Serological and molecular detection of foliar fungal pathogens of *Persea bombycina* Kost and activation of defense response using bioinoculants

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DECLARATION

I declare that the thesis entitled "**Serological and molecular detection of foliar fungal pathogens of *Persea bombycina* Kost and activation of defense response using bioinoculants"** has been prepared by me under the joint supervision of Dr. Shilpi Ghosh, Department of Biotechnology and Professor B.N. Chakraborty, Immuno Phytopathology Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

It is a well known fact that conventional methods for identifying fungal plant pathogens rely on the interpretation of visual symptoms and/or the isolation, culturing and laboratory identification of the pathogen. The accuracy and reliability of these methods depend largely on the experience and skill of the person making the diagnosis. Diagnosis requiring culturing can be time consuming and can be impractical when rapid results are required. Hence newer methods that are increasingly being applied to the diagnosis of plant pathogens, include immunological methods, DNA/RNA probe technology and polymerase chain reaction (PCR) amplification of nucleic acid sequences.

The present study deals with the serological and molecular detection of foliar fungal pathogens (*Colletotrichum gloeosporioides* and *Pestalotiopsis disseminata*) of muga host plant *Persea bombycina* Kost, most commonly known as som plant causing leaf blight and grey blight diseases respectively. Muga sericulture is an integral part of North-Eastern India and is a major economic threshold. One of the main reason for decrease in muga culture is due to the various foliar diseases that affects the quality and quantity of the leaves and in turn affects the silk production. It is hence essential to detect these foliar diseases at an early stage and also to minimise these disease using eco-friendly technologies by application of different bioinoculants.

At the onset, two major foliar fungal diseases of som plant, leaf blight and grey blight was recorded, their causal organism isolated and their morphological characteristics studied to identify and understand them. Screening of resistance of eight different morphotypes against these pathogens was carried out. Polyclonal antibodies against these pathogens were raised separately in male white rabbits and immunological assays were optimized for easy and early detection of these pathogens in som leaf tissues. Cross reactive antigens (CRA) shared between som plant and fungal pathogens were demonstrated following indirect immunofluorescence and immunogold labelling. Detection of pathogen in infected leaf was carried out using Plate trapped antigen coated Enzyme Linked Immunosorbent Assay (PTA-ELISA)

and Dot-immunobinding assay. Cellular localization of pathogen in these infected tissues was also studied using indirect immunofluorescence technology as well as immunogold labelling. Early detection of infection in artificially inoculated leaves was studied using PTA-ELISA and Dot-blot technique. It was noted that using these immunotechniques the fungal diseases could be detected as early as 24hrs after inoculation whereas the disease symptoms appeared only after 90-120hrs after inoculation.

Molecular detection of the two major fungal pathogens, *C. gloeosporioides* (SOM/Cl/02) and *P. disseminata* (IPL/SOM/P/01) was carried out using 18S rDNA sequencing of their conserved region using ITS1/ITS4 primer pair. The BLAST query of the 18S rDNA sequence of the isolates against GenBank database confirmed the identity of the isolate SOM/Cl/02 as *Colletotrichum gloeosporioides* and IPL/SOM/P/01 as *Pestalotiopsis* sp. The sequences have been deposited in NCBI GenBank database, under the accession no KM491736 for *C. gloeosporioides* and KT697994 for *Pestalotiopsis* sp. Species specific identification of other isolates was done using specific primer pair for *C.gloeosporioides* –CgINT/ITS4. Diversity analysis among the different fungal isolates was carried out using RAPD and DGGE techniques.

In vitro antagonistic effect of two selective Plant growth promoting rhizobacteria (PGPR) and two selective Plant growth promoting fungus gave positive result against both the pathogens. These PGPR (*Bacillus pumilus* and *B. altitudinus*) and PGPF (*Trichoderma harzianum* and *T. asperellum*) were mass multiplied and applied to the som plants to evaluate their effects on growth promotion and biochemical changes. Dominant arbuscular mycorrhizal fungi (AMF) isolated from the rhizosphere of som plants were mass multiplied and used for application as bioinoculant for root colonization in som plants. Besides, these bioinoculants were also used as value addition with vermicompost.

Growth enhancement was evaluated in terms of height, no. of leaves and no. of branches. Results revealed that growth promotion occurred in all morphotypes of som plants following application of bioinoculants, singly or jointly and in different combinations. Activities of defense enzymes (peroxidase, phenylalanine ammonia

lyase, chitinase and β-1,3glucanase) following treatment were analysed. HPLC profile of phenolic acids were also determined. Enhanced increase in activities of these defense enzymes were also noted in leaf as well as root of two morphotypes of som plants (S5 and S6) that were grown in field condition after joint application of bioinoculants. Disease incidence was found to be decreased in treated plants in comparison with untreated control plants. Immunological tests like indirect immunofluorescence confirmed the induction of defense enzymes in both root and leaves after application of bioinoculants.

Immunogold localization of defense enzymes (glucanase and chitinase) in som leaves and roots following colonization with AMF and treated with bioinoculant was studied using transmission electron microscopy. Deposition of gold particles was observed near the cell wall of treated roots and leaves. Induction of resistance in som plants against foliar fungal pathogens was confirmed using bioinoculants.

PREFACE

At the very outset, I express my profound sense of gratitude and indebtedness to my supervisor Dr. Shilpi Ghosh, Department of Biotechnology and Professor B.N. Chakraborty, Immuno-Phytopathology Laboratory, Department Of Botany, University of North Bengal for their constant guidance throughout my research work. Without their encouragement and tireless mentorship, I would not have completed my thesis. Professor Usha Chakraborty of the Department of Botany needs special mention for her supportive attitude whenever I was in need of her help. I would like to thank her for supporting and guiding me.

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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)].

Chapter 2
LITERATURE REVIEW

Disease diagnosis and pathogen identification by conventional methods, which involve isolating the pathogen and characterizing it by inoculation tests, are labour-intensive and time-consuming. Over the past few decades, immunological and molecular diagnostic methods have increasingly received attention as an alternative or complement to conventional methods (Schaad *et al.,* 2003). Serological methods (enzyme linked immunosorbent assay) are routinely used in several laboratories for these purposes because they allow sensitive and simultaneous analysis of many samples in a single micro plate. One major drawback of serological assays, however, is false positives caused by cross-reaction of antibodies with plant debris or unrelated organisms (De Haan *et al.,* 2000). Molecular methods based on polymerase chain reaction (PCR) are being used more frequently for detecting fungal pathogens in plant tissues (Bonants *et al.*, 1997; Zhang *et al.*, 1999; Kong *et al.*, 2003; Shen *et al.*, 2005; Wang *et al.*, 2006), owing to increased specificity and sensitivity compared with more traditional techniques. Also, large numbers of samples can be processed in a short time by these methods.

Disease diagnosis is an art as well as a science. We use the scientific method to perform and interpret tests for the detection of pathogens. The art lies in synthesizing information on symptom development, case history, and results of laboratory tests to determine the most likely causes of disease. Understanding the difference between the terms ‘diagnosis’ and ‘detection’, which are often mistakenly used interchangeably, is crucial. Detection of a pathogen does not necessarily prove that it caused the disease at hand. For example, one can usually culture, or induce by incubation, several pathogens from a plant that are not actively causing the observed symptoms. Considering complex etiology of plant disease it can be stated that the ‘phenomenon of “one cause-one disease” is rare in nature’. The diagnostician must identify the various causal factors and determine their relative importance. This task can be difficult, as different factors may cause similar symptoms and different symptoms may be caused by the same factor. Just as a positive test result does not prove that the pathogen detected caused the disease, a negative result does not definitely rule out the presence of a particular pathogen. The test itself may fail, or improper selection of

plant tissues can lead to erroneous conclusions. Attempts to use culture techniques to recover a vascular pathogen, such as *Fusarium oxysporum* f.sp.*lycopersici*, from wilted tomato foliage rather than vascular tissues would give erroneous negative results. False negative results can occur with any assay, and the diagnostician must be aware of the likelihood of their occurrence.

Obviously, the choice of diagnostic test(s) can greatly influence the diagnosis. Many factors influence the interpretation of test results and the value of the final diagnosis. The quality and case-history of the sample provided, available resources, and expertise of the diagnostician all play a role in the accuracy of the diagnosis. Sample quality is of paramount importance and is probably the most common limiting factor to accurate diagnosis. The best diagnosis would be one for which the affected plant(s) could be observed both in the field and in the laboratory. Unfortunately, expense, time and logistics usually preclude field visits by the diagnostician. Case history information provided with a specimen can be more important to the diagnosis than the specimen itself. This is particularly true when biotic agents play a minor role in the disease. Indeed, many plant specimens submitted to diagnostics labs are afflicted with abiotic problems, such as nutritional imbalances, chemical injury, temperature or moisture extremes, or air pollution. Most abiotic problems must be diagnosed from case-history information and symptom directions in the literature. Inadequate or incorrect information often leads to an inaccurate diagnosis.

The competent diagnostician must be able to synthesize information about many different aspects of plant health and know the plant in health as well as disease. The diagnostician has historically been a generalist rather than a specialist, well versed not only in pathology, but also in agronomy, horticulture, entomology, soil science and weed science. To understand and interpret modern diagnostic techniques, he or she must also have working knowledge of immunology and molecular biology. In addition to familiarity with pathology, the diagnostician must develop expertise in production practices, response of plants to environmental factors, and characteristics of specific cultivars or hybrids of many different crops. To this extent the individual must become a crop specialist. The difficulties inherent in becoming both a specialist and a generalist in a field that deals with hundreds of plant species and exponentially more pathogens or abiotic agents are obvious. Pathogens most readily detected or identified by various techniques are as follows:

Table 1: Serological and molecular techniques for detection of plant pathogens

Serological and Molecular techniques	Plant Pathogens
Multiwell ELISA	Fungi, Bacteria, Viruses
Flow-through	Fungi, bacteria
Dipstick	Fungi
Dot-Blot	Fungi, bacteria, Viruses
Tissue print	Fungi, viruses
Immunofluorescence	Fungi, bacteria, virus inclusions
Serologically specific electron microscopy (SSEM)	Viruses
Agglutination	Viruses, bacteria
Nucleic acid hybridization	Fungi, bacteria, viruses, viroids
Dot blot/Squash blot	Fungi, bacteria, viruses, viroids
Polymerase chain reaction (PCR)	Fungi, bacteria, viruses, viroids
Tissue print hybridisation	Viruses
Fatty acid analysis	Bacteria
dsRNA analysis	Viruses
Polyacrylamide gel electrophoresis (PAGE)	Viroids and viruses
Nutritional test kits	Bacteria
Light microscopy	Fungi, bacteria, nematodes, virus inclusions
Culture	Fungi, bacteria
Baiting	Fungi
Host inoculation	Viruses, viroids, fungi, bacteria, nematodes
Leaf dips	Viruses
Extraction/Identification	Nematodes

2.1. Immunodetection of phytopathogens

Recent trends in detection of plant pathogens include the development of more rapid diagnostic techniques with high specificity for the target organism (Chakraborty, 1988). These techniques can be used to detect fungi, bacteria and viruses present in low amounts in 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. Some of these rapid sensitive techniques are enzyme linked immunosorbent assay (ELISA), immunofluorescence (IF) and the polymerase chain reaction (PCR). Commercial developments of such techniques are expensive.

Although most plant pathogenic fungi can be detected by microscopy and other conventional means, but serological techniques have extra advantage where:

(1) The fungus in question is not readily is not identified by morphological characteristics

(2) Species identification is important and difficult by conventional means
(3) Detection of root pathogens prior to development of foliar symptoms is necessary
(4) Large number of samples must be processed for a particular disease for which conventional methods are time consuming
(5) Rapid, on-site detection is necessary for making disease management decisions for high-value crops
(6) Regulations governing the use of pesticides require demonstration of presence of a particular pathogen
(7) The fungus causes disease are low, difficult-to-detect populations in plant tissue; or
(8) Plant material is subject to quarantine regulation.

Before the development of serological techniques, laborious and time-consuming assays such as transmission to indicator host were used for the routine detection of viruses in many diagnostics labs. Early serological techniques were less sensitive and more time-consuming than those more widely used today. Conventional techniques for viral detection have largely been replaced by the more rapid and sensitive modern techniques, however, some of the conventional techniques still have a place in the diagnostic lab.

The diagnosis of bacterial diseases has been aided by many contributions from both basic and applied research, directed to the rapid detection and identification of plant pathogenic bacteria. Many selective and semi-selective media have been developed. Serology continues to become more useful as sensitivity increases and assay time decreases. Several test formats similar to those developed for fungi and viruses have been developed for bacteria, including multiwell ELISA, dot blot and immunofluorescence. Fatty acid analysis has also proven to be a reliable method of identification, and new techniques, such as nucleic acid probes and PCR, have extended the limits of specificity.

Many techniques that differ in sensitivity, specificity, reliability and cost are available for the detection of plant pathogens. The most desirable methods for diagnosticians are those that give the least number of false negative or false positive over time and among workers, are relatively rapid, cost-effective and detect the

broadest range of pathogens. Sensitivity is not a priority in the diagnostic lab as it is in the regulatory labs or indexing programmes, although a technique must be sensitive enough to detect a pathogen in symptomatic tissue. A diagnostician depends heavily on reliable, accurate, standardized assays. Rapidity is important, although techniques that may take an entire day to complete but do not require diagnosticians full attention are preferably acceptable. Cost is always a consideration, and diagnostic labs will invest in technology that is applicable to the greatest number of pathogens. Analysis of the interpretation of the techniques currently available for pathogen detection will serve a s a guide to those who must use the art as well as the science for disease diagnosis. The diagnostician should not lose sight of the fact that the main goal is to interpret results. With difficult cases, the diagnostician must integrate many lines of 'hard' and 'soft' empirical evidence in order to make a reliable diagnosis.

Immunological methods are being used increasingly in agricultural research and ELISA is now routine practice for plant virus detection and diagnosis. Application to the study of bacteria and fungi came much later presumably because they are generally much more complex in their makeup. Routine testing of large number of samples will only be possible when specific, sensitive, easy and reproducible methods of diagnosis are available. Various modifications and amplifications, as used in ELISA for viruses and bacteria can also be used to increase the specificity and sensitivity for fungi. Background reactions can be reduced or eliminated by the addition of different blocking agents which saturate non-specific binding areas. These include for fungi skimmed milk, gelatine, bovine serum albumin, albumin from chicken protein and serum of non-immunised animals. Modifications and amplifications can only improve the results of ELISA when sufficient specific antibodies are available for the particular application. Therefore of all the variables in ELISA procedure, antiserum quality is undoubtedly the most important (Chakraborty and Chakraborty, 2003).

Most of the currently available serological tests for fungi rely on direct or indirect ELISA with either polyclonal or monoclonal antibodies. ELISAs for fungi have been developed in a variety of formats, some of which are more appropriate for diagnostic labs than others. Available formats include: multiwell, flow-through, dipstick, dot blot and tissue print assays. In multiwall ELISA, the wells of commercial kits are precoated with antibodies specific to the target organisms. Addition of tissue homogenate to the wells allows binding of antigens to the antibodies, which can be

detected by antibody-enzyme conjugate. Multiwall ELISA has been used successfully to detect a wide range of plant pathogenesis fungi (Table-1). Sensitivity of multiwall ELISA is high. The main advantage of multiwall ELISA over conventional techniques is rapidity. In flow-through ELISA diagnostics assay is performed on a device composed of absorbent plastic material with antibodies immobilized on the surface. The sample, added dropwise to the top surface, flows past immobilised antibodies that trap the antigen. Antigen is detected by enzyme conjugate. The flow through kits, which are rapid, easy to use, and self-explanatory are particularly suited for field diagnosis. The membrane on the dipstick, which is precoated with specific antibodies, is soaked in buffered crude plant extracts to allow antigen-antibody binding. After rinsng the dipsticks, bound antigens are detected with enzyme conjugate. The dot-blot format differs from DAS-ELISA in that the antigen or plant sap is spotted directly onto a nitrocellulose or polyvinylidene membrane that has not been pretreated with antibodies. For tissue-print ELISA a cross section of the plant tissue is pressed directly onto a membrane, thus eliminating a need for an extraction step. If the target pathogen is present, the antigen is transferred from the plant tissue to the membrane. Membrane-bound antigen is detected by antibody-enzyme conjugate. Tissue-print ELISA is simple to perform, requires minimal equipment and is faster than dot-blot. Prints of many different sections of plant tissue can be done rapidly for a high probability of detection. As with dot blot high background colour from healthy plant sap may occur, but modification of ELISA procedure can reduce background colour. Different immunological formats used for detection of plant pathogenic fungi have been listed in Table 2.

2.1.1. Foliar Pathogens

***Colletotrichum* sp.**

Infection of sugarcane stalks by *Colletotrichum falcutum* could be very rapidly detected by an indirect antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) technique using polyclonal antiserum raised against the pathogen, well before symptom expression. Among the different host tissues, root eyes, buds and internal white spots were determined by ELISA to show high pathogen colonization. Differential host pathogen interaction caused by different isolated of the pathogen could also be effectively and more rapidly determined by ELISA procedures. The same technique was also utilized to differentiate host pathogen as resistant,

moderately resistant, and susceptible based on the pathogen colonization in the cane stalks (Viswanathan *et al*., 1998, 1999).

***Pestalotiopsis* sp.**

Polyclonal antibody raised against mycelia and cell wall antigens of *Pestalotiopsis theae* and IgG were further purified by ammonium sulphate fractionation and chromatography on DEAE-Sephadex and the immunoglobin fractions were used for DAC-ELISA, DAS-ELISA and Competition ELISA tests (Chakraborty *et al*., 1995b, 1996b). Pathogen was detected in tea leaves as early as 12h after inoculation with *P.theae*. At antiserum dilution upto 1:125, the pathogen could be detected in inoculated leaf extracts up to antigen concentration of 2mg/L. The antiserum reacted with two other isolates of *P.theae* but not with the antigens from mycelia extracts of *Glomerella cingulata* and *Corticum invisum* or with tea leaves inoculated with these pathogens.

***Glomerella* sp.**

Serological cross reactivity between *Glomerella cingulata* and *Camellia sinensis* has been demonstrated by Chakraborty *et al.* (1996a). PAb raised against antigen preparation of mycelia and cell wall of *G. cingulata* were used for detection of pathogen in tea leaf tissues. Antisera raised against cell wall preparations gave better recognition than that against mycelia preparation as observed in DAC-ELISA and DAS-ELISA test (Chakraborty *et al*., 2002a)

***Exobasidium* sp.**

Early detection of *Exobasidium vexans* causal agent of blister blight of tea were achieved using immunoassays by Chakraborty *et al.* (1997) in order to develop field management strategies. PTA-ELISA formats and immunofluorescence techniques were developed for early detection of the pathogen in tea leaf tissues (Chakraborty *et al*., 2009)

***Pythium* sp.**

DAS-ELISA with a monoclonal antibody directed against *Pythium* sp. was developed to detect this pathogen in turfgrass. The monoclonal antibody was the product of the hybridoma cell line PA5, produced using *P. aphanidermatum* as

immunogen. The antibody bound *P. aphanidermatum*, *P. graminicola*, *P. myriotylum* and *P. ultimum* all of which were involved with *Pythium* blight (Miller *et al*., 1986). Isolation of *Pythium* sp. from different soils by conventional methods revealed *P.violae* to be most common, while *P.sulcatum* was isolated less frequently. Competition ELISA using polyclonal antibodies against *P.violae* and *P.sulcatum* confirmed the results of conventional techniques. With cavities developed on field grown carrots, ELISA confirmed the predominance of *P.violae*. In one sample, *P.sulcatum* was isolated from a small number of lesions but was not detected in ELISA. The competition ELISA did not indicate the presence of *Pythium* in a range of non-cavity spot lesions from which attempts at isolation were negative.

***Bipolaris* sp.**

Major cross reactive antigens shared between *Bipolariscarbonum* and tea were demonstrated by Chakraborty and Saha (1994) using indirect ELISA and its cellular location was also detected in epidermal and mesophyll tissues of tea varieties. Recently, serological and molecular detection of *Bipolaris sorokiniana* causing spot blotch disease of wheat has been demonstrated in a susceptible genotype (CWL-6726) using PTA-ELISA, Dot immunobinding assay, Western Blot analysis and immunofluorescence (Chakraborty *et al*., 2016a)

***Alternaria* sp.**

During investigation of serological characteristics of *Alternaria* spp. isolated from plant leaves, seeds and soil, which were pathogenic mainly to carrot and parsley, firstly a polyclonal antiserum was prepared against one isolate from Serbia, identified as *A. dauci*. This antiserum was specific to *Alternaria* genus, while there was no reaction with antigens from other phytopathogenic fungi genera (*Fusarium, Rhizoctonia* and *Agaricus*). Antigenic characteristics of *Alternaria* genus fungi were examined by Electro-Blot-Immunoassay serological method (EBIA, Western blot), i.e. their protein profiles were compared. Investigated *Alternaria* spp. isolates showed different protein band profiles in gel and on nitrocellulose paper, and the observed differences were in complete correlation with the results of the previous identification. All investigated isolates, both domestic and the standards, were similar to each other, and they could be correctly identified to the species level using EBIA. Besides grouping to the species level, antigenic characteristics indicated similarities and

differences among the isolates within the same and different species, showing their complex relationships which properly reflect their diversity in nature (Bulajic *et al.*, 2007).

2.1.2. Root pathogens

***Phythophthora* sp.**

Phytophthora fragariae was detected by ELISA in roots of strawberry cv. Teniva. Because of high sensitivity of ELISA, presence of fungal antigens was demonstrated before symptoms could be detected in microscopical observations. Antiserum raised against pooled mycelium suspensions from five isolates representing five physiological races of *P. fragariae,* used in an ELISA detected soluble antigens at protein concentrations as low as 2ng/ml. Fungal antigens could also be detected in extracts of strawberry plants infected with *P. fragariae*. Root extracts prepared from the alpine strawberry *Fragaria vesca* and *F. ananassa* cv. Cambridge Favourite infected with any of the five isolates studied, produced strong reactions in ELISA (Mohan, 1988). Development of an agri diagnostic *Phytophthora* multiwall ELISA kit for detection of *Phytophthora* in plant tissue, which also readily detected *Phytophthora* in soil where soyabeans were damaged by *P. megasperma* f.sp. *glycinea* was also reported by Schmitthenner, 1988. A polyclonal antiserum prepared in rabbit immunized with a mycelium extract of *Phytophthora infestans*, reacted in ELISA with mycelium extracts of two *Phytophthora* species but not with those of 10 other microorganisms found on potato. Benson (1991) compared two serological assay kits to a culture plate method for detection of *Phythophthora cinnamomi* in root samples from inoculated azaleas. Both the multiwall E kit and rapid assay F kit detected *P. cinnamomi* in azalea roots beginning one week after inoculation. Agreement between immunoassay kits and culture plate results for detection of *P. cinnamomi* was most consistent beginning one week after inoculation. Root symptoms, and not foliar symptoms, of *Phytophthora* root rot was evident at this time. Although colour reactions in the rapid assay detectors became increasingly darker after completion of the test, results after 5 min were as reliable as those after 60 min, since reading for uninoculated controls used for determining test thresholds also increased with time. The multiwall kit detected *P. cinnamomi* in root samples containing as little as 1% infected root tissues. In a commercial nursery survey, 5 and 15% of the azalea root samples at two nurseries had positive ELISA values that were

unconfirmed by culture plate. The rapid assay kit detected *P. cinnamomi*, was easy to use and gave results in a short time. Pscheidt *et al.* (1992) tested the sensitivity of *Phytophthora* specific immunoassay on 17 species of *Phythophthora* collected throughout the world, including 8 isolate each of *P. cinnamomi* and *P. cactorum*. Kits were also used in the diagnosis of plant specimens with symptoms characteristics of *Phytophthora* infection. *Phythophthora* isolates tested produced a positive reaction with the immunoassay kit. The lowest absorbance relative to other species was obtained from *P. cinnamomi* and *P. megasperma*. Variation in absorbance was high among isolates of *P.cinnamomi* but low among *P.cactorum*. Clinic samples with typical symptoms of *Phythopthora* infection produced a positive reaction with the immunoassay, as did pure cultures of *Phythophthora* sp. isolated from these samples. Cross reactions occurred with several *Phythium* sp. isolated from clinic samples and with several specimens infected with *Peronospora* sp. Cross reactivity with some of the *Pythium* sp. made interpretation difficult, but when kit results were combined with field histories and symptomatology, the immunoassays proved to be a useful tool in clinical diagnosis. A dipstick immunoassay that was specific for *Phythophthora cinnamomi* was developed for use in soils. Azo dye detection of monoclonal antibody-labelled cysts attached to a nylon membrane provided a rapid, sensitive assay suitable for field use. There was no crass reaction with other *Phythophthora* and *Pythium* species in controlled enzyme assays or with soil or with organic matter that adhered to the membrane. The assay was used successfully to detect *P. cinnamomi* in a wide range of soil samples collected from beneath a diverse range of host species. According to the authors the dipstick assay offers several advantages compared with traditional procedures- i.e., familiarity with *Phythophthora* taxonomy is not required, the assay can be performed by unskilled personnel; and soil, rather than infected plant tissues can be assayed. Field testing of the assay showed that in kit form, it could be used as a reliable diagnostic tool to replace or augment current isolation and detection methods. ELISA can also be used for the quantitative determination of fungal biomass in infected tissues.

***Fusarium* sp.**

Competitive types of two novel ELISAs for *Fusarium* species were developed by Kitagawa *et al.* (1989). Antiserum against a strain (F504) of *F.oxysporum* was elicited in rabbits, and a highly specific, sensitive, and accurate ELISA for the

homologous strains was developed by using the antiserum with β-D-galactosidase-labelled anti-rabbit IgG as the secondary antibody and cell fragments of the strain attached to Amino-Dylark balls as the solid-phase antigen. All other microorganisms tested, including nine other strains of *Fusarium*, showed little cross reactivity. When cell fragments of *F.oxysporum* F501 attached to the balls were usedas a solid phase antigen in a heterologous competitive ELISA, the modified assay was a general assay for 10 strains of four *Fusarium* species with a high sensitivity. The specificity of the heterologous competitive ELISA was shown to be limited to *Fusarium* species. Detection of fusarial toxins using ELISA has also been reported by some authors. Phelps *et al.* (1990) developed an ELISA for detection of isomarticin, a naphthazarin toxin produced by *Fusarium solani*. A carbodimide procedure was used to couple the haptenisomartic in to BSA for the immunogen and to alkaline phosphatise for the enzyme-linked tracer. The resulting assay had a detection limit of 2ng/ml for isomerticin; other napthazarin toxins were detectable at less than 10 ng per well in ELISA plates. The assay was specific for naphthazarins. The cross reactivity for a number of phenolic compounds, including the closely related naphthoquinones, was three orders of magnitude less sensitive. Naphthazarin toxins of *Fusarium solani* were also detected by competitive ELISA analysis in Xylem fluid of roots rotted by *F. solani* and symptom less scaffold roots and branches of healthy appearing and diseased citrus trees (Nemec and Charest, 1991). In healthy appearing roots of trees with blight symptoms, 11.4 times more toxins were detected than roots of healthy trees; rotted roots contained significantly higher toxins than other roots. Using indirect ELISA detection of *Fusarium oxysporum* in soybean root tissue was demonstrated by Chakraborty *et al.* (1997)

***Macrophomina* sp.**

Serological relationship between *Macrophomina phaseolina* and soybean root was demonstrated by Chakraborty and Purkayastha (1983). Major cross reactive antigens were located in soybean root tissue mainly in the susceptible variety. Quick and accurate detection of *Macrophomina phaseolina* causing root rot disease of *Citrus reticulata* from soil was carried out using polyclonal antibodies raised against fungal mycelia of *M. phaseolina* using immunological formats such as immunodiffusion, PTA-ELISA, dot immunobinding assay, Western blot analysis and indirect immunofluorescence (Chakraborty *et al*., 2012)

***Sclerotinia* sp**.

Jamaux and Spire (1994) developed a serological test that allows the early detection of infection of young petals of *Sclerotinia sclerotiorum*, an important pathogen of rapeseed. Soluble mycelia extracts of S. *Sclerotiotum* were used to produce the first generation antiserum. This was not specific for *S. sclerotiorum* in DAS-ELISA and allowed the screening of cross reacting fungal species such as *Botrytis cinera*, a pathogen commonly present on rapeseed petals. Use of a polyclonal anti-*B. cinera* serum enabled the absorptions by serial cycles, of *S. sclerotiorum* antigens common to *B.cinera*. residual antigens were used as immunogens for the production of second generation antisera (S1 and S2) which were then tested by DAS-ELISA. Cross reaction with *B.cinera* decreased with purification cycles of immunogen whereas cross-reactions with some unrelated fungi slightly increased.

***Fomes* sp**.

Polyinst mycelia clonal antibody (PAb) against mycelial antigen of *Fomes lamaoensis* causal agent of brown root rot disease of tea was raised and analysed following DAC-ELISA formats. Such format was very useful in detecting the pathogen in infested soil which enables disease prevention at an early stage (Chakraborty *et al,* 2001)

ELISA has become established as a standard diagnostic procedure in laboratories throughout the world. The versatility of the technique, the sensitivity, speed, and precision with which results can be obtained, and the scale of operation that are possible are attributes of ELISA which, when combined, cannot be matched with any other sero-diagnostic method. Undoubtedly, the impact of ELISA has been greatest in the large-scale diagnosis of diseases of perennial and vegetatively propagated crops such as trees, bulbs and ornamentals and possibly of most significance, potatoes and foliar and root diseases of tea (*Camellia sinensis*). The potential of the ELISA technique for obtaining quantitative data has been exploited for various purposes, including studies on host-pathogen interactions, epidemiological investigations and characterization studies. With increasing interest shown in possible use of immunosorbent assays by plant health inspection and certification agencies the need to standardize reagents and operational procedures, as well as methods for analysis and presentation of results, becomes correspondingly greater. International

agreement is difficult to achieve in any field, and the formulation of certificate standards, based on immunosorbent assay procedures, is not likely to be easy. However, the relative ease and speed with which plant populations can be assayed for specific pathogens, at the same time enabling quantitative determination of the proportion of the infected individuals facilitates classification of the disease status of the population, if not the formulation of nationally and internationally acceptable standards. There are two principal technical aspects to be considered – (a) provision of calibrated biological standards (antiserum and antigen) against which test samples can be measured and classified, and (b) the establishment of accepted protocols and analytical procedures.

Immunofluorescence (IF) has also been used to identify various microorganisms (Table 3). Plant samples applied to microscopic slides as thin tissue sections or soil samples are fixed by a short period of drying or by treatment with chemicals. The optimal fixing method for each sample (antigen) must be determined empirically. To date, indirect immunofluorescence has been preferred for in situ analyses of phytopathogenic fungi. It has proved more suitable for detection in plant material or soil than direct immunofluorescence. IF is particularly suitable for detection and localisation of antigens in plant tissue and soil because the cells can be viewed directly. Thus in plants latently infected with phytopathogenic fungi, the pathogen could be detected by IF. Not only identification of the pathogen but quantitative assessments are possible by measuring and counting hyphae as well as resting spores (Wakeham and White,1996). The availability of sufficient specific antibodies is vital to the success of IF. Interpretation of IF is frequently made more difficult by other sources of fluorescence, such as cross reactions of other organisms with the antibody, autofluorescence of the plant material and fungicides in soil samples, and non-specific adsorption of marked antibodies to soil particles. The importance of such interference has to be individually determined for each system and requires repeated observations of suitable controls, non-coloured samples or possibly healthy tissue. Different techniques have been tested to eliminate interfering fluorescence in fungi.

Immunological techniques will become increasingly important for the detection, diagnosis and epidemiology of pathogens and non-pathogenic fungi. Of primary importance for immunological studies is selection and preparation of an appropriate antigen and the production of antibody. For most host-pathogen combination,

selection and specific modification of test method will be essential. Choice of a fungal antigen will be determined by the relevant structures or metabolic products to be detected for the pathogen under investigation. This requires appropriate knowledge of the biology and epidemiology of the pathogen. The antigen must be present in sufficient amount, stable and have the highest possible immunogenicity. Generally fungal antigenic determinants are concentrated in young hyphal tips and those metabolites in the category of surface components may produce specific antibodies of high affinity. For routine use with large sample numbers, time saving and easy to handle methods is essential. ELISA is a good example, since to a large extent the process may be automated. Even more convenient are methods such as dot blot and dip stick assays. Where it is necessary to process large number of samples, as in the plant protection services, immunological techniques will become increasingly important. Some methods which are at present have limited use will undoubtedly become more important in work with fungi.

2.1.3. Microtitre immune spore trapping method

Airborne spores of fungal plant pathogens have commonly been detected and enumerated by microscopic examination of surfaces on which spores have been impacted (Aylor, 1998; Hunter *et al.,* 1999). However, technological advances in fungal diagnostics in which either antibody or nucleic acid probes are used offer the potential for developing rapid systems for detecting and quantifying airborne spores of fungal plant pathogens. An immunoassay system developed by Spore View (Chaparral Diagnostics, Burlington, Vt.) utilizes passive deposition of ascospore of *Venturia inaequalis*, the causal agent of apple scab, on a membrane surface. Similarly, studies to develop an antibody based immunoassay for early detection of *Sclerotinia sclerotiorum* (Jamaux and Spire, 1994), a major fungal pathogen of oilseed rape (*Brassica napus*), have relied solely on passive deposition of ascospores on rapeseed petals and subsequent mycelial growth.

Table 2: Different immunological formats used for detection of plant pathogenic fungi

Crop	Pathogen	Serological formats
Tea	*Armillariamellea*	PTA indirect, Dot Blot
	Bipolariscarbonum	PTA indirect
	Corticumtheae	PTA indirect, Dot blot
	Exobasidiumvexans	PTA indirect, Dot blot
	Fomeslamaoensis	PTA indirect
	Glomerellacingulata	PTA indirect
	Pestalotiopsistheae	PTA indirect
	Poriahypobrumea	PTA indirect, Dot blot
	Roselliniaarcuata	PTA indirect, Dot blot
	Sphearostilberepens	PTA indirect
	Ustulinazonata	PTA indirect, Dot blot
Wheat	*Fusariumcalmorum*	PTA indirect,
	Gauemannomycesgraminis	DAC
	Pseudocercosporellaherpotrichoides	PTA indirect,
	Bipolarissorokiana	PTA Indirect, Dot blot
Rice	*Penicilliumislandicum*	PTA Indirect
Barley	*Erysiphegraminis*f.sp.*hordei*	PTA indirect
Potato	*Phomaexigua*	PTA indirect,
	Phytophthorainfestans	PTA indirect
Tomato	*Phytophthoranicotianae*	PTA indirect
Soybean	*Fusariumoxysporum*	PTA indirect
	Fusariumgraminearum	PTA indirect
	Phomopsislongicolla	PTA indirect,
	Phytophthoramegasperma	DAC
	Sclerotiumrolfsii	PTA indirect
Cotton	*Thielaviopsisbasicola*	PTA indirect
Sugarcane	*Colletotrichumfalcatum*	DAC
	Ustilagoscitaminea	DAC
Sunflower	*Sclerotiniasclerotiorum*	DAC
Strawberry	*Phytophthorafragariae*	PTA indirect
Grapes	*Botrytis cinerea*	PTA indirect
Turf grass	*Leptosphaeriakorrae*	PTA indirect
	Pythiumaphanidermatum	Competition
Pine, Birch	*Phialophoramutabilis*	PTA indirect
Elm	*Ophiostomaulmi*	PTA indirect
Anemones	*Colletotrichum sp.*	PTA indirect
Tall fescue	*Acremoniumcoenophialum*	Protein A Sandwich
Mandarin	*Macrophominaphaseolina*	PTA ELISA, Dot Blot
Soil	*Phytophthoraparasitica*	DAC

Table 3: *In situ* analysis of phytopathogenic fungi using immunofluorescence

Pathogen	Crop	Immunofluorescence assay
*Alternaria*sp.	Barley (grain)	Indirect
*Aspergillus*sp.	Barley (grain)	Indirect
Bipolariscarbonum	Tea (leaf)	Indirect
Corticumtheae	Tea (leaf)	Indirect
Eutypaarmeniacea	Grapevines (wood)	Direct, Indirect
Exobasidiumvexans	Tea (leaf)	Indirect
Fomeslamaoensis	Tea (root), soil	Indirect
Fusariumgraminearum	Soybean (root), soil	Indirect
Fusariumoxysporum	Soybean (root), soil	Indirect
Fusariumvasinfectum	Cotton (root), soil	Direct
Ganodermalucidum	Cashew (root)	Direct
Glomerellacingulata	Tea (leaf)	Indirect
Ophiostomaulmi	Elm (wood)	Indirect
Pestalotiopsistheae	Tea (leaf)	Indirect
*Penicillium*sp.	Barley (grain)	Indirect
Penicilliumcyclopium	Barley (grain), soil	Indirect
Phaeolusschweinitzii	Soil	Indirect
Phomaexigua	Potato (tuber)	Indirect
Phytophthoramegasperma	Soil	Indirect
Phythophthoracinnamomi	Soil	Direct, Indirect
Plasmodiophorabrassicae	Cabbage (root), soil	Indirect
Sclerotiumrolfsii	Soybean (root), soil	Indirect
Sphaerostilberepens	Tea (root), soil	Indirect
Ustulinazonata	Tea (root), soil	Indirect

A new microtiter immune spore trapping device (MTIST device) was designed by Horticulture Research International, Warwickshire, which uses a suction system to directly trap air spores by impaction in microtiter wells, and this device can be used for rapid detection and quantification of ascospores of *M. brassicicola* and conidia of *Botrytis cinerea* by an enzyme-linked immunosorbent assay (ELISA). The MTIST device can be used to rapidly differentiate, determine, and accurately quantify target organisms in a microflora. The MTIST device is a portable, robust, inexpensive system that can be used to perform multiple tests in a single sampling period, and it should be useful for monitoring airborne particulates and microorganisms in a range of environments. For ascospores of *M. Brassicicola* correlation coefficients ($r2$) of 0.943 and 0.9514 were observed for the number of MTIST device-impactedascospores per microtiter well and the absorbance values determined by

ELISA, respectively. These values were not affected when a mixed fungal spore population was used. There was a relationship between the number of MTIST device-trapped ascospores of *M. brassicicola* per liter of air sampled and the amount of disease expressed on exposed plants of *Brassica oleracea* (Brussels sprouts). Similarly, when the MTIST device was used to trap conidia of *B. cinerea*, a correlation coefficient of 0.8797 was obtained for the absorbance values generated by the ELISA and the observed number of conidia per microtiter well. The relative collection efficiency ofthe MTIST device in controlled plant growth chambers with limited airflow was 1.7 times greater than the relative collection efficiency of a Burkard 7-day volumetric spore trap for collection of *M. brassicicola* ascospores (Kennedy *et. al.,* 2000)

2.2. Molecular Detection of phytopathogens

The presence of nucleic acids (DNA and/RNA) is one of the important characteristics of all living organisms. These characteristics of nucleic acids and other organelles of organisms have been studied for detection, identification and differentiation of the microbial plant pathogens. Today in the 21st century scientists are becoming increasingly able to diagnose and manage diseases at the molecular level. Molecular methods offer an entirely new approach to the plant disease diagnosis, however many a times molecular methods may be an improvement over conventional microbiology testing in many ways. Over the last ten years much effort has been devoted to the development of methods for detecting and identifying plant pathogens based on DNA/RNA probe technology and PCR amplification of nucleic acid sequences. Perhaps the greatest advantage these techniques have over conventional diagnostic methods is the potential to be highly specific. They can distinguish between different fungal species, and within a single species (Ward, 1995 and Ward and Adams, 1998). DNA-based diagnostics are also used to determine particular genetic properties of the pathogen, for example, they can be used to determine whether the pathogen is resistant or sensitive to particular fungicides and to determine its virulence characteristics. The rapid development in the fields of molecular plant pathology has provided new insights into the genetic and structural features of a large number of pathogens. These results obtained through intensive basic research are further leading to improvement in diagnosing procedures. As more information becomes available on fungal genomics and gene function, the scope for

using molecular-based diagnostic will also increase. Automation and high-density oligonucleotide probe arrays (DNA chips) also hold great promise for characterizing microbial pathogens.

2.2.1. Hybridization-Based Nucleic Acid Techniques

The characteristic genetic constitutions of individual organisms are due to many generations of mutations and recombinations. It is generally accepted that closely related organisms share a greater nucleotide similarity than those that are distantly related. Techniques based on nucleic acid hybridization involve the identification of a highly specific nucleotide common to a given strain or isolate of the microbial plant pathogen species, but absent in other strains or isolates or species and this selected sequence of the organism is used to test for the presence of the target organism. Likewise, a highly conserved sequence present in all strains or species in a genus may be employed to probe for the presence of any member of that genus. The selection of a specific sequence as a probe is distinctly derived from the sequential data and screening of related organisms to determine its specificity.

Detection of microbial plant pathogens by nucleic acid hybridization techniques is based on the formation of double-stranded (ds) nucleic acid molecules by specific hybridization between the single-stranded (ss) target nucleic acid sequence (denatured DNA or RNA) and complementary single-stranded nucleic acid probe. Sequences of either RNA or DNA have been used as probes. If the probe strand in the duplex is labeled with a detectable marker like ^{32}P, information of the duplex can be assayed after removal of unhybridized sequences. Hybridization reaction may be performed in solution (solution hybridization), in situ (in situ hybridization) and on solid filter supports (filter hybridization). The filter and in situ hybridization methods have been more frequently employed for detection of microbial pathogens.

Detection of fungal plant pathogens by employing nucleic acid (NA)-based techniques provides certain distinct advantages over immune detection methods. The fungus-like and fungal pathogens are complex antigens, the nature of which may vary, depending on the stage in their life cycle. The antisera produced against one type of spores or mycelium formed at a particular stage may not actively react with spores or mycelium produced at all stages in the life cycle of the pathogen. However, the presence or absence of spore-bearing structures or the slow growing nature of some fungal pathogens will not affect their detection by NA-based techniques, since the

nature of the genomic elements remains constant, irrespective of the stages of life cycle of the pathogen to be detected. It is possible to detect, identify, differentiate and quantify the fungal pathogens concerned, using appropriate DNA probes, even in the case of pathogens that are not amenable for detection by other methods. For example, fungal pathogens causing nonspecific, generalized rotting and death of plants and obligate fungal pathogens that cannot be cultured may be rapidly detected and differentiated by employing suitable probes. (Narayanasamy, 2011)

2.2.2. Polymerase Chain Reaction (PCR)

The polymerase chain reaction **(**PCR) allows the amplification of millions of copies of specific DNA sequences by repeated cycles of denaturation, polymerisation and elongation at different temperatures using specific oligonucleotides (primers), deoxyribonucleotide triphosphates (dNTPs) and a thermostable *Taq* DNA polymerase in the adequate buffer (Mullis and Faloona, 1987). The amplified DNA fragments are visualized by electrophoresis in agarose gel stained with EtBr, SYBR Green or other safer molecule able to intercalate in the double stranded DNA, or alternatively by colorimetric (Mutasa *et al.,* 1996) or fluorometric assays (Fraaije *et al.,* 1999). The presence of a specific DNA band of the expected size indicates the presence of the target pathogen in the sample. Advances in PCR-based methods, such as real-time PCR, allow fast, accurate detection and quantification of plant pathogens in an automated reaction. Main advantages of PCR techniques include high sensitivity, specificity and reliability. Moreover, it is not necessary to isolate the pathogen from the infected material reducing the diagnosis time from weeks to hours, and allowing the detection and identification of non-culturable pathogens (Capote *et al*., 2012). This is particularly useful in studying systemic infections, or in the early detection of disease, before symptoms are visible. Compared to culturing, molecular methods are relatively fast; results are often possible within one or two days of sampling. They are potentially more reliable than identification of visual symptoms, as they do not rely on the skills needed to distinguish subtle differences in disease symptoms (McCartney *et al.,* 2003).

The products of PCR can be used for three different purposes: (i) as a target for hybridization, (ii) for direct sequencing of the DNA to determine strain variations and (iii) as a specific probe. Rasmussen and Reeves (1992) appear to be the first to apply PCR approach for the detection of a bacterial pathogen in diseased plants. PCR assay

may be preferred by researchers because of several advantages over conventional methods involving isolation and examination of cultural characteristics. The pathogen(s) need not be isolated in pure culture before detection in infected plant materials. It is enough, if the pathogen DNA is extracted. High levels of sensitivity and specificity, in addition to simplicity, have made the PCR-based assays as the technique of choice for routine and large scale application in quarantine and certification programs.

Although PCR is a highly sensitive technology, the presence of inhibitors in the plant tissues and soil, greatly reduces its sensitivity. The inhibitors are believed to interfere with the polymerase activity for amplification of the target DNA. Another problem with the conduct of PCR arises from the possible DNA contamination leading to false negative results. Hence, it is essential to adopt stringent conditions during all operations and to have proper negative controls. Further, it would be desirable to allot separate dedicated areas for pre- and post-PCR handling. The DNA-based detection methods have yet another limitation. In addition to determining the presence or absence of the pathogen in the plants or in the environment, the pathogen detection system has the principal goal of ascertaining the viability of pathogen propagules. In the event of positive result, it is necessary to know whether the pathogen detected poses a threat to crop production, public health or food safety. The lack of discriminating viable from dead cells is a pitfall commonly recognized, while applying nucleic acid-based systems, including PCR and microarrays (Keer and Birceh 2003; Call 2005). Development of the method involving enrichment culturing (BIO-PCR) prior to PCR, addresses this problem to some extent (Schaad *et al.* 2003). Designing suitable primers is a critical step in PCR assay. Generally, short sequences (100–1,000 bp) are more efficiently amplified and resolved by agarose electrophoresis. Specific primers are derived from sequences of either amplified or cloned DNA (cDNA) or RNA from target pathogen species or strains or isolates. Primer specificity for target sequences is affected by many factors which include primer length, annealing temperature, secondary structure of target and primer sequences. Ribosomal genes and the spacers between them provide targets of choice for molecular detection and phylogenetic investigations, since they are present in high copy numbers, contain conserved as well as variable sequences and can be amplified and sequenced with universal primers based on their conserved sequences (Bary *et al.* 1991; Bruns *et al.* 1991; Stackebrandt *et al.* 1992; Ward and Gray 1992).

A PCR assay, based on the amplification of 5.8S rDNA gene and ITS4 and ITS5 primers, was employed for the rapid detection and identification of economically important *Phytophthora* spp. belonging to six taxonomic groups. The pathogens detected include *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. erythroseptica*, *P. fragariae*, *P. infestans*, *P. megasperma*, *P. mirabilis* and *P. palmivora* (Liew *et al.* 1998).

Sometimes, PCR using a single pair of primers does not always give a sufficiently specific or sensitive test. This can be overcome by using DNA probes in conjunction with PCR (Mutasa *et al*., 1995) A second approach is to use nested primers. Here, after an initial PCR, the product is subjected to a second round of PCR using primers which recognise a region within that amplified by the first pair (Foster *et al.,* 2002, Schesser *et al.,* 1991). However, this procedure is more labour-intensive, more costly and more prone to contamination than the single primer pair method. Another approach to overcoming sensitivity and specificity problems is by using antibodies in conjunction with PCR, ie. immuno-PCR or immunocapture PCR. However, although this approach has been used to detect a few plant pathogenic viruses (Jacobi *et al.,*1998, Shamloul and Hadidi., 1999) and phytoplasmas (Pollini *et al.,* 1997), we are not aware of any examples of its use to detect fungi. Where there is a need to detect several different pathogens simultaneously, multiplex PCR, involving several pairs of primers in the same PCR reaction, can be used (Fraaije *et al*., 2001). This can save time and reduce costs, but care is needed to optimise the conditions so that all of the different amplicons can be generated efficiently (McCartney *et al.,* 2003).

2.2.3. PCR-ELISA

This serological-based PCR method uses forward and reverse primers carrying at their 5' end biotin and an antigenic group (e.g. fluorescein), respectively (Landgraf *et al.,* 1991). PCR amplified DNA can be immobilized on avidin or streptavidin-coated microtiter plates via the biotin moiety of the forward primer and then can be quantified by an ELISA specific for the antigenic group of the reverse primer (e.g. anti-fluorescein antibody detected by colorimetric reactions). PCR-ELISA method is as sensitive as nested PCR. In addition, it does not require electrophoretic separation and/or hybridisation, and can be easily automated. All reactions can be performed in 96-well microtiter plates for mass screening of PCR products making them very

suitable for routine diagnostic purposes. This procedure has been used for detection and differentiation of *Didymella bryoniae* from related *Phoma* species in cucurbits (Somai *et al.*, 2002) and for detection of several species of *Phytophtora* and *Pythium* (Bailey *et al.*, 2002).

2.2.4. PCR-DGGE

This method is mainly applied for the analysis of the genetic diversity of microbial communities without the need of any prior knowledge of the species (Portillo *et al.*, 2011). DGGE (Denaturing Gradient Gel Electrophoresis) and its variant TGGE (Temperature Gradient Gel Electrophoresis) use chemical gradient such as urea (DGGE) or temperature (TGGE) to denature and separate DNA samples when they are moving across an acrylamide gel. In PCR-DGGE target DNA from plant or environmental samples are firstly amplified by PCR and then subjected to denaturing electrophoresis. Sequence variants of particular fragments migrate at different positions in the denaturing gradient gel, allowing a very sensitive detection of polymorphisms in DNA sequences. In addition, PCR-DGGE primers contain a GC rich tail in their 5' end to improve the detection of small variations (Myers *et al.*, 1985). The bands obtained in the gel can be extracted, cloned or reamplified and sequenced for identification, being even possible to identify constituents that represent only 1% of the total microbial community. These techniques are very suitable for the identification of novel or unknown organisms and the most abundant species can be readily detected.

This method is however time-consuming, poorly reproducible and provides relative information about the abundance of detected species. Interpretation of the results may be difficult since the micro heterogeneity present in some target genes may appear as multiple bands in the gel for a single species, leading to an overestimation of the community diversity. Furthermore, fragments with different sequences but similar melting behaviour are not always correctly separated. In other cases, the analysis of complex communities of microorganisms may result in blurred gels due to the large number of bands obtained.

A PCR-DGGE detection tool based in the amplification of the ITS region has been recently applied to detect multiple species of *Phytophthora* from plant material and environmental samples (Rytkönen *et al.*, 2012). Other authors have used this technique to compare the structure of fungal communities growing in different

conditions or environments, e.g. to study the impact of culture management such as biofumigation, chemifumigation or fertilisation on the relative abundance of soil fungal species (Omirou *et al.*, 2011; Wakelin *et al.*, 2008).

2.2.5. Fingerprinting

Fingerprinting approaches allow the screening of random regions of the fungal genome for identifying species-specific sequences when conserved genes have not enough variation to successfully identify species (McCartney *et al.*, 2003). Fingerprinting analyses are generally used to study the phylogenetic structure of fungal populations. However, these techniques have been also useful for identifying specific sequences used for the detection of fungi at very low taxonomic level, and even for differentiate strains of the same species with different host range, virulence, compatibility group or mating type.

2.2.5.1Restriction fragment length polymorphism (RFLP)

RFLP involves restriction enzyme digestion of the pathogen DNA, followed by separation of the fragments by electrophoresis in agarose or polyacrilamide gels to detect differences in the size of DNA fragments. Polymorphisms in the restriction enzyme cleavage sites are used to distinguish fungal species. Although DNA restriction profile can be directly observed by staining the gels, Southern blot analysis is usually necessary. DNA must be transferred to adequate membranes and hybridised with an appropriate probe. However, the Southern blot technique is laborious, and requires large amounts of undegraded DNA. RFLPs have been largely used for the study of the diversity of mycorrhizal and soil fungal communities (Thies, 2007; Kim *et al.*, 2010; Martínez-García *et al.*, 2011). Although used for differentiation of pathogenic fungi (Hyakumachi *et al.*, 2005) this early technique has been progressively supplanted by other fingerprint techniques based in PCR.

PCR-RFLP combines the amplification of a target region with the further digestion of the PCR products obtained. PCR primers specific to the genus *Phytophthora* were used to amplify and further digest the resulting amplicons yielding a specific restriction pattern of 27 different *Phytophthora* species (Drenth *et al.*, 2006). PCR-RFLP analysis of the ITS region demonstrated the presence of different anastomosis group (AG) within isolates of *Rhizoctonia solani* (Pannecoucque and Höfte, 2009); It also allowed the differentiation of pathogenic and non pathogenic

strains of *Pythium myriotolum* (Gómez-Alpizar *et al.*, 2011). In other cases, the analysis of the ITS region by this technique failed in differentiating closely related species (e.g., clade 1c species such as *Phytophthora infestans* and *P. mirabilis*) (Grünwald *et al.*, 2011).

2.2.5.2 Random amplified polymorphic DNA (RAPD)

RAPD (random amplified polymorphic DNA), AP-PCR (arbitrarily primed PCR) and DAF (DNA amplification fingerprinting) have been collectively termed multiple arbitrary amplicon profiling (MAAP; Caetano-Annolles, 1994). These three techniques were the first to amplify fragments from any species without prior sequences information. These three techniques produce markedly different amplification profiles, varying from quite simple (RAPD) to highly complex (DAF) patterns. The key innovation of RAPD, AP-PCR and DAF is the use of a single arbitrary oligonucleotide primer to amplify template DNA without prior knowledge of the target sequence. The amplification of nucleic acids with arbitrary primers is mainly driven by the interaction between primer, template annealing sites and enzymes, and determined by complex kinetic and thermodynamic processes (Caetano-Annolles, 1997).

The RAPD protocol usually uses a 10 bp arbitrary primer at constant low annealing temperature (generally 34 – 37^{o}C). RAPD primers can be purchased as sets or individually from different sources. Although the sequences of RAPD primers are arbitrarily chosen, two basic criteria indicated by Williams *et al.* (1990) must be met: a minimum of 40% GC content (50 - 80% GC content is generally used) and the absence of palindromic sequence (a base sequence that reads exactly the same from right to left as from left to right). The resulting PCR products are generally resolved on 1.5- 2.0% agarose gels and stained with ethidium bromide. Most RAPD fragments result from the amplification of one locus, and two kinds of polymorphism occur: the band may be present or absent, and the brightness (intensity) of the band may be different. Band intensity differences may result from copy number or relative sequence abundance (Devos and Gale, 1992) and may serve to distinguish homozygote dominant individuals from heterozygotes, as more bright bands are expected for the former.

Random amplified polymorphic DNA (RAPDs) analysis has attracted a lot of attention after its advent during the 90's .This marker system was developed by Welsh and McClelland (1990). Manulis *et al.*,(1994), applied RAPDs to the carnation wilt fungal pathogen *Fusarium oxysporum* f. sp. *Dianthi* and they were able to identify specific banding patterns that were subsequently used as probes to distinguish between races of the pathogen. In another study, genetic relationships could be inferred among the wheat bunt fungi using RAPD markers (Shi *et al*., 1995). *Colletotrichum gloeosporioides* isolates from mango and cashew plants were separated in different groups based on their RAPD band profile (Serra *et al,* 2011). Nithya *et al.* (2012) identified new isolate of red rot in the Tamil Nadu regions by RAPD analysis. The RAPD primer OPE-01 amplified a ~ 560 bp fragment to from most of the *C. falcatum* isolates tested, regardless of the geographic origin. He also developed a sequence characterised amplified region (SCAR) marker based on the RAPD data for accurate and sensitive detection of *C. falcatum* in infected sugarcane setts by using PCR. The *Pestalotiopsis* isolates causing grey blight disease in *Camellia sinensis* showed diverse morphological characters. Their diversity was also studied genetically using molecular markers RAPD and ISSR. The results indicated that, within southern India, the diversity of *Pestalotiopsis* was high both morphologically and genetically (Joshi *et al;* 2009).

2.2.5.3 Amplified fragment length polymorphism (AFLP)

AFLP analysis (Vos *et al.*, 1995) consists in the use of restriction enzymes to digest total genomic DNA followed by ligation of restriction half-site specific adaptors to all restriction fragments. Then, a selective amplification of these restriction fragments is performed with PCR primers that have in their 3' end the corresponding adaptor sequence and selective bases. The band pattern of the amplified fragments is visualized on denaturing polyacrylamide gels. The AFLP technology has the capability to amplify between 50 and 100 fragments at one time and to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As with other fingerprinting techniques, no prior sequence information is needed for amplification (Meudt and Clarke 2007). The disadvantages of AFLPs are that they require high molecular weight DNA, more technical expertise than RAPDs (ligations, restriction enzyme digestions, and

polyacrylamide gels), and that AFLP analyses suffer the same analytical limitations of RAPDs (McDonald 1997).

Depending on the primers used and on the reaction conditions, random amplification of fungal genomes produces genetic polymorphisms specific at the genus, species or strain levels(Liu *et al.,* 2009). As a result, AFLP has been used to differentiate fungal isolates at several taxonomic levels e.g. to distinguish *Cladosporium fulvurn* from *Pyrenopeziuz brassicae* species (Majer *et al.,* 1996),*Aspergillus carbonarius* from *A. ochraceus* (Schmidt *et al*., 2004), and *Colletotrichum gossypii*from *C. gossypii* var. *cephalosporioides* (Silvar *et al*., 2005); also to differentiate *Monilinia laxa* that infect apple trees from isolates infecting other host plants (Gril *et al*., 2008); and to separate non-pathogenic strains of *Fusarium oxysporum* from those of *F. commune* (Stewart *et al.,* 2006). AFLP markers have also been used to construct genetic linkage maps e.g. of *Phytophthora infestans* (VanderLee *et al*., 1997). Specific AFLP bands may also be used for SCAR markers development used in PCR-based diagnostic tests. Using SCAR markers Cipriani*et al.* (2009) could distinguish isolates of *Fusarium oxysporum* that specifically infect the weed *Orobanche ramose*. AFLP profiles have also been widely used for the phylogenetic analysis of *Fusarium oxysporum* complex (Baayen *et al*., 2000; Fourie *et al*., 2011; Groenewald *et al*., 2006).

2.2.6. Sequencing

Morphological characteristics are not always enough to identify a pathogen. One of the most direct approaches to do that consists in the PCR amplification of a target gene with universal primers, followed by sequencing and comparison with the available publicly databases. In addition, new fungal species have been described by using sequencing approaches. However, the use of sequence databases to identify organisms based on DNA similarity may have some pitfalls including erroneous and incomplete sequences, sequences associated with misidentified organisms, the inability to easily change or update data, and problems associated with defining species boundaries, all of them leading to erroneous interpretation of search results. An effort for generating and archiving high quality data by the researcher's community should be the remedy of this drawback (Kang *et al*., 2010). Other limitation of sequencing as diagnostic tool is the need to sequence more than one

locus for the robustness of the result, and the impractical of this method in cases when rapid results are needed such as for the control or eradication of serious plant disease outbreaks. Nevertheless, the increase of sequencing capacity and the decrease of costs have allowed the accumulation of a high numbers of fungal sequences in publicly accessible sequence databases, and sequences of selected genes have been widely used for the identification of specific pathogens and the development of sequence-based diagnostic methods.

2.3. Induction of defense responses in higher plants

Plants are compelled to withstand stresses of all kinds, be it biotic, abiotic or anthropogenic as a consequence of their immobility (Chakraborty, 1996). Higher plants protect themselves from various stresses, such as pathogen attacks, wounding, application of chemicals including phyto-hormones and heavy metals, air pollutants like ozone, ultraviolet rays, and harsh growing conditions. This reaction is known as the defense response of higher plants, and a series of proteins actively synthesized with this reaction is called defense-related proteins or PR-proteins (PRPs). Such constraints lead to production of a wide array of defense compounds, which are either induced or preformed (Chakraborty, 2012). PRPs have been grouped in to five main classes consisting of the 10 major acidic PRPs of tobacco characterized both by biochemical and molecular biological techniques and designated as PR-1 to -5. A unifying nomenclature was proposed based on their grouping into eleven families, classified for tobacco and tomato, sharing amino acid sequences, serological relationships and/ or enzymatic or biological activity (Neuhaus, 1999). The criteria for inclusion of new families of PRps were (a) protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and (b) induced expression must have shown to occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combination must have been confirmed independently in different laboratories. So far, 17 families of PRps have been recognized. However, the properties of all these proteins have not yet been elucidated (Chakraborty, 2013).

PRPs might be involved in recognition processes, releasing defense-activating signal molecules from the walls of invading pathogens. This would hold particularly for chitinases and glucanases that could liberate elicitor-type carbohydrate molecules from fungal and bacterial cell walls. Such elicitors could help stimulate defense

responses in adjacent cells and thus accelerate and enhance these reactions, as well as induce acquired resistance to further infection. A role of PRPs as specific internal signal generating enzymes would be consistent both with their occurrence in specific organs and with their induction during the development and in response to stressful pathogen infections.

The versatile multicomponent defense system of plants is adequate to provide them protection against most of their potential pathogens, (Chakraborty *et al.*, 2005 a, b c) only a few of them can overcome this defense and cause disease. Just before or concomitant with the appearance of a hypersensitive reaction (HR) the synthesis of PR-proteins is increased. In addition to the localized HR, many plants respond to pathogen infection by activating defences in uninfected parts of the plant (systemic acquired resistance, SAR). As a result, the entire plant is more resistant to secondary infection. SAR is long lasting and confers broad based resistance to a variety of pathogens. The synthesis of antimicrobial products, including phytoalexins and PR proteins, correlates well with the development of both HR and SAR.

2.3.1. Management of plant health

Soil microbes offer largely unexplored potential to increase agricultural yields and productivity in a low-input manner (Chakraborty, 2013a). Soil biota provides a number of key ecological services to natural and agricultural ecosystems. Increasingly, inoculation of soils with beneficial soil biota is being considered as a tool to enhance plant productivity and sustainability of agricultural ecosystems. In the development of sustainable crop production practices, the use of microbal inoculants as replacement for chemical fertilizers and pesticides is receiving attention (Chakraborty and Chakraborty, 2013).

Many of the microbes isolated and classified as biocontrol agents (BCAs) can be considered facultative mutualists, because survival rarely depends on any specific host and disease suppression will vary depending on the prevailing environmental conditions. Further down the spectrum, commensalism is a symbiotic interaction between two living organisms, where one organism benefits and the other is neither harmed nor benefited. Most plant-associated microbes are assumed to be commensals with regards to the host plant, because their presence, individually or in total, rarely results in overtly positive or negative consequences to the plant. And, while their presence may present a variety of challenges to an infecting pathogen, an absence of

measurable decrease in pathogen infection or disease severity is indicative of commensal interactions. Competition within and between species results in decreased growth, activity and/or fecundity of the interacting organisms. Biocontrol can occur when nonpathogens compete with pathogens for nutrients in and around the host plant. Direct interactions that benefit one population at the expense of another also affect our understanding of biological control. Significant biological control most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens (Chakraborty *et al.,* 2012a).

In most research to date, biocontrol agents are applied singly to combat a pathogen. Although the potential benefits in the application of a single biocontrol agent has been demonstrated in many studies, it may also partially account for the reported inconsistent performance, because a single biocontrol agent is not likely to be active in all kinds of soil environments and agricultural ecosystems (Raupach and Kloepper, 1998). This may have resulted in inadequate colonization, limited tolerance to changes in environmental conditions and fluctuations in production of antifungal metabolites (Wellerand and Thomashow, 1994; Dowling and O'Gara, 1994). Several approaches have been used to overcome these problems, including combined application of two or more biocontrol strains to enhance the level and consistency in disease control (Pierson and Weller, 1994; Schisler *et al*., 1997; Raupach and Kloepper, 1998). Multiple strain mixture of microbial agents has been employed with some success against plant pathogens in previous studies.These include mixtures of fungi (Paulitz *et al.,* 1990; Budge *et al*.,1995; Schisler *et al.,* 1997), mixtures of bacteria (Pierson and Weller, 1994;Raupach and Kloepper, 1998) mixtures of yeasts (Janisiewicz,1996), bacteria and fungi (Duffy *et al.*, 1996; Leibinger *et al.,* 1997), and bacteria and yeast (Janisiewicz and Bors, 1995).In addition to disease control, strain mixtures enhanced the plant growth interms of increased seedling emergence (Dunne *et al.,* 1998), plant height (Raupach andKloepper, 1998) and yield (Nandakumar *et al.,* 2001; Pierson and Weller, 1994; Duffy *et al.*,1996). Enhancing biocontrol activity by using mixtures of antagonist may have advantages: (i) it may broaden the spectrum of activity, (ii) it may enhance the efficacy and reliability of the biocontrol, and more importantly (iii) it may allow the combination of various traits without employment of genetic engineering (Janisiewicz, 1996). Moreover the designing of combination of strains and making use of multiple antifungal traits exhibited by them may prove to be

advantageous by ensuring that at least one of the biocontrol mechanisms will be functional under the unpredictable field conditions faced by the released PGPR strains (Niranjan Raj *et al*., 2005).

2.3.1.1 Plant growth promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-Aminocyclopropane-1- carboxylate deaminase (ACC), quorum sensing (QS) signal interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promoting beneficial plant-microbe symbioses, interference with pathogen toxin production etc. (Bhattacharyya and Jha,2012). The concept of PGPR has now been confined to the bacterial strains that can fulfil at least two of the three criteria such as aggressive colonization, plant growth stimulation and biocontrol (Weller *et al*. 2002; Vessey 2003). Most rhizobacteria associated with plants are commensals in which the bacteria establish an innocuous interaction with the host plants exhibiting no visible effect on the growth and overall physiology of the host (Beattie 2006). In negative interactions, the phytopathogenic rhizobacteria produces phytotoxic substances such as hydrogen cyanide or ethylene, thus, negatively influence on the growth and physiology of the plants. Counter to these deleterious bacteria, there are some PGPRs that can exert a positive plant growth by direct mechanisms such as solubilization of nutrients, nitrogen fixation, production of growth regulators, etc., or by indirect mechanisms such as stimulation of mycorrhizae development, competitive exclusion of pathogens or removal of phytotoxic substances (Bashan and de-Bashan 2010). The bacterial genera such as *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* belongs to ePGPR (Gray and Smith 2005), i.e Extracellular Plant growth promoting rhizobacteria wherein these bacteria may reside in the rhizosphere, on the rhizoplane or in the spaces between the cells of the root cortex. On the other hand, the intracellular plant growth promoting rhizobacteria (iPGPR) locates generally inside the specialized nodular structures of root cells. These include the endophytes (*Allorhizobium, Azorhizobium, Bradyrhizobium,*

Mesorhizobium and *Rhizobium*) and Frankia species both of which can symbiotically fix atmospheric N2 with the higher plants (Verma *et al.* 2010).

The export oriented agricultural and horticultural crops depends on the export of residue free produce and has created a great potential and demand for the incorporation of biopesticides in crop protection. To ensure the sustained availability of biocontrol agent's mass production technique and formulation development protocols has to be standardized to increase the shelf life of the formulation. It facilitates the industries to involve in commercial production of plant growth promoting rhizobacteria (PGPR). PGPR with wide scope for commercialization includes *Pseudomonas fluorescens, P. putida, P. aeruginosa, Bacillus subtilis* and other *Bacillus* spp. The potential PGPR isolates are formulated using different organic and inorganic carriers either through solid or liquid fermentation technologies. They are delivered either through seed treatment, bio-priming, seedling dip, soil application, foliar spray, fruit spray, hive insert, sucker treatment and sett treatment. Application of PGPR formulations with strain mixtures perform better than individual strains for the management of pest and diseases of crop plants, in addition to plant growth promotion. Supplementation of chitin in the formulation increases the efficacy of antagonists (Nakkeeran*et al*., 2005)

Nandakumar *et al*. (2001) developed talc based strain mixture formulation of fluorescent pseudomonads. It was prepared by mixing equal volume of individual strains and blended with talc as per Vidhyasekaran and Muthamilan (1995). Talc based strain mixtures were effective against rice sheath blight and increased plant yield under field conditions than the application of individual strains. Vidhyasekaran and Muthamilan (1995) stated that soil application of peat based formulation of *P. fluorescens* (Pf1) at the rate of 2.5 Kg of formulation mixed with 25 Kg of well decomposed farm yard manure; in combination with seed treatment increased rhizosphere colonization of Pf1 and suppressed chickpea wilt caused by *Fusarium oxysporum* f.sp. *ciceris*. Bioformulations of saw dust, rice husk and tea waste of two different PGPR *Serratia marcescence* and *Bacillus megaterium* was applied to five different tea varieties TV-18, TV-23, TV-25, TV-26 and T/17/1/154 in experimental field as well as in nursery. Application of these bioformulations increased height, emergence of new leaves and branches in all five varieties though all varieties did not show similar responses (Chakraborty *et al*., 2012).

Application of *P. fluorescens* on to foliage (1kg of talc based formulation /ha) on 30, 45, 60, 75 and 90 days after sowing reduced leaf spot and rust of groundnut under field conditions (Meena *et al.,* 2002). Preharvest foliar application of talc based fluorescent pseudomonads strain FP7 supplemented with chitin at fortnightly intervals (5g/l; spray volume 20l/ tree) on to mango trees from pre-flowering to fruit maturity stage induced flowering to the maximum, reduced the latent infection by *C. gloeosporioides* beside increasing the fruit yield and quality (Vivekananthan *et al*., 2004).

Plant pathogens establish host parasite relationships by entering through infection court such as spermosphere, rhizosphere and phyllosphere. Hence, protection of sites vulnerable for the entry and infection of pathogens would offer a better means for disease management. Combined application of talc based formulation of fluorescent pseudomonads comprising of Pf1 and FP7 through seed treatment, seedling dip, soil application and foliar spray suppressed rice sheath blight and increased plant growth better than application of the same strain mixture either through seed, seedling dip or soil (Nandakumar *et al*., 2001). Seed and foliar application of talc based fluorescent pseudomonas reduced leaf spot and rust of groundnut under field conditions (Meena *et al*., 2002). Delivering of rhizobacteria through combined application of different delivery systems will increase the population load of rhizobacteria and thereby might suppress the pathogenic propagules.

2.3.1.2. Plant growth promoting fungus (PGPF)

As ubiquitous and often predominant components of the mycoflora in native and agricultural soils throughout all climatic zones, *Trichoderma* species play an important role in ecosystem health (Klein and Eveleigh, 1998). Adapted to virtually every ecosystem, these fungi live in marine and terrestrial sites. They colonize aboveground and belowground plant organs and grow between living cells (endophytes), and they appear in plant litter, soil organic matter (saprophytes), and mammalian tissues (human pathogens). However, the ability of these fungi to sense, invade, and destroy other fungi has been the major driving force behind their commercial success as biopesticides (more than 60% of all registered biopesticides are *Trichoderma*-based) (Verma*et al.,* 2007). These fungi not only protect plants by killing other fungi and certain nematodes but induce resistance against plant

pathogens, impart abiotic stress tolerance, improve plant growth and vigor, solubilise plant nutrients, and bioremediate heavy metals and environmental pollutants (Hermosa *et al.*, 2012, Lorito *et al.*, 2010, Mastouri *et al.*, 2012, Shoresh *et al.*, 2010). In addition, this genus comprises fungi that produce secondary metabolites of clinical significance and enzymes with widespread industrial application. As *Trichoderma* has had a major impact on human welfare, recent genome sequencing projects have targeted seven species: *Trichoderma reesei*, *Trichoderma virens*, *Trichoderma atroviride*, *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderm alongibrachiatum*, and *Trichoderma citrinoviride.* The genome sequencing of *Trichoderma* species has stimulated the development of systems biological approaches, initiated and enhanced whole-genome expression studies, and provided unique data for phylogenetic and bioinformatic analyses toward understanding the roles of these opportunists in ecosystems.

Recent years have witnessed a wave of interest in plant disease resistance [induced systemic resistance (ISR); to some extent, systemic acquired resistance (SAR)] induced by the *Trichoderma*-root symbiosis (Harman *et al*., 2004, Hermosa *et al*., 2012). These plant-centered mechanisms have rivalled mycoparasitism as an explanation for how *Trichoderma* controls plant diseases (Lorito *et al*., 2010, Shores *et al*., 2010). The combined ability to attack soil borne pathogens while priming plant defenses, however, is what promotes *Trichoderma* as such a promising partner for sustainable management of plant diseases. The first field success of biological control (target *Sclerotium rolfsii*) using *Trichoderma* was not until the 1970s (Wells *et al*., 1972). Applied and fundamental research on these fungi has continued unabated since.

Mycoparasitism, wherein a fungus derives nutrients living in association with another fungus, is an ancestral trait of *Trichoderma* (Kubicek *et al.*, 2011). Mycoparasitic *Trichoderma* species can destroy the host, but there are biotrophic mycoparasites that do not kill the host. Druzhinina *et al.* (2011) broadened the concept of mycoparasitism to other nutritional relationships between fungi and defined mycotrophy to include the ability of *Trichoderma* to feed on dead fungi as well. *Trichoderma* can also kill plant-parasitic nematodes, expanding their range of biocontrol. The production of chitinases, glucanases, and proteases facilitates the flow of nutrients into the mycoparasite and of degradation of the host. *Trichoderma* parasitizes not only active hyphae but also resting structures/propagules, such as

sclerotia and perithecia (Inch *et al.*, 2011, Mukherjee *et al*., 1995). Mycoparasitism-related genes respond transcriptionally to the prey, and several studies identified genes expressed during interactions of *Trichoderma* species with plant pathogens.

The saprophytic fitness of *Trichoderma* species has enabled their establishment in soil and rhizosphere and often within roots where hyphae grow between cortical cells. *Trichoderma*-root interactions involve recognition, attachment, penetration, colonization, and nutrient transfer from the root. This source-sink communication may be central to the mutualistic interaction, influencing proliferation of *Trichoderma* in the rhizosphere and root (Vargas *et al*., 2011, 2009). Although well known for their ability to colonize the rhizosphere with limited root penetration, some *Trichoderma* species are known to reside in plants as typical endophytes, entering through trichomes by producing appressoria-like structures. Penetration into the plant would initially imply activation of plant immunity. Symbionts, however, circumvent or remodel the plant immune response (Zamioudis and Pieterse, 2012). Whether the *Trichoderma*-induced defense response is typical of the ISR, which is induced by plant growth–promoting rhizobacteria, or of the SAR, which is induced by necrotrophs, is debatable. The defense pathways triggered by *T. asperellum* and *Pseudomonas fluorescens* were found to be highly similar. *Trichoderma*-induced defense response has been traditionally treated as ISR (Shoresh*et al*., 2010). ISR, SAR, or both could be activated depending on the timing of interactions and the applied inoculums load of *Trichoderma* (Segarra *et al*., 2007). Infection of roots by *T. asperellum* did not provoke major transcriptomic changes in *Arabidopsis* leaves, but genes for pathogenesis-related (PR) proteins were upregulated upon challenge inoculation by the pathogen *Pseudomonas syringae* (Brotman *et al*., 2012).

Trichoderma species produce several proteinaceous elicitors, including a xylanase, peptaibols, and the small cysteine-rich secreted protein Sm1/Epl1,that can trigger defense responses in plants when produced in planta or applied in pure form (Djonovic*et al*., 2006, Seidl*et al*., 2006).

In the past year, the genomes of *T. harzianum* and *T. asperellum*, which are biocontrol species that belong to other phylogenetic branches have become available. Postgenome experiments have been conducted to examine mycoparasitism on a genomic scale. Chitinases, which are considered highly important in mycoparasitism, have been the main focus. A comparative analysis of chitinases revealed that *Trichoderma* genomes harbor between 20 and 36 different genes that encode

chitinases, with chi18-13 and chi18-17 significantly expanded in the mycoparasites *T. Atroviride* and *T. virens* (Ihrmark *et al*., 2010). The availability of *Trichoderma* genomes coupled with those of severalplants permits the bidirectional interactionsat the genome scale to be studied. Analysis of transcriptome changes in *T. harzianum*, *T. virens*, and *Trichoderma hamatum* during interactions with tomato plants revealed that1,077 genes were regulated when the fungi were grown with tomato (593 in *T. harzianum*, 336 in *T. virens*, and 94 in *T. hamatum*, six genes being common to all three). Interestingly, genes encoding enzymes needed for chitin degradation (N-acetylglucosamine-6-phosphate deacetylase,glucosamine-6-phosphate deaminase, and chitinase) were significantly over expressed in all three *Trichoderma* spp. during early interactions with tomato plants (Rubio *et al*., 2012). From the plant side, studies on the transcriptomic response of *Arabidopsis* to *T. harzianum* inoculation revealed that after 24 h of incubation SA- and JA-related genes were down regulated, whereas several genes related to abiotic stress response were upregulated (Moran-Diez *et al*., 2012). Secreted proteins are likely to play a role in the communication between *Trichoderma* and plants, just as they do in plant-pathogen and plant-mycorrhizae interactions. The *Trichoderma* genomes have now been shown to contain a large repertoire of small cysteine-rich secreted protein-like genes (Kubicek *et al*., 2011), potentially encoding hundreds of possible elicitors.

A thorough understanding of the processes involved in production of secondary metabolites is important for effective, harmless utilization of *Trichoderma* spp. Similar to other fungal genomes, most of the secondary metabolism–related genes in *Trichoderma* reside in clusters, with many acquired by horizontal gene transfer, and the majority appear to be silent under standard laboratory conditions. *Trichoderma* secondary metabolites may be grouped into peptaibols, small NRPs (e.g., gliotoxin, siderophores), polyketides, terpenes, or pyrones. Iron is an essential element for survival, and siderophores are important for the acquisition of iron in a competitive environment. On average, *Trichoderma* spp. produced 12–14 siderophores (Lehnar *et al*., 2013). The genomes of *T. virens* and *T. reesei* contain a gene for extracellular siderophore production (Sid Dortholog) in addition to the functional NPS6 cluster that is conserved across the three species (Mukherjee *et al*., 2012).

Bigirimana *et al* (1997) showed that treating soil with *Trichoderma harzianum* strain T-39 made leaves of bean plants resistant to diseases that are caused by the fungal pathogens *B. cinerea* and *Colletotrichum lindemuthianum*, even though T-39

was present only on the roots and not on the foliage. The same group extended their findings from *B. cinerea* to other dicotyledonous plants (De Meyer *et a*l., 1998).The efficacy of biocontrol agents on *Macrophomina phaseolina* (Tassi) Goid., which causes dry root rot of pigeonpea were studied. In dual culture technique both *Trichoderma virens* (PDBC TVS-2) and *Pseudomonas fluorescens* (PDBC Pf1) significantly inhibited the mycelial growth of *M. phaseolina*by 78.22 per cent and 76.66 per cent respectively. Among the three methods by which, the talc based formulations of these bioagents were applied, seed treatment (4g/kg seed)along with soil application (2 g talc powder mixed with FYM/pot) supported the maximum plant stand and less root rot incidence compared to other treatment and found significantly superior to seed treatment (2g/kg seed). However, it was on par with soil drenching with carbendazim (0.1%). Seed treatment + soil application with *T. virens* recorded less root rot incidence (2.89%) among the four bioagents used (Lokesha*et al*; 2007). Application of *T. harziamun* T-22 on roots of maize plants caused 44% reduction of lesion size in wounded leaves caused by *Colletotrichum graminicola*. These state that *T. harzianum* causes induced systemic resistance in plants thereby defending the plant against various disease (bacterial, fungal and viral) in different parts of plants even though they are present in rhizospheric zone of the plant (Harman *et al*., 2004).

2.3.1.3. Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizas are mutualistic symbiotic associations between the roots of most vascular plants and a small group of fungi in the new phylum Glomeromycota (Schüßler *et al.*, 2001). It provides phosphorus and nutrition to the plants and itself obtains carbon from the plant to support itself. The association between arbuscular mycorrhizal fungi and plants improves the fitness of both plant and AMF symbionts constituting a mutualism. Although some structural variation exists in this category, most arbuscular mycorrhizas are characterized by the presence of intraradical hyphae (intercellular or intracellular in location), arbuscules (finely branched hyphae involved in nutrient exchange), extraradical mycelium (hyphae that connect the root to the soil), and spores formed in the extraradical mycelium (Smith and Smith, 1997). It has been estimated that over 80% of all vascular plants form arbuscular mycorrhizas.

Root colonization with AMF is a dynamic process, which influenced by several edaphic factors such as nutrient status of soil, seasons, arbuscular mycorrhizal (AM)

strains, soil temperature, soil pH, host cultivar susceptibility to AM colonization and feeder root condition. There has been growing appreciation of the importance of plant and fungal interaction especially AMF on terrestrial ecosystem (Giovannetti *et al.*, 2006, Rodrigues, 2008). The assumed primary benefit to plants of the Mycorrhizal symbiosis is an increased uptake of immobile nutrients, especially phosphorus that are mobilised by the fungus. However, there is increasing evidence that AMF have a range of other effects, for example, protection against plant parasites (Aggarwal *et al.*,2006, Bhargava *et al.*,2008), water stress tolerance (Newsham *et al.*,1995) alleviation of salt stress (Evelin *et al.*, 2009) and in sustainable maintenance of plant health and soil fertility (Jeffries *et al.*,2003,Wright and Upadhyaya, 1998). Very little experimental work has been performed on mutual recognition of mycorrhizal fungi and their hosts. Their specificity is not close; a single fungal isolate may form vesicular-arbuscular mycorrhiza with a wide range of species of host of all the phyla of land plants in laboratory experiments. Specificity seems to be closer in the competition of natural vegetation than in pure culture. The extent to which this impression is real is questionable. At all events, any mycorrhizal host is usually compatible with a wide range of fungi and each mycorrhizal fungus with a wide range of hosts. Moreover at a single time one individual plant may associate with several species of fungi. Mycorrhizal fungi have extensive compatibility with potential hosts which is perhaps only limited by the inhibitory properties in the host, which itself can be universal or selective in response to the fungi (Chakraborty and Chakraborty, 2012).

The endosymbioses formed between plants and micro-organ-isms play an important role in agriculture natural ecosystems. The most widespread mutualistic endosymbiotic interactions are formed between plant roots and AMF. The successful establishment of this mutualistic association constitutes a strategy to improve the nutritional status of both partners. The fungi receive fixed carbon compounds from the host plant, while the plant benefits from the association by the increased nutrient uptake of phosphorus, enhanced tolerance to abiotic stress, and resistance to pests and pathogens (Bhargava *et al.*, 2008, Smith and Read, 2008). Generally, AMF show little or no specificity and the factors that determine whether mycorrhiza are formed or not appear to depend on the genotype of the host plant (Koide and Schreiner, 1992). Evidence for this is provided by the existence of non-host plant species (Giovannetti

and Sbrana, 1998) and Myc mutant plants unable to form AM symbiosis (Gollotte *et al.*, 1993).

Many works have been done to conclusively prove that VAM fungi are involved in biocontrol of fungal pathogens. Blight disease of tomato caused by *Phytophthora parasitica* has been controlled using *Glomus mossae* (Cordier *et al*., 1998). *G. mossae* has also reduced brown root rot disease in tea caused by *Fomes lamaoensis and Sclerotial* blight (Chakraborty *et al*., 2007,2009). This particular genus of VAM has also controlled the blight of soybean caused by *Phytophthora megasperma* var. *Sojae* (Graham 2001).

The use of molecular diagnostics as a tool in plant disease management is in its infancy. There is increasing economic and environmental pressure to reduce the use of agrochemicals to control crop diseases. Disease management systems are now being developed that aim to reduce agrochemical use bymore efficient targeting of sprays, for example by optimising the timing and dose of sprays applied. For many crop diseases control measures need only be taken when disease levels exceed some economically damaging threshold. Conventionally, disease pressure in crops is estimated by assessing visual symptoms. Molecular diagnostics offer alternative, more accurate, methods for determining disease or pathogen inoculums thresholds. Incorporating these new tools into disease management systems is a challenge; however, the benefits through efficient use of fungicides could be considerable. Molecular diagnostics are already making a considerable impact on research in Plant Pathology. In time they will become increasingly important in the management and control of plant disease.

Chapter 3
MATERIALS AND METHOD

3.1. Plant Material

3.1.1. Collection

Eight different morphotypes of *Persea bombycina* (viz. S1, S2, S3, S4, S5, S6, S7 and S8) were collected from Central Muga Eri Research and Training Institute (CMER&TI), Jorhat, Assam. These different morphotypes of som are classified based on their leaf shape. Commonly they are called as Ampotia (S1 and S2), Naharpotia (S3), Jampotia (S4), Belpotia (S5), Kothalpotia (S6) etc.

3.1.2. Maintenance in glass house

The eight different morphotypes of som plant were maintained in glass house of Immuno-phytopathology Laboratory, Department of Botany, University of North Bengal. Minimum 15 plants of each morphotypes were maintained in earthenware pots (12" dia) (Figure 1)

3.1.3. Maintenance in field condition

One year old saplings of all eight morphotypes were transferred to Experimental field in Padmaja Park of University of North Bengal where all suitable management practices were taken into consideration for proper growth of the plants. Two different morphotypes (S5 and S6) that showed the best growth activity under nursery and glass house and nursery condition were transferred to experimental field of immune-phytopathology Laboratory, NBU for close monitoring and further experimentation. (Figure 2)

Figure1: Maintenance of *Persea bombycina* morphotypes in glass house condition

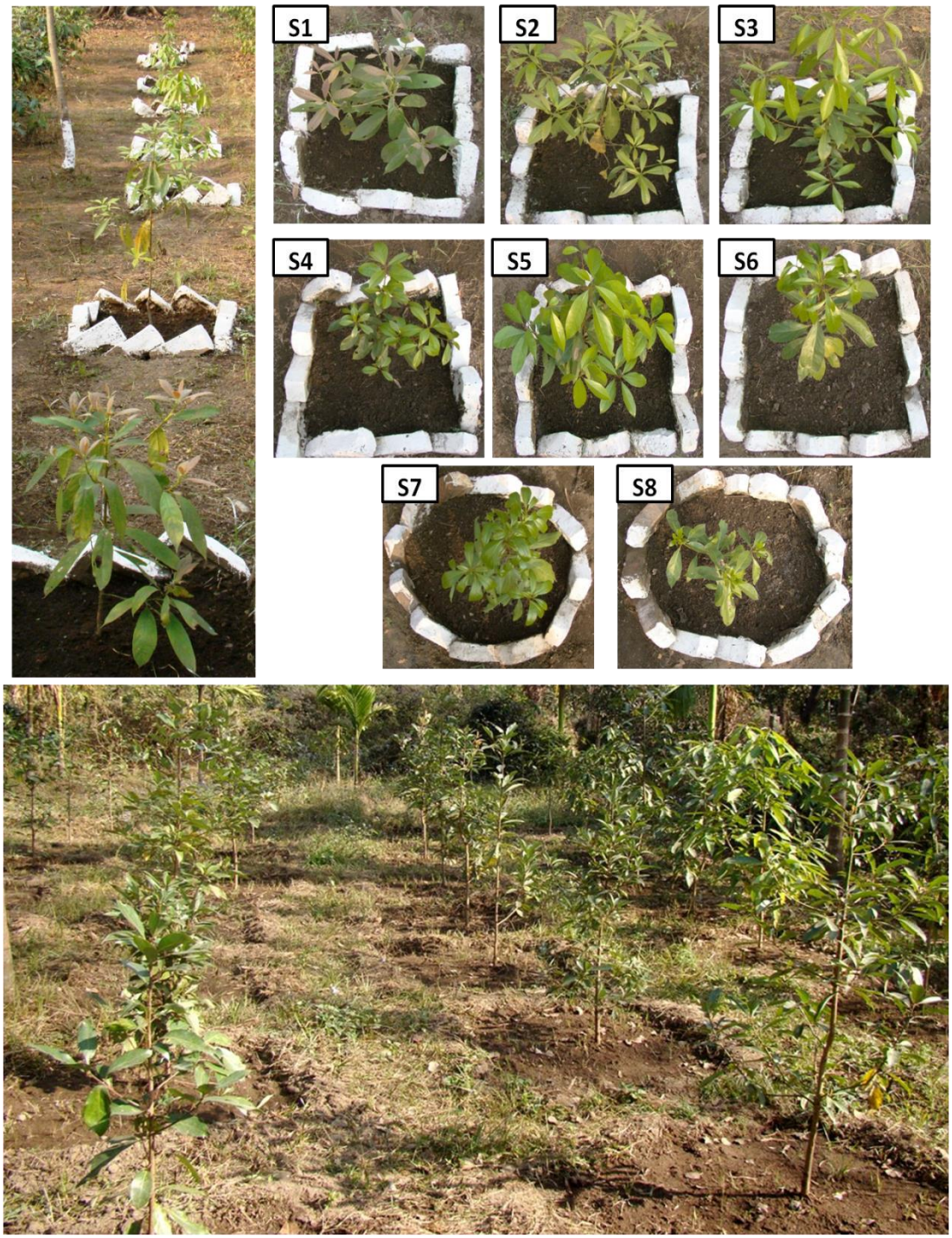


Figure2: Maintenance of *Persea bombycina* morphotypes in field condition

3.2. Fungal Culture

3.2.1. Isolation and maintenance

The fungal pathogens which they are present in deep seated tissue of infected leaves were isolated by culturing pieces of internal tissues. Infected tissues were thoroughly washed in sterile water and then swabbed with cotton wool dipped into 80% ethanol, followed by exposure to an alcohol flame for a few seconds. The outer layer of tissues was quickly removed by a flame sterilized scalpel. Small pieces from the central core of tissue in the area of the advancing margin of infection were removed by a sterilized scalpel and sterilized by dipping into 90% alcohol then flamed for a few seconds. The sterilized tissues were transferred to potato dextrose agar in Petri dishes and incubated at 28ºC for 1 week. The fungal mycelium grown was transferred to PDA slants and kept for further identification.

3.2.2. Morphological and microscopic observation

The isolated fungi were allowed to grow in Petriplates (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspension was prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lacto phenol- cotton blue, covered with cover slip and sealed with wax. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

3.2.3. Completion of Koch's Postulate

Fresh young som leaves were collected from experimental field of Immuno-Phytopathology lab and inoculated with conidial suspension of the isolated fungal pathogen following detached leaf inoculation technique. After 96h of inoculation, the infected som leaves were washed thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ solution for 3-5 min washed several times with sterile distilled water and transferred aseptically into Potato Dextrose Agar (PDA) slants. These isolates were examined 15 days of inoculation at 30°C and identity of the organism was confirmed by comparing with the stock culture.

3.2.4. Assessment of mycelia growth

Mycelial growth of the fungal cultures was assessed in both solid media and liquid media to study and evaluate their cultural characteristics.

3.2.4.1. Solid Media

To assess the growth of fungal culture in solid media, the fungus was first grown on petri dishes, each containing 20ml of PDA followed by incubation for 7 days at 30ºC. Agar blocks (6mm diameter) containing the mycelium was cut with sterile cork borer from the actively growing region of mycelial mat and transferred to each Petri dish containing 20ml of different sterilized solid media. The colony diameter was studied at regular interval of time. The media were as follows:

A. Potato Dextrose Agar (PDA)

Peeled potato - 40.00g, Dextrose – 2.00g, Agar - 2.00g, Distilled water - 100ml

B. Richard's Medium (RMA)

KNO_3 - 1.00g, KH_2PO_4 - 50g, $MgSO_4.7H_2O$ - 0.25g, $FeCl_3$ - 0.002g, Sucrose - 3.00g, Agar- 2.00g, Distilled water – 100ml

C. Oat Meal Agar (OMA)

Oat meal – 3.00g, Agar – 2.00g, Distilled water – 100ml

3.2.4.2. Liquid Media

To assess the mycelial growth in liquid media the fungus was first grown on petriplates, each containing 20ml of PDA and incubated for 5-8 days at 28ºC. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) containing 50 ml of sterilized Potato dextrose broth (PDB) and Richards medium and incubated for 6 - 8 days with constant stirring at room temperature. After incubation the mycelia were harvested through muslin cloth, collected in aluminium foil cup of known weight and dried at 60ºC for 96 h, cooled in desiccators and weighed.

3.3. Soluble proteins

3.3.1. Extraction of soluble protein

3.3.1.1. Fungal mycelia

Mycelial protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250 ml Erlenmeyer flask each containing 50 ml of potato dextrose broth (PDB) and incubated for 10 days at 30+ 1ºC. for extraction of antigen, mycelial mats were harvested washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortor and pestle and homogenized with cold 0.05m sodium phosphate buffer (PH-7.2) supplemented with 0.85% NaCl, 10mM sodium metabisulphite, PVPP (Polyvinyl pyrollidine Phosphate) and 0.5mM magnesium

chloride in ice bath. The homogenated mixture was kept for 2h or overnight at 4ºC and then centrifuged at 10.000rpm for 30 min, at 4ºC to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4ºC. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4º C, the precipitate was dissolved in the same buffer (pH 7.2). The preparation was dialysed for 72h through cellulose tubing (sigma chemical co., USA) against 1L of 0.05 M sodium phosphate buffer (pH 7.2) with six changes. The dialysate was stored at -20ºC and used as antigen from the preparation of antiserum and other experiment.

3.3.1.2. Leaf

Soluble protein was extracted from som leaves following the method of Chakraborty *et al*., (1995). Leaf tissues were frozen in liquid nitrogen and ground in 0.05mM Sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.5mM $MgCl_2$and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.3.2. Estimation of soluble protein content

Soluble proteins were estimated following the method as described by Lowry *et al.*, (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% CuSO4 and1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH) wasadded. This was incubated for 15 min at room temperature and then 0.5ml of 1N FolinCiocalteau reagent was added and again incubated for further 15 min following whichoptical density was measured at 720 nm. Quantity of protein was estimated from thestandard curve made with bovine serum albumin (BSA).

3.4. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detailed analysis of protein profile following the method of (Laemmli, 1970). For the preparation of gel the following stock solution were prepared.

3.4.1. Preparation of stock solution

Following stock solution were prepared

A. Acrylamide and N'N' – methylene bis acrylamide

Stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water, as both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light. The pH of the solution was kept below 7.0 and the stock solution was

then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4ºC and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C. Tris Buffer

i) 1.5M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4ºC for further use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4ºC for use.

D. Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris- Glycine electrophoresis buffer

Tris running buffer consists of 25mM Tris base, 250mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1L distilled water.

F. SDS gel loading buffer

This buffer contains 50 mM Tris –HCl (pH 6.8), 10 mM ß- mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4 M ß- mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water.

3.4.2. Preparation of gel

Mini slab gel (plate size 8cm x10cm) was prepared for analysis of proteins patterns through SDS-PAGE. For gel preparation, two glass plates (8 cmx10 cm) were washed with dehydrated alcohol and dried to remove any traces of grease. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the three sides of the glass plates were sealed with gel sealing tape or wax, clipped tightly to prevent any leakage and kept in the gel casting unit. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb +1cm). After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel was complete,

overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris – Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	10% Resolving Gel (ml)	5% Stacking Gel (ml)
Distilled Water	2.85	2.10
30% Acrylamide	2.55	0.50
Tris*	1.95	0.38
10%SDS	0.075	0.030
10%APS	0.075	0.030
TEMED**	0.003	0.003

*For 1.5 M tris pH 8.8 in resolving gel and for 1M Tris pH 6.8 in stacking gel

** N,N,N‘,N‘ –Tetramethyl ethylene diamine

3.4.3. Sample preparation

Sample (50µl) was prepared by mixing the protein extract (35 µl) with 1xSDS gel loading buffer (16 µl) in cyclomixer. All the samples were floated in a boiling water bath for 30 minutes to denature the proteins samples. After boiling, the sample was loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97,4000: Biovine Serum Albumin - 68,000; Albumin -43,000; Carbolic Anhydrase -29.000; Soybean Trypsin inhibitor- 20,000; Lysozyme - 14,300) was treated as the other samples and loaded in separate well.

3.4.4. Electrophoresis

Electrophoresis was performed at 18mA current for a period of two to three hours or until the dye reached the bottom of the gel.

3.4.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally fixed in

glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. When the stain was completely dissolved, 45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper. The gel was removed from the fixer and stained in this stain solution for 4 h at 37 ºC with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5:1) at 40 ºC with constant shaking until the background become clear.

3.5. Preparation of antigen

3.5.1. Fungal antigen

Mycelial protein was prepared following the method as outlined by (Chakarborty and Saha 1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4 ºC and then centrifuged at 10,000rpm for 30 min at 4 ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

3.5.2. Leaf Antigen

3.5.2.1. Healthy leaf

Antigens from healthy leaves were prepared following the method of Chakraborty and Saha (1994). Fresh, young healthy leaves were collected from the experimental garden and kept at 4°C. Then the leaves were weighed and crushed in mortar and pestle with 0.05M Sodium phosphate buffer supplemented with 10mM Sodium metabisulphite, 2mM PVPP 10,000 (soluble) and 0.5mM magnesium chloride (pH 7.2). At the time of crushing with sea-sand insoluble PVPP of equal weight was used. The leaf slurry was strained through a muslin cloth and then centrifuged (15,000g) for 30 min at 4°C. the supernatant was used as healthy leaf antigen and was kept at -20°C until required.

3.5.2.2. Artificially inoculated leaf

Antigen from *C. gloeosporioides* and *P. disseminata* inoculated leaves were extracted following the method of Alba & DeVay (1985) with modification. Fresh, young leaves were collected from experimental garden and kept in plastic trays as described in detached leaf inoculation technique. Leaves were inoculated with conidial suspension of both the pathogens separately. Control sets were prepared by mounting the leaves with drops of sterile distilled water. Antigens were prepared from inoculated leaves as well as control leaves as described earlier. The prepared antigens were stored at -20°C until further experimental purposes.

3.5.2.3. Naturally infected leaf

For the extraction of naturally infected leaf antigens, the infected leaves were collected from the experimental garden and kept at 4°C. Then the infected portion of leaf was cut into small pieces, weighed and antigens were prepared as before.

3.5.3 AMF antigen

AMF spores were isolated from rhizosphere soil of som by wet sieving and decanting method (Gerdemann and Nicolson, 1963). With the help of a dissecting microscope parasitized spores, plant debris etc were separated and clean AMF spores were isolated. These spores were sonicated with 0.1% normal saline under the frequency range of 70-75 mhz as impulse. The supernatant was used as antigen source.

3.6. Serology

3.6.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal and bacterial antigens in New Zealand white male rabbits of approximately 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera reserves for plant pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. twice a day. After each bleeding they were given saline water for three consecutive days and kept in proper hygienic conditions.

3.6.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera , intramuscular injections of 1ml antigen(protein extracted) mixed with 1ml of Freund‘s complete adjuvant (Genei) were given into each rabbit 7 days after pre-immunization bleeding and repeating the doses at 7 days intervals for consecutive

week followed by Freund‘s incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Method of (Alba and Devay, 1985) and (Chakraborty and Saha, 1994) were followed for immunization.

3.6.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they were placed on their back on a wooden bored fixed at an angle of 60º, and held the rabbits tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 -10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at 37ºC for 1hr for clotting. After clotting; the colt was loosened with a sterile needle. Finally, the serum was classified by centrifugation. (2000g for 10 minute at room temperature) and distributed in 1 ml vials and stored at -20ºC as crude antisera. The serum was used for double diffusion analysis, dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.6.4. Purification of IgG

3.6.4.1. Precipitation

IgG was purified as described by (Clausen, 1988). Crude antiserum (2ml) was diluted with two volume of distilled water and an equal volume of 4M (NH_4SO_4) ammonium sulphate was taken and pH adjusted to 6.8, stirring the mixture for 16h at 20º C in magnetic stirrer. The precipitate thus formed was collected by centrifugation at 12,000 rpm for 1h at 22 º C for 1 h. Supernatant was discarded and pellet was used for further steps.

3.6.4.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30cm height) and allowed to settle for 2h. After the column material had settled 25ml of buffer (0.02M sodium phosphate, pH 8.0) washing was given to the column material.

3.6.4.3. Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.03 M. the initial elution buffer (1) was 0.02 M sodium phosphate (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column buffer (2) was soaked into buffer (1) thereby producing a continuous raise in morality. Ultimately, 40 fractions each of 5ml were collected and the optical density values were recorded at 280nm using UV-Vis spectrophotometer (DIGISPEC-200GL).

3.7.Immunological assays

3.7.1. Agar gel double diffusion

3.7.1.1. Preparation of agarose slides

The glass slides (6cm x 6cm) were degreased using ethanol 90%v/v: diethyl ether (1:1v/v) and ether, then dried in hot air oven. After drying the plates were sterilized inside the petriplate each containing one plate. Conical flask with Tris-Barbiturate buffer (pH 8.6) is placed in boiling water bath. Agar/ agarose (0.9%) was boiled over water bath to dissolve the agar at 90 º C for next 15 min. Then pinch of 0.1% (w/v) sodium azide was added and mixed well. For the preparation of agarose gel, the molten agarose is poured (6 to 10 ml) on the grease free sterilized slide with the help of a sterile pipette in laminar air flow chamber and allow it to solidify, after solidification cut 3-7 wells (6mm diameter) with sterilized cork borer distance of 1.5 to 2cm away from central well and 2.0 to 2.5 cm from well to well.

3.7.1.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1967). Antigen plus undiluted antisera appropriately diluted were poured into wells with micropipette (50µl/well) antisera in middle. Slides were kept in moist chamber at 25ºC for 72h. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

3.7.1.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 1% NaN_3) for 72 h with 6

hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then the slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25g Coomassie blue, 45ml methanol, 45ml distilled water and 10ml glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratios) with changes until background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50º C.

3.7.2. Plate trapped antigen coated (PTA) - ELISA

Plate trapped antigen coated (PTA)-ELISA was performed following the method as described by (Chakraborty *et al.*., 1995) with modifications. Antigen were diluted with coating buffer and the antigens were loaded (200µl per well) in ELISA plate (Coaster EIA/RIA, strip plate, USA) arranged in12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25 ºC for 4 h. then the plate was washed four times under running tap water and twice with PBS-Tween and each time shaken well to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and plate was incubated at 25 ºC for 1h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200 µl per well) and incubated at 4ºC overnight. After a further washing, antirabbit IgG goat antiserum labeled with alkaline phosphatase diluted 10000 times in PBS, was added to each well (100 µl per well) and incubated at 37 ºC for 2 h. The plate was washed, dried and loaded with 200 µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 1 h. color development was stopped by adding 50 µg per well of 3 N NaOH solution and the absorbance was determined in an Multiscan Ex (Thermo Electron) ELISA Reader (Multiskan, Thermo Labsystems) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.7.3. Dot immunobinding assay

Dot blot was performed following the method suggested by Lange *et al.*,(1989) with modifications. Following buffers were used for dot immunobinding assay.

A. Carbonate –bicarbonate (0.05 M, pH 9.6) coating buffer.

B. Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.

C. Blocking solutions 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3.

D. Alkaline phosphatase buffer (100 mM tris HCl, 100 mM NaCl, 5mM $MgCl_2$)

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate-bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Antigen (5µl) was loaded on to NCM and allowed to dry for 30 min at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker. Respective polyclonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at 4 ºC for overnight. The membrane was then washed gently in running tap water for three min, thrice followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at 37ºC. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.7.4. Western blot analysis

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electro transferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of the pathogen (*C. gloeosporioides* and *P. disseminata*) following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-choloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of the proteins was visualized as violet coloured bands on the NCM

3.7.5. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia, cross- section of mandarin roots and leaves were done using FITC labeled goat antirabbit IgG following the method of (Chakraborty and Saha, 1994). Both FITC and RITC were done to locate AMF spores in soil and observe root colonization and cellular location of AMF which was mass multiplied in mandarin roots following colonization with AMF.

3.7.5.1. Fungal mycelia

Fungal mycelia were grown in liquid potato dextrose medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in

Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. The mycelia was washed thrice with PBS- Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 min at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were observed and photograph under both phase contrast and UV fluorescence condition using Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.7.5.2. Conidia

Fungal conidia were collected from 15 day-old culture and a suspension of this was prepared with PBS, pH 7.2. Conidial suspensions were taken in micro-centrifuge tubes and centrifuged at 3000g for 10min and the PBS supernatant was discarded. Then 200µl of diluted antisera (in PBS pH 7.2) in the ratio 1:125 was added into microcebtrifuge tube and incubated for 2h at 27°C. After incubation, the tubes were centrifuges at 3000g for 10 min and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween pH 7.2 by centrifugation as before and 200µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26°C for 1h. After the dark incubation excess FITC-antisera was removed by repeated washing with PBS-Tween pH 7.2 and the spores were mounted on glycerol jelly and observed under Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.7.5.3. Cross section of som roots and leaves

Initially, cross section of healthy mandarin roots and leaves were cut and immersed in PBS (pH 7.2). These section were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1 hour at room temperature. After incubation, cross sections were washed thrice with PBS- Tween (pH 7.2) for 15 minute and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC).The sections were incubated for 45 minutes in dark. After that sections were washed thrice with PBS- Tween as mentioned above and then mounted on a grease free slide with 10%glycerol. Fluroescence of the root and leaf sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.7.5.4. AMF in som root

Roots of maize plants (4 months) and som plants (2 years old) in which AMF spores of som were mass multiplied were macerated according to Philips and Hayman method as described by with a few modification. Antigen was given in the dilution 1:50 goat antirabbit IgG after washing with PBS pH 7.2 thrice. The roots were incubated overnight in dark. The next day, the roots were again washed thrice with PBS Tween and fluorescein isothiocyanate(FITC) and rhodamine isothiocyanate (RITC) was added in the dilution 1:10 and incubated for 45 minutes in dark. The roots were again washed thrice in PBS and mounted in 10% glycerol in grease free slides. Fluroescence of the root section were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.8. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. Liquid nitrogen was used for crushing the cell mass for both cases.

3.8.1. Preparation of genomic DNA extraction buffer

The following buffer for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

DNA Extraction buffer

1M Tris-HCl pH 8.0

5M Nacl

0.5 mM EDTA, pH 8.0

10% SDS

3.8.2. Genomic DNA extraction

The fungal mycelia was grown in PDB for 6-7 days and then harvested. Total genomic DNA was extracted as described by Kuramae and Izioka (1997). The mycelium was ground into the fine powder under liquid nitrogen and suspended in 700 µL extraction buffer . Upon homogenization, the tubes were incubated for 30 minutes at 65ºC. DNA samples were purified with equal volumes of chloroform: isoamyl alcohol (24:1) mixture (1X), and precipitaded with isopropanol. The tubes were centrifuged at 15400g for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried, suspended in TE buffer (pH 8.0) and stored at 4° till further use.

3.8.3. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAase treatment. Genomic DNA was

resuspended in 100 µl 1 X TE buffer and incubated at 37$_o$C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.8.4.Agarose gel eletrophoresis to check DNA quality

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample.

3.8.4.1. Preparation of DNA samples for electrophoresis

Agarose (0.8%) in 1X TBE buffer was melted, cooled and poured into the gel casting tray with ethidium bromide. Gels solidify in 15-20 min.

3.8.4.2. Run gel electrophoresis for DNA fraction

15µl of sample and 5µl of DNA loading dye mixed properly was loaded in each well of agarose gel (1%).The electrical head of the gel tank was attached firmly and electric supply was applied at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 min. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.9. ITS PCR analysis

All isolates of *Colletotrichum* and *Pestalotiopsis* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus

96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

3.9.1. ITS- PCR primers

The following primers were used to amplify ITS regions

Seq Name	Primer Seq 5'-3'	Mer	TM	%GC
ITS-1	TCTGTAGGTGAACCTGCGG	19	63.9	57
ITS-4	TCCTCCGCTTATTGATATGC	20	61.5	45
CgInt	GGCCTCCCGCCTCCGGGCGG	20	84.4	90

3.9.2. Amplification conditions

Temperature profile, 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.9.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the ITS primer pairs by SciGenom, Kerala. DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares. The chromatogram of the DNA sequence was analysed by the software Chromus. All the DNA sequences was edited by using the software BioEdit and aligned with Clustal W algorithms.

3.10. BLAST analysis of the sequences

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence.

3.11. Submission of rDNA gene to NCBI genbank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.12. Multiple sequence alignment and Phylogenetic analysis

The sequenced PCR product was aligned with ex-type STRAIN SEQUENCES FROM ncbi Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson et al; 1994), included in the Megalign module (DNASTAR Inc.). Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were done manually in order to artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01). Neither gaps (due to indertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic interference was performed by the UPGMA method (Sneath and Sokal, 1973). Bootstrap tests with 1000 replications were conducted to examine the reliability of the interioir branches and the validity of these trees obtained. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura et al., 2007.

3.13. RAPD PCR analysis

For RAPD, random primers were selected (Table-1). PCR was programmed with an initial denaturing at 94oC for 4 min. followed by 35cycles of denaturation at 94oC for 1 min, annealing at 36oC for 1 min and extension at 70oC for 90 s and the final extension at 72oC for 7 min. in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

3.13.1. RAPD primers

The following primers were used for RAPD analysis in the study

Seq Name	Primer Seq 5'-3'	Mer	TM	%GC
OPA-1	CAGGCCCTTC	10	38.2	70
OPD-6	GGGGTCTTGA	10	32.8	83
OPD-12	TGAGGGGAGA	10	36.9	60

3.13.2. Amplification conditions

Temperature profile, 94oC for 4 min followed by 35cycles of denaturation at 94oC for 1 min, annealing at 36oC for 1 min and extension at 70^{o}C for 90 s and the final extension at 72oC for 7 min in a Primus 96 advanced gradient Thermocycler.

3.13.3. Analysis of RAPD band patterns

RAPD band patterns were initially assessed by eye and isolates were grouped according to their shared band patterns.

3.13.4. Scoring of individual bands

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme NTSYSPc and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods, photographs of the gels were scanned into a computer and saved as graphics files.

3.13.5. Reconstruction of the phylogenetic tree

As with sequence data, RAPD data can be analysed in a number of different ways. The simplest form of analysis is to group isolates with identical band patterns for a given primer. More complex analyses involve cladistic analysis of data and reconstruction of the phylogenetic tree.

3.13.5.1. UPGMA method

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *InSilico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11W). The SIMQUAL program was used to calculate the Jaccard‘s coefficients. The RAPD patterns of each isolate was evaluated, assigning character state “1” to indicate the presence of band in the gel and “0” for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

3.14. Denaturing Gradient Gel Electrophoresis (DGGE)

3.14.1 PCR amplification of genomic DNA of the isolates for DGGE analysis

Denaturing Gradient Gel Electrophoresis was performed according to the method of Zhao et al; 2006. 18S DNA (200bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end) F352T : 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C-3' and 519r: 5'- ACC GCG GCT GCT GGC AC-3') in 25µl of reaction mixture containing 1X PCR buffer, 2.5mM MgCl2 (Bangalore Genei, India), 100 ng of the template DNA,25.0pmol each of the forward and reverse primers, 250µM each of the dNTPS, and 1U of Taq DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 5min, followed by 6 cycles of 95°C for 1min,65°C for 1min, and 72°C for 1min, in which the annealing temperature was reduced by 0.5°C per cycle from the preceding cycle, and then 24 cycles of 95°C. Perpendicular DGGE was performed with "the decode universal mutation detection system" (Bio Rad laboratories, USA).

A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimise suitable concentration and finally 20% to 60% denaturant was found optimal for the best result.

3.14.2. Denature Gradient Gel Electrophoresis of the PCR products

3.14.2.1. Reagents and solutions required for DGGE analysis

40% Acrylamide:bisacrylamide (37.5:1)

50X DGGE/TAE buffer solution

Trizma-Base:	484.4g
Sodium-Acetate:	272.0g
Trisodium EDTA	37.2g
H_2O	2 litres

pH 7.40 adjusted with about 230ml of glacial acetic acid

Preparation of Denaturants

100% Denaturant:

Urea	42.0g
38.5% Acrylamide (makes a 6.5% gel)	16.9ml
50X DGGE/TAE	2.0 ml
Formamide	40.0ml

Filled up to 100ml with distilled water.

0% Denaturant:

38.5% Acrylamide (makes a 6.5% gel)	16.9ml

50X DGGE/TAE	2.0 ml
Ammonium persulphate	10% (w/v)
TEMED	20µl

3.14.2.2. Creating the gel sandwich (DCode System BioRad)

Large glass plates were cleaned with soap and a soft sponge and rinsed with tap water. After drying, they were cleaned with 96% ethanol. Both 1mm spacers were also cleaned with 96% ethanol and placed on the large glass plates. The clamps were scrwed to the sides of the sandwich, in order to be sure that the spacers, 2 glass plates and especially the glass plates were aligned at the bottom side of the sandwich and placed in the holder. The clamps were unscrewed and the alignment card slid between the glass plates to align the spacers. The clamps were screwed and the alignment of the glass plates were checked. Then the sandwich was placed on top of the rubber gasket and the handles pressed down.

3.14.2.3. Preparation of the gel

One tube of APS 10% per gel was prepared. The tubing needle was replaced with a new one, the screw between the compartments was opened and the compartments rinsed with water using pump at a uniform speed. The system was completely drained and flushed with compressed air. The gel solutions were prepared as required. Stacking gel was also prepared according to the following:

UF solution	UF (%)	Acrylamide/ bis (%)	Volume UF solution (ml)	Volume APS 10% (µl)	Volume TEMED (µl)
Low	30-45	6	13	78	6
High	60	6	13	78	6

APS and TEMED was added to the high and low solutions according to the table, stirred gently by hand and proceeded immediately for pouring the high concentration solution in the compartment closest to the outlet of the gradient mixer and the low concentration solution in the other compartment by the delivery system. The whole system was kept for polymerization.

3.14.2.4. Running a gel

Fresh 0.5X TAE buffer was added to the buffer tank to the mark "Fill". The DCode^{TM} Universal Mutation Detection System (Bio-Rad) was switched on at least 60 minutes before electrophoresis, so that the buffer can heat up to 60°C. After 2-3 hours of

polymerization, the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel. The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the other side to get a closed upper buffer compartment. (A dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode^{TM} was then switched off and the lid taken off after 1 min. The sandwich holder was then slided into the buffer tank, with the red dot of the cathode at the right side. The DCode^{TM} pump and the stirrer underneath the tank were switched on (300 rpm) until the samples were loaded.

3.14.2.5. Staining of gels and photography

Before taking DGGE units out from the tank, the run-evaporated H2O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50X TAE buffer and the gel was stained for 5 minutes and destained with running buffer. The gel was photographed under UV-transilluminator.

3.14.2.6. Data Analysis

3.14.2.6.1. Scoring of individual band

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme BioProfil 1D and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods protograph the gels were scanned into a computer and saved as graphic files.

3.14.2.6.2. UPGMA analysis of the DGGE bands.

Variability among the different groups of isolates were detected on the basis of the banding pattern obtained on denature gradient gel. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *in silico* into similarity matrix using NTYSYpc (Version 2.11W) as in case of RAPD analysis. However, a more complex analysis involved cladistic analysis of data and reconstruction of phylogenetic tree. A two (2-D) and three dimension (3-D) principal component analysis was constructed to provide another means and test the relationship among different tested groups using EIGEN programme (NTSYS-PC).

3.15. Assessment of disease caused by fungal pathogens on som plants

3.15.1. Detached leaf inoculation

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded som leaves detached from plants were placed in trays lined with moist blotting paper. Wounds were made on adaxial surface of each leaf with 26 G½ needle and inoculated with 20µl droplets of spore suspension (1.2 X 10^6 conidia ml^{-1}) of the fungus (prepared from 14 days old culture in PDA). Spore suspension was placed (2-4 drops $leaf^{-1}$) on the adaxial surface of each leaf with a hypodermis syringe on the wounds. In control sets drops of sterile distilled water were placed on the wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation.

Percent drops that resulted in lesion production were calculated after 48, 72 and 96 hours of incubation as described by Chakraborty and Saha (1994). Observations were made based on 50 inoculated leaves for each treatment in average of three separate trials.

3.15.2. Whole Plant inoculation

Whole plant inoculation was carried out essentially as described by Mathur *et al* (2000) with minor modifications. The fungus was grown in PDA for 14 days at 30°±2°C and spore suspension was prepared (1.2 X 10^6 conidia ml^{-1}). Tween-20 was added @ 2ml l^{-1} to facilitate adhering of the spores to leaf surface. 2 year old plants were spray-inoculated with an atomizer @ 100ml per plant so as to wet both ventral and dorsal surfaces. The plants were immediately covered with polythene bags so as to maintain high relative humidity and kept overnight. Next day, the polythene bags were removed and transferred to glass house benches and maintained at 30°±2°C.

The disease severity on plant leaves was recorded using a 0-5 rating scale (Lakshmi et al 2011), where 0 = no infection; 1 = up to 5%; 2 = 6-10%; 3 = 11 20%; 4 = 21-50% and 5 = > 50% leaf area affected by the disease. Based on this numerical ratinga Percent Disease Index (PDI) was calculated using the formula:

PDI = [total numerical ratings/(number of leaves examined × max rating scale)]× 100.

Results were always computed as the mean of observations of 25 well-established and branched 7 month old som plants in average of three separate experiments.

3.16. In vitro testing for antagonism to fungal pathogens

3.16.1. Antifungal test of Plant growth promoting rhizobacteria (PGPR)

The obtained PGPR isolates were evaluated against leaf pathogen *C. gloeosporioides* and *P. disseminata* in dual culture using NA medium. The bacteria were streaked on one side of the Petri plate and 4mm fungal pathogen block was placed at the other side of theplate, incubation was undertaken for 5-7 days at 28º±2ºC and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates. For each test three replicate plates were used. Those bacteria, which were antagonistic to *C. gloeosporioides* and *P. disseminata*, were selected for further evaluation and identification.

3.16.2. Antifungal test of Plant growth promoting fungus (PGPF)

The efficacy of PGPF (*Trichoderma* sp.) isolates was tested *in vitro* for inhibiting growth of the pathogen (*C. gloeosporioides* and *P. disseminata*) in dual culture using PDA. Each fungal isolate was placed at one side of the agar plate about 1cm away from the edge and a 4mm diameter block of the pathogen, taken from growing edge replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28ºC and inhibition zone towards the fungus colony in individual plate was quantified. Results were expressed as mean % of inhibition in presence of the fungal isolate.

3.17. Mass multiplication and application of bioinoculants and pathogen

3.17.1 Arbuscular Mycorrhizal Fungi (AMF)

3.17.1.1. Isolation of AMF spores

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil of som by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Approximately 250 g of soil was suspended in 1 L water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200). Pores are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through these sieves was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve and spores collected by fine brushes and were kept in different Petri plates according to their size and colours. Moreover for further observations or purification of AMF spores sucrose gradient centrifugation method was used. In sucrose gradient centrifugation (Daniels and Skipper, 1982),

spores and minimal amount of organic particles were further purified by suspending sieving in 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) was passed through a sieve of 400 mesh and rinsed with distilled water to remove sucrose residue.

With the help of a simple microscope (20X) parasitized spores, plant debris etc were separated. Spores were sonicated at 30 Hz for two minutes to remove the debris adhered to the spores then clean spores were stained with Melzar's reagent (50% aqueous solution of chloral hydrate with 2.5-3.75% potassium iodide and 0.75-1.25% iodine) and studied microscopically. For further use, the AMF spores were stored in Ringer's Solution (8.6g NaCl, 0.3g KCl, 0.33g $CaCl_2$ in 1 L of boiled distilled water) at -15ºC to - 20 ºC or in sterile distilled water. Identification of genera and species was done microscopically using the specific spore characters such as size, colour, shape, wall structure, surface ornamentation and bulbous suspensor by using identification manuals (Trappe, 1982; Schenck and Perez, 1990).

3.17.1.2 Histopathology of som roots

Fungal association of AM fungi within the root tissues was observed according to Philips & Hayman (1970). Young roots from mandarin plants were dug out manually. Roots were cut into 1cm or smaller pieces and washed in tap water gently to free them from soil particles. It was boiled in 2% KOH in hot water bath for 1 hour. The KOH was decanted and the roots washed with water for 2-3 times. 1% HCL was added and kept for 30 minutes. After decanting the HCL the sample was washed thrice in tap water and cotton blue, lactic acid and glycerol was added in the ratio 1:1:1 to stain the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells, and boiled in water bath for 1 hour. The excess stain was decanted and sample placed in 50% glycerol for destaining. The roots were then crushed under pressure in slide and covered with cover slip for microscopic observation. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.17.1.3 Mass multiplication of AMF

AMF spores were multiplied in roots of maize as host plant. AMF spores were isolated from rhizosphere of all eight morphotypes of som plant using decanting and sieving method as described earlier. The mass of spores were washed with distilled water several times to remove the adhered debris. Filter paper was cut into small bits about the size of 1 cm. With the help of fine tweezers, 45-50 AMF spores were placed in the filter paper bits. They were then carefully placed onto the roots of the 7-10 days

old host seedling in plastic pots (12inch) having autoclaved soil to discard the presence of other fungal propagules. Maize plants were grown both in the field and pots. After 45 days, the presence of spores was verified and inocula were prepared by mixing the chopped roots of maize plants with the potted soil where extra radical spores of AMF were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.17.2 Plant Growth promoting Fungi (PGPF)

3.17.2.1. Selection of PGPF

One isolate of *Trichoderma harzianum* (BRHS/480) and another isolate of *Trichoderma asperellum* (RHS/S569) were selected as Plant growth promoting fungi based on their performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.*, 2014)

3.17.2.2 Mass multiplication

3.17.2.2.1 Wheat bran media

Inoculum of *Trichoderma asperellum* and *Trichoderma harzianum* were prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28ºC for 10 days. To each pot of soil (2000 g), 10 g of the wheat bran colonized by *T.asperellum* and *T. harzianum* was mixed to give a concentration of 105 cfu / g of soil as described by Chakraborty *et al.*, (2003).

3.17.2.2.2 Tricho-compost

Six layers of compost materials (each layer about 25 cm thick) was made. 3 parts cellulosic waste (rice straw, grass, corn stalk, spent mushroom substrate) and 1 part mixture of leguminous plant materials (Mungbean, Peanut, Soybean) and animal manure was mixed. Each layer of piled compost materials was sprinkled with 30 litres of Tricho inoculants solution. Additional water is sprinkled to keep the compost heap moist. It was covered with plastic sheet or sack to increase temperature and prevent too much water in case of rainfall. Compost heap was turned from top to bottom after two weeks. The Tricho compost was ready for harvest four weeks after preparation. The compost was stored in sacks or applied directly into the soil.

3.17.3. Plant Growth promoting Rhizobacteria (PGPR)

One isolate of *Bacillus pumilus* (BRHS/C1) and one isolate of *Bacillus altitudinus* (BRHS/S73) were selected as Plant growth promoting rhizobacteria based on their

performance as potential growth promoters as well as their biocontrol efficiency in field grown crops (Sunar *et al.*, 2013)

3.17.3.1. Selection of PGPR

3.17.3.2 Mass multiplication

3.17.3.2.1 Soil drench

The bacteria were grown in NB for 48 h at 28ºC and centrifuged at 12,000rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3 $X10^6$ cfu ml^{-1}.The bacterial suspension was applied to the pots during transplantation of seedlings. Applications were done @ 100 ml per pot at regular interval of one month for three months subsequently. The rhizosphere of two year‘s old potted plant was inoculated twice at an interval of 20-25 days.

3.17.3.2.2 Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of 3x106 cfu ml-1 after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the ten year old bushes after pruning. The spraying was done forth nightly till the new shoots started appearing.

3.17.3.2.3 Talc based formulation

Ten g of carboxy methyl cellulose sodium salt (Himedia) was mixed with one kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized twice for 30 min each. The bacterium was first grown in nutrient broth and after 48 h the actively growing cells in log phase were harvested by centrifugation at 21 000 g, and aqueous suspension was made to achieve a concentration of $3x10^9$ CFU ml^{-1} which was determined spectrophotometrically. To 1 kg of sterilized talcum powder 400 ml of bacterial inoculum was added and mixed well under sterile condition. The talc mix was dried under shade to bring moisture to less than 20%. The formulation was packed in milky white colour polythene bags to eliminate UV exposure, sealed and stored at room temperature for future use. The talcum based formulation was applied in the field at the rate of 100 g per pot (12 $x10^{10}$ bacterial cells).

3.17.4 Vermicompost preparation

Vermicompost was prepared in plastic beds using organic waste materials collected from the local area. 15-20 cm layer of this waste was covered with another 2-3 cm of

dried aquatic plants. *Eisenia foetida*, the earthworm used for vermicomposting was added on the top. The final top layer was made of dried cow dung and the vermin bed was sealed with plastic cover. This set was kept undisturbed for 15-20 days, after which the bed was stirred and shaken to release the organic gas produced during vermicomposting process and for proper mixing of the materials. The compost was ready after 40-45 days when it turned into black light weight powder with no odour. After its completion the earthworms are separated from the final product and the manure was dried and sieved for further use. This vermicompost was applied to the field at the rate 200g per pot.

3.17.5 Application of different bioinoculants under pot and field condition

In case of pot treatment, initially Vermicompost is added to the soil @ 200g/pot. Then AMF spores were added to the roots of som sapling using filter paper disc. After 15 days of treatment, *T. asperellum* in wheat bran medium was added to the pots. Two weeks after application of PGPF, foliar spray as well as soil drench application of PGPR was done for 15 days at 5 days interval. In case of joint treatment with all three bioinoculants, they were added to the pots sequentially but in case of dual treatments (AMF+PGPF, AMF+PGPR, PGPF+PGPR) application was done accordingly.

For field inoculation, chopped maize roots colonized with dominant spores of *G. constrictum* (AMF) were applied in the root rhizosphere following transplantation in the field from nursery- grown 7 month old plants. One month following application of AMF, root colonization status was examined. Then mass multiplied *T. asperellum* made with wheat bran was applied in soil. Two weeks after application of *T. asperellum* (PGPF), further soil application of talc based formulation as well as foliar spray of *B. altitudinus* (PGPR) was done. Growth parameters were finally recorded after 6 months of application of last treatment.

3.17.6. Inoculum preparation of pathogen

The leaf blight pathogen, *Colletotrichum gloeosporioides* and the grey blight pathogen *Pestalotiopsis disseminata* were grown in 100ml PDA medium for 7-10 d till sporulation occurred. The spores were then scraped off from the surface of the media with the help of inoculating needle and the spores were collected insterile distilled water. The spore suspension containing3×10^4 spores/ml with 0.01% Tween 20 was sprayed on to the treated as well untreated potted plants and covered with plastic bags for 48 h.

3.18. *In vivo* assessment of plant growth promotion

3.18.1. Assessment of plant growth following application of bioinoculants

Plant growth promotion was recorded after 30 and 60 d of application of bioinoculants in potted plants and after four months in the field grown plants. The growth parameters such as number of leaves, branches and height were observed.

3.18.2. Assessment of disease severity.

The disease severity on plant leaves was recorded using a 0-5 rating scale (*Lakshmi et al 2011*), where 0 = no infection; 1 = up to 5%; 2 = 6-10%; 3 = 11 20% 4 = 21-50% and 5 = > 50% leaf area affected by the disease. Based on this numerical rating a Percent Disease Index (PDI) was calculated using the formula:

PDI = [total numerical ratings/(number of leaves examined × max rating scale)]× 100.

3.18.3. Assessment of soil phosphate mobilization

3.18.3.1. Extraction of Soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich, 1984). Quantitative estimation of phosphate was done following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988).

3.18.3.2. Estimation of soil phosphate

For estimation, 2 ml aliquot of the soil extract was transferred to test tube along with the transfer of 2 ml aliquots of each of the working standards. Then 8 ml of the colorimetric working solution (25 ml conc. ammonium paramolybdate solution, 10 ml ascorbic acid soln., final volume- 1000ml) was added to each test tube and mixed thoroughly. They were allowed to wait for 20 mins for colour development. Finally, % transmittance of all standards and samples on a colorimeter with wavelength set at 882 nm was recorded.

3.19. Extraction and Assay of defense enzyme activities after application of bioinoculants

3.19.1. ß-1, 3- glucanase (E.C. 3.2.3.39)

Extraction of ß-1,3- glucanase (E.C. 3.2.3.39) was done following the method described by Pan et al.. (1991). Mandarin root and leaf samples (1g) were crushed in

liquid nitrogen and extracted using 5ml of chilled 0.05 M sodium acetate buffer (pH 5,0) by grinding at 4 ºC using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4 ºC and the supernatant was used as crude enzyme extract.

Estimation of the ß-1,3-glucanase was done by following the Laminarin dinitrosalicylate method (Pan et al., 1991): The crude enzyme extract of 62.5µl was added to 62.5 µl of laminarin (4%) and then incubated at 40ºC for 10 minutes. The reaction was stopped by adding 375µl dinitrosalicylic reagent and heating for 5 min in boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissues

3.19.2. Chitinase (E.C. 3.2.1.14)

Extraction of chitinase (E.C. 3.2.1.14) was done by following the method described by Boller and Mauch (1988) with modifications. 1g root and leaf sample from mandarin plants were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1M Sodium Citrate buffer (pH 5). The homogenate was centrifuged for 10minutes at 12,000rpm and the supernatant was used as enzyme source.

Chitinase activity was measured according to the method described by (Boller and Mauch, 1988). The assay mixture consisted of 10µl Na-acetate buffer (1M) pH 4, 0.4ml of enzyme solution, 0.1ml of colloidal chitin (1mg). Colloidal chitin was prepared as per the method of (Roberts and Selitrennikoff, 1988). After 2h of incubation at 37 ºC the reaction was stopped by centrifugation at 10,000g for 3minutes. An aliquot of supernatant(0.3ml) was pipetted into a glass reagent tube containing 30µl of potassium phosphatebuffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzymeHelicase (Sigma) for 1hour. After 1h, the pH of the reaction mixture was brought to 8.9by addition of 70µl of sodium borate buffer (1M) pH9.8. The mixture was incubated in a boiling water bath for 3minutes and then rapidly cooled in an ice water bath. After addition of 2ml of DMAB (ρ-dimethyl aminobenzaldehyde) reagent, the mixture was incubated for 20 min at 37 ºC. There of absorbance value at 585nm was measured using a UV-VISspectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as µg GLcNAc min^{-1} mg^{-1} fresh tissues.

3.19.3. Phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5)

Extraction of PAL (E.C. 4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1gm root and leaf sample was crushed in 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß mercaptoethanol in ice cold temperature. The slurry was centrifuged in 15000 rpm for 20 minutes at 4ºC. Supernatant was collected and after recording its volume, was immediately used for assay or stored at -20ºC.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 3 µM L- phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1 h at 40 ºC the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min g -1 fresh weight of tissues.

3.19.4.Peroxidase (E.C. 1.11.1.7)

For the extraction of peroxidase (E.C.1.11.1.7) the plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) containing 2 mM ß mercaptoethanol under ice cold conditions, the homogenate was centrifuged immediately at 15000 rpm for 20 minutes at 4 ºC. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20 ºC (Chakraborty *et al..*, 1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100µl of 4mM H2O2, 100 µl O-dianisidine (5mg ml-1 methanol) and 1.7ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in presence of H_2O_2 (Chakraborty *etal..*, 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1}.

3.19.5. Isozyme analysis of peroxidase

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold condition as described by Davis (1964) and used immediately for the isozyme analysis.

3.19.5.1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N‘ N‘ methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of N‘ N‘ bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of N, N, N‘, N‘-tetramethyl ethylene diamine (TEMED) was added. The pH was adjusted to 8.9 with concentrated HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g glycine in 1 L of distilled water.

3.19.5.2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E:

distilled water in the ratio of 1: 1: 4: 1 by pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2: 1: 1: 4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30- 45 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoretic apparatus. Tris- Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.19.5.3. Sample Preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

3.19.5.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3 - 4 h at 4°C until the dye front reached the bottom of the gel.

3.1.95.5. Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Gasber (1973). The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7 % Acetic acid. After the appearance of clear blue colored bands, analysis of isozyme was done immediately.

3.19.6. Extraction and estimation of phenol content

3.19.6.1. Extraction of phenol

Phenol was extracted from the fresh young leaves and roots following the method of Mahadevan and Sridhar (1982). One g of sample were cut into pieces and

immediately immersed in 10 ml of boiling alcohol. After 15 minutes of boiling it was cooled and crushed in mortar using pestle thoroughly at room temperature. The extract was filtered through Whatmann No. 1 filter paper. Final volume was adjusted with 80 % ethanol. The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

3.19.6.2. Estimation of Total phenol

Total phenol content was estimated by Folin Ciocalteau‘s reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin Ciocalteau‘s reagent followed by 2 ml of 20 % sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.19.6.3. Estimation of Ortho-phenol

O-dihydroxy phenol was also estimated following the method of Mahadevan and Sridhar (1982). 1 ml of alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow‘s reagent ($NaNO_2$ - 10 g, Na_2MoO_4 - 10 g, distilled water - 100 ml) and 2 ml of 1 N NaOH and mixed thoroughly at room temperature following which the volume of the reaction mixture was raised to 10 ml. Absorbance of the colored solution was recorded at 515 nm. Quantity of the O- dihydroxy phenol was estimated using caffeic acid as standard.

3.20. Analysis of antifungal compounds

3.20.1. Collection of leaf diffusates and their bioassay

Leaf diffusates were obtained by a modified drop diffusate technique of Muller (1958). Forty young leaves, each of 8 different morphotypes, were collected from green house, washed and placed on moist filter paper in separate trays. Wound was made in the leaves using needle and spore suspension of *C. gloeosporioides* was placed on the wounds using Pasteur pipette and incubated for 2 days. In case of control, distilled water was placed on the wounds in place of spore suspension. Drops of spore suspension were collected from each leaf of the eight morphotypes separately, centrifuged and supernatants collected. These were passed through

sintered glass filter and their biological activities were assayed on spore germination and appressoria formation (Chakraborty *et al*., 1995).

3.20.2. HPLC analysis of phenolic compounds

Fresh leaves of som plant were chopped into pieces and soaked overnight in methanol in the ratio 1:3 (w/v), filtered through Buckner's funnel and the solvent was evaporated using lyophilizer as described by Pari and Latha (2004). The dried powder was finally mixed in HPLC graded methanol and stored at 4°C for further analysis. HPLC analysis of phenolic compounds present in the extracts was done using SPD-10A VP Shimadzu UV-VIS Detector. A flow rate of 1 mL/min, and gradient elution of acetonitrile-water-acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0– 50 min solvent B from 0 to 100%; and injection volume of 20 µl were applied; whereas the separation of compounds was monitored at 280 nm (Pari *et al.*, 2007).

3.21. Scanning Electron Microscopy

Spores of fungal pathogens were examined under scanning electron microscopy (SEM). Selected fungal spores were sonicated under 35 MHz to followed by washing five times in sterile distilled water, surface disinfected with 4% (wt/vol) chloramine-T and 300 ppm of streptomycin for 1 h, and then rinsed a further five times in sterile distilled water and were stored in eppendorf‟s tube in room temperature. Each sample was placed within separate aluminium "disc cup" (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

3.22. Transmission Electron Microscopy

3.22.1. Specimen preparation

3.22.1.1. Fixation

Control and inoculated root samples (1-2 mm) were excised in 0.1M sodium phosphate buffer pH 7.4. They were immediately transferred to 2.5% Glutaraldehyde in eppendorf tubes for 2-12 hours at room temperature.

3.22.1.2. Dehydration

Dehydration was done in ascending grades of alcohol at intervals of 30 mins in 4° C (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 hr interval each at 4° C in PLT-272(M) Fume Hood (Tanco).

3.22.1.3. Infiltration

Infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1 hr each at 4° C.

3.22.1.4. Embedding

The samples were dipped in LR White and kept overnight at 4° C. They were keptat room temperature for 3 hrs. A fresh change of LR white was done and kept at 56° C for36 hrs.

3.22.2. Viewing preparation

3.22.2.1. Trimming

Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM60) fitted with a rotating milling cutter

3.22.2.2. Sectioning

A series of thick sections of the selected blocks were cut with Belgium glass strips in microtome (Leica EM UC7) to observe under an optical microscope. These semithin sections are stained with 1% aqueous toluidine blue solution. These sections can be viewed in light microscope.

3.22.3. Immunogold labeling

Ultrathin sections (60nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labeling.

3.22.3.1. Primary antibody

The grids containing ultrathin sections were floated in blocking solution containing 2% skimmed milk agar for 30 min. Primary antibody was diluted in 1% fish gelatin in the ratio 1:20. Grids were incubated the PAbs for 24 hrs at 4° C. Grids were washed on drops (100 µl) of fish gelatin pipetted on to parafilm 10X2 min.

3.22.3.2. Secondary antibody

Grids were incubated with anti-rabbit IgG (Whole Molecule) gold antibody produced in goat affinity isolated antibody (Sigma-G7402) diluted in 1:5 in fish gelatin at room temp for 3 hrs.

3.22.4. Staining

Sections were stained with 2% uranyl acetate for 15 min. The sections were washed in double distilled water. Post stain was done in 0.2% lead acetate for 5 min. Washed again in double distilled water.

3.22.5. Viewing

Ultrastructural analysis of the section was performed with Morgagni 268D with iTEM Imaging System. Specificity of labeling was assessed by the control test by incubating sections with rabbit pre-immune serum instead of the primary antibody.

Chapter 4
RESULTS

4.1 Foliar fungal Disease incidence of som plants (*Persea bombycina* Kost)

Som plant is vulnerable to many foliar diseases that affect the normal growth of the plant, quantity and quality of leaves and ultimately cocoon yield production. Leaf spot, red rust, leaf blight and grey blight are the major foliar diseases of som.

4.1.1 Leaf Blight

Leaf blight disease is one of the major foliar fungal diseases of som plant, caused by *Colletotrichum gloeosporioides.* It leads to premature leaf fall and causes approximately 6.3% of the total leaf yield loss. The disease appears on young and mature leaves in the form of brown colour, round to oval spots that irregularly spread to the entire leaf. As the disease progresses, the spots get collapsed and malformed. The infected areas dry up and become brown to black in colour. At the advanced stage of disease development, brownish colour (stromatic masses or sclerotia) lesions / streaks appears on the twigs also. The top of the branch or the entire brunch may wither away (Figure 3). Maximum blight infection was noted during the month of April-August in nursery grown plant at experimental station. The disease incidence was recorded for all the eight morphotypes of som plants which were maintained in nursery as well as grown in the experimental field. Results as described in figure 5 revealed that the percentage disease index was lowest in S7 morphotype and highest in S3 and S6morphotype.

4.1.2 Grey Blight

Grey blight caused by *Pestalotiopsis disseminata* has been reported as a major epidemic disease of muga host plant, causing 13.8-41.6% leaf yield loss. Main symptoms were noticed as appearance of small, oval and discolour lesions scattered irregularly on young and mature leaves. In subsequent days following infection, brown or grey spots developed irregularly and in advanced stages the spots gets collapsed, malformed and the entire leaf withered and dried off (Figure 4).Under nursery condition, presence of grey blight disease was recorded and percentage disease incidence (PDI) was determined and presented in figure 5. It was recorded that establishment of disease was highest in S5 morphotype and lowest in S3 morphotype.

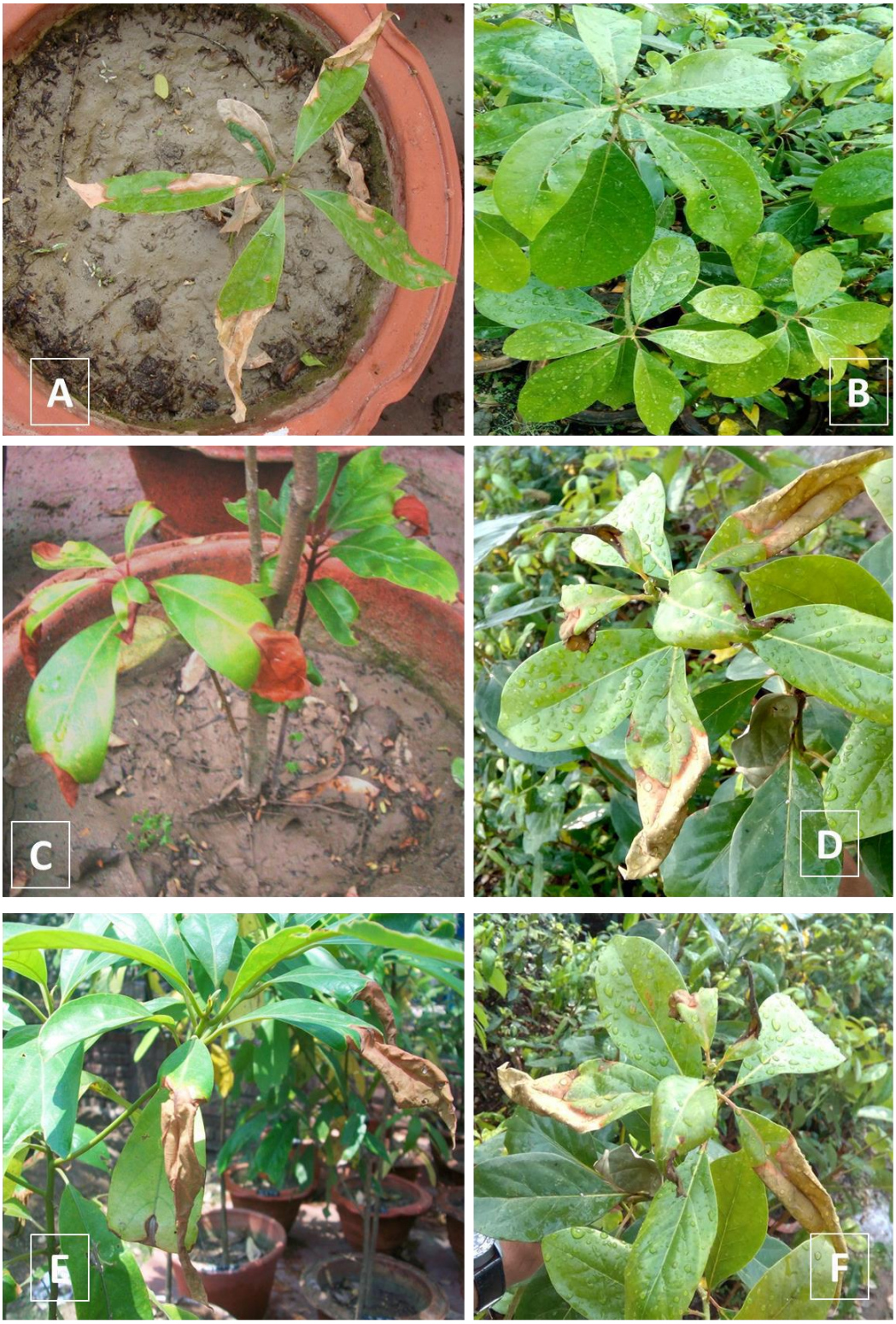


Figure 3: Leaf blight disease incidence of nursery grown (A,C,E) and field grown (B,D,F) som plants. Healthy leaf (B), Naturally infected leaf (A,C,D,E,F); Morphotype: (A) – S3, (B, D, F) – S6, (C) – S5, (E) – S4

Figure 4: Grey blight disease incidence in field grown som plants. Healthy leaf (A) naturally infected (B-D); Morphotype: (A) – S3, (B) – S5, (C) – S6 and (D) – S7

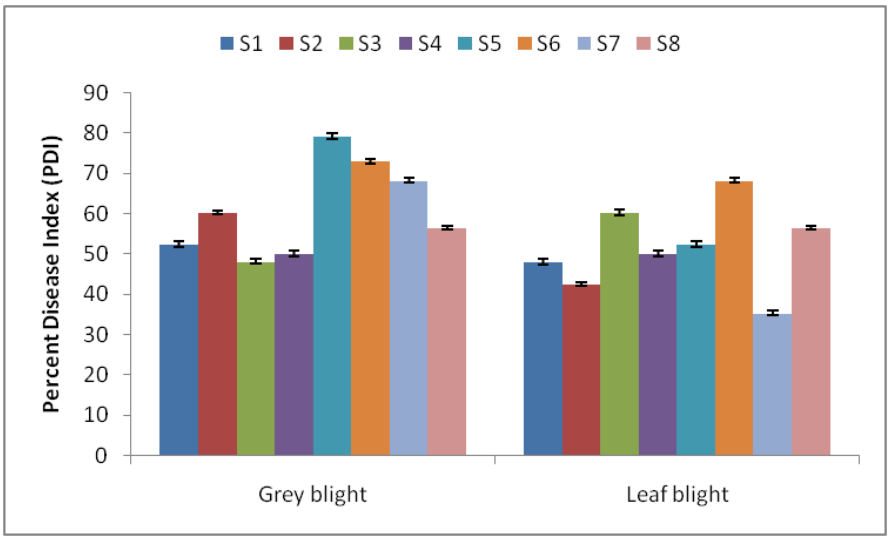


Figure 5: Percent Disease Index (PDI) of leaf blight and grey blight disease in nursery grown som plants

4.1.3 Completion of Koch's postulate

Isolation of pathogen was carried out from leaf blight and grey blight infected leaf samples according to the methods mentioned in earlier section. Three different isolates from leaf blight infected leaves were isolated from S2, S4 and S6 morphotype of som plants and were coded as – SOM/Cl/01, SOM/Cl/02 and SOM/Cl/03. Similarly in case of grey blight infected leaf samples, three more isolates were isolated from S5, S6 and S7 morphotype and coded as – IPL/SOM/P/01, IPL/SOM/P/02 and IPL/SOM/P/03.

Out of these six isolates, two isolates namely, IPL/SOM/P/01 and SOM/Cl/02 were used for artificial inoculation of som leaves of all eight morphotypes using detached leaf inoculation technique. Disease symptoms were noted after 48hrs of inoculation. Pathogen was re-isolated from these infected leaves and was confirmed with the original isolates using morphological identification techniques.

Hence these two isolates, IPL/SOM/P/01 and SOM/Cl/02 was taken into consideration for further morphological identification and molecular classification using 18S rDNA sequencing technique and phylogenetic analysis.

4.2 Growth and spore characters of *Pestalotiopsis disseminata*

The three isolates of *P. disseminata* obtained from three different morphotypes (S5, S6 and S7) of som plants and these were coded as IPL/SOM/P/01, IPL/SOM/P/02 and IPL/SOM/P/03. These isolates were maintained in PDA slants. Koch's postulate was performed in order to confirm the disease causing capacity of the isolates. For assessment of their growth rate and sporulation, three different media (PDA, OMA and RMA) were used. Mycelial dry weight was measured for all three isolates grown in Richard's medium. Spore characters of the isolates were noted and measured. Total soluble protein was extracted and estimated from mycelia grown in Richard's medium.

4.2.1 Growth

The fungus was grown in three different media, Potato Dextrose Agar (PDA), Richard's Synthetic Agar (RMA) and Oat meal agar (OMA). In each medium mycelia growth was recorded after 4,6,8 and 10 days of incubation at 25±2°C. The result as presented in table 4records that the growth rate on PDA was highest and on OMA was lowest. The growth rate ranged from 9.6mmto 13.5mm on the 10^{th} day of incubation.

The fungus generally exhibited white mycelia growth from which black acervuli developed (Figure 8 A,B,C).

Growth in liquid synthetic medium (RM) was measured for the three isolates separately by taking dry weight. The resulting data is presented in table 5. The dry weight accumulated was highest 20 days after inoculation in *P. disseminata*, irrespective of the isolate. Maximum growth (300mg g^{-1} tissue) was recorded for IPL/SOM/P/01 isolate whereas IPL/SOM/02 has the lowest weight (200mg g^{-1} tissue).

Table 4: Mycelial growth of *P. disseminata* isolates in different solid media

Isolate	Diameter of mycelia (mm)[a]		
	Media		
	Potato Dextrose Agar	Richard's Agar	Oat Meal Agar
IPL/SOM/P/01	13.5±0.5	11.2±0.5	10.1±0.2
IPL/SOM/P/02	11.5±0.3	10.8±0.4	9.2±0.5
IPL/SOM/P/03	10.6±0.4	10.2±0.2	9.6 ±0.5

[a]10 days after incubation, ± Standard Error, Average of three replicates, Incubation at 25±2°C

Table 5: Mycelial growth of *P. disseminata* isolates in Richard's solution

Days after inoculation	Growth rate (mg)		
	Isolates		
	IPL/SOM/P/01	IPL/SOM/P/02	IPL/SOM/P/03
5	115±1.02	92±1.08	106±1.12
10	229±1.14	115±1.02	152±1.11
15	285±1.15	178±1.06	204±1.19
20	300±1.23	200±1.02	285±1.14

± Standard Error, Average of three replicates, incubation at 25±2°C

4.2.2 Sporulation

Sporulation was assessed in all the three isolates of *P. disseminata* in three different media (PDA, OMA and RMA). Highest sporulation was observed in PDA medium for all the three isolates whereas lowest sporulation was observed in OMA media (Figure 6). Isolate IPL/SOM/P/01 showed highest sporulation among all the three isolates.

4.2.3 Spore morphology

Large no. of conidia were produced within the acervuli. Conidial morphology was studied under Scanning electron microscope (Figure 7) as well as bright field Leica microscope (Figure 8 D,E.F). Conidia is long, fusiform, straight, rarely curved, 4-celled, slightly constricted at septa, concolourous median cells, apical and basal cells hyaline, apical cell cylindrical with 2 apical appendages (setulae) 25µm long,

basal cell conical with a short basal appendage. Presence of relatively long apical appendages that are unbranched and unknobbed and attached to the tip of apical cells.

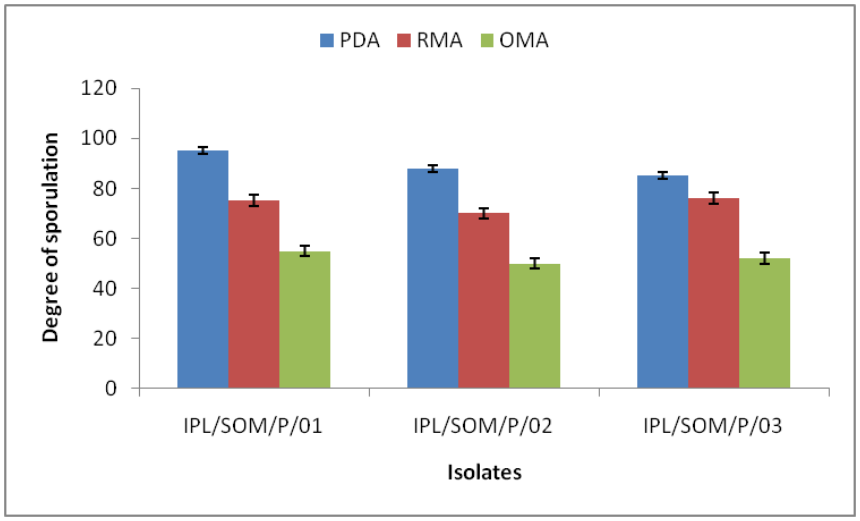


Figure 6: Percentage sporulation of *P. disseminata* isolates in three different solid media (PDA – Potato Dextrose Agar, RM – Richard's Medium, OMA – Oat Meal Agar)

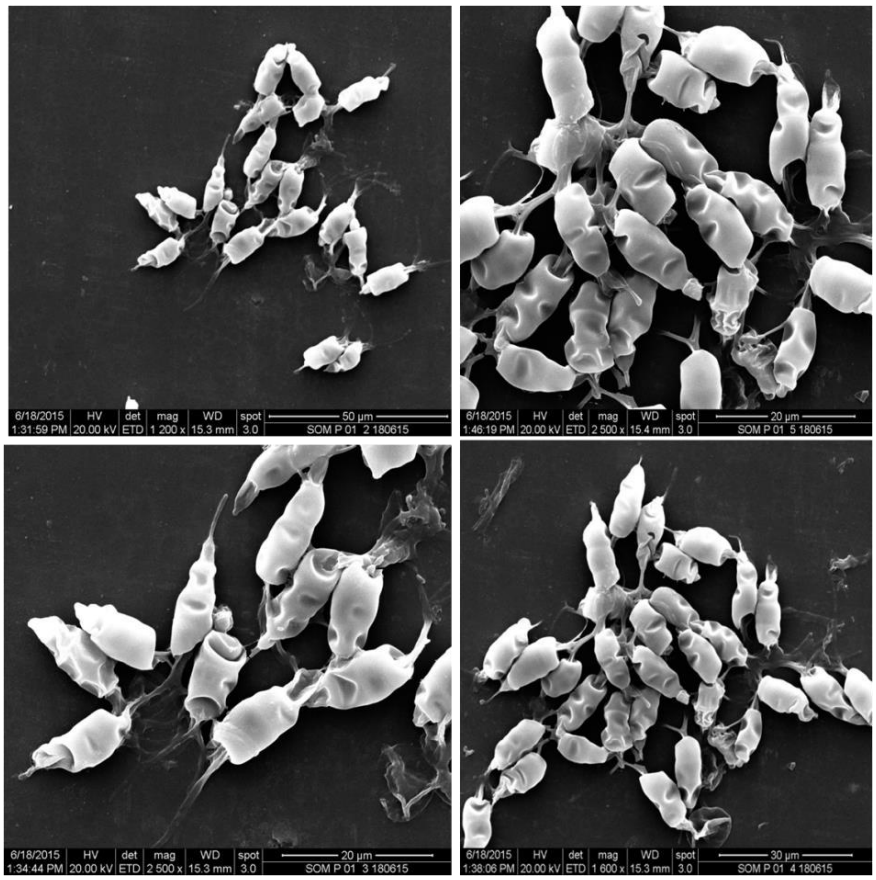


Figure 7: Scanning electron microscopic observations of *P. disseminata* spores (isolate IPL/SOM/P/01)

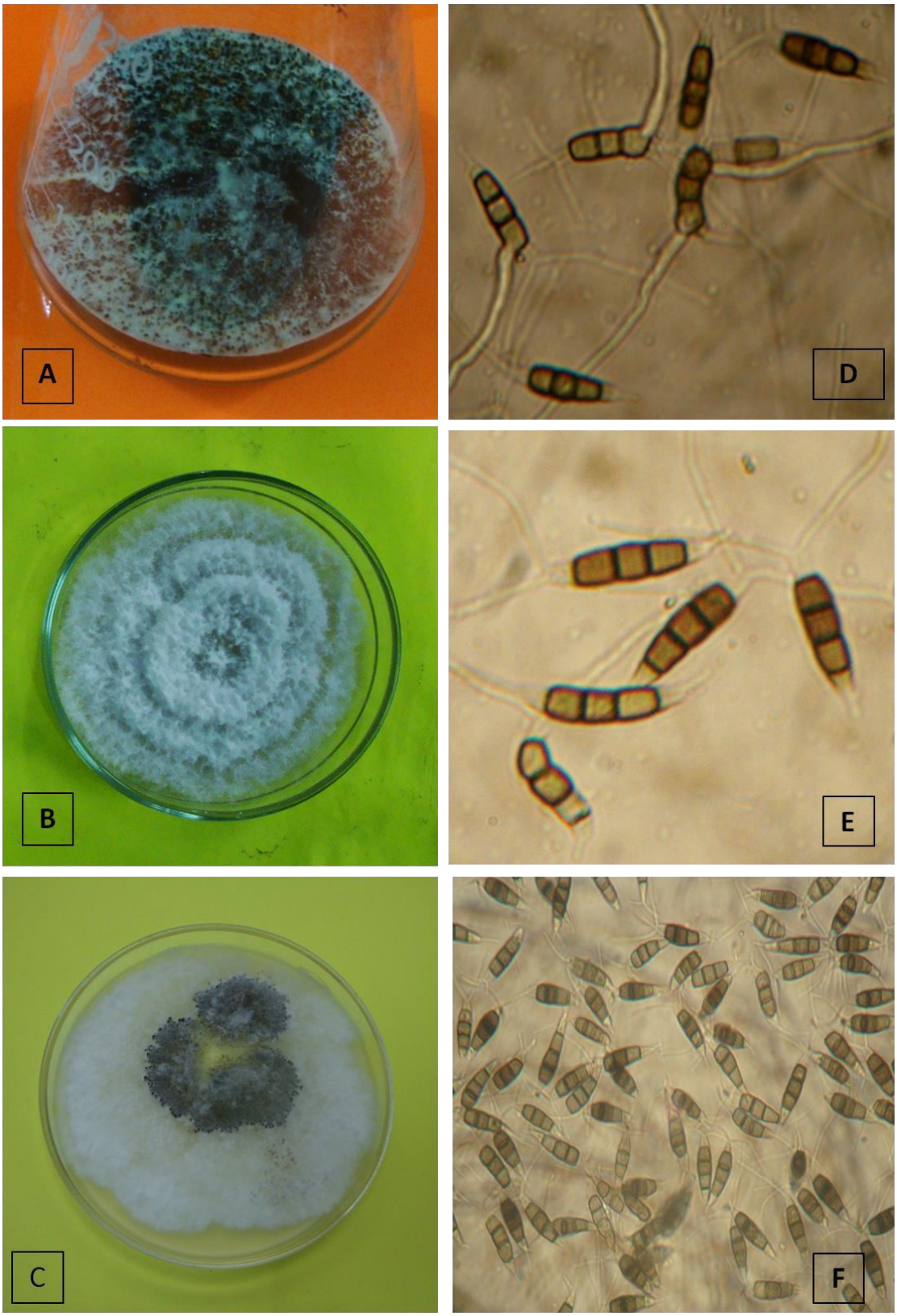


Figure 8: Growth of *P. disseminata* isolates in PDA media (A-C) and spore characteristics (D-F); (A,D) – IPL/SOM/P/01; (B,E) – IPL/SOM/P/02; (C,F) – IPL/SOM/P/03

4.3 Growth and spore characters of *Colletotrichum gloeosporioides*

Three isolates of *C. gloeosporioides* were obtained from three different morphotypes (S2, S4 and S6) of naturally leaf blight infected som plants and were coded as SOM/Cl/01, SOM/Cl/02 and SOM/Cl/03. These were all maintained in PDA slants and Koch's postulates were completed for confirmation of the disease causing capacity.

For assessment of growth rate and sporulation, different solid media, Potato dextrose Agar (PDA), Richard's Media(RMA) and Oat Meal Agar (OMA) in petridishes were inoculated with 4mm dia plugs taken from the periphery of young fungal culture of specified media, incubated at 25°C, and growth rate was measured by taking readings at every 24hrs. Mycelial dry weight was measured for all three isolates grown in liquid Richard's Solution. Spores of the isolates grown on PDA medium after 14 days of incubation were measured with the help of ocular micrometer. The spores of each isolate were germinated separately on glass slides at 25±2°C, and relative humidity at around 90% and their appressorial dimensions measured after 24hrs.

4.3.1 Growth

Growth rates were measured in the three strains of *C. gloeosporioides* in three different solid media – PDA, RMA and OMA. The results are presented in table 6 it is evident that the isolates did not differ significantly with respect to their growth rates in solid media, however there was some difference observed with respect to the type of the solid media used. For all the three isolates growth rate was highest in PDA and lowest in OMA. The growth rates ranged from 8.4 mm day^{-1} to 11.5 mm day^{-1}.The patterns of growth differed depending upon the media used. In PDA mycelial colour was white to greyish white to black on the upper surface. In RMA the mycelial colour was white to orangish white. In OMA the mycelial growth was always digging and sparse (Figure 10 A-C).

Growth in liquid synthetic Richard's solution (RM) was measured for the three isolates separately by taking dry weight. The resulting data is presented in table 7. The dry weight accumulated was highest 25 days after inoculation in *C. gloeosporioides*, irrespective of the isolate. However the dry weight values at that point differed significantly among the isolates. SOM/Cl/02 has the highest weight (423 mg) and SOM/Cl/01 has the lowest weight (235 mg).

4.3.2 Sporulation

Sporulation was assessed in *C. gloeosporioides* isolates in three different media (PDA, OMA and RMA). Highest degree of sporulation was observed in PDA which was similar for all the isolates. Lowest sporulation was observed in OMA. Results are shown in Figure 9. SOM/Cl/02 exhibited highest amount of sporulation among the three isolates.

4.3.3 Spore morphology

Conidial morphology was studied under bright field Leica Microscope (Figure 10D-F). Conidia are cylindrical with rounded apex and truncated base, hyaline and aseptate. Spore dimensions were always measured from spores grown in PDA. Between the isolates, the mean width of conidia did not vary much, the widest being in SOM/Cl/03 (4.5µm) and the narrowest in SOM/Cl/01 (3.2µm). The mean conidial length also didn't vary much among the isolates, the shortest being SOM/Cl/03 (10.5µm) and the longest being SOM/Cl/02 (13.4µm) as shown in Table 8.Conidia germinated by irregularly round brown appressoria.

Table 6: Mycelial growth of *C. gloeosporioides* isolates in different solid media

Isolate	Diameter of mycelia (mm)[a]		
	Media		
	Potato Dextrose Agar	Richard's Agar	Oat Meal Agar
SOM/Cl/01	11.2±0.85	9.6±0.89	8.4±0.79
SOM/Cl/02	11.5±0.78	10.5±0.96	8.9±0.68
SOM/Cl/03	11.4±0.84	10.2±1.04	8.6±0.84

[a]10 days after incubation, ± Standard Error, Average of three replicates, Incubation at 25±2°C

Table 7: Mycelial growth of *C. gloeosporioides* isolates in Richard's solution

Days after inoculation	Growth rate (mg)		
	Isolates		
	SOM/Cl/01	SOM/Cl/02	SOM/Cl/03
5	95±1.02	112±1.08	108±1.12
10	129±1.14	245±1.02	168±1.11
15	185±1.15	378±1.06	259±1.19
20	235±1.23	423±1.02	356±1.14

± Standard Error, Average of three replicates, incubation at 25±2°C

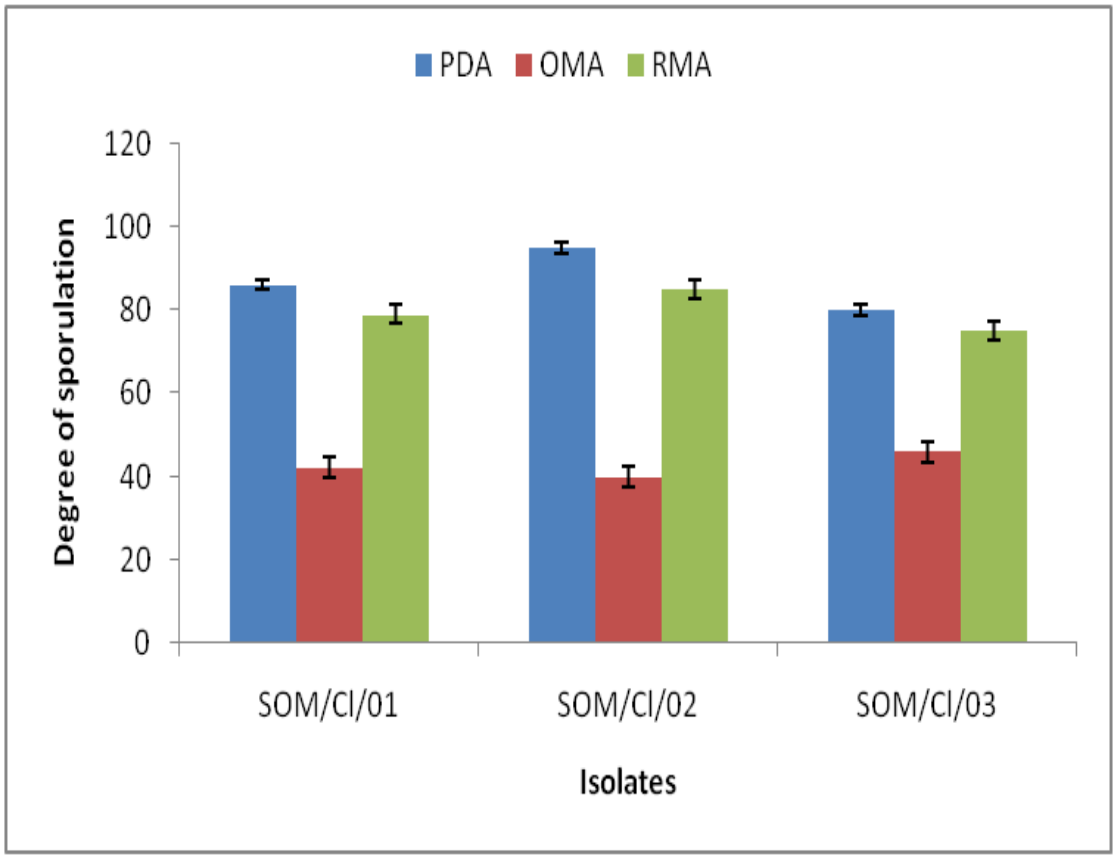


Figure 9: Percentage sporulation of *C. gloeosporioides* isolates in three different solid media (PDA – Potato Dextrose Agar, RM – Richard's Medium, OMA – Oat Meal Agar)

Table 8: Spore characters of *C. gloeosporioides* isolates

Isolate	Spore size (µm)*	
	Length	Width
SOM/Cl/01	11.9±1.12	3.2±1.05
SOM/Cl/02	13.4±1.16	3.9±1.20
SOM/Cl/03	10.5±1.11	4.5±1.15

± Standard Error, *mean of 50 spores, Isolates were grown in Richard's solution

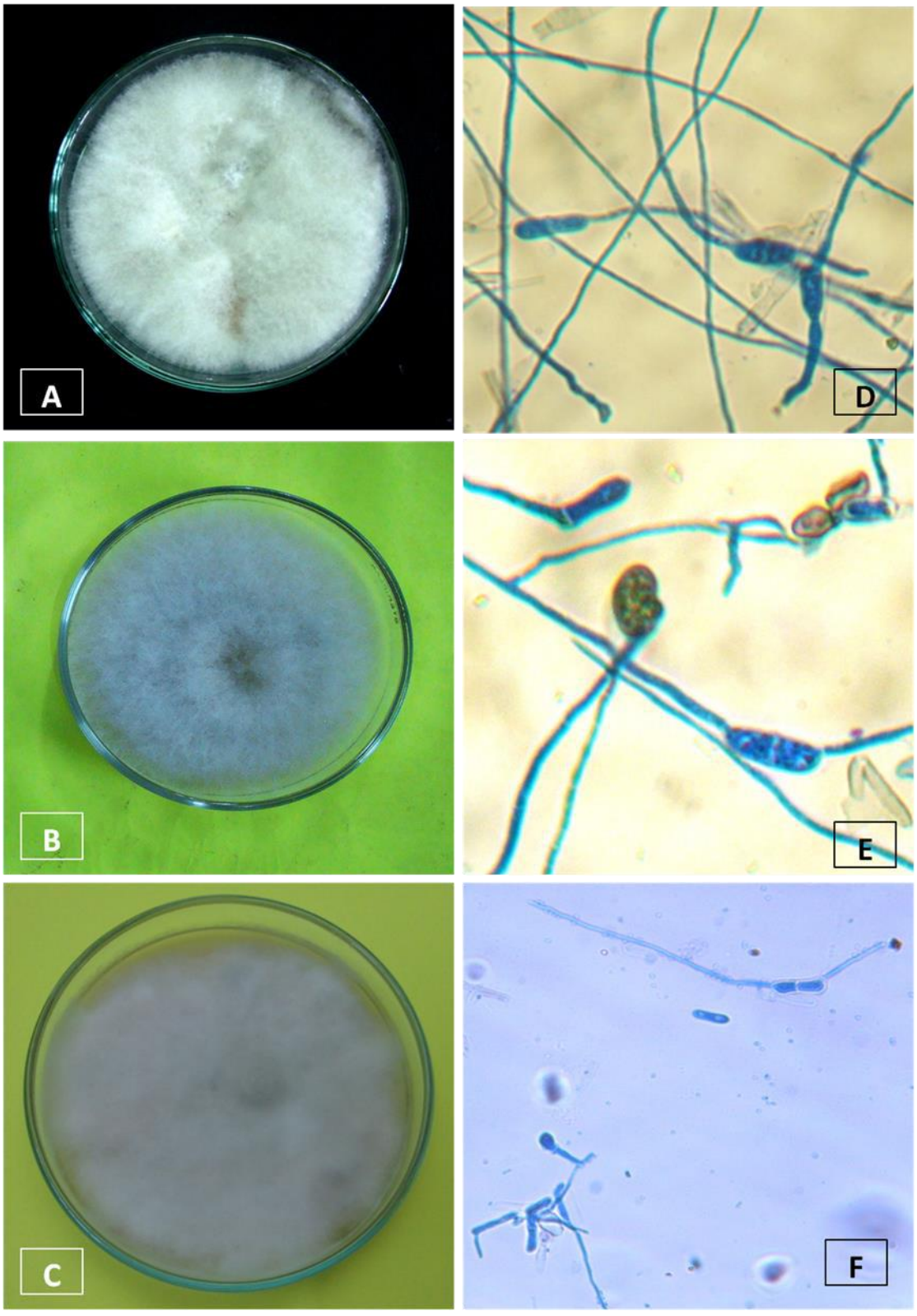


Figure 10: Growth of *C.gloeosporioides* isolates in PDA media (A-C) and spore characteristics (D-F); (A,D) – SOM/Cl/01; (B,E) – SOM/Cl/02; (C,F) – SOM/Cl/03

4.4Screening of resistance of som plants towards foliar fungal pathogens

Resistance of som plants against *Colletotrichum gloeosporioides* and *Pestalotiopsis disseminate* was carried out among eight different morphotypes released by Central Muga and Eri Research and training Institute (CMER&TI), Jorhat, Assam, following detached leaf and whole plant inoculation technique. Methods of inoculation, incubation conditions and disease assessment procedures have been described in details in Materials and methods.

4.4.1. Detached leaf inoculation

Detached leaf inoculation of eight different morphotypes of som plant was carried out. Disease assessment and symptom development were done after 48, 72 and 96 h of inoculation on the basis of percent drop that resulted in lesion production.

4.4.1.1 *P. disseminata*

Leaves of all eight morphotypes of som plants were inoculated with spores of *P. disseminata*. Results as shown in table 9 reveal that after 96h of incubation 72% lesion production was obtained in S5 morphotype while in S3 morphotype approximately 30% lesion production was observed. Hence it can be said that S5 morphotype is highly susceptible to grey blight disease followed by S6, S7 and S2. Least susceptible is S3 morphotype (Figure 11).

4.4.1.2 *C. gloeosporioides*

In case of leaves inoculated with *C. gloeosporioides* spore suspension, results revealed that S6 morphotype was most susceptible, followed by S3, S8 and S5. The least susceptible was S7 morphotype followed by S2, S1 and S4. After 96h of inoculation 62% lesion production was obtained in S6 morphotype while in S7 morphotype approximately 25% lesion production was observed (Table 10 and Figure12).

Table 9: Pathogenicity test of *P. disseminata* isolate on *Persea bombycina* following detached leaf inoculation technique

Morphotype	Percentage lesion production[a]		
	Hours after inoculation		
	48	72	96
S1	32.16±0.6	36.15±1.1	45.12±0.8
S2	46.25±0.4	51.26±1.8	58.24±0.8
S3	18.11±0.5	22.32±0.6	**30.16±0.6**
S4	23.50±0.6	31.15±0.4	39.45±1.2
S5	49.16±0.8	58.42±1.6	**72.15±1.2**
S6	43.26±1.2	55.36±1.2	65.23±1.6
S7	43.45±0.5	50.21±1.2	63.16±1.4
S8	41.29±0.6	46.14±0.6	52.11±1.6

[a] Average of three separate trials, 50 leaves inoculated in each trial, ± Standard error

Table 10: Pathogenicity test of *C. gloeosporioides* on *Persea bombycina* following detached leaf inoculation technique

Morphotype	Percentage lesion production[a]		
	Hours after inoculation		
	48	72	96
S1	30.63±1.2	35.23±0.8	45.23±1.2
S2	26.52±1.6	30.25±0.9	36.12±0.6
S3	42.25±0.6	50.85±1.5	59.25±1.4
S4	36.25±0.5	43.23±1.2	48.52±1.6
S5	40.12±0.3	49.25±1.6	56.30±1.5
S6	49.56±1.2	55.23±1.4	**62.06±1.2**
S7	20.12±1.5	25.23±0.5	**30.12±1.4**
S8	36.52±1.4	42.23±1.5	50.23±0.8

[a] Average of three separate trials, 50 leaves inoculated in each trial, ± Standard error

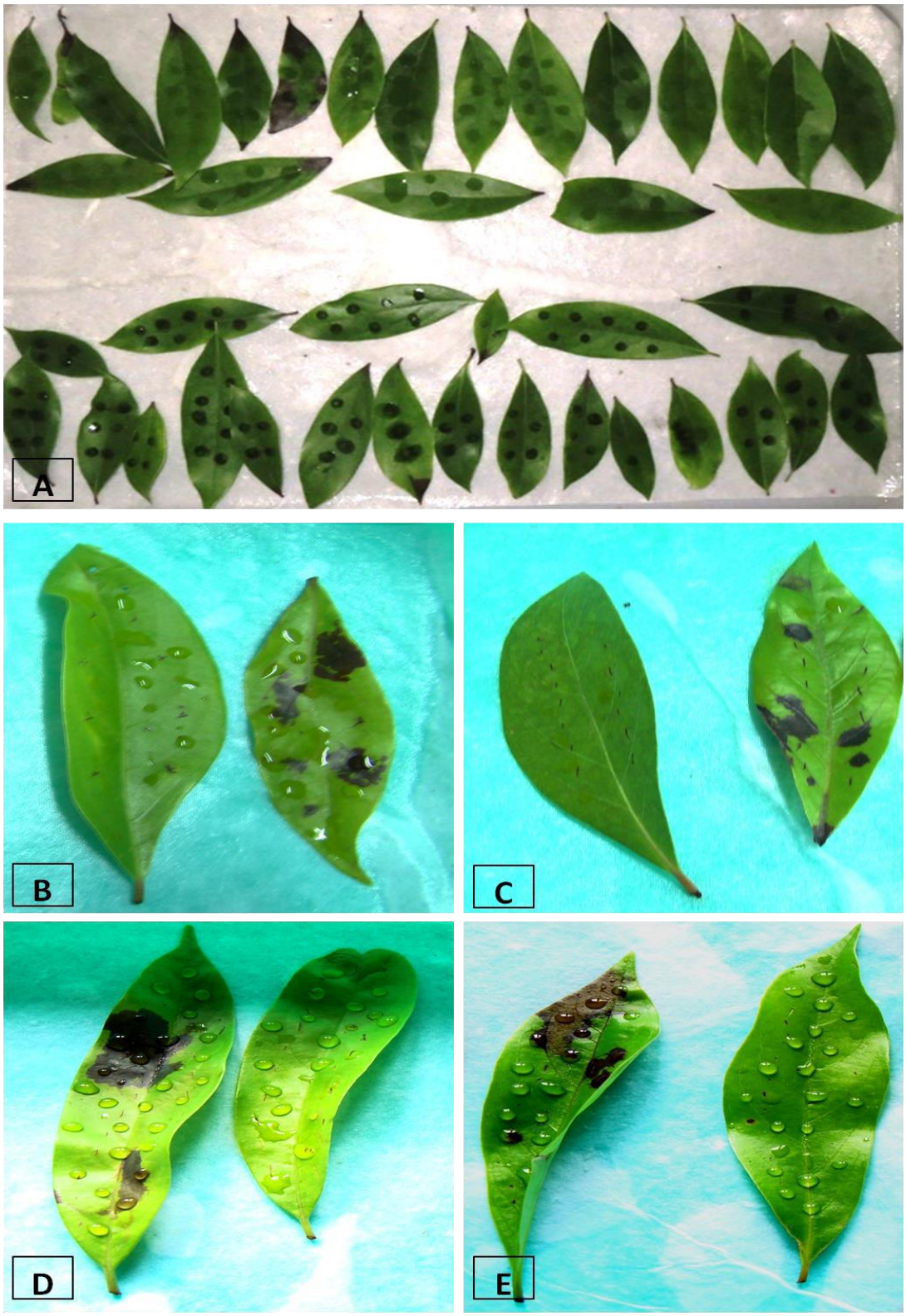


Figure 11: Pathogenecity test of *P. disseminata* isolate following detached leaf inoculation technique. (A) – Experimental setup after 48h of inoculation, (B,C) – Leaves of S5 morphotype showing disease symptoms after 96h of inoculation, (D,E) – Leaves of S6 morphotype showing disease symptoms after 96h of inoculation

Figure 12: Pathogenecity test of *C. gloeosporioides* isolate following detached leaf inoculation technique. (A) – Experimental setup after 48h of inoculation, (B,C) – Leaves of S5 morphotype showing disease symptoms after 96h of inoculation, (D,E) – Leaves of S6 morphotype showing disease symptoms after 96h of inoculation

4.4.2 Whole Plant inoculation

Eight morphotypes of well established pot grown som plants were inoculated with spore suspension of *C. gloeosporioides* and *P.disseminata* separately and were incubated with plastic cover for 48h. The pots were observed at 15, 20 and 30 days interval and appearance of disease symptoms were noted. Accordingly percentage disease index was measured (Table 11).

4.4.2.1. *P. disseminata*

In case of pots inoculated with *P. disseminata* spores disease intensity was highest at each interval in S5 morphotype followed by S6, S7 and S2. It was least in S3 morphotype followed by S4, S1 and S8 as shown in Figure 13(A).

4.4.2.2. *C. gloeosporioides*

In case of pots inoculated with *C.gloeosporioides* spores disease intensity was high in S6, S3, S5 and S8 at each interval in comparison to other morphotypes like S4, S1, S2 and S7 as shown in figure 13(B).

Results obtained from varietal resistance test performed on 8 som morphotypes against *C. gloeosporioides* and *P. disseminata* following detached leaf and whole plant inoculation technique indicated that S6 morphotype is highly susceptible to *C. gloeosporioides* and S7 morphotype is least susceptible. On the other hand S5 morphotype is highly susceptible to *P. disseminata* whereas S3 morphotype is least susceptible.

Table 11: Grey blight and leaf blight disease incidence of som following whole plant inoculation with *P. disseminata* and *C. gloeosporioides*

Morphotype	Percent Disease Index (PDI)*	
	P. disseminata	***C. gloeosporioides***
S1	50±0.08	48±0.05
S2	58±0.06	42±0.06
S3	42±0.05	65±0.07
S4	48±0.09	50±0.08
S5	72±0.12	60±0.06
S6	69±0.06	70±0.04
S7	60±0.07	35±0.06
S8	55±0.06	56±0.08

*After 30days of inoculation, ±Standard error

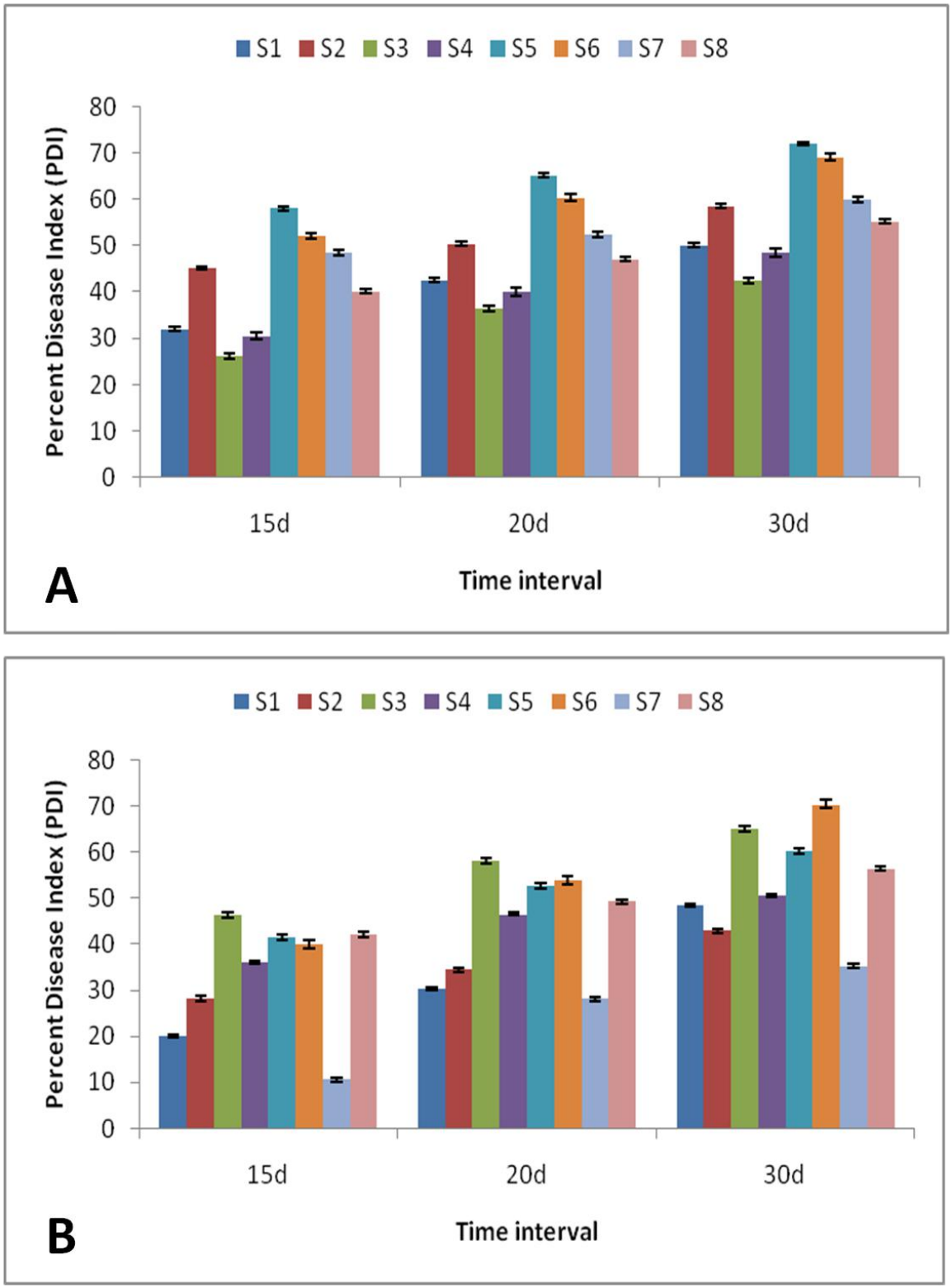


Figure 13: Screening of resistance of eight morphotypes of som plants against (A) *P. disseminata* and (B) *C. gloeosporioides* following whole plant inoculation

4.5 Immunoassays for detection of *Pestalotiopsis disseminata* and *Colletotrichum gloeosporioides*

4.5.1. Soluble protein

Quantitative protein analysis of the mycelial antigen of *C. gloeosporioides and P. disseminata* isolates was done at 4 day intervals for 20 days. Results as shown in table12 and 13 reveal that 12 day-0ld culture of SOM/Cl/02 and IPL/SOM/P/01 had the highest protein content of 25.6 mg/g^{-1} fresh tissue weight and 20.5 mg/g^{-1} fresh tissue weight respectively. The results are prepared in tables. It is clear that SOM/Cl/02 and IPL/SOM/P/01exhibited high total protein content from 4^{th} to 12^{th} day of incubation than the other isolates of both the pathogen. Hence mycelial antigen of these two isolatesof *P. disseminata* and *C. gloeosporioides* was analysed by SDS-PAGE. The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker. Bands of varying intensities ranging from 97 Kda to 14.3 Kda were present in both the pathogens. Bands of lower molecular weight were present especially in *P. disseminata*.

Table 12: Protein content of *C. gloeosporioides* isolates

Isolates	Protein content (mg g^{-1})				
	Incubation period (days)[a]				
	4	8	12	16	20
SOM/Cl/01	8.2±1.12	10.6±1.22	13.5±1.04	11.5±1.14	10.2±1.14
SOM/Cl/02	14.0±1.09	20.5±1.14	25.6±1.11	22.5±1.03	20.3±1.02
SOM/Cl/03	9.5±1.06	11.3±1.06	10.5±1.13	8.6±1.04	7.2±1.05

[a] Incubation temperature 25°C, ±Standard Error, n=3

Table 13: Protein content of *P. disseminata* isolates

Isolates	Protein content (mg g^{-1})				
	Incubation period (days)[a]				
	4	8	12	16	20
IPL/SOM/P/01	12.2±1.12	20.6±1.22	28.9±1.04	25.5±1.14	20.2±1.14
IPL/SOM/P/02	9.5±1.06	11.3±1.06	10.5±1.13	8.6±1.04	7.2±1.05
IPL/SOM/P/03	10.5±1.06	18.3±1.06	25.5±1.13	20.6±1.04	17.2±1.05

[a] Incubation temperature 25°C, ±Standard Error, n=3

4.5.2 Immunological assays

Immunological assays were performed using Polyclonal antibodies (PAb) raised against mycelia protein of *P. disseminata* and *C. gloeosporioides* in rabbit. Effectiveness of antigen in raising antibodies were checked initially using agar gel

double diffusion technique followed by dot immunobinding assay and western blot analysis. Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity.

4.5.2.1 Optimization of PTA-ELISA

Optimization of ELISA was done considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain the maximum sensitivity. Antiserum dilutions ranging from 1:125 to 1:16000 were tested against homologous antigen at a concentration of 5mg/L. Absorbance values in ELISA decreased from the dilution of 1:125 to 1:2000 after which it levelled off. Dilutions of antigen concentration in two fold series ranging from 25 to 1600µg/L were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with a concomitant increase of antigen concentrations. Concentration as low as 25µg/L can be easily detected by ELISA at both antisera dilutions.(Figure 14).

4.5.2.2 PTA-ELISA

PTA-ELISA was used to check the effectiveness of homologous and heterologous antigens with PAbs of both the pathogens. It was observed that titre values of ELISA were significantly higher in case of homologous antigen-antibody reaction in comparison with heterologous antigen-antibody reaction. (Table 14). In case of heterologous reaction, antigen of *Dreschleraoryzae* and *Bipolarissorokiniana* was taken into consideration.

Table 14: Indirect ELISA values (A_{405}) of mycelial antigens reacted with PAbs of *C. gloeosporioides* and *P. disseminate*

Fungal Antigen (40µg/ml)	PAb of *C. gloeosporioides*	PAb of *P. disseminata*
Colletotrichumgleoesporioides	1.912±0.008	0.789±0.009
Pestalotiopsisdessiminata	0.658±0.008	1.820±0.005
Dreschleraoryzae	0.107±0.012	0.156±0.006
Bipolarissorokiniana	0.112±0.006	0.109±0.003

Absorbance at 405nm, PAb at 1:125 dilution

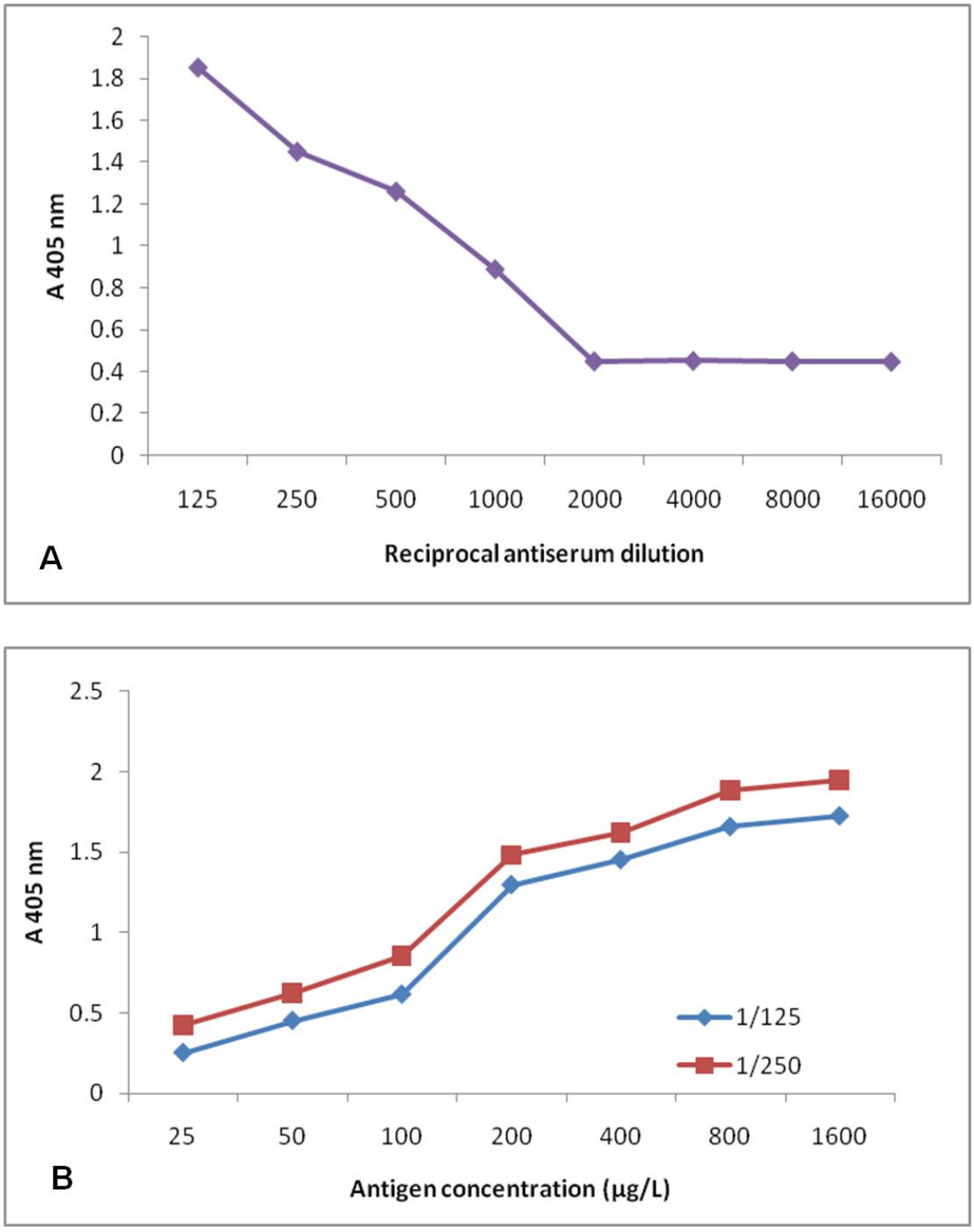


Figure 14: Optimization of ELISA by considering two variable, (A) dilution of the antiserum and (B) dilution of the antigen extract

4.5.2.3 Dot-Immunobinding Assay

Dot immunobinding assay using mycelial antigen and PAb of *P. disseminata* and *C. gloeosporioides* was also standardized. Dot immunobinding assays confirm the effectiveness ofraising antibodies against *P. disseminata* and *C. gloeosporioides*. Soluble protein obtained fromseven-day old mycelia of *P. disseminate* and *C. gloeosporioides* were reacted on nitrocellulose paper with PAb-Pt and PAb-Cg. Result shows development of deep purplecolour in homologous reactions (Table 15) indicating a positive reaction suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen. However, light pinkish colour was observed in heterologous reactions (Figure 15A and 17A)

Table 15: Dot immunobinding assay of mycelial antigens reacted with PAbs of *C. gloeosporioides* and *P. disseminate*

Antibody Source	Antigen Source	Intensity of dots
Colletotrichum gloeosporioides	*C.gloeosporioides*	++++
	P. disseminata	++
	D. oryzae	+
	B. sorokiniana	+
Pestalotiopsisdisseminata	*P. disseminata*	++++
	C.gloeosporioides	++
	D. oryzae	+
	B. sorokiniana	+

++++ Very deep purple; +++ Deep purple; ++ Light pinkish; + Very light purple

4.5.2.4 Western Blot

Western blot analysis using PAb of *P.disseminata*and *C. gloeosporioides*was also performed to develop strategies for rapid detection of the pathogens. For this total soluble protein of young mycelia was used as antigen source and SDS-PAGE was performed followed by probing with alkaline phosphatase conjugate. The bands on nitrocellulose membrane were compared with bands on SDS-PAGE. Bands of varying molecular weights (14 KDa to 97 KDa) were seen in SDS-PAGE. The bands on nitrocellulose membrane were compared with those present in SDS-PAGE. Bands with lower molecular weights were more in numbers.

In case of Western blot of mycelia of *P. disseminata* 7 different bands of varying molecular weights, mainly lower molecular weights were observed on

nitrocellulose membrane. These bands therefore contain epitopes of antigen that were present in the mycelial protein (Figure 15 C). Similarly in case of *C. gloeosporioides*the nitrocellulose membrane spotted 5 different bands of higher molecular bands, corresponding to the epitopes of pathogenic antigen (Figure 17 C)

4.5.2.5 Indirect Immunofluorescence

Indirect immunofluorescence of young hyphae and spores of *P. disseminata* and *C. gloeosporioides*was carried out with homologous antibody and reacted with fluorescein isothiocyanate (FITC) labelled antibodies of goat specific for rabbit globulin.

4.5.2.5.1 Mycelia

Young mycelia of both the pathogen was treated with their respective homologous antibody and labelled with FITC. Strong apple green fluorescence was seen in both mycelia which was confirmation of the homologous reaction of the pathogen and the antibody (Figure 15 B and 17 B).

4.5.2.5.2 Spores

Spores of both the pathogens were also treated with their respective homologous antibody and then labelled with FITC. In case of *P. disseminata* spores only the setulae and appendages showed apple green fluorescence as the conidia are dark septate, confirming the identity of the pathogen (Figure 16). On the other hand *C. gloeosporioides* spores showed apple green fluorescence throughout the structure as it is hyaline in nature and easily gets labelled with FITC (Figure 18). This also confirms homologous antigen-antibody reaction between pathogen and their corresponding antibody.

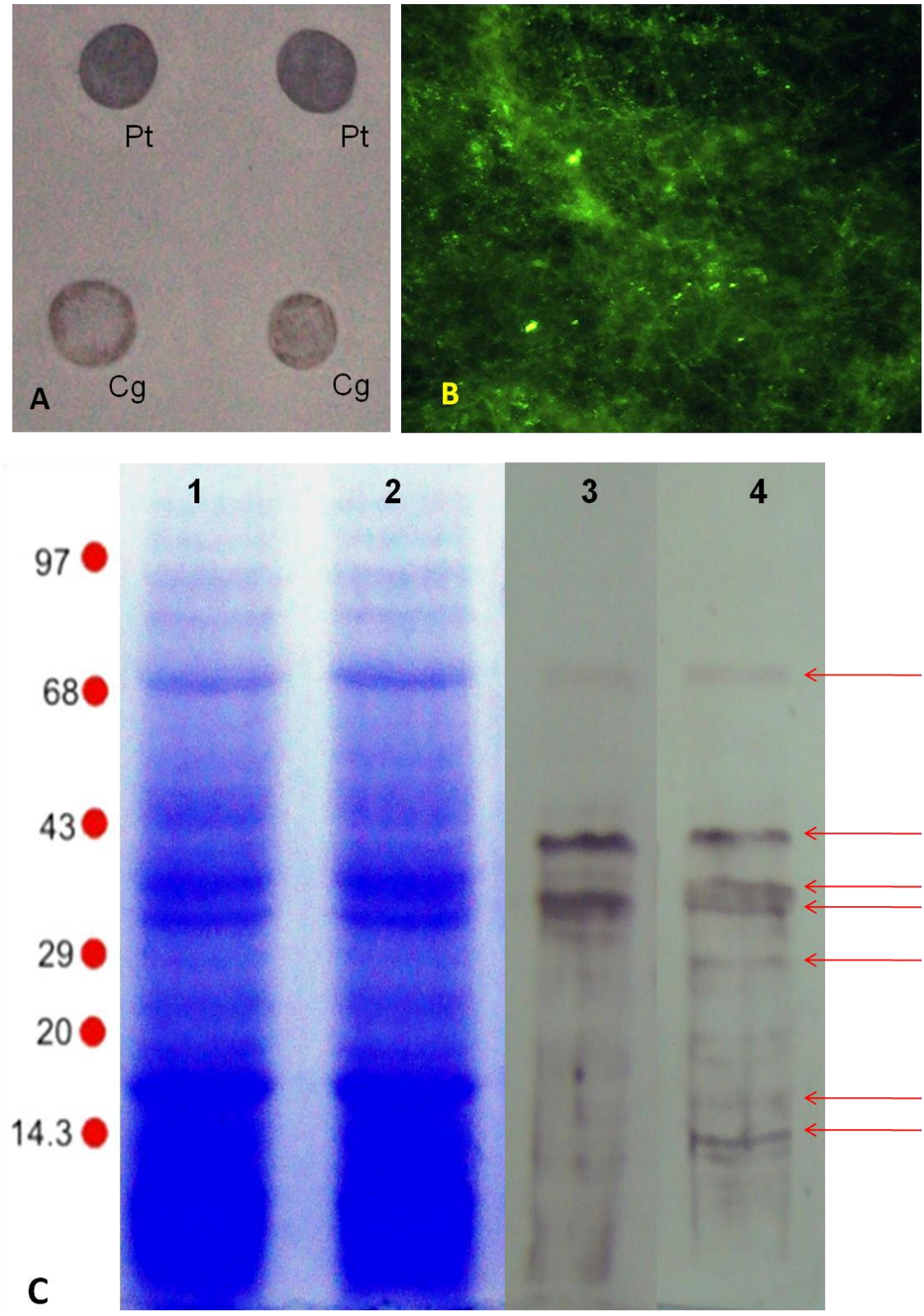


Figure 15: Serological assay of *Pestalotiopsis disseminata*; (A) - Dot blot; (B)Immunofluorescence of young mycelia of *P. disseminata* reacted with PAb-Pt and labelled with FITC; C– Western blot analysis of mycelial antigen of *P. disseminata* probed with PAb-Pt on nitrocellulose membrane using NBT/BCIP as substrate

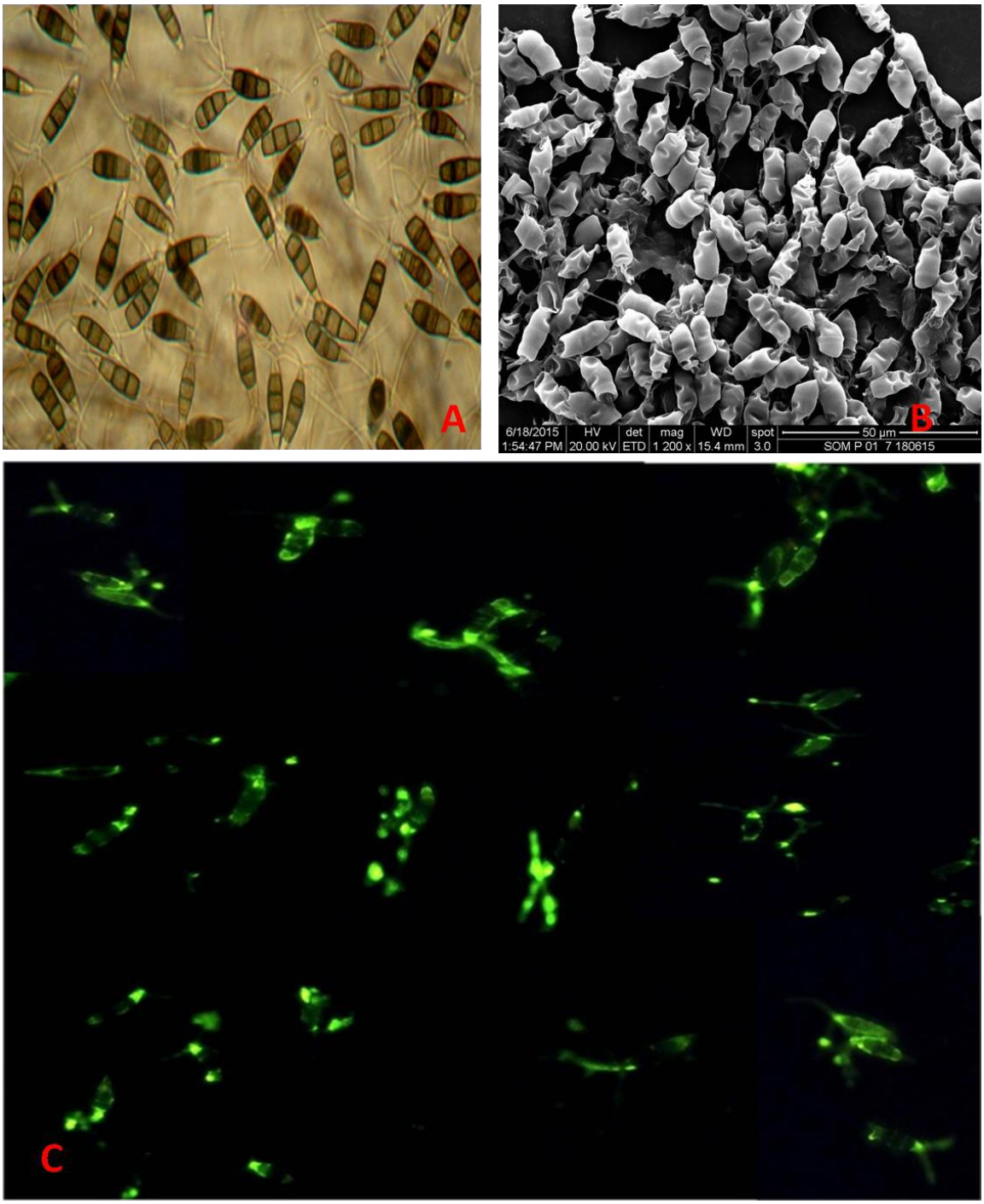


Figure 16: Spore morphology of *P. disseminata*. (A) – Spore under bright field, (B) – Scanning electron micrograph of spores, (C) – Indirect immunofluorescence of spores of *P. disseminata* treated with PAb-Pt and labelled with FITC

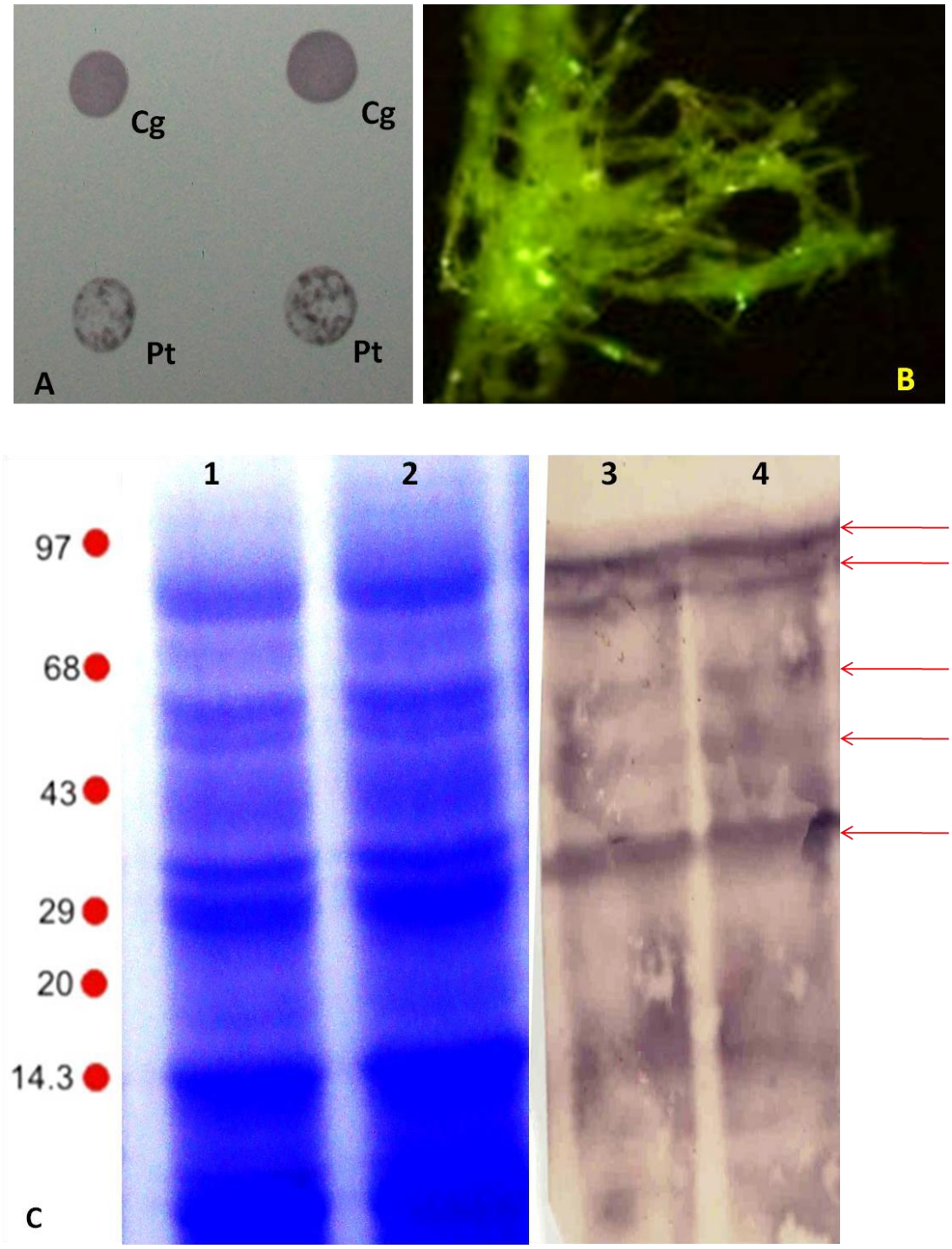


Figure 17: Serological assay of *Colletotrichum gloeosporioides*; (A) – Dot blot; (B) - Immunofluorescence of young mycelia of *C. gloeosporioides* reacted with PAb-Cg labelled with FITC; C - Western blot analysis of mycelial antigen of *C. gloeosporioides* probed with PAb-Cg on nitrocellulose membrane using NBT/BCIP as substrate

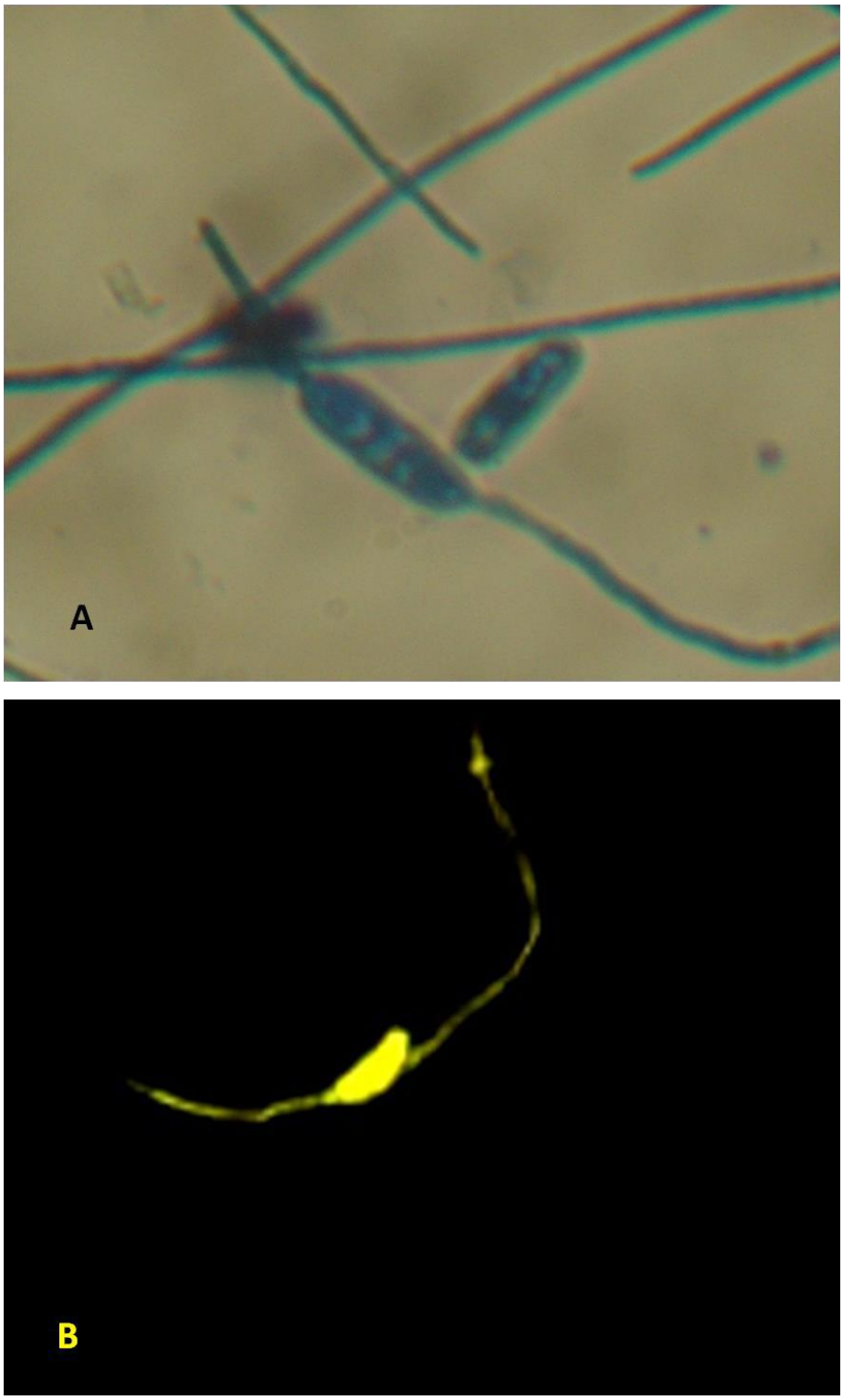


Figure 18: Spore morphology of *C. gloeosporioides*; (A) – Spore characteristics under bright field microscope, (B) – Indirect immunofluorescence of spores of *C. gloeosporioides* probed with PAb-Cg and labelled with FITC

4.6 Detection of major cross reactive antigens shared by*Persea bombycina* and foliar fungal pathogens

In phytopathological studies it is important to learn the host parasite relationship at the cellular level. The presence of cross reactive antigens (CRA) between plant host and parasite reflect degrees of compatibility in the parasite association. The unique presence of CRA in hosts and parasites continues to suggest a regulatory role of CRA in host-parasite specificity. To achieve this antibodies labelled with fluorescein isothiocyanate (FITC) were used to determine the location of CRA in cross sections of som leaves and fungal cells. PTA-ELISA was also used to check the amount of cross reactive antigens present in healthy leaf tissues.

4.6.1 PTA-ELISA

PTA-ELISA was carried out using antigen of three different age groups of healthy som leaves using PAb-Pt as well as PAb-Cg to check the presence of cross reactive antigens in healthy leaves. It was recorded in table 16 and 17 that CRA is present in young leaves more than the medium or mature leaves and PAb-Cg gave better result suggesting a susceptibility of som morphotypes towards leaf blight pathogen more than grey blight pathogen.

Table 16: Indirect ELISA values (A_{405}) of som leaf antigens reacted with PAbs of *C. gloeosporioides*

Antigen	**PTA-ELISA values at 405nm***		
Leaf Antigen (40µg/ml)	**Young**	**Medium**	**Mature**
Morphotype S1	0.85±0.23	0.72±0.05	0.45±0.01
S2	0.76±0.31	0.63±0.05	0.39±0.03
S3	1.18±0.20	1.07±0.03	0.97±0.35
S4	0.92±0.08	0.81±0.10	0.55±0.20
S5	1.00±0.05	0.91±0.08	0.60±0.10
S6	1.26±0.13	1.10±0.23	1.00±0.17
S7	0.65±0.02	0.52±0.06	0.25±0.10
S8	1.05±0.15	1.00±0.07	0.85±0.11
Mycelial antigen *C. gloeosporioides*	1.93±0.07		

* PAb of *C. gloeosporioides* at 1:125 dilution, ± Standard error

4.6.2 Cellular localization of CRA in som leaf tissue usingindirect immunofluorescence

Indirect immunofluorescence using PAb–Cgand PAb-Pt labelled with FITC was used to determine the location of CRA in som leaf tissues. Fresh cross-sections of healthy som leaves of S5 and S6 morphotype was cut through the midrib and treated with PAb-Cg antibody as well as PAb-Pt antibody, labelled with FITC conjugate and observed under UV fluorescence conditions. The leaf sections which were not treated with antibodies, when observed under UV fluorescence microscope exhibited a natural bright yellow autofluorescence mainly on the cuticle. Leaf sections treated with the antibodies reacted much more strongly to FITC and exhibited apple green fluorescence. PAb-Cg was most reactive with leaf sections of S6 morphotype whereas PAb-Pt was more reactive with S5 morphotype (Figure 19 and 20).Here reaction with FITC developed fluorescence that was distributed throughout the leaf tissues. It appears that CRA may form a continuum between the cells of host and parasite, which favours the growth and establishment of the pathogen in the host tissue.

Table 17: Indirect ELISA values (A_{405}) of som leaf antigens reacted with PAbs of *P. disseminate*

Antigen		**PTA-ELISA values at 405nm***		
Leaf antigen (40µg/ml)		**Young**	**Medium**	**Mature**
Morphotype	S1	0.85±0.06	0.76±0.05	0.51±0.08
	S2	0.96±0.11	0.88±0.07	0.71±0.09
	S3	0.65±0.04	0.52±0.03	0.32±0.14
	S4	0.76±0.11	0.65±0.05	0.45±0.06
	S5	1.37±0.03	1.07±0.12	0.97±0.03
	S6	1.12±0.03	1.00±0.12	0.92±0.21
	S7	1.06±0.14	0.95±0.07	0.81±0.13
	S8	0.90±0.04	0.80±0.08	0.65±0.17
Mycelial antigen *P. disseminata*		1.82±0.07		

*****PAb of *P. disseminata* at 1:125 dilution, ± Standard error

4.6.3 Cellular localization of CRA in som leaf tissue using immunogold labelling

The purpose of the ultrasturctural immunocytochemical studies was to locate cross-reactive antigenic sites in som leaf tissue shared by *C. gloeosporioides* and *P. disseminata*. Ultrathin sections of healthy leaf tissues of S5 and S6 morphotypes were treated with PAb-Cg, labelled with gold conjugate of 5nm followed by uranyl-acetate staining. Electron microscopic observations of som leaf tissues showed specific localization of the antibody in certain intercellular structures (Figure 21 and22). The

gold particles were scattered in the cytoplasm. In some areas the particles were concentrated to show the accumulation of CRA in the region. This showed the compatibility of the pathogen with the host tissue.

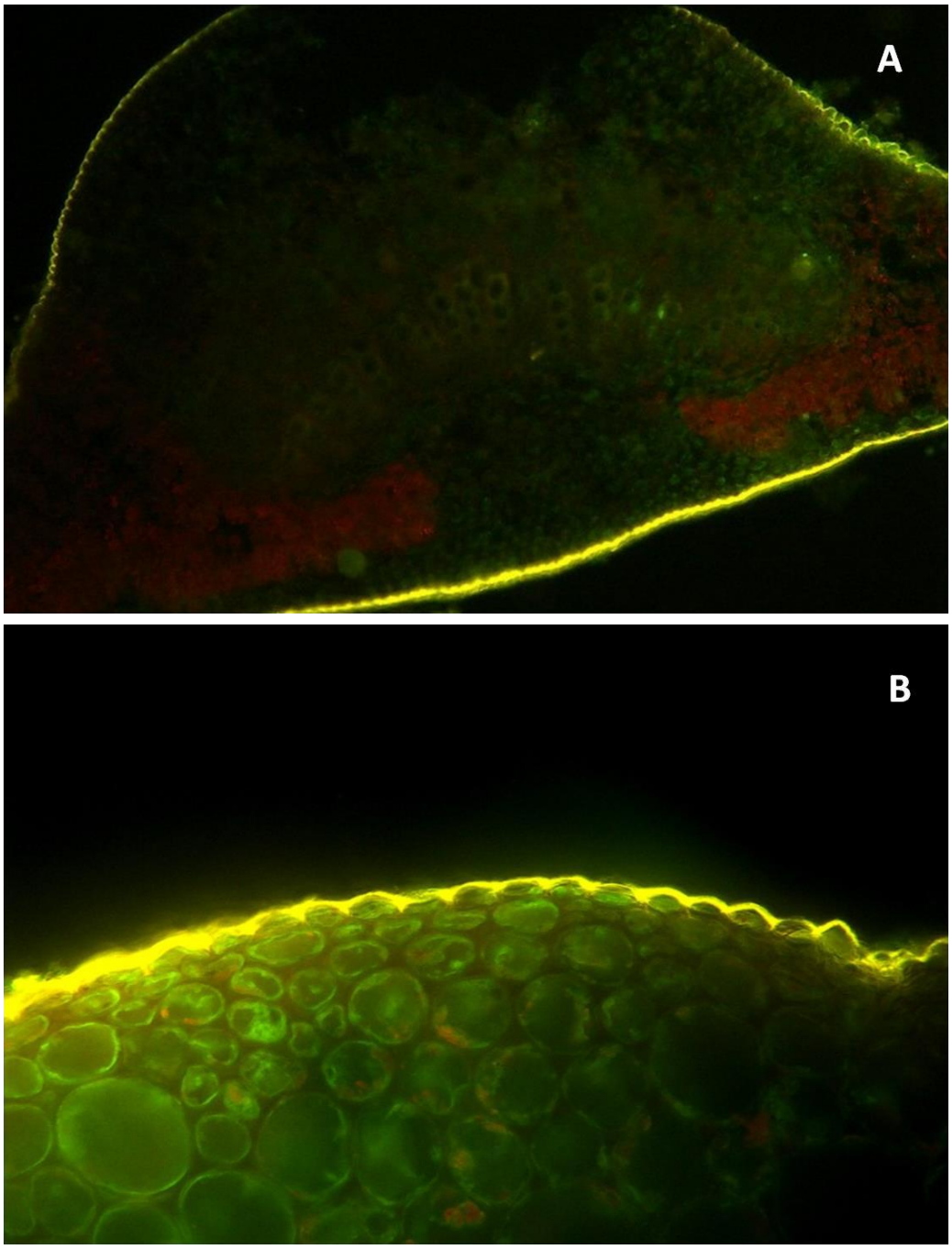


Figure 19: Cellular localization of CRA shared by som plant and *C. gloeosporioides.* (A) Autofluorescence; (B) Cellular localization of CRA in som leaf tissue treated with PAb-Cg and labelled with FITC

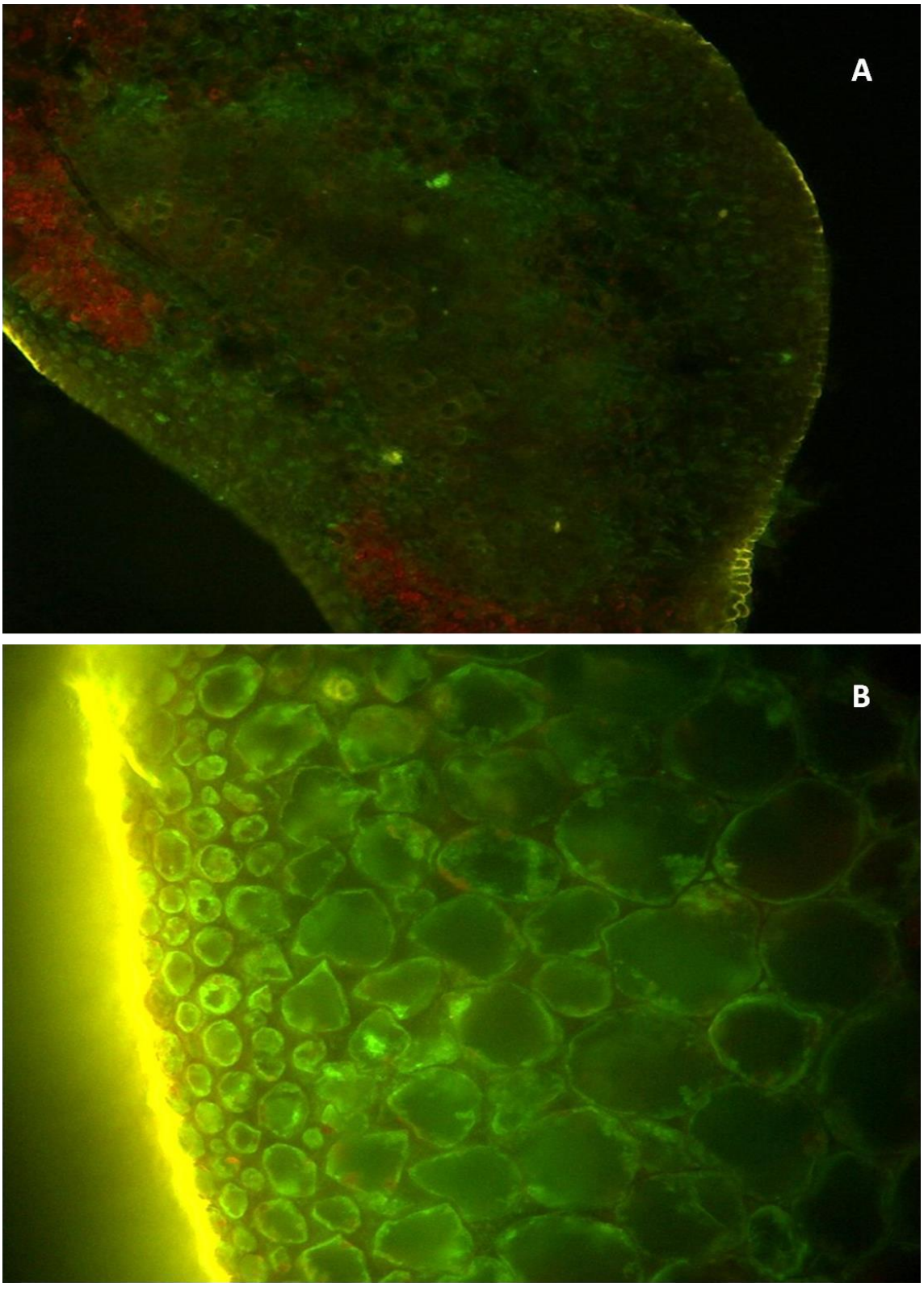


Figure 20: Cellular localization of CRA shared by som plant and *P. disseminata* (A) Autofluorescence; (B) Cellular localization of CRA in som leaf tissue treated with PAb-Pt and labelled with FITC

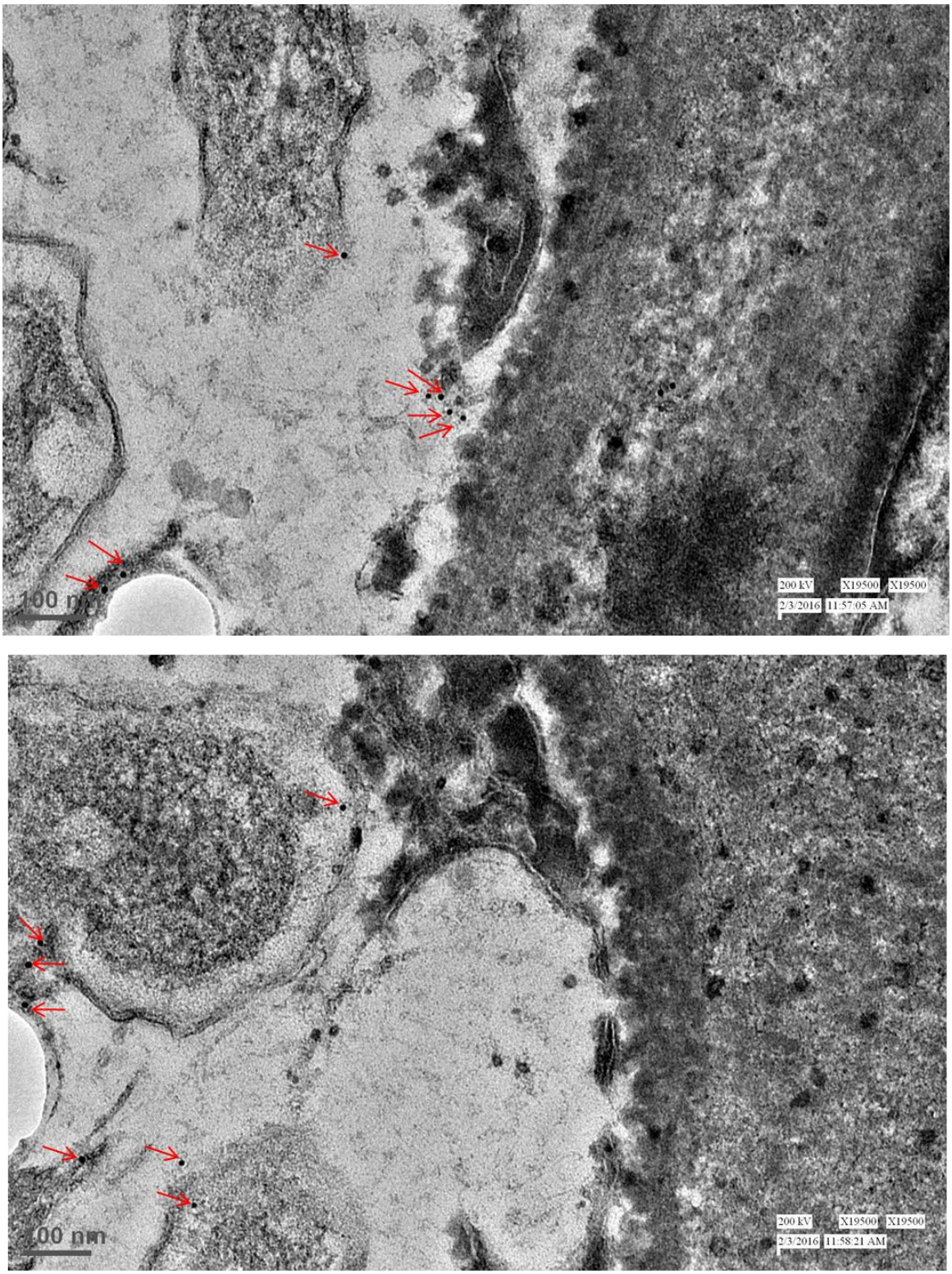


Figure 21: Transmission electron micrographs showing immunogold localization of CRA in som leaf tissue reacted with PAb-Cg and labelled with antirabbit-IgG (whole molecule) gold conjugate

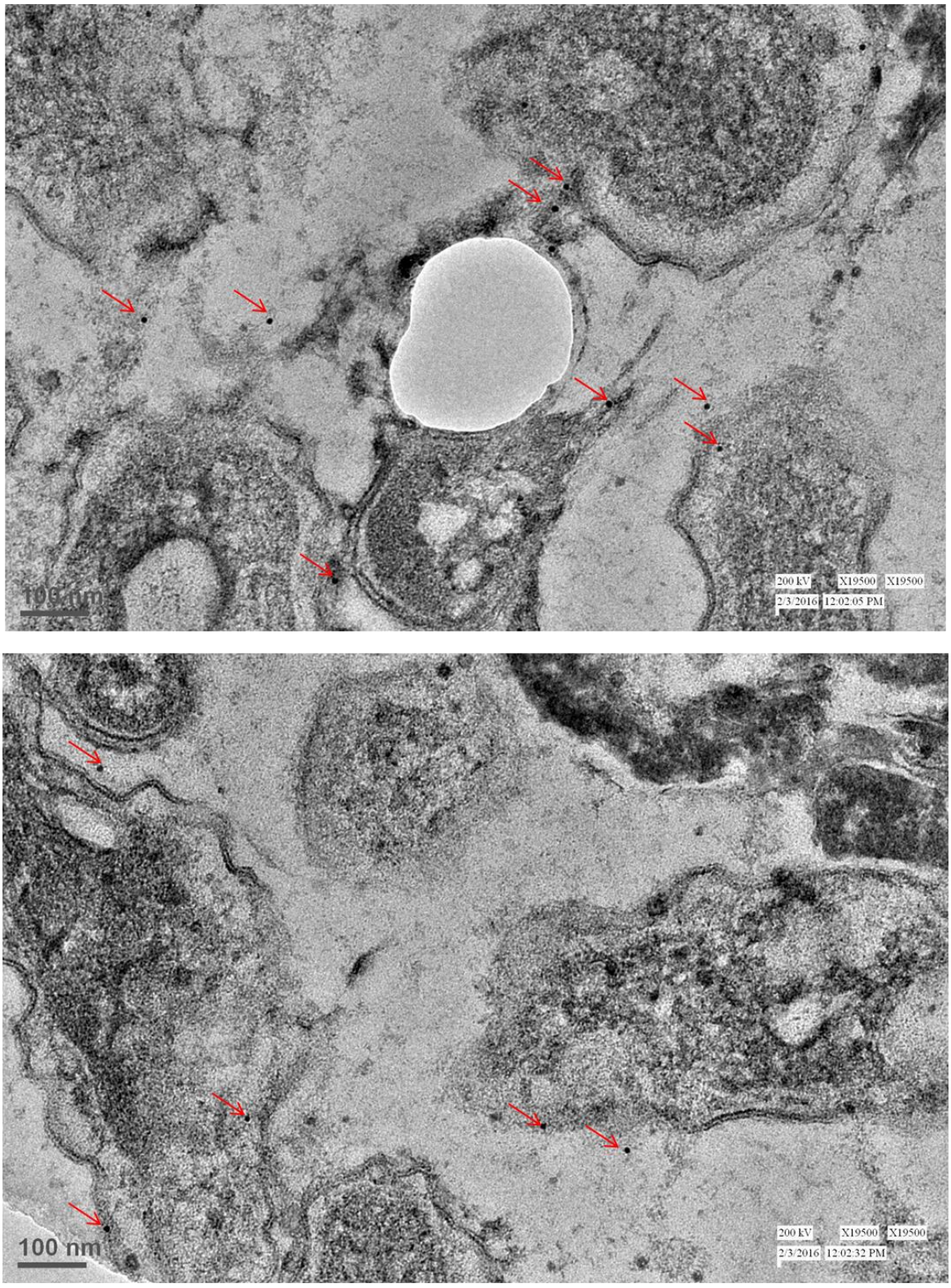


Figure 22: Transmission electron micrographs showing immunogold localization of CRA in som leaf tissue reacted with PAb-Pt and labelled with antirabbit-IgG (whole molecule) gold conjugate

4.7 Serological detection of *P. disseminata* and *C. gloeosporioides* in som leaf tissues

The PTA-ELISA is very sensitive and has proved valuable in detecting infection before macroscopic symptoms appear. Such detection techniques make it possible to detect microquantities of pathogen within a few hours of infection which is much more advantageous than conventional techniques involving pathogen inoculation, visible symptoms and microscopy. These have tremendous potential for plant disease management strategies since detection of pathogens at the initial stages of infection can lead to formulation of control measures before much harm has been done. In the present study immunoassays are also being used for various other purposes such as localization of pathogen within tissues and identifying specific antigens in electrophoretically separated components. It was of interest to determine whether P. disseminata and C. gloeosporioides infections were detectable in som leaf tissues using immunoassays such as PTA-ELISA, Dot-blot and Western blot.

4.7.1 Natural infection

Natural grey blight and leaf blight infected som leaves of eight different morphotypes were collected from experimental garden of Immuno-phytopathology Laboratory. Percentage disease incidence of these blight diseases was also recoreded during the time of collection.

4.7.1.1 PTA-ELISA

Antigens prepared from blight infected leaves of the different morphotypes and corresponding healthy samples were tested in PTA-ELISA at antigen coating concentrations of 40µg protein ml^{-1} and probed with PAb-Cg and PAb-Pt. PTA-ELISA values of blight infected extracts of all morphotypes were higher than the healthy controls at the same antigen concentration (table 18 and 19)

4.7.1.2 Dot immunobinding assay

Healthy som leaf antigens and antigens prepared from blight infected leaves from all eight morphotypes of som plant were coated on nitrocellulose membranes and reacted with PAb-Cg and PAb-Pt following the protocol of Dot-blot assay. Employing NBT/BCIP as substrates reaction produced violet coloured dots. Results revealed the healthy samples had lighter dots when compared with those of infected and homologous sample (table 20 and 21).

Table 18: Detection of pathogen in naturally leaf blight infected som leaves using PTA-ELISA formats

Morphotypes	PAb-Cg		
	Healthy	Infected	PDI
S1	0.079±0.008	0.089±0.009	38.1±0.02
S2	0.085±0.009	0.095±0.015	32.5±0.01
S3	0.092±0.012	0.125±0.016	60.3±0.008
S4	0.087±0.014	0.105±0.011	40.1±0.007
S5	0.156±0.011	0.198±0.009	52.5±0.01
S6	0.096±0.009	0.155±0.008	68.2±0.006
S7	0.078±0.015	0.088±0.014	25.32±0.02
S8	0.081±0.008	0.098±0.016	56.5±0.008

Note: PTA-ELISA values at 405nm; Leaf antigen concentration 40µgml^{-1}; PAb dilution 1:125; ± Standard error

Table 19: Detection of pathogen in naturally grey blight infected som leaves using PTA-ELISA formats

Morphotypes	PAb-Pt		
	Healthy	Infected	PDI
S1	0.084±0.021	0.090±0.015	30.5±0.001
S2	0.084±0.004	0.089±0.005	38.2±0.002
S3	0.080±0.011	0.098±0.011	20.4±0.008
S4	0.076±0.008	0.088±0.014	25.6±0.015
S5	0.082±0.011	0.108±0.002	55.2±0.009
S6	0.095±0.03	0.123±0.009	50.3±0.014
S7	0.087±0.003	0.098±0.006	42.5±0.018
S8	0.081±0.004	0.090±0.008	35.6±0.008

Note: PTA-ELISA values at 405nm; Leaf antigen concentration 40µgml^{-1}; PAb dilution 1:125; ± Standard error

Table 20: Dot immunobinding assay of healthy and leaf blight infected leaf antigens of som plants using PAb of *C. gloeosporioides*

Antigen (40µg/ml)	**PAb-Cg**	
Leaf antigen	**Healthy**	**Infected**
Morphotype S1	+	+++
S2	++	++++
S3	++	+++++
S4	++	++++
S5	++	+++++
S6	++	+++++
S7	+	+++
S8	+	+++++
Mycelial antigen: *C.gloeosporioides*	+++++	

Colour intensity of dots: + insignificant; ++ light violet; +++ violet; ++++ deep violet; +++++ deeper violet; NBT/BCIP used as substrate; PAb dilution 1:125.

Table 21: Dot immunobinding assay of healthy and grey blight infected leaf antigens of som plants using PAb of *P. disseminata*

Antigen (40µg/ml)	**PAb-Pt**	
Leaf antigen	**Healthy**	**Infected**
Morphotype S1	+	++++
S2	++	+++++
S3	+	+++
S4	+	+++
S5	++	+++++
S6	++	+++++
S7	+	++++
S8	+	++++
Mycelial antigen: *P. disseminata*	+++++	

Colour intensity of dots: + insignificant; ++ pink; +++ light violet; ++++ violet; +++++ deep violet; NBT/BCIP used as substrate; PAb dilution 1:125.

4.7.2 Detection of foliar fungal pathogens in leaf tissues following artificial inoculation

4.7.2.1 PTA-ELISA

PTA-ELISA could readily detect reaction between leaf antigens and PAb of pathogens. Antigens extracted from healthy and artificially inoculated with *P disseminata* and *C. gloeosporioides* were tested against PAbs of the pathogens separately. Infection could be detected from 24 h onwards in ELISA on the basis of significantly higher absorbance values of infected leaf extracts in comparison with healthy leaf extracts (table 22).

Table 22:PTA-ELISA values (A_{405}) showing reaction of PAbs of *C. gloeosporioides* and *P. disseminata* with antigens of healthy and inoculated som leaves

Som morphotypes	Leaf antigen concentration (40 µg/ml)		
	Healthy	Inoculated[a]	
		PAb-Cg	PAb-Pt
S1	0.090±0.015	0.165±0.064	0.152±0.010
S2	0.084±0.004	0.172±0.081	0.169±0.008
S3	0.080±0.011	0.146±0.029	0.177±0.009
S4	0.076±0.008	0.153±0.026	0.189±0.120
S5	0.082±0.011	0.149±0.042	0.135±0.160
S6	0.090±0.008	0.136±0.106	0.148±0.005
S7	0.098±0.0006	0.159±0.061	0.167±0.014
S8	0.095±0.009	0.171±0.059	0.172±0.014

Absorbance at 405 nm, PAb of *C.gloeosporioides* and *P. disseminata*, [a]3 days after inoculation

4.7.2.2 Dot immunobinding assay

For DIBA, total soluble protein extract was prepared from healthy and artificially inoculated leaves of eight different morphotypes of som plant. Dot immunobinding assay was performed using these antigen preparations with IgG of *C. gloeosporioides* and *P. disseminata*. Antigens were spotted carefully on nitrocellulose paper and probed with these IgGs. Results have been presented in table 23. Clear and intense colour reactions were observed with homologous antigens, as noted in

previous chapter. In case of non-homologous reactions there was vide variations among the dots.

Greater colour intensity was noted in S5 and S6 morphotype with both the IgGs which showed susceptible reaction to both the pathogens in varietal tests. On the other hand S3 and S7 morphotypes showed insignificant colour reaction with *P. disseminata* IgG and *C. gloeosporioides* IgG respectively. These were incompatible with the respective pathogens.

The eight different morphotypes of som plant showed differences in disease reaction with both the pathogen infections. The results obtained were similar whether assessed by traditional methods or by immunological techniques, which conclusively proved that S5 and S6 morphotypes are susceptible to both the pathogens.

Table 23: Detection of foliar fungal pathogens in artificially inoculated Som leaves using Dot immunobinding assay

Antigen (40µg/ml)	**PAb of *P. disseminata***		**PAb of *C. gloeosporioides***	
Morphotype Leaf antigen	**Healthy**	**Inoculated with *P. disseminata****	**Healthy**	**Inoculated with *C. gloesporioides****
S1	+	+++	+	++
S2	++	++++	++	+++
S3	+	++	++	++++
S4	+	++	++	+++
S5	++	++++	++	++++
S6	++	++++	++	++++
S7	+	+++	+	++
S8	+	+++	+	++++
Mycelial antigen *P. disseminata*	++++		++	
C.gloeosporioides	+++		++++	

Colour intensity of dots: + pink; ++ light violet; +++ violet; ++++ deep violet; NBT/BCIP used as substrate; PAb (1:125); * 48hrs after inoculation.

4.7.3 Immunolocalization of fungal pathogens in blight infected som leaf tissues

4.7.3.1 Indirect immunofluorescent antibody staining of infected leaves

Naturally blight infected som leaves were considered for this experiment. Cross section of leaves were treated with PAb-Cg and PAb-Pt separately and labelled with FITC. Healthy cross sections were autofluorescent along the cuticle (Figure 23A and24A). Blight infected leaves showed stong apple green fluorescence with respective antibody labelling in the palisade and spongy parenchymatous tissues (Figure 23B,Cand 24B,C).

4.7.3.2 Immunogold labelling of blight infected leaves

Ultrastructural and immunocytochemical studies on leaves affected by the grey blight pathogen (*P. disseminata*) and leaf blight pathogen (*C. gloeosporioides*) showed specific localization of antibody on the fungal cell wall and certain intercellular structures. The purpose of ultrastructural immunocytochemical studies was to determine sites of antibody binding on the fungus and also to determine whether extracellular binding of the antibody occurred in som leaf tissues infected by both the pathogens separately. Fixation had an important effect on the ultrastructural quality and antigenic response. Post embedding immunocytochemical labelling of healthy and blight infected leaf segments were performed on segments of LR-white embedded tissues, previously fixed with 0.1 M Sodium phosphate buffered-glutaraldehyde (3%) and using PAb-Cg and PAb-Pt and labelled with antirabbit-IgG (whole molecule) gold conjugate (5nm).

Fungal mass in the cellular compartments was intensely labelled by PAb-Cg as well as PAb-Pt. In the host cells different degrees of labelling was observed. However, in infected tissue, gold particles were predominantly localized (Figure 25 and 26). The gold particles observed on the surface appeared as either as individual spherical particles covering the fungal surface varied in an even distribution or as clusters of particles. Sections of host cells severely blight infected were strongly labelled confirming the presence of fungus in the cell. Gold labelling in the sections showed a high amount of labelling in host cytoplasm and lesser amount in vacuoles, mitochondria and walls.

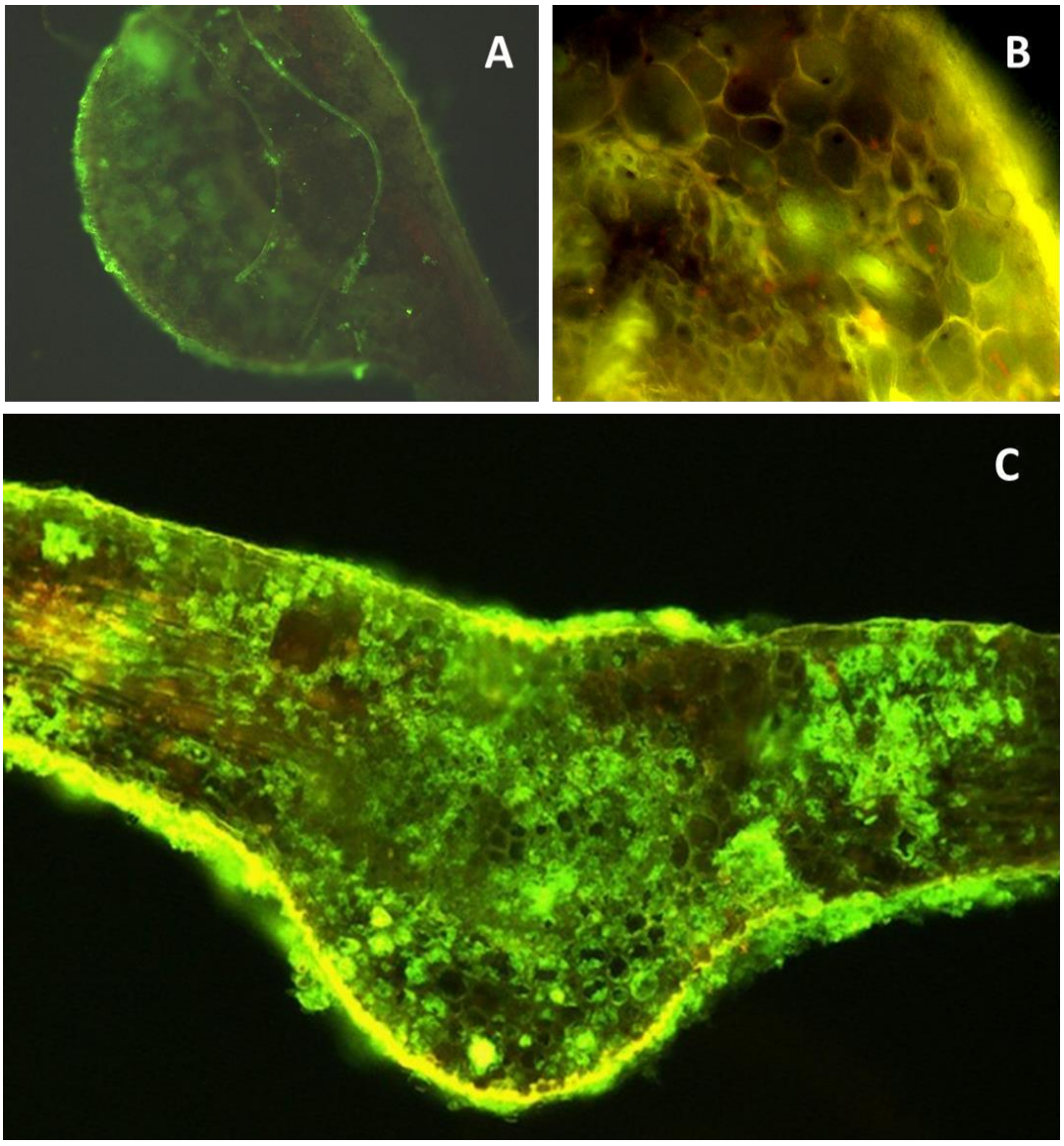


Figure 23: Immunofluorescence of naturally infected som leaf tissue treated with PAb-Cg and labelled with FITC; Transverse section of (A) Healthy and (B and C) infected leaf

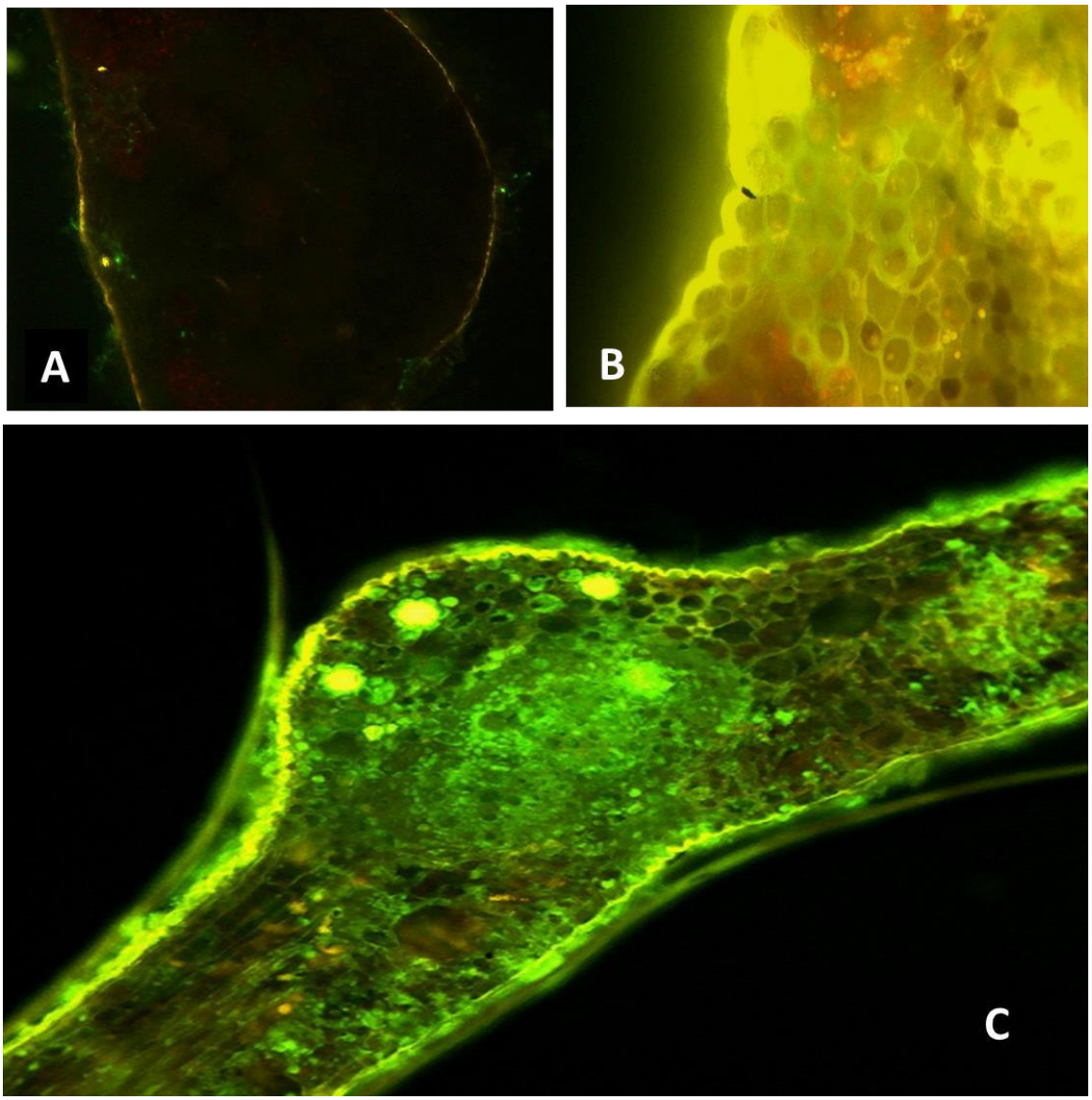


Figure 24: Immunofluorescence of naturally infected som leaf tissue treated with PAb-Pt and labelled with FITC;Transverse section of (A) Healthy and (B and C) infected leaf

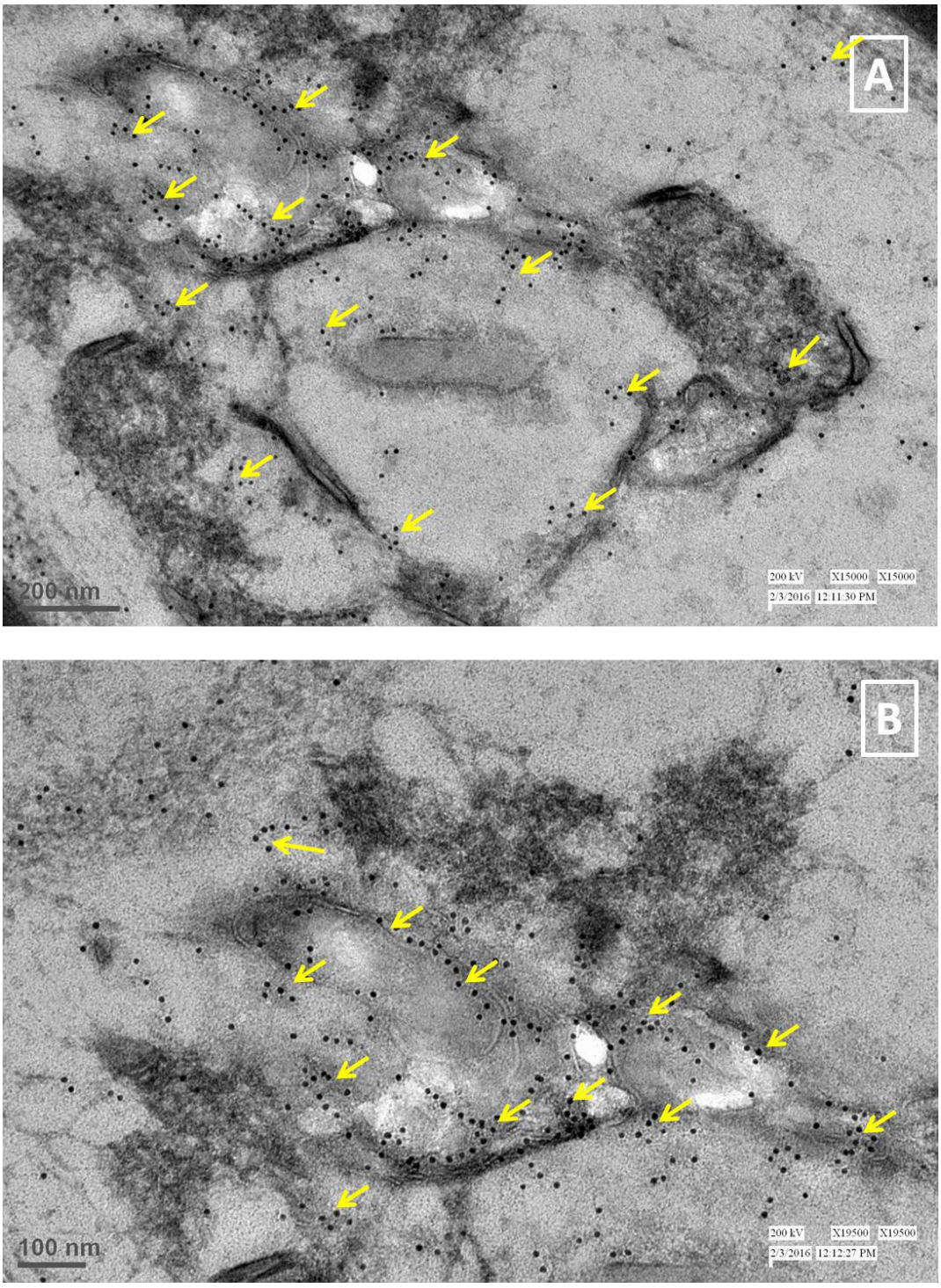


Figure 25: Transmission electron micrograph of infected leaf tissue treated with PAb-Cg and labelled with antirabbit-IgG (whole molecule) gold conjugate

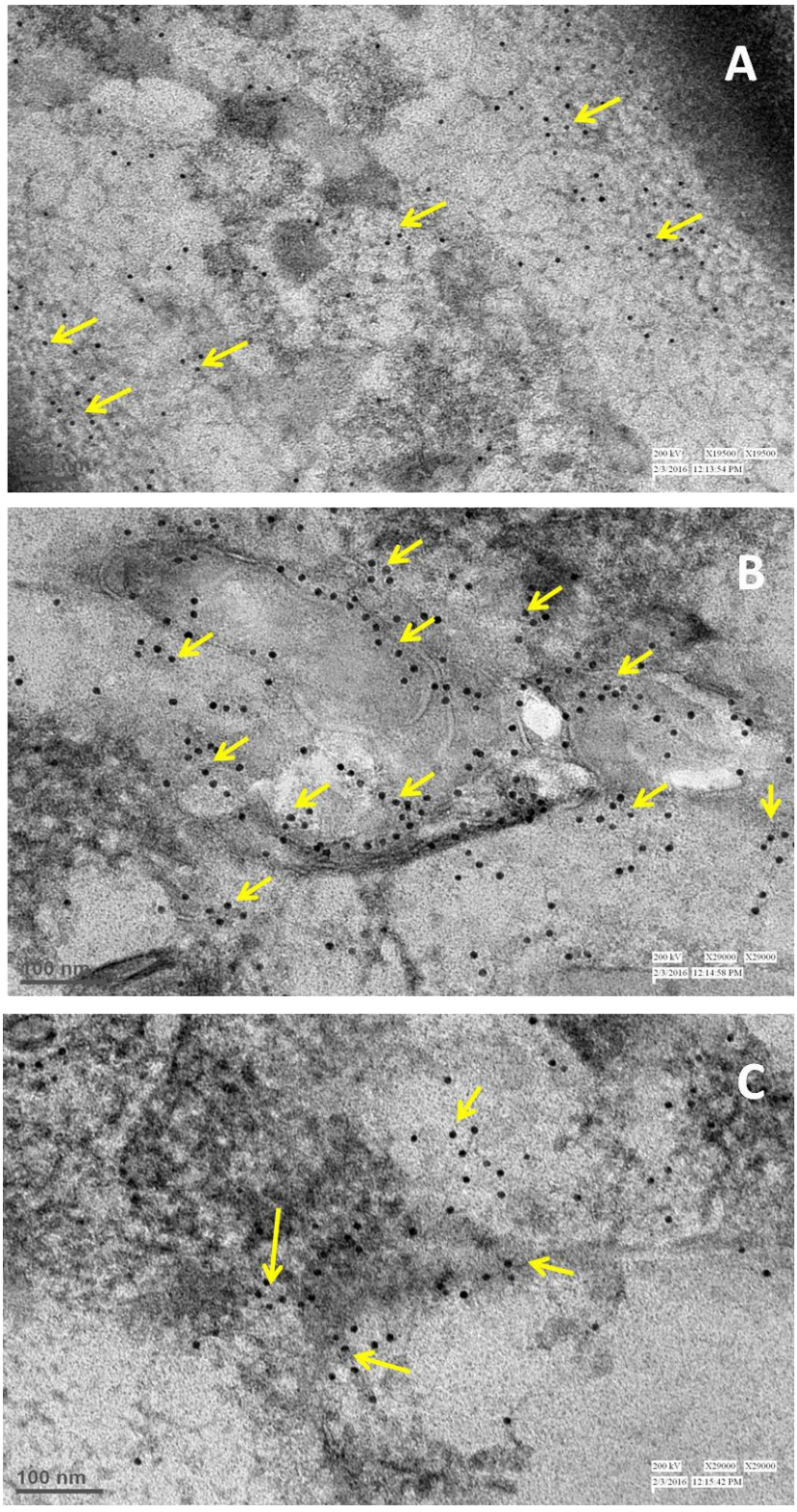


Figure 26: Transmission electron micrograph of infected leaf tissue treated with PAb-Pt and labelled with antirabbit-IgG (whole molecule) gold conjugate

4.8 Mycorrhizal association of som plants and immunolocalisation of Arbuscular Mycorrhizal Fungi in root tissues

4.8.1 Mycorrhizal association in roots of som plants

Arbuscular Mycorrhizal Fungal spores collected fromthe rhizospheric region of all eight morphotypes (S1-S8) of Som plant were critically examined.Morphological features of isolated AMF sporeswere minutely examined with special referenceto variation in size, colour, wall thickness, shapeand wall layers. Presence of huge diversity inthe population included *Glomus*sp, *Scutellospora*sp, *Acaulospora*sp and *Gigaspora*sp. Onobservation it was found that presence of *Glomus*sp dominated the AM population followed by*Scutellospora*sp, *Acauolospora*sp and *Gigaspora*sp (Figure 27). The detailed description of themicroscopic characters of the isolated AMF spores is given in Table 24. The spore populationwas highest in S4 morphotype (82%) and least in S8 morphotype (63%) (Table 25).

4.8.2 Histopathology and colonization of roots with AMF in som plants

Mycorrhization pattern of the roots and percent root colonization of all eight som plants were determined.Plant species differ in the pattern of AMF rootcolonization. Since variations in vesicles, hyphal branching patterns, structure of hyphaeand staining intensity of hyphae are different for each genus, it is possible to identifyGlomeromycota fungi upto genus level but it is difficult to separate species.

It was recorded that colonization of roots with AMF was highest in S5 morphotype and lowest in S8 morphotype of som plants as shown in Figure 28. Histo-pathological analysis of the roots also revealed the presence of intra and inter-radical hyphae, arbuscules and vescicles in the root segments indicating the fact that infection of the AM spores have taken place (Figure 29).

Table 24: Microscopic characters of AMF spores associated with som root

Genus and Species	Colour	Shape	Spore Layer	Spore size (µm)	Other description
Glomusfasciculatum	Pale yellow to bright brown	Globose to subglobus	3	70-120	Spore layer continuous
Glomus mosseae	Brown to orange brown	Globose to subglobose	3	200	Hyphae are double layered
Glomusaggregatum	Pale yellow	Globose to oval	1-2	200-1800 x 200-1400	Sporocarps formed in loose clusters
Glomus constrictum	Brownish orange to dark brown	Globose to subglobose, sometimes ovoid	2	110-130 x 150-160	Subtending hyphae straight or curved, usually markedly constricted at the spore base.
Gigaspora gigantea	Greenish yellow	Globose to Subglobose	2	250-270 x 265-370	Formed terminally or laterally on a bulbous sporangenous cell
Gigaspora margarita	Yellowish white to sunflower yellow	Globose to Subglobose	2	300-340 x 360-380	Spores produced singly in the soil, blastically at the tip of a bulbous sporangenous cell
Acaulospora bireticulata	Brownish	Globose	3	280-410	Surface ornamentation is prominent. Spores are borne laterally from the neck of soporiferous saccule
Acaulospora capsicula	Orange red to capsicum red	Globose to subglobose	3	220-310 x 290-440	Sporiferoussaccule pale yellow to brownish yellow which usually falls off when spores mature
Scutellosporapellucida	Hyaline white to yellow brown	Globose to subglobose	3	120-240	Two bi-layered hyaline flexible inner walls are formed during germination that readily separate from each other and from the spore wall.
Scutellospora rubra	Dark orange brown to red brown	Globose to subglobose	3	140-220	Germinal walls are formed completely separate from the spore wall

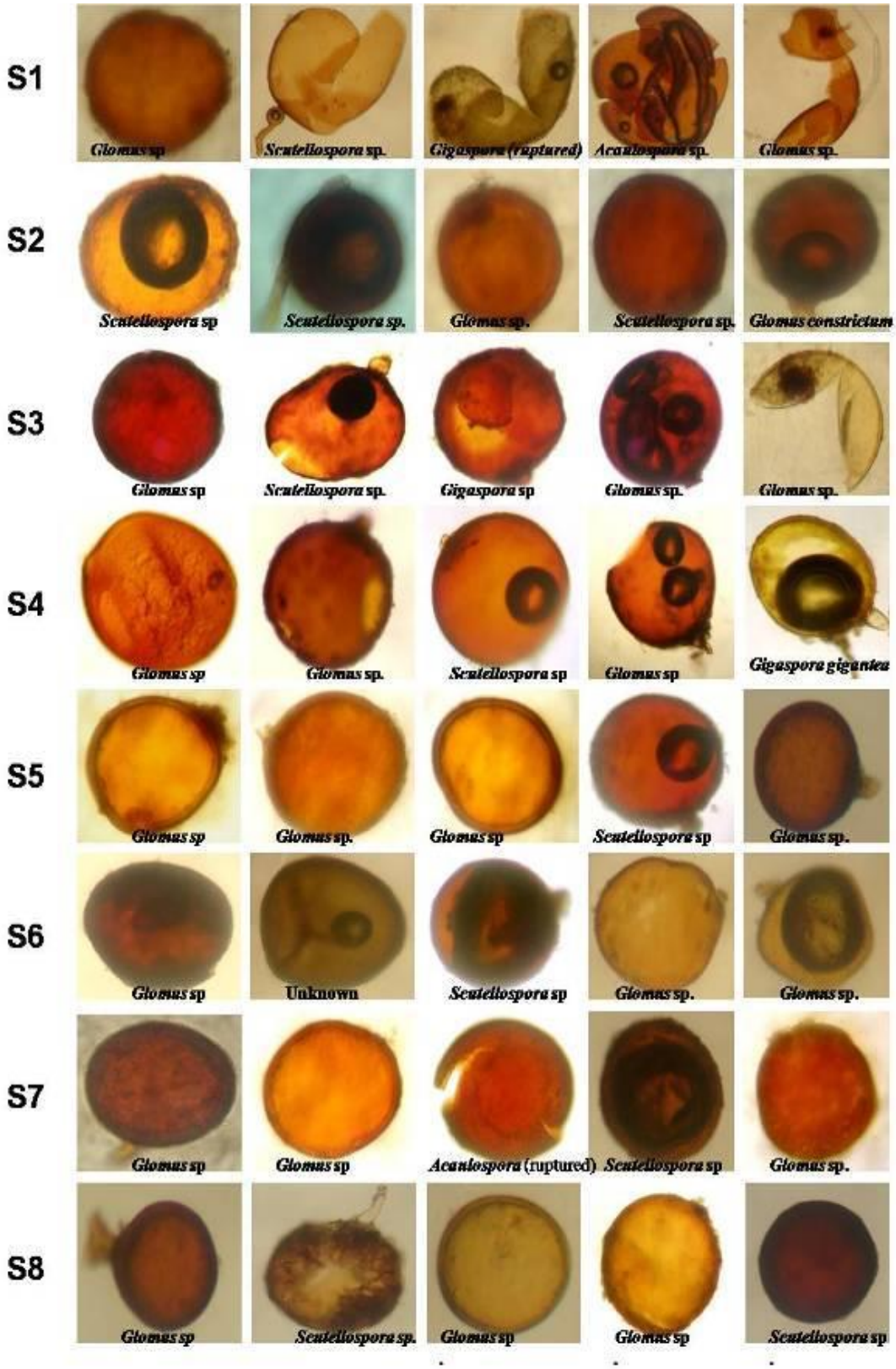


Figure 27: Mycorrhizal association with eight morphotypes of Som plants grown in the experimental field

Table 25: **AMF spore population in field grown Som plants**

Morphotypes	***Glomus* sp**	***Scutellospora* sp**	***Acaulospora* sp**	***Gigaspora* sp**	**Spore count (spores/100g of soil)**
S1	+++	++	+	+	72
S2	+++	++	+	-	70
S3	++	+++	+	+	79
S4	+++	++	+	+	82
S5	+++	+++	-	-	75
S6	+++	++	-	-	80
S7	++	++	+	+	77
S8	+++	++	-	-	63

Key: + = 0-25%, ++ = 25-50%, +++ = 50-75%, - = Absent

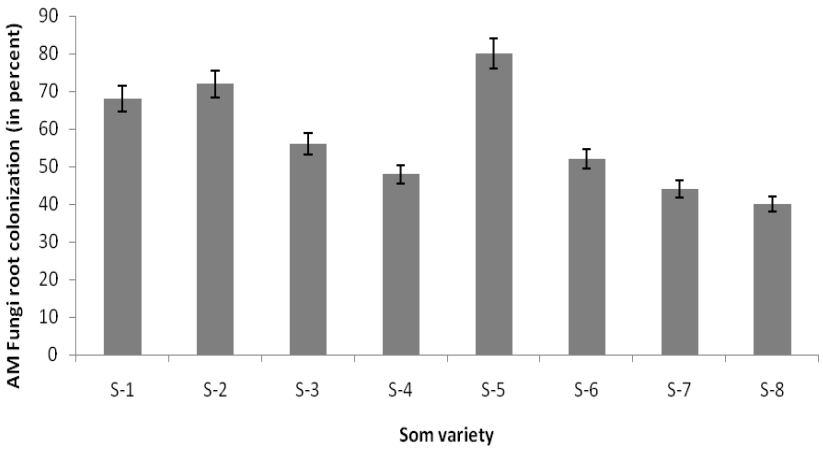


Figure 28: Root colonization (in percent) with arbuscular mycorrhizal fungi in som plants

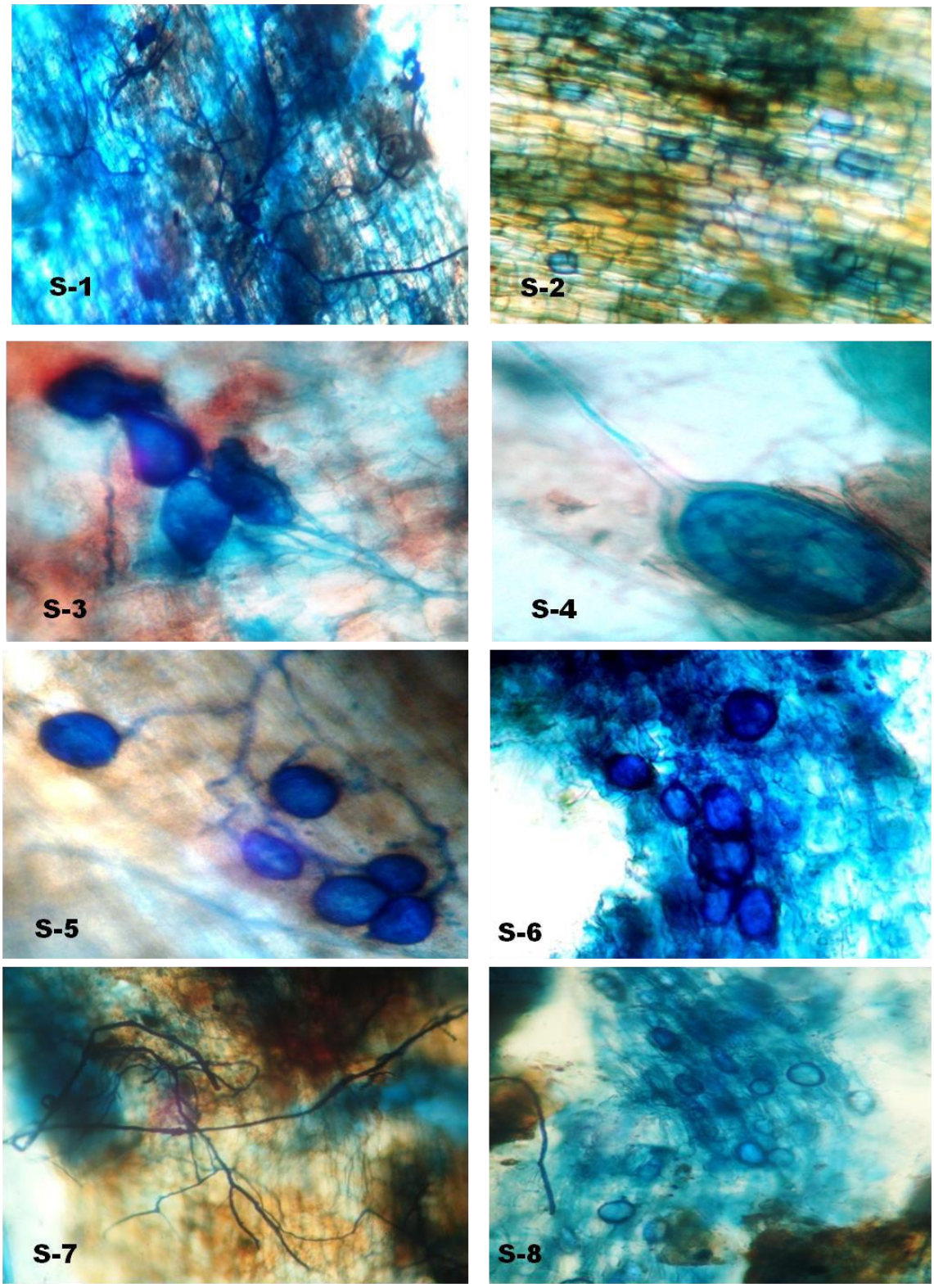


Figure 29: **Root colonization of different morphotypes of Som plant with AM fungi in experimental field of NBU showing intraradical hyphae (S-1and S-7), arbuscules and vesicles embedded in root cells(S-2 and S-8), branched vesicles (S-3,S-5 and S-6) and spore with long hyphal attachment (S-4).**

4.8.3 Immunolocalization of AMF in som root tissues

4.8.3.1 Indirect immunofluorescence

PAbs of AMF andgoat antisera specific to rabbitglobulins conjugated with FITC were used for indirectimmunofluroscence study to detect the AMF hyphae colonization in roottissues of som plants.Roots of som saplings were carefully separated, cleaned and inoculated withAMF spores extracted from the rhizosphere of som plant. The present investigation was designed to locate colonization of AMF in the rhizosphere as well as their cellular localization in root tissues of som.

On observation under UV- microscope brightapple green fluorescence of the hyphae, vesicles and arbuscules within the host tissuewere shown. Fluorescence was more prominent towards the cortex layer in most of thetissues which tells us their successful colonization in som roots leading towardsinduction of resistance(Figure 30)

4.8.3.2 Immunogold labelling

Labeling of AMF treated root segments was performed onsections of LR-white embedded tissues, previously fixed with 0.1 M sodium phosphate buffered-glutaraldehyde (2.5%) and using PAb of AMF and labelled with antirabbit-IgG (whole molecule) gold conjugate (10nm). AMF treated roots showed the presence of gold particles scattered around the cell wall. Gold particles were concentrated mostlynear the cell wall and interfacial matrix (Figure 31). Presence of gold particles proved that colonization of AMF in som root tissue was viable and that it could be easily detected.

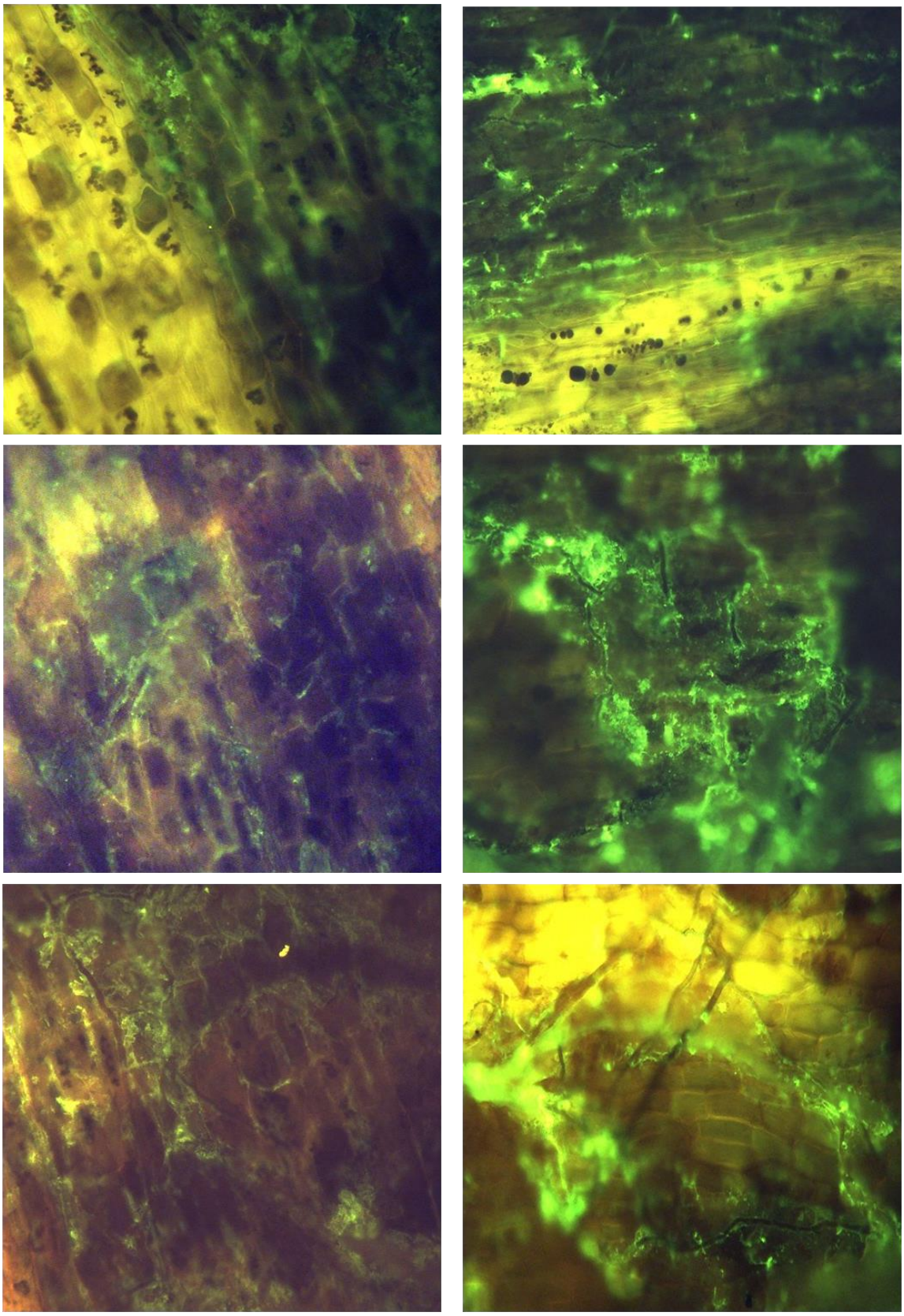


Figure 30: Indirect immunofluorescence of som root tissue treated with PAb of AMF and labelled with FITC

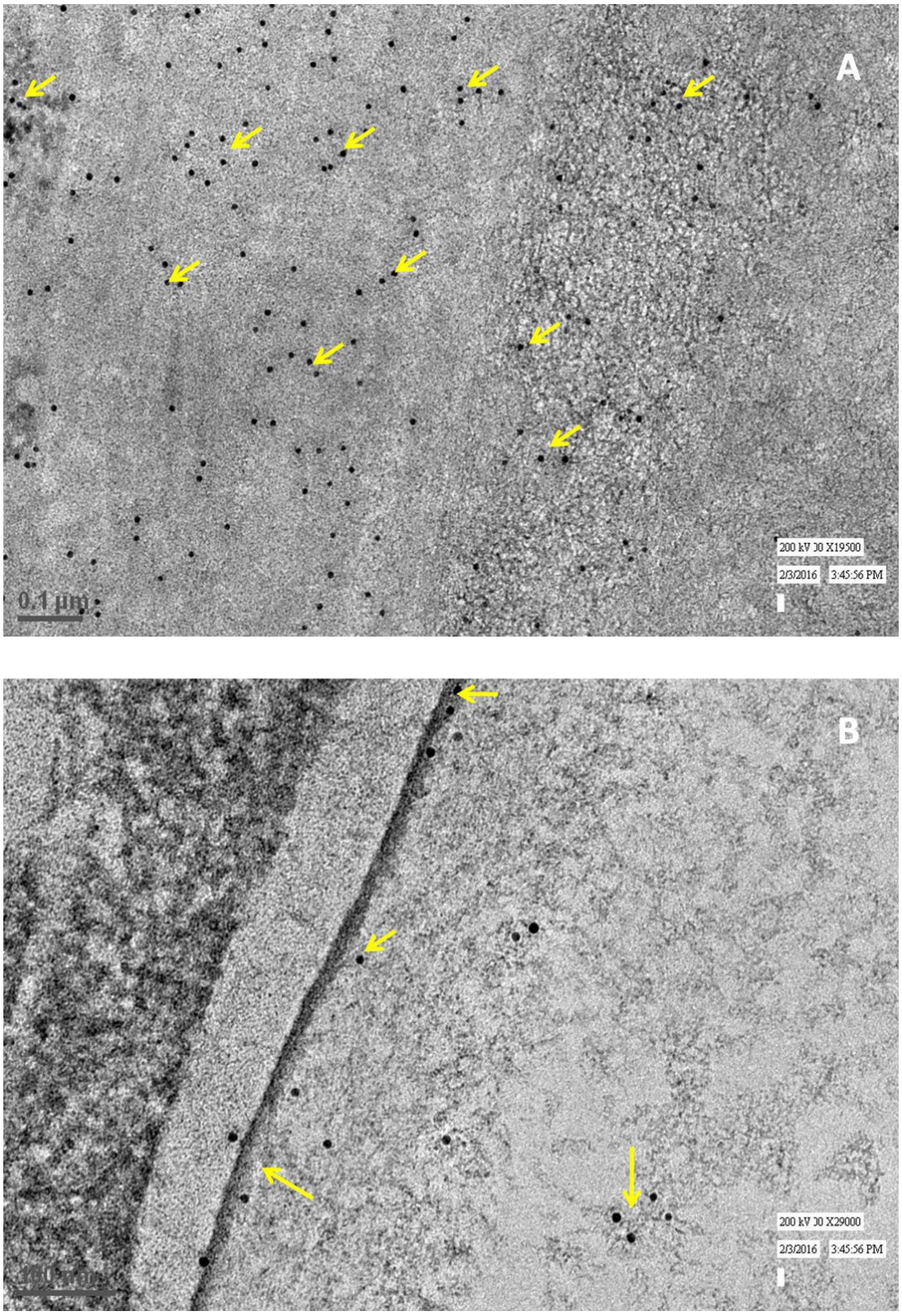


Figure 31: Transmission electron micrograph of som root tissue treated with PAb-AMF and labelled with antirabbit-IgG (whole molecule) gold conjugate

4.9 Molecular detection of foliar fungal pathogens of som plants

Genomic DNA of both the fungal pathogens *Colletotrichum gloeosporioides* and *Pestalotiopsis disseminata* weresuspended in 100µl 1X TE buffer treated with RNAse (60 µg) until further use. Agarosegel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNAevaluated in terms of the ratio between absorbance of A260 and A280 showed that genomicDNA was ~1.8. PCR amplification of ITS region of 18 S rDNA was carried out using ITS1 and ITS4, universal primers.

4.9.1*Colletotrichumgloeosporioides*

4.9.1.1 18 S rDNA sequence analysis

The BLAST query of the 18S rDNA sequence of *C.gloeosporioides*against GenBankdatabase confirmed its identity. The sequences have been deposited in NCBI, GenBankdatabase under the accession no. KM491736.The sequence chromatograms have beenrepresented in Figure 32 .

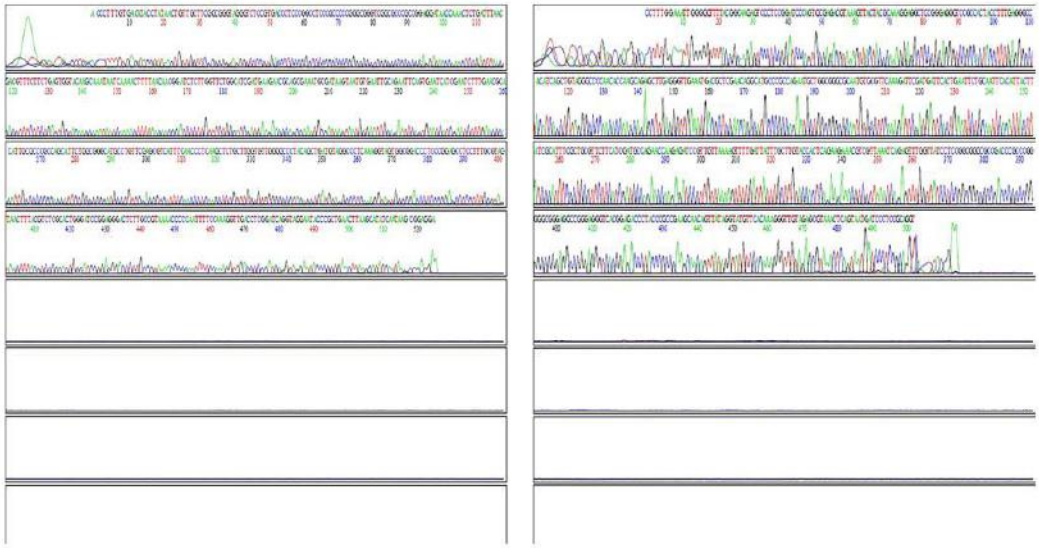
4.9.1.2 Multiple Sequence Alignment

A multiple sequence alignment of ITS gene sequences of *C.gloeosporioides*was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Figure 33).

4.9.1.3 Phylogenetic analysis

Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *C.gloeosporioides*(KM491736) (Table 26). The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 1.32880262 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates)are shown next to the branches.

Chromatogram



Partial sequence of 18S RNA gene

ACCTGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCTTTGTGAACATACCTATAACTGTT
GCTTCGGCGGGTAGGGTCTCCGTGACCCTCCCGGCCTCCCGCCCCCGGGCGGGTCGGCGCCC
GCCGGAGGATAACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAA
AACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCT
GGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACA
GCTGATGTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTTA
CGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTTTCCAAAGGTTGACCT
CGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Sequence deposited: NCBI

Accession: KM491736
Version: KM491736.1
GI: 695614263
DNA LINEAR: 563bp

Title: *Colletotrichum gloeosporioides* strain SOM.CL.02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ORIGIN

```
      1 acctgcggag ggatcattac tgagtttacg ctctacaacc ctttgtgaac atacctataa
     61 ctgttgcttc ggcgggtagg gtctccgtga ccctcccggc ctcccgcccc cgggcgggtc
    121 ggcgcccgcc ggaggataac caaactctga tttaacgacg tttcttctga gtggtacaag
    181 caaataatca aaacttttaa caacggatct cttggttctg gcatcgatga agaacgcagc
    241 gaaatgcgat aagtaatgtg aattgcagaa ttcagtgaat catcgaatct ttgaacgcac
    301 attgcgcccg ccagcattct ggcgggcatg cctgttcgag cgtcatttca accctcaagc
    361 tctgcttggt gttggggccc tacagctgat gtaggccctc aaaggtagtg gcggaccctc
    421 ccggagcctc ctttgcgtag taactttacg tctcgcactg ggatccggag ggactcttgc
    481 cgtaaaaccc ccaattttcc aaaggttgac ctcggatcag gtaggaatac ccgctgaact
    541 taagcatatc aataagcgga gga
//
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Figure 32: Chromatogram and sequence deposition of 18S r DNA region of *C. gloeosporioides* (KM491736).

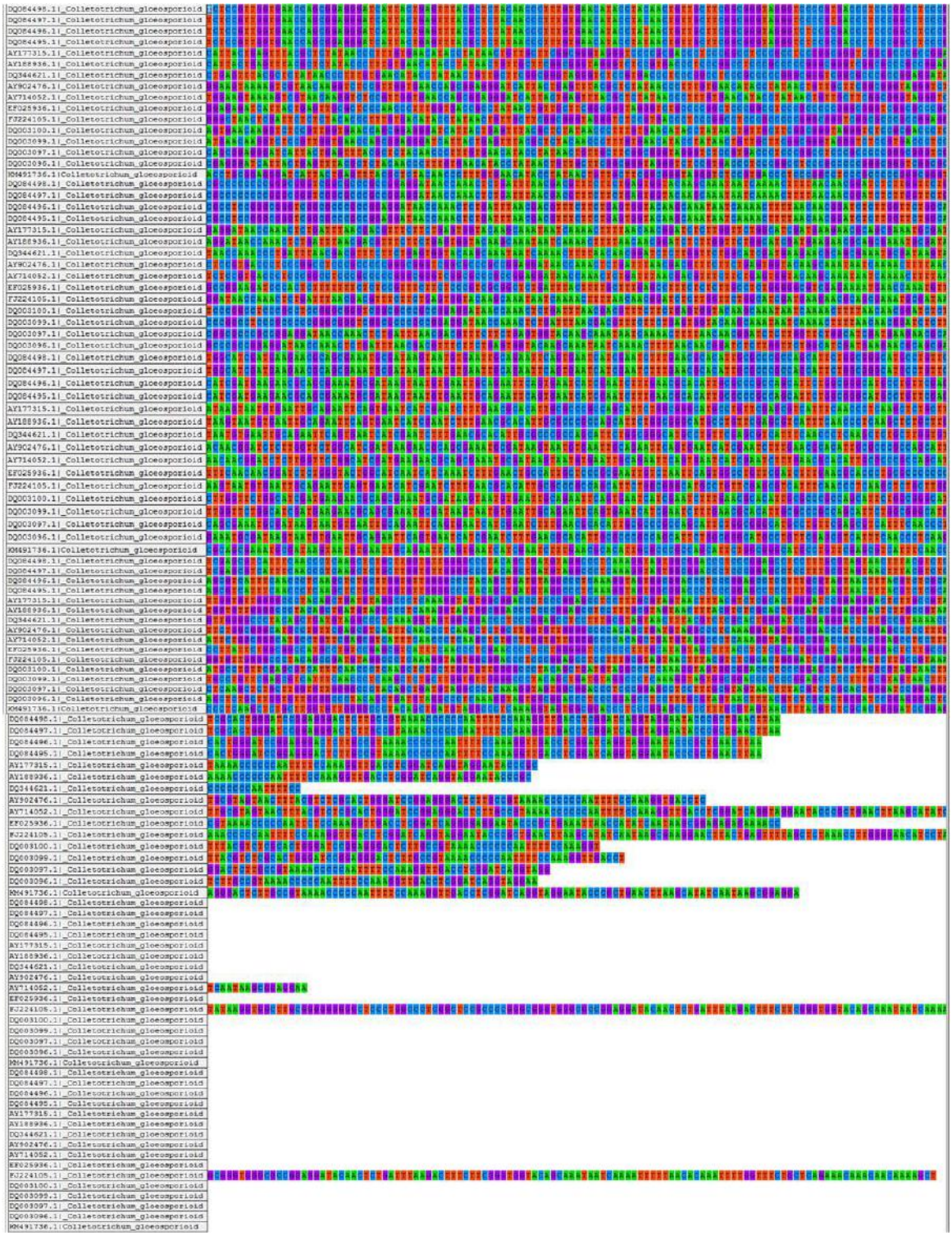


Figure 33: Multiple sequence alignments of *C. gloeosporioides*(KM491736) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

Table 26: GenBank accession numbers and geographic location of ex-type strains of *Colletotrichum* species that showed homology with isolate SOM/CL/02

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Country
1	KC790943	LC0600	592 bp	India
2	KC790936	MTCC10324	583 bp	India
3	JX076858	IMI176619	432 bp	India
4	KU498272	BRIP 49125	602 bp	Australia
5	KF772134	F10PGBYS01	562 bp	China
6	KM105221	IMI 387103	549 bp	Germany
7	KM105189	Abr 3-1	548 bp	Germany
8	KM105180	CBS 102189	546 bp	Germany
9	KC425661	F15	534 bp	Italy
10	KT285382	COUFAL7300	548 bp	Brazil
11	KU933924	LC03	581 bp	China
12	KC790960	MTCC10326	586 bp	India
13	DQ084498	P-1	560 bp	Israel
14	DQ084497	L38	560 bp	Israel
15	DQ084496	L60	557 bp	Israel
16	DQ084495	L50	557 bp	Israel
17	EF025936	Cm 8	560 bp	India
18	FJ224105	178	752 bp	China
19	**KM491736**	**SOM/CL/02**	**563 bp**	**India**

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 398 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Figure 34)

4.9.1.4 Species specific primer for identification of *C. gloeosporioides*

Specific characterization of *C. gloeosporioides* isolates (SOM/Cl/01, SOM/Cl/02, SOM/Cl/03 and SOM/Cl/04) were carried out using Colletotrichum gloeosporioides specific primer pair CgInt-ITS4. PCR amplification of 14 different isolates of fungal pathogens of som plant was carried out using this specific primer pair. Result as presented in figure 35B shows the presence of a single band in lanes containing the PCR products of 4 isolates of *C. gloeosporioides* when run in 1% Agarose gel. On comparison with the molecular ladder it was observed that a single

band of 480bp was produced by 4 isolates only. According to the literature, these 4 isolates are *C. gloeosporioides* isolates since this specific primer pairs only amplifies *C. gloeosporioides* isolates.

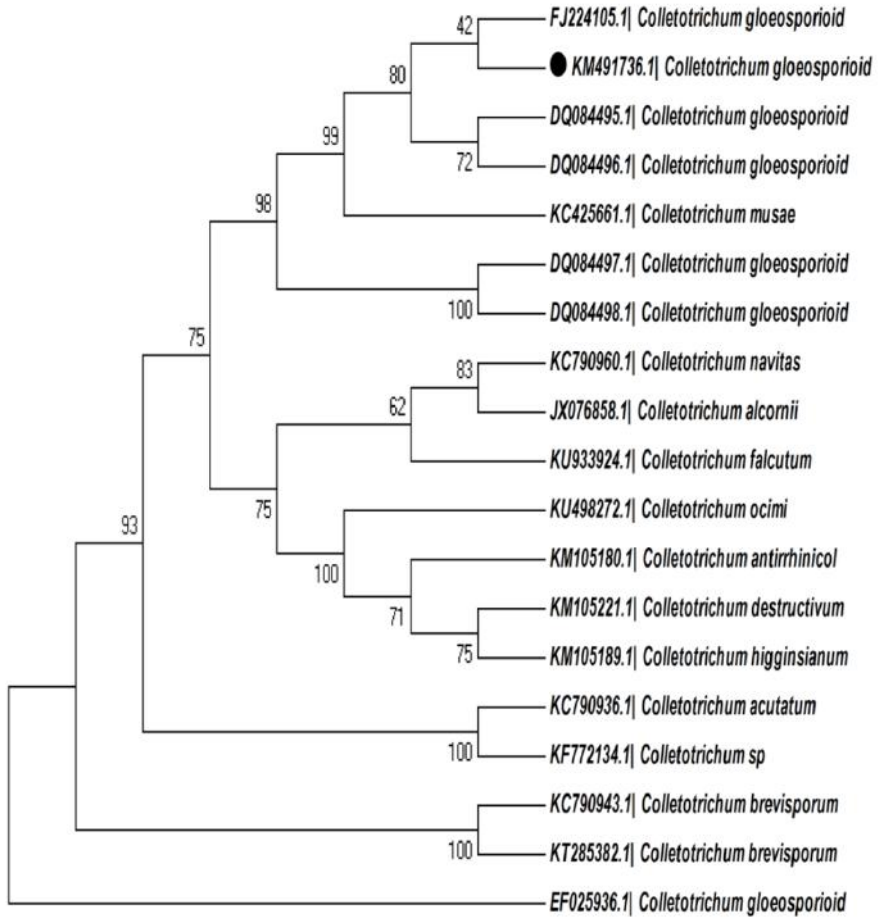


Figure 34: Phylogenetic placement of *C. gloeosporioides* (KM491736) with other ex-type strain sequences obtained from NCBI GenBank Database

Figure 35: PCR amplification of genomic DNA of foliar fungal pathogens, (A) using ITS1/ITS4 primer (M – DNA ladder, Cl/02 – *C. gloeosporioides* isolate, P/01 – *P. disseminata* isolate) (B) using primer pair CgINT/ITS4 (Lane 1-4: *Pestalotiopsis* isolates, Lane 5-10: *Bipolaris* isolate; Lane 11- 14*: Colletotrichum* isolates)

4.9.2 *Pestalotiopsis disseminata*

4.9.2.1 18 S rDNA sequence analysis

The BLAST query of the 18S rDNA sequence of *P.disseminata* against GenBankdatabase confirmed its identity. The sequences have been deposited in NCBI, GenBankdatabase under the accession no. KT697994.The sequence chromatograms have beenrepresented in Figure 36.

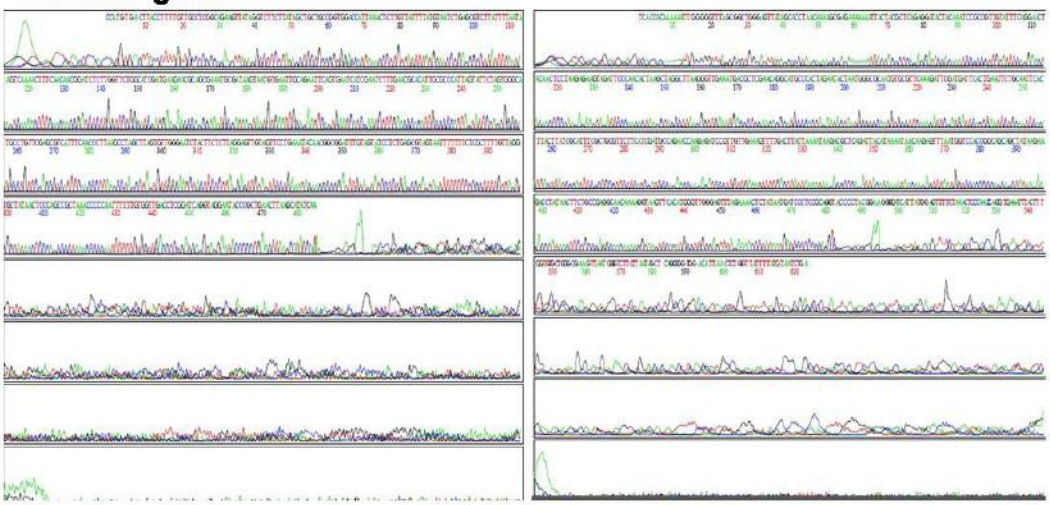
4.9.2.2 Multiple Sequence Alignment

A multiple sequence alignment of ITS gene sequences of *P.disseminata* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Figure 37.).

4.9.2.3 Phylogenetic analysis

Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *Pestalotiopsis* sp (KT697994) (Table 27). The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 1.46762707 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 436 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Figure 38).

Chromatogram



Partial sequence of 18S ribosomal RNA gene

GCGGAGGGATCATTATAGAGTTTTCTAAACTCCCAACCCATGGAACTTACCTT
TTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTATAGCTGCTGCCGGTGGACCA
TTAAACTCTTGTTATTTTATGTAATCTGAGCGTCTTATTTTAATAAGTCAAAACTT
TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
TGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCT
TAAGCCTAGCTTAGTGTTGGGAATCTACTTCTCTTAGGAGTTGTAGTTCCTGA
AATACAACGGCGGATTTGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTCGCTT
TTGTTAGGTGCTATAACTCCCAGCCGCTAAACCCCCAATTTTTTGGGTTGACC
TCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT

Sequence deposited NCBI

Accession: KT697994
Version: KT697994.1
GI: 941063000
DNA LINEAR: 521 bp

Title: *Pestalotiopsis* sp. IPL/SOM/P/01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

ORIGIN

1 gcggagggat cattatagag ttttctaaac tcccaaccca tgtgaactta ccttttgttg
61 cctcggcaga agttataggt cttcttatag ctgctgccgg tggaccatta aactcttgtt
121 attttatgta atctgagcgt cttattttaa taagtcaaaa ctttcaacaa cggatctctt
181 ggttctggca tcgatgaaga acgcagcgaa atgcgataag taatgtgaat tgcagaattc
241 agtgaatcat cgaatctttg aacgcacatt gcgcccatta gtattctagt gggcatgcct
301 gttcgagcgt catttcaacc cttaagccta gcttagtgtt gggaatctac ttctcttagg
361 agttgtagtt cctgaaatac aacggcggat ttgtagtatc ctctgagcgt agtaattttt
421 ttctcgcttt tgttaggtgc tataactccc agccgctaaa cccccaattt tttgtggttg
481 acctcggatc aggtaggaat acccgctgaa cttaagcata t

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Figure 36: Chromatogram and sequence deposition of 18S rDNA region of *Pestalotiopsis sp* (KT697994)

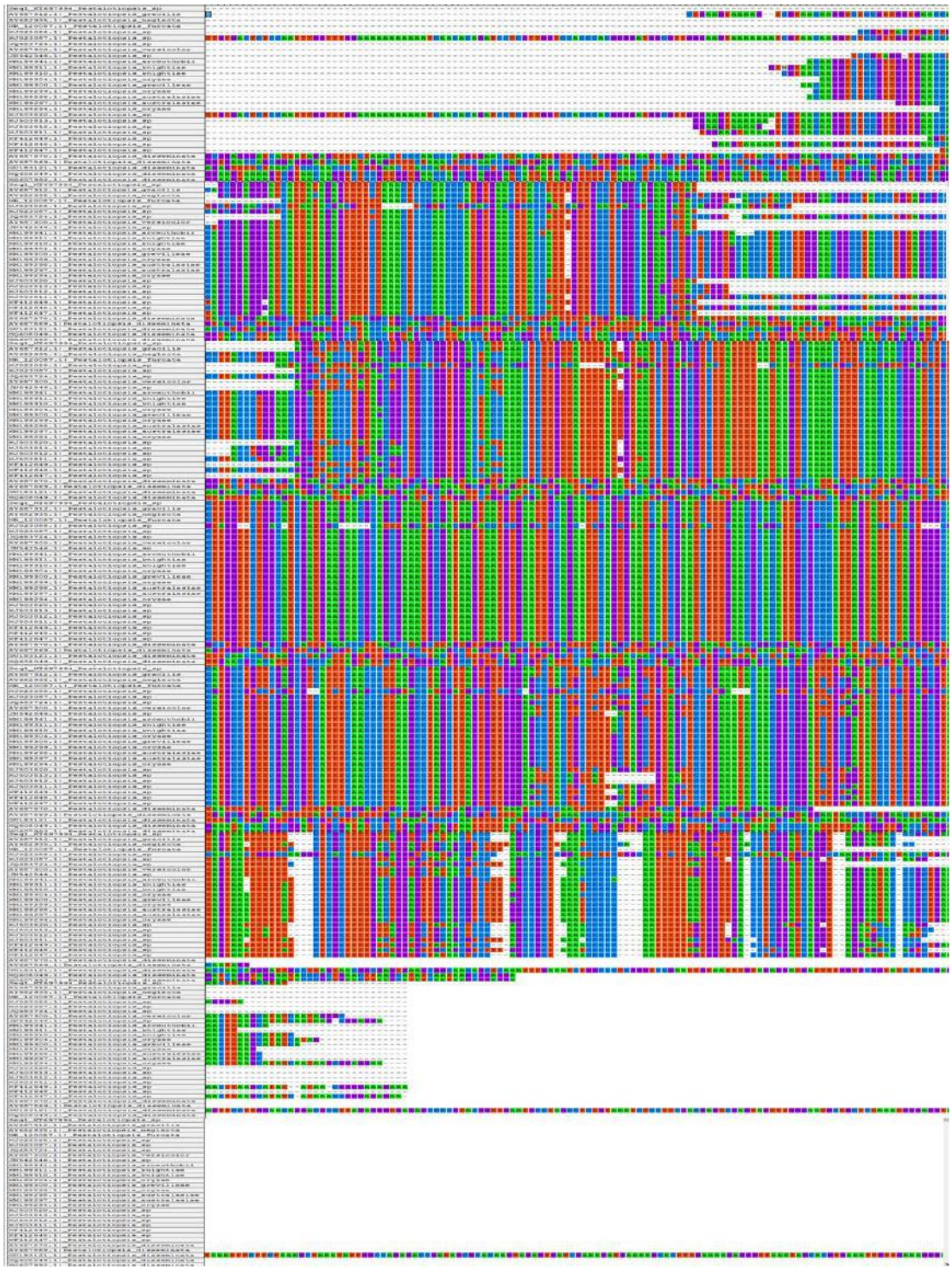

Figure 37: Multiple sequence alignment of *Pestalotiopsis*sp(KT697994) with other extypes isolate. The conserved regions of the gene are demonstrated in different colour

Table 27: GenBank accession numbers and geographic location of ex-type strains of *Pestalotiopsis* species that showed homology with isolate IPL/SOM/P/01

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Country
1	AY682935	sop753-1023	564 bp	China
2	AY687312	sop766-1026	542 bp	China
3	NR120087	MFLUCC 12-0054	538 bp	Thailand
4	KJ023088	HGUP4301	534 bp	China
5	KJ023087	HGUP4301	622 bp	China
6	JQ683724	MFLUCC12-0054	538 bp	Thailand
7	AY687300	162417	530 bp	China
8	JN542546	L1	548 bp	-
9	KM199341	CBS 434.65	593 bp	USA
10	KM199310	CBS 114138	604 bp	New Zealand
11	KM199298	CBS 114141	596 bp	Australia
12	KJ503820	MFLUCC13-0915	622 bp	China
13	KJ503813	MFLUCC12-0125	553 bp	Thailand
14	KJ503812	MFLUCC12-0121	551 bp	Thailand
15	KJ503811	MFLUCC12-0314	598 bp	Thailand
16	KF412648	1443274	598 bp	China
17	AY687870	PSH2000I-066	560 bp	China
18	GU183121	NRRL 36915	1113 bp	Georgia
19	HQ607992	CY152	629 bp	Texas
20	**KT697994**	**IPL/SOM/P/01**	**521 bp**	**India**

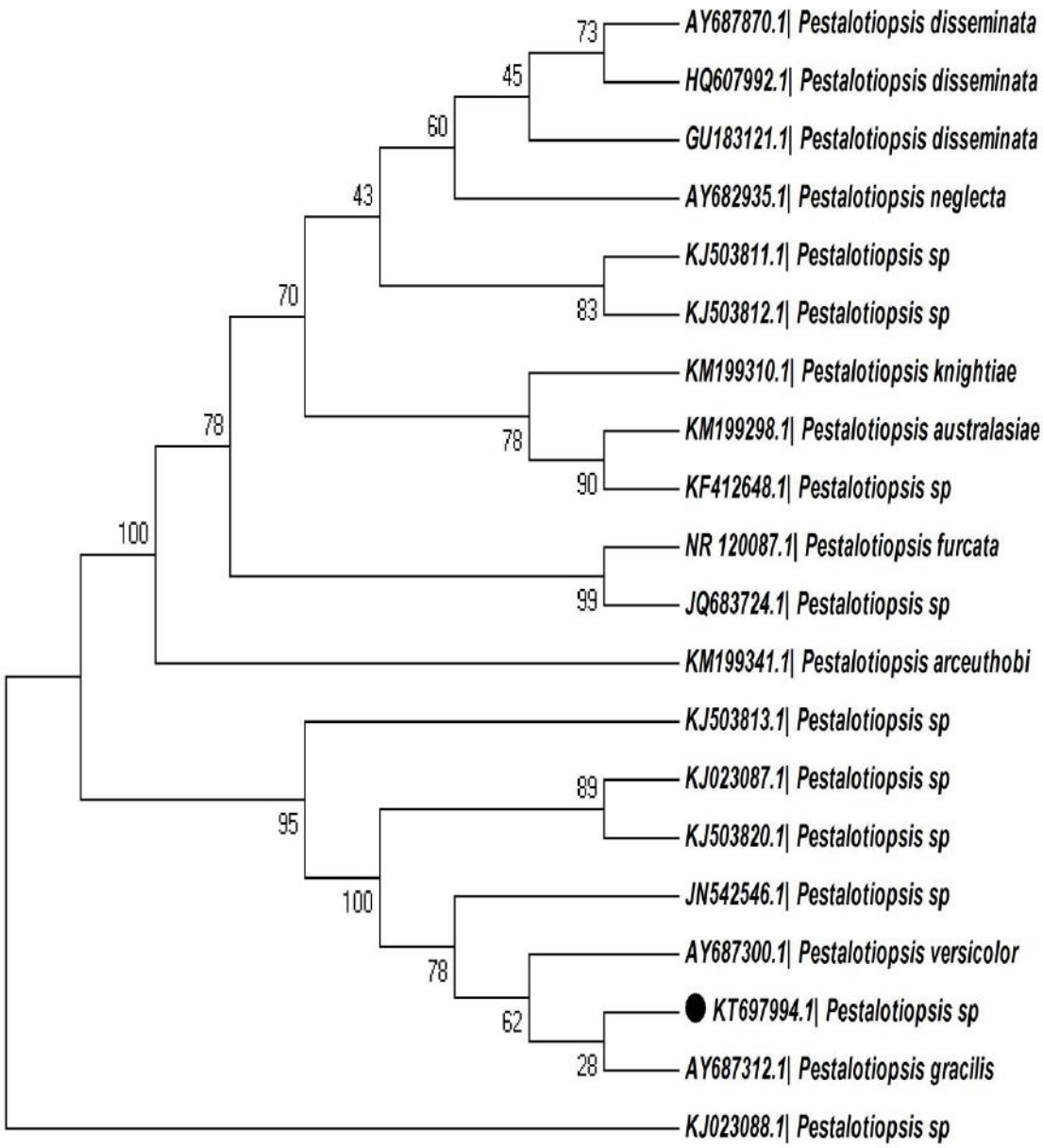

Figure 38: Phylogenetic placement of *Pestalotiopsis* sp(KT697994) with other ex-type strainsequences obtained from NCBI GenBank Database

4.10 Molecular characterization of isolates of foliar fungal pathogens

4.10.1 RAPD analysis

Genomic DNA of different isolates of *C. gloeosporioides* and *P. disseminata* was amplified using ITS1/ITS4 primers. Polymerase Chain Reaction wasperformed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taqpol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mMdNTPs, 1.5 µl of 100 mMreverse and forward primers and 1 µl of 50 ng template DNA.The genetic relatedness among the isolates were analysed by random primers to generate reproducible polymorphism. Among the three different primers (OPA-1, OPD-6 and OPD-12), OPD-6 and OPD-12 did not produce any polymorphic bands. However OPA-1 produced a total of 22 bands out of which 6 bands were polymorphic. The RAPD profile showed that primer OPA-1 showed maximum polymorphism among the different isolates of *C. gloeosporioides* and *P. disseminata* (Table 28). Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix. Based on the results obtained all the eight isolates can be grouped into two main clusters. RAPD banding pattern revealed that the isolates of *C. gloeosporioides* and *P. disseminata* were genetically different and showed polymorphism among each other (Figure39).

Table 28: Total number of polymorphic bands produced by different RAPD primers

Primer	No. of RAPD products (bands)		
	Total bands	Polymorphic bands	% polymorphism
OPA-1	22	6	27%
OPD-6	16	0	-
OPD-12	16	0	-

4.10.2 DGGE analysis

DGGE is an electrophoretic technique that efficiently separates DNA molecules according to their size, as wellas sequence differences. Analysis of genetic diversity among different isolates of *Pestalotiopsis* and *Colletotrichum* was evaluated on the basis of the sequence difference between their conserved sequences.

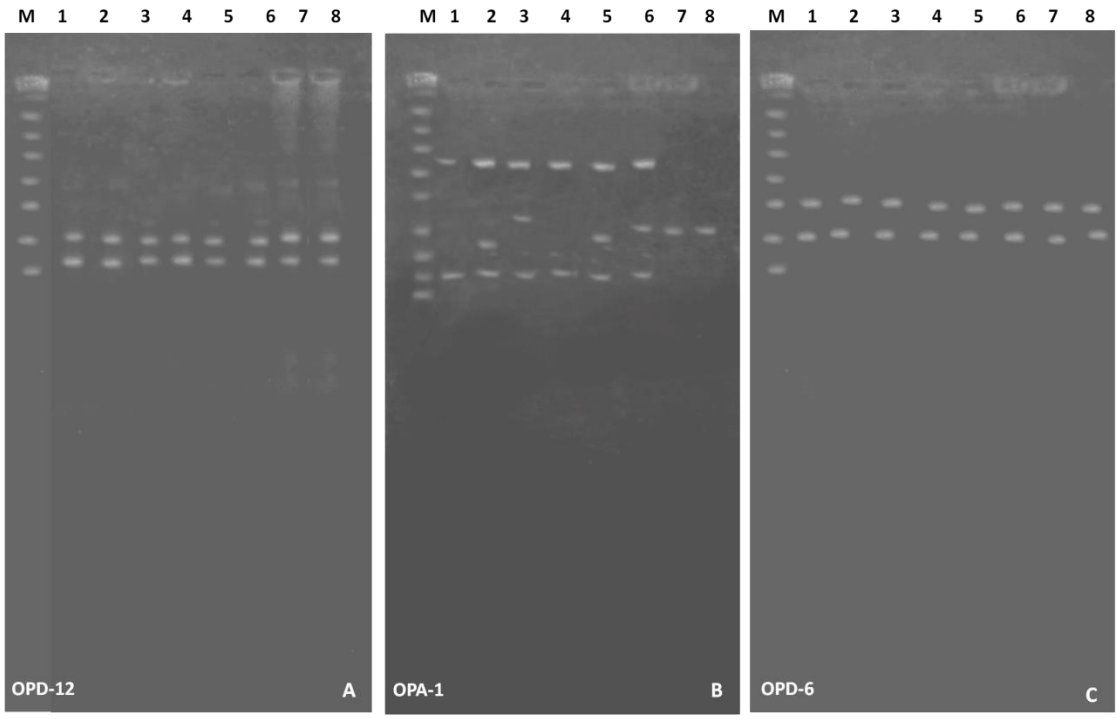

Figure 39: RAPD-PCR analysis of *Colletotrichum* and *Pestalotiopsis* isolates of som plant using primers (A) – OPD-12, (B) - OPA-1 and (C) - OPD-6. Lane M – DNA ladder, Lane 1-4 *C.gloeosporioides* isolates (SOM/Cl/01, SOM/Cl/02, SOM/Cl/03, SOM/Cl/04), Lane 5 – 8 *P. disseminata* isolates (IPL/SOM/P/01, IPL/SOM/P/02, IPL/SOM/P/03, IPL/SOM/P/04)

For this the ITS (18S rDNA gene sequences of fungal genome) were amplified using a special set of primers, forward primer containing GC clamp at 5' end (F352T: 5' – CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C – 3' and 519r: 5' – ACC GCG GCT GCT GGC AC – 3').The amplified products were then electrophoresed in a perpendicular DGGE performed with "The Decode Universal Mutation Detection System" (BioRad Laboratories, USA). A series of gradient ranging from 0-100%, 0-50%, 10-80% and 20-60% was utilized for optimizing a suitable concentration for analyzing the amplicons.

Denaturing Gradient Gel electrophoresis analysis of four different isolates of *Colletotrichum gloeosporioides* (SOM/Cl/01, SOM/Cl/02, SOM/Cl/03, SOM/Cl/04) and four isolates of *Pestalotiopsis disseminata* (SOM/P/01, SOM/P/02. SOM/P/03, SOM/P/04) was assessed using the GC flung primers as mentioned above. The uniform PCR product of 300bp was obtained. The DGGE electrophoresis yielded a uniform and unique banding pattern of each group of organism. In this uniform

gradient gel of 20-60%, the banding pattern among the four isolates each of *P. disseminata* and *C. gloeosporioides* were identical to that of the reference isolates used for confirmation (Figure 40). Out of these eight isolates, only one of *P. disseminata* (SOM/P/01, KT697994) and one of *C. gloeosporioides* (SOM/Cl/02, KM491736) has been sequenced and their GC content known. These two isolates were taken as reference isolates. The eight isolates have been separated into two distinct groups based on the migration rate of their amplified 18S fragment following DGGE. The amplified bands of *C. gloeosporioides* migrated faster than those of *P. disseminata*. This is due to the GC content of the isolates where the GC content of *C.gloeosporioides* is 52% and *P. disseminata* isolate is 42%. DNA with higher GC content will always migrate faster in denaturing gel. Themigration of amplified 18S rDNA samples within each group was similar, suggesting that therewas little intraspecific variation among the isolates.

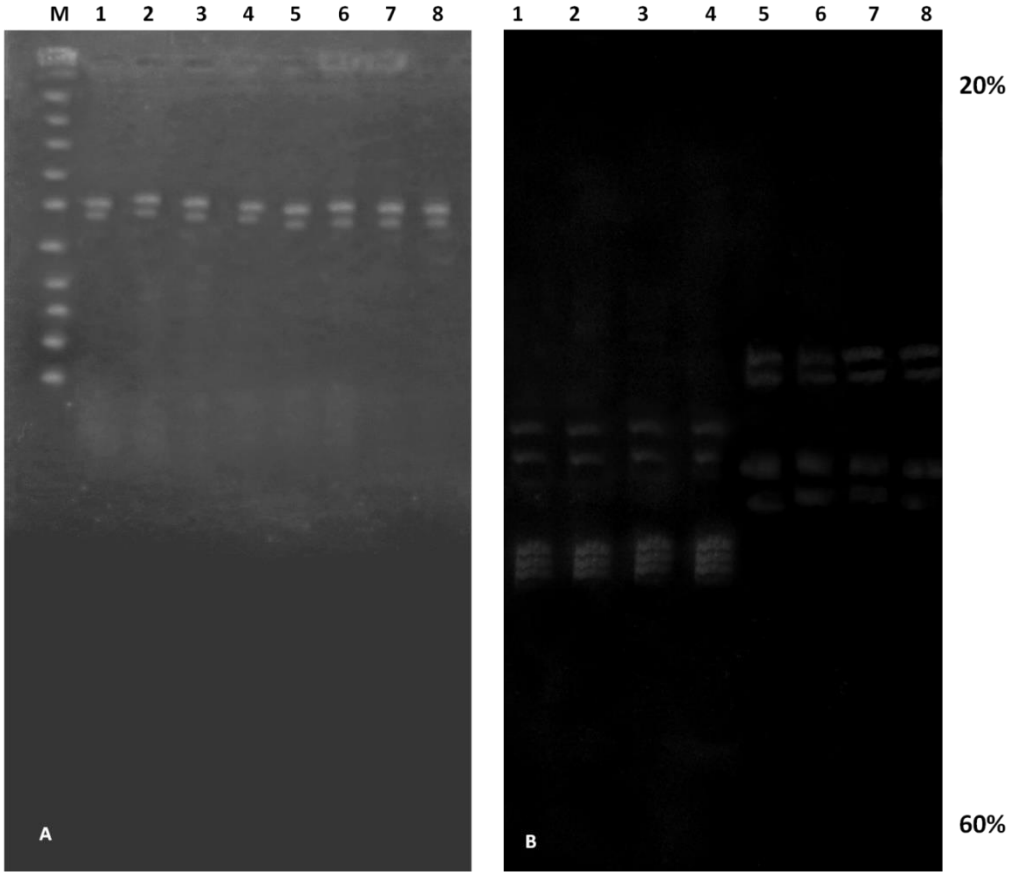

Figure 40: Denature Gradient Gel Electrophoresis of the ITS-PCR amplified products of isolates of *Colletotrichum gloeosporioides* (Lane 1-4; SOM/Cl/01, SOM/Cl/02, SOM/Cl/03, SOM/Cl/04) and *Pestalotiopsis disseminata* (Lane 5-8; IPL/SOM/P/01, IPL/SOM/P/02, IPL/SOM/P/03, IPL/SOM/P/04). (A) – ITS-PCR products, 300bp; (B) – Gradient 20-60%, 8hrs run, 100V

4.11 Determination of activity of defense enzymes in som leaves infected naturally with foliar fungal diseases

4.11.1 Peroxidase

Peroxidases are members of a large group of heme-containing glycoproteins that catalyze oxidoreduction between hydrogen peroxide and various reductants. They have an absolute requirement of hydrogen peroxide as electron donor. Peroxidases are implicated to play multiple roles in plant-pathogen interactions. In case of peroxidise activity (quantitative analysis) o-dianisidine was used as substrate and its oxidation was monitored spectrophotometrically. For qualitative profiling of POX isozymes benzidine, another aromatic diamine, was used as a substrate. It gave rise to distinct blue coloured bands after separation of enzyme extract by Native PAGE and staining. Peroxidase specific activity was assessed in healthy and naturally infected som leaf tissues for the eight different som morphotypes. The results are presented in figure 41(A) where it can be noticed that peroxidase activity has increased in all infected leaf samples when compared with their respective healthy samples. Highest amount of activity was seen in infected leaves of S7 morphotype and lowest in S8 morphotype.

4.11.1.1 Peroxyzyme analysis

Peroxidase variations have been reported to be used as genetic markers at different levels within a taxon. Hence at the onset peroxyzyme analysis for all the eight morphotypes of som was studied. It was seen that there was a lot of variations among the different isozymes present in each morphotypes shown as different bands present. However two bands with R_m value 0.28 and 0.53 were seen common in all morphotypes.

In order to reveal changes in the isozyme patterns on infection, Native PAGE was performed in 4 morphotypes (S3, S5, S6 and S7) according to the percentage disease incidence. Three bands (3, 4 and 5) with Rm values 0.23, 0.45 and 0.57 respectively were present in both healthy as well as in infected leaf samples of S3, S5 and S6 morphotypes but extra bands (1, 2 and 6) with Rm values 0.11, 0.17 and 0.82 respectively were present only in infected leaf samples. (Figure 42A).However peroxyzyme analysis of healthy and infected leaves of S7 morphotype revealed the presence of a new band in infected leaf samples correspondingto Isozyme 1 being produced in infected leaves and not in healthy samples, shown in Figure 42B.Isozyme 2 was produced in both healthy and infectedsamples.

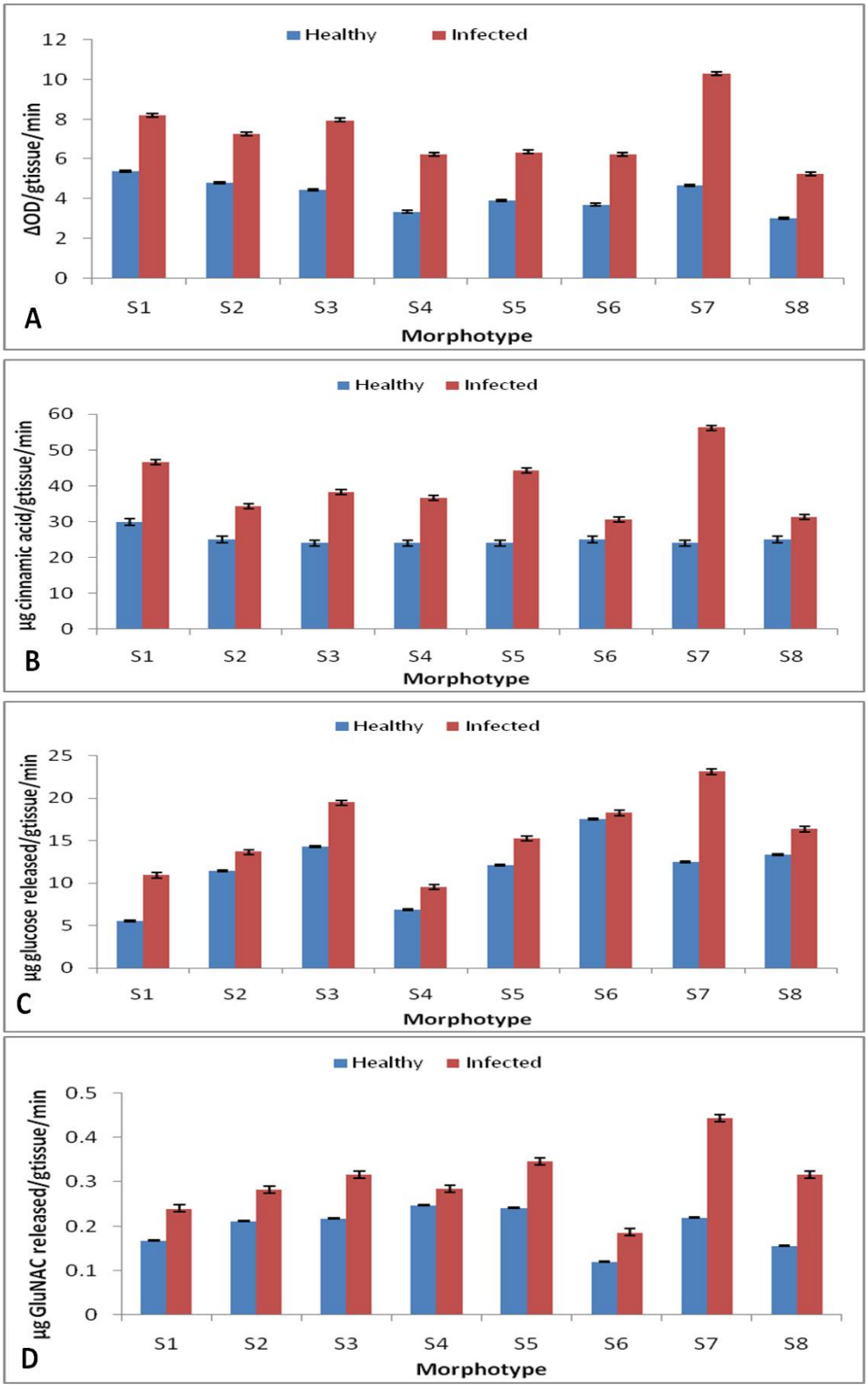

Figure 41: Analysis of defense enzymes in healthy and *C. gloeosporioides* infected leaves of som morphotypes; A – Peroxidase; B – PAL; C – Chitinase, D - β 1,3Glucanase

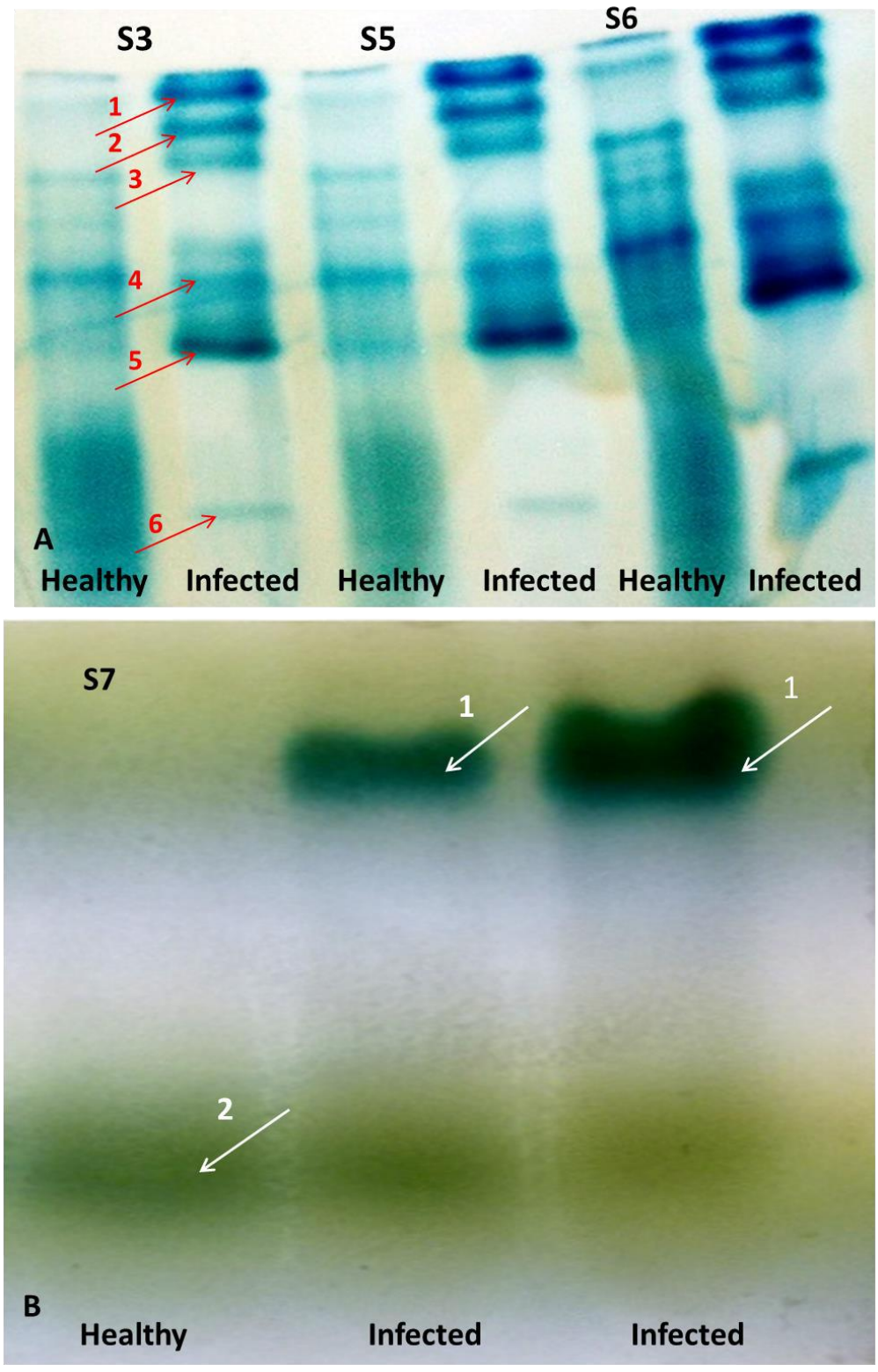

Figure 42: (A and B) – Peroxyzyme analysis of healthy and infected leaves of som morphotypes.

4.11.2 Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) enzyme activity was measured in healthy and infected som leaves for all the eight morphotypes. As shown in the figure 41(B). PAL activity was more in infected leaves than in healthy leaves. Highest activity was seen in S7 morphotype and least in S6 morphotype. The enzyme activity correlates with disease incidence in all the morphotypes.

4.11.3 Chitinase

Chitinase enzyme is one of the important PR proteins involved in defense mechanism of plants. This enzyme was also analysed for healthy and infected leaves of all eight morphotypes of som plants. It was seen that infected leaves had increased activity of the enzyme than the healthy leaves. However in S6 morphotype the levels were almost same for both the samples. Highest increase of enzyme was seen in S7 morphotype correlating the fact the disease establishment was also high in this particular morphotypes (Figure 41C).

4.11.4 β 1,3 Glucanase

β 1,3 Glucanase activity was also measured in healthy and blight infected leaves. In this case also increase of enzyme activity was noted in infected leaves of all morphotypes but the highest was in S7 morphotype. However the enzyme activity did not increase much in infected leaves when compared with their corresponding healthy leaves (Figure 41D).

4.12 Analysis of diffusible compounds in som following natural infection with pathogens

4.12.1 Changes in the levels of phenolics

4.12.1.1 Total Phenol

Total phenols were estimated in healthy and naturally infected som leaves. It was observed that total phenol content increased in infected leaves in al morphotypes. However accumulation of total phenol was more in those morphotypes where disease incidence was less. Highest accumulation of total phenol was seen in S7 morphotype and lowest was seen in S6 morphotype as evident in table 29.

4.12.1.2 Orthophenol Content

Orthodihydroxy phenol content was also estimated for healthy and infected som leaves of all morphotypes. Table 29 revealed that the highest accumulation of

Ortho phenol was seen in S7 morphotype and least was seen in S6 morphotype. These results are similar to total phenol variation in healthy and infected leaves.

Table 29: Total and Ortho-phenol content in healthy and infected leaves

Morphotype	Total Phenol Content (mg/g tissue)		Ortho-phenol Content (mg/gtissue)	
	Healthy	Infected	Healthy	Infected
S1	3.90±0.12	5.60±0.15	2.35±0.05	3.75±0.03
S2	2.50±0.36	5.20±0.11	1.80±0.03	3.50±0.03
S3	2.90±0.22	3.20±0.11	1.25±0.05	2.25±0.02
S4	3.60±0.16	4.50±0.12	2.25±0.04	3.25±0.05
S5	2.80±0.19	4.20±0.16	1.25±0.03	3.25±0.01
S6	2.20±0.11	2.50±0.14	1.36±0.02	2.75±0.04
S7	3.25±0.12	6.65±0.13	2.00±0.04	4.50±0.01
S8	3.29±0.14	4.80±0.11	1.63±0.05	3.25±0.01
CD(P=0.05)	Treatments 0.912 Morphotype 1.824		Treatments 0.421 Morphotype 0.843	

4.12.2 Studies on biological activities of leaf diffusates

The diffusates were collected from leaves of all morphotypes of som plants showing variable degree of susceptibility towards the leaf blight disease. Their effect on spore germination and appressoria formation of *Colletotrichum gloeosporioides*was tested *in vitro*. The diffusates from all morphotypes were fungitoxic (Table 30). Percentage inhibition of spore germination as well as appresoria formation was highest in S7 morphotypes indicating the fact that the diffusate of that morphotypehas some potential fungitoxicity that is inhibiting the growth of the particular organism.

Table 30: Effect of leaf diffusate of som morphotypes on spore germination and appressoria formation of *C. gloeosporioides*

Morphotype	Spore germination[a], %	Inhibition of spore germination, %	Appressoriaformation[a], %	Inhibition of appressoria formation, %
Control	83.5±1.2	-	65.0±1.8	-
S1	9.5±1.6	89	4.2±1.6	94
S2	6.5±1.3	92	0	100
S3	43.2±1.4	48	20.5±1.2	68
S4	24.2±1.8	73	8.3±1.1	87
S5	39.5±1.1	53	13.8±1.6	79
S6	45.0±1.5	46	24.6±1.8	62
S7	4.5±1.2	94	0	100
S8	35.2±1.6	58	10.2±1.1	84

[a]Average of 200 spores

4.12.3 High performance Liquid Chromatography

Phenolic acids present in healthy and infected leaves were further analysed using HPLC. Peak 1,2 and 3 are present both in healthy and infected leaves but the height of these peaks increased markedly in infected leaves in comparison with healthy leaves indicating an increase of these phenolic acids following infection. Peak 4 is present in healthy sample but disappeared in infected leaf samples. It is interesting to note that five new peaks viz 5,6,7,8 and 9 were evident in infected samples but all these peaks were absent in healthy leaf samples indicating that these phenolic acids might play an important role in defense mechanism of the plant against infection. When compared to standard phenolic acid it was confirmed that Peak 1, 2, 3 and 4 represented Resorcinol, Catechol, Morin and Chlorogenic acid respectively whereas peak 5 and 7 represented ferulic acid and peak 6 represented salicylic acid. (Table 31, Figure 43). Peak 8 and 9 was present only in infected samples and not in healthy samples. When compared with authentic phenolic acids these two peaks could not be identified.

Table 31: Retention time and peak heights of phenolics from healthy and infected leaf sample of S7 morphotype analyzed by HPLC

Sample	Peak no	Retention time	Height
Healthy	1	9.960	79.284
	2	13.310	190.922
	3	14.890	508.657
	4	21.400	42.346
Infected	1	9.990	339.562
	2	13.250	698.064
	3	14.720	945.181
	5	25.650	237.487
	6	26.680	491.245
	7	29.340	89.340
	8	30.820	68.346
	9	34.890	46.420

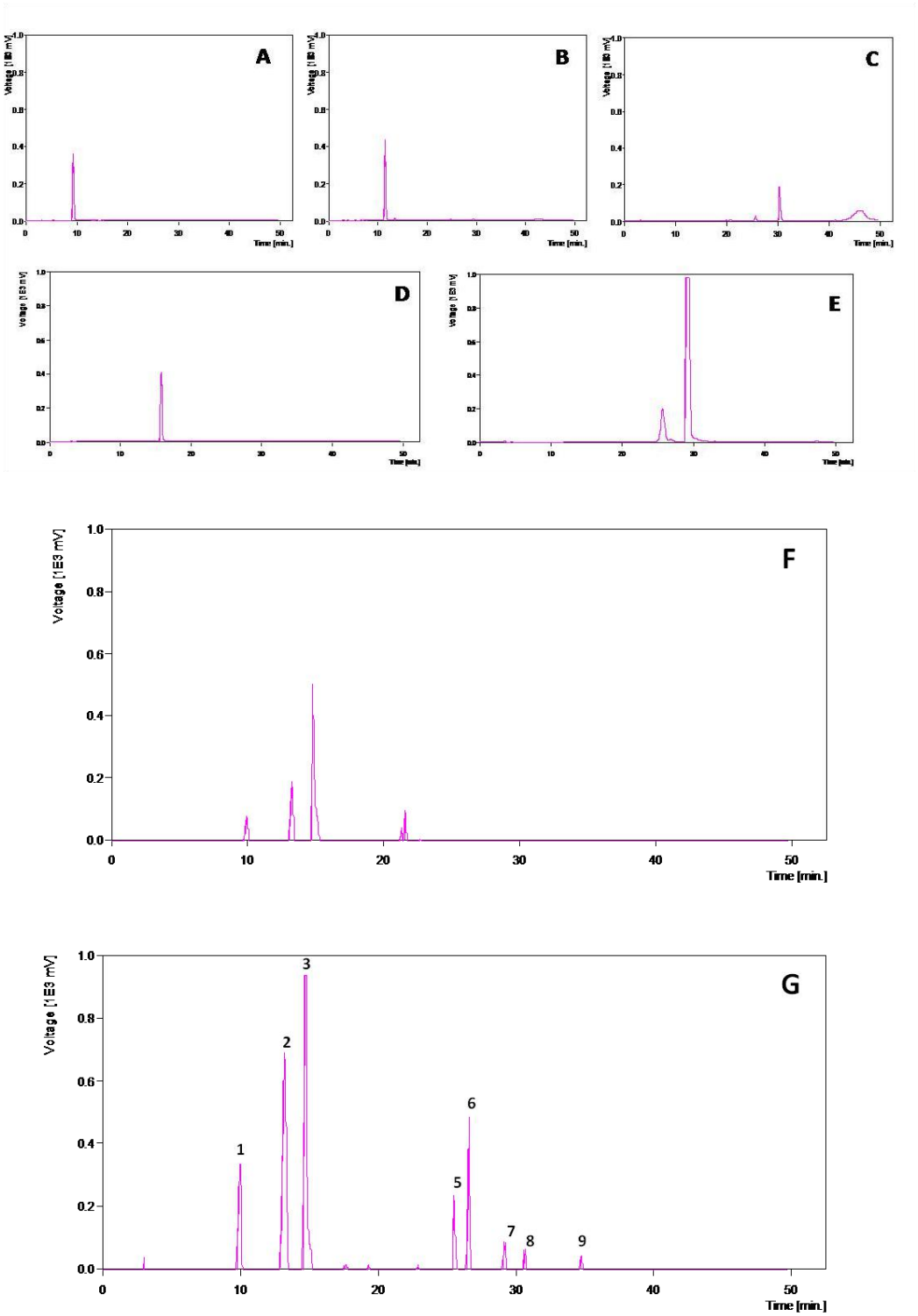

Figure 43: (A-E) HPLC profile of standard phenolic acids; A – Resorcinol, B – Catechol, C – Salicylic acid, D – Chlorogenic acid, E – Ferulic acid; (F-G)HPLC analysis of phenolic acids in leaves of Som plant; F – Healthy leaf, G – Infected leaf (1 – Resorcinol, 2 – Catechol, 3 – Chlorogenic acid, 4 – Morin, 5 and 7 – Ferulic acid, 6 – Salicylic acid

4.13 Antagonistic activity of selected bioinoculants against pathogens

4.13.1 Plant Growth Promoting Rhizobacteria(PGPR)

Two isolates of PGPR, *Bacillus pumilus* (BRHS/C1) and *B. altitudinus* (BRHS/S 73) showed growth promoting activity for eight different morphotypes of som plants. Hence these bacterial isolates were taken into consideration for testing their antagonistic activity against the two foliar fungal pathogens *C. gloeosporioides* and *P. disseminata*. For each of the antagonistic tests, 5mmdisc of pathogen isolates were taken from 5 days old culture and placed at the periphery of the petri plate and the bacterial isolate was streaked on the other side of the plate. The result of interaction is presented in Table 32 and figure 44. Both bacterial isolates could inhibit the growth of fungal pathogens markedly, however *B. pumilus* (BRHS/C1) inhibited the growth of *C.gloeosporioides* and *B. altitudinus* (BRHS/S 73) could inhibit the growth of *P. disseminata* more prominently than the other pathogen respectively.

Table 32: *In vitro* antagonistic test of selected PGPR isolates against foliar fungal pathogens

Interacting microorganisms	Diameter of fungal colony	% inhibition
C. gloeosporioides	8.6±0.09	-
C. gloeosporioides + *B. pumilus* (BRHS/C1)	3.8±0.12	55.8
C. gloeosporioides + *B. altitudinus* (BRHS/ S73)	5.6±0.18	34.8
P. disseminata	8.8±0.10	-
P. disseminata + *B. pumilus* (BRHS/C1)	3.6±0.16	59.0
P. disseminata + *B. altitudinus* (BRHS/ S73)	6.2±0.14	29.5

Average of three replicates; ± Standard error

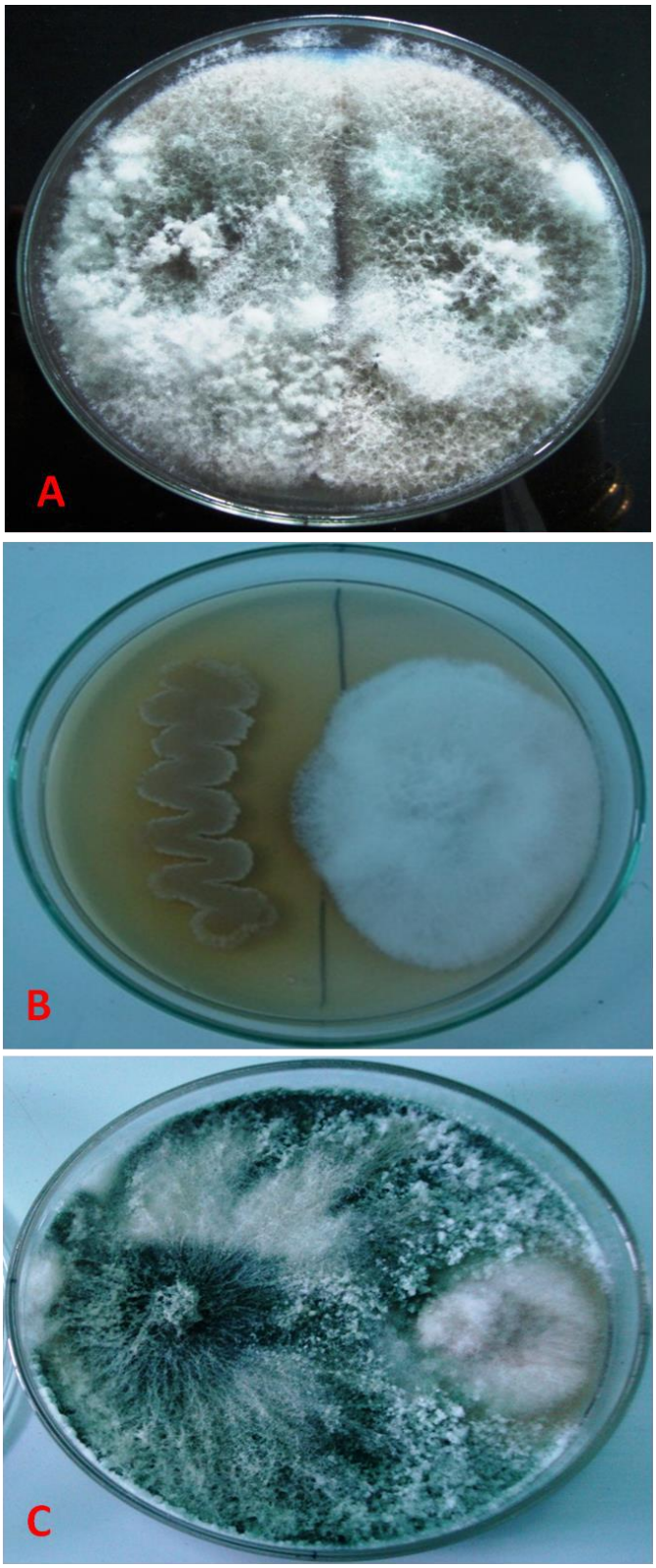

Figure 44: *In vitro* antagonistic test of PGPR and PGPF against C. *gloeosporioides* isolate (SOM/Cl/02), (A) – *C. gloeoesporiodes* culture grown alone in PDA, (B) – interaction with *B. pumilus,* (C) interaction with *T. harzianum*

4.15.2 Antagonistic effect of PGPF

Two isolates of Trichoderma, one *T. harzianum* and another of *T. asperellum* was taken into consideration for checking their antagonistic effect against the two foliar pathogen of som *C. gloeosporioides* and *P. disseminata*. For each of the antagonistic tests, 5mm disc of fungal isolates were taken from 5 days old culture and placed at the periphery of the petri plate. Similarly, agar disc of 5 mm from pathogen culture was placed in the same petri plate on the opposite end. The percent inhibition in the radial colony wascalculated by the following formula-

Percent inhibition = C-T/CX100,

Where C= radial growth in control set and T= radial growth in treated set.

Their interactions in inhibition percentage was recorded and enlisted in Table 33. *T. harzianum* isolate showed more profound inhibitory effect against the fungal pathogens than *T.asperellum* isolate (Figure 45).

Table 33: *In vitro* antagonistic test of selected PGPF isolates against foliar fungal pathogens

Interacting microorganisms	Diameter of fungal colony	% inhibition
C. gloeosporioides	8.6±0.09	-
C. gloeosporioides + *T. harzianum* (RHS/480)	1.8±0.11	79
C. gloeosporioides + *T. asperellum* (RHS/S569)	3.2±0.15	62.7
P. disseminata	8.8±0.10	-
P. disseminata + *T. harzianum* (RHS/480)	2.2±0.16	75
P. disseminata + *T. asperellum* (RHS/S569)	1.4±0.14	84

Average of three replicates; ± Standard error

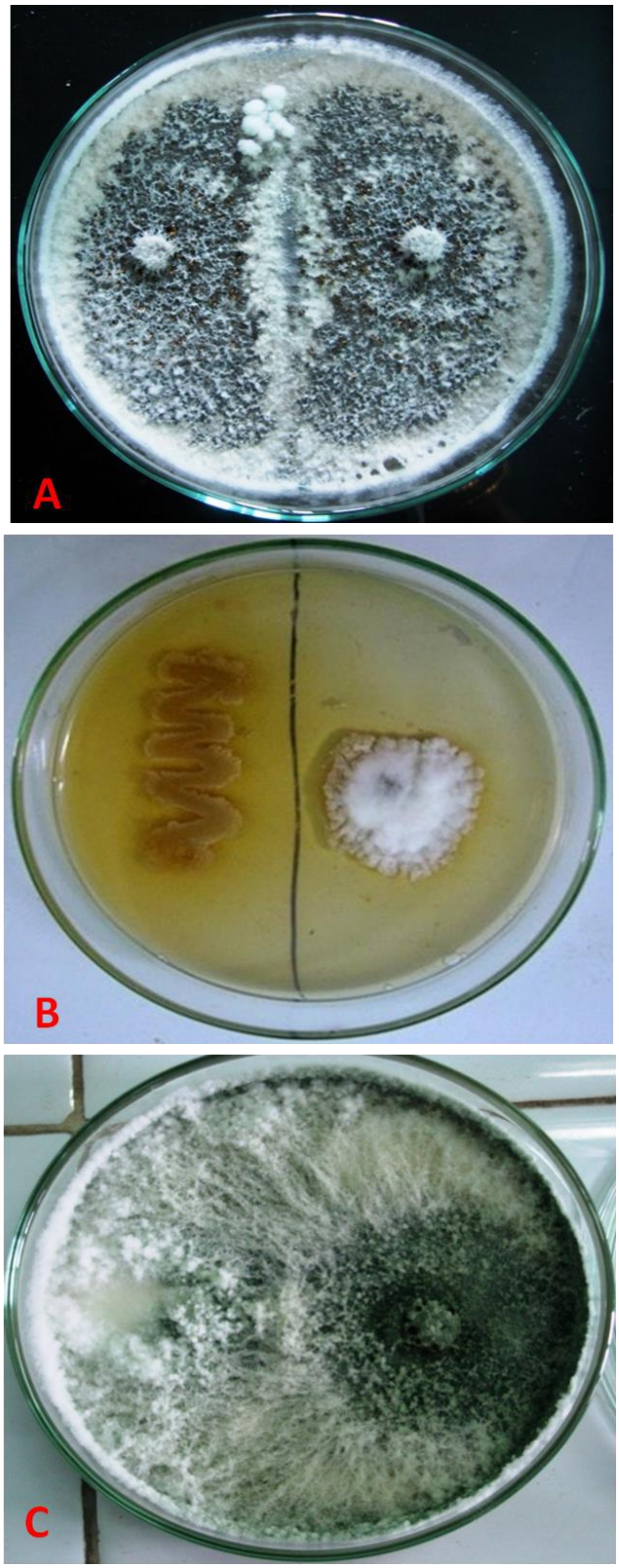

Figure 45: *In vitro* antagonistic test of PGPR and PGPF against against *P. disseminata* isolate (IPL/SOM/P/01), (A) – *P. disseminata* culture grown alone in PDA, (B) – interaction with *B. pumilus*, (C) interaction with *T. harzianum*

4.14 Growth promotion and biochemical changes in som plants following application of bioinoculants

Growth promotion in eight different morphotypes of som plants were checked following their treatment with bioinoculants viz. PGPR (*B.pumilius, B. altitudinus*), PGPF (*T.hazianum, T.asperellum*) and AMF (Consortium of *Glomus and Gigaspora*). These different bioinoculants were added to the soil at different time interval as mentioned in Materials and Methods. Effect of their application on growth and biochemical changes in som plants were noted under both nursery and field conditions.

4.14.1 Application of PGPR and AMF

4.14.1.1 Growth promotion

The effect of treatment with *B. pumilus*(BRHS/C1) and AMFpopulation, either alone or in combination, on growth ofeight different morphotypes of the som plants wasvariable. Significant increase in height, number of leavesand lateral branches were observed in all the treatmentsin comparison to their control 30 and 60 d afterinoculation in potted plants (Table 34). In field grownplants growth was recorded after 60 d of inoculationwith PGPR and AMF, either alone or in combination. Allbio treated plants showed enhanced growth over the control plants (Figure 46).Under the field condition, the growth was highest inthe plants treated with both AMF and *B. pumilus*60dtreatment compared to the control and other treatments. Among the different morphotypes, significant increase inheight was observed in plants treated with combinationof AMF and PGPR whereas number of leaf was morewhen plants were inoculated with either of thebioinoculants alone. However, differences among thedifferent morphotypes were not significant indicatingthat all morphotypes responded similarly to PGPR andAMF.

4.14.1.2 Biochemical changes

The protein content wassignificantly higher in plants with dual treatment than thesingly treated plants and control (Table 35). In case ofphenolics, both the total and orthophenol content in theleaves of the treated plants were found to be almostsimilar when either treatment was applied. However,significantly higher phenol content was recorded inplants following dual application of the inoculants.Analysis of variance revealed significant differencesbetween morphotypes and treatments.

Table 34: Growth promotion of pot grown som plants following treatment with PGPR and AMF

Morphotype	Treatment	Height (cm) After		No. of leaves after		No. of Branches After	
		30 days	60 days	30 days	60 days	30 days	60 days
S1	Control	24	29	17	22	2	3
	PGPR	52 (116)	54 (86)	49(188)	60(172)	4(100)	5(66)
	AMF	30 (25)	66(127)	26(52)	52(136)	5(150)	6(100)
	PGPR+AMF	42 (75)	97(234)	29(70)	35(59)	3(50)	4(33)
S2	Control	26	28	12	10	1	1
	PGPR	53(103)	59(110)	37(208)	52(420)	3(200)	4(300)
	AMF	29(11)	31(10)	16(33)	42(320)	4(300)	5(400)
	PGPR+AMF	36(38)	69(146)	18(50)	32(220)	3(200)	4(300)
S3	Control	26	28	10	25	0	3
	PGPR	59(126)	67(139)	42(320)	77(208)	5(500)	8(166)
	AMF	48(84)	69(146)	22(120)	51(104)	2(200)	6(100)
	PGPR+AMF	53(103)	94(235)	45(350)	62(148)	3(300)	6(100)
S4	Control	24	26	9	15	2	2
	PGPR	39(62)	50(92)	35(288)	76()406	3(50)	5(150)
	AMF	30(25)	49(88)	21(133)	62(313)	2(0)	4(100)
	PGPR+AMF	46(91)	95(265)	30(233)	65(333)	4(10)	8(300)
S5	Control	26	31	9	22	3	3
	PGPR	60(130)	72(132)	35(288)	46(109)	5(66)	5(66)
	AMF	53(103)	70(125)	32(255)	50(127)	5(66)	6(100)
	PGPR+AMF	69(165)	93(200)	40(344)	55(150)	5(66)	5(66)
S6	Control	10	14	6	16	0	0
	PGPR	41(310)	48(242)	30(400)	55(243)	2(100)	4(400)
	AMF	23(130)	55(292)	19(216)	58(262)	1(200)	4(400)
	PGPR+AMF	33(220)	71(335)	21(250)	52(225)	2(100)	4(400)
S7	Control	30	30	17	18	2	2
	PGPR	45(50)	48(60)	22(29)	31(752)	2(0)	3(50)
	AMF	34(13)	40(33)	22(29)	40(122)	2(0)	4(100)
	PGPR+AMF	44(46)	82(173)	48(182)	55(205)	4(100)	8(300)
S8	Control	10	25	5	10	0	0
	PGPR	59(490)	63(152)	18(260)	35(250)	5(500)	5(500)
	AMF	21(110)	61(144)	12(140)	30(200)	3(300)	4(400)
	PGPR+AMF	23(130)	68(172)	15(200)	43(330)	2(200)	3(300)
CD (P=0.05)	**Treatments**	**7.62**	**9.21**	**10.06**	**10.04**	**1.14**	**1.54**
	Varieties	**10.78**	**13.02**	**14.23**	**14.20**	**1.62**	**2.17**

( ) Parenthesis denotes percentage increase in relation to control

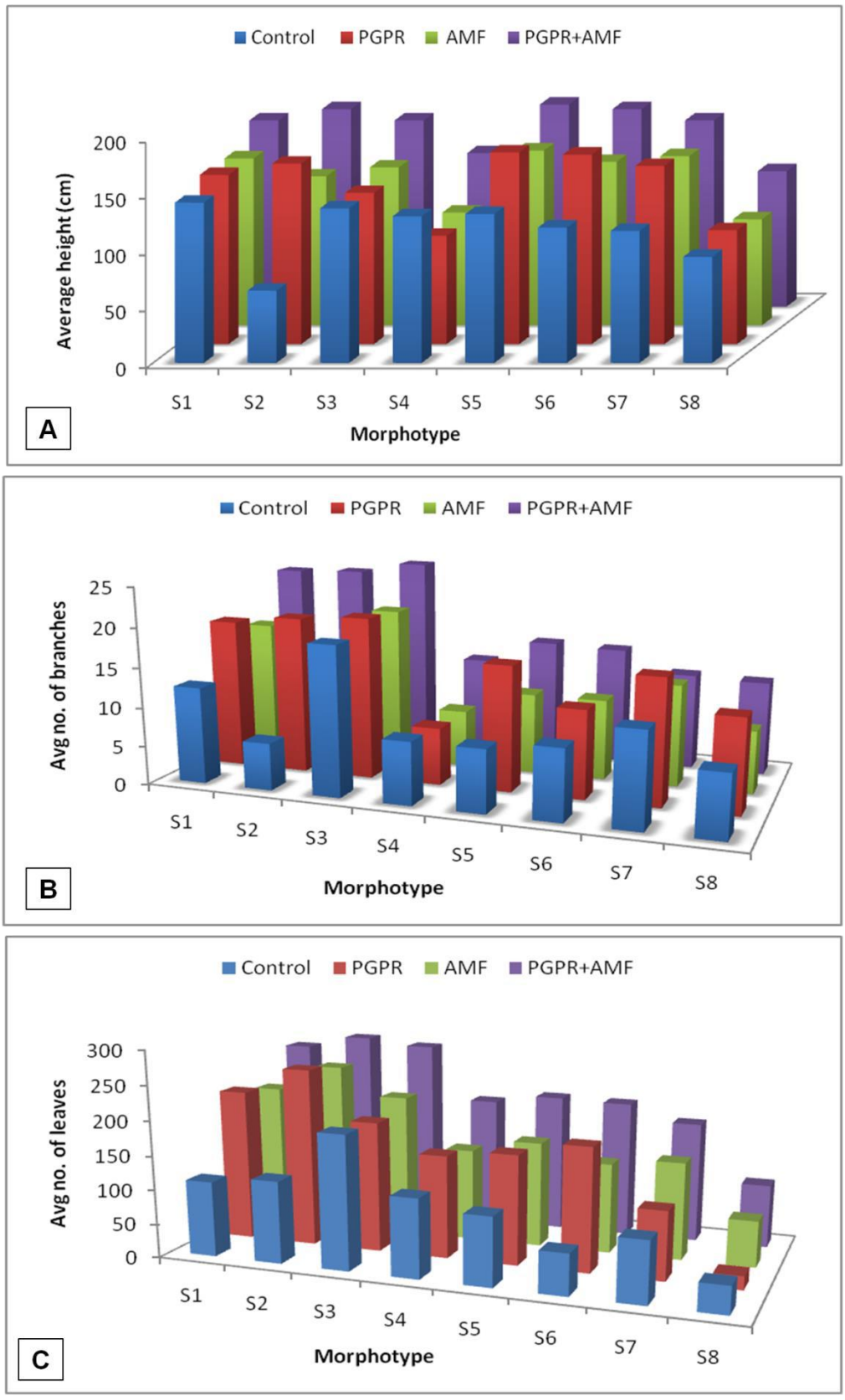

Figure 46: Growth enhancement in som plants in field following treatment with AMF and PGPR singly and jointly

Table 35: Protein and phenol content in som plants following treatment with PGPR and AMF

Morphotype	Treatment	Total Phenol content (mg/ gtissue)	Orthophenol Content (mg/gtissue)	Total Soluble protein Content (mg/gtissue)
S1	Control	3.90	2.35	42.00
	PGPR	6.50	5.25	65.00
	AMF	6.25	5.55	85.00
	PGPR+AMF	8.75	6.75	115.00
S2	Control	2.50	1.90	36.00
	PGPR	4.25	2.65	72.00
	AMF	5.00	2.05	90.00
	PGPR+AMF	5.69	3.00	107.00
S3	Control	2.90	1.25	35.40
	PGPR	4.25	2.26	77.00
	AMF	4.75	2.95	90.00
	PGPR+AMF	5.83	3.25	125.00
S4	Control	3.60	2.25	43.60
	PGPR	5.65	3.25	64.00
	AMF	4.80	3.45	82.80
	PGPR+AMF	6.80	4.50	97.50
S5	Control	2.80	1.25	34.00
	PGPR	4.50	2.75	72.00
	AMF	4.25	3.30	90.00
	PGPR+AMF	5.00	4.00	115.00
S6	Control	4.20	2.00	32.90
	PGPR	7.65	4.65	75.00
	AMF	6.50	5.80	92.00
	PGPR+AMF	8.19	6.50	105.00
S7	Control	3.25	1.63	44.80
	PGPR	5.35	3.78	69.00
	AMF	4.65	2.45	84.00
	PGPR+AMF	6.25	4.00	95.00
S8	Control	3.29	2.36	35.40
	PGPR	6.39	4.04	65.00
	AMF	6.00	3.96	77.00
	PGPR+AMF	7.36	5.00	86.00
CD (P=0.05)	**Treatments**	**0.49**	**1.05**	**7.14**
	Varieties	**0.69**	**1.49**	**10.10**

4.14.2Application of Vermicompost with value addition(PGPR and AMF)

Som plants were also treated with Vermicompost, PGPR (*B.altitudinus*) and AMF (*G.constrictum*) singly and in combination to check their effect on growth promotion and biochemical changes of som plants.

4.14.2.1 Growth promotion

Growth promotion in terms of height, no. of leaves and no. of lateral branches was recorded after 45 days of treatment. In all morphotypes, growth was significantly increased in treated plants in comparison to their respective control plants. Growth was observed to be highest in case of triple treatment of bioinoculants (Table 36,Figure 47 and 48).

4.14.2.2 Biochemical changes

Total soluble protein was quantified in leaves of control and treated som plants where it was noticed that protein content increased in leaves following the treatments. However the content was more in case of dual treatment of Vermi and PGPR as well as triple treatment when compared to control and single treatment.Total phenol content also increased in leaves following treatment and it was recorded to be highest in S3 and S4 morphotypes as shown in figure 49.

Four major defense enzymes were also studied in leaves of som plants following different treatments. It was observed that PAL, POX, CHT as well as GLU increased in all treatments than in control sets. Highest increased was seen in dual and triple treatment irrespective of morphotypes (Figure 50).

Table 36: Growth promotion in som plants following treatment with Vermicompost, PGPR and AMF, singly and jointly

Morphotype	Treatment	Height (cm)	No. of leaves	No. of Branches
S1	Control	29	22	3
	Vermi	54(89)	60(172)	5(66)
	G. constrictum	66(127)	52(136)	6(10)
	B.altitudinus	64(120)	35(59)	5(66)
	B.altitudinus +Vermi	97(234)	35(59)	4(33)
	Vermi+*G.constrictum* +*B.altitudinus*	99(241)	63(186)	8(166)
S2	Control	28	10	1
	Vermi	59(110)	33(330)	4(300)
	G. constrictum	31(10)	42(320)	5(400)
	B.altitudinus	31(10)	43(330)	6(500)
	B.altitudinus +Vermi	59(110)	32(220)	4(300)
	Vermi+*G.constrictum* +*B.altitudinus*	69(146)	52(420)	6(500)
S3	Control	28	25	3
	Vermi	67(139)	63(152)	8(166)
	G. constrictum	69(146)	51(104)	6(100)
	B.altitudinus	67(139)	55(120)	7(133)
	B.altitudinus +Vermi	67(139)	62(148)	6(100)
	Vermi+ *G.constrictum*+*B.altitudinus*	94(235)	77(208)	8(166)
S4	Control	26	15	2
	Vermi	50(92)	64(326)	5(150)
	G. constrictum	50(92)	63(320)	5(150)
	B.altitudinus	49(88)	62(313)	4(100)
	B.altitudinus +Vermi	54(107)	65(333)	8(300)
	Vermi+*G.constrictum* +*B.altitudinus*	95(265)	76(406)	9(350)
S5	Control	31	32	3
	Vermi	72(132)	46(43)	5(66)
	G. constrictum	71(129)	53(65)	6(100)
	B.altitudinus	70(125)	55(71)	6(100)
	B.altitudinus +Vermi	73(135)	50(56)	5(66)
	Vermi+*G.constrictum* +*B.altitudinus*	93(200)	57(78)	7(133)
S6	Control	14	16	1
	Vermi	48(242)	55(243)	4(300)
	G. constrictum	55(292)	54(237)	4(300)
	B.altitudinus	56(300)	58(262)	3(200)
	B.altitudinus +Vermi	60(328)	52(225)	4(300)
	Vermi+*G.constrictum* +*B.altitudinus*	71(407)	59(268)	5(400)
S7	Control	30	18	2
	Vermi	48(60)	31(72)	3(50)
	G. constrictum	40(33)	40(122)	4(100)
	B.altitudinus	41(36)	43(138)	5(150)
	B.altitudinus +Vermi	49(63)	55(205)	8(300)
	Vermi+*G.constrictum* +*B.altitudinus*	82(173)	56(211)	9(350)
S8	Control	25	20	1
	Vermi	64(156)	54(170)	4(300)
	G. constrictum	61(144)	60(200)	5(400)
	B.altitudinus	62(148)	55(175)	6(500)
	B.altitudinus +Vermi	63(152)	53(165)	3(200)
	Vermi+*G.constrictum* +*B.altitudinus*	68(172)	63(215)	8(700)
CD (P=0.05)	**Treatment** **Morphotype**	**9.308** **10.748**	**8.890** **10.265**	**1.395** **1.611**

( ) Parenthesis denotes percentage increase in relation to control

Figure 47: Growth promotion of som plants in glass house conditions after 45days of treatment with *Bacillus altitudinus*

Figure 48: Growth promotion in som plants after 45 days of treatment with bioinoculants [Row 1 – Vermi+*B.altitudinus*+*G.constrictum*; Row 2 – Vermi+*B.altitudinus*; Row 3 - Vermi; Row 4 – *B.altitudinus*; Row 5 – *G.constrictum*; Row 6 – Control]

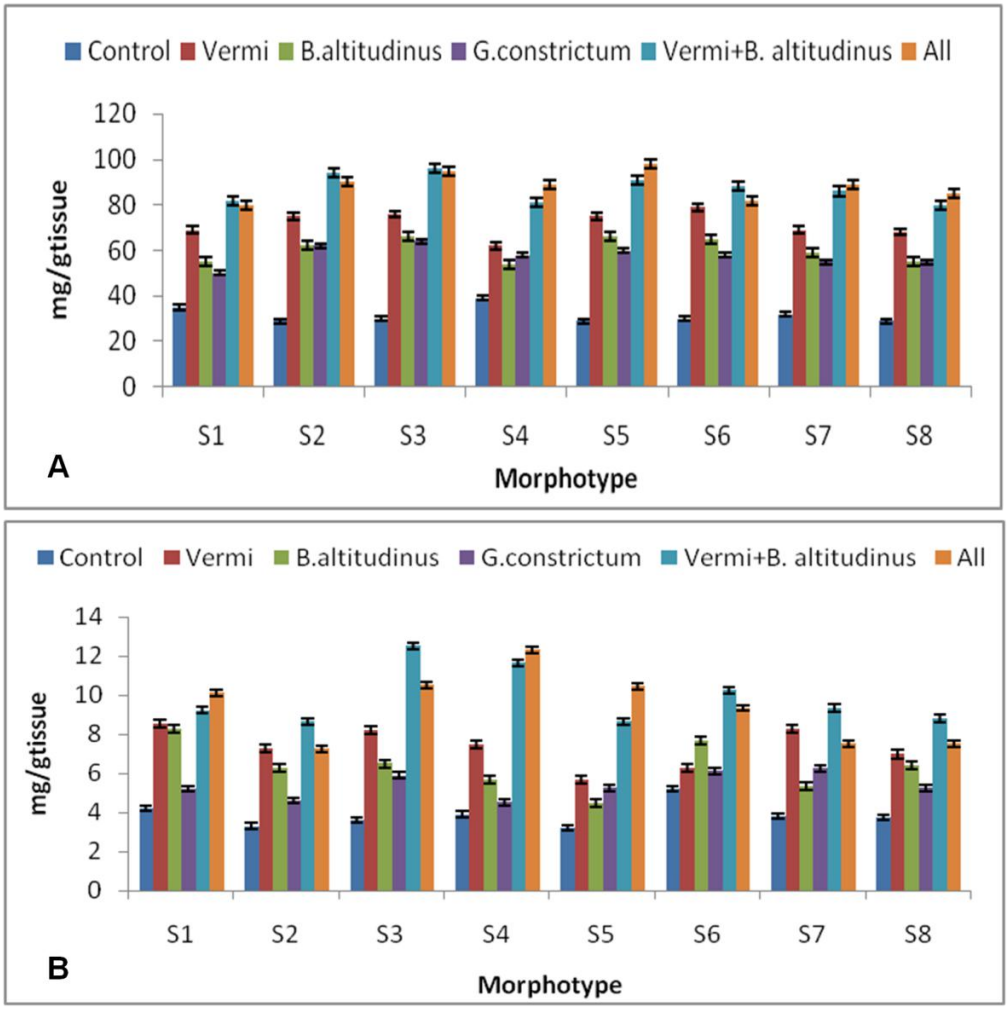

Figure 49: Total soluble protein (A) and Total phenol (B) contents in som plants following treatments with Vermicompost, PGPR and AMF

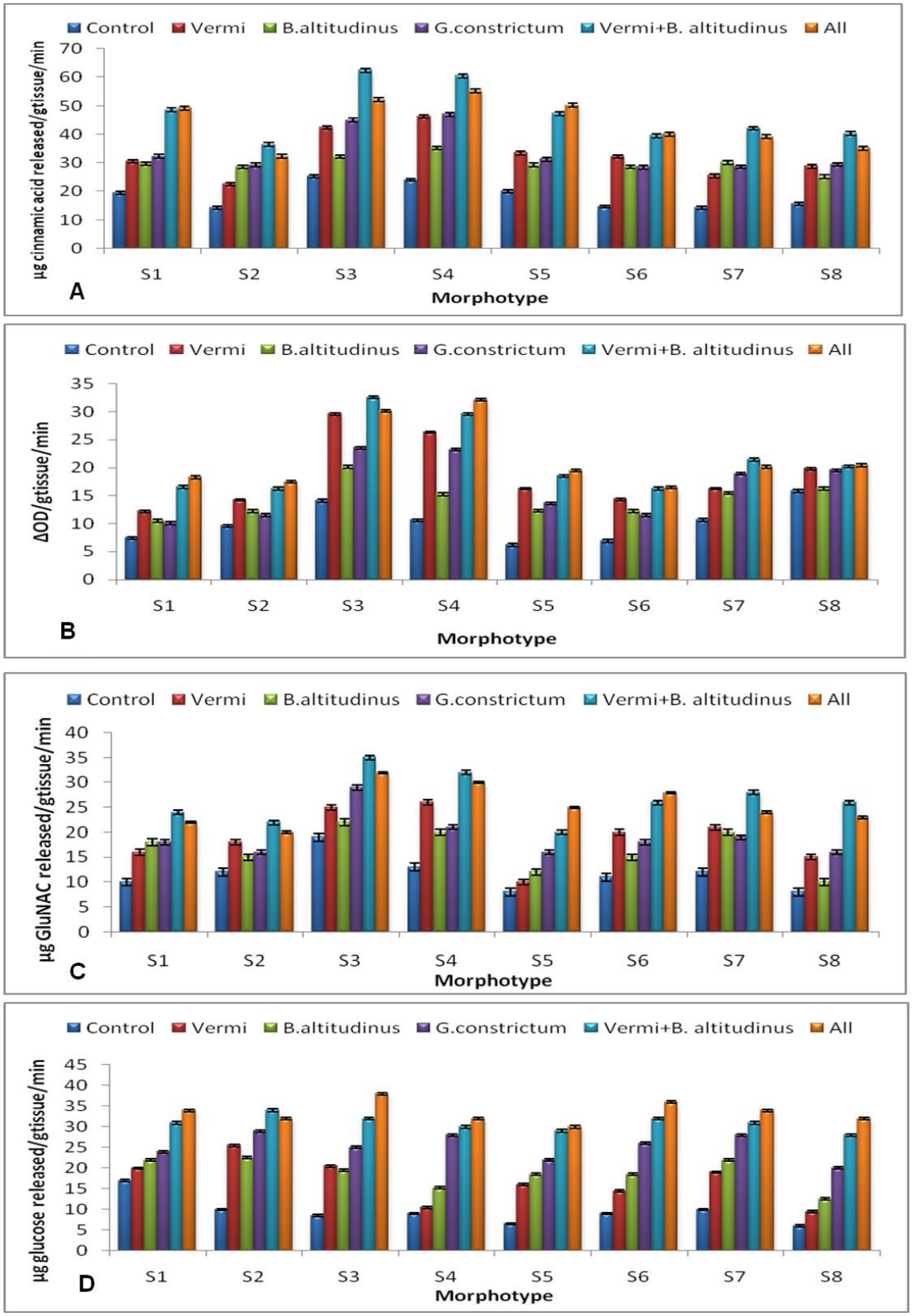

Figure 50: Activities of defense enzymes (A) PAL, (B) POX, (C) CHT and (D) GLU in leaves of som plants following treatment with value added Vermicompost

4.14.3 Application of PGPR, PGPF and AMF

Eight different morphotypes of Som plant were grown in pots under nursery conditions. They were treated with bioinoculants, viz, PGPR, AMF and PGPF in single, dual and triple combinations.

4.14.3.1 Growth promotion

Growth enhancement, in terms of height, no. of leaves and no. of branches were measured after 30 and 60 days of the final PGPR treatment. The result as shown in Table 37 revealed that growth was significantly improved after each treatment but best growth was obtained in S5 and S6 morphotypes after application of joint treatment with the three bioinoculants. Growth promotion was almost 4 fold increased following treatment in these two morphotypes.

4.14.3.2 Biochemical changes

Changes in levels of total and ortho-phenols were observed in plants treated with all the different inoculants under potted condition. Total soluble protein content also increased in treated plants in comparison to control sets. Triple treatment showed better results than dual or single treatment as shown in Figure 51.

Activities of four important defenceenzymes (POX, PAL, CHT and GLU), 48h following last application of PGPR from leaves grown in pot revealed that activity of these enzymes increased following treatment in comparison to untreated control plants. Joint treatment of all bioinoculants yielded the best results than the other combinations (Figure 52).

4.15 Activation of defense response of som plants against phytopathogens following application of bioinoculants

4.15.1 Effect of PGPR, PGPF and AMF against *Colletotrichum gloeosporioides*

The disease management approach for leaf blight disease of som plants was initially done using different combinations of bioinoculants such as PGPR (*B. pumillus*), PGPF (*T.harzianum*) and AMF (combination of *Glomus* and *Gigaspora*). These bioinoculants were used singly, jointly as well as in triple combination to check whether they reduce the disease in the plants. After 48 hrs of artificial inoculation of spore suspension of *C. gloeosporioides*, the activity of defense enzymes were checked and percent disease index (PDI) was calculated after 21 days of spray.

Table 37: Growth promotion in different morphotypes of som plants following application of PGPR, AMF and PGPF

Morphotype	Treatment	Height (cm) After		No. of leaves after		No. of Branches After	
		30 days	60 days	30 days	60 days	30 days	60 days
S1	Control	24	29	17	22	2	3
	PGPR	52(116)	54(86)	49(188)	60(172)	4(100)	5(66)
	AMF	30(25)	66(127)	26(52)	52(136)	5(150)	6(100)
	PGPF	31(29)	64(120)	25(47)	35(59)	4(100)	5(66)
	PGPR+AMF	42(75)	97(234)	29(70)	35(59)	3(50)	4(33)
	PGPF+AMF	43(79)	68(134)	32(88)	36(63)	3(50)	5(66)
	PGPR+PGPF	53(120)	95(227)	31(82)	53(140)	4(100)	6(100)
	PGPR+PGPF+AMF	55(129)	99(241)	52(205)	63(186)	6(200)	8(166)
S2	Control	26	28	12	10	1	1
	PGPR	53(103)	59(110)	37(208)	52(420)	3(200)	4(300)
	AMF	29(11)	31(10)	16(33)	42(320)	4(300)	5(400)
	PGPF	28(7)	31(10)	17(41)	43(330)	5(400)	6(500)
	PGPR+AMF	36(38)	69(146)	18(50)	32(220)	3(200)	4(300)
	PGPF+AMF	37(42)	59(110)	31(158)	33(230)	4(300)	6(500)
	PGPR+PGPF	54(107)	67(139)	31(158)	54(440)	5(400)	7(600)
	PGPR+PGPF+AMF	56(115)	69(146)	38(216)	55(450)	6(500)	8(700)
S3	Control	26	28	10	25	1	3
	PGPR	59(126)	67(139)	52(420)	77(208)	5(400)	8(166)
	AMF	48(84)	69(146)	22(120)	51(104)	2(100)	6(100)
	PGPF	47(80)	67(139)	23(130)	55(120)	3(200)	8(166)
	PGPR+AMF	53(103)	94(235)	45(350)	62(148)	3(200)	6(100)
	PGPF+AMF	54(107)	67(139)	41(310)	63(152)	5(400)	7(133)
	PGPR+PGPF	61(134)	92(228)	43(330)	57(128)	6(500)	9(200)
	PGPR+PGPF+AMF	64(146)	94(235)	64(540)	78(212)	7(600)	12(300)
S4	Control	24	26	9	15	2	2
	PGPR	39(62)	50(92)	45(400)	76(406)	3(50)	5(150)
	AMF	30(25)	49(88)	21(133)	62(313)	2(0)	4(100)
	PGPF	31(29)	50(92)	25(177)	63(320)	3(50)	5(150)
	PGPR+AMF	46(91)	95(265)	30(233)	65(333)	4(100)	8(300)
	PGPF+AMF	45(87)	54(107)	33(266)	64(326)	6(200)	9(350)
	PGPR+PGPF	42(75)	76(192)	35(288)	65(333)	8(300)	10(400)
	PGPR+PGPF+AMF	44(83)	90(246)	57(533)	79(426)	10(400)	12(500)
S5	Control	26	31	9	12	3	3
	PGPR	60(130)	72(132)	35(288)	46(283)	5(66)	5(66)
	AMF	53(103)	70(125)	40(344)	55(358)	5(66)	6(100)
	PGPF	54(107)	71(129)	41(355)	57(375)	6(100)	7(133)
	PGPR+AMF	69(165)	93(200)	32(255)	50(316)	5(66)	5(66)
	PGPF+AMF	69(165)	73(135)	34(277)	53(341)	7(133)	6(100)
	PGPR+PGPF	61(134)	92(196)	36(300)	59(391)	9(200)	8(166)
	PGPR+PGPF+AMF	64(146)	93(200)	53(488)	73(508)	11(266)	13(333)
S6	Control	10	14	10	16	1	1
	PGPR	41(316)	48(242)	40(300)	55(243)	2(100)	4(300)
	AMF	23(130)	55(292)	19(90)	58(262)	1(0)	4(300)
	PGPF	24(140)	56(300)	22(120)	59(268)	3(200)	3(200)
	PGPR+AMF	33(230)	71(407)	21(110)	52(225)	2(100)	4(300)
	PGPF+AMF	34(240)	60(328)	22(120)	54(237)	3(200)	5(400)
	PGPR+PGPF	43(330)	85(507)	23(130)	61(281)	5(400)	7(600)
	PGPR+PGPF+AMF	45(350)	96(585)	44(340)	65(306)	7(400)	9(800)
S7	Control	30	30	17	18	2	2
	PGPR	45(50)	48(60)	22(29)	31(72)	2(0)	3(50)
	AMF	34(13)	40(33)	22(29)	40(122)	2(0)	4(100)
	PGPF	33(10)	41(36)	23(35)	43(138)	4(100)	5(150)
	PGPR+AMF	44(46)	82(173)	48(182)	55(205)	4(100)	8(300)
	PGPF+AMF	45(50)	49(63)	48(182)	56(211)	6(200)	9(350)
	PGPR+PGPF	46(53)	81(170)	49(188)	59(227)	9(350)	10(400)
	PGPR+PGPF+AMF	48(60)	84(180)	52(205)	64(255)	11(450)	11(450)
S8	Control	10	25	5	17	1	1
	PGPR	59(490)	63(152)	38(660)	45(164)	5(400)	5(400)
	AMF	21(110)	61(144)	12(140)	50(194)	3(200)	5(400)
	PGPF	22(120)	62(148)	13(160)	53(211)	4(300)	6(500)
	PGPR+AMF	23(130)	68(172)	15(200)	53(211)	2(100)	3(200)
	PGPF+AMF	24(140)	64(156)	16(220)	54(217)	4(200)	4(300)
	PGPR+PGPF	60(500)	66(164)	42(740)	58(241)	5(400)	7(600)
	PGPR+PGPF+AMF	62(520)	67(168)	45(800)	65(282)	8(700)	9(800)
CD (P≤ 0.05)	**Treatment** **Varieties**	**6.798** **6.798**	**8.173** **8.173**	**7.394** **7.394**	**7.279** **7.279**	**1.152** **1.152**	**1.250** **1.250**

( ) Parenthesis denotes percentage increase in relation to control

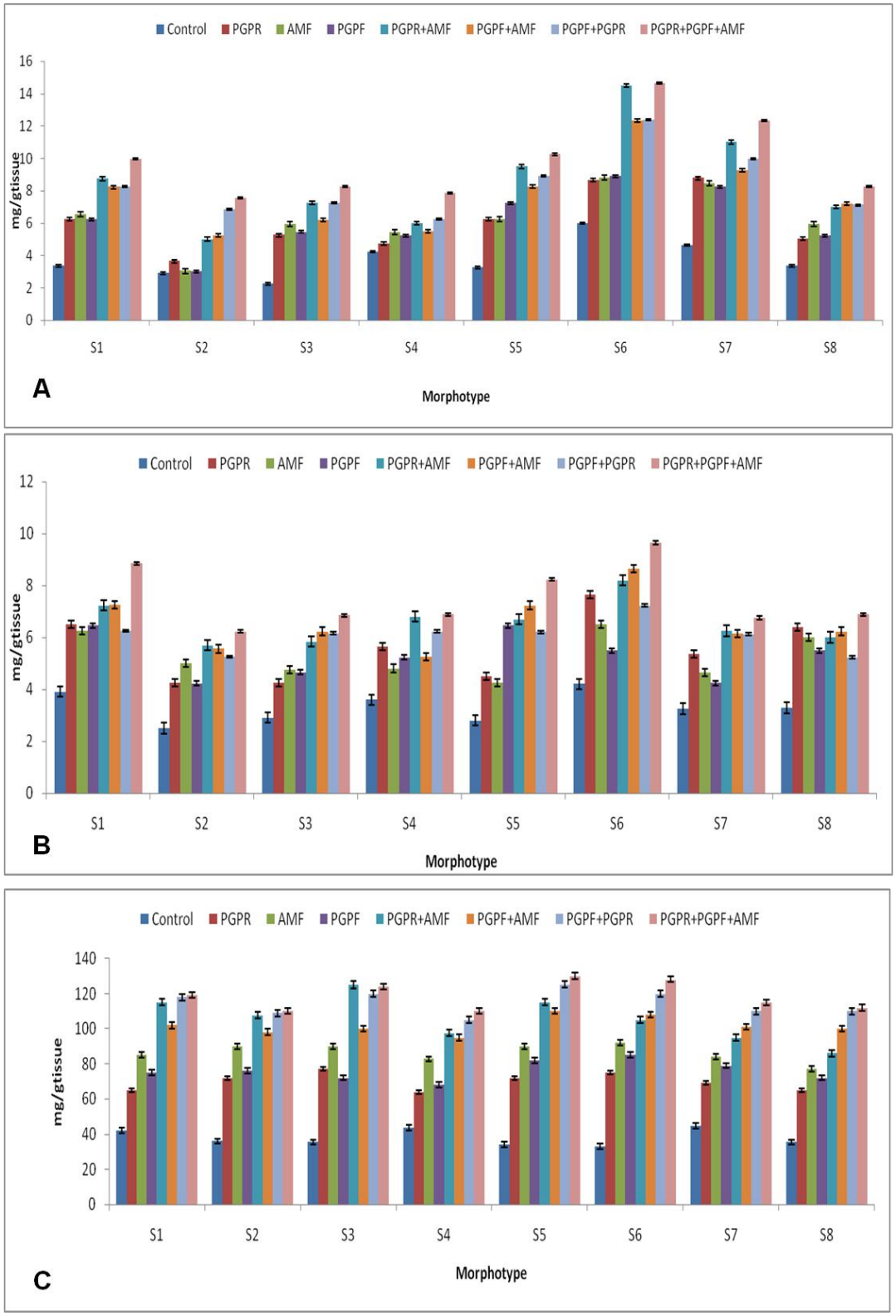

Figure 51: Analysis of Total Phenol (A), Orthophenol (B) and Total Soluble protein (C) content in som plants after treatment under nursery conditions

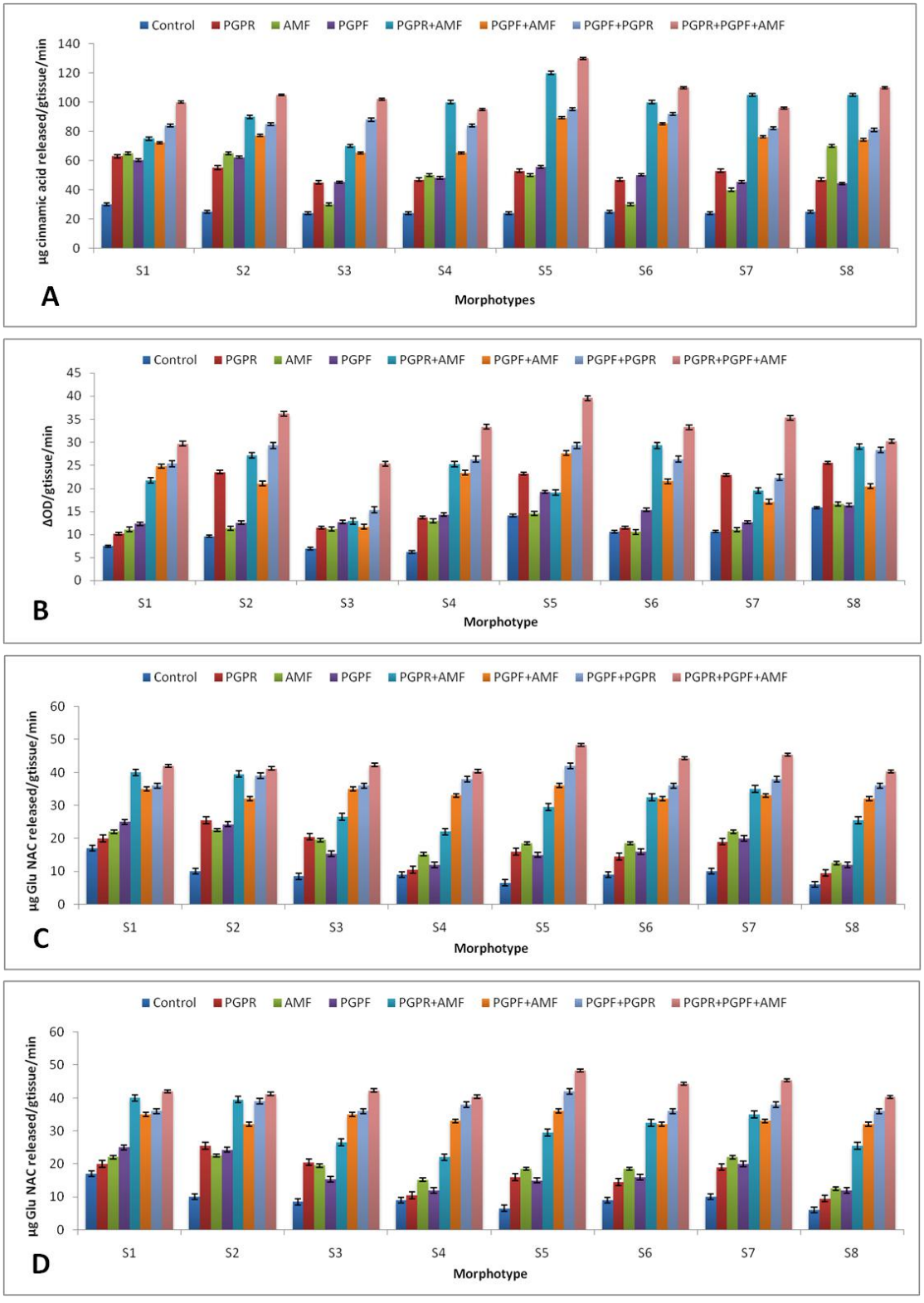

Figure 52: Activities of defense enzymes, PAL (A), Peroxidase (B), Chitinase (C) and β-1,3Glucanase (D) in control and treated som plants under nursery conditions.

4.15.1.1 Disease suppression

The spore suspension of *Colletotrichum gloeosporioides* isolate was sprayed on the leaves of eight different morphotypes of som plants following their treatment with PGPR, PGPF and AMF. The disease establishment was noted after 21 days of treatment. The result as presented in figure 53 showed that the percentage of disease index varied among the different morphotypes. However the percentage was much less in the plants treated with bioinoculants than the untreated control plants. The result as shown in figure also revealed that even though disease incidence was reduced in singly treated plants, but maximum suppression of the disease was due to mixed inoculation of all the three different bioinoculants.

4.15.1.2 Changes in defense enzymes

The changes in levels of four different defense enzymes viz. Peroxidase, Phenylalanine ammonia lyase, Chitinase and β-1,3 Glucanase was analysed after 48 hrs of artificial inoculation of *C. gloeosporioides* spore suspension. The following result as shown in figure 54 revealed that levels of defense enzymes were increased to a larger extent in bioinoculant treated plants of all morphotypes in comparison with their untreated control sets. This collaborates with the fact the disease incidence was suppressed in treated plants where the defense enzymes were increased.

4.15.2 Effect of PGPR, AMF and Vermicompost against *Pestalotiopsisdisseminata*

The disease management approach in case of grey blight of som was done using Vermicompost, PGPR (*Bacillus altitudinus*) and AMF (*Glomus constrictum*). These bioinoculants were used singly as well as in combination to treat two different morphotypes of Som (S5 and S6) that are highly susceptible to this disease. After treatment with bioinoculants, treated as well as untreated control plants were inoculated with spore suspension of the pathogen and percent disease incidence (PDI) was recorded after 7,14,21 and 28 days of inoculation. Activity of defense enzymes were analysed after 48h of pathogen spray.

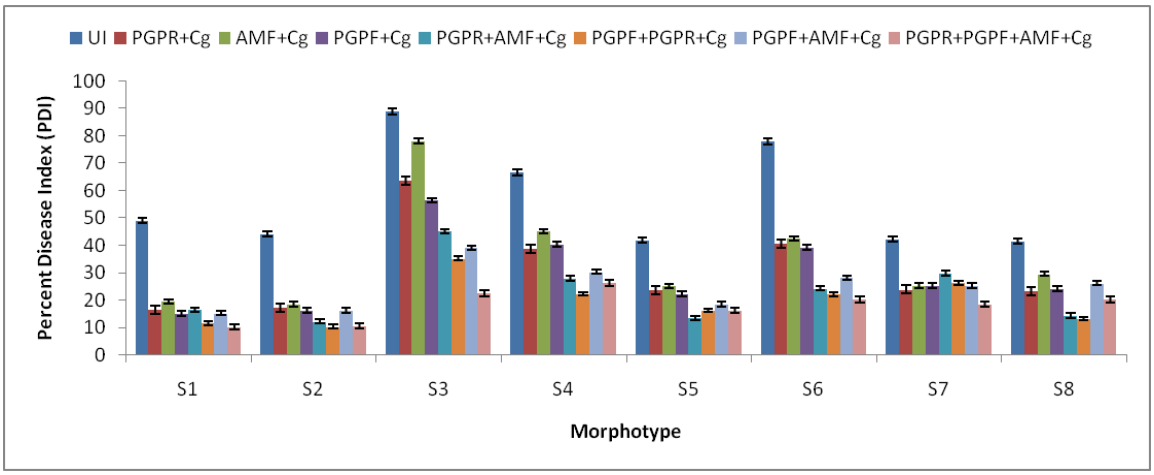

Figure 53: Percent disease index in som plants after treatment with bioinoculants and inoculated with pathogen [UI=Untreated Inoculated, Cg = *C. gloeosporioides*]

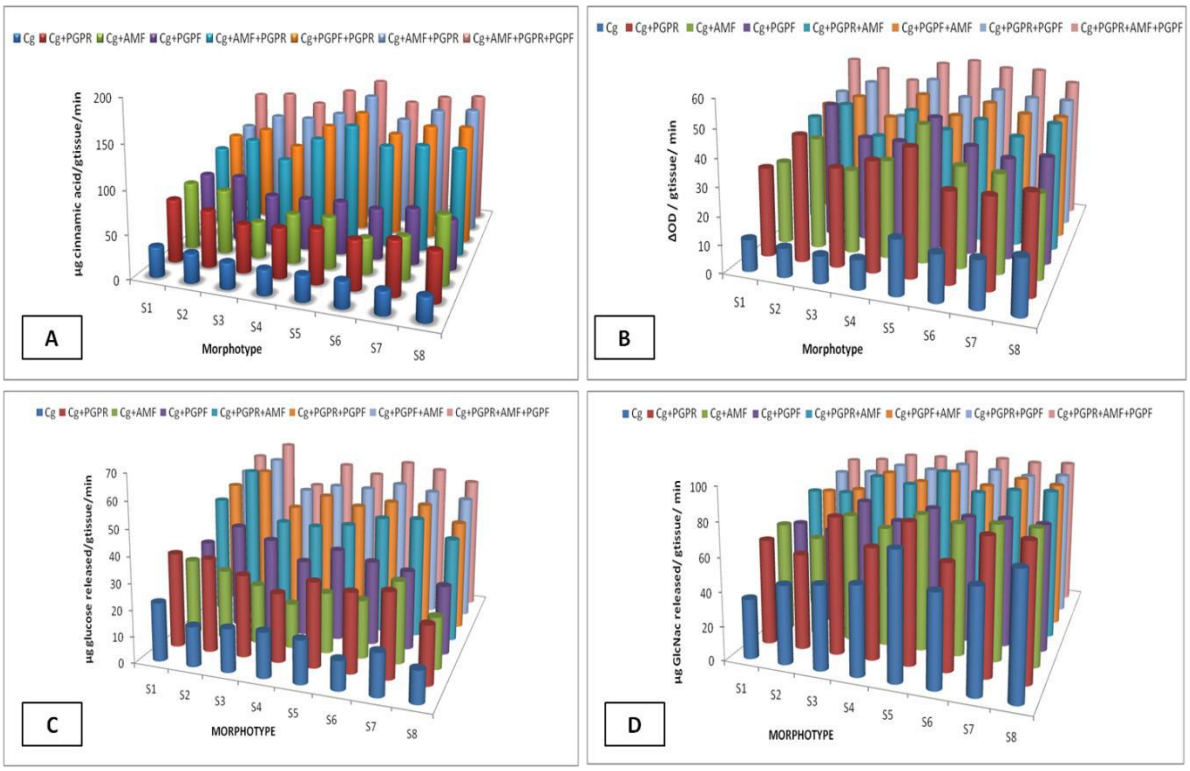

Figure 54: Activity of defense enzymes in bioinoculants treated som plants following artificial inoculation with pathogen (A – Peroxidase, B – PAL, C- Chitinase , D – β-1,3 Glucanase, Cg – Treated with *C. gloeosporioides*)

4.15.2.1 Disease suppression

Upon pathogen spray, the percent disease index was recorded after 7, 14, 21 and 28 days. It was observed that disease incidence was much less in treated inoculated plants in comparison to untreated inoculated (UI) plants for all the intervals (Figure 55). Among the various treatments a consistent decrease in disease incidence was observed in plants treated withVermi+PGPR followed by Vermi+PGPR+AMF and then by PGPR treatment when compared to the untreated inoculated plants. It was seen that disease progression in treated plants were very slow when compared with untreated plants.

4.15.2.2 Defense enzymes

The changes in levels of four different defense enzymes viz. Peroxidase, Phenylalanine ammonia lyase, Chitinase and β-1,3 Glucanase was analysed after 48 hrs of artificial inoculation of *P. disseminata* spore suspension. The following result as shown in Table 38 revealed that levels of defense enzymes were increased in bioinoculant treated inoculated plants of all morphotypes in comparison with their untreated inoculated sets. The levels of enzyme increased mainly in Vermi+PGPR treated and Vermi+PGPR+AMF treated plants. This collaborates with the fact the disease incidence was suppressed in these treatment plants where the defense enzymes were increased.

Table 38: Activity of defense enzymes in som plants following treatment with bioinoculants and artificial inoculation with *P. disseminate*

Morphotype	Treatments	PAL	POX	CHT	GLU
S5	Untreated Inoculated	20.12±0.12	8.56±0.11	10.25±0.06	9.60±0.11
	Vermicompost	33.45±0.16	16.25±0.16	16.00±0.09	18.00±0.03
	B. altitudinus	29.25±0.15	12.32±0.19	12.30±0.08	20.50±0.06
	G. constrictum	25.32±0.12	10.25±0.15	13.25±0.10	17.00±0.10
	Vermi+*B.altitudinus*	47.25±0.10	18.52±0.14	20.00±0.05	29.00±0.12
	Vermi+*B.altitudinus*+ *G.constrictum*	50.12±0.11	19.45±0.11	25.00±0.06	30.00±0.09
S6	Untreated Inoculated	14.65±0.16	9.25±0.16	11.00±0.04	9.00±0.05
	Vermicompost	32.25±0.13	14.32±0.11	20.23±0.06	19.60±0.08
	B. altitudinus	28.52±0.12	12.23±0.15	15.56±0.05	18.50±0.12
	G. constrictum	20.12±0.11	11.50±0.12	16.00±0.09	16.00±0.11
	Vermi+*B.altitudinus*	39.54±0.15	18.25±0.13	26.00±0.06	32.00±0.06
	Vermi+*B.altitudinus*+ *G.constrictum*	40.12±0.15	20.56±0.14	28.00±0.06	36.00±0.05
CD(P=0.05)	Treatments Morphotype	6.566 3.791	2.147 1.239	3.153 1.820	5.438 3.139

± Standard Error, Average of three replicates

4.16 Induction of resistance in field grown som plants against *Colletotrichum gloeosporioides*

4.16.1 Growth promotion

Among the eight morphotypes of som plants, S5 and S6 showed improved growth status in pot experiments, and these were selected for field application. For field inoculation, chopped maize roots colonizedwith dominant spores of *G. constrictum* (AMF) were applied in the rootrhizosphere following transplantation in the field from nursery- grown 7 month old plants. One monthfollowing application of AMF, root colonization status was examined. Then mass multiplied *T. asperellum* made with wheat bran was applied in soil. Two weeks afterapplication of *T. asperellum* (PGPF), further soil application of talc based formulation as wellas foliar spray of *B. altitudinus*(PGPR) was done. When the overall growth of the plants in terms of height, no. of leaves and no. of branches were measured after six months of transplantation, it was observed that application of the bio-inoculants improved the growth of the treated plants in comparison to the control plants (Table 39, Figure 56). Maximum growth enhancement was recorded as 83.4% increase in height, 150% increase in no. of branches and 238.7% increase in no. of leaves in comparison to control.

Table 39: Growth promotion of field grown som plants following application of bioformulation

Growth parameters	S5 Morphotype		S6 Morphotype	
	Control	Treated	Control	Treated
Height (in m)	1.42 ± 0.02	1.79 ± 0.09 (26%)*	1.81±0.02	3.32 ± 0.01 (83.4%)*
No. of branches	15 ± 0.44	27 ± 0.94 (80%)*	20±0.91	50 ± 0.45 (150%)*
No. of leaves	310 ± 0.47	1050 ± 0.97 (238.7%)*	450±0.48	1250 ± 0.94 (177.7%)*

±indicates standard error; *Parenthesis denotes percentage increase in relation to control; Differences between control and treated significant at p=0.01 for all three growth parameters in both morphotypes.

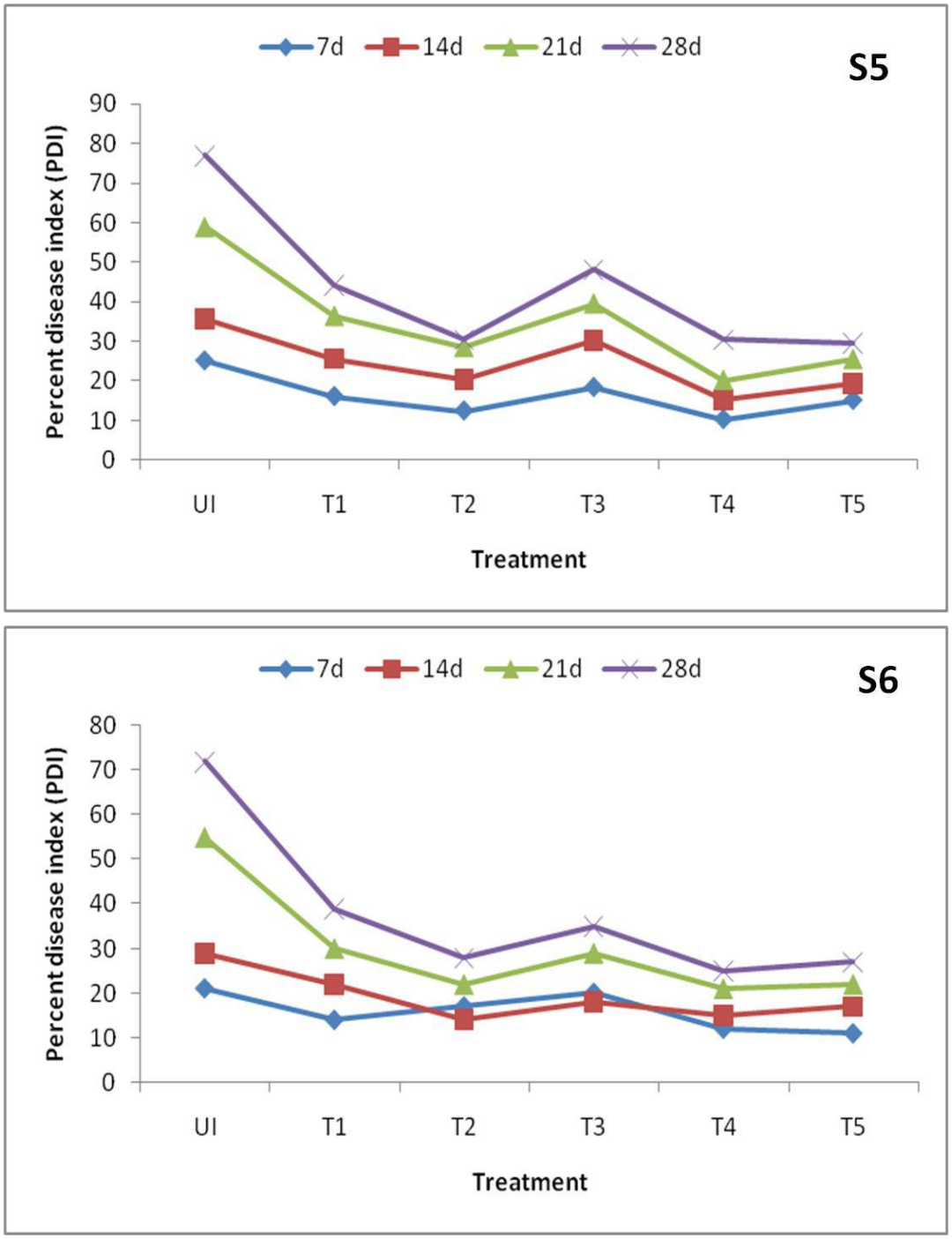

Figure 55: Percent Disease Index in som plants following treatment and artificial inoculation with *P. disseminata*; UI – untreated inoculated, T1 – Vermicompost, T2 – *B. altitudinus*, T3 – *G.constrictum*, T4 – Vermi+*B. altitudinus*, T5 – Vermi+*B. altitudinus*+*G. constrictum*

4.16.2 Biochemical changes

In field condition, phenol contents (total and orthophenols) in both morphotypes increased following the treatments. Estimation of protein and chlorophyll content of the leaves of the control and treated plants revealed that though there was not much difference in chlorophyll content, protein content increased in treated plants in relation to control sets (Table 40). Activities of defence enzymes were also analysed in leaves following treatment. It was observed that treated plants had more activity than the control plants. In case of peroxidase, the activity was higher in treated leaves than the control leaves. However not much changes were observed in chitinase and glucanase activity in control and treated plant.

4.16.3 HPLC analysis of phenolics

The changes in phenolic compounds in the leaves of control and treated S5 and S6 morphotype were measured using HPLC analysis. Analysis of the samples revealed the presence of different peaks showing a variety of phenolic acids present in treated and control som plants of S5 and S6 morphotypes (Figure 57). In case of S5 morphotype, control leaves revealed the presence of six main peaks (viz. 1-6) of which 1 & 2 were not detected in case of treated samples. However, absorbance values of the other4 peaks increased in treated samples. In case of S6 morphotypes, eight different peaks were observed in both control and treated sets, wherein absorbance value of two peaks (4 and 5) increased in treated sets. Comparison with standards revealed two of the phenols to be chlorogenic and ferulic acids in both morphotypes. Increase in absorbance values in treated samples confirm enhancement of phenolics contents following treatment with bioinoculants

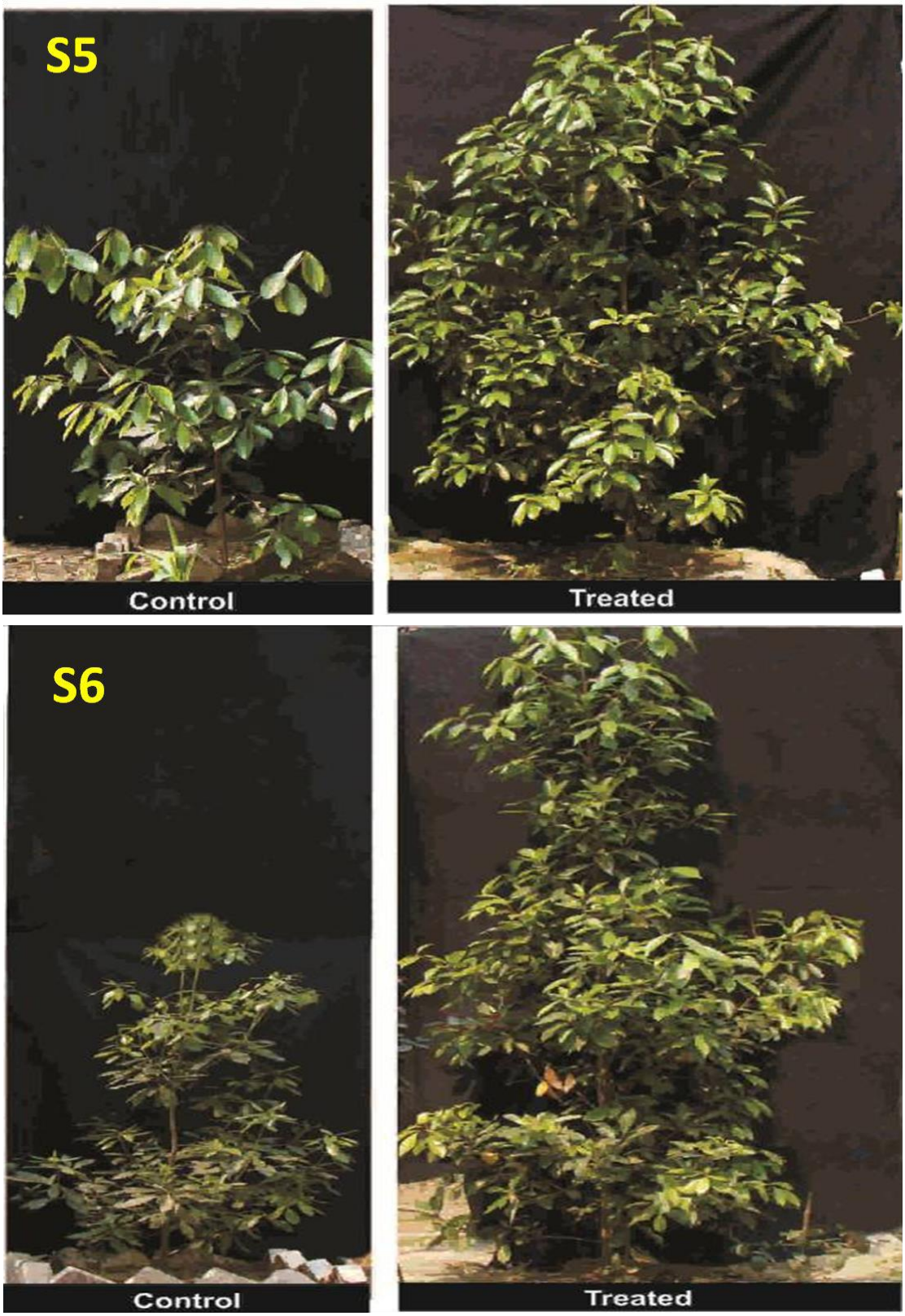

Figure 56: Growth promotion following application of bioinoculants in 18 month-old som plants under field condition. A&B – Morphotype S5; C&D – Morphotype S6; A&C – Untreated Control; B&D – AMF, PGPF and PGPR treated plants

Table 40: Effect of joint application of AMF, PGPF and PGPR on biochemical components and defense enzymes of som plants

Biochemical components	S5 morphotype		S6 morphotype	
	Control	Treated	Control	Treated
Total Chlorophyll (µg/g tissue)	30.2±0.54	30.4±0.52	26.6±0.51	27.5±0.56
Soluble Protein (mg/g tissue)	32.5±0.25	65.4±0.22	29.9±0.32	44.3±0.36
Total Phenol (mg/g tissue)	8.3±0.12	12.7±0.16	8.3±0.11	10.2±0.15
Ortho Phenol (mg/g tissue)	4.3±0.02	6.8±0.05	4.2±0.03	6.5±0.07
Phenylalanine ammonia lyase (µg cinnamic acid/gtissue/min)	60.0±1.23	66.3±1.35	54.6±1.02	76.0±1.23
Peroxidase (ΔOD/g tissue/min)	1.8±0.12	4.9±0.65	2.8±0.12	5.7±0.32
Chitinase (µg Glu NAC/g tissue/min)	60.2±1.59	66.9±1.22	84.0±1.05	91.2±1.19
β-1,3 Glucanase (µg glucose/g tissue/min)	54.9±1.16	68.6±1.19	54.2±0.45	61.5±0.23

±indicates standard error; Differences between control and treated significant at p=0.01 for all biochemical components in both morphotypes

.4.16.4 Changes in defense enzyme after artificial inoculation

Leaves from treated and untreated plants of S5 and S6 morphotypes were artificially challenge inoculated following detached leaf inoculation with spore suspension of *C. gloeosporioides* and percent lesion production was assessed 48, 72 and 96h of inoculation. Results revealed that approximately 44.73% and 39.02% reduction in disease development occurred in S5 and S6 leaves of bioinoculants treated plants in relation to untreated control respectively (Table 41). Assay of defence enzymes -PAL, POX, CHT and GLU as well as total phenol content in the leaves of the inoculated plants (S5 and S6) was carried out after every 24 hr intervals up to 72 hrs. Enzyme activities increased following treatments and enhanced markedly after challenge inoculation with *C. gloeosporioides* (Figure 58).

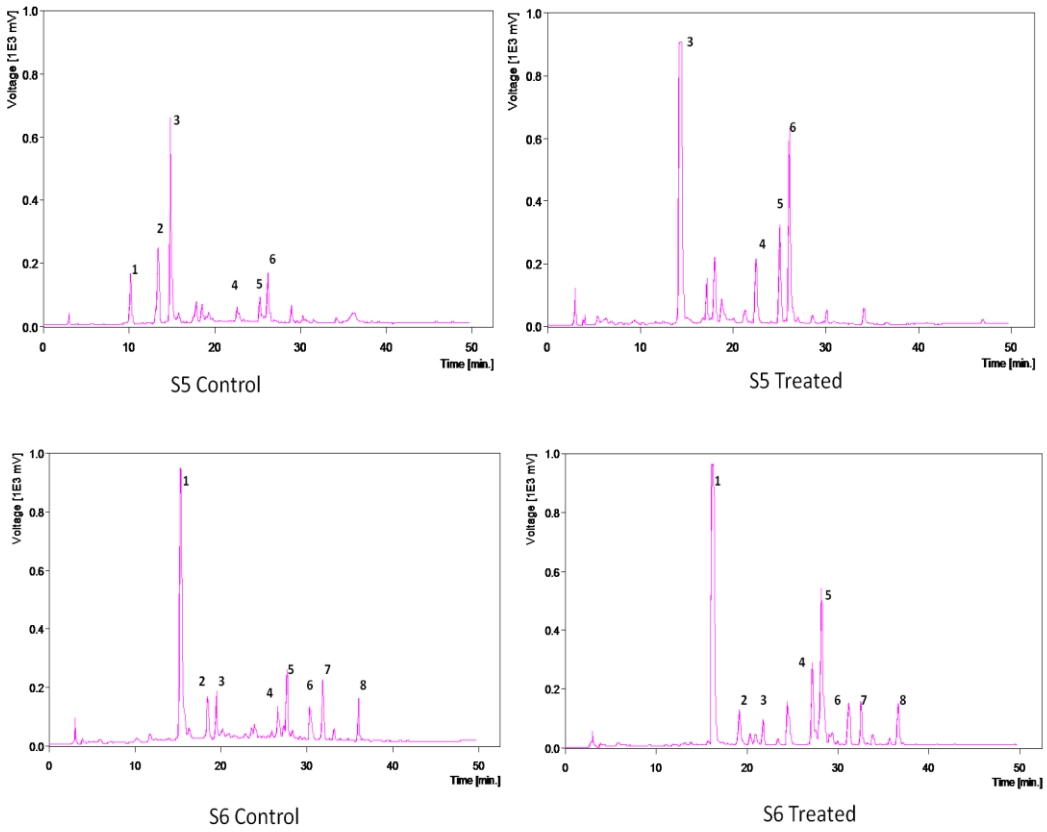

Figure 57: HPLC analysis of the phenolic acid content in control and treated som plants under field condition

Table 41: Percent lesion production in som plants after joint application with AMF, PGPF and PGPR following detached leaf inoculation with *C. gloeosporioides*

Hours after inoculation	Percent (%) lesion production			
	S5 morphotype		S6 morphotype	
	Untreated inoculated	Treated inoculated	Untreated inoculated	Treated inoculated
48	53±0.48	28±0.95 (47.16%)*	58±0.92	38±0.95 (34.48%)*
72	69±0.23	37±0.47 (46.37%)*	75±1.43	47 ±0.49(37.33%)*
96	76±0.94	42±0.48 (44.73%)*	82±0.44	50±0.95 (39.02%)*

±indicates standard error; Average of three separate trials, 50 leaves inoculated for each treatment,

*Parenthesis denotes percentage reduction in disease development in leaves of bioinoculants treated plants in relation to untreated control

Differences between untreated inoculated and treated inoculated significant at p=0.01 for all hours after inoculation in both morphotypes.

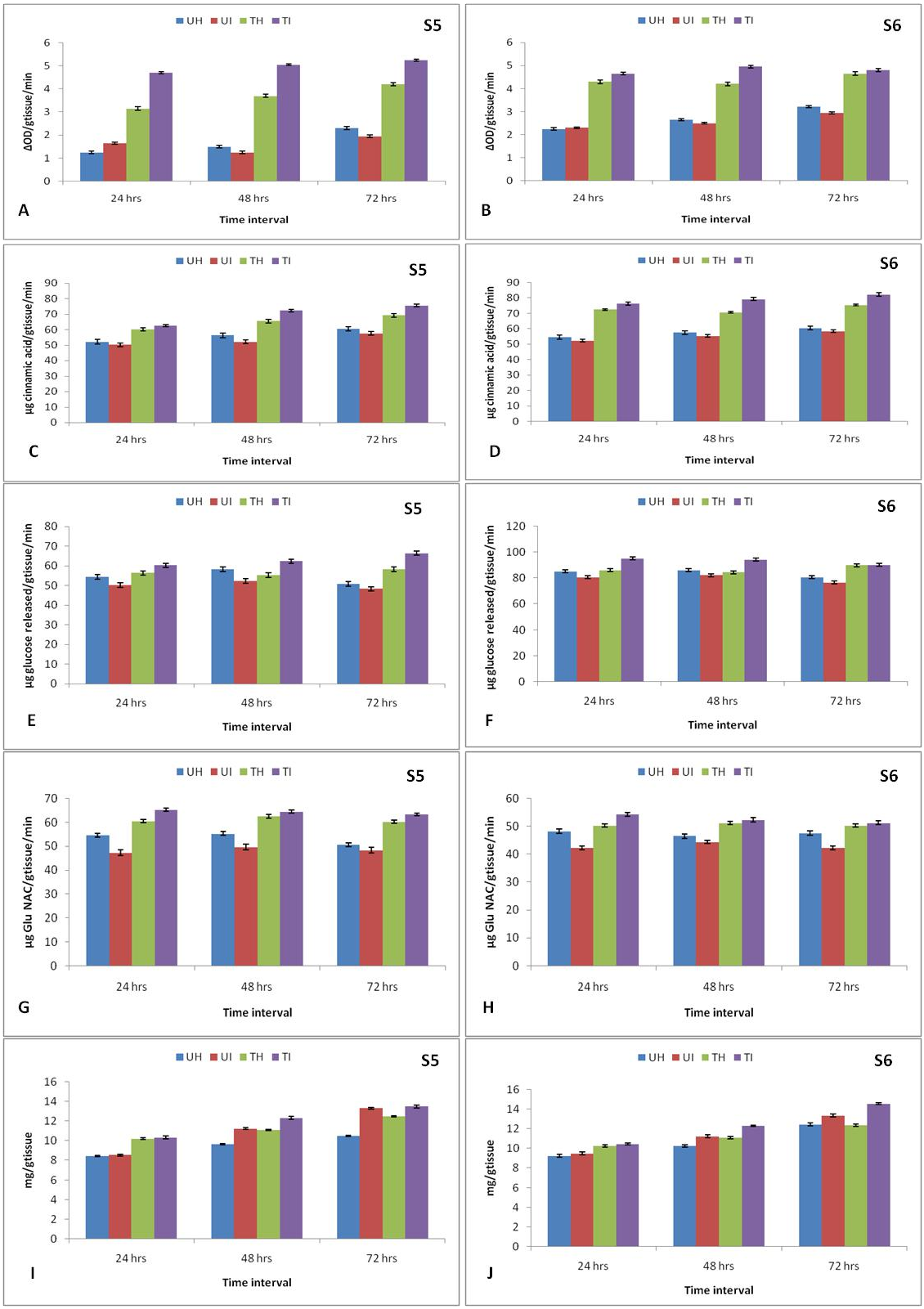

Figure 58: Changes in levels of defense enzymes: Peroxidase (A & B), PAL (C & D), Chitinase (E & F) and Glucanase (G & H) as well as changes in total phenol content (I & J) in S5 and S6 plants following artificial inoculation with *C.gloeosporioides*

4.17 Cellular localization of glucanase and chitinase in leaf and root tissues of som plant following induction of resistance using bioinoculants

Induction of defense enzymes in leaves of som plants following treatment of bioinoculants has already been established in earlier sections. Enhanced activities of defense enzymes such as peroxidase, phenylalanine ammonia lyase, chitinase and glucanase were reported following application of PGPR as well as AMF and PGPF in S6 morphotype. Glucanase and chitinase being an important PR protein related to induction of resistance in plants, cellular localization of these enzymes in root and leaf tissue was studied in this section.

4.17.1 Cellular localization of glucanase

4.17.1.1 Indirect Immunofluorescence

Cellular localization of glucanase enzyme in leaves and roots of som plants was determined following indirect immunofluorescence test using FITC binding and treatment with PAb raised against glucanase. Leaf sections from untreated control plants and *B. pumilus* treated plants were taken and root sections from AMF and *T. harzianum* inoculated plants were taken. Immunolocalization of glucanase in treated leaves and root sections of som plants were observed using FITC after treatment with PAb raised against glucanase. Positive reaction withFITC was observed in cellular localization which gave indication of the induction of glucanase in som leaf and root tissues (Figure 59 and 60). Bright apple green fluorescence was observed intreated leaves and

4.17.1.2 Immunogold localization

The accumulation of PR protein β-1,3 Glucanase was investigated in LR-embedded cross sections of leaf tissues of som plant following their treatment with *B.pumilus* using PAb of glucanase andlabeled with antirabbit-IgG (whole molecule) gold conjugate (10nm). Microscopic observations revealed intense labelling corresponding to glucanase deposition in leaf tissues of PGPR treated plants. Gold labelling in the sections showed a high amount of labelling in chloroplast and host cytoplasm (Figure61 and 62). This indicates the induction of defense enzyme glucanase following their treatment with PGPR.

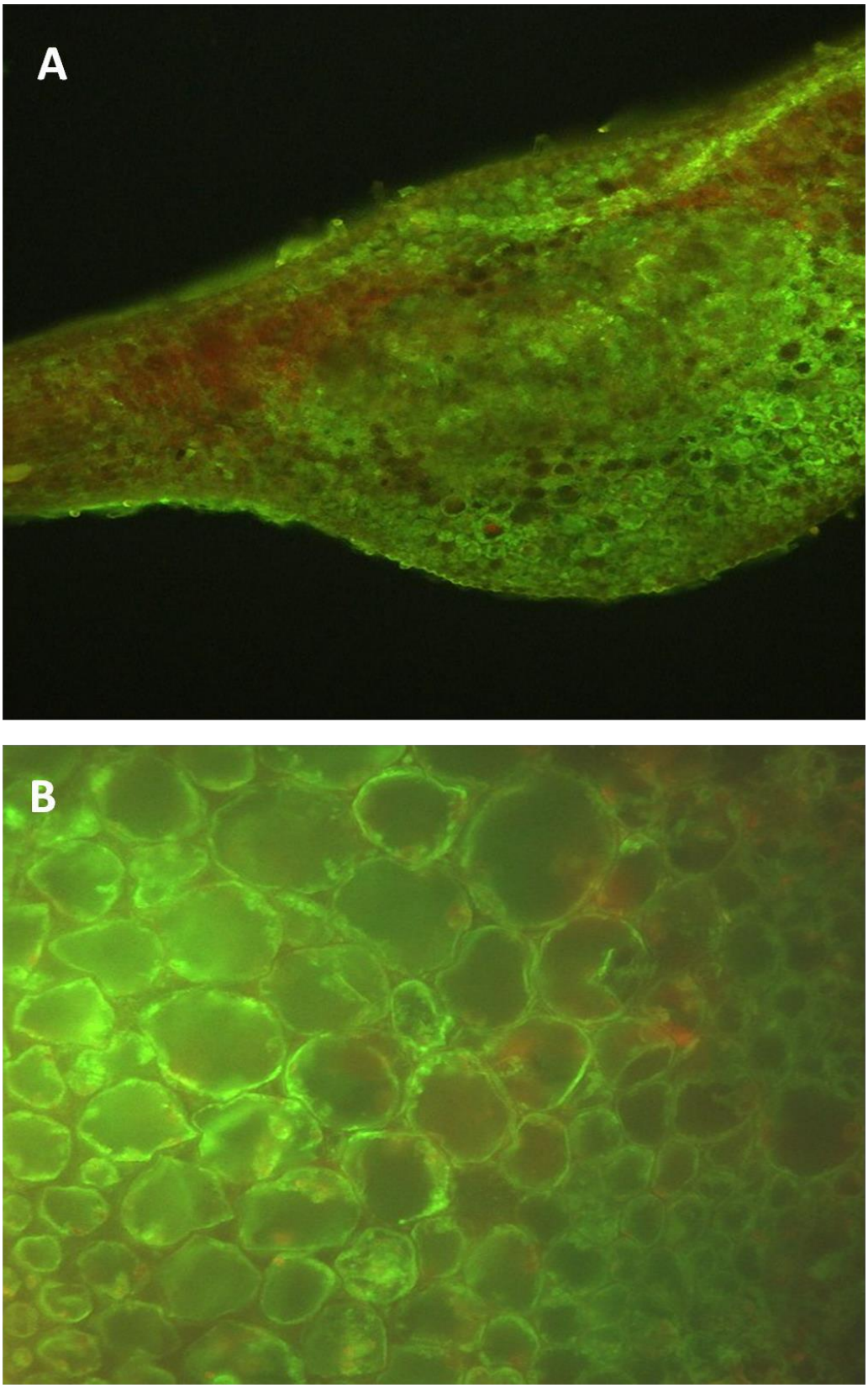

Figure 59: Cellular localization of glucanase in som leaf tissues following treatment with bioinoculants, probed with PAb of glucanase and labelled with FITC (A&B)

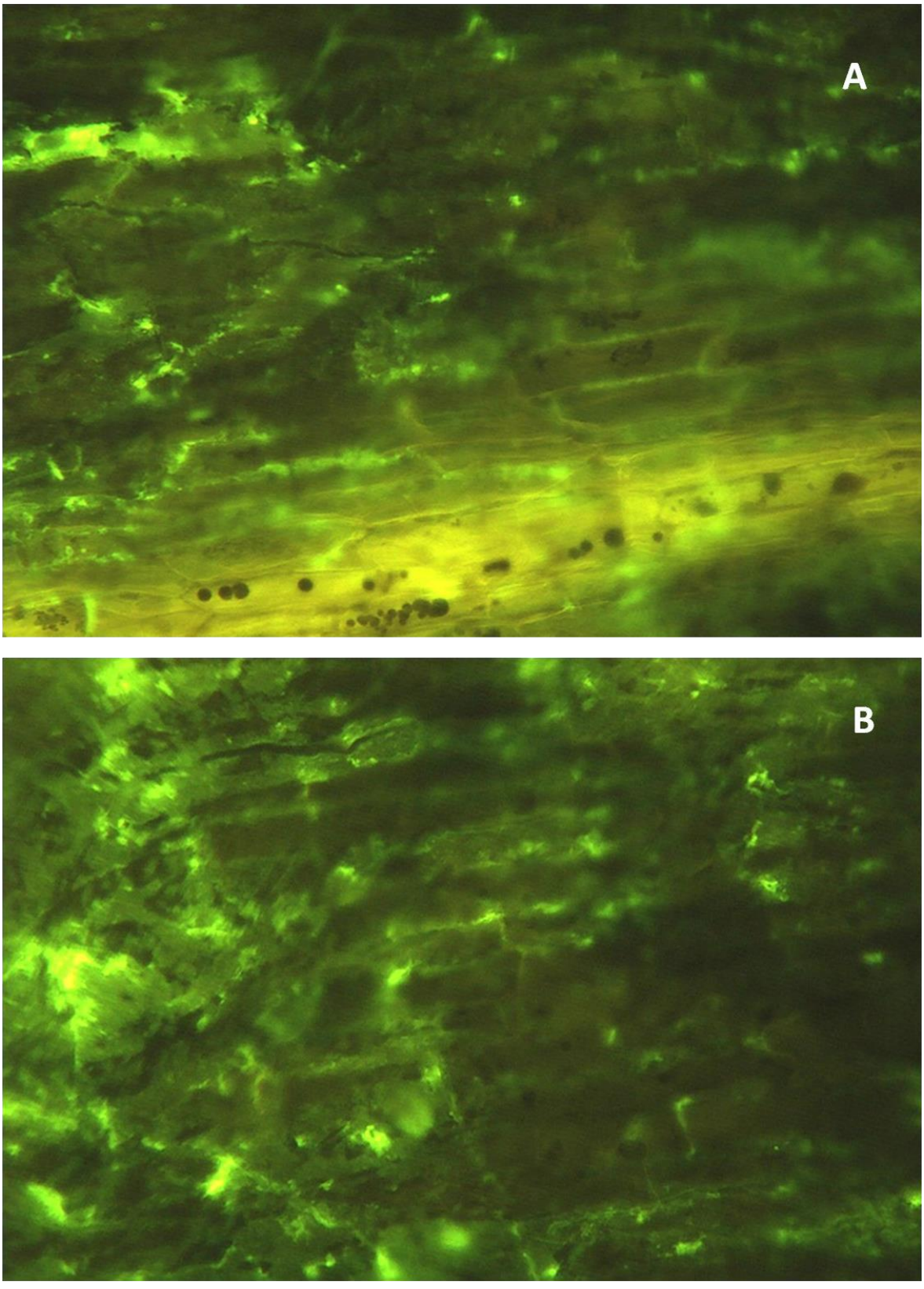

Figure 60:Cellular localization of glucanse in roots following treatment with bioinoculants, probed with PAb of glucanase and labelled with FITC (A&B).

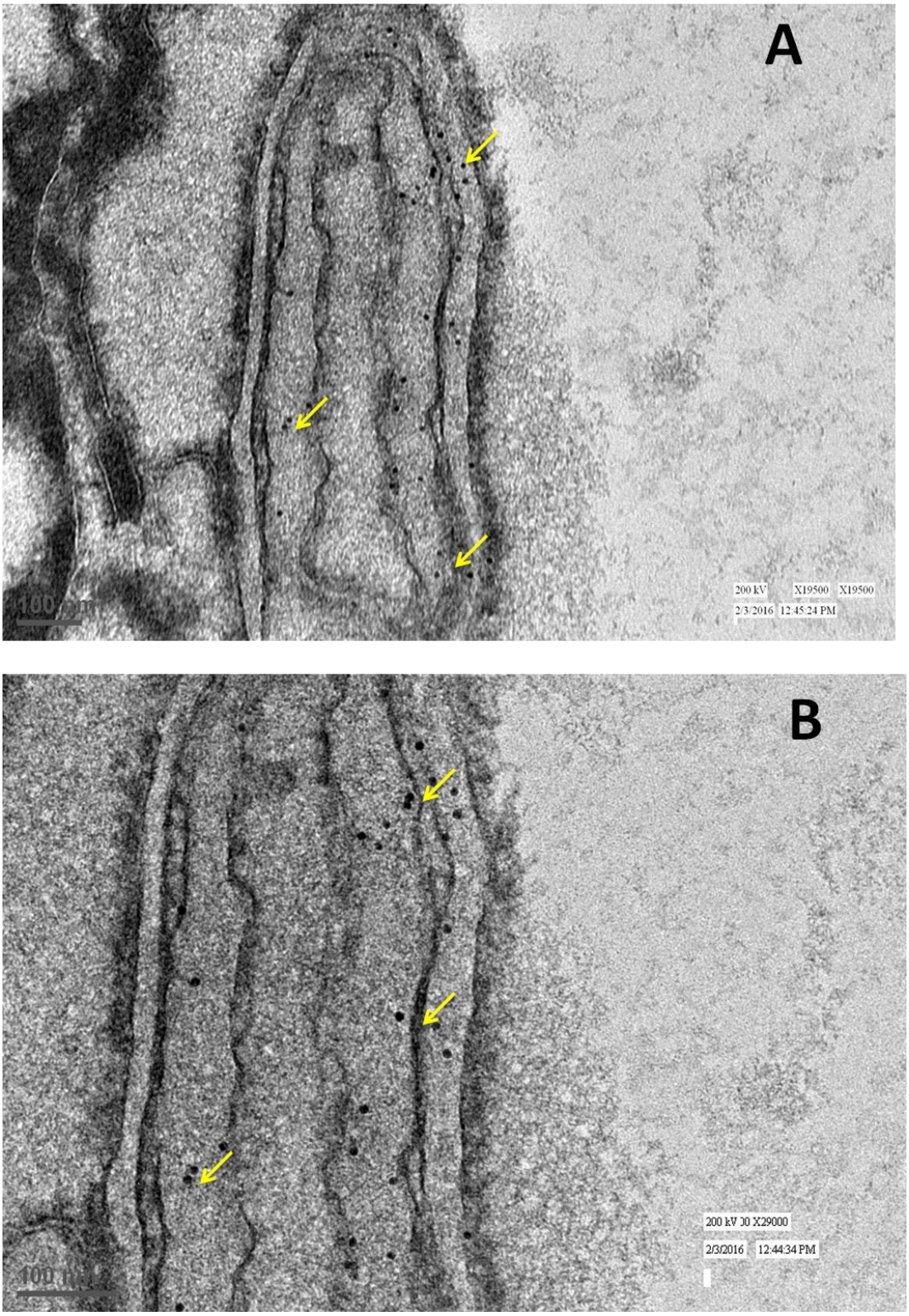

Figure 61: Transmission electron micrographs of bioinoculant treated leaf tissues of som plant (S6) reacted with PAb of glucanase and labelled with antirabbit goat IgG (whole molecule) gold conjugate

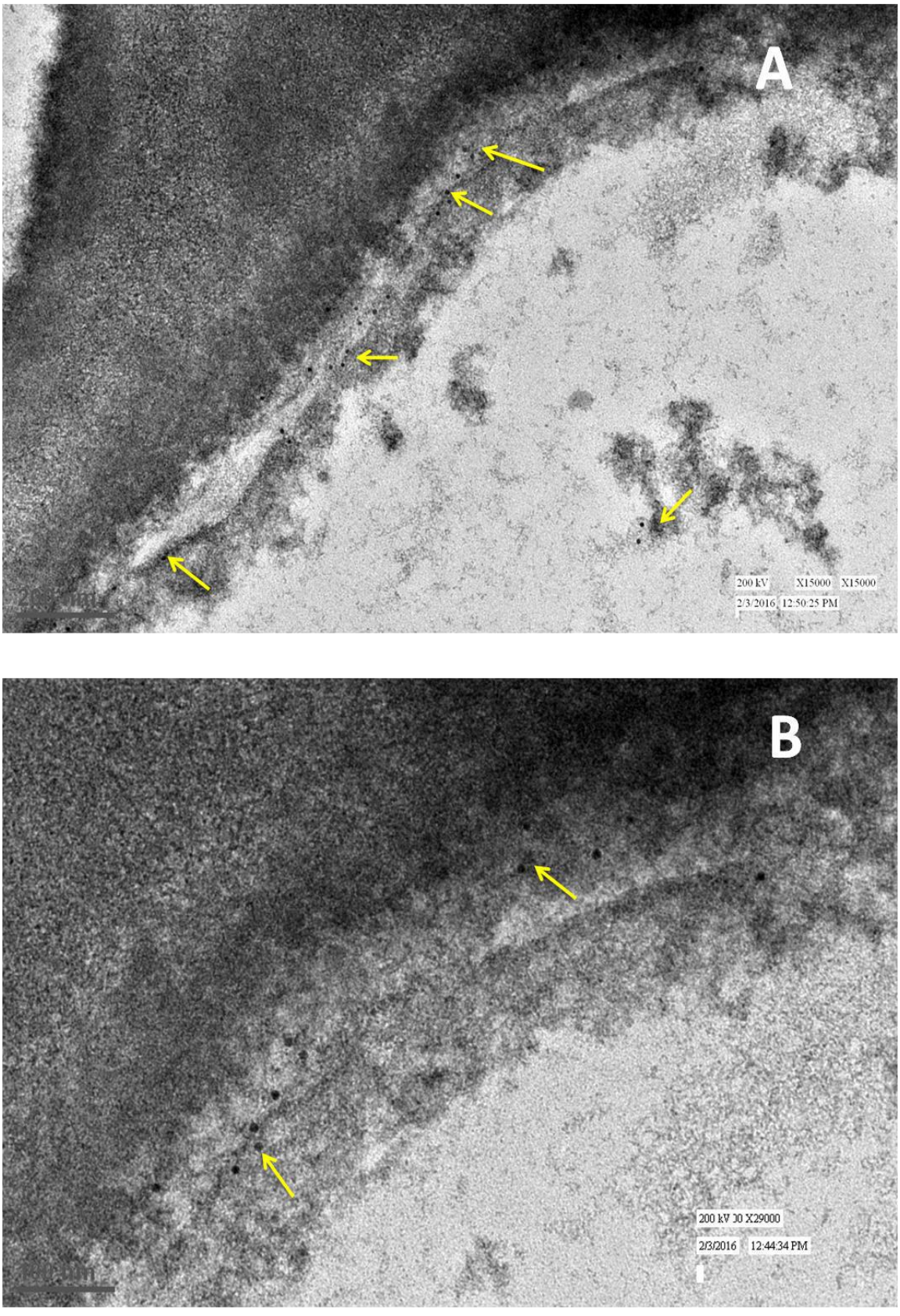

Figure 62: Transmission electron micrographs of bioinoculant treated root tissues of som plant (S6) reacted with PAb of glucanase and labelled with antirabbit goat IgG (whole molecule) gold conjugate

4.17.2 Cellular localization of chitinase

4.17.2.1 Indirect immunofluorescence

Cellular localization of chitinase enzyme in leaves and roots of som plants was determined following indirect immunofluorescence test using FITC binding and treatment with PAb raised against chitinase. Leaf sections from untreated control plants and *B. pumilus*t reated plants were taken and root sections from AMF and *T. harzianum* inoculated plants were taken. Immunolocalization of chitinase in treated leaves and root sectionsof som plants were observed using FITC after treatment with PAb raised against chitinase. Positive reaction withFITC was observed in cellular localization which gave indication of the induction of chitinase in som leaf and root tissues (Figure 63 and 64). Bright apple green fluorescence was observed intreated leaves and roots which testified the increased accumulation of chitinase enzyme in treated leaves and root sections.

4.17.2.2Immunogold localization

The accumulation of chitinase was investigated on cross sections of LR-white embedded leaf tissues of som plants treated with *B.pumilus* and root tissues of som plants treated with AMF and *T. harzianum*, previously fixed with 0.1 Msodium phosphate buffered-glutaraldehyde (2.5%) and using PAb of chitinase andlabeled with antirabbit-IgG (whole molecule) gold conjugate (10nm).Microscopic observations revealed intense labelling corresponding to chitinase deposition in leaf as well as root tissues of PGPR and AMF treated plants. Gold labelling in the sections showed a high amount of labelling in chloroplast and host cytoplasm and less in vacuoles, mitochondria and cell wall (Figure 65 and 66). This indicates the induction of defense enzyme Chitinase following their treatment with PGPR, AMF and PGPF.

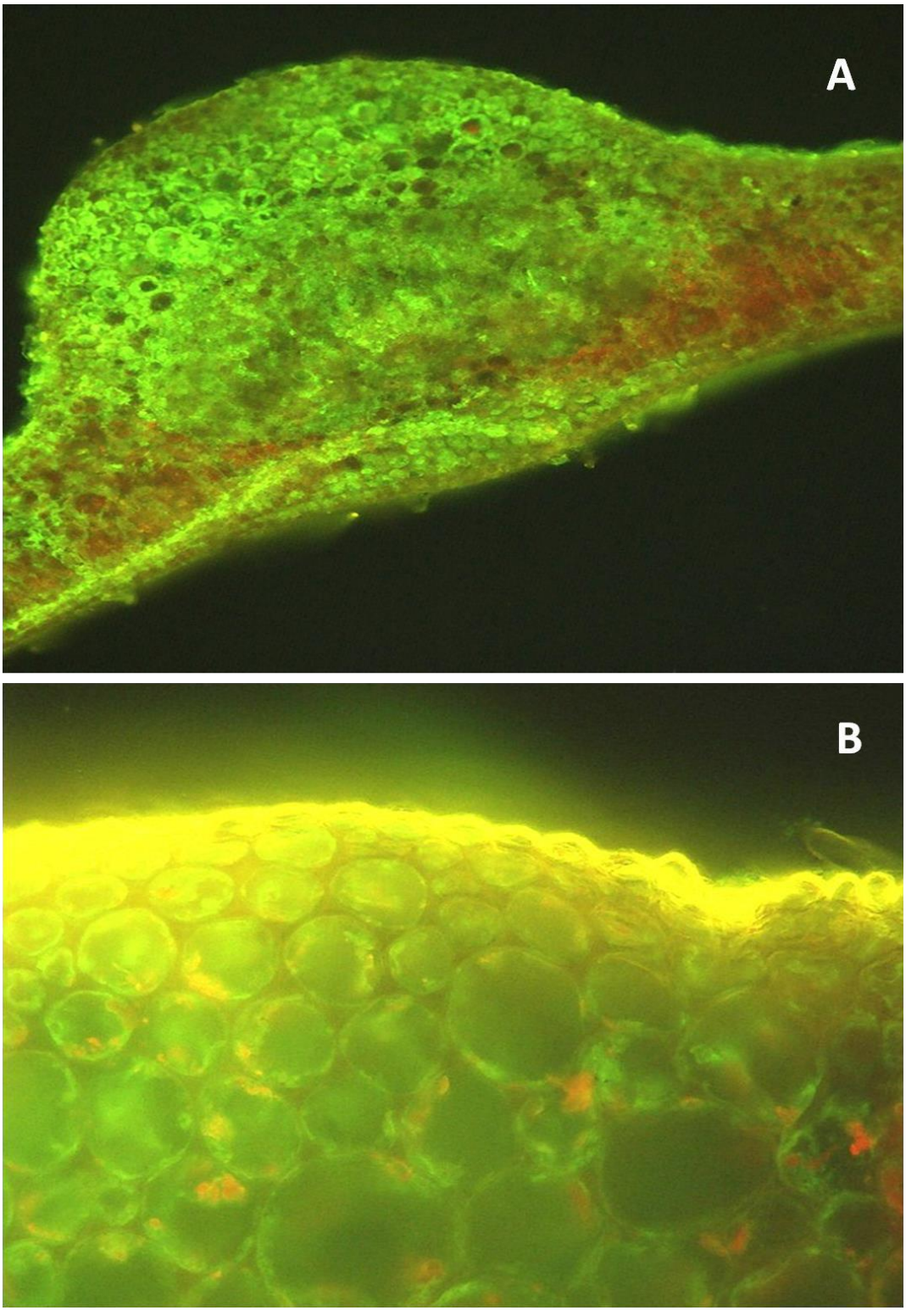

Figure 63: Cellular localization of chitinase in som leaf tissues following treatment with bioinoculants, probed with PAb of chitinase and labelled with FITC (A&B)

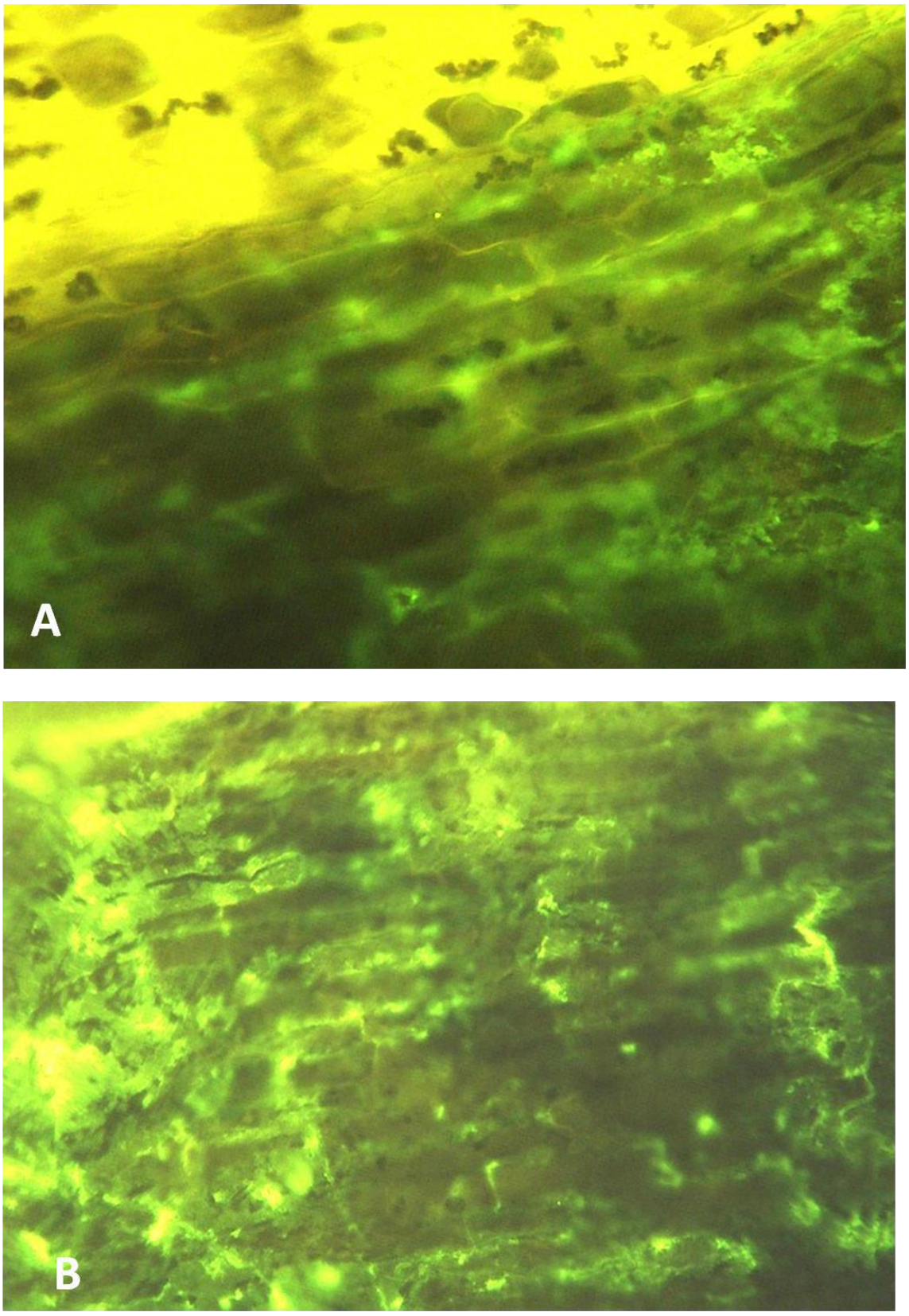

Figure 64: Cellular localization of chitinase in roots following treatment with bioinoculants, probed with PAb of chitinase and labelled with FITC (A&B)

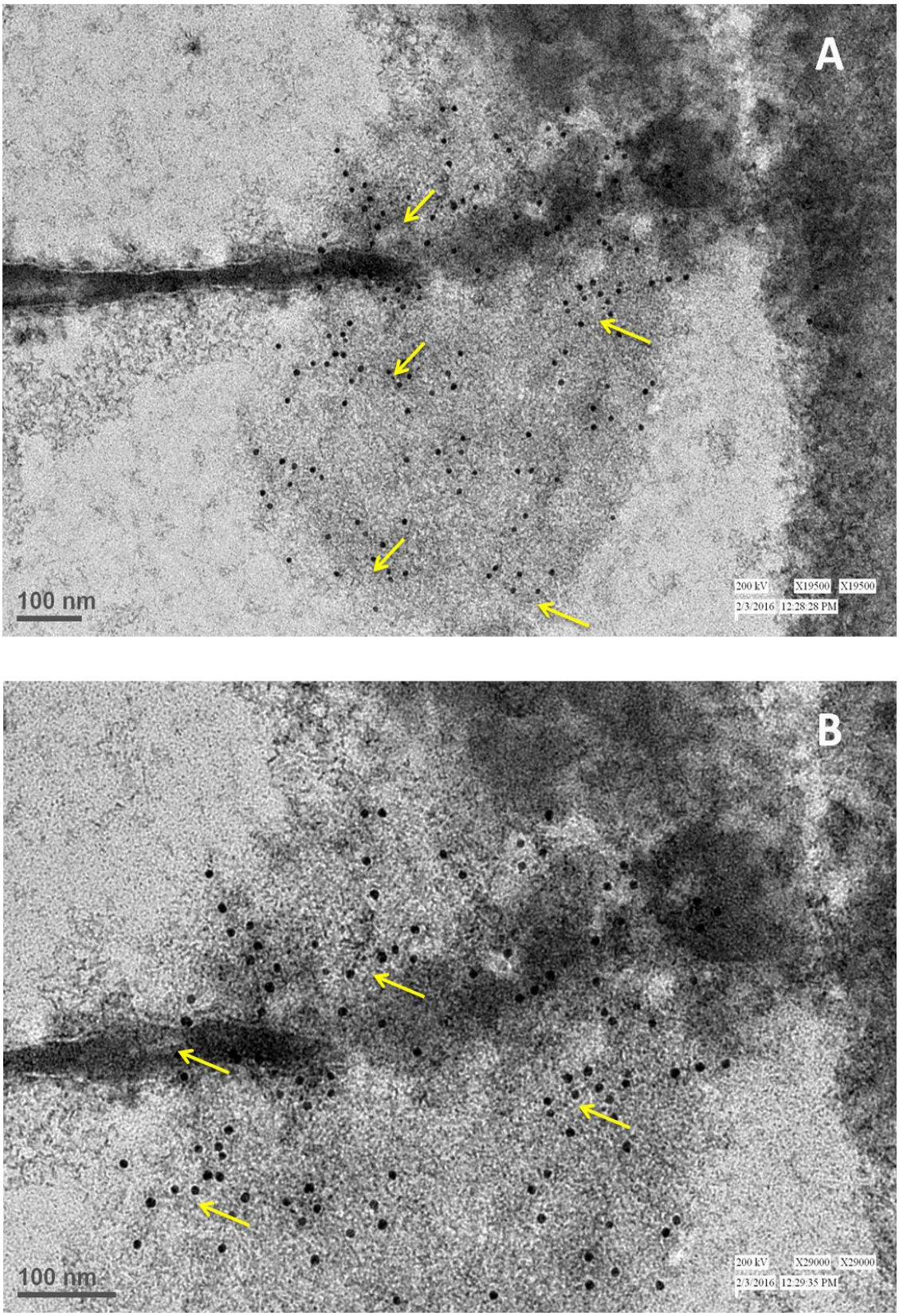

Figure 65: Transmission electron micrographs of bioinoculant treated leaf tissues of som plant (S6) reacted with PAb of chitinase and labelled with antirabbit goat IgG (whole molecule) gold conjugate

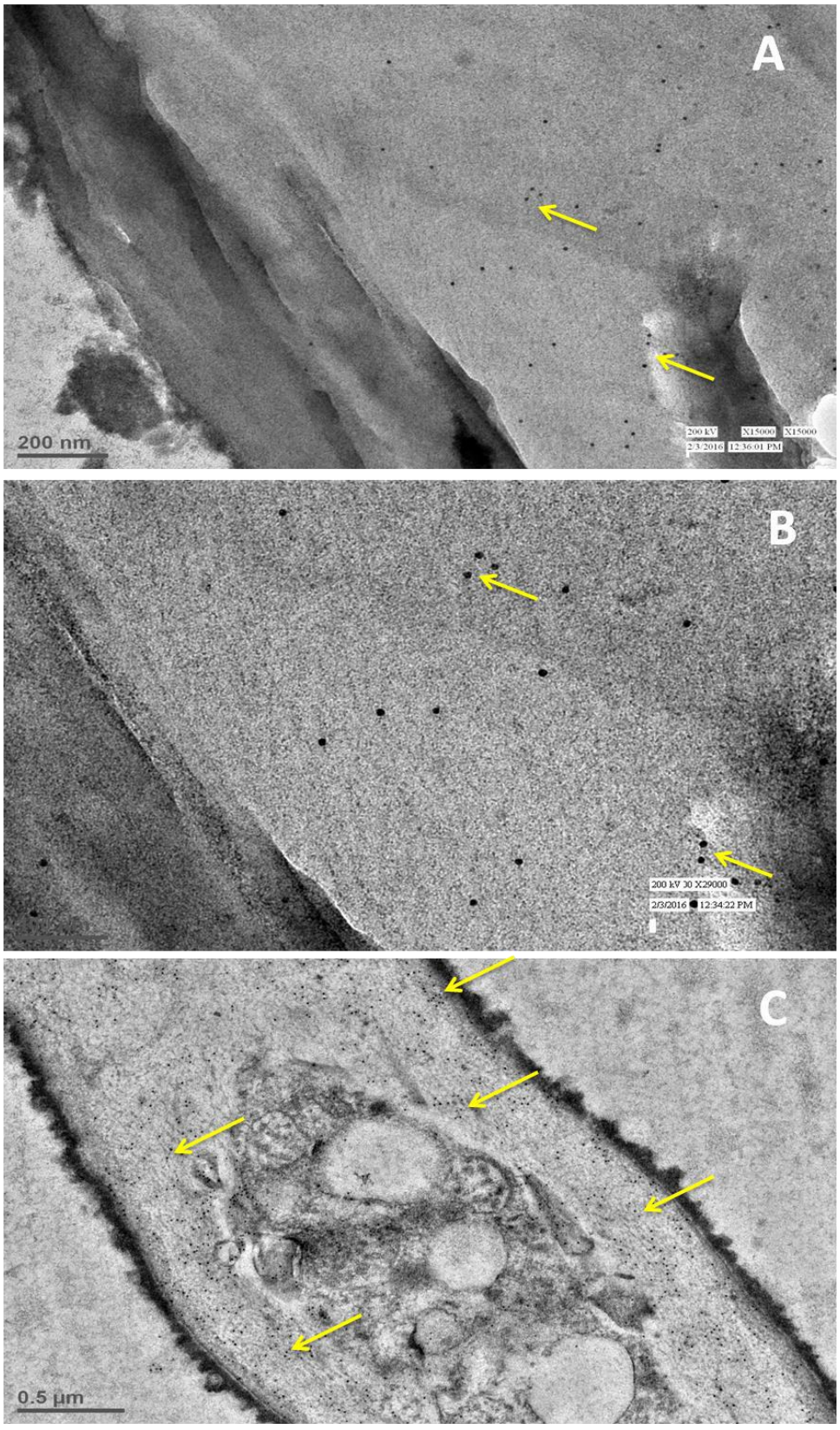

Figure 66: Transmission electron micrographs of bioinoculant treated root tissues of som plant (S6) reacted with PAb of chitinase and labelled with antirabbit goat IgG (whole molecule) gold conjugate

Chapter 5
DISCUSSION

Muga food plants have wide distribution throughout the North-Eastern India and in some parts of Northern India. These plants are widely available in the states of Assam, Meghalaya, Manipur, Mizoram, Nagaland, Tripura, Sikkim, Himachal Pradesh, Uttaranchal, Uttar Pradesh, Gujarat, West Bengal and Pondicherry and sporadically available in Arunachal Pradesh. It was also reported that they are present in Nepal, Myanmar, Malaysia, Indonesia, Bhutan and Srilanka (Tikader and Kamble, 2010). All the muga host plants grow well in wet and warm climatic conditions with high rainfall and soil pH range of 4.0- 6.8. Muga silkworm host plant, som (*Persea bombycina* Kost.) is a heterogeneous wild deciduous tree available abundantly in natural forest of northeast India. It exhibits variability in several desirable and undesirable traits.

Although, collection of muga plant genetic resources was initiated in 1988 by the Regional Muga Research Station (RMRS), Boko, Kamrup, Assam, only fourteen cultivars of som and ten cultivars of soalu could be collected from Assam and Meghalaya. After initial evaluation, 8 som and 10 soalu genotypes were selected for further studies. These genotypes were characterized based on morphology, floral biology, propagation, chemo-assay and bioassay (Hazarika*et al*., 1996; Paliwal andDas, 1989; Raja Ram, 1998; Siddiqui *et al*., 1998, 2000; Singh *et al*., 2000; Thangavelu*et al*., 2005). In another effort, 39 som genotypes were collected by the Central Muga Eri Research and Training Institute, Lahdoigarh, Assam. These genotypes were characterized based on a set of characters viz. Plant height(m), No. of branches, Inter nodal distance (cm), Laminar length (cm), Laminar width (cm), no. of leaves and Leaf yield/plant (kg).In general, som trees have the potential of yielding 22-24 MT /ha/year leaf but presently only 16-18 MT/ha/year is obtaining. So, there is an yield gap of more than 8MT/ha/year. To fill up this gap, agronomic and cultural practices need to be improved.

On the basis of leaf size and shape, stem, inflorescence, colour of the sprouted leaves and size of the plants of som, Raja ram *et. al.* (1993) described eight morphotypes of som and named them as Som cultivars -S-1, S-2, S-3, S-4, S-5, S-6,

S-7 and S-8. Kataky and Hazarika (1996), however, categorized the som plant into three groups viz. most preferred, preferred and least preferred based on feeding performance of *A. assama.* On the basis of bio-chemical analysis, bioassay and post cocoon parameters Siddiqui *et al* (2000) reported that S-6, S-4, S-3 and S-5 were palatable and superior for sustainable yield of muga cocoon, whereas three morpho variants viz. S-l, S-2 and *S*-7 were less preferred by the muga silkworm. Therefore in the present study eight different Som morphotypes viz, S1, S2, S3, S4, S5, S6, S7 and S8 were taken into consideration for different experimental work.

Jolly *et al*., (1976) reported that nutritionally rich food plants are required for healthy growth of silkworm. Production of muga silk depends upon the quality of leaves. The nutritional value of leaf, however, varies greatly owing to a number of factors among which the diseases play an important role (Dandin *et al.,* 2003). Being a perennial plant, som plants are exposed to different environmental vagaries and pathogens prevailing in the region throughout the year. As a result, fungi cause number of diseases (Das *et al.,* 2003). Babulal *et al.,*(2000) reported that a considerable amount of leaf yield is lost regularly due to foliar diseases in som plant. Feeding of such infected leaves yielded poor Effective Rate of Rearing (ERR). An extensive survey of literature revealed that a limited quantum of work has been done on pathological aspects i.e. eco-pathology, epidemiology and management of diseases of som plant *(Persea bombycina).*

The present study deals with two major foliar fungal diseases of som plant, Leaf blight and Grey blight. Das and Benchamin (2000) reported that 13.8% - 33.5% leaf yield loss is caused by leaf blight, leaf spot, red rust and leaf curl diseases of som plant. Das *et al* (2005) first reported the occurrence of leaf blight in som plants caused by *Colletotrichum gloeosporioides* that caused approximately 6.3% of the total yield loss in leaf yield. Bharali (1969) and Das and Bechamin (2000) reported Grey blight caused by *Pestalotiopsis disseminata* as a major epidemic disease of muga host plant, som causing 13.8% - 41.6% leaf yield loss. Similarly in the present investigation it was observed that under nursery condition leaf blight was prominent from month of April to August and was highest in S6 morphotype causing 68.2% PDI. On the other hand establishment of grey blight disease was highest in S5 morphotype causing around 72% of disease incidence.

At the outset of the present study symptamology and etiology of the leaf blight and grey blight disease of som plant was studied. Das *et al* (2010) reported the

symptoms and the epidemiological parameters that causes the grey blight disease of som plant. It was recorded that young leaves (leaf no. 1-4 from the top) are more susceptible to the disease than the older leaves. This is in accordance with the results recorded in the present study where the young leaves were more prone to disease and the disease symptoms clearly indicate grey blight disease. It was also discussed that sporulation of *P. disseminata* spores were optimum at 25±2°C and it started at 8h of incubation and increased until 20h of incubation. Conidial morphology is the most widely used taxonomic character for the genus *Pestalotiopsis*. Colour of the median cells is still a widely used character, and all species separate into three groups based on this- concolorous, versicolorous umber olivaceous and versicolorous fuliginous olivaceous. Molecular evidence indicates that it is more precise to group species according to concolorous and versicolorous rather than the above three groups (Jeewon *et al*., 2003).The length of the apical appendages and the number of the apical appendages are also widely used characters for species identification. Some species can also be identified by the presence of knobbed apical appendages. The apical appendages can arise from the top, middle, bottom or different positions in the apical hyaline cells and such characters are widely used in species identification. Furthermore the apical appendages can be divided into branches; in some species presence or absence of the basal appendages is another character for species diagnosis (Maharachchikumbura *et al*; 2011). Based on these different taxonomic classifications, the conidial morphology of the fungal isolates of grey blight was studied and they could be placed under *P. disseminata* group.

On the other hand the growth and sporulation of *Colletotrichum gloeosporioides* causing leaf blight disease of som plant was studied. Chakraborty *et al* (1995a) studied the factors influencing spore germination, appressoria formation and disease development in *Camellia sinensis* by *Glomerella cingulata*. They reported that spore germination and appressoria formation were optimum at a temperature of 25°C, pH 5.0 and a 24h incubation period. In the present investigation optimum condition necessary for growth and sporulation of *Colletotrichum gloeosporioides* was tested in vitro. Maximum growth occurred in Potato Dextrose Agar medium at an incubation period of 10days. Sporulation behaviour and appressoria formation varied with the different media on which the fungus was grown.

In the present study varietal resistance of eight different morphotypes of som plants, against the fungal pathogens *P.disseminata* and *C. gloeosporioides* was carried

out by detached leaf and whole plant inoculation techniques. Responses exhibited by the morphotypes were essentially the same in both the techniques. Of the 8 morphotypes, S5 and S6 morphotypes were more susceptible to the fungal diseases. Chakraborty *et al* (1995b) tested the pathogenecity of three different isolates of *Pestalotiopsis theae* on 12 tea varieties with detached leaf inoculation technique to reveal the susceptible and resistance variety of tea to grey blight disease. Chakraborty *et al* (1996a) also tested pathogenecity of *Glomerella cingulata* towards tea varieties using both detached leaf and cut shoot method.

Studies have been undertaken for detection of fungal pathogens in host tissues by immunological methods by Chakraborty and Chakraborty (2003). The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseases plant tissues. These rely on solid or soluble antigenic materials by antibodies raised against the organisms and subsequent use of an enzyme labelling system. The possible involvement of cross reactive antigens (CRA) in determining the degree of compatibility has been reported by several workers in different host-pathogen systems, viz., potato-*Phytophthora infestans*(Alba and De Vay, 1985), soybean – *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983), tea – *Bipolaris carbonum* (Chakraborty and Saha, 1994), groundnut - (Purkayastha and Pradhan, 1994), tea- *Ustulina zonata* (Chakraborty *et al.*, 2002b) and tea – *Exobasidium vexans* (Chakraborty *et al.*, 2009).
In the present study serological relationship between som morphotypes and the two different fungal pathogens, *P. disseminata* and *C. gloeosporioides* were determined using indirect immunofluorescence and indirect immunogold labelling techniques. Cross sections of healthy som leaves exhibited a natural bright yellow autofluorescence mainly on the cuticle. PAb-Cg was most reactive with leaf sections of S6 morphotype. On the other hand leaf sections that were treated with PAb-Pt were more reactive with S5 morphotype. Here reaction with FITC developed fluorescence that was distributed throughout the leaf tissues. The cellular location of CRA in tea leaves shared by *Pestalotiopsis theae* (Chakraborty *et al*., 1995b), *G.cingulata* (Chakraborty *et al*., 1996a) and *E. vexans* (Chakraborty *et al*., 2009) has been studied by immunofluorescent techniques.

Effectiveness of PAbs raised against pathogen was confirmed by immunodiffusion. IgGs were purified before application in immunoenzymatic assays to minimize non-specific binding. Optimization of ELISA was done considering two

variables – dilution of antigen and antiserum. The PTA-ELISA format was employed for the detection of pathogen in artificially inoculated som morphotypes using PAb-Cg and PAb-Pt. Absorbance value (A_{405}) was always higher in infected leaf extracts than healthy ones thereby allowing easy and early detection of infection, as early as 24 hrs of inoculation. Chakraborty *et al* (2009) reported that PTA-ELISA format could easily detect pathogen *Exobasidium vexans* in susceptible variety of *Camellia sinensis* (AV-2) as early as 24h after artificial inoculation whereas the disease symptoms were not visible before 12 days. The results of PTA ELISA was confirmed by Dot-Immunobinding Assay in which intensity of dots widely varied among different morphotypes of som plants artificially inoculated with the pathogens. Early and rapid diagnosis of red rot disease in sugarcane caused by *Colletotrichum falcatum* was also performed using DIBA technique where infected samples depicted dark blue precipitate on the nitrocellulose membrane due to the antigen-antibody reaction (Hiremath *et al*., 2004). Early detection of grey blight pathogens in som morphotypes using PTA-ELISA format and DIBA has already been reported by Acharya *et al* (2015a).

Effectiveness of mycelial antigen of pathogens in raising antibodies was assessed using DIBA. Development of deep violet colour following homologous reaction with antigen and antibody confirmed its identity. Western blot analysis using PAb-Pt and PAb-Cg was also carried out in the present study to develop strategies for rapid detection of pathogens. Here the bands on SDS-PAGE gel were compared with bands on nitrocellulose membrane. Bands of varying molecular weights were seen in SDS-PAGE out of which some bands were also seen on nitrocellulose membrane suggesting these to be the respective epitopes of the antibodies. Chakraborty *et al* (2012) raised polyclonal antibodies against mycelial antigens of *Macrophomina phaseolina* and further used them in immunological formats such as immunodiffusion, PTA-ELISA, dot immunobinding assay, Western blot analysis and indirect immunofluorescence for quick and rapid detection of the pathogen.

In the present investigation indirect immunofluorescence study of young mycelia and fungal spores was carried out with homologous antibody labelled with FITC. Strong apple green fluorescence was seen in both mycelia and spores which confirmed homologous reaction of the pathogen and antibody. The present study also reports the use of indirect immunofluorescence tests using PAb-Cg and PAb-Pt as a suitable technique for localization of the pathogen and could be employed for

immunodetection of pathogen in som leaf tissues. Kratka et al (2002) reported the use of a polyclonal antibody IgG K91 to detect a quarantine pathogen of strawberry, *Colletotrichum acatatum* using four different immunotechniques, PTA-ELISA, dot-blot, immunoprint and immunofluorescent microscopy.

In the current investigation, an attempt was also made to conduct ultrastucturalimmunocytochemical studies to locate cross reactive antigens shared by *Persea bombycina* and its fungal pathogens through transmission electron microscopy (TEM). Encouraging results were obtained following immunogoldcytochemical staining of ultrathin som leaf sections. Electron microscopic observations of healthy leaf tissues treated with PAb-Cg and PAb-Pt showed specific localization of antibody associated with epidermal and mesoplhyll cells. In addition, immunogold labelling of *P. disseminata* and *C. gloeosporioides* in infected som leaf tissues was also performed and intense labelling was observed on fungal mass that was established inside the tissue.

The polymerase chain reaction is undoubtfully the most important technique in diagnostics and has found wide application as a powerfulmolecular tool mostly due to the development of thermotolerant DNA polymerases and automated thermocyclers.PCR is preferred over classical or other moleculartechniques in the diagnosis of plant pathogens for a number of advantages that makes it very popular. Since high quality of DNA is not generally required, there is no needfor culturing the target pathogen. PCR cycles are completedin much shorter time than other molecular techniques, thusallowing a very fast screening of a large number of samples.Because of its high sensitivity, minute amounts of the target DNA are required. The ribosomal DNA gene cluster (rDNAs) is an extensively used target sequence for PCR detection of fungal plant pathogens because of a number of useful features.rDNAs bear common sequences found in the nucleus and themitochondria of eukaryotes. The nuclear rDNA cluster ispresent as tandem repeats of several hundred copies in cell, which allows high sensitivity of detection. The rDNA geneis consisted of three subunits: a large (LSU) of 28S and asmall (SSU) of 18S that are separated by a much smallergene of 5.8S. The three subunits are connected together withtwo internal transcribed spacers (ITS1 and ITS2). This whole gene cluster is repeated in the genome many timesthus being an appealing target for PCR amplification (Paplomatas *et al*., 2006). ITS sequences have gained popularity for being more variable regions and therefore allow selectivedetection of closely related organisms. Universal

primersdesigned on conserved sequences found on the small andlarge subunits, have been extensively used for the amplification of ITS regions. The amplified sequences arebetween 500-800 bp, a relatively small amount of target DNA is required for PCR, while the PCR products have been used as species-specific probes (Bruns *et al*.,1992; Gardes and Bruns,1993; White *et al*., 1990).Moreover, determination of ITS sequences afteramplification by universal primers, has allowed thedetection, identification and taxonomy of unculturable orunknown fugal species.

In the present study, ITS regions of ribosomal genes for construction of primers were used to identify *C. gloeosporioides* and *P. disseminata*. ITS region of rDNA of the pathogens was amplified using genus specific ITS1 and ITS4 primers. Amplified products of size in range of 550bp were produced by the primer pairs. These PCR products were used for sequencing of 18S rDNA region of both the pathogens. The sequence information of the pathogens was then analysed through BLASTn program one at time. The information generated for *C. gloeosporioides* isolates indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *C. gloeosporioides* with 100% similarity. On the other hand the sequence information of *P. disseminata* isolates analysed using BLAST n program indicated that the sequences contained genetic information of internal transcribed spacer region of rDNA gene of *Pestalotiopsi*s sp. with 100% similarity.

Identified *C. gloeosporioides* rDNA gene sequences obtained from NCBI Genbank of various host plants were selected for comparisons of rDNA gene sequences of *C.gloeosporioides* isolate of som plant. Similarly, identified different *Pestalotiopsis*r DNA gene sequences obtained from NCBI Genbank of various host plants were also selected for comparison of rDNA gene sequence of *Pestalotiopsis* sp isolate of som plant. Phylogenetic tree was constructed separately for the two different pathogens of som plants to infer the evolutionary history of these isolates. Maharachchikumbura *et al* (2011) discussed that due to overlapping morphological charecters between the different species of *Pestalotiopsis* genus, identification to the species level is rather difficult. For this reason, naming of species is difficult and highly subjective and many sequences for *Pestalotiopsis* deposited in GenBank are likely to be wrongly named. Therefore the Pestalotiopsis isolate in the present investigation could be identified at the genus level only using the molecular identification tools.

On the other hand the present study also conducted specific characterization of *C. gloeosporioides* isolates with primers CgInt-ITS4. The amplification using this primer pair was positive for all the *C. gloeosporioides* isolates of som plant, generating fragments of approximately 450bp and negative for all the *Pestalotiopsis*sp isolates. The use of these primers confirmed the identity of the isolates of *C. gloeosporioides* identified by traditional method. Mills *et al* (1992), Freeman *et al* (2000), Maciel *et al* (2010) and Chowdappa *et al* (2012) used these markers for identification of the *C. gloeosporioides* isolates which showed fragments of 450-bp. The results were similar to those obtained in the present study.

In the present investigation amplified fragments of *Colletotrichum* isolates and *Pestalotiopsis* isolates were distinguished using DGGE analysis. This analysis clearly separated the Colletotrichum isolates from Pestalotiopsis isolates based on their GC content and hence the banding patthern. Fagbola and Abang (2004) distinguished *Colletotrichumcircinans* and *C. coccodes* isolates based on DGGE analysis of their amplified fragments in spite of the failure of previous attempts at genetic differentiation of the two species based on RFLP analysis of the rDNA ITS region.

In the present study *C. gloeosporioides* and *Pestalotiopsis* isolates were evaluated for their genetic variability using RAPD markers. The random primers OPA-1, OPA-4, OPD-6 and OPD-12 were used. Out of these 4 primers used, OPA-1 produced reproducible banding pattern. Xiao *et al* (2004) used random amplified polymorphic DNA (RAPD) markers to determine genetic relationships among isolates recovered from noncultivated hosts and diseased strawberry plants. Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from noncultivated hosts into two separate clusters. One cluster contained 50 out of the 52 isolates and a second cluster contained 2 isolates that were homothallic in culture. Isolates from strawberry were interspersed within the cluster containing the 50 isolates that were recovered from the noncultivated hosts. The results were not inconsistent with the hypothesis that *C. gloeosporioides* isolates obtained from strawberry and non-cultivated hosts adjacent to strawberry fields are from the same population. Gupta *et al* (2010) collected twenty-five isolates of *Colletotrichum gloeosporioides* causing mango anthracnose from different agroclimatic zones of India. These isolates were evaluated for their pathogenic variability on mango seedlings and genetic characterization using random amplified polymorphic DNA (RAPD molecular techniques). The random primers OPA-1, 3, 5, 9, 11, 15, 16 and 18

were used and the twenty five isolates were grouped into two. Out of eight primers in RAPD, OPA-1, 3 and 18 were able to produce reproducible banding pattern. Each of these primers generated a short spectrum of amplicons, located between 661 and 2291-bp markers, indicative of genetic polymorphism. Dendogram revealed more than 75% level of similarity. The data suggest that RAPD may be of value by virtue of its rapidity, efficiency and reproducibility in generating genetic fingerprints of *C. gloeosporioides* isolates. Joshi *et al* (2009) studied the genetic diversity of 22 *Pestalotiopsis*isolates causing grey blight disease of tea (*Camellia sinensis*) using two molecular marker system, RAPD and ISSR. The UPGMA clustering of both the methods were comparable and the results indicated that, within the southern India, the diversity of Pestalotiopsis was high both morphologically and genetically.

The next phase of our study was to check for antagonistic activity of selected PGPR and PGPF against the fungal pathogens of som plants. Two different *Trichoderma* isolate – *T. harzianum* and *T. asperellum* was used for in vitro antagonistic study where T. harzianum isolate RHS/480 showed better antagonistic effect against both the pathogens *C. gloeosporioides* and *P. disseminata* than *T. asperellum*. Hence further study was carried out using *T. harzianum* isolate. Rabha et al (2015) studied in vitro antagonistic activity of *T. harzianum* isolate PBR1, isolated from native rhizosphere of som plant against two important foliar fungal pathogen *Pestalotiopsis disseminata* and *Phylostictapersea*. Sunar *et a l*(2014a) tested three species of *Trichoderma* viz. *T. harzianum, T. erinaceum*and *T. asperellum* for their *in vitro* effects against *S. rolfsii*and theinhibition percentage was up to 75 percent in *T. harzianum* which was taken up forfurther *in vivo* assay and was effective in suppressing sclerotial rot of *Glycine max* causedby *S. rolfsii*. Similarly two different PGPR viz. *Bacillus pumilus* and *Bacillus altitudinus* was also taken into consideration for checking their *in vitro* antagonistic activity against the fungal pathogens. *B.pumilus* showed better activity against *C. gloeosporioides* and *B. altitudinus* showed better antagonism towards *Pestalotiopsis* sp. Hence these PGPR were further utilised for their in vivo assay against their respective pathogens.

Changes in levels of different defense related enzymes, viz. Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Chitinase (CHT) and β-1,3Glucanse (GLU) following infection with *Colletotrichumgloeosporioides*was also studied during this investigation. Presence of new peroxidase isozyme in infected leaf samples was also recorded (Chakraborty *et al.,* 2016b).Correlation of this result was also made with the

study undertaken by Parihar*et al* (2012) where it was seen that biochemical analysis of genotypes of *Brassica juncea*infected with *Alternaria* blight revealed an increase in PAL, PPO and peroxidase activity. Singh *etal* (2014) reported that preformed phenolic compounds as well as Peroxidase enzyme play important role in resistance of Chili against *Colletotrichum capsici.* Analysis of peroxidase isozymes by polyacrylamide gel electrophoresis showed four isozymes in healthy tea leaf samples and five in tea leaves infected with *Exobasidium vexans*. They suggested that the appearance of new bands following infection can be correlated with the induction of the catalytic activity of more isozymes, leading also to an overall increase in peroxidase activity (Chakraborty *et al.*, 2002)

In the present study it was observed that total as well as ortho-dihyroxy phenol content increased in infected plants, more in plants with less infection. Infection by *Venturiainequalis* in apple caused an accumulation of phenolic compounds wherein Folin-Ciocalteu values increased by 1.4 to 2.4 fold (Petkovsek *et al*., 2008). Taware *et al* (2010) studied that there was significant increase in total phenolic content of grape leaves due to foliar powdery mildew infection. These results are in accordance with the result obtained in the present study.

Thin layer chromatography has been used by various workers to study the phenol profile of different plants like tea (Chakraborty and Saha, 1994), som (Neog *et al.*, 2011), different medicinal plants (Maobe *et al*., 2012). Studies on secondary metabolites of som plant by Neog *et al* (2011) revealed the presence of four major phenolic acids – Chlorogenic acid, Catechol, Morin and Gallic Acid. In the present study HPLC analysis revealed the presence of Resorcinol, catechol and chlorogenic acid in both healthy and infected leaves (*Colletotrichum gloeosporioides*) but the height of these peaks increased in infected samples. In addition presence of two new peaks in infected sample could be identified as ferulic acid and salicylic acid. Results revealed that the pathogen triggered the production of resorcinol, catechol, chlorogenic acid, ferulic acid and salicylic acid in the muga host plant as biochemical defense strategy (Chakraborty *et al*., 2016 b). Presence of chlorogenic acid as part of defense system has been studied by different workers in different crops, such as Potato tubers (Malamberg and Theander, 2011), apples (Petkovsek 2003), coffee (Rodrigues 2011) and tomato (Lopez-Gresa*et al.*, 2011). Presence of salicylic acid and ferulic acid in infected leaves and not in healthy leaves indicate the role of this phenolic acid in defense against pathogen. When biochemical charecterization of

maize plants infected with *Drechslera dactylidis* was done, it was found that salicylic acid increased 2-fold in infected leaf samples (Ghany 2012).

Activation of defense response in som plants was observed after application of bioinoculants against *C.gloeosporioides* and *P. disseminata*. The association of AMF with the Som plants was studied and their diversity was assessed in eight morphotypes. Among these, *Glomus* sp, *Scutellospora* sp, *Acaulospora* sp and *Gigaspora* sp were foundassociated with the different morphotypes of Som plants, wherein *Glomus* sp was predominant. Growth promotion in Som plants following successful root colonization with AMF was noted in terms of increase in height, no of leaves and no of branches. Two main defense enzymes in plants, Phenylalanine Ammonia Lyase (PAL) and Peroxidase (POX) were found higher in AMF inoculatedplants in comparison to control plants indicating induced resistance in Som plants(Chakraborty *et al*., 2013). Application of *B. pumilus* and AMF, singly or jointly increased growth of som morphotypes in pot as well as in field condition, but better result was obtained when both were co-inoculated. Enhanced accumulation of total protein, phenolics as well as increased activities of major defense enzymes peroxidase, phenylalanine ammonia lyase, chitinase and β1,3 glucanase were also observed following the above mentioned treatments in som plants. Dual application of AMF and PGPR also suppressed the disease incidence of leaf blight of som plants following artificial inoculation of treated plants with *C. gloeosporioides*(Chakraborty *et al*; 2014). A significant improvement in growth measured in terms of increase in shoot length, number of leaves and branches was observed when one year old som saplings were bacterized with a strain of *Pseudomonas* under pot conditions (Rabha *et al.*, 2014). Increased activity of chitinase, β-1, 3-glucanase and peroxidase were alsodetermined in tea plants following treatments with Josh - a bioformulation of AMF (Chakraborty *et al.,* 2007).

Growth and yield of various crops have been reported to increase following application of Vermicompost along with other bioinoculants such as tomato (Bhattacharjee*et al*., 2015), Chickpea (Sahni *et al*., 2008), guava (Pathak *et al*., 2013). In accordance with these observations, in the present study it was observed that application of Vermicompost, PGPR and AMF, alone and in combination increased growth of eight different morphotypes of som plants. Enhancement of total phenol as well as defense related enzymes was seen in dual and triple treatments irrespective of the morphotypes. Among the various treatments a consistent decrease in disease

incidence of grey blight after artificial inoculation of P. disseminata spores was observed in plants treated with PGPR followed by Vermi + PGPR treatment when compared to the untreated inoculated plants. It was seen that disease progression in treated plants were very slow when compared with untreated plants (Acharya *et al*., 2015b).

Effect of combined application of three different bioinoculants - PGPR, AMF and PGPF, on growth as well as suppression of leaf blight disease of som plant was also studied in this investigation. It was recorded that under nursery condition, growth enhancement was noticeable after 60 days of combined treatment of all bioinoculants. Two morphotypes S5 and S6 were shown highest growth and was transferred to field where the soil was amended with bioformulation of PGPR, PGPF and mass multiplied AMF. Total protein, chlorophyll content as well as total and ortho-phenol content of treated plants was increased than control plants. Unni*et al*(2008) found that five different PGPRs helped improve the growth of the som plant by increasing chlorophyll content, free amino acid, total protein, reducing sugar, carbohydrate and dry weight.Balamurugan*et al* (2013) explained in his studies with *Camellia sinensis* that during the first six months after application of inorganic fertilizers (IOF) and bioinoculants (BF), the results revealed that recommended dosage of the fertilizers provided significantly higher values of biometric characteristics in terms of stem height, no. of leaves, biomass production in tea seedlings. Results of chlorophyll content of the leaves sampled after six months revealed that application of 100% IOF+BF had shown higher total chlorophyll content followed by 50% IOF+BF and 100% IOF alone. But after 12 months, chlorophyll content was higher after application of 100% IOF+BF followed by IOF alone.

Disease symptoms were established in the plants sprayed with spore suspension of *C. gloeosporioides* well after 96 hrs of inoculation. It was noted that symptoms appeared in untreated inoculated leaves much faster than in treated inoculated plants. Results of Percent disease index correlate with the results obtained from the analysis of defense enzymes where the enzyme activity of PAL, POX, CHT and GLU was much higher in treated inoculated leaves indicating their increased defense against the pathogen. But the healthy leaves showed more levels of enzymes suggesting that the inoculated leaves are prone to disease development where the enzyme levels were less. Application of *G. mosseae*and *T. hamatum*singly or jointly suppressed root rot of mandarin caused by *F. solani*. Defense enzymes, chitinase, glucanase and peroxidase

showed enhanced activitiesduring disease suppression (Allay and Chakraborty, 2010). Chakraborty *et al*., 2016 reported that dual application of *Bacillus pumilus* and *Rhizophagusfasciculatus* caused induction of resistance in *Camellia sinensis* against *Sclerotiumrolfsii*. It is now clear that microbes in small consortia enhance the defense signalling cascades leading to enhance transcriptional activation of several metabolic pathways (Sarma*et al*., 2015).

In the next phase of our study, induction of defense enzymes mainly chitinase was studied using fluorescent antibody staining technique. Leaves of som plants treated with PGPR and roots treated with AMF and *T. harzianum* were reacted with Pab of chitinase followed by labelling with FITC. Strong bright apple green fluorescence was observed in the epidermal and homogenously in mesophyll tissues inleaves and homogenously in cortical cells and epidermal cells in roots. Enhancement ofchitinase was revealed in both leaves and roots following induction.Chakraborty *et al* (2009) studied the expression of chitinase in leaves of treated tea plants following induction with salicylic acid using immunofluorescent techniques.

Simultaneously immunogold localization of defense enzyme chitinase in leaves of som plants treated with PGPR as well as roots treated with AMF and PGPF was studied through Transmission Electron Microscopy. Heavy deposition of gold particles was observed in cytoplasm of leaf section confirming the expression of chitinase following treatment with bioinoculants. Yedida*et al*(1999), observed that application of *Trichoderma* to rhizosphere of cucumber seedlings initiated a series of morphological and biochemicalchanges. Gold labeling was done with β-1,4-exoglucanase and β-1,3-glucanase whereintense gold labeling was observed over both the cell walls and wall appositions. The deposition of the chitinase following treatment with salicylic acid was found predominantly in cellular compartments of ultrathin sections of tea leaves. Gold labelling in the sections showed a high amount of labelling in chloroplasts and hostcytoplasm and lesser amount in vacuoles, mitochondria and walls(Chakraborty *et al*., 2009). This suggests the potential of salicylic acid for protection of tea against foliar pathogens.

It is evident from the results that observed growth promotion and enhanced resistance to pathogen due to joint application of bioinoculants (AMF, PGPF and PGPR) can be attributed to activation of defense responses in som plants such as increased accumulaton of phenolics and defense enzyme activities.

Chapter 6
CONCLUSION

- Disease incidence of two major foliar fungal diseases – Grey blight and leaf blight were evaluated in eight morphotypes of *Persea bombycina.* Occurrence of leaf blight disease was much frequent in the area than grey blight disease.
- Isolation of pathogen from infected leaves resulted in three isolates from leaf blight infected leaves – SOM/Cl/01, SOM/Cl/02, SOM/Cl/03 and three more isolates from grey blight infected leaves – IPL/SOM/P/01, IPL/SOM/P/02, IPL/SOM/P/03. Following completion of Koch's postulate two isolates – IPL/SOM/P/01 for *Pestalotiopsis disseminata* and SOM/Cl/02 for *Colletotrichum gloeosporioides* were further taken into consideration for molecular identification.
- Growth characters and spore morphology of these six isolates were studied. Scanning electron microscopy of the spore structures was also studied.
- Screening of resistance of eight morphotypes towards foliar fungal pathogens was studied using two techniques – detached leaf and whole plant inoculation technique. Both the techniques gave similar result where it was observed that S5 morphotype is highly susceptible to grey blight disease followed by S6, S7 and S2. On the other hand S6 morphotype was most susceptible to leaf blight disease, followed by S3, S8 and S5.
- Polyclonal antibody was raised separately against mycelial antigen of both the fungal pathogens. These antibodies were used for serological characterization of the fungal pathogens by PTA-ELISA, Dot-blot, Western blot and Indirect immunofluorescence of mycelia and spore. Western blot analyses using polyclonal antibody of *C. gloeosporioides* and *P. disseminata* revealed that the PAb could show different levels of homologous reactions with the antigens of *C. gloeosporioides* and *P. disseminata* respectively. Sharp and intense bands were produced on the nitrocellulose membrane after enzymatic reaction with NBT BCIP. Efficacy of polyclonal antibodies raised against the mycelial antigen was further tested with the help of indirect immuno fluorescence of young mycelia of *C. gloeosporioides* and *P. disseminata.* The mycelia treated with PAbs and labeled with FITC showed apple green fluorescence.

- Presence of cross reactive antigen (CRA) in host tissues was detected using PTA-ELISA. Cellular localization of these CRA in host leaf tissues were checked using indirect immunofluorescence as well as immunogold labelling .
- Immunodetection of foliar fungal pathogens in naturally infected leaf tissues was carried out using PTA-ELISA as well as Dot immunobinding assay.. Cellular localization of the pathogens in infected som leaf tissues was studied using indirect immunofluorescence with PAb-Pt and PAb-Cg followed by labelling with FITC. Transmission electron microscopy of ultrathin sections of infected leaf tissues using the polyclonal antibodies and labelling with antirabbit goat IgG (whole molecule) gold conjugates to confirm the presence of the pathogen in infected leaf samples.
- Molecular detection of foliar fungal pathogens *P. disseminata* (IPL/SOM/P/01) and *C. gloeosporioides* (SOM/Cl/02) was carried out using 18S rDNA sequencing using ITS1/ITS4 primers. The BLAST query of the 18S rDNA sequence of the isolates against GenBank database confirmed the identity of the isolate IPL/SOM/P/01 as *Pestalotiopsis* sp and SOM/Cl/02 as *Colletotrichum gloeosporioides*. The sequences have been deposited to NCBI, Genbank database under the accession no KT697994 for *Pestalotiopsis* sp and KM491736 for *C. gloeosporioides*.
- Species specific primer pair for *C. gloeosporioides* (CgINT/ITS4) was used to identify the other isolates. Electrophoresis led to the appearance of 480 bp single band only for *C. gloeosporioides* isolates separating them from the other isolates of *P. disseminata*.
- Molecular characterization of the fungal isolates was carried out using RAPD and DGGE analysis. The genetic relatedness among the different isolates of *Colletotrichum* and *Pestalotiopsis* was analyzed separately using different random primers for producing reproducible polymorphism. RAPD banding pattern revealed that the isolates of *C. gloeosporioides* and *P. disseminata* were genetically different and showed polymorphism among each other. On the other hand DGGE analysis could differentiate the isolates into two separate groups based on the migration rate of their amplified DNA. The migration of amplified 18S rDNA samples within each group was similar, suggesting that there was little intraspecific variation among the isolates.

- Activity of various defense enzymes such as PAL,POX,CHT and GLU were assayed in healthy as well as in infected leaf samples. It was recorded that the activity of these enzymes were more in infected leaves than in healthy samples. Isozymes of peroxidase was also checked using native PAGE and it was revealed that appearance of new peroxyzyme was seen in infected leaf samples.
- Changes in levels of phenolic in healthy and infected samples were also noted. The phenolic acids were checked using High Performance Liquid Chromatography and it was revealed that Catechol, Morin and Chlorogenic acid were present in both healthy and infected leaf samples, however their intensity increased in infected samples. On the other hand appearance of new peaks representing ferulic acid and salicylic acid were recorded in infected leaves. Hence it was evident that chlorogenic acid, ferulic and salicylic acids play an important role in defense mechanism of these plants against foliar fungal pathogens.
- Selected bioinoculants such as PGPR (*Bacillus pumilus* and *B. altitudinus*) and PGPF (*Trichoderma harzianum* and *T. asperellum*) were evaluated for their antagonistic effect against the fungal pathogens and it was recorded that these two bioinoculants could easily prevent the growth of the fungal pathogens *in vitro*.
- These bioinoculants were mass multiplied and applied to the som plants under nursery and field condition. Association of Arbuscular mycorrhizal fungi (AMF) with som roots was checked and these AMF were further mass multiplied for application. Vermicompost with value addition with bioinoculants was also prepared and applied accordingly. All these bioinoculants were applied alone and in combinations to determine their effects on growth promotion and biochemical changes in som plants.
- It was recorded that bioinoculants applied in different combinations enhanced the growth of som plants, irrespective of the morphotypes. Biochemical changes including defense enzymes were also seen to increase in treated plants in comparison to untreated control plants.
- Activation of defense response against *C. gloeosporioides* and *P. disseminata* in plants treated with bioinoculant was observed. Application of PGPR, PGPF and AMF in combination decreased the disease incidence of leaf blight.

Besides application of value added Vermicompost reduced the disease incidence of grey blight.

- Growth promotion of two different morphotypes (S5 and S6) of som plant was studied under field condition following application of bioinoculants. HPLC analysis of their phenolic content was also studied where it was observed that bioinoculant treated plants showed presence of more phenolic acids than control sets. On the other hand disease incidence of leaf blight decreased and levels of defense enzymes increased after artificial inoculation of these plants with *C. gloeosporioides*.
- Following these studies, cellular localization of two important defense enzymes – Glucanase and Chitinase was studied using indirect immunofluorescence and immunogold labelling. Expression of these enzymes were noted in treated leaf and root sections, confirming earlier results obtained.
- Results of the present study indicate that application of bioinoculants promotes growth and bioprimes the som plant against foliar fungal pathogens by up-regulation of defense activities. These findings could be helpful in protecting plants against fungal pathogens and improving the quality and quantity of the foliage of *P. bombycina* that would in turn provide quality yield of cocoon of muga silkworm.

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APPENDIX A: List of Thesis related publications

In Journals

1. Chakraborty BN, **Acharya A** and Chakraborty U (2016) Biochemical changes in *Persea bombycina* following infection with *Colletotrichum gloeosporioides*. *Journal of Mycopathological Research* 54(1):127-133

2. **Acharya A**, Ghosh S and Chakraborty BN (2015) Serological detection of *Pestalotiopsis disseminata* in *Persea bombycina* causing grey blight disease. *International journal of Advanced biological Research* 5(4): 327-333

3. **Acharya A**, Chakraborty U, Ghosh S and Chakraborty BN (2015) Management of grey blight disease of Som plants using value added vermicompost with *Glomus constrictum* and *Bacillus altitudinis*. *NBU Journal of Plant Sciences* 9(1): 46-53

4. Chakraborty B.N, **Acharya A**, Chakraborty U, Jha DK, Rabha J , Sharma HK (2014) Growth Promotion and Induction of Defense Enzymes in *Persea bombycina* following application of AMF and PGPR against *Colletotrichum gloeosporioides* causing Leaf Blight Disease. *Journal of Mycology and Plant Pathology* 44(3): 249-256

5. Chakraborty BN, **Acharya A**, Chakraborty U, Rabha J, Jha D.K (2013) Screening of Arbuscular Mycorrhoizal Fungi associated with *Persea bombycina* Kost and their improvement of plant health. *Journal of Plant Disease Science*. 8(2): 141-147

APPENDIX B: List of Abbreviations

APS- Ammonium per sulphate
BLAST- Basic local alignment search tool
BSA- Bovine serum albumin
BSS-2- Biclonal seed stock- 2
$Ca_3(PO_4)_2$ - Tri-calcium phosphate
$CaHPO_4$ - Calcium phosphate
CAS- Chrome azurol S
CAT- Catalase
CDA- Chitinase detection agar
cfu- Colony forming unit
CHT- Chitinase
$CuSO_4$- Copper sulphate
DAC-ELISA- Direct antigen coating- Enzyme linked immune-sorbent assay
DAPG- 2,4-Diacetylphloroglucinol
dATP- Deoxy adenosine tri-phosphate
dCTP- Deoxy cytosine tri-phosphate
DEAE cellulose- diethyl aminoethyl cellulose
dGTP- Deoxy guanosine tri-phosphate
DMAB- Di methyl amino benzaldehyde
DNA- Deoxyribonucleic acid
dNTPs- Deoxy nucleotide tri-phosphates
DNSA- Dinitro salicylic acid
dTTP- Deoxy thymidine tri- phosphate
EDTA- Ethylene diamine tetra acetic acid
ELISA- Enzyme linked immune- sorbent assay
ER- Root endosphere
$FeCl_3$- Ferric chloride
FITC- Fluorescein isothiocyanate
g- gram
Gfp- Green fluorescent protein
GlcNAc- N-acetyl glucosamine
H_2O_2 - Hydrogen peroxide
H_2SO_4- Sulphuric acid
HCl- Hydrochloric acid
HCN- Hydrocyanic acid
HDTMA- Hexa-decytrimethyl ammonium bromide
HPLC- High performance liquid chromatography
IAA- Indole acetic acid
ISR- Induced systemic resistance
KCl- Potassium chloride
KH_2PO_4 – Potassium dihydrogen phosphate

MEGA 4- Molecular Evolutionary Genetics Analysis 4
mg- Mili gram
$MgCl_2$- Magnesium chloride
ml- Mili litre
Na_2CO_3- Sodium carbonate
Na_2HPO_4- Di sodium hydrogen phosphate
Na_2MoO_4- Sodium molybdate
NaCl- Sodium chloride
NaN_3- Sodium azide
$NaNO_2$- Sodium nitrite
NaOH- Sodium Hydroxide
NB- Nutrient Broth
NBT/BCIP substrate- Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
NCBI- National Center for Biotechnology Information
NCM- Nitrocellulose membrane
NH_4Cl- Ammonium chloride
PAL- Phenylalanine ammonia lyase
PBS-Tween- Phosphate buffer saline- Tween
PCA- Phenazine-1-carboxylic acid
PCI- Water saturated phenol: Chloroform: Isoamyl alcohol
PCR- Polymerase chain reaction
PCR–RFLP- Polymerase chain reaction- Restriction fragment length polymorphism
PGPR- Plant Growth Promoting Rhizobacteria
PGPTs- Plant growth promoting traits
pNPP- p- nitrophenyl phosphate
POX- Peroxidase
PPO- Polyphenol oxidase
PR proteins- Pathogenesis related proteins
PRN- Pyrrolnitrin
PSB- Phosphate solubilising bacteria
PVK- Pikovskaya's agar
PVLG – Poly vinyl Lacto Glycerate
PVP- Poly vinyl- pyrollidone
RNA- Ribonucleic acid
RP- Rock phosphate
RS- Rhizosphere soil
SA- Salicylic acid
SAR- Systemic acquired resistance
SDS- Sodium dodecyl sulphate
SDS-PAGE- Sodium dodecyl sulphate- Poly-acrylamide gel electrophoresis
SKM- Skim milk agar
TAE buffer- Tris Acetic Acid and EDTA buffer
TE buffer- Tris-EDTA buffer

TEMED- N,N,N',N'-Tetramethylethylenediamine
Tris Hcl- Tris hydrochloric acid
UPGMA- Unweighted Pair Group Method with Arithmetic Mean
yr- Year
β-1,3-GLU- β-1,3 glucanase
µl- Micro litre

APPENDIX C: List of Chemicals

Ammonium chloride
Ammonium per sulphate
Bovine serum albumin
Calcium phosphate
Carboxy methyl cellulose
Chrome azurol S
Colloidal chitin
Copper sulphate
Deoxy nucleotide tri-phosphates
Di methyl amino benzaldehyde
Di sodium hydrogen phosphate
Diethyl aminoethyl cellulose
Dinitro salicylic acid
Ethylene diamine tetra acetic acid
Ferric chloride
Fluorescein isothiocyanate
Helicase (3%)
Hexa-decytrimethyl ammonium bromide
Hydrochloric acid
Hydrocyanic acid
Hydrogen peroxide
Indole acetic acid
LR White resin
Magnesium chloride
N,N,N',N'-Tetramethylethylenediamine
N-acetyl glucosamine
Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
O-dianisidine (5 mg/ml methanol)
p- nitrophenyl phosphate
Phosphate buffer saline- Tween
Poly vinyl- pyrollidone
Potassium chloride
Potassium dihydrogen phosphate
Sodium azide
Sodium carbonate
Sodium chloride
Sodium dodecyl sulphate
Sodium Hydroxide
Sodium molybdate
Sodium nitrite
Sulphuric acid

Tri-calcium phosphate
Tris Acetic Acid and EDTA buffer
Tris hydrochloric acid
Tris-EDTA buffer
Water saturated phenol: Chloroform: Isoamyl alcohol
0.05(M) sodium phosphate buffer (pH 6.8)
0.1M sodium phosphate buffer pH (7.4)
0.1(M) sodium acetate buffer (pH 5.0)
0.2M Na-phosphate buffer (pH 5.4)
0.3mM borate buffer (pH 8.0)
1 M K-PO4 buffer (pH 7.1)
1-amino-cyclopropane-1-carboxylic acid hydrochloride
1M Na-acetate buffer (pH 4)
Sodium borate buffer (pH 8.8)
2 mM β- mercaptoethanol
1 M Na-borate buffer (pH 9.8)
2% L-phenylalanine
2,4-Diacetylphloroglucinol
4 mM H_2O_2.
4% laminarin
2.5% Glutaraldehyde
30%-90% absolute alcohol
1% aqueous toluidine blue solution
2% uranyl acetate
0.2% lead acetate

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SEROLOGICAL DETECTION OF *PESTALOTIOPSIS DISSEMINATA* IN *PERSEA BOMBYCINA* CAUSING GREY BLIGHT DISEASE

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ABSTRACT
Polyclonal antibody was raised in white rabbit against *Pestalotiopsis disseminata*, causal agent of grey blight of *Persea bombycina* Kost. Effectiveness of mycelial antigen raising antibody was confirmed by Dot immunobinding assay as well as Western Blot analysis. The titre value of the antibody was determined by PTA-ELISA format. Leaf antigens from eight morphotypes of healthy and naturally infected som plant were also analysed by Dot Blot and PTA-ELISA to detect the presence of the pathogen in the leaves. Detection of pathogens in leaf tissue was also confirmed by indirect immunofluorescence test. Early detection of pathogens in leaf tissues after artificial inoculation with spores of *P. disseminata* was also performed using PTA-ELISA where it was found that presence of pathogen could be detected as early as 24 hrs after inoculation whereas the symptoms of the disease was not established in the plant before 96hrs at the earliest. Thus by using these serological tools easy and early detection of the pathogens in the leaves can be done that would help to develop better and faster management strategies for the grey blight disease.

KEYWORDS: *Persea bombycina*, Grey blight, early detection, PTA-ELISA, DIBA, Immunofluorescence.

INTRODUCTION

Muga silk is an exclusive prerogative of the North East India and more particularly in the Brahmaputra valley of Assam. The people of more than 38,000 villages in this region fully depend upon sericultural activities. The muga silkworm is a multivoltine and polyphagous insect. Som *(Persea bombycina* Kost) and Soalu *(Litsea monopetela* (Roxb.) Pers, are the primary food plants of muga silkworm (Choudhury, 1970). 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). Diseases, unfavorable weather conditions, insect pests, poor agronomical practices, un-wanted weeds are the main reasons for low productivity. 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). Grey blight is one of the major foliar fungal diseases of som caused by *Pestalotiopsis disseminata* (Thum) Stey (Bharali, 1969). The symptoms of the disease are the appearance of small, oval, and discolour lesions which are irregularly scattered on the leaves. The brown or grey spots develop irregularly in the subsequent days after infection (Das *et al*., 2010). As the disease development is in progress, the spots get collapsed, malformed and ultimately the entire leaf dries up (Keith *et al*., 2006). This 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.

Recent trends in detection of plant pathogens include the development of more rapid diagnostic techniques with high specificity for the target organism (Chakraborty, 1988). 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). Since *P. disseminate* causes huge losses to the farmers, the present study deals with the early detection of the disease by the help of various serological methods that might lead to certain degree of management of grey blight disease.

MATERIALS & METHODS

Plant material

Eight different morphotypes of som plant *viz*. S1 to S8, was collected from Central Muga and Eri Research and Training Institute (CMER&TI), Jorhat Assam and maintained in experimental field condition of Immuno-phytopathology lab, Department of Botany, University of North Bengal

Fungal culture

The pathogen (Som/P/01) was isolated in PDA media from naturally infected som leaves of S1 morphotype and the

fungal culture was purified using hyphal tip method in PDA slants. These slants were maintained for further studies. For light microscopic studies, hyphae and spores were stained with Lactophenol-cotton blue.

Inoculation technique and disease assessment

Artificial inoculation of som leaves were done following the method of Chakraborty *et al*. (1996). Conidial suspension of *P. disseminata* (3 X 10^6 conidia ml^{-1}) was placed on adaxial surface of each leaf (2-6 droplets/ leaf) with a hypodermic syringe. In control sets drops of sterile water were placed on the leaves. Humid conditions were maintained by covering each tray with a glass lid and sealing with petroleum jelly. Trays were kept at 25°C.

Assessment of inoculums infectivity and symptom development were done on the basis of percentage of drops that resulted in lesion production after 72hrs (Chakraborty & Saha, 1994). Observations were based on 50 inoculated leaves for each morphotype.

Preparation of antigen

Antigens were prepared from mycelia of *P. disseminata*, healthy, naturally infected and artificially infected leaves of som plant following the method described by Chakraborty and Purkayastha (1983). They were stored at -20°C till further use.

Production and purification of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelia antigen of *P. disseminata* following the method of Chakraborty & Purkayastha (1983). Normal sera were collected by ear vein puncture from the rabbits before immunization. The antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant was injected subcutaneously at weekly interval for six consecutive weeks. The blood samples were collected after six weeks following injection and kept for 1h at 37°C. The clots were loosened and stored at 4°C. The antisera was clarified by centrifugation and then stored at -20°C till further use. IgGs were purified by DEAE-Sephadex column chromatography following the protocol of Clausen (1988).

Dot immunobinding Assay

Mycelial antigens prepared from *P. disseminata* were loaded on nitrocellulose membrane filters using Bio-Dot Apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *P. disseminata* as outlined by Lange *et al*. (1989).

Western Blotting

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electro transferred to NCM using semi-dry Trans-blot unit (Bio-Rad) and probed with PAbs of *P. disseminata* following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indoylphosphate (NBT-BCIP) as substrate. Immuno-reactivity of the proteins was visualized as violet-coloured bands on the NCM.

PTA-ELISA

Optimization of ELISA was done using purified IgGs of known concentration which was predetermined using the referred formula. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (100mg/ml) were used for PTA-ELISA as enzyme substrate (pNPP), reaction was terminated after 60 mins and the absorbance values were recorded as mean of five adjacent wells measured at 405nm essentially as described by Chakraborty & Sharma (2007). Antigens from fungal pathogens as well as antigens from healthy and infected leaves were diluted with coating buffer and IgGs were diluted to 1:125 with PBS-Tween containing 0.5% BSA. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (100mg/ml) was used for PTA-ELISA as enzyme substrate (pNPP) in PTA-ELISA test. Absorbance values were measured at 405 nm in an ELISA reader (Microplate Reader, Analytical technologies Ltd). Absorbance values in wells not coated with antigens were considered as blanks.

Fluorescence antibody staining and microscopy

Five days old mycelia and conidiospores of *P. disseminate* were treated with PAbs of *P. disseminata* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC). At the same time thin cross sections of healthy and grey blight infected leaf tissues were also treated in similar manner. Observations were made using a Biomed microscope (Leitzz) equipped with an I-3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Moticam Pro 285B.

RESULTS & DISCUSSION

Identification of the pathogen

The pathogen was isolated in Potato Dextrose Medium (PDA) from infected leaves. It was purified using hyphal tip method and further grown in petriplates. The pathogen grew as a sheet of white mycelium and after 7 days black acervulus was formed. Identification of the pathogen was based on the microscopic view of the spores present in the acervuli. The spores contained dark septate cell and transparent setulae and appendages, characteristic feature of *Pestalotiopsis* sp. (Fig 1).

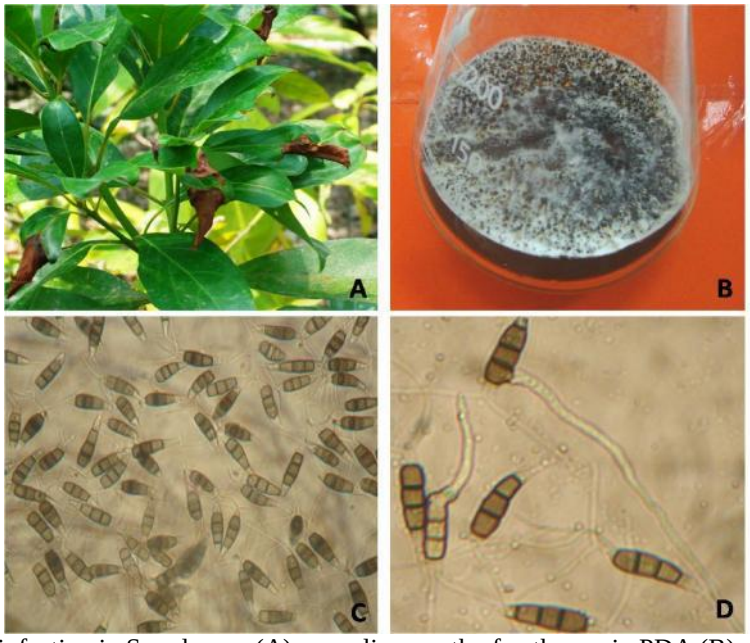

FIGURE 1: Grey blight infection in Som leaves (A), mycelia growth of pathogen in PDA (B), spores of pathogen (C-D)

Dot Immunobinding Assay (DIBA)

Effectiveness of mycelia antigen of *P. disseminata* in raising antigen was assessed using Dot immunobinding assay (DIBA). Development of deep violet colour following homologous reaction with antigen and antibody confirm its identity (Fig. 2) Chakraborty *et al.* (2012) studied the effectiveness of mycelia antigen *Macrophomina phaseolina* in raising antibody against the pathogen by confirming a positive reaction between the antigen and antibody on nitrocellulose membrane.

FIGURE 2: DIBA showing reaction of *P. disseminata* antigen with PAb of *P. disseminata* (Homologous reaction)

Optimization of ELISA

Optimization of ELISA was done considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain the maximum sensitivity. Antiserum dilutions ranging from 1:125 to 1:16000 were tested against homologous antigen at a concentration of 5mg/L. Absorbance values in ELISA decreased from the dilution of 1:125 to 1:2000 after which it levelled off. Dilutions of antigen concentration in two fold series ranging from 25 to 1600µg/L were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with a concomitant increase of antigen concentrations. Concentration as low as 25µg/L can be easily detected by ELISA at both antisera dilutions (Fig 3).

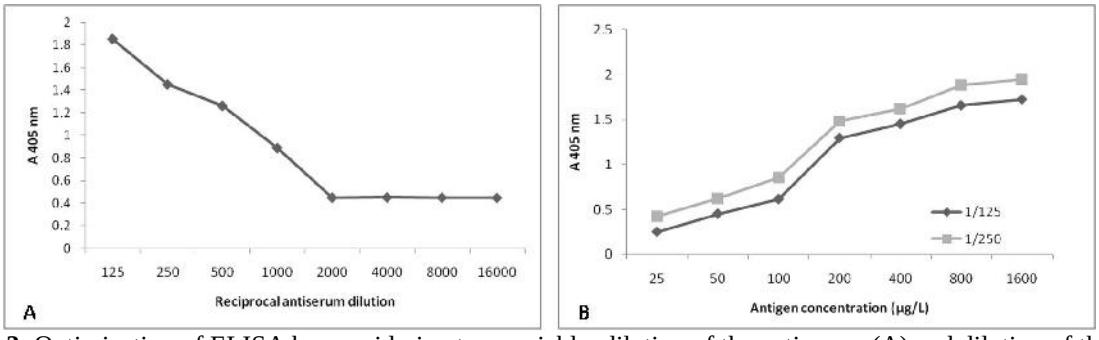

FIGURE 3: Optimization of ELISA by considering two variable, dilution of the antiserum (A) and dilution of the antigen extract (B).

Detection of pathogen using PTA-ELISA format and DIBA

Leaf antigen of both healthy and infected samples of eight different morphotypes was reacted with PAb of *P. disseminata* on nitrocellulose membrane. Results revealed development of deep violet colour in infected leaf samples indicating the presence of grey blight disease in som plants. In the next set of experiments eight different morphotypes of som plants, viz. S1 to S8 were used for the detection of infection. Antigen preparations from both healthy and naturally infected leaves of som plants were tested against the 1:125 dilution of antiserum of *P. disseminata*. Results are presented in the Table 1. Absorbance values for healthy leaf samples were significantly lower than corresponding infected leaf samples. This technique can therefore be used to detect the presence of the fungal pathogen in the plant easily and fast. Early and rapid diagnosis of red rot disease in sugarcane caused by *Colletotrichum falcatum* Went was also performed using DIBA technique where infected samples depicted dark blue precipitate on the nitrocellulose membrane due to the antigen-antibody reaction (Hiremath *et al*., 2004).

TABLE 1: ELISA values showing reaction of antiserum of *P. disseminata* with antigens of healthy and naturally infected som leaf samples

Morphotype	Antigen concentration at 40µg/L		Colour intensity on Nitrocellulose membrane	
	Healthy	Infected	Healthy	Infected
S1	0.084±0.021	0.890±0.015	+	++
S2	0.084±0.004	0.989±0.005	+	++
S3	0.080±0.011	0.898±0.011	+	++
S4	0.076±0.008	0.988±0.014	+	++
S5	0.082±0.011	1.108±0.002	+	+++
S6	0.095±0.03	1.123±0.009	+	+++
S7	0.087±0.003	0.998±0.006	+	++
S8	0.081±0.004	0.890±0.008	+	++

*Antisera used at 1:125 dilutions, absorbance taken at 405nm, '±' Standard error
Colour reaction '+' – Pink, '++' – Violet, '+++' – Deep violet

Leaves of two different morphotypes, S5 and S6, showing highest absorbance values in case of natural infection, were artificially inoculated with *P. disseminata* as described in materials and methods. Antigens were extracted at 24hr interval for 4 days. These antigens (40µg/L) were tested against *P. disseminata* antisera at 1:125 dilution Infections could be detected from 24hrs onwards in ELISA on the basis of higher absorbance values of infected leaf extracts in comparison to healthy leaf extracts (Table 2). Here we could see that detection of infection by ELISA was possible before the symptoms generally appeared on detached leaves after 3days of inoculation. In order to facilitate implementation of disease management strategies effectively, early and reliable detection of pathogen is important. Indirect ELISA format was employed for the detection of *Phytophthora infestans* causing the potato late blight disease even before the first appearance of visible symptoms (Narayanasamy, 2010), *Pestalotiopsis theae* causing grey blight disease in tea (Chakraborty *et al.*, 1996), *Exobasidium vexans* causing blister blight disease in *Camellia sinensis* (Chakraborty and Sharma, 2007) as well as *Macrophomina phaseolina* causing root rot disease of *Citrus reticulate* (Chakraborty *et al*., 2012). Chakraborty *et al*. (2009) also reported that PTA ELISA format could easily detect pathogen *Exobasidium vexans* in susceptible variety of *Camellia sinensis* (AV-2) as early as 24h after artificial inoculation whereas the disease symptoms were not visible before 12 days. Similar strategy can be used to detect grey blight pathogen in som plants at an early stage using this ELISA format.

Table 2: ELISA values showing reaction of antiserum of *P.disseminata* with antigens of healthy and artificially inoculated som plants at different time intervals

Morphotype	Time interval	Healthy	Inoculated
S5	24hrs	0.045±0.003	0.152±0.010
	48hrs	0.049±0.001	0.369±0.008
	72hrs	0.041±0.006	0.877±0.009
	96hrs	0.042±0.008	1.189±0.120
S6	24hrs	0.029±0.002	0.135±0.160
	48hrs	0.030±0.001	0.448±0.005
	72hrs	0.036±0.004	0.767±0.014
	96hrs	0.040±0.002	1.172±0.014

* Antisera used at 1:125 dilution, Antigen concentration at 40µg/L, absorbance taken at 405nm, '±' Standard error

Western Blot analysis

Western blot analysis using PAb of *P. disseminata* was also performed to develop strategies for rapid detection of the pathogen. For this total soluble protein of young mycelia was used as antigen source and SDS-PAGE was performed followed by probing with alkaline phosphatase conjugate. The bands on nitrocellulose membrane were compared with bands on SDS-PAGE. Bands of varying molecular weights was seen in SDS-PAGE but two bands of molecular weights around 68 and 65KDa respectively were seen on nitrocellulose membrane suggesting these two to be the respective epitope of the antibody (Fig 4). Hence the results suggest that Western Blot format can be used as a refined tool for detection of the pathogen. Kitagawa et al (1989) also used Western blot analysis to identify *F. oxysporum* f. sp. *cucumrium* among other *Fusaria* by first analysing the mycelia antigen of the pathogen on SDS-PAGE and then performing Western blot analysis using homologous PAb.

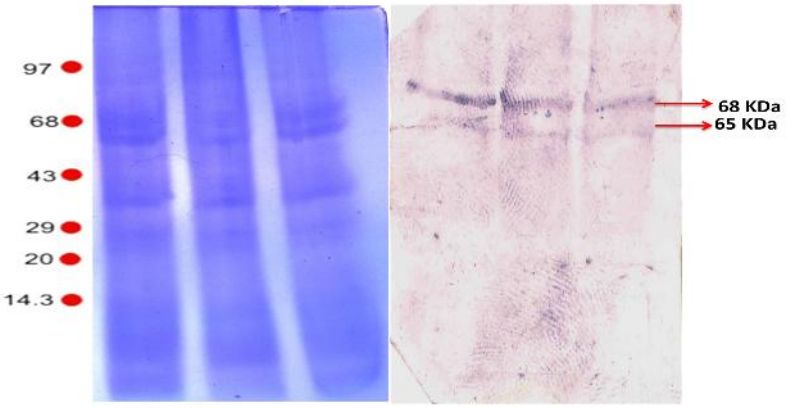

FIGURE 4: Western Blot analysis of protein profile (SDS-PAGE) of *P.disseminata* antigen with homologous PAb

Indirect immunofluoresecence assay

In the present study indirect immunofluorescence of young hyphae and spores of *P. disseminata* was carried out with homologous antibody and reacted with fluorescein isothiocyanate (FITC) labelled antibodies of goat specific for rabbit globulin. Strong apple green fluorescence was seen in both mycelia and spores which was confirmation of the homologous reaction of the pathogen and the antibody (Fig 5).

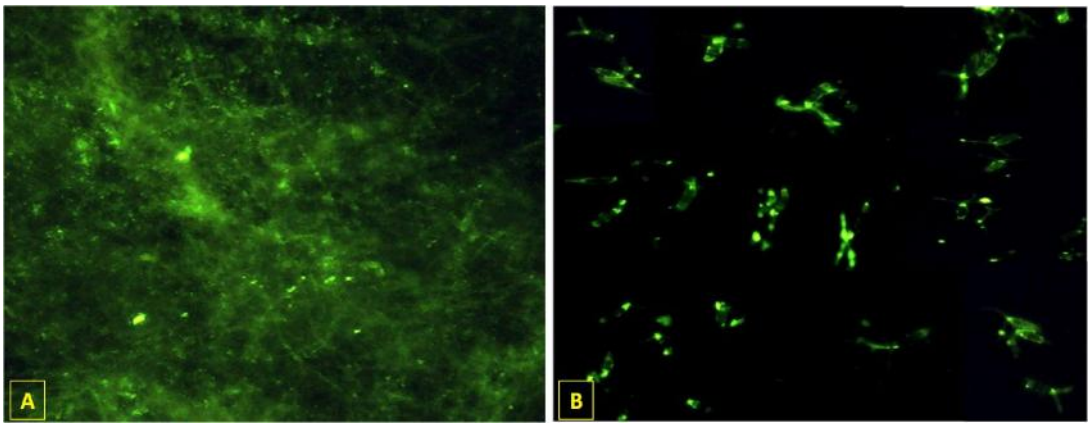

FIGURE 5: Indirect immunofluorescence of *P. disseminata* mycelia (A) and spores (B)

In case of *P. disseminata* spores only the setulae and appendages showed apple green fluorescence as the conidia are dark septate, confirming the identity of the pathogen. Chakraborty *et al*. (2012) conducted indirect immune fluorescence with young hyphae and sclerotia of *M.phasolina* with homologous PAb to obtain apple green fluorescence confirming the pathogen. Similarly, treatment of mycelia and conidia of *P. theae* with its own antiserum followed by FITC labelling developed a general fluorescence that was more intense on young hyphal tips and on the setulae and appendages of the conidia (Chakraborty *et al*., 1995). In this study indirect immunofluorescence of healthy leaf tissue segement with PAb of *P. disseminata* showed autofluorescence of the cuticle layer of the segment that indicates that pathogen is not present in the healthy section. Whereas in case of infected leaf section, apple green fluorescence is seen at the centre at the same time present in small quantities throughout indicating the spread of infection in the leaf (Fig 6).

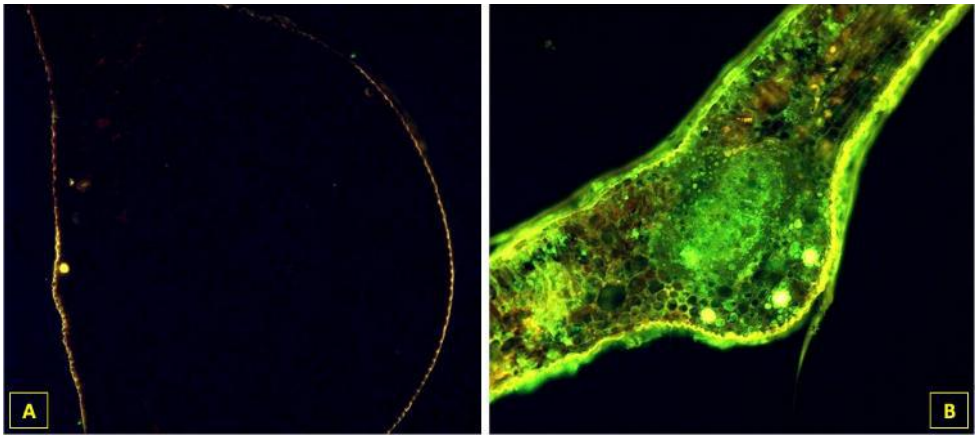

FIGURE 6: Indirect immunofluorescence of healthy leaf tissue (A) and blight infected leaf tissue (B) treated with PAb of *P. disseminata*

CONCLUSION

From the present study it can be concluded that serological techniques are easy, fast and reliable methods to detect grey blight pathogen in som plants. Early detection of pathogen will help in developing of management strategies against grey blight disease that will in turn help to improve the leaf quality and health status of som plant.

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Management of grey blight disease of Som plants using value added vermicompost with *Glomus constrictum* and *Bacillus altitudinis*

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Abstract

Grey blight disease caused by *Pestalotiopsis disseminata*, is one of the major foliar fungal diseases that constantly affects *Persea bombycina* Kost, a primary host plant of muga silkworm. Under nursery condition, grey blight disease was recorded mostly in S5 and S6 morphotypes of som plants. Vermicompost, PGPR and AMF, alone and in combination were applied for the improvement of the growth of eight morphotypes of som plant as well as to reduce the disease incidence. Growth in terms of height (cm), no. of leaves and no. of branches were studied. Analysis of some major defense related enzymes such as POX, PAL, CHT and GLU was also carried out to check induction of resistance after treatment. Artificial inoculation of som plants under nursery condition with spore suspension of *P. disseminata* was performed and disease progression noted for 7, 14, 21 and 28 days. It was clearly seen that disease progression was slow and less in treated inoculated plants. The results emphasize the fact that application of bioinoculants can be studied in larger scale for the upliftment of the health status of muga host plants.

Keywords: *Persea bombycina*, Vermicompost, PGPR, AMF, foliar fungal diseases.

Introduction

Persea bombycina, commonly called as 'Som' plant is an evergreen tree that belongs to the family Lauraceae. Som is the primary host plant of the silkworm *Antharea assamensis* that produces the golden yellow silk famously called as Muga silk. Cultivation of muga silk is an all year round practice in North Eastern India, mainly Assam. Som plants are mostly grown in the wilds. Hence these plants received very little attention of the scientific community and very less is known about their biochemical and genetic composition. High demand of muga silk has led to the domestication of som plants and rearing of silkworms in closed area. These plants are now-a-days grown significantly in West Bengal, mainly in Coochbehar disrict. 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.

A major problem in cultivation of healthy som plants that reduces the quantity and quality of the leaves are the various foliar fungal diseases of this plant**.** One of the major foliar fungal diseases of som plant is grey blight caused by *Pestalotiopsis disseminata* (Das *et al.,* 2010). These diseases are usually controlled through application of various fungicides. But application of fungicides and pesticides cause decline in the quality of the leaves. Use of bioinoculants along with vermicompost have caused decline in the disease incidence as well as improvement in the growth of plants in several cases that has been reported earlier by many researchers in the field (Sahni *et al.,* 2008, Theunissen *et al.,* 2010., Asciutto *et al.,* 2006). Vermicomposting is the simple biotechnological process by which organic material is consumed by earth worms and in the process of their digestion it enhances the process of degradation of the material and converts it into a nutrient-rich end product, called vermicompost. Vermicompost contains most nutrients in plant available forms such as nitrates, phosphates and exchangeable calcium and soluble potassium (Bhattacharjee *et al,* 2015). These help the plant to easily assimilate the required nutrition for its growth and development. Plant growth promoting bacteria (PGPR) can act as an added value. It is considered that as PGPR are soil borne bacteria they can easily mix well with the vermicompost and enhance the effect of the compost. Arbuscular mycorhizal fungi (AMF) are soil microbes forming symbiotic association with plant root system of all most all plant species. Chakraborty *et al* (2013) reported the presence of various AMF spores associated with the different morphotypes of som plants and effect of these spores of improvement of plant health. Keeping

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these findings in mind the following study was undertaken to understand the effect of vermicompost, PGPR as well as AMF, singly as well as jointly on the growth as well as disease establishment of the muga host plant.

Materials and methods

Plant sample

Eight different morphotypes of som plants (S1–S8) were collected from Boko, Assam and maintained under net house condition in Immuno-Phytopathology laboratory, Dept. of Botany, University of North Bengal.

Assessment of disease severity

The disease severity on the leaves was recorded on the basis of a 0-5 rating scale and calculated following the method of Chakraborty *et al* (2014).

Isolation of pathogen and morphological identification

The causative agent of grey blight disease was isolated from infected leaves on PDA slant after surface sterilization with 0.01% $HgCl_2$. The slants were incubated at 28°C for 5-7days till development of black acervulus. The spores were then observed under light microscope for identification of the pathogen. The fungal culture was further maintained in PDA slants.

Preparation and application of bioinoculants

Vermicompost was prepared in plastic beds using organic waste materials collected from the local area. 15-20 cm layer of this waste was covered with another 2-3 cm of dried aquatic plants. *Eisenia foetida*, the earthworm used for vermicomposting was added on the top. The final top layer was made of dried cow dung and the vermin bed was sealed with plastic cover. This set was kept undisturbed for 15-20 days, after which the bed was stirred and shaken to release the organic gas produced during vermicomposting process and for proper mixing of the materials. The compost was ready after 40-45 days when it turned into black light weight powder with no odour. After its completion the earthworms are separated from the final product and the manure was dried and sieved for further use.

The selected PGPR strain (BRHS/ P 73) of *B. altitudinus* was grown in nutrient broth for 48h and then centrifuged at 15000 rpm for 15min. The pellet obtained was re-suspended in sterile distilled water. The optical density of this suspension was measured using a UV-VIS Spectrophotometer at 600nm, to obtain a final density of 1×10^6 cfu/ml. For the preparation of bio-formulation, 10gm of Carboxy methyl cellulose (CMC) was added to 1 kg of talc and the pH was adjusted to 7 by adding calcium carbonate. They were sterilized by autoclaving at 15lbs.p.s.i for 30 mins at 120°C twice. 100ml of bacterial suspension was added to the talc formulation in a mass mixture and mixed for 5 mins. The resultant mixture is packed in polythene bags, labelled and kept at room temperature for further use.

The arbuscular mycorrhizal fungi (AMF) spores obtained from the rhizosphere of Som plants were mass multiplied in maize plant. The spores were washed several times with distilled water. They were then inoculated in roots of 7-10 d-old maize seedlings which were grown in Petri plates. After inoculation they were transferred to black plastic pots (30-cm) having autoclaved soil to eliminate other fungal propagules. The presence of AM spores was confirmed 45 d after inoculation. The maize roots were cut into small pieces and these shredded roots along with soil was added to the rhizosphere of the som plants for treatment.

The selected som plants for study were first treated with 200 g of vermicompost @per pot followed by a mixture of shredded roots and soil containing AMF. 200 g of talc based formulation of PGPR was then added to the treated pots. Aqueous suspension of selected PGPR was also sprayed on the leaves of som plant for three times with an interval of three days after each spray.

Inoculum preparation and application of pathogen

The grey blight pathogen *P. disseminata* was grown in 100ml PDA media in 250 ml flask for 10 days till black acervulus is formed. The spores are then scrapped off the surface of the medium with the help of inoculating media and collected in sterile distilled water. This suspension was filtered through muslin cloth and the filtrate containing $2X10^6$ spores/ml was used further as inoculum source.

This suspension was mixed with few drops of Tween 20 and sprayed onto the leaves of bioinoculant treated as well as untreated potted plants. These plants were kept covered with moist polythene bags for 48hrs to provide adequate temperature for the spores to germinate and establish disease.

Evaluation of Growth

Growth promotion in terms of height, no. of leaves and no. of lateral branches was recorded after every 15 days in both treated and control plants.

Biochemical analyses of leaves

Determination of total soluble protein

Total soluble protein was extracted from the leaves using phosphate buffer (pH 7.2) and was estimated following the method of Lowry *et al.,* 1951.

Estimation of total phenol content of the leaf

Phenol was extracted from the leaves of som plants by boiling in 100% alcohol and crushing in 70% alcohol and filtered. The filtrate is used for estimation of total phenol content following the method of Mahadevan and Sridhar (1978).

Extraction and estimation of defense enzymes

Four major defense enzymes were estimated in the leaves of som plants following the treatments. Phenylalanine ammonia lyase (PAL) enzyme was extracted from the leaves using sodium borate buffer (pH 8.8) and Peroxidase (POX) was extracted from the leaves using phosphate buffer (pH 6.8). Both enzyme extracts were estimated following the method of Chakraborty *et al* (1993). Chitinase and β-1,3-Glucanase both were extracted from leaves using acetate buffer (pH 5). Chitinase was assayed from the enzyme extract following the method of Boller and Mauch (1988) and Glucanase was assayed following the method of Pan *et al* (1991).

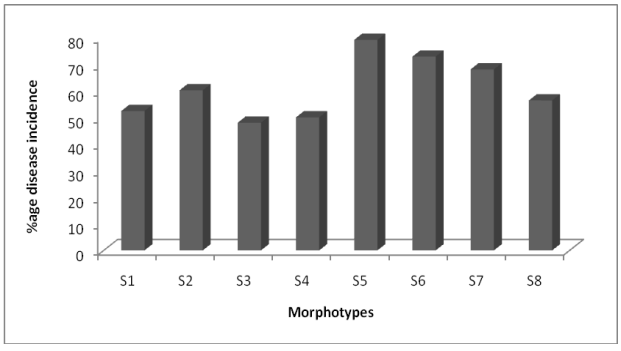

Fig 1: Percent disease incidence in leaves of som plants under nursery condition

Result

Analysis of disease occurrence in nursery condition

Under nursery condition presence of grey blight disease was recorded and percentage disease incidence (PDI) was calculated accordingly. It was recorded that establishment of disease was highest in S5 morphotype and lowest in S3 morphotype (Fig 1). So it can be assumed that morphotype S5 is susceptible to this disease.

Identification of the causal organism

The causal organism of the disease was isolated from the infected leaves in PDA slants. After proper growth, morphological examination showed the growth of white mycelia and presence of black acervulus, typical characters of *Pestalotiopsis* sp. Spores were examined under light microscope and based on the spore characters the organism was identified as *Pestalotiopsis disseminata* (Fig. 2).

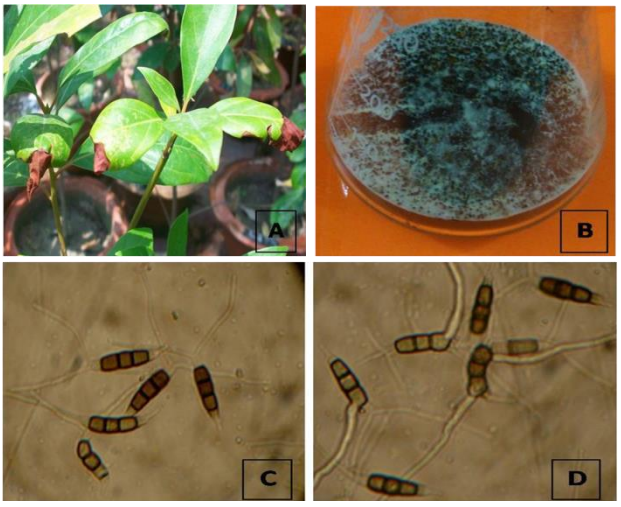

Fig 2: (A) Som plant showing symptom of Grey blight; (B-D) *Pestalotiopsis disseminata* - grown in PDA (B), Spores (C) and Germinated spores (D)

Effect of bioinoculants on growth

Application of different bioinoculants was carried out accordingly as outlined in materials and methods. Growth promotion in terms of height, no. of leaves and no. of lateral branches was recorded after 45 days of treatment. In all morphotypes, growth was significantly increased in treated plants in comparison to their respective control plants. Growth was observed to be highest in case of triple treatment of bioinoculants (Table 1, Fig. 3 & 4).

Quantification of different biochemical components of leaf

Total soluble protein was quantified in leaves of control and treated som plants where it was noticed that protein content increased in leaves following the treatments. However the content was more in case of dual treatment of Vermi and PGPR as well as triple treatment when compared to control and single treatment. Total phenol content also increased in leaves following treatment and it was recorded to be highest in S3 and S4 morphotypes. (Fig. 5).

Fig 3: Growth promotion of som plants in glass house conditions after 45days of treatment with *Bacillus altitudinus*

Fig 4: Growth promotion in som plants after 45 days of treatments with bioinoculants (Row 1 Vermi+*B. altitudinus*+ *G. constrictum*, Row 2- Vermi+*B. altitudinus*, Row 3- Vermi, Row 4- *B. altitudinus*, Row 5- *G. constrictum* and Row 6- control)

Table 1: Growth promotion in som plants after 45 days of treatment with bioinoculants

Morphotype	Treatment	Height (cm)	No. of leaves	No. of Branches
S1	Control	29	22	3
	Vermi	54	60	5
	G. constrictum	66	52	6
	B.altitudinus	64	35	5
	B.altitudinus+Vermi	97	35	4
	Vermi+*G.constrictum+B.altitudinus*	99	63	8
S2	Control	28	10	1
	Vermi	59	52	4
	G. constrictum	31	42	5
	B.altitudinus	31	43	6
	B.altitudinus+Vermi	69	32	4
	Vermi+*G.constrictum+B.altitudinus*	59	33	6
S3	Control	28	25	3
	Vermi	67	77	8
	G. constrictum	69	51	6
	B.altitudinus	67	55	8
	B.altitudinus+Vermi	94	62	6
	Vermi+*G.constrictum+B.altitudinus*	67	63	7
S4	Control	26	15	2
	Vermi	50	76	5
	G. constrictum	50	63	5
	B.altitudinus	49	62	4
	B.altitudinus+Vermi	95	65	8
	Vermi+*G.constrictum+B.altitudinus*	54	64	9
S5	Control	31	32	3
	Vermi	72	46	5
	G. constrictum	71	57	7
	B.altitudinus	70	55	6
	B.altitudinus+Vermi	93	50	5
	Vermi+*G.constrictum+B.altitudinus*	73	53	6
S6	Control	14	16	0
	Vermi	48	55	4
	G. constrictum	55	58	4
	B.altitudinus	56	59	3
	B.altitudinus+Vermi	71	52	4
	Vermi+*G.constrictum+B.altitudinus*	60	54	5
S7	Control	30	18	2
	Vermi	48	31	3
	G. constrictum	40	40	4
	B.altitudinus	41	43	5
	B.altitudinus+Vermi	82	55	8
	Vermi+*G.constrictum+B.altitudinus*	49	56	9
S8	Control	25	7	0
	Vermi	64	54	4
	G. constrictum	61	60	8
	B.altitudinus	62	63	6
	B.altitudinus+Vermi	68	53	3
	Vermi+*G.constrictum+B.altitudinus*	63	55	5
CD (P=0.05)	Treatment	9.308	8.890	1.395
	Morphotype	10.748	10.265	1.611

Assay of different defense enzymes

Four major defense enzymes were studied in leaves of som plants following different treatments. It was observed that PAL, POX, CHT as well as GLU increased in all treatments than in control sets. Highest increased was seen in dual and triple treatment irrespective of morphotypes. (Fig 6)

Artificial inoculation of the pathogen and disease establishment

As disease incidence under nursery condition was highest in S5 followed by S6 morphotype, these two particular morphotypes were taken for further study. After treatment with bioinoculants, treated as well as healthy plants were inoculated with spore suspension of the pathogen and percent disease incidence (PDI) was recorded after 7,14,21 and 28 days of inoculation. It was observed that disease incidence was much less in treated inoculated plants in comparison to untreated inoculated (UI) plants (Table 2). Among the various treatments a consistent decrease in disease incidence was observed in plants treated with PGPR followed by Vermi + PGPR treatment when compared to the untreated inoculated plants. It was seen that disease progression in treated plants were very slow when compared with untreated plants.

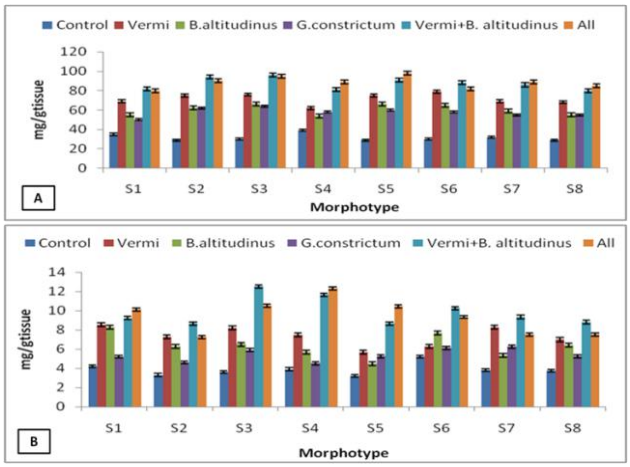

Fig. 5: Total soluble protein (A) and Total phenol (B) contents in som plants following different treatments

Discussion

In the present study growth of all the eight morphotypes was noted following treatment but combined application of vermicompost, PGPR and AMF showed the best result. In an earlier study by Bhattacharjee *et al* (2015) it was found that maximum enhancement of growth and yield was observed in tomato plants treated with vermicompost alone followed by vermicompost along with all microorganisms and then vermicompost with plant growth promoting bacteria (PGPR). An integrated approach by using vermicompost and a strain of *Pseudomonas syringae* (PUR46) containing plant growth promoting traits was adopted under green house condition by Sahni *et al* (2008) where it was recorded that 25% vermicompost and seed bacterization of *Cicer arietinum* resulted in an increased plant growth and also reduced plant mortality against Collar rot disease of chickpea. Pathak *et al* (2003) reported that height of guava plant was stimulated by different bioinoculants (PGPR, VAM, Azotobacter) in combination with farm yard manure as well as vermicompost. VAM inoculation with vermicompost also positively affected number of leaves per plant.

Table 2: Percent Disease Incidence (PDI) in S5 and S6 morphotype after artificial inoculation with *P. disseminata*

Treatments	S5 morphotype				S6 morphotype			
	7d	14d	21d	28d	7d	14d	21d	28d
Untreated Inoculated	25.3±0.82	35.6±0.65	68.2±0.42	85.2±0.25	21.2±0.12	29.6±0.22	55.2±0.21	72.5±0.32
Vermicompost	16.2±0.42	25.6±0.62	36.5±0.22	44.2±0.29	14.2±0.11	22.6±0.23	30.5±0.36	39.2±0.35
B. altitudinus	12.5±0.45	20.3±0.35	28.6±0.26	30.5±0.32	17.5±0.24	14.3±0.12	22.6±0.24	28.5±0.36
G. constrictum	18.5±0.84	30.2±0.25	39.6±0.45	48.2±0.25	20.5±0.32	18.2±0.19	29.6±0.24	35.2±0.34
Vermi+*B.altitudinus*	10.3±0.65	15.3±0.28	20.2±0.48	30.5±0.31	12.3±0.15	15.3±0.18	21.2±0.25	25.5±0.24
Vermi+*B.altitudinus*+ *G.constrictum*	15.2±0.52	19.5±0.56	25.5±0.52	29.5±0.36	11.2±0.16	17.5±0.16	22.5±0.32	27.5±0.48

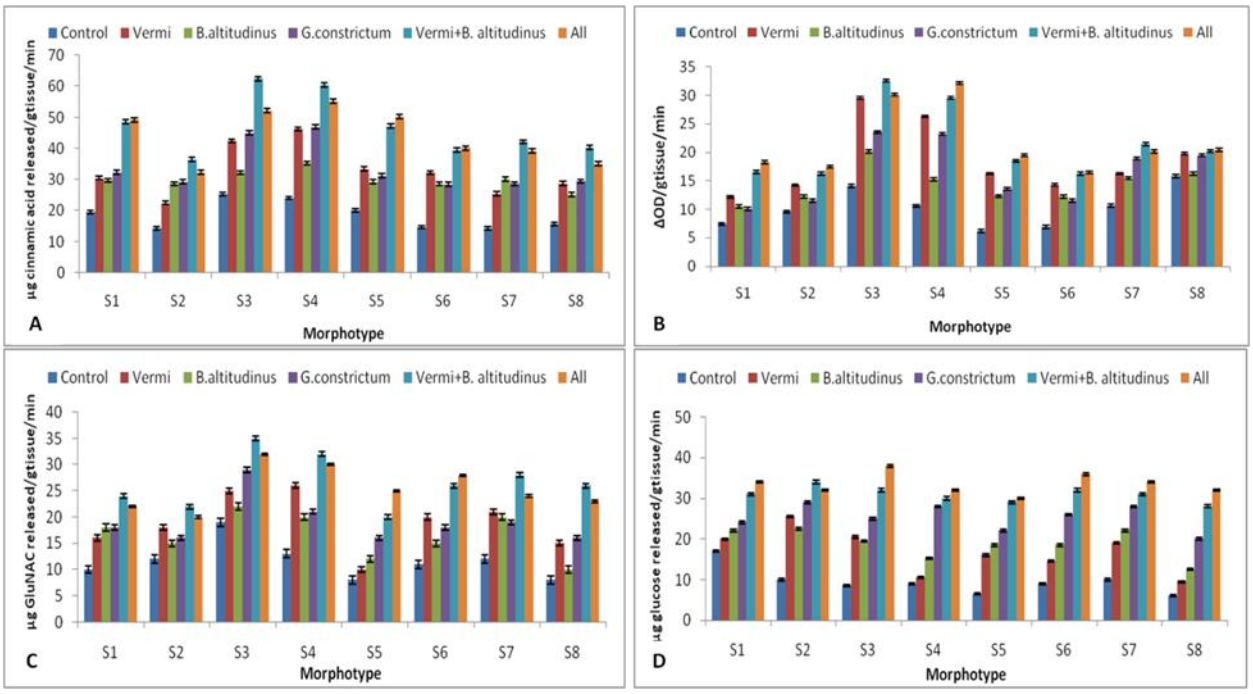

Fig. 6: Activities of defense enzymes (A) PAL, (B) POX, (C) CHT and (D) GLU in leaves of som plants following treatment

Patil (2010) reported that a combined treatment of biofertilizer and chemical fertilizer increased chlorophyll, growth, carbohydrates and proteins content in *Stevia rebaudiana* Var Bertoni compared to control. Similarly, in our study it was observed that treatment with vermicompost and other biofertilizers increased the protein and phenol content in treated plants than in control. However no treatment with chemical fertilizers was carried out.

In an earlier study by Chakraborty *et al,* 2014, it was recorded that dual application of AMF and PGPR increased phenolics as well as other defence enzymes in som plants. Treatment with these bioinoculants also decreased disease incidence in som plants artificially inoculated with *Colletotrichum gleoesporioides*, the causal agent of leaf blight disease of the plant. Similar results were also observed in our study were different defense enzymes were increased following treatment with vermicompost and other bioinoculants. Decrease of disease incidence of grey blight in som plants was also noted in the present study following treatment and artificial inoculation. Therefore it can be concluded that the treatment of som plants with value added vermicompost can lead to sustainable agriculture of such plants related to sericulture.

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Biochemical changes in *Persea bombycina* following infection with *Colletotrichum gloeosporioides*

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Colletotrichum gloeosporioides was isolated from the blight infected blighted leaves of *Persea bombycina* Kost which was confirmed by Koch's postulate as well as by bright field microscopy of the spores. Diffusates were collected from detached leaves inoculated with the pathogen and effect of these diffusates on spore germination and appressoria formation by the spore were observed. Quantification of total and O-dihydroxyphenol from healthy and infected leaves revealed that phenol contents were higher in the infected leaves. Activity of defense enzymes such as peroxidase (POX), phenylalanine ammonia lyase (PAL), chitinase (CHT) and glucanase (GLU) revealed that they were enhanced in infected leaves than in healthy leaves. TLC and HPLC analysis of phenolic compounds present in healthy and naturally infected leaves was also performed. HPLC analysis revealed the presence of Resorcinol, catechol and chlorogenic acid in both healthy and infected leaf samples but the height of these peaks increased in infected samples. In addition presence of two new peaks in infected sample could be identified as ferulic acid and salicylic acid. Results revealed that the pathogen triggered the production of resorcinol, catechol, chlorogenic acid, ferulic acid and salicylic acid in the muga host plant as biochemical defense strategy.

Key words: Leaf blight, *Persea bombycina*, *Colletotrichum gloeosporioides*, HPLC analysis

INTRODUCTION

Leaf blight disease is one of the major foliar fungal diseases of *Persea bombycina* Kost, commonly known as "som" plant. The plant is mainly infected with the disease during the rainy and humid months.The symptoms appear as brown spots in the tip which moves towards the base of the leaf. The leaf withers, dries and finally falls off. This causes an estimated leaf yield loss of about 2932 kg/ha/yr. Since the leaves of this plant are vital for the feeding and growth of the silkworms, they should be maintained properly and the disease should be irradicated. But management of disease is not easy without the proper knowledge of the biochemical constituents of the plant as well as the details of the causal organism. Proper knowledge will help in formulating the correct management practise. C*olletotrichum gloeosporioides* Penz can attack leaves, petioles and blooms during periodsof prolonged leaf moisture and high humidity. The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important post-harvest pathogens (Chowdappa *et al*, 2012). In this study attempts have been made to determine biochemical changes in leaves of som plants following infection with *C. gloeosporioides* causing leaf blight disease.

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MATERIALS AND METHODS

Plant material

Eight morphotypes (S1 – S8) of Som plants (which are the genotypes of PB001-PB008) were collected from Central Muga Eri Research and Training Institute (CMER&TI),

Jorhat, Assam for our study which are being maintained under net house condition as well as in experimental field of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. These common genotypes are classified based on their leaf shape like Ampotia (Morphotypes S1 and S2), Nahorpotia (S3), Jampotia (S4), Belpotia (S5), Kothalpotia (S6) etc.as mentioned in their website (http://cmerti.res.in/faq.html).

Isolation and identification of pathogen

The pathogen was isolated from infected leaf of som plants showing typical symptoms of leaf blight in potato dextrose agar (PDA) medium and the fungal culture thus obtained was purified using hyphal tip method in PDA slants. These slants were maintained for further studies. For light microscopic studies, hyphae and spores were stained with lactophenol-cotton blue.

Preparation of spore suspension

The fungal pathogen was grown in PDA medium in 100 ml flask till sporulation occurred. The spores were then scrapped off using sterile distilled water from the surface of the medium, filtered through muslin cloth and finally spore concentration was measured by hemocytometer. The concentration was approximately $3x10^4$ spores/ml.

Completion of Koch's postulate

Koch postulate was confirmed following detached leaf inoculation technique as described by Chakraborty *et al,* (1995). The disease assessment of the leaves was carried out by the process described by Chakraborty and Saha (1994).

Assessment of disease incidence

The occurrence of blight disease was recorded in case of all the eight morphotypes under nursery condition and the disease incidence was calculated according to the method of Chakraborty *et al,* (2014).

Collection of leaf diffusates and their bioassay

Leaf diffusates were obtained by a modified drop diffusate technique of Muller (1958). Forty young leaves, each of 8 different morphotypes, were collected from net house, washed and placed on moist filter paper in separate trays. Wound (1mm dia scratch) was made on the adaxial surface of the leaves using needle and 20 µl droplets of spore suspension of *C. gloeosporioides* was placed on the scratch (4-6 per leaf) using Pasteur pipette, the trays were covered with glass plates properly sealed with petroleum jelly and incubated for 48 hours. In case of control, distilled water was placed on the scratch in place of spore suspension. Drops of spore suspension were collected from each leaf of the eight morphotypes separately, centrifuged and supernatants collected. These were passed through sintered glass filter and their biological activities were assayed on spore germination and appressoria formation (Chakraborty *et al*, 1995).

Analysis of defense enzymes

Four major defense enzymes of plants were assayed during infection with healthy and naturally infected leaf samples. The leaf samples of healthy and infected plants were crushed using different buffers specific for the different enzymes, centrifuged and the supernatant collected which was used as crude enzyme source. Phenylalanine Ammonia lyase (PAL) as well as Peroxidase (POX) was performed following the method of Chakraborty *et al,* (1993).Chitinase was assayed following the method Boller and Mauch (1988) and â-1,3Glucanase was assayed following the method of Pan *et al*, (1991).

Isozyme analysis of peroxidase by PAGE

The healthy and infected leaves were used to prepare the enzyme extract in 0.1 M sodium phosphate buffer (pH 7). Polyacrylamide gel electrophoresis was performed according to the method of Davis (1967), followed by staining of the gel using Benzidine dye and hydrogen peroxide.

Estimation of phenols

Total Phenol: For estimation of total phenol, Folin Ciocalteau's method as described by Bray and Thorpe (1954) was followed. One ml of alcohol extract was taken, 1ml of Folin Ciocalteau was added and 2ml of Na_2CO_3 was added. The tube was shaken and heated in a boiling water bath for 1min , cooled and finally the volume was raised to 25ml. Quantity of total phenol was measured at 515 nm and calculated using caffeic acid as standard.

Ortho-dihydroxy Phenol: This was estimated by

addition of 1ml of alcohol extract, 2ml of 0.05 N HCl, 1ml of Arnows reagent and 2ml of 1N NaOH. The final volume was raised to 25 ml and the absorbance was recorded at 515 nm.

Thin Layer Chromatography of phenolic extracts

Thin layer chromatography technique was used to separate the different phenols present in healthy and infected leaves. Extraction of the sample was done following the method described by Neog *et al.* (2011), where the sample was extracted twice using petroleum ether and dichloromethane. After concentrating, it was dissolved in methanol and run in TLC plates using two different solvent systems, Acetic-acid and chloroform (1:9) and Ethyl-acetate and benzene (9:11).

HPLC analysis of phenolic compounds

Fresh leaves of som plant were chopped into pieces and soaked overnight in methanol in the ratio 1:3 (w/v), filtered through Buckner's funnel and the solvent was evaporated using lyophilizer as described by Pari and Latha (2004). The dried powder was finally mixed in HPLC graded methanol and stored at 4°C for further analysis. HPLC analysis of phenolic compounds present in the extracts was done using SPD-10A VP Shimadzu UV-VIS Detector. A flow rate of 1 mL/min, and gradient elution of acetonitrile-water-acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0– 50 min solvent B from 0 to 100%; and injection volume of 20 ìl were applied; whereas the separation of compounds was monitored at 280 nm (Pari *et al*, 2007).

RESULTS AND DISCUSSION

Assessment of Disease incidence

Maximum blight infection was noted during the month of April-August (Figure 1A). The disease incidence was recorded in nursery condition for all the eight morphotypes. Results (Table 1) revealed that the percentage disease index was lowest in S7 morphotype and highest in S3 and S6morphotype.The pathogen isolated from infected som leaf was identified as *Colletotrichum gloeosporioides* based on the spore characters, germination behaviour, appressoria formation (Fig-1 C-D) followingcompletion of Koch postulate using detached leaf technique (Fig 1B) and finally confirmed by 18S rDNA sequencing which has been deposited in NCBI data base (KM491736).

Table 1 : Percentage disease index of leaf blight disease in different morphotypes of som plants under green house condition

Morphotype	Percent disease index
S1	48.1±0.02
S2	42.5±0.01
S3	60.3±0.008
S4	50.1±0.007
S5	52.5±0.01
S6	68.2±0.006
S7	35.32±0.02
S8	56.5±0.008

Effect of leaf diffusates on spore germination and appressoria formation

The diffusates were collected from leaves of all morphotypes of som plants showing variable degree of susceptibility towards the disease. Their effect on spore germination and appressoria formation was tested *in vitro*. The diffusates from all morphotypes were fungitoxic (Table 2) but the activity of the diffusates from S7was higher than those from S3 and S6.

Assay of defense enzymes in healthy and infected leaves

Levels of four major defense enzymes or PR-proteins were assayed in healthy and infected leaves where it was found that levels of these enzymes were significantly increased in infected leaves in comparison to healthy leaves. However the level was much higher in infected leaves of S7 morphotype and less in S6 leaves the results are shown in Figure 2.

Peroxyzyme analysis revealed the presence of a new band in infected leaf samples corresponding to Isozyme 1 being produced in infected leaves and not in healthy samples, shown in Figure 3. Isozyme 2 was produced in both healthy and infected samples.

Estimation of phenol content in healthy and infected leaves

Total and ortho-dihydroxyphenol content of healthy and infected leaves were estimated following the

Table 2: Effect of leaf diffusate of different sommorphotypes on spore germination and appressoria formation of *C. gloeosporioides*

Morphotype	Spore germination[a], %	Inhibition of spore germination, %	Appressoria formation[a], %	Inhibition of appressoria formation, %
Control	83.5±1.2	-	65.0±1.8	-
S1	9.5±1.6	89	4.2±1.6	94
S2	6.5±1.3	92	0	100
S3	43.2±1.4	48	20.5±1.2	68
S4	24.2±1.8	73	8.3±1.1	87
S5	39.5±1.1	53	13.8±1.6	79
S6	45.0±1.5	46	24.6±1.8	62
S7	4.5±1.2	94	0	100
S8	35.2±1.6	58	10.2±1.1	84

[a]Average of 200 spores

above mentioned procedure and it was recorded that both the phenol content increased in infected leaves than their healthy counterparts but the level of phenol changes varied among the different morphotypes. It was observed that phenolics were highest in infected leaves of S7 morphotype and lowest in S6 morphotype (Table 3)

Analysis of phenols using thin layer chromatography

Analysis of phenols through TLC showed the presence of blue coloured spots with different Rf value when sprayed with Folin Ciocalteau's reagent as well as applying ammonia fumes. In infected samples two different spots were prominent at Rf 0.85 and 0.21 whereas two spots at Rf 0.72 and 0.15 were evident in healthy leaf samples.

Analysis of phenolic acids using HPLC

Phenolic acids present in healthy and infected leaves were further analysed using HPLC. Peak 1,2 and 3 are present both in healthy and infected leaves but the height of these peaks increased markedly in infected leaves in comparison with healthy leaves indicating an increase of these phenolic acids following infection. Peak 4 is present in healthy sample but disappeared in infected leaf samples. It is interesting to note that five new peaks viz 5,6,7,8 and 9 were evident in infected samples but all these peaks were absent in healthy leaf samples indicating that these phenolic acids might play an important role in defense mechanism of the plant against infection. When compared to standard phenolic acid it was confirmed that Peak 1, 2, 3 and 4 represented Resorcinol, Catechol, Morin and Chlorogenic acid respectively whereas peak 5 and 7 represented ferulic acid and peak 6 represented salicylic acid. (Table 4, Figure 4). Minor Peaks 8 and 9 were present only in infected samples and not in healthy samples; when compared with authentic phenolic acids, these two minor peaks could not be identified.

In the present study it was seen that almost all the morphotypes of som plants were infected with leaf blight under nursery condition. However morphotype S7 was least infected and morphotype S6 and S3 were highly infected. After isolation and microscopic study, *Colletotrichum gloeosporioides* was found to be the causal agent. Completion of Koch postulate also validated our findings. Das *et al*. (2005) reported the occurrence of *Colletotrichum* infection in Assam.

Table 3 : Total and Ortho-phenol content in healthy and infected leaves

Morphotype	Total Phenol Content (mg/g tissue)		Ortho-phenol Content (mg/gtissue)	
	Healthy	Infected	Healthy	Infected
S1	3.90±0.12	5.60±0.15	3.35±0.05	7.25±0.03
S2	2.50±0.36	5.20±0.11	4.80±0.03	8.50±0.03
S3	2.90±0.22	3.20±0.11	2.25±0.05	3.25±0.02
S4	3.60±0.16	4.50±0.12	4.25±0.04	6.25±0.05
S5	2.80±0.19	4.20±0.16	3.25±0.03	5.25±0.01
S6	2.20±0.11	2.50±0.14	3.36±0.02	2.75±0.04
S7	3.25±0.12	6.65±0.13	6.00±0.04	10.50±0.01
S8	3.29±0.14	4.80±0.11	4.63±0.05	5.25±0.01

Diffusates collected from all morphotypes of som plants inhibited spore germination but the diffusates from less infected morphotypes were much more effective than highly infected morphotypes. Earlier Chakraborty *et al,* (1995) also reported that diffusates of phenolic nature collected from susceptible and resistant varieties of *Camellia sinensis* to *Glomerella cingulata* inhibited spore germination and appressoria formation. These findings suggest the presence of some metabolites in the diffusates that is fungitoxic in nature and can be used by the plants for their innate immunity against the fungal pathogen.

Table 4: Retention time and peak heights of phenolics from healthy and infected leaf sample of S7 morphotype analyzed by HPLC

Sample	Peak no	Retention time	Height
Healthy	1	9.960	79.284
	2	13.310	190.922
	3	14.890	508.657
	4	21.400	42.346
Infected	1	9.990	339.562
	2	13.250	698.064
	3	14.720	945.181
	5	25.650	237.487
	6	26.680	491.245
	7	29.340	89.340
	8	30.820	68.346
	9	34.890	46.420

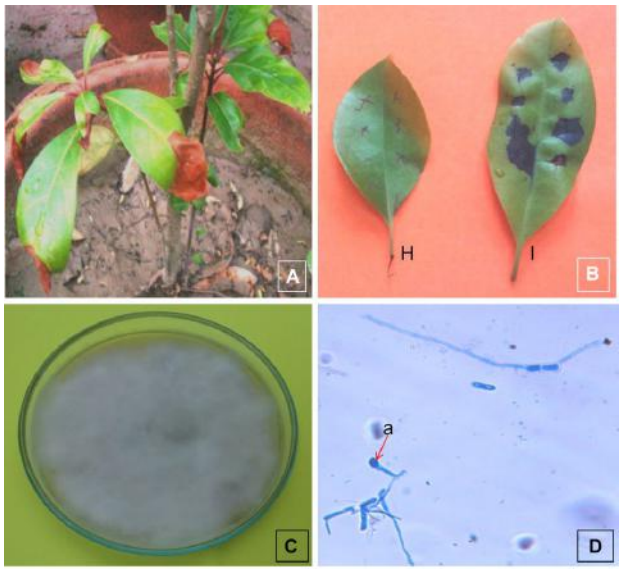

Fig 1: (A) – Leaf blight of Som plant, (B) – Untreated Healthy (H) and artificially inoculated leaf (I), (C) Mycelial growth of *Colletotrichum gloeosporioides* on PDA medium, (D) – Spore germination and appressoria formation(a) of *C. gloeosporioides*

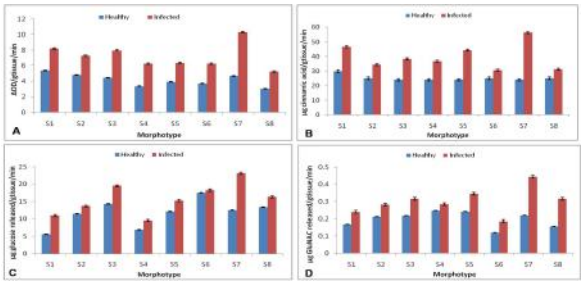

Fig 2: Activities of defense enzymes in healthy and blight infected leaves; A – Peroxidase, B – Phenylalanine ammonia Lyase, C – Chitinase, D – â-1,3 Glucanase

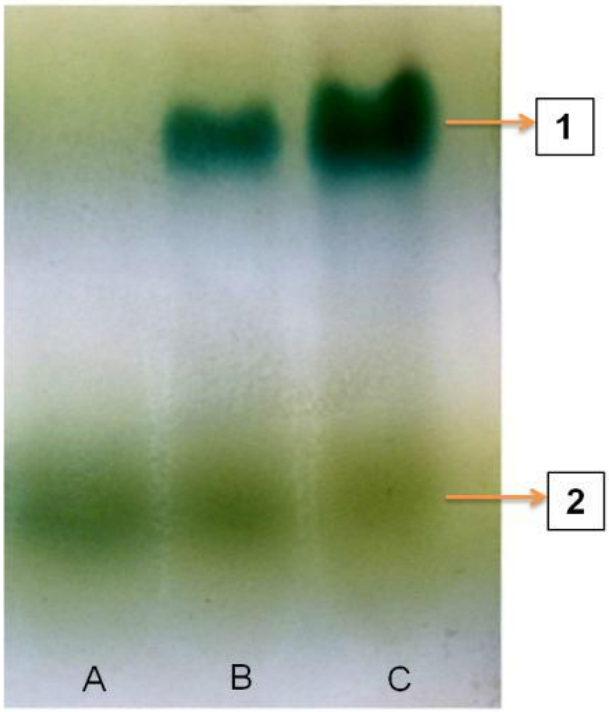

Fig 3: Peroxyzyme analysis of healthy and infected leaves of S7 morphotype in native PAGE. Lane A- Healthy, Lane B-C – Infected

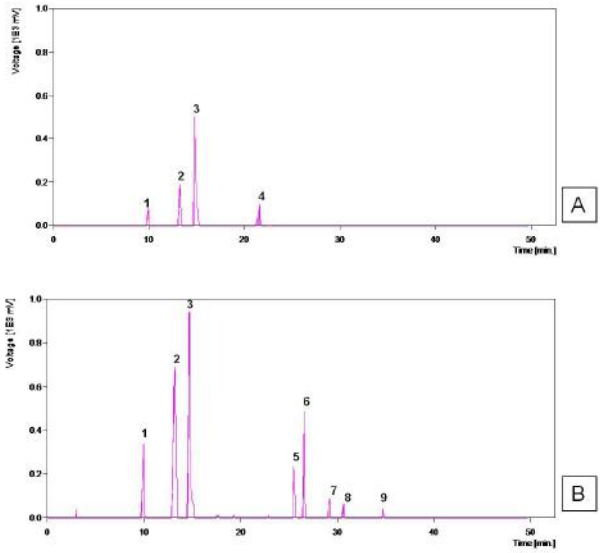

Fig 4: HPLC analysis of phenolic acids in leaves of S7 morphotype (A – Healthy, B – Infected leaf; 1 – Resorcinol, 2 – Catechol, 3 – Chlorogenic acid, 4 – Morin, 5 and 7 – Ferulic acid, 6 – Salicylic acid).

In connection with the disease establishment in the plants, different defense enzymes were also analysed and it was recorded that defense enzymes like PAL, POX, CHT and GLU showed an increased activity in infected samples. Moreover their activity was much higher in plants showing less disease index. Correlation of this result was also made with the study undertaken by Parihar *et al.* (2012) where it was seen that biochemical analysis of genotypes of *Brassica juncea* infected with *Alternaria* blight revealed an increase in PAL, PPO and peroxidase activity. Singh *et al.* (2014) reported that preformed phenolic compounds as well as Peroxidase enzyme play important role in resistance of Chili against *Colletotrichum capsici.*Analysis of peroxidase isozymes by polyacrylamide gel electrophoresis showed four isozymes in healthy tea leaf samples and five in tea leaves infected with *Exobasidium vexans*. They suggested that the appearance of new bands following infection can be correlated with the induction of the catalytic activity of more isozymes, leading also to an overall increase in peroxidase activity (Chakraborty *et al*, 2002). In the present study it was observed that total as well as ortho-dihyroxy phenol content increased in infected plants, more in plants with less infection. Infection by *Venturia inequalis* in apple caused an accumulation of phenolic compounds wherein Folin-Ciocalteu values increased by 1.4 to 2.4 fold (Petkovsek *et al*., 2008).Taware *et al,* (2010) reported that there was significant increase in total phenolic content of grape leaves due to foliar powdery mildew infection. These results are in accordance with the result obtained in the present study.Thin layer chromatography has been used by various workers to study the phenol profile of different plants like tea (Chakraborty and Saha, 1994), som (Neog *et al*, 2011), different medicinal plants (Maobe *et al*, 2012). Studies on secondary metabolites of som plant by Neog *et al.* (2011) revealed the presence of four major phenolic acids – chlorogenic acid, catechol, morin and gallic Acid. In the present study also HPLC analysis of healthy and blight infected som plants revealed the presence of Chlorogenic acid and catechol which was highly increase in infected leaf samples, indicating the function of this metabolite in defense mechanism of plant. Presence of chlorogenic acid as part of defense system has been studied by different workers in different crops, such as Potato tubers (Malamberg and Theander, 1985), apples (Petkovsek, *et al,* 2003), coffee (Rodrigues, *et al,* 2011) and tomato (Lopez-Gresa*et al.*, 2011). Presence of salicylic acid and ferulic acid in infected leaves and not in healthy leaves indicate the role of this phenolic acid in defense against pathogen. When biochemical charecterization of maize plants infected with *Drechslera dactylidis* was done, it was found that salicylic acid increased 2-fold in infected leaf samples. (Abdel Ghany 2012).

Thus in conclusion it can be said that, studies on effect of diffusates on spore germination and appressoria formation of *C. gloeosporioides* as well as HPLC profile of the phenolic acids in healthy and infected leaf samples shows presence of some metabolites in less infected plants that might have certain activity that helps the plant in resisting the disease establishment and progression. This might be the key in analysing the phytoalexin component of the som plant against various fungal pathogens which will help in strategising for integrated management of the disease.

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