Molecular Biology in Plant Pathogenesis and Disease Management

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Microbial Plant Pathogens

Volume 1

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Contents

Table of Contents for Volumes 2 and 3

Volume 2

Volume 3

Preface

Molecular biology has emerged as one of the most important branches of biological sciences, helping them in achieving rapid and significant advancements. This branch has been able to provide the essential information on the basic cell behavior patterns such as growth, division, specialization, movement and interaction in terms of various molecules responsible for them. In the early stages of molecular biological research, simple organisms such as viruses and bacteria formed the preferred test materials. The realization that the function of whole organism can be fully understood, only when it is dissected and analyzed at molecular levels became widespread. Hence, the structure and function of nucleic acids (DNA and RNA) and proteins were extensively examined. It has been well demonstrated that individual molecules function generally as components of complex gene expression mechanisms in metabolic pathways or as structural elements invariably in concert with other molecules. Although the concept of the gene as a unit of hereditary information was introduced by Gregor Mendel, the term "gene" was coined later by Wilhelm Johansen to describe a heritable factor responsible for the transmission and expression of a particular trait. The one gene-one enzyme model paved the way for the finding that the gene is a length of DNA in the chromosome and it encodes the information required to produce a single enzyme. These early discoveries laid the foundation for the initiation of scientific inquiries to understand the structure and functions of genes in higher organisms including plants and mammals.

Interactions between biotic and abiotic factors and crop plants determine their health and consequent reproductive capacity. Plants, as they develop from seeds or propagative materials, encounter various kinds of microorganisms capable of favoring or adversely affecting their development. Microbial plant pathogens have evolved, over a period of time, strategies to overcome the defense responses of and to breach successfully different barriers formed by plant hosts, leading to initiation of infection and subsequent colonization of tissues of a compatible plant species. However, the plants also step up their defense-related activities, as soon as the presence of the pathogen is perceived, at different stages of plant-pathogen interaction by activating defense-related genes, leading to the formation of structural barriers and biosynthesis of antimicrobial compounds. Plant defense responses involve complex biochemical pathways and functions of multiple signal molecules.

Studies on molecular biology of plant pathogens, infection process and disease resistance have provided information essentially required to understand the vulnerable stages at which the microbial pathogens can be effectively tackled and to workout novel strategies to incorporate disease resistance genes from diverse sources and/or to enhance the levels of resistance of cultivars with desirable agronomic attributes. New molecules, without any direct inhibitory effect on the pathogen, but capable of eliciting plant defense responses have been developed or discovered. Transgenic plants with engineered genes encoding viral coat proteins, HR-elicitors, such as harpins or overexpressing *R* genes or PR proteins, such as chitinases with enhanced levels of resistance to pathogens show promise for effective exploitation of this approach. It is possible to complement or replace the chemicals- fungicides and bactericides- by adapting new disease-management technologies emerging from the basic knowledge of plant-pathogen interactions at molecular and cellular levels. Constant, cooperative and comprehensive research efforts undertaken to sequence the whole genomes of plants and pathogens can be expected to result in the development of better ways to manipulate resistance mechanisms enabling the grower to achieve higher production levels and the consumer to enjoy safer food and agricultural products.

This book presents updated and comprehensive information in an easily understandable style, on the molecular biology of plant-pathogen interactions in three volumes: 1. Microbial plant pathogens, 2. Molecular biology of plant disease development and 3. Molecular biology in crop disease management. The usefulness and effectiveness of molecular techniques to establish the identity of pathogens precisely, to have a better understanding of the intricacies of the success or failure of pathogen infection respectively in compatible and incompatible plant species and to develop more effective crop disease management systems is highlighted with suitable examples. Appendices containing protocols included in appropriate chapters will be useful for students and researchers of various departments offering courses and pursuing research programs in molecular biology in general and plant pathology in particular.

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Chapter 1
Introduction

Agricultural and horticultural crop production is hampered by several limiting factors, of which diseases caused by microbial pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids – still remain a formidable one. Losses due to diseases are estimated to be about 30% in developing countries, whereas European and North and Central American countries may lose about 15–25% of the produce (James 1981; Pimentel 1997). A later assessment by Pimentel et al. (2000) indicated that losses caused by natural introduction of invasive plant pathogens and insect pests accounted for about $10 billions in the USA alone annually. The imperative need to save the crops from the attack of potential pathogens was realized and research efforts were intensified in the later half of the twentieth century. Various aspects of the pathogens and the diseases induced by them were investigated. The conventional methods involving cultural and microscopic methods provided the basic knowledge on plant-pathogen interactions leading to development of disease symptoms on compatible host plant species or cultivar. In contrast, the pathogen development was partially or entirely inhibited depending on the levels of resistance in incompatible interaction. Furthermore, epidemiological factors favoring disease incidence and spread under experimental and natural conditions were determined to develop disease prediction models and forewarning systems. Short-term and long-term strategies were planned based on the results of conventional techniques. However, the inherent limitations of the conventional methods necessitated to look for techniques with higher levels of precision and reliability (Narayanasamy 2002, 2006).

1.1 Molecular Biology as a Research Tool

Studies relating to molecular biology and biotechnology have provided an impetus to the rapid development of different branches of biological sciences, resulting in accumulation of information which could not have been gathered using conventional procedures. The importance of discovery that *Tobacco mosaic virus* (TMV) causing tobacco mosaic disease could be crystallized, was duly recognized by the award of Nobel Prize to Stanley (1935) and this work signaled the commencement

P. Narayanasamy, *Molecular Biology in Plant Pathogenesis and Disease Management: Microbial Plant Pathogens, Vol. 1*.

of application of molecular methods in Plant Pathology. Equally important was the finding of Bawden et al. (1936) that TMV was a nucleoprotein, opening up the field of Plant Virology and of research on virus diseases of humans and animals for molecular analysis. The presentation of crucial evidence for gene-for-gene interaction in flax-rust pathosystem by Flor (1942, 1946) established the concept that the genes conditioning the reaction of the host may be identified by their interaction with specific strains of the pathogen. On the other hand, those genes that condition pathogenicity may be identified by their interaction with specific host varieties. The concept of Flor, demonstrated to be true in several pathosystems, was hailed as one of the most important contributions during the last century, providing a firm footing for studying the host plant-pathogen interactions more critically.

Development of techniques for isolation, cloning and sequencing the deoxyribose nucleic acid (DNA) of various organisms including plants and pathogens marked the period of significant revolution in biological sciences. Braun and Pringle (1959) demonstrated that the crown gall bacterium *Agrobacterium tumefaciens* induced permanent transformation of plant host cells resulting in the autonomous and rapid growth of the transformed cells in culture. The *A. tumefaciens*-mediated transformation system is still widely adopted for transforming plants with genes from diverse sources. *Arabidopsis thaliana*, a small dicotyledonous cruciferous plant species has been adopted as a model species for most aspects of plant biology, because of its small genome size, short life cycle, small stature and ability to produce large number of progenies. These attributes have made this plant species ideally suited for genetic and mutational analyses. *Arabidopsis* has been shown to have a relatively high proportion of genes involved in metabolism and defense. Many of the physiological processes investigated in *Arabidopsis* and crop plants appear to share many common features, especially related to disease and pest resistance and salt tolerance. As the genome of *A. thaliana* has been fully sequenced, it provides the ideal tool to have a more clear insight into the molecular bases of various physiological processes, including resistance to diseases caused by microbial pathogens. However, the conclusions arrived at based on experiments performed on *A. thaliana* need to be translated into practical outcomes in various crops. Application of genomics to molecular resistance breeding is considered as the most important area of promise, leading to development of crop cultivars with built-in resistance to one or more diseases (Slater et al. 2003; Lucas 2004).

Some of these early breakthrough-findings obtained by applying the molecular techniques revealed the effectiveness and potential of these approaches. Evidences in support of numerous suggestions and hypotheses proposed earlier have been obtained by using molecular methods. These methods have been shown to be very useful to investigate various aspects of plant-pathogen interaction resulting in either disease progress or restriction due to effective elicitation of host defense responses nullifying the adverse effects of the pathogen-derived products. Interaction between avirulent strains of pathogens and resistant hosts generally results in hypersensitive response (HR). Bacterial pathogens and their host plant species have been shown to be preferable systems for molecular approaches. An avirulence gene *avrA* from a race 6 strain of *Pseudomonas syringae* pv. *glycinea* was cloned. This avirulent

gene, when transferred to other races, conferred the ability to elicit a resistance response on soybean cultivars with *Rpg 2* gene for resistance (Alfano and Collmer 1996). By cloning of virulence and *hrp* genes from bacterial pathogens, significant progress was made in understanding various phenomena relating to pathogenesis such as virulence, plant recognition and host range in many pathosystems. The discovery of genes involved in the production of host-specific toxins (HSTs) by fungal pathogens elucidated the molecular bases of symptom induction and resistance to the pathogens producing the HSTs (Durbin 1981; Baker et al. 2006). Furthermore, the effectiveness and reliability of the molecular techniques in hastening the application of different disease management strategies particularly for the development of cultivars with built-in resistance to diseases caused by microbial pathogens has been well recognized as a significant advantage over conventional methods.

1.2 Application of Molecular Techniques

The characteristics of microbial pathogens have been studied to identify and to classify them into different classes, families, genera and species based on morphological variations and this approach was primarily applied in the case of oomycetes and true fungi. Biochemical studies are also required for classifying bacterial pathogens, since the variations in the morphological features alone are not enough to differentiate genera and species. As the viral pathogens are extremely small in size, the virus particle morphology offers no dependable basis for differentiation, necessitating the use of molecular biological approaches for their detection and differentiation. Even in the case of fungal pathogens differentiation based on morphological characteristics may not be possible, if their development is affected by environmental conditions and the presence of other fast-growing saprophytic microorganisms. The effectiveness and applicability of molecular techniques in studying the pathogen characteristics, disease development and formulation of suitable crop disease management systems are discussed in three volumes that include ten chapters, in addition to this introductory chapter.

In Volume 1, the information on the molecular techniques to study the characteristics of microbial pathogens is presented. Rapid detection, precise identification and unambiguous differentiation of various microbial pathogens or variants of a pathogen species are of paramount importance to initiate effective strategies of crop disease management. The effectiveness of molecular techniques to meet this requirement is discussed in Chapter 2. The genetic diversity of plant pathogens has to be assessed to understand the different levels of pathogenic potential (virulence) of strains, races or biotypes of a pathogen species, so that the occurrence of more virulent strain(s) can be detected, identified and quantified rapidly (Volume 1, Chapter 3).

Various phases of disease development in susceptible plants under in vitro and factors influencing disease incidence and spread under in vivo resulting in occurrence of epidemics have been examined in detail by using molecular techniques.

The intricacies of host-pathogen interactions at molecular and cellular levels are elucidated in two chapters in Volume 2 of this treatise. It has been possible to visualize and monitor various steps from pathogen adhesion to tissue colonization and symptom expression during different phases of pathogenesis by applying suitable and sensitive molecular methods (Volume 2, Chapter 2). Factors influencing plant disease incidence and spread have been studied using conventional methods for identification and quantification of pathogen populations in epidemiological investigations. With the availability of molecular techniques and specific monoclonal antisera or primers that can amplify specific sequences of pathogen DNA to precisely identify pathogens up to strain/varieties level, it has been possible to determine the distribution and components of pathogen populations and spatial and temporal variations in pathogen populations, in addition to locating the different sources of inoculum (Volume 2, Chapter 3).

Management of crop diseases successfully is the ultimate aim to provide reasonable margin for the grower for his efforts to produce food to meet the requirements of the consumers who need food free of pathogens and their toxic metabolites or chemical residues. Volume 3 encloses six chapters which provide information on the short- and long-term disease management strategies that can be made effective by application of appropriate molecular methods. It has been well demonstrated that molecular assays are highly efficient in detecting and identifying pathogens present in plant materials that may or may not exhibit symptoms of infection by pathogens of quarantine importance. Application of these techniques can effectively prevent introduction of exotic pathogen(s) that may find suitable conditions for development and spread in new geographical location(s) (Volume 3, Chapter 2).

Use of crop cultivars with built-in resistance to different diseases is considered as the economical and ecologically safe strategy of disease management. Locating disease resistance genes not only in plants, but also in diverse sources including insects and rapid selection of resistant genotypes or lines using genetic markers at early growth stage, have been possible due to the application of appropriate molecular methods. Furthermore, the visual scoring methods to assess the disease intensities exhibited by different genotypes have not provided consistent results, because environmental factors are likely to influence the symptom expression. In contrast, molecular methods employed to determine the quantum of pathogen DNA have proved to be more accurate, reliable and rapid (Volume 3, Chapter 3).

It is very difficult to transfer resistance gene(s) into cultivars from distantly related or unrelated plant species, because of the sterility of progenies associated with interspecific or intergeneric crosses. But biotechnological approaches have offered the possibility of transferring resistance gene(s) from plants and also from diverse sources such as fungi, insects, and frogs. Genetic engineering techniques have provided wide options which are not available, if conventional breeding procedures are to be followed. Pathogen-derived resistance (PDR) approach has been shown to be successful in developing virus disease resistant crop cultivars (Volume 3, Chapter 4).

Development of disease-resistant cultivars through genetic engineering technology has been attempted in the case of a few crops such as tomato, tobacco,

potato and rice, leaving out a large majority of crops untouched. Nevertheless, the possibility of enhancing the levels of resistance of desired cultivars by the application of effective inducers of resistance has been demonstrated to be a practical proposition. Both biotic and abiotic inducers have been tested on a wide range of agricultural and horticultural crops. As resistance inducers activate the natural disease resistance (NDR) mechanisms existing in the plants, it is apparent that the plants treated with inducers, behave like genetically resistant plants. Furthermore, the inducers act on plants, but not on the pathogens. Hence, the chances of development of resistance to these agents are remote, indicating the usefulness and practicability of this disease management strategy (Volume 3, Chapter 5).

Various fungal and bacterial species existing in the rhizosphere, phyllosphere or spermosphere have been found to have the potential of protecting plants against microbial pathogens. The molecular bases of protection to plants against microbial pathogens by the activities of biocontrol agents (BCAs) have been investigated. The genes controlling the production of antibiotics and enzymes capable of inhibiting the growth and development of the pathogen have been isolated, cloned and characterized. Efforts have been made to enhance the biocontrol potential of the selected BCAs by transferring genes from other microorganisms. In addition, monitoring the spread, persistence and survival of the introduced BCAs has been effectively carried out by using appropriate molecular techniques (Volume 3, Chapter 6).

Various kinds of chemicals have been used at different stages of crop growth and storage for the control of microbial pathogens causing different diseases in agricultural and horticultural crops and the produce. Although chemicals are able to reduce the disease incidence and spread significantly, the emergence of resistant or less sensitive strains of fungal and bacterial pathogens has been of great concern for the growers and the industry. Further, the growing awareness of the general public of the possible effects of pollution and persistence of residues, due to chemical application resulted in considerable difficulty in marketing the produce with higher levels of chemical residues. This situation led to restricted use or withdrawal of certain fungicides/chemicals from the market. Rapid identification and differentiation of resistant and sensitive strains and monitoring of appearance of new strains resistant to fungicides may be possible using molecular techniques that can detect the changes in the sequences of specific gene(s) of the pathogens (Volume 3, Chapter 7).

This book aims to provide the latest information to gain comprehensive knowledge on various aspects of plant-pathogen interaction leading either to development of symptoms induced by the pathogen or restriction of development and consequent elimination of the pathogen. The study of plant-pathogen interaction at cellular and molecular levels needs no emphasis, since molecular biological investigations have opened up the avenues that could not be accessed through conventional procedures. The information presented in this volume is expected to be useful for researchers, teachers and upper level graduate students, pursuing investigations in biological sciences in the Departments of Plant Pathology, Molecular Biology and Biotechnology, Microbiology, Biochemistry, Plant Physiology and Plant Breeding and Genetics and also personnel of Plant Quarantine and Certification Programs.

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Chapter 2
Molecular Techniques for Detection of Microbial Pathogens

Abstract Detection, identification and differentiation of microbial plant pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids – infecting various crops constitute the basic step for the development of effective crop disease management systems. The conventional cultural methods involving isolation and studying the morphological characteristics using microscope are labor intensive and cumbersome, yielding sometimes, inconclusive results. The molecular techniques, on the other hand, are able to provide precise, reliable and reproducible results rapidly, facilitating early disease management decisions. Biochemical, immunological and nucleic acid-based assays have been preferred, because of the distinct advantages over the conventional methods. The molecular techniques have been very useful in the identification of obligate pathogens causing diseases such as downy mildews, powdery mildews and rusts and also fungi that grow very slowly in the culture media, taking several weeks to produce spore forms that can be used for identification. The availability of antisera, primers and commercial kits has led to widespread application of the molecular techniques for on-site detection during field surveys for assessing the distribution of existing pathogens, occurrence of new/introduced pathogens or strains and for preventing introduction of new pathogens through seeds or planting materials. Furthermore, molecular techniques have been demonstrated to be useful in breeding programs to identify sources of resistance to disease(s). Several protocols for molecular techniques, useful for researchers and students are presented in the Appendix.

Depending on the levels of resistance/susceptibility of plant species to different microbial pathogens, the nature of the interaction between host and pathogen may vary. In a compatible (susceptible) interaction, development of symptoms characteristic of the disease may be observed, facilitating the identification of the pathogen inducing such symptoms. On the other hand, in an incompatible interaction (resistant/immune), the pathogen development may be hampered to different degrees resulting in the production of small necrotic (hypersensitive) flecks or complete absence of any visible symptom. In another type of interaction, the plant species is susceptible to the pathogen in question, but no symptom of infection may be discernible. This type of interaction known as latent infection is frequently seen

P. Narayanasamy, *Molecular Biology in Plant Pathogenesis and Disease Management: Microbial Plant Pathogens, Vol. 1*.

in the case of plants infected by viruses. These plants are known as symptomless carriers, posing potential danger for crop production. In yet another type of interaction designated quiescent infection, the pathogen remains dormant in infected immature fruits till they ripen. Such quiescent infections are seen in bananas and mangoes frequently. Nevertheless, the characteristic symptom may provide, however inaccurate it may be, a basis for the identification and differentiation of some microbial pathogens.

Disease diagnosis relates to the identification of the nature and cause of the disease, whereas detection deals with establishing the presence of causative target organism with the test sample. It has been found to be very difficult, frequently unsuccessful, if the symptom expression in a pathosystem is not clear. Further, if a host plant is subject to attack by many pathogens simultaneously or successively one after another, the symptom picture may be very different from those symptoms induced by pathogens individually. Reliability on symptoms alone may lead to erroneous identification of the causative agent(s). Rapid and precise identification of the cause of the disease is the basic requirement for the development of effective disease management systems. A wide range of diagnostic procedures has been employed for identification, differentiation and quantification of microbial plant pathogens. The sensitivity, reliability and reproducibility of the techniques have been constantly improved by the intensive research efforts in different countries.

Identification of fungi and fungi-like, oomycetes infecting plants has been made primarily on the basis of morphological characteristics such as type, shape and color of sexual and/asexual spore forms. Oomycetes lack taxonomic affinity with true fungi. The taxonomic position of the oomycetes with a unique lineage of eukaryotes unrelated to true fungi, but closely related to brown algae and diatoms has been established based on molecular phylogenetic and biochemical investigations. Oomycetes are included in the Kingdom Stramenopila, group Stramenopiles which encloses golden-brown algae, diatoms and brown algae such as kelp (Sogin and Silberman 1998; Baldauf et al. 2000). The fungi-like oomycetes are included under different sections for fungal pathogens for discussion of various aspects. Tests to determine physiological and biochemical characteristics were the basic tools used to identify and differentiate between bacterial pathogens for several decades from 1930s. These tests were occasionally applied for the identification of filamentous fungi which in general exhibit greater phenotypic plasticity than bacteria (Bridge 2002). The physiological and biochemical tests applied for the bacterial and fungal pathogens cannot be employed for the identification and differentiation of plant viruses, since they are extremely small and do not have any detectable physiological activity.

The physiological and biochemical properties vary widely between different groups of bacterial pathogens. No single standard set of tests can be used for all bacteria. Hence, different sets of tests have to be employed to identify isolates of different bacteria. However, commercial kits have been developed for Gram-positive and Gram-negative bacteria based on assimilation tests by Biolog Inc., USA. As some metabolites like mycotoxins (aflatoxin, ochratoxin) are produced only by a narrow range of fungal species, this property may be of significance in the systematics of

certain filamentous fungi like *Aspergillus* spp. and *Penicillium* spp. (Frisvad et al. 1998). Production of mycotoxins may be more precisely detected by using serological methods (Narayanasamy 2005).

The conventional methods depending on the isolation of microbial pathogens from infected plant tissues, multiplication on suitable media and determination of morphological characteristics and physiological and biochemical features require substantially long periods. Furthermore, the results are significantly influenced by cultural conditions and the interpretation of observations requires considerably long experience. Attempts were made to develop methods that depend on the intrinsic characteristics of the microbial pathogens. In addition, the identification of the isolates, strains, races/biotypes has to be done very rapidly and precisely, if the incidence of a new disease is seen. This will facilitate initiation of measures to prevent or restrict further spread of the disease(s). Diagnostic techniques based on the molecular characteristics of the microbial pathogens have been demonstrated to fulfill these requirements.

During the three decades and more, rapid advances made in the study of molecular biology of microbial pathogens have provided adequate information for the development of several sensitive and rapid methods for characterization of microbial pathogens and determination of molecular variability of and relationship between fungal, bacterial and viral pathogens. Molecular techniques have been shown to be very useful in studying various aspects of plant-pathogen interactions, epidemiology of crop diseases and to assess the effectiveness of different disease management strategies. A wide range of techniques has been employed to suit the pathosystem (Narayanasamy 2001, 2005). The relative usefulness of some of the basic molecular techniques widely applied, are presented in this chapter.

2.1 Detection of Microbial Pathogens by Biochemical Techniques

2.1.1 Electrophoresis

Microbial pathogens may be detected and identified based on their specific physiological and biochemical activities in the case of some fungal and bacterial pathogens. Manipulating and analyzing DNA are fundamental procedures in the study of molecular biology of organisms. DNA is isolated intact and treated with restriction enzymes to generate pieces small enough to be resolved by electrophoresis in polyacrylamide or agarose. Separating complex mixtures of DNA into different sized fragments by electrophoresis has been a well established method for over three decades. Analysis of total protein profiles generated by separating whole cell protein extracts by electrophoresis has been shown to be useful. Isozyme electrophoresis has been found to be effective in species/strain differentiation. Isozyme electrophoresis of enzymes such as esterases, phosphatases and dehydrogenases of fungal and bacterial origin provides different patterns according to their relative mobility. Each band is considered as an allele of a specific locus in the pathogen genome. The bands are labeled alphabetically from the slowest to the fastest.

2.1.1.1 Fungal Pathogens

Isozyme electrophoresis can be used for defining groups within species of oomycetes and fungi. Based on the analysis of isozyme patterns, *Phytophthora cambivora, P. cinnamomi* and *P. cactorum* could be clearly separated. Further, it was possible to further subdivide each species into electrophoretic types (ETs). By using cellulose acetate electrophoresis (CAE) for fractionation of phosphoglucose isomerase malate dehydrogenase and lactate dehydrogenase, intraspecific diversity and interspecific relatedness of different papillate species of *Phytophthora* were assessed. A very close genetic relatedness between *P. medii* and *P. botryosa* that clustered together was evident. Likewise, *P. katsurae* and *P. heveae* were together in a cluster, while *P. capsici* and *P. citrophthora* formed another cluster (Oudemans and Coffey 1991a, b).

Within a morphologic species of *Leptosphaeria maculans,* highly virulent and weakly virulent strains inducing black leg or stem canker disease in canola (*Brassica napus*) were differentiated based on the fast or slow movement of isozymes of glucose phosphate isomerase (GPI). The highly virulent strains contained fast isozyme and they were placed in electrophoresis Type 1 (ET1). The isozymes of weakly virulent strains moved only short distance and they formed a distinct group (ET2) (Sippell and Hall 1995). The presence of GPI in leaf lesions induced by *L. maculans* was also detected by CAE which could be also employed to differentiate the pathogen from *Pseudocercosporella capsellae*. In addition to ET1 and ET2 electrophoretic patterns of highly virulent and weakly virulent strain, ET3 allozyme was present in a few typical and atypical lesions caused by *L. maculans*. The lesions induced by *P. capsellae* had the fastest allozyme ET4 (Braun et al. 1997). The isolates (726) of *Phytophthora infestans*, causing late blight disease of potato and tomato collected in Canada were classified into eight genotypes depending on the allozyme patterns at two loci GPI and peptidase (Pep) with markers for mating type, metalaxyl sensitivities and cultural morphology. Differences in the banding patterns for the allozymes of the GPI locus were significant leading to differentiation of seven of the eight genotypes (Peters et al. 1999). Four allozyme genotypes at GPI and Pep loci were distinguished among the 85 isolates of *P. infestans* present in North Carolina. These isolates predominantly belonged to US-7 genotype and US-8 genotype (Fraser et al. 1999).

Isozyme polymorphisms among different isolates of closely related species of *Fusarium* such as *F. cerealis, F. culmorum, F. graminearum* and *F. pseudograminearum* occurring around the world, were investigated by using cellulose-acetate electrophoresis (CAE). Remarkably uniform isozyme patterns were obtained intraspecifically irrespective of the geographical origin of the isolates or the host/substrates from which they were isolated. Comparison of the elctrophoretic types (ETs) of adenylate kinase (AK), NADP-dependent glutamate dehydrogenase (NADPGDH) peptidase B (PEPB), peptidase D (PEPD) and phosphoglucomutase (PGM) proved to be diagnotic for at least one of the four species examined. However, PEPD alone was useful as a marker to distinguish the four taxa studied, providing a rapid and simple CAE-based diagnostic protocol. The results based

on similarity values indicated that *F. graminearum* was more closely related to *F. cerealis* and *F. culmorum* than to *F. pseudograminearum.* The morphological similarity of *F. graminearum* and *F. pseudograminearum* did not reflect their genetic relatedness, suggesting the need for supporting evidence(s), for classification of fungal pathogens (Làday and Szécsi 2001).

2.1.1.2 Bacterial Pathogens

The usefulness of SDS-PAGE technique for the differentiation of bacterial pathogens has been demonstrated. Silver staining of SDS-lysed cells of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) electrophoretically separated into dark gray bands with a MW of 32–35 kDa and 25–27 kDa. The band with larger MW designated α was present in 192 of 197 tomato race/strain and the band with smaller MW designated β, was present in all 55 strains of tomato race 2. Race/strains expressing an α band could not hydrolyse starch (Amy^-) and very few degraded pectate (Pec^-). In contrast, most of race 2 strains were Amy^+ and Pec^+. Silver staining of protein profiles and testing for amylolytic activity of *Xcv* may be useful to assign uncharacterized strains of *Xcv* to appropriate phenotypic group (Bouzar et al. 1994) [Appendix 1]. In the case of *Pseudomonas syringae* pv. *pisi* (*Psp*) the envelope protein profiles of bacterial cells formed the dependable basis for identification and differentiation of the strains. *Psp* strains showed three unique protein bands (60, 65 and 150 kDa) which were absent in 29 strains of *P. syringae* pv. *syringae* tested (Malandrin et al. 1996). Some of the bacterial pathogens have been identified using their isozyme profiles. The suitability of applying the patterns of enzymes of esterase (EST) and superoxide dismutase (SOD) for the identification, differentiation of strains and diagnosis of diseases caused by *Psp* was assessed. Two EST zymotypes specifically present in the profiles could be used for the identification of *Psp*. Furthermore, there was significant correlation between these EST patterns and race structure of this pathovar. Races 2, 3, 4 and 6 exhibited patterns similar to zymotype 1, whereas races 1, 5 and 7 were included in zymotype 2 based on the similarity of isozyme profiles (Malandrin and Samson 1998). The whole-cell protein analyses of *Xylella fastidiosa* (causing Pierce's disease of grapevine) strains by employing SDS-PAGE technique indicated variations in the protein banding patterns among the strains. It was possible to detect and identify the strains (75) based on the presence, absence or intensity of 10 protein bands and assign them to 6 different groups. This technique has been shown to be a rapid and consistent method of identifying the strains of *X. fastidiosa* (Wichman and Hopkins 2002).

A new procedure known as pulsed field gel electrophoresis (PFGE) to separate DNA was introduced by Schwartz and Cantor (1984). With further studies, PFGE has reached a level for routine application and commercial pulsed field units have been manufactured for large scale use. Now PFGE permits cloning and analysis of a small number of very large pieces of a genome. The genomic analysis of strains of *Erwinia amylovora,* (causing fire blight disease), from the Mediterranean region and European countries was performed. The PFGE patterns were determined by assaying the *Xba1* digests of bacterial genomic DNA. The strains from Austria and

Czech were grouped with the central European type (Pt1). The strains from the eastern Europe and Mediterranean region were placed in the second group Pt2. Italy has strains showing patterns of all three types (Zhang et al. 1998). The isolates of *Acidovorax avenae* subsp.*citrulli* infecting cucurbits in Israel were subjected to PFGE analysis which revealed 23 unique DNA fragments and five different profiles, each of which contains 9–13 bands. The isolates were grouped into two classes. The distinct advantage of PFGE over rep-PCR assay was the greater levels of reproducibility of results and the genetic diversity of the isolates may be reliably assessed PFGE analysis (Burdman et al. 2005).

2.1.1.3 Viroid Pathogens

The distinct nature of the causal agent of potato spindle tuber disease was first established by using PAGE system. The cellular nucleic acids from both healthy and infected plants were extracted and separated by PAGE. The causative agent *Potato spindle viroid* (PSTVd) appeared as a distinct band only in the samples from infected tissues and it was absent in the comparable healthy potato tissues. The mild and severe strains of PSTVd could be detected and differentiated. Further, PAGE was successfully employed to free several elite or basic seed stocks of potato in certification programs (Morris and Wright 1975). The differences in the electrophoretic mobility of isolates of *Coconut cadang-cadang viroid* (CCCvd) were used as the basis of differentiating the different isolates (Randles 1985). *Coconut tinangaja viroid* (TiVd) in coconut leaf samples was detected by analytical agarose gel electrophoresis (Hodgson et al. 1998).

The presence of citrus viroids – *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viorid* (CBLVd), *Hop stunt viroid* (HSVd) and *Citrus viroid III* (Cvd-III) – in citrus samples from greenhouse was detected by sequential polyacrylamide gel electrophoresis (sPAGE). Sample extracts (20 µl equivalent to 300 mg fresh weight) are subjected to nondenaturing 5% PAGE at 60 mA for 2.5 h. After staining the gel with ethidium bromide, a segment (1.5 cm) of the gel between CEVd and 7S RNA is excised and subjected to a second 5% PAGE containing 8 M urea. After silver staining, the viroid bands can be viewed (Barbosa et al. 2005).

2.1.1.4 Viral Pathogens

Electrophoretic techniques have been employed to a limited extent as a step in the process of virus purification from crude plant extracts or suspensions containing mixture of viruses or their strains. Plant virus suspensions in suitable buffers are layered into appropriate buffered density gradients of gels formed in a tube and separation of components of the suspension takes places over a period of several hours in a zonal density gradient. Other methods based on the principle of electrophoresis, using of pH gradient or paper curtains have been used for the purification of *Southern bean mosaic virus* and for separation of *Tobacco mosaic virus* strains. For unstable viruses like *Citrus infectious variegation virus*, electrophoretic technique is especially valuable (Narayanasamy and Doraiswamy 2003). The chitinase isozymes

patterns of healthy and virus-infected leaves may form a basis for the detection of virus infection by SDS-PAGE technique. The chitinase isozymes were detected by SDS-PAGE technique in the crude extracts from leaves of healthy and TMV-infected tobacco plants. There were eight dominant chitinase isozymes detected in tobacco extracts. One of them was present only in the TMV-infected leaves, while another accumulated at a greater concentration in TMV-infected than in mock-inoculated leaves (Pan et al. 1991).

SDS-PAGE technique was applied to detect the presence of a unique protein band (32–34 kDa) present in leaves of maize infected by a new virus designated *Wheat yellow head virus* (WYHV). This protein was not present in extracts from healthy maize leaves [Appendix 2]. The amino acid sequences of their protein was most closely related to the nucleoprotein of *Rice hoja blanca virus*, indicating that WYHV is a tenuivirus (Seifers et al. 2005). The relative molecular mass of *Apricot latent virus* coat protein (CP) was determined by using SDS-PAGE technique. The dissociated CP migrated as a single band with an estimated size of ca 50 kDa (Ghanen-Sabanadzovic et al. 2005) (Fig. 2.1). The nucleocapsid proteins (Nps) of *Calla lily chlorotic spot virus* (CCSV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Watermelon silver mottle virus* (WSMoV) were dissociated from the purified nucleocapsids and subjected to SDS-PAGE analysis for the estimation of their relative molecular mass. CCSV NP had a molecular mass of 31 kDa which was similar to that of WSMoV, but slightly larger than those of TSWV and INSV which had molecular mass of 29 kDa (Lin et al. 2005).

SDS-PAGE technique was applied to determine the molecular masses of coat proteins in purified preparation of *Peanut chlorotic streak virus* that has two polypeptides with approximate relative molecular masses of 51 and 58 kDa (Reddy et al. 1993). The virus coat proteins may be expressed in the bacterium *Escherichia coli*. The expressed coat protein can be purified by SDS-PAGE technique for use as

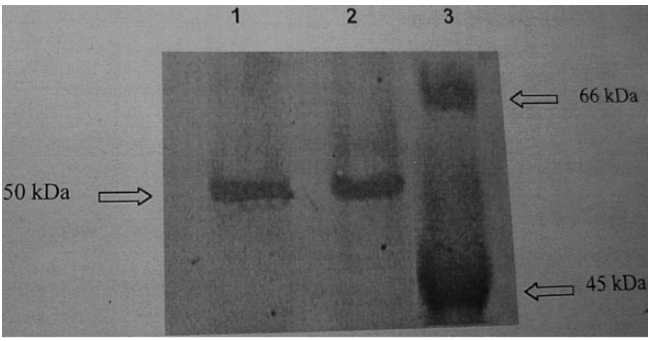

Fig. 2.1 Electrophoretic analysis of dissociated *Apricot latent virus* (ApLV)-strain Apr47 coat protein (CP)
Note the single band of 50-kDa protein in lanes 1 and 2 representing viral CP. Molecular markers [albumin egg (MW 45,000) and albumin bovine (MW 60,00)] are placed in lane 3. (Courtesy of 2005 2005; Journal of Plant Pathology, Edizioni ETS, Pisa, Italy)