CHAPTER 3

MATERIALS AND METHODS

Study site:

Goalpara district is situated on the south bank of river Brahmaputra, and it covers an area of 1,824 square kilometres which is bounded by West and East Garo Hills districts of Meghalaya on the South, Kamrup district on the East, Dhubri district on the West and, River Brahmaputra all along the North. The geographical location of the district is between latitudes 25.53° to 26.30° North and longitudes 90.07° to 91.05° East. Except for few low forested hills, the topography of this district is generally characterised by an almost flat plain. The agroclimatic conditions of the district are suitable for various agricultural activities.The climate in the district is moderate during the winter and hot in the summer. During the month of April rain makes its first appearance with occasional and irregular light showers and at times heavy downpour followed by cyclonic storm. The irregular rainfall continues upto the end of May. From the early part of June, the monsoon rain normally begins and heavy of very heavy rains occur in the district till the month of October. About 80% rainfall is from South West monsoon. The area coverage in the district composed of eight development block and twenty six sericulture cycle. Sericulture in Goalpara district existed as a practice amongst people since long. Around 290 villages in the district are involved in seiculture activities. (Statistical Handbook, Assam, 2006). Five major muga silkworm rearing villages of Goalpara district, Assam were selected depending upon the direction for collecting the leaves, soil and air samples during six muga crop seasons. The study was conducted from February, 2014 to January, 2016.

The study area Dorapara Agia located at centre of the district with latitude 26°5'31.525"N and longitude of 90°33'57.109"E. The Som plantation covering an area of 12.65 bighas of land with a total number of 1325 plants aging between 8-10 years.

While Budlung pahar is located on the North with a latitude of 25°59'48.254"N and longitude of 90°57'15.076"E. The plantation cover an area of 7.25 bighas of land with a total no. of 775 plants aging in between 12-15 years.

Lengopara is situated on the South of the district with latitude of 26°6'7.699"N and longitude of 90°47'9.044"E. The plantation covers an area of 11.95 bighas of land with a total no. of 1370 plants aging in between 8-10 years.

Buraburi is located on the East with latitude of 25°58' 47.057"N and longitude of 90°47'32.217" East. The plantation covers an area of 6.1 bighas of land with a total no. of 685 plants aging in between 9-11 years.

Bhalukdubi Kalyanpur is located with latitude 26°5'38.357"N and longitude of 90°33'43.882" E on the West. The plantation covers an area of 6.65 bighas of land with a total no. of 575 plants aging in between 10-12 years.

Different age of the leaves depending upon the size and shape viz. tender, semi mature and mature were randomly collected during rearing (outdoor) season from February, 2014 to January, 2016 in sterile polybags and taken back to the laboratory from the 5 study sites of Goalpara district mentioned above during the 6 muga crop seasons. Isolation and identification of fungi were done by using "A manual of soil fungi" by Gilman (1995), "Illustrated genera of imperfect fungi" by H.L. Barnett (1960) and the diseases were identified with the help of "Diagnostic manual for diseases & pests of muga silkworms and their host plant" by CMER&TI, CSB, Lahdoigarh, Jorhat.

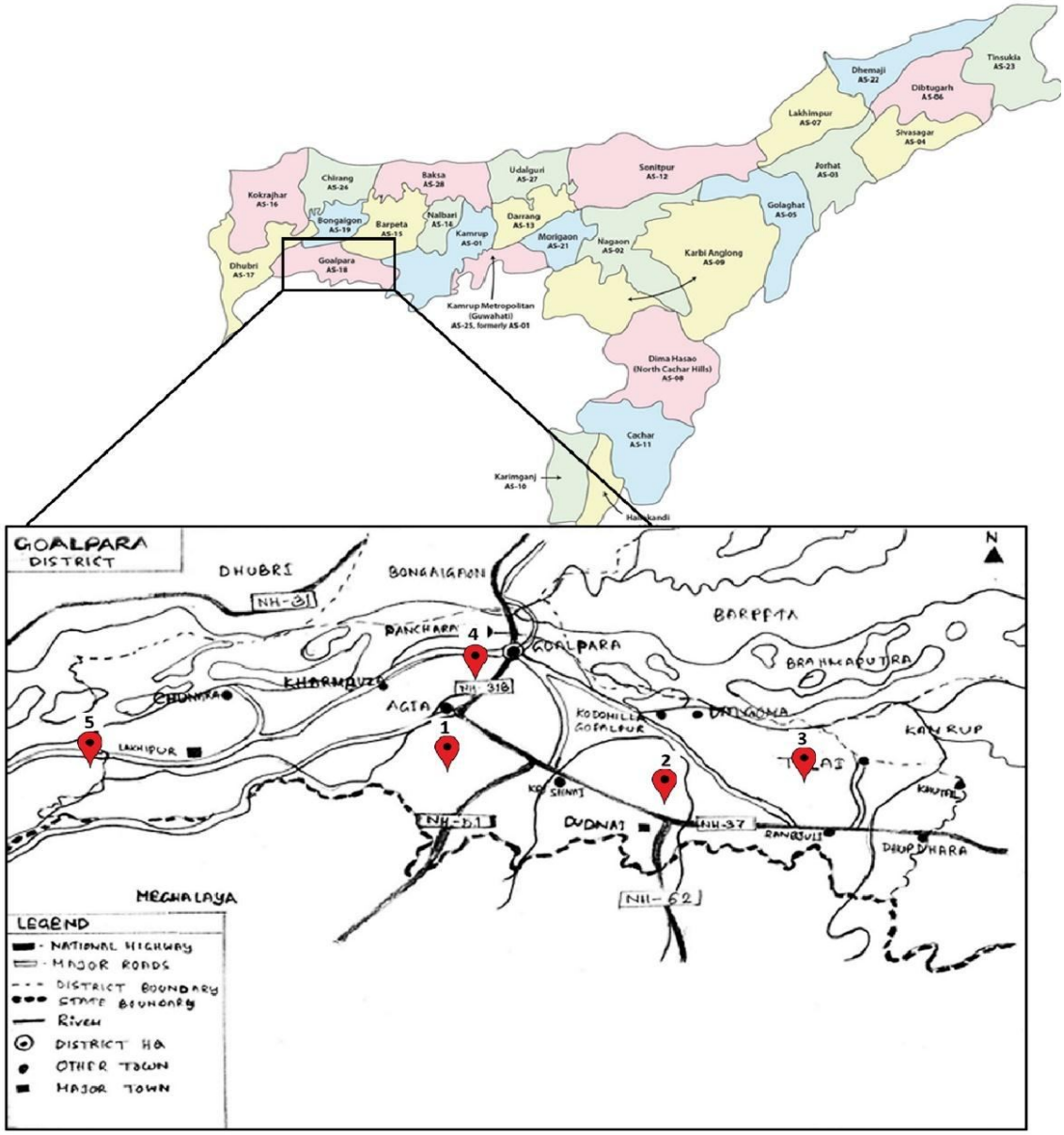

Fig. 1. Study sites. The red dots with number denotes the places selected for the respective study within the Goalpara district. (1) Dorapara Agia; (2) Buraburi; (3) Budlungpahar; (4) Bhalukdubi Kalyanpur; (5) Lengopara

Disease intensity was determined by randomly selecting 25 plants, five in four corners & five in the centre of the garden (total 5 × 5 = 25 plants). In each plant, total no. of leaves infected with various diseases was counted. Various foliar diseases was recorded on the basis of the methods Thangavelu *et* al. (1998) and Das & Benchamin(2000) and with the help of "A diagnostic manual for diseases & pests of muga silkworms and their host plant" by CMER&TI, CSB, Lahdoigarh, Jorhat. Disease epidemiology study was conducted in relation to Seasonal variation of incidence of the major foliar disease of Som with the various climatic factors such as Temperature, Humidity and rainfall of the district as well as age of the plant and types of leaf infected.The infected leaves were categorized into five grades as suggested by Dubey and Dwivedi (1991) .

Grade 1 – No infection

Grade 2 – 0-5 % of leaf lamina infected

Grade 3 – 6 – 25 % of leaf lamina infected

Grade 4 – 26 -50 % of leaf lamina infected

Grade 5 --------- 51- 100 % of leaf lamina infected

The Percentage disease index (PDI) was calculated using the standard formula:

$$\text{Percent Disease Index (PDI) \%} = \frac{\text{Sum of numeric grades}}{\text{Total number of plants x Maximum grade}} \times 100$$

Soil samples of the muga food plantation area were collected following standard sampling method. Five major muga silkworm rearing villages of Goalpara district were selected depending upon the direction namely Dorapara agia on the Centre, Budlung pahar on the North, Lengopara on the South, Buraburi on the East and Bhalukdubi Kalyanpur on the West respectively for collecting the soil samples during six muga crop seasons. For collection of rhizosphere soil sample each Som plantlet was carefully uprooted and the soil adhering to the roots was gently shaken into a strerile polythene bag; the bag was tied and labelled. The non rhizosphere soil samples were collected by digging a few centimeters deep into the field with a sterile hand trowel; the soil collected was then tied and labelled. Isolation and evaluation of microfungi from soil and rhizosphere will be done by serial dilution agar

plating method (Atlas and Parks, 1997).The petridishes were incubated at 28±1 ° C for 7 days and then the plates are examined for the development of fungal colonies. The isolated fungi were identified. The mycelia and spore characters of fungi were studied under microscope (Labomed, Germany) using Lactophenol cotton blue staining and with the help of " A manual of soil fungi by Gilman (1995) and "Illustrated genera of imperfect fungi" by H.L. Barnett (1960).

Air samples were collected using the culture (gravitational setting) method with petridishes containg Potato Dextrose Agar (PDA), Martins Rose Bengal Agar (MRBA) and Czapek's Dox Agar medium supplemented with Chloramphenicol (250mg/ml) to prevent bacterial growth. Five major muga silkworm rearing villages of Goalpara district were selected depending upon the direction namely Dorapara agia on the centre, Budlung pahar on the north, Lengopara on the south, Buraburi on the east and kalyanpur on the west respectively for collecting the air samples during six muga crop seasons. After exposing the plate for 10-15 minutes at 2-3 meter height above the ground level they were transferred to the laboratory and kept for incubation at 28° ± 1° C for a period of 7 days and then the the plates are examined for the development of fungal colonies. The isolated fungi were identified. The mycelia and spore characters of fungi were studied under microscope (Labomed, Germany) using Lactophenol cotton blue staining and with the help of "A manual of soil fungi" by Gilman (1995) and "Illustrated genera of imperfect fungi" by H.L. Barnett (1960).

Different age leaves depending upon the size and shape viz. tender, semi mature and mature were randomly collected during rearing (outdoor) season from February,2014 to January, 2016 in sterile polybags and taken back to the laboratory from the study sites for collecting the samples during the 6 muga crop seasons .Serial washing technique as described by Williams *et* al. (1965) and leaf sectioning and plating method described by Dickinson and Preece (1976) were employed. Leaf discs were cut for each leaf categories with the help of sterilized borer.Pieces from leaf categories were placed separately in 20 ml of sterile distilled water in 250 ml of Erlenmeyer flask shaken for 20 minutes at 120 rpm. The extract of the detachable fungal propagules from the leaf surface was determined by plating 1 ml solution from washing to the petriplates containing PDA media. The cut out leaf discs dorsal and ventral surface were impringed on the surface of PDA media containing petridishes. The petridishes were incubated at 28° ± 1° for 7 days and then the plates are

examined for the development of fungal colonies. The isolated fungi were identified. The mycelia and spore characters of fungi were studied under microscope (Labomed, Germany) using Lactophenol cotton blue staining and with the help of " A manual of soil fungi by Gilman (1995) and "Illustrated genera of imperfect fungi" by H.L. Barnett (1960).

Plate.1. A newly pruned Som garden of Dorapara Agia from Goalpara district of Assam

Physicochemical properties of soil

Determination of soil pH:

The pH of the soil sample was determined in distilled water and 1N Kcl solution taking soil solution ratio of 1:1 using Systronics digital PH meter.

Determination of organic carbon (Walkley and Black, 1934)

- 0.5gm of soil was taken in a conical flask, 5ml 1N $K_2Cr_2O_7$ and 10ml conc.H_2SO_4 were added and mixed them well
- Kept it for 30 minutes for the reaction to occur and cooled
- 100ml of distilled water were poured to the mixture and 5 ml of orthophosphoric acid and 5ml of sodium fluoride were added and mixed
- 2-3 drops of diphenyl amine indicator were added
- Titrated with 0.5 N $Fe(NH_4)_2(SO_4)_2.6H_2O$ till the colour changes to bright green through blue
- The reading was noted
- A blank was carried out without the soil sample.

Determination of total nitrogen content (Jackson, 1958)

- 5g of processed soil sample were weighed in a 500ml Kjeldahl flask.
- 30ml of Conc. H_2SO_4 containing 2g of salicylic acid were added
- The contents of the flask were shaken until thoroughly mixed, and allowed to stand for, at least , 30 minutes with frequent shaking.
- Added 5g of sodium thiosulphate, heated the solution for 5 minutes and cooled.
- 20ml of water, 10g of K_2SO_4 and 1g of the digestion accelerator were added and digest the contents till light green colour appears.
- Heat was gradually raised until the acid reached its boiling point
- Cooled the contents and diluted to about 100ml with distilled water, swirld the flask for about 2 minutes, and transfered the fluid part to a 1000ml distillation flask
- Washed the residues left in the Kjeldahl flask with 4-5 lots of 50-60 ml distilled water, decanting the washing into the distillation flask.
- Few glass beads were added to prevent bumping

- The other end of the condenser were connected with a tube which dips into 50ml of 0.1 N H_2SO_4, contained in a conical flask, with two drops of methyl red indicator
- Added 125ml of 40% NaOH solution along the side of the flask till the contents were alkaline. Then the flask were heated
- The NH_3 formed was allowed to get absorbed in the standard H_2SO_4. When no more NH_3 is received, the distillation was stoped and proceeded for titration.
- Titration were performed with 0.1 N NaOH solution till the pink colour changes to yellow.

Determination of available phosphorous in soil (Bray and Kurtz, 1945).

- 5gm soil was taken in 100ml conical flask, add 50ml NH_4F working solution and 1 st. Spoon of ash.
- Shaking was done for 5 minutes and filtered through Whatman 42 paper.
- 5 ml of aliquot into 25 ml volumetric flask were taken and 5 ml of NH_4F working solution + 5ml of Dickman reagent + 1ml of Stannous chloride solution were added.
- The volume were made upto 25ml with distilled water
- A blank with H_2O was prepared
- Absorbance was taken at 660 nm after 5 minutes by using colorimeter
- The reading was recorded and ploted in st. Curve.

Determination of Available Potassium (Toth and Prince ,1949)

- Weighd 5gm of soil in a 150ml of conical flask
- 25ml of neutral N NH4OAc solution were added to it.
- The contents of the conical flask were shaken on an electric shaker for 5 minutes and filter.
- Feeded the filtrate into the atomizer of the flame photometer, 100g of which had been set with 40 ppm K solution and noted the reading.
- Reading was located on the standard curve

Determination of Water Holding Capacity (Lal, 1977):

- Two funnels were taken and lined them with filter paper and put them in a measuring cylinder.
- Two soil samples were put in two separate funnels
- Pour equal amount of water in both funnels
- Let the water dripped in the cylinder
- Volume of water collected were recorded.

Determination of Soil moisture content (Wilde *et* al.,1985):

- To determine the soil moisture content, soil samples were collected from the study sites and were sieved through 2mm mesh sieve and 20gm of fresh soil samples were taken in the moisture boxes of known weight. The moisture boxes with soil samples were then oven dried at 105°C for 24 hrs to a constant weight. The moisture content was determined by the following formula:

$$\text{Moisture content} = \frac{\text{Weight of fresh soil} - \text{Weight of oven dried soil}}{\text{Weight of fresh soil}} \times 100$$

Determination of Soil texture (Black, 1965):

- 20 g of air dried soil was taken and H_2O_2 were added (10ml 30% H_2O_2) and boil till the OM is completely destroyed till the effervescence was stopped.
- Allowed to cool and filtered it. The filtrate was discarded.
- The soil that remains on the filter paper washed clearly with water and collected in a beaker.
- $Na(PO_3)_6$ (5gm in 100ml distilled water)was added to the above and shaked it with a mechanical stirrer for 10 minutes.
- The contents were poured into a 1000ml cylinder and made up the vol. with water.
- Shaking was done for 30 minutes (1min)
- 10 ml aliquot was taken at 10 cm depth after 5 min of stirring.
- Put the aliquot in a glass plate and dried it on the hot plate.
- Cooled in the dessicator and it to 0.001gm (W_1)

- After expiry of the requisite time , 2nd pipetting of 10ml at 10cm and dried it.
- After cooled down ,weight was taken.
- The rest of the content of the cylinder was decanted in the 45μ sieve. The sand was washed and cleaned several times with water and then dried.
- Calculation:

  (W1-W) x 500= Silt + Clay (%)

  (W2-W) x 500= Clay (%)

  Sand (%)= Wt. of sand x 5

  (Silt + Clay) – Clay = Silt

Biochemical estimation of leaves:

Total Nitogen:

Total nitrogen was determined by Micro-Kjeldahl method (Jackson, 1958) as mentioned above for soil samples.

Crude protein:

It was determined by methods used by Wood *et* al., (1964). Where crude protein was calculated as amount of Nitrogen ×6.25

Total carbohydrate:

Total carbohydrate was determined by Anthrone method (Yem and Willis, 1954). Where 100 mg of dried leaf powder was extracted into 25 ml of 80% ethanol and the mixture was heated in a water bath for 10 min. After cooling, the mixture was centrifuged at 2000 rpm for 15 min. and the supernatant was collected and the pellet was extracted thrice. The supernatant was discarded and the residue was saved and 3 ml of distilled water and 6.5 ml of 52% perchloric acid was added. The contents were stirred constantly for 5 min. and occasionally for the next 15 min., and then 20 ml of water was added and centrifuged at 3000 rpm for 10 min. The supernatant was made upto 100 ml with distilled water. Optical density was measured at 625 nm. Glucose was used as standard and a conversion factor of 0.9 was used to obtain the value of starch from glucose.

Crude fibre (AOAC, 1975):

Crude fibre was obtained from the loss in weight on ignition of dried residue remaining after digestion of fat free samples with 1.25% each of sulphuric acid and sodium hydroxide solutions under specified conditions.

$$\% \text{ of fibre} = \frac{\text{Loss of weight in ignition}}{\text{Weight of sample used}} x\ 100$$

Moisture content(AOAC, 1975):

Moisture content was determined by washing a known weight of leaf sample with clean and distilled water and drying to a constant weight at 60°C in hot air oven.

The pathogen, *Pestalotiopsis disseminata* causing grey blight disease of som was isolated from freshly infected som leaves, collected randomly from various places of Goalpara district of Assam, India which is the area of study. Pure culture of the fungus were maintained by subculturing periodically on fresh potato dextrose agar (PDA) medium. Four systemic fungicides i.e chemicals namely Bavistin, Copper oxychloride, Mancozeb and Topsin M and leaf extracts from five locally available plant species i.e. *Azadirachta indica*, *Lantana camara*, *Eupatorium odoratum*, *Lucas aspera* and *Bouganvillea spectabilis* were selected as nonsystemic fungicides. Both the treatments were made 1:100(0.01), 5:100(0.05), 10:100(0.10), 15:100(0.15) and 20:100(0.20) concentration and tested against *P. disseminata* by the Poisoned food method (Nene and Thapliyal, 1993). Equal volume of PDA are mixed with the fungicides in a conical flask. Then the medium was poured in sterilised petridishes and kept for solidification. A mycelial disc of 4 mm in diameter of the test fungus was taken from 7 days old culture with the help of sterilised cork borer and placed at the centre of the petridishes containg the media and the fungicide. A mycelia disc of pathogen on PDA without adding fungicides served as control.

Plant extracts were made by using method of Singha *et* al. (2004). Fresh leaves of each plant species were washed in distilled water and separately homogenised with sterile water in 1:1 (W/V) in a sterilised mortar and pestle. The homogenate was filtered through muslin cloth and was considered a stock solution. Five dilutions as mentioned earlier i.e. 1:100(0.01), 5:100(0.05), 10:100(0.10), 15:100(0.15) and 20:100(0.20) were prepared from the stock solution using distilled water. 20 ml of PDA along with 2 ml of plant extracts from each dilution was poured separately with the medium. Actively growing 7 days old culture of

P.disseminata was cut into 4 mm in diameter and treatment were made using the same procedures used in case of chemical fungicides. A mycelia disc of pathogen on PDA without adding plant extracts served as control. Each treatment were made triplicate and linear mycelial growth were recorded after 5-7 days.

The percentage growth inhibition was calculated using the formula suggested by Vincent (1927).

$$I = \frac{C - T}{C} \times 100$$

Where, I = Percent inhibition

C = Radial growth in control

T= Radial growth in treatment