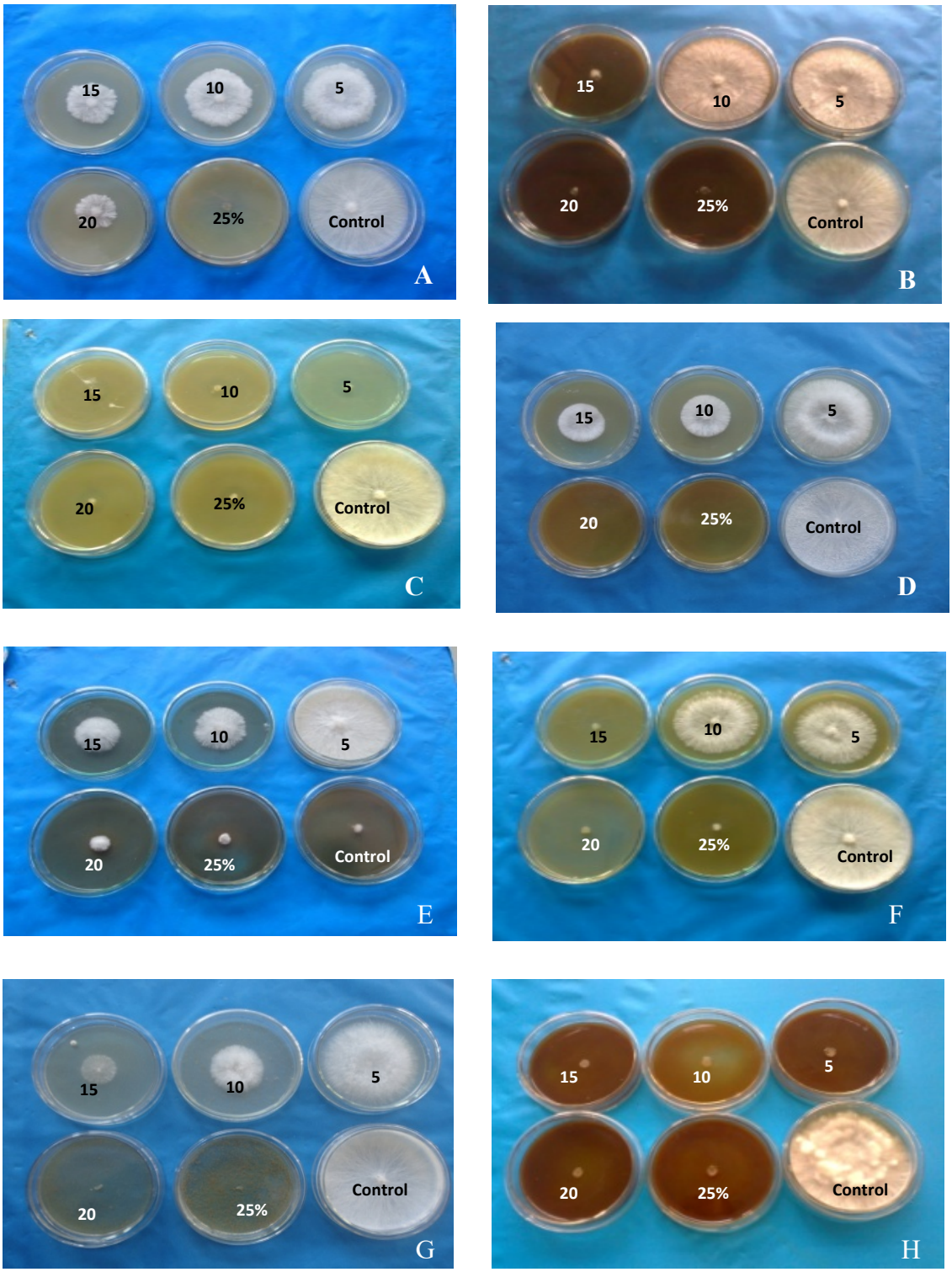


Plate 4.2 Photographs showing effect of aquous extract of *M. oleifera* (A), *A. paniculata* (B), *D. metel* (C) *C. longa* (D), *T. populnea* (E), *A. vasica* (F), A. *indica* (G) *and* *L. inermis* (H).

Effect of ethanol extracts on mycelial growth of *S. rolfsii*

To study the effect of ethanol extracts of eight medicinal plants namely *L. inermis, D. metel, A. paniculata, A. vasica, A. indica M. oleifera*, *T. populnea* and *C. longa* were evaluated against *S. rolfsii* and the results are presented in Table 4.4 and Plate 4.3. *L. inermis* and *D. metel* were showed hundred percent inhibition at all concentrations. Next of *A. paniculata, A. vasica, C. longa*, *A. indica* and *T. populnea* showed hundred percent inhibition at 15%, 20% and 25% concentrations and *M. oleifera* showed hundrad percent inhibition at 20% and 25% concentrations.

Table 4.4 Effect of ethanol extracts of medicinal plant on mycelial growth of *S. rolfsii* on PDA after 7 days of incubation.

Name of plants	**Radial growth(mm) of mycelium in different concentrations**					
	5%	**10%**	**15%**	**20%**	**25%**	
L. inermis	0.0 (100*) i	0.0(100*) h	0.0(100*) c	0.0(100*) b	0.0 (100*) b	
D. metel	0.0(100)c	0.0(100)	0.0(100) c	0.0 (100) b	0.0(100) b	
A. paniculata	38.0 (57.77) f	20.0 (77.77) f	0.0(100) c	0.0(100) b	0.0(100) b	
A.indica	21.30(76.33) h	8.00(91.11) g	0.0(100) c	0.0(100) b	0.0(100) b	
A. vasica	80.0 (11.11) c	56.0((37.77) c	0.0(100) c	0.0 (100) b	0.0 (100) b	
M. oleifera	59.5 (33.38) d	33.0 (63.33) e	20.0(77.77) b	0.0 (100)b	0.0(100) b	
C. longa	33.66(62.6) g	19.0(78.88) f	0.0(100) c	0.0(100) b	0.0(100) b	
T. populnea	46.47(48.36) e	40.0(55.55) d	0.0(100) c	0.0(100) b	0.0(100) b	
Control	90.0(0.0) a	90.0 (0.0) a	90.0(0.0) a	90.0(0.0) a	90.0(0.0) a	
± SE	± 5.69	± 5.79	± 5.53	± 5.54	± 5.54	
F value	6.18	3.93	5.02	3.99	3.99	
LSD(P=0.05)	31.62	31.623	20.81	22.24	22.24	

*=Inhibition percentage

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

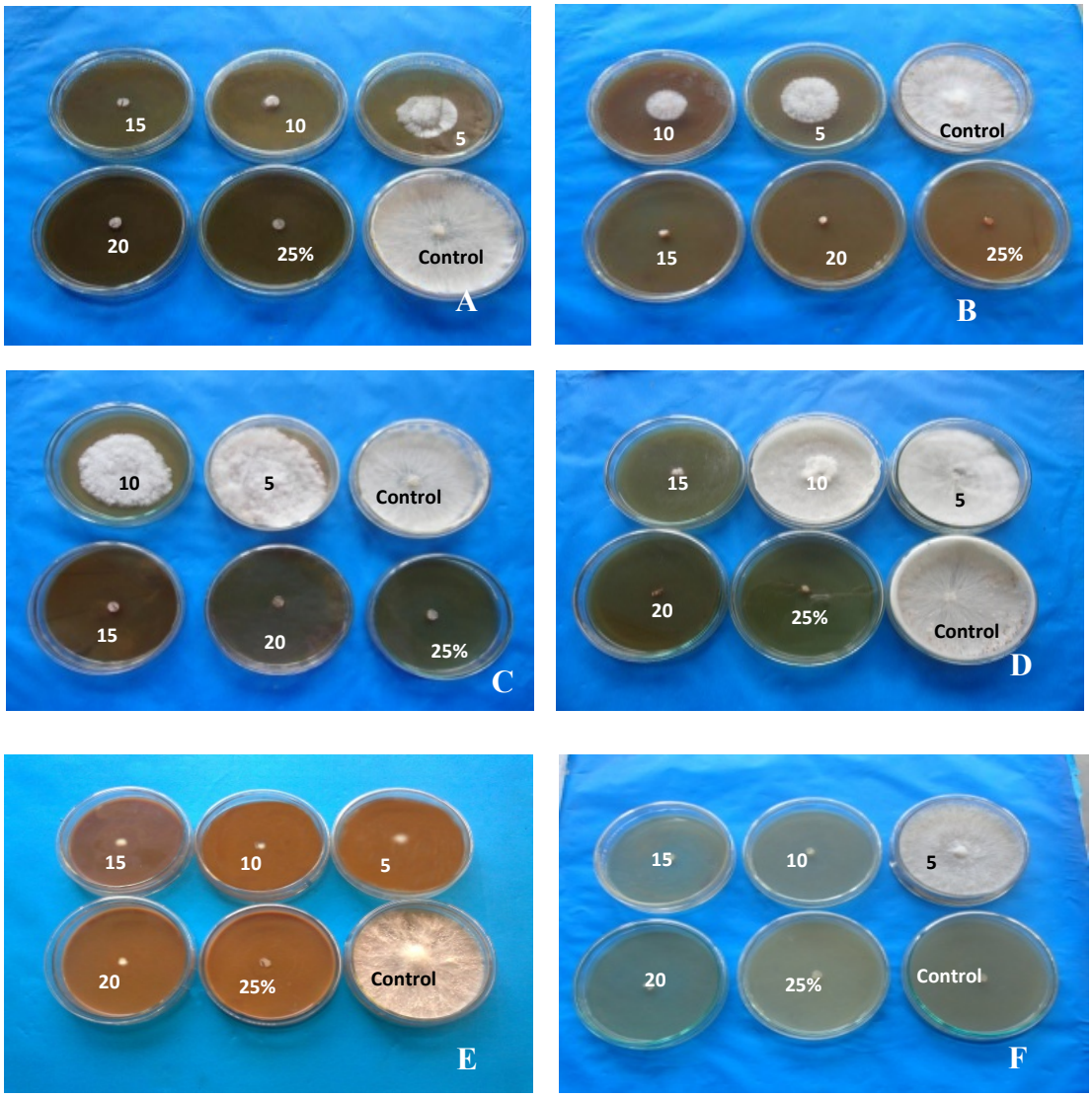


Plate 4.3 Photographs showing effect of ethanol extract of *A. paniculata* (A), *C. longa* (B), *A.vasica* (C), *T. populnea* (D), *L. inermis* (E) and *D. metel* (F).

Effect of aceton extracts on mycelial growth of *S. rolfsii*

The effect of aceton extracts of eight medicinal plants namely *L. inermis, D. metel, A. paniculata, A. indica, A. vasica, M. oleifera*, *T. populnea* and *C. longa* were evaluated against *S. rolfsii* and the results are presented in Table 4.5 and Plate 4.4. Hundred percent inhibitions were showed in *L. inermis* and *D. metel* at all concentrations. Next of *A. indica* and *T. populnea* showed hundred percent inhibitions at 10, 15, 20 and 25% concentrations while *C. longa* completly inhibited at 15, 20 and 25% concentrations. *A. vasica* showed hundred percent inhibitions at 20 and 25% concentrations. *A. paniculata* and *M. oleifera* did not show complete inhibition at any concentration.

Table 4.5 Effect of aceton extract of medicinal plant on mycelial growth of *S. rolfsii* on PDA medium on 7 days of incubation.

Name of plants	**Radial growth (mm) of mycelium at different concentrations.**				
	5%	**10%**	**15%**	**20%**	**25%**
L. inermis	0.0(100*) g	0.0 (100*) e	0.0 (100*) e	0.0(100*) d	0.0(100*) d
D.metel	0.0 (100) g	0.0(100) e	0.0 (100) e	0.0(100) d	0.0(100) d
A. paniculata	47.5(47.22) c	39.0(56.66) c	20.5(77.22) d	8.5 (90.55) c	8.5(90.55) c
A.indica	10.59(88.23) f	0.0(100) e	0.0(100) e	0.0(100) d	0.0(100) d
A. vasica	78.0 (13.33) b	60.0(33.33) b	35.0(61.11) c	0.0(100) d	0.0(100) d
M. oleifera	90.0 (0.0) a	89.0(1.11) a	80.0(11.11) b	77.0(14.44) b	77.0 (14.44) b
C. longa	31.75(64.72) d	21.0(76.66) d	0.0(100) e	0.0(100) d	0.0(100) d
T. populnea	26.0 (71.11) e	0.0(100) e	0.0 (100) e	0.0(100) d	0.0 (100) d
Control	90.0(0) a	90.0(0) a	90.0(0) a	90.0(0) a	90.0 (0) a
± SE	± 6.80	± 7.05	± 6.70	± 6.75	± 6.75
F value	14.71	12.82	10.04	15.03	15.03
LSD (p=0.05)	22.33	21.30	22.29	20.84	20.84

*=Inhibition percentage

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

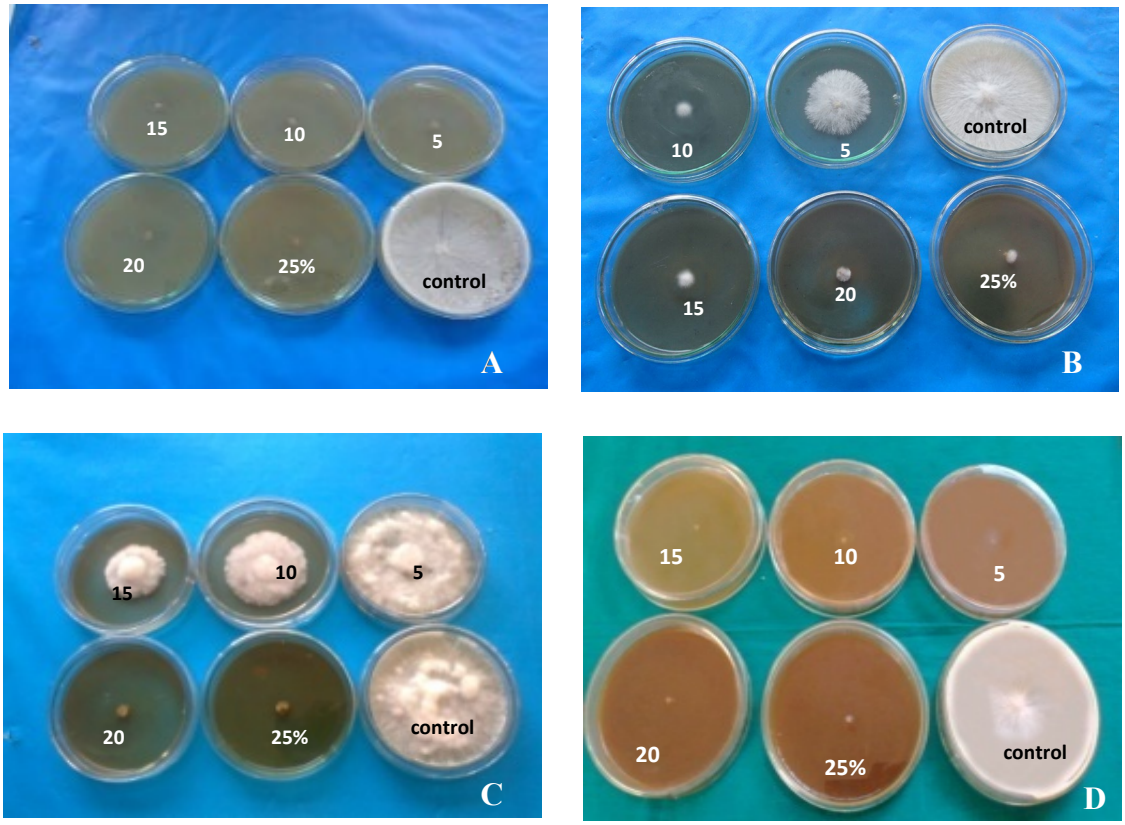


Plate 4.4: Photographs showing effect of acetone extract of *D.metel* (A), *A.indica* (B), *A. vasica* (C) and *L.inermis* (D) on mycelial growth of *S. rolfsii*.

4.3 Effect of bio-control agents on percentage inhibition of radial growth (PIRG) of *S. rolfsii*

Dual culture

Six bio-agents namely *T. harzianum, Botrytis* sp., *T.virens*, *F.oxysporum*, *Aspergillus* sp. and *Penicillium* sp. were tested against *S. rolfsii* and the results are presented in Table 4.6 and Plate 4.5. From the result it was observed that the highest mycelial length of the pathogen was recorded in *F. oxysporum* (68.78 mm) followed by *Aspergillus* sp (53.04mm), *Penicillium* sp. (44.50mm), *Botrytis* sp. (40.33mm) and *T. virens* (35.04 mm) and the lowest was recorded in *T. harzianum* (30.55mm) treated plat after 7 days of incubation. On the other hand the highest percentage inhibition of radial growth (66.10%) was recorded in *T. harzianum* followed by *T. virens* (61.06%), *Botrytis* sp. (55.18%), *Penicillium* sp. (50.55%) and *Aspergillus* sp. (41.06%) and the lowest was in *F. oxysporum* (23.57%). The time of over growth of colony was recorded from 6 to 10 days. The degree of antagonisms was measured according to Bell's scale. None of the antagonist's completely overgrew the pathogen. *T. harzianum*, *T. virens* and *Penicillium* sp. were covered two third of the medium surface after 7 days and placed in scale- 2, whereas *Botrytis* sp. covered one half of the medium surface (more than one third and less than two third) after 7days and placed in scale-3. Rest of antagonists placed in scale-4. (Table-4.7)

From the result it was observed that in dual culture the most effective antagonists were *T. harzianum* and *T. virens* So that these two antagonists were considered for poison agar technique.

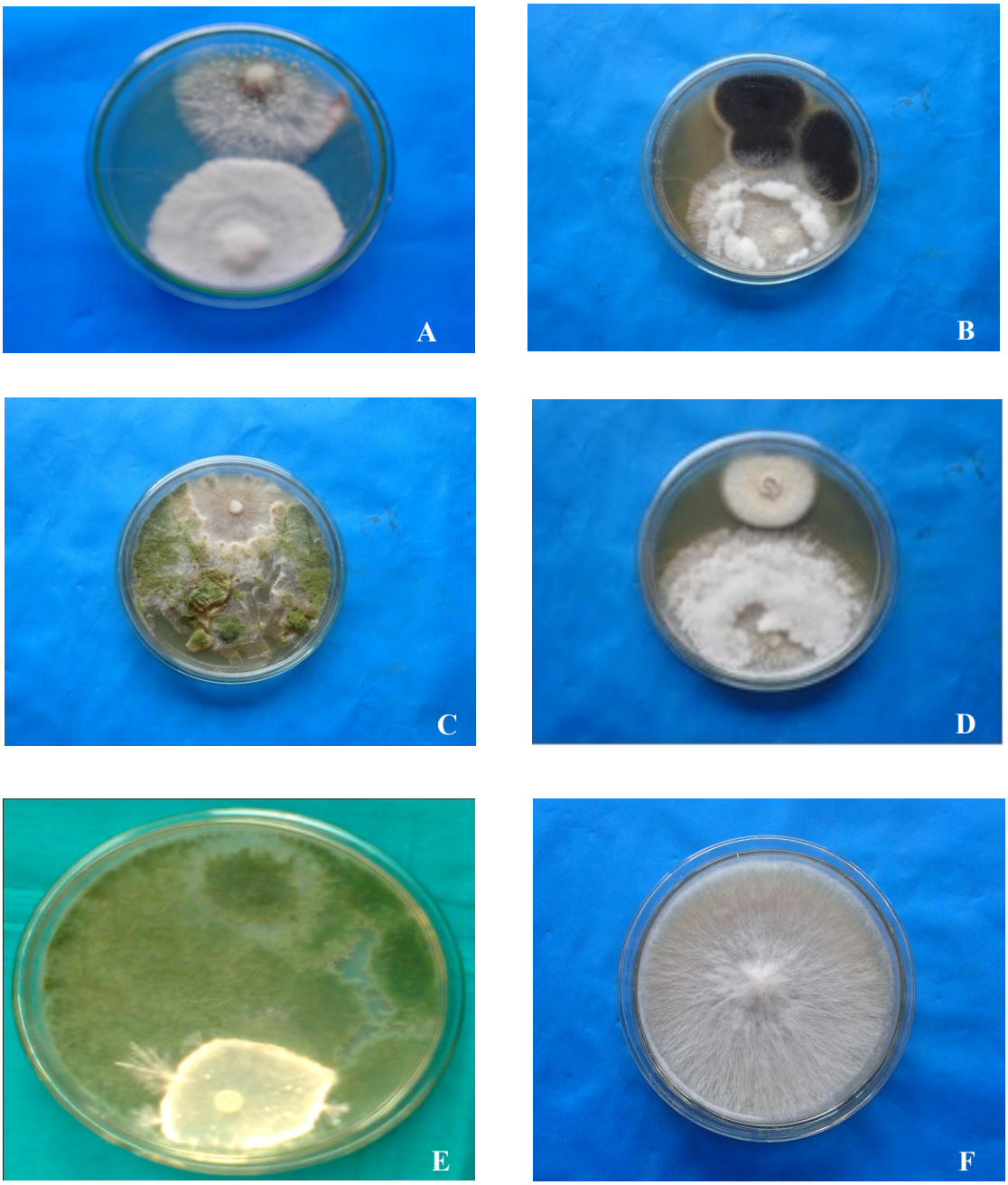


Plate 4. 5 Photographs showing antagonistic effect of *Botrytis* sp. (A), *Aspergillus* sp. (B), *Penicillium* sp. (C), *F.oxysporum* (D), *T. harzianum* (E) against *S. rolfsii* and Control (F).

Table 4.6 Antagonistic effects of six bio agents on percentage inhibition of radial growth (PIRG) of *S. rolfsii* after 7 days of incubation at 28±2^{0}C in dual culture.

Antagonists	Percentage inhibition of radial growth (PIRG) in different days.					Time of over growth of colony (day)
	3rd	4th	5th	6th	7th	
T. harzianum	7.21 bc (91.9*8)	15.02cde (83.31*)	19.23f (78.63*)	25.29 f (66.05*)	30.55 g (66.05*)	7 ab
T. virens	15.30 a (83)	20.50b (77.22)	25.35 de (71.83)	30.20e (66.44)	35.04f (61.06)	6 b
Botrytis sp.	9.53b (89.41)	17.33bcd (80.74)	30.40 bc (66.22)	35.25d (60.83)	40.33e (55.18)	7 ab
Aspergillus sp.	8.03bc (91.07)	11.60 e (87.11)	22.15e f (75.38)	28.50ef (68.33)	53.04c (41.06)	7 ab
Penicillium sp.	10.90 b (87.88)	19.32 b c (78.53)	31.09 c (65.45)	44.02c (51.08)	44.50 (50.55)	7 ab
F. oxysporum	5.30c (94.11)	15.9 cde (82.33)	27.33 cd (69.63)	48.55b (46.05)	68.78b (23.57)	10a
Control	15.40 a	29.00 a	52.80 a	76.32 a	90.00 a	5 b
± SE	± 0.88	± 1.19	± 2.30	± 3.66	± 2.48	± 0.43

*=Inhibition percentage.

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

Table 4.7 Evaluation of six antagonists against fungal pathogen S. *rolfsii* in dual culture following Bell' s scale* (1982)

Antagonists	*S. rolfsii*
T. harzianum	2
T. v*irens*	2
Botrytis sp.	3
Aspergillus sp.	4
Penicillium sp.	2
F. oxysporum	4

***Degree of antagonism as proposed by Bell *et al. (*1982)**

Poison agar technique

In poison agar test 7, 14 and 21 days of metabolites of *T. harzianum* and *T. virens* were tested against *S. rolfsii* and the results are presented in Table 4.8-4.9 and Plate 4.6 - 4.7. The highest percentage of inhibition was recorded as 68.47, 61.98, and 43.01 % o f 7, 14 and 21 days old metabolites, respectively at 90% concentration while lowest inhibition was recorded as 33.20, 11.11 and 12.98% of 7, 14 and 21 days old metabolites, respectively at 30% concentration for *T. harzianum.*

On the other hand in *T. virens,* the highest percentage of inhibition was recorded as 57.77, 44.44, and 41.85 % of 7, 14 and 21 days old metabolites, respectively at 90% concentration while loewst inhibition was recorded as 33.33, 16.66 and 8.27 % of 7, 14 and 21 days old metabolites respectively at 30% concentration.

Table 4.8 Effect of metabolites of *T. harzianum* against *S. rolfsii* in poison agar technique.

No. of Days	7 days old metabolites			14 days old metabolites			21 days old metabolites		
	30%	60%	90%	30%	60%	90%	30%	60%	90%
4	33.13(63.18*)c	30.22(66.42*) d	28.37(68.47*)b	60.95(32.27*)d	45.72(49.2*)c	34.21(61.98*)d	76.03(15.52*)a	66.93(25.63*)b	51.29(43.01*)a
5	48.29(46.34) b	40.16(55.37)c	35.20(60.88)a	65.23(27.52)c	62.35(30.72)b	50.45(43.94) c	77.62(13.75)a	70.33(22.22)a,b	65.00(27.77) ab
6	60.12(33.20) a	48.08(46.57) b	35.20(60.88)a	73.00(18.88) b	67.00(25.55)a	57.56(36.04) b	77.62(13.75)a	74.67(17.03) b	71.00(21.11)a
7	60.12(33.20)a	52.21(41.98) a	35.20(60.88)a	73.00(11.11) a	72.11(16.54)a	69.09(23.23) a	78.31(12.98)a	77.62(13.75)	75.00(16.66)b
± SE	± 3.37	± 2.57	± 1.01	± 1.63	± 3.27	± 3.85	± 0.58	± 1.23	± 3.07

*=Inhibition percentage

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

Table 4.9 Effect of metabolites of *T. virens* against *S. rolfsii* in poison agar technique.

No. of Days	7 days old metabolites			14 days old metabolites			21 days old metabolites		
	30%	60%	90%	30%	60%	90%	30%	60%	90%
4	55.39(38.45*)b	42.00(53.33*)b	38.00(57.77*)b	60.00(33.33*)b	55.12(38.75*)c	50.00(44.44*)c	80.30(10.77*)a	68.25(24.16*)b	52.33(41.85*)c
5	58.22(35.31)a,b	54.00(40.00)b	42.00(53.33)b	72.00(20.00)b	65.30(27.44)b	62.20(30.88)b	82.55(8.27)a	75.20(16.44)a	64.90(32.33)b
6	60.00(33.33)a,b	57.00(36.66)a	51.00(38.88)a	75.00(16.66)a	67.12(25.42)a,b	64.00(28.88)a,b	82.55(8.27)a	75.20(16.44)b	70.00(16.66)a
7	60.00(33.33)a,b	57.00(36.66)a	53.00(38.88)a	75.00(16.66)a	67.12(25.42)a,b	66.00(26.66)a	82.55(8.27)a	75.20(16.44)b	70.00(16.66)a
± SE	± 2.60	± 1.92	± 2.35	± 1.92	± 1.76	± 1.94	± 0.57	± 0.92	± 2.37

*=Inhibition percentage

[Values in a column having same leletter don't differ significantly (P=0.05) according to DMRT]

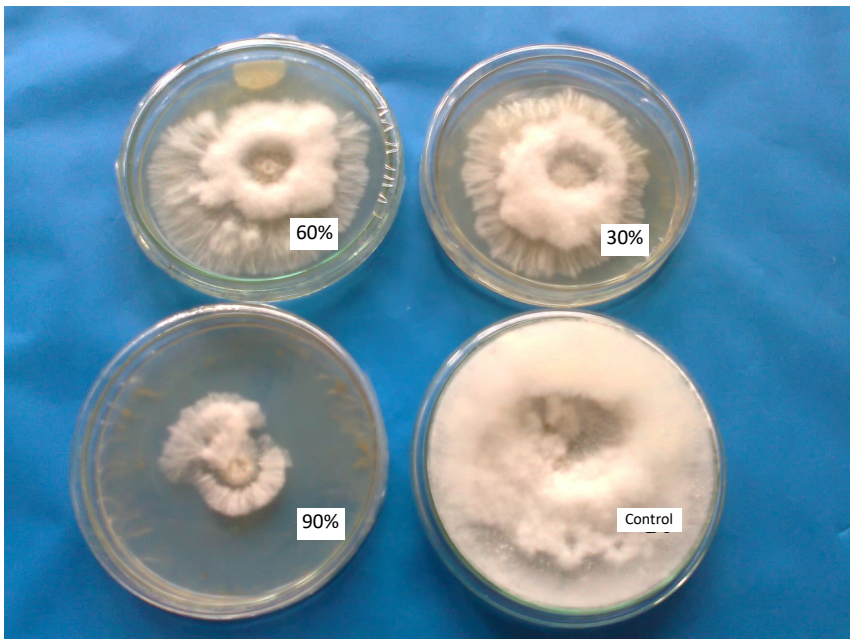


Plate 4.6 Photographs showing inhibition of mycelial growth of *S. rolfsii* by metabolities of *T. harzianum.*

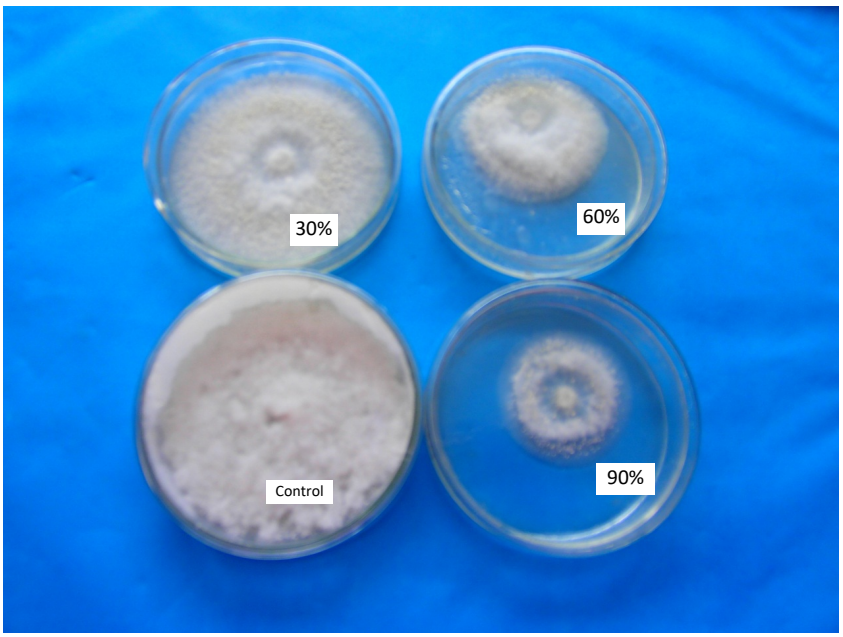


Plate 4.7 Photographs showing inhibition of mycelial growth of *S. rolfsii* by metabolites of *T. viride.*

4.4 Effect of organic amended soil on germination of sclerotia of *S. rolfsii*

In this study eleven types of amended soil were used to observed thier effect on *S. rolfsii.* Effect of organic amended soil on germination percentage and mycelial length of sclerotia of *S. rolfsii* was tested by two methods as surface culture and pot culture.

Surface culture

Different types of amended soil were used to control the growth of *S.rolfsii* and the results are presented in Table 4.10 and plate 4.8. In surface, culture technique the maximum sclerotia germination was observed in T_1 (sawdust amended soil) and T_{12} (control) and the lowest was T_4 (cowdung amended soil). There was no sclerotia germination was observed in T_9 (datura leaf dust) and T_{10} (neem leaf dust) amended soil. Hundred percent inhibition was exibited in T_9 (datura leaf dust) and T_{10} (neem leaf dust) treatment. Next of inhibition 80.00% was measured in T_{11} (Henna leaf dust) amended soil followed by 75.00 % in T_7 (*T.harzianum*), 71.05 % in T_8 (Tricho-compost), 69.75% in T_4 (cowdung), 66.77% in T_6 (compost), 54.17% in T_5 (mustard oil cake), 16.67% in T_2 (rice husk) and 3.34% in T_3 (ash) amended soil. No inhibition was recorded in T_1 (sawdust) treatment andT_{12} (control).

In case of mycelial growth the highest mycelial growth 70.2mm was recorded in T_{12}(control) and the lowest mycelial growth 25.50 mm was recorded in T_{11} (Henna leaf dust) there was no mycelial growth was observed T_9 (datura leaf dust) and T_{10} (neem leaf dust) treatment. Hundred percent inhibition was exibited in T_9 (datura leaf dust) and T_{10} (neem leaf dust) treatment. Next of inhibition 63.85% was recorded in T_{11} (henna leaf dust) followed by 62.49% was in T_7 (*T.harzianum*), 57.23% in T_5 (mustard oil cake), 56.55% in T_8 (Tricho-compost), 43.01% was in T_4 (cowdung), 37.32 % in T_6 (compost), 33.64.58% in T_3 (ash), 20.22% was in T_2 (rice husk) and the lowest inhibition 14.52% was recorded in T_1 (Sawdust) amended soil.

Table 4.10 Effect of organic amended soils on germination (%) of sclerotia and mycelial growth of *S. rolfsii* in surface culture teccnique after 7 days of incubation periods.

Treatement	Mean of germination percentage of sclerotia	Mean radial growth of mycelial length(mm)
T_1(Saw dust)	100.00 (0.0 *)a	60.00(14.52*)b
T_2(Rice husk)	83.33(16.67) b	56.0(20.22) c
T_3(Ash)	96.66(3.34)a	37.5(33.64)f
T_4(Cowdung)	30.25(69.75)e	40.00(43.01)e
T_5(Mustard oil cake)	45.83(54.17) c	30.02(57.23) g
T_6(Compost)	33.23(66.67) d	44.00(37.32)d
T_7(*T. harzianum*)	25.00(75.00)ef	26.33(62.49) h
T_8(Tricho-compost)	28.5(71.05) e	30.5(56.55)g
T_9(Datura leaf dust)	0.00 (100.00) h	0.00(100.00)i
T_{10}(Neem leaf dust)	0.00(100.00) h	0.00(100.00)i
T_{11}(Henna leaf dust)	20.00 (80.00)g	25.5(63.85)h
T_{12}(Untreated control)	100.00(0.00) a	70.2(0.00)a
± SE	± 1.15	± 3.48
F value	0.87	1.2
LSD(P<0.05)	20.02	19.22

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

*= Inhibition percentage

Plate 4.8 Photographs showing effect of organic amended soil on germination of sclerotia of *S. rolfsii* in Surface culture [T_1=soil+sawdust; T_2=soil+rice husk; T_3=soil+ash; T_4=soil+Henna leaf dust; T_5=soil+Datura leaf dust; T_6=soil+ Tricho-compost T_7=soil+*T.harzianum*; T_8=soil+Neem leaf dust; T_9=soil+Mustard oil cake, T_{10}=control (only soil)]

Pot Culture Technique

In pot culture, it was observed that the hundred percent germination of sclerotia of *S. rolfsii* was recorded in T_1 (sawdust) and T_{12} (control. Hundred percent inhibition was exibited in T_9 (datura leaf dust) and T_{10} (neem leaf dust) treatments after 10-40 days of incubation period. Next of inhibition 79.05% was recorded in T_{11} (henna leaf dust), followed by 77.77% in T_8 (Tricho-compost), 75.00% in (*T. harzianum*) 70.00 % in T_4 (cowdung), 67.00% in T_6 (compost), 46.00% in T_5 (mustard oil cake) and 40.00% in T_2 (rice husk) amended soil after 40 days of incubationin soil. No inhibition was recorded in T_1 (sawdust) treatment and T_{12} (untreated control).

In case of mycelial growth the highest mycelial length of *S. rolfsii* was recorded 85.0 mm in T_{12}(untreated control) and the lowest 6.00mm was in T_{11}(henna leaf dust). Hundred percent inhibition was recorded in T_9 (neem leaf dust) and T_{10} (datura leaf dust) after 40 days of incubation in soil. Next of inhibition was recorded 92.94% in T_{11} (henna leaf dust) followed by 64.58% in T_7 (*T.harzianum),* 64.0% in T_8 (Tricho-compost), 58.82% in T_6 (compost), 55.29% in T_5(mustard oil cake), 53.88% in T_4(cowdung), 47.50% in T_2 (Rice husk) and 41.66% in T_3(ash). The lowest inhibition was recorded 11.76% in T_1 (sawdust). The results are presented in Table 4.11-12 and plate 4.9-10.

Table 4.11 Effect of organic amended soil on germination % of sclerotia of *S. rolfsii* after 10-40 days of incubation period in pot culture technique.

Treatement	Mean of germination percentage of sclerotia			
	10th day	**20th day**	**30th day**	**40th day**
T_1(Saw dust)	100(0.0 *)a	100(0.0 *)a	100(0.0*)a	100(0.0*)a
T_2(Rice husk)	90(10) b	75.5(24.5) c	67.4(32.6)b	60.0(40.0) b
T_3(Ash)	90(10) b	80.5(19.5) b	70(30.0) b	65(35.5) b
T_4(Cowdung)	90.1(9.9)b	77.3(22.7) c	65.0(35) b	30.0(70) cdef
T_5(Mustard oil cake)	70.3(29.27d)	60.0(40) e	59.1(40.9) b	54.0(46) bc
T_6(Compost)	80.3((19.7 c	70.5(29.5)d	60.7(39.3) b	33.0(67) bcd
T_7(*T. harzianum*)	58.0(42) e	42.33(57.67f)	26.22(76.78) d	25.0 (75.00) def
T_8(Tricho-compost)	50.0(50) f	45.00(54.67)f	31.1(68.9) d	27.02 (77.77) de
T_9(Datura leaf dust)	00.00(100.0)	0.00 (100.00) h	0.00 (100.00) e	0.00 (100.00) f
T_{10}(Neem leaf dust)	0.00 (100.00)h	0.00 (100.00) h	0.00 (100.00) e	0.00 (100.00) f
T_{11}(Henna leaf dust)	41.2(59.0) g	36.0(64.00) g	31.1(68.9) d	20.5(79.5) cdef
T_{12}(Untreated control)	100.00(0.0) a	100.00(0.0) a	100.00 (0.0) a	100.00(0.0) a
± SE	± 5.75	± 5.45	± 5.41	± 5.45
F value	10.14	9.65	9.62	10.96
LSD(p=0.05)	20.28	25.38	22.82	22.83

*=Inhibition percentage

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

Table 4.12 Effect of organic amended soil on mycelial growth (mm) of *S. rolfsii* after 10-40 days of incubation in pot culture technique.

Treatments	Mean radial growth(mm) of the fungus			
	10th day	20th day	30th day	40th day
T_1(Saw dust)	85.0(0.0*)a	82.0(3.52*)a	78.00(8.2*)b	75.0(11.76*)b
T_2(Rice husk)	80.0(1.1)b	78.0(4.7)b	65.3(23.52)c	45.5(47.05)d
T_3(Ash)	80.0(5.8)b	68.5(9.1)c	75.0(11.76)b	60.0(41.66)c
T_4(Cowdung)	77.0(11.7)bc	68.0(40.8)c	40.0(57.84)f	39.2(53.88)e
T_5(Mustard oil cake)	70.3(9.4)d	60.0(20.0)d	45.0(52.9)e	38.0(55.29)ef
T_6(Compost)	75.0(11.76)c	50.3(41.17)e	60.0(29.41)d	35.0(58.82)fg
T_7*(T. harzianum*)	66.0(22.35)e	40.3(52.58)f	35.2(58.58)g	30.1(64.58)h
T_8(*Tricho*-compost)	50.0(41.17)f	40.0(57.84)f	45.0(47.0)e	34.50(64.0)g
T_9(Datura leaf dust)	25.0(70.58)g	23.5(72.35)g	4.2(95.05)i	0.0(100.00)j
T_{10}(Neem leaf dust)	25.0(70.58)g	20.0(76.47)h	6.5(92.0)i	0.0(100.00)j
T_{11}(Henna leaf dust)	75.0(11.76)c	50.4(41.0)e	12.0(85.88)h	6.0(92.94)i
T_{12}(Untreated control)	85.00(0.00)a	85.0(0.00)a	85.0(0.00)a	85.0(0.00)a
± SE	± 3.47	± 3.54	± 4.55	± 4.40
F value	1.43	5.40	6.60	6.43
LSD(p=0.05)	25.40	22.33	22.35	22.31

*=Inhibition percentage

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

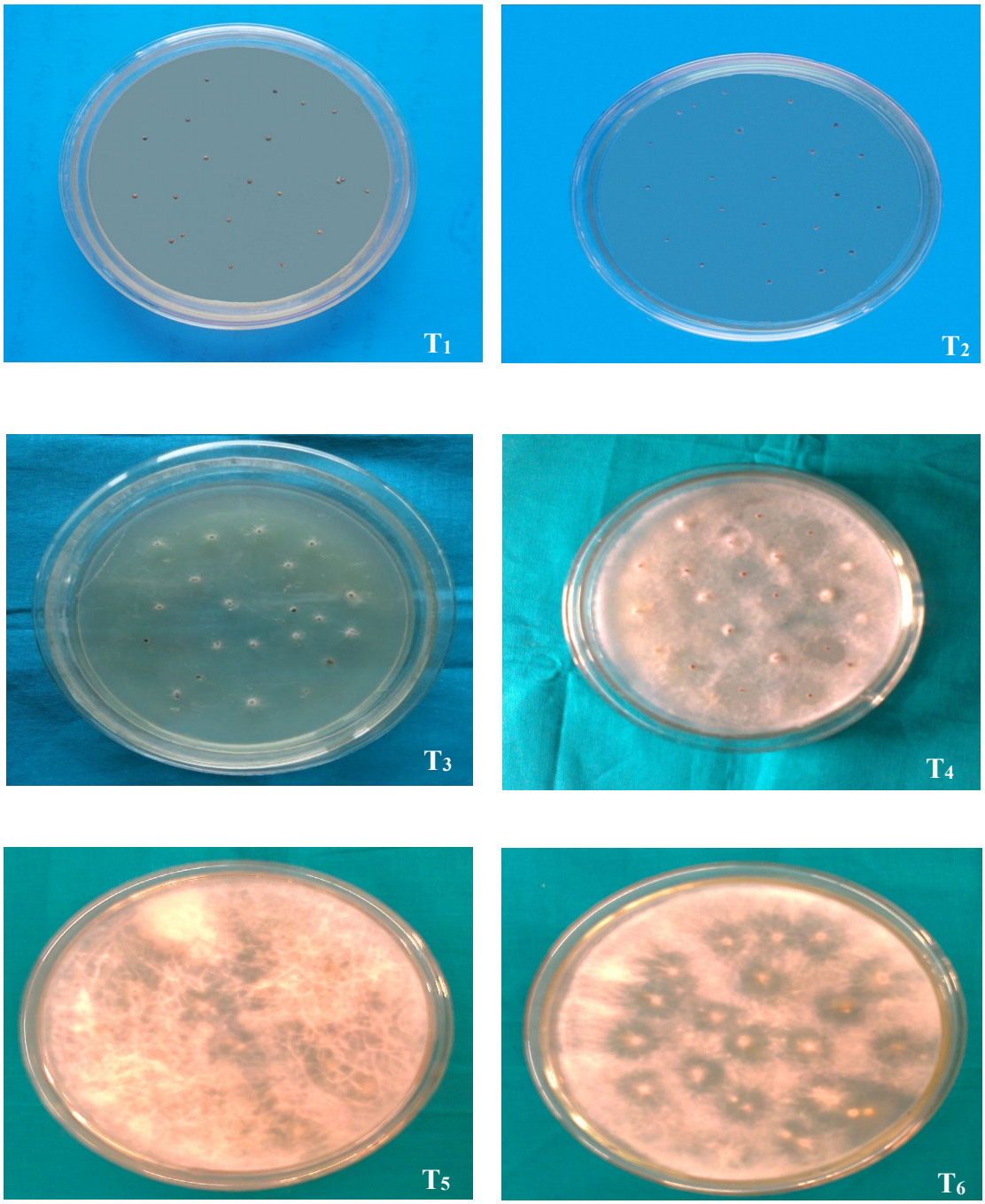


Plate 4.9 Photographs showing effect of organic amended soil on germination of sclerotia of *S. rolfsii* in pot culture after 40 days, T_1=datura leaf dust, T_2= neem leaf dust, T_3= henna leaf dust, T_4 = *T.harzianum* T_5=Sawdust; T_6 Control.

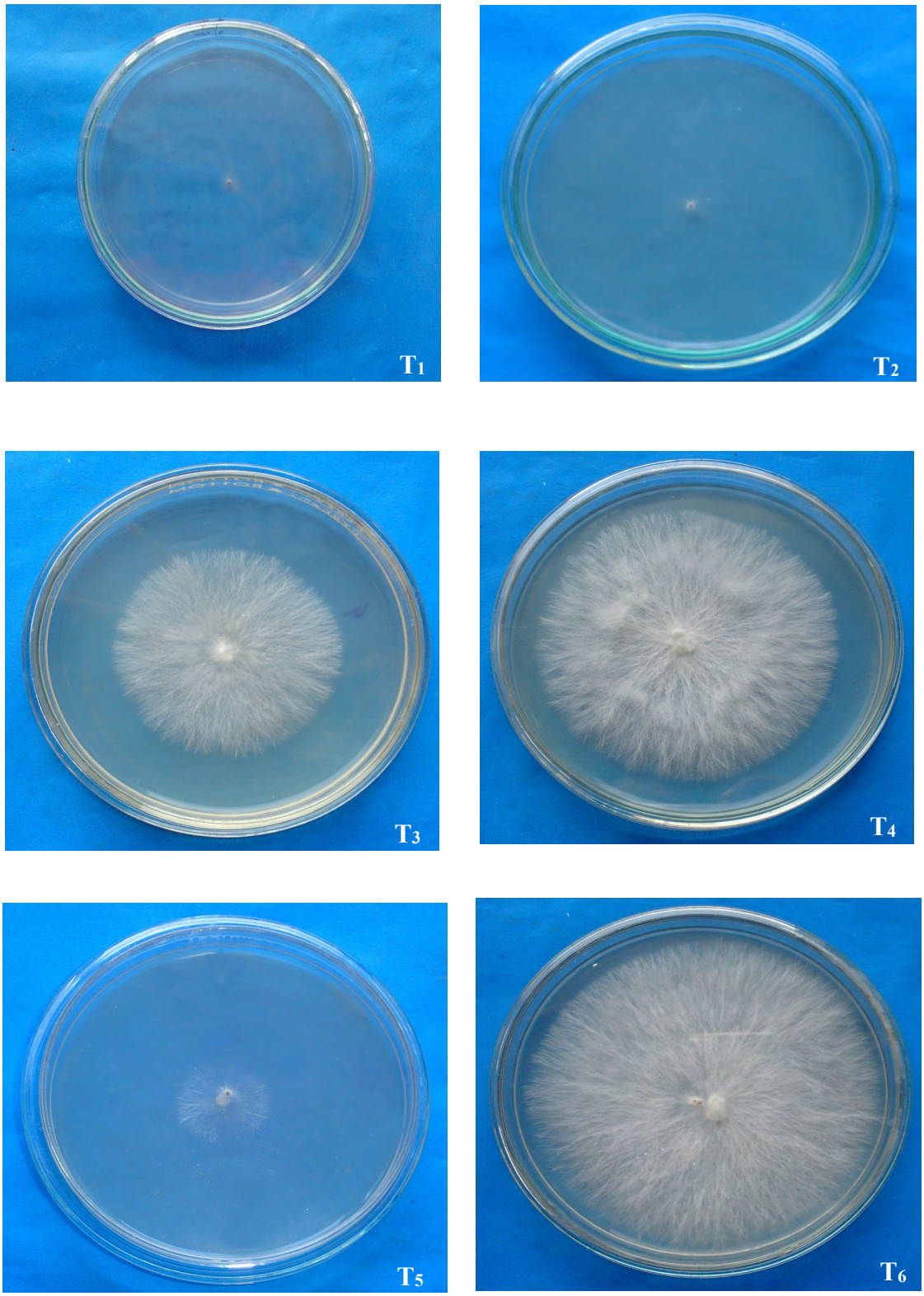


Plate 4.10 Photographs showing effect of organic amended soil on mycelial length of *S. rolfsii* in pot culture after 40 days, T_1=neem leaf dust, T_2= daruta leaf dust, T_3= *T. harzianum*, T_4=sawdust, T_5 = henna leaf dust and T_6= control.

DISCUSSION

In the present study different types of fungicides, plant extracts, bio-agents and amended soil were evaluated to control of *S.rolfsii* in *in vitro* condition. Total eight fungicides were evaluated, among them the most effective fungicides were Dithan-M45 and Bavistin which showed 100% inhibition of mycelial growth at 100, 200, 400 and 800 ppm concentrations while no inhibition was exhibited in rovral at 100 ppm concentration. Mahmood *et al.* (1977) observed that formaldehyde completely inhibited the fungal growth of *S. rolfsii* and formaldehyde was the most effective in killing sclerotia of the test fungus. Siddaramaiah *et al*. (1979) observed *in vitro* conditions that calixin completely inhibited the mycelial growth of *S .rolfsii.* Okonkwo (1988) reported effectiveness of formaldehyde at various concentrations against stem-rot fungus, *S. rolfsii*. Santosh and Dasgupta (1989) reported that Rhizolex 50 WP a new fungicide to control the foot rot of betel vine (*Piper betle* L.) caused by *S. rolfsii.* Rondon *et al*, (1995) used copper oxychioride, vinclozolin (as Ronilan), Iprodione (as Rovral), Metalaxyl (as Ridomyl), Chiorothalanil (as Daconil), PCNB [Quinto zene], Captan, Benomyl, Carboxin + Thiram and Thiabendazole at five concentrations against the growth and sclerotia formation of *S. rolfsii.* Carboxin + Thiram, Copper Oxychioride and Quintozene were found to be most effective, both inhibitmg mycelial growth and scierotia formation at low concentration. Dubey and Kumar (2003) reported the fungicidal effect of azadirachtin as good as the fungicides bavistin. Fouzia-Yaqub and Saleem-Shahzad (2015) studed the effect of fungicides on *in vitro* growth of *S. rolfsii* and reported that at low concentration, no fungicide inhibited the growth of *S. rolfsii*, however, at high concentration Dithane M-45 and Sencozeb significantly reduced the growth of *S. rolfsii.* Parven *et al.* (2016) evaluated *in vitro* efficacy of some fungicides, plant extracts and bioagents against *S.rolfsii* to control foot and root rot disease of betel vine. They found that out of six fungicides bavistin was very effective to inhibit the mycelial growth of *S. rolfsii* in culture medium. In this study it was also found that bavistin inhibit (100%) the pathogen at all concentrations so, this findings support by the observations of Fouzia-Yaqub and Saleem-Shahzad (2015) and Perven *et al* (2016).

In vitro effect of different plant extracts on mycelial growth of *S. rolfsii* was observed. Out of twelve medicinal plants *D. metel* and *L. inermis* were most efective and

hundred percent inhibitions was recorded in *D. metal* and *L. inermis* aquouas extracts followed by *A. indica, A. vasica*, *A. paniculata, T. populnea, M. olefera*, *C. longa*, *O. sanctum, R. serpentina* and *V. rosea.* The minimum inhibition was showed by *H. indicum.*The significant decrease in mycelial growth of the fungus treated with various plant leaf extracts was probably due to presence of antifungal compounds or ingredients in plant leaf extracts. Ethanolic extract of mycellial growth of *S. rolfsii L. inermis* showed hundred percent inhibitions and the lowest inhibition showed in *C. longa* at 10% concentration. On the other hand eight aceton plant extracts screened for their antifungul activity. *L. inermis* and D. *metel* showed hundred percent inhibitions at all the concentrations and lowest inhibition showed at *A. paniculata* at 25% concentration. Use of barks and corks of commonly grown trees and shrubs is also an economical and feasible way of controlling major plant diseases. Iqbal *et al*. (2006) evaluated *in vitro* efficacy of plant leaf extracts against *S. rolfsii.* Farooq *et al.(*2010) reported the maximum inhibition of mycelial growth of *S. rolfsii* causing southern sclerotium rot in sugar beet, was recorded by *A. indica* (73.8%) followed by *Cassia fistula* (73.5%) and *Cannabis sativa* (67.1%). Darvin (2013) evaluated effect of plant extracts on radial growth of *S. rolfsii* causing stem rot of groundnut. Sharma and Basandrai (1997) found leaf extract of *A. indica* effective in reducing sclerotial viability of *Sclerotinia sclerotium* isolated from beans. Inhibitory effects of plant leaf extracts have also been observed on viruses and other soil fungi (Baker, 1981; Kuc and Sham, 1977). Now a day's use of chemical for management of crop disease is being discouraged due to health hazards and environmental pollution. So, control of the pathogen through botanical pesticides or plant extracts and natural biocides might be a good alternative. Botanical extracts are biodegradable (Devlin and Zettel, 1999) and their use in crop protection is a practical sustainable alternative. It reduces environmental contamination and health hazards (Grange and Ahmed, 1988). Botanical fungicides are unique because they can be produced easily by the farmers and small industries (Roy *et al*., 2005). Few works have been done by using tobacco, neem, garlic and some other plant extracts to control some other fungi. Antifungal activities of garlic, neem, and allamanda have been reported by many researchers (Islam, 2005; and Arun *et al*., 1995). Jalal and Ghaffar (1992) studied antifungal characteristics of *O. sanctum* L. and found that its leaf extract completely inhibited the growth of *S. rolfsii* and other fungi. Amin *et al.* (2013) evaluated effect of some botanical extracts and cow's urine on *Sclerotium rolfsii* causal agent of foot and root

rot of betel vine. Khanzada *et al.* (2006) evaluated the effect of plant leaf extract concentration in reducing the mycelial growth of *S. rolfsii* and found that *D.alba* and *C. procera* were most effective @ 1.5 and 2%. In this study *L. inermis* and *D. metel* completely inhibit the growth of *S. rolfsii.* So, the present study supports the observations of previous study.

In the present research, six antagonists were evaluated against *S.rolfsii* in *in vitro* condition. The maximum inhibition percentage was observed in *T. harzianum* both dual culture and poison agar method. In dual culture method, *T. harzianum* showed around 66.00% inhibition of mycelial growth against *S. rolfsii*. On the other hand in poison agar method 68.88% inhibition of mycelial growth was recorded. Biswas and Sen (2000) tested 11 isolates of *T. harzianum* against *S. rolfsii* in dual culture where isolates T_8, T_{10} and T_{12} were effective and they over grew the pathogen up to 92%, 85% and 79% respectively *in vitro*. Murtaza *et al*. (2012) tested the effect of *Trichoderma* culture filtrate with different concentrations grown in different media and they observed 60 to 80% of inhibition at 100% concentration and low inhibition observed at low concentration. Lim and Thee, (1990) reported that, the isolates of *T. harzianum* inhibited the growth of *S. rolfsii* up to 67% in dual culture on malt agar and up to 100% using a cellophane overlay technique and 68.88% inhibition was recorded in poison agar. The findings of the present study also support the observation of Lim and Teh (1990).

The effect of organic amended soils on germination and mycelial length of *S. rolfsii* was tested by two methods i.e in surface culture and pot culture. In surface culture, hundred percent sclerotia germination was observed in T_1 (sawdust) and T_{12} (control) and no sclerotia germination was observed in T_9 (*A. indica)* and T_{10} (*D.metel)* amended soil. In pot culture, the highest germination of sclerotia of *S. rolfsii* was recorded in T_{12} (untreated control) and T_1 (Saw dust) and the lowest percentage of germination was recorded in T11 (Henna leaf dust) at 10, 20, 30 and 40 days of incubation in soil. On the other hand the highest mycelial length of *S. rolfsii* was recorded in T_{12} (control) and T_1 (sawdust) at 10 days of incubation in soil and no mycelial growth was recorded in T9 (neem leaf dust) and T_{10} (datura leaf dust) after 10 to 40 days of incubation in soil. It is necessary to study the effects of amendments on sclerotial germination and mycelial growth of *S. rolfsii* for management of diseases. This knowledge helps to identify some of the reasons for success or failure

of amendments to control this fungus. *In vitro* studies on eleven organic amendments against *S. rolfsii* revealed that, there was a significant difference in percent germination of mycelial growth of *S. rolfsii* by all the soil amendment tested. Results from this study indicate that organic amendments can be used to manage *S. rolfsii* and disease caused by this pathogen. Certainly stimulation of microbial activities, determined by physical and chemical properties of materials used for amendments has an important role. Each amendment was associated with an optimal time to decrease sclerotia germination and increase survival and growth. In this study neem leaf dust is a very effective amendment. Neem contains a variety of chemical constituents such as nimolicinol, isolimolicinolide, azadirachtin, azadirachtol, nimlinone, nimbocinol, nimbocinone, nimocin, etc. (Tewari, 1992). Shivpuri *et al.* (1997) reported the fungitoxic properties of neem leaf extract against several fungi. The present study neem and datura leaf dust were completely inhibit the germination of *S. rolfsii.* In this study *T. harzianum* also showed better performance in both surface culture and pot culture. Compost, cowdung, musterd oil cake were more effective amendments compare to sawdust, ash, rice husk to control *S. rolfsii* Mukherjee *et al.* (1995) observed that *T. harzianurn* was effective in suppressing *S. rolfsii* and *Rhizoctonia solani. T. harzianum* was found to be effective in destroying the sclerotia of both fungi. Chamswamg (1992) reported that isolates of *Trichoderma* species increased the survival rate of tomato seedlings infected by *S. rolfsii* the causal pathogen. Hadar and Gorodecki (1991) found an inhibitory effect of grape marc compost on sclerotial germination and viability, and associated this effect with high numbers of *Penicillium* isolated from sclerotia. Blum and Kabana (2004) evaluated the effect of organic amendment on sclerotia germination, mycelial growth, and *S. rolfsii* induced diseases. Khattabi *et al.* (2004) reported that horse manure inhibited the mycelial growth of *S. rolfsii.* The present results also suport the previous fndngs and suggests using effective organic amendments for field trial to manage foot rot disease of betelvine.

CHAPTER -5

In vivo control of *S. rolfsii* Sacc. causal agent of foot rot disease of Betel vine

INTRODUCTION

Bangladesh is the second largest grower country of betel vine at about 1400 hectares area are cultivated betel vine (Anonymous 2006). But every year this crop suffer from many diseases such as anthracnose, wilt, leaf spot, and stem rot, foot rot etc. Among the diseases foot rot caused by *S. rolfsii* is the most drastic disease which losses the production of betel leaf to a great extent (Islam, 2005). *S. rolfsii* is a serious soil borne pathogenic fungus and harmful to many economically valuable crops in most of the tropical and subtropical region of the world (Aycock, 1966). Botanical extract, are biodegradable and their use in crop protection is a practical sustainable alternative. It reduces environmental contamination and health hazards (Grange and Ahmed, 1988). Plants are the sources of natural pesticides that make excellent leads for new pesticide development (Arokiyaraj *et al*. 2008; Shanmugavalli *et al*. 2009; Swarna and N. Reddy, 2009). Many species of fungi and bacteria are reported to be effective bio control agents against soil borne plant pathogens (Papavizas, 1985; Mukhopadhyay, 1994). Several strains of *Trichoderma* and *Chaetomium* have been found to be effective as bio-control agent of various soil and seed borne plant pathogenic fungi (Chet and Inbar 1994). The use of fungicide, plant extracts, bio-control agent, organic and inorganic amendments has been suggested by several workers to control the soil borne pathogen. Integrated management system can play an important role to control foot rot disease of betel vine caused by *S. rolfsii*.

MATERIALS AND METHODS

5.1 Location of the experiment

In vivo experiments were conducted at the boroj of Baghmara Upazila in Rajshahi district under natural Boroj condition. The experiment was carried out during the period from June 2014 to July 2015 season.

5.2 Land preparation

In vivo experiment was carried out with two boroj with well drainage system. Weeds and stubbles were removed. The soils are rich and are characterized by high lime content. Provide drainage trenches of 0.5 m width by 2.5m depth in between two adjoining beds. The texture varies from clay loam to light sandy loam according to its formation from the silt of the various tributaries of the Ganges. The experiment was carried out in Randomized Complete Block Design (RCBD) with three replications.

5.3 Plantation of betel vine cuttings

Healthy and disease free betel vine cuttings were planted in the experimental plot. At first, hole is made with khurpi, so that the internodes below the bud point is dipped in soil, but must be touching with surface soil. The hole is completely packed with the help of thumb finger. After that, planted material is covered with straw. This planted betel vine plant was watered twice a day with the help of watering cane or sprinkler.

5.4 Irrigation and weeding

Irrigation boroj immediately after planting and afterwards once in a week. Weeding was done oftenly during the visit of boroj. Fertilizers and manures were applied to the plot for betel vine cultivation. First application at 15 days after lifting the vines and second and third dose at 30 - 45 days intervals.

5.5 Application of fungicides to control foot rot disease of betel vine *in vivo* condition

In *in vitro* conditions, total eight fungicides with dfferent concentrations (100, 200, 400 and 800 ppm) were evaluated against the pathogen of foot rot disease of betel

vine and the most effective three fungicides were selected in this experiment and are mentioned below:

T_1=Bavistin (2%)

T_2= Dithan M-45(2%)

T_3= Rovral (2%)

T_4=Control (Without fungicide)

5.6 Preparation of plant extracts

The plant extracts were prepared using the method of Bhatti (1988) as described in earlier experiment *(in vitro).*

5.7 Application of plant extracts to control foot rot disease of betel vine in *in vivo* condition

Application of plant extracts

In *in vitro* condition total 12 plant extracts with their different concentrations were evaluated and the most effective plants extracts were noted as *A. indica, D. metel* and *L. inermis* against the pathogen. These three effective plants were selected for *in vivo* experiment and 10% concentration was used for each plant. The experiment was laid out of RCBD (Randomized Complete Block Design) with three replications. Data were collected on different yield and yield bearing characters. One field trials were conducted on 2014- 2015 cropping season the name of treatments were as follows:

T_1=*D. metel* (10 %)

T_2=*L. inermis* (10%)

T_3=A. indica (10 %)

T_4=Control (Without plant extracts)

5.8 Application of bio-agent to control foot rot disease of betel vine in *in vivo* condition

In *in vitro* experiment six antagonists were evaluated against *S. rolfsii* and the most effective two *Trichoderma* spp. (*T. harzianum* and *T.virens*) were selected for *in vivo* experiment.

Preparation and application of *Trichodera* spp.

Mycelial disc (5 mm) of *Trichoderma* spp. (*T. harzianum* and *T.virens*) were obtained from 4-5days old culture and transferred to 500-ml conical flask seperately and incubated at 28ºC for 4-5 days in rotary shaker with gentle shaking. At the end of incubation period the conical flask were shaken at 50 rpm for 35min in an orbital shaker. Then the content of each conical flask was filtrate through sterile muslin cloth. The filtrate with the spores @ the concentration of 2×10^6 was incorporated with boroj soil to 100 ml/hill.

5.9 Application of soil amendments to control foot rot disease of betel vine in *in vivo* condition

Total 11 different soil amendments were screened against *S. rolfsii*. causal agent of foot rot disease in *in vitro* condition and most effective soil amendments were selected for *in vivo* experiment as following treatments

T_1=Neem leaf dust (500g/plot)

T_2=Compost (500g/plot)

T_3=Datura leaf dust (500g/plot)

T_4= Mustard oil cake (500g /plot)

T_5=Control (Without amendment)

5.10 Integrated management to control foot rot disease of betel vine

Integrated disease management strategy was followed in two borojes at Baraigram village, Baghmara Thana in Rajshahi district for controlling foot rot disease of betel vine. Total six treatments were laid out in randomized block design with three

replications. Before starting the experiment all the infected plants in treatment rows were removed. Then antagonists were applied within the rows of vines and slightly covered with the soil. The treatment of the IDM (Integrated Disease Management) strategy were as follows

T_1 = Bavistin (2%)

T_2 =Neem leaf dust amended (500g/plot)

T_3 = Datura leaf extract (10%/ ml)

T_4 = Spore of *T. harzianum* (100ml/hill)

T_5 = $T_1 + T_2+T_3$

T_6 = $T_1 + T_2+T_3+T_4$

T_7 = Control (untreated)

5.11 Methods of data collection

Each plant was marked with proper labeling with the treatment and data on following quantitative characters were measured. At growth period plant height, number of leaves/plant, number of branching, fresh weight of leaves, disease incidence, yield per plant were recorded. Plant height was measured in centimeter (cm) by a meter scale from the ground level up to the top level. Twenty leaves were selected at random from the plant and their weight was taken in gram by an electric balance. Total number of plant and total number of infected plants were counted and disease incidence was calculated by adopting the grading formula of Siddaramaiah *et al.* (1978).

$$\text{Percentage of disease incidence} = \frac{\text{Total No. of Infected Plants}}{\text{Total No. of Plants}} \times 100$$

Data were collected on individual plant basis and mean data were used for analysis.

5. 12 Statistical analysis of data

For *in vivo* experiments Randomized Completely Block Design (RCBD) was followed data on yield and yield contributing parameters were recorded and analyzed and the significant difference of the treatment means were compared by Duncan's Multiple Range Test (DMRT), with the help of SPSS computer software programs version (20).

5.13 Phytochemical analysis of treated betel vine leaf through HPLC (High-performance liquid chromatography).

To monitor the changes of plant metabolites during the treatment and cropping season phytochemical analysis of betel vine leaves were conducted. Fresh leaves were different experimental (T_1 – T_7) plant (Betel vine) as before.

Preparation of extract

Harvested leaves (1g) were grinded with mortar and pestle using 10 ml deionized water and centrifuged at 1500 g for 10 min. The filtrates were separated and storing at -20^{o}C. The extracts were then 100 times diluted in demonized water and filtered through nylon membrane before using for HPLC analysis.

HPLC condition and Equipments

The HPLC analysis were carried out using waters binary gradient HPLC system (Milford, Massachusetts, USA) comprising a quaternary pump, an on- line degasser AF and a UV visible detector Mede 2489 and Empower 2 ^{TM} software were used for analysis.

Column: 18 reverse phase column were used (particle size : 5 µm, pore size : 300 A, pH ranges : 1.5 – 10, dimension: 250 mm × 10 mm).

Experimental condition: The gradient analysis was performed with buffer A (water and 0.1% TFA) and buffer B (80% acetonitrile and 0.1% TFA) as mobile phase at the gradient of : 1-24 min 100% A, 25-34 min 100% B, 35-40 min 100% A and flow rate 0.5 ml/min.

Standard preparation

Standard for each amino acid and organic acids were purchased from Sigma-Aldrich, Co., St. Louis and Carl Roth. Amino acid/organic acid stock solutions (0.5 mM) were prepared in LC-MS grade water. In addition, samples were diluted 100 times in LC-MS grade water before injection.

Detection of metabolites

Both standards and samples were filtered using 0.22 µm Minisart Syringe Filters (Sartorius Stadium Biotech, Germany) before injection. Metabolites were detected with a Waters 2489 dual absorbance detector (Waters Corporation, Milford, Massachusetts, USA) at 280 and 360 nm. Peak identifications were achieved by comparing retention times and mass spectra of sample peaks with those of authentic standards.

RESULTS

The present research was conducted to control foot rot disease of betel vine in *in vivo* condition. For this reasons different types of plant extracts, fungicides, bio-control agents and organic amendments were used to control of the pathogen. The results are presented under the following subheading:

5.1 Effect of fungicides to control foot rot disease of betel vine in *in vivo* condition during 2014-15 season

In 2014-15 cropping season three fungicides were used to control foot rot disease of betel vine and the results are presented in Table 5.1. Among the three fungicides T_1 (bavistin) showed the most effective performance because the highest plant height (215cm) was recorded and the lowest plant height was recorded in T_4 (control). The highest (18) number of branches was recorded in T_1 (bavistin) and the lowest (8) was in T_4 (control). On the other hand the highest (30) number of leaves was recorded in T_1(bavistin) treated plot and lowest (15) was recorded in T_4 (control). There was no significant difference between T_1 (30) and T_3 (29) treated plot. The highest fresh wt. (72.03gm) of leaves was recorded in T_1 (bavistin) and the lowest fresh wt.(46.88gm) of leaves was recorded in control. The highest yield (25.09*) was recorded in T_1 (bavistin) treated plot and the lowest yield (12.65*) was recorded in T_4 (control). On the other hand the highest percent disease incidence (79.33%) was recorded in untreated plot and the lowest (19.52%) was recorded in T_1 (bavistin).

Table 5.1 Effect of fungicides against foot rot disease of betel vine (*in vivo)* during 2014-15 season.

Treatments	Plant height (cm)/plant	No. of branches	No. of leaves/plant	Fresh weight of 20` leaves (gm)	Yield (*)	Disease incidence (%)
T_1 (bavistin)	215.30a	18a	30a	72.03a	25.09a	19.52c
T_2(dithan M-450)	200.50a	15b	27b	62.33c	19.00b	22.21b
T_3(rovral)	208.60a	12c	29a	66.90b	25.32a	20.92c
T_4 (control)	150.22b	8d	15c	45.55d	12.33c	79.33a
± SE	± 6.42	± 1.21	± 1.87	± 9.93	± 1.44	± 7.91

[* = lakh/ha/year]

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

5.2 Effect of plant extracts to control foot rot pathogen of betel vine in *in vivo* condition

In 2014-15 cropping season three plant extracts were used to control foot rot disease of betel vine and the results are presented in Table-5.2. Out of three treatments the highest plant height (211.10cm) was recorded in T_1 (*D. metel*) treatment and the lowest (175.00cm) was in control. On the other hand the highest number of branches (15) was recorded in T_3 (*L. inermis*) and the lowest (3) was recorded in T_4 (control). There was no significant difference between T_1 (*D. metel*) and T_3 (*L. inermis*) treatment for leaf branch and no. of leaves. The highest fresh weight (75.59gm) of leaves was recorded in T_2 (*A. indica*) treatment and the lowest fresh wt. (41.30gm) of leaves was recorded in control. Among the three treatments the highest number of leaves was (30) recorded in T_2 (*A. indica*) and the lowest was recorded in control and there was no significant difference between T_2 and T_3 treatment. Performance of yield data was recorded the highest yield (21.58*) was recorded in T_2 (*A. indica*) and the lowest (3.00*) was in control. On the other hand the highest disease incidence (75.80%) was recorded in control plot and the lowest (35.22%) was recorded in T_3 (*L. inermis*) treated plot. It was observed that all yield bearing characters were significantly increased at all treatments compare to the control and the highest disease incidence was observed in untreated control.

Table 5.2 Effect of plant extracts against foot rot disease of betel vine (*in vivo*) during 2014-15 season.

Treatments	Plant height (cm)/plant	No. of branches	No. of leaves/plant	Fresh weight of 20 leaves (gm)	Yield (*)	Disease incidence (%)
T_1(*D.metel*)	211.10a	14a	28a	55.03b	19.0a	40.2b
T_2(*A. indica)*	205.72b	12a	30a	75.59a	21.58a	30.03b
T_3(*L.inermis)*	206.35b	15a	27a	53.55b	20.09a	35.22b
T_4(control)	175.02c	3b	19b	35.00c	3.00b	75.80a
± SE	± 4.34	± 1.32	± 1.44	± 4.04	± 2.31	± 4.84

[*= lakh/ha/year]

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

5.3 Effect of bio-control agent to control foot rot disease of betel vine (*in vivo)* during 2014-2015 season

Two *Trichoderma* spp. were evaluated in *in vivo* condition and the results are presented in table-5.4. The highest plant height (220.72 cm) was recorded inT_1 (*T. harzianum*) and the lowest plant height (138.02 cm) was recorded in T_3 (control). On the other hand the highest number of branches (18) was recorded in T_1 (*T. virens)* and lowest (4) was recorded in T_3 (control). Out of two treatments the highest number (31) of leaves was recorded in T_1 (*T. harzianum*) and the lowest (19) was recorded in T_3 (control). Between the two treatments the highest fresh wt. (75.30 gm) of leaves was recorded in T_1 (*T. harzianum*) treated plot and the lowest fresh wt. (45 gm) of leaves was recorded in control plot. The highest yield (27.34) was recorded in T_1 (*T. harzianum*) treated plot and the lowest yield (4.09) was recorded in T_3 (control plot). On the other hand the highest disease incidence (78.33%) was recorded in T_4 (untreated plot) and the lowest

was recorded in T_3 (25.05%). Disease incidence was significantly (p=0.05) varied among the treatment.

Table 5.3 Effect of bio-control agents against foot rot disease of betel vine *in vivo* condition during 2014 -15 season

Treatments	Plant height (cm)/plant	No. of branches	No. of leaves/plant	Fresh weight of 20 leaves (gm)	Yield (*)	Disease incidence (%)
T_1(*T.harzianum*)	220.72a	14b	31a	75.23a	27.34a	25.05c
T_2(*T.virens*)	216.35b	18a	30a	64.50b	25.52b	30b
T_3(control)	138.02c	4c	19b	40.33c	4.09c	78.33a
± SE	± 13.42	± 2.16	± 1.94	± 5.21	± 1.31	± 8.51

[*= lakh/ha/year]

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

5.4 Effect of organic amendments to control foot rot disease of betel vine in *in vivo* condition during 2014 – 2015 season

Total four types of soil amendments were evaluated and the results are presented in Table-5.4. The highest plant height (223.22 cm) was recorded in T_4 (datura leaf dust) and the lowest plant height (190.01 cm) was recorded in T_5 (control). On the other hand the highest number of branches (15) was recorded in T_3 (neem leaf dust) and lowest (7) was recorded in T_5 (control). In other treatments T_1, T_2, and T_4 the number of branches was 14, 11 and 12 respectively. There was significant difference between all the treatments compare to the control. Among the four treatments the highest number (30) of leaves was recorded in T_3 (neem leaf dust) and lowest (17) was recorded in T_5 (control). Similar result was observed in T_2 (compost) and T_4 (datura leaf dust). Out of four treatment the highest fresh wt. (80.90 gm) of leaves was recorded in T_3 (neem leaf dust) treated plot and the lowest fresh wt. (41.00 gm) of leaves was recorded in control plot. The highest yield (19.03) was recorded in T_3 (neem leaf dust) treated plot and lowest yield (3.25) was recorded in T_5 (control plot).

On the other hand the highest disease incidence (78.03%) was recorded in T_4 (untreated plot) and the lowest (38.66) was recorded in T_4 (datura leaf dust).

Table 5.4 Effect of soil amendment against foot rot disease of betel vine (*in vivo)* during 2014 -15 season.

Treatments	Plant height (cm)/plant	No. of branches	No. of leaves/plant	Fresh weight of 20 leaves (gm)	Yield (*)	Disease incidence (%)
T_1 (Mustard oil cake)	205.30d	14a,b	27a	75.33b	13.23b	50.05c
T_2(compost)	210.50c	11b	28a	78.01a,b	16.33a	59.2b
T_3(Neem leaf dust)	218.00b	15a	30a	80.9a	19.03a	62.02b
T_4(datura leaf dust)	223.22a	12ab	28a	74.25b	13.2b	38.66d
T_5Control	190.01e	7c	17b	41.00c	3.25c	78.03a
± SE	± 3.09	± 0.86	± 1.30	± 3.93	± 1.49	± 3.51

[*= lakh/ha/year]

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

5.5 Effect of integrated management to control foot rot disease of betel vine in *in vivo* condition.

Six treatments were evaluated and the results are presented in Table-5.5. From the results it was evident that the highest plant height (227.11cm) was recorded in T_6 (T_1+T_2+T_3+T_4) and the lowest (170.00 cm) was recorded in T_7 (control). On the other hand the highest number of branches (18) was recorded in T_6 and lowest (4) was recorded in T_7 (control). The highest fresh wt. (86.50 gm) of leaves was recorded in T_6 (bavistin + *Datura* leaf extracts + neem leaf dust+ *T. harzianum)* treated plot and lowest fresh wt. (40.22 gm) of leaves was recorded inT_7 (control plot). Among the six treatments the highest number (31) of leaves was recorded in T_6 (T_1+T_2+T_3+T_4) treated plot and lowest (19) was recorded in T_7 (control plot). There was no significance difference between T_2 (*Datura* leaf extracts) and T_5 (bavistin + *Datura* leaf extracts +

neem leaf dust) treated plot. The highest yield (38.50*) was recorded in T_6 (T_1+T_2+T_3+T_4) treated plot and the lowest yield (3.09*) was recorded inT_7 (control plot). On the other hand the highest disease incidence (75%) was recorded in T_7 (untreated control plot) and the lowest (9.34) was recorded in T_6 (T_1+T_2+T_3+T_4) treated plot. The significant (p=0.05) difference was observed among the treatments compare to control in all yield bearing characters.

Table 5.5 Integrated management to control of foot rot disease of betel vine (*in vivo)* during 2014-15 season.

Treatments	Plant height (cm)/plant	No. of branches	No. of leaves/plant	Fresh weight of 20 leaves (gm)	Yield (*)	Disease incidence (%)
T_1(Bavistin)	217.10c	15b	30a	82.33b	33.35b	19.33b
T_2(*Datura* leaf extracts)	219.55bc	17c	26b	71.01d	27.11d	40.30c
T_3(Neem dust)	211.60d	14b	30a	82.20b	29.20cd	50.02b
T_4 (*T. harzianum*)	219.72bc	16c	28ab	79.12bc	20.59e	30.39d
T_5(T_1+T_2+T_3)	221.33b	17c	29ab	78.11c	31.25bc	10.25e
T_6(T_1+T_2+T_3+T4)	227.11a	18d	31a	86.50a	38.50a	9.34f
T_7(untreated control)	170.00e	3a	19c	40.22e	3.09f	75.03a
± SE	± 4.00	± 1.12	± 0.92	± 3.27	± 2.41	± 4.92

[* = lakh/ha/year]

[Values in a column having same letter do not differ significantly (P=0.05) according to DMRT]

A

B

C

D

E

F

Plate 5.1 Photographs showing effect of different treatment plot of betel vine; A (bavistin), B (datura leaf extracts), C (neem leaf dust) D (bavistin + datura leaf extracts + neem leaf dust), E (bavistin + datura leaf extracts + neem leaf dust + *T. harzianum*), and F (Untreated control).

5.6 Phytochemical analysis of betel vine leaf through HPLC (High-performance liquid chromatography)

To study the biochemical changes among the treated plants, phytochemicals were analyzed through HPLC. In this experiment only integrated management plot was selected. Aqueous extract of plant leaves were analyzed by HPLC and the results are presented in Table-5.6 and Fig 5.1-5.7. The total number of phytocompounds was varied with the treatments. The maximum number (31) of compounds was detected in T_6 (T_1+T_2+T_3+T_4) treatment while minimum (16) was detected in T_7 (foot rot affected leaf). Twelve standards namely oxalic acid, ascorbic acid, arginine, methionine, citrate, proline, penylalanin, trieonine, valin, leucine, malate, GA3 were used and compared with HPLC profile of treated plant extracts.

In T_1 treated betel leaf, total 19 numbers of phytocompounds were observed and the peak profile is presented in Figure 5.1 and Table-5.6. Out of 19 compounds only 3 phytocompounds were identified. The name of identified phytocompound was as oxalic acid, GA3 and citrate. Out of them oxalic acid detected the highest 0.71% area at the $t/_R$ of 2.983 followed by GA3 0.62 % area at the $t/_R$ of 32.753 and Citrate 0.35% at the $t/_R$ of 28.618.

In T_2 treated betel leaf total 27 phytocompounds were observed and the peak profile is presented in figure-5.2. Out of 27 phytocompounds, only 10 phytocompounds were identified. The name of identified phytocompounds were as oxalic acid, Ascorbic acid, phenylalanine, threonin, valin, leucine, arginin, citrate, GA3 and tryptophan . Out of them arginin, detected the highest 5.87 % area at $t/_R$ of 19.73 followed by tryptophan 4.19% at the $t/_R$ of 14.28, valin 2.95% at the $t/_R$ of 19.30 , ascorbic acid 2.38% at the $t/_R$ of 4.15, phenylalanine 2.24% at $t/_R$ of 24.29, oxalic acid 0.99% at the $t/_R$ of 2.73, GA3 0.86% at $t/_R$ of 31.49, leucine 0.24% at the $t/_R$ of 27.38 and citrate 0.07% at the $t/_R$ of 28.35.

In T_3 treated betel leaf, total 25 phytocompounds were observed and the peak profile is presented in Figure-5.3. Out of 25 phytocompounds only 9 phytocompounds were identified. The name of identified phytocompounds was as oxalic acid, citrate, methionine, arginin, valin, leucine, ascorbic acid and GA3. Out of them, tryptophan

detected the highest 7.48 % area at the $t/_R$ of 15.55 followed by oxalic acid, 2.12 % area at the $t/_R$ of 2.727, leucine 2.59% at the $t/_R$ of 27.339, methionine 2.18% area at the $t/_R$ of 25.867, arginin 1.24% area at the $t/_R$ of 19.74, valin 2.43% at the $t/_R$ of 20.626, ascorbic acid 1.13 % at the $t/_R$ of 4.0 96 , citrate 1.23% at the $t/_R$ of 28.625, GA3 0.39% at the $t/_R$ of 32.117.

In T_4 treated betel leaf total 23 phytocompounds were observed and the peak profile is presented in Figure-5.7. Out of 23 phytocompounds only 7 phytocompounds were identified. The names of identified phytocompounds were as oxalic acid, Citrate, Methionine, phenylalanine, leucine, prolin and GA3. Out of them Citrate detected the highest 5.69% area at the $t/_R$ of 30.521 followed by oxalic acid 3.07 % area at the $t/_R$ of 29.41, GA3 0.67% at the $t/_R$ of 32.81, leucine 0.36% at the $t/_R$ of 27.45, prolin 0.14% at the $t/_R$ of 32.24, and metheonin 0.13% at the $t/_R$ of 25.81.

In T_5 treated betel leaf, total 25 phytocompounds were observed and the peak profile is presented in Figure-5.4. Out of 25 phytocompounds, 8 phytocompounds were identified. The names of identified phytocompounds were as oxalic acid, phenylalanine, ascorbic acid, asparagine, citrate, valin, leucine, and metheonin. Out of them metheonin detected the highest 10.38% area at the $t/_R$ was 25.545, followed by citrate 5.42% at the $t/_R$ of 28.781, leucine 2.61% area at the $t/_R$ 19.348, phenylalanine 1.25% at the $t/_R$ of 31.653, oxalic acid 0.66% area at the $t/_R$ of 2.671, ascorbic acid 0.23% at the $t/_R$ of 4.233, asparagine 0.02% at the $t/_R$ of 2.671 and valin 2.92% at the $t/_R$ of 19.348 .

In T_6 treated betel leaf, total 31 phytocompounds were observed and the peak profile is presented in Fig-5.5. Out of 31 phytocompounds only 8 phytocompounds were identified. The name of identified phytocompound was as oxalic acid, valine, leucine, arginin, methionin, citrate, prolin and GA3. Out of them oxalic acid detected the highest 31.37% area at the $t/_R$ of 2.983 followed by methionin13.02% area at the $t/_R$ of 25.75, prolin 10.94% area at the $t/_R$ 35.64, leucine 5.28% at the $t/_R$ of 27.33, citrat

5.07 % at the $t/_R$ of 30.32, GA3 3.31% $t/_R$ of 32.74, valin 2.09% at the $t/_R$ of 20.61 and arginin 0.72% at the $t/_R$ of 19.75.

In T_7 treated betel leaf total 16 phytocompounds were identified and the peak profile is presented in Figure-5.6. Out of 16 compounds, 9 phytocompound were identified. The name of identified phytocompounds was as oxalic acid, phenylalanine, threonin, valin, leucine, arginin, metheonin, prolin and malate. Out of them phenylalanine detected the highest 4.07% area at the $t/_R$ was 25.067 followed by leucine 2.48% area at the $t/_R$ of 27.357, metheonin 2.7 % at the $t/_R$ of 25.783, arginin 1.99% at the $t/_R$ of 20.292, valin 1.31% at the $t/_R$ of 20.799, malate 0.65% at the $t/_R$ of 36.733, threonin 0.08% at the $t/_R$ of 16.733, GA3 0.4% at the $t/_R$ of 32.823, and proline 0.0% at the $t/_R$ of 34.95.

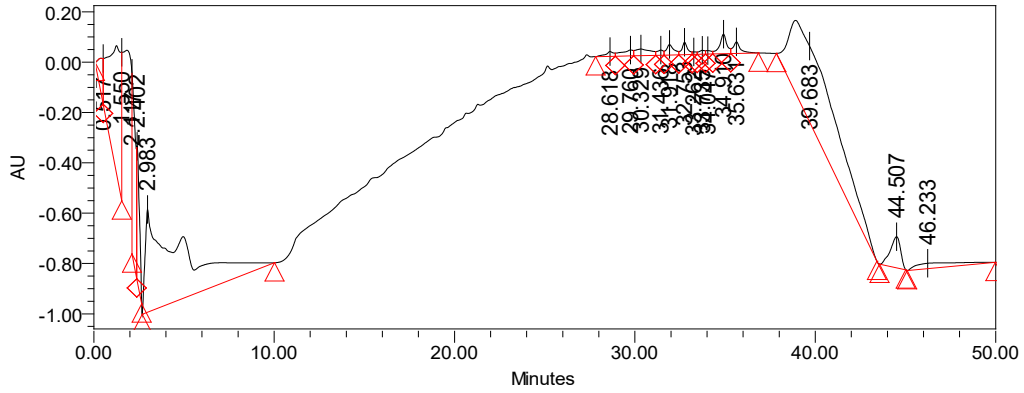


Fig. 5.1: HPLC chromatograph of aquous extract of T_1 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

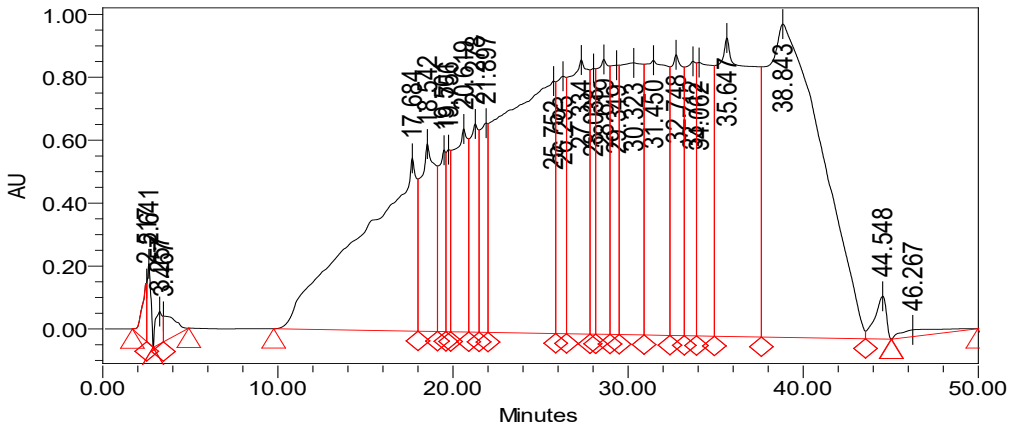


Fig. 5.2: HPLC chromatograph of aquous extract of T_2 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

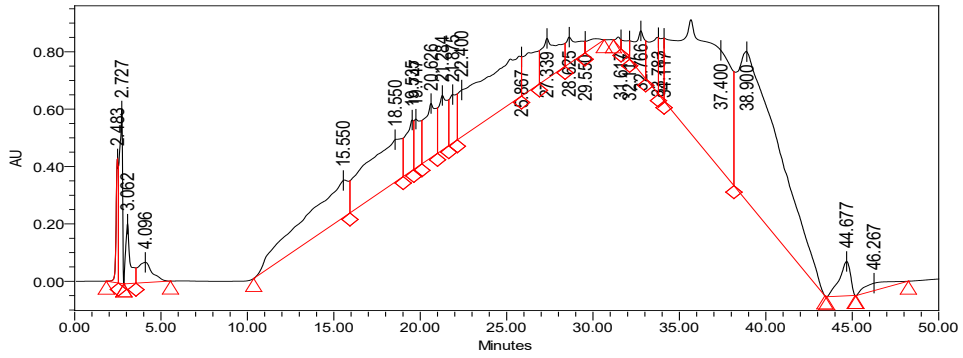


Fig. 5.3: HPLC chromatograph of aquous extract of T_3 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

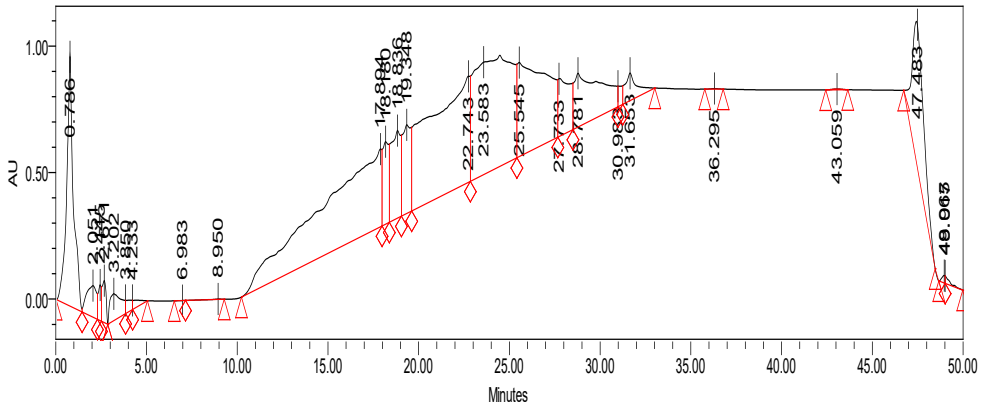


Fig. 5.4: HPLC chromatograph of aquous extract of T_4 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

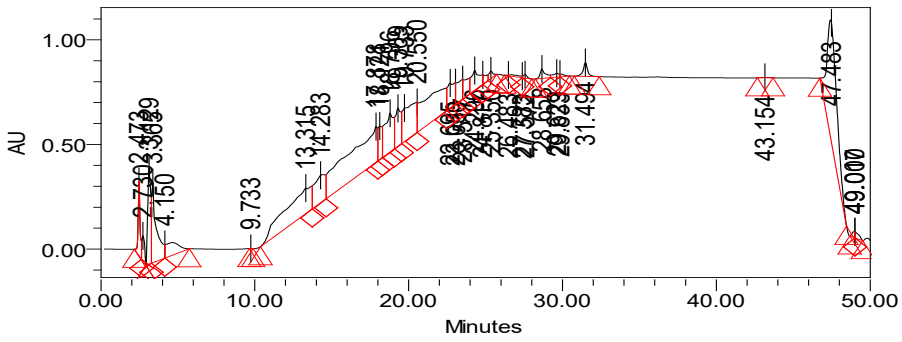


Fig. 5.5: HPLC chromatograph of aquous extract of T_5 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

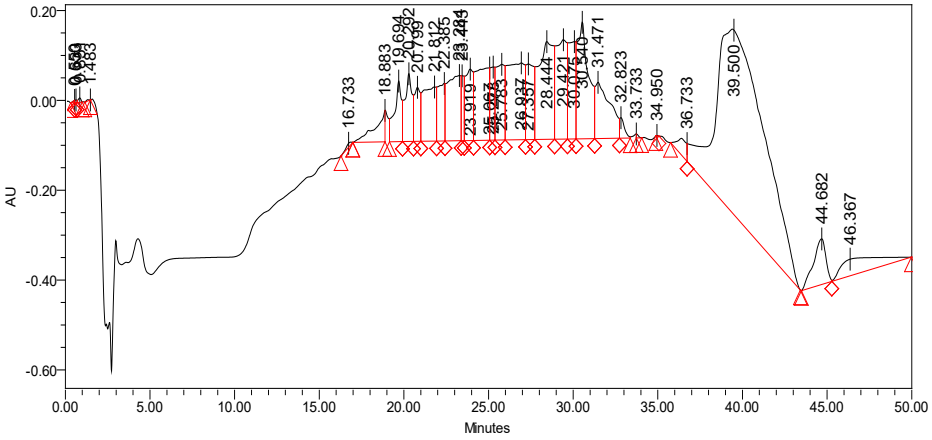


Fig. 5.6: HPLC chromatograph of aquous extract of T_6 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

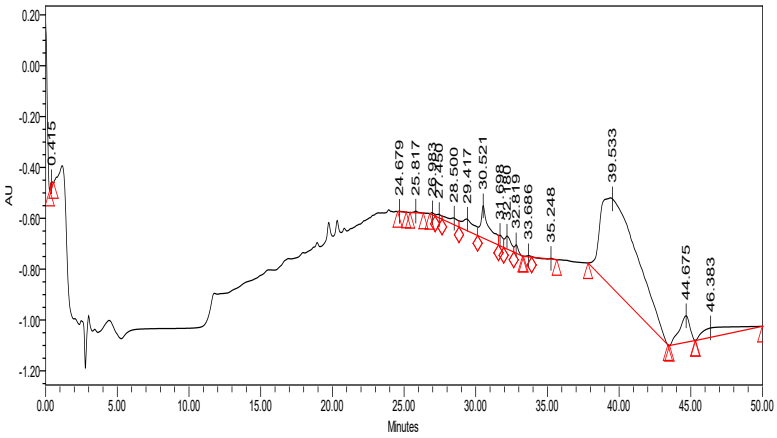


Fig. 5.7: HPLC chromatograph of aquous extract of T_7 treated plant. HPLC analysis with C_{18} reverse column. UV detector at 280nm. The mobile phase was water- acetonitrile. Flow rate 0.5 ml/min and injection vol: 20 µl.

Table 5.6: List of identified phytocompounds of treated betel vine leaves by HPLC analysis with comparing different standards.

Treatment	Total compound by HPLC	Oxalic acid t/R=2.739	Ascorbic acid t/R =4.149	Phenylalanin t/r = 24.5	Treonine t/R =16.7	Valin t/R =25.1	Leucine t/R =27.4	Arginine t/R =19.659	Methionine t/R =25.133	Citrate t/R =30.705	Proline t/R =35.965	Malate t/R =37.161	GA3 t/R =32.81	Tryptophan t/r = 15.5
T_1	19	0.28	NI	NI	NI	NI	NI	NI	NI	0.35	NI	NI	0.62	NI
T_2	27	0.99	2.38	2.24	0.08	2.95	0.24	5.87	NI	0.7	NI	NI	0.86	4.19
T_3	25	2.12	1.13	NI	NI	2.43	2.59	1.24	2.18	1.23	NI	NI	0.39	7.48
T_4	23	3.07	NI	0.04	NI	NI	0.36	NI	0.13	0.69	0.14	NI	0.67	NI
T_5	25	0.66	0.23	1.25	NI	2.92	2.61	4.68	10.38	5.42	NI	NI	NI	NI
T_6	31	31.37	NI	NI	NI	2.9	5.28	0.72	13.2	5.7	10.94	NI	3.31	NI
T_7	16	0.71	NI	4.07	0.08	1.31	2.48	1.99	2.7	NI	0.0	0.65	NI	NI

[Key: NI=Not identified, T_1= bavistin, T_2= neem leaf dust, T_3=datura leaf extracts, T_4= *T. harzianum*, T_5= $T_1+T_2+T_3$, T_6= $T_1+T_2+T_3+T_4$, T_7 = Foot rot affected leaf, treated leaf.]

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DISCUSSION

In these study different fungicides, plant extracts biocontrol agents and organic amendments were evaluated for *in vivo* experiment to control foot rot disease of betel vine. It was observed that all the treated fungicides showed better performance compared to the control. Out of three fungicides most effective fungicide was bavistin because it increased all yield contributing characters and decreased disease incidence. Madhovi and Bhattiprolu (2011) evaluated *in vivo* soil drenching with nine fungicides and found tuberconazole and combination of carbendazim + mancozeb effective for controlling *S. rolfsii* at 1000-3000 ppm. Anilkumar and Pandurangegowa (1984) evaluated the efficacy of vitavax as soil drenching fungicide against *S.rolfsii* in reducing the seedling mortality. Patil *et al.* (1986) observed soil drenching of captan, copperpoxychloride and difoliton were found effective for controlling wilt disease of betelvine caused by *S. rolfsii.* Vanitha and Suresh (2002) reported that, seed treatment with carbendazim recorded significantly the lowest incidence (10.83%) of collar rot of brinjal caused by *S. rolfsii* compared to control (39.30%). Tiwari and Ashok (2004) reported that fungicides like carboxin, epoxiconazole, hexaconazole, propiconazole and triadimefon which were found highly effective against *Rhizoctonia solani* and *S. rolfsii*, and can be formulated as seed dresser either with thiram or mancozeb to control both collar rot and root rot as well as seed mycoflora, effectively. Harlapur and Kulkarni (1992) and Virupaksha Prabhu *et al*. (1997) observed complete inhibition of growth of *S. rolfsii* with Dithane M-45 and agallol. Siddaramaiah *et al*., (1979), Kulkarni *et al*.(1986) and Virupaksha (1994) reported that bavistin was found to be effective against the growth of *S. rolfsii*. In the present study it was also observed that bavistin was most effective for control of foot rot pathogen *S.rolfsii* which agreed with the findings of Siddaramaiah *et al*. (1979), Kulkarni *et al*. (1986) and Virupaksha (1994).

Chemicals are harmful for environment and management of soil born plant pathogens due to the wide spared host range, abundant growth of the pathogens and its capability of producing excessive sclerotia that may persist in soil for several years. For this purpose, plant extracts may be used as an alternative source for controlling *S. rofsii* since they comprises a rich source of bioactive substance. Plant extracts are eco-friendly

and safe for our environment so, management of soil born pathogen such as *S. rolfsii* through plant extracts or botanical pesticides and natural biocides might be a good alternative. From the results it was evident that *A. indica* is the most effective plant because this plant showed better preference for all yield contributing characters such as plant height, number of leaves /plants, leaf weight, leaf yield and reduced disease incidence. Many reports has been published about different parts of neem plants. Daynaram and Tewari (1994) evaluated the soil application of *Azadirchca indica (*neem*), Adhatoda vasica, Aegle marmelos, Anisomeles ovata, Cymboposon flexuos leaves, Curcuma amada* rhizomes and *Ferula foetida* resin for pre and post emergence collar rot of chickpea caused by *S. rolfsii*. Okereke and Wokocha (2006) reported that, the inhibition of damping off disease of tomato incited by *S. rolfsii* was heighest with soil drenching with neem seed (62%) followed by ginger (57.4%). In this study it was also found that neem plant extracts was more effective for reduction of foot rot disease incidence and better for increasing yeild of betel vine. So, the present findings supported those previous reports.

Biological control relay developed as an academic discipline during the 1970s and is now a mature science supported in both the public and private sector. Research related to biological control is published in many different scientific journals, particularly those related to plant pathology and entomology. *Trichoderma* is a well-established genus, and is extensively studied for its high potential as biological control of fungal induced plant diseases. *T.harzianum*, whether as a single species inocula or in combination with other *Trichoderma* species had been used in the control of several diseases (Samuels, 1996). Some species of *Trichoderma* i.e., *T. hamatum*, *T. harzianum, T. koningii, T viride* and *T. virens* showed promising results in controlling *Phytophthora* crown and root rot of apple seedlings under experimental conditions, and thus recommended as a potential biological control agent against the disease on apple trees in orchard (Roiger and Jeffers, 1991). Studies in India used *T. virens* and *T. longibranchiatum* in the control of groundnut root and stem rot disease caused by *Rhizoctonia solani* (Sreenivasaprasad and Manibushanrao, 1993). In the narrowest sense, biocontrol involves suppressing pest organisms with other organisms.

In this study two *Trichoderma* spp. were evaluated in *in vivo* experiments for controlling foot rot disease of betel vine caused by *S. rolfsii. T. harzianum* was the most effective because this strain increased all yield contributing characters and decreased disease incidence. In earlier study Krutova (1987) reported from the laboratory and field experiment that *T. harzianurn* showed hyper parasitic activity on sclerotia of *S. sclerotium* and were capable of destroying its sclerotia in soil. Sharma and Sing (1990) reported that *T. harzianum* was effective against *S. sclerotium* when added to sterilized and unsterilized soils of pea in green house condition. Mycelial preparation of *T. harzianum* was the most effective against *S. sclerotium*. D'Souza *et al*. (2001) observed significant reduction of foot rot disease of betel vine with the use of *T. harzianum.* Dasgupta *et al* (2011) observed minimum foot rot disease of four application of *Trichoderma* at quarterly intervals. In this study *T. harzianum* showed better performance to control foot rot disease incedence of betel vine causal agent of *S. rolfsii* so, that early reports supported this results.

In this study four types of soil amendments were evaluated in *in vivo* experiment and out of them neem leaf dust showed better performance because it increased leaf yield and decreased the incidence of foot rot disease of betel vine. In earlier study it was reported that four application of a *T. harizanum* inoculated oil cake in soil at quarterly interval significantly reduced the disease incidence as well as increase in leaf yield and fresh weight of 100 leaves but was less effective than Bordeaux mixture (1%) application at monthly intervals for four times starting at the onset of monsoon in all the AICRP centres of betel vine (Anonymous, 1997-98, 1998-99 and 1999-2000). Similarly use of corn straw and til oil cake also reduced disease (Mehrotra and Tiwari,1976). Rahman (2005) reported that application of mustard oil cake @ 3 ton/ha and half decomposed poultry manure @ 5 ton/hac weeks before sowing seeds performed better in reducing incidence of collar rot of Chickpea. Faruq and Islam (2007) evaluated the effect of selected soil amendments on seed germination, seedling growth and control of damping off of eggplant and tomato seedlings. Uddin *et al (*2011) evaluated the effect of *T. harzianum* and some selected soil amendment against damping off disease complex of potato and chilli. Haque *et al*. (2001) observed that the disease severity was more at the early flowering and peak fruiting stage. He also reported that organic amendment with sesame oil cake as preventing and ash as curative measures and

spraying of chemical at later stages gave better results for the achievement of full success in controlling the foot rot disease of brinjal.

In the present study integrated disease management approaches was conducted with the combination of plant materials, fungicides, biocontrol agents and soil amendments and found that treatment T_6(bavistin +datura leaf extracts + Neem leaf dust+*Trichoderma harzianum)* showed better peformance. Maiti (1999) has meticulously identified the component of IDM that are now available for the *Phytophthora* disease of betel vine, including disease resistance, plant nutrition, sanitation, chemical and biological control. Efforts on IDM of betelvine were initiated under the AICRP betel vine project at several centers and some prima facie improvement in disease management and yield increase were reported (Anonymous, 1998, 1999, 2000). Madhavi *et al.* (2011) evaluated integrated disease management of dry root rot of chilli incited by *S. rolfsii.* Sultana *et al.(*2012) conducted integrated management of mitigating root rot of chilli caused by *S. rolfsii*. Mohanty *et al.* (2011) conducted field experiment for two consecutive years at the betel leaf garden (Pan barejas) at Virus Research Farm, Kalyani, India, to test the efficacy of sanitation, two fungicides viz. Fosetyl-Al, Bordeaux mixture, biocontrol agent *T. harzianum* used in different combinations for the control of two major diseases namely foot rot and leaf rot of betel vine. The results showed that integrated use of sanitation, Bordeaux mixture and *T. harzianum* gave the best control of foot rot and leaf rot of betel vine. The present investigation support the previous report as integrated management is the best means of controlling of foot rot disease of betel vine caused by *S. rolfsii.*

To observe biochemical changes, phytoconstitents among the treated betel vine leaves were analyzed through HPLC In the present study it was observed that the highest (31) number of phytocompounds was detected in T_6 (bavistin + datura leaf extracts + neem leaf dust + *T. harzianum*) treated leaf and the lowest (16) was in foot rot affected leaf. On the other hand the highest percentage area (31.37%) of oxalic acid was observed in T_6(bavistin + datura leaf extracts + neem leaf dust + *T. harzianum)* treated leaf. *Piper betel* contains a wide variety of biologically active compounds whose concentration depends on the variety of the plant, season and climate. Sugumaran *et al.* (2011) described that *Piper betel* leaf has piperol-A, piperol-B, methyl piper 22 betlol and they also have been isolated. Dwivedi *et al.*

(2014) and Chahal *et al.* (2011) reported that *Piper betel* leaves have an essential oil composing of terpinen4-ol, safrole, allyl rocatechol monoacetate, eugenol, eugenyl acetate, hydroxyl chavicol, ugenol, *Piper betel* and the betel oil contains cadinene carvacrol, allyl catechol, chavicol, p-cymene, caryophyllene, chavibetol, cineole and estragol as the major components. Periyanayagam *et al.* (2012) reported hydroxychavicol acetate, allylpyrocatechol piperbetol, isoeugenol, anethole, stearic acid, methyl eugenol, carvacrol, polyphenol, alkaloids, saponin, tannin, steroids and other compounds are also found in *Piper betle.* Sugumaran (2011) reported the active ingredient of *piper betel* oil which is obtained from the eaves is primary a class of allyl benzene compounds, chavibetol, chavicol, estragole, eugenol, methyl eugenol and hydroxycatechol. Rathee *et al.*(2006) studied antioxidant activity of *Piper betle* leaf extract and its constituents. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of the ethanol extracts of three varieties (Bangla, Sweet, and Mysore) of *Piper betle* revealed the Bangla variety to possess the best antioxidant activity that can be correlated with the total phenolic content and reducing powers of the respective extracts. Column chromatography of the extract of the bangla variety led to the isolation of chavibetol (CHV), allylpyrocatechol (APC) and their respective glucosides. The HPTLC analyses of the extracts revealed similar chemical properties of three *Piper betle* varieties. Syahidah *et al.* (2017) evaluated phytochemical analysis, identification and quantification of antibacterial active copounds in betle leaves, (*Piper betle)* methanolic extract and showed the presence of alkaloids, phenols, flavonoids, tannins, saponins, glycosides, terpenoids and steroids. Therefore, in that study, HPLC method to quantify the content of hydroxychavicol and eugenol in *P. betle* methanolic extract was employed. The linear regression for both analytes has showed good linearity in the investigated ranges with correlation coefficients of 0.9990 for hydroxychavicol and 0.9959 for eugenol. The average retention time of hydroxychavicol and eugenol was found at 4.02±0.002 and 7.61±0.005 min, respectively. Foo et al (2015) evaluated and reported that HPLC is an efficient method in terms of simplicity, precision, rapid and accurate for the simultaneous determination. In the present investigation it was noted that number of total compounds were varied with the treatment and amino acids were decreased foot rot affected leaf.

CHAPTER -6

SUMMARY

SUMMARY

Betel vine is a cash crop of economic importance and extensively grown on large scalein different parts of Bangladesh. Rajshahi is one of the major betel vine growing district in Bangladesh but the production of betel vine is decreasing rapidly due to different fungal diseases. Foot rot disease caused by *S. rolfsii* is one of the major disease of betel vine which spread widely all over the year.The present project has been undertaken for the first in Bangladesh for detail investigation on the biology of *S. rolfsii* specially about morphological and moleculer characteristics and its control with multidisciplinary approach.

The incidence of foot rot disease of betel vine at different boroj of Northern Parts of Bangladesh were survyed during June2011-Dcember 2013. From the survey result it was observed the highest disease incidence was recorded in Mohonpur area in 2011-2012 cropping season. On the other hand 2012-13 cropping season the highest disease incidence was recorded in Baghmara area.Total 22 isolates were isolated from the foot rot affected portion of plants from betel vine boroj at different locations.Pathogenicity test of the isolates were conducted by artificially inoculating of betel vine plantlet and disease severitywas measured. Theheighest disease severitywas recorded in isolate BA-1 (Baghmara-1). These isolates were identified as *Sclerotiumrolfsii*following the standard refference. Morphological diversity was observed among the isolates by studingmycelial growth rate, number, shape, size and color of sclerotia.The isolates of *S. roifsii* varied in all of the characters.Out of twenty two isolates the highestmycelal growth rate was recorded in BA-1 isolate and the lowest was in Du-1. The sclerotiaof these isolates were mostly round and oval in shape. The number of sclerotia produced by different isolates were varied and most of the sclerotia were dark brown.Combined morphological data of twenty two characters were analyzed by MVSP and the results showed six groups at 62% similarity level.Moleculer characterization of these 22 isolates of *S. rolfsii*was conducted with RAPD PCR technique using RAPD-1, RAPD-2, RAPD-3 and RAPD-4 primer. Dendogram constructed using PAST software which separated the isolates into two groups and each groups further divided by different sub groups.

Representative isolates of betel vine were identified as *S. rolfsii* (teleomorph: *Atheliarolfsii*) based on phylogenetic analysis of the internal transcribed spacer (ITS). According to the multiple sequence analysis of the six isolates under the present study it is suggested that there is a genetic variability prevailing among the *S.rolfsii* isolates in Bangladesh.

In the present study different types of fungicides, plant extracts,bio-agents and amended soil were evaluated against the growth of *S.rolfsii*in *in vitro* condition.Total eight fungicides were evaluated and out of them the most effective fungicides were DithanM-45 and bavistin which showed hundred percent inhibition at different concentrations. Out of twelve medicinal plants *D.metel* and *L.inermis* were most efective at all concentrations.On the other handethanol extract of *L.inermis* showed hundred percent inhibition at all the concentrations and in case of aceton extracts*L. inermis,* and *D. metel* showed hundred percent inhibition. Among the six antagonists the maximum inhibition percentage was observed in *T. harzianum*forboth dual culture and poison agar method. The Effect of organic amended soil on germination and mycelial growth of *S. rolfsii*were tested by two methods i.e surface culture method and pot culture method. In surface culture method, the maximum sclerotiagermination was observed inT_1 (sawdust amended soil) and T_{12}(control) and the lowest was T_4(cowdung amended soil). There was no sclerotia germination was observed in T_9(neem leaf dust*)* and T_{10}(datura leaf dust)amended soil. In pot culture, it was observed that the highest germination of sclerotia of *S. rolfsii*was recorded in T_{12} (untreated control) and T_1 (control) and the lowest was T_{11}(henna leaf dust)after 10 days of incubation in soil. On the other hand the highest mycelial length of *S. rolfsii*was recorded in T_{12}(control) and T_1(sawdust) after 10 days of incubation in soil and hundred percent inhibition was recorded in T_9 (neem leaf dust) and T_{10} (datura leaf dust) after 40 days of incubation in soil. From the result it was observed that neem leaf dust was very effective amendment. Many research has been done from the different parts of neem but no reports are available in previous literature about the use of neem leaf dust. On the other hand datura leaf dust was another effective amendment in both technique as surface culture and pot culture. This two plant leaf dust was completely inhibit the germination of *S. rolfsii.* In this study *T.*

harzianum also showed better performance against the pathogen. Sawdust, ash, rice husk were not so effective to control sclerotial germination and mycelial growth of *S. rolfsii.* From the results it is revealed that organic amendmentsdatura and neem leaf dust were more effective thus may be usefor field trial to manage foot rot disease of betelvine.

In*in vivo* experiment it was observed that all the treatment showed better performance compared to control. Among the fungicides the most effective fungicide wasbavistin because it increased all yield contributing characters and decreased foot rot disease incidence.*A.indica*extracts alsoshowed better performence for all yield contributing characters such as plant height, number of leaves /plants, leaf weight, leaf yield and reduced the incidence of foot rot disease. *T. harzianum*was the most effective because this strain increased all yield contributing characters and decreased disease incidence. In this study four types of soil amendments were evaluated and out of them neem leaf dust showed better performance because it increased leaf yield and decreased the incidence of foot rot disease of betel vine. In case of integrated disease management combined treatmentT_6 (bavistin+neem leaf extracts + neem leaf dust +*T.harzianum)* performed best results to control foot rot disease of betelvine.In phytochemical analysis of treated betel leaf it was observed that the highest number of phytocompounds was showed in T_6 (bavistin + datura leaf extracts + neem leaf dust + *T. harzianum*) treated leaf and the lowest (16) was in foot rot affected leaf. On the other hand the highest percent area of prolin was observed in T_6(bavistin + datura leaf extracts + neem leaf dust +*T. harzianum)* treated. *Piper betle* contains a wide variety of biologically active compounds whose concentration depends on the variety of the plant, season and climate.From the results it is revealed that the number and the amount of phytocompounds were varied with the treatments. It was remarkly noticed that the amount of amino acids which are vital compounds of the plants are gradually reduced in foot rot infected leaf but raised in combined treatment T_6(bavistin + datura leaf extracts + neem leaf dust + *T. harzianum*. Hence it may be concluded that integrated management is fruitful means to control of foot rot disease of betel vine and as well as to improved phytoconstituents of the betel vine leaf.

From the results it may be concluded that revealed that the isolates of *S. rolfsii*in Northern Parts of Bangladesh are morphologicallyvaried and showed distingly six groups. Molecular characterization also supports the morphological results when the isolates were tested by ITS region sequencing. In *in vitro* control fungicides bavistin and dithan M-45, *Daturametel , Lowsoniainermis*leaf extracts, neem and datura leaf dust amendment completely inhibited the pathogen. On the other hand in boroj condition integrated treatment T_6 (bavistin + datura leaf extracts + *T. harzianum* + neem leaf dust) showed better performance as contributing maximum yield and less disease incidence.Phytochemical analysis of betel vine leaf also indicate that the integrated treatment enriched the amount phytoconstituents of the plants. Hence, Integrated management may be fruitful means to control of foot rot disease of betel vine and as well as to improved phytoconstituents of the betel vine leaf.

CHAPTER -7

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Appendix

Appendix 8.1: Morphological Characteristics of *S. rolfsii*isolates

	Isolates																					
Morphological characters	BA-1	CH-1	BA-2	BA-3	BA-4	BA-5	BA-6	MA-1	MA-2	MO-1	MO-2	PO-1	PO-2	DU-1	DU-2	PU-1	PU-2	N-1	N-2	PA-1	CH-2	PA-2
Mycelial color on PDA	3	1	3	1	3	1	3	1	1	1	1	1	3	1	1	1	1	3	3	3	3	3
Mycelial growth on lid	3	1	3	1	1	1	1	2	2	1	2	2	1	2	1	2	2	1	1	2	1	3
Arial mycelial quantity	3	2	5	2	3	1	1	3	2	1	2	2	3	1	3	1	2	1	3	3	2	1
Fluffy mycelium	1	1	0	1	0	0	1	1	0	0	0	0	0	1	1	1	0	1	0	1	1	1
Quantity of Fluffy mycelium	1	3	2	1	2	1	3	1	2	2	2	1	3	1	2	2	2	1	2	3	2	3
Color of sclerotia	5	5	5	5	1	8	6	6	1	7	1	1	7	7	6	5	1	7	1	5	5	5
Quantity of sclerotia	3	3	1	2	2	1	2	3	2	2	3	3	3	1	2	2	2	2	1	3	2	2
Topography of sclerotia	1	1	4	1	1	1	1	1	2	1	1	2	1	2	1	1	1	2	1	1	1	1
Shape of sclerotia	4	3	2	1	1	3	3	4	3	3	1	3	5	3	1	3	3	1	1	1	4	3
Superficial Sclerotia (ss)discrete(<1mm)	2	4	2	2	2	3	4	4	1	3	4	4	4	2	2	3	2	4	2	4	2	3
SS discrete(>1mm)	3	3	1	3	3	1	1	3	1	3	3	3	3	1	1	1	3	3	1	3	2	2
SS discreate (2mm)	1	1	3	1	1	1	1	2	2	1	1	2	2	1	1	2	1	1	0	1	2	1
SS discrete(>2mm)	0	0	0	1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	0	0	1	1
SS aggregated	0	0	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	1	0	1	1	0
SS Scattered	3	1	1	3	3	1	1	1	3	1	1	3	3	1	1	1	2	1	1	1	1	1
SS near inoculum	1	2	3	2	2	1	1	2	1	1	1	2	1	2	2	1	1	2	1	2	1	2
SS near margin	2	1	2	1	1	1	2	1	3	3	2	3	3	0	1	1	1	3	2	2	1	1
SS dispersed on whole colony	2	3	1	3	3	2	2	1	1	2	3	3	3	1	1	2	2	3	1	3	2	2
Exudate droplets on sclerotium surface	3	1	1	1	2	1	2	2	1	2	1	2	1	2	2	1	1	2	1	2	1	1
Colony reserve pigment	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	1	1	1
Pseudo sclerotia	2	3	1	1	1	1	2	2	1	1	1	1	3	2	1	1	1	1	1	1	3	3
Sclerotia on lid	1	1	2	1	2	1	1	1	2	1	2	2	1	2	1	1	2	2	2	2	1	2
Zonation	1	1	1	1	1	1	1	1	2	1	1	2	2	1	1	1	2	2	2	1	1	1
Zonation type	2	2	2	2	2	2	2	2	1	2	3	1	1	3	2	2	1	1	1	2	3	2

(Data were taken as follow as Table- 3.2)

Appendix 8.2: Somatic Compatibility and Incompatibilityreactions among the isolates of *S.rolfsii* in all possible combinations.

*** NB = No barrage zone and B = Barrage zone present**

Isolate	BA-1	CH-1	BA-2	BA-3	BA-4	BA-5	BA-6	MA-1	MA-2	MO-1	MO-2	PO-1	PO-2	DU-1	DU-2	PU-1	PU-2	N-1	N-2	PA-1	CH-2	PA-2
BA-1		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
CH-1			B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
BA-2				B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
BA-3					B	B	B	B	B	NB	B	NB	B	NB	B	NB	B	NB	B	B	NB	B
BA-4						B	B	B	B	B	B	B	B	B	B	B	NB	B	B	B	B	B
BA-5							B	B	B	NB	B	B	NB	NB	B	B	B	B	B	NB	B	NB
BA-6								NB	B	B	B	B	B	B	B	NB	B	B	B	NB	B	B
MA-1									B	B	B	B	NB	B	B	B	B	NB	B	B	NB	B
MA-2										NB	B	B	B	B	NB	B	B	B	NB	B	B	B
MO-1											B	NB	B	B	B	B	NB	B	B	B	NB	B
MO-2												B	B	B	NB	B	B	B	NB	B	B	NB
PO-1													NB	B	B	B	NB	B	B	NB	B	B
PO-2														NB	B	B	B	B	B	B	B	B
DU-1															B	B	NB	B	B	B	B	B
DU-2																B	B	B	B	B	NB	B
PU-1																	NB	B	NB	B	B	B
PU-2																		B	NB	B	B	NB
N-1																			NB	B	B	B
N-2																				B	B	NB
PA-1																					B	B
CH-2																						B
PA-2																						

Appendix 8.3: Genetic similarity index between Paris of isolated in RAPD analysis

Isolate	BA-1	CH-1	BA-2	BA-3	BA-4	BA-5	BA-6	MA-1	MA-2	MO-1	MO-2	PO-1	PO-2	DU-1	DU-2	PU-1	PU-2	N-1	N-2	PA-1	CH-2	PA-2
BA-1	0																					
CH-1	1	0																				
BA-2	0	1	0																			
BA-3	1	0	1	0																		
BA-4	1	0	1	0	0																	
BA-5	0	1	0	1	1	0																
BA-6	1.41	1	1.41	1	1	1.41	0															
MA-1	0	1	0	1	1	0	1.4142	0														
MA-2	1	1.41	1	1.41	1.41	1	1	1	0													
MO-1	0	1	2.5	1	1	0	1.41	0	1	0												
MO-2	0	1	1.1	1	1	0	1.41	0	1	0	0											
PO-1	0	1	0.2	1	1	0	1.41	0	1	0	0	0										
PO-2	0	1	0.1	1	1	0	1.41	0	1	0	0	0	0									
DU-1	0	1	0.9	1	1	0	1.41	0	1	0	0	0	0	0								
DU-2	1	1.41	1	1.41	1.41	1	1.73	1	1.41	1	1	1	1	1	0							
PU-1	1.41	1.73	1.41	1.73	1.73	1.41	2	1.41	1.73	1.41	1.41	1.41	1.41	1.41	1	0						
PU-2	1.41	1.73	1.41	1.73	1.73	1.41	2	1.41	1.73	1.41	1.41	1.41	1.41	1.41	1	0	0					
N-1	1.41	1.73	1.41	1.73	1.73	1.41	1.41	1.41	1	1.41	1.41	1.41	1.41	1.41	1	1.41	1.41	0				
N-2	1	1.41	1	1.41	1.41	1	1.73	1	1.41	1	1	1	1	1	1.41	1	1	1.73	0			
PA-1	1.73	2	1.73	2	2	1.73	2.23	1.73	2	1.73	1.73	1.73	1.73	1.73	1.41	1	1	1.73	1.41	0		
CH-2	1	1.41	1	1.41	1.41	1	1.73	1	1.41	1	1	1	1	1	1.41	1.73	1.73	1.73	1.41	2	0	
PA-2	1.41	1.73	1.41	1.73	1.73	1.41	2	1.41	1.73	1.41	1.41	1.41	1.41	1.41	1	0	0	1.41	1	1	1.73	0

Appendix8.4 :*Sclerotium* species and ITS sequences Gene Bank Accession number used in this study

Species	Isolate	Host	Gene Bank Accession No
S rolfsii	CH-1	Betel vine	MH514002
S.rolfsii	CH-2	Betel vine	MH514003
S.rolfsii	DU-1	Betel vine	MH513999
S.rolfsii	BA-1	Betel vine	MH514000
S.rolfsii	BA-3	Betel vine	MH514001
S .rolfsii	MA-1	Betel vine	MH514004
A.rolfsii	NM	Onion	MH382882
A.rolfsii	Sc-03	Sweet potato	KX186998
A.rolfsii	QNGC17T1	*groundnut*	HQ895937
A.rolfsii	SR001	*Commelinacommunis*	HQ420816
A.rolfsii	MHGNU F118	*Arctiumlappa*	KU760984
S.delphinii	ZM130296	*Lonicera japonica*	KJ145328
S.delphinii	CICR-NGP	*Gossypiumhirsutum*	KC565737
S.coffeicola	CBS115.19	*Coffeasp*	AB075319
S.coffeicola		*Annona sp.*	MF170962
S.coffeicola	SC1	NM	KP176676
S.hydrophilum	WIN(M)723	NM	DQ875597
S.hydrophilum	Msh1	Rice	FJ595944
S.rhizodes	CBS126.13	NM	FJ231394
S. rhizodes	CBS321.68	NM	FJ231395
S. rhizodes	CBS276.69	NM	FJ231393
S.cepivorum	NKI 286	*Allium cepa*	FJ460433
S.denigrans	CBS396.54"	NM	FJ231405
S.perniciosum	CBS275.93	NM	FJ231408
S.perniciosum	CBS274.93	NM	FJ231407
S.perniciosum	CBS268.30	NM	FJ231406
R.solani	ATCC 10183"	NM	DQ369819