Chapter 1
INTRODUCTION

Silk Industry is an agro based industry, the end product of which is silk. Silk is a protein fiber produced by silkworm for spinning cocoon. Sericulture is both an art and science of raising silkworms for silk production. India is the second largest producer of raw silk after China and the biggest consumer of raw silk and silk fabrics. It is a farm-based, labour intensive and commercially attractive economic activity falling under the cottage and small-scale sector. India is a producer of all the five commercially traded varieties of natural silks namely, Mulberry, Tropical Tasar, Oak Tasar, Eri and Muga. Assam enjoys irrefutable reputation for its exquisite silk product. The silk industry of Assam mainly comprises the culture of eri, muga, and mulberry silk. Eri and muga are the exclusive monopoly of the people of Assam. The production of eri and muga silk are mainly concentrated in the Brahmaputra valley and is predominantly based in the rural areas (Mahan, 2012).

Muga silk, popularly known as the "golden silk", is one of the most precious silk fibers available on earth due to its uniqueness in silk fibers and rarity in presence, as this silkworm is present only in the North-Eastern region of India. Although muga culture has a rich tradition and heritage, it is increasingly being threatened for its very survival due to the rampant and irrational exploitations (Tikader *et al*., 2011a). Muga silk is produced by the silkworm *Antheraea assamensis* Helfer, a polyphagous insect feeding on a wide range of plants viz., som (*Persea bombycina*) and soalu (*Litsea monopetala*) being the primary host plants, and dighloti (*Litsea salicifolia*) and mejankori (*Litsea citrata*) as the secondary host plants (Bhattacharya *et al*., 1993; Tikader and Rajan, 2012). Although, efforts have been made to domesticate this silkworm by rearing them under captivity, not much success could be obtained, hence, still left in the wilderness of the North eastern India which has distinct tropical humid climatic conditions with evergreen and deciduous forests. In order to provide a better shelter for this silkworm, efforts have been made to cultivate the host plants in the border regions of the forest (Thangavelu *et al*., 2005). Since being left in the wilderness, these host plants received little attention from the scientific community. Consequently, agronomic, biochemical and morphological traits of these plants have not been studied well to formulate strategies necessary to improve the leaf

productivity as well as adaptability to make muga silk production adequate enough to meet even the domestic demand of India.

"Som" belongs to the family Lauraceae, a medium size evergreen tree with spreading branches, bark and foliage usually aromatic, alternate leaves grows abundantly in its natural habitat in Assam particularly Brahmaputra Valley up to an elevation of about 500 meters, apart from its distribution extend to Khasi and Jayantia Hillls in India, along the Lower Himalaya and as far as to the west of Nepal (Rahman *et al*., 2012). Muga culture has been confined to the north eastern states of India and to a small extent to the Coochbehar district of West Bengal. Due to evergreen nature of this food plant, muga silkworm can be reared on it throughout the year. The plants become suitable for rearing of muga silkworm after 3-5 years of growth and can be used until 20-25years (Singh and Sen, 2001).

Som leaves improves silk producing ability whereas, soalu leaves enhances egg laying capacity of muga silkworm. The nutrition of silkworm entirely depends upon the quality of leaves. The food plants (leaves) have significant effect on health and survival of silkworms. Better the quality of leaves greater the possibility of obtaining good quality cocoons (Khanikar and Unni, 2006). Growth of silkworm, cocoon quality and quantity of raw silk entirely depends upon the quality of leaves (Chakravorty *et al*., 2006). Since leaf quality has significant impact on quantity and quality of the silk fiber, for sustaining muga culture it is important to ensure availability of adequate quantity of qualitatively superior leaves (Tikader *et al*., 2013).

The muga food plant som is vulnerable to many foliar diseases that affect the normal growth of the plant, quantity and quality of leaves and ultimately cocoon yield production (Das *et al.,* 2003). Philip *et al.* (1994) reported an estimated annual leaf yield loss of about 20-30% due to major diseases. Leaf spot, red rust, leaf blight and grey blight are the major foliar diseases of som (Bharali, 1969; Thangavelu *et al.,* 1988; Das and Benchamin, 2000).

The occurrence of leaf blight disease on muga food plant was first reported by Das *et.al.* (2005). Leaf blight disease is caused by *Colletotrichum. gloeosporioides.* It leads to premature leaf fall and causes 1273 Kg acre *4* year"1 that is approximately 6.3% of the total yield loss in leaf yield. Grey blight caused by *Pestalotiopsis disseminata* has been reported as a major epidemic disease of muga host plant, som causing 13.8-41.6% leaf yield loss (Bharali, 1969; Das and Benchamin, 2000). The

disease is so severe that it leads to shortage of quality leaves for rearing of muga silkworm finally causing severe economic loss to farmers.

Detection of propagules of plant pathogens in plants, seed, vegetative propagating materials and in plant products is an essential component of disease management strategies. Detecting and identifying pathogens provides the basis for understanding their biology and selecting appropriate control strategies (Goulter and Randles, 1996). Traditionally, diagnosis of plant diseases has been based on recognizing characteristic symptoms presented by diseased plants and looking for the presence of pathogens on their surface. This, together with other observations and evaluation of the environmental conditions, generally allows the causative agent to be classified as a virus-like organism, a bacterium, a fungus or some environmental factor. Successful diagnosis of many fungal and bacterial plant diseases depends on knowledge of plant pathology and experience in detecting and identifying the pathogen on the surface. In some cases these methods are still the cheapest, simplest and most appropriate, conventional methods do, however, have a number of drawbacks, which has prompted the search for alternative diagnostic techniques. Traditional methods generally require skilled and specialised microbiological expertise, which often takes many years to acquire. There is a need to use more generic techniques that can be taught quickly and easily to relatively unskilled staff. Methods that involve culturing can often take days or weeks to complete and this is not acceptable when rapid, high-throughput diagnosis is required. The results are not always conclusive, e.g. where similar symptoms can be caused by different pathogens or physiological conditions. Closely related organisms may be difficult to discriminate on the basis of morphological characters alone. It may also be necessary to discriminate between populations of the same pathogen that have specific properties, e.g. fungicide resistance, toxin production or differences in virulence. Traditional methods may not be sensitive enough (e.g. where the detection of presymptomatic infection is needed) and as such much effort has been devoted to the development of novel methods for detecting and identifying plant pathogens over the last decade (Ward *et al*., 2004). Recent trends in detection of plant pathogens include the development of more rapid diagnostic techniques with high specificity for the target organism (Chakraborty, 1988).The techniques can be divided into serology based methods and nucleic acid based methods. These techniques can be used to detect fungi, bacteria and viruses present in low quantities and on plant tissues and

therefore in many cases the pathogen can be detected at an earlier stage of disease development than was previously possible (Chakraborty and Chakraborty, 2003).

The earliest serological techniques in plant pathology used polyclonal antisera prepared by centrifugation of clotted blood of immunized animals. For classical enzyme-linked immunosorbent assay (ELISA), this is further refined to a serum fraction that is predominantly IgG, which is obtained by ammonium sulphate precipitation, followed by passage over an ion-exchange cellulose column (Clark and Adams, 1977). Although polyclonal antisera are still used regularly, the use of monoclonal antibodies in plant pathology is becoming progressively more routine. The principal aim of an immunodiagnostic assay is to detect or quantify the binding of the diagnostic antibody with the target antigen. There are a number of different ways of detecting antibody/antigen binding, but often these involve coupling the antibody to an enzyme that can be used to genera tea colour change when a substrate is added (Ward *et al*., 2004).The most commonly used serological methods for detection for fungal pathogens includes Double immunodiffusion techniques, ELISA and indirect immunofluorescence assays.

Diagnosis using serological methods has many advantages. Although antibodies may take several weeks to produce, they are generally stable for long periods if stored correctly and produce results quickly. They have wide application for general and specific recognition of unique epitopes of many micro-organisms but have been under-utilised in the diagnosis of plant pathogens other than viruses. Tests using antibodies have improved greatly. They are now suitable for both laboratory and field conditions, can identify strains within species, are sensitive to the nanogram level and take less time to carry out. There are some limitations to the use of antibodies in pathogen diagnosis. Firstly, the nature of the cross reactions between heterologous antibody-antigen complexes are not well understood so the degree of relatedness between cross reacting isolates cannot be estimated. Secondly, diagnosis is based on only part of the organism's structure such as the coat protein of a virus which represents only a small proportion of the information about the virus. Thirdly, serology is only useful when the antiserum has been prepared or when an antigen is available for producing an antiserum. Finally, serology is of no use for identifying previously undescribed pathogens (Goulter and Randles, 1996).

Nucleic acid-based methods (using probes and/or PCR) have increasingly been used in recent years to develop diagnostic assays for plant pathogens (Schots *et*

al., 1994; Ward, 1994; Martin *et al.*, 2000).These methods, particularly those based on PCR, are potentially very sensitive. In addition they also offer the potential to be highly specific. Most assays developed for bacteria and fungi have detected pathogen DNA, which is easier to prepare, and more stable than RNA.PCR, a method for rapidly synthesising (amplifying) millions of copies of specific DNA sequences, is the most important technique used in diagnostics (Dieffenbach and Dveksler, 1995; Edel, 1998; Kidd and Ruano, 1995). Sometimes nested PCR is used to improve the sensitivity and/or specificity of the assay (Schesser *et al.*, 1991; Mutasa *et al.*, 1995; Foster *et al.*, 2002). This involves two consecutive PCR reactions, the second one using primers that recognise a region within the PCR product amplified by the first set. PCR products can also be detected with a probe (Mutasa *et al.*, 1995). This can improve the sensitivity and specificity of the assay, particularly when the amplified product may not be sufficient to be seen on an agarose gel. An alternative approach involves screening random regions of DNA to search for one which is specific for the target organism (Henson, 1989; Mutasa *et al.*, 1993). In recent years, the most common strategy used for this is to first use a technique such as random amplified polymorphic DNA (RAPD) PCR (Nicholson *et al.*, 1996, 1998) to identify differences between the organism of interest and other related organisms. RAPD-PCR uses short primers of random sequence in a PCR with fairly non-specific, non-stringent conditions. Using this method, some DNA fragments are usually amplified from any organism tested, but the pattern of bands may be specific for a particular organism. Many different primers are tested until a band is found that is present only in the organism of interest. Potential diagnostic bands are then sequenced and used to design specific SCAR (sequence characterised amplified region) primers (Nicholson *et al.*, 1996, 1998). Extensive screening must then be done to ensure the specificity of the assay.

Both immunological and nucleic acid techniques offer considerable advantages over traditional diagnostic methods. However, the choice between these newer methods will depend on several factors including the application involved, the skills of the staff, the costs, the facilities needed and how many samples are to be analysed. Sometimes, a combination of diagnostic techniques is the best approach, for example immunocapture can be used to improve the sensitivity or specificity of PCR assays and overcome problems with inhibitors in the sample. Culturing for a short time can be combined with PCR-detection (BIO-PCR) to increase the quantity of

pathogen present, and to ensure that only viable microorganisms are detected (Schaad *et al.*, 1999).

In order to suppress the fungal diseases in the plants, natural and eco-friendly techniques can be utilized since use of excess chemicals and fertilizers as well as different fungicides can cause damage to the yield of the silk. Use of consortia of helpful bacteria and fungus can improve the health status as well as induce systemic resistance in these plants. Several instances has been reported where different bioinoculants have induced systemic resistance in several crop plants (Chakraborty *et. al*., 2006, De Meyer *et al.*1998; Yedidia *et al*. 1999; Meena *et al*. 2000; Oostendorp *et al*. 2001; Bargabus *et al*. 2004; Bharati *et al*. 2004). Unni *et. al* (2008) have isolated some PGPR from the rhizosphere of Som plants and have evaluated for their plant growth promoting traits. It has already been established that the growth, development and economic characters of silkworms are influenced to a great extent by nutritional content of their food plants (Neog *et al*, 2011). Acharya *et al*., 2013 have also studied improvement of health status of Soalu plants (*Litsea monopetala*) using five different plant growth promoting rhizabacteria.

Hence the present work will deal with early and easy detection of foliar fungal pathogens of Som plants using serological and molecular methods as well as activation of defense responses in the infected plants using bioinoculants that can lead to a eco-friendly and sustainable sericulture cultivation.

Objectives

- Detailed studies of the growth, morphology, sporulation and other characters of different isolates of *Pestalotiopsis disseminata* and *Colletotrichum gloeosporioides* causing grey blight and leaf blight disease of Som plants.
- Biochemical analysis (protein, phenols, and chlorophyll content) of healthy and naturally infected Som plants.
- Preparation and partial purification of antigens from healthy and infected leaves as well as from *P.disseminata* and *C.gloeosporioides*.
- Raising of polyclonal antibody (PAb) against virulent isolates of *P. disseminate* and *C. gloeosporioides,* their immunological detection and identification using immunoassays.

- Molecular identification of the pathogens using species specific primers and ITS-PCR.
- *In vitro* testing of selected biocontrol agents (PGPF, PGPR and AMF) for suppression of fungal pathogens.
- Determination of biochemical changes in Som plants following treatment with bioinoculants and/or inoculated with foliar fungal pathogens with special reference to phenolics, proteins and defense enzymes [Chitinase (CHT), β-1,3 Glucanase (GLU), Phenylalanine ammonia lyase (PAL) and Peroxidase (POX)].