Morphological and Molecular identification of major fungal disease of large cardamom (*Amomum subulatum* Roxb.) in Sikkim and its management using botanicals

A Thesis Submitted

To

Sikkim University

In Partial Fulfillment of the Requirement for the

Degree of Doctor of Philosophy

By

KABITA GURUNG

Department of Horticulture

School of Life Sciences

Sikkim University

Gangtok Sikkim - 737102

INDIA

Dedicated to my precious

"My Family"

CONTENTS

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(KabitaGurung)

Date: 17.11.2020

DECLARATION

I declare that the thesis entitled **"Morphological and Molecular identification of major fungal disease of large cardamom (*Amomum subulatum* Roxb.) in Sikkim and its management using botanicals"** submitted by me for the award of degree of **Doctor of Philosophy** in Horticulture to the Sikkim University under the supervision of Dr. Niladri Bag, Associate Professor, Department of Horticulture is my original research work solely carried out by me in the Department of Horticulture, School of Life Science, Sikkim University. The content of this thesis is based on the experiments performed by me. The thesis has not been submitted for any other degree in any other University.

Kabitaf

Place: Gangtok
Date: 17.11.2020

(Kabita Gurung)
Regd. No. –16/PhD/HOR/01
Department of Horticulture
Sikkim University

6 माइल, सामदुर, तादोंग - 737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स - 251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur, Tadong-737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax : 251067
Website : www.cus.ac.in

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SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
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CERTIFICATE

This is to certify that the thesis entitled **"Morphological and Molecular identification of major fungal disease of large cardamom (*Amomum subulatum* Roxb.) in Sikkim and its management using botanicals"** submitted to the Department of Horticulture, Sikkim University, Gangtok in partial fulfillment of the requirement for the degree of **Doctor of Philosophy in Horticulture,** embodies the results of bonafied research work carried out by **Miss Kabita Gurung** under my guidance and supervision. The results are original and no part of the thesis has been submitted anywhere for any other degree, diploma.

It is recommended that the Ph.D. thesis be placed before the examiners for the evaluation.

Niladri Bag 17.11.2020

Place: Gangtok

Date: 17.11.2020

(Dr. Niladri Bag)
Chairman
Associate Professor
Department of Horticulture
School of Life Sciences
Sikkim University
Gangtok-737102, India

6 माइल, सामदुर, तादोंग - 737102
गंगटोक, सिक्किम, भारत
फोन-03592-251212, 251415, 251656
टेलीफैक्स - 251067
वेबसाइट - www.cus.ac.in

6th Mile, Samdur, Tadong-73
Gangtok, Sikkim,
Ph. 03592-251212, 251415, 25
Telefax : 25
Website : www.cus

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

(भारत के संसद के अधिनियम द्वारा वर्ष 2007 में स्थापित और नैक (एनएएसी) द्वारा वर्ष 2015 में प्रत्यायित केंद्रीय विश्वविद्यालय)
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Submitted by: **Miss Kabita Gurung** under the supervision of Dr. Niladri Bag, Associate Professor, Department of Horticulture, Sikkim University, Gangtok, India.

Kabita

(Kabita Gurung)
Research Scholar

Niladri Bag 17.11.2020

(Dr. Niladri Bag)
Supervisor

17/11/2020

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It is recommended that the Ph.D. thesis be placed before the examiners for the evaluation.

Place: Gangtok
Date: 17.11.2020

Niladri Bag 17.11.2020
(Dr. Niladri Bag)
Associate Professor & Head
Department of Horticulture
School of Life Sciences
Sikkim University
Gangtok-737102, India

DEPARTMENT OF HORTICULTURE * SIKKIM UNIVERSITY *

अध्यक्ष
Head
उद्यानिकी विभाग
Department of Horticulture
सिक्किम विश्वविद्यालय
Sikkim University

ABBREVIATION

%	Percentage
°C	Degree centigrade
µg/ml	Microgram per milliliter
µm	Micro meter
AMSL	Above mean sea level
ANOVA	Analysis Of Variance
BLAST	Basic Local Alignment Sequencing Tool
Bp	Base pair
CTAB	Hexacetyl trimethylammonium bromide
Cm	Centimeter
CRD	Completely Randomized Design
CWA	Coconut watery endosperm
CZ	Czapek Dox
DCM	Di chloromethane
DNA	Deoxyribonucleic acid
dNTPS	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g	Example
Etc	Et cetera
et al	et alia

G	Gram
g/l	Gram per liter
GPS	Global Positioning System
HCL	Hydrochloric acid
i.e	That is
ICRI	Indian Cardamom research institute
ITS	Internal Transcribed Spacer
ITK	Indigenous traditional knowledge
Km	Kilometer
LBA	Lima bean agar
LSD	Least Significant Difference
MEGA	Molecular Evolutionary Genetic Analysis
mg/ml	Milli gram per milliliter
mg/l	Micro gram per liter
$MgCl_2$	Magnesium chloride
MIC	Minimum Inhibitory concentration
M	Meter
Mm	Millimeter
Ml	Milliliter
Mm	Milli molar
MT	Metric Ton
NA	Nutrient Agar

NaOH	Sodium hydro oxide
NCBI	National centre for Biotechnology Institute
NFCCI	National Fungal culture Collection of India
PCA	Potato Carrot Agar
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PDI	Percent Disease Index
PDC	Percent Disease Control
pH	Hydrogen ion concentration
RAPD	Random Amplified Polymorphic DNA
RNase	Ribonucleic acid
Rpm	Rotation per minute
SDA	Sabourad Dox Agar
SDS	Sodium Dodecyl Sulfate
Sp.	Species
TAE	Tris-acetate- EDTA
TE	Tris- EDTA
TYE	Tryptone Yeast Agar

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Publication

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CHAPTER 1

INTRODUCTION

1.1. Brief account on the state, Sikkim

Sikkim is a tiny beautiful hill state lying in the lap of the Eastern Himalaya with lash green vegetations, high mountains, glaciers, rivers and streams. Nature is generous to the region with treasures of natural resources. Sikkim is one of the richest biodiversity- hot spots in the world. The state is inhabited by 14 hill tribes, and harbors 4458 species of flowering plants, 515 varieties of orchids, 36 types of rhododendron species, 11 oak varieties, 23 bamboo varieties, 16 conifers species, 362 types of ferns and fern's allies, 8 tree ferns and more than 24 medicinal plants owing to varied agro- climatic conditions.

It is a small multi-ethnic state, located in the Eastern Himalayas covering a geographical area of 7096 sq km (1,09000 hectares), which is 0.22% of India's geographical area and it is the second smallest state in the country. Sikkim is bordering with Nepal in the West, Tibet Autonomous Region (TAR) in the North, China in the north and North East, Bhutan in the South East, West Bengal in the South. According to the census 2011, the population in the state is 6.10 lakhs which is least in the country. The State has four districts, North, West, East and South. Out of the total population in the state, the rural population is 74.85% who live in the villages (Census of India 2011).

Agriculture is considered to be the major economic activity in the rural area. More than 64 percent of the population depends upon the agriculture for their livelihood. Main crops cultivated in the state are maize, rice, large cardamom, ginger and mandarin. Large cardamom is considered to be an important and most profitable among other crops grown in the state. The state is also known to be the largest producer of the crop and contributes to the lion share, i.e., 88% of the national production and also played a significant role in the world trade of large cardamom.

1.2. Agro diversity of Sikkim

Sikkim is blessed with diversified ecosystem comprising of five different climatic zones, six different forest types, three soil orders, 21 glaciers, 28 mountain peaks and 227 lakes and wetlands and more than 104 rivers and stream in the range of small geographical area of 7096 sq km. Hence, the state is considered as the richest biodiversity region of India (Anonymus 1994). The state is also enriched with diverse agro biodiversity including the different farming cultures, agro climatic zones, traditional landraces and indigenous farming and livestock culture, etc. which directly or indirectly linked up for the food and agriculture system.

Sikkim is basically an agrarian state with the net cultivable area of 79,000 ha which follows traditional farming. The farming is done in hill slopes as terrace farming which is fully organic. The region houses a different crop genetic diversity including 5,580 plant species, among which 550 species are categorized as food crops and 50% are cultivated in the region. A total of 126 landraces of cereals including 77

races of rice, 26 races of maize and 26 races of millet are available in Sikkim. Apart from cereal crops, the region also grows 18 cultivars of oilseeds and 34 cultivars of pulses. The region is also rich in vegetable and spices with 132 and 38 different species respectively (Sharma *et al.* 2016). The crops are grown in three seasons, i.e., pre kharif, kharif and rabi season. The pre kharif eventually starts during February with the onset of first shower. Maize is the only pre kharif crop grown in the region. Similarly, the crops like rice, soybean, ginger and few vegetables are grown during May to June as the kharif cops. Finally the rabi season crops includes cauliflower, radish and carrot which are grown during September to October.

The region is also known to put into practice indigenous traditional farming, where the local communities still believe in preserving and strategizing the farming system for food security and sustainable livelihood management. It is believed that the several ethnic communities with diverse social cultures and preferences have contributed a lot to the biodiversity enrichment of the state. The living example for traditional farming is the large cardamom based agro forestry system which still prevails in the region. Large cardamom is one of the important cash crops of Sikkim. Studies revealed that Himalyan alder (*Alnus nepalensis*) based large cardamom farming has been considered as the legacy farming. The crop is endemic to the region and the state is also rich in genetic diversity of large cardamom. According to reports and documents etc. different genotypes of large cardamom found in Sikkim include *Amomum linguiforme, Amomum. kingii, Amomum aromaticum, Amomum corynostachyum, Amomum dealbatum, Amomum costatum,* and *Amomum*

plauciflorum. Interestingly, with the help of traditional farming, Sikkim represents an example for the subsistence and sustainable farming through cardamom based agro forestry, floriculture and fruit growing in the marginal area. So the preservation of this agro biodiversity system is very crucial to allow the ecosystem to flow in a sustainable manner (Table 1.1).

Sikkim officially has announced the adoption of organic farming in the year 2003 to convert entire state into organic practice of farming through a declaration in legislative assembly. Now, it is fully certified organic state of India as per the guidelines laid down by national programme on organic farming and in January 2016 Prime Minister, Govt. of India, announced Sikkim officially as the 1st organic state in the country. So chemical pesticide, fungicide or inorganic chemicals are strictly prohibited in the state for any kind of field activity or pest and disease control.

1.3. Brief account of the crop

The large cardamom (*Amomum subulatum* Roxb.) is considered to be the oldest spices known to the mankind. It is mentioned in the Sashruta that since the early days of Ayurveda and Unani system of treatment for several aliments of human beings. The genus *Amomum* was introduced to Greeks and Romans during the 4th BC which later on was recorded by Theophrastus (Mukherjee 1972*)*. Roxburgh (1820) was first to illustrate and describe this plant in his 'Plants of the Coast of Coromandel' and in 'Flora Indica' (Roxburgh 1820). Large cardamom (*A. subalatum*Roxb.) belongs to the

Table 1.1: Different ecological zone with references to the agro biodiversity found in Sikkim

Ecological zone	Altitude (m amsl)	Climate	Attributes	Crop species
Pastoralism zone	4,000–5,500	Alpine	Grazing areas for yak and sheep	Potato, cabbage, leafy vegetable, medicinal and aromatic crops
Mixed farming zone	2,500–4,000	Temperate	Agro pastoralism	Medicinal plants, potato, cabbage, some oilseeds, and some beans/peas
Traditional agro forestry zone	600–2,500	Warm temperate	Agro diversification, traditional farming (intercropping, crop rotation, green manuring).	Rice, maize, buckwheat, beans, pulses, finger millets, yams, tubers, ginger, large cardamom
Terrace rice cultivation-based mixed farming zone	above 300	Sub tropical	Live stock, wet and dry agriculture	Mandarin, ginger

Source: Sharma *et al.* 2012

Fig. 1.1: Large cardamom plantation in Sikkim
A. Large cardamom field with lush green plants grown under canopy at West Sikkim,B. Large cardamom cultivation in an open condition at Gaucharan, Assam Linzey, East Sikkim,C. Weeding and cleaning of the field at the time of fruiting.

family Zingiberaceae under the order Scitaminae. It is a perennial herb which is monoceious and monocotyledon (Sajini *et al.* 1997).

'Queen of spices' - the large cardamom (*Amomum subulatum* Roxb.) is one of most significant cash crops, highly priced with high economic returns and indigenous to Sikkim - a tiny north eastern organic state of India. Large cardamom is locally known as bada elaichi in Hindi and alainchii inNepali.In the ancient time indigenous tribe (Lepchas) of Sikkim used to collect large cardamom from forest for use as medicine and spice. Since then, gradually the crop has been domesticated and being cultivated in Sikkim. With its high return than any other crop and increasing demand, the crop gradually became attractive and popular among people in the state. Now it is one of the most important cash crops and about 88% population of Sikkim are dependent on it for source of income and livelihood (Sharma *et al.* 2000, Bhattarai *et al.* 2013). Sikkim contributes maximum, i.e., 85%of large cardamom production in India. It is also cultivated in parts of Uttarakhand and some other North-eastern States like Arunachal Pradesh, Manipur, Nagaland, Mizoram and Assam (Sharma *et al.,* 2000). The crop is popularly known as "Alainchii" in nepali.

Large cardamom is a perennial, shade-loving crop found at between 600–2,400 m amsl. It requires a high level of humidity in air (>90%) and soil moisture (>70%) and, therefore, grows best in areas with annual rainfall of 2,000–4,000 mm and ambient air temperature of 10–22ºC (Sharma 2012, Sharma *et al.* 2000). Generally matured large cardamom plants are about 1.5 to 2.5 m in height, and possess 9-13 leaves in each tiller (Fig.1.2 A, B,C). The plant is characterized with the subterranean

Fig. 1.2: Large cardamom plant at reproductive stage

A.Fully mature large cardamom plant at the time of flowering, B. The plant at its flowering stage, C. The close view of the flower, D. The plant at its fruiting stage, E. The close view of the fruits in the clump.

rhizome where the initiation of the leafy shoots and spike takes place. Total number of such rhizome found in single plant varied between15 to 140. The leaves are long, liner, green to darker green in colour, with smooth texture in both the surfaces, pointed apex and prominent midribs. Inflorescence is a condensed spike on a short peduncle (Fig. 1.2A). The flower of large cardamom is bracteates, bisexual, zygomorhic epigynous and cuspinated (Fig. 1.2B, C). Generally an inflorescence bears 40 to 50 flower buds in the peduncle in an acropetal sequence. In the spring plants flower that last for three days and sometimes more. The inflorescence remains intact with the flower for a considerable period during April to May (Sharma *et al.* 2000). Each spike bears 10-15 capsules depending upon the cultivar. Fruits of the large cardamom are round to oval shaped, trilocular with many seeded in a capsule. The capsule wall is reddish brown to pink in color densely covered with spines (Fig. 1.3A). Seeds of large cardamom are white or greenish in colour in immature stage (Fig. 1.3B) which become black on maturity the capsule (Fig. 1.4A, B). The plant bears flower after the third year of plantation. The flowering habit varies based on the altitudinal range and vigor of the plant. In general flower bud differentiation takes place during August in lower altitudes and October in higher altitudes. The capsule matures during the month of September to October (Rao *et al.* 1993).

The crop is found growing in all the four districts of Sikkim ranging with different altitude from 800 - 3000 m amsl. The East district is considered as the largest producer amongst the rest. In the year 2017-2018, large cardamom production in Sikkim was recorded 4385.28MT from the 17735.15 hectares area out of which

Fig. 1.3: Large cardamom capsules (Immature)

A.Immature fruits, B. Close view of the immature capsules with greenish seeds

Fig. 1.4: Large cardamom capsules (Mature)

A.Mature and cured fruits, B.Close view of mature capsule with black seeds

1487.8MT form East district - the largest producer among all the four districts of Sikkim followed by south with 983.94MT. The two other districts north and west contributed 939 MT and 974.54 MT, respectively (Table 1.2).

The farming of large cardamom is an agro forestry based system as it requires tree species as a shade tree. Several studies showed that, there are many advantages of this agroforestry based farming as it helps to conserve the tree species biodiversity with the additional income like the production of fodder and fuel for the local people. Studies also showed that the cultivation of large cardamom could provide three to four times higher income to that of other traditional crops grown in the region specially rice, maize, zinger, etc. (Chettri *et al.* 2013).

In Sikkim, there are mainly six local varieties of large cardamom in cultivation namely *Swaney, Ramsey, Ramla, Golsey, Varlangely and Sermna.* Most popular, important and wildly cultivated variety is*Swaney*. The word "*Swaney*" has come from the "Sawan" – a month of Nepali calendar (August) when the crop matures. This variety is an early maturing crop. The cultivar is adapted to wide range of altitude. Plants are robust, 1.5 to 2.0 m tall, with ovate and broad leaves. This cultivar bears reddish-brown to maroonish, variable in size from medium to bold round capsules with 40 to 50 seeds. The variety is susceptible to chirkey and foorkey (viral diseases).

Ramsey is named after the "Bhutia" word "Ram" which means "mother" and "sey" means "gold". This cultivar is well suited to the high altitudes (1500 m) and could be cultivated in steep slopes as well. This cultivar is tall about 1.5-2.0 m high, with maroon tiller and narrow leaves. The plant bears reddish-brown to maroonish,

Table 1.2: District wise area and production of large cardamom in Sikkim

Year	East		North		South		West	
	Area(Ha)	Production(MT)	Area(Ha)	Production(MT)	Area(Ha)	Production(MT)	Area(Ha)	Production(MT)
2017-18	6514	1487.80	3868	939.00	3707.95	983.94	3645.20	974.54
2016-17	6784	1584.60	4050	1044.00	3661.50	996.33	3641.70	1008.02
2015-16	6558	1561.00	3850	1001.00	3580.85	966.40	3531.40	937.234
2014-15	6565	1309.60	3830	959.00	1066.00	935.70	1139.00	870.00
2013-14	4950	1188.00	3800	784.78	3531.00	935.70	3480.00	835.20
2012-13	4870	1096.00	3720	691.80	3569.00	899.06	3491.00	794.20
2011-12	4866	1036.00	3642	655.00	3640.00	824.00	3354.00	722.00
2010-11	5455	1069.00	3642	626.00	3697.00	910.00	3354.00	705.00
2009-10	5925	1129.90	4284	848.00	3741.00	818.00	3461.00	746.00
2008-09	5925	1187.00	4184	836.00	3777.00	861.00	3485.00	794.00

(Source: Anonymous 2009-2018, Spice Board, Govt. of India)

Smaller capsule with 16 to 30 seeds in a capsule (Karibasappa *et al.* 1987).

The other popular cultivar is Ramla resembling the similar characteristics to Ramsey but restricted to the high altitude areas of North Sikkim. It is believed that this cultivar is the natural hybrid of cultivar Golsey and Ramsey. Plants of this cultivar are 1.5 to 2.0 m tall with long and broad leaves. Capsules are dark pink in colour, medium-bold in size with 25-35 seeds in each.

Varlangely is considered as the good yielding cultivar which is suited for high altitude plantation. Plants are tall and robust with 1.5 to 2.5 m long, high yielding and with medium to bold capsules having 40-50 seeds each. This cultivar also resembles the cultivar Ramsey with the similar narrow leaves and wavy margins.

The cultivar Golsey is well suited to low and medium altitudes. The word "golsey" came from "Bhutia" and "Hindi" which means round and yellow. It is less vigorous with erect leaves. This cultivar is identified with greenish to maroonish stem and bears a large capsule with 30 to 40 seeds per capsule (Biswas *et al.* 1986). Further, this cultivar is tolerant to chirkey and susceptible to foorkey and leaf steak diseases.

Sermna is a local cultivar originated from Hee-goan (West Sikkim) by the local tribe "Limbo" which is believed to be high yielding and is resistant to diseases and pest as well. The plant features are similar to cultivar Golsey except the leaves which are drooping. Because of its drooping characteristics the cultivar is named as "Sermna" meaning droop "in limbo".

Apart from the six local varieties as described above, in 2004 Indian Cardamom Research Institute, Regional Station, Spices Board, Tadong, Gangtok has released two high yielding varieties suitable for cultivation in Sikkim and

Darjeeling. Varieties are named as ICRI Sikkim 1 and ICRI Sikkim 2. These varieties are high yielding in comparison to the local varieties and basically the selected from the cultivar Sawney (Gudade *et al.* 2013, Vijayan *et al.* 2018).

ICRI Sikkim 1: This variety is suitable for medium to high altitudes (1500 - 1650 m amsl) plantation. Plants are robust with ovate broad leaves and maroon coloured tillers. After 5 years of plantation about 60% spikes bear capsules. Capsules are maroon in colour, bold, 2.2 cm long and 2.0 cm in diameter with 40-50 numbers seeds in each capsule. Average yield per hector is approximately 840 kg (Vijayan *et al.* 2018).

ICRI Sikkim 2: This variety is also suitable for medium altitude (1500 m amsl) plantation. Plants are robust with ovate broad leaves and deep maroon in coloured tillers. About 60% spike bears capsules in a 5 years old plant. Capsules are also maroon in colour, bold, 2.7 cm long and 2.2 cm in diameter with an average 45 seeds in each capsule. Average yield per hector is around 898 kg (Vijayan *et al.* 2018).

Large cardamom is a low volume, high valued, non-perishable, perennial spice crop. It is a major export oriented cash crop of most large cardamom growing Southeast Asian countries (Sharma *et al.* 2009, Srivastava and Verma 2089a, b). For years, India has been recognized as a major producer, exporter and consumer of large cardamom. But now a day in India, large cardamom consumption is quite high and mostly consumed domestically as a result of which exportation is declining substantially. Export data showed that the large cardamom export from India has been severely affected during the last five years and in 2018-19 India exported 860 Tonnes which is only 9.92% of the national production (Table 1.3). When we look back, in 2008-09 India exported 1875.04 Tonne which was 43.61%

of national production. Thereafter drastic declining in export was recorded, i.e., in 2009-10 export was 1000 Tonnes which was only 23.91% of national production. The trend maintained till 2012-13 and during this period export was 20-25% of national gross production. Again after 2013-14, export started declining further and in 2014-15 India exported only 665 Tonnes of large cardamom worth Rs. 8403.90 lakhs (Table 1.3). Reports show that this is due to poor growth of the crop where the foliar parts are being severely damaged by several pathogens. Even the seed of such poor plants are discolored especially whitish in color which did not fetch a good market price. This drastic downfall of the production led to the reduction in the economy which became the main concern for the concerned department and the farming communities.

Table 1.3: Production, export and cost of large cardamom in international market grown in India

Year	Total production (Tonne)	Export Quantity (Tonne)	% Export in respect to production	Value Rs. (Lakh)
2007-08	4920	1325.03	26.93	1500.01
2008-09	4300	1875.04	43.61	2280.74
2009-10	4180	1000	23.92	1788.60
2010-11	3916	775	19.79	4462.90
2011-12	3860	935	24.22	6830.00
2012-13	4145	1100	26.53	7366.192
2013-14	4465	1110	24.86	7961.15
2014-15	4850	665	12.51	8403.90

2015-16	5315	600	10.77	7332.50
2016-17	5572	780	13.20	8265.45
2017-18	5906	760	12.87	5646.60
2018-19	8669	860	9.92	6106.00

Source: Anonymous 2009-2019 (Annual Reports Spice Board, Govt. of India).

1.4. Indigenous traditional knowledge

The diverse ethnic communities and the species richness make Sikkim, a treasure house of indigenous traditional knowledge (ITK) which has major role in agriculture system management (Chhetry *et al.,* 2013, Talukdar *et al.,* 2012). Traditional indigenous knowledge (ITK) is one kind of actual knowledge that has been passed on from the ancestors to the coming generation so that the practices involved would remain alive. It can be considered as a hereditary knowledge that was once practiced by the ancestors to control many diseases and pests. It is a local knowledge that is unique to the culture of a society in a particular region. Other names for it are local knowledge, folk knowledge, people's knowledge, traditional wisdom or traditional science, indigenous traditional knowledge (ITK) etc. This knowledge or the practice is passed from generation to generation, usually by word of mouth and cultural rituals, and has been the basis for agriculture, food preparation, health care, education, conservation and wide range of other activities, and still that sustain societies in many parts of the world. Traditional knowledge is an unwritten form of science techniques that needs a scientific validation and documentation to encourage sustainable farming practices. The indigenous traditional knowledge belongs to the particular communities that have been able to

use their minds and sense of understanding to cope up with the different kinds of diseases and pests in the agriculture system.

North Eastern region of India is rich in ITK and nourished by hundreds of ethnic tribes and communities (Kumar *et al.* 2009). Use of plants and animal parts and products are important components of ITK in the management of pests and diseases of crops in this region as well as in Sikkim. Likewise, improved ITK based farming is an important and relevant tool for Sikkim as it has already adopted an organic farming system. Ashes of fire wood, lime, neem seed powder, peels of citrus, lemon and pomelo and other botanical preparations for both storage and the management of field pests are very common among the people of Assam and other North Eastern states. Therefore the indigenous traditional knowledge found and practiced in Sikkim has to be identified and refined with the improved techniques that could be brought into practices as far as the organic farming is concerned. This kind of practice is purely an eco friendly and sustainable practice. It helps to retain the fertility of soil along with preserving the natural ecosystem. Knowing the fact, Sikkim is blessed with several effective botanicals, the practices of the ITK based farming is/will be the most useful practice for the farmers of the region. It is already documented and proved that botanicals are rich source of many active ingredients that prevent the infestation and cure from pests and diseases organically. Several botanicals like leaf extract of Titepati (*Artemisia vulgaris*) Chilowney (*Schima wallichii*), Banmara (*Chromolaena odorata*) and Tobacco are being used against the sucking pests by a number of farmers in the region. Bookey timur (*Zanthoxylum allatum*), Datura, Neem, Angeri and fermented leaf extract are the list of a few botanicals being used by the farmers of the north eastern regions to control numerous pest and diseases of the crops (Anonymous 2014).

There are many rural areas where wise farmers practice and prepare the botanical formulation with the mixture of cow dung and cow urine which are uniformly sprayed over the crops for their healthy and diseases free growth. These formulations are either prepared from one botanical or more than one which are fermented for a month before use. Uniqueness of the practice is the botanical used in these formulations are the one which are locally available in and around the area such as *Artemisia*, Neem, wild fern, Datura, *Schima wallichi* and many more. The practices like using of ashes, lime, neem seed powder, peels of citrus and other botanical preparations for both storage and management of filed pests are the prime component of ITK followed among the people of northeast region.

The indigenous traditional practices have been used in this region for different crops against various diseases since long time. However, these practices and formulations have not been used against the large cardamom diseases. In order to improve, and manage the disease of large cardamom, it is therefore necessary to execute the ITK based management practices to control the blight diseases of large cardamom.

1.5. Organic Sikkim

Sikkim officially announced adoption of organic farming in the year 2003 to convert entire state into organic practice of farming through a declaration in legislative assembly. Now, it is fully certified organic state of India as per the guidelines laid down by National programme on Organic farming. In January 2016 Prime minister, Govt. of India announced officially as the 1st organic state in the country. So chemical pesticide, fungicide or inorganic chemicals are strictly prohibited in the state for any kind of field activity or pest and disease control.

Hence, there are huge scopes of application of organic products for growth enhancement for agricultural crops and disease management. Unfortunately many farmers are not aware of different scientific disease management practices in an organic way. Sikkim is the hot spot of biodiversity at eastern Himalaya and is endowed with rich floral and faunal diversity. Plants are valuable sources of biologically active molecules possessing antimicrobial property. A few botanicals have been reported from Sikkim for management of some fungal diseases (Belbase*et al*. 2018) but the availability of the potential botanicals is still unknown. The present investigation will help to explore different botanicals and their derivatives, having good potential to manage diseases of several crops in varied agricultural practices for sustainable development and economic growth of the local farmers.

Organic farming of Sikkim comprises of many Indigenous knowledge technologies which are generally used by local farmers for plant disease management practices. But the study on the documentation of traditional practices, most importantly ITKs on disease management, has not yet been done systematically and scientifically. In the present study ITK based formulations have been collected based upon reports available and by conducting survey. The techniques have been screened on the basis of their effectiveness and finally identification of active ingredients has been carried out.

1.6. Origin of the problem

Overall large cardamom production in Sikkim, with respect to area, total production and yield per hector in the past two decades is depicted in the table 3. It shows that in the first phase of five years a rise in area and production was

recorded but the increment was negligible. In 2000-01 total cultivated area under large cardamom in Sikkim was 18148 hector, with total production 3540 Tonnes. In the year 2003-04, the state experienced maximum area (23513 ha) with large cardamom plantation, and maximum production (5037 Tonnes) in 2004-05. But during this period a wide spread viral disease out broke in the area and affected the production (Gopi *et al.* 2018). To improve the situation the Spice Board, Govt. of India and the Horticulture and Cash Crops Development Department, Govt. of Sikkim initiated and implemented several welfare programmes in the region. Under those programmes, good quality planting materials were produced which were made available to the growers with subsidy. Growers were encouraged to take up replantation of old and uneconomic gardens with certified saplings. Further, the board supported small and marginal growers with subsidy for replanting. Subsidy also provided for initial three years of gestation period for new plantations (Anonymous 2010-18). Despite all the incentives and activities after 2004-05 a sharp decline in terms of area and production in the large cardamom sector was recorded and continued till 2013-14, for a span of 10 years. During this bad spell, least plantation was recorded in 2011-12 with 15502 ha production area, i.e., 34% lower to that of 2003-04 and production 3237 Tonnes, 36% lower to that of 2004-05. Hence, the fall in the yield and quality of large cardamom are creating an apprehension about the future of the crop in the state. The climatic conditions and fertile soil of Sikkim once were the best to grow this lucrative crop. But at present cardamom cultivation in Sikkim is facing disease problem mainly due to various types of viral and fungal pathogen resulting in drastic reduction in production and quality (Sharma *et al.* 2000, Gurung *et al.* 2020, Joshi and Piya 2019). Reports say various factors are responsible for the situation and this may be

a cumulative effect of climate change, lack of irrigation facilities during dry season, open cultivation, inadequate nutrient management, unscientific methods of cultivation, pests and, diseases, like chirkey, foorkey, leaf blight, leaf spot, anthracnose, wilt, collar rot, capsule rot and leaf streak, etc. (Sharma *et al.* 2000, 2008, Gurung *et al.* 2020, Gopi *et al.* 2018, Anonymous 2010-19).

The emerging climatic changes always brought disadvantages to the ecosystem. Gradually the changed climatic factor becomes critical for many unusual variations in natural condition which ultimately becomes the breeding ground for many harmful pathogens. Likewise with reference to the farming community, there may be emergence of new and different diseases and pest causing the destruction of the crops. As mentioned, the large cardamom, one of the important cash crops of the Sikkim is now facing severe difficulty due to the climatic change in the region. The crop is slowly losing its productivity experiencing the increased infestation of pest and new diseases. The production has been brought down to the half of what it used to be in the last decade of the nineteenth century (Table 1.3). (Sharma *et al.* 2008, Anonymous 2010, Saju *et al.* 2011**).** Apart from the climatic factors, there are other reasons such as alteration in cultivation practices, inadequate pollination system, incidence of pests and diseases (viral and fungal) etc. those have destabilized the total production of the crop. There has been drastic reduction in production and quality of large cardamom for which the farming communities in the region are facing huge monetary loss.

Reports document that the reduction of large cardamom plantation in the region is due to different kind of diseases, like chirkey, foorkey, leaf blight, leaf spot, anthracnose, wilt, collar rot, capsule rot and leaf streak, etc (Sharma *et al.*

2000, 2008, Saju *et al.* 2011, 2013, Gopi *et al.* 2018, Gurung *et al.* 2020). Studies also revealed that poor agronomic conditions and practices have resulted in an increase of several foliar diseases including leaf blight, leaf spot and leaf streak (Sharma *et al.* 2008, Gopi *et al.* 2018, Gurung *et al.* 2020). If we look back to the history, in the year 1989, leaf streak disease was first noticed in the region and causal organism was identified as *Pestalotiopsis royenae* (Srivastava and Verma 1989). The cited study reported that *P. royenae* caused foliar damages with typical symptoms showing crinkled leaf pattern. Interestingly, the *Golsey* variety having smooth leaf texture was more sensitive to the disease. Initially, the disease symptoms were observed as rectangular spots on the leaf veins showing streak appearance mainly on young leaves and gradually turned into reddish brown with necrotic area (Srivastava and Verma 1989). Recently, another group of scientists also reported leaf streak damage in large cardamom (Gopi *et al.* 2018).

Reports reveal reduction of large cardamom plantation in the region due to various rot diseases among which blight is highly destructive in Sikkim and Darjeeling (Saju *et al.* 2011, 2013, Gopi *et al.* 2019). Initially due to this infection lesions are formed on leaves and pseudostem and then gradually affect pseudostems and become necrotic resulting in lodging and death of tillers. Rot disease was first noticed in large cardamom in the region about three decades ago in late 1990s and was described as some mysterious disease. After more than two decades in 2013 a group of scientists from the Indian Cardamom Research Institute, Gangtok reported that *Colletotrichum gloeosporioides* is responsible for theleaf blight (Saju *et al.* 2013), but microscopic or molecular characterizations were not found in the report. Another group of scientists tried to control the disease using a chemical pesticide, like copper oxychloride with limited success. So far

pathogens like *Fusarium* sp., *Rhizoctonia* sp. are also reported for other fungal diseases of large cardamom (Anonymous 2014).

Since the disease is widespread in these areas, availability of healthy mother plants for the production of healthy planting material is also very difficult. Therefore, new plantations with healthy looking planting materials are also being infected quickly as they may contain germ spores of the fungus. However, till date only few survey and scientific reports are available mainly describing the severity of the problem with no proper identification of the causal organism. Hence, keeping the above mentioned facts in mind, the present study was focused on identification of major fungal pathogenbased on the morphological and molecular characterization so that effective measures could be developed to protect this important crop, the life line for rural hill economy of north east India.

Therefore, the present study was undertaken for identification of causal organism causing the leaf blight disease of large cardamom and management of the same with the microbial agents and ITK based formulation and potential botanicals with the following the objectives:

1. Isolation and identification of causal organism of fungal disease of large cardamom using morphological and molecular markers.
2. *In vitro* screening of botanicals and indigenous knowledge based formulations to be used to control the fungal disease.
3. Effect of botanicals/formulation to control fungal disease in pot experiment in nursery.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Origin of the problem

Agriculture is the backbone of rural India and is the primary economic activity for most of the rural population of the Indian Himalayan states. About 80% of the rural population of Sikkim is dependent on agricultural activity and it serves as the source of livelihood and economic security of the state. Sikkim is enriched with high agro-biodiversity and is home of different kind of major crops such as Ginger, Dalle, Large cardamom, several green vegetables etc. Moreover, the local inhabitants are fond of wild edible vegetables, spices, fruits etc. It is also believed that some wild species of the genus *Amomum* are found only in Sikkim, which suggested that the origin of *Amomum subulatum* is Sikkim Himalayas. Sikkim is home to several crop plants and is also considered as the treasure house of many medicinal plants.

Sikkim is known for large cardamom (*Amomum subulatum* Roxb.), a unique crop indigenous to the region. It is an age old spice crop grown in natural forest in Sikkim. The crop has a pleasant aromatic odor for which it is used in culinary purposes. The crop also possesses some medicinal activity which is used in several diseases and disorders. The state is the largest producer of large cardamom in the country and accounts for almost 85% of total national production. Till the end of nineteenth century, Sikkim enjoyed monopoly in large cardamom cultivation, tread of the crop (Sharma *et al.* 2016, Avasthe *et al.* 2011). Moreover, Sikkim was credited with the distinction of being the largest cardamom growing

area in the country as well as in the world. But for the last few decades large cardamom cultivation in the region is passing through a very bad phase. During this period fall in the yield and quality of large cardamom are creating an apprehension about the future of the crop in the state. The favourable climatic conditions and fertile soil of the mountain regions of Sikkim once was the best to grow for this lucrative crop. But at present cardamom cultivation in Sikkim is facing challenge from disease caused mainly due to various types of viral and fungal pathogen, and resulting in drastic reduction in production and quality (Sharma *et al.* 2000, Joshi and Piya 2019, Gurung *et al.* 2020). Reports say that various factors are responsible for this situation and this may be an effect of climate change, lack of irrigation facilities during dry season, open cultivation, inadequate nutrient management, unscientific methods of cultivation, diseases and pests, and non-availability of quality planting materials for infilling and new plantation, etc. (Srivastava and Verma 1989, Saju *et al.* 2013, Gopi *et al.* 2018, Gurung and Bag 2018, Gurung *et al.* 2020).

Several national agencies like Spice Board, Govt. of India, Regional center of Indian Agriculture Research Institutes (IARI) and Department of Science & Technology, Govt. of Sikkim, and Horticulture department, Govt. of Sikkim etc. are working to control the problem, however, with negligible success. Only few survey and scientific reports are available those mainly describe the severity of the problem but there is no recommendation towards controlling the problem. And the disease persists in the region with the same intensity or even more.

Therefore, keeping the above mentioned facts in mind, the present study was focused on identification of major fungal pathogenbased on the morphological

and molecular characterization so that effective measures could be developed to protect this important crop, a life line for rural hill economy of north east India.

2.2. National status

Large cardamom is one of the major plantation crops in the North Eastern Himalayan region including Sikkim and the Darjeeling hills in India, the eastern part of Nepal, and southern Bhutan (Sharma *et al.* 2000). Sikkim is the largest producer of large cardamom in the country. India is the second largest in the world, after Nepal. Large cardamom is a perennial, shade-loving crop that grows well in high rainfall areas with moderate temperature (10-22°C) (Sharma and Rai 2012, Sharma *et al.* 2000). Generally in this region it is cultivated as an understory crop in association with nitrogen fixing Himalayan alder (*Alnus nepalensis*) and other forest tree species that provide shade. Overall performance of the large cardamom is poor when cultivated under direct sun light.

In terms of economic return, the crop is quite beneficial for the mountain people as it provides high return with low inputs (Sharma *et al.* 2000, 2016).The cardamom based agro forestry is a sustainable land- use practice at various landscape level supporting multiple functions and eco system services. It has major contribution in socio-economic sustainability, functional, cultural, educational and recreational value in addition to employment opportunities in eco-tourism.

Sharma *et al.* (2012) has reported sharp decline of large cardamom production system in Sikkim Himalayas. 60% of the cardamom plantations in Sikkim have become barely productive, resulting in a tremendous decline in cultivated area as well as total production in the state (Anonymous 2002, 2006 & 2010). The income of marginal and cardamom-dependent farmers in the eastern

Himalayan region has dramatically declined, jeopardizing their livelihoods (Sharma *et al.* 2009). Climate change is becoming a well-known phenomenon in the Himalayas and is causing unpredictable and erratic rainfall, warmer weather, early flowering, less snow in the mountains and rapid melting of snow, early onset of summer and monsoon, and the drying up of water sources (Partap and Partap 2009, Chaudhary *et al*. 2011, Sharma and Rai 2012), which is also impacting severely on cardamom-based farming systems.

As per the records of the Spices Board and Horticulture and Cash Crop Development Department, Govt. of Sikkim, during 1999 and 2001 in Sikkim a total 23,484 hectares land was under large cardamom cultivation. Further in 2003 Food Security and Agriculture Department, and the Horticulture and Cash Crop Development Department, Govt. of Sikkim, brought 26,734 hectares land under new plantation under large cardamom expansion mission. As a result Sikkim achieved a record production of 5,152 metric tones in 2004. Thereafter, however, the region is experiencing long dry spells in all successive years and disease incidents also have increased substantially. As a result large cardamom productivity and area under cultivation is decreasing rapidly. In 2007/08 total area under large cardamom plantation was only 12,500 hectares, which is about 45% less than that of 2003 (Anonymous 2019). Experts are in opinion that it is due to various factors like lack of quality planting materials, climate change, lack of irrigation facilities, open cultivation, inadequate nutrient management, non adoption of scientific methods of cultivation, diseases and pests etc. Blight disease of large cardamom is considered one of the major causes leading to this decline in productivity.

The pathogen causing blight with severe and devastating effect in large cardamom plantation in Sikkim and Darjeeling Hills was isolated and identified as *Colletotrichum gloeosporioides.* According to a survey report conducted in 1996, about 44.2-62.9% incidence of the disease was observed in all four districts of Sikkim and Darjeeling Hills. The disease appears generally with the beginning of premonsoon showers followed by clear sunny days during March-May and spreads rapidlyduring rainy season.

Along with various factors, fungal disease in large cardamom is the most serious problem now-a-days causing substantial loss in productivity and economy. Fungal disease in large cardamom was first reported in 1989 (Srivastava and Verma 1989a, b). It was reported that leaf streak and minute grey spots are caused by *Pestalotiopsis versicolor* and *Pestalotiopsis royenae* respectively. There after in 2010 a group of scientists from the Indian Cardamom Research Institute reported that *Colletotrichum gloeosporioides* is responsible for causing the blight and is highly destructive in Sikkim and Darjeeling (Saju *et al.* 2011 & 2013). This fungal disease causes leaf and pseudostem lesions followed by necrosis resulting in death of tillers. In 2011 other group of scientists tried to control the disease using botanicals and chemical pesticide, like Copper Oxychloride with limited success (Saju *et al.* 2011). Viral diseases namely chirke and foorkey of large cardamom are also reported (Vijayan *et al.* 2014). There are other fungal diseases found in large cardamom that have affected the productivity sharply. They are wilt, leaf rot, leaf spot, anthracnose, rust, leaf blight and leaf streak (Srivastava 1995, Srivastava & Verma 1989a). So far other pathogens like *Fusarium* sp., *Rhizoctonia* sp. have been reported for other fungal diseases of large cardamom (Anonymous 2014).

From the very beginning a large number of tribal farmers in Sikkim are in practice of the traditional methods of curing large cardamom. The fungal diseases, particularly the *Colletotrichum* blight and viral diseases *Chirke* and *Foorkey* are major threat to this crop. The farmers in this region have been following indigenous pest and diseases management practices. Basically they follow various cultural practices like, the uprooting of the diseased plants, destroying and taking them to an isolated place, chopping into small pieces and burring in pits for quick decomposition. Traditional pest management practice was followed by the farmers in large cardamom. Ash and leaf extracts are commonly used to manage pest. Leave extract of Titepati, Chillowney, Indreni and Tobacco are used. However, reports on such studies are only a few (Sharma *et al.* 2009, Saju *et al.* 2011, Gopi *et al.* 2016).

Gudade *et al.* 2013has reported the traditional practices of large cardamom in Sikkim and Darjeeling**.** About 3863 MT of large cardamom are being produced annually from 26,459 ha of Sikkim region that is emerging as India's organic large cardamom hub. The cultivation practices adopted by local farmers are traditional and organic. It is eco-friendly, less expensive due to utilization of local resources, knowledge and labour. Six local cultivars *viz*, *Ramsey, Ramla, Sawney, Varlangey, Seremna*, *Dzongu Golsey* are grown as per location and altitude respectively.

Therefore, a concerted effort from all stake holders and scientific interventions are necessary for the survival of this highly valued crop. But very few reports are available about the control measure of the disease. So, urgent research interventions are required to control the fungal blight with botanicals and Indigenous Knowledge based formulations and scientific validation of botanicals.

To precede further work done on the topic has been reviewed in the following paragraphs.

2.3. International status

No such scientific report regarding the problem has been found in the literature. In 2017 Sony *et al.* have reported severe disease infestation in large cardamom in eastern Nepal and decline in production. They have described the impact of disease on the farmers' livelihoods and strategies accomplished to manage the disease (Sony *et al.* 2017). In another study which was basically based on survey was conducted in Teharthum District, eastern Nepal to investigate the status of marketing and socioeconomics aspects of large cardamom production in that area (Bhandary and Bhandary 2018). A report appeared in 2019 discussed about the dynamics of large cardamom production, marketing and trade in Bhutan, Nepal, and India (Joshi and Priya 2019). The study was mainly compilation of information based on the data retrieved from several government statistical data base and publications of different researchers. They also conducted a brief field visit in Birtamod, Nepal during September 2017 and reported the on-field primary experiences on the issue. Finally, report highlighted the hardship faced by the farmers and other people associated with activities on large cardamom of Nepal and India due to extensive loss in cardamom yield due to disease infestation, old plantation, poor crop management, and changing climate (Priya and Joshi 2019). Few more activity reports are available published by International Centre for Integrated Mountain Development, Kathmandu, Nepal (ICIMOD), Nepal (Pratap *et al.* 2014).

2.4. Large cardamom farming in Sikkim

There are a number of studies on large cardamom cultivation in Sikkim mostly with reference to the disease management. But very few reports are available about the control measure of the disease. In a detailed study extensive survey was conducted by Sharma *et al.* (2016) covering cardamom growing areas of East, West and South Sikkim. During this study they surveyed Hee-Pechreak and Hee-Martam in West Sikkim, Sumik-Khamdong, Sang-Martam and Dhanbari-Tumin in East Sikkim and Lingee-Shokpay in the South Sikkim. The survey was basically to understand about the role of large cardamom among different ethnic communities living in these areas, with respect to their social, economic and farming relationship, etc. The study revealed that the large cardamom cultivation was considered to be the second largest activity for the family income after the government services. The highest production and household income from the crop were recorded in the Hee-Pechreak village of West Sikkim. This was due to high production of the local cultivar "Sermna" which is considered to be the robust and disease tolerant along with the high yielding capacity. The variety "Dzongugolsey" planted in Lingee- Shokpay was also found to be the effective cultivar that provided the significant off-market acceptability and returns. In addition it was also observed that several areas were suffering with low yield due to the poor-quality planting material, disease and pest incidence etc. According to the growers, drying up of the streams and irrigation sources were the main declining factor followed by inappropriate agronomic practices such as shade trees, low quality planting saplings, poor soil fertility, pollinators, incidence of diseases, pest and old exhausted farms, etc.

In a study Bhattarai *et al.* (2013) conducted a baseline survey in the North Sikkim with the objective to the livelihood improvement through sustainable large cardamom farming among the locals. They observed that with that availability of healthy planting sucker provided by the "Spice Board", ICRI, Govt. of India farmers were able to produce quality cardamom and fetched good market return. The phyto-sanitation of large cardamom fields was also made by using the two bio-agents namely *Pseudomonas fluorescens* and *Bacillus subtilis.* Further, the use of the improved and scientific 'Bhatti' prepared by ICRI, Tadong also helped the local farmers to get high market price as the capsules produced were with good colour. Likewise, Chhetri *et al.* (2014) conducted a field survey in Sikkim and Darjeeling hills about the perception of large cardamom among the local people. They reported that the crop is the main cash crop of these sub Himalayan regions, and luxuriant growth was observed under shade trees. The crop was seen declining at an alarming rate lately.

Since the crop grows is an agroforestry-based system, it is considered to be the ecological and sustainable cultivation system. There are many tree species that serves as the shade tree for large cardamom plantation. The preferred tree species among the farmers are *Alnus nepalansis, Machilus edulis, Melia composite, Schimawallichi, Ficus cunia and Saurauvia nepalensis.* Among the shade trees *Alnus nepalnensis* is one of the excellent shade tree species which is found to be popular as it helps in nitrogen fixing activities and provides nutrient rich litter. The species also provides the additional income to the locals, as fodder, fuel and timber (Singh *et al.*1989).

2.5. Medicinal property of large cardamom

Large cardamom is not only an excellent spice crop, a basic element in the various culinary preparations, confectionaries as flavoring agents but also blessed with many medicinal properties. The fruit of large cardamom is effective against cardiac stimulant, stomachache, throat ache, respiratory problems and several other ailments. It can be used in the field of cosmetic and pharmaceutical industries as well. The seed of large cardamom contains essential oil which is rich in cineol that makes the crop a medicinal one. The seed on steam distillation yields a dark brown essential oil with the odor of cineol. The oil is an excellent anti-microbial agent (Rout *et al.* 2003). Nadkarni *et al.* (1976) reported that the seeds contain the glycosides Petunidin 3,5-diglucoside and leucocynidin-3-O-β-D-glucopyranoside and a new aurone glycoside, subulin along with the presence of chalcone, cardamonin and a flavanone, alpinetin. Gupta *et al.* (1984) have analyzed oil derived from different strains of *A. subulatum* growing wild in Sikkim and found that the 1,8-cineole content varied from 77 to 89%. The large cardamom has a great impact on the human health as it contains many compounds which are anti-oxidant along with many important minerals like potassium, calcium, magnesium, manganese and iron. This essential mineral is known to be the important cell component that acts as a good cardiac stimulant and a powerful free radical scavenger. The anti-oxidant properties are known to prevent different kind of diseases which are widely used in many traditional medicines like antiseptic, carminative, digestive, diuretic, expectorant, simulative and tonic to heart and liver as well. Moreover, the seed of large cardamom can be consumed to get rid of bad breath as it helps in treating the tooth abscesses, gum infection, throat infection and lung congestion. Ravichandran *et al.* (2005) reported that the anti-oxidant present

in large cardamom was proven to have anti-wrinkle activity which can be an important component in the facial cream. Large cardamom is therefore considered as the popular and oldest ingredient in ancient Indian remedies and Ayruveda. The seeds can also be used as against the antidote for scorpion sting and snake bite (Bisht *et al.* 2010). The essential oil and oleoresin are important flavoring agents in flavored liquor, confectionaries etc.

2.6. Economic prospects of large cardamom

In a very important study Sharma *et al.* (2000) showed that the income generation potential of large cardamom based farming is almost double in comparison to maize and potato based farming. They have also compared income potential of cardamom with another important cash crop grows in the area, i.e., ginger and have shown that although the contribution of ginger to the rural economy has increased considerably, still the income from large cardamom farming is much higher.

It is one of the main cash crops cultivated in Sikkim, Darjeeling district of West Bengal, and few other north eastern states in India. The spice crop has played an important role in the rural economy of Sikkim and other north eastern states in the country. More than 80% rural population in the region depends directly or indirectly on large cardamom cultivation for their livings. It grows well in areas with a rainfall of 2000- 4000 mm per annum and air temperature between 10- 22°C (Sharma *et al.* 2000, Sharma and Rai 2012). It can grow in hilly stapes and steep terrains, while land with a moderate slope is better for cultivation of the crop. Deep and well drained loam soil is good for cardamom cultivation.

India is the largest producer of the large cardamom as it contributes 54 percent share in the world production. Sikkim alone produces 80% of India's share

in the production of large cardamom. The crop has high demand in other countries as well and is exported to countries like Pakistan, Singapore and Middle East with good market price (Economic Survey of Sikkim 2006-07). India serves as the biggest market for Nepal and Bhutan for large cardamom. In India important markets for this crop are Kolkata, Guwahti, Amritsar, Kanpur and Delhi. The crop is also grown in some other parts of India like Arunachal Pradesh, Manipur, Nagaland, Mizoram, Assam, some parts of Uttarakhand and Darjeeling hills that have directly or indirectly helped in the income generation for the people in these areas (Avasthe *et al.* 2014, Pratap *et al.* 2014).

2.7. Decline of large cardamom in Sikkim

In agronomy point of view, large cardamom is a shade loving crop that grows healthy under canopy in diffused filtered light. But now-a-days, the open field plantation has become popular in the region where several foliar diseases are very frequent and several new foliar diseases have also been observed. According to the Spice Board, Govt. of India report, in the year 1999 in Sikkim total cultivated area under large cardamom was 23,484 hectares and actual production area was 19,912 ha. Then in 2003 under large cardamom expansion mission, Food Security and Agriculture Department and the Horticulture and Cash Crop Development Department, Govt. of Sikkim, brought 26,734 hectares land under new plantation. This led to achieve a record production of 5,152 metric tonnes in 2004. Thereafter, the region is experiencing long dry spells during winter in successive years and till date the trend is continuing. Due to this drastic change in the weather condition disease incidents also may have increased substantially. As a result, large cardamom productivity and area under cultivation has been decreased rapidly. In

2006 & 2007 total area under large cardamom plantation was only 12,500 hectares, which was 53% lower to that of 2004. Experts are of opinion that there are multiple factors responsible for this drastic decrease in the production in the region, like climate change, alteration in cultivation practices, inadequate pollination system, incidence of pests and diseases (viral and fungal), etc.(Srivastava 1995, Saju *et al.* 2013, Sharma *et al.* 2016, Sony and Upreti 2017, Gopi *et al.* 2008).

Viral disease of large cardamom

The incidence of the disease and pest were always problematic to the large cardamom faming. In 1958 Raychaudhuri and Chatterjee first reported the viral *Chirke* disease from the Darjeeling district of West Bengal (Raychaudhuri and Chatterjee 1958). Then few more reports were also published regarding the viral disease reported from Darjeeling and also from the Sikkim (Raychaudhuri and Chatterjee 1961, Varma and Capoor 1964, Raychaudhuri and Ganguly 1965, Mandal *et al.* 2012, Vijayanandraj*et al.* 2013). In 2014Vijayan and his group evaluated the viral disease in various nurseries and main plantations in Sikkim and Darjeeling district of West Bengal and reported that the *Chirke* disease infection in lower side varied from 0.5% to 6.5% and infection *of foorkey* disease varied from low as 0.5% to high 37.5% (Vijayan *et al.* 2014). Gradually with the erratic climatic behavior disease incidence of Chirkey and Foorkey adversely affected the crop in Sikkim and reduced the productivity substaintially (Sharma *et al.* 2016, Gurung *et al.* 2020).

2.9. Fungal disease of large cardamom

Besides the viral diseases, the crop were witnessing the fungal diseases such as collar and seedling rot caused by *Fusarium oxysporum*, leaf streak caused by *Pestalotiopsis versicolor* and leaf rust caused by *Phakospora elettariae.* Since these fungal diseases were damaging the crop to the extreme level therefore the management of the diseases became a major concern. Lately during 1990s the large cardamom field started to decline at an alarming rate. In the year 1999 the diseases were referred to as *Colletotrichum* blight that really devastated the cultivation of large cardamom (Saju *et al.* 2011, Sharma *et al.* 2016, Gurung *et al.* 2020).

Experts are in opinion that it is due to various factors like lack of quality planting materials, climate change, lack of irrigation facilities, open cultivation, inadequate nutrient management, non-adoption of scientific methods of cultivation, diseases and pests etc. Blight disease of large cardamom is considered as one of the major causes leading to the decline in productivity. The pathogen causing blight with severe and devastating effect in large cardamom plantation in Sikkim and Darjeeling Hills was isolated and identified as *Colletotrichum gloeosporioides.* According to the survey report conducted in 1996 about 44.2-62.9% incidences of the disease was observed in all four districts of Sikkim, and Darjeeling Hills. The disease appears generally with the beginning of pre-monsoon showers followed by clear sunny days during March-May and spreads rapidly during rainy season (Gurung *et al.* 2020).

The leaf blight of large cardamom is considered to be the major fungal diseases that have affected the production and yield of the crop to the substantial level. The disease was later described as the anthracnose in large cardamom from

Sikkim where the crop was cultivated (Srivastava 1989). The disease then increased drastically and started spreading with the high intensity and incidence in the various districts of Sikkim, and Darjeeling hills of west Bengal (Saju *et al.* 2011). The symptoms observed were brownish lesions on the psuedostem at the initial phase that gradually became black. The black psuedostem tends to develop a necrotic region and the leaves get dried and eventually the whole plantation dried up. The pathogen was isolated in the Potato dextrose agar (PDA) and on the basis of growth characteristics was identified as the *Colletotrichum* sp. (Saju *et al.* 2013).

Colletotrichum species are known to cause severe diseases in many crops. The species is particularly endophytes that has been isolated and identified to cause damaging symptoms like dark lesions on the fruit and leaves giving a necrotic appearance with concrete rings. One of the widespread diseases caused by *Colletotrichum* species is anthracnose which is basically a seed and soil borne pathogen for several crops throughout the world. The pathogen is known to cause the damage during seedling phase as well (Kaya *et al.* 2006). The pathogen requires 25 -28°C with the pH of 5.8-6.5 for better growth. The typical feature of the pathogen is that it can survive and remain dormant as long as the season is dry and gets activated as soon as there is a favorable condition. The mode of infection caused was hemibiotrophic mode where biotrophic and necrotrohpic occurs sequentially (Kumari *et al.* 2017). The pathogen grows and sporulates well in various medium like Potato dextrose agar (PDA), Lima bean agar (LBA), malt extract and oat meal agar. Rajeendran *et al.* 2017 has reported that the *Colletotrichum gleoeosporioides* is known to cause infection during seedling emergence and seed germination stages in peanut.

Moral *et al.* (2017) reported that the anthracnose in almond caused by *Colletotrichum* species is reemerging and is endemic to the almond cultivating region. The pathogen was reported with the symptoms associated with the fruit ripening stage, mummification of the fruit and also the premature fruit drop.

The genus *Colletotrichum* is considered to be the one of the important pathogens in the entire world. The pathogen is generally known to cause a disease called anthracnose in wide range of horticultural crops (Hyde *et al.* 2009). Anthracnose is destructive disease that affects the plant in the field as well as after the harvest. There are many reports regarding the post harvest losses caused by the anthracnose. Likewise, anthracnose caused by the different species of *Colletotrichum* is a huge threat and a loss in the pepper cultivation. (Liu *et al.* 2016). The pathogen is the causal agent for anthracnose worldwide, including the anthracnose in mango which is regarded as the dangerous as it has reduced the yield drastically (Zakaria *et al.* 2015).

Another minor pathogen i.e., *Robillarda sessizis* is also known to cause leaf spot in the large cardamom during rainy season. The symptoms observed include the spot those are smaller in size and are surrounded by chlorotic haloes. The favorable atmosphere makes the spot spread very fast giving the soaked appearance on the affected portion. The spot are seen in varying shape and size with the elongated and wavy margin covering the entire leaf. The brown colored spots eventually turns to black spot making the field barren with only the diseased plants. The large cardamom plantation is also affected by different kinds of rot such as spike rot, root rot and collar rot. They are known to be caused by *Rhizoctonia solani* which is considered as the most damaging pathogen in the cultivation. The initial symptoms include water soaked appearance with the brown

margin on the corolla or calyx. This infection later on spreads and fails the flower to bloom from the capsules itself. Hereby the whole spike becomes slimy, soft and watery. The green colour with dull appearance and yellowing of the seedling is the main symptoms of the diseases which lead to the complete drying of roots; collars, spikes, and gradually the plant die in the field (Srivastava 1991).

Studies also revealed that poor agronomic conditions and practices have resulted in an increase of several foliar diseases including leaf blight, leaf spot and leaf streak (Sharma *et al.* 2008, Gopi *et al.* 2018, Gurung *et al.* 2020). Further, if we look back the history, in the year 1989, leaf streak disease was first noticed in the region and causal organism was identified as *Pestalotiopsis royenae*n (Srivastava and Verma 1989). The cited study reported that *P. royenae* caused foliar damages with typical symptoms showing crinkled leaf pattern. Interestingly, the *Golsey* variety having smooth leaf texture was more sensitive to the disease. Initially, the disease symptoms were observed as rectangular spots on the leaf veins showing streak appearance mainly on young leaves and gradually turned into reddish brown with necrotic area (Srivastava and Verma 1989). Recently, another group of scientists also reported the leaf streak damage in large cardamom (Gopi *et al.* 2018). Authors reported that the damages were due to the active involvement of tea mosquito bugs (*Helopeltis theivora*), and not by the pathogen *P. royenae.* Interesting part of the report is that they isolated the pathogen *P. royenae* but the pathogencity test observed no leaf streak disease in the inoculated leaves. It was also reported that the symptoms of leaf streak were initiated in the second day of release of tea mosquito bug on the test plant (Gopi *et al.* 2018). However, till date there is no report regarding the severity of the problem with proper identification of the causal organism.

2.10. Identification of fungal phytopathogen

2.10a. Isolation of fungal pathogens

Phytopathogenic fungi are causing the most damaging diseases in all kind of plants, including economically important crops. Fungal infestation of crops or plants is very dangerous and may cause severe damage to the overall productivity. As a result whole world is witnessing considerable yield losses every year. Fungal pathogens infect almost all the plant parts i.e., roots, stems, leaves, flowers, fruits, seeds etc. (Maheswari and Komalavalli 2013, Sartori *et al.* 2013, Rebecca *et al*. 2012) and induce characteristic disease symptoms like spots, blights, rots, smuts anthracnose, wilts, downy mildew etc. In order to control the disease proper diagnosis, isolation and identification of the pathogens are few essential steps. For the isolation of the pathogen sampling is another important aspect to be performed. Collected samples are brought to the laboratory and then by plant parts of the infected areas are cut into small pieces for the initiation of isolation of the pathogen. Then washing of the explants and followed by surface sterilization and then transferred to PDA media for the fungal growth. The resulted fungi are then purified using the hyphal tips technique on PDA or other specified media for further study.

Media composition, incubation temperature and pH of the media are important characteristics for the growth of fungal phytopathogen. Generally, the most widespread established culture-based morphological techniques are used to identify plant pathogens. Isolation of fungal pathogen and growth, specific culture media are another important requirement in the laboratory. Apart from the composition of the culture media other physiological factors like response of the pathogen to the incubation temperature and pH of the media are also major factors

for the fungal growth (Von Arx 1957, Johnson and Sparrow 1961, Alderman 1982, Okamoto *et al.* 1985, D. J. S. Barr 1987, Smith and Black 1990, Rand 1990, Gunnel and Gubler 1992, Gaddeyya *et al*., 2012,) Generally, nonspecific media supplemented with antibiotics, glucose, yeast extract, peptone, etc. are used and samples are incubated at ambient temperature from which the samples are collected (about 25-30°C). Most common culture media include malt extract agar, potato dextrose agar (PDA), dilute potato dextrose agar, Potato Carrot Agar (PCA), Sabouraud Dextrose Agar (SDA), V8 Juice Agar (VJA), Czapek Dox Agar (CDA) and water agar etc. Medium is selected based on the response and growth of the pathogen (Johnson and Sparrow 1961, Alderman 1982, Okamoto *et al.* 1985, D. J. S. Barr 1987).

In a study Zainab *et al*. (2016) performed the isolation of the fungi causing leaf spots in mango (*Magnifera indica*). They used PDA medium and incubated at 25-28°C under light for 6 days for optimum growth of the fungal pathogen. Anthracnose is an important and major diseases encountered in the banana especially during the ripening stage.

In 2012 a group of researchers isolated the endophytes from the orchid using leaves, petioles and blooms as sample showing the anthracnose symptoms. The pure cultures were obtained by repeated subculture and incubated at 24±1ºC for 3-7 days (Chowdappa *et al.* 2012).

2.10b. Microscopic study

Another most important step includes the purification of isolated fungi using the hyphal tips technique on the suitable medium and then repeated subculture of each isolated fungus on slant medium for future studies and storage at 4˚C. The fungi

are then subjected to identification. Finally, identification of fungal isolates are done on the basis of cultural characteristics, colony morphology, conidia and spores, etc. as described by several scientists (Gilman 1957, Barnett and Hunter 1972, Nelson *et al.* 1982, Pitt and Hocking 1997).

Ghosh *et al.* (2016) reported microscopic characteristics of *Colletotrichum* isolate with fusiform, falcate with acute apices. The conidium of the isolate was recorded with the size ranging from 19.65–21.00 µm in length and 3.0–3.5 µm in breadth, respectively. It was one-celled with cylindrical shape. Similar report stating the morphological characteristics were recorded where the colony was white to orange with slight shades of pink and light grey aerial mycelium. According to Von Arx 1957, *Colletotrichum gloeosporioides* is known to have a wide variation when isolated on different media and different physiological conditions. The fungus was also described with irregular spores with brown to black dots. The conidial spores produced were in variable shape and size with pinkish in colour under the moist condition. The conidia were found to be cylindrical, straight and sometimes oval attached with hyaline conidiophores (Sattar and Malik 1939).

2.10c. Molecular characterization of pathogen

With the current knowledge it is well recognised that the morphological study is not sufficient for authenticated identification of any pathogen including fungal phytopathogens. On the basis of morphological data it is quite difficult to distinguish between the two related species.

As sciences progressed, more and more molecular techniques are being developed. Molecular identification techniques based on the total fungal DNA give

a unique barcode of a particular isolate and are used for the identification of different fungal isolates up to the species level (Landeweert *et al.* 2003). Due to its high accuracy, identification using DNA barcode has become an essential tool for most of the mycologists studying fungal taxonomy, molecular evolution, population genetics or fungus-plant interactions, etc. (Moller *et al*. 1992). The method is based on the sequencing of PCR amplified part of 18S rRNA genes with universal primers specific to the fungal species (Monod *et al*. 2005, Hensel and Holden 1996, Alsohaili and Bani-Hasan 2018). At present several advanced molecular diagnostic techniques are available for the identification of pathogenic fungi. Among which following methods are used extensively by several researchers:

Conventional PCR: Discovery of PCR brought a revolution in the field of phytopathology. It is highly specific for the detection of diseases caused by the microorganism. It is quite rapid and gives precise results, when primer of the specific species is used (Fang and Ramasamy 2015, Henson and French 1993, Compton 1991.

Nested PCR: In this method, two sets of primers are used for multiplication, which increases the yield and specificity of amplification of the target DNA (Tsai *et al.* 2006, Bhat and Browne 2010, Aslam *et al.* 2017).

Multiplex PCR: In this method several pairs of primers are used in a reaction that allows the simultaneous detection of different target sequences of DNA. The

method is quite relevant in plant pathology when plants are infected with more than one pathogens (Dasmahapatra and Mallet 2006, Cho *et al.* 2016).

Reverse transcriptase (RT) PCR: The method gives quantitative data about the pathogen. It is more sensitive than conventional PCR. In this method initially the RNA is reversely transcribed into cDNA with the help of random primers, RT enzyme which is followed by amplification by a PCR (Tang *et al.* 1997, Capote *et al.* 2012). RT-PCR has been used for the detection and quantification of the *Fusarium graminearum* fungi which cause the Fusarium ear blight (FEB) disease in several cereals i.e., wheat, maize, barley, rye, and oat etc. (Brown *et al.* 2011).

Real-time PCR (qPCR): This method is an improved form of the conventional PCR. In this process quantification of DNA and amplification is done simultaneously (Mackay 2004). In this process SYBR Green I or sequence-specific fluorescent-labeled probes are used as the Taq Manprobe for the reactions during amplification (Badali and Nabili 2012). These probes are connected with reporter and quencher dyes attaches to the 5′ end and other on 3′ end, respectively (Dasmahapatra and Mallet 2006).

Serial analysis of gene expression (SAGE): It is comprehensive and sequence-based method for the analysis of quantitative gene expression profile and simultaneous identification of multiple transcripts. The method is based on sequencing and quantification of 15 bp or longer oligonucleotides and similarity of sequences against the available genome sequences to find the corresponding expressed genes (Velculescu *et al.* 1995).

In continuation with the above paragraphs, nowadays, several molecular methods have been developed for the identification of fungal species. DNA barcoding is considered as an important, rapid and accurate method to identify unknown fungal samples. In this approach Internal Transcribed Spacer (ITS) region of nuclear DNA (rDNA) has been designated as primary DNA barcode for the identification of fungi. ITS region is a highly polymorphic noncoding region with adequate taxonomic units. Hence, it is useful to distinguish sequences of fungi upto the species level (Schoch *et al.* 2012, Anonymous 2015, Fajarningsih 2016). The sequences of ITS region of the pathogens are compared with sequences deposited in the GenBank(Benson *et al.* 2012) using BLAST tool. The BLAST provides sequence similarity available in the NCBI GenBank and other sequence databases (Benson *et al.* 2013).

Nowadays multiple genes have been identified such as the internal transcribed spacer rDNA (ITS region), b-tubulin (*TUB, ACT, CAL, CHS, GAPDH* etc. those are successfully used to obtain clear divergence between the closely related species in *Colletotrichum* and other fungal pathogens (Fu *et al.* 2013, Guo *et al.* 2014, Majid et al. 2015, Sun *et al.* 2019, Ramos *et al.* 2019, Gurung *et al.* 2020). For molecular characterization of fungal pathogens, ITS- restriction fragment length polymorphism technique, random amplified polymorphic DNA and sequence analysis of intergenic spacer are used for validation of the genus as well as the species (Gautam *et al.* 2012, Al-Wadai *et al.* 2013, Karthikeyan *et al.*2013, Hadrich *et al.* 2013, Chiotta *et al.* 2011, Kana *et al.* 2013, Spadaro *et al.* 2012, Kizis *et al.* 2014). In 2013, Fu *et al.,* Garibaldi *et al.* in 2020 used the b-tubulin gene for species identification and phylogenetic analysis of b-tubulin sequence as a method for describing new species of *Coletotricum* sp. The use of

the ITS regions along with other gene like β-tubulin sequences was used for the identification. The BLAST analysis revealed that the isolates were identified with 100% similarity with *Colletotrichum asianum*. Similar molecular work was reported for the identification of *Colletotrichum* species causing leaf spot in soybean where random amplified polymorphic DNA (RADP) primers were used for the identification of *Colletotrichum* isolates. The data was supported with the genetic evidence where the isolate had 100% similarity with the *Colletotrichum capsici* isolated from chilly (Ghosh *et al.* 2016). The molecular characterization of the *Colletotrichum gloeosporioides* causing destruction in orchid was reported by Chowdappa *et al.* 2012.

2.11. Botanicals and indigenous knowledge based formulations to control the fungal disease

2.11a. Indigenous traditional knowledge in Sikkim

Sikkim, a small multi-ethnic state, is unique in terms of cultural and biological heritage. The state is inhabited by 14 hill tribes and harbours 4458 species of flowering plants, 506 species of lichens, 480 pteridophytes and 17 gymnosperm owing to varied agro-climatic conditions and farming system. Such diverse ethnic communities, the species richness, rich in culture, customs, and language and traditional practices make Sikkim, a treasure house of indigenous traditional knowledge (ITK) which has major role in agriculture system management (Hussain and Hore 2009, Chhetry *et al.* 2013, Talukdar *et al.* 2012).

According to Wang (1988), ITK is the sum total of knowledge and practices based on people's accumulated experience for dealing with the situations and problems in various aspects of their daily life. In an addition, Warren (1989)

elegantly described indigenous knowledge as kind of information which is acquired from generation after generation as a word of mouth. Sharma *et al.* (2009) described the indigenous technical knowledge as an art of using the natural resources for the improvement of productivity taught by the elderly people in the region. In the other reports scientists elaborated that the indigenous based knowledge is a time tested over a period of time, sustainable, affordable for section of people with less risk to the environment and the farming communities. Therefore, it is very precious in the present scenario of overpopulation, pollution; insecticidal pressure in the environment has to be conserved as the natural resources so that agricultural problems especially for diseases pest management could be managed in an effective way (Arunachalam 2008).

As civilization progressed, this unique and knowledge based farming was disturbed during the green revolution time when several new varieties were introduced as well as application of chemicals started and increased with the time and ages. Gradually farmers were attracted towards the application of chemicals in various forms and at different stages of farming for the early and lucrative return as a result ITK based farming slowly disappeared. According to Atte (1989) the indigenous knowledge is still alive and being practiced within the different ethnic communities worldwide. The indigenous traditional knowledge is unwritten form of knowledge captured by the brains, language, skills that are present in today's world and passed on from generation to generation.

Sikkim is fully organic state and use of synthetic chemicals for agricultural activities is banned. Hence, several indigenous practices are used by the farmers for plant protection measures of major agricultural crops. With the accumulated knowledge and experiences the local inhabitants generally utilize the resources

available in the area especially botanicals. These are eco-friendly, sustainable and relatively cheaper when compared to other options being adopted to mitigate the pest and diseases prevalent in the region. Being as an organic state, there is a strict prohibition and stringent laws are in place on the use of any kind of chemical for management of the major diseases and pest of the crops.

Avasthe *et al.* (2004) reported the use of wood ash for storing of cereals and pulses and concluded that the ash act as a desiccation agent and fills the gaps that restricts the movement of pests during the storage as a result insects gets killed. Further Chandols *et al.* (2011) revealed that the seeds of maize, rice, green gram and other pulses maintained their viability when stored with ash powder. They also observed that the ash not only keeps the pest away but can be used as repellent against the ants, grubs, cut worms in the field. Ash mixed with common salt is the major ingredient used by many farmers of Sikkim to control variety of major pests. The mandarin trunk painted with the white colour is one of the popular practices seen as it helps to keep the orchard away from the infestation of *Phytopthora sp.* responsible for causing gummosis in the plant. It helps to control bark eating caterpillar, trunk borer away from the orchard. Using of salt against the leech and African snail is also another popular techniques followed in Sikkim. Apart from salt, kerosene oil is also considered as an important component as it can be applied for the management of the trunk and stem borer in mandarin. Narayanasamy (2002) illustrated the effect of cow urine against the management of fruit borer and leaf beetle with its powerful repelling activities. The mixture prepared from cow dung extract is also found effective against the ear head bug, leaf folder, stem borer and caterpillar. The cow dung and urine is also considered to be essential nutrient source along with significant repellent potential against the

major pest and diseases. The storage structure made up with bamboo plastered with cow dung and clay known as “bhakari” is meant for storing the grains and large cardamom in the villages. These structures are very helpful and popular in other parts of India as well (Rizwana *et al.* 2011).

Rahman *et al.* (2009) revealed that the mulching practice is considered as a traditional approach due to its effectiveness against the weeds and it protects the main crop from pest and diseases too. Apart from it, mulching also helps to improve germination per cent in ginger crop in Sikkim. The commonly used mulches are forest litter collected from *Artemisia vulagris, schima wallachi* and C*hromolaena odorata,* etc. Litters of *Schima wallachi* used as a mulching material is believed to control the soft rot and bacterial wilt in ginger cultivation (Kumar *et al.* 2012).

The indigenous traditional knowledge based farming also involves the use of local and traditional varieties of superior quality as most farmers in Sikkim prefer to grow their own local cultivar than the high yielding hybrid varieties. Few aromatic rice varieties such as *Krishna bhog, Bhrimphul, Gujribhog, Thaparey, Kalonunia and Nunia* are still being grown by the farmers as they are diseases tolerant (Rahman *et al.* 2011). In case of large cardamom, the variety *Dzongu Golsey* is relatively tolerant to Chirkey disease. Apart from it Sermna, the local cultivar of large cardamom introduced and selected by the local tribe of West Sikkim district is believed to be high yielding and proven to be tolerant to leaf blight disease caused by *Colletotrichum gloeosporioides* (Sharma *et al.* 2016).

The ethnic farming communities are the said source of indigenous traditional practices which are relatively safe for the ecosystem, as well as socially acceptable. The use of plant and animal parts; their derivatives, byproducts etc. are

important ingredients used in traditional practices. The 'Jhum' cultivation is one of the activities of traditional method that has been followed over ages which helps in promoting soil ferity, provide shelters to many bird species, recycling of waste product of the crops etc. (Chhetry *et al.* 2009).

In a study Pradhan *et al.* (2017) documented numerous indigenous traditional knowledge practiced regarding the pest control activities in agricultural system. The plant extract of *Cestrum aurantiacum* mixed with cow dung and fermented over one month was very effective against the underground pest of the crops. Similarly the extract prepared from *Litsaea citrate* (Siltimur) can be applied to the plants to keep the pest away due to its unique odor. It was also been observed that the extract of *Pieris ovalifolia* locally known as Angeri and *Artmesia vulgaris* locally known as titaypati mixed with cow urine were quite effective spray to control the harmful pests in the crop field.

2.11b. Botanicals as the anti fungal agent in *in-vitro* condition

Sikkim is the treasure house of many medicinal and aromatic plants. It is well known fact that, most medicinal and aromatic plants have numerous bio-active compounds that act as anti microbial agents (Mahesh *et al.* 2008). In Sikkim, local people based on their ITK's use several plant based formulations to protect their crops from many diseases in the region. Botanicals are safe due to its biodegradability and non phytotoxicity properties (Talibi *et al.* 2012). Therefore, botanical are the new ray of hope for emerging bio-pesticide industries where the use of the synthetic pesticides can be reduced to some extent. The natural product from the plants can be a source of new compound to the industry for the development of natural pesticides (Satishs *et al.* 2008). Since many plants are

unexplored in the region, so their effectiveness against diseases is still unknown. So, there is an urgent need to undertake systematic study to explore plants to be used as bio-pesticide for management of harmful pest and diseases (Gangadevi *et al.* 2008). The potential of plants for their antifungal properties could be used against the harmful pathogen in the organic farming system and as result; it can become an area of interest for the eco friendly model of disease management.

The traditional practices being followed for the effective crop improvement mainly focus upon the management of pest and diseases so that the crop grows healthy results in high yield. The utilization of the plant parts is one of the important tools used in traditional agricultural practices due to its easy availability among the natural resources. Botanicals can be defined as the plant extract or plant volatile which has a unique property to exhibit the pest and pathogen control activities (Regnault *et al.* 2008). The botanicals can be used in the form of extract, paste and formulation with other substances. The extract can be prepared from water and some organic solvents like ethanol, methanol etc. The plant parts used may differ from the plant species to species (Trucksess *et al.* 2008). The use of the botanicals in form of bio-pesticides has been followed from the Egyptian dynasties (Isman 2006).

At the present time, people are aware about the harmful effect of the chemical pesticides on human health and environment. Hence, the use of botanicals in crop protection is being preferred as it is safe and eco friendly (Regnault *et al.* 2008). The botanical used against for a particular pathogen creates an unfavorable environment to the pathogen and restricts its growth and reproduction cycle (Zaker 2016). Phenols and essential oil obtained from the plant resources caused the damage in the cell membrane of the pathogen and inhibit their

metabolic activities and finally killed the pathogen (Gujar *et al.* 2012). Here it should be mentioned that some botanicals are also harmful to the animal and human health as they contain toxic substances. Few plant species secret rotenone which is harmful to the fish (Cavoski *et al.* 2011); likewise ryanodine is also unsafe compound for mammals as it weakens the cardiac muscles (Hajdu *et al.* 1961).

With the current knowledge and experience, botanicals and its formulations are the most effective alternative which reduces the use of synthetic pesticides and fungicides. Sometimes compounds present in the botanical may be toxic to the insects, and animals therefore the use of these botanical may be utilized as repellent and fumigant.

Konecha *et al.* (2018) examined the effect of *Brassica alba* against the lepidopteran pests namely *Cydiapo monella, Dendro limuspini* and *Spodoptera exigua*. They prepared the different solution out of mustard oil and tween 80 at different concentration. The result revealed that the application of 2-2.5% of the oil is effective as the 100 percent population of the insects was killed. This product can be further used as an alternative for chemical product and used as bio insecticide. In addition to the above statement, Bharadwaj (2012) elaborated the study by using the aqueous extract of twenty different plants against the *Fusarium solani*. The results revealed that the leaf extract of *Lawsonia alba* and *Acacia catechu* has shown a strong suppression against the mycelium growth of the *F. solani*. Likewise the seed of *Dedonia viscosa* also showed a strong anti fungal activity. On the other hand, the extract prepared from *Mimosa hamata, Ocimum sanctum, Acacia arabicae, Jacaranda mimosaefolia* showed a good inhibitory effect against the test fungi.

Senguttuvan *et al.* (2013) reported anti fungal activities of the extract prepared from the medicinal plant i.e., *Hypochaeris radicata* against the two fungal species namely *Aspergillus niger* and *Mucor sp*. Extract was prepared by using leaves and roots in various solvents such as petroleum ether, chloroform, ethyl acetate and methanol and water. The anti fungal activity was performed using the disc diffusion method where the minimum inhibitory concentration (MIC) was determined. They revealed that ethyl acetate extract was the best among the other solvent used against both the fungal species and root extract was more effective than the leaf extract.

Rongai *et al.* (2015) screened the anti fungal activity of some botanical extracts against the pathogen *Fusarium oxysporum*. They have screened twenty four different botanical extracts in the preliminary test, out of which fifteen botanicals proved to have anti fungal properties and six botanicals (*Rivina humulis, Brassica carinata*, *Brunfelsia calyicina, Salvia guaranitica* and *Punica granatum*) showed complete inhibition of mycelium. The extracts with good antifungal extract were enriched with high level of phenols.

The herbs and spices are known to mankind used to enhance the flavor and aroma to the food products. In the experiments it may be concluded that they can preserve food products. Besides these properties, the spices and herbs are known to have anti microbial activities as well (Shelef 1983, Zaikka 1988).

Erturk (2006) tested the anti microbial activity of the some spices using the ethanol extraction against *Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa* along with the fungal species *viz., Candida albicans* and *Aspergillus niger* using agar dilution method. The spices used were *Melissa officinalis, Mentha piperita, Laurus*

nobilis, Rhuscoriaria, Dianthus coryophyllum, Piper nigrum, Capsicum annuum, Juniperus oxycedrus, Erica arborea, Colutea arborescens, and Cuminum cyminum. The *Piper nigrum* and *Erica arborea*were found to be the most effective against the *Pseudomonas aeruginosa* likewise the extractsfrom*Laurus nobilis, Juniperus oxycedrus* and *Colutea arborescens* inhibited the growth of *Candida albicans* and *Aspergillus niger*. The extract performed well and found to be significantly better than the standard fungicide nystatin in effective control.

Similarly, Askarne *et al.* (2012) determined the anti fungal activity using the botanical extract in species like *Anvillea radiate, Asteriscus graveolens, Bubonium odorum, Haliminumum bellatum, Hammada scoparia, Thymus leptobotrys* and *Inula viscose* using the Soxhlet apparatus in different solvent. Their results revealed that extract prepared from *Inula viscose, Asteriscus graveolens, Buboniu modorum* and *Thymus lepto botrys* inhibited the fungal growth with the highest zone of inhibition. The product obtained from botanicals is safer to the environment, human and other animals as well. The bio active compound present in the botanicals are reported to possess the anti fungal potential against many harmful phyto-pathogens (Meepagala *et al.* 2002). The evaluation of different plant extract against the fungal activity was conducted by Ademe *et al.* (2003) prepared ethyl acetate extract from the *Lantana camera* which inhibited growth of *Colletotrichum gloeosporioides*. Rathod *et al.* (2015) reported the anti fungal activity of the medicinal plant namely *Santalum album* and *Terminalia arjuna* against *Candida albicans* and revealed that aqueous and acetonic extract of these plant species were known to have ethno botanical properties.

Therefore, they possess the anti fungal activities and can be an amazing bio fungicide in near future. The presence of phyto-chemical compound like alkaloids,

tannin, flavnoids, and lactones are the probable reason for their anti fungal activity. Timur (*Zanthoxylum armatum*) is considered to be one of the important medicinal cum spice crop in the hilly regions. The crop is indigenous spice of Nepal and it is cultivated in mid western regions. The berry is usually reddish in color with 4-5 mm in diameter. The seed is solitary, globose, shining with bitter taste. The plant is especially used as the flavoring agent in culinary preparations (Kunwar*et al.* 2010). The plant is also used as an anti fungal agent against the fungal species like *Aspergillus sp., Alternanria sp., Pencillium sp., Cladosporium sp. and Helminthosporium sp*. The essential oil obtained from the timur is believed to posses anti fungal activity where the zone of inhibition was recorded the maximum when applied against the *Cladosporium sp.* (Prajapati *et al.* 2014*).*

Zanthoxylum armatum is considered to be one of the most important medicinal plants used in India with ten more related species. The plant is known to have medicinal properties as it contains active ingredient to relieve pain. It plays an important role in therapeutic and pharmaceutical field. Alam *et al.* (2016) determined the antimicrobial, cyto-toxic, phyto-toxi and insecticidal activity of *Zanthoxylum aramatum* and revealed that the plant is an excellent and safe anti microbial and cyto-toxic agent which could be of a great significance in diseases control with respect to human, animal and plant. Maneji *et al.* (2017) demonstrated the antifungal activity of important botanicals like *Lantana camera, Moringa oleifera* and *Tagetes minuta* against the *Rhizopus stolonifer* in *in-vitro* condition. These botanicals were dried and the extract was prepared for the use against the fungal strain following the poisoned food technique. Among the plants *Lantana camera* was found to be most effective as it inhibited the 60% of the

mycelium growth of the test fungi because of phenols, alkanes, and alkenes anhydride and alkyl halide presence.

2.12. Effect of botanicals/formulation to control fungal disease in pot experiment in nursery.

2.12.1Efficacy of botanicals in the field and green house condition

The insects and pests visit on the crops directly or indirectly lead to transmission of many harmful diseases on the field. There are several measures for the management of the diseases by using the synthetic chemicals that helps to control and manage the diseases like blight. But the loss cannot be fully compensated as the field is still getting deteriorated due to the accumulation of the toxicity on field as well as on food products by the chemical pesticides and insecticides (Stangarlin *et al.* 2011). By keeping in the view of human and environment health, the management of the diseases is to be done by an eco-friendly manner. The use of the botanicals and its formulation has to be done on the field as well apart from *in-vitro* condition to observe the direct effect on crop plants. Lengal *et al.* (2017) observed the efficacy of the botanicals and bio control agents on the tomato plants against the blight disease. The plant extract used were capable enough to reduce the population of pest causing blight i.e., whiteflies by 63%. The use of the plant extract proved to be an effective against the early and late blight as the intensity of these two blights were reduced more than the effect of the synthetic pesticides. It is further revealed that the plant extract and bio control agents could be important alternatives to the chemical pesticides and may be incorporated in integrated pest and diseases management of the crops.

The management of harmful pathogens can be done by using the chemical pesticides but due to its residual toxicity to both environment and human health, it therefore becomes harmful to use. The use of plant extracts, their formulations and biological agents such as microbes can be a good and safer choice (Nautiyal 2001). There are various biological agents like *Pseudomonas sp.* which are been used extensively for the control of soil borne pathogens causing damage to the plants (Weller 1988). The use of the *Pseudomonas sp.* has been used extensively and considered as the superior bacteria. The reason for their use is the properties they possess like the adaptive metabolism along with the ability to secrete the compounds that inhibit the several phyto-pathogens (Thomashow and Weller 1990).

Nashwa *et al.* (2012) evaluated different plant extracts and examined their antifungal activity against the late blight disease in greenhouse condition. Total six different plant extracts used in the study were *Ocimum basilicum, Azadirachta indica, Eucalyptus chamadulonsis, Datura stramonium,Nerium oleander* and *Allium sativum*. The powered samples were dissolved in sterile distilled water which was further diluted to 1% and 5% respectively. It was revealed that the extracts were effective against the pathogen in *in-vitro* condition and the most effective result was obtained by the leaf extracts of *Datura stramonium, Azadirachta indica* and *Alliumsativum* at 5% concentration as it caused the highest reduction of mycelial growth of *Alternaria solani*. Likewise, in *in-vivo* condition, the highest reduction of disease severity was recorded by the extracts of *Allium sativum* at 5% concentration and *Daturastramonium* at 1% and 5% concentration. These plant extract can be further analyzed for their compound purification to identify their compound responsible for their antimicrobial potential.

There are investigations under going in order to identify the mechanism responsible for the disease suppression by the use of the plant extract. Vijayan (1989) opined that the presence of the active principle compound in plants directly acts upon the pathogen which might suppress their growth and activity. Bowers and Locke (2004) demonstrated the use of different plant extracts along with some essential oil on the population of *Phytophthora nicotianae* in open as well as in greenhouse condition. The plant extract prepared from pepper and cassia plant were made into a formulation using cinnamon oil and mustard oil and applied under greenhouse condition which reduced the disease significantly.

Amienyo *et al.* (2015) reported the antifungal potential of *Carica papaya* and *Lantana camera* extract for controlling the late blight of potato caused by *Phythopthora infestans.* The extract of these plant were prepared in different concentration ranging from 20, 40 and 60 g/l respectively. It was obvious from the study that the *Carica papaya* extract was more effective than *Lantana camera* with mycelium growth reduction up to 75 %. Similarly, the extract of *Lantana camera* at highest concentration reduced the growth at the rate of 57%.

Trivediv *et al.* (2006) evaluated the *Pseudomonas corrugate* for their antagonistic properties in *in-vitro* condition. The bacterium was basically isolated from the Indian Himalayan Region and the antagonistic properties were examined against the two pathogens namely *Alternaria alternate* and *Fusarium oxysporum*. It was quite clear from the results that the bacterium could not inhibit the growth of fungal species but was successfully reducing the growth of both the species. The production of siderophore, ammonia, lipase and chitinase by the bacterium in the growth medium contributed to the antagonistic activities. Chanda *et al.* (2018) reported the use of bio control agent *viz., Trichoderma hazarium* and *Pseudomonas*

fluroscens for the control of the *Cercospora* leaf spot disease. Both bio control agents showed the good efficacy on the disease with low disease severity in comparison to the fungicide applied plots. *Colletotrichum* species is considered to be the most destructive pathogen that causes blight, anthracnose in many horticultural crops. The management of the species is very difficult and the use of chemical is still a concern for the health issues of both human and the soil. Therefore, there is an urgent need for an effective alternative option which is eco friendly and sustainable for both human and environment. Gwade *et al.* (2009) demonstrated the use of botanical extracts, bio control agents like *Trichoderma viride* and *Verticillium lecanii* and chemical fertilizer against the *Colletotrichum truncatum*. The antifungal activity was performed by following the poisoned food technique in the Potato dextrose agar. The results obtained from the study showed a significant inhibition of mycelium growth with 41.79 and 23.75 %.

The bio-control agents comprise of both fungal and bacterial species. The antagonistic bacteria produce a diverse range of secondary metabolites which help them in various mechanisms like enzyme activity and catalyzing biochemical reaction (Das *et al.* 2006). One of the most popular genus that has received much attention as bio control is *Bacillus* species. The species is considered to produce active antagonistic metabolites, which is also readily available in abundant in soil and is known to survive the adverse environmental condition (Silo-suh*et al.* 1994).

The use of the species i.e., *Bacillus subtilis* has been reported by Collins *et al.* (2003), where the inhibition of the pathogen like *Cercospora beticola* and *Colletotrichum gleosporioides* was done successfully. The *Bacillus* species are used in an extensive way in the formulation form and used especially against the fungal strain in both *in-vitro* and field condition (Chumthong *et al.* 2008). The use

of bio control agents especially *Trichoderma hazarium* against the damping off caused by *phythium aphanidermatum* has been found (Fajola *et al.* 1974). Likewise the other important bio control agent, *Bacillus subtilis* is also being used equally and reported in controlling many pathogens in crops (Asaka 1996). The combination of diseases is known to cause the severe damages in tobacco plant like bacterial wilt caused by *Ralstonia solanacearum*, damping off by *Pythium aphanidermatum* and leaf spot by *Cercospora nicotiana*. The disease in tobacco is reported to be managed by the application of *Bacillus subtilis* and *Trichoderma harzianum* individually as well as in combination dose. The combined effect of these two bio control was appreciable against all the disease. However, the individual effect was not seen and recorded at appreciable level (Maketon *et al.* 2008).

Large cardamom being one of the important cash crops of Sikkim is losing its yield and production at an alarming rate. The state is moreover an organic state where the chemical fertilizer is strictly prohibited for the use in agro technology. Therefore, the use of biological control can be treated as best alternative measure to control the fungal diseases in the major cash crop large cardamom. *Psuedomanas fluroscens* is a plant growth promoting rhizobacteria found in the root zone of the plants known to accelerate the growth and multiplication capacity of plants, cause hyper parasitism and competition to other microbes and antagonistic activity against the fungal pathogen. Vijayan *et al.* (2013) reported the use of the bio control agent *i.e.Pseudomonas fluorscens* against the fungal disease in large cardamom and revealed that the bio control was able to suppress the pathogen growth.

CHAPTER 3

MATERIALS AND METHODS

3.1. Isolation and identification of causal organism of fungal disease of large cardamom using morphological and molecular markers.

3.1.1. Survey to large cardamom farming areas in Sikkim

The survey was conducted in 50 villages distributed in all the four districts of Sikkim during 2017-18. The main purpose of the study was to explore the present condition of the infestation of the diseases focusing mainly on the fungal infestation in the large cardamom cultivation in the region. During the field survey blight disease causing damage to the crop and infection index were recorded.

For this study quadrates measuring $1\times1m^2$ was used for scoring. In each plot, five quadrates were made randomly with at least 3-4 plants in each. Infection was recorded by taking account of five infected leaves from each plant of the quadrate. The infection index or disease severity was recorded based on the severity scale as described by Sharma and Kolte 1994, with slight modification. Periodic observations at one month interval for a year were also recorded in a cardamom field at the village Assam Linzey, East Sikkim, to understand the dynamics of cyclic epidemics of blights with seasonal variation.

Secondary data used in the study were collected from various reports of government agencies, Spice Board, institutions, etc. In addition to this data were compiled from various literatures, research findings etc.

Table 3.1: Disease descriptive scale

Rating	Description	Infection stage
0	No visible symptom observed on leaf (Disease free healthy plants)	stage –0
2	Visible brown spot with <1% leaf area affected (Infection)	stage –I
4	Brown sunken spots with 1-10% leaf area affected (Infection)	stage –II
6	Brown spots with 11-25% leaf area affected (Moderately infected)	stage –III
8	Spot seen with 26-50% leaf area affected (Highly infected)	stage –IV
10	>51% leaf area affected with circular to irregular spots (Severely infected)	stage –V

$$\textbf{Disease severity or Infection index (\%)} = \frac{\text{Sum of all the disease rating}}{\text{Total no. of rating x maximum disease grade}} \times 100$$

3.1.2. Sample collection:

For sampling, infected leaves of large cardamom showing the blighted or the streak appearance was considered as a sample. During the study samples were collected from 50 villages of all the four districts of Sikkim following random sampling method. Infected leaves were cut from the diseased plants with sterilized scissors, kept in air tight sample bags with proper numbering and brought to the laboratory and stored at 4°C until further study.

3.1.3 Isolation of pathogenic fungi:

The pathogenic fungi were isolated from the rectangular spots of leaves of large cardamom having symptoms of streak appearance, by culturing on potato dextrose agar (PDA, Hi-media, India) (nutrient medium) and incubated at 25°C following the protocol as described by Pandey and Palni (1998) and Gurung et al. (2020). For isolation, leaves were cut into small pieces, washed thoroughly with running tap for about 10 min, then were taken to the laminar air flow chamber in a 500 ml beaker (Borosil, India). Samples were then treated with 0.1% mercuric chloride solution for 1 min. After this treatment, repeated washing was performed with sterilized distilled water at least for three times to remove traces of mercuric chloride from leaf pieces. Finally, these treated leaf pieces were further trimmed from all the margins and inoculated on Petri plates (100mm, Borosil) containing nutrient medium. After inoculation, plates were sealed with Parafilm-M (Tarson, India) and kept for incubation for five days at 25°C in dark. Six-seven repeated sub-culturing using single mycelium as inoculum were performed on PDA in Petri plates to obtain the pure culture. Thus obtained pure culture was then transferred to the fresh PDA slants in 60 ml culture tube, tightened with screw cap (Borosil, India) containing 25 ml medium each, incubated at 25°C for one day and then kept at 4°C for storage for further use.

3.1.4. Morphological characterization

Effect of different medium on growth and colony morphology of the fungal isolate

Growth characteristics on solid media

The morphological characterization of the isolate (AsLES-2a) was done on the basis of colony features like size, colour, pigmentation, and growth response on

different growth medium. In the study five different types of media, i.e., Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Sabouraud Dextrose Agar (SDA), V8 Juice Agar (VJA), and Czapek Dox Agar (CDA) (Hi-media, India) were tested for the morphological study of the isolate. Colony diameter was measured everyday at 2 pm for 7 days and growth rate was calculated as mean daily growth (mm per day). After 7 days, colony diameter, colony colour (front and back view) and pigmentation of culture were also recorded.

3.1.5 Physiological characteristics:

3.1.5a. Temperature requirement of the fungal isolates

Among the physical requirement, temperature is considered to be the most important factor affecting the growth of isolates. The Potato dextrose agar (PDA) was used as the medium for the experiment. Then isolates were further incubated at wide range of temperature ranging from 5 to 45° C. The growth of the isolates was observed and the data were taken after 5 days of incubation.

3.1.5b. pH requirement

To determine the best pH requirement of the isolate a detail experiment was conducted using a range of pH of the (PDA). The different pH levels were set ranging from 3-13.0 on the PDA plates expect 3, 11 and 13 were prepared in PD broth. The pH levels were adjusted using 1N Hydrogen chloride (HCL) and sodium hydro oxide (NaOH). The medium was autoclaved for sterilization and the cultures were inoculated and incubated at 25°C for 5 days. After five days, the maximum growth of colony was recorded for the appropriate results.

3.1.6. Microscopic characterization of the fungal isolates

Slide preparation

The fungal morphology was studied macroscopically. For the preparation of the slide, clean sterilized slides, cover slips, lop and needles were used. To determine the vegetative and reproductive structures, fungal isolate was grown for 72 h on PDA. A small portion of the mycelium was taken from the freshly grown isolate and mounted on microscopic glass slides, stained with lacto phenol cotton blue and observed under compound microscope (Labophot-2, Nikon, Japan). Microscopic structures i.e., hyphae, conidia, conidiophores and arrangement of spores were characterized, measured and photographed. Detail procedure is as follows:

Procedure

a) A loop filled with lacto phenol was taken and placed on the centre of clean glass slide.
b) After that a pinch of fresh culture from the PDA plate was transferred to the glass slide.
c) Mycelia were separated with sterilized needle.
d) Then lacto phenol cotton blue stain was added to the slide and mixed well.
e) A cover glass was placed over the sample and pressed gently so that mycelia and spores spread uniformly.
f) Now the slide was placed under the compound microscope (Labophot-2, Nikon, Japan) for observation.
g) Then microscopic structures like hyphae, conidia, conidiophores and arrangement of spores were characterized, measured and photographed.

h) Finally, isolates were sent to the Agharkar Research institute Fungus Culture Collection (ARIFCC), Pune, India for physical authentication, molecular characterization and identification.

3.1.7. Pathogenicity confirmation:

The pathogenicity conformity experiment was conducted on healthy grown large cardamom in the poly house, at 6th mile, Gangtok (25.85°N, 93.77°E, and 1120 m amsl) following the Koch' postulates (Koch 1876). The temperature of the poly house was normal ambient temperature (25-28°C) as available in the region.

Fresh conidial suspension was prepared from growing culture to initiate infection on healthy test cardamom plants. Lawn of five days old fully grown culture was poured with 5 ml sterilized distilled water; the suspension was then harvested by agitating the fungal lawn with the help of sterile glass rod. Then suspension was sieved with a clean muslin cloth and removed agar and mycelium from the suspension. Spores available in the suspension were determined by hemocytometer. The final conidial concentration in the suspension was maintained 1×10^4 ml^{-1} for inoculation. Then healthy test plants were sprayed with conidial suspension. The control plants were sprayed with distilled water, kept at a safe distances to prevent any kind of contamination. After about 8-10 days of inoculation, a first symptom of infection was noticed, and gradually number of stark lesions increased on the leaf surface. Then, after 15 days of inoculation, infected leaves with spots were collected for isolation of pathogen as mentioned in the previous section.

3.1.8 Molecular identification of fungal isolate

Molecular identification of isolated fungal pathogen was carried out based on Internal Transcribed Spacer (ITS) DNA amplification and sequence analysis.

3.1.8a. Isolation of genomic DNA

The genomic DNA was extracted from seven days old fungal culture grown in potato dextrose agar plate. The fungus mass from culture agar plate was scraped out with the help of sterilized fine spatula. The fungal mass was then placed in a 2mL centrifuge tube containing a ceramic pestle, 60-80 mg sterile glass beads and lysis buffer (100mM Tris HCL (pH 8.0), 50mM EDTA, 3% SDS). Homogenization of fungal mass was done twice in a vortex machine (Eppendorf, India) at 10 minutes. The resulting fungal tissue homogenate was centrifuge at 13,000 rpm for 10 minutes and the supernatant was transferred to a new micro centrifuge tube. To the supernatant, 2 µL of RNase A (10mg/mL) was added and incubated at 37°C for 15 minutes. After that equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) was added and mixed, followed by centrifugation at 13, 000 rpm for 10 minutes. The upper aqueous layer was taken into a fresh microcentrifuge tube and then adds equal volume of 100% ethanol was added. Following precipitation at -20°C for 30 minutes, the whole content was again centrifuge at 12,000 rpm for 10 minutes to pellet down the DNA. The DNA pellets was final was with 70% ethanol and centrifuge at 12,000 rpm for 5 minutes. The DNA pellet was air dried and dissolves in 1x TAE buffer.

3.1.8b. PCR Amplification of ITS regions

Polymerase chain reaction (PCR) is basically a technique to produce high copy number of DNA fragments from the source DNA which is mainly carried out by the polymerase enzyme that synthesizes the specific DNA segments by the provided primers (short DNA sequences Complementary to the desired gene). It is followed by three main steps. The first step requires high temperature near about 95°C to melt the DNA in the single strands. It is then followed by the involvement of comparatively lower temperature near about 50°C to facilitate the binding of primers to the desired DNA fragment. In the third step, the polymerase starts copying the DNA fragment optimally at 72°C. The three steps cycles are repeated to achieve the higher copy number. After the reaction, the amplified products are run on agarose gel for the verification and analysis.In the present study, internal Transcribed Spacer (ITS) genes were PCR amplified using two universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Fu et al. 2013; Guo et al. 2014; Duan et al. 2019). PCR was carried out in a total volume of 50 µl using 2.5 µl each dNTPs, 2 µl $MgCl_2$, 2 µl template DNA, 1 µl each primer (ITS4 and ITS5), 1 µl Taq DNA polymerase, and 33 µl nuclease free water (Himedia). Reactions were performed in the Master cycler gradient (Eppendorf, Germany) with following reaction conditions; 95 °C for 5 min. for initial denaturation followed by 30 cycle of 95°C for 30 sec., 55°C for 1 min, 72°C for 1 min and the final extension at 72°C for 10 minutes. The PCR product was purified with the Hi-PurA^{TM} PCR clean up kit (Himedia, India) and sequenced by ABI Applied Biosystems (35000 Genetic Analyzer, SeqGen, Inc. 1725 Del Amo BlvdTorrance, CA 9001, USA) using each of ITS4 and ITS5.

3.1.8c. Evolutionary analysis by Maximum Likelihood method

Raw sequence obtained was manually edited for inconsistency. The retrieved sequence was assembled and aligned using Coden-Code Aligner software and compared using NCBI BLASTn tool (Altschul et al. 1990). The phylogenetic tree was created by using the Maximum Likelihood (ML) method and Kimura 2-parameter model (Kimura 1980) using MEGA X software (Kumar et al. 2018). Clade stability of the phylogenetic tree was according to bootstrap analysis with 1000 replicates (Kishino and Hasegawa 1989). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 516 positions in the final dataset. The sequences of ITS region of the present isolate were compared with *Colletotrichum* spp. sequences deposited in the GenBank(Benson et al. 2012). The identified sequence was submitted to the NCBI gene bank for the isolate accession number.

3.2. *In vitro* screening of botanicals and indigenous knowledge based formulations to control the fungal disease of large cardamom

3.2. 1. Collection of the botanicals

The sample for the experiment was collected from different places of Sikkim. The parameters for the selection of the botanicals were based on the farmer's perception recorded through structured questionnaire(Table 3.2),availability of the plant in the region, and secondary sources. Initially, fifty botanicals were collected, and screened for their anti-fungal potential. Based on the basis of the efficacy of anti fungal effects finally 24 botanicals were taken for further study (Table 4.28).

Table 3.2: Questionnaire used for field survey to understand farmer's perception, and identification of botanicals/ plants are in use to control disease of large cardamom grown in Sikkim

Date:

Personal information:

Sl. No.	Particular	Response of the farmer
1.	Name of the respondent/ farmer:	
2.	Age and gender:	
3.	Community:	
4.	Village Name:	Altitude
5.	Education qualification:	
6.	Family members:	Male: Female:
7.	Source of income (Rs.):	Farming: Other :

Large cardamom details:

8.	Total agricultural land owned by you?	
9.	What are the crops grown in your field?	
10.	What is the total area under cardamom cultivation?	
11.	Mention the cardamom cultivation variety grown in your field?	
12.	From where did you get the saplings?	
13.	Is the saplings distributed by the government?	If yes Year Price/sapling
14.	Mention the yield and price	

15.	Describe the present condition of large cardamom cultivation?	
16.	Describe the changes in the cultivation compared to 10-20 years back in respect to the health, yield and price?	
	Is the plants are healthy at present?	If no What is the problem?
	Have you noticed any disease/ pest?	Yes No
17.	Categorized the disease?	Viral Bacterial Fungal
18.	Mention the specific symptoms regarding the disease?	
19.	Which month is the most prevalent month for the diseases?	
20.	What is the probable cause of the diseases in the crop?	
21.	How it spreads, if possible?	
22.	% crop loss occurred due to the disease?	

Management details:

25.	What is the management practices being followed to control disease? Please specify?	
26.	What is the organic solution(s) are	

	being used to mitigate the disease?	
27.	What is the ITK being practiced for the diseases management?	
28.	How did you get the idea?	
29.	From whom you learned this ITK?	
30.	How many farmers of your village practice the ITK you follow?	
31.	Any idea regarding the botanical used for the disease management in large cardamom?	Yes No
32.	If yes, mention name of botanicals/ plants are being used?	
33.	If yes, procedure for preparation of the botanicals and the formulation?	
34.	Status of plants used in ITK (Abundant/ Rare/ Least available	
35.	Are the botanicals/ formulations applied for other crops also?	
36.	Rate of effectiveness of the botanicals? Advantages / disadvantages?	
37.	Please mention government initiative to improve large cardamom cultivation?	
38.	Training for the preparation of biopesticides or botanicals?	

39.	Opinion about the crop	
40.	Any other information	

(Kabita Gurung) (Signature of the respondent)

Declaration and No Objection:

I ……..…., residence of Village ……..……..., Dist. …… have shared my experience and knowledge regarding Indigenous Traditional Knowledge (ITK) in complete consciousness and sense to Miss Kabita Gurung. I do not have any objection for using this information for her research or other activities.

Date: (Signature with full name)

घोषणा एवम् अनापत्ति

म ....., गाउँ..... जिल्ला ..... निवासी, आफ्नो खुसी अनि स्वस्थ्य मानसिकताले जातीय पारम्परिक ज्ञानका (ITK) केही जानकारी सुश्री कविता गुरूङ, शोधार्थी सिक्किम विश्वविद्यालयसँग साझा गरिरहेको/गरिरहेकी छु। मैले दिएका जानकारीहरू उनले आफ्नो शोध कार्यमा प्रयोग गर्न सक्छिन् र यसमा मलाई कुनै आपत्ति छैन भनी घोषणा गर्दछु।

दिनाङ्क पूरा नाम अनि हस्ताक्षर

3.2.2. Preparation of sample for the extraction

Collected botanicals were brought to the laboratory for further study. At first all the botanical(s) were washed thoroughly with the running tap water to remove the dirt particles. They were further rinsed with distilled water, placed on blotting papers and kept in shade for drying. After the samples were dried completely, were grinded to form powder with the help of mortar and pestle. The powdered samples were then stored in air tight sample containers for the further use.

3.2.3. Preparation aqueous and solvent extraction

The extracts were prepared following the maceration method using distilled water and five different solvents. The solvents used in this experiment wereethanol, dichloromethane (DCM), acetone, ethyl acetate and chloroform (Merck, India).

Extracts of these samples were prepared by maceration method. For maceration, 2g powdered sample of each botanical was taken separately in 30 ml culture tube having screw cap (Borosil, India).Five solvents and distilled water (10 ml each) were added into the culture tubes andtightened with screw caps. The culture tubes were then placed on a rotary shaker at 150 rpm (REMI, India)for 24 hours to dissolve the powders uniformly. After 24 hours shaking extracts were taken out from the culture tubes and filtered through Whatman No.1 filter papers. Filtrates were collected in sterilized tubes. Final volume of the aliquots was makeup each to 10 ml by adding appropriate quantity of the respective solvents, tightened with screw caps and kept at 4°C for further use.

3.2.4. Determination of antifungal potential of different botanicals *in vitro*

3.2.4a. Disc diffusion method

To determine the qualitative estimation of antifungal potential of different extracts, PDA plate based bioassays were performed using disc diffusion method as described by Adhikari *et al.* 2018.For this experiment *Colletotrichumgloeosporioides* was used as test pathogen. In the first step of the experiment, fungal suspension of*C.gloeosporioides* was prepared form the freshly grown pathogen in potato dextrose broth. 100 µl of the test organism was spread uniformly on the PDA plates with the help of a sterile glass spreader.

Sterilized filter paper (Whatman No. 1) discs of 5 mm diameter were placed on the PDA surface with the help of autoclaved forceps. Then 15 µl of extract was loaded over the filter disc already kept on the PDA plates. All the plates were kept at 25ºC for incubation. The results were recorded after 120 hours of incubation on the basis of zone of inhibition (mm). All the experiments were performed in triplicate and repeated once.

3.2.4b. Determinationof Minimum Inhibitory Concentration (MIC)

For the quantitative analysis, Minimum Inhibitory Concentration(MIC) of the extracts was determined using different concentration of extracts in the medium.Procedure as described by the Clinical and LaboratoryInstitute Methodology (Phongpaichit*et al.* 2005, Rex *et al.* 2008) was followed to determine the MIC. During this experiment, concentration of extract used was 100-1000 µg/ml for MIC determination.In a sterilized test tube1 ml extract of required concentration, 1 ml test pathogenand 8 ml potato dextrose broth (PDB) were taken,

mixed uniformly and incubated at 25°C for 120 hours. In this experiment controls were of two sets, one with the broth and the test pathogen and other with the broth and the extract. After 24 h incubation the MIC value was determined on the basis of the lowest concentration of the extract that showed complete inhibition of growth of the test pathogen in the culture tubes. The absolute inhibition of growth of the pathogen was again confirmed by taking inoculums from experimental tubes and plating on PDA media.

3.2.5. Use of the bio control agents against the test pathogen *C. gloeosporioides*

Bacterial cultures used for the experiment:

The cultures of *Bacillus subtilis, Psudomonas corrugata* and *Tricodermagamsii* used in this study. These antagonistic strains were obtained from the microbial culture collection centre of the microbiology laboratory, GBPHIHED, Almora (Curtsy: Dr. Anita Pandey). The bacterial cultures were revived taking the isolates from slants maintained at 4°C by using repeated subculturing at 4 days interval on the tryptophan yeast extract (TYE) agar medium incubated at 25°C. After five subcultures the cultures were revived with normal growth and then these cultures were used for further experiments.

3.2.5a.Dual cultures for determination of the production of diffusible anti fungal compound

The isolates were tested for the production of antagonistic compound by dual culture method as described by Chawrasia *et al.*(2005). To test the ability of the isolates for inhibition of the pathogen, a disc of test fungus mycelium of about 8.0

mm diameter from the freshly grown 5-days old culture was placed off the centre on each Potato Carrot Agar (PCA) plate. Then, on the same plate the inoculation of the test pathogenwas done at about 2.0-2.5 cm away from the fungal disc. Petri plates were incubated at 25°C for 5 days.Datawere recorded after 5 days of culture by measuring the percentage of inhibition.

Growth inhibition (%) was calculated using the following formula:

$$\frac{R1 - R2}{R1} \times 100$$

Where R1 represents the radius of colony growth of the test fungal pathogen in the control plate.R2 represents radius of fungal pathogen colony in the plate co-cultivated with the bacterial colony.

3.2.5b. Production of volatile anti-fungal compound

Each bacterial isolate was inoculated on one half of a Petri dish containing Potato Carrot Agar (PCA)and a 6.0mm disc of 5 days old culture of the test fungal pathogen was placed in the center of other half of the plate. Both the half dishes were placed face to face preventing any physical contact between the pathogen and the bacterial suspension, sealed with parafilm.Inoculated plates were incubated at 25°C for 5 days.Growth of the pathogen was measured and compared with the controls developed in absence of the antagonistic bacteria. Results were expressed as means of percent inhibition of growth of the pathogen in the presence or absence of any antagonistic bacterial isolates.

3.3. Effect of botanicals/ formulations to control fungal disease in pot experiments in nursery

3.3.1. Experimental material

Botanicals and microbial isolates

The botanicals foundmaximum promising antagonistic effect on the test pathogen in the *in vitro*assay, were used for this experiment.Similarly promising microbial isolates were taken for the experiment; those had shown the ability of inhibition of the pathogen. Two botanicals and two microbial isolates were used for the poly house experiment in the nursery at 6th mile, Gangtok (25.85°N, 93.77°E, 1120 m amsl).

a) Botanicals: Plant extracts of *Zanthoxylum aramatum* and *Lantana camera*.
b) Bacterial isolated: *Bascillus subtilis* and *Trichoderma gamsii*
c) Test pathogen: *Colletotrichum gloeosporioides* (NFCCI4542).

Plant material

Tissue cultured raised large cardamom plantlets were used as an experimental material during the present study. The plants were raised using the MS media supplemented with phytohormones (Courtesy: Tissue culture laboratory, Govt. of Sikkim). The plants were hardened in the pot trays with the mosses as the medium. One month old hardened plantswere brought to the nursery and keptin a sanitized poly house to prevent any kind of insect or microbial attack. In the beginning all the plantswere transplanted to plastic pots (9×9 cm) filled with sterilized soil (pH 6.0). The soil was sterilized by autoclaving at 121°Cfor 15 psi for one and halfhours. After three days of the soil preparation plantlets were transplanted and

allowed to grow for the further experiment. Regular watering of the plants was done at 15 days interval and whenever required.

3.3.2. Challenge inoculation of experiment under ploy house condition

The experiment was conducted for a period ofone and half years started in the May 2019. The experimental design was laid in randomized block design (RBD). Four different treatments were performed for this study, with threereplications. In each treatment a block of 15 plants (5 plants in a row x 3 rows) were used. Distance between pots was 20 cm. and between the blocks was45cm. For each experiment total five blocks were taken including one controlblock. The control block was kept in isolation and 100 cm away from the rest of the blocks to avoid any kind of contamination.

The temperature of the poly house was recorded everyday and it was maintained at 25-28°C throughout the experiment. Sometimes during the bright sunny day, when rise intemperature was noticed, the soil of the poly house was watered to cool it down to 25-28°C.

Treatment detail

Lay out of treatments are as follows:

a. Control: (i.e.) sterilized soil + TC plants.
b. Sterilized soil +TC plants +pathogen spore (*C.gloeosporioides*)
c. Sterilized soil +TC plants +pathogen+ treatments-1 (*Zantoxylum aramatum)*
d. Sterilized soil +TC plants +pathogen+ treatments-2 (*Lantana camera*)
e. Sterilized soil +TC plants +pathogen+ treatments-3 (*Trichoderma gamsii*)
f. Sterilized soil +TC plants +pathogen+ treatments-4 (*Bacillus subtilis*)

3.3.3. Challenge inoculation of pathogen and effect of botanicals and bio-control agents to control the pathogen

Fresh spore suspension of the test pathogen (*C. gloeosporioides*) was prepared as described in the previous subsection (3.1.6 Pathogenicity confirmation). The suspension of the pathogen having 1×10^4 ml^{-1} conidial spores was used to create infections on the healthy TC raised cardamom saplings. Initially about $1cm^2$on the upper surface of healthy leaves of the plants were rubbed with dry sterilized absorbent cotton.A smear of conidial suspension of the pathogen was taken with sterilized cotton and stroke on the same portion of the already rubbed leaves. Inoculated leaves were allowed to dry for 10 minutes. All the inoculated plants were covered with the polythene bags each for 48 hours. All the experiments were performed in triplicates and five plants were taken in each replication. For control rubbing of the leaves was performed and the leaves were sprayed with distilled water. The block of the control plants was kept in an isolation to avoid any kind of contamination. In another block plants infected with the pathogenic inoculum but without any treatment were considered as negative control.

Treatments ofbotanical and bio-control agents against pathogen were givenas foliar spray. In this study two sets of spray were designed, in one set all the botanical and bio-control agents were prayed 4 days before inoculation. In the other set first spray was given after the removal of the polythene cover after inoculation of pathogen, i.e., 2 days after the inoculation. Then for both the sets treatments were repeated in 15 days interval and continued for six months.

Disease incidence and pathogenicity of the pathogen were performed as described earlier. Percent diseases in control was determined using the formula described by Walker 1988 as follows:

$$PDC = \frac{\text{PDI in control} - \text{PDI in treatment}}{\text{PDI in control}} \times 100$$

(PDC = Percent Disease Control; PDI = Percent Disease Incidence)

Data were recorded in one month interval and continued for 11 months. The growth parameters of the plant were also recorded with response to each treatment.

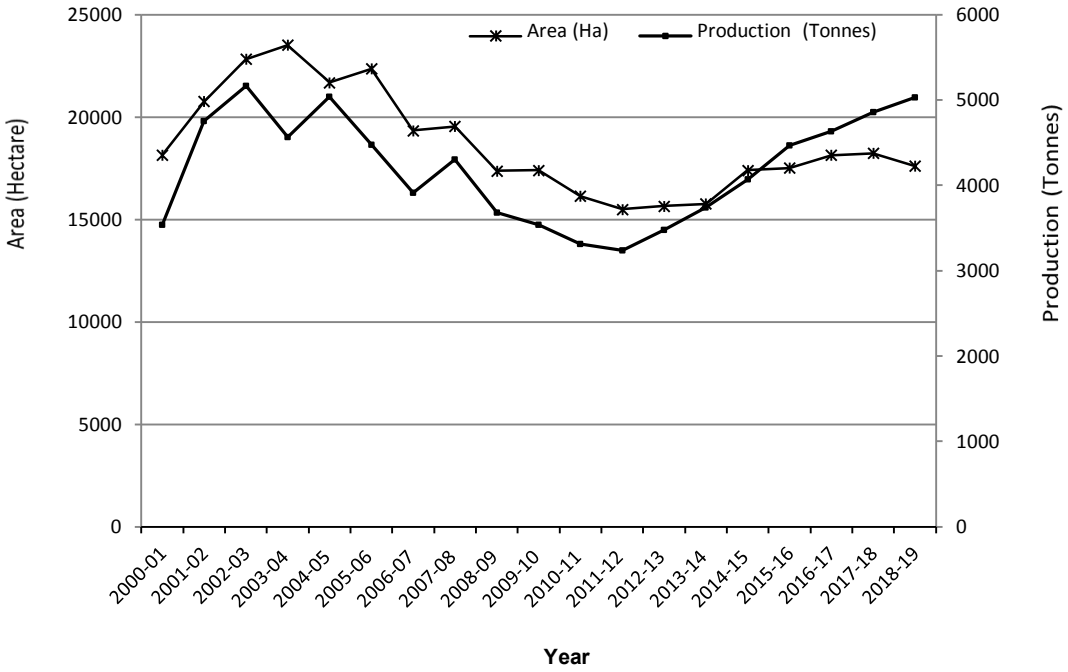
CHAPTER 4

RESULTS

4.1. Isolation and identification of causal organism of fungal disease of large cardamom using morphological and molecular markers

4.1.1. Status of Large cardamom (*Amomum subulatum* Roxb.) grown in Sikkim

Overall large cardamom production in Sikkim, with respect to area, total production and yield per hectare in the past two decades is depicted in the figure as given bellow (Fig. 4.1).It shows that in the first phase of five years rise in area and productions were recorded but the increment was negligible. In 2000-01 total cultivated area under large cardamom in Sikkim was 18148 hector, with total product 3540 Tonne. In the year 2003-04, the state experienced most area (23513 ha) with large cardamom plantation, and in 2004-05 maximum production (5037 Tonnes). But during this period a wide spread viral disease problem out broke in the area and impacted the production. To improve the situation the Spice Board, Govt. of India and the Horticulture and Cash Crops Development Department, Govt. of Sikkim have initiated and implemented several welfare programmes in the region. Under those programmes, production of good quality planting materials and were made available to the growers with subsidy. Growers were encouraged to take up replantation of old and uneconomic gardens with certified saplings.



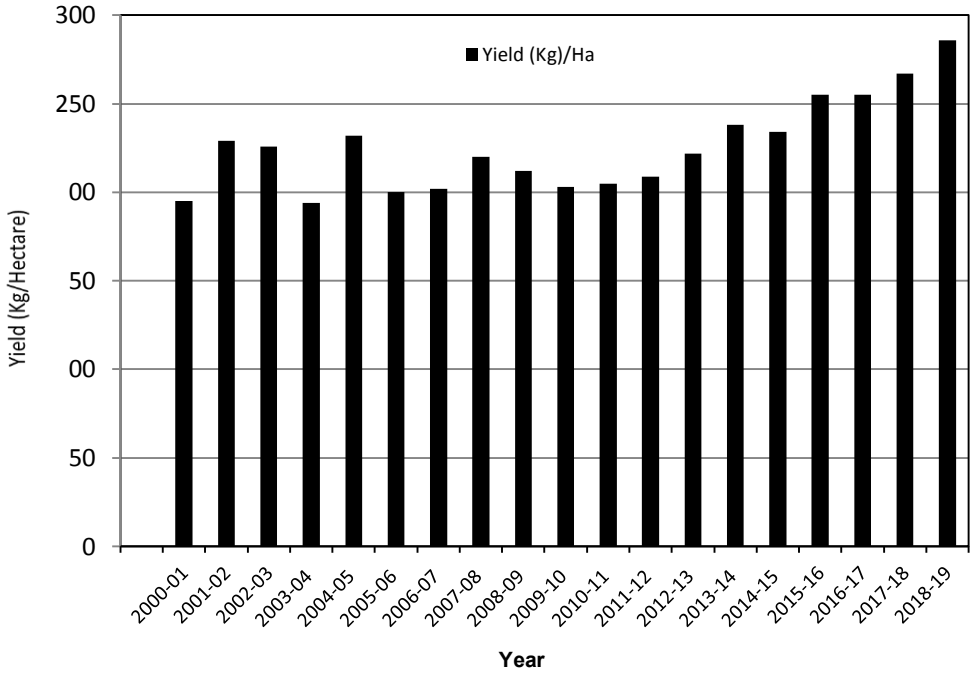


Fig 4.1: Area, production and yield of large cardamom grown in Sikkim

(Source: Anonymous 2009-19, Annual Reports of Spice Board, Govt. of India)

Table 4.1: District wise area and production of large cardamom in Sikkim

Year	**East**		**North**		**South**		**West**	
	Area (Ha)	Production (MT)	Area (Ha)	Production (MT)	Area (Ha)	Production (MT)	Area (Ha)	Production (MT)
2017-18	6514	1487.8	3868	939	3707.95	983.94	3645.2	974.54
2016-17	6784	1584.60	4050	1044	3661.5	996.33	3641.7	1008.02
2015-16	6558	1561	3850	1001	3580.85	966.40	3531.4	937.234
2014-15	6565	1309.60	3830	959	1066	935.7	1139	870
2013-14	4950	1188	3800	784.78	3531	935.70	3480	835.20
2012-13	4870	1096	3720	691.80	3569	899.06	3491	794.20
2011-12	4866	1036	3642	655	3640	824	3354	722
2010-11	5455	1069	3642	626	3697	910	3354	705
2009-10	5925	1129.90	4284	848	3741	818	3461	746
2008-09	5925	1187	4184	836	3777	861	3485	794

(Source: Anonymous 2008-18, Annual Report, Spice Board, Govt. of India)

Further, the board supported to small and marginal growers with subsidy for replanting. Subsidy also provided by the Spice Board of India for initial three years of gestation period for new plantations. Despite all the incentives and activities after 2004-05 a sharp decline in terms of area and production in the large cardamom sector recorded and continued till 2013-14, for a span of 10 years.During this bad spell, least plantation was recorded in 2011-12 with 15502 ha production area, i.e., 34% lower to that of 2003-04 and production 3237 Tonnes, 36% lower to that of 2004-05 (Fig. 4.1).

To cope up with the situation, National and state agencies in the region have introduced a number of strategies, i.e., training to the growers, better farm management, etc. Under this programme application of Farm Yard Manures (FYM) before flowering and after harvesting, irrigation during dry winter months, etc. has initiated. Integrated pest management strategies were also adopted which included manual pest management; application of bio-pesticide, bio-formulations, etc. Uprooting of infected plants and burning of diseased plants were done. Further new areas were brought under cardamom plantation. Old infected fields were kept as fallow land for few years for natural sanitization. In 2013-14 once again slight rise in area, total production and yield were noticed and till now the trend is maintained. In 2017-18 total cultivated area under large cardamom in Sikkim was 18232 hector, with total 4682 Tonne product and yield 267 Kg/Ha (Fig. 4.1).

4.1.2. Disease symptoms assessment in large cardamom

During the study 30-60% severity of leaf blight incidence has been recorded. The survey showed that disease condition is alarming and majority of the plantations in the state are affected with 40-45% blight incidence (Table 4.2).Affected leaves

from diseased cardamom plantations with blight symptoms were characterized by sunken appearances with the necrotic areas and yellowish-brown irregular spots (Fig. 4.2). It was observed that necrotic symptoms spread from the tip and sometimes from the leaf margin. Gradually leaves tend to dry out from the tip resulting drying of the whole planteventually causing death of plants. Fruiting and fruits were abnormal, immature with whitish brown seeds. Initially the disease was noticed with the commencement of pre-monsoon showers in the month of April-May which progressed rapidly during the rainy season. However, in some areas the incidence was started during winter months (January-March). The symptoms shown were water-soaked lesions which appeared either at margins or tips or any other point on the leaves which enlarged rapidly, coalesced and covered major portion or the entire leaf lamina giving a blight appearance. The advancing lesions were blackish brown in colour and margins give a yellow halo. In some cases, the entire lamina became yellowish with blight symptoms. The affected area became necrotic and dried up of plantation. It was also noticed that, among the six varieties grown in the state, *Varlangey, Swaney, Ramla* and *Ramsey* were found with blight symptoms. Further it was also quite prominent that new plantations in open field conditions were worst affected with the disease in comparison to those grown under canopy cover. Among the four districts studied, west district of Sikkim was found to be the severely affected with the disease and with33-45% disease incidence.

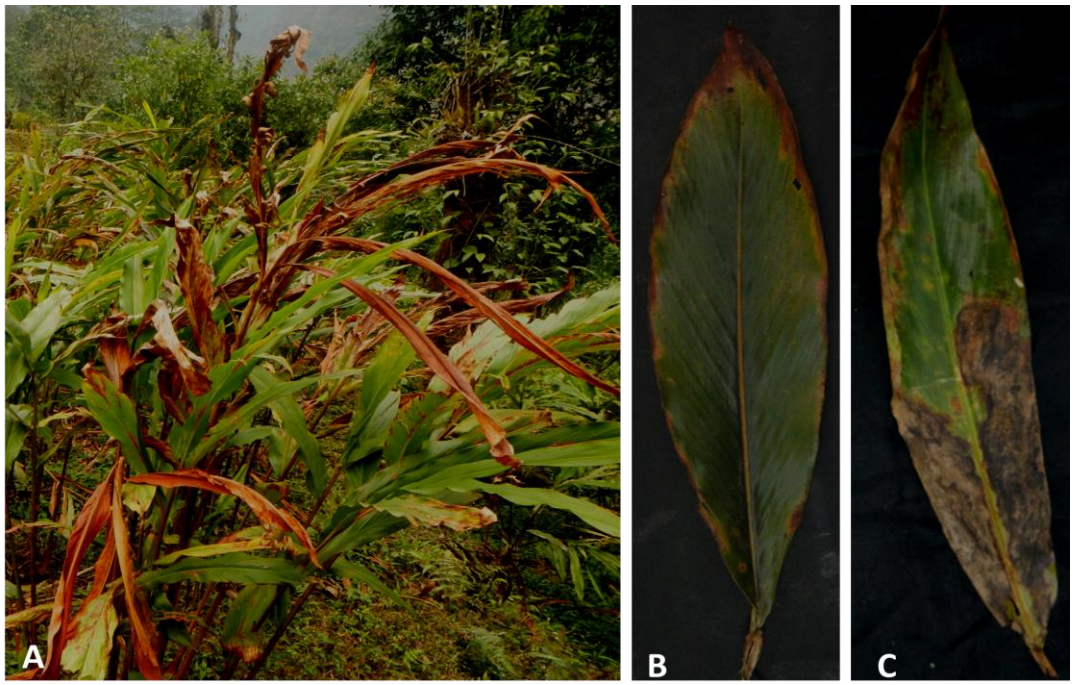


Fig 4.2: The large cardamom field with the typical blighted symptoms caused by the phytopathogens.

A-The large cardamom plant with blighted symptoms in the field; B- Close up view of the leaf with the blighted margin; C-the leaf with the sunken spots in the entire leaf.

Table 4.2: Details of leaf blight disease severity in different villages of Sikkim

District	**Name of village**	**Altitude (m amsl)**	**Varietie(s)**	**% Disease incidence**
East Sikkim				
	Pakyong	1364	*Swaney*	47.5
	Assam Linzey	*1335*	*Swaney*	46
	Lamaten	*1200*	*Varlangey, Swaney*	45.5
	Bauchand	*1350*	*Varlangey*	35
	Chujachen	*1150*	*Varlangey*	44.5
	Kopchey	*1050*	Varlangey	46

	Dalapchand	1080	Varlaney	42.5
	Simik-khamdong	985	Swaney	43.5
	Ray	950	Swaney, Ramsey	41
	Namchey bong	1250	Swaney	49
	Lingtam	1300	*Varlangey*	39
	North regu	1280	*Varlangey, Swaney, Ramsey*	41.5
	South regu	1200	*Varlangey, Ramsey*	43.5
	Rumtek	1500	*Swaney*	44
	Aho	1150	*Varlangey*	56
	Andheri	1180	*Varlangey*	60
	Dickhu	1020	*Swaney*	52.5
West Sikkim				
	Hee-pechreak	1650	*Sermna, Ramsey*	45
	Dentam	1500	*Sermna*	36
	Sribadam	1485	*Sermna*	41.5
	Hee-goan	1750	*Sermna*	40
	Yuksom	1438	*Ramla, ramsey*	35
	Ramjethang	1428	*Varlangey*	32
	Tashiding	1440	*Varlangey , Ramla*	31.5
	Kechuparli	1975	*Varlangey*	35
	Tshong	1875	*Ramsey*	32.5
	Hee bermoik	1789	*Sermna, Varlangey*	34.5
	Kaluk	1713	*Sermna, swaney*	40
	Middle Geyzing	1800	*Swaney, varlangey*	32.5
	Tekjek	1850	*Varlangey*	33
North Sikkim				
	Mangan	1143	*Golsey, Swaney*	42.5
	Lower Singhik	1100	*Golsey, Ramsey*	42
	Upper Shinghik	1112	*Swaney*	31.5

	Naga	1250	*Ramsey ,Swaney*	45
	Mangshila	1150	*Ramsey*	30
South Sikkim				
	Yangyang	851	*Swaney, Golsey*	44.5
	Chuba	2300	*Swaney*	40
	Tumin	1720	*Swaney, Golsey*	43
	Dhanbari	1714	*Swaney, Golsey*	43.5
	Kolkaley	1825	*Swaney, Ramsey,Golsey*	45
	Nyna	790	*Swaney*	43.5
	Tokal bermoik	851	*Swaney*	39
	Rang rang	822	*Ramsey*	51
	Damthang	2030	*Varla*	35
	Ravongla	2230	*Varlangey*	39
	Kewzing	2050	*Varlangey, ramla*	45
	Bikmut	1714	*Ramla*	45
	Lingmoo-sokpay	1300	*Swaney, varlangey*	40

4.1.3. Isolation of fungal isolates

During the study initially total fifty isolates were obtained pathogenic isolates. Based on the similar colony morphology and growth characteristics of isolates these isolates were grouped into nine (09) categories and were taken up for characterization and identification. All the nine isolates were sent to National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune for authentication. Based on the basis of morphological features, both gross and microscopic the isolate no LC02 was identified as *Pestalotiopsis maculans*, isolate LC03 *Verticillium lecani*, isolate LC04 *Curvulariaeragrostidis*, isolate LC05

Colletotrichum gloeosporioides, isolate LC11 *Phoma cava,*isolate LC12 *Epicoccum nigrum* and LC06, LC08, LC09 were identified as *Fusarium* sp. Out of these total nine fungi eight were identified as highly pathogenic and one non-pathogenic beneficial fungus. Detail characterizations of eight pathogenic fungi have been described in detail in the subsequent sections.

Table 4.3: Identification of isolates

Isolate No.	**Identified fungalspecies/ pathogens**	**NFCCI-ARI, Pune accession number**
LC02	*Pestalotiopsis maculans*	NFCCI4698
LC03	*Verticillium lecani* (Non-pathogenic beneficial fungus)	NFCCI 4540
LC04	*Curvulariaeragrostidis*	NFCCI 4541
LC05	*Colletotrichum gloeosporioides*	NFCCI 4542
LC06, LC08, LC09	*Fusarium* sp.	NFCCI 4543, 4544
LC11	*Phoma cava*	NFCCI 4663
LC12	*Epicoccum nigrum*	NFCCI 4545

Morpho-molecular characterization of isolate No. LC02:

The isolate was isolated from leaf stark lesions of large cardamom grown in Sikkim. It was noticed that 4-12%plantations from all the four districts in the state, were infected by leaf stark incidence showing leaves with numerous circular to rectangular spots with varying sizes (Fig.4.3).Spots were characterized by rectangular to circular shape with smaller size, brown or translucent colour,

scattered all over the leaf on the upper surface parallel to the leaf veins. These tiny spots eventually form cluster(s). The disease was noticed in four varieties i.e., *Swaney, varlangey, Ramsey* and *Golsey*, grown in the region.

Growth pattern of fungal pathogen in different medium

Initially, the isolation of the endophytic pathogen was done on potato dextrose agar (PDA) at 25°C and incubated for seven days. For the further characterization, the isolate was grown in five different solid media namely, PDA, czapek dox agar (CDA), V8 juice agar (VJA), sabourd dox agar (SDA) and potato carrot agar (PCA). The maximum colony growth was observed on PDA with 67.3±0.88 mm colony diameter and found to be significantly different $P < 0.05$) to that of V8 juice with 60.3±0.33 mm and CDA with 60±1.15 mm. There was no significant difference of colony growth grown on SDA with 65±1.15 mm, PCA with 63.3±0.88 (Table 4.5). Growth pattern and colony color were also recorded. The colony of the isolate observed from the front was white cottony mass (Fig. 4.4) where as colony observed from the reverse side of the plate, found to be light brown with blackish central portion (Fig. 4.4).

Fine septate mycelium and 5-celled smooth conidia was observed under a microscope. Apical and basal cells of the conidia were hyaline and median three cells were brown to blackish, out of which the upper two were observed darker in comparison to the lower one (Fig. 4.4). Conidia were measured 17 to 22 µm long and 5 to 7 µm wide (Table 4.4). Based on the macroscopic and microscopic characterization, the isolate was identified as *Pestalotipsis maculans* (NFCCI4698).

Fig.4.3. Leaf streak of Large Cardamom (*Amomum subulatum* Roxb.) var. *Varlangey* in Sikkim caused by isolate no. LC02

(A) The plant observed with the symptoms of leaf streak in the field.

(B) Close up view of a leaf infected with leaf streak diseases.

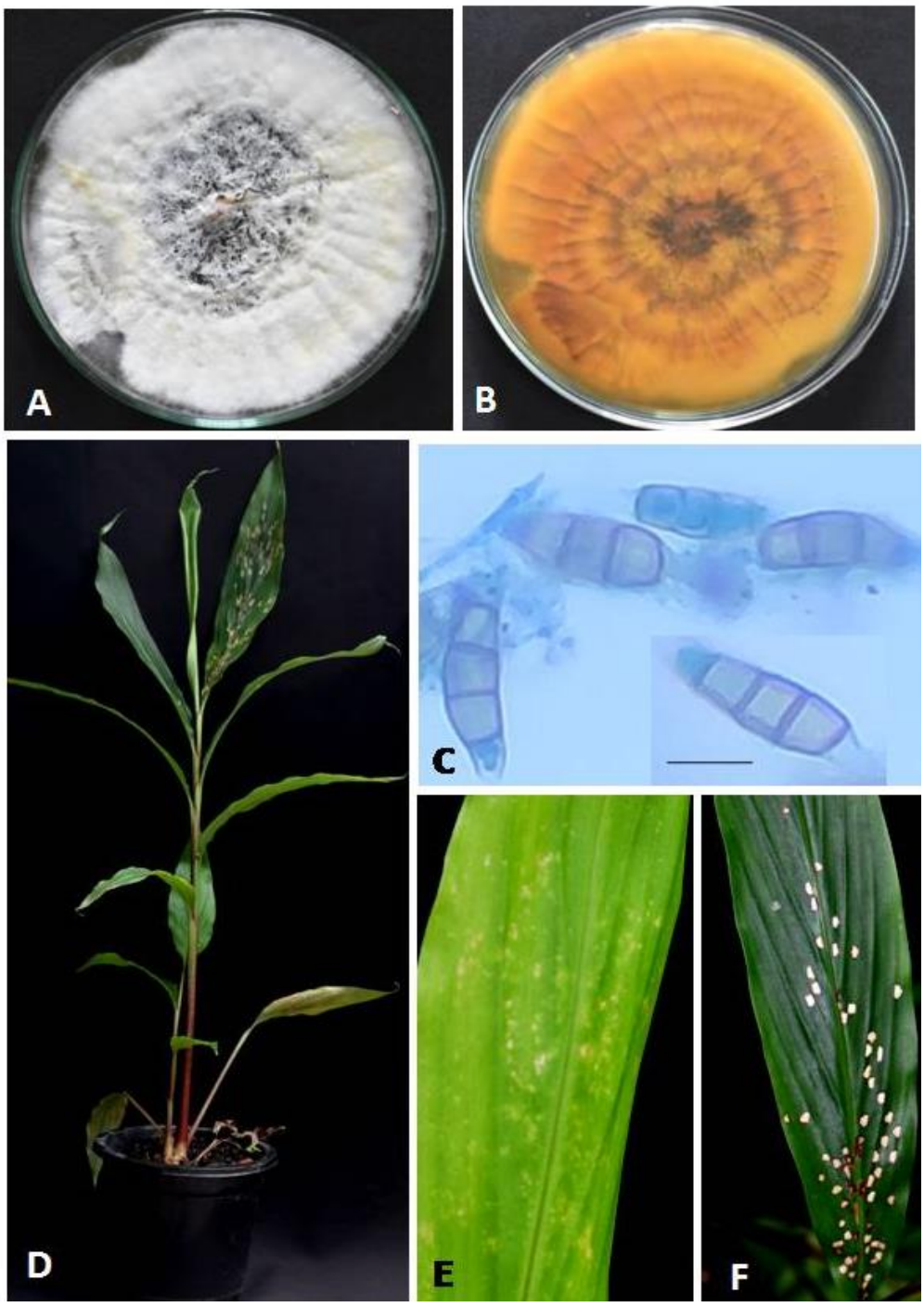


Fig. 4.4: Leaf streak of Large Cardamom (*Amomum subulatum* Roxb.) var. *Varlangey* in Sikkim caused by isolate no LC02

(A-D) *In vitro* growth of of the isolate(A- front view, B- reverse side view), (C) Conidiospores (Bar = 15µm), (D) The plant seen with the leaf streak in pot experiment, (E) initiation of the minute spots seen after 21 days of the inoculation, (F) Prominent and visible leaf streaks recorded after 33 days of infection.

Table 4.4: Effect of different nutrient media on growth and colony characteristics of the *Pestalotipsis maculans*(Isolate LC02)

Description	Potato Dextrose Agar (PDA)	Sabouraud Dextrose Agar (SDA)	Czapek Dox Agar (CDA)	V8 Juice Agar	Potato Carrot Agar (PCA)	LSD *(P= 0.05)*
Size (mm diameter)	67.3±0.88	65±1.15	60±1.15	60.3±0.33	63.3±0.88	5.73
Front view	White colour with cottony appearancewith slight orange tinge	White glossy colony	White colour with cottony appearance	White colour	White colour with cottony appearance	
Reverse view	Pale yellow with blackish tinge	Light yellow	Light cream	White with yellow tinge	Off white	
Sporulation	High	High	Low	Low	Low	
Exudates	Less	Less	No exudates	Less	Less	

Mean value (n =3) with ± Standard error (SE)

Influence of temperature and pH on the growth of the *Pestalotipsis maculans* (isolate LC02)

In the present study, the mycelial growth of the isolate followed linear trends of response with the changes in temperature up to 30°C and then decreased sharply as temperature increased thereafter. Colony growth was least at 35°C and at 10°C with colony diameter about 15.0 mm and 27.0 mm respectively. The optimum temperature for colony growth was found to be 25°C with colony diameter 67.33±0.88 mm after 5 days of incubation.

Medium pH also significantly affected radial mycelia growth of the isolate. Colony growth of the isolate was observed in a wide range of pH (5.0 – 10.0) of medium. Maximum colony growth was obtained at medium pH 6.0 - 8.0 with 53.0-55.0 mm colony diameter. Then asharp decline of the growth occurred when medium pH increased to 11 or more. No colony growth was observed whenmedium pH was 3 and 13.

Pathogenicity test

The pathogenicity of the isolate (LC02) *Pestalotiopsis maculans* was confirmed by causing infection by the isolated pathogen, then re-isolation, identification and confirmation of the fungus. For this, healthy large cardamom leaves were wounded with sterilized blade and infected with inoculums. Infected leaves started to show the symptoms of tiny brown lesions. Eventually after 8-9 days of inoculation spots were visible with characterized symptoms, surrounded by brown colored rings. The symptoms were only observed on the inoculated plants (Fig. 4.4F-H) where as no symptoms were observed in control plants.

Table 4.5: Effect of temperature on the growth of the*Pestalotipsis maculans*

Temperature (°C)	**Colony growth (mm)±SE**
5	0±0
10	27±0.88
20	31.33±0.33
25	67.33±0.88
35	15±1.73
45	0±0
50	0±0
LSD	3.88

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media with pH 5.5.
SE = Standard error; LSD = Least significant difference.

Sequence analysis

The PCR analysis of the genomic DNA of pathogenic fungus isolated from the infected large cardamom leaves was performed by using ITS 4 and 5 primers. The tested fungal strain showed 100% sequence similarity with *Pestalotiopsis maculans*sequence available in the public domain i.e., with NCBI accession number MN710582. Top five hits with 100% homology of the sequences upon BLASTn analysis are*Pestalotiopsis maculans* (MN710582), *Pestalotiopsis sp.* isolate P4 (MN180879.1), *Neopestalotiopsis sp.* (LC412067.1), *Pestalotiopsiscoffeae-arabicae* isolateSL74 (MN105556.1), *Pestalotiopsis sp*.

strain LCM 817.01 (MF495382.1) and *Neopestalotiopsis clavispora* strain 17GDNS14 (MK278906.1) (Fig. 4.5).

Table 4.6: The effect of pH on the growth of the *Pestalotipsis maculans*

pH	**Colony growth (mm) ±SE**
3	0±0
5	53±1.15
6	55±1.53
7	56±0.58
8	53±0.58
9	50±0.88
10	40±0.58
11	22±1.15
13	0±0
LSD	2.53

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media at 25°C. The pH value of the media was adjusted before autoclaving.

SE = Standard error; LSD = Least significant difference.

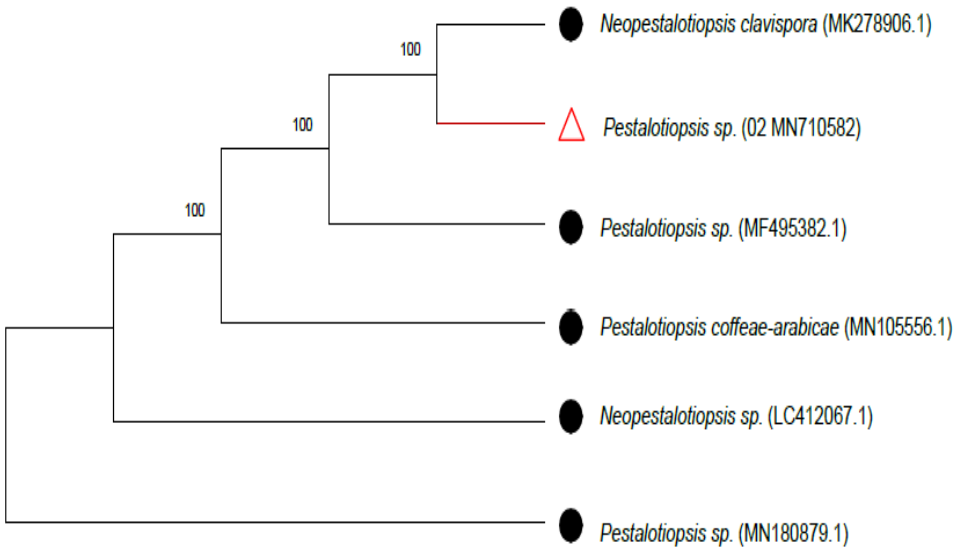


Fig 4.5: Phylogenetic tree showing the relationship of the pathogen with the previously reported isolated *Pestalotipsis* sp. Bootstrap values are indicated for each branch divergence of 1,000 replicates.

Further the, Maximum Composite Likelihood (MCL) phylogenies generated in this study with eleven (15) isolates of *Pestalotiopsis* spp. comprising the isolate of the present study and ten (14) reference isolates taken from the GenBank (Fig. 4.5). The tree with the highest log likelihood is -4436.67. Further, isolateswere associated with infection of different hosts such as *Buxus megistophylla, Camellia sinensis, Camellia japonica, Myrica rubra*, *Macadamia integrifolia, Mangifera indica, Fragaria ananassa,* blue berry, date palm *etc.* and different geographical origin (Table 4.8). The molecular phylogenetic results showed that there is no general pattern observable while compared with host plant families, (Fig. 4.5). The pathogen, as defined by the analysis, show no host specificity, but each

Table 4.7: Phenotypic and Genotypic description of the *Pestalotipsis maculans*(Isolate LC02)

Character	Description
Colony morphology on PDA	Front view- white colony with slight orange tinge; Reverse view- pale yellow with black tinge
Microscopic features	Septate with apical appendages and the conidia were seen with 4-5 celled in brown to black colour, 17 to 22 µm long and 5 to 7 µm wide
Physiology characters (pH and temperature)	Temperature ranges between 5°C to 50°C, optimum 25°C and pH 3to 13, optimum 7
Accession number and nucleotide sequence number	NFCCI-4698 and MN710582
Phylogenetic relationship (18S rRNA analysis) and top hits upon BLASTn analysis	100 % similarity with *Pestalotiopsis maculans* (MN710582), *Pestalotiopsis sp.* isolate P4 (MN180879.1), *Neopestalotiopsis sp.* (LC412067.1), *Pestalotiopsiscoffeae-arabicae* isolateSL74 (MN105556.1), *Pestalotiopsis sp*. strain LCM 817.01 (MF495382.1) and *Neopestalotiopsis clavispora* strain 17GDNS14 (MK278906.1)

Table 4.8: Isolates of *Pestalotiopsis* spp. causing leaf disease in different host and geographical origin used in this study for phylogenic analysis

GenBank accession number	Causal organism	Host plant	Disease symptoms	Geographical origin	Source	Gene	Reference
MN710582	*Pestalotiopsis maculans*	Large cardamom	Leaf streak	Sikkim, India	This study	ITS	This publication
KY928287	*Pestalotiopsis maculans*	*Buxus megistophylla*	Leaf blight	China	Genbank	ITS	Chen *et al*. 2017 (unpublished)
AF405296	*Pestalotiopsis maculans*	*Camellia sp.*	Leaf spot	France	Genbank	ITS	Jeewon*et al*. 2002
AB482217	*Pestalotiopsis maculans*	*Camellia japonica*	Leaf blight	Okinawa	Genbank	ITS	Watanabe*et al*. 2010
KX757718	*Neopestalotiopsis clavispora*	*Camellia sinensis*	Brown blight	China	Genbank	β-tubulin ITS, TEF	Chen*et al.* 2018
KY319134	*Neopestalotiopsis clavispora*	*Camellia sinensis*	Gray blight	China	Genbank	β-tubulin	Wang*et al.* 2019
KM402033	*Pestalotiopsis*	Myrica rubra	Brown leaf	China	Genbank	ITS	Lu*et al*. 2015

	clavispora		spot				
EF152585	*Pestalotiopsis clavispora*	Blue berry	Leaf Spot	China	Genbank	β-tubulin	Luan*et al.* 2008
AB453860	*Pestalotiopsis maculans*	*Camellia japonica*	Leaf blight	Japan	Genbank	EF1a	Watanabe*et al.* 2010
MG729679	*Neopestalotiopsis clavispora*	Blue berry	Blight	Sichuan	Genbank	ITS	Ren*et al.* 2013
JX875595	*Pestalotiopsis clavispora*	*Mango (Mangifera indica)*	Grey leaf spot	Italy	Genbank	ITS	Ismail*et al.* 2013
MH685414	*Neopestalotiopsis clavispora*	Date palm (*Phoenix dactylifera)*	Leaf spot	Iran	Genbank	ITS	Wei and Zhao 2016
KM264343	*Neopestalotiopsis clavispora*	Strawberry (*Fragaria ananassa*)	Leaf spot	China	Genbank	ITS	Basavand*et al.* 2020
JX875596	*Pestalotiopsis clavispora*	*Mango (Mangifera indica)*	Grey leaf spot	Italy	Genbank	ITS	Ismail*et al.* 2013
JX398978	*Neopestalotiopsis clavispora*	*Macadamia integrifolia*	Brown spots of leaf	Brazil	Genbank	ITS	Santos*et al.* 2019

Source: GenBank (https://blast.ncbi.nlm.nih.gov/)

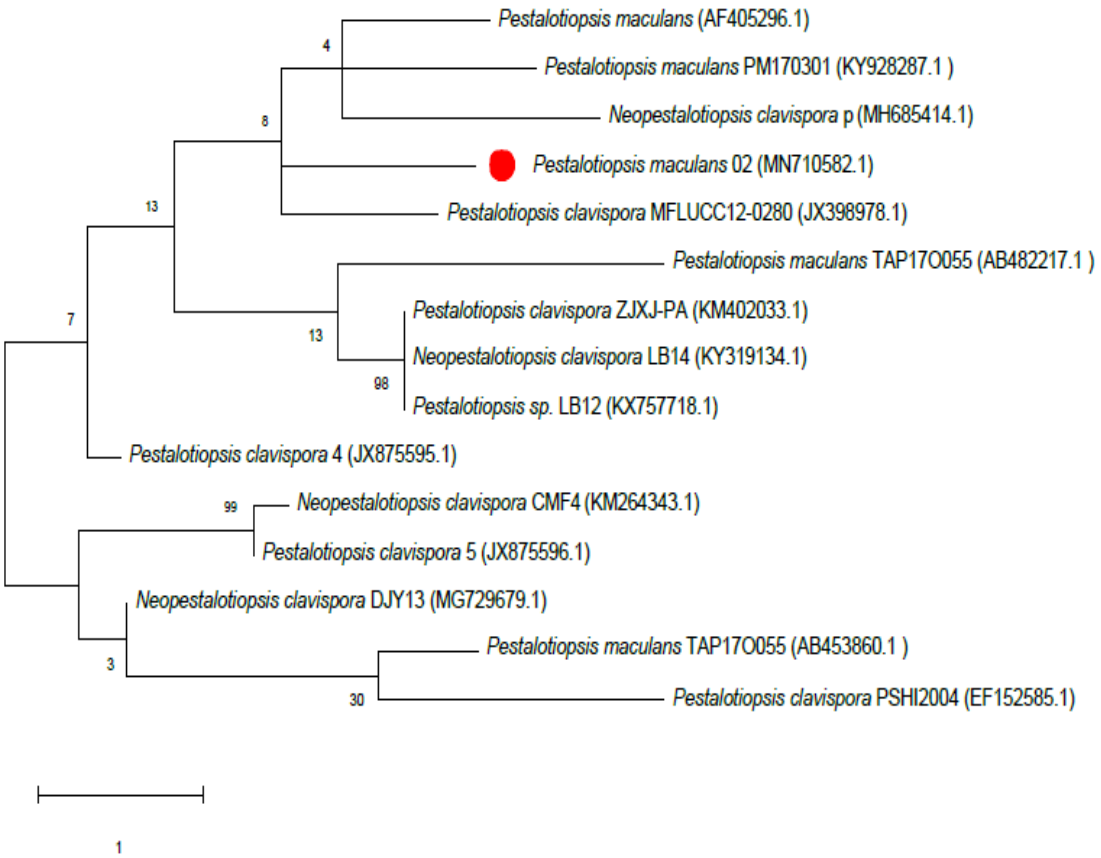


Fig. 4.6: Phylogenetic tree builtusing neighbor-joining (NJ) methods with ITS nucleotide sequence of *Pestalotiopsis maculans* isolated from large cardamom (*Amomum subulatam* Roxb.) and 14 other reference ITS sequences of *Pestalotiopsis* spp. retrieved from the GenBank reported causing similar types of leaf disease (Leaf streak, blight and spot etc.) in various host plants and geographical origin using MEGA X. Accession numbers of sequences are given in parenthe-ses. Bar, 1.0 substitution per nucleotide position.

The pathogen, as defined by the analysis, show no host specificity, but each species occurs on a variety of different host plants with similar effect. The tree presented showed that present isolate *Pestalotiopsis maculans* (MN710582) performed the close clade with *Pestalotiopsis clavispora* (JX398978.1).On the contrary, *Pestalotiopsis maculans* (AF405296.1, KY928287.1) showed a distinct clade with *Pestalotiopsis clavispora* (MH685414.1) strain available in the database. The analysis suggesting that nucleotide variation among the *Pestalotiopsis* spp. was not only related to geographical origin but also host associated (Fig.4.6).

Morpho-molecular characterization of isolate No. LC04

Phenotypic characteristics of the disease

This is a typical leaf blight disease initially started with the symptoms of 7-12 dark brown spots per leaf. Leaf spots are usually scattered over the leaf surface, circular or irregular with variable shape, light brown center and reddish brown margins (Fig. 4.7). At early stage, these spots were very tiny with 1.0-2.5 mm diameter, yellow central core with gradual discoloration towards the leaf surfaces. In due course of time, these spots increased in size and spread to the entire leaves and turned to dark brown (Fig.4.7) causing severe foliar damage to the plants. Eventually, these spots turn necrotic blight appearance and finally the infected leaves are dry out.

Radial mycelial growth of the isolate was significantly ($P < 0.05$) affected by the culture media. Results revealed that out of five different media tested for the fungal isolate, three, i.e., Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar and V8 Juice Agar were found to be significantly different ($P < 0.05$) and gave satisfactory performance. In Potato Carrot Agar and Czapek Dox Agar medium, colony growth was significantly low ($P > 0.05$) and with 60.5$\pm$0.41mm and 51.5$\pm$0.41 mm average colony diameter in comparison to 64.5$\pm$0.41 mm, 63.5$\pm$1.24 mm and 64.5$\pm$0.41 mm when grown on (PDA), Sabouraud Dextrose Agar (SDA) and V8 Juice Agar respectively. As PDA is the most common and widely used medium for fungal experiment, PDA was used for further study (Table 4.9).

Temperature was found to be another important physical factor influencing growth of fungal isolates. In the present study, the mycelial growth of the isolate followed

linear trends of response with the changes in temperature up to 35°C and then decreased sharply as temperature increased thereafter. Colony growth was least at 45°C and at 10°C with colony diameter about 20.5 mm. The optimum temperature for colony growth was found to be between 25°–35°C with colony diameter 64.5±0.41 mm after 5 days of incubation. At 20°C, moderate growth with average 41±0.83 mm colony diameter was recorded which was significantly low ($P > 0.05$) in comparison to the growth at 25-35°C. No colony growth was observed at 5°C (Table 4.10).

Medium pH also significantly affected radial mycelial growth of the isolate. Colony growth of the isolate was observed in a wide range of pH (5.0 – 9.0) of medium. Maximum colony growth was obtained at medium pH 6.0 - 8.0 with 64.5-63.5 mm colony diameter (Table 4.11). Then a sharp decline of the growth occurred when medium pH increased to 9 or more. No colony growth was observed when medium pH was 3 and 13.

Table 4.9: Effect of different solid media on the growth of the isolate no LC04

Colony diameter (mm)±SE					
Potato Dextrose Agar (PDA)	**Sabouraud Dextrose Agar (SDA)**	**Czapek Dox Agar**	**V8 Juice Agar**	**Potato Carrot Agar**	**LSD**
64.5±0.41	63.5±1.24	51.5±0.41	64.5±0.41	60.5±0.41	1.18 (*P= 0.05*)

Each treatment consisted of three replications. The results were recorded after incubation for 5days at 25°C, and media pH 5.5.
SE = Standard error; LSD = Least significant difference.

Fig. 4.7: Leaf blight of Large Cardamom (*Amomum subulatum* Roxb.)var. Varlangey in Sikkim caused by isolate no. LC04A. Leaf blight effected large cardamom field. B. Close view of infected plant. C-D. Leaf blight symptoms in large cardamom caused by *C. eragrostidis*(C- Bar= 10 cm; D- Bar= 30 cm).

Table 4.10: The effect of temperature on the growth of the *Curvularia eragrostidis*

Temperature (°C)	Colony growth (mm)±SE
5	0±0
10	20.5±0.41
20	41±0.83
25	86±1.66
35	89.5±0.41
45	20.5±2.07
50	0±0
LSD	2.73

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media with pH 5.5.

SE = Standard error; LSD = Least significant difference.

Table 4.11: The effect of pH on the growth of the *Curvularia eragrostidis*

pH	Colony growth (mm) ±SE
3	0±0
5	78.5±0.41
7	82.5±0.41
9	83.5±1.24
10	58±1.66
11	54.5±1.24
13	0±0
LSD	2.83

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media at 25°C. The pH value of the media was adjusted before autoclaving.

SE = Standard error; LSD = Least significant difference.

Table 4.12: Phenotypic and genotypic characters of the endophytic fungi (Isolate No LC04)

Sl. No.	Character	Description
1.	Colony morphology	Greenish with white margin and dark green and white from invert plate view along with wavy margins with 64.5±0.41 mm colony diameter
2.	Microscopic features	Mycelium septate, hyaline and 4-8 µm wide. Size of conidia: length 78.81± 1.65 µm; width in the middle 42.16±1.51 µm.
3.	Physiological characterization (pH and temperature)	Temperature requirement between 10°C to 45°C, optimum 25°C and pH 5 to 11 optimum 7
4.	Accession number and nucleotide sequence number	NFCCI 4541 and MN710527, respectively
5.	Phylogenetic relationship of the isolate (18rRNAs analysis) and list of top five hits upon BLASTn analysis	Maximum (100%) similarity with *Curvularia eragrostidis* (KU856617.1), *C. eragrostidis* (KU232927.1),*C. eragrostidis* (KU232931.1),*C. eragrostidis* (KU232929.1) and *C. eragrostidis* (KU232927.1)

Colony morphology of the fungus obtained on PDA is presented in(Fig. 4.8).Colony diameter at 25°C was 64.5 mm, greenish with white margin from the top view, and dark green and white from invert plate view, with wavy margins. The isolated fungal culture was observed to be fast growing on PDA with scanty mycelia, and cottony with fast fungal growth. The mycelium was septate, hyaline,

and 4-8 µm wide. The conidiospores were simple oval shaped, septate, pale brown with hyaline base and fertile tip. The conidia were olive brown, 3-septate, more or less curved at the 3rd cell or nearly straight in the middle. Size of conidia is: length 78.81± 1.65 µm; width in the middle 42.16±1.51 µm (Table 4.12.; Fig. 4.8). The 3rd cell from the base was larger and darker than the basal and apical cells which were hyaline with smooth tip. On the basis of colony morphology and microscopic features, the isolate was identified as *Curvularia* sp by National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune.

Based on ITS sequences and phylogenetic analysis top five hits upon BLASTn analysis are *Curvularia eragrostidis* (KU856617.1), *C. eragrostidis* (KU232927.1), *C. eragrostidis* (KU232931.1), *C. eragrostidis* (KU232929.1) and *C. eragrostidis* (KU232927.1) with maximum (100%) similarity with the isolate. The tree has shown the highest log likelihood (-769.39). The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. The submission number of the gene sequence alignment at NCBI is MN710527, was identified as *Curvulariaeragrostidis* (Fig. 4.9). The ITS rDNA sequence data of the fungal species showed 100% similarity with sequences of *Curvularia eragrostidis* available in GenBank. Total score and query coverage showed 100% similarities between the DNA sequences of ITS gene of the isolate and *C. eragrostidis* from NCBI database. The fungal isolate and nucleotide sequence has been deposited in National Fungal Culture Collection of India (*NFCCI*) and GenBank (Benson *et al.* 2012) with accession numbers NFCCI 4541 and MN710527, respectively (Fig. 4.9). To the best of our knowledge, it is the first report on *Curvularia eragrostidis* of family pleosporaceae causing leaf blight, and is likely to be a new foliar threat to large cardamom in the region.

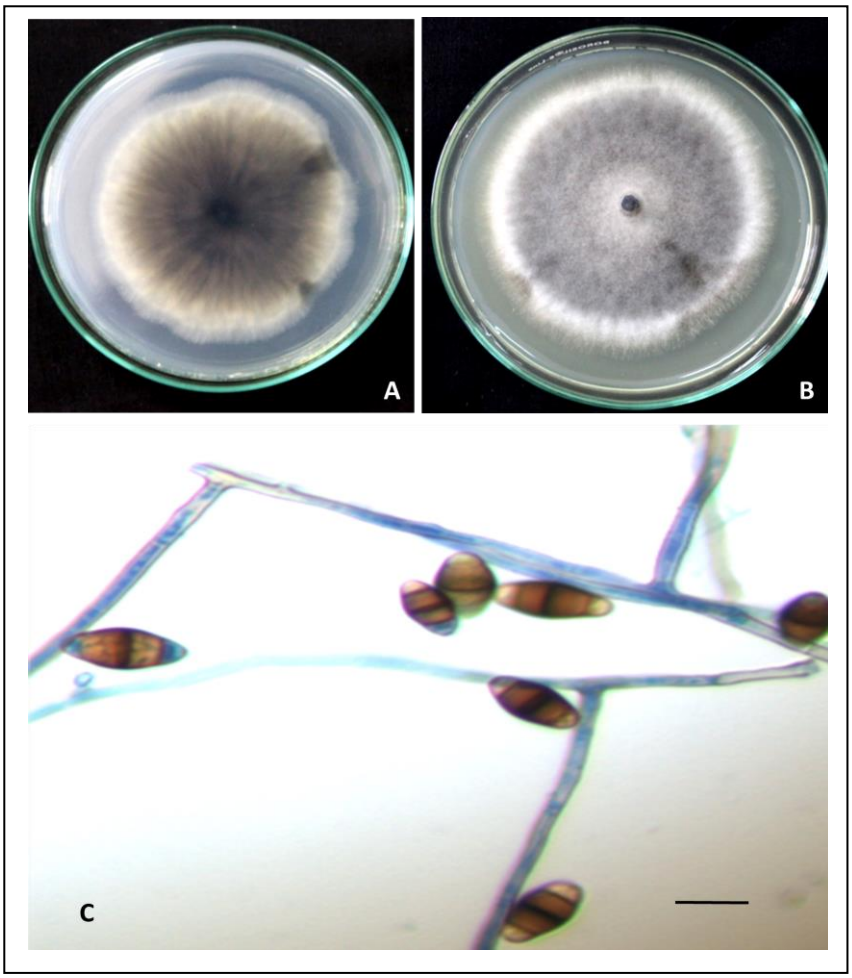


Fig. 4.8: Leaf blight of Large Cardamom (*Amomum subulatum* Roxb.)var. Varlangey in Sikkim caused byisolate no. LC04

A-B. *In vitro* growth of the isolate(A- front view, B- view from lower side). C. Mycelium and conidiospores (Bar= 50µm).

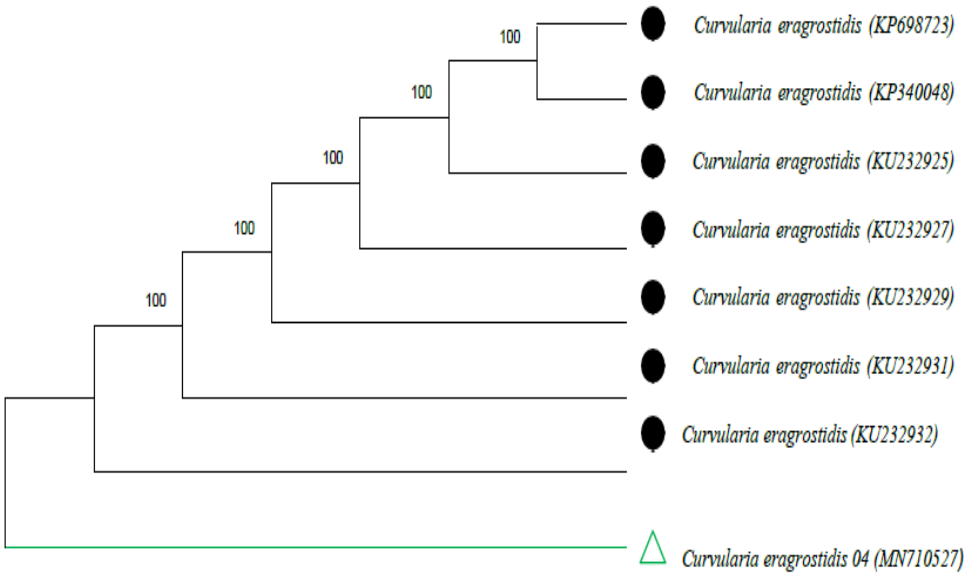


Fig.4.9. Phylogenetic tree constructed with Neighbour-Joining method using MEGA X. Bootstrap values = 1000

Morpho-molecular characterization of isolate No. LC05

Phenotypic characteristics of the disease

Affected leaves from diseased cardamom plantations with blight symptoms were characterized by sunken appearances with the necrotic areas and yellowish-brown irregular spots (Fig. 4.10A). It was observed that necrotic symptoms spread from the tip and sometimes from the leaf margin (Fig. 4.10 B). Gradually leaves tend to dry out from the tip resulting drying of the whole plant (Fig. 4.10 C), eventually causing death of plants. Fruiting and fruits were abnormal, immature with whitish brown seeds. Initially the disease was noticed with the commencement of pre-monsoon showers in the month of April-May which progressed rapidly during the rainy season. However, in some areas the incidence was started during winter

months (January-March) (Unpublished observation by the group). The symptoms shown were water-soaked lesions which appeared either at margins or tips or any other point on the leaves which enlarged rapidly, coalesced and covered major portion or the entire leaf lamina giving a blight appearance. The advancing lesions were blackish brown in colour and margins give a yellow halo. In some cases, the entire lamina became yellowish with blight symptoms. The affected area became necrotic and dried up of plantation. It was also noticed that, among the six varieties grown in the state, *Varlangey, Swaney, Ramla* and *Ramsey* were found with blight symptoms. Further it was also quite prominent that new plantations in open field conditions were worst affected with the disease in comparison to those grown under canopy cover. Among the four districts studied, west district of Sikkim was found to be the severely affected with the disease and with33-45% disease incidence (Gurung and Bag 2020).

Growth pattern of fungal pathogen in different medium

Initially isolation and growth of the fungi on PDA at 25^0C was found normal. Physico-morphological characteristics of the isolate were observed on five different solid media, namely PDA, CDA, VJA, SDA and PCA. Interestingly maximum mean colony diameter (61.33±0.88mm) was recorded when isolate was grown on PDA and CDA. This was followed by VJA and SDA with 55.33±0.88 mm colony diameter. Least colony growth was recorded (47±0.58 mm) on PCA medium (Table 4.13). The culture produced white colony with an orange shade at the centre (Fig. 4.10 D). From the back side of the culture plates orange pigmentation was observed and this character was common on all the solid media used (Fig. 4.10 E).

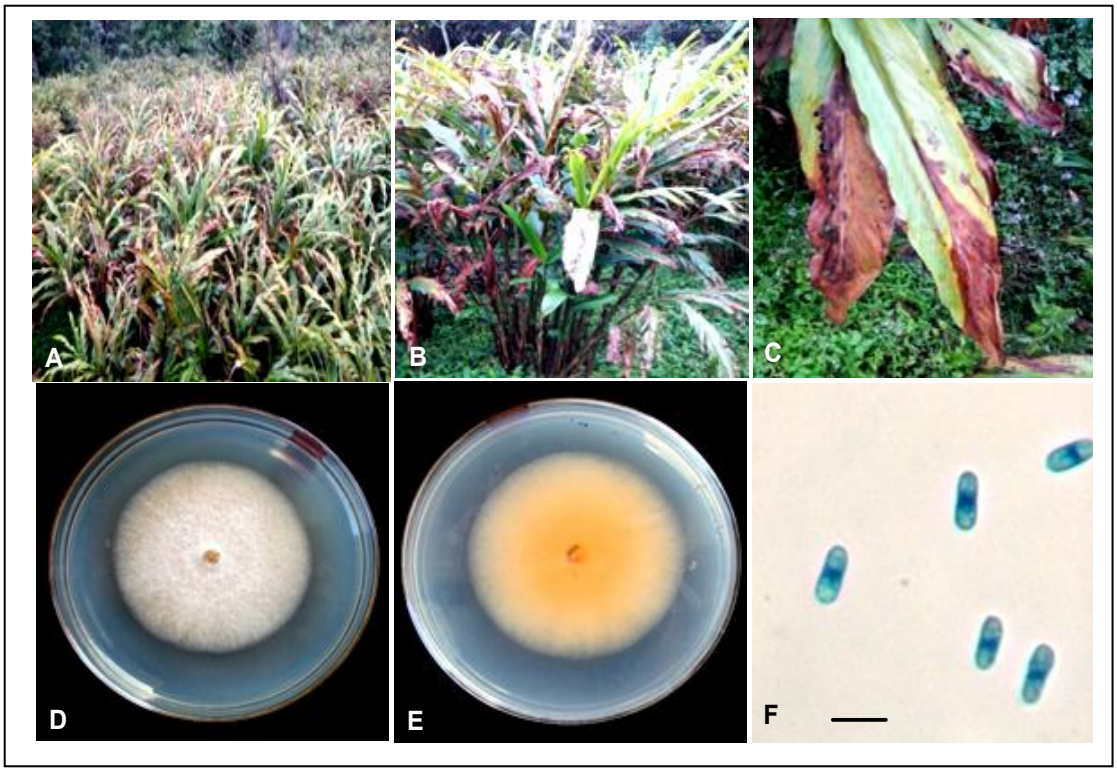


Fig. 4.10: Leaf blight of Large Cardamom (*Amomum subalatum* Roxb.) var. Varlangey in Sikkim caused by *Colletotrichum gloeosporioides*. (A) Leaf blight affected large cardamom field, (B) Close view of infected plant, (C-D) Leaf blight symptoms in large cardamom caused by *C. gloeosporioides*, (E-F) *In vitro* growth of *C. gloeosporioides* (E- front view, F- view from reverse side), (G) Mycelium and conidiospores of *C. gloeosporioides* (Bar = 10 µm).

Table 4.13Effect of different media on the growth of *Colletotrichum gloeosporioides*

PDA	SDA	CDA	VJA	PCA	LSD *(P= 0.05)*
59.6±0.69	53.33±0.88	59.6±0.69	52.33±0.88	47.00±0.58	2.42

Each treatment consisted of three replications. PDA = Potato Dextrose Agar, SDA = Sabouraud Dextrose Agar, CDA = Czapek Dox Agar, VJA = V8 Juice Agar, PCA = Potato Carrot Agar, Mean value (n =3) with ± Standard error (SE), LSD = Least significant difference (*P= 0.05*).

Mycelium of the isolate was septate, hyaline, and 2-4 µm wide. The conidiospores were cylindrical with both ends rounded and sometimes oblong (Fig. 4.10 F). Length and breadth were 11-12 µm and 3-4 µm, respectively. On the basis of colony morphology, microscopic features, and molecular characterization of the isolate (LC05) was identified as *Colletotrichum gloeosporioides* (NFCCI 4542) by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune. Molecular characterization and identification of *C. gloeosporioides,* causing leaf blight to large cardamom in this region is the first detailed confirmatory report about the pathogen. The pathogenicity of *C. gloeosporioides* was also confirmed during the study by causing similar infection on large cardamom in the experimental field by the isolated pathogen, then through re-isolation, identification, and confirmation of the fungus. On the basis physical characters, i.e., colony morphology, growth and microscopic observations, out of the total 48 samples collected and analysed during this study, *C. gloeosporioides* was identified in 14 samples. Detail molecular analysis was carried with the isolate

(LC05) isolated from the large cardamom variety *Varlangey* collected from the village Assam Linzey, East Sikkim.Remaining isolates are stored at 4°C.

Effect of temperature and pH on the growth

Temperature and pH of medium are very important physical factors influencing growth of fungi. In this study, it was observed that the pathogen could grow in a wide range of temperature between 10-35°C with varying growth rate. Least growth was observed at low temperature (10°C) and at high temperature (40°C), although the overall colony growth was suppressed significantly. No colony growth was observed at 5°C and 45°C temperature or beyond. Maximum colony growth (86.89 mm) was recorded at 25°C temperature. It was significantly high in comparison to that of other temperature tested, and was followed by 20°C with 29.6±0.50 mm colony growth (Fig. 4.11-A).

Effect of different pH on the mycelium growth of the isolate is depicted in (Fig.4.11-B). No mycelium growth was noticed when medium pH was maintained at 3, 11 and 13. Maximum growth of the mycelium (60±0.69 mm) was obtained at pH 7, followed by pH 5.5 with 47±0.58 mm colony growth. Minimum growth was recorded (22±0.88mm) at medium pH 10 (Fig. 4.11-B)**.**Previous studies also confirmed that 25°C is suitable for growth of *Colletotrichum sp.*

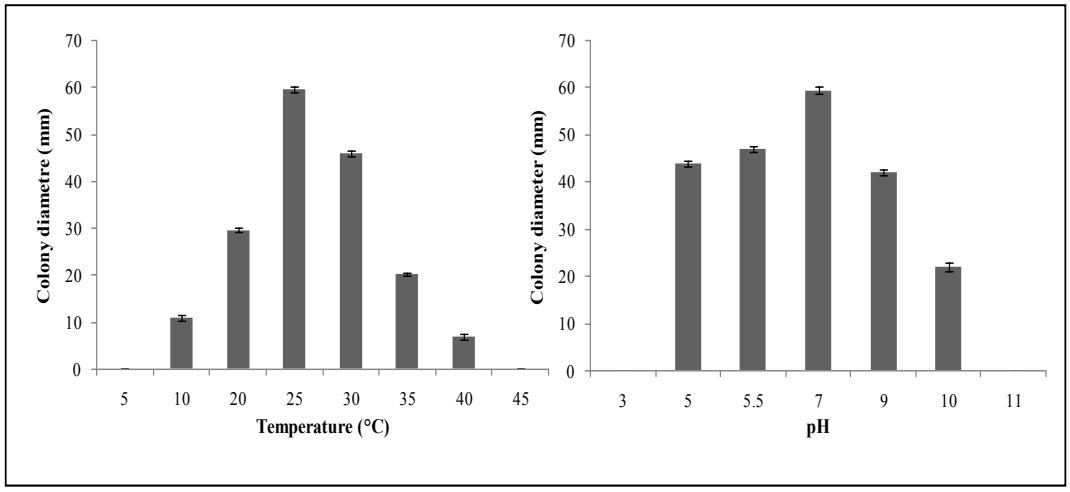


Fig 4.11: (A)-The effect of temperature and (B)- pH on the growth of the *Colletotrichum gloeosporioides* on PDA medium

Pathogenicity and sequence analysis

The pathogenicity of *C. gloeosporioides* was also confirmed by causing infection to the healthy large cardamom plant(s) by the isolated pathogen (Fig. 4.12 A-F), then re-isolation, identification and confirmation of the fungus. During the study first infection was noticed on inoculated plant after 17-18 days (Fig. 4.12-B), showing wilting symptoms. Initially black blight appearance of the leaf margin along with the yellowish dot was noticed (Fig. 4.12-C).

The phylogenetic analysis of ITS regions of *Colletotrichum* sp. sequence isolated from leaf blight of large cardamom showed 100% sequence similarity with the top five hits of *Colletotrichum gloeosporioides* sequence available in the public domain, i.e., isolate SFD-3 (MN498096.1), *Colletotrichum* sp. isolate – FLJ (MN498092.1), *Colletotrichum* sp. isolate – YN17 (MN486560.1) and *C. gleosporioides* isolate MKC5 (MN427973.1). Results of BLASTn analysis is

depicted in (Table 4.15). The phylogenetic tree was generated based on neighbour-joining method with the bootstrap support by taking 1,000 replicates and the resulted one major clad. The phylogenetic tree based on multiple sequence alignment analysis (Fig. 4.13) showed that the sequence share 100% homology with ITSsequences of five *Colletotrichum* sp., i.e., *C. gloeosporioides* (MN710587.1), (MN625177.1), (MN714388.1) and *C. fructicola* (MN608177.1), (MN598653.1) with 100% bootstraps value. The present isolate *C. gloeosporioides* (05) MN710587 performed the close clade with *C. gloeosporioides* (MN625177.1) with similarity coefficient .Total score and query coverage showed the comparison between the DNA sequences of ITSgene of *C. gloeosporioides* and other species of *Colletotrichum* from NCBI database (Table 4.15). The ITS region of the isolate isolated from large cardamom and the combined tree supported by close clade with *C. gloeosporioides* confirmed the identity of the isolate as *C. gloeosporioides* (Fig. 4.13).

Further to complement the above result another ML phylogenies generated in this study with eleven (11) isolates of *Colletotrichum* spp. comprising the isolate of the present study and ten (10) reference isolates taken from the GenBank gave identical sequences with 88%, 94%, 98%, and 99% bootstraps value, respectively (Fig. 4.13). Further, each isolate was associated with infection of different hosts such as *Oxalis corniculata*, tea, Spider lily and Chinese bean tree etc. and different geographical origin (Fu *et al.* 2013; Guo *et al.*2014;Sun *et al.* 2019; Ramos *et al.* 2019).The tree presented showed that present isolate *C. gloeosporioides* (MN710587.1) performed the close clade with *C. gloeosporioides* (JN165746.1). Onthe contrary*, C. gloeosporioides* (MN710587.1) also showed some distinctclade with other strains available in the database.

Table 4.14: Phenotypic and genotypic characters of the fungi isolate

Character	Description
Colony morphology on PDA	Fast growing, white colony with scanty mycelium and orange shade at the centre, reverse white, but turns orange on 5 days incubation at 25 °C with maximum mean 61.33±0.88 mm colony diameter.
Microscopic features	Mycelium of the isolate was septet, hyaline, and 2-4 µm wide. The conidiophores were cylindrical with both ends rounded and sometimes oblong. Length and breadth of conidiophores were 11-12 µm and 3-4 µm, respectively.
Physiological characterization (pH and temperature)	Can endure wide range of temperature between 10°C to 40°C (optimum 25°C), and pH 5 to 10 (optimum 7)
Culture accession number	NFCCI 4542
Nucleotide sequence Accession number	MN710587.1
Phylogenetic relationship (18S rRNA analysis)and top hits upon BLASTn analysis	Maximum (100%) similarity with *Colletotrichum gloeosporioides* (MN710587.1), *C. gloeosporioides* (MN625177.1), *C. fructicola* (MN608177.1), *C. gloeosporioides* (MN714368.1), *C. gloeosporioides* MN427973.1, *C. fructicola* (MN608177.1) and *C. cereal* (MN486560.1)

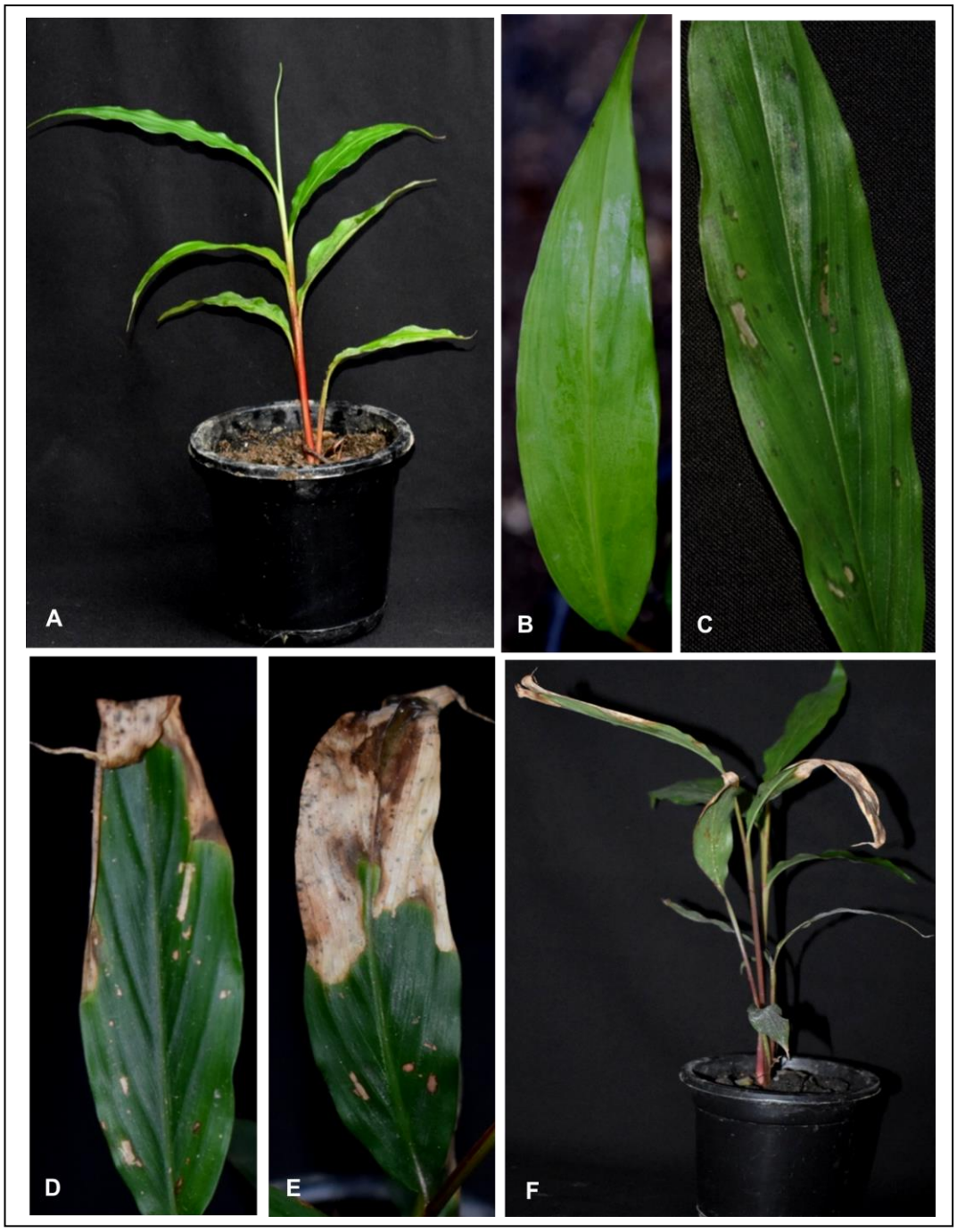


Fig. 4.12: The experiment on confirmation of pathogenicity of *C. gloeosporioides* on healthy large cardamom plant(s) in the nursery:(A) Control plant (Disease free large cardamom plant grown in the nursery , (B) The leaf after inoculation of the inoculum (Spores of *Colletotrichum gleosporioides*), (C) Initiation of distinct disease symptoms on the 18th day of inoculation, (D)Tip blight observed on 28th day of the diseases development on the leaf, (E) Enhanced leaf blight covering almost half of the leaf at 33rdday of inoculation, (F) The infected diseased plant

Table 4.15: Isolates of *Colletotrichum*spp. causing leaf disease in different host and geographical origin used in this study for phylogenic analysis

GenBank accession number	Causal organism	Host plant	Disease symptoms	Geographical origin	Source	Gene	Reference
MN710587.1	*C. Gloeosporioides*	Large cardamom	Leaf blight	Sikkim, India	This study	ITS	Present isolate
MK673858.1	*C. gloeosporioides*	*Hymenocallis littoralis* (Spider lily)	Leaf spot	China	GenBank	ITS	Sun *et al.* 2019
1JN165746.1	*C. gloeosporioides*	Chinese bean tree	Leaf spot	China	GenBank	ACT, CAL, CHS, GAPDH, TUB	Fu *et al.* 2012
MK639180	*C. gloeosporioides*	*Oxalis corniculata*	Leaf with anthracnose	Brazil	GenBank	ITS	Ramos *et al.* 2019
KC913204.1	*C. gloeosporioides*	Yellow Mountain fuzz tip, a cultivar of tea, (*Camellia sinensis* (L.) Kuntze)	Leaf Brown blight	China	GenBank	ITS	Guo *et al.*2014

MK2515971.1	*C. graminicola*	Maize	Leaf blight	China	GenBank	ITS	Duan *et al.* 2019
MN520417.1	*C. fioriniae*	*Mahonia aquifolium*	Leaf blight	Italy	GenBank	ITS, ACT, and TUB	Garibaldi *et al.* (2020)
HQ731491.1	*C. lineola*	*Swallow worts* (Cynanchum)	Leaf blight	Russia	GenBank	ITS	Berner *et al.* 2011
DQ839609.1	*C. acutatum*	*Myrica cerifera*	Leaf spot	Florida	GenBank	ITS	MacKenzie *et al.* 2006
EU859957.1	*C. cereal*	Creeping bent grass	Leaf blight	Mississippi and Alabama	GenBank	ITS	Young *et al.* 2008
EU000060.1	*C. linicola*	Bindweed	Leaf spot	Turkey	GenBank	ITS	Tunali *et al.* 2008

Source: GenBank (Benson *et al.* 2012)

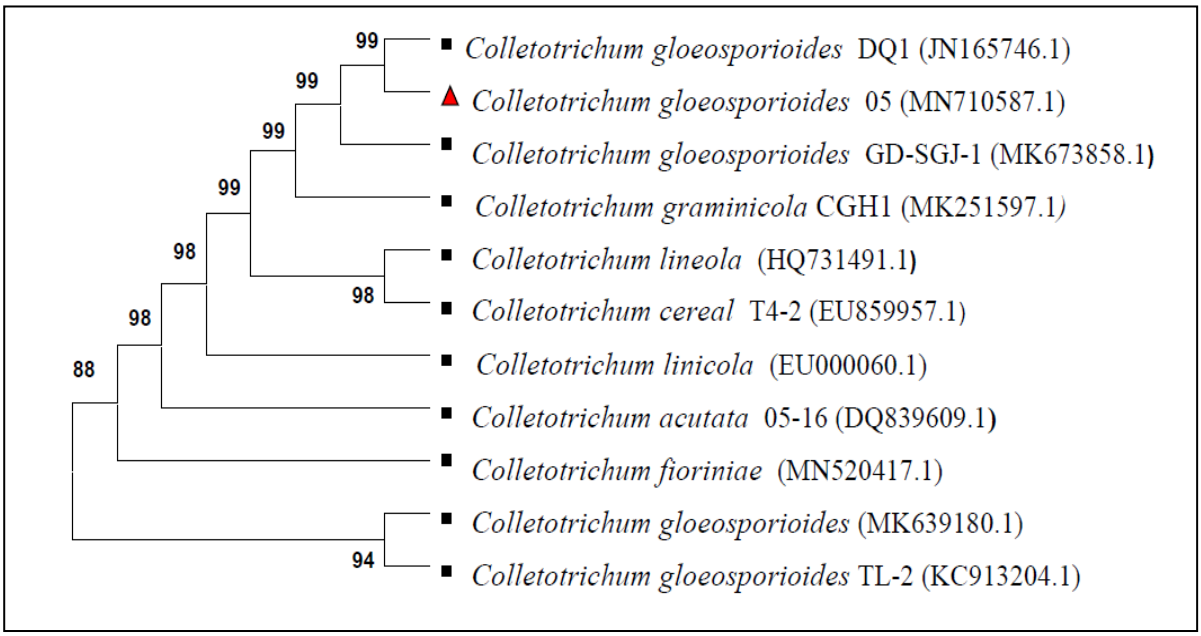


Fig 4.13: Phylogenetic tree builtusing neighbor-joining (NJ) methods with ITS nucleotide sequence of *Colletotrichum gloeosporioides* isolated from large cardamom (*Amomum subulatam* Roxb.) and other ITS sequences *Colletotrichum* spp. of plants species using MEGA X. Bootstrap values are indicated for each branch divergence of 1,000 replicates

(KC913204.1) and *C. gloeosporioides (*MK639180.1) causing leaf diseases in tea and *Oxalis corniculata*, respectively showed a distinct clade with the present strain with 94% bootstraps value. The analysis revealed that the homology rate between some different species, such as *C. graminicola* (MK2515971.1), *C. lineola* (HQ731491.1) and *C. cereal* (EU859957.1) was even higher (99% and 98% respectively) than that along with different host accessions of the same species, suggesting that nucleotide variation among all the *Colletotrichum* spp. was not only related to geographical origin but also host associated (Fig. 4.13).

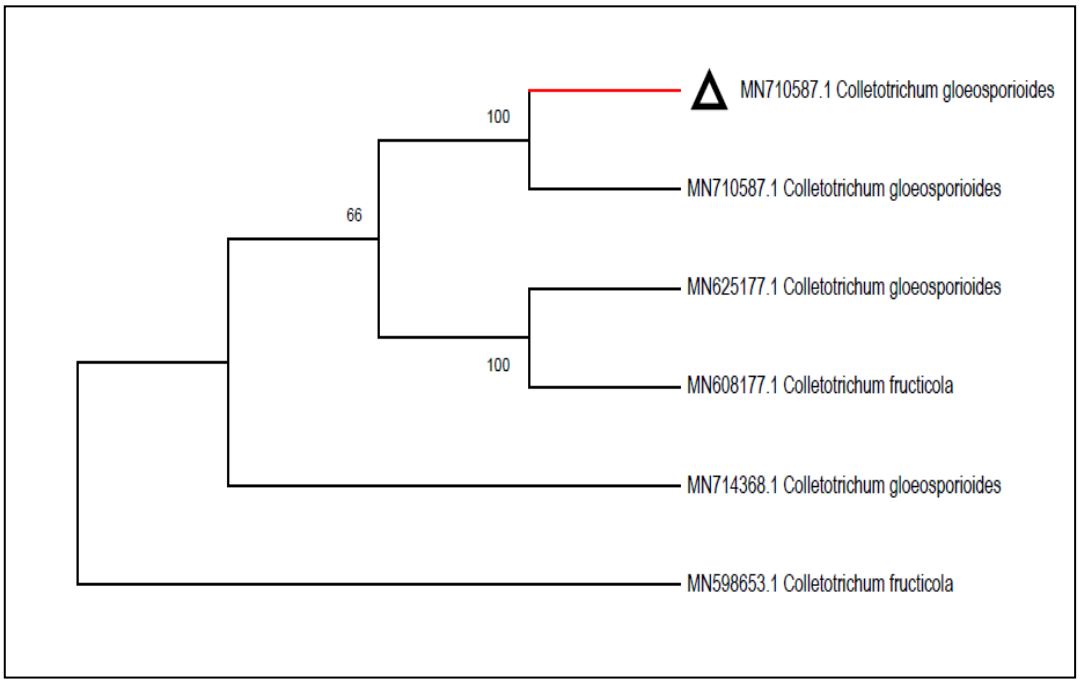


Fig 4.14: Phylogenetic tree builtusing neighbor-joining (NJ) methods with ITS nucleotide sequence of *Colletotrichum gloeosporioides* isolated from large cardamom (*Amomum subulatam* Roxb.) and ten (10) other reference ITS sequences of *Colletotrichum* spp. retrieved from the GenBank reported causing similar types of leaf disease (blight, spot and anthracnose etc.) in various host plants and geographical origin using MEGA X. Bootstrap values are indicated for each branch divergence of 1,000 replicates

Hence, based on the colony characteristics like growth, colour, morphology, cylindrical conidia with round ends and application of species-specific PCR using ITS primers developed for *C. gloeosporioides* confirm the pathogen as *C. gloeosporioides* isolated from diseased large cardamom grown in Sikkim. The fungal culture has been deposited at the NFCCI-ARI, Pune with an accession number NFCCI 4542 and the sequences have been deposited in NCBI GenBank with accession number MN710587. To the best of our knowledge, this is the first

confirmatory molecular characterization of *C. gloeosporioides* causing blight disease of large cardamom in Sikkim as a major pathogen. The study mirrored the importance of future research on this pathogen to alleviate the risk to the large cardamom cultivation in Sikkim.

Morpho-molecular characterization of isolate No. LC06, LC08, LC09

Phenotypic characteristics of the disease

During the study three isolates (No. LC06, LC08, LC09)were collected from different cardamom growing regions in Sikkim causing wilt and rot in the large cardamom. Thedisease was a typical leaf blight followed by wilting and in rot ofthe cardamom plants.The wilting of the plant and the individual leaf is the characteristics symptoms of the pathogen. The grayish color on the leaves accompanied by the sudden wilt where the whole becomes water soaked which becomes black in color and ultimately rots (Fig. 4.15 A, B). At first the disease starts from the tip with blighted appearance and gradually moves towards the basal region of the plant.

The disease was especially could be observed in large cardamom in the month of June –September when humidity in the air is quite high. If the weather condition is dry, the white portion seen on the leaves does not spread instead becomes dry and the remaining portion of the leaves becomes discolored with yellowish to pale. The diseases is also accompanied by the larger spots staring from the tip which eventually spreads on the entire leaves especially on the older one which results in the blighted tip.

Growth pattern of fungal pathogen in different medium

Growth of all the isolates was different irrespective of varied solid media used for the morphological characterization. Colony colour varied from white, white colony with cottony mass, pink, dark-pink, pinkish-white etc. when observed from the

front and inverted plate (Table 4.16).List growth was recorded for the isolate LC01 in the (SDA) medium with colony diameter 17.00±1.15 mm and maximum LC07 58.00±1.53 mm in PDA medium. Among the media tested in terms of overall growth PDA was found to be the most suitable one (Table 4.17).

Influence of temperature and pH on the growth of the fungal isolate

It is known that temperature and pH of the medium requirement are important physical factors affecting growth of any microbial isolate in culture. Hence the morphological characterization of all the isolates was then followed by physiological characterization where the two basic parameters i.e., temperature and pH of the isolates was studied. The wide range of the temperature were set ranging from the lowest i.e., 5°C to the highest i.e., 50°C. The result revealed that the maximum colony growth was recorded at 25°C for all the isolates (Table 4.18).

Likewise, growth of the isolates was also recorded in different pH ranges of the medium. Best and maximum growth was observed in the medium with pH 5-9(Table 4.18). Isolates failed to grow at very low to high pH of the medium (i.e., pH 3 and 13, respectively).

The mycelium was septate, hyaline, and 3-7 µm wide. The conidiospores were simple cylindrical, with two to several celled, fusiform to sickle shaped with elongated apical cells(Fig. 4.15 E). Hence, the above mentioned three pathogenic isolates (No. LC06, LC08, LC09) which had different morphological features, gross growth responses but on the basis of overall colony characteristics and microscopic features, those isolates were similar to those of*Fusarium* sp. (Leslie and Summerell 2006)and which was further confirmed by the National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune with accession number NFCCI- 4543, NFCCI- 4544, NFCCI- 4699.

Table 4.16: Colony morphology of fungal isolates on five different media

Isolates No.	Potato Dextrose Agar	Sabouraud Dextrose Agar	Czapek dox Agar	V8 juice Agar	Potato Carrot Agar
LC06	White with cottony mass colony.Invert plate color-yellowish at centre with white margin	Fully cottony mass white colony. Invert plate color- white	White colony, invert plate color- white	White cottony mass colony.Invert plate color – white	White colony, invert plate color- yellowish at the centre with white margin
LC08	Dark-pink with white margin with cottony mass colony.Invert plate color-dark-pink	Pinkish-white colony with cottony mass. Pinkish at the centre along with white margin.Invert plate color-pink	Cottony mass with white colony. Invert plate – white	Whitish cottony mass colonyInvert plate color- off white	Pink cottony mass colony with white margin.Invert plate color- pink
LC09	Light pink and white colony with cottony mass. Invert plate color – dark pink	White and pink colony with cottony mass Invert plate color- off white	White colony with cottony mass. Invert plate color- white	White colony with cottony mass. Invert plate color- white	White and pink at the centre colony with cottony mass. Invert plate color- white

Table 4.17: Effect of different solid media on the growth of different fungal isolates

Isolate no.	Potato Dextrose Agar (PDA)	Sabouraud Dextrose Agar (SDA)	Czapek Dox Agar	V8 Juice Agar	Potato Carrot Agar
	Colony diameter (mm)±SE				
LC06	27.67±0.88	32.33±0.67	26.67±0.89	32.33±0.89	22.66±0.67
LC08	45.00±1.15	42.67±0.88	52.67±0.88	56.00±1.53	39.33±0.88
LC09	51.00±0.58	36.67±0.88	59.33±1.45	59.67±1.45	48.00±0.58

Each treatment consisted of three replications. The results were recorded after incubation for 5days at 25°C, and media pH 5.5.

SE = Standard error.

Table 4.18: Temperature and pH requirement of fungal isolates

Isolate No	Temperature range (°C)	pH range
LC06	5-45, opt. 25	3-13,opt.7-9
LC08	5-45, opt. 25	3-13,opt.7-9
LC09	5-45, opt. 25	3-13,opt.7-8

Table 4.19: Conidial characteristics of different isolates

Isolate No.	Length (µm)	Width (µm)	Shape
LC06	1.28	0.44	Cylindrical
LC08	0.98	0.44	Cylindrical
LC09	1.45	0.54	Cylindrical

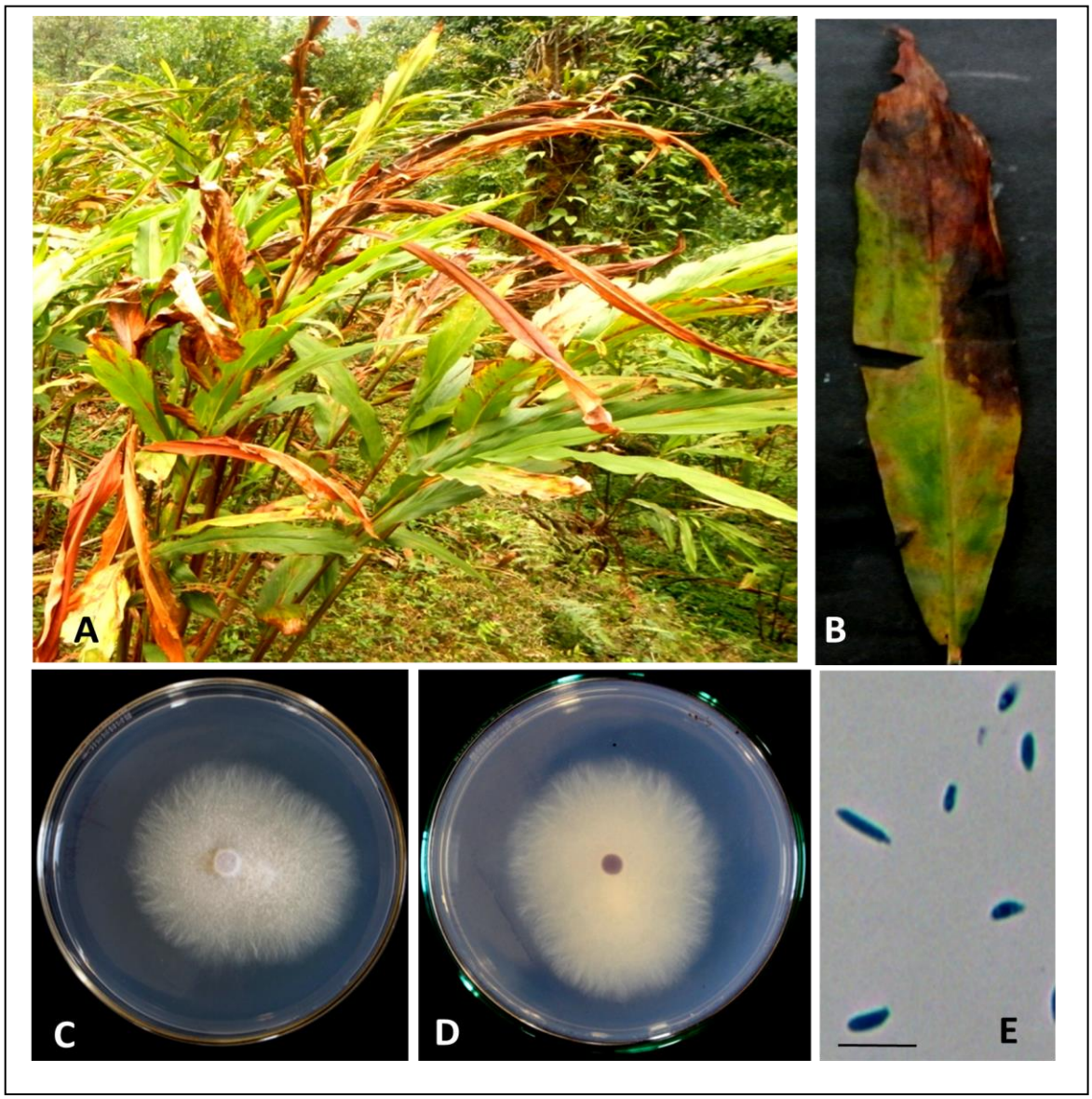


Fig: 4.15: A- Close view of infected plant caused by *Fusarium sp.* B-The leaf with the typical symptoms of rot caused by *Fusarium sp.* C-D *In vitro* growth of *Fusarium sp* (C- front view, D- view from lower side). E- conidiospores of *Fusarium sp.* (Bar= 100µm).

Morphological characterization of isolate No. LC11

Growth pattern of fungal pathogen in different medium

This is a typical leaf disease where it is accompanied by the symptoms of small lesion on the younger leaves. Leaf spots are usually observed with numerous water soaked lesions, round in shape with greyish margin which eventually develops into a prominent yellowish spots. (Fig. 4.16 A,B). The symptoms are seen mostly in the nursery stages. It has also been recorded in Arunachal Pradesh along with Sikkim and Darjeeling hills. In due course of time, these spots increase in size, turning yellow to brown and black spots. Numerous spots coalesce to form a bigger patch which resembles as the blighted leaf and dries out.

The infected leaves particularly with the spot or blight were taken as an experimental sample for the isolation procedure. The isolate obtained from the sample were subjected to different physico-morphological characterization. The colony growth was maximum when grown on potato dextrose agar and Sabourauddox agar with 26.6 mm and 25.00 mm respectively. Similarly the growth performance of the isolate was moderate when grown on V8-juice agar and potato carrot agar with 18.6mm and 21.3mm respectively**.** Colony morphology of theisolate obtained on PDA is presented in**(**Fig.4.16). Colony morphology, microscopic characteristics,and physiological requirements of the fungus are presented in Table (4.20).The fungus was found to have the growth in a wide range of temperature 10°C to 35°C (optimum 25°C) and tolerated wide range of pH (5.0–13., optimum 6.0). On the basis of colony morphology, microscopic features the fungus was identified as *Phoma cava Schulzer* **.** The culture has been accessioned as NFCCI 4663.

Table 4.20 : Phenotypic and Genotypic description of *Phoma cava* (LC11)

Character	Description
Colony morphology	Front view-White colony with cottony mass Invert plate color-light yellowish
Microscopic features	Septate with dark round to oval shaped conidia with the approximately length and width of 50 µm and 3 µm respectively.
Physiology characters (pH and temperature)	Temperature requirement between 5°C to 50°C optimum 25°C and pH 5 to 13 optimum 6
Accession number	NFCCI-4663

Table 4.21: Effect of different solid media on the growth of *Phoma cava* (LC11)

Colony diameter (mm) ±SE					
Potato Dextrose Agar (PDA)	**Sabouraud Dextrose Agar (SDA)**	**Czapek Dox Agar**	**V8 Juice Agar**	**Potato Carrot Agar**	**LSD**
26.65±0.33	25.00±0.58	18.67±0.88	20.00±0.58	21.33±0.88	2.15

Each treatment consisted of three replications. The results were recorded after incubation for 5days at 25°C, and media pH 5.5.

SE = Standard error; LSD = Least significant difference.

Table 4.22: The effect of temperature on the growth of the *Phoma cava* (LC11)

Temperature (°C)	Colony growth (mm) ± SE
5	0±0
10	27.66±0.88
20	31.33±0.33
25	47.33±1.33
35	25±1.73
45	0±0
50	0±0
LSD	3.88

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media with pH 5.5.
SE = Standard error; LSD = Least significant difference.

Table 4.23: The effect of pH on the growth of the *Phoma cava*(LC11)

pH	Colony growth (mm) ±SE
3	0±0
5	33.67±0.88
6	31.0±1.15
7	30.0±0.58
8	29.8±0.88
9	23.67±0.88
10	0±0
11	0±0
13	0±0
LSD	2.58

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media at 25°C. The pH value of the media was adjusted before autoclaving.
SE = Standard error; LSD = Least significant difference

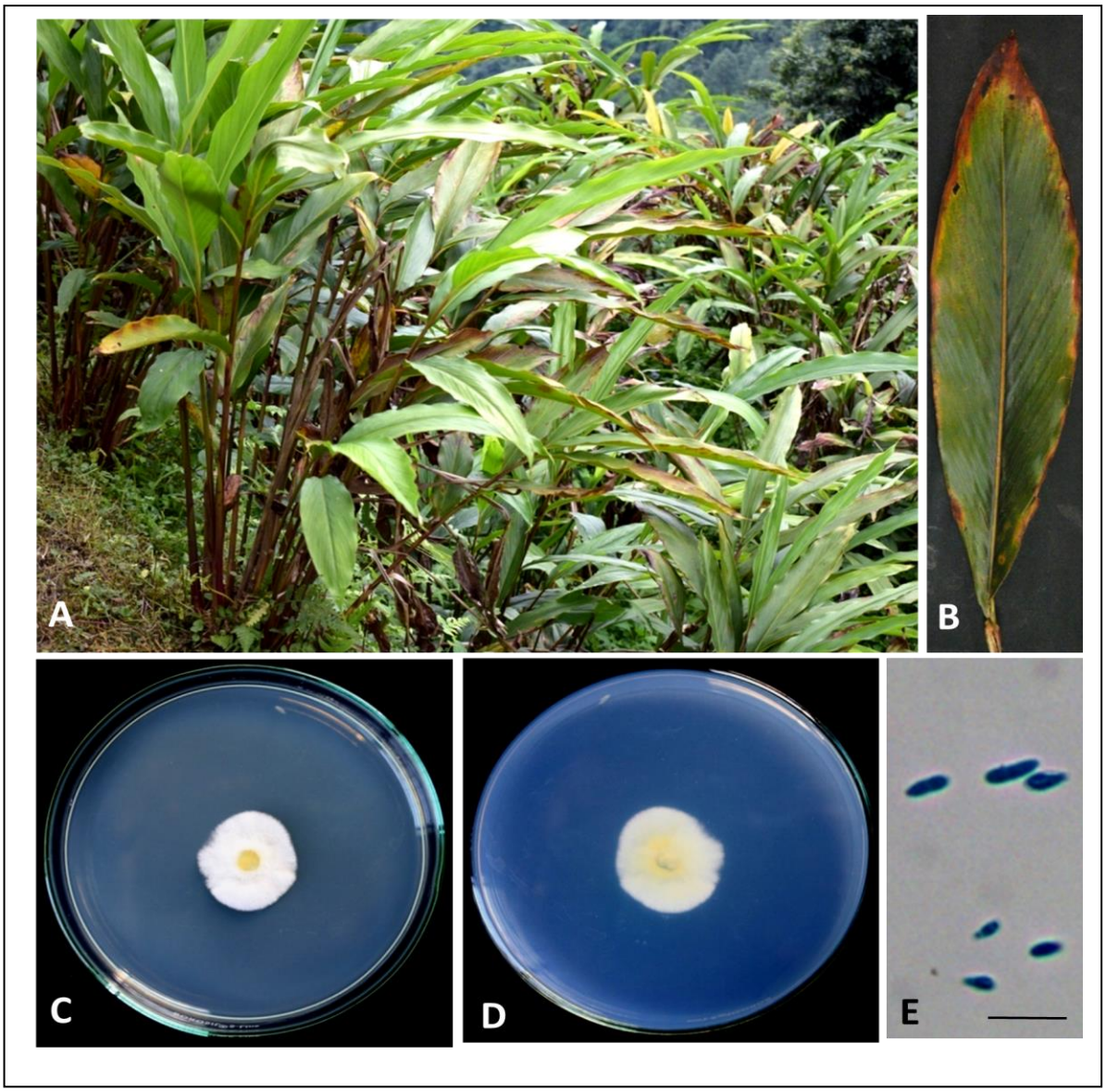


Fig: 4.16: A- the close view of the large cardamom plant with the *Phoma* leaf blights; B- the leaf with the *Phoma* leaf blight. C&D- *In vitro* growth of *Phoma cava* (C- front view, D- view from lower side). E- conidiospores of *Phoma cava*(Bar = 100µm).

Morpho-molecular characterization of isolate No. LC12

Growth pattern of fungal pathogen in different medium

Among the seven different pathogens isolated during the study, *Epicoccum nigrum* is one of the new recorded pathogen encountered during the study. The pathogen is known to cause a leaf disease accompanied by the leaf spots in several crops in the field. The symptoms are seen with the chlorotic spots especially on the upper side of the leaf (Fig. 4.17A,B). These spots were irregular in shape with 2-6mm diameter on the leaf margin which was reddish to brown in color. In some, these spots were encountered in a larger necrotic lesion which was spreaded sparsely on the leaf surface giving the blighted appearance. In large cardamom, the spots were seen in the *Varlangey* and *Sermna* varieties grown in the open condition.

Radial mycelial growthof the isolate was significantly(P <0.05) affected by the culture media. Results revealed that out of five different media tested for the fungal isolate, three, i.e., Potato Dextrose Agar (PDA) and V8 Juice Agar were found to be significantly different (P <0.05) and gave satisfactory performance with colony growth 63.17 and 62.33 mm respectively (Table 4.25).

Temperature was found to be another important physical factor influencing growth of fungal isolates. In the present study, the mycelial growth of the isolate followed linear trends of response with the changes in temperature up to 35°C and then decreased sharply as temperature increased thereafter. Colony growth was least at 45°C and at 10°C. The optimum temperature for colony growth was found to be between 25°–35°C with colony diameter 63.17mm after 5 days of incubation. No colony growth was observed at 5°C and 50°C temperature (Table 4.26).

Medium pH also significantly affected radial mycelial growth of the isolate. Colony growth of the isolate wasobserved in a wide range of pH (5.0 – 9.0) of medium. Maximum colony growth was obtained at medium pH 6.0 - 8.0 with 59.3-43.4 mm colony diameter (Table 4.27). Then asharp decline of the growth occurred when medium pH increased to 10 or more. No colony growth was observed whenmedium pH was 3 and 13.

The mycelium was septate, hyaline, and 3-6 µm wide. The Conidia were globose to pyriform, with 1.11µm length and 0.44µm width. (Table 4.24; Fig. 4.17). On the basis of colony morphology and microscopic features, the isolate was identified as *Epicoccum nigrum* (NFCCI-4545) by National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune.

Based on ITS sequences and phylogenetic analysis top five hits upon BLASTn analysis are *Epicoccum nigrum* isolate 09-B1 (*Neopestalotiopsis sp.* (MK370650.1), *Epicoccum nigrum* strain AG22 (MF188977.1), Uncultured fungus clone ZSY201307-21 (KX515742.1) and Uncultured fungus clone ZBJ201307-58 (KX515326.1) with maximum (100%) similarity with the isolate. The tree has shown the highest log likelihood. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. The submission number of the gene sequence alignment at NCBI is MN712328, was identified as *Epicoccum nigrum* (Fig. 4.18).

Table 4.24: Phenotypic and Genotypic description of *Epicoccum nigrum* (LC12)

Character	Description
Colony morphology	Front view - white cottony mass with pink at the centre Invert plate - white color with pink at the centre with white margin
Microscopic features	Conidia are globose to pyriform, with 1.11µm length and 0.44µm width.
Physiology characters (pH and temperature)	Temperature requirement between 5°C to 50°C optimum 25°C and pH 3to 13 optimum 7-8
Accession number and nucleotide sequence number	NFCCI-4545 and MN712328 respectively
Phylogenetic relationship (18rRNAs analysis) with the top five hits upon BLASTn analysis	100 % similarity with *Epicoccum nigrum* isolate 09-B1 (*Neopestalotiopsis sp.* (MK370650.1), *Epicoccum nigrum* strain AG22 (MF188977.1), Uncultured fungus clone ZSY201307-21 (KX515742.1) and Uncultured fungus clone ZBJ201307-58 (KX515326.1).

Table 4.25: Effect of different solid media on the growth of *Epicoccum nigrum* (LC12)

Colony diameter (mm) ± SE					
Potato Dextrose Agar (PDA)	**Sabouraud Dextrose Agar (SDA)**	**Czapek Dox Agar**	**V8 Juice Agar**	**Potato Carrot Agar**	**LSD**
62.67±1.45	47.33±0.88	54.00±0.58	62.33±1.45	47.00±0.58	3.35

Each treatment consisted of three replications. The results were recorded after incubation for 5days at 25°C, and media pH 5.5.

SE = Standard error; LSD = Least significant difference.

Table 4.26: The effect of temperature on the growth of the *Epicoccum nigrum* (LC12)

Temperature (°C)	**Colony growth (mm)±SE**
5	0±0
10	24.3±0.41
20	37.0±0.83
25	47±1.66
35	32.5±0.41
45	11.5±2.07
50	0±0
LSD	2.73

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media with pH 6.0

SE = Standard error; LSD = Least significant difference.

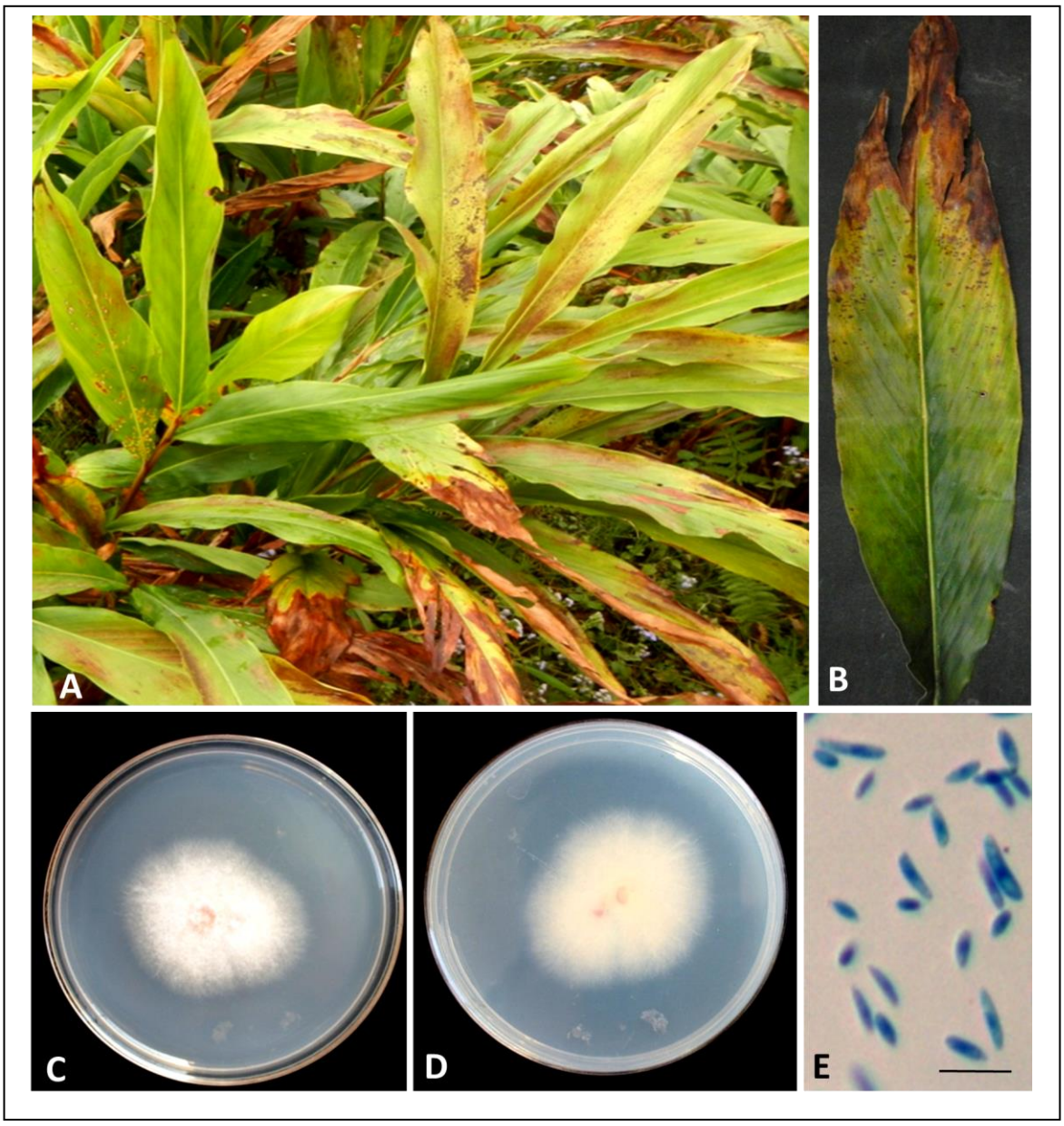


Fig 4.17: A- close view of the plant with leaf diseases caused by *Epicoccum nigrum*; B-the leaf with the typical numerous leaf spots. C &D - *In vitro* growth of *Epiccocum nigrum* (C- front view, D- view from reverse side). E- Conidiospores of *Epicoccum nigrum* (Bar= 100µm).

Table 4.27: The effect of pH on the growth of the *Epicoccum nigrum* (LC12)

pH	Colony growth (mm) ±SE
3	0.0±00
5	34.0±0.58
6	59.3±0.58
7	54.0 ±0.58
8	37.3±0.58
9	43.4±0.58
10	32.4±0.88
11	21.3±1.20
13	0.0±00
LSD	2.52

Each treatment consisted of three replications. The results were recorded after incubation for 5days on PDA media at 25°C. The pH value of the media was adjusted before autoclaving. SE = Standard error; LSD = Least significant difference

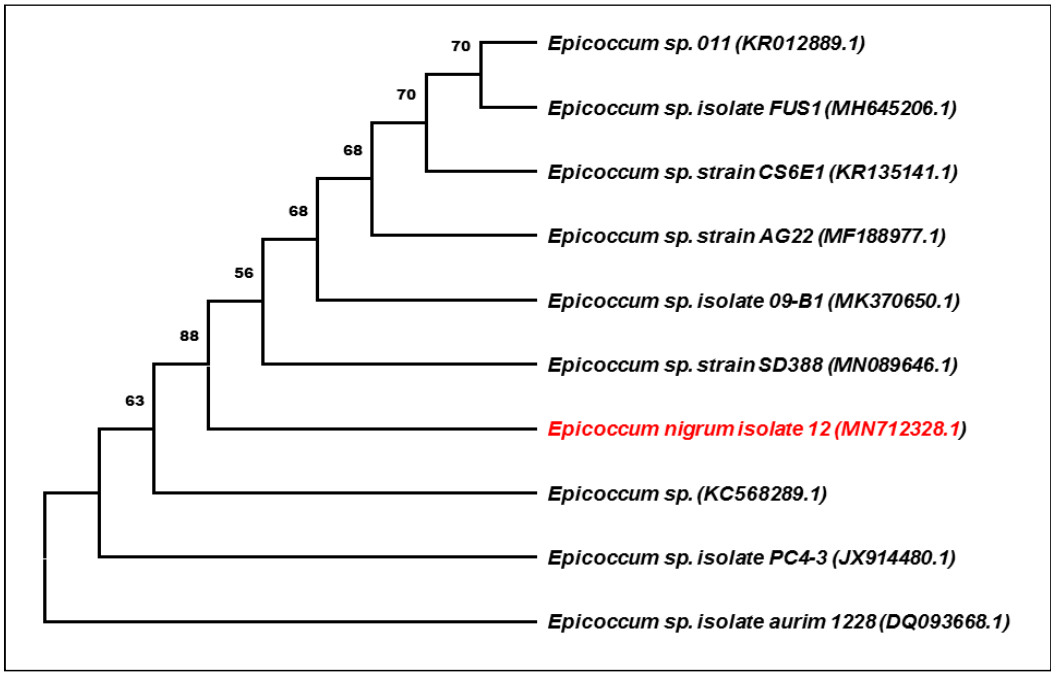


Fig 4.18: Phylogenetic tree builtusing neighbor-joining (NJ) methods with ITS nucleotide sequence of*Epicoccum nigrum* isolated from large cardamom (*Amomum subulatam* Roxb.) using MEGA X. Bootstrap values are indicated for each branch divergence of 1,000 replicates.

4.2. *In vitro* screening of botanicals and indigenous knowledge based formulations to control the fungal disease

4.2.1. Collection of the botanical

Based on the primary survey conducted during this study and secondary information 50 botanicals were collected from all four districts of Sikkim. Survey was conductedbetween May- June 2017, the peak growing season of vegetations in the region. The botanicals were collected based on traditional use as practiced by the local inhabitant. Initially in a rapid screening, based on the anti fungal activity, 24 botanicals were shortlisted for further study (Table 4.28).

4.2.2. *In vitro* antifungal activity of botanicals against *Colletotrichum gloeosporoidies*

Antifungal efficiency of the selected 24 botanicals was tested against the *C. gloeosporioides* under *in vitro* conditionin the plant pathology laboratory, Deptt. of Horticulture, Sikkim University, Gangtok. The antifungal potential of these selected botanicals was determined following the disc diffusion method.For this study total six solvents i.e., water, ethanol, dichloromethane (DCM), acetone, ethyl acetate and chloroform were used for the preparation of plant extract. In the beginning botanicals were extracted in the distilled water, but none of the botanicals tested showed any response against the pathogen and no inhibition zone was noticed. Interestingly when extraction was done using solvents all the botanicals showed positive response against the pathogen with varied responses depending upon the botanicals and solvents used. The result revealed that the ethanol as a solvent was found to be the best solvent for extraction of botanicals in comparison to the other solvents used during the experiment. Ethanol extract of the botanicals showed maximum zone of inhibition when applied against the test

Table 4.28: List of plant species studied along with their traditional uses and location of collection

Sl. No	Plant species	Local name	Family	Parts used	Indigenous traditional use/ practices	Place of collection	Altitude (m amsl)	Latitude (N)	Longitude (E)
1	*Capsicum annum var .cerciformae*	Dalle khorsani	Solanaceaae	Fruit	Reduces cholesterol	Nandok , East Sikkim	1155	27° 16' 51"	88° 36' 27"
2	*Sechium edule* (Leaf)	Iskus	Curcurbitaceae	Fruit and leaf	Good for thyroid health, Prevents kidney stones, anaemia, anti aging properties	Nandok , East Sikkim	1133.5	27° 18' 51"	88° 36' 49"
3	*Sechium edule* (Fruit)	Iskus	Curcurbitaceae	Fruit and leaf	Good for thyroid health, Prevents kidney stones, anaemia, anti aging properties	Nandok , East Sikkim	1133.5	27° 18' 51"	88° 36' 49"
4	*Nasturtium officinale*	Simrayo	Brassicaceae	Leaf	Jaundice, relief from Hypertension	Gaucharan ,East Sikkim	1335	27° 11' 35"	88° 40' 15"
5	*Nictotiana tabacum*	Kacho paat	Solanaceae	Leaf	Used as an insecticide	Geyzing, West Sikkim	1430	27° 19' 02"	88° 14' 53"

6	*Lantana camera*	Aulay Banmara	Verbenaceae	Leaf	Antiseptic, anti spasmodic , helps in healing of wounds	6th mile, tadong, East Sikkim	1650	27° 10' 48"	88° 12' 10"
7	*Azadirachta indica*	Neem	Meliaceae	Leaf	Used against boils, antiseptic, decoction	Belbotey,South Sikkim	828	27^0 07' 74	88^0 20' 73
8	*Zanthozylum aramutum*	Timur	Rutaceae	Berries	Treat sore, fever and colds, malaria, measles, skin infections and coughs	Mangalbaria, West Sikkim	1142	27° 19' 24"	88°19' 18"
9	*Tupistra nutans*	Nakima	Asparagaceae	Flower	Used to treat chickenpox, Slow-healing wounds, antidiabetic	Geyzing, West Sikkim	823	27° 16 ' 14"	88°18' 27"
10	*Curcuma longa*(leaf)	Haldi	Zingiberaceae	Leaf	Anti-parasitic for skin infection, blood purifier, applied on wounds	Chisopani, south Sikkim	913	27^0 09'.50	88^0 18' 09
11	*Allium sativum*	Lasun	Alliaceae	Bulb	Skin diseases, flatulence, colic	Kitam, South Sikkim	891	27° 06' 03"	88° 23' 49"

12	*Brassica oleracea var.capitata*	Banda gobi	Cruciferous	Leaf	Bio-fumigation	Assam linzey, East Sikkim	1335	27° 14' 37"	88° 35' 58"
13	*Brassica junacea*	Tori ko sag	Cruciferous	Leaf	Good during the stomach upset	Assam linzey East Sikkim	1335	27° 14' 37"	88° 35' 58"
14	*Cannabis sativa*	Ganja	Cannabaceae	Leaf	Spasmolytic, analgesic	4th mile, South Sikkim	876	27° 11' 53"	88° 21' 49"
15	*Glaphylopteriopsis erubenscens*	Fern	Thelypteridaceae	Leaf	Rheumatism	Metro point, tadong East Sikkim	1120	25° 85, 11,,N,	93.77°E
16	*Litsea citrita*	sil timur	Lauraceae	Berries	Sprains, indigestion, paralysis and even mental disorders like hysteria	Mangalbaria,West Sikkim	1142	27° 17' 23"	88°15' 15"
17	*Ocimum sanctum*	Tulsi	Labitaceae	Leaf	Applied for curing ring worm and other skin diseases	6th mile, tadong East Sikkim	1650	27° 10' 51"	88° 12' 05"
18	*Curcuma amada*	Mango ginger	Zingiberaceae	Rhizome	Laxative and expectorant	Kitam, South Sikkim	818	27^0 07' 38"	88^0 21'11"
19	*Schima wallichi*	Chilawney	Theaceae	Leaf	uterine disorders	Namcheybong, East Sikkim	1250	27° 18' 51"	88° 36' 49"

20	*Pieris Formosa*	Angeri	Ericaceae	Leaf	Intestinal worms as well as scabies	Pakyong, East Sikkim	1364	27° 18' 53"	88° 36' 34"
21	*Phytolacca acinossa*	Jaringo	Phytolaccaceae	Leaf	Treat bleeding infection, cuts, mastitis	Namcheybong, East Sikkim	1250	27° 18' 51"	88° 36' 49"
22	*Moringa olerifera*	Sajana	Moringaceae	Leaf	Urinary disorders, piles, asthma	Chisopani, South Sikkim	913	27^0 09´50"	88^0 18' 09"
23	*Heracleum wallichi*	Chimphing	Apiaceae	Flower	Stomach disorders, tuberculosis	Padamchen, East Sikkim	1350	27° 14' 41"	88° 36' 15"
24	*Clematis buchnaniana*	Pinaasay lahara	Ranunculaceae	Leaf	Cure sinusitis	6^{th} mile, tadong, East Sikkim	1650	27° 06' 03"	88° 23' 49"

pathogen (Table 4. 29). All the botanicals tested in the study extracted in ethanol showed zone of inhibition with varying intensity depending upon the plant species used. On the other hand, the Di-chloromethane (DCM) extract showed least effect and the zone of inhibitions found to be the minimum for extracts of 15 botanicals and no response was observed in 8 botanicals.

When compared the efficiency of the botanicals extracted in ethanol *Zanthozylum aramutum* was found to be the most effective and showed maximum inhibition zone (14.5±0.41 mm) among other botanicals tested. This was followed by the ethanol extract of *Litsea citrita* and *Heracleum wallichi* showing 14±0.94 mm and 13.6±0.57 mm inhibition zones respectively. Likewise the zone of inhibition was recorded to be quite promising in *Litsea citrita* acetoneextract with inhibition zone 13.5±1.41 mm followed by *Nictotiana tabacum* extract with 11.5±1.2 mm.

When ethyl acetate was used as a solvent to extract the botanicals maximum zone of inhibition was obtained with the extract of *Zanthozylum aramutum* followed by *Nictotiana tabacum* with the inhibition zone 12±0.41 mm and 11±0.94 mm respectively. Interestingly when chloroform was used as solvent, theextract of *Lantana camera* showed the highest zone of inhibition with 14±0.83 mm followed by the extract of *Zanthozylum aramutum* with 9±0.83 mm inhibition zone.

The result as depicted in the table – 4.29 regarding the anti fungal activity of botanicals tested in the present investigation revealed that irrespective of the solvents used, extract of *Zanthozylum aramutum* was found to be the most effective against the test pathogen and maximum inhibition zone (14.5±0.41 mm) was obtained when extracted in ethanol. This was followed by the extract of *Lantana camera* that showed significant zone of inhibition irrespective of

Table 4.29: Antifungal efficacy of the different botanicals and solvents used for extraction on *Colletotrichum gloeosporoidies*

		Diameter of the zone of inhibition (mm) ± SE				
Sl. No.	**Plant extract**	**Ethanol**	**Dichloromethane (DCM)**	**Acetone**	**Ethyl acetate**	**Chloroform**
1	*Capsicum annum var .cerciformae*	2.5±0.47	1.9±0.09	3.2±0.23	3.3±0.14	2.2±0.23
2	*Sechium edule* (Leaf)	1.9±0.09	0.9±0.09	1.65±0.14	1.65±0.14	0.9±0.09
3	*Sechium edule* (Fruit)	2.75±0.23	1.65±0.14	2.15±0.14	1.75±0.23	1.25±0.23
4	*Nasturtium officinale*	2.5±0.47	1.5±0.47	2.25±0.23	2.75±0.23	1.25±0.23
5	*Nictotiana tabacum*	11.6±0.88	6±0.94	11.5±1.2	11±0.94	7.5±0.47
6	*Lantana camera*	11.5±1.24	5.5±0.41	7±0.83	6±0.83	14±0.83
7	*Azadirachta indica*	9.5±0.41	4.5±0.41	12±0.83	5.5±0.41	3.75±0.62
8	*Zanthozylum aramutum*	14.5±0.41	7.5±0.41	9±0.83	12±0.41	9±0.83
9	*Tupistra nutans*	1.25±0.23	1.6±0.09	1.1±0.09	1.25±0.23	0.9±0.09
10	*Curcuma longa*(leaf)	5±0.58	NA	1.16±0.44	1.33±0.33	1.16±0.44
11	*Allium sativum*	7.33±0.33	4.33±0.33	4.66±0.67	2±0.58	5.16±0.61
12	*Brassica oleracea var.capitata*	11±0.58	NA	5.33±0.33	7.33±0.67	1.66±0.67
13	*Brassica junacea*	4.33±0.89	NA	1±0.58	7.33±0.89	4.33±0.89

14	*Cannabis sativa*	8.6±0.67	NA	1.83±0.94	5.66±0.44	0.66±0.16
15	*Glaphylopteriopsis erubenscens*	6±0.94	3.5±0.47	4.5±0.47	5.5±0.47	2.75±0.23
16	*Litsea citrita*	14±0.94	5.5±0.47	13.5±1.41	6±0.94	3.5±0.47
17	*Ocimum sanctum*	8.33±0.33	3.66± 0.33	6.25±0.20	5±0.57	4±0.57
18	*Curcuma amada*	6.6±0.33	NA	4.6±0.33	2.6±0.33	NA
19	*Schima wallichi*	9.6±0.66	NA	3.1±0.16	4±0.0	5.33±0.33
20	*Pieris Formosa*	4.3±0.33	NA	5.3±0.33	5.5±0.47	NA
21	*Phytolacca acinossa*	10.3±0.5	NA	3.9±5.2	NA	4±0.57
22	*Moringa olerifera*	12.6±1.5	0.8±0.5	7.3±5.5	11.6±0.5	11.3±1.5
23	*Heracleum wallichi*	13.6±0.57	6.6±1.5	7.2±5.6	0.8±0.1	0.6±0.1
24	*Clematis buchnaniana*	6.6±0.33	2.2±0.23	3.2±0.4	2.4±0.2	2.2±0.23
	LSD(P=0.05)	1.37	0.91	1.32	1.24	0.94

*All the experiment was performed in triplicates; Values are mean inhibition zone (mm) ± SE. The zone of inhibition was recorded after five days, incubated at 25°C, SE= Standard error, NA - No activity.

solvents used in this study. Chloroform extract of the *Lantana camera* was found to be the most effective and showed 11±0.94 mm inhabitation zone against the *C. gloeosporioides*.

4.2.3. Minimum Inhibitory Concentration (MIC) determination

The Minimum Inhibitory Concentration(MIC) was determined to record the quantitative estimation of the extract used in the study. The MIC values are depicted in the table 4.30. Results showed a wide range of MIC, which indicates that the activity of botanical extracts varied depending on the plant species and solvent used. Out of the 6 different solvents ethanol extract showed the highest inhibition effect followed by acetone and ethyl acetate extracts respectively. No response was observed for aqueous extract. The result also confirmed lowest MIC against *C. gleosporioides* of the two most effective botanicals i.e., *Zanthozylum aramutum* and *Lantana camera* in ethanol extract were 600 µg /ml and 700 µg /ml respectively.

Table 4.30: Minimum inhibitory concentration (MIC) of different botanical extracts and solvents used for extraction

Sl. No	**Plant used for extraction**	**Minimum inhibitory concentration (MIC), µg /ml**				
		Ethanol	**DCM**	**Acetone**	**Ethyl acetate**	**Chloroform**
1	*Capsicum annum* var. *cerciformae*	600	600	700	700	800
2	*Sechium edule* (Leaf)	400	NA	600	700	800

3	*Sechium edule* (Fruit)	600	800	600	400	700
4	*Nasturtium officinale*	800	NA	800	900	900
5	*Nictotiana tabacum*	600	700	600	600	800
6	*Lantana camera*	700	900	800	800	800
7	*Azadirachta indica*	600	800	700	700	800
8	*Zanthozylum aramutum*	600	900	600	800	900
9	*Tupistra nutans*	700	NA	700	700	900
10	Curcuma longa(leaf)	800	NA	600	NA	800
11	*Allium sativum*	400	700	500	500	700
12	*Brassica oleracea var.capitata*	800	NA	600	800	NA
13	*Brassica junacea*	600	700	600	600	700
14	*Cannabis sativa*	500	700	600	700	800
15	*Glaphylopteriopsis erubenscens*	400	600	600	500	700
16	*Litsea citrita*	500	700	600	700	600
17	*Ocimum sanctum*	400	600	800	400	600
18	*Curcuma amada*	800	NA	700	800	NA
19	*Schima wallichi*	600	NA	600	700	NA
20	*Pieris Formosa*	700	NA	800	600	NA
21	*Phytolacca acinossa*	500	NA	600	NA	800
22	*Moringa olerifera*	600	800	700	700	800
23	*Heracleum wallichi*	400	600	400	600	600

24	*Clematis buchnaniana*	800	NA	700	800	NA
	LSD (P=0.05)	77.98	55.38	89.48	100.92	89.88

*The experiment was conducted using the *Colletotrichum gloeosporioides*in Potato Dextrose Broth, incubated at 25°C for 5 days.

With critical examinations of*in vitro* screening results and performances as described above ethanol extracts of the *Zanthozylum aramutum* and *Lantana camera* were used to control the *C. gloeosporioides*for the field experiment under green house condition.

4.2.4. Antagonistic effect of the bio control agents against the test pathogen *C. gloeosporioides*

In this study the efficiency of antagonistic microbial agents was tested against the pathogenic test fungi*Colletotrichum gloeosporioides.*Productions of diffusible and volatile anti fungal compounds from the antagonistic microbial agents were evaluated using the dual culture method and is depicted in the table 4.31.

The results revealed the positive effect of diffusible and volatile compounds produced by all the three antagonistic bacteria with varying effectiveness. Antagonistic suppression of fungal growth was noticeable after 24 h incubation. Percent inhibition in radial growth caused by diffusible compounds of *Bacillus subtilis, Trichoderma gamsii* was 8.66 and in case of *Psudeomanas corrugate* was 10.66.Percent inhibition in radial growth due to the production of volatile compounds from the *Trichoderma gamsii, Bacillus subtilis* and *Psudeomanas corrugate* were 7.33±0.33, 8.00±0.57 and 10.00±0.57 respectively.The inhibition of the growth of pathogenic fungi (*C. gloeosporioides*) continued to increase with the increase in incubation period. Fungal inhibition after

Table 4.31: Inhibition of radial growth of *Colletotrichum gloeosporioides*by diffusible and volatile compounds produced by different antagonistic microbes

	% inhibition in radial growth ± SE							
Microbial agents	**24 hours**		**72 hours**		**96 hours**		**120 hours**	
	Diffusible	**Volatile**	**Diffusible**	**Volatile**	**Diffusible**	**Volatile**	**Diffusible**	**Volatile**
Bacillus subtilis	8.66±0.88	8.00±0.57	22.5±0.41	26.5±0.41	29.0±0.83	38.0±0.33	34.5±0.41	45.5±0.41
Trichoderma gamsii	8.66±0.88	7.33±0.33	29.5±1.24	26.0±0.83	33.0±0.83	39.5±0.41	34.0±0.88	50.0±1.66
Psudeomanas corrugata	10.66±0.88	10.00±0.57	26.5±2.07	35.5±0.41	33.5±1.24	44.0±0.83	43.0±1.66	56.0±0.83
Control	11.33±0.33	10.33±0.33	33.5±0.41	35.0±1.66	46.4±1.24	46.0±0.83	55.0±0.83	56.0±0.83

Each treatment was performed in triplicates. n=3; SE = Standard error

120 h of incubation due to diffusible compounds of *B. subtilis*, *T. gamsii andP. corrugata* were 34.5±0.41, 34.0±0.88 and 43.0±1.66 respectively andfor volatile substances 45.5±0.41, 50.0±1.66 and 56.0±0.83 respectively.With detailed assessment and performances as described in above paragraphs *B. subtilis* and*T. gamsii* were selected for further study tocontrol the *C. gloeosporioides*in the field experiment under green house condition.

4.3. Effect of botanicals/formulations to control fungal disease in pot experiment in the nursery

4.3.1. Efficacy of the botanicals and the bio-agent to control disease following challenge inoculation of *Colletotrichum gloeosporioides*

Field trials werecarried out for a period of 12 months under green house condition with disease free tissue culture raised plantlets of large cardamom. Results showed all the treatments used for the experiment showed significant inhibition of the pathogen.

Results revealed that tissue culture raised healthy plants initially treated with botanicals and the bio agents 3 days before the inoculation of the pathogenic spores led to some significant protection against leaf blight of large cardamom caused by the *C. gloeosporioides*(Table 4.32).Initial treatments before the inoculation successfully defended the initiation of the test pathogen for longer period of time. The symptoms on these treated plants were not visible for about one and half month from the date of inoculation. Whereas in case of control plants disease symptoms were visible by 17 days and disease incident increased substantially as the period of infection progressed. During the experiment maximum disease incident was 93.6% and many plants died due to the rust disease (Fig. 4.32). It appears that the initiations of disease symptoms on such treated plants was not easy and lead to the healthy growth of the plant even after the spraying of pathogenic inoculums. The first clear and visible symptom was noticed

after the 42 days of the spraying of the extract prepared from *Zanthoxylum aramtum*. Similarly in case of *Lantana camera*treated plantlets, the first clear symptom was seen on the 46th days of the spraying of pathogenic inoculums. On the other hand the application of the biological agent was found to be more efficient as compared to the plant extracts tested with the limitation of the experiment. Both the biological agents delayed the infection for about 3 months. The plantlets were initially sprayed with *Bascillus subtilis* and *Trichoderma gamsii* and were treated as per the schedule. First clear and visible symptom was noticed after 90 and 88 days of the spraying of the pathogenic inoculums, respectively. The bio control agent successfully helped in the suppression of the rust symptoms and plants remain healthy. It was also noticed that the disease incidence were less and the disease control was significantly more as compared to the control plants.

In the other sets of experiment the prepared biological agents were sprayed on the infected leaves initially after 2 days of inoculation, then treatment continued at 15 days interval as per the schedule. On the 17th day of inoculation of pathogen the symptoms were clearly visible. The result of the study revealed that the disease severity gradually decreased as time elapsed and the treatment progressed. It was also recorded that the disease was not spreading on to the new emerging leaves in comparison to the untreated i.e., control plants. The most effective extract was *Lantana camera* as the disease severity was 68.00% and as a result disease wascontrolled for29.16% whereas in case of the treatment of*Zanthoxylum aramatum* the diseases severity was recorded 76.00% and disease control obtained20.80%. Similarly the result for the application of the bio control agents revealed that the treatment with *Bacillus subtilis* led to the disease severity of 53.00% and the disease control of 44.79% followed by the diseases severity of 61.30% and the control of 36.14% using *Trichoderma gamsii* treatment. The result revealed that the extract prepared from *Lantana camera* was protective and good in comparison to the extract prepared from *Zanthoxylum aramtum*.

Table 4.32: Effect of pre- inoculation spray of selected botanicals on % diseases incidence caused by *Colletotrichum gloeosporioides*

SL.NO	**PERCENT DISEASES INCIDENCE (%)**									
TREATMENTS	July.2019	Aug.2019	Sept.2019	Oct. 2019	Nov.2019	Dec.2019	Jan.2020	Feb. 2020	Mar.2020	Apr. 2020
Zantoxylum aramatum	6.6±0.45	12.3±0.44	16.0±0.29	23.0±0.58	29.6±0.61	33.3±0.59	37.0±0.16	42.6±0.42	49.3±0.50	53.3±0.73
Lantana camera	10.3±0.21	16.6±0.44	20.6±1.55	23.6±1.35	27.3±0.44	31.2±0.23	38.3±0.59	44.7±0.59	49.0±0.44	56.6±0.15
Trichoderma gamsii	NS	NS	10.3±0.44	12.6±0.15	18.6±0.32	25.0±0.21	29.3±0.89	33.3±0.73	37.3±0.61	39.5±0.61
Bacillus subtilis	NS	NS	12.0±0.58	15.6±0.11	22±0.29	27.3±0.73	33.6±0.44	39.0±0.74	42.0±0.50	45.0±0.44
Control	10.6±0.44	18.6±0.45	30.6±0.45	41.3±0.46	53.3±0.21	64.0±0.61	76±0.58	82.6±0.78	86.6±0.58	93.6±0.79
LSD (P=0.05)	0.98	0.98	0.96	0.92	1.60	1.70	1.30	1.26	1.45	1.83

Each treatment consisted of three plants in triplicates. The data was recorded at every 30 days interval after the application of the different treatments. The control was only inoculated with the inoculum whereas all the treatments are being sprayed with the various extracts. NS- No symptoms.

Table 4.33: Effect of post inoculation spray of selected botanicals on % diseases incidence caused by *Colletotrichum gloeosporioides*

TREATMENTS	June.2019	July.2019	Aug.2019	Sept.2019	Oct. 2019	Nov.2019	Dec.2019	Jan.2020	Feb.2020	Mar.2020	Apr.2020
Zantoxylum aramatum	10.6±0.89	13.3±0.40	24±0.06	33±0.29	46.6±0.44	53.3±0.29	60±1.19	62.6±0.16	69.3±0.44	73.3±0.14	76±0.58
Lantana camera	13.3±0.29	16.6±0.21	22.6±0.29	30±0.16	37.3±0.35	44±0.31	53.3±0.29	58±0.31	64±0.61	66.6±0.35	68±0.44
Trichoderma gamsii	6.6±0.20	14.6±0.15	21.3±0.35	30.6±0.44	34.6±0.033	40.0±0.29	49.3±0.30	57.3±0.37	57.3±0.21	60±0.29	61.3±0.88
Bacillus subtilis	8.00±0.11	12.0±0.44	18.6±0.15	30.6±0.44	33±0.44	37.3±0.27	46.6±0.41	49±0.29	50.0±0.44	52±0.29	53.3±0.21
LSD(P=0.05)	0.72	1.25	0.87	0.80	1.39	1.03	2.31	1.22	1.06	1.50	1.78

Each treatment consisted of three plants in triplicates. The data was recorded at every 30 days interval after the application of the different treatments. The control was only inoculated with the inoculum whereas all the treatments are being sprayed with the various extracts.

Table 4.34: Percent diseases control (%) of pre and post spraying of the different treatments

Sl. No.	**Post spraying**		**Pre spraying**	
TREATMENTS	**PDI (%)**	**PDC (%)**	**PDI (%)**	**PDC (%)**
Zantoxylum aramatum	76.00	20.8	53.3	43.0
Lantana camera	68.00	29.16	56.6	39.5
Trichoderma gamsii	61.30	36.14	39.5	57.7
Bacillus subtilis	53.00	44.79	45.0	51.9
Control	96	0	93.6	0

Each treatment consisted of three plants in triplicates. The data were recorded at every 30 days interval.

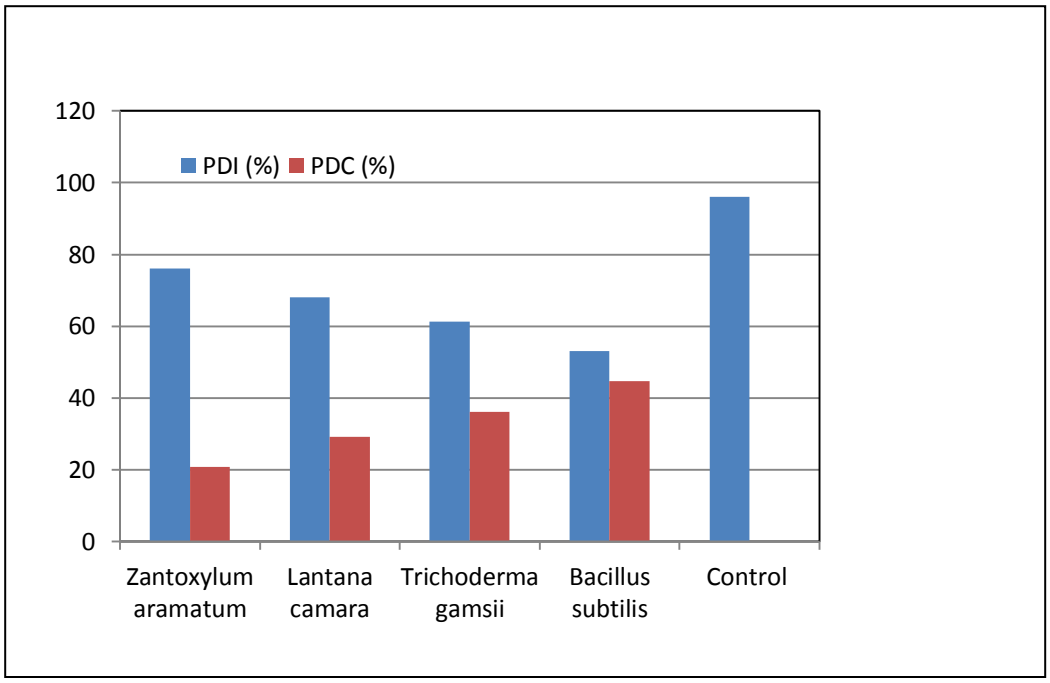


Fig 4.19: The representation of the percent disease incidence (PDI %) and percent disease control (PDC %) for the post spraying of the different treatments.

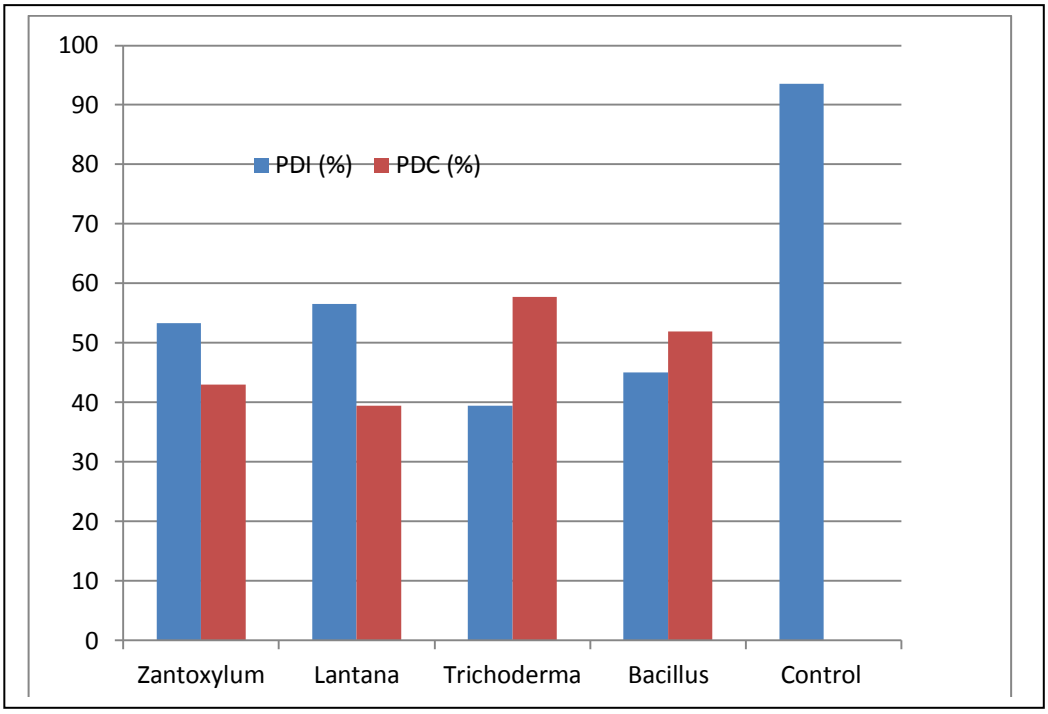


Fig 4.2: The representation of the percent disease incidence (PDI %) and percent disease control (PDC %) for the pre spraying of the different treatments

The results indicated that all the treatments as tested during the experiment were quite efficient. Foliar application as spray started before inoculation of the pathogen was more effective and found to be the best timing for treatment showing longer resistance rather than treatments started after the inoculation of pathogen. Among the botanicals and bio-inoculums tested *Lantana camera* and *Bacillus subtilis* were most effective and promising treatment to control *C. gloeosporioides.*

CHAPTER 5

DISCUSSION

5.1. Isolation and identification of causal organism of fungal disease of large cardamom using morphological and molecular markers

5.1.1. Disease assessment

With the advancement in the science and knowledge, it is now established that with estimated 1.5 million species fungi are having massive diversity on the earth. They are also one of the significant principal decomposers of the ecosystem (Capote *et al.*2012,Aslam *et al.* 2017). Several fungi are beneficial to human, animal and environment with their medicinal, economical and ecological services. On the other hand a large number of fungi are harmful, pathogenic and cause diseases in plants and animals resulting in huge economic loss to the society. Hence to overcome the problem, identification of the fungi causing plant diseases is the first and most important step in plant pathology (Borman *et al.* 2008).

Large cardamom is one of the most important and popular cash crop grown in Sikkim. It is also considered as the black gold for the region. But, it is gradually losing its productivity and quality due to biological and climatic pressure in the region. Now a day, the large cardamom belts in the state are rarely seen with the healthy plantations, which, once used to grow as healthy large cardamom plants. So, with the current scenario rapid decline in the production of large cardamom is a matter of concern in the state. During the present study, multiple foliar diseases such as viral

diseases (namely *chirke* and *foorkey*), leaf blight, leaf spot, leaf streak etc. were noticed in the large cardamom plantations in the state (Gopi *et al.* 2018; Saju *et al.* 2011, Gurung *et al.* 2020).

During the study 30-60% severity of leaf blight incidence has been recorded. The survey showed that disease condition is alarming and majority of the plantations in the state are affected with 40-45% blight incidence. From the literature it is evident that in 1958 a group of scientists (Raychaudhuri and Chatterjee 1958, Vijayan *et al.* 2014) for the first time reported about the occurrence of a new virus disease (chirkey) of large cardamom in Darjeeling district of West Bengal. Then in 1964 Varma and Capoor have reported Foorkey disease – a viral disease of large cardamom (Varma *et al.* 1964). In 1984 another group of scientist (Prasad *et al.* 1984) reported leaf blight of large cardamom caused by *Colletotrichum* sp., followed by another study on leaf blight (Srivastava 1985). In due course of time leaf streak caused by *Pestalotiopsis royenae* (Guba) was reported as a new disease of large cardamom from Sikkim. Then capsule rot and wilt of large cardamom were also been reported (Srivastava and Verma 1989a, b). Initially people were unaware about the rot-disease and described it as some mysterious disease. Then after about two decades, in 2010 a group of scientists from the Indian Cardamom Research Institute reported that *Colletotrichum gloeosporioides* is responsible for causing the leaf blight (Prasad *et al.* 1984). So far pathogens as mentioned above, and other pathogens like *Fusarium* sp., *Rhizoctonia* sp. etc. also reported for other fungal diseases of large cardamom (Sharma *et al.* 2016, Gurung *et al.* 2020).

5.2. Morphological and molecular identification of major fungal pathogen of large cardamom

Now a day, the large cardamom belts in Sikkim are rarely seen with the healthy plantations, which, once used to grow as healthy large cardamom plants. So, rapid decline in the production of large cardamom is a matter of concern in the state. Reports show various factors are responsible for the situation and this may be an effect of climate change, lack of irrigation facilities during dry season, open cultivation, inadequate nutrient management, unscientific methods of cultivation, pests and, diseases, like chirkey, foorkey, leaf blight, leaf spot, anthracnose, wilt, collar rot, capsule rot and leaf streak, etc. (Sharma *et al.* 2000, Saju *et al.* 2013, Gopi *et al.* 2018, Gurung *et al.* 2020). During the present study, multiple foliar diseases such as viral diseases (namely *chirke* and *foorkey*), leaf blight, leaf spot, leaf streak etc. were noticed in the large cardamom plantations in the state.

If we check the time line about the disease in the region, about three decades ago for the first time leaf streak in large cardamom caused by *Pestalotiopsis royenaea* was noticed in Sikkim (Srivastava and Verma 1989). Then after 20 years in 2011 and 2013 the disease was discussed and reported by two different groups of scientists (Saju *et al.* 2011, Vijayan *et al.* 2013). During the present survey, leaf streak symptoms were recorded in large cardamom plantations and *Pestalotiopsis maculans* has been identified as causal organism responsible for the disease. In recent times, the disease again have been reported as leaf streak on the tender leaves of large cardamom transmitted by tea mosquito bug, although, they have isolated the causal organisms and identified as *Pestalotiopsis royneae* from diseased leaf (Gopi *et al.* 2018). They

have commented that, the leaf streak of large cardamom is not a disease rather it was a feeding result of the tea mosquito bug.

It is known that fungi exhibit variable morphological characters under the influence of different nutritional composition of the medium (Kim *et al.* 2005, Saha *et al.* 2008, Cagigal and Sanchez 2017). In the present study, the fungal isolate was found to grow maximum on the PDA among all other media tested. Similar results on the growth of the *Pestalotiopsis* sp. isolated from guava was reported by Keith and co-researchers (Keith *et al.* 2006, Saha *et al.*2008) where the PDA as the medium was found to be the best suited. The influence of the two significant physical factors i.e., temperature and pH were also recorded during the present study. The fungal isolate when incubated at 25°C was found to have obtained maximum growth. Previous findings also reported that the optimum temperature for growth of *Pestalotiopsis* sp. lies between the ranges of 22-28°C (Keith *et al.* 2006). Another study also revealed that the optimum temperature for the growth of *Pestalotiopsis theae* was 25±2°C (Suwannarach *et al.* 2013). Similarly, in the present study 25°C incubation temperature was found optimum for the growth of *Pestalotiopsis* sp. Further, in the present study, it was observed that the *Pestalotiopsis maculans* could tolerate a wide range of pH levels (5-11 pH) of the medium while maximum growth was observed when pH of the medium was 7-8. However the report by Keith and group reveals that the pH 5.5 was the most suitable for the growth of *Pestalotiopsis* sp. causing the scab disease in guava(Keith *et al.* 2006). Likewise, the optimum mycelia growth of *Pestalotiopsis* sp. was observed in the pH 5.5 (Hopkins and McQuilken 2000).

The pathogenicity of*Pestalotipsis* sp., was also reported in many other crops such as apple, blueberry, coconut, grapevine, guava, hazelnut, litchi and tea, etc. (Crocus *et al.* 2011, Zhang *et al.* 2012, Maharachchikumbura *et al.* 2016). The pathogen is reported as majorpathogen that causes leaf blight - an economically important disease in tropical region (Zhang *et al.* 2012, Selmaoui *et al.* 2014) and now it is reported causing leaf streak disease in large cardamom.

To the best of our knowledge, the present finding is the first report to provide, a combination of morpho- molecular characterization of the *Pestalotiopsis maculans* that is causing the leaf streak on large cardamom leaf grown in Sikkim. The molecular application of PCR using rRNA gene ITS primers confirms that the pathogen causing leaf streak is by *Pestalotiopsis maculans.* The pathogen, *P. maculans*, has been observed in large cardamom growing regions in Sikkim and till now damage is negligible. But as it is a fungal infection, etiology, prevalence of the disease has to be carried out in the state so that the diseases does not become a major, uncontrolled and unmanaged in days to come.

During the present study attempts were also made for scientific evaluation and identification of causal organisms for blight of large cardamom in the region. Interestingly, out of the four districts of Sikkim, samples collected from three villages of East district was distinct with symptoms. The pathogen was identified as *C. eragrostidis*, which is first report of the pathogen causing blight disease to large cardamom in three villages of East district of Sikkim.

In the late 1990s rot disease was first noticed in the region and people described it as some mysterious disease. Then after more than two decades in 2010 a

group of scientists from the Indian Cardamom Research Institute reported that *Colletotrichum gloeosporioides* is responsible for causing the leaf blight (Saju *et al.* 2011). Another group of scientists tried to control the disease using a chemical pesticide, like Copper Oxychloride with limited success. So far other pathogens like *Fusarium* sp., *Rhizoctonia* sp. are also reported for other fungal diseases of large cardamom (Anonymous 2014). On the basis of the morphological behavior of the isolate and species characteristics the pathogen has been identified as *C. eragrostidis.* Further in addition to morphological parameters and characterization using two partial ITS rDNA sequence evaluated in the NCBI database also confirmed the isolate as *C. eragrostidis* with 100% sequence similarity - a newly identified pathogen causing blight disease to large cardamom grown in Sikkim.

The genus *Curvularia* is having of more than 40 species which are distinguished by differences by conidia structure, number of septa and the colony morphology (Zhang *et al.* 2004, Chung and Tsukiboshi 2005). Most of species of *Curvularia* are pathogenic and give rise to substantial loss losses in agricultural produced all over the world. Generally the genus caused leaf spot disease which is necrotic for several plant families (Dasgupta *et al.* 2005). For example the pathogen *Curvularia* causes leaf spot disease in rice, wheat, grass, maize, tea, and sorghum, etc. (Dasgupta *et al.* 2005, Fajolu *et al.* 2012, *Dey et al. 2016,* Garcia-Aroca *et al.* 2018; Seephueak *et al.* 2019). It is also reported that the *Curvularia* causes leaf spot disease is one of the major draw backs in rice cultivation as well, as it inhibits the germination of the rice seeds also. The disease is characterized by the symptoms of long and wide grayish white spot with brown color surrounding around it with irregular shape (Bawa

et al. 2018) with the brown border surrounded by a yellow halo (Fajolu 2012, Sarkar *et al.* 2018, Seephueak *et al.* 2019). Surprisingly, in the present study, phenotypic characteristics are similar as reported above and were recorded with distinct conidiospores causing blight of large cardamom and gradually infected leaves damaged severely and dried up.

There are other fungal diseases (i.e., wilt, leaf rot, leaf spot, anthracnose, rust, leaf blight and leaf streak) found in large cardamom that have affected the productivity sharply (Sharma and Rai 2012,Saju *et al.* 2013, Gopi *et al.* 2018). So far, other pathogens like *Fusarium* sp., *Colletotrichum* sp. and *Rhizoctonia* sp. have reported for fungal diseases of large cardamom (Anonymous 2014). The study indicated that this disease with distinct symptoms caused by *C. eragrostidis* is a new threat for the crop but till now restricted within the East district of Sikkim. But the presence of the disease indicated that the *C. eragrostidis* is a new minor fungal pathogen associated with leaf blight of large cardamom growing in the region. So further studies are needed to determine the distribution and severity of the disease in the region. Hence, the present study will help to develop strategy to prevent crop losses due to *C. eragrostidis* infection.

Now it is evident that numerous factors are responsible for the decline in overall productivity of large cardamom grown in Sikkim, among which the fungal rot infestation is considered as most important one. In late 1990s rot disease was first noticed in large cardamom in the region and people described it as some mysterious disease. Reason was unknown at that time or thereafter for about two decades and till

date there is no report of molecular identification of the causes or causal organisms and proper controlling measures so far.

Then in 2010 a group of scientists from the Indian Cardamom Research Institute reported that *Colletotrichum gloeosporioides*is responsible for the leaf blight (Saju *et al.,* 2011). Another group of scientists tried to control the disease using a chemical pesticide, like Copper Oxychloride with limited success. So far other pathogens like *Fusarium* sp., *Rhizoctonia* sp. are also reported for other fungal diseases of large cardamom (Anonymous 2014). In 2013 Saju *et al.* analyzed the situation in detail and reported the *leaf blight*caused by *C. gleosporiodies*. In the plant pathology *blight* caused by *C. gleosporiodies* is considered to be the most devastating disease that results in huge crop loss and even decline in plant population for numerous crops worldwide. The situation in Sikkim is same in case of large cardamom plantation.

It is known that fungi exhibit variable morphological characters under the influence of different nutritional composition of the medium (Kim *et al.* 2005, Saha *et al.* 2008, Cagigal and Sanchez 2017). In the present study, colony characters and growth were found to be varied on different media. When *C. gloeosporioides* was cultured on most conventional PDA medium for fungal isolates, normal growth was obtained. Czapek dox agar medium was found compatible and obtained maximum growth as compared with PDA although statistically non-significant. Similar response on growth of *C. gloeosporioides* isolated from diseased guava was reported by Singh et al. 2006 and PDA was found to be the best suited medium. In an earlier study the

colony colour of the*C. gleopsoorides* was found to be blackish on water agar medium, white colony was observed when cultured on Richards, oat meal agar and PDA while grayish white on Czapek Dox Agar medium (Hubballi 2010, Saha *et al.* 2008, Cagigal and Sanchez 2017). The present study colony colour was found to be green and white cottony colour masses in front view and white colour when observed from inverted side of the plate on PDA medium.

The temperature is one of the important physical environmental factors that affect the growth of fungi in culture. It is important for regulating the growth and reproduction of fungi. The present study revealed that the good growth of *C. gloeosporioides* was observed in a wide range of temperature (20 to 35°C) and maximum growth was recorded at 25°C. The present study is supported by the finding of Devi (2008) stated that the maximum growth of *Colletotrichum* sp. was found at 25°C. Likewise the *C.gloeosporioides* isolated from mango, almond and avocado plants was found to have maximum growth at 25°C (Gonzalez 2003, Hubballi 2010, Kim *et al.* 2005, Saha *et al.* 2008). Like temperature, pH also plays an important role in the growth of fungus. In the present study, the maximum growth was recorded at pH 5.5 to 7. Similarly pH also plays an important role in the growth of *Colletotrichum* sp. and in the present investigation the maximum growth of the pathogen was recorded at pH 5.5 to 7. The finding is similar to the results reported earlier, where it was reported that the growth of *C. gloeosporioides* was maximum at pH 7 followed by 6 (Hubballi et al. 2010, Nitzan and Tsror 2003).

In the present study, the symptoms produced by the pathogen on the plant based bio assay were similar to the symptoms appeared in the field. The symptoms

appeared to be blight one, with the necrotic spots along with the die back symptoms, could be observed on the 16th to 18th days of inoculation. Tip burn of leaves was also noticed along with the blighted appearance from the leaf margins. It is known that different species of *Colletotrichum* causes disease in many crops with necrotic tissue and sunken appearance symptoms (Bailey and Jeger 1992, Sharma and Tamta 2015). Colony morphology with faster growth, cylindrical conidia with round ends are typical identifying characters for the *C. gloeosporioides* (Quimo and Quimo 1975, Chowdappa *et al.* 2009) and similar morphology and the microscopic features were observed in the present study.

The important purpose of the study was to confirm that blight of large cardamom is caused by *C. gloeosporioides*by using molecular markers and pathogenecity assay. Colony characteristics like faster growth, colour, morphology, cylindrical conidia with round ends and application of species-specific PCR using ITS primers developed for *C. gloeosporioides* confirm the pathogen as *C. gloeosporioides* isolated from diseased large cardamom grown in Sikkim. To the best of our knowledge, this is the first confirmatory molecular characterization of *C. gloeosporioides* causing blight disease and serious damage to large cardamom as a major pathogen. Hope, this will enable effective targeted measures to protect this important crop – life line for rural hill economy of north east India and Sikkim in particular.

Fusarium is one of the important fungal pathogens reported responsible for blight disease of large cardamom and causes considerable economic damage to the farmers (Anonymous 2014, Sharma *et al.* 2016, Gurung *et al.* 2020). During the

present investigation out of the nine samples identified, three samples were confirmed as *Fusarium* wilt and rot disease in large cardamom. There were basically two most prevalent species reported namely *Fusarium oxysporum* and *Fusarium avenaceum* which are known to cause wilt and leaf rot in many plants. The wilting of the plant and the individual leaf are the basic characteristic symptoms of the pathogen.

The wilt caused by *Fusarium* sp. was found to be severe in the nursery as well as in field condition. It was also noticed that intensity of the disease was quite high during October to February in each year. During this period sudden wilt of the saplings and infected crops were noticed. The white cottony mass on the leaf surface is the indication of leaf rot.The infection expands and forms a chlorotic portion that gradually increases and leads to the drying up the plant. The humid climate during June to September in a year is considered to be the favorable breeding condition for the pathogens (Srivastava 1989). Apart from large cardamom, the genus *Fusarium* is considered as one of the most common pathogen responsible for the destructions in many major crops.

It is well known that *Fusarium* species are abundant in the soil and are considered as soil borne fungiassociated with plantroots as parasites or saprophytes. *Fusarium*sp. are significant plant pathogens causing vascular wilts on a wide range of horticultural crops,root rot diseases of many crops, legumes, head blight of cereals and other plants, etc.(Booth 1971, McGee and Kellock 1974, Kelloc *et al*. 1978, Lamprecht *et al*. 1988, Satyaprasad *et al*. 2000, Kang *et al*. 2005).

Generally *Fusarium* causes characteristic wilt symptoms frequently in the seedling stage (Bakar *et al.* 2019). The genus *Fusarium* comprises of approximately

300 phylogenetically distinct species and 20 species complexes. The most common *Fusarium* species are *F. solani* complex, *F. oxysporum* complex and *F. fujikuroi* complex (Balajee *et al.* 2009, O'Donnell *et al.* 2015). Fusarium causes leaf diseases in many crops which is the major drawback in the farming community. The leaf spot and leaf rot caused by the *Fusarium* is responsible for the downfall of the production in many crops. The disease is characterized by the chlorosis symptoms of the older leaves and the sudden collapse of the plant while still greenish in color. The symptoms were observed in the present investigations on large cardamom wilt also. The disease is basically a die back as it starts from the tip of the leaf that gradually spreads towards the base of the plant. The plant looks discolored with the wilting appearance at the end.It has been studied that warm, dry weather is most suitable for rapid spreading of Fusarium and therefore, it is a big problem in Indian subcontinent and infect a large number crops including large cardamom in Sikkim (Sharma *et al.* 2016, Bakar *et al.* 2019).

In the present study leaf blight in large cardamom caused by *Phoma cava* was also confirmed. Based on the phenotypic characters, the endophytic fungus, isolated from the leaf of large cardamom was identified as *Phoma sp.* The genus *Phoma* has been known as the plant pathogen causing leaf blight disease in various crops. This genus is also responsible for causing leaf spot and leaf blight disease inlarge cardamom. This is a typical leaf disease where it is accompanied by the symptoms of small lesions on the younger leaves. Leaf spots were usually observed with numerous water soaked lesions, round in shape with greyish margin which eventually developed into prominent yellowish spots.The symptoms were seen mostly in the nursery stages.

It has also been recorded in Arunachal Pradesh along with Sikkim and Darjeeling hills (Anonymous 2014, Sharma *et al.* 2016). In due course of time, these spots increased in size, turning yellow to brown and black spots. Numerous spots coalesced to form a bigger patch which resembled the blighted leaf and dried out. Thus, it has become a major drawback in the cultivation of many crops including flowers. The characteristic symptom of the blight caused by*Phoma cava* was small necrotic spots on both the sides of the infected leaves. These spots later on enlarged to form bigger blighted regions on leaves resulting in the wilting (Patil *et al.*2012).

In the present investigation another pathogenic fungal strain *Epiccocum nigrum* has been isolated from the blighted leaves of large cardamom grown in Sikkim. *Epicoccum nigrum* is considered as a pathogenic and endophyte fungi belonging to the phylum ascomycota (Anderson *et al*. 1981). There are reports where the pathogen is known to cause severe leaf spot disease in *Lablab purpureus, Lotus corniculatus* (Mahadevakumar *et al*. 2014, Wu *et al*. 2017and Colavolpe *el al*. 2018). Despite being pathogenic fungi, it is also considered as a bio-control agent against many pathogens as it produces pigmented as well as non pigmented antimicrobial compounds (Brown *et al.* 1987, Gribanovski-sassu and Foppen 1967).

During the study, different samples with the symptomatic appearance were collected, among which blighted leaf spots a major concern. On the basis of the morphological behaviour of the isolate, and species characteristics the pathogen has been identified as *Epicoccum nigrum*. To the best of our knowledge it is the first report of isolation and identification of *Epiccocum nigrum*as pathogen causing blight disease in large cardamom. For the authenticated identification, the two partial ITS

rDNA sequenceswere evaluated in the NCBI database.The isolate was confirmed as *Epicoccum nigrum* with 100% sequence similarity with that present in the NCBI domain.

The genus *Epiccocum*sp. is a microscopic mould and a common causative agent of leaf spot in various economically important crops. Despite its pathogen city recorded in many crops, it is reported as potential biological control agents against the wide range of plant pathogens (Elmer and Reglinski 2006, Brown *et al.* 1987). The colony morphology observed in the present study was similar to the study conducted by Hashmeen (2004) where off white colony was seen on PDA with reddish centre when viewed from back side of the plate.

Most of the species of *Epicoccum* are pathogenic and are known to cause a devastating leaf disease on a wide range of crops. Generally, *Epicocccum* is responsible in causing leaf spot disease which has chlorotic lesion that increases into the necrotic lesion leading to a bigger spot on the leaf.The disease is characterized by the symptoms of a small chlorotic lesion on the upper surface of the leaf with yellowish irregular spot which tend to increase into a larger portion of leaf resulting in necrotic appearances. The pathogen *Epicoccum* has affected and caused leaf spot disease in tea, snow pea,mango, paw paw, lily, flowering cabbage, Indian bean etc. (Chen *et al.* 2020, Aigbe *et al.* 2019, Zeng *et al.* 2018, Yu *et al.* 2019, Mahadevakumar *et al.*2014).

5.3. Management of blight disease of large cardamom using botanicals, indigenous knowledge based methods and biocontrol agents

Management of fungal blight diseases of large cardamom continuesto be one of the important challenges in Sikkim. Till date, only a few measures for thecontrol of fungal blight, in organic agriculture,are available and most of them are based on agronomic practices. In this study, the antifungal activity of different botanical extracts from twenty four plant species and three bacteria as biocontrol agents were evaluated against pathogenic *C. gloeosporioides* under both *in vitro* and nursery conditions.

Sikkim is an organic state and to meet the crop protection through an organic way,there can be no better option than the use of the available plant resources and the bio agents. As far as the state is concern, it is an agriculturedominated state. The population here dwells their living through farming along with large cardamom farming. The crop is one of the main cash crops and an income generator for the rural peoplein Sikkim. Since, the crop is declining at an alarming rate due to several pathogenic problems, protection of this highly valued crop from the pathogen is very urgent. Therefore, it is now a high time to develop protection measuresof the crop from the fungal pathogens with the help of the locally available plant extracts, formulations prepared on the basis of indigenous knowledge (ITK) and the bio control agents.

Plants are considered as natural healers as they possess many bio-active components which help them to survive and defend their enemy in an ecosystem. Plants are considered as an accessible source of therapeutics against the pathogens in

this area. Further the use of biological agents for managing the diseases hasenormous advantage as generally they are non toxic, eco-friendly, locally available and inexpensive. There are many reports which have already described the efficacy of several plant extracts and bio-control agents. It is now a well established fact that the natural products can help to control the population of various pathogens and could be included as potential component(s) in the integrated diseases management programme (Bowers and Locke 2004). Therefore, there is a dire need to screen the plants for the development of the bio-fungicides to protect crops from the pathogen. The search for the potential plant for making the bio-fungicide must consider persistent activity which can be a good source of resistant against the pathogens.

Studies have already shown that the plants with antifungal properties have great advantages as they can be explored and recognized as a source of disease resistance drug against many harmful phyto-pathogens (Siva *et al.* 2008). Although Sikkim isenrichedwith biodiversity, the use of the plant extract against the pathogen causing leaf blight in large cardamom is not yet carried out. Till date the leaf blight caused by fungal pathogenin large cardamom is only managed through the agro-techniques and with some locally available biocontrol measures with limited application and success. The present study was therefore carried out to make the best utilization of the bio-resources available in the region by preparation of the plant extract, formulation prepared usingthose extractsusing the processas developed and followed by the local people with scientific validation.

The present investigation was carried out to screen the best botanical extract which can be used as the potential biofungicide against the leaf blight caused by

C.gloeosporioides. These botanical extracts were screened in the laboratory under*in vitro* condition and was found to be the effective one. In the present study twenty four different plant extracts were screened against the test fungus. The plant extracts were prepared infive different solvents. The study revealed thatthe highest antifungal activity was exhibited by the ethanol extract and the least by the dichloromathane (DCM) extract. It was also found that the aqueous extract did not show any anti fungal activity.

Zantoxylum armatum is basically a medicinal plant that has been used in the tradition medicine since ages for the treatment of various diseases like toothache, pneumonia and tick infestation etc. (Sindhu *et al.* 2010). It is a shrub that bears a fruit which is enriched with antimicrobial ingredients and also been used in traditional system of medicine for the treatment of fever, skin sensitivity, anti-inflammation, chest infection, dental problems, and digestive problems etc. (Srivastava *et al.* 2006).The plant is enriched with alkaloids, flavonoids, terpenoids etc. which makes the plant activein antimicrobial, antipyretic, anti-inflammatory, and with cytotoxic effects (Yang *et al.* 2008, Islam *et al.* 2001, Bafi *et al.* 2005).

This is evident from the present investigation that the *Zanthoxylum aramtum* has a good antifungal potential. The study revealed that the ethanolic extract of the plant had maximum zone of inhibition with 14.5 mm followed by ethyl acetate extract. The results in the present study matches with the earlier findings of Alam *et al.*(2016)where antifungal activity of *Z. aramatum* which was demonstrated through the extracts made in ethanol and n-hexane. In the cited study, ethanol extracts of *Z.*

aramatum showed highest inhibition activity against few selected test fungus (*Fusarium solani, Candida albicans, Aspergillus flavus, Trichophyton longifusis*).

Similar study was demonstrated with the *Z. aramatum* oil of the fruit extracted through hydro-distillation techniqueand fund effective against *Aspergillus sp., Penicillium sp., Alternaria sp., Cladosporium Sp.* and *Helminthosporium sp.* (Prajapati *et al.,* 2015). Several other studies also confirm the antifungal activities of essential oil extracted from *Zanthoxylum* species (Nanasombat and Wimuttigosol 2011, Prakash *et al.* 2012). Scientists also confirmed that the antifungal property of the essential oilof fruit of *Zanthoxylum* species was due to the presence of linalool and limonene (Tiwary *et al.* 2007, Soares *et al.* 2012, Rancicl *et al.* 2003).

In the present investigation extract of *Lantana camera* also showedthe reduction in the mycelia growth of the test fungi *C. gloeosporioides*in the *in vitro* experiment.*Lantana camera*is basically a weed having useful secondary metabolites. The plant is used as herbal medicine for the treatment of cancers, chicken pox, measles, tumors etc. (Mandal *et al.* 2011). Other uses of the plant are as firewood and mulching material (Saraf *et al.* 2011).

In 2018 Girish *et al.* (2018) reported that*Lantana camera*poses several qualities like anti fungal, anti proliferative, antimicrobial, fungicidal, insecticidal and nematicidal activity. They have demonstrated the antifungal activity of the *Lantana camera* extract against*Alternaria* sp., *Aspergillus niger, Cladosporium* sp*., Curvularia* sp. and *Fusarium* sp. Similar response of the *Lantana camera* was also observed against *Aspergillus fumigates, Curvularia lunata* and *Aspergillus flavus*(Saraf *et al.* 2011, Srivastava and Singh 2011, Yuan *et al.* 2012, Naz *et al.* 2013,Singh *et al.* 2014).

The probable reason for the inhibition may be attributed to the presence of alkaloids, terpenoids, saponins, tannins and essential oils. In the year 2012 Elansary *et al.* analyzed in detail the chemical composition of the essential oilextracted from the *Lantana camera.* They observed that it posesesstrans-caryophyllene (15.57%), α-humulene (9.16%), bicyclogermacrene (6.69%), farnesol (6.38%), spathulenol (6.02%), germacrene-D (5.23%), α-cyclocitral (5.08%), sabinene (4.28%), trans-photocitral (4.4%), bicycloelemene (3.78%), camphor (3.57 %), calamanene, (3.22%), calarene (3.19%), Δ-cadinene (2.9%), α-pinene (2.11%) and trans-sabinene (1.09%). Further Ganjewala *et al.*(2009) showed that the lantadenes is the compound which is actually responsible for the biological activities as documented by several researchers. Further *Lantana camera*showed an effective antifungal activity against *Colletotrichum falcatum* causing red rot disease in sugarcane (Sreermallu *et al.* 2017). The finding also supported that the extract of *Lantana camera*had strong antifungal activity against *C. gloeosporioides.*

In the present study the MIC value of the plant extracts was also worked using the pathogen *C. gleosporidies*. The result of the study revealed that the MIC value of the *Zanthoxylum aramutm* was found to be 600(µg/ml) when extracted in ethanol and acetone. Further, the MIC of *Lantana camera* inethanolic extract was obtained 700 (µg/ml). Likewise, 50% of inhibition of the pathogen was observed with methanol extract and no inhibition recorded in aqueous extract. Similar type of resultswere alsoreported by Alam *et al.*(2017) where the extract of *Zanthoxylum aramtum* was prepared in the aqueous, methanol, n-hexane and chloroform. They observed the antifungal activity of *Zanthoxylum aramtum*extracts against the pathogen

Aspergillus flavus, Fusarium solani and*Trichophyton longifusus.*Sreermulu *et al.*(2017)also studied antifungal activityof *Lantana camera*extract against *Colletotrichum falacatum* using the different solvents. In the same way, Ademe *et al.* (2013) also reported that the extract of the *Lantana camera* gave the lowest spore germination percentageof*Colletotrichum gloeosporioides*. In 2012 other study demonstrated antagonistic effects of *Lantana camera* extracts prepared in methanol, acetone, ethanol and water against *Alternaria alternate.* Among these extract, complete inhibition of the fungal pathogen was obtained with ethanol and acetone extracts and no effect was noticed in aqueous extract (Singh and Srivastava 2012).

Now-a-days bio-control of pathogens using antagonistic microbial strains is considered as another effective, ecofriendly and alternative approach for disease management practices. In the present exploration, three bacterial biocontrol agents were tested against *C. gloeosporioides*. Production of diffusible and volatile antifungal compounds from the antagonistic microbial agents were evaluated using the dual culture method on *C. gloeosporioides.* It wasrevealed that, all the microbial strains tested during the study showed clear antagonism and significantly reduced the growth of *C. gloeosporioides* with varying efficiencies. Percent inhibition in radial growth caused by diffusible compounds of *Bacillus subtilis, Trichoderma gamsii* was 8.66 and in case of *Psudeomanas corrugate*it was 10.66.Percent inhibition in radial growth due to the production of volatile compounds from the *Trichoderma gamsii, Bacillus subtilis* and *Psudeomanas corrugate* were 7.33±0.33, 8.00±0.57 and 10.00±0.57, respectively.

Thus, during the study antifungal activity was confirmed in all the 24 botanicals and three microbial isolates tested, even though the results showed that different plant extracts varied in their efficacy in inhibiting *C. gloeosporioides* growth under *in vitro* condition. The inhibitory potential of the plant extracts was found to vary with the plant species as well as the different solvent used for preparation of extract. *In vitro* experiments showed that ethanol extracts of *Zanthozylum aramutum* and chloroform extracts of*Lantana camera*were the best mycelial growth inhibitors. Further, with detailed assessment and performances as recorded during the *in vitro* experiment *B. subtilis* and *T. gamsii* were found to be most effective to control the *C. gleosporioides*. Hence, this is a clear indication that the extracts of two plants, i.e., *Zanthozylum aramutum* and *Lantana camera* contain antifungal compounds and bacterial strains *B. subtilis* and *T. gamsii* are responsible for the antifungal activity, and were effective to control the growth of important fungal pathogen *C. gloeosporioides* causing leaf blight of large cardamom in Sikkim.

5.4. Effect of botanicals and microbial agents to control *Colletotrichium gloeosporioides* in pot experiment in the nursery

*Colletotrichum gloeosporioides*has been considered as one of the important fungal pathogens causing leaf blight of large cardamom grown in Sikkim. Further,*Pestalotiopsis royenae, Fusarium* sp., *Phoma* sp., *Epicoccum nigrum,Rhizoctonia* sp. etc. are also reported for other fungal diseases of large cardamom grown in Sikkim (Sharma *et al.* 2016, Gurung *et al.* 2020). Thus the large cardamom grown in Sikkim Himalaya is now facing a devastating leaf blight disease

caused by above fungal pathogens that has brought down its yield drastically. Therefore the crop needs to be protected from the fungal pathogens, and a solution for the management of the fungal diseases is very urgent for the organic Sikkim.Thus the best and effective solution can be the use of the botanical extract and the microbial agent.

In general plant diseases caused by the fungal pathogens are controlled by chemical fungicides. This is quite expensive and also hazardous to the environment. Indiscriminate use of these chemical fungicides has caused several problems, like toxic chemicals residues in food, water and soil have killed several beneficial insects and microorganisms (Pimentel and Levitan, 1986). Further, repeated use of these chemicals may result into development of resistance in the pathogens and help to develop the new races of pathogens. Therefore, to overcome the problem, alternative bio-control methods should be developed on priority basis. Thus antifungal activity of several plant extracts has gained the attention to a great extent. A wide range of secondary metabolites are present in the plants, namely phenols, flavonoids, quinines, tannins, essential oils, alkaloids, saponins and steroids, etc. and they have a major defensive role for plants (Islam *et al*. 2001, Bafi *et al.* 2005).

During the present investigation two botanicals and two biological agents have shown very good results to control the pathogen in the laboratory under *in vitro* conditions. There are many reports and literature which supports these botanical extract as antifungal agents under*in vitro* condition. But, the efficacy and validation of these products in the nursery conditions (*in vivo)* are insufficient. In the present investigation two promising botanical extracts,thoroughly screened *in vitro* were used

to examine their potentiality against the leaf blight in the nursery.To the best of our knowledge, these botanical extractshave not been used against the leaf blight diseases in large cardamom farming till date.

Results of the present challenged experiment under the nursery conditions showed that all the treatments tested were efficient for inhibition of the pathogen *C. gloeosporioides*. It was observed that when disease free tissue culture raised healthy plants treated with botanicals and the bio agents 3 days ahead of the inoculation of the pathogenic spores, successfully defended the test pathogen for longer period of time. During the experiment maximum disease incident was 93.6% and many plants died due to the rust disease. It appears that the initiations of disease symptoms on such treated plants was not easy as the treatment led to the healthy growth of the plants even after the spraying of pathogenic inoculums. The first clear and visible symptom was noticed after 42 days of spraying the extract prepared from *Zanthoxylum aramtum*. Similarly in case of *Lantana camera*treated plantlets, the first clear symptom was seen on the 46^{th} days of spraying of the pathogenic inoculums.

On the other hand the application of the biological agent was found to be quite efficient as compared to the plant extracts tested. Both the biological agents delayed the infection for about 3 months. The plantlets were initially sprayed with *Bascillus subtilis* and *Trichoderma gamsii* and were treated as per the schedule. First clear and visible symptom was noticed after 90 and 88 days of the spraying of the pathogenic inoculums, respectively. The bio control agent successfully helped in the suppression of the rust symptoms and plants remained healthy. It was also noticed that the disease

incidences were less and the disease control was significantly more as compared to the control plants.

During the study *Lantana camera* extract was found to bethe most effective one. With the treatment of *Lantana camera* extract disease severity was recorded 68.00% and as a result disease was controlled for 29.16%. Whereas in case of the treatment of*Zanthoxylum aramatum* the diseases severity was 76.00% and disease control obtained20.80%. Similarly the result for the application of the bio control agents revealed that the treatment with *Bacillus subtilis* led to the disease severity of 53.00% and the disease control of 44.79% followed by the diseases severity of 61.30% and the control of 36.14% using *Trichoderma gamsii* treatment.

In a study Nair *et al.*(1982) showed antimicrobial, larvicidal and cytotoxic activity of *Zanthoxylum.*The likely reason for their antifungal activities is mainly due to the presence of various biologically active compounds. According to Tiwary *et al.*(2007) the inhibitory effect of the *Zanthoxylum aramtum* might be attributed to the presence of linalool and limonene.As reported by Prakash *et al.*(2012)the essential oil of *Zanthoxylum* species exhibit broad range of antifungal activitiesagainst different fungii.e.,*Fusarium* sp., *Aspergillus* sp. and *Pencillium* sp.etc. This was further confirmed by the few more reports that the *Zanthoxylum aramtum* possess the antifungal activity against a wide range of fungal species (Islam *et al*. 2001, Bafi *et al.* 2005).

The present work revealed that an extract prepared from the *Lantana camera* can also be used as the potential antifungal agent against the leaf blight. *Lantana camera* is generally a wild plant which is known to possess a significant medicinal

property due to the presence of abundant phytochemicals. The plant is known to exhibit significant antibacterial and antifungal activities (Grish *et al.,* 2017). According to Cartwright *et al.*(1977) the important and major compound responsible for the antifungal activity found in *Lantana camera* is cyclopropane. Further all parts of the*Lantana camera* such as flowers, stem and leaves are known to possess anti fungal properties(Boughalleb *et al.* 2005). The findings of the present work are supported by the similar kind of work performed by Bashir *et al.,* 2019 where the extract prepared from the *Lantana camera* has been found to be successful in inhibiting the mycelium growth of *Colletotrichum gloeosporioides*causing the anthracnose. The methanolic extract of the *Lanatana camera* with different concentrations ranging from 1-5% were found to be efficient in suppressing the growth of the test fungi in *invitro* condition.

According to Das *et al.*(2006) the antagonistic bacteria and fungi are known to produce a diverse range of secondary metabolites, enzymes, biochemicals and antibiotics which work as enzymatic activity and therapeutics against the another group of micro organism. The present work utilizes two most widely used microbial agents against the leaf blight viz., *Bacillus subtilis and Trichoderma gamsii.*

The antagonistic bacteria used in the present work revealed the potential efficacy against the leaf blight of large cardamom caused by the *C. gloeosporioides* in the green house condition. The blight observed in the leaf of large cardmom was suppressed and did not allow the blight to increase. The application of the microbial agent proved that the blight diseases can be controled about with 40-45% efficiency. Similar type of work was reported by Srimai *et al.* (2014) where they had applied

Bacillus subtilis to control the leaf spot disease caused by *Cercospora lactucae-sativae*in Lettuce in a green house experiment. The result revealed that the formulation was effective against the pathogen which suppressed the leaf spot disease. Earlier reports also demonstrated theantagonistic activity of *Bacillus subtilis*and showed the growth inhibition of the wide range of pathogens such as *Cercospora beticola*, *Colletotrichum gloeosporioides*, *Pseudocercospora purpurea* and *Rhizoctonia solani* (Lindow and brandl 2003, Collins *et al.* 2003, Eeden and korsten 2006 and Kai *et al*.2007). Another study by Arunyanant *et al*. (2008) alsosupported the present work,they have observed similar response of *Bacillus subtilis.*They demonstrated that,when formulation of the antagonistic *Bacillus subtilis* was applied against fungi *Curvularia lunata,Fusarium semitechium*, *Cercospora oryzae* effective inhibitionof growth of the pathogens was recorded.

Trichoderma genus is a well knownbiological agent that has been used for the management of several pathogens. As reported earlier (Ahned 2011, Parveen 2012), the *Trichoderma* sp. has the property to reduce the growth of pathogens and it has been widely used against *Fusarium solani* and *Fusarium oxysporum*. Dennis and Webster (1971) have reported that the *Trichoderma* sp. have the ability to inhibit the pathogenic fungi and act as an effective antagonistic bio-control agent, as they are capable of producing acetaldehyde which is known to possess antimicrobial activities. The present findings were also supported by the findings of several researchers (Gawade *et al.* 2009,Devis *et al*.2003, Raheja and Thakur 2002, Kaur *et al.* 2006) and confirmed that the *Trichoderma* sp. viz., *viride*, *harzinum* and *virens* work as effective antagonists against *Colletotrichum* species**.** Maketon *et al.*(2008) have reported

antagonistic properties of *Bacillus subtils* and *Trichoderma harzianum*against the tobacco diseases in greenhouse experiment. The two microbial agents were used against the diseaseslikebacterial wilt (*Ralstonia solanacearum*), damping-off (*Pythium aphanidermatum*), and frogeye leaf spot (*Cercosporanicotiana*).

Finally, the present study describes, for the first time, morphological and molecular characterization of six fungal pathogens ie., *Pestalotiopsis maculans, Curvulariaeragrostidis, Colletotrichum gloeosporioides, Fusarium* sp., *Phoma cava* and*Epicoccum nigrum* isolated from the large cardamom. To the best of our knowledge, out of these six pathogens*Curvularia eragrostidis* and *Epicoccum nigrum* are being reported for the first time as the pathogen of leaf blight, and they are likely to be new foliar threats to large cardamom in the region.

The results indicated that all the treatments as tested during the experiment were quite efficient. Foliar application as spray started before inoculation of the pathogen was more effective and found to be the best timing for treatment showing longer resistance rather than treatments started after the inoculation of pathogen. Among the botanicals and bio-inoculums tested *Lantana camera* and *Bacillus subtilis* were most effective and promising treatment to control *C. gloeosporioides.* Hence, as reported in this study, further research is necessary for the adoption of different botanicals, bio-inoculums and formulations those will be usefulto control the disease,and healthy large cardamom plantation will emerge again in the region.

CHAPTER 6

SUMMARY AND CONCLUSION

Large cardamom (*Amommum subulatum* Roxb.), originated and endemic at Sikkim, India, is a shade loving spice crop, grown under the forest trees with wide ranges of altitudes varying from 600 to 2400 m amsl. It is one of the main cash crops cultivated in Sikkim, Darjeeling district of West Bengal, and few other north eastern states in India. The spice crop has played an important role in the rural economy of Sikkim and other north eastern states in the country. More than 80% rural population in the region depends directly or indirectly on large cardamom cultivation for their livings.

Sikkim is known for large cardamom and the tiny hill-state, produces 90% of the country's large cardamom and in 2017- 2018 total large cardamom plantation area in Sikkim was 17735.15 ha that produced 4385.28 MT cardamom. But for the past two decades large cardamom cultivation in the region is passing through a critical phase. The mountain regions that once offered good climatic conditions like, soothing temperature, fertile soil, distributed rain fall, humidity, etc. for this important crop, have turned into a breeding ground for pathogenic diseases. According to the studies, various factors are responsible for the situation including non-availability of quality planting materials, an effect of climate change, lack of irrigation facilities during dry season, open cultivation, inadequate nutrient management, unscientific methods of cultivation, diseases, pests, etc. Most importantly large cardamom production has gone down drastically due to various types of fungal rot diseases among which blight is

highly destructive one in Sikkim. As a result large cardamom plantation is disappearing in the region at an alarming rate. Despite several incentives and activities after 2004-05 a sharp decline in terms of area and production in the large cardamom sector recorded and continued till 2013-14, for a span of 10 years. During this bad spell, least plantation was recorded in 2011-12 with 15502 ha production area, i.e., 34% lower to that of 2003-04 and production 3237 Tonnes, 36% lower to that of 2004-05.Since the disease is widespread in these areas, availability of healthy mother plants for the production of healthy planting material is also very difficult. Therefore, new plantations with healthy looking planting materials are also being infected quickly as they may contain germ spores of the fungus. However, till date only few survey and scientific reports are available mainly describing the severity of the problem with no proper identification of the causal organism. Hence, keeping the above mentioned facts in mind, the present study was focused on identification of major fungal pathogenbased on the morphological and molecular characterization so that effective measures could be developed to protect this important crop, the life line for rural hill economy of north east India.

During the study 30-60% severity of leaf blight incidence has been recorded. The survey showed that disease condition is alarming and majority of the plantations in the state are affected with 40-45% blight incidence. Affected leaves from diseased cardamom plantations with blight symptoms were characterized by sunken appearances with the necrotic areas and yellowish-brown irregular spots. It was observed that necrotic symptoms spread from the tip and sometimes from the leaf margin. Gradually leaves tend to dry out from the tip resulting drying of the whole

plant, eventually causing death of plants. The affected area became necrotic and dried up of plantation. It was also noticed that, among the six varieties grown in the state, *Varlangey, Swaney, Ramla* and *Ramsey* were found with blight symptoms. Further it was also quite prominent that new plantations in open field conditions were worst affected with the disease in comparison to those grown under canopy cover. Among the four districts studied, west district of Sikkim was found to be the severely affected with with 33-45% disease incidence.

In the present study initially total fifty pathogenic isolates were obtained. Based on the similar colony morphology and growth characteristics of isolates these isolates were grouped into nine (09) categories and were taken up for characterization and identification. All the nine isolates were sent to National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune for authentication. Based on the basis of morphological features, both gross and microscopic the isolate no LC02 was identified as *Pestalotiopsis maculans*, isolate LC03 *Verticillium lecani*, isolate LC04 *Curvulariaeragrostidis*, isolate LC05 *Colletotrichum gloeosporioides*, isolate LC11 *Phoma cava,*isolate LC12 *Epicoccum nigrum* and LC06, LC08, LC09 were identified as *Fusarium* sp. Out of these total nine isolates eight were identified as highly pathogenic and one non-pathogenic beneficial fungus.

Further, it was observed that the crop was infested with several fungal diseases, among which leaf streakwas considered as one of them. Effect of different media, tolerance to different temperature, and pH level on growth and of the pathogen was studied. For the molecular analysis, ITS region 1 and 4 along with the BLASTn analysis have been done. Based on the colony morphology, microscopic features and

molecular characterization, the isolate was identified as *Pestalotiopsis maculans*. The pathogenicity of the pathogen was also confirmed during the study. To the best of our knowledge, it is the first confirmatory molecular characterization of *P. maculans* causing leaf streak disease of large cardamom.The fungal culture has been deposited at the NFCCI-ARI, Pune with an accession number (NFCCI-4698) and the sequence has been deposited in NCBI GenBank with accession number MN710582.

At present, leaf blight is considered as a major threat to cardamom cultication in Sikkim. During this survey, a typical symptom of leaf blight was observed on cardamom leaves in many locations. The leaves with blights were collected, surface sterilized, and inoculated on potato dextrose agar (PDA). The pathogen was isolated as pure culture, and on the basis of morphological and microscopic characteristics, the fungus was identified species of *Curvularia*. Molecular characterization of the fungal isolate with ITS-rDNA partial gene amplification using universal primers (ITS4 & ITS5), showed 100% similarity with *Curvularia eragrostidis* (family: Pleosporaceae). The fungal isolate and nucleotide sequence was deposited in National Fungal Culture Collection of India (NFCCI), Pune and NCBI with accession numbers NFCCI 4541 and MN710527, respectively. This is the first report on the occurrence of *C. eragrostidis* pathogen causing leaf blight of large cardamom grown in Sikkim.

In another observation it was found that the crop is now facing with a devastating disease that has brought down the yield to the modest level. The pathogen is known to cause severe foliar diseases with blight symptoms. Leaves of affected plants were characterized by sunken appearances with the necrotic areas, yellowish-brown color with irregular spots, spread rapidly to the whole plants, resulting drying

up and death of plants. The infected leaves were then further studied and the pathogen was obtained using the Potato dextrose agar medium, incubated at 25 °C for five days. The mycelium was septet, hyaline, and 2-4 µm wide. The conidiospores were cylindrical with both ends rounded, sometimes oblong. Length and breadth were 11-12 µm and 3-4 µm, respectively. Based on the colony morphology, microscopic features and molecular characterization the isolate was identified as *Colletotrichum gloeosporioides*. The pathogenicity of *C. gloeosporioides*was also confirmed during the study. The fungal isolate and nucleotide sequence have been deposited in National Fungal Culture Collection of India (NFCCI), Pune and NCBI with accession numbers NFCCI 4542 and MN710587, respectively.

During the present investigation three isolates (No. LC06, LC08, LC09) were isolated explants collected from different cardamom growing regions in Sikkim causing wilt and rot in the large cardamom. The disease was a typical leaf blight followed by wilting and in rot of the cardamom plants. The wilting of the plant and the individual leaf was the characteristics symptoms of the pathogen. The grayish color on the leaves accompanied by the sudden wilt where the whole becomes water soaked which becomes black in color and ultimately rots. Interestingly growth of all the isolates was different irrespective of varied solid media used. Colony colour varied from white, white colony with cottony mass, pink, dark-pink, pinkish-white etc. when observed from the front and inverted plates. The mycelium was septate, hyaline, and 3-7 µm wide. The conidiospores were simple cylindrical, with two to several celled, fusiform to sickle shaped with elongated apical cells. Finally, the above mentioned three pathogenic isolates (No. LC06, LC08, LC09) which had different morphological

features, gross growth responses but on the basis of overall colony characteristics and microscopic features, those isolates were similar to those of*Fusarium* sp. and which was further confirmed by the National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune with accession number NFCCI- 4543, NFCCI- 4544, NFCCI- 4699.

In other observation a typical leaf disease was observed, where the symptoms were small lesion on the younger leaves and are usually observed with numerous water soaked lesions, round in shape with greyish margin which eventually develops into a prominent yellowish spots. In due course of time, these spots increase in size, turning yellow to brown and black spots. Numerous spots coalesce to form a bigger patch which resembles as the blighted leaf and dries out.

The isolate obtained from the sample were subjected to different physico-morphological characterization. The colony growth was maximum when grown on potato dextrose agar and Sabourauddox agar medium. The fungus was found to have the growth in a wide range of temperature 10°C to 35°C (optimum 25°C) and tolerated wide range of pH (5.0–13., optimum 6.0). On the basis of colony morphology, microscopic features the fungus was identified as *Phoma cava* Schulzer The culture has been accessioned as NFCCI 4666.

Among the seven different pathogens isolated during the investigation, *Epicoccum nigrum* was one of the new recorded pathogen encountered during the study. The symptoms were seen with the chlorotic spots especially on the upper side of the leaf. These spots were irregular, reddish to brown in color, 2-6mm diameter

found on the leaf margin. Interestingly, these spots were seen in the *Varlangey* and *Sermna* varieties grown in the open condition.

Results revealed that out of five different media tested for the fungal isolate, three, i.e., Potato Dextrose Agar (PDA) and V8 Juice Agar were found to be significantly different ($P < 0.05$) and gave satisfactory performance with colony growth 63.17 and 62.33 mm respectively. In the present study, the optimum temperature for colony growth was found to be between 25°–35°C with colony diameter 63.17 mm after 5 days of incubation. Medium pH also significantly affected radial mycelial growth of the isolate. Colony growth of the isolate was observed in a wide range of pH (5.0 – 9.0) of medium. Maximum colony growth was obtained at medium pH 6.0 - 8.0 with 59.3-43.4 mm colony diameter. No colony growth was observed when medium pH was 3 and 13. The mycelium was septate, hyaline, and 3-6 µm wide. The Conidia were globose to pyriform, with 1.11µm length and 0.44µm width.On the basis of colony morphology and microscopic features, the isolate was identified as *Epicoccum nigrum* (NFCCI-4545) by National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune. Based on ITS sequences alignment at NCBI and phylogenetic analysis 100% similarity the isolate was identified as *Epicoccum nigrum* with accession numberMN712328.

Sikkim is fully organic state and use of synthetic chemicals for agricultural activities is banned. Hence, several indigenous practices are used by the farmers for plant protection measures of major agricultural crops. With the accumulated knowledge and experiences the local inhabitants generally utilize the resources available in the area especially botanicals. These are eco-friendly, sustainable and

relatively cheaper when compared to other options being adopted to mitigate the pest and diseases prevalent in the region.

It is well known fact that, most medicinal and aromatic plants have numerous bio-active compounds that act as anti microbial agents. In Sikkim, local people based on their ITK's use several plant based formulations to protect their crops from many diseases in the region. Botanicals are safe due to its biodegradability and non phytotoxicity properties. Therefore, botanical are the new ray of hope for emerging bio-pesticide industries where the use of the synthetic pesticides can be reduced to some extent.

In the present investigation antifungal efficiency of the selected 24 botanicals was tested against the *C. gleosporidies* under *in vitro* condition. The antifungal potential of these selected botanicals was determined following the disc diffusion method.When compared the efficiency of the botanicals extracted in ethanol *Zanthozylum aramutum* was found to be the most effective and showed maximum inhibition zone (14.5±0.41 mm) among other botanicals tested. This was followed by the extract of*Lantana camera* that showed significant zone of inhibition irrespective of solvents used in this study. Chloroform extract of the *Lantana camera* was found to be the most effective and showed 11±0.94 mm inhabitation zone against the *C. gleosporidies*. The result also confirmed lowest MIC against *C. gleosporioides* of the two most effective botanicals i.e., *Zanthozylum aramutum* and *Lantana camera* in ethanol extract were 600 µg /ml and 700 µg /ml respectively.

In this study the efficiency of antagonistic microbial agents was also tested against the pathogenic test fungi *C. gloeosporioides.*Productions of diffusible and

volatile anti fungal compounds from the antagonistic microbial agents were evaluated using the dual culture method. The results revealed the positive effect of diffusible and volatile compounds produced by all the three antagonistic bacteria with varying effectiveness. Antagonistic suppression of fungal growth was noticeable after 24 h incubation. Percent inhibition in radial growth caused by diffusible compounds of *Bacillus subtilis, Trichoderma gamsii* was 8.66 and in case of *Psudeomanas corrugate* was 10.66.Percent inhibition in radial growth due to the production of volatile compounds from the *Trichoderma gamsii, Bacillus subtilis* and *Psudeomanas corrugate* were 7.33±0.33, 8.00±0.57 and 10.00±0.57 respectively.The inhibition of the growth of pathogenic fungi (*C. gleosporoids*) continued to increase with the increase in incubation period. Fungal inhibition after 120 h of incubation due to diffusible compounds of *B. subtilis*, *T. gamsii andP. corrugata* were 34.5±0.41, 34.0±0.88 and 43.0±1.66 respectively and for volatile substances 45.5±0.41, 50.0±1.66 and 56.0±0.83 respectively. With detailed assessment and performances as described in above paragraphs *B. subtilis* and *T. gamsii* were selected for further study to control the *C. gleosporioides* in the field experiment under green house condition.

Field trials were also carried out for aperiod of 11 months under green house condition with disease free tissue culture raised plantlets of large cardamom.

Results revealed that tissue culture raised healthy plants initially treated with botanicals and the bio agents 3 days before the inoculation of the pathogenic spores led to some significant protection against leaf blight of large cardamom caused by the *C. gloeosporioides.* Further the results indicated that all the treatments as tested during the experiment were quite efficient. Foliar application as spray started before

inoculation of the pathogen was more effective and found to be the best timing for treatment showing longer resistance rather than treatments started after the inoculation of pathogen.

In conclusion, for the last two decades large cardamom cultivation in the region is passing through a very bad phase. The survey showed that the disease severity is alarming and majority of the plantations in the state are affected with 40-45% blight incidences. The present study describes, for the first time, morphological and molecular characterization and identification of six fungal pathogens (i.e., *Pestalotiopsis maculans, Curvulariaeragrostidis, Colletotrichum gloeosporioides, Fusarium* sp., *Phoma cava* and*Epicoccum nigrum*) isolated from large cardamom grown in Sikkim. To the best of our knowledge, it is also the first report of *Curvularia eragrostidis* and *Epicoccum nigrum* causing leaf blightand are likely to be new foliar threats to large cardamom in the region.

Further this is the first report on field trials to observe the effect of botanicals and bio-control agents against leaf blight caused by *C. gloeosporioides.*Experiments were conducted under green house condition with disease free tissue culture raised plantlets of large cardamom. Results showed that all the treatments used for the experiments significantly inhibited the pathogen.Foliar application as spray started before inoculation of the pathogen was more effective and found to be the best timing for treatment showing longer resistance rather than treatments started after the inoculation of pathogen. Among the botanicals and bio-inoculums tested *Lantana camera* and *Bacillus subtilis* were found to be the most effective and promising treatment to control the *C. gloeosporioides* causing leaf blight disease in large

cardamom. It is expected that the findings of this research will help to enable effective targeted measures to protect this important crop – life line for rural hill economy of north east India and Sikkim in particular.

CHAPTER 7

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