INVESTIGATION ON PESTALOTIA LEAF SPOT OF BETELVINE (*Piper betle* L.) AND ITS MANAGEMENT

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INVESTIGATION ON PESTALOTIA LEAF SPOT OF BETELVINE (*Piper betle* L.) AND ITS MANAGEMENT

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This is to certify that the thesis entitled**, "INVESTIGATION ON PESTALOTIA LEAF SPOT OF BETELVINE (*Piper betle* L.) AND ITS MANAGEMENT"** submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in PLANT PATHOLOGY** embodies the result of a piece of bona fide research work carried out by Registration No**. 18-09236** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma elsewhere in the country or abroad.

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

Dated: 15 September, 2020
Place: SAU, Dhaka, Bangladesh

…….....
Prof. Dr. Md. Rafiqul Islam
Supervisor

Dedicated
to
My Beloved Parents

List of Abbreviations of Technical Symbols and Terms

Full Words	**Abbreviation/ Symbol**
Agro-Ecological Zone	AEZ
And	&
And others	*et al.*,
Bangladesh Bureau of Statistics	BBS
Centimeter	cm
Commonwealth Mycological Institute	CMI
Complete Randomized Design	CRD
Coefficient of Variance	CV
Days After Inoculation	DAI
Degree Centigrade	^{o}C
Duncan's Multiple Range Test	DMRT
Emulsifiable Concentrate	EC
Gram	g
Hectare	ha
Journal	*J.*
Kilogram	kg
Least Significant Difference	LSD
Litre	L
Metric Ton	MT
Millimeter	mm
Muriate of Potash	MoP
Namely	viz.
Negative Logarithm of Hydrogen Ion Conc.	p^{H}
Parts per million	ppm
Percentage	%
Percent Disease Index	PDI
Percentage of Disease Incidence	% DI

Per Square Inch	PSI
Potato Dextrose Agar	PDA
Randomized Complete Block Design	RCBD
Sher-e- Bangla Agricultural University	SAU
Sodium Hypo chloride	NaOCl
That is	i.e
Triple Super Phosphate	TSP
Water Dispersible Granule	WDG
Wettable Powder	WP

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ABSTRACT

Betelvine (*Piper betle* L.) is an important cash crop grown throughout the year in Bangladesh. Isolation and identification of the causal organism, pathogenicity test and development of management practices against *Pestalotia* sp. were the main purposes of the investigation. The *in vitro* experiments were conducted at MS Laboratory, Department of Plant Pathology and the field experiment was conducted in the Central Farm of Sher-e-Bangla Agricultural University (SAU), Dhaka-1207. *Pestalotia* sp., the causal organism that caused leaf spot of betelvine is a new record in Bangladesh. A survey was conducted in four upazilas viz. Kaligonj, Debhata, Shymnagar under Satkhira district and Debidwar upazila under Cumilla district to investigate the prevalence of *Pestalotia sp.* causing pestalotia leaf spot of betelvine. The survey study revealed that the disease incidence of pestalotia leaf spot of betelvine ranged from 8.70% to 42.77%. The maximum 42.77% disease incidence was recorded in Kaligonj, Satkhira and the minimum 8.70% disease incidence was recorded in Debidwar, Cumilla. The maximum 13.66% disease severity was recorded in Shymnagar, Satkhira where minimum 2.65% disease severity was recorded in Debidwar, Cumilla. The organism isolated from the diseased leaf was identified as *Pestalotia* sp. based on its characteristics (CMI Description no. 515) and the pathogenicity was confirmed by following Koch's Postulates. In the disease mitigation study, four chemical fungicides viz. Autostin 50 WDG, Tilt 250 EC, Goldton 50 WP and Dithane M-45, four plant extracts viz. Garlic clove extract, Ginger Rhizome extract, Onion bulb extract and leaf extract were evaluated. The fungicide Tilt 250 EC and Autostin 50 WDG @500ppm concentration completely (100%) inhibit the mycelial growth of the pathogen in *in vitro.* In the field condition, maximum 81.74% reduction of disease severity over control was recorded in case of Tilt 250EC, followed by Autostin 50 WDG (67.83%). In case of plant extracts, Garlic clove extract resulted the highest reduction 86.68% of mycelial growth at 1:1 concentration in *in vitro* and the reduction of disease severity was 60.87% over control in field experiment. In case of 1:2 concentration, the reduction of mycelial growth was less than 1:1 concentration.

CHAPTER-1
INTRODUCTION

CHAPTER I

INTRODUCTION

Betelvine (*Piper betle* L.) is the most important and useful horticultural crop in Bangladesh. The deep green heart-shaped leaves of betelvine are perennial, dioecious evergreen creeper belongs to the family Piperaceae and grown in tropical and sub-tropical regions in the world. The betel leaf is familiarly known as 'pan' is cultivated largely for its leaves, which is an important cash crop of Bangladesh and mainly used for chewing. Betelvine is a climbing plant with shiny, broadly ovate and heart-shaped with bleaching quality, softness, pungency and aroma. Betelvine plants have two types of branches namely, orthotropic (vegetative) and plagiotropic (reproductive) branches. The stems are semi-woody, climbing by many short adventitious roots arising from the nodes.

Betelvine leaves are chewed along with areca nut as a masticator in social, cultural and religious events for hospitality. Usually, the people of South-Asia, Southeast Asia, Gulf states and Pacific islands chew betel leaves. All classes of people of Bangladesh chew betel leaf not only as a habit but also as an item of rituals, etiquette and manners.

Betelvine leaves have various medicinal values and also used as an antiseptic. Betel leaves are also known for its medicinal attributes containing some vitamins, enzymes, thiamine, tannin, iodine, iron, calcium, riboflavin, minerals, protein, essential oil and medicine for liver, brain and heart diseases (Chopra *et al.*, 1956). The leaves are found to contain enzyme diastase and catalyzes and vitamins A and C. It is supposed to be tonic to the brain, liver and heart in human beings. Betel leaf is also anti-rheumatic, antirhodant, anti-cough and antinoctornel emission. Volatile oil extract from betel leaf has been found to have antiseptic (Chattopadhyay, 1967).

Betelvine is grown under tropical conditions where humidity and temperature do not fluctuate abnormally and high humidity with moderate sunshine prevails

throughout the year. Geographically, it belongs to the region bounded by 68° E to 118° W longitudes and 30° N to 12° S latitudes. It is grown from sea level to an altitude of about 900 m (Chaurasia, 2001). It is grown in the area where rainfall about 2250 – 4750 mm, relative humidity and temperature ranging from 40 – 80% and 15 – 90^{o}C, respectively (Guha and Jain, 1997).

Bangladesh is the second-largest betelvine growing country in the world. The total cultivated area under the crop in Bangladesh in 2016-17 was about 23,813 ha and the total annual production was about 2,14,252 metric tons. The average yield per hectare is 9.00 metric tons (BBS, 2017). Pan leaf is usually plucked in the month of *Kartik*, *Phalgun* and *Ashar*. The Kartik pan is considered by consumers to be the best and Ashar pan the worst.

There are about 100 varieties of betel leaf (pan) across the world of which 40 are encountered in India and 30 in West Bengal and Bangladesh (Guha, 1997). A several numbers of betelvine cultivars viz., Desi Bangla, Bangla, Kali Bangla, Jhali, Sanchi, Goyeshi, Bhabna, Mitha, Geso, Bonhoogly, etc. are found in Bangladesh. The most probable place of origin of the pan is Malayasia (Chattopadhyay and Maity, 1967). 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 of Bangladesh. It is an important economic crop in Bangladesh and exported to many countries of Asia and Europe including India, Pakistan, Saudi Arabia, United Arab Emirates, England, Italy and Germany (Khaleque, 1998). Export quality betel leaves are presently grown in the districts of Natore, Kushtia, Rajshahi, Barisal, Khulna and Chuadanga. In the year of 1974-75 and 1991, Bangladesh started exporting of betel leaf in Europe and Saudi Arabia, respectively.

In Bangladesh, the betelvine is cultivated basically under an artificially erected structure, that locally known as Baroj, Bareja or Bheet. The baroj is a kind of garden which fenced by jute straw and the roof is covered by coconut leaf or wheat/rice straw on a framework of bamboo. To cultivate the betel vine, low

light intensity, mild temperature (10°C to 30°C), high humidity with moderate sunshine & 1450-1700 mm rainfall and frequent irrigation are needed throughout the year.

Different diseases are the prominently known limiting factors in betelvine cultivation (Rahman, 2017). The betelvine is highly susceptible to diseases, pests and natural calamities (Sayeeduzzaman, 1988). Environmental factors play an important role in the development of disease as they help the pathogen for growth, dissemination and infection as well as influences on the expression of susceptibility or resistance of the host plant after infection (Walker, 1965). Report reveals that among the environmental factors, temperature, humidity and rainfall are the most crucial for the development of leaf spot of betelvine (Maiti and Sen, 1982).

Among the diseases of betelvine, leaf spot is the most destructive disease which creates a spot in leave and decreases the production of betel leaf to a great extent. Leaf spot of betel vine was first identified by Roy (1948) in Bangladesh. It is also known as anthracnose of 'Pan'. The epidemic form of the disease in the country has also been reported (Hossain *et al.*, 1986). It may cause 10-60% yield loss (Maiti and Sen, 1982) and reduces the market value of the crop.

Leaf spot appears light to dark brown surrounded by diffuse chlorotic yellow halo and marginal leaf tissue becomes black, necrotic and gradually spreads towards the leaf centre. In the anthracnose stage circular, black lesions that occur rapidly increase in size and girdle the stem culminating in the death of the vine.

Colletotrichum capsica, *C. gloeosporioides* and *C. piperis* were reported as the causal agents of leaf spot disease of betelvine (Huq, 2011). In course of a survey of plant diseases prevalent in the crop, leaf spot disease caused by *Pestalotia* sp. was observed in two districts including Satkhira and Cumilla during 2019.

Pestalotia sp. is common in tropical and temperate regions, usually occur as saprobes or endophytes and may cause plant disease (Maharachchikumbura *et al.*, 2011). Acervuli are formed dark, disc or cushion-shaped under the plant epidermis which then splits open revealing the fruiting structures. Conidia are produced conidiophores within the acervulus. Conidia are divided into multi-celled with usually three darkly pigmented center cells and clear pointed end cells. Conidia are ellipsoid or fusoid (football-shaped). *Pestalotia* has two or more clear, whisker-like appendages arising from the end cell. *Pestalotia* was carried out the sections quadriloculare, quinqueloculatae and sexloculatae for 4-celled conidia, 5-celled conidia and 6-celled conidia, respectively (Guba, 1961).

Pestalotia sp. is very difficult to control even by the use of chemical fungicides. Some fungicides such as Tilt 250 EC, Autostin 50 WDG, Goldton 50 WP, Dithane M-45, etc. have been recommended to use against control of leaf spot pathogen (Khalequzzaman, 1998).

The continuous and indiscriminate uses of chemicals result in the accumulation of harmful chemical residues in the soil, water and plants. In a developing country like Bangladesh, farmers are illiterate and they rarely follow the proper handling of chemicals, which created health hazards. The injudicious use of chemicals not only hazardous to living beings but also hamper the natural ecological balance by killing the beneficial and/or antagonist microorganisms. The continuous and spontaneous chemical application also induced the development of resistant isolates of the pathogens, which sometimes become more virulent. Hence, efforts have to be made to retain pathogen activity below the economic threshold level by choosing alternatives to chemical pesticides.

Botanical extracts are biodegradable and their use in crop protection are a sustainable alternative. It reduces environmental contamination and health hazards (Grange and Ahmed, 1988). Research on the bio-active ingredients, application methods, rate of fungicides and environmental impact of botanical fungicides is a pre-requisite for sustainable agriculture. Few works have been done by using Onion, Ginger, Neem, Garlic, and some other plant extracts to

control some fungi. Different natural biocides also used separately or in combination with plant extracts to control some fungi. Antifungal activities of Garlic, Neem, Onion, Zinger have been reported by many researchers against plant pathogens (Islam, 2005).

At present, betelvine has a worldwide market. But in competition with India and other betelvine producing countries, Bangladesh has a very small share of the world betelvine market for lower production of quality betelvine due to the various insect pests and pathogenic attack of this export-oriented crop (Goswami *et al*., 2002).

In Bangladesh, leaf spot of betelvine is a major disease and the incidence of the disease is increasing day by day. The betelvine growers are presently discouraged to cultivate betelvine as they have no suitable approach for controlling the disease of betelvine. A huge amount of betel leaf in different 'Baroj' become ruined every year due to the severe attack of leaf spot disease. If such a situation is continued, betelvine cultivation would face a great threat and the country will loss a huge income of foreign currency. Thus, the problem needs to give argent attention.

Based on the above facts the present piece of research has been undertaken to achieve the following Objectives:

1. To find out the disease prevalence of leaf spot of betelvine.
2. To isolate, identify and test of pathogenicity of the causal pathogen(s) of leaf spot of betelvine.
3. To find out the management options for pestalotia leaf spot of betelvine.

CHAPTER-2
REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Pestalotia sp., the causal organism of betelvine is a new record in Bangladesh and therefore no literature is available on its incidence, severity and management in the country. However, infection of this pathogen has been reported on a number of other hosts and attempts have been made to collect literature on this fungus. The present review includes an overview on epidemiology, incidence, severity, method of inoculation and management causing leaf spot disease of betelvine. In addition, other relevant reports are also included in this chapter.

2.1. History of the pathogen

Mohanty (1980) first reported that leaf spot of betelvine was caused by *Pestalotia* sp. in India and it was the serious pathogen for crop losses.

De Notaris (1839) introduced the genus *Pestalotia* De Not. based on the generic type *Pestalotia pezizoides* De Not., which occurred on the leaves of grape plants (*Vitis vinifera*) in Italy.

According to Menon and Pandalai (1958), grey leaf blight of coconut caused by *Pestalotiopsis palmarum* is a wide spread disease found in almost all the coconut growing countries of the world. The disease was first reported from British Guyana and later from Malaysia, New Hebrides, Sri Lanka, India, Trinidad, etc.

Pestalotia sp. is a common pathogen in tropical and temperate regions, usually occur as saprobes or endophytes and may cause plant disease (Arrhenius and Langenheim, 1986; Maharachchikumbura *et al.*, 2011). *Pestalotia* sp. is primarily a secondary pathogen. It is saprophytic on dead and dying tissues and is weakly parasitic infecting wounds under moist conditions.

Amrutha and Reshmy (2018) reported that leaf spot/blight disease caused by *Neopestalotiopsis clavispora* was a major problem in various strawberry

growing areas in India and caused mild to severe damage to plants and also reduced fruit quality and market value.

According to Feng *et al*. (2014), *Pestalotia* sp. was the causal agent of leaf blotch in *Rosa chinensis*, in China. Mahadevakumar & Janardhana (2014) stated that *Pestalotiopsis* sp. causing leaf spot in *Vigna unguiculata* in India.

Rahman *et al.* (2013) found that grey leaf spot disease caused by *Pestalotia palmarum* (Cooke.) attacks the gardens and decreases the growth and development of the tree as well as the yield of the fruit.

According to Kowalik (2013), the symptoms usually include discrete spots with tan to brown centers surrounded by a darker border. Leaf spot is a common disease of azalea which is caused by *Pestalotia* sp. A comparison of *P. sydowiana* (syn. *Pestalotia sydowiana*) and *Truncatella truncata* (syn. *Pestalotia truncata*) in causing necrotic symptoms on the leaves of azalea and evergreen rhododendron leaves.

Lazarotto *et al.* (2012) stated that a new type of leaf spot disease associated to *Pestalotiopsis* genus fungi has been occurring in the state of Rio Grande do Sul, Brazil. In severe cases, it culminates in fallen leaves and nut production losses, due to a decrease of photosynthetic area.

Chuanqing *et al*. (2010) observed that *P. microspora* is a causal agent of nut black spot in *Carya cathayensis*.

According to Dayan (2004), Leaf spot caused by *Pestalotia* sp. was a common disease of Narra (*Pterocarpus indicus* Wild), an endemic tree species in the Philippines and also associated with other diseases of forest trees such as seed disease in *Agathis philippinensis*, *Eucalyptus deglupta* and *Acacia mangium.*

Islam *et al*. (2004) reported that naturally infected leaf samples of betelnut having characteristic symptoms of spots caused by *Pestalotia palmarum* was noticed from the sample collected from the campus of Khulna University, Bangladesh.

Ghose and Dasgupta (2000) found that coconut mainly suffered from grey leaf spot or blight caused by (*Pestalotiopsis palmarum*) in 22 countries. He described distribution, alternative hosts, epidemiology, varietal reaction and management of the disease. He also reported that grey leaf spot or blight caused by *Pestalotiopsis palmarum* had no immune or resistant sources against the disease.

Sharma *et al*. (1987) and Patel and Patel (1981) reported that leaf disease of Chicku (*Achras sapota*) is caused by *Pestalotia Sapotae* L.

According to Rajendran (1971), leaf spot of sapota caused by *Pestalotia* species is the most common and serious disease in India.

2.2. Symptoms of leaf spot disease

According to Ata (2014), leaf spot caused by *Pestalotia indicus* was characterized by brown lesions in the leaf blade. Black fruiting bodies were also visible in some of the lesions.

Rahman *et al.* (2013) found that symptoms developed in the mature leaves in the form of grayish white spots surrounded by brown margin. Several spots coalesce together and form irregular grey necrotic patches and show burnt or blighted appearances. The upper surface of the affected leaves reveals dark grey eruptions like pin heads.

Joshi (2005) reported that symptoms were observed in the form of minute irregular necrotic patches of brick red color at leaf margins on cashew. These patches enlarged, turn gray-silvery in color covering the major portion of the leaf lamina. Severely blighted leaves defoliated prematurely.

Islam (2004) found the infected leaf sample was yellowish or brownish or dark spot surrounded by yellow halo. The centers of the spot were brown to black; spots were more or less circular.

Nambiar (1994) reported that in adult coconut palm, first symptom of grey leaf spot appears on the outer whorl of the lower most leaves as small yellowish-brown spots. Spots gradually become oval in shape encircled by greyish brown

band. Centre of the spot turns greyish white, brown bands darkens and gets surrounded by a yellowish halo. In advance stage spots coalesce to form large irregular necrotic patches. Leaflets shrivel, dry completely and show a burnt and blighted.

Sharma *et al*. (1987) and Patel and Patel (1981) observed symptoms appear as numerous small, reddish-brown specks on the leaf lamina. These gradually enlarge to form more or less circular spots measuring 1-3 mm in diameter. Fully developed spots have greyish center lesions.

2.3. Morphological characteristics of the Pathogen

Naeimi *et al.* (2015) reported that *Pestalotia disseminata* colony was colorless, turning cottony white with abundant scattered acervuli containing black, slimy spore masses. The black acervular conidiomata containing abundant conidia that were five-celled, straight or slightly curved, fusiform, smooth, constricted at septa. Olive to brown and end cells were hyaline; two to three unbranched appendages (setulae) on the apical cell.

Ata (2014) observed, the fungi produced a white cottony mycelium. Under the microscope, the species identified as *Pestalotia* sp. has fusiform and septate conidia with branched appendages.

According to Ismail (2013), *Pestalotia* isolates five-celled conidia were observed, of which apical and basal cells were thin-walled hyaline to pale olivaceous and the three median cells were thick-walled light to dark brown. In the first group of the isolates resembling to *Pestalotia uvicola* conidia were smooth, fusiform, straight.

Sutton (1973) separated the two genera and placed *Pestalotiopsis* in order Melenconiales and *Pestalotia* in order Sphaeropsidales under Coleomycetes.

According to Guba (1961), acervuli are formed dark, disc or cushion-shaped under the plant epidermis which then splits open revealing the fruiting structures. Conidia are produced conidiophores within the acervulus. Conidia are divided

into multi-celled with usually three darkly pigmented center cells and clear pointed end cells. Conidia are ellipsoid or fusoid (football-shaped). *Pestalotia* has two or more clear, whisker-like appendages arising from the end cell. *Pestalotia* was carried out the sections quadriloculare, quinqueloculatae and sexloculatae for 4-celled conidia, 5-celled conidia and 6-celled conidia, respectively.

Steyaert (1949) reported genus *Pestalotiopsis* was segregated from *Pestalotia* mainly on the basis of conidial septation and number of apical appendages. He accepted 45 species in *Pestalotiopsis* Sect.

De Notaris (1839) introduced the genus *Pestalotia* is characterized by 6-celled conidia with four deeply olivaceous central cells, distosepta, hyaline terminal cells and simple or branched appendages arising from the apex.

2.4. Epidemiology

According to Karthikeyan *et al*. (2002), the disease caused 10.4% reduction in height, 20.1% in leaf production and 12.5% in collar girth in the coconut seedlings.

Noriega *et al*. (1991) found that *Pestalotiopsis* leaf spot was very common in all the orchards and the fungus caused maximum damage to the pre-bearing palms.

Suryachandra selvan *et al*. (1991) reported that grey leaf blight intensity in East Coast was maximum in December (40.5%) and minimum in June (23.9%) at Vepangulam, Tamil Nadu.

Rao *et al.* (1975) found that maximum infection of *Pestalotiopsis palmarum* causing grey leaf blight of coconut occurred during August and November in Andhra Pradesh.

2.5. Environmental factors on the disease development

Xianshu and Han (1994) found the disease occurred all the year round, the leaf spot disease incidence increasing with rainfall, relative humidity and low temperature during August to December. They also reported from China that high humidity and monthly mean temperature of 17 to 24^0 C were favorable for *Pestalotiopsis palmarum* disease epidemic. They observed that high seedling density, continuous cloud, rainy weather and heavy dew triggered rapid disease spread in Hainan province of China.

Das *et al.* (1976) reported that pH 4.5 and 25^0 C temperature was most suitable for germination of *P. palmarum* spores. Conidial germination was very high 80.05% in rain water against 58.0% after 12 hours in sterilized distil water as control in an experiment with six different sources of water.

2.6. Pathogenicity of *Pestalotia* sp. isolates

Rahman *et al.* (2013) proved the pathogenicity of *Pestalotiopsis palmarum* on coconut leave. They were injured softly by flame sterilized pointed needles. Advanced hyphae and acervuli were cut from the margin of pure cultures carefully with the help of flame burned inoculation needle and placed at three places (two at both margins and one at the center) onto the injured excised leaf of the host and incubated at 27°C±1 for ten days. After fifteen days *Pestalotiopsis palmarum* produced characteristic symptoms of grey leaf spot of coconut.

Islam *et al.* (2004) observed the pathogenicity test of leaf spot caused by *Pestalotia palmarum* on betelnut. He noticed that this species was found pathogenic excised leaves on betelnut.

Cardoso *et al.* (2003) could reproduce the typical symptoms of grey leaf blight in the pathogenicity experiments in Brazil.

Balakrishnan *et al*. (1982) reported severe grey leaf blight damage on *Palmyra palm* caused by *Pestalotiopsis palmarum* and confirmed pathogenicity.

2.7. *In-vitro* evaluation of chemical fungicides against *Pestalotia* sp.

Rahman *et al.* (2013) reported that the fungicides were tested against *Pestalotiopsis palmarum* at 1000, 2000 and 3000 ppm concentrations. All fungicides at all concentrations inhibited the growth of the pathogen and their effect differed significantly (≤ 0.01). No growth was observed in Hexaconazol, Propiconazol, Hepridion and Carbendazim at any concentrations. Mancozeb 2000 ppm and Mancozeb + Metalexil 2000 and 3000 ppm was inhibited 78.67%, 79.02% and 77.62%, respectively. Mancozeb + Metalexil 1000 ppm inhibited the lowest was 60.49%.

Moshayedi (2017) reported that Mancozeb and Carbendazim at 1000, 2000 and 3000 ppm concentrations were inhibited 100% of radial growth of *Pestalotia* sp.

Islam *et al*. (2004) found that *in vitro* experiment Bavistin of three doses (100, 200, 300 ppm) and Tilt 250 EC (100 and 200 ppm) were more effective in inhibition mycelium growth of *Pestalotia palmarum.* All doses of Bavistin (100, 200, 300 ppm) and Tilt 250 EC (100 and 200 ppm) were inhibited 100% mycelium growth of *Pestalotia palmarum.* Dithane M-45 at 100 ppm was inhibited 26% of radial growth of *P. palmarum*.

2.8. *In-vitro* evaluation of botanicals against *Pestalotia* sp.

Islam *et al*. (2004) found that Garlic extract at 4 - 5% concentration were the best plant extracts, which inhibited 100% of radial growth of *Pestalotia palmarum*. Neem extract at 5% concentration inhibited 12.82% but no inhibition of radial growth was founded in case of Onion and Ginger plant extracts.

CHAPTER-3
MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1. Experimental site

3.1.1. *In vitro* Experiments

In vitro experiment was conducted at the MS Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University (SAU), Sher-e-Bangla Nagar, Dhaka- 1207.

3.1.2. *In vivo* Experiments

The *in vivo* experiment was conducted in the temporarily built betelvine garden (Baroj) at the Central field of Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh.

3.1.3. Soil type

The soil of experimental site belongs to the Agro-ecological region of ‘‘Madhupur Tract’’ (AEZ No. 28). The zone was Deep Red Brown Terrace soil and belongs to ‘‘Nodda’’ cultivated series. The top soil is silty clay loam in texture. Organic matter was very low (0.82%) and soil pH varied from 5.47 - 5.63. The information about AEZ 28 (Anonymous, 1994) is stated below:

Characteristics of AEZ-28

Land type	Medium high land
General soil type	Non-Calcareous Dark gray floodplain soil
Soil series	Tejgaon
Topography	Upland
Elevation	8.45
Location	SAU Farm, Dhaka
Field Level	Above flood level
Drainage	Fairly good
Firmness (consistency)	Compact to friable when dry

3.2. Experimental period

The experiments were conducted during the year 2018-2020. *In vivo* experiments were done all around the year starting from November 2018 to June 2020.

3.3. Experimental design

Completely Randomized Design (CRD) was exercised with 3 replications for *in vitro* experiments and Randomized Complete Block Design (RCBD) with 3 replications was used for *in vivo* experiments.

3.4. Materials

3.4.1. Equipment and instruments

The common laboratory equipment and instruments used were autoclave, hot air oven, incubator, laminar flow air chamber, refrigerator, electronic weighing balance, research microscope, physical balance, etc.

3.4.2. Glassware

Different types of Corning and Borosil glassware were used in experimental work. The common glasswares were petri-plates, test tubes, conical flasks, measuring cylinder, glass rods, coverslips, beakers, pipette, etc.

3.4.3. Other materials

Miscellaneous materials viz., marking pencils, rubber bands, sticky labels, muslin cloth, blotting paper, inoculating needle, forceps, spirit lamp, filter paper, cork borer, funnel, glass jar, thin wrapping tape, polypropylene bags, mercuric chloride and sodium hypochlorite stock solutions etc. were used.

3.5. Experiments

A set of four experiments were conducted during the study period to formulate an investigation and integrated approach for the management of leaf spot disease of betelvine**.** The experiments are as follows:

1. Study on disease incidence and disease severity of leaf spot disease of betelvine in major betelvine growing areas of Bangladesh.

2. Isolation, identification, multiplication and preservation of the pathogenic isolates of the causal pathogen that causing leaf spot disease collected from different betelvine growing regions of Bangladesh.
3. Pathogenicity study of the isolates of *Pestalotia* sp. causing leaf spot disease of betelvine.
4. *In vitro* evaluation of different treatments against radial mycelial growth of *Pestalotia s*p.

Experiment 1. Study on disease incidence and disease severity of leaf spot disease of betelvine in major betelvine growing areas of Bangladesh

3.5.1.a. Location of survey

Surveys were conducted at major betelvine growing areas of selected four upazilas of Bangladesh. The upazilas were Kaligonj, Debhata, Shymnagar in Satkhira district and Debidwar under the Cumilla district. Three betelvine gardens (baroj) of each upazila were considered for recording disease data of betelvine.

3.5.1.b. Period of survey

Survey data were collected during the late summer (August) and late winter (February) seasons of the year 2019-2020.

3.5.1.c. Data collection

In each growing upazilas, three barojes were selected for data recording. Each spot or baroj were covered an area of approx. 200 sq. m. Cultivars of betelvine available in those areas were considered for investigation. Plants were selected randomly from the central part of the baroj. Altogether 10 plants of each baroj and 30 plants of each location were randomly considered for the survey. Data were collected on the parameters mentioned below:

Disease data

a) Disease incidence of leaf spot of betelvine
b) Disease severity of leaf spot of betelvine

3.5.1.d. Calculation of disease incidence/ disease severity

The occurrence of disease in the field were calculated as follows:

$$(\%) \text{ disease incidence} = \frac{\text{Number of infected leaves in the area covered}}{\text{Number of inspected leaves}} \text{ X } 100$$

The disease severity of leaf spot was calculated as PDI (Percent Disease Index) using '0-5' scale proposed by Goswami *et al*. (2002).

PDI scale of leaf spot

% Leaf area diseased	Grade
0 (Healthy)	0
1-5	1
6-15	2
16-30	3
31-50	4
> 50	5

Percent disease index were measured by using the following formula (Islam, 2005).

$$\text{Disease severity (PDI)} = \frac{\text{Sum of total disease rating X } 100}{\text{Number of observations X Highest grade in the scale}}$$

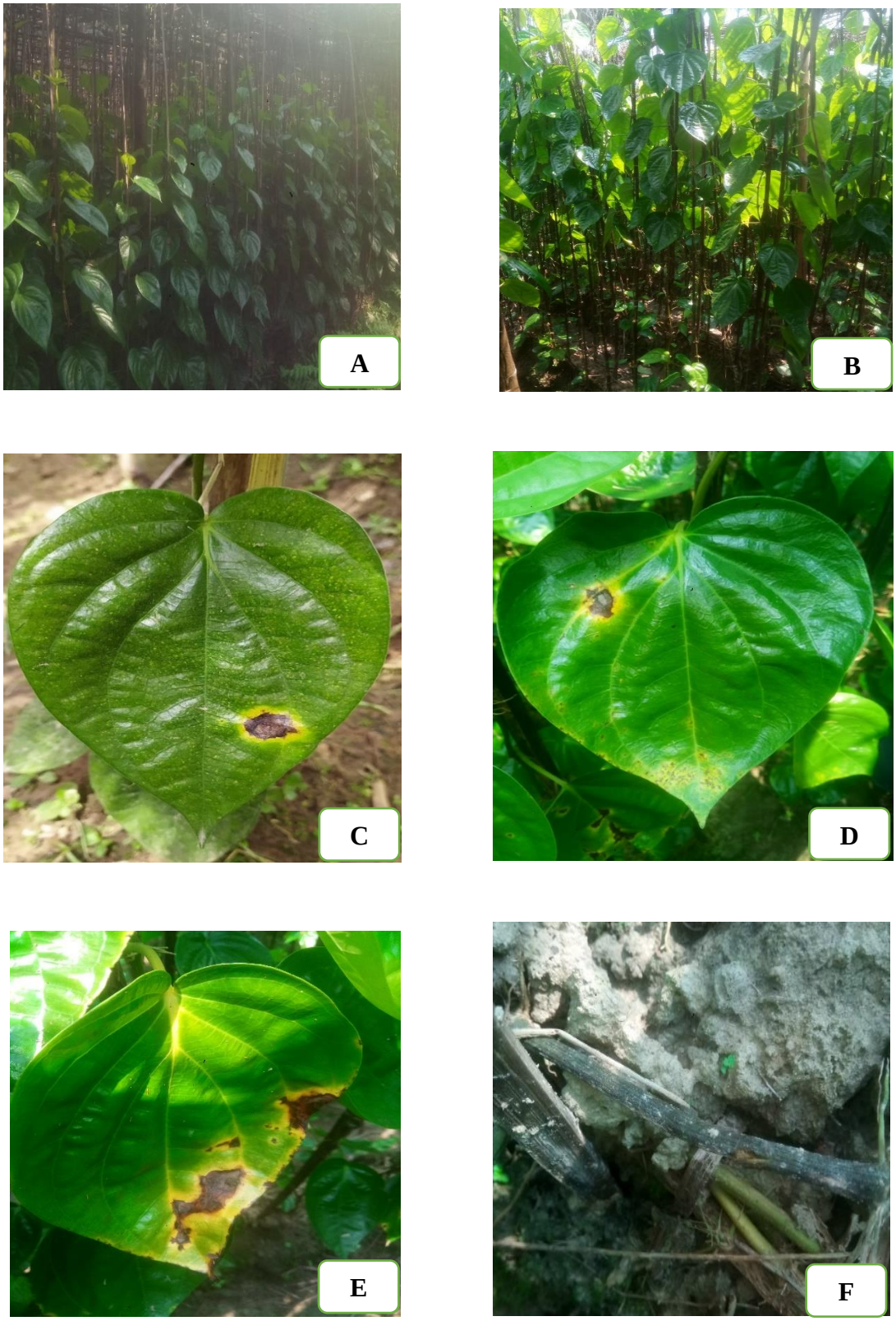


Plate 1. Orchard view of healthy betelvine (A & B), diseased betelvine (C & D) caused by *Pestalotia* sp., anthracnose (E) and foot and root diseases (F)

Experiment 2. Isolation, identification, multiplication and preservation of causal pathogen(s) of leaf spot disease collected from different betelvine growing regions of Bangladesh.

3.5.2.a. Collection of disease specimens

Affected leave samples of betelvine (*Piper betle* L.) were collected from different "Baroj" in Satkhira and Cumilla district. Collected samples were put in polyethylene bags immediately after collection to protect them from drying. Then the samples were preserved at 4°C in refrigerator.

3.5.2.b. Sterilization of materials and equipment's

Liquid materials, such as media and distilled water were sterilized in an autoclave at 121°C and 15 PSI for about 30 min. For surface sterilization, 1% sodium hypochlorite (NaOCl) was used for plant materials such as leaf and rectified spirit used for other equipment like inoculation-needles, forceps, inoculation chamber, hands etc.

3.5.2.c. Isolation of causal organism

Affected leaves samples were first washed with tap water to remove dust particles. Diseased tissues were cut into small bits of convenient size and disinfected by dipping in 1% sodium hypochlorite (NaOCl) solution for 30 seconds to 1 minutes followed by thorough washing in three changes of sterile water to remove the traces of sodium hypochlorite. The bits were then put on filter papers in sterilized petri dishes in order to absorb excessive water present on them. These bits were then plated on potato dextrose agar (PDA) medium in 90 mm petri dishes, which were already sterilized, poured and cooled under aseptic conditions. These plates were then incubated at 25°C ± 1. The petri plates were observed daily for the growth of fungi. When the growth of the fungus was observed, individual mycelial tip was carefully transferred with inoculating needle to PDA plates as well as slants and incubated at 25°C ± 1 for getting pure culture.

3.5.2.d. Identification, multiplication and preservation of the pathogen

Pure culture of the isolates was prepared following hyphal tip methods (Mian, 1995) and the individual cultures were purified by repeated sub-culturing on PDA medium in plates. Isolated fungi were sub-cultured on PDA slants and kept at 25℃ ±1 for seven days. When maximum growth was noticed, such slants were preserved in refrigerator at 4°C. The cultures were sub-cultured once in a month to maintain the viability of fungus. The fungi were then identified on the basis the morphological characteristics with the help of identifying key book (Barnett and Hunter, 1972).

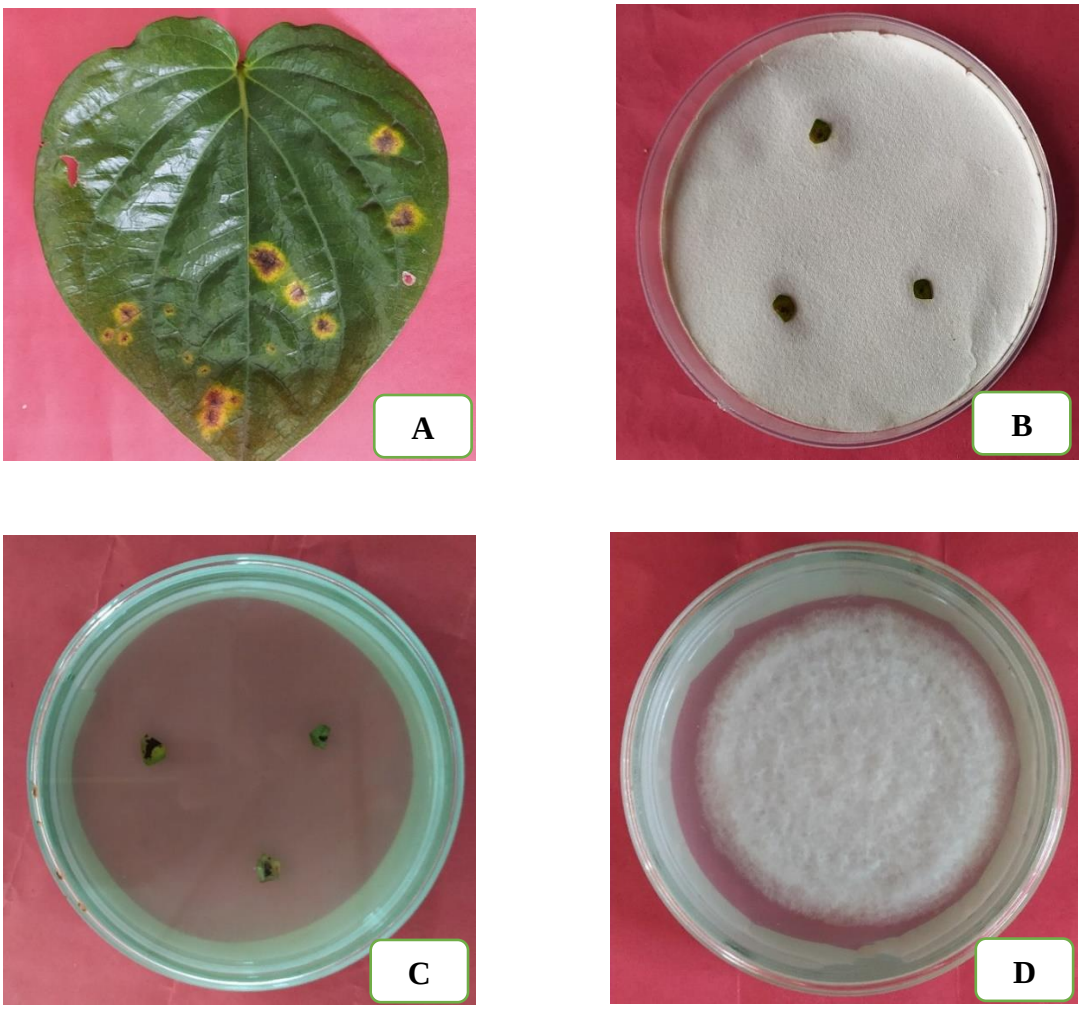


Plate 2. Leaf spot disease sample of betelvine (A), Infected leaf segment on moist blotter (B), Infected leaf segment on PDA media (C) and Pure culture of isolated *Pestalotia* sp. (D)

3.5.2.e. Preparation of Potato Dextrose Agar (PDA) media

PDA media were used to stimulate sporulation (slide preparations), maintain stock cultures of certain dermatophytes and differentiate atypical varieties of dermatophytes by pigment production (MacFaddin, 1985). Composition of PDA media preparation:

Peeled potato (decoction)	: 200g
Dextrose	: 20g
Agar	: 20g
Distilled water	: 1000 ml

At first, 200g potato was taken and cleaned followed by washing with tap water. Then the potato was peeled and cut in a small slice and boiled about 30-40 minutes in one-liter water. When potato was soft fully, it was sieved. After that, 20g dextrose and with a few minutes' interval, 20g Agar were mixed slowly with it and stirred properly so that it cannot be coagulated. Then the media was sterilized in an autoclave at temperature of 121^0C temperature under 15 PSI pressure for about 30 minutes.

After autoclaving, the media was kept 20-30 minutes for cooling in laminar air flow cabinet. Then 25-30 drops lactic acid and Phenoxymethyl Penicillin tablet (Oracyn-k) were added with the media in order to maintain slightly acidic condition for the growth of the fungus and preventing bacterial contamination. The works were done aseptically inside the laminar air flow cabinet.

Experiment 3. Pathogenicity test of the isolates of *Pestalotia* sp. causing leaf spot disease of betelvine

3.5.3.a. Inocula preparation and inoculation with the causal pathogen

The pathogenicity test was conducted by spraying spore suspension on leaves. For spore suspension preparation, fungi cultures were grown on PDA media and after 10-12 days of growth, 20 mL of distilled water were added to each plate. Then, the surface of the medium was scraped with a glass rod, and the suspension was drained into a beaker.

Pestalotia sp. isolate was tested. Inoculation was performed by spraying the suspension on leaves of betelvine. Plants remained for 72 hours in a humid chamber, under shade and irrigation was done when necessary. After symptom expression, isolation was carried out in order to confirm the pathogen identification. The assessments conducted after 10 days, and disease incidence (%) was assessed, with subsequent pathogen re-isolation in PDA medium and to identify the pathogen caused leaf spot.

3.5.3.b. Confirmation of the disease

After appearance of disease symptoms, it was necessary to confirm the disease and that is why Koch's postulate (re-isolation and identification of the pathogen) was performed following the standard procedure (Mitchell *et al*., 1997).

Experiment 4. *In vitro* evaluation of different treatments against radial mycelial growth of *Pestalotia s*p.

The efficacy of IPM components viz. chemical fungicides and botanical extracts were assayed by growth inhibition technique as follows:

1. Cup /Groove method

3.5.4.a. *In vitro* screening of fungicides against the pathogen causing leaf spot disease of betelvine

Four fungicides reported effective against *Pestalotia* sp**.** were evaluated by Growth Inhibition Technique using Cup /Groove method (Table 1, Plate 3).

3.5.4.b. Preparation of fungicides solution

Fungicide solutions were prepared dissolving required amount of water with the weighed amount of fungicide. 150 ml Erlenmeyer flask was used for each concentration. Flasks were labeled and shaken thoroughly before use.

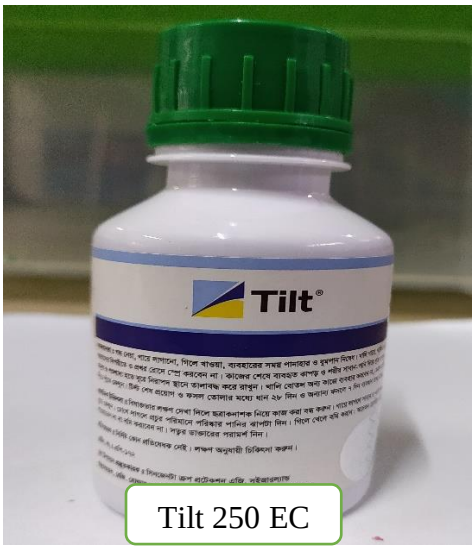
3. 5.4.c. *In vitro* screening of fungicides

Cup /Groove Method

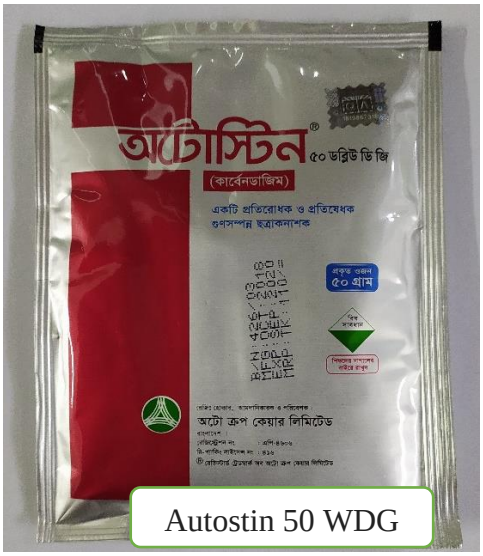
In this method, petri plates were prepared with 15 ml PDA media. After solidification, 5 mm discs of the medium were scooped from PDA plate, three places maintaining an equal distance from the centre by a sterilized disc cutter. One milliliter of chemical fungicide was put into each hole and the plates were stored overnight in laminar air flow cabinet for diffusion of the input in the medium around the hole before resumption of fungal growth. The next day, one 5 mm block of 5 days old fungal culture (pathogen) cut by sterilized disc cutter and it was placed at the centre of the plate. The plates were then placed at 25°C ± 2 in incubation chamber. The linear growth (cm) of mycelium of *Pestalotia* sp**.** was recorded at 24 hrs. interval until the control plates were filled in (Islam, 2001; Islam, 2005).

Table 1. List of fungicides used in the bioassay *in vitro* against *Pestalotia* sp.

Sl. No.	Tread name	Chemical name	Active ingredient	Conc. used
01	Tilt 250 EC	1-[2-(2,4-Dicholorophenyl4-propyle- 1,3-diooxalane-2EI-Methyl]-IH, 1,2,4-Triyazole	25% Propiconazole	100 ppm 200 ppm 300 ppm 500 ppm
02	Autostin 50 WDG	Mythyl-2-Benzimidazole Carbamate	50 % Carbendazim	100 ppm 200 ppm 300 ppm 500 ppm
03	Goldton 50WP	Copper oxychloride	50 % Copper	100 ppm 200 ppm 300 ppm 500 ppm
04	Dithane M-45	Manganous ethylene bisdithiocarbamate-ion	80% Mancozeb	100 ppm 200 ppm 300 ppm 500 ppm



Tilt 250 EC



Autostin 50 WDG

Goldton 50 WP

Dithane M-45

Concentration of different chemical fungicides prepared for evaluation

Plate 3. Different chemical fungicides used in the study against *Pestalotia* sp**.**

3.5.4.d. *In vitro* screening of botanicals against the pathogen causing leaf spot disease of betelvine

Four different plant extracts were evaluated against *Pestalotia* sp**.** were evaluated by Growth Inhibition Technique using Cup /Groove method (Table 2, Plate 4).

3.5.4.e. Preparation of plant extracts solution

The plant extracts were prepared by using the method of Islam (2005). Collected leaves/rhizome/bulb/clove were weighed in an electric balance and then washed in the running tap water. After washing, the leaves/rhizome/bulb/clove was cut into small pieces. For getting extract, plant parts were blended in an electric blender adding equal amount of sterile water for 1:1 solution (100 ml water for 100 gm plant parts). The blend was filtered through sterile cheesecloth. For getting 1:2 solution, (100 ml water for 50 gm plant parts) of distilled water was added with plant parts.

Table 2. List of the particulars of selected indigenous plant species assayed against *Pestalotia* sp.

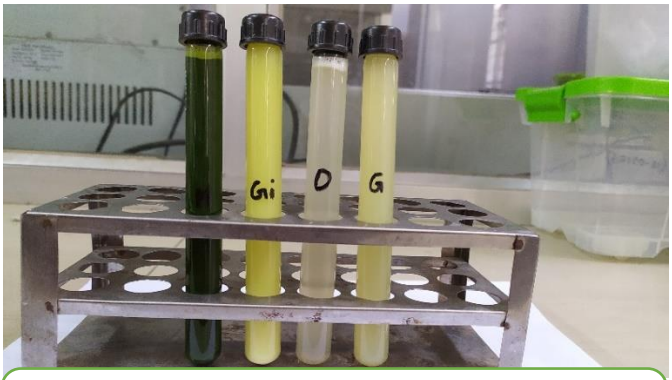
Sl. No.	Local name	English name	Scientific name	Plant parts used	Conc. used
01	Roshun	Garlic	*Allium sativum*	Clove	1:1 & 1:2
02	Ada	Ginger	*Gingiber officinales*	Rhizome	1:1 & 1:2
03	Peaz	Onion	*Allium cepa*	Bulb	1:1 & 1:2
04	Neem	Margosa	*Azadiracta indica*	Leaf	1:1 & 1:2

Onion

Garlic

Ginge

Neem



Concentration of different Plant extracts prepared for evaluation

Plate 4. Plant parts used to test antifungal activity against *Pestalotia* sp**.**

3.5.4.f. Measurement of radial growth (cm) and determination of percent Inhibition

After 24 hours of inoculation, radial growth (cm) of *Pestalotia* sp**.** in petri dishes was recorded. The mycelium growth (cm) of each plate was measured by taking average of the two diameters taken right angles for each colony. The linear growth (cm) of mycelium of the causal pathogens were recorded at 1 day's interval until the control plates were filled in (Islam, *et al*. 2001; Nene and Thaplial, 1979).

Inhibition of radial growth was computed based on colony diameter on control plate using the following formula shown below:

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

X= Average radial growth (cm) of *Pestalotia* sp. in control petri dishes.

Y= Average radial growth (cm) of *Pestalotia* sp. in treated petri dishes.

3.6. Field experiment for leaf spot disease management

3.6.1. Experimental site

The experiment was conducted in the betelvine garden (baroj) in the central field of Sher-e-Bangla Agricultural University.

3.6.2. Experimental design

The experiment was carried out with Randomized Complete Block Design (RCBD) with 3 replications maintaining plot size (3 x 1) m^2, block to block distance 50 cm, row to row distance 90 cm and plant to plant spacing 20 cm.

3.6.3. Treatments explored in the experiment

Nine different treatments comprising different fungicides and plant extracts were explored in this experiment. The treatments were stated below:

T_1 = Autostin 50 WDG

T_2 = Dithane M-45

T_3 = Goldton 50 WP

T_4 = Tilt-250 EC

T_5 = Onion bulb extract

T_6 = Garlic clove extract

T_7 = Ginger rhizome extract

T_8 = Neem leaf extract

T_9 = Control

3.6.4. Land preparation

A piece of medium high land with well drainage system was selected and deep ploughing was done during at the middle of November month. After ploughing, upper soil is left exposed in sun for one months. During the last week of December, two or three ploughing was done for well pulverized tilth condition. Weeds and stubbles were removed. Provide drainage trenches of 90 cm width by 15 cm depth in between two adjacent beds.

3.6.5. Collection of betelvines

The susceptible betelvine cultivar Lal-dinghi cutting were collected from a betelvine orchard in kishoreganj. The cuttings were forty-centimeter-long with three to five nodes.

3.6.6. Plantation of betelvine

The vine was raised in the experimental field by vegetative propagation from the cuttings under partially shaded and humid environment in the betelvine orchard which (baroj) was a small hut like structure of approximately 2-meter height. It was constructed with the locally available materials like bamboo stems, jute sticks, paddy straw and rope etc. Planting was done with the help of khurpi. For planting, a hole was made with khurpi, so that the internodes below the bud point is dipped in soil, but rest part of cutting must be touching with surface soil. The cuttings of 3-5 years old vines were planted in the furrows (8-10 cm deep). The hole was completely packed with the help of thumb finger.

3.6.7. Fertilizers and manures

Fertilizer dose was used as Urea - 130, TSP - 220, MoP - 36, Zypsum - 50 and Zinc Sulphate - 15 kg/ha. Cow-dung and Mustard oil cake used @ 20t and 6t, respectively (Haque *et al*., 2013). All the fertilizer except urea were applied during land preparation. Urea was applied in three splits at 60, 90 and 120 days after plantation. Vermi compost was also applied @ 10kg after three months of plantation.

3.6.8. Intercultural operations

Sticking of vines, irrigation and fertilization/manuring were given as per requirement of the orchard. Weeding was done as and when necessary.

3.6.9. Data collection

Data were recorded five days after interval for number of infected leaves and then converted to percent infected leaf at the end of study. The leaf showing apparent spot was considered as infected leaf and the leaf without these symptoms were considered healthy.

The data were recorded on following parameters:

i. Days required for appearance of 1st disease symptom
ii. % leaf area infection

3.7. Statistical analysis of data

Completely Randomized Design (CRD) was followed with 3 replications for *in vitro* experiments and Randomized Complete Block Design (RCBD) with 3 replications was used for *in vivo* experiments. Duncan's Multiple Range Test (DMRT) was explored for comparison of means (Gomez and Gomez, 1983). Statistical package program 'Statistic 10' was used for analysis of the experimental data.

CHAPTER-4
RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

Experiment 1. Study on disease incidence and disease severity of leaf spot disease of betelvine in major betelvine growing areas of Bangladesh

Survey experiments were conducted at major betelvine growing areas of selected four upazilas of Bangladesh. The upazilas were Kaligonj, Debhata, Shymnagar in Satkhira district and Debidwar under the Cumilla district. Three betelvine gardens (baroj) of each upazila were considered for recording disease data. Incidence (% leaf infection) and severity (% leaf area infection) were recorded.

4.1.1. Disease incidence (%) of pestalotia leaf spot of betelvine

Disease incidence of leaf spot of betelvine were significantly varied from one upazila to another upazila and one baroj to another baroj (Table-3). The maximum disease incidence 42.77% was recorded in Kaligonj upazila under Satkhira district where disease incidence ranged from 26.45% to 42.77%. The minimum disease incidence 8.70% was found in Debidwar upazila under Cumilla district where disease incidence ranged from 8.70% to 11.34%. The highest disease incidence 42.77% was found in boroj-2 in Kaligonj and the lowest disease incidence 8.70% was found in boroj-3 in Debidwar. Suryachandra selvan *et al*. (1991) reported that grey leaf spot intensity in East Coast was maximum 40.5 % in December and minimum 23.9 % in June at Vepangulam, Tamil Nadu, India.

Table 3. Disease incidence of leaf spot of betelvine in different upazilas of Satkhira & Cumilla district.

Location	% Diseases Incidence			Average
	Baroj-1	Baroj-2	Baroj-3	
Kaligonj, Satkhira	39.62 a	42.77 a	26.45 a	36.28
Debhata, Satkhira	26.21 b	34.73 ab	23.17 ab	28.04
Shymnagar, Satkhira	32.37 ab	20.89 bc	35.27 a	29.51
Debidwar, Cumilla	11.34 c	9.92 c	8.70 b	9.99
CV (%)	23.63	29.35	35.22	-
LSD (0.05)	12.93	15.88	16.47	-

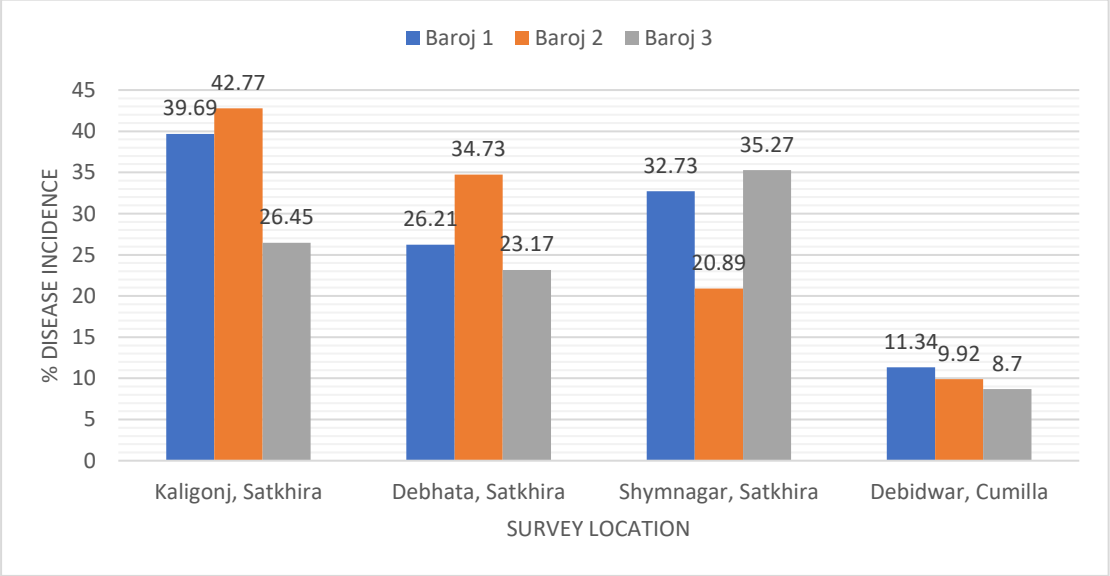


Figure 1: Mean disease incidence of leaf spot of betelvine in different upazilas of Satkhira & Cumilla district.

4.1.2. Disease severity (%) of pestalotia leaf spot of betelvine

Disease severity of leaf spot of betelvine were significantly varied from one upazila to another upazila and one baroj to another baroj (Table-4). Maximum disease severity 13.66% was recorded in Shymnagar upazila under Satkhira district where disease severity ranged from 8.11% to 13.66%. The minimum disease severity 2.65% was found in Debidwar upazila under Cumilla district where disease severity ranged from 2.65% to 3.61%. The highest disease severity 13.66% was found in boroj-3 in Shymnagar and the lowest disease severity 2.65% was found in boroj-2 in Debidwar.

Table 4. Disease severity of leaf spot of betelvine in different upazilas of Satkhira and Cumilla district.

Location	% Diseases Severity			Average
	Baroj-1	Baroj-2	Baroj-3	
Kaligonj, Satkhira	11.56 a	13.47 a	7.73 bc	10.92
Debhata, Satkhira	7.87 b	11.85 ab	10.57 ab	10.10
Shymnagar, Satkhira	10.26 ab	8.11 b	13.66 a	10.68
Debidwar, Cumilla	3.02 c	2.65 c	3.61 c	3.09
CV (%)	20.56	22.32	28.61	-
LSD (0.05)	3.36	4.02	5.08	-

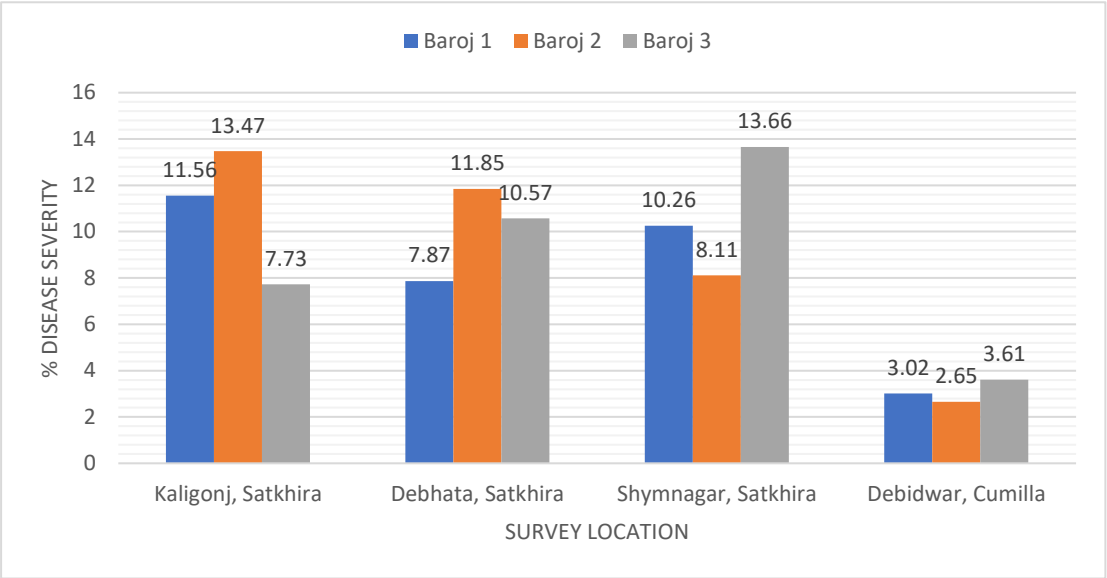


Figure 2: Mean disease severity of leaf spot of betelvine in different upazilas of Satkhira & Cumilla district.

Experiment 2. Isolation and identification of causal pathogen(s) of leaf spot disease collected from different betelvine growing regions of Bangladesh.

The samples upon incubation on PDA media, the fungus produced white cottony embedded mycelium. After 10-12 days of incubation, the fungus produced numerous acervuli. The acervuli were black in color and present moderately coagulated in the centre of the plate. The microscopic observation revealed that acervuli produced conidia containing 3-4 cell. Two hyaline apical appendages and one basal appendage were observed. Based on the key characteristics of the isolated studied in the experiment and the fungus was identified as *Pestalotia* sp. (CMI Description No. 515).

Ata (2014) observed the fungi produced a white cottony mycelium. Under the microscope, the species identified as *Pestalotia* sp. has fusiform and septated conidia with branched appendages.

Naeimi *et al.* (2015) reported that *Pestalotia disseminata* colony was colorless, turning cottony white with abundant scattered acervuli containing black, slimy spore masses. The black acervular conidiomata containing abundant conidia that were five-celled, two to three unbranched appendages on the apical cell.

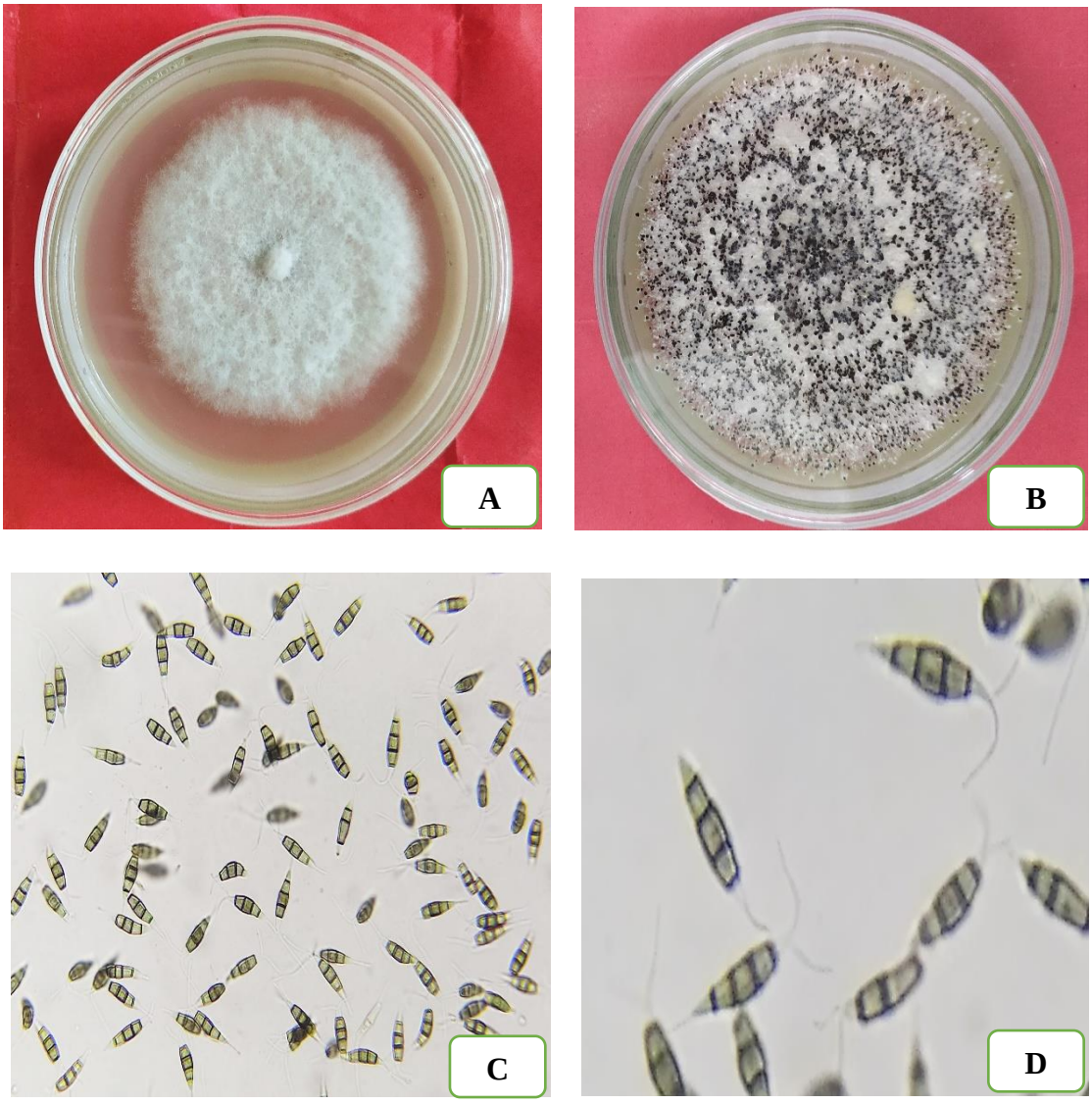


Plate 5. Colony texture on PDA media (**A&B**), Conidia of *Pestalotia* sp. observed under compound microscope (**C**. 10X; **D**. 40X)

Experiment 3. Pathogenicity test of the isolates of *Pestalotia* sp. causing leaf spot disease of betelvine

4.3.1. Symptomatology study

The result of the pathogenicity test of the fungal isolates showed that *Pestalotia* sp. was pathogenic on betelvine. It produced the characteristic symptom of yellowish to dark brown spots on leaves surrounded by yellow halo with concentric rings. The symptoms were first appeared at 10 days after inoculation. *Pestalotia* sp. was re-isolated from the infected leaves of the betelvine by observing. The pathogen under microscope and the key characteristic yellowish to dark brown spots with concentric rings on the leaves. The re-isolated pathogen from infected betelvine leaves showed the similar characteristic of the fungus causes disease of betelvine. 38.33% betelvine leaves were infected at 20 days after inoculation.

Treatments	Percent Disease Index (PDI)		
	10 DAI	15 DAI	20 DAI
Control	11.67	23.33	38.33

Islam *et al.* (2004) found the infected leaf sample was yellowish or brownish or dark spot surrounded by yellow halo. He also observed the pathogenicity test of leaf spot caused by *Pestalotia palmarum* on betelnut. He noticed that this species was found pathogenic on leaves of betelnut. Rahman *et al.* (2013) proved the pathogenicity of *Pestalotiopsis palmarum* on coconut leave. He observed, after fifteen days inoculation *Pestalotiopsis palmarum* produced characteristic symptoms of grey leaf spot of coconut.

Nambiar (1994) reported that in adult coconut palm, first symptom of grey leaf spot appears as small yellowish-brown spots. Centre of the spot turns greyish white, brown bands darkens and gets surrounded by a yellowish halo.

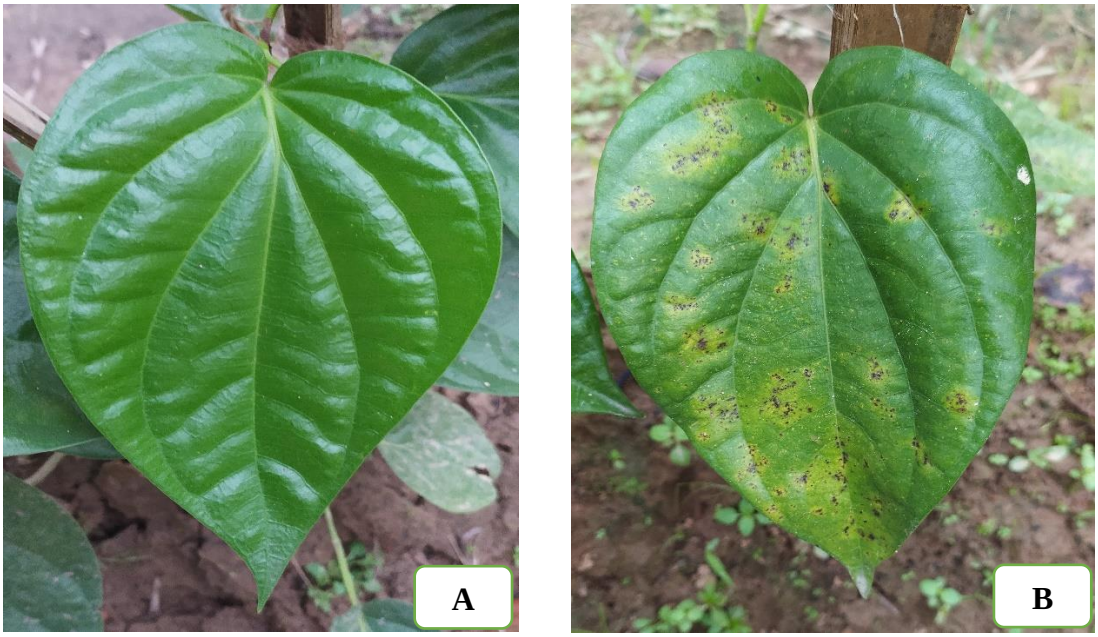


Plate 6. Infected betelvine leaves showing characteristic symptoms of pestalotia leaf spot (A) compare to healthy leaves (B) after 20 days inoculation.

Experiment 4. *In vitro* evaluation of different treatments against radial mycelial growth of *Pestalotia* sp.

Evaluation of the selected treatments in controlling *Pestalotia* sp. causing leaf spot of betelvine was done *in vitro*. The results were compiled based on the inhibition of radial mycelium growth.

4.4.1. *In vitro* evaluation of chemical fungicides at different concentrations against *Pestalotia* sp. by Poisoned Food Technique

The efficacy of 4 fungicides viz. Autostin 50 WDG, Dithane M-45, Goldton 50 WP, Tilt 250 EC were evaluated against *Pestalotia* sp. at 100 ppm, 200 ppm, 300 ppm and 500 ppm concentration by Food Poisoned Technique (Cup method). All the fungicides assayed in the experiment showed significantly promising results in comparison to control. Among the fungicides, Tilt 250 EC showed the highest performance against the pathogen irrespective of concentration followed by Autsotin 50 WDG, Dithane M-45 and Goldton 50 WP. The performances of the chemical fungicides distinctly differenced with the increase of the concentration. Tilt 250 EC and Autostin 50 WDG completely inhibited the

mycelium growth even at 200 ppm concentration. While the Dithane M-45 and Goldton 50 WP allowed little mycelium growth of the fungus. Based on the performance at 500 ppm concentration, the 100% mycelial growth were reduced in case of Tilt 250 EC and Autostin 50 WP while was 66.76% and 57.96% in case of Goldton 50 WP and Dithane M-45, respectively.

Islam *et al*. (2004) found that *in vitro* experiment Bavistin of three doses (100, 200, 300 ppm) and Tilt 250 EC (100 and 200 ppm) were more effective in inhibition mycelium growth of *Pestalotia palmarum.* All doses of Bavistin (100, 200, 300 ppm) and Tilt 250 EC (100 ppm and 200 ppm) were inhibited 100% mycelium growth of *Pestalotia palmarum.* Dithane M-45 at 100 ppm was inhibited 26% of radial growth of *P. palmarum*.

Table 5: *In vitro* efficacy of chemical fungicides at different concentrations in inhibition of mycelial growth at 3DAI, 5DAI and 7DAI of *Pestalotia* sp.

Treatments	Radial mycelium growth (cm) at 3DAI, 5DAI and 7DAI in different concentration												% inhibition of mycelium growth at 500 ppm concentration (Based on 7 DAI)
	100 ppm			200 ppm			300 ppm			500 ppm			
	3DAI	5DAI	7DAI	3DAI	5DAI	7DAI	3DAI	5DAI	7DAI	3DAI	5DAI	7DAI	
Autostin 50 WDG	1.25 d	1.42 d	1.68 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	100%
Dithane M-45	3.00 b	4.18 b	5.78 b	2.93 b	4.00 b	5.17 b	2.58 b	2.95 b	3.40b	2.17 b	2.74 b	3.25 b	57.96%
Goldton 50 WP	2.73 c	3.85 c	5.00 c	2.82 c	3.80 c	4.40 c	2.20 c	2.58 c	2.75 c	1.73 c	2.30 c	2.57 c	66.76%
Tilt 250 EC	1.07 e	1.20 e	1.37 e	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	100 %
Control	3.37 a	5.00 a	7.73 a	3.37 a	5.00 a	7.73 a	3.37 a	5.00 a	7.73 a	3.37 a	5.00 a	7.73 a	-
CV (%)	**3.53**	**1.70**	**3.08**	**2.45**	**1.51**	**4.05**	**3.80**	**1.94**	**4.92**	**3.44**	**2.47**	**5.13**	-
LSD (0.05)	**0.15**	**0.10**	**0.24**	**0.08**	**0.07**	**0.26**	**0.11**	**0.07**	**0.25**	**0.09**	**0.09**	**0.25**	-

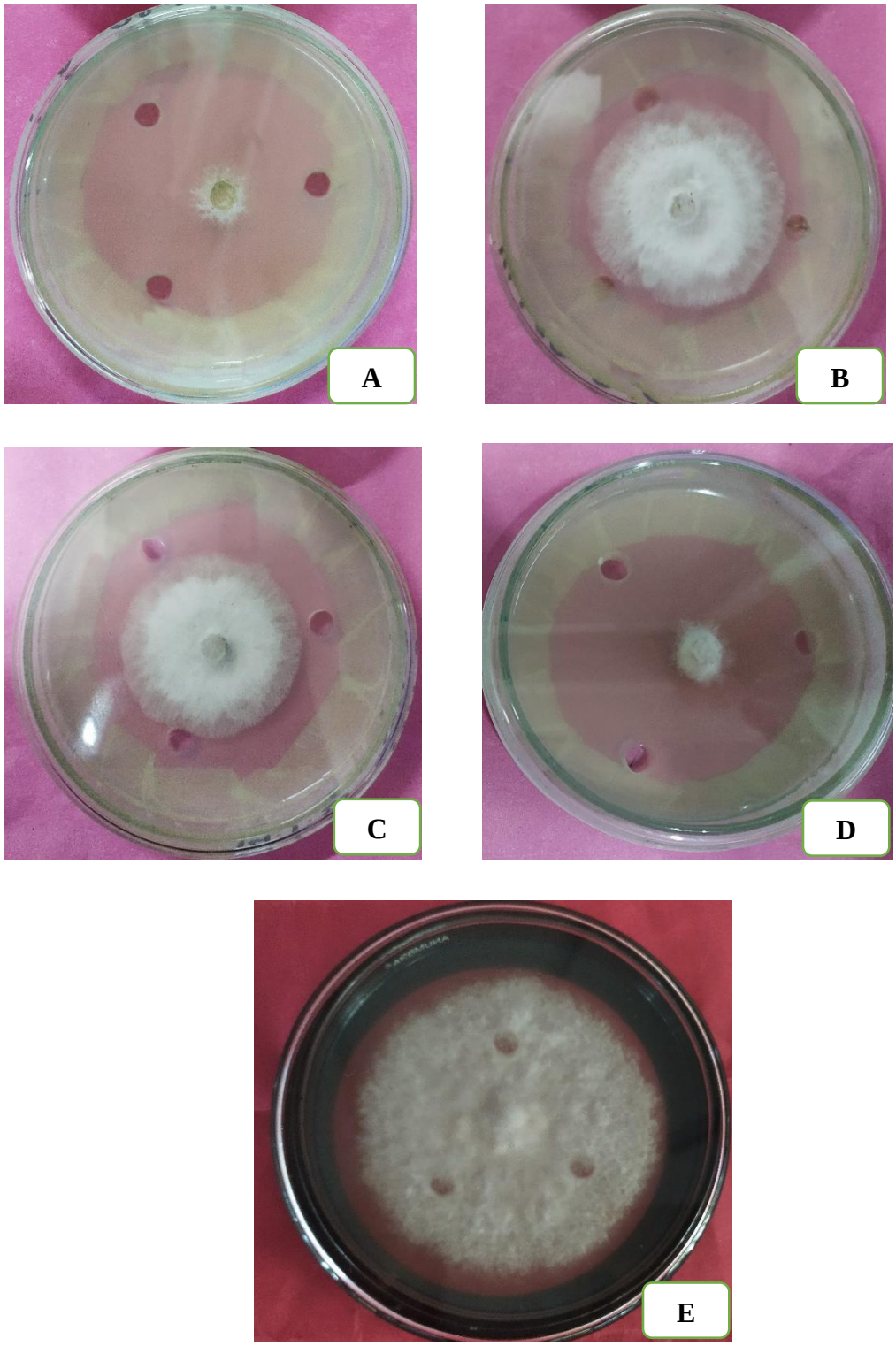


Plate 7. Radial mycelial growth of *Pestalotia* sp. at 100 ppm concentration of chemical fungicides after 7 DAI (A) Autostin 50 WDG, (B) Dithane M-45, (C) Goldton 50WP, (D) Tilt-250 EC and (E) Control

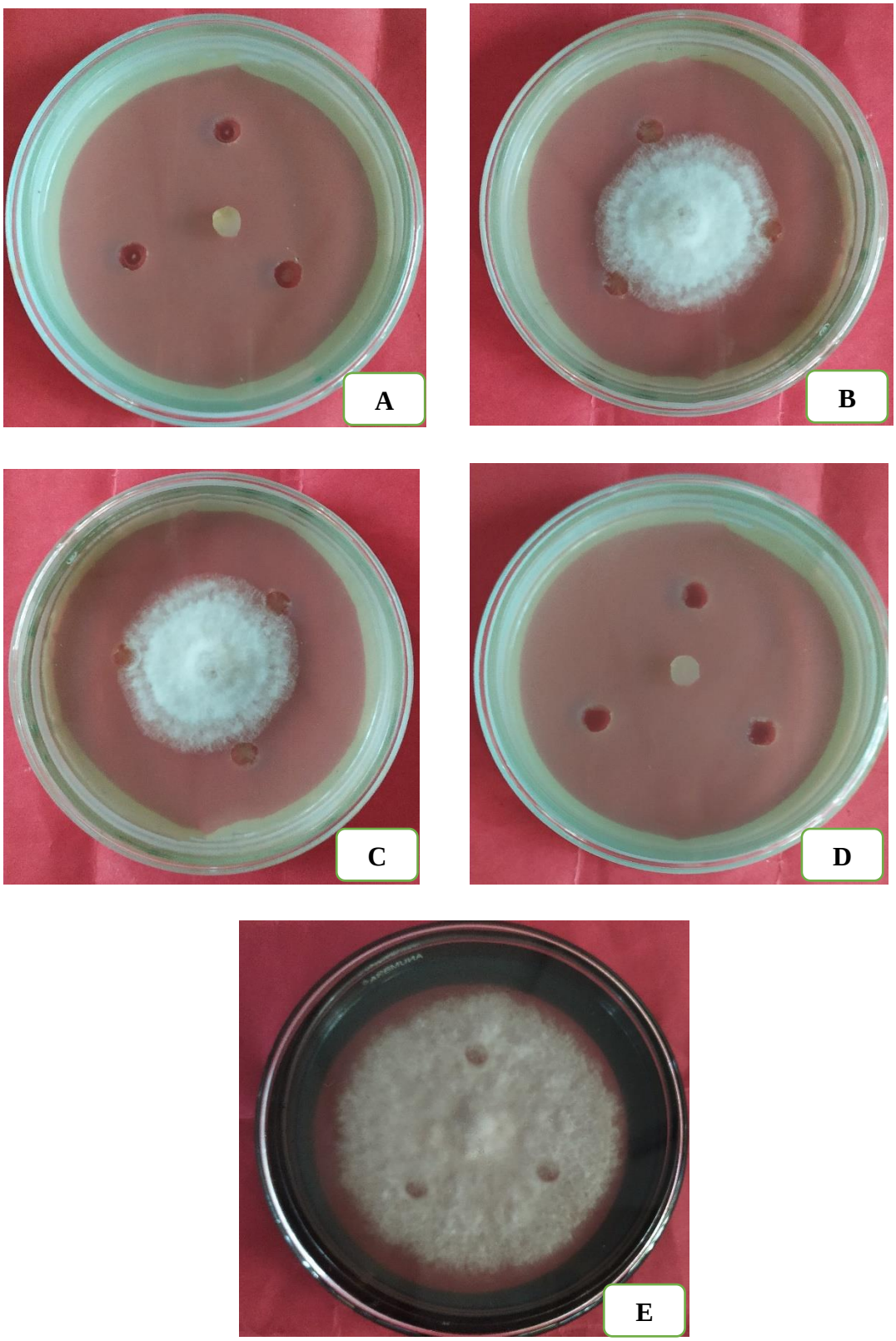


Plate 8. Radial mycelial growth of *Pestalotia* sp. at 200 ppm concentration of chemical fungicides after 7 DAI (A) Autostin 50 WDG, (B) Dithane M-45, (C) Goldton 50WP, (D) Tilt-250 EC and (E) Control

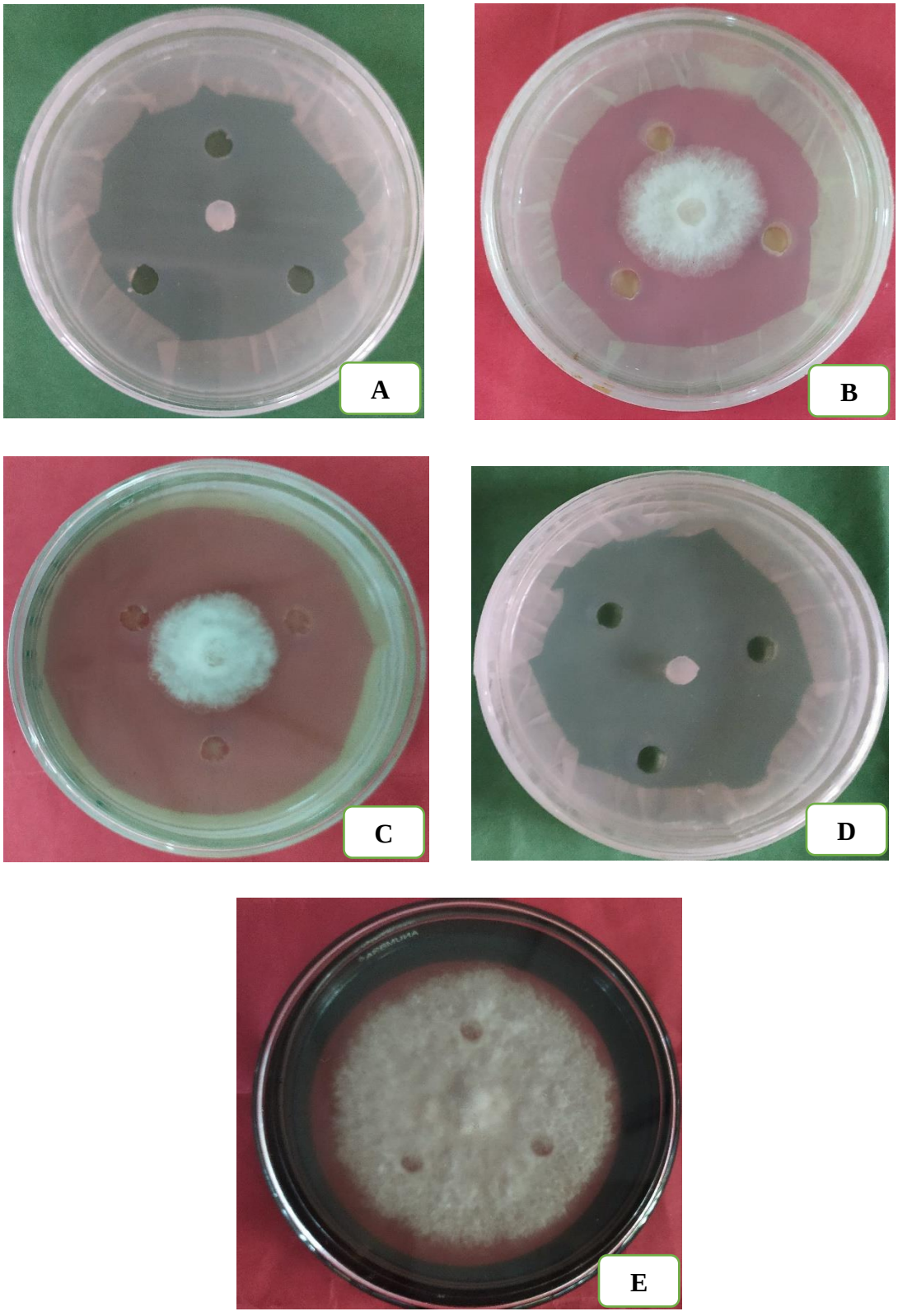


Plate 9. Radial mycelial growth of *Pestalotia* sp. at 300 ppm concentration of chemical fungicides after 7 DAI (A) Autostin 50 WDG, (B) Dithane M-45, (C) Goldton 50WP, (D) Tilt-250 EC and (E) Control

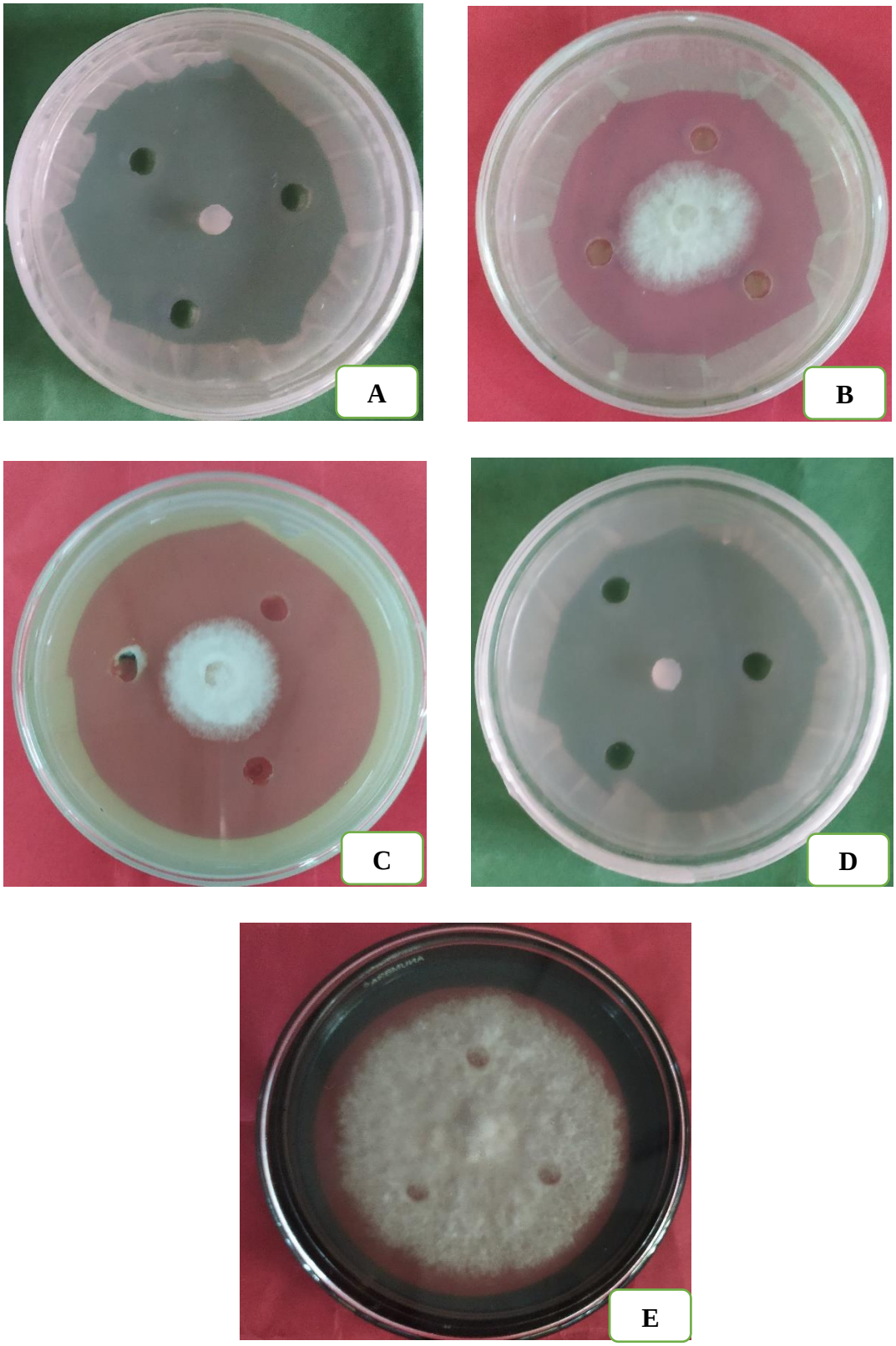


Plate 10. Radial mycelial growth of *Pestalotia* sp. at 500 ppm concentration of chemical fungicides after 7 DAI (A) Autostin 50 WDG, (B) Dithane M-45, (C) Goldton 50WP, (D) Tilt-250 EC and (E) Control

4.4.2. *In vitro* efficacy of botanical extracts at different concentrations in inhibition of mycelial growth of *Pestalotia* sp. in Poisoned Food Technique (Cup method)

Efficacy of plant extracts on radial mycelial growth of *Pestalotia* sp. is presented in Table 6 and Plate 10. Plant extracts have profound and significant effect on reduction of radial mycelial growth of the fungus. Radial mycelial growth for all the tested plant extracts ranged from 1.03 cm to 7.73 cm recorded after incubation of 7 days. The lowest radial mycelial growth 1.03 cm of *Pestalotia* sp. was recorded in case of Garlic extract irrespective of concentration. The reduction of radial mycelial growth was the highest in case of garlic extract 86.68% followed by Onion (81.63%), Ginger (74.13%), Neem (70.63%) irrespective of 7 days after inoculation at 1:1 concentration. The lowest 70.63% reduction of mycelial growth was recorded in Neem preceded by Ginger 74.13% at 7 days after inoculation.

In case of 1:2 concentration, the trend of result was more or less similar but the reduction of mycelial growth was less than 1:1 concentration.

Islam *et al*. (2004) reported that *in vitro* experiment, Garlic extract at 4-5% concentration were the best plant extracts, which inhibited 100% of radial growth of *Pestalotia palmarum*. Neem extract at 5% concentration inhibited 12.82% but no inhibition of radial growth was founded in case of Onion and Ginger plant extracts.

Table 6: *In vitro* efficacy of botanical extracts at different concentrations in inhibition of mycelial growth at 3DAI, 5DAI and 7DAI of *Pestalotia* sp.

Treatments	Radial mycelium growth (cm) at 3DAI, 5DAI and 7DAI in different concentration						% inhibition of mycelium growth (Based on 7 DAI) at 1:1 concentration
	1: 2 Concentration			1: 1 Concentration			
	3 DAI	5 DAI	7 DAI	3 DAI	5 DAI	7 DAI	
Onion bulb extract	1.77 c	2.62 cd	3.00 c	1.17 c	1.40 d	1.42 c	81.63%
Garlic clove extract	1.52 d	2.28 d	2.43 d	0.70 d	0.87 e	1.03 d	86.68%
Ginger rhizome extract	1.85 c	2.92 bc	3.37 b	1.29 c	1.58 c	2.00 b	74.13%
Neem leaf extract	2.07 b	3.10 b	3.63 b	1.47 b	1.80 b	2.27 b	70.63%
Control	3.37 a	5.00 a	7.73 a	3.37 a	5.00 a	7.73 a	-
CV (%)	**4.77**	**6.44**	**3.85**	**5.17**	**3.74**	**5.60**	-
LSD (0.05)	**0.18**	**0.37**	**0.28**	**0.15**	**0.15**	**0.15**	-

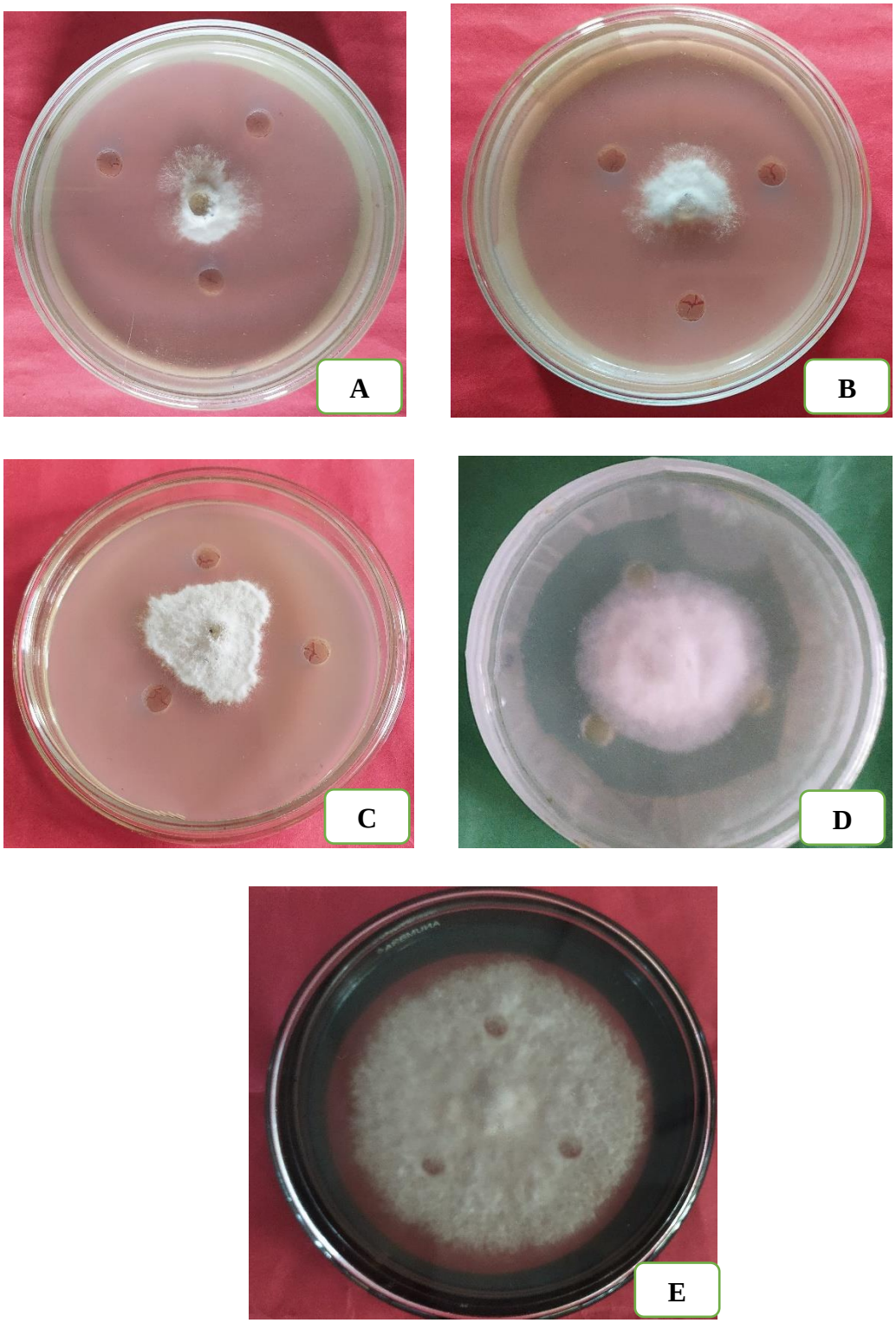


Plate 11. Radial mycelial growth of *Pestalotia* sp. at 1:2 concentration of plant extracts after 7 days after inoculation (A) Onion extracts, (B) Garlic extracts, (C) Ginger extracts, (D) Neem extracts and (E) Control

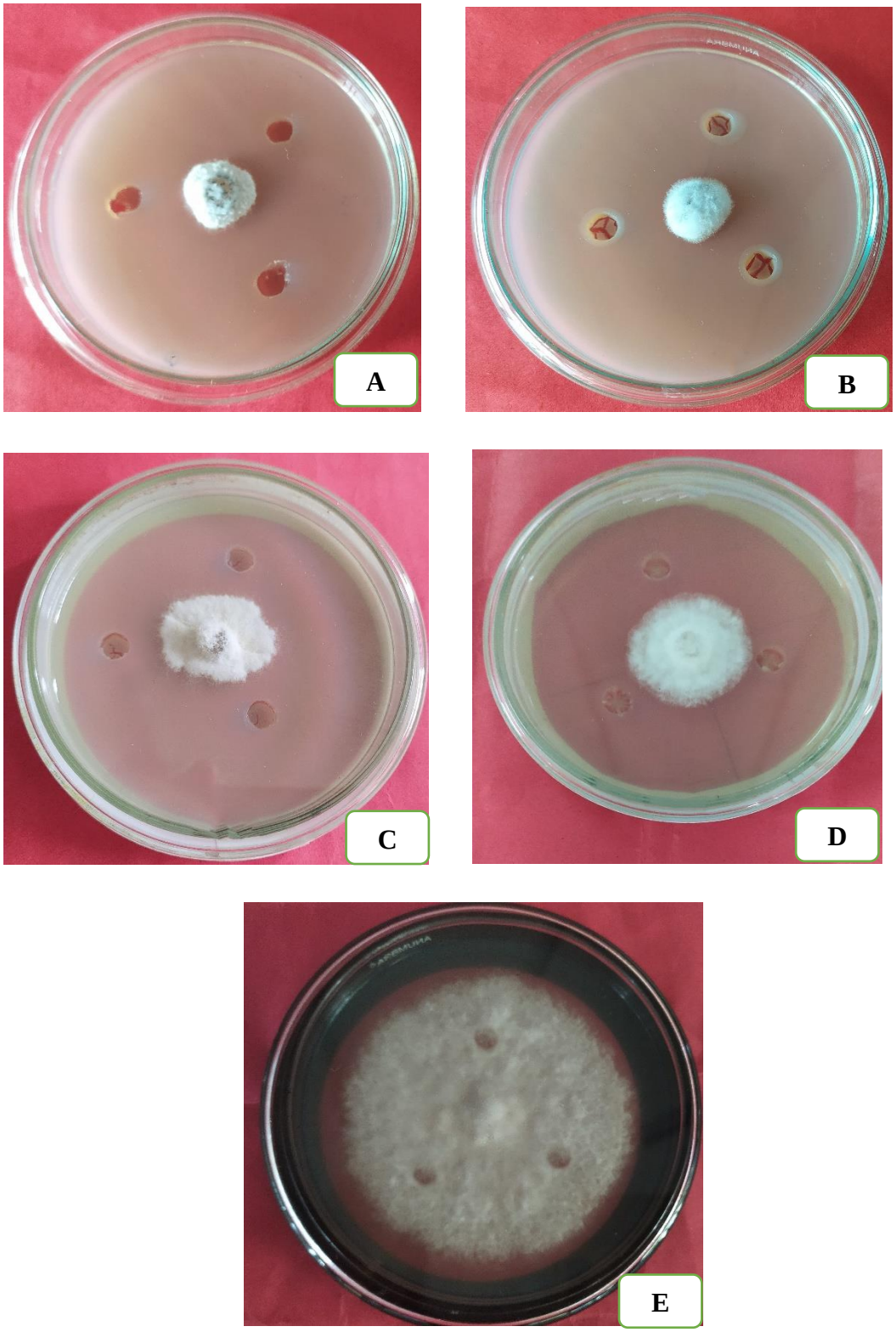


Plate 12. Radial mycelial growth of *Pestalotia* sp. at 1:1 concentration of plant extracts after 7 days after inoculation (A) Onion extracts, (B) Garlic extracts, (C) Ginger extracts, (D) Neem extracts and (E) Control

4.5. Evaluation of selected chemical fungicides and plant extracts on Percent Disease Index (PDI) of leaf spot disease of betelvine in field condition

Data recorded on disease severity of pestalotia leaf spot of betelvine as effected by the application of different chemical fungicides and plant extracts were summarized and presented in Table 7. The effects of different treatments recorded at different days after inoculation (DAI) differed significantly as compared to control. The results showed that the spraying of Tilt 250 EC showed the lowest disease severity 7.00% at 20 DAI followed by Autostin 50 WDG (12.33%). The highest disease severity 38.33% was recorded in control treatment at 20 DAI while the garlic clove extract and Ginger rhizome extract treated leaves showed 30.00% and 29.33% disease severity, respectively at 20 DAI. Dithane M-45, Goldton 50 WP, Onion bulb extract and Garlic clove extract treated leaves were showed 20.00%, 18.00%, 23.33%, 15.00% disease severity, respectively. Among all the treatments, Tilt 250 EC was found the best for reducing Percent Disease Index 81.74% of betelvine followed by Autostin 50 WP 67.83% and Garlic clove extract 60.87%.

Table 7. Evaluation of selected chemical fungicides and plant extracts on Percent Disease Index of leaf spot disease of betelvine in field condition

Treatments	Percent Disease Index (PDI)			% Reduction of PDI over control
	10 DAI	15 DAI	20 DAI	
T_1 = Autostin 50 WDG	2.67 de	7.67 e	12.33 ef	67.83 %
T_2 = Dithane M-45	5.67 bc	12.00 cd	20.00 cd	47.82 %
T_3 = Goldton 50WP	3.33 cd	9.33 de	18.00 cd	53.04 %
T_4 = Tilt-250 EC	0.00 e	3.67 f	7.00 f	81.74 %
T_5 = Onion bulb extract	7.33 b	13.33 c	23.33 c	39.13 %
T_6 = Garlic clove extract	4.67 b-d	11.33 cd	15.00 de	60.87 %
T_7 = Ginger rhizome extract	6.67b	12.67 cd	29.33 b	23.48 %
T_8 = Neem leaf extract	5.33 b-d	17.00 b	30.00 b	21.73 %
T_9 = Control	11.67 a	23.33 a	38.33 a	-
CV (%)	**32.75**	**17.11**	**14.69**	-
LSD (0.05)	**2.99**	**3.63**	**5.46**	-

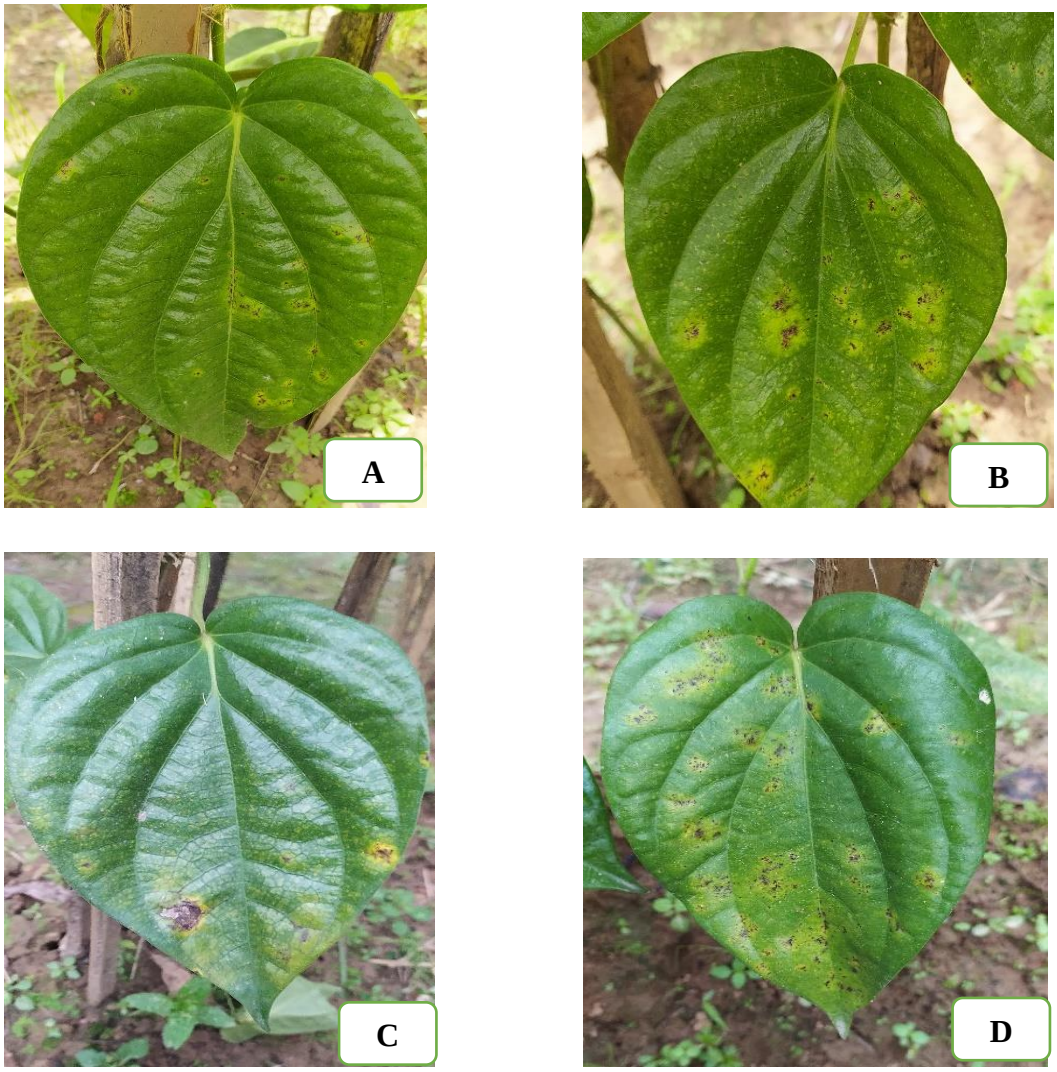


Plate 13. Betelvine leaves showing disease severity as affected by application of treatments (A) leaves treated with Tilt 250 EC, (B) Autostin 50 WDG, (C) Garlic clove extract and (D) Control

CHAPTER-5
SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCLUSION

Betelvine (*Piper betle* L.) is an important cash and horticultural crop in Bangladesh. *Pestalotia* sp. is a new causal organism that caused leaf spot of betelvine in Bangladesh. The *in vivo* experiment was conducted in the Central farm and *in vitro* experiment was conducted in MS Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural university (SAU), Dhaka-1027 during the period from November 2018 to June 2020. The survey study of the disease was conducted at major betelvine growing areas of four upazilas viz., Kaligonj, Debhata, Shymnagar under Satkhira district and Debidwar upazila under Cumilla district. The experiments were aimed to find out the disease incidence and disease severity, identify the causal pathogen(s), test the pathogenicity and find out the management options for *Pestalotia* leaf spot of betelvine.

Disease incidence of leaf spot of betelvine ranged from 8.70% to 42.77% in the surveyed area. The maximum disease incidence 42.77% was recorded at Kaligonj, Satkhira and the minimum 8.70% at Debidwar, Cumilla. Disease severity of leaf spot of betelvine ranged from 2.65% to 13.66%. The maximum disease severity 13.66% was recorded at Shymnagar, Satkhira and the minimum 2.65% at Debidwar, Cumilla.

Pathogenicity test showed *Pestalotia* sp. produced characteristic symptoms on betelvine and proved to be the causal pathogen of leaf spot disease of the betelvine.

The *in vitro* evaluation of chemical fungicides and plant extracts were done based on mycelial growth inhibition. Among four chemical fungicides, Tilt 250 EC and Autostin 50 WDG performed the best result in inhibition of mycelial growth of *Pestalotia* sp in all concentration (200 ppm, 300 ppm, 500 ppm). Tilt 250 EC and Autostin 50 WDG inhibited 100% mycelial growth of *Pestalotia* sp. Among four botanical extracts, Garlic clove extracts showed better performance

than other plant extracts in inhibition of mycelial growth 86.68% of *Pestalotia* sp. at 1:1 concentration. In case of 1:2 concentration, the reduction of mycelial growth was less than 1:1 concentration.

In case of *in vivo* experiment, the disease severity (PDI) was observed at different DAI. The lowest disease severity 7.00% was recorded in case of Tilt 250 EC at 20 DAI. The highest percent disease severity 38.33% was recorded in case of untreated control while garlic clove extract treated leaves showed 15.00% disease severity at 20 DAI. The maximum reduction of PDI were recorded by Tilt 250 EC treated leaves 81.74% followed by Autostin 50 WDG 67.83% and Garlic clove extract 60.87%.

From the findings of the present study, it may be conculcated that Tilt 250 EC had a promising effect in reducing the disease incidence and disease severity of leaf spot disease of betelvine caused by *Pestalotia* sp. Autostin 50 WDG also showed better performance in reducing the disease infection in field condition. Garlic clove extract showed significantly better performances than other plant extracts.

CHAPTER-6
REFERENCES

CHAPTER VI

REFERENCES

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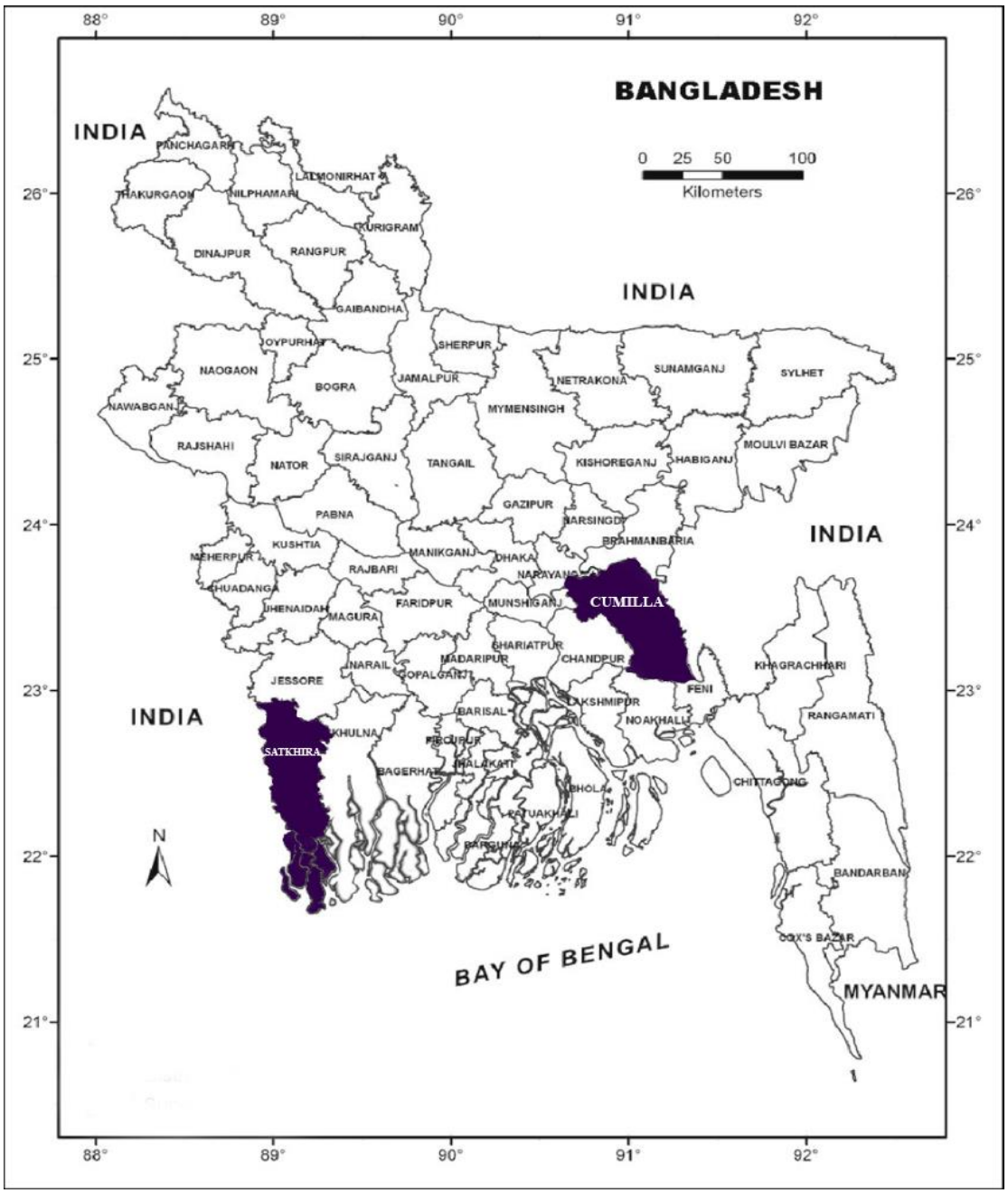
CHAPTER-7
APPENDICES

CHAPTER VII

APPENDICES

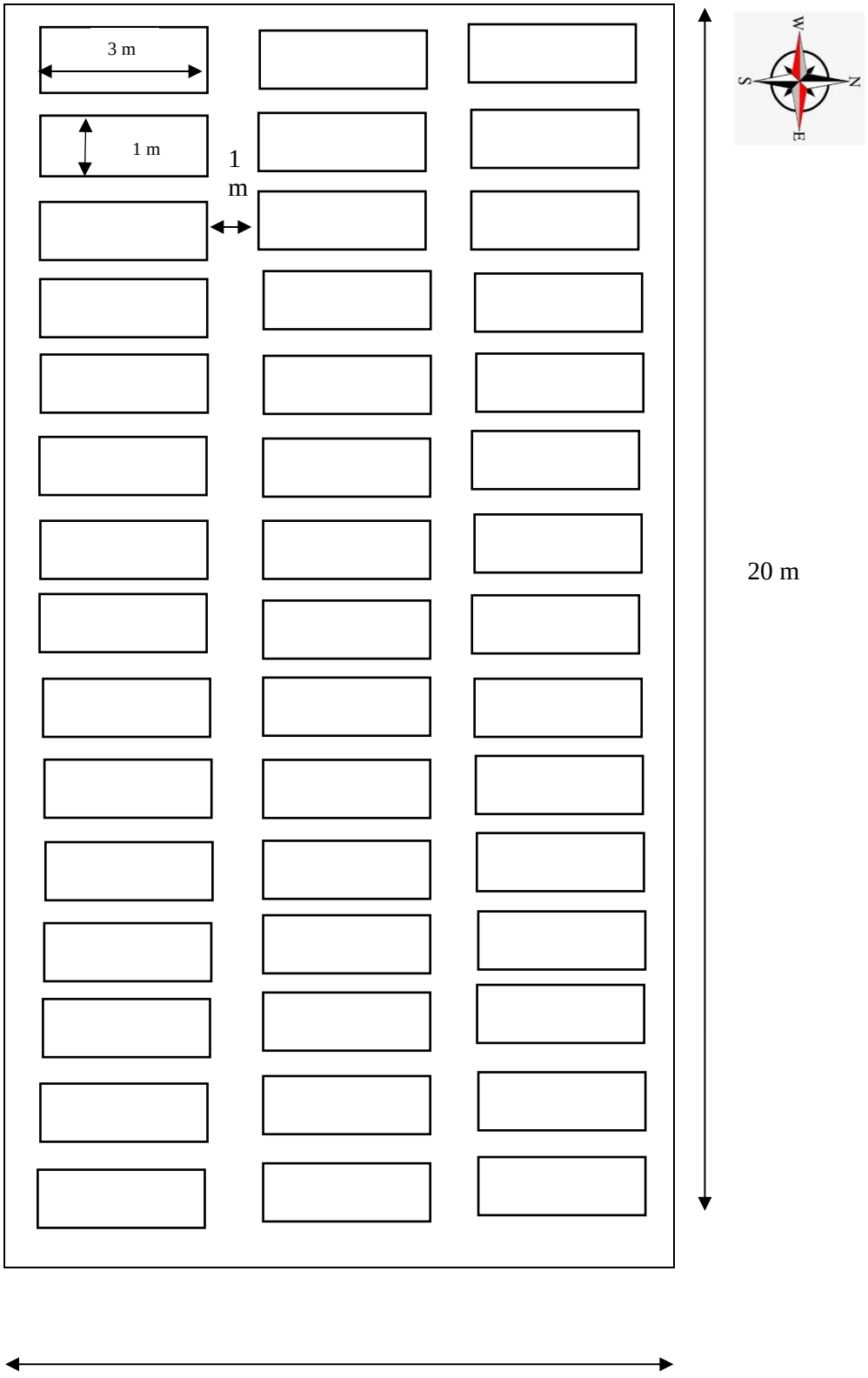
Appendix I. Map showing the experimental survey location under study



District: **Satkhira**- Kaligonj, Debhata, Shymnagar

District: **Cumilla**- Debidwar

Appendix II: Experimental Layout for betelvine garden



Appendix III: Nutritional Composition of fresh betel leaf

Sl. No.	Constituents	Approximate composition
01	Water	85-90%
02	Protein	3-3.5%
03	Fat	0.4-1.0%
04	Minerals	2.3-3.3%
05	Fibre	2.30%
06	Chlorophyll	0.01-0.25%
07	Carbohydrate	0.5-6.10%
08	Nicotinic acid	0.63-0.89 mg/100g
09	Vitamin C	0.005-0.01%
10	Vitamin A	1.2-2.9 mg/100g
11	Thiamin	10-70 µg/100g
12	Riboflavin	1.9-30 µg/100g
13	Tannin	0.1-1.3%
14	Nitrogen	2.0-7.0%
15	Phosphorus	0.05-0.6%
16	Potassium	1.1-4.6%
17	Calcium	0.2-0.5%
18	Iron	0.005-0.007%
19	Iodine	3.4 µg/100g
20	Essential oil	0.08-0.2%
21	Energy	85-90%

Source: Guha, 2006.