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.